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Identification of an Emaravirus in a Common Oak (*Quercus robur* L.) Conservation Seed Orchard in Germany: Implications for Oak Health

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Abstract: We observed the health status of oak trees in a conservation seed orchard for over twenty years, focusing on characteristic virus-suspected symptoms. The orchard was established in 1992 in Kreuztal, North Rhine-Westphalia (Germany) with 1302 seedlings in 186 clusters. The number of seedlings showing chlorotic ringspots and mottle on leaves has fluctuated annually, but has increased from 3.3% to 12.1% in the last 20 years; the number of affected clusters has risen from 8% to 25.9%. A scientific breakthrough was the identification of a novel virus related to members of the genus *Emaravirus* in diseased oak by high-throughput sequencing (HTS). Screening of the oak seedlings in three consecutive years, using a newly established virus-specific diagnostic reverse transcription polymerase chain reaction (RT-PCR), confirmed the virus infection and revealed a close to 100% association between the observed leaf symptoms and the novel virus. As no other plant virus could be identified in the HTS-datasets, we assume the novel virus is primarily causing the symptoms. To reliably detect the novel virus in oaks, RT-PCR targeting the viral RNA3 or RNA4 should be applied in routine testing of symptomatic leaf tissue. It was obvious that most groups with virus-infected plants cluster, with only five out of the 42 affected groups being offside, not bordering on other affected groups of plants. There was no clear correlation between the detection of the virus and the overall vitality of the seedlings. There was no relation between seedling performance and presence or absence of viral infection. Forecasts on the future growth behavior of these virus-infected oak trees are therefore not possible.

Keywords: novel virus; chlorotic ringspots; long-term survey; RT-PCR detection

1. Introduction

Quercus robur (L.), commonly known as common oak, pedunculate oak, or European oak, is a very widespread species which is native to most of Europe and described as a vigorous tree with a large ecological amplitude [1]. Oaks are amongst the most economically and ecologically important deciduous trees in Europe providing wood for fuel, bark for tanning, timber for construction, and acorns for livestock. Across 34 European countries, pedunculate oak covers approximately 49,000 km^2 [2]. In Germany, oak species can be found on around 10% of forested land, making oaks the second most important deciduous tree species after European beech (*Fagus sylvatica* L.) [3]. The production of high-quality wood is associated with long rotations and high labor costs.

Dieback and decline in oak have been reported in Europe since the early 1900s as well as in the most recent decades [4,5]. On the basis of historical records and dendrochronological measurements, oak decline in Central Europe has been attributed to single or combined effects of various abiotic factors such as air pollution, nitrogen eutrophication, soil chemical stress and climatic extremes (winter frost, summer drought), defoliating insects, and pathogenic fungi. However, little attention has been paid to plant viruses, despite [6] classifying this group of organisms as a presumed contributing factor in tree decline as early as 1985. The author stated "We have to live with viruses and microorganisms. Most probably they have more or less slowly invaded our forests during hundreds of years without causing serious damage by their own, except in a few regionally restricted cases. There is no indication that they are the actual cause of the sudden epidemic-like spread of the new decline of our forests. We have to realize, however, that they play their role as an important predisposing factor in the decline disease spiral." Plant viruses are rumored to lead to early senescence of trees, which is known to reduce the regeneration capacity of the host plants, and the juvenile metabolic vigor is lost prematurely. Thereby, virus-infected trees have a reduced potential for recovery from omnipresent abiotic stress conditions compared to non-infected trees.

Most plant diseases have adverse effects on plant growth and productivity, which can range from relatively low tree mortality rates to 100% losses for certain plant species. Co-infection with multiple viruses may enhance pathogenicity. Although the impact of plant viruses on forest trees remains to be investigated, limited information concerning fruit trees is available. For instance, [7] showed that yield efficiencies of peach infected with a mild isolate of plum pox virus (PPV) did not differ statistically although trees produced slightly more fruit of smaller size that ripened earlier than non-infected trees. Mixed infections of PPV, prune dwarf virus (PDV), and prunus necrotic ringspot virus (PNRSV) have been shown to reduce growth and to exhibit bark canker, trunk malformation, and tree mortality in some peach cultivars [8]. The synergistic effect of PPV with other viruses resulted in a growth reduction of the seedlings by 2.9 to 69.1%.

