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Wall proteins of *Vitis vinifera* pollen I. Constancy of the phenotype[1])

by

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Proteine der Pollenwand bei *Vitis vinifera* I. Konstanz des Phänotyps

Zusammenfassung: Es wird vorgeschlagen, zur Charakterisierung verschiedener *Vitis-vinifera*-Sorten die isoelektrische Fokussierung pufferlöslicher Wandproteine der Pollenkörner zu verwenden. Die Proteinmuster umfassen etliche Dutzend Banden; die meisten sind allen Proben gemeinsam. Es wurden einige klonspezifische Komponenten ermittelt, deren Expression zeitlich stabil und unabhängig von Umwelteinflüssen war.

Key words: pollen, protein, analysis, variety of vine, clone, ampelography.

Introduction

Biochemical methods are being widely applied to taxonomic problems, most effectively so when dealing with interspecific comparisons or when relating low-ranking taxa (CRONQUIST 1981; DEL BELIN PERUFFO *et al.* 1983; PRUS-GLOWACKI 1983). Among these methods, electrophoretic techniques that characterize a given phenotype by the chemicophysical properties of the constituent proteins are more and more relied upon for identification and comparison (STEGEMANN *et al.* 1973; STEGEMANN 1983; RIGHETTI 1983).

Pollen wall proteins are implied in the highly specialized pollen-stigma recognition mechanism (HESLOP-HARRISON *et al.* 1975; HOWLET *et al.* 1975; CLARKE *et al.* 1979). This role implies a powerful constraint upon their pattern for stability and species specificity (JOHNSON and FAIRBROTHERS 1975). That is why we have selected the pollen as the most suitable sample for our taxonomic studies. As further advantage, this material corresponds to a well defined step in the vegetable cycle and can be easily collected and stored (JOHNSON and FAIRBROTHERS 1975).

In order to characterize different biotypes of *Vitis vinifera* we have thus analized by electrophoretic techniques the pollen wall proteins. As a first step of our investiga-

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tion we have assessed the proposed protocol by checking the constancy of the protein pattern (i) over time by comparing samples collected in different years, and (ii) upon environment influence by varying the growing conditions of the vine.

Materials and methods

Samples

The pollen was collected in 3 subsequent years (1983—1985) for seven clones from two varieties of *Vitis vinifera,* listed in Table 1.

Extraction protocol

The pollen was stirred at room temperature for varying periods of time in a solution 30 mM Tris/EDTA, pH 8.5 containing 150 mM NaCl, with a sample : buffer ratio of 1 : 20 w/v. The suspension was then spinned 20 min at 45,000 *g* (18,500 rpm with a JA20 rotor in a centrifuge J2-21 Beckman).

Table 1

Vitis vinifera clones used for pollen sampling

Die *Vitis-vinifera*-Klone, von denen Pollenproben entnommen wurden

Variety	#	Biotype	Origin		
Cabernet franc	a	Ferrari	Az. Tilatti	Corno di Rosazzo	(UD)
Cabernet franc	b	lato strada	Az. Ersa	Codroipo	(UD)
Cabernet franc	c	lato pinot	Az. Ersa	Codroipo	(UD)
Cabernet franc	d	Bagnoli	Az. Dominio	Bagnoli	(PD)
Merlot	e	Tilatti	Az. Tilatti	Corno di Rosazzo	(UD)
Merlot	f	lato strada	Az. Ersa	Codroipo	(UD)
Merlot	g	lato frutteto	Az. Ersa	Codroipo	(UD)

Protein and sugar quantitations

Protein concentration was evaluated according to MARKWELL *et al.* (1978) both on the raw extracts and on the pellet collected after TCA precipitation (PETERSON 1983). Sugars were quantified in the TCA supernatant according to HODGE *et al.* (1962). The activity of the enzyme malic dehydrogenase (MDH) released in the medium was assayed according to BERGMEYER and BERNT (1963).

