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Influence of stress metabolite stilbenoids of Shavkapito vine trunk (*Vitis vinifera* L.) on the activity of Crown gall agent Agrobacterium *tumefaciens in vitro condition*

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A B S T R A C T

It was investigated the impact of stress metabolite stilbenoids of Shavkapito vine trunk (*Vitis vinifera* L.) on the activity of crown gall agent *Agrobacterium tumefaciens "in vitro"* condition. Health and infected vine trunks were brought from the east part of Georgia (village Mukhrani) on the Eutric cambisols soil of 15 years old vineyard. It was isolated strong, medium and weak bacterial strains from the naturally diseased with Agrobacterium tumefaciens Shavkapito trunk. It was established stress-metabolite stilbenoids from the healthy and infected trunks by HPLC analyze. Main stilbenoids turned out trans-resveratrol and trans-ε-viniferin. It was investigated inhibitory impact of trans-resveratrol, trans-e-viniferin and stilbenoids total preparation on strong, medium and weak bacterial strains. It occurred that stilbenoids inhibitory impact depends on concentration and detect differently according to the strains strength.

Key words: vine, stilbenoids, Shavkapito, crown gall

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Introduction

The most important physiological role of vine stilbenoids are phytoalexin treatment. According to previous studies developed by the Institute of Viticulture and Enology of the Agricultural University of Georgia, the vine stilbenoids are involved for responses toward bacterial (*Agrobacterium tumefaciens*) and fungal diseases in grape varieties. Crown gall infected vines of the grape varieties – Rkatsiteli, Saperavi, Cabernet Sauvignon, Tsitska and Tsolikouri were identified in East and West regions of Georgia; healthy vines were considered, as well. Stilbenoid- containing fractions were isolated from the trunk of the infected and healthy vines and the single compounds were identified (trans-resveratrol and trans -ε- viniferin). The grape variety had a crucial role in the amount of detected stilbenoids. The obtained results were important for identifying the correlation of the immunity of the grape varieties with the phytoalexin stilbenoids [1]. Healthy and crown gall infected vines of *V. vinifera* L, cvs. Saperavi and Rkatsiteli were selected from vineyards in East region of Georgia, in 2018 and 2019. Samples of infected and healthy vines were taken in February – March 2018 in a 16-year-old vineyard and in January 2019 in a 24-year-old vineyard, both located on an alluvial soil. Stilbenoids (trans-resveratrol and trans-ε-viniferin) were isolated from the infected and healthy vine trunks and analyzed by HPLC/MS, with three replicates. The concentrations of *trans*-ε-viniferin in healthy trunks of the 24- year-old Saperavi and Rkatsiteli, were higher than the concentration of *trans*-resveratrol. On the other hand, in the healthy trunks of 16-year-old vines, the concentration of *trans*-resveratrol exceeded the concentration of trans- ε- viniferin. In the crown gall infected 24-year-old vines, the concentration of trans-resveratrol increased while the concentration of trans- ε- viniferin decreased [2].

The trunk stilbenoids of healthy and crown gall infected vines of Georgian red vine variety - Ojaleshi was studied and identified as stress-metabolite compounds: cis-piceid, trans-resveratrol, trans-ε-viniferin, cis-miyabenol C, cis-miyabenol. The concentrations were higher in infected vines compared to the healthy ones. Among the stress-metabolite stilbenoids were dominating trans-resveratrol and trans-e-viniferin, which concentrations increase under bacterial cancer disease condition. In concrete: trans-resveratrol-2,45g/kg - 3,28g/kg; for trans-ε-viniferin 7,18g/kg - 8,35g/kg [3].

It was established stress-metabolite stilbenoids from Tavkveri grape vine variety trunk in condition bacterial cancer disease. Received results are following: trans-resveratrol increases from 1.65 g/kg to 4.54 g/kg and cis—δ- viniferin, which decreases from 4.27 to 2.47 g/kg [4].

