



Detection of *Beet Necrotic Yellow Vein Virus* (BNYVV) in some common weeds of sugar beet fields in Fars province

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ABSTRACT

Rhizomania is one of the most important sugar beet diseases in Iran and some other parts of the world. In this study, the infection rate of the most important weeds of sugar beet fields under natural condition to the causal agent of the BNYVV (*Beet necrotic yellow vein virus*) disease was investigated at Fars Agricultural Research Center, Iran. Roots were collected from the infected weeds and BNYVV detection was carried out using ELISA test. Among the studied weeds, the highest rate of infection was detected in wild beet, *Beta vulgaris* subsp. *maritima* and for the rest of weeds including *Amaranthus retroflexus*, *Portulaca oleracea*, *Chenopodium album*, *Solanum nigrum*, *Convolvulus arvensis*, *Hibiscus trionum*, and *Heliotropium europaeum* no infection was detected. *B. maritima* seedlings were planted in infected soil in the greenhouse, and after two months, systemic leaf symptoms including mosaic, vein yellowing, short bunchy habit of growth (Rosette), and severe stunting were observed. Mechanical inoculation of BNYVV to *B. maritima* plants resulted in the emergence of disease symptoms. Upon the inoculation of infected *B. maritima* leaves on the three susceptible sugar beet cultivars (IC, PP8, and 7233 cultivars), yellow local and necrotic lesions were observed. The virus concentration in *B. maritima* was higher than the three aforementioned cultivars. This is for the first time to detect BNYVV in *B. maritima* and report it as a systemic host for BNYVV in Iran. Compared with other weeds grown in areas infected to rhizomania, this population of *B. maritima* (accession number 8901) had higher inoculum increment ability in the soil.

Keywords: *Beta maritima*, ELISA, mechanical inoculation, rhizomania, weed

INTRODUCTION

Rhizomania is one the most important disease of sugar beet (McGrann et al. 2009; Rush et al. 2006). The causal agent of the disease is *Beet necrotic yellow vein virus* (BNYVV, Tamad 1975; Tamada and Baba 1973). The virus belongs to *Benyvirus* genus (Rush 2003). The disease was first reported in 1996 in Fars province (Izadpanah et al. 1996) and later its distribution was reported in most sugar beet planting areas (Darabi et al. 2003; Mehrvar et al. 2009). The disease damage depends on sugar beet genotype, virus pathogenity, inoculum rate, BNYVV interaction with other pathogens, infection time, and climate condition (McGrann et al. 2009; Rush et al. 2006; Scholten

and Lange 2000; Stevens and Asher 2005). Severe infection decreases sugar yield to 50-60% and in some cases, up to 90% in susceptible cultivars (Asher 1993; Henry 1996; Johansson 1985). The natural host of the virus is mainly limited to the *Beta* species and sugar beet is the only and most important host. Spinach (*Spinacia oleracea* L.) and some *Chenopodium* species, including *C. polyspermum* L., *C. murale* L. and *C. capitatum* (L.) Asch. are also infected by the virus (Abe and Tamada 1986; Hugo et al. 1996). Currently, the only known vector of BNYVV in nature is *Polymyxa betae* Keskin. The fungus is the mandatory parasite of the root and produces resistant zoospore and spores in its life cycle (Barr and Asher 1996; Keskin 1964). The *P. betae* host range is relatively limited and mainly restricted to *Chenopodiaceae*, *Ama-*

