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Phloem sap exudates as a criterion for sink strength appreciation in *Vitis vinifera* cv. Pinot noir grapevines

by

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Les exsudats de sève phloémique comme critère d'appréciation de la force de puits de la grappe chez *Vitis vinifera* cv. Pinot noir

Résumé : L'évolution temporelle des principaux constituants de la sève libérienne alimentant la grappe de *Vitis vinifera* Pinot noir a été étudiée au cours de la floraison, après adaptation d'une technique de prélèvement par exsudation facilitée. La composition de la solution d'exsudation retenue est la suivante: HEPES (10 mM, pH 7,5), EDTA (10 mM). Sur la grappe maintenue *in situ*, l'extrémité de la première ramification est sectionnée puis immergée dans le milieu précédemment défini pour permettre la récupération des assimilats. Les composés organiques prédominants dans la sève libérienne sont les glucides solubles, les acides aminés et les acides organiques (saccharose, glutamine et tartrate respectivement). Pour chacun de ces groupes métaboliques, les quantités moyennes exsudées sont voisines de 300 nmol par grappe en 4 heures. Les quantités croissantes de glucides, d'acides aminés et de potassium collectées entre le début de l'anthèse et la nouaison reflètent l'augmentation de flux de masse parvenant à la grappe et soulignent l'évolution de la force d'appel de ce puits. De plus, la part croissante de la glutamine et des hexoses dans les exsudats suggère une régulation dans la distribution des assimilats aux organes reproducteurs.

Key words: phloem sap, Vitis, grapevine, translocation, grape, nutrition.

Introduction

In fruit crops the quantity and quality of yield depend largely on the amount and nature of the assimilates allocated to the harvested organs over their whole period of development (GIFFORD and EVANS 1981). Phloem transport in sieve tubes ensures the transfer of C and N assimilates from source organs (mature leaves) to the different sink tissues, i.e. fruits, growing shoots, and perennial woody parts (PATE 1980; STITT and QUICK 1989). Flowers and fruits are, more or less, favoured by the source assimilate supply, according to their metabolic activity (sink strength) (HO 1988). In grapevine cluster, particularly, metabolic disorders can lead to flower (and/or young fruit) abortion, even in favorable environmental conditions. This phenomenon called 'physiological coulure' is largely interpreted in terms of temporary depletion in the carbohydrates feeding the clusters (RIVES 1961) but the assessment of phloemic substances translocated to the bunch through the pedicel has not yet been fully assessed even, as suggested COOMBE (1973), numerous studies have dealt with berry development.

Thus, the aim of the present work was to study the biochemical composition of the phloem sap allocated to the vine cluster, over the flowering time-course. An improved technique of phloem sap collection was developed to optimize the buffer composition for exudate release.

Material and methods

Plant material: Twenty-year-old grapevines of *Vitis vinifera* L. cv. Pinot noir grafted onto 41 B rootstock (*V. berlandieri* × *V. vinifera* cv. Chasselas) trained in the

field at Ambonnay (Marne, France), have been investigated. These experimental vines were selected with regard to their homogeneity and sampled in relation to the rate of flowering as follows (0, 10, 70, 100 % of opened flowers, and beginning of fruit set), from 27.06. to 5.07.91.

Collection technique: The phloem sap translocated to the cluster was collected by the facilitated exudation technique (KING and ZEEVAART 1974), where the excised organ dips in a buffered solution comprising of HEPES (10 mM, pH 7.5) and EDTA (10 mM). The presence of EDTA in the buffer is required to chelate the calcium, which is implicated in the plugging of P-proteins of the sieve plates and/or callose formation. The presence of EDTA thus favours exudation. As previously reported by HANSON and COHEN (1985) for *Pisum sativum* plantlets, this agent increases the collection of carbohydrates and amino acids.

For all selected clusters, maintained *in situ*, the first branch was sectionned under a drop of the EDTA solution, then rinsed for 5 min with the same medium. An Eppendorf tube containing 1.5 ml of this solution was then attached to the tip of the severed organ (the part remaining on the plant) and fixed with 'terostat' mastic.

After 4 h of exudation (from 10.00 to 14.00) samples were collected and then frozen immediately at −20 °C prior to analysis. Before assay samples were freeze-dried and resolubilized by 100 µl of deionised water. They were then mixed in pairs (corresponding to 5 replicates) in order to obtain measurable quantities of the exuded substances.

