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Relationship between endogenous hormone levels of grapevine callus cultures and their morphogenetic behaviour

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Summary

Dark callus cultures from leaves and anthers of three grapevine genotypes (Seyval blanc, Riesling and Trollinger) were propagated *in vitro* for almost two years on medium deprived of growth regulators. Three different callus lines originated from these initial callus cultures: A dark non-embryogenic one, another composed by clusters of somatic embryos and a friable, whitish callus line that can produce somatic embryos under appropriate circumstances. Endogenous hormone levels *viz.* indole-3-acetic acid (TAA), abscisic acid (ABA), gibberellins A_1, A_3 and A_{20}, zeatin/zeatin riboside and N^6(Δ^2-isopentenyl) adenine/ N^6(Δ^2-isopentenyl) adenosine, were analysed in the different callus types. Only higher ABA levels correlated with the morphogenetic capacity of the cultures. When endogenous hormone levels were analysed in the line constituted by conglomerates of somatic embryos at different dates during the whole culture period, higher levels of TAA and ABA were found during the winter months even if the cultures were maintained permanently under constant temperature and photoperiod conditions. A 4-week chilling period led to a reduction of the endogenous ABA level.

K e y w o r d s : somatic embryogenesis, *in vitro* culture, phytohormones, radio-immunoassay, chilling.

Tntroduction

Since the first report of somatic embryogenesis from nucellus of *Vitis vinifera* L. cv. Cabernet-Sauvignon (Mullins and Srinivasan 1976), embryogenic cultures have been reported for more than 40 *Vitis* genotypes using different explant sources: *e.g.* leaf discs (Matsuta and Hirabayashi 1989; Reustle 1989; Robacker 1993; Harst 1995), zygotic embryos (Stamp and Meredith 1988; Gray 1989, 1992; Emershad and Ramming 1994), anthers (Mauro *et al.* 1986; Mullins *et al.* 1990; Le Gall *et al.* 1994), and petioles (Robacker 1993). Tt has been observed that the ability of a particular explant to undergo somatic embryogenesis is highly genotype dependent (Mauro *et al.* 1986; Matsuta and Hirabayashi 1989).

Somatic embryogenesis in grapevine and its potential application to biotechnology are well recognised; *e.g.* genetic transformation to reduce the impact of biotic and abiotic stresses (Mullins *et al.* 1990; Colby and Meredith 1993; Le Gall *et al.* 1994) or to obtain viable protoplasts for eventual somatic hybridisation (Reustle *et al.* 1994; Reustle and Natter 1994). However, a prerequisite for the utilisation of these strategies in recalcitrant genotypes is to understand the mechanisms by which competent tissues are included.

Among the endogenous factors that may play a role in the induction and further development of somatic embryos in grapevine, extracellular macromolecules set free by the cells (polysaccharides, glycoproteins and proteins with a molecular weight >10 kDa (Coutos-Thevenot *et al.* 1992 a, b) and plant hormones, *e.g.* indoleacetic acid (TAA) (Faure *et al.* 1998), abscisic acid (ABA) (Rajasekaran *et al.* 1982; Faure *et al.* 1998) and gibberellins (Takeno *et al.* 1983), have been investigated.

The aim of the present work was to measure the endogenous hormone levels (TAA, ABA, gibberellins A_1, A_3 and A_{20} (GAs), zeatin/zeatin riboside (Z/ZR) and N^6(L'^{2}-isopentenyl) adenine/ N^6(L'^{2}-isopentenyl) adenosine (iP/iPA)), in different grapevine callus types and genotypes and to relate them to their ability to induce somatic embryogenesis. The endogenous levels of these hormones were also evaluated along a period of almost two years, which was followed by a 4-week chilling treatment (4 °C), in embryogenic callus cultures of cv. Seyval blanc.