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ranthaceae, *Caryophyllaceae* and *Portulacaceae* families. The *P. betae* population is heterogeneous and has a variation in host specificity so that *P. betae* isolated from a plant species may infest susceptible plants in other families or even in the same family (Abe and Tamada 1986; Barr and Asher 1992). Barr and Asher (1992) identified three *P. betae* biotypes in UK. The first biotype infests a wide range of *Chenopodium* species, the second biotype has a narrow host range and the third biotype only infests *Silene alab* L. species. Also, some specialized forms of *P. graminis* L. that normally infect cereals also infect sugar beet plants but it is not clear whether they are able to transfer BNYVV too (Rush 2003). The disease severity is directly dependent on virus concentration in infected plants which also depends heavily on the disease inoculum rate (spores of the fungus-resistant virus) in the soil (Asher et al. 2002; Buttner et al. 1995; Giunchedi et al. 1987). Therefore, with increase in inoculum, the disease severity and consequently its damage will increase. One of the most important factors that cause a significant increase in the inoculum rate, is the presence of susceptible host for BNYVV (Buttner et al. 1995; Hugo et al. 1996; Tuitert et al. 1994).

Several studies were conducted to determine the role of weeds and secondary hosts in BNYVV transmission and the disease widespread in contaminated areas. In Japan, BNYVV was only detected in resistant spores of *P. betae* which were formed in sugar beet, spinach, *Chenopodium murale* and *C. capitatum*. Therefore, field weeds were not involved in virus storage and the disease widespread (Abe and Ui 1986). Weeds were also collected in fields contaminated to rhizomania in Germany and their infection to BNYVV was evaluated. In these studies, remarkable concentration of virus was not detected in plant species including *Chenopodium* (Hess et al. 1982). In Germany and also in the USA, although some plant species such as *Gomphrena globose* L. was identified as host for the virus but the virus concentration in this plant was lower than sugar beet (Al Musa and Mink 1981). In Turkey, among the studied plants, chicory (*Cichorium intybus* L.), plantain (*Plantago major* L.), grass knotweed (*Polygonum aviculare* L.), datura (*Datura stramonium* L.), nightshade (*Solanum nigrum* L.) and chameleons were infected to BNYVV (Yanar et al. 2006). In UK, among the studied weeds, only *C. polyspermum* species was detected as a host for BNYVV (Hugo et al. 1996). In general, most of the weeds including

important sugar beet weeds such as *Chenopodium* species, pigweed, purslane and bindweed were not naturally infected to the virus but others such as nightshade irrespective of infection to BNYVV did not have a significant concentration of virus (Hugo et al. 1996; Mouhanna et al. 2008; Yanar et al. 2006).

In this study, except *B. maritima*, no infection to BNYVV was detected among the studied weeds. Therefore, it is essential to identify and control the natural hosts of the virus which act as a source of infection and cause increase in the disease inoculum. The aim of this study was to investigate the susceptibility of some important sugar beet weeds to BNYVV in order to identify alternative hosts of the disease. The susceptibility of *B. maritima* to rhizomania was also evaluated. This species has a diversity in susceptibility to rhizomania and in some of its populations the resistance source was detected (Biancardi et al. 2002; Geyl et al. 1995). The identified sources were widely used in sugar beet breeding programs for producing sugar beet varieties resistant to the disease. This species is widespread in some parts of Iran and is known as a weed in these regions (Mir Kamali 1999).

MATERIALS AND METHODS

Different weeds including amaranth, purslane, orach (*Chenopodium album* L.), nightshade (*Solanum nigrum* L.), field bindweed (*Convolvulus arvensis* L.), wild kenaf (*Hibiscus trionum* L.) and chameleon (*Heliotropium europaeum* L.) were evaluated. The abovementioned weeds were collected from sugar beet fields infected to rhizomania in Fars Research Center for Agriculture and Natural Resources, Zarghan. Consecutive planting of sugar beet for resistant genotype selection not only increased inoculum rate but also increased weed density. Weed roots were collected from moisturized soil and 20-25 seedlings per species were evaluated. Together with a selective weed root harvest, the adjacent sugar beet root was harvested as a positive control and evaluated by ELISA test. Weeds' roots were also collected from the control (disease free) field. The seeds of *B. maritima* were collected from Marbooyeh village in Darab city (20 km Northwest of Darab).

