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ONION YELLOW DWARF VIRUS RELATIVE QUANTIFICATION USING REAL-TIME POLYMERASE CHAIN REACTION IN 'ROSSA DI TROPEA' ONION BY A ΔΔ Ct METHOD.

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Abstract:	As part of a plant-pathogen interaction study between Onion yellow dwarf virus (OYDV) and onion cultivar Rossa di Tropea, a ΔΔCt-based relative quantification of OYDV was investigated to relate OYDV titer to accumulation of secondary metabolites in onion bulbs. An appropriate reference gene (RG) was required to achieve data normalization. Since no single internal control gene is universally used as an RG, multiple stably expressed reference genes were investigated. In particular, elongation factor (Elf), protein phosphatase 2A (PP2A), helicase (Hel-1), 5.8S rRNA, ubiquitin (UBQ) and ß-Actin (ß-Act) were compared one to another in both leaf and bulb tissues, at different growth and development stages, and with different infection status (healthy/OYDV-infected). Preliminary gene screening was carried out using an RT-qPCR assay (SYBR chemical), assessing both Ct values and melting curves. Expression stability of the reference genes in the sample sets was independently determined by three different software packages: geNorm, NormFinder and Bestkeeper. In contrast to Elf, PP2A, Hel-1 and ß-Act, 5.8S rRNA and UBQ proved to be the most stable RGs. An OYDV specific RT-qPCR TaqMan® assay was also developed and validated for relative quantification of OYDV titer. The assay was shown to be specific and sensitive, able to identify virus presence up to 10-6 dilution, representing a rapid and sensitive diagnostic tool for OYDV detection for application in field surveys. Finally, a ΔΔCt method was

	developed, to be applied in future studies describing the molecular interaction between OYDV and onion cv. 'Rossa di Tropea'. This approach was used to provide relative quantification of OYDV titer in samples obtained from different experimental trials.
Response to Reviewers:	Response to Reviewers Dear EJPP, Dr. Margaria and Reviewers: Please to find herein attached the final revision of the manuscript. L61: remove “()”L66: which could then be correlatedL257: “to quantify the level of expression…”… remove relative, as RealTime can provide relative or absolute quantificationL266: space in “RGunder”L278: delete “,” before “The”L295: put “.” Before “this study”L320: space in “thatthey”L324: remove “)”L329: “ the will represent…” or “they will continue to ….”L331: “may impact bulb quality”L335: I imagine the Authors mean “colturally”L337: “a reference gene” We amended the manuscript according to the minor text revisions. I would like to thanks you all again for all the effort to improve this manuscript I hope that now it could be considered suitable to be published Kind regards Antonio Tiberini

ONION YELLOW DWARF VIRUS ΔΔCt-BASED RELATIVE QUANTIFICATION OBTAINED BY USING REAL-TIME POLYMERASE CHAIN REACTION IN 'ROSSA DI TROPEA' ONION.

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ABSTRACT

As part of a plant-pathogen interaction study between *Onion yellow dwarf virus* (OYDV) and onion cultivar Rossa di Tropea, a ΔΔCt-based relative quantification of OYDV was investigated to relate OYDV titer to accumulation of secondary metabolites in onion bulbs. An appropriate reference gene (RG) was required to achieve data normalization. Since no single internal control gene is universally used as an RG, multiple stably expressed reference genes were investigated. In particular, elongation factor (Elf), protein phosphatase 2A (PP2A), helicase (Hel-1), 5.8S rRNA, ubiquitin (UBQ) and ß-Actin (ß-Act) were compared one to another in both leaf and bulb tissues, at different growth and development stages, and with different infection status (healthy/OYDV-infected). Preliminary gene screening was carried out using an RT-qPCR assay (SYBR chemical), assessing both Ct values and melting curves. Expression stability of the reference genes in the sample sets was independently determined by three different software packages: geNorm, NormFinder and Bestkeeper. In contrast to Elf, PP2A, Hel-1 and ß-Act, 5.8S rRNA and UBQ proved to be the most stable RGs. An OYDV specific RT-qPCR TaqMan® assay was also developed and validated for relative quantification of OYDV titer. The assay was shown to be specific and sensitive, able to identify virus presence up to 10^{-6} dilution, representing a rapid and sensitive diagnostic tool for OYDV detection for application in field surveys. Finally, a ΔΔCt method was developed, to be applied in future studies describing the molecular interaction between OYDV and onion cv. 'Rossa di Tropea'. This approach was used to provide relative quantification of OYDV titer in samples obtained from different experimental trials.

Keywords: Plant Virology, Onion bulb, Rossa di Tropea, OYDV, reference gene, ΔΔCt

INTRODUCTION

Onion yellow dwarf virus (OYDV, genus *Potyvirus*, family *Potyviridae*), with a ssRNA(+) genome, is one of the 14 viruses reported to cause infection in onion and other *Allium* spp. worldwide, affecting yield of crops and quality of fresh-market and dry bulbs (Katis *et al.*, 2012). It is the most economically important virus in onion (Van Dijk, 1993). OYDV was first described in 1929 in Iowa, USA (Melhus *et al.,* 1929). It has since been identified in most countries where onions and shallots are cultivated (Bos, 1976) and was first detected in Italy in 1993 (Marani and Bertaccini, 1993). Symptoms can be severe and range from leaf yellowing and dwarfing to stem twisting. Infection with OYDV can also reduce bulb weight and size up to 40%, with seed loss up to 50% (Elnagar *et al*., 2011; Kumar *et al.,* 2012). The virus is naturally spread from plant to plant by several aphid species in a non-persistent manner (Drake *et*

al., 1933; Abd El-Wahab, 2009; Kumar *et al.,* 2011). Seed transmission of OYDV has not been reported, only detection in seeds from infected plants (Abd El-Wahab *et al*., 2009), in literature. OYDV persists in bulb and crop residues (Schwartz and Mohan, 2008; Katis *et al.,* 2012).

In 2005, OYDV was reported for the first time in the Calabria region (Southern Italy) on the speciality onion cultivar 'Rossa di Tropea' (Parrella *et al.,* 2005) – an economically important local crop. This cultivar, a particular pink-red coloured onion type, was awarded the EU Protected Geographical Indication (PGI) trademark, it is well-known worldwide for its mild, sweet flavor and it represents an upcoming "functional food" due to its high content in nutraceuticals (i.e. phenolics, flavonoids, fructoligosaccharides, alk(en)yl cysteine sulphoxides). In particular, the cultivar 'Rossa di Tropea' shows anti-inflammatory, anti-cholesterol, anticancer and antioxidant properties (Grzelak *et al.,* 2009; Benmalek *et al.,* 2013; Shon *et al.,* 2004; Lanzotti, 2006). A previous unpublished study showed an alarming incidence of OYDV at early growing stages maintained up to bulb or seed harvesting time in which an incidence of 95%, or even 100%, could be observed (Manglli, personal communication). OYDV represents one of the most limiting biotic stresses for cv. 'Rossa di Tropea'.

