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Influence of Raspberry bushy dwarf virus on pollination of red raspberry (*Rubus idaeus* L.) cultivars

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Abstract Raspberry bushy dwarf virus (RBDV) is one of the major pollen-borne pathogens of the genus *Rubus* that causes drastic reduction of yield and degradation of berry quality. The aim of the study was to evaluate the quality of raspberry pollen and the effect of RBDV on pollination process. The research was carried out at the Institute of Horticulture. Within two years, 2017 and 2018, the pollen viability and pollen germination capacity of nine raspberry cultivars were analysed. The cross-pollination was done and the pollination quality of 31 crossing combinations was evaluated. The study found that although the pollen viability of cultivars infected with RBDV was higher than that of uninfected cultivars, there were no statistically significant differences. The viral contamination of the mother plant played a more important role in the pollination process. Pollination was better on uninfected mother plants and pollen germinated was faster than on infected plants. However, when the virus-infected cultivars were pollinated with infected pollen, the virus had an effect on the growth rate of pollen tubes, that decreased and the pollen tubes did not reach the ovary.

Key words: pollen germination, pollen viability, hybridization, fluorescence microscopy.

INTRODUCTION

Most of commercially grown raspberries are self-fertile (Daubeny, 1971). Insect activity helps to distribute pollen, increase the number of drupelets, and thus affect the size of the fruit (Cane, 2005). The number of drupelets may also be increased if different cultivars are crossed (Colbert & de Oliveira, 1990; Żurawicz et al., 2018), though the increase is largely dependent on the cultivars, which are used in crossing (Żurawicz, 2016).

Pollination is an essential step in the reproduction of flowering plants, while plant viruses can be spread via pollen during pollination process. There are known 46 plant viruses that may spread via pollen, where 18 of these can cause both: mother plant (horizontal transmission by pollen) and seed (vertical transmission by pollen) infection during pollination and fertilization (Card et al., 2007; Isogai et al., 2014). Raspberry bushy dwarf virus (RBDV) is one of them (Murant et al., 1974; Isogai et al., 2014).

The RBDV is widespread and infects wild and cultivated plants of the genus *Rubus* worldwide (Murant, 1976; Murant, 1987; Martin et al., 2013). The economic importance of RBDV is difficult to assess (Murant, 1987), but it significantly affects the yield and quality of raspberries (Murant et al., 1974; Daubeny et al., 1982). In addition, the virus spreads very rapidly and susceptible cultivars may be contaminated during the first two to three flowering seasons (Murant et al., 1974) and within five to six years the plants may be 100% infected with the virus (Bulger & Martin, 1990; Martin, 2002).

The virus spreads by transferring RBDV infected pollen with bees and other insects to uninfected plants during the pollination process (Chard et al., 2001). Infected pollen first land on the stigma and infect it, and then lead to systemic infection of the plant (Jones et al., 1982; Isogai et al., 2014; Isogai et al., 2015). Drupelet abortion is one of the most visible signs of virus infection in berries, called 'crumbly fruit', which consists of a few large, irregularly shaped drupelets, and, when picked, it crumbles into individual drupelets due to poor drupelet adhesion (Murant et al., 1974; Daubeny et al., 1978; Moore & Robbins, 1990; Martin et al., 2013).

Virus spreads very rapidly. Most of the reported susceptible cultivars can became infected during the first two to three flowering seasons, if the source of infection is close (Murant et al., 1974; Jones et al., 1982). Therefore the planting of resistant cultivars is the only method to control the virus (Murant, 1987). Studies on the mechanism of infection with RBDV in raspberry plants are available in the literature, while there is a lack of studies on the effect of the virus on viability of pollen and the pollination process. The aim of our study was to evaluate the viability of pollen infected with RBDV and the pollination process itself.

MATERIALS AND METHODS

The research was carried out at the Institute of Horticulture (LatHort) in 2017 and 2018. During previous studies done by the Unit of Plant Pathology and Entomology of LatHort, the infection by RBDV virus was determined for nine raspberry cultivars: 'Lubetovskaja', 'Ina', 'Glen Ample', 'Glen Rosa', 'Glen Moy', 'Glen Rosa', 'Glen Magna', 'Glen Doll', which were used in this study.

