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GRAPEVINE PLASTICITY AND TERROIR: A MULTIDISCIPLINARY APPROACH FOR DISSECTING THE SINGULAR EFFECT OF SOIL ON GRAPEVINE GROWTH AND BERRY QUALITY

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ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops since it is largely used for wine production. It also represents the best paradigm for what concern phenotypic plasticity, meant as the capability of a single genotype to give rise to a range of different phenotypes. In fact, the complex interplay between a cultivar and the agri-environmental factors of a certain area, nowadays recognizable with the term *terroir*, has a crucial impact on grape traits that ultimately affects wine quality. Despite the high importance that scientists and traders give to the *tipicity* of each wine, not much is known about the singular effect that each terroir-factor has on the final product traits. To shed light on it, controlled experiments are needed for maintaining all the inputs as constant as possible, except one.

In this research, we set an on-field experiment for studying the singular effect that soil – one of the main components of terroir – has on the grapevine growth and on the grape quality during two consecutive years by a multidisciplinary approach. Indeed, soil provides the grapevine with water and nutrients, but any chemical imbalance ends up influencing the vine growth and the quality of the grapes. Moreover, the influence of soil (regarding its texture, depth, mineral compositions, and microbiological community) has not been studied so widely, mainly due to its great variability even inside the same field. We chose three different soils from three Italian viticultural areas of Veneto region (Valpolicella, Valdobbiadene, and Legnaro), and set them in the same place but keeping them separated by means of cement boxes. *Vitis vinifera* cv. Corvina and cv. Glera were chosen for the study, hence exposing them to the same climatic and agronomical conditions.

Specifically, we focused on the soil characterization by physico-chemical and microbiological analyses, and on phenological and physiological analysis of vines at both vegetative and reproductive stages. We also sampled berries throughout the maturation for metabolomic and transcriptomic analyses.

The three soils differed from each other mainly by texture composition and the content of skeleton, organic matter, and potassium. Phenological differences emerged throughout years and between cultivars even if the effect of the soil was more evident considering the 2017 vintage. Plants grown in Valdobbiadene (VV) soil were delayed with respect to the others. This effect resulted less clear in 2018 vintage, possibly due to the interaction with the plant age and/or climate. Valpolicella (F) soil positively affected berry weight, whereas Valdobbiadene

(VV) soil always produced lighter berries. In 2017, we also observed a stronger soil effect on the berry sugar accumulation pattern when compared to the latest vintage (2018).

To study the molecular and biochemical plasticity, berries were collected in 2018 and processed for metabolomic and transcriptomic analyses. Transcriptomic analyses on both berry skin and pulp revealed a strong cultivar-tissue plasticity at harvest. Metabolomic analysis on berry skin revealed a significant effect of soil on several metabolites belonging to the phenylpropanoid pathway (especially flavonols and stilbenes) and high plasticity of metabolites accumulated in Corvina berry skin at harvest. Interestingly, the analysis revealed many interaction effect (cultivar*soil), especially for the class of flavan-3-ols, flavonolos, and hydroxycinnamic acids. Moreover, many different soil-metabolites correlations emerged when considering the two cultivars separately, suggesting, once again, a strong cultivar-specific response.

This research can be meant as a first step in the study of the singular effect of the soil factor on grape traits, revealing its potential importance on the final product quality and supporting the hypothesis of a delicate balance which exists between a cultivar and its typical area of production.

RIASSUNTO

La vite (*Vitis vinifera* L.) è una delle colture frutticole più importanti dal punto di vista economico poiché è ampiamente utilizzata per la produzione di vino. Rappresenta anche il miglior paradigma per quanto riguarda la plasticità fenotipica, intesa come la capacità di un singolo genotipo di dare origine a una gamma di fenotipi diversi. In effetti, la complessa interazione tra una cultivar e i fattori agroambientali di una determinata area, oggi riconoscibile con il termine *terroir*, ha un impatto cruciale sui tratti dell'uva che influiscono in definitiva sulla qualità del vino. Nonostante la grande importanza che scienziati e commercianti danno alla tipicità di ciascun vino, non si sa molto del singolo effetto che ogni fattore del terroir produce sulle caratteristiche del prodotto finale. Per far luce su ciò, sono necessari esperimenti controllati al fine di mantenere tutti gli input il più costanti possibile, tranne uno.

Con questa ricerca, ci siam proposti di studiare l'effetto che il suolo - uno dei componenti principali del terroir - ha sulla crescita della vite e sulla qualità dell'uva mediante un approccio multidisciplinare. In effetti, il suolo fornisce alla vite acqua e sostanze nutritive, ma ogni squilibrio chimico finisce per influenzare la crescita della vite e la qualità dell'uva. Inoltre, l'influenza del suolo (per quanto riguarda la sua tessitura, profondità, composizione minerale e microbiologica) non è stata studiata così ampiamente, principalmente a causa della sua grande variabilità spaziale, anche all'interno dello stesso appezzamento. Abbiamo scelto tre diversi suoli provenienti da tre aree viticole italiane della regione Veneto (Valpolicella, Valdobbiadene e Legnaro), collocandoli nello stesso posto ma separandoli per mezzo di cassoni di cemento. Le cultivar Corvina e Glera sono state scelte per lo studio, esponendole quindi alle stesse condizioni climatiche e agronomiche.

Nello specifico, ci siamo concentrati sulla caratterizzazione del suolo mediante analisi fisico-chimiche e microbiologiche e sull'analisi fenologica e fisiologica dello sviluppo delle viti. Inoltre, nel secondo anno, le bacche sono state campionate durante la maturazione per analisi metabolomiche e trascrittomiche.

I tre terreni differivano l'uno dall'altro principalmente per tessitura e contenuto di scheletro, materia organica e potassio. Differenze fenologiche sono emerse nel corso degli anni e tra le cultivar anche se l'effetto del terreno era più evidente considerando l'annata 2017. Le piante coltivate nel suolo di Valdobbiadene (VV) risultavano più in ritardo nello sviluppo. Questo effetto è risultato meno evidente nell'annata 2018, probabilmente a causa dell'interazione con l'età della pianta e le condizioni climatiche stagionali. Il terreno di Valpolicella (F) ha influito

positivamente sul peso della bacca, mentre il terreno di Valdobbiadene (VV) ha sempre prodotto bacche più leggere. Nel 2017, abbiamo anche osservato un forte effetto del suolo sull' accumulo di zucchero, meno marcato nell'ultima annata (2018).

Le bacche raccolte nel 2018 sono state anche processate per analisi metabolomiche e trascrittomiche per studiarne la plasticità a livello molecolare e biochimico. Le analisi trascrittomiche sia su buccia che su polpa hanno rivelato una forte plasticità del tessuto al momento della raccolta. Le analisi metaboliche su buccia hanno rilevato un effetto significativo del suolo su numerosi metaboliti appartenenti alla via dei fenilpropanoidi (in particolare flavonoli e stilbeni) e un'elevata plasticità in buccia di Corvina al momento del raccolto. È interessante notare come dall'analisi siano emersi alcuni effetti di interazione (cultivar * suolo), in particolare per le classi di flavanoli, flavonoli, e acidi idrossicinnamici. Inoltre, sono emerse molte correlazioni tra componenti del suolo e metaboliti di buccia quando considerate le due cultivar separatamente, suggerendo, ancora una volta, una forte risposta cultivar-specifica.

Il nostro studio può essere inteso come un primo passo nella comprensione dell'effetto del fattore suolo sui tratti dell'uva, grazie anche alla sua peculiarità multidisciplinare, ricordando la sua potenziale importanza sulla qualità del prodotto finale e sostenendo l'esistenza di un delicato equilibrio tra cultivar area di produzione.

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Abbreviations

Units

°C	degrees Celsius
aa	amino acid
bp/kb	base pairs
g; mg; µg	gram(s); milligram(s); microgram(s)
m; km; mm; cm	meter(s); kilometer(s); millimiter(s); centimeter(s)
L; ml; µl	liter(s); milliliter(s); microliter(s)
M	molar (moles per L)
s	second(s)

Elements

EC	Electrical Conductance
C_tot	total carbon
C_org	organic carbon
N_tot	total nitrogen
C_N	carbon-to-nitrogen ratio
Al	aluminum
Ca	calcium
Fe	iron
K	potassium
Mg	magnesium
Mn	manganese
Na	sodium
P	phosphorous
S	sulfur
Ti	titanium
As	arsenic
B	baron
Ba	barium
Be	beryllium
Cd	cadmium
Co	cobalt

Cr	chromium
Cu	copper
Li	lithium
Mo	molybdenum
Ni	nickel
Pb	lead
Sn	tin
Sr	strontium
V	vanadium
Zn	zinc
Ca_ex	exchangeable calcium (mg/kg s.s.)
K_ex	exchangeable potassium (mg/kg s.s.)
Mg_ex	exchangeable magnesium (mg/kg s.s.)
Na_ex	exchangeable sodium (mg/kg s.s.)
CEC_ex	cation exchangeable capacity (mg/kg s.s.)
Ca_mol	exchangeable calcium (cmoli(+)/kg s.s.)
K_mol	exchangeable potassium (cmoli(+)/kg s.s.)
Mg_mol	exchangeable magnesium (cmoli(+)/kg s.s.)
Na_mol	exchangeable sodium (cmoli(+)/kg s.s.)
CEC_mol	cation exchangeable capacity (cmoli(+)/kg s.s.)

Metabolites

Cyan-3-glu	Cyanidin-3- O-glucoside
Pet-3-glu	Petunidin-3-O-glucoside
Peo-3-glu	Peonidin-3-O- glucoside
Mal-3-glu	Malvidin-3-O-glucoside
Delph-3-glu	Delphindin-3-O- glucoside
Delph-3-acet	Delphinidin-3-O-(6′′-acetyl-glucoside)
Cyan-3-acet	Cyanidin-3-O-(6′′-acetyl-glucoside)
Pet-3-acet	Petundin-3-O-(6′′-acetyl- glucoside)
Mal-3-acet	Malvindin-3-O-(6′′-acetyl-glucoside)
Peo-3-acet	Peonidin-3-O-(6′′-acetyl-glucoside)
Delph-3-coum	Delphinidin-3-O-(6′′- p-coumaroyl-glucoside)
Mal-3-caffe	Malvidin-3-O-(6′′-caffeoyl-glucoside

Cyan-3-coum	Cyanidin-3-O-(6′′-p-coumaroyl-glucoside)
Pet-3-coum	Petunidin-3-O-(6′′-p-coumaroyl-glucoside)
Peo-3-coum	Peonidin-3-O-(6′′-p- coumaroyl-glucoside)
Mal-3-coum	Malvidin-3-O-(6′′-p-coumaroyl-glucoside)
Myr-3-glr	Myricetin-3-O-glucuronide
Rutin	Quercetin-3-O-rutinoside
Myr- 3-glu	Myricetin-3-O-glucoside
Quer-3-glr	Quercetin-3-O-glucuronide
Quer-3-glu	Quercetin-3-O-glucoside
Kaemp-3-glr	Kaempferol-3-O- glucuronide
Kaemp-3-glu	Kaempferol-3-O-glucoside
Narin-chalc-glu	Naringenin-chalcone-4-O-glucoside
Hex.	Hexoside

General

G x E	Genotype per Environment
F	Fumane (soil)
VV	Vittorio Veneto (soil)
L	Legnaro (soil)
DOCG	Controlled and Guaranteed Designation of Origin
DOC	Controlled Designation of Origin
IGT	Indicazione Geografica Tipica
cv.	cultivar
K5BB	Kober 5BB (rootstock variety)
ARPAV	Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto
ISTAT	Istituto Nazionale di Statistica
ANOVA	Analysis of Variance
PCA	Principal Component Analysis
FEM	Fondazione Edmund Mach
NGS	Next Generation Sequencing
DAFNAE	Department of Agronomy, Food, Natural resources, Animals and Environment
TSS	total soluble solids
TA	titratable acidity
MI	maturation index

LAI	leaf area index
ASE	allele specific expression
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
USA	United States of America
VST	variance stabilizing transformation
GO	Gene Ontology
FDR	False Discovery Rate
SEA	Singular Enrichment Analysis
DEGs	Differentially Expressed Genes
HCL	Hierarchical clustering
GS	gas chromatography
UPLC	Ultra Performance Liquid Chromatography
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
NMR	nuclear magnetic resonance
MS	mass spectrometry
rpm	revolutions per minute
STS	stilbene synthase
TF	transcription factor

Chapter 1

Introduction and outline of the thesis

1.1 INTRODUCTION

Individual organisms can alter their development, physiology, and life history depending on environmental conditions. It means that even the plant growth behaviors and the final product features, in nature as well as in agriculture, deeply depend on the external conditions. These environmental responses represent evolved characteristics that vary among species, populations, and genotypes (Sultan, 2000). In this context, we talk about Genotype-per-Environment interactions (GxE interactions), that may be defined as a change in the relative performances of a "character" of two or more genotypes measured in two or more environments (Bowman, 1972). Interactions may therefore involve changes in rank order for genotypes between environments and changes in the absolute and relative magnitude on the genetic, environmental and phenotypic variances between environments (Bowman, 1972). In this perspective, GxE interactions might thus represent an important source for the intraspecific phenotypic variability that, in an evolutionary dynamics, generally allows species to withstand and adapt to novel biotic and abiotic environmental changes (Nicotra et al., 2010). Instead, from an agronomical point of view, the array of different phenotypes has always allowed farmers to select the organisms (plants and animals) that best suited the conditions of an area, in terms of survival and final product quality. Some authors have also proposed the existence of a close relationship between crop plant variety and its environment, stressing that the presence of a variety in a given locality is not just a chance occurrence, rather there is a genetic component that helps the individual to adapt to that area. They also pointed out the role of selection in directing the 'genotypical differentiation of the population in a given locality' (Baye et al., 2011).

In molecular biology, the plant phenotype can be meant as the outcomes of complex synergistic developmental systems, influenced by multiple interacting genes and gene products as well as by organism's internal and external environments (Sultan, 2000). These "outcomes" reflect the *ability* of a single genotype to give rise to different phenotypes when exposed to different environmental conditions, to date known as "*phenotypic plasticity*" (Sultan, 2000), whereas the *set* of phenotypes, that can be produced by an individual genotype when exposed to different environmental conditions is known as "*norm of reaction*" (Pigliucci and Schlichting, 1998). Several different mechanisms might be involved in different aspects of plasticity; these are believed to include environmentally dependent regulatory loci as well as

non-epistatic loci at which allelic expression varies with the environment (Sultan, 2000). In fact, the phenotype can't be meant as the cumulative result of genotype and environment, but rather it should be considered as the result of the genetic makeup of the active genes and the intensity with which the active genes create gene products and changes in response to environmental factors and developmental time. Therefore, it is insightful to think of alleles for gene expression as each contributing (additively or non-additively) to a reaction norm across environments rather than to a static expression level or fitness (Hodgins-Davis and Townsend, 2009).

In the past, most of the studies focused on the simple behavior of plants, in terms of growth and morphology, as response to contrasting light, moisture, and nutrient environments (Sultan, 2000). Other studies also included directly functional aspects of plasticity such as the proportional resource allocation to different plant tissues or assimilation rate (Sultan, 2000), as well as many other key functional traits (Nicotra et al., 2010). For the last 20 years, new disciplines, such as transcriptomics and metabolomics, have been developing and gaining a sophisticated understanding of the mechanisms of plasticity, as recently it has already proposed for the transcriptome profiling of some model animal organisms such as the fruit fly *Drosophila melanogaster* (Zhou et al., 2012), the mouse *Mus musculus* (Hamilton and Yu, 2012), and the nematode *Caenorhabditis elegans* (Li et al., 2006). In fact, the availability of high-throughput expression profiling technologies now makes it possible to analyze gene expression (activity and spatiotemporal characteristics) on a global scale, so that transcriptome plasticity can be investigated directly (Gilad et al., 2006; Leakey et al., 2009; Ranz and Machado, 2006). Eventually, the integration of different 'omics' technologies will make it possible to better understand the total network of plant responses to various external factors; in this way, a systems biology approach will be developed to explore and exploit the possible synergism between genes, proteins and metabolites (Ali et al., 2009).

While phenotypic variation between species and organism of the same species may reflects differences in gene structure as well as differences in gene expression, phenotypic plasticity among clones of a given genotype is likely to be much more dependent on differential gene expression in different environments (Gilad et al., 2006). This provides the possibility to better study how plant organisms behave in different conditions and what are the molecular mechanisms at the basis of the phenotype array, which to date remains still poorly characterized (Gianoli and Valladares, 2012). Among the cultivated plants, grapevine (*Vitis* spp)

is one of the best paradigm for what concern studying GxE interactions and phenotypic plasticity based on the clone responses (Keller, 2010). Moreover, it is globally the most important fruit species due to the numerous uses of its fruit in the production of wine, grape juice and other foods. Among the Vitis species, *V. vinifera* is currently the most cultivated fruit crop around the world because of its use in wine production (Lodhi and Reisch, 1995), with 75.1 million tons of berries produced in 2014 (http://www.oiv.int/). Omics' approaches have been utilizing in grape to understand the environmental effects on plant and fruit development (Fortes and Gallusci, 2017), but we are just at the beginning to understand the patterns and mechanisms involved in plastic responses of this plant to single environmental factors (Valladares et al., 2007). Besides, such approach in grapevine is even difficult due to its perennial biological cycle and the complexity in well isolating a single factor in an open field context.

The general aim of this section is to investigate the "state of arts" of what the current literature offers about the behavior of grapevine under different environmental and human inputs such as climatic conditions, soil type and agricultural practices. Indeed, all of them can have a profound impact on the berry transcriptome, in turn affecting ripe berry and wine quality traits (Dal Santo et al., 2013). The first part focuses on some viticultural and oenological concepts coming from the interaction between the "agri-environment" and the grapevine varieties, plus some following implications into the wine world. In the second part, we tried to analyze the single effect that the main agri-environmental factors have on the grape traits, both by a transcriptional and a metabolomic approach. Eventually, we tried to summarize the most insightful concepts and highlight the research perspectives that might be followed up.

1.2 GRAPEVINE AND TERROIR: from plasticity to terroir

Grapevine berries are characterized by considerable phenotypic plasticity, with the same clone showing variability for many traits within individual berries, among berries within a cluster, between cluster on a vine, and among vines in the vineyard, depending on both environmental factors and viticultural practices (Keller, 2010). This can be considered a burden because berries may unevenly mature and display large inter-seasonal fluctuations in quality, but it also offers some advantages such as the ability of existing cultivars to adapt to specific growing

regions and the production of different wines even from the same cultivar (Dai et al., 2011). Furthermore, it has been shown that subtle geographical differences can have a significant effect on grape/wine composition even when variability within vineyards was reported to be relatively high (Mulas et al., 2011). It means that fruit and wine composition is strongly influenced by the interactions between the plant's genome, the local growing conditions (including the vine management system), and the oenological practices of each winery, which could explain why is so difficult to replicate a wine from a region outside that area (Fabres et al., 2017). These interactions were firstly defined by French winemakers with the single word of "*terroir*" (from the word '*terre*', meaning 'soil') (Gladstones, 2011). It is also frequently used to relate wine sensory attributes to its geographical origin (Van Leeuwen and Seguin, 2006) as it were possible to put a signature on the wine bottle depending of the area of production. The notion of terroir in viticulture precisely refers to this complex interplay of factors: it involves the vine and its environment, including phenology, geography, geology, pedology and the local climate of a vineyard, along with human activity (Seguin, 1986). Albeit numerous authors have proposed varying definitions of the concept of terroir, they all agree on its geographical dimension (Foroni et al., 2017; Roullier-Gall et al., 2014). On this basis, it could be proposed that if grape composition is marked by chemical fingerprints from a given terroir, wines made from these grapes should also reflect related fingerprints (Roullier-Gall et al., 2014). Clearly, the relationship between the concepts of terroir and the phenotypic plasticity is quite strict, and understanding the genetic and molecular mechanisms underlying this "genotype per environment" interactions is of pivotal importance not only for researchers, but also for winemakers. The economic importance of grapevine has prompted the investigation to the molecular factors that regulate growth, development, berry ripening, and particularly the impact of gene expression on quality traits (Tornielli et al., 2012). Despite that, less work has been done so far to elucidate the molecular mechanisms involved in the grapevine response to the terroir. The problem is that the elements affecting grapevine growth and fruit composition are complex and multifarious (Fabres et al., 2017). Moreover, as the number of environments and the number of genotypes increase, the number of possible GxE interactions increases tremendously, which would certainly make their implication more difficult to comprehend (Allard, 1999; Allard and Bradshaw, 1964). A strategy to better understand the interaction between genome and environment could be by isolating as much as possible each terroir factor by a scientific approach. Then, by the integration of environmental information

with genomic, epigenomic, transcriptomic, and metabolomic data, it will be possible to better understand the effect of terroir at a molecular level (Fabres et al., 2017).

At this point, it will be worth to study how deeply the terroir might affect the final grape traits, mentioning which are the metabolites more terroir-responsive, and investigating the possibility to distinguish grapes and wines depending on the area of origin, both in terms of uniqueness and traceability.

1.3 THE WEIGHT OF TERROIR (The power of terroir)

According to the strictest definition of terroir, similar grape varieties – as well as plants of the same clone – from different terroirs tend to express significantly different characteristics (Foroni et al., 2017; Frost et al., 2015). In other words, the wine produced in a given region can be considered unique and cannot be reproduced elsewhere even though the grape and winemaking techniques are carefully duplicated (Anesi et al., 2015; Seguin, 1988). Grape and wine quality reflects the levels and composition of many primary and secondary metabolites that shapes the overall sensory experience of its derived product (Anesi et al., 2015; Reshef et al., 2017). Specifically, grape berries contain the major compounds contributing to wine flavour, resulting from metabolic changes that occur during the growth of berries up until harvest (Lund and Bohlmann, 2006; Roullier-Gall et al., 2014). The rate of biochemical changes during berry development has been demonstrated to be varietal dependent as well as influenced by climatic factors, soil, irrigation level, and other viticultural practices (Degu et al., 2014). In addition, the different components of the metabolome and transcriptome can also respond to unique interactions of factors within each terroir (Anesi et al., 2015).

As expected, the effect of terroir on metabolites is noticeable in wines and grape berries, even though it’s been demonstrated to be more evident in musts (Roullier-Gall et al., 2014). Many authors have proposed a notable effect on the amount of phenolics that, although depending on the cultivar (Dimitrovska et al., 2011; Fernández-Marín et al., 2013; Guerrero et al., 2009; Katalinić et al., 2010; Navarro et al., 2008; Yang et al., 2009), are highly influenced by the terroir (de Andrés-de Prado et al., 2007; Fernández-Marín et al., 2013; Tarko et al., 2010) in both

qualitative and quantitative composition (Bautista-Ortin et al., 2012; Talaverano et al., 2016). For example, it has been demonstrated that grape anthocyanin composition depends not only on the maturity of the grapes but also on different vine growing parameters like soil and climate along with practices such as pruning, fertilization, or watering (Ali et al., 2009; Gonzalez-Neves et al., 2002). The strong terroir effect can also be demonstrated in the syntheses and accumulation of aromas in the grapes (Alessandrini et al., 2016), the compounds responsible for the wine appreciation. In addition, some other parameters that seem to be affected by both terroir and variety are the date of harvest, based on the total soluble solids content (TSS), and the total acidity (Fernández-Marín et al., 2013) of grapes.

In their recent study, Anesi et al. (2015) performed a metabolite analysis on a single Corvina clone cultivated in seven different vineyards for three consecutive years. Among the various metabolite classes they found, as well as for anthocyanins, a clear plastic response were seen for the classes of stilbenes, flavonoids, and some volatile organic compounds (especially for sesquiterpenes). In this case, such compounds gave proving to be the most plastic components of the metabolome. Other studies as well demonstrated the importance of such classes when comparing different growing conditions: the stilbene content has also been demonstrated to considerably vary in wines due to several factors such as climate, grape variety, fungal infection (Perrone et al., 2007), UV light, metal ions (Püssa et al., 2006), and oenological methods (Ali et al., 2009; Gambuti et al., 2004). Contrary, there are also some reports suggesting that the type of soil, its chemical composition, and the geographical exposure of the vineyards have no effect on the perceived quality of wines (Cross et al., 2011; Foroni et al., 2017; Gergaud and Ginsburgh, 2001).

Metabolite results can be confirmed by transcriptomic data: previous works have indicated that the berry-specific expression of genes related to anthocyanin synthesis shows substantial plasticity and is greatly influenced by the environment (Anesi et al., 2015; Azuma et al., 2012; Pastore et al., 2013; Teixeira et al., 2013). In line with other studies, Dal Santo et al. (2013) found that the phenylpropanoid pathway, especially the resveratrol biosynthesis, was one of the most environmentally-dependent metabolic components, with a good correlation between metabolite levels and the induction of gene expression. Working with the red cultivar Corvina, they also verified that it reflected a deep plasticity of the berry transcriptome at harvest, as well as the all ripening phase could be modified by the growing conditions. They estimated that the 5 % of the Corvina transcriptome is used for terroir-specific adaptation, in

which the plastic genes are particularly enriched in ontology categories such as transcription factors, translations, transport, and secondary metabolism.

When trying to correlate specific terroir features with the berry metabolome, it has been demonstrated that it makes much more sense to associate environmental changes to many small metabolic changes in composition rather than to a small number of major metabolic shifts (Anesi et al., 2015). Many authors also suggested that the most vivid effect is due to the vintage (Anesi et al., 2015; Dal Santo et al., 2013). Anyway, they also demonstrated that the strong effect of vintage on sample correlation can fade during berry ripening, suggesting that the impact of agronomic practices and other environmental conditions (as the soil composition) on the berry transcriptome becomes more important at this stage. Despite the strong vintage-specific effect on both metabolome and transcriptome, it has been proposed the existence of a clear terroir-specific effect of the transcriptome and metabolome in phenolic composition, which persists over several vintages and allow each vineyard to be recognizable by the unique profile of specific metabolites. Fernandez-Marin et al. (2013) focused on stilbene composition of four red cultivars in four different Andalusian terroirs, suggesting that, in some cases, the terroir can be more important than the variety for the traits they considered in the study. In fact, they even noticed how samples grouping was better observed according to terroirs rather than to varieties. Besides molecular analyses, the importance of terroir has been highlighted in Foroni et al. (2017) where participants of an olfactory survey could "smell the terroir": they could distinguish wines from different terroirs and cultivars, with the terroir origin parameter being more easily discriminated than the cultivar parameter. In another study, conducted in the Loire Valley (France), a good relationship has been found between the perception of the growers of wine behaviour in relation to terroir (precocity, vigour, water status, quality potential...) and characteristics of the physical environment (e.g. soil, climate, and topography) (Morlat and Guilbault, 2001).

On these basis, it could be proposed that if grape composition in marked by chemical fingerprints from a given terroir, wines made from these grapes should also reflect related fingerprints (Roullier-Gall et al., 2014), since the metabolome (as well as the transcriptome) can be associated to the growing site both in grapes (Anesi et al., 2015) and wines (Fabres et al., 2017; Gambetta et al., 2016, 2017). Moreover, other authors suggest the utilization of epimarks for distinguishing agronomic practices and terroir certification of wines (Crisp et al., 2016; Fortes and Gallusci, 2017), a branch of the discipline which is not yet well explored.

1.4 CLIMATE, SOIL, AND HUMAN PRACTICIES: the terroir factors

The term terroir involves so many factors that all the studies available in the scientific literature are often difficult to assess and compare with each other. In fact, the conditions are so different for each terroir that it is difficult to make a direct comparison (Fernández-Marín et al., 2013; Li et al., 2011). Nevertheless, there is an increasing interest to define and quantify the contribution of individual factors to a specific terroir objectively. Specifically, since grapes provide the basis for many wine aromas, flavors and colors, there is much interest in factors affecting the composition of ripe berries (Anesi et al., 2015; Lund and Bohlmann, 2006). Defining factors that contribute to this typicality is important for preserving the diversity and enhancing the value of wines and other regional agricultural commodities. Among all the factors that contribute to the terroir effect, soil, climate, and farming practices might represent the three most important "macro-factors" defining the signature/fingerprint of a wine-region (Dal Santo et al., 2018).

1.4.1 CLIMATE

Supported by numerous studies, the climate can be considered as the most crucial terroir factor (de Andrés-de Prado et al., 2007). It exerts the strongest effect on berry composition (Robinson et al., 2012) acting on numerous metabolic pathways during berry ripening, thus influencing the commercial value of the grapes (Dal Santo et al., 2013) at harvest. Secondary metabolisms in grapes showed to be highly sensitive to different climates, whether from transcriptomic and metabolomic basis (Dal Santo et al., 2013). For example, recent researches showed that light, water deficit, and a high temperature fluctuation between daytime and night-time led to the up-regulation of genes belonging to the flavonoid metabolism, hence the flavonoid amount (Kennedy et al., 2002; Mateus et al., 2001). Instead, tannins have been shown to be less sensitive to either terroir and variety, but more affected by climate influence in their composition than in their content (Mateus et al., 2001). Moreover, high variations in climatic conditions can stimulate the natural biosynthesis of stilbenes (Fernández-Marín et al.,

2013), as it was shown for the resveratrol content (de Andrés-de Prado et al., 2007; Nikfardjam et al., 2006; Yasui et al., 2002).

Climate involves so many factors which can be summarized in temperature, wind, rainfall, humidity, and radiation. Each of them depends on the vine region, considered as a specific place characterized by a specific latitude, altitude (elevation) and surrounding topography. The mentioned factors together contribute to determine the cluster surrounding, also meant as microclimate (Fabres et al., 2017). Specifically, among the climate elements, temperature has a great impact on grape ripening, with warmer weather conditions promoting faster ripening (Alessandrini et al., 2016; Falcão et al., 2010; Mira de Orduña, 2010). Besides, the accumulation of some grape compounds like anthocyanins showed to be highly dependent on temperature conditions (Downey et al., 2006). For example, high differences of temperature between day and night were correlated to an high anthocyanin content (Mateus et al., 2001; Yamane et al., 2006); instead, it has been also demonstrated that different temperature regimes led to the accumulation of anthocyanins (low temperatures) and the inhibition of both anthocyanins and flavonoids accumulation (high temperatures) (Azuma et al., 2012; Mori et al., 2005). Similarly, some authors found that cold climatic conditions seem to promote stilbene biosynthesis (Abril et al., 2005; Fernández-Marín et al., 2013). Even hydroxycinnamic acids, precursors of many volatile compounds, hence contributing to the aromatic profile of ripe berries, showed to be strongly influenced by the temperature during the growing season (Jackson and Lombard, 1993; Jones and Davis, 2000).

Altitude is another climate parameter deeply studied and is directly associated with temperature (Alessandrini et al., 2016), but also with humidity, sunlight exposure, and other environmental factors that influence grape maturation (Mateus et al., 2002). Recent works showed that altitude was strongly associated with different responses in the ripening process, meant as soluble solid concentration, as well as pH and acidity (Alessandrini et al., 2016), and differences in the volatile content at grape maturity (terpenes and norisoprenoids). Dal Santo et al (2013) also reported the secondary metabolism as strongly influenced by elevation, with genes representing terpenoid, lignin, carotenoid metabolism, and genes encoding GSTs found to be differently modulated. Moreover, it has been shown that elevation can affect the composition of other non-volatile metabolites such as stilbenes (Dal Santo et al., 2016), resulting in their accumulation at higher elevations, although in a cultivar-dependent manner (Bavaresco et al., 2007).

Water status, depending on the amount and frequency of precipitations, is considered an important factor for the resulting quality of the wine. Water stress has been reported to induce the accumulation of many compounds like anthocyanins and aroma precursors (Bonfante et al., 2016). Vine water deficit also affects stilbenes and flavononol content (Castellarin et al., 2007; Deluc et al., 2009, 2011; Hochberg et al., 2015), in some extreme drought cases even reducing the stilbene concentration (de Andrés-de Prado et al., 2007).

Solar irradiance effect on berry metabolites accumulation has been analyzed in stable meteorological conditions by Reshef et al. (2017). They could link differences in sugar, organic acids, amino acids, and phenylpropanoids content to the different distribution of irradiation across the grape cluster. Among the primary and secondary berry metabolites, flavonoids were considered an highly responsive group to light (Chalker-Scott, 1999; Winkel-Shirley, 2002). For example, anthocyanin composition has found to be affected by fruit sunlight in the proportion of acetylated and coumarylated forms, and of the di-hydroxylated and tri-hydroxylated anthocyanin ratio (Chorti et al., 2010; Tarara et al., 2008). Many studies also focused on the induction of flavonols biosynthesis and accumulation as response to increasing solar irradiance levels (Azuma et al., 2012; Matus et al., 2009; Pereira et al., 2006). Reshef et al. (2017) also reported less changes in stilbene content (piceid and delta-viniferin) from berries under different solar irradiance levels.

1.4.2 SOIL

Providing the vine with water and nutrients, soil is considered one of the major components of viticultural terroir (de Andrés-de Prado et al., 2007). In addition, any imbalance in chemical composition leads to affect the vine growth (www.oiv.int), the organoleptic properties of grapes (de Andrés-de Prado et al., 2007; Foroni et al., 2017) and, hence, the resulting wine (Foroni et al., 2017). Unlike other terroir factors, such as climate, soil is characterized by a great spatial variability, therefore it's not uncommon to find several soil types in a single one hectare plot (www.oiv.int). In-field soil variability makes also difficult to relate vine behavior to geology or geomorphology, even though, in some regions, it has been observed a correspondence

between the type of geological outcrop and the typicity of the wine (www.oiv.int). Recently, some authors supported the contribution of the soil, in terms of physicochemical properties, as the reason of berry and wine uniqueness in composition (Cheng et al., 2014; Zerihun et al., 2015), also considering this factor as the key for explaining some differences in wine quality within the same region and climate classification (de Andrés-de Prado et al., 2007). These authors, with regard to sensory analysis, also reported significant differences between the wines produced from two vineyards exposed to the same climatic condition but differing in soil characteristics. Interestingly, such sensory differences were confirmed throughout vintages and between vineyards, but few differences were found between years in the same vineyard, indicating that the wines' sensory attributes remained constant from one year to the following one.

Among all the soil features, soil texture has been proposed to have a major influence on vine development and, consequently, on the wine characteristics (de Andrés-de Prado et al., 2007). Soil depth is another aspect considered to deeply influence the final quality (www.oiv.int). From soil texture and soil depth depend many other important aspects such as nitrogen supply and water supply (Coipel et al., 2006). Soil nitrogen availability is determined by soil mineral composition, pH, and cation exchange capacity which is also influenced by the level of organic matter and the mineralization speed of a certain area; water availability is determined by the soil water holding capacity. The current literature proposes the effect of water and fertile elements as necessary for a good vine development, even though excessive quantities can be detrimental for grape composition, increasing vine vigor and production, promoting rot development, but reducing harvest quality (Boulton et al., 1999). By contrast, more restricted water availability combined with low fertility levels has been shown to benefit grape quality (de Andrés-de Prado et al., 2007), as well as high sugar and high anthocyanin content in the berries have been related to low vine water and low nitrogen status. Along with anthocyanin synthesis, phenolic content has been reported to increase in limited drought conditions (Koundouras et al., 2006; Salón et al., 2005). In line with these results, more fertile soil condition, which was reported to be the one with higher water-holding capacity, produced wines with lower color intensity, lower amount of total phenolic, as well as lower hydroxycinnamic compounds quantity. In a recent work, Anesi et al. (2015) could correlate the berry composition of non-volatile metabolites to some soil properties such as pH, total lime, active lime, clay percentage, organic matter and exchangeable potassium; they also reported

a relationship between the berry composition of volatile metabolites and certain soil properties (active lime and pH). Up to date, few data have been reported about the soil effect on stilbene synthesis in grapes. Sometimes, soil has been proved to be as important as the influence of the climate even when considering the indirect effect on compounds concentration, as demonstrated for trans-resveratrol by de Andrés-de Prado et al. (2007). Moreover, other authors proposed a positive effect of high soil water-holding capacity on stilbene biosynthesis stimulation in grapes (Bavaresco et al., 2009; Koundouras et al., 2006).
Despite its importance, the influence of soil (regarding its texture, depth, chemical composition, fertility, and water availability) on the characteristics of a wine has not been studied so widely (Van Leeuwen et al., 2004; Seguin, 1986), due to the complication in isolating such terroir factor in an open field context (as the vineyard is). Moreover, it might be worthy to investigate the effect on the final product (grapes and wine) as consequence of the interaction between soil and other agro-environmental inputs, as already reported for the interaction with seasonal low and moderate rainfall (de Andrés-de Prado et al., 2007).

1.4.3 AGRONOMICAL PRACTICIES in viticulture

Viticultural practices are another aspect known to affect the composition of the berries (Anesi et al., 2015; Reynolds and Heuvel, 2009). In spite of the growth year exerts a strong effect on berry development, it has been also demonstrated that it can fade during berry ripening, suggesting that the impact of the other external condition (as agronomical practices) on the berry transcriptome becomes more important at this stage (Dal Santo et al., 2013). Some of these practices regard the training system, vine spacing, pruning level, fertilization, irrigation, soil management, intercropping, and canopy management (Fabres et al., 2017). Some of them are considered to influence the vine status in terms of water and mineral supply, some others also contributing to the microclimate changes around the cluster. For example, some authors clearly demonstrated that the accumulation level of primary and secondary metabolites, which are responsible of the berry sensorial profile, vary as a function of the microclimatic conditions in the immediate vicinity of the cluster (Downey et al., 2006; Jackson and Lombard, 1993). As demonstrated for cv. Sauvignon Blanc, even carotenoid metabolism is highly responsive to the microclimate (Young et al., 2016), as well as several heat shock proteins have been reported

as highly responsive to the microclimatic changes around the cluster (Pastore et al., 2013), for instance when resulting from two different training system (Guyot and parral system) (Dal Santo et al., 2013). It has also been reported that viticultural practices can affect the phenolic compounds, color and morphology of grapes during seed and grape development (Roby and Matthews, 2008), as well as the composition of volatile metabolites (Anesi et al., 2015).

All the practices that somehow modify the cluster exposition to the light affect the temperature and the metabolism of the berries. In a recent work, Reshev et al. (2017) compared the berry metabolite composition under different levels of filtered solar irradiance and orientation in the cluster. They found that cluster shading affected the pattern of accumulation of several primary and secondary metabolites in both berry pulp and skin, such as aminoacids, acids, some alchools, and hormones, as well as flavanols, anthocyanins, and flavonols. Another common practice influencing the level of solar irradiance is the leaf removal around the cluster during berry maturation (early or late leaf removal). The practice demonstrated to significantly advance maturation by increasing the soluble solids and anthocyanin concentrations (Talaverano et al., 2016). Other components seemed to be affected by this technique are the total phenolic compounds and tannins, which can reach higher values if more exposed to the light throughout the berry maturation phase. In addition, these higher concentrations were related to a smaller berry size trend in all stages induced by the practice. Studies showing no improving effect on soluble solids of must in some cultivars, suggest the cultivar-depending relationship with the 'leaf removal' practice (Main and Morris, 2004; Zoecklein et al., 1992). Moreover, several authors proposed that those agricultural practices that somehow "stress" the vineyard, cause resveratrol content to increase (Bavaresco, 2003; Dani et al., 2007; Fernández-Marín et al., 2013).

In another study, (Garrido et al., 2016) focused on human practices by comparing the effect of different levels of cluster thinning with different levels of water deficit. Thinning, already known as viticultural practice for improving the quality of the grapes by increasing the total soluble solids, and the anthocyanins and phenolic content, was found to confirm these findings, increasing the berry dry weight, and speeding the ripening progress. Similar results were found as response of water deficit (no irrigation) and furthermore such effect was enhanced when the two practices were combined.

