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Flavanols in grapevine: *in vitro* accumulation and defence reactions in shoots

by

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S u m m a r y : Callus of two grapevine cultivars was cultivated for 4 weeks on MS-medium, half strength, which was additionally supplemented with abscisic acid (ABA), (+)-catechin or a combination of both. (+)-Catechin did not elevate the pool size of the callus flavanols. Application of 3 % sucrose caused a significant increase of the callus flavanols, whereas the response to ABA was quite variable. Leaves and shoots of cv. Spätburgunder (Pinot noir) were examined histologically on the mode of flavanol deposition. The flavanols of the leaf mesophyll were spread evenly throughout the central vacuole whereas those of the outer shoot cortex were confined to special intravacuolar globules. The amorphous leaf flavanols were converted into globular ones in the vicinity of fungal infections. Apparently, the central vacuole acquires special adjustment under the influence of infection stress. When paraquat was applied to the shoots, amorphous material was attached towards the cell walls. Additionally, the globular flavanols disappeared which could be reversed by addition of (+)-catechin. Thus, (+)-catechin diminished the oxidative damage caused by the oxygen radical producing herbicide. (+)-Catechin and epicatechin are the dominant flavanols of the leaves, whereas the dimeric proanthocyanidins B3 and B1 (PAs) predominate in the callus.

K e y w o r d s : tissue culture, flavanols, proanthocyanidins, fungal infection, vacuoles, globules, histology.

Introduction

Shoots and berries of grapevine are known to contain a wide range of different polyphenols. Synthesis and localization of these phenols appear to be primarily associated with resistance against diseases (Hoos and Blaich 1990; Scalbert 1991).

Among the phenols, the oligomeric flavanol group is of unique biological importance by virtue of its complexing and enzyme inactivating properties (Hagerman and Butler 1981, Haslam 1994). These phenols having a catechol group were shown to be prominent compounds of grapevines (Lee and Jaworski 1987, Ricardo da Silva *et al.* 1992, Santos-Buelga *et al.* 1995).

In a number of tree crops maximal flavanol concentrations occurred in the outer protective layers of the fruits (Feucht *et al.* 1994). There appears to be a parallel with the flavanols deposited in the exocarp and seed coat of grape berries (Amrani Joutei *et al.* 1994; Escribano Bailon *et al.* 1995). These features advocate for a special protection by flavanols of functionally important tissues. However, specific cytological events, as for example lignification (Edwards 1992), and developmental allocation patterns (Forrest and Bendall 1969, Stafford *et al.* 1989) may also interfere in synthesis and deposition of flavanols.

For an understanding of host-parasite interactions and the role of flavanols therein more basic knowledge is needed. In the present paper, *in vitro* experiments on possible regulatory factors of the accumulation of flavanols in callus are described. Further emphasis is laid on the mode of flavanol deposition in the highly differentiated leaves and shoots and the changes produced by fungal infection.

Materials and methods

C a l l u s c u l t i v a t i o n *i n v i t r o* : Explants of leaf petioles and young shoot internodes from the cultivars Dornfelder and Riesling were excised and cultivated in 22 x 160 mm culture tubes containing 10 ml liquid MS-medium, half strength. The standard medium contained 8.5 µM IAA and 1.6 µM BA. Special manipulations with different concentrations of hormones and sucrose are given in the Table. In 4 experiments, 68 µM (+)-catechin was added alone or in combination with 20 or 40 µM ABA. Sixty callus pieces distributed on 20 culture tubes were used for one treatment. The tissues were placed for 4 weeks in a growth room with a 16 h light period (long day, 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

P a r a q u a t t r e a t m e n t : Paraquat (methylviologen, 10 mg/l) was used for imbibing 2 mm long shoot discs of young elongating internodes of Spätburgunder (Pinot noir). The imbibition was performed for exactly 3 d under long day condition. Controls were treated with distilled water. The experiments were repeated 4 times.