Surveys in North German nurseries and of several German forest districts on the health status of European oak (*Quercus robur* L.) led to the observation of many seedlings and trees with characteristic virus-like symptoms such as chlorotic ringspots, chlorotic spots, and mottling [9]. Some of these plants had degenerated twigs and suffered from a distinct loss of vigor. Already in the 1970s, these symptoms had been described and following visual inspection assumed to be induced by a viral pathogen [10,11]. However, neither the etiology nor the epidemiology of a viral agent has so far been described. In order to demonstrate the biotic nature of the virus-like symptoms and to identify the putative agent, we initially focused on symptomatic trees. Virus-like symptoms on European oak as well as therewith confusable discolorations are described by [12]. In 1996, [9] carried out experiments testing graft-transmission of the assumed agent and stated that the symptoms are induced by an infectious agent. Transmission to herbaceous plants by mechanical inoculation failed in these and later experiments. The authors ruled out the presence of tobacco mosaic virus (TMV), tobacco necrosis virus (TNV), cherry leaf roll virus (CLRV), and brome mosaic virus (BMV), which are known to occur not only in forest ecosystems but are also associated with *Quercus* sp. [11]. Recent advances in high-throughput sequencing (HTS) technologies and bioinformatics have generated unique opportunities for discovering and diagnosing plant viruses and viroids. Thus, during the last decade the application of this technology has led to the discovery of more than one hundred new plant viruses, new virus variants, or new plant hosts of known viruses [13], which has shed light on the complex world of microbial communities in environmental samples [14].

Advances in knowledge on the causes and impact of oak decline have been slow, being partly due to the complex nature of the problem. Therefore, we aimed to (i) observe the health status of oak trees in a conservation seed orchard over a period of more than twenty years by visual means, (ii) document the characteristic virus-suspected symptoms, (iii) identify a novel virus by HTS in diseased oak, and (iv) verify the association of the novel virus with the chlorotic ringspots via the development and application of a virus-specific reverse transcription-polymerase chain reaction (RT-PCR).

2. Materials and Methods

2.1. Conservation Seed Orchard

The selected orchard with common oak was established in 1992 in Kreuztal, North Rhine-Westphalia (Germany) with three-year-old seedlings (origin 817 01, provenience "von Plettenberg/Hovestadt"). The 3.1 ha oak cluster planting established on former farmland comprises 186 groups consisting of seven seedlings each (Figure 1). These seedlings were planted with an initial spacing of 1 m and at a distance (center to center) of 12 m between the groups. The site is 281 m above sea level. The long-term mean annual rainfall is 859 mm, and the mean annual temperature is 8.5 °C.

Figure 1. Conservation seed orchard in Kreuztal (North Rheine-Westfalia, Germany). (**right top**) top view, 2008; (**right below**) in the front group with three trees (yellow arrows), 2015.

Since 1995, the orchard was inspected at least once a year, focusing on the characteristic virus suspected leaf symptoms (Figure 2). Inspection and sampling took place in late spring/early summer. During this period leaf symptoms were mostly very clearly visible and not yet masked by other biotic or abiotic factors. Symptomatic leaf samples were always taken from individual trees. For comparison, we included leaf material from trees not exhibiting virus-suspected symptoms.

2.2. RNASeq of Diseased Oak Leaves and Data Analyses

In 2014, new molecular tools enabled further steps to unravel the causal agent inducing the disease. Leaf material from a single diseased common oak (E53309, *Quercus robur* L.) tree from the seed conservation orchard was sampled. The leaves of this tree had shown characteristic chlorotic ringspots and mottling over several years. For RNA-sequencing (RNASeq) (Illumina, HiSeq), total RNA was isolated from 50 mg of tissue showing chlorosis and necrosis, excised from leaves (Invitrap Spin Plant RNA Mini Kit, Invitek Molecular, Berlin, Germany), followed by mRNA enrichment (Dynabeads mRNA purification Kit, Thermo Fisher Scientific, Hennigsdorf, Germany), purification, and DNAse digest. Single-read sequencing was performed with Illumina's sequencing-by-synthesis approach using an RNA sample preparation kit, a single-read cluster generation kit v3, and the TruSeq mRNA sample

prep kit v3 (Illumina Inc., San Diego, CA, USA). Sequencing of barcoded libraries was performed on an Illumina HiSeq 2500 system (Illumina Inc., San Diego, CA, USA). De novo assembly of reads of this sample was performed by selecting standard parameters and using a CLC Genomics Workbench V7.0.1 (Qiagen, Aarhus, Denmark). Taxonomic binning was performed using MEGAN (MEtaGenome Analyzer, version X, University Tübingen, Germany) [15] and by applying one read support parameter, while the Basic Local Alignment Search Tool X (BLASTX, National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) comparison against the nr protein database (NRPROT) of assembled contigs with a minimal size of 300 nucleotides was run under default parameters for identifying nucleotide entries for viruses in GenBank (NCBI). In 2016, a high-throughput sequencing (RNASeq, Illumina Inc., San Diego, CA, USA) of a diseased tree from the same seed orchard was performed for emaraviral sequences amplified by use of the generic PDAP213 primer [16]. RNA-extraction from a pooled sample of leaf material collected from a diseased tree in June 2016 (E54889) showing chlorotic ringspots and mottle and ds-cDNA preparation for library generation for RNASeq carried out by the Company BaseClear B.V. (Leiden, Netherlands); data analyses, including assembly of contigs, were then conducted as described in [17].

