

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 (IAA), abscisic acid (ABA), gibberellins A₁, A₃ and A₂₀, zeatin/zeatin riboside and N⁶(Δ²-isopentenyl) adenine/ N⁶(Δ²-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 IAA 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.

Key words: somatic embryogenesis, *in vitro* culture, phytohormones, radio-immunoassay, chilling.

Introduction

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; ROBACHER 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 (ROBACHER 1993). It 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.* ge-

netic 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 (IAA) (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 (IAA, ABA, gibberellins A₁, A₃ and A₂₀ (GAs), zeatin/zeatin riboside (Z/ZR) and N⁶(Δ²-isopentenyl) adenine/ N⁶(Δ²-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

In vitro culture of grapevine callus cultures: Callus cultures (kindly provided by Dr. G. M. REUSTLE and Dr. A. MATT, Fachgebiet Weinbau, Institut 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. In 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.

Sampling for hormone determination in the different callus types: The determination of IAA, ABA, GAs, Z/ZR and iP/iPA was always performed on the same 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.

Chilling: 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.

Extraction and purification of plant hormones: 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 µm; Schott, Mainz, Germany). To estimate the losses that can occur during purification an internal standard of 1-¹⁴C-IAA (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 C₁₈ Sep-Pak cartridge (reversed phase, Waters, Eschborn, Germany) (modified by BERTLING and BANGERTH 1995).

Quantification of hormones: Hormones were quantified by radio-immunoassay using polyclonal antibodies. Fractions containing IAA, ABA and GAs were methyl-

ated with diazomethane. Antibodies used were raised in rabbits against free IAA, free ABA, GA₃, ZR and iPA and radioimmunological hormone analysis was performed according to BOHNER and BANGERTH (1988). Cross reactions of the GA₃ antibody used were determined according to BERTLING and BANGERTH (1995), to be about 90 % with GA₁ and GA₂₀. Therefore, the GAs determined by means of this antibody are expressed as GA₃ 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 IAA, >92 % for ABA, >89 % for GAs and >80 % for both cytokinins. Therefore, the IAA internal standard was the only one used regularly and the IAA levels obtained were adjusted to the corresponding recovery value for each sample, due to the high variation.

Statistical analysis: Endogenous hormonal levels were determined in at least three biological replications and analysed using the STATISTICA for Windows (StatSoft Inc., Tulsa, Oklahoma, USA) Version 5.1 Student-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

In vitro response: The callus cultures of Seyval blanc and Riesling were characterized by their dark colour and by the spontaneous formation of whitish 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. In 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.

Endogenous hormone levels in the different callus lines: 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 IAA 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. Intermediate 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.

Annual changes in the endogenous hormone levels in the embryogenic line: 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 IAA seemed to exhibit a cyclic behaviour, reaching maximum values in winter. In the second year, the increase in IAA started already in summer. The concentration of ABA behaved similar to that of IAA, but displaced by one or two months: In 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. In 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.

Effect of chilling on endogenous hormone contents: 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 IAA 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 IAA 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

In vitro response: The genotypes used in these experiments were chosen because of their distinct *in vitro* behaviour under the culture conditions tested. Seyval blanc

