SUBSTRATE INFLUENCE ON FLAVONOID GENE EXPRESSIONS DURING EXPOSURE OF RED RASPBERRY PLANTS TO WATER DEFICIT

INFLUENȚA SUBSTRATULUI ASUPRA EXPRESIEI GENELOR SINTEZEI FLAVONOIZILOR IN TIMPUL EXPUNERII PLANTELOR DE ZMEUR LA DEFICITUL DE APA DIN SOL

EFROSE Rodica[1], CIOBOTARI Gh.[1], MORARIU Aliona[1], PASCU D.D.[1], BRÂNZĂ Maria[1], SFICHI-DUKE Liliana [1]
e-mail: lilianasfichi@gmail.com

Abstract. *Red raspberry is an economically important crop worldwide and represents an invaluable source of health-related compounds. Drought stress is one of the factors that greatly affect plant growth and agricultural productivity. The aim of the present study was to gain insights into the transcription profile of flavonoid-related genes in response to water-deficit stress. For this purpose, the expression of PALs, 4CLs and CHS genes in red raspberry plants grown in soil and in a soil: peat mixture was investigated. Transcriptional profiling was performed on leaf tissues collected from plants grown in semi-controlled greenhouse conditions and exposed to different soil water levels such as full water supply (90% FC), moderate (50% FC), and severe water stress (35% FC). Furthermore, we investigated whether the changes in gene expression were reflected on the metabolite level. Our data showed that transcript accumulation was affected by both water stress and substrate conditions.*
Key words: *raspberry, qRT-PCR, PAL, 4CL, CHS, water deficit, flavonoids.*

Rezumat. *Zmeurul este o specie importanta la nivel mondial din punct de vedere economic intrucat reprezintă o sursă bogata de compusi importanti pentru sanatatea umana. Seceta este unul dintre factorii cu efecte negative asupra creşterii plantelor şi productivitatii agricole. Scopul studiului de faţă a fost acela de a obţine informatii cu privire la schimbarile survenite în profilul de transcriere a genelor responsabile de sinteza flavonoizilor ca răspuns la stresul indus de deficitul de apă din sol. În acest scop, expresia genelor PALs, 4CLs şi CHS în culturi de zmeur cultivate în sol şi într-un amesteec de sol: turba a fost investigata. Profilul transcriptelor a fost efectuat pe tesuturi de frunze colectate de la plantele cultivate în condiţii semi-controlate de seră şi expuse la diferite grade de hidratare a substratului de crestere si anume hidratare optima (90% FC), hidrare moderată (50% FC), şi deficit hidric sever (35% FC). Mai mult, am investigat daca schimbarile in expresia genelor s-au reflectat in profilul fenilpropanilor. Datele obtinute au aratat ca atat acumularea transcriptelor cat si a metabolitilor respectivi a fost afectata atat de deficitul hidric cat si de caracteristicile substratului de crestere.*
Cuvinte cheie: *zmeur, qRT-PCR, PAL, 4CL, CHS, deficit hidric, flavonoizi*

[1] University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania

INTRODUCTION

The phenylpropanoid pathway leads to the synthesis of many important secondary metabolites such as lignins, flavanols and anthocyanins. Former studies have reported the accumulation of flavanols under different stress situations such as drought (Gansh et al., 2009). Drought stress is one of the factors that greatly affect plant growth and agricultural productivity. It induces changes in water status, concentrations of compatible solutes and other osmoprotectants, cell membranes, oxidative conditions, and in antioxidative metabolism (Tahkokorpi et al., 2007).

Raspberries (*Rubus idaeus* L.) are very rich sources of bioactive compounds such as phenolics, anthocyanins, organic acids, minerals, and more. It has been confirmed that the antioxidant ability of raspberry fruit is derived from the contribution of phenolic compounds in raspberries (Liu et al., 2002). However, the composition in bioactive compounds depends on the environmental conditions and genotype. In this study we aimed to investigate the effects of water deficit on phenylpropanoid genes and the accumulation of anthocyanins, flavanols and total phenolics in raspberry plants (Ruvi cv) grown in soil and a mixture of soil: peat.

