UNIVERSITÀ DEGLI STUDI FIRENZE

Study of Rootstock-Scion Interactions in Grapevine

Ph.D. in AGRICULTURAL AND ENVIRONMENTAL SCIENCES

CYCLE XXXII

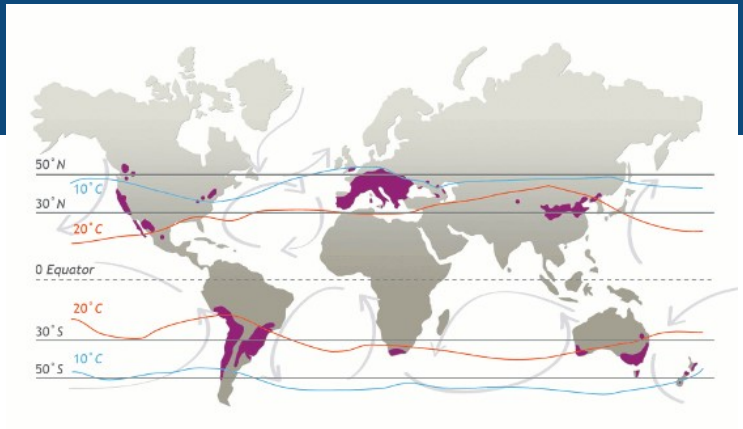
Ph.D. student: Alessandra Zombardo

Tutor: Prof. Giovan Battista Mattii

Final Discussion – Florence, 28-02-2020

Viticulture & Climate change

"Wine is proving to be a canary in the coalmine for climate change"
Goode, Nature 2012



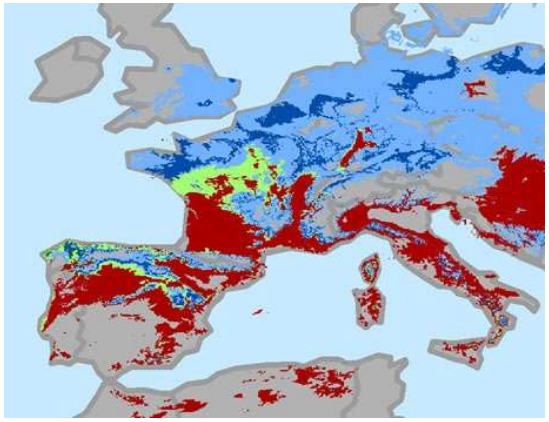
Wine districts: 30° - 50° North latitude, 30°- 50° South latitude

→ Crop very sensitive to deviations in climate,
need of specific temperatures for vegetative development, despite phenotypic plasticity

Suited areas: semi-arid climates, with possible events of drought and water deficit

Global warming: Viticulture expanded to new cool-climate districts,
problems for quality wine-producing countries!

→ up to 56% of current wine-growing lands may no longer be suitable for vineyards if the planet warms by 2°C (PNAS)


A snapshot of European viticulture in 2050. Red: drought areas; green: suitable areas; blue: new potential areas. Adapted from Hannah (*et al*., 2013).

«*Viticulture by its nature is complicated. As the world's climates are transformed, it is only becoming more so*»

The New York Times

The impact of climate change on grapevine

Severe multiple summer stresses: strong effects on grape quality
→ irregular yields, decoupling of technological and phenolic maturity, atypical aroma profiles…

Adaptive strategies:

1) Modifications of viticultural techniques

2) Modifications of plant materials:

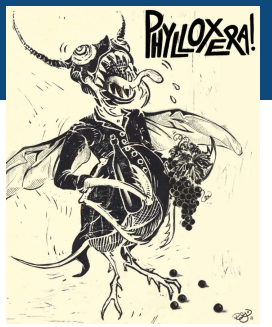
- grapevine CVs/clones
- Exploitation of **ROOTSTOCKS**

Example of climate change damage on cultivated grapevines

ROOTSTOCK: required against phylloxera

from 1863, France → vine epidemic, vineyard devastation in EU

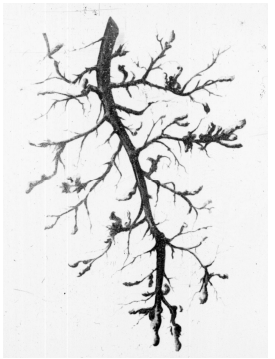
Daktulosphaira vitifoliae (US):
soil dwelling aphid, feeds solely on *Vitis* species



"The bug that almost ended wine"

- American *Vitis*: almost resistant, leaf lesions
- *V. vinifera* and Eurasian *Vitis*:

high radical sensitivity, formation of nodosities/tuberosities
Stop H_2O/mineral uptake, stop source/sink translocation
→ rapid decline of vigour leading to plant death

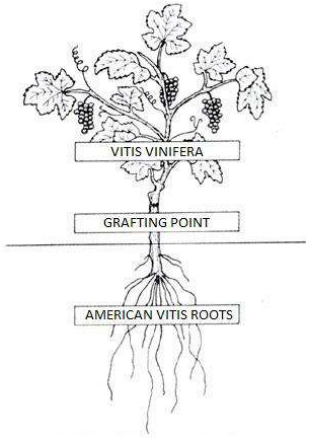


Symptoms of phylloxera attack on leaf or roots

Solution: GRAFTING!

Resistant root system (hybrids of American *Vitis*)
+
Shoot of *Vitis vinifera*

→ Composite vine: 2 genotypes merged – 1 final phenotype

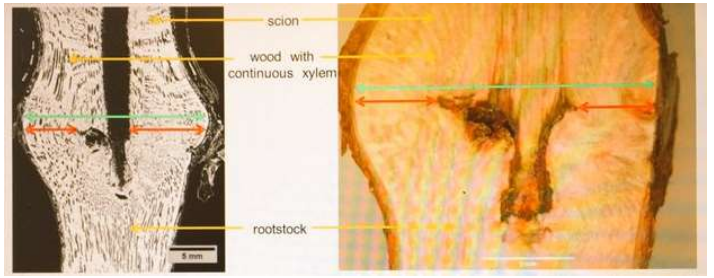


A composite vine

Rootstock-scion interaction

The rootstock can confer to the scion additional positive traits
→ enhanced tolerance to different kind of abiotic stresses (drought, water deficit)

GRAFTING: surgical union
welding of graft junction
reconnection of vascular system



Longitudinal sections and tomography views of Omega graft zones, Pinot gris on 110 Richter (Deloire *et a*l., 2019)

Transcriptomic reorganization:

→ differential expression of huge number of genes, effective through out the vine's life
→ grafting triggers defense and stress response mechanisms (phenylpropanoid pathway)
(Corso et al., 2015; Berdeja et al., 2015; Maré et al., 2016; Chitarra et al., 2017)

Molecular interaction networks: STILL LARGELY UNKNOWN
Few info about the influence on grape quality

Recent evidence:

- Macromolecules are mobile through graft union (mRNAs, miRNAs) (Yang *et al.*, 2015; Pagliarani *et al*., 2017)
- Phloem: pathway for the systemic translocation of macromolecules → long-rang movement, exchange of information between tissues (MarMarìn-Gonzales *et al.*, 2012)

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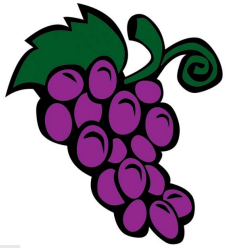
AIM OF THE PROJECT

Investigate the rootstock influence on grape quality
in conditions of optimal irrigation or water deficit
using an integrated molecular and biochemical approach



Research Labs:

CREA – Research Centre for Viticulture and Enology (Arezzo)

CREA – Research Centre for Genomics and Bioinformatics (Fiorenzuola d'Arda – PC)