D e t e r m i n a t i o n o f f l a v a n o l s : Extraction of 100 mg freeze-dried material was performed with acetone/H_2O (80/20, v/v). Separation, identification and quantification of the diverse flavanols were based on the methods of Treutter *et al.* (1994) and on cochromatography with authentic samples from grape seeds (Santos-Buelga *et al.* 1995). The reagent used for post-column derivatization was *p*-dimethylaminocinnamaldehyde (DMACA).

Total soluble flavanols from callus material were determined colorimetrically by use of DMACA (Feucht and

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SCHMID 1983). An aliquot of 0.5 ml was mixed with 2 ml of the reagent and the blue colour complex of the test solution was estimated at 640 nm after 25 min in an Uvikon photometer and calculated as (+)-catechin. The soluble proanthocyanidins (PAs) were estimated from an aliquot of the same acetone extract by boiling for 20 min in butanol/HCl (5/1, v/v). The bound proanthocyanidins were determined from the insoluble residue employing the same boiling procedure. Both, the soluble and insoluble compounds were estimated at 550 nm and calculated as cyanidin.

Histochemistry: Small tissue sections were excised from leaves and shoots of Spätburgunder vines at several dates during the growing period. The samples were fixed in 2.5 % glutaraldehyde, dehydrated, embedded and stained with DMACA or with toluidine blue O as described by GUTMANN and FEUCHT (1991). The tissues were sectioned at 5 µm using a sliding microtome (Leitz 1400) equipped with a tungsten carbid knife.

Table

Effects of hormones, sucrose and (+)-catechin (Cat) on the content of flavanols (calculated in mg (+)-catechin/g DW) and PAs (calculated in mg cyanidin/g DW) of callus. The values for callus FW are the means of 60 callus pieces from 4 repetitions

Treatment	Total flavanols	Soluble PAs	Insoluble PAs	Callus mg FW*)
Riesling				
Control	3.6	2.6	3.0	88 a
ABA 40 µM	5.4	2.7	2.7	265 b
Cat	3.1	2.4	2.6	115 a
ABA + Cat	3.8	2.4	2.3	262 b
Sucrose 1 %	0.2	0.7	1.3	86 a
" 2 %	3.5	3.2	4.2	135 b
" 3 %	3.9	3.9	4.1	302 b
" 4 %	0.8	1.2	2.2	158 b
BAP 0.8 µM	2.4	2.6	4.4	132 a
" 1.6 µM	2.4	2.5	4.0	95 a
" 2.4 µM	5.2	3.5	4.7	91 a
" 3.2 µM	2.7	2.4	3.3	67 b
Dornfelder				
Control	14.4	8.2	4.7	144 a
ABA 20 µM	11.0	6.6	5.6	156 a
Cat	8.5	5.8	6.2	178 a
ABA + Cat	6.8	3.4	5.3	138 a
Control	12.7	10.2	6.9	91 a
ABA 20 µM	20.3	15.3	5.9	53 b
Cat	16.4	13.6	7.3	98 a
ABA + Cat	20.0	14.5	6.7	57 a
Control	9.9	5.0	3.3	170 a
ABA 20 µM	4.0	2.0	2.9	60 b
Cat	8.5	4.2	4.1	171 a
ABA + Cat	12.5	6.3	5.3	209 a

*) means followed by the same letter are not significantly different at $P < 5$ %.

Results

Variability of callus cultures: Callus cultures were performed with the cvs Riesling and Dornfelder (Table). Generally, Riesling showed lower amounts of flavanols and soluble PAs as compared with Dornfelder. Thus, the genotype may play a role in flavanol synthesis. Addition of (+)-catechin did not influence the pool size of the 3 flavanol fractions in both cultivars and the effects of ABA varied greatly. When ABA and (+)-catechin were given together the results were likewise inconsistent. Sucrose at 2 % and 3 % elevated notably the total flavanols as well as the soluble and insoluble PAs.