A strain of *Agrobacterium tumefaciens*, isolated from the trunk of infected *V. vinifera* L. cv. Rkatsiteli was studied microscopically and its pathogenesis was established. The goal of the research was to study the role of stilbenoids on the bacterial growth. The bacterium strain was inoculated in two ways: a) the surface of the growth was covered by a watery suspension of stilbenoids; b) stilbenoids were added to the growth medium before sterilization. In both experimental protocols the concentrations of the stilbenoids were: 1 mg/100 ml, 2 mg/100 ml, 3 mg/100 ml, 4 mg/100 ml, 5mg/100ml, 10 mg /100 ml, 15mg/100ml, 20 mg /100 ml and 30 mg /100 ml. The control version was the same medium without stilbenoids. The incubation period was 14-15 days at 27 °C and all the treatments completely (100%) inhibited *Agrobacterium tumefaciens* growth over the control. A second experiment was set up in order to study the bacterial growth inhibition under stilbenoid concentrations lower than 1 mg/100 ml (ranging from 0.1 mg/100 ml to 0.9 mg/100 ml): the bacterial growth inhibition increased from 0.0 % to 88.0% by increasing the stilbenoid concentrations [5.].

Also, it was studied the concentrations (in the berry skin) of stilbenoids of white wine variety Rkatsiteli under grey mould (*Botrytis cinerea*) attack. Samples of healthy and infected grapes – with 60% gray mould, were taken in 2018 during the technological maturity, from the same vineyard (16-year-old) planted in eastern Georgia. The vineyard soil belongs to meadow cinnamonic – Calcariccambisols and calcic kastanozems type. The stilbenoids profiles of healthly and infected skins were detected by HPLC/MS analysis. The dominant stress-metabolites were trans-resveratrol and its derivatives: trans-piceid, cis-piceid, trans-piceatannol, trans-ε-viniferin. The concentration variability of these stilbenoids under gray mould infection was different, as follows (healthy vs. infected): trans-resveratrol 39.27mg kg^{-1}→57.33mg kg^{-1}; trans-piceid 13.72mgkg^{-1}→29.43mgkg^{-1}; trans-piceatannol 5.37mg kg^{-1}→19.45mg kg^{-1}; trans-ε-viniferin 7.22 mg kg^{-1}→5.13mg kg^{-1}. These are the first evidences of the link stilbenoids –gray mould in the Rkatsiteli variety [6.].

Another trial on the interaction between grey mould infection and stilbene production of the white cv. Tsolikouri was carried out; the vineyard (30-year-old) was cultivated in the west part of Georgia (Zestafoni region) on raw humus calcareous-rendzic-leptosols soil. The production of trans-resveratrol, ε-viniferin, trans-piceid, cis-piceid, trans-piceatannol was elicited by grey mould. These are the first evidences of the link stilbenoids –grey mould in the Tsolikouri variety [7.].

Moreover, a study on the effect of powdery mildew (*Uncinula necator*) infection in the synthesis of stibenoids in the grape white variety Rkatsiteli was developed. Samples of healthy and infected grapes (50% of powdery mildew attack) were taken at the beginning of September 2018 (technological maturity), from the same vineyard (32-year-old) planted in eastern part of Georgia; the vineyard soil belongs to meadow cinnamonic –Calcaric cambisols and calcic kastanozems type. The concentration of stilbenoids increased during the disease and the dominant stress-metabolites were trans- resveratrol and trans-ε-viniferin, while the minor compounds were trans-piceid, cis-piceid, trans-piceatannol and oligomeric stilbenoids. The concentrations of these stilbenoids changed from healthy to infected grapes, as follows: trans-resveratrol 27.7mg/kg→58.92 mg/ kg(53,0%); trans ε-viniferin 11.22 mg /kg→32.55mg /kg(65,5%); trans-piceid 5.36 mg/ kg→7.27mg /kg(26,3%) ; trans-piceatannol 1.45mg/ kg→2.04 mg/kg(28,9%); cis-piceid 17.75 mg/kg→17.79 mg/kg(0,2%); trans-astringin 14.45mg/kg →16.93mg/kg(12,9%); cis-astringin 15.02 mg/kg→16.78 mg/kg(10,5%). These are the first evidences of the link stilbenoids –powdery mildew in Rkatsiteli grape variety [8].

Another trial included the relationship between leaf stilbenoids of the white grape variety Tsitska, grown in the west part of Georgia and downy mildew infection. The stilbenoids trans-resveratrol and ε- viniferin were higher in the infected vines leaves than in the healthy ones. This is the first study done on Tsitska grape variety concerning this subject [9].

