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Mineral stress affects the cell wall composition of grapevine (*Vitis vinifera* L.) callus

João C. Fernandes[a], Penélope García-Angulo[b], Luis F. Goulao[c], José L. Acebes[b], Sara Amâncio[a,*]

[a] DRAT/CBAA, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Tapada da Ajuda, 1349-017 Lisbon, Portugal
[b] Departamento de Biología Vegetal, Área de Fisiología Vegetal, Universidad de León, E-24071 León, Spain
[c] BioTrop – Instituto de Investigação Científica Tropical (IICT, IP), Av. da República, Quinta do Marquês, 2784-505 Oeiras, Portugal

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ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops in the world. Deficit in nitrogen, phosphorus and sulfur nutrition impairs essential metabolic pathways. The influence of mineral stress in the composition of the plant cell wall (CW) has received residual attention. Using grapevine callus as a model system, 6 weeks deficiency of those elements caused a significant decrease in growth. Callus CWs were analyzed by Fourier transform infrared spectroscopy (FT-IR), by quantification of CW components and by immunolocalization of CW epitopes with monoclonal antibodies. PCA analysis of FT-IR data suggested changes in the main components of the CW in response to individual mineral stress. Decreased cellulose, modifications in pectin methyl esterification and increase of structural proteins were among the events disclosed by FT-IR analysis. Chemical analyses supported some of the assumptions and further disclosed an increase in lignin content under nitrogen deficiency, suggesting a compensation of cellulose by lignin. Moreover, polysaccharides of callus under mineral deficiency showed to be more tightly bonded to the CW, probably due to a more extensive cross-linking of the cellulose–hemicellulose network. Our work showed that mineral stress impacts the CW at different extents according to the withdrawn mineral element, and that the modifications in a given CW component are compensated by the synthesis and/or alternative linking between polymers. The overall results here described for the first time pinpoint the CW of *Vitis* callus different strategies to overcome mineral stress, depending on how essential they are to cell growth and plant development.

1. Introduction

The structural and mechanical support of plants is provided by cell walls (CWs), which are load-bearing, extensible viscoelastic structures that surround the cells, acting as an "exoskeleton". The CW plays a vital role in the regulation of the rate and direction of growth and the morphology of plant cells and organs [1]. The plant CW is a dynamic complex with further functions such as control of the diffusion through the apoplast, signaling, regulation of cell-to-cell interactions, storage of carbohydrates, or protection against biotic [2] and abiotic stress agents [3].

In the primary CW, cellulose is the main load-bearing polysaccharide which interlinks with cross-linking matrix glycans, predominantly xyloglucan in dicots [4], to form an extensive framework that provides most of the tensile strength to the CW matrix. This network is embedded in a surrounding phase constituted by pectic polysaccharides, forming hydrophilic gels that determine the regulation of the hydration status and ion transport, the definition of the porosity, stiffness and control of the wall permeability [5]. These features are, in turn, defined by the chemical structure of pectic polysaccharides, particularly the branching degree and pattern, the decoration with neutral sugars and the degree and pattern of acetyl-and methyl-esterification, which can lead to either stiffening or loosening of the CW [6]. The occurrence of micro-domains inside the pectic polysaccharides means the localization of precise areas with distinct properties, providing a highly fine-tuned regulation of the wall properties to cope with the cell functioning. In addition to polysaccharides, a third network composed by structural glycoproteins contributes to the biophysical properties of the primary CW and cell adhesion [7,8]. In some tissues, after cell growth has ceased, a secondary CW is formed with higher cellulose content and a different organization of its deposition. After cellulose, lignin is

Abbreviations: 2.4-D, 2,4-dichlorophenoxy-acetic acid; CDTA, cyclohexane-*trans*-1.2-diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid sodium salt; CW, cell wall; FT-IR, Fourier-transform infrared; GC, gas chromatography; HRP, horseradish peroxidase; MS, Murashige and Skoog Medium; -N, nitrogen deficient callus; -P, phosphorus deficient callus; PBS, phosphate buffered saline; PCA, principal component analysis; PVP-40T, polyvinylpyrrolidone; RG-I, rhamnogalacturonan-I; -S, sulfur deficient callus; SD, standard deviation; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +351 213653418; fax: +351 33653383.
E-mail address: samport@isa.utl.pt (S. Amâncio).

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the second most abundant plant polymer in vascular plants [9]. In secondary CWs, lignin is deposited within, around or among the cellulose microfibrils establishing covalent bonds with carbohydrates, providing additional strength and rigidity that, along evolution, allowed plants to grow upward [10].

The most consensual dicot primary CW model has been the "tethered network", a representation in which the hemicellulose polymers link cellulose through hydrogen bonds to create a load bearing tether, inserted in an amorphous cement-like pectin matrix [11]. However, recent results disclosed the presence of covalent linkages between rhamnogalacturonan-I (RG-I)-arabinan side-chains and cellulose microfibrils [12] and covalent linkages between xyloglucan and pectins *in muro*, [13,14] providing structural links between two major cell wall domains. Moreover, since not all of the cellulose microfibril surfaces are covered with xyloglucans and not all xyloglucans are adsorbed to cellulose [15–17], the existence of such other linkages within the CW is expected to maintain its structure.

During development, the fine structure of the plant CW matrix is extensively modified. The amount and composition of specific molecules and their arrangements differ among plants, organs, cell types and even in different micro-domains of the wall of a given individual cell [18].

