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Titolo della tesi

"*Vitis vinifera* and drought stress: physiological and anatomical responses”

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I. Summary

Climate change represents a threat to natural ecosystems as well as to agricultural production, due to the increase in the frequency and intensity of drought. Like many other crops, grapevine, despite being considered a drought tolerant crop, is threatened by climate change which can lead to a reduction in yields and grape quality and to the modification of the geographical distribution of the wine-growing areas. Multiple mechanisms and responses to counteract the negative effects of the water deficit are put in place by grapevine. Stomatal regulation, osmoregulation, reduction of growth and modification of xylem architecture are some of these acclimations. To support the adoption of strategies and actions in vineyard to face drought, the study of these mechanisms and responses is crucial. The purpose of this thesis fits into this context. In particular, it aims to contribute to the understanding and verifying any involvement of structural anatomical characters of the xylem or of non-structural carbohydrates (NSCs) in the response to the water deficit, in different *Vitis vinifera* cultivars. To this end, this thesis envisaged the design and implementation of experiments in which modulation of the NSCs content and the eventual relationship of this with other variables (i.e., xylem anatomy, degree of xylem embolism, growth) were investigated by imposition of water deficit with variable intensity and duration. What emerged from this work is that in young potted plants of *Vitis vinifera*, there is a NSCs-mediated response to short and severe drought stress, although there are slight differences between cultivars with different hydraulic behaviors (*i.e.*, isohydric and anisohydric). The accumulation of low molecular sugars in the period of stress could represent an acclimation strategy carried out by the grapevine with a potential role of osmoregulation, and / or xylem refilling and / or energetic support, consistently with what proposed by other authors. The response to drought stress in anisohydric cultivars would also involve short-term anatomical modifications at the xylem level (*i.e.*, reduction of the lumen of the newly produced tracheary elements) and a recovery mechanism of embolized vessels that could be driven by a possible thickening of the wall due to the adhesion of hygroscopic aqueous gels mediated by maltose-maltodextrin accumulation. The involvement of NSCs as osmo-compatible solutes has been blurred in the case of less

intense and more lasting drought stress, where decrease of plant growth appears to be the key adaptive mechanism.

II. Publications, Conferences, Training activities

PUBLICATIONS

Vuerich, M., Petrussa, E., Boscutti, F., Braidot, E., Filippi, A., Petruzzellis, F., Tomasella, M., Tromba, G., Pizzuto, M., Nardini, A. & Casolo, V. Contrasting responses of two grapevine cultivars to drought: the role of non-structural carbohydrates in xylem hydraulic recovery. Manuscript submitted for publication.

Vuerich, M., Braidotti, R., Sivilotti, P., Alberti, G., Casolo, V., Braidot, E., Boscutti, F., Calderan, A., & Petrussa, E. (2021). Response of Merlot grapevine to drought is associated to adjustments of growth and nonstructural carbohydrates allocation in above and underground organs. *Water*, 13(17), 2336. https://doi.org/10.3390/w13172336

Filippi, A., Braidot, E., Petrussa, E., Fabro, M., **Vuerich, M.**, & Boscutti, F. (2021). Plant growth shapes the effects of elevation on the content and variability of flavonoids in subalpine bilberry stands. *Plant Biology*, 23(2), 241–249. https://doi.org/10.1111/plb.13194

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Falchi, R., Petrussa, E., Braidot, E., Sivilotti, P., Boscutti, F., **Vuerich, M.**, Calligaro, C., Filippi, A., Herrera, J. C., Sabbatini, P., Zancani, M., Nardini, A., Peterlunger, E., & Casolo, V. (2020). Analysis of non-structural carbohydrates and xylem anatomy of leaf petioles offers new insights in the drought response of two grapevine cultivars. *International Journal of Molecular Sciences*, 21(4), 1457. https://doi.org/10.3390/ijms21041457

Filippi, A., Petrussa, E., Boscutti, F., **Vuerich, M.**, Vrhovsek, U., Rabiei, Z., & Braidot, E. (2019). Bioactive polyphenols modulate enzymes involved in grapevine pathogenesis and chitinase activity at increasing complexity levels. *International Journal of Molecular Sciences*, 20(24), 6357. https://doi.org/10.3390/ijms20246357

Vuerich, M., Ferfuia, C., Zuliani, F., Piani, B., Sepulcri, A., & Baldini, M. (2019). Yield and quality of essential oils in hemp varieties in different environments. *Agronomy*, 9(7), 356. https://doi.org/10.3390/agronomy9070356

CONFERENCE - ORAL COMMUNICATION

Vuerich, M.; Cingano, P.; Petrussa, E.; Braidot, E.; Pellegrini, E.; Mestroni, M.; Boscutti, F. Effects of flooding stress on the saltmarsh halophyte *Salicornia fruticosa* (L.) L.: upscaling perspectives. In: 116th annual congress of the Italian Botanical Society (VII International Plant Science Conference) 8-10 September 2021.

Vuerich, M.; Boscutti, F.; Braidot, E.; Filippi, A.; Nardini, A.; Petrussa, E.; Petruzzellis, F.; Pizzuto, M.; Casolo, V. Contrasting responses of two grapevine cultivar with different hydraulic behaviour to drought: the role of nonstructural carbohydrates in xylem embolism. Extended elevator speech in: Plant Biology Europe, 28 June - 1 June 2021.

Casolo, V.; Agliasso, C.; Cavalletto, S.; Morabito, C.; **Vuerich, M.**; Boscutti, F.; Secchi, F. Soluble carbohydrates metabolism sustains energetics and xylem hydraulic functionality upon relief from drought in *Populus tremula x alba*. In: 4th Xylem International Meeting, Padua, 25-27 September 2019.

CONFERENCE - POSTER COMMUNICATION

Rainis, S.; Fabro, M.; Danielis, R.; Dorigo, G.; Carnio, A.; Sigura, M.; Lami, F.; **Vuerich, M.**; Boscutti, F. Effectiveness of cover crops for supporting biodiversity and agronomic and ecosystem services in agricultural and rural landscapes. In: 24th ASPA Congress, Padua, 21-24 September 2021.

Scarpin, D.; **Vuerich, M.**; Braidot, E.; Petrussa, E.; Fellet, G.; Honsell, G.; Sivilotti, P.; Marchiol, L.; Degli Esposti, L.; Adamiano, A.; Iafisco, M. Calcium phosphate nanoparticles doped with copper ions as efficient tools for downey mildew prevention. In: Plant Biology Europe, 28 June - 1 June 2021.

Vuerich, M.; Boscutti, F.; Braidot, E.; Calderan, A.; Casolo, V.; Falchi, R.; Filippi, A.; Herrera, J.C.; Nardini, A.; Peterlunger, E.; Petrussa, E.; Zancani, M.; Sivilotti, P. Investigation of non-structural carbohydrates and xylem anatomy in petiole of grapevine varieties during water limitation and after re-irrigation. In: 4th Xylem International Meeting, Padua, 25-27 September 2019.

Filippi, A.; Petrussa, E.; Rabiei, Z.; **Vuerich, M.**; Braidot, E. Can agriculture be eco-friendly? Plant extracts as grapevine defense inducers. In: Jointed SIBV-SBI Congress, Padua, 4-6 September 2019.

OTHER PUBLICATIONS

Rainis, S.; Fabro, M.; Danielis, R.; Dorigo, G.; Lami, F.; **Vuerich, M.**; Boscutti, F. (2021) Potenziale contributo in termini di servizi agronomici ed ecosistemici delle colture di copertura (cover crop) - notiziario ERSA.

Calderan, A.; Petrussa, E.; Filippi, A.; **Vuerich, M.**; Nassivera, F.; Iseppi, L.; Butinar, L.; Reščič, J.; Bariviera, L.; Natale, S.; Nardini, A.; Mihelčič, A.; Vanzo, A.; Lisjak, K.; Sivilotti, P. (2020) Quale apporto idrico permette di ottenere una buona qualità delle uve di Terrano con un buon rapporto benefici/costi? in Atti della conferenza finale Progetto Agrotur II - Štanjel, 24 luglio 2020.

TRAINING ACTIVITIES

31st Dendroecological Fieldweek - Sta. Maria, Val Müstair, Switzerland, 28 August - 5 September 2021.

Agricultural Science and Biotechnology PhD winter school 2021 - Systems Biology.

Agricultural Science and Biotechnology PhD winter school February 2020 - Statistics and R.

Agricultural Science and Biotechnology PhD winter school February 2019 - Microscopy school.

1. Introduction

Among fruit crops, *Vitis vinifera* L. plays a role of primary importance at an economic and cultural level. Due to the large number of cultivars and their combinations with rootstocks, which allow adaptation to different environmental niches (Ollat *et al.*, 2019), it is grown worldwide, typically in temperate zones. According to OIV estimation (OIV, 2019), world vineyards cover a surface of approximately 7.4 million hectares and grape production stands at 77.8 million tonnes. Fifty-seven percent of these grapes are destined for wine production, equal to 292 million hectolitres, of which half is attributed to just three countries, Italy, France and Spain (OIV, 2019). Focusing on the areas intended for wine production, many of these are characterized by Mediterranean climates (*i.e.*, warm and dry summers) (Gambetta *et al.*, 2020) and by seasonal droughts (Chaves *et al.*, 2010). Albeit at the expense of a reduction in yield, moderate drought stress is functional to the qualitative improvement of the grapes, and therefore an irrigation deficit strategy is sought by producers (Gambetta *et al.*, 2020). However, an increase in the frequency and intensity of drought due to global warming, a phenomenon already observed in various regions of the world, and which will be exacerbated with a further increase in temperature, could compromise the balance between yield and quality. Indeed, as highlighted by the Intergovernmental Panel on Climate Change (Masson-Delmotte *et al.*, 2018), there is substantial evidence that human activities have had impacts on the global climate and that the Earth's temperature will increase by a further 1.5° C between 2030 and 2052 if the rate of increase remains equal to that current. Together with the soil, the climatic characteristics of a wine-growing area contribute to the determination of its terroir. Therefore, further climatic changes, in addition to compromising yield and quality, could also influence the geographical distribution of the wine-growing areas, leading in extreme cases to the loss of suitability for viticulture in some areas (Santos *et al.*, 2020). Therefore, the preservation of the current location of the wine regions and the maintenance of the productions in terms of quality and yield are issues of emerging concern (Ollat *et al.*, 2019; Gambetta *et al.*, 2020). To address these problems, there are several strategies. Short-term strategies involve changing cultivation practices, soil management and the adoption of irrigation. The change of training systems as well as the choice of different cultivars and rootstocks, on the other hand, fall within the long-term strategies (Santos *et al.*, 2020). However, to support the adoption of these strategies with a view to a more targeted and sustainable choice, the study of the physiology of adaptation of the different grapevine cultivars to drought is crucial (Gambetta *et al.*, 2020).

1.1. Water transport in grapevine: anatomy and physiology

Xylem anatomy

Together with the phloem, the xylem constitutes the vascular system of the grapevine, as well as of all other tracheophytes. Using the effective analogy proposed by Jensen and co-workers (2016), the xylem "is the water highway" with the task of carrying water from the roots to the leaves, where the phloem consists of "small country roads passing through the villages" along which sugars move from sources to sinks. As in other flowering plants (Jacobsen, 2015), in grapevine the secondary xylem in woody organs is a tissue made up of different types of cells such as fibers, parenchymatic cells, tracheids and vessel elements. The last two types of cells form the tracheary elements, which are the real subjects of water transport (Keller, 2015). The vessels are the largest tracheary elements and are basically hollow columns that can exceed one meter in length, and hundreds of micrometers in diameter. They are composed of modules, called vessel elements, which are stacked axially, separated by porous perforation plates. Vessels formed in the beginning of the growing season have simple perforation plates, differently from those forming the latewood, which have scalariform perforation plates (Evert *et al.*, 2006; Keller, 2015). Tracheids are less frequent and, differently from vessels, are single cells, smaller in both diameter and length, and occur mostly in the latewood. A secondary cell wall impregnated with lignin and the absence of protoplasm at maturity are characteristics common to both vessels and tracheids. These features make these cells suitable for transporting water in the grapevine. Before becoming active in the transport of water, these cells die, and organelles and cytoplasm are removed creating a lumen where the water can be transported. The lignification of the secondary cell wall makes the conduit almost impermeable and gives it the mechanical strength necessary to avoid collapsing under the negative pressure necessary for the translocation of water. However, to ensure the exchange of material between other tracheary elements and to the surrounding parenchyma cells, lack of secondary wall occurs in oval regions, called pits, which generate water flow resistance, but play an important role as capillary safety valve. In grapevine, pits are generally scalariform and bordered (*i.e.*, the secondary wall overarches the pit chamber reducing its aperture) (Keller, 2015).

Water transport physiology

Grapevine must acquire carbon dioxide from the atmosphere to carry out photosynthesis, and it does so simply by opening the stomata (Keller, 2015; Jensen *et al.*, 2016). However, the diffusion of CO_2 inside the leaf has an aftermath, the loss of water from the leaf into the atmosphere: in a C_3 plant such as grapevine, for each carbon dioxide molecule fixed through photosynthesis, about 500 molecules of water evaporate from the leaf mesophyll cells (Taiz and Zeiger, 2010). The outflow of water from plants is called transpiration (Keller, 2015). However, this counter-effect of the photosynthetic process is the triggering of water absorption from the roots to the soil and the water movement within the xylem (Tyree and Zimmermann, 2002). Indeed, the recognition to transpiration of the role of the driving force in water absorption and the peculiar chemical and physical properties of water (*i.e.*, high surface tension, cohesion or the strong tendency of H_2O molecules to "stick" together) are the principles on which the Cohesion-Tension theory, first articulated by Dixon and Joly (1895) and modified later by other authors (Steudle, 2001; Tyree and Zimmermann, 2002), is based. According to this theory, the water forms a *continuum* along the environment and whole plant, joining the roots from the soils, therefore the stem, to the leaves, therefore the atmosphere, in the so-called soil-plant-air *continuum* (SPAC) (Honert, 1948; Steudle, 2001). The tension generated by the evaporating surface of the leaf is transmitted along this *continuum* due to the cohesion property of water and therefore the water lost in transpiration is continuously replaced by water pulled up from the lower parts of the plant. The tension continues at ground level, where the roots absorb the water available in the soil to them, which in turns, becoming drier, draws water from more distant and wetter areas (Keller, 2015). In this way plants do not need active pump for moving water, but this process is passive and is determined by a gradient in water potential that is established along the SPAC. The water potential (Ψ, expressed in units of pressure Pa) is the measure of the free energy of water per unit molar volume of liquid water and it indicates the availability of water in any aqueous system (Keller, 2015). Water moves spontaneously from area with higher towards regions of lower water potential (Tyree and Zimmermann, 2002). The free energy of the outside air at a temperature of 25 °C and a relative humidity content of 50% is equal to -95.2 MPa (Taiz and Zeiger, 2010), whereas the water potential of the air spaces in a grapevine leaf in well watering condition is around -1.4 MPa: this huge gradient in terms of water potential is responsible for the transpiration. Going down the plant, the potential increases until it reaches values close to -0.4 in the root. Such value is still low enough to generate a potential gradient with the surrounding soil which in wet conditions has a water potential close to zero (Keller, 2015).

The tension triggered by the transpiration is only one of the components that contribute to the water potential. Indeed, the Ψ is described by the following equation:

$$\Psi = \Psi_P + \Psi_\pi + \Psi_M + \Psi_g$$

where Ψ_P is the pressure potential, Ψ_π the osmotic potential, Ψ_M the matrix potential and Ψ_g the gravitational potential.

Usually, the last two components are negligible: Ψ_M is in fact close to zero in plant tissues, while Ψ_g assumes significance only in the case of tall plants, therefore not in the case of *Vitis vinifera* which rarely exceeds a height of 2 m. On the other hand, Ψ_π should be considered since it measures the decrease of the water potential due to the presence of solutes in water. It is thus caused by the presence of dissolved solutes and decreases linearly as the solute concentration increases (Keller, 2015). A gradient in Ψ_π has basically no influence on the rate of bulk flow of water in the xylem, but it does play a role in the water flow at the membrane level: the movement of water is always from a region of lower solute concentration (*i.e.*, less negative water potential) to one of higher solute concentration (*i.e.*, more negative water potential) (Tyree and Zimmermann, 2002). Therefore, plant cells use osmolytes such as non-structural carbohydrates (NSCs), organic acids and inorganic ions to lower their Ψ and to attract water into the cells (Keller, 2015).

The convenience of the term water potential is due to its applicability at different scales, not only at the cellular level but to any medium with variable water content such as the soil-plant-air *continuum*.

1.2. Drought stress: responses and consequences in grapevine

The amount of water lost due to the transpiration and the amount of water absorbed by the roots determine the water balance of the grapevine. This balance can be rationalized and described by the following equation (Keller, 2015):

$$(\Psi_{leaf} - \Psi_{air})(r_s + r_b)^{-1} = (\Psi_{soil} - \Psi_{leaf})r_h{}^{-1}$$

The left side of the equation represents the flow of transpired water. This is a function of the water potential gradient between the air and the leaf, while it is inversely proportional to the resistance that the water molecules encounter to exit the leaf. This resistance is determined by r_s, the stomatal resistance (*i.e.*, degree of stomata aperture) and by r_b, the boundary layer resistance (*i.e.*, thin film of relatively moist air at the surface of the leaf). The right part of the equation instead describes the flow of absorbed water which is directly related to the water potential gradient between soil and leaf, while it is inversely proportional to the resistance that water encounters during translocation (hydraulic resistance, r_h), mainly determined by the friction of the water with the walls of the vessels and by the distance of the leaf bundles, and therefore by the xylem architecture (Tyree and Zimmermann, 2002; Keller, 2015).

The grapevine must absorb more water than the amount lost in transpiration to allow growth: even if 97% of the water is “simply” transpired, its growth is indeed mainly caused by cellular expansion due to the import of water (Taiz and Zeiger, 2010).

During drought stress conditions the Ψ_{soil} decreases and more negative root potentials are needed to absorb water. To maintain the water potential gradient that drives the flow of water, the Ψ in the shoot and in the leaves must decrease too. Frequently the conditions of water shortage are also accompanied by high air temperatures which determine a reduction in the relative humidity of the air and therefore a decrease in Ψ_{air} which, in cascade, determines a greater tension on the leaf and therefore an increase in transpiration (Taiz and Zeiger, 2010). In these conditions grapevine can no longer maintain a sufficient absorption to sustain transpiration and becomes drought stressed. Furthermore, the great tension generated applied to the water column inside the xylem can cause its rupture. Indeed, during translocation water is in a metastable state, and this condition is maintained by its property of cohesion and adhesion to walls of xylem conduits. Like a rope, if the water column inside the xylem is put under too much tension, high enough to cause vapor bubble formation, it can break, causing the expansion of air within the xylem conduits and the lumen of the xylem conduit is suddenly filled with air making the vessels no longer functional for the water transport. This sudden phase change, known as cavitation, has been experimentally observed to happen spontaneously in water-filled glass capillary tubes at -25 MPa (Briggs 1950; Caupin and Herbert 2006), far from the lowest tension values measured in plants and therefore spontaneous cavitation is rarely a common phenomenon (Nardini *et al.*, 2018). Actually, in plant the primary source of gas leading to embolism formation is the air entering from the pit membrane. If on the one hand the pits are fundamental for the creation of a network of connections between the vessels, on the other they represent potential air entry points. When the xylem tension reaches a critical threshold, air is unavoidably aspirated into the functioning conduit from the pits membrane of the functioning water-filled conduits neighbouring air-filled compartment. This process is known as air-seeding (Venturas *et al.*, 2017, Avila *et al.*, 2022, Nardini *et al.*, 2018). Among anatomical features of xylem conduits, pit structure and in particular pit membrane thickness represents a key feature in the embolism resistance: it has been observed that angiosperms having thick pit membranes are less prone to drought-induced embolism occurrence than species with thin membrane (Kaack *et al.*, 2021, Li *et al.*, 2016). The presence of gas-filled conduits may act as a source of embolism propagation if drought persists: from pits membrane of embolized vessels entry of gas can occur in the neighbouring vessels, making more vessels embolized (Avila *et al.*, 2022, Tyree and Zimmermann, 2002; Brodersen *et al.*, 2013). An excess of embolism can lead to a too low water supply, which can ultimately lead to hydraulic failure and cause plant death (McDowell *et al.*, 2008). The presence of embolism in fact leads to an increase in the resistance that water encounters in the plant (r_h) and therefore exacerbates the reduction of the already decreasing absorption (Tyree and Zimmermann, 2002).

However, to prevent or to tolerate the onset of xylem embolism, adaptive mechanisms are put in place by the grapevine in response to drought stress. These responses are complex and of different by their nature and temporal scale *i.e.*, hydraulic, physiological, morphological and anatomical (Simonneau *et al.*, 2017; Gambetta *et al.*, 2020).

The reduction of transpiration is one of short term actions that the plant carries out to avoid water loss. In order to do this, the plant increases stomatal resistance by closing the stomata and thus conserves water and avoids a drop in water potential at the leaf level (Keller, 2015). The triggers of this phenomenon are both hydraulics and abscisic acid (ABA) although the relative contribution of these two modes remains unclear (Gambetta *et al.*, 2020). During stress, ABA is synthesized at the root level, the first organ to perceive the scarcity of water, and translocated to the foliar apparatus. The increase in ABA at the leaf level in fact determines the closure of the stomata. However, this only happens when the stomatal conductance (*i.e.*, the reciprocal of r_s) reaches very low values. In fact, although the production of ABA at the root level is rapidly induced by water stress, its translocation to the leaves can be too slow in the case of sudden stress. Therefore, in this scenario, early stomatal closure is not driven by ABA, but is instead driven by changes in xylem tension, which is virtually instantaneously transmitted from root to leaf, where pressure turgor is altered. Only following the perception of this rapid signal does the leaf synthesize on site the ABA necessary for stomatal closure (Keller, 2015; Dal Santo *et al.*, 2016).

Depending on the degree of severity of the stress, this reduction in stomatal conductance has differential effects on photosynthesis. In fact, in the grapevine, only severe and long water stresses lead to an apparent inhibition of photosynthetic activity, whereas an increase in the intrinsic water use efficient is observable in the case of mild stress (Gambetta *et al.*, 2020). However, even in mild stress conditions, the reduction in plant growth, which in these cases occurs before stomatal closure, can still lead to a reduction of the photoassimilates due to the reduction of the canopy, the photosynthetic factory (Tarara *et al.*, 2011). Nonetheless, the still lasting photosynthetic activity, combined with this biomass reduction of the plant, guarantees a greater concentration of newly photoassimilated carbon in the different organs (McDowell, 2011). When the conditions of water stress worsen, the inhibition of photosynthesis and the reduction of growth do not allow the accumulation of reserves, causing the starch to run out, with the persistence of drought. However, the decrease in grape growth is not homogeneous among its organs: the root slows down its growth with a ratio lower than that of the epigeal part, causing an increase in the root-shoot ratio and guaranteeing the exploration of deeper soils horizons looking for water supply (Keller, 2015).

A further acclimation strategy carried out by the grape is osmotic adjustment, or osmoregulation, which permits continuous water transport at low water potentials, thus increasing plant drought resistance. The accumulation of osmotically active solutes, or osmolytes, in leaf cells is a response to the drought conditions that grapevine adopt to decrease the osmotic potential (Gambetta *et al.*, 2020). This leads to a lowering of their point of loss of turgor (Ψ_{TLP}), the Ψ at which the leaf cells lose turgor, allowing them to maintain the positive cell turgor despite the decrease of the water potential and the stomatal and hydraulic conductance in drier conditions (Bartlett *et al.*, 2014). Inorganic ions, non-structural carbohydrates, to a lesser extent amino acids and organic acids are the compatible solutes accumulated in the expanding leaves and root tips (Patakas *et al.*, 2002; Cramer *et al.*, 2007). The osmoregulatory capacity of the tissues decreases during their expansion while increases during the season, in other words the tissues developed at the end of the season have a better capacity for osmoregulation than those advanced at the beginning of the vegetative season (Patakas and Noitsakis, 1999).

While the previous regulation mechanisms act, within the water balance, mainly on the water potential of the leaf and on the stomatal resistance, the modification of the xylem architecture is an acclimation response to water stress that acts on the resistance of the plant. Under conditions of water deficit grapevine develop narrower tracheary elements than well-watered plants (Lovisolo and Schubert, 1998; Hochberg *et al.*, 2015). The reduction in the diameter of the vessel, although it increases the friction of the water as it flows inside the vessel (*i.e.*, the increase in r_h), which can lead to a reduction of the water flow absorbed, represents a response to water stress as smaller vessels are less susceptible to cavitation (Tyree and Dixon, 1986; Davis *et al.*, 1999). Still remaining in the context of xylem architecture, *Vitis vinifera* constitutively presents a peculiar characteristic defined as hydraulic vulnerability segmentation. Basically, in a plant that has this trait, annual organs are more vulnerable to xylem embolism than the perennial organs and this protects the latter from levels of water stress that lead to embolism. Indeed, leaves and petioles have narrower and shorter tracheary elements than those of the rest of the plant and with perforation plates that do not allow air to pass into the next vessel, functioning as hydraulic safety valves (Choat *et al.*, 2008). In this way the grapevine prefers to abandon the canopy of the current year rather than spreading embolisms in the xylem network of perennial structure (Gambetta *et al.*, 2020).