BNYVV detection in samples

DAS-ELISA test was conducted based on Clark and Bar-Joseph (1984) method to detect BNYVV.

The anti-serum applied in this study belonged to the Iranian isolate of the virus (Darabai et al. 2010). Different stage of the test was conducted and absorption was measured at 405 nm after 30 min. Roots were rinsed with water and dried. Then, 0.2 g of lateral roots or root tip was weighed and extracted in 1.5 ml buffer. For leaf sampling, 0.2 g of the tissue with disease symptoms (infected to BNYVV) was selected and extracted at the same amount of buffer. In each ELISA plate, six wells were considered as negative control (including sugar beet and weed root extract), and eight wells as positive control (extract from *Chenopodium quinoa* leaves infected to BNYVV and sugar beet root extract infected to rhizomania). Absorption rates which were three times more than the average absorption of healthy roots extract were considered as positive control (infected with BNYVV, Wisler et al. 2003).

Evaluation of B. maritima response to rhizomania under greenhouse and field conditions

To evaluate *B. maritima* response to BNYVV, seeds were sown in infected soil to rhizomania in the greenhouse. Infected soil was composed of one-third infected soil to rhizomania, one-third healthy soil, and one-third of leaf and sand mixture. Plants were kept at 20-30 °C in the greenhouse. Five to six weeks after planting, roots of 50 *B. maritima* seedlings were evaluated by ELISA test. Seeds were also sown in infected fields to rhizomania in Fars Research Center for Agriculture and Natural Resources, Zarghan. Four to five weeks after sowing, 40 seedlings were harvested and evaluated by ELISA test.

Mechanical inoculation

In this study, the possibility of the mechanical transmission of BNYVV to *B. maritima* and its susceptibility was evaluated. Therefore, the seeds of *B. maritima* were sown in healthy soil in the greenhouse. Six weeks after planting, seedlings were subjected to three different virus inoculations. The inoculations included sugar beet leaf extract infected to BNYVV with yellowing and vein necrosis, the *C. quinoa* leaf extract infected to BNYVV with leaf spot symptoms and sugar beet root extract (susceptible cultivar IC) infected to rhizomania. Each virus inoculation was applied on 30-40 healthy *B. maritima* seedlings. The response of three sugar beet cultivars to mechanical transfer of the virus from *B. maritima* was evaluated. Seedlings of three sugar beet cultivars susceptible to rhizomania (PP8, IC, and 7233) were mechani-

cally inoculated at 6-8-leaf stage by leaf extract of *B. maritima* infected to BNYVV. In addition, leaf extract of *B. maritima* was also applied on *C. quinoa*. For inoculation, 1 g of tissue containing the virus was crushed in 6-7 ml of 0.1 M phosphate buffer with 7.1 acidity in mortar. The extract was mechanically inoculated on seedling leaf sprayed with carbide powder. Inoculated plants were kept in the greenhouse at 21-30 °C.

Comparison of the virus concentration in B. maritima root with three susceptible cultivars

Seeds of the three susceptible sugar beet cultivars to rhizomania (PP8, IC, and 7233), *B. maritima*, and the commercial cultivar Dorothea were sown in randomized complete block design with four replications in an infected soil to rhizomania in the greenhouse. Each cultivar was sown in four pots each containing 3-4 seedlings. After eight weeks, 6-8 seedlings were harvested from each treatment. Then, from each seedling, 0.2 g tissue of the tap root was taken, weighed and extracted in 1.5 ml extraction buffer. With ELISA-test, the virus concentration was measured in individual roots. Data were analyzed using SAS software and Duncan's multiple range test was used for mean comparison.

