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THE IMPACT OF GRAPEVINE FANLEAF VIRUS (GFLV) ON QUANTITY AND QUALITY PARAMETERS OF GRAPEVINE (*Vitis vinifera* L.)

DISSERTATION

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VPLIV VIRUSA PAHLJAČAVOSTI LISTOV VINSKE TRTE (GFLV) NA KOLIČINO IN KAKOVOST GROZDJA (*Vitis vinifera* L.)

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TABLE OF CONTENTS

LIST OF TABLE V

LIST OF FIGURES VI

ABBREVIATIONS AND SYMBOLS XI

1 INTRODUCTION 1

1.1 AIMS 4

1.2 HYPOTHESIS 6

2 THEORETICAL BACKGROUND 7

2.1 The importance of viticulture 7

2.2 Winegrowing regions in Slovenia and Italy 8

2.3 Cultivars 11

2.3.1 ‘Refošk’ 11

2.3.2 ‘Schioppettino’ 12

2.3.3 ‘Volovnik’ 13

2.4 Grapevine training systems 14

2.5 Grapevine pathogens 15

2.6 Nepoviruses 17

2.7 Grapevine fanleaf virus (GFLV) 18

2.7.1 Genome of GFLV 18

2.7.2 Symptomatology 21

2.7.3 Transmission and control 24

2.8 Grapevine ripening and quality parameters 27
2.8.1 Soluble solids 29
2.8.2 Organic acids and pH 29
2.8.3 Phenolic compounds 30
2.8.3.1 Nonflavonoid phenolics 32
2.8.3.2 Flavonoid phenolics 32
2.8.3.3 Anthocyanins 34
2.8.3.3.1 The anthocyanin biosynthesis pathway 36

3 EXPERIMENT 40

3.1 Selection of vineyards and cultivars 40

3.2 Sampling 44

3.3 ELISA test 45
3.3.1 Buffers for ELISA test 45
3.3.2 Homogenisation of plant material 46
3.3.3 DAS – ELISA test 47
3.3.4 DASI – ELISA test 48

3.4 METHOD OF QUALITY AND QUANTITY ANALYSES 49
3.4.1 Yield and berry weight 49
3.4.2 Quality parameters 49

3.5 METHOD FOR HPLC ANALYSIS 50
3.5.1 Extraction of phenolic compounds and sample preparation 50
3.5.2 Quantification of grape anthocyanins 50

3.6 GENE EXPRESSION ANALYSIS 52
3.6.1 Sample preparation and RNA isolation 52
3.6.2 DNase treatment 53
3.6.3 Reverse transcription 53
3.6.4 Quantitative polymerase chain reaction (qPCR) 54
3.6.5 Relative quantification 56

3.7 Microvinification **56**

3.8 Statistical analysis **57**

4 RESULT AND DISCUSSION **58**

4.1 Presence of grapevine viruses **58**

4.1.1 DAS and DASI ELISA test for virus detection 58

4.2 Impact of GFLV on quantity parameters of grapevine **60**

4.2.1 Clusters number 60

4.2.2 Cluster weight 62

4.2.3 Berry weight 63

4.2.4 Yield 64

4.3 Impact of GFLV on quality parameters of grapevine **67**

4.3.1 Soluble solids 67

4.3.2 Titratable acids and pH 69

4.4 Anthocyanin profile in grapevine berries **72**

4.5 Impact of GFLV on expression of genes in flavonoid biosynthetic pathway **79**

4.5.1 Chalchone synthase (CHS) 79

4.5.2 Flavanone 3-hydroxylase (F3H) 80

4.5.3 Flavonoid 3´ hydroxylase (F3’H) and flavonoid 3´5´-hydroxylase (F3´5´H) 83

4.5.4 Leucoanthocyanidin dioxygenase (LDOX) 85

4.5.5 Flavonoid-3-o-glucosyltransferase (UFGT) 87

4.6 MapMan **88**

4.7 Impact of GFLV on wine quality parameters **90**

5 CONCLUSIONS **94**

6 SUMMARY **96**

7 POVZETEK 100

8 REFERENCES.. 102

LIST OF TABLE

Table 1: Phenolic compounds produced and accumulated in the grape berry (Teixeira et al., 2013) 34

Table 2: Selected vines of cultivar ‘Refošk’.......... 41

Table 3: Selected vines of cultivar ‘Schioppettino’.......... 42

Table 4: Selected vines of cultivar ‘Volovnik’.......... 43

Table 5: Chromatographic conditions for HPLC analyses 51

Table 6: Primer pairs used in analysis of gene expression. 55

Table 7: Effect of cultivar, virus infection and season on the composition of monomeric, acetylated, p-coumarated anthocyanins and on the relative proportions of OH-, OCH3-, di- tri-substituted forms. Data analysed through three-ways ANOVA of and interaction of effects computed (*, p<0.05; **, p<0.01; ***, p<0.001). 77

Table 8: Wine quality parameters in cv. ‘Schioppettino’ pruned in single Guyot training system.......... 90

Table 9: Wine quality parameters in cv. ‘Schioppettino’ pruned in double Guyot training system.......... 91

Table 10: Wine quality parameters in cv. ‘Refošk’ trained in single Guyot training system 92

Table 11: The average points of sensory evaluation.......... 93

LIST OF FIGURES

Figure 1: Winegrowing regions and districts in Slovenia (foto: www.sloveniavino.com). 9

Figure 2: Winegrowing districts of Friuli-Venezia Giulia region (foto: www.barriquefinewines.com). 11

Figure 3: Cluster of cultivar ‘Refošk’ (foto: Denis Rusjan). 12

Figure 4: a). Vineyard of cv. ‘Schioppettino’ in Prepotto and b). ‘Schioppettino’ cluster (Foto: Maja Cigoj). 13

Figure 5: Single (a) and double (b) Guyot training system (Vršič and Lešnik, 2005). 15

Figure 6: Schematic presentation of genetic organization of genomic (RNA1 and RNA2) and satellite RNAs of GFLV. ORFs are represented by open boxes and the 5´ and 3´ UTR regions by narrow lines. ProCo – proteinase cofactor, Hel – helicase, VPg - viral protein, Pro – proteinase, Pol – polymerase, HP - homing protein, MP - movement protein, CP - coat protein, polyA – polyA tail (Fuchs et al., 1989; Belin et al., 2001). 20

Figure 7: Typical GFLV symptoms: a) leaf yellowing; b) fanleaf deformations; c) symptoms on cluster and d) double nodes (foto: Maja Cigoj). 22

Figure 8: Clusters of GFLV infected vines of cultivar ‘Schioppettino’ (foto: Maja Cigoj). 23

Figure 9: GFLV transmission (Gollino, 2013). 24

Figure 10: Diagrammatic representation of berry development (Keller, 2010). 27

Figure 11: Shematic structure of a ripe grape berry and phenolic biosynthesis distribution between several organs and tissues (Teixeira et al., 2013). 31

Figure 12: Flavonoid ring structure and numbering (Teixeira et al., 2013). 33

Figure 13: Structural identification of anthocyanidins (Castañeda-Ovando et al., 2009). 35

Figure 14: Structures and numbering scheme of primary anthocyanins (Košir et al., 2004). 36

Figure 15: Schematic representation of the flavonoid biosynthetic pathway (Matus et al., 2009). 37

Figure 16: The location of experimental vineyard in Komen (Google maps…, 2014). 41

Figure 17: The location of experimental vineyard in Prepotto (Google maps…, 2014). 42

Figure 18: The location of experimental vineyard in Slap (Google maps, 2014). 43

Figure 19: HPLC chromatogram of anthocyanins at 520 nm. The peak number 1 correspond to Del-3-Glu, peak number 2 to Cy-3-Glu, peak number 3 to Pet-3-Glu, peak number 4 to Peo-3-Glu and peak number 5 to Mal-3-Glu. 51

Figure 20: GFLV titre in leaves of cultivar ‘Volovnik’ measured by ELISA. 59

Figure 21: The cluster number in GFLV infected and healthy vines of the varieties ‘Refošk’ and ‘Schioppettino’, trained with single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$). 61

Figure 22: The cluster weight (g) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 62

Figure 23: The 100-berry weight (g) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 64

Figure 24: The yield (kg) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 65

Figure 25: The soluble solids (Brix°) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 67

Figure 26: Average content of titratable acids (g/L) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 70

Figure 27: The average pH in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 71

Figure 28: The anthocyanins content (mg/kg berry) in berry skin of cultivar 'Schioppettino', trained in single Guyot training system. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).......... 73

Figure 29: The contribution of non-methoxylated (OH), methoxylated (OCH_3), di-substituted (DI SUB) and tri-substituted (TRI SUB) anthocyanins of cultivar 'Schioppettino', trained in single Guyot in GFLV infected and healthy vines in 2011 and 2012. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$). 73

Figure 30: The anthocyanins content (mg/kg berry) in berry skin of cultivar 'Schioppettino', trained in double Guyot training system. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$)................... 74

Figure 31: The contribution of non-methoxylated (OH), methoxylated (OCH3), di-substituted (DI SUB) and tri-substituted (TRI SUB) anthocyanins of cultivar 'Schioppettino', trained in double Guyot in GFLV infected and healthy vines in 2011 and 2012. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$). 75

Figure 32: The anthocyanins content (mg/kg berry) in berry skin of cultivar 'Refošk', trained in single Guyot training system. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$).. 76

Figure 33: The contribution of non-methoxylated (OH), methoxylated (OCH3), di-substituted (DI SUB) and tri-substituted (TRI SUB) anthocyanins of cultivar 'Refošk' in GFLV infected and healthy vines in 2011 and 2012. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$). 76

Figure 34: Gene expression of CHS2 in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar 'Schioppettino'. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$)............................. 79

Figure 35: Gene expression of F3H1 in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar 'Schioppettino'. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$)............................. 81

Figure 36: Gene expression of F3H2 in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 82

Figure 37: Gene expression of F3´H in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 83

Figure 38: Gene expression of F3´5´H in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 84

Figure 39: a) the cumulative expression of F3`H and F3`5`H gene during berry development and b) the content of TRI SUB and DI SUB anthocyanin (g/kg berry). 84

Figure 40: Gene expression of LDOX in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 86

Figure 41: Gene expression of UFGT in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001). 87

Figure 42: MapMan visualization of the phenylpropanoid pathway during berry development. Green colour represents down-regulation and red colour up-regulation in infected vs. healthy samples. 89

ABBREVIATIONS AND SYMBOLS

°Baume	Baume degrees
°Brix	Brix degrees
°Oechsle	Oechsle degrees
1A	protein of putative proteinase cofactor
$1B^{Hel}$	putative helicase
$1C^{Vpg}$	viral protein genome-linked
$1D^{Pro}$	proteinase
$1E^{Pol}$	putative RNA-dependent RNA polymerase (RdRp)
$2A^{HP}$	homing protein
$2B^{MP}$	movement protein
$2C^{CP}$	coat protein
AILV	Artichoke Italian latent virus
ArMV	Arabis mosaic virus
BBLMV	Bluberry leaf mottle virus
CHS2	chalchone synthase
CLRV	Cherry leafroll virus
COX	cytochrome oxidase
CP	coat protein
Cy-3-Glu	cyanidin-3-glucoside
Del-3-Glu	delphinidin-3-glucoside
DOC	Original location certified
F3´5´H	flavonoid 3´ 5´ hydroxylase
F3´H	flavonoid 3´ hydroxylase
F3H1	flavanone 3-hydroxylase 1
F3H2	flavanone 3-hydroxylase 2
GARSV	Grapevine Antolian ringspot virus
GBLV	Grapevine Bulgarian latent virus
GCMV	Grapevine chrome mosaic virus
GDefV	Grapevine deformation virus
GFkV	Grapevine fleck virus
GFLV	Grapevine fanleaf virus

GLRaV	Grapevine leafroll associated virus
GRSPaV	Grapevine rupestris stem pitting virus
GTRSV	Grapevine Tunisian ringspot virus
GVA	Grapevine virus A
GVB	Grapevine virus B
Hel	helicase
HP	homing protein
LDOX	leucoanthocyanidin dioxygenase
Mal-3-Glu	malvidin-3-glucoside
MeOH	methanol
MP	movement protein
Peo 3-Glu	peonidin-3-glucoside
Pet 3-Glu	petunidin-3-glucoside
Pol	polymerase,
polyA	polyA tail
PRMV	Peach rosette mosaic virus
Pro	proteinase
ProCo	proteinase cofactor
RNA	ribonucleic acid
RpRSV	Raspberry ringspot virus
RW	rugose wood
satRNA	satellite RNA
SLRSV	Strawberry latent ringspot virus and
TBRV	Tomato black ring virus
ToRSV	Tomato ringspot virus
ToRSV	Tomato ringspot virus
TRSV	Tobacco ringspot virus
TRSV	Tobacco ringspot virus
UBI_CF	ubiquitin-conjugating factor
UFGT	flavonoid 3-O-glucosyltransferase
VPg	viral protein

1 INTRODUCTION

Vitis vinifera L. is one of the most widely cultivated plant, and one of the most economically important species worldwide (Vivier and Pretorius, 2002). Grapevine is susceptible to several plant pathogens which cause significant damage to crops with impact on vine longevity and yield (Espinoza et al., 2007; Pinto et al., 2014).

There are more than 80 infectious agents including viruses, viroids, phytoplasmas, bacteria and fungi that have been reported in grapevines, some with extremely high incidences (Martelli and Boudon-Padieu, 2006). To date, 67 viruses that belong to eight families and 21 genera have been isolated from grapevines (Martelli, 2012). The occurrences of viruses in vines affects all the vegetative organs, as well as the quality and quantity of grape yield (Engel et al., 2010). The most efficient way to control the detrimental effects of grapevine viruses is sanitary selection. The aim of sanitary selection is to propagate vines which are healthy and to prevent them from infection in the mother plants for propagation. The Slovenian certification scheme recommends testing the grapevines for 12 viruses (Rules on the demarcation, 2003).

One of the oldest known viral diseases of grapevines is grapevine degeneration disease, caused by Grapevine fanleaf virus (GFLV), which occurs in all winegrowing regions of the world (Raski et al., 1983). GFLV is a *Nepovirus* (Hewitt et al., 1962; Pinck et al., 1988; Fuchs et al., 1989) and is transmitted from vine to vine by the ectoparasitic nematode *Xiphinema index* (Hewitt et al., 1958). GFLV has been reported to cause significant economic losses by reducing grape yield and shortening the longevity of vines (Andret-Link et al., 2004).

A reduction in grape yield caused by GFLV could be from moderate (10%) to very high (>80%) (Andret-Link et al., 2004) and can even result in a total loss of yield (Raski et al., 1983). Regarding bunches; the virus affects an average weight and a number of clusters per vine. Furthermore, the ripening of the berries can be irregular among clusters, and/or even on the same bunch (Martelli and Savino, 1990). It was reported that also fruit quality is affected by GFLV due to a decrease in sugar content

and titratable acids (Andret-Link et al., 2004). But most reports were not supported with experimental data, except Cretazzo et al. (2009), who observed the influence of GFLV on growth and production parameters of grapevine but on relatively small number of vines.

Phenolic compounds are secondary metabolites that strongly affect the quality of the grapes and wines; among them, anthocyanins and related copygments are particularly important since they contribute to the red/blue colouration of the grapes and wines (Figueiredo-González et al., 2012). It is well known that anthocyanin biosynthesis is strongly affected by biotic stresses caused by pathogenes (Gould and Lister, 2006), where also by virus infections (Guidoni et al., 1997). Moreover, few abiotic factors, such as drought and agro-ampelotechnic practices that modify light environmental of canopy or crop load may trigger significant changes in anthocyanin abundance in grapes (Downey et al., 2006; Guidoni et al., 2008). A few papers report that GFLV infection decreases the total anthocyanin content (Cretazzo et al., 2009) in berries, but there are no papers, that report how GFLV infection affects the individual monomeric anthocyanin content and relative proportions among them.

Several observations were also published on the impact of other grapevine viruses (mainly GLRaV) on the expression of targeted genes implicated in phenylpropanoid biosynthesis pathway (Vega et al., 2011; Lecourieux et al., 2014; Guidoni et al., 1997; Cabaleiro et al., 1999). Nowadays, there are no reports regarding an effect of GFLV infection on the expression of genes involved in phenylpropanoid biosynthesis pathway, however only a few scientific studies focused on the gene expression in phenylpropanoid biosynthesis pathway are regarding other viruses.

Our focus was to identify GFLV infected and healthy vines of cultivars 'Refošk' (*V. vinifera* L.), 'Schioppettino' (*V. vinifera* L.) and 'Volovnik' (*V. vinifera* L.). We investigated the effect of GFLV infection on grape quantitaty and quality at harvest of cultivar 'Schioppettino' trained on single and double Guyot and of cultivar 'Refošk' trained on single Guyot system and cultivated under controlled conditions. In this study, a special emphasis was given to the analyses of the individual anthocyanins in berry skin from GFLV infected and healthy vines, where an expression of targeted

genes involved in anthocyanin biosynthesis pathway during berry ripening was also performed.

At harvest time, the grapes of GFLV infected and healthy vines were collected separately and microvinification was made in order to state the effect of virus infection on the organoleptic characteristics of wines.

1.1 AIMS

The vines included in the experiment were tested for the presence of GFLV and other important viruses, included in Slovenian certification scheme. From EPPO it is recommended to test the presence of viruses which occur in the EPPO region, where Tomato black ring virus (TBRV) and Grapevine chrome mosaic virus (GCMV) were included for Slovenia and Strawberry latent ringspot virus (SLRSV) and TBRV for Italy. Until now in our laboratory the ELISA test for GCMV, Tomato ringspot virus (ToRSV), SLRSV and Tobacco ringspot virus (TRSV) was not preformed, therefore our aim was:

- **Introduction of new diagnostic DAS-ELISA methods for four important grapevine viruses: GCMV, ToRSV, SLRSV and TRSV.**

Autochthonous cultivars have an important role to maintain cultural heritage. In our preliminary experiments, all vines of cultivar 'Volovnik' sampled in vineyard were infected with GFLV, therefore our aim was:

- **Searching of healthy plants of the old Slovenian cultivar 'Volovnik' or to obtain them in laboratory.**

A lot of vineyards in winegrowing region are infected with viruses, mostly with GFLV. In the literature, most reports about impact of GFLV on quality and quantity of grapes are not supported with experimental data, therefore our aims were:

- **Evaluation of the impact of GFLV infection on quantitative parameters of grapevine, such as yield and berry weight.**

- **Evaluation of the impact of GFLV infection on quality parameters of grapes: pH, total soluble solids, titratable acids and profil of phenolic compounds.**

In the literature, no studies of impact of GFLV on expression of genes involved in anthocyanin biosynthetic pathway were reported, therefore our aim was:

- **Studying of gene expression of six targeted genes involved in anthocyanins biosynthetic pathway.**

1.2 HYPOTHESIS

New diagnostic methods for 4 important grapevine viruses (GCMV, ToRSV, SLRSV and TRSV) could be introduced in our laboratory.

The majority of cultivated vines of ‘Volovnik’ are infected with GFLV. In the case of absence of healthy vines of ‘Volovnik’ in vineyards, they could be obtained in laboratory with thermotherapy.

GFLV infection significantly reduces the grapevine yield and the berry weight.

GFLV infection increases the grape quality (soluble solids, pH, titratable acids, anthocyanins content in berry skin) in comparison to healthy content.

GFLV infection affects the expression of targeted genes involved in anthocyanin biosynthesis pathway in comparison to healthy content.

2 THEORETICAL BACKGROUND

2.1 The importance of viticulture

Grapevine (*Vitis* sp.) is globally one of the most important plants, and in different winegrowing areas the produced grape is destined to wine production or other uses; as fresh fruits, as withered fruits, for juice production and distillation. In 2011, the total surface devoted to vineyards was approximately 7.6 million hectares throughout the world, and the total grape production reached 69.2 Mt (International Organisation of Vine and Wine, 2013).

In Slovenia, there are around 15.973 ha of vineyards with an annual production of 54.3 million litters of wine, 62 % of white and 38 % of red wines. The surface is administrated by 27.802 winegrowers, meaning that an average surface cultivated by each grower is around 0.57 ha (Register of grape and wine producers, 2014).

Grapevine cultivation is linked with human civilisation since ancient times. In the earliest writings and archives associated to all kind of agricultural and religious activities, a significance importance was given to grapevine. To date, the oldest record mentioning the use of the grapevine derivatives by humans dates back to 7.400 – 7.00 B.C. (This et al., 2006).

The grapevine belongs to the family Vitaceae, which comprises tens of wild *Vitis* species distributed in Asia (app. 40 species), North America (app. 40 species) and Europe (one species) under subtropical, Mediterranean and continental-temperate climatic conditions. *Vitis vinifera* L. is the only species that acquired significant economic interest over time. Some other species, notably the North American *Vitis rupestris* Scheele, *Vitis riparia* Michaux or *Vitis berlandieri* Planchon, since they reported interesting tolerance to limestone, drought and pathogens, such as Phylloxera (*Viteus vitifoliae* Fitch), powdery mildew (*Erysiphe necator* Schw) and downy mildew (*Plasmopara viticola* Berk. & Curtis, Berk. & De Toni) were widely used not only as rootstock, but also in new rootstock breeding programs (Terral et al., 2010).

Among the *Vitis* species, *Vitis vinifera* L. is currently the most cultivated grapevine around the world, except in few federal states in Nord America. However, *V. vinifera* is successfully cultivated only in temperate climate regions characterised by sufficient rain, warm and dry summers, and relatively mild winters (Jones et al., 2005)

2.2 Winegrowing regions in Slovenia and Italy

Slovenia has three main winegrowing regions (Figure 1):

- Posavje (winegrowing district of: Dolenjska, Bizeljsko-Sremič and Bela Krajina)
- Podravje (winegrowing district of: Štajerska Slovenia and Prekmurje).
- Primorska (winegrowing district of: Vipavska dolina, Slovenska Istra, Goriška brda and Kras)

The winegrowing region of Primorska is situated in the west of Slovenia. The southern part of the region extends to the Adriatic Sea and the Istrian peninsula up to the border with Croatia. Towards the west it borders Italy, and to the east and north is limited by the harsh continental climate of higher hills and plateaus (Kerma, 2010).

The winegrowing district of Kras occupies approx. 575 hectares of vineyards and it is surrounded by the Trieste Bay, the Vipava Valley and the Brkini hills. The most widely planted cultivar was 'Refošk', also known under synonyms: 'Refosco d` Istria', 'Refosco del Carso', 'Refošk istrski', 'Teran', 'Istrijanec', 'Teranovka' (Vertovec, 1844). 'Refošk' is cultivated mainly in the winegrowing districts of Kras and Slovenska Istra winegrowing districts where represents the 73 and 45 percent of the vineyards area (Register of grape and wine producers, 2014).

The winegrowing district of Kras is subdivided in two sub-districts, Kraška planota and Vrhe. The recommended varieties for the sub-district of Kraška planota are: Malvazija' and 'Refošk', while permitted varieties are 'Vitovska grganja', 'Chardonnay', 'Sauvignon', 'Sivi pinot', 'Beli pinot', 'Merlot' and 'Cabernet sauvignon'. The recommended varieties in Vrhe are 'Rebula', 'Malvazija', 'Laški

rizling', 'Sauvignon', 'Pinela', 'Zelen', 'Beli pinot', 'Sivi pinot', 'Chardonnay', 'Merlot', ''Barbera'' and 'Cabernet sauvignon', while permitted varieties are 'Zeleni Sauvignon', 'Rumeni muškat', 'Pikolit', 'Vitovska grganja', 'Prosecco', 'Modri pinot', 'Cabernet franc', 'Refošk', 'Syrah', 'Glera', 'Klarnica', 'Pergolin' and 'Poljšakica' (Rules on the demarcation…, 2003).

Figure 1: Winegrowing regions and districts in Slovenia (foto: www.sloveniavino.com).

The winegrowing district of Vipavska dolina of the winegrowing Primorska is denominated after the River Vipava occupies approx. 2,100 hectares of vineyards and is located in the western part of Slovenia. The valley is surrounded by the high plateaus of Trnovski gozd and Nanos on the north and by Karst on the south. The Vipavska dolina is subdivided in two winegrowing sub-district: Zgornja Vipavska dolina and Spodnja Vipavska dolina. In Primorska winegrowing regions there are preserved the oldest traditional and also a few autochthonous Slovenian grapevine cultivars (Škvarč, 2005).

Recommended varieties in Vipavska dolina are: ‘Rebula’, ‘Malvazija’, ‘Laški rizling’, ‘Sauvignon’, ‘Pinela’, ‘Zelen’, ‘Beli pinot’, ‘Sivi pinot’, ‘Chardonnay’, ‘Merlot’, ‘‘Barbera’’ and ‘Cabernet sauvignon’, while permitted varieties are ‘Zeleni sauvignon’, ‘Rumeni muškat’, ‘Pikolit’, ‘Vitovska grganja’, ‘Prosecco’, ‘Modri pinot’, ‘Cabernet franc’, ‘Refošk’, ‘Syrah’, ‘Glera’, ‘Klarnica’, ‘Pergolin’ and ‘Poljšakica’. The cultivar ‘Volovnik’ is included in the list of domestic and local cultivars in Slovenia (Rules on the demarcation…, 2003).

Italy has twenty wine regions: Veneto, Tuscany, Piedmont, Emilia-Romagna, Lombardy, Umbria, Abruzzo, Trentino Alto-Adige, Marche, Puglia, Lazio, Sicily, Sardinia, Campania, Liguria, Calabria, Molise, Basilicata, Valle d´Aosto and Friuli-Venezia Giulia.

The region Friuli-Venezia Giulia (Figure 2) occupies approx. 10,000 hectares of vineyards; yearly wine production is 4 million hectolitres of wine; 43 % white and 57 % red wines. Among them 21 % are classified in ″Denominazione di Origine Controllata″ (DOC) categories (Commission Regulation, 2007).

The region Friuli-Venezia Giulia is located in the far north-eastern corner of Italy, just across the border from Austria and Slovenia. There are some excellent vineyard sites in the sloping foothills of the Alps, but most of Friuli´s vineyards are located on the flat plains extending inland from the Adriatic Sea. The unique combination of mountain air and maritime breezes and humidity make an optimal conditions for viticulture - warm sunny days and cool evenings. The most important variety is Sauvignon and from red varieties ‘Schioppettino’, which is made into a full body wine with aggressive spice and flavour reminiscence of cherry.

Figure 2: Winegrowing districts of Friuli-Venezia Giulia region (foto: www.barriquefinewines.com).

2.3 Cultivars

2.3.1 'Refošk'

'Refošk'is of economic importance as the leading red grapevine and the fourth most frequent cultivar in Slovenia. 'Refošk'grapes grown in the district Kras are used to produce the highly appreciated wine PTP Teran, which is protected with a recognised traditional denomination (Rules on wine, 2013). 'Refošk'represents one of the earliest cultivated cultivars in this region and due to several clones, the ampelographers are still not in agreement on the basic traits of the cultivar. Italian varieties of 'Refošk'known as 'Refosco del peduncolo rosso', 'Refoscone', 'Refosco grosso', 'Refosco nostrano', are morphologically and genetically different from 'Refošk' grown in Slovenia (Cipriani et al., 1994; Calo, 2004; Rusjan et al., 2015).

