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# Detection of beet necrotic yellow vein virus in Pakistan using bait-plant bioassay, ELISA and RT-PCR

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**The Northwestern plains of Pakistan are the major sugar beet producing region in the country, providing an important alternative to sugar cane for sugar production when sugar cane is absent in the fields. We surveyed this region for four consecutive years and found that Beet necrotic yellow vein virus (BNYVV) is prevalent in at least five of these districts (Peshawar, Charsadda, Nowshera, Mardan and Swabi). An increase in virus incidence was observed in 2012 as compared to previous years (2009 to 2011) in all the sugar beet growing districts surveyed. The identity of the virus was confirmed using bait bioassay, enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR) and infectivity assay in roots and leaves of bait plants and sugar beet commercial cultivars. The results indicate that the virus was detected in at least 17 (out of 20) locations and all the four sugar beet cultivars commercially grown in the region were found susceptible to the virus. Our results indicate that bait plant bioassay, ELISA, RT-PCR and infectivity assay can efficiently detect BNYVV in roots and leaves of baited plants, field samples and sugar beet cultivars commercially grown in the region. This is the first report of BNYVV in Pakistan using both conventional and molecular techniques.**

**Key words:** Detection, BNYVV, plant bioassay, ELISA, RT-PCR, sugar beet, Pakistan.

## INTRODUCTION

*Beet necrotic yellow vein virus* (BNYVV) is one of the most economically important pathogen of sugar beet (*Beta vulgaris* L.) (Putz et al., 1990; Richards and Tamada, 1992; Tamada, 2002; Rush, 2003). The virus was first reported from Italy (Canova, 1959) and has since been reported from sugar beet producing countries of Europe (Richard-Molard, 1985; Henry et al., 1986; Hill, 1989; Lindsten, 1989; Paczuski and Szyndel, 1994; Lennefors et al., 2000; Harju et al., 2002; Borodynko et al., 2009), North America (Duffus et al., 1984; Rush and Heidel, 1995; Rush et al., 2006; Liu and Lewellen, 2007) and Asia (Tamada and Baba, 1973; Koenig and Lennerfors, 2000; Sohi and Maieki, 2004; Mehrvar et al., 2009). BNYVV is transmitted by soil-borne

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**Abbreviations: BNYVV,** *Beet necrotic yellow vein virus*; **DAS-ELISA,** double antibody sandwich-enzyme-linked immunosorbent assay; **RT-PCR,** reverse transcription-polymerase chain reaction; **dNTPs,** deoxynucleotide triphosphates; **IgG,** immunoglobulin G.

plasmodiophorid (protozoan) vector, *Polymyxa betae* Keskin (Abe and Tamada, 1986; Tamada, 2002). The virus is cystosorus-borne and survives many years in soils even without susceptible plants (Tamada, 2002).The disease appears as foci (patches) in low laying areas of sugar beet fields. The infected sugar beet plant shows mild to severe chlorosis particularly in vascular bundle followed by venial necrosis (necrotic yellow vein). The virus also produces characteristic symptoms on roots by exhibiting proliferation of lateral rootlets (rhizomania) and stunting (Tamada, 2002).

BNYVV is type species of the genus *Benyvirus* (Tamada, 2002). It has rigid rod-shaped particles of five modal lengths (390, 265, 105, 90 and 80 nm) and a constant diameter of 20 nm (Putz, 1977; Tamada, 2002). BNYVV genome contains five distinct species of plus-sense single stranded RNAs with lengths of 6746 (RNA 1), 4612 (RNA 2), 1773 to 1774 (RNA 3), 1465 to 1467 (RNA 4) and 1342 to 1347 (RNA 5) nucleotides depending on strains (pathotypes) (Bouzoubaa et al., 1985; 1986; 1987; Tamada et al., 1989; Saito et al., 1996; Kiguchi et al., 1996; Tamada, 2002). Three pathotypes of the virus has been reported world-wide, namely A, B and P (Kruse et al., 1994; Koenig et al., 1995; Koenig and Lennefors, 2000). The pathotype A has been reported from many European countries, North America, Japan, China and Iran, whereas pathotype B has been reported frequently from France and Germany, and occasionally from Sweden, China and Japan (Saito et al., 1996; Kiguchi et al., 1996; Miyanishi et al., 1999; Dawei et al., 1999; Lennefors et al., 2000; Sohi and Maleki, 2004). Pathotypes A and B are distributed worldwide and contain only four genomic RNAs. The pathotype P contains a fifth RNA species and has been reported from France, the UK, Kazakhstan, China and Japan (Kruse et al., 1994; Koenig et al., 1995; Koenig et al., 1997; Koenig and Lennefors, 2000; Harju et al., 2002).

In the BNYVV genome, RNAs 1 and 2 encode genes involved in replication, virion assembly, cell-to-cell movement, silencing suppression and vector transmission (Tamada and Kusume, 1991; Richards and Tamada, 1992; Tamada et al., 1996) whereas RNAs 3, 4 and 5 are associated with acquisition and transmission of the virus by its vector and disease development in sugar beet roots (Lemaire et al., 1988; Jupin et al., 1991; Richards and Tamada, 1992). It has been reported that the p25 protein encoded by RNA 3 is involved in classical root symptoms on natural hosts and the N protein expected to be associated with the induction of necrosis in leaves (Jupin et al., 1992; Klein et al., 2007; Chiba et al., 2008). The core region from 1033 to 1257 nt of RNA 3 is essential for the vascular movement of the virus in *Beta macrocarpa* (Lauber et al., 1998). BNYVV RNA 4 encoded p31 gene is associated with efficient vector-transmission, virulence and RNA silencing suppression in sugar beet roots (Tamada and Abe, 1989; Rahim et al., 2007). The BNYVV pathotype P contains RNA 5, encoding a 26-kDa protein, involved in symptom severity in a synergistic mechanism with RNA 3. Thus, pathotype P appears to be more virulent than others (A, B) (Koenig et al., 1997; Covelli et al., 2009; Link et al., 2005; Miyanishi et al., 1999; Dawei et al., 1999; Kiguchi et al., 1996). BNYVV has been detected using serological and molecular techniques elsewhere in the world (Torrance et al., 1988; Koenig et al., 1995; Koenig et al., 1997; Morris et al., 2001; Meunier et al., 2003; Bushehri et al., 2006; Mehrvar et al., 2009; Wang et al., 2011). The plains of Northwest of Pakistan are the major sugar beet growing areas in the country. The sugar beet is grown during spring (February to June) and exclusively used for sugar production to fill up the gap in sugar industries in Pakistan when sugar cane is not available for crushing. The presence of BNYVV has been suspected on the basis of symptom expression in sugar beet crops present in the Northwestern plains of Pakistan (Arif, 2000) but no research work has been done on its detection, identification and characterization in Pakistan. In this paper, we report the detection and identification of the virus using both conventional and molecular techniques.