RESULTS

ELISA results showed that weed's root was not infected to BNYVV. Average of the absorption rate for weed roots collected from infected field to rhizomania is shown in Table 1. No difference was

Table 1. Absorption results of ELISA-test for BNYVV detection in weed roots

Family	Scientific name	Average of absorption*
Amaranthaceae	<i>Amaranthus retroflexus</i>	0.009
Portulacaceae	<i>Portulaca oleracea</i>	0.016
Solanaceae	<i>Solanum nigrum</i>	0.011
Convolvulaceae	<i>Convolvulus arvensis</i>	0.007
Malvaceae	<i>Hibiscus trionum</i>	0.012
Boraginaceae	<i>Heliotropium europaeum</i>	0.017
Chenopodiaceae	<i>Chenopodium album</i>	0.003
Chenopodiaceae	<i>Beta maritima</i> ¹	0.748
Chenopodiaceae	<i>Beta maritima</i> ²	0.812
Chenopodiaceae	<i>Beta maritima</i> ³	0.886
Chenopodiaceae	<i>Beta vulgaris</i> ⁴	0.618
Chenopodiaceae	<i>Beta vulgaris</i> ⁵	0.017
Chenopodiaceae	<i>Chenopodium quinoa</i> ⁶	0.892

¹ Wild beet root sown in infected field to rhizomania at Fars Research Center for Agriculture and Natural Resources, ² Wild beet root sown in infected soil to rhizomania in the greenhouse, ³ Wild beet leaf infected to BNYVV with systematic mosaic symptoms, ⁴ Infected sugar beet root (IC cultivar, positive control), ⁵ Healthy sugar beet root (IC cultivar, negative control), and ⁶ *C. quinoa* leaf infected to BNYVV with yellow spots and necrotic symptoms (positive control).

* Absorption at 405 nm wavelength

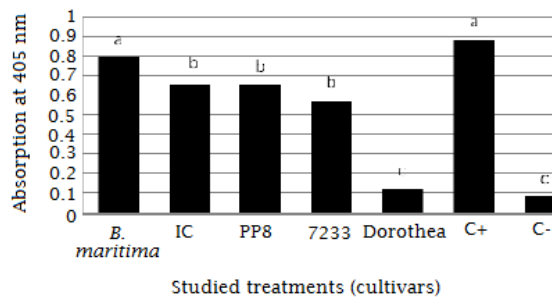


Figure 1. Mean comparison of average absorption in ELISA test for cultivars [c⁺: *C. quinoa* leaf infected to BNYVV (positive control), c⁻: healthy *C. quinoa* leaf (negative control)]



Figure 2. Systematic mosaic and rosette formation in *B. maritima* resulted from natural infection to BNYVV.



Figure 3. Localized spot and systematic mosaic formation in *B. maritima* 12 days after mechanical inoculation by infected sugar beet leaf extract



Figure 4. Systematic mosaic and rosette in *B. maritima* 14 days after mechanical inoculation by infected sugar beet leaf to *C. quinoa* extract

observed between absorption rate of the weed roots collected from disease-free and infected fields. However, sugar beet plants adjacent to infected weed roots showed high infection to BNYVV. In this study, the average absorption rate of the *B. maritima* roots collected from the infected field at Fars Research Center for Agriculture and Natural Resources, Zarghan, was also high (0.747, Table 1).

ELISA results showed high infection to BNYVV in seedling roots of *B. maritima* samples collected from infected soil to rhizomania in the greenhouse with an average rate of 0.812 (Table 1). In addition to high infection to BNYVV, a few of the seedlings (20 seedlings) showed foliar symptoms of systematic mosaic, vein yellowing, tiny leaf formation and stunting (Figure 1). ELISA results confirmed BNYVV presence in seedlings with foliar symptoms and high infected seedling extract absorption (0.886, Table 1). Systematic foliar symptoms formation was not simultaneous in seedlings and emerged 4-6 weeks after planting. Seedlings with foliar symptoms did not have the ability to produce seeds and most of them disappeared after a short time.