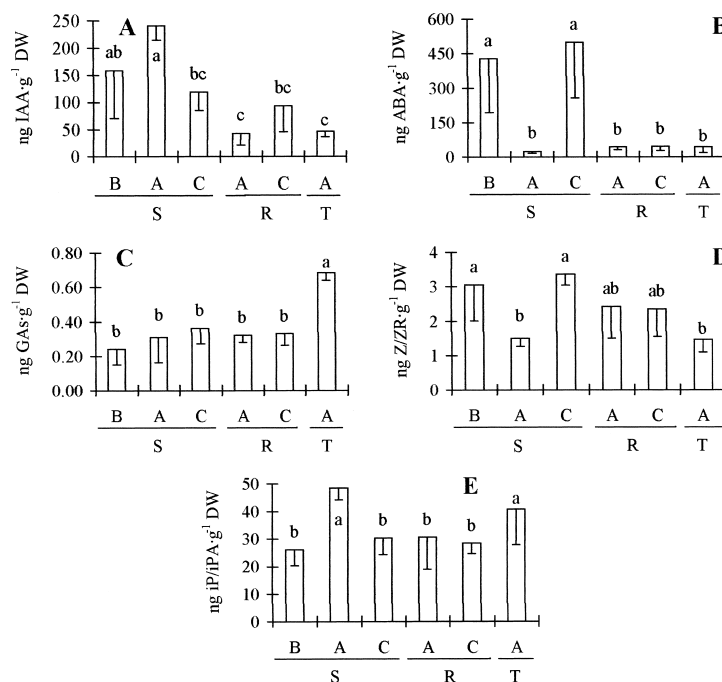


Fig. 1: Endogenous levels of IAA (A), ABA (B), GAs (C), Z/ZR (D) and iP/iPA (E) in the B-, A- and C-callus types of grapevine (S: Seyval blanc; R: Riesling and T: Trollinger). Significant differences (<0.05) are marked by distinct letters.

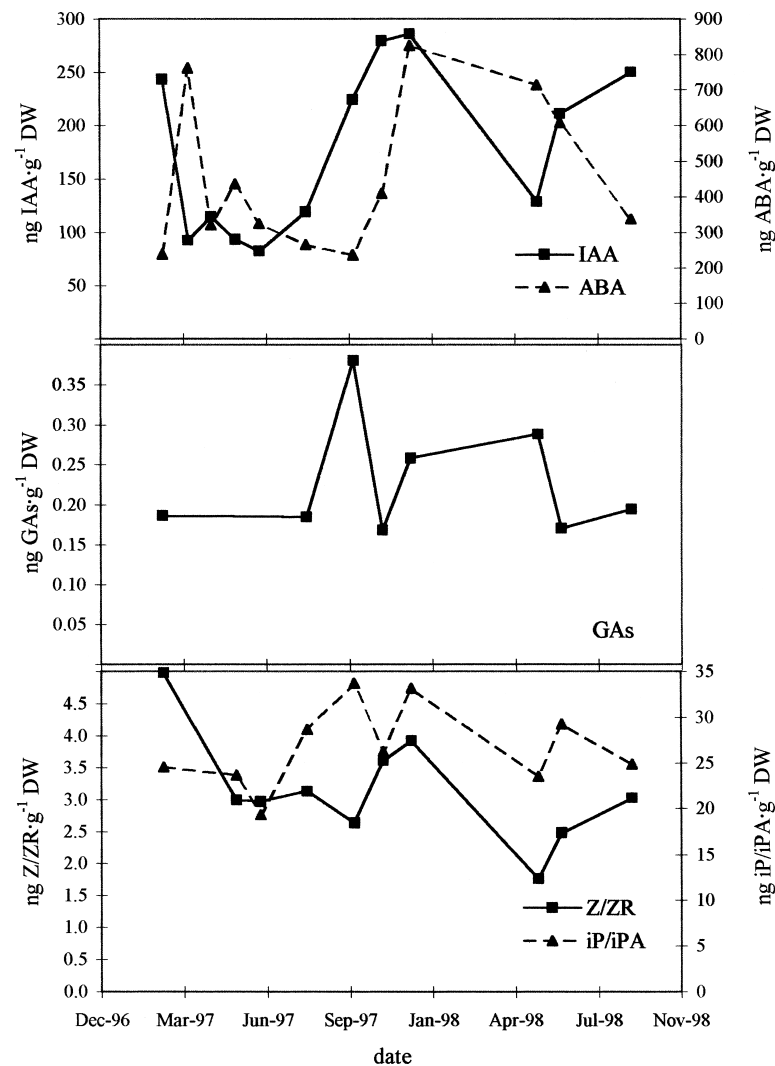


Fig. 2: Endogenous hormone levels of B-type calli (Seyval blanc), analysed at different dates during almost two years of culture.