## MATERIALS AND METHODS

### Collection of samples

Soil and plant samples were collected from five districts of Northwest of Pakistan: Peshawar, Charsadda, Nowshera, Mardan and Swabi (Table 1). Four fields were selected in each of the five districts and plant samples were collected from an estimated area of four meter square of each selected site/field. Approximately 2 to 3 kg soil samples were collected from cropping layer (5 to 10 cm) below the surface of each of the three sites/ locations, about 20 to 25 m intervals and 3 to 4 m inside the boundaries of each field. The soil was mixed thoroughly and kept moist at 4°C until used for air drying.

### Soil-bait test

Field soil samples were spread out on paper sheet and air-dried for seven to ten days at 22 to 24°C. The dried soil samples were ground into fine powder using pestle and mortar and passed through 300 and 200 µm sieves, respectively. Twenty grams of fine soil powder was placed in each of four depressions in steam-sterilized potting mixture (sand: clay: compost, 1:1:1 ratio v/v) in one liter plastic pots. The pots were soaked for 10 to 15 h with tap water in a steel tray (four pots/ tray/ treatment). Four *B. vulgaris* cv. Kewiterma seedlings were transplanted into each pot and were placed in a glasshouse at 18 to 20°C. The pots were flooded to half their height in water for three days, allowed to drain and then left without water for four days. The plants were uprooted after eight weeks for virus testing through back inoculation of sap from roots and leaves to indicator plants (*Chenopodium quinoa*, *Chenopodium amaranticolor*, *B. vulgaris cv* Kewiterma, *Spinacea oleracea, Tetragonia expansa* and *Nicotiana benthamiana)*. Both root and leaves samples of baited *B. vulgaris* were also tested by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) (Koenig et al., 1997; Arif et al., 2014) and by reverse transcription polymerase chain reaction (RT-PCR) (EPPO, 2004)

**Table 1.** Detection of *Beet necrotic yellow vein virus* in Northwestern plains of Pakistan using DAS-ELISA

| District | Number of samples | | | | | | | | Mean % Incidence |
|---|---|---|---|---|---|---|---|---|---|
| | 2009 | | 2010 | | 2011 | | 2012 | | |
| | Infected/ Tested | % Incidence[a] | Infected/ Tested | % Incidence[a] | Infected/ Tested | % Incidence[a] | Infected/ Tested | % Incidence[a] | |
| Peshawar | 250/30 | 12.0 | 210/28 | 13.3 | 245/40 | 16.3 | 260/55 | 21.1 | 15.6 |
| Charsadda | 280/43 | 15.3 | 200/34 | 17.0 | 260/50 | 19.2 | 250/60 | 24.0 | 18.8 |
| Nowshera | 240/26 | 10.8 | 205/25 | 12.1 | 250/54 | 21.6 | 230/51 | 22.1 | 16.6 |
| Mardan | 290/53 | 18.2 | 240/49 | 20.4 | 300/64 | 21.3 | 262/66 | 25.1 | 21.2 |
| Swabi | 285/48 | 16.8 | 230/42 | 18.2 | 230/58 | 25.2 | 245/62 | 25.3 | 21.3 |

[a]Percent incidence of the virus was calculated as: number of plant infected multiplied by 100 and divided by total number of plant tested.

**Serological detection of BNYVV in bait plant through DAS-ELISA**

DAS-ELISA was done in root and leaf samples of bait plants for the detection of BNYVV as described by Koenig et al. (1997) and Arif et al. (2014). The tests were performed in polystyrene micro-plates (NUNC, Immunoplate II, Thermal Scientific, USA) following manufacturer's instructions. The plates were coated with 200 µl aliquots of BNYVV–specific immunoglobulin G (IgG) (Bioreba, Switzerland) with Coating buffer, pH 9.6 (1000 ml of the buffer contains $Na_2CO_3$ and $NaHCO_3$, 1.59 and 2.93 g, respectively). The plates were kept at room temperature (24 to 25°C) in a humid box for 3 to 4 h. Leaf samples were extracted by crushing through pestle and mortar in extraction buffer, pH 7.4 (1000 ml of extraction buffer contains Tris 2.40 g, NaCl 8.00 g, PVP K25 (MW 24000) 20.00 g, Tween 20 0.50 g and KCl 0.20 g) at ratio of 1:5 (w/v) which was 1:1 (w/v) for root samples. 200 µl of prepared sample were dispensed in each well after three times washing with 1 × PBST, pH 7.4. Controls, provided in the kit were used as recommended by the supplier (Bioreba, Switzerland). ELISA plates were incubated inside a humid box either for 4 h at room temperature or 4 to 6°C overnight in refrigerator. After washing, 200 ul of diluted enzyme conjugate (Bioreba, Switzerland at 1:1000 dilutions) was dispensed in each well of plate and incubated in a humid box for 3 to 4 h at room temperature. The plates were washed again three times with washing buffer. 200 µl of the freshly prepared substrate solution (para-nitrophenyl-phosphate (pNPP) tablets at 1 mg/ml) was dispensed in each well of the microtitration plates and incubated at RT in the dark. Absorbance at 405 nm ($A_{405}$) was measured using the Titertek Multiskan, Model MC (Flow Laboratories, Covina, CA, USA) after 1 or 2 h of substrate incubation at room temperature, and overnight at 4°C. Samples were considered positive when the $A_{405}$ values exceeded the value of virus-free samples by at least a factor of three.

**Molecular detection of BNYVV in bait plants**

***RNA Extraction and first cDNA strand synthesis***

Total RNA extracts were obtained from roots and leaves of *B. vulgaris* (bait plants) and sugar beet commercial cultivars using RNA extraction kit, following manufacturer's instruction (Invitrogen (PureLink ® RNA Mini Kit; Life Technologies, USA). RNA pellets were dissolved in RNase-free water (50 µl) and the final RNA concentration was measured with a GeneQuart™ photometer (Pharmacia, Sweden). First strand cDNA was synthesized by mixing 2 µg of total RNA with 1 µg of downstream primer, 1 µl 10 X PCR buffer (10 mM Tris-HCl, pH 8.4 containing 50 mM KCl and 2 mM $MgCl_2$), and 2 mM each of four deoxynucleotide triphosphates (dNTPs) in a total volume of 10 µl. The mixture was heated to 65°C for 2 min followed by cooling to 42°C on heating block and 20 Units of both RNase inhibitor and reverse transcriptase (Boehringer-Mannheim, Germany) were added. The mixtures were incubated at 42°C for 2 h and 1 µl of the total cDNA product was then used for PCR amplification.