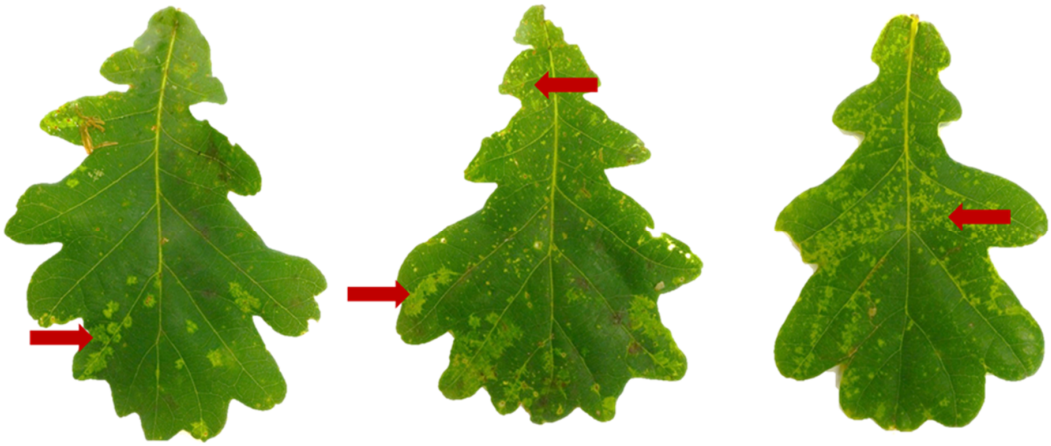

Figure 2. Oak leaves with characteristic virus-suspected leaf symptoms. (**Left**) chlorotic ringspots (arrow left); (**middle**) chlorotic ringspots (arrow left) and distinct chlorotic lesions (arrow right); (**right**) distinct chlorotic lesions (arrow right).

2.3. RT-PCR for Plant Virus Diagnosis

Starting in 2016, we conducted RT-PCR analysis to both confirm viral sequences identified by HTS in diseased oaks and to study the association of the virus with observed symptoms. To this end, leaf material from the seed conservation orchard was sampled and was immediately used for isolation of total RNA or stored at −20 °C beforehand.

Total RNA was isolated according to [18] and reverse transcribed into cDNA by the use of random hexamer primer and Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) following manufacturer's instructions. Synthesized cDNA was used as template for conventional RT-PCR. The mitochondrial expressed *nad5* gene was verified according to [19] as an internal control. RT-PCR-based detection of the different viral genome components applied the genus-specific primer pair motif A sense and motif C antisense [20] as well as HTS-derived species-specific primers (RNA2-Primer C1291-1029F: CCATTCTTAAGTATGTGTGAA/C1291-1609R: TATGATCATGATGGATTCACAGAG; RNA3-Primer Emara-oak-NC-F2: CAGAGCTATGGCTATCTGCA/ Emara-oak-NC-R2: GTTGCTATCACTTCTGCAGG; RNA4-Primer C755-514F: CAAGCTCCTGAAGCTTATTCAACA /C755-826R: GAATCAATTGTTCAGATGAGCATG). PCR amplification was conducted in a thermal cycler (BioRad Laboratories GmbH, Feldkirchen, Germany) with an initial denaturation at 94 °C

for 3 min, followed by cyclic amplification for 35 cycles at 94 °C for 30 s, at 50 °C (RNA1 and RNA2)/54 °C (RNA3)/55 °C (RNA4) for 30 s, and at 72 °C for 30 s. The PCR products were separated by 1–1.5%-agarose gel electrophoresis. Amplified PCR products were subsequently sequenced by Sanger Sequencing in both directions using PCR product specific primers.

3. Results

3.1. Identification of a Novel Virus in Oak

RNA-seq of mRNA, enriched from an oak tree exhibiting chlorotic ringspots (E53309) from the conservation seed orchard, showed a total of 2,585,326 reads (1,753,138 pairs) at an average length of 100 nucleotides (nt), reaching a total read sequence length of 258,532,600 nt. BLASTX search of contigs performed in the NCBI database identified six contigs relating to RNA1 to RNA4 encoded by different emaraviruses (Table 1). Contigs relating to other plant viruses were not identified in this dataset.

Table 1. Assembled contigs of the novel virus identified in oak samples by HTS in 2014 (E53309) and 2016 (E54899), compared to known emaravirus RNA segments. Query coverage, E value, and sequence identity are given in comparison with the best match in BlastX.

