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The role of the secondary phloem during the development of the grapevine Berry Shrivel ripening disorder

Sara Crespo-Martínez[a,*], Mirosław Sobczak[b], Elżbieta Różańska[b], Astrid Forneck[a], Michaela Griesser[a]

[a] University of Natural Resources and Life Sciences Vienna, Department of Crop Sciences, Division of Viticulture and Pomology. UFT Tulln, Konrad Lorenzstrasse 24, A-3430, Tulln, Austria
[b] Warsaw University of Life Sciences (SGGW), Faculty of Agriculture and Biology, Department of Botany, Nowoursynowska 159, Building 37, 02-776, Warsaw, Poland

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ABSTRACT

Berry Shrivel (BS) is a post-veraison physiological ripening disorder of grapevine berries. Its symptoms encompass low pH, reduced content of sugars and anthocyanins, and loss of turgor leading to berries shriveling. Evidence for the primary causes of BS is still speculative and anatomical studies are scarce. So far, anatomical studies have determined necrotic cells, degraded primary phloem cells and hardening of secondary phloem cells in the rachis of BS affected grapes. The picture is far from being complete. Herein we report in-depth analyses of the ultrastructure, anatomy and spatial elementary analysis of rachis and pedicel tissues of BS symptomatic grape clusters with different symptom severity. We hypothesize that structural changes in the vascular system of BS affected grape clusters could alter transport functions of the phloem tissue and contribute to the appearance of BS symptoms. By applying different microscopic techniques (LM, SEM, TEM and EDS) we found a number of anatomical differences in both, rachis and pedicels, between H and BS symptomatic grapes, which include: (i) extended areas of collapsed cells and cell wall thickenings in the secondary phloem in BS samples; (ii) reduced number of cell layers in the cambium in BS samples; (iii) higher rate of callose deposition on sieve plates that are additionally covered with a carbohydrate-like material in BS samples; and (iv) reduced (up to 60%) estimated sieve tube conductivity in BS samples.

1. Introduction

Grapevine is a fruit crop of high economic importance worldwide. The ripening of grapevine berries encompasses complex morphological and physiological processes, which disturbance leads to physiological ripening disorders such as bunch stem necrosis (BSN), late-season dehydration and Berry Shrivel (BS) that is sometimes named sugar accumulation disorder (SAD) (Krasnow et al., 2009) or suppression of uniform ripening (SOUR) (Bondada, 2014). The most popular red grape cultivar in Austria "Blauer Zweigelt" (*Vitis vinifera* L.) is specifically prone to develop the BS ripening disorder (Griesser et al., 2012a, 2017, 2018; Knoll et al., 2006, 2010). The first symptoms, such as reduced sugar accumulation, inhibited anthocyanin biosynthesis and high pH values appear shortly after veraison (start of grape berry ripening). Further development exacerbates the aforementioned symptoms and affected berries additionally lose turgor and elasticity, undergo enhanced rate of mesocarp cell death and produce more tannins. Finally, BS berries show the typical shrinking or shriveling (Bondada and Keller, 2012a; Griesser et al., 2012a; Knoll et al., 2010; Krasnow et al., 2009). Although shriveling berries are a common symptom for different ripening disorders, the causes as well as the consequences appear to be different (Bondada and Keller, 2012b; Griesser et al., 2012a; Krasnow et al., 2010). Bunch stem necrosis (BSN) starts with necrotic patches on rachis and pedicels leading to extended necrosis disrupting water and solute transport towards berries (Bondada and Keller, 2012b), whereas no necrotic tissue is observed with BS or late season dehydration (LSD) on the rachis surface. Sugar accumulation in BS berries stops shortly after veraison, keeping values for soluble solids consistently low during

Abbreviations: A, area; BS, Berry Shrivel affected clusters; BSN, bunch stem necrosis; EDS, energy-dispersive X-ray spectroscopy; FDA, fluorescein di-acetate; GA, gibberellic acid; H, healthy clusters; IAA, indole-3-acetic acid; $k_{sieve\ element}$, sieve tube-specific conductivity; l, sieve tube element length; LM, light microscopy; l_p, sieve plate thickness; n_p, number of sieve pores per sieve plate; r, sieve tube element radius; r_p, sieve pore radius; SD, standard deviation; SEM, scanning electron microscopy; TEM, transmission electron microscopy

* Corresponding author.
E-mail address: saraolexoa@hotmail.com (S. Crespo-Martínez).

