DOI: 10.22620/agrisci.2017.22.002

RHIZOCTONIA SOLANI – SHOOT ROT CAUSAL AGENT ON GRAFTED VINE CUTTINGS DURING THE STRATIFICATION PROCESS

Emil Balashkov[1*], Petya Christova[2], Neli Prodanova-Marinova[1], Slavtcho Slavov[2]**

[1]Institute of Viticulture and Enology – Pleven
[2]AgroBioInstitute – Sofia

E-mail: emo_bl87@abv.bg*; sbslavov@abi.bg**

Abstract

A study was carried out to determine the causal agent of the shoot rotting observed during the stratification of grafted vine cuttings at the Institute of Viticulture and Enology – Pleven. It was found that the described disease symptoms were caused by *Rhizoctonia solani* fungus, based on the morphology of mycelium colonies and DNA sequencing. The inhibition effect of some fungicides on the mycelia growth of two *R. solani* isolates derived from naturally infected plant material was studied *in vitro*.

The radial growth inhibition of EM-10 isolate colonies was demonstrated after treatment with *Quadris* (*250 g/l azoxystrobin*) at a dose of 0.075% *in vitro*. *In vivo* application of both fungicides, *Quadris* and *Proplant 722 SL* (at a dose of 20 ml/m^2) inhibited efficiently *Rhizoctonia solani* infection during the stratification process. Both fungicides did not inhibit the callus formation and did not express unfavorable effect on the graft between the cutting and the rootstock.

Keywords: grafted vine cuttings, stratification, rot, *Rhizoctonia solani*, fungicides.

INTRODUCTION

The proper stratification process for the precise grafting and obtaining of standard rooted vines and high quality of starting plant material is essential for the quality and quantity of potential grape yield. During this process, the grafted cuttings placed under specific conditions (temperature mode, moisture, access to air) form a callus between the rootstock and the cutting. Wood sawdust, forest moss, sand, etc. have been most commonly used as a moisture-retaining substrate (Abrasheva et al., 2008; Braykov et al., 2005). Maintaining a constant temperature of 24-26 °C in the zone of the graft as well as the high substrate moisture and air humidity created favorable conditions for pathogenic fungi development. A brown rot with a heavy gray-brown spore formation coating the diseased tissue was observed regularly during the stratification.

Until now *Botrytis cinerea* was reported as a causal agent of shoots rotting of grafted cuttings during the stratification (Kolev, 1962; Raykov and Nachev, 1968, 1971; Radulov and Georgieva, 1984; Vanev, 1995; Masheva et al., 2004; Bobev, 2000; Abrasheva et al., 2008; Harizanov et al., 2009). *B. cinerea* was postulated as a causal agent of similar symptoms on the shoots in the nurseries too. In some cases, the infection was so heavy that it damaged wholly the vine shoots (Malenin, 2003; Stancheva, 2006).

Rhizoctonia solani was previously reported as a grapevine pathogen. *Macrophomina phaseolina* (Tassi) Goid. [*Rhizoctonia bataticola* (Taub.) Briton-Jones] and *Rhizoctonia solani* Kiihn were associated with root rot in dying or dead grapevines in the Western Cape of South Africa (Marais, 1979). The virulence of *R. solani* isolates obtained from roots of diseased grapevines also was determined both alone and in combination with *Meloidogyne incognita* (Walker, 1997). The same pathogen was detected in vineyards in Sonora, Mexico, and showed a high degree of genetic variability based on Amplified Fragment Length Polymorphism (Meza-Moller et al., 2011). However, no reports of *R. solani* infecting young vine shoots in the nurseries have been found.

The objective of this study was to determine the causal agent of the vine shoots rotting of the grafted cuttings during the process of stratification as well as investigation of the disease control options using appropriate pesticides.

MATERIALS AND METHODS

The trials under production conditions were carried out during the production of vine propagation material at in the period 2015-2016 years. Some of the laboratory trials were partially conducted in IVE – Pleven and others (mainly DNA analyses of the pathogen identification) were carried out in AgroBioInstitute – Sofia.