Although effects of viral infection on onion agronomic traits (and crops in general) are well documented, little information is available about viral effects on host cellular metabolism and compounds synthesis. The content of these phyto-chemicals varies depending on cultivar type, environmental and growth conditions, time of harvesting, storage conditions, and, possibly, the phytosanitary status of the crops. To investigate these effects the project "SI.ORTO - *Study on Interaction between Onion Yellow Dwarf Virus and Nutraceutical Compounds of 'Rossa di Tropea' Onion* ~~()~~" was funded by the Italian Ministry of Education, University and Research (MIUR) with the purpose of identifying and evaluating modulation and variation of onion secondary metabolites such as flavonols, flavonoids, anthocyanines and diallylsulfides, through both standard and novel approaches i.e. -*omics* sciences –. In the framework of SI.ORTO, the present study aimed to establish a method to achieve relative quantification of OYDV titer in onion bulbs which could ~~then to be~~then be correlated to absolute quantification of secondary metabolites.

To achieve this quantitative Reverse transcription real time polymerase chain reaction (RT-qPCR) was chosen applying a ΔΔCt method (Livak *et al.,* 2001). Both a housekeeping gene and an OYDV-specific RT-qPCR assays were selected, developed and validated. Further, the developed RT-qPCR - ΔΔCt method was used for preliminary relative quantification of OYDV in samples collected from experimental trials.

MATERIAL AND METHODS

Plant material for Reference Gene selection

Samples were selected by collecting different onion tissues (bulbs and leaves) from both healthy and OYDV-infected cv.'Rossa di Tropea' plants, at various growth stages and conditions according to the biennial onion production cycle. This consists of a first year starting from seedling to fresh bulb production and a second year when bulbs deriving from the first year are transplanted to obtain flower stems and seeds.

Two collecting sites were included for sampling: a commercial field at a farm located in Campora San Giovanni (CS), Calabria, Italy, in the main production area; and an experimental trial of post-bulb plants established at the Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca Difesa e Certificazione (CREA-DC) in Rome, Italy. Bulk samples (3 plants each), consisting of leaves and bulbs from both the first and second cycle, were collected in a period ranging from October 2015 to February 2016 and from March 2016 to September 2016 respectively. In particular, leaf samples were collected from post-bulb plants in October 2015, November 2015 and February 2016, and from pre-bulb plants in March, April and June 2016. Bulb samples were collected at time of harvest

and after storage (June and September 2016). For each sample, OYDV presence was assessed by endpoint RT-PCR (Manglli *et al.,* 2014). The samples were gathered in groups and subgroups in relation to tissue type, growth stage, OYDV presence/absence, collection date and site (See Table 1).

Plant material for Onion yellow dwarf virus relative quantification

OYDV isolates from both *Allium cepa* and *A. sativum* (CREA-DC collection) were used as positive control for molecular assay validation. For OYDV relative quantification analysis, plant material deriving from two different experimental trials. One trial was located in Campora San Giovanni (CS-Italy) in field, the other one took place at the *Mediterranean* University in Reggio Calabria, in pots, both trials were conducted under an insect proof greenhouse. Trials included two conditions (healthy and OYDV-infected), each conducted in triplicate randomized blocks. Three-month-old healthy onion seedlings were transplanted and then, three months later, plants of three out six blocks were mechanically inoculated with OYDV. Experimental inoculation was accomplished through 10 needle punctures along two leaves of each plant; before each puncture, the needle was soaked in sap of OYDV-infected leaves extracted in presence of phosphate buffer (0.1 M; 1:5 w:v). Approximately one-month post-inoculation, all plants were assayed by OYDV serological assay (ELISA), following manufacturer's instruction (BIOREBA, Switzerland). ELISA assays were performed in duplicate, using the healthy and positive controls provided by ELISA kit. Reactions were measured by spectrophotometer Multiscan FC (Thermo Scientific, Carlsbad, CA) at 405 nm, and samples showing an optical density twice that of the healthy control were considered positive. ELISA testing indicated that 80% of inoculated plants were infected with OYDV. Bulbs collected from either healthy or OYDV-infected plants were harvested and sub-grouped in relation to three different time points overlapping the production season. Bulbs were selected at harvesting time, with fresh leaves (T_0); when ready for storage with dried leaves (T_1); and as dry bulbs post-storage (T_2) sampling points corresponding to 2, 3 and 5 months post-inoculation respectivley. In total, for each experimental trial, 18 bulk samples, each consisting of three plants) were obtained: half of them representing healthy plants for each the three time-points, and half of them representing the OYDV-infected plants. All 18 bulk samples were investigated through the ΔΔCt method, as reported in Table 7.

Total RNA and cDNA synthesis

Leaf and bulb tissues were initially ground with liquid nitrogen using mortar and pestle. Total RNA extraction was performed by using Real Kit (Durviz, Valencia, Spain), according to manufacturer's protocol. The concentration and purity of RNA samples were measured by NanoDrop ND1000 spectrophotometer.

As described by Pasquini *et al.* (2008), the cDNA employed for the selection of candidate reference genes (RGs) was synthesized (final volume 30 µl) from approximately 300 ng of total RNA by using 2 µM random hexamers, 6 µM oligo dT, and SuperScript IIITM (Invitrogen, Carlsband, CA, USA) as reverse transcriptase.

From total RNA and cDNA, tenfold dilution series were prepared to assess the primer amplification efficiencies and to determine standard curves for both OYDV and RGs.

Selection of candidate Reference Genes (RGs)

Six genes commonly used in qPCR data normalization – namely, helicase (Hel-1), ubiquitin (UBQ), 5.8S rRNA, elongation factor (Elf), protein phosphatase 2A (PP2A), and ß-Actin (ß-Act) were compared in qPCR expression analysis specifically in onion cv. 'Rossa di Tropea'. These genes had been identified in previous studies where they were commonly used as internal controls (Brunner *et al*., 2004; El Morsi *et al.,* 2015, Obrero *et al*., 2011; Robene *et al*.,

2015). Table 2 shows primer sequences of the six genes included in this study. These primer sets were used in the qPCR assay using SYBR Green I Mix (TaKaRa, Dalian, China) in a 20 µl reaction volume and performed in a 96-well plate on a ABI PRISM 7500 Fast (Applied Biosystems). Reaction mixtures contained 10 µl SYBR Green I Mix, 2 µl of cDNA, ddH_2O, and a final primer concentration of 0.4 µM. The following amplification conditions were applied: an initial denaturation step of 95°C for 30 s; 40 cycles at 95 °C for 5 s; and 60°C for 20 s. The final melt curve was obtained from 65°C to 95°C to verify primer specificity. Each assay included three technical replicates. The general quality assessment of the qPCR results was based on the amplification and melting curve profiles of the samples in relation to assay controls and no template controls as well as the different sample conditions (growth phases, leaf and bulb tissues, healthy/OYDV-infected plants). The specificity of the amplicons was verified by the presence of a single peak in the qPCR melting curve.

