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VITRIFICATION AND BA-INDUCED ANOMALIES IN IN VITRO CHESTNUT CULTURES

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RESUMEN

VITRIFICACION Y ANOMALIAS INDUCIDAS POR BENZYLADENINA EN CASTAÑO CULTIVADO IN VITRO

Se indujo vitrificación en brotes de castaño cultivados in vitro mediante subcultivos sucesivos en el medio de Murashige y Skoog (MS). Por otra parte, se indujeron cultivos anómalos cuando brotes de castaño se cultivan en un medio de Heller modificado incrementando la concentración de la citokinina 6-benzyladenina (BA) durante sucultivos sucesivos. Se estudiaron y compararon las alteraciones morfológicas, anatómicas y metabólicas de los dos tipos de cultivos.

Aunque alguna de estas modificaciones parecen similares, la vitrificación producida por elevadas concentraciones de amonio en el medio MS es diferente de las alteraciones producidas por los elevados niveles de citokininas. Estos resultados parecen indicar que, en castaño, los cultivos vitrificados se producen como consecuencia de la composición del medio mineral más que de la concentración de reguladores de crecimiento.

Palabras clave: ***Castanea,*** castaño, cultivo de tejidos, vitrificación.

SUMMARY

Vitrification of in vitro chestnut shoot cultures was induced by subsequent subcultures in Murashige and Skoog (MS) medium. Anomalous tissues were also induced by using increasing concentration of 6-benzyladenine (BA) in successive subcultures in a modified Heller medium. The morphological, anatomical and metabolic alterations in both types of cultures were studied and compared.

Although some of these alterations appear to be similar to the vitrification produced by high ammonium content medium such as MS they are different to the alterations produced by high levels of cytokinins (BA). These findings seem to indicate that, in chestnut, vitrified tissues are the consequence of mineral medium composition rather than growth regulators.

Key Words: ***Castanea,*** chestnut, tissue culture, vitrification.

INTRODUCTION

Vitrification is a problem that often arises during in vitro culture of a great number of herbaceous and woody species. Such tissues rapidly lose their capacity for propagation, showing low rates of multiplication, rooting and survival on transfer to soil.

Vitrified tissues are characterized by thickened and translucent stems and leaves which are thick, brittle, wrinkled, curled and frecuently very elongated with no differentiated palisade (Vieitez *et al.*, 1985). Biochemical disorders such as increased water content, deficiency in lignification, lower activity of enzymes related to lignification, decrease in phenols content, less chlorophyll than normal plants and alteration of endogenous growth regulators levels are also associated to vitrification (Gaspar *et al.*, 1987). On the other hand, the biosynthesis of lignin and cellulose as well as phenolic production are closely associated since defficiency in both substances may result from the decreased C/N ratio produced by an excess of nitrogen. It is possible that the rapid uptake of ammonium ions (asimilated faster than others such as NO_3^-) increases the consumption of glycosides sufficiently to divert them from the metabolic patways leading to the synthesis of lignin.

The mechanism of vitrification induction is not well understood. One of the mentioned causes is continuous use of Murashige and Skoog's mineral medium (MS), which has a high concentration of ammonium ions (Gaspar *et al.*, 1987). We have found (Vieitez *et al.*, 1985) that two successive subcultures on MS are sufficient for micropropagated chestnut to acquire the symptoms of vitrification, whereas normal morphology is maintained indefinitely when subculturing on a modified Heller's medium. The combination of high concentrations of cytokinins and low concentrations of agar has also been reported to cause vitrification-like anomalies in certain cultures (Debergh *et al.*, 1981; Bornman, Vogelman, 1984). More recently, the induction of vitrification in melon by cytokinin treatments has been reported (Leshem *et al.*, 1988).

This article compares the morphological, anatomical and metabolic alterations brought about in chestnut shoots subcultured on media with high concentrations of the ammonium ion to those induced by high concentrations of benzyladenine (BA).