***Polymerase chain reaction***

For RT-PCR detection, primer pairs were synthesized according to the protocol for the diagnosis of BNYVV (EPPO, 2004). Each 50 µl PCR reaction mixture contained 1 µl cDNA (template), 5 µl of 10X PCR buffer for *Taq* polymerase, 2 µl of the 2 mM dNTPs, 1 µl of the *Taq* DNA polymerase and 0.5 pmol of each of the downstream primer (BNYVV 017 (R) 5'-ACTCGGCATACTATTCACT(T)-3' and upstream primer (BNYVV 016 (F) 5'-CGATTGGTATGAGTGATTT(A)-3'. After the initial denaturation at 94°C for 2 min, PCR was performed for 35 cycles, each at 94°C for 45 s, 58°C for 30 s and 72°C for 1 min, followed by a final extension step at 72°C for 3 min. The PCR products (10 µl) were electrophoresed in 1% agarose gels in Tris-borate-EDTA buffer containing 0.5 µl/ml ethidium bromide. Molecular size markers (DRIgest ™, Promega, USA) were used to indicate size of the PCR product and specific bands were visualized under UV light.

## RESULTS

### Prevalence of BNYVV in plains of Northwest of Pakistan

BNYVV was detected consistently from 2009 to 2012 and was prevalent in sugar beet crop of farmer's fields of Northwestern plains in all the five districts viz. Peshawar, Charsadda, Nowshera, Mardan and Swabi (Table 1). Our results indicate that virus incidence increased in 2012 as compared to previous years (2009 to 2011) in all the districts surveyed (Table 1).

### Detection of BNYVV through soil-bait tests

Root and leaves samples from each of the four *B. vulgaris* cv Kewiterma plants (in one pot/location) were

**Table 2.** Detection of *Beet necrotic yellow vein virus* (BNYVV) in *Beta vulgaris*-bait plants grown in putative viruliferous soils collected from five districts of Northwestern plains of Pakistan.

| District | Locations /fields[a] | Detection of BNYVV through: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Systemic symptoms *B. vulgaris*-baited plants[b] | | ELISA | | RT-PCR | | Infectivity assay[e] | |
| | | Root[c] | Leaf[d] | Root | Leaf | Root | Leaf | Root | Leaf |
| Peshawar | 1 | + | + | 1.110[f,g] (0.504) | 0.950 (0.640) | + | + | + | + |
| | 2 | - | - | .080 (0.040) | 0.091 (0.045) | - | - | - | - |
| | 3 | + | + | 1.010 (0.610) | 0.710 (0.580) | + | + | + | + |
| | 4 | + | + | 1.000 (0.721) | 0.988 (0.860) | + | + | + | + |
| Charsadda | 1 | + | + | 0.740(0.510) | 0.690 (0.465) | + | + | + | + |
| | 2 | + | + | 0.780 (0.615 ) | 0.971 (0.690) | + | + | + | + |
| | 3 | + | + | 0.930 (0.856) | 1.010 (0.640) | + | + | + | + |
| | 4 | - | - | 0.078 (0.040) | 0.080 (0.045) | - | - | - | - |
| Nowshera | 1 | + | + | 1.110 (0.710) | 0.913 (0.510) | + | + | + | + |
| | 2 | + | + | 1.010 (0.810) | 0.970 (0.530) | + | + | + | + |
| | 3 | + | + | 0.981 (0.641) | 1.210 (0.688) | + | + | + | + |
| | 4 | - | - | 0.089 (0.040) | 0.071 (0.046) | - | - | - | - |
| Mardan | 1 | + | + | 0.910 (0.980) | 0.960 (0.821) | + | + | + | + |
| | 2 | + | + | 0.890 (0.865) | 0.870 (0.794) | + | + | + | + |
| | 3 | + | + | 0.889 (0.695) | 0.781 (0.498) | + | + | + | + |
| | 4 | + | + | 1.121 (0.860) | 0.981 (0.992) | + | + | + | + |
| Swabi | 1 | + | + | 1.010 (1.120) | 0.891 (0.745) | + | + | + | + |
| | 2 | + | + | 1.110 (0.960) | 0.980 (0.970) | + | + | + | + |
| | 3 | + | + | 0.990 (0.870) | 0.881 (0.810) | + | + | + | + |
| | 4 | + | + | 0.911 (0.960) | 0.971 (0.890) | + | + | + | + |
| Control[h] | | - | - | 0.071 (0.045) | 0.072 (0.048) | - | - | - | + |
| Healthy[i] | | - | - | 0.070 (0.046) | 0.075 (0.051) | - | - | - | - |
| Infected[i] | | - | - | 1.440(1.210) | 1.501 (1.150) | + | + | + | - |

[a]Soil samples were collected from farmer's fields at district: Peshawar (1, Bara; 2, Urmar; 3, Wasak-1; 4, Warsak-2); Charsadda (1, Charsadda-1; Charsadda-2; 3, Anwar Khan Kalay; 4, Bahadar Kalay); Nowshera (1, Nowshera; 2, Akora Khattak; 3, Zandae; 4, Sardheri); Mardan (1, Mardan-1; 2, Mardan-2; 3, Sugar Crops Research Institute-experimental fields; 4, Asota); Swabi (1, Swabi-1; 2, Swabi-2; 3,Toppi; 4, Karnal Sher Kalay). [b]Symptoms of BNYVV in *B. vulgaris*-bait plants grown 8 week in putative viruliferous soils collected from four fields each of five districts of Northwestern plain of Pakistan. [c]Symptoms were characterized by root stunting and proliferation of lateral rootlets on main tap root and necrosis of vascular bundles. [d]Symptoms were characterized by mild to severe chlorosis (spots) followed by vein-banding, venial necrosis, leaf deformation and stunting. [e]Symptoms of BNYVV in diagnostic hosts (*Chenopodium quinoa*, *C. amaranticolor*, *B. vulgaris cv* Kewiterma, *Spinacea oleracea, Tetragonia expansa* and *N. benthamiana* ) upon mechanical inoculation of sap from roots and leaves samples of *B. vulgaris*-baited plants (see Table 3 for details).[f]Mean values ($A_{405nm}$) of four replicates. [g] Values ($A_{405nm}$) after 2 h and 1 h at room temperature (25°C) (in parentheses). [h]*Beta vulgaris- cv* Kewiterma plants were grown as control without adding putative viruliferous soils powder;[i] Known negative and positive controls of BNYVV.

evaluated after 8 weeks on the basis of symptom expression, DAS-ELISA, RT-PCR and back inoculation of sap to indicator plants (Table 2). Bait plants grown in 17 out of 20 soil samples from locations in five districts were BNYVV positive on the basis of symptoms, ELISA, RT-PCR and reaction of back-inoculation on series of diagnostic hosts. The bait plants (*B. vulgaris* cv Kewiterma) exhibited mild to severe chlorosis (spots) in leaves followed by vein-banding, venial necrosis, leaf deformation and stunting. The symptoms on baited plants were characterized as root stunting and proliferation of lateral rootlets on main tap root and necrosis of vascular bundles. Transverse cutting of randomly selected root samples showed brown discoloration (necrosis). The $A_{405}$ values in samples considered positive was quite higher than that of the values of healthy sap (Table 2). RT-PCR analysis of pooled samples for both roots and leaves of four *B. vulgaris* cv Kewiterma bait plants indicated that out of 20 locations, BNYVV was detected in 17 locations (Table 2 and Figure 1). A band size of 500 bp was visible

