



THE INFLUENCE OF VIRAL INFECTIONS ON ANTIOXIDANT LEVELS IN THE GENETICALLY MODIFIED PLUM VARIETY “HONEY SWEET” (PRUNUS DOMESTICA L.)

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ABSTRACT

It is well-known that polyphenolic compounds are found abundantly in fruit, but various kinds of diseases lower these levels. This work measures total polyphenolic content, antioxidant activity and the levels of specific important antioxidants in fruits of the genetically modified (GM) plum variety HoneySweet, trees which were previously inoculated with a range of different virus infections. These were the Plum Pox virus (PPV), Prune Dwarf virus (PDV) and Apple Chlorotic Leaf-Spot virus (ACLSV). Uninoculated trees were used as controls. Antioxidant activity was measured using four different photometric methods – DPPH (2,2-diphenyl-1-picrylhydrazyl), DMPD (N-dimethyl-p-phenylenediamine), ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (Ferric reducing antioxidant power). Total polyphenol content was measured using the Folin–Ciocalteu method. The profiles of 10 specific antioxidant constituents in the fruits of the GM plum variety HoneySweet were detected and analyzed, since these are of interest for their role in human diets and could play a role in the resistance of plants to viruses. Detection was made using HPLC with UV-VIS detection. They were: gallic acid, p-coumaric acid, 4-aminobenzoic acid, chlorogenic acid, caffeic acid, ferulic acid, vanillin, rutin and quercetin. The compound with the highest concentration was chlorogenic acid (587 mg/100 g), and that with the lowest was p-coumaric acid (0.95 mg/100 g). Of the four methods of antioxidant activity used, in three the lowest levels of antioxidant activity were seen where the PPV virus was combined with ACLSV, and in three the highest levels were seen in the un-inoculated control without any infection. The highest values of total polyphenols were seen in the control (65.3 mg/100 g), followed by infection of PPV, then treatment PPV, PDV and ACLSV, then treatment PPV and PDV and finally the lowest levels were seen in treatment PPV and ACLSV (44.2 mg/100 g), which was also that with the lowest antioxidant activity.

Keywords: *Prunus domestica* L; viral infection; antioxidants

INTRODUCTION

The levels of antioxidants in fruit is dependent on many factors, but mainly on the species and the level of ripeness (Los et al., 2000). It is also influenced by the rootstock employed (Forcada et al., 2014) and the method used for processing fruits (Miletic et al., 2014). Many scientific studies have now been published analyzing the antioxidants in plums (Gao et al., 2014, Morabbi Najafabad & Jamei, 2014, Venter et al., 2014), and it has been established that plum extracts can have an inhibiting effect on cancers (Vizzotto et al., 2014). The anti-inflammatory and antioxidant properties of plums have also been studied (Popov et al., 2014). Other studies have shown them to have anti-hyperglycemic properties (Utsunomiya et al., 2005). However, very little has been written about the changes in levels of these important constituents of tree fruits growing under the influence of viral infections. "HoneySweet" is a plum variety developed through genetic engineering to be highly resistant to plum pox potyvirus (PPV) the causal agent of sharka disease that threatens stone-fruit industries world-wide, and most specifically in Europe

(Ravelonandro et al., 2013). Polak et al., 2012 have reported that after co-infections of PPV with PDV and/or ACLSV, there was practically no effect on the quantity and quality of HoneySweet fruits, which were large, sweet, and of a high eating quality. Fruit compositional studies are continuing in the USA and Europe since quality and nutrient composition is affected by the time of harvest and environmental factors that may vary within and between years. Nevertheless, the studies to date show that "Honey – Sweet" fruit are of high quality and nutritious (Capote et al., 2008).

However, the aim of this work was to highlight the changes in the levels of polyphenols and antioxidant activity which can occur in the GM plum HoneySweet following inoculation with various viral infections.

MATERIAL AND METHODOLOGY

Biological material

Abbreviation of viruses: ACLSV – Apple chlorotic Leaf Spot Virus, PPV – plum pox virus, PDV – Prune dwarf virus.

The GM plum variety HoneySweet was first inoculated with three viruses, PPV, ACLSV and PDV, and the fruits of *Prunus domestica* – transgenic plum variety HoneySweet, were harvested at the normal level of harvest ripeness in 2011 (Prag, Ruzyne).