The shoot tip of 'Refošk' is light green with high density of hairs. The edge of young leaf is reddish. The mature leaf is three partial or pentagonal, circular to cordate, leaf sinus forms a ″V″. The cluster (Figure 3) is a medium to big, compact with medium pedicels and low berry weight. The berries are dark blue with thick skin (Hrček in Korošec-Koruza, 1996).

Figure 3: Cluster of cultivar 'Refošk' (foto: Denis Rusjan).

2.3.2 'Schioppettino'

The cultivar 'Schioppettino' (Figure 4a and b) derives from Friuli-Venezia Giulia region, from the area between Prepotto and Goriška brda. The historical references of the 'Schioppettino' are from 1282. In 1863 the cultivar 'Schioppettino' was described in the wine grape catalogue for Friuli Venezia Giulia. Like other old varieties, in the early 20th century, 'Schioppettino' was also replaced with other varieties from France and in that time it was almost lost. Thanks to vine-growers and researchers, in 1981, the cultivar 'Schioppettino' is a recommended cultivar in the region Udine. Nowadays, the most famous location, where the cultivar 'Schioppettino' grows is Prepotto in the region Friuli-Venezia Giulia (Pucciarelli, 2010). Also in the winegrowing region Primorska, the cultivar is known as 'Pokalca' and is classified as permitted cultivar. (Rules on the demarcation, 2003).

a) b)

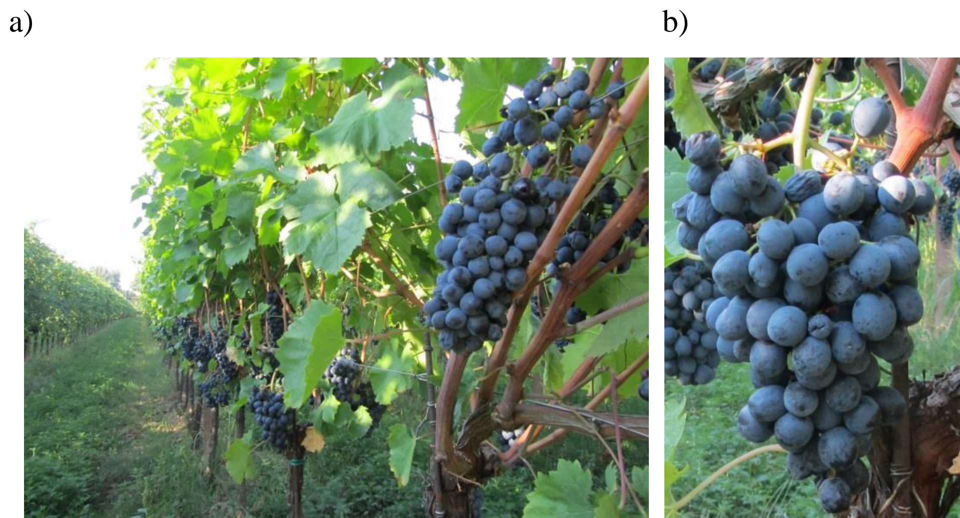


Figure 4: a) Vineyard of cv. ‘Schioppettino’ in Prepotto and b) ‘Schioppettino’ cluster (Foto: Maja Cigoj).

The synonyms of cultivar ‘Schioppettino’ are ‘Pokalca’, ‘Ribolla nera’ and ‘Črna rebula’. The shoot tip is fully open, green with high density of hairs. The mature leaf is pentagonal, leaf sinus forms a ″V″, the bottom side of leaf has a low hairiness. The cluster is a medium, cylindrical, very compact with short pedicels. The berries are dark blue with thick skin.

2.3.3 ‘Volovnik’

The long viticulture tradition in Vipavska dolina maintained some local grapevine cultivars such as ‘Volovnik’. The cultivar ‘Volovnik’ is an autochthonous cultivar, mainly planted in the Vipavska dolina, around the village Slap. A cultivar ‘Volovnik’ was first described by Matija Vertovec in the book Vinoreja in 1844 as a well known, but rarely planted cultivar. The synonym of the cultivar ‘Volovnik’ is ‘Drenik’ (Vertovec, 1844). The ampelographic description of the cultivar ‘Volovnik’ was not found in any literature.

2.4 Grapevine training systems

Grapevines need to be trained onto a trellis in order to spread the vine and provide light to the leaves and clusters. Training is the physical manipulation of a plant´s form. Training systems, regardless of their complexity can be distilled to four basic combinations:

- head/spur, basically a short trunk and several two-buds bearing units
- head/cane, a short trunk with one or more longer bearing units (Guyot)
- cordon/spur, horizontal extensions of the trunk with several two-node spurs
- cordon/cane, similar to head/spur but with longer bearing units (Sylvoz). Canes are usually tied in head-trained systems but can be free-hanging in conjunction with cordons.

Training a grapevine accomplishes many objectives. First, the perennial wood and canes can be disposed in such a way as to manipulate the exposure of leaf area to maximize the interception of light, leading to higher yield potential, optimization of the leaf area to fruit ratio, higher quality, and better disease control. Second, bearing units are distributed on a trellis to facilitate movement of equipment through the vineyard operations. Third, trunks and canes are disposed so as to avoid competition for light between vines. Fourth, proper training can provide that a renewal zone is formed, which ensures that the vine from is perpetuated and yield is maintained. Lastly, the amount of perennial wood can be varied to reduce the hazard of winter injury (Reynolds and Vanden Heuvel, 2009). The difference in training systems and pruning techniques is due to variability in fruitfulness of different grape varieties.

The Guyot training system was named after dr. Jules Guyot, a 19th century French scientist. In the ″Single Guyot″ (Figure 5a), each vine has one cane preserved each year, for the generation of the next year fruiting canes, and one spur, which is for the generation of the replacement cane. In ″Double Guyot″ (Figure 5b), each vine has two canes trained in opposite directions along wires.

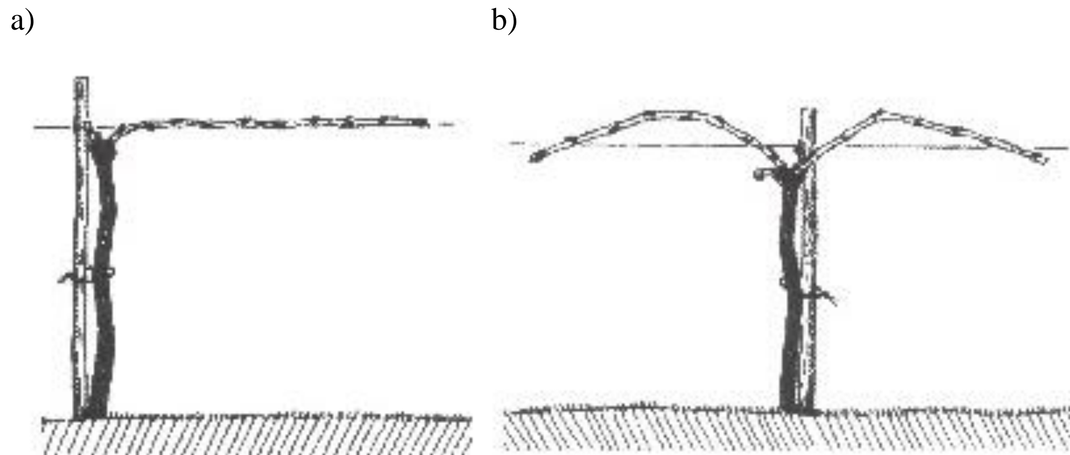


Figure 5: Single (a) and double (b) Guyot training system (Vršič and Lešnik, 2005).

2.5 Grapevine pathogens

Environmental stresses represent the most limiting factors for agriculture. Common abiotic stresses around the world that affect the grapevine are drought (water deficit), temperature, and acidity of the soil. Rarely there is a single abiotic stress affecting a plant, there are almost always interacting factors (Cramer, 2010). Besides abiotic stresses, grapevine is also exposed to many biotic stresses caused by insects, fungi, bacteria, phytoplasmas and viruses, which are responsible for great economic losses throughout the world, and for entraining the extensive use of agrochemicals that could cause biotic stresses (Laimer et al., 2009).

The accidental introduction of the root-attacking insect Phylloxera from North America into Western Europe resulted in massive destruction of vineyards. Consequently, Phylloxera resistant North American *Vitis* species and their hybrids were used as rootstocks onto which *V. vinifera* varieties were grafted (King and Rilling, 1985). Furthermore, grapevine scion and rootstock varieties are exchanged frequently between growers, breeders, and researchers across the world. Perhaps the long history of cultivation, grafting between different scion and rootstock varieties, and introduction of new viruses via vectors such as mealybugs, scale insects and nematodes are responsible for the fact that grapevines are known to be host to a vast number of taxonomically diverse pathogens.

Grapevine is susceptible to a wide variety of plant pathogenic fungi, bacteria and viruses, which affect the plant growth conditions, decreased yield and grape quality (Nicol et al., 1999; Singh Brar et al., 2008; Hren et al., 2009; Mannini et al., 2011).

There are more than 80 infectious agents including viruses, viroids and phytoplasmas that have been reported in grapevines, some with extremely high incidences (Martelli and Boudon-Padieu, 2006). To date, 68 viruses that belong to eight families and 21 genera have been isolated from grapevines (Martelli, 2012); a single grapevine plant can be infected by a mixture of distinct virus species (Gugerli et al., 1997; Credi, 1997; Pompe-Novak et al., 2007; Komar et al., 2008) and viral variants (Meng et al., 1999; Goszczynski and Jooste, 2003; Vigne et al., 2004; Turturo et al., 2005; Meng et al., 2006; Pompe-Novak et al., 2007).

Virus diseases spread by insects (such as Pierce´s disease), or by nematode (such as fanleaf degeneration disease) are the most destructive and are difficult to control.
Virus caused diseases rank as the most economically damaging of any grapevine diseases, because in contrast to most fungal and bacterial diseases, once infected, the canes remain systemically infected for life with no prospect for a cure. Viruses seriously disrupt the structure and all functions of infected grapevine plants. Damaging effects of viral infections are expressed by various types of symptoms. First and most important is the reduction of grape yield and quality, often also reducing the productive life of grapevine canes. In the production of grapevine stock, some viruses prevent rootstock and scion unions (incompatibility). The extent of damage depends on the characteristic of individual viruses and their strains, the susceptibility of a grapevine variety and the mode of virus transmission and spread (Woodham et al., 1983; Clingeleffer and Krake, 1992; Wolpert and Vilas, 1992).

Plant secondary metabolism provides a line of defence in cellular response to biotic and abiotic stress and changes the grape quality, as secondary metabolites contribute to colour, taste and aroma of fresh and dried grapes and they are involved in wine stabilization and aging processes (Ferrandino and Lovisolo, 2014).

An important quality indicator of red grapes and wine is the colour, which is impacted by biotic stresses such as pathogen attack (Lee and Martin, 2009). Among biotic stresses, viral infections produce an important impact in grapevine physiology, causing significant economic losses every year (Vega et al., 2011).

2.6 Nepoviruses

Grapevine fanleaf virus (GFLV) is a biparticulate and bipartite member of the genus *Nepovirus* in the family *Secoviridae* (Sokhandan-Bashir and Melcher, 2012). *Secoviridae* is a newly assigned family of plant viruses in the order *Picornavirales,* that includes the genera Comovirus, Fabavirus, Nepovirus, Cheravirus, Sadwavirus, Sequivirus and Waikavirus (Sanfaçon et al., 2009).

Nepoviruses are divided into three subgroups based on the sizes of their RNA2. Species that infect grapevine in subgroup A are grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV), Tobacco ringspot virus (TRSV), Grapevine deformation virus (GDefV) and Raspberry ringspot virus (RpRSV). In Subgroup B there are Artichoke Italian latent virus (AILV), Grapevine chrome mosaic virus (GCMV), Grapevine Antolian ringspot virus (GARSV) and Tomato black ring virus (TBRV). In subgroup C there are Bluberry leaf mottle virus (BBLMV), Cherry leafroll virus (CLRV), Grapevine Bulgarian latent virus (GBLV), Grapevine Tunisian ringspot virus (GTRSV), Peach rosette mosaic virus (PRMV) and Tomato ringspot virus (ToRSV) (Digiaro et al., 2007).

Additional linear or circular satellite RNAs, which sometimes modulate symptoms, are found associated with several *Nepoviruses* of all three subgroups. They are either linear (1100-1800 nts) with a 5′-linked VPg, a 3′ poly(A) tail encoding a 36-48 kDa polypeptide, or circular (300-460 nt) and apparently non-coding (King et al., 2012).

2.7 Grapevine fanleaf virus (GFLV)

Grapevine fanleaf degeneration caused by GFLV is one of the oldest known viral diseases of grapevines. It occurs in all winegrowing regions of the world (Liebenberg et al., 2009). In the European theory literature, records of the disease date back some 50 years, and grapevine leaves with typical symptoms were found in herbaria established before the introduction of American rootstock hybrids (Martelli and Boudon-Padieu, 2006). The virus is found on its natural woody host, *Vitis* ssp., all over the world and has also been reported on Bermuda grass (*Cynodon dactylon* L.) in Iran (Izadpanah et al., 2003; Zarghani et al., 2013).

2.7.1 Genome of GFLV

The genome of GFLV is composed with two single-stranded, positive-sense RNAs, termed RNA1 and RNA2 and sat RNA (Figure 6) (Mekuria et al., 2009).

RNA1 is 7,342 nt long and contains a single open reading frame of 6,855 nt, extending from nts 243 to 7097 (Ritzenthaler et al., 1991). RNA1 encodes polyprotein P1 (253 kDa), which is a processed by an embedded proteinase activity into five proteins required for replication, including a putative proteinase cofactor (1A), a putative helicase ($1B^{Hel}$), a viral protein genome-linked or VPg ($1C^{Vpg}$), a proteinase ($1D^{Pro}$) and a putative RNA-dependent RNA polymerase (RdRp) ($1E^{Pol}$) (Ritzenthaler et al., 1991; Andret-Link et al., 2004; Liebenberg et al., 2009) (Figure 6). These proteins are required for RNA1 replication, and function in trans to ensure RNA2 replication (Ritzenthaler et al., 2002).

RNA2 consists of 3774 nts and codes for a polyprotein of Mr 122K, which is cleaved by the RNA1 encoded viral proteinase into three individual proteins, including a homing protein ($2A^{HP}$) necessary for RNA2 replication, a movement protein ($2B^{MP}$) and a coat protein ($2C^{CP}$) (Serghini et al., 1990; Ritzenthaler et al., 1991; Margis et al., 1993; Gaire et al., 1999) (Figure 6).

The analysis of the RNA content of the F13 GFLV isolate revealed the presence of extra RNA, RNA3, which has been found to have properties of a satellite RNA (Pinck

et al., 1988). This RNA is dependent on the presence of the two genomic RNAs for its multiplication. The structure obtained was 1114 nucleotides in length (Fuchs et al., 1989).

Lamprecht and collaborators, 2012 detected satRNA in field samples of Cabernet sauvignon, collected in the South Africa. The full length sequence of GFLV-SACH44 satRNA is 1,104 nt in length excluding the poly(A) tail. This isolate is more similar to ArMV satRNA (86-88% identity) than to GFLV-F13 (82% identity) (Lamprecht et al., 2012).

In field samples of Zinfandel and Cabernet sauvignon collected in California by Gottula and collaborators, 2013, detected satRNA, which showed at least 94% identity with each other, but only up to 78% with the satRNA of GFLV-F13. Samples collected from germplasm collection in New York, showed a satRNA with 94 to 98% identity at the nucleotide level with satRNAs collected in California and 77.5% with the GFLV F13 satRNA. These GFLV satRNA variants had a higher nucleotide sequence identity with satRNAs of ArMV strains NW and J86 (93.8 to 94.6%) than with the satRNA of GFLV- F13 and those of other ArMV strains (68.3 to 75.0%) (Gottula et al., 2013).

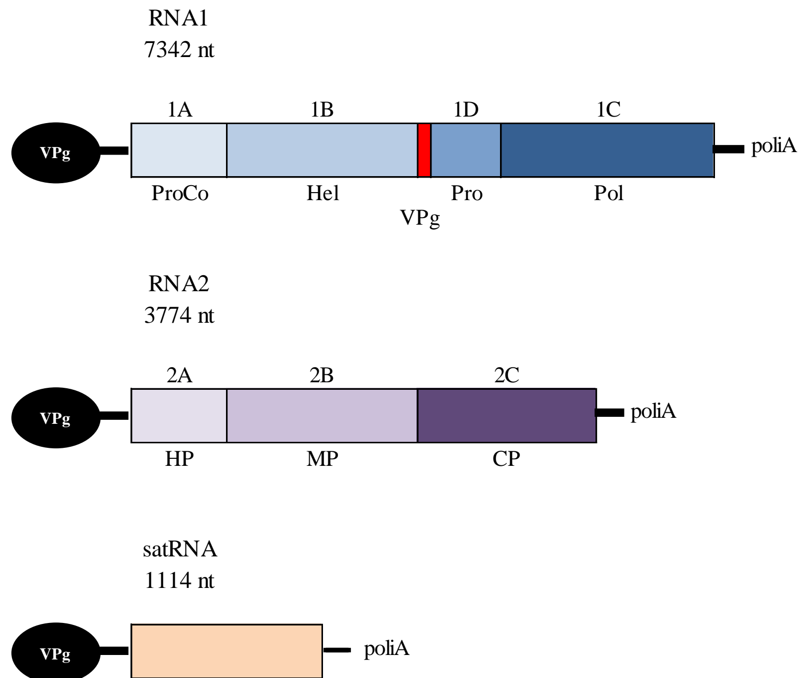


Figure 6: Schematic presentation of genetic organization of genomic (RNA1 and RNA2) and satellite RNAs of GFLV. ORFs are represented by open boxes and the 5´ and 3´ UTR regions by narrow lines. ProCo – proteinase cofactor, Hel – helicase, VPg - viral protein, Pro – proteinase, Pol – polymerase, HP - homing protein, MP - movement protein, CP - coat protein, polyA – polyA tail (Fuchs et al., 1989; Belin et al., 2001).

Multiple infections by divergent GFLV isolates can occur in a single grapevine (Vigne et al., 2004; Pompe-Novak et al., 2007), as well as mixed infections with other *Nepoviruses* and viruses from different genera (Laimer et al., 2009). Recombination can be an important factor in viral evolution (García-Arenal et al., 2000; García-Arenal et al., 2001; Moury et al., 2006) and in the case of GFLV, recombination have been reported to occur within RNA2, both between distinct genetic variants of GFLV (Boulila, 2007; Pompe-Novak et al. 2007; Vigne et al. 2004, 2008, 2009), and between GFLV and other closely related viruses from the genus *Nepovirus,* including ArMV (Vigne et al., 2008; Jawhar et al., 2009; Mekuria et al., 2009) and GDeFV (Mekuria et al., 2009).

2.7.2 Symptomatology

GFLV got its name from the fan-like leaf shape that may be exhibited on infected vines and the gradual decline in growth and vigour of infected vines over time (Oliver & Fuchs, 2011).

GFLV can cause symptoms on leaves, shoots and bunches (Figure 7). The infection with GFLV affects vine growth; affected vines may be smaller than healthy ones, particularly if the nematode vector is present. The canes and foliage appear clustered because of stunting. Internodes may develop secondary shoots or split. Tendrils occasionally develop into lateral shoots (Golino et al., 2013).

Three commonly leaf symptoms are associated with vine infection:

Fanleaf deformations: leaves are asymmetric with an open petiole sinus. The main veins are drawn close together and teeth along the margin of the leaf blade are elongated, giving the leaf the appearance of a fan. Leaves become distorted and asymmetrical with sharply toothed margins and closer primary veins (Andret-Link et al., 2004).

Yellow mosaic: leaf blades develop a bright yellow colour over the entire leaf or in irregular patches across the leaf blade. The intense yellow appears in early cool spring and fades rapidly with rising temperatures. Other foliar symptoms include chlorotic mottling, yellow mosaic with partially or completely chrome-yellow leaves (Raski et al., 1983).

Vein banding: bright yellow bands may develop along the major veins starting in early or midsummer and persist through most of the vegetative season (Martelli, 1993). In summer, the vegetation resumes its optimal colour (Pearson and Goheen, 1988).

Canes can also be malformed, showing short internodes, double nodes and zigzag growth between nodes (Raski et al., 1983).

Difference in symptomatology caused by GFLV (e.g. bushy-like growth or yellow mosaic symptoms) may reflect also in different physiological response of the grapevine (Martelli and Savino, 1990). Variability in symptom expression may depend on the host (*Vitis* species or cultivar) and on the virus strain (Legin et al., 1993). No clear association was observed among different GFLV isolates and expressed symptoms (Pompe-Novak et al., 2007).

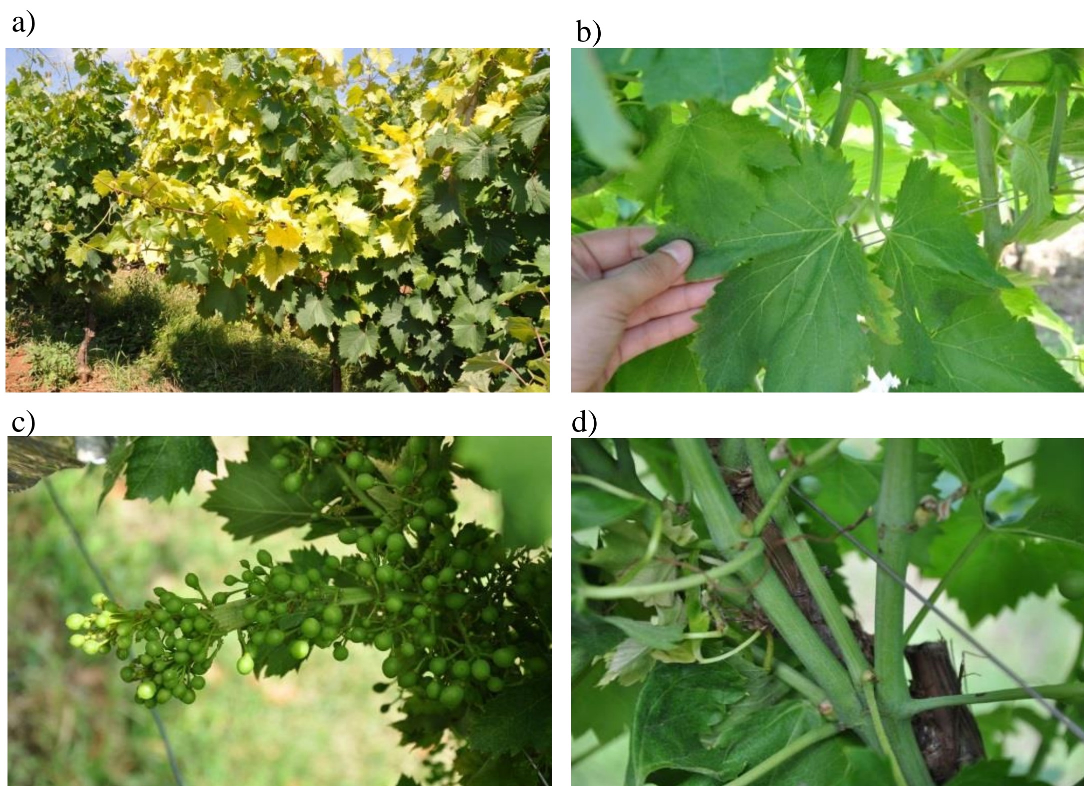


Figure 7: Typical GFLV symptoms: a) leaf yellowing; b) fanleaf deformations; c) symptoms on cluster and d) double nodes (foto: Maja Cigoj).

Yield losses, caused by GFLV infection, could be moderate (10 %) to very high (>80 %), depending on the virulence of the virus isolate, the susceptibility of the grapevine variety, and environmental factors (Andret-Link et al., 2004). The reduction in yield can even result in a total loss of production (Raski et al., 1983). GFLV virus is estimated to affect around 2,000 hectares (6 % of the total acreage cultivated with grapes) in the Champagne region of France. The productive life of GFLV infected vineyards is also significantly reduced, 15-20 years instead of 30-40 years or longer (Andret-Link et al., 2004). The rooting ability of rootstock and the graft take of scions

are both substantially reduced in GFLV infected grafts. Clusters and berries are reduced in size and number, their ripening is irregular (Martelli and Savino, 1990) (Figure 8). It was reported that also grape quality is affected by GFLV due to a decrease in sugar content and titratable acids (Andret-Link et al., 2004).

Figure 8: Clusters of GFLV infected vines of cultivar 'Schioppettino' (foto: Maja Cigoj).

Several findings demonstrate the negative influence of Grapevine leafroll-associated viruses (GLRaV) and the viruses linked to the rugose wood (RW) complex on grapevine physiology (Guidoni et al., 1997; Bertamini et al., 2004), growth (Credi and Babini, 1997; Cabaleiro et al., 1999), and must quality of wines such as colour intensity (Lider et al., 1975), soluble solid accumulation and titratable acids in berries (Cretazzo et al., 2009).

Several reports demonstrate also the effect of mixed infection with GFLV and other viruses. Credi and Babini, (1997) observed the reduction in yield in vines infected with mixed infection with GFLV and GLRaV, lower titratable acids, and pH. The mixed infection with GFLV and grapevine fleck virus (GFkV) affected vines performance and chemical composition of grape juice (Cretazzo et al., 2009; Santini et al., 2011). Studies have reported that infection is often associated with reduced vegetative vigour (Walter and Martelli, 1996; Credi & Babini, 1997; Cabaleiro et al., 1999; Kovacs et al., 2001), but most reports regarding of impact of GFLV on production parameters are quite generalised and not supported by detailed studies, except of Cretazzo et al. (2009), who observed the influence of GFLV on growth and production parameters of grapevine on relatively small number of vines.

2.7.3 Transmission and control

GFLV is transmitted from grapevine to grapevine by the ectoparasitic dagger nematode *Xiphinema index* of the family *Longidoridae* (Hewit et al., 1958; Raski et al., 1983; Brown & Weischer, 1998). The long-distance spread of grapevine viruses occurs primarily by the propagation of infected plant material (Gambino et al., 2005; Oliver and Fuchs, 2011) (Figure 9).