Raspberry pollen grains were collected shortly before pollination when the white petals appeared and the buds were still closed (BBCH 59). Pollen viability was determined by using the method of staining with acetic carmine solution, where the fertile pollen coloured in carmine red colour, whereas the sterile pollen remained light brown or pale pink and had an atypical shape.

Pollen germination capacity test was carried out *in vitro* on solid nutrient medium in various germination conditions. Two types of medium were used in 2017and 2018:

- 10% sucrose medium = 0.5 g agar + 5 g sucrose + 50 mL distilled water;
- 15% sucrose medium = 0.5 g agar + 7.5 g sucrose + 50 mL distilled water + 0.015 g boric acid.

The medium with pollen was placed in a box with moistened filter paper, which retains air humidity, and then placed in an incubation chamber (Memmert, Germany) at 22 °C and incubated for four hours. Leica DMLS microscope (objective 40×) was used for counting live but ungerminated and germinated pollen (a small tube longer than pollen length was visible). In order to evaluate whether pollen germination increases over time, pollen grains were germinated for more than 12 hours on 10% sucrose

medium in Petri dishes. The temperature increase by 2 °C and 20% sucrose medium (0.5 g agar + 10 g sucrose + 50 mL water + 0.02 g boric acid) was used additional in 2018 to assess the difference between cultivars in diverse growing conditions.

In the study, two RBDV-infected cultivars: 'Ina' and 'Glen Doll', and two non-RBDV-infected: 'Lubetovskaja' and 'Glen Ample' were selected for cross-pollination. The flowers were counted on the canes of open pollination. On the canes evaluated for self-pollination, isolators were put on flowers and the flowers were not emasculated. In the treatment with cross-pollination, the flowers under isolators were emasculated. Emasculated flowers were pollinated according to the crossing plan 2 to 3 days after emasculation. In study, 31 crossing combinations were performed, including open pollination and self- pollination.

Fluorescence microscopy was used to determine the biocompatibility. Pollinated flowers were picked on days 1, 2, 3, 4 and 5 after pollination and then used for laboratory analysis. The flowers were fixed in FAA solution (80% ethyl alcohol, 37% formalin, 100% glacial acetic acid; ratio 8:1:1) for 24 hours, then rinsed for 3.5 h in water, 0.5 h in distilled water, and 70% ethyl alcohol was added after the final rinse. Preserved flowers began to be prepared 33 hours prior to microscopy. Firstly they were macerated in 8N NaOH for 12 h, then rinsed for 8 h in water and 1 h in distilled water. The flowers were stained in 0.1% aniline blue and 0.1N K_3PO_4 aqueous solution for at least 12 hours. Fluorescence was monitored by adjusting the settings of the Leica DMLS microscope, adding an additional device for fluorescent light, and working in the dark. Samples were viewed at 10× and 40× magnification. How far the pollen tubes have grown was evaluated visually (evaluated as: no pollination; fluorescent pollen, where the pollen tubes has grown to ¼; ½; ¾ or full length of the style). The percentage of non-pollinated stigmas was determined relative to the total number of counted pistils for every crossing and standard deviations calculated. The percentage of pollen germination rates on each sampling day were determined as well. Average values of 5 days for each crossing were used for further analysis. The averages were grouped into four groups based on a viral infection of the female and male plants:

1) uninfected mother plant pollinated with infected pollen;
2) uninfected mother plant pollinated with uninfected pollen;
3) infected mother plant pollinated with infected pollen;
4) infected mother plant pollinated with uninfected pollen.

To evaluate the potential impact of environmental factors on the pollen development process, meteorological data (air temperature, air relative humidity, precipitation and mean wind speed) in May and June of 2017 and 2018 were included in the data analysis. Meteorological data were collected by station 'Lufft' located in the orchard.

Data analysis was performed using Microsoft Office Excel 2007. The mean viability of the pollen and standard deviation between readings were calculated, the one-factor analysis of variance was performed. Mean germination rate and standard deviations were calculated for each growing condition and for each studied cultivar. One-factor analysis of variance was performed.

