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Growth enhancement of grapevine callus by catechin on auxin-free media

by

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Summary: Proliferating Spätburgunder cells cultured *in vitro* were found to synthesize the monomeric and oligomeric flavanols catechin, C2, B3, B1, B2 prodelphinidin, ECG (epicatechin gallate), B2G, B2-gallate and B5. Catechin and prodelphinidin were the predominating compounds of all calli studied. Growth promotion of calli from Spätburgunder and Romulus was studied using MS media containing benzyladenine (BA) alone (control), BA plus catechin, and BA combined with two different concentrations of indole-3-acetic acid (IAA). Growth on BA alone was notably reduced. Catechin (68 µM) stimulated callus growth several-fold when combined with 0.8 µM BA; however, this effect was only observed with explants excised from internodes of vigorously growing young shoots.

Key words: grapevine, in vitro culture, callus, flavanols, catechin, growth promotion.

Introduction

Phenols, such as stilbenes play a role in defence mechanisms against pathogens of grapevine (Hoos and Blaich 1990, Kindl 1994). In the *Vitis vinifera* cv. Spätburgunder flavanols were found to accumulate in newly dividing mesophyll cells surrounding the infection site (Feucht *et al.* 1996). Cell division near infection sites might depend on IAA produced either by fungi (Mayer 1989, Isaac 1992) or by the host tissue (Audus 1959). However, decarboxylation of IAA near infection sites has also been reported (Antonelly and Daly 1966). Under the chaotropic conditions of cellular degradation, inhibition by *o*-dihydroxyphenols of IAA oxidases as proposed by Stonier and Yang (1973) would be advantageous. The catechol group of phenols is active in preserving IAA, hence much work has been focused on caffeic acid in this respect (Krylov *et al.* 1994, Volpert *et al.* 1995).

In the past substantial progress has been made in determining the proanthocyanidin structures of grapevine (Ricardo da Silva *et al.* 1991). Catechin a basic unit of proanthocyanidins was found in various fruit crops to be involved in cell division (Feucht *et al.* 1992). This study deals with basic aspects of growth promotion of grapevine cells by catechin.

Material and methods

Tissue culture: Explants from Spätburgunder were excised from vigorously elongating shoots in spring and from mature shoots in June and July. Romulus (*V. vinifera*) shoots were only sampled in spring and handled identically. The sections, 1 mm long, were transferred to and maintained on liquid MS medium (Murashige and Skoog 1962) in test tubes (22 mm x 160 mm, pH 5.7) for 4 weeks. The tubes were sealed with glass caps. Macroelements were supplemented at half strength. IAA (indole-3-acetic acid; 1 $mg \cdot l^{-1}$ (5 µM) or 5 $mg \cdot l^{-1}$ (26 µM)), benzyladenine (BA; 0.2 $mg \cdot l^{-1}$ (0.8 µM)) and sucrose (4 %) were added. In several treatments catechin was added (20 $mg \cdot l^{-1}$ (68 µM)). All cultures were maintained at 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance and day/night temperatures of 26/20 °C provided by Grox Lux lamps.

Three explants each were placed into one test tube and 20 test tubes were used per treatment. Mean values of one treatment are based on a total of 60 callus pieces. The significance of differences between the calli of paired test tubes was tested by the student's t-test.

Separation and determination of flavanols: The freeze-dried material was extracted exhaustively with 80 % acetone. The combined extracts were evaporated *in vacuo* to dryness dissolved in methanol and an aliquot was used to determine the flavanols. The individual flavanols including the proanthocyanidins were determined by HPLC-CRD (Treutter 1989, Treutter *et al.* 1994).

Histology: The tissue was fixed in 2.5 % glutaraldehyde buffered by phosphat buffer (pH 7.5). After fixation, the material was dehydrated by alcohol. The tissue was then infiltrated with historesin. Sections (4 to 5 µm thick) were cut with a carbid knife on a sliding Leitz microtome. The sections were stained with acid *p*-dimethylaminocinnamaldehyde (DMACA) turning grapevine flavanols into reddish-blue (300 mg DMACA were dissolved in 100 ml n-butanol containing 3.5 ml conc. HCl). The sections were transferred in canada balsam, examined and photographed using a Zeiss Axioskop microscope (Gutmann and Feucht 1991).