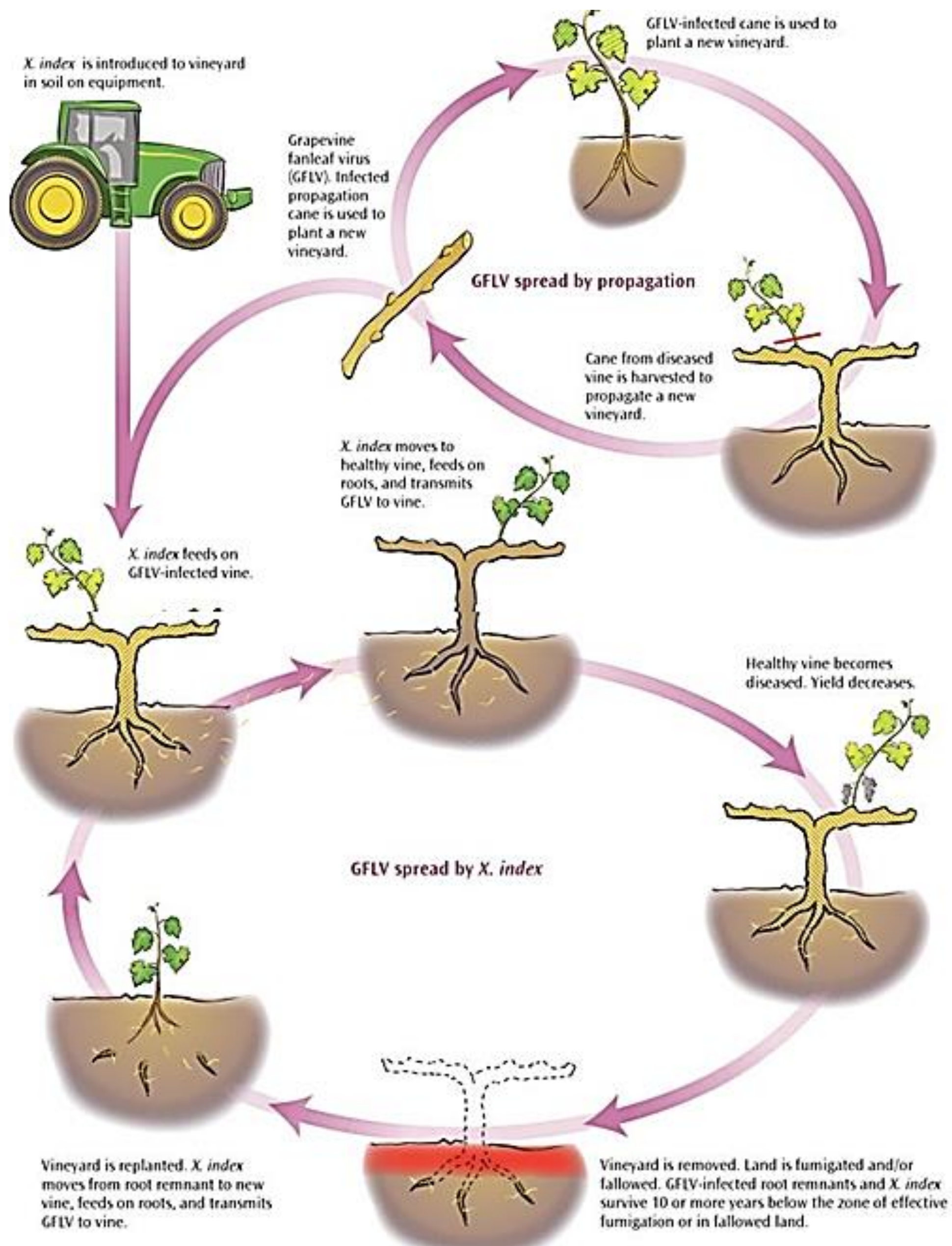


Figure 9: GFLV transmission (Gollino, 2013).

Xiphinema index males are rare, females reproduce parthenogenetically, and adults develop through four juvenile stages (Raski et al., 1983). Like other *Nepoviruses*, GFLV can be acquired and transmitted by both juvenile and adult forms of the vector. GFLV is not passed transovarily through nematode eggs (Taylor and Raski, 1964; MacFarlane, 2003).

Xiphinema index feeds on growing root tips and acquires GFLV particles upon feeding (Hewit et al., 1958; Raski et al., 1983; Wyss, 2000). A single brief feeding on an infected vine root can make nematodes viruliferous for up to 9 months in moist soil in the absence of host plants. The nematode can retain the virus for up to eight months in the absence of host plants, or up to three months when feeding on resistant host plants (Taylor and Raski, 1964). *Xiphinema index* has been reported to survive for 4.5 years in a fallowed vineyard, although remaining grapevine roots were suspected to have maintained nematode viability by providing feeding sources (Raski et al., 1965). A period of at least 10 years is necessary to ensure the elimination of *Xiphinema index* populations.

The use of nematicides and fumigants to control the nematode has not been successful, because of nematode´s ability to exist on detached grape roots deep in the soil profile and because of the relatively poor penetration of fumigants (Raski and Goheen, 1988).

The use of resistant rootstock upon which fruiting cultivars are grafted is often the best way to overcome nematode problems in perennial crop. The new rootstock named VR 039-16, a hybrid between *V. vinifera* and *V. rotundifolia*, was discovered resistant to *X. index* and tolerant to fanleaf virus (Ferris et al., 2012).

The transgenic approach to obtain virus resistance could be a useful strategy to control the infection. Several transgenic attempts against GFLV were made to achieve resistance by expressing the viral coat protein (CP) gene (Gambino et al., 2005; Valat et al., 2006). Other GFLV derived construct such as the MP gene were introduced into rootstock 41B (Valat et al., 2006). Resistance to GFLV was also reported in the cultivar Chardonnay grafted onto transgenic 41B rootstock clones expressing the

GFLV CP gene (Vigne et al., 2004). These transgenic grapevines had no detectable effect on the emergence of recombinant GFLV species over a three year tested period in naturally GFLV infected vineyards (Fuchs et al., 2007).

The propagation of uninfected material is one of the most effective ways for controlling grapevine virus disease. The certification scheme for grapevine provides detailed guidance on the production of pathogen tested material of grafted grapevine varieties and rootstocks. For the production of certified grapevine varieties and rootstock, the following successive steps should be taken:

- selection for healthy quality of individual plants of each scion variety or rootstock;
- the assessment of health status of visually selected plants by testing the production of healthy plants (nuclear stock) by thermotherapy and/or meristem-tip (shoot-tip) culture followed by testing;
- the maintenance of the nuclear stock under conditions ensuring freedom from re-infection by aerial or soil vectors;
- multiplication of the nuclear stock in one phase (propagation stock), under conditions ensuring freedom from re-infection;
- distribution of propagation stock to nurseries, and
- production of certified (virus tested) plants.

Plant material produced according to this certification scheme is derived from nuclear stock plants that have been tested and found free from the viruses.

In Slovenia certification scheme is recommended to test the presence of: GFLV, ArMV, TBRV, Grapevine leafroll associated virus (GLRaV) -1, -2, -3, 4-9, GVA and Grapevine fleck virus (GFkV) (Rules on the demarcation, 2003).

GFLV free material is readily obtained through conventional or slightly modified thermotherapy, grafting or in vitro meristem and shoot tips culture. Sanitary selection combined with thermotherapy is a most effective tool to reduce the incidence of fanleaf virus in new established vineyards. Healthy plants, when planted in nematode free soil or in soils with populations of nonviruliferous vectors, remain uninfected for the productive life of the vineyard. Vineyards planted with GFLV free plants are very

homogeneous in morphology and productivity, the yield is improved from 40 to 70 % and the berries contain higher amount of sugar (Pearson and Goheen, 1988).

2.8 Grapevine ripening and quality parameters

Ripening is characterized by a number of changes, including berry volume increase, berry colouration, flesh softening, catabolism of organic acids, formation of flavour and aroma compounds, and intense accumulation of soluble solids (Coombe and McCarthy, 2000; Terrier et al., 2001).

Grape is a non-climacteric fruit. Berry development and ripening can be divided into three phases according to the berry formation (Figure 10) (Coombe and McCarthy, 2000). During stage I, starting at fruit set, berries grow through cell division; stage II, called lag phase, is characterized by a pause in berry growth while seed embryos start to form and grow. Stage III starts at véraison , when berries change colour, soften, accumulate sugars and metabolize acids (Coombe, 1959; Harris et al., 1968).

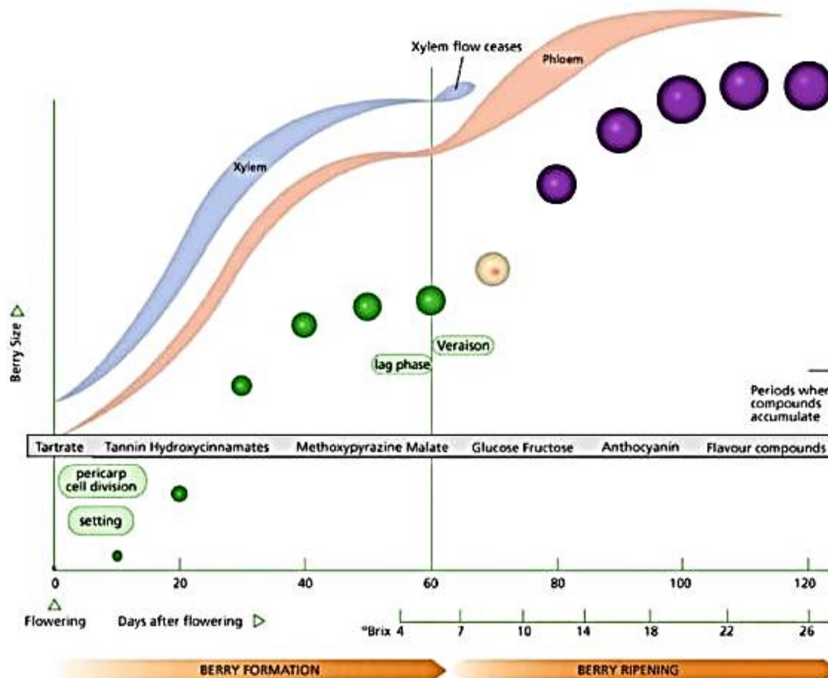


Figure 10: Diagrammatic representation of berry development (Keller, 2010).

Stage I starts at bloom and lasts for approximately 60 days afterwards, corresponding to a phase of fast cell division and elongation with rapid accumulation of organic acids. The berry expands in volume and starts to accumulate solutes such as tartaric and malic acid. Tartaric acid has the highest accumulation in the flesh. It accumulates during the initial stages of berry development and provides acids. Malic acid has the highest content in the flesh and it is also important in the final wine making process. Other important acids that start to accumulate in flesh and skins of the berry at that time are hydroxycinnamic acids. They are important because of their involvement in browning reactions and because they are precursors to volatile phenols such as tannins. The tannins are present in the skins and seeds, and are responsible for bitterness and astringency of wine (Keller, 2010).

Stage II, called the lag phase is distinguished by a pause in berry growth, during which seed embryos start to grow rapidly. At the start of lag phase, berries have reached at least half of their final size. Following the five to ten day lag, cells expand and continue to accumulate acids and tannins, which reach their maximum levels at verasion (Coombe and Bishop, 1980; Keller, 2010).

The stage III starts with véraison which is marked by berry softening and an increase in sugar content, followed by a rapid change in skin colour from green to red, the most abundant accumulation of soluble solids and the dilution of tartaric acid. During third phase, the berry doubles in size (Keller, 2010) and significant changes occur in the fruit both at level of gene expression as well as physiology and structure: the cell wall softenes, sugars and anthocyanins accumulate, metabolism of organic acids, accumulation of flavour compounds and changes in the level of growth substances (Robinson and Davies, 2000; Terrier et al., 2005; Deluc et al., 2007; Lund et al., 2008). During the ripening phase a number of major physiological and biochemical changes occur simultaneously in the grape berry, and these changes determine the quality of the fruit at its harvest.

2.8.1 Soluble solids

During the first period of rapid growth of the berries the percentage of sugars is low, usually less than 2 % of the berry fresh weight. During ripening, the sugars increase rapidly (Winkler et al., 1974). The primarily sugar of grapes are glucose and fructose, which determine fruit and wine quality because, they contribute to the sweet taste of the fruit, decrease the perception of acidity, bitterness and astringency (Keller, 2010). The ratio of glucose and fructose in grapes changes considerably between fruit set until fruit maturity. Glucose predominates during the green berry and early ripening stages; during the latter part of berry ripening glucose and fructose are present in about equal concentration whereas in overripe grapes fructose generally exceeds glucose. Sugars represent more than the 90 % of the soluble solids in mature berries. In berries of most *Vitis* cultivars, 95-99 % of these sugars are present in the form of the hexoses glucose and fructose, the remainder is mainly sucrose (Keller, 2010). Soluble solids are expressed as °Brix, °Baume, or °Oechsle and their content can be measured by several methods. Sugar content in berries is related to the potential alcohol volume (% vol.) after alcohol fermentation and the likelihood of residual sugars remaining (Jackson and Lombard, 1993).

2.8.2 Organic acids and pH

The content of organic acid is one of the most important quality characteristics of grapes for wine production, and has an important impact on wine colour, flavour and stability (Mato et al., 2005). The dominant organic acids in grape are tartaric acid and malic acid, which represent 70 to 90 % of total grape titratable acids. In comparison with tartaric and malic acid, citric acid is present in grape juice and wine in relatively low content (Ruffner, 1982). Although it has a minor direct impact on the organoleptic properties of wine, the content of citric acid is important in the control and development of flavour during and after malolactic fermentation (Nielsen and Richelieu, 1999). Tartaric acid is the primary non fermentable soluble acid in grape and the principal acid in wine, contributing an important aspect to taste, and aging

potential of the wine (Preiner et al., 2013). The level of tartaric acid rises during ripening (Jančářová et al., 2013).

Juice, pressed from ripening grape, generally has a pH between 3.0 and 3.5, but sometimes can exceed 4.0 in overripe berries. Values of pH in excess of approximately 3.6 are undesirable because they lead to decreased colour intensity and microbial stability and increased susceptibility to oxidation in wine and other grape products (Keller, 2010).

The skin colour is reddish and brilliant in grapes of moderate to high acids and low pH, and tends to be bluish and dull in grapes of low acids and high pH. The pH is also a determining factor for the duration and start of alcohol fermentation. At a low pH, other conditions being equal, the fermentation will be cleaner and the wine less liable to attack by spoil organisms (Winkler 1974).

2.8.3 Phenolic compounds

Phenolic compounds can be defined as molecules naturally derived from plants or microbes consisting of a phenyl ring backbone with hydroxyl group or other substitutes (Teixeira et al., 2013).

Phenolic compounds are secondary plant metabolites that are one of the major quality factors in grapevine and in the resulting wine due to their contribution to red wine colour (Figueiredo-González et al., 2012) and taste (bitterness and astringency); in addition, they have exhibited potential benefits to human health (Cheynier, 2005).

The World Health Organization (WHO) emphasizes the importance of antioxidant activity of phenolic component for the most important health problems prevention, namely, cardiovascular diseases, diabetes, cancer and obesity (Paredes-López et al., 2010).

Phenolic compounds of the grape are divided between:

- nonflavonoid compounds: hydroxybenzoic acid, hydroxycinnamic acids and stilbens
- flavonoid compounds: flavanones and flavones, flavonols (flavan-3-ols) and anthocyanins

The skin represents around 10-15 % of the berry weight and it is the principal source of aromatic compounds and flavour precursors. It also contains flavonoid phenolic compounds (30 % of the total berry phenolics). The seeds, which represent about 4 % of berry fresh weight, contain both non-flavonoid and flavonoid phenolic compounds, including a relatively large amounts of tannin. Seed phenolics represent 60 % of those compounds found in the berry. The flesh accounts for about 80 % of the berry weight; its primary constituents are hexose sugars, organic acid and non-flavonoid phenolic. Phenolic compounds in the flesh represent around 10 % of the total phenolic content of berries (Hornsey, 2007). The schematic representation of phenolic compound distribution in a grape berry is presented in Figure 11.

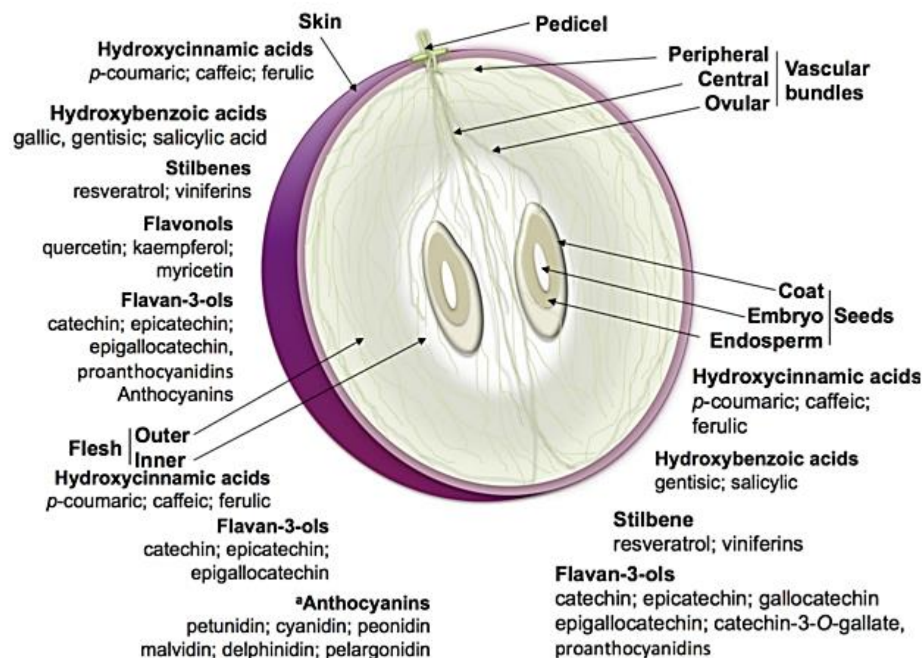


Figure 11: Shematic structure of a ripe grape berry and phenolic biosynthesis distribution between several organs and tissues (Teixeira et al., 2013).

2.8.3.1 Nonflavonoid phenolics

Nonflavonoid phenolics are found in grapes and wine, but with exception of hydroxycinnamic acid, they are present at low concentrations.

Hydroxycinnamic acids are the major phenolic compounds in white wine and are important in white wine colour (Kennedy et al., 2006). Their synthesis occurs mainly before véraison and during ripening (Table 1). The content of hydroxycinnamic acids decreases with increasing berry size and dilution of solutes, through its content per berry remains almost constant. Although its accumulation occurs predominantly in the flesh, they are present in all berry tissue (Teixeira et al., 2013). In terms of concentration, *p*-coumaric, caffeic, and ferulic acids are predominant. These three hydroxycinnamic acids differ by the type and number of substituents on the aromatic ring (Figure 12). They are present primarily as *trans* isomers, but traces of *cis* isomers have also been detected. Hydroxycinnamic acids are esterified with tartaric acid, and thus named coutaric acid (*trans-p*-coumaroyl-tartaric acid), caftaric acid (*trans*-caffeoyl-tartaric acid), and fertaric acid (*trans*-feroulyl-tartaric acid) (Castellarin et al., 2012).

Stilbenes are polyphenolic secondary metabolites (Jeandet et al., 2002), whose skeleton is based on the 1,2-diphenylethylene structures (Moreno-Labanda et al., 2004). These compounds are present in soft tissues (fruits, leaves, root tips and other herbaceous organs) as phytoalexins induced by biotic and abiotic stress (Bavaresco et al, 2007). In grapes, two of major stilbene phytoalexins are *trans*-reservatrol (*trans*-3,4´5-trihydroxistilbene), *trans*- and *cis*-piceid (*trans*- and *cis*-resveratrol-3-O-β-D-glucopyranoside) (Mattivi et al., 1995). Stilbenes are located essentially in skins (Table 1) and mainly in glucosylated form (Creasy and Coffee, 1988), but were also reported to be present in grape seeds (Pezet and Cuenat, 1996).

2.8.3.2 Flavonoid phenolics

Flavonoids are localized mainly in the berry skin and in some layers of the seed coat (Table 1). Most of the skin flavonoids are abundant in the inner thick-walled layers of

hypodermis. In this fraction, the major class of flavonoids is represented by anthocyanins, proanthocyanidins (also known as tannins) and flavan-3-ols and flavonols (Teixeira et al., 2013).

Flavonoids are C6-C3-C6 polyphenolic compounds in which two hydroxylated benzene rings, A and B, are joined by a three-carbon chain that is part of a heterocyclic ring (Figure 12).

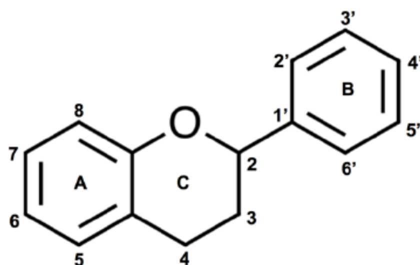


Figure 12: Flavonoid ring structure and numbering (Teixeira et al., 2013).

Flavonols are a class of flavonoids with 3-hydroxyflavone backbone. They differ by the number and type of substituents on the B ring (Figure 12), and occur conventionally as glucosides, galactosides, rhamnosides, and glucuronides with the sugar bond attached to the position 3 of the flavonoid skeleton. The grape berry synthetize kaempferol, quercitin, myrcetin and the methylated forms isoharmnetin, laricitrin and syringetin (Teixeira et al., 2013). Flavonols protect plants against UV light. It was reported that sunlight and UV-B light increase concentration of quercetin glycosides in grapevine berries, petunia and soybean (Czemmel et al., 2009).

Flavan-3-ols are a complex subclass of flavonoids encompassing the simple monomers (+) catechin and its isomer (-) epicatechin, and the oligomeric and polymeric procyanidins, commonly known as condensed tannins (Tsang et al., 2005).

Proanthocyanidins are oligomers and polymers of flavan-3-ols units linked by C4-C6 and C4-C8 carbon-carbon bonds. They are known to accumulate in grape skins and seeds but to be negligible in flesh. Proanthocyanidins contribute to the astringency and bitterness of grape and wine and play a very important role in the quality of red wine (Fujita et al., 2005).

Table 1: Phenolic compounds produced and accumulated in the grape berry (Teixeira et al., 2013)

	Level of synthesis[a]				Berry phonological stage[b]			
Compound	Skin	Flesh	Seed	Location	Blooming	Green stage	Véraison	Ripening
Nonflavonoids								
Hydroxycinnamic acid	++	+++	++	Hypodermal cells and placental cells of the pulp; primarily in the vacuoles of mesocarp cells.	+++	+++	+	+
Hydroxybenzoic acids	+	-	++					
Stilbenes	+++	+	++	Berry skin and seeds.	-	+	++	+++
Flavonoids								
Flavonols	++	+	+++	Dermal cell vacuoles of the skin tissue and cell wall of skin and seeds.	++	+	+++	++
Flavan-3-ols	++	+	+++	Specific vacuoles of hypodermal skin cells and seeds coat soft parenquima.	+	++	+++	++
Anthocyanins	+++	-*	-	Cell layers below the epidermis; storage confinedto the vacuoles and cytoplasmic vesicles named anthocyanoplasts.	-	-	+	+++

Legend: [a,b] Very abundant compound (+++) to absent (-); *Teinturiers contain anthocyanin in mesocarp cells.

2.8.3.3 Anthocyanins

During ripening, the phenolic composition of the skin changes as the berry loses chlorophyll (Giovanelli & Brenna, 2006) and begins to synthesize and accumulate phenolic compounds (Watson, 2003).

Anthocyanins are phenolic plant metabolites belonging to the flavonoid family. They are water-soluble pigments that are responsible for the red, blue, and purple colours of most flowers and fruits. They play an important role in wine quality, contribute to the sensory characteristic of wine (Košir et al., 2004) and also in protecting plants against abiotic and biotic stresses. They are also known as potential antioxidants. The beneficial health roles of anthocyanins have received considerable attention as they are potentially protective factors against cancer and heart disease (Guo et al., 2014).

The anthocyanidins are the basic structure of the anthocyanins. The anthocyanidins (or aglycons) consist of an aromatic ring (A) bonded to an heterocyclic ring (C) that

contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring (B) (Figure 13). When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety), they are known as anthocyanins (Castañeda-Ovando et al., 2009).

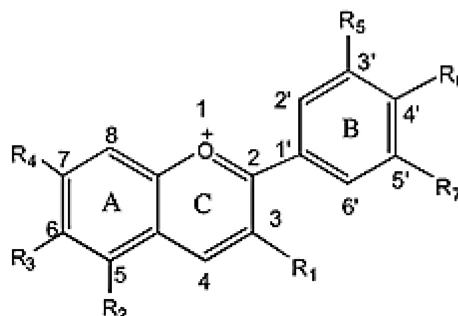


Figure 13: Structural identification of anthocyanidins (Castañeda-Ovando et al., 2009).

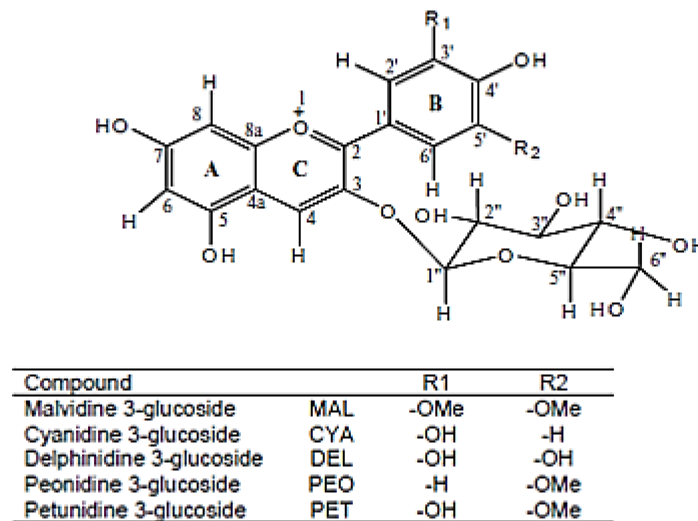
There are six common anthocyanidins in plants: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Each of these anthocyanidins can be glycosylated and acylated at different sites and with different sugars and acyl groups (Boss and Davis, 2001).

Vitis vinifera varieties usually produce 3-monoglucoside, 3-acetylglucoside and 3-p-coumaroyglucoside derivatives of the anthocyanidins delphinidin, cyanidin, peonidin, petunidin and malvidin (Figure 14) (Boss and Davis, 2001).

Chemically, anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts. These compounds strongly absorb visible light and are responsible for many of the colours seen in plant tissues, ranging from red through to blue. There have been many different types of anthocyanins in plants. The different anthocyanins are distinguished by:

- the number and position of hydroxyl groups attached to the rings
- the degree and position of methylation of the hydroxyl groups
- the nature and the number of sugars attached, and the position of their attachment
- the nature and number of aliphatic or aromatic acids attached to these sugars

Anthocyanin accumulation commences at véraison and continues throughout ripening (Kuhn et al., 2013). The extent of this accumulation is influenced by several variables including differences in cultivar, season, growing region and viticultural practices.



Compound		R1	R2
Malvidine 3-glucoside	MAL	-OMe	-OMe
Cyanidine 3-glucoside	CYA	-OH	-H
Delphinidine 3-glucoside	DEL	-OH	-OH
Peonidine 3-glucoside	PEO	-H	-OMe
Petunidine 3-glucoside	PET	-OH	-OMe

Figure 14: Structures and numbering scheme of primary anthocyanins (Košir et al., 2004).

