New natural weed host *Raphanus raphanistrum* L. (Brassicaceae) for *Beet necrotic yellow vein virus* and its vector *Polymyxa betae* Keskin

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Abstract: Rhizomania is an important virus disease of sugar beet (*Beta vulgaris*) caused by *Beet necrotic yellow vein virus* (BNYVV). The virus is transmitted to the roots of host plants by the plasmodiophorid *Polymyxa betae*. During survey studies in September and October 2009, yellow vein banding symptoms were observed on wild radish (*Raphanus raphanistrum*) plants growing in spinach (*Spinacia oleracea*) fields with a history of rhizomania in Samsun Province in the Black Sea Region of Turkey. To verify possible alternative hosts for BNYVV and *P. betae, R. raphanistrum* and spinach plants and soil samples were collected. BNYVV was detected in the leaf samples of field-collected *R. raphanistrum* using the DAS-ELISA test. This result was confirmed by RT-PCR and the *Raphanus* isolate was determined to be from the type A strain based on restriction fragment length polymorphism analysis. The presence of *P. betae* cystosori was not detected in the roots of *R. raphanistrum* plants using a light microscopy technique, but some objects resembling sporogenic plasmodia of *P. betae* were observed in the infested root cells. To confirm this result, total RNA was extracted from the roots of these samples and tested by RT-PCR using *Polymyxa*-specific primers. In contrast to the microscopy method, all samples tested positive for *P. betae* using RT-PCR. Healthy seeds of *R. raphanistrum* were planted in plastic pots containing soil from the same fields infested with BNYVV and the seedlings showed similar symptoms to those growing in natural conditions. To our knowledge this is the first report of a BNYVV infection of *R. raphanistrum* under natural conditions.

Key words: Rhizomania, *Polymyxa betae*, Brassicaceae, *Raphanus raphanistrum*, host

1. Introduction

*Beet necrotic yellow vein virus* (BNYVV), which is the agent of rhizomania disease, is transmitted by *Polymyxa betae* Keskin and causes significant losses in sugar beet fields all over the world. The disease is characterized in sugar beet plants by extensive rootlet proliferation from the main taproot and leaf chlorosis. BNYVV is the type species of the genus *Benyvirus* and it contains four or five ssRNAs (Koenig et al., 1986). Molecular analyses (restriction fragment length polymorphism, single-strand conformation polymorphism, and sequencing) of BNYVV isolates have revealed the existence of three strain groups (A, B, and P type) in Europe (Kruse et al., 1994; Koenig et al., 1995; Koenig and Lennefors, 2000; Ward et al., 2007). Rhizomania disease was first reported in sugar beet in Turkey in 1987 (Koch, 1987). Since then it has spread to many major sugar beet-growing areas within the country, especially the Western and Central Black Sea Region and the Central Anatolia and Marmara Regions (Erdiller and Özgür, 1994; Kaya, 2009; Kutluk Yilmaz and Arli Sokmen, 2010). At present, the disease is controlled by cultivating BNYVV-resistant sugar beet cultivars. However, resistance-breaking BNYVV strains have been found in cultivars carrying the *Rz1* gene in the United States, Europe (Liu and Lewellen, 2007; Bornemann et al., 2015), and Turkey (Kutluk Yilmaz et al., 2012).

Both BNYVV and its vector *P. betae* have limited host ranges. A few members belonging to Amaranthaceae, Asteraceae, Caryophyllaceae, Chenopodiaceae, Portulacaceae, Solanaceae, and Poaceae were recorded as alternative hosts for BNYVV (Tamada and Baba, 1973; Barr and Asher, 1992; Liu and Lewellen, 2007; Bornemann et al., 2015), and Turkey (Kutluk Yilmaz et al., 2012).

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belonging to Brassicaceae. The broad ecological tolerance of these weeds, such as quick germination, rapid growth, and abundant seed production, make it a successful weed in many countries. This weed, a native of northern Europe and northern Asia, is found in cultivated fields around the world (Cheam and Code, 1995; Blackshaw, 2001).

