Effects of Watersoluble Boron and Aluminium Compounds on the Synthesis of Flavanols in Grape Vine Callus

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Dedicated to Professor Hans-Ludwig Schmidt at the occasion of his 70th birthday

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Internode explants of grape vine were used to produce proliferating callus cells *in vitro*. The tissues were grown on a modified M/S – medium supplemented with different concentrations of boron (H_3BO_3) in the range of 0 to 600 μM and aluminium ($AlCl_3$) in the range of 0 to 85 μM. With increasing concentrations of boron the content of the following flavanols declined: procyanidin B3 (catechin-(4α → 8) catechin), procyanidin B1 (epicatechin-(4β → 8) catechin), procyanidin B2 (epicatechin-(4β → 8) epicatechin), and B2–3-O-gallate, catechin and epicatechin. ECG (epicatechingallate) showed increased values in dependance on boron supply. Procyanidin B5 (epicatechin-(4β → 6)-epicatechin) showed an indifferent behaviour. In the case of aluminium the concentrations of flavanols were generally increased up to a maximum of 46% with the exception of ECG and B5 where no significant change was observed. While the total sum of flavanols was decreased by boron up to about 30% in comparison to the control (no boron addition) the content of flavanols was basically increased by aluminium up to about 25%. We conclude that the addition of watersoluble boron and aluminium compounds to the culture can significantly modify the synthesis of special monomeric and oligomeric flavanols.

Introduction

The role of flavanols in diverse processes, including ecological interactions and plant protection is a continuing goal of chemists and plant physiologists. In grape vine tissues secondary metabolites such as phenols are constitutively present (Hoos and Blaich, 1990; Santos-Buelga *et al.,* 1995). Accumulation of flavanols in mesophyll tissues was attributed to fungal infection (Feucht *et al.,* 1996). There is evidence that catechin and gallocatechin derivates are implicated in the resistance of grape vine against fungi (Dai *et al.,* 1995 a; Dai *et al.,* 1995 b).

During the last decade the antioxidant activity of grape vine phenols including flavanols towards LDL oxidation has gained much attention (Frankel *et al.,* 1993; Haslam, 1998). In plants, the flavanols were qualified as redox regulators controlling cell division (Stonier and Yang, 1973). In view of the fairly general importance of flavanols in basic cell physiology more experiments are needed to evaluate the conditions which modify their synthesis. The influence of watersoluble boron and aluminium compounds on the content of flavanols in grape vine callus is reported.

Experimental

Explant source

Internode segments were collected from the young elongating sprouts of grape vine, cv. 'Spätburgunder'. The best type of explant from an internode was a 1 mm thick cross-sectional slice.

In vitro culture

The media used (Murashige and Skoog, 1969) half strength, pH 5.8, and supplemented with 'boron' (added as boric acid, H_3BO_3) and 'aluminium' (added as $AlCl_3$) in a range of concentration as indicated in "Results" (Table I). The hormones BA (benzyladenine) at 1.6 μM and IAA (indoleacetic acid) at 8.5 μM were added before autoclaving at 121 °C for 20 min.

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Three internode segments were placed in each culture tube (22 × 160 mm) on paper bridges covering the liquid medium. For each treatment of the boron and aluminium series a total of 30 slices in ten test tubes each were cultivated. In addition to controls 5 different concentrations of boron and 5 concentrations of aluminium were tested (Table I). Cultures were kept in growth chambers for 4 weeks in a 16-h photoperiod using a photon flux density of 250 μmol m^{-2} s^{-1}.

Determination of flavanols

From freeze-dried and powdered material 100 mg dry substance were exhaustively extracted in pure acetone by use of an ultrasonic bath 30 min under ice-cooled conditions. The vacuum-dried material was dissolved in 0.5 ml methanol and injected into a HPLC equipment (Kontron). The column (250 × 4 mm i.d.) was prepacked with Shandon Hyperil ODS, 3 μm. The solvents were 5% formic acid and gradient grade methanol with a flow rate of 0.5 ml/min. This technique is coupled with a post column reaction detection system, described by Treutter (1989).

Postcolumn derivatization was performed with a PTFE capillary equipment (length 9 m and diameter 0.5 mm). The colour reaction was performed with the p-dimethylaminocinnamaldehyde reagent (DMACA). The blue colour products had a maximal absorption near 640 nm. Further details regarding these procedures and the standards used for identification and quantification are given by Treutter *et al.* (1994) and Pascual-Teresa *et al.* (1998). Monomeric catechins were purchased from Merck (Darmstadt). Proanthocyanidins were isolated from horse chest nut shells and grape seeds (Treutter *et al.*, 1994; Pascual-Teresa *et al.*, 1998).

The standard deviation of the individual flavanols as calculated from 4 experiments was between 6 to 8%.

Results

A number of flavanols were separated from the callus tissues. They were identified according to their retention time in HPLC chromatography and are presented in Table I. The fast migrating B3 consists of two catechin units linked at the C-4 and C-8 positions. The dimeric B1 consists of epicatechin and catechin likewise linked through the C-4 and C-8 positions. The following monomeric catechin (Cat) is the most abundant flavanol of the callus tissues. B2 is a dimeric compound consisting of 2 units epicatechin, linked at C-4 and C-8 position. B2 occurs in moderate concentrations and the next compound of the elution profile, epicatechin (Epicat), is similar in amount. The slowly migrating B2–3-O-gallate (B2-G) is followed by epicatechingallate (ECG). The last compound eluted from the column is B5 consisting of 5 epicatechin units and occurring in rather minute amounts.

