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Biochemistry

Change of Phytoalexin Stilbenoids in the Vine Trunk (*Vitis vinifera* L.) under Crown Gall Infection (*Agrobacterium tumefaciens*)

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Stilbenoids (grapevine main phytoalexins) have important biological activities against bacterial and fungal diseases. In this paper, we investigate the correlation between the extent of bacterial attacks (in some Georgian white and red winegrape varieties) and the stilbenoids production. The experiment, run in 2022 and 2023, considered Saperavi and Rkatsiteli winegrape varieties artificially infected by *Agrobacterium tumefaciens* and grown in different locations, as follows: Saperavi in Mukuzani and Napareuli; Rkatsiteli in Tsarapi and Tibaani. Untreated vines were considered as control. A water suspension of the pure culture of *Agrobacterium tumefaciens* was used as inoculum in April and the trunk stilbenoids levels were checked (in both years). In 2023, a subset of infected vines was previously treated by stilbenoids and the development/multiplication of bacterial cancer in the period from April to September was observed both in the vineyard and in the lab. Trans-resveratrol was found to be dominant compared to trans-ε-viniferin in both the healthy and infected vines of Saperavi and Rkatsiteli. In 2022, the levels of stilbenoids of healthy and infected vines changed depending on the variety and the location, while in 2023 the infected vines had higher concentrations of the stibenoids as compared to the healthy ones in all areas, except for Rkatsiteli in Tibaani, where the opposite occured. The pre-treatment by stilbenoids in the infected vines reduced the development/multiplication of the bacterium. The results of the experiment are important for further research to better understand the interaction between crown gall infection and stilbenoids production in Saperavi and Rkatsiteli winegrape varieties. © *2024 Bull. Georg. Natl. Acad. Sci.*

Saperavi, Rkatsiteli, stilbenoids, phytoalexins, crown gall, *Agrobacterium tumefaciens*

Stilbenoids are a wide class of phenolic compounds, which include resveratrol, piceatannol and their glucosides, dimers, trimers, tetramers, etc. in *cis*- and *trans*-isomeric forms [1]. Stilbenoids are characterized by a number of high biological activities and among them is important the phytoalexin activity for the plant, especially for the grapevine. The most valuable phytoalexin stilbenoids are:

resveratrol [2], pterostilbene [3], piceid[4], viniferins [5]. Phytoalexins, under plant infection, are actively synthesized and behave against disease-causing microorganisms (for instance *Botrytis cinerea* and *Plasmopara viticola*). Alongside the biotic factors, phytoalexins also react to abiotic stresses such as UV rays and $AlCl_3$ [6]. Adrian et al. [7] studied the levels of stilbenoids in the berry skin of red (Pinot noir, Gamay) and white (Chardonnay) winegrape varieties infected by *Botrytis cinerea* under UV rays treatment. All samples infected with *B. cinerea* revealed a decreased amount of resveratrol and an enlarged concentration after UV irradiation; pterostilbene was found in low concentrations in infected berries of Chardonnay and Gamay. Pterostilbene was also detected in low concentrations in grape skins by other authors [8]. As mentioned by Pezet and Pont [9] pterostilbene plays a dominant role in the resistance of grapes against disease-causing microorganisms. Other authors studied the interaction between stilbenoids and *Botrytis cinerea* in grapevine. According to Bezhuashvili et al [10] stilbenoids have been identified in healthy and naturally infected Georgian winegrape varieties – Rkatsiteli (white), Tsolikouri (white), Alexandrouli (red), Mujuretuli (red). *Trans*-resveratrol and *trans*-ε-viniferin were dominant for red varieties; moreover, *trans*-resveratrol was lower than *trans*-ε-viniferin in healthy grape skins, and the concentration of *trans*-resveratrol was significantly higher under gray mold infection than *trans*-ε-viniferin. In white wine grape varieties (Rkatsiteli and Tsolikouri), the main stress metabolite was *trans*-resveratrol, which increased significantly under gray mold attack [11]. The inhibitory effect of *trans*-resveratrol on *Botrytis cinerea* activity and consequently the spread of gray mold on grapes, has been established under lab conditions (in petri dishes) [12]. Stilbenoids had an inhibitory effect on *Botrytis cinerea* pure culture in food areas placed in petri dishes and there was a negative correlation between the fungal propagation and the stilbenoids concentration. According to Adrian et al. [13], resveratrol concentration of 100 μg/ml completely inhibited the development of *B. cinerea* mycelium, while concentrations of pterostilbene at 20-40-60 μg/ml caused inhibition of 50%, 80%, and 100%, respectively. According to Pezet and Pont [9], the concentration of pterostilbene of 18 μg/ml caused 50% inhibition of *B. cinerea* mycelium development while the concentration of 52 μg/ml resulted in an inhibition of 52%.