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the ripening process (Griesser et al., 2018). In contrast, BSN or LSD berries usually reach higher soluble solid values as processes are disturbed later during ripening (Krasnow et al., 2010). The causes of LSD have recently been proposed as a combination of enhanced cell death and disturbed hydraulic conductance (Tilbrook and Tyerman, 2009).

Although the understanding of BS has increased recently (Bondada, 2016; Bondada and Keller, 2012a,b, 2013; Griesser et al., 2012a, 2017, 2018; Hall et al., 2011; Keller et al., 2016; Krasnow et al., 2009, 2010) the causes of BS are still obscure. The hypothesis that BS is caused by a nutrient deficiency was challenged by different approaches, which provided highly variable results. In general, lower content of phloem-delivered mineral nutrients, especially potassium (K^+), in BS berries (Bachteler et al., 2015b; Bondada and Keller, 2012b; Krasnow et al., 2009), rachis and pedicels of affected clusters was reported (Griesser et al., 2017). In contrast, calcium (Ca^{2+}), a xylem transported element, was present in significantly higher concentrations in BS affected berries (Bachteler et al., 2015b; Griesser et al., 2017; Krasnow et al., 2009). Experiments with soil (Bachteler et al., 2015b) or foliar (Bachteler et al., 2015a) application of fertilizers provided inconsistent results of BS incidence and BS grapes mineral nutrient content. Therefore this hypothesis seems to be unlikely as a primary cause of BS, nevertheless the determined effects on nutrient content could indicate a reduced or even lost functionality of the vascular tissue (Bondada, 2016).

Indeed, many studies focused on the integrity and functionality of the vascular tissue in BS affected grape clusters (Bondada and Keller, 2012a; Hall et al., 2011; Zufferey et al., 2015). Cell death coincided with the appearance of visible symptoms in the grape berry mesocarp and increases progressively with symptom severity (Bondada and Keller, 2012a; Krasnow et al., 2008, 2009). BS berries have a weak vascular connection through the brush with the pedicel and disorganized location of vascular tissues in the berries (Bondada and Keller, 2012b). Some studies have been focused on the xylem showing that the xylem mobile dye Basic Fuchsine could not penetrate the peduncles of BS clusters in contrast to H clusters when applied to the shoot base (Hall et al., 2011). Also xylem hydraulic conductance in rachis was not different between H and BS grapes (Zufferey et al., 2015). Viability stains (FDA) of the phloem of BS clusters gave first indications of a possible role of impeded phloem import towards berries as a crucial factor of BS development (Hall et al., 2011). Similarly, degradation of primary phloem and the appearance of hard non-functional secondary phloem within the rachis of severely BS affected samples were observed (Zufferey et al., 2015).

Although previous studies point towards a potential disturbance of the vascular tissue in BS development, the picture remains incomplete. Herein we report a comprehensive anatomical study of rachis and pedicels of BS grape clusters differing in their symptom severity to discriminate between early events in symptom development and potential follow up secondary symptoms. Our hypothesis sees the degradation of the vascular tissue as cause for the decreased support of BS grape clusters with assimilates. Thereby we used light, transmission and scanning electron microscopy, and energy-dispersive X-ray spectroscopy techniques to study the phloem, cambium and xylem in rachis and pedicels of H and BS affected grape clusters.