MATERIALS AND METHODS

Cultures from two clones were used: clone 812 derived from juvenile *Castanea sativa* Mill. and clone 431 from adult tissues of a *C. sativa x C. crenata* hybrid. Both were originally established in vitro 2 years ago from shoots tips using the procedure previously described (Vieitez, Vieitez, 1982/83; Vieitez *et al.*, 1983). They have both been subsequently subcul-

tured every month on a medium consisting of Heller's (Heller, 1953) macronutrient formula with the concentration of all macronutrients multiplied by 1.25 and 1 mM $(NH_4)_2SO_4$ added (refered as $H + SO_4$) together with the micronutrients and FeEDTA of Murashige and Skoog's (1962) medium (MS), 1 mg L^{-1} thiamine-HCl, 0.1 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} pyridoxine-HCl, 100 mg L^{-1} m-inositol, 0.2 mg L^{-1} 6-benzyladenine (BA), 30 g L^{-1} sucrose and 6 g L^{-1} agar-agar.

Vitrified cultures were obtained after 5 subsequent subcultures on a medium containing the MS macronutrients. Among other components, the MS medium contains 25 mM of NO_3NH_4. The effects of 5 successive subcultures on the usual medium supplemented with 0.2, 0.5, 1, 2 or 5 mg L^{-1} of BA were also determined.

All media were brought to pH 5.5-5.6 before autoclaving at 1.05 kg cm^{-2} for 15 min. Twenty-four cultures per treatment were grown under 30 μE m^{-2} s^{-1} delivered during a 16 h photoperiod by cool-white fluorescent lamps, with day and night temperatures of 25° C and 18° C respectively.

Anatomical examination

The material examined comprised 5 mm segments of the basal part of the stem, 5 mm segments from the central region of the leaves and 3-5 mm shoot tips. Tissue pieces were fixed in a 18:1:1 mixture of 50 v/v ethanol, glacial acetic acid and formalin, dehydrated by passage through ethanol-butyl-alcohol series, and embedded in paraffin wax (Jensen, 1962). Sections 10 μm thick were cut and stained with safranin fast green. Polarized light microscopy was used to observe lignification of the tissues.

Determination of water content and C/N ratio

After removal of any basal callus, the whole shoots (leaves, buds and stems) were dried with filter paper and immediately weighed to determine their fresh weight (FW). They were then dipped in liquid nitrogen, lyophilized and re-weighed to determine their dry weight (DW). The ratio FW/DW was calculated.

The C/N ratios for both normal and anomalous shoots were determined in lyophilized material using an automatic nitrogen analyzer.

In both cases, water content and C/N ratios, three replications of each sample were done.

Pigment content

10 mg of vitrified and normal material lyophilized as above was extracted for 2 h in the dark with 1.5 ml of HPLC grade ethanol at room temperature, after which 30 μl of the extract (equivalent to 0.2 mg dry weight) was analysed in a Waters HPLC chromatograph equipped with a programmable pump in a μ-bondapak C 18 column using 98:2 methanol-water as solvent flowing at 1 ml min^{-1} rate. The chromatographic conditions are similar to those proposed by Braumann and Grimme (1981). Peaks with the same retention times to those of chlorophyll *a*, chlorophyll *b* and violaxanthin were collected from the chromatograph and individual UV-visible spectra were recorded in a Pye-Unicam spectrophotometer.

RESULTS

Anomalies caused by high concentrations of BA

1) Morphology and Anatomy

When subcultured in the presence of high BA concentrations (1, 2 or 5 mg L^{-1}), the two clones under study produced shoots with very short internodes that were much shorter than shoots grown with just 0.2 mg L^{-1} BA, which were considered normal. At the same time, the very large number of short axillary buds gave the cultures the appearance of a rosette. Leaves too, were shorter and thicker than normal (Fig. 1 a). All these features increased in intensity as the concentration of BA was increased from 0.5 to 5 mg L^{-1}.