There were four treatments using various combinations of the viruses, plus an untreated control group: I- PPV, PDV and ACLSV, II- PPV and PDV, III- PPV and ACLSV, IV- PPV alone, and V – the controls, which were not inoculated with any virus.

Preparation of samples

Representative samples (2 g) were taken from individual fruits, transferred to three bowls and homogenized with 8 ml water. Precise volumes of the homogenized samples were placed in test tubes and automatically agitated for 30 minutes and then centrifuged for 30 minutes at 16400 rpm·min⁻¹. The supernatant fluid was then removed by pipette and used for the individual analyses.

Assesment of antioxidant components by HPLC

For the determination of the HPLC profiles of the individual cultivars, high performance liquid chromatography (HPLC) with electrochemical and UV-VIS detection was used. The system consisted of two Model 582 ESA chromatographic pumps (ESA Inc., Chelmsford, MA, USA) with a working range from 0.001 to 9.999 mL min⁻¹ and a Zorbax SB C18 (150 × 4.6; size of particles 5 µm, Agilent Technologies, USA) reverse phase chromatographic column. For UV detection, a Model 528 ESA UV detector was used. A twelve-channel CoulArray detector (ESA) was used for electrochemical detection. Samples were injected automatically by an autosampler (Model 542, ESA), which has incorporated a thermostatic space for a column. This method is described in detail in article Zitka et al., 2011.

Determination of antioxidant activity

Spectrophotometric measurements of antioxidant activity were carried out using the BS-400 automated chemical analyser (Mindray, Shenzhencity, China). Transfer of samples and reagents was provided by a robotic arm equipped with a dosing needle (error of dosage not exceeding ±5 % of volume). Cuvette contents were mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples.

Determination of antioxidant activity by the DPPH test

This procedure for the determination was taken from publications by Sochor et al., 2010a. A 150 µL volume of reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl – DPPH•) was incubated with 15 µL of the sample. Absorbance was measured at 505 nm for 10 minutes.

Determination of antioxidant activity by the ABTS test

The procedure for the determination was taken from a publication by Sochor et al., 2010a. A 150 µL volume of reagent. Seven mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•) and 4.95 mM potassium peroxydisulphate was mixed with 3 µL of the sample. Absorbance was measured at 660 nm for 10 minutes.

Determination of antioxidant activity by the FRAP method

The procedure for this determination was taken from a paper by Sochor et al., 2010b. A 150 µL volume of reagent was injected into a plastic cuvette with subsequent addition of a 3 µL sample. Absorbance was measured at 605 nm for 10 minutes.

Determination of antioxidant activity by the DMPD method

Procedure for the determination was taken from a publication by Pohanka et al., 2012. A 160 µl volume of reagent is injected into a plastic cuvette with subsequent addition of 4 µl sample. Absorbance is measured at 505 nm for 10 minutes.

Determination of total polyphenol content

The total level of polyphenols was determined using the Folin-Ciocalteu method, in which a 0.5 ml sample is diluted with 1.5 ml ACS water and 0.05 ml of Folin-Ciocalteu reagent (Sigma Aldrich, USA) added. Absorbance was measured after 30 minutes (at 22°C) using a double-beam spectrophotometer SPEKOL 210 (Carl Zeiss Jena, Germany) with a wavelength $\lambda = 640$ nm. The results were expressed as gallic acid equivalents, in mg·kg⁻¹.

RESULTS AND DISCUSSION

When evaluating antioxidant status, antioxidant activity was evaluated using DPPH test, methods FRAP methods DMPD and ABTS. Resulting values of antioxidant activities were converted to 1 gram of protein.

The antioxidants were studied using 1) HPLC, 2) measures of antioxidant activity and 3) measurements of total polyphenol content.

