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Grafting Genotype Combination Effect of *Vitis* ssp. on Roots Phylloxeration Degree and Vigor of Grapevine

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Abstract: The present study investigated whether different grafting combinations (hypobiont-epibiont) of *Vitis* spp. influence root infection with phylloxera and thus vine biomass on potted 2-year-old plants. The study was conducted simultaneously at two locations in Slovenia (VEM) and Hungary (GF). The dormant canes of 'Johanniter' (JOH), 'Riesling' (RR) and *Vitis berlandieri* × *Vitis riparia* 'Teleki 5C' (5C) were hetero-grafted (each with each) and autografted (each with itself), so that nine plant combinations were used for the trial. The roots of the experimental plants at different ages (1–2 years) were infested with two phylloxera populations originating from two locations (VEM, GF). Plant growth was quantified 120 days after inoculation by measuring root and shoot biomass, while the extent of phylloxera infestation was assessed by the number of feeding sites (nodosities, tuberosities) and the number of larval stages of phylloxera. In most cases, the genotype of the hypobiont influenced the degree of phylloxera infestation on all roots of the two-year-old root system. At both locations, the highest number of nodosities and the highest increase in phylloxera population was observed on the autografted Teleki 5C (5C/5C). The phylloxera biotype derived from *Vitis vinifera* roots (GF) induced tuberosities, especially on roots of combinations where JOH and RR were used as hypobionts. No correlation was found between biomass and phylloxera infestation. The hypobiont genotype had no effect on cane biomass at the end of the growth cycle at either experimental site.

Keywords: grapevine; phylloxera; hypobiont-epibiont; phylloxeration

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1. Introduction

Worldwide viticulture is based on domesticated varieties of *Vitis vinifera* L., which are highly susceptible to infestation by root phylloxera (*Daktulosphaira vitifoliae* Fitch). Grape phylloxera is an important historical pest in viticulture, which is controlled by partially resistant and tolerant rootstock varieties [1] or by quarantine measures in wine-growing areas where the vines are grown with their own roots [2]. Phylloxera causes galls on primary root tips (nodosities) and on physiologically older lignified root sections (tuberosities), from which it spreads to the other parts of the *Vitis* host plants [3,4]. Nodosities are described as hook-shaped galls caused by a combination of cell hypertrophy of the distal cells and lack of radial expansion of the root cells near the insertion zone of the stylet. Physiologically, phylloxera nodosities have been shown to compete with other plant organs for plant nutrients and are therefore considered heterogeneous sink organs that modulate the displacement of sinks and sources within the grapevine [5] towards the feeding site. Nodosities accumulate carbohydrates, starch, non-structural sugars and amino acids, which are thought to serve as nutrient stores for larval development and egg production [6] and increase sink activity of the

feeding site [7]. It has also been shown that phylloxera infestation leads to altered metabolic pro-files in the roots and leaves of the host plant [8–11]. A heavy infestation of phylloxera roots can lead to a decline in the vines and ultimately to the death of the plants, mainly due to secondary infections with fungal pathogens [12]. The sting-sucking and gall-forming insect was originally introduced to Europe from America in the mid-nineteenth century. In Europe, phylloxera is mainly controlled by the use of tolerant rootstocks developed by conventional breeding of hybrids of American *Vitis* species with root resistance (*V. riparia* Michx., *V*. *berlandieri* Planc., *V. rupestris* Scheele), which have been successfully used to solve the problem [2,13]. In commercial viticulture in cool climates, most high-value scion varieties (*V. vinifera* L.) are grafted onto *V. berlandieri* × *V. riparia* hybrids, which are currently used as rootstock varieties. Breeding programs aiming at phylloxera resistance use hybridized *V. cinerea* Arnold [14–16] or *Muscadinia rotundifolia* Michx. [13,17], which are intended to prevent phylloxera feeding and suppress gall formation. Among them, 'Börner' (*V. riparia* × *V. cinerea*), a relatively new rootstock hybrid, shows a hypersensitive response to phylloxera attack, resulting in localized necrosis upon insect bite and high or absolute tolerance of roots [18]. Therefore, these genotypes are extremely attractive genetic resources for further rootstock breeding [19].