The anatomy of normal in vitro chestnut tissues (those cultivated under 0.2 or 0.5 mg L^{-1} BA) has been previously described by Vieitez *et al.* (1985). The leaves of shoots cultured with 0.2 mg L^{-1} show the anatomy of Fig. 1 b. However the leaves of shoots cultured with 1, 2 or 5 mg L^{-1} BA lacked the normal clear differentiation between palisade and spongy parenchyma. With 2 mg L^{-1} BA, palisade parenchyma was present but was arranged in a less ordered fashion than in normal leaves. The thickening of the leaves of anomalous cultures was due to the proliferation of spongy parenchyma, which consisted of irregularly distributed cells at the thickest part of the leaf next to the midvein, whose abaxial parenchyma was highly developed and formed by larger cells than in normal leaves (Fig. 1 c). The lignification of xylem elements was less than normal in both the midvein and lateral veins, especially on the adaxial face.

Media with 1 mg L^{-1} or more of BA gave rise to thicker stems than those with 0.2 or 0.5 mg L^{-1} BA. This was largely due to the greater development of the pith, which was made up of large parenchyma cells. As in the leaves, xylem and sclerenchyma were less lignified than normal, although 5 mg L^{-1} BA induced the differentiation of lignified tracheid-like structures in the pith. The cortex also possessed larger parenchyma cells than normal, as did the many stipules which, because of the shortening of the internodes, almost covered the stem.

The apical meristem was very much altered in shoots cultured with high BA concentrations, becoming broader and flatter as the concentration of cytokinin increased. With 2 or 5 mg L^{-1} BA it often exhibited a depression, which was probably a consequence of a mechanical effect due to the production of a larger number of axillary buds on a very short stem segment (Figs. 1 d, e). Sections very close to the dome of meristem showed pith with very large cells.

2) Water content, C/N ratio and pigments

Table 1 lists the FW/DW and C/N ratios of cultures for four subcultures on the modified Heller's medium used with various concentrations of BA. Water content rose with BA concentration and also, with the number of subcultures. The C/N ratio fell during the first subculture by increasing the BA concentration, but the-