Material and Methods

I n v i t r o c u l t u r e o f g r a p e v i n e c a l l u s c u l t u r e s : Callus cultures (kindly provided by Dr. G. M. Reustle and Dr. A. Matt, Fachgebiet Weinbau, Tnstitut für Obst-, Gemüse- und Weinbau, Universität Hohenheim, Stuttgart), obtained from leaf discs of the genotypes Seyval blanc and Trollinger, and from anthers of Riesling after a procedure described by Reustle (1989), were transferred every 4 weeks to 25 ml of solid fresh medium and maintained at 26 °C under permanent darkness. Callus was cultivated on a solid NN-69 culture medium with mineral salts and organic addenda according to Nitsch and Nitsch (1969) with 2 % sucrose and a pH of 5.8 in 90 x 15 mm sterile disposable Petri dishes. Tn every subculture, callus segments of each genotype were classified according to their morphogenetic characteristics and the culture continued in different Petri dishes.

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The categories used for classification were: (A_B) dark callus producing somatic embryos, (A) brown to dark callus without embryogenic ability, (B) embryo clusters producing secondary embryos, and (C) friable whitish-yellowish callus.

After 16 months of culture, callus samples were subcultured on 25 ml of LS/R solid medium (Linsmaier and Skoog 1965), supplemented with 0.4 mg l^{-1} thiamine HCl. 100 mg l^{-1} myo-inositol and 3 % sucrose (pH adjusted to 5.8 with KOH). They were incubated at 26 °C at a 16 h photoperiod (4.75 W m^{-2} provided by 40 W fluorescent lamps, Philips, Eindhoven, Netherlands) in order to characterise their regeneration ability, which was evaluated after 4 and 8 weeks. The plant material was cultured for a total period of 24 months.

S a m p l i n g f o r h o r m o n e d e t e r m i n a t i o n i n t h e d i f f e r e n t c a l l u s t y p e s : The determination of TAA, ABA, GAs, Z/ZR and iP/iPA was always performed on the ame sample. Samples of the different callus types (A, B and C) were surface dried and cleaned with a paper towel, immediately frozen in liquid nitrogen and stored at -20 °C. The frozen samples were then freeze-dried and stored again at -20 °C.

The B-type callus of Seyval blanc was sampled 13 times during the experiment and evaluated for their endogenous hormone content. Since GAs were not analysed from the onset of the study, there are some data missing in the analysis of this group of hormone.

C h i l l i n g : To evaluate the effect of chilling on the endogenous hormone levels of the Seyval blanc B-type callus line, after the 22nd subculture, some samples were maintained at 4 °C in the dark for 4 weeks.

E x t r a c t i o n a n d p u r i f i c a t i o n o f p l a n t h o r m o n e s : Approximately 300 mg dry weight (DW) per sample were ground in liquid nitrogen, homogenised with an Ultra-Turrax (Janke and Kunkel, Staufen, Germany) in 80 % methanol below 0 °C and then extracted overnight with 50 ml 80 % aqueous methanol in the darkness at 4 °C. The extracts were then filtered through G4-glassinter-filters (max. pore size 10-16 !Jm; Schott, Mainz, Germany). To estimate the losses that can occur during purification an internal standard of 1-^{14}C-TAA (spec. act. 14 mCi·$mmol^{-1}$; Amersham, Braunschweig, Germany) was added. The extract was dried under vacuum and dissolved in 12 ml 0.1 M ammonium acetate (pH 9.0) by using an ultrasonic bath and were subsequently frozen at -20 °C overnight. After thawing, the extract was centrifuged at 22,000 rpm at 4 °C for 25 min.

For purification, the supernatant obtained by centrifugation was passed through a preconditioned column combination of polyvinylpyrrolidone (Sigma Chemical Co., Deisenhofen, Germany), DEAE-Sephadex A-25 (Pharmacia, Freiburg, Germany) and a C_{18} Sep-Pak cartridge (reversed phase, Waters, Eschborn, Germany) (modified by Bertling and Bangerth 1995).

Q u a n t i f i c a t i o n o f h o r m o n e s : Hormones were quantified by radio-immunoassay using polyclonal antibodies. Fractions containing TAA, ABA and GAs were methylated with diazomethane. Antibodies used were raised in rabbits against free TAA, free ABA, GA_3, ZR and iPA and radioimmunological hormone analysis was performed according to Bohner and Bangerth (1988). Cross reactions of the GA_3 antibody used were determined according to Bertling and Bangerth (1995), to be about 90 % with GA_1 and GA_{20}. Therefore, the GAs determined by means of this antibody are expressed as GA_3 equivalents and called GAs in the following text.

