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Phytoalexin Stilbenoids of Saperavi and Rkatsiteli(*Vitis vinifera* L.) as Biomarkers of Resistance to Crown Gall Infection (*Agrobacterium tumefaciens*)

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ABSTRACT---- *The manuscript investigates the correlation between the production of phytoalexin stilbenoids—specifically trans-resveratrol and trans-ε-viniferin—and the resistance of Georgian grapevine varieties, Saperavi and Rkatsiteli , to Crown gall infection caused by Agrobacterium tumefaciens. The study takes place in multiple vineyard locations in Georgia, where experimental grapevines are artificially infected and monitored under varying environmental conditions. The article discusses the accumulation of stilbenoids in healthy and infected vine trunks, observing that these compounds act as stress metabolites that are actively involved in defense mechanisms against bacterial infection. Laboratory and vineyard experiments assess the inhibitory effect of stilbenoids on A. tumefaciens, showing that stilbenoids significantly inhibit bacterial growth, supporting their role as potential biomarkers for vine resistance.*

Keywords--- Saperavi, Rkatsiteli, Stilbenoids, Phytoalexins, Crown gall

1. INTRODUCTION

The resveratrol and its derivatives (glucosides, dimers, trimers, tetramers, etc.) are presente in the plant as *cis*- and *trans*-isomeric forms[1]. Stilbenoids are characterized by the phytoalexin activity for the plant, especially for the grapevine. The most important phytoalexin stilbenoids are: resveratrol[2], pterostilbene [3], piceid[4], viniferins [5]. Phytoalexins, under plant infection conditions, are synthesized and act against disease-causing microorganisms (for example *Botrytis cinerea* and *Plasmopara viticola*). Besides the biotic factors, phytoalexins also respond to abiotic stresses such as UV rays and $AlCl_3$ [6,7]. All samples infected with *B. cinerea* showed a decreased amount of resveratrol and an increased concentration after UV irradiation. Pterostilbene was found in low concentrations in infected berries of Chardonnay and Gamay. Pterostilbene was also observed in low concentrations in grape skins by other authors [8].According to Pezet and Pont [9] , pterostilbene is important in the resistance of immature grapes against disease-causing microorganisms. A concentration of pterostilbene of 18μg/ml caused a 50% inhibition of *B. cinerea* mycelium development while a concentration of 52 μg/ml resulted in an inhibition of 52%.

Other authors described the interaction between stilbenoids and *Botrytis cinerea* in grapevine.

According to Bezhuashvili et al [10] stilbenoids have been identified in healthy and naturally diseased Georgian vinegrape varieties - Rkatsiteli (white), Tsolikouri (white), Alexandrouli (red), Mujuretuli (red).*Trans*-resveratrol and *trans*-ε-viniferin were dominant for red varieties; *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*-e-viniferin; it decreased under disease conditions. In white wine grape varieties (Rkatsiteli and Tsolikouri), the main stress metabolite was *trans*-resveratrol, which increased significantly in gray mold disease conditions [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 of the fungus- *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].

Evidences have been obtained on the capability of some highly pathogenic *B. cinerea* strains to circumvent the defense by detoxifying resveratrol through an oxidative process [14].Other stilbenoids can be detoxified by enzymatic (laccase) activity of *B. cinerea*, resulting in the release of compounds like pterostilbene *trans*-dehydrodimer, pterostilbene *cis*-dehydrodimer, resveratrol *trans*- dehydrodimer [15]. All the physiopathological aspects of stilbenoids are addressed in the review written by Jeandet et al [16]. Stress metabolite phytoalexin stillbenoids are expressed by trans-resvatrol and its derivatives, under bacterial (17) and fungal attack (18,19) of Saperavi and Rkatsiteli.The aim of the study was to study the changes of the stilbenoids in Saperavi and Rkatisteli trunks and investigate the stress metabolite stillbenoids influence on "*Agrobacterium tumefaciens''* in lab– "in vitro'' and in vineyard - "in vivo'' conditions.