T a b l e

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. In 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. It is not clear if the latter is the result of an embryogenic or an organogenic process. It 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. In 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.

Identical 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 IAA 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 IAA levels are determinant, had already passed.

IAA 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 IAA levels decreased sharply during the expression of somatic embryogenesis, due to an increase

in weight and also to an absolute decrease of the IAA 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. It 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. If concentrations are high, somatic embryos are inhibited to germinate precociously, and rather proliferate, probably through secondary embryogenesis. If, 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 where Z is considered the active cytokinin and iP its immediate non-active precursor (KAMÍNEK *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 IAA 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 IAA 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 IAA and ABA accumulation in the tissue determined in the present work (Fig. 2). It seems possible that the higher IAA 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; JIMÉNEZ 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 α -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 IAA 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.

Chilling: Chilling is an effective practice used to increase the germination rate of grapevine somatic embryos (RAJASEKARAN *et al.* 1982). In 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. It 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 IAA levels remained unchanged. In 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 (IAA, GAs, Z/ZR and iP/iPA). However, even if statistically not significant, the chilled samples contained twice as much endogenous IAA compared to those cultured under normal conditions. The role that endogenous IAA may play during dormancy breakdown has received little attention, compared to the more conspicuous effect of ABA and GAs. In 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 IAA 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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References

- BERTLING, I.; BANGERTH, F.; 1995: Changes in hormonal pattern of the new growth of *Sclerocarya birrea* after rejuvenation treatment with GA₃ and 'heading back'. *Gartenbauwissenschaft* **60**, 119-124.
- BOHNER, J.; BANGERTH, F.; 1988: Effects of fruit set sequence and defoliation on cell number, cell size, and hormone levels of tomato fruits (*Lycopersicon esculentum* Mill.) within a truss. *Plant Growth Regul.* **7**, 141-155.
- COLBY, S. M.; MEREDITH, C. P.; 1993: Transformation in grapevine (*Vitis* spp.). In: Y. P. S. BAJAJ (Ed.): *Plant Protoplasts and Genetic Engineering IV*. Biotechnol. Agric. For. **23**, 375-385. Springer Verlag, Berlin.
- COOKE, T. J.; RACUSEN, R. H.; COHEN, J. D.; 1993: The role of auxin in plant embryogenesis. *Plant Cell* **5**, 1494-1495.
- COUOTOS-THEVENOT, P.; GOEBEL-TOURAND, I.; MAURO, M. C.; JOUANNEAU, J. P.; BOULAY, M.; DELOIRE, A.; GUERN, J.; 1992 a: Somatic embryogenesis from grapevine cells. I. Improvement of embryo development by changes in culture conditions. *Plant Cell Tissue Organ Cult.* **29**, 125-133.
- ; MAES, O.; JOUENNE, T.; MAURO, M. C.; BOULAY, M.; DELOIRE, A.; GUERN, J.; 1992 b: Extracellular protein patterns of grapevine cell suspensions in embryogenic and non-embryogenic situations. *Plant Sci.* **86**, 137-145.
- EMERSHAD, R. I.; RAMMING, D. W.; 1994: Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). *Plant Cell Rep.* **14**, 6-12.
- ERNST, D.; OESTERHELT, D.; SCHAFFER, W.; 1984: Endogenous cytokinins during embryogenesis in an anise cell culture (*Pimpinella anisum* L.). *Planta* **161**, 240-245.
- FAURE, O.; AARROUF, J.; 1994: Metabolism of reserve products during development of somatic embryos and germination of zygotic embryos in grapevine. *Plant Sci.* **96**, 167-178.
- ; DEWITTE, W.; NOUGARÉDE, A.; VAN ONCKELEEN, H.; 1998: Precociously germinating somatic embryos of *Vitis vinifera* have lower ABA and IAA levels than their germinating zygotic counterparts. *Physiol. Plant.* **102**, 591-595.