However, most available rootstocks are only partially resistant or tolerant to phylloxera [1]. Knowledge about the interaction between root and leaf in phylloxera is still limited. The manipulative effects of phylloxera attack on roots and leaves have been described [7,9], as well as the effects on root morphology, the regulation of phytohormonal defense mechanisms and the content of secondary metabolites in phylloxera-infested plants [20]. A rarely discussed fact is the effect of the combination of hypobiontand epibiont (root-stock-scion) on phylloxera infestation. The resilience of tolerant rootstocks as a primary management strategy may also be challenged in the future by host-plant interactions with different phylloxera biotypes [21,22] and by possible effects of climate change on the spread of grapevines and phylloxera [2].

The aim of this study was to determine whether graft combinations (hypobi-ont-epibiont) of *Vitis* ssp. affect the performance of root-feeding phylloxera and whether biomass production is influenced by the insect's feeding performance. We hypothesized that tolerance to root-feeding phylloxera is influenced by a direct genotype interaction of the grafted hypobiont-epibiont combination. The plant biomass as a measure for the resistance of the rootstock against phylloxera infestation is influenced by the grafting combination. The phylloxera biotypes have different effects on the inoculation combinations, which is due to adaptation mechanisms of the host plant.

2. Materials and Methods

2.1. Plant Material and Study Site

In order to obtain data to test this hypothesis, a potted plant trial with grafted grapevines was carried out in two consecutive years (2014–2015) at two locations in Hungary (GF) and Slovenia (VEM). The plant material used to determine the influence of graft genotypes on phylloxera performance consisted of graft combinations (hypobiont-epibiont) of *Vitis berlandieri* × *Vitis riparia* 'Teleki 5C', the interspecific hybrid 'Johanniter' and the grape variety 'Riesling'. Hetero- and autografted grafts were used to control the effects of the genotype combinations. Vine inocula from two different regional locations were used to achieve the broadest possible spectrum of interactions (Figure S1). It is expected that an effect of the graft genotype (hypobiont-epibiont) will be either positive or negative compared to the autografted grafts (e.g., 5C/5C). The data used to measure the interaction are the number of insects, the number of feeding sites (nodosities and tuberosities) and the biomass of the plants as a measure of the host suitability of the host plant combinations.

The grafted plant material for all trials was developed simultaneously and later divided as follows. The dormant canes of 'Riesling' cl. 239 (*V. vinifera*) and 'Johanniter' were collected in the collection vineyard of the University Centre of Viticulture and Enology

of Meranovo (VEM), Faculty of Agriculture and Life Sciences, Slovenia. The canes of 'Teleki 5C' cl. 6 Gm (5C) were obtained from the Georgikon Faculty (GF), Hungary.

2.2. Experiment Set Up

Prior to grafting, the canes were soaked in water for 12 h, disinfected for 12 h in a 0.5% solution of Chinosol W (8-hydroxyquinoline sulphate, Bayer CropScience Ltd., Leverkusen, Germany) and stored in plastic bags at 2 °C. Hetero- and autografted hypobiont-epibiont combinations (Table 1) were grafted in 2014 with mechanical omega grafting (40 grafts per combination) from a commercial nursery near Ptuj (46°50′88.8″ N, 15°97′74.3″ E, 280 m AMSL) in northeastern Slovenia. The grafts were waxed with "Plastigreffe 6535 rossa" and before potting with "Plastiffina 7321 top blu", both from Agrichem Barozzi, (Revere, Italy).

Table 1. The grafting combinations set up in the trials, 40 grafts per combination.

