Alma Mater Studiorum Università di Bologna

SCUOLA DI AGRARIA E MEDICINA VETERINARIA
Corso di Laurea in
International Master in Horticultural Sciences

Viticoltura Generale

Effects of Sink-Source Manipulation of Grapevine Plants on the Activity of Carbohydrate Enzymes in leaves

Tesi di laurea di: Jedi Joy Belmonte Mahilum

Relatore
Chiar.mo Prof. Astrid Forneck

Correlatore
Dott./Prof. Ilaria Filippetti

Supervisor
Dott. Michaela Griesser

Visto, si approva la tesi

_____
(firma del relatore)

Anno Accademico 2013/2014
Sessione Straordinaria

Technische Universität München

Center of Life and Food Sciences Weihenstephan
Horticultural Science

Master Thesis

Submitted in Partial Fulfillment of the Requirements for the Academic Degree of Master of Science

Effects of Sink-Source Manipulation of Grapevine Plants on the Activity of Carbohydrate Enzymes in leaves

Jedi Joy Belmonte Mahilum

First Examiner:
Prof. Dr. Astrid Forneck

Second Examiner:
Prof. Dr. Ilaria Filippetti

Supervisor:
Dr. Michaela Griesser

Start Date:
March 2014

Date Submitted:
August 2014

Effects of Sink-Source Manipulation of Grapevine Plants on the Activity of Carbohydrate Enzymes in leaves

Master Thesis submitted by

Jedi Joy Belmonte Mahilum

University of Natural Resources and Life Sciences, Vienna
Department of Crop Sciences, Division of
Viticulture and Pomology

Univ. Prof. Dr. Astrid Forneck
Supervisor: Dr. Michaela Griesser

August 2014

Acknowledgements

Firstly, I would like to thank Prof. Dr. Astrid Forneck for making me a part of her working team in the Crop Sciences department.. To my supervisor, Dr. Michaela Griesser for entrusting me a part of her big project, guidance and sharing her expertise. Many thanks also to Sara Crespo, for her untiring support and encouragement, who have been there for me and teaching the protocols of my experiment. To Markus Eitle for also sharing his insights and previous experiences in this master program. To Sigrid Hensler who was there for me whenever I need some stuff and my co-master students who cheered me up in the laboratory.

I would also like to thank the organizers of this International Master program. Studying in 3 high standard European universities provides me a unique experience. I know this will be a springboard for my career and future endeavors. To Prof. Dr. Guglielmo Costa, Dr. Francesco Spinelli, Dr. Ilaria Filippetti and Ms.Irene Donati of the University of Bologna (UNIBO); Prof. Dr. Dieter Treutter, Prof. Dr. Joachim Meyer and Dr. Sybille Michaelis of Technical University of Munich (TUM) and; Prof. Dr. Johannes Balas and Bakk. Agnes Hajek of the University of Natural Resources and Life Sciences, Vienna (BOKU).

I also thank my family back in the Philippines, especially to my father Jun and my mom, who were my great supporter and encourager. To my hubby Jimmy, for being there for me all the way to the end, never giving up on me,...Thank you for your love asawa ko. To ate Monet, Joan and family, my VCCFF(W8) family who provide me a second family here in Vienna even far away from my homeland.

And most of all to God Almighty. I dedicate this piece of work, for without Him, I am nothing in this world. I owe everything to the Lord….To God be the glory!!!

Table of Contents

PAPER FORMAT

I. General Information of the Topic

I.1. Introduction and Objectives of the Master Thesis

Vitis (grapevines) is a genus of about 60 species of vining plants in the flowering plant family Vitaceae. It is native to Europe and Central Asia which appeared approximately 65 million years ago. The species occur in widely different geographical areas and show a great diversity of form. Grapevine is one of the most important fruit crop grown in the world. It is economically important as source of grapes for direct consumption and for fermentation to produce wine (GeoChemBio). The sugar content of grapes is of commercial importance for making wine. Grape sugars can be fermented by yeast to produce alcohol as well as augment flavour profile to the final wine products (Hayes et al., 2007). Grapes contain a large amount of sugar approximately 15 to 25%. Glucose and fructose are more or less of the same amount and only trace amount of sucrose. It contains 3mg/100g of vitamin C. The berries also have tartaric and malic acids both at approximately 0.5g/100g (EOL, 2006). Seedless grapes such as "Thompson seedless' and 'Black Corinth 'are also made into raisins or dried grape fruit. The berry fruit can also be processed into grape juice. Grapes are also an important source of grape seed oils and ethanol. (http://www.uky.edu/Ag/Horticulture/comfruit.html#grapes).

Grapevine shoot is a succulent stem bearing the leaves, tendrils and flower cluster. These organs are contained within a bud. All vines share the same basic, physiological features. The function of photosynthesis in the grapevine is to produce hexoses and sucrose which can be combined with other molecules to form larger carbohydrates (such as cellulose) that can be used to create other structures in the vine, energy reserves for the plant and, for fruiting grapevines, can be concentrated in grape berries which contain the reproductive seeds of the vine and are more attractive to birds and other animals. Their growth makes leaf collection challenging and polymorphic leaves make identification of species difficult. Mature grapevines can grow up to 48 cm in diameter at breast height and reach the upper canopy of trees more than 35 m in height (Everhart, 2010). During the development of grapevines, the leaves undergo a gradual transition from importing importing assimilates (sink) to exporting of photosynthetic products (source). One of the factors affecting photosynthesis is leaf age. The rate of photosynthesis increases rapidly at a younger age during the leaf expansion stage and declines as it matures. More of the photosynthetic products is exported and contributes to vine growth when it is 1/3 of its full size. When it reaches its full size, it is photosynthesizing at its peak (Retallack, 2012).

High quality wines can only be produced from high quality grapes and this requires proper cultural management practices. The combination of climate, soils, and vine vigor often leads to excessive vegetative growth. Too thick canopy can reduce vine fruitfulness, decrease varietal character, degrade other components of fruit quality and hamper efforts at disease control. Canopy management practices can help alleviate these problems. Canopy management provides a set of tools that improve the structure of canopy and microclimate (Vasconcelos and Castagnoli, 2001). Canopy management entails decisions regarding row and vine spacing, choice of rootstock, training and pruning practices, irrigation, fertilization, and summer activities such as shoot hedging, shoot thinning, and selective leaf removal (Smart et al. 1990). Canopy management and production practices can impact directly vine balance by affecting yield through flowering and fruit set (Vanko and Skinkis, 2013). The growth of vines is dependent both on carbon dioxide fixation and the integration of processes such as carbon allocation, accumulation and utilization. Early leaf removal prior to flowering, elimination up to approximately 80%, increased canopy efficiency, induced better performance of WUE and tolerance to photoinhibition (Pallioti, 2011). Preflowering leaf defoliation also showed significant improvement in final grape composition and greater wine appreciation (Poni et al 2008).

Several studies have been made on metabolic control of carbon metabolism from measurements of key metabolites and enzymes or gene sequences in tissue extracts but less is known in about the significance of partitioning between different tissues within the leaf (Pollock, et al., 2000). The aim of this study is to explore the changes of enzymatic activity in the leaves of a 1-year old fruiting grapevine plants by manipulating their canopy through defoliation.

Hypothesis:

In this study investigated the different levels of leaf removal on the sink-source balance of grapevine plants. Thereby we focused on the contents of glucose and fructose in leaves and berries and on the activity of important enzymes within the carbohydrate metabolism, invertases and sucrose synthase.

Hypothesis 1: Severe leaf removal is strongly influencing the leaf to fruit ratio and has a strong influence on fruit quality. We expect effects on hexoses contents and activity in leaves and berries due to treatment.

Hypothesis 2: The insertion level of leaves (also reflecting leaf age) is influencing the enzyme activity, thereby reflecting the main function of the leaf as either supplier or consumer of the photosynthetic products.

Objectives of the study

1. To establish a method to determine the enzymatic activity of cytosolic, cell wall and vacuolar invertases and sucrose synthase in the leaves of grapevine plants.
2. To establish a method to determine glucose, fructose, and starch in leaves of grapevine plants.
3. To analyze the enzymatic activity and the contents of hexoses and starch in leaves obtained from an experiment in 2013, where the sink-source balance of fruiting 1-year old grapevine plants was manipulated by different levels of defoliation.

I.2. Review of Literature

Effects of Vineyard Management

Vineyard management can help vine balance inorder to sustain productive yield, achieve fruit quality and maintain health of vines (Vanko and Skinkis, 2013). In the study of Campostrini and Bertamini (2014), there was strong evidence that canopy manipulation can strongly modify physiological activity by affecting microclimatic conditions and the sink-source relationship. Removal of leaves influences growth and photosynthetic capacity of plants, remobilizes carbon and nitrogen reserves and accelerates sink metabolism that leads to improved sink-source relations. Defoliation can manipulate source-sink relations by removing lower and senescing leaves to obtain greatest photosynthetic capacity and efficient carbon and nitrogen metabolism under optimal and stressful environments (Iqbal et al., 2012).

In 2010, Patakas and Noitsakis studied the effect of leaf age on water relations, organic solute, and total ion accumulation of mature and immature leaves of two-year-old grapevines under water stress conditions. The concentration of starch decreased significantly in both mature and immature leaves during drought periods. On the other hand, relative proportion of monosaccharides and sucrose

in immature leaves was significantly different compared to mature ones. Total inorganic ions also increased significantly with leaf age.

Variation in leaf area is a powerful determinant of crop growth rate. Carbon dioxide is fixed in the mature leaves specifically in the palisade parenchyma of chloroplasts of mesophyll cells via the Calvin cycle wherein net products are triose phosphates and can be fed into several biosynthetic pathways like as starch, lipids or amino acid biosynthesis in the chloroplast or sucrose and amino acid synthesis in in the cytosol. (Schulz et al., 1993). Several stages exist in partitioning of photosynthesis products. From a developing leaf, photosynthetic products are partitioned within the leaf in exported materials and for further leaf growth or temporary storage. The exported assimilates are partitioned between sinks such as meristematic, elongation or storage sinks (Gifford and Evans, 1981).

The leaves and photosynthesis

The most efficient leaves in the canopy are those that are at its youngest full-grown size. Photosynthetic rate increases until leaves attain full size which is approximately 40 Days after unfolding. At the early stage, 50 to 80% of their final sizes, leaves are net importers of carbohydrates and lateral shoots become net exporters when at fully expanded size (Vasconcelos and Castagnoli, 2001). In all cultivars of grapevine, leaf photosynthesis increases from bud break until flowering and then regularly decreases until leaf senescence (Stoev, 1952). Although grapevine leaves may assimilate CO_2 as early as bud break (Griffon, 1905; Kriedemann, 1968), leaves remain sink organs during the early steps of their development.

Photosynthesis fluctuates during the day and year depending on environmental conditions. Temperature variations influence stomatal opening and the rate of net photosynthesis (Schultz, 2003). Under natural conditions, photosynthesis is greatest during the first hours of the day, in the morning reaching approximately 10–15 μM CO_2 m^{-2} s^{-1} (Chaumont *et al.,* 1994; Medrano *et al.,* 2003). From flowering to veraison, the flux of fixed CO_2 is oriented towards both developing annual organs and perennial organs. During veraison, the pool of starch reserves is restored in the wood of most varieties (Stoev and Ivantchev, 1977) and the main part of photoassimilates is then directed to berry maturation such as in Pinot noir and Sauvignon blanc (Candolfi-Vasconcelos et al., 1994; Caspari et al., 1998). At a later stage of plant development, photosynthetic activity progressively declines in parallel with leaf senescence (Stoev, 1952; Bertamini and Nedunchezhian, 2003). This decline is mostly due to a

reduction in stomatal conductance (Schultz et al., 1996) and in protein content (Bettner et al., 1986), especially Rubisco (Hunter et al., 1994).