For the RGs selected from this primary selection, a standard curve was obtained by plotting mean Ct values of the tenfold dilution series against the logarithm of the pooled cDNA dilution factors. The Ct values and the following equation were used to determine efficiency (E) of each gene with the slope of a linear regression model: $\% E = (10^{[-1/slope]} - 1) \times 100\%$, including linear correlation (R^2). In addition, three different Microsoft Excel-based software packages – namely, geNorm (Vandesompele *et al*., 2002), NormFinder (Andersen *et al.,* 2004), and BestKeeper (Pfaffl *et al.,* 2004) – were used to rank expression stability of reference genes across all experimental sets. These data were either used directly for stability calculations (BestKeeper analysis) or were converted into relative quantities $2^{-\Delta Ct}$ (in which ΔCt = the corresponding Ct value - minimum Ct) and, later, imported into geNorm and NormFinder. The data set submitted to the software was grouped in accordance to both tissue type (bulbs and leaves) and OYDV presence/absence and then, submitted *in toto* to obtain relative and general ranking respectively. According to methods already in use (Petriccione *et al.,* 2015; Rubio-Pina *et al*., 2011; Radonic *et al*., 2004), the most stable reference gene is that with both the lowest expression stability value (SV) (geNorm and NormFinder), and the lowest standard deviation (SD) and coefficient of variance (CV) value. A TaqMan® probe was then synthesized for the selected most stable gene.

Development of an OYDV TaqMan® assay and evaluation of its specificity and analytical sensitivity

Several OYDV nucleotide sequences of two genes (nuclear including gene b (NIb) and coat protein gene (CP)) were retrieved on GenBank and aligned by Clustal X software, including the sequence previously reported in Calabria (Manglli *et al.,* 2014, Acc. N° KF623540.1). The most conserved region was used as the template for TaqMan® probe and primer set design (Table 3) by Primer Express® Software Version 3.0 (Applied Biosystems). The TaqMan® probe and primer set were synthetized by BioFab Research (Rome, Italy). RT-qPCR reactions were performed using the ABI PRISM 7500 Fast (Applied Biosystems). Probe and primer assay conditions were optimized using various primer and probe concentrations (data not shown). Optimized amplifications were carried out in 20-µl reaction volumes containing 10 µl of 2× Mastermix (Applied Biosystems), 1.6 µM of OYDV-F and OYDV-R primers, 0.075 µM of 5′FAM-labeled OYDV TaqMan® probe, and 2 µl of template RNA (0.4–43 ng) extracted from either leaf or bulb tissues of both healthy and OYDV-infected samples. The cycling conditions included: a preliminary RT step at 48 °C for 30 min, and an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation and annealing/elongation for 15 s at 95 °C and 1 min at 60 °C, respectively. Analyses were performed using the ABIPRISM software version v2.2.2. Each sample was run in duplicate. The general quality assessment of the PCR results was based on the amplification curve pattern and melting curve profiles. The amplification efficiency (E) was calculated as reported above for the reference genes. In order to assess the assay specificity, other viruses which naturally infect onion, were included. In particular, *Iris yellow spot virus* - IYSV, *Garlic common latent virus* - GCLV and *Shallot yellow stripe virus* – SYSV (belonging to *Tospovirus*, *Carlavirus* and *Potyvirus* genus, respectively) were used as no template control to assess

possible cross reactions. Analytical sensitivity (sample limit detection - LOD) of RT-qPCR was determined using tenfold serial dilution of infected OYDV leaves and bulbs which, to compare efficacy, were used simultaneously in serological (DAS-ELISA) and molecular (endpoint RT-PCR) methods commonly used in OYDV detection (Manglli *et al.,* 2014). For these assays, the samples were prepared as follows: 0.5 g of plant material (both infected and healthy leaves and bulbs) were ground in 5 ml extraction buffer (1X phosphate buffered saline, 0.1 % Tween 20). The dilution series were obtained by adding 100 µl from infected extract to 900 µl of healthy crude plant extract. From each dilution, 200 µl were directly tested by ELISA and 200 µl were employed for total RNA extraction, using RNase plant mini kit (Qiagen), and, afterwards, total RNA aliquots were assayed by endpoint RT-PCR and RT-qPCR, respectively.

ELISA was performed as described above. Endpoint RT-PCR was performed by using specific OYDV primer sets according to Manglli *et al*. (2014). The TaqMan® RT-qPCR assay was performed in an ABI PRISM 7500 Fast instrument (Applied Biosystems) according to the above-mentioned procedure. Two replicate reactions were run for each sample and their Ct values averaged.

OYDV and Reference Gene amplification efficiency validation

The amplification efficiency in RT-qPCR, for OYDV and the selected reference gene, that should be equal to be suitable for applying in ΔΔCt method, were both determined. As mentioned above, the cDNA was obtained by tenfold serial dilution in water (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution). For each dilution, the ΔCt (Ct target – Ct RG) was calculated and plotted in a dispersion graph against the logarithmic (base 10) value of input cDNA concentrations. The amplification efficiency is to be considered equal or equivalent if the slope value is less than 1.

RESULTS

Selection and validation of candidate Reference Genes

The gene expression experiment carried out with the six genes in 'Rossa di Tropea' onion leaves and bulbs (healthy or OYDV-infected), showed good amplification curves for most of them (Fig. 1 and Table 4). All assays were performed using the same RNA extracts whose quality was spectrophotometrically evaluated. Except for Hel-1 and β-Act (where an amplification signal was not obtained in all of the samples included in the analysis), UBQ, Elf, PP2A and 5.8S rRNA showed satisfactory Ct values (Fig. 1), with Ct mean value ranging from 18.29 (5.8S rRNA) to 28.09 (UBQ) and low Ct corresponding to high levels of expression. Analyzing the RT-qPCR melting curves allowed to observe a single peak for UBQ, Elf, and 5.8S rRNA, confirming the specificity of all amplicons, except for PP2A (Fig. 1). Amplification efficiencies for UBQ, Elf and 5.8S rRNA were calculated from standard curves with satisfactory linear relationships ($R^2 > 0.99$) ranging from 101% to 108% (*Cf.* Table 4). Whereas UBQ and 5.8S rRNA Ct values did not show major discrepancies with their mean value (29.62 and 16.09 respectively), Elf showed a much wider range of Ct values, with minimum and maximum value ranging from 25.71 to 30.94 (mean value = 28.76), and a high standard deviation (SD = 1.97). These data were confirmed by analyzing the plotted Ct mean value vs. each sample subgroup (Fig. 2); as a result, 5.8S rRNA and UBQ showed a more linear trend. All the results concerning RGs stability derive from the independent use of the three above-mentioned softwares and are summarized in Annex 1 (Supplementary material). This analysis included UBQ, Elf and 5.8S rRNA, and excluded Hel-1, ß-Act (no amplification signal) and PP2A (no-specificity); the samples labelled LH, LI, BH and BI were grouped according to tissue type (leaves/bulbs-L/B), OYDV presence/absence (I/H), and a general rank was established in each group.