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EXPERIMENTAL SYSTEM @CREA-VE Arezzo

set up to simulate the growing conditions of a real vineyard

→ advantages over open field trials

- Plastic pots filled by clay-loam texture soil (Chianti Classico district - Tuscany)
- Adult vines in the pots: cv *Pinot noir,* clone ENTAV 115
- Training: upward vertical shoot positioned trellis, spur cordon pruning
  Placement in rows, orientation N-S, outdoor area
- Irrigation: drip emitters, regulated water supply
- Experimental design: randomized blocks with 9 repetitions *per* each root system

ROOT SYSTEMS CONSIDERED

1) **P: 1103 Paulsen** (*Vitis berlandieri* x *V. rupestris* – Paulsen, 1865)
   high vigor, drought-tolerant

2) **M: M 101-14** (*V. riparia* x *V. rupestris* - Millardet and De Grasset, 1882)
   low vigor, drought-sensitive

3) **NGC**: not grafted vines (control)

Rootstock	Resistance to phylloxera	Limestone tolerance	Drought tolerance	Vigour	Stagnation tolerance	Salinity tolerance
1103 Paulsen	****	17%	****	****	***	***
Mgt 101-14	****	9%	**	**	***	***

Cosmo, 1958

Palliotti et al., La nuova viticoltura, 2015

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Ph.D. Research work built on:

RINGO

"**R**ootstock-scion **IN**teraction in **G**rapes: an **O**mics perspective"

Italian – Israeli bilateral project

2012-2013: TRANSCRIPTOMICS by Next-Generation Sequencing

→ Elaboration and implementation of already obtained data (qRT-PCR)
→ Enrichment of PHENOTYPING about grape quality

2018:
Same experimental protocol of 2017
+ *pre*-veraison water stress trial

2017:
Same conditions of RINGO
alongside molecular (qRT-PCR) and chemical analyses (HPLC)
→ accurate measurements on vine phenology, physiology, productivity

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Transcriptomic and biochemical investigations support the role of rootstock-scion interaction in grapevine berry quality

Zombardo A, Crosatti C, Bagnaresi P, Bassolino L, Reshef N, Puccioni S, Faccioli P, Tafuri A, Delledonne M, Fait A, Storchi P, Cattivelli L, Mica E

Research paper accepted for publication
on ***BMC Genomics***

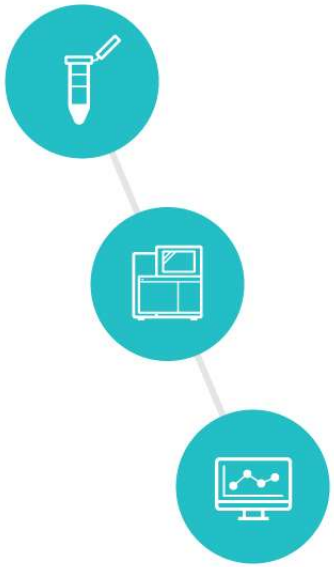
BMC Series

Methods

Pot system: vines maintained in equal agronomic conditions, with abundant water supply

2 growing season: 2012, WARMER (1450 GDDs - DOY 92-235)
2013, COOLER (1276 GDDs - DOY 92-236)

Berry sample collections: Veraison = **T1**
Maturity = **T2**



18 samples *per* each vegetative season
(3 root systems x 2 ripening times x 3 biological replicates)

Separation of berry skins

Analysis of transcriptional and biochemical scenario:

- RNA- and small RNA-seq analyses + gene expression by qRT-PCR
- Chemical analyses (HPLC) → accumulation of phenolic compounds

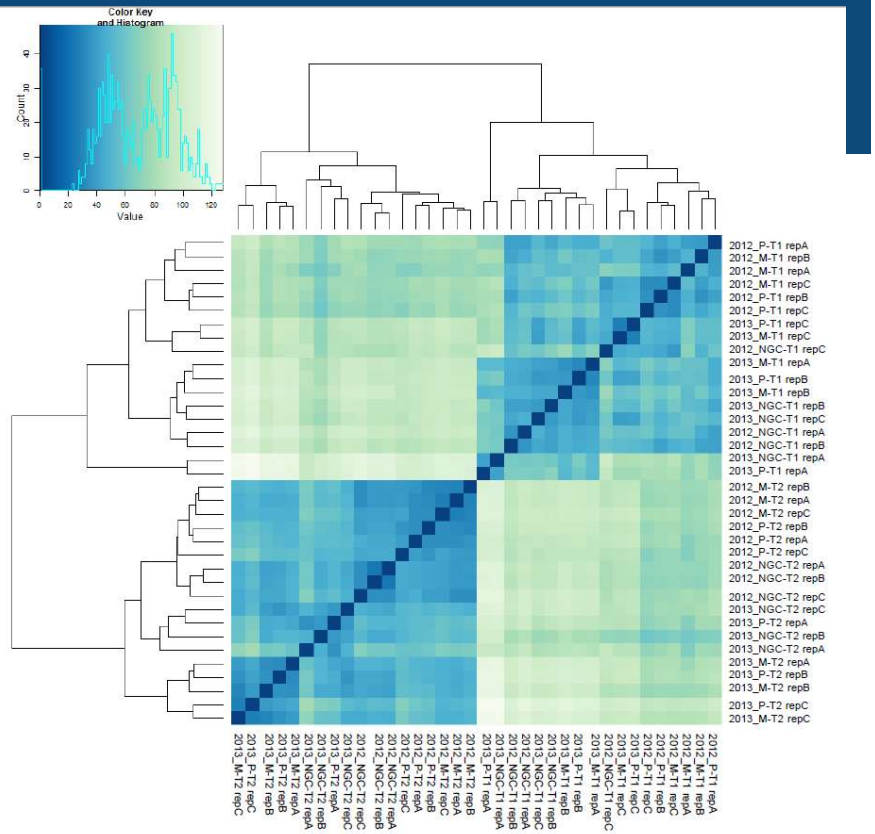
Illumina RNA-Sequencing

36 RNAseq libraries constructed with total RNAs
→ Quality filtered reads mapped
to *Vitis vinifera* 12x25 reference genome

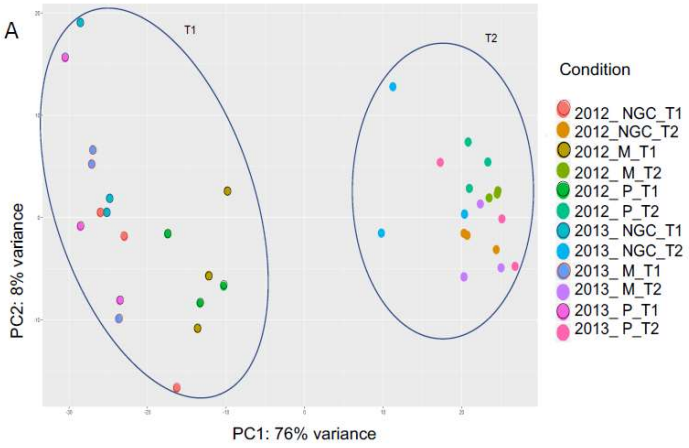
Sample correlation
Samples at T1 separated from samples at T2, independently from the year

Other results:
- Within each developmental group (T1 and T2): clear distinction 2012/2013