Fruit development

Grape berry is an independent biochemical factory. It has the ability to synthesize the primary metabolites and all other berry components such as flavour and aroma compounds that define a particular quality of wine. Increasing berry volume is associated with increase in sugar content after veraison (Gholami et al., 1995). Berry development consists of two sigmoidal growth periods separated by a lag phase (Coombe and McCarthy, 2000). First phase (stage I) is manifested with seed formation and cell division. Rapid cell division appears in the first few weeks and the total number of cells within the berry is established. This affects the eventual size of the berry (Harris et al., 1968). The berry also expands in volume as solutes accumulate and reach its maximum around veraison.

The lag phase is another stage of berry development that takes place before veraison. During this stage there is very low or no increase in berry fresh weight or size. The highlight of this stage is the beginning of accumulation of sugars into the fleshy parenchyma cells (Deluc et al., 2007) .

The second growth phase of berry development is characterized by softening and coloring of the berry. The berry approximately doubles its size between the beginning of the second growth period and harvest stage (Kennedy, 2002). Increase in berry size is the result of water and sugar accumulation in vacuoles which is accompanied by disassembly of the mesocarp cells leading to berry softening (Keller, 2010). Many of the solutes accumulated in grape berry during the early stage of development remain at harvest, but the increase in berry volume significantly reduces its concentration (Kennedy, 2002). The major impact during the second growth period is the tremendous increase in compounds, major ones being glucose and fructose, occurs as a result of a total biochemical shift to fruit ripening. Sucrose produced from photosynthesis in the leaves is imported into the berries during fruit ripening. In the fruit, sucrose is hydrolized into constituent sugars glucose and fructose (Robinson and Davies, 2000). This is affected by external factors such as crop load, canopy size and disease status of the plant. The shift of phloem unloading from symplasmic to apoplasmic pathway during fruit development was also evident in the anatomical study of Zhang, et al. (2006). The turning point of phloem unloading pathways was further detected at veraison which is an important developmental checkpoint of grapeberry. They also observed increase in levels of expression and activities of cell wall and acid

invertase around the onset of ripening and reached high level in the later stage. This reveals evidence an apoplasmic unloading pathway after the onset of ripening.

Sink-Source Relations

The partitioning of current assimilates in plants involves movement of material between chloroplasts, cytosol, mitochondria and vacuoles (Pollock and Kingston-Smith, 1997). Sucrose imported in the cytoplasm of recipient sink cells is chemically unaltered or hydrolysed by extracellular invertase into hexoses and can then enter a number of metabolic pathways or stored in the vacuoles.

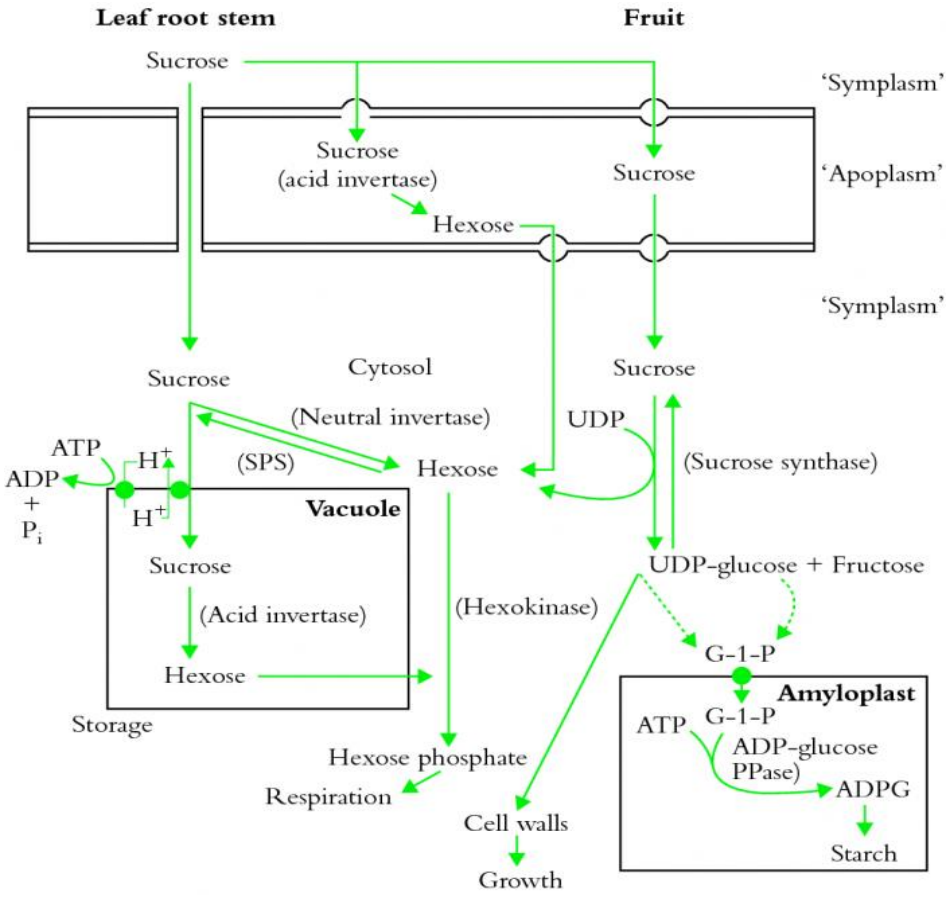

Figure 1. Pathways of sugar metabolism and compartmentation within sink cells (http://plantsinaction.science.uq.edu.au/edition1).

Sugar metabolism and transport

In most species, sucrose is the most prevalent sugar produced in photosynthetic organs (source) and transported via the phloem over long distances to sinks. In sink organs, sucrose is either directly imported or cleaved by cell-wall bound invertases into monosaccharides, glucose and fructose or stored in vacuoles (http://plantsinaction.science.uq.edu.au). These two monosaccharides can be taken up by sink cells (Bastien, 2010). Hexoses that are produced from the source (leaves) are transported to sink cytoplasm and are rapidly phosphorylated to hexose-6-phosphate by glucose and fructose specific kinases. From these forms hexoses can be used as substrates for respiration, for synthesis of new constituents or converted to sucrose by sucrose phosphate synthetase. In sugar beet tap roots and sugar cane stems, the sucrose synthesized by this reaction can be accumulated in the vacuoles while in grape berries, sucrose is rehydrolysed into hexoses by vacuolar acid invertase. Sugar transport and allocation of photosynthetic assimilates between source and sinks are the major determinants for crop productivity (Gifford, et.al, 1984).

Invertase and sucrose synthase are the two enzymes capable of hydrolysing sucrose in green plants. Invertase catalyzes the irreversible hydrolysis of sucrose into hexoses, glucose and fructose. The activity of enzyme invertase varies depending on the plant species, organ type and stage of development. Both acid and neutral invertases are present in plant cells. According to Wardlaw (1990), acid invertases are usually active in rapidly growing leaves which makes hexoses available for respiration and biosynthesis (Zrenner *et al*. 1995). The enzymatic paths of sucrose cleavage in plants are catalysed by invertases and sucrose synthases. Invertases produce glucose and form twice as many hexoses. Sucrose metabolism lies at the heart of a sensitive, self-regulatory development system in plants (Koch, 2004). Sucrose is the main transport form of photoassimilates in most plants, unlike hexoses that do not circulate over long distances. Sucrose therefore has to pass through several membranes involving specific sucrose carriers (Kuhn et al., 1998). Because of its physicochemical properties and chemical stability, sucrose has a high translocation rates (0.3 to 3 m h-1) even in solutions of high concentration such as phloem sap having a sucrose concentration of 200-1600mM (van Bel, 1996). Glucose and fructose are the main sugars in grape berry and are present in similar concentrations while sucrose in trace amounts (Kliewer, 1965). Generally, glucose and fructose may range from 45.9 to 131.0 mg per liter while sucrose accounts for less than 2.0% of total sugars (Shiraishi, 1993 and Liu et al., 2006).

Sucrose and products like glucose and fructose are the central molecules for carbohydrate translocation, metabolism and sensing in higher plants (Roitsch and Gonzalez, 2004). It's role is to supply energy and serves as a carbon backbone for plant growth and development. Sugars also control a wide range of plant physiological processes such as photosynthesis, sugar transport, nitrogen uptake, defence reactions, secondary metabolism and hormonal balance (Smeekens et al., 2010).

I.3 Literature Cited

Afoufa-Bastien, D., Medici A., Jeauffre J., Coutus-Thevenot P., Lemoine R., Atanassova R. and M. Laloi. (2010). The Vitis vinifera sugar transporter gene family: phylogenetic overview and macroarray expression profiling. BMC Plant Bilology 10:245.

Bertamini, M. Muthuchelian K. and N. Nedunchezian. (2004). Effect of grapevine leafroll on the photosynthesis of field grown grapevine plants (Vitis vinifera L. cv. Lagrein). Journal of Phytopathology 152:145-152.

Bettner W, Mu¨ller U, Schall B. (1986). Untersuchungen zum Wachstumsrhythmus und zum Gehalt an Na¨hrstoffen in einja¨hrigen Rebtrieben bei den Sorten Kerner und Optima. Teil 1: Wachstumsrythmus; Teil 2: Nährstoffgehalt. Wein Wissenschaft 41:147-169.

Candolfi-Vasconcelos, M.C., M.P. Candolfi, and W. Koblet. (1994). Retranslocation of carbon reserves from the woody storage tissues into the fruit as a response to defoliation stress during the ripening period in Vitis Vinifera L. Planta 192:567-573.

Caspari H, Lang A, Alspach P. (1998). Effects of girdling and leaf removal on fruit set and vegetative growth in grape. American Journal of Enology and Viticulture 49:359–366.

Chaumont, M. et al., (1994). Seasonal and diurnal changes in photosynthesis and carbon partitioning in Vitis vinifera leaves in vines with and without fruit. I. Exp. Bot. 45:1235-1243

Cierezko, I. (2009). Sucrose metabolism in plant tissues under stress conditions: key enzymes, localization and function. ISBN: 978-81-7895-422-6:193-218.

Coombe, B.G., McCarthy, M.G. (2000) "Dynamics of grape berry growth and physiology of ripening," Australian Journal of Grape & Wine Research 6: 131-135.

Davies, C. and S. Robinson. (1996). Sugar Accumulation in grape berries. Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. Plant Physiology. 11(1):275-283.

Deluc, L. et al., (2007). Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. BMC genomics. 8:429.

EOL. (2006). *Vitis vinifera*. Wine grape.

Everhart, S.E. (2010). Upper canopy collection and identification of grapevines (Vitis) from selected forests in the Southeastern United States. Castanea 75(1):141-149

Gifford, R.M., Thorne J.H. Hitz W.D. and R.T. Giaquinta. (1984). Crop productivity and assimilate partitioning. Science 1225:801-808.

Gifford, R.M and L.T. Evans. (1981). Photosynthesis, carbon partitioning and yield. Ann. Rev. Plant PhysioL 32:485-509

Gholami, M., Hayasaka, Y., Coombe, B.G., Jackson, J.F., Robinson, S.P. Williams, P.J. (1995) "Biosynthesis of flavour compounds in Muscat Gordo Blanco grape berries," Australian Journal of Grape & Wine Research 1: 19-24.

Griffon E. (1905). L'assimilation chlorophyllienne chez les jeunes pousses des plantes. Application a` la vigne. Comptes-Rendus de l'Acade´mie des Sciences. 140:1148–1151

Harris, J.M., Kriedemann, P.E., Possingham, J.V. (1968) "Anatomical aspects of grape berry development," Vitis 7: 106-109.

Hayes, M.A. et al., (2007). Isolation, functional characterization, and expression analysis of grapevine (Vitis vinifera L.) hexose transporters: Differential roles in sink source tissues. Journal of Experimental Botany 58: 1985-1997.

Hayes, M.A. et al., (2010). Involvement of abscisic acid in the coordinated regulation of a stress-inducible hexose transporter (VvHT5) and a cell wall invertase in grapevine in response to biotrophic fungal infection. Plant Physiology 153: 211-221.