Stilbenoids act against different vine diseases caused by bio factors. The following stilbenoids were identified in the extract of vine (*Vitis vinifera*) trunks, roots and annual shoots: Ampelopsin A, (E)-piceatannol, Pallidol, E-resveratrol, hopeaphenol, isohopeaphenol, (E)-ε-viniferin, (E)-miyabenol C, (E) –w-viniferin, r- and r2-viniferin. It was established that the extract inhibits the growth of sporulation of fungus *Plasmopara viticola* by 50%, while the most active inhibitor of it turned out to be r2-viniferin [10]. Under the influence of *Botrytis cinerea* on the mixture of pterostilbene and resveratrol 7 new stilbens were formed, while 5 new stilbenes were formed from pterostilbene under the same terms. The anti-fungal effect of these stilbenoids was fixed against *Plasmopara viticola* [11]. At three stages of the grape (*Vitis vinifera*) berry development, the berries were infected with *Botritis cinerea* spores „in vitro“. In the infected berries, pterostilbene, (E)-ε-viniferin and trans-resveratrol were detected, being (E)-ε-viniferin the most produced [12].

Berries of *Vitis vinifera* L. cv. Barbera in the ripening period were infected with conidial suspension of *Aspergillus japonicus*, *A. ochraceus*, *A. fumigatus* and *A. carbonarius*. The process of formation of ochratoxin A and stilbenoids was assessed. It was found out that all fungi except for *A. fumigatus* significantly increased the concentration of trans-resveratrol and at the same time, trans-piceid stays unchanged. In the berries damaged by *A. ochraceus*, the concentration of piceatannol increased significantly. A large amount of ochatoxin A was synthesized in the berries infected by *A. carbonarius* isolate and the anti-fungal activity of stilbenoids was tested under the following concentrations: 300 mg/g resveratrol and 20 mg/g piceatannol, which were effective for an inhibition of the fungal (*A. carbonarius*) growth [13].

Objects and Methods

The objects of research were: a). healthy and naturally diseased by crown gall (*Agrobacterium tumefaciens*) vine trunks 15-year-old shavkapito (red grape wine variety, *Vitis vinifera* L.) cultivated in Eastern Georgia (village of Mukhrani) on Eutric cambisols type soil; b). Pathogenic bacterial strains of *Agrobacterium tumefaciens* isolated from naturaly diseased by crown gall vine trunk of Shavkapito vine variety. From stilbenoids we used water suspensions of the total preparation, trans-resveratrol and trans-e-viniferine released from the vine at the concentrations indicated in the table.

Isolation bacterial strains and establishing their pathogenesis. We took infected Shavkapito trunk cut peace by bacterial cancer. We crushed it in Rodin, added sterile water and made a bacterial suspension. Prepared in petri dishes (8 cm in diameter) sowed the bacterial suspension to the food area and put it in a thermostat at a temperature of 25-27^{0}C. After 12 hours of incubation, the bacterial colonies developed in the food area. We carefully removed and transplanted it to the same food area in the tubes. Closed tubes were placed in a thermostat at a temperature of 25-27^{0}C. To determine the pathogenesis bacterial colonies developed after 24 hours of incubation were removed and transplanted to sliced healthy carrot rings and were placed in a desiccator. Bacterial growth was observed in the laboratory at a temperature of 22-23^{0}C. According to the intensity of development, we identified strong, medium-strength and weak bacterial strains. We transplanted them into food area prepared in tubes and stored them in appropriate conditions for subsequent studies [14].

Isolation of stilbenoids from healthy and infected with bacterial cancer trunks was made according to the scheme (Fig.1).

The effect of stilbenoids on the activity of bacterial strains was determined by the degree of inhibition of their growth and development "***in vitro***." Prepared in advance food areas in petri dishes were treated with water suspensions of stilbenoids and then transplanted with strong, medium-strength and weak strains of *Agrobacterium tumefaciens*.

As a control variant we used bacterial strains sown in the same food area treated with sterile water. Their growth and development took place under the conditions described above.

Stress-metabolite stilbenoids were analyzed by HPLC method. For this purpose, we used the Varian chromatograph, SupelcosilPM LC18 Column, 250x4,6mm, eluents: A. 0,025% trifluoroacetic acid, B.Acetonitrile:A 80/20. Gradient mode: 0-35 min, 20-50% B, 48-53min, 200% B. Flow rate of the eluent- 1 ml/min; wavelength-306 and 285nm. Isolated stilbenoid-containing fractions were filtered using a membrane filter (0,45μ) before the chromatographic procedure [15].