The great variability in accumulation of flavanols points to dynamic and transient metabolic changes occurring during cultivation. Grapevine callus is apparently critically unbalanced with regards to storage and conversion of flavanols.

A comparison of the effects of the media on callus growth is likewise given in the Table. FW increment of callus differed greatly with respect to responsiveness to ABA, BA and sucrose. In the case of Riesling ABA given singly resulted in a three-fold increase of FW compared to control. This callus was extremely watery (DW was 2.4 % of FW compared to 5.0 % in controls). Application of (+)-catechin in the same experiment elicited no significant increase in FW. However, combining ABA and (+)-catechin resulted again in significant growth promotion. Riesling tissue showed likewise significant growth

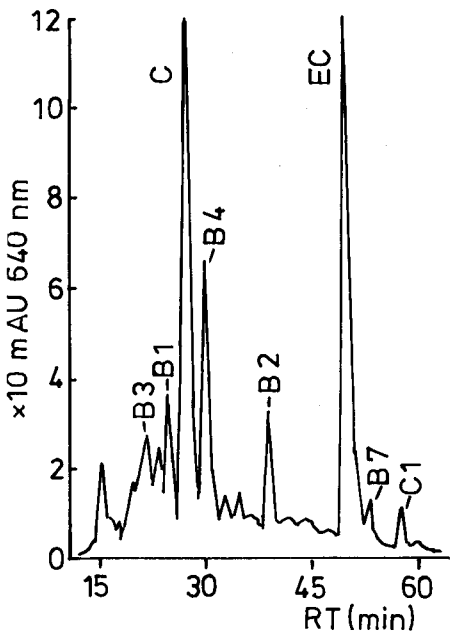

Fig. 1: HPLC-CRD profile of flavanols from leaves (Spätburgunder, Pinot noir) detected at 640 nm and expressed in absorption units (AU). C, (+)-catechin; EC, epicatechin.

promotion at sucrose levels up to 3 %. BA did tentatively reduce FW at 1.6 and 2.4 µM. 3.2 µM BA reduced growth to approximately one-half of that of 0.8 µM BA.

In the case of Dornfelder ABA reduced FW in two of the 3 experiments by 42 % and 65 %, respectively. (+)-Catechin, alone or combined with ABA, dit not affect FW. The qualitative pattern of leaf flavanols as evaluated by HPLC-CRD is shown in Fig. 1. The compounds B3, B1, (+)-catechin (C), B4, B2, epicatechin (EC), B7 and C1 were detected in Spätburgunder. Some minor peaks have not yet been identified.

Effects of sucrose on flavanol content: Riesling callus cultures were treated with sucrose at 4 different levels (Fig. 2). Already at day 4, after starting the treatment, 2, 4 and 6 % sucrose increased flavanols to about two times that of 1 % (control). By day 8, during the early exponential growth phase, both 2 and 3 % sucrose increased the flavanols for over 600 % compared with day 4. However, 1 and 6 % were less effective. By day 12 a further significant sucrose response on flavanols was observed at 2 and 3 %, whereas 1 and 6 % sucrose were almost equal to day 8.

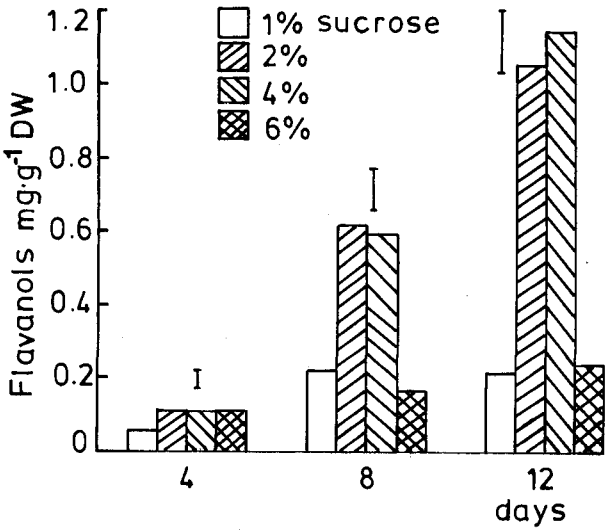

Fig. 2: Effect of sucrose on levels of total flavanols after 4, 8 and 12 d of tissue culture. Vertical bars, GD 5 %.