Hunter, J.L. Skrivan, R. and H.P. Ruffner. (1994). Diurnal and seasonal physiological changes in leaves of Vitis vinifera L.: CO2 assimilation rates, sugar levels and sucrolytic enzyme activity. Vitis 33:189-195.

Iqbal, N., Masood A. and N.A. Khan. (2012). Analyzing the significance of defoliation in growth, photosynthetic compensation and source-sink relations. Photosynthetica 50(2):161-170.

Keller, M. (2010). The science of grapevines: anatomy and physiology. Elsevier Inc. Chapter 2: 78-83.

Kennedy, M. (2010). Understanding grapeberry development. Practical Winery and Vineyard. July/August. 14-23.

Kliewer, W.M. (1965). Changes of concentration of glucose, fructose and total soluble solids in flowers and berries of Vitis vinifera. Am. J. Enol. Vitic. 16:101-110.

Koch, K. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current opinion in plant biology. 7, 235-246.

Kriedemann PE. (1968) . Photosynthesis in vine leaves as a function of light intensity, temperature and leaf age. Vitis. 7: 213–220.

Lecourieux, F. et al. (2013). An update on sugar transport and signalling in grapevine. Journal of Experimental botany. 65(3), 821-832.

Liu, H. F. et al., (2006). Sugar and acid concentrations in 98 grape cultivars analysed by principal component analysis. J. Sci. Food Agric. 86:1526-1536.

Medrano H, Escalona J.M., Cifre J, Bota J, and J. Flexas. (2003). A ten-year study on the physiology of two Spanish grapevine cultivars under field conditions: effects of water availability from leaf photosynthesis to grape yield and quality. Functional Plant Biology 30: 607–619.

Nguyen-Quoc, B. and C.H. Foyer. (2001). A role of 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. Journal of experimental biology. 52(358). 881-889.

Pallioti, A. et al., (2011). Early leaf removal to improve vineyard efficiency: Gas exchange, source-sink balance, and reserve storage responses. American Journal for Enology and Viticulture. 62(2): 219-228.

Pastore, C. et al., (2013). Selective defoliation affects plant growth, fruit transcriptional ripening program and flavonoid metabolism in grapevine. BMC Plant biology. 13(30): 1-16.

Patakas, A. and B. Noitsakis. (2001). Leaf age effects on solute accumulation in water stressed grapevines. Journal of Plant Physiology. 158:63-69

Pollock, C.J. et al., (2000). Intracellular and intercellular compartmentation of carbohydrate metabolism in leaves of temperate gramineae. Brazilian Journal of Botany, 20. ISSN 1806-9959.

Pollock, C. J. and A.H. Kingston-Smith. (1997). The vacuole and carbohydrate metabolism. Advances in Botanical Research. 25:195-215.

Poni, S., F. Bernizzoni, and S. Civardi. (2008). The effect of early leaf removal on whole-canopy gas exchange and vine performance of Vitis vinifera L. 'Sangiovese'. Vitis 47:1-6.

Retallack, M. (2012). Grapevine biology. Wine growing for the future 1-5pp.

Roitsch, T. and M. Gonzalez. (2004). Function and regulation of plant invertases: sweet sensations. Trends in plant science. 9(12), 606-613.

Schulz, A. (1994). Phloem transport and differential unloading in pea seedlings after source sink manipulations. Planta 192:239-248.

Schultz, H.R. (2003) . Extension of a Farquhar model for limitations of leaf photosynthesis induced by light environment, phenology and leaf age in grapevines (Vitis vinifera L. cv. White Riesling and Zinfandel). Functional Plant Biology. 30:673–687.

Schultz HR, Kiefer W., and W. Gruppe. (1996). Photosynthetic duration, carboxylation efficiency and stomatal limitation of sun and shadeleaves of different ages in field-grown grapevine (Vitis vinifera L.). Vitis 35:169–176

Shiraishi, M. (1993). Three descriptors for sugars to evaluate grape germplasm. Euphytica. 71:99-106.

Smart, R. et al., (1985). Canopy microclimate modification for the cultivar Shiraz II effects on must and wine composition. Vitis. 24: 119-128.

Smeekens, S. Ma J, Hanson J., and F. Rolland. (2010). Sugar signals and molecular networks controlling plant growth. Current opinion in plant biology. 13:274-279.

Smith, A. M. and Ceeman S. (2006). Quantification of starch in plant tissues. Nature protocols. 1(3), 1342-1345.

Stoev, KD. (1952). Analyse biochimique de la Vigne au cours du développement de son cycle annuel. Viticulture and Enology 12:42-49.

Stoev, K. and V. Ivantchev. (1977). Données nouvelles sur le problème de la translocation descendante et ascendante des produits de la photosynthèse de la vigne. Vitis. 16:253-262.

Sturm, A. and G. Tang. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in plant science. 4(10), 401-407.

Walker, A.J., Ho, L. and D.A. Baker. (1978). Carbon translocation in tomato: pathway of carbon metabolism in fruit. Ann. Bot. 42: 901-909.

Wardlaw, I.F. (1990). The control of carbon partitioning in plants. Translay Review. New Phytologist 116:341-381.

Wu, B.H. et al. (2011). Carbohydrate metabolism in grape cultivars that differ in sucrose accumulation. Vitis. 50(2), 51-57.

Van Bel AJE. (1996). Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons. Journal of experimental botany. 47: 1129-1140.

Vasconcelos, C. and S. Castagnoli. (2001). Leaf canopy structure and vine performance. American journal of enology and viticulture 51(4).

Zhang, B. et al. (2013). Synergistic and antagonistic effects of α-amylase and amyloglucosidase on starch digestion. Biomacromolecules 14,1945-1954.

Zrenner, R., Salanoubat M., Willmitzer L., and U. Sonnewald. (1995). Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (Solanum tuberosum L.). Plant J 7:97-107.

Http://www.GeoChemBio.com/biology/organism/Vitis

Http://www.plantsinaction.science.uq.edu.au

Http://www.uky.edu/Ag/Horticulture/comfruit.html#grapes

II. PUBLICATION FORMAT

Effects of Sink-Source Manipulation of Grapevine Plants on the Activity of Carbohydrate Enzymes in leaves

Jedi Joy Belmonte Mahilum, Sara Crespo Martinez, Michaela Griesser, Astrid Forneck

Department of Crop Science, Division of Viticulture and Pomology, University of Natural Resources and Applied Life Science, Vienna, Austria.

Abstract

Sugar content is an important determining factor of grape berry quality. Among the sugars present in grapevines are sucrose, glucose and fructose. Sucrose is synthesized in the source organs (leaves) and transported to sink organs which is further metabolised into simple sugars such as fructose and glucose that can be used in the carbon skeleton of the plant during its growth and development or stored in organs for reserve as starch. This degradation of sugars is catalysed with enzymes namely invertases (vacuolar, cytosolic and cell wall invertases) and sucrose synthase. Enzymatic activities are regulated by genes and are also affected by environmental factors or stress. The canopy of the plant plays a big role in the photosynthetic activity of the plant which is the driving mechanism for its growth rate. Canopy management is one of the cultural management practices that affect physiological aspects of the plant by improved micro-climatic conditions and manipulating sink-source relationships. This experiment aimed at investigating the fluctuations in sugars present in grapevine tissues as well as the changes in enzymatic activities involved in sugar metabolism through canopy management at groat-sized (BBCH 73) and ripening (BBCH 89) stage. Results showed that defoliation of leaves and shoot increased starch content with decreasing amount of leaf canopy at an early stage of development, 3 days after removal. But at ripening stage, trend of starch content in leaves decreased with decreasing canopy. On the other hand, glucose and fructose concentrations have no significant difference between treatments as affected by leaf defoliation. Enzymatic activities in the leaves also did not show evident differences among treatments. The results suggest that high starch content in the leaves entails low enzymatic activities and conversion of sucrose into glucose and fructose despite the difference in amount of canopy (shoot and leaves).

Key words: grape, sucrose, sink organs, micro-climatic conditions, sink-source relationships, invertases, sucrose synthase

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is a non-climacteric fruit species and among one of the most important fruit crop in the world. Vitis is a genus of about 60 species of vining plants in the flowering plant family Vitaceae. It is native to Europe and Central Asia which appeared approximately 65 million years ago. The species occur in widely different geographical areas and show a great diversity of form. Grapevine is one of the most important fruit crop grown in the world. It is economically important as source of grapes for direct consumption and for fermentation to produce wine (GeoChemBio.com). The sugar content of grapes is of commercial importance for making wine. Grape sugars can be fermented by yeast to produce alcohol as well as augment flavour profile to the final wine products (Hayes et al., 2007). High quality wines can only be produced from high quality grapes. Producing high quality grapes requires proper cultural management practices. The combination of climate, soils, and vine vigor often leads to excessive vegetative growth. Too thick canopy can reduce vine fruitfulness, decrease varietal character, degrade other components of fruit quality and hamper efforts at disease control. Canopy management practices can help alleviate these problems.

Over the last two decades, canopy management of vineyards has received much attention leading to many excellent contributions. Previous research focused on the general principles for optimal light distribution, nowadays, the effects of climate change effects and in more detail how canopy management impacts cluster microclimate with emphasis in color and flavor compounds have been addressed (Poni, 2014). Canopy management entails decisions regarding row and vine spacing, choice of rootstock, training and pruning practices, irrigation, fertilization, and summer activities such as shoot hedging, shoot thinning, and selective leaf removal (Smart et al. 1990). Canopy management and production practices can impact directly vine balance by affecting yield through flowering and fruit set (Vanko and Skinkis, 2013). The growth of vines is dependent both on carbon dioxide fixation and the integration of processes such as carbon allocation, accumulation and utilization. Early leaf removal prior to flowering, elimination up to approximately 80%, increased canopy efficiency, induced better perforfance of WUE

and tolerance to photoinhibition (Pallioti, 2011). Preflowering leaf defoliation also showed significant improvement in final grape composition and greater wine appreciation (Poni et al 2008).

Higher plants are photosynthetically active and can generate energy that supply organs for its growth and development through carbon fixation in leaves. Organs in plants are generally divided in to sink and source (Turgeon, 1989). Source organs are mostly net exporters of assimilates produced through photosynthesis such as mature leaves while sink organs are net exporters of photoassimilates and are usually photosynthetically inactive. Sinks can either be utilization and storage sinks. Utilization sinks are highly metabolically active and found in rapidly growing tissues like immature leaves while storage sinks are organs such as tubers, seeds and roots where the imported carbohydrates are deposited in the form of storage compounds like starch, sucrose, fatty acids or proteins (Sonnewald and Willmitzer, 1992). Metabolic sinks, immature leaves for example, is under developmental control because they become photosynthetically active sources after maturation (Gifford et al., 1984). Aside from allocation of assimilates between sink and source organs, photoassimilates as has to be partitioned also into either starch synthesis in the choloroplast for temporary storage or sucrose synthesis in the cytosol for export within the mesophyll cell (Stitt et al., 1987; Stitt 1990b; Stitt, 1996).

In grapevines, the berry fruits become very active sink organs as it approaches ripening stage. Berry size rapidly increases as a result of water and sugar accumulation in vacuoles which is accompanied by disassembly of the mesocarp cells leading to berry softening (Keller, 2010). During this period, there is a tremendous increase in major compounds, such as glucose and fructose as a result of total biochemical shift to fruit ripening. Sucrose produced in the leaves is transported to the berries and hydrolysed into glucose and fructose (Robinsons and Davies, 2000). Sugars in plants can reach the sink cells in two different ways, either apoplastic or symplastic pathways and within the sink apoplasm, sucrose can be hydrolysed by extracellular invertases into hexoses. Another pathway is through symplastic path. Within the sink cytoplasm, sucrose can be hydrolysed or stored in the vacuoles (http://plantsinaction.science.uq.edu.au). The enzymatic paths of sucrose cleavage in plants are catalysed by invertases and sucrose synthases. Invertases produce glucose and form twice as many hexoses. Sucrose metabolism lies at the heart of a sensitive, self-regulatory development system in plants (Koch, 2004). Sucrose is the main transport form of photoassimilates in most plants, unlike hexoses that do not circulate over long distances. Sucrose therefore has to pass through several membranes involving specific sucrose carriers (Kuhn et al., 1998).