- T2: not grafted plants (NGC) grouped together, divided by grafted vines



HCA of the 36 samples sequenced by RNA-seq



PCA of the 36 samples in the RNA-seq dataset

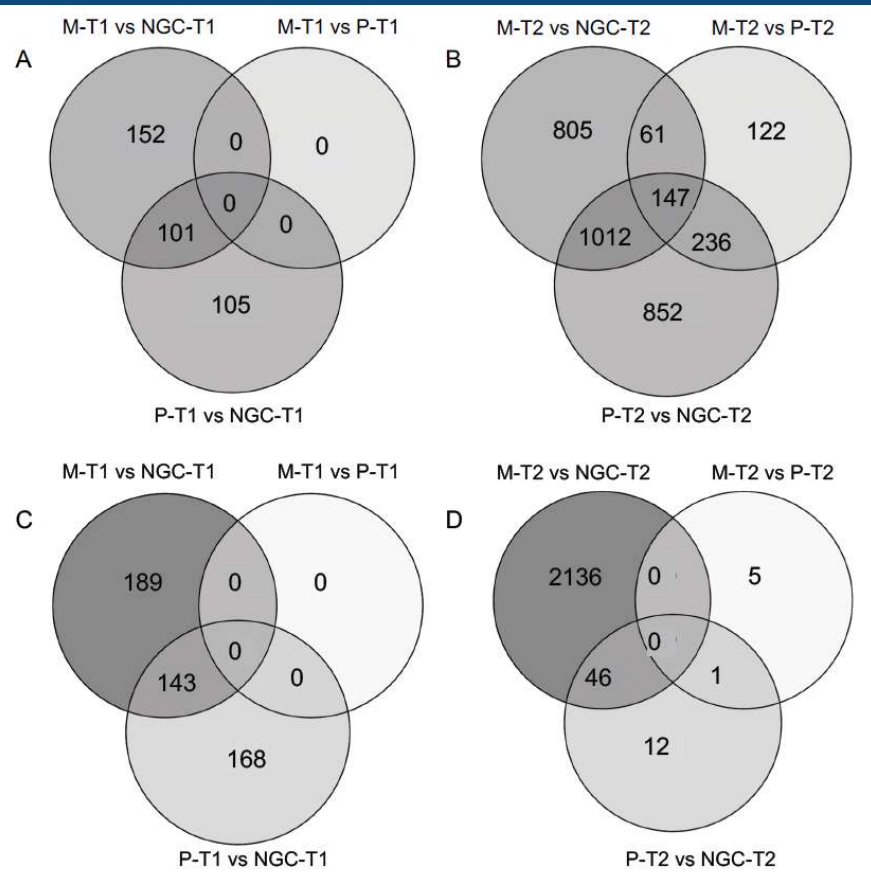
Differential expression analyses

DEGs in 6 comparisons: from 0 to 2247

Two major trends in both years:

- lower number at T1, higher at T2

- Grafted vines (M and P) were more similar to each other than to NGC vines



Venn diagrams of DEGs in the three root systems. A, B = 2012; C, D = 2013.

- **2012**

T1: most genes were up-regulated in NGC compared to M or P, no differences between grafted vines

T2: most genes were down-regulated in NGC compared to M or P. Among grafted, most genes were up-regulated in P compared to M → subsequent analyses on DEGs.

- **2013**

the variability among samples not sufficient to perform additional analyses on DEGs → less stressful environmental conditions?

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MapMan and GO enrichment analyses

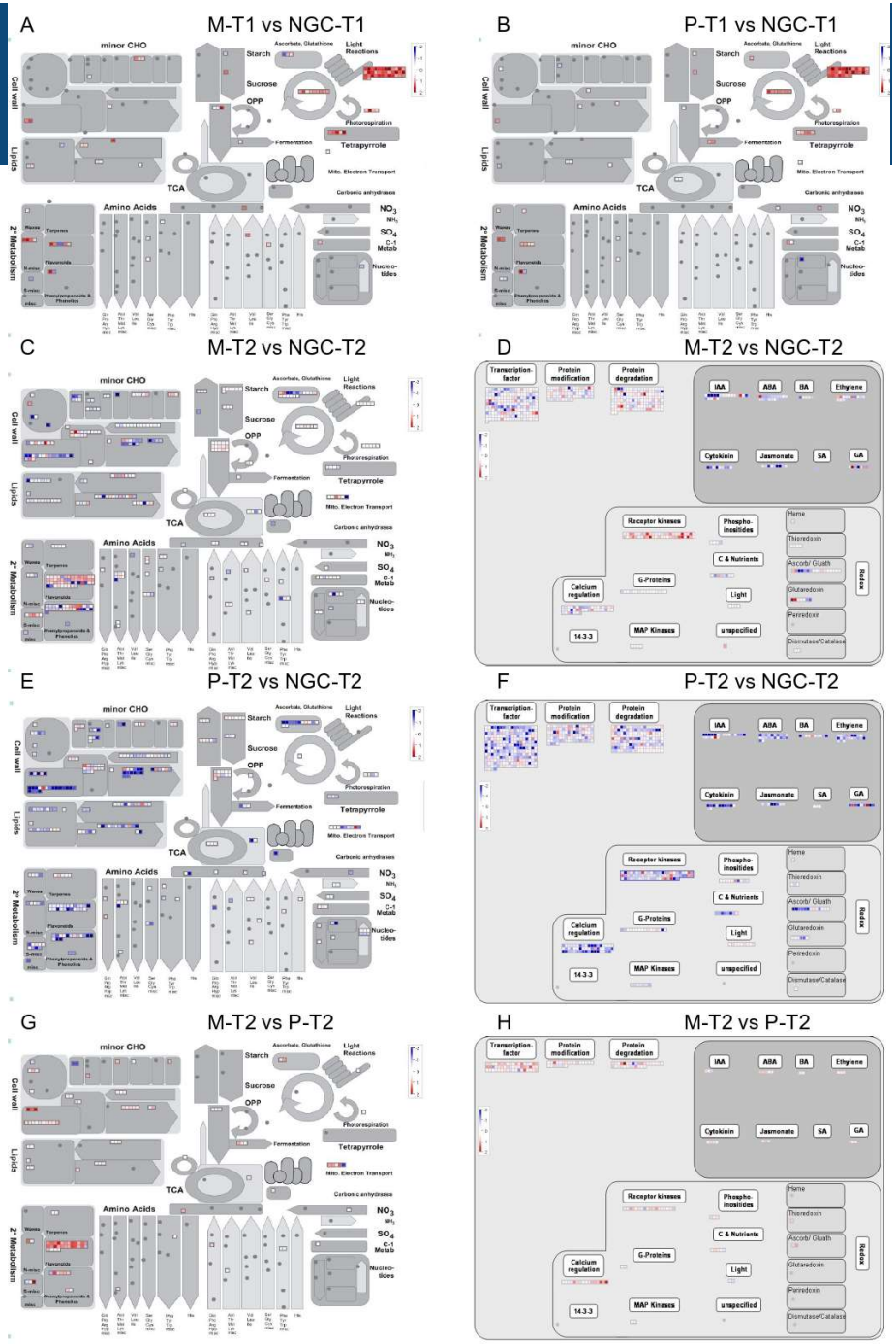
performed to evaluate metabolic pathways and cellular functions among DEGs

T1: DEGs mainly related to photosynthesis.
Most up-regulated genes in NGC

T2: DEGs related to secondary metabolism, stress response, hormonal regulation.
Most up-regulated genes in P

T2: DEGs related to TFs
(including many miRNA predicted targets)
Up-regulated in P

Results confirmed by GO terms!



Differences in the expression of genes involved in the cellular metabolism visualized by MapMan.