Despite the strategies to prevent xylem embolism seen above, embolism appears to be a condition to live with for many vascular plants (Secchi and Zwieniecki, 2011). Mechanisms to restore xylem transport functionality when water is ended are therefore needed for avoiding hydraulic failure. A mid-to-long term strategy for hydraulic recovery is the production of new xylem conduits by cambial activity to replace no longer non-functional conduits (Secchi *et al.*, 2017). Other mechanisms to restore water transport may act through the formation of a positive root pressure, water uptake through leaves and/or bark, and recovery under low residual xylem tension (Tomasella *et al.*, 2019). Xylem embolism removal due to the generation of positive root pressure is a mechanism that has long been known to occur when the soil is fully saturated and the transpiration has ceased: a positive pressure throughout the xylem is produced and the embolized tracheary element is filled again (Cochard *et al.*, 1994). The recovery of embolized conduits while water remains under tension in adjacent functioning conduits is defined refilling. Despite the refilling process is still controversial due to its apparent contradiction to laws of thermodynamics, it is more accepted and considered a paradigm for plant physiology (Secchi and Zwieniecki, 2011). Indeed, in support of this theory, modern techniques of imaging have allowed to see *in vivo* the ability of plants to refill embolized vessels under low tensions (Brodersen *et al.*, 2010; Zwieniecki *et al.*, 2013; Knipfer *et al.*, 2015). The same mechanism has been observed in grapevine using X-ray Micro Computed Tomography (Micro-CT) analysis without presence of root pressure (Knipfer *et al.*, 2016; Brodersen *et al.*, 2018). Despite the great research activity in this context, a definitive mechanistic explanation for xylem hydraulic recovery from embolism under tension has not yet been provided. However, what has been pointed out by multiple studies is that the refilling process requires both a source of energy to overcome existing energy gradients acting against it, and a source of water to fill empty conduits (Secchi *et al.*, 2017). Indeed, recovery from xylem embolism under tension, cannot be a spontaneous process but necessitates of energy-driven and active processes in the xylem to promote water flow into embolized conduits. Existing models of xylem repair suggest that embolism formation is sensed by the plant, although the mechanism is not known yet, and that this acts as a trigger for xylem parenchyma cells to load solutes into the residual droplets of water still present in the adjacent embolized conduit. This generates a drop in osmotic potential which thus reaches water potentials sufficiently negative to allow water to flow into tracheary elements (Zwieniecki and Holbrook, 2009; Nardini *et al.*, 2011). Parenchyma cells with less solutes and water become sink and this induce a discharge of sugars and water from the phloem. Therefore, living parenchyma cells can function as bridges between phloem and embolized conduits (Nardini *et al.*, 2011).

In this context starch metabolism seems to be at the forefront of plant response to embolism. In fact, xylem parenchyma cells were often reported to have less starch content during water scarcity and embolism formation and this was associated with a contemporary increase in stem sucrose content (Salleo *et al.*, 2004; Regier *et al.*, 2009; Secchi and Zwieniecki, 2011; Tomasella *et al.*, 2019). This can lead to speculation that the solutes used as osmolytes to lower the osmotic potential, and therefore of the water potential, within the embolized vessels are soluble non-structural carbohydrates deriving from local starch hydrolysis as well as from phloem export. Moreover, the energy required for overcoming the energy gradients opposing the refilling process could be provided by the starch mobilization (Salleo *et al.*, 2004; Zwieniecki and Holbrook, 2009). Non-structural carbohydrates therefore play a fundamental role in contrasting the risk of hydraulic failure, whether they are used both directly or indirectly for the production of new tracheary elements or as solutes or energy sources. This scenario highlights how the maintenance of high levels of non-structural carbohydrates during stress and their availability at the time of the recovery phase are essential for vigor and survival of the plant (Tomasella *et al.*, 2019).

One of the peculiarities that make *Vitis vinifera* particularly attractive for research in the field of drought stress is the fact that the same species includes cultivars that respond in different ways, along all the nuances of the iso/anisohydric spectrum, classification introduced for the first time in grapevine by Schultz (2003). At one edge of this spectrum, cultivars with an isohydric behavior react to drought with a very sensitive stomatal closure response, regulated by a faster transcriptomic response of ABA-related genes which leads to avoid a drop in the water potential. On the other hand, anisohydric cultivars maintain higher stomatal conductance and transpiration rates, and their Ψ_{leaf} markedly decrease consistently with decreasing soil water availability or increasing atmospheric water demand, showing a slower sensitivity (Schultz, 2003; Lovisolo *et al.*, 2010; Dal Santo *et al.*, 2016; Simonneau *et al.*, 2017). These differences among cultivars are also reflected in hydraulic resistance (Hochberg *et al.*, 2015) and activity of aquaporins in the roots, which are regulated to adjust water flow to transpiration rate (Vandeleur *et al.*, 2009). Despite the iso- or anisohydric could presumably indicate the cultivar performance under drought or the underlying physiological responses, it is still unclear which hydraulic behaviour is the best adapted to drought (Gambetta *et al.*, 2020). This is also due to the fact that the same cultivar can behave as iso- or anisohydric, depending on the conditions (Lovisolo *et al.*,

2010) and the environmental factors, being at least as significant as the genotype, should be considered (Hochberg *et al.*, 2018).

The variability in the drought response strategies shown by different grape cultivars under mild or severe drought could be also ascribed to specific anatomical parameters of the xylem vessel network and/or to different non–structural carbohydrate-involved mechanisms, nonetheless a definitive comprehension of these factors influencing the plant susceptibility or tolerance to drought-induced embolism has far to be fully elucidated.

1.3. Aim of the thesis

In this context, this thesis aims to contribute to the understanding of the response mechanisms of different cultivars of *Vitis vinifera* to water deficit. In particular, considering that there are few and not definitive scientific works aimed at verifying any involvement of structural anatomical characters of the xylem or of non-structural carbohydrates in the response to the water deficit, during the three-year research activity several experiments have been designed and conducted in order to try to clarify these aspects of investigation.

The common denominator of these experiments was the imposition of a water deficit, which had a variable intensity and duration in the different experiments, and the choice of the investigation variable, *i.e.,* the content of the main carbohydrates, according to the evidence of their role in stress tolerance and recovery. The various experiments then aimed at evaluating the possible relationship between the modulation of the NSCs content and other acclimations and stress responses, in particular anatomical traits, embolism and morphological traits.

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2. NSCs and xylem anatomy

2.1. Introduction to the study | Chapter 2

Being more vulnerable to embolism than perennial organs, the petioles play a very important role in *Vitis vinifera* during water stress, acting as safety valves according to the hydraulic vulnerability segmentation model.

By virtue of this peculiarity, it has been asked whether at the level of this organ variations in the non-structural carbohydrates content and anatomical modifications induced by water stress were recordable, and if these modifications had a different trend and intensity in two distinct grapevine cultivars, classified as iso- and anisohydric.

To verify these aspects, one-year-old grapevines belonging to the Syrah and Cabernet Sauvignon cultivars were subjected to a short - *i.e.*, 10 days - and intense stress - *i.e.*, removal of irrigation - followed by the resumption of watering. Before the start of the experiment and two days after the resumption of irrigation, samples were collected for anatomical analysis and for the quantification of non-structural carbohydrates, which were also quantified at the end of the drought phase.

2.2. Analysis of Non-Structural Carbohydrates and Xylem Anatomy of Leaf Petioles Offers New Insights in the Drought Response of Two Grapevine Cultivars

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Keywords: Cabernet Sauvignon; Syrah; glucose; maltose; starch; sucrose; conduits area; drought; recovery

ABSTRACT

In grapevine, the anatomy of xylem conduits and the non-structural carbohydrates (NSCs) content of the associated living parenchyma are expected to influence water transport under water limitation. In fact, both NSC and xylem features play a role in plant recovery from drought stress. We evaluated these traits in petioles of Cabernet Sauvignon (CS) and Syrah (SY) cultivars during water stress (WS) and recovery. In CS, the stress response was associated to NSC consumption, supporting the hypothesis that starch mobilization is related to an increased supply of maltose and sucrose, putatively involved in drought stress responses at the xylem level. In contrast, in SY, the WS-induced increase in the latter soluble NSCs was maintained even 2 days after re-watering, suggesting a different pattern of utilization of NSC resources. Interestingly, the anatomical analysis revealed that conduits are constitutively wider in SY in well-watered (WW) plants, and that water stress led to the production of narrower conduits only in this cultivar

INTRODUCTION

The health decline of woody plants subjected to drought has been ascribed to two main processes: hydraulic failure and carbon starvation (McDowell *et al.*, 2008). The first process results in impaired water transport due to xylem embolism formation. In some plants, the occurrence of early embolism in distal sectors leads to the sacrifice of short-lived organs (*i.e.*, leaves), thus reducing transpiring surface and energy needs. This strategy was postulated for the first time by Zimmerman (1983), and it is known as "vulnerability segmentation". This concept was later tested using grapevine as a model plant, because of its marked susceptibility to petiole xylem embolism, and leaf shedding has been suggested to be a strategy to prevent embolism propagation from petioles to stem vascular tissues (Hochberg *et al.*, 2016; Charrier *et al.*, 2018).

Plants suffering reduced hydraulic efficiency also undergo a decrease in photosynthesis due to stomatal closure, leading to the consumption of non-structural carbohydrate (NSC) reserves. It has been suggested that the hydraulic behavior of different plant species influences the occurrence of this phenomenon (McDowell, 2011; Kannenberg *et al.*, 2019). In particular, isohydric species are considered to be more exposed to carbon starvation risk when compared to anisohydric ones, due to the prompt stomata closure in the case of water deficit (Tardieu and Simonneau, 1998; McDowell *et al.*, 2008). However, this assumption needs to be taken with caution, considering that the isohydric/anisohydric definition has been recently revised, pointing out that this classification can be significantly affected by the environment and other intrinsic traits of the plant (Garcia-Forner *et al.*, 2016; Martínez-Vilalta and Garcia-Forner, 2017; Hochberg *et al.*, 2018).

In woody plants, xylem water transport can be restored after a water stress period by producing new xylem conduits (Zhang *et al.*, 2019), and/or retrieving water in the xylem to refill embolized conduits (Trifilò *et al.*, 2014; Savi *et al.*, 2016*b*; Yoshimura *et al.*, 2016; Gleason *et al.*, 2017; Barbara Beikircher *et al.*, 2017; Love and Sperry, 2018). It has been hypothesized that the latter process involves soluble NSCs decreasing the osmotic potential in the embolized xylem, thus promoting water movement from the nearest parenchymatic cells to the gas-filled conduits (Secchi and Zwieniecki, 2011, 2016; Nardini *et al.*, 2011). The degradation of starch seems to play a major role in embolism repair by providing high levels of sucrose in parenchyma cells adjacent to xylem conduits (Bucci *et al.*, 2003; Salleo *et al.*, 2004, 2009; Secchi and Zwieniecki, 2011). Furthermore, enhanced concentrations of glucose, fructose, sucrose and other products of starch degradation (e.g., maltose) have been recently observed in the xylem sap of poplars subjected to drought (Pagliarani *et al.*, 2019).

Grapevine is cultivated worldwide in several contrasting climates. Increasing mean daily temperatures have been recorded over the past decades in many vine-growing regions, with seasonal trends characterized by milder winters and more frequent summer droughts. Due to the economic importance of this crop, such changes have stimulated investigations of grapevine responses to drought (Lovisolo *et al.*, 2010; Hochberg *et al.*, 2017; Perrone *et al.*, 2012; Cramer *et al.*, 2007; Tyerman *et al.*, 2012). Indeed, the global scarcity of water resources is becoming a major limiting factor in grapevine cultivation (Vinocur and Altman, 2005). Drought stress responses are strictly dependent on the specific adaptation of each cultivar to environmental conditions (Catacchio *et al.*, 2019). Chouzouri et al. (2005) reported that xylem anatomical traits significantly influenced gas exchange and vulnerability to embolism. In particular, the number and the size of xylem conduits are key factors in modulating the hydraulic responses in grapevines. Recent studies confirmed that NSCs have a crucial role in sustaining grapevine metabolism under drought conditions (Quintana-Pulido *et al.*, 2018). Falchi et al. (2019) reported that dormant canes of cv. Syrah (SY) and Cabernet Sauvignon (CS), subjected to an early or late drought stress in the previous growing season changed their soluble NSC pools in both wood and bark. Accordingly, after recovering from water stress, drought affected the leaf petiole transcriptome and, in particular, genes related to sugar metabolism (Perrone *et al.*, 2012).

Among the adaptive strategies adopted by anisohydric grapevine cultivars under drought stress, leaf osmotic adjustment has been proposed to occur by active accumulation of carbohydrates and other osmolytes together with anatomical trait modulation (Lovisolo *et al.*, 2010). However, contrasting results have been reported in the literature regarding the contribution of NSC to active osmotic adjustment in drought-stressed grapevines (Rodrigues *et al.*, 1993; Patakas and Noitsakis, 2001; Patakas *et al.*, 2002). The present study aimed at investigating the impact of a short and severe drought stress on petiole anatomical traits and NSC content of SY and CS grapevines, in order to outline the cultivar-specific drought response strategies to cope with drought at petiole level.

MATERIALS AND METHODS

Plant Material and Experimental Design

The experiment was carried out at the experimental farm of the University of Udine, located in the Friuli Venezia Giulia region (North-Eastern Italy; 46°02’ N, 13°13’ E; 88 m a.s.l.). Twenty-four vines of cv. Cabernet Sauvignon R5 (*Vitis vinifera* L.) and cv. Syrah ISV-R1 (*Vitis vinifera* L.), two years old, both grafted on S.O.4, were planted in March 2016 in 20 L plastic pots, filled with field sieved soil supplemented with 10% perlite. The plants were grown under a clear plastic film tunnel (EVA ethylene-vinyl-acetate) opened on the sides as described in Herrera et al. (2015). The plants were organized in a completely randomized experimental design encompassing three rows. During the growing season, the vines were trained vertically and when the shoot length exceeded the height of the trellising system (2.1 m), the shoot was positioned horizontally in the last catching wire. Water was supplied by a drip irrigation system with three emitters per pot (PCJ 2 L h^{-1}, Netafim, Israel) and a set of vines was positioned under weighting mini-lysimeters to measure evapotranspiration (ETc), as described in Hochberg et al. (2017). During the season, the irrigation was maintained at 100% ETc, until treatments were imposed from 8–18 August: WW, well-watered vines; WS, water-stressed vines (irrigation withheld by removing the drippers from the pots). At the end of the stress treatment, the water-stressed vines were re-irrigated at field capacity and samples were harvested two days later. The predawn leaf water potential (Ψ_{pd}) and stem water potential (Ψ_{stem}) were measured (at 5 time-points) on fully expanded leaves at 6 am and 1 pm, respectively. To measure the water potential, the leaves were bagged immediately (for Ψ_{pd}) or 1 h for (Ψ_{stem}) before measurement and they were excised from the shoot using a sharp blade. The bagged leaves were placed into the pressure chamber (Soil Moisture Co., Santa Barbara, CA, USA), pressurized using a nitrogen tank, and Ψ was recorded when the initial xylem sap was observed emerging from the cut end of the petiole. The LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) device was used to measure photosynthesis (P_N; mmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) and transpiration (E; mmol H_2O m^{-2} s^{-1}) using a constant light intensity (1000 µmol photons m^{-2} s^{-1}) and CO_2 concentration (400 µmol mol^{-1}). The leaf water potential and gas exchange measurements (three replicates x treatment) were carried out on clear and sunny days at the beginning of the trial before the imposition of stress, during water stress and after recovery on both SY and CS vines and both WW and WS treatments. K_{LEAF} was calculated as $E/(\Psi_{pd} - \Psi_{stem})$.

Anatomical Measurements and Theoretical Hydraulic Conductivity

Petioles from leaves sampled for Ψ_{pd} measurement were separated into two portions; the basal portion was used for that quantification of non-structural carbohydrates (NSCs), and the distal portion was used for anatomical analysis. Sampling was performed before dawn, and therefore the glucose and maltose/sucrose levels in petioles were not dependent on photosynthetic activity. Consistently, after a whole night, the starch level was also at a minimum (Weise *et al.*, 2006), being the sum of residual transitory starch and of secondary starch. After sampling, the cross-sections of petioles were processed as described by Falchi et al. (2019). Briefly, 6-µm microtome sections (stained with safranin/alcyan blue) were prepared (see Figure 2 as example) and three digital slides for each cultivar and for each treatment were acquired with an Aperio CS scanner (Leica, model AT2, Wetzlar, Germany) at 5X magnification and anatomical parameters were analyzed by open-source Fiji software (Schindelin *et al.*, 2012). The following parameters were analyzed: conduit number, total conduit area, average conduit area. The theoretical hydraulic conductivity (K_t; Kg MPa^{-1} s^{-1}) was estimated using the Hagen–Poiseuille's modified by Tyree and Ewers (1991) as $\rho\pi\ 128\ \eta^{-1}\ \Sigma\ D^4$, where D is the radius of the conduit in meters, ρ is the fluid density (assumed to be 1000 Kg m^{-3}) and η is the viscosity (assumed to be 8.9×10^{-10} MPa). The theoretical specific hydraulic conductivity (K_{ts}; Kg MPa^{-1} s^{-1} m^{-1}) was calculated by dividing K_t by the cross-sectional area of the petiole (PA).

Non-Structural Carbohydrates (NSC)

The extraction and analysis of carbohydrates followed the protocol of Quentin et al. (2015) adapted to small amounts of material, as described by Savi et al. (2016*a*). Petioles were immediately microwaved for 2 min at 600 W to block enzymatic activities. To avoid the loss of NSC due to tissue burning, the operation was made in a water-sutured space. Further samples were dried at 60 ◦C overnight. Then, they were ground into fine powder in a mortar under liquid nitrogen. Aliquots of 20 ± 2 mg of powder were suspended in 0.3 mL ethanol 80% (v/v) twice, incubated at 80 °C for 30 min, and then dried at 60 °C overnight. A third suspension was made with 0.5 mL of 50 mM Tris-HCl pH 7.5 and used to re-suspend the crystalized carbohydrates resulting from previous steps. The supernatants obtained after incubation at 80 °C and a 14,000× g centrifugation for 5 min were used for soluble sugar measurement, while the pellet was re-suspended in 1 mL of acetate buffer (0.4 M $NaCH_3COO$, pH 4.6), and used for starch quantification. Glucose was quantified in a VICTOR3 Multilabel Counter Plate Reader (Perkin Elmer, Boston, MA, USA) after enzymatic conversion to NADPH by 0.1 U per sample of hexokinase and glucose−6-P dehydrogenase at 37 °C for 10 min in 100 µL of assay buffer (50 mM Tris-HCl pH 7.5 with 5 mM $MgCl^2$ and 125 µM $NADP^+$). For sucrose analysis, 100 µL of the supernatant was placed in a 1.5 mL Eppendorf tube with 300 µL of acetate buffer with 25 U of invertase to break down sucrose into fructose and glucose. The same procedure was followed for soluble starch degradation products—maltose disaccharide fraction and maltodextrin oligosaccharides—using 25 U of α-amyloglucosidase per sample (in the text maltose was used to indicate this pool of sugars coming from starch degradation). The amount of soluble carbohydrates was then expressed as mg glucose g^{-1} DW by using a calibration curve of known amounts of glucose. The starch contained in the pellets was digested overnight at 55 °C with 100 U α-amylase and 25 U α-amyloglucosidase per sample. The amount of starch (mg g^{-1} DW) was calculated by means of a calibration curve made with the known amounts of amylose from potato starch (all the chemicals was form Sigma-Aldrich, Milan, Italy).

Statistical Analysis

The effect of treatment on the functional parameters of the two cultivars was tested using a three-way analysis of variance (ANOVA). As all the parameters related to plant water status were highly correlated ($r > 0.7$; $p < 0.001$; Figure S1), we used the predawn leaf water potential (Ψ_{pd}) as the dependent variable and the treatment, cultivar, time and their interactions as fixed factors.

The distribution of xylem conduit size was compared between grapevine cultivars, before and after the treatment, and within each cultivar, for water-stressed vs. well-watered plants. The xylem conduit distribution was represented by density plots. Paired differences in conduit size distribution were tested by the two-sample Kolmogorov-Smirnov test ($p < 0.05$).

Variations in K_{ts} between cultivar, treatment, time after treatment and their interactions were tested using a three-way analysis of variance (ANOVA).

Preliminarily, the correlations of measured NSC (*i.e.*, glucose, maltose, sucrose and starch) were assessed by Pearson test. Due to the high collinearity between sucrose and maltose ($r > 0.7$, $p < 0.001$) and sucrose and starch ($r > 0.4$, $p < 0.001$) (Figure S2), sucrose was considered as interchangeable with maltose content and was not included in the box plot of NSC concentration (Figure 2). The variations in NSC between cultivar, treatment, time after treatment and their interactions were tested using a three-way analysis of variance (ANOVA).

The relationships between NSC content (*i.e.*, glucose, maltose and starch) and petiole xylem anatomy in the two cultivars were tested using linear models. The conduit number was used as a proxy of anatomy variation, as it showed high correlation with the other parameters (Figure S2). Each model included NSC as the dependent variable and grape cultivar, number of conduits and their interaction as fixed effects. Consistently, the content of NSC was also related to predawn leaf water potential, comparing the interaction with the two cultivars. The models were simplified with a manual backward stepwise procedure, where significance was missing ($p > 0.05$).

All the statistical analyses were performed in R statistical software (R Core Team, 2020) and model assumptions (*i.e.*, linear models and ANOVA) were checked by diagnostic plot of residuals. Outlier records were detected by the function “outlier Test” of the ‘car’ package (Fox and Weisberg, 2019) and removed from models (where $p <0.01$).

RESULTS

Water Status of Cabernet Sauvignon and Syrah Subjected to Water Deficit

Predawn leaf water potential (Ψ_{pd}) was chosen as a representative of grapevine water status, with all other functional parameters (Section 4.1) being highly correlated (Figure S1). Water stress (WS) significantly affected the predawn leaf water potential (Ψ_{pd}). No difference emerged between SY and CS, but the interaction treatment x cultivar evidenced contrasting responses of the cultivars to WS (Table 1). Ψ_{pd} in well-watered (WW) vines was similar in both cultivars over the whole experiment (Figure 1). As expected, drought induced a time-dependent decrease in Ψ_{pd}. In particular, CS underwent a mild reduction in water status, since at Day 5 and 8 the Ψ_{pd} was similar, whereas SY suffered a larger and significant drop in Ψ_{pd}. At the end of the water stress period (10th day), SY had more negative Ψ_{pd} than CS. Re-irrigation allowed a prompt recovery of Ψ_{pd} in both cultivars.

Petiole Xylem Anatomy and Theoretical Hydraulic Conductivity

At the end of the experiment, the difference in the distribution of xylem conduit dimensional classes between CS and SY in WW vines was statistically significant, showing that SY had larger conduits than CS (Figure 2a,c,e,g; D = 0.14; $p<0.001$). Nonetheless, in WS vines, the mean size of conduits was similar in both cultivars (90 and 100 μm^2 for CS and SY, respectively—Figure 2b,d,f,h; D = 0.29, $p < 0.001$). In detail, the CS conduit size distribution was similar in WW and WS (Figure 2a,b,e,f; D = 0.02, $p = 0.43$). The distribution of conduit size in SY was significantly affected by the treatment (Figure 2c,d,g,h; D = 0.14, $p<0.001$) as WW vines had larger conduits, with higher mean size when compared to WS vines (~140 vs. 100 μm^2, respectively).

Consistently with the above-described differences in conduit size, the theoretical specific hydraulic conductivity (K_{ts}) also displayed a significant difference in the interaction time x cultivar, but did not reveal significant differences due to cultivar or time (Table 2). However, whereas in CS the K_{ts} was similar in all treatments, in SY it increased from 0.20 ± 0.08 Kg MPa^{-1} s^{-1} m^{-1} at time zero to 0.41 ± 0.22 Kg MPa^{-1} s^{-1} m^{-1} and 0.35 ± 0.04 MPa^{-1} s^{-1} m^{-1} in WW and WS, respectively, at the end of the experiment.

Non-Structural Carbohydrates in Petioles

The glucose concentration in petioles was different between the cultivars (Table 3). The concentration was higher in CS as compared to SY, and it significantly decreased during the experiment in both cultivars, especially in SY (Figure 3a).

Since sucrose and maltose concentrations were highly correlated (Figure S2), maltose was also used to represent sucrose. Maltose significantly changed in response to all variables and in several interactions (*i.e.*, time × treatment, time × cultivar and treatment × time × cultivar) (Table 3). A decreasing trend in WW samples of both CS and SY was detected, with overall lower concentration in CS (Figure 3b). On the contrary, WS treatment induced an increase in maltose concentration in both cultivars compared to the control, with a larger increase in CS. However, after re-watering (2 days of recovery) the two cultivars showed opposite behaviour, as maltose concentration returned to the same level as the controls in CS, while it did not change in SY. The starch content also underwent significant changes in response to both treatment and cultivar (Table 3). In WS vines, starch concentration was significantly lower than in the control, regardless of the cultivar (Figure 3c), and even after 2 days from re-watering.