Imbibition of tissue sections in proanthocyanidins: Tissue sections of Spätburgunder (5 µm thick) were placed on glass slides and dried at 40 °C;

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then they were imbibed for 2 h in a fraction of *Aesculus hippocastanum* containing different proanthocyanidins. The "Aesculus fraction" was washed off with water, the tissue was dried again and stained for flavanols with the DMACA reagent. Staining was ceased after 20 min and the excess DMACA was washed off with ethanol.

Results

Influence of explant source on callus growth: In spring transversal sections were excised from different plant tissues and cultured in the presence of IAA and BA (Fig. 1). After 4 weeks the internode segments were most responsive in growth while the leaf veins were least responsive. Explants from rapidly elongating tendrils showed rather low callus proliferation and the leaf petioles appeared to be intermediate between tendrils and internodes. Therefore, under the conditions of the medium used the kind of tissue has a strong effect on callus growth.

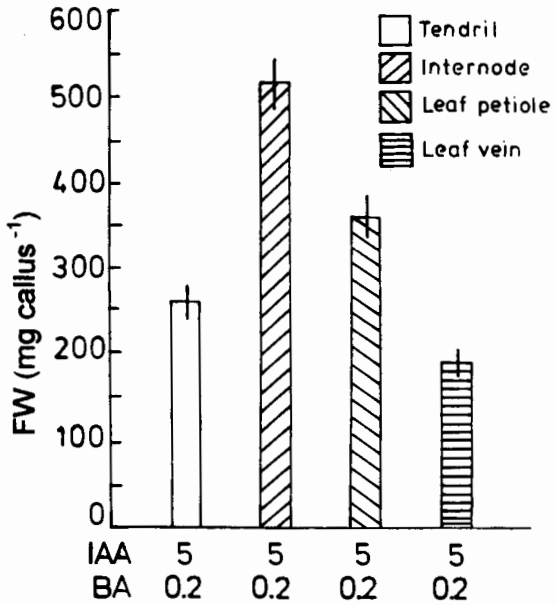

Fig. 1: Growth of callus (FW, fresh weight) of various grape organs (cv. Spätburgunder). Vertical bars represent ± SE (n = 60). IAA and BA in mg·l⁻¹.

Influence of catechin on callus growth: To study growth promotion by catechin tissue was excised from rapidly growing young shoot internodes. Catechin, when added together with IAA, was not effective in stimulating growth compared with IAA alone (data not shown).

Explants (Spätburgunder) sampled in early June and cultivated with BA yielded poor growth reaching 80 mg per callus (Fig. 2 A). However, BA in combination with 68 µM catechin enhanced the fresh weight (FW) increase 5-fold up to nearly 400 mg per callus. In the same set of experiments the addition of IAA (1 and 5 mg·l⁻¹) increased FW to roughly twice that of calli cultivated on BA together with catechin. Because of the enormous growth obtained with IAA after 4 weeks of culture we decided to maintain the cultures up to 10 weeks on the same medium. Thus, a final callus FW up to 800 mg was obtained in the presence of IAA.

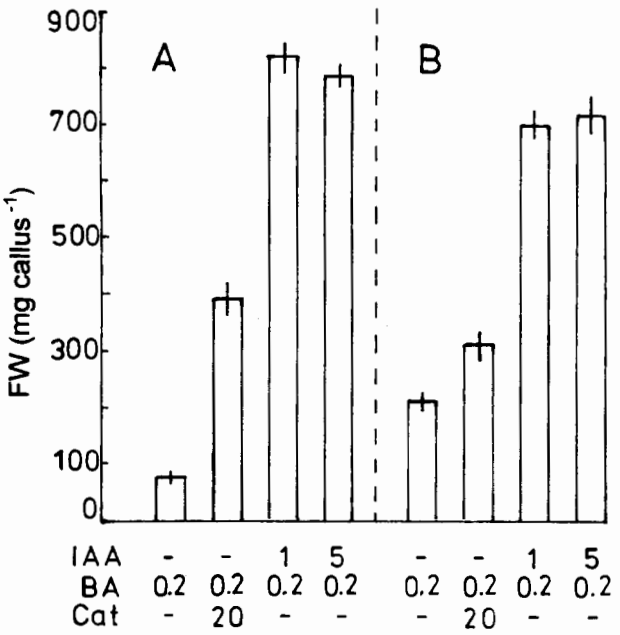

Fig. 2: **A**. Effects of catechin and IAA on callus growth (Spätburgunder) as compared with BA alone. Explants were sampled in spring. **B**. Explants (Spätburgunder) sampled in late July. The basic growth potential of the explants was high as shown on routine medium with IAA and BA. IAA, BA and catechin in mg·l⁻¹. Vertical bars represent ± SE (n = 60).