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Gene expression (qRT-PCR)

10 genes selected to validate RNA-seq data
→ involved in the phenylpropanoid pathway:
5 structural genes
(PAL, DFR, F3'H – 2 isoforms, FLS)
5 coding for transcription factors
(MYBC2-L3, MYB14, MYB4R1, NAC44, NAC60)

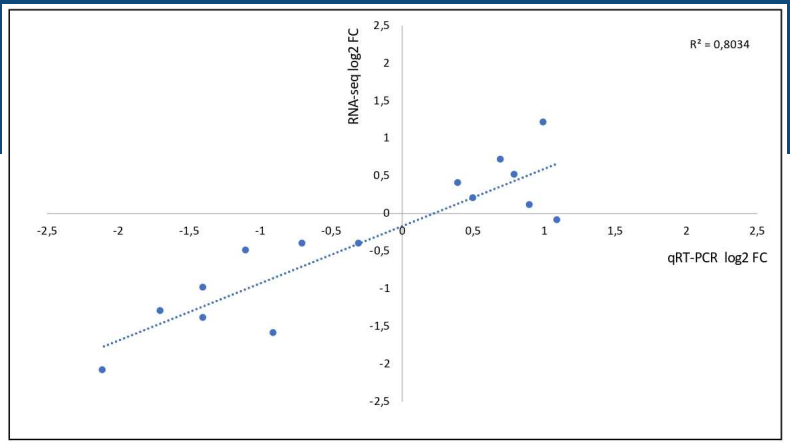
→ The fold change values confirmed RNA-seq results and technique

Interesting results:

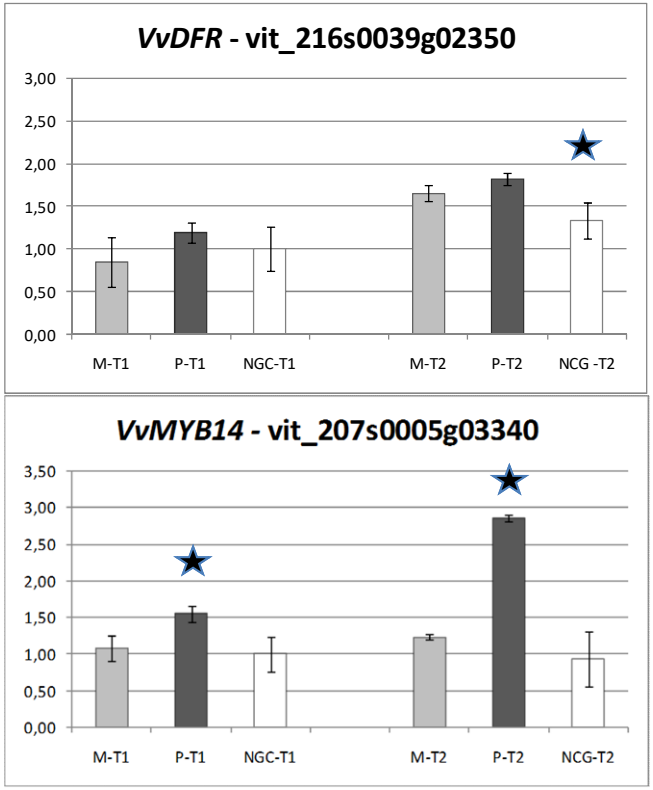
- DFR: more expressed in P and M at T2 → higher anthocyanins concentration in skins at maturity (accordance with HPLC results)

- MYB14: feedback regulation of resveratrol synthesis. Up-regulated in P at T2 (doubled): higher trans-piceid concentration in P

→ greater tolerance to drought?



Scatter Plot showing correlation between log2FC obtained via RNAseq (Y axis) and qRT-PCR (X axis) data.

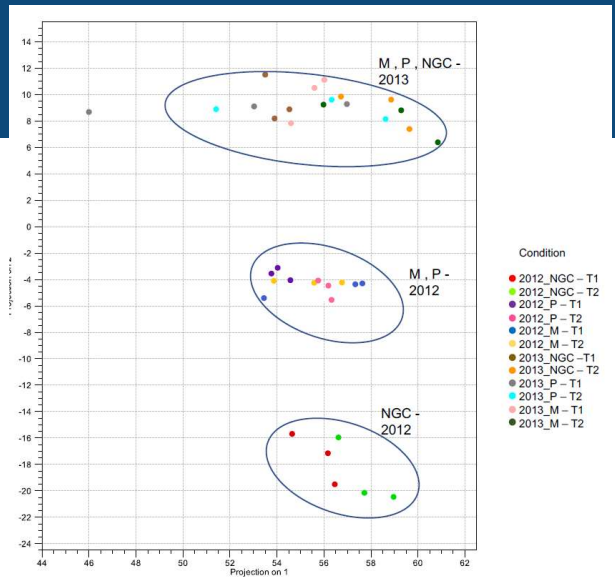


Expression profiles of DFR and MYB14 genes (qRT-PCR). Ct value with $2^{-\Delta\Delta Ct}$ method.

Illumina smallRNA-Sequencing

36 small RNA-seq libraries constructed from total RNAs

Library size distibutions: peaks 21 - 24 nt
→ lengths consistent with DICER derived products



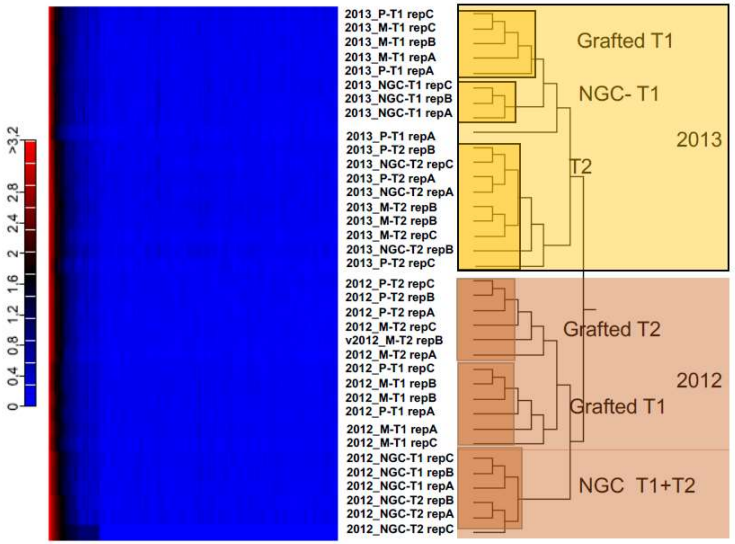
PCA of the 36 samples in the smallRNA-seq dataset

miRNA identification and analysis

Clean and trimmed reads compared to all plant species miRNAs deposited in miRBase
→ 159 (2012)/164(2013) annotated MIR families, all 48 grapevines MIR families retrieved

Correlation:
Samples primarily divided by the year effect

Moreover:
separation grafted/not grafted,
then separation T1/T2



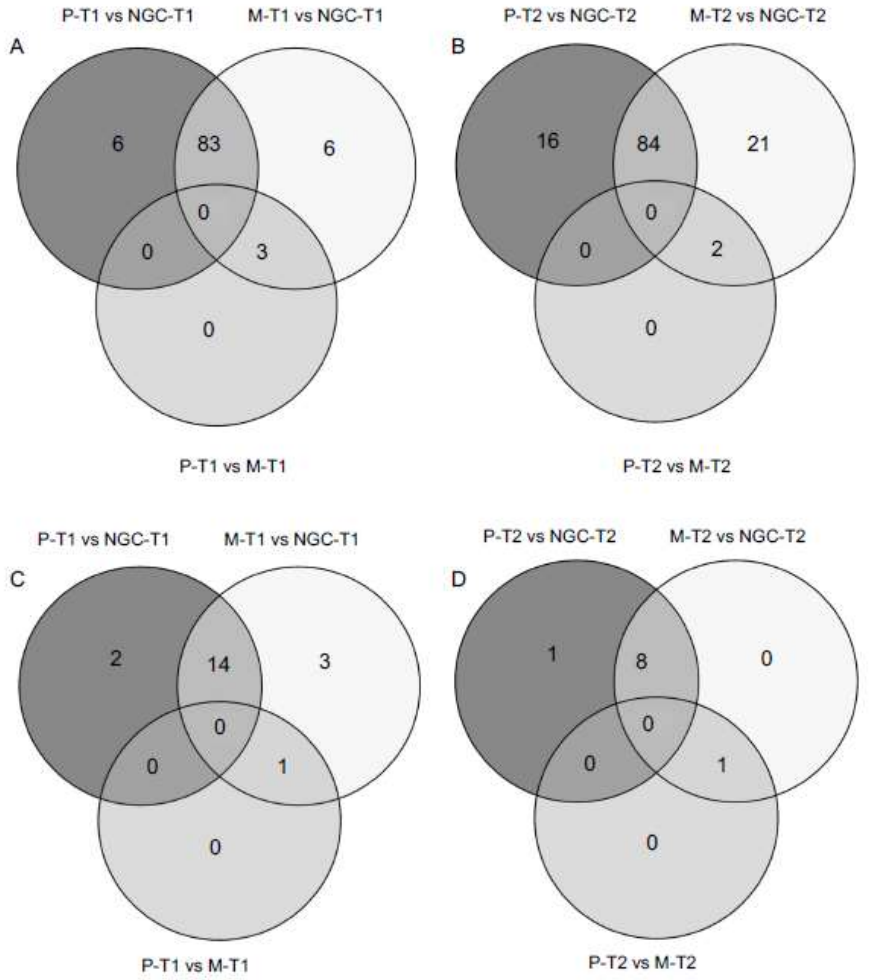
HCA of the 36 samples sequenced by smallRNA-seq