A major problem is that the virus particles survive inside resting spores of \textit{P. betae} for at least 15 years (Abe and Tamada, 1986). In this long period, some weed species act as alternative hosts for the virus and vector (Hugo et al., 1996; Legreve et al., 2005; Mouhanna et al., 2008; Rysanek et al., 2008). In 2009, several spinach fields with a history of rhizomania in Samsun Province in the Black Sea Region of Turkey exhibited yellow vein banding symptoms in wild radish and yellow-green mottling in spinach leaves. The presence of these kinds of virus symptoms on plants in these fields raises the question of where BNYVV is present in wild radish and spinach crops. Like sugar beet, spinach (\textit{Spinacia oleracea} L.) also belongs to the family Chenopodiaceae. In Italy, BNYVV was identified in spinach plants in 1995 (Autonel et al., 1995) and then the virus was found in spinach crops in California in 2010 (Liu et al., 2010). More recently, BNYVV was also detected in field-grown spinach in the western part of Turkey (Gumus et al., 2014).

Improved control strategies for BNYVV require further knowledge of potential reservoirs of infection in sugar beet fields. However, reports on natural hosts of BNYVV based on large-scale surveys are scarce. Maintenance of virus inoculum in weed species might provide a means for viruses to survive through the seasonal cycle and explain their prevalence during epidemics. Therefore, discovering the weed reservoirs of BNYVV may help us understand their epidemiology and provide basic information for implementation of effective management strategies. Therefore, the objective of this study was to test \textit{R. raphanistrum} for reservoirs of BNYVV and its vector \textit{P. betae} by using ELISA and RT-PCR tests.

2. Materials and methods

2.1. Plant and soil material
In 2009, typical virus-like symptoms such as yellow-green mottling in spinach plants and yellow vein banding on \textit{R. raphanistrum} were observed in a spinach field in the Bafra district of Samsun Province in Turkey. This area is well known from our previous studies to be highly contaminated by \textit{P. betae} and BNYVV. For this reason, a total of 25 \textit{R. raphanistrum} and 9 spinach plants were collected. Soil samples were also taken from patches with plants displaying typical virus symptoms. Additionally, three soils infested with \textit{P. betae} and BNYVV and a noninfested soil (from the Ondokuz Mayis University, Plant Protection Department collection) were used to grow sugar beets in a bait plant test as positive and negative controls in serological and molecular studies. Samples were homogenized and used for bioassays under greenhouse conditions.

2.2. Seed material
Seeds from Chenopodiaceae, Brassicaceae, Amaranthaceae, and Portulacaceae were used in this study and supplied from Ondokuz Mayis University, Plant Protection Department collection.

2.3. Bait plant technique
Seeds were sown separately into 500-mL plastic pots containing rhizomania-infested soil in three replications with ten seeds per plastic pot. The pots were placed in a controlled glasshouse at 19 °C (night) and 25 °C (day). After 8 weeks, plants were harvested and their roots were carefully washed in running tap water. One section of the roots from each bait plant was checked for the presence of \textit{P. betae} cystosori by light microscope. Another part was tested for the presence of BNYVV by ELISA. Plants that tested positive for BNYVV or \textit{P. betae} were assessed again in a second test to determine if the virus and vector could be transmitted back to sugar beets. For this purpose, dried roots of infected plants were cut into small fragments and mixed with equal parts sterile sand and soil, into which a BNYVV-susceptible sugar beet seed (Arosa) was sown. After 8 weeks, infections by \textit{P. betae} and BNYVV were assessed.

Besides this, sugar beet seeds of rhizomania-susceptible Arosa were planted into 300-mL pots of three BNYVV- and \textit{P. betae}-infested soils. Noninfested soil samples mixed with sterile sand (1:1) were used as positive and negative controls in serological and molecular studies.

2.4. Serological tests
DAS-ELISA was performed for BNYVV using polyclonal antiserum (Sediag, France) according to Clark and Adams (1977). A sample was considered positive when the absorbance value at 405 nm was more than two times the mean of the negative controls (Meunier et al., 2003).

2.5. Microscopy analysis of \textit{Polymyxa betae}
Root samples were stained with lactophenol containing 0.1% acid fuchsin and examined under a light microscope (Leica, Switzerland) to detect the presence of \textit{P. betae} cystosori (Abe and Tamada, 1986).