Tree	RNA Genomic Segment	Contig Number	Size (nt)	Best Match in BlastX (Accession Number)	Query Coverage (%)	E Value	Sequence Identity (%)
E53309	RNA1	C1727	2314	TiRSaV * (QAB47307.1)	99	1×10^{-139}	56
		C447	1290	TiRSaV (QAB47307.1)	99	0.0	48
		C329	1509	TiRSaV (QAB47307.1)	84	2×10^{-90}	42
	RNA2	C1291	2295	TiRSaV (QAB47308.1)	76	0.0	44
	RNA3	C881	1170	TiRSaV (QAB47309.1)	71	2×10^{-41}	39
	RNA4	C755	1388	TiRSaV (QAB47310.1)	71	2×10^{-158}	65
E54899	RNA1	S71	527	TiRSaV (QAB47307.1)	88	8×10^{-18}	33
		S134	346	RLBV ** (YP_009237274.1)	97	4×10^{-28}	62
		S36	805	RLBV (YP_009237274.1)	90	1×10^{-39}	40
	RNA2	S18	1138	TiRSaV (QAB47308.1)	89	4×10^{-105}	42
	RNA3	S28	990	TiRSaV (QAB47309.1)	75	2×10^{-47}	41
	RNA4	S26	1008	TiRSaV (QAB47310.1)	64	2×10^{-104}	70

* Ti ringspot-associated virus, ** raspberry leaf blotch emaravirus.

HTS of a second tree with chlorotic leaf ringspots and mottle (E54889) delivered a total of 1,071,338 reads with 1,024,219 mapped reads in pairs that were assembled into 155 contigs with a minimum size of 304 nucleotides. We confirmed the presence of four genome segments of the novel virus in the assembled contigs by searching all contigs with BLASTX against the NRPROT database. Altogether, six contigs were identified revealing highest sequence identities for proteins encoded by different emaraviruses, with four of them providing additional sequence information of the novel virus relating to RNA1 (S71, S36), RNA3 (S28), and RNA4 (S26) of emaraviruses (Table 1). Amino acid identities of the 12 contigs queried in BLASTX varied between 33 and 70% compared to known emaraviral protein sequences (Table 1). The contigs for RNA2, RNA3, and RNA4 assembled from sample E53309 delivered completely covered coding sequences (cds), and we obtained fully covered cds of RNA3 from second HTS dataset (sample E54899). BLASTX analysis of cds-derived amino acid sequences resulted in 43% identity to putative glycoprotein precursor of ti ringspot-associated virus (TiRSaV), 39% identity to putative nucleocapsid protein of TiRSaV, and 65% identity to putative

movement protein of TiRSaV for RNA2, RNA3, and RNA4, respectively. The nucleotide sequences reported here have been deposited in GenBank with accession numbers LR824572–LR824583. Sequence comparison of overlapping regions of the six contigs, assembled from the diseased oak leaf material received by the two different HTS strategies, respectively, show high sequence identities of at least 96% at the nucleotide level (data not shown). We therefore deduce that a novel virus related to emaraviruses is present in ringspot-diseased oak trees.

3.2. Detection of the Novel Virus in Ringspot Diseased oak Trees

We derived primer pairs from HTS-based sequence information for RT-PCR targeting the different genome components of the new virus identified in oak trees from a conservation seed orchard. In three consecutive years, detection of the novel virus was associated with observed virus-suspected symptoms on diseased oaks (Table 2).

Table 2. Overview of reverse transcription polymerase chain reaction (RT-PCR)-based detection of the novel virus targeting the viral RNA3 and RNA4 in leaf material of individual oaks collected in June or July of three consecutive years.

Tree Year	Chlorotic Ringspots [No. Detected/No. Tested]	No Symptoms [No. Detected/no. Tested]
2016	40/40	0/4
2017	20/20	0/0
2018	52/53	0/0
Total (no.)	112/113	0/4
Total (%)	99,1	0

We detected the genome components of the new virus specifically in samples from trees exhibiting chlorotic ringspot symptoms, whereas no viral RNAs were detectable in samples collected from trees without virus-like symptoms (Figure 3).

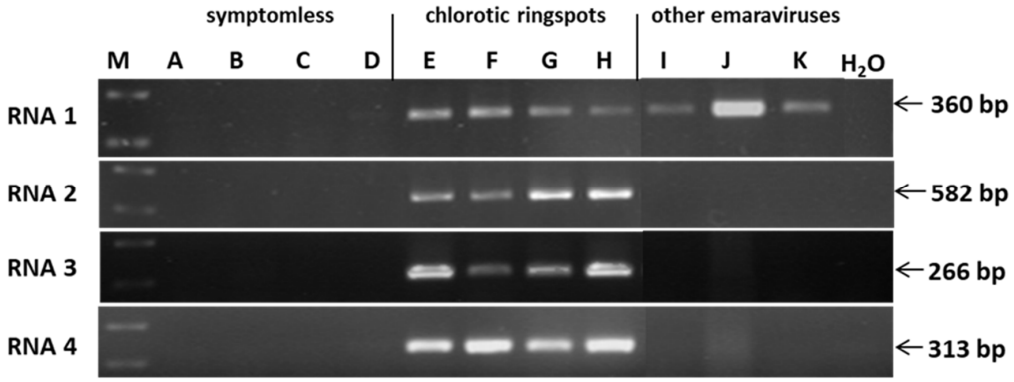

Figure 3. Establishment of virus-specific RT-PCRs for the detection of the novel virus in diseased oaks. Detection of emaraviruses by generic primers targeting viral RNA1 [20], including symptomless trees (A–D), trees showing chlorotic ringspots in leaves (E–H), Fig mosaic emaravirus (I), European mountain ash ringspot-associated emaravirus (J), and an unassigned emaravirus in maple (K). Detection of the novel virus in diseased oaks by species-specific primers targeting the viral RNA2, RNA3, and RNA4. Fragment lengths of amplified PCR products are indicated on the right. M—GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Hennigsdorf, Germany).