Hypobiont	Epibiont		
	Teleki 5C	**Riesling**	**Johanniter**
Teleki 5C	5C × 5C	5C × RR	5C × JOH
Johanniter	JOH × 5C	JOH × RR	JOH × JOH
Riesling	RR × 5C	RR × RR	RR × JOH

The trials were conducted in the greenhouse in 2014 and 2015 at the two trial locations in Meranovo, Slovenia (VEM) and Keszthely, Hungary (GF) with 10 replicates per grafting combination. After callus formation (end of April 2014), the grafted vines were waxed and planted in 3-L plastic pots filled with 1 kg of drainage stones (from the riverbed) at the bottom and 2 L of peat substrate (potting substrate, Klas-mann-Dielmann, Germany) at the top. After budbreak, the vines were pruned back to a single vertical shoot and the side shoots were removed weekly. Soil moisture was adjusted daily with irrigation through the tubes and using capillarity (GF) to 50% of the water stor-age capacity, while in VEM irrigation was done with a drip irrigation system (one drop per pot). In both experiments, 100 phylloxera eggs per pot were transferred to 3 × 3 cm filter papers and placed next to the root of the plants (the first time in June 2014 and the second time in June 2015). Finally, the entire top of the pots was tightly sealed with aluminum foil.

2.3. Sampling and Measurements

To determine the vigor of the vines, weekly measurements of the growth of the main shoots (lateral shoots were continuously removed during the trial) were carried out, starting with the inoculation of the phylloxera roots at the beginning of June 2014. In September 2015, the vines were destructively sampled by separating the plant organs. Biomass production was determined by shoot and root development (based on dry weight at 65 °C) at VEM. The roots were freed from adhering soil particles. The number of nodosities and the number of tuberosities were counted for all replicates at both locations (VEM and GF). The number of insects (all feeding stages) was counted with an optical microscope in VEM (Euromex Z-series, Arnhem, Holland) and GF (Nikon SMZ800, Tokyo, Japan).

2.4. Grape Phylloxera Populations

The experiments were carried out in two environments using local phylloxera populations (Daktulosphaira vitifoliae) for inoculation at two different plant ages (first and second year of graft growth). The phylloxera inocula were either leaf-feeding field populations of the rootstock 'Binova' (in VEM) or root-feeding populations on *V. vinifera* cultivars (excised root system) (GF). The second biotype was preselected due to its aggressiveness in in vitro root bioassays and propagated on root pieces. All leaf-feeding populations were considered as "biotype C", while the root-feeding populations (used only in the GF experiments) were considered as "biotype A" according to Forneck et al. [23]. Biotype A is adapted to root feeding on *Vitis vinifera* roots and induces the formation of tuberosities on susceptible host

plants. The samples of the experimental Phylloxera populations were genotyped according to Forneck et al. [23] before use. This showed that the phylloxera populations differed between the experiments carried out in VEM and GF, as they were clustered according to their place of origin (Figure S1).

2.5. Statistical Analysis

The statistical differences between the graft combinations were tested using a one-way analysis of variance (ANOVA). Statistical analysis of the data was performed using the SPSS 25.0 program (IBM) with $p \leq 0.05$. The mean values were compared with Duncan's MRT test. Correlations were calculated between the number of nodosities and the number of insects as well as the number of insects and plant biomass (dry weight) at the VEM site.

3. Results

3.1. Grape Phylloxera Infestation Level

The number of nodosities varied considerably within the graft combinations and between all experimental plants. At both experimental locations (VEM and GF, 192.2 ± 33.42 and 83.3 ± 16.85 mean nodosities per plant, respectively), we found the highest mean number of nodosities on two-year-old root systems of 5C. The differences were significant compared to RR and JOH (Table 2) ($p \leq 0.01$). At both locations, the number of nodosities was highest on the autgrafted 5C with 273 ± 78.10 (VEM) and 161 ± 26.35 (GF) mean nodosities per plant. The differences were significant compared to all graft combinations with RR and JOH as hypobionts and to the graft combinations 5C/JOH and 5C/RR on GF. The lowest mean number of nodosities was observed in the combination JOH/5C (VEM) and RR/JOH (GF) with an mean of 1.0 ± 0.58 and 6.33 ± 4.22 nodosities per plant, respectively ($p \leq 0.05$).

Table 2. Effect of grafting genotype combination on average number of nodosities and number of individuals per grafting combination (10 repetitions) in 2015 at both experimental locations (VEM and GF).