As far as UBQ is concerned, SV equaled 0.39 and 0.08 with geNorm and NormFinder respectively, whereas with BestKeeper CV was 4.44 and SD 0.84; in the case of 5.8S RNA, SV reached 0.39 and 0.07 with geNorm and NormFinder respectively, while with BestKeeper CV equaled 3.95 and SD 0.40; as for Elf, SV was 0.54 and 0.18 with geNorm and NormFinder respectively, whereas with BestKeeper CV reached 4.89 and SD 1.97.

As shown above, the general ranking top first position of 5.8S rRNA was confirmed in all the sample groups, with the exception of BH (representing the healthy bulbs). When using BestKeeper, though, UBQ and 5.8S rRNA switched their positions. In contrast, Elf showed good SV value in bulb but not in leaf samples; such values were characterized by a general high variability as it can easily be deduced from its high SD values.

As independently confirmed by all the three softwares in use, almost in all groups 5.8S rRNA proved to be the reference gene with the most stable expression level in the pathosystem 'Rossa di Tropea'/OYDV. For this reason, its corresponding TaqMan®® probe was synthesized (BioFab research, Rome, Italy) and labeled Cy3 and BHQ2 as reporter and quencher, respectively.

Development of an OYDV TaqMan® assay and evaluation of its specificity and analytical sensitivity

The OYDV-specific primer and probe set (Table 3) produced optimal amplification curves in all the OYDV-infected samples – including OYDV isolates from *Allium sativum* –, independently of collection time (T_0, T_1 and T_2) and tissue matrix (bulbs and leaves). In leaves and bulbs, the Ct values ranged from 16 to 18 and from 18 to 21 respectively. Having been calculated from the standard curve with satisfactory linear relationships ($R^2 > 0.99$), the OYDV assay amplification efficiency reached 99% both in leaf and bulb samples (Figure 3). No amplification was observed in negative controls (healthy samples and water) and no reaction was observed in the GCLV, SYSV and IYSV-infected samples confirming the specificity of the assay.

Analytical sensitivity results are summarized in Figure 4 and Table 5. In comparison to DAS-ELISA and endpoint RT-PCR, the RT-qPCR assay diagnostic sensitivity showed the best results, allowing detection of OYDV up to 10^{-6} and 10^{-5} for leaves and bulbs, respectively. In endpoint RT-PCR, LOD was up to 10^{-4} (leaves) and 10^{-3} (bulbs); whereas ELISA assessed the presence of the virus up to 10^{-2} dilution in both tissues.

OYDV and Reference Gene amplification efficiency evaluation

The primer amplification efficiency was determined using tenfold serial dilutions of cDNA by qPCR with OYDV and 5.8S rRNA primer and probe sets. The plotted ΔCt (Ct OYDV - Ct RG) values for each dilution series were used to obtain the graph and slope value, as shown in Figure 5 and further summarized in Table 6.

Since the slope value in the graph was less than 0.1 (-0.0157), OYDV primers and probe and the endogenous internal control 5.8S rRNA primers and probe were considered to have the same priming efficiency, therefore, they resulted to be suitable for ΔΔCt-based OYDV relative quantification.

OYDV relative quantification in preliminary analysis

OYDV titer in the bulk samples (each consisting of three bulbs and deriving from the two experimental trials) were quantified applying the ΔΔ Ct method. The virus titers in each sample, expressed as fold differences and calculated based on T_0 time-point mean ΔCt value, are summarized in Table 7. The T_1 and T_2 collection time-point data were normalized to T_0. All the samples showed amplification with an average Ct value around 15 for the reference gene (5.8S rRNA). No Ct and amplification reactions were obtained for all the samples of non- OYDV inoculated plants (healthy bulbs) at both experimental sites. The comparison between the OYDV relative quantification (expressed in ΔΔCt value ranging from 0.3 to 1.5) in inoculated T_1/ T_2 vs T_0 samples did not highlight variability in virus replication

from harvesting time to three-months after storage. By comparing virus titer at T_1 and T_2 with those at T_0, a significant modulation (decrease or accumulation) could not be observed.

DISCUSSION AND CONCLUSION

Selection and validation of candidate Reference Genes

Quantitative real-time PCR (qPCR) is a powerful technique to quantify ~~relative~~ the level of expression of target genes or sequences. It has become one of the main diagnostic tools in molecular plant pathology and, in particular, in gene expression studies in order to understand the biological process involved in host-pathogen interactions. Moreover, qPCR has become one of the most used diagnostic techniques for plant virus identification, showing robust relative quantification values and data. As suggested by the "Minimum Information for publication of Quantitative real-time PCR Experiments" (MIQE) guidelines (Bustin *et al*., 2009), in qPCR assay the requirement of a normalization method against reference genes (RGs) is important for reliable, validated results. Ideal RGs should be stably expressed in all cells and tissues and remain stable under different experimental conditions (Robledo *et al*., 2014; Huggett *et al*., 2005). Many RGs have been determined in plant cells and across different plant species, growth stages, and biotic and abiotic stresses (Lilly *et al.,* 2011). However, no gene can be said to be used as a universal RG under different experimental conditions. Even though a few studies had already been carried out to assess RGs in virus-plant pathosystems, no OYDV RT-qPCR specific assay had been developed. This study assessed six RGs for their use as internal controls in gene expression analysis and relative quantification assay of *Allium cepa* cv.'Rossa di Tropea' responses to OYDV infection. A specific OYDV RT-qPCR assay was developed and compared with the suitable selected RG so that it could be employed for ΔΔCt-based relative quantification of OYDV. Three different statistical algorithms were used to identify the best RGs; then, geNorm, NormFinder, and BestKeeper independently provided a final ranking. As confirmed in many other studies (Mafra *et al.,* 2012; Zhu *et al*., 2013; Guo *et al.,* 2014), combining these results in order to select and validate the best RGs can generate substantial discrepancies in the final ranking due to the different mathematical models associated with each algorithm. Nonetheless, the final ranking appearing in this study showed identical results. Preliminary selection reduced the number of candidate RGs from six to three. Candidates PP2A (aspecific amplicon), ß-Act (no amplification reaction) and Hel-1 (low quality in amplification curves pattern and amplification missing in a few samples) were discarded, selecting 5.8S rRNA, UBQ and Elf for further analysis~~.~~, The analysis of the Ct values of the first two genes, in opposition to that of Elf, showed a similar and a more constant trend (Figure 2). 5.8S rRNA appeared to be the RG with the lowest mean Ct value. In order to better evaluate the candidate RGs, the data-set submitted to geNorm, NormFinder, BestKeeper were grouped in accordance to tissue type (Leaves-L or Bulbs-B) and OYDV absence/presence (H/I). The final ranking appeared to be almost identical among each data-set, independently of tissue type and/or OYDV presence, and indicated 5.8S rRNA as the most suitable candidate RG. The only exception was represented by the BH group when analyzed by BestKeeper, in which UBQ was ranked highest. In spite of very slight differences occurring between the results related to these two genes, in the BI group all the three software packages indicated that the stability value (SV) was higher in Elf than in UBQ. As for the SV, both 5.8S rRNA and UBQ could be used as RGs for RT-qPCR normalization in the cv.'Rossa di Tropea' OYDV pathosystem. Confirming the results previously obtained in other onion cultivars (Robene *et al.,* 2015) and in plant-virus interactions (Mascia *et al.,* 2010; Moreno *et al.,* 2011), these values show that 5.8S rRNA and UBQ are stable RGs in the onion cv. 'Rossa di Tropea'. By contrast, Elf gene should only be used as RG in bulb samples (Figure 2). As far as the other genes are concerned, PP2A has been reported to be suitable for normalization in *Cassava brown streak virus* (CBSV)-infected tissues (Jarošová and Kundu, 2010), ß-Actin was used as an RG in the *Iris yellow spot virus* (IYSV)-onion

