NESTED RT-PCR AND IMMUNOCAPTURE RT-PCR FOR DETECTION OF *BEET NECROTIC YELLOW VEIN VIRUS* ON SUGAR BEET IN LAKE DISTRICT OF TURKEY

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ABSTRACT

Rhizomania is a viral disease, caused by beet necrotic yellow vein furovirus (BNYVV) which was detected in Lake District of Turkey sugar beets in 2006-2007. A total of 203 soil samples were collected from 24 different sugar beet production areas of this region. Plants of Rhizomania-susceptible cultivar Kasandra were grown in these soil samples using bait plant techniques. Bait plants root samples were analysed by DAS-ELISA, RT-PCR, Nested Reverse Transcription-Polymerase Chain Reaction (nPCR) and Immunocapture - Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR) using specific primers. ELISA test results showed that 85 sample were infected with BNYVV. Fifty samples which were negative in the DAS-ELISA tests were used for RT-PCR for the identification of BNYVV. As a result of RT-PCR, 25 samples were determined as positive. RT-PCR was carried out using the 016 and 017 specific primers which amplify a 500 base pair (bp) fragment of the read-through region of the coat protein gene of BNYVV. BNYVV detected in bait plant root products of the expected size were amplified by Nested Reverse Transcription-Polymerase Chain Reaction (nRT-PCR) and Immunocapture-Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR) (326 bp for nPCR). Twenty five samples which were negative in the RT-PCR tests were used for nRT-PCR for the identification of BNYVV. As a result of nRT-PCR 19 samples were determined as positive. At last, 6 samples which were negative in the nRT-PCR tests were used for IC-RT-PCR. As a result of IC-RT-PCR 2 samples were determined as positive. Because ELISA method is simple, quick, efficient and not expensive, its main use can be to analyse BNYVV of the seedlings in the early days. RT-PCR methods are complex and expensive, but they are more sensitive than ELISA.

Key words: sugar beet, Rhizomania, BNYVV, ELISA, RT-PCR, nRT-PCR, IC-RT-PCR.

INTRODUCTION

Rhizomania (root madness) is a very damaging disease leading to a major reduction in root yield and quality of sugar beet. This disease of sugar beet was first reported in Italy in 1952 by Canova. At present, it is widely distributed on a large sugar beet growing area in Asia, Europe, and America. It still continues to spread to the other areas (Putz et al., 1990; Suarez et al., 1999). Rhizomania is caused by beet necrotic yellow vein *Benyvirus* (BNYVV) (Tamada and Baba, 1973; Koenig and Tamada, 2000). The virus is transmitted by a soil-borne plasmodiophorid *Polymyxa betae* (Keskin, 1964) and is known to survive in the soil for at least 15 years, protected by resting cystosori of the fungus (Tamada, 1975). Thus, the disease can not be controlled by agronomical practices. *Polymyxa betae* has an important role on transmission, and distribution of rhizomania (Blunt et al., 1991). The main symptom of rhizomania is root bearding (known as root madness). In addition stunting, chlorosis of leaves, yellowing of veins and necrosis of leaf veins can often be observed.

The disease causes economic loss to sugar beet by reducing yield. It can cause a decrease in root yield of 30-90% (Clover et al., 1999; Asher and Kerr, 1996) and it reduces sugar yield up to 70% (Putz et al., 1990; Whitney and Duffus, 1991; Rush and Heidel, 1995). In Turkey, rhizomania was first detected in the beet samples sent from Alpullu and Amasya to Germany in 1987

(Koch, 1987). Later the presence of the disease was reported in different beet growing areas of Turkey (Vardar and Erkan, 1992). Rhizomania is presently one of the most important sugar beet diseases in Turkey (Kaya, 2009; Kıymaz and Ertunç, 1996; Kutluk Yılmaz and Yanar, 2001).

Even though Lakes region is one of the important sugar beet production areas in Turkey, viral diseases in sugar beet growing in this region are largely unknown because of a lack of attention to viral diseases and absence of any virus diagnosis programme in the region. In this research, detection and identification of *Beet necrotic yellow vein virus* was investigated in Lake District sugar beet plantations of Turkey, using DAS-ELISA, RT-PCR, nPCR and IC-RT-PCR.