Evidences have been obtained on the capability of some highly pathogenic *B. cinerea* strains to circumvent the defence by detoxifying resveratrol through an oxidative process [14]. Other stilbenoids can be detoxified by enzymatic (laccase) activity of *B. cinerea*, causing the release of compounds similar to pterostilbene *trans*-dehydrodimer, pterostilbenecis-dehydrodimer, resveratrol *trans*-dehydrodimer [15]. All the physiopathological characteristics of stilbenoids are described in [16].

The aim of the study was to determine the variation of phytoalexin stilbenoids of Georgian vine grape varieties of Saperavi (red grape) and Rkatsiteli (white grape) trunks, under infection by bacterial cancer (crown gall).

Materials and Methods

The experiment, run in 2022 and 2023, considered Saperavi (red) and Rkatsiteli (white) winegrape varieties artificially infected by *Agrobacterium tumefaciens* and grown in commercial vineyards of different locations of eastern Georgia, as follows:

- Saperavi in: a) Mukuzani – (17 year-old vineyard on eutric cambisols and calcic kastanozems type of soil); b) Napareuli (40 year-old vineyard on eutric cambisols and Calcic kastanozems type of soil);
- Rkatsiteli in: a) Tsarapi (40 year-old vineyard on meadow cinnamonic-calcaric cambisols and Calcic Kastanozems type of soil); b) Tibaani (17 year-old vineyard on cinnamonic – calcaric cambisols and calcic kastanozems type of soil).

Untreated vines (not inoculated) were considered as control, for the same varieties in the same different locations.

Stilbenoids analysis: these compounds were detected by the method of high performance liquid chromatography (HPLC) [17].

Vineyard experiment: water suspension of pure culture of *Agrobacterium tumefaciens* (a strong pathogenic strain) was used as inoculum on the trunk (5 plants/variety/location) of the above mentioned vines, in April. A subset of vines, just before inoculation, was treated by three different products, as follows: a) a suspension of *trans*-resveratrol; b) a suspention of ε-viniferin; c) a suspension of their mixture. The source of these compounds was not lab chemicals but one-year-old canes. The tests done were as follows:

- *Trans*-resveratrol and ε-viniferin concentrations in the healthy and infected (without pre-treatment) vine trunks (in both years, at September), according to the scheme of Fig.
- Development/multiplication of the bacterium (only in 2023 in vines from the vineyards and in the lab), in the infected vines, without pre-treatment and with pre-treatment; the observation period was from April to September. Development of the bacterium (on the vine trunk): artificially infected vine trunks were cut and brought to the lab where a part of the bacterium developed from the trunk was taken and sown on a pre-prepared food area in a petri dish and placed in a thermostat at 27°C. The bacteria multiplied here are then sown on the receiving object – a pre-cut round carrot. These carrot rings are placed in a desiccator whose bottom is covered with water. The desiccator is closed with a lid and placed in a thermostat at 27°C; the incubation period is 5-7 days-night. The pathogenicity of the bacterial strain is selected based on the intensity of bacterial development on the carrot rings. Specifically, 80-100% of development is a strong strain; 50-80% is a medium strain and 30 - 50% is a weak strain [18].

***In vitro* experiment**: The experiment was carried out on the nutrient – Potato Dextrose Agar. In the control variant, a water suspension of a pure culture of *Agrobacterium tumefaciens* (a strong pathogenic strain) was applied to the food area. In the treatment variants, the food area was pre-treated with stilbenoids in a similar way as in the vineyard and then applied a water suspension of the pure culture of the bacterium. We placed closed Peter plates in a thermostat at 27°C and observed the growth and development of bacteria during the incubation period.