2.6. Total RNA isolation
Total RNA was extracted from the roots and leaves of \textit{R. raphanistrum} and the roots of sugar beet that had previously tested positive for BNYVV in ELISA. Total RNA was extracted from 100 mg of frozen tissue using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions.
2.7. RT-PCR for detection of BNYVV and *Polymyxa betae*

One-step RT-PCR was performed as described in the manufacturer's manual (QIAGEN). For the detection of BNYVV, the primers proposed by Kruse et al. (1994) were used (forward primer: GTGATATATATGTGAGGACGCT and reverse primer: CCGTGAAATACGTGATGTT), which amplified RNA-3. The RT-PCR reaction included the following: 15.5 µL of RNase-free water, 1 µL of dNTPs mix (400 µM), 5 µL of 5X Q solution, 0.25 µL each of forward and reverse primers (0.6 µM), 1 µL of QIAGEN OneStep RT-PCR enzyme mix, and 2 µL of RNA sample. Reverse transcription consisted of 30 min at 50 °C and 15 min at 95 °C. We then ran 35 cycles composed of: denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C, and elongation for 1 min at 72 °C. A final elongation of 7 min at 72 °C was also performed.

For the detection of *Polymyxa* in roots of *R. raphanistrum* and sugar beet, oligonucleotides flanking 5.8s-rDNA and internal transcribed spacers (ITS) were used. This includes upstream 5'-GAGGCATGCTTCCGGAGGGCTCT-3' and downstream primer 5'-CTGCGGAAGGATCATTAGCGTT-3' (Ward and Adams, 1998). The RT-PCR reaction included the following: 15.2 µL of RNase-free water, 1 µL of dNTPs mix (400 µM), 5 µL of 5X Q solution, 0.15 µL each of forward and reverse primers (0.6 µM), 1 µL of QIAGEN OneStep RT-PCR Enzyme mix, and 2.5 µL of RNA sample. Reaction conditions were the same as used for the amplification of RNA-3 except for annealing temperature (55 °C).

After PCR amplification, 2 µL of loading buffer was added to the PCR products. The samples were analyzed on 1.2% ethidium bromide agarose gel in 1X Tris-borate-EDTA buffer using the Gel Doc 2000 Systems (Bio-Rad, USA) (Ward and Adams, 1998).

2.8. Restriction fragment length polymorphism analysis

To produce a PCR product for restriction fragment length polymorphism (RFLP) analysis, the primers proposed by Kruse et al. (1994) were used to amplify the RNA-3 as described above, which was then cut separately with enzymes EcoRI, BamHI, and MspI.

3. Results and discussion

3.1. Detection of BNYVV

A total of 9 spinach plants and 25 *R. raphanistrum* samples were collected from the spinach field and tested by DAS-ELISA. In this study, BNYVV was detected in 20 samples of *R. raphanistrum* collected from the field (Figure 1A). On the other hand, 7 of the field-grown spinach samples were infected with BNYVV. Generally, BNYVV-positive spinach plants have yellow-green mottling symptom on their leaves. Although spinach is grown in many regions of Turkey, BNYVV has only been recently recorded in spinach production areas in İzmir and Manisa provinces located in the western part of Turkey (Gumus et al., 2014). In the ELISA tests with the *R. raphanistrum* samples, color development was very slow. For this reason, the DAS-ELISA test microtiter plates were read following 2 h substrate incubation at room temperature and then again following overnight substrate incubation at 4 °C. Even after overnight incubations, relatively low A405 values were obtained for most of the samples. The positive controls supplied by the Sediag Biochemica and root samples from the sugar beet bait plants grown in BNYVV-infested soils gave satisfactory positive results at 2 h and 16 h substrate incubations. The reason for the low ELISA values could be low virus titers in *R. raphanistrum* samples. In addition, RT-PCR assay to detect BNYVV and *P. betae* was carried out on total RNA extracted from leaves and root samples from *R. raphanistrum* and root samples from the sugar beet bait. All samples tested positive for BNYVV.
in this RT-PCR detection assay (Figure 2). Until now, BNYVV host studies on Brassicaceae members such as *R. raphanistrum*, *R. sativus*, *B. napus*, and *S. arvensis* have failed to demonstrate infection by this virus or its vector (Barr and Asher, 1992; Hugo et al., 1996; Legreve et al., 2005). However, BNYVV and *P. betae* were both detected in *Capsella bursa-pastoris* (L.) Medik. and *Thlaspi arvense* L., which belong to the family Brassicaceae, in a bait plant test using RT-PCR by Legreve et al. (2005). However, in this study, neither BNYVV nor its vector were found in these species by ELISA or light microscopy, respectively (Legreve et al., 2005). Later on, Mouhanna et al. (2008) found that *C. bursa-pastoris* was infested with *P. betae* and BNYVV using bait plant test and back transmission studies. To our knowledge, this is the first report of a BNYVV infection of *R. raphanistrum* in the family Brassicaceae under natural conditions.