MATERIAL AND METHODS

A total of 203 soil samples were collected from different locations of in Lake District of Turkey between 2006 and 2007. The soil samples were used in bait plant tests. In bait plant studies the rhizomania-susceptible cultivar (Kasandra) was used. The root samples were used to test for the presence of virus by ELISA, RT-PCR, nPCR and IC-RT-PCR studies. First, all bait plant root samples were tested for the presence of BNYVV with specific ELISA detection kits (Agdia Company, Elkhart, United States), using the previously reported DAS-ELISA method (Clark and Adams, 1977). Absorbance

values were measured at 405 nm with a microplate reader (Versa Max Microplate Reader). Samples with DAS-ELISA values at least twice higher than those of the healthy control were considered to be positive (Mouhanna et al., 2002). Total RNA was extracted from 100 mg of root tissue, essentially based on RNeasy RNA extraction Kit protocol (QIAGENE®, Germany).

Fifty ELISA negative root samples were then tested by RT-PCR for BNYVV. The RT-PCR, using 016 and 017 specific primers, which amplified a 500 bp fragment of the read-through region of the coat protein of BNYVV (Morris et al., 2001), showed that 25 out of the 50 soil samples (50%) were contaminated with BNYVV (Figure 2). One step RT-PCR was undertaken in 50 μL reaction volumes containing 1 μL of template RNA, 2.5 μL of forward and reverse primers (016 and 017, 5 μM), 5 μL of 10X Taq reaction buffer (100 mM Tris-HCl, 500 mM KCl, pH 9.0), 3.75 μL of $MgCl_2$ (20 mM), 1 μL of dNTPs (10 mM), 0.05 μL of MMLV reverse transcriptase, 0.5 μL RNasin and 33.2 μL distilled water and 0.5 μL of Taq polymerase (Bio Basic, D0081). Thermocycling was carried out as follows; 37ºC for 30 min., 94ºC for 2 min., then 30 cycles of 94ºC for 1 min., 55ºC for 1 min. and 72ºC for 1 min., followed by 72ºC for 3 min. (Harju, 2003). Amplified products were observed after electrophoresis in ethidium bromide-stained agarose gels (Figure 1).
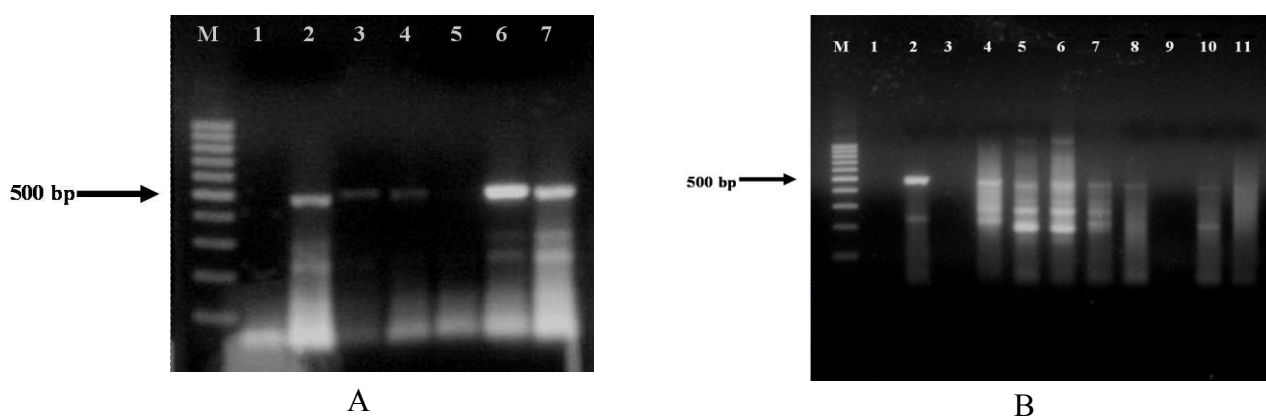

A   B

Figure 1. RT-PCR 500 bp bands (arrow) of Lake District in Turkey sugar beet samples proved the presence of BNYVV virions in plant roots
A) M: marker; 1: negative control; 2: positive control; 3-7: samples of sugar beet roots;
B) M: marker (100 bp DNA Ladder, Fermentas); 1: negative control; 2: positive control;
3-11: samples of sugar beet roots.