Isolation of the causal agent

Samples of cuttings of different vine varieties with manifested symptoms of rotting during the stratification were collected for isolating the pathogen. A standard methodology was used to isolate the causal agent, consisted in: washing the shoots under running water and liquid soap for removing the microflora on their surface; successive sterilizing with 75% ethanol for 1 minute and 5% sodium hypochlorite for 5 minutes, followed by triple washing in sterile water (Liu, 2001*).* Plant parts taken from the border between healthy and diseased tissue were transferred in Petri dishes under sterile conditions on Difco Potato Dexstrose Agar medium (39 g/L, BD, France) and were incubated in Petri dishes at 24 °C in the dark. The correct isolation of the pathogen was proved by application of the Koch's postulates (Stancheva, 2004) by detached leaf bioassay. Young vine leaves were pre-washed and decontaminated with 0.5% potassium permanganate solution for 5 minutes and washed with sterile water according to Tafradzhiyski (1967). Inoculation of the leaves was done with the mycelium downward after slight damage to the leaf epidermis by needle on the inoculation spot. Following the Koch's postulates re-isolation of the pathogen was done after the appearance of the specific symptoms (Stancheva, 2004).

Identification of the causal pathogen

The causal agent of shoot rot on grafted vine cuttings was identified by mycelial characteristics and DNA analysis. A sequence analysis of the ITS ribosomal DNA of the investigated fungal pathogens was used for the molecular identification of two isolates EM-4 and EM-10. DNA was extracted from 10-day mycelium culture (100 mg) of both studied isolates using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol.

The PCR amplification of the ITS region of the isolates was performed by a primer pair ITS5/ITS4. The PCR program was as follows: 96°C – 2 min; followed by 35 cycles of 96°C – 1 min, 55°C – 1 min, 72°C – 2 min; and final elongation at 72°C – 10 min. The result of the PCR analysis was visualized on a 1% agarose gel. The obtained PCR products were sequenced in the European Custom Sequencing Center (Germany). The analysis of the result was processed by BLAST software.

Laboratory assay for fungicides' effect

The response of two isolates EM-4 and EM-10 to some fungicides was studied *in vitro* in a laboratory by measuring the radial growth of the colonies growing on PDA. The following fungicides were used: Proplant 772 SL (*722 g/l propamocarb hydrochloride*) and Quadris (*250 g/l azoxystrobin*) for EM-4; Proplant, Quadris and Topsin M (700 *g/kg thiophanate – methyl*) for EM-10. The applied fungicide doses are pointed in the following variants:

V1 – Proplant at a dose of 0.1%;
V2 – Proplant at a dose of 0.2%;
V3 – Quadris at a dose of 0.075%;
V4 – Quadris at a dose of 0.15 %;
V5 – Topsin M at a dose of 0.1%;
V6 – Topsin M at a dose of 0.2 %;
K – untreated control.

Each variant was set in four replicates.

The fungicide application on the growth media was done as 20 µl drops of the respective fungicide concentration was placed of the four places on the medium in a Petri dish, and the isolate was inoculated in the middle of the dish. The growth of the mycelium colony was measured on the first, second and third day after inoculation.

Fungicide control under production conditions (during stratification)

The experiment was carried out under common stratification production conditions. Wooden containers sized 82 x 60 x 50 cm were used for this purpose. Nonsterile moist wood sawdust was the substrate for taking the place of the process. About 1000 plants of Naslada variety grafted to Berlandieri X Riparia SO4 rootstock were arranged for stratification in each container. A constant temperature of 24-28°C and air humidity 80–85% was maintained in the dark during the entire stratification process.

Application of Proplant, Quadris and Switch products (*375 g/kg fludioxonil + 250 g/kg cyprodinil*) were performed during the trial. Each variant included one container. Four replicates of 10 cuttings per variant were prepared. The status of the callus at the end of the stratification and the ratio of the cuttings with diseased shoots were determined.

The variants with fungicide doses applied in the trial during stratification were the following:

V 1 – Proplant at a dose of 0.1%;
V 2 – Proplant at a dose of 0.2%;
V 3 – Proplant at a dose of 20 ml/m^2;
V 4 – Quadris at a dose of 0.075%;
V 5 – Quadris at a dose of 0.15%;
K – control (untreated);
E – standard (Switch at a dose of 0.08%).

The fungicides were sprayed, and only in variant three, the Proplant was applied as irrigation of seedlings.

Data were processed by Data Analysis MS Excel (2007), and analysis of variance (Dimova and Marinkov, 1999).