Glycoprotein fractionation

Affinity chromatography was performed on a 3 ml column of immobilized Concanavalin A (ConA-Sepharose, Pharmacia, Uppsala, S); 0.5 ml of sample were fractionated in each run. The elution was with (i) a 20 mM Tris/HCl buffer pH 7.4, containing 0.5 M NaCl, added with (ii) 0.4. M α-methylglucopyranoside and followed by (iii) 0.1 M H_3BO_3 pH 6.4. Fractions of 2 ml were collected with a flow rate of 0.17 ml/min. The optical density of the effluent was monitored at 280 nm.

Electrophoretic analysis

Isoelectric focusing was run on a non-linear 3.5—10 pH gradient (1 % Ampholine in the range 3.5—10 and 1 % Ampholine 4—6) in a polyacrylamide matrix T % = 5, C % = 4 (HJERTÉN 1962). The slabs (125 × 260 × 0.5 mm) were run in a Desaga Desaphor HF cell (Heidelberg, FRG) at 4—6 °C, while delivering a constant power of 15 W (Desaga Desatronic 3000/200 power supply) for 1.5 h. The focused pattern was stained with silver nitrate according to MERRIL *et al.* (1981).

Table 2

Protein and sugar concentrations (µg/100 µl) of the pollen extracts · Pollen/buffer ratio 1 : 20 w/v

Protein- und Zuckerkonzentration der Pollenextrakte (µg/100 µl) · Verhältnis Pollen/Puffer 1 : 20 (w/v)

Sample	Protein concentration I	II	Sugar concentration III
a	374	215	146
b	198	121	182
c	216	146	104
d	154	116	83
e	181	112	242
f	163	62	442
g	145	49	292

I: Evaluation according to MARKWELL *et al.* (1978) in the raw extract.
II: Evaluation on the pellet from a precipitation with 10 % TCA.
III: Evaluation on the TCA supernatant, according to ASHWELL (1957).

Table 3

Time dependence of protein extraction from pollen · Sample a, pollen/buffer ratio 1 : 20 w/v

Die zeitliche Abhängigkeit der Proteinextraktion aus Pollen · Probe a, Verhältnis Pollen/Puffer 1 : 20 (w/v)

Sample	µg Protein/ 10 µl extract	U.E. MDH[1])/ 10 µl extract	U.E. MDH[1])/ 1 µg protein extract
Intact pollen:			
Extraction time (min)			
10	24.00	189.0	7.85
20	25.14	312.0	12.43
30	26.56	333.2	12.55
60	26.94	498.5	18.00
Pollen extracted for			
60 min, then ground	224.00	43470	194.00
Ground pollen	136.00	27216	200.00

[1]) U.E. MDH = Units of the malyc dehydrogenase enzyme.

Results

Table 2 records protein and sugar concentrations for the pollen extracts (the pollen was stirred for 30 min at room temperature) from the seven biotypes of *Vitis vinifera* var. Cabernet franc and var. Merlot, listed in Table 1.

The entries in column I and II correspond to the protein quantitation on the raw extracts and on the pellets after TCA precipitation, respectively. The discrepancy between the two sets of data may be due either to the influence from non-protein components or to the incomplete TCA precipitation of short peptides (BENSADOUN and WEISTEIN 1975; PETERSON 1983). Column III lists the sugar concentrations as evaluated on the TCA supernatants. From the data above the quantitative composition of the pollen extracts appears to vary among the seven vine biotypes under investigation.

Table 3 shows the kinetics of wall component extraction (as monitored by the whole protein concentration in the medium) as well as the release of cytoplasmatic proteins (through the activity of the representative enzyme MDH). Specificity and at the same time high yield may be obtained with short incubations, the lower limit being set by the operational constraints: in fact after 10 min already 89 % (24 µg/10 µl) of the proteins obtained after 60 min (26.94 µg/10 µl) but only 38 % (189 U.E. MDH/10µl) of the