pathosystem (El Morsi *et al.,* 2015); and Hel-1 was selected as an RG in *Cucurbita pepo* (Obrero *et al.,* 2011). In preliminary studies conducted on commercially available onion bulbs (red, white and yellow varieties) these RGs showed non-homogenous amplification patterns (data not shown), thus implying a dissatisfactory performance.~~,~~ T~~t~~his study on the OYDV-'Rossa di Tropea' pathosystem does not consider them as suitable RGs.

Development of an OYDV TaqMan® assay and evaluation of its specificity and analytical sensitivity

Diagnosis of OYDV in onion is mainly performed by serological (ELISA) testing or conventional endpoint RT-PCR. Within the SI.ORTO project neither of these methods was suitable because OYDV relative quantification was required to correlate virus titer to absolute quantification of secondary metabolites of nutraceutical interest. Thus, specific real-time PCR primers and probe were designed and assessed. Results indicate that this OYDV protocol was specific, allowing the virus diagnosis in all the infected samples included in the study, without nonspecific host reactions (healthy samples) or cross reactions (no target viruses). Moreover, the method had a good analytical sensitivity in comparison to the commonly used RT-qPCR assays designed for plant viruses (Liu *et al*., 2013; Dai *et al*., 2013; Harper *et al*., 2011). As expected, OYDV RT-qPCR gave enhanced sensitivity over DAS-ELISA and endpoint RT-PCR. Analytical sensitivity data highlighted that the molecular assays showed a tenfold less efficiency (Table 5) to detect OYDV in bulbs, in comparison to leaf samples. Despite all, the assay efficiencies in leaves and bulbs showed R^2 values higher than 0.99 (0.99617 and 0.9953 for leaves and bulbs respectively), thus demonstrating that it could be reliably used in both tissue types. In addition, the enhanced sensitivity indicates the ability to detect the virus in emerging plantlets with a low virus titer allow earlier detection of infection which could facilitate the early removal of inoculum sources.

OYDV 5.8S rRNA-based ΔΔCt Method

The use of a comparative Ct (ΔΔCt) method (Livak *et al.,* 2001) for relative quantification of OYDV viral RNA requires: *i)* selection of an RG; *ii*) selection of OYDV RT-qPCR assay; and *iii*) OYDV and RG primer efficiency validation. As reported here, the first two requisites were successfully demonstrated. By plotting ΔCt (cycle threshold [Ct] of OYDV – Ct of 5.8S rRNA) against the log concentration of cDNA, a slope absolute value of 0.015 was obtained. It could, therefore, be assumed that OYDV primers have the same priming efficiency as the 5.8S rRNA assay and that they were suitable for ΔΔ Ct-based quantification of OYDV. The inclusion of a three time-point strategy (T_0, T_1, and T_2 corresponding to cv. 'Rossa di Tropea' onion production phases) allowed further investigation of the effect of OYDV infection on cv. 'Rossa di Tropea' onion bulbs. The bulbs included in the analysis derived from two different experimental trials (field and pots), from two geographic areas. Surprisingly, the results in Table 7 showed that there is no significant correlation of time after mechanical inoculation~~)~~ to OYDV relative quantification. Each of the three set of samples showed a different modulation of virus titer at the three reference time-points. Nevertheless, these results highlight how quickly OYDV is translocated in the bulb, which represents an elective site for virus replication after leaf. The stable presence of OYDV in bulb tissue during all production phases, from harvesting to storage, could indicate a potential phytosanitary risk. While bulb tissues enter dormancy, OYDV is still active and in replicative state, maintaining constant titer. If infected bulbs are used for 2nd year onion production (seed production), they will continue to ~~contain~~ represent~~ing~~ a ready source of inoculum to other onion crops. Additionally, a constant OYDV virus titer in bulb tissues may impact ~~upon~~ bulb quality, which may have implications on modulation of primary and secondary metabolites accumulation in bulbs.

For the first time in literature, this study describes an assay based on RT-qPCR methodology for detection and quantification of OYDV – the most economically important and limiting biotic stress in onion and, more specifically, in

~~the~~ a co~~u~~lturally important cultivar 'Rossa di Tropea'. The results presented here give important clues about OYDV translocation pattern in bulbs at the end of the 1st year growing cycle and describe an intensive viral activity during storage stages. The ΔΔCt method developed by using 5.8S rRNA as a~~n~~ reference gene for OYDV quantification represents a fundamental tool to better investigate the 'Rossa di Tropea'/OYDV interaction in future studies.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical statement. This research did not involve any animal and/or human participant. The authors declare that they have no conflict of interest.

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Figure Legend

Figure 1. Amplification and melting curves obtained for elongation factor (Elf), protein phosphatase 2A (PP2A), helicase (Hel-1), 5.8S rRNA, ubiquitin (UBQ) and ß-Actin (ß-Act) reference genes in the preliminary selection in a RT-qPCR assay using SYBR green chemistry.

Figure 2. Trends for three out of six reference genes (RG), elongation factor (Elf), 5.8S rRNA and ubiquitin (UBQ), overcoming the preliminary selection on the base of amplification and melting curves pattern (Figure 1). For each RG is reported the cycle threshold (Ct) mean value in each sample included in the analysis.

Figure 3. OYDV assay efficiency. OYDV TaqMan® assay standard curves in leaves and bulbs samples obtained from tenfold dilution series. The curve formula in the graph reports slope and R^2 values.

Figure 4. Endpoint RT-PCR and RT-qPCR analytical sensitivity comparison in leave and bulb samples, panel a) and b) respectively. For each assay is reported the amplification curve obtained by RT-qPCR [upper side of panel a) and b)] and pictures of agarose gel (1.5%) of amplicons (bottom side of panel) obtained from the tenfold dilution series of *Onion yellow dwarf virus* infected leaves (a) and bulbs (b).