RESULTS AND DISCUSSION

Symptoms

The observations carried out during the stratification of the grafted vine cuttings showed the same symptoms development throughout all years of the study. The tissues of the shoots that were grown over the sawdust originally turned brownish from top to bottom and disintegrated under their own weight or when touched. The diseased tissue was jelly-like and wet, and brown spots of different shape and size appeared (Fig. 1).

The disease developed on plants in the wooden containers as expanded areas of circles with different size. It spread extremely fast from plant to plant, and very high number of completely healthy looking plants could express symptoms and be heavy damaged in one-two days (Fig. 2).

Isolation of the causal agent

The shoot rot developed on all of the vine cultivars in the stratification containers. Totally nine isolates were collected from the diseased plants of different cultivars (Table 1). All of them are collected in 2015 mainly from infected vine cuttings in which the rot progressed from the cutting top or symptoms were visible in the middle of the cutting stem as elongated slightly carved brown lesions.

Fig. 1. *Grafted cuttings with shoots rotten in varying degrees compared to healthy ones (in the middle of the left picture)*

Fig. 2. *Circle areas of infected plants in the stratification containers, with vine cultivars differing in level of susceptibility*

Table 1. List of isolates obtained from vine shoot rot, originating from different vine cultivars

Number of isolate	Vine cultivar of isolate origin
EM-1	Bolgar
EM-3	Merlot
EM-4	Naslada
EM-5	Storgozia
EM-6	Muscat Ottonel
EM-7	Muscat Plevenski
EM-8	Cabernet Sauvignon
EM-9	Slava
EM-10	Muskat Kaylashki

Identification of the causal agent

Using visual observation on the pathogen colonies development on artificial media in Petri dish, it was found that young mycelium colonies were whitish and became grayish-white gradually. Later, they grew darker and turned brownish in color. The microscope observations showed that the mycelium tends to branch at right angles, a septum near each hyphal branch and a slight constriction at the branch was visible, which istypical for *Rhizoctonia solani* fungus (Fig. 3).

Well developed colony of *Rhizoctonia solani* formed sclerotia (Fig. 4). The sclerotia were brownish, irregular in shape, and size was varying from 1 to 4 mm, scattered and not very dense. They were formed on the 8-10th day of their development, grown on potato dextrose agar (PDA) and cultured at 24 °C in the dark.