Combination Hypobiont/Epibiont	Nr. of Nodosities (Average ± SE)				Nr. of Individuals (Average ± SE)			
	VEM		GF		VEM		GF	
5C/5C	273.5 ± 78.10	a	161.50 ± 26.35	a	60.7 ± 21.24	a	54 ± 7.39	a
5C/RR	195.7 ± 34.85	ab	35.50 ± 16.46	bc	31.2 ± 13.30	ab	25.7 ± 8.39	abc
5C/JOH	107.5 ± 36.36	bc	52.83 ± 8.02	b	30.3 ± 10.24	ab	49.3 ± 12.13	ab
5C	192.2 ± 33.42	A	83.28 ± 16.85	A	40.7 ± 9.14	A	43.0 ± 5.98	A
RR/RR	7.5 ± 2.10	c	44.50 ± 12.29	bc	0.75± 0.48	b	20.7 ± 4.42	bc
RR/5C	2.0 ± 2.00	c	27.83 ± 13.89	bc	0.25 ± 0.25	b	28.0 ± 11.94	abc
RR/JOH	52.0 ± 14.59	c	6.33 ± 4.22	c	19.0 ± 6.76	ab	15.7 ± 7.75	c
RR	25.0 ± 8.83	B	26.22 ± 7.06	B	8.4 ± 3.74	B	21.4 ± 4.82	B
JOH/JOH	53.8 ± 49.19	c	22.17 ± 9.75	bc	21.40 ± 19.70	ab	8.5 ± 7.58	c
JOH/5C	1.0 ± 0.58	c	40.00 ± 4.56	bc	0.25 ± 0.25	b	40.0 ± 4.35	ab
JOH/RR	15.2 ± 7.50	c	8.50 ± 5.98	c	6.25 ± 2.90	b	22.2 ± 14.64	abc
JOH	25.69 ± 18.93	B	23.56 ± 4.96	B	10.23 ± 7.57	B	32.5 ± 6.30	AB

Different letters (lowercase between the grafting combnations, upercase between the hypobionts) indicate significant differences between the average of grafting combinations with standard error (±SE) (Duncan MRT test, $p \leq 0.05$).

Similar ratios were observed for the number of phylloxera individuals at both locations. Significantly more phylloxera individuals (VEM) were found on the roots of 5C (40.7 ± 9.14 individuals per plant), compared to RR and JOH with 8.4 ± 3.74 and 10.23 ± 7.57 phylloxera individuals per plant, respectively ($p \leq 0.05$). In GF, the differences were only significant between 5C (43.0 ± 5.98 individuals per plant) and RR (21.4 ± 4.82 individuals per plant). The highest phylloxera infestation was found in the combination 5C/5C with

60.7 ± 21.24 (VEM) and 54 ± 7.39 (GF) individuals per plant. In VEM, it was significantly different from the graft combinations in which RR and JOH were used as hypobionts, except for the RR/JOH and JOH/JOH graft combinations. In GF, it was significantly different from the graft combinations RR/RR, RR/JOH and JOH/RR ($p \leq 0.05$). In general, the number of nodosities and the number of insects on two-year-old roots were higher on autografted 5C (5C/5C) at both locations. However, this does not show the actual situation of phylloxera population in the whole root system of the plant at the GF site, as part of the phylloxera population was on tuberosities (shown as low), which we could not detect at the VEM site.

The highest average number of tuberosities (GF) was found in the JOH combination (15.5 ± 3.01 per plant) and was significantly from the 5C combination (on average less than one tuberosity per plant). However, the differences between the combinations were not significant ($p \leq 0.05$). The lowest number of tuberosities was observed in the combinations where 5C was used as a hypobiont (Figure 1). Tuberosity-feeding phylloxera larvae (Figure 1) was also highest in the combinations where JOH was used as hypobiont (63.4 ± 17.43 per plant) and significantly higher than in 5C (1.8 ± 1.83 per plant) ($p \leq 0.05$). A significantly higher number of phylloxera infestations on tubersities was counted in the combination JOH/5C (82.2 ± 4.9 ind. per plant) compared to all combinations with 5C as hypobiont and to the combination RR/5C. Due to the low number of tuberosities on the 5C roots, the number of phylloxera infestations was significantly lower. Most of the plants with 5C (the first three combinations in Figure 1) had neither tubersities nor phylloxera, with the exception of the 5C/5C combination, which had on average less than one tuberosities and 5.5 ± 5.5 phylloxera individuals per plant.