RESULTS AND DISCUSSION

Meteorological data

The data on meteorological conditions in May-June of 2017 and 2018 is presented in Fig. 1. The significantly lower average air temperature (11.78 °C) was recorded in May 2017 compare to May 2018. During this period the air temperature was between 1.71 and 19.85 °C, whereas in May 2018, the air temperature was between 10.38 and 21.74 °C. In both years, the minimum and maximum air temperatures during June were similar: 10.00 to 19.58 °C in 2017, and 10.30 to 21.23 °C in 2018. in June 2017, air humidity was 74.23% and it was significantly higher than in June 2018. Wind speed, which can affect pollen transport from plant to plant, was significantly higher in June 2017 (2.5 m s^{-1}) compare to June 2018, but significantly lower in May 2018 (1.54 m s^{-1}). The amount of precipitation was significantly higher (1.97 mm m^{-2}) in June 2017.

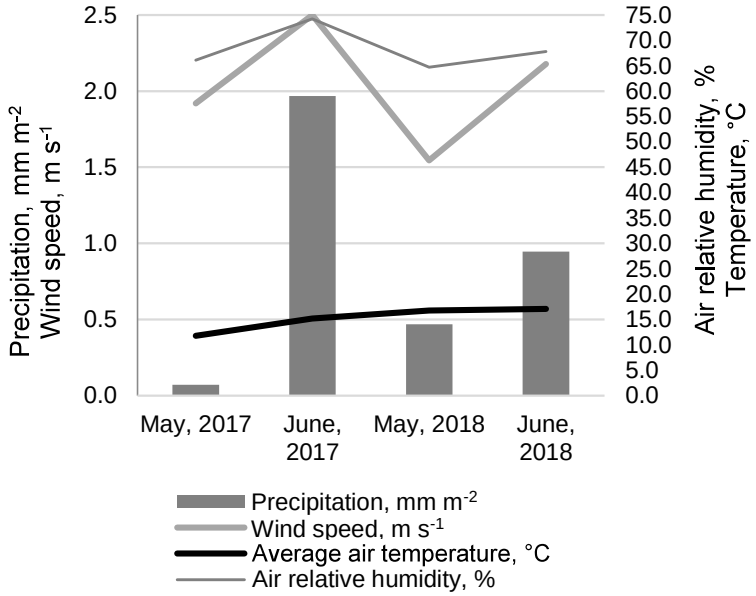

Figure 1. Precipitation (mm m^{-2}), wind speed (m s^{-1}), average air temperature (°C) and air relative humidity (%) in May and June of 2017 and 2018.

Viability

The average pollen viability of five cultivars used in the study was 92%, (*SD* 6%) in 2017 (Fig. 2). 'Glen Ample' (93.8%), 'Alvi' (94%), and 'Ina' (94.5%) infected with RBDV showed highest pollen viability, but no significant difference was found between cultivars in 2017 ($p = 0.09$).

Pollen viability of seven cultivars studied in 2018 ranged from 93% to 99%, the average viability was 97% (*SD* 3.1%) (Fig. 3). Significant difference was found between cultivars ($p = 3.65 \times 10^{-5}$). 'Lubetovskaja' (viability of pollen 93%) had significantly lower amount of viable pollen than other cultivars.

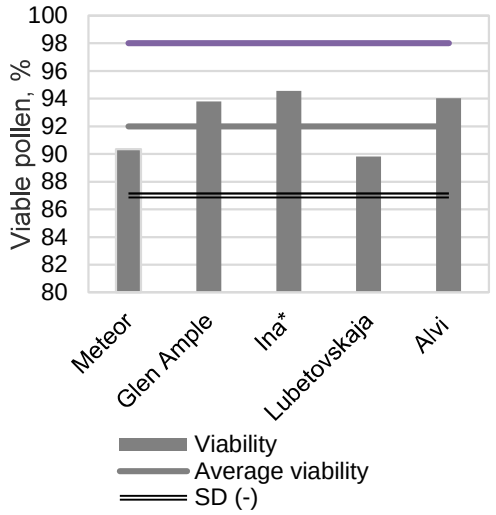

Figure 2. Pollen viability of raspberry cultivars 'Ina', 'Alvi', 'Glen Ample', 'Meteor' and 'Lubetovskaja' in 2017, expressed as a percentage of evaluated pollen grains.