A few studies have been made on metabolic control of carbon metabolism from key measurements of metabolites and enzymes or gene sequences of the tissue extracts (Pollock, et al., 2000). This experiment main objective is to explore the changes of enzymatic activity in the leaves of a 1-year old fruiting grapevine plants by defoliation and manipulating its canopy. To study the effect of sink-source manipulation on the dynamics of enzyme activity as well as to quantify monosaccharaides and sugars present in the tissues of grapevines by FTIR or spectralphotometer.

MATERIAL AND METHODS

Experimental design

The experiment was conducted in a greenhouse of the University of Applied Life Sciences Vienna (BOKU) at the location Tulln (UFT) in Lower Austria during the vegetation period 2013. In total 132 fruiting one-year old potted grapevine plants cultivar Zweigelt St9 grafted on Kober 5BB were used for the experiment. The plants were potted 2012 in 80% standard soil substrate (Einheitserde ED73) supplemented with 20% sand of 0.5-2 mm size. The plants were placed into the greenhouse on 09.04.2013 and pruned to one shoot and reduced to one grape cluster per plant after bloom. All plants were fertigated with a drip watering system (FERTY 3 Mega with 50g per 100L end concentration; Control 90mL per plant and day, T1 and T2 63ml per plant and day, T3 27ml per plant and day).

The experiments started with begin of the treatment on 11.06.2016 by applying four different levels of leaf removal (Figure 1). Control plants were standardized to 18-20 leaves per plant with removal of the shoot tip. Every second leaf was removed in treatment 1 and the half of the shoot was removed in treatment 2 representing 50% of the leaves of the control plants. With treatment 3 only the leaf opposite and below the grape cluster was left. Three leaves per plant (L1, L3 and L7) were labelled and used for measurement and analyses thereafter. To determine the effect of the treatment over time samples of the plants were harvested at four developmental stages: Harvest 1, 3 days after start of the treatment (groat-sized; 14.06.2013), harvest 2 at berry touch (03.07.2013), harvest 3 at veraison (23.07.2013) and harvest 4 at ripening (13.08.2014). Each plant was harvested only one time. Half of the plants were used to determine growth parameters, like weight of leaves (FW and DW), size of leaves, weight of shoot (FW and DW), weight of grape cluster and weight of 10 berries (FW and DW). Leaves, berries and shoots of the remaining half of the plant was immediately frozen in liquid nitrogen and stored at -80°C for further analyses.

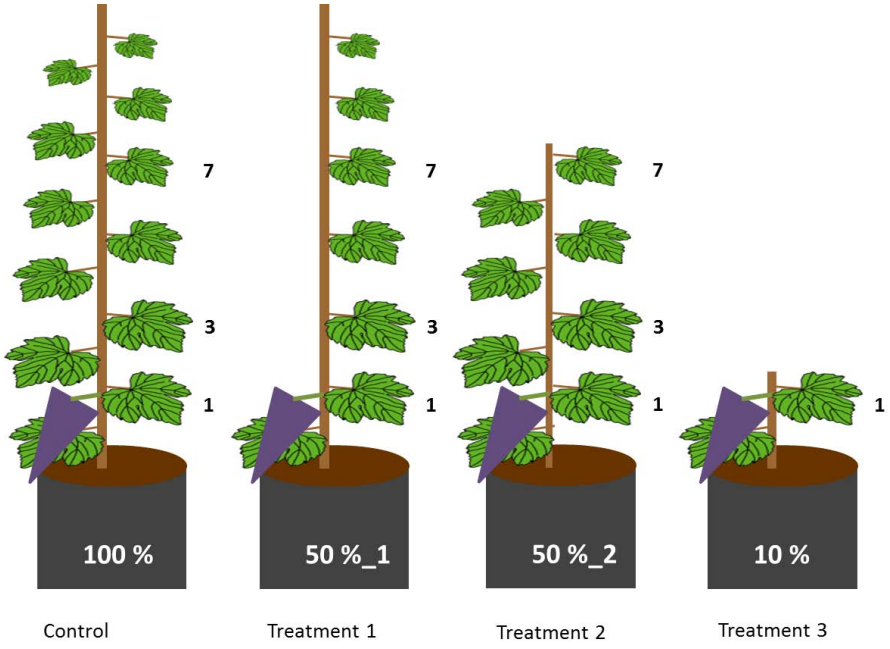

Figure 1: Experimental design of the sink-source treatments applied on one-shoot grapevine plants in 2013

Enzyme assay of grapevine leaves and berries

Homogenization: The leaf samples were homogenized with a ball mill (Retsch, Haan, Germany) for 1 min at 30 Hz and kept in liquid nitrogen to prevent thawing. 2x250 mg of the homogenized sample was weight into a cold cold 2-ml eppendorf tubes. The exact weight was recorded and samples were stored at -80°C till further analysed.

Extraction: A protocol established by Elisabeth Covington and Roitsch et al 2012 (personal communication) was applied. Three types of extracts were obtained: Crude extract, R-extract and Z-extract. 500mg of samples were extracted in a buffer consisting of 0.5 M MOPS (pH=7.5), 5 mM MgCl2, 0.5 mg/mL BSA, 0.05% (v/v) Triton X-100, 25 µM dithiothreitol, 0.1 mM PMSF, 1 mM benzamidine, and 3% (w/v) PEG-4000 (Takayanagi and Yokotsuka 1997, modified). It was then centrifuged at 4 °C, 13 000rpm for 10 minutes. The supernatant were then transferred into cold eppendorf tubes and labelled as crude extract. 1ml of the crude extract was desalted in GE PD minitrap G-25 (GE Biosciences) and allowed to flow through. It was then eluted with the addition of K-PO4 buffer. The flow through was collected in a 2-ml eppendorf tube and labelled as R-extract. The

precipitate was then added with high salt extraction buffer and stored overnight at 4°C. The next day, it was centrifuged at 13 000rpm, 4°Cfor 10min. Supernatant was desalted again in GE PD miditrap G-25 and allowed to flow through. It was then eluted with K-PO4 buffer, flow through collected in 2-ml eppendorf tube and labelled as Z-extract.

Starch determination: Soluble sugars were extracted following starch protocol (Smith and Zeeman, 2006). From a starting plant material of 100mg, 1-ml of 80% ethanol was added and incubated in a thermoincubator at 99°C for 3 minutes. This step was repeated twice. 1-ml of water was then added and mixed. 0.20mL of homogenate was then transferred into 4 eppendorf tubes, each labelled as S1, S2, B1 and B2. It was then heated to 100°C for 10minutes to gelatinize starch granules and allowed to cool. Each tube was then added with 0.20mL of 200mM Na acetate, pH 5.5. S1 and S2 were added with 6 units of α-amyloglucosidase and 0.5 units of α-amylase while B1 and B2 are without enzymes. The samples in tubes were incubated at 37 °C for 4 hours, centrifuged at 10000g for 5 min and the supernatant collected in new eppendorf tubes for detection. 20µL extract with assay reagent: double distilled water, 0.5mM ATP, 1mM NAD, 4mM MgCl2 and enzymes, 1unit of hexokinase and 1unit glucose-6-phospahte was pipetted in microtiter plate. OD was then recorded using a microtiter plate reader (Omega-MARS BMG LabTech).

Glucos21e-Fructose Quantification: A standard curve containing glucose and fructose was prepared with the following concentrations; 3mM, 2mM, 1mM, and 0.6mM. In every hole of the titer plate, a 100µL mixture of mastermix (39µl of 0.2mM HEPES, 0.75µl of 0.25M EDTA, 0.4µl of 1M MgCl2, 1 µl of 1M DTT, 2.5µl of 0.1M ATP, 1µl of 0.1M NAD and 55.5µl double distilled water) was added and 20µL of crude extract from each samples. Absorbance was then measured at 340nm in microtiter plate reader (Omega-MARS BMG LabTech) then samples and blanks were added with enzymes hexokinase (0.7U) and glucose-6-phosphate dehydrogenase (0.3U) and absorbance was measured again at 340nm for glucose detection. After the second reading, all the holes of microtiter plate were added with another enzyme, glucophosphate isomarase (1U) and absorbance measured in microtiter plate reader for glucose and fructose. This protocol was adapted from Megazyme.

Protein amount determination: The amount of protein in both R-and Z-extracts were determined by Bradford protocol. A calibrating curve was made containing different concentrations (0,5, 10, 15 and 20µL) of BSA. For the R-extract, 1µL of sample plus 99µL of double distilled water was used in each hole of the microtiter plate. Each hole was then added with 150µL of staining solution (Bradford stain)

and incubated at room temperature for 10 minutes. Absorbance was then measured at 595nm in microtiter plate reader (Omega-MARS BMG LabTech) and amount of protein calculated. The same steps were done with Z-extract with 2µL sample and 98µL of double distilled water (Bradford protocol by Jammer, 2013).

Invertase activity**:** This protocol has 2 parts namely; enzyme reaction and glucose detection as adapted from invertase assay protocol (E.D. Covington, 2012). For the enzyme reaction, A reaction master mix and blank mastermix were made. The reaction master mix contained 5 µl sucrose, 25 µl of 0.2M sodium acetate buffer at pH4.0, and 20 µl double distilled water while the blank master mix contained only 25 µl sodium acetate buffer at pH4.0 and 25 µl double distilled water. 10 µL of R-extract or Z-extract was transferred in 2 µl safe-lock eppendorf tubes and added with 40µL of mastermix or blank mastermix and kept on ice. Immediately after, all tubes were incubated in thermoblock at 37 °C for 30minutes. For glucose detection, a mixture of 25 volumes of Nelson's A (192.3 µl) and 1 volume of Nelson's B (7.7 µl) were mixed and added to the eppendorf tubes with samples. The tubes were then heated in thermoblock for 20 minutes at 99 °C. All the tubes were allowed to cool down for 15minutes and added with 200 µl arsenomolybdate. This step was done in the fumehood. The mixture was centrifuged at 14 000rpm for 5minutes. After centrifugation, 130µL of each standard and sample was transferred to a Greiner 96-F well plate and absorbance measured at 660nm in microtiter plate reader (Omega-MARS BMG LabTech).

CytInv and SuSy determination: A mastermix with the following composition: 69.80µl double distilled water, 8µl of 1M HEPES (pH 7.0), 0.64µl of 250mM EDTA, 0.32µl 1M $MgCl_2$, 0.8µl of 1M DTT, 2.08µl 100mM ATP, 0.8µl of 100mM NAD, 0.32µl of 1000U/ml of glucose-6-phosphate dehydrogenase, 0.672µl of 1000U/ml hexokinase and 0.56µl of 1000U/ml PGI was prepared. 3 premixes (-UDP control, +UDP and no substrate control) containing incomplete reaction were also prepared. –UDP control contained a mixture of 34 µl per reaction mastermix, 40 µl per reaction of 1M sucrose and 16 µl double distilled water; +UDP mix with 34 µl mastermix, 40 µl sucrose and 16 µl per reaction UDP; and no substrate control with only 34 µl mastermix and 56 µl double distilled water. All of these mixes were prepared in eppendorf tubes. Then, 50 µL of the master mix and 20µL of glucose-fructose mixture or double distilled water or R-extract were added in each hole of the microtiter plate. Both the plate with samples and master mix and the three premixes were incubated at 30 °C for

30minutes. After 30 minutes, 150µL premix was added to each well and absorbance measured at 340nm in a microtiter plate reader (Omega-MARS BMG LabTech).