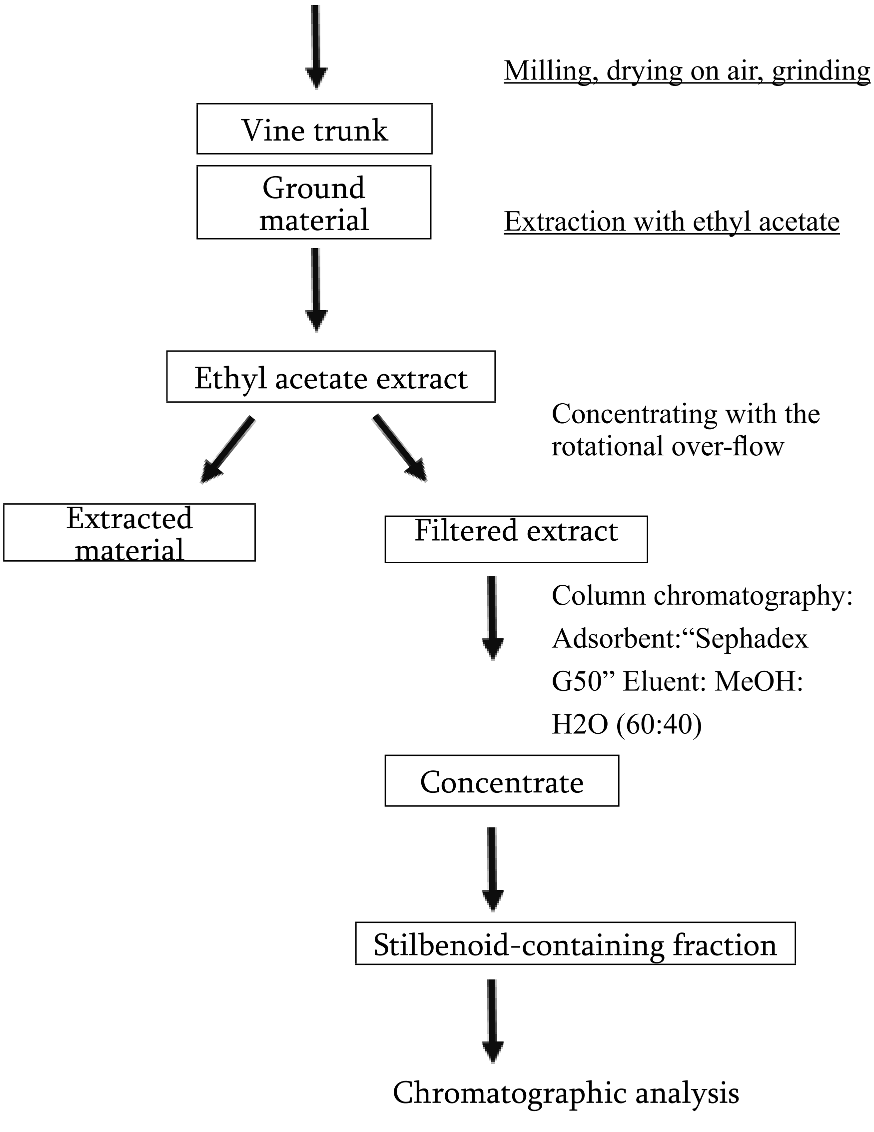

Fig. 1. *Chart of isolating a stilbenoid-containing fraction from vine trunk*

Aim of the research: Aim of the research was Georgian endemic red vine variety Shavkapito trunk health and infected by crown gall (*Agrobacterium tumefaciens*). Determination of phytoalexin stress metabolite stilbenoids. Identification there pathogen strains and study impact of stilbenoids on it activity -inhibition it development.

Results and Discussion.

The difference in the content of stylbenoids of healthy and diseased by bacterial cancer vine trunks of Shavkapito was determined by HPLC analysis. Based on this, the stress metabolite phytoalexin stylbenoids were identified. Main stress metabolite stilbenoids turned out trans-resveratrol and trans-ε-viniferin. In the experiments, we used isolated by us individual trans-resveratrol, trans- ε-viniferine and a total preparation of stilbenoids from the vine.

It was isolated strong, medium and weak bacterial strains from the naturally diseased with Agrobacterium tumefaciens Shavkapito trunk. In the control variants - on food areas not treated with stilbenoids, pathogenic strains of *Agrobacterium tumefaciens* were development as follows: strong -100%, medium- 55%, weak -30%. The development of pathogenic strains on food areas treated with stilbenoids is given in Tables -1,2.