*Infected cultivars by RBDV, *SD*(-) and *SD*(+) – min and max borders of mean standard deviation of pollen viability.

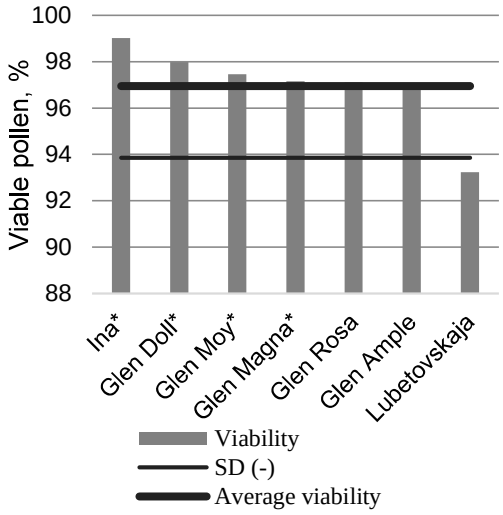

Figure 3. Pollen viability of raspberry cultivars 'Ina', 'Glen Doll', 'Glen Moy', 'Glen Magna', 'Glen Rosa', 'Glen Ample' and 'Lubetovkaja' in 2018, expressed as a percentage of evaluated pollen grains.

*Infected cultivars by RBDV, *SD*(-) and *SD*(+) – min and max borders of mean standard deviation of pollen viability.

The difference in results between years could be affected by the different meteorological conditions during the raspberry flowering and pollination time in May and June. For example, in May 2017, the average air temperature was significantly lower compare to May 2018. Minimum and maximum temperatures in May were also relatively different between the both years, 1.71 and 19.85 °C in 2017, respectively, while in May 2018, it was 10.38 and 21.74 °C, so in 2017, the pollen viability may had been affected by relatively much lower temperatures during pollen development and raspberry flowering, which were close to zero at some periods. Though, Otterbacher et al. (1983) is concluded that the reduction in pollen viability is caused directly by high-temperature stress. In our experiment, the pollen viability was above 93% for all cultivars in 2018, which is considered as high. In similar studies, fresh pollen viability for species *Rubus ellipticus* ranged from 32.5 to 97.7% (Pawar et al., 2017) and *Rubus paniculatus* S. 73.3% (Hiregoudar et al., 2019). In the study of Gercekcioglu et al. (2000), pollen viability in *Rosaceae* trees ranged from 71.5 to 81.8%.

Germination

Studied cultivars showed different pollen germination rate on 10 and 15% sucrose medium at 22 °C for 4 hours in 2017 and 2018 (Table 1).

Cultivated for more than 12 hours on 10% sucrose medium, 'Lubetovskaja' showed the best germination capacity (29%). The average germination rate of cultivars was 25.3%, with standard deviation 2.4%. There was no significant difference in the percentage of germinated pollen compared to cultivation for 4 hours on 10% medium.

However, it was visually observed that the length of the pollen tubes of all cultivars were significantly longer after maintenance of 12 hours.

Table 1. Average pollen germination rate of raspberry cultivars, expressed as a percentage of the listed pollen on 10 and 15% sucrose medium at 22 °C for 4 hours in 2017 and 2018

Cultivar	10% sucrose		15% sucrose		Average for cultivar	$SD_{cultivar}$
	2017	2018	2017	2018		
Meteor	15.1	-	24.9	-	20.0	6.9
Alvi	15.9	-	10.6	-	13.3	3.7
Glen Ample	13.9	23.9	18.0	40.0	23.4	11.5
*Ina**	22.6	24.4	27.2	62.5	34.1	18.0
Lubetovskaja	16.6	22.7	14.1	55.3	27.2	19.1
Glen Doll	-	30.0	-	33.6	31.8	2.5
Glen Moy	-	25.4	-	18.8	22.1	4.7
Glen Rosa	-	30.5	-	44.2	37.4	9.7
Glen Magna	-	22.5	-	37.7	30.1	10.7
average	16.8	25.6	19.0	43.3	26.2	
SD_{medium}	3.4	3.3	6.9	16.4	7.2	

*RBDV infected cultivars are presented in italic.