25 samples which were negative in the DAS-ELISA and RT-PCR tests were used for nRT-PCR for the identification of BNYVV. Nested primer pairs, designated rhzn15/rhzn17, should give products of 326 bp. Nested nRT-PCR method, as one step, was carried out according to Harju (2003). For nPCR reactions, 0.5 μl of product from the first round of amplification was used as template in 50 μl reactions containing 2.5 μl of forward and reverse primers (rhzn 16 or rhzn17, 100 μM), 5 μl of 10×*Taq* reaction buffer (100 mM Tris-HCl,

500 mM KCl, pH 9.0), 3.75 μl of MgCl$_2$ (20 mM), 1 μl of dNTPs (10 mM) and 2.5 units of *Taq* DNA polymerase (Bio Basic, D0081). Thermocycling was performed as follows: 94°C for 2 min., then 30 cycles of 94°C for 1 min., 58°C for 1 min. and 72°C for 1 min., followed by 72°C for 3 min. In all reactions, healthy plant material and sterile distilled water controls from the first round of PCR were used. Amplified products were observed after electrophoresis in ethidium bromide-stained agarose gels (Figure 2).
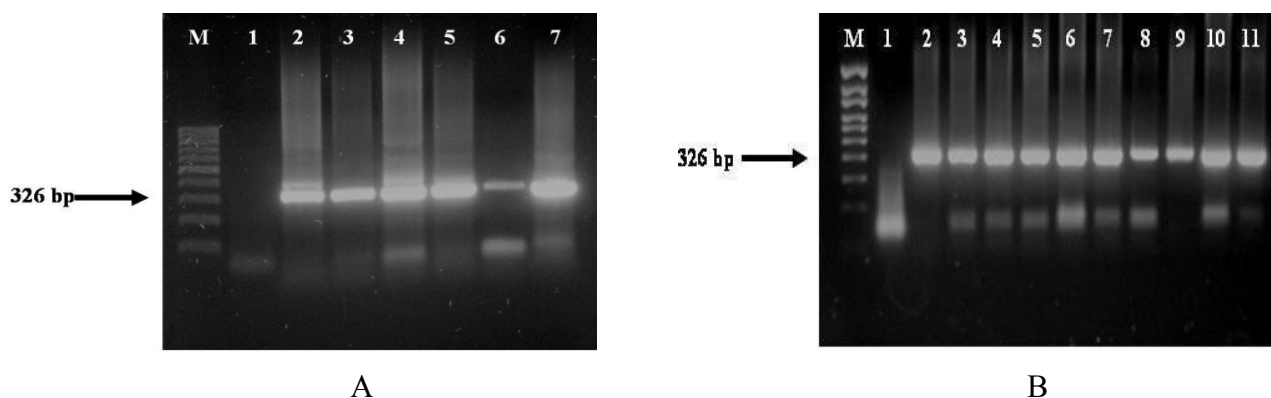

Figure 2. nRT-PCR 326 bp bands (arrow) of Lake District in Turkey sugar beet samples proved the presence of BNYVV virions
in plant roots
A) M: marker; 1: negative control; 2: positive control; 3-7: samples of sugar beet roots;
B) M: marker (100 bp DNA Ladder, Fermentas); 1: negative control; 2: positive control;
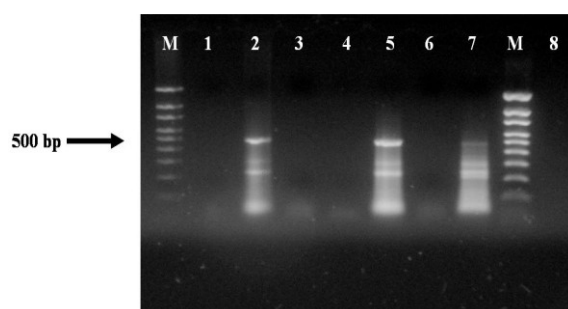3-11: samples of sugar beet roots.