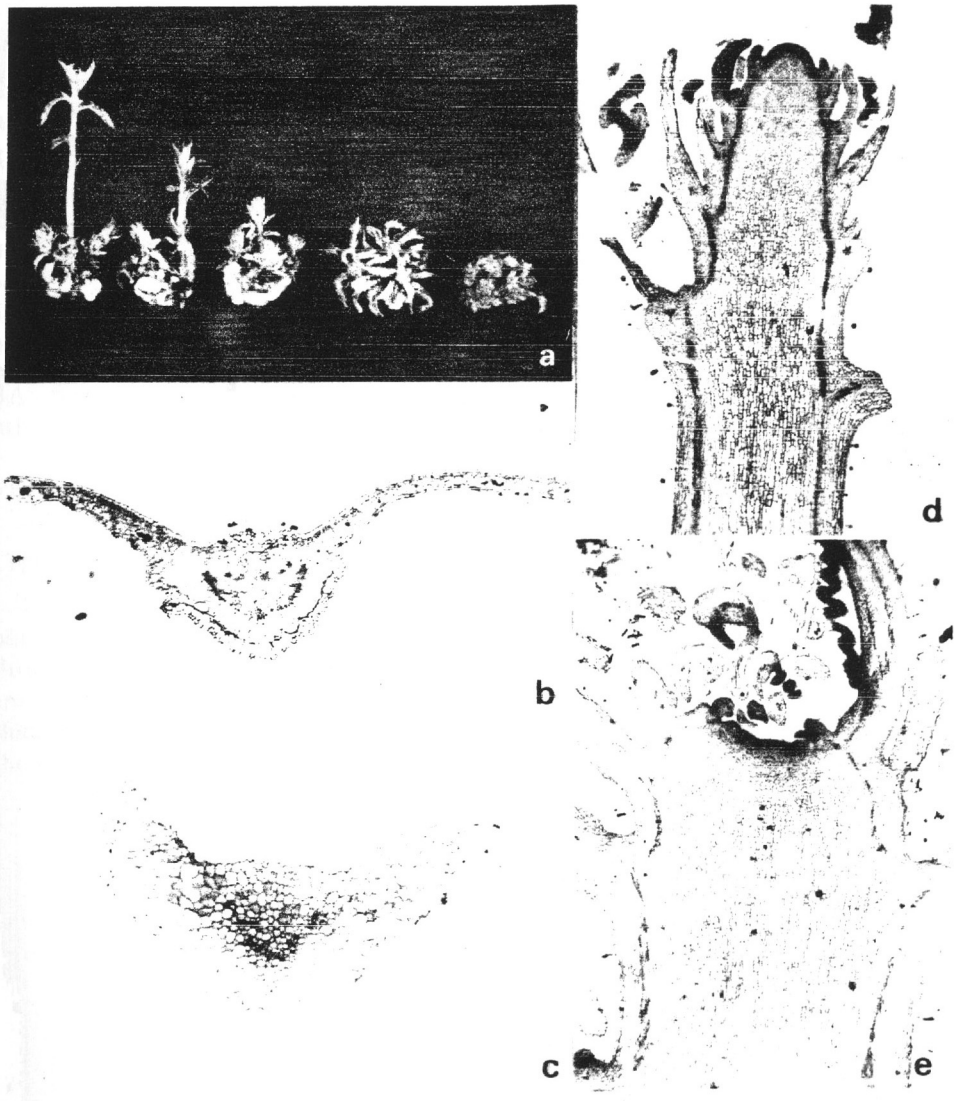

FIG. 1.—*(a) Effect of BA on shoot proliferation on H + SO_4 medium. From left to right 0.2, 0.5, 1, 2, and 5 mg L^{-1}. (b-c) Transections of leaves of shoots grown on 0.2 mg L^{-1} BA (b) and 2 mg L^{-1} (c) (x 56). (d-e) Longisections of a normal (d) and anomalous (e) apex of shoots grown on 0.2 and 2 mg L^{-1} BA respectively (x 35).*

TABLE 1

Mean values of FW/DW and C/N ratios of tissues (clone 431) maintained for four subcultures in $H + SO_4$ medium with various concentrations of BA. ± Standard error.

	FW/DW				C/N			
	Subculture number				Subculture number			
Treatment BA (mg L^{-1})	1	2	3	4	1	2	3	4
0.2	9.1 ± 0.8	10.9 ± 0.8	13.2 ± 1.0	11.4 ± 1.1	16.3 ± 2.0	16.1 ± 2.0	21.0 ± 2.5	21.9 ± 2.7
0.5	11.7 ± 1.1	12.4 ± 1.4	13.1 ± 1.5	12.8 ± 1.1	16.0 ± 2.0	15.4 ± 1.9	17.2 ± 2.1	20.2 ± 2.4
1.0	12.1 ± 0.9	12.8 ± 1.5	13.2 ± 1.5	14.8 ± 1.7	13.4 ± 1.7	13.2 ± 1.7	17.6 ± 2.1	17.9 ± 2.1
2.0	12.8 ± 1.4	14.7 ± 1.6	16.8 ± 1.9	18.3 ± 2.2	13.3 ± 1.4	14.2 ± 1.5	14.9 ± 1.5	14.8 ± 1.6
5.0	14.1 ± 1.5	15.6 ± 1.6	16.5 ± 1.9	17.4 ± 2.0	12.5 ± 1.1	13.9 ± 1.4	14.4 ± 1.5	14.1 ± 1.5

reafter increased with the number of subcultures.

The pigment content was followed using HPLC. As a general model it was found that increasing the concentration of BA caused a marked increase in violaxanthin content and a very slight increase in chlorophyll levels. The same effect was produced by increasing the number of subcultures for a given BA concentration. Figures 2 a and b show the chromatograms of shoots cultured in the presence of 0.2 and 2.0 mg L^{-1} of BA respectively during the third subculture.

Vitrification caused by MS mineral salts

1) Morphology and anatomy

In keeping with our previous paper (Vieitez *et al.*, 1985), vitrified shoots had stems that were shorter and thicker than normal and leaves that were longer than normal, and the shoot apices showed retarded development of leaf primordia. Vitrification appeared in the third or fourth subculture on MS medium, according with the morphology exhibited.

Anatomically, vitrified shoots were less differentiated than normal, the xylem tissue was poorly lignified and large intercellular spaces appeared. In vitrified leaves there were no clear differentiation between palisade and spongy parenchyma, and in the spongy mesophyll large intercellular spaces developed. Vitrified apices typically had domes with few meristematic cells.