Detection of certain specific antioxidant components using HPLC

Jaiswal et al., 2013 identified forty-one comprising by *Prunus salicina* and *prunus domestica*. Were identified: caffeoylquinic acids, feruloylquinic acid, p-coumaroylquinic acids, methyl caffeoylquinates, methyl p-coumaroylquininate, caffeoylshilcemic acids, catechin, epicatechin, rutin, esculin, quercetin, quercetin-3-O-hexosides, dimeric proanthocyanidins, trimeric proanthocyanidins, caffeoyl-glucoside, feruloyl-glucoside, p-coumaroyl-glucoside, vanillic acid-glucosides, 3,4-dihydroxybenzoyl-glucoside, quercetin-3-O-pentosides, quercetin-3-O-rhamnoside, quercetin-pentoside-rhamnosides, and 3-p-methoxycinnamoylquinic acid LC-MSn method.

The profiles of 10 specific antioxidant constituents in the fruits of the transgenic plum variety HoneySweet were detected and analyzed, since these are of interest for their role in human diets and could play a role in the resistance of plants to viruses. Detection was made using HPLC with UV-VIS detection. They were: gallic acid, p-coumaric acid, 4-aminobenzoic acid, chlorogenic acid, caffeic acid, ferulic acid, vanillin, rutin and quercetin

The compound with the highest concentration was chlorogenic acid (587 mg/100 g in fruit with virus combination PPV and PDV), and that with the lowest was p-coumaric acid (0.95 mg/100 g in fruit with virus PPV).

Table 1. Concentrations of selected polyphenolic compounds (mg·100g⁻¹). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

Treatment Number	1	2	3	4	5
Virus combinations	PPV, PDV ACLSV	PPV, PDV	PPV ACLSV	PPV	Control
Gallic acid	29.8 ±2.7	34.5 ±3.4	22.16 ±2.1	33.5 ±3.4	19.9 ±3.2
p-coumaric acid	1.25 ±0.19	3.46 ±0.22	2.59 ±0.15	0.95 ±0.07	1.45 ±0.12
4-aminobenzoic acid	1.91 ±0.07	2.56 ±0.22	3.58 ±0.26	2.75 ±0.18	1.56 ±0.13
Chlorogenic acid	356 ±9.87	587 ±14.2	113 ±2.32	112 ±8.2	376 ±9.1
Caffeic acid	9.54 ±0.65	7.69 ±0.87	5.65 ±0.28	6.87 ±0.85	4.39 ±0.41
Vanillin	7.65 ±0.88	5.97 ±0.59	6.42 ±0.29	3.87 ±0.19	5.39 ±0.25
Rutin	38.8 ±1.15	42.6 ±2.4	29.9 ±1.26	26.8 ±0.21	28.6 ±0.91
Ferulic acid	2.4 ±0.13	9.8 ±0.85	5.9 ±0.58	6.5 ±0.90	3.5 ±0.12
Quercetin	11.9 ±0.12	19.7 ±1.11	8.6 ±0.54	9.6 ±0.84	7.6 ±0.65

These results are in accord with **Donovan et al., 1998** and **Rop et al., (2009)** determined chlorogenic acids and proanthocyanidins were the major phenolics present in plums. On the other hand, **Miletic et al., 2013** noticed that major phenolic compound in fresh plums and prunes (cvs. "Valjevka" and "Mildora") is neochlorogenic acid, followed by caffeic acid and chlorogenic acid. **Piga et al., 2003** noticed lower value of chlorogenic acid content 58 mg/100 g DM. In our study we determined the higher level of gallic acid (19.87 mg/L - control variant) and 28.62 rutin (28.62 mg/L - control variant) in comparison with studies of **Miletic et al., 2013** (2.56 mg/100g; 5.25 mg/100 g).

The lowest levels of the antioxidants under investigation were found in the controls (with the exception of chlorogenic acid), and the highest were found in the variant inoculated with PPV and PDV. Concretely results are in Table 1.

Assessment of antioxidant activity

The methods for measuring antioxidant activity are usually based on the reactions caused by free radicals and then their inhibition by the compounds under investigation.

The advantages are their simplicity, stability, low cost and reliability. The results of all methods are expressed in gallic acid equivalents (GAE) to make comparisons easier. Since five fundamentally different methods were used to measure antioxidant activity, the results and conclusions can be seen with some confidence (**Sochor et al., 2011**).

In our study we have used four different methods for measuring antioxidant activity. Two based on the ability of the antioxidant to destroy synthetic radicals (tests DPPH and DMPD), the method FRAP, based on the reduction of ferric iron complexes to ferrous, and the method TEAC, based on assessing sample activity in terms of its gallic acid equivalent.

