# ANTIOXIDANT ACTIVITY AND PHENOLICS IN TOMATO LEAVES EXTRACTS INFECTED WITH LATE BLIGHT

**Marijana Peić Tukuljac[1], Dejan Prvulović[1], Slađana Medić-Pap[2], Dario Danojević[2]**

[1]*University of Novi Sad, Faculty of Agriculture, Trg D.Obradovića 8, 21000 Novi Sad, Serbia*
[2]*Institute of Field and Vegetable Crops Novi Sad, M. Gorkog 30, 21000 Novi Sad, Serbia*
*e-mail:marijanapeictukuljac@yahoo.com*

**Abstract**
Tomato is one of widely cultivated vegetable food crops in the world, because of its significant importance in human nutrition. Late blight is a serious tomato disease caused by *Phytophthora infestans*, which could lead to complete crop loss. The aim of this paper was to analyse the content of bioactive compounds in leaves of tomato genotype Bizon and their connection with late blight infection rate. It was found that at the beginning of late blight infection plant generated a high amount of polyphenols and flavonoids, but their concentration and antioxidant activity were reduced with the increasing rate of infection. Statistically significant differences ($p<0.01$) were observed between infection rate and sampling date as well as in their interaction on the measured biochemical parameters.

## Introduction

Plants are constantly exposed to many abiotic and biotic stress conditions such as high temperature, drought as well as the attack of the pathogens. In these stressful conditions, the production of reactive oxygen species (ROS) is increased and induces cellular damage. Despite their ability to cause harmful oxidation, in low concentration ROS are important signalling molecules. Different enzymatic and non-enzymatic defence mechanisms, are involved in ROS scavenging and prevention of oxidative stress [1,2]. Phenolic compounds are the most important non-enzymatic antioxidants. Flavonoids are a large group of natural phenolic compounds which are capable of chelating free radicals by donating a hydrogen atom or by single-electron transfer [3].

Tomato is one of the most important vegetable cultivated throughout the world that is consumed fresh as well as processed. It has a big influence in nutrition because provide a sufficient amount of dietary antioxidants and vitamin, especially vitamin A and C [4]. *Phytophthora infestans* is an oomycete which cause one of the most serious disease of tomato known as late blight (LB). This destructive disease is responsible for significant economical loses of tomato crops [5].

The goal of this paper was to investigate the effect of the late blight intensity in tomato leaves on antioxidant capacity, by measuring the content of total phenols and flavonoids, and by using six different antioxidant assays.

## Experimental

The material for this study was lower leaves of tomato genotype Bizon that was cultivated in the experimental field of the Institute of Field and Vegetable Crops, Novi Sad, Serbia, during 2014. Sowing for seedlings production in a glass house was done on 3$^{rd}$ of April and the plants were transplanted on 27$^{th}$ of May into the open field. Space between rows was 140 cm, while space between plants in the row was 50 cm. There was no fungicide application. The first sampling date was on 4$^{th}$ of August and the second was on 18$^{th}$ of August. Ten fully expanded lower leaves were taken from different plants and evaluation of infection rate was done according to the EPPO modified scale: 0-without infection, 1-less than 5% of leaf affected, 2- spots covering 5-10%, 3-spots covering 10-25%, 4- spots covering 25-50%, 5-spots covering more than 50% of the leaf.[6]