As table 1 shows, stilbenoids inhibitor behavior on the different strength of the bacterial cancer strain is different. In concret, analyzed stilbenoids same concentations in different strength pathogenic strains characterized by different inhibition quality: for totally inhibation strong strain stilbenoids concentration decreises . The concentration of stilbenoid for total inhibition of the strong strain decreases for the pathogenic strain of medium and weak strength.

Above mentioned full dynamic results is given in table 2. In concret, to inhibit totally strong bacterial cancer strain is needed trans-resveratrol concentration 2.8mg/100ml, for medium strength pathogenic strain- 2.6 mg/100ml, weak pathogenic strain-1.6 mg/100ml. Trans-ε-viniferin 2.4 mg/100 ml is enough to inhibit totally strong pathogen strain. For medium strength-2.2 mg/100ml, for weak – 1.4 mg/100ml. Total stilbenoids preparate concentration dynamic is following: for strong pathogenic strain-2.2 mg/100ml, for medium-1.6 mg/100ml, for weak pathogen strain -1.2 mg/100ml.

Conclusion

Theafore, during the carried out research were identified pathogenic strains: strong, medium, weak – from infected by Agrobacterium tumefaciens trunk, of the Georgian red vine grape variety Shavkapito. It was established inhibitor impact of the phytoalexin stilbenoids: trans-resveratrol, trans-ε-viniferin and total stilbenoids preparate on the pathogen strains. Above mentioned stilbenoids, regarding to there inhibition quality are positioned for the following way: stilbenoids complex preparate > trans-ε-viniferin>trans-resveratrol. Research results are important for determine the immunity correlation of Shavkapito vine variety to the phytoalexin stilbenoids.

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Table 1. *Impact of Phytoalexin stilbenoids inhibitor behavior on the pathogen strains development*

N	Stilbenoids	Concentration, mg/100 ml	Bacterial growth Inhibition, %
	Strong Pathogen strain		
1.	Trans-resveratrol	1.0	87.0
		2.0	95.0
		3.0	100.0
		4.0	100.0
		5.0	100.0
2.	Trans-ε-viniferin	1.0	89.0
		2.0	97.0
		3.0	100.0
		4.0	100.0
		5.0	100.0
3.	Total preparat	0.5	82.0
		1.0	90.0
		2.0	99.0
		3.0	100.0
		4.0	100.0
		5.0	100.0
	Medium Pathogen strain		
1.	Trans-resveratrol	1.0	91.0
		2.0	98.0
		3.0	100.0
2.	Trans-ε-viniferin	1.0	93.0
		2.0	99.0
		3.0	100.0
3.	Total preparate	1.0	96.0
		2.0	100.0
	Weak Pathogen strain		
1.	Trans-resveratrol	1.0	95.0
		2.0	100.0
2.	Trans-ε-viniferin	1.0	97.0
		2.0	100.0
3.	Total preparate	1.0	99.0
		2.0	100.0

Table 2. *Dependence of inhibition of bacterial strains development on stylbenoid concentrations*

Stilbenoids concentration, mg/100ml	Inhibition of development,%	Inhibition of development,%	Inhibition of development,%
	for trans-resveratrol	for trans-ε-viniferin	for total preparate
Strong pathogen strain			
1.2	88.5	89.9	91.5
1.4	90.0	91.5	93.0
1.6	91.0	94.0	95.5
1.8	93.0	96.0	97.5
2.0	95.0	98.0	99.0
2.2	96.0	99.0	100.0
2.4	97.5	100.0	100.0
2.6	99.0	100.0	100.0
2.8	100.0	100.0	100.0
3.0	100.0	100.0	100.0
Medium pathogen strain			
1.2	92.0	93.5	97.0
1.4	93.5	94.5	99.0
1.6	95.0	96.0	100.0
1.8	96.5	97.5	100.0
2.0	98.0	99.0	100.0
2.2	98.5	100.0	"—"
2.4	99.5	"—"	"—"
2.6	100.0	"—"	"—"
2.8	100.0	"—"	"—"
3.0	100.0	"—"	"—"
Weak pathogen strain			
1.2	95.0	98.0	100.0
1.4	98.0	100.0	100.0
1.6	100.0	100.0	100.0
1.8	100.0	100.0	100.0
2.0	100.0	100.0	100.0

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