RT-PCR with genus-specific primers targeting the viral RNA1 in combination with direct sequencing of the PCR products support the assumption of the new virus in oak being a member of the plant pathogenic genus *Emaravirus*. Moreover, RT-PCRs targeting the genome segments RNA2 to RNA4 allowed the detection of the virus solely in the diseased oaks expressing chlorotic ringspots

(Figure 3E–H), and not in symptomless oaks (Figure 3A–D) nor woody plants infected with other emaraviruses (Figure 3I–K). Primer pairs targeting RNA3 and RNA4, respectively, showed highest sensitivity in the established RT-PCR.

A sequence comparison of Sanger sequenced PCR products amplified with primers targeting the RNA3 and RNA4 of the novel virus revealed sequence identities of at least 93% and 88%, respectively, with the respective HTS generating reference sequences at the nucleotide level. All trees investigated in 2016–2018 were infected by the same novel virus. We highly recommend RT-PCR targeting the viral RNA3 or RNA4 for the routine testing of oaks exhibiting virus-suspected ringspot symptoms for the reliable detection of the new virus in symptomatic leaf material.

3.3. Monitoring

Out of 186 groups planted in 1992, only 162 remain to date (Table 3). Within 27 years of the orchard being established, the total number of trees has decreased from 1302 to 422. A natural selection of ideally one tree per group is desired and was reached by 44 (data not shown). The number of seedlings showing the characteristic virus associated symptoms (Figure 2) is subject to large fluctuations. For example, in 2013, only 17 trees were classified as virus-infected, but 43 and 51 were classified thusly in 2008 and 2018, respectively. During the last 20 years, the number of affected groups increased significantly from 14/175 (8%) to 42/162 (25.9%). During this period, the number of individual trees with characteristic symptoms increased from 19/577 (3.3%) to 51/422 (12.1%). In 2016, a molecular diagnostic system was set up and enabled the detection of the novel agent causing these symptoms in following years.

Table 3. Overview of number of groups and individual seedings displaying symptoms associated with the novel emaravirus from 1992 to 2018 in the conservation seed orchard planted in 1992 (ne: not evaluated).

		1992	**1998**	**2003**	**2008**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**
Groups	total	186	175	171	164	162	162	162	162	162	162
	with characteristic symptoms	ne	14	24	34	17	19	33	38	33	42
Seedlings	total	1302	577	455	440	435	432	432	427	425	422
	with characteristic symptoms	ne	19	28	43	17	21	38	42	39	51

In recent years there have been fluctuations in the number of diseased single trees with visible characteristic leaf symptoms (Table 3). While 2015 to 2017 show a relatively constant occurrence of emaravirus-infected trees, 2014 and 2018 stand out, with the number of diseased tress being clearly below and above the average, respectively. This phenomenon applies to both infected individual trees and the number of affected groups.

The number of established oak groups in 1992 was anticipated as also being the future number of crop trees. A first thinning, to remove all other remaining oaks in the group to provide growing space for the single prevailing crop tree, has yet to be applied. To date, 44 of the remaining 162 groups consist of one prevailing crop oak. Of these oaks, seven (15.9%) were infected with the novel emaravirus. In another 43 groups, neither of the last two remaining trees were able to prevail. Remarkably, in nine of those groups, one oak tree was infected with the virus (Figure 4A), in four groups both were infected. A total of 64 groups consist of one or two healthy oak trees (Figure 4B). These groups are distributed throughout the site and cluster; only three single crop trees stand individually. The 24 groups being lost, without a viable prevailing oak, cluster in a northeast and southwest orientation (Figure 4C). In four of these groups, trees with characteristic virus-associated symptoms were observed in previous years. When analyzing the distribution of the 42 groups affected by the virus (Figure 4A), it was obvious that

most of these groups cluster. Of these, only five are offside and do not border on affected groups of plants. Interestingly, only at the center of the site, an area of about 20 groups consists of symptomless seedlings. We did not detect any virus symptoms within the entire observation period in these groups.