Statistical analysis

All statistical analyses were performed using the Software PASW 18 (SPSS, Hong Kong). Data were tested for normal distribution and homogeneity of variance. One-way Anova was conducted using Scheffe's test to determine if there are significant differences among treatments.

RESULTS

I. Changes in sugar content and enzyme activities in grapevine

1. Detection of sugars

The starch content of leaves at groat-sized berries (BBCH 73) harvested 3 days after treatment application and at ripening (BBCH 89) stage is shown in Figure 2. There was no significant difference between values obtained from leaf 1 and leaf 7 (data not shown), therefore results from both leaves were combined and analyzed. It is evident that starch content increased as the number of leaves per plant is decreased with treatment 3 (10% shoot) as the highest followed by treatment 2 (50% shoot), then treatment 1 (every 2nd leaf removed) while control treatment showed the least amount of starch already three days after the start of the treatment Both treatments T2 and T3 showed a significant difference with the control. At ripening stage (BBCH 89), the trend in the amount of starch in grapevine leaf is reversed compared with groat-sized (BBCH 73stage). The amount of starch is decreasing with treatment 3 as the least. Treatment 3 also depicts that starch content is significantly lower compared to other treatments (control, T1 and T2).

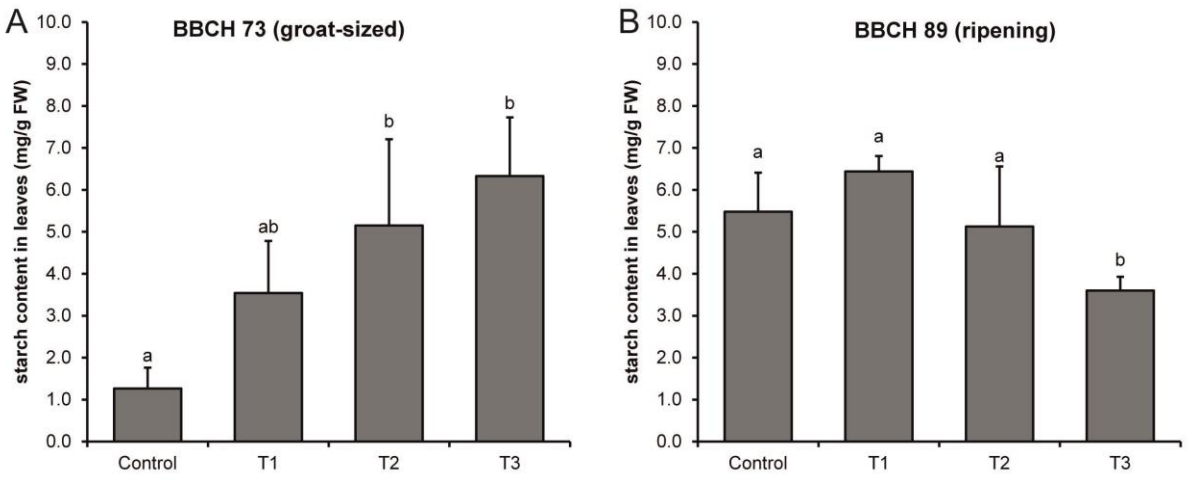

Figure 2. Starch content in leaves at BBCH 73(groat-sized) and BBCH 89 (ripening) stages. Data represent mean and standard deviation tested for significance by Scheffe.

At ripening stage (BBCH 89), the trend in the amount of starch in grapevine leaf is reversed compared with groat-sized (BBCH 73stage). The amount of starch is decreasing with treatment 3 as the least. Treatment 3 also depicts that starch content is significantly lower compared to other treatments (control, T1 and T2).

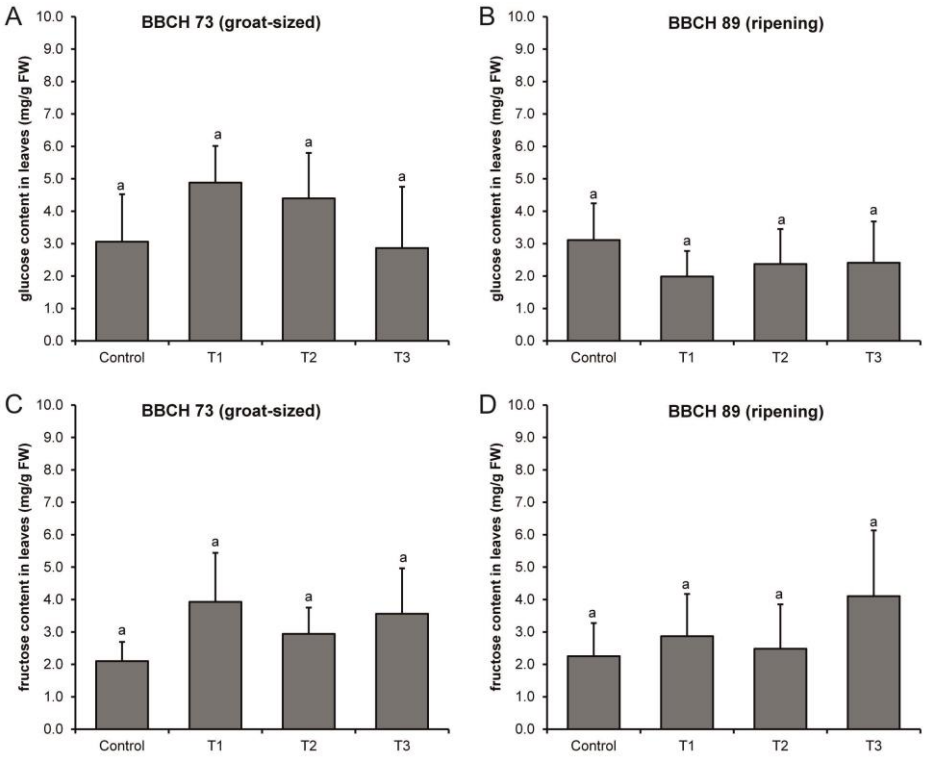

Figure 3. Mean values of glucose and fructose content in leaves at BBCH 73(groat-sized) and BBCH 89(ripening) stages calculated in Scheffe's test.

Glucose and fructose content of grapevine leaves were also quantified. At groat-sized (BBCH 73)stage, both monosaccharides glucose and fructose were observed highest in treatment 2. Other treatments have a quit a little difference but in the statistical analysis, all treatments are not significantly different from each other as indicated by the same letters. On the other hand, glucose and fructose content at ripening stage did not show significant difference among treatments. Nonetheless, the control treatment showed the highest amount of glucose and fructose and treatment 3 (10% shoot) with the least amount of canopy has the lowest amount.

Figure 4. Mean values of glucose and fructose content in berries at BBCH 73(groat-sized) and BBCH 89(ripening) stages calculated in Scheffe's test.

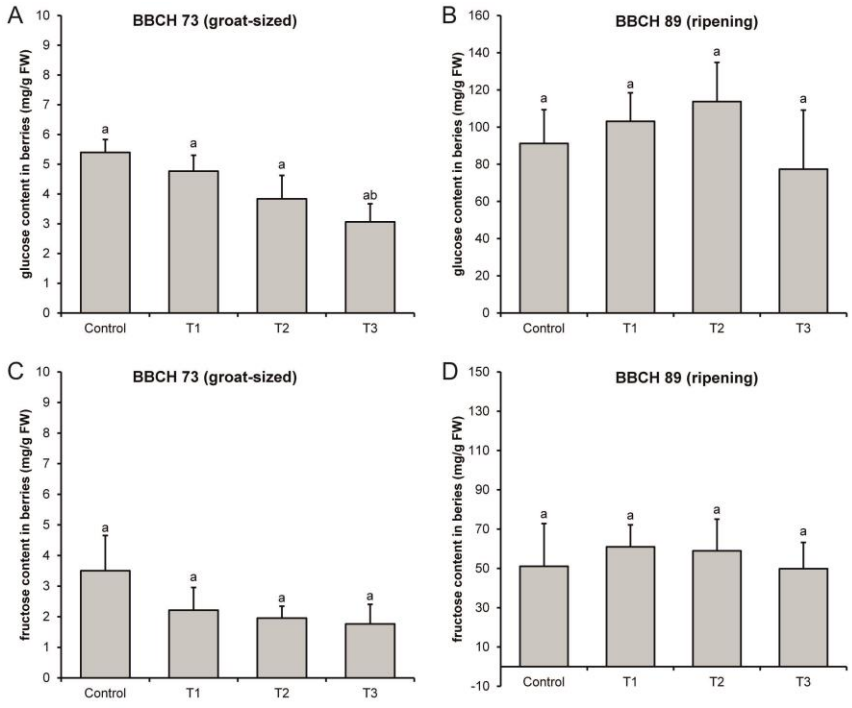

Figure 4 shows the glucose and fructose content of berries both in groat-sized (BBCH 73) and ripening (BBCH 89) stages. For glucose content at groat-sized or BBCH 73, T3 (10% shoot) showed the least amount of sugars and comparably lower among all other treatments while control (intact leaves) showed the highest. On the other hand, at ripening stage (BBCH 89), T1 (every 2nd leaf removed) and T2 (50% shoot) showed the highest amount of glucose. Despite the difference there was no significant difference detected among all treatments as indicated by the same letter annotation. Fructose content also showed the same trend in amount as with glucose in both groat-sized or BBCH 73 stage and ripening or BBCH 89 stages, control being highest and the all other treatments have more or less comparable amounts. The difference in quantity in each treatment also did not show any significance difference according to Sheffe's test. It is evident that glucose and fructose contents increase at ripening stage (BBCH 89) compared with groat-sized (BBCH 73) stage. In here, we see the shift in trend of sugar transport from source (leaves) to sink (berries).

Detection of enzymatic activities

Among the enzymes studied in this experiment were invertases (vacuolar, cell wall and cytosolic) and sucrose synthase. Invertase is the key enzymes for the hydrolysis of starch into glucose and fructose.

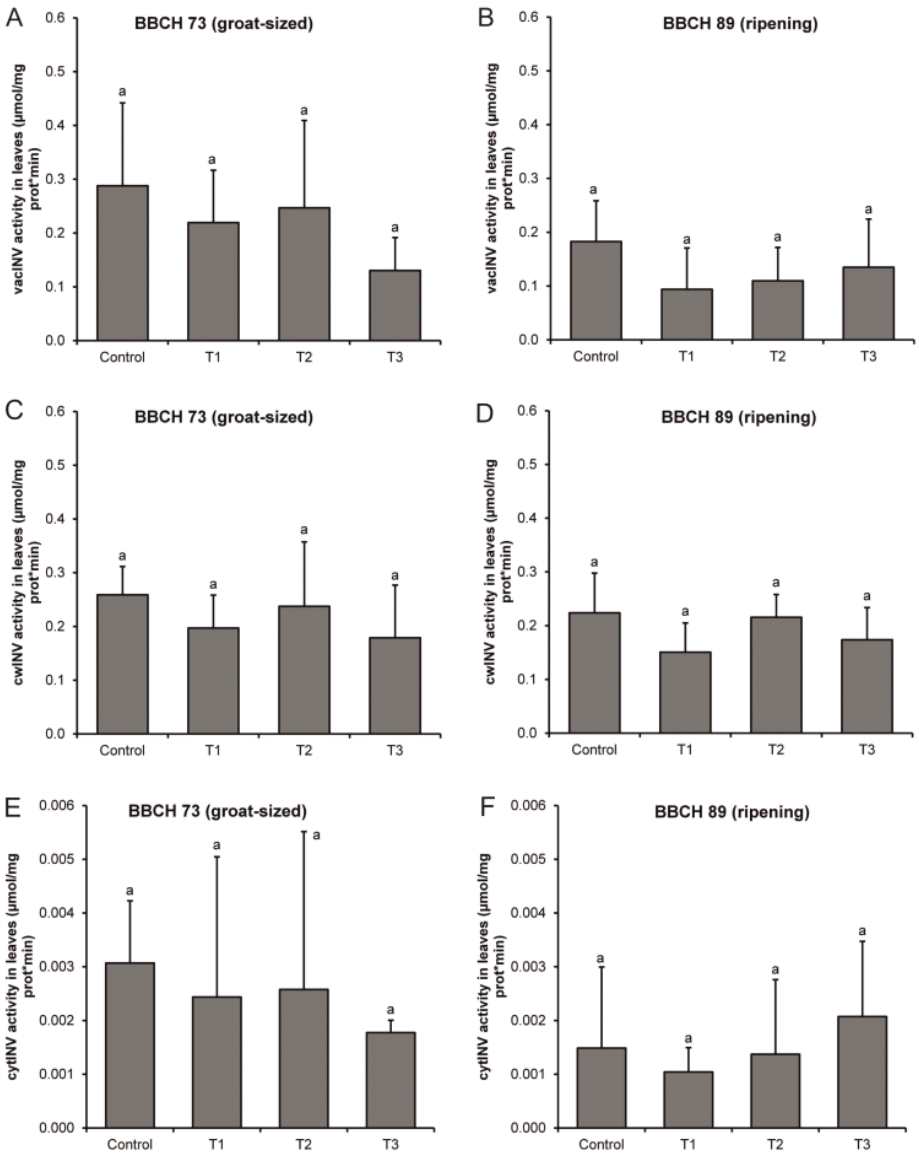

Figure 5. Enzyme activity in grapevine leaves (µmol/mg protein*min) both at BBCH 73 and BBCH 89 satges. Data represent mean and standard deviation calculated in Scheffe's test. vacINV = vacuolar invertase, cwINV = cell wall invertase, cytINV = cytosolic invertase

