COBISS Code 1.01

DOI: 10.14720/aas.2014.103.1.07
Agrovoc descriptors: vitis vinifera, grapevines, land varieties, plant diseases, viroses, grafting, disease transmission, stress

Agris category code: h20, f02

Autografted vines of cultivar 'Refošk' (*Vitis vinifera* L.) reveal symptoms of the rugose wood disease

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Received Janury 23, 2014; accepted March 10, 2014.
Delo je prispelo 23. januarja 2014, sprejeto 10. marec 2014.

ABSTRACT

Rugose wood disease complex is one of the most important graft-transmissible grapevine diseases and it is considered to be a viral disease. With the aim to obtain more information about appearance of rugose wood disease observed on cultivar 'Refošk', 'Refošk' vines from collection vineyard in Komen were used for green grafting on SO4 rootstock and autografts for control were made as well. Rugose wood symptoms were observed on grafts of two 'Refošk' biotypes, which confirmed graft transmissibility. Appearance of rugose wood symptoms on autografts excluded the impact of incompatibility in rugose wood disease, but at the same time it could be proposed that stress caused by grafting has an important role.

Key words: rugose wood complex disease, green grafting, graft indexing

IZVLEČEK

POJAV ZNAMENJ RAZBRAZDANJA LESA NA CEPLJENKAH S SPOJENIMI LASTNIMI DELI TRSOV SORTE *Vitis vinifera*'Refošk'

Kompleks bolezni razbrazdanja lesa je ena od najpomembnejših bolezni vinske trte, ki se prenaša s cepilnim materialom in za katero velja, da naj bi jo povzročali virusi. Da bi pridobili več podatkov o razvoju znamenj bolezni razbrazdanja lesa smo cepiče sorte 'Refošk' s kolekcijskega vinograda iz Komna s tehniko cepljenja zeleno na zeleno cepili na podlago SO4. Za kontrolo smo mladike prerezali in jih ponovno spojili. S pojavom znamenj razbrazdanja lesa na cepljenkah pri dveh biotipih sorte 'Refošk' smo potrdili ugotovitev, da se bolezen prenaša s cepljenjem, medtem ko lahko zaradi pojava znamenj na cepljenkah s spojenimi lastnimi deli mladik sklepamo, da na razvoj bolezni ne vpliva inkompatibilnost cepiča in podlage, temveč bi lahko imel pomembno vlogo stres, ki ga izzove cepljenje.

Ključne besede: kompleks bolezni razbrazdanja lesa, zeleno cepljenje, indeksiranje

1 INTRODUCTION

Rugose wood disease complex is one of the most important graft-transmissible grapevine diseases, however despite numerous studies (Credi, 1997b; Meng et al., 1999; Nakaune et al., 2008) its etiology is still largely unknown. The disease is spread worldwide (Martelli, 1993) and has been found on many grapevine cultivars *V. vinifera* and

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on other species of the genus *Vitis* (Bonfiglioli et al., 1998; Credi, 1997a; Nakaune et al., 2008).

Rugose wood complex is a disease complex usually characterized by modifications of the woody cylinder that is typically marked by pits and/or grooves (Martelli, 1993). The ridges of the cortex consist of hyperthrophied rays extending from the bark into the functional xylem (Martelli, 1993). Anatomical abnormalities originate from the altered behavior of the vascular cambium (Martelli, 1993). The symptoms can appear either on scion, rootstock or on both (Martelli, 1993). The rugose wood complex of diseases are essentially diseases of grafted vines, since appearance of symptoms for non-grafted vines is unusual (Bonfiglioli et al., 1998).

Rugose wood disease is considered to be a viral disease, although this assumption is based only on its graft transmissibility and in part on its vector transmissibility (Martelli, 1993). Rupestris stem pitting, the most widespread disease of the rugose wood complex, is consistently associated with *Grapevine Rupestris stem pitting associated virus* (GRSPaV) (Meng et al., 1999; Nakaune et al., 2008). On the other hand GRSPaV was also detected in asymptomatic vines (Meng et al., 2005; Nakaune et al., 2008). Recently GRSPaV was observed to be closely associated with vein necrosis symptoms (Bouyahia et al., 2005). The exact etiological role of GRSPaV in different diseases remains unknown. *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) are thought to be involved in Kober stem grooving and Corky bark, respectively (Bonavia et al., 1996; Garau et al., 1994) and they are transmitted by mealybug (*Phenacoccus aceris*) (Le Maguet et al., 2012). However, due to the complexity of the disease, a general causal agent has not been identified yet.