During ripening, red grapes accumulate anthocyanin pigments in skin cells (Robinson and Davies, 2000). In a few teinturier varieties, accumulation in the berry skin is paralleled by accumulation in flesh (Falginella et al., 2012; Teixeira et al., 2013). The quantity and quality of colour in grape berries at harvest are crucial factors that influence wine making (Boss et al.,1996).

2.8.3.3.1 The anthocyanin biosynthesis pathway

Anthocyanin synthesis is part of the flavonoid pathway (Figure 15) that also produce flavonols, catechins, and proantocyanidins through specific enzymes that utilise the same metabolic intermediates (Falginella et al., 2012).

Phenylalanine ammonia lyase (PAL) is the first enzyme involved in anthocyanin production: it catalyses the synthesis of cinnamic acid from phenylalanine.

The first flavonoid produced is a chalcone, and the enzyme involved is chalcone synthase (CHS). Chalcone is produced by the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA. CHS is a member of the plant polyketide synthase superfamily, which also includes stilbene synthase, acridone synthase, pyrone synthase, bibenzyl synthase and *p*-coumaroyltriacetic acid synthase (Dao et al., 2011). In grapes, the three upstream enzymes are encoded by multi-copy genes; three copies of CHS (CHS 1, CHS2 and CHS3) were reported (Sparvoli et al., 1994).

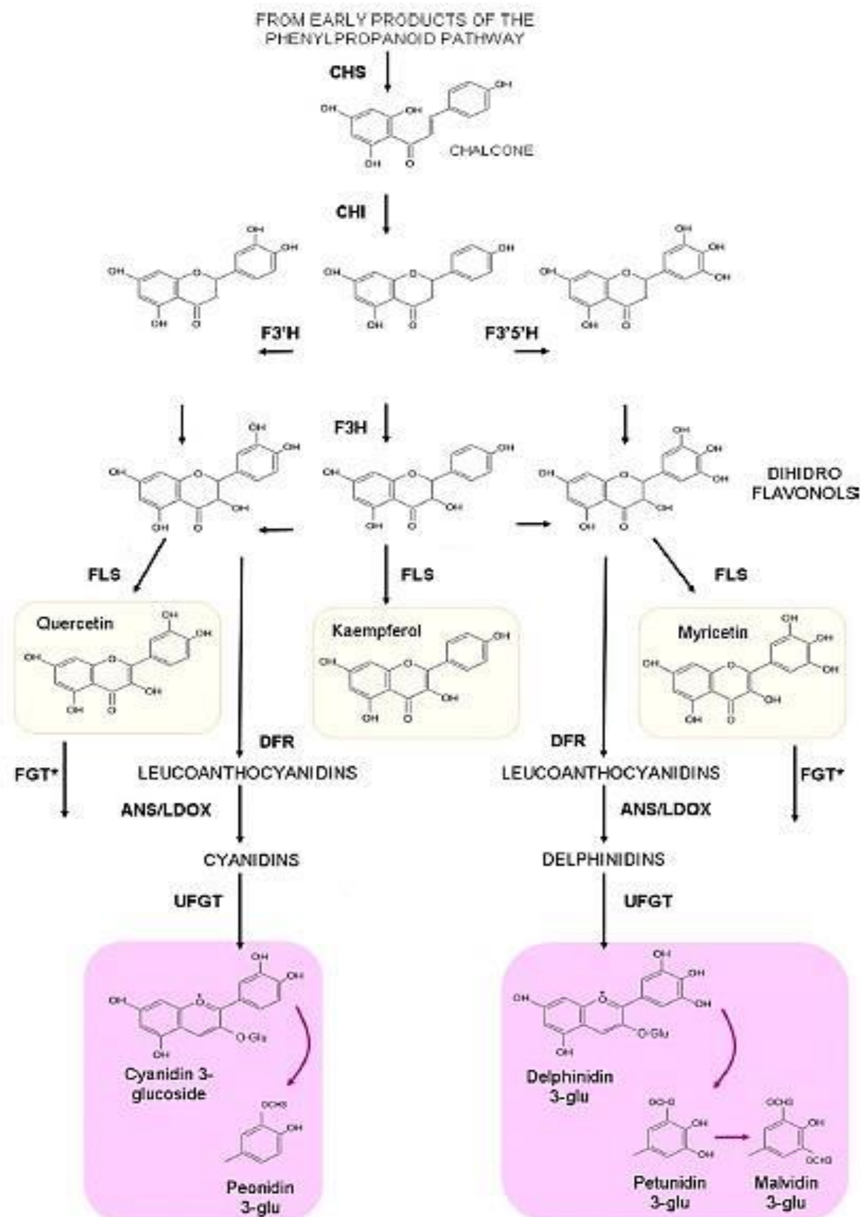


Figure 15: Schematic representation of the flavonoid biosynthetic pathway (Matus et al., 2009).

Calchones are then converted to flavanones by chalcone isomerase (CHI), which catalyses a stereo-specific ring closure (Boss and Davis, 2001). From these central intermediates, the pathway diverges into several branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyses the stereospecific 3β-hydroxylation of (2S)-flavanones to dihydroflavonols.

F3´H and F3´5´H play a key role in determining the pattern of anthocyanin. Whereas F3´H in necessary for the synthesis of 3´-hydroxylated anthocyanins (cyanidin, peonidin), F3´5´H participates in the synthesis of 3´5´-hydroxylated anthocyanins (delphinidin, petunidin and malvidin) (Pascual-Teresa and Sanchez-Ballesta, 2007). F3´H and F3´5´H catalyse hydroxylation at the 3´ and 3´, 5´ positions, respectively of B-ring of flavonoids. Thus, these enzymes are presumed to control the branching points of the parallel pathways producing the compositionally different flavonoids with a B-ring hydroxylation pattern (Koyama and Goto-Yamamoto, 2008).

For the biosynthesis of anthocyanins, dyhidroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS) (Dao et al., 2011). The exact chemical nature of the subsequent conversion of leucoanthocyanidins to anthocyanidins is somewhat uncertain. It is widely accepted that the enzyme responsible for the next step in anthocyanin synthesis is the leucoanthocyanidin dioxygenase (LDOX), also called ANS. The LDOX encodes an enzyme belonging to the class of 2-oxoglutarate-dependent dioxygenases (Boss and Davis, 2001) and it catalyses the conversion of leucoanthocyanidins to anthocyanidis in the anthocyanins pathway (Gollop et al., 2001). The LDOX gene has been shown to be expressed in Shiraz grapevine in all the plant organs: leaves, tendril, green can, root, seeds, flowers, berry skin and berry flesh (Boss et al., 1996; Gollop et al., 2001).

The final and specific step for the anthocyanin biosynthesis pathway is the formation of 3-glucosides by uridine 5´-diphosphate (UDP)-glucose: flavonoid 3-O-glucosyltransferase (UFGT). Steps earlier than UFGT are common to the biosynthetic pathway of epicatechin, another flavonoid of grapevine (Poudel et al., 2008). In grape of *Vitis vinifera* L. cultivars, anthocyanidins can only be O-glycosylated at the C3

position with the addition of glucoses by the activity of UFGT. Normally, the UFGT expression is only detected in berry skin after the onset of véraison specifically, whereas most of the upstream genes may express constitutively in different organs and tissues at diverse levels. The UFGT enzyme shows the highest activity with cyanidin as acceptor, but it can also use delphinidin as well as peonidin, petunidin and malvidin at lower levels at its optimal pH 8.0 (He et al., 2010).

The last biosynthetic step of UFGT-mediated anthocyanin synthesis does not occur in white fruited grapevine cultivars and hence these cultivars do not express colour in their berries (Gutha et al,. 2010). In the case of red berries, anthocyanins are transported from cytosol into vacuoles and ultimately accumulated in berry skin cells (Braidot el al., 2008; Hichri et al., 2011).

After methylation was catalysed by O-methyltransferases (OMT) it modifies cyanidin at the 3´ position leading to peonidin, and modifies delphinidin at the 3´ and sequentially 5´ position leading to petunidin and malvidin (Wang et al., 2013).

Northern analysis of the anthocyanin pathway gene expression in berry skin during development revealed two distinct patterns of expression (Boss et al., 1996). All the anthocyanin genes, except UFGT, are highly expressed in early development, followed by a decrease of the expression of all the genes during the lag phase of berry development. After véraison, there is a coordinate induction of all the genes, including UFGT and this coincides with the accumulation of anthocyanins in the skin. These results suggest that UFGT is under a different regulatory regime than genes from the rest of the anthocyanin pathway. The anthocyanin pathway is tightly controlled by the regulatory genes as has been observed in other plants (Boss and Davis, 2001).

3 EXPERIMENT

3.1 Selection of vineyards and cultivars

Samples were collected from vines of cultivar 'Refošk'in a vineyard in Komen belonging to the winegrowing district Kras (Figure 16), from vines of cultivar 'Schioppettino' in a vineyard in Prepotto in Friuli-Venezia Giulia winegrowing district (Figure 17) and from vines of cultivar 'Volovnik' in Slap in Vipavska dolina winegrowing district (Figure 18).

In this study, the cultivars 'Refošk'and 'Volovnik' were trained in single Guyot training system, while cultivar 'Schioppettino' was trained in single and double Guyot training system.

In the vineyard in Komen, 7 healthy and 7 GFLV infected vines of cultivar 'Refošk'were selected (Table 2). In the vineyard in Prepotto, 11 healthy and 14 GFLV infected vines of cultivar 'Schioppettino' trained on single Guyot were selected (Table 3), while for double Guyot, 9 healthy and 9 GFLV infected vines were choosen (Table 4). In vineyard in Slap, 1 healthy and 6 GFLV infected vines of cultivar 'Volovnik' were selected. The vines were selected according to visual inspection and to previous testing of virus presence

The vines were selected from a large part of vineyard, to get the most representative results of quantity and quality of grapes. The vines were marked as (SCH 8/15), where the abbreviation means cultivar name (SCH – 'Schioppettino', REF – 'Refošk'and VOL – 'Volovnik'), the numbers means the successive number of row and the planting site (8/15 = eighth row / fifteenth vine).

Data of the vineyard in Komen:

Owner:	Vinakras z.o.o.
Location:	Komen
Area:	4 ha
Altitude:	280 m

Cultivar: ‘Refošk’

Training system: single Guyot

Establishing year: 1996

Figure 16: The location of experimental vineyard in Komen (Google maps…, 2014).

Table 2: Selected vines of cultivar ‘Refošk’

Selected vines	
Healthy vines	**GFLV infected vines**
REF 18/12	REF 19/22
REF 18/15	REF 21/17
REF 20/11	REF 22/12
REF 21/23	REF 22/18
REF 38/33	REF 38/31
REF 38/35	REF 21/14
REF 39/04	REF 39/09

Data of the vineyard in Prepotto:

Owner: Vigna Petrussa

Location: Prepotto

Area: 2 ha

Altitude: 150 m

Cultivar: ‘Schioppettino’

Training system: single Guyot and double Guyot

Establishing year: 2000 (single Guyot) and 1994 (double Guyot)

Figure 17: The location of experimental vineyard in Prepotto (Google maps…, 2014).

In case of ‘Schioppettino’, both training systems were selected due to different ratio between canopy and yield. Therefore, there it was supposed a different response to viral infection.

Table 3: Selected vines of cultivar ‘Schioppettino’

Selected vines			
Single Guyot		**Double Guyot**	
Healthy vines	GFLV infected vines	Healthy vines	GFLV infected vines
SCH 7/24	SCH 10/16	SCH 22/17	SCH 22/10
SCH 7/31	SCH 10/24	SCH 22/19	SCH 22/16
SCH 7/35	SCH 10/32	SCH 22/20	SCH 24/13
SCH 7/45	SCH 11/36	SCH 22/21	SCH 25/13
SCH 7/47	SCH 11/44	SCH 24/07	SCH 25/18
SCH 8/23	SCH 11/8	SCH 25/21	SCH 25/40
SCH 8/26	SCH 7/05	SCH 25/27	SCH 25/43
SCH 8/27	SCH 7/07	SCH 25/29	SCH 25/44
SCH 8/41	SCH 7/21	SCH 26/24	SCH 26/09
SCH 9/39	SCH 8/08		
SCH 12/23	SCH 8/15		
	SCH 8/19		
	SCH 8/22		
	SCH 9/21		

Data of the vineyard in Slap:

Owner:	STS Vrhpolje
Location:	Slap
Area:	1.3 ha
Altitude:	150 m
Cultivar:	'Volovnik'
Training system:	single Guyot
Establishing year:	2012/2013

Figure 18: The location of experimental vineyard in Slap (Google maps, 2014).

Table 4: Selected vines of cultivar 'Volovnik'

Selected vines	
Healthy vines	**GFLV infected vines**
VOL 5/19	VOL 5/8_1_4
	VOL 5/8_5_9
	VOL 5/12_1
	VOL 5/12_2
	VOL 5/12_3
	VOL 5/12_4

3.2 Sampling

Samples of young and mature leaves were randomly collected in June 2011 in vineyards in Komen and Prepotto from all 59 selected vines. After sampling, the samples were stored at -80°C until analysis. Collected samples were analysed by ELISA for the presence of Grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV), Grapevine leafroll associated virus (GLRaV)-1, -2, -3, -4-9, Grapevine virus B (GVB), Grapevine virus A (GVA), Grapevine fleck virus (GFkV), Tomato black ring virus (TBRV), Grapevine chrome mosaic virus (GCMV), Toamto ringspot virus (ToRSV), Raspberry ringspot virus (RpRSV), Strawberry latent ringspot virus (SLRSV) and Tobacco ringspot virus (TRSV).

Samples of mature laves were collected in September 2014 in vineyards in Vipavska dolina from all 7 selected vines. After sampling, the samples were stored at -80 °C until analysis. Collected samples were analysed only for the presence of GFLV, because previous testing in our laboratory showed that the majority of the vines of cultivar 'Volovnik' were infected with GFLV.

Every two weeks from véraison to harvest 2012 in the vineyard in Prepotto, the samples of berries were collected separately from three GFLV infected and three healthy vines of cultivar Scioppettino trained in single Guyot, to analyse targeted gene expression. Berries were randomly collected from the top, bottom and centre of the bunch, both from shaded and sun exposed parts of the clusters in falcon tubes and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until analysis.

The study of the impact of GFLV on yield parameters and grape quality was performed in three subsequent years (2011, 2012 and 2013). At harvest time the samples of grape were separately collected from 30 GFLV infected and 27 healthy vines of cultivars 'Refošk'and 'Schioppettino', together with the assessment of yield/plant and number of clusters. After weighing the total yield, berries were randomly collected from the different clusters to weight the mass of 100 berries. From these 100 berries, the content of soluble solids, pH and titratable acids were measured immediately after crushing.

At harvest time in 2011 and 2012, 100 berries were collected randomly from GFLV infected and healthy vines for the determination of anthocyanin content. The samples were immediately stored at -80 °C until analysis.

At harvest time in 2012 and 2013, all grapes from both infected and healthy vines of cultivars 'Refošk'and 'Schioppettino' were collected for small-scale vinification.

3.3 ELISA test

Collected samples were analysed for the presence of viruses by ELISA test with slightly modified protocol, as performed Hren et al. (2009). The leaf samples of individual vines were analysed for the presence of GFLV, ArMV, GLRaV-1, -2, -3, -4-9, GFkV, GVB, GVA, GCMV, ToRSV, SLRSV, GCMV and TRSV with double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) and for GVB with double antibody sandwich indirect (DASI) ELISA test.

3.3.1 Buffers for ELISA test

<u>Extraction buffer (pH = 8.2):</u>

TRIS (Sigma, Germany)	264 mM
TRIS-HCl (Sigma, Germany)	236 mM
NaCl (Merck, Germany)	137 mM
PVP K25 (Fluka, Germany)	2 %
PEG 6000 (Merck, Germany)	2 mM
Tween 20 (Sigma, Germany)	0.05 %

<u>PBS Washing buffer (pH = 7.4):</u>

NaCl (Merck, Germany)	137 nM
KH_2PO_4 (Ridel, Germany)	1.5 nM
Na_2HPO_4 (Merck, Germany)	8 nM
KCl (Merck, Germany)	3 nM
Tween 20	0.05%

Coating buffer (pH = 9.6):

Na_2CO_3 (Merck, Germany)	15 nM
$NaHCO_3$ (Merck, Germany)	35 nM

Conjugate buffer for DAS ELISA (pH = 7.4):

TRIS (Sigma, Germany)	20 nM
NaCl (Merck, Germany)	137 nM
PVP K25 (Fluka, Germany)	2%
Tween 20	0.05%
BSA (Sigma, Germany)	0.2%
$MgCl_2$ x $6H_2O$ (Merck, Germany)	1nM
KCl (Merck, Germany)	3 nM

Substrate buffer (pH = 9.8):

Dietanolamin	9.7%

Conjugate buffer for DASI-ELISA (pH = 7.4):

NaCl (Merck, Germany)	137 nM
KCl (Merck, Germany)	3 nM
PVP K25 (Fluka, Germany)	2%
BSA	0.02%
Tween 20	0.05 %
KH_2PO_4 (Ridel, Germany)	1.5 nM
Na_2HPO_4 (Merck, Germany)	8 nM

3.3.2 Homogenisation of plant material

The leaves from apical shoots were ground in Bioreba bags with extraction buffer at ratio 1:10 (w/v) using a Homex grinder (Bioreba, Nylon, Switzerland).

3.3.3 DAS – ELISA test

200 µl of antibodies (Bioreba AG, Switzerland or Agritest, Italy), diluted in coating buffer in ratio 1 : 1000, were added to 96 plates (Greiner). The plates were covered and placed in incubation for 4 hours at 30 °C in the case where use antibodies produced by Bioreba and for 2 hours at 37 °C in the case where use antibodies produced by Agritest. After incubation, the plates were washed for 4 times with washing buffer.

200 µl of homogenised plants material were added to plates and placed to overnight incubation at 4 °C.

The next day, the plates were washed for 4 times with washing buffer. After washing, 195 µl antibodies (Bioreba AG, Switzerland Agritest, Italy), diluted in conjugate buffer, at ratio 1 : 1000, were added to 96 plates (Greiner). After 5 hours of incubation at 30 °C in the case where use antibodies produced by Bioreba and 2 hours of incubation at 37 °C in the case where use antibodies produced by Agritest, the plates were washed for 4 times with washing buffer.

After that, 200 µl of para-nitrophenyl-phosphate with 1 mg/ml concentration in substrate buffer were added. The plates were incubated at room temperature.

Optical density (OD) was measured after 30 min, 1 hour, 2 hours and 18 hours of incubation with substrate at 405 nm using a plate reader (Tecan Sunrise™, Männedorf, Switzerland). Data were processed using Magellan™ data analysis software.

ELISA reads were considered positive, when they reached values higher than 2-fold of the value of the negative controls.

3.3.4 DASI – ELISA test

200 µl of homogenised plants material were added to 96 plates (Greiner), covered and placed to overnight incubation at 4 °C. After incubation, the plates were washed for 4 times with washing buffer.

200 µl of antibodies (Agritest, Italy), diluted in conjugate buffer in ratio 1 : 1000, were added to plates. The plates were covered and placed to incubation for 2 hours at 37°C. After the incubation, the plates were washed for 4 times with washing buffer.

200 µl of antibodies (Agritest, Italy), diluted in conjugate buffer, at ratio 1 : 1000, were added to plates. After 2 hours incubation at 37 °C, the plates were washed for 4 times with washing buffer.

After that, 200 µl of para-nitrophenyl-phosphate with 1 mg/ml concentration in substrate buffer were added. The plates were incubated at room temperature.

Optical density (OD) was measured after 30 min, 1 hour, 2 hours and 18 hours of incubation with substrate at 405 nm using a plate reader (Tecan Sunrise™, Männedorf, Switzerland). Data were processed using Magellan™ data analysis software.

ELISA reads were considered positive, when they reached values higher than 2-fold of the value of the negative controls. For each sample, an average of optical density value was calculated. The inhibition was excluded by diluting a pool of extracts in the extraction buffer in the ratios 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$.

3.4 METHOD OF QUALITY AND QUANTITY ANALYSES

3.4.1 Yield and berry weight

At harvest, the number of clusters per vine was recorded; a total yield of individual vine and 100 berries were weighted. From the total yield and cluster number, an average weight of cluster was calculated.

3.4.2 Quality parameters

Sampled grape was crushed in the plastic bag by hand and the obtained grape juice was filtered through filter paper. The sugar content of the grape juice was measured with digital refractometer (ATAGO WM-7) in °Brix units. In pre-cleaned glass prism of the refractometer a drop of grape juice was added to measured soluble solids. There were done three replicates for each sample.

The pH of the grape juice was measured with Titrino plus (Metrohm 848 Titrino plus, USA). Before measurement, the pH meter was calibrated with standard solutions: pH = 4.0, pH = 7.0 and pH = 9.0. The electrode was added in 10 mL of grape juice and the pH was measured.

The content of titratable acids of the grape juice was measured with automatic neutralizer Titrino plus (Metrohm 848 Titrino plus, USA). 10 mL of grape juice was diluted with 40 mL of water. The laboratory glass cup was placed on automatic magnetic stirrer (Metrohm 801 Titrino plus, USA). During the stirring, the content of titratable acids (g/l) was measured by titration with 0.1 M NaOH to the end point of pH 8.2.

3.5 METHOD FOR HPLC ANALYSIS

3.5.1 Extraction of phenolic compounds and sample preparation

The phenolic compounds from grape berry skin were extracted according to the method by Mattivi et al. (2006). From each group of samples, three samples of 20 frozen berries were peeled in three repetitions and subjected to extraction for 24 hour at room temperature in 100 mL of methanol (MeOH, Sigma, Germany). After the first extraction, the liquid phase was separated from skins and 50 mL of methanol (Sigma, Germany) was added to the same skins, which were subjected to a further extraction for 2 hours at room temperature. Both liquid phases were combined in dark glass bottles and stored at -20 °C until preparation for HPLC analyses.

The extracts of berry skins were filtered through a 0.45µm PTFE syringe filter (Chromafil Xtra, Macherey-Nagel, Düren, Germany). The filtered extracts were diluted with 1% trifluoroacetic acid (TFA, Sigma, Germany) in water at ratio 1 : 9 and transferred directly into the HPLC vials.

3.5.2 Quantification of grape anthocyanins

The separation and quantification of individual anthocyanins delphinidin-3-glucoside (Del-3-Glu), cyanidin-3-glucoside (Cy-3-Glu), petunidin-3-glucoside (Pet-3-Glu), peonidin-3-glucoside (Peo-3-Glu) and malvidin-3-glucoside (Mal-3-Glu) were performed using gradient high performance liquid chromatography (HPLC) with UV-VIS detection at 520 nm. The analysis was carried out with a Waters chromatographic system (Waters, Milford, MA, USA) comprising two Waters 510 pumps, a Waters 717+ autosampler, and a Waters 2487 UV – visible (VIS) dual wavelength detector. Individual anthocyanins were separated using a Phenomenex Luna C18, 4.6 mm x 150 mm, 5 µm column (Phenomenex, USA) under defined chromatographic conditions (Table 5).

Table 5: Chromatographic conditions for HPLC analyses

Time	Flow	%A	%B
0	0.90	60	40
20	0.90	35	67
28	0.90	25	75
40	0.90	25	75
41	0.90	0	100
44	0.90	0	100
45	0.90	60	40
55	0.90	60	40

Legend: A, B – mobile phase A, B

The separation gradient of mobile phases was used. Mobile phase A contained methanol (Sigma, Germany) with 0.2 % trifluoroacetic acid (TFA, Sigma, Germany), mobile phase B contained water with 0.2 % TFA (Sigma, Germany). The injection volume was 10 μl. All analyses were carried out in biological triplicates and technical duplicates. Commercially available standards of peonidin-3-glucoside, malvidin-3-glucoside, delphinidin-3-glucoside and cyanidin-3-glucoside were separately dissolved in MeOH and used as standard stock solution for generating calibration curves. The stock solutions were diluted with 1 % TFA (Sigma, Germany) in water. These standard solutions were injected to generate the calibration curve for the standards compounds.

In Figure 19, an example of the HPLC chromatogram of skin methanolic extract at 520 nm is shown.

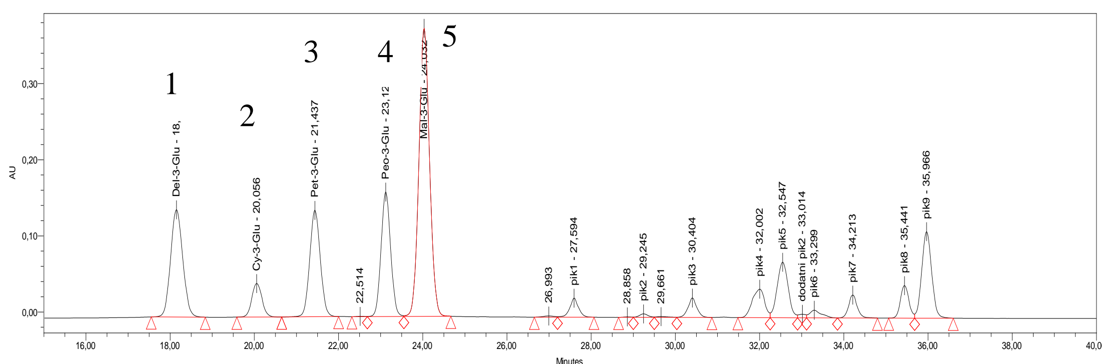


Figure 19: HPLC chromatogram of anthocyanins at 520 nm. The peak number 1 correspond to Del-3-Glu, peak number 2 to Cy-3-Glu, peak number 3 to Pet-3-Glu, peak number 4 to Peo-3-Glu and peak number 5 to Mal-3-Glu.

The contribution of non-methoxylated (OH) (delphinidin and cyanidin), methoxylated (OCH_3) (peonidin, petunidin and malvidin), di-substituted (DI SUB) (cyanidin and peonidin) and tri-substituted (TRI SUB) anthocyanins (delphinidin, petunidin and malvidin) was calculated for GFLV infected and healthy vines.

3.6 GENE EXPRESSION ANALYSIS

3.6.1 Sample preparation and RNA isolation

Berries stored at -80 °C were peeled and split into skin, flesh and seeds. Samples of skin, flesh and seeds were ground to a fine powder in liquid nitrogen and subsequently 300 mg were weighted and placed into falcon tubes.

For the total RNA extraction, RNeasy plant mini kit (Qiagen) was used applying modified protocol described by Hren et al. (2009); 1 ml of RLC extraction buffer (Qiagen) preheated to 56 °C and containing 10 mg/ml PVP MW 40000 (Sigma) at a ratio 1:10 (w/v) was added to 300 mg of ground frozen plant material, vortexed vigorously, incubated for 3 min at 56 °C and centrifuged 30 s at 10,000 *g*.

500 µl lysate (supernatant) were transferred to a QIAshredder spin column (purple) and centrifuged for 2 min at 14,000 *g*. This step was done twice. After centrifugation, both lysates were combined in a new microcentrifuge tube and 0.5 volume of ethanol (Sigma, Germany) was added and mixed immediately by pipetting.