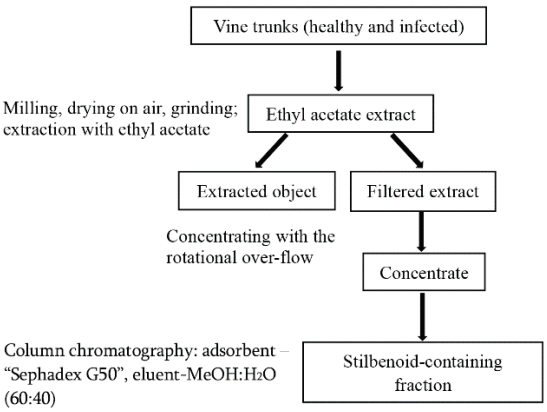

Fig. The chart of isolating a stilbenoid-containing fraction from vine trunk.

Results and Discussion

Table 1 shows the climatic conditions (temperatures) in the different locations between 2022 and 2023. Under these natural conditions, the development of bacterial cancer in Saperavi and Rkatsiteli.

Table 1 shows the climatic conditions (temperatures) in the different locations between 2022 and 2023. Under these natural conditions, the development of bacterial cancer in Saperavi and Rkatsiteli vine trunks artificially inoculated with *Agrobacterium tumefaciens* led to produce phytoalexin stilbenoids. The main stress-metabolite stilbenoids were identified based on HPLC analysis: *trans*-resveratrol and *trans* ε-viniferin. In all experimental vineyards, the concentration of *trans*-resveratrol

Table 1. Daily air temperature range (°C) in the experimental vineyards (between 2022 and 2023)

Months	Locations							
	Mukuzani		Napareuli		Tsarapi		Tibaani	
	2022	2023	2022	2023	2022	2023	2022	2023
April	12- 22	13-24	8-25	9-23	10-21	11-23	11-25	14-25
May	11- 27	12-26	10-24	12-26	10-24	11-28	11-27	13-28
June	17-25	18-27	18-27	19-28	18-28	16-26	18-27	20-28
July	17-34	17-35	17-36	16-35	16-35	17-35	17-35	20-36
August	27-35	21-37	27-38	21-38	25-36	21-37	24-37	24-38
September	22-28	14-28	20-30	15-31	21-38	15-26	23-29	15-27

Table 2. Change of concentration of *trans*-resveratrol and *trans*-ε-viniferin (g/kg) in vine trunks of healthy and infected vines, depending on the year, the variety and the location

Stilbenoids	Location							
	Mukuzani		Napareuli		Tsarapi		Tibaani	
	Health	Infected	Health	Infected	Health	Infected	Health	Infected
2022 year *Trans*-resveratrol	5.31	7.95	4.83	9.12	8.32	11.53	12.40	5.43
Trans- ε-viniferin	4.42	2.37	1.60	5.12	6.31	3.43	3.65	2.24
2023 year *Trans*-resveratrol	5.84	6.33	5.52	6.83	7.52	10.74	11.34	7.13
Trans- ε-viniferin	5.14	5.72	3.81	6.25	3.85	4.92	5.11	3.23

exceeds the concentration of *trans*-ε-viniferin in healthy and infected vine trunks of Saperavi and Rkatsiteli. In Tibaani area Rkatsiteli vine trunk shows a very high content of trans-resveratrol. It is noteworthy that the concentration of *trans*-resveratrol, in both years, increases in the infected vines, except for Rkatsiteli in Tibaani. The values of *trans*-ε-viniferin in healthy and infected vines, changed depending on the year, the variety and the location (Table 2).