Figure 5. Primer efficiency validation using tenfold serial dilutions of cDNA of *Onion yellow dwarf virus* (OYDV) and 5.8S rRNA assays The cycle threshold (Ct) (Ct of OYDV – Ct of 5.8S rRNA) was plotted against the log concentration of cDNA.

Table 1. Samples included in this study to evaluate candidate reference gene (RG). Samples were gathered in subgroups according to tissue type (leaves-L/bulb-B), growth phase (pre-bulb/post bulb-plants), collection time (harvesting/after storage) *Onion yellow dwarf virus* (OYDV) presence/absence (infected-I/healthy-H) and site. Each subgroup includes 3 plants each.

Tissue type, growth/collection phases	Healthy/ Infected	Collection Date	Location	Subgroup code	Group code
leaves from post-bulb plants	H	Oct 2015	CREA-DC	LH Oct 15	LH
leaves from post-bulb plants	H	Nov 2015	CREA-DC	LH Nov 15	LH
leaves from post-bulb plants	H	Feb 2016	CREA-DC	LH Feb 16	LH
leaves from post-bulb plants	I	Oct 2015	CREA-DC	LI Oct 15	LI
leaves from post-bulb plants	I	Nov 2015	CREA-DC	LI Nov 15	LI
leaves from post-bulb plants	I	Feb 2016	CREA-DC	LI Feb 16	LI
leaves from pre-bulb plants	H	Mar 2016	Campora	LH Mar 16	LH
leaves from pre-bulb plants	H	Apr 2016	Campora	LH Apr 16	LH
leaves from pre-bulb plants	H	Jun 2016	Campora	LH Jun 16	LH
leaves from pre-bulb plants	I	Mar 2016	Campora	LI Mar 16	LI
leaves from pre-bulb plants	I	Apr 2016	Campora	LI Apr 16	LI
leaves from pre-bulb plants	I	Jun 2016	Campora	LI Jun 16	LI
bulb tissues - harvesting time	H	Jun 2016	Campora	BH Jun 16	BH
bulb tissues - after storage	H	Sep 2016	Campora	BH Sep 16	BH
bulb tissues - harvesting time	I	June 2016	Campora	BI Jun 16	BI
bulb tissues - after storage	I	Sep 2016	Campora	BI Sep 16	BI

Table 2. Primer pairs for RT-qPCR amplification of candidate reference genes used in this study.

Gene	Forward	Reverse	Reference
Helicase	GCGGGCACTTGGAGATTATC	ACACTGGTCCCTCCCACACA	Obrero *et al.* 2011
Ubiquitin	GTTGATTTTTGCTGGGAAAGC	GATCTTGGCCTTCACGTTGT	Brunner *et al*., 2004
5.8S rRNA	GCGAAATGCGACACTTGGTGTGA	GCGCAACTTGCATTCAAAGA	Robene *et al.* 2015
Elongation factor	ACTGTGCAGTAGTACTTGGTG	AAGCTAGGAGGTATTGACAAG	Obrero *et al.* 2011
Protein phosphatase 2A	GAGCCCAGAACAGGAGCTAACA	CCACATTACCTGTATCGGATGACA	Obrero *et al.* 2011
ß-Actin	CTCGCCTTTGCCGATCC	GATCTTCATGAGGTAGTCAGTC	ElMorsi *et al.* 2015

Table 3. Primers and probe set for RT-qPCR designed for *Onion yellow dwarf virus*.

Name	Position	Sequence	nt
OYDV qRTFW	2216	CACGTTACGCATTCGACTTTTATG	24
OYDV qRTRev	2298	TGCCGCCTTCATCTGCAT	18
OYDV qRTProbe	2252	5' FAM -AAACCCCAACAAGGGCTAAGGAGGCAC - 3' BHQ-1	27

Table 4. Amplification efficiencies (E) for ubiquitin (UBQ), elongation factor (Elf) and 5.8S rRNA reference genes overcoming primary selection; cycle threshold (Ct) values (minimum, maximum and mean), standard deviation (SD), and linear regression (R^2) are reported.

Gene	Ct min	Ct max	Ct mean -SD	R^2	Efficiency (E) %
Elf	25.71	30.94	28.052 - 1.84	0.999	106.2%
UBQ	28.42	30.652	29.48 - 0.70	0.999	101.2%
5.8S rRNA	15.49	16.67	16.09 - 0.33	0.997	108.8%

Table 5. Analytical sensitivity of ELISA, RT-PCR and RT-qPCR assays using ten-fold dilution series of *Onion yellow dwarf virus* infected leaves and bulbs.

	Dilution series										
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Assay on leaves											
ELISA	+	+	+	-	-	-	-	-	-	-	-
Endpoint RT-PCR	+	+	+	+	+	-	-	-	-	-	-
RT-qPCR	+	+	+	+	+	+	+	-	-	-	-
Assay on bulbs											
ELISA	+	+	+	-	-	-	-	-	-	-	-
Endpoint RT-PCR	+	+	+	+	-	-	-	-	-	-	-
RT-qPCR	+	+	+	+	+	+	-	-	-	-	-

+ = positive result; - = negative results

Table 6. Cycle threshold (Ct) results for the tenfold dilutions series, reported in logarithmic values. Each dilution was assessed in triplicate and mean value is reported including standard deviation (SD). ΔCt value was obtained comparing *Onion yellow dwarf virus* (OYDV) and 5.8S rRNA Ct values to validate primer efficiency.

	1	0.1	0.01	0.001	0.0001	0.00001
OYDV	16.149	19.218	21.967	24.976	28.289	31.212
	16.176	19.152	22.016	25.269	28.173	31.439
	16.112	19.170	22.208	25.203	28.274	31.389
Mean	**16.146**	**19.180**	**22.064**	**25.149**	**28.245**	**31.346**
SD	0.026	0.028	0.104	0.125	0.052	0.097
5.8S rRNA	15.494	18.462	21.405	24.659	27.577	30.719
	15.587	18.574	21.438	24.544	27.497	30.575
	15.582	18.537	21.560	24.592	27.683	30.621
Mean	**15.554**	**18.524**	**21.467**	**24.598**	**27.586**	**30.638**
SD	0.043	0.047	0.067	0.047	0.076	0.060
	0	-1	-2	-3	-4	-5
ΔCt	**0.591**	**0.656**	**0.596**	**0.551**	**0.660**	**0.708**

SD.	0.001	0.001	0.008	0.009	0.004	0.007

Table 7. *Onion yellow dwarf virus* **(**OYDV) quantification in bulk samples derived from SI.ORTO project experimental trials n field (F) or pots (P). Each bulk (representing three samples each) is assayed in triplicate. Samples of T_1 and T_2 time points were quantified as fold difference in relation to average ΔCt (3.605) obtained at T_0 time point samples.