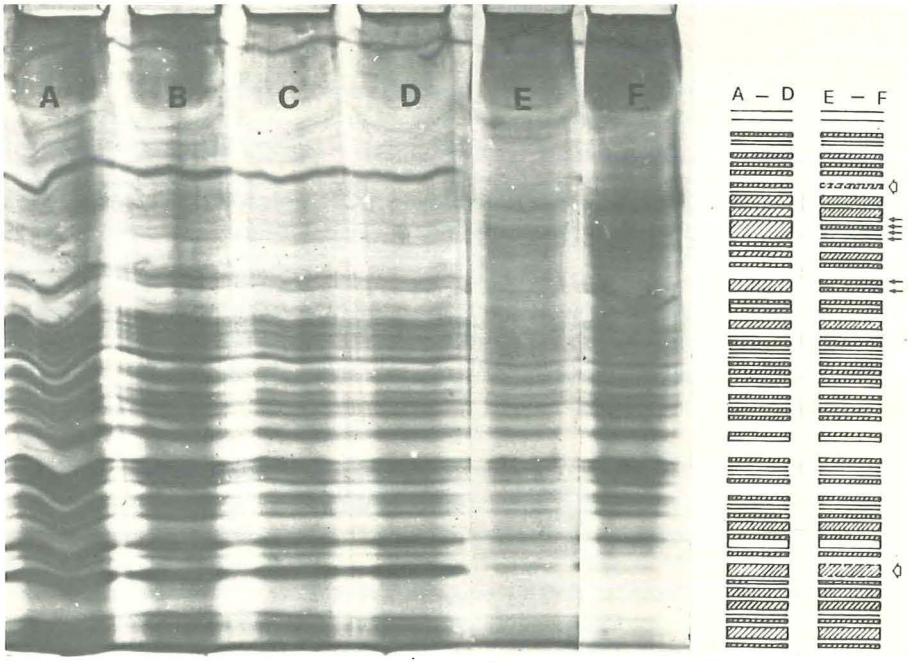

Fig. 1: IEF on a non-linear pH gradient of pollen wall proteins compared upon extraction for 10 to 60 min (A, B, C, D), upon crushing the sample after buffer extraction (E), directly upon intact pollen grinding (F). — ← Components present only after pollen crushing. ⇦ Marks quantitative differences.

IEF (nichtlinearer pH-Gradient) von Pollenwandproteinen bei Steigerung der Extraktionsdauer von 10 auf 60 min (A, B, C, D), aus einer nach Pufferextraktion zerquetschten Pollenprobe (E) sowie unmittelbar aus gemahlenem intaktem Pollen (F). — ← Komponenten, die nur auftreten, wenn der Pollen gequetscht wurde; ⇦ bezeichnet quantitative Unterschiede.

MDH activity measured after 60 min (498.5 U.E. MDH/10 µl) are found in the pollen extract.

In Fig. 1, the protein patterns for the pollen wall (sample a) are compared (i) upon extraction for 10 to 60 min (lanes A, B, C, D), (ii) upon crushing the sample after buffer extraction (lane E) and (iii) directly upon pollen grinding (lane F). Within each group (ground vs. intact pollen), the patterns A, B, C, D and E, F are virtually identical, whereas the two sets of samples differ by at least six major protein bands, that appear *ex novo,* or increase in their relative concentration as the pollen is ground.

Fig. 2 shows the IEF profile of the extracts from the three clones of cv. Merlot. While clones e and g exhibit some specific components of their own (3 such markers in e, 2 in g), in f all the recorded protein bands are shared with either e or g.

On the contrary, the patterns of the three clones of the cultivar Cabernet (shown in Fig. 3) differ slightly and only in quantitative terms.

The same results are obtained for each clone (cv. Merlot and cv. Cabernet franc), when analysing pollen collected in 3 different years (Fig. 4, samples a and e).

The glycoprotein components of the wall pollen were studied in some more detail. The extract from clone g was fractionated by affinity chromatography on ConA-Sepharose. Three fractions were collected: (1) unbound material, (2) bound material eluted with 0.4 M α-methyl-glucoside, (3) strongly bound material released with 0.1 M borate pH 6.6. The three pools, as well as the starting material, were then run in IEF (Fig. 5). Many bands were found to be shared by the three fractions, which should imply that unbound or weakly bound proteins differ by the strongly bound components, with identical pI, by loss of neutral sugars.