2. Materials and methods

2.1. Collection of plant material

Plant material for LM, SEM and TEM analyses was collected in commercial vineyards in Lower Austria and Burgenland (*Vitis vinifera* L. cv. Zweigelt grafted on Kober 5BB (*V. riparia* x *V. berlandieri*) rootstock) between 2013 and 2015. Samples (3–5 mm in length) for LM and TEM were collected from basal, middle and distal parts of rachis (having a diameter of ~4 mm) and randomly selected pedicels of H and BS affected berry clusters in 2013 and 2014 (Supplementary Fig. S1). In 2013, samples were collected from eight H and eight BS affected clusters taken from 16 individual plants and in 2014 from seven H and 11 BS affected clusters taken from 18 plants. Collected BS samples were categorized into two groups ('early' and 'late' symptoms) according to symptom severity on the berries. Samples with 'early' symptoms showed first symptoms of turgor loss but not yet shrinking berries, whereas samples with 'late' symptoms had obviously shrunken berries. Berries of all BS clusters had values for soluble solids between 10-15°Brix, whereas berries from H clusters reached values between and in excess of 17–20°Brix and (Table 1). Rachis and pedicel samples were collected and immediately transferred into a modified Karnovsky's fixative (2% (w/v) paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and 2% (v/v) glutaraldehyde (Fluka, Buchs, Switzerland)) in 100 mM sodium cacodylate buffer, pH 7.2. Samples for SEM and EDS analyses (H (n = 5) and BS (n = 6)) were collected from a commercial vineyard in Burgenland (Austria) in 2015. The BS samples were also divided into two batches, with 'early' or 'late' symptoms, as described above.

Table 1
Characteristics of H and BS samples. Samples were collected at two different time-points: BS samples showing early symptoms (BBCH 85) and those showing late or very severe symptoms (BBCH 89).

	BS samples	Healthy samples
Early symptoms	- Turgor loss - 10-12° Bx - Reddish colored	- Turgid - 17-20° Bx - Dark blue colored
Late symptoms	- Shrinkage - 12-15° Bx - Reddish colored	- Turgid - > 20° Bx - Dark blue colored

2.2. Microscopy procedures

2.2.1. Light microscopy

In the laboratory, H and BS samples were further fixed in a modified Karnovsky's fixative in cacodylic buffer for 2 h at room temperature under -0.4 MPa vacuum. Afterwards samples were washed four times in the same buffer for 10 min each at room temperature. They were dehydrated in a 10% graded ascending series of aqueous mixtures of ethanol for 30 min each. Thereafter, they were infiltrated and embedded in 2-hydroxyethyl methacrylate (HEMA, also known as glycol methacrylate or JB4 (Polyscience Inc., Warrington, PA, USA) following the manufacturer's instructions. Resin-infiltrated samples were embedded in molds (Polyscience Inc.) filled with freshly prepared embedding medium. Slots in molds were covered with block holders to remove air and stored at 4 °C overnight to control the exothermic resin polymerization. Polymerized resin cubes were mounted on wood holders and sectioned (5 µm thick sections) on a Leica rotatory microtome equipped with a D-knife (Leica Biosystems, Wetzlar, Germany) (Forneck et al., 2002; Sullivan-Brown et al., 2011; Vorwerk et al., 2008).

Three staining procedures enabled us to analyze the anatomy of the vascular system in rachis and pedicels: (1) single staining with 0.05% (w/v) aqueous solution of Toluidine Blue (Sigma-Aldrich) for 5 min at room temperature following a washing step in distilled water (Gerlach, 1984); (2) double staining with 0.1% (w/v) aqueous solution of Safranin (Sigma-Aldrich) for 1 min, washed with 70% ethanol, thereafter stained with a 1% (w/v) aqueous solution of Astra Blue (Sigma-Aldrich) for 10 min and washed with distilled water (Kraus et al., 1998); or (3) Fuchsin-Chrysoidin-Astral Blue (FCA or Etzold) triple staining performed with a mixture of 0.01% (w/v) new Fuchsine (Sigma-Aldrich), 0.014% (w/v) Chrysoidin (Sigma-Aldrich) and 0.012% (w/v) Astra