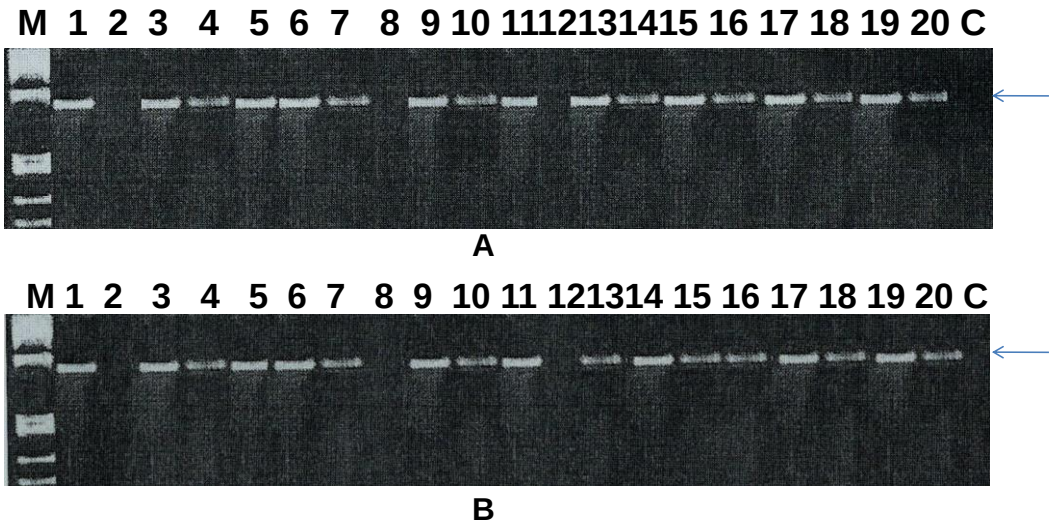


**Figure 1.** Detection of *Beet necrotic yellow vein virus* (BNYVV) by RT-PCR in roots and leaves of sugar beet bait plants (A) roots (B) leaves after 8 weeks of growing in infested soils collected from five districts Northwest of Pakistan. Lanes contain: M, DNA ladder PhiX17RFI DNA/HaeIII digest; each lane represents sampling areas as: Lane 1-4, Peshawar (1, Bara; 2, Urmar; 3, Wasak-1; 4, Warsak-2); lane 5-8, Charsadda (1, Charsadda-1; 2, Charsadda-2; 3, Anwar Khan Kalay; 4, Bahadar Kalay); lane 9-1, Nowshera (1, Nowshera; 2, Akora Khattak; 3, Zandae; 4, Sardheri); lane 13-16, Mardan (1, Mardan-1; 2, Mardan-2; 3, Sugar Crop Research Institute-experimental fields; 4, Asota); Lane 17-20, Swabi (1, Swabi-1; 2, Swabi-2; 3,Toppi; 4, Karnal Sher Kalay); C; virus free control. Arrow indicates the position of BNYVV specific 500 bp band.

in agrose gel from both roots (Figure 1A) and leaves (Figure 1B) of *B. vulgaris* cv Kewiterma bait plants. Back inoculation of sap both from roots and leaves of baited plants on series of indicator plants (*C. quinoa*, *C. amaranticolor*, *B. vulgaris cv* Kewiterma, *S. oleracea, T. expansa* and *N. benthamiana*), further confirmed the presence of BNYVV in 17 out of 20 locations (Tables 2 and 3). However, BNYVV was consistently not detected from soils samples collected from farmers fields at three locations (Peshawar-Urmar; Charsadda-Bahadar Kalay; Nowshera-Sardheri) on basis of symptoms, ELISA, RT-PCR and back inoculation of sap from both roots and leaves of bait plants (Table 2 and Figure 1)

### Detection of BNYVV in commercial sugar beet cultivars grown in Northwestern plains of Pakistan

BNYVV was detected through symptomatology, ELISA, RT-PCR and infectivity assay in root and leaf samples of four sugar beet cultivars commercially grown in Northwestern plains of Pakistan (Table 4). Composite sampling was made from both roots and leaves of sugar beet cultivars (Kewiterma, Pamella, Polyplus, Polyrex) bait plants (4 to 6 plants) eight weeks grown in viruliferuious soil powders. In all the four cultivars, root proliferation (bearding) and internal browning (necrosis) were positively correlated with the presence of the virus (Table 4). However, the virus was also detected, in some cases, in symptomless leaves of the sugar beet cultivars grown as bait plants. The results were further confirmed by RT-PCR and back inoculation of sap from both root and leaf samples of sugar beet commercial cultivars (Kewiterma, Pamella, Polyplus, Polyrex) with one of the aggressive isolate of BNYVV from each district of Northwestern plains. RT-PCR amplified a 500 bp band against each selected isolates in all four sugar beet cultivars commercially grown in the region (Table 4 and Figure 2). Back inoculation of sap from the root samples of bait plants to diagnostic hosts (*C. amaranticolor*, *C. quinoa* and *N. benthamiana*) further confirmed the reaction of the cultivars against BNYVV (Table 4).

## DISCUSSION

BNYVV is one of the most economically important viruses infecting sugar beet in Pakistan. It is transmitted by a soil-borne vector (*Polymyxa betae*) and it persists inside the thick-walled resting spores (cystosori) for many years. It has been reported that the environmental conditions and cultural practices do not alter the viability of the virus inside the resting spores (Tamada, 2002; Rush, 2003). Therefore, the virus detection and timely diagnosis is utmost important. The results of this investigation indicates that BNYVV and its vector, *P. betae* is prevalent in selected fields of major districts of Northwest, presumably extended to the areas where surveys were

**Table 3.** Detection of *Beet necrotic yellow vein virus* (BNYVV) using plant-bioassay and assessment of reaction of local lesion hosts on inoculation of sap from root and leaf samples of *Beta vulgaris*-bait plants grown in putative viruliferous soils collected from five districts of Northwestern plains of Pakistan.