miRNA Differential Expression Analysis

2012:

- Strongest differences in grafted *vs* NGC
- Almost all DE miRNAs more expressed in NGC, both at T1/T2
- Few DE miRNAs between M and P

2013: Reduced number of DE miRNAs
→ less stressful environmental conditions?



Venn diagrams of DE miRNAs in the three root systems. A, B = 2012; C, D = 2013.

Small RNA in silico target identification

DE miRNA, both T1/T2 → miRNAs regulating secondary metabolism and stress response

- mir858: known to be master regulator of TFs

→ 34 MYB genes in grapevine berries predicted as targets
→ MYB174,MYB175,MYB13 identified as DE in M vs NGC and P vs NGC
→ opposite expression profiles of MYB target genes in RNA-seq and mir858
→ miR858 confirmed as negative regulators of MYB TFs expression

Journal of Experimental Botany, Vol. 70, No. 18 pp. 4775–4791, 2019
doi:10.1093/jxb/erz264 Advance Access Publication May 30, 2019
This paper is available online free of all access charges (see https://academic.oup.com/jxb/pages/openaccess for further details)

RESEARCH PAPER

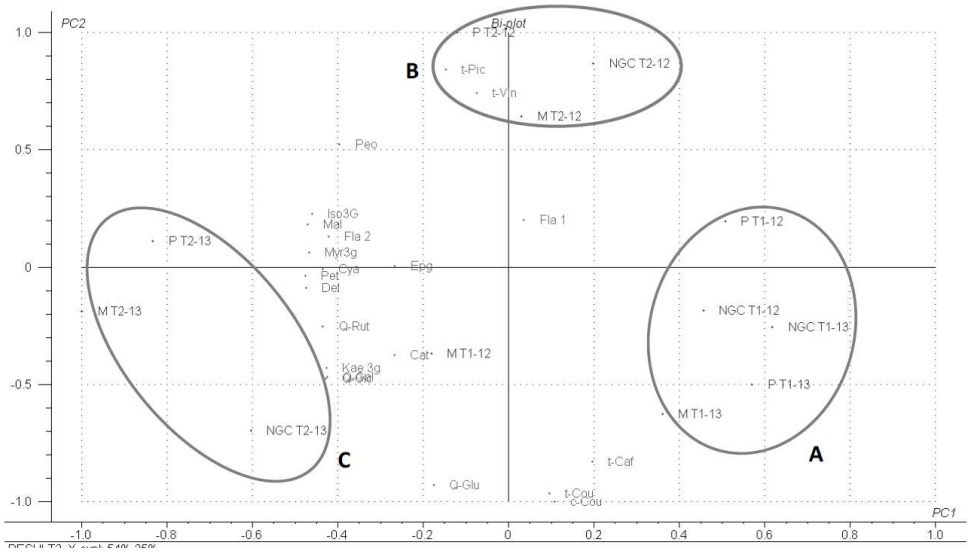
miR828 and miR858 regulate VvMYB114 to promote anthocyanin and flavonol accumulation in grapes

Varsha Tirumalai[1,2], Chenna Swetha[1,2], Ashwin Nair[1,2], Awadhesh Pandit[1], and Padubidri V. Shivaprasad[1,*]

qRT-PCR for some miRNAs: data of RNA-seq not confirmed.
Presence of isomiR (1-2 nt shorter) more expressed? Primers not able to distinguish!

Grape phenolic composition (HPLC)

- Main discriminating factor:
grape ripening stage → general phenolic composition very different at T1/T2
- Year effect: 2012/2013 separated at T2
- T2: grafted more similar compared to NGC



PCA of the 36 samples based on their chemical composition

Other results (2012, only - ANOVA):

- T1: higher diversity in the accumulation of several phenolic compounds between M, P, and NGC
- Anthocyanins: greater similarity between M and P → high concentration of anthocyanins (total and disubstituted) → up-regulation of DFR
- Resveratrol: trans-piceid detected as significantly different at T2 in P vines

→ up-regulation of MYB14

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Influenza del portinnesto sul metabolismo secondario di uve Pinot nero

Zombardo A, Mica E, Puccioni S, Bassolino L, Perria R, Mattii GB, Cattivelli L, Storchi P

Poster presented @Enoforum 2019 – Vicenza

Extended abstract published on www.infowine.com
Internet Journal of Viticulture and Enology, 2019:1/10




<u>GROWING SEASON 2017</u>: SAME CONDITIONS OF RINGO PROJECT

→ All the vines under optimal irrigation (midday stem Ψ above -1,0 MPa)

AIM: Check additional differences or confirm the previous results between the root systems considered

Results:

- No water stress or drought damage, despite hot vegetative season
- No differences in phenology, gas exchange, photosynthetic efficiency
- No differences in yield and technological maturity

→ Absence of limiting factors: no rootstock-effect on vine's primary metabolism

HPLC → some differences confirmed in the accumulation of secondary metabolites

- Anthocyanin profile: alterations due to the rootstock
  P → higher concentration of disubstituted anthocyanins
- Differences in other phenolic compounds:
  P → Higher concentration of trans-piceid (resveratrol)

qRT-PCR → 10 genes involved in secondary metabolism, DE mainly at maturity

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Berry quality of grapevine under water stress as affected by rootstock-scion interactions through gene expression regulation

Zombardo A, Mica E, Puccioni S, Perria R, Valentini P, Mattii GB, Cattivelli L, Storchi P

Research paper in preparation for publication on
Agronomy - Special Issue
"Tackling Grapevine Water Relations
in a Global Warming Scenario"

agronomy

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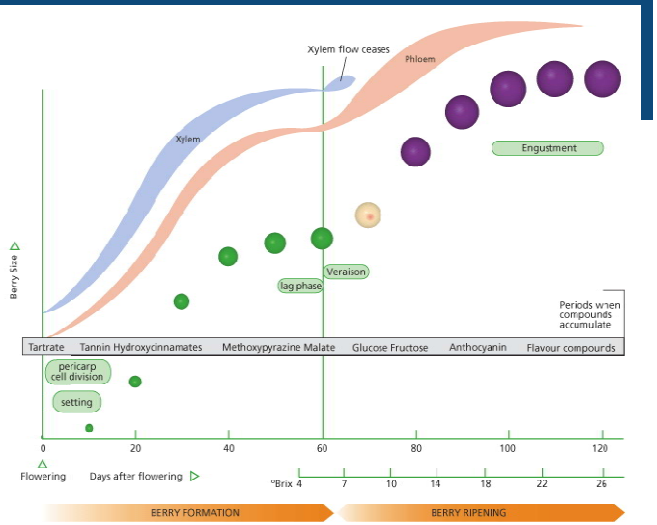
GROWING SEASON 2018