Of the four methods used, in three the lowest levels of antioxidant activity were seen where the PPV virus was combined with ACLSV (treatment 3), and in three the highest levels were seen in the un-inoculated control without any infection. The results from methods ABTS, FRAP and DPPH were strongly correlated: ABTS and DPPH r = 0.96, ABTS and FRAP r = 0.95.

Assessment of total polyphenol content

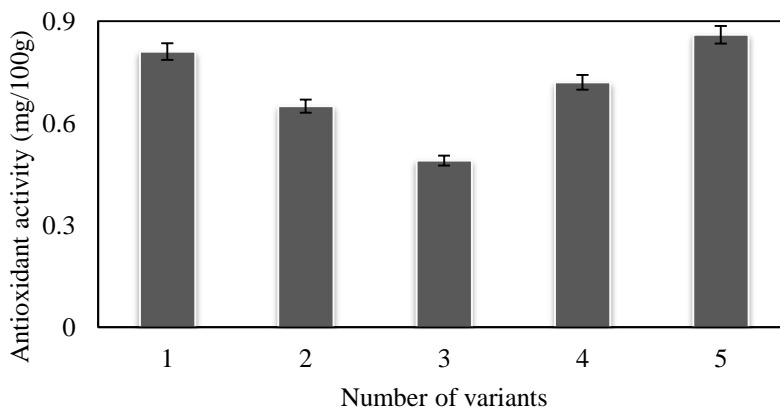


Figure 1. Antioxidant activity as measured by method DPPH and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

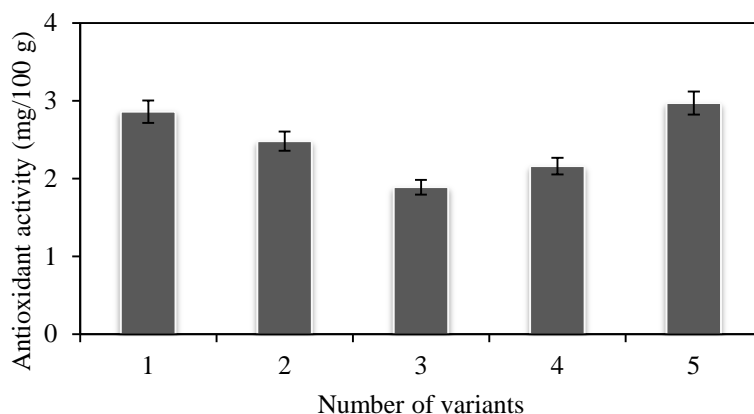


Figure 2. Antioxidant activity as measured by method ABTS and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

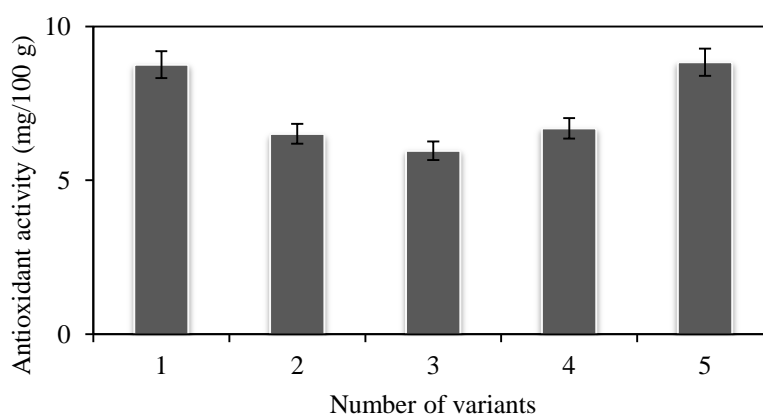


Figure 3. Antioxidant activity as measured by method FRAP and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