MATERIAL AND METHOD

Experimental conditions. *Rubus ideaus* plants (Ruvi cultivar) were divided in two lots and grown under greenhouse environment for 3 weeks. One lot (control) was maintained by irrigation at 90%FC. The other lot was exposed to water stress by withholding water until the field capacity decreased to 35%. The pots were kept at the above drought stress levels by weighting. Control plants were watered daily. Plant leaves were collected from three biological replicates ground to a fine powder in liquid nitrogen and stored at -80^{o}C to preserve full-length RNA.

RNA isolation and quantification. Total RNA extraction and purification was performed with Spectrum Plant Total RNA kit. RNA quality was verified by Agilent Bioanalyzer analysis using an RNA 6000 Nano Labchip kit. To remove any trace of genomic DNA contamination, RNA samples were treated with DNase (Promega).

cDNA synthesis and qRT-PCR analysis. Equal aliquots of RNA-DNAse treated samples were reverse transcribed with SuperScript II Reverse Transcriptase kit (Invitrogen). The resulted first-strand cDNA was amplified using gene-specific primers for *Rubus idaeus spp*. listed in Table 1 (Efrose et al.,2012), using Primer Express 1.5 software (Applied Biosystems, Darmstadt, DE). Quantitative real-time PCR analysis was performed on the Rotor-Gene 6000 (Corbette) using MyTaqTMRedMix (Bioline).The temperature cycle used comprised 40 cycles at 95°C for 15 sec and 60°C for 1 min. Relative transcript levels of the gene of interest (X) were calculated using a modification of the comparative threshold cycle method, as a ratio to the histone H3 gene transcripts (U), as $(1+E)^{-\Delta Ct}$, where ΔCt was calculated as $(Ct_X – Ct_U)$. PCR efficiency (E) for each amplicon was calculated employing the linear regression method (Ramakers et al. 2003).

Determination of Total Phenolics. The content of total phenolics was determined by Folin-Ciocalteu method using gallic acid as a standard compound (Singleton et al., 1999).

Determination of Total Anthocyanins. Anthocyanin quantitation was performed in leaf samples by the pH differential method of Giusti and Wrolstad (2003). Values were expressed in terms of mg anthocyanin/100 g FW.

Determination of Flavonols. The total flavanol content was estimated using the p-dimethylaminocinnamaldehyde (DMACA) method as described by Arnous et al. (2002). Results were expressed as catechin equivalents (mgCTE/100 g FW). At least three analyses were run for each experimental category. Each analysis consisted of triplicate measurements of each sample and data were averaged over the three measurements.

RESULTS AND DISSCUSIONS

Expression levels of phenylpropanoid genes in red raspberry cultivar during progressive drought. The expression of six phenylpropanoid pathway genes (*pal1, pal2, chs, 4cl1, 4cl2* and *4cl3*) was investigated in leaves collected from *Rubus* plants exposed to different water deficit conditions.

Table 1.

Primers used for qRT-PCR analysis

Target genes	Gene-specific primers	
Phenylalanine ammonia-lyase1 (pal1)	pal1F	5'-TCGACAATGCCAGGATCGA-3'
	pal1R	5'-CAACGGATAAGACCTGCATTCC-3'
Phenylalanine ammonia-lyase2 (pal2)	pal2F	5'-ACCTCTTCCGATCTGCTAGCC-3'
	pal2R	5'-CGAAGTGGAATGGAATGACACA-3'
4-coumarate:coA ligase1 (4cl1)	4cl1F	5'-TGCTCGTCACCCATCCTAACA-3'
	4cl1R	5'-TCACGACAAATGCAACCGG-3'
4-coumarate:coA ligase2 (4cl2)	4cl2F	5'-CGGCTACTTTCCCAAATCGATA-3'
	4cl2R	5'-TCACCCCGGCCATTATAGAA-3'
4-coumarate:coA ligase3 (4cl3)	4cl3F	5'-TCCGCAAAAAGATGATGCTG-3'
	4cl3R	5'-GCTCATTGCCGCCATTAGAT-3'
chalcone synthase (chs)	chsF	5'-TCACAGTGTGGCAGCTTCAAC-3'
	chsR	5'-ACTGATCAAGGAGATCACCCAA-3'
histoneH3 (his)	hisF	5'-TTCCAGAGCCATGCAGTTTTG-3'
	hisR	5'-TGGCATGAATGGCACAGAGA-3'