1.4.4 MICROBIOME

In the last decades, the focus has also significantly shifted to the microbiological profiles of different soils and environments. Indeed, many authors have been stressing the importance of the role of the soil's microflora in the terroir expression (Bourguignon, 1995). Bacteria and fungi live in complex co-associations with plants and have important roles in shaping the characteristics of the soil (e.g. by mineralization of organic matter) and in promoting the productivity and health of the plant itself. In fact, the commensal microbial flora that coexists with the plant may be one of the key factors that influence different vine-traits (grape size, shape, color, flavor, yield, and so forth) (Gilbert et al., 2014), playing an important role determining wine quality (Bokulich et al., 2016; Burns et al., 2015). Interest in this type of research has been developed in parallel to technological advances for the taxonomic identification of microorganisms. If in the past the presence of certain microorganisms was only been ascertained thanks to the in-vitro cultivation of specific culture media, today it is possible to return a complete bacterial and fungal profile by metagenomic analysis which determines the types and the relative abundances of the present ecotypes by means of next-generation sequencing of 16S rRNA and internal transcribed spacer (ITS) ribosomal sequence (Bokulich et al., 2014). The technique, as well as reducing the time and resources, allows to identify all those taxa that are not in-vitro cultivable, and therefore not detectable by traditional techniques, on average representing around 90% of the known ecotypes. Indeed, wine grapes harbor on the skin surface a wide range of microbes originating from the surrounding environment, many of which are recognized for their role in grapevine health and wine quality. The correlation between microbial assemblages and specific climatic conditions suggests the existence of nonrandom biogeographic model definable as "microbial terroir" (Bokulich et al., 2014). Grape variety can play a significant role in shaping microbial community patterns across all regions and vintages, and furthermore, climatic factors can also shape the bacterial and fungal communities of wine grapes across multiple growing years (Bokulich et al., 2014). Notably, the biological activity on soils run by bacteria, fungi, and their drivers – such as endemic anecic earthworms – will deeply influence terroirs together with the strains of Saccharomyces circulated by wasps and possibly by other insects too (Foroni et al., 2017). However, information on the impact of soil microbial communities on soil functions, grapevine plants, and wine quality is still lacking (Fabiani et al., 2016). Nevertheless, it has been

demonstrated how grape and wine microbiota present regionally defined patterns associated with vineyard and climatic conditions. Both grape microbiota and wine metabolite profiles distinguish viticultural area designations and individual vineyards. Still Bokulich et al. (2016) also demonstrated that the grape-and-wine microbiota and metabolites are regionally distinct, the must and wine microbiota correlate with the wine metabolome and fermentation performance, and grape must microbial composition predicts the metabolite composition of the finished wine. This suggests that microbial dispersion patterns may really contribute to regional wine characteristics but the degree to which these microbial patterns associate with the chemical composition of wine is unclear (Bokulich et al., 2016). Hence, wine production methods and wine style may ultimately determine the degree to which different microbial activities might contribute to the wine chemical composition. The close correspondence between must microbial composition and putative wine metabolite profiles may indicate that the grape microbiota influences the chemical properties of the finished wine and/or that both are strongly shaped by the same regional factors (Bokulich et al., 2016). Grape and wine microbiota that exhibits regional patterns correlated with grape and wine chemical composition suggests that each terroir can carry a sort of "microbiological signature" whose effects, as a part of the vineyard-specific signature, contribute to define the terroir and hence the characterization of the final product.

1.5 CONCLUDING REMARKS and outline of the thesis

The information reported in this chapter denotes a complex interplay among the agri-environmetal factors defining the quality of berries – and wine – of a certain area. What's more, the interaction between a specific cultivar and its 'terroir' – meant as the contribution of climate, soil, agronomical practices, microbial community, etc. – might represent the key for obtaining a product with unique and unduplicable characteristics. The "power" of terroir is observed by the consistency of its uniqueness despite the deep influence that the vintage conditions exert. In other words, terroir might be conceived as a weak balance – a signature – that somehow remains recognizable over the years, overcoming the deep influence of the vintage. Terroir factors and their interactions represent an open field in science for studying the mechanisms at the basis of this uniqueness. The best and simple approach for dissecting and studying the contribution of each terroir factor on grapes and wine is by isolating one of

the variables, maintaining all the others as constant as possible. A step forward is represented by the integration of the knowledge coming from different disciplines, for instance by the integration of multi-omics data.

In this thesis, in the next chapters, we'll try to study the effect that the soil – one of the main components of terroir – has on the vegetative growth and grape traits of two typical Italian cultivars: Glera and Corvina. Such cultivars are strictly linked to our territory (Veneto Region, Italy), also known for being the basis of important wines as Prosecco and Amarone respectively. Since the 'soil' represents the only one variable in our experiment, we assumed that each difference among plants will be potentially related to differences among soils with respect to their physical, chemical, and microbiological composition.

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http://www.oiv.int/.

Chapter 2

General Materials and Methods

2.1 Materials

2.1.1 Experimental plan

The experiment was conducted during two consecutive growing seasons (2017 and 2018) in a tailored experimental vineyard located at the L. Toniolo experimental farm of the University of Padua (Legnaro, Italy). The vineyard was planted in 2016 using two-years-old grafted vines. The area is characterized by a humid subtropical climate (www.it.climate-data.org) with hot and humid summers and cool to mild winters. Three different soils were collected from three different areas of the Veneto region: one typical for Corvina production (DOCG Valpolicella; Valpolicella area), one typical for Prosecco production (DOCG Prosecco; Valdobbiadene-Conegliano area), whereas the third one representing the typical soil of the L. Toniolo experimental farm in Legnaro (**Fig. 2.1**) which is still within the borders of the DOC Prosecco area.

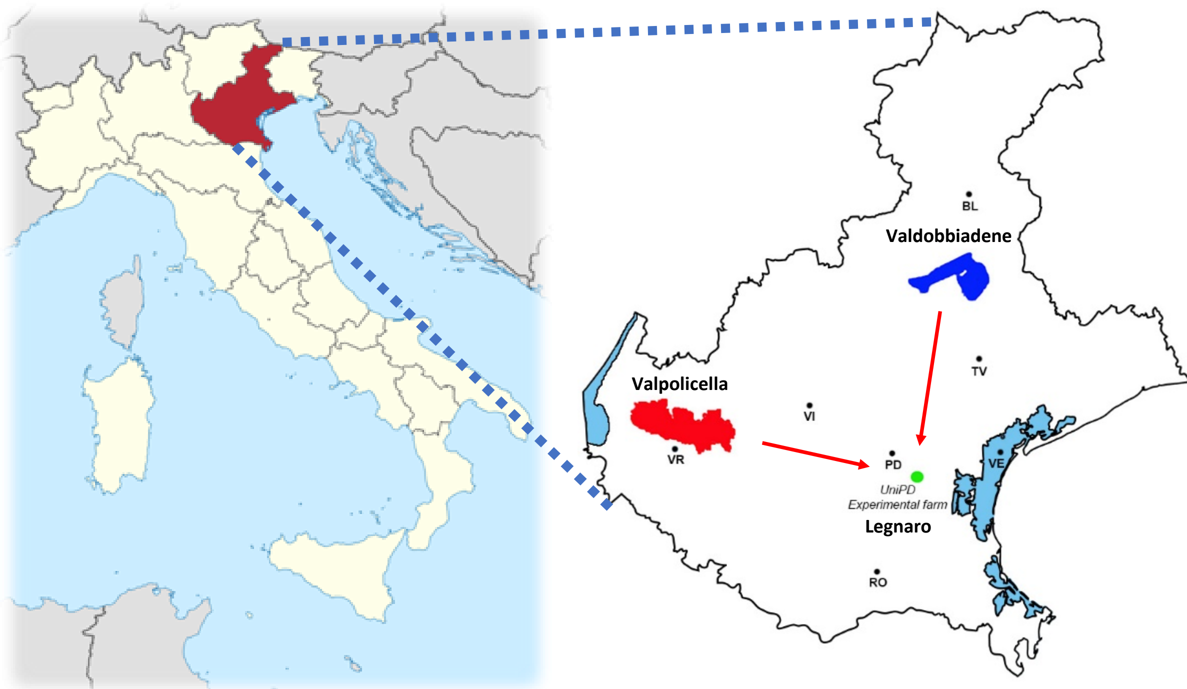

Figure 2.1 – The three areas where the soils where collected from: Valpolicella (red) from the locality of Fumane (F), Valdobbiadene-Conegliano (blue) from the locality of Vittorio Veneto (VV), and Legnaro (green; L) where the experiment is located at the L. Toniolo experimental farm of the University of Padua (PD, Italy).

In this thesis, we named the soil according to the specific localities where they were collected from: “F” stands for Fumane (from Valpolicella area), “VV” stands for Vittorio Veneto (from Valdobbiadene-Conegliano area), and “L” stands for Legnaro. To keep the soils isolated from each other and the surrounding local substrate, they were set into cement boxes (2m x 2m x 1,5m). Two certified grapevine varieties, *Vitis vinifera* cv. ‘Corvina’ and cv. ‘Glera’, grafted onto the same type of rootstock (Kober 5BB), were chosen to grow on the three selected soils, hence exposed to the same climatic and agronomic conditions. The choice of the Kober 5BB is due to the fact this genotype performance is quite stable and uniform upon many different conditions, avoiding to facilitate the growth of plants in a substrate rather than in another one. The plants were obtained from a certified nursery (Vitis Rauscedo, Pordenone, Italy). Each cultivar-soil combination was set in three replicates constituted of three independent boxes, each one containing four plants for a total number of 18 boxes and 72 plants (two cultivars x three soils x three biological replicates), following a randomized disposition (**Fig. 2.2**).

The experimental plan herein described allowed us to minimize the effect linked to the agronomic and climatic variability. We considered that as a good way for isolating and studying in more detail the singular effect that different soils can have on the grape quality and plant growth behavior.

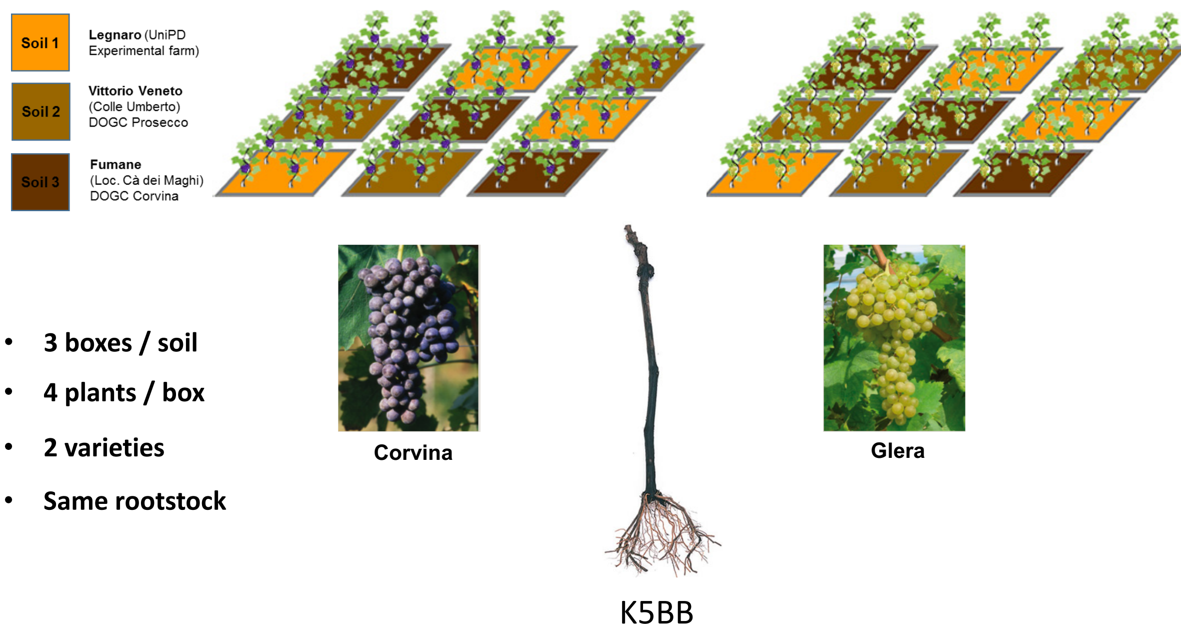

Figure 2.2 – Experimental design: three soil boxes each thesis, four plants each box, two cultivars (Glera and Corvina), same type of rootstock (Kober 5BB).

2.1.2 Soil classification according to ARPAV

2.1.2.1 Fumane soil (*"F"*)

According to the Veneto Land Map (ARPAV, www.arpa.veneto.it), the soil taken from the Valpolicella area, in the locality of Fumane, can be identified by the code 'AR2.2'. It refers to valley and conoid fills, characterized by mixed, fine and gravelly, extremely calcareous deposits, derived from the alteration of rocks of sedimentary origin. They are gently sloping surfaces (1-3% slope), with traces of intertwined channels not very evident. Land use is mainly made up of arable land (corn), vineyards and meadows. The parental material consists of clay and lime mixed with gravel, extremely calcareous. However, they are deep soils, with a moderately fine texture, a common skeleton, an alkaline reaction, and good drainage.

2.1.2.2 Legnaro soil (*"L"*)

The soil taken from the locality of Legnaro, precisely within the boundaries of the experimental farm L. Toniolo of the University of Padua, can be identified by the code BR4.6. The description refers to the modal plain formed by the Brenta river in recent times. They are flat surfaces that develop with an NW-SE course, starting from the meandering plain, up to the lagoon edge, and are connected to the bumps. Land use is mainly made up of arable land (maize). The parental material consists of a good fraction of silt. The soils are deep, of medium texture, alkaline reaction, very calcareous, strongly calcareous in depth, mediocre drainage, deep brim.

2.1.2.3 Vittorio Veneto soil (*"VV"*)

The land taken from the Valdobbiadene-Conegliano area of Vittorio Veneto can be identified by the code AA2.2. It refers to ancient surfaces of the Piave river and the proglacial plain of the amphitheater of Vittorio Veneto (coneides of Nervesa and Vittorio Veneto), with traces of intertwined canals, sub-flat (0.5-1% slope). The starting material is extremely calcareous, sandy and gravelly. The soils are deep, moderately fine texture, coarse in depth, with an abundant skeleton that allows moderately rapid drainage, the reaction is alkaline. Generally, the soil is used for arable crops (corn), vineyards and meadows.

2.1.3 Cultivars

2.1.3.1 Glera

Vitis vinifera cv. Glera is grown mainly in the Veneto region (Italy), traditionally in a hilly area in the north of Treviso, near Conegliano and Valdobbiadene. In such area, the altitude varies between 50 and 500m above sea level. In Italy, total cv. Glera's growth surface consisted of 18,255 ha in 2010 (from ISTAT data). Cv. Glera is a highly productive grape that ripens late in the season. Bunch at harvest is large-sized (20-25 cm long), elongated, pyramidal-shaped, winged (**Fig. 2.3**). The peduncle is long, thin and herbaceous. The berry is medium-sized and spherical-shaped. The skin is waxy, yellow-gold-colored and thin. It has high acidity and a fairly neutral palate, making it ideal for sparkling wine production. Glera's aromatic profile is characterized by white peaches, with an occasional soapy note. The wine is light-bodied and low in alcohol (8.5% is the minimum permitted alcohol level for Prosecco), well suited to be drinking in the summer months or as an aperitif. Italian wine produced from Glera is usually either slightly fizzy (frizzante) or sparkling (spumante). A few still wines are also made from Glera, but on nowhere near the same scale as the sparkling wines that are so exported around the globe. The worldwide popularity of Prosecco has resulted in many imitations of the style. (https://www.wine-searcher.com/grape-392-glera-prosecco)

Figure 2.3 – Typical cv. Glera bunch in post-veraison.

2.1.3.2 Corvina

Corvina (**Fig. 2.4**) is an Italian red wine grape most famous as a key constituent of Valpolicella wines, along with Rondinella. It is well-known as the base grape variety for Amarone and Recioto wines. Its most commonly cited characteristic is its sour cherry flavor, as well as its lack of color and tannin. Wines from this cultivar tend to be bright red and lighter in structure. The variety also lends itself well to the wilting, process used to make the famous Amarone wine. Corvina is widely planted in Italy's northeastern corner, for the production of DOC, DOCG and IGT wines. In blends, cv. Corvina's high level of acidity and distinctive cherried, herbaceous flavors are essential to the character of the wine. The variety ripens very late, which can be an issue for growers, but thick skins means that Corvina lends itself well to air-drying. Grapes are spread on straw mats after picking and can develop phenols and sugars while drying out to concentrate flavors. This is the method employed in the production of Amarone and Recioto of Valpolicella wines and to a lesser extent the region's Ripasso wines.
https://www.wine-searcher.com/grape-117-corvina

Figure 2.4 – Typical cv. Corvina bunch in post-veraison.

2.1.4 Soil sampling

Soils were sampled from each box (18) at the end of March of three consecutive years (2016, 2017, 2018). Specifically, 2016 also corresponded to the planting year of the experiment. The sampling consisted in getting a vertical slice of soil from the surface to 20 cm depth, at the center of each box. In this way, we wanted to get the least contaminated soil samples possible

from the external environment. The material was then used for physicochemical analysis such as texture and mineral composition, and soil microbiological analysis such as soil respiration and metagenomics.

2.1.5 Grapevine tissue

For the biochemical and molecular assays (metabolomic and transcriptomic analyses), berries were collected throughout the ripening phase of 2018 accounting for the grapevine growth stages based on the modified E-L system (Lorenz et al., 1995). The phenological phases taken into consideration were the 'softening' (34 E-L), 'post-véraison' (36 E-L), and 'harvest' (38 E-L). Three berries were collected at the same time of the day (around 11 a.m.) from the central part of a representative cluster of each plant, avoiding those with visible damage and/or signs of pathogen infection. Berries from plants grown on the same soil-box were pooled together to represent a single biological replicate and immediately frozen into liquid nitrogen. In the lab, berry skin was carefully separated from pulp and seeds while kept frozen in dry ice, placed in polypropylene tubes, then stored at -80°C until further processing. For the transcriptomic analysis, we focused on both skin and pulp separately, whereas exclusively on grape skin for the metabolomic analysis.

2.1.6 Meteorological data

Meteorological data were retrieved by the Veneto Regional Agency for Prevention and Protection (ARPAV, http://www.arpa.veneto.it). Temperature measurements were obtained from a recording station located within 500m from the experimental field, 2m from the ground level. Average daily temperatures were used to define average monthly and seasonal temperature trends. Monthly precipitations were also obtained from the same source. **Figure 2.5** depicts the monthly temperature trend of 2017 and 2018, in comparison with the average monthly temperature calculated from the last 25 years (1994-2018). Similarly, **Figure 2.6** shows the monthly precipitation of the same considered periods (2017, 2018, and 1994-2018).

We notice that, during the 2017 vegetative season, climatic condition were rather extreme: the temperature was always above the 20-years average, especially during summer, and the precipitation events were not considered enough for a good vine water supply (206 mm from March to August). Indeed, in order to avoid stress responses, during summer 2017 we decided to occasionally water the vineyard. The climatic conditions during summer 2018 were less

extreme when compared with those of 2017. Specifically, monthly temperatures in 2018 were still above the last 25-years average trend but precipitations were more constant and abundant with respect to 2017 (538.4 mm from March to August), leading us to never water the plants in 2018. Moreover, it's worth noticing that in April 2017 a quite serious freezing event damaged many vineyards across Italy; Legnaro site seemed to not report any damage or stress response (minimum temperature during the two extreme days was recorded at 3.5°C and 2.8°C on 18th and 19th April respectively).

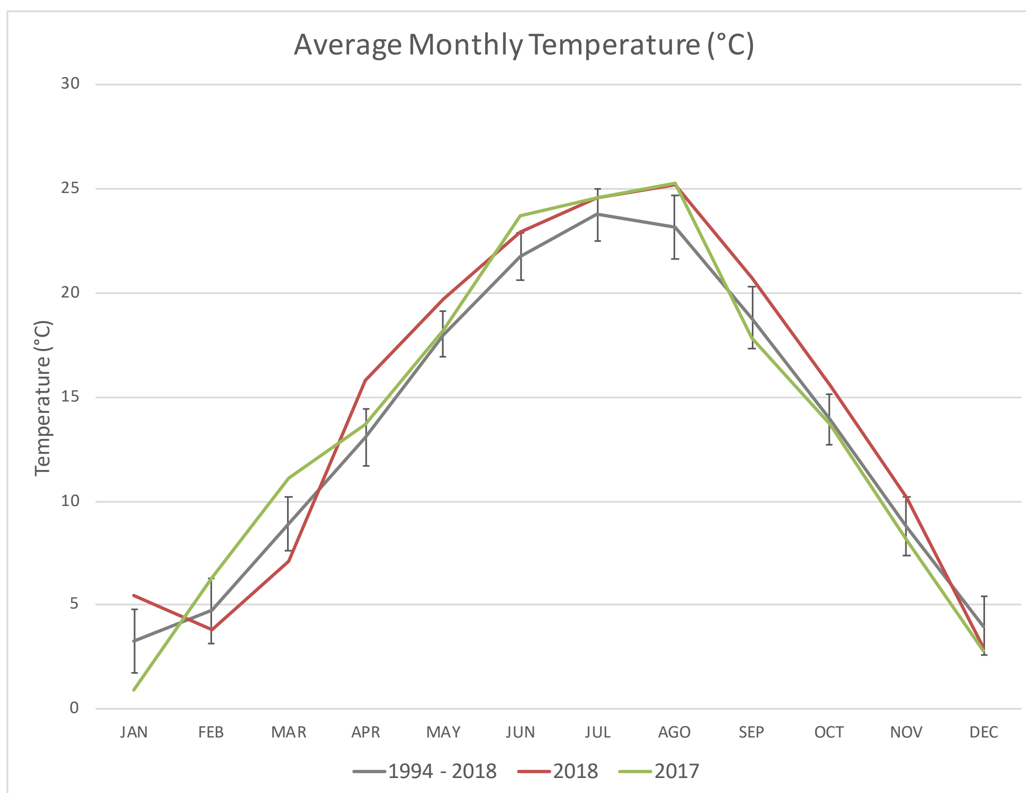

Figure 2.5 – Monthly temperature (°C) related to the 2017 (green line), 2018 (red line), and the average trend of the period between 1994 and 2018 (grey line). Error bars represent the standard deviation (n=25).

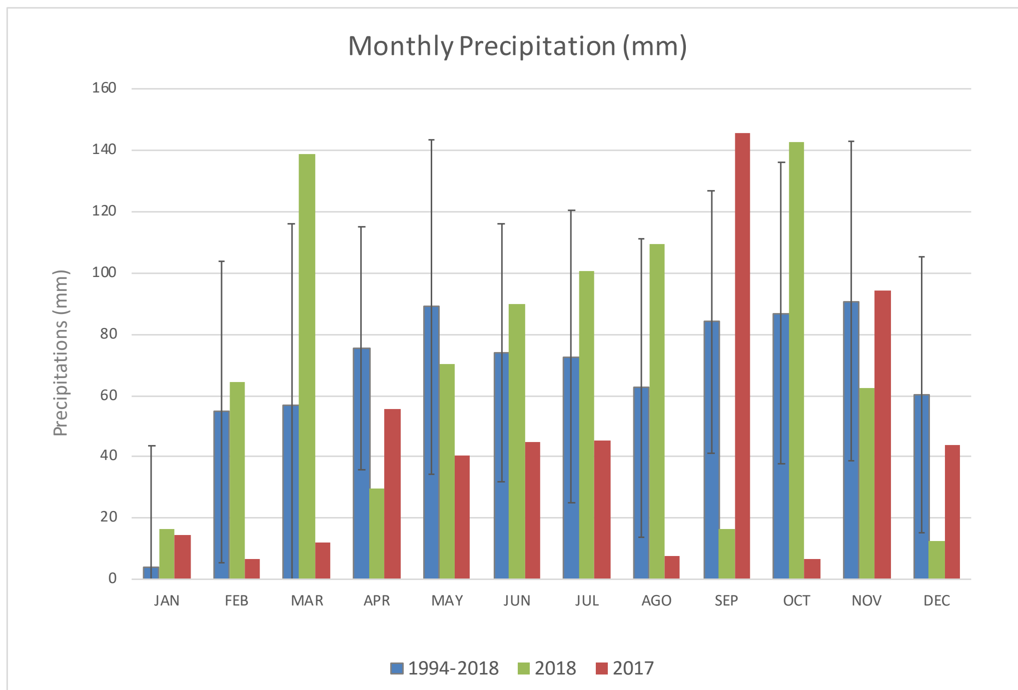

Figure 2.6 – Monthly precipitations (mm) related to 2017 (red bars), 2018 (green bars), and the period between 1994 and 2018 (blue bars). Error bars represent the standard deviation (n=25).

2.1.7 Vineyard management

Vine rows were set in a north-south orientation. Vines were pruned at the beginning of March before the bud breaking phase for maintaining a spurred cordon growing system. In order to limit the influence of both vine growing and any microbiological selection by using chemical herbicides, weeds were periodically and mechanically removed throughout the two seasons (2017 and 2018). No chemical fertilization was provided to not overshadow any possible difference on the plant phenotype due to the differences in the soil chemical composition. To avoid visible plant stress symptoms, which might heavily alter the vegetative growth and berry maturation, watering was provided by a drip irrigation system only when necessary (especially in 2017, see '2.1.6 Meteorological data'). For maintaining an appropriate canopy ventilation and promoting light penetration, shoot trimming (green pruning) were carried out every 20 days from the end of May to September (harvest time) Pesticides were applied according to the current legislation against the *Scaphoideus titanus* (Insecta, Cicadellidae), and against the development of the powdery mildew and the downy mildew.

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https://www.wine-searcher.com/grape-392-glera-prosecco

https://www.wine-searcher.com/grape-117-corvin

Chapter 3

Physico-chemical and microbiological analyses of the selected soils

3.1 Introduction

In agriculture, soil is the primary production factor without which any plant cultivation could not be possible. Each soil has its own characteristics accounting for its mineral elements and organic substance, as well as for physical and microbiological traits.

In grapevine cultivation, soil is one of the most important environmental factors since affects the vine development (Ubalde et al., 2010) and contributes to determine the typicity of the wine (Deloire et al., 2005). Anyway, its effect must be conceived as the result of the interaction with other viticultural factors, especially with the climate (Ubalde et al., 2010). Among its properties, soil depth and texture have been reported to exert the strongest effect, mainly because they have the major control on soil water availability (Seguin, 1986). Soil mineral content, instead, has been reported to not have a direct relationship with the final wine quality (Poni et al., 2018), but when considering the content of nitrogen or some mineral unbalanced conditions (Choné et al., 2001; Van Leeuwen et al., 2004). Moreover, importance is being given to the relationship between grape-wine quality and the soil microbial community. Surely, it is determined by the soil characteristics, the climate, and the surrounding environment, but it can also have a direct effect on the vine development (Bokulich et al., 2016).

To shed light on the soil contribution in the terroir expression, we start from a complete analysis of the soils involved in our experiment, giving information about their physical, chemical, and microbiological composition. Eventually it might be possible to find out which are the differences among soils which determined differences in vine and grape development.

3.2 Materials and Methods

3.2.1 Soil sampling

Soils were sampled from each of the 18 cement boxes at the end of March of three consecutive years (2016, 2017, 2018). Specifically, 2016 corresponded to the planting year of the experiment. The sampling consisted in getting a vertical slice of soil from the 0 to 20 cm depth from five points at the center of each box and bulked to obtain a sample of about 3 kg per box. In this way, we aimed at getting the most representative box-soil condition and restricting as much as possible any contamination from the external environment. After sorting out the litter and large plant root fragments, samples were placed in labeled plastic bags, sealed, and

transported to the laboratory. Samples were air-dried in a controlled room at 20 °C, crushed by a rolling pin to break up clods, passed through a 2-mm sieve, and stored at low humidity. The proportion of soil skeleton was determined by weight after sieving the bulk samples at 2 mm. The material was then used for physicochemical analysis such as soil texture and mineral composition, and soil microbiological analysis such as soil respiration and metagenomics.

3.2.2 Soil chemical and physical analyses

Soil analyses were in accordance with the methods of the Italian Society of Soil Science (SISS - International Union of Soil Science; Violante, 2000). In brief, pH was measured potentiometrically using 1:2.5 soil/water extracts and electrical conductivity in 1:5 soil/water extracts. Calcium carbonate equivalent was determined by the calcimeter method and gravimetric loss of CO_2. Particle size analysis was performed according to the hydrometer method, using sodium hexametaphosphate as a dispersant (Gee and Bauder, 1986). Exchangeable bases, including calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na), were extracted using barium chloride (Sumner and Miller, 1996), and their concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) on a SPECTRO CIROS (Spectro Analytical Instruments, Kleve, Germany). Organic C was determined by dry combustion in a CNS Vario Macro elemental analyzer (Elementar, Hanau, Germany) and correcting for the inorganic C. Plant available phosphorus (P) was calculated using the Olsen method (Olsen and Sommers, 1982). Olsen extractable-P (POls) was obtained by shaking 1.0 g of soil with 20mL of 0.5 mol L−1 sodium bicarbonate solution (pH 8.5) for 30 min. After filtration through Whatman No. 42 filter paper, P in the extracts was determined by ICP-OES. Macroelements (Al, Ca, Fe, K, Mg, Mn, Na, P, S) were extracted with DTPA solution whereas microelements (B, Ba, Cd, Ch, Cu, Mo, Ni, Pb) by digestion with an HNO3 plus HCl solution. After filtration through a 0.45 µm syringe filter, the elements in the extracts were determined by ICP-OES. All the soil analyses were carried out at the DAFNAE laboratories (Legnaro, Italy) of the University of Padua.

3.2.3 Soil respiration estimation

To measure the soil respiration and record its kinetics in time, we followed a method designed by Squartini et al. (2017), which consists of a simple tool set up just using agarose, cotton wool, a plastic test tube, and a pH indicator. The principle is based on the fact that CO_2 produced by

respiration dissolves in the water of the agarose-gel and dissociates into carbonic acid, lowering the pH in proportion to its intensity. In practice, a hot solution of 5 ml of 0.7% agarose in water at pH 7, with 1% Carlo Erba universal pH indicator dye is poured into the 13 ml graduated falcon tube, then a small piece of cotton is put as spacer and holder in order to sustain the un-sieved air-dried soil sample (3 g) and to prevent the contact between soil and agarose solution. After that, the tube is accurately closed and incubated.

The pH variation was daily observed by comparing samples with the reference scale and taking note of the volumes assuming the different pH over time. The low cost and versatility of the principle allow to carry out environmental research monitoring in any country irrespective of laboratory facilities or complex supplies. The result is proportional to soil vitality, fertility and overall attitude to be responding to stimuli or to indicate pollution and overall impacts affecting its properties. On our samples, we applied both a basal and a substrate-induced test. The first one consisted in re-wetting the air-dried soil by adding 100 µl of demineralized water; in the second one a Substrate-Induced Respiration (S.I.R.) test was applied, in which 100 µl of glucose solution (at a concentration of 18 mg/100 µl) were added. For each test, a control tube of gel plus cotton septum but without soil was also present. Each box sample represented a biological replicate, for a total of six replicates for each of the two tests. Despite the original protocol takes into account only the values as liberation of protons (dH^+) reached at the end of the experiment, we also focused on the all values representing the kinetics of the overall period.

3.2.4 Soil microbiota analysis by means of 16S and ITS amplicon sequencing

Soil samples from each box (18) collected at the end of March 2017 were processed for DNA genomic purification. Amplicon library preparation, quality and quantification of pooled libraries and high throughput sequencing by Illumina technology were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy), according to the 16S Metagenomic Sequencing Library Preparation kit (Illumina) and reported in Arrigoni et al. (2018). Preprocessing and data analysis were performed using the MICCA, MICrobial Community Analysis, software (Albanese et al., 2015) v 1.7.

3.2.5 Statistical tools

Data for the statistical analysis were processed by analysis of variance (ANOVA) and differences amongst means were compared using the post-hoc Tukey's test. When ANOVA assumptions were not met, Kruskal-Wallis test was used. p-value <0.05 was considered statistically significant. Multifactorial ANOVA (II-way ANOVA) was performed when including both cultivar and soil as statistical factors to reveal any interaction effect. Principal Component Analysis (PCA) was performed with R software in RStudio, and the results of this analysis are presented also as Biplots. The matrix for the analysis consisted of all 18 box-soil samples (6 replicates each type of soil). Heatmaps based on hierarchical clustering were performed by ClustVis website (https://biit.cs.ut.ee/clustvis/)

3.3 Results

3.3.1 Soil description based on soil-chemical analyses

The soils utilized for the experiment derived from three well-known Italian viticultural areas so they can be assumed to be suitable for vine growing. All the soils presented alkaline pH values around 8.0. The soil skeleton, which can favor water drainage and reduce water-holding capacity, was almost absent in Legnaro soil ("L"; 0.1%), but much more present in Fumane soil ("F"; 21.8%) and Vittorio Veneto soil ("VV"; 40.0%). Other notable differences regarded soil texture, organic carbon content, and potassium level (**Fig 3.1**). The coarse fraction was much higher in VV (54%) and L (46%) in comparison with F (36%) which, instead, presented a considerably high clay level (44%). According to the International Society of Soil Science, Fumane ("F") soil can be identified as a clayey soil, whereas Legnaro ("L") and Vittorio Veneto ("VV") as sandy-silty (**Fig. 3.2**). Organic carbon was three times higher in VV soil (3.33%) which, being closely related to organic matter, might increase soil fertility and consequently improve vine nutrition. Nevertheless, the VV soil presented the lowest potassium level (5.74 g/kg s.s.). Possibly due to its high slate fraction, F was characterized by a high level of cation-exchange capacity (40.9 cmol/kg s.s.), followed by VV and L whose levels were lower (32.7 and 21.9 cmol/kg s.s. respectively). This characteristic might overcome any low-fertility problem due to the important skeleton content. These and other soil details/characteristics are shown in Supplementary materials.

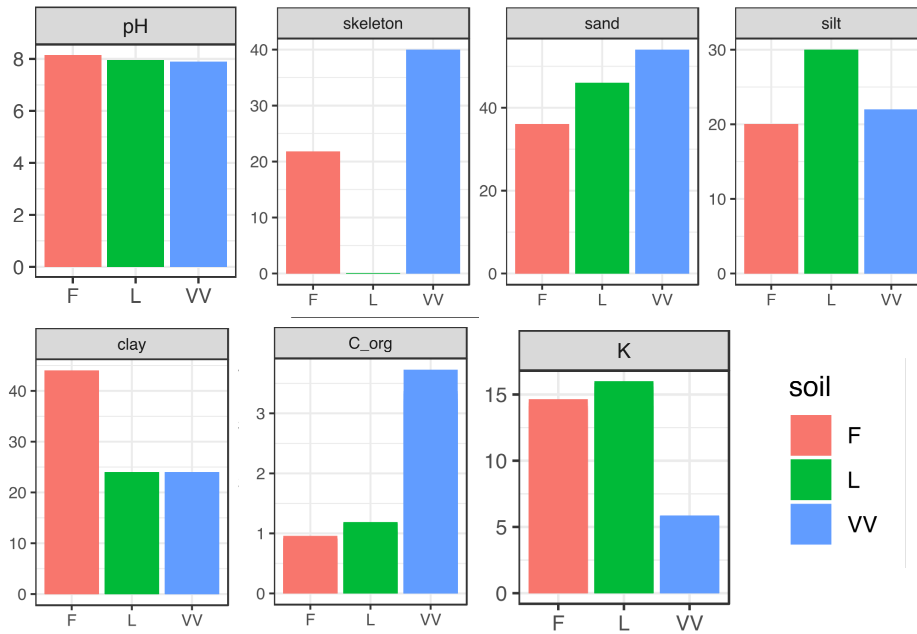


Figure 3.1 – Some of the physico-chemical characteristics of the selected soils (Fumane soil from Valpolicella area, "F";, Vittorio Veneto soil from Valdobbiadene-Conegliano area, "VV"; Legnaro soil, "L"). Skeleton, sand, silt, clay, and organic carbon (C_org) content are reported as percentage (%). Potassium (K) content is reported as g/Kg s.s.

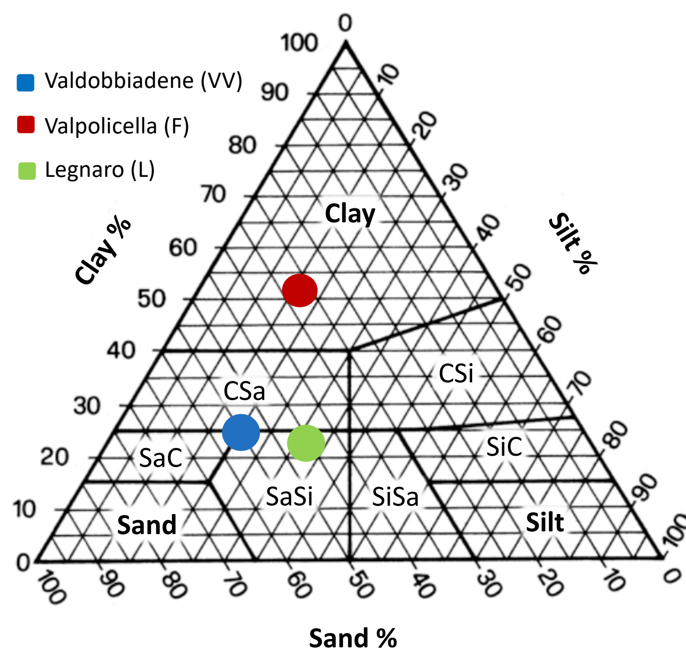


Figure 3.2 – Texture triangle according to the ISSS (International Society of Soil Science). Soils ("VV" from Valdobbiadene-Conegliano area, "F" from Valpolicella area, and "L" from Legnaro) are categorized based on the percentage (%) of sand, silt, and clay.

3.3.2 Principal Component Analysis (PCA) of soil components

When using the physicochemical characteristics for plotting a Principal Component Analysis (PCA), soils clearly clustered according to their origin (F, L, VV) (**Fig 3.3**). PC1 explained 54.5 % of the total variance and well divided VV from F, while L was graphically in between. PC2 explained 36 % of the variance and clearly separated F and VV from L. Moreover, PC3 (not graphically represented) explained just 4 % of the total variance.

Biplot graph (**Fig 3.4**) also revealed the contribution of each soil characteristic to the soil grouping disposition. Specifically, for what concerns PC1, the most discriminant components were represented by sand, Ba, Be, Al, Co, Li, exchangeable Mg and K, Ti, total C, Clay, pH, Ni, Pb, Sr, S, Cd, total N, organic C, Cr, Mo, EC, As. Instead, Mn, C-N ratio, V, silt, Ca, Stones, B, CSC, Zn, Mg, humidity, P, exchangeable Ca, Fe, K, Na, Sn, available P, were the most discriminant elements for PC2.

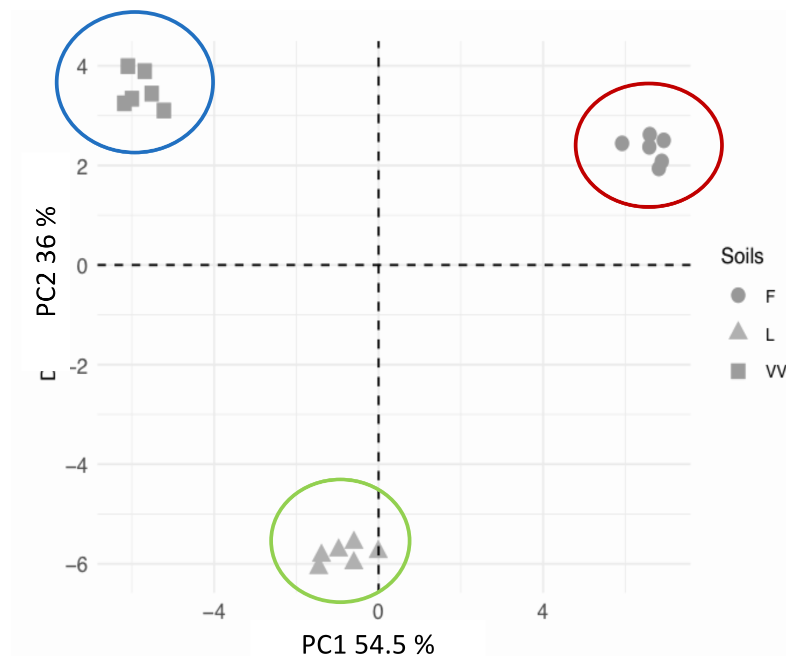


Figure 3.3 – Principal Component Analysis (PCA) based on all the soil physico-chemical characteristics (including microelements, macroelements, and exchangeable cations). Soils are represented as "F" (Fumane), "VV" (Vittorio Veneto), and "L" (Legnaro).

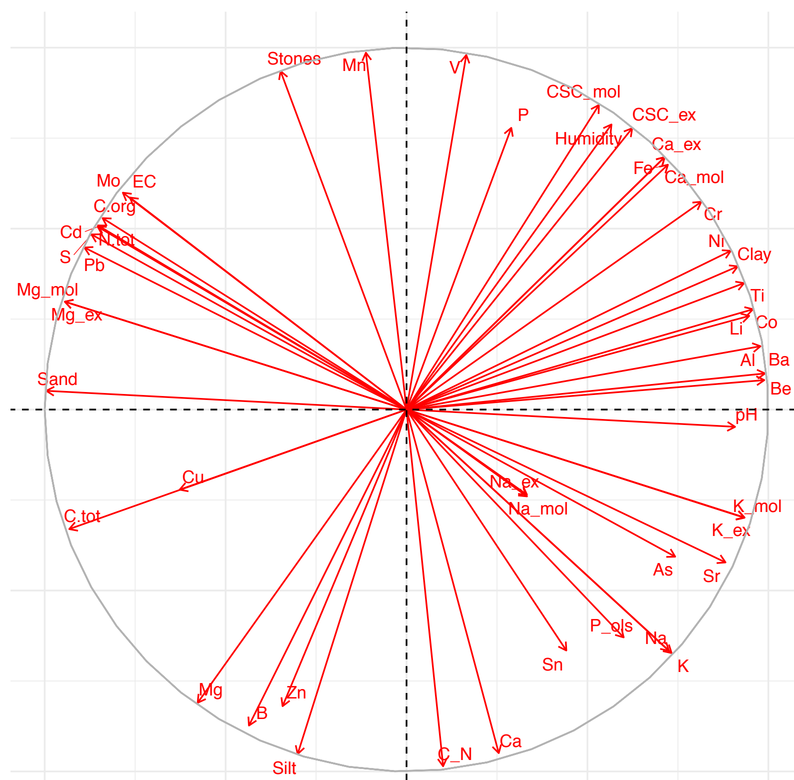


Figure 3.4 – Biplot display of the Principal Component Analysis (PCA) reveals the contribution of each element to the soil grouping.