The data in Table 3 clearly shows the inhibitory effect of phytoalexin stilbenoids on the reproduction and development of *Agrobacterium tumefaciens* in Saperavi and Rkatsiteli vine trunks. In the artificially infected vines pretreated with stilbenoids, bacterial cancer developed differently depending on the grapevine variety and the location. For example, in Mukuzani, we got 2 strong strains, 1 with a medium strength pathogen and 2 without development in Saperavi grape trunks. We obtained a similar result in Napareuli on Saperavi vine trunks, but at the same time, a significant difference was observed in the period of disease development. In Mukuzani *Agrobacterium tumefaciens* began to multiply 1 month after infection, in Napareuli the result was reached 3 weeks after infection. Rkatsiteli grape variety showed strong and quick infection symptoms in Tsarapi – with 4 strong and 1 medium strength pathogenic strains. It is noteworthy the result of Rkatsiteli variety in Tibaani, where the highest concentration of *trans*-resveratrol was observed in healthy vine trunks. Bacterial cancer developed slowly and without a strong activity. As concerning the role of the variety (without pre-treatment), in Rkatsiteli the symptoms were present 1 week after infection, while in the case of Saperavi, the bacteria did not develop. The reproduction and development of *Agrobacterium tumefaciens* in the same experimental vineyards on vine trunks pre-treated with stilbenoid suspension was different. In all variants, we obtained significantly weakened pathogenic strains compared to the control. This effect was evident using a water suspension of *trans*-resveratrol and *trans*-ε-viniferin. In the trunks pretreated with suspension of *trans*-ε-viniferin (P-2), everywhere

Table 3. Multiplication of *Agrobacterium tumefaciens* on artificially infected vines trunks under vineyard condition

Vine variet & Location	Time of infection	Monitoring stages the 1st	stage2nd	stage3rd stage
	26.04.2023	04.05.2023	16.06. 2023	08.09.2023
Rkatsiteli		No pre-treatments on vine trunks with developed different strengths of the pathogen		
Tsarapi Tibaani	+ +	start of multiplication start of multiplication	4-strong,1-medium 3-medium,2-without	4-strong, 1-medium 3-medium,2-weak
Saperavi Mukuzani Napareuli	 + +	 was not found was not found	 it was not found 2-strong, 1-medium,2-without	 2-strong, 1-medium, 2-without 2-strong, 1-medium, 2-without
Rkatsiteli	Vine trunks pre-treatments by stilbenoids suspension			
Tsarapi Pre-treatment by P-1 Pre-treatment by P-2 Pre-treatment by P-3	 + + +	 has not started “----------” “----------”	 2-medium,2-weak 1-strong,2-medium 2-medium, 1-weak	 2-medium,2-weak, 1-without 1-strong,2-medium,2-weak 2-medium,1-weak, 2-without
Tibaani Pre-treatment by P-1 treatment by P-2 Pre-treatments by P-3	 + + +	 has not started “----------” “----------”	 1-medium, 1-weak 1-strong, 1-medium,1-weak 2-medium,1-weak	 1-medium,1-weak , 2-without 1-strong,1-medium,2-weak,1without 2-medium,1-weak,2-without
Saperavi	Vine trunks pre-treatments by stilbenoids suspension			
Mukuzani Pre-treatment by P-1 Pre-treatment byP-2 Pre-treatment by P-3	 + + +	 has not started “-------” “-------”	 has not started “-------” “-------”	 2-medium,1-weak, 2-without 1-strong,1-medium,2-weak, 1-without 2-weak,3-without
Napareuli Pre-treatment by P-1 Pre-treatment byP-2 Pre-treatment by P-3	 + + +	 has not started “-------” “-------”	 2-medium,1-weak,2-wihthout 1-strong,1-medium,2-weak, 2-weak,3-without	 2-medium,1-weak,2-wihthout 1-strong,1-medium,2-weak,1-without 2-weak, 3-without

P-1 – *Trans*-resveratrol suspension; P-2- *Trans*-ε-viniferin suspension; P-3-*Trans*-resveratrol and *Trans*-ε-viniferin mixture suspension.

strong: 80-100% development; medium: 50-80% development; weak: 30-50% development; without bacterium development. The numbers in front of these expressions indicate, how many samples (out of five) had that evaluation.

we obtained only 1 sample with a strong pathogen strain; this result is due to the relatively low concentration of *trans*-ε-viniferin in the suspension. Thus, the results of the experiment conducted in the vineyards revealed the importance of phytoalexin stilbenoids of the vine trunks in “in vivo” conditions to fight against the development of crown gall. Phytoalexin characteristics of stilbenoid *trans*-resveratrol and *trans*-ε-viniferin identified in Saperavi and Rkatsiteli vine trunks under the crown gall infection were confirmed by “in vitro” experiments conducted in lab. The reproduction and development of *Agrobacterium tumefaciens* placed on the food area treated with the same stilbenoid suspension was inhibited within 90-98%.