As shown in figures 3, vacuolar invertase content in the leaves of grapevines at groat-size stage was highest in the control treatment or when the leaves were not defoliated and it is comparable also to treatment 2 (50% shoot,). On the other hand, treatment 3 (10% shoot,) showed the least amount of vacuolar invertase. At ripening stage, control treatment also showed the highest amount of vacuolar invertase and treatment 1 (every 2nd leaf removed) showed the least amount. By comparing both graphs, it is evident that the enzymatic activity of vacuolar invertase slowed downed at ripening stage as compared to groat-sized (BBCH 73) stage. Despite the difference in amount, statistical analysis showed no significant difference among all treatments as indicated with the same letter.

There was also no much changes detected in the activity of cell wall invertase in both stages. At groat-sized (BBCH 73) stage, cell wall invertase activity in the leaves of grapevines was observed highest in both control and T2. On the other hand, it was detected lower in treatments 1 and 3. Yet, no significant difference among treatments was observed in the activity of cell wall invertase during this stage. The same trend was observed during ripening (BBCH 89) stage, control and treatment 2 are comparable and have the highest in activity. No significant difference was detected among treatments with cell wall invertase activity.

For cytosolic invertase, the same trend was detected as compared to cell wall invertase. Although highest values were also detected with the control treatment in both groat-sized (BBCH 73) and ripening (BBCH 89) stages, statistical tests showed that there is no significant difference among treatments as indicated with the same letter annotation.

Very low activity of sucrose synthase was detected in the leaves of grapevines for all samples and treatments. The results showed no significant difference among all treatments.

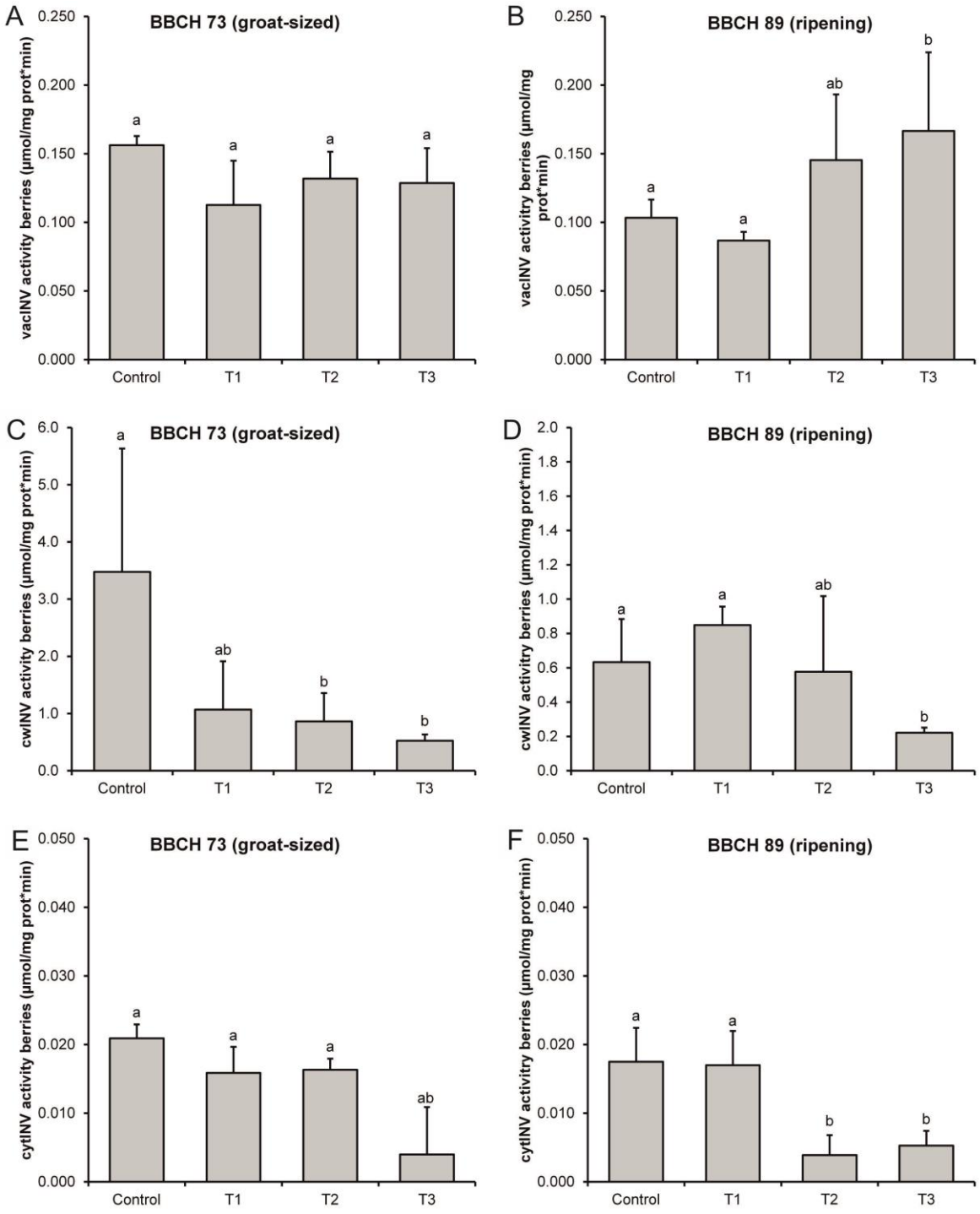

Figure 6. Enzyme activity in grapevine berries (µmol/mg protein*min) both at BBCH 73 and BBCH 89 satges. Data represent mean and standard deviation calculated in Scheffe's test. vacINV = vacuolar invertase, cwINV = cell wall invertase, cytINV = cytosolic invertase

In berries, vacuolar invertase activity was detected highest in control treatment at groat-sized (BBCH 73) stage (Figure 6). All the other treatments are comparable and are of the same amounts with T1 as the lowest but there was no significant difference detected between treatments as analysed in Scheffe's test. While at ripening stage (BBCH 89), it is evident that vacuolar invertase is significantly higher in lesser amounts of canopy (T2 and T3) than control and T1. Treatment 3 (10% shoot) showed the highest activity and T1 (50% shoot) the lowest. For cell wall invertase (cwINV), a different trend was observed. It was lowest with T3 (10%) and significantly different in treatments with lesser higher amount of canopy, control and T1 both at groat-sized and ripening stages. Cytosolic invertase was also detected highest in control treatment in both groat-sized (BBCH 73) and ripening (BBCH 89) stages. As with Sheffe's test, treatments 2 and 3 are significantly lower in cytINV (cytosolic invertase) activity than other treatments (control and T1). This reveals that decreasing the amount of canopy also reduces cytosolic activity in berries.

Discussion

The selective leaf defoliation of grapevine leaves around berry clusters influences berry sugar and anthocyanin content at harvest depending on the timing of defoliation (Pastore et al., 2013). Starch is the primary storage carbohydrate in plants. It is important in the carbon economy of plant organs, tissues and cell types. Starch occurs in plants from 1-100µm. Quantification of starch is important to determine the rates of accumulation and degradation in comparison with other fluxes (Smith and Zeeman, 2006). It is evident that starch concentration is higher in defoliated leaves compared to non-defoliated ones (Figure 1). The same result was also obtained in the study of Pallioti et al. in 2011, where leaf removal prior to flowering showed a higher starch content. An increase in starch concentration of basal leaves was also detected during the most active vegetative growth period until the inception of veraison through the effect of canopy management (Hunter et al.,1995).

Sucrose is the major photoassimilate transported from photosynthetic leaves to the developing fruit (Walker and Ho, 1978). The fate of sucrose in sink organs can be in two ways, it's either directly imported or cleaved by cell wall-bound invertases into monosaccharides such as glucose and fructose. Sugars can also be allocated in the different organelles of source and sink cells. Some hexoses can be transported into the chloroplasts, vacuole and golgi apparatus. The rate of sucrose import is regulated by sucrose concentration gradient between leaves and fruit and there is an inverse relationship between import rates and sucrose levels in the fruit (Walker et al. 1978). Sucrose is metabolically inert and needs to be converted into glucose and fructose inorder to be metabolized. In the results, there was no significant difference detected in glucose and fructose concentrations of grapevines both at groat-sized (BBCH 79) and ripening (BBCH 89) despite the higher concentration of sugars at an early stage (Figure 2). A similar result was also detected wherein partial defoliation (33% and 66%) showed no significant differences on the sugar and acid contents during the developmental stages of berries (Hunter, 1990; Souza et al., 2014). Partial defoliation of fully expanded leaves that have high photosynthetic rates resulted in increased photosynthesis in older leaves (Hodgkinson, 1974; Kaschuk et al., 2010). The plant compensated for the initial loss of photosynthetic capacity by increasing photosynthetic rates in older leaves to maintain the same export capacity under constant sink demand (Ainsworth and Bush, 2011). In complementary study, photosynthetic rates in young leaves remained high for longer period of time during fruit development and photosynthetic decline of older leaves was reversed due to the increased demand by filling fruit (Hall and Brady, 1977). Leaves are net importers of photoassimilates

at the early stage of development until it attains its full size and shifts to become net exporters especially at the inception of fruit development to ripening stage. Carbohydrates synthesized in the leaves are transported to the sink (berries) as sucrose especially at the fully expanded leaf stage of the plant.

The sugars accumulate in berries in a linear manner especially if they reach veraison stage. These sugars that accumulate in grape berries account for 65-91% of the fresh weight in mature berries (Kuhn et al., 2014). In the later stage of fruit development, production of sucrose sugars heightened and transported to the sink organs (berries) in order to compensate its high sugar requirement. This is evident with the increase in glucose and fructose concentrations in berries at ripening (BBCH 89) stage as compared to leaf sugar content in both stages and early stage of fruit development (BBCH 73). The increased sink demand enhances photosynthetic activity in the leaves (Hodgkinson, 1974; Hall and Brady, 1977; Kaschuk et al., 2010). Export of carbohydrates from sources (leaves) provide substrate for the growth and maintenance of non-photosynthetic tissues sinks (Ainsworth and Bush, 2010). Low content of sucrose both in glucose and fructose forms was also affected to destruction of leaves through defoliation; this is obvious in the early stage of fruit development during the onset of leaf removal (Figure 3). The changes of sucrose and hydrolysing enzymes are affected by environmental stresses (Cierezko, 2009). In apple, sugar metabolism and accumulation is highly regulated by developmental processes. The early stage of fruit development showed high enzymatic activities which also resulted with rapid sugar metabolism to satisfy the energy requirement for cell division and fruit development (Li, 2012). As a result of these activities in plant tissues, low fructose, sucrose and starch concentrations were detected in grapevine leaves and also coincide with the results of this study (Figures 2 and 3).