2. MATERIALS AND METHODS

The study considered healthy and Crown gall-infected vine trunks from Saperavi (red) and Rkatsiteli (white) vineyards located in viticulture areas in Eastern Georgia. As concerning to the experiment Saperavi trunks were sampled, as follows: a)Mukuzani area - from a 17-year-old vineyard located on Eutric Cambisols and calcic Kastanozems type of soil. b) Napareuli area cultivated with 40-year- old vineyard on Eutric Cambisols and calcic Kastanozems type of soil; Rkatsiteli trunks varieties were sampled from a) Tsarapi area cultivated with 40 year-old- vineyard grown on meadow cinnamonic-calcaric cambisols and calcic kastanozems type of soil, b) Tibaani area – with 17 year-old- vineyard grown on cinnamonic calcareous-calcaric cambisols and calcic kastanozemstype of soil.

2.1 Methods. Stilbenoids containing fractions were isolated from healthy and infected vine trunks according to Fig.1. Trans-resveratrol and ε-viniferin were individually isolated from one-year-old vine shoots by ethylacetate extraction and column separation as shown in Fig.1.

2.2 Stilbenoids were determined by the method of high performance liquid chromatography (HPLC) [20]. For this purpose, we used the chromatograph UHPLC focused. DIONEX Ultimate 3000; Column- Supelcosil PM LC18, 250x4,6mm; Eluents: A. 0,025% trifluoroacetic acid,B.Acetonitrile: A, 80/20. Gradient mode: 0-35 min, 20-50% B; 35-40 min. 50-100% B ; 41-46min. 100% B ; 46-48min 100-20% B; 48-53min, 20% B. Wave length: 306 nm for trans-stilbenoids, 285 nm for cis-stilbenoids. Flow rate of the eluent- 0,8ml/min; fractions were filtered using a membrane filter (0.45μ)before the chromatographic procedure.

3. DETERMINING THE EFFECT OF STILBENOIDS ON THE ACTIVITI OF *Agrobacterium tumefaciens* UNDER LAB AND VINEYARD CONDITION.

3.1 Lab experiments. We isolated stilbenoids containing fractions from the healthy and artificially infected trunks of Saperavi and Rkatsiteli vines from experimental vineyard . We determined the impact of trans-resveratrol, trans-ε-viniferin and their total preparation on the activity of *Agrobacterium tumefaciens* "in vitro". The experiment was carried out in the petri dishes on the food area - Potato Dextrose Agar. As a control variant, we directly applied a water suspension of a pure culture of *Agrobacterium tumefaciens* (a strong pathogenic strain) on the food area. In the experimental variants, we used pre-treated food area with stilbenoids. Then we applied a water suspension of the pure culture of the bacteria on the food area, we placed closed Peter dishes in a thermostat at 37^{0}C and observed the growth and development of bacteria during the incubation period.

3.2 Vineyard experiment. We carried out artificially infected Saperavi and Rkatsiteli vine trunks under natural conditions - in experimental bio vineyards located in the above-mentioned areas . We made cut of each variety, 5-10 trunks, and infected them in 2 variants with a water suspension of a pure culture of *Agrobacterium tumefaciens* (a strong pathogenic strain). 1. Control variant - without pre-treatment with stilbenoids of the trunk. 2. Variant of pretreatment with stilbenoids. Specifically, we used water suspensions of certain concentrations of trans-resveratrol, trans-ε-viniferin and their total preparation. We observed the development of grapevine bacterial cancer in the period of April-September 2024. In September, we took the trunks of healthy and diseased vines from the vineyards to the lab for research.