Localized changes in CW composition and structure also provide the cell with a notable ability to tolerate abiotic stresses, such as osmotic [19] and chemical [20,21].

Deficiencies in mineral nutrition, particularly nitrogen (N), phosphorus (P), potassium (K) and sulfur (S), which are required in relatively large amounts by the plant, strongly affect the plant metabolism with subsequent impact on the plant growth, crop yield and in both nutritional and organoleptic quality of the agronomic product [22–25]. Essential nutrients are major regulators of plant growth and development due to their involvement in primary metabolic pathways, e.g. amino acid and nucleotide biosynthesis, protein phosphorylation or disulfide bonds between cysteine.

Plant development and anatomy are impacted by abiotic stresses and a common "stress-induced" set of responses have been reported: prompting of localized cell division, arrestment of cell elongation, and modifications in cell differentiation status [26].

Limited mineral nutrient availability has been reported to affect organ growth rates, through inhibition of the production of new cells and/or cell expansion [27] via reduction of CW plasticity [28,29]. It has been proposed that nutrient-induced stress act by modifying xylem tension which then signals the onset of CW rearrangements in growing tissues [30,31]. These components are determined by the dynamic regulatory properties of the CW. Nevertheless, and even though the importance of mineral nutrition in plant development has been widely recognized, only residual attention has been given to its influence on the CW dynamics. More recently, global transcriptomic studies involving nutrient depleted plants revealed differential regulation of CW-related genes and proteins in various species [32,33], emphasizing the CW role in survival response mechanisms.

Despite the grapevine (*Vitis vinifera* L.) economic value and scientific relevance as a model species, there is little information about the CW structure and polysaccharide composition in this species. Investigation has been mainly focused to the economic important organ, the fruit, both berry pulp and skin, reviewed in [34].

The aim of the present work was to investigate the response of the CW to mineral depletion of individual major nutrients, nitrogen, phosphorus and sulfur, using *Vitis* callus as experimental model. Here, an integrated approach employing complementary methodologies was followed. Fourier-transform infrared (FT-IR) spectroscopy coupled with chemometrics was used to detect changes in CW polymers and putative cross-links [35,36] to retrieve the major candidate events occurring in the CW in response to the imposed conditions. Candidate events were further tested by chemical methods and immunochemical staining using monoclonal antibodies [37] and through the determination of monosaccharide composition of fractionated CWs. The combined use of these methodologies allowed drafting a map of CW responses to specific changes in the mineral health in *Vitis* callus.

2. Material and methods

2.1. Cell culture and mineral stress imposition

V. vinifera cv Touriga Nacional callus tissue was maintained in the dark at 25 °C, as described in Jackson et al. [38]. Four and a half grams of callus tissue was used as initial explant in medium containing MS basal salts [39] (DuchefaBiochemie, Haarlem, NL) supplemented with 2.5 μM 2.4-D (2,4-dichlorophenoxy-acetic acid); 1 μM kinetin; 5 g l^{-1} PVP-40T; 20 g l^{-1}, sucrose; 2 g l^{-1} Gelrite®, pH 5.7. The calluses were sub-cultured every 3 weeks. Four treatments were applied: full nutrients (control), nitrogen deficiency (-N), phosphorus deficiency (-P) and sulfur deficiency (-S). Commercial MS was used to obtain control samples while modified MS media in which nitrates, phosphates and sulfates were substituted for chlorides were considered -N, -P and -S treatments respectively. Calluses were sub-cultured to the respective medium after 3 weeks of growing. After each culture cycle in the respective treatment medium each sample, corresponding to 10 Petri dishes (9 cm Ø) containing four calluses, was collected to monitor growth. Based on the results obtained, 6 weeks grown callus (2 × 3 weeks) samples were used for CW analyses.

2.2. Cell wall isolation

Twenty grams of callus samples were homogenized in liquid nitrogen using a mortar and pestle, washed with cold 100 mM potassium phosphate buffer pH 7.0 (2×), and treated overnight with 2.5 U ml^{-1} α-amylase VI from hog pancreas (Sigma–Aldrich Co., St. Louis) at 37 °C. The suspensions were centrifuged and the pellet was sequentially washed with distilled water (3×), acetone (3×), methanol:chloroform (1:1; v/v) (3×), diethylether (2×), and then air-dried [40].

2.3. FT-IR spectroscopy and multivariate analysis

FT-IR analysis was performed according to the methodology described in Alonso-Simón et al. [36]. Tablets for FT-IR spectroscopy were prepared in a Graseby-Specac Press, using 2 mg of CW samples mixed with potassium bromide (KBr) (1:100, w/w) from a minimum of 11 biological replicates per treatment. Spectra were obtained on a Perkin-Elmer System 2000 FT-IR at a resolution of 1 cm^{-1}. In order to tackle CW structure modifications, a window between 800 and 1800 cm^{-1}, which contains information of polysaccharide characteristic linkages, was selected for analysis. Normalization and baseline-correction were made using the Perkin-Elmer IR Data manager software and the data exported to Microsoft Excel for area normalization. Principal component analysis (PCA), using Pearson coefficient for distance estimation, was performed with a maximum of four principal components using the Statistica 6.0 software package (StatSoft, Inc., USA).

2.4. Cellulose quantification

Cellulose was quantified by the Updegraff method [41], using the hydrolytic conditions described by Saeman et al. [42] and quantifying the glucose released by the anthrone method [43].