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SPECIFIC ACCUMULATION OF *o*-DIPHENOLS IN STRESSED LEAVES OF *PRUNUS AVIUM*

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Abstract—As a result of graft incompatibility epinastic sweet cherry leaves exhibited a specific accumulation of chlorogenic acid, catechin and quercetin 3-*O*-glucoside. In contrast the concentrations of rutin, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-rutinoside and prunin remained unchanged.

The term graft incompatibility describes a physiological disorder which includes many symptoms which depend on the plant species [1]. Despite the fact that some theories exist [2, 3] the primary causes of graft incompatibility remain still unknown. Many biochemical events are affected such as enzyme activity [4] and the phenolic metabolism near the graft union [5, 6]. However, in our opinion, the first visible symptoms occur in the leaves.

Recent results indicated an altered carbohydrate metabolism and an accumulation of total phenols in *Prunus* leaves [7, 8]. Therefore we studied in depth the variation of phenolic compounds of cherry leaves with respect to stress symptoms. The occurrence of these phenols in *Prunus* tissues had been described by several authors [e.g. 9].

For this purpose green leaves were harvested during June, July, August and October of two consecutive years from healthy trees (homospecific graftings *P. avium* cv Sam upon *P. avium* rootstock) and incompatible heterografts (*P. avium* cv Sam upon *P. cerasus* rootstock). The latter showed an epinastic leaf position as the only visible stress symptom. The stress situation of the epinastic leaves—in contrast to the healthy ones—was confirmed by their decreased chlorophyll content (Table 1), which could hardly be detected visually. In addition the stress-indicating amino acid proline showed a strong increase. However, the nearly identical percentages of the dry matter (healthy leaves 43.5%, epinastic ones 45.2%) indicated that there had been no water loss in the epinastic leaves as compared to the healthy status.

The accumulation of starch could be interpreted as a consequence of the reduced export of triose-P from the chloroplasts [10] or of lowered phloem transport rates [11]. The reduced chlorophyll content may be indirectly related to the starch accumulation. Sequences starting with free radical formation and resulting in chlorophyll destruction might operate [12].

From this point of view, the observed accumulation of phenolic compounds (Table 1) is not simply as a consequence of the metabolism of excessive carbohydrate. Moreover, chlorogenic acid, catechin and quercetin-derivatives are known as antioxidants and radical scavengers [13, 14]. Their efficiency depends particularly on the *o*-diphenol-structure of the B-ring. Chlorogenic acid and quercetin have also been shown to inhibit lipoxygenase activity in leaves [15]. In contrast to this, *p*-hydroxylated phenolics catalyse lipid peroxidation [16]. The enrichment of the *o*-diphenols in epinastic leaves (Table 1) could thus be considered as a protective mechanism. This view is strengthened by the fact that not all phenolic compounds increase simultaneously.

EXPERIMENTAL

General. Leaves were harvested at 3 different dates during the growth period from 4 homogenetic graftings (*P. avium* cv Sam grafted on *P. avium* cv F12/1) and 11 heterografts (*P. avium* cv Sam grafted on *P. cerasus*) always at midday when the transpiration rates were highest (measured with a porometer). At each date a mixed leaf sample per tree was divided into 4 parts for dry matter estimation and chemical analysis respectively.

Quantitative analysis. Fresh leaves were homogenized (Ultra Turrax, sonification) and rapidly extracted with 80% aq. Me_2CO and centrifuged. The chlorophyll content was directly estimated by its absorbance at 667 nm. Exhaustive extraction of soluble phenolic compounds followed during the next 10 days at 4°. The solvent was changed × 3. The combined extracts were concd to dryness, redissolved in MeOH and analysed by rpHPLC [17]. Proline was extracted from one third of the leaf-material and estimated photometrically [18]. The fourth part of the sample was extracted with hot 80% aq. ethanol, the solvent was evapd, the residue resolved in water, centrifuged and the concentration of soluble sugars determined enzymatically, using glucose-6-phosphate dehydrogenase for glucose and fructose (the latter after its isomerization) as NADH equivalents. Sucrose was cleaved by β-fructosidase and glucose was estimated as above. Sorbitol was oxidized to fructose by sorbitol-dehydrogenase. After oxidizing NADH by lactate-dehydrogenase and pyruvate fructose was isomerized and determined as glucose. Starch was cleaved by amyloglucosidase and the glucose was estimated again as indicated above.

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Table 1. Content of selected organic constituents (μM/g dry wt) of healthy and epinastic *Prunus avium* leaves

Compounds	Healthy leaves mean	s.e.	Epinastic leaves mean	s.e.
chlorophyll	2.1	0.3	1.03	0.3
proline	1.3	0.2	3.9	0.6
Carbohydrates				
glucose	37.2	6.3	36.7	4.5
fructose	38.1	11.0	40.2	5.4
sucrose	93.7	9.8	59.3	7.5
sorbitol	402.1	49.6	372.4	12.3
starch*	214.7	57.6	319.6	50.3
Phenolic compounds				
prunin	1.7	0.2	2.2	0.2
kaempferol 3-*O*-rutinoside†	11.1	0.9	11.7	1.5
kaempferol 3-*O*-glucoside†	15.3	3.5	18.7	2.2
rutin	15.2	0.5	15.7	1.9
quercetin 3-*O*-glucoside†	15.9	2.3	27.1§	2.7
catechin	23.7	3.9	49.0‡	6.7
chlorogenic acid	48.2	1.8	115.7‡	10.9

*As glucose equivalents

†Calculation based on the molar extinction of rutin.

‡,§The mean values in a row are significantly different by Student's *t*-test at the 1% and 5% levels, respectively.

s.e. standard error of the mean.

Isolation and identification. The phenolic compounds were isolated by CC (Polyamid SC-6, 600 × 15 mm I.D.) and subsequent prep. HPLC (LiChrosorb RP18, 5 μm, 250 × 20.5 mm i.d.). The structures were confirmed by their UV-absorbance spectra [19] before and after acid hydrolysis (boiling in 2 M methanolic HCl, 20 min) and comparison of spectral properties and chromatographic behaviour (TLC, cellulose 10 × 10 cm, *n*-BuOH–HOAc–H_2O 4:1:5, upper phase; rpHPLC [13] with authentic samples (chlorogenic acid, (+)-catechin, prunin, rutin, quercetin, kaempferol). In the case of flavonols the degree of glycosylation was determined according to [20] on polyamid TLC. The rutinosides were partially hydrolysed by the H_2O_2-method [21] which yielded rutinose. The sugar moieties were identified by TLC (cellulose, as above and silica gel, EtOAc–*iso*-PrOH–H_2O, 65:32:13; visualized with aniline hydrogen phthalate) co-chromatographing with authentic sugars (glucose, rhamnose, rutinose). For monoglucosides the type of linkage was confirmed by unspecific β-glucosidase. Chlorogenic acid was additionally hydrolysed by esterase and the resulting quinic acid detected on TLC (cellulose, as above) following the method of ref. [22].

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