Symptoms similar to those of rugose wood were observed and recorded in Slovenia as well. 'Refošk' vines from the collection vineyard in Komen, Slovenia, that have shown diverse rugose wood symptoms, like swelling above the grafted site, deep grooves, pitting, thicker scion vines; were already tested by ELISA, ISEM, Western blot and RT-PCR for the presence of the rugose wood disease related viruses (Petrovic et al., 2003; Tomazic et al., 2005a; Tomazic et al., 2005b; Tomazic et al., 2008) but none of the tested viruses could be correlated with rugose wood disease on the cultivar 'Refošk'.

According to EPPO certification scheme for the production of healthy plants for planting, graft indexing is still a compulsory step, since rugose wood disease can be identified solely on woody differential hosts; in case of *Grapevine virus A* and *Grapevine virus B* molecular testing is recommended (EPPO, 2008). However, it was already shown that biological indexing tests may not be completely reliable, because results can be affected by various elements, such as a possible synergistic effect of various causal pathogenic agents and possible latency of wood disorders (Credi, 1997a). Due to extreme complexity of rugose wood disease complex much more research is needed to enable the use of modern technologies to clarify the etiology of this disease. We believe the work we are presenting in the present paper will contribute to enhanced understanding of the etiology of rugose wood disease complex.

The first objective of this study was to find out if rugose wood symptoms are expressed in green grafts and to determine when they appear after green grafting. Indexing tests are most frequently performed with the dormant chip budding method, which may take two to several years before rugose wood symptoms become visible (Credi, 1997b; Martelli, 1993). The green grafting method has several advantages in comparison with woody grafts: for several of the graft-transmitted diseases, symptoms develop in a matter of weeks instead of months; less space is required for green-grafted indicators and it can be done throughout the growing season (Walter et al., 1990). Green graft indexing is used in Slovenia in grapevine sanitary selection as well. However, Walter et al. (1990) didn't manage to detect stem pitting when using green grafting on the indicators Kober 5BB and *V. rupestris* after 3 months. Besides that, when they compared results of green grafting and dormant budding, they discovered more positive indicators of corky bark on grafts with the dormant budding technique. The second objective of this work was to test if rugose wood symptoms are expressed on autografted 'Refošk' vines to determine if the appearance of rugose wood symptoms could be due to a physiological response of the plant to grafting stress, rather than or in addition to the transmission of a virus or virus-like agents.

2 MATERIAL AND METHODS

2.1 Plant material

Plant material for indexing was obtained from the collection vineyard in Komen (N45 48.917 E13 44.692), established in 1989 when old 'Refošk' vines from the field were propagated by grafting on SO4 (*V. berlandieri* x *V. riparia*) and planted in blocks of 3 to 35 vines per biotype. Vines are trained as double guyot and cultivated according to the instructions of integrated pest management.

Fifteen percent of vines from collection vineyard have shown rugose wood symptoms on rootstocks and/or scions, while 18 % of plants with rugose wood symptoms died in 10 years after planting (Tomazic et al., 2005b). The virus status of 'Refošk' vines used in this study was already reported (Petrovic et al., 2003; Tomazic et al., 2005a; Tomazic et al., 2005b; Tomazic, 2002; Tomazic et al., 2008). Results were summarized in Table 1. However, virus status could be changed in case of transmission.

'Refošk' vines used for indexing are listed in Table 1. As a putative source of rugose wood, vines labeled as 'Refošk' 20, 38, 48 and 51 were used. From vineyard observation 'Refošk' biotypes 38 and 48 develop symptoms on itself, on the other hand 'Refošk' biotypes 20 and 51 induce apparent rugose wood symptoms on SO4 rootstock while scion parts maintain a healthy appearance (Table 3). Regarding the differences in symptoms expression, there could be two different types of rugose wood. 'Refošk' biotypes 43 and 61 never showed symptoms in the vineyard and were used as controls. In June 2009, shoots of 'Refošk' vines were collected in the morning and immediately transported to the Vine Selection Center in Vrhpolje, where green grafting on SO4 rootstock and autografting were carried out.