3.3.3 Soil respiration estimation

Two tests, re-wetted and Substrate-Induced Respiration (S.I.R.), were applied to soils collected in 2016, 2017, and 2018, and the kinetics were daily monitored for at least 20 days (or at least until the values appeared stable for more than three days). Soils under test produced different amount of H+, but the effect was different when comparing the three years (**Fig 3.5**).

In 2016, we noticed many differences among soils throughout the whole period (18 days) in both re-wetted and SIR tests. Since the beginning of the experiment, the three soils produced different amount of H+, evidently higher in L than in F. Interestingly, after 12 and seven days in re-wetted and SIR test respectively, some differences were maintained but only comparing L and VV with F, the latter still having the least values.

Similarly to 2016, the re-wetted test showed the same rank-trend for soils collected in 2017, which means that they statistically differed from each other for most of the period with H+ content in L samples higher than VV, in turn higher than F. Since the second day of the experiment, L could be distinguished from VV and F. Instead, when analyzing H+ production in

soils under SIR test, differences amongst soils were maintained until the 11th day of the experiment, showing F value statistically lower than VV and L ones.

In 2018, re-wetted soils gave statistical differences only during the first eight days of the experiment. Since from the beginning, Tukey's test showed that L and VV soils grouped, differently to F soil which showed the lowest H+ content.

S.I.R. test showed statistical differences during the first twelve days, then any difference among means couldn't be explained by ANOVA. At the beginning, the mean values of L and VV were higher compared to F. Subsequently, the mean values among soils grouped differently, and VV value was always lower than L and F ones.

Soils under re-wetted test showed to be less responsive throughout the years. It's clear how the number of differences in 2018 (6) is lower in comparison with 2017 and 2016 (19 and 17 respectively). Anyway, the rank trend was always the same, with L higher than VV and both higher than F (L > VV > F). Similarly, soils under SIR test showed a reduction in responsiveness when comparing 2019 and 2018 with 2016. In fact, 2016 year showed statistical differences for each day of the experiment considered in the analysis (18 times), whereas 2018 and 2017 only 12 and 9 respectively. Moreover, throughout the years, in the cases differences were statistically confirmed, the soil rank considerably changed from L > VV > F to VV > L > F.

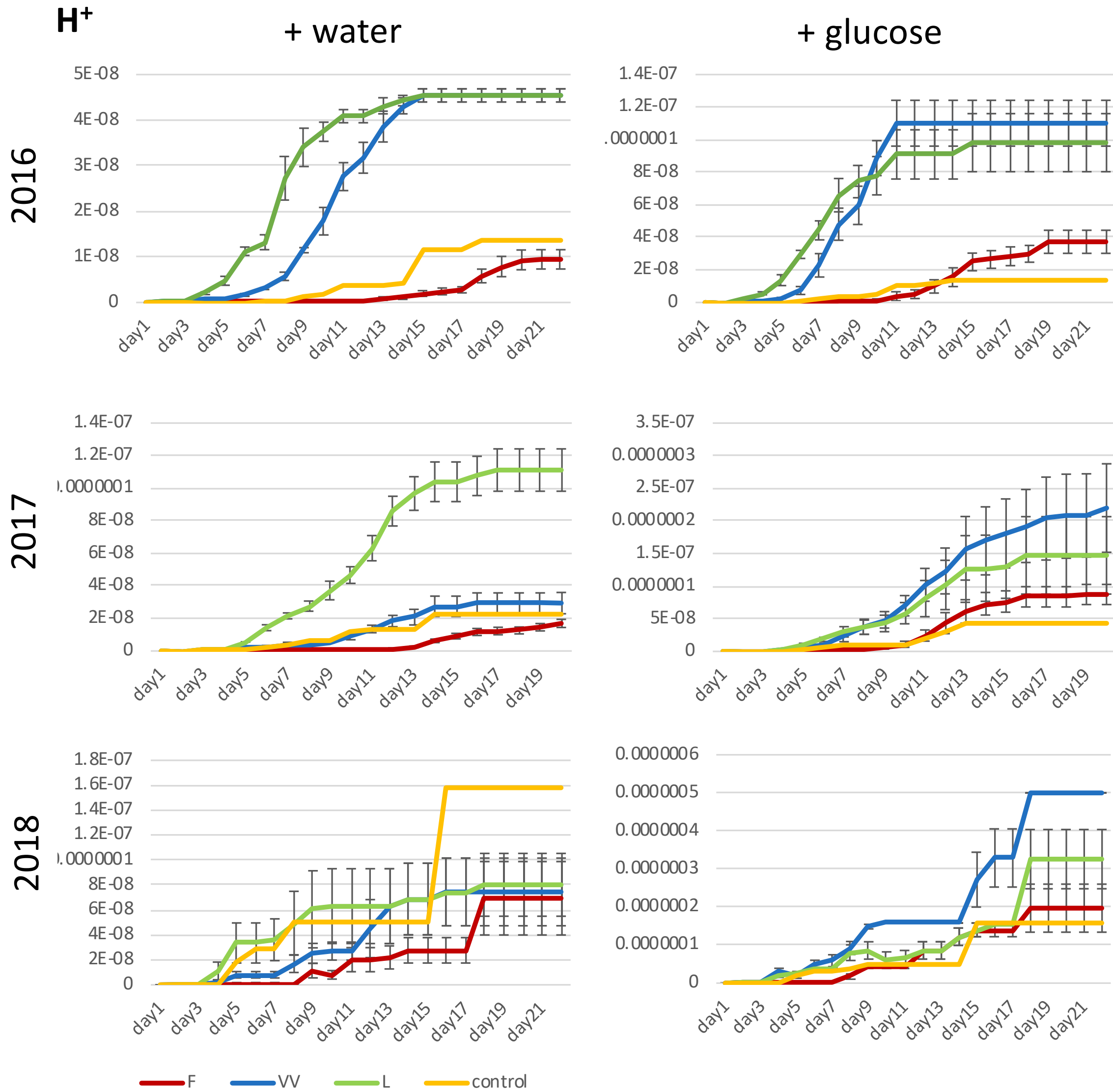
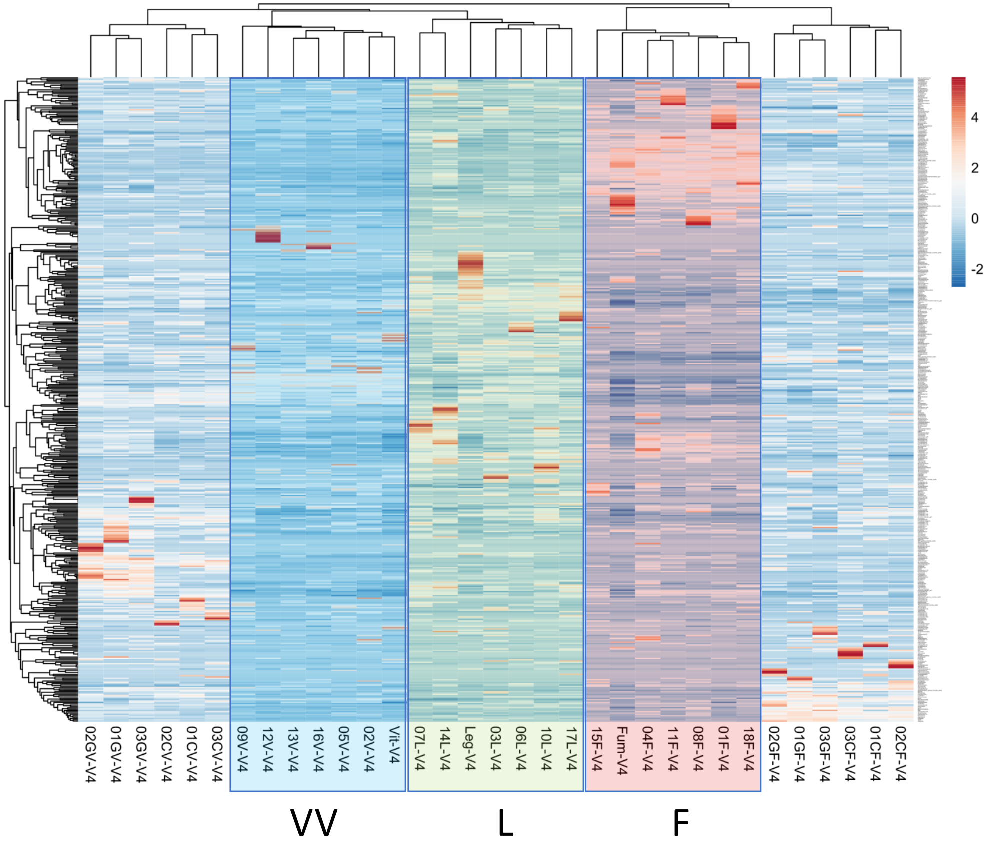


Figure 3.5 – Soil respiration assay. Values represent the H+ accumulation during the experiments in three different years (2016, 2017, 2018) from the first day of the experiment (day1) for two conditions (water-added represented as "+ water" and S.I.R. represented as "+ glucose"). Soils are indicated with the initials "F" (Fumane), "L" (Legnaro), and "VV" (Vittorio Veneto). Values are the means of six biological replicates corresponding to the soil boxes. Error bars are standard errors (n=6).

3.3.4 Analysis of the soil microbiota

The three soils were analyzed in six biological replicates (six boxes each type of soil). Besides the soil-boxes involved in the experiment, where grapevine plants were planted, we also considered soils collected from boxes without plants, considered as controls, and samples of soil from the areas of origin (Fumane and V. Veneto) where the soils were collected from.

Figure 3.6 illustrates the graphical heatmap with hierarchical clustering based on the OTUs identified by NGS. At first sight, the analysis showed a clear grouping based on the biological replicates both for 16S and ITS amplicons. Interestingly, we could appreciate two different behaviors between bacterial and fungal communities. In the first case, the six biological replicates of each type of soil ended up for clustering also with both the respective "virgin soil" (i.e. soils without grapevines growing in them) and the "in-situ" soils which correspond to the same kind of soil but still placed in the zone of origin. In the second case instead, grouping was still observed based on the box-biological replicates but the analysis also showed a separation according to the current soil location. In other words, regardless of the type of soil, all the replicates collected from the experiment in Legnaro were separated from the "in-situ" ones which, instead, clustered together.


16S (bacteria)
02GV-V4
01GV-V4
03GV-V4
02CV-V4
01CV-V4
03CV-V4
09V-V4
12V-V4
13V-V4
16V-V4
05V-V4
02V-V4
Vit-V4
07L-V4
14L-V4
Leg-V4
03L-V4
06L-V4
10L-V4
17L-V4
15F-V4
Fum-V4
04F-V4
11F-V4
08F-V4
01F-V4
18F-V4
02GF-V4
01GF-V4
03GF-V4
03CF-V4
01CF-V4
02CF-V4
VV
L
F
4
2
0
-2

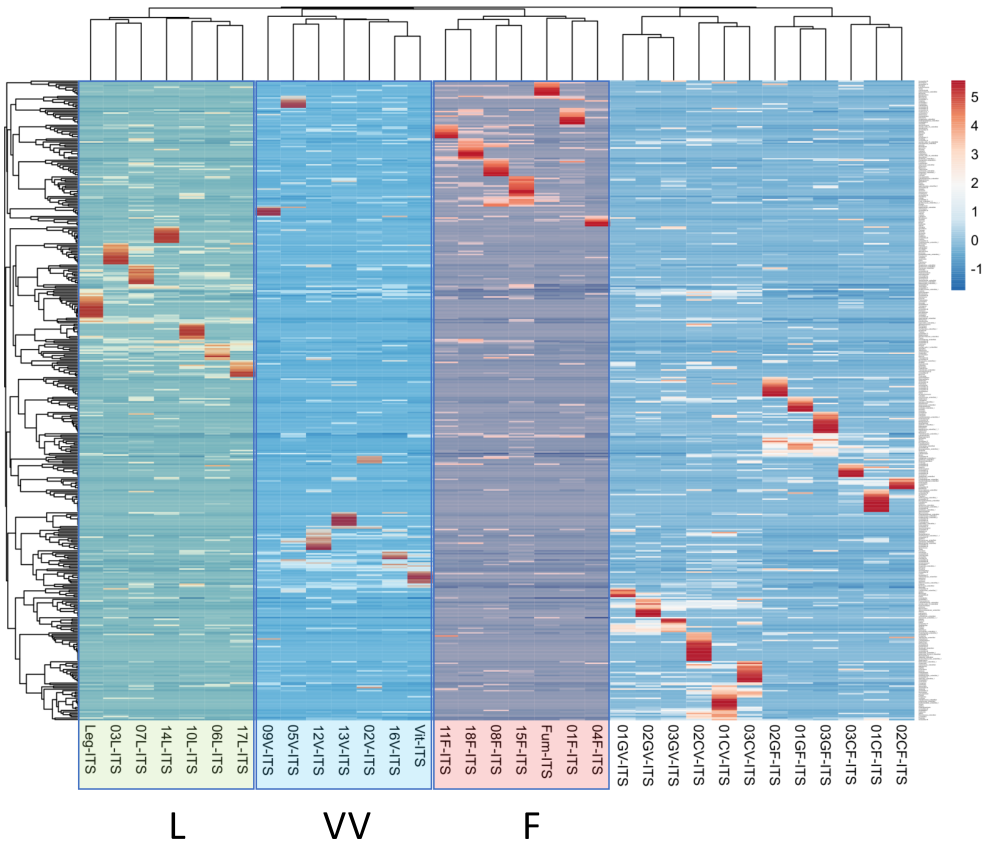

ITS1 (fungi)
Leg-ITS
03L-ITS
07L-ITS
14L-ITS
10L-ITS
06L-ITS
17L-ITS
09V-ITS
05V-ITS
12V-ITS
13V-ITS
02V-ITS
16V-ITS
Vit-ITS
11F-ITS
18F-ITS
08F-ITS
15F-ITS
Fum-ITS
01F-ITS
04F-ITS
01GV-ITS
02GV-ITS
03GV-ITS
02CV-ITS
01CV-ITS
03CV-ITS
02GF-ITS
01GF-ITS
03GF-ITS
03CF-ITS
01CF-ITS
02CF-ITS
L
VV
F
5
4
3
2
1
0
-1

Figure 3.6 – Heatmaps with hierarchical clustering of soil replicates based on OTUs detected by means of 16S and ITS1 amplicon sequences. ID number corresponds to box position at the L. Toniolo experimental farm; letters (F, VV, L) correspond to the type of soil; Vit-V4, Fum-V4, and Leg-V4 correspond to soils in boxes at the L. Toniolo experimental farm without any plant. CF, GF: soils collected in the Fumane area planted with cv. Corvina and cv. Glera respectively. Similarly, CV and GV are soils in Vittorio Veneto area planted with cv. Corvina and cv. Glera respectively.

Soil microbial identification was performed by analyzing the composition of bacterial and fungal communities by means of a meta-barcoding approach. The barcoding regions of ITS1 and ribosomal 16S DNA were amplified for fungal and bacterial identification respectively.
The number of fungal OTUs was consistent among soil samples, accounting for almost 50,000 reads per sample (**Tab 4.1**). Fungal communities of the three selected soils were dominated by Ascomycota phylum, followed by Zygomycota phylum. Other top-50 most abundant OTUs involved Basidiomycota and Chytridiomycota phyla as well. Nectriaceae (Fusarium equiseti) and Pleosporaceae, Davidiellaceae were the most abundant families. Anyway, the number of fungal OTUs differed according to the soil. For example, the Pluteaceae family (Basidiomycota; 77.21% similarity) was greatly present in L soil but almost absent in the other two. Interestingly, zero OTUs were found in the Legnaro virgin sample (soil without grapevine growing in it) as well. OTUs corresponding to Cladosporium cucumerinum (Ascomycota, Davidiellaceae; 100 % similarity) were highly present in F soil but almost absent in L and VV.Another example is represented by an identified OUT of the Mortierellaceae family (Zygomycota) which was most present in VV, followed by L, but pretty absent in F.

Table 4.1 – Top 50 ITS1 OTUs based on the general average amount. Letters indicate statistical significance about their amount among the three soils (Fumane, Legnaro, and Vittorio Veneto) based in ANOVA and Tukey's test. Virgin soil (soils without plants growing in them) values are reported too. OTU's identification is reported in Supplementary materials.

OUTs	average	F	VV	L	F_virgin	VV_virgin	L_virgin
DENOVO1	4288.8	6035.4	3858.2	2972.7	12519.0	2732.0	855.0
DENOVO2	3028.4	4824.7	2818.7	1441.7	3539.0	289.0	1618.0
DENOVO3	2102.0	610 b	4438.4 a	1257.5 b	130.0	2661.0	1976.0
DENOVO5	2023.0	233.5 b	3887.1 a	1948.1 a	21.0	3600.0	1587.0
DENOVO4	1372.3	2144.8	1079.8	892.2	3182.0	50.0	169.0
DENOVO33	1153.6	0.2 b	0.7 b	3459.7 a	0.0	0.0	0.0
DENOVO6	934.8	1367.0	321.4	1116.0	1014.0	91.0	852.0

DENOVO9	884.2	1643.5	460.4	548.5	474.0	149.0	101.0
DENOVO12	804.0	756.5	1475.5	179.8	878.0	140.0	31.0
DENOVO8	768.0	466.4	1134.0	703.7	73.0	3160.0	324.0
DENOVO17	742.3	44.7 c	1580.1 a	602.1 b	0.0	1685.0	572.0
DENOVO11	730.2	2125.5	31.8	33.1	583.0	0.0	8.0
DENOVO7	724.4	186.4 b	1514.8 a	471.8 a	108.0	3528.0	693.0
DENOVO10	689.5	473.5	920.4	674.5	93.0	809.0	378.0
DENOVO22	670.0	1840.4 a	147.4 a	22.2 b	5215.0	0.0	0.0
DENOVO15	662.3	1048.7 a	761.5 a	176.5 b	1.0	365.0	0.0
DENOVO25	655.3	489.4 a	1454 a	22.4 b	0.0	0.0	0.0
DENOVO20	540.8	1179.4	234.2	208.5	2.0	0.0	0.0
DENOVO18	533.0	7 b	0.1 b	1591.8 a	0.0	0.0	562.0
DENOVO19	494.7	326.8	551.0	606.1	0.0	0.0	37.0
DENOVO27	489.4	1186.1	248.0	34.0	0.0	0.0	0.0
DENOVO23	482.3	40.1 b	1199.8 a	207 a	0.0	800.0	263.0
DENOVO13	477.2	57.5 b	39.7 b	1334.4 a	0.0	0.0	1773.0
DENOVO36	468.2	86.1 b	826.8 a	491.7 a	1.0	184.0	641.0
DENOVO56	460.4	84.4 b	55.4 b	1241.4 a	0.0	0.0	380.0
DENOVO70	427.7	38 b	12.8 b	1232.2 a	0.0	0.0	0.0
DENOVO41	405.1	308.8	710.7	195.7	0.0	53.0	153.0
DENOVO40	404.3	38 b	181.5 a	993.4 a	0.0	281.0	3889.0
DENOVO30	392.3	1152 a	13 b	11.8 b	0.0	0.0	0.0
DENOVO29	376.0	585.4	379.5	163.0	335.0	1.0	1.0

The number of bacterial OTUs was consistent among soil samples, accounting for almost 42,000 reads per sample (**Tab 4.2**). The composition of bacterial communities was different among the three soils. It was dominated by Archaea, Cyanobacteria, Acidobacteria, Actinobacteria, and Protobacteria. Even the number of bacterial OTUs differed depending on the type of soil. For example, the Archaea identified as one of the most abundant OTUs was much more present in VV and L soils as compared to F one. Some OTUs corresponding to Proteobacteria were typical of F soil, which is known to include many denitrifying bacteria. Interestingly, the high clay content of F soil promotes an asphyxial environment which favors denitrification phenomena. Moreover, two specific OTUs corresponding to an Acidobacteria and a Cyanobacteria (Streptophyta) were found much more present in L soil as compared to the other two.

Table 4.2 – Top 50 16S OTUs based on the general average amount. Letters indicate statistical significance about their amount among the three soils (Fumane, Legnaro, and Vittorio Veneto) based in ANOVA and Tukey's test. Virgin soil (soils without plants growing in them) values are reported too. OTU's identification is reported in Supplementary materials.

OUTs ID	average	F	VV	L	F_virgin	VV_virgin	L_virgin
DENOVO1	2451.2	381.5 b	4152 a	2820.2 a	385	6075	2818
DENOVO2	1784.6	4138.7 a	256.4 b	958.8 b	9016	7	3

DENOVO4	515.7	516.5	434.2	596.4	289	534	630
DENOVO3	491.8	1230.8 a	72.8 c	171.8 b	740	85	80
DENOVO8	411.5	58.2 b	751.8 a	424.4 a	31	919	356
DENOVO13	406.7	96.4 b	1037.8 a	85.8 b	23	1089	67
DENOVO15	346.9	80.8 b	380 a	580 a	19	479	357
DENOVO51	334.2	115.2 c	283.1 b	604.2 a	17	307	627
DENOVO6	326.1	743.8 a	87.7 b	147 b	632	60	79
DENOVO5	315.3	848.7 a	54.7 b	42.5 b	278	15	18
DENOVO9	306.7	72.1 c	582.5 a	265.5 b	23	617	235
DENOVO30	302.3	688.4 a	100.1 b	118.5 b	802	56	97
DENOVO17	300.3	606.8 a	108.8 b	185.4 b	400	78	85
DENOVO7	300.1	275.4 ab	455.2 a	169.7 b	285	457	168
DENOVO105	261.7	432.8 a	83.4 b	269 a	312	65	179
DENOVO21	247.1	539.7 a	76.4 c	125.4 b	259	52	44
DENOVO9913	240.4	77.8 c	466.2 a	177.1 b	7	447	192
DENOVO23	231.9	70.5 c	436.8 a	188.4 b	22	406	89
DENOVO28	223.1	231.4 a	153.5 b	284 a	32	42	99
DENOVO10	189.8	263.4 a	112.8 b	193.1 ab	99	66	116
DENOVO16	184.5	22.5 c	456.5 a	74.5 b	12	619	75
DENOVO14	178.1	90.5 b	310.4 a	133.4 b	168	527	192
DENOVO11	173.9	29 b	471 a	21.8 b	8	424	7
DENOVO102	160.6	66.8 b	234.7 a	180.4 a	48	287	193
DENOVO34	157.9	69 b	213.5 a	191.1 a	18	195	72
DENOVO46	146.3	60.4 c	128.4 b	250.1 a	4	88	126
DENOVO191	146.2	238.5 a	47.2 b	152.8 a	253	16	115
DENOVO42	139.9	169.8 a	63.1 b	186.7 a	87	55	138
DENOVO27	139.9	66 b	216.7 a	137 a	105	462	195
DENOVO110	138.5	192.1	88.4	135	101	7	19

3.4 Discussion

Both chemical and microbiological analysis of the soils considered in the experiment were conducted to give a first explanation about the soils predisposition to vine growth and, especially, to highlight their diversity since it is considered the key point of our project. First of all, the soils utilized for the experiment derived from well-known Italian viticultural areas (Valpolicella and Conegliano-Valdobbiadene areas) so they can be assumed to be suitable for vine growing. Comparing the soil chemical analysis with the local vine guidelines (“L’interpretazione delle analisi del terreno”, ARPAV, 1990), the soils were found to not express any extreme or detrimental chemical condition.

From an agronomical point of view, the mineral availability of the three selected soils could be conceived as comparable since the pH levels were similar (8.1, 7.9, 7.8 for F, L, VV respectively; ARPAV, 1990). Besides, pH is also determinant for the microbiological activity and plant adaptability. Based on the results, we’ve been working with alkaline soils (pH > 7.5) which, in comparison with acid soils, are characterized by a lower availability of microelements, a

retrogradation of phosphorus with formation of insoluble phosphates, an increase in the amount of calcium as to induce antagonisms with magnesium (Mg) and potassium (K), and an increase in the availability of molybdenum (Mo). Anyway, the choice of the type of rootstock (Kober 5BB) seems good due to the fact that this genotype is known to facilitate the mineral absorption even in presence of alkaline soil and active limestone (http://vivairauscedo.com/en/portinnesti). Soil texture differences worth to be studied since the texture is considered one of the most important soil feature affecting grapevine growth (de Andrés-de Prado et al., 2007). The most evident difference is represented by the clay content which is 44 % in F soil, whereas 24 % in both L and VV. In line with the literature, clayey soils are also characterized by high CEC and hence strong retention power of exchangeable elements. Moreover, it has a high water retention capacity, expressed as humidity in our analysis, potentially causing a delaying of soil heating up in spring, hence delaying the start of microbiological processes and the absorption of nitrogen too. Clayey soils are also known to be predisposed to radical asphyxia, affecting vine growth and deeply shaping the soil microbiological composition. Instead, L and VV soil textures were much closer to the loam typology. Although the latter is considered the best condition for crop development, many authors proposed that good grape quality for wine production comes from not optimal environmental conditions, even whether determined by the soil factor (Koundouras et al., 2006; Roby et al., 2004; Salón et al., 2005). Another remarkable difference we want to highlight concerned the skeleton content which is quite present in F (21.8%) and VV (40.0%) but almost absent in L soil (0.1%). It generally favors water drainage and reduces the total water-holding capacity (in our case meant as water absolute content). Anyway, water drainage in L and F soils is guaranteed by their sand content (40%) and skeleton content (21.8%) respectively. VV soil seems to present the most adverse conditions for the plant nutrition but contains an high organic matter level (3%) as compared to the others (1%). Indeed, organic matter favors soil water and chemical compounds holding. According to the analysis, N and available P were good enough for plant nurture in all the soil analyzed. The overall differences among soils were then confirmed by both Principal Component Analysis based on all the physicochemical elements detected and microbiological analysis. In both cases, the clustering observed based on the biological replicates supported the robustness of our experimental plan.

As already mentioned, in parallel to physico-chemical analysis, microbiological assays were performed. Among them, the measurement of soil respiration is considered tightly linked to

the assessment of soil microbial activity and biomass (Squartini et al., 2017). The analysis of variance on the respiration kinetics suggested us that the three soil microbial communities are potentially different from each other, even though such differences were more pronounced in the analysis of the soil collected at the first year of the trial (2016). The 2018 essay, which showed the least differences, might represent the evolution of the microbial community towards fewer differences as a result of an environmental/climate influence as well as slight but continuous soil contamination due to the routine farming practices over the years. The statistical analysis also revealed the absence of any interaction soil-cultivar effect on the microbial respiration kinetics. It might mean that either the cultivars scarcely influence the soil microbial composition or the time required for supporting such hypothesis is longer than three years. We even didn't focus the sampling on the rhizosphere, which might represent the best point where to highlight any difference. What worth noting is that in all the year considered, H^+ production from F soil looked to be lower even than the blank control (sample treated without soil). It means that either, somehow, the H^+ production in the control was higher or the CO_2 liberation from F soil might have been associated to the production of NH_3, which in a water matrix becomes NH_4^+,consequently increasing the pH value. Indeed, NH_3 liberation is typical of clayey soils, as is F, which favor anaerobic conditions (asphyxia) and hence the proliferation of denitrifying bacteria.

To evaluate the robustness of the soil biological replicates in different boxes, to check that the microbiological profile of the soils was similar to that of the areas they were collected from, and to investigate about the differences amongst the different substrates, we also conducted an amplicon sequencing analysis of 16S and ITS1 DNA regions. This metabarcoding analysis allowed us to identify the whole soil microbiota, both in term of bacterial (16S amplification) and fungal (ITS1 amplification) populations. The analysis was conducted in collaboration with FEM (San Michele all'Adige, TN, Italy) and was performed by Next Generation Sequencing (NGS) platforms (Illumina Myseq, 2 x 300bp). The heatmaps clearly showed that all the replicates collected from the cement boxes clustered together, representing good replicates and indicating that the experimental plan is robust. The fact that both F and VV soil replicates in boxes were closely related to the soils collected from the areas of origin indicate that, although the rearrangements they experimented during transportation, soils mostly maintained their bacteriological profile and our boxes are representative of the microbiota profile of the place of origin. Anyway, this phenomenon has been shown only for the bacteria

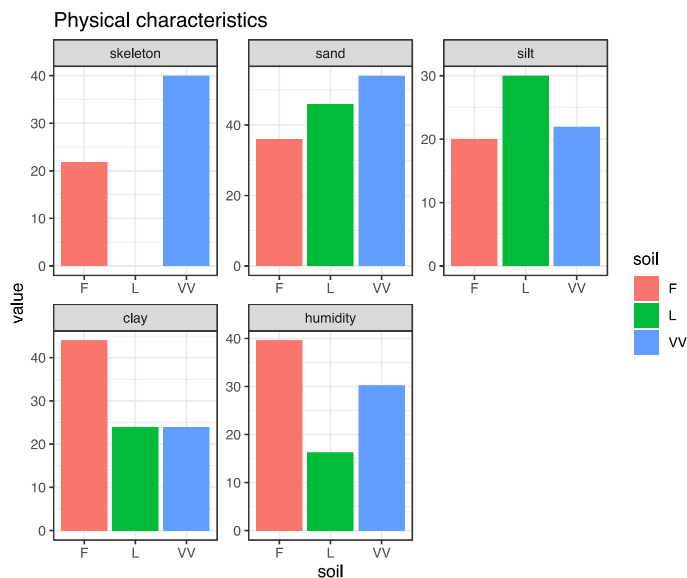
community. We can deduce that, in comparison with fungi, bacteria either are slower and the environmental contamination takes more time or their community development deeply depends on the soil features (mainly texture, organic carbon and the mineral composition). It follows that fungi spread more rapidly in the environment and their community is more susceptible to environmental perturbations as compared to bacteria.

Moreover, several and complex bacterial and fungal communities were identified in the three soils when using a meta-barcoding approach. On the one hand, differences in microbial composition contributed to differentiate the three selected soils. On the other hand, such differences might have a direct and/or indirect effect on the vine development, eventually in terms of grape and wine quality (Bokulich et al., 2016).

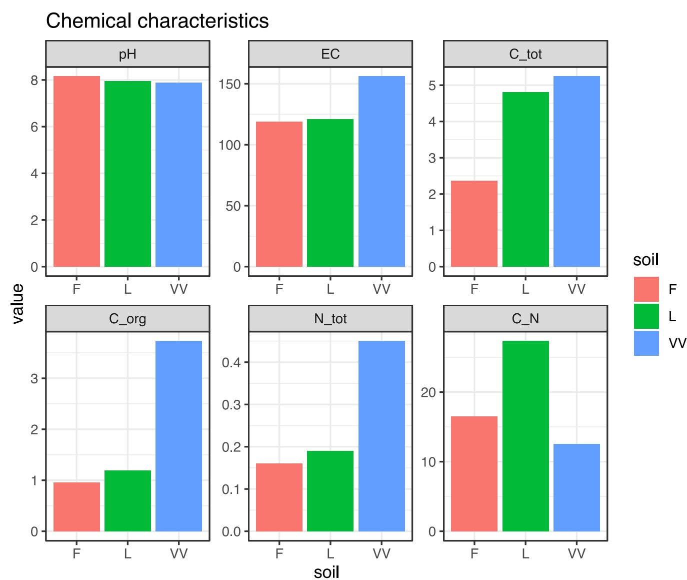
In conclusion, even though the analyses revealed different characteristics amongst the three soils, they can all be still conceived as potentially fertile and suitable for studying the effect of the grapevine growth and grape quality.

3.5 Supplementary materials

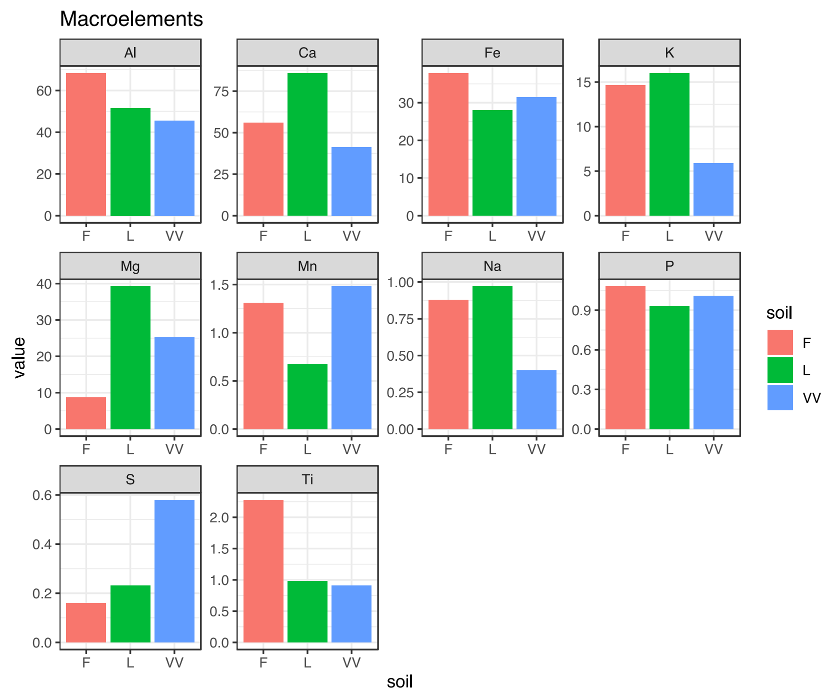
Supplementary Figure 1: physical soil constituents. Skeleton, sand, silt, and clay values are reported as percentage (%). Humidity is reported as g/kg.



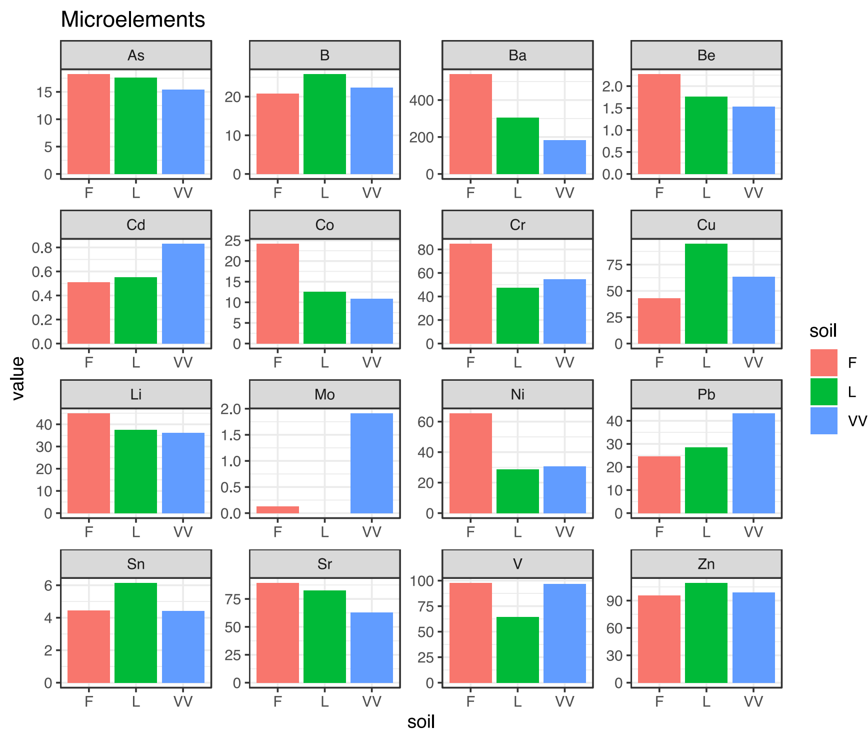
Supplementary Figure 2: some chemical soil characteristics: pH, electrical conductivity (EC; µS/cm), total carbon (C_tot; % s.s.), organic carbon (C_org; % s.s.), total nitrogen (N_tot; % s.s.), carbon-nitrogen ratio (C_N).



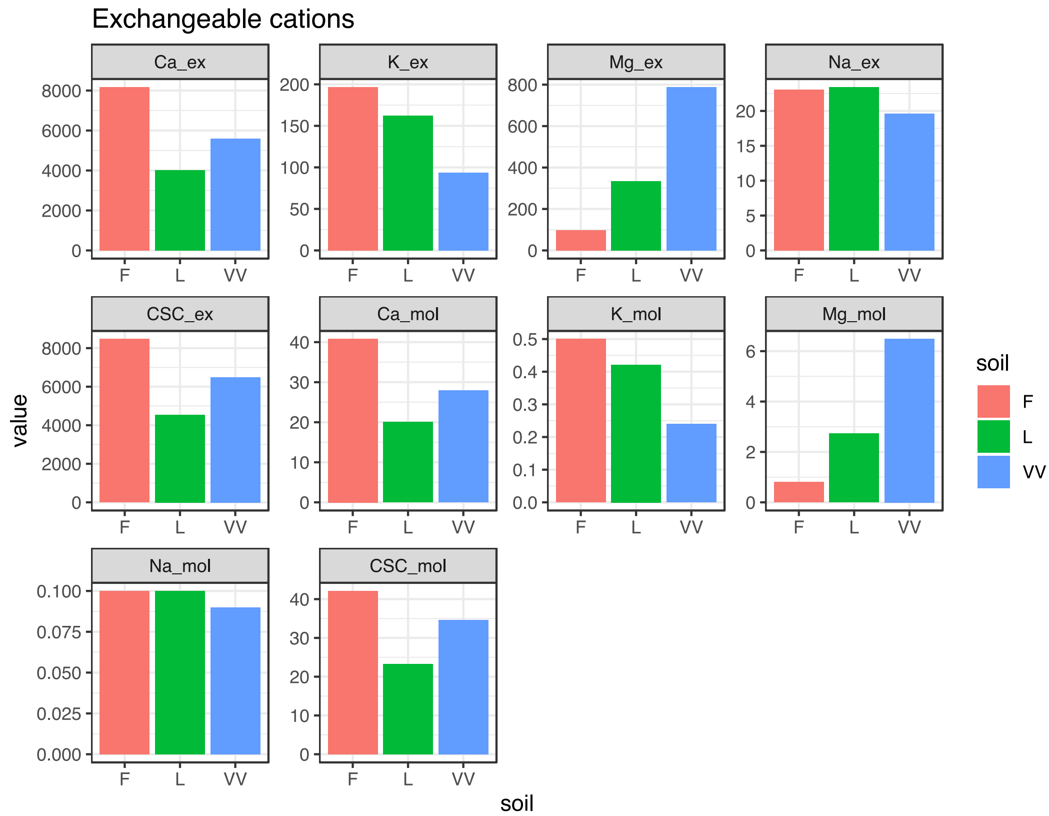
Supplementary Figure 3: Macroelements (g/kg s.s.): aluminum (Al), Calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorous (P), sulfur (S), and titanium (Ti).



Supplementary Figure 4: Microelements (mg/kg s.s.): arsenic (As), boron (B), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lithium (Li), molybdenum (Mo), nickel (Ni), lead (Pb), tin (Sn), strontium (Sr), vanadium (V), and zinc (Zn).



Supplementary Figure 5: Exchangeable cations (mg/kg s.s.): exchangeable calcium (Ca_ex), .potassium (K_ex), magnesium (Mg_ex), sodium (Na_ex), and cation exchangeable capacity (CSC_ex). Values are reported also as cmol/kg s.s. (Ca_mol, K_mol, Mg_mol, Na_mol, CSC_mol).



Supplementary Table 1: Top 50 ITS OTUs identified based on the total average amount and their taxonomic identification.