As to the effect of increasing application of 'boron' the registered flavanols showed in nearly all

Table I. Effects of the addition of watersoluble boron ('B' = H_3BO_3) and aluminium ('Al' = $AlCl_3$) compounds on the content of flavonols in callus tissue from *Vitis vinifera*. The data of content is referring to μg/g dry weight. (B3 = catechin-(4α→8)-catechin, B1 = epicatechin-(4β→8)-catechin, Cat = catechin, B2 = epicatechin (4β→8)-epicatechin, Epicat = epicatechin, B2–G = B2-gallate, ECG = epicatechin-gallate, B5 = epicatechin-(4β→6)-epicatechin.

	μM		B3*		B1*		Cat		B2		Epicat		B2-G*		ECG	B5*		Total
Control	0	100%	99	100%	113	100%	188	100%	16	100%	38	100%	93	100%	75	3	100%	625
	49		69		111		167		14		37		96		94	5		593
'B'	97		89		112		187		12		37		99	136%	102	3		641
	194		67		86		151		10		32		81		83	5		515
	307	49%	49		69	72%	136		12	76%	29		70		80	4	72%	449
	599		54	54%	61		146	63%	10		35	66%	61		88	3		458
Control	0	100%	50	100%	47	100%	109	100%	12	100%	30	100%	39		76	2	100%	365
	2		63		55		110		13		34		36		47	3		361
'A'	8	134%	67	138%	65	133%	145		14		28	146%	57		77	2	125%	455
	21		48		45		117		14		37		50		85	2		398
	41		57		46		102	133%	16	147%	44		43		68	3		379
	83		46		45		89		14		31		44		77	3		349
'B+Al'	97+8		37		33		83		11		26		44		65	2		301

* Calculated as B2. ECG = epicatechin-3-0-gallate. B2-G = B2-3-0-gallate.

treatments a general reduction expressed for the most striking results in% with regards to the control (see Table I). Only in the case of ECG an increase (of 36%) was determined. Maximal decrease was found in the sequence for B3 51%, for B1 46%, for B2 37%, for B2–3-O-gallate 34% and for catechin 28%. Generally, the most effective reduction of most flavanols took place near the concentration of 307 μM H_3BO_3. The decrease of content is also essentially reflected in the integral sum of flavanols (see Table I, 'total').

'Aluminium' included into the nutrient medium tended to promote most of the flavanols as compared with controls, the only exception being epicatechingallate. Expressed in% with respect to the control values at a given concentration of aluminium, the increase of flavanols was in the following order: epicatechin (Epicat) 47%, B2 -3-O-gallate (B2-G) 46%, B1 38%, B3 37%, B2 34% and catechin 33%. The data is consistent with the total content of flavanols (see 'total' in Table I). B5 yielded amounts too small to allow a significant conclusion. Some 'aluminium treatments' showed slightly increased amounts of flavanols which were thought to be of minor physiological significance.

In a final experiment 97 μM H_3BO_3 and 8 μM $AlCl_3$ were combined (last line of Table I). It is evident that the addition of boron decreased the values for nearly all individual flavanols to levels lower than those of the Al-series.

Discussion

The results give strong support to the concept that elevated levels of boron reduce special flavanols and that increased levels of aluminium tend to increase most of the flavanols tested. Thus, there is an opposite effect of boron and aluminium on the metabolic potential of grape vine callus. The differential response obtained might be due to a slightly toxic boron effect whereas alumimium appears to favour a stress-type promotion of flavanols. The effectiveness of aluminium might be sought in a slight stress imposed on DNA (Morimura *et al.*, 1978) and/or cellular membranes. Phenylalanine ammonia-lyase the key enzyme for the phenol metabolism is located at the endoplasmatic reticulum (Hrazdina, 1994). Treatment of leaves with aluminium fosetyl resulted in elevated tannin levels and increased resistance against *Phytophthora* (Durand and Salle, 1981).

Aluminium ions binds tighter to cell walls and cell membranes than calcium, and thus disturbs the cation balance (Bengtsson *et al.*, 1988). Intracellularly, owing to its high affinity to phosphate aluminium disturbs important metabolic pathways based on phosphorylation reactions (Petersson *et al.*, 1985).

More significant, however, may be the induction of oxidative stress genes by aluminium (Richards *et al.*, 1998). Here, using a grape vine test system, very low levels of aluminium impose apparently a mild oxidative stress which promotes the synthesis of antioxidant flavanols.

The most conspicuous effects of boron and aluminium are found in the range 300–600 μM H_3BO_3 (20–40 ppm) and 8–40 μM $AlCl_3$ (2–10 ppm). These values are similar to those which were found for optimal pollen growth (Polster *et al.*, 1992). Even boron contents in the range of 100–1000 μM can influence the symptom expression of *Belladonna mottle virus* infection on tobacco plants (Bengsch *et al.*, 1989) or the genetic transposition in *Antirrhinum majus* (unpubl. results). These facts and the results presented here supply strong indications that boron and aluminium compounds play an important role in the secondary metabolisms. Flavanols are involved in natural defence mechanism, and these results are to our knowledge the first observations indicating a possible role of aluminium compounds in increased plant resistance.

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