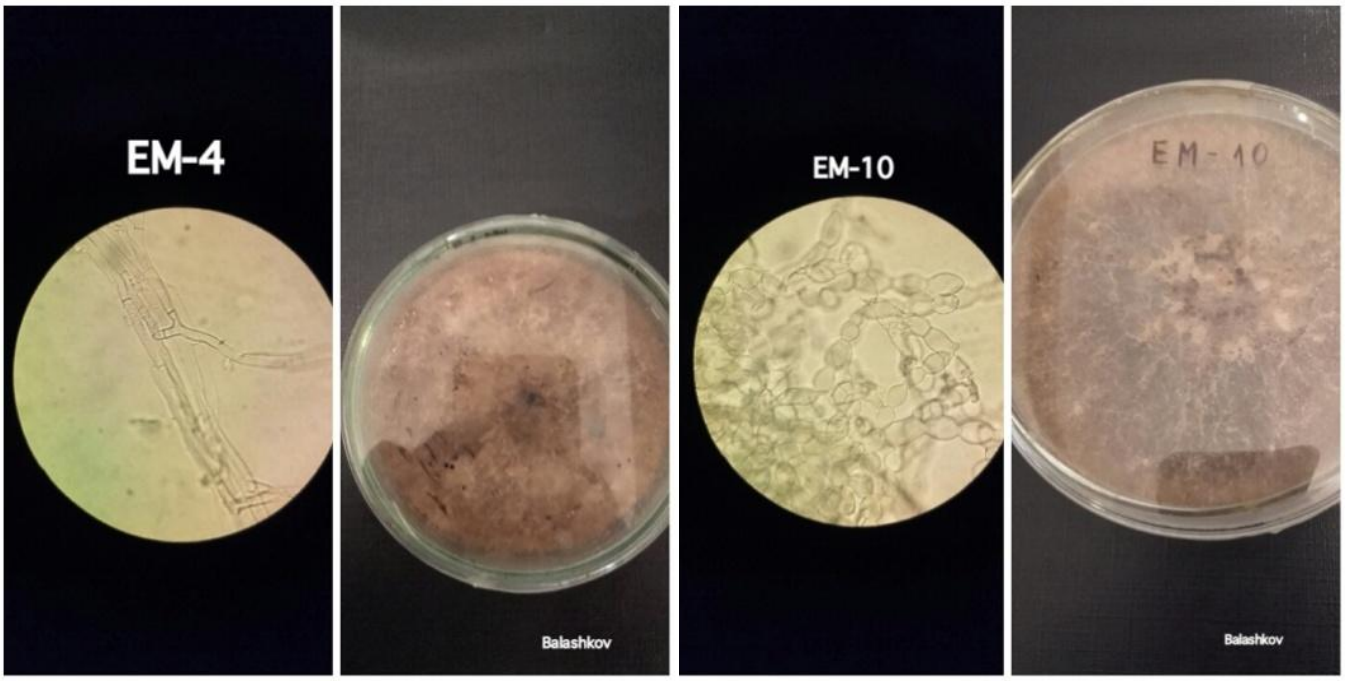

Fig. 3. *Microscopic image of isolates EM-4 and EM-10 and the colonies from which the microscopic preparations were made*

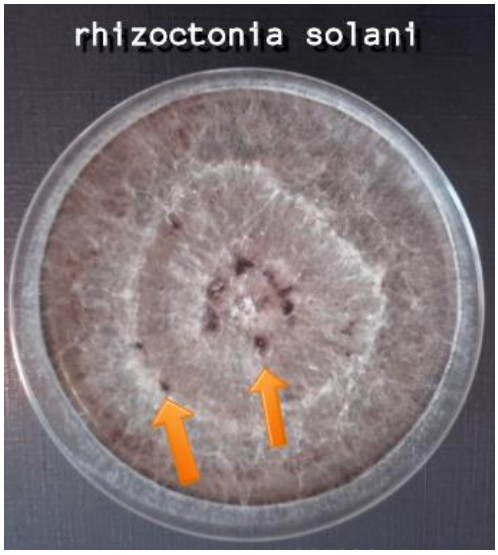

Fig. 4. *Rhizoctonia solani* colony with formed sclerotia

The result of amplification of the ITS region of both tested isolates with ITS5 and ITS4 primers was a 700 bp PCR product (Fig. 5). The sequencing analysis and BLAST search showed that the ITS sequence of the isolate EM-10 agreed 100% with sequences of *R. solani* deposited in GenBank (JQ669932.1, JQ616873.1, KX118331.1, LC017861.1, etc), whereas the isolate EM-4 shared 95% ITS identity with other published isolates of *R. solani* as EU730821.1, FJ492111.2, KX468074.1, KX631318.1, ect. That confirmed the isolation of two genetically diverse isolates *R. solani* from the rotting vine shoots.

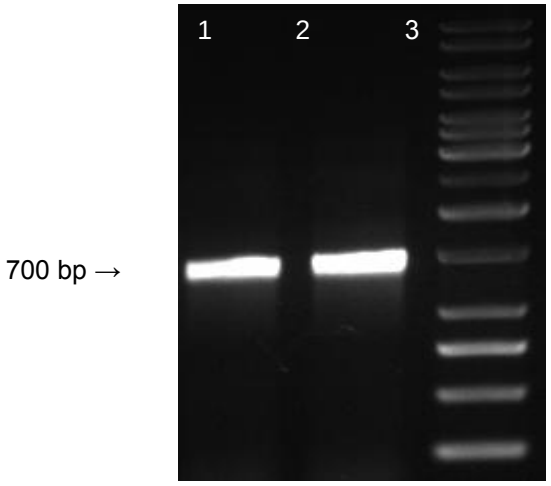

Fig. 5. *PCR amplification of the ITS region of isolates EM-4 and EM-10 with ITS5 and ITS4 primers. 1 – EM-4; 2 – EM-10; 3 – 1 kb DNA marker*

Regarding the mycelium characteristics of the isolated fungal pathogen and results from DNA analyses performed it is referred as *Rhizoctonia solani*. In the past, its sexual form *Corticium solani* belonged to *Basidiomycetes* class (Krastev and Beleva, 1974).