3.2. RFLP analysis of BNYVV
Kruse et al. (1994) showed that RNA-3 of B type strains is cut by *Bam*HI, *Eco*RI, *Sty*I, and *Msp*I. In contrast, the A type strains were not cut by any of these restriction enzymes. Our BNYVV isolates were not digested by these restriction enzymes, suggesting that the isolate from *R. raphanistrum* belongs to the A type strain (Figure 3). The A type, the most widespread strain (Schirmer et al., 2005), has been previously detected in Turkey (Kruse et al., 1994; Kutluk Yilmaz et al., 2007); the B type strain is more restricted and mainly found in Germany, France (Kruse et al., 1994; Koenig et al., 2008), Japan (Miyanishi et al., 1999), and China (Li et al., 2008).

3.3. Detection of *P. betae* in infested roots
Roots of *R. raphanistrum* that were collected from a spinach field were inspected for the presence of *P. betae* using light microscopy. *P. betae* cystosori were not detected in roots of any of these plants, though some objects resembling sporogenic plasmodia of *P. betae* were described in the root cells of *R. raphanistrum* (Figure 4). To confirm this result, sugar beet bait plant and field-grown *R. raphanistrum* samples were subjected to RT-PCR assay with *Polymyxa*-specific primers (Ward and Adams, 1998) to determine whether they were infected with *Polymyxa* spp. Ward and Adams (1998) emphasized that the products of amplification using these primers on isolates of *Polymyxa graminis* types I and II and *P. betae* could all be distinguished from one another on 1.2% agarose gels. Therefore, a 250-bp DNA fragment expected for the ITS...
between the 5.8 s and the 18 s rRNA of *P. betae* was obtained from the roots of the susceptible sugar beet cultivar grown in rhizomania-infested soils and *R. raphanistrum* in this assay (Figure 5). Rysanek et al. (2008) suggested that the PCR method would be useful for verifying the presence of *P. betae* in the case of low numbers of cystosori in host range studies. Moreover, in some plant species, plasmodia or zoosporangia could be present without cystosori; PCR could reveal such hidden infections. Similarly, Legreve et al. (2008) found that BNYVV and *P. betae* were detected by RT-PCR in some species belonging to different families, while the virus and vector went undetected by ELISA and microscopy, respectively. We suggest that viruliferous *P. betae* zoospores were able to penetrate the roots of *R. raphanistrum* plants and introduce the virus, but the protozoa were unable to produce cystosori and complete their life cycle in the roots of wild radish. An incomplete infection, similar to our result, was reported with *P. betae* in *Gomphrena globosa* (Al Musa and Mink, 1981) and *P. graminis* in *Arachis hypogaea* (groundnut) (Thouvenel and Fauquet, 1981). Later on, Barr et al. (1995) reported similar observations about early *P. betae* developmental stages without cystosori in two wild beet species, *Beta patellaris* and *Beta procumbens*. Although the wild radish leaves became infected naturally with BNYVV in this field, they were unable to provide sources of virus infection to other plants, whereas comparable sugar beet roots were able to do so. There are various factors, such as environmental conditions, the lack of vital nutritional factors, or insufficient nutrients, which might have limited the development of pathogen structures (Barr et al., 1995).

3.4. Bait plant and back inoculation tests

Among all Brassicaceae species tested for *P. betae* and BNYVV host status, sugar beet, spinach, *Chenopodium album* L., *Chenopodium murale* L., *Amaranthus retroflexus* L., and *Portulaca oleracea* L. were added to the bait plant test. The Table shows the species that became infected with either *P. betae* or BNYVV. In this study, *P. betae* cystosori were found by light microscopy only in sugar beet, *C. album*, *A. retroflexus*, and *P. oleracea*. Interestingly, we could not detect either *P. betae* or BNYVV in spinach plants. From all tested species, infection by BNYVV was only detected in sugar beet and *R. raphanistrum* using ELISA. *R. raphanistrum* plants also showed symptoms of yellow vein banding under controlled conditions similar to natural conditions (Figure 1B). However, *P. betae* was not detected using the light microscopy technique in these plant roots. Neither BNYVV nor *P. betae* was detected in the other tested Brassicaceae species: *R. sativus*, *E. sativa*, *B. rapa*, *B. juncea*, and *B. oleracea* (Table).