For biochemical analyses, plant material (200 mg) was milled to a fine powder and samples were prepared by extraction in 70% aqueous acetone solution (50 ml) during 24 h then centrifuged at 5000 rpm for 15 min. The supernatant was separated and kept in cold storage.
The total polyphenolic content (TP) was determined according to the Folin-Ciocalteu procedure [7]. Determination of total flavonoid content (TF) was carried out using a method based on the flavonoid characteristics to build metal-complexes with aluminium chloride ($AlCl_3$) [8]. The calibration curve was constructed with Quercetin and the results of TP and TF were expressed as catechin equivalents in mg per g of dry weight (mg QE/g DW). The total antioxidant activity (TAA) of extracts was determined according to the phosphomolybdenum method described by Kalaskar and Surana [9]. Using the method reported by Saha et al. [8] was assayed the total reduction capacity (TRC). Determination of DPPH radicals scavenging activity was measured as described by Lai and Lim [10]. Ferric reducing antioxidant power (FRAP) of extracts was estimated with the method developed by Valentão et al. [11]. The ABTS radical scavenging assay was performed according to the method by Miller et al. [12]. The calibration curve was constructed with a standard Trolox solution and the results of TAA, TRC, DPPH, FRAP and ABTS were expressed as Trolox equivalents in mg per g of dry weight (mg Trolox/g DW). The nitroblue tetrazolium (NBT) assay was used to determine the superoxide free radical scavenging activity [9] and the results were expressed as a percentage of inhibition of superoxide anion. All experimental measurements were performed in triplicate and the results of the total polyphenols, flavonoids and antioxidant activity assayed with the total antioxidant activity, total reduction capacity, FRAP, DPPH, ABTS and NBT test were expressed as the mean ± standard deviation (Table 1).
The data obtained were processed by applying the analysis of variance (Factorial ANOVA) using software STATISTICA ver. 13.2 (StatSoft, Inc., USA) and significant differences between the groups were determined using the Bonferroni test ($p<0.01$). The correlation coefficients were calculated according to Spearman.

**Results and discussion**

The content of polyphenols, flavonoids and antioxidant activity of tomato leaves extracts are presented in Table 1. Except for the FRAP test, two-way ANOVA suggested that the rate of infection, sampling date and interaction between these two factors have statistically significant ($p<0.01$) influence on all measured biochemical parameters.
In both sampling dates, the content of total phenols and flavonoids of analysed extracts was reduced with increasing of infection rate. *Post-hoc* Bonferroni test ($p<0.01$) for all assays showed the most pronounced difference between non-infected plants and plants attacked by late blight in the cases when LB' s infected area covering 25% and more than 50% of the leaf. At the beginning of infection, the amount of polyphenols and flavonoids is increased, since these secondary metabolites have a role in the plant response to abiotic and biotic stress [3].

Table 1. Polyphenolic compounds and antioxidant activity of tomato leaves extracts

| Parametar | Infection rate | TP[1] | TF[1] | TAA[2] | TRC[2] | DPPH[2] | ABTS[2] | FRAP[2] | NBT[3] |
|---|---|---|---|---|---|---|---|---|---|
| Sampling date 1 | 0 | $21.91^{ai} \pm 0.145$ | $21.37^{abcdf} \pm 0.298$ | $57.93^{abd} \pm 2.867$ | $6.96^{a} \pm 0.116$ | $2.04^{a} \pm 0,150$ | $5.44^{a} \pm 0,197$ | $3.45 \pm 0.062$ | $54.98^{ad} \pm 0.554$ |
| | 1 | $23.36^{a} \pm 0.417$ | $22,73^{abcd} \pm 0,428$ | $60.94^{ad} \pm 0.720$ | $8.27^{cd} \pm 0,072$ | $3.09^{c} \pm 0,196$ | $6.32^{c} \pm 0,181$ | $4.95 \pm 0.046$ | $52.99^{a} \pm 0.575$ |
| | 2 | $13.18^{bc} \pm 0.431$ | $5.85^{bdfg} \pm 0,150$ | $51.84^{de} \pm 0.735$ | $7.94^{ac} \pm 0.128$ | $1.59^{ad} \pm 0.225$ | $4.92^{ad} \pm 0.135$ | $2.57 \pm 0.050$ | $33.85^{ce} \pm 1.354$ |
| | 3 | $12.07^{cb} \pm 0.176$ | $5.77^{fg} \pm 0.348$ | $48.57^{de} \pm 0.960$ | $7.44^{ac} \pm 0.104$ | $1.57^{ad} \pm 0.149$ | $4.20^{d} \pm 0,379$ | $2.54 \pm 0.043$ | $30.25^{e} \pm 1.730$ |
| | 4 | $4.57^{d} \pm 0.053$ | $0.89^{g} \pm 0.030$ | $26.97^{c} \pm 1.732$ | $3.58^{f} \pm 0.492$ | $0.75^{d} \pm 0.202$ | $2.30^{b} \pm 0.478$ | $0.81 \pm 0.036$ | $10.98^{f} \pm 0.695$ |
| Sampling date 2 | 0 | $59.40^{e} \pm 1.357$ | $30.44^{ac} \pm 17.279$ | $55.53^{abd} \pm 0.910$ | $11.67^{be} \pm 0.487$ | $5.23^{b} \pm 0.227$ | $1.75^{b} \pm 0.041$ | $8.57 \pm 0.082$ | $62.7^{b} \pm 0.242$ |
| | 1 | $67.55^{f} \pm 0.421$ | $32.04^{cd} \pm 0.180$ | $59.19^{ad} \pm 1.600$ | $10.36^{be} \pm 0.275$ | $5.02^{b} \pm 0.362$ | $2.07^{b} \pm 0.090$ | $6.59 \pm 0.289$ | $60.52^{b} \pm 0.714$ |
| | 2 | $53.35^{g} \pm 2.108$ | $23.69^{ad} \pm 1.052$ | $54.79^{ae} \pm 1.342$ | $9.89^{e} \pm 0.355$ | $3.65^{c} \pm 0.223$ | $1.46^{be} \pm 0.069$ | $5.70 \pm 0.228$ | $56.71^{d} \pm 0.479$ |
| | 3 | $48.72^{h} \pm 0.926$ | $13.79^{aefg} \pm 2.057$ | $52.09^{be} \pm 4.362$ | $10.43^{be} \pm 0.666$ | $3.63^{c} \pm 0.427$ | $1.41^{be} \pm 0.021$ | $5.26 \pm 0.177$ | $52.36^{e} \pm 0.905$ |
| | 4 | $19.57^{i} \pm 0.728$ | $1.95^{fg} \pm 0.172$ | $25.89^{c} \pm 1.145$ | $9.50^{de} \pm 0.160$ | $0.774^{d} \pm 0.035$ | $0.78^{e} \pm 0.060$ | $1.31 \pm 0.129$ | $8.49^{f} \pm 1.566$ |