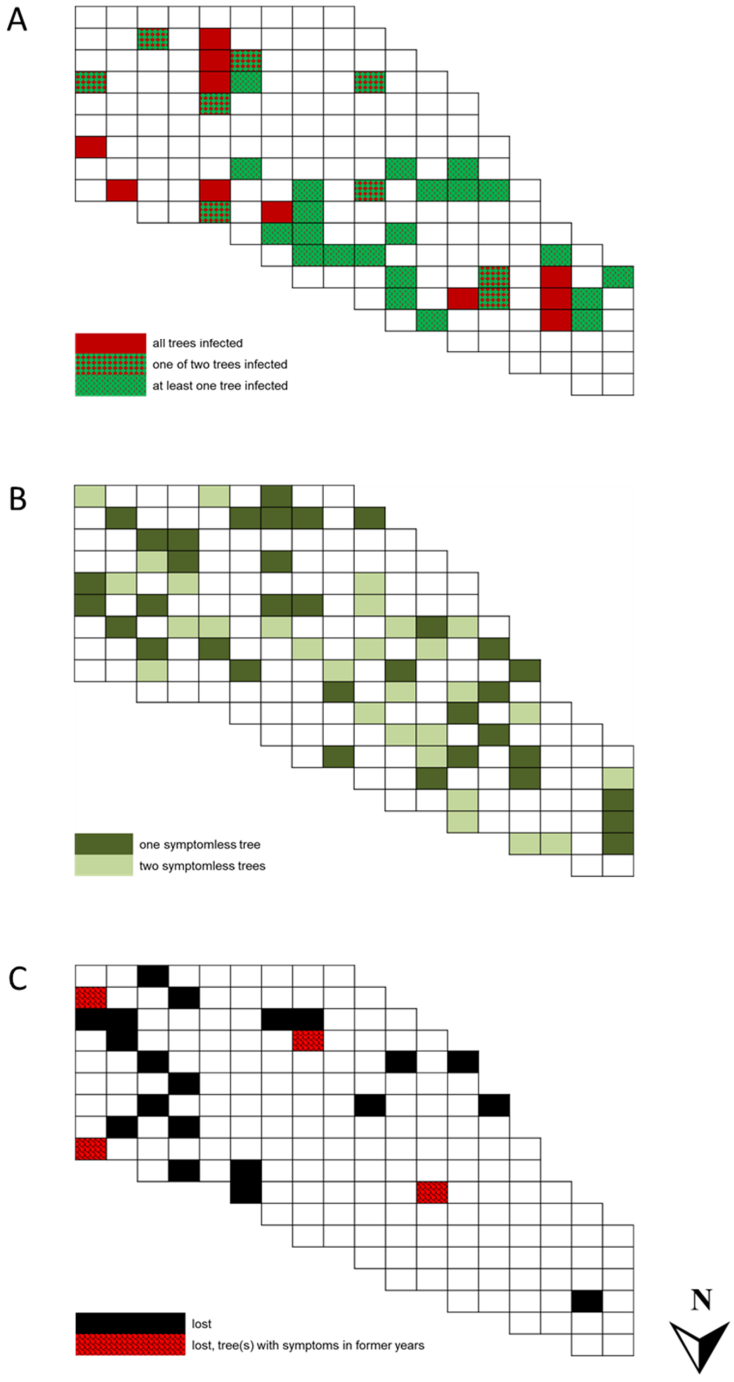

Figure 4. Distribution of lost groups and groups with symptomless and virus-infected trees in the orchard in July 2018. (**A**) groups with virus-infected tree(s); (**B**) groups consistent of one or two symptomless trees; and (**C**) lost groups.

4. Discussion

Seed orchards of forest plants such as the monitored common oak orchard in this study supply tree nurseries to cover seed demand. Although natural regeneration is the preferred option for stand

establishment in many regions of central Europe, seeding and planting also play a major role. In Germany, nearly 50% of oak forests are established by planting due to the difficulties in regenerating oaks naturally [21]. This is particularly the case where competition from herbaceous and woody vegetation hinders the natural regeneration of oak or when coniferous stands are to be converted to oak forests and acorn sources are lacking. The main role of seed orchards is to preserve endangered populations and provide valuable seed.

In central Europe, cluster planting to establish an oak stand or orchard became popular in the 1970s. Oaks are planted in more or less denser groups, which are spaced at a distance that represents the target density of future crop trees. It was proposed that uniformly distributed clusters of sown or planted oaks would enable a more regular early spacing of trees, thereby resulting in a structural homogeneity of the crown layer [22]. As shown by a meta-analysis of oak cluster planting trials located in Germany, Switzerland, and Austria, the mean survival rate of oaks in group plantings was determined to be about 80% after one decade [23]. Nest planting trials, which were established earlier, showed survival rates estimated at less than 50% after two decades.

Our observations also fall into this order of magnitude. Our survey revealed that about a third of the oak seedlings in the conservation seed orchard survived the first two decades after stand establishment. Major differences in the health status between the groups are remarkable. Twenty-five years after planting, in about 22% of the clusters, seedlings prevailed, whereas 13% of them did not produce a single tree. In 12%, more than four trees still have to share the confined space. In our opinion, a whole complex of factors certainly contributes to lower survival, unfavorable growth, and reduced quality of oaks in the orchard. Abiotic factors like climatic extremes (extreme winter frost, late and early frosts) and general water stress, in particular summer drought, can cause severe damage by reducing tree growth and causing root damage and senescence [24]. Furthermore, competition from ground vegetation; infection with microorganisms, rodents, and insect borers; and deer-browsing of terminal shoots may limit growth and satisfactory development of the trees. Although the direction contribution of nutritional deficiencies remains to be investigated, it can definitely exacerbate the impact of the aforementioned stress factors, thereby increasing the chances of decline or even death of oak trees.