Repeating these experiments with original explants of Spätburgunder excised in late July showed the catechin effect to be not significantly different from the BA control (Fig. 2 B). Instead, both treatments with 5 and 26 µM IAA increased callus FW to about twice of that of BA together with catechin.

Subculturing of the calli of the Spätburgunder failed to show any response to added catechin as well (Fig. 3), de-

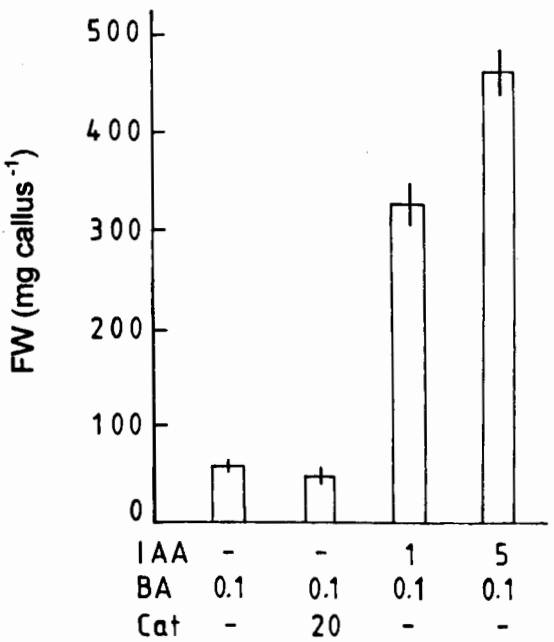

Fig. 3: Catechin without effects on growth of subcultured callus (Spätburgunder) despite a high growth potential on routine medium. Concentrations of IAA, BA and catechin: see Fig. 2. Vertical bars represent ± SE (n = 60).

spite the high growth potential of these subcultures as evidenced by cultivation on 26 µM IAA and even on 5 µM IAA. Summarizing, the regulation of callus growth by catechin is largely determined by the developmental stage at the time of explanting.

As a consequence of the foregoing experiments in early June only young elongating internodes were excised from terminal shoots of Romulus (Fig. 4 A). Overall, callus of Romulus grew less vigorously than that of Spätburgunder. Poor callus growth occurred with BA alone. When BA together with 68 µM catechin were added the rate of callus growth was nearly 6-fold that of BA alone. Both IAA concentrations behaved alike when combined with catechin but they were somewhat less effective in growth promotion than BA with catechin.

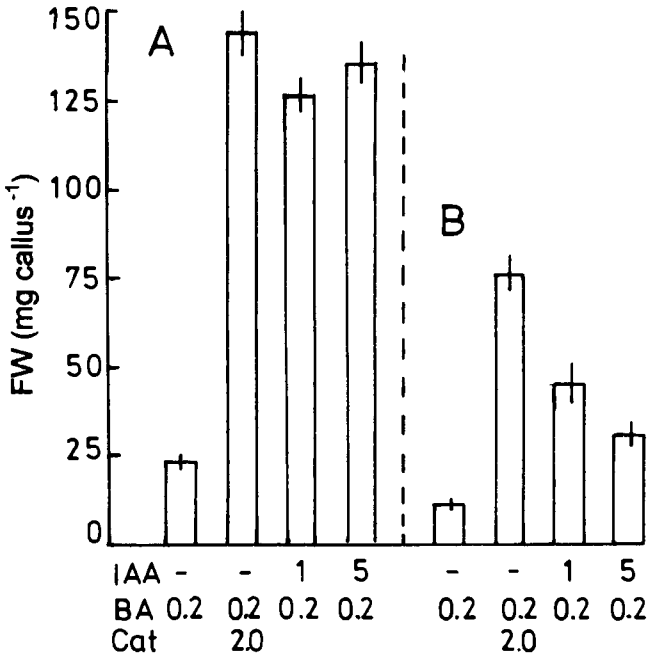

Fig. 4: **A.** Explants sampled from vigorously growing terminal shoots of Romulus. The callus growth rate between BA alone and BA combined with catechin was significantly different at the $P<0.001$ level. Data are means of two independent experiments. **B.** Explants sampled from moderately growing lateral shoots (Romulus). The growth rate between BA alone and BA combined with catechin was significantly different at the $P<0.001$ level. Vertical bars represent ± SE (A, n = 120; B, n = 60).