Flavanol pattern of callus tissue: The qualitative pattern of the callus (Spätburgunder) flavanols was not completely identical with that of the leaves. In the callus B4, B7 and C1 were not present and 2 peaks, Pa and Pb, have not yet been identified (Fig. 3). As to the relative levels, B1 was dominating in the callus, whereas in the leaves it was rather a minor compound. Reversely, in the leaves the content of epicatechin was lower, but in the callus it was dominating. Comparing the effects of 1 % and 3 % sucrose on the content of the individual flavanols the highly significant accumulation found at the higher sugar level was striking.

Change of flavanol deposition near fungal infections: Staining with DMACA gave a distinct bluish colour characteristic of flavanols. The healthy leaf sector of Spätburgunder thus showed an over-

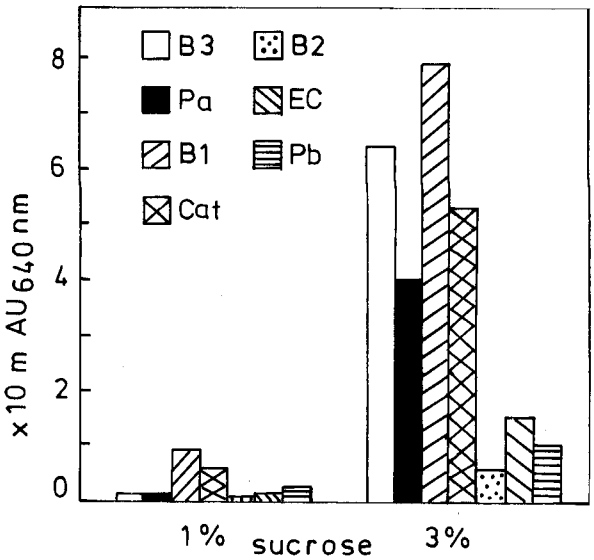

Fig. 3: Qualitative pattern of callus flavanols and effects of 1 % and 3 % sucrose on the amounts of the individual flavanols. AU; B1, B2, B3, EC: see Fig. 1; Cat: (+)-catechin; Pa, Pb: compounds not identified. Values are means of 2 repetitions.

all flavanol staining of the entire palisade cells except for the plasmic centre (Fig. 4 A). Some bundle sheath cells and several spongy parenchyma cells stained additionally, however with decreasing intensity towards the lower epidermis.