Based on observations from conventional row plantings, it has previously been suggested that to achieve a final number of 70–80 harvestable crop trees, 150–250 potential future crop trees per hectare would be required at age 20 [25]. No significant difference was observed in oak survival rates comparing group and row planting [26]. This calculated number of potential future crop trees is slightly lower in the observed conservation seed orchard. Information is lacking regarding mortality rates of future crop trees from cluster plantings, as existing trials have not yet reached an age for the first commercial thinning [22]. As there will be at the most only one future oak crop tree for each initially planted cluster, any further mortality of crop trees or downgrading will affect the overall silvicultural aim and likely the economic outcomes.

To operate the orchard economically in the medium as well as in the long term, the oak trees must be kept healthy and vital. For example, European oak powdery mildew (*Erysiphe alphitoides*) can have a significant role in decline of mature trees, especially when leading to defoliation [27]. Although severe infection leads to a serious decline in leaf life-span, the fungus has only a moderate impact on leaf physiology [28]. However, the decrease in carbon assimilation in infected leaves was not compensated by an increase in healthy leaves of the same seedling.

It is often assumed that virus-infected plants show an increased susceptibility to negative environmental impacts such as climate change and pathogen/pest attack or a reduced adaptive capacity to respond to such negative site conditions. Although both cases result in a decline of the tree over time, understanding the causes of such decline is required to predict its consequences and to make recommendations for preventative actions in forestry. This is a great challenge for the scientific community, as reliable data on the cause and effects of viruses in woody plants are so far rare. One of the few exceptions is Sharka disease caused by plum pox virus (PPV). It is considered one of the most

detrimental diseases of stone fruits and is among the most studied viral diseases [29]. Severe losses may result in premature fruit drop, deformed, and discolored fruits, or reduce yield and quality of alcohol and spirits produced from diseased fruits. Ref. [30] identified the chloroplast and the photosynthetic machinery of the plant as the most affected parts. The authors attribute an imbalance in the antioxidant machinery with an accompanying accumulation of reactive oxygen species as contributing to the viral symptoms as well as to the deleterious effects of the infection.

In diseased common oak trees from the seed orchard we identified several virus-related contigs showing high sequence similarities to genome components of emaraviruses using two independent HTS approaches. The genus *Emaravirus* of the family *Fimoviridae*, order *Bunyavirales*, is relatively new. The type member *European mountain ash ringspot-associated emaravirus* (EMARaV) was identified in 2007 [31]. So far, EMARaV, *Actinidia chlorosis ringspot-associated emaravirus* 1, *Blackberry leaf mottle associated emaravirus*, *Fig mosaic emaravirus*, *High plains wheat mosaic emaravirus*, *Pigeonpea sterility mosaic emaravirus*-1, *Pigeonpea sterility mosaic emaravirus*-2, *Pistacia emaravirus B*, *Raspberry leaf blotch emaravirus*, *Redbud yellow ringspot-associated emaravirus*, and *Rose rosette emaravirus* are members of that genus. Others putative emaraviruses such as blue palo verde broom virus, Ti ringspot-associated virus, and jujube yellow mottle-associated virus, which possess characteristic features, are not yet officially classified [32–34]. Typically, they have a genome composed of four to eight negative-sense RNAs, encoding at least RNA-dependent RNA polymerase (RdRp), a glycoprotein (GP) precursor, a nucleocapsid protein (NP), and a nonstructural movement protein (MP) as core elements [35]. The genome organization of the novel virus identified in oak resembles that of emaraviruses, in that contigs relate to four core genome segments, each encoding a single open reading frame in negative orientation. Based on similarities of deduced protein sequences to other emaraviruses and established species demarcation criteria from the International Committee on Taxonomy of Viruses (ICTV), we suggest that the newly identified virus in oak represents a new member of the genus *Emaravirus* [35]. This assumption is further strengthened by the RT-PCR based amplification of a PCR product with a genus-specific primer pair targeting RNA1 [20]. We deduced the functions of the RNA1 to RNA4 encoded proteins of the novel virus as described above and derived species-specific primer pairs targeting the different genome segments to establish an RT-PCR based detection system for the novel virus in oaks. Over three years (2016–2018), we carried out a comprehensive screening of oak trees in the seed orchard and confirmed (i) the suitability of RT-PCR targeting the viral RNA3 and RNA4 for routine testing and (ii) a clear association between the detection of viral genome segments and chlorotic ringspot on the leaves of diseased trees. Since HTS datasets gave no hints to the involvement of other plant viruses, we assume that the novel virus is the primary cause of the observed symptoms. As the genetic composition of emaraviruses is variable with respect to genome segments, we cannot rule out the possibility that the novel virus may have other genome segments. Further molecular characterization is essential. The illumination of biological features is also required.