650 µl of the samples were transferred to an RNeasy spin column (pink) and centrifuged 20 s at 10,000 *g*. The flow-through was discarded and the steps were repeated until the whole volume of each sample was used.

The spin column membrane was washed once with 700 µl RW1 buffer and twice with 500 µl RPE buffer to remove all remaining proteins and impurities.

RNA was eluted twice using 30 µl of RNase free water, preheated to 65 °C each time, with 5 min incubation at room temperature (T = 23 °C ± 2) in between and stored at -80 °C until analysis were carried out.

3.6.2 DNase treatment

DNase treatment was done with DNase I, Amplification Grade kit (Invitrogen, USA)

The reaction mixture for 1 sample contained:

DNase I, Amp Grade	0.1 µl
10x DNase I reaction buffer	2 µl
RNase free water	8 µl
RNA	10 µl

Samples were incubated at room temperature (T = 23 °C ± 2) for 15 min. After incubation, the reaction mixture was inactivated with 2 µl of 25nM EDTA solution and heated for 10 min at 65 °C. The 12.5 µl sample was denatured for 5 min at 80 °C and placed on ice.

3.6.3 Reverse transcription

Reverse transcription was done with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

The 2x Reverse Transcription master mix for 1 sample contained:

10x RT Buffer	2.5 µl
25x dNTP Mix	1 µl
RT Random Primers	2.5 µl
RNase free water	4.25 µl
RNase Inhibitor	1 µl
MultiScribe™ Reverse Transcriptase	1.25 µl

The master mix (12.5 µl) was added to DNase treated samples and carried out on a GeneAmp® PCR System 9700HT (Applied Biosystem) as following: 25 °C for 10 min, 37 °C for 120 min and 4 °C for ∞.

3.6.4 Quantitative polymerase chain reaction (qPCR)

For gene expression analysis, the qPCR method was selected. Each reaction (5 µ) contained 2 µl of cDNA and 3 µl of mastermix (SYBR® Green or TaqMan), containing 300 nM of each primers in case of SYBR Green chemistry and 300 nM primers and 150 nM probes in case of TaqMan chemistry. In case of SYBR® Green chemistry, the Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) was used. In case of TaqMan chemistry, the TaqMan Universal PCR Master Mix (Applied Biosystems, USA) was used.

As reference genes for normalisation, cytochrome oxidase (COX) and ubiquitin-conjugating factor (UBI_CF) were selected. The validation of the stability of their expression was made using geNorm (Pfaffl, 2001; Vandesompele et al., 2002), which calculated the gene-stability measure for both reference genes in a given set of samples.

For genes chalchone synthase (CHS2), flavanone 3-hydroxylase 1 (F3H1), flavanone 3-hydroxylase 2 (F3H2), flavonoid 3´ hydroxylase (F3´H), flavonoid 3´ 5´ hydroxylase (F3´5´H), leucoanthocyanidin dioxygenase (LDOX), flavonoid 3-O-glucosyltransferase (UFGT) and UBI_CF, the SYBR® Green chemistry was selected, while for COX, the TaqMan chemistry was used.

The primer pairs and probe characteristics for qPCR are shown in Table 6.

Table 6: Primer pairs used in analysis of gene expression.

Gene name	Nucleotide sequence 5´ - 3´	Sequence	Final concentration (nM)	Reference
CHS2	Forward	TCTGAGCGAGTATGGGAACA	200	Goto-Yamamoto et al., 2002
	Reverse	AGGGTAGCTGCGTAGGTTGG	200	
F3H1	Forward	CCAATCATAGCAGACTGTCC	300	Sparvoli et al., 1994
	Reverse	TCAGAGGATACACGGTTGCC	300	
F3H2	Forward	CTGTGGTGAACTCCGACTGC	300	Sparvoli et al., 1994
	Reverse	CAAATGTTATGGGCTCCTCC	300	
F3´H	Forward	GGCGGAAGGTTTCCTTGAT	300	Castellarin et al., 2006
	Reverse	GCACGTTGATCTCGGTGAG	300	
F3´5´H	Forward	TGTACCAACGACCCCAAAAT	300	Castellarin et al., 2006
	Reverse	GAACCTTCCTCGTGTCTCAG	300	
LDOX	Forward	AGGCTCTACTCTCCAAATGA	300	Goto-Yamamoto et al., 2002
	Reverse	GAAGCTTGAAACACAGACCA	300	
UFGT	Forward	AATCTGAGAGCCCTAAGAGA	300	Goto-Yamamoto et al., 2002
	Reverse	GGTGGTACAAGCAACAGTTC	300	
UBI_CF	Forward	CTATATGCTCGCTGCTGACG	300	Castellarin et al., 2007a
	Reverse	AAGCCAGGCAGAGACAACTC	300	
COX	Forward	CGTCGCATTCCAGATTATCCA	300	Weller et al., 2000
	Reverse	CAACTACGGATATATAAG AGCCAAAACTG	300	
	Probe	TGCTTACGCTGGATGGAATGCC CT	150	

3 µl of mastermix was pipetted on 384 well plate (384 Well Clear Optical Reaction Plates, Applied Biosystems, USA) and then 2 µl of cDNA was added. For each sample, 10-fold and 100-fold diluted cDNA was used in technical duplicates. For each amplicon, the non templated control (NTC) was done, using water instead of cDNA.

After pipetting, the well was covered with adhesive cover (Thermo Scientific) and centrifuged for 1 min at 1000 *g*. The qPCR was carried out in LightCycler® 480 instrument (Roche, Applied Systems, USA). The qPCR cycles were performed as following: 50 °C for 2 min, 95 °C for 10 min (polymerase activation) and then 40 cycles at 95 °C for 10 s and 60 °C for 1 min. In case of SYBR Green chemistry, the dissociation curve (95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s) was preformed to verify the specificity of products and primer dimers.

The initial data analysis was performed on Roche LightCycler Software and then the Cq values were exported to Excel file for further analysis.

3.6.5 Relative quantification

The relative quantification with calibration curve was used. For each amplicon, the calibration curve was done. The relative expression ratio was calculated based on efficiencies of amplification (Equation 1) of each amplicon in each sample,

$$E = 10^{(1/slope)} \qquad \ldots(1)$$

where slope represents ΔC_q between 10- and 100-fold dilutions; and the differences of normalised C_q values between each individual sample and control sample. C_q values were normalised to the geometric mean of both reference genes (COX and UBI_CF) expression.

3.7 Microvinification

In the 2012 and 2013 vintages, the grapes from selected vines of both cultivars, 'Refošk' and 'Scihoppettino' were collected separately from infected and healthy vines for small-scale winemaking. After grape crushing and destemming, must were placed in plastic containers. Potassium metabisulfit (10 g/hl), fermentation activators, and active dry yeast (*Saccharomyces cerevisiae*) were added for simultaneous start of the process. After fourteen days of maceration, the wine was drawn off, and allowed to sediment for four days. At the end of sedimentation, wine was added into the bottles, where 1.4 ml/L of SO_2 was added.

Chemical evaluation was done on the wines immediately after the end of fermentation. The main analytical parameters of wine (alcohol, dry extract, titratable acids, pH, tartaric acid, malic acid) were assessed by the Agricultural and Foresty Institute of Nova Gorica (Slovenia) using a WineScan FT 120 spectrometer (Foss, Hillerød, Denmark) fitted with a Michelson interferometer generating Fourier transform infrared (FT-IR) spectra.

3.8 Statistical analysis

The statistical analysis was done in Microsoft Excel 2007. The data were divided in six groups, depending on cultivar, training system and health status. For each, quality and quantity parameters of grapevine, the average values and confidence interval were calculated. The confidence interval was calculated as 1.96 times standard error. The statistically significant differences between healthy and GFLV infected plants were calculated by *t*-test (*, $p<0,05$; **, $p<0,01$; ***, $p<0,001$).

4 RESULT AND DISCUSSION

4.1 Presence of grapevine viruses

4.1.1 DAS and DASI ELISA test for virus detection

European and Mediterranean Plant Protection Organization (EPPO) recommend to test the presence of viruses and virus-like disease in grapevine occurring in the EPPO region. In Slovenia, the Rules for marketing and vegetative propagation of vines (2005) recommends, to test the vines for the presence of Grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV), Raspberry ringspot virus (RpRSV), Tomato black ring virus (TBRV), Grapevine leafroll associated virus (GLRaV) –1, -2, -3, 4-9, Grapevine virus A (GVA), Grapevine virus B (GVB) and Grapevine fleck virus (GFkV). For Slovenia EPPO recommended, that in addition to the viruses including in the certification scheme, to test the presence of (TBRV) and Grapevine crown mosaic virus (GCMV). For other countries, such as Italy it is recommended to test the presence of Strawberry latent ringspot virus (SLRSV) and TBRV. The occurrence of GFLV, ArMV, GLRaV-1, -2, -3, -4-9, GVB, GVA, GFkV, GCMV, Tomato ringspot virus (ToRSV), RpRSV, SLRSV and Tobacco ringspot virus TRSV was tested by DAS-ELISA test.

In total 45 samples of ‘Schioppettino’ collected in the vineyard in Prepotto and 15 samples of ‘Refošk’ collected in vineyard in Komen, were tested for 14 viruses.

In all tested samples of cultivars ‘Schioppettino’ and ‘Refošk’, we could not detected the presence of any other viruses, except the infection with GFLV.

In last decade many ELISA test were made on a cultivar ‘Refošk’ in winegrowing region Kras, where GFLV, GLRaV-1, GFkV and GVA viruses were found.

Among some native Slovenian varieties, which have an important role to maintain cultural heritage, the cultivar ‘Volovnik’ was almost lost. Only a few vines were preserved, whose descendants are current vines. In nature, it was difficult to find healthy plant material of native cultivars. With regard to the autochthonous cultivar

‘Volovnik’, seven vines collected in vineyard in Slap were tested for the presence of GFLV. Only one GFLV free vines of cultivar Vol 5/9 was found in vineyard in Slap (Figure 20). Nevertheless, it is important to preserve the healthy vines to maintain the cultivar for the future and also for scientific purposes.

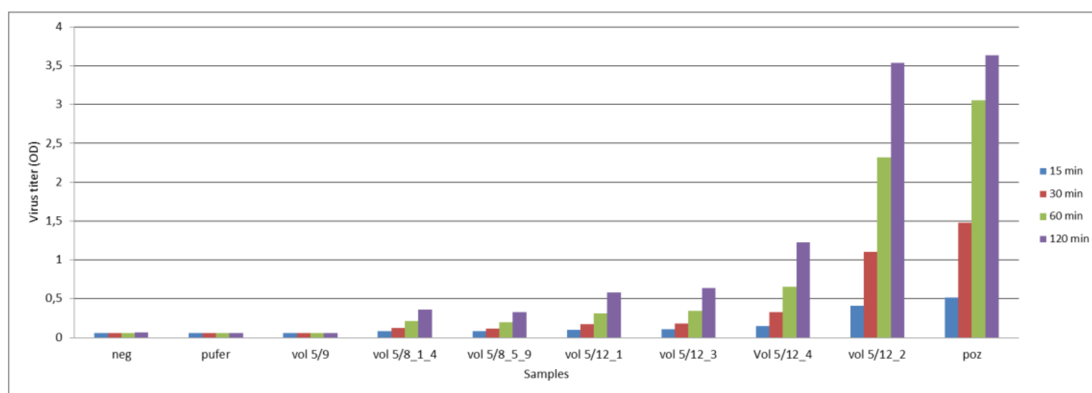


Figure 20: GFLV titre in leaves of cultivar ‘Volovnik’ measured by ELISA.

4.2 Impact of GFLV on quantity parameters of grapevine

4.2.1 Clusters number

Irrespective to virus infection, the average cluster number per vine of cultivar 'Refošk' was raging from 9 to 17, while of 'Schioppettino' trained on single Guyot from 7 to 10 and for double Guyot from 5 to 11. Differences in the number of clusters per vine between seasons were more evident in case of 'Refošk', but anyway, for both 'Refošk' and 'Schioppettino' the lower values were observed in the last season 2013.

In the years 2011 and 2012, the impact of GFLV infection on the number of clusters per vine was not clear, and no significant differences were found in neither the cultivar or training system. Cluster thinning, as normally applied by the winegrowers, most probably partly contributed to eliminate the difference between GFLV and healthy plants.

In 2013 the grape production in the wine region Primorska was reduced due to quite severe drought and cluster thinning was not performed in both vineyards. In the same year an average cluster number per vine for the cultivar 'Refošk' and 'Schioppettino', trained on double Guyot, was lower in GFLV infected vines, as compared with the healthy ones, while for the 'Schioppettino' trained on single Guyot, statistically significant lower cluster number was observed in GFLV infected vines as compared with healthy ones (Figure 21). In the cultivar 'Refošk', the reduction in cluster number was approx. 30 %, but for 'Schioppettino' was approx. 54 % and 32 %, respectively for single and double Guyot.

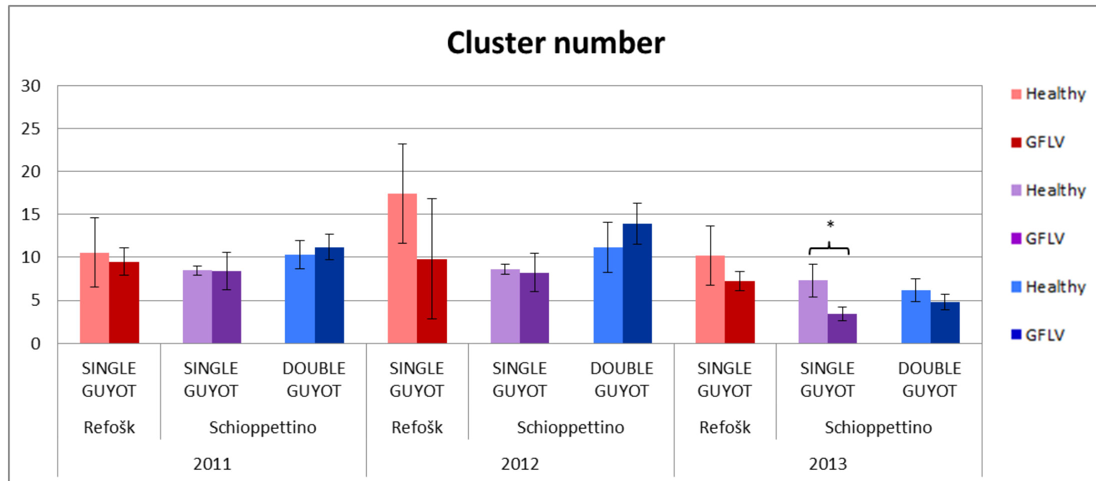


Figure 21: The cluster number in GFLV infected and healthy vines of the varieties ‘Refošk’ and ‘Schioppettino’, trained with single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

What it can be speculated is that the occurrence of GFLV virus infection can impact on the bud fertility, resulting in a reduced number of clusters. Our results also match what found by Cretazzo et al. (2009), who observed lower cluster numbers in GFLV infected vines, as compared with healthy ones in cultivar ‘Callet’, and statistically significant less clusters (-26%) were observed in cultivar Moll. Not only GFLV was found to affect the number of clusters, Endeshawet al. (2014) observed that GLRaV-3 infection significantly altered not only the cluster number per vine but also the berry weight.

The reduction of bud fertility is also related with availability of carbohydrates in the permanent wood (cordon and roots), and Ruhl and Clingeleffer (1993) showed a reduction of carbohydrates in both young and old tissues when the vines were infected with GLRaV.

By comparing cultivars and training systems, the most sensitive cultivar was ‘Schioppettino’ especially when trained on Single Guyot.

Why single and double Guyot responded in a different way? Looking at the age of the plants, single Guyot was adopted in younger plants, while double in older, more robust plants. The older plants could probably, partially, better compensate the effect

of virus infection and maintain unchanged bud fertility since more carbohydrate could accumulate in the permanent organs of the vine.

4.2.2 Cluster weight

The average cluster weight (g) was lower in GFLV infected vines in both cultivars and in both training systems (Figure 22). Statistically significant lower cluster weight was observed in GFLV infected vines of cultivar ‘Schioppettino’ trained in single and double Guyot in 2011. The same trend was also observed in 2012 and in 2013, but the differences were not statistically significant. In cultivar ‘Refošk’, in the first two years of observation, 2011 and 2012, the effect of GFLV infection on cluster weight was noticed, but the differences were not statistically significant, while in 2013, GFLV infection significantly reduced cluster weight.

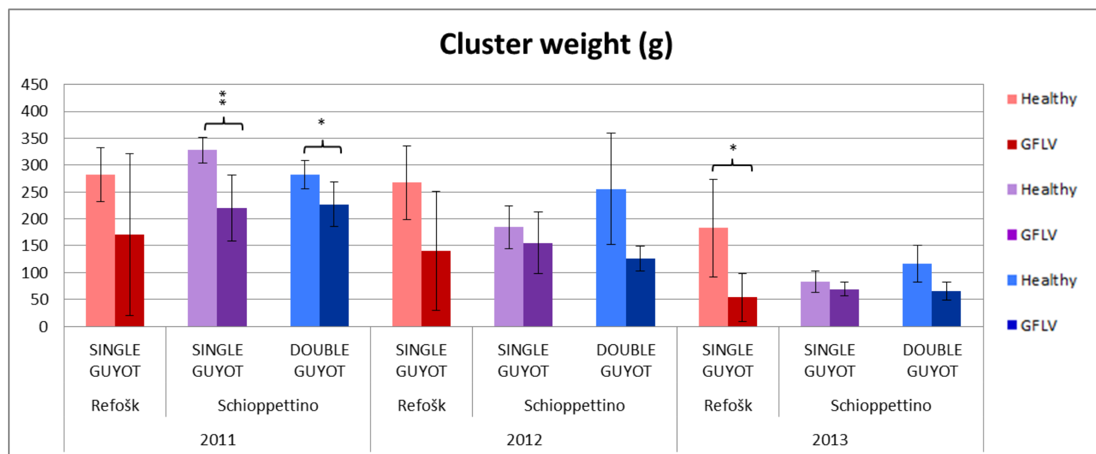


Figure 22: The cluster weight (g) in GFLV infected and healthy vines varieties ‘Refošk’ and ‘Schioppettino’, trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

In case of the cultivar ‘Refošk’, the average cluster weight for healthy vines ranged between 180 to 280 g, while for GFLV infected vines was between 55 and 170 g. The weight found in healthy plants was slightly lower than the value of 300 g reported by Koruza et al. (2012), who reported that the average cluster weight for ‘Refošk’ clone SI-35.

Cluster weight was most variable component of yield from year to year. It seems affected by season and environmental conditions. Other factors that may affect cluster weight include variety, cultural practice (irrigation and fertilizers), diseases and insects (Dami & Sabbatini, 2011). In our research, the reduction in cluster weight was observed due to GFLV infection in infected vines. Although the cluster thinning was performed, lower cluster weight was observed due to smaller berries, which is a common symptom in GFLV infected vines (Andret-Link, et al., 2004). Komar et al. (2008) observed in 7 years of observations a cumulative 20 % reduction in cluster weight in vines infected by GFLV-GHu. The cluster weight is also negatively affected by other viruses. The infection with GLRaV-1 significantly decreased the number of cluster per vine in cultivar ‘Refošk’ (Tomažič et al., 2005). Moutinho-Pereira et al. (2012) evaluated in three years the yield of GLRaV-1 and -3 infected vines. The results showed the decrease in yield per vine in plants infected with GLRaV-1 and -3 compared with healthy ones. This was mainly linked with a significant decrease in the average cluster weight.

4.2.3 Berry weight

The berry weight was in general affected by GFLV infection, thus a reduction was shown in all years and both cultivars. As regard the cultivar ‘Schioppettino’ trained on both single and double Guyot, the berry weight was lower in GFLV infected vines, as compared to the healthy ones. Significant differences were found in case of single and double Guyot in the season 2011 and 2013, respectively. For the cultivar ‘Refošk’, in the first year of observation the effect of GFLV infection on berry weight was not clear, since there was a slight increase and in 2012 a reduction, but both statistically not significant. Only in the season 2013, the reduction of berry weight due to GFLV infection was significantly (Figure 23).

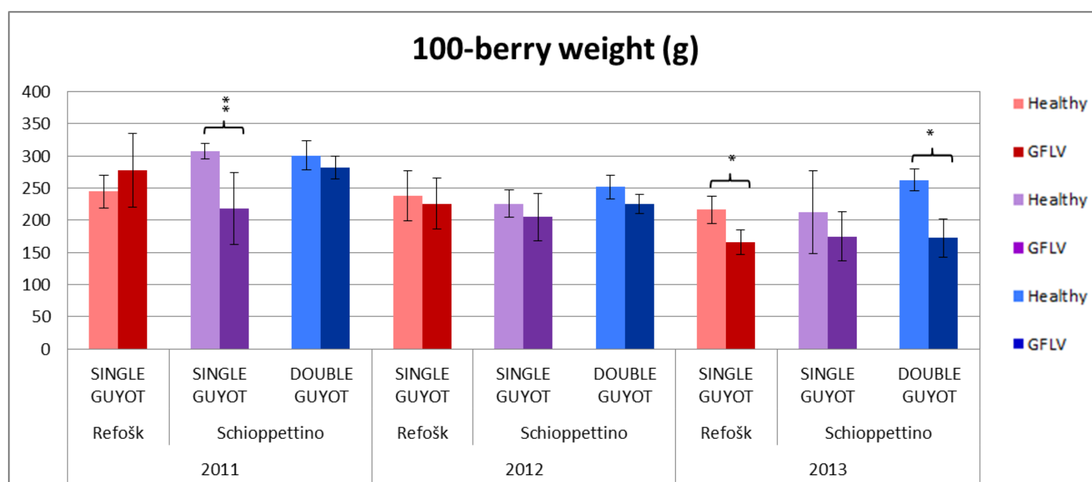


Figure 23: The 100-berry weight (g) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

Andret-Link et al. (2004) reported that the infected berries were uneven in size with numerous small and seedless individuals, some of which did not mature. Cretazzo et al. (2009) reported statistically significant lower berry weight in GFLV infected vines of cultivars 'Manto Negro', 'Callet' and Moll as compared to healthy ones.

4.2.4 Yield

The yield amount is affected by the number of clusters, the average cluster weight, and also by the berry weight. During the three years study, in general, the yield was lower in GFLV infected vines (*, p>0.05) compared to healthy ones (Figure 24). In year 2011, the reduction of a total yield at the cultivars 'Refošk' and 'Schioppettino' trained in single Guyot was approx. 30 %, while compared to 'Schioppettino' trained in double Guyot, the reduction was around 13 %. In the following season, in 2012, the reduction in yield ranged between 27 % and 32 % for both cultivars and without any differences between training systems.

The highest decrease of yield was observed in 2013 for both cultivars and both training systems. At cultivar 'Refošk', significantly lower yield was observed in GFLV infected vines when compared with healthy ones. The yield was lower for approx. one kg in GFLV infected vines as compared with healthy one. The reduction was higher than 60 %. At cultivar 'Schioppettino' trained in single Guyot, similarly

to what shown in 2011 and 2012 was observed also in 2013. In all three years, significantly lower yield was observed in GFLV infected vines as compared to healthy one. For cultivar 'Schioppettino' trained on double Guyot, the reduction in yield in 2013 was around 50%. The average values of yield were lower in GFLV infected vines in all three years, but the differences were not statistically significant.

These results indicate that GFLV caused a great yield decrease in both cultivars ('Refošk' and 'Schioppettino') and training systems (single Guyot and double Guyot) during three years of observation. Moreover, the GFLV showed a greater impact in case of 'Schioppettino' trained in single Guyot, where the significantly lower yield was observed.

As reported above, most probably the older age of the vines could support more easily the accumulation of carbohydrates in the permanent structures thus compensating the effect of GFLV infection. In addition, double Guyot develops wider leaf area, meaning that there are more leaves that can supply photosynthates to the plant also for reserves in the permanent structures of the vine.

The lower yield of 'Refošk' could also be due to flower-shedding, since the variety is highly susceptible to that (Plahuta and Korošec-Koruza, 1996).

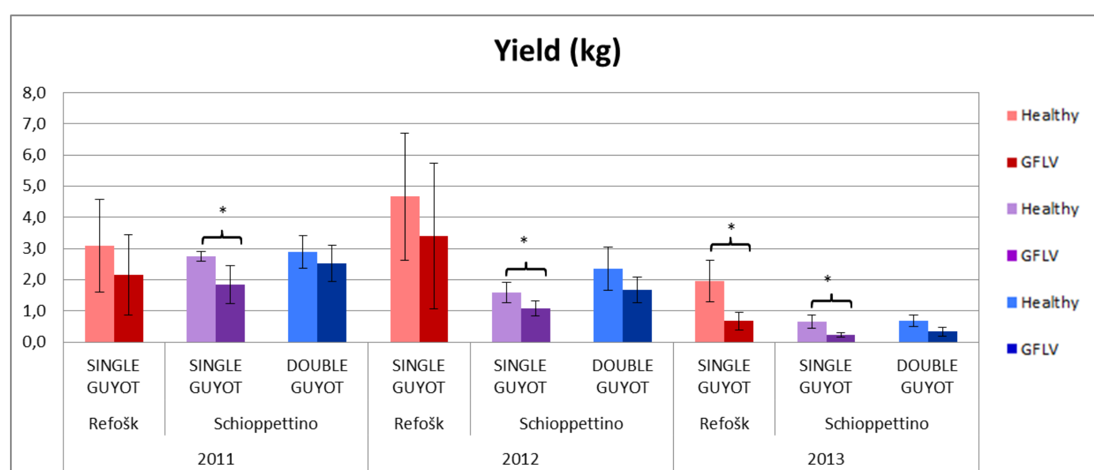


Figure 24: The yield (kg) in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

Another explanation could be that the viral infection has a different impact on young or old vines. As already discussed above, the vines trained in single Guyot training

system were younger than the vines trained in double Guyot. This fact would indicate that the young vines were more sensitive to GFLV infection resulting in a reduction of yield. During the three seasons, it was possible to ascertain that there was a different effect of the GFLV on the two canes of double Guyot and usually one was more vigorous than the other. This probably means that the distribution of the virus is not homogeneous within the plant, thus allowing different responses in shoot growing. The research was done in the vineyards where normally the winegrowers perform cluster thinning in order to reduce the production and obtain better grape quality at harvest. In both season 2011 and 2012 this operation was performed in the vineyard of 'Schioppettino' located in Prepotto. As normally happens, the reduction of the yields was much larger for the high-yielding plants and much poorer for low-yielding ones, and if the production was really low no clusters were removed. What we can only speculate – since both healthy and GFLV-infected plants were subjected to cluster thinning – is, that the difference in yield between healthy and infected plants was lowered mainly because less production was present in GFLV vines. To prove that, we can compare the data obtained in the season 2013; in that year the yield was really low due to the occurrence of drought, thus the winegrowers decided not to perform cluster thinning. Here it is evident that there is a strong reduction of yield in GFLV plants, at least for single Guyot and in both 'Refošk' and 'Schioppettino' cultivars.