Bulk ID	**Time point**	**Trails Field (F) Pots (P)**	**Target avarage Ct**		**△Ct**	**$2^{-(\triangle\triangle Ct)}$**
			OYDV	**5.8S rRNA**		
2	T_1	P1	20.28	16.362	3.918	0.825019429
3	T_2	P1	20.478	15.349	5.129	0.356382895
11	T_1	P2	20.13	15.221	4.909	0.415091127
12	T_2	P2	23.45	15.561	7.889	0.052610698
14	T_1	P3	19.047	16.017	3.03	1.526788262
15	T_2	P3	19.576	15.32	4.256	0.652703644
23	T_1	F4	19.854	15.525	4.329	0.62049866
24	T_2	F4	19.027	15.772	3.255	1.306312442
29	T_1	F5	19.308	15.564	3.744	0.930772183
30	T_2	F5	19.08	15.372	3.708	0.954290225
35	T_1	F6	18.665	15.611	3.054	1.501599422
36	T_2	F6	20.053	15.545	4.508	0.548095806

Figure 1

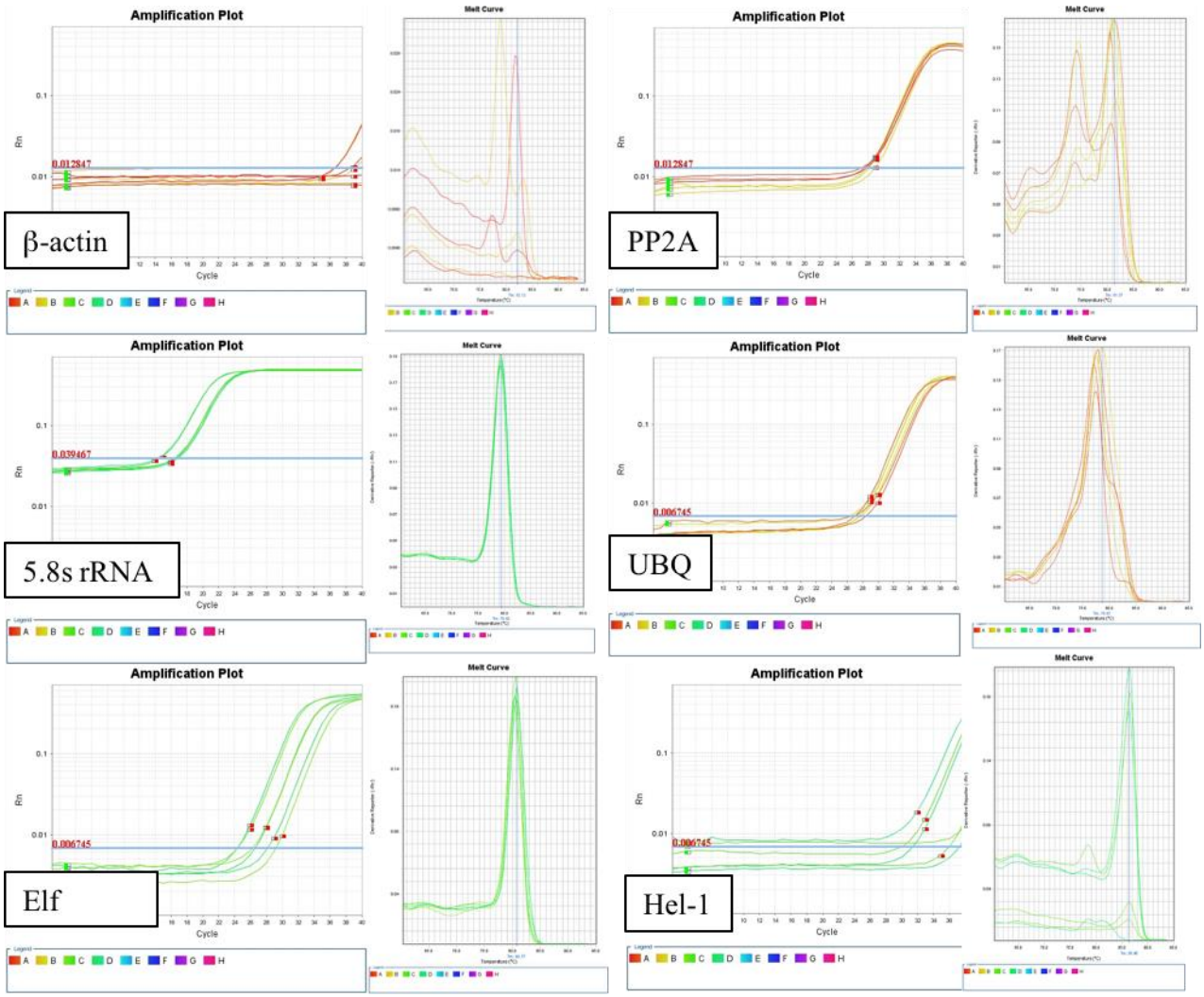
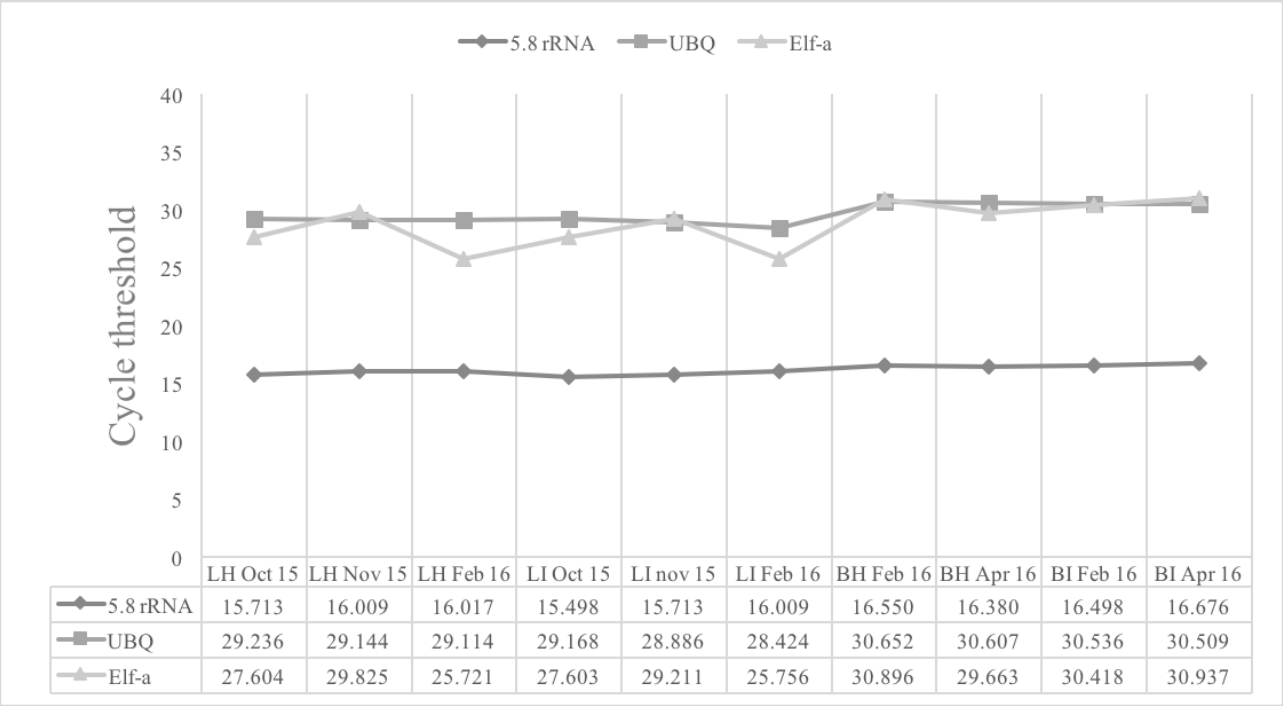