Value is a mean of three replicates ±standard deviation (SD)
[1]Expressed as mg Quercetin/g DW [2]Expresed as mg Trolox/g DW [3]Expresed as % inhibition
Value without the same superscript within each row differ significantly at $p< 0.01$ (Bonferroni post-hock test)
0-without infection, 1- infected area covering less than 5% of leaf, 2- infected area covering 5-10% of leaf, 3- infected area covering 10-25% of leaf, 4- infected area covering 25-50% of leaf , 5- infected area covering more than 50% of leaf.

This fact suggested that polyphenols and flavonoids are involved in the first response of plants to late blight attack. The results of this study are in agreement with report of other authors [12]. Taking into consideration that FRAP and ABTS tests are specific for flavonoids, and that FRAP did not show the statistically significant difference between infected plants, it is suggested that some other polyphenolic compounds, like terpenoids and flavonones ,may be involved in antioxidant protection mechanisms in tomato.[13] According to other authors, the amount of polyphenols increases during the early phases of infection because the plant is generating some precursors of lignin, which also belongs to this large group of molecules. Lignin is known as a physical barrier against initial pathogen colonization [5,14,15,16].
Spearman coefficient for all measured biochemical parameters showed strong negative correlation with LB infection rate, but the only correlation with TRC was not significant (Table 2). Except for the correlation between ABTS and TP as well as TF, all used tests were in strong positive and significant correlation.

**Table 2.** The Correlation between biochemical parameters and leaf infection intensity

| | TP[1] | TF[1] | TAA[2] | TRC[2] | DPPH[2] | ABTS[2] | FRAP[2] | NBT[3] |
|---|---|---|---|---|---|---|---|---|
| Infection rate | -0.61** | -0.76** | -0.85** | -0.27 | -0.68** | -0.46** | -0.71** | -0.85** |
| TP[1] | | 0.86** | 0.65** | 0.84** | 0.93** | -0.27 | 0.95** | 0.86** |
| TF[1] | | | 0.82** | 0.58** | 0.89** | 0.09 | 0.90** | 0.89** |

[1]Expressed as mg Quercetin/g DW, [2]Expresed as mg Trolox/g DW, [3] Expressed as % inhibition
**Statistically significant at $p < 0.01$ (Spearman correlation)

## Conclusion

The results of this study suggested that infected tomato plant in early phase of infection with *Phytophthora infestans,* generated a higher amount of polyphenols and flavonoids. These natural antioxidant substances are involved in earlier defence mechanism against the pathogen. The results of ABTS and FRAP indicate that some other polyphenolics compounds, apart from flavonoids, are involved in mechanisms of antioxidant protection of tomato.