Analytical techniques

For the major organic compounds, soluble carbohydrates (glucose, fructose and sucrose) were assayed by enzymatic methods (Kit Boehringer, Mannheim, F.R.G.) and quantified by spectrophotometric detection according to BERGMEYER *et al.* (1974). Organic acids were separated by ion exchange chromatography (column HPX-87H) and then determined by U.V. detection (206 nm). The total amino acid content was assessed colorimetrically using the cyanide-ninhydrin reagent (ROSEN 1957). The different amino acids were separated by ion exchange chromatography (Biotronik LC 5001 analyser, lithium, citrate buffers), identified and quantified by comparison with a mixture of standard amino acids (Benson standard PANB). Their ninhydrin coloration was then detected by spectrophotometry (570 nm). Potassium was measured by flame emission spectrophotometry (Eppendorf). The nitrate content was assessed by ion exchange chromatography (Dionex 4000i, column HPIC AS4A).

Results

I. Improvement of the exudation technique

Different compositions of the dipping solution were tested in order to improve the phloem sap collection technique described in the chapter 'Material and methods'. In preliminary studies a non-metabolised sugar (mannitol), which was originally thought to facilitate the exudation phenomenon by increasing the osmotic gradient was added. Our results (data not presented) revealed no increases in gathered exudates when using this compound. Furthermore, after freeze drying, the mannitol precipitate renders pellet resolubilization with deionised water rather difficult.

Table shows the total soluble carbohydrates exuded (per cluster) during a 4-h collection period using different buffer solutions. The highest quantity of sugars released,

Table

Influence of the dipping solution composition on quantitative exudation of total soluble carbohydrates, expressed in glucose equivalent (nmoles exudated in 4 hours); values are means ± SD, N = 3

Influence de la composition de la solution de récupération sur la quantité de glucides solubles totaux exsudés, exprimés en équivalent glucose (nmoles exsudées en 4 heures); valeurs moyennes ± SD, N = 3

HEPES	EDTA			
	1 mM	5 mM	10 mM	Mean
10 mM	350 ± 105	538 ± 53	**721 ± 126**	536 ± 181
20 mM	505 ± 425	478 ± 107	998 ± 696	659 ± 482
Mean	427 ± 289	508 ± 79	857 ± 471	

with a maximum of reproducibility, was obtained using the buffer containing: 10 mM HEPES (pH 7.5), 10 mM EDTA.

II. Biochemical features of the phloem sap distributed to the cluster

Quantitative (nmol metabolite exudated/4 h/cluster) and qualitative estimation of the metabolite composition in the phloem exudates gave the following results (mean values): The soluble carbohydrates corresponded to 290 nmol, mainly sucrose (70 % of the mean glucidic pool); glucose and fructose being present in the ratio 2:1. The amino acids and amides comprised 300 nmol, mainly glutamine (60 % of the amino-acid pool). Proline was detected in relatively high amounts (almost 10 % of the total) whereas the other amino acids were present in lower proportions, each one representing less than 2 % of the total. For organic acids, the concentration reached 305 nmol, tartaric acid (170 nmol), malic acid (110 nmol), citric acid (25 nmol). These results were assessed at mid-flowering. Potassium exuded amounts were close to 800 nmol/sample. Nitrate was not detected in significant amounts in the exudates the pH value of which was 7.7.

III. Temporal evolution of the phloem sap composition over the flowering time-course

Figs. 1 and 2 show the quantitative and qualitative release of the major organic components (soluble carbohydrates and amino acids, respectively) present in the phloem sap over all the 1991 blooming period. Similar profiles were also obtained in 1990 using the same grapevines, grown under identical conditions (data not shown).

After a sharp initial decrease (24 %, at 10 % anthesis), the amount of total soluble carbohydrates exhibited a 60 % increase until fruit set. In the meantime the ratio of sucrose/total soluble sugars decreased significantly, correlating with the number of opened flowers. At the beginning of the flowering period, sucrose represented 85 % of the total and diminished steadily reaching 60 % of the total at the beginning of fruit set. The glucose/fructose ratio was constant and equivalent to 2:1 over the whole experimental time-course.

Total amino acid allocation to the cluster exhibited a 120 % increase during the same period (Fig. 2). The 2 major amino acids translocated in the sieve tubes were glutamine and proline as previously described. Their respective proportions increased in an inverse pattern during the blooming period. For glutamine an increase from 50 to 65 % of the total amino acid content was measured, while for proline a 15 to 8 %

decrease was observed during the experiment. The relative concentrations of other amino acids remained constant during the flowering time-course.

A net increase in potassium flux was observed in the exudates until fruit set. The amount of potassium translocated to the cluster was directly correlated with the rate of flowering: 420 nmol (before anthesis); 360, 740, and 820 nmol (at 10, 70 and 100 % anthesis, respectively); 1270 nmol at beginning of fruit set.