2) Water content, C/N ratio and pigments

Table 2 lists the FW/DW and C/N ratios of clones 431 and 812 when maintained on modified Heller's medium with 0.2 mg L^{-1} BA and after 4 consecutive subcultures on MS also with 0.2 mg L^{-1} BA. Water content was maintained at the same

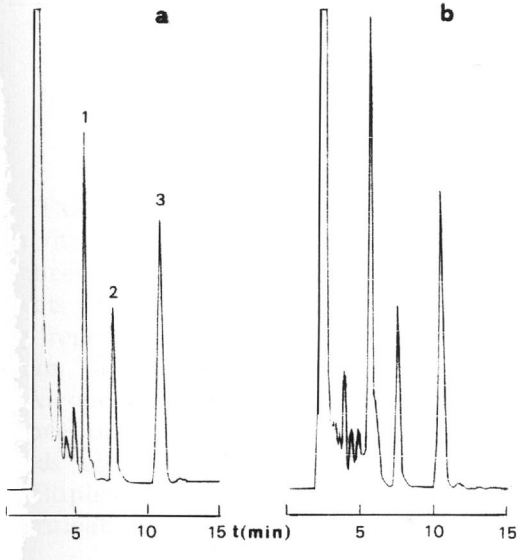

FIG. 2.—*HPLC chromatograms of extracts of shoots (clone 431) grown on (a) 0.2 mg L^{-1} BA and (b) 2 mg L^{-1} BA (third subculture). Peaks tentatively identified as: 1, Violaxanthin; 2, chlorophyll* b; *3, chlorophyll* a.

TABLE 2

Mean values of FW/DW and C/N ratios of tissues (clones 431 and 812) maintained in $H + SO_4$ or MS media plus BA 0.2 mg L^{-1}. Results were taken during four consecutive subcultures. ± Standard error.

		FW/DW				C/N			
		Subculture number				Subculture number			
Clone	Treatment BA 0.2 mg L^{-1}	1	2	3	4	1	2	3	4
431	$H + SO_4$	8.6 ± 0.6	8.5 ± 0.6	8.6 ± 0.5	8.7 ± 0.7	16.2 ± 2.6	16.7 ± 2.0	16.6 ± 2.1	16.3 ± 2.1
431	MS	10.6 ± 0.8	12.1 ± 1.0	12.7 ± 1.1	12.8 ± 1.0	12.8 ± 1.6	5.4 ± 0.5	6.2 ± 0.8	5.9 ± 0.6
812	$H + SO_4$	8.9 ± 0.6	8.4 ± 0.5	8.6 ± 0.8	8.6 ± 0.6	17.8 ± 2.1	16.9 ± 1.9	17.2 ± 2.0	17.4 ± 2.1
812	MS	9.8 ± 0.7	10.4 ± 1.0	14.8 ± 1.5	14.4 ± 1.5	14.8 ± 1.6	6.9 ± 0.9	8.1 ± 0.8	7.9 ± 0.8

level in both clones after 4 subcultures on Heller's medium. This content was higher when shoots were subcultured on MS medium, not only in the first subculture but also on successive subcultures, the amount of water increasing as the number of subcultures increased.

Also the C/N ratio remained more or less constant in cultures subcultured on Heller's medium, but fell sharply in the second subculture on MS medium although it remained more or less constant thereafter.

Chlorophyll content decreased with increasing vitrification, i.e. the greater the number of subcultures. The height of the chromatogram peaks suggest that the chlorophyll *a/b* ratio remains constant once vitrification has set in, with more chlorophyll *b* than chlorophyll *a* (Fig. 3 a, b).