Overall recovery during the described purification procedures was previously determined by radiolabelled internal standards, and was found to be between 40 and 70 % for TAA, >92 % for ABA, >89 % for GAs and >80 % for both cytokinins. Therefore, the TAA internal standard was the only one used regularly and the TAA levels obtained were adjusted to the corresponding recovery value for each sample, due to the high variation.

S t a t i s t i c a l a n a l y s i s : Endogenous hormonal levels were determined in at least three biological replications and analysed using the STATTSTTCA for Windows (StatSoft Tnc., Tulsa, Oklahoma, USA) Version 5.1 Studenten-Version; '97 Edition. The Post-Hoc Tukey's Honest-Significant-Difference-Test (HSD) for unequal N (Spjotvoll/Stoline) was used to determine significant differences in hormone level means (<0.05).

Results

I n v i t r o r e s p o n s e : The callus cultures of Seyval blanc and Riesling were characterized by their dark colour and by the spontaneous formation of whithish structures on their surface, therefore they were classified as type A_B. After continuous subculture, some of these A_B callus cultures lost their ability to produce somatic embryos, and just continued growing in size, without showing any sign of organised development (they were then graded as type A-callus). When the whitish structures formed on the surface of the A_B callus cultures were isolated and cultured separately, they could form clusters of somatic embryos (type B) in Seyval blanc and friable, whitish-yellowish callus (type C) in Seyval blanc and Riesling; some of them also germinated and were discarded. On the other side, the Trollinger callus cultures behaved similarly during the whole experiment: They never regenerated shoots of somatic embryos. Tn summary, type A callus was typical for all three genotypes, types A_B and C for Seyval blanc and Riesling, and type B just for the former.

Four weeks after transfer to the regeneration conditions mentioned above, a further development of the somatic embryos, that compose the clusters of the B-type structures, was observed. At that moment it was possible to recognise, using a stereo microscope, the presence of torpedo-shaped embryos. However, those embryos did not develop further, as observed 4 weeks later. A similar phenomenon, but to a lesser extent, was noticed after transferring the A_B- and C-type callus cultures to the same regeneration conditions. None of the A-type callus cultures showed any sign of morphogenetic competence.

E n d o g e n o u s h o r m o n e l e v e l s i n t h e d i f f e r e n t c a l l u s l i n e s : The average endogenous hormone levels of the different callus types from the distinct genotypes are presented in Fig. 1. The A_B-type callus could not be analysed, because its amount tended to diminish with time of culture, through to its transformation into A- and B-callus, in detriment of its own growth. The highest TAA concentration was found in the A-type callus of Seyval blanc, and the lowest in the same callus type of the other two genotypes. Medium concentrations were obtained in the B- and C-type callus cultures of Seyval blanc and the C-type callus of Riesling. Higher ABA levels were found in B- and C-type callus cultures of Seyval blanc than in any other callus culture evaluated. Compared to the analysed material, no differences were found. The concentration of GAs in the Trollinger A-callus was almost twice as much.

Higher Z/ZR levels were found in B- and C-type callus cultures of Seyval blanc compared to A-type callus cultures of the same genotype and of Trollinger. Tntermediate values were obtained in both callus types of Riesling (not significantly different from any other type). For iP/iPA, an almost opposite behaviour to what was observed for Z/ZR was found: A-type callus cultures of Seyval blanc and Trollinger contained the highest levels of these hormones, compared to lower levels in the other callus lines. Considering the levels of all hormones analysed, no differences were found neither between the B- and C-type callus cultures of Seyval blanc, nor between both callus types in Riesling.

A n n u a l c h a n g e s i n t h e e n d o g e n o u s h o r m o n e l e v e l s i n t h e e m b r y o g e n i c l i n e : As mentioned above, the B-type callus of Seyval blanc was the only callus type with sufficient increase in growth to allow the collection of tissue for periodical evaluations of their endogenous hormone levels. These evaluations were conducted at different dates within almost two years (Fig. 2).