OTU ID	taxonomy
DENOVO1	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Nectriaceae,Fusarium,equiseti
DENOVO2	Fungi;Ascomycota,Dothideomycetes,Pleosporales,Pleosporaceae,Alternaria,alternata
DENOVO3	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Nectriaceae,Fusarium,oxysporum f. sp. vasinfectum
DENOVO5	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Nectriaceae,Fusarium,sp. r316
DENOVO4	Fungi;Ascomycota,Dothideomycetes,Capnodiales,Davidiellaceae,Cladosporium,oxysporum
DENOVO33	Fungi;Basidiomycota,Agaricomycetes,Agaricales,Pluteaceae,Volvariella,hypopithys
DENOVO6	Fungi;Ascomycota,Dothideomycetes,Pleosporales,,Phoma,sp.
DENOVO9	Fungi;Ascomycota,Dothideomycetes,Pleosporales,Pleosporales_incertae_sedis,Epicoccum,nigrum
DENOVO12	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Bionectriaceae,Myrothecium,sp. 14016
DENOVO8	Fungi;Zygomycota,Zygomycetes,Mortierellales,Mortierellaceae,Mortierella,alpina
DENOVO17	Fungi;Zygomycota,Zygomycetes,Mortierellales,Mortierellaceae,Mortierella,sp. CBS 118520
DENOVO11	Fungi;Ascomycota,Dothideomycetes,Capnodiales,Davidiellaceae,Cladosporium,cucumerinum
DENOVO7	Fungi;Zygomycota,Zygomycetes,Mortierellales,Mortierellaceae,Mortierella,alpina
DENOVO10	Fungi;Ascomycota,Ascomycota,Ascomycota,Ascomycota,Plectosphaerella,cucumerina
DENOVO22	Fungi;Chytridiomycota,Chytridiomycetes,Rhizophlyctidales,,Rhizophlyctis,rosea
DENOVO15	Fungi;Ascomycota,Dothideomycetes,Pleosporales,Pleosporaceae,Curvularia,inaequalis
DENOVO25	Fungi;Chytridiomycota,Chytridiomycetes,Rhizophlyctidales,,Rhizophlyctis,rosea
DENOVO20	Fungi;Ascomycota,Leotiomycetes,Helotiales,Sclerotiniaceae,Botryotinia,fuckeliana
DENOVO18	Fungi;Ascomycota,Ascomycota,Ascomycota,Ascomycota,Phialophora,cyclaminis
DENOVO19	Fungi;Ascomycota,Leotiomycetes,Helotiales,,Tetracladium,sp.
DENOVO27	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Bionectriaceae,Hydropisphaera,erubescens
DENOVO23	Fungi;Zygomycota,Zygomycetes,Mortierellales,Mortierellaceae,Mortierella,sp.
DENOVO13	Fungi;Ascomycota,Sordariomycetes,Sordariales,Chaetomiaceae,,
DENOVO36	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Nectriaceae,Cylindrocarpon,sp.
DENOVO56	Fungi;Ascomycota,Sordariomycetes,Hypocreales,Bionectriaaceae,Emericellopsis,sp. s012
DENOVO70	Fungi;Ascomycota,Dothideomycetes,Pleosporales,,,
DENOVO41	Fungi;Ascomycota,Ascomycota,Ascomycota,Ascomycota,Verticillium,
DENOVO40	Fungi;Ascomycota,Sordariomycetes,Microascales,Microascaceae,Lophotrichus,sp.
DENOVO30	Fungi;Ascomycota,Sordariomycetes,Xylariales,Amphisphaeriaceae,Bartalinia,robillardoides
DENOVO29	Fungi;Ascomycota,Dothideomycetes,Pleosporales,,Phoma,multirostrata
DENOVO49	Fungi;Basidiomycota;Agaricomycetes;Agaricales;Agaricaceae
DENOVO34	Fungi;Ascomycota;Dothideomycetes;Pleosporales;Sporormiaceae;Preussia;Preussia_sp
DENOVO37	Fungi;Ascomycota;Ascomycota;Ascomycota;Ascomycota;Ascomycota;Ascomycota
DENOVO50	Fungi;Fungi_unidentified
DENOVO21	Fungi;Ascomycota;Dothideomycetes;Pleosporales;Pleosporales;Pleosporales_sp
DENOVO51	Fungi;Basidiomycota;Agaricomycetes
DENOVO31	Fungi;Ascomycota;Sordariomycetes;Sordariales
DENOVO32	Fungi;Basidiomycota;Agaricomycetes;Cantharellales;Ceratobasidiaceae;Ceratobasidiaceae
DENOVO26	Fungi;Ascomycota;Sordariomycetes;Sordariomycetes;Sordariomycetes;Sordariomycetes_sp
DENOVO43	Fungi;Ascomycota;Sordariomycetes;Sordariales;Chaetomiaceae;Humicola;Humicola_nigrescens
DENOVO58	Fungi;Basidiomycota;Agaricomycetes;Cantharellales;Ceratobasidiaceae;Ceratobasidiaceae;Ceratobasidiaceae_sp
DENOVO2456	Fungi;Zygomycota;Incertae_sedis_10;Mortierellales;Mortierellaceae;Mortierella;Mortierella_sp
DENOVO1997	Fungi;Ascomycota;Dothideomycetes;Pleosporales;Incertae_sedis_13;Phoma;Phoma_brasiliensis
DENOVO512	Fungi;Zygomycota;Incertae_sedis_10;Mortierellales;Mortierellaceae;Mortierella
DENOVO52	Fungi;Ascomycota;Sordariomycetes;Sordariales;Lasiosphaeriaceae;Podospora;Podospora_sp
DENOVO44	Fungi;Ascomycota;Sordariomycetes
DENOVO45	Fungi;Ascomycota;Ascomycota;Ascomycota_sp
DENOVO2651	Fungi;Ascomycota
DENOVO60	Fungi;Ascomycota;Sordariomycetes;Sordariales;Lasiosphaeriaceae;Lasiosphaeris
DENOVO28	Fungi;Zygomycota;Incertae_sedis_10;Mortierellales;Mortierellaceae;Mortierella;Mortierella_sp|SH218045.06FU

Supplementary Table 2: Top 50 16S OTUs identified based on the total average amount and their taxonomic identification.

OTU ID	taxonomy
DENOVO1	Archaea
DENOVO2	Bacteria;Cyanobacteria/Chloroplast;Cyanobacteria
DENOVO4	Bacteria;Acidobacteria;Acidobacteria_Gp16;Gp16;Gp16;Gp16
DENOVO3	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Micrococcaceae;Arthrobacter
DENOVO8	Archaea;Crenarchaeota;Thermoprotei
DENOVO13	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Microlunatus
DENOVO15	Bacteria;Acidobacteria;Acidobacteria_Gp4;Gp4;Gp4;Gp4
DENOVO51	Archaea;Crenarchaeota;Thermoprotei
DENOVO6	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Geodermatophilaceae;Blastococcus
DENOVO5	Archaea;Crenarchaeota;Thermoprotei
DENOVO9	Archaea
DENOVO30	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae
DENOVO17	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Massilia
DENOVO7	Bacteria
DENOVO105	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Sphingomonas
DENOVO21	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae
DENOVO9913	Archaea
DENOVO23	Bacteria;Actinobacteria;Actinobacteria;Solirubrobacterales
DENOVO28	Bacteria;Acidobacteria;Acidobacteria_Gp6;Gp6;Gp6;Gp6
DENOVO10	Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Myxococcaceae;Corallococcus
DENOVO16	Bacteria;Actinobacteria;Actinobacteria
DENOVO14	Bacteria;Actinobacteria;Actinobacteria
DENOVO11	Bacteria;Actinobacteria;Actinobacteria;Rubrobacterales;Rubrobacteraceae;Rubrobacter
DENOVO102	Bacteria
DENOVO34	Bacteria;Acidobacteria;Acidobacteria_Gp6;Gp6;Gp6;Gp6
DENOVO46	Bacteria;Acidobacteria;Acidobacteria_Gp6;Gp6;Gp6;Gp6
DENOVO191	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Nocardioidaceae;Nocardioides
DENOVO42	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae
DENOVO27	Bacteria
DENOVO110	Bacteria;Proteobacteria
DENOVO40	Bacteria;Acidobacteria;Acidobacteria_Gp4;Gp4;Gp4;Gp4
DENOVO54	Bacteria;Actinobacteria;Actinobacteria
DENOVO47	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae;Skermanella
DENOVO19	Bacteria
DENOVO41	Bacteria;Proteobacteria;Betaproteobacteria
DENOVO45	Bacteria;Verrucomicrobia;Spartobacteria;Spartobacteria_genera_incertae_sedis
DENOVO57	Bacteria
DENOVO122	Bacteria;Actinobacteria;Actinobacteria;Solirubrobacterales;Solirubrobacteraceae;Solirubrobacter
DENOVO70	Bacteria;Proteobacteria;Betaproteobacteria
DENOVO73	Bacteria;Verrucomicrobia;Opitutae;Opitutales;Opitutaceae;Opitutus
DENOVO91	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Intrasporangiaceae;Janibacter
DENOVO44	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium
DENOVO4395	Archaea
DENOVO146	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Microbacterium
DENOVO26	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Methylobacteriaceae;Microvirga
DENOVO143	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Massilia
DENOVO22	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Sinobacteraceae;Steroidobacter
DENOVO84	Bacteria;Actinobacteria;Actinobacteria
DENOVO12	Archaea;Crenarchaeota;Thermoprotei
DENOVO18	Bacteria

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Chapter 4

Phenology and physiological traits

4.1 Introduction

Once verified the robustness of the experimental plan both in terms of soil health and replicability, we aimed to analyze the plant behavior as response to the soil effect. In particular, a first question we wanted to answer was whether different soils, hence whether differences in soil composition, already described in Chapter 3, might lead to differences in plant phenology and physiological traits throughout the seasonal development.

It is well known that soil properties can influence grapevine performance (Pickering and Wheeler, 2003). Specifically, nitrogen content and water availability are two of the main factors reported to modulate grapevine development. On the one hand, they have an effect on vine vigor, usually promoting canopy shading and resulting in a low fruit quality. On the other hand, grapevine with inadequate growth produces low yield and low quality. In turn, canopy status, structure, and leaf development have been reported to affect the ripening progress in all stages, to influence the yield, grape quality, and being determinant in health conditions as response to diseases (Grantz and Williams, 1993; Pickering and Wheeler, 2003). For example, shaded canopies have been reported to reduce anthocyanin, sugar, phenol levels, aromatic compounds, but increase titratable acidity.

Soil can also influence the root growth and distribution, resulting in different root-to-shoot ratio. Roots can adsorb and store nutrients which are important both in the early growing season and in ripening when fruits develops. Moreover, roots are responsible for the hormone balance in the whole plant: cytokinins and gibberellins are produced in the root apex but targeting the shoot part, even affecting the ripening process (Grantz and Williams, 1993).

4.2 Material and methods

4.2.1 Phenological stages

Starting from March of both years (2017 and 2018), grapevine phenological stages were weekly monitored for both vegetative and reproductive phases, till the harvest date (in September). The “Modified E-L system” scale (Coombe BG, 1995) (Supplementary materials Figure 1) was used throughout the whole period, even though at the beginning of the vegetative season (between bud breaking and shoot development) the BBCH scale was also used (Lorenz et al., 1995) (Supplementary materials Figure 2).

4.2.2 Physiological analyses on grapevine canopy

The one-year-old shoots used for the horizontal guyot framework were chosen for measuring the shoot size at the curvature by means of a caliber. From bud breaking to flowering, the internode growth and the leaf number of a representative shoot per plant were also weekly measured as biometric indicators for assessing the vegetative growth. Later, the leaf area index (LAI) parameter was used as canopy growth indicator, and was weekly calculated for each plant by using a free-download App developed by the "School of Agriculture, Food and Wine (University of Adelaide, Australia)" (De Bei et al., 2016). Other analyses, in collaboration with the research group of Prof. Vamerali (DAFNAE, University of Padua, Italy), focusing on the period from flowering to *véraison* of 2018, included leaf gas exchanges, stomatal conductance and, as consequence, the net photosynthesis rate using a newly-released photosynthesis system (LI-6800, LICOR, USA).

4.2.3 Physiological analysis on grapevine berries

The number of bunches per plant was taken into account as fertility parameter, and was also related to the number of shoots for computing the bunches-shoots ratio.

Throughout berry maturation, total soluble solids (TSS) in °Brix were assessed from four berries each plant at three time points from *véraison* to harvest, using a hand refractometer (Palette PR-100, Atago USA). Titratable acidity measurement (g/L tartaric acid equivalents) was performed for each biological replicate (box) according to the standard procedures used in (Guymon and Ough, 1962). With the same juice, pH was determined by means of a pH-meter. For each sampling date, maturation index was also computed from the ratio between the TSS value and the titratable acidity value of each biological replicate. At harvest, berry weight was averaged from at least 15 berries selected from the middle part of a representative bunch of each plant.

4.2.4 Statistical analyses

Statistical analyses were performed using the R v3.3.1 software, in RStudio. Comparisons among treatments, involving different cultivars and soils, were performed using II-way ANOVA. Kruskall-Wallis test was used for the phenological data comparison at each time point and when ANOVA assumptions were not met. Tukey's HSD multiple comparison was used as *post-*

hoc test to compare means between treatments when found significantly different. The phenological value obtained from the average of the four plants of each box (E-L scale) represented a biological replicate of the dataset. Data were then normalized by the dataset median value and used for dendrogram construction by using the hierarchical clustering tool of JMP®, Version 13 (SAS Institute Inc., Cary, NC, 1989-2007).

4.3 Results

4.3.1 Phenological stages

Phenological differences, based on average developmental rate (BBCH and modified E-L scale), were observed among soils and between cultivars throughout both years. Interestingly, the soil effect was more evident in 2017 compared to 2018 vintage.

Generally, the soil factor showed to have a significant effect on both cultivars at the beginning of the vegetative growth (bud breaking and shoot development), at flowering, and during the phases covering the period between berry development and harvest (**Tab. 4.1**).

Table 4.1 – Phenological development of plants grown in different soils in 2017 (A) and 2018 (B) based on E-L scale. Values are reported as the mean of the biological replicates (n=3); Statistical significance (Kruskall-Wallis test) is represented by the p-value (“.” P<0.1; “” P<0.05; “**” P<0.01; “***” P<0,001);*

A			mean value Glera					mean value Corvina		
date	***sign***	***p value***	***F***	***VV***	***L***	***sign***	***p value***	***F***	***VV***	***L***
03_03_2017	ns	0.264	2.0	2.0	1.9	**	0.002	2.0	2.0	1.5
07_03_2017	ns	1.000	2.0	2.0	2.0	ns	1.000	2.0	2.0	2.0
10_03_2017	ns	1.000	2.0	2.0	2.0	ns	1.000	2.0	2.0	2.0
16_03_2017	ns	0.164	2.2	2.0	2.0	ns	0.472	2.1	2.0	2.0
23_03_2017	*	0.016	3.0	2.6	2.3	ns	0.309	2.4	2.2	2.1
30_03_2017	ns	0.141	3.8	3.6	3.2	ns	0.202	2.7	2.3	2.4
06_04_2017	ns	0.193	8.3	7.2	7.0	*	0.022	5.0	3.3	3.8
13_04_2017	ns	0.486	12.3	12.3	11.9	**	0.001	10.5	5.8	6.5
19_04_2017	ns	0.853	13.9	13.8	13.6	**	0.006	12.7	8.9	9.5
28_04_2017	ns	0.877	14.9	14.9	14.7	ns	0.123	13.9	12.3	12.8
04_05_2017	ns	0.232	15.3	14.8	15.6	ns	0.271	14.5	13.6	13.4
09_05_2017	*	0.022	16.1	15.8	16.7	ns	0.118	15.7	14.6	15.0
17_05_2017	.	0.061	17.0	16.8	17.0	.	0.069	16.2	15.6	15.6

22_05_2017	ns	0.425	18.8	18.3	18.7	.	0.078	18.7	17.9	18.4
24_05_2017	*	0.044	21.6	21.0	22.9	.	0.081	20.4	18.6	20.4
26_05_2017	ns	0.300	25.0	24.3	25.0	ns	0.188	21.5	21.2	22.9
29_05_2017	ns	0.370	26.3	26.2	26.4	*	0.011	26.0	24.4	26.0
31_05_2017	ns	0.461	27.5	27.2	27.4	**	0.008	26.6	25.7	26.5
08_06_2017	*	0.014	29.3	29.4	29.9	**	0.001	29.3	27.7	28.8
14_06_2017	*	0.043	31.5	31.1	31.6	***	0.001	31.7	30.5	31.5
21_06_2017	*	0.026	32.1	32.0	32.6	**	0.005	32.4	32.1	33.0
29_06_2017	**	0.003	32.1	32.1	32.7	.	0.068	32.7	32.4	33.0
05_07_2017	***	0.000	32.4	32.1	33.0	ns	1.000	33.0	33.0	33.0
11_07_2017	ns	1.000	33.0	33.0	33.0	ns	1.000	33.0	33.0	33.0
18_07_2017	ns	1.000	33.0	33.0	33.0	ns	1.000	33.0	33.0	33.0
25_07_2017	**	0.006	33.8	33.3	33.9	***	0.001	34.3	33.0	34.1
26_07_2017	na	na	34.4	33.8	34.4	***	0.000	34.8	33.0	34.7
28_07_2017	***	0.000	35.1	34.3	35.0	***	0.000	35.0	33.6	34.8
31_07_2017	***	0.000	35.5	34.8	35.6	*	0.018	35.3	34.6	35.2
02_08_2017	***	0.000	35.8	35.3	35.9	***	0.000	35.5	34.7	35.6
04_08_2017	***	0.000	35.9	35.3	35.9	***	0.001	35.9	35.2	35.8
08_08_2017	***	0.000	36.0	35.5	36.0	***	0.000	35.9	35.4	35.9
16_08_2017	ns	0.651	36.8	36.7	36.7	*	0.017	36.8	36.4	36.5
30_08_2017	ns	0.587	37.4	37.3	37.4	**	0.004	37.5	36.9	37.3
06_09_2017	.	0.078	37.7	37.4	37.8	.	0.085	37.7	37.2	37.4
13_09_2017	na	na	na	na	na	*	0.021	37.7	37.1	37.4

B			mean value Glera					mean value Corvina		
date	***sign***	***p value***	***F***	***VV***	***L***	***sign***	***p value***	***F***	***VV***	***L***
14_03_2018	.	0.082	1.5	1.8	1.8	*	0.026	1.3	1.5	1.7
21_03_2018	ns	0.598	2.0	2.0	2.0	ns	0.680	1.7	1.7	1.8
27_03_2018	ns	0.465	2.6	2.7	2.4	ns	0.314	2.0	2.0	2.2
30_03_2018	ns	0.368	3.0	3.0	2.9	ns	1.000	2.0	2.0	2.0
03_04_2018	ns	0.306	3.4	3.2	3.2	ns	0.899	2.5	2.5	2.4
06_04_2018	*	0.041	4.3	4.0	3.9	ns	0.268	2.9	2.6	2.7
10_04_2018	ns	1.000	5.0	5.0	5.0	ns	0.183	3.5	3.0	3.4
13_04_2018	ns	1.000	5.0	5.0	5.0	*	0.042	4.8	4.1	4.4
17_04_2018	ns	0.593	10.0	9.8	9.5	*	0.011	7.5	6.1	6.2
20_04_2018	ns	0.245	11.7	11.4	10.9	.	0.076	10.2	9.4	9.4
24_04_2018	ns	0.444	13.3	12.8	13.3	ns	0.801	11.8	11.6	11.6
27_04_2018	ns	0.346	15.0	14.8	14.9	ns	0.274	12.0	11.7	12.0
02_05_2018	ns	0.144	15.9	15.3	15.9	ns	0.970	12.6	12.7	12.6
07_05_2018	.	0.061	18.0	17.7	18.0	ns	0.188	16.6	16.2	16.5
10_05_2018	ns	0.417	18.0	17.9	18.0	ns	0.272	17.1	16.8	17.0

14_05_2018	ns	1.000	18.0	18.0	18.0	ns	0.427	18.2	18.0	18.1
17_05_2018	*	0.041	19.2	18.4	18.4	**	0.009	19.8	18.4	19.1
21_05_2018	**	0.004	25.7	25.1	25.0	ns	0.108	25.5	24.5	25.3
24_05_2018	*	0.020	26.4	26.3	26.9	.	0.064	26.3	25.7	26.4
28_05_2018	ns	0.361	27.6	27.3	27.4	.	0.065	27.5	26.9	27.6
31_05_2018	ns	0.279	29.0	29.0	28.9	ns	0.143	28.9	28.4	28.9
08_06_2018	ns	0.435	30.9	31.0	31.0	ns	0.799	31.1	31.0	30.9
14_06_2018	***	0.001	31.4	31.1	32.0	ns	0.216	32.0	31.8	31.8
20_06_2018	*	0.033	31.6	31.4	31.9	ns	0.261	31.9	31.7	32.0
28_06_2018	**	0.006	31.8	31.5	32.2	ns	0.748	32.3	32.5	32.4
03_07_2018	***	0.000	32.2	32.2	32.9	ns	0.279	33.0	33.0	32.9
13_07_2018	ns	0.243	32.7	32.6	32.9	ns	1.000	33.0	33.0	33.0
20_07_2018	ns	0.316	32.8	32.8	33.0	ns	0.279	33.0	33.0	33.1
23_07_2018	*	0.012	34.3	34.0	34.6	*	0.011	34.7	34.1	34.7
27_07_2018	***	0.000	34.2	34.3	34.9	ns	0.627	34.9	34.8	35.0
30_07_2018	ns	0.301	35.4	35.2	35.5	ns	0.143	35.5	35.2	35.3
03_08_2018	ns	0.251	35.7	35.5	35.7	ns	0.270	35.8	35.9	35.7
08_08_2018	ns	0.167	35.9	35.7	35.8	ns	0.426	36.1	36.0	36.1
16_08_2018	ns	0.161	36.7	36.6	36.5	ns	0.121	36.6	36.4	36.4
29_08_2018	ns	0.762	37.4	37.3	37.3	.	0.064	37.1	36.9	36.8
11_09_2018	ns	0.785	37.4	37.3	37.4	ns	0.320	37.5	37.4	37.2

In 2017, F soil seemed to anticipate the buds development with respect to L and VV soil, but showed to be the most delaying one in 2018. During all the flowering period, it was evident how plants in VV soil were delayed compared to the ones in L and particularly in F soil. Differences were statistically maintained even during berry development in which VV soil led to a slight delaying development in 2017, while in 2018 it was more evident how the plants in L soil clearly anticipated the stage. At *véraison* (the beginning of the ripening period) VV soil was still the last one promoting the berry color changing. The same trend was maintained even after veraison, but close to the harvest point the different effect of the soils on the phenological stages seemed to shade, especially in 2018 than in 2017.

When studying the two cultivars separately, cv. Corvina looked more responsive to soil differences throughout all the 2017, but less than cv. Glera in 2018. In comparison with Glera, Corvina revealed clear differences during the first part of grapevine seasonal development, showing plants on F soil clearly anticipating the stages when compared to the ones on VV and L soils.

Although the two cultivars similarly behaved in 2017, in 2018 Corvina plants grown in different soils showed differences also during shoot development, while Glera plants showed clear differences only during berry development before veraison.

The dendrogram built from the average developmental value (E-L scale) of each biological replicate (**Fig. 4.1**) depicted a sharp separation between years and cultivars. According to the hierarchical clustering, the soil effect was more evident considering the 2017 vintage than 2018, since the sub-clusters were consistent with the type of soil. At first sight, the plants grown in VV soil were clearly separated from the others. This effect resulted less clear in 2018, where vines clearly clustered by cultivar but the soil component hasn't shown to be discriminant.

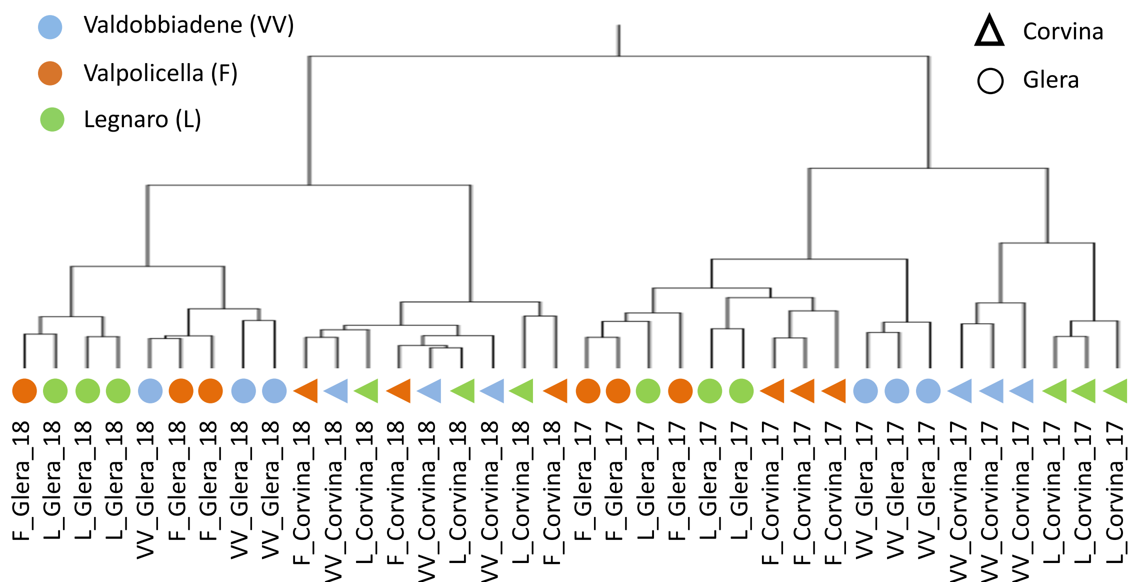


Figure 4.1 – Dendrogram built from the average developmental value (E-L scale) of each biological replicate (n = 3). Data were normalized by the dataset median value. ID samples refer to the type of soil (F, VV, L), the cultivar (Corvina, Glera), and the growth year (17 and 18 as 2017 and 2018 respectively).

What we also noticed is that BBCH scale was more sensitive at the beginning of the vegetative stage (from bud breaking to shoot development). In fact, the phenological stage subdivision looked more detailed, and differences among treatments could be better discriminated (data not shown). However, the "Modified E-L system" scale was still used till the end of the season since is considered clear and easy to consult.

4.3.2 Physiology on canopy traits

One-year-old shoot size

The assessment of shoot circumference and diameter can provide useful information about the growing conditions of the previous year. For both years (2017 and 2018), the statistical analysis revealed significant differences among soils, showing the least values for plants grown on F soil. Despite it involved both cultivars, the differences were more evident in cv. Glera than cv. Corvina. (**Fig. 4.2**).

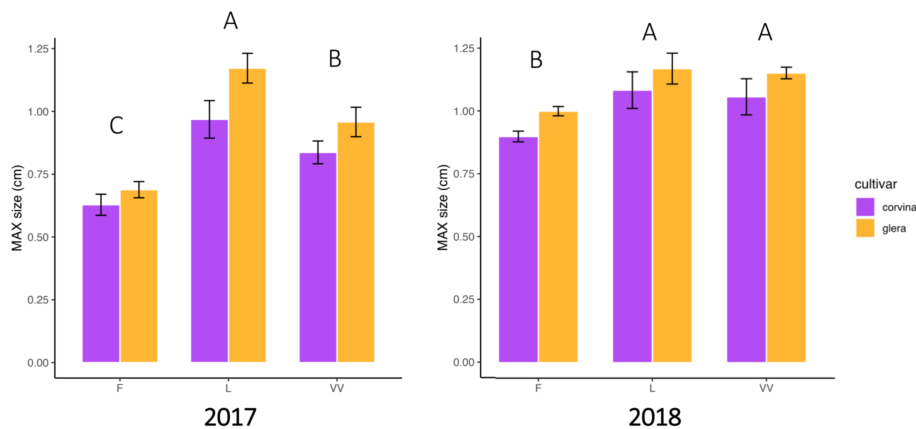


Figure 4.2 – One-year shoot size and diameter for 2017 and 2018 respectively. Error bars are standard errors (n = 12). Bars marked by different letters represent significantly different values.

Leaf Area Index (LAI)

The Leaf Area Index (LAI) is commonly defined as the total one-sided area of leaf tissue per unit ground surface area (Watson, 1947). It is defined as a common and important biophysical parameter used to estimate agronomical variables such as canopy growth, light interception and water requirement of plant and trees (De Bei et al., 2016). LAI is difficult to measure and estimate since it traditionally requires destructive techniques that are even labor intensive. Instead, data collected by the smartphone-tailored VitiCanopy application allowed us to estimate the parameter by an easy and non-destructive way.

Throughout the vegetative 2018 growing season, the analysis of variance did not show any significant difference among soils in both cultivars. Instead, during 2017, canopy differently developed among the three soils with L soil as the most performing one, and F soil as the least performing one. (**Fig.4.3**).

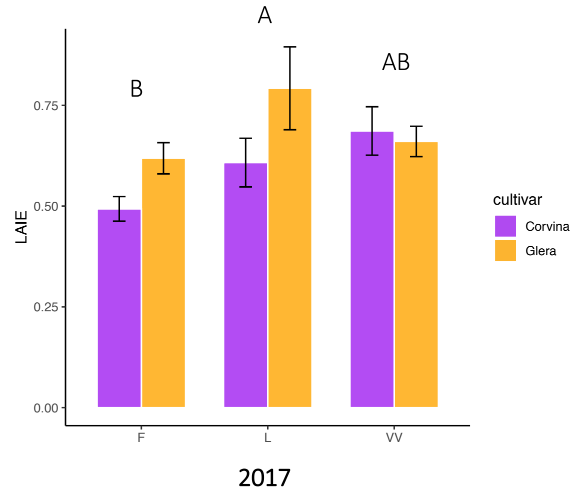


Figure 4.3 – Leaf Area Index measured in June 2017. Error bars are standard errors (n = 12). Bars marked by different letters represent significantly different values.

Shoot length and leaves number

Other two parameters for describing the canopy growth were the shoot length and the leaves number. They can be considered closely related to each other since both depend on shoot developing. Differences were evident in 2017 season, mainly concerning the cv. Corvina at the beginning of the vegetative growth and during the phase before the bunch closure till ripening. In the first case, F soil promoted the growth, whereas, during berry development, the same soil was the last one in the ranking for the traits considered. The opposite trend was instead observed in plants form VV soil. In 2018, significant differences were observed only after flowering ended, in which plants in L soil developed the longest shoots.

Transpiration – stomatal conductance – PhiPS2

During the 2018 season, we also focused on leaf transpiration and photosynthetic parameters by using Licor6800 photosynthesis measurer. We noticed some significant differences assessing the stomatal conductance and the transpiration only after veraison. The plants grown on F soil reached the highest transpiration values, whereas the ones from VV soil the lowest values. Moreover, at flowering, PhiPS2 values were significantly different among soils, with L significantly higher than VV (**Fig. 4.4**).

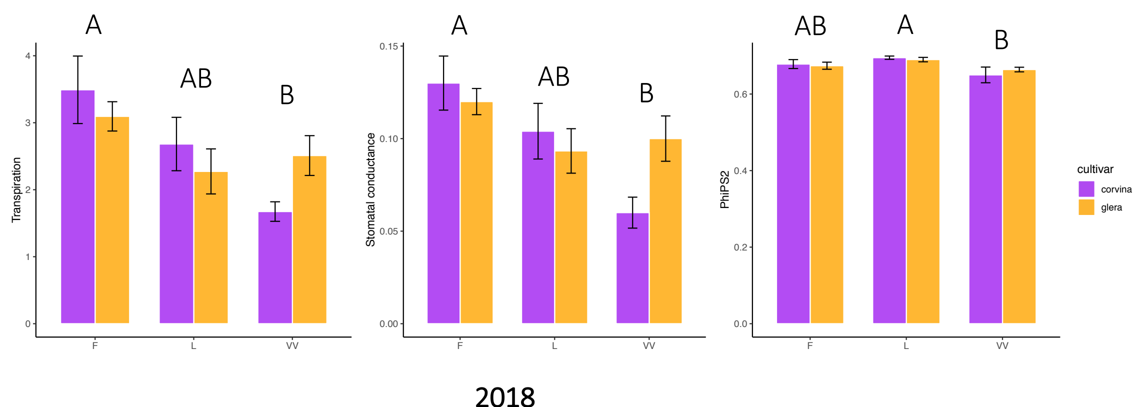


Figure 4.4 – Transpiration, Stomatal conductance, and PhiPS2 (photosystem 2 activity) measured in August 2018. Error bars are standard errors (n = 12). Bars marked by different letters represent significantly different values.

4.3.3 Physiology on berry traits

(Statistical analysis are shown in Supplementary materials, table 1 & 2)

Number of bunches

The analysis of variance showed a significant effect in both 2017 and 2018 for the number of bunches per plant (P<0.001). Precisely, plants on L soil always showed the highest values both in terms of bunches number and bunches-shoot ratio. In 2017, the trait considered showed to be affected also by the interaction between the cultivar and soil factors. The ranking of treatments was different between cultivars when comparing the effect of F soil: from being the least promoting for cv. Glera (5.83 ± 1.14), it reached an intermediate value for what concern the Corvina bunches number (7.33 ± 1.56), whose mean value was between L (the highest; 12.50 ± 1.10) and VV (the lowest; 5.90 ± 1.16) ones (**Fig. 4.5**).

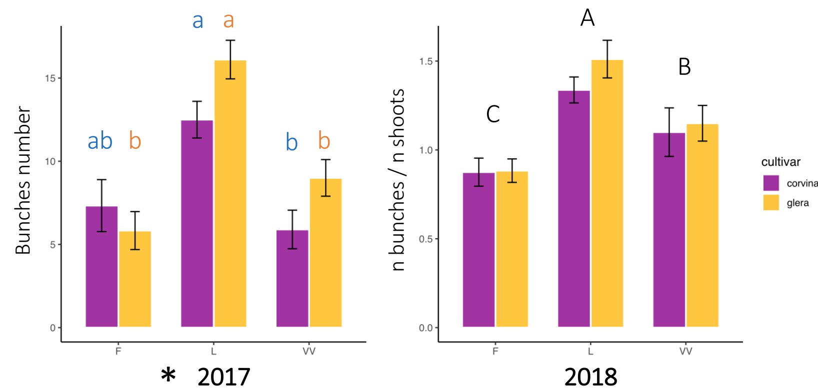


Figure 4.5 – Number of bunches / bunches-shoot ratio. Error bars are standard errors (n = 12). Bars marked by different letters represent significantly different values.

Sugar accumulation (°Brix)

Grape sugar concentration is considered an important characteristic since determines the potential alcoholic degree of the future wine. The sugar content was detected as total soluble solids (TSS) at three time points starting after veraison phase (full-veraison, mid-ripening, harvest; **Tab 4.2**). The statistical analysis revealed significant differences among soils in both years and for both cultivars. As a general effect, the plants grown on F soil were always related to the highest sugar content even though such differences were less remarkable in 2018 as compared to 2017, and they statistically decreased throughout the ripening process.

Also for this parameter (TSS), cv Corvina was more plastic than cv. Glera since more significant differences were observed. Interestingly, close to the harvest of 2017, the analysis revealed an interaction effect between soil and cultivar: the highest value of F soil on cv Corvina was not maintained on cv Glera in which ,instead, TSS value was mostly promoted by L soil.

Table 4.2 – Brix values expressed as the mean of the biological replicates ± standard error (n=12). Letters indicate the mean separation according to Tukey's test whether ANOVA revealed significant difference for the soil factor (with "a" standing for the highest value).

	mean value Glera			**mean value Corvina**		
date	***F***	***VV***	***L***	***F***	***VV***	***L***
08_08_2017	12.20 ± 0.32 a	10.75 ± 0.50 b	12.15 ± 0.45 ab	13.76 ± 0.65 a	11.05 ± 0.43 b	13.58 ± 0.47 a
16_08_2017	14.42 ± 0.31	14.00 ± 0.47	14.14 ± 0.34	17.46 ± 0.53 a	15.07 ± 0.70 b	16.09 ± 0.51 ab
30_08_2017	17.13 ± 0.32	16.83 ± 0.35	17.13 ± 0.36	20.69 ± 0.25 a	18.53 ± 0.39 b	19.73 ± 0.31 a
06_09_2017	17.96 ± 0.21 a	17.10 ± 0.28 b	18.14 ± 0.23 a	21.04 ± 0.29	19.64 ± 0.68	20.09 ± 0.42

13_09_2017	na	na	na	21.02 ± 0.19 a	19.13 ± 0.49 b	20.25 ± 0.37 ab
16_08_2018	14.30 ± 0.42	13.68 ± 0.57	12.83 ± 0.38	16.37 ± 0.45 a	14.85 ± 0.21 b	14.86 ± 0.40 b
29_08_2018	16.97 ± 0.27	16.77 ± 0.31	16.58 ± 0.35	19.16 ± 0.32	18.36 ± 0.26	17.84 ± 0.52
11_09_2018	17.17 ± 0.26	16.85 ± 0.38	16.71 ± 0.36	20.36 ± 0.50	20.00 ± 0.42	19.46 ± 0.70

Total Acidity (TA) and pH

Organic acids are one of the main compounds of grapevine berry and must. Tartaric, malic, and citric acids represent 90% of the total acidity in grapes. Tartaric acid is not common in nature but is important in grape. Due to the precipitation, acid concentration changes from 15g/L on the first maturation phase up to 6 g/L on the last stages. As expected, in all the thesis considered the acidity level decreased throughout ripening, while pH increased. In parallel, pH is becoming increasingly recognized for its important contribution to wine quality because it plays a key role in prevention of microbiological spoilage, malolactic fermentation occurrence and color stability of wines (Faclao et al., 2008).

Considering the titratable acidity (TA; **Tab 4.3**) and pH (**Tab 4.4**) values, no significant differences were observed among soils in 2018. Instead, in 2017, we observed how pH value was significantly higher in grapes from plant in L soil, especially at the beginning of the ripening period. Such difference was more evident in cv Corvina than Glera ($P<0.05$ and $P<0.1$ respectively). Similarly to pH, titratable acidity was found to be significantly different just at the beginning of the ripening stage (full-veraison), but treatments between cultivars had different trend: TA value from L soil was considerably lower in Glera, whereas TA value from F soil was considerably lower in Corvina. In both cultivars, we observed that such differences faded throughout the ripening process, especially in cv. Glera than in cv. Corvina.

Table 4.3 – Titratable acidity values expressed as the mean of the biological replicates ± standard error (n=3). Letters indicate the mean separation according to Tukey's test whether ANOVA revealed significant difference for the soil factor (with "a" standing for the highest value).

	mean value Glera			**mean value Corvina**		
date	***F***	***VV***	***L***	***F***	***VV***	***L***
08_08_2017	14.68 ± 0.53	14.98 ± 0.80	12.88 ± 0.33	13.45 ± 0.88	19.18 ± 0.30	17.38 ± 3.00
16_08_2017	9.50 ± 0.20	9.58 ± 0.78	9.78 ± 0.81	9.58 ± 0.55	12.15 ± 0.98	10.65 ± 0.72
30_08_2017	6.23 ± 0.31	6.05 ± 0.68	5.98 ± 0.67	7.33 ± 0.37	8.08 ± 0.22	7.88 ± 0.40
06_09_2017	5.43 ± 0.37	4.93 ± 0.32	5.28 ± 0.30	na	na	na

13_09_2017	na	na	na	6.53 ± 0.30	6.83 ± 0.04	6.85 ± 0.38
16_08_2018	6.72 ± 0.39	6.63 ± 0.52	7.80 ± 0.19	10.25 ± 1.17	7.28 ± 0.72	8.50 ± 0.24
29_08_2018	4.50 ± 0.19	4.32 ± 0.54	5.15 ± 0.73	6.12 ± 0.86	6.27 ± 0.09	6.57 ± 0.54
11_09_2018	4.22 ± 0.87	4.67 ± 0.74	4.68 ± 1.05	5.00 ± 0.32	4.81 ± 0.68	4.99 ± 1.34

Table 4.4 – pH values expressed as the mean of the biological replicates ± standard error (n=3). Letters indicate the mean separation according to Tukey's test whether ANOVA revealed significant difference for the soil factor (with "a" standing for the highest value).

	mean value Glera			**mean value Corvina**		
date	***F***	***VV***	***L***	***F***	***VV***	***L***
08_08_2017	2.94 ± 0.02	2.95 ± 0.03	3.02 ± 0.01	2.92 ± 0.03 ab	2.86 ± 0.01 b	3.00 ± 0.02 a
16_08_2017	3.24 ± 0.06	3.24 ± 0.02	3.29 ± 0.02	3.15 ± 0.04	3.10 ± 0.03	3.19 ± 0.02
30_08_2017	3.49 ± 0.06	3.50 ± 0.02	3.57 ± 0.04	3.36 ± 0.03	3.34 ± 0.03	3.44 ± 0.03
06_09_2017	3.61 ± 0.04	3.62 ± 0.03	3.67 ± 0.04	na	na	na
13_09_2017	na	na	na	3.50 ± 0.02	3.47 ± 0.03	3.58 ± 0.03
16_08_2018	3.53 ± 0.08	3.48 ± 0.05	3.54 ± 0.03	3.51 ± 0.04	3.50 ± 0.04	3.50 ± 0.01
29_08_2018	3.46 ± 0.07	3.43 ± 0.03	3.45 ± 0.02	3.36 ± 0.07	3.27 ± 0.01	3.30 ± 0.03
11_09_2018	3.50 ± 0.10	3.47 ± 0.02	3.49 ± 0.04	3.45 ± 0.09	3.41 ± 0.10	3.42 ± 0.14

Maturation Index (MI)

Another way for representing the maturation pattern of berries consists on the ratio computation between sugar content (TSS) and titratable acidity (TA). This parameter value tends to increase throughout ripening as the sugar content increases and the acidity decreases. It might better explain differences not emerged with the single sugar assessment.