Although not all emaraviruses have been biologically characterized, various modes of transmission have been proven, including eriophyid mites, grafting, mechanical means (inoculation and contaminated cutting tools), and (rarely) seeds [33]. In the current study the proportion of virus-infected trees increased from 3 to 12% during the observation period. The distribution of these trees suggests a vector transmission of the virus, as already shown for some other viruses from the genus *Emaravirus*. However, so far neither a vector nor an artificial mechanical transmission has been confirmed. Furthermore, it remains unclear why the virus is not inevitably more often transmitted to adjacent seedlings within the same cluster, rather than to neighboring cluster. In this context, virus resistance has to be considered, with future investigations exploring plant defense.

Both biotic and abiotic conditions shape plant responses to stress events. The environment is rarely ideal for plant growth and even mild; episodic stresses can predispose plants to inoculum levels they would otherwise resist [36]. Most plant viruses are described as pathogens causing diseases in agricultural and horticultural crops. They are harmful to their host as they are dependent on host resources to support their own reproduction and dissemination. It is becoming evident that the

nature of the virus–host relationship is dependent on the environment. For example, a virus being pathogenic under normal environmental conditions can become beneficial to the host under stressful conditions [37]. This is true when the virus can ameliorate the impacts of biotic stress and can help plants to combat abiotic stress. White clover plants, for example, are less attractive to fungal gnats when they are infected with *White clover mosaic virus* [38], and wild gourds are less attractive to beetles when they are infected with *Zucchini yellow mosaic virus* [39]. The capacity of a mild strain of a virus can also be used to protect the plant against subsequent infection by a severe strain of the virus. This is known as mild strain cross-protection and has proven to be a powerful approach in combating devastating pathogens such as *Citrus tristeza virus* and *Pepino mosaic virus* [40]. As shown by [41], infection with plant viruses, independent of the width of their host range, can actually provide their host protection from drought stress. In virus-infected tobacco, beet, and rice, drought symptoms appeared later and leaves were able to maintain water longer than uninfected counterparts. Barley yellow dwarf virus-infected wheat was also associated with higher leaf water potential when water inputs were low and infected plants surpassed control plants in performance traits such as above-ground growth, seed set, seed yields, and seed germination [42]. The physiological mechanisms responsible for virus-conditioned resistance to drought stress still remain to be elucidated. It was suggested that the protection may be due to a virus-induced accumulation of osmoprotectants and antioxidants such as anthocyanins [41]. Likewise, proline, a proteinogenic amino acid acting on osmotic adjustment that helps subcellular structure stabilization and the elimination of free radicals, invariably had a higher concentration in sweet orange (*Citrus sinensis*) infected with citrus tristeza virus [43]. Survival of infected plants under extreme drought stress represents a conditional difference in fitness, assuming the surviving virus-infected plants can subsequently produce offspring. The authors of [37] have already recognized the importance of viruses that benefit their plant hosts and are contributing members of robust ecosystems. It is therefore essential to investigate the influence of viruses on tree fitness and populations in environmental contexts further. However, demonstrating that a virus benefits a plant host is difficult as in an ecosystem the virus and host do not exist in isolation. The benefit or harm of a virus for the plant probably depends therefore on how interactions between the virus and other players in the community affect their composition and, ultimately, the fitness of the host.

Although the association between the observed leaf symptoms and the novel emaravirus in the oak conservation seed orchard is close to 100%, the association between the detection of the virus and the overall performance of the seedlings is non-existent or at best weak. Thus, a virus-infected seedling may even emerge to a clearly prevailing tree. Reliable data are currently lacking for forecasts on the growth behavior of virus-infected oak trees in the future.

5. Conclusions

Investigations on the epidemiology and functional genomics of the novel emaravirus in oak have to be intensified to quicken the evaluation of the effect of that new virus on its host and microbiome. It is not clear whether the virus is in the long-term detrimental to oaks or beneficial. The cumbersome sexual regeneration of oak species may further jeopardize the ability of oak forests to adapt to our changing environment and the increase in extreme weather conditions. Oak seed orchards support the conservation as well as the development of forest genetic resources. High-quality seedlings are necessary for successful reforestation in a time of climate change. Quality traits must be considered in particular if a seedling has to have a high survival and growth capacity after transplanting in an unfavorable environment. Unfortunately, it is not possible to ascribe a universal set of structural or physiological high-quality attributes to plants. Traits of seedlings conferring high performance on one site do not necessarily maximize seedling outplanting performance on another. Finally, it is conceivable that plant viruses may extend survival of their hosts under conditions of abiotic stress that could benefit hosts if they can subsequently recover and reproduce. Such beneficial effects of viruses have been poorly studied so far. Perhaps we need to break completely new ground to enhance silviculture.

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