The observations confirms that GFLV infection is responsible for a reduction of yield as previously reported by other authors that found crop losses between 20 to 90 % (Raski et al., 1983; Walter and Martelli, 1996; Mannini, 2003; Andret-Link et al., 2004), depending on the cultivar and the environment of cultivation. Also Cretazzo et al. (2009) showed similar trends in their results; for the cultivar 'Callet' they observed 21 % lower yield in GFLV infected plants as compared with healthy ones. Moreover, for the cultivar Moll they observed 42 % lower yield in GFLV infected plants than in healthy plants, while in the cultivar 'Manto Negro' no reduction in the total yield was reported. In another experiment carried out on the cultivar 'Nebbiolo', Santini et al. (2011) observed higher vigour (19 %) and yield (27 %) in healthy vines as compared with vines infected with mixed infection with GFLV and GFkV.

For other viruses, such as GLRaV-3 and GLRaV-1, yield was also affected. Endeshawet al. (2014), working with vines infected with GLRaV-3, showed a significant reduction in yield up to 40 % as compared with the healthy vines. Similar results were also showed by Moutinho-Pereira et al. (2012) on vines infected with GLRaV-1 and GLRaV-3.

4.3 Impact of GFLV on quality parameters of grapevine

4.3.1 Soluble solids

The occurrence of GFLV infection did not significantly impact differences in the content of soluble solids in grape berries (Figure 25). Looking at the cultivar ‘Refošk’, in all three years, the average content of soluble solids was 10 % higher (more than 1.3 Brix) in GFLV infected vines as compared to healthy vines. On the other hand, in the cultivar ‘Schioppettino’ trained in double Guyot the average values of the content of soluble solids were even slightly lower in GFLV infected vines in all three years, as compared to healthy ones.

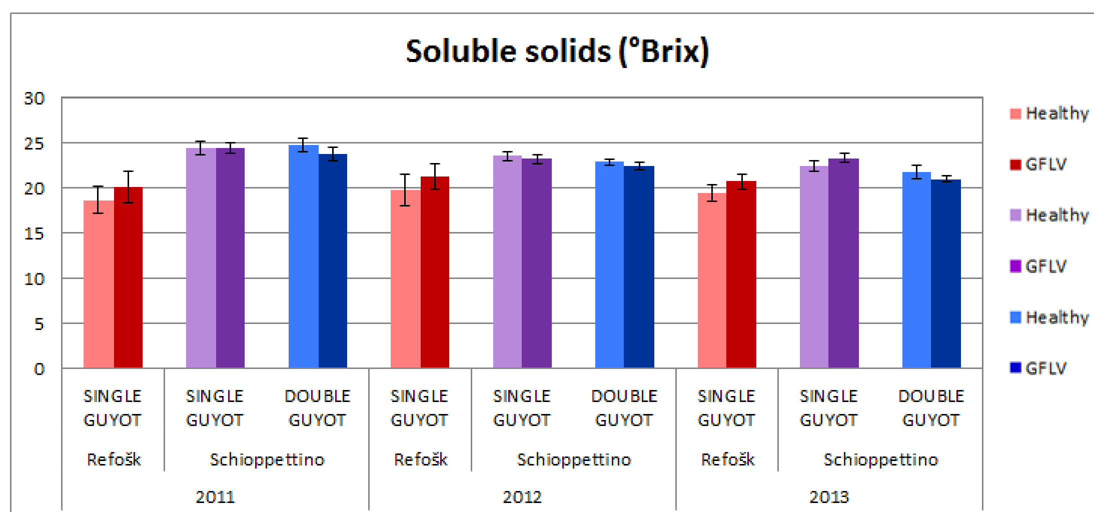


Figure 25: The soluble solids (Brix°) in GFLV infected and healthy vines varieties ‘Refošk’ and ‘Schioppettino’, trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$).

The sugar concentration is an indicator of berry maturity (Conde et al., 2007) and indicates potential alcohol yield after fermentation (Jackson & Lombard, 1993). Many factor such as canopy management, row orientation, pruning, etc. may affect the berry size and modify proportions of skin, flesh and seed in grapevine berry (Hunter et al., 1995; Haselgrove et al., 2000; Bindon et al., 2008). Variability in berry size can be mitigated by cultural practices and environmental factors, that occur during and after flowering (Gray and Coombe, 2009). Sugar content per berry (sugar loading) seemed proportional to size (Roby and Matthews, 2004; Barbagallo et al., 2011), whereas the content was shown to be negatively related. Pisciotta et al. (2013) reported that the sugar concentration value decreased approximately 12 % from smaller to larger berries. Our results are in agreement with previous reports in case of cultivar 'Refošk' and 'Schioppettino' trained in single Guyot. The GFLV infection impaired a decrease in berry mass, and consequently the soluble solid content was slightly higher as compared to healthy plants. In case of the cultivar 'Schioppettino' trained in double Guyot, the berry weight was lower, but also the soluble solids were slightly lower in GFLV infected vines as compared with healthy ones.

In general, when comparing cultivars 'Refošk' and 'Schioppettino' the soluble solids (Brix) were higher in cultivar 'Schioppettino' than in cultivar 'Refošk' for app. 1 – 3 Brix. In the cultivar 'Refošk' GFLV infection increased the soluble solids content by 1.3 – 1.5 Brix, which is an important improvement in must quality parameters for winemakers. Irrespective to the virus infection, the average soluble solids for cultivar 'Refošk' ranged from 19.3 to 20.3 Brix, what is in accordance with the reports of Koruza et al. (2012). The average soluble solids for cultivar 'Schioppettino' ranged from 21.5 to 24 Brix, irrespective to the virus infection and training systems. This is in accordance with the average soluble solids content (22.3 Brix) determined for winegrowing sub-district Friuli Colli Orientali (Bigot et al., 2014). The similar values were also reported for the clones VCR 412 (Vivai Cooperativi Rauscedo, 2011) and FVG 430 (Pecile et al., 2015) of cultivar 'Schioppettino'.

The presented results are in agreement with Cretazzo et al. (2009), who did not observe a significantly differences in soluble solids in cultivar 'Manto Negro' and

‘Callet’, between healthy and GFLV infected vines. On the other hand, the same authors observed significant differences in cultivar Moll, where the soluble solids were for 2 Brix higher in GFLV infected vines, than in healthy one.

In contrast to GFLV, other viruses negatively affected the soluble solids content in berries. Kovacs et al. (2001) observed a 6 % decrease in fruit soluble solids in GLRaV-3 and GFkV infected plants of cultivar Vidal blanc. The plants infected with GLRaV-3 show 2 % decrease in soluble solids in the cultivar Vidal blanc and a 4 % decrease in soluble solids in cultivar St. Vincent. The same trend was observed by Endeshaw et al. (2014) in GLRaV-3 infected vines at harvest time. Differently, Giribaldi et al. (2011) showed that the mixed infection with GLRaV-1, GLRaV-3 and RSPaV did not affect berry composition in terms of soluble solids. Moreover, Tomažič et al. (2005) reported an increase of soluble solid content in plants of ‘Refošk’ infected with GLRaV-1.

4.3.2 Titratable acids and pH

In general, the titratable acids (g/L) were slightly lower and pH was slightly higher in GFLV infected vines compared to healthy ones, but the influence of GFLV infection on titratable acids was different from year to year, especially in cultivar ‘Schioppettino’ trained in double Guyot (Figure 26).

The titratable acids (g/L) in cultivar ‘Refošk’ ranged from 8 in 2011 to 15 g/L in 2013 and in cultivar ‘Schioppettino’ 2.8 to 5.4 g/L, irrespective to virus infection or training systems. The variation in titratable acids content was in accordance with interyear variations, as for example the average titrable acids content at harvest in winegrowing region Kras in 2013 was 17.7 g/L (KGZ Nova Gorica), what is slightly higher that what found in our grape samples in the same year. Such high titratable acids content was probably to relate to too early harvest. Koruza et al. (2012) reported that for cultivar ‘Refošk’ the normal average titratable acid pointed at 9.4 g/L (no data about the years of measurements is reported).
In case of the cultivar ‘Schioppettino’, observed titratable acids content in was even lower as compared to titratable acids (g/L) of clones VCR 412 (Vivai Cooperativi

Rauscedo, 2011) and FVG 430 (Pecile et al., 2015). The content of titratable acids (g/L) observed for winegrowing sub-district Friuli Colli Orientali (Bigot et al., 2014), was in accordance with our results obtained in 2012 and 2013. In 2011, the really late harvest that led to overripe grapes, probably accounted for such lower content of titratable acids.

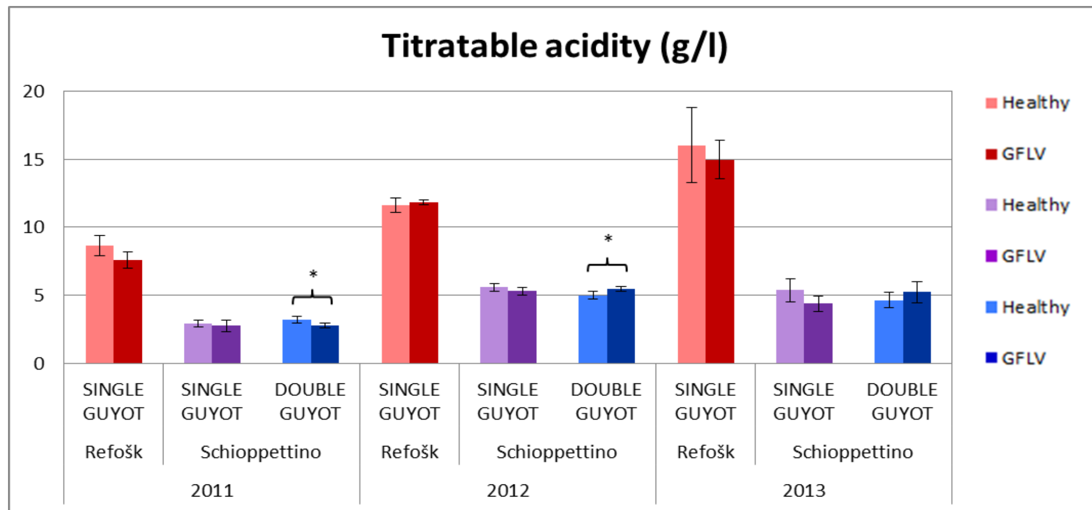


Figure 26: Average content of titratable acids (g/L) in GFLV infected and healthy vines varieties ‘Refošk’ and ‘Schioppettino’, trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$).

The influence of GFLV infection on pH was more constant (Figure 27). The influence of the GFLV on pH in 2011 was statistically significant in both cultivars and in both training systems, and in 2013 the influence was noticed in the cultivar ‘Schioppettino’ trained in single Guyot.

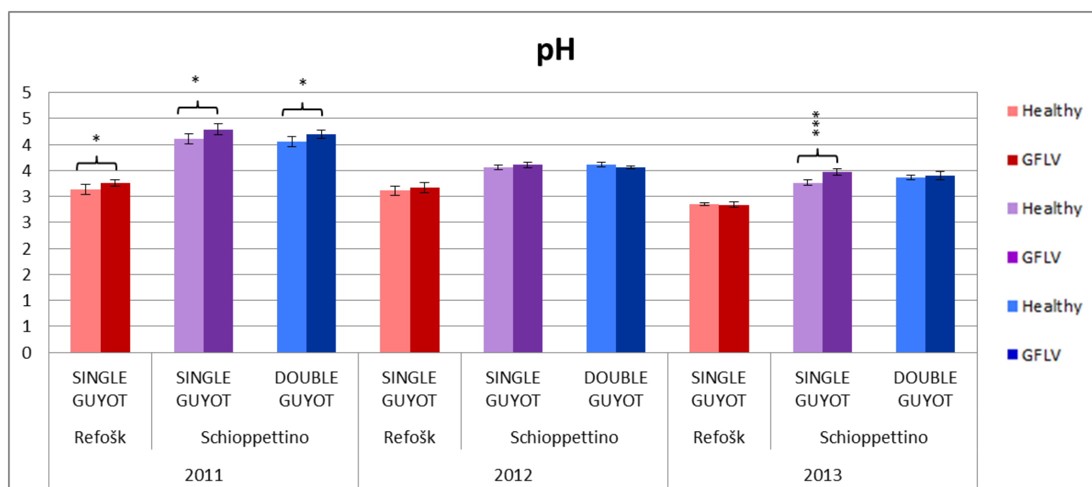


Figure 27: The average pH in GFLV infected and healthy vines varieties 'Refošk' and 'Schioppettino', trained in single Guyot and double Guyot training systems. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

When comparing the cultivars, regardless the health status, lower pH was observed for the cultivar 'Refošk', as compared with the cultivar 'Schioppettino'. The pH in the cultivar 'Refošk' ranged between 2.80 and 3.20 in accordance with the results obtained in winegrowing region Kras in 2013 (KGZ Nova Gorica), where the average pH was 2.90. For the cultivar 'Schioppettino', pH ranged between 3.2o and 4.20, irrespective to training systems and virus infection. The results obtained in 2012 and 2013 were in accordance with results observed for the winegrowing sub-district Friuli Colli Orientali (Bigot et al., 2014) and also with the results obtained for clones VCR 412 (Vivai Cooperativi Rauscedo, 2011) and FVG 430 (Pecile et al., 2015). In 2011 the pH reached over 4, what is probably due to overripe grapes or due to hot weather. As explain above, in the season 2011 the grapes were harvested really late with hot temperatures, and the values of pH overcame the 4.00. It is well known, that pH increases during the maturation time and harvest when the temperatures are high (Spayd et al., 2002; Sadras et al., 2013).

Sampol et al. (2003) showed reduction in photosynthesis due to occurrence of viruses. The development of grape acids is dependent on photosynthesis, lack of which seldom limits titratable acids. The reduction of titratable acids during maturation is related to the respiration rate of the berry and is a function of temperature (Jackson and Lombard, 1993). In the present experiment, meanly a reduction of acidity at harvest was shown in GFLV infected plants. Thus, we can

speculate that GFLV infection reduces photosynthesis, but the reduction of the yield compensate any delay grape maturation mainly in case of single Guyot. To support this idea, in the season 2012 and 2013 a higher titratable acids was observed in case of double Guyot, where probably the higher yield vanished any compensation effect on titratable acids degradation.

Working on thecultivar ‘Manto Negro’, Cretazzo et al. (2009) observed a slightly higher titratable acids in GFLV infected vines, while for the cultivar Moll, the titratable acids was slightly lower in GFLV infected vines.

Higher titratable acids were observed in the fruit of GLRaV-3 positive cultivr St. Vincent and cultivar Vidal blanc, in plants, infected with mixed infection of GLRaV-3 and GFkV and also 14 % higher titratable acids were observed in cultivar Vidal blanc (Kovacs et al., 2001). Similar findings were reported by Wolpert and Vilas, (1992), who studied the effect of latent leafroll strains in cultivar Zinfandel and cultivar White Riesling. Leafroll infected vines reported higher titratable acids as compared with healthy ones. Opposite results were found by Mannini et al. (2011) in GLRaV-3 infected vines, with lower titratable acids as compared with healthy ones. In a viticultural experiment, Pereira-Crespo et al. (2012) found that when leaves were removed around the bunches in GLRaV-3 infected vines, the quality of virus infected plants was similar to those of virus-free plants.

4.4 Anthocyanin profile in grapevine berries

In general, the anthocyanin content in grape berries was increased by GFLV infection in both cultivars. In case of the cultivar ‘Schioppettino’ trained on single Guyot all individual anthocyanins, except Mal 3-Glu, and total anthocyanins were significantly more abundant in GFLV infected berries in 2011, compared to the healthy berries. The same trend was observed in 2012, when the concentrations of all individual anthocyanins were higher in GFLV infected berries, but only the differences in the content of petunidin-3-glucoside and total anthocyanins were significantly higher in GFLV infected berries, as compared the healthy berries (Figure 28).

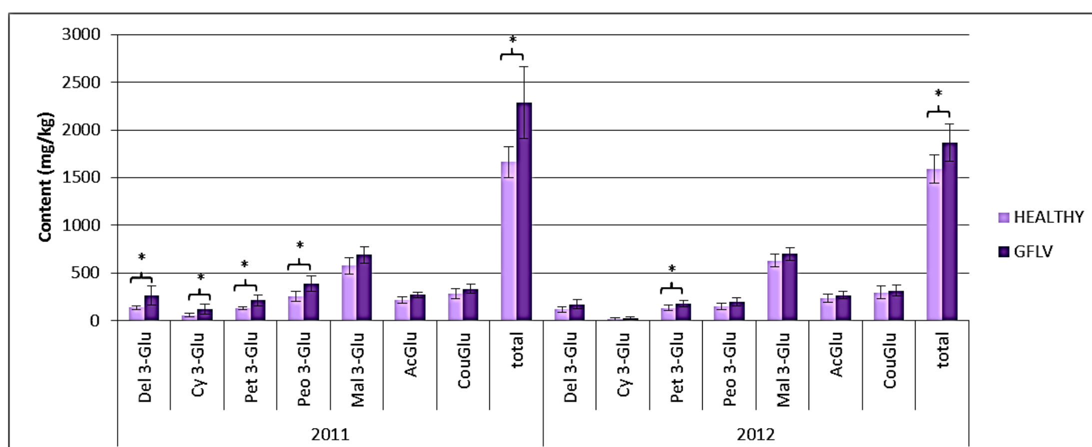


Figure 28: The anthocyanins content (mg/kg berry) in berry skin of cultivar 'Schioppettino', trained in single Guyot training system. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

The OH-, OCH_3-, di- and tri- substituted anthocyanins reported statistically significant higher values in GFLV infected grape berries in 2011 as compared to the healthy berries. The same trend was observed also in 2012; the profile of OH-, OCH_3-, di- and tri-substituted anthocyanins was higher in GFLV infected berries, but only the differences in profile of OCH_3- and tri-substituted anthocyanins were statistically significant higher in GFLV infected berries (Figure 29).

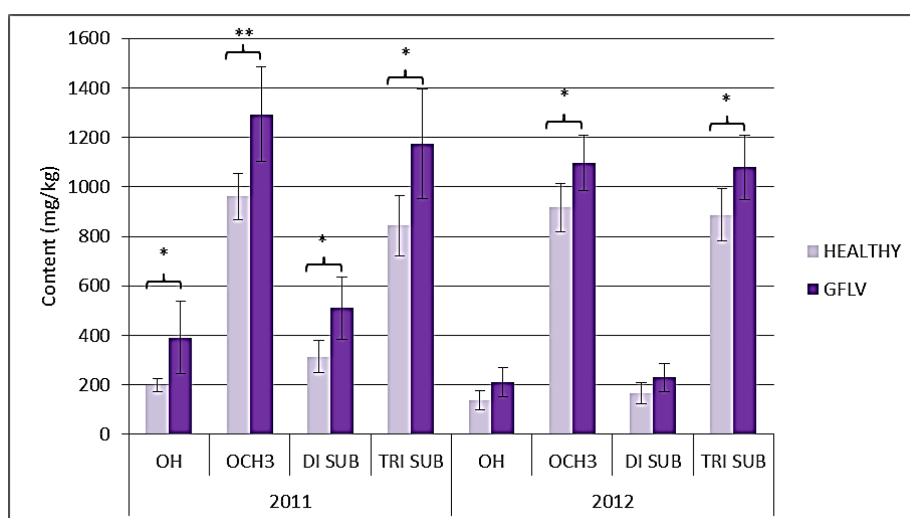


Figure 29: The contribution of non-methoxylated (OH), methoxylated (OCH_3), di-substituted (DI SUB) and tri-substituted (TRI SUB) anthocyanins of cultivar 'Schioppettino', trained in single Guyot in GFLV infected and healthy vines in 2011 and 2012. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

On the average of two consecutive years, the GFLV infected vines had the 43 % more hydroxylated anthocyanins and 20 % more methoxylated anthocyanins, as compared with healthy ones. Also the content of di-substituted anthocyanins (35 %) and tri-substituted anthocyanins (21 %) was higher in berry skins of grapes from GFLV infected plants as compared to healthy controls.

The same trend was observed also for 'Schioppettino' trained in double Guyot. All averages of individual and total anthocyanins were slightly higher in GFLV infected berries in 2011 and 2012 as compared to the healthy berries, but no significant differences were found (Figure 30).

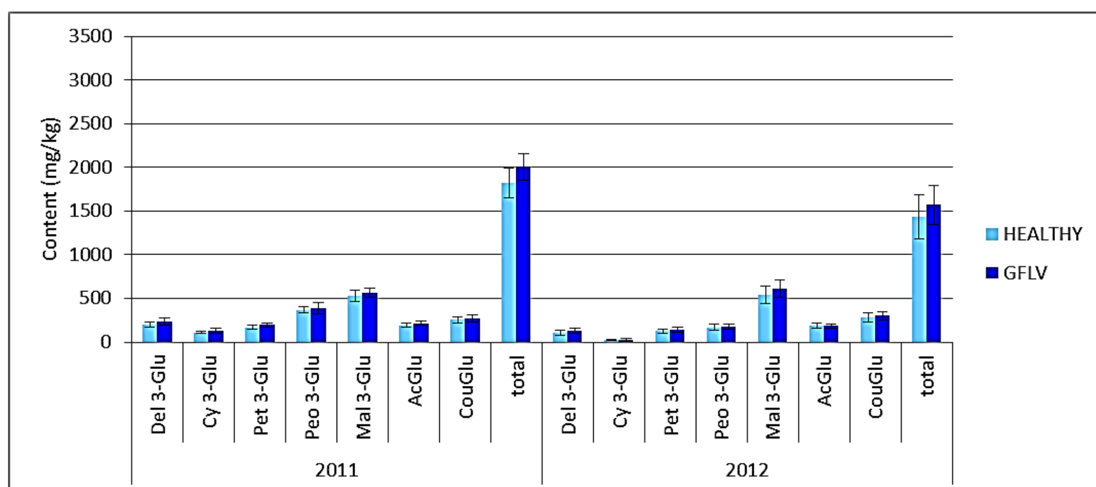


Figure 30: The anthocyanins content (mg/kg berry) in berry skin of cultivar 'Schioppettino', trained in double Guyot training system. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$).

A slightly higher content of OH-, OCH_3-, di- and tri-substituted anthocyanins were observed in GFLV infected berries in 2011 and 2012 in 'Schioppettino' trained in double Guyot, as compared to the healthy berries, but as shown above, the differences were not statistically significant (Figure 31).

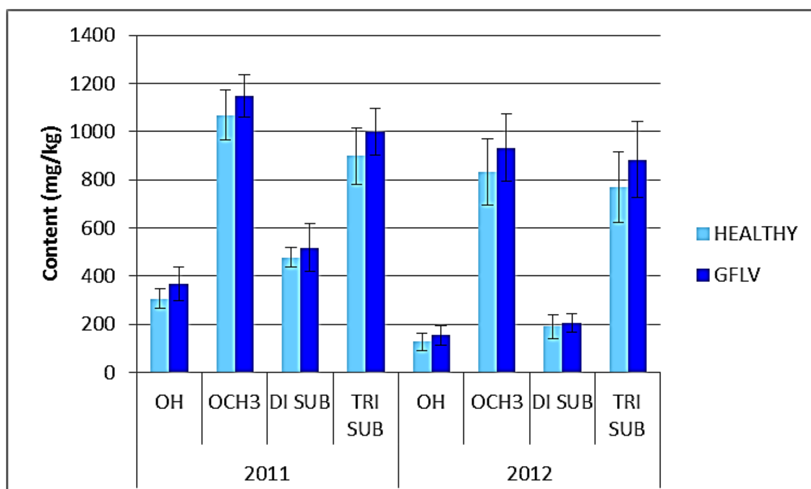


Figure 31: The contribution of non-methoxylated (OH), methoxylated (OCH3), di-substituted (DI SUB) and tri-substituted (TRI SUB) anthocyanins of cultivar 'Schioppettino', trained in double Guyot in GFLV infected and healthy vines in 2011 and 2012. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

On the average of two consecutive years, the GFLV infected vines showed 13% more hydroxylated anthocyanins, 7 % more methoxylated anthocyanins, 3 % more di-substituted anthocyanins and 11 % more of tri-substituted anthocyanins in berry skin of grapes from GFLV infected plants as compared healthy ones.

Similarly to 'Schioppettino', aslo for the cultivar 'Refošk', the slightly higher content of individual and total anthocyanins was observed in GFLV infected vines as compared to healthy controls (Figure 32). In 2011, the differences in content of Peonidin-3-glucoside in GFLV infected and healthy vines was ststistically significant.

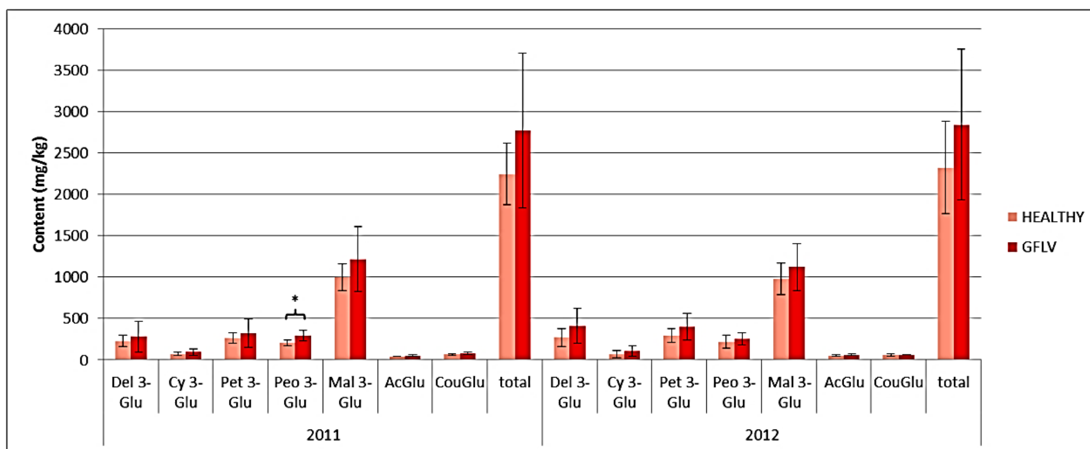


Figure 32: The anthocyanins content (mg/kg berry) in berry skin of cultivar 'Refošk', trained in single Guyot training system. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

The relative contribution of OH-, OCH_3-, di- and tri-substituted anthocyanins observed for the cultivar 'Refošk' was similar to what explained in case of the cultivar 'Schioppettino'. The contribution was slightly higher in GFLV infected berries in 2011 and 2012, as compared to the healthy berries (Figure 33), but none of the differences were statistically significant.