In general, comparing the data of both study years and pollen germination on different media in different growing conditions, it was observed that the best pollen germination was on 15% sucrose media, at 22 °C for 4 hours - 43.3% (SD = 16.4%). Cultivars showed the lowest germination capacity on 20% sucrose medium at 24 °C for 12 hours - 10.9% (SD_{medium} = 2.1%).

It was observed that in each germination condition one of the cultivars studied showed better pollen germination than the other, so it can be concluded that each cultivar has its own optimal pollen germination conditions. However, the hypothesis at the beginning of the study, that pollen from the RBDV infected cultivar has a lower pollen germination capacity, was not confirmed. The composition of the medium and the germination time had a greater influence on the pollen germination capacity. Pollen germination was performed on 10, 15 and 20% sucrose medium. Although there are studies that have shown that increasing the concentration of sucrose in the medium promotes pollen germination (Hiregoudar et al., 2019), the best medium for raspberry pollen in this study was the 15% sucrose medium. This medium is also optimal option for germinating of pollen from other *Rosaceae* plants, such as apple tree *(Malus)* (Abdel, 1999). For cherry laurel (*Prunus laurocerasus* L.) the best results were observed after 24h of germination on 15% sucrose medium (Sulusoglu & Cavosuglu, 2014). This germination time was also optimal to *Rubus paniculatus* S. (Hiregoudar et al., 2019), but in this study the optimal medium was 25% Sucrose + 5ppm Gibberellic acid.

In N. Pawar study (Pawar et al., 2017), 25% sucrose medium was also determined as optimal for *Rubus ellipticus* pollen germination, while there was added 0.4% boric acid to medium and the germination was observed after 48 h.

Interestingly, the cultivars 'Ina' and 'Lubetovskaja', which showed the highest and lowest levels of pollen viability, respectively, showed the highest susceptibility to germination media in the germination test. For cv. 'Ina' the standard deviation between the media was 17% and for cv. 'Ļubetovskaja' the standard deviation was 16.2%.

Cross-pollination

More non-pollinated stigmas were found in crosses, where an infected cultivar was used as a female plant (Fig. 4). By using self-pollination the uninfected cultivars had slightly more non-pollinated stigmas (20.6 ± 1.7%) than infected cultivars (16.4%), while the standard deviation was higher - 12.2%. The least amount of non-pollinated stigmas was in open pollination, 8.2 ± 0.3% for uninfected cultivars and 2.9 ± 3.3%for infected cultivars.

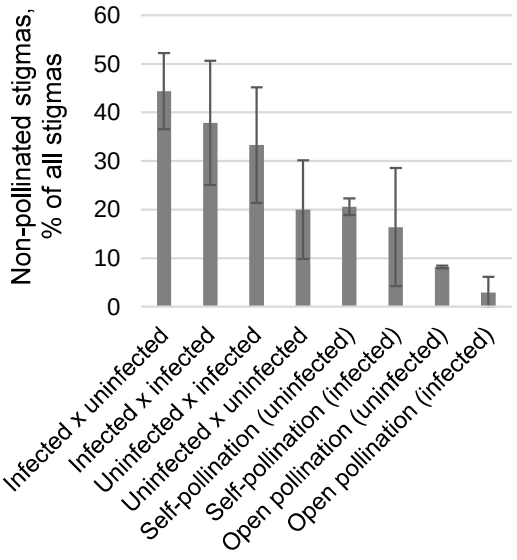

Figure 4. Relative amount of non-pollinated stigmas for different crossing combinations, compared to self-pollination and open pollination of infected and uninfected cultivars.