Significant differences emerged in both years, and particularly already in post veraison (**Tab 4.5**). F soils showed to promote the berry maturation with respect to VV and L soils. Anyway, cv Corvina showed to be more plastic for the trait considered, especially in 2017, whereas cv. Glera showed more significant differences in 2018. Besides the statistical soil effect, the analysis of variance revealed a slight interaction effect (P=0.069) at the first day of sampling in 2017 (immediately post-veraison). Indeed, grapes from plants in F soil showed higher MI values in cv Corvina than in cv Glera, resulting in a different soil-trend between cultivars.

Table 4.5 – Maturation index values expressed as the mean of the biological replicates ± standard error (n=3). Letters indicate the mean separation according to Tukey's test whether ANOVA revealed significant difference for the soil factor (with "a" standing for the highest value).

	mean value Glera			**mean value Corvina**		
date	***F***	***VV***	***L***	***F***	***VV***	***L***
08_08_2017	0.84 ± 0.03 ab	0.72 ± 0.03 b	0.96 ± 0.03 a	1.03 ± 0.14	0.58 ± 0.02	0.82 ± 0.10
16_08_2017	1.52 ± 0.04	1.47 ± 0.09	1.48 ± 0.12	1.84 ± 0.20	1.22 ± 0.11	1.54 ± 0.14
30_08_2017	2.76 ± 0.15	2.85 ± 0.31	2.92 ± 0.26	2.84 ± 0.16 a	2.25 ± 0.02 b	2.50 ± 0.11 ab
06_09_2017	3.34 ± 0.17	3.49 ± 0.14	3.49 ± 0.23	na	na	na
13_09_2017	na	na	na	3.24 ± 0.18	2.76 ± 0.06	2.96 ± 0.12
16_08_2018	2.20 ± 0.13 a	2.11 ± 0.17 a	1.64 ± 0.04 b	1.84 ± 0.21	2.06 ± 0.08	1.73 ± 0.05
29_08_2018	3.77 ± 0.07 ab	3.87 ± 0.16 a	3.34 ± 0.18 b	3.19 ± 0.16 a	2.93 ± 0.04 ab	2.68 ± 0.13 b
11_09_2018	4.23 ± 0.27	3.76 ± 0.21	3.81 ± 0.30	4.07 ± 0.09	4.18 ± 0.17	4.28 ± 0.49

Berry weight at harvest

Several traits of harvest berries were screened, including berry weight and size. About these, in 2017, the soil from Valpolicella (F) and Legnaro (L) positively affected the berry weight (1.57g ± 0.04 and 1.64g ± 0.04 respectively), whereas Valdobbiadene soil (VV) always produced lighter berries (1.34g ± 0.06). Interestingly, in 2018, the analysis of variance revealed a slight interaction effect ($P<0.1$) between cultivar and soil in which F soil was the most performing for cv Corvina berries, even though the general effect showed F soil as the least performing one. The berry weight was also affected by the interaction between years and cultivars. In fact, the ranking of the two cultivars for the trait considered was not maintained comparing the two years (**Fig. 4.6**).

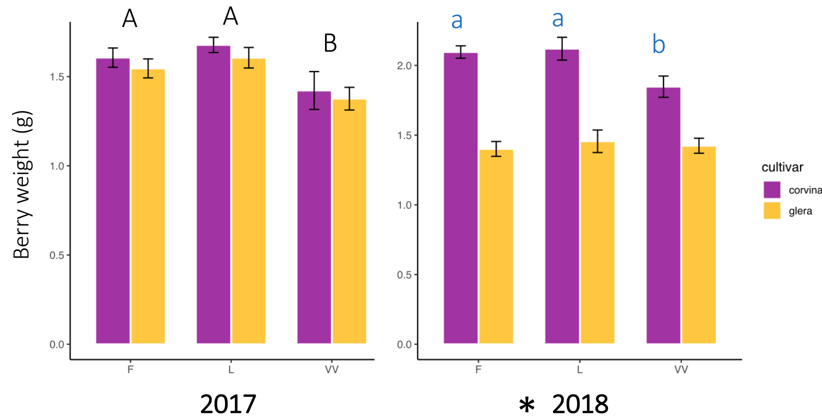


Figure 4.6 – Berry weight at harvest. Error bars are standard errors (n = 12). Bars marked by different letters represent significantly different values.

4.4 Discussion

In the experiment, the effect due to the agronomical and climatic variability was minimized, allowing us to isolate and study in more detail the singular effect that the different soils had on the grapevine growth and grape traits. Indeed, every difference observed can be related to, and potentially explained, by the characteristics which distinguish the three soils, previously described in Chapter 3. Soil effect can be meant as the result of both intrinsic and extrinsic soil features. On the one hand, soil mineral composition, pH, along with microbiology activity determine soil nutrient availability. On the other hand, soil texture, structure, and depth determine soil water holding capacity and grapevine water supply (Le Bulletin De L'Oiv, 2010). Nevertheless, such distinction is not always clear since each soil characteristic might have many effects on the plant phenotype, even depending on the interaction with other soil features. For example, soil organic matter helps to hold soil water, promotes the microbiological activity, and provides nutrients to the plant (Pickering and Wheeler, 2003). Its content also depends on the velocity of mineralization which is linked to the soil texture (Ripoche et al., 2010). Despite this complex interplay among soil features, we'll try to explain as much as possible the effect we observed on the plants of our experiment.

This study showed that the soil factor, here studied comparing three different soils, can potentially affect the plant growth and grape quality, as shown by the numerous significant

differences found for the traits considered in this chapter. Anyway, comparing the two years (2017 and 2018), it seemed that the soil effect also depended on the interactions with other terroir factors (e.g. cultivar and year).

The grapevine phenological development were affected by soil differences in both seasons examined, but this effect took place at different times throughout crop growth, without observing a sharp distinction of one of the three soil-thesis (F, L, VV). As an explanation, either soil differences were too weak for determining stable differences or the predominant influence is driven by the climate. Indeed, despite phenology is known to be also affected by the interaction between climate and soil, namely vintage, (Pereira et al., 2005), climate is still considered the most incisive terroir factor on phenology exerting its effect on the sum of temperature (Pereira et al., 2006).

High spring temperature are known to anticipate the plant reawakening up. In our case, since the air temperature was exactly the same for all thesis, differences must be investigated in soil features. The different behavior we observed from plants in different soils and comparing the two years might find a reason in the soil temperature and water content (Coipel et al., 2006). In 2017, the dark color of F soil might have promoted a higher heat degree accumulation, anticipating the bud breaking. Instead, in 2018 the effect was not replied and plants didn't show any significant difference possibly due to the affection of the rainy period close to the predicted reawakening phase which delayed the budbreak in the soil with the higher level of humidity (F).

Throughout the season, the ranking of the three soils, still considering the phenological stages, changed between the first and the second developmental phases (i.e. before and after flowering). This is consistent with the existence of a sequence of two growth phases, autotrophic and heterotrophic, from bud breaking to veraison. Precisely, between bud breaking and flowering shoot growth mainly depends on the resource mobilization from reserves accumulated during the previous season, particularly during the post-harvest period (Conradie, 2017; Zapata et al., 2004). In line with this principle, we noticed that from flowering plants on VV soil showed to be delayed with respect to the others.

Similar consideration might be done when analyzing other parameters linked to the shoot development: number of leaves per shoot and shoot length ranking were found to change after flowering when comparing the effect of the three soils. When analyzing the cv Corvina separately, this effect was clear also for vines grown in F soil, which seemed to promote the

vegetative growth at the beginning of the season, whereas were found to be the less vigor after flowering.

The analysis of variance also showed differences in the one-year-shoot size parameter. The results were similar in both years where L soil seemed to promote the shoot size development. This can be explained by the water condition and mineral nutrition of the previous year. L soil indeed is characterized by the highest level of nitrogen and water availability. Moreover, such results are in line with the analysis of the Leaf Area Index (LAI): the good development observed in vines from L soil, significantly higher in 2017, might have been the proof of a better soil nutrition in 2016. Instead, significant differences were not observed in 2018, maybe masked by the periodic shoot trimmings: the elapsed time between one trimming to the other one could not have been enough for highlighting any possible difference in canopy growth among treatments.

The PhiPS2 is defined as the quantum yield of PSII calculated from fluorescence computed from the re-emission of photons by chlorophyll associated with photosystem II, hence it is considered a good parameter for the photosynthetic activity interpretation. At flowering, it has been found to be higher in grapevine leaves form L soil, possibly due to the better soil condition which improved shoot nurture. Interestingly, such result is not strictly linked to the transpiration level, which was higher in F treatments (at least at veraison), possibly since photosynthesis does not only depend on the grapevine water balance.

Indeed, PSII is sensitive to environmental stresses such as temperature, drought, and radiation. Stresses that affect PSII efficiency will cause a decrease in Fv/Fm. As for other plant measurements, there are many plant-related and environmental factors that affect fluorescence results, including leaf age, health, and environmental conditioning (Licor handbook manual – Using the LI-6800 Portable Photosynthesis System). The leaf transpiration, particularly influenced by the mesophyll conductance, is the main factor supporting the leaf photosynthesis, thus an improvement of its rate represents a strategy that potentially allows to increase the final crop yield (Ort et al., 2015).

The Total Soluble Solids (TSS) concentration in grape berries mainly depends on the sugar content. It is also considered the most used parameter for assessing the ripening progress (Poni et al., 2018) and for determining the date of harvest. Therefore, data have been utilized for both the phenological assay and sugar content comparison among treatments. Despite significant differences emerged immediately after veraison from plants grown in different soils,

such differences apparently faded throughout the ripening process, more markedly in 2018 than in 2017. The sugar accumulation is known to be under genetic control (Shiraishi et al., 2012), also depending on the agri-environmental conditions (Clingeleffer, 2010) and the berry development (Coombe, 1992). In this case, the weak differences observed might have been due to either a slight anticipation of the phenological phase (assessed by the sugar accumulation) or an actual consequence of the soil mineral composition. In fact, as reported by many authors, there is a significant positive relationship between K content and berry sugar accumulation, particularly after veraison, due to the K redistribution from leaves (Blouin and Cruège, 2013). During this stage, K plays an important role in the accumulation of sugars (Conde et al., 2007) since for both components the accumulation depends on the osmotic potential generated. In line with this piece of information, in our experiment, the soil VV soil was the one accumulating the least level of sugar in both years, being also characterized by the lowest level of soil K.

In parallel to the vegetative growth, the yield components and the grape quality were also evaluated. In L soil, the highest number of bunches per plant could be due to the soil fertility conditions. Indeed, the inflorescence initiation occurs in the latent buds during the previous season (Lebon et al., 2008; May, 2000) when the number of future inflorescences is regulated by the physiological status of the grapevine during summer (Lebon et al., 2008) promoted by the good fertility condition of L soil (nitrogen and water availability).

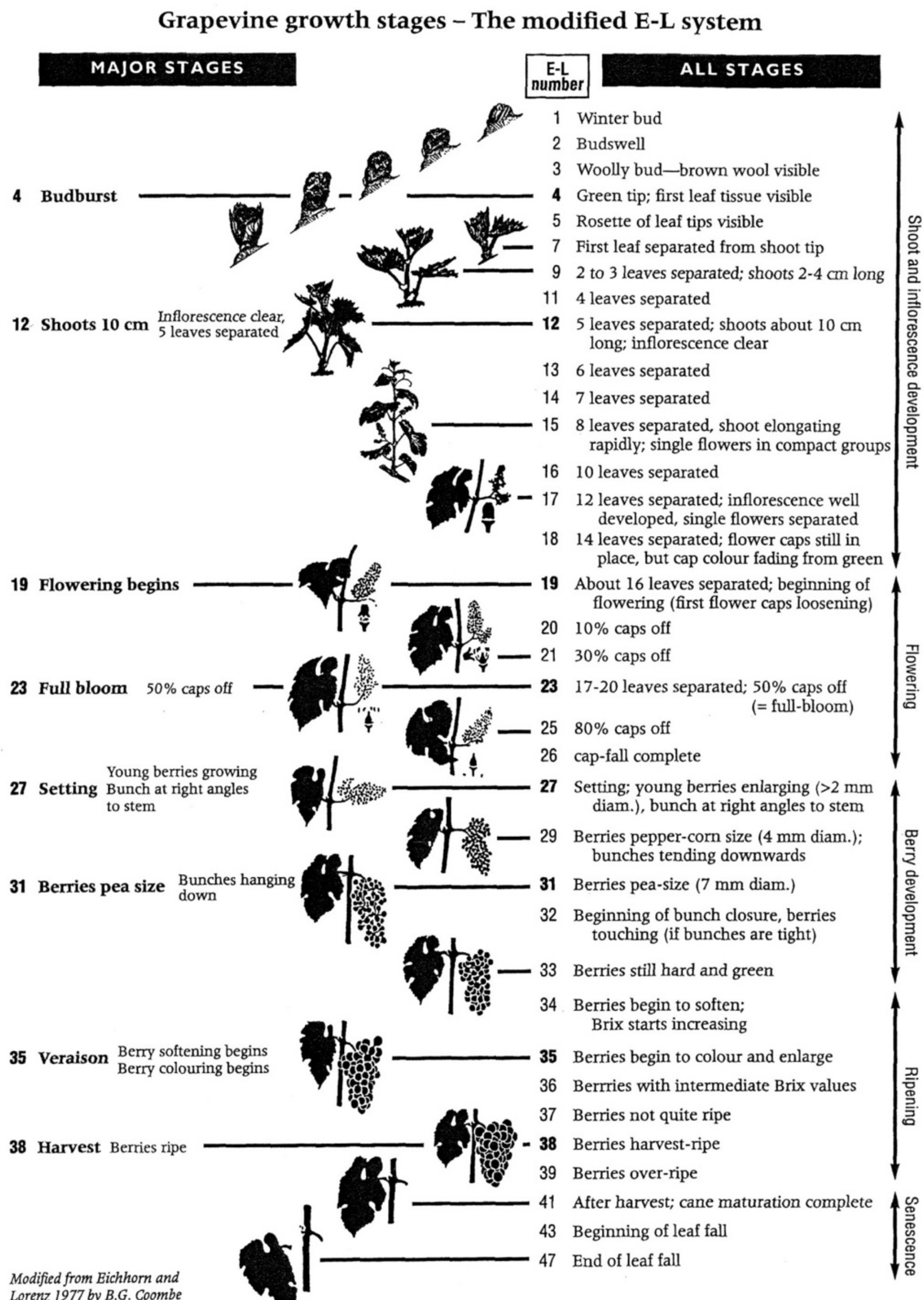
Moreover, the results for yield components were more intriguing as the number of bunches was also affected by the interaction between cultivar and soil factors: In 2017, the value of plants grown in F soil changed from being the lowest one in cv Glera, to be the intermediate value in cv Corvina, demonstrating that such parameter is potentially influenced by the interaction of many factors.

The high number of bunches in L soil was also associated by the high value of berry weight at harvest in both years. Berry weight is determined to a large extent at veraison, and it is particularly affected by water stress occurring between flowering and veraison (Deloire et al., 2001; Gómez-del-Campo et al., 2002). Apparently, L soil guaranteed the best water supply as compared with the other two soils, possibly due to the absence of skeleton, which determined less drainage and the greatest water content in absolute terms. Berry weight is another parameter apparently affected by the interaction of many factors: the value was reported to be high in the F soil treatment in 2017, but was found to be the lowest one in 2018.

When analyzing the two cultivars separately, cv. Corvina has shown to be more plastic than cv. Glera for most of the traits considered. This might support the idea by which different cultivars differently respond to environmental changes not only in terms of plasticity but GxE interaction (Dal Santo et al., 2018). In this case the studied involved the effect of the soil, but other works observed the different responsiveness also to other treatments. For example, Fernández-Marín et al. (2013) comparing the stilbene composition of different cultivar in different terroirs, founding how one of the considered genotype were less responsive than the other one, in this case cv. Merlot and cv. Syrah respectively.

4.5 Supplementary materials

Supplementary Figure 1: Grapevine growth stages according to the modified E-L system (Combe, 1995).



Supplementary Figure 2: Phenological grapevine growth stages, BBCH scale from bud break to shoot development.

Grapevine Lorenz et al., 1994

Phenological growth stages and BBCH-identification keys of grapevine
(*Vitis vinifera* L. ssp. *vinifera*)

Code	Description
Principal growth stage 0: Sprouting/Bud development	
00	Dormancy: winter buds pointed to rounded, light or dark brown according to cultivar; bud scales more or less closed according to cultivar
01	Beginning of bud swelling: buds begin to expand inside the bud scales
03	End of bud swelling: buds swollen, but not green
05	"Wool stage": brown wool clearly visible
07	Beginning of bud burst: green shoot tips just visible
08	Bud burst: green shoot tips clearly visible
Principal growth stage 1: Leaf development	
11	First leaf unfolded and spread away from shoot
12	2nd leaves unfolded
13	3rd leaves unfolded
1 .	Stages continuous till . . .
19	9 or more leaves unfolded

Supplementary Table 1: Physiological parameters assessed in 2017 and analyzed by ANOVA. Factors were "soil", "cultivar", and their interaction. Statistical significance is represented by the p-value ("." P<0.1; "*" P<0.05; "**" P<0.01; "***" P<0,001). Tukey's test, as *post-hoc* test, compared means between soil-treatments when found significantly different (with "a" representing the highest value).

								post-hoc test		
date	**parameter**	***soil***	***p value***	***cultivar***	***p value***	***interaction***	***p value***	***F***	***VV***	***L***
03_03_2017	one-year shoot size (max)	***	0.000	**	5.4E-03	ns	0.297	c	b	a
03_03_2017	one-year shoot size (min)	***	0.000	**	5.0E-03	ns	0.411	c	b	a
29_05_2017	SPAD	ns	0.172	***	4.2E-06	ns	0.731			
09_06_2017	LAIE	*	0.023		3.8E-02		0.169	b	ab	a
17_05_2017	leaves number	ns	0.901	*	1.1E-02	ns	0.261			
24_05_2017	leaves number	ns	0.655	ns	8.1E-01	ns	0.218			
31_05_2017	leaves number	ns	0.621	ns	3.6E-01	ns	0.239			
08_06_2017	leaves number	ns	0.858	ns	2.5E-01	ns	0.303			
14_06_2017	leaves number	ns	0.918	.	8.2E-02	ns	0.575			
21_06_2017	leaves number	ns	0.552	*	2.9E-02	ns	0.832			
29_06_2017	leaves number	ns	0.171	.	7.6E-02	ns	0.745			
05_07_2017	leaves number	.	0.069	ns	1.9E-01	ns	0.953			
11_07_2017	leaves number	.	0.059	.	7.6E-02	ns	0.633			
18_07_2017	leaves number	*	0.015	.	5.6E-02	ns	0.722	b	a	ab
25_07_2017	leaves number	*	0.016	.	9.5E-02	ns	0.419	b	a	ab
01_08_2017	leaves number	.	0.073	*	3.7E-02	ns	0.741			
13_04_2017	shoot length	**	0.003	***	2.1E-12	ns	0.743	a	b	b
19_04_2017	shoot length	**	0.009	***	2.2E-09	ns	0.499	a	b	ab
28_04_2017	shoot length	*	0.034	***	1.5E-07	ns	0.359	a	b	ab
04_05_2017	shoot length	ns	0.111	***	1.5E-05	ns	0.318			
12_05_2017	shoot length	ns	0.205	***	9.5E-05	ns	0.484			
17_05_2017	shoot length	ns	0.367	**	2.3E-03	ns	0.394			
24_05_2017	shoot length	ns	0.670	ns	1.4E-01	ns	0.473			
31_05_2017	shoot length	ns	0.828	ns	9.9E-01	ns	0.303			
08_06_2017	shoot length	ns	0.513	ns	2.0E-01	ns	0.361			
14_06_2017	shoot length	ns	0.453	ns	1.2E-01	ns	0.864			
21_06_2017	shoot length	ns	0.286	*	2.3E-02	ns	0.773			
29_06_2017	shoot length	ns	0.105	ns	1.1E-01	ns	0.600			
05_07_2017	shoot length	*	0.035	*	4.8E-02	ns	0.612	b	a	ab
11_07_2017	shoot length	*	0.033	*	1.8E-02	ns	0.348	b	a	a
18_07_2017	shoot length	*	0.010	*	3.0E-02	ns	0.295	b	a	a
25_07_2017	shoot length	**	0.008	.	7.2E-02	ns	0.209	b	a	ab
01_08_2017	shoot length	*	0.026	.	5.2E-02	ns	0.464	b	a	ab
31_05_2017	bunches number		0.000		1.1E-01		0.027	b	b	a
08_08_2017	Babo	***	0.000	**	7.1E-03	ns	0.366	a	b	a
16_08_2017	Babo	*	0.035	***	1.4E-06	ns	0.124	a	b	ab
30_08_2017	Babo	*	0.012	***	2.9E-13	*	0.029	a	b	a
06_09_2017	Babo	**	0.004	***	7.4E-13	ns	0.233	a	b	a
13_09_2017	Babo									
08_08_2017	Brix	***	0.000	**	7.0E-03	ns	0.366	a	b	a
16_08_2017	Brix	*	0.035	***	1.4E-06	ns	0.124	a	b	ab
30_08_2017	Brix	*	0.012	***	2.9E-13	*	0.029	a	b	a
06_09_2017	Brix	**	0.004	***	7.4E-13	ns	0.235	a	b	a
13_09_2017	Brix									
08_08_2017	pH	**	0.003	**	7.3E-03	ns	0.185	b	b	a
16_08_2017	pH	ns	0.206	**	3.4E-03	ns	0.779			
30_08_2017	pH	.	0.073	***	5.5E-04	ns	0.880			
06_09_2017	pH									
13_09_2017	pH									

08_08_2017	acidity	.	0.083	*	4.4E-02	.	0.070			
16_08_2017	acidity	ns	0.223	.	6.8E-02	ns	0.245			
30_08_2017	acidity	ns	0.834	***	9.9E-04	ns	0.582			
06_09_2017	acidity									
13_09_2017	acidity									
08_08_2017	maturation index	**	0.002	ns	3.6E-01	.	0.054	a	b	a
16_08_2017	maturation index	.	0.059	ns	6.8E-01	ns	0.109			
30_08_2017	maturation index	ns	0.447	.	7.1E-02	ns	0.232			
06_09_2017	maturation index									
13_09_2017	maturation index									
29_09_2017	berry weight	***	0.000	ns	1.4E-01	ns	0.895	a	b	a
29_09_2017	berry diameter	***	0.000	ns	3.2E-01	ns	0.899	a	b	a
29_09_2017	seed number	*	0.013	***	3.8E-07	ns	0.839	b	a	a

Supplementary Table 2: Physiological parameters assessed in 2018 and analyzed by ANOVA. Factors were "soil", "cultivar", and their interaction. Statistical significance is represented by the p-value ("." P<0.1; "*" P<0.05; "**" P<0.01; "***" P<0,001). Tukey's test, as *post-hoc* test, compared means between soil-treatments when found significantly different (with "a" representing the highest value).

								post-hoc test		
date	**parameter**	***soil***	***p value***	***cultivar***	***p value***	***interaction***	***p value***	***F***	***VV***	***L***
02_05_2018	shoot_size	**	*0.008*	*	*0.020*	ns	*0.942*	*b*	*a*	*a*
24_05_2018	LAIE	ns	0.918	***	0.000	ns	0.601			
20_06_2018	LAIE	ns	0.454	ns	3.046	ns	0.826			
28_06_2018	LAIE	ns	0.528	***	0.000	ns	0.912			
03_07_2018	LAIE	ns	0.175	ns	0.145	ns	0.640			
13_07_2018	LAIE	ns	0.817	*	0.014	ns	0.494			
20_07_2018	LAIE	ns	0.881	*	0.014	ns	0.595			
14_05_2018	leaves_number	ns	0.726	**	0.007	ns	0.797			
24_05_2018	leaves_number	ns	0.404	ns	0.810	.	0.080			
31_05_2018	leaves_number	ns	0.116	ns	0.597	ns	0.829			
08_06_2018	leaves_number	ns	0.204	ns	0.894	ns	0.108			
14_06_2018	leaves_number	ns	0.892	ns	0.997	ns	0.801			
27_04_2018	shoot_length	.	0.098	***	0.000	ns	0.883			
02_05_2018	shoot_length	ns	0.294	***	0.000	ns	0.954			
07_05_2018	shoot_length	ns	0.607	*	0.029	ns	0.850			
14_05_2018	shoot_length	ns	0.398	ns	0.886	ns	0.676			
24_05_2018	shoot_length	ns	0.245	ns	0.268	ns	0.597			
31_05_2018	shoot_length	*	0.013	ns	0.118	ns	0.775	b	b	a
08_06_2018	shoot_length	ns	0.145	*	0.031	ns	0.635			
14_06_2018	shoot_length	ns	0.544	**	0.003	ns	0.954			
14_05_2018	bunches_number	***	0.000	ns	0.224	ns	0.341	b	b	a
14_05_2018	shoot_number	ns	0.585	ns	0.745	ns	0.270			
14_05_2018	bunches/shoots	**	0.004	ns	0.394	ns	0.697	c	b	a
16_08_2018	Brix	**	0.003	**	0.005	ns	0.518	a	b	b
29_08_2018	Brix	*	0.049	***	0.000	ns	0.380	a	ab	b
11_09_2018	Brix	ns	0.331	***	0.000	ns	0.861			
16_08_2018	pH	ns	0.589	ns	0.584	ns	0.698			
29_08_2018	pH	ns	0.134	***	0.000	ns	0.617			
11_09_2018	pH	ns	0.852	ns	0.259	ns	0.981			
16_08_2018	acidity	ns	0.486	ns	0.153	ns	0.473			
29_08_2018	acidity	ns	0.283	***	0.000	ns	0.790			
11_09_2018	acidity	ns	0.934	ns	0.429	ns	0.863			
16_08_2018	maturation_index	*	0.015	ns	0.312	ns	0.267	a	a	b
29_08_2018	maturation_index	**	0.003	***	0.000	ns	0.388	a	a	b
11_09_2018	maturation_index	ns	0.780	ns	0.327	ns	0.422			
11_09_2018	berry_weight	.	0.066	***	0.000		0.082	b	ab	a
11_09_2018	barry_weight_CV	ns	0.365	***	0.000	.	0.088			
11_09_2018	seed_n/berry_n	ns	0.672	ns	0.697	ns	0.936			
03_07_2018	Stcond_Licor1600	ns	0.205	ns	0.760	ns	0.195			
08_08_2018	Stcond_Licor1600	**	0.003	ns	0.525	ns	0.107	a	b	ab
24_05_2018	Transp_Licor1600	ns	0.101	**	0.008	.	0.099			
03_07_2018	Transp_Licor1600	ns	0.209	ns	0.520	ns	0.363			
08_08_2018	Transp_Licor1600	**	0.007	ns	0.946	ns	0.200	a	b	ab
24_05_2018	PhiPS2_Licor6800	*	0.012	ns	0.954	ns	0.688	ab	b	a

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Chapter 5

Transcriptomic analysis on berry pulp and skin

5.1 Introduction

In the previous chapters the effect of different soils in Glera and Corvina cultivars was studied at both phenological and physiological level. Our analyses revealed that this terroir factor has a significant impact on grapevine growth, influencing the phenology and the developmental processes at both vegetative and reproductive stages, and effecting several berry traits, such as sugar content, acidity, and berry weight (Chapter 4).

Although phenotypic plasticity is an important ecological phenomenon, the underlying genetic and molecular mechanisms still remain poorly characterized (Fusco and Minelli, 2010). Phenotypic variation between species and organism of the same species may reflect differences in gene structure as well as differences in gene expression, but phenotypic plasticity among clones of the same genotype is likely to be much more dependent on differential gene expression in different environments (Gilad et al., 2006). Transcriptome plasticity has been described in model organisms such as the fruit fly *Drosophila melanogaster* (Zhou et al., 2012), the mouse *Mus musculus* (Hamilton and Yu, 2012), the nematode *Caenorhabditis elegans* (Li et al., 2006) and other non-model organisms in wild or controlled environments (Bay et al., 2009; Cheviron and Brumfield, 2009; Debes et al., 2012). Nevertheless only a few studies have been conducted in plants cultivated in open fields, where they are exposed to multiple environmental stimuli that induce complex responses in terms of gene expression, metabolic activity, and epigenetic modifications. Concerning grapevine (*Vitis vinifera* L.), the growing interest of scientific community and wine producers, on GxE interactions, together with the availability of a fairly good reference genome and more than 100 sequenced varieties, led to a boost in studies on this issue. Recently, in a first of a kind study, the phenotypic plasticity in grapevine was evaluated by comparing the berry transcriptome of a single clone of Corvina throughout 3 consecutive vintages cultivated in 11 different vineyards in the Verona area. This study led to the identification of approximately 1400 plastic transcripts, which responded to different environments, meant as the combination of pedo-climatic, agronomical and geographical conditions (Dal Santo et al., 2013). More recently, Dal Santo et al. (2018) tried to dissect the basis of grapevine GxE interactions characterizing the berry plasticity at level of transcriptome, methylome and allele specific expression (ASE), in two varieties cultivated in three different environments over two vintages. Using a novel approach, they were able to identify genes with expression profiles that

were: unaffected by genotype or environment, genotype-dependent but unaffected by the environment, environmentally dependent regardless of genotype, and GxE-related.
Our challenge in the present study is to go further, trying to underly the genetic and molecular mechanisms at the base of phenotypic plasticity in response to a single terroir factor and not to a combination of variables difficult to dissect. Taking advantage of the high-throughput expression profiling technologies now available, we analyzed gene expression on a global scale, trying to investigate the berry transcriptome plasticity in response to different soils considered and described in the previous chapters. Although grapevine plasticity has been described also in other studies, to our knowledge, this is the first study transcriptomic study focusing on a singular terror factor keeping all the other ones as constant as possible.

5.2 Materials and Methods

5.2.1 Sampling

For the molecular analysis, three phases throughout berry maturation were considered: softening (34 E-L stage, corresponding to 'berries begin to soften'), mid-ripening (36 E-L stage, corresponding to 'intermediate Brix value'), and harvest (38 E-L stage, corresponding to 'berries harvest-ripe'). For each plant, three berries were collected from the central part of a representative cluster at the same time of the day (from 10.00 to 11.00 am). Berries from the same soil-box were pooled together, thus a singular biological replicate was constituted of 12 berries sampled from 4 plants. Once collected, berries were immediately frozen in liquid nitrogen and transported to the lab where they were removed of the seeds and peeled out of the skin. The two tissues, skin and pulp, were separately ground by mortar and pestle in liquid nitrogen and stored in polypropylene tubes at -80°C until further processing.

5.2.2 RNA extraction, quantification, quality check

Total RNA was extracted from approximately 400 mg of skin and pulp tissue. We used the Spectrum^{TM} Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions with some modifications (Fasoli et al., 2012).

RNA quantity and quality were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and a capillary electrophoresis in agarose gel respectively.

5.2.3 mRNA sequencing

A total number of 108 RNA samples (3 soils x 3 biological replicates x 2 cultivars x 2 tissues x 3 time points) were obtained and processed for mRNA-seq by Illumina technology. RNA-seq library preparation, quality and quantification of pooled libraries and high throughput sequencing by Illumina technology were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy). Libraries were prepared starting from 1 ug of total RNA using the HiSeq 2500 platform (Illumina) sample preparation protocol (Illumina Inc., https://www.illumina.com/) according to the manufacturer's instructions. Paired-end reads of 100 nucleotides (nt) were obtained using an Illumina HiSeq 2500 sequencer, and sequencing data were generated using the base-calling software Illumina Casava v1.8.2. After RNA Sequencing the raw data (FASTQ) underwent quality control analysis using FastQC Version 0.11.5 (Leggett et al., 2014) and the Illumina reads were pre-processed to remove low-quality reads and sequencing adapters with Trimmomatic Version 0.39 (Bolger et al., 2014). The resulting pre-processed reads were aligned to the reference genome of Vitis vinifera 12X (Ensembl Plants Genome release 43) with the Subread aligner Version 1.6.4 (Liao et al., 2014). Raw read counts were extracted from the Subread alignments using the featureCount Version 1.6.4 read summarization program (Liao et al., 2014). Summarized read count data were normalized using the Trimmed Mean of M-values (TMM) method, which provides between-sample normalization while correcting for variations in sequencing depth and sample variation (Robinson and Oshlack, 2010).

5.2.4 Statistical analysis

Row read counts were transformed and normalized using a "variance stabilizing transformation" (VST), which leads different samples to have a comparable variance between them and also normalizes on the size of the library (Love et al., 2014). The VST normalized read counts were used to identify DEGs among various treatments by using EdgeR Version 3.26.5 (Robinson et al., 2010). Results were analyzed by I-way ANOVA for the investigation of the soil effect on both cultivars taken together and separately. In order to functionally classify the

genes affected by soil factor the Gene Ontology terms were retrieved and imported in Blast2GO software v2.5.0 (Conesa and Götz, 2008). Alternatively, the Plant_slim GO term assignation to DEGs was performed by means of the AgriGO v2.0 on line tool (http://systems biology.cau. edu.cn/ agriGOv2/; Tian et al., 2017).

Using the same tool, Gene Ontology (GO) Singular Enrichment Analysis (SEA) was applied to the different lists of DEGs (depending on the variables chosen). To do that we used the gene ID of the Gramene release 50 (Tello-Ruiz et al., 2018), a Fisher statistical test with a Yekutely multi-test adjustment method (FDR under dependency) and a statistical significance lower than 0.05.

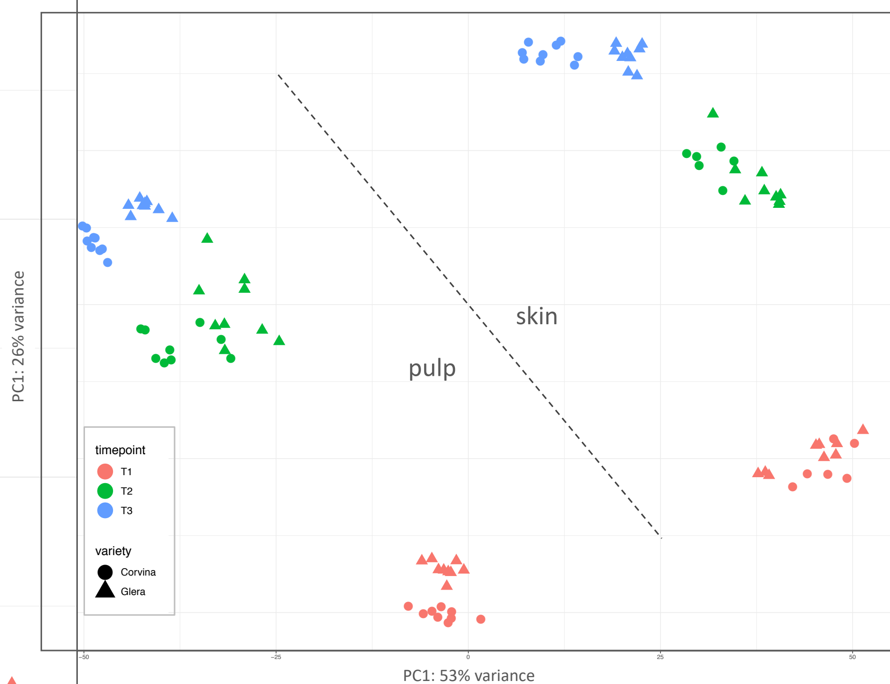
Graphical representation of genes expression, hierarchical, and k-Means (KPM) clustering were performed using the Multi Experiment Viewer software (MeV; Saeed et al., 2006) using the Pearson correlation coefficient. Venn diagrams and identification of common and specific DEGs were performed using Venny on line tool (https://bioinfogp.cnb.csic.es/ tools/venny/) (Oliveros, 2018). The choice of the optimal k-value in k-means clustering was performed using different estimation methods including the Elbow method, the Silhouette method, the Sum of squares method and the NbClust tool. All these analyses were performed using the R software.

5.3 Results and Discussion

5.3.1 RNA-seq an read mapping on *V. vinifera* reference genome

With the aim of gaining a better comprehension of the molecular mechanisms underlying the plastic responses of Glera and Corvina genotypes to different soils considered, an mRNA-seq analysis by means of NGS technologies was performed. The whole transcriptome sequencing was achieved using a HiSeq 2500 platform (Illumina). The analysis was accomplished on 108 samples described in Chapter 5.2, in order to obtain a detailed screenshot of the transcriptome modulation in both cultivars throughout the entire developmental kinetics. Sequencing produced a total number of 852.988.367 paired-end reads (100 bp length for both forward and reverse reads respectively), with a number of reads produced for each sample ranging from 3 to 13 millions, an average value of 8 mln/sample and a median of 7.4 million reads. An average of 76 % total reads passed the quality control test (filtered based on read length after low quality base trimming) and were mapped onto the PN40024 v1 prediction of the grapevine

reference genome (Jaillon et al., 2007) producing a number of unique reads ranging from 2 to 11 million depending on the sample. Data were transformed using a "variance stabilizing transformation" (VST), which leads different samples to have a comparable variance between them and also normalizes on the size of the library and used to build a Principal Components Analysis (PCA). As illustrated in **Figure 5.1**, PC1 explained 53 % of the total variance and clearly divided the samples based on the two tissues (flesh or skin). The PC2 explained 26 % of the variance and well divided the three developmental stages considered (softening, mid-ripening, and harvest). Moreover, in both tissues and in each singular time point considered, especially at harvest, the two cultivars were clearly separated. Conversely, when looking at the soil effect on a whole transcriptome scale, the PCA did not clearly separate flesh and skin samples on both varieties, suggesting that transcriptional rearrangements due to this terroir factor are limited to a reduced number of transcripts (**Fig 5.2**).



al Component Analysis (PCA) based on VST-normalized data obtained from 108 RNA-
tuted of skin and flesh tissues of Glera and Corvina varieties at softening (T1), mid-
pening (T3) phases. Colors indicate different developmental stages.

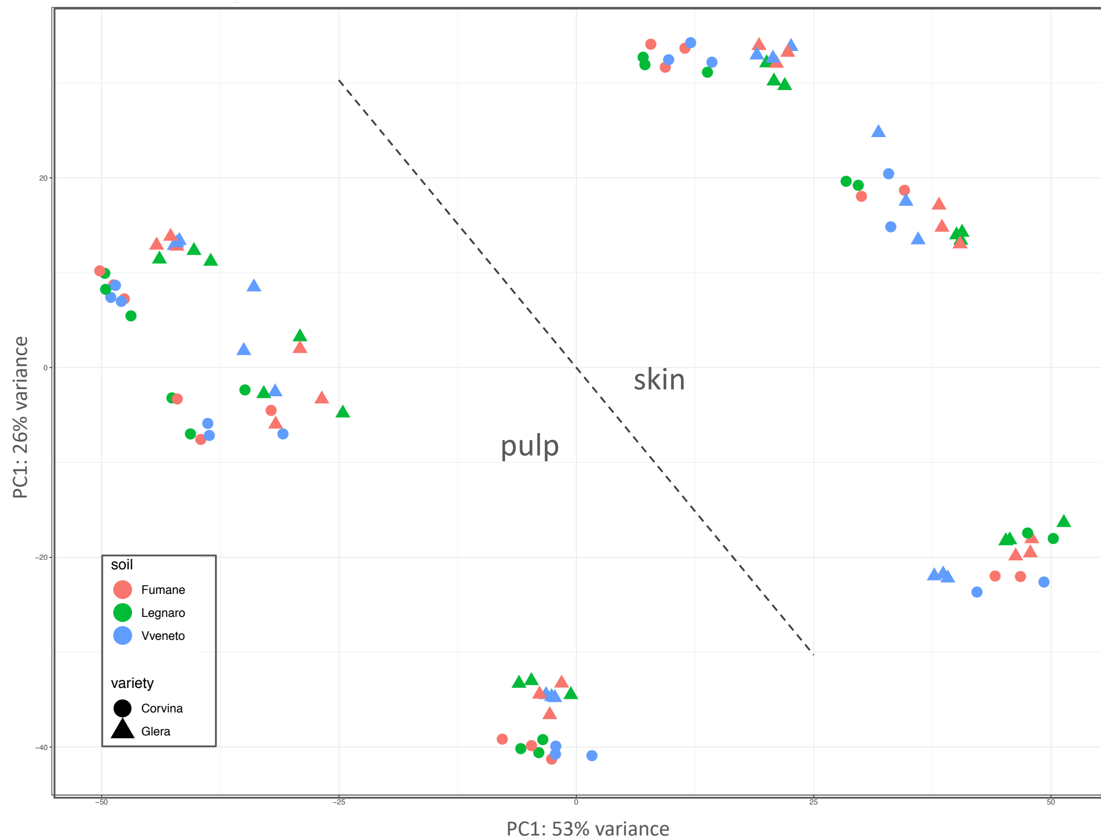


Figure 5.2 – Principal Component Analysis (PCA) based on VST normalized data obtained from 108 RNA-seq samples constituted of skin and pulp tissues of Glera and Corvina varieties in softening (T1), mid-ripening (T2) and ripening (T3) phase. Colors indicate different soils considered in the experiment, namely Fumane, Legnaro and V. Veneto.