2.2 Grafting experiment

For the rootstock, shoots of 'Refošk' vines and SO4 were cut on two buds (right below lower bud and in the middle of internode above the next bud), while the scion cuttings had one bud. Leaves on upper rootstock node and on the scion were trimmed to about half of their original size before grafting. The method used for indexing was machine splice grafting. The assembled graft was wrapped with white first aid tape. Rootstocks were treated with naphthalene acetic acid (NAA) (Germon Bewurzelungspuder H per talee legnose, Conc. E. Gerlach GmbH, Germany). Grafts were planted in vermiculite and kept for 34 days in humid chamber on 28 °C and 85 % relative humidity. After that period rooted grafts were transplanted into universal substratum in flowerpots and transferred to the green house to the controlled water table for constant irrigation where they were maintained till September 2012.

The grafts were visually examined monthly for the presence of the rugose wood symptoms on the scion and rootstock parts in 2009 and 2010, while the last two years only at the end of active vegetation at the beginning of September. At final examination all grafts were autoclaved and bark was peeled away.

Table 1. Virus status of 'Refošk' vines from collection vineyard in Komen, Slovenia, as reported in previous studies.

Virus / 'Refošk' vine	20 (IV[d]/110[e])	38 (VIII/44)	43 (VIII/113)	48 (IX/43)	51 (IX/69)	61 (XII/68)
ArMV[a]	-	-	-	-	-	-
GFkV[a]	+	-	-	-	-	-
GFLV[a]	-	-	-	-	-	-
GLRaV-1[a]	-	-	-	-	+	-
GLRaV-2[a]	-	-	-	-	?	+
GLRaV-3[a]	-	-	-	-	-	-
GLRaV-6[a]	+	+	-	-	-	-
GLRaV-7[a]	-	-	-	-	?	-
GVA[a]	-	-	-	-	-	-
GVB[a]	-	-	-	-	-	-
RSPaV-1[a, d]	+[a,b,d]/-[c]	+[a,b,d,c]	+[a,b,d]/-[c]	+[a,b,d]/-[c]	+[a,b,d]/-[c]	+[a,b,d] / -[c]

[a] ELISA testing, [b] PCR testing, [c]ISEM testing, [d]Western blot testing, [d] Raw in collection vineyard, [e] Vine number in raw

? unable to confirm infection since threshold was difficult to determine due to higher background in ELISA testing

3 RESULTS AND DISCUSSION

A major part of research on rugose wood disease on 'Refošk' was done in the field of virology, but no causal agent was identified that could be used in sanitation for rugose wood affected vines (Tomazic et al., 2005a; Tomazic et al., 2005b; Tomazic et al., 2008). In the collection vineyard in Komen it is observationally evident that vines, propagated by grafting from the same mother vines are showing similar rugose wood symptoms, which indicates that rugose wood is transmitted with grafting. This is consistent with rugose wood complex in general (Martelli, 1993).

Indexing for rugose wood associated diseases is usually carried out using standard indicator vines, i.e. *V. rupestris* St. George, LN 33 (Coudero 1613 x *V. berlandieri*) and Kober 5BB (*V. berlandieri* x *V. riparia*). In our study, grafting on rootstock SO4 was performed, since it is frequently used rootstock in Kras and Slovenska Istra winegrowing districts, it develops rugose wood symptoms and it was used in the collection vineyard in Komen.

3.1 Survival rate of grafted material

The results of autografting and grafting 'Refošk' on SO4 are summarized in Table 2. At final monitoring from 27.3 % to 80 % of 'Refošk' autografts were still growing. The highest mortality rate at last monitoring was observed in 'Refošk' 43, 51 and 61, reaching 60 % to 72.7 %. 'Refošk' 43 and 61 biotypes are symptomless in the vineyard. On the other hand only 30 % of 'Refošk' 38 and 20 % of 'Refošk' 48 autografts died, while both biotypes show the most severe rugose wood symptoms in the vineyard.

Considering the results of grafting 'Refošk' on SO4 from 25 % to 40 % of grafts were viable at final monitoring, except the 'Refošk' 51 grafts with survival rate of 60 %.

The survival rate of grafted material could not be explained by different grafting treats, especially due to low number of survived 'Refošk' 43 and 61 autografts, higher number of survived 'Refošk' 38 and 48 autografts and very variable survival rate of 'Refošk' 20 and 51 autografts and grafts; therefore it could be assigned to random effect. In the case that grafts, putatively affected with rugose wood, show lower survival rate, they could be eliminated in early stages of planting material production in vine nursery.

