Identification of *Beet necrotic yellow vein virus* isolate detected in Lithuania

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Abstract. Sugar beet plants with rhizomania symptoms in their roots were detected in the central region of Lithuania. The study was undertaken to ascertain the possible presence of beet necrotic yellow vein virus (BNYVV) in the rootlets. DAS-ELISA, lateral flow tests, electron microscopy and mechanical transmission to indicator plantsconfirmed the presence of BNYVV. BNYVV was detected in leaf tissue of indicator plants with local lesions, and products of the expected size were amplified by RT-PCR, immunocapture RT-PCR (IC-RT–PCR) and nested PCR (nPCR) (500 bp for RT-PCR and 326 bp for nPCR). This is the second location where the presence of BNYVV quarantine pathogen in Lithuania has been proved.

Key words: Rhizomania, RT–PCR, nPCR, IC–RT–PCR, IEM

INTRODUCTION

Rhizomania of sugar beet (*Beta vulgaris*) is caused by *Beet necrotic yellow vein virus* - a member of the genus *Benyvirus* - which is a quarantined organism in all European countries. BNYVV is transmitted by fungus vector (*Polymyxa betae* Keskin.) which survives in infested soil for many years; the disease cannot be controlled by crop rotation or by having a beet-free period (Rush, 2003). First described in Italy in 1952 (Canova, 1959), rhizomania spread to many other sugar beet growing countries in Europe, Asia and North America (Morris, 2001; Ratti et al., 2005). The main symptom of rhizomania is root bearding (known as "root madness"). Other symptoms - stunting, chlorosis of leaves, yellow veining and necrosis of leaf veins – often can be observed. Severe economic losses can result, due to reduction of root yield, sugar content and purity (Scholten & Lange, 2000; Rush, 2003).

Sugar beet crops in Lithuania cover more than 20 000 Ha. Surveys for the presence of *Beet necrotic yellow vein virus* (BNYVV) in sugar beet crops in Lithuania have regularly been carried out since 1998 using DAS-ELISA test. The first hotbed of the disease was detected in 2004 and the causal agent was identified (Jackeviciene et al., 2005);anew seat of rhizomania was detected in one sugar beet plot in the Kaunas region in 2005. The purpose of this work was to present results of the identification and some biological properties of the causal agent.

MATERIALS AND METHODS

Samples of sugar beet roots with bearded appearance were tested in express

lateral flow (Strube-Dieckmann Spot-check LF[TM]) and DAS ELISA tests (Clark & Adams, 1977) using the DSMZ immunological kit. Virus particles were observed by immunosorbent electron microscopy (IEM). Isolation of BNYVV was performed by mechanical inoculation of indicator plants using infected sap of bearded roots.

Total RNA was extracted from plant tissue according to the instruction of "QuickPrep[TM] Total RNA Extraction Kit for the direct isolation of total RNA from most eukaryotic tissues or cells" (Amersham Biosciences, UK).

RT-PCR was carried out using specific primers, which amplify a 500 bp fragment of the read-through region of the coat protein gene located on RNA-2 of BNYVV (Henry et al., 1995; EPPO Bulletin, 2004). Temperatures used for the cycling reaction were as follows: 30 min at 37°C; 2 min at 94°C; followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; finally, 3 min at 72°C.

In order to increase the BNYVV detection level, nPCR method was also used. It improves the sensitivity of virus detection by 1000-fold compared with the standard RT-PCR assay (Morris et al., 2001). nPCR was performed with internal primers, which annealed to the amplicon, produced from standard PCR, and specifically amplified a 326 bp product (Morris et al., 2001; EPPO Bulletin, 2004). Cycling conditions were 94°C for 2 min; 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min; finally, 72°C for 3 min.

For the IC-RT-PCR method the pathogen was partially purified by adsorption with specific antibodies (DSMZ) on a solid-phase (in microtubes). Then tubes were washed with PBST buffer and extracts like those used for DAS-ELISA were added to the tubes. Finally the RT-PCR reaction mix was added to each sample tube after washing and was thermocycled at the temperatures used for RT-PCR, above. It has been noted that that using the one tube RT-PCR+nPCR method is a 100-fold improvement on conventional IC-RT-PCR (Olmos et al., 1999). n IC-RT-PCR was conducted using 0.5 μl of the IC-RT-PCR product, using specific primers for nPCR.