| District | Locations/ fields [a] | *C. quinoa*[b,d] | | *C. amara-nticolor*[b,d] | | *B. vulgaris* cv. Kewiterma[c,d] | | *S. oleracea*[c,d] | | *T. expansa*[c,d] | | *N. benthamiana*[c,d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Root | Leaf | Root | Leaf | Root | Leaf | Root | Leaf | Root | Leaf | Root | Leaf |
| Peshawar | 1 | CL [e] | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 2 | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| | 3 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 4 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| Charsadda | 1 | CL, NL | CL, NL | CL, NL | CL | CS, LD | CS, LD | LD, SI | LD, SI | CL, NL | CL, NL | LD, SI | LD, SI |
| | 2 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 3 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 4 | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Nowshera | 1 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 2 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 3 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 4 | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Mardan | 1 | CL | CL | CL | CL | CS, VN | CS, VN | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 2 | CL, NL | CL, NL | CL, NL | CL, NL | CS, YVB | CS, YVB | LD, SI | LD, SI | CL, NL | CL, NL | LD, SI | LD, SI |
| | 3 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 4 | CL, NL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL, NL | CL | LD, SI | LD, SI |
| Swabi | 1 | CL, NL | CL | CL, NL | CL | CS, LD, YVB | CS, LD | LD, SI | LD, SI | CL, NL | CL | LD, SI | LD, SI |
| | 2 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| | 3 | CL, NL | CL, NL | CL, NL | CL, NL | CS, LD, VN | CS, LD | LD, SI | LD, SI | CL, NL | CL, NL | LD, SI | LD, SI |
| | 4 | CL | CL | CL | CL | CS | CS | LD, SI | LD, SI | CL | CL | LD, SI | LD, SI |
| Control Healthy[f] | | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Infected[g] | | CL, NL | CL | CL, NL | CL | CS, LD, YVB | CS, LD | LD, SI | LD, SI | CL, NL | CL | LD, SI | LD, SI |

[a]Soil samples were collected from farmer's fields in Northwestern plains of Pakistan (details in under Table 1). [b]Diagnostic hosts with local lesions only after one week of mechanical inoculation. [c]Diagnostic hosts with systemic infection after two weeks of mechanical inoculation. [d] *Beta vulgaris and S. Oleracea* exhibited symptoms two weeks and *Chenopodium* spp. *and N. benthamiana* one week whereas *Tetragona expansa* 10 days after mechanical inoculation. [e]CL: chlorotic lesion, NL: necrotic lesions, CS: chlorotic spotting, YVB: yellow vein-banding, VN: vein necrosis, LD: leaf distortion, SI= Systemic infection. NS: apparently no symptom and plant look healthy. [f]Reaction of local lesion hosts on inoculation of sap from known healthy root and leaf samples of *B. vulgaris* plants. [g]Reaction of local lesion hosts on inoculation of sap from root and leaf samples of *B. vulgaris* plants previously infected with BNYVV.

not conducted. The prevalence of BNYVV in Northwestern plains of Pakistan is extremely important and needs immediate attention of the government agencies. Further spread of the virus and the vector could be avoided by strictly applying quarantine regulations to the pockets that are still free from BNYVV

**Table 4.** Detection of BNYVV in sugar beet cultivars commercially grown in Northwestern plains of Pakistan.

| Sugar beet Cultivars | District/ locations/fields[a] | Detection of BNYVV through | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Symptoms[b] | | ELISA | | RT-PCR | | Infectivity assay[e] | |
| | | Root | Leaf | Root | Leaf | Root | Leaf | Root | Leaf |
| Kewiterma | Peshawar-1 | RP | NS | 0.990[c,d] (0.604) | 0.890 (0.540) | + | + | + | + |
| | Charsadda-1 | RP | CS, VC | 0.810 (0.470) | 0.790 (0.423) | + | + | + | + |
| | Nowshera-1 | RP | CS, VC | 0.910 (0.580) | 0.913 (0.490) | + | + | + | + |
| | Mardan-4 | RP | CS, VC, YVB | 1.101 (0.650) | 0.992 (0.662) | + | + | + | + |
| | Swabi-2 | RP | CS, VC, LD | 1.123 (0.845) | 1.210) (0.890) | + | + | + | + |
| Pamella | Peshawar-1 | RP | CS, VC | 0.860 (0.405) | 0.930 (0.620) | + | + | + | + |
| | Charsadda-1 | RP | CS, VC | 0.788 (0.449) | 0.772 (0.465) | + | + | + | + |
| | Nowshera-1 | RP | NS | 0.730 (0.650) | 0.819 (0.445) | + | + | + | + |
| | Mardan-4 | RP | CS, VC | 0.987 (0.680) | 0.970 (0.550) | + | + | + | + |
| | Swabi-2 | RP | CS, VC, LD | 0.890 (0.690) | 1.090 (0.770) | + | + | + | + |
| Polyplus | Peshawar-1 | RP | CS, VC | 0.930 (0.504) | 0.890 (0.640) | + | + | + | + |
| | Charsadda-1 | RP | CS, VC | 0.830 (0.460) | 0.740 (0.420) | + | + | + | + |
| | Nowshera-1 | RP | CS, VC | 0.920 (0.620) | 0.900 (0.580) | + | + | + | + |
| | Mardan-4 | RP | CS, VC, LD | 1.201 (0.871) | 0.990 (0.612) | + | + | + | + |
| | Swabi-2 | RP | CS, VC, YVB | 1.100 (0.670) | 0.890 (0.590) | + | + | + | + |
| Polyrex | Peshawar-1 | RP | CS, VC | 0.812 (0.522) | 0.940 (0.560) | + | + | + | + |
| | Charsadda-1 | RP | CS, VC | 0.860 (0.490) | 0.710 (0.405) | + | + | + | + |
| | Nowshera-1 | RP | NS | 0.986 (0.589) | 0.970 (0.430) | + | + | + | + |
| | Mardan-4 | RP | CS, VC | 1.110 (0.660) | 1.210 (0.592) | + | + | + | + |
| | Swabi-2 | RP | CS, VC | 1.000 (0.690) | 0.880 (0.620) | + | + | + | + |
| Healthy[f] | | - | - | 0.070 (0.046) | 0.075 (0.051) | - | - | - | - |
| Infected[g] | | - | - | 1.440 (0.820) | 1.501 (0.880) | + | + | + | + |

[a]Soil samples were used in the experiment were collected from farmer's fields at district: Peshawar (1: Bara); Charsadda (Charsadda-1); Nowshera (Nowshera,-1); Mardan (4: Asota); Swabi (2: Swabi-2) . [b]Symptoms of BNYVV on roots and leaves of sugar beet cultivars commercially grown in Northwest of Pakistan; roots symptoms-RP: root proliferation (bearding); transverse cutting of the root exhibited brown discoloration; Further details of leaf symptoms of BNYVV are given under Table 3. [c]Mean values ($A_{405nm}$) of three replicates. [d]Values ($A_{405nm}$) after 2 h and 1 h at room temperature (25°C) (in parentheses).[e] Symptoms of BNYVV in diagnostic hosts (*Chenopodium quinoa*, *C. amaranticolor* and *N. benthamiana* ) upon mechanical inoculation of sap from root and leaf samples of plants of sugar beet cultivars commercially grown in Northwest of Pakistan. [f] *Beta vulgaris- cv* Kewiterma plants were grown as control without adding putative viruliferous soils powder. [g]BNYVV infected plants of *Beta vulgaris-cv* Kewiterma were previously isolated from Mardan-4 putative viruliferous soil powder and the virus was confirmed by ELISA, RT-PCR and Infectivity assay.

and its vector, *P. betae*.