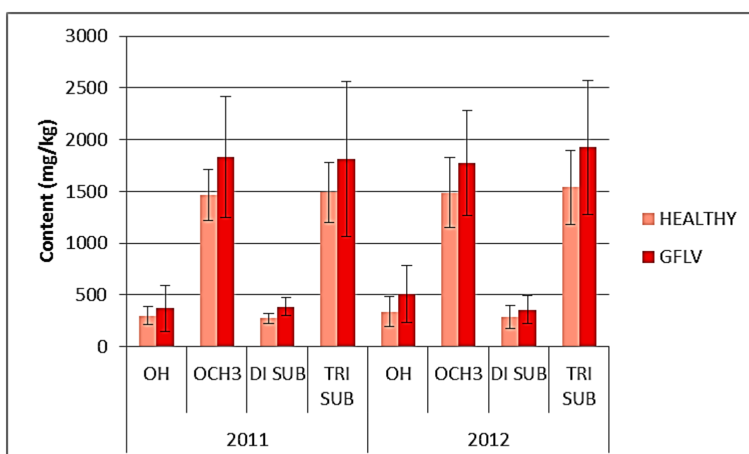


Figure 33: The contribution of non-methoxylated (OH), methoxylated (OCH3), di-substituted (DI SUB) and tri-substituted (TRI SUB) anthocyanins of cultivar 'Refošk' in GFLV infected and healthy vines in 2011 and 2012. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

On the average in two consecutive years, the GFLV infected vines had the 29 % more non-methoxylated anthocyanins and 18 % more methoxylated anthocyanins, comparing healthy one. Also the content of di-substituted anthocyanins (+25 %) and

the content of tri-substituted anthocyanins (+19 %) was higher in berry skin of grapes from GFLV infected plants as compared to healthy controls.

In both cultivars, in both training systems and in both year, the most abundant anthocyanin was malvidin-3-glucoside.

All data were processed through three ways ANOVA with the aim to understand the relative importance of cultivar (irrespective of the training system), virus infection and year on the anthocyanin content and relative proportion of OH-, OCH_3-, di- and tri-substituted monomers. Moreover also interactions between factors were ascertained (Table 7).

Table 7: Effect of cultivar, virus infection and season on the composition of monomeric, acetylated, p-coumarated anthocyanins and on the relative proportions of OH-, OCH3-, di- tri-substituted forms. Data analysed through three-ways ANOVA of and interaction of effects computed (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

	Del-3-Glu	Cy-3-Glu	Pet-3-Glu	Peo-3-Glu	Mal-3-Glu	Ac Glu	Cou Glu	total	OH	OCH_3	DI SUB	TRI SUB
Cultivar (G)	0,000*	0,038*	0,000*	0,533	0,000*	0,064	0,009*	0,000*	0,000*	0,000*	0,729	0,000*
Virus (V)	0,001*	0,013*	0,000*	0,002*	0,001*	0,013	0,033+	0,000*	0,002*	0,000*	0,004*	0,000*
Year (Y)	0,937	0,001*	0,545	0,000*	0,609	0,126	0,089	0,234	0,31	0,119	0,000*	0,919
G*V	0,592	0,948	0,373	0,931	0,23	0,612	0,877	0,448	0,685	0,306	0,935	0,306
G*Y	0,001*	0,000*	0,016*	0,000*	0,359	0,068	0,050*	0,062	0,001*	0,183	0,000*	0,241
V*Y	0,856	0,509	0,816	0,135	0,544	0,693	0,225	0,51	0,934	0,43	0,217	0,836
G*V*Y	0,121	0,249	0,233	0,862	0,77	0,434	0,82	0,515	0,138	0,837	0,593	0,524

Irrespectively to other factors, the ANOVA results show that GFLV infection has statistically significant impact on content of all individual anthocyanin, total anthocyanin and on OH-, OCH_3-, di- and tri-substituted anthocyanins. The cultivar also impaired significantly by impacts on anthocyanins except on Peo-3-Glu, Acetil-3-Glucoside derivatives and di-substituted anthocyanins. These two factors accounted for most of the differences, while the year reported less importance, only showing changes of the content of Cy-3-Glu, Peo-3-Glu and di-substituted anthocyanins.

The lack of interaction between the cultivar and virus infection (G*V), virus infection and season (V*Y) and among all factors together (G*V*Y) can be explained easily since the relative importance of the virus infection masks the effect of the other combined factors on anthocyanin content. Another explanation can be found in the interaction between cultivar and year (G*Y), that showed statistically significant differences in the content of Del 3-Glu, Cy-3-Glu, Pet-3-Glu, Peo-3-Glu, Coumaryl-3-Glucosides, OH- and di-substituted anthocyanins.

The anthocyanin composition is an important quality parameter of red grapes, due to the importance of these compound in the colour of the wines (Orak, 2007), and the data of the experiments here presented provide evidence that the GFLV infection had a positive effect on anthocyanin content in both cultivars and both training systems. Increased concentrations of anthocyanins in grapes can significantly improve the final quality of the wine.

Not many results can be found in literature as regard the effect of GFLV on the enhancement of anthocyanin content in grapes.

Contrary to our observations, Cretazzo et al. (2009) observed lower content of total anthocyanins in the cultivar 'Manto Negro' and ''Callet'' in GFLV infected vines, as compared to healthy ones, while in the same cultivars a mixed infection with GFLV, GLRaV and GFkV, resulted in a higher total anthocyanin content as compared to healthy ones. Similar to our observations, Santini et al. (2011) observed in the cultivar 'Nebbiolo' a higher percentage peonidin derivatives in vines infected by a mixed infection of GFLV and GFkV, as compared with healthy ones and lower percentage of malvidin derivatives.

In the contrast to GFLV infection, GLRaV infection had negative impact on anthocyanin content. In cultivar 'Pinot noir' all five individual anthocyanins (glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin) tended to be lower in GLRaV infected samples, compared to the healthy ones (Lee et al., 2009). The lower anthocyanin accumulation was observed also in cultivar Cabernet Sauvignon in GLRaV-3 infected berries as compared to uninfected ones at véraison.

In other time points no differences between healthy and infected berries were observed (Vega et al., 2011).

4.5 Impact of GFLV on expression of genes in flavonoid biosynthetic pathway

As regard the cultivar ‘Schioppettino’ pruned on single Guyot, the differences in anthocyanin content in GFLV infected and healthy berries were the most pronounced; because of that, the studies were completed also coupling them together with the studies of the expression of the selected targeted genes involved in the flavonoid biosynthetic pathway. The analyses were carried out separately in skin, flesh and seed of berries.

4.5.1 Chalchone synthase (CHS)

At the beginning of the flavonoid biosynthesis pathway, the early gene chalchone synthase 2 (CHS2), which is involved in recruitment of flavonoid precursors to enter the pathway, showed different behaviour in all tissues. A lower expression was observed in GFLV infected skin at first point of sampling (véraison), but thereafter no differences were evaluated in its expression in GFLV infected and healthy skins during the following period of berry development (Figure 34).

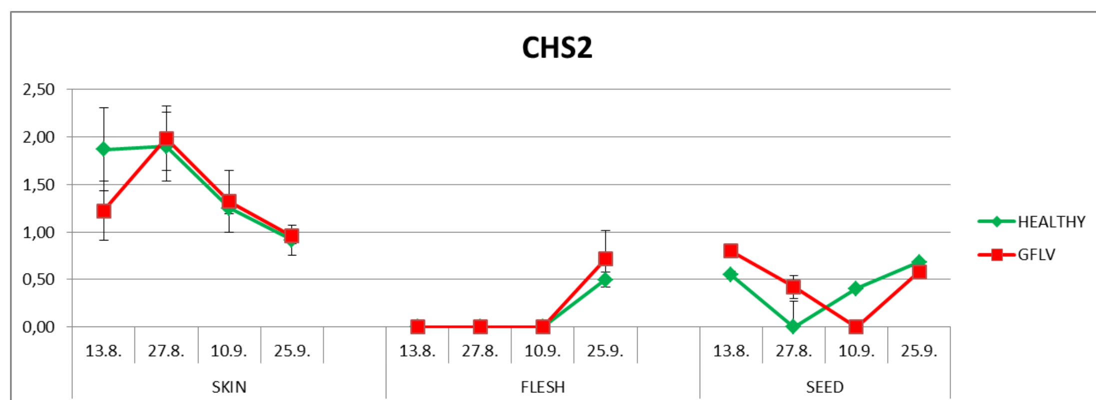


Figure 34: Gene expression of CHS2 in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

In the flesh we could not detected the expression of CHS2 in the first three point of sampling (13. 8., 27. 8. and 10. 9.), while at harvest time, the expression increased and it was slightly higher in case of GFLV infected berries as compared to healthy one (Figure 39).

At the beginning of berry development, especially at véraison and 14 days after véraison, the CHS2 gene was more expressed in GFLV infected seeds, while in the third time point of sampling (10. 9.) and at harvest time (25. 9.), the relative expression in GFLV infected seeds was lower (Figure 39).

Vega et al. (2011) showed that GLRaV-3 infection affected the expression of CHS2. The CHS2 was up-regulated in immature infected berries, but repressed at the ripening stage. The 3- and 4- folds higher expression levels of CHS2 gene were observed also in leaves infected with GLRaV-3 by Gutha et al. (2010).

The picture of CHS is not complete, since there is a small family of chalcone synthase (CHS1, CHS2 and CHS3) that all together could help to explain the overall expression at that stage in the phenylpropanoid pathway. In our experiment also CHS1 and CHS3 could have been triggered by GFLV infection. A similar expression behaviour of CHS2 as we found after GFLV infection was observed by Castellarin et al. (2007) comparing water stress and well-watered plants. Moreover, Ban et al. (2003), reported that ABA treatment of Kyoho grapes at véraison enhanced the accumulation of anthocyanin and the expression of CHS genes in berry skin.

4.5.2 Flavanone 3-hydroxylase (F3H)

A family of two Flavanone 3-hydroxylase (F3H) genes are known, the F3H1 and the F3H2. In our research both copies were analysed in GFLV infected and healthy berries.

In berry skins, the gene F3H1 was highly expressed at the beginning of berry development, with a following reduction in the expression going through ripening, in both GFLV infected and healthy berry skins. Looking at the trends during maturation, the F3H1 gene was up regulated in GFLV infected skins in the first two

dates of sampling, at véraison and 14 days after, but regardless the virus infection the expression of F3H1 gene decreased in skin and increased in flesh during the berry development.

In the flesh, the relative expression of F3H1 was slightly higher in GFLV infected berries, as compared with healthy ones. From véraison through harvest, the expression increased in both GFLV and healthy flesh, reporting slightly higher values in GFLV infected tissues at harvest (Figure 35).

Regarding the seeds, the expression was slightly higher at véraison in GFLV infected berries as compared with healthy ones (Figure 35). Therefore no differences in the relative expression were found through harvest.

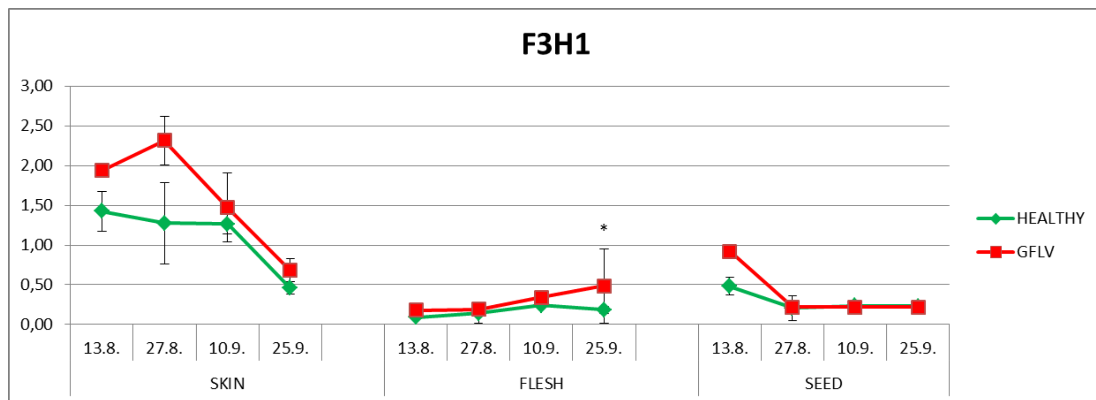


Figure 35: Gene expression of F3H1 in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

Similarly as F3H1 gene, the F3H2 gene, regardless the virus infection, was also more expressed in skins than in seeds and flesh, but on the opposite the expression of the gene F3H1 was quite stable during the season. The F3H2 gene was more expressed in the skin of healthy berries compared to GFLV infected berries, except for the second point of sampling (27. 8.), when higher expression was observed in GFLV infected berries. In the flesh, the expression of F3H2 gene was more or less equal in GFLV infected and healthy berries (Figure 36) as observed for F3H1.

Regarding the seeds, the relative expression for F3H2 gene was similar to the expression of CHS2 gene in seeds. At the beginning of berry development, at véraison and 14 days after it, the F3H2 gene was more expressed in GFLV infected

seeds. From the third point of sampling (10. 9.) until harvest time (25. 9.), the expression was turned, thus the F3H2 gene was more expressed in healthy seeds (Figure 36).

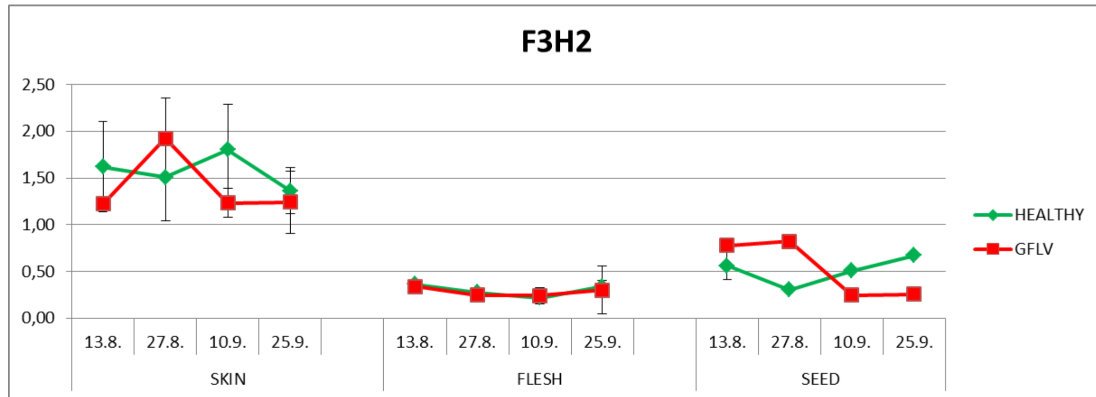


Figure 36: Gene expression of F3H2 in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar 'Schioppettino'. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$).

Both copies of F3H genes were also checked for their relative expression by Falginella et al. (2012) in red grape cultivar (Alicante Bouschet) characterised by pigmented flesh. F3H1 and F3H2 showed similar transcriptional trends, but the F3H1 expression was more correlated with anthocyanin genes and F3H2 was more correlated with proanthocyanidins genes (Falginella et al., 2012). In our research, F3H1 was differently expressed in skins of GFLV infected and healthy berries, what might lead to high expression of genes involved in anthocyanin biosynthetic pathway and consequently to higher accumulation of anthocyanins that was measured by HPLC in infected skins as compared to healthy controls. As we already speculated above, also the expression of F3H gene in grapevine berry was induced by water deficit (Castellarin et al., 2007), remaining constantly higher under drought conditions and was strongly correlated with anthocyanin content.

4.5.3 Flavonoid 3´ hydroxylase (F3'H) and flavonoid 3´5´-hydroxylase (F3´5´H)

The F3´H and F3´5´H play a key role in the branching of the phenylpropanoid pathway, determining the ratio between di-substituted and tri-substituted anthocyanins or other polyphenols. The F3´H is basically required for the synthesis of di-substituted anthocyanins (cyanidin and peonidin), while the F3´5´H is necessary for the synthesis of tri-substituted anthocyanins (delphinidin, petunidin and malvidin) (Pascual-Teresa and Sanchez-Ballesta, 2007). Regardless the viral infection the expression of the F3'H gene decreased and the expression of the F3´5´H increased during the berry development in the skin, while in the flesh the expression of both genes increased during the berry development. In several experiments, the relative accumulation of tri-substitute anthocyanins increases with maturation, and so we can speculate that a change in the relative expression of F3´H (Figure 37) and F3´5´H (Figure 38) change during the maturation.

In general, the F3´H gene was down-regulated by GFLV infection in skin and flesh; and the F3´5´H gene was up-regulated by the GFLV infection in the same tissues. These results confirmed that the gene F3´5´H show significant correlation at the transcriptional level with the accumulation of 3´4´5´OH anthocyanins.

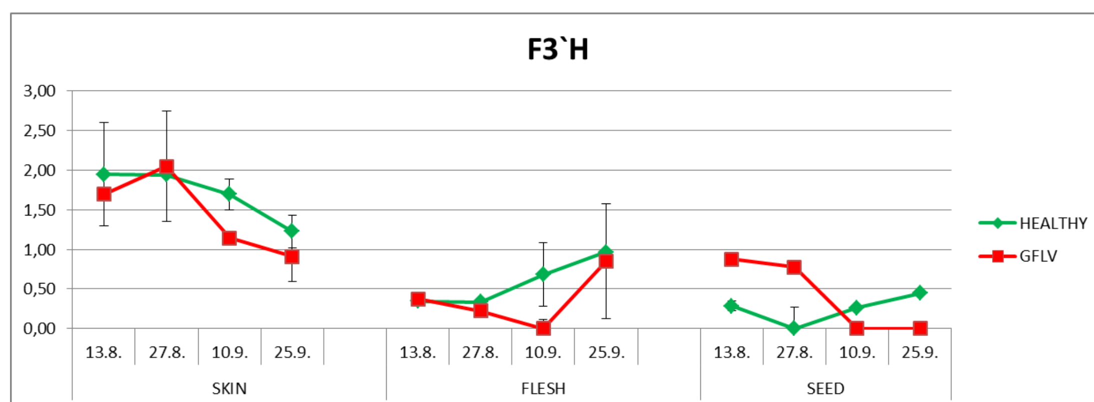


Figure 37: Gene expression of F3´H in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar 'Schioppettino'. Significances between means were checked with *t*-test (*, $p<0,05$; **, $p<0.01$; ***, $p<0.001$).

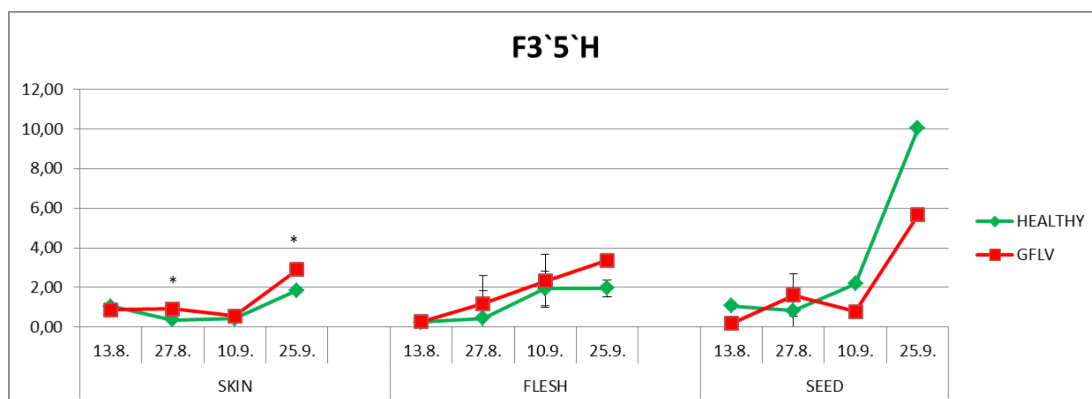


Figure 38: Gene expression of F3´5´H in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar 'Schioppettino'. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

On average the F3´H gene was 20 % less expressed and the F3´5´H gene was 30 % more expressed in skins of GFLV infected berries during the berry development as compared to healthy controls (Figure 39a). Consequently, at harvest in berry skins of the same GFLV infected vines, the ratio between tri- and di-substituted anthocyanins (measured by HPLC) was 18 % higher than in healthy controls (Figure 39b).

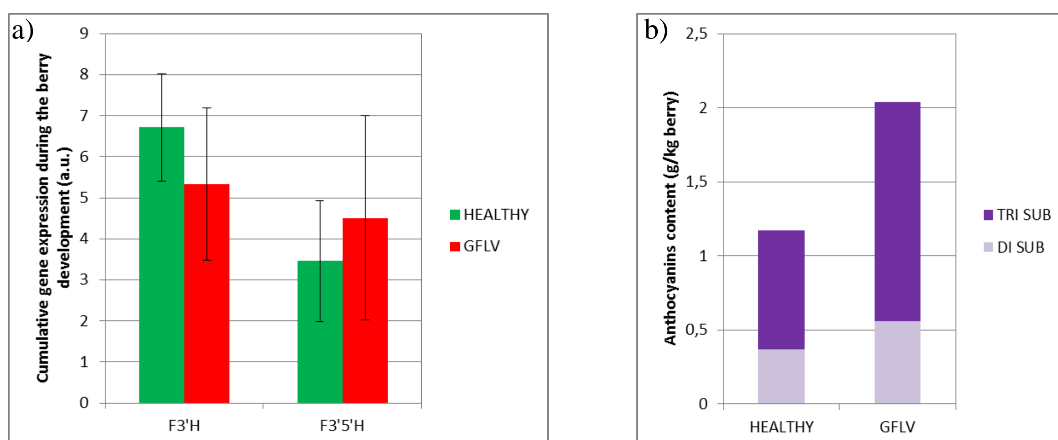


Figure 39: a) the cumulative expression of F3`H and F3`5`H gene during berry development and b) the content of TRI SUB and DI SUB anthocyanin (g/kg berry).

Falginella et al. (2012) reported that the transcription levels of several gene copies of the F3´5´H gene family affect anthocyanin composition. If the F3´5´H activity is up-regulated and F3´H activity is down-regulated, the product of flavonoid hydroxylases are predominately channelled into the branch of the pathway that leads to the synthesis of tri-substituted anthocyanins at the expense of those channelled into the

synthesis of di-substituted anthocyanins. Transcript abundance of the F3´5´H and the level of anthocyanin hydroxylation during maturation are also positively correlated as reported by Bogs et al. (2006), Castellarin et al. (2006), Jeong et al. (2006) and Castellarin et al. (2007).

Gutha et al. (2010) reported that the two flavonoid hydroxylases, the F3´H and the F3´5´H were significantly highly expressed in GLRaV-3 infected leaves as compared to healthy ones. Moreover, it was recently reported that F3´H gene was only slightly detectable and F3´5´H gene was expressed at non detectable levels in green, fully expanded grapevine leaves (Castellarin et al., 2006; Kobayashi et al., 2009).

The transcription of the F3´5´H was up-regulated under drought conditions from the completion of véraison onwards, peaking up in concomitance with the increase biosynthesis of 3´4´5´-OH anthocyanins in water stress plants. In contrast, the expression of F3´H was down-regulated in water stress plants through the phase of colour transition, while from full véraison onwards, F3´H was up-regulated by drought (Castellarin et al., 2007). These results are similar to our findings on GFLV infected vines. This coincidence of changing indicates that both abiotic (drought) and biotis stresses (GFLV infection) could affect in a similar way the anthocyanin biosynthesis pathway.

4.5.4 Leucoanthocyanidin dioxygenase (LDOX)

The conversion of leucoanthocyanidins to anthocyanidis is catalysed by the LDOX gene. Regardless the vine infection, the LDOX gene was highly expressed in the skin at véraison, but its expression decreased at the end of the berry development. The GFLV infection slightly up-regulated the expression of the LDOX gene in the berry skin (Figure 40). Regarding the flesh and seeds, in both GFLV infected and healthy berries, the expression of the LDOX gene was really lower. In the seeds, GFLV infection caused even a down-regulation of the LDOX gene in the second half of the berry development (Figure 40).

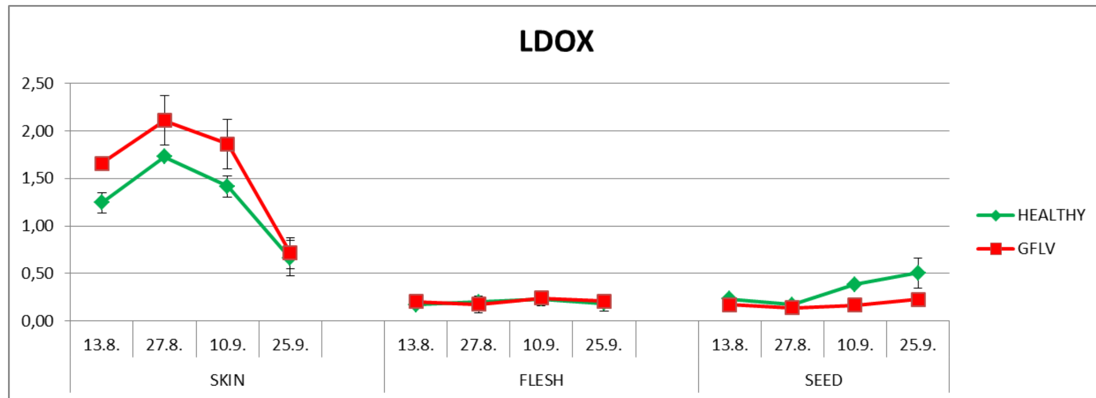


Figure 40: Gene expression of LDOX in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar ‘Schioppettino’. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

Mori et al. (2005) observed the expression of genes in grape berries grown under elevated night temperature conditions. The expression levels of LDOX gene in berries grown under high night temperatures was less than that in berries grown under low night temperatures at véraison, while in the later stage of ripening (from 30 to 45 days after véraison) the transcript levels were unaffected by night temperatures.

Ban et al. (2003) reported that ABA treatment of Kyoho grapes at véraison enhanced the accumulation of anthocyanin and the expression of the LDOX gene. Similarly, Castellarin et al. (2007) reported that the transcription of the LDOX gene was promoted by water deficit at the beginning of véraison, but as ripening progressed, these differences were not consistently maintained. It is well known that in condition of water stress ABA is promoted, thus we can easily understand while the previous two experiments provided the same results.

Margaria et al. (2014) working with healthy plants or infected by Flavescence dorée phytoplasma, found different expression of the LDOX genes in grapevines ‘Nebbiolo’ and ‘Barbera’. Specifically, an increase in expression of the LDOX gene in infected ‘Barbera’ vines was shown while only a lower higher expression was observed in the ‘Nebbiolo’ grapevine.

4.5.5 Flavonoid-3-o-glucosyltransferase (UFGT)

The flavonoid-3-o-glucosyltransferase (UFGT) gene is involved in the final step of the anthocyanin biosynthesis. It showed very similar expression pattern as the LDOX. Regardless the viral infection, the UFGT was highly expressed in skin at véraison, but its expression decreased until harvest time. The UFGT was slightly up-regulated in skins of GFLV infected berries at véraison. The expression of UFGT gene in flesh and seeds was low and GFLV infection even down-regulated it in seed (Figure 41).