The concentration in TAA seemed to exhibit a cyclic behaviour, reaching maximum values in winter. Tn the second year, the increase in TAA started already in summer. The concentration of ABA behaved similar to that of TAA, but displaced by one or two months: Tn February 1997 a low initial ABA content was followed by a peak which then declined within the following 5-6 months. Finally, it increased again to a maximum in December 1997, before it started to decline again. Tn the case of GAs, no definite pattern could be detected; in most of the samples the level of GAs was very low and just above the detection limit. Only in September 1997 a maximum concentration was observed. The levels of the two classes of cytokinins in these samples were also low, showing a similar pattern for Z/ZR and iP/iPA concentration.

E f f e c t o f c h i l l i n g o n e n d o g e n o u s h o r m o n e c o n t e n t s : The endogenous hormone content in B-type callus cultures of Seyval blanc, either chilled at 4 °C for 4 weeks or cultured under normal conditions (26 °C) are presented in the Table. Except for ABA and TAA the endogenous levels of all other hormones were similar in the chilled and untreated samples. The chilling process caused a considerable decline in the ABA content and, although statistically not significant, a doubling in the TAA concentration in the tissues. When transferred to regeneration conditions, 100 % of the chilled callus cultures produced somatic embryos that developed further, compared to only 40 % in the untreated callus cultures.

Discussion

I n v i t r o r e s p o n s e : The genotypes used in these experiments were chosen because of their distinct *in vitro* behaviour under the culture conditions tested. Seyval blanc

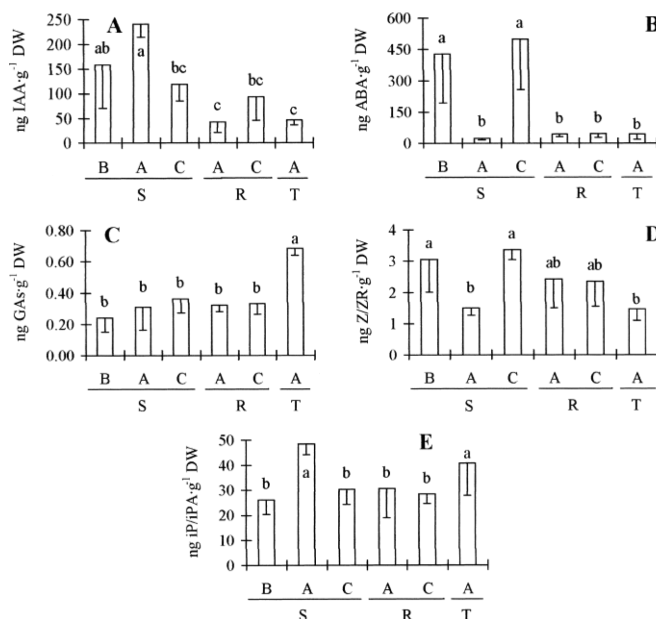

Fig. 1: Endogenous levels of IAA (**A**), ABA (**B**), GAs (**C**), Z/ZR (**D**) and iP/iPA (**E**) in the B-, A- and C-callus types of grapevine (S: Seyval blanc; R: Riesling and T: Trollinger). Significant differences (<0.05) are marked by distinct letters.