The reduction of leaf are through different treatments of defoliation after flowering period showed large reductions in the sugar content of 'Riesling' berries at harvest (Stoll et al., 2013). This also conforms to the results of this study wherein, the least amount of canopy or shoot showed lower amounts of glucose and fructose at harvesting (BBCH 89) stage (Figure 4).

Organs of most plant species require carbon and energy in the form of sugars. These require regulation of enzymatic activities such as sucrose synthase and several isoforms of invertases including vacuolar, cytosolic and cell wall invertases in the extracellular tissues. Invertases are extracellular enzymes which cleave sucrose in the apoplast into glucose and fructose. The resulting hexoses are transported by hexose transporters into the cell (Roitsch, 2003). Vacuolar and cell wall invertases also (acid invertases) have the same biochemical properties. They cleave sucrose most efficiently between

pH 4.5 and 5.0 and attack the disaccharide from fructose residue while cytoplasmic invertase with pH optima, cleave sucrose in the neutral or slightly alkaline range (Sturm, 1999).

The genes of enzymes are spatially and temporally expressed during plant development and are regulated by several environmental factors. Leaf and shoot defoliation do not significantly affect the enzymatic activities of grapevine tissues both in groat-size (BBCH 73) and ripening (BBCH 89) stages in the leaves of grapevines (Figure 5). There were too low enzymatic activities detected as affected by canopy disturbance through defoliation. In berries, activities of the different isoforms of invertases showed some differences as affected by the different levels of defoliation (Figure 6). At ripening stage, vacuolar invertase activity increased significantly in T3 (10% shoot). While the other isoforms of invertases, cell wall and cytolosic, activities were significantly lower when lesser amount of canopy was left (T2 and T3). At fruit maturation, starch deposited during the early stage of development is reconverted into sucrose depending on enzymatic activities such as acid invertase in the vacuole (Sturm and Tang, 1999). This conforms that only low concentration of starch was converted into sugars as indicated by the low enzymatic activities of invertases and sucrose synthase in the leaves except for vacuolar invertase in berries at ripening stage where it significantly increase when lower amount of canopy (10%) was left..

Concluding remarks

This study underlines the complexity of sink-source relations in grapevines as affected by canopy management through defoliation. Leaf defoliation significantly affects starch content of grapevine leaves especially at the early stage of removal. Amount of starch in leaves is increasing with the decreasing canopy left in grapevines at groat-sized (BBCH 73) stage but later decrease in trend as fruit development progress at ripening or BBCH 89 stage. At an early stage of berry development, starch concentration is low as compared to leaf tissues. More of the sugars are present in the leaves and stored as starch. During ripening stage (BBCH 89), starch content in leaves is decreasing with the least amount of leaves having the lowest. In the berries, it is also evident that sugar content increases in amount as compared with the early stage of development. Starch and sugars are transported to the berries and become net importers of photosynthetic products. Despite the differences detected in starch concentration, enzymatic activities showed no significant effect on sugar concentration as affected by

canopy management. It can be concluded that low amount of sugars are converted into glucose and fructose and merely stored as starch.

III. Recommendations and considerations

Further studies are recommended to support the present study on the enzymatic changes of grapevine leaves manipulated through defoliation of canopy. A detailed investigation is highly recommended such as several harvest dates of tissue samples from prior to fully expanded leaf stage, fruit setting stage until ripening or harvesting stage. An in-depth study such as molecular techniques must also be done to support the results of the present experiment. Sugar concentrations and the genes governing as such must be investigated through transcriptional and protein regulation. Various genes encoding key enzymes or transporters involved in sugar metabolism and accumulation must also be given attention to support this preliminary work. Several cultivars must also be investigated for changes in sugar concentrations and enzymatic activities through canopy management since it is also influenced by genotype.

IV. Summary

Over the last decades problems in grapevine fields arise. One example is the thick canopy which gives a major problem to grape growers. Thick cover of grapevines impacts negative effects like reduced fruitfulness, low fruit quality and hamper efforts of disease control on berry fields. Canopy management includes some techniques to address this problem through improvement of canopy structure and microclimate of grapevines as well as upgrade quality and composition of the fruit. Among the techniques used in canopy management includes plant spacing, selection of rootstock, training and pruning practices, irrigation, fertigation, shoot hedging, thinning and selective leaf removal.

A study on canopy management through different levels of defoliation was done to determine the effect on sink-source mechanism of the leaves in a 1-year old grapevine plants. It also aimed to study the enzymatic changes involved in sugar metabolism such as vacuolar, cell wall, cytosolic invertases and sucrose synthase. Two different harvests were done, one at groat-sized or BBCH 73 and another at

ripening or BBCH 89 stage. Two types of leaves were also collected, leaf 1 and leaf 7 but statistical analysis showed that there was no significant difference between the two leaves therefore results of the two leaves were combined and compared with other treatments. Starch content was tested with the steps followed in starch protocol (Smith and Zeeman, 2006). In the results, it showed that there was a great increase of starch content in the leaves especially at bloom or BBCH 73 stage, which was 3 days after treatment application. Starch content was detected highest at treatment 3 (10%) shoot) and least when the canopy remained intact (undefoliated). On the other hand, at ripeng (BBCH 89) stage, the trend on starch content in defoliated leaves was reversed. The ones with the higher amount of canopy (leaves and shoot) showed higher starch content, control being highest and T3 lowest. Hexose sugars such as glucose and fructose were also determined by Gluoce-Fructose protocol (by megazyme). In the leaves, tests showed that sugars, glucose and fructose, content have no significant difference both at groat-sized (BBCH 73) and ripening (BBCH 89) stages among treatments affected by the different levels of canopy management. In berries, sugar content also did not show any significant difference across treatments but an increase in amount was detected as it approached ripening stage.

The enzymatic activities of invertases also showed no significant difference across treatments and harvest dates in leaves whereas for berries, invertases showed fluctuations of activities. At ripening stage, vacuolar invertase is significantly higher with 10% canopy while cell wall and cytosolic invertases are significantly lower compared to control and T1 (every 2^{nd} leaf removed). Sucrose synthase showed very low activity that difference computed was nil as tested SuSy enzymatic test.

V. Appendix

Detailed presentation of the different protocols and tests used in the experiment

Starch protocol

1. From a starting plant material of 100grams, add ml 80% aq. Vol/vol ethanol. Incubate in boiling water bath for 3 min. Spin samples in centrifuge 3 000g for 5 to 10 min at room temperature. Repeat it twice more.
2. Leave the ethanol to evaporate for 10mins and add 1ml of water to homogenize.
3. Prepare 4 eppendorf tubes (1.5ml) for every sample, each labelled as S1, S2, B1 and B2.
4. Transfer 2 ml of the homogenate to each tube.
5. Heat to 100°C for 10min. to gelatinize starch granules.
6. Allow to cool. Add 0.2ml 200mM Na acetate (pH5.5) to each tube. Incubate the tubes (S1 and S2 with enzymes; B1 and B2 without enzymes) at 37 deg for 4 hours.
    a. S1 and S2: add 6 units of α-amyloglucosidase and 0.5 units of α-amylase.
    b. B1 and B2: without enzymes
7. Spin tubes in a microcentrifuge to remove the particulate material. 10 000g for 5 min at room temperature.
8. Put the supernatant in new eppendorf tubes (1.5ml).
9. Use the supernatant for glucose assay. Mix 20 µl extract with the assay reagents and add 150 µl of master mix in every hole.

    Assay reagents (prepare freshly the mixture): 72.15µl ddH20, 100mM HEPES, 0.5mM ATP, 1mM NAD and 4mM MgCl2

10. Add 0.1U hexokinase and 0.1U glucose-6-phosphate dehydrogenase (G6PDH) into all the holes (standards, samples and blanks).
11. Record OD 340 in the microtiter plate reader (Omega-MARS BMG LabTech).

Protocol for Glucose-Fructose Quantification (Adapted from Megazyme)

Standard Curve

1. Prepare solutions of 3mM, 2mM, 1mM and 0.6mM concentrations (standard curve).
2. Use the crude extract from the extraction protocol.

Plate Running

3. Prepare first the design of your plate and write contents in the laboratory book.
4. Add in every hole 20µl of samples, standards or water.
5. Add in every hole 100µl of the freshly prepared mastermix.
   Mastermix contains: 39µl HEPES pH 7.5 (0.2M), 0.75µl EDTA (0.25M), 0.4 µl MgCl2 (1M), 1 µl DTT (1M), 2.5 µl ATP (0.1M) and 55.5 µl ddH20.
6. Measure at 340nm the plate ODblanks. Then add 0.7U hexokinase and 0.3U G6PDH to all holes (samples and standards).
7. Measure at 340nm the plate ODglucose till the absorbance is constant then add 1U GPI (glucophosphate isomarase to all holes (samples and blanks).
8. Measure at 340nm the plate ODglucose + fructose in microtiter plate reader (Omega-MARS BMG LabTech) till the absorbance is constant.

BRADFORD PROTOCOL (determination of protein amount) by A. Jammer (2013)

1. Prepare staining solution (Bradford stain): Rotiquant (Roth); working solution prepared according to manufacturer's instructions. Prepare also stock solution of BSA (0.1g/L). Prepare a calibrating curve in a 96 well-plate.

Well	A1	B1+B2	C1+C2	D1+D2	E1+E2	D1+D2

Concentration (µg per 100 µl reaction)	Blank	0	0.5	1.0	1.5	2.0
BSA (µl)	0	0	5	10	15	20
Water (µl)	100	100	95	90	85	80

2. Measure 4 replicates of each sample and use mean values for evaluation. The amount of sample used in the assay needs to be adjusted to the amount of protein in the sample in order to obtain measured values that are within the range of the calibrating curve. Thus, if you expect high amounts of protein (R-extract), 1µl of sample or even less will be sufficient. If the amount of protein in your sample is very low (Z-extract), you will have to use larger volumes of sample. Whichever volume is used, dilute each sample with water to 100 µl before adding the staining solution. For R-extract, add 3µl + 97µl ddH20; for Z-extract, add 10µl + 90µl ddH2). Add 150µl of staining solution (Bradford stain).
3. Incubate at room temperature for 10min.
4. Measure absorbance at 595nm in microtiter plate reader(Omega-MARS BMG LabTech) .
5. Calculate protein amount in samples according to calibrating curve and give the values in µg protein per µl assay mix.

Invertase Assay Protocol (E.D. Covington)

A. Enzyme reaction
    1. Preheat the eppendorf thermoblock at 37 °C.
    2. Prepare a reaction mastermix and a blank mastermix. Reaction master mix per sample contains: 5µl of 1M sucrose, 25µl of 0.2 M Na acetate buffer (pH4.0), 20µl water while blank mastermix contains: 25µl of 0.2 sodium-acetate buffer (pH4.0) and 25µl water.
    3. Transfer 3µl R-extract or 10µl Z-extract or H2O into pre-chilled 2ml safe-lock tubes. Use large tubes because the mixture will bubble in later steps.
    4. Add 40µl mastermix or blank mastermix to each tube, while keeping tubes on ice.
    5. Immediately transfer all tubes to a 37°C thermoblock for 30 minutes.
    6. Prepare standards for calibration curve: Make a 50:50 mixture of 10mM glucose with 10mM fructose.