Transcription profiling of the above mentioned genes was performed using relative quantification of target gene transcripts in comparison to the appropriate reference gene. The expression levels of the control gene, was used as internal standards to normalize small variations in cDNA template amounts. The relative transcript levels of the gene of interest were calculated as a ratio to the histone H3 gene transcripts (Fig.1). At 50% FC the transcript levels of flavonoid genes decreased on both substrates. The only gene upregulated by moderate water deficit was *pal1* in plants grown in soil:peat. With the exception of *chs* and *4cl1* the plants cultured in soil showed lower transcript levels than those cultured in soil:peat. When FC decreased at 35% the genes were upregulated and the transcript levels increased on both substrates. Among all genes, the expression of *pal2* in plants

cultured in soil and *pal2*, *4cl2* and *4cl3* in plants cultured in soil:peat showed the highest rate of transcript accumulation. However, under 35% FC, the plants cultured in soil accumulated higher levels of *chs* transcripts than plants grown in soil:peat (Fig.1). These results show that phenylpropanoid genes are upregulated by severe drought stress mainly when plants are grown in soil:peat although this subsstrate has an inhibitory effect on the accumulation of *chs*.

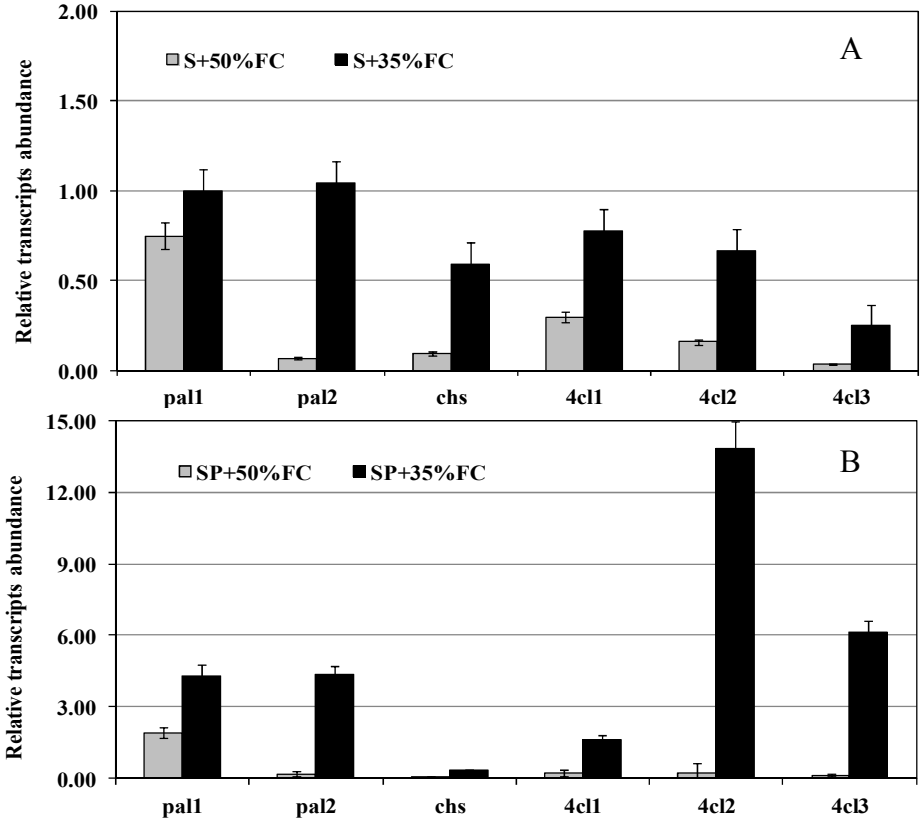

Fig. 1 - Expression levels of phenylpropanoid genes in red raspberry cultivar during progressive drought. Plants were grown in soil (A) or in a soil:peat mixture (B). Total RNA was isolated from leafs, treated with Dnase. reverse transcribed to cDNA, and subjected to real-time quantitative PCR. Transcript levels in the different samples were normalized to those of the reference gene, histone H3. Relative mRNA level was calculated with respect to the level of histone H3 transcripts. Values are given as the ratio between stress and control plants. Bars show means +SD (n = 3)

Changes in metabolites accumulation during progressive drought. Total phenolics accumulation was not influenced by growth substrate or moderate water stress. At 35% FC there was a decrease in the accumulation of total phenolics irrespective of substrate.

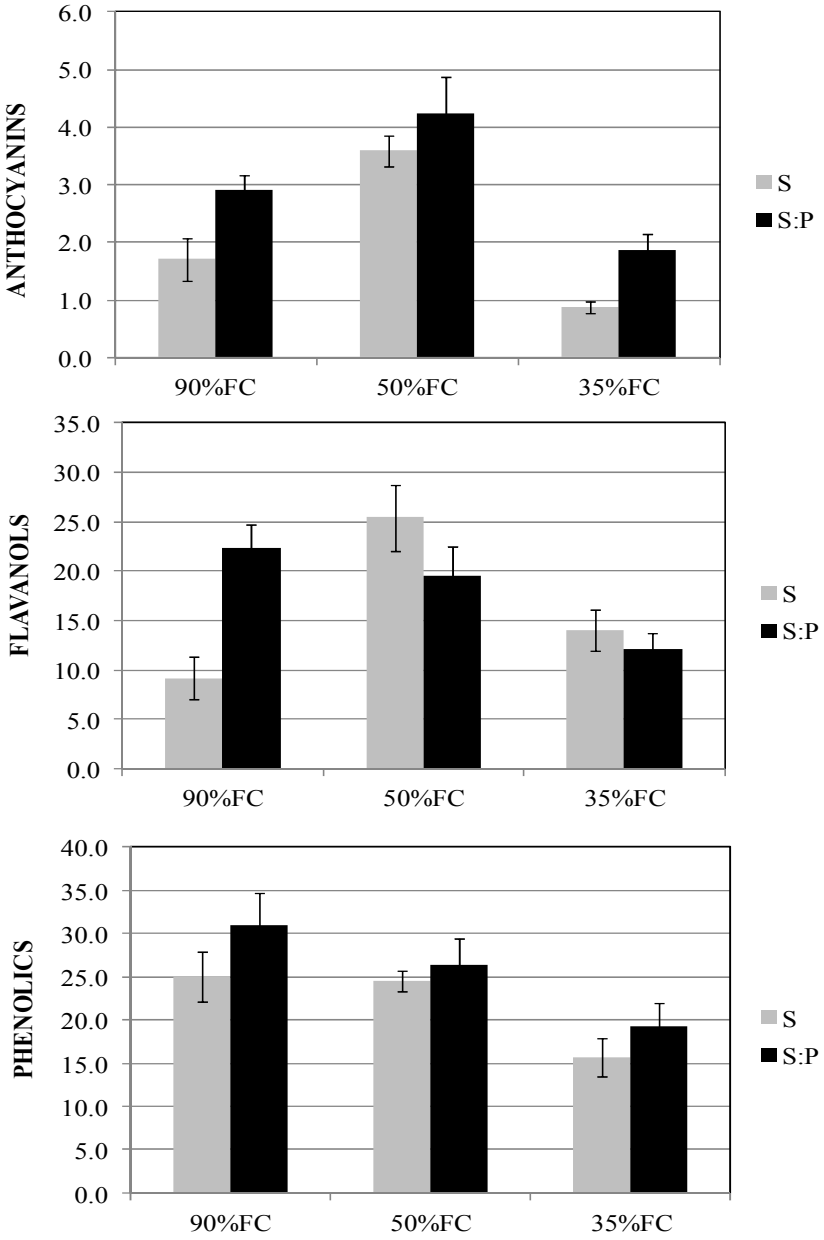

Fig. 2 - Changes in total phenolics, anthocyanins, and flavonols in red raspberry cultivar during progressive drought.