Two weeks later in June, these experiments were repeated using explants from axillary shoots which grew less vigorously than the main shoots (Fig. 4 B). This resulted in a generally restricted callus growth and a very poor growth response to BA alone. However, in accord with the foregoing experiments BA combined with catechin gave a striking roughly 6-fold growth promotion.

Structures and quantities of flavanols: A typical flavanol profile of Spätburgunder callus is shown in Fig. 5. The data presented in the Table correspond to the calli shown in Fig. 2 A. Tissues grown on BA alone had higher contents of B 2, prodelphinidin and B5 than the other three treatments showing vigorous growth. However, BA

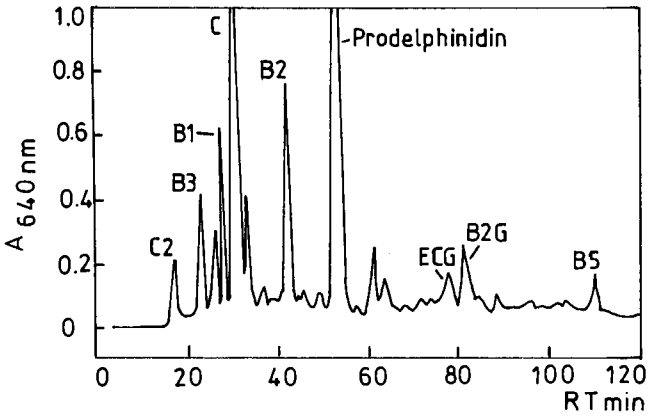

Fig. 5: Typical HPLC-CRD profile of flavanols from callus (Spätburgunder) based on a blue colour complex (A = 640 nm) with DMACA.

Table

The content of flavanols (mg·g^{-1} dry weight) of callus tissue (Spätburgunder) grown on different media (see Fig. 2 A)

Flavanol	BA	BA+ catechin	IAA 1 + BA	IAA 5 + BA
C2	0.23	0.39	0.39	0.44
B3	0.54	1.01	1.17	0.87
B1	0.63	1.81	1.20	1.28
C, catechin	5.01	6.98	6.10	5.72
B2	0.88	0.50	0.29	0.33
Prodelphinidin	6.68	3.94	3.69	3.78
Epicatechin-gallate (ECG)	0.26	0.19	0.29	0.23
B2G, B2-gallate	0.43	1.21	1.50	1.43
B5	0.20	0.06	0.04	0.02

alone had lower contents of B1 and B2-gallate than the other three treatments. Thus, callus growth rate is not generally related to alterations of the content of individual flavanols.

Structure of flavanol deposits in growing and non-growing tissues: Flavanols of Spätburgunder callus were deposited in rapidly growing tissues at the cellular periphery in rather small inclusions or globules (arrow), about 1 to 2 µm in diameter (Fig. 6 A). After imbibing of the callus in ABA these deposits disappeared and the flavanols leached away towards the cell walls (Fig. 6 B). They also occurred in the intercellular spaces (arrow). Some of the flavanols were dispersed within the disorganized cell.

Sections from wounded shoot internodes (Fig. 6 C) revealed a mixture of both foregoing figures, i.e. the globular type around the wound where cell division occurs and the extracellular deposition within the senescing wound area (asterisk).

Addition of oligomeric *Aesculus* flavanols to lesioned berry tissues of Romulus (Fig. 6 D) confirmed a strong flavanol binding capacity of the cell walls, the amorphous masses extruding toward the wound surface (asterisk). The cell walls of two suberizing cell layers failed to stain with DMACA (arrows). The Romulus berries rarely contain flavanol globules.