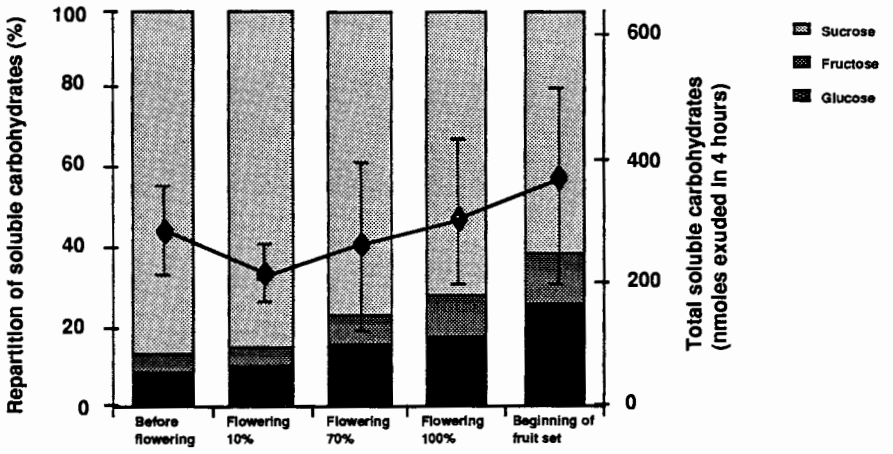

Fig. 1: Temporal evolution of quantity (◆) and repartition of soluble carbohydrates in the phloem sap of *Vitis vinifera* Pinot noir over the flowering time-course. Values are means ± SD, N = 5. Quantity is expressed in glucose equivalent.

Evolution temporelle de la quantité (◆) et de la répartition des glucides solubles dans la sève phloémique de *Vitis vinifera* Pinot noir au cours de la floraison. Valeurs moyennes ± SD, N = 5. Quantité exprimée en équivalent glucose.

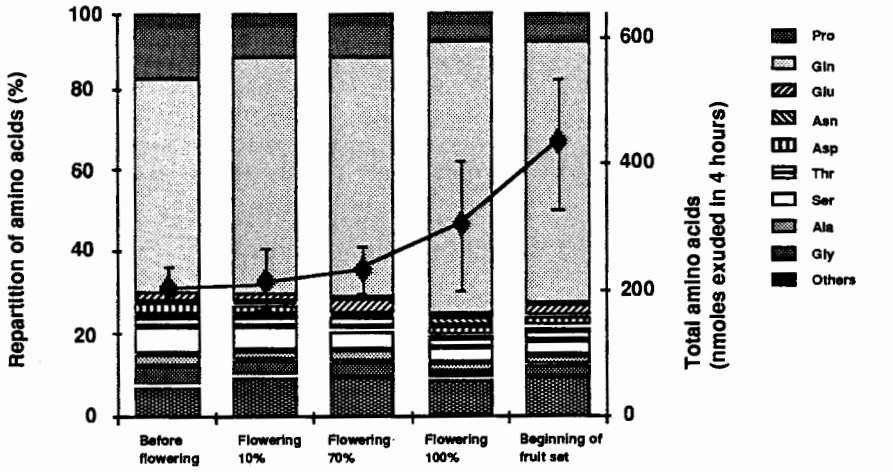

Fig. 2: Temporal evolution of quantity (◆) and repartition of amino acids in the phloem sap of *Vitis vinifera* Pinot noir over the flowering time-course. Values are means ± SD, N = 5. Quantity is expressed in glutamine equivalent.

Evolution temporelle de la quantité (◆) et de la répartition des acides aminés dans la sève phloémique de *Vitis vinifera* Pinot noir au cours de la floraison. Valeurs moyennes ± SD, N = 5. Quantité exprimée en équivalent glutamine.

Discussion

The pH value of the exudate (7.7), together with the high quantities of potassium, sucrose, amino acids and the absence of nitrate suggest that the technique developed to collect preferentially the phloem sap was successful. Indeed, according to PATE *et al.* (1974), the phloem characteristics are a slightly alkaline pH, high concentrations of soluble sugars (mainly sucrose), amino acids and potassium. Moreover, in the case of phloem contamination by xylem, a more acidic pH value, and a drastically reduced concentration of sucrose and potassium would be expected (VREUGDENHIL and KOOT-GRONSVELD 1989).

The different collection techniques revealed that stylectomy is the most efficient when requiring an accurate biochemical determination of sap components (GIROUSSE *et al.* 1991) while facilitated exudation allows the routine assessment of qualitative phloem sap features (WEIBULL *et al.* 1990) and dynamic phenomena in a continuous fashion (GROUSSOL *et al.* 1986). In grapevine, the impossibility of using aphids at the cluster level justifies the choice of the EDTA-facilitated exudation technique. Even if quantitative values must be interpreted with caution, because of the injury due to the partial section of the cluster, they can reflect the true mass flow of nutrients supplied to the cluster.

Tartaric acid, the major form of organic acid found in cluster exudates at blooming time, confirming KLIEWER (1965) experiments, corresponds to 60 % of the total acid composition whereas malic and citric acids represent 28 and 13 % of the total, respectively. Between berry set and veraison, the ratio tartrate/malate was approximately 1:1, malic acid becoming more important (KLIEWER 1965).