B. Glucose detection

1. Prepare Nelson's reagent by mixing 25 volumes of Nelson's A with 1 volume of Nelson's B (in the fume hood), and warm up the thermoblock to 100 deg (7.7µl B + 192.3 µl A).
2. At the end of the 30-minute incubation, add 200µl of arsenomolybdate reagent to each tube (in the fume hood). Arsenic is highly toxic! Wear nitrile gloves and work in the fume hood.
3. Centrifuge them at 14 000rpm for 5 min to pellet out any precipitate.
4. Transfer 130µl of each standard and sample to a Greiner 96-F well plate. Cover the plate with an adhesive plate cover in order to avoid breathing arsenic fumes.
5. Measure absorbance at 660nm in plate reader. (Omega-MARS BMG LabTech) Remove plate cover immediately before reading absorbance, and replace cover immediately after the read is finished.

<u>Sucrose synthase (SuSy) activity determination</u>

1. Prepare the mastermix recipe. Master mix for 160µl reaction volume contains: 69.80µl H20, 8µl 1M HEPES/NaOH pH 7.0 , 0.64µl 250mM EDTA, 0.32µl 1M MgCl2, 0.8µl 1M DTT, 2.08µl 100mM ATP, 0.8µl 100mM NAD, 0.32µl of 1000U/ml G6PDH, 0.672 of 1000U/ml hexokinase and 0.56µl of 1000U/ml PGI.
2. After preparing the mastermix, prepare mixes of incomplete reaction mixtures:
    a. –UDP: 34µl/rxn mastermix, 40µl/rxn 1M sucrose and 16µl/rxn H2O.
    b. +UDP: 34µl/rxn mastermix, 40µl/rxn 1M sucrose and 16µl/rxn 10mM UDP)
    c. No substrate control: 34µl/rxn mastermix and 56µl/rxn H2O.
3. Add 50µl of the basic mastermix in every hole of the 96-well reaction plate and add 20µl of the correspondent samples/standard in each hole.
4. Incubate both the 96-well reaction plate with mastermix, sample/standards and all premixes at 30°C for 30minutes in the microtiter plate reader and eppendorf thermoblock, respectively.
5. After incubation, add 90µl of each control premixes to the appropriate well.
6. Measure absorbance at 340nm, at 30 °C in microtit er plate reader(Omega-MARS BMG LabTech) every 1 minute, for as long as necessary to see activity. An hour is sufficient.

VI. References

Ainsworth, E.A. and D.R. Bush. (2011). Carbohydrate export from the leaf: A highly regulated process and target to enhance photosynthesis and productivity. Plant Physiology 155(1):64-69.

Alison, A.M. and S.C. Zeeman. (2006). Quantification of starch in plant tissues. Nature Protocols 1(3):1342-1345.

Afoufa-Bastien, D., Medici A., Jeauffre J., Coutus-Thevenot P., Lemoine R., Atanassova R. and M. Laloi. (2010). The *Vitis vinifera* sugar transporter gene family: phylogenetic overview and macroarray expression profiling. BMC Plant Bilology 10:245.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Bertamini, M. Muthuchelian K. and N. Nedunchezian. (2004). Effect of grapevine leafroll on the photosynthesis of field grown grapevine plants (*Vitis vinifera* L. cv. Lagrein). Journal of Phytopathology 152:145-152.

Candolfi-Vasconcelos, M.C., M.P. Candolfi, and W. Koblet. (1994). Retranslocation of carbon reserves from the woody storage tissues into the fruit as a response to defoliation stress during the ripening period in *Vitis Vinifera* L. Planta 192:567-573.

Chaumont, M. et al., (1994). Seasonal and diurnal changes in photosynthesis and carbon partitioning in *Vitis vinifera* leaves in vines with and without fruit. I. Exp. Bot. 45:1235-1243

Cierezko, I. (2009). Sucrose metabolism in plant tissues under stress conditions: key enzymes, localization and function. ISBN: 978-81-7895-422-6:193-218.

Coombe, B.G., McCarthy, M.G. (2000). Dynamics of grape berry growth and physiology of ripening. Australian Journal of Grape & Wine Research 6: 131-135.

Davies, C. and S. Robinson. (1996). Sugar Accumulation in grape berries. Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. Plant Physiology. 11(1):275-283.

Deluc, L. et al., (2007). Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. BMC genomics. 8:429.

Gifford, R.M., Thorne J.H. Hitz W.D. and R.T. Giaquinta. (1984). Crop productivity and assimilate partitioning. Science 1225:801-808.

Gholami, M., Hayasaka, Y., Coombe, B.G., Jackson, J.F., Robinson, S.P. Williams, P.J. (1995). Biosynthesis of flavour compounds in Muscat Gordo Blanco grape berries. Australian Journal of Grape & Wine Research 1: 19-24.

Harris, J.M., Kriedemann, P.E., Possingham, J.V. (1968). Anatomical aspects of grape berry development. Vitis 7: 106-109.

Hayes, M.A. et al., (2007). Isolation, functional characterization, and expression analysis of grapevine (Vitis vinifera L.) hexose transporters: Differential roles in sink source tissues. Journal of Experimental Botany 58: 1985-1997.

Hayes, M.A. et al., (2010). Involvement of abscisic acid in the coordinated regulation of a stress-inducible hexose transporter (VvHT5) and a cell wall invertase in grapevine in response to biotrophic fungal infection. Plant Physiology 153: 211-221.

Henschke, J. (2011). Impact of various canopy defoliation and anti-transpirant treatments on fruit maturation in grapevines. Vinifera.

Hodgkinson, K.C. (1974). Influence of partial defoliation on photosynthesis, photorespiration and transpiration by Lucerne leaves of different ages. Aust. J. Plant Physiology 1:561-578.

Hunter, J.L. Skrivan, R. and H.P. Ruffner. (1994). Diurnal and seasonal physiological changes in leaves of *Vitis vinifera* L.: CO2 assimilation rates, sugar levels and sucrolytic enzyme activity. Vitis 33:189-195.

Hunter, J.J, Ruffner, H.P. and E.O. Volschenkl. (1995). Starch concentrations in grapevine leaves, berries and roots and the effect of canopy management. S. Afr. J. Enol. Vitic., 16(.2):35-39.

Kaschuk, G. et al. (2010). Differences in photosynthetic behaviour and leaf senescence of soybean (*Glycine max* L.) dependent on N2 fixation or nitrate supply. Plant Bio 12:60-69.

Keller, M. (2010). The science of grapevines: anatomy and physiology. Elsevier Inc. Chapter 2: 78-83.

Kennedy, M. (2010). Understanding grapeberry development. Practical Winery and Vineyard. July/August. 14-23.

Kliewer, W.M. (1965). Changes of concentration of glucose, fructose and total soluble solids in flowers and berries of Vitis vinifera. Am. J. Enol. Vitic. 16:101-110.

Koch, K. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current opinion in plant biology. 7, 235-246.

Kuhn, C. et al. (1999). Update on sucrose transport in higher plants. Journal of experimental botany. 50, 935-953.

Lecourieux, F. et al. (2013). An update on sugar transport and signalling in grapevine. Journal of Experimental botany. 65(3), 821-832.

Li, M., Feng F. and L. Cheng. (2012). Expression patterns of genes involved in sugar metabolism and accumulation during apple fruit development. PLoS One 7(3): e33055.doi:10.137/journal.pone.0033055.

Liu, H. F. et al., (2006). Sugar and acid concentrations in 98 grape cultivars analysed by principal component analysis. J. Sci. Food Agric. 86:1526-1536.

Nguyen-Quoc, B. and C.H. Foyer. (2001). A role of 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. Journal of Experimental Biology. 52(358). 881-889.

Pallioti, A. et al., (2011). Early leaf removal to improve vineyard efficiency: Gas exchange, source-sink balance, and reserve storage responses. American Journal for Enology and Viticulture. 62(2): 219-228.

Pastore, C. et al., (2013). Selective defoliation affects plant growth, fruit transcriptional ripening program and flavonoid metabolism in grapevine. BMC Plant Biology. 13(30): 1-16.

Poni, S., F. Bernizzoni, and S. Civardi. (2008). The effect of early leaf removal on whole-canopy gas exchange and vine performance of *Vitis vinifera* L. 'Sangiovese'. Vitis 47:1-6.

Retallack, M. (2012). Grapevine biology. Wine growing for the future 1-5pp.

Roitsch, T. and M. Gonzalez. (2004). Function and regulation of plant invertases: sweet sensations. Trends in Plant Science. 9(12), 606-613.

Schulz, A. (1994). Phloem transport and differential unloading in pea seedlings after source sink manipulations. Planta 192:239-248.

Shiraishi, M. (1993). Three descriptors for sugars to evaluate grape germplasm. Euphytica. 71:99-106.

Smart, R. et al., (1985). Canopy microclimate modification for the cultivar Shiraz II effects on must and wine composition. Vitis. 24: 119-128.

Smeekens, S. Ma J, Hanson J., and F. Rolland. (2010). Sugar signals and molecular networks controlling plant growth. Current opinion in plant biology. 13:274-279.

Smith, A. M. and Ceeman S. (2006). Quantification of starch in plant tissues. Nature protocols. 1(3), 1342-1345.

Stoll, M. et al., (2013). Impact of various leaf area modifications on berry maturation in *Vitis vinifera* L. 'Riesling'. Acta Hort. (ISHS) 978:293-299.

Sturm, A. (1999). Invertases, primary structures, functions and roles in plant development and sucrose partitioning. Plant Physiology 121(1):1-8.

Sonnewald, U. and L. Willmitzer. (1992). Molecular approaches to sink-source interactions. Plant Physiol. 99(4): 1267–1270.

Souza, E.R. et al. (2014). Defoliation levels on gas exchange and composition of grape clusters cv. Italia. Brazilian Journal of Applied Technology for Agricultural Science 7(1):29-37.

Stitt M, Huber SC, Kerr PS (1987) Control of photosynthetic sucrose formation. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants. Academic Press, New York, pp 327–409.

Stitt M. (1990b). Fructose 2,6-biphosphate. In: Rey PM, Harborne J. Methods in Plant Biochemistry. 3:87-92.

Stitt, M. (1996). Metabolic regulation of photosynthesis. *In* Photosynthesis and the environment. (N.R. Baker, ed.). Kluwer Academic Publishers, Dordrecht, p.151-190.

Sturm, A. and G. Tang. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in plant science. 4(10), 401-407.

Turgeon, R. (1989). The sink-source transition in leaves. Annual Review of Plant Physiology and Molecular Biology. 40:119-138.

Walker, A.J., Ho, L. and D.A. Baker. (1978). Carbon translocation in tomato: pathway of carbon metabolism in fruit. Ann. Bot. 42: 901-909.

Wu, B.H. et al. (2011). Carbohydrate metabolism in grape cultivars that differ in sucrose accumulation. Vitis. 50(2), 51-57.

Van Bel AJE. (1996). Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons. Journal of experimental botany. 47: 1129-1140.

Vasconcelos, C. and S. Castagnoli. (2001). Leaf canopy structure and vine performance. American Journal of Enology and Viticulture 51(4).

Zhang, X.Y. et al. (2006). A shift of phloem unloading from symplastic to apoplastic pathway is involved in developmental onset of ripening in grape berry. Plant Physiology 142(1):220-232.

Zhang, B. et al. (2013). Synergistic and antagonistic effects of α-amylase and amyloglucosidase on starch digestion. Biomacromolecules 14:1945-1954.

Zrenner, R., Salanoubat M., Willmitzer L., and U. Sonnewald. (1995). Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (Solanum tuberosum L.). Plant J. 7:97-107.

Http://www.GeoChemBio.com/biology/organism/Vitis

Http://www.plantsinaction.science.uq.edu.au

VII. Affidavit

Name: Jedi Joy Belmonte Mahilum

Date of birth: 30.11.1981

I declare under penalty of perjury that I have produced this work independently, without unauthorized assistance of third parties and without the use of any other person than the the specified resources. Data and concepts acquired directly or indirectly from other sources are identified through citations and listed in the references. This also applies to figures, graphs, illustrations and the like, as well as to the internet sources and unpublished sources.

The work has not been published either in this country or another country in the same or similar version.

_____ Date:_____

Signature