According to the recent classification of fungal plant pathogens, teleomorph of *Rhizoctonia solani* is referred as *Thanatephorus cucumeris*, division *Basidiomycota*, class *Agaricomycetes*, order *Cantharellales*, family *Ceratobasidiaceae* (Gónzalez et al., 2016).

Fungicides' effect on mycelia growth and shoot rot on grafted vine cuttings development.

The experiments were performed with two of the collected *Rhizoctonia solani* isolates – EM-4 and EM-10.

While tracing the radial mycelium growth dynamics of the colonies of EM-4 isolate in laboratory conditions, none of the applied products showed a significant inhibition effect on pathogen mycelia development compared to the untreated control at any of the doses tested. (Fig. 6). Mathematical significance among the differences in mycelium growth rate had not been established, but in all measurements days, the mycelium colony radius of the untreated control is smaller than those in the variants with fungicides.

The mycelium growth rate of the isolate EM-4 was more intensive during the first 24 hrs of development and at the third day, as the fastest-growing rate had the colony treated with Proplant at a dose of 0.2% (V2) (Table 2). Throughout the second day, the mycelia growth rate slowed down, however, its rate in the treated variants corresponded to the control. During the third day, the most active growth was observed compared to the growth rate in the first two days, and a delay in variants V1, V2 and V3 were already recorded in comparison with the control.

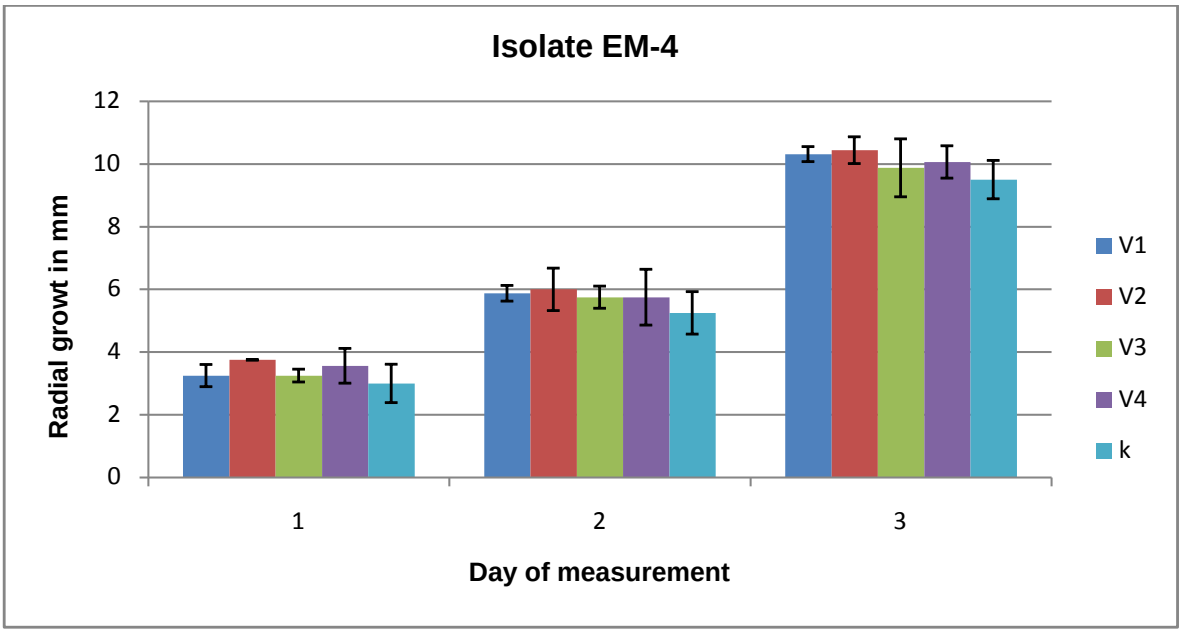

Fig. 6. *Radial growth of colonies from isolate EM-4.*
The radius of the colonies in mm is shown. V1 – Proplant (0.1%); V2 – Proplant (0.2%); V3 – Quadris (0.075%); V4 – Quadris (0.15 %); K – untreated control

Table 2. EM-4 growth rate per day (in mm)

Variant	Day of fungi development		
	1-st	2-nd	3-rd
V1	3.25	2.1	4
V2	3.75	2.2	3.4
V3	3.25	2.5	4.1
V4	3.5625	3	4.8
K	3	2.2	4.5

V1 – Proplant (0.1%); V2 – Proplant (0.2%); V3 – Quadris (0.075%);
V4 – Quadris (0.15 %); K – untreated control

The isolate EM-10 revealed significant growth inhibition compared to the control in variants V3 and V4 during the second and the third day (Fig. 7). At the time of the first recording, the difference was insignificant, and it could not be stated to have a considerable effect.