The study has revealed that BNYVV can be successfully detected in infested soil by bait test supplemented with ELISA or RT-PCR or both. Back inoculation of the sap from both roots and leaves of baited plants (especially roots) on test plants served as a successful bio-assay for the detection of BNYVV and other soil-borne viruses transmitted through plamodiophorid vector (Arif et al., 2014). We did not carry out any assay for the confirmation of *Beet soil-borne virus* (BSBV) due to unavailability of antibodies, but mix infection of BSBV with BNYVV were observed on the basis symptoms during field visits. Researchers reported BSBV mixed infection with BNYVV elsewhere during field studies (Rush and Heidel, 1995; Wisler et al., 2003). Both viruses are vectored by *P. betae* in soil (Abe and Tamada, 1986; Rush and Heidel, 1995).

The results also indicated an increasing trend in virus incidence in almost all areas surveyed for BNYVV in Northwestern plains. This may be due the continuous cultivation of sugar beet in same field year after year resulting in an increased inoculum pressure for both

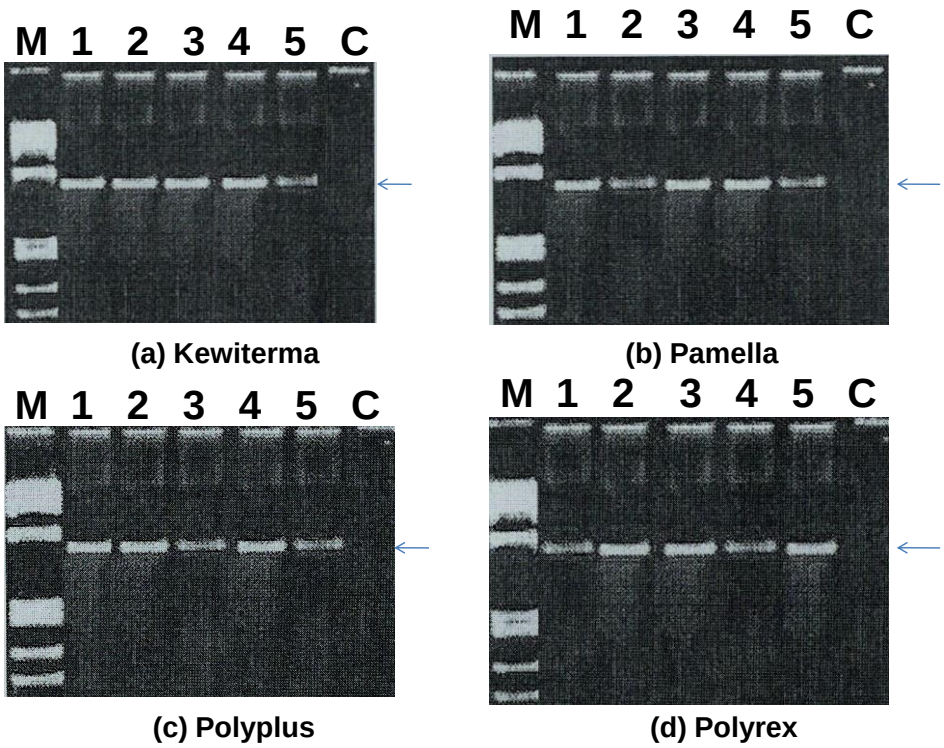


**Figure 2.** Detection of *Beet necrotic yellow vein virus* (BNYVV) RNA sequence by RT-PCR in sugar beet roots of four cultivars commercially grown in Northwestern plains of Pakistan. The sugar beet cultivars were grown in bait test for 8 wk against one aggressive isolate from each of five districts. (a) Kewiterma. (b) Pamella. (c) Polyplus. (d) Polyrex. Lane M, DNA ladder PhiX17RFI DNA/HaeIII digest; 1, Peshawar-1; 2, Charsadda-1; 3, Nowshera-1; 4, Mardan-4; 5, Swabi-2; the 500 bp band is indicated; C, virus-free control. Arrow indicates the position of BNYVV specific 500 bp band.

vector and virus, thus enhancing the incidence and distribution of the pathogens as reported elsewhere previously (Abe and Tamada, 1986). In Northwestern plains, sugar beet has been cultivated since the era of British India. This continuous planting of sugar beet with a combination of favorable conditions including high moisture content and high inoculum pressure has resulted in high virus incidence and severity in this area. Water table is high in sugar beet fields in District Mardan and Swabi due to the presence of Tarbela Dam (one of the largest dam in the world) and it may promote vector motility of plasmodiophorid vector, enabling the vectors to be more rapidly acquire and transmit viruses such as BNYVV.

The molecular studies based on sequence analysis of RNA 3 and RNA 5 of local isolates of BNYVV is currently in progress. However, preliminary work based on CP-encoding nucleotide sequences analysis indicates that percentage identity is highly conserved for all the isolates reported worldwide, suggesting that either the virus has a very stable genome or this might be the incidence of a recent introduction of the virus in different sugar beet growing areas. The comparison of all reported isolates at nucleotide as well as amino acid levels shows very low degree of variation. The result is quite in agreement with the findings of Bouzoubaa et al. (1987) where high level of genome conservation was reported for BNYVV reported isolates. The phylogram and multiple sequence alignment of coat protein encoding sequence also showed very little variability among the reported isolates. Two clades were formed with isolates reported from Kazakhstan and Italy and which deviates from other isolates. Within the second clade, two sub groups were formed. Pakistani isolate clustered with Iranian and Chinese isolates indicating a possible common entrance or introduction from these regions. We also found that coat protein gene of BNYVV-Pak was closely related to other sequences of different BNYVV isolates available in the database; supporting the idea that coat protein is relatively stable sequence in BNYVV genome. There is a possibility that the virus was introduced along with the viruliferous zoospores or resting spores of *P. betae*. BNYVV has been reported in China since 1978 (Gao et al., 1983). Both China and Pakistan has direct trade passing through Northern parts of Pakistan to rest of the country. The possibility of transportation of contaminated plant material (beet, etc) or adhering infested soil from China to Pakistan could not have been avoided. Further

investigation of Pakistani isolates of BNYVV with Chinese and Iranian virus isolates will provide information on the origin and distribution of the virus within the country.