Relationships between Petiole NSC Content and Anatomy

Significant relationships emerged between the concentration of both glucose and maltose, and the total number of petiole xylem conduits, with cultivar-specific trends (Table 4). In CS, the number of conduits was negatively correlated with the concentration of both glucose and maltose (Figure 4a,b), whereas in SY maltose and glucose were nearly unrelated to this anatomical trait.

On the contrary, in both cultivars the relationship between starch and the number of conduits showed similar trends, with a decreasing number of conduits along with increasing starch levels and starch concentration in SY always being higher than in CS (Figure 4c). Consistently, for starch, no significant interaction between the number of conduits and cultivar was found (Table 4).

Relationships between Petiole NSC and Ψ_{pd}

In the two cultivars, maltose content was related to changes in Ψ_{pd} with similar trends but different magnitudes (Table 5). Maltose concentration increased in petioles with low Ψ_{pd}, especially in CS (Figure 5a). On the contrary, petiole starch content strongly decreased at low Ψ_{pd} in both cultivars.

DISCUSSION

Water Status and Petiole Anatomy

Grapevine cultivars respond to water limitation by adopting specific hydraulic strategies, ranging from conservative to dissipative water use (Lovisolo *et al.*, 2010). These contrasting adaptations involve various degrees of stomatal closure, xylem anatomical traits (size and number of xylem conduits), hormonal signaling, and eventual capacity for embolism recovery (Dal Santo *et al.*, 2016).

In grapevine, the leaves and petioles are the sectors most vulnerable to xylem embolism, acting as safety valves to protect xylem integrity in other organs, like the roots and stem (Zufferey *et al.*, 2011; Hochberg *et al.*, 2016; Charrier *et al.*, 2016). As a result, hydraulic segmentation might lead to daily or seasonal cycles of embolism events during water shortage periods in leaves and petioles, and the subsequent recovery of hydraulic functioning.

Regardless of the mechanisms proposed (Charrier *et al.*, 2016), xylem refilling is an energy-demanding process and it is supposed to require starch reserve mobilization (Salleo *et al.*, 1996; Pagliarani *et al.*, 2019). In the present study, we compared petiole anatomical traits and NSC content of two grapevine cultivars (CS and SY) subjected to either water stress (WS) or well-watered (WW) regimes.

The results confirm that a ten-day-long water limitation imposed to CS and SY potted grapevines strongly influenced leaf gas exchanges (result not shown) and water status, as shown by the drop in Ψ_{pd}. Although the isohydric/anisohydric classification should be cautiously assumed (Lavoie-Lamoureux *et al.*, 2017), a more marked Ψ_{pd} drop under drought would be expected in SY, since it is supposed to be a near-anisohydric cultivar (Schultz, 2003; Soar *et al.*, 2006; Hochberg *et al.*, 2014). In contrast, CS is generally recognized as isohydric (Schultz, 2003; Hochberg *et al.*, 2013; Tramontini *et al.*, 2014; Quintana-Pulido *et al.*, 2018) and hence should show higher Ψ_{pd} during stress treatment. Consistently, our analysis revealed some varietal differences, since SY exhibited a more negative Ψ_{pd} compared to CS, supporting the hypothesis that SY is less prone to rapid stomatal closure under water shortage.

According to the literature (Munitz *et al.*, 2018; Netzer *et al.*, 2019), a close relationship between xylem architecture and response to water stress has been proposed, although other factors could also play significant roles. In grapevine, a strong correlation was demonstrated between stem diameter, xylem conduit diameter and hydraulic properties by comparing young shoots and mature trunks (Jacobsen *et al.*, 2015). In the case of petioles, a high proportion of small conduits is expected to lead to lower theoretical hydraulic conductivity. However, at time zero, there was no significant difference in K_{ts} between CS and SY, since the small size of conduits in CS was counterbalanced by their higher number, as compared to SY (see also Figure S2, being average conduit area inversely related to conduit number). On the other hand, SY petioles showed wider conduits, as compared to CS. In response to drought, SY showed a significant reduction in the frequency of large xylem conduits, which could be ascribed to a shift in the petiole cambium differentiation. As a result, new conduits formed with a size similar to that observed in CS. These observations would support the hypothesis that SY could be more vulnerable to embolism when exposed to water limitation (Shelden *et al.*, 2017), whereas CS could be anatomically pre-adapted to water stress, exhibiting constitutively smaller conduits. These findings are consistent with reports by Netzer et al. (2019) and Munitz et al. (2018), which described cambial activity, conduit diameter and hydraulic conductance in response to limited water availability. On the other hand, SY showed increased K_{ts} in both WW and WS at the end of the experiment. Nevertheless, this parameter was not different between cultivars, because the drought-driven production of smaller conduits in SY was compensated by their increased number. Our results suggest the existence of a cultivar-specific plasticity of cambial cells, leading to the production of different dimensional classes of xylem conduit. This also implies that a well-coordinated developmental process in SY is likely to contribute to the maintenance of the water balance when drought episodes occur during the season. Taken together, these analyses confirm not only that the petiole xylem anatomy in SY is different from that of CS, but also that the varietal responses to hydraulic impairment could be ascribed to a change in cambial differentiation at the bundle level, ultimately affecting conduits dimension and distribution.

Petiole Non-Structural Carbohydrates

The controversial role of NSC pools in the plant response to drought, due to the interaction and balance of these pools between plant organs and tissue partitioning at different time scales (Nardini *et al.*, 2018; Tomasella *et al.*, 2020), prompted us to compare petiole NSC content in water stressed and well-watered grapevines, in order to elucidate their possible changes under drought and recovery. Our results confirm significant differences in NSC concentration in the petioles of the two cultivars. Starch storage represents the overall metabolic response, but it is apparently not directly related to glucose (Sulpice *et al.*, 2009). Correspondingly, we show that glucose and starch pools are not related and, since glucose did not even correlate with maltose/sucrose (Figure S2), we could argue that its concentration mirrors the signaling (Ehness *et al.*, 1997; Smeekens *et al.*, 2010) and energetic status (Petrussa *et al.*, 2018) requirements. Therefore, the decrease in glucose observed during the experiment could indicate a reduction in metabolic activity in the tissues.

The relationship between starch reserves and maltose content is crucial to explaining the physiological drought response based on the osmotic control of the decreasing water potential, as already investigated by Patakas et al. (2002). In our experiment, the drop in the leaf water potential in stressed plants was counteracted by an increase in maltose/sucrose in both cultivars, which may modify the osmotic potential, thus allowing turgor maintenance. Interestingly, CS-stressed plants exhibited an almost complete depletion of maltose at the recovery stage, whereas SY displayed steady levels of the same sugar. This observation supports the hypothesis that CS stress response could be mainly based on NSC utilization, whereas, in SY, anatomical adjustments would also account for drought tolerance. On the other hand, in both SY and CS stressed plants, the levels of starch did not show any significant change at the recovery stage. In fact, the modifications induced by drought could not be reversed in a short time by the restoration of the water supply, similarly to what was observed in Mediterranean trees recovering from severe drought stress (Lloret *et al.*, 2018).

NSC Content, Water Status and Xylem Architecture as an Integrated Cultivar-Specific Response to Drought

The relationships between NSC content and predawn leaf water potential (Table 5 and Figure 5) supported the putative role of NSC dynamics in plant responses to drought. Both cultivars displayed a similar trend in petiole starch concentration in response to Ψ_{pd}

variations. Conversely, maltose/sucrose accumulation showed a significant difference between cultivars (Table 5) and the concentration of these sugars, being related to the magnitude of stress, displayed in CS a much more pronounced negative relationship. On the other hand, SY exhibited an intermediate behavior between anisohydric and isohydric extremes, possibly taking advantage of the osmotic effect of maltose/sucrose to face drought stress, although this was not the only response strategy adopted by this cultivar. It could suggest the involvement of maltose in the recovery process; this hypothesis is supported by the significantly higher content of maltose in SY during the recovery stage. Starch reserves and the number of conduits were negatively correlated in both cultivars (Figure 4). This can be explained by the inverse correlation, found in petioles, between xylem conduit number and the area occupied by parenchyma (not shown, $r = -0.61$; $p < 0.05$). In fact, a solid relationship between total parenchyma and stem starch reserves has been demonstrated in trees (Plavcová *et al.*, 2016). This feature could indicate that a higher carbon investment in xylem conduits determines a lower capacity for the storage of reserves.

Variations in xylem architecture have been discussed in relation to hydraulic behavior in response to water shortage (Venturas *et al.*, 2017). Grapevine cultivars showing wider xylem conduits are believed to have higher hydraulic conductance and xylem vulnerability (Schultz, 2003). In CS, low levels of glucose and maltose/sucrose corresponded to a higher number of conduits (Figure 4) and consequently to a higher translocation area (Figure S2). Conversely, in SY, where the increase in conduit number was counterbalanced by the decrease in their diameter and starch reserves were not limiting, it is likely that both metabolic substrates and osmolytes remained almost unchanged.

AUTHOR CONTRIBUTIONS

Conceptualization, E.P. (Elisa Petrussa), V.C. and P.S. (Paolo Sivilotti); Methodology, V.C, P.S. (Paolo Sivilotti) and E.P. (Elisa Petrussa); Validation, F.B. and M.Z.; Formal Analysis, F.B., E.B. and M.V.; Investigation, R.F., P.S. (Paolo Sivilotti), E.P. (Elisa Petrussa), M.V., A.F., C.C. and J.C.H.; Data Curation, F.B. and M.V.; Writing—Original Draft Preparation; R.F., V.C., E.P. (Elisa Petrussa) and E.B.; Writing—Review & Editing, All authors; Supervision, A.N., P.S. (Paolo Sivilotti), V.C. and E.B.; Funding Acquisition, E.P. (Elisa Petrussa), E.P. (Enrico Peterlunger), and P.S. (Paolo Sivilotti). All authors have read and agreed to the published version of the manuscript.

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TABLES

Table 1. Outcomes of the analysis of variance (three-way ANOVA) applied to predawn leaf water potential (Ψ_{pd}) in relation to drought stress (treatment), time, cultivar and their interactions.

Factor	**Df**	**F value**	***p*-value**
treatment	1,33	447.92	<0.001
time	4,33	75.74	<0.001
cultivar	1,33	0.80	0.386
treatment:time	4,33	89.22	<0.001
treatment:cultivar	1,33	9.27	0.004
time:cultivar	4,33	7.36	<0.001
treatment:time:cultivar	4,33	2.74	0.045

Table 2. Theoretical specific hydraulic conductivity (K_{ts}) in relation to drought stress (treatment), time, cultivar and their interactions. Data were analyzed with a three-way ANOVA.

Factor	**Df**	**F value**	**p-value**
treatment	1,16	0.004	0.952
time	1,16	0.998	0.332
cultivar	1,16	0.134	0.719
treatment:time	1,16	0.004	0.952
treatment:cultivar	1,16	0.467	0.504
time:cultivar	1,16	**64.909**	**0.021**
treatment:time:cultivar	1,16	0.467	0.504

Table 3. Analysis of variance (three-way ANOVA) applied to leaf petiole non-structural carbohydrates content in relation to drought stress (treatment), time, cultivar and their interactions. Statistically significant values are marked in bold.

Factor	**Df**	**F value**	**p-value**
Glucose			
treatment	1,24	0.193	0.664
time	**2,24**	**13.239**	**<0.001**
cultivar	**1,24**	**18.454**	**<0.001**
treatment:time	2,24	0.355	0.705
treatment:cultivar	1,24	0.787	0.384
time:cultivar	2,24	3.007	0.068
treatment:time:cultivar	2,24	0.197	0.823
Maltose			
treatment	**1,22**	**23.922**	**<0.001**
time	**2,22**	**13.237**	**<0.001**
cultivar	**1,22**	**16.455**	**<0.001**
treatment:time	**2,22**	**8.667**	**0.002**
treatment:cultivar	1,22	0.823	0.374
time:cultivar	**2,22**	**13.442**	**<0.001**
treatment:time:cultivar	**2,22**	**3.504**	**0.048**
Starch			
treatment	**1,2**	**24.259**	**<0.001**
time	2,22	1.287	0.296
cultivar	**1,22**	**11.684**	**0.002**
treatment:time	**2,22**	**6.383**	**0.006**
treatment:cultivar	1,22	1.029	0.321
time:cultivar	**2,22**	**10.935**	**<0.001**
treatment:time:cultivar	**2,22**	**3.651**	**0.043**

Table 4. Outcomes of the linear models (ANOVA table) applied to non-structural carbohydrates (*i.e.*, glucose, maltose, starch) in relation to xylem conduit number, cultivar and their interaction.

Explanatory variables	**Df**	**F value**	***p*-value**
Glucose			
vessel number	1,13	7.91	**0.015**
cultivar	1,13	15.38	**0.002**
vessel number:cultivar	1,13	5.21	**0.040**
Maltose			
vessel number	1,13	0.25	-
cultivar	1,13	26.01	**0.000**
vessel number:cultivar	1,13	16.23	**0.001**
Starch			
vessel number	1,14	4.33	-
cultivar	1,14	8.39	**0.012**
vessel number:cultivar	-	-	-

Table 5. Outcomes of the linear models (ANOVA) applied to non-structural carbohydrates in relation to predawn leaf water potential (leaf potential), cultivar and their interaction.

Explanatory variables	**Df**	**F value**	***p*-value**
Glucose			
leaf potential	-	-	-
cultivar	1,26	7.13	**0.013**
leaf potential:cultivar	-	-	-
Maltose			
leaf potential	1,23	19.21	**0.000**
cultivar	1,23	5.59	**0.027**
leaf potential:cultivar	1,23	4.45	**0.046**
Starch			
leaf potential	1,26	11.21	**0.002**
cultivar	1,26	5.59	**0.049**
leaf potential:cultivar	-	-	-

FIGURES

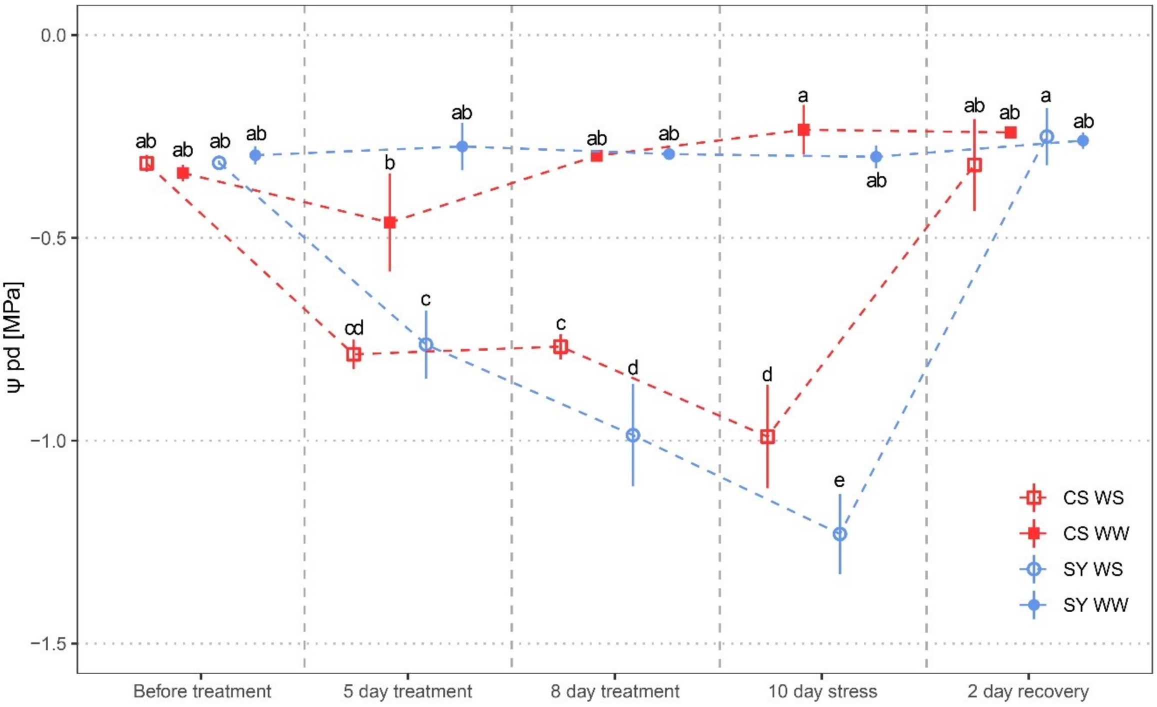

Figure 1. Predawn leaf water potential (Ψ_{pd}) measured in Cabernet Sauvignon (CS) and Syrah (SY) vines under well-watered (WW) and water stress (WS) conditions. Measurements were carried out the day before water stress imposition, during stress and 2 days after rehydration, respectively. Different letters correspond to statistically significant differences ($p < 0.05$).

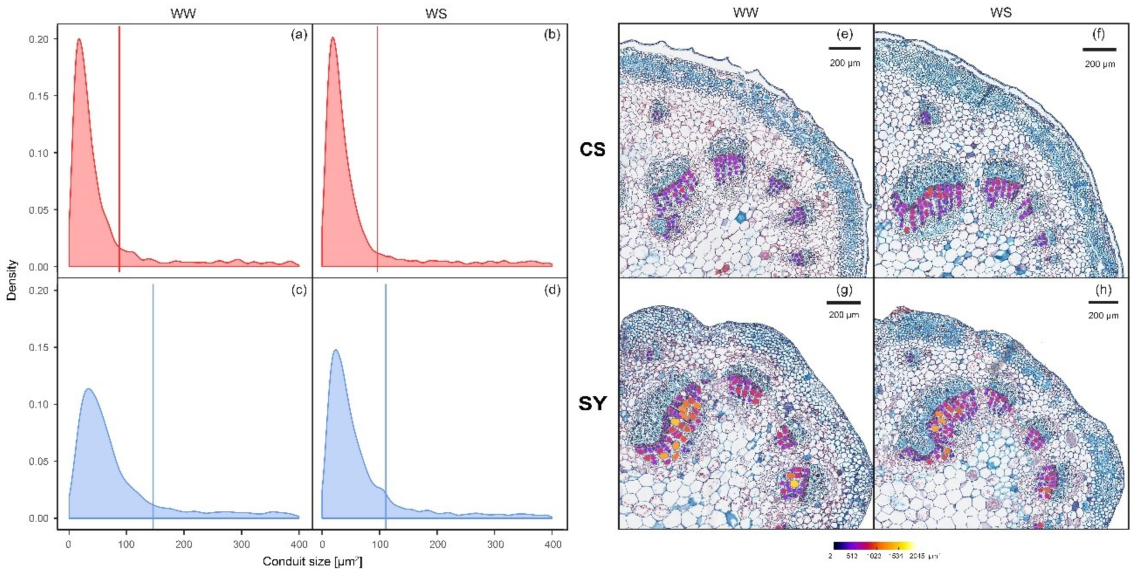

Figure 2. Left panel: frequency of xylem conduit size in petioles of Cabernet Sauvignon (CS) (a,b) and Syrah (SY) (c,d) vines under well-watered (WW) and water stress (WS) conditions. Lines correspond to the mean values of each distribution curve. Right panel: representative cross-sections of Cabernet Sauvignon (CS) (e,f) and Syrah (SY) (g,h) petioles, in well-watered (WW) and water stress (WS) conditions. The color scale indicates different conduit sizes (from dark blue for the narrower to white for the wider).

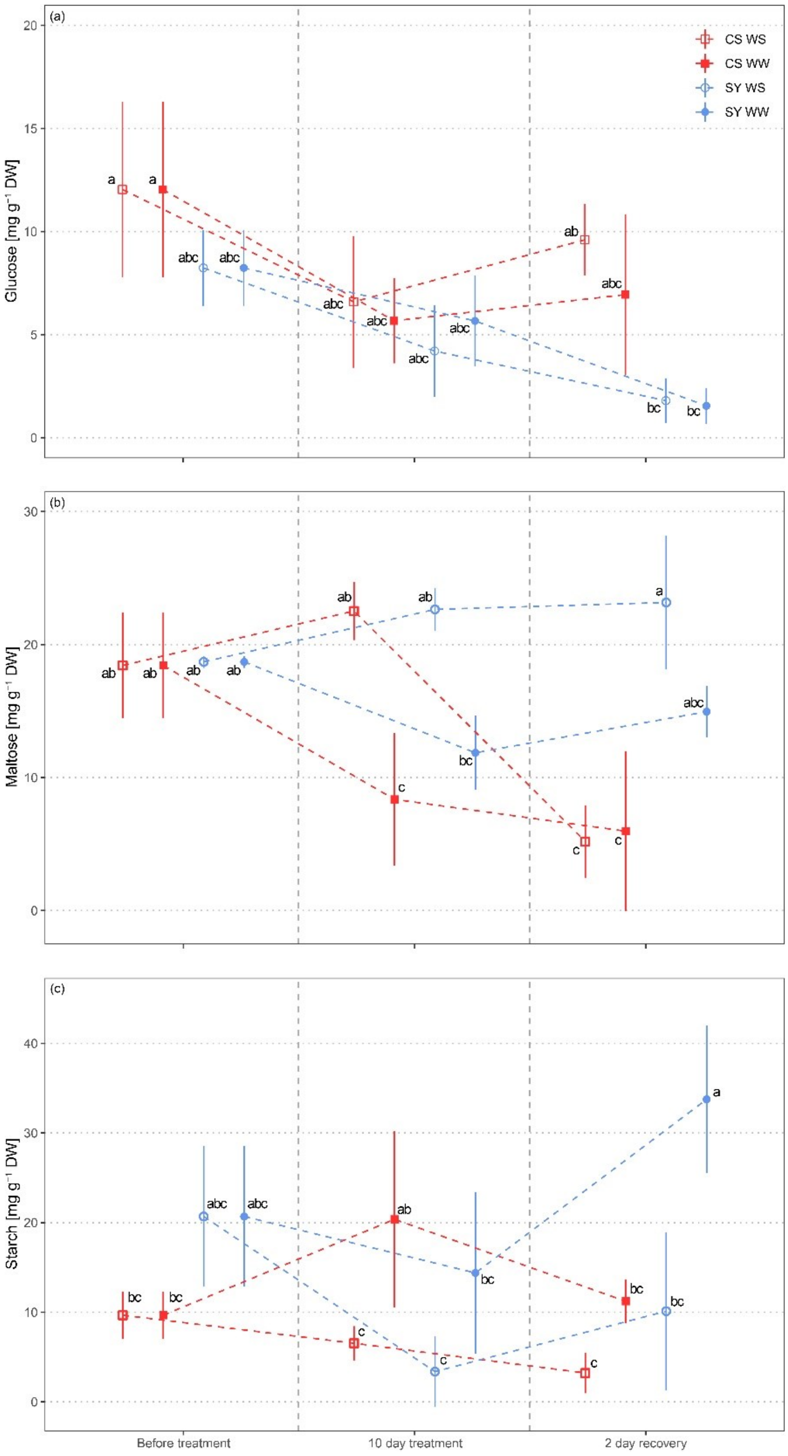

Figure 3. Non-structural carbohydrate (panel (a), glucose; panel (b) maltose; panel (c), starch) concentration in leaf petioles of Cabernet Sauvignon (CS) and Syrah (SY) vines under well-watered (WW) and water stress (WS) conditions. Different letters correspond to statistically significant differences ($p < 0.05$).

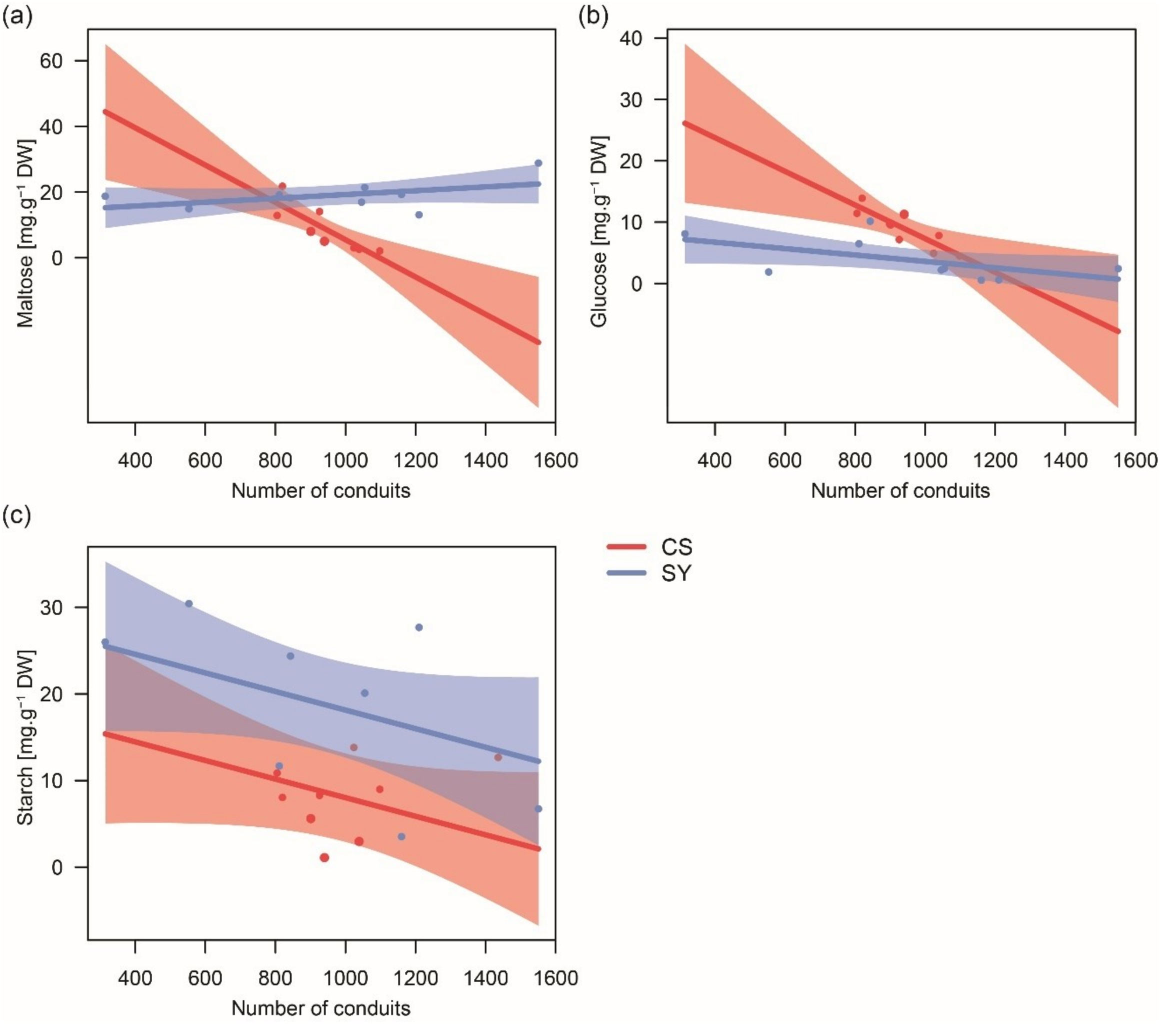

Figure 4. Relationships between the content of glucose (a), maltose (b) and starch (c) in leaf petiole and the number of conduits in Cabernet Sauvignon (CS) and Syrah (SY), according to the outcomes of applied linear models. Shaded areas represent the confidence intervals (0.95).