For variants V1 and V2 during the first recording no difference could be made in comparison with the control, but during the second recording, there was a slight effect in both variants towards the control.

During the last recording, the difference between V1 and V2 was again non-significant. Variants V5 and V6 had non-significant higher growth than the control. The only fungicide that shows some inhibition effect on mycelium growth of isolate EM-10 is Quadris in both applied doses.

Correspondingly, the growth rate of colonies V3 and V4 in 24 hours was the lowest during the first 24 hours compared to the other treated variants and the control (Table 3). In V4 the rate remained the same over the next 24 hours, while in V3 (Quadris at a dose of 0.075%) the growth rate dropped to the lowest level of 2.6 mm, recorded in the trial. The growth inhibition in this variant continued during the third day but significantly smaller.

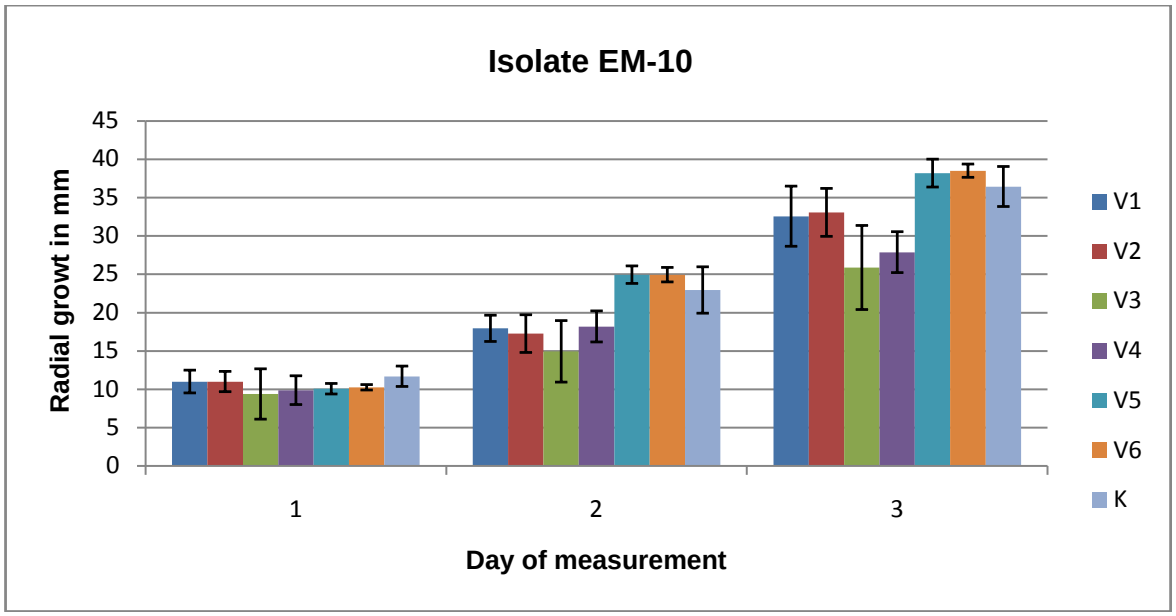

Fig. 7. *Radial growth of colonies from isolate EM-10.*
The radius of the colonies in mm is shown. V1 – Proplant (0.1%); V2 – Proplant (0.2%); V3 – Quadris (0.075%); V4 – Quadris (0.15 %); V5 – Topsin M (0.1%); V6 – Topsin M (0.2 %); K – untreated control

Table 3. EM-10 growth rate per day (in mm)

Variant	Day of fungi development		
	1-st	2-nd	3-rd
V1	11	7.9	14.5
V2	11	7.5	19.3
V3	9.375	2.6	7.8
V4	9.875	9.9	12.6
V5	10.0625	14.1	12.7
V6	10.25	14.1	14.5
K	11.6875	10.2	16.1

V1 – Proplant (0.1%); V2 – Proplant (0.2%); V3 – Quadris (0.075%); V4 – Quadris (0.15 %); V5 – Topsin M (0.1%); V6 – Topsin M (0.2 %); K – untreated control

When comparing the growth rates of EM-4 and EM-10 colonies shown in Tables 2 and 3, it could be concluded that EM-10 isolate induced an active colony growth and therefore it was significantly, approximately three-fold more, fast growing.