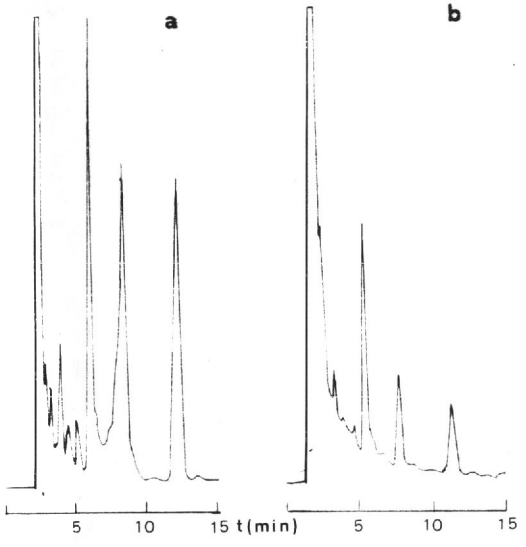

FIG. 3.—*HPLC chromatograms of extracts of shoots (clone 812) grown on MS medium during successive subcultures to induce vitrification. (a) first subculture; (b) fourth subculture. Peaks as in Fig. 2.*

DISCUSSION

The anomalous growth induced in in vitro chestnut cultures by high BA concentrations differs in several aspects from the vitrification induced by repeated subcultures on MS medium. In the first place, increasing BA concentration favours the appearance of large numbers of axillary buds all down the stem, whereas multiplication rates fall sharply upon vitrification by MS. This effect of BA on axillary bud development has also been observed in embryonic chestnut axis cultures (Vieitez, Vieitez, 1980). The "high-BA syndrome" resembles vitrification in that leaves are abnormally thick and lack of clear differentiation between palisade and spongy parenchyma, but in high-BA syndrome the thickening is due to proliferation of the spongy parenchyma cells, whereas vitrifica-

tion is the result of the development of large intercellular spaces. Another difference is that there is less lignification in vitrified cultures than in those with high-BA syndrome, which when 5 mg L^{-1} BA was used, developed tracheid-like structures in the pith that recalled similar structures observed in chestnut epicotyl sections cultured in the presence of 2 or 5 mg L^{-1} BA (San-José, 1983; San-José, Vieitez, 1984).

Both vitrification and high-BA syndrome increase water content, but their effects on the C/N ratio are quite different. The C/N ratio drops immediately upon subculture from modified Heller to MS (which has a high ammonium content as it can be see in Material and Methods) and remains low thereafter, whereas the initial decrease suffered upon increasing the concentration of BA in Heller tends to be gradually recovered from, if the same BA concentration is maintained thereafter. This difference is undoubtely due to the culture with high BA-syndrome being more lignified than vitrified cultures.

Vitrified and high-BA cultures also differ as regards their chlorophyll content and the chlorophyll *a/b* ratio is greater than the unit in cultures with high-BA syndrome and less than the unit in vitrified cultures. Crevecoeur *et al.* (1987) reported finding more chlorophyll *b* than *a* in normal and habituated sugar beet callus.

Gaspar *et al.* (1987) state that among the metabolic alterations occurring in vitrification, those involving growth regulators can have serious consequences for the development of cultures. Some authors (Debergh, 1983; Leshem *et al.*, 1988; Kevers *et al.*, 1984; Hussey, 1986; Chiariotti, Antonelli, 1987) have been more specific in pointing to cytokinins, either alone or in conjunction with other substances, as the agent causing or triggering the onset of vitrification. This does not seem to be the case in chestnut, however. Not only have we found in previous work that endogenous cytokinin levels determined by immunoassay are lower in vitrified than in normal shoots (Vieitez *et al.*, 1988), but that this present study shows that the vitrification brought about by the ammonium-rich culture medium MS (25 mM as against 1 mM in modified Heller medium) which is exactly as described by Debergh *et al.* 1981), involves anomalies that are quite different from those caused by high concentrations of the cytokinin BA. We accordingly agree with Beauchesne (1981), who suggested that the role of growth regulators in vitrification, if any, is to accelerate rather than induce it, and that the anomalies they bring about when used in large doses, though similar to those involved in vitrification, are not necessarily identical.

CONCLUSIONS

It has been demosntrated that the morphological, anatomical and metabolic alterations produced when chestnut is cultivated in vitro with increasing concentrations of cytokinins are different than those exhi-

bited with high ammonium content in the culture medium.

This indicate that vitrification produced for ammonium-rich medium such as Murashige and Skoog (MS) is not related with the alterations produced by high amounts of 6-benzyladenine.

It is possible to overcome vitrification in chestnut cultures by using mineral media with lower ammonium amounts than that of MS.

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