Table 2: Graft success and number of 'Refošk' autografts and grafts on SO4 rootstock at final examination. 'Refošk' vines used for green grafting were from collection vineyard in Komen, Slovenia.

	Autografted 'Refošk' vines					'Refošk' grafted on SO4				
'Refošk' vine	Total number of autografts (12th June 2009)	Successfully rooted autografts at the time of transplanting (17th August 2009)		3rd September 2012		Total number of grafts (12th June 2009)	Successfully rooted grafts at the time of transplanting (17th August 2009)		3rd September 2012	
		n	%	n	%		n	%	n	%
'Refošk' 20 IV/110	11	10	90,9	7	63,6	12	11	91,7	3	25,0
'Refošk' 38 VIII/44	10	9	90,0	7	70,0	10	7	70,0	3	30,0
'Refošk' 43 VIII/113	10	9	90,0	4	40,0	10	8	80,0	4	40,0
'Refošk' 48 IX/43	10	10	100,0	8	80,0	8	7	87,5	3	37,5
'Refošk' 51 IX/69	11	8	72,7	3	27,3	10	9	90,0	6	60,0
'Refošk' 61 XII/68	10	9	90,0	3	30,0	10	8	80,0	3	30,0
Total / Average (%)	62	55	88,9	32	51,8	60	50	83,3	22	36,7
SO4	10	7	70,0	4	40,0	10	8	80,0	3	30,0
Total / Average (%)	72	62	86,1	36	50,0	10	8	80,0	4	40,0

3.2 Expression of rugose wood symptoms on 'Refošk' 38 and 'Refošk' 48 autografts and grafts on SO4 rootstock

The first typical symptoms of rugose wood were observed on 'Refošk' 38 and 48 vines at last examination, i.e. 39 months after grafting. Fine grooving was observed on the scion part of 'Refošk' 38 and 48 vines grafted on SO4 rootstock, while no evident symptoms were seen on SO4 rootstock (Table 3 and Figure 1). When 'Refošk' 38 and 48 were autografted, the symptoms of rugose wood were observed on the scion part of 'Refošk' 38 autografts, while on 'Refošk' 48 autografts symptoms were observed on the scion and rootstock (Table 3 and Figure 1). According to the literature this is the first report of observed rugose wood symptoms on autografts.

Appearance of rugose wood symptoms on grafts and autografts of 'Refošk' 38 and 'Refošk' 48 confirmed that rugose wood is transmitted by grafting. Since the symptoms were observed on autografts then the effect of incompatibility in the development of rugose wood disease could be eliminated. Possible reasons could therefore be reaction of plant as response to stress and wound healing or synergistic effect of stress and pathogen agents. The low number of 'Refošk' 38 and 'Refošk' 48 grafts and autografts with symptoms could be explained in two ways: a) in the case that pathogen agents causing the development of rugose wood are viruses, then it is possible that they were not already transmitted to green canes. It is known that viruses are unevenly distributed throughout the canopy (Fiore et al., 2009; Rowhani and Uyemoto, 1997); b) the second reason is possible latency of symptoms as reported by (Credi (1997a); Martelli, 1993). It should be also taken into consideration that symptoms could become visible if plants would be left in green house for additional one year or if different growing conditions were applied.

Although green-grafting was used, more than three years were needed in order to symptoms of rugose wood became visible what is similar to graft indexing with chip budding method (Credi, 1997b; Martelli, 1993).

Table 3: The results of graft indexing and the results of vineyard monitoring for rugose wood symptoms.

'Refošk' vine	Symptoms observed in collection vineyard		Graft indexing results	
	'Refošk' (scion)	SO4 (rootstock)	Appearance of rugose wood symptoms on autografts	Appearance of rugose wood symptoms on 'Refošk'/SO4 grafts
'Refošk' 20 IV/110	-	+	-/-	-/-
'Refošk' 38 VIII/44	+	-	+/- (2/7)	+/- (1/3)
'Refošk' 43 VIII/113	-	-	-/-	-/-
'Refošk' 48 IX/43	+	-	+/+ (2/8)	+/- (1/3)
'Refošk' 51 IX/69	-	+	-/-	-/-
'Refošk' 61 XII/68	-	-	-/-	-/-