Fungicide control of shoot rot on grafted vine cuttings during the stratification.

The standard Switch was a fungicide recommended for gray rot control, which acted on all three races spread on the vine (Gabriolotto et al., 2009; Baroffio et al., 2003).

The lack of effect during the stratification revealed that damage to young shoots was due to a pathogen different from *Botrytis cinerea* The isolates of *Rhizoctonia solani* EM-4 and EM-10 isolated during the stratification demonstrated different susceptibility to the tested fungicides, but in the production conditions, the impact of the high doses resulted in an increase of grafted cuttings not affected by rotting. Their ratio in all treated variants exceeded the control and the standard, but for V2 (Proplant 0.2%) and V4 (Quadris 0.075%), the difference compared to the control was proven and in V3 (Proplant 20 ml/m^2) and V5 (Quadris 0.15%) it was very well provided. The most obvious effect against *Rhizoctonia solani* showed Quadris at a dose of 0.15% (Table 4).

The tested fungicides did not adversely affect the callus formation and the grafting of the cuttings. The cuttings with full circular callus from V4 were practically equal with the control while the cuttings from the other treated variants exceeded them significantly.

The highest ratio of full circular callus was reported again with Quadris at a dose of 0.15%. The difference was very well provided. Results obtained after treatment with the standard were unsatisfactory.

Table 4. Effect of some fungicides on callus formation and healthy shoots development during the grafted vine cuttings stratification

Variant	Callus at the graft (%)						Cuttings with healthy shoots	
	0	1/3	1/2	2/3	Full circular			
V1	-	-	-	45,0	55,0	+	35,0	ns
V2	2.5	-	-	40,0	57,5	+	37,5	+
V3	-	27.5	-	25,0	47,5	ns	47,5	++
V4	-	15.0	-	52,5	32,5	ns	37,5	+
V5	-	27.5	-	25,0	80,0	+++	72,5	+++
K	-	17.5	-	50,0	32,5	x	22,5	x
E		25.6		47,5	27,5	ns	22,5	ns
					GD(5.0%)=20.525 *GD(1.0%)=28.116* *GD(0.1%)=38.315*		*GD(5.0%)=13.786* *GD(1.0%)=18.884* *GD(0.1%)=25.735*	

V1 – Proplant (0.1%); V2 – Proplant (0.2%); V3 – Quadris (0.075%); V4 – Quadris (0.15 %); V5 – Topsin M (0.1%); V6 – Topsin M (0.2 %); K – untreated control

(+) – the difference is proven; (++) – the difference is well secured; (+++) – the difference is very well provided; (n. s) – the difference is unproven

CONCLUSIONS

Based on the mycelium characteristics and DNA analyses performed of the isolated fungal pathogen it is determined that *Rhizoctonia solani* is the causal agent of rotting of the shoots of the grafted vine cuttings during the stratification at the Institute of Viticulture and Enology – Pleven.

In this study, we have found differences among the two isolate of Rhizoctonia solani to fungicides tested. It seems that in natural conditions during the stratification process, the pathogen possess isolates of different susceptibility to different fungicides. It is a base for fast selection of resistant pathogen forms to the applied fungicides for the control of the disease.

In our experiments as a most promising fungicides for the effective control was found Quadris at a dose of 0.075%, which inhibited the mycelium growth both *in vitro* and in vivo during the stratification of the grafted cuttings. The highest effect occurred at a dose of 0.15%. Proplant 722 SL at a dose of 20 ml/m^2 that also successfully inhibited the growth of *Rhizoctonia solani* during the stratification.

The tested fungicides did not adversely affect the callus formation and did not reduce the quality of the graft between the cutting and the rootstock.