All PCR products were detected after 5% bisacrylamide gel electrophoresis, stained with ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

The presence of BNYVV in the rootlets of rhizomania-affected sugar beet was ascertained by immunological methods of an express lateral flow (screening) test and the DAS ELISA test, using alkaline phoshatase as the enzyme and nitrophenyl phosphate as the substrate. The ELISA test was positive if absorptance of the investigative sample was more than 2–3 times greater than absorptance of the negative control (Clark et al., 1977). Later, samples positive for BNYVV were mechanically transmitted to indicator plants (*Chenopodium quinoa, C. amaranticolor* and *Tetragonia expansa*), which displayed only local reaction (Fig.1). The presence of characteristics indicating BNYVV - numerous trapped,, straight, very short and longer rod-shaped virus particles about 20 nm in diameter in beet rootlets extracts and experimentally inoculated plants - was confirmed by IEM (Fig. 2).
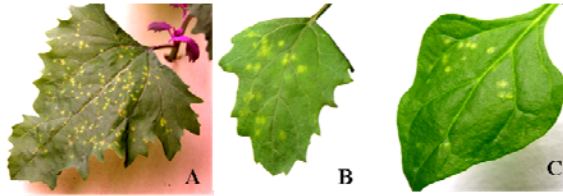
Fig. 1. Local chlorotic lesions on indicator plants: (**A.** *C. amaranticolor* **B.** *C. quinoa* **C.** *T. expansa*).
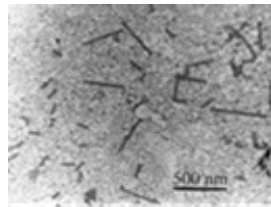

Fig. 2. IEM (magnification 20 000 x): BNYVV characteristic virus particle.
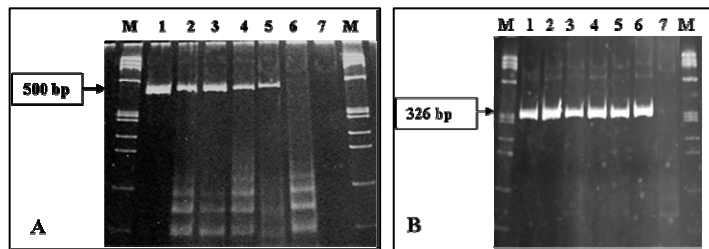

Fig. 3. PCR products: (**A. RT-PCR:** M—marker; 1—4 samples of indicator plants extract; 5—K+; 6—K-; 7—Kw; **B. nPCR:** M—marker; 1—K+; 2—6 samples of indicator plants extract; 7—K-).
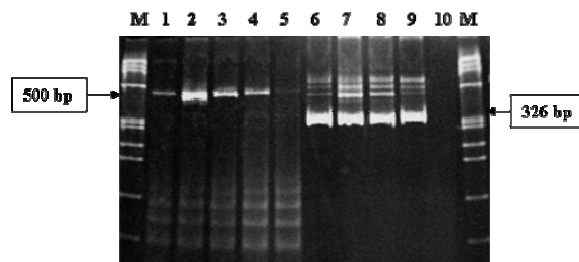

Fig. 4. PCR products: M—marker; 1—3 IC-RT-PCR samples of indicator plants extract; 4—K+; 5—K-; 6—8 nIC-RT-PCR samples of indicator plants extract; 9—K+; 10—Kw.

The presence of BNYVV in tissue extracts of indicator plants with local lesions was proved by RT-PCR or nPCR and IC-RT-PCR or nIC-RT-PCR methods using specific primers, which were mentioned above. 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 (Figs 3A, 4). No products were obtained from negative (healthy plant tissue) and water controls. Primers designed for nPCR (or nIC-RT-PCR) (Morris et al., 2001) to complement the internal sequence of the 500 bp product amplified of RT-PCR produced the expected 326 bp product (Figs 3B, 4).

CONCLUSIONS

BNYVV infected sugar beet plants showed very mild symptoms on leaves and typical symptoms on roots in comparison with classical symptoms of rhizomania disease. Characteristic symptoms on inoculated plants, morphology of detected virus particles by IEM, positive ELISA tests, and detection of BNYVV by PCR methods confirm the presence of BNYVV in sugar beet plants with rhizomania symptoms detected in the central region of Lithuania.

This investigation confirms the detection of the second BNYVV hotbed in Lithuania and indicates the need for appropriate measures to be taken for its control.

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