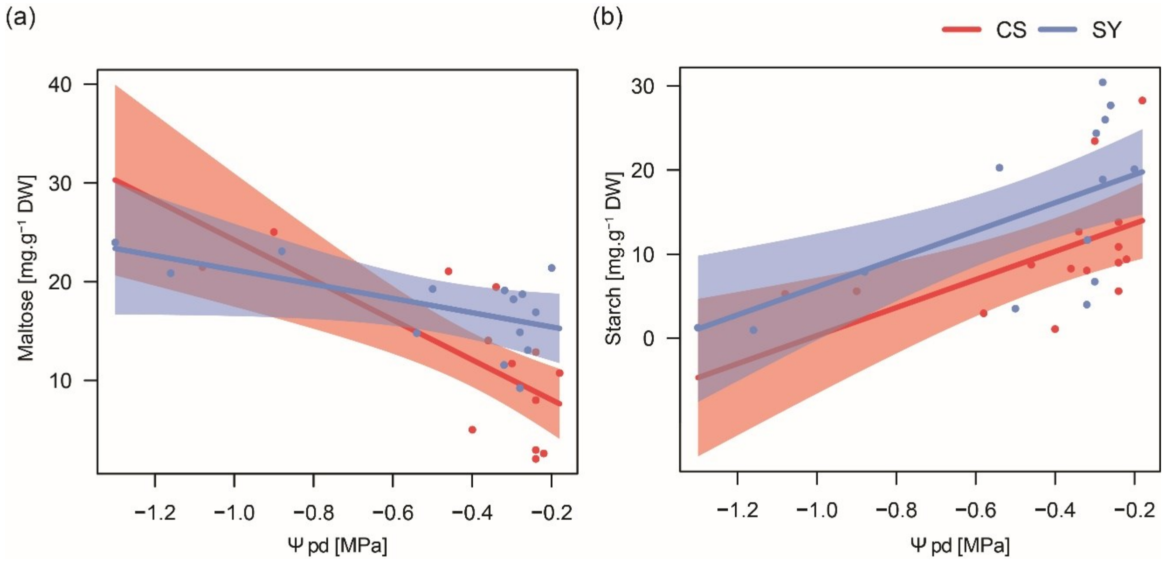

Figure 5. Relationships between predawn leaf water potential (Ψ_{pd}) and maltose (a) and starch (b) content in leaf petioles of Cabernet Sauvignon (CS) and Syrah (SY), according to the outcomes of applied linear models (see text for details). Shaded areas represent the confidence intervals (0.95).

SUPPLEMENTARY MATERIAL

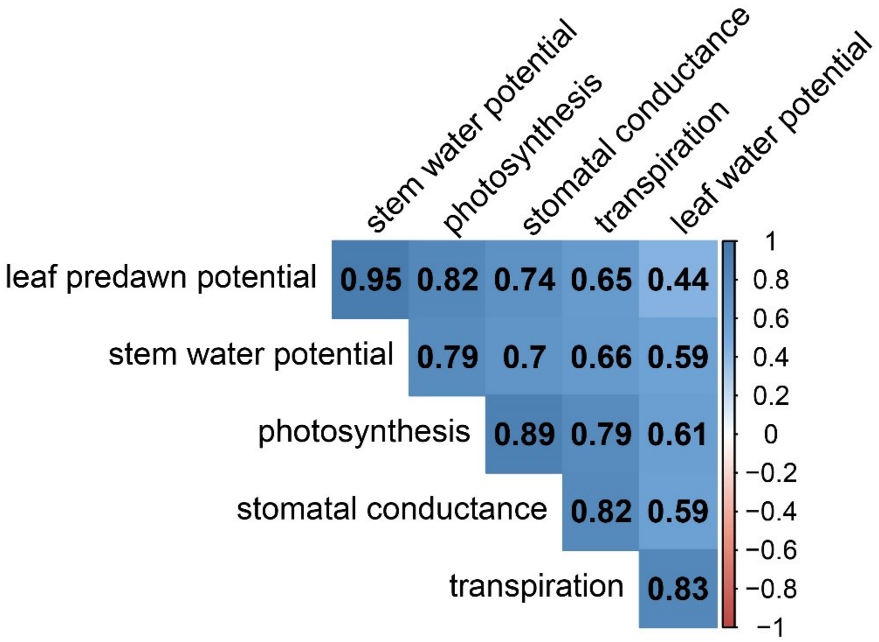

Figure S1. Correlation between the measured hydraulic parameters. Numbers represent Pearson’s r ($p < 0.001$).

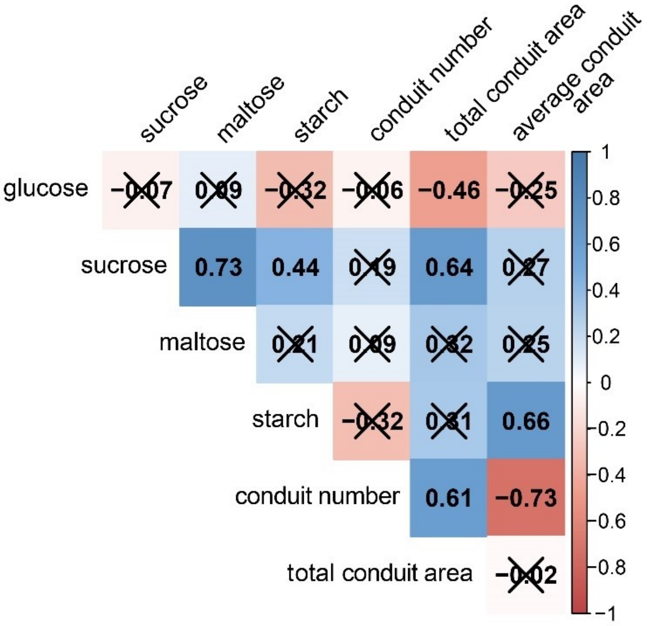

Figure S2. Correlations between the concentration of non-structural carbohydrates and xylem conduit measurements. In blue, positive correlations; red negative correlations. Numbers represent Pearson's r, not significant correlations are also marked with a cross symbol ($p < 0.05$).

3. NSCs and xylem embolism

3.1. Introduction to the study | Chapter 3

Micro computed tomography - Micro CT - has become one of the most valid techniques in research to evaluate the degree of embolism in plants *in vivo*: thanks to the different density due to the presence / absence of water, the embolized vessels can be easily identified by distinguishing them from those filled with water.

The work described below exploited this analysis technique to evaluate whether one-year-old plants belonging to two grapevine cultivars with different hydraulic behavior - Barbera and Grenache - showed different degrees of embolism when subjected to severe and intense stress and whether at resuming irrigation were able or not to recover the embolized vessels.

Parallel to these investigations, the non-structural carbohydrate content was quantified in order to evaluate any correlation between this variable and the degree of embolism.

3.2. Contrasting responses of two grapevine cultivars to drought: the role of non-structural carbohydrates in xylem hydraulic recovery

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Keywords: embolism, maltodextrins, maltose, sucrose, starch, *Vitis vinifera*, water stress.

ABSTRACT

Xylem embolism is one of the possible outcomes of decreasing xylem pressure when plants face drought. Some species can recover from embolism, and recent studies have proposed a role of non-structural carbohydrates (NSCs) in the generation of osmotic pressure required for refilling embolized conduits. Potted cuttings of grapevine cultivars Grenache and Barbera were selected for their putative contrasting hydraulic behaviour. Plants were subjected to an intense drought stress followed by re-irrigation. Stem embolism rate and its recovery were monitored *in vivo* by X-Ray Micro Computed Tomography (Micro-CT) analysis. In parallel, the same plants were analyzed for xylem conduits features and NSC content. Both cultivars exhibited a near-anisohydric response to drought, and both recovered from xylem embolism after re-irrigation. However, Barbera plants were more prone to embolism, despite mean vessel diameter – Micro-CT detected – was similar between cultivars, and in Barbera was further reduced during the recovery phase.

Modulation of soluble NSC patterns during water stress and recovery at the expenses of starch was observed in both cultivars. However, the two cultivars adopted different NSC-use strategies in response to drought, suggesting two possible scenarios at the base of conduit refilling. In Grenache, the hydraulic recovery appeared to be mediated by sugars, with a positive relationship between the soluble NSC content and the degree of embolism. In Barbera, maltose/maltodextrins could be primarily involved in conduit refilling, via formation of cell-wall pectin-hydrogels, which could be responsible for the reduction of conduit lumen detected by Micro-CT imaging.

Key message: cultivars of *Vitis vinifera* can repair embolized conduits via differential use of non-structural carbohydrates

INTRODUCTION

Over recent decades, terrestrial plants have faced increasing frequency and intensity of drought events (Dai, 2011). Intense or prolonged water deficit can threat plant growth and survival, with direct effects on crop production (Lesk *et al.*, 2016) and natural vegetation (Adams *et al.*, 2017). Vascular plants can sustain photosynthesis and growth by maintaining the balance between the water absorbed by roots from the soil, and the water lost by transpiration (Venturas *et al.*, 2017; Nardini *et al.*, 2018). However, severe drought events can lead to a drop of xylem pressure below species- and organ-specific critical thresholds, leading to embolism-induced disruption of the continuity of water columns in xylem conduits (Tyree and Sperry, 1989; Mayr *et al.*, 2014), and ultimately causing plant dehydration and dieback (Tyree, 1988; Tyree and Zimmermann, 2002; Nardini *et al.*, 2013; Choat *et al.*, 2018). In woody angiosperms, such detrimental consequences are thought to occur when xylem hydraulic conductance drops by more than 80% compared to the well-hydrated status (Urli *et al.*, 2013; Nardini *et al.*, 2013).

Plants experiencing drought undergo a decline of water potential, which can be more or less pronounced depending on the hydraulic strategy and stomatal behaviour along an iso (conservative)- to anisohydric (dissipative) *continuum* (Klein *et al.*, 2014; Charrier *et al.*, 2018). It is now clear that this classification does not really capture the whole spectrum of possible stomatal responses (Martínez-Vilalta and Garcia-Forner, 2017; Hochberg *et al.*, 2018), also due to the importance of interaction with environmental conditions and drought intensity. However, contrasting stomatal regulation and photosynthetic behaviours of iso- vs anisohydric plants have been claimed to lead to different trajectories of non-structural carbohydrates (NSCs) content and plant hydraulic functioning under drought. These would range from i) a progressive decline of energy sources (carbon starvation) (McDowell *et al.*, 2008) if carbon demand strongly exceeds carbon supply, to ii) a complete failure of hydraulic conductance depending on the species-specific vulnerability to embolism (Garcia-Forner *et al.*, 2017). The first scenario is more likely for plants displaying an isohydric strategy, whereas the second is expected in species showing anisohydric behaviour. However, most drought events do not suddenly reach critical thresholds leading to hydraulic failure, and woody plants can respond to water deprivation with medium-long term acclimation responses including morpho-anatomical changes such as production of new xylem conduits (Zhang *et al.*, 2019), increased conduit wall thickness and wood density, and pit membrane modifications (Hacke *et al.*, 2001; Sperry *et al.*, 2006, 2007). When drought ends and water supply is restored, plants can also recover their hydraulic functioning by means of rapid physiological responses to restore water transport. Indeed, it has been suggested that, under certain conditions, plants can refill embolized conduits (Trifilò *et al.*, 2015; Yoshimura *et al.*, 2016; Savi *et al.*, 2016; Beikircher and Mayr, 2017; Gleason *et al.*, 2017; Love and Sperry, 2018).

The actual occurrence of embolism repair has been intensively investigated and debated (Martorell *et al.*, 2014; Secchi and Zwieniecki, 2016; Ooeda *et al.*, 2016; Trifilò *et al.*, 2017; Nardini *et al.*, 2018; Brodersen *et al.*, 2018). This phenomenon is frequently accompanied by the increase of the sugars pool at the expense of starch (Yoshimura *et al.*, 2016; Beikircher and Mayr, 2017; Pagliarani *et al.*, 2019; Wang *et al.*, 2019; Tomasella *et al.*, 2021), suggesting an active osmotic role of NSCs as during the rehydration mechanism (Nardini *et al.*, 2011; Brodersen and McElrone, 2013; Knipfer *et al.*, 2016), to generate the local pressure driving water flow into gas-filled conduits (Secchi and Zwieniecki, 2011, 2012).

Despite experimental evidence supporting hydraulic recovery in tracheophytes based on refilling of embolized conduits, the validity of these findings is still under debate, in particular for reports suggesting refilling under residual moderate xylem tension (Cochard and Delzon, 2013). Synchrotron X-ray Computed Micro Tomography (Micro-CT) has been used to visualize *in vivo* embolism formation and subsequent refilling (Brodersen *et al.*, 2013). In particular, several Micro-CT experiments on embolism repair were performed on *V. vinifera,* with some studies confirming the occurrence of this phenomenon (Holbrook *et al.*, 2001; Brodersen *et al.*, 2013; Knipfer *et al.*, 2015), but others reporting lack of conduit refilling after rehydration (Charrier *et al.*, 2016).

These apparently contrasting results do not surprise, considering the critical role of plant physiological status at the end of drought in determining whether embolism repair is energetically possible (Savi *et al.*, 2016; Tomasella *et al.*, 2019, 2021; Trifilò *et al.*, 2021). Moreover, the actual mechanisms of water-stress response in *V. vinifera* are complicated by several anatomical and physiological peculiarities due to its vine habitus and ecology, features that are also variable among cultivars (Lovisolo *et al.*, 2010). In particular, a critical point is the regulation of stomatal closure in different cultivars that can range between isohydricity and anisohydricity (Hochberg *et al.*, 2018; Villalobos-González *et al.*, 2019). Such contrasting stomatal responses might reflect also in NSC metabolism under drought, and thus possibly on the occurrence of xylem refilling process upon rehydration. To date, the literature reports contrasting results on the contribution of NSCs to active osmotic adjustment in drought-stressed grapevines (Rodrigues *et al.*, 1993; Patakas and Noitsakis, 2001; Patakas *et al.*, 2002). Recent investigation on NSC content related to water stress in both canes and leaf petioles, suggested that the observed variation of NSCs depends on the type of drought stress (*i.e.*, short and severe vs mild and prolonged), and on the hydraulic behaviour displayed during stress (Falchi *et al.*, 2019, 2020; Morabito *et al.*, 2021).

Here, we specifically tested the relationship between hydraulics and carbon metabolism under drought and recovery in two grapevine cultivars reported to display contrasting stomatal behaviours. Based on Micro-CT analysis, we checked whether grapevine cuttings exposed to a severe drought inducing xylem embolism could recover their xylem functionality by refilling vessels upon rehydration. Concurrently, we investigated the role of NSCs along the drought stress and subsequent recovery, focusing on different compounds potentially important for osmoregulation and for energetic metabolism (*i.e.* glucose, sucrose, maltose/maltodextrins and starch). We specifically aimed at describing eventual cultivar-based differences in xylem embolism formation/recovery, as coupled to NSC metabolism.

MATERIAL AND METHODS

Plant material and growth conditions

One-year old cuttings of *Vitis vinifera* L. cv. Barbera and Grenache (VCR 207 and VCR3 clones, respectively), both grafted on 15 cm-long SO4 rootstock, were gently provided by Vivai Cooperativi Rauscedo VCR (Rauscedo, Italy). Cuttings were planted in late May 2019 in 1 L pots filled with field sieved soil supplemented with 10% perlite and randomly allocated in a growth chamber of the University of Udine (Italy) (mean temperature 22°C, light:dark photoperiod 12:12 h, 92 µmol $s^{-1}m^{-2}$ of photons, relative humidity of 40%). Plants were irrigated to field capacity every 1–3 days, until early September, when the experiment started. Plants were subjected to later shoot removal and shoot trimming from

July to early August to maintain them within dimensions compatible to fit the sample holder at Micro-CT beamline.

Experimental design

The experimental treatments were applied in early September 2019. At the beginning of the experiment, 7 plants per cultivar were kept as controls and daily irrigated for the whole duration of experiment (W, well-watered), while 14 plants per cultivar were subjected to drought stress by suspending irrigation for 6 days (S, water stress). At the end of the drought period, half of the stressed plants were re-watered to field capacity (R, water stress treatment followed by recovery) and allowed to recover over 48 h (Fig. 1).

Water potential (Ψ_{pd}) measurement

Predawn leaf water potential (Ψ_{pd}) was measured on the second fully expanded leaf of each plant. Leaves were bagged immediately before measurement and they were excised from the shoot using a sharp blade. The bagged leaves were placed into a pump-up Pressure Chamber (PMS Instrument Company, Albany, OR, USA), and Ψ_{pd} was recorded when the initial xylem sap emerged from the cut end of the petiole.

X-ray Micro-CT observations

All control, stressed and recovered plants of each cultivar were analysed via Micro-CT scans at the Elettra synchrotron radiation source (Trieste, Italy), using the phase contrast facility available at the SYRMEP beamline (www.elettra.trieste.it). Plants were transported from the growth chamber to the Micro-CT station one night before the start of analysis and kept in the dark.

To reduce sample movement during the scan rotation, each plant was removed from the pot, root and soil were secured in a plastic bag and the whole plant was wrapped in plastic film and secured to a wooden axis fixed to the beam-line sample holder such that stem distance was 12 cm from the detector. Grapevine stem was observed at the 2nd-4th node from the rootstock using a pixel size of 2.9 μm at mean energy of 22 keV, with 1800 projections acquired during the sample rotation over 180°. Scans were performed in free propagation phase contrast modality and an exposure time of 100 milliseconds. In total 7 plants per treatment and cultivar were scanned, and each plant was scanned only once to avoid possible damage to living cells, as already verified by Petruzzellis et al. (2018). The Micro-CT slices were reconstructed applying a phase retrieval pre-processing filter (Paganin *et al.*, 2002), before the use of the conventional filtered back projection algorithm implemented in the SYRMEP TOMOPROJECT (STP) software (Brun *et al.*, 2015). One slice per sample was selected and analyzed by means of the Fiji software (Schindelin *et al.*, 2012). For each sample, the transverse areas of all gas-filled (dark gray) and water-filled (light gray) xylem conduits were measured. The embolism rate (as a proxy of percentage loss in conductivity, PLC) was calculated as percentage of the transversal area of embolized xylem conduits with respect to the total xylem conduits area.

Non-structural carbohydrate content

Non-structural carbohydrates (NSCs) content was measured on the same samples used for X-ray Micro-CT observations. After the scan, a 4 cm stem segment was collected just above the scanned portion and microwaved at 700 W for 3 min in a water saturated atmosphere to stop enzymatic activities. Samples were then ground into fine powder in a mortar under liquid nitrogen and oven dried at 56 °C for 24 h. Then, 15 ± 1 mg of powder were suspended in 0.3 mL ethanol 80% (v/v) twice, incubated at 80 °C for 30 min, and then the resulting supernatant dried at 60 °C overnight. A third suspension was made with 0.5 mL of 50 mM Tris-HCl pH 7.5 and used to re-suspend the crystalized carbohydrates resulting from previous steps. The supernatants obtained after incubation at 80 °C and a 14,000 × *g* centrifugation for 5 min were used for soluble NSC measurement, while the pellet was re-suspended in 1 mL of sodium acetate buffer (0.2 M $NaCH_3COO$, pH 4.6), and used for starch quantification. Total soluble NSC fraction was quantified by a VICTOR3 Multilabel Counter Plate Reader (Perkin Elmer, Boston, MA, USA) by means of the anthrone-sulfuric acid assay (Yemm and Willis, 1954). A glucose standard curve was used to compare the colorimetric response of the samples, whose absorbance was read at 620 nm, and the NSC content was expressed as mg [glucose] g^{-1} [DW].

Glucose was analyzed by enzymatic hexokinase/glucose-6-P dehydrogenase assay (Landhäusser *et al.*, 2018), sucrose and maltose/maltodextrins, followed the same process, after conversion into glucose by 25 U Invertase or 25 U Amyloglucosidase for the latter sugars, respectively. Starch digestion was performed overnight at 55 °C, using 100 U of α-Amylase and 25 U of Amyloglucosidase per sample. To prevent further degradation, the samples were boiled for 3 min. Starch fraction was quantified by means of enzymatic assay, as previously mentioned. Known amounts of amylose were also processed and analyzed to obtain a calibration curve. The final concentration of starch in the sample was then expressed as mg [starch] g^{-1} [DW]. All the chemicals were purchased from Sigma-Aldrich, Milan, Italy.

Statistical analyses

All the statistical analyses were performed in R statistical software (R Core Team, 2021). The effect of the different treatments, cultivars and their interaction was tested separately for each parameter using two-way ANOVA analysis through 'anova' function in 'stats' R package. The relationships between NSC content (*i.e.*, glucose, sucrose, maltose/maltodextrins and starch), Ψ_{pd}, and embolism rate in the two cultivars were tested using linear models. Model assumptions (*i.e.*, linear models and ANOVA) were checked by diagnostic plot of residuals. Outlier records were detected by the function "outlier Test" of the 'car' package (Fox and Weisberg, 2019) and removed from analysis (where $P < 0.01$). For significant tests ($\alpha = 0.05$), Tukey's Honestly Significant Differences post hoc analysis was carried out through 'TukeyHSD' function in 'stats' R package. Xylem conduit size distribution was compared within each cultivar, between treatments. The xylem conduit distribution was represented by density plots. Paired differences in conduit size distribution were tested by the two-sample Kolmogorov-Smirnov test ($P < 0.05$).

RESULTS

Water status

Experimental treatments significantly affected the pre-dawn water potential (Ψ_{pd}) (Table 1) whereas the interaction between treatment and cultivars was not significant. When grapevine plants were exposed to drought, in both cultivars a similar and significant decrease of Ψ_{pd} was observed (Fig. 2A) (average -1.0 MPa, min. -1.5 MPa). After re-watering, Ψ_{pd} returned to pre-drought values. A similar negative relationship between PLC and Ψ_{pd} was observed for the two cultivars (Fig. S1 Panels A and B).

PLC was statistically influenced by treatments (Table 1). Both cultivars showed a two-fold increase of PLC during drought, followed by a recovery after re-watering (Fig. 2B), as documented by Micro-CT images in terms of number of gas- vs water-filled conduits (Fig. S2). When evaluating the effect of treatment on both cultivars, the PLC was significantly different in W versus S ($P = 0.014$), while it was marginally significant when comparing S to R samples ($P = 0.079$). Despite the similar behavior in response to the treatment, a statistical difference between the two cultivars was observed (Table 1), with Grenache displaying a significantly lower level of embolism rate.

Non-structural carbohydrates

The content of total soluble NSCs in the stem followed the same trend of embolism rate, being affected by the factor treatment (Table 2). In both cultivars, NSCs increased in S and decreased in R plants (Fig. 3A). Overall, the differences of sugars found between W and S and the drop observed between S and R vines were both significant, ranging from 20 to 30% ($P = 0.035$ and $P = 0.009$, respectively). Despite the lack of significance of the interaction between cultivar and treatment, a higher sugar level in R plants compared to W ones was observed in Barbera, while the opposite pattern was observed in Grenache displaying a low concentration in R respect to W. A positive relation between soluble NSCs and PLC in both cultivars was found (Fig. 3 Panels B and C), though it was significant only for Grenache.

Between the analyzed single sugar components (*i.e.*, glucose, sucrose, and maltose/maltodextrins), sucrose showed to be the most abundant soluble component in both cultivars, representing about 2/3 of the total NSCs (Fig. 4A). Its content was not significantly affected by the factors and their interaction (Table 2). However, a positive relationship with PLC was found only in Grenache (Fig. 4 Panels C and E), consistently with the previous results on total soluble NSCs.