The studies on BNYVV worldwide revealed that there are three pathotypes, the A, B and P (Kruse et al., 1994; Koenig et al., 1995; Schirmer et al., 2005). The pathotype A reported mostly from European countries, North America, Japan, China and Iran while pathotype B reported frequently from countries such as France, Germany and rarely from Sweden, China, Japan and Poland (Saito et al., 1996; Miyanishi et al., 1999; Lennefors et al., 2000; Li et al., 2008; Borodynko et al., 2009, Mehrvar et al., 2009). A severely pathogenic pathotype P has been reported from Japan (Tamada et al., 1996), France (Koenig et al., 1997), Kazakhstan and China (Koenig and Lennefors, 2000), and the UK (Harju et al., 2002). This pathotype P contains RNA 5 in addition to normal BNYVV genome. Further work is required to determined pathotype(s) in Pakistan. We suspect a mix population of pathoptype A and B in sugar beet growing areas of the Northwest of Pakistan (results not shown). However, the possibility of the existence of pathotype P could also not be avoided because in some cases, BNYVV infected sugar beet exhibited severe symptoms in screen house conditions and rapidly kill the plants.

Aspects which require further investigations include determining the density of *P. betae* carry BNYVV, and the phenomenon whether BNYVV infected sugar beet plant through secondary infection plays a role in making *P. betae* viruliferous or a viruliferous vector delivers virus in plant through primary infection, or both. Also, stimulation of cystosori in the soil to release zoospores in absence of the host or the needed trap plants to attract zoospores before plantation of sugar beet in infected soils need to be investigated. Answers to these questions may help to develop control strategies against BNYVV and other viruses transmitted by plasmodiophorid vectors.

## Conflict of interests

The authors did not declare any conflict of interest.

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### REFERENCES

Abe H, Tamada T (1986). Association of *Beet necrotic yellow vein virus* with isolates of *Polymyxa betae* Keskin. Ann. Phytopathol. Soc. Jpn. 52:235-247.

Arif M, Ali M, Rehman A, Fahim M (2014). Detection of potato mop- top virus in soils and potato tubers using bait-plant bioassay, ELISA and RT-PCR. J. Virol. Methods 195:221-227.

Arif M (2000).Fungally-transmitted rod-shaped viruses: Biology, transmission and molecular pathology. Pak. J. Biol. Sci. 3:1194-1212.3.

Borodynko N, Hasiow-Jaroszewska B, Pospieszny H (2009). Evidence for the presence of beet necrotic yellow vein virus type A and B in Poland. J. Plant Dis. Prot. 166:106-108.

Bouzoubaa S, Guilley H, Jonard G, Richards K, Putz C (1985). Nucleotide sequence analysis of RNA-3 and RNA-4 of Beet necrotic yellow vein virus isolates F2 and G1. J. Gen. Virol. 66:1553-1564.

Bouzoubaa S, Quillet L, Guilley H, Jonard G, Richards K (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. J. Gen. Virol. 68:615-626.

Bouzoubaa S, Ziegler V, Beck D, Guilley H, Richards K, Jonard G (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. J. Gen. Virol. 67:1689-1700

Bushehri AS, Adel J, Dehkordi MKH (2006). Detection of beet necrotic yellow vein virus with reverse transcription-polymerase reaction. Inter. J. Agric. Biol. 8:280-285.

Canova A (1959). On the pathology of sugar beet. Info. Fitopatol. 9:390-396.

Chiba S, Miyanishi M, Andika IB, Kondo H, Tamada T (2008). Identification of amino acids of the beet necrotic yellow vein virus p25 protein required for induction of the resistance response in leaves of *Beta vulgaris* plants. J. Gen. Virol. 89:1314-1323.

Covelli L, Klein E, Gilmer D (2009). The first 17 amino acids of the beet necrotic yellow vein virus RNA-5-encoded p26 protein are sufficient to activate transcription in a yeast one-hybrid system. Arch. Virol. 154:347-351.

Dawei I, Jialin Y, Chenggui H, Toa L, Shucai Q, Koenig R, Yi L (1999). Detection and nucleotide analysis of RNA5 of *Beet necrotic yellow vein virus* isolated in China. Chin. J. Biotechnol. 15(4):253-257.

Duffus JE, Whitney ED, Larson, RC, Liu H –Y, Lewellen RT (1984). First report in Western hemisphere of rhizomania of sugar beet by beet necrotic yellow vein virus. Plant Dis. 68: 251.

EPPO (2004) Diagnostic protocols for regulated pests-beet necrotic yellow vein virus. EPPO Bull. 34:229-237.

Gao JL, Deng F, Zhai HQ, Liang XS, Liu Y (1983). The occurrence of sugar beet rhizomania caused by beet necrotic yellow vein virus in China. Acta Phytopathol. Sin. 13:1-4.

Harju VA, Mumford RA, Bockley A, Boonham N, Clover GRG, Weekes R, Henry CM (2002). Occurrence in the United Kingdom of beet necrotic yellow vein virus isolates which contain RNA 5. Plant Pathol. 51:811.

Henry CM, Jones RAC, Coutts RHA (1986). Occurrence of a soil-borne virus of sugar beet in England. Plant Pathol. 35:85-591.

Hill SA (1989). Sugar beet rhizomania in England. EPPO Bull. 19:501-508.

Jupin I, Guilley H, Richards K, Gonard G (1992). Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. EMBO J. 11:479-488.

Jupin I, Richards KE, Tamada T (1991). Pathogensis of beet necrotic yellow vein virus. Sem. Virol. 2:121-129.

Kiguchi T, Saito M, Tamada T (1996). Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. J. Gen. Virol. 77: 575-580.

Klein E, Link D, Schirmer A, Erhardt M, Gilmer D (2007). Sequence variation within beet necrotic yellow vein virus p25 protein influences its oligomerization and isolate pathogenicity on *Tetragonia expansa*. Virus Res. 126:53-61.

Koenig R, Haeberle A, Commandeur U (1997). Detection and characterization of a distinct type of beet necrotic yellow vein virus RNA 5 in sugar beet growing areas in Europe. Arch. Virol. 142:1499-1504.

Koenig R, Lennefors B-L (2000). Molecular analyses of European A, B and P type sources of beet necrotic yellow vein virus and detection of the rare P type in Kazakhstan. Arch. Virol. 145:1561-1570.

Koenig R, Luddecke P, Kaeberle AM (1995). Detection of beet necrotic yellow vein virus strains, variant and mixed infections by examining single-strand confirmation polymorphisms of immunocapture RT-PCR products. J. Gen. Virol. 76:2051-2055.