Figure 3. IC-RT-PCR 500 bp bands (arrow) of Lake District in Turkey sugar beet samples
proved the presence of BNYVV virions in plant roots
(M: marker (100 bp DNA Ladder, Fermentas); 1: negative control; 2: positive control;
3-8: samples of sugar beet)

One step immunocapture RT-PCR was carried out as follows (Morris et al., 2001): Coat RT-PCR tubes with polyclonal anti-BNYVV antiserum were incubated 3 h at 33°C. Then tubes were washed with PBST buffer three times. Grind sample roots in ELISA extraction buffer was added to the

tubes and incubated overnight at 4°C, washed three times with PBST and twice with sterile distilled water. Finally the RT-PCR reaction mix was added to each sample tube. The 50 μL reaction volumes contained 2.5 μL of forward and reverse primers (016 and 017, 100 μM), 5 μL of 10X *Taq* reaction buffer

(100 mM Tris-HCl, 500 mM KCl, pH 9.0), 3.75 µL of MgCl2 (20 mM), 1 µL of dNTPs (10 mM), 0.05 µL of MMLV reverse transcriptase, 0.5 µL RNasin and 34.2 µL distilled water and 0.5 µL of *Taq* polymerase (Bio Basic, D0081). Thermocycling was carried out as follows: 37ºC for 30 min., 94ºC for 2 min., then 30 cycles of 94ºC for 1 min., 55ºC for 1min and 72ºC for 1 min., followed by 72 ºC for 3 min. (Harju, 2003). Amplified products were observed after electrophoresis in ethidium bromide-stained agarose gels (Figure 3).

RESULTS AND DISCUSSION

During field surveys in the 2006 to 2007 in sugar beet production of Lake District in Turkey rhizomania symptoms of leaves and roots were observed. Leaves of infected plants were then pistachio green, wilted and with reduced growth. Additionally, the plants showed typical overgrowth of lateral roots and production of smaller beet roots. 203 soil samples were collected in sugar beet production fields in these areas. These soils were used to detect the presence of BNYVV and *P. betae* using the bait plant test. ELISA tests were performed nine weeks later on the young sugar beet roots. The samples were first tested by double antibody sandwich enzyme linked immunosorbent assay for BNYVV. DAS-ELISA showed that 85 of 203 soil samples (41%) from 22 localities were contaminated with BNYVV.

50 ELISA negative root samples were then tested by RT-PCR for BNYVV. RT-PCR showed that 25 of the 50 soil samples (50 %) were contaminated with BNYVV (Figure 2).

BNYVV detected in bait plant root products of the expected size were amplified by Nested Reverse Transcription-Polymerase Chain Reaction (nRT-PCR) and Immuno-capture-Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR)(326 bp for nPCR). The 25 samples which were negative in the RT-PCR tests were used for nRT-PCR for the identification of BNYVV. As a result of nRT-PCR, 19 samples were determined as positive. At last, 6 samples which were negative in the nRT-PCR tests were used for IC-RT-PCR. As a result of IC-RT-PCR, 2 samples were determined as positive.

Nested RT-PCR and IC-RT-PCR are very sensitive methods, even at lower virus concentration (Webster et al., 2004). Nested RT-PCR improves the sensitivity of virus detection by 1000 fold compared with the standard RT-PCR (Morris et al., 2001). Although RT-PCR tests are very specific and sensitive test when compared to DAS-ELISA, they are not convenient for routine tests with lots of samples. Simple, quick, efficient and not expensive, DAS-ELISA is suitable for analysing more samples in epidemiological studies (Wisler et al., 1994; Rush and Heidel, 1995).

CONCLUSIONS

Detection and identification of BNYVV by different PCR methods confirmed the presence of BNYVV in sugar beet plants with rhizomania symptoms detected in the Lakes region of Turkey.

The presence of BNYVV in many bait plant roots found negative by DAS-ELISA test was proved by RT-PCR, nRT-PCR and IC-RT-PCR methods using specific primers. RT-PCR and IC-RT-PCR reactions resulted in specific amplification of the 500 bp fragment of the read-through region of the coat protein gene of BNYVV RNA-2. Primer design for nPCR to complement the internal sequence of the 500 bp product amplified of RT-PCR produced the expected 326 bp product.

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