REFERENCES

Abrasheva, P., K. Bambalov, A. Georgiev, 2008. Viticulture and Enology. Matkom, Sofia, 344.

Baroffio, C.A., W. Siegfried, U.W. Hilber, 2003. Long-Term Monitoring for Resistance of *Botryotinia fuckeliana* to Anilinopyrimidine, Phenylpyrrole, and Hydroxyanilide Fungicides in Switzerland. Plant Disease. 87, 6, 662-666.

Bobev, S., 2000. Guidebook on Crop Diseases. Sofia, PSSE 194-196.

Braykov, D., S. Pandeliev, L. Masheva, Ts. Mievska, A. Ivanov, V. Roychev, P. Botyanski, 2005. Viticulture. Academic Publishing House of the University of Agriculture, Plovdiv, 408.

Dimova, D., E. Marinkov, 1999. Experimental Works and Biometrics. Plovdiv, Academic Publishing House of the Higher Institute of Agriculture, 263.

Gabriolotto, C., M. Monchiero, M. Nègre, D. Spadaro, M. Gullino, 2009. Effectiveness of Control Strategies against Botrytis cinerea in Vineyard and Evaluation of the Residual Fungicide Concentrations. *Journal of Environmental Science and Health Part B.* 44, 389–396.

Gónzalez, D., M. Rodriguez-Carres, T. Boekhout, J. Stalpers, E. E. Kuramae, A. K. Nakatani, R. Vilgalys, M. A. Cubeta, 2016. Phylogenetic relationships of *Rhizoctonia* fungi within the Cantharellales. Fungal Biology**,** vol. 120, Issue 4, 603–619.

Harizanov, A., M. Todorova, V. Harizanova, 2009. Vine Diseases and Pests. Videnov and Son, Sofia, 221.

Kolev, V., 1962. Viticulture. Zemizdat, Sofia, 148.

Liu, C. H., W. X. Zou, H. Lu, R. X. Tan, 2001. Antifungal Activity of Artemisia annual endophyte Cultures against Phytopathogenic Fungi. Journal of Biotechnology. 88, 277–282.

Malenin, I., 2003. Vine Diseases. Enyovshe, Sofia, 167.

Marais, P. G., 1979. Fungi Associated with Root Rot in Vineyards in the Western Cape. Phytophylactica. 11, 65-68.

Masheva, A. Ts. Mievska, A. Ivanov, V. Roychev, P. Botyanski, P. Kirovski, 2004. Practical Manual in Viticulture. Academic Publishing House of the University of Agriculture, Plovdiv, 186.

Meza-Moller, A., M. Esqueda, F. Sanchez-Teyer, G. Vargas-Rosales, A.A. Gardea, M. Tiznado-Hermandez, 2011. Genetic Variability in *Rhizoctonia solani* Isolated from *Vitis vinifera* Based on Amplified Fragment Length Polymorphis. American Journal of Agricultural and Biological Sciences. 6, 3, 317-323.

Orozco-Avitia, A., M. Esqueda, A. Meza, M. Tiznado, A. Gutierrez, A. Gardea, 2013. Temperature Effect on *Rhizoctonia solani* Analyzed by Microcalorimetry. American Journal of Agricultural and Biological Sciences. 8, 2, 162-166.

Radulov, L., P. Georgieva, 1984. Production of Vine Propagation Material. Zemizdat, Sofia, 76.

Raykov, E., P. Nachev, 1968. Vine Diseases and Pests. Zemizdat, 177.

Raykov, E., P. Nachev, 1971. Vine Diseases and Pests. Zemizdat, Sofia, 198.

Stancheva, Y 2004. General Pathology of Plants, Pensoft, Sofia, 325, 409.

Stancheva, Y., 2006. Atlas of Agricultural Crop Diseases. Pensoft, II, 178-179.

Tafradzhiiski, I., S. Dimitrov, S. Karov, 1967. Practical Mannual to Phytopathology. Hristo G. Danov, Plovdiv, 135.

Vanev, S. 1995. Parasitic fungi on vine on the Balkan Peninsula. Al “Prof. Marin Drinov”, Sofia, 115.

Walker, G. E., 1997. Effects of Meloidogyne spp. and Rhizoctonia solani on the Growth of Grapevine Rootings. Journal of Nematology. 29, 2, 190-198.