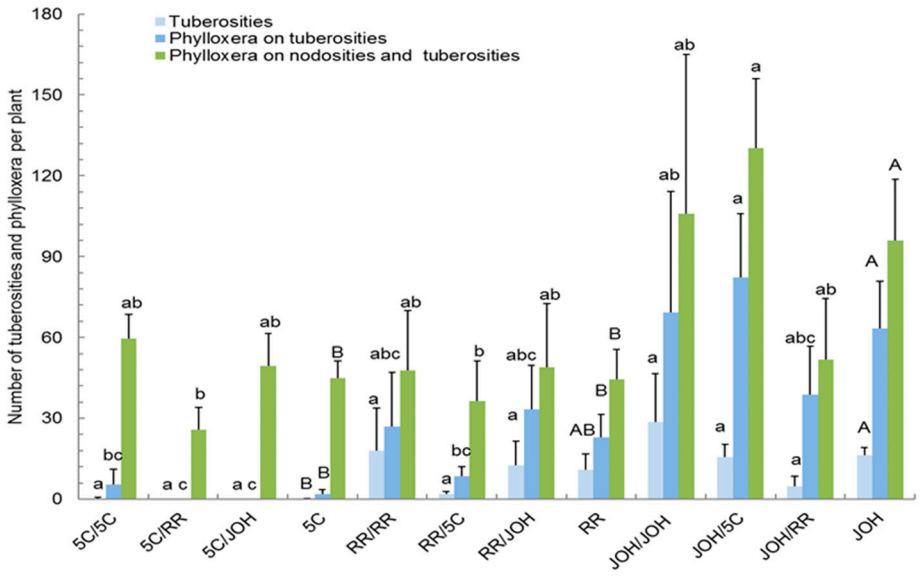

Figure 1. Number of tuberosities per plant on a two-year-old root system at Georgikon Faculty (GF), Kesthely, Hungary, number of individuals on tuberosities and number of all individuals of phylloxera (on nodosities and tuberosities). Different letters (lowercase between the grafting combinations, uppercase between the hypobiont) indicate significant differences between the average of grafting combinations with standard error (±SE) (Duncan MRT test, $p \leq 0.05$).

However, a comparison of the total number of phylloxera individuals on the roots (nodosities and tuberosities together) shows different ratios (Figure 2). On average, the highest phylloxera population was found on plants where JOH was used as hypobiont (95.9 ± 22.84 individuals per plant), and it was significantly different from RR and 5C ($p \leq 0.05$) with 44.4 ± 11.28 and 49.3 ± 12.13 individuals per plant, respectively. The combination JOH/5C showed the highest phylloxera infestation on the roots (130.2 ± 25.74 per plant) and was significantly from 5C/RR and RR/5C ($p \leq 0.05$) with 25.7± 8.39 and 36.5 ± 14.9 individuals per plant, respectively ($p \leq 0.05$).

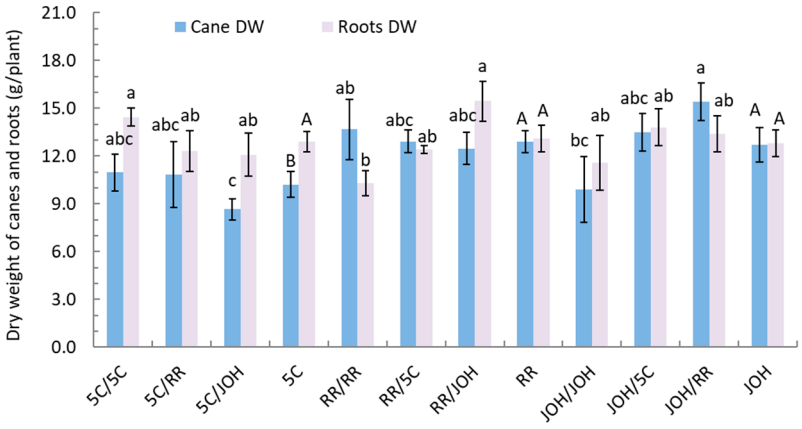

Figure 2. Dry weight of canes and roots (g/plant) of different grafting combinations ($p \leq 0.05$), University centre of Viticulture and Enology Meranovo (VEM), Slovenia. Different letters (lowercase between the grafting combinations, upercase between the hypobionts) indicate significant differences between the average of grafting combinations with standard error (±SE) (Duncan MRT test, $p \leq 0.05$).