The highest relative amount of fluorescent pollen was found for infected cultivars using the open pollination - 57.6% (Fig. 5). The average relative amount of fluorescent pollen for all groups was 35.6 ± 12.2%. The highest relative amount of pollen germinated up to ¼ was in crosses, where the infected cultivar was pollinated with both uninfected pollen (32.3%) and infected pollen (31.6%). The least relative amount of pollen at this stage was in the open pollination, 4.3% for infected cultivars and 6.8% for uninfected cultivars. On average for all groups, 25.1 ± 6.7% of the pollen tubes were germinated to ½ of the style length. There was significantly more pollen at this stage if uninfected mother plant was pollinated with uninfected pollen (33.7%), but significantly less in open pollination of infected cultivars (14.3%). On average, 15.6 ± 4.6% of the all pollen was germinated up to ¾ of the style length. The least relative amount of germinated pollen at this stage was detected if an infected cultivar was crossed with an infected cultivar (7.1%). On average, 4.2 ± 2.3% of the pollen tubes were germinated to the ovule. The highest relative amount of pollen at this stage were in the open pollination of the infected cultivar (7.6%), but the least relative amount of if the infected cultivar was used as the mother plant.

Initially looking at the pollen germination process at each crossing on each sampling day, it was expected that at each subsequent day, the most of pollen tubes would have germinate further on the stylus and in the day 5, the most pollen would have reached the ovule. One of the reasons why this correlation did not materialize is due to the limited number of flowers in isolators. During first days collected flowers were of visually better quality, whereas the quality of the samples collected in subsequent days was not so high. Considering that air temperatures at the time of pollination in late May, early June, reached 30 °C, and isolators may have raised the temperature even further, the quality of the samples may have been affected by high temperature stress (Otterbacher et al., 1983), which may have caused a reduction in pollen viability and germination capacity. A negative effect of increased air average temperature on pollination was observed also in another Latvian study, about sour cherry (Feldmane

et al., 2017). The results may also have been influenced by the characteristics of the cultivars used in the study, as there may be cultivars that are genetically or physiologically incompatible with each other.

It was hypothesized that infectivity by pollen plays an important role in pollination. This hypothesis was partially confirmed in our study, because the infectivity of mother plant played a more important role in the pollination process. The pollination was better and pollen tubes grew faster for uninfected mother plants than for infected. However, if pollination was done with infected pollen on the infected mother plant, the rate of pollen tubegrowth decreased, the pollen tubes did not reach the ovule and fertilization did not occur. This could be explained by the delay in the effective pollination period (EPP) the ovule is aged when the pollen tubes reach it (Williams, 1965). EPP and ovule aging are also mediated by high temperatures (Sanzol & Herrero, 2001).

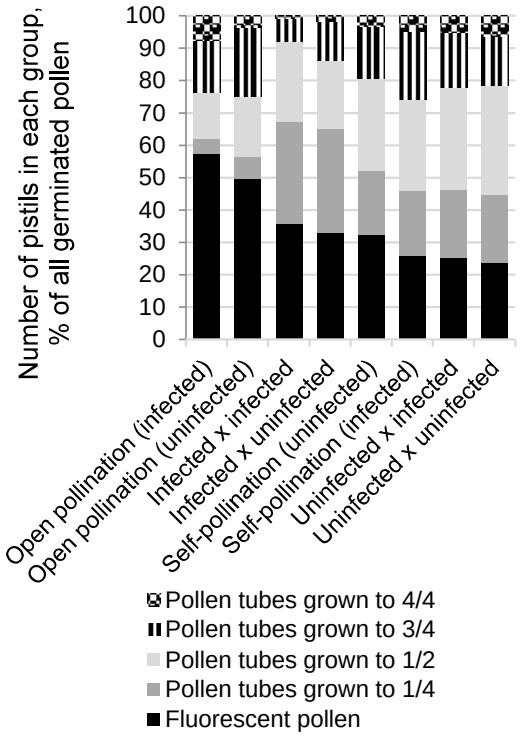

Figure 5. Development of pollen tubes in style and amount of florescent pollen in different crossing combinations, expressed as percentage of all pollinated pistils.

CONCLUSION

The results of the study showed that pollen infected with RBDV germinates equally well to uninfected ones in the studied cultivars. They have the same or slightly higher viability, but during the pollination process, the virus has an effect on pollen tubes growth rate.

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