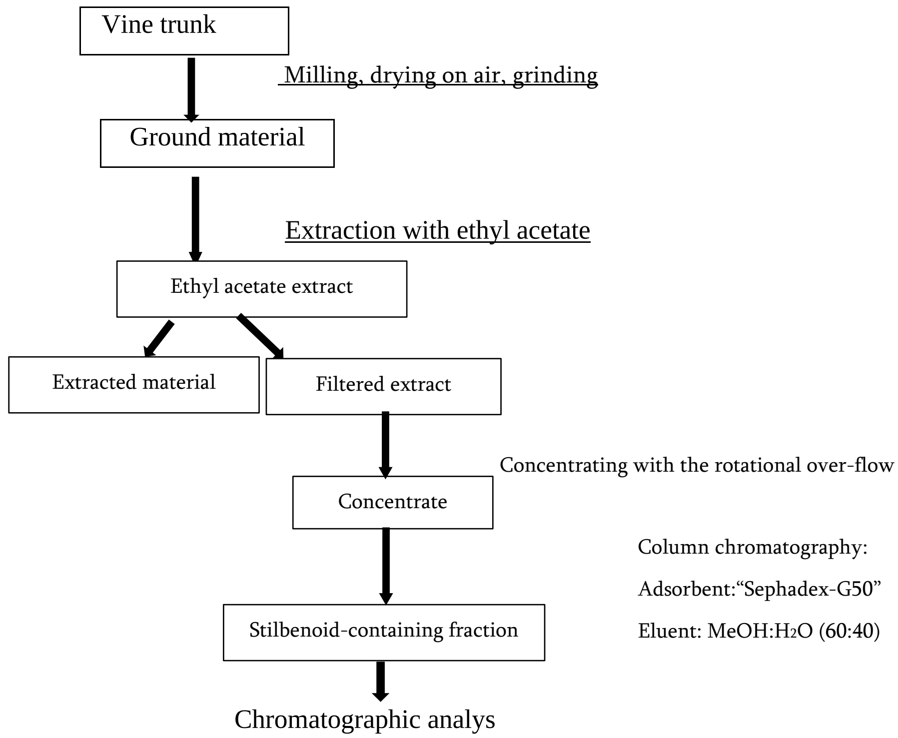

Figure 1: Chart of isolating a stilbenoid-containing fraction from vine trunk and grape skin

We took healthy and diseased grapevine stems separately, ground them in an electric grinder, and air-dried. We extracted the ground object with ethyl acetate under hot conditions. We filtered the extract and concentrated it on a vacuum condenser under pressure of 40^0C degrees. For separation, the received concentrate was applied to a chromatographic column containing the adsorbent "Sephadex -50". We used eluent MeOH:H_2O (60:40). We obtained a stilbenoid-containing fraction and also the individual required stilbenoids.

4. RESULTS AND DISCUSSION

It should be noted that the accumulation of phytoalexin stilbenoids in the vine trunks of Saperavi and Rkatsiteli are studied under the influence of abiotic (soil, air temperature) and biotic (crown gall) factors. In April-October of 2024 year , in the experimental areas , under normal rainy weather, the daily air temperature varied in the intervals indicated in Table 1.

Table 1: Daily air temperature (^{0}C) range in experimental viticulture areas

Months	**Areas**			
	Mukuzani	**Napareuli**	**Tsarapi**	**Tibaani**
April	11-23	15-22	14-23	
May	11-24	10-27	10-26	11-23
June	16-26	17-27	19-28	19-28
July	17-33	17-37	16-36	17-36
August	21-37	23-39	23-37	23-38
September	17-30	16-30	17-28	16-30
October	12-26	13-27	12-26	14-27

Temperature is one of the important abiotic factor impacting the biosynthesis of stilbenoids in grapevine. The inhibitory effect of high temperature on the biosynthesis of stilbenoids has been established by a number of researchers [21-23]. It is known that the temperature of normal biosynthesis of resveratrol is 15-20^{0}C for prolonged biosynthesis 5^{0}C, and for inhibition -20^{0}C and heat treatment at 65^{0}C for 2 hours[24]

In the experimental vineyards at the indicated temperature resveratrol and its derivatives were accumulated in the trunk of healthy Saperavi and Rkatsiteli grapes. *Trans*-resveratrol and *trans*- ε-viniferin are significantly dominant (table 2).

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

Stilbenoids	**Location**							
	Saperavi				**Rkatsiteli**			
	Mukuzani		**Napareuli**		**Tsarapi**		**Tibaani**	
	Health	**Infected**	**Health**	**Infected**	**Health**	**Infected**	**Health**	**Infected**
Trans-resveratrol	6.25	7.87	5.05	6.27	7.88	9.65	9.90	7.15
Trans- ε-viniferin	4.81	5.22	4.15	5.83	3.22	3.08	5.64	4.21