- minus or plus before slash indicates absence or presence of rugose wood symptoms on scion, while sign after slash indicates absence or presence of symptoms on rootstock
- numbers in parenthesis indicate the number of autografts or grafts, out of total number of viable vines observed at last monitoring

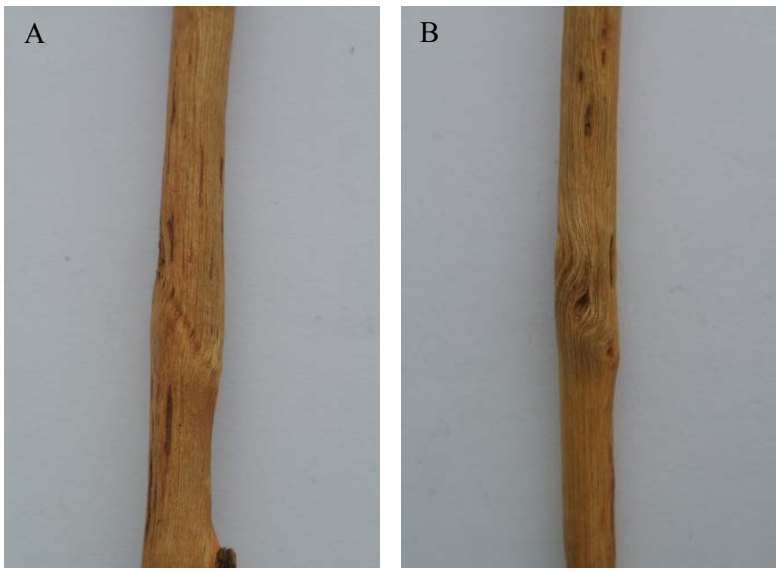

Figure 1: 'Refošk' 48 autograft showing fine grooving on rootstock and on scion part (A); 'Refošk' 38 grafted on SO4 rootstock (B). Symptoms of rugose wood are visible on scion part.

3.3 Symptomless 'Refošk' 20 and 'Refošk' 51 autografts and grafts on SO4 rootstock

The rugose wood symptoms were not detected on 'Refošk' 20 and 51 grafs and autografts (Table 3). Therefore, we were not able to confirm transmittance of rugose wood on SO4 with 'Refošk' 20 and 'Refošk' 51. We proposed that the source of rugose wood should be 'Refošk' vines since infection of rootstock in collection vineyard could be eliminated because rugose wood is present only in specific biotypes and not randomly across vineyard as would be expected in case of the infected rootstock. The possibility of symptomless SO4 could be that a different SO4 clone was used for grafting than the one used in the collection vineyard. Differential sensitivity of clones was observed for example in 'Syrah' clones for Syrah decline, which is also a disease causing

degeneration of the woody cylinder (Renault-Spilmont et al., 2007).

As hypothesized by Credi (1997b) development of rugose wood is dependent also on abiotic factors, as in the case of leafroll where the choice of indicator is based on climatic conditions (EPPO, 2008). For optimum symptoms expression in green grafting effects of temperature and light conditions could be optimized (Walter et al., 1990). This could also improve the development of symptoms in 'Refošk' 38 and 'Refošk' 48 grafts and autografts. However, it is possible, that symptoms would become expressed in the green grafts after one additional year. Walter et al. (1990) did not manage to detect stem pitting disease symptoms on indicators Kober 5BB and *V. rupestris* a few months after green grafting and concluded that perhaps a longer incubation period is required for classic symptoms to develop.

4 CONCLUSIONS

With this experiment we were able to confirm graft-transmissibility of rugose wood. However, graft success of grafts with vines showing rugose wood symptoms in the collection vineyard was not affected, which means that they are not excluded in the process of planting material production. The appearance of rugose wood symptoms on autografts supports the statement that effect of incompatibility is not involved in rugose wood. On the other hand stress caused by grafting could have notably impact on the development of symptoms what makes rugose wood even more complex. Proper growing conditions of graft indexing trials should, therefore, be defined in order to maximize the expression of rugose wood symptoms.

5 ACKNOWLEDGEMENTS

The authors thank the Slovenian Research Agency for financial support to MH, contract No. 1000-08-310189. Many thanks to Andreja Škvarč, head of the Vine Selection Centre Vrhpolje for kindly offering a place in greenhouse and to Matej Vrčon for their technical help and for growing the greenhouse plants. The authors thank dr. Jernej Jakše for the suggestions that improved the manuscript.

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