Kruse M, Koenig R, Hoffmann A, Kaufmann A, Commandeur U, Solovyev

AG, Savenkov I, Burgermeister W (1994). Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. J. Gen. Virol.75:1835-1842.
Lauber E, Guilley H, Tamada T, Richards K, Gonard G (1998). Vascular movement of beet necrotic yellow vein virus in *Beta macrocarpa* is probably dependent on an RNA 3 sequence domain rather than a gene product. J. Gen. Virol. 79:385-393.
Lemaire O, Merdinoglu D, Valentin P, Putz C, Ziegler-Graff V, Guillyey H, Jonard G, Richards, K (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by *Polymyxa betae*. Virology 162:232-235.
Lennefors BL, Lindsten K, Koenig R (2000). First report of A and B type *Beet necrotic yellow vein virus* in sugar beets in Sweden. Eur. J. Plant Pathol. 106:199-201.
Li M, Liu T, Wang B, Han C, Li D, Yu J (2008). Phylogenetic analysis of beet necrotic yellow vein virus isolates from China. Virus Gen. 36:429-432.
Lindsten K (1989). Investigations concerning soil-borne viruses in sugar beet in Sweden. EPPO Bull. 19:531-537.
Link D, Schmidlin L, Schirmer A, Klein E, Erhardt M, Geldreich A, Lemaire O, Gilmer D (2005). Functional characterization of the *Beet necrotic yellow vein virus* RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants. J. Gen. Virol. 86:2115-2125.
Liu H-Y Lewellen RT (2007). Distribution and molecular characterization of resistance-breaking isolates of beet necrotic yellow vein virus in the United States. Plant Dis. 91:847-851.
Mehrvar M, Valizadeh J, Koenig R, Bragard CG (2009). Iranian beet necrotic yellow vein virus (BNYVV): pronounced diversity of the p25 coding region in A-type BNYVV and identification of P-type BNYVV lacking a fifth RNA species. Arch. Virol. 154:501-506.
Meunier A, Schmit JF, Stas A, Kutluk N, Bragard C (2003). Multiplex reverse transcription-PCR for simultaneous detection of beet necrotic yellow vein virus, beet soil-borne virus, and beet virus *Q* and their vector *Polymyxa betae* KESKIN on sugar beet. Appl. Environ. Microbiol. 69:2356-2360.
Miyanishi M, Kusume T, Saito M, Tamada T (1999). Evidence for three groups of sequence variants of *Beet necrotic yellow vein virus* RNA 5. Arch. Virol. 144:879-892.
Morris J, Clover J R G, Harju VA, Hugo SA, Henry CM (2001). Development of highly sensitive nested RT-PCR method for *Beet necrotic yellow vein virus*. J. Virol. Methods 95:163-169.
Paczuski R, Szyndel MS (1994). The occurrence of rhizomania in Poland. *Ochrona Roslin* 38:9-10.
Putz C (1977). Composition and structure of *Beet necrotic yellow vein virus*. J. Gen. Virol. 35: 397-401.
Putz C, Merdinoglu D, Lemaire O, Stocky G, Valentin P, Wiedemann S (1990). Beet necrotic yellow vein virus, causal agent of sugar beet rhizomania. Rev. Plant Pathol. 69:247-254.
Rahim MD, Andika IB, Han C, Kondo H, Tamada T (2007). RNA4-encoded p31 of *Beet necrotic yellow vein virus* is involved in efficient vector transmission, symptom severity and silencing suppression in roots. J. Gen. Virol. 88:1611-1619
Richard-Molard M (1985). Rhizomanie-situation en (1985). In: compte Rendu du 48e Congres d'hiver de l'Insstitut International deRecherches Betteraviers, Institut International de Recherches Betteravieres, Brussels, Belgium. pp. 347-360.
Richards KE, Tamada T (1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Ann. Rev. Phytopathol. 30:291-313.
Rush CM (2003). Ecology and epidemiology of benyviruses and plasmodiophorid vectors. Ann. Rev. Phytopathol. 41:567-592.
Rush CM, Heidel GB (1995). Furovirus diseases in the United States. Plant Dis. 79:868-875.
Rush CM, Liu H-Y, Lewellen RT, Acosta-Leal R (2006). The continuing saga of rhizomania of sugar beets in the United States. Plant Dis. 90:4-15
Saito M, Kiguchi T, Kusume T, Tamada, T (1996). Complete nucleotide sequence of the Japanese isolate S of beet necrotic yellow vein virus RNA and comparison with European isolates. Arch. Virol. 141:2163-2175.
Schirmer A, Link D, Cognat V, Moury B, Beuve M, Meunier A, Bragard C, Gilmer D, Lemaire O (2005). Phylogenic analysis of isolates of *Beet necrotic yellow vein virus* collected from worldwide. J. Gen. Virol. 86:2897-2911.
Sohi HH, Maleki M (2004). Evidence for presence of types A and B of *Beet necrotic yellow vein virus* (BNYVV) in Iran. Virus Gen. 29:353-358.
Tamada T (2002). Beet necrotic yellow vein virus. CMI/AAB Descriptions of Plant Viruses. No. 391. Commonwealth Mycological Institute/Association of Applied Biologists, Kew, Surrey, England.
Tamada T, Abe H (1989). Evidence that *Beet necrotic yellow vein virus* RNA-4 is essential for efficient transmission by the fungus *Polymyx betae*. J. Gen. Virol. 70:3391-3398
Tamada T, Baba T (1973). *Beet necrotic yellow vein virus* from rhizomania-affected sugar beet in Japan. Ann. Phytopath. Soc. Jpn. 39:325-332
Tamada T, Kusume T (1991). Evidence that the 75K readthrough protein of *Beet necrotic yellow vein virus* RNA-2 is essential for transmission by the fungus *Polymyx betae*. J. Gen. Virol. 72:1497-1504.
Tamada T, Schmitt C, Saito M, Guilley H, Richards K, Jonard G (1996). High resolution analysis of the read-through domain of beet necrotic yellow vein virus read-through protein: a KTER motif is important for efficient transmission of the virus by *Polymyxa betae*. J. Gen. Virol. 77:1359-1367
Tamada T, Shirako Y, Abe H, Saito M, Kigushi T, Harada T (1989). Production and pathogenicity of isolates of *Beet necrotic yellow vein virus* with different number of RNA components. J. Gen. Virol. 70:3399–3409
Torrance L, Pead M, Buxton G (1988). Production and some characteristics of monoclonal antibodies against beet necrotic yellow vein virus. Ann. Appl. Biol.113:519-530.
Wang Y, Fan H, Wang X-B, Li M, Han C, Li D, Yu J (2011). Detection and characterization of spontaneous internal deletion mutants of beet necrotic yellow vein *virus* RNA3 from systemic host *Nicotiana benthamiana*. Virol. J. 8:335-344.
Wisler GC, Lewellen RT, Sears JL, Wasson JW, Liu HY, Wintermantel WM (2003). Interactions between beet necrotic yellow vein virus and beet soil-borne mosaic virus in sugar beet. Plant Dis. 87:1170-1175.