In mechanical inoculation of BNYVV with *C. quinoa* isolates, leaf and root of infected sugar beet to BNYVV were initially inoculated on *B. maritima* seedlings and yellow spots followed by systematic mosaic were formed (Figure 2, 3). Symptoms formed on *B. maritima* as a result of inoculation with *C. quinoa* isolates, leaf and root of infected sugar beet were appeared after six, nine, and 14 days, respectively, and later expanded. In this case, infected seedlings had stunted growth and usually died after a short time. Also, in mechanical inoculation of the virus from the extract of infected leaf of *B. maritima* on its healthy plants, the abovementioned symptoms were appeared after six days. Contrary to natural infection of the seedlings (infection of the seedlings planted in soil infected to rhizomanai by *P. betae*) in which systematic symptoms appeared gradually and in some seedlings, in mechanical inoculation, the symptoms were formed in most seedlings simultaneously. Yellow spots followed by necrosis were formed in mechanical transmission of BNYVV from the extract of infected *B. maritima* leaf on three sugar beet cultivars (Figure 4). Sometimes restricted yellowing symptoms were formed around certain veins but in the abovementioned cultivars, systematic disease symptoms were not formed. In mechanical inoculation of the virus from the infected extract of *B. maritima* on



Figure 5. Yellow localized spot and necrosis in sugar beet leaf (cultivar IC), 12 days after BNYVV mechanical inoculation by infected sugar beet leaf to *B. maritima* extract



Figure 6. Localized spot and necrosis in *C. quinoa* leaf, 12 days after BNYVV mechanical inoculation by infected sugar beet leaf to *B. maritima* extract

C. quinoa seedlings, yellow spot followed by necrosis were appeared. Yellow symptoms were usually developed around veins (Figure 5). By comparing virus concentration in *B. maritima* root with three susceptible sugar beet cultivars (IC, PP8, and 7233) sown in infected soil to rhizomania in the greenhouse, it was shown that the virus concentration in *B. maritima* root was significantly higher than in the three cultivars ($P < 0.05$). Average absorption rate of ELSA test results is shown in Figure 6. The lowest infection rate was belonged to Dorothea cultivar.

DISCUSSION

Results of this study are similar to other studies. However, considering the variation in the isolates of vector fungi for BNYVV transfer (Gerik and Duffus 1988; Kastirr et al. 1994) and also different types and variants of the virus in Iran (Mehrvar et al. 2009), for reliable conclusion on the secondary hosts of BNYVV, it is essential to evaluate other plants (especially weeds of sugar beet fields) in other infected regions of the coun-

try. It is also likely that BNYVV rate in weed root would be less than the threshold which could be detected by ELISA test. Therefore, using sensitive diagnostic methods for virus detection such as molecular methods is essential (Mouhanna et al. 2008). Mouhanna et al. (2008) results illustrated that in some plants sown in infected soil to rhizomania (such as few monocotyledons), no infection to rhizomania was detected in ELISA test; however, under natural infection, causal agent was transferred from the root of these plants to susceptible sugar beet cultivars. This status illustrates low virus concentration in the root of the above-mentioned plants which was not detectable by ELISA test. These authors suggested natural virus transfer (by *P. betae*) as the most proper method for the evaluation of plant susceptibility to BNYVV. In general, weed role in disease widespread was low compared with sugar beet and only sugar beet can be considered as a natural host for the virus (Hess et al. 1982; Hugo et al. 1996). Therefore, *P. betae* population can be increased to 10000 times of its initial population in a growing season by planting susceptible sugar beet (Asher 2003). Wild sugar beet, *B. maritima*, which is also called sea beet is mainly a coastal plant which grows in Mediterranean and North Atlantic Ocean coast from Britain to Canary island (Doney et al. 1990). This plant has wide diversity according to latitude and life cycle so that its biennial and perennial populations are found in northern areas of UK, Netherland, and Belgium and its annual population in Mediterranean areas. *Beta maritima* cross with agronomical beets is done simply (Hjerdin et al. 1994). Resistance to cercospora leaf spot (*Cercospora beticola* Sacc.) and rhizomania is the most important result of the gene transfer from this species to sugar beet (Skaracis and Biancardi 2000; Biancardi et al. 2002). So far, several rhizomania resistance genes have been identified in *B. maritima* (Biancardi et al. 2002; Geyl et al. 1995). One of the most important sources of the resistance was taken from Denmark and a wild sugar beet (wild beet 42, WB42) known as *Rz2* (Scholten et al. 1999). Currently, this gene is considered as the most effective source of resistance against the disease. Other resistance sources including *Rz3* and *Rz5* were also obtained from the accession numbers 41 (WB41) and 258 (WB258) from wild beet (Gidner et al. 2005; Grimmer et al. 2008). In addition, wild beet was used for the transfer of powdery mildew, *P. betae* and root-knot nematode (*Meloidogyne* spp) resistance genes (Asher et al. 2008; Lewellen and Schrandt 2001; Yu et al.