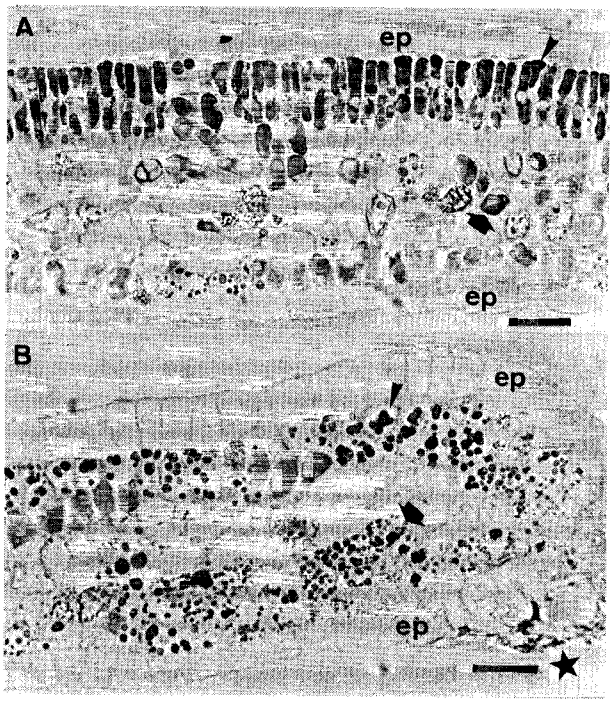

Fig. 4: Staining with DMACA. **A.** Healthy leaf section showing deep flavanol staining in the upper palisade cells (arrowhead) and to a lesser extend in the spongy parenchyma cells (arrow). Each palisade cell contains 2 elongated large vacuoles which are mostly separated by non-staining plasmic material. Both epidermal layers (ep) were without flavanols. **B.** Infected leaf sector indicating numerous flavanol globules in both palisade cells (arrowhead) and spongy parenchyma cells (arrow). In this way the flavanols were compartmented away from the vacuolar sap. The infection site is indicated by the asterisk. Bars = 50 µm.

The healthy pattern was drastically changed near a fungal infection caused by *Phomopsis viticola* (asterisk, Fig. 4 B). The elongate palisade cells had divided periclinally, the upper halves showing a dramatic vacuolization along with forming several flavanol globules therein. This pattern of dedifferentiation was intensified towards the necrotic margin and was likewise found to be pronounced in the spongy parenchyma cells.

Toluidine staining of the same leaf sector has the advantage to clearly reveal the cell wall structures (Fig. 5). However, this non-specific stain gives greenish phenol/flavanol colouration which is difficult to distinguish from the plasmic material giving a grey staining.

In accordance with DMACA staining the palisade cells showed large central vacuoles being enriched with phenols (Fig. 5 A). About 250 µm away from the necrotic margin the palisade cells changed their normal status (arrowhead) by attaining competence for cell division, as was already shown by DMACA. By a periclinal plane of division, two equal-sized cells arose from one palisade cell (Fig. 5 B, arrowhead). However, halving the volume led to an unequal qualitative pattern of the daughter cells. The upper cell abandoned the homogenous flavanol structure to form 4 discrete flavanol inclusions within the main vacuole. The lower daughter cell continued to display the original amorphous flavanol deposition besides less densely staining plasma and a prominent nucleus. As many as 4 neighbouring palisade cells showed the same synchrony of a periclinal division pattern (right from the arrowhead). Advancing towards the infection site, cellular enlargement, vacuolization and formation of flavanol globules proceeded more intensely (Fig. 5 C).

Along with the dedifferentiation into parenchymatic cells a resumption of meristematic activity and formation of cambium-like cell layers took place (arrowhead) which in turn were rapidly transformed to giant cells beneath the necrotic boundary (Fig. 5 C).

Reaction of cortical tissue to paraquat- and wound stress: The subepidermal layer of the shoot cortex contained regularly large flavanol inclusions (Fig. 6 A, toluidine staining). Beneath the subepidermis they were less frequent, however, they increased again towards the inner cortex.