5.3.2. Identification of plastic genes as a response to soil factor at harvest

Successfully mapped read counts were used to identify plastic transcripts as a response to soil factor at harvest (T3) since , for this stage, grape berry transcriptome has been reported to be characterized by deep plasticity which is responsible for the diverse qualitative traits (Dal Santo et al., 2013). The number of transcripts showing significant modulation among different soils was assessed separately in pulp and skin tissues and separately for both genotypes (Glera and Corvina) by one-way analysis of variance (ANOVA). To define which genes were differently expressed as response to soil factor, we set a threshold p-value lower than 0.01 identifying a total number of 1408 differently expressed genes (DEGs). This number represents the sum of those genes significantly affected by different soils in each of the four variety/tissue combination considered, i.e. Corvina skin, Corvina pulp, Glera skin, and Glera pulp. In detail, 964 plastic genes were identified in *V. vinifera* cv. Glera, with 401 DEGs detected in skin tissue

and 653 ones in flesh (**Figure 5.3a**). Amongst these, 90 transcripts were common between the two tissues considered, whereas 521 genes were flesh-specific (80% of the total Glera pulp) and 297 genes were skin-specific (74%; **Figure 5.3b**). In *V. vinifera* cv. Corvina we detected a lower number of DEGs, with 505 plastic genes identified. Unlike Glera, in this variety the highest number of genes with statistical significance was in skin (297) compared to flesh (216; **Figure 5.3a**). Only 8 of them were in common between the two tissues, whereas 256 genes were specifically modulated in skin (86%), and 182 ones were pulp-specific (84%; **Figure 5.3b**). Thus, transcriptomic data at harvest suggest a higher plasticity of Glera compared to Corvina variety and a different sensitivity of flesh and skin tissues in the two genotypes, with Glera showing a higher transcriptome plasticity in pulp and Corvina in skin respectively. Looking at the common genes between the same tissue in both varieties, Glera and Corvina flesh shared 25 genes, while Glera and Corvina skin shared 12 genes. None of the 1408 genes was common to all the four variety/tissue combinations (**Figure 5.3b**). Among the 25 commonly expressed genes in Corvina and Glera pulp, we found genes involved in the primary metabolism of protein, carbohydrate and nucleobase, as well as in the regulation of gene expression (e.g. zinc finger), response to stimulus (disease and stress resistance such us R protein). We also noticed genes related to the ethylene and kinase protein signaling pathways. Seven of them were unrecognized (“No Hit”) or uncharacterized (“Unknown” and “Unknown protein”). Among the 12 commonly expressed genes in Corvina and Glera skin, we found genes involved in the response to stimuli, primary metabolism, as well as in the regulation of the gene expression (a zinc finger) and some unknown protein.

Hierarchical clustering (HCL) analysis on the 1441 soil-responsive genes detected, revealed five major clusters (**Figure 5.3c**). A first cluster (cluster 1) included 155 genes showing higher expression levels in both skin and flesh of cv Glera respect to Corvina. Cluster 2 encompassed 197 genes expressed in pulp of both Glera and Corvina varieties. Cluster 3 included 490 genes expressed in the skin of both varieties. Cluster 4 had 321 genes expressed exclusively in both Corvina tissues and finally cluster 5, which was less clearly defined respect to the others, included 313 genes which were strongly expressed in Corvina pulp (but partially also in skin and in Glera pulp) and strongly downregulated in Glera skin.

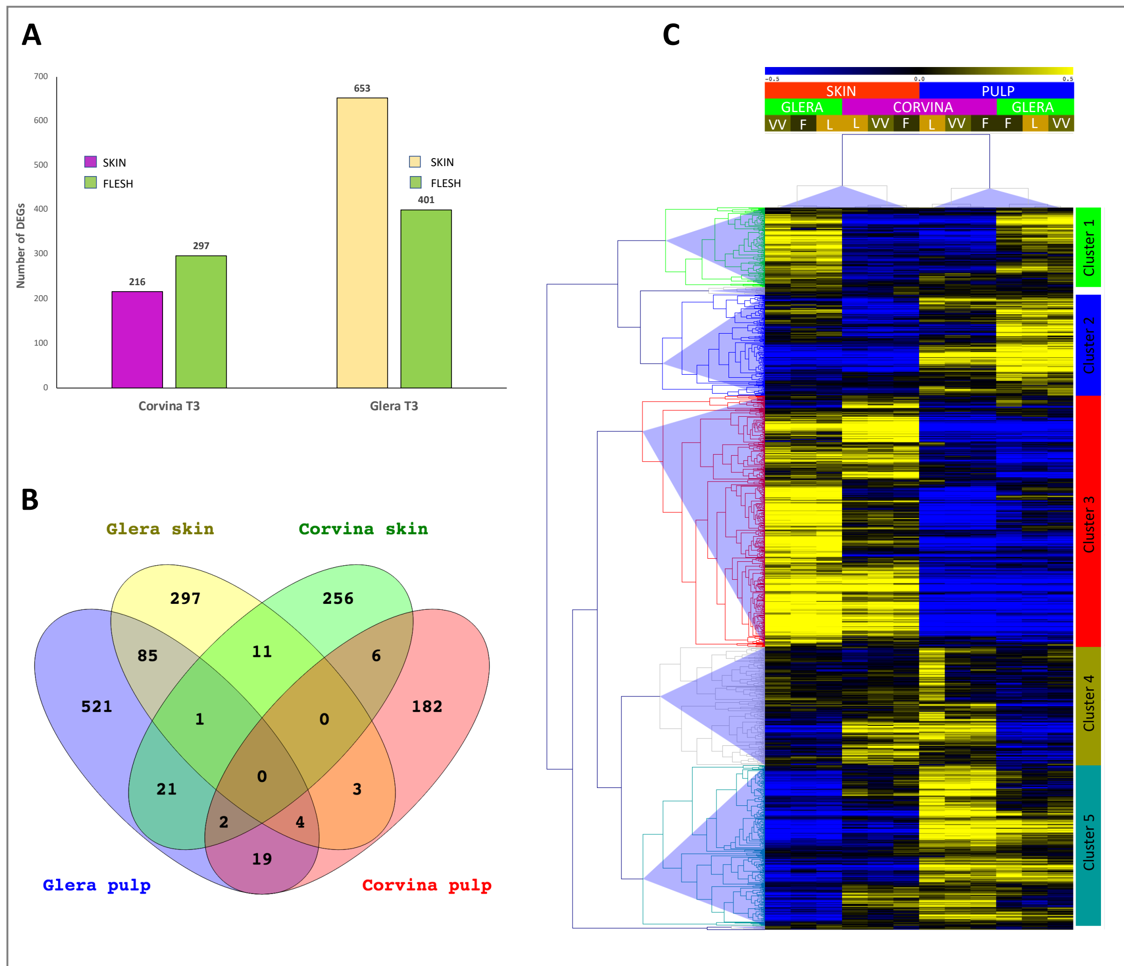


*Figure 5.3 – Differentially expressed Genes (DEGs) identified by means of one-way ANOVA in both skin and flesh of Glera and Corvina varieties. **A**, histograms showing the different numbers of significant genes in each condition; **B**, Venn diagram indicates the number of common and specific genes at ripening phase (T3); **C**, Hierarchical clustering analysis of transcripts that were differentially modulated among different soils in both variety (Glera and Corvina) and tissues (flesh and skin) at harvest (T3). One-way analysis of variance (P <0.01) was used to define transcripts whose expression is modulated in at least one variety/tissue combination. Pearson's correlation distance was used as the metric to create the transcriptional profile dendrogram. Data are the average of the three biological replicates (n=3).*

5.3.3 Functional classification and enrichment analyses of plastic genes

The 1408 genes identified by the one-way ANOVA analysis were assigned to the respective GO terms using the Go_Slim database, a reduced subset of the ontology designed specifically for plants. Given the low number of statistically significant genes in each singular tissue/variety combination (Glera skin, Glera pulp, Corvina skin, Corvina pulp), the number of enriched GO categories in each combination was quite low (data not shown). For this reason, we proceeded

with a general preliminary approach to provide an overview of the main biological processes involved in response to soil independently by the tissue and/or variety considered. Among the 1408 DEGs, one-thousand-one-hundred forty-one (1141) IDs were functionally annotated and grouped into the following functional categories based on the GO biological process domain: “regulation of biological process”, “reproduction”, “biological regulation”, “developmental process”, “multicellular organismal process”, “cellular process”, “reproductive process”, “metabolic process”, “growth”, “localization”, “multi-organism process”, and “response to stimuli” (**Figure 5.4**).

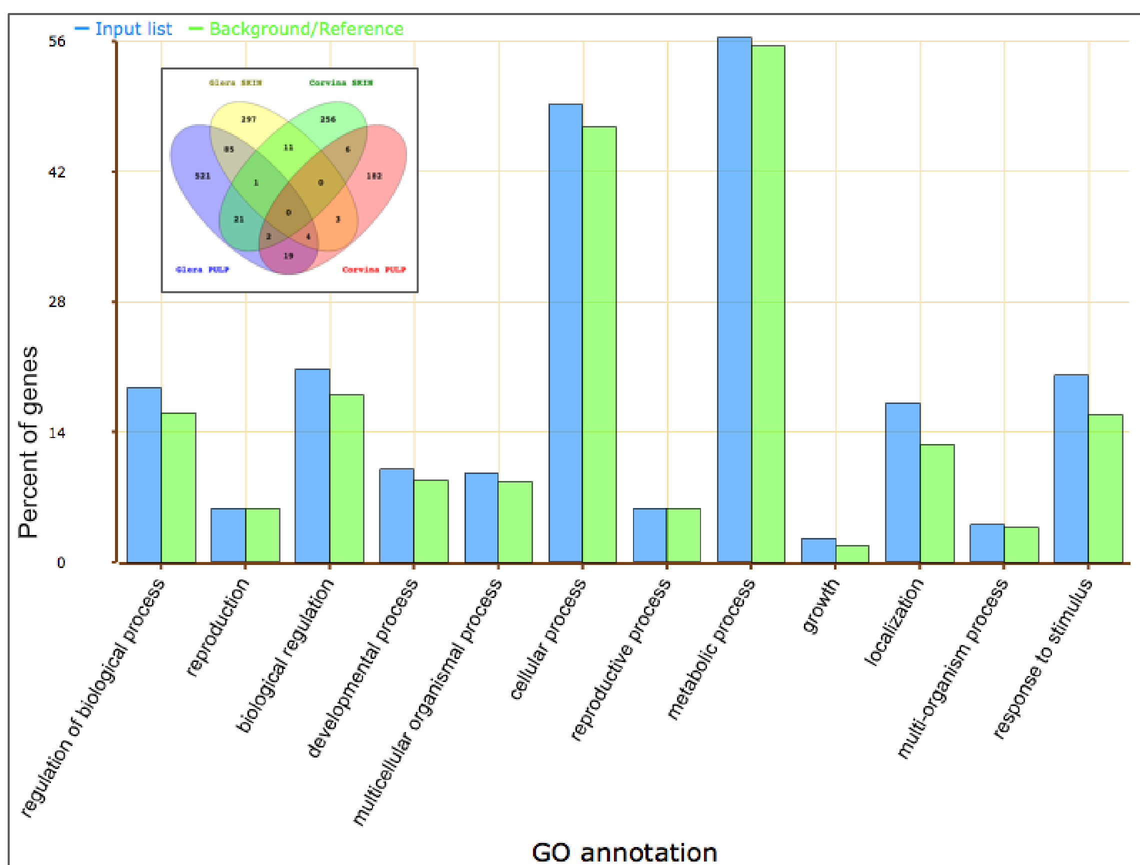


Figure 5.4 – GO Plant-Slim flash chart of 1141 (out of 1408) plastic transcripts detected in both tissues (skin and flesh) of Glera and Corvina varieties. Blue bars indicate the percentage of terms identified in our subset of significant transcripts whereas green bars indicate the percentage of terms associated to the GO-annotated genes in the whole PN40024 12x v1 prediction (22.924 genes).

The analysis of transcript functional categories revealed that 14% of such plastic genes were unrecognized (“No Hit”) or uncharacterized (“Unknown” and “Unknown protein”). In order to

gain insights into the biological processes involved in the soil response plasticity, the 1141 GO-annotated plastic genes were analyzed for over-represented functions using a Singular Enrichment Analysis (SEA). Nine GO categories were found to be significantly enriched respect to the whole genome annotation: “response to abiotic stimulus” (GO:000968), “response to external stimulus” (GO:0009605), “response to stimulus” (GO:0050896), “transport” (GO:0006810), “regulation of anatomical structure size” (GO:0090066), “regulation of cell size” (GO:0008361), “establishment of localization” (GO:0051234), “localization” (GO:0051179), and “regulation of cellular component size” (GO:0032535). Amongst these macro categories those ones showing the highest significance (FDR < 0.05) belonged to categories “regulation of anatomical structure” (195 DEGs), “localization” (19 DEGs) and “response to stimulus” (229 DEGs), for what concerns the Biological Process domain. Regarding the cell component domain, enriched GO categories belonged to “membrane”, “cytoplasm”, “Vacuole” and “thylakoid”. Finally, the only category enriched in the Molecular function domain was “transport activity” (**Figure 5.5**).

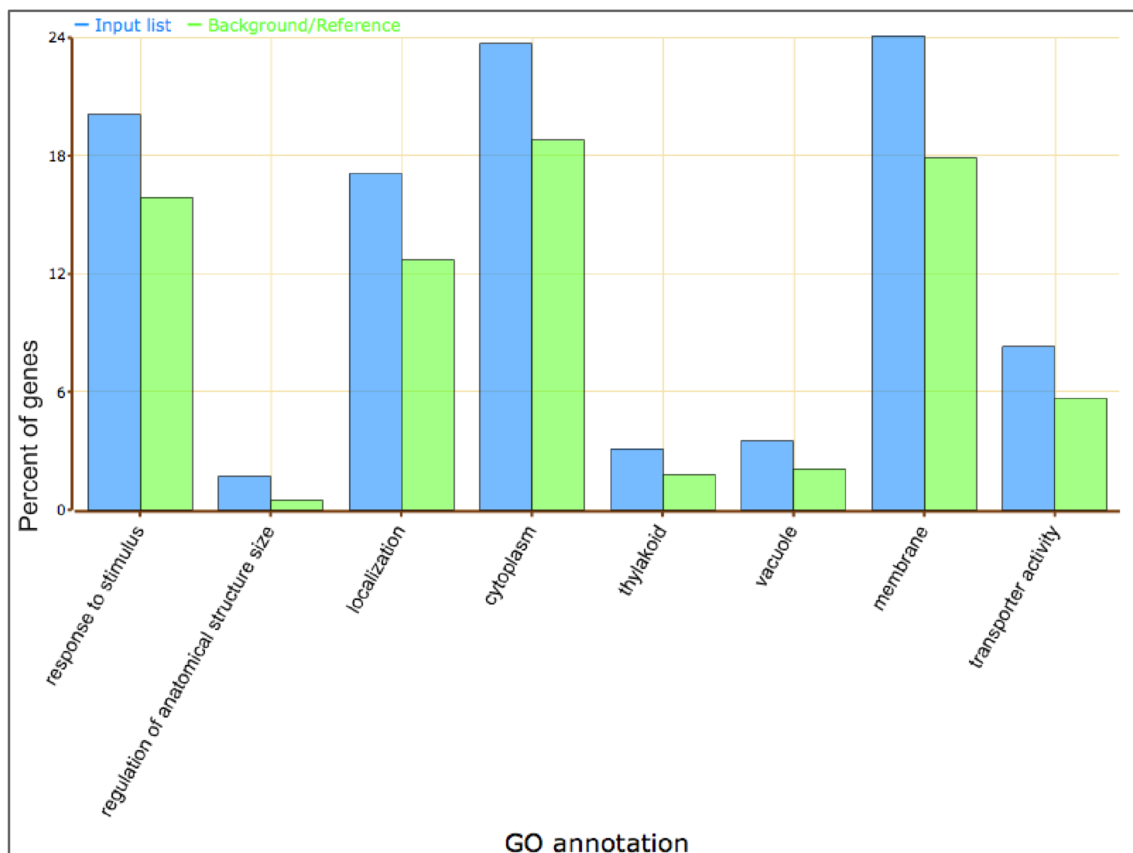


Figure 5.5 – Singular Enrichment Analysis (SEA) on 1141 GO-annotated DEGs genes identified in both skin and flesh tissues of Glera and Corvina varieties. Concerning the Biological process domain, the terms

"response to stimulus", "regulation of anatomical structure" and "localization" were significantly enriched (FDR < 0.01). For Cellular compartment enriched terms were "cytoplasm", "thylakoid", "vacuole" and "membrane", whereas only the "transporter activity" was enriched within the Molecular function domain.

Of particular interest is the category related to the regulation of anatomical structure for its highest statistical significance. In fact, although only 19 out of 1141 GO-annotated DEGs belonged to this ontology (1.6%), it should be considered that over the 20.929 GO-annotated genes in the whole grapevine genome, only 112 genes are associated to this term (0.5%). Looking more in detail at the distribution of these genes between the different varieties and tissues considered, we found that they were equally distributed between Glera and Corvina, with 11 genes significantly modulated in Corvina (5 in skin, 5 in pulp and one in both tissues) and 9 in Glera (4 in skin, 3 in pulp and 2 in common).

Table 5.1 – List of the 19 DEGs belonging to the enriched GO category "regulation of anatomical structure". The functional annotation based on Grimplet et al. (2012), and the variety-tissue combination where they were detected (indicated with an X) are reported.

Gene ID	Functional annotation	Corvina skin	Corvina pulp	Glera skin	Glera pulp
VIT_00s0227g00200	formin protein AHF1	x			
VIT_02s0012g01410	TRN2 (TORNADO 2)	x			
VIT_02s0025g02020	Abl interactor 3 (ABIL3)			x	x
VIT_08s0007g02030	AUX1 protein				x
VIT_08s0007g05060	ABC transporter B member 1	x			
VIT_08s0058g00780	DISTORTED3/SCAR2		x		
VIT_10s0116g01740	ATP synthase gamma chain 1t (ATPC1)	x			
VIT_11s0016g00100	Adapter protein SPIKE1 (SPK1)		x		
VIT_11s0016g00160	forminy 2 domain-containing protein		x		
VIT_14s0006g03280	Nuclear matrix constituent protein 1		x		
VIT_14s0060g02220	F-actin capping protein alpha subunit				x
VIT_14s0108g00430	ABC transporter B member 16			x	
VIT_15s0046g01640	Abl interactor protein 1 (ABIL1)			x	

VIT_16s0022g00470	Peroxisomal biogenesis factor 11 (PEX11)			x	
VIT_17s0000g02420	Auxin transport protein (PIN3)		x		
VIT_17s0000g05070	Phytochelatin synthetase	x		x	
VIT_17s0000g10010	Callose synthase catalytic subunit	x	x		
VIT_18s0001g13360	Nodulin MtN21 family				x
VIT_19s0014g02740	Metallothionein			x	x

Just one gene was shared by the two varieties (VIT_17s0000g05070), specifically only in skin tissue. It encodes for a phytochelatin synthetase and was also recently reported as a meta-QTL candidate gene differentially expressed across veraison (Delfino et al., 2019). In our experiment it was differently modulated when comparing the skin of the two cultivars: it was found to be downregulated in F compared to L soil but upregulated in F compared to VV in Corvina and Glera respectively.

Table 5.1 summarizes the distribution and functional annotation of the 19 size-related genes in all condition considered at ripening whereas **Figure 5.6** shows the expression pattern of these genes in the different soils considered. Although a clear molecular function for most of these genes is still lacking, and we cannot track a straight relation with the regulation of anatomical structures in grapevine, it's worth to notice that the physiological analyses related to berry growth showed statistically significant differences between the size of berries in plants grown in different soils, thus confirming a modulation of berry organ development at both molecular and physiological level. Moreover, those genes showing the highest differences between soils appear to be related to heavy metals, such as *VIT_19s0014g02740* which encodes for a metallothionein and is upregulated in pulp of Corvina plants grown in Vittorio Veneto (CPVVT3A) or *VIT_17s0000g05070*, encoding for a phytochelatin synthase, which is downregulated in the pulp of Glera grown on the same soil (GPVVT3A). Two other genes involved in the regulation of anatomic structure are *VIT_08s0007g02030* and *VIT_17s0000g02420*. *VIT_08s0007g02030* encodes for an auxin influx protein whereas *VIT_17s0000g02420* for an auxin efflux protein. Auxin are known to be involved in the control and regulation of berry development and berry size, as previously reported in Corso et al. (2016), Cookson et al. (2013), and Gillaspy et al. (1993). In other species such as apple (*Malus domestica*), it has been shown that IAA applications increase fruit size and reduces abscission in apple, while an excess of IAA results in reduced growth and fruit drop (Devoghalaere et al.,

2012). Another gene that could be grouped with auxin-related genes is *VIT_02s0012g01410* encoding an orthologous of the protein TORNADO 2 (TRN2), a putative transmembrane protein belonging to the family of tetraspanins. These proteins, widely distributed throughout the animal and plant kingdoms, interact with a variety of proteins, such as other tetraspanins, integrins, proteoglycans, growth factors, growth factor receptors, and signaling enzymes (Hemler, 2001, 2003; Maecker et al., 1997) and seem to be involved in auxin distribution and homeostasis, and seem to be essential for the establishment of development, thereby controlling the interplay between cell cycle progression and differentiation (Cnops et al., 2006).

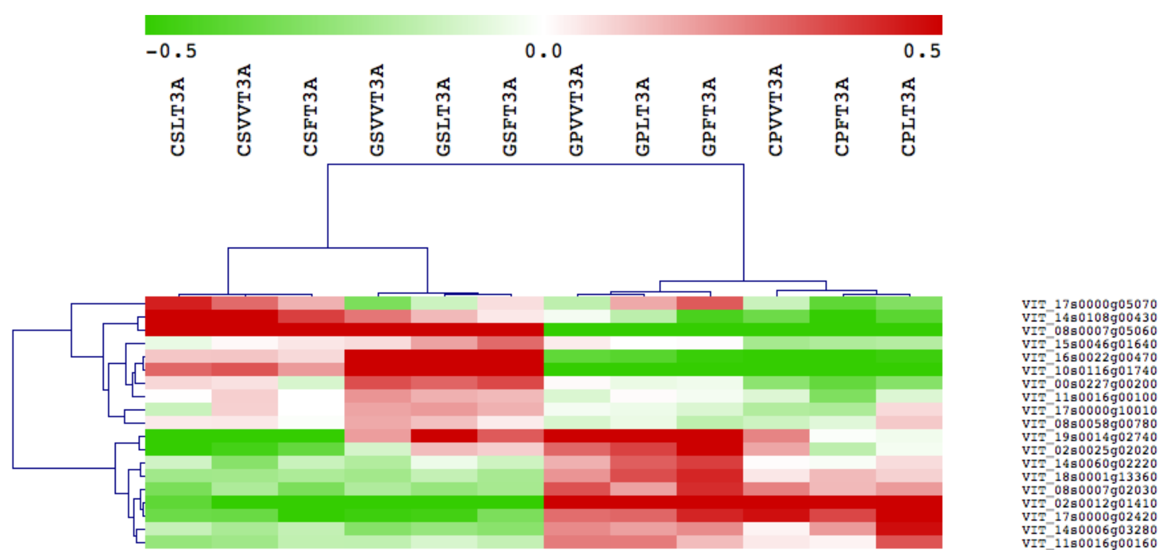


Figure 5.6 – Heatmap showing the expression of 19 DEGs associated to the GO term “regulation of anatomical structure” in both tissues (skin and pulp) of Corvina and Glera varieties at harvest (T3). Samples are identified as follows: variety: C (Corvina) or G (Glera); tissue: S (skin) or P (pulp); soil: L (Legnaro), VV (Vittorio Veneto) and F (Fumane).

5.3.4. K-means clustering of berry pulp and skin DEGs

In order to achieve an unimodal distribution of the 844 DEGs identified in pulp of both Corvina and Glera variety by the ANOVA, we performed a k-means clustering analysis of the average VST-normalized count data applying increasing values of k (k=1 to 10; **Figure 5.7**) and we selected the right number of clusters using different methods, including the Elbow and the NbClust methods (**Figure 5.7**). We identified k=8 as the optimal number of clusters.

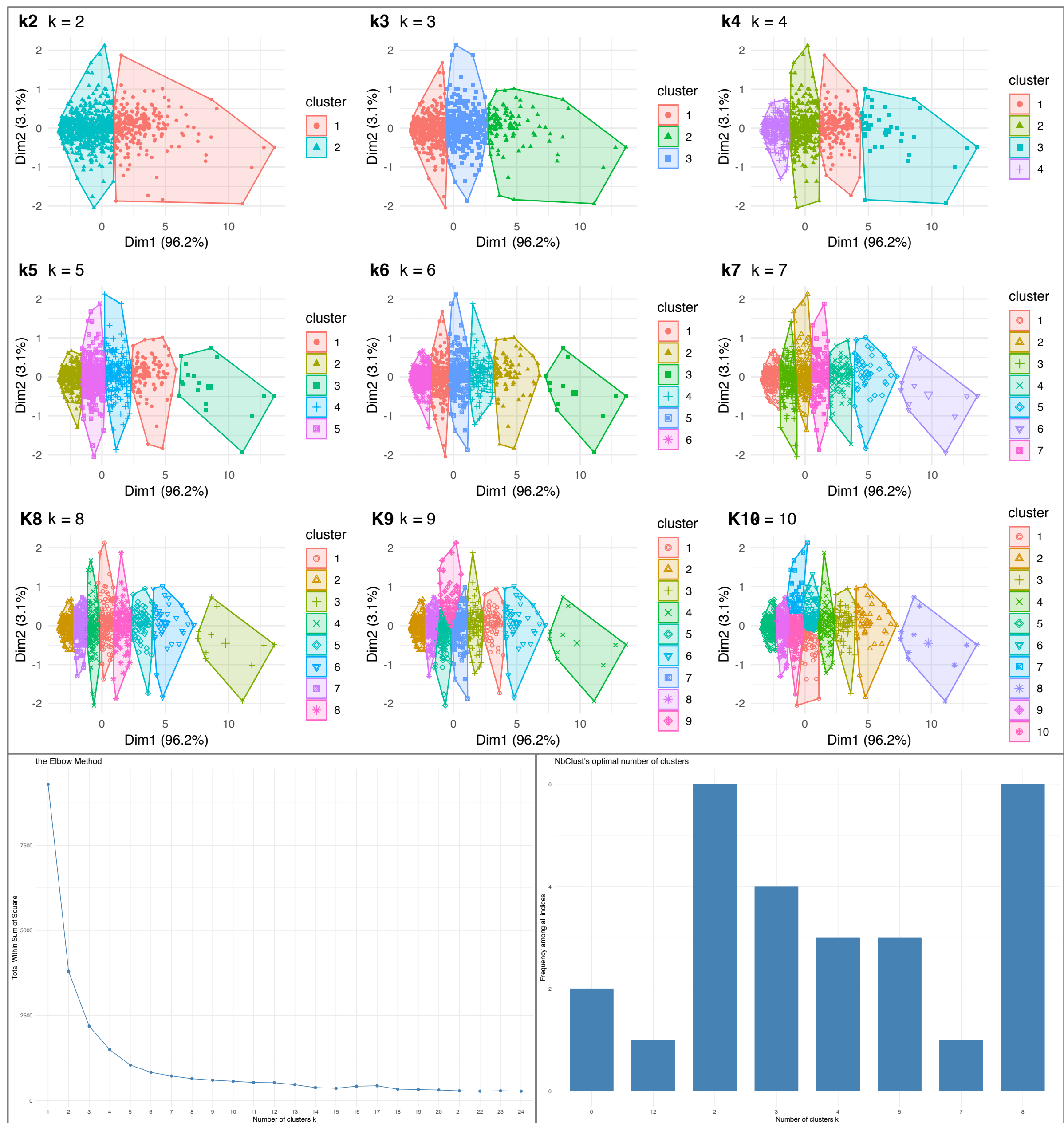


Figure 5.7 – Setting the number of clusters for different values of k. Increasing values of k (2 to 10) were applied to the VST-normalized read count of the 844 DEGs detected in pulp of both Corvina and Glera varieties. Visual assessment (upper window), the Elbow method (down-left window), and the histograms (down-right window) based on the 30 indices of the NbClust R package. Based on both methods we selected k = 8.

Figure 5.8 reports the heatmap showing the expression pattern of transcripts belonging to the 8 clusters identified by the k-means analysis, together with their visual assessment, indicating where delineations occur between clusters. Amongst the 8 cluster, we did not take into account those showing a clear separation in terms of gene expression between the two

varieties (cluster 1, cluster 5, cluster 8). Conversely, we considered of great interest all other clusters, which showed a clear effect of the soil in the two genotypes. The genes belonging to Cluster 2 showed an increased expression in Corvina pulp, which was limited to the Legnaro and soil and not to the Fumane and Legnaro ones. Cluster 3 is composed of a few genes showing an induction limitedly to the pulp of Corvina berries grown in Legnaro soil and of Glera in V. Veneto one. In cluster 4 genes were induced exclusively in the pulp of Glera berries grown in V. Veneto soil.

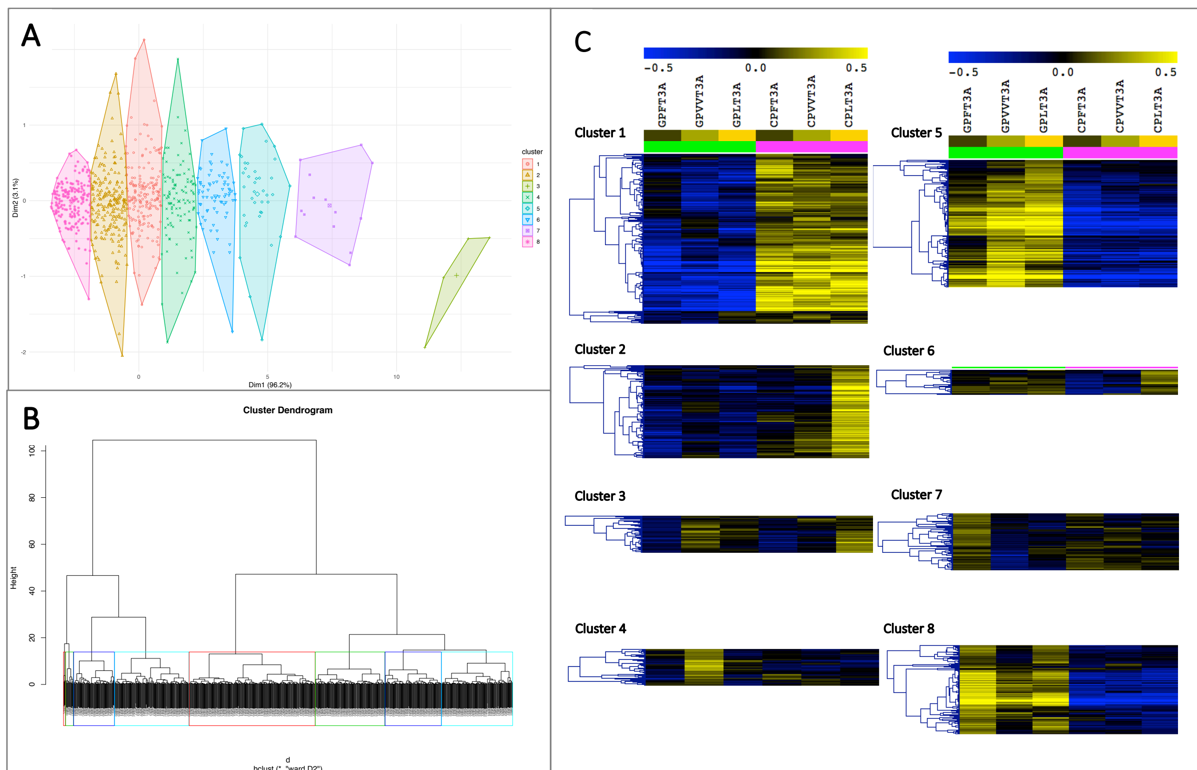


*Figure 5.8 – **A**, Visual assessment indicating where delineations occur between the 8 clusters identified by means of k-means analysis for the merged DEGs of Corvina and Glera pulp; **B**, dendrogram showing the clusters of different genes from a topological point of view; **C**, heatmap showing the expression pattern of transcripts belonging to the 8 clusters identified by the k-means analysis. Pearson's correlation distance was used as the metric to create the transcriptional profile dendrogram in panels B and C. Data are the average of the three biological replicates. Thesis code indicates the cultivar ("G" Glera, "C" Corvina), the tissue ("S" Skin), the type of soil ("L" Legnaro, "VV" V. Veneto, "F" Fumane), and the time point ("T3" harvest).*

For what concern the skin tissues, the Elbow and NbClust methods indicated k=4 as the optimal number of clusters from the 686 skin DEGs, as illustrated in **Figure 5.9**. Also in this case we did not consider those clusters that clearly divided the two varieties but only those ones showing differences in gene expression patterns amongst different soils within the same variety (**Figure 5.10**).

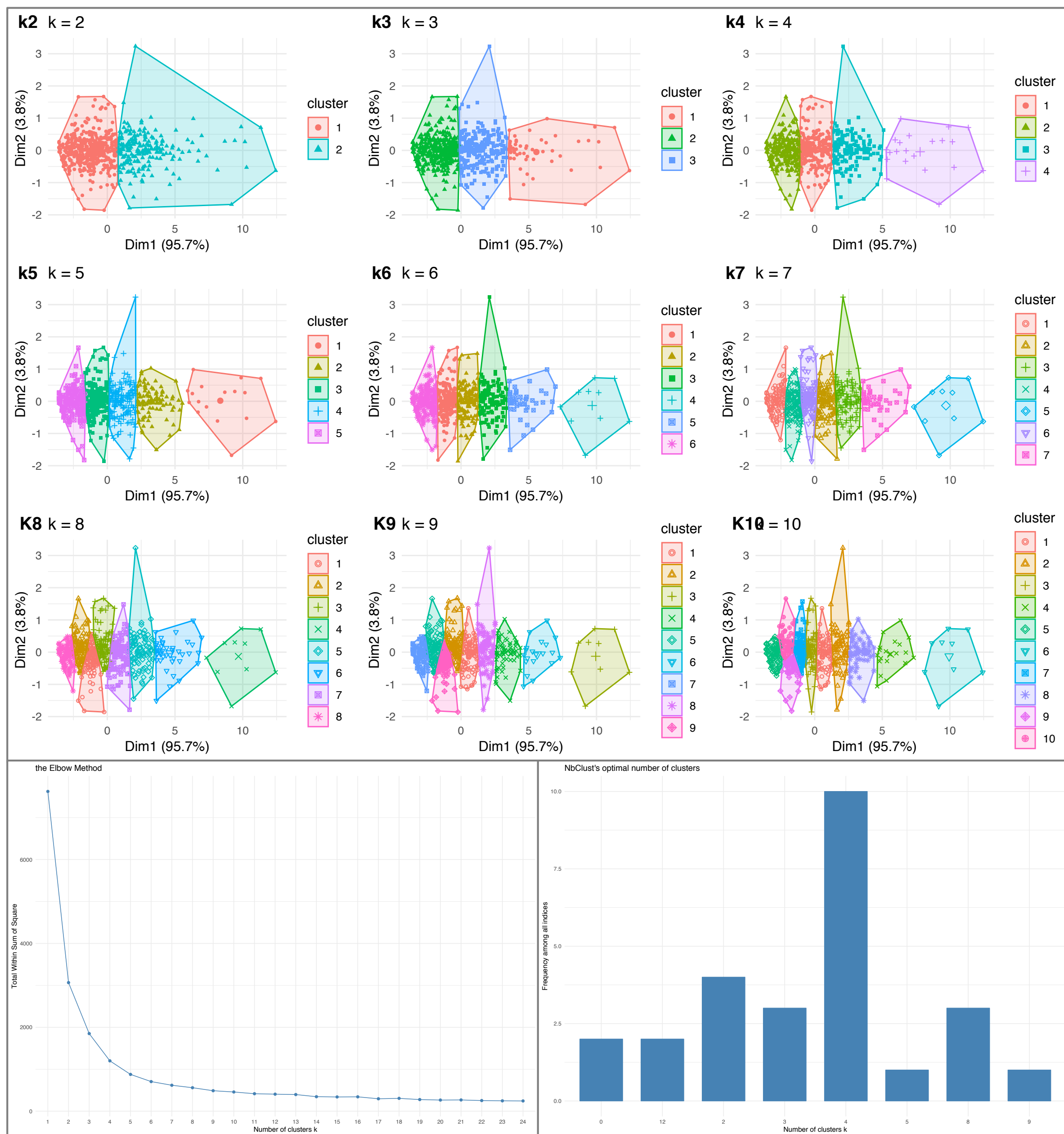


Figure 5.9 – Setting the number of clusters for different values of k. Increasing values of k (2 to 10) were applied to the VST-normalized read count of the 686 DEGs detected in skin of both Corvina and Glera varieties. Visual assessment (upper window), the Elbow method (down-left window), and the histograms

(down-right window) based on the 30 indices of the NbClust R package. Based on both methods we selected k = 4.

In this particular case, the expression patterns were less clear. Cluster 3 was composed of genes showing a down regulation in the skin of Corvina plants grown in Legnaro and V. Veneto soils, whereas cluster 4 showed a marked induction of genes in Glera grown in V. Veneto and in general a downregulation in both varieties grown in Legnaro soil.

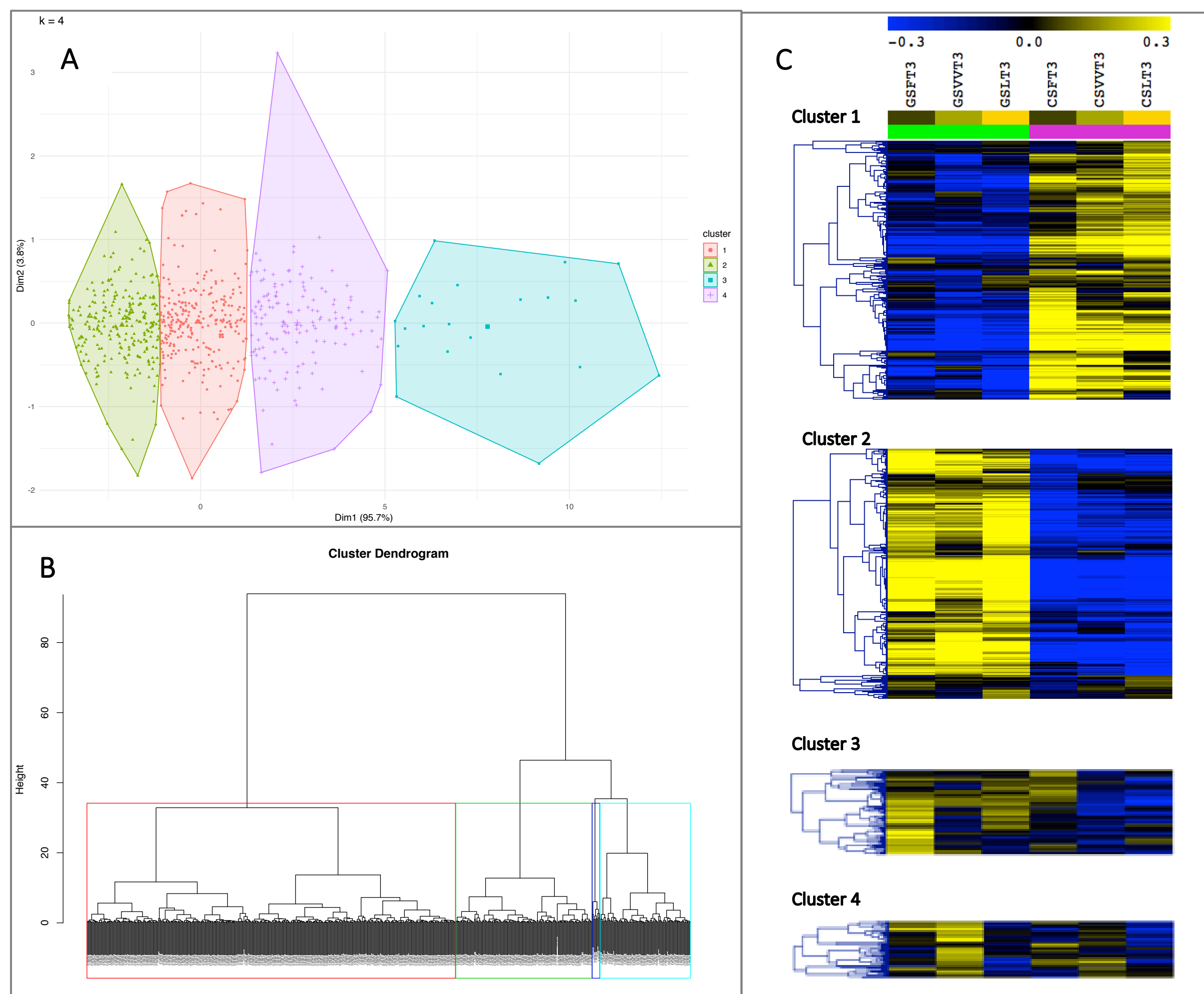


*Figure 5.10 – **A**, Visual assessment indicating where delineations occur between the 4 clusters identified by means of k-means analysis for the merged DEGs of Corvina and Glera skin; **B**, dendrogram showing the clusters of different genes from a topological point of view; **C**, heatmap showing the expression pattern of transcripts belonging to the 4 clusters identified by the k-means analysis. Pearson's correlation distance was used as the metric to create the transcriptional profile dendrogram in panels B and C. Data are the average of the three biological replicates. Thesis code indicates the cultivar ("G"*

Glera, "C" Corvina), the tissue ("S" Skin), the type of soil ("L" Legnaro, "VV" V. Veneto, "F" Fumane), and the time point ("T3" harvest).