Conclusion

Trans-resveratrol and *trans*-ε-viniferin were established as the main stress metabolites (stilbenoids) under crown gall infection of Saperavi and Rkatsiteli vine trunks. It was determined a dominant concentration of *trans*-resveratrol compared to *trans*-ε-viniferin in all locations in healthy vine trunks. Under bacterial infection, their variation occurred depending on the locations. The phyto-

alexin activity of the mentioned stilbenoids was confirmed by the results of bacterium development, in the vineyard (“in vivo”) and in the lab (“in vitro”). The results indicate the importance of stilbenoids as the biomarkers of Saperavi and Rkatsiteli varieties as a characteristic of the resistance to the bacterial cancer.

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ბიოქიმია

ვაზის შტამბის (*Vitis vinifera* L.) ფიტოალექსინი სტილბენოიდების ცვალებადობა ბაქტერიული კიბოთი (*Agrobacterium tumefaciens*) ინფიცირების პირობებში

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(წარმოდგენილია აკადემიის წევრის რ. გახოკიძის მიერ)

ვაზის სტილბენოიდების ფიტოალექსინური თვისებები მნიშვნელოვანი ბიოლოგიური აქტივობის მახასიათებელია ბაქტერიული და სოკოვანი დაავადებების წინააღმდეგ. ჩვენ მიერ გამოკვლეულია საქართველოს ზოგიერთი თეთრ- და წითელყურძნიანი საღვინე ვაზის ჯიშის იმუნიტეტის კორელაცია ფიტოალექსინ სტილბენოიდებთან ბაქტერიული და სოკოვანი დაავადებების პირობებში. წინამდებარე მასალები წარმოადგენს საფერავის და რქაწითელის ვაზის შტამბების ფიტოალექსინი სტილბენოიდების ცვალებადობის კვლევის შედეგებს ბაქტერიული კიბოთი (*Agrobacterium tumefaciens*) ინფიცირებისას ნიადაგურ-კლიმატური პირობებით განსხვავებულ მევენახეობის მიკროზონებში. კონკრეტულად, საფერავისთვის – მუკუზნის და ნაფარეულის, რქაწითელისთვის – წარაფის და ტიბაანის მიკროზონებში. აღნიშნული ვაზის ჯიშების ხელოვნური დაინფიცირება ჩავატარეთ *Agrobacterium tumefaciens*-ის წმინდა კულტურის წყლიანი სუსპენზიით 2023 წლის აპრილში და ბაქტერიული კიბოს განვითარებას/გამრავლებას დავაკვირდით აპრილი-სექტემბრის პერიოდში. ხელოვნურად დავაინფიცირეთ საფერავის და რქაწითელის ჯანსაღი და, ასევე, წინასწარ სტილბენოიდების წყლიანი სუსპენზიით დამუშავებული ვაზის შტამბები. საექსპერიმენტო მიკროზონების ვენახებში საფერავის და რქაწითელის ჯანსაღი ვაზის შტამბში ტრანს-რესვერატროლი აღმოჩნდა დომინანტი ტრანს-ε-ვინიფერინთან შედარებით. ტრანს-რესვერატროლი დიდი

რაოდენობით დაფიქსირდა ტიბაანის მიკროზონის რქაწითელის ვაზის შტამბში. ბაქტერიული კიბოთი ინფიცირებულ საფერავის და რქაწითელის ვაზის შტამბებში გამოვლინდა ძირითადი სტრეს მეტაბოლიტი სტილბენოიდები: ტრანს-რესვერატროლი და ტრანს-ε-ვინიფერინი. დადგინდა მათი ცვალებადობის დამოკიდებულება ვაზის ჯიშის, ასაკის და ნიადაგურ-კლიმატურ ფაქტორებზე. ექსპერიმენტის შედეგები მნიშვნელოვანი მონაცემებია შემდგომი კვლევების გასაგრძელებლად-საფერავის და რქაწითელის სტილბენოიდური ბიომარკერის, როგორც ვაზის ბაქტერიული კიბოს მიმართ რეზისტენტობის დასადგენად.

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