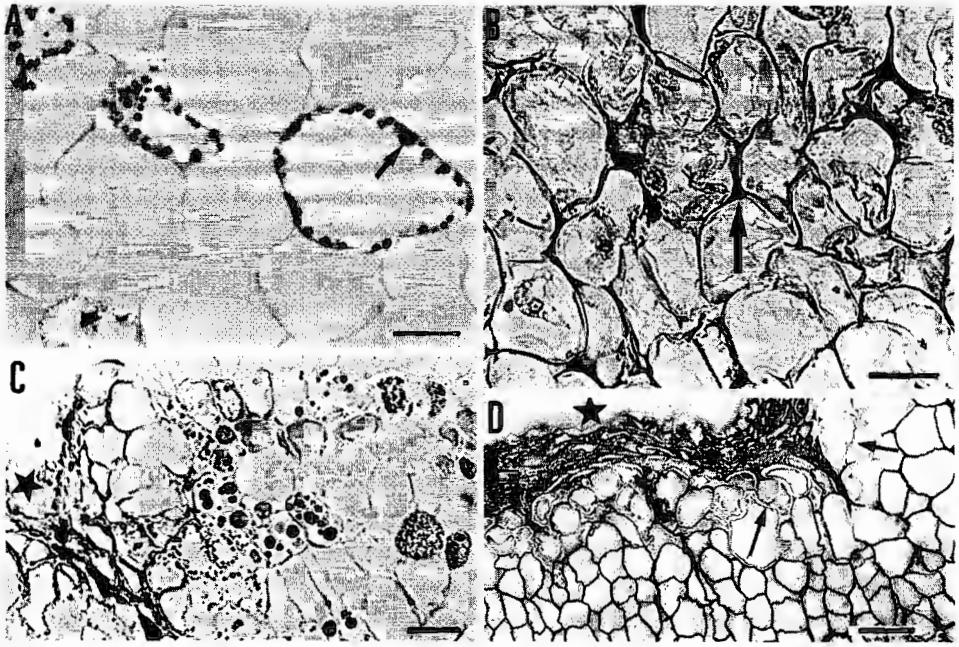

Fig. 6: Tissue sections of Spätburgunder. **A**. Callus tissue with small flavanol globules located at the cellular periphery inside the tonoplast (arrow). **B**. Callus treated with ABA showing damaged cellular structures and leaching of flavanols towards cell walls and into intercellular spaces (arrow). **C**. Cross section of a wounded shoot internode with flavanol globule and necrotic material (asterisk). **D**. Wound site of a Romulus berry treated with *Aesculus hippocastanum* extract. Outside the wound surface the extruded material stained dark blue because of complexation with added proanthocyanidins (asterisk). Arrows indicate two suberized phellem layers lacking the ability of complexing with proanthocyanidins. Cell walls of the underlying parenchyma showed a high affinity for added flavanols. Bars: 25 µm.

Discussion

Cell division and the formation of phenols have been described for wounded sites of woody species (ACHOR *et al.* 1991, ADASKAVEG 1992, FEUCHT *et al.* 1993). Fungal infection changes the permeability of host cell membranes (BECKMAN 1966) and flavanols at physiological levels diffusing out of cells (FEUCHT *et al.* 1997) may be considered to act as antioxidant shields (ELSTNER *et al.* 1994). ABA which increased after fungal infection (TAYLOR *et al.* 1996) might enforce the flavanol inclusions of the grapevine to dissolve whereby the flavanols are extruded to the cellular periphery.

Experimentally, cell walls of grape showed a notable binding affinity to flavanols and might constitute a final flavanol deposit of senescing cells.

However, the most exciting fact is that catechin is a substance capable to promote cell division of callus on growth media free of IAA. Plant systems showing an increased peroxidase activity in wounded and infected tissues had an IAA deficiency (FARKAS and STAHMANN 1966, HAMMERSCHMIDT *et al.* 1982). Antioxidants like catechin or other flavanols may compensate such a deficiency.

In several fruit tree species catechin was found to increase the growth effect of IAA (FEUCHT and SCHMID 1980, FEUCHT and TREUTTER 1996). LAVEE *et al.* (1994) working with olive tissues were the first to demonstrate that chlorogenic acid is capable to induce growth in IAA-free media. More recently, an outstanding effect of catechin in inhibiting IAA decomposition of fungus-infected fruit of *Prunus domestica* was served by FUCHS and SPITELLER (1997). In these experiments, catechin was found to be 20- to 100-fold more efficient in preventing IAA oxidation than chlorogenic acid. Oxygen radicals destroying IAA (PARUPS 1984) were scavenged by catechins (TOREL *et al.* 1986) and a number of other phenols (ELSTNER *et al.* 1994).

On IAA-free media such mechanisms may preserve the endogenous IAA molecules of the explant itself. At present, however, it is not known why BA combined with catechin produces the great long-term growth response of grapevine callus compared to BA alone.

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