Glucose represented a minor component of NSC (approx. 1 mg g^{-1} DW), and did not display significant changes between treatments, cultivar or their interaction (Fig. S3A and Table 2). Interestingly, a positive significant relationship between glucose and embolism rate was found only in Barbera (Fig. S3 Panels B and C).

The portion of water soluble starch (Landhäusser *et al.*, 2018), derived from starch degradation, represents maltose and short-chain carbohydrate polymers identified as maltodextrins. Maltose and maltodextrins content was significantly different between cultivars (Table 2), reaching approx. 20% and less than 10% of total NSCs in Barbera and Grenache, respectively (Fig. 4B). The two cultivars exhibited contrasting trends (Table 2). Maltose and maltodextrins increased during recovery in Barbera and decreased in Grenache. No significant relationship between maltose/maltodextrins and PLC was observed in both cultivars (Fig. 4 Panels D and F).

Starch level in green stems did not show any significant changes with respect to treatment (Fig. 5A), but, consistently to maltose/maltodextrins content, it was influenced by cultivar factor (Table 2), as its content was double in Barbera compared to Grenache. Finally, in Barbera a negative significant relationship emerged between starch and embolism rate (Fig. 5B), while in Grenache the same relationship was positive, but not significant (Fig. 5C).

Vessel size

Barbera and Grenache differed in the distribution of vessel size classes in their stems ($P < 0.001$). In fact, Grenache showed a significantly higher number of wide vessels (Fig. S4 Panel A). The very short recovery time (2 days) likely did not allow the production of new vessels (Fig. S4 Panel B). Nonetheless, when comparing the diameter of vessels in stressed vs recovered samples, a significant difference was surprisingly observed in Barbera, that showed an apparent increase in the percentage of narrower vessels ($P < 0.001$) (Fig. 6).

DISCUSSION

Water status

Young cuttings of *V. vinifera* subjected to drought underwent a decrease of Ψ_{pd} to values consistent with those recorded in field-growing grapevine plants experiencing severe water shortage (Lovisolo *et al.*, 2010). Despite Barbera and Grenache are generally considered as opposite in terms of hydraulic behavior (anisohydric and isohydric, respectively) (Schultz, 2003; Shelden *et al.*, 2017), young cuttings from these cultivars exhibited very similar water potential drops and hydraulic vulnerability, suggesting a substantial overlap of their hydraulic strategies (Martínez-Vilalta and Garcia-Forner, 2017). Hence, both cultivars did not differ in their leaf water status under drought, and their response suggested a near-anisohydric regulation of water potential. Grenache showed a higher number of wide xylem vessels compared to Barbera independently from the treatment, which however translated, somehow unexpectedly, in significantly lower PLC under drought.

In both cultivars, the observed decrease in water potential under water shortage was coupled to increasing PLC. However, the recovery phase allowed plants to return to the same values of Ψ_{pd} measured before water stress was imposed. In this case, a complete recovery of PLC was also observed, indicating that grapevine might recover from embolism through the refilling of empty vessels as already shown in previous studies in the genus Vitis (Brodersen *et al.*, 2013, 2018; Knipfer *et al.*, 2016) and in other woody species (Wheeler *et al.*, 2013; Secchi and Zwieniecki, 2014; Trifilò *et al.*, 2014). We also showed that in grapevine this phenomenon occurred independently on the hydraulic strategy previously claimed for Barbera and Grenache.

Non-structural carbohydrates

The level of soluble NSCs measured in both cultivars confirmed that grapevine responds to water stress with an accumulation of sugars, as recently observed in leaf petioles of cultivar Merlot (Vuerich *et al.*, 2021). On the contrary, Savi et al. (2019*a*) reported a more complex scenario, depending on organ and carbohydrate species, in the cultivar Riesling. The observed accumulation of NSCs is compatible with the role that soluble carbohydrates play in osmoregulation when woody plants are exposed to drought (Tomasella *et al.*, 2021; Peltier *et al.*, 2021), low temperature (Lintunen *et al.*, 2016; Casolo *et al.*, 2020; Baffoin *et al.*, 2021) or salinity (Gil *et al.*, 2013; Pellegrini *et al.*, 2020). This has also been reported for *V. vinifera* exposed to water stress (Patakas and Noitsakis, 2001; Degu *et al.*, 2019), although inorganic ions like K^+ (Monder *et al.*, 2021) are considered the major drivers of this process in grapevine, since the contribution of organic compounds was suggested to be minor (Patakas *et al.*, 2002). Interestingly, Morabito et al. (2021) showed that in potted cuttings of Grenache, the hydraulic strategy is dependent on the progression of water stress. In our study, despite the fast development of drought induced by the interruption of irrigation, the increase in stem soluble NSCs observed in both cultivars was consistent with what reported when a slow developing drought was imposed in Grenache. This result underlines that the type of stress may not be the only driver modulating the response of grapevines to drought, but other traits such as rootstock genotype and scion size might be involved.

When total soluble NSCs were plotted vs PLC, both cultivars showed a positive relationship, though this was significant only in Grenache. This finding is consistent with the relationships between soluble NSCs and PLC reported for other woody plants (Trifilò *et al.*, 2019), as well as with observations of NSC consumption coupled to embolism recovery at the end of the water stress (Tomasella *et al.*, 2019)

Previous studies have reported that the ratio of NSC in plants subjected to drought is variable depending on sugar type (Pagliarani *et al.*, 2019; Savi *et al.*, 2019*b*) as observed also in grapevine leaf, stem, root (Savi *et al.*, 2019*a*) and petioles (Falchi *et al.*, 2020). In our experiment, when separately considering sucrose, glucose and maltose (including maltodextrins), it appeared that while in Grenache the sucrose sustained NSCs response to drought (positive relationship with PLC), in Barbera the glucose exhibited such a positive relationship, although its very low relative concentration could not explain the observed increase in total soluble NSCs under drought. On the contrary, when the relationship between starch and PLC was considered, the scenario was the opposite with Barbera displaying a significant negative relationship between starch and PLC. Our data suggest that only in Grenache the refilling model proposed by Secchi and Zwieniecki (2016) was likely operating. In fact, in Grenache, embolism recovery was apparently coupled to sucrose levels, which were directly correlated with total NSCs. In the above cited model, soluble NSCs were proposed to derive from starch by acid invertase pathway producing glucose and fructose (Pagliarani *et al.*, 2019). Our data in Grenache confirm a direct involvement of sucrose that probably derives from the maltose/maltodextrin pool (representing soluble, ready-to-use starch), rather than starch. The low content of maltose and starch in stressed and recovered Grenache plants also indicate that sucrose production guaranteed from photosynthesis is limited under such conditions. On the contrary, in

Barbera free sugars are also necessary for recovery, though being not related to embolism, and it may be rationalized that embolism repair could occur with a different mechanism, being starch hydrolyzed from stress to recovery through amylase, producing maltose and then increasing glucose which is consumed during recovery. The opposite trends found between glucose and PLC (positive relationship) and starch and PLC (negative relationship) further corroborate this scenario.

Vessel size

It is well known that reducing the vessel dimensions is a widespread drought acclimation strategy in woody plants (Blackman *et al.*, 2010; Jupa *et al.*, 2021) and also in grapevine (Lovisolo and Schubert, 1998), to mitigate the risk of embolism-induced loss of hydraulic conductance. Nevertheless, the time lapse of the present experiment was not long enough to allow for production of new xylem during or after drought stress, as observed in leaf petioles of grapevines subjected to different water treatments by Falchi et al. (2020). Hence, the recorded reduction in vessel diameter observed in Barbera rises some interesting questions. In fact, in this cultivar, an evident increase in soluble NSCs was detected during stress, though not significantly correlated with PLC, suggesting that it could be not correlated to an ongoing refilling process. Furthermore, maltose/maltodextrins content increased during recovery, as water-soluble short chain carbohydrates polymers produced by breakdown of starch (Beck and Ziegler, 1989). Maltodextrins are known to produce highly hygroscopic aqueous gels (Kennedy *et al.*, 1995; Chronakis, 1998; Loret, 2004), also in combination with pectin (Evageliou, 2000). We suggest that, during recovery, maltodextrins could be transferred into the apoplast, soaking xylem vessels. This could lead to a density change of cell walls, detected by Micro-CT as a cell wall swelling and leading to apparently reduced diameter of conduit lumen. Pectin-hydrogel involvement in modulation of xylem hydraulics was suggested through an alteration of pit microchannels (Zwieniecki *et al.*, 2001), but an even more fascinating hypothesis involving carbohydrate polymers in the control of vessel refilling, named ‘pit membrane osmosis’, was proposed by Hacke and Sperry (2003). Maltodextrins might be released into embolized vessels, lowering Ψ and producing a hydrogel held in the cell wall which, due to its polymerization with pectins, cannot move across pits that would thus act as an osmotic ‘membrane’. The hygroscopic capacity of maltodextrin gel accumulated in the cell wall of embolized conduits might drive water inflow from both neighbouring parenchyma cells and water-filled conduits, thus allowing reconnection to the transpiration stream. Alternatively, modification of surface tension induced by maltodextrins might favour hydraulic isolation of refilling conduits, via interaction with pit membrane features, making possible local generation of positive pressure via osmotic mechanisms, while the bulk of still water-filled conduits are under residual negative pressure (Zwieniecki and Holbrook, 2000).

In conclusion, our results showed that one-year old cuttings of *V. vinifera* can recover from xylem embolism under moderate residual tension, through an active refilling process likely mediated by soluble NSCs in a direct (Grenache) or indirect (Barbera) way. Both strategies require starch depletion, but in the first case soluble NSC content (in particular sucrose) is directly correlated with the degree of embolism, consistently with the hypothesis proposed for poplar by Secchi & Zwieniecki (2016), in which sugar accumulation in the xylem sap is necessary to generate a local positive pressure forcing water in the embolized vessels. In the second case, we suggest the possible formation of cell-wall pectin-hydrogel, due to maltose/maltodextrins accumulation deriving from starch degradation, that could act as a hygroscopic sponge unable to move through pits but drawing water from neighboring parenchyma cells.

AUTHOR CONTRIBUTIONS

VC, AN and EP conceived and designed the research. MP projected and prepared specific grape cuttings; MV, VC, FB, FP, EP, AF, MT and GT, were involved in Micro-CT observations; MV and FB performed the image reconstruction; EB and FP evaluated hydraulics and anatomy; MV, EP, and VC performed the NSCs measurements. MV and FB performed the statistical analysis. VC, EP, MV and AN wrote the manuscript. All the authors contributed to data discussion, revised and approved the manuscript.

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TABLES

Table 1. Analysis of variance (two-way ANOVA) applied to Ψ_{pd} and PLC in relation to cultivar, treatment and their interactions.

Factor	**Df**	**F Value**	***p*-Value**
Ψ_{pd}			
Cultivar	1,39	0.20	0.655
Treatment	2,39	36.38	< 0.001 ***
Cultivar*Treatment	2,39	0.25	0.786
PLC			
Cultivar	1,32	4.72	0.037 *
Treatment	2,32	4.84	0.015 *
Cultivar*Treatment	2,32	0.02	0.981

*, ** and *** significance at the $p < 0.05$, 0.01 and 0.001 levels, respectively.

Table 2. Analysis of variance (two-way ANOVA) applied to Total soluble NSCs, Sucrose, Glucose, Maltose/maltodextrins and Starch content in relation to cultivar, treatment and their interactions.

Factor	**Df**	**F Value**	**p-Value**
Total soluble NSCs			
Cultivar	1,38	1.35	0.252
Treatment	2,38	5.89	< 0.006 **
Cultivar*Treatment	2,38	1.35	0.272
Sucrose			
Cultivar	1,39	4.72	0.858
Treatment	2,39	2.11	0.134
Cultivar*Treatment	2,39	0.46	0.632
Glucose			
Cultivar	1,33	0.005	0.942
Treatment	2,33	0.026	0.974
Cultivar*Treatment	2,33	0.107	0.342
Maltose/maltodextrins			
Cultivar	1,35	36.80	< 0.001 ***
Treatment	2,35	0.026	0.209
Cultivar*Treatment	2,35	0.107	0.004 *
Starch			
Cultivar	1,33	13.77	< 0.001 ***
Treatment	2,33	1.15	0.330
Cultivar*Treatment	2,33	1.13	0.337

*, ** and *** significance at the $p < 0.05$, 0.01 and 0.001 levels, respectively.

FIGURES

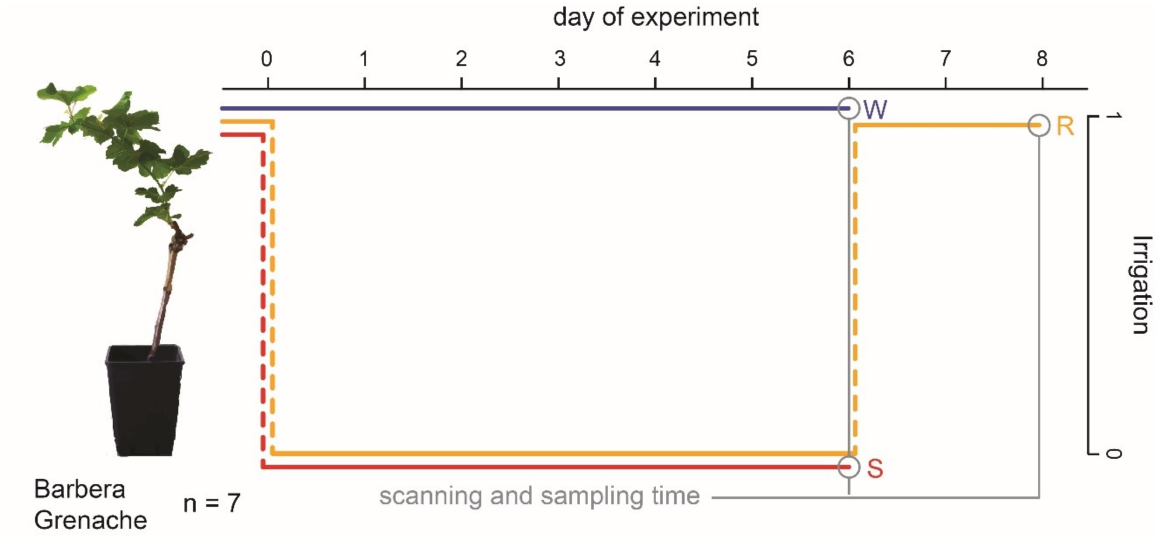

Fig. 1. Experimental design. Plants of cv. Barbera and Grenache were divided in three experimental groups (n = 7): well-watered (W, blue line) plants were well irrigated at field capacity for 6 days, whereas water stressed (S, red line) and recovered (R, orange line) plants were not irrigated. Recovered plants were then re-watered at field capacity for the following 2 days. W and S plants were harvested after 6 days, while R plants were harvested after 2 days of re-irrigation, when pre-dawn leaf water potential, PLC by Micro-CT analysis and NSC content in stem were measured.

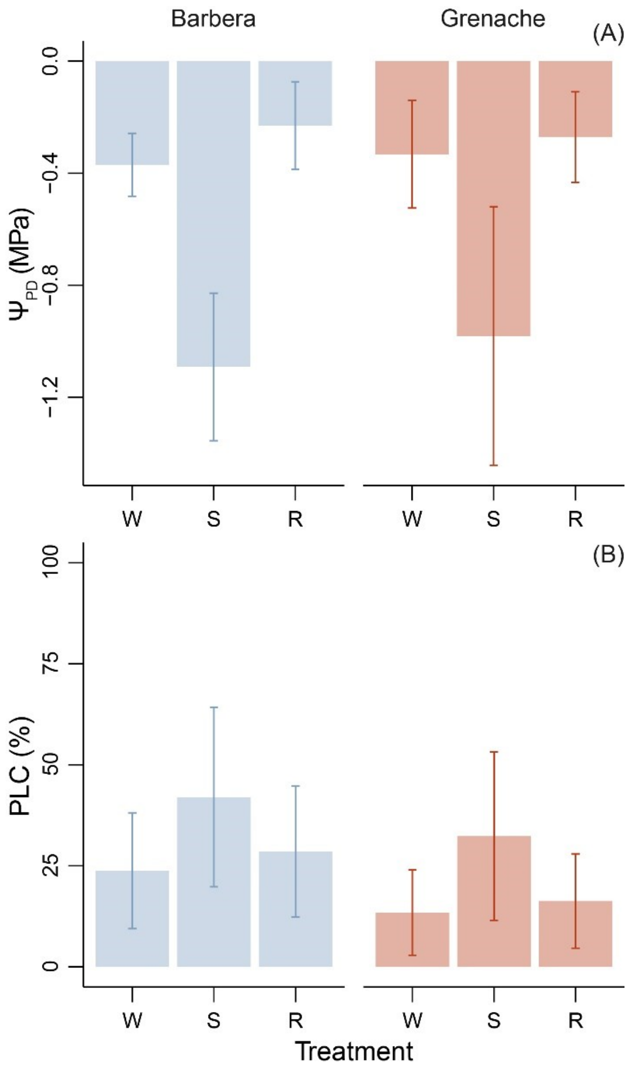

Fig. 2. Predawn leaf water potential (Ψ_{pd}) and percentage loss in conductivity (PLC) in cv. Barbera and Grenache under different water status. Ψ_{pd} (panel A) and PLC (panel B) in well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Means ± SD (n = 7) are shown.).

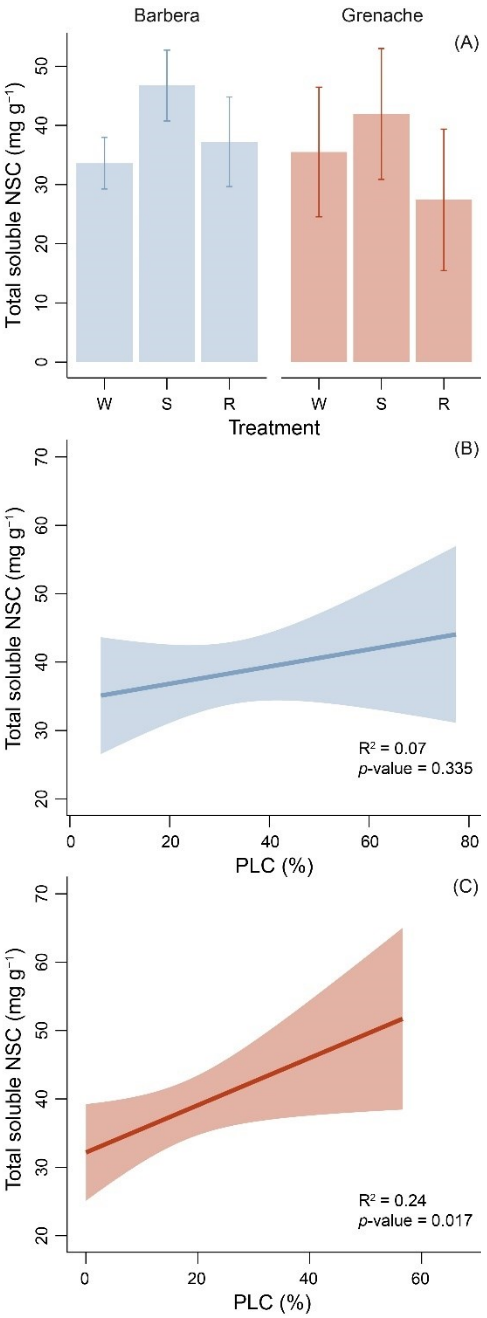

Fig. 3. Total soluble non-structural carbohydrates (NSC) in stems of cv. Barbera and Grenache plants under different water status and relationship with PLC. Total soluble NSC (panel A) in stems of well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Means ± SD (n = 7) are shown. Relationships between the content of total soluble NSC in cv. Barbera (panel B) and Grenache (panel C) and PLC, according to the outcomes of applied linear models. Shaded areas represent the confidence intervals (0.95).

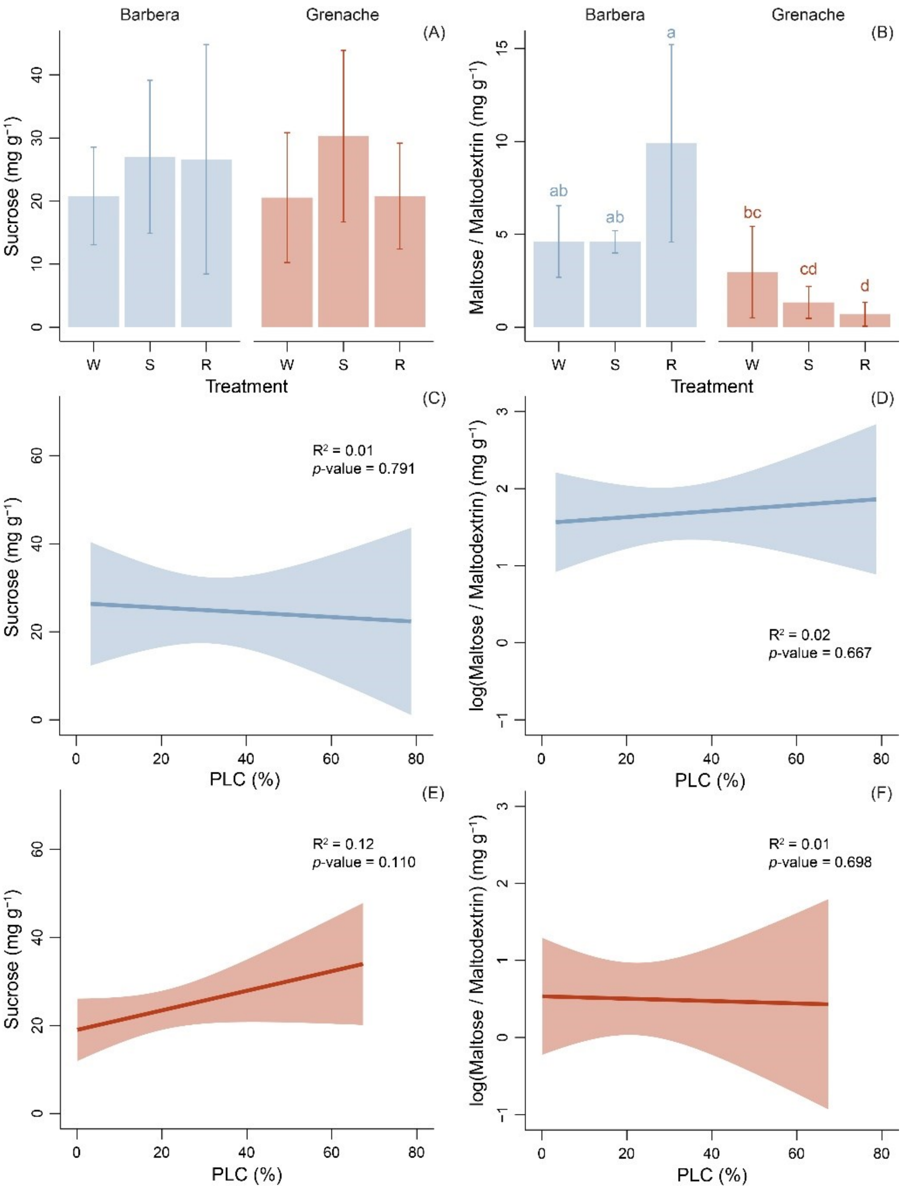

Fig. 4. Sucrose and maltose/maltodextrin content in stems of cv. Barbera and Grenache plants under different water status and relationship with PLC. Sucrose (panel A) and maltose/maltodextrins (panel B) content in stems of well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Means ± SD (n = 7) are shown. Different letters correspond to statistically significant differences (*$P < 0.05$*). Relationships between the content of sucrose or maltose/maltodextrins and PLC in cv. Barbera (blue pattern, panels C and D, respectively) and in cv. Grenache (red pattern, panels E and F, respectively), according to the outcomes of applied linear models. Shaded areas represent the confidence intervals (0.95).

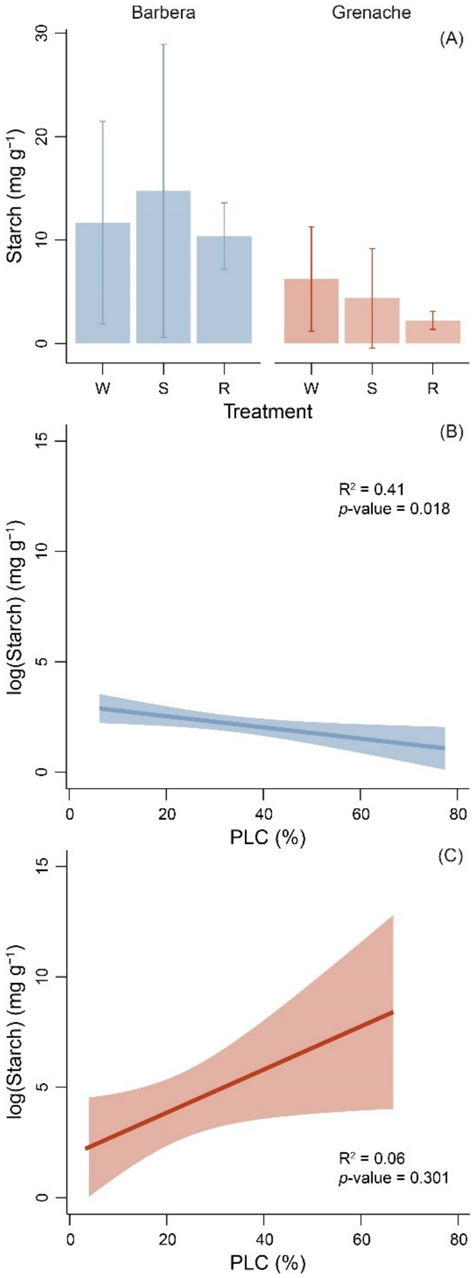

Fig. 5. Starch content in plant stems of cv. Barbera and Grenache under different water status and relationship with PLC. Starch content (panel A) in stems of well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Means ± SD (n = 7) are shown.