Anthocyanins increased under moderate drought but they decreased when FC reached 35%. In general, the amount of anthocyanins was higher in plants grown in soil: peat than in soil.The amount of flavanols increased at 50% FC only in plants cultured in soil. At 35% FC flavanol content decreased but in soil they

still were higher than at 90% FC. Interestingly, at 90% FC flavanol levels on soil:peat were higher than in soil (Fig. 2). The data obtained from the analysis of metabolite accumulation show that moderate drought stimulates the accumulation of anthocyanins and flavanols but severe water stress downregulates the production of phenylpropanoids even if the expression of phenylpropanoid pathway genes is upregulated.

CONCLUSIONS

Transcripts of phenilpropanoid genes were differentially expressed during progressive drought and were influenced by the growth substrate.

Moderate drought (50% FC) increased the transcription of *pal1*, whereas severe drought (35% FC) increased the transcription of pal2. *4cl* genes, mostly *4cl2* and *4cl3* were upregulated in plants grown in soil:peat mixture, under the influence of severe drought conditions while *chs* transcripts were more abundant in plants grown in soil.

The level of flavanols and anthocyanins increased at 50%FC but they decreased at 35% FC. Their accumulation was influenced by growth substrate being more abundant in plants grown in soil:peat.

***Acknowledgement**: The present contribution was supported by the EU-funding grant POSCCE-A2-O2.1.2-2009-2, I.D. 524, cod SMIS-CSNR 11986.*

REFERENCES

1. **Arnous A., Makris D. P., Kefalas P.**, **2002** - *Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece*. J. Food Compos. Analysis, 15, p. 655-65.
2. **Barrs H.D., Weatherley P.E.**, **1962** - *A re-examination of the relative turgidity technique for estimating water deficit in leaves.* Austr. J. Biol. Sci., 15, p. 413-428.
3. **Efrose R., Ciobotari G., Morariu A., Pascu D., Brinza M., Sfichi-Duke L.**, **2012** - *The use of qRT-PCR approach for comparative phenylpropanoid gene expression studies in two Rubus spp.,* The "Ion Ionescu de la Brad" Publishing House, Scientific works, Series Horticulture, p. 57-62.
4. **Gansch H., Weber C. A., Lee C. Y.**, **2009** - *Antioxidant capacity and phenolic phytochemicals in black raspberry.* NY Fruit Quarterly, 17, p. 20-23.
5. **Giusti M.M., Wrolstad R.E.**, **2003** - *Acylated anthocyanins from edible sources and their applications in food systems.* Biochem. Eng. J., 14, p. 217-225.
6. **Liu, M., Li X.Q., Weber C.A., Lee C.Y., Brown J., Liu R.H., 2002** - *Antioxidant and antiproliferative activities of raspberries.* J. Agric. Food Chem., 50, p. 2926-2930.
7. **Ramakers C., Ruijter J.M., Deprez R.H., Moorman A.F., 2003** - *Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data.* Neurosci. Lett. 339, p. 62–66.
8. **Singleton V.L., Orthofer R., Lamuela-Raventos R.M.**, **1999** - *Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent.* Methods Enzym., 299, p. 152–178.
9. **Tahkokorpi M., Taulavuori K., Laine K., Taulavuori E.**, **2007** - *After-effects of drought-related winter stress in previous and current year stems of Vaccinium myrtillus L.* Environ. Experim. Bot., 61, p. 85–93.