Pre-veraison water stress trial (DOY185 – DOY210)
Berry growth phase I

3 Irrigation Protocols:

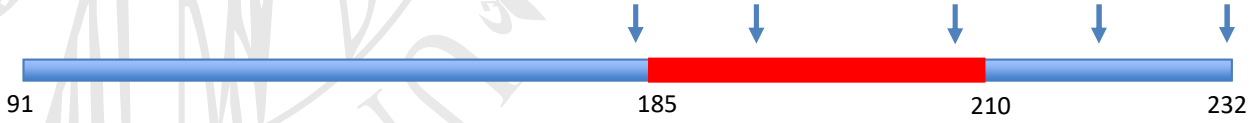
1) Severe Water Deficit (WS-1; 25% of field capacity)
2) Intermediate Water Deficit (WS-2; 40% of field capacity)
3) Control (WW; 90% of field capacity)



Graphic representation of the double sigmoid pattern of berry development (Coombe, 2001).

AIM: To test the rootstock influence on plant physiology and grape quality in the event of water shortage

- Measurements on grapevine physiology: leaf gas exchanges, chlorophyll fluorescence



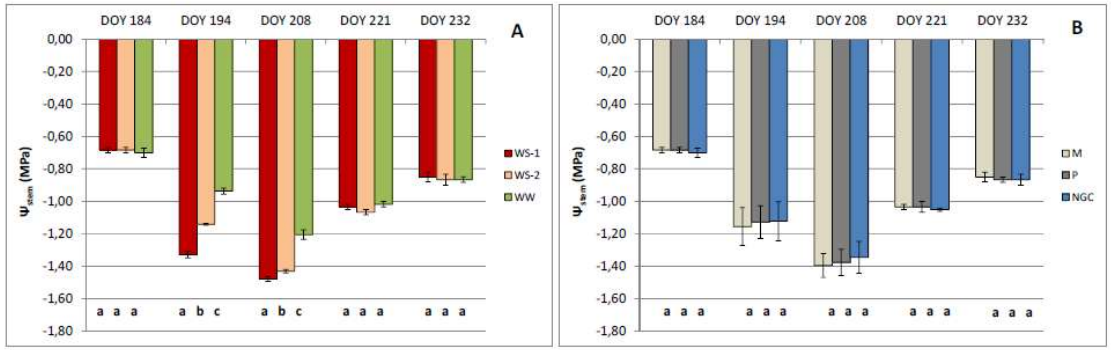
- Yield, Technological maturity, phenolic compound contents by HPLC
- Grape sampling for qRT-PCR analyses on genes and miRNAs (harvest): WS-1 e WW, only

Water status (midday Ψ stem)

WS-1 and WS-2 → water status alterations during the trial (DOY 194 – DOY 208), recovery when water supply restored

WW → more uniform values

Root systems: no rootstock influence



Leaf Water Potentials (Ψstem, MPa) in adult leaves

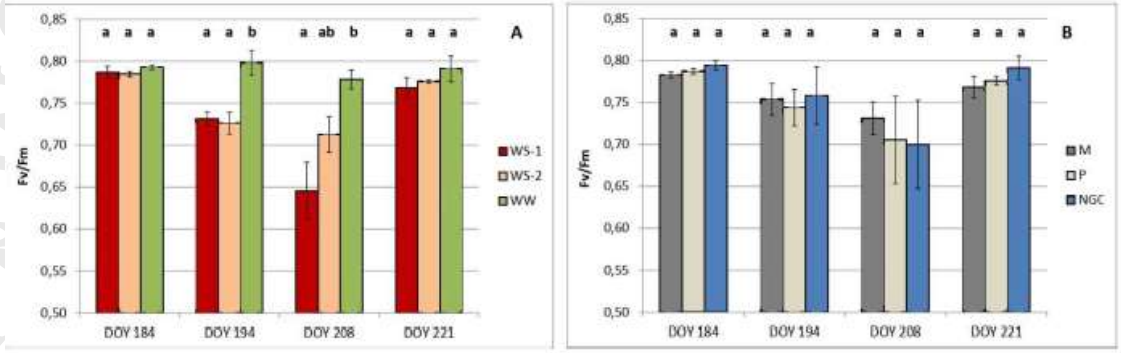
Photosynthetic efficiency (Fv/Fm)

Identical starting conditions (DOY 184)

During water stress: significantly lower in WS-1 and WS-2

After water stress (DOY 221): restored values, slightly lower in the vines that suffered from water deficit

Root systems: no significant differences



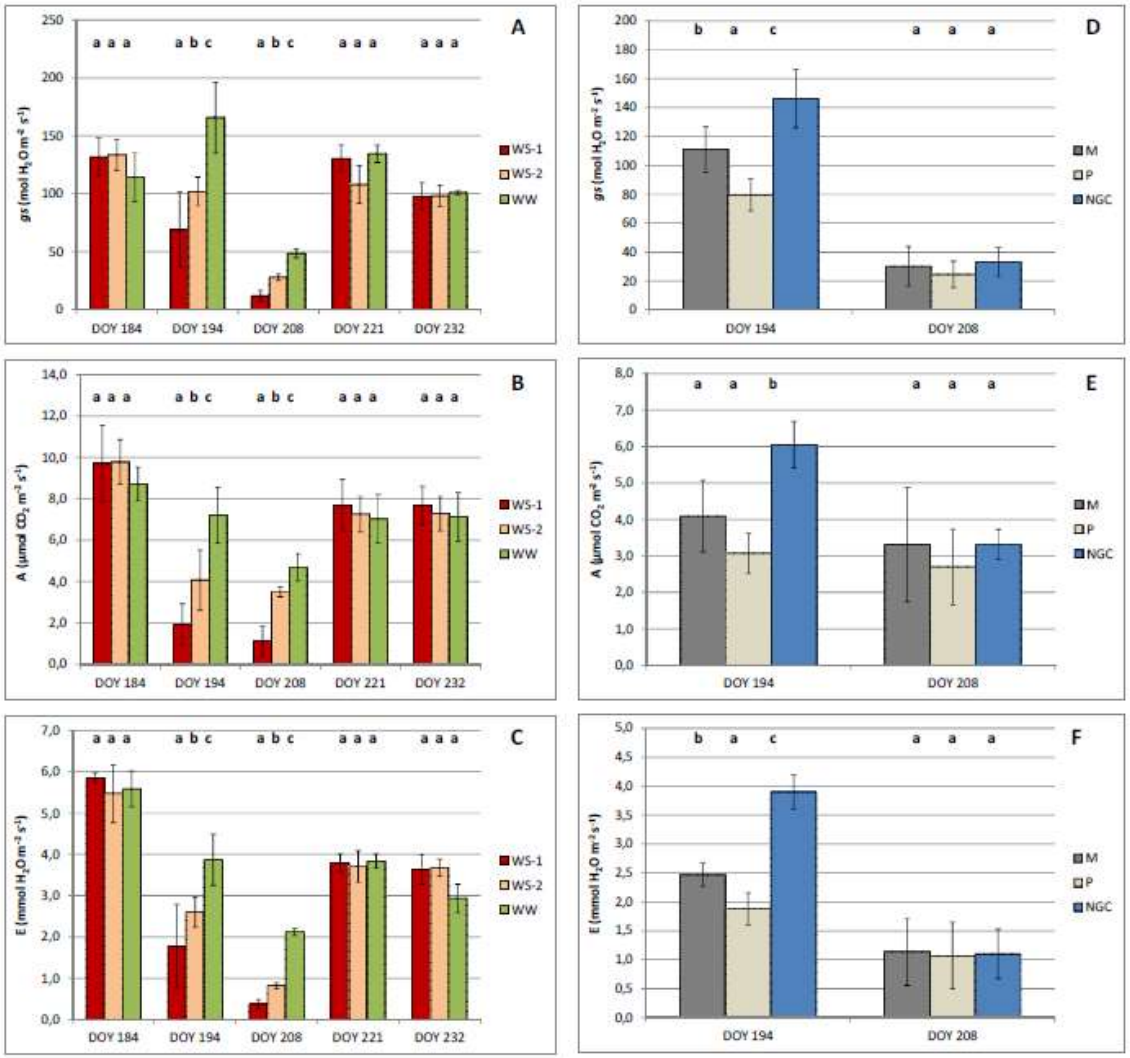
Chlorophyll fluorescence (Fv/Fm) in adult leaves