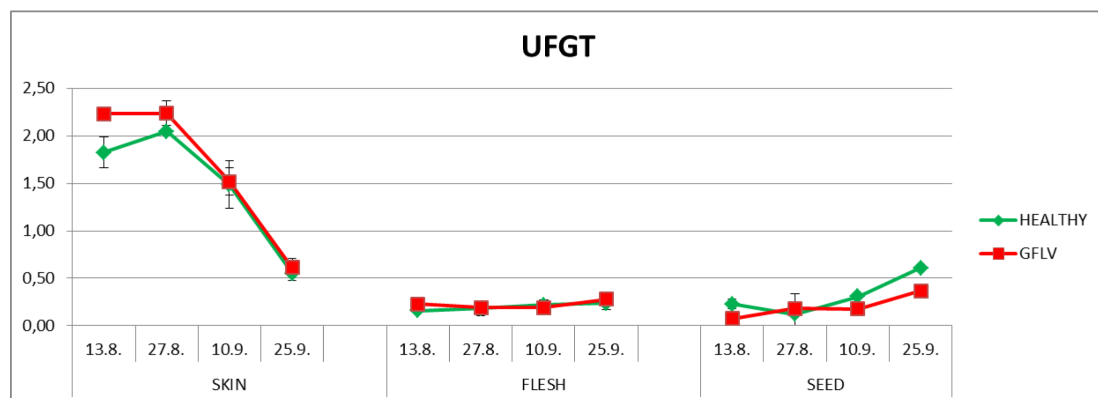


Figure 41: Gene expression of UFGT in grape berry skin, seed and flesh of healthy and GFLV infected vines of cultivar 'Schioppettino'. Significances between means were checked with *t*-test (*, p<0,05; **, p<0.01; ***, p<0.001).

In contrast to our results, the expression of UFGT gene was strongly repressed in skin of berries infected with GLRaV-3 virus (Vega, et al., 2011).

Castellarin et al. (2007) reported that the expression profile of the UFGT was higher in water stressed fruits that in control fruit. Higher expression levels of the UFGT gene were observed in GLRaV-3 infected symptomatic leaves, which indicate enhanced synthesis of anthocyanins and proanthocyanidins in GLRaV-3 infected leaves as compared to healthy one (Gutha et al., 2010).

Neither of the investigated genes is a key point in the transcriptional regulation of the anthocyanin pathway in grape, what is consistent with the hypothesizes of Boss et al. (1996), but the F3H1 gene was identified to be the most strongly regulated by the

GFLV infection, indicating that F3H1 gene has an important role in the increase of total anthocyanin content caused by the GFLV infection. Besides that, up-regulation of F3´5´H gene and down-regulation of F3´H gene were shown to have an important role in changing the ratio between tri-substituted and di-substituted anthocyanins caused by GFLV infection.

Taken together we can conclude that transcriptional regulation is an important part in the regulation of anthocyanin biosynthesis pathway in vines influenced by GFLV infection.

4.6 MapMan

The final list of differentially expressed genes was imported into the MapMan visualization tool (Rotter et al., 2009) where genes are organized in graphically represented metabolic pathway and the corresponding log2-fold change for each gene is colour coded. The log2-fold change represents the difference in level of gene expression between GFLV infected and healthy vines. The intensity of gene expression was showed with colour coded (in the upper left corner of the image). Highly expressed genes in GFLV infected plants were coloured red, while genes highly expressed in healthy plants were coloured green. Each coloured box (near each gene) in the figure, represented sampling point (Figure42).

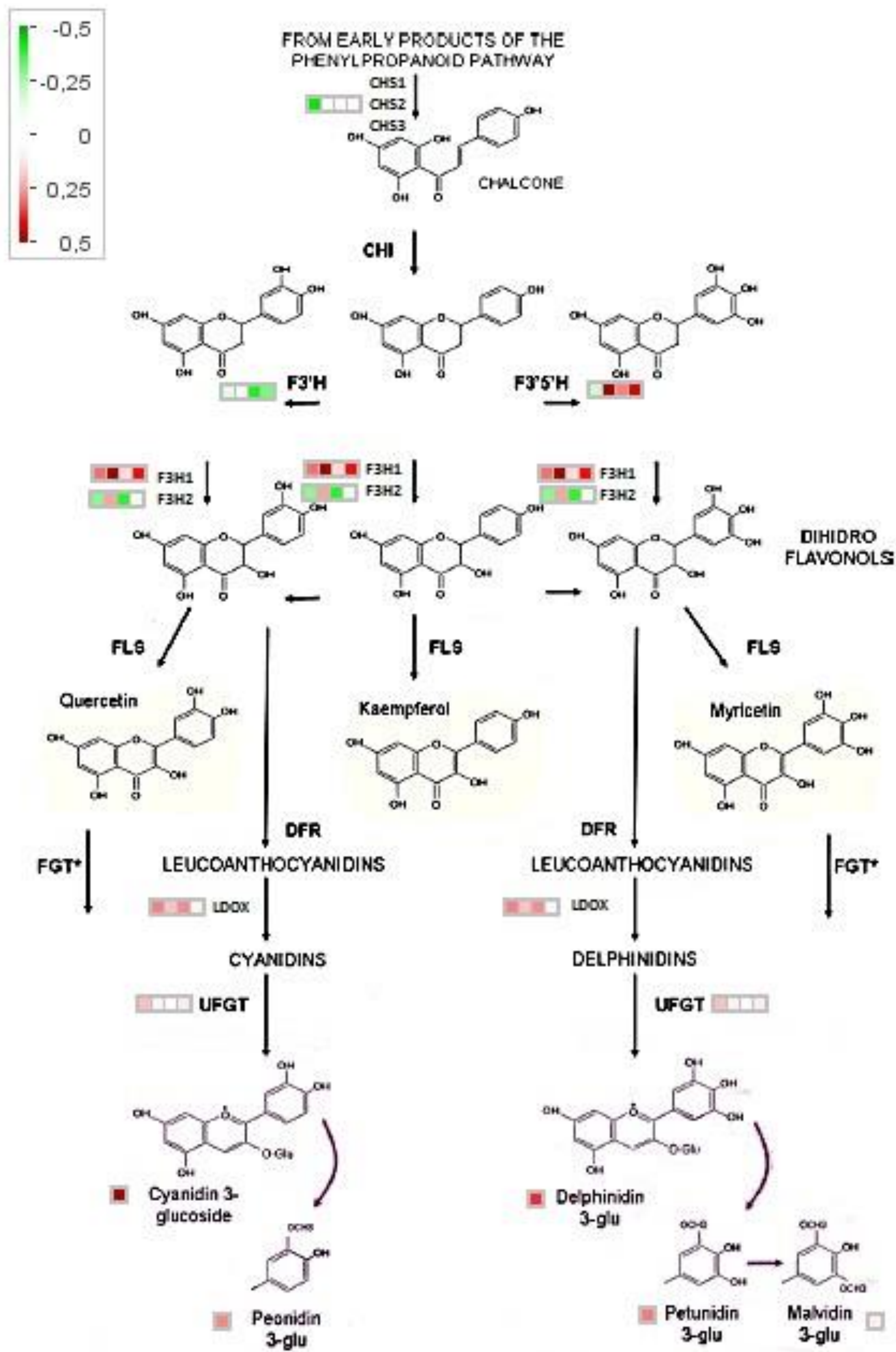


Figure 42: MapMan visualization of the phenylpropanoid pathway during berry development. Green colour represents down-regulation and red colour up-regulation in infected vs. healthy samples.

4.7 Impact of GFLV on wine quality parameters

Microvinifications were performed from GFLV infected and healthy vines of cultivars ‘Refošk’ and ‘Schioppettino’ trained in single and double Guyot training system in 2012 and 2013. The results of wine quality parameters of cultivar ‘Schioppettino’ trained in single Guyot (Table 8), double Guyot (Table 9) and of cultivar ‘Refošk’ (Table 10) are present.
In general, if we compare the years 2012 and 2013, there was some inter-year variation, an usual outcome, since the season 2013 was much drier than the season 2012.

Much stronger and statistically significant impact on wine quality parameters was caused by GFLV infection. The strongest influence was observed on total extract, the content of which was up to 100 % higher in the wine from GFLV infected vines as compared to the wine from healthy wines. The influence of GFLV infection on total extract content was statistically significantly higher in wines from both cultivars (‘Refošk’ and ‘Schioppettino’) and wines from vines trained in both training systems (single and double Guyot).

Table 8: Wine quality parameters in cv. ‘Schioppettino’ pruned in single Guyot training system

Training system	SINGLE GUYOT					
Year	2012			2013		
Vine status	healthy	GFLV	sig.	healthy	GFLV	sig.
Titratable acids (g/L)	6.3	6.4	***	5.1	5.2	ns
Ph	3.5	3.5	**	3.6	3.9	***
Dry extract (g/L)	2.0	2.4	***	2.2	2.1	ns
Alcohol (vol. %)	13.1	13.0	*	13.7	13.4	ns
Total extract (g/L)	29.1	31.0	***	26.8	31.4	**
Malic acid (g/L)	2.4	2.4	ns	1.7	1.9	***
Citric acid (g/L)	0.2	0.2	ns	0.2	0.2	*
Tartaric acid (g/L)	0.8	1.0	ns	1.5	1.1	**

Legend: Significances (sig.) between means were checked with *t*-test (ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

The influence of the GFLV infection was observed also on dry extract content with one exception. No influence was observed in wine from cultivar ‘Schioppettino’ trained in single Guyot training system in the year 2013. Othervise, the dry extract content was statistically significantly higher in wines from GFLV infected vines of both cultivars (‘Refošk’ and Scioppettino) and both training systems (single and double Guyot).

Table 9: Wine quality parameters in cv. ‘Schioppettino’ pruned in double Guyot training system

Training system	DOUBLE GUYOT					
Year	2012			2013		
Vine status	healthy	GFLV	sig.	healthy	GFLV	sig.
Titratable acids (g/L)	5.97	6.10	ns	5.93	6.03	ns
Ph	3.54	3.49	**	3.82	3.92	***
Dry extract (g/L)	2.03	2.80	**	1.77	2.30	**
Alcohol (vol. %)	12.97	12.50	**	12.83	12.07	***
Total extract (g/L)	27.30	30.57	***	28.60	30.23	**
Malic acid (g/L)	2.03	1.90	ns	1.57	1.90	**
Citric acid (g/L)	0.16	0.16	ns	0.13	0.20	*
Tartaric acid (g/L)	1.00	1.20	***	1.43	1.27	*

Legend: Significances (sig.) between means were checked with *t*-test (ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

The GFLV infection had also an impact on alcohol content in wines. The content of alcohol in wine from GFLV infected vines was 1,5% - 5,9% lower than in wine from healthy vines. The decrease in alcohol content caused by the GFLV infection was statistically significant in wines from both cultivars of vines and from vines trained in both training systems.

The influence of GFLV on the titratable acids depended on cultivars. In the wine from GFLV infected vine of the cultivar ‘Refošk’, titratable acids were significantly lower and pH significantly higher than in wines from healthy vines of cultivar ‘Refošk’. On the contrary, in wines from GFLV infected vines of cultivar ‘Schioppettino’, titratable acids was higher than in wines from healthy vines of the cultivar ‘Schioppettino’. Besides that, the influence of GFLV infection on the ratio between different wine acids was observed, but the pattern of the influence was not the same in different cultivars and in different years.

Table 10: Wine quality parameters in cv. 'Refošk' trained in single Guyot training system

Training system	SINGLE GUYOT					
Year	2012			2013		
Vine status	healthy	GFLV	sig.	healthy	GFLV	sig.
Titratable acids (g/L)	9.7	9.33	**	10.53	9.30	***
Ph	3.06	3.10	**	3.15	3.32	***
Dry extract (g/L)	2.3	5.66	*	3.07	5.93	ns
Alcohol (vol. %)	11.27	11.00	*	10.77	10.53	ns
Total extract (g/L)	25.03	28.10	*	26.73	53.37	**
Malic acid (g/L)	3.13	3.10	ns	4.90	4.23	**
Citric acid (g/L)	0.22	0.26	*	0.32	0.53	**
Tartaric acid (g/L)	3.73	3.50	*	3.53	3.37	ns

Legend: Significances (sig.) between means were checked with *t*-test (ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001).

Similarly to our observations of the influence of GFLV, also Legorburu et al. (2009), reported in cultivar Tempranillo that the effect of the GFLV or GLRaV-3 infection on wine quality resulted in a more concentrated wine than that from healthy vines, with higher titratable acids content and colour intensity. GFLV infected plants suffered from severe fruit yield loss, mediated by virus-induced poor set. Like any other factor limiting vine vigour, this decrease in yield was counterbalanced by higher sugar content. However, this counterbalancing did not completely compensate for the overall yield loss due to the virus, and resulted in less alcohol, tartaric acid and anthocyanins produced per hectar. The effect of GFLV or GLRaV-3 infection on wine quality was smaller than that of the vineyard or the vintage, but still statistically significant (Legorburu et al., 2009).

Mannini et al. (2011) reported significant fewer intense violet nuances in colour, a poorer bouquet (fruity flavours), and a darker body, which resulted in a higher astringency and lower softness in wine produced from GLRaV-3 infected vines as compared to wine from healthy vines. In other words, the wine obtained from the vines from virus free plants was slightly more drinkable than the wines from GLRaV-3 infected plants. On the contrary, the wine produced from GLRaV-3 infected vines, showed brighter violet nuances in colour (probably due to lower pH) and a richer body with a consequent stronger astringency in taste. These findings are

in contrast with the results reported on ‘Nebbiolo’ and tempranillo wines, where the wines from GLRaV-3 free plants showed an increase in colour intensity.

The sensory evaluation of ‘Refošk’ and ‘Schioppettino’ wines was performed, following a 100 points scale, with the participation of 10 testers. The higher overall evaluation was given to the scioppettino wine trained in single and double Guyot produced from vines infected with GFLV. The opposite was for ‘Refošk’ wines, which got lower overall evaluation for the wine derived from GFLV infected vines as compared the wine from healthy vines (Table 11).

Table 11: The average points of sensory evaluation

	2012	2013
REF healthy	78.8	81.8
REF GFLV	77.3	75.5
SCH SG healthy	80.0	69.2
SCH SG GFLV	82.3	70.2
SCH DG healthy	75.3	72.7
SCH DG GFLV	82.5	73.8

Legend: REF – ‘Refošk’; SCH – ‘Schioppettino’

5 CONCLUSIONS

A large number of vines of cultivars ‘Schioppettino’ and ‘Refošk’ were tested for the presence of viruses that are included in Slovenian certification scheme (GFLV, ArMV, GLRaV -1, -2, -3, 4-9, GVA, GVB and GFkV) and for 5 other important grapevine viruses (GCMV, ToRSV, SLRSV, TRSV and RpRSV), to find healthy vines and vines infected only with GFLV. We could not detect the presence of any other viruses, instead of infection with GFLV. Besides, seven vines of cultivar ‘Volovnik’ were tested to find GFLV-free plant, among which, only one vine (Vol 5/9) was considered as a GFLV free.

The infection with the GFLV decreased a yield of grapevines, berry and cluster weight in both cultivars, ‘Refošk’ and ‘Schioppettino’ trained on single and double Guyot, while the cluster number was not affected by the GFLV in none of the cultivars and training systems. The reduction in yield was due to smaller berries and due to lower cluster weight. In both training systems, the impact of GFLV on quantity parameters was observed. Greater impact was observed on vines trained on single Guyot as on vines trained on double Guyot training system.

The GFLV infection did not significantly affect the content of soluble solids in berries in none of studied cultivars and training systems. The influence of GFLV infection on titratable acids was different from year to year, while the influence of GFLV infection on pH was more constant; slightly higher pH was observed in GFLV infected vines as compared to healthy ones.

Anthocyanin contents of individual and total anthocyanin in berries increased by virus infection in both cultivars and training systems. The greater impact of virus infection was observed on anthocyanin content in berries of cultivar ‘Schioppettino’, trained in single Guyot training system, than in double Guyot and in cultivar ‘Refošk’.

In the cultivar ‘Schioppettino’ trained in single Guyot, the studies of anthocyanin content were complemented also by the studies of expression of genes involved in

flavonoid biosynthetic pathway. The grates impact of the GFLV on gene expression was observed in berry skin. The GFLV infection affected the expression of genes involved in anthocyanin biosynthesis. Under the influence of GFLV infection, the biosynthesis of 3´5´ hydroxylated anthocyanins (delphinidin, petunidin, malvidin) was increased due to up-regulation of F3H1. Under the influence of GFLV infection the ratio between tri- and di-substituted anthocyanins increased due to up-regulation of F3´5´H and down-regulation of F3´H.

The GFLV infection increased total extract and decreased alcohol content in the wine from GFLV infected vines as compared to wine from healthy vines. The higher overall sensory evaluation was given to the 'Schioppettino' wine trained in single and double Guyot produced from vines infected with GFLV, while 'Refošk' wines got lower overall evaluation for the wine derived from GFLV infected vines as compared the wine from healthy vines

6 SUMMARY

Grapevine (*Vitis vinifera* L.) is one of the most widely cultivated fruit crops and is globally one of the important fruit species due to the numerous uses of its fruit in the production of wine, grape juice and for fresh and dry consumption. The grapevine is exposed to many abiotic and biotic stresses caused by insects, fungi, bacteria, phytoplasmas and viruses. The Grapevine fanleaf virus (GFLV) is one of the most economically important viral disease affecting grapevines in all vine-growing regions of the world. It is naturally spread by the nematode vector *Xiphinema index* and through the use of infected planting material. The productive life of GFLV infected vineyards is significantly reduced, 15-20 years instead of 30-40 years or longer.

In this study the influence of GFLV on agronomical important quality and quantity parameters of grapes at harvest time was investigated on a large number of vines of two cultivars, ‘Refošk’ and ‘Schioppettino’, trained in two training systems, single and double Guyot. Among quality parameters, special attention was given to analyses of individual anthocyanin in berry skin.

The infection with the GFLV decreased the yield of grapevines in both cultivars (‘Refošk’ and ‘Schioppettino’) and in both training systems (single and double Guyot). The statistically significant lower yield was observed in Schiopettino trained in single Guyot in all three years of observation. The infection with the GFLV decreased the berry weight. In the cultivar ‘Schioppettino’, trained in single Guyot, the berry weight was statistically significantly lower in GFLV infected vines in 2011 and 2013. The lower berry weight was also observed in ‘Schioppettino’ trained in double Guyot, while in cultivar ‘Refošk’ in 2011 and 2012, the effect of the GFLV infection on berry weight was not observed, even if in 2013 the GFLV infection statistically reduced berry weight. The reduction in yield was due to smaller berries and of course to the related lower cluster weight. Statistically significant lower cluster weight was observed in GFLV infected vines of cultivar ‘Schioppettino’ trained in single and double Guyot in 2011. The same trend was observed also in 2012 and in 2013. In cultivar ‘Refošk’, the effect of the GFLV infection on cluster

weight was observed in all three years, but only in 2013 the differences were statistically significant.

The GFLV infection did not significantly affect the content of soluble solids in berries. However, in all three years in cultivar ‘Refošk’, the average soluble solids content was slightly higher in GFLV infected vines than in healthy vines. In the cultivar ‘Schioppettino’ in both training systems, the significant differences were not observed between GFLV infected and healthy vines. The influence of GFLV infection on titratable acids was different from year to year, especially in cultivar ‘Schioppettino’ trained in double Guyot training system. The GFLV infection increased pH more constantly and even statistically significant in both cultivars and both training systems.

The HPLC method was performed to analyse the individual anthocyanins in grapevine berries. Anthocyanin concentration in berries was increased by virus infection in both cultivars and both training systems. Among both cultivars and training systems the virus infection had the greatest impact on anthocyanin content in the cultivar ‘Schioppettino’, trained on single Guyot. All individual anthocyanins and total anthocyanins were significantly higher in GFLV infected berries in 2011, as compared to the healthy controls. The same trend was observed also in 2012, but only the differences in content of petunidin-3-glucoside and total anthocyanins were significantly higher in GFLV infected berries, compared to the healthy controls. For the cultivar ‘Schioppettino’ trained in double Guyot and cultivar ‘Refošk’, all the average amounts of individual and total anthocyanins were slightly higher in GFLV infected berries in 2011 and 2012, compared to the healthy berries, but the differences were not statistically significant.

In cultivar ‘Schioppettino’ trained in single Guyot training system, the studies of anthocyanin content were complemented with the studies of the expression of genes involved in flavonoid biosynthetic pathway. Quantitative real-time PCR was used to analyse the gene expression in grapevine berries, divided in seed, flesh and skin. The F3H1 gene was identified to be the most strongly regulated by GFLV infection, indicating that the F3H1 gene has an important role in the increase of total

anthocyanin content caused by the GFLV infection. Besides up-regulation of the F3´5´H gene and down-regulation of F3´H gene, both genes showed to have an important role in changing the ratio between tri-substituted and di-substituted anthocyanins caused by the GFLV infection. The results indicate that the transcriptional regulation is an important part in theof anthocyanin biosynthesis pathway regulation in vines influenced by GFLV infection.

In wine, the strongest influence was observed on the total extract, where the content was up to 100 % higher in the wine from GFLV infected vines as compared to the wine from healthy vines. GFLV infection decreased alcohol content in wine from GFLV infected vines as compared to wine from healthy vines. The higher sensory overall evaluation was given to the scioppettino wine trained in single and double Guyot produced from vines infected with GFLV. The opposite was for 'Refošk' wines, which got lower overall evaluation for the wine derived from GFLV infected vines as compared the wine from healthy vines

The presence of GFLV virus on grapevines promoted an improvement of the anthocyanin content and modification of the relative proportion between di-, tri-, OH and OCH_3 forms, with little changing in basic maturation parameters (less sugars and high acidity). In the frame of global warming, lower alcohol wines are searched, together with a high occurrence of secondary metabolites. In the future will be advisable to reconsider the presence of viruses, such as GFLV, on particular grape cultivars like 'Schioppettino', since the vigour of the plant can overcome the presence of the virus, balancing the productivity and resulting in a better grape quality at harvest.

In future experiments will be advisable to consider the effects of GFLV virus infection on water stress tolerance, following leaf water potential and modifications in root hydraulic conductivity and xylem cavitation. Moreover, since we found up-regulation of the phenylpropanoid pathway, will be of interest to investigate the role of GFLV on the other classes of polyphenols (flavones, flavan-3-ols…) and the whole plant metabolome.

Key words: Grapevine, Vitis vinifera, Grapevine fanleaf virus, GFLV, quality, quantity, anthocyanins, gene expression

7 POVZETEK

VPLIV VIRUSA PAHLJAČAVOSTI LISTOV VINSKE TRTE (GFLV) NA KOLIČINO IN KAKOVOST GROZDJA

Vinska trta (*Vitis vinifera* L.) je ena izmed najpomembnejših in najbolj razširjenih gojenih rastlin. V svetovnem merilu spada med pomembnejše ″sadne″ vrst zaradi številne uporabe tako v pridelavi in predelavi kot tudi za svežo uporabo. Tako kot vse druge rastline, je tudi vinska trta izpostavljena vplivom okolja ter boleznim in škodljivce. Med biotske faktorje vključujemo žuželke, glive, fitoplazme, bakterije in viruse. Eden izmed ekonomsko najpomembnejših virusov, ki okužujejo vinsko trto v vseh vinorodnih regijah po svetu je virus pahljačavosti listov vinske trte (GFLV), ki povzroča bolezen imenovano kužna izrojenost vinske trte. V vinogradih se na kratke razdalj prenaša s talno ogorčico (*Xiphinema index*), ki se hrani na koreninah vinske trte, na daljše razdalje pa ga prenašamo ljudje z okuženim sadilnim materialom. Kot posledica okužbe z virusom GFLV se lahko na grozdu pojavi močno osipanje, grozd je redek. Grozdi zorijo nepravilno, jagode so drobne, slabo obarvane in nedozorele, kar lahko povzroči zmanjšanje količine pridelka (tudi do 80%) ter skrajšano življenjsko dobo trsov. Klub poročilom o vplivu okužbe z GFLV na kakovost pridelka, je zelo malo eksperimentalnih podatkov o vplivu okužbe z GFLV na količinske in kakovostne parametre grozdja. V okviru doktorske disertacije smo analizirali vpliv okužbe z GFLV na količino (skupni pridelek, maso jagod, teža grozda in število grozdov) in kakovost grozdja z merjenjem osnovnih kakovostnih parametrov (količina skupnih sladkorjev, vrednost pH, količina titracijskih kislin) ter posameznih in skupnih antocianov v grozdju pri sorti ʻRefošk' in ʻSchioppettino', gojeni na dveh gojitvenih oblikah, enojni in dvojni Guyot.

Okužba z GFLV vpliva na zmanjšanje pridelka pri obeh sortah (ʻRefošk' in Scioppettino) in obeh gojitvenih oblikah (enojni in dvojni Guyot). Zmanjšanje pridelka se ni zgodilo zaradi manjšega števila grozdov, saj so imeli trsi okuženi z GFLV celo večje število grozdov, ampak na račun manjših grozdov in posledično

tudi manjših jagod. Vpliv na zmanjšanje pridelka pa je imelo tudi osipanje, ki je pogosto bolezensko znamenje pri okužbi z GFLV.

Okužba z GFLV ni imela statistično značilnega vpliva na vsebnost skupnih sladkorjev, kljub temu pa je bila količina skupnih sladkorjev višja v trsih okuženih z GFLV. Vpliv okužbe z GFLV na titracijske kisline, nismo zaznali, saj se je le ta spreminjala iz leta v leto, medtem ko je bila vrednost pH višja v trsih okuženi z GFLV.

Z metodo HPLC smo analizirali posamezne antociane v jagodnih kožicah. Okužba z GFLV vpliva na povišanje posameznih in skupnih antocianov v grozdju. Večji vpliv okužbe smo zaznali pri sorti 'Schioppettino', gojeni na gojitveni obliki enojni Guyot kot pa pri gojitveni obliki dvojni Guyot in pri sorti 'Refošk'. Vendar kljub temu je bila vsebnost antocianov tudi pri sorti 'Schioppettino' gojeni na gojitveni obliki dvojni Guyot in pri sorti 'Refošk' višja v grozdju iz trsov okuženih z GFLV.

Pri sorti 'Schioppettino', gojeni na gojitveni obliki enojni Guyot, so se pokazale statistično značilne razlike v vsebnosti antocianov med zdravimi in z GFLV okuženimi trtami, zato smo raziskave nadgradili z analizo vpliva okužbe z GFLV na izražanje genov, vključenih v metabolno pot antocianov. Okužba z GFLV vpliva na izražanje genov, ki vodijo v povečano sintezo delphinidin-3- glukozida, petunidin-3-glukozida in malvidin-3-glukozida. Sklepamo, da ima gen F3H1, kateri je bil bolj izražen v jagodnih kožicah trsov okuženih z GFLV, pomembno vlogo pri povečanju antocianov v kožicah, medtem ko imata gena F3´H in F3´5`H, pomembno vlogo pri spreminjanju razmerja med di- in tri- substituiranimi antociani.

Ob trgatvi smo iz ločeno pobranega pridelek zdravih in z GFLV okuženih trsov opravili mikrovinifikacijo, da bi analizirali vpliv okužbe z GFLV na kakovost vina. Okužba z GFLV je vplivala predvsem na skupni ekstrakt in alkohol. Vino narejeno iz grozdja trsov okuženih z GFLV ima višji skupni ekstrakt in nižjo stopnjo alkohola.

Ključne besede: Virus pahljačavosti vinske trte, GFLV, količina, kakovost, antociani, izražanje genov

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