- GOEBEL-TOURAND, I.; MAURO, M. C.; SOSSOUNTZOV, L.; MIGINIAC, E.; DELOIRE, A.; 1993: Arrest of somatic embryo development in grapevine: Histological characterization and the effect of ABA, BAP and zeatin in stimulating plantlet development. *Plant Cell Tissue Organ Cult.* **33**, 91-103.
- GRAY, D. J.; 1989: Effects of dehydration and exogenous growth regulators on dormancy, quiescence and germination of grape somatic embryos. *In Vitro Cell. Dev. Biol.* **25**, 1173-1178.
- -; 1992: Somatic embryogenesis and plant regeneration from immature zygotic embryos of muscadine grape (*Vitis rotundifolia*) cultivars. *Amer. J. Bot.* **79**, 542-546.
- GUIDERDONI, E.; MÉROT, B.; EKSOUMTRAMAGE, T.; PAULET, F.; FELDMANN, P.; GLASZMANN, J. C.; 1995: Somatic embryogenesis in sugarcane (*Saccharum* species). In: Y. P. S. BAJAJ (Ed.): *Somatic Embryogenesis and Synthetic Seed II*. Biotechnol. Agric. For. **31**, 92-113. Springer Verlag, Berlin.
- HARST, M.; 1995: Development of a regeneration protocol for high frequency somatic embryogenesis from explants of grapevines (*Vitis* spp.). *Vitis* **34**, 27-29.
- HATZPOULOS, P.; FONG, F.; SUNG, Z. R.; 1990: Abscisic acid regulation of DC8, a carrot embryogenic gene. *Plant Physiol.* **94**, 690-695.
- JIMENEZ, V. M.; BANGERTH, F.; (2000): Endogenous hormone levels in explants and in embryogenic and non-embryogenic cultures of carrot. *Physiol. Plant.* (in press).
- KAMÍNEK, M.; MOTYKA, V.; VANKOVÁ, R.; 1997: Regulation of cytokinin content in plant cells. *Physiol. Plant.* **101**, 689-700.
- KIYOSUE, T.; NAKAJIMA, M.; YAMAGUCHI, I.; SATOH, S.; KAMADA, H.; HARADA, H.; 1992: Endogenous levels of abscisic acid in embryogenic cells, non-embryogenic cells and somatic embryos of carrot (*Daucus carota* L.). *Biochem. Physiol. Pflanzen* **188**, 343-347.
- KOMAMINE, A.; KAWAHARA, R.; MATSUMOTO, M.; SUNABORI, S.; TOYA, T.; FUJIWARA, A.; TSUKUHARA, M.; SMITH, J.; ITO, M.; FUKUDA, H.; NOMURA, K.; FUJIMURA, T.; 1992: Mechanisms of somatic embryogenesis in cell cultures: Physiology, biochemistry, and molecular biology. *In Vitro Cell. Develop. Biol.* **28**, 11-14.
- LE GALL, O.; TORREGROSA, L.; DANGLOT, Y.; CANDRESSE, T.; BOUQUET, A.; 1994: *Agrobacterium*-mediated genetic transformation of grapevine somatic embryos and regeneration of transgenic plants expressing the coat protein of grapevine chrome mosaic nepovirus (GCMV). *Plant Sci.* **102**, 161-170.
- LINSMAIER, E. M.; SKOOG, F.; 1965: Organic growth factors requirements of tobacco tissue culture. *Physiol. Plant.* **18**, 120-127.
- MATSUTA, N.; HIRABAYASHI, T.; 1989: Embryogenic cell lines from somatic embryos of grape (*Vitis vinifera* L.). *Plant Cell Rep.* **7**, 684-687.
- MAURO, M. C.; NEF, C.; FALLOT, J.; 1986: Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. *Plant Cell Rep.* **5**, 377-380.
- MULLINS, M. G.; SRINIVASAN, C.; 1976: Somatic embryos and plantlets from an ancient clone of the grapevine (cv. Cabernet-Sauvignon) by apomixis *in vitro*. *J. Exp. Bot.* **27**, 1022-1030.