5.3.5 K-means clustering of berry pulp and skin DEGs in both Corvina and Glera separately

When analyzing the four conditions separately (Corvina skin, Corvina pulp, Glera skin, Glera pulp) it became clear how DEGs of each tissue-cultivar combination were differently modulated among the three soils involved in the experiment (Fumane, Legnaro, Vittorio Veneto; **Fig 5.11**).

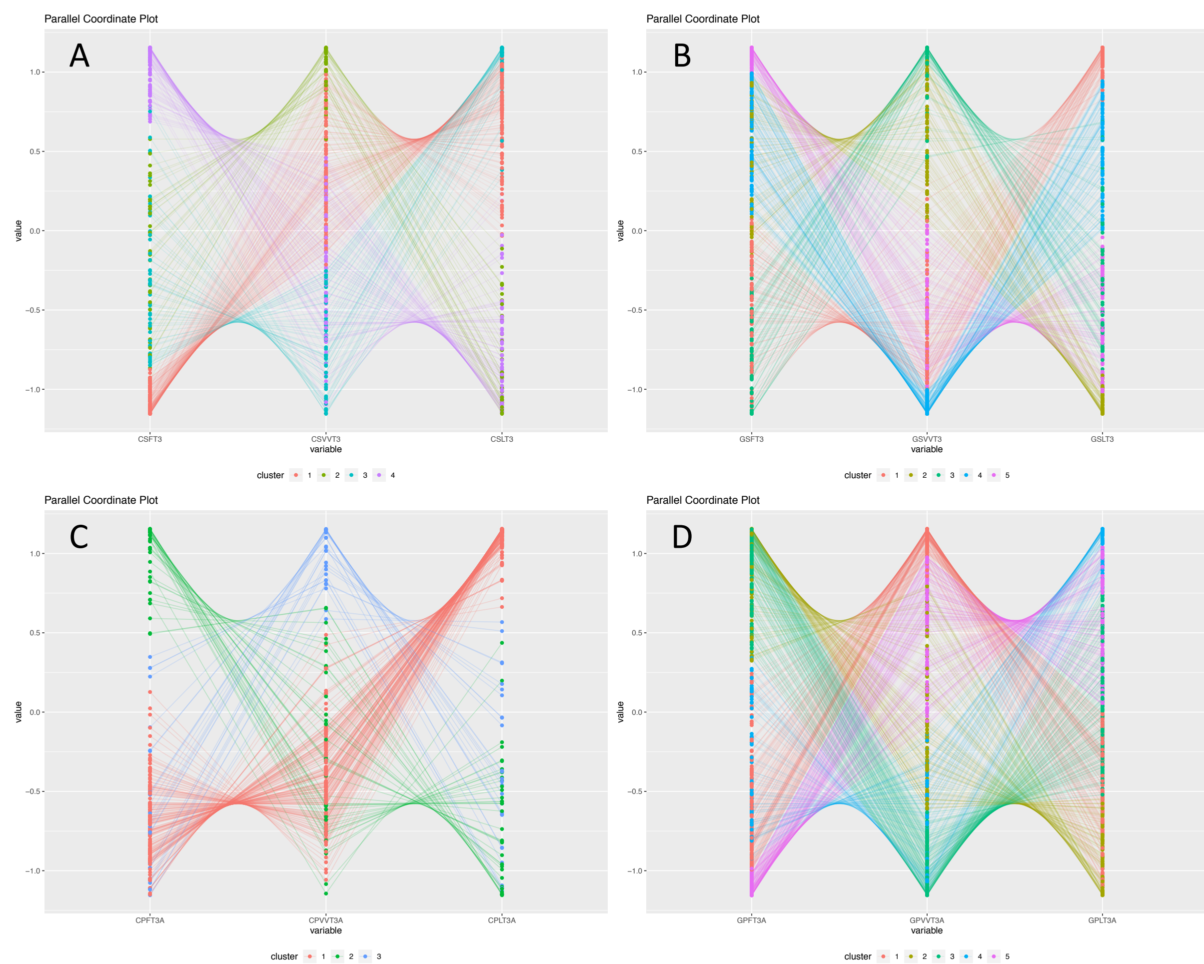


Figure 5.11 – K-means cluster analysis of DEGs when dividing the four tissue-cultivar conditions at harvest (T3): Corvina skin (A), Glera skin (B), Corvina pulp (C), and Glera pulp (D). In the abscissa, names are composed by the cultivar (Corvina or Glera) followed by the type of tissue (Skin or Pulp), the type of soil (F, VV, L), and T3 standing for harvest point. Different clusters are represented by different colors.

The 297 DEGs related to Corvina skin were divided into four clusters according to the methods already described. Each cluster was characterized by a specific profile among soils (**Tab 5.2**). Specifically, in cluster 1 genes were down-regulated in F when compared to L, in cluster 2 genes were up-regulated in VV when compared to L, in cluster 3 genes in L soil were up-regulated when compared to VV, and in cluster 4 genes from F were up-regulated with respect to L.

It is worth highlighting some genes involved in the flavonoid biosynthesis: UDP-glucuronic acid/anthocyanin glucuronosyltransferase (VIT_04s0044g01540; cluster 1) which was recently classified as UFGT by Rienth et al. (2014); a leucoanthocyanidin dioxygenase (VIT_08s0105g00380; cluster 1), also found highly modulated as response to cluster thinning in Sangiovese (Pastore et al., 2011), but down-regulated in water deficit condition in Savoi et al. (2016). The latter was also proposed to be regulated exclusively by MYBA1 (Matus et al., 2009). Two anthocyanin 3-O-galactosyltransferase (VIT_06s0009g01990 and VIT_06s0009g02010; cluster 2) also proposed to be regulated by MYBA1 (Matus et al., 2009); an anthocyanin permease AM3 (VIT_16s0050g00900; cluster 2), which being an AM3, it contributes to specifically transport acetylated forms (Gomez et al., 2009; Muñoz et al., 2014). An anthocyanin membrane protein 1 (Anm1; VIT_08s0007g03570) was found in cluster 4 and it has been recently highly correlated with WRKY26 (Amato et al., 2017); it is potentially involved in the role of anthocyanin amount and composition, anyway reported to be more expressed in the skin than in the pulp (Costantini et al., 2015). An isoflavone 2'-hydroxylase was also found (VIT_07s0129g00860; cluster 3). Some auxin associated genes were found to be differently modulated in Corvina skin, which expression was always lower in Fumane compared with Legnaro (cluster 1). VIT_14s0060g01720 encodes for a dormancy/auxin associated family protein which was found in other studies to be upregulated in *V. pseudoreticulata*, a Chinese variety resistant to the downy mildew (Liu et al., 2019). In the same paper the auxin efflux carrier protein (PIN7) was found to be downregulated; however we found VIT_01s0011g04640 as auxin efflux carrier. Genes belonging to the MYB family were found in cluster 4: Myb TKI1 (TSL-kinase interacting protein 1; VIT_07s0031g01930) which is known to be a transcription factor gene identified as switch gene in both red and white cultivars (Massonnet et al., 2017). Switch genes are related to the transition phase from herbaceous to the ripening phase (corresponding to veraison point), and are likely involved in such phenomena (Palumbo et al., 2014). Myb domain protein 24 (VIT_14s0066g01090) and MYBPA1 protein (VIT_15s0046g00170) coding genes were also detected. The first one is a

transcription factor coding gene recently found to be up-regulated in an early ripening mutant (Ma and Yang, 2019), while the second one is a gene was found to be overexpressed at harvest as result of a leaf defoliation treatment at veraison (Pastore et al., 2013). It is known to control the expression of proanthocyanidin pathway, especially in early berry development (herbaceous phase). Its different modulation in ripening and late-ripening (harvest) might be due to its role in anthocyanin biosynthesis as well, by controlling the regulation of some genes upstream of UFGT as suggested by Falginella et al. (2012). These transcription factors were also found to be up-regulated in an early ripening mutant (Ma and Yang, 2019). These results might suggest a slight anticipation of ripening promoted by F soil as well as a different amount of anthocyanidins and tannins produced in Corvina skin in F soil-treatment.

Table 5.2 – Mean center profiles of the four clusters identified for Corvina skin DEGs as response to the three soils at harvest (T3). Soils are identified by the letter (F, VV, L). "C" and "S" stand for "Corvina" and "skin" respectively. Gene expression values were row standardized by subtracting the row-wise (gene) mean and then dividing it by the row-wise SD.

cluster	CSFT3	CSVVT3	CSLT3
1	-1.0802621	0.3722714	0.7079907
2	-0.1655687	0.9989906	-0.8334218
3	-0.2992192	-0.7303731	1.0295923
4	1.0191567	-0.2471977	-0.771959

The 401 DEGs related to Glera skin were divided into five clusters (**Tab 5.3**). In cluster 1 genes from L soil were up-regulated when compared to those ones from the other two soils, contrary to cluster 2 where genes from L were down-regulated instead. In cluster 3 genes from VV were down-regulated when compared to F, in cluster 4 genes from VV were down-regulated, in cluster 5 genes from F were up-regulated if compared to the ones from the other two soils.

Among them we found genes related to flavonoid biosynthesis such as isoflavone 2'-hydroxylase (VIT_07s0129g00730; cluster 1), flavodoxin-like quinone reductase 1 (VIT_00s0271g00110; cluster 3), flavonol synthase (VIT_13s0047g00210; cluster 3), leucoanthocyanidin dioxygenase (VIT_11s0118g00360; cluster 3), isoflavone reductase protein 2 (VIT_03s0088g00140; cluster 4), UDP-glucose/anthocyanidin 5,3-O-glucosyltransferase (VIT_16s0050g01580; cluster 4), another Isoflavone reductase

(VIT_03s0038g04700; cluster 5). Genes related to auxin (VIT_07s0031g02200, VIT_10s0003g00090, VIT_09s0002g05160, VIT_14s0083g00940, VIT_18s0001g08090, VIT_18s0001g12100, VIT_05s0020g04680, VIT_13s0067g00330) from different clusters were identified, as well as gene from the transcription factor MYB family (VIT_14s0060g02640, VIT_07s0197g00060, VIT_01s0026g01050, VIT_07s0005g01210, VIT_17s0000g04130, VIT_18s0001g09850), potentially involved in the ripening process (auxin related genes) and in the biosynthesis of flavonoids (MYB family). The gene encoding for WRKY DNA-binding protein 51 (VIT_04s0069g00970, cluster 3) was recently found to be up-regulated in an early ripening mutant (Ma and Yang, 2019), co-expressed with VviSST genes (Vannozzi et al., 2018), and up-regulated under ultraviolet-C treatment (Suzuki et al., 2015). An ethylene responsive gene was also found in cluster 3 (VIT_16s0013g00900) that, as supported by Cramer et al. (2014), the expression of this gene is supposed to decrease throughout ripening, and the fact that it is still more expressed in VV soil might be related to the slight delaying of VV berries ripening. Moreover, it was reported that ethylene signaling and biosynthesis might be involved in the production of grape aroma. In Ma & Yang (2019) it was one of the transcripts down-regulated in the early ripening mutant, and its function was confirmed to be related to the ripening process (Licausi et al., 2010). It was found positive correlating with genes related to stilbene biosynthesis such as to cis-piceid content in vines resistant to downy mildew (Vezzulli et al., 2019), as well as up-regulated by high temperature and correlating with heat stress response activation genes (Carbonell-Bejerano et al., 2013).

Table 5.3 – Mean center profiles of the five clusters identified for Glera skin DEGs as response to the three soils at harvest (T3). Soils are identified by the letter (F, VV, L). "G" and "S" stand for "Glera" and "skin" respectively. Gene expression values were row standardized by subtracting the row-wise (gene) mean and then dividing it by the row-wise SD.

cluster	GSFT3	GSVVT3	GSLT3
1	-0.441324	-0.6432673	1.0845913
2	0.477939	0.6078067	-1.0857458
3	-0.74574	1.0367165	-0.2909765
4	0.5962218	-1.1092786	0.5130567
5	1.1089984	-0.539279	-0.5697194

The 216 DEGs of Corvina pulp were divided into three clusters: genes were found to be up-regulated in L, F, and VV for cluster 1,2, and 3 respectively, when compared to their down-regulation in F, L, and VV respectively (**Tab 5.4**).

Among them, we mention two genes related to auxin metabolism: AP2-like ethylene-responsive transcription factor (VIT_08s0007g08580; cluster 1), auxin transport protein (PIN3; VIT_17s0000g02420; cluster 1). Auxin transporter genes were recently related to a greater number of final berries per cluster due to their interaction with the flower and fruitlet abscission, specifically reducing the phenomena (Grimplet et al., 2017). But their effect at harvest is still unclear. Anyway, in *Solanum lycopersicum*, SlPIN4 expression was related to a delaying in early fruit development, especially in young fruits (Mounet et al., 2012), suggesting us slight delay of berry maturation in L and VV soils. Some genes involved in flavonoid biosynthesis were identified: flavodoxin-like quinone reductase 1 (VIT_19s0014g04660, cluster 2), and chalcone-flavanone isomerase (VIT_13s0067g02870, cluster 3) which was recently found to be down-regulated in small berries both in berry development and ripening when compared to large berries (Wong et al., 2016).

Table 5.4 – Mean center profiles of the three clusters identified for Corvina pulp DEGs as response to the three soils at harvest (T3). Soils are identified by the letter (F, VV, L). “C” and “P” stand for “Corvina” and “pulp” respectively. Gene expression values were row standardized by subtracting the row-wise (gene) mean and then dividing it by the row-wise SD.

cluster	CPFT3A	CPVVT3A	CPLT3A
1	-0.6941458	-0.4035526	1.0976983
2	0.996423	-0.3093701	-0.6870529
3	-0.6107002	0.9888156	-0.3781154

The 653 DEGs of Glera pulp divided into five clusters (**Tab 5.5**). In cluster 1 and cluster 4 genes were up-regulated in VV and L respectively, while in cluster 5 genes from F were down-regulated. In cluster 2 and 3, F soil was related to the up-regulation of the belonging genes when compared to those ones from L and VV respectively.

Many genes related to auxins, ethylene, abscisic acid, flavonoid biosynthesis, transcription factors were found. Among them, Myb domain protein 14 gene (VIT_05s0049g01020; cluster

1) is a TF involved in the regulation of the stilbene biosynthesis in grapevine (Höll et al., 2013; Vannozzi et al., 2018) even though other TFs might take its role over in certain condition (Suzuki et al., 2015) and its expression is not always linked with a different modulation in STSs expression (Savoi et al., 2016). It was also found to be down-regulated as response to red blotch disease (Blanco-Ulate et al., 2017), up-regulated in an early ripening mutant (Ma & Yang, 2019), and strongly modulated by water deficit (Savoi et al., 2017). Its expression was also found consistent between small and large berries (Wong et al., 2016). Other MYB related genes were VIT_04s0043g00340 (KANADI 1; cluster 1), VIT_07s0197g00060, VIT_12s0028g00980, VIT_18s0001g09850, VIT_08s0040g00900, VIT_17s0000g07510. Some of them (VIT_04s0043g00340, VIT_05s0049g01020, VIT_18s0001g09850, VIT_08s0040g00900) were reported to be involved in the ripening process (Ma & Yang, 2019), but one identified as switch gene correlating with some NACs in Zenoni et al. (2019). MYB44 (VIT_18s0001g09850, cluster 2) is likely related to the repression of sucrose transporters expression in berries (Agudelo-Romero et al., 2013). Since this fact, sucrose accumulation in Glera pulp might have been stopped in F soil but not in L at harvest, mirroring the slight advancing of ripening in the first soil. The same result was found both for in skin and pulp tissues od such cultivar.

WRKY DNA-binding protein 51 (VIT_07s0031g01710, cluster 1), WRKY DNA-binding protein 75 (VIT_17s0000g01280; cluster 1), WRKY DNA-binding protein 70 (VIT_08s0058g01390; cluster 5) encoding genes were also found. They are related to the modulation of STS genes since VIT_17s0000g01280 is considered capable of activating the promoter of elicitor-responsive genes (Lijavetzky et al., 2012), while VIT_07s0031g01710 was found co-expressed with VviSST genes in Vannozzi et al. (2018) and up-regulated under ultraviolet-C treatment (Suzuki et al., 2015). This gene had also similar expression profile among soils in both Glera pulp and skin. In cluster 1 we also noticed an ethylene-responsive element binding factor coding gene (VIT_16s0013g00890) which was reported to be possibly involved in the ripening (Ma & Yang, 2019). Indeed, ERF TFs coding genes are known to play an important role in the ripening process.

Table 5.5 – Mean center profiles of the five clusters identified for Glera pulp DEGs as response to the three soils at harvest (T3). Soils are identified by the letter (F, VV, L). "G" and "P" stand for "Glera" and

"pulp" respectively. Gene expression values were row standardized by subtracting the row-wise (gene) mean and then dividing it by the row-wise SD.

cluster	GPFT3A	GPVVT3A	GPLT3A
1	-0.5980798	1.09157196	-0.4934922
2	0.935012	-0.026491	-0.9085211
3	0.9197496	-0.9763176	0.05656794
4	-0.3796684	-0.6855482	1.06521662
5	-1.0973014	0.54512242	0.55217894

5.3.6 Comparison of the modulation of the DEGs shared among the two cultivars

As reported in the Venn diagram previously described, some DEGs were found in common between Corvina skin and Glera skin, and between Corvina pulp and Glera pulp (**Fig 5.12**). Nevertheless, their modulation among the three soils were not always the same.

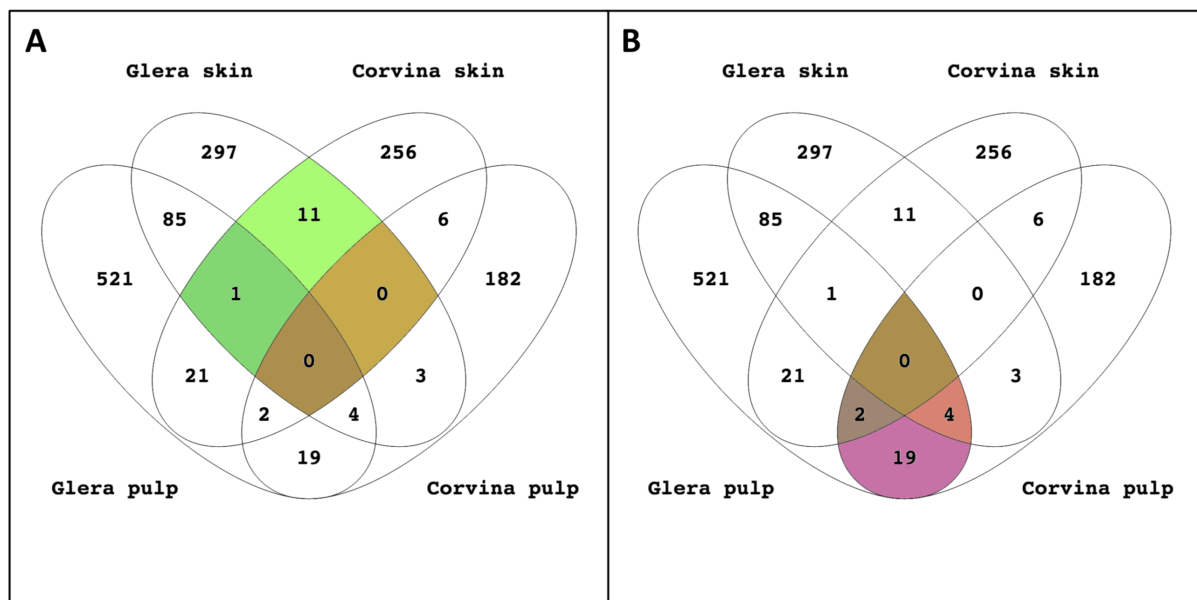


Figure 5.12 – Venn diagrams highlighting the number of common DEGs between Glera and Corvina skin (A), and between Glera and Corvina pulp (B) as response to different soils at harvest (T3).

In **Table 5.6** we reported the list of genes differently modulated in both Corvina and Glera skin, and the clusters where they were grouped into according to the k-mean analysis (see previous paragraph). The list refers to genes mainly involved in the primary metabolism, two unknown proteins, one regulation of gene expression and one response to stimuli. For the most part,

common DEGs had different expression pattern among soils with an array of different combinations. For example, the gene coding for the abscisic stress ripening protein 2 (ASR2; VIT_18s0072g00380) was significantly up-regulated in L soil in both Corvina and Glera skin but its lower expression was more clear in F and VV soils respectively. In the literature, it was reported as one of the most abundant transcripts found in five cultivars at harvest (Ghan et al., 2015), as well as one of the gene responsive to water deficit (Medici et al., 2014) and with a potential role in heat-induced berry responses (Lecourieux et al., 2019). The gene coding for the zinc finger protein (VIT_06s0004g02630) was up-regulated in the skin of Corvina berries from L soil, but in F soil of Glera skin. Other genes showed different patterns: down-regulated in F from Corvina but in VV from Glera (VIT_17s0000g05070); up-regulated in F from Corvina but down-regulated in F of Glera (VIT_08s0007g01630). Just one of them had a clearl similar pattern among soils. It encoded for an endochitinase A2 precursor (VIT_05s0077g01250) which were highly expressed in L soil when compared to the other two soils in both Corvina and Glera skin.

Table 5.6 – list of the common DEGs between Glera and Corvina skin. The number of cluster refers to the k-means clusterization previously described of each cultivar-tissue condition. Functional annotation according to Grimplet et al. (2012).

Gene ID	Functional annotation	Corvina cluster	Glera cluster
VIT_00s1937g00010	Amine oxidase	3	4
VIT_03s0110g00200	Apocytochrome b	4	2
VIT_04s0008g01380	Unknown protein	3	5
VIT_05s0077g01250	Endochitinase A2 precursor	3	1
VIT_06s0004g02630	Zinc finger (C3HC4-type ring finger)	3	5
VIT_08s0007g00540	Haloacid dehalogenase hydrolase	3	5
VIT_08s0007g01630	Tubulin beta-2 chain	1	5
VIT_08s0007g03110	Transposon protein, Mutator sub-class	1	3
VIT_14s0060g00250	Unknown	3	4
VIT_16s0039g00820	CYP89A5	2	1
VIT_17s0000g05070	Phytochelatin synthetase	1	4
VIT_18s0072g00380	Abscisic stress ripening protein 2 (ASR2)	1	1

In **Table 5.7** we reported the list of genes differently modulated in both Corvina and Glera pulp, and the clusters where they were grouped into according to the k-mean analysis. They mainly belonged to the primary metabolisms as well as to regulation of gene expression, signaling, and response to stimuli. Similarly to skin tissue, also common genes in pulp followed different

expression patterns among soils when comparing the two cultivars, but in this tissue we mainly noticed similar combinations: down-regulated in F in both cultivars but differently in the other two soils (1-5 combination), up-regulated in F in both cultivars but differently in the other two soils (2-3 combination), up-regulated in L and VV in Corvina and Glera respectively.

Table 5.7 – list of the common DEGs between Glera and Corvina pulp. The number of cluster refers to the k-means clusterization previously described of each cultivar-tissue condition. Functional annotation according to Grimplet et al. (2012).

Gene ID	Functional annotation	Corvina cluster	Glera cluster
VIT_00s0246g00140	NADH-plastoquinone oxidoreductase subunit 5	1	1
VIT_00s0379g00020	Transducin protein	1	5
VIT_00s2512g00010	No hit	1	1
VIT_02s0012g00240	Unknown protein	1	5
VIT_02s0012g00250	Vacuolar protein sorting 13C protein	1	5
VIT_02s0012g00270	Pleckstriny (PH) domain-containing protein	1	5
VIT_05s0020g00410	C2 domain-containing protein	1	1
VIT_05s0020g02000	SYD (splayed)	1	5
VIT_06s0004g02600	MOM1 (maintenance of methylation1)	1	1
VIT_06s0004g04470	Heat shock protein 70	2	3
VIT_07s0031g01830	Unknown protein	1	5
VIT_10s0003g00260	DnaJ homolog, subfamily B, member 4	2	3
VIT_13s0067g02320	CTV.22	1	1
VIT_13s0147g00120	TIR-NBS-LRR-TIR disease resistance protein	1	5
VIT_13s0158g00210	RPM1 (resistance to p. syringae pv maculicola 1)	1	1
VIT_14s0060g01970	F-box domain containing protein	2	3
VIT_15s0021g02540	CCR4-NOT transcription complex, subunit 1	1	5
VIT_15s0021g02580	CCR4-NOT transcription complex, subunit 1	1	5
VIT_15s0048g01650	WD-40 repeat family protein / beige-related	1	1
VIT_17s0000g07240	Activating signal cointegrator 1 complex subunit 3	1	5
VIT_18s0001g01620	AarF domain containing kinase 1	1	5
VIT_18s0001g01670	Zinc finger (CCCH-type) family protein	1	1
VIT_18s0001g07740	SABRE	1	5
VIT_18s0089g00100	R protein L6	1	1
VIT_19s0015g00670	E3 ubiquitin-protein ligase HUWE1	1	5

5.4 Conclusions

A deep Genotype x Environment interaction was confirmed by the analysis of the berry transcriptome. We noticed that most of the DEGs found as response to different soils were tissue- specific and cultivar-specific, suggesting us that the effect of the soil, in terms of gene expression, deeply depends on the type of tissue and on the genotype. Besides the low sharing rate of common DEGs between cultivars, the importance of the soil-cultivar interaction effect

was amplified when revealing the different profile of the shared genes among the three soils and between Glera and Corvina. It is worth to noting how many combinations emerged for the common skin DEGs, but only a few were reported for the pulp DEGs, suggesting us that the network of gene modulation in berry skin is less dense than that one in berry pulp. Indeed, berry skin is known for harboring many metabolites responsible for the sensory attributes of grape and wine, mainly belonging to the secondary metabolism. Such metabolism is less essential than the primary one for the basic cell functions, hence it can afford to experience more variability in its expression. Such feature becomes interesting when working with different cultivars exposed to a specific combination of external factors, namely terroir, for the wide array of phenotypic results we could obtain.

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Chapter 6

The biochemical plasticity of the berry skin metabolome in two Italian grapevine cultivars grown in different soils

6.1 INTRODUCTION

The quality of grapevine berries mainly depends on its metabolites which are known to be sensitive to external conditions (Zhang et al., 2005). In particular, the chemical diversity is mostly affected by secondary metabolites playing a very important role in the human taste perception, although present in low concentrations (Roullier-Gall et al., 2014). Grape secondary metabolites predominantly include the phenylpropanoids, typically found in the berry skin, and comprise flavonoids, phenolic acids, stilbenes, and viniferins (Anesi et al., 2015). Particularly, the plasticity of phenylpropanoid metabolism is a well-known feature of grape berries and confers many of the wine quality traits that represent specific terroirs (Teixeira et al., 2013). The chemical composition of grapes and wine has been intensely studied in the recent decades and the number of compounds identified increased exponentially since the development of different analytical techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) coupled with nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Brun et al. 1986).
In this chapter, we analyzed the berry skin metabolites by means of LC-MS technology, hence focusing on the secondary metabolism, as response to soil differences.

6.2 MATERIALS AND METHODS

6.2.1 Berry sampling

For this biochemical assay, two ripening stages after veraison were considered in accordance with the Modified E-L System: stage II corresponding to 'intermediate Brix value' (36 E-L stage – 12 and 9 °Brix for cv. Corvina and cv. Glera respectively), and stage III or harvest corresponding to 'berries harvest-ripe' (38 E-L stage – 24 and 18 °Brix for cv. Corvina and cv. Glera respectively). Three berries were collected at the same time of the day (around 11 a.m.) from the central part of a representative cluster of each plant. Berries from plants grown in the same soil-box were pooled together to represent a single biological replicate. Berries were immediately frozen in liquid nitrogen and then transported to the lab where skin and pulp were carefully separated, but discarding the seeds, while kept frozen in dry ice, placed in polypropylene tubes, then stored at -80°C until further processing.

Analyses were focused exclusively on the grape skin corresponding to the different theses (cultivar*soil).

6.2.2 Biochemical analyses on berry skin

Berry skins were analyzed by LC-MS technology at Sede Boqer campus of Ben-Gurion University of the Negev (Israel) in collaboration with the research team of Prof. Aaron Fait.

6.2.2.1 Extraction of the samples

Before processing, samples were pulverized by means of mortar and pestle. The powder was weighed (about 200 mg), lyophilized, and metabolites were extracted in a 1 ml pre-chilled methanol:chloroform:water extraction solution (2.5:1:1 v/v). Internal standards, i.e., 300µl of 1 mg/ml ampicillin in water, and 380µl of 1mg/ml corticosterone in methanol, as described in (Weckwerth et al., 2004), were subsequently added. The mixture was briefly vortexed, 100µl of methanol was added and then placed on a horizontal shaker for 10 min at 1000 rpm. The samples were later sonicated for 10 min (Elmasonic S30, Elma, Singen, Germany) and centrifuged for 10 min (14000 rpm, microcentrifuge 5417R, Eppendorf, Hamburg, Germany). The supernatant was decanted into new tubes, mixed with 300µl of chloroform and 300µl of MiliQ water (Millipore, MA, USA), vortexed for 10 s and then centrifuged at 14000 rpm for 5 min.

6.2.2.2 UPLC analysis

The water/methanol phase, obtained from the extraction protocol, was separated and filtered in vials (0.22 µm Millipore, MA, USA) for UPLC analysis. The samples were run in a Ultra Performance Liquid Chromatography coupled to a Quadrupole Time-of-Flight Mass-Spectrometer (UPLC- QTOF MS, Waters, MA, USA) system operating in positive and negative ion modes. MassLynxTM software (Waters) version 4.1 was used as the system controlling the UPLC and for data acquisition. The raw data acquired were processed using MarkerLynx application manager (Waters) as described in Hochenberg et al. (2013). Metabolites were also annotated based on the fragmentation patterns crossed with the ChemSpider metabolite database (http:// www.chemspider.com/), the consistency of their retention times with those of identified metabolites, and the comparison with the data in the current scientific literature.

6.2.2.3 Data normalization

The value of each metabolite detected by the machine was normalized to the internal standard which reported less variability long the runs. Further accuracy was acquired by normalizing data samples to the relative dry weight of each metabolite. The values refer to the metabolite relative abundance based on ion count.

6.2.3 Statistical analyses

Statistical analyses were performed using R v3.3.1, in RStudio. The Shapiro test was used to test data for normality. The effect of the treatments, involving two cultivars and three soils, were assessed by means of multifactorial ANOVA. Significant difference between treatments was assessed by the Tukey post-hoc test at the 95% confidence level ($P<0.05$). Kruskall-Wallis test was utilized when ANOVA assumptions were not met. The principal component analysis (PCA) was based on mean centered and standardized data (unit variance scaled); results were pictured as bi-plots of scores (treatments) and loadings (variables) plots. The Pearson correlation were carried out by using the "corrplot" package in order to construct separate metabolite correlation matrices for cv. Corvina, cv. Glera, and the joint dataset, based on the entire set of samples from each treatment. Correlations were considered strong with $r > 0.5$ and $r < -0.5$.

6.3 RESULTS

6.3.1 Biochemical analyses on both cultivars – G x E

Fifty-two metabolites were detected, normalized to the internal standards and dry weight, then subjected to statistical analysis (Supplementary materials Table 1). The detected metabolites mainly belonged to the secondary metabolism, including one flavanonol and some anthocyanins (limitedly to cv. Corvina), flavanols, flavonols, hydroxycinnamic acids, and stilbenes. The analysis of variance revealed that different soils had a significant effect on the metabolite relative abundance. Such differences have to be meant as consequence of the general effect of the soil factor on the two cultivars and on both the sampling dates considered (3 soils * 2 cultivar * 2 sampling dates). Specifically, some differences regarded the anthocyanin delph-3-coum, the flavanol gallocatechin, the hydroxybenzoate hex. We also found differences in the flavonols content (rutin, myricetin, and myr-3-glu), and in stilbenes content (piceatannol, cis-piceid and trans-piceid). Soils also influenced the skin tartaric acid content. For the most part, we found that Fumane (F) soil was associated with

the highest metabolites accumulation level in berry skin, whereas Valdobbiadene soil (VV) was the one with the least values, except for the cis-Piceid content. Differently to other metabolites, the ranking of the three soils for the stilbenes content was not the same: the highest accumulation of piceatannol was observed in L soil treatment, whereas cis-piceid content was higher in berry skins from plants grown in VV soil.

The analyses also revealed several soil-cultivar interaction effects. They mainly concerned the flavonols content (myricetin and myr-3-glu), some acids (p-coumarate and caff-tart), and 4 out of 6 stilbenes detected (piceatannol, trans-piceid, cis-piceid, and delta-viniferin) (**Fig. 6.1**). The analysis of variance also allowed us to focus on the interaction effect involving all the three factors: soil, cultivar, and phenological stage (sampling date). In this case, the metabolite content that seemed to be affected by the interaction of all the three factors mostly belonged to the flavanols class (proc-B1 and gallocatechin). The effect was also noticed for delph-3-glu, caff-tart, and the stilbene delta-viniferin.

In post veraison, different soils seemed to differently affect the berry skin phenylalanine content, in which F and VV soils produced a higher values than L soil. Focusing on the date of harvest, an interaction effect between cultivar and soil was also observed for the ferulate and delph-glu content.

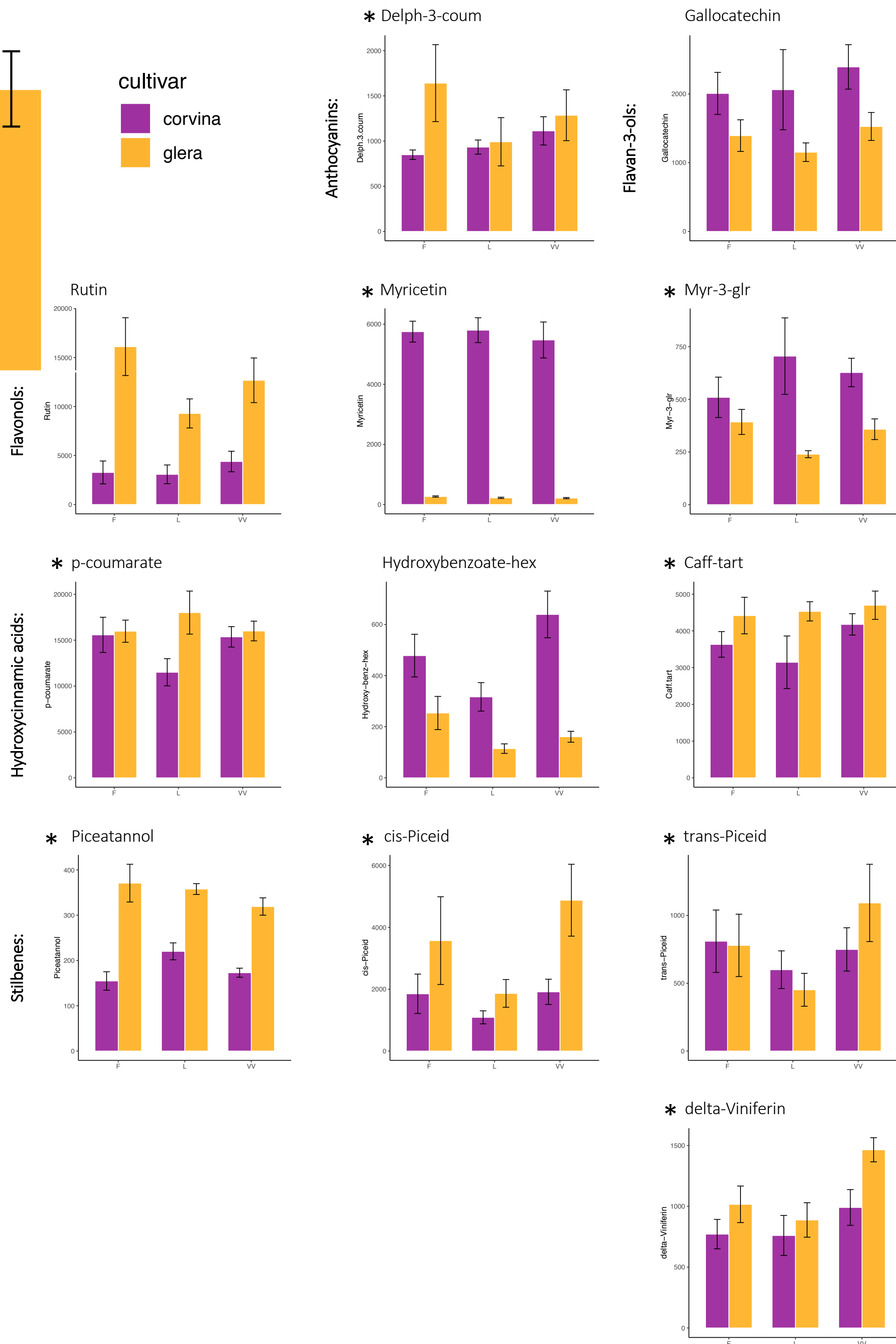

*Figure 6.1 – Metabolites found significantly different (P < 0.05) to soil factor in both time points. Levels represent relative abundance based on ion count. Asterisks indicate the metabolites affected by the cultivar*soil interaction. Error bars are standard errors (n = 6).*

6.3.2 Biochemical analyses on cv. Corvina

In comparison with the analysis on both cultivars, in cv. Corvina the soil did not significantly affect the piceid content when considering the sampling points together. Nevertheless, the piceatannol content was still higher in berry skins from plants grown in L soil. Also trans-resv content was found to be slight different among soil treatments even though not supported by statistical significance ($P<0.1$). Differences were also observed in the hydrocinnamic acids content, with L soil leading to the least value of p-coumarate. Even courtarate content almost reached significant difference for the soil factor. Other compounds with the same trend ($P<0.1$) were the amino acids phenylalanine and tryptophan, and some anthocyanins such as peo-3-glu and peo-3-acet. When analyzing the two dates of sampling separately, it was clear how F soil led to a higher content of phenylalanine at post-veraison but lower of tryptophan at harvest. At post-veraison, among the anthocyanins, delphinidins were the most affected by the soil factor ($P<0.1$). At harvest, cv. Corvina showed high plasticity: 14 out of 52 detected metabolites were found to be affected by the soil factor ($P<0.1$). Among the anthocyanins, peo-3-glu was higher in VV and F soils compared to L soil. Differences were observed also for the peo-3-acet content and for other coumaric forms such as mal-3-coum and peo-3-coum. Among the flavanols, procyanidin B1 and gallocatechin reached the highest value in F soil and always the lowest value in L soil. The same trend was observed also for the caff-tart content. Among stilbenes, cis-piceid showed to be plastic at harvest with the highest content reached in F soil. See Supplementary materials Table 2.