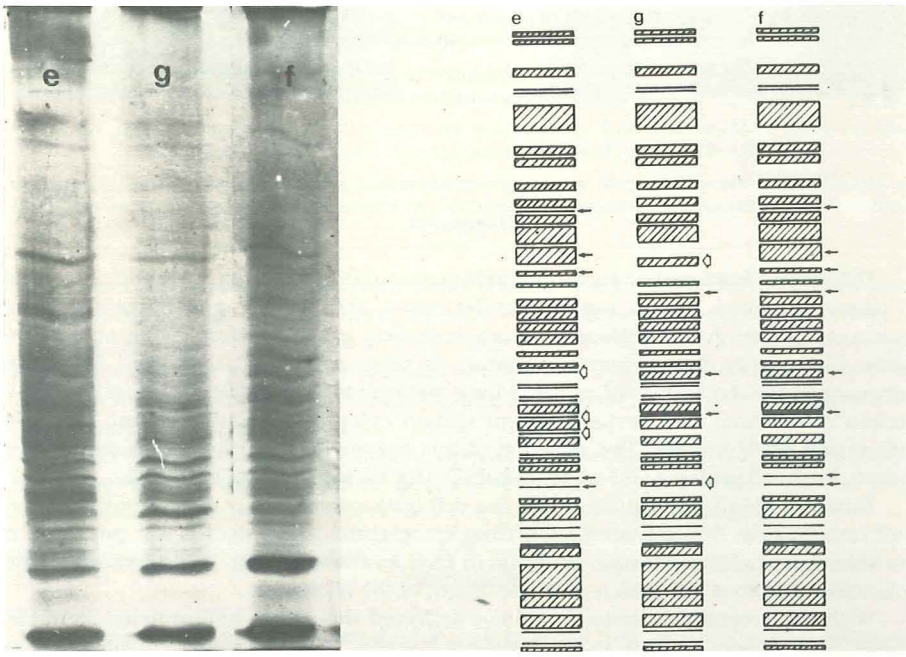

Fig. 2: IEF on a non-linear pH gradient of pollen wall proteins from the three clones of cv. Merlot (e, f, g). — ⇦ Specific components. ← Non specific components.

IEF (nichtlinearer pH-Gradient) von Pollenwandproteinen der drei Merlot-Klone e, f, g. — ⇦ bezeichnet spezifische, ← nichtspezifische Komponenten.

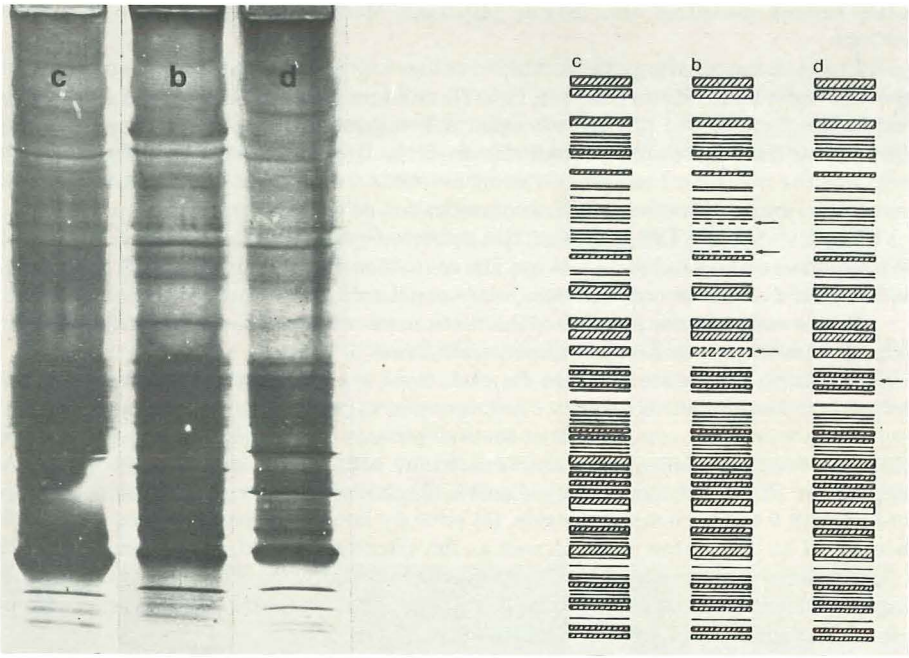

Fig. 3: IEF on a non-linear pH gradient of pollen wall proteins from the three clones of cv. Cabernet franc (clones b, c, d). — ← Quantitative differences.