Figure 2



	LH Oct 15	LH Nov 15	LH Feb 16	LI Oct 15	LI nov 15	LI Feb 16	BH Feb 16	BH Apr 16	BI Feb 16	BI Apr 16
5.8 rRNA	15.713	16.009	16.017	15.498	15.713	16.009	16.550	16.380	16.498	16.676
UBQ	29.236	29.144	29.114	29.168	28.886	28.424	30.652	30.607	30.536	30.509
Elf-a	27.604	29.825	25.721	27.603	29.211	25.756	30.896	29.663	30.418	30.937

Figure 3

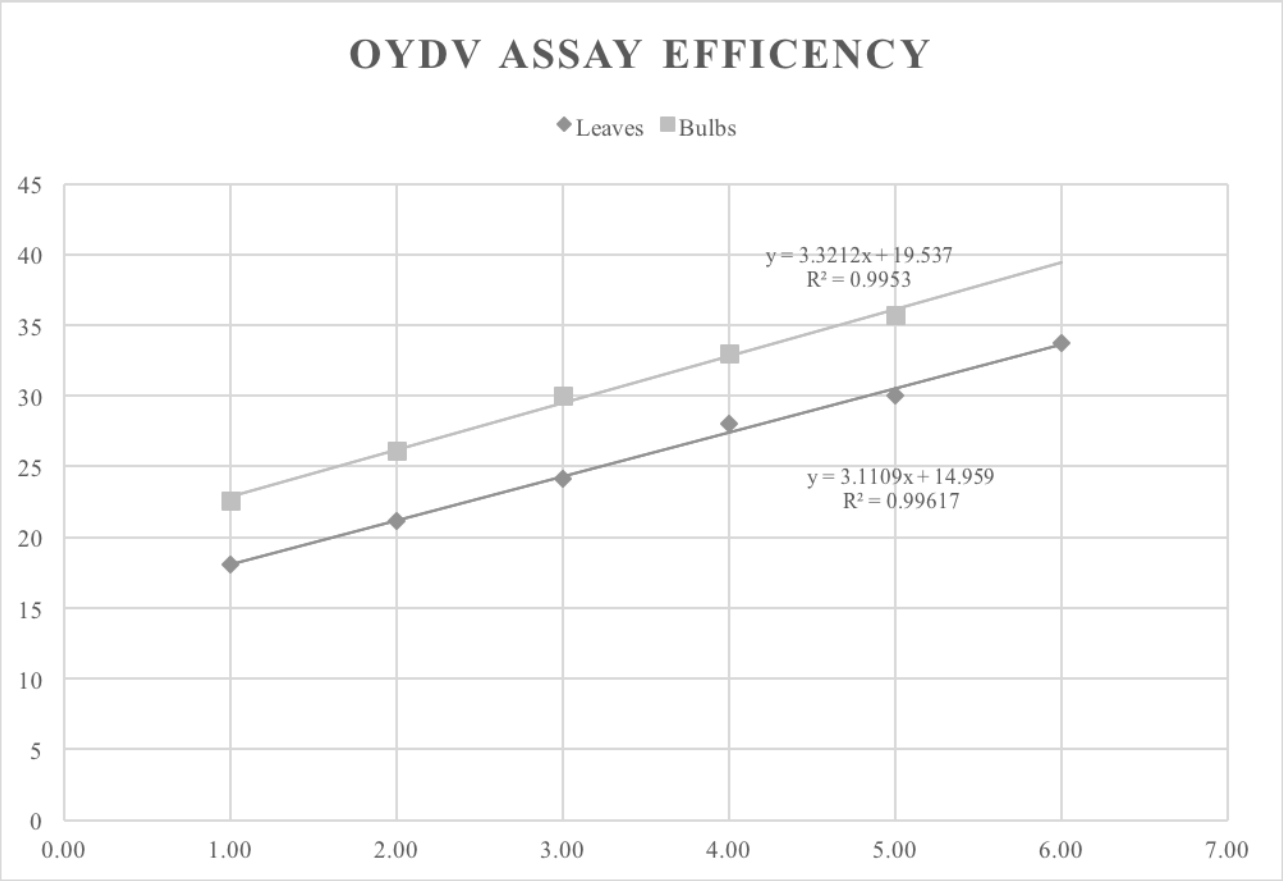

OYDV ASSAY EFFICENCY
Leaves
Bulbs
y = 3.3212x + 19.537
R² = 0.9953
y = 3.1109x + 14.959
R² = 0.99617
45
40
35
30
25
20
15
10
5
0
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00

Figure 4

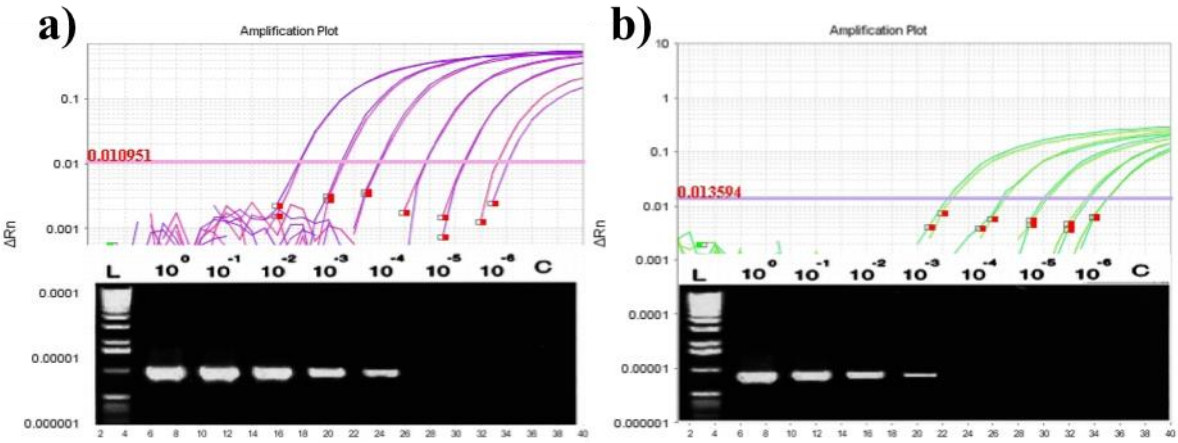