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

Vine variety & Location	**Time of infection**	**Degree of infection% /Biological efficiency% of stilbenoids**		
		Monitoring Stages		
		The first stage	**2nd stage**	**3rd stage**
	16-18.04.2024	25-27.06.2024	16-18.09. 2024	15-17.10.2024
Rkatsiteli	No pre-treatments on vine trunks with developed different strengths of the pathogen			
Tsarapi	+	50/50	100/0	100/0
Tibaani	+	50/50	100/0	100/0
Saperavi				
Mukuzani	+	50/50	100/0	100/0
Napareuli	+	50/50	100/0	100/0
Rkatsiteli	Vine trunks pre-treatments by stilbenoids suspension			
Tsarapi				
Pre-treatment by P-1	+	it has not started	100/0	100/0
Pre-treatment by P-2	+	“-----------"	80/20	80/20
Pre-treatment by P-3	+	“-----------"	80/20	80/20
Tibaani				
Pre-treatment by P-1	+	it has not started	100/0	100/0
Pre-treatment by P-2	+	“-----------"	100/0	100/0
Pre-treatment by P-3	+	“-----------"	80/20	80/20
Saperavi	Vine trunks pre-treatments by stilbenoids suspension			
Mukuzani				
Pre-treatment by P-1	+	it has not started	60/40	80/20
Pre-treatment by P-2	+	“-------”	60/40	80/20
Pre-treatment by P-3	+	“-------”	60/40	60/40
Napareuli				
Pre-treatment by P-1	+	it has not started	80/20	60/40
Pre-treatment by P-2	+	“-----------"	60/40	60/40
Pre-treatment by P-3	+	“-----------"	60/40	60/40

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

Table 4: Variation of pathogenicity of *Agrobacterium tumefaciens* during vine trunk infection

Vine variety & Location	**Time of infection**	**Monitoring Stages**		
		The first stage	**2nd stage**	**3rd stage**
	16-18.04.2024	25-27.06.2024	16-18.09. 2024	15-17.10.2024
Rkatsiteli	No pre-treatments on vine trunks with developed different strengths of the pathogen			
Tsarapi	+	Start of multiplication	5-strong, 5-medium	5-strong, 5-medium
Tsarapi	Vine trunks pre-treatments by stilbenoids suspension			
Pre-treatment by P-1	+	It has not started	3-medium, 2-weak	3-medium,2-weak
Pre-treatment by P-2	+	“-----------"	3-medium,1-weak, 1- without	3-medium,1-weak, 1-withaut
Pre-treatment by P-3	+	“-----------"	2-medium, 2-weak, 1- without	2-medium, 2-weak, 1-without
Saperavi	No pre-treatments on vine trunks with developed different strengths of the pathogen			
Mukuzani	+	5- without, 5- weak	2-strong, 5-medium, 3-weak	2-strong, 5-medium, 3-weak
Mukuzani	Vine trunks pre-treatments by stilbenoids suspension			
Pre-treatment by P-1	+	it has not started	2-medium, 1-weak, 2-without	2- medium, 2-weak, 1-without
Pre-treatment by P-2	+	“-----------"	1-medium, 2-weak, 1-without	2-medium, 2-weak, 1-without
Pre-treatment by P-3	+	“-----------"	1-medium, 2-weak, 2-without	1- medium, 2-weak, 2-without

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

Under the conditions of artificial infection of bacterial cancer in Saperavi and Rkatsiteli vine trunks, the following stress metabolite phytoalexin stillbenoids were detected: trans-resveratrol and trans-viniferin. In healthy trunks trans-resveratrol concentration exceeds trans-ε-viniferin concentration. *Agrobacterium tumefaciens* infection causes a change of stilbenoids physiological concentrations. For example: *Trans*-resveratol and *trans*- *ε* -viniferin content in Saperavi trunks in both areas are increasing, while in Rkatsiteli variety a different situation occurred (Table2).

Phytoalexin characteristics of stilbenoids 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 suspensions was inhibited within 90-98%.

Stillbenoids phytoalexin activity was revealed regarding *Agrobacterium tumefaciens's* activity and its spread inhibition in lab – „in vitro" and „in vivo" condition in vineyard. The dominant effect of stillbenoids total prepate was confirmed by the experiment(Table 3). Non -treated trunks of Saperavi and Rkatsiteli varieties were fully affected by the bacterial cancer. In addition, Saperavi grape resistance to Rkatsiteli relatively is dominant, which presented a weak growth of *Agrobacterium tumafaciens* pathogenic strains. Pre-treatment of trunks with stillbenoids suspension is very important to produce a different effect. Especialy, highly effective is the total stilbenoids preprate (table 3,4).

5. CONCLUSION

The results obtained clearly demonstrate the active inhibitory activity of the stress metabolite stilbenoids(*trans*-resveratrol and *trans*- *ε* -viniferin) of Saperavi and Rkatsiteli vine trunks on the development of crown gall. Our studies confirm that stilbenoids are an important biomarker to characterize the resistance of Saperavi and Rkatsiteli vine trunks toward crown gall.

6. AKNOWLEGEMENT

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