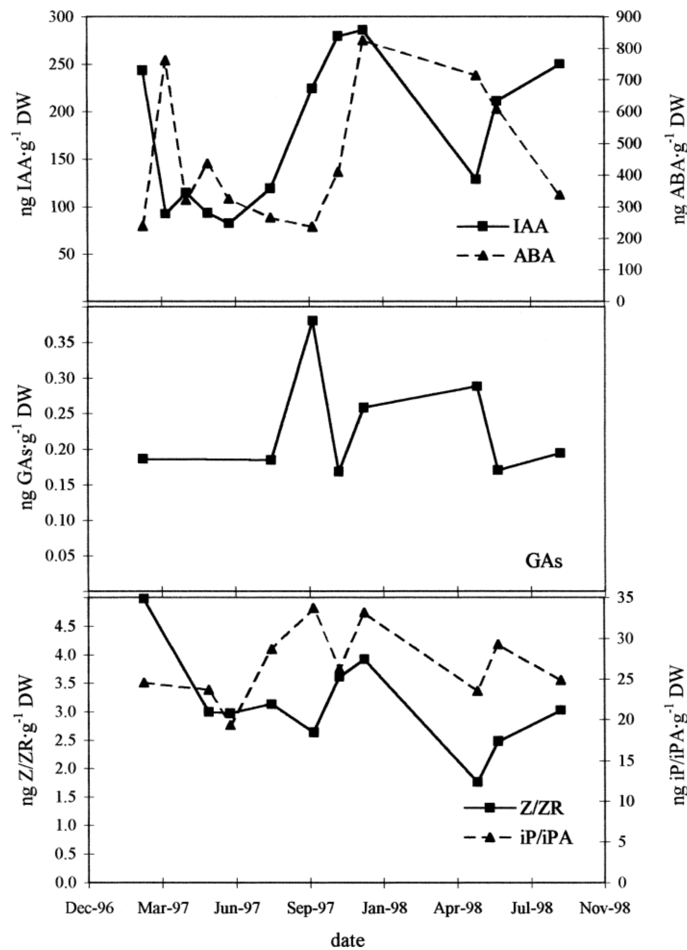

Fig. 2: Endogenous hormone levels of B-type calli (Seyval blanc), analysed at different dates during almost two years of culture.

Table

Endogenous hormone content (ng g^{-1} DW) of B-type callus of Seyval blanc kept at 4 °C for 4 weeks (chilling), or at 26 °C (control). Significant differences (<0.05) are marked by distinct letters

	IAA	ABA	GAs	Z/ZR	iP/iPA
26 °C	240 ± 30 a	398 ± 48 a	0.13 ± 0.01 a	2.44 ± 0.75 a	27.0 ± 0.0 a
4 °C	480 ± 108 a	251 ± 29 b	0.15 ± 0.01 a	2.89 ± 0.26 a	31.5 ± 3.8 a

and Riesling are known for their competence for somatic embryogenesis, and the former genotype also for its ability to form embryo clusters through secondary embryogenesis. On the other hand, Trollinger lacks this capacity and has never been reported to generate shoots or somatic embryos (G. M. REUSTLE and A. MATT, pers. comm.).

The main difference between Seyval blanc and Riesling genotypes was the inability of the latter to form B-type callus. Tn this genotype, the continuous growth of the somatic embryos, without a developmental arrest, led to the formation of abnormally germinated embryos, similar to what was observed in caraway (*Carum carvi* L.), soybean, alfalfa and grapevine (c.f. references in FAURE *et al.* 1998), and in consequence, impaired the development of B-type callus. This phenomenon can possibly be related to the endogenous hormone levels in the different genotypes (see below). The other extreme was found in Trollinger, whose callus did not show any sign of morphogenetic capacity. This kind of genotypic differences, regarding somatic embryogenesis, has been found for grapevine before (MAURO *et al.* 1986; MATSUTA and HIRABAYASHI 1989; GRAY 1992).

The constant removal of the somatic embryos and other structures formed on the surface of the A_B-type callus segments, led to a cease in the production of such structures

with the consequence of A-type callus establishment being unable to carry out any morphogenetic development when transferred to regeneration conditions. After the C-type callus cultures of both cultivars were transferred to regeneration conditions, different morphogenetic developments were observed: While in Seyval blanc some embryos were formed on the surface of the callus cultures which developed further, in Riesling root and shoot formation occurred. Tt is not clear if the latter is the result of an embryogenic or an organogenic process. Tt has been observed earlier that both embryogenic and organogenic modes of plant regeneration are commonly induced *via* somatic embryos (Popescu 1996).

Once transferred into regeneration conditions, the arrested somatic embryos present in the B- and A_B-type callus cultures of Seyval blanc developed further, but only up to the torpedo stage. Thereafter they formed abnormal structures or stopped growing. Faure and Aarrouf (1994) and Faure *et al.* (1998) also found a blockage in the further development of grapevine somatic embryos, once they reached the torpedo stage. Rajasekaran *et al.* (1982) assumed this developmental arrest to be a type of dormancy.