3.2. Vigor of Scion-Graft Combinations

The vigor of the host plant combinations was evaluated as phylloxera can be positively influenced by higher shoot and root biomass, which may affect the translocation of carbohydrates to the roots. At VEM, the combinations with 5C as hypobiont had an average dry weight (DW) of 10.2 g/graft, which was 20 to 21% less than JOH (12.7 g/graft) and RR (12.9 g/graft), respectively. The JOH/RR combination had the highest DW and was significantly different from 5C/JOH and JOH/JOH. Even between these two combinations, the differences in the DW of the rods were significant ($p \leq 0.05$) (Figure 3). The average root dry weight was about 13 g/plant for all hypobionts (5C, RR and JOH), and the differences between hypobionts were not significant. The grafting combinations RR/JOH and 5C/5C had significantly higher root dry weight (15.4 and 14.4 g DW of roots per plant, respectively) than RR/RR (10.3 g/plant). All other differences were not significant ($p \leq 0.05$) (Figure 2). The grafting combinations had a minor effect on vigor, which is also confirmed by the correlation between the number of phylloxera individuals and the dry weight of roots and canes. Although an increase in phylloxera individuals was expected with an increase in nodosities, the number of individuals had no effect on the dry weight of the canes and roots, and the correlation is low (Figure 3).

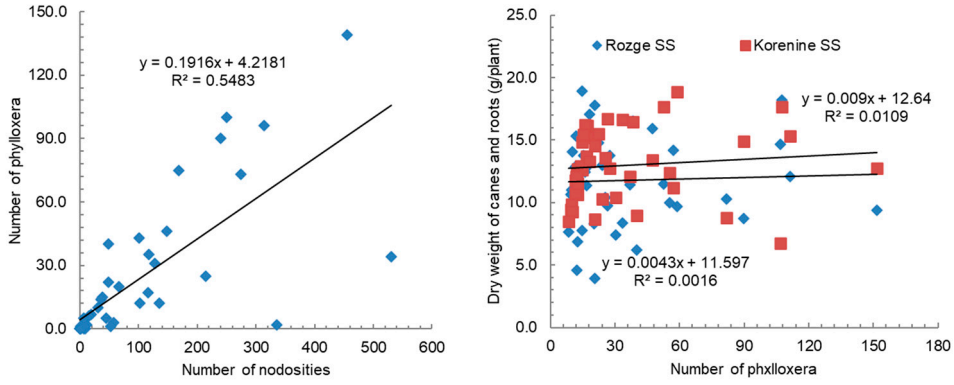

Figure 3. Relation between number of nodosities and number of phylloxera (left) and number of phylloxera and dry weight of canes and roots in g/plant ($p \leq 0.05$), at University Centre of Viticulture and Enology Meranovo (VEM), Slovenia.

4. Discussion

Currently, plant tolerance to root-feeding phylloxera is being studied in different genotypes of *Vitis* ssp. while the interactions between hypobiont and epibiont are more

focused on root biomass [24]. Here, we hypothesized an interaction between hypobiont and epibiont reflected in the effect on tolerance to phylloxera as measured by the number of insects and the number of feeding sites (nodosities and tuberosities). Our results show that phylloxera populations at both experimental locations attacked the roots of the tested *Vitis* host genotypes studied forming nodosities. In addition, tuberosities were formed (in the two-year trials of GF) on the roots of RR and JOH, which are susceptible to both grafting combinations. We did not expect tuberosities formation on the 5C roots, as neither biotype A nor biotype C are considered to induce tuberosities on them [25]. On 5C, we also did not expect so high number of nodosities. The hypobiont genotype has the strongest influence on phylloxeration of root-feeding phylloxera—regardless of the geographical population of origin of the phylloxera. No significant effects of the epibiont on the development of phylloxera infection or the biomass production of the host plants were found. It was demonstrated that phylloxera biotypes adapted to *V. berlandieri* × *V. riparia* hybrids are predominant in these areas. This could be important for winegrowers both in the areas concerned and in other areas where rootstocks with this pedigree are used. However, we observed that 5C in particular coped with phylloxera infestation and did not reduce biomass, which is why it is considered phylloxera tolerant, as also found by Clarke et al. [26]. Previous studies have investigated the extent to which phylloxera infestation affects the vigor of the vines. The results indicate that root-feeding phylloxera populations are able to attract assimilates from the leaves and increase host photosynthetic rates, leading to a systemic compensatory effect within the vine [5,9,27].