Leaf gas exchange

Before water stress (DOY 184):
similar behavior

During water stress:
(DOY 194 – DOY 208)
WS-1 and WS-2: sharp drop in stomatal conductance (gs), net photosynthesis (A), transpiration (E)

After water stress:
(DOY 221 – DOY 232)
The vines resumed their functionality, at a lower level than *pre*-stress conditions



Stomatal conductance (*gs*), Net Assimilation (A), Leaf Transpiration (E), in adult leaves

Root system:

- Statistically significant effect on gas exchanges only at the beginning of water stress trial

→ In general, NGC showed better performances

Yield and technological maturity assessment

Production traits:

- no alteration caused by water supply or root system

Technological maturity:

- The water supply significantly affected sugar content, titratable acidity, pH
- The root system did not affect the primary metabolism → previous results confirmed

	Yield *per* vine		Clusters *per* vine		Average cluster weight		pH		Titratable acidity		Sugars	
	g		n		g				g/L tartaric acid		° Brix	
Root system												
M	1028	a	12,4	a	79,31	a	3,21	a	5,69	a	20,7	a
P	872	a	10,3	a	74,95	a	3,19	a	5,73	a	20,2	a
NGC	715	a	9,1	a	75,04	a	3,17	a	5,62	a	21,0	a
Water protocol												
WS-1	943	a	11,4	a	79,59	a	3,30	c	5,35	a	22,4	c
WS-2	768	a	10,2	a	72,81	a	3,20	b	5,32	a	21,0	b
WW	904	a	10,2	a	76,89	a	3,07	a	6,38	b	18,5	a
Root system	ns		ns		ns		ns		ns		ns	
Water protocol	ns		ns		ns		***		***		***	
A x B	ns		ns		ns		ns		ns		ns	

Production parameters and technological analyses at harvest

Phenolic compound contents

Method: Di Stefano and Cravero (1991), total extracts of berry skin and seeds

→ Significant differences in total skin anthocyanins
→ Significant differences in total skin polyphenols
→ Significant differences in total seed polyphenols

Due to root system and water supply
→ Higher contents in case of water stress, and due to grafting on P rootsock

	Skin anthocyanins		Skin polyphenols		Seed polyphenols	
	mg/Kg grapes		mg/Kg grapes		mg/Kg grapes	
Root system						
M	871	a	1556	a	4117	a
P	1034	b	1753	b	4872	b
NGC	945	ab	1583	a	4188	a
Water Protocol						
WS-1	1146	c	1738	b	4782	b
WS-2	1002	b	1798	b	4431	b
WW	703	a	1357	a	3963	a
Root system	*		*		**	
Water protocol	***		***		**	
A x B	ns		ns		ns	

Berry phenolic compound contents at harvest.

Anthocyanin profiles

- Trisubstituted or disubstituted anthocyanins: significantly different contents

→ due to both water supply and root system

- WS-2/WW and P:

→ highest content in trisubstituted anthocyanins (higher malvidin-3-G)

- WS-1 and M/NGC:

→ highest content of disubstituted anthocyanins (higher cyanidin and peonidin-3-G)

	Delphindin		Cyanidin		Petunidin		Peonidin		Malvidin		Trisubstituted anthocyanins		Disubstituted anthocyanins		Trisubstituted Disubstituted Ratio	
	%		%		%		%		%		%		%			
Root system																
M	4,47	b	2,21	b	6,25	b	29,15	b	57,93	a	68,64	a	31,36	b	2,21	a
P	3,69	a	1,43	a	5,56	a	23,96	a	65,36	b	74,61	b	25,39	a	3,19	b
NGC	3,77	a	2,03	b	5,56	a	28,71	b	59,94	a	69,26	a	30,74	b	2,41	a
Water Protocol																
WS-1	3,61	a	1,92	a	5,40	a	30,24	b	58,83	a	67,84	a	32,16	b	2,20	a
WS-2	4,15	b	1,85	a	6,03	b	26,16	a	61,81	ab	71,99	b	28,01	a	2,74	ab
WW	4,15	b	1,90	a	5,94	b	25,42	a	62,59	b	72,68	b	27,32	a	2,87	b
Root system	*		***		*		*		*		*		*		*	
Water protocol	*		ns		*		*		*		*		*		*	
A x B	ns		ns		ns		ns		ns		ns		ns		ns	

Anthocyanin profiles of berry skins

HPLC analyses

- Flavonols (not shown): No significant differences
- Flavanols (not shown): Few differences, due to the root system only

→ M had the highest concentration of procyanidin B1 and epicatechin

- HCTA: Some differences, due to the root system only

→ M had the highest concentration of trans-caftaric acid and trans-fertaric acid

- Stilbenes: Significant differences due to both water supply and root system

→ WS-1 vines had the highest concentration of resveratrol and trans-ε-viniferin

→ M had the highest concentration trans-ε-viniferin

	Protocatechuic acid		Trans-caftaric acid		Cis-cutaric acid		Trans-cutaric acid		Trans-fertaric acid		Polydatin		Resveratrol		Trans-ε-viniferin	
	HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area	
Root system																
M	17,89	a	26,75	b	154,94	a	112,27	a	38,72	b	354,84	a	127,73	a	7,31	b
P	14,78	a	24,18	ab	112,91	a	94,27	a	28,89	ab	287,15	a	100,94	a	5,00	a
NGC	16,36	a	21,64	a	111,19	a	98,29	a	26,39	a	373,48	a	105,65	a	6,18	ab
Water Protocol																
WS-1	19,40	a	24,62	a	119,76	a	103,75	a	34,38	a	348,30	a	136,86	b	7,58	b
WS-2	16,10	a	23,69	a	124,01	a	98,39	a	28,42	a	320,27	a	85,96	a	5,15	a
WW	13,53	a	24,27	a	135,26	a	102,70	a	31,19	a	346,90	a	111,50	ab	5,76	ab
Root system	ns		*		ns		ns		*		ns		ns		*	
Water protocol	ns		ns		ns		ns		ns		ns		*		*	
A x B	ns		ns		ns		ns		ns		ns		ns		ns	