Relationships between the content of starch and PLC in cv. Barbera (panel B) and Grenache (panel C), according to the outcomes of applied linear models. Shaded areas represent the confidence intervals (0.95).

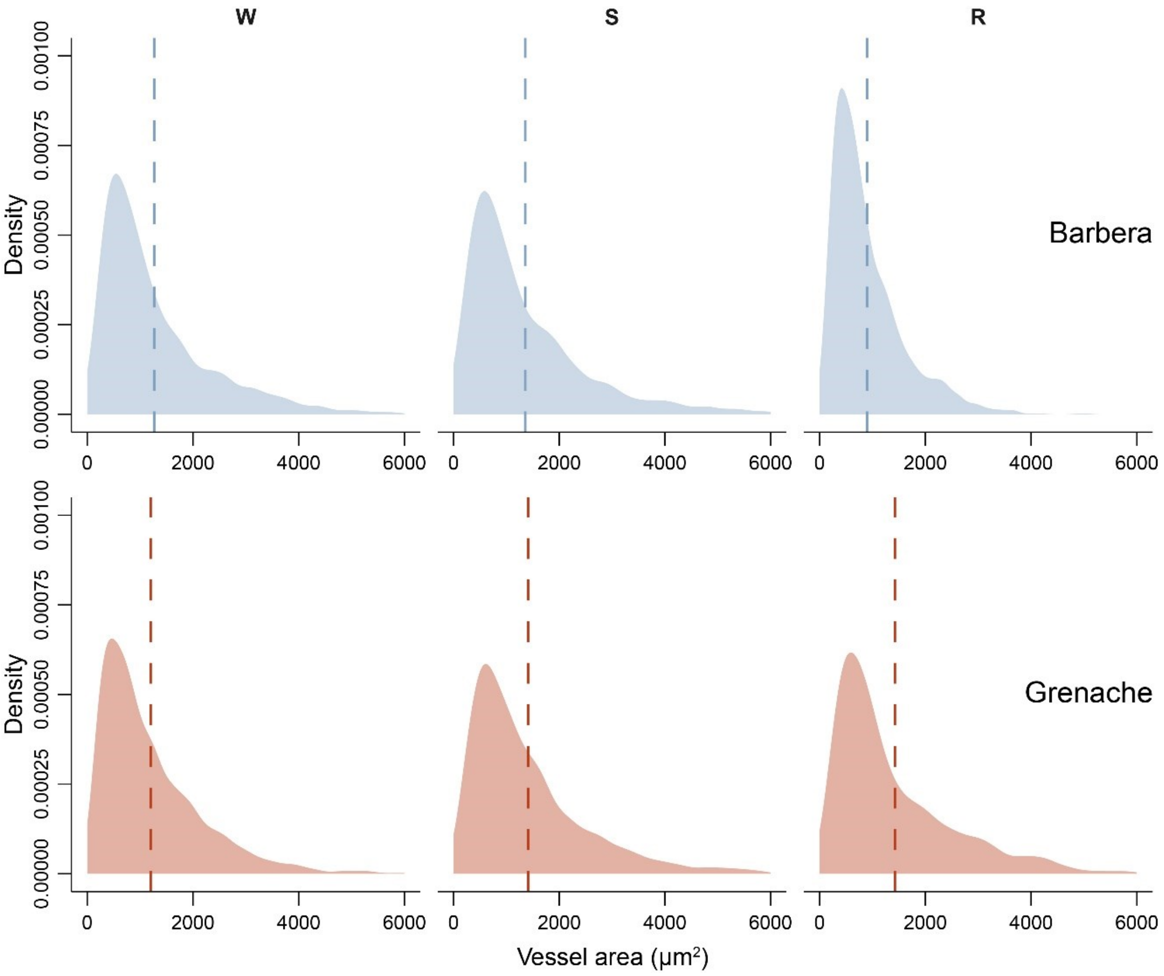

Fig. 6. Xylem vessel size class distribution in stems of cv. Barbera and Grenache under different water status. Distribution of vessel lumen size classes, as measured on the basis of Micro-CT observations in stems of well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Lines correspond to the mean values of each distribution curve.

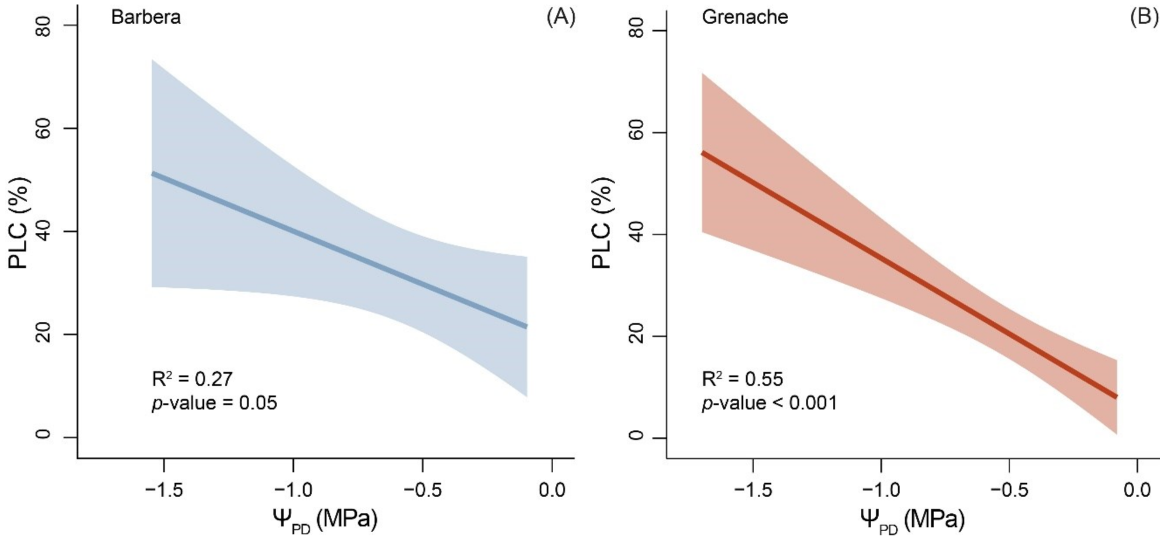

Fig. S1. Vulnerability curves in plants of cv. Barbera and Grenache. Vulnerability to drought-embolism (PLC) in plants of cv. Barbera (Panel A, blue pattern) and Grenache (Panel B, red pattern). PLC was determined by Micro-CT observations of embolized conduits of xylem and plotted against Ψ_{pd} values (n = 7), according to the outcomes of applied linear models. Shaded areas represent the confidence intervals (0.95).

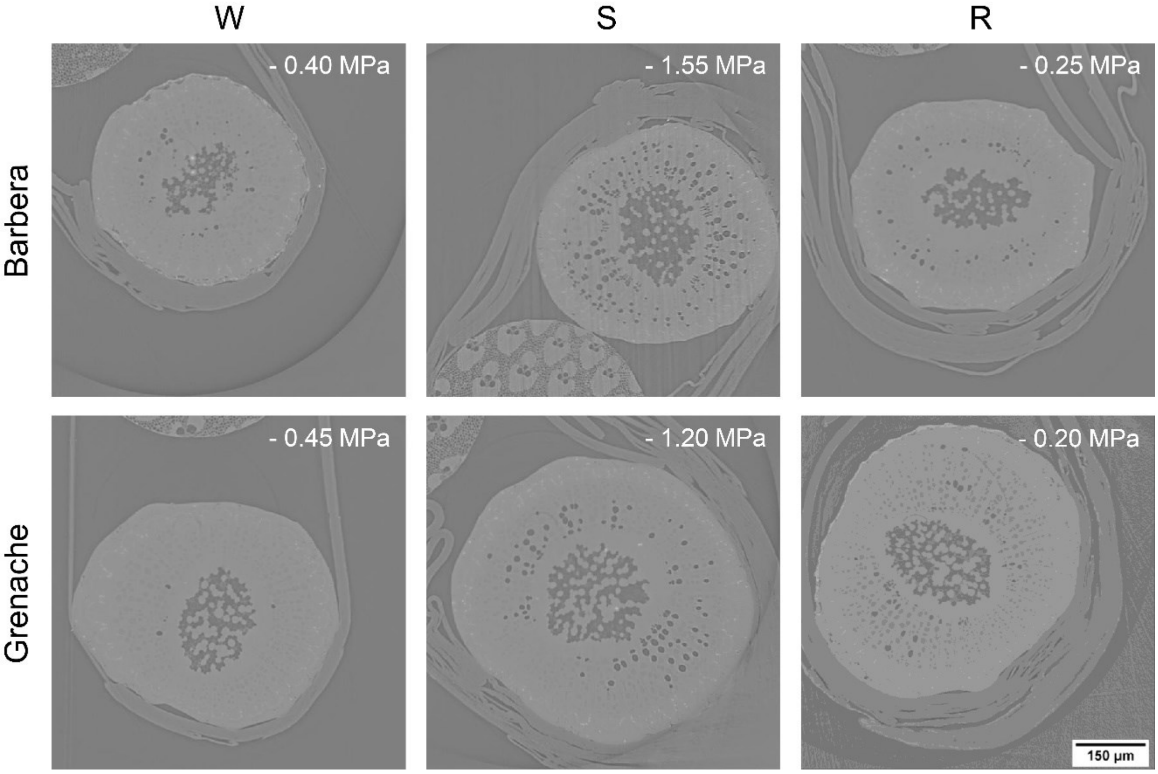

Fig. S2. Xylem embolism visualization by Micro-CT image analysis in plant stems of cv. Barbera and Grenache under different water status. Imaging of transverse sections of Micro-CT scans showing stem xylem functionality as occurrence of water filled vessels (light gray) or embolized conduits (appearing as black gray) in well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera and Grenache. Ψ_{pd} values of these samples at the time of scanning were shown in the images. Bars = 150 µm.

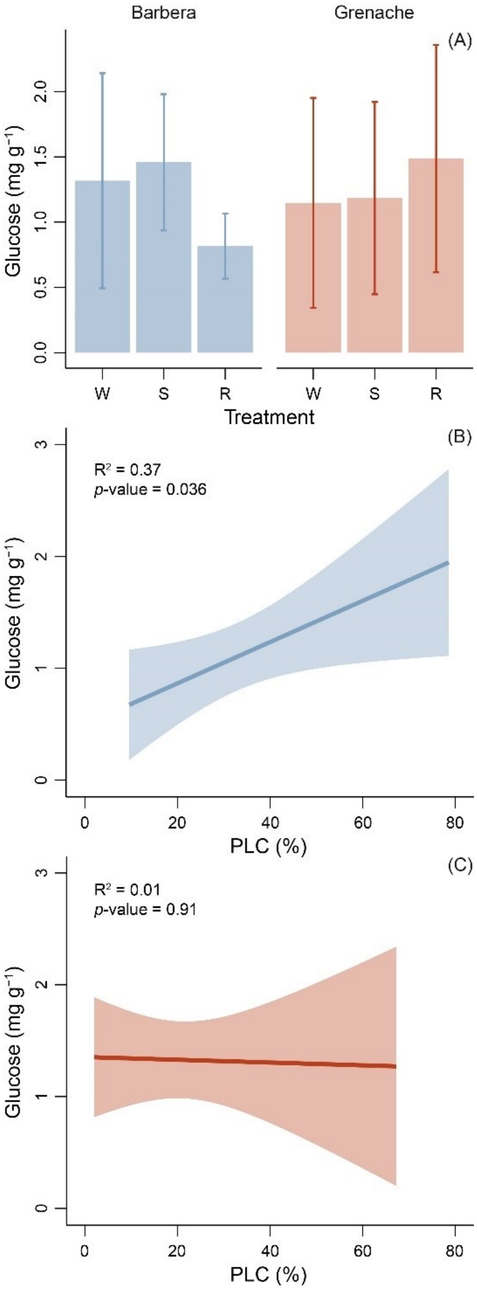

Fig. S3. Glucose content in plant stems of cv. Barbera and Grenache under different water status. and relationship with PLC. Glucose content (panel A) in stems of well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Means ± SD (n = 7) are shown.

Relationships between the content of glucose and PLC in cv. Barbera (panel B) and Grenache (panel C), according to the outcomes of applied linear models. Shaded areas represent the confidence intervals (0.95).

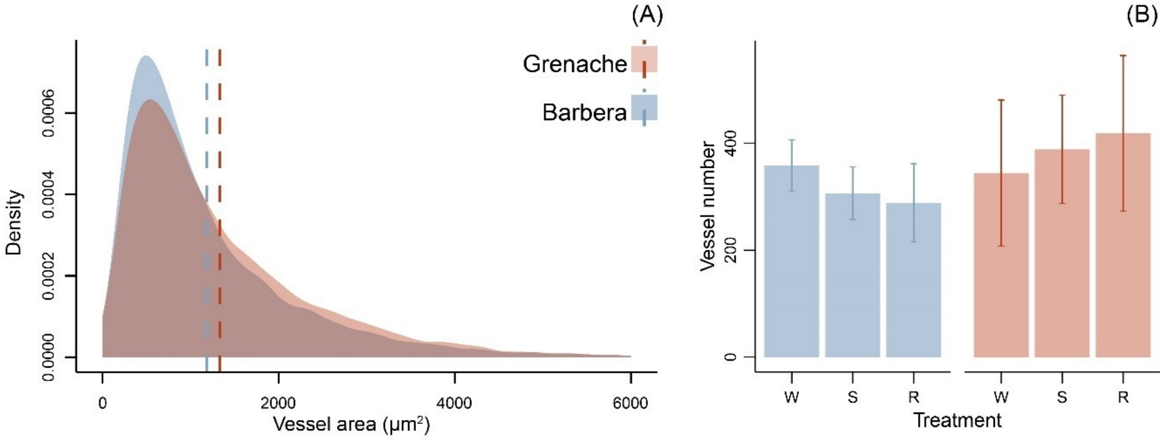

Fig. S4. Xylem vessel size class distribution and vessel number in stems of cv. Barbera and Grenache under different water status. Distribution of vessel lumen size classes (panel A), as measured on the basis of Micro-CT observations, in stems of cv. Barbera and Grenache, independently from treatment. Dashed lines correspond to the mean values of each distribution curve. Vessel number (panel B) in stems of well-watered (W), water stressed (S) and recovered (R) plants of cv. Barbera (blue pattern) and Grenache (red pattern). Means ± SD (n = 7) are shown.

4. NSCs and growth

4.1. Introduction to the study | Chapter 4

With the aim to evaluate rapid adjustments of the plant to the water deficit, the previously reported experiments were characterized by drought stresses that were short and intense and therefore could not have such a marked effect on growth. However, NSCs in addition to being involved in osmoregulation, play a pivotal role in growth and plant development, since they are the substrate for all plant metabolic activity.

In this context, an experiment was set up to investigate the multifaceted role of NSCs: one-year old grapevines (cv. Merlot) were subject to progressive and increasing drought stress, creating favorable conditions for the implementation of adaptive strategies. During and at the end of drought stress NSCs content and growth traits were measured to establish the trade-off between accumulation and growth.

4.2. Response of Merlot Grapevine to Drought Is Associated to Adjustments of Growth and Nonstructural Carbohydrates Allocation in above and Underground Organs

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Keywords: drought; *Vitis vinifera*; NSC; Merlot; dry matter allocation

ABSTRACT

Studying changes in partitioning of dry matter and nonstructural carbohydrates (NSC) content in both aboveground and underground perennial tissues in drought-affected grapevines could provide insights into plant response and carbon allocation strategies during stress periods. The analysis of soluble NSC and starch content in leaf petioles, due to their role in hydraulic segmentation, should also be considered. In the present research, these aspects have been investigated in Merlot grapevines grown in pots and subjected to progressive and increasing soil dehydration, and in well-irrigated vines. Drought conditions caused drastic reduction of shoot elongation and total plant leaf area development in favor of a greater biomass allocation and partitioning towards roots, where most of the NSC reserves were also conserved. Dry matter content of the perennial organs increased in stressed vines due to growth reduction, allocation of carbon reserves and possible anatomical modifications. Vines subjected to drought showed a higher NSC content in petioles, supporting the hypothesis that they are involved as compatible solutes in osmotic adjustments.

INTRODUCTION

It has been ascertained that future global warming scenarios will increase the drought risk in most of the cultivated regions in the next several decades (Cramer *et al.*, 2018; Santos *et al.*, 2020; Masson-Delmotte *et al.*). Drought can seriously affect crop yield and quality, and, in exceptional cases, crop survival (Farooq *et al.*, 2009; Poni *et al.*, 2018; Sorek *et al.*, 2021). Where applicable, irrigation practices are largely adopted to compensate seasonal water deficit, even though such practices may also generate negative environmental aftermaths (Perret and Payen, 2020). In this context, agricultural research must deal with this emerging problem by either optimizing water usage or creating more tolerant/resistant breeds. To achieve these objectives a deeper understanding on the responses and on the mechanisms implemented by plants under stress conditions is needed. Grapevine (*Vitis vinifera* L.) is mostly cultivated in Mediterranean climate areas but has been also gradually expanding to other climatic regions. Grapevine has demonstrated a high phenotypic plasticity and is considered as a model perennial plant in many studies in plant science. Under water deficit conditions, stomatal closure is one of the main physiological responses adopted by plants and could have strong effects on plant carbon balance. The regulation of stomatal closure in relation to the water potential has been proposed as a crucial discriminant between two distinct behaviors: isohydricity and anisohydricity (Tardieu and Simonneau, 1998). Moreover, Schultz (2003) demonstrated that in grapevines both of these behaviors are present, but this categorization is not strictly genotype-dependent and could be influenced by environmental factors (Hochberg *et al.*, 2018; Villalobos-González *et al.*, 2019). Thus, there is no clear evidence on which of the two behaviors is most performant under drought (Gambetta *et al.*, 2020). Even though Hochberg *et al.* (2018) suggested that the terminology should be reconsidered, it is still considered convenient. Isohydric plants are more prone to stomatal closure and therefore are more subject to carbon starvation; as opposite, the anisohydric varieties, accepting a major risk of dehydration, can maintain high photosynthetic rates during water shortages. The sugars produced by photosynthesis are mainly allocated in three ways: i. consumed by the primary or secondary metabolism; ii. invested in new growth; or iii. accumulated in the reproductive organs or stored in the perennial organs. As far as plant metabolism is concerned, there are many chemical compounds and pathways involved in water stress responses that may create complex and aggregated modulations, most of which are not yet fully understood (Hochberg *et al.*, 2013;

Degu *et al.*, 2019; Yadav *et al.*, 2021). The different carbon allocation between sinks (new vegetation or storage organs), and therefore investments in the brief or long term, could have a consistent impact on the carbon balance. Due to water deficit, modifications on the root to shoot ratio have been observed in many plant species (Benjamin *et al.*, 2014; Ledo *et al.*, 2018). In fact, shoot growth is often reduced, favoring root growth, in order to increase water absorption and to explore deeper soil layers (Anjum *et al.*, 2017). Starch is an amorphous glucose polymer and by far the major carbon reserve compound in the plant kingdom. Starch accounts for the main part of the nonstructural carbohydrates (NSC), and can be efficiently converted in soluble sugars, if necessary (MacNeill *et al.*, 2017). Soluble sugars are primarily involved in plant metabolism but can also play an important role in signaling and osmoprotective processes (Dong and Beckles, 2019). This study aims at parsing the multifaceted role of NSC metabolism in plants, investigating the interplay between NSC allocation and partitioning and plant growth as drivers of plant plasticity during water deficit events. We hypothesized that NSC are involved as compatible solutes in osmotic adjustments in vines subjected to drought. We tested such a hypothesis in a trial designed to oblige vines to experience a progressive and increasing soil dehydration, creating favorable conditions for the implementation of adaptive strategies.

MATERIALS AND METHODS

Plant Material and Modulated Drought Stress Treatment

The experiment was set up in the experimental farm “A. Servadei” of the University of Udine (Udine, Italy) (46°02’ N, 13°13’ E; 88 m a.s.l.) in the summer season 2020. At DOY (day of year) 128 (7 May), 112 one-year-old vines (*Vitis vinifera* L., VCR—Rauscedo, Italy) cultivar Merlot (clone R18 grafted onto SO4 rootstock) were bed out on 20 L pots filled with a mixture of soil (80%) collected at the farm site (2 cm-sieved, 50% sand and 20% clay) and peat (20%). The experiment was conducted under a tunnel with open sides covered with a polyethylene film to exclude rain watering. At the beginning of June, the tunnel film was whitened with a shade paint (ca 30% light attenuation) to avoid excessive radiation-induced overheating of the air and pots. Vines were arranged on four single rows (1.3 x 0.4 m) with a NW-SE orientation. To limit environmental differences, the rows were divided in two plots (with alternated treatments) consisting of 12 plants each and 2 additional plants at the row heads were disposed as buffers. One shoot was trained vertically, whereas bunches and lateral shoots were regularly removed. For each vine row, a drip irrigation line was placed with four emitters per pot (PCJ 2 L h^{-1}, Netafim, Hatzerim, Israel), keeping all plants well-watered until the beginning of the experiment. At DOY 205 (23 July) the treatments were set as follows: the control (well-watered, WW) plants were irrigated with 100% of ET_{LYS} (lysimeter evapotranspiration) throughout the experimental period; the stressed vines (water stressed, WS) received 50% of ET_{LYS} up to DOY 237 and then the regime was halved (25%) until DOY 260 (16 September). Irrigation was supplied every day during the night. The daily ET_{LYS} was directly determined as the average water loss calculated on four potted vines (of the group WW) placed each on a scale (self-constructed) that served as a lysimeter. The same system was previously described by Hochberg et al. (2017). Moreover, four other scales were used to monitor the ET in WS plants throughout the experiment.

Plant Water Status and Gas Exchange Monitoring

On weekly basis, stem water potential (Ψ_s) and gas exchange parameters were measured on fully expanded leaves throughout the experiment. To assess the Ψ_s, the leaves were bagged and covered with aluminum foil 1 h before the measures, which took place during sunny days around midday (between 12 am and 2 pm). After that time, the leaves were collected singularly, and the petioles were excised using a razor blade. While still bagged, the leaves were immediately placed into a Scholander's pressure bomb (Soil Moisture Co., Santa Barbara, CA, USA) and pressurized using a nitrogen cylinder. The Ψ_s was determined when the xylem sap was visibly spurting out from the cut end of the petiole. The weekly data of Ψ_s were computed to calculate the integral of the accumulated water deficit using the model proposed by Fernández et al. (2009). The plants were transferred to the laboratory the day before the sampling date and maintained in the dark until processing started (at 7 am). The gas exchange parameters were measured using an infrared gas analyzer (LI-6400, LI-COR corp., Lincoln, NE, USA). The measurements were taken at a constant light intensity (1000 µmol m^{-2} s^{-1}) as well as CO_2 concentration (400 µmol ml^{-1}) and ambient temperature and humidity. The intrinsic water use efficiency ($_i$WUE) was calculated by dividing net photosynthesis (P_N) by stomatal conductance (g_s).

Morphometric and Biomass Measurements

The evaluations were performed at three sampling times: T0 (pre-treatment); T1, 32 DOE (day of experiment); and T2, 55 DOE. Shoot (at internode between 4th and 5th leaf) and petiole (at 4th leaf) portions were sampled for the NSC analysis. Then, the whole root of each grapevine was discarded from the pot, quickly washed under tap water and thoroughly cleaned with compressed air from soil particles. A pool of three fine root portions (cut approx. 5 cm from the tip, corresponding to the maturation zone of the primary root (Gambetta *et al.*, 2013), and with 2.5–3 cm length) was immediately sampled for biochemical analysis.

The remaining roots, main leaves and the whole annual shoot were separately weighed to assess fresh weight (FW). After this step, all leaves of each vine were placed on an optical scanner to estimate their area, enabling the assessment of three morphological characteristics: total leaf area, mean leaf area and leaf number per each vine. Root, leaf and shoot samples were then dried at 70 °C for 48 h to provide the dry biomass weight (DW) and the measurements of their dry matter content (DMC) expressed as mg DW g^{-1} FW (Pérez-Harguindeguy *et al.*, 2013) as well as the root-to-shoot ratio (R/S).

Measurements of Non-Structural Carbohydrates

The extraction of starch and total soluble NSC was performed as described in (Falchi *et al.*, 2020), with the following modifications: after extraction with 80% ethanol, soluble carbohydrates were recovered in the alcoholic supernatant, while the pellet (containing both starch and maltodextrin oligosaccharide fractions) was incubated in 0.5 mL 2 mM HEPES-Tris pH 6.7 at 25 °C overnight. After a 14,000 x *g* centrifugation for 5 min, solubilized maltodextrin oligosaccharides were recovered in the suspension and combined with all soluble carbohydrate fraction (hereinafter referred to as total soluble NSC), whereas the remaining starch in the pellet was processed as already detailed. Starch and total soluble NSC were quantified by the Anthrone assay method (Yemm and Willis, 1954), as outlined in Tomasella *et al.*, (2020), and expressed as mg of glucose equivalent g^{-1} DW by using standard solutions of amylose and glucose for calibration curves of starch or soluble sugars, respectively.