Besides this, we tried using roots of a plant infected with a vector or the virus (sugar beet, *C. album*, *A. retroflexus*, *P. oleracea*, and *R. raphanistrum*) to see if they had the potential to act as an alternative host for *P. betae* and BNYVV to transmit to susceptible sugar beet plants. Transmission back to sugar beet occurred only from *B. vulgaris* (Table).

In rhizomania disease, the virus–vector–host relationships are very complex. Over the last decades, the existence of different genotypic groups has become obvious, and they correspond to differences in pathogenicity and specific geographical region. BNYVV isolates fall into three strain groups: A, B, and P types (Kruse et al., 1994; Koenig et al., 1995; Koenig and Lennefors, 2000; Ward et al., 2007). In the last years, resistance-breaking BNYVV strains have been found in cultivars carrying the *Rz1* gene in the United States, Europe (Liu and Lewellen, 2007; Bornemann et al., 2015), and Turkey (Kutluk Yilmaz et al., 2013). Beside this, the performance of viruliferous *P. betae* in soils is influenced by many different biotic and abiotic factors. Gerik and Duffus (1988) described differences in vectoring abilities of *P. betae* populations depending on their origin. There are also subgroups of *P. betae* with specific host ranges, such as betae, amaranthi, and the family Portulacaceae (Barr, 1979; Abe and Tamada, 1986; Abe and Ui, 1986). Moreover, weeds are populations with a wide range of genetic variability. In this respect, this appears to be the first report of *R. raphanistrum* from the family Brassicaceae acting as a host of BNYVV under natural conditions. The *Raphanus* isolate was assigned to the A type strain based on RFLP analysis. Although *P. betae* cystosori were not found in the roots of

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**Figure 5.** RT-PCR analysis for detection of *P. betae*. M: 1-kb DNA ladder (Promega). The arrow indicates the *P. betae*-specific 250-bp DNA fragment. Lane 1: root RNA from *R. raphanistrum*. Lane 2: negative control (healthy sugar beet roots). Lanes 3–5: positive controls (BNYVV-containing sugar beet roots).
R. raphanistrum, it may infect wild radish roots without cystosori development. Therefore, R. raphanistrum seems to be a relatively inefficient host for rhizomania compared to sugar beet. Nonetheless, knowing that this weed is a host of BNYVV may help to understand its epidemiology and could provide basic information for implementation of effective management strategies. Further work is needed in the search for natural hosts of BNYVV and P. betae.

Table. ELISA and microscopy results for the bait plant and back inoculation to B. vulgaris tests.

<table>
<thead>
<tr>
<th>Family</th>
<th>Plant species</th>
<th>Presence of P. betae cystosori (microscopy)</th>
<th>Infection by BNYVV (ELISA)</th>
<th>Back inoculation B. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenopodiaceae</td>
<td>Beta vulgaris L. cv. Arosa</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>Spinacia oleracea L.</td>
<td>-</td>
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<td>nt</td>
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<tr>
<td>Chenopodiaceae</td>
<td>Chenopodium album L.</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Chenopodiaceae</td>
<td>Chenopodium murale L.</td>
<td>-</td>
<td>-</td>
<td>nt</td>
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<tr>
<td>Amaranthaceae</td>
<td>Amaranthus retroflexus L.</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Portulacaceae</td>
<td>Portulaca oleracea L.</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>Raphanus raphanistrum L.</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Brassicaceae</td>
<td>Raphanus sativus L.</td>
<td>-</td>
<td>-</td>
<td>nt</td>
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<tr>
<td>Brassicaceae</td>
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<td>-</td>
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<td>nt</td>
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<td>Brassicaceae</td>
<td>Brassica napus (B-2 accession)</td>
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<td>nt</td>
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<tr>
<td>Brassicaceae</td>
<td>Brassica juncea (L.) Czern (B-4 accession)</td>
<td>-</td>
<td>-</td>
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<td>Brassica juncea (B-5 accession)</td>
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<tr>
<td>Brassicaceae</td>
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<td>-</td>
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<tr>
<td>Brassicaceae</td>
<td>Brassica rapa L.</td>
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<tr>
<td>Brassicaceae</td>
<td>Eruca sativa L.</td>
<td>-</td>
<td>-</td>
<td>nt</td>
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</table>

nt: not tested.

References