## Acknowledgements

This research was a part of project TR 31030 "Developing vegetable varieties and hybrids for outdoor and indoor production" supported by the Ministry of Education, Science and Technological Development of Republic of Serbia.

## References

[1] B. Poljsak, D. Šuput, I. Milisav. Achieving the Balance between ROS and Antioxidants: When to Use the Synthetic Antioxidants. *Oxidative Medicine and Cellular Longevity*, 2013, 2013:11
[2] A. Krishnamurthy, B. Rathinasabapathi, Oxidative stress tolerance in plants: Novel interplay between auxin and reactive oxygen species signaling. *Plant Signaling & Behavior*, 2013;8(10):e25761
[3] D. Procházková, I. Bousová, N. Wilhelmová, Antioxidant and prooxidant properties of flavonoids, *Fitoterapia*, 2011;82(4):513-23.
[4] P. Adhikari, Y. Oh, D.R. Panthee, Current Status of Early Blight Resistance in Tomato: An Update.*International Journal of Molecular Sciences*, 2017; 18(10):2019

[5] M. Nowicki, M.R. Foolad, M Nowakowska, E.U. Kozik, Potato and Tomato Late Blight Caused by Phytophthora infestans : An Overview of Pathology and Resistance Breeding, *Plant Disease*, 2012, 96: 4-17.
[6] EPPO/OEPP Standards 2013. Efficacy evaluation of fungicides PP 1/65 (3), Downy mildew of lettuce and other vegetables, 83-87
[7] V Nagavani, Raghava Rao T, Evaluation of antioxidant potential and identification of polyphenols by RP-HPLC in *Micheliachampaca* flowers, *Advances in Biological Research*, 2010, 4 (3): 159-168
[8] A. Saha., R. Rahman., M. Shahriar., S. Saha., N. Al Azad., S. Das, Screening of six Ayurvedic medicinal plant extracts for antioxidant and cytotoxic activity, *Journal of Pharmacognosy and Phytochemistry*, 2013, 2 (2): 181-188
[9] M. Kalaskar, S. Surana, Free radical scavenging, immunomodulatory activity and chemical composition of *Luffa acutangula* var. *amara* (Cucurbitaceae) pericarp, *Journal of the Chilean Chemical Society*, 2014, 59(1): 2299-2302
[10] H.Y. Lai, Y.Y. Lim, Evaluation of antioxidant activities of the methanolic extracts of selected ferns in Malaysia, *International Journal of Environmental Science and Developmen*t, 2011, 2(6): 442-447
[11] P. Valentão, E. Fernandes, F. Carvalho, P.B. Andrade, R.M. Seabra, M.L. Bastos, Antioxidative properties of cardoon (*Cynara cardunculis* L.) infusion against superoxide radical, hydroxyl radical, and hypochlorus acid, *Journal of Agricultural and Food Chemistry*, 2002, 50 (17), 4989-4993
[12] D. Prvulović, S. Medić-Pap, D. Danojević, S. Tančić-Živanov, D. Latković: *Polyphenolic content and antioxidant capacity of tomato leaves extracts infected with late blight*, XXIII savetovanje o biotehnologiji sa međunarodnim učešćem, Book of abstracts, 2018, 333-338
[13] M. Henriquez , L. Adam, F. Daayf, Alteration of secondary metabolites' profiles in potato leaves in response to weakly and highly aggressive isolates of *Phytophthora infestans*, *Plant Physiology and Biochemistry*,2012, 57: 8-14
[14] N. Miller, C. Rice-Evans, M. Davies, V. Gopinathan, A. Milner, A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates, *Clinical Science*, 1993, 84 (4): 407-412
[15] K. Kulbat, The role of phenolic compounds in plant resistance, *Biotechnology and Food Sciences*, 2016, 80(2): 97-108
[16] E. Miedes, R.Vanholme, W. Boerjan, A. Molina, The role of secondary cell wall in plant resistance to pathogens, *Frontiers in Plant Science*, 2014, 5: 358