Phenolic compounds detected by HPLC in berry skins

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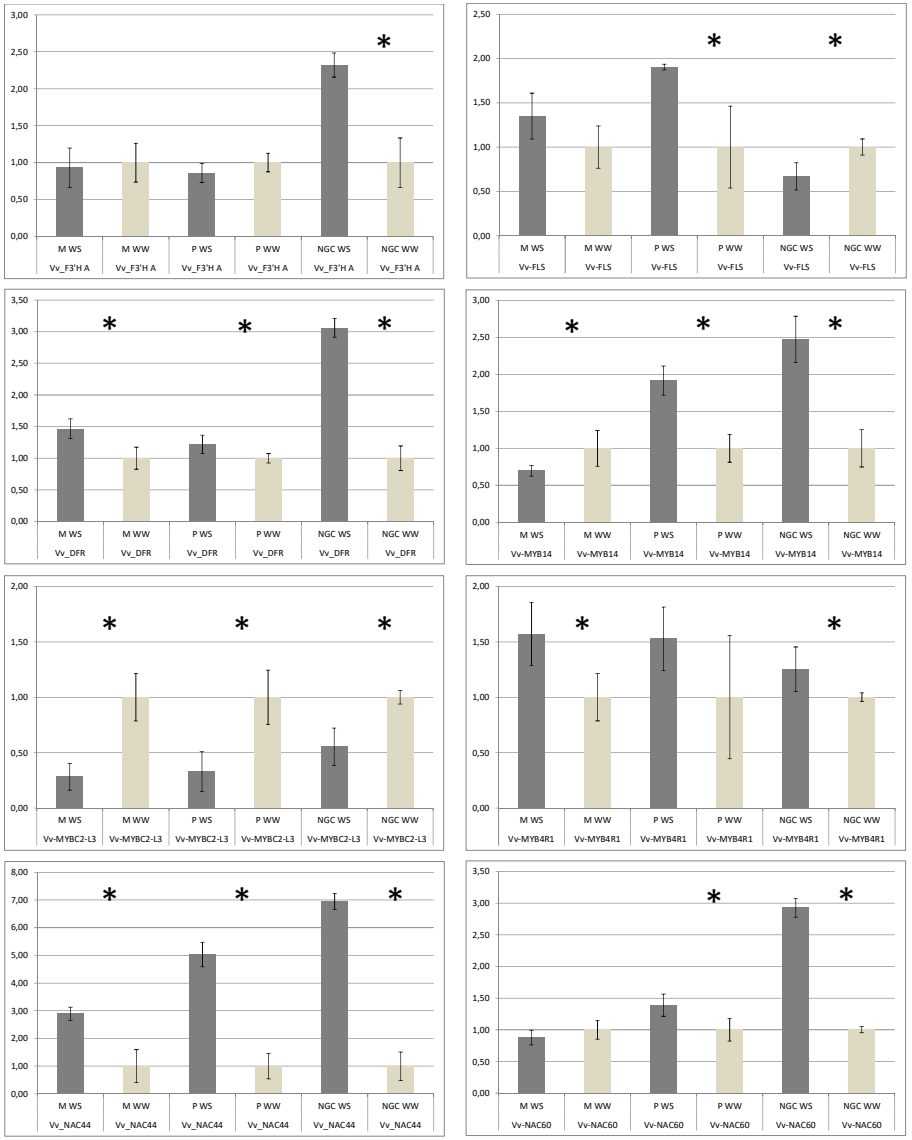
Gene expression (qRT-PCR)

only WS1 and WW at maturity
REFERENCE → WW in each root system

5 structural genes
- PAL: No differences
- F3'H (A): up-regulated in NGC-WS
- F3'H (B): No differences
- FLS: up-regulated in P-WS, NGC-WW
- DRF: WS always up-regulated

5 genes coding for TFs
- MYB 14: up-regulated in P-WS, NGC-WS, M-WW
- MYBC2-L3: WS always down-regulated
- MYB4R1: up-regulated in M-WS, NGC-WS
- NAC 44: WS always up-regulated
- NAC 60: up-regulated in P-WS, NGC-WS

→ application of early water stress caused lasting effects altering gene expression in berry skins at maturity



Expression profiles of 8 selected genes (qRT-PCR).
Ct value with $2^{-\Delta\Delta Ct}$ method.

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miRNA expression (qRT-PCR)

- **miR395:**

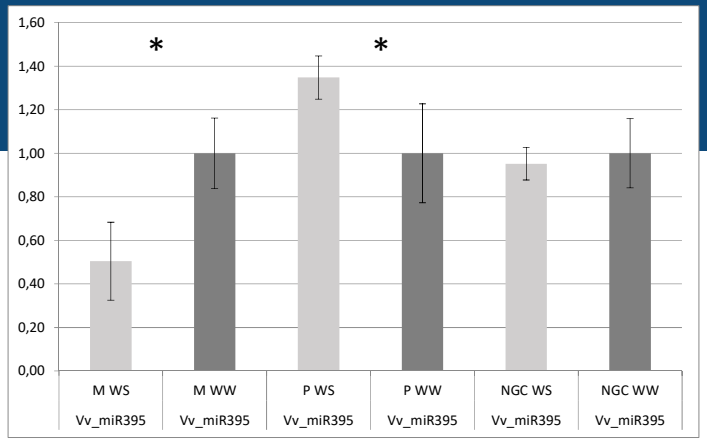
Up-regulated in P-WS, down-regulated in M-WS,
→ up-regulated in the presence of
drought stress in *Oryza sativa* (Zhou *et al.*, 2010)

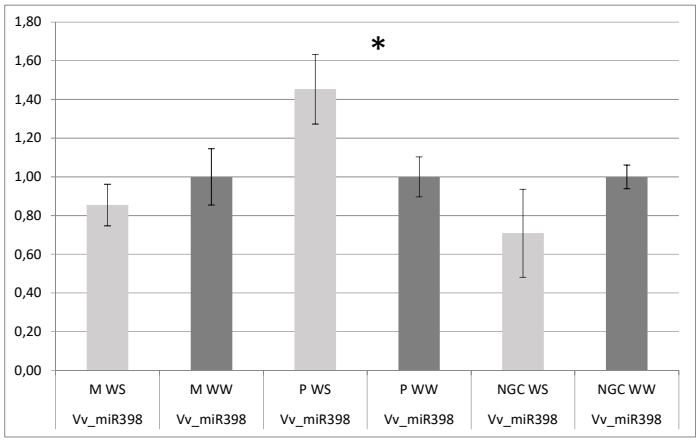
- **miR398:**

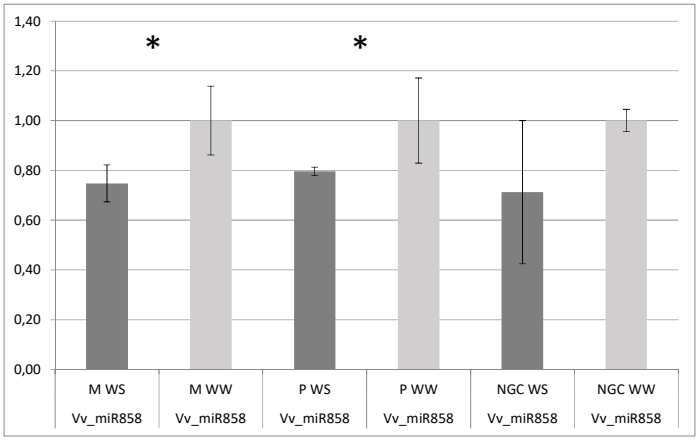
Up-regulated in P-WS,
Normally, down-regulated to dissipate
oxidative stress in plant tissues.
(Sunkar *et al.*, 2006; Zhu *et al.*, 2011)

- **miR858:**

Down-regulated in M-WS, P-WS
→ level of mRNAs coding for MYB TFs is
up-regulated WS grafted vines







Expression profiles of 3 selected miRNAs (qRT-PCR).
Ct value with $2^{-\Delta\Delta Ct}$ method.

GENERAL CONCLUSIONS:

- Some genetic determinants (both genes and miRNAs) involved in the phenylpropanoid pathway and stress response were identified as influenced by the rootstock

- Main effects on grape quality charged to the secondary metabolism

→ anthocyanins, stilbenes

→ more significantly modulated in the vines grafted on 1103 Paulsen

- Early water stress modulated the expression of genes and miRNAs involved in secondary metabolism

→ **further investigation is needed!**

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Thanks for your attention!

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SAVE WATER DRINK WINE