Endogenous hormone levels in the different callus lines: To the best of our knowledge, there is no previous report, in which the endogenous hormone levels in grapevine callus cultures varying in their degree of competence to conduct somatic embryogenesis were compared. Tn the present study the B- and C-type callus cultures of Seyval blanc had identical hormonal patterns. Even if both callus lines had distinct organisation patterns, as described above, they shared the fact, that when cultured under regeneration conditions, further development of somatic embryos occurred in both lines.

Tdentical hormone patterns were also found between both callus types in Riesling. However, in this case, the morphological and morphogenetic differences between them were more conspicuous. While the A-type callus cultures did not show any sign of morphogenesis, the appearance of roots and shoots was obvious in the C-type callus cultures. Since roots and shoots originated independently, and did not form part of the same axis, they were considered to be organogenetic in nature.

Endogenous TAA levels did not seem to be a very determinant indicator of embryogenic competence in the grapevine model system evaluated. Contrary to carrot and cereal systems described elsewhere (Vasil 1987; Komamine *et al.* 1992), in the case of grapevine, the competent callus lines are formed by somatic embryos inhibited in their development (Reustle 1989), and not by proembryonal or competent cells, that need to be induced to form somatic embryos (Komamine *et al.* 1992; Nuti Ronchi and Giorgetti 1995). At the time of hormone analysis, the critical step, from a proembryonal mass of cells (with radial symmetry), to an oblong embryo (with bilateral symmetry), in which TAA levels are determinant, had already passed.

TAA levels seem to be more dependent from genotype than related to the characteristics of the callus cultures (Fig. 1). Only Faure *et al.* (1998) found that in grapevine somatic embryos the TAA levels decreased sharply during the expression of somatic embryogenesis, due to an increase in weight and also to an absolute decrease of the TAA content. However, they did not evaluate endogenous hormone levels in incompetent tissues.

Endogenous ABA levels seemed to be more related to the embryogenic competence of the grapevine callus cultures, than any other hormone evaluated. Higher levels of endogenous ABA were typical of competent callus lines (B- and C-type Seyval blanc callus cultures). Since these callus lines originated from the multiplication of the whitish structures formed on the surface of A-type callus, their endogenous hormone levels should be representative for these structures. Tt was mentioned above that the inability of Riesling to form B-type callus cultures might be due to the fact that all somatic embryos, formed on the surface of the A-type callus, germinated instantly, not permitting their proliferation through secondary embryogenesis to form the B-type callus. Probably the ABA synthesised in the somatic embryos formed on the surface of the A-type callus cultures determines their further development. Tf concentrations are high, somatic embryos are inhibited to germinate precociously, and rather proliferate, probably through secondary embryogenesis. Tf, however, ABA concentrations are low, precocious germination occurs, impairing the establishment of a callus-type tissue. Faure *et al.* (1998) related low levels of endogenous ABA to high levels of precocious germination of grapevine somatic embryos, as compared to zygotic embryos, in which normally a quiescent period occurs previous to germination (Rock and Quatrano 1995).

Few can be concluded in relation to the role of GAs as an indicator of embryogenic competence from the results obtained. Since no differences in the GAs levels were observed among the various callus types in Seyval blanc and Riesling; the higher levels in Trollinger seem to be genotype dependent.

The influence of cytokinins seems to be more related to cell division than to the embryogenic process, as also proposed previously for *Pimpinella anisum* (Ernst *et al.* 1984). Those callus types with a higher growth rate (B and C, data not shown) contained higher levels of Z/ZR and lower ones of iP/iPA (Fig. 1). These results coincide with the characteristics of the different cytokinins were Z is considered the active cytokinin and iP its immediate non-active precursor (Kaminek *et al.* 1997; Strnad 1997).

The low growth rate and lack of competence of A-type callus cultures are reflected in the low endogenous levels of most hormones analysed. Exceptions are the TAA levels in Seyval blanc and GAs and iP/iPA levels in Trollinger. No explanation can be presented for the first case, and genotypic differences may be related to the latter.