6.3.3 Biochemical analyses on cv. Glera

The effect of the three soils on the single cultivar Glera was in line with the results of the analysis on both cultivars. Interestingly, the relative amount of both trans- and cis-piceid was found significantly higher in VV soil compared to L soil, with F soil having an intermediate effect. At post-veraison, the effect of the three soils was clear in phenylalanine content, in which VV showed the highest value while L the least value. At the date of harvest, a slight difference was also observed for the quer-3-glr content ($P<0.1$).

6.3.4 Correlation analysis

In total, three correlation matrices were constructed for the two cultivars separately plus the merged dataset, based on samples obtained at harvest against the soil physico-chemical features. The analysis revealed three different behaviors for the three datasets considered. Specifically, we found

a considerable number of good correlations (r > 0.5 and r < -0.5) when analyzing the dataset concerning the cv. Corvina, less in cv. Glera, and a little number in the merged dataset (Corvina and Glera joint). In all cases, the number of negative and positive correlations was comparable and mainly involved entire classes of metabolites.

6.3.4.1 cv. Glera

Comparing cv. Glera skin metabolites to soil properties, it was clear how the group of anthocyanins, flavonols, and some stilbenes well correlated to soil characteristics (**Fig. 6.2**). The three detected anthocyanins (delph-3-glu, delph-3-coum, and cyan-3-coum) showed similar behavior to the group of flavonols to which quercetin, rutin, the myricetin-forms, and the kaempferol-forms belong to. These two groups were characterized by positive correlations (almost always with r > 0.5) with the soil parameters clay, humidity, pH, Al, Fe, P, Ti, Ba, Be, Co, Cr, Li, Ni, Sr, ex-Ca, and still positive but less strong correlations with K, Na, As, ex-K, and CEC. Instead, they were found to negatively correlate (almost always with r < -0.5) with sand, EC, total carbon, organic carbon, total N, Mg, S, B, ex-Mg, ex-Na, and still but less strongly with silt, Cd, Cu, Mo, and Zn. About stilbenes, trans- and cis-piceid were the two out of the six compounds found to strongly correlate with the soil characteristics. In detail, they positively correlated with soil skeleton, EC, organic C, total N, Mn, Mo, V, and CEC, but negatively with silt, C/N ratio, Ca, K, Mg, Na, B, Sr, Zn, and P-Olsen. Other interesting correlations regarded the amino acid tryptophan with Zn and Na-ex (-0.53 and -0.55 respectively), and the procyanidin B1 and catechin which positively correlated with ex-Na (0.86 and 0.60 respectively). Exchangeable Sodium (Na-ex), particularly, had the highest number of correlations (17 out of 39), showing negative values with anthocyanins, flavanones, flavanonols, flavonols, and stilbene groups, but positive with hydroxycinnamic acids and flavanols (catechins).

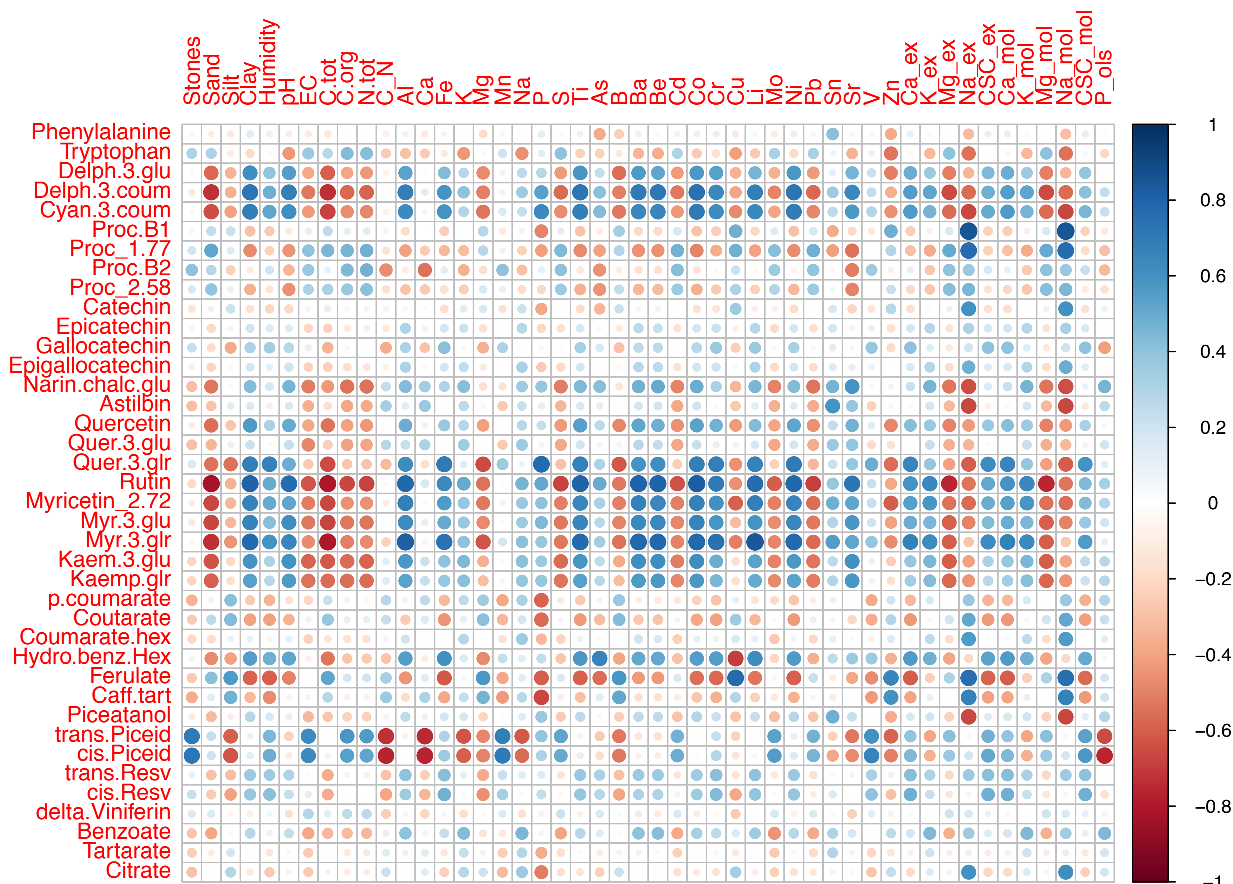

Figure 6.2 – Correlation analysis of grape skin secondary metabolites of cv. Glera at harvest against the soil physico-chemical characteristics. The analysis was generated using the Pearson's correlation.

6.3.4.2 cv. Corvina

About the cv. Corvina metabolites, all the compound groups were found to high correlate with soil components, but flavonols (the yellow pigments) which only positively correlated with soil P content (**Fig. 6.3**). On the one hand, the analysis showed that flavanols and hydroxycinnamic acids had similar behavior: they positively correlated with skeleton, clay, humidity, Al, Fe, Mn, P, Ti, Ba, Co, Cr, Li, Ni, V, ex-Ca, CEC, Ca-mol, CEC-mol. Epicatechin (another flavanol) also with K, Na, As, Be, Sr, K-ex, Na-ex, K-mol, and Na-mol. Instead, the two groups negatively correlated with silt, total C, C/N ratio, Ca, Mg, B, Sn, and Zn, and only flavanols also with sand, EC, organic C, total N, S, Cd, Cu, Mo, Pb, ex-Mg, and Mg-mol. On the other hand, anthocyanins were found to have an opposite trend compared to flavanols. This was noticed for 30 soil elements out of 48 analyzed, in which the first group positively correlated – while the second negatively – with sand, total C, Mg, S, Cd, Cu, Mo, Pb, ex-Mg, and Mg-mol, and vice versa with clay, Al, Fe, K, Na, Ti, As, Ba, Be, Co, Cr, Li, Ni, Sr, Ca-ex, K-ex, Na-ex, CEC-ex, Ca-mol, K-mol, and Na-mol. Some anthocyanins positively correlated also with skeleton, EC, organic

C, total N, Mn, but negatively with silt, pH, C/N ratio, Ca, B, Sn, Zn, CEC-mol, and P-Olsen. In comparison with other groups, stilbenes were found to not follow a specific trend. Among them, the most correlating metabolites were piceatannol and the piceids (both trans and cis forms), in most cases observing an opposite trend. Piceatannol highly correlated with stones, clay, humidity, Fe, Mn, P, Ti, Co, Cr, Li, Ni, V, Ca-ex, CEC-ex, Ca-mol, CEC-mol, as well as with silt, total C, C/N ratio, Ca, Mg, B, Cu, and Zn, negatively and positively respectively. Piceids, instead, positively correlated with clay, humidity, pH, Al, Fe, K, Na, Ti, As, Ba, Be, Co, Cr, Li, Ni, Sr, K-ex, Na-ex, CEC-ex, Ca-mol, K-mol, Na-mol, P-ols, and negatively with sand, EC, total C, organic C, total N, S, Cd, Cu, Mo, Pb, Mg-ex, Mg-mol. Moreover, trans-resv strongly negatively correlated with Sn. Delta-viniferin had a strong positive correlation with P. The two amino acids phenylalanine and tryptophan behaved similarly with significant positive correlations with silt, C/N ratio, Ca, Mg, B, Zn. Also phenylalanine singularly with K, Na, As, Sn, whereas tryptophan singularly only with total C. The negative correlations for both the amino acids regarded stones, humidity, Mn, P, V, CEC-ex, and CEC-mol, plus singularly with clay, Fe, Ti, Co, Cr, Li, Ni, Ca-ex, Ca-mol for tryptophan. P was the soil element with the highest number of correlations (19 out of 52). In particular, it never correlated with anthocyanins but positively with the flavanones, some flavanols (procyanidin B1, catechin, and gallocatechin), many flavonols and hydroxycinnamic acids, and the stilbene delta-viniferin. Negative correlations were found between P and piceatannol, the amino acids phenylalanine and tryptophan.

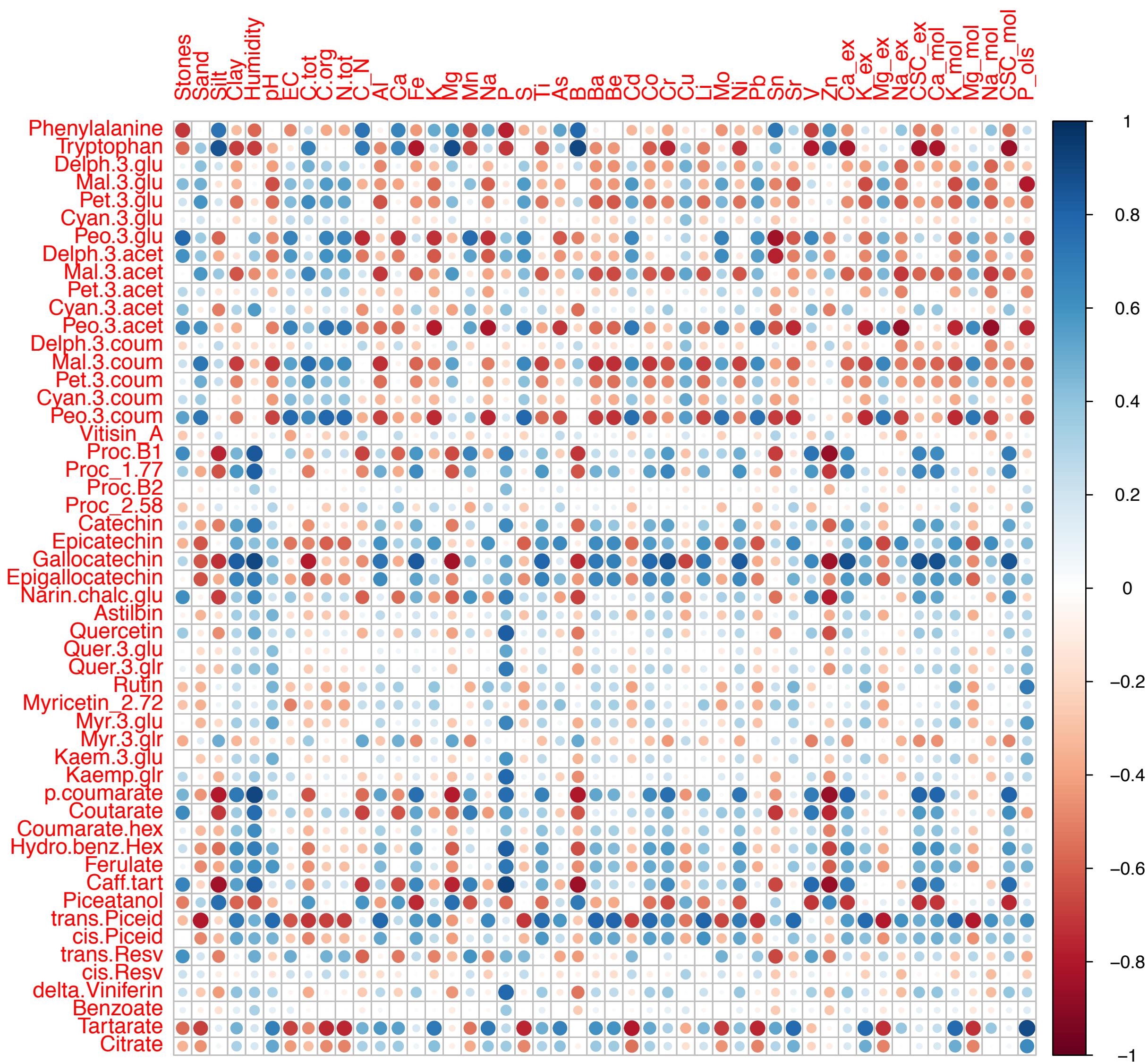

Figure 6.3 – Correlation analysis of grape skin secondary metabolites of cv. Corvina at harvest against the soil physico-chemical characteristics. The analysis was generated using the Pearson's correlation.

6.3.4.3 Combined dataset

We also tried to correlate soil components with the combined dataset of cv. Corvina and cv. Glera (**Fig. 6.4**). In this case, only the shared metabolites were taken into consideration (i.e. most of the anthocyanins were excluded from the analysis). Interestingly, the number of significant correlations found was considerably smaller compared to the ones of the two separated datasets (cv. Corvina and cv. Glera). Flavonol group was found to be the most involved, with quer-3-glr and myr-3-glu showing the highest and statistically significant correlations. They were positively correlated with clay, P, and Ni. Myr-3-glu also with pH, Ti, Ba, Be, Co, but negatively with sand, total C, Mg-ex and Mg-mol; whereas quer-3-glu also with humidity and Cr, but negatively with B. The flavanol gallocatechin

similarly behaved to flavonol metabolites, positively correlating with clay, humidity, Ti, Ba, Be, Co, Cr, Ni, and negatively with total C and B. Moreover, it was found enriched of many other strong correlations such as with silt, Mg, Zn, and positively with Al, Fe, Li, V, Ca-ex, CEC-ex, Ca-mol, and CEC-mol. Other spotted significant correlations regarded procyanidin B1 with Sn (r = -0.56), epicatechin with Na-ex and Na-mol (r = 0.48 and 0.49 respectively), trans-piceid with CEC-ex and CEC-mol (r = 0.47 and 0.48 respectively). Indeed, among the detected stilbenes, the two piceid forms were found to be the most responsive to soil variations in the correlation analysis. They also tended to negatively correlate with silt, Mg, B, and Zn. It is worth noticing that, among the soil components, P was the element showing the highest number of strong correlations (specifically only positive ones). As well as with the already mentioned flavonols (quer-3-glu, myr-3-glu), it also correlated with kaemp-glr, the flavanone narin-chalc-glu, and the hydroxycinnamic acid hydroxy-benz-hex.

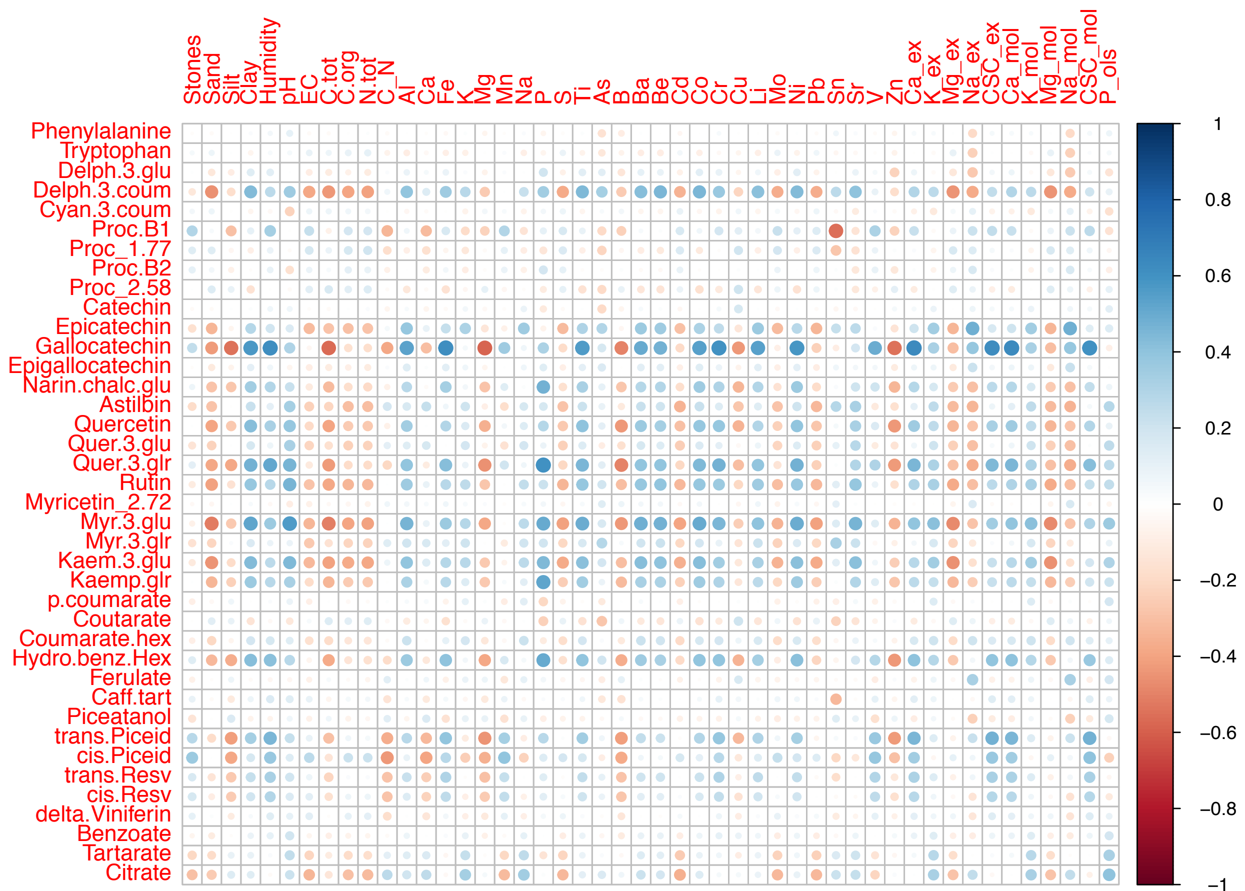

Figure 6.4 – Correlation analysis of grape skin secondary metabolites of both cv. Corvina and cv. Glera at harvest against the soil physico-chemical characteristics. The analysis was generated using the Pearson's correlation.

6.4 DISCUSSION

Of the many factors affecting grape quality, the balance of phenolic compounds plays a major part, providing the basis for many aromas, flavors, and colors (Pereira et al., 2005). It has also been reported that phenolic compounds have potential good benefits on human health (Carrieri et al., 2013; Guerrero et al., 2009) leading to an increase in the interest in studying the berries composition as response to different terroir factors (Lund and Bohlmann, 2006; Swiegers et al., 2005). Indeed, berry phenolic composition depends on multiple environmental factors including cultivar, climate, and soil (Bautista-Ortin et al., 2012), the latter known to exert a determinant effect (Coelho et al., 2006; Ubalde et al., 2010; Vilanova et al., 2007) even though its contribution on the final product has not been studied so widely (Van Leeuwen et al., 2004; Seguin, 1986).

In this study we focused on the effect that three different soils had on the berry skin secondary metabolites of two cultivars. On the one hand, we wanted to give some insights into the biological modulation of the metabolites when studying the two cultivars together and separately, and, on the other hand, to find out which metabolites were modulated in a cultivar dependent manner. The scientific community has already been focusing on the cultivar-specific regulation of the specialized metabolism (Degu et al., 2014) and on the basis of the grapevine berry transcriptome plasticity concerning the G x E interactions (Dal Santo et al., 2018). The metabolite amount, based on the ion count, was detected by means of LC-MS technology and subjected to statistical analysis. The three soils were considered as the experimental treatments and were proven to affect the metabolites in berry skin, even in a cultivar dependent manner.

The analysis of variance showed that different soils produced different amount of secondary metabolites in the berry skins of both cultivars. Statistically, the effect was proved when including both time points in the analysis (post-veraison and harvest) but was more evident at post-veraison stage. Indeed, the pedoclimatic conditions are known to strongly influence the ripening dynamics in terms of phenolic compounds synthesis, as also proposed by Dal Santo et al. (2016). Among the phenylpropanoids, flavonols and stilbenes were the two groups mainly involved in this different modulation. Flavonols are reported in grape berries in the form of 3-O-glycoside of quercetin, myricetin, kaempferol, isorhamnetin, laricitrin, and syringetin, both in red and white cultivars (Mattivi et al., 2006; Talcott and Lee, 2002). Their accumulation is also known to be responsive to light (Cortell and Kennedy, 2006; Reshef et al., 2017) and temperature variations (Del-Castillo-Alonso et al., 2016). Stilbenes (1,2-diarylethenes) are another class of phenolic compounds (non-flavonoids) belonging to

the phenolic group, and are mainly located in the grape skin (Creasy and Coffee, 1988; Roggero and Garcia, 1995). Their importance comes from their potential valuable effects on human health (Guebailia et al., 2006), attracting considerable interest due to the fact that grape and wine are considered two of the main sources of bioactive stilbenes in the diet (Fernández-Marín et al., 2013). Our results are consistent with previous studies demonstrating the capability of stilbenes to be environmentally plastic. Dal Santo et al. (2016) reported that the stilbenes biosynthesis, especially resveratrol, is one of the most metabolic component to be dependent to the environment. They also attributed significant differences in trans-resveratrol concentration to soil differences, supporting the idea about a relevant soil importance on the modulation of this type of metabolites class. Despite few data are nowadays available, soil factor has been proposed to be as important as climate on berry stilbene amount (de Andrés-de Prado et al., 2007). Once again, as reported in the previous chapters, it seems that stilbene biosynthesis in grape depends on soil features, especially on its water holding capacity which may lead to a stimulation effect (de Andrés-de Prado et al., 2007; Bavaresco et al., 2009; Koundouras et al., 2006). In our experiment, the piceatannol content was consistent with such statements since it was found to be higher in grapes from the soils characterized by the highest water contents (L and F soils). Instead, piceids accumulation seemed to have an opposite trend, with the highest amount found in the supposed most stressing treatment represented by VV soil (lowest water content, unbalanced soil Mg/K ratio). Indeed, stilbenes are also known for being produced as response to stress conditions (either abiotic and biotic) (Bavaresco, 2003; Dani et al., 2007; Deluc et al., 2011), which might be the case of VV soil.

Some metabolites from the two groups herein described (flavonols and stilbenes) were reported to be also affected by the interaction between the soil and cultivar factors. This could mean that, in a certain cultivar, the amount of a certain metabolite had a specific trend among soils, which was not maintained in the other cultivar. Such results, besides supporting the hypothesis by which stilbenes and flavonols are quite plastic, lead to think that each cultivar can be characterized by a specific response in terms of metabolites balance, which, in turn, depends on the overall interaction among the agri-environmental inputs. This is not trivial since farmers can obtain specific results, in terms of grape flavors, aromas and color, only from a specific cultivar, cultivated in a specific area. In other words, it sounds like each cultivar wants its own environment for expressing the best commercial traits in the grapes and, in turn, in the wine. Curiously, accounting for the analysis of variance, the anthocyanins content was not responsive to soil differences except for the peonidin forms (peo-3-glu, peo-3-acet, and peo-3-coum) and myr-3-coum. In fact, many studies reported that anthocyanin

accumulation is more related to climate factor (Ubalde et al., 2010) even though other authors have proposed a noticeable effect due to the soil type on many berry traits, also involving the total anthocyanin concentration (Van Leeuwen et al., 2004). Once again, it might be a consequence of the vine water and nitrogen status, whose deficit leads to the reduction of the vine vigor and yield, limiting the size of the berries and increasing the grape's sugar content and phenolic components (Choné et al., 2001; Coipel et al., 2006). In fact, this was not our case since soil nitrogen and water supply levels didn't exceed the stress level considered for vine cultivation (see Chapter 3). This effect was evident for the low anthocyanin content in skin berries from L soil which is absent in skeleton and harbors the highest content of water, as well as for the lower delph-3-coum content found from VV soil which is high in nitrogen and soil organic matter content. On the contrary, F soil led to high production of anthocyanins and flavonols thanks to its lower soil nitrogen, organic carbon, and water content than L soil. The results are in line with previous reports showing that the higher is the soil fertility, especially in terms of water-holding-capacity (L soil), the lower is the color intensity and shade, mainly due to a lower phenolic composition, and smaller is the amount of hydroxycinnamic compounds, as found in our experiment for p-coumarate and caff-tart content in L soil (de Andrés-de Prado et al., 2007).

The analysis showed that cv. Glera could be high responsive to pedoclimatic conditions as was reported for another white variety, cv Garganega, in terms of the accumulation of phenolic compounds during the overall ripening period in different environments (Dal Santo et al., 2016). They also observed that cv. Garganega was much more plastic than cv. Corvina for the trait considered. Contrary, when analyzing the skin secondary metabolites at harvest, cv. Corvina was characterized by a deep plasticity as also proposed by Dal Santo et al. (2013). In fact, considering a quite significant level ($P<0.1$), the analysis revealed 14 out of 52 metabolites significantly differently accumulated among treatments, with respect to the 4 out of 52 in cv. Glera. This is consistent with what reported in Chapter 4: Glera could be considered more "elastic", whereas cv. Corvina more plastic to the external stimuli. Such effect might give an explanation to the extension area of Amarone DOCG and Prosecco DOC, the latter about three times wider in comparison to the former.

Three correlation matrices were constructed to elucidate any possible relationship between skin metabolites of two cultivars, treating them separately plus the merged dataset, and the soil physico-chemical features. This also allowed us to compare the response of a red cultivar vs. a white cultivar as response to changes in soil composition, and, in the merged dataset, to find the correlations putatively definable as no-cultivar dependent. It is worth noting that the metabolites differently

accumulated among soil treatments were found to be also well correlated with many soil components. Therefore, it seems that such differences in metabolites composition are possibly due to differences in soil composition. This is not trivial since the main message from the current literature is that the soil effect is observed only for unbalanced conditions (Pereira et al., 2005; Seguin, 1986) and that the chemical/mineral uptake provided by the soil does not exert a significant influence on the fruit (Van Leeuwen et al., 2004) and wine quality (Poni et al., 2018). Grape composition, indeed, has always been reported to be more affected by physical soil properties (texture and porosity), mainly conditioning the water supply and drainage (Choné et al., 2001; Seguin, 1986). Only few studies reported good correlations between some soil properties and the volatile and non-volatile metabolites composition (Anesi et al., 2015). The same authors also proposed that changes in soil composition and other terroir features reflect on the berry metabolome as many small changes rather than a small number of major metabolic shifts. Similarly, in our results, we observed that besides the metabolites found significantly different there were many other remarkable correlation concerning metabolites belonging to the same classes. As they reported, soil pH, percentage of clay, organic carbon, and exchangeable potassium correlated with the metabolite composition. The current literature also suggests nitrogen as one of the mineral component which is well correlated with grape and wine quality (Choné et al., 2001), mostly affecting the grape sugar and phenolic composition when in excess. As general knowledge, soil nitrogen availability is known to reduce grape and wine anthocyanin concentration (Brunetto et al., 2009; Pérez-Álvarez et al., 2016), and this effect was also shown in our analysis for the two main classes of pigments (anthocyanins and flavanols). This might be explained by the higher transcription level of the related genes in berries from plants cultivated without nitrogen supply (Soubeyrand et al., 2014).

In our analysis, the number of good correlations were higher when analyzing the two cultivar separately as compared to that from the merged dataset. This may lead to think that, assuming they might be only partially spurious correlations, the response of metabolites accumulation to changes in soil composition deeply depend on the type of cultivar. This concept is also supported by the significant interactions revealed by the analysis of variance. On the one hand, from the merged dataset, we found out a set of metabolites whose modulation is putatively less dependent on the cultivar (mainly gallocatechin and some flavonols). On the other hand, we observed which are the chemical soil elements mostly involved in the metabolite accumulation. About that, phosphorous seemed to be the most correlating elements with the secondary metabolites on berry skin of both cultivars. This element is known for being involved in several physiological processes in the cell

(membrane formation, carbohydrate metabolism, protein synthesis, energy storage and transfer) but there is still little information about its impact on grape traits (Poni et al., 2018).

6.5 Supplementary materials

Supplementary Table 1: Analysis of variance (ANOVA) on detected metabolites. Factors are soil, cultivar, and their interaction. The analysis refers to the dataset considering both the time points. Statistical significance is represented by the p-value ("." P<0.1; "*" P<0.05; "**" P<0.01; "***" P<0,001). Tukey's test compared means between soil-treatments when found significantly different (with "a" representing the highest value).

		Glera & Corvina								
		soil		cultivar		soil*cultivar		Tukey's Test		
		sign	p-value	sign	p-value	sign	p-value	F	VV	L
	Phenylalanine	ns	0.1485	***	0.0000	ns	0.2730			
	Tryptophan	ns	0.3568	***	0.0000	ns	0.1783			
Anthocyanin	Delph-3-glu	ns	0.6299	***	0.0000	ns	0.2604			
	Mal-3-glu	ns	na	ns	na	ns	na			
	Pet-3-glu	ns	na	ns	na	ns	na			
	Cyan-3-glu	ns	na	ns	na	ns	na			
	Peo-3-glu	ns	na	ns	na	ns	na			
	Delph-3-acet	ns	na	ns	na	ns	na			
	Mal-3-acet	ns	na	ns	na	ns	na			
	Pet-3-acet	ns	na	ns	na	ns	na			
	Cyan-3-acet	ns	na	ns	na	ns	na			
	Peo-3-acet	ns	na	ns	na	ns	na			
	Delph-3-coum	*	0.0121	ns	0.6758	.	0.0544	a	b	ab
	Mal-3-coum	ns	na	ns	na	ns	na			
	Pet-3-coum	ns	na	ns	na	ns	na			
	Cyan-3-coum	ns	na	ns	na	ns	na			
	Peo-3-coum	ns	na	ns	na	ns	na			
	Vitisin_A	ns	na	ns	na	ns	na			
	Delph-3-ferur	ns	na	ns	na	ns	na			
Flavanols	Proc-B1	ns	0.2500	*	0.0474	ns	0.5073			
	Proc_1.77	ns	0.7261	***	0.0005	ns	0.3872			
	Proc-B2	ns	0.8500	***	0.0000	ns	0.4254			
	Proc_2.58	ns	0.9963	ns	0.9891	ns	0.5769			
	Catechin	ns	0.7326	ns	0.1497	ns	0.6312			
	Epicatechin	ns	0.8556	***	0.0000	ns	0.2646			
	Gallocatechin	*	0.0102	***	0.0000	ns	0.9832	a	b	ab
	Epigallocatechin	ns	0.5317	***	0.0000	ns	0.3512			
Flavanones	Narin-chalc-glu	ns	0.3255	***	0.0001	ns	0.4386			
Flavanonol	Astilbin	ns	0.2649	ns	0.3855	ns	0.6683			
Flavonols	Quercetin	ns	0.2177	ns	0.1096	ns	0.5646			
	Quer-3-glu	ns	0.6230	.	0.0679	ns	0.5744			
	Quer-3-glr	ns	0.1579	*	0.0146	ns	0.6668			
	Rutin	**	0.0066	***	0.0000	ns	0.3420	a	b	ab
	Myricetin_2.72	**	0.0054	***	0.0000	*	0.0354	a	b	ab
	Myricetin_3.87	ns	na	ns	na	ns	na			
	Myr-3-glu	ns	0.3231	ns	0.2795	ns	0.7499			
	Myr-3-glr	*	0.0237	***	0.0000	**	0.0088	a	b	ab
	Kaem-3-glu	ns	0.1460	**	0.0048	ns	0.7974			
	Kaemp-glr	ns	0.3923	**	0.0065	ns	0.5671			
Hydroxycinnamic acids	p-coumarate	ns	0.4360	**	0.0035	*	0.0477			
	Coutarate	ns	0.9648	ns	0.1644	ns	0.2664			
	Coumarate-hex	ns	0.4539	***	0.0006	ns	0.9648			
	Hydro-benz-Hex	**	0.0030	***	0.0000	ns	0.3830	a	b	b

	Ferulate	ns	0.3024	***	0.0000	ns	0.1051			
	Caff-tart	ns	0.4284	***	0.0004	*	0.0453			
Stilbenes	Piceatannol	**	0.0088	***	0.0000	.	0.0737	ab	b	a
	trans-Piceid	.	0.0670	ns	0.2059	*	0.0103			
	cis-Piceid	*	0.0480	***	0.0001	.	0.0676	ab	a	b
	trans-Resv	ns	0.2751	**	0.0050	ns	0.4679			
	cis-Resv	ns	0.1625	**	0.0086	ns	0.9610			
	delta-Viniferin	ns	0.5348	***	0.0008	.	0.0866			
	Benzoate	ns	0.5174	***	0.0000	ns	0.8323			
	Tartarate	.	0.0587	***	0.0000	ns	0.6036			
	Citrate	ns	0.9355	**	0.0023	ns	0.7736			

Supplementary Table 2: Analysis of variance (ANOVA) on detected metabolites related to the two cultivars separately at harvest as response to soil factor. Statistical significance is represented by the p-value ("." P<0.1; "*" P<0.05; "**" P<0.01; "***" P<0,001). Tukey's test compared means between soil-treatments when found significantly different (with "a" representing the highest value).

		Glera					Corvina				
		sign	p-value	Tukey's Test F	VV	L	sign	p-value	Tukey's Test F	VV	L
	Phenylalanine	ns	0.960				.	0.075			
	Tryptophan	ns	0.642				*	0.011	b	ab	a
Anthocyanin	Delph-3-glu	ns	0.168				ns	0.556			
	Mal-3-glu	ns	na				ns	0.332			
	Pet-3-glu	ns	na				ns	0.244			
	Cyan-3-glu	ns	na				ns	0.884			
	Peo-3-glu	ns	na				*	0.048	ab	a	b
	Delph-3-acet	ns	na				ns	0.277			
	Mal-3-acet	ns	na				ns	0.196			
	Pet-3-acet	ns	na				ns	0.743			
	Cyan-3-acet	ns	na				ns	0.392			
	Peo-3-acet	ns	na				.	0.090			
	Delph-3-coum	.	0.087				ns	0.807			
	Mal-3-coum	ns	na				.	0.071			
	Pet-3-coum	ns	na				ns	0.408			
	Cyan-3-coum	ns	0.150				ns	0.630			
	Peo-3-coum	ns	na				.	0.052			
	Vitisin_A	ns	na				ns	0.801			
	Delph-3-ferur	ns	na				ns	0.773			
Flavanols	Proc-B1	ns	0.750				*	0.048	a	a	b
	Proc_1.77	ns	0.447				ns	0.166			
	Proc-B2	ns	0.543				ns	0.978			
	Proc_2.58	ns	0.597				ns	0.770			
	Catechin	ns	0.863				ns	0.301			
	Epicatechin	ns	0.872				ns	0.212			
	Gallocatechin	ns	0.618				*	0.016	a	ab	b
	Epigallocatechin	ns	0.871				ns	0.165			
Flavanones	Narin-chalc-glu	ns	0.280				ns	0.125			
Flavanonol	Astilbin	ns	0.675				ns	0.670			
Flavonols	Quercetin	ns	0.231				ns	0.490			
	Quer-3-glu	ns	0.630				ns	0.863			
	Quer-3-glr	.	0.093				ns	0.743			
	Rutin	*	0.011	a	b	ab	ns	0.608			
	Myricetin_2.72	ns	0.143				ns	0.614			
	Myricetin_3.87	ns	na				ns	0.406			
	Myr-3-glu	ns	0.202				ns	0.794			
	Myr-3-glr	*	0.039	a	b	b	ns	0.433			
	Kaem-3-glu	ns	0.205				ns	0.759			
	Kaemp-glr	ns	0.327				ns	0.675			
Hydroxycinnamic acids	p-coumarate	ns	0.563				*	0.028	a	ab	b
	Coutarate	ns	0.479				.	0.093			
	Coumarate-hex	ns	0.878				ns	0.573			
	Hydro-benz-Hex	ns	0.328				ns	0.186			
	Ferulate	ns	0.220				ns	0.337			
	Caff-tart	ns	0.462				*	0.025	a	ab	b
Stilbenes	Piceatannol	ns	0.733				*	0.042	b	ab	a
	trans-Piceid	ns	0.123				*	0.021	a	b	ab
	cis-Piceid	ns	0.102				ns	0.336			
	trans-Resv	ns	0.854				ns	0.215			

cis-Resv	ns	0.532	ns	0.847			
delta-Viniferin	ns	0.896	ns	0.495			
Benzoate	ns	0.585	ns	0.917			
Tartarate	ns	0.831	*	0.036	a	b	ab
Citrate	ns	0.731	ns	0.446			

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Chapter 7

Concluding remarks

7.1 Summary and conclusions

In the experiment, the effect due to the agronomic and climatic variability was minimized, allowing us to isolate and study in more detail the singular effect that three different soils had on the grapevine growth and grape traits. Indeed, since 'soil' was the only one variable in our experiment, we assumed that each difference among plants was potentially related to differences among soils with respect to their physical, chemical, and microbiological composition. For giving a complete overview of such effect, the experiment was conducted in a multidisciplinary way, gaining from soil science, plant physiology, transcriptomics, and metabolomics.

The statistical analyses showed that the three soils had a noticeable effect on grapevine growth and berry traits. Such effect was also highlighted with the molecular and biochemical profile of berries at harvest. As many authors proposed, the main effect could be related to the physical soil features such as skeleton content and texture, which deeply influence soil water availability. Canopy growth, phenological development, berry sugar accumulation, and berry weight seemed to be the parameters mostly affected by soil physical differences. Transcriptomic data at harvest revealed a deep Genotype x Environment interaction, in which the effect of the soil was found to be strongly cultivar-dependent. The metabolomic analysis also suggested a direct relationship between skin metabolites and soil mineral components. Correlations between these parameters were clear for the classes of pigments – anthocyanins and flavonols –in the red and white cultivar respectively. This is not trivial since the main message from the current literature is that the soil mineral effect is observed only for unbalanced conditions (Pereira et al., 2005; Seguin, 1986) and that the chemical/mineral uptake provided by the soil does not produce a significant influence on the fruit (Van Leeuwen et al., 2004) and wine quality. Molecular analysis also confirmed previous results supporting deep plasticity of transcription berry reprogramming at harvest (Anesi et al., 2015; Dal Santo et al., 2013, 2016), and, along with biochemical results, a cultivar-specific response to soil differences.

Based on our results, we can state that the 'soil factor' plays an important role in the quality of the final grapes, in turn mirroring such uniqueness to the derived wine. Such effect can be meant also as the result of a complex interplay among all the terroir factors, in our experiment investigated by the cultivar-soil interaction. We also hypothesize that "the higher the

phenotypic plasticity of a cultivar, the smaller is the area of production of a certain wine". In our case, we found out that *V. vinifera* cv. Corvina at harvest was more plastic than cv. Glera concerning the skin metabolites. Based on this fact, we suppose that a high responsive cultivar – as Corvina showed to be –might deeply change its pattern of metabolites accumulation whether cultivated outside its typical area of production, resulting in a great changing of its berry metabolomic profile, hence sensory profile. Valpolicella area is one-third Prosecco production area, indicating that, on the one hand, Corvina cultivar might exert its best performance only in a restricted area and, on the other hand, the less phenotypic plasticity of cv. Glera allows farmers to obtain suitable grapes for "good" Prosecco even in a wider area. Yet Turesson (1922) articulated the existence of a close relationship between varieties of crop plants and their environment and stressed that the presence of a variety in a given locality is not just a chance occurrence, rather there is a genetic component that helps the individual to adapt to that area.

Our experiment represents a step forward of a deeper understanding of how and how much the soil is determinant for the final grape quality, hence how and how much it is involved in the terroir concept. Nonetheless, only by the integration of multiple 'omics', hence through the integration of environmental information with genomic, epigenomic, transcriptomic, and metabolomics data, it'll be possible to shed light on how plants modulate their response to different environments and understanding the effect of terroir at a multi-level (Fabres et al., 2017).

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