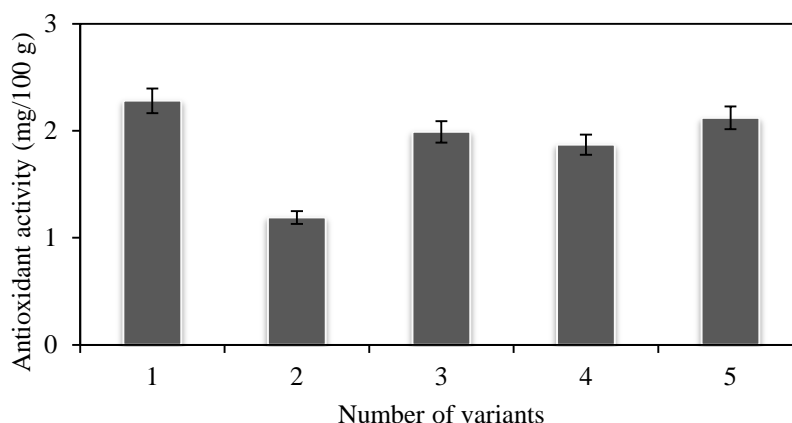


Figure 4. Antioxidant activity as measured by method DMPD and expressed in gallic acid equivalents (mg/100g). 1 – PPV, PDV and ACLSV, 2 – PPV and PDV, 3 – PPV and ACLSV, 4 – PPV alone, 5 – the controls.

Polyphenols are one of the most often measured and widely discussed groups of compounds in our diet today, and at the time of writing over 8000 have been identified. The fruits of the European plum *Prunus domestica* exhibit

a great diversity in appearance including skin colors. Their skin contains many polyphenolic compounds (Treutter et al., 2012).

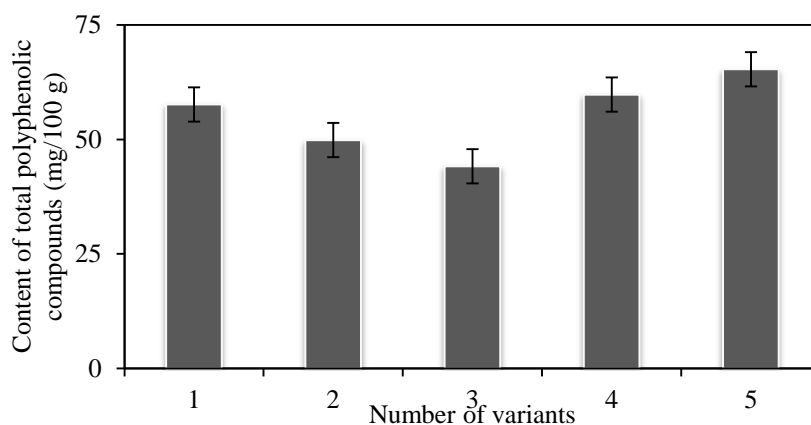


Figure 5. A comparison of total polyphenol content for each treatment.

Overall totals were measured using colorimetric methods using Folin-Ciocalteu reagent. The reagent does not only measure phenols, and will react with any reducing substance. It therefore measures the total reducing capacity of a sample, not just phenolic compounds. This method is widely used for its simplicity and reliability (Sanchez-Rangel et al., 2013).

The highest values were seen in the control (treatment 5), followed by treatment 4 (PPV), then treatment 1 with all three infections (PPV, PDV and ACLSV), then treatment 2 (PPV and PDV) and finally the lowest levels were seen in treatment 3 (PPV and ACLSV), which was also that with the lowest antioxidant activity.

In comparison, the total polyphenol content measured in plums by Luxembourg scientists showed the variety Kirk to have the highest levels, of $185 \text{ mg} \cdot 100 \text{ g}^{-1}$ (Kaulmann et al., 2014). In the USA fruit composition including study of total polyphenols content was evaluated comparing "HoneySweet" to a range of conventional plum cultivars). These analyses showed that "HoneySweet" fruit composition is generally in the range of the other plum cultivars tested (Ravelonandro et al., 2013). However, in the mentioned study HoneySweet reached up higher level of polyphenols $118 \text{ mg}/100 \text{ g}$.

CONCLUSION

It is evident from these results that the type of virus infection has an influence on the levels of antioxidants in the variety HoneySweet. The controls displayed the highest level of antioxidant activity and also had the highest total polyphenol content, from which it can be concluded that the virus infections were probably the cause of the reduced levels of antioxidants and polyphenols seen in the inoculated trees. This can only be confirmed by further studies, however.

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