1999). Also, resistance source to environmental stresses (salt and drought) was identified in *B. maritima* (Luterbacher and Smith 1998). In addition to *B. maritima* population, different populations with varied degree of susceptibility were identified. For example, one sample of *B. maritima* collected from Turkey were susceptible to rhizomania and showed systematic mosaic and stunting symptoms under mechanical inoculation. This sample was named as M8 line (*Beta vulgaris* subsp. *maritima* M8, Tamada 2007). No comprehensive information is available about *B. maritima* distribution in Iran. This genus was only reported from sugar beet fields in Khuzestan but is currently reported from wheat field in Fars, Bushehr, Hormozgan, and Semnan (Mir Kamali 1999). It seems that *B. maritima* has wide distribution in Iran. *Beta maritima* has annual nature and bolts in the first year of vegetative growth which guarantees its survival. This genus is known as weed beet which makes significant problem in sugar beet fields because of its morphological and physiological similarity with sugar beet. In addition, because of *B. maritima* distribution in prone areas to autumn sugar beet planting (south and south-west of Iran), the disease inoculation will increase in both plant and soil. It is clear that with the disease widespread, damage to autumn sugar beet growing will increase. BNYVV cannot be systemic in most sugar beet species, therefore, wild beet species and other studied hosts were mechanically inoculated (Tamada 1999). However, mechanical transfer of the virus on healthy seedlings of *Beta macrocarpa* Guss. usually causes yellow spot and systemic yellow mottle. BNYVV is naturally systemic in spinach which results in yellow mottle and stunting (Tamad 1975). Systemic symptoms of BNYVV in sugar beet are yellowing and necrosis (Figure 6) and are distinguished from systemic virus symptoms in *B. maritima*. The abovementioned symptoms related to virus are rarely seen in susceptible sugar beet cultivars due to limitation of the virus presence on the root (Kaufmann *et al.* 1992; Tamada and Baba 1973; Tamada 2002). In this study, the inoculation of the virus extracted from *B. maritima* leaf on three sugar beet cultivars restricted the disease symptoms to the inoculated leaves and only yellow spots and necrosis were formed. This study was the first report of *B. maritima* identification as a systemic and experimental host of BNYVV. According to these results and considering the lack of systemic host for BNYVV, *B. maritima* can be used as a proper systemic host in disease studies for the

proliferation and recognition of BNYVV and also evaluation of the disease severity of different virus isolates (Tamada 2007). Seeds of this plant were collected from sugar beet fields in Fars province and registered under 8901 accession number in seedbank of Sugar Beet Seed Institute. In general, *B. maritima* has special role in proliferation and distribution of rhizomania disease owing to different characteristics such as high rate of seed production, high susceptibility to the disease and large distribution in some regions (especially in prone areas for autumn sugar beet growing compared to weed population). Therefore, determination of its distribution areas and the effective control of it is recommended. In addition, the susceptibility of different *B. maritima* isolates to rhizomania disease can be evaluated and resistant populations can be used in breeding programs.

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