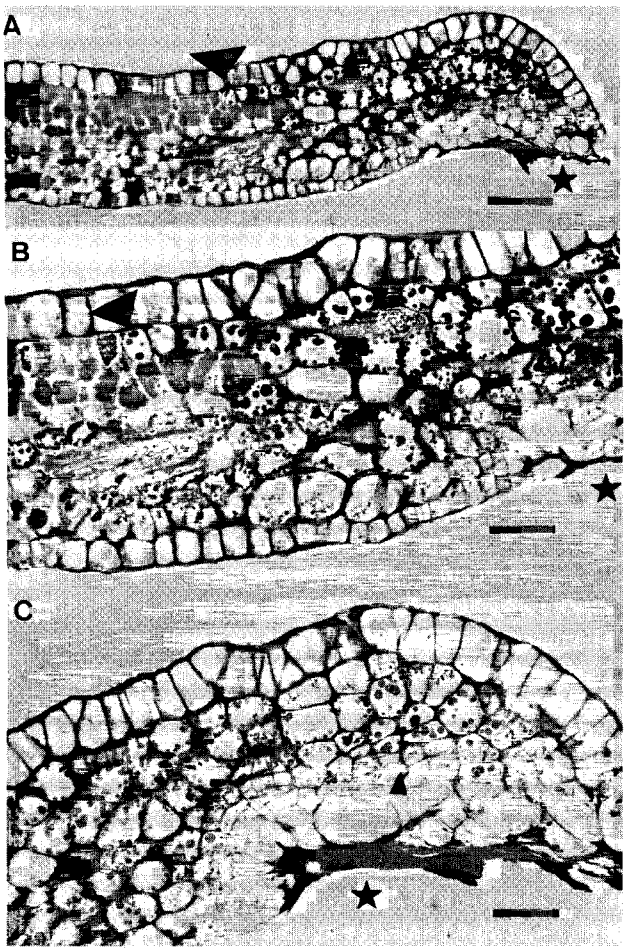

Fig. 5: Staining with toluidine. All three leaf sections were sampled near the infection site. **A.** About 250 µm away from the infection site (asterisk) the palisade cells showed anticlinal divisions (arrowhead). **B.** (Enlargement of A). The flavanol globules appeared as clearly defined ball-shaped structures in the upper daugther cells of the dividing palisade cells (arrowhead) and in the spongy parenchyma. The cell walls were dense in appearance. **C.** Above the necrotic infection site (asterisk) a meristem was formed with ordered periclinal divisions (arrowhead). The newly formed cells released from the meristem showed a notable surface expansion. Bars: A = 100 µm, B and C = 50 µm.

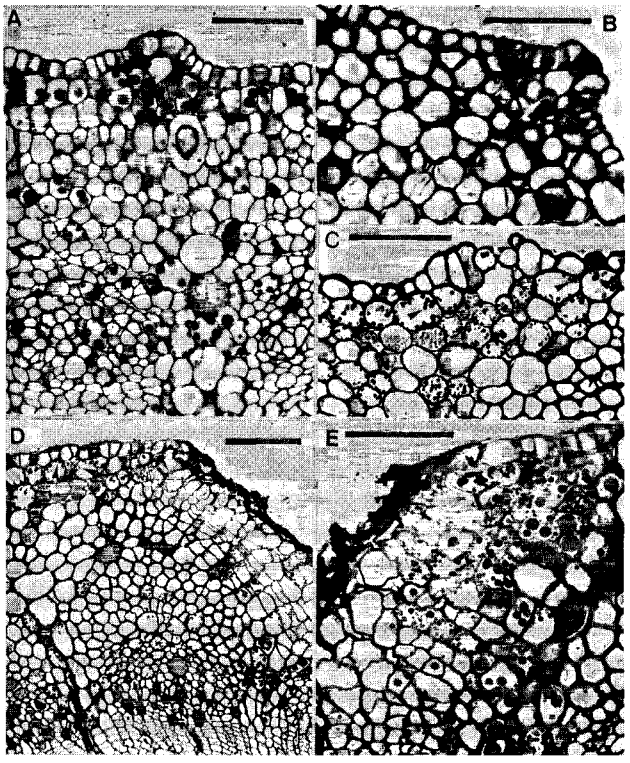

Fig. 6: Staining of shoot sections with toluidine. **A.** Controls with normal-sized flavanol globules especially around a developing lenticell and in the phloem. **B.** Treatment with paraquat resulting in a loss of the globules around the lenticell. **C.** Combined treatment with paraquat and (+)-catechin resulting in formation of rather small flavanol globules (arrowheads). **D.** Infected shoot section showing a cambium-like meristem and small flavanol globules nearby. Again, small-sized globules were formed (arrowheads). **E.** Infected shoot section showing dividing parenchyma cells (asterisks). The larger globules are more distant from the wound surface. Bars = 100 µm.