Statistical Analysis

The effect of treatment, time and their interaction on morphometric and biochemical parameters were tested using a two-way analysis of variance (ANOVA). ANOVA assumptions were checked by diagnostic plot of residuals. When necessary, variables were subjected to logarithmic transformation. *A posteriori* pairwise comparison of means was performed using Tukey's test ($p < 0.05$) only where significant interactions were found.

Correlations between morphometric and biochemical variables were also assessed by Pearson test.

All the statistical analyses were performed in R statistical software (v. 4.0.3) (R Core Team, 2020). Outlier records were detected by the function "outlier Test" of the 'car' package (Fox and Weisberg, 2019) and removed from models (where $p < 0.01$).

RESULTS

Water Plant Status and Use Efficiency

The Ψ_s is a reliable measure to assess plant water status (Choné et al., 2001). During the experiment stressed vines reached a minimum value of -0.88 MPa, suggesting that severe dehydration levels were avoided. The trend of the plant water status shows an increasing difference between WW and WS during the experiment (Figure 1A).

WW ETLYS trend (Figure 1B) was closely related to the variability in the environmental conditions. Differently, in case of the WS plants, the daily variation of the ETLYS tended to stabilize as a consequence of the drought progression. Thus, the limited water availability during the stress period obliged WS vines to optimize their water consumption. Intrinsic WUE at leaf level (Figure 1C) was constantly higher in WS plants except for the final measurement when, due to the prolonged water deficit conditions, the values of PN in WS plants suddenly dropped down. Concerning this point, all the considered gas exchange parameters (PN, g_s and E) were significantly reduced in WS during the water deficit interval (Figure S1).

Biomass and Morphometric Traits in Different Organs in Response to Modulated Drought Stress

Root and shoot biomass parameters were consistently correlated in both treatments whereas a lack of a consistent correlation pattern was observed when comparing these variables and leaf morphometric traits (Figure S2).

Morphometric Parameters in Leaf

As it is shown in Table 1, a highly significant effect was observed when considering drought-modulated stress factor and the interaction between treatment and time. Water restriction caused a significant reduction in number of leaves per plant at both stages analyzed (Figure 2A). In WS plants the decrease in leaf number at T1 was significant in respect to WW. In September the number of leaves per plant in WS vines was further reduced, whereas an inverse behavior was observed in WW, where this trait remained substantially stable.

Mean leaf area was highly related to the extent of expansion of the foliar lamina in canopy and, hence, theoretically also to the water status of the plant. The ANOVA results showed that variance in mean leaf area was significantly affected by water supply (Table 1). Notably, mean leaf area changed in an opposite way during the experiment when considering WW and WS, respectively (Figure 2B). Nevertheless, leaves were always constantly smaller in their average size in WS plants, regardless the stress intensity. The difference in leaf size was larger at the first level of stress and then it reduced in September, after the second drought treatment. Distinctly, WW exhibited an enhanced average leaf area only in the early season (starting from the initial value in July of 5870 ± 1164 mm2, dotted grey line in Figure 2B), which declined from August.

Dry Matter Content and Morphometric Parameters in Shoot

Shoot DMC was mainly predicted by time, drought and their interaction (Table 1). Accumulation of dry mass in annual shoot increased throughout the season in WW: compared to T0 (dotted grey line, Figure 3A), it showed ~21% growth at T1 in August and +80% at the final sampling time T2. When grapevines were subjected to increasing drought levels over time, shoot DMC was always significantly higher than in irrigated vines at both sampling times, as compared to the control. Similar to what was observed in WW, shoot DMC also continued to significantly grow throughout the season, despite water stress, with a larger change (dry mass was even doubled at the end of the experiment compared to the initial level in July, where in WW the increase was equal to ~80% more of the initial state).

When we consider total shoot DW, chosen as good predictor of organ growth, it was evident that treatment strongly explained most of the variance, while timing was not significant (Table S1). More in detail, in control vines a significant gradual development of the whole annual shoot (as total DW) was detected (Figure S3A). In contrast to what was assessed for DMC, drought stress caused a different effect on total shoot DW, since all stressed vines resulted in an overall reduction in their values compared to well-watered ones. Nonetheless, WS vines continued to grow in their total shoot mass over the whole experiment time, although at a lesser extent, compared to WW. Overall, shoot DW increased by ~176% from the initial level to the one measured at the end of experiment, despite the ongoing severity of drought stress.

Dry Matter Content and Morphometric Parameters in Root

DMC was highly affected over time and by drought in root, though their interaction was not significant, differently to what was shown in shoots (Table 1).

WW plants exhibited an increasing root DMC throughout the period of the experiment (Figure 3B), as well as WS vines. It is noteworthy that water limitation caused a strong significant enhancement in root DMC, as evidenced by the large increase observed in WS in August (+ 64%) and in September (+59 %) compared to their relative control.

Regarding the effect on root growth, estimated as total DW, the variance was significantly described by both time and treatment factors, without any significant interaction among them (Table S1). Specifically, a steep increase of root DW in WW samples was also observed (Figure S3B) over time, reaching three and five-fold greater values than T0 in T1 and T2, respectively. As in shoot, WS vines exhibited a strong reduction in root development, though accompanied by some increase over time. In August, root DW was 19.8 ± 2.76 g (2.5-fold more compared to initial level and -20.5% compared to the respective control) and during the second stress grew to a value of 30.48 ± 4.00 g (a 3.9-fold increase compared to initial level and -27% with respect to its control).

Root-to-Shoot Ratio

ANOVA results highlighted clearly that this variable displayed important changes between treatment and time but not for their interaction (Table 1). Grapevines distributed more biomass in their underground organs than in leaves and shoots at the end of growing season in September, and this physiological aspect was evident in both WS and WW (Figure 3C). In WS samples the ratio was greater than in WW, in both T1 (+33%) and T2 (+20%).

A stronger direct correlation was observed among the R/S ratio and root DMC observed in WS compared to WW ($r = 0.853$, Figure S2). We also observed a significant negative correlation between root DMC and leaf number found only in WS ($r = -0.789$, Figure S2).

Non-Structural Carbohydrate Content in Different Organs in Response to Modulated Drought Stress

A lack of a consistent correlation pattern between soluble NSC and starch in the various organs considered was observed in both treatments (Figure S2).

NSC Content in Leaf Petiole

Time and treatment differently influenced petiole starch and soluble NSC content (Table 2). Petiole starch concentration significantly changed over the season timing, while no differences were found due to water stress. Conversely, soluble NSC content was affected by the water treatment and time; no significant interaction between the two factors was observed.

The pattern of starch pool was similar in both WW and WS along the experimental period (Figure 4A), showing a gradual increase from the beginning of the drought imposition, throughout T1 and T2.

The pattern in total soluble NSC changes was different to what was observed in starch, as drought always induced a significant accumulation of these carbohydrates, despite the intensity level of imposed stress (Figure 4B). More in detail, the increase in total soluble NSC in WS petiole was +29% at T1 and +24% at the second period, as compared to WW values. Again, a gradual increase in their concentration was also appreciable, encompassing the entire period of experiment, reaching higher values at T2 with respect to what was detected at the previous stage (+13.4% in WS, whereas it corresponded to +8% in WW).

NSC Content in Shoot

When comparing the effect of drought and time on the specific pools of NSC in shoot, a more complex situation could be observed. ANOVA analysis revealed that the differences in starch content could be significantly attributed to the two experimental factors and their interaction (Table 2). Total soluble NSC was similar, as highlighted by the lack of significance for all factors including their interaction. As can be observed in Figure 4C, starch was increasingly accumulated throughout the considered period in shoot from both WW and WS. In WW, starch concentration ranged from an initial concentration of 46.00 ± 16.14 to 50.06 ± 8.83 mg g-1 at T1, and then it doubled by the final stage. In WS vines, the increase was 1.85 times more with respect to the initial value at T1 and even 3.6 times more at T2. As a result, at the last stage, when WS vines were subjected to the more severe drought level, starch content in shoot was significantly higher compared to WW, reaching 82% of increase. Unlike starch, the total soluble NSC pool changed in both WS and WW plants during time and over treatment with an inverse pattern (Figure 4D). Specifically, a slight increase (6.5%) in NSC content in WW shoots was ascertained, reaching the level of 126.18 ± 8.86 and 134.49 ± 16.33 mg g-1 at T1 and T2, respectively. WS vines accumulated high levels of soluble NSC at the first sampling time (140.40 ± 14.19 mg g-1, +11% with respect to control), but later at T2 they reduced their shoot concentration to the far lower value of 129.78 ± 17.35 mg g-1, similarly to what was found in WW. Finally, observing the correlation pattern between shoot morphological traits and NSC, we found that shoot DMC and shoot starch in both WW and WS grapevines were significantly correlated (r_WS = 0.639; r_WW = 0.828, Figure S2).

NSC Content in Root

In roots, starch was neither influenced by any of the considered factors, nor by their combination, whereas in the case of total soluble NSC both sampling time and the interaction (time x treatment) became significant (Table 2).

In WW vines, starch was accumulated in the first period of experiment (+32% from July to August). Further, starch reserve levels were not affected on T2. WS plants followed a similar pattern (+23% compared to the basal concentration) (Figure 4E,F). Afterwards, starch content did not change in more stressed roots at T2, quite similar to the same level previously seen in WW.

When considering the total soluble NSC content in roots at T1, a lack of significance was found due to water supply treatment. Then, the level of NSC in WS roots remained quite the same, whereas in well-watered samples it drastically decreased (28% less than in WS).

A lack of any significant correlation of root DMC (or any other morphological traits) with neither root starch nor soluble NSC content, respectively, was found (Figure S2). Finally, differently from petiole or shoot, root starch amount was predominant over total soluble NSC pool. It was overall three times more than total soluble carbohydrates independently of the experimental factors.

DISCUSSION

The present study shows that WS plants were exposed to an increasing level of drought, ranging from mild ($50 < g_s < 150$ mmol H2O m^{-2} s^{-1}) to moderate (<50 mmol) intensity levels, as defined on the stomatal conductance basis (Lovisolo *et al.*, 2010; Hochberg *et al.*, 2013) (Figure S1), and displayed also by increasing accumulated water stress values (Figure 1A). Given that stomatal closure is one of the early well-known responses adopted by vine plants to protect from the risk of hydraulic failure (Cochard *et al.*, 2002; Gambetta *et al.*, 2020), we expected that WS would respond to drought by drastically reducing gas exchanges and net photosynthesis. However, it has been found that WS vines regulated their water use by largely enhancing $_i$WUE (Figure 1C) from the imposition of the mild water restriction on-wards. This was in accordance to what was recently stated by authors (Dayer *et al.*, 2020; Gambetta *et al.*, 2020), that different genotypes may exhibit substantial variations in the extent of their control on stomatal closure, depending also on environmental conditions and intensity of stress. In some varieties, the plant exhibits a mild restriction of gas exchange under drought conditions (*i.e.*, anisohydric), sustaining photosynthesis even at lower leaf water potential (Schultz, 2003). This leads to a trade-off between carbon assimilated and water lost by transpiration and represents some degree of tolerance of the plant under drought. Generally, this hydraulic behavior causes an improvement of the plant WUE under moderate stress, as found in the present work. This behavior, characterized by a gradual significant lowering of midday Ψ_s, confirmed that Merlot grapevines maintained their stomata only partially closed under drought conditions, in agreement with what was reported by other researchers (Schultz, 2003; Williams and Baeza, 2007; Shellie and Glenn, 2008; Degu *et al.*, 2019). Since stomatal closure is usually less susceptible to water stress or temporally delayed in anisohydric-like response, photosynthesis is also less constrained in comparison with isohydric response. Thus, some authors suggested that anisohydric response could protect against carbon starvation (McDowell *et al.*, 2008; Sade *et al.*, 2009, 2012) and, hence, is more adapted to survive under conditions of mild, prolonged drought (Gerzon *et al.*, 2015). Altogether, in our trial we can assume that the levels of carbon assimilation under drought did not sacrifice allocation of NSC reserves in vine perennial organs, at the expense of plant growth. In accordance with the above suggestions, we found that both drought conditions reached at T1 and T2 greatly affected plant growth through a differential biomass allocation in root vs. shoot and leaf canopy (Figures 2 and 3). Indeed, although total root biomass was significantly smaller, WS plants sacrificed mainly above-ground growth with respect to their underground organ. This resulted also in the improvement of their water use efficiency, as mentioned above, given that the restricted transpiration area was better equilibrated to the limited water supply. Bringing all the results together, mild to moderate stress induced the vines to stop the elongation of the annual shoots, thus reducing the number of principal leaves compared to vines under well-watered regime. It is well known that soil water scarcity has a strong impact on grapevine shoots (Pellegrino *et al.*, 2005; Lovisolo *et al.*, 2010) by blocking shoot internode extension, as well as leaf lamina expansion (Hochberg *et al.*, 2017) and, when the drought is particularly severe, even shedding leaves, to some extent (Degu *et al.*, 2019). In our experimental conditions, WS was shown to face increasing drought imposition by maintaining on the shoot a small, but large enough, number of mature leaves, characterized by a smaller leaf area if compared to WW. When drier conditions were reached at T2, WS vines also experienced a significant loss of leaves. We speculate that at this stage the plant did not develop the apical, less-expanded leaves of the shoot, as we found a strong inverse relation among number of leaves and mean leaf area only in WS samples (r = -0.667 and r = 0.620 in WS compared to WW, Figure S2). In this regard, several studies have demonstrated that under abiotic stresses, elevated levels of endogenous abscisic acid (ABA) inhibited shoot growth (Cardoso *et al.*, 2020), in addition to the best-known role of controlling stomatal closure and promoting root elongation against lateral root formation (Rosales *et al.*, 2019; Cardoso *et al.*, 2020). Moreover, dry mass allocation was expected to be sensitive to environmental constraints, such as nutrient or water limitation, being changing over certain phenological stages of a plant (Brunner *et al.*, 2015). In our experiment, WS vines largely increased their DMC in roots and shoots with respect to WW. DMC partitioning was favored more in roots than in shoots, thus resulting in an increased root-to-shoot ratio in WS (Figure 3). These results were also confirmed by a stronger direct relationship observed among R/S ratio and root DMC observed in WS compared to WW (r = 0.853, Figure S2), or the significant inverse relation between root DMC and leaf number found only in WS (r = -0.789, Figure S2). Our results can be rationalized by the strategy that grapevines exploit to mitigate the risk of hydraulic failure in case of water shortage, limiting canopy vigor in favor of the underground organ. Preferential development of roots with respect to shoots is important for more exploration into deeper soil layers, as already reported by various authors across various species (Dry *et al.*, 2000; Lovisolo *et al.*, 2010; Brunner *et al.*, 2015). Additionally, a higher density of roots measured in WS could suggest that grapevine roots under dehydration could afford other strategies to cope with stress conditions, such as modifications of their anatomical tissues (e.g., increasing suberin deposition in cell walls) (Lovisolo *et al.*, 2010; Barrios-Masias *et al.*, 2015; Yıldırım *et al.*, 2018) and/or by reducing vessel size or building thicker walls of fibers (Moura *et al.*, 2010; Brunner *et al.*, 2015). Both these anatomical strategies cooperate in maximizing water absorption (Steudle, 2000), lowering grapevine root hydraulic conductivity. These acclimation-induced anatomical adjustments, in association with modulated aquaporin expression or activation at various extent in different varieties, could synergistically interact improving $_{i}$WUE under drought (Vandeleur *et al.*, 2009). Interestingly, starch and soluble NSC content in roots were not affected by drought imposition. Relatively elevated pools of soluble sugars were also preserved in both WW and WS over time in order to push forward biochemical reactions for their interconversion to starch. This was confirmed by the lack of any significant correlation of root DMC (or any other morphological traits) with neither root starch nor soluble NSC content, respectively (Figure S2). Nevertheless, it is also conceivable that root DMC increased in Merlot grapevines under water deficit due to xylem anatomical changes, as it is well-known that drought affects xylogenesis by reducing cambial cell layers and vessel lumen (Savi *et al.*, 2015; Nardini *et al.*, 2017; Eckert *et al.*, 2019). These acclimatory modifications have been already demonstrated to occur in some grapevine varieties in organs such as dormant cane stem (Falchi *et al.*, 2019), mature stem (Jacobsen *et al.*, 2015; Munitz *et al.*, 2018) or leaf petiole (Hochberg *et al.*, 2014; Falchi *et al.*, 2020). As opposite, shoot DMC and shoot starch in both WW and WS grapevines were significantly correlated (r_WS = 0.639; r_WW = 0.828, Figure S2), suggesting that in annual shoot dry matter partitioning was mainly devoted to sustain starch reserves along the vegetative season (from T1 to T2) and even along drought intensification (Table 2 and Figure 4). The latter result thus point out that in Merlot water shortage firstly arrested or decrease stem elongation and then delayed the depression of photosynthetic rate. Consistently with this result, previous studies attributed NSC accumulation in drought-stressed organs to a lag between decrease in growth and carbon assimilation (Mitchell *et al.*, 2013). This may cause an improvement of carbohydrate allocation to the shoot, which protects the plant against future abiotic or biotic challenges. Given the importance of leaf petiole as one of the most vulnerable organs of the grapevine plant to embolism occurrence during drought (Hochberg *et al.*, 2016), NSC concentration was followed in this organ during the modulation of stress. Differently from root and shoot organ, NSC pools in leaf petioles were mainly ascribed to relatively higher concentrations of soluble sugars (>100 mg g^{-1} DW), instead of the starch counterpart (Figure 4). Considering the leaf petiole, we could speculate that starch reserves are mainly ascribed to storage in amorphous form, and soluble, osmo-compatible NSC fractions could be derived from both photosynthesis and starch remobilization. Noticeably, in contrast to perennial organs, soluble NSC in petioles were strongly affected by drought with a high level of significance, being always higher in WS compared to WW (Table 2 and Figure 4). Moreover, a direct relationship among soluble NSC and petiole starch (r = 0.683, respectively, Figure S2) was observed in WS petioles, indicating that starch reserves sustained the high concentration of soluble sugars. For instance, high levels of sucrose strongly correlated with starch content in sprouting tubers of Arum species when its reserve pools started to be remobilized (Petrussa *et al.*, 2018). All together, these results could support the hypothesis that petiole soluble sugars could be involved in osmotic regulation. Consistent with the present study, we recently demonstrated that in Syrah variety showing a near-anisohydric response to a short severe stress, maltose/sucrose levels increased over the whole drought imposition and also two days after re-irrigation, suggesting the important contribution of the latter to turgor maintenance (Falchi *et al.*, 2020). Indeed, these sugars together with organic acids, proline, polyamines and other osmolytes decrease leaf osmotic potential as already described in leaf mesophyll (Chaves and Oliveira, 2004; Degu *et al.*, 2019; Gambetta *et al.*, 2020; Gurrieri *et al.*, 2020). Additionally, besides their contribution to osmotic adjustment, elevated soluble sugars contribute as nutrients or energy reserve, antioxidant activities, membrane protection and cell integrity in parenchyma tissues, as well as favor an improved capacity of hydraulic recovery (Secchi *et al.*, 2017; Tomasella *et al.*, 2020). In any case, it could not be excluded that in Merlot variety also anatomical adjustments such as vessel size restriction at the petiole level actually could simultaneously cooperate contributing to improve resistance to water limitation. In this regard, in comparing petiole xylematic traits among Cabernet Sauvignon and Syrah varieties, we have evidenced that anisohydric-like Syrah also produced narrower vessels after experiencing a short severe drought stress (Falchi *et al.*, 2020).

CONCLUSIONS

The progressive water stress imposed on pot-grown Merlot grapevines caused a drastic restriction in shoot elongation and total leaf area development in favor of a more active biomass allocation and partitioning towards the underground organ. Overall, the mitigation of the evaporative demand at the plant level operated by these long-term adjustments is essential for drought tolerance and therefore vines could preserve, though reduced, a sufficient C assimilation increasing their water use efficiency, even in front of a risky lowering of water potential. The data clearly show that C fixation was mainly devoted to preserving NSC (mainly starch) pools in roots at safe thresholds or even to enhance them in woody stems, suggesting their pivotal role as strategic reserves in preparation for facing future stresses. Nevertheless, investigation of anatomical changes and NSC patterns in roots affected by drought is still scarce, and further studies are needed to better understand the modifications imposed by water stress. Moreover, the elevation of soluble NSC observed at the level of petioles suggests that they also potentially play the role of osmoprotectants and osmocompatible compounds and not only as merely metabolic C supply. To date, this strategy applies to anisohydric behaviour, confirming Merlot to belong to this hydraulic category. Other experiments are ongoing and will be carried out in the next future with the aim to examine the responses of varieties with different hydraulic behaviour subjected to various scenarios of water constrains.

AUTHOR CONTRIBUTIONS

Conceptualization, P.S., E.P., R.B. and M.V.; methodology, all authors; formal analysis, F.B., E.B. and M.V.; investigation, E.P., R.B., M.V., V.C., E.B., A.C.; data curation, M.V. and F.B.; validation, G.A., M.V. and F.B.; writing—original draft preparation, E.P., R.B. and M.V.; writing—review and editing, all authors; visualization, M.V.; supervision, E.P. and P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

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TABLES

Table 1. Outcomes of the analysis of variance (two-way ANOVA) applied to leaves number, mean leaf area, shoot and root DMC, and R/S ratio in relation to drought stress (treatment), time and their interactions.

Factor	**Df**	**F value**	***p*-Value**
Leaves number			
Time	1,22	2.89	0.103
Treatment	1,22	48.40	< 0.001 ***
Time x Treatment	1,22	5.76	0.025 *
Mean Leaf Area			
Time	1,22	0.41	0.528
Treatment	1,22	34.67	< 0.001 ***
Time x Treatment	1,22	3.56	0.072
log(Shoot DMC)			
Time	1,22	213.21	< 0.001 ***
Treatment	1,22	53.49	< 0.001 ***
Time x Treatment	1,22	8.17	0.009 **
log(Root DMC)			
Time	1,23	27.77	< 0.001 ***
Treatment	1,23	204.32	< 0.001 ***
Time x Treatment	1,23	0.22	0.639
log(R/S ratio)			
Time	1,24	60.35	< 0.001 ***
Treatment	1,24	16.60	< 0.001 ***
Time x Treatment	1,24	0.69	0.411

Table 2. Outcomes of the analysis of variance (two-way ANOVA) applied to starch content and total soluble NSC content in petiole, shoot and root in relation to drought stress (treatment), time and their interactions.

Factor	**Df**	**F value**	***p*-Value**
PETIOLE log(Starch)			
Time	1,17	6.14	0.024 *
Treatment	1,17	0.47	0.504
Time x Treatment	1,17	0.02	0.883
PETIOLE Total soluble NSC			
Time	1,15	6.20	0.025 *
Treatment	1,15	27.21	< 0.001 ***
Time x Treatment	1,15	0.05	0.824
SHOOT Starch			
Time	1,24	104.48	< 0.001 ***
Treatment	1,24	83.73	< 0.001 ***
Time x Treatment	1,24	11.08	0.003 **
SHOOT Total soluble NSC			
Time	1,24	0.04	0.835
Treatment	1,24	0.75	0.395
Time x Treatment	1,24	2.96	0.098
ROOT Starch			
Time	1,21	0.41	0.528
Treatment	1,21	1.36	0.256
Time x Treatment	1,21	0.10	0.756
ROOT Total soluble NSC			
Time	1,23	15.91	< 0.001 ***
Treatment	1,23	2.66	0.116
Time x Treatment	1,23	13.77	0.001 **

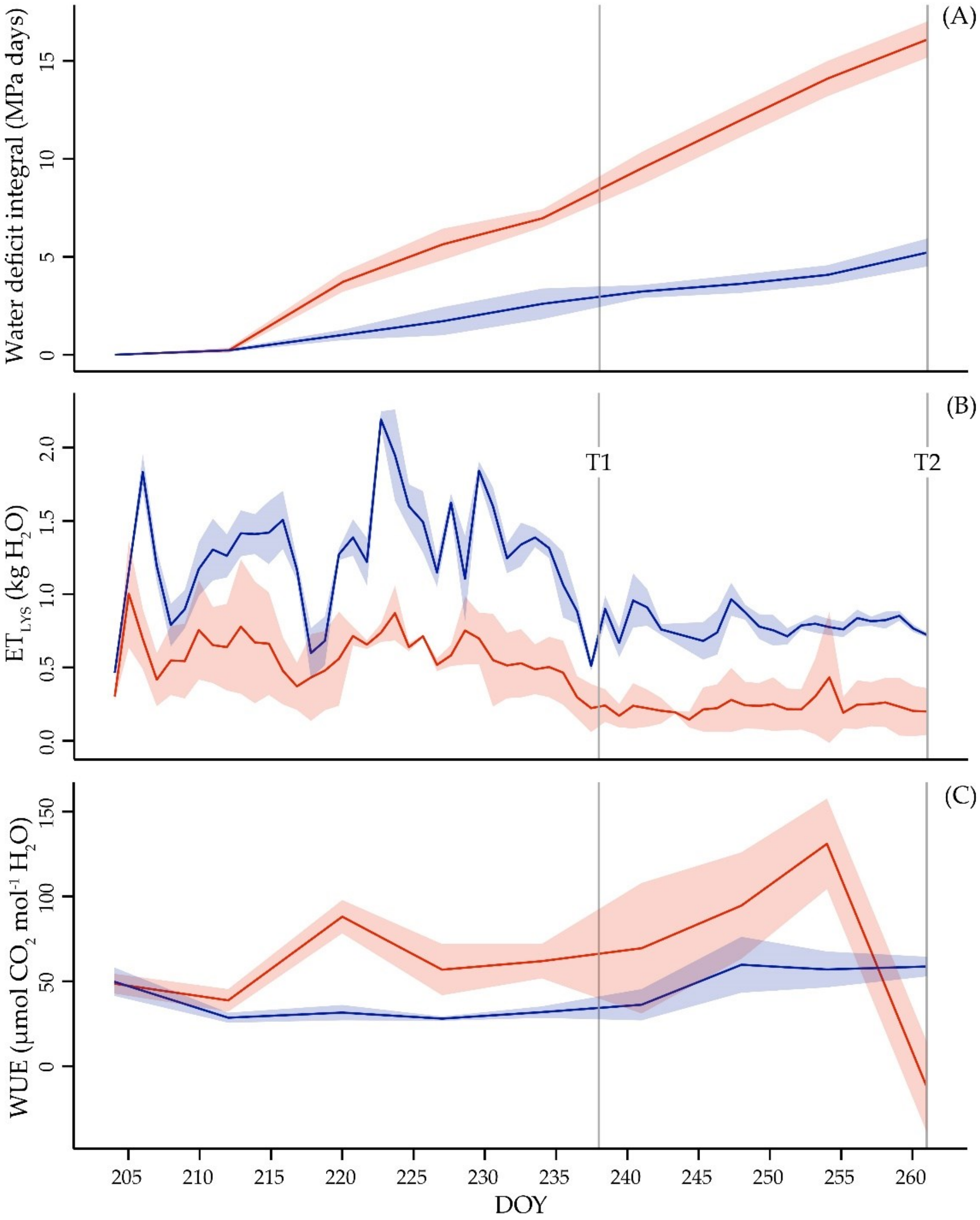

Figure 1. Split lines interpolating mean value (full color line) ± standard deviation (shaded area) of water deficit potential (panel A), crop evapotranspiration (panel B) and water use efficiency (panel C), measured in Merlot vines under well-watered (WW – blue line) and water stress (WS – red line) conditions. Data are referred to the whole experimental period.