The final finding for these two locations is that GF, where the root-feeding phylloxera (radicole population) infestation was carried out, had a higher incidence of tuberosities on the two-year-old root system. The highest phylloxera population was found on the plants with JOH hypobionts, while in the VEM experiment using the leaf gall eggs, only one tuberosity was observed and the highest population was found on the nodosities. The total population size of phylloxera on the nodosities was similar on 5C in both trials (VEM and GF) (Table 2), but on the tuberosities it was on average five and nine times higher on RR and JOH, respectively, than on GF (Figure 1). On the roots of RR and JOH (which were infected with the radicole population) in GF, an average of 52 to 66% of the phylloxera population was found on tuberosities, while on 5C only 4% of the population was found on them. The rest of the phylloxera population was found on nodosities.

Overall, the genotype of the hypobiont has the greatest influence on phylloxera infestation. There is no evidence that the epibiont influences phylloxera infestation through biomass or other unknown effects. Even though the first study on the effects of genotype and grafting did not show significantly measurable infection rates on potted vines, effects on the whole plant can be observed that influence the physiology of the vine. Certainly, further trials need to be conducted to investigate the hypobiont-epibiont interaction on performance under phylloxera infestation and possible compensatory effects on the biomass of the epibiont (scion). The effects on leaf-galling phylloxera may also be influenced by hypobiont-epibiont interaction and should be considered in future experimental studies.

5. Conclusions

Resistance to phylloxera is still the most important characteristic of rootstocks for modern viticulture. Our results show that phylloxera populations at both trial locations attack the roots of the tested *Vitis* host hypobiont-epibiont combinations and form nodosities on all of them. The genotype of the grafting has the strongest influence on the phylloxeration of the root-feeding phylloxera—regardless of the geographical origin of the phylloxera population. In addition, tuberosities developed on both graft combinations on RR and JOH (in the two-year trials of GF), which are susceptible to this pest. We did not expect tuberosities to develop on 5C, but the number of nodosities was highest on 5C at both locations (GF, VEM). We detected the prevalence of phylloxera biotypes adapted to *V. berlandieri* × *V. riparia* hybrids at these locations. We could not detect any significant effects of the epibiont on the development of phylloxera infection or on the biomass production of

the host plants. Although the initial study on the effects of genotype grafting did not show significant measurable levels of infection in potted grapevines, effects on whole plants can be observed to influence grapevine physiology. Certainly, further experiments need to be conducted to investigate the interaction between hypobiont-epibiont on performance under phylloxera infestation and possible compensatory effects on biomass of the epibiont (scion). The effects on leaf-galling phylloxera may also be influenced by the interaction between hypobiont-epibiont and should be considered in future experimental studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050445/s1, Figure S1: Principal component analysis of 14 MLGs (MultiLocusGenotypes) within the experimental Phylloxera populations employed (SLO = Slovenian genotypes employed in the VEM experiments, H = Hungarian genotypes employed in the GF experiments. AT1, CH5, DE single founder lineages kept as reference biotypes at the Institute of Viticulture and Pomology, Vienna (BOKU) and serve as defined genotype controls. Genotypic Diversity displayed per Axis: x-axis reflects to 44.78% and y-axis 42.05%. In total 35 samples (VEM: 14, GF: 18, BOKU-standard: 3) were individually genotyped and further modified according to Forneck et al. [23] based on seven SSR markers (Phy_III_55, Phy_III_30, Phy_III_36, Dvit6, DV4, DV8 and DVSSR4). In this set of samples three control genotypes were included to keep allele calling.

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