- -; TANG, F. C. A.; FACCIOTTI, D.; 1990: *Agrobacterium*-mediated genetic transformation of grapevines: Transgenic plants of *Vitis rupestris* Scheele and buds of *Vitis vinifera* L. *Bio/Technology* **8**, 1041-1045.
- NITSCH, J. P.; NITSCH, C.; 1969: Haploid plants from pollen grains. *Science* **163**, 85-87.
- NUTI RONCHI, V.; GIORGETTI, L.; 1995: The cell's commitment to somatic embryogenesis. In: Y. P. S. BAJAJ (Ed.): *Somatic Embryogenesis and Synthetic Seed I*. Biotechnol. Agric. For. **30**, 3-19. Springer Verlag, Berlin.
- PAASCH, K.; LEIN, C.; NESSIEM, M.; NEUMANN, K. H.; 1997: Changes in the concentration of some phytohormones in cultured root explants and in intact carrot plants (*Daucus carota* L.) during the day. *J. Appl. Bot.* **71**, 85-89.
- POPESCU, C. F.; 1996: Somatic embryogenesis and plant development from anther culture of *Vitis vinifera* (L.). *Plant Growth Regul.* **20**, 75-78.
- RAJASEKARAN, K.; HEIN, M. B.; DAVIS, G. C.; CARNES, M. G.; VASIL, I. K.; 1987 a: Endogenous growth regulators in leaves and tissue cultures of *Pennisetum purpureum* Schum. *J. Plant Physiol.* **130**, 12-25.
- -; - -; VASIL I. K.; 1987 b: Endogenous abscisic acid and indole-3-acetic acid and somatic embryogenesis in cultured leaf explants of *Pennisetum purpureum* Schum. *Plant Physiol.* **84**, 47-51.
- -; VINE, J.; MULLINS, M.G.; 1982: Dormancy in somatic embryos and seeds of *Vitis*: Changes in endogenous abscisic acid during embryogeny and germination. *Planta* **154**, 139-144.
- REUSTLE, G.; 1989: Untersuchungen zur Protoplastenkultur und zur somatischen Embryogenese bei Reben. Diss. Univ. Hohenheim. 112 p.
- -; HARST, M.; ALLEWELDT, G.; 1994: Regeneration of grapevine (*Vitis* sp.) protoplasts. *Vitis* **33**, 173-174.
- -; NATTER, I.; 1994: Effect of polyvinylpyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.). *Vitis* **33**, 117-121.
- ROBACKER, C.; 1993: Somatic embryogenesis and plant regeneration from Muscadine grape leaf explants. *HortScience* **28**, 53-55.
- ROCK, C. D.; QUATRANO, R. S.; 1995: The role of hormones during seed development. In: P. J. DAVIES (Ed.): *Plant Hormones*. Physiology, Biochemistry and Molecular Biology, 671-697. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- STAMP, J. A.; MEREDITH, C. P.; 1988: Proliferative somatic embryogenesis from zygotic embryos of grapevine. *J. Amer. Soc. Hort. Sci.* **113**, 941-945.
- STRNAD, M.; 1997: The aromatic cytokinins. *Physiol. Plant.* **101**, 674-688.
- TAKENO, K.; KOSHIOKA, M.; PHARIS, R. P.; RAJASEKARAN, K.; MULLINS, M. G.; 1983: Endogenous gibberellin-like substances in somatic embryos of grape (*Vitis vinifera* x *Vitis rupestris*) in relation to embryogenesis and the chilling requirement for subsequent development of mature embryos. *Plant Physiol.* **73**, 803-808.
- VASIL, I. K.; 1987: Developing cell and tissue culture systems for improvement of cereal and grass crops. *J. Plant Physiol.* **128**, 193-218.

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