



# Quantification of rhizomania virus by automated RNA isolation and PCR based methods in sugar beet

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**Abstract** Rhizomania is a grave disease affecting sugar beet (*Beta vulgaris* L.). It is caused by the Beet Necrotic Yellow Vein Virus (BNYVV), an RNA virus transmitted by the plasmodiophorid vector *Polymyxa betae*. Genetic resistance to the virus has been accomplished mostly using phenotype-genotype association studies. As yet, the most convenient method to ascertain plant resistance has been the quantification of viral titer in roots through the ELISA test. This method is particularly time-consuming and clashes with the necessities of modern plant breeding. Here, we propose an alternative and successful phenotyping method based on the automatic extraction of the viral RNA from sugar beet roots and its relative and absolute quantification by quantitative real-time PCR (qRT-PCR) and digital PCR (dPCR), respectively. Such a method enables an improved standardization of the study, as well as an accurate quantification of the virus also in those samples presenting low virus titer, with respect to the ELISA test.

**Keywords** Sugar beet · Rhizomania virus · High-throughput phenotyping · Quantitative real-time PCR · Digital PCR

## Abbreviations

qRT-PCR Quantitative real-time PCR

dPCR Digital PCR  
RT-PCR Reverse transcriptase PCR

Rhizomania is the most destructive disease affecting sugar beet: it can decrease sugar yield by up to 70% [24, 31, 33]. Rhizomania is caused by the Beet Necrotic Yellow Vein Virus (BNYVV), a virus transmitted by the soil-borne plasmodiophorid vector *Polymyxa betae*. The BNYVV is a single-stranded rod-shaped virus consisting of 4 or 5 ssRNAs [28]. According to the RNA structure, the A, B, and P forms of BNYVV have been distinguished. Strains A and B have four RNAs while the P-type also has a fifth RNA strand. The function of each RNA strand has been well characterized [11]. In particular, the third one is involved in the long-distance movement of the virus in the plant [12]. The P25 virulence factor is located on this strand and is responsible for the development of one of the main root symptoms: the extensive proliferation of lateral roots [10, 32]. This gene is the one displaying the highest variability: mutations at positions 67 to 70 (hypervariable tetrad) are unique for each strain of the virus [22]. The variability within the P25 gene allows the virus to bypass the resistance. Koenig et al. [16] described the most common mutations of this tetrad in each strain: in the A-type ACHG, AHHG, AFHG, ALHG, AYHG, VCHG; in the B-type AYHR, AHHR; in the P-type SYHG have been identified. More recently, a new A-strain has been characterized, where the hypervariable tetrad presents the AYPR motif. This strain is less common than the others but more aggressive [4].

Rhizomania virus is spread worldwide, and it causes enormous damage to the crop. The only approach to control the disease is the use of host genetic resistance. The

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first source of resistance (Alba) was identified in Italy in 1978 [3] just by observing which plants were not showing the typical symptoms of infection (yellowing leaves, hairy roots, low sugar content, and processing quality). But the symptoms appear in quite a wide range, so visual inspection alone is not sufficient for a proper diagnosis [1]. Thus, between 1984 and 1985, researchers started to utilize the enzyme-linked immunosorbent assay (ELISA) test for the detection of BNYVV [17, 23]. Both double and triple antibody sandwich (DAS-, TAS-) ELISA tests are suitable for its detection [2]. Since its introduction in the 1980s, this method has been the indisputable basis for genotype–phenotype association studies. Research shifted from the visual identification of resistant individuals to the identification of resistant genotypes using molecular markers associated with resistant phenotypes recognized through ELISA. This pipeline allowed several markers associated with resistant genotypes to be identified [5, 21, 27]. However, the ELISA test is unable to detect little infection levels [6], and there have been no functional updates to the method since its introduction. There have been efforts to make the phenotyping of rhizomania more sensitive and precise using reverse transcriptase PCR, but these attempts did not streamline the workflow. Some studies proving how PCR-based workflows are more sensitive and specific than ELISA-based phenotyping are those from Henry et al. [14], Morris et al. [20], and Harju et al. [13]. However, the before-mentioned experiments were not able to work independently of the ELISA test. Yardımcı and Çulal [31] state that the reverse transcriptase PCR is preferable to ELISA, but RT-PCR with subsequent gel electrophoresis is still slower than qRT-PCR.