IEF (nichtlinearer pH-Gradient) von Pollenwandproteinen der drei Cabernet-franc-Klone b, c, d. — ← bezeichnet quantitative Unterschiede.

Discussion

Differently from earlier suggestions (MAKINEN and BREWBAKER 1967) and according to recent literature (KNOX and HESLOP-HARRISON 1971; GHOSH and SHIVANNA 1984), moistened pollen grains release proteins, including enzymes, from exine and intine layers and, mostly, from germination pores. In other reports (CLARKE *et al.* 1979), the only control for the release of proteins from the wall was to check the integrity of the plasma membrane. As a further control against cytoplasmic contamination we have thus tested MDH activity. The selection of this enzyme as a marker relies on its high ratio to total cell proteins and on the feasibility for its easy and reliable essay.

Data in Table 3 give evidence that the diffusate we analyze contains mostly pollen wall components. Any contamination from cytoplasmic proteins does not interfere in the subsequent electrophoretic analysis: in fact, as shown in Fig. 1, the pattern of the pollen extracts does not change with the length of the treatment.

With the proposed technique we have analyzed the pollen wall proteins from the clones of cv. Merlot as well as of cv. Cabernet franc. In each group, two clones were grown in similar conditions in adjacent areas, while the third one was raised in a different region with a dissimilar environment. For the Merlot variety, specific protein bands were observed for each biotype, even when grown in the same area (Fig. 2). On the contrary, no differences were noticed between the different clones of Cabernet

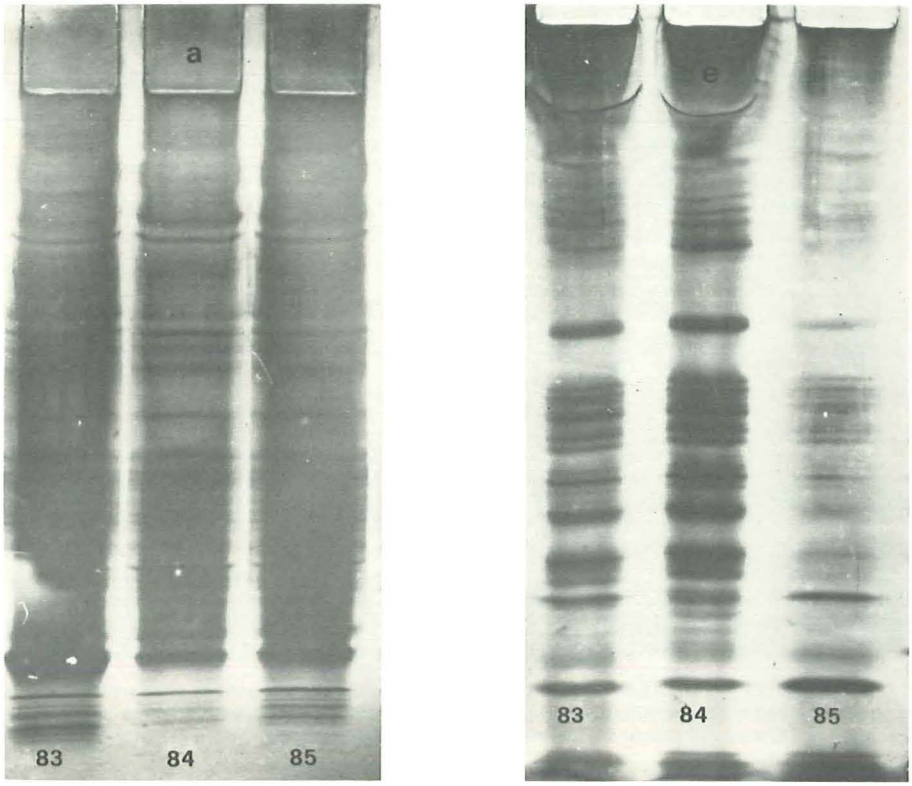

Fig. 4: IEF on a non-linear pH gradient of pollen wall proteins from sample (a) cv. Cabernet franc and sample (e) cv. Merlot collected in 3 subsequent years (1983—1985).