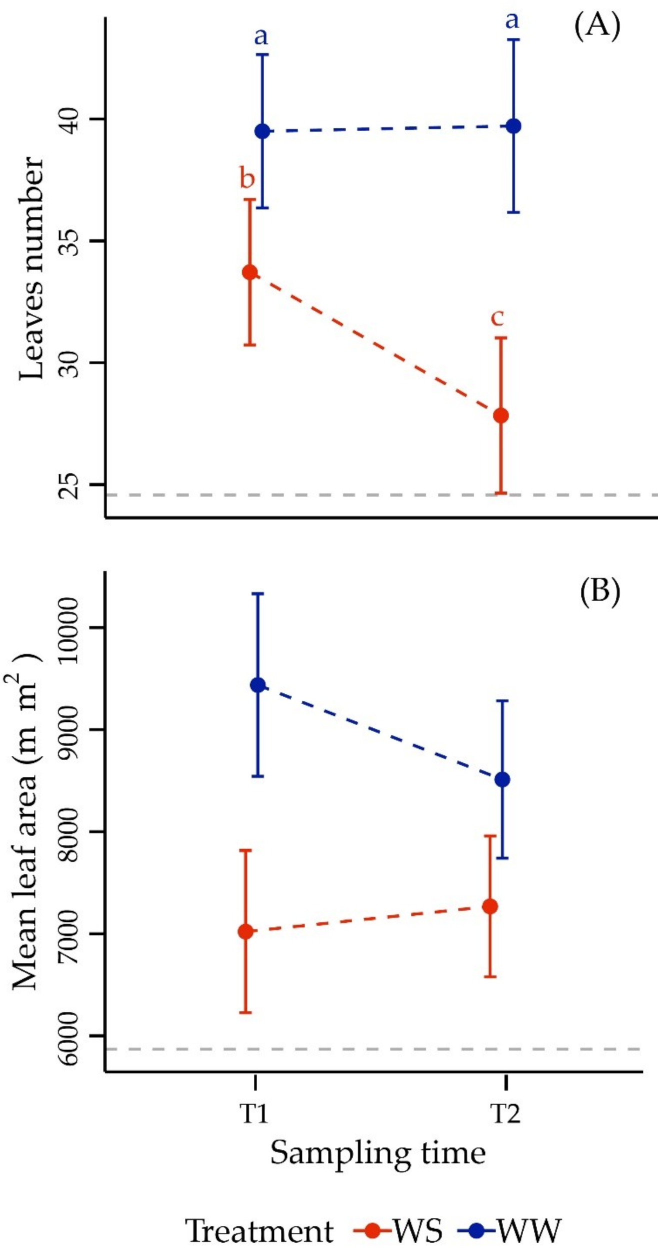

Figure 2. Mean values ± standard deviation of leaves number (panel A), and mean leaf area (panel B) in Merlot vines under well-watered (WW) and water stress (WS) conditions at sampling times T1 and T2 (237 and 260 DOY). The horizontal dotted line indicates the value of the variables at T0 (205 DOY). Different letters correspond to statistically significant differences ($p < 0.05$).

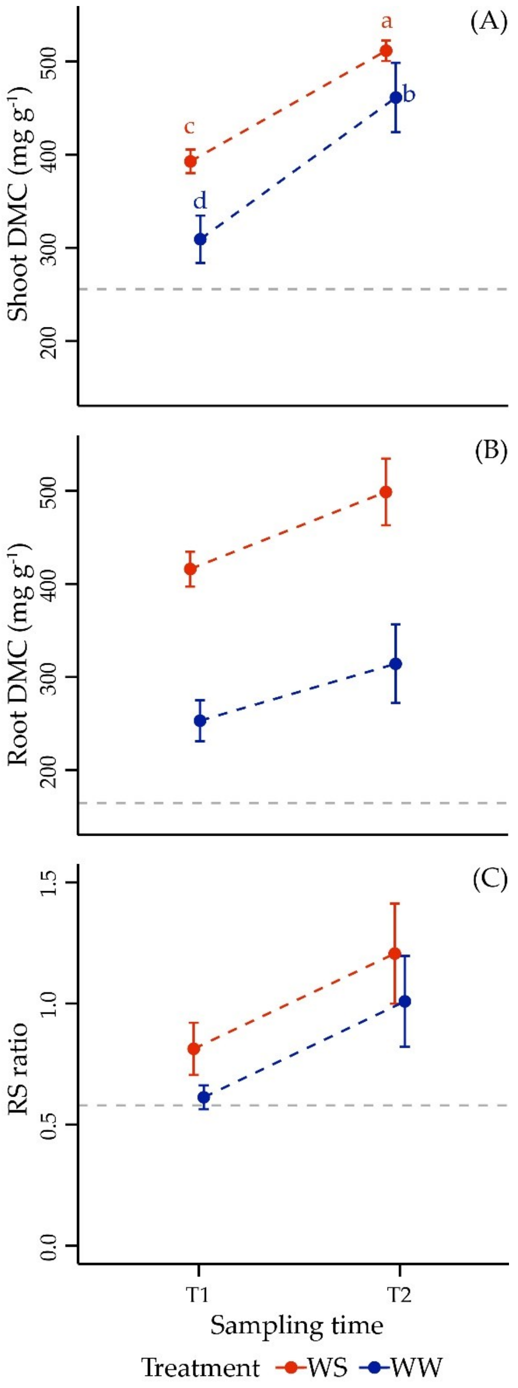

Figure 3. Mean values ± standard deviation of dry matter content (DMC) of shoot and root (panel A and B, respectively), and of RS ratio (panel C) in Merlot vines under well-watered (WW) and water stress (WS) conditions at sampling times T1 and T2 (237 and 260 DOY). The horizontal dotted line indicates the value of the variables at T0 (205 DOY). Different letters correspond to statistically significant differences ($p < 0.05$).

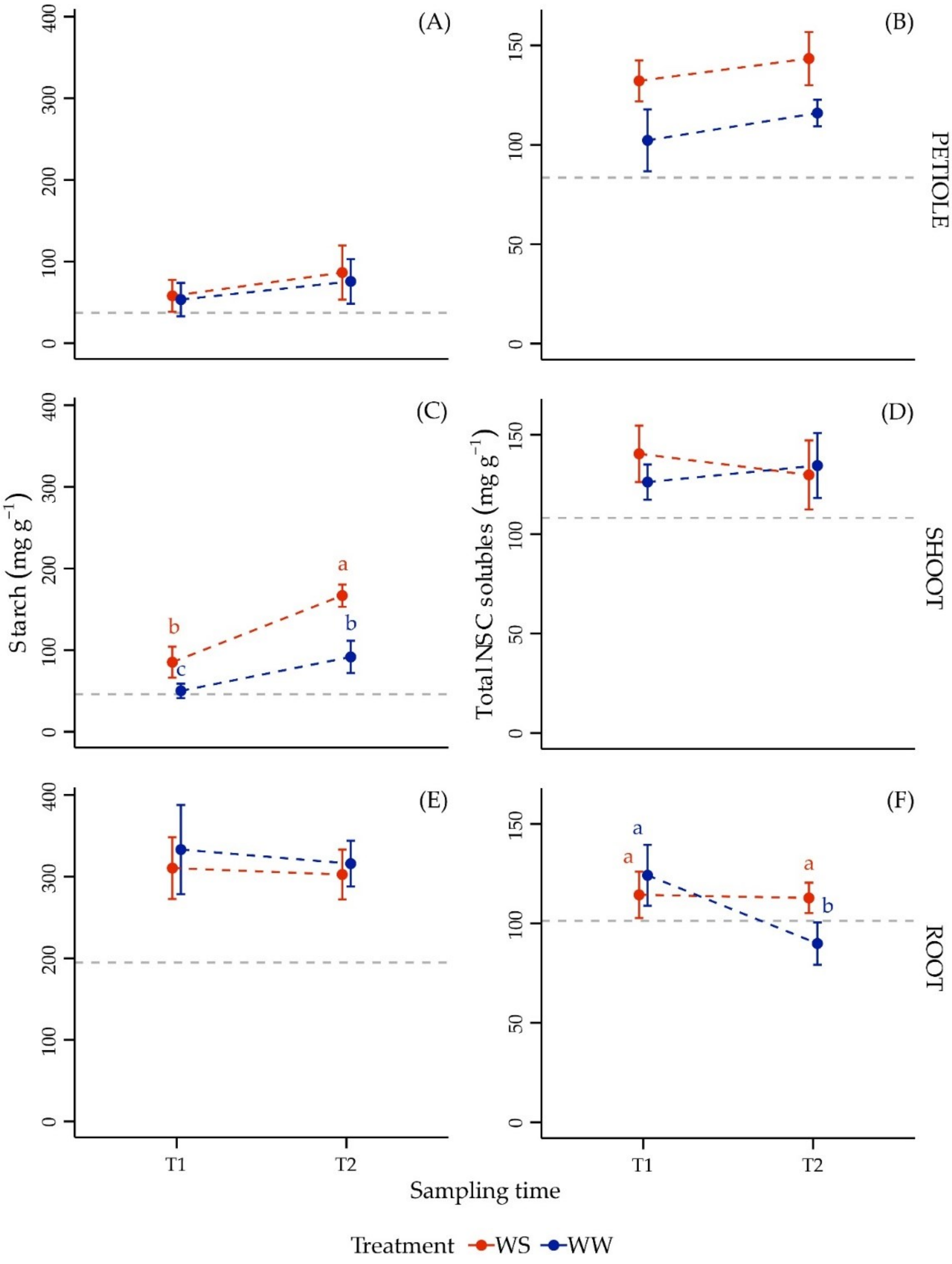

Figure 4. Mean values ± standard deviation of starch and total NSC soluble concentration in petiole (panels A and B), in shoot (panels C and D) and root (panel E and F) in Merlot vines under well-watered (WW) and water stress (WS) conditions at sampling times T1 and T2 (237 and 260 DOY). The horizontal dotted line indicates the value of the variables at T0 (205 DOY). Different letters correspond to statistically significant differences ($p < 0.05$).

SUPPLEMENTARY MATERIAL

Table S1. Outcomes of the analysis of variance (two-way ANOVA) applied to shoot and root DW, in relation to drought stress (treatment), time and their interactions.

Factor	**Df**	**F value**	***p*-Value**
Shoot DW			
Time	1,24	3.07	0.093
Treatment	1,24	24.21	< 0.001 ***
Time x Treatment	1,24	0.07	0.796
Root DW			
Time	1,24	47.89	< 0.001 ***
Treatment	1,24	13.68	< 0.011 **
Time x Treatment	1,24	0.42	0.522

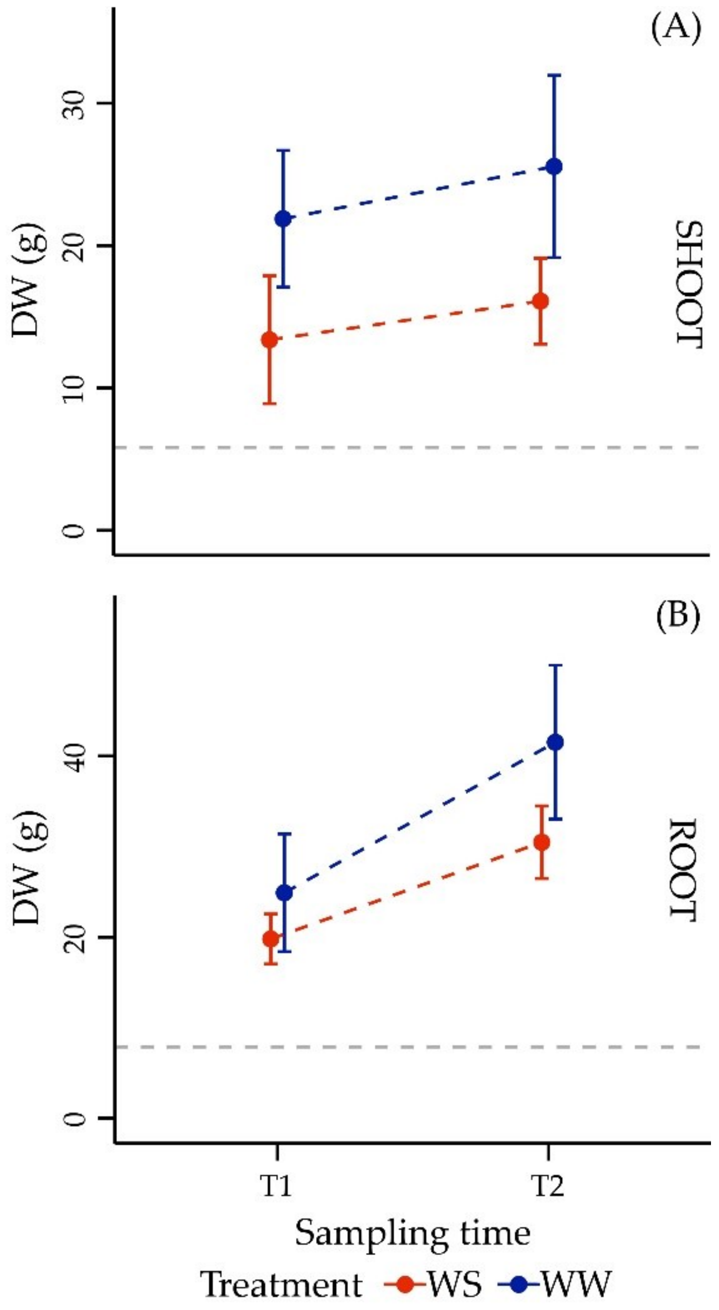

Figure S1. Mean values ± standard deviation of dry weight (DW) of shoot and root (panel A and B, respectively) in Merlot vines under well-watered (WW) and water stress (WS) conditions at sampling times T1 and T2 (237 and 260 DOY). The horizontal dotted line indicates the value of the variables at T0 (205 DOY). Different letters correspond to statistically significant differences ($p < 0.05$).

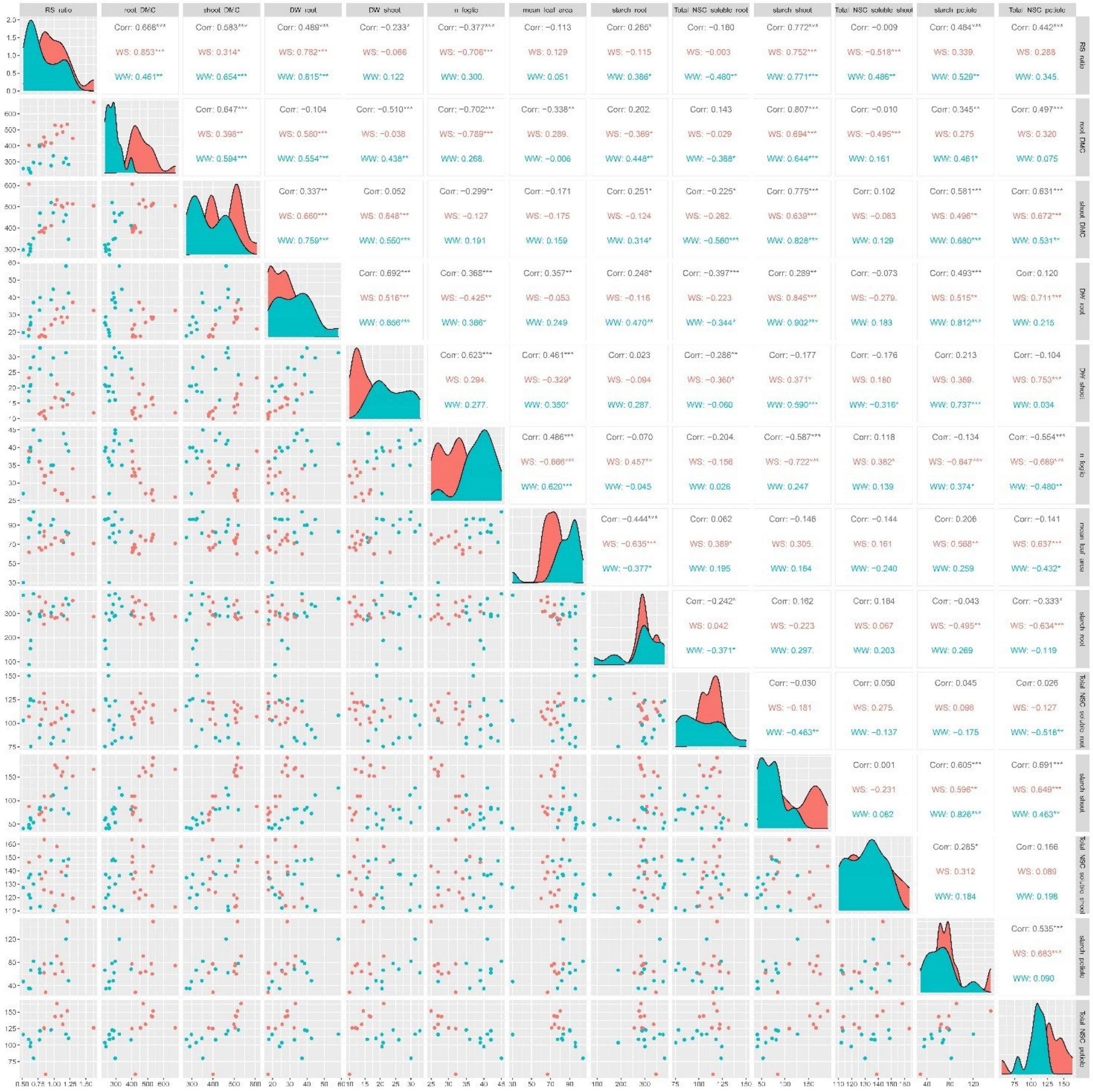

Figure S2. Correlation matrix showing the correlation coefficients between the considered variables divided by treatment. The Pearson correlation is shown on the left, the distribution of the variables is shown along the diagonal, scatter plots with the relationships between each variable are shown on the right.

5. Final dissertation

The results obtained during the three-year research activity presented in this thesis have contributed to providing new information useful for understanding the response mechanisms of *Vitis vinifera* to water deficit, as a function of the considered cultivar and of the stress level. This information on physiological processes can be considered fundamental as a support for the choice of the best strategies to be adopted in the vineyard to counteract the climate change already underway.

The findings of this study confirm the fact that in *Vitis vinifera*, as well as in other species, the content of NSCs is involved in tolerance and survival to drought stress. In fact, in all the experiments conducted, regardless of whether the cultivars investigated were iso- or anisohydric, the water deficit has always led to an increase in the content of low molecular weight sugars, with a certain degree of variability attributable to the cultivar, to the organ subject to investigation, and to the intensity and duration of the stress. This result, consistent with that of other authors (Salleo *et al.*, 2009; Nardini *et al.*, 2011; Secchi and Zwieniecki, 2016; Tomasella *et al.*, 2019*b*), supports the hypothesis that the accumulation of soluble NCS in the period of stress represents an acclimation strategy carried out by the grapevine with a potential role of osmoregulation, and / or refilling and / or energetic support.

If the stress was followed by a recovery period, the soluble NSCs content varied according to the distinct hydraulic behaviors to which the putatively investigated cultivars belonged. In species considered to be near-isohydric, the content of soluble NSCs decreased when irrigation was resumed, suggesting a possible recovery mechanism close to the model proposed by Secchi and Zwieniecki (2016), according to which the NCSs accumulated in the embolized tracheary elements are carried away by the restored water flow at the end of the water deficit. On the other hand, in the near-anisohydric species the decrease in soluble NSCs was not so marked, and, above all, in both studies the maltose content remained high in the recovery phase, which led to speculating on its implication in this stage, although its role has not yet been definitively clarified.

It was evident, however, that the response in the anisohydric cultivars seemed to be more complex, not only of hydraulic type and mediated by the NSCs, as in the case of the isohydric ones, but it also envisaged other acclimation mechanisms. Specifically, it was observed that in the petioles of the Syrah cultivar, when subjected to a short and intense stress, a short temporal lag was sufficient for newly produced tracheary elements having narrower lumen compared to well-watered plants, less vulnerable to embolism occurrence.

The *in vivo* investigation of the degree of embolism in plants subjected to water stress and the subsequent resumption of watering also revealed a more 'complex' response in the anisohydric cultivar. In fact, what emerged is that, although cultivars with hydraulic behaviors are both able at the restoration of irrigation to have a functional recovery of the xylem vessels embolized in the previous stress phase, in Barbera, generally considered as anisohydric, an apparent reduction in lumen of stem xylem vessels has been observed. Considering the absence of an increase in the number of vessels that could therefore justify this modification with a shift towards smaller dimensional classes, and considering that the lignified vessels cannot shrink, this reduction in lumen could be attributable to the fact that in the still embolized vessels there is a possible thickening of the wall due to the adhesion of hygroscopic aqueous gels determined by the interaction between pectin and maltose/maltodextrins, whose content remains high even in the recovery phase.

In the case of drought of lesser intensity and more lasting, the content of low molecular weight soluble sugars was influenced more markedly by growth, which undergoes an organ-specific decrease, and therefore, by virtue of this trade-off, the role of soluble sugars seems to be more nuanced, or rather masked, in these conditions.

Differently to what is reported in the literature (Bucci *et al.*, 2003; Salleo *et al.*, 2009; Secchi and Zwieniecki, 2011; Tomasella *et al.*, 2019*a*,*b*), the increase in soluble NSCs resulting from the water deficit has not always been accompanied by an evident decrease in starch. This could be due to the fact that changes in the starch content due to its catabolism for the production of simple sugars may have been too slight for the detection method used in these experiments.

Despite the necessary revision of the iso- anisohydric terminology suggested by different authors, the present thesis has shown that in young potted grapevine, there is a different NSC-mediated response to drought depending on whether the same cultivars belong to one or to the other category. Therefore, the great interspecific variety of *Vitis vinifera* deserves attention and it is more than ever necessary to conduct comparison experiments between cultivars to understand which one presents the best responses under certain conditions.

FUTURE PROSPECTIVE

In order to better understand the NSCs dynamics during and after water stress, both on the spatial scale - between the various organs of the grapevine - and on the temporal scale - their evolution during the vegetative and dormant season - new investigations are necessary and new methodologies required. Transcriptomic analysis could prove useful to understand the regulatory mechanisms driving these processes, which have not been addressed directly if the investigation is based solely on the content variations of certain compounds. The regulation of key enzymes of photosynthesis, as well as those of starch metabolism could prove useful in this regard, as well as those related to the regulation of cambial activity could elucidate the anatomical response mechanisms seen in petiole of the Syrah cultivar.

Furthermore, modern isotopic tools could be used for tracing the origin of NSCs and their translocation within the plant.

In order to approach the natural grapevine growth conditions, the investigations conducted in this thesis should undergo an upscaling process passing from laboratory / semi-controlled conditions to the open field context, where other environmental components (edaphic, microclimatic, competition etc.) enter at stake.

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