In shoots of Spätburgunder especially the subepidermal layer contains flavanol globules as a typical pattern. Around the developing lenticels, 2 or 3 subepidermal cell layers showed large vacuoles with some flavanol globules therein. These were dissolved by treatment with paraquat and non-flavanolic depositions became appressed to the cell walls (Fig. 6 B). This deleterious paraquat effect was largely overcome by (+)-catechin (Fig. 6 C). However, the resulting inclusions were not as large as in the controls.

Wounding injury (fungal destruction) resulted likewise in the formation of the small-sized flavanol globules just beneath the newly formed wound cambium (Fig. 6 D). Occasionally, also the large-size type was formed at other sites of the wound (Fig. 6 E). There was no cambium-like tissue but parenchyma cells were in division. The large globules were located near the thick-walled collenchyma, while the smaller ones were found in the vicinity of the necrotic border zone.

Discussion

(+)-Catechins and proanthocyanidins, being key constituents of grapevine berries, mainly occur in the outer protective layers of both fruit skin and seeds (Amrani Joutei *et al.* 1994), in addition to a number of flavonolglycosides (Moskowitz and Hrazdina 1981). Cells of two fungus-resistant *Vitis* species showed a more rapid accumulation of flavonoids than those of a susceptible species (Dai *et al.* 1995). High levels of PAs were found in fungus-resistant grapevine cultivars by Bachmann and Blaich (1979).

Callus from excised leaf petioles has been utilized to study possible effects of external (+)-catechin on the endogenous pool size of flavanols and PAs. A promotive role of (+)-catechin in establishing rich PA pools could not be verified. Carbon atoms deriving from sucrose appear more apted to promote flavanol synthesis. The important point is that prolific cell growth does not exclude the establishment of secondary metabolism as indicated by flavanols.

As to the problem of the great growth variability of the seemingly uniform callus cultures it must be stated that each set of subcultured callus constitutes an individual sensory system for growth factors either from inside or outside the cells (Bornman 1983). It is also generally accepted that hormones are inefficient when present in cells lacking sensivity and responsiveness (Davies 1995).

The unexpected three-fold increase in callus fresh weight by ABA in only one of the 4 subcultures supports this view. This callus was extremely watery and ABA is principally capable to promote water uptake, as was indicated earlier by Glinka and Reinhold (1972).

Under field conditions, ABA accumulates near fungal infections (Pegg 1976) and mediates wound responses in the host tissue (Hetherington and Quatrano 1991).

In field-grown grapevines different modes of flavanol deposition could be found. Upon fungal infection there was a change from amorphous flavanols to a globular type of deposition along with an enlargement of the central vacuole.

Formation of flavanol globules allows to establish a flavanol-free phase in the remainder vacuole with totally changed metabolic properties and ion fluxes. Up to 18 different enzymes were found in vacuoles (Wink 1993). Vacuolar peroxidases of grapevine cells (Garcia-Florenciano *et al.* 1991) would be precipitated if they had a direct access to complexing PAs. Formation of flavanol globules goes along with dedifferentiation of the highly specialized leaf mesophyll into parenchymatous cells which is a prerequisite of subsequent resumption of cell division and wound healing at the infected site.

Paraquat, producing toxic oxygen radicals in chloroplasts (Elstner and Osswald 1980), lipids and proteins (Peleg *et al.* 1992), caused dispersion of the flavanol globules of grapevine. Those radicals accumulate near fungal infection sites (Sutherland 1991). In grapevine shoots, paraquat damage was largely overcome by addition of (+)-catechin. Their oligomeric condensation products are known to be potent radical scavengers (Ricardo da Silva *et al.* 1991).

The profound metabolic changes associated with the formation of wound callus involving sucrose and growth hormones may be basically modified by phenols such as flavanols to fulfill vital requirements of defence.

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