IEF (nichtlinearer pH-Gradient) von Pollenwandproteinen der Sorten Cabernet franc (Klon a) und Merlot (Klon e); die Pollenproben wurden in 3 aufeinanderfolgenden Jahren (1983—1985) entnommen.

franc, even when grown in different areas (Fig. 3). The protein pattern from a given clone was found to be identical when the pollen was collected in 3 subsequent years.

This set of observations lead us to conclude that, as required by their physiological role, the pollen wall proteins are constant and specific components for each vine clone. They are thus the sample of choice for the characterization of the various cultivars and for comparative studies.

A further development we plan is to restrict our comparison to the glycoproteins of the pollen wall, by exploiting their differential affinity to immobilized lectins. These components, in fact, are primarily involved in the pollen-stigma recognition.

Summary

Isoelectric focusing of buffer-soluble wall proteins from pollen grains is suggested for the characterization of different *Vitis vinifera* cultivars. The protein pattern includes several tens of bands, most of which common to all samples. A few clone-specific components are identified, whose expression is stable over time and independent from the influence of environment.

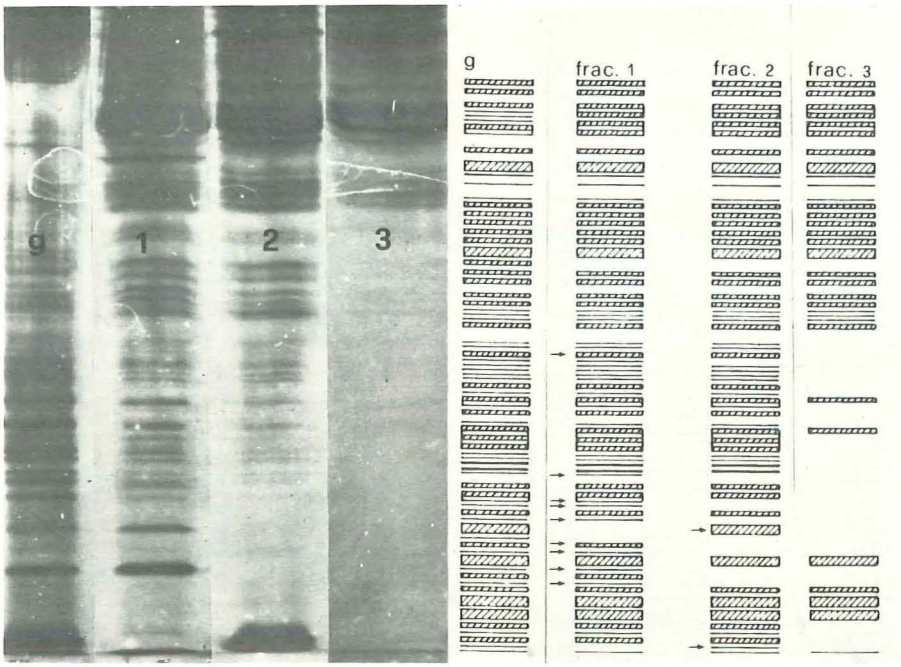

Fig. 5: IEF profiles of the raw extract from clone (g) and of the three fractions obtained by affinity chromatography on ConA-Sepharose: (1) Unbound material, (2) material eluted with α-methyl-glucoside 0.4 M, (3) material released with borate 0.1 M pH 6.6. — ← Specific components of each fraction.

IEF-Profile des Rohextraktes aus Klon (g) und der drei Fraktionen, die durch Affinitätschromatographie auf ConA-Sepharose erhalten wurden: (1) Nichtgebundenes Material, (2) mit 0,4 M α-Methylglucosid eluiertes Material, (3) mit 0,1 M Borat von pH 6,6 freigesetztes Material. — ← bezeichnet die spezifischen Komponenten der einzelnen Fraktionen.

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