Annual changes in the endogenous hormone levels in embryogenic lines: Endogenous hormone levels in B-type callus cultures of Seyval blanc were analysed during 19 months of culture under homogeneous maintenance conditions. Samplings were conducted always at the end of a 4-5 weeks culture cycle to avoid fluctuations in the endogenous hormone levels, caused by activation of growth after transfer to fresh medium. While the endogenous TAA and ABA contents followed a more or less cyclic pattern, those of GAs and

cytokinins were more irregular. PAASCH *et al.* (1997) reported variations in the endogenous hormone contents in carrot tissue cultures during the day, even if the cultures were maintained under constant conditions. Although seasonal variations of hormone concentrations in tissue culture to the best of our knowledge, have not yet been reported, their occurrence is not to be excluded. Using the same Seyval blanc B-type clusters, G. M. REUSTLE and A. MATT (pers. comm.) found seasonal changes in their ability to produce secondary embryos and to germinate and convert to plants. They observed peaks of maximum morphogenetic competence during winter which coincided with peaks of high TAA and ABA accumulation in the tissue determined in the present work (Fig. 2). Tt seems possible that the higher TAA levels impair the formation of an auxin gradient during the initial steps of embryogenic development. Establishment of such a gradient has been demonstrated to be a prerequisite for development of somatic embryos from competent carrot cells (COOKE *et al.* 1993). The ABA concentrations were higher in embryogenic callus lines when compared to non-embryogenic lines of callus cultures in carrot (KIYOSUE *et al.* 1992; JIMENEZ and BANGERTH, in press), *Pennisetum purpureum* (RAJASEKARAN *et al.* 1987 b) and sugarcane (GUIDERDONI *et al.* 1995). The role of ABA in somatic embryogenesis may be exerted through regulation of certain genes (*e.g.* DC8) that are thought to be involved in desiccation and maturation phases of embryogenesis (HATZOPOULOS *et al.* 1990). RAJASEKARAN *et al.* (1987 a, b) proposed that ABA can exert its role on somatic embryogenesis by regulating carbohydrate metabolism, *via* inhibiting a-amylase activity. There are several examples where applied ABA has been shown to stimulate cell division and DNA synthesis, callus production and shoot morphogenesis, and to inhibit peroxidase activity, a probable reason for TAA inactivation (RAJASEKARAN *et al.* 1987 a and references therein).

The lack of coincidence between peaks of maximal or minimal concentration of the other hormones, with seasonal changes in the embryogenic competence described above, together with the lack of a more detailed knowledge concerning the role of these hormones during the embryogenesis from somatic cells, makes it difficult to postulate a role for them in this phenomenon.

C h i l l i n g : Chilling is an effective practice used to increase the germination rate of grapevine somatic embryos (RAJASEKARAN *et al.* 1982). Tn the present experiment chilling treatment induced the further development of the inhibited somatic embryos of the B-type callus of Seyval blanc with a concomitant reduction in the ABA levels. RAJASEKARAN *et al.* (1982) reported, that in maturing grapevine somatic embryos, ABA accumulation might be involved in dormancy, and that chilling of these embryos lowered the ABA levels and induced germination. Tt has been reported that high levels of endogenous as well as applied ABA slows down the growth of somatic embryos and inhibits the precocious germination of grapevine somatic embryos (GOEBEL-TOURAND *et al.* 1993). FAURE *et al.* (1998) found that chilling of grape seeds resulted in a dramatic decrease in the ABA content of embryos, while the TAA levels remained unchanged. Tn the present work, no significant differences between treated and non-treated callus cultures were found in the endogenous levels of any of the other hormones (TAA, GAs, Z/ZR and iP/iPA). However, even if statistically not significant, the chilled samples contained twice as much endogenous TAA compared to those cultured under normal conditions. The role that endogenous TAA may play during dormancy breakdown has received little attention, compared to the more conspicuous effect of ABA and GAs. Tn a recent investigation, HERRERA *et al.* (in prep.) found that breaking dormancy in oil palm zygotic embryos by hydrogen cyanamide caused an increase in the endogenous TAA levels in the treated seeds.

TAKENO *et al.* (1983) analysed different GA-like substances in mature grape somatic embryos after one, two and 4 weeks of chilling at 4 °C. They observed that chilling produced a rapid increase in free GA-like substances one week after chilling, but did not find differences in total free GA-like substances during chilling. However, an increase in $GA_{1/3/20}$ was observed after 4 weeks when compared to the corresponding levels after one and two weeks.

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