Sucrose is the main carbohydrate translocated in the phloem sap of various species (SWANSON and EL-SHISHINY 1958; GIROUSSE *et al.* 1991; WEINER *et al.* 1991). Its high level is closely linked to an active loading into the sieve elements energized by proton release into the external medium (ATPases pump system). This proton transfer is counter-balanced by the import of potassium ions into the bundle aperture (DELROT and BONNEMAIN 1979), explaining the large amount of potassium obtained in the dipping solution (1000 nmol/cluster in 4 h). The absence of neutral invertase activity in the sieve tubes (BROVCHENKO 1970; ESCHRICH and HEYSER 1975) supports the predominance of sucrose found in our experiment.

Hexoses, mainly glucose and fructose, were significantly represented, reaching nearly 40 % of the carbohydrate pool at fruit set, suggesting that an increasing proportion of sucrose is hydrolyzed in the sieve tubes. In addition to the induced-injury effect of cutting the cluster branch, which could involve the release of sucrose splitting enzymes in the dipping solution (GROUSSOL *et al.* 1986), we suggest that an induction of invertase and other enzymes may occur over the flowering period (see below).

Total amino acids or carbohydrates released are close to 300 nmol/cluster in 4 h, determining a 1:1 molar ratio already reported in *Medicago sativa* (GIROUSSE *et al.* 1991). In the phloem sap of *Vitis vinifera* we showed glutamine as the major nitrogen transported form. In the maize, it is glutamine and aspartate (MUHITCH 1989; CLIQUET *et al.* 1991), asparagine in alfalfa (GIROUSSE *et al.* 1991), and O-acetylhomoserine in the developing hull of pea (ROCHAT and BOUTIN 1989). The main translocated forms of reduced nitrogen thus seem to be species-specific. The amino acids present in the sieve tubes result either from an active loading process (BROVCHENKO 1963; WEINER *et al.* 1991) and/or from passive diffusion (WINTER *et al.*, in press). The rather large amount of proline that was measured, as 10 % of amino acid pool, could be the consequence of the mechanical stress of stalk sectioning, and/or to hydric stress (HSIAO 1973) occurring in the field under sunny conditions.

The sharp rise in amino acids translocated into the cluster, particularly glutamine, as the enhanced allocation of carbohydrates reveal the increasing sink strength of this organ over the flowering time-course. Amino acids influx is associated with an active protein synthesis within the grapevine ovary tissues between anthesis and fertilization (SLOCUM and GALSTON 1985), or further, until harvest (CONRADIE 1980). In parallel, the rising sugar allocation, already reported in this plant by COOMBE (1989) and RUFFNER *et al.* (1990) can reflect the metabolic activities linked to cell division and growth (HO 1988; DOEHLERT and CHOUREY 1991).

All these processes could be supported by enzymatic activities which gradually take place within the stalk as shown by BROVCHENKO (1963) or in the pedicel tissues (BURGER and SCHAFFER 1991) as flowering and berry set proceed. These enzymes could be involved in (i) the splitting of sucrose (invertase, sucrose synthase) as recently shown by HUBBARD *et al.* (1991) for different fruit species, and/or (ii) in nitrogen metabolism, as demonstrated by MUHITCH (1989), who reported a concomitant increase of GS activity and nitrogen assimilation during maize kernels filling.

Unfortunately, to our knowledge, no previous study has been carried out in *Vitis vinifera*, treating about the enzymatic activities present in the pedicel of young berries. Particularly, there is a lack of information on nitrogen metabolism at young fruit set. The modifications in the organic composition of the phloem sap during the blooming period, complemented by enzymatic studies, should allow the appraisal of the mechanisms which regulate the berry set.

Summary

The temporal evolution of the main compounds present in the phloem sap feeding the cluster of *Vitis vinifera* Pinot noir has been determined from the beginning of flowering until fruit set, after improvement of the facilitated exudation technique. The retained composition for the dipping solution was: HEPES (10 mM, pH 7.5), EDTA (10 mM). The first ramification of the cluster, maintained *in situ*, was sectionned then immersed in the dipping solution in order to favour the phloem exudation. The major organic components of the phloem sap were carbohydrates, amino acids and organic acids (i.e. sucrose, glutamine and tartrate, respectively). For each metabolic group, the mean exuded quantities correspond to 300 nmol per cluster in 4 h. The sharp increase in both organic compounds and potassium released over the flowering time-course reflects the rise of the mass flow supplying the cluster and underline the increasing sink strength of this organ. Moreover, the increasing contents of glutamine and hexoses in the exudate suggest a regulation in the allocation of assimilates to the reproductive organs.

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