In this paper, we provide improvements in rhizomania virus detection based on automated RNA extraction from seedling roots, followed by relative and absolute quantification by real-time PCR and digital PCR, respectively.

Sugar beet pollinator lines L1 and L2 (resistant and susceptible to rhizomania, respectively), were provided by DAFNAE-University of Padova, Italy. Seeds were rinsed in ethanol 96% and then steeped in H<sub>2</sub>O<sub>2</sub> 3% overnight to stimulate germination. They were then placed in the folded paper for germination in dark conditions at room temperature. After a couple of days, only germinated seeds were transplanted into rhizomania contaminated soil. This soil was a mixture of 50% contaminated soil (collected in Montagnana, Padova, Italy), 25% organic soil, and 25% sand. Seeds were grown in two transparent boxes with a depth-filtration system (Microbox TP5000-TPD5000, Micropoli, Italy) using a final volume of 1 l of soil, one box for L1 and one for L2. The two boxes contained a total of 8 plants each. At transplanting, 100 ml of purified water was added to the box. No more water was added during the growth.

Plants were sampled after 4 weeks of growth. Whole roots (main root and lateral roots) were meticulously washed to remove any trace of soil that can interfere with the next PCR step [7]. Each root was homogenized, and only those weighing at least 0.22 g were kept (12 out of 16). A double sampling was done on the roots of each plant: 0.07 g of the root was taken for RNA extraction and qRT-PCR phenotyping, 0.15 g of the root was used for a backcheck ELISA test. Total nucleic acid extraction was conducted using a BioSprint 96 (QIAGEN, Germany) with the protocol optimized for the purification of total RNA from plant tissue. Collection microtubes (1 ml, 96 racked tubes, QIAGEN) with roots were filled with 200 µl RLT (Guanidine thiocyanate buffer under patent protection) and one 3 mm Ø tungsten bead. Tubes were loaded in a TissueLyser II (QIAGEN) for cell lysis (5 min, 30 Hz) and centrifuged (20,000 g × 5 min). 300 µl of lysate from each tube was transferred into the first 96-deep-well plate (S-Blocks, QIAGEN). Other 4 deep-wells plates were used for the extraction together with one 96-deep-well plate (96-Well Microplates MP, QIAGEN). Plates were filled as shown in Table 1 and loaded into the BioSprint 96 robotic station for nucleic acid extraction. Additional enzymatic treatment with DNase is recommended. In our case, DNase I (Thermo Fisher Scientific, US) was used following the manufacturer's instructions.

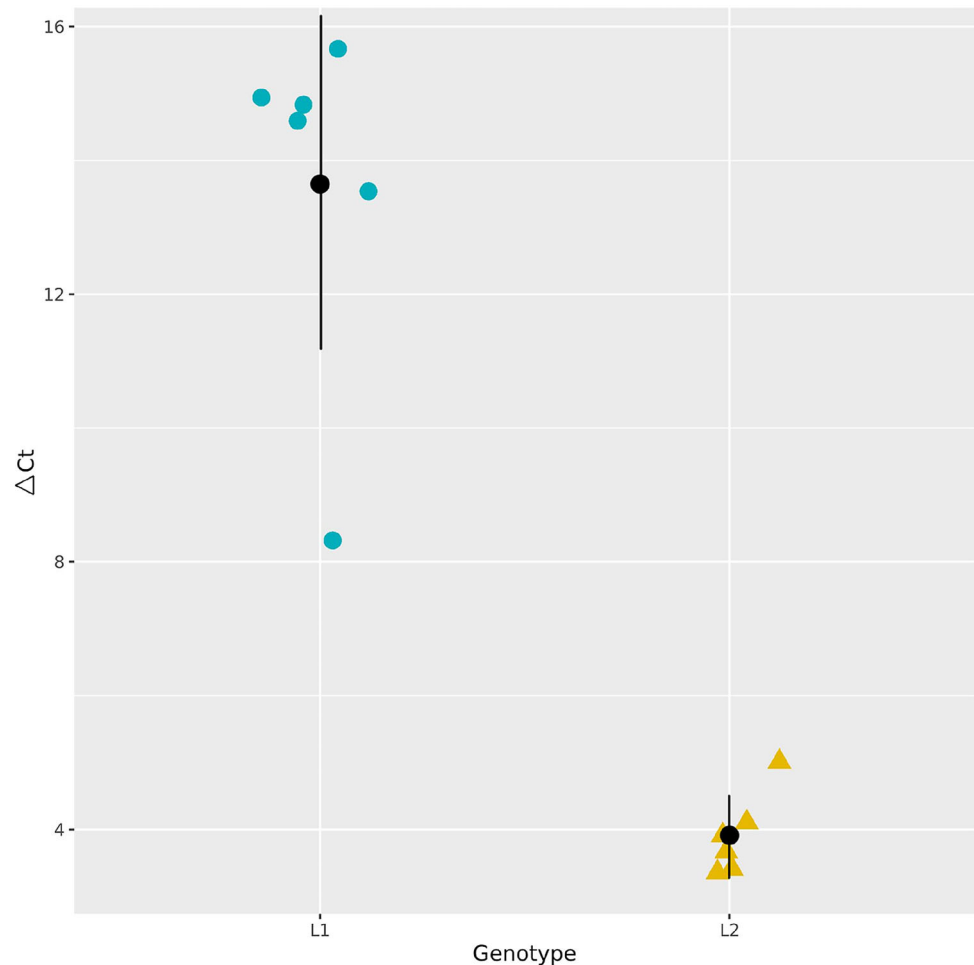
A quantitative real-time PCR targeting the BNYVV was used to estimate the quantity of virus in each sample. The reaction kit (PCRBIO SYSTEMS, UK) was modified to run in 6 µl volumes on the QuantStudio 12 K-Flex (Thermo Fisher Scientific, USA) using 384-well plates. The mix was composed of 0.2 µl each of forward and reverse primer and 0.1 µl of TaqMan probe (Supplementary Material 1), 2.5 µl 2xqPCR BIO Probe 1-Step Go Mix, 0.5 µl 20xRTase Go, 1 µl PCR-grade water, and 1.5 µl template RNA. Cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 60 s, and 72 °C for 15 s. All reactions were run in duplicate.

As a reference check, we used an internal susceptible control (SC, Ct = 13.15). The qRT-PCR analysis highlighted a significant difference ( $P < 0.01$ ) of around 9 Ct between the two L1 and L2 lines (Supplementary Material 2) (Fig. 1). Not all the cultivated genotypes present the same level of resistance to rhizomania: our results show a range of resistance, and they have been relativized to our internal SC.

We also conducted an ELISA test on the same samples, using juice extracted from the roots, as a backcheck to establish a correlation between molecular (qRT-PCR) and serological (ELISA) results. Therefore, samples were analyzed by triple-antibody sandwich (TAS)-ELISA using a BNYVV kit supplied by Agdia EMEA (France) according to the manufacturer's instructions. Results recorded

**Table 1** List of reagents and volumes to be added to the 96-deep-well plates for automated RNA extraction on the BioSprint robotic station

Plate Nr	Plate type	Volumes	Reagents
1	S-Blocks	300 $\mu$ l	Root lysate
		200 $\mu$ l	Isopropanol
		25 $\mu$ l	MagAttract Suspension G (QIAGEN)
2	S-Blocks	500 $\mu$ l	RPW (Guanidine hydrochloride buffer under patent protection)
3	S-Blocks	500 $\mu$ l	96% ethanol
4	S-Blocks	500 $\mu$ l	96% ethanol
5	S-Blocks	500 $\mu$ l	0.02% (v/v) of TWEEN 20 (AMRESCO, US)
6	MP	150 $\mu$ l	PCR-grade H <sub>2</sub> O (elution plate)

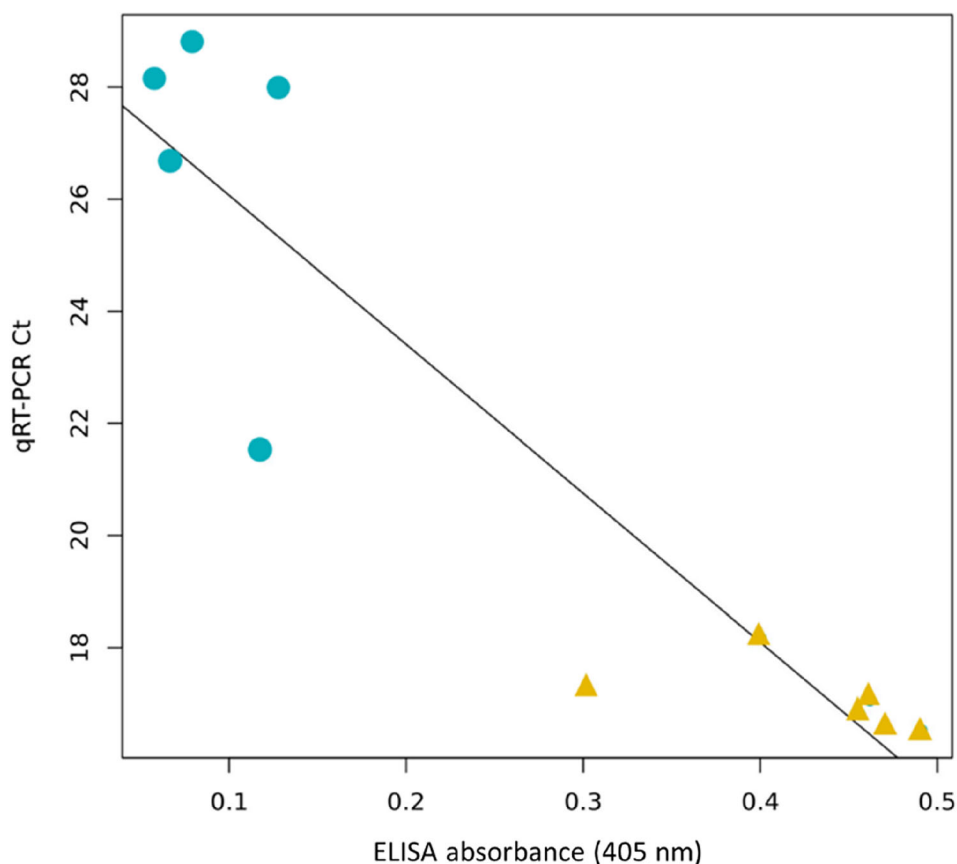
**Fig. 1**  $\Delta$ Ct values ( $C_{t_{\text{sample}}} - C_{t_{\text{ref}}}$ ) obtained from qRT-PCR are shown. The graph shows means (black dots) and standard errors (vertical bars) inside the two groups. Blue dots represent the resistant (L1) samples and the yellow triangles represent the susceptible (L2) samples to the BNYVV

using a Jenway 640 S UV/Vis Spectrophotometer at 405 nm are shown in Supplementary Material 3. Due to the sampling method, the analysis of individual associations between the serological and molecular tests was possible only for 11 out of the 12 root samples. A significant correlation ( $r = -0.93$ ;  $P < 0.01$ ) was found between molecular (qRT-PCR) and serological (ELISA) results (Fig. 2).

Digital PCR analysis was further carried out to support qRT-PCR with absolute quantification of the virus. dPCR quantification was conducted using the QuantStudio 3D

Digital PCR System (Thermo Fisher Scientific). The dPCR mix was composed of 8  $\mu$ l of QuantStudio 3D Digital PCR Master Mix (Thermo Fisher Scientific), 1.44  $\mu$ l of both forward and reverse primers, 0.8  $\mu$ l of the probe, and 2.82  $\mu$ l of nuclease-free water. The primers and the probe used in the analysis were the same as those used for the qRT-PCR. The thermal profile was described by Stevanato and Biscarini [26]. Digital PCR data were analyzed with the QuantStudio 3D AnalysisSuite Cloud software (Thermo Fisher Scientific, USA). The absolute levels of the

**Fig. 2** Correlation scatter plot between molecular (qRT-PCR) and serological (ELISA) results. The resistant samples L1 (blue dots) and the susceptible samples L2 (yellow triangles) were sampled twice to do the two types of analysis. The two different analyses present a significant correlation ( $r = -0.93$ )

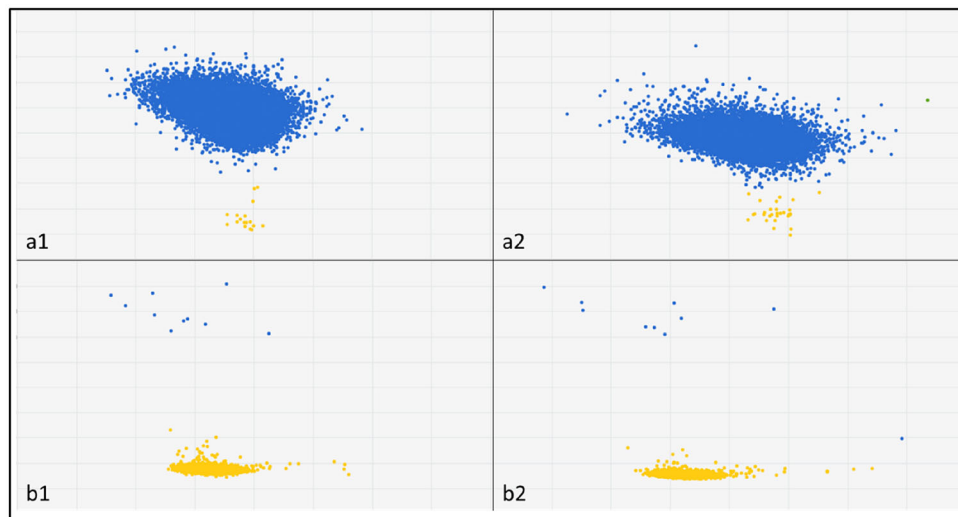


target gene were expressed as the number of copies per microgram of RNA. Confidence interval and precision of dPCR analysis were calculated using Poisson statistics directly by the QuantStudio 3D AnalysisSuite Cloud software. Four samples were analyzed, two resistant ones with the highest Ct, and two susceptible ones with the lowest Ct, to draw a calibration line. As result, the two resistant samples present fewer events of amplification (dots) than the two susceptible samples. Scatter plots obtained by QuantStudio 3D Analysis Suite Cloud Software using the relative quantitation application are represented in Fig. 3. The whole experiment has been replicated three times anyway we have been using this method for 3 years.

The research displays an alternative approach to the ELISA test. Differently from the foregoing protocols, ours not only makes the test more sensitive and specific, but it works independently of ELISA. Furthermore, we included the automated extraction of RNA and ran the qRT-PCR on 384-well plates, which jointly clearly speed up the protocol. This method enables the extraction of up to 96 RNA samples in about 30 min and to quantify the virus in up to 384 samples in two and a half hours. The qRT-PCR is remarkably sensitive: this allows not only to discriminate between susceptible and resistant samples, as ELISA does but also to select the most resistant samples among the

resistant ones. This aspect is critical for breeders who already have resistant materials but are constantly seeking more resistant ones. The high-throughput of the analyses also makes it feasible to rapidly screen wide collections of plant materials.

For this protocol, the one-step chemistry has been chosen, which offers some inherent advantages: joining the reverse transcriptase step with the PCR step reduces the time required for the analysis and possible pipetting mistakes. Besides, the qRT-PCR-based assay is supported by the dPCR: this detection method is well-known for its sensitivity, even higher than qRT-PCR [30]. The further value from the dPCR depends on several factors: (I) qRT-PCR, being a relative quantification, relies on an external reference, while dPCR does not [15]; (II) dPCR is more tolerant to inhibitory substances than qRT-PCR [8] and this is useful given that RNA is extracted from the roots, and some traces of soil, rich in inhibitors, can remain on the samples despite the washing steps; (III) both qRT-PCR and dPCR work with similar fluorescence chemistry for nucleic acid detection, so it is not necessary to design two different assays for the two technologies; (IV) dPCR is extremely powerful in detecting minute traces of nucleic acids [15, 29], allowing to discriminate between resistant plants and apparently resistant plant with minimal virus titers;



**Fig. 3** Scatter plot from dPCR analysis of the BNYVV in 2 susceptible (**a1**, **a2**) and 2 resistant (**b1**, **b2**) RNA samples. Wells with yellow dots indicate ROX signal (passive reference), while wells with blue dots indicate FAM signal, correlating with the presence and quantity of target. QuantStudio 3D Digital PCR Analysis Suite Cloud Software calculates the absolute number of targets and performs statistics, providing the confidence interval and precision. Blue dots

are described as the number of dots converted by the software into copies  $\mu\text{L}^{-1}$  of the target. The susceptible samples (**a1**, **a2**) show a high level of amplification of the target (8911.4 copies/ $\mu\text{L}$  and 9344 copies/ $\mu\text{L}$  respectively) while the resistant **b1** and **b2** samples show almost no amplification (0.738 copies/ $\mu\text{L}$  and 0.812 copies/ $\mu\text{L}$  respectively). The four scatter plots are created by the QuantStudio 3D Digital PCR Analysis Suite Cloud Software

(V) dPCR has already been tested for the detection of human viruses, such as HIV but also on RNA viruses, and the measurement of low copy RNA targets has been satisfactory [25].

Many BNYVV mutations have already been identified and many others could occur. The qRT-PCR relies on specific primers and probes for the virus: in the case of new mutations or whenever discrimination among different strains of the virus is needed, the use of new or different probes would be sufficient to accurately detect the strain. It has also been challenging, in some cases, to distinguish between BNYVV and BSBMV (Beet soil-borne mosaic virus) applying the ELISA test, because the structure of the two viruses is similar [9]. This problem no longer exists with the qRT-PCR targeting specific nucleic acids.

Interestingly, the detection method introduced for the BNYVV is analogous to the virus-detection method in the clinical field. For example, the detection protocol for COVID-19 on swabs is qRT-PCR-based and extremely similar [19]. Also, dPCR is used for the screening of human pathogens, such as viruses but also cancer. In fact, plant virology opened the way for medical virology: the first virus ever discovered was the Tobacco mosaic virus (TMV), which formed the basis for subsequent virologic studies [18]. It is thus not surprising that protocols used in plant biology can be easily reconverted for medical biology. Indeed, this is proof of the efficiency of such protocols.

As proved from previous studies, the sugar beet community has been demanding for some time for an alternative high-throughput method to the ELISA test. Nevertheless, the best outcome was only achieved by reverse transcriptase PCR, with subsequent gel electrophoresis. With the arrival of one-step qPCR mixes, we gather it is required to introduce a new phenotyping method, better performing, precise, and also able to work in the presence of small quantities of virus. Anyway, we are not suggesting that the ELISA-based phenotyping is not good or sensitive enough but offering an alternative that can be useful also for high-throughput needs.

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**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Human and animal rights statement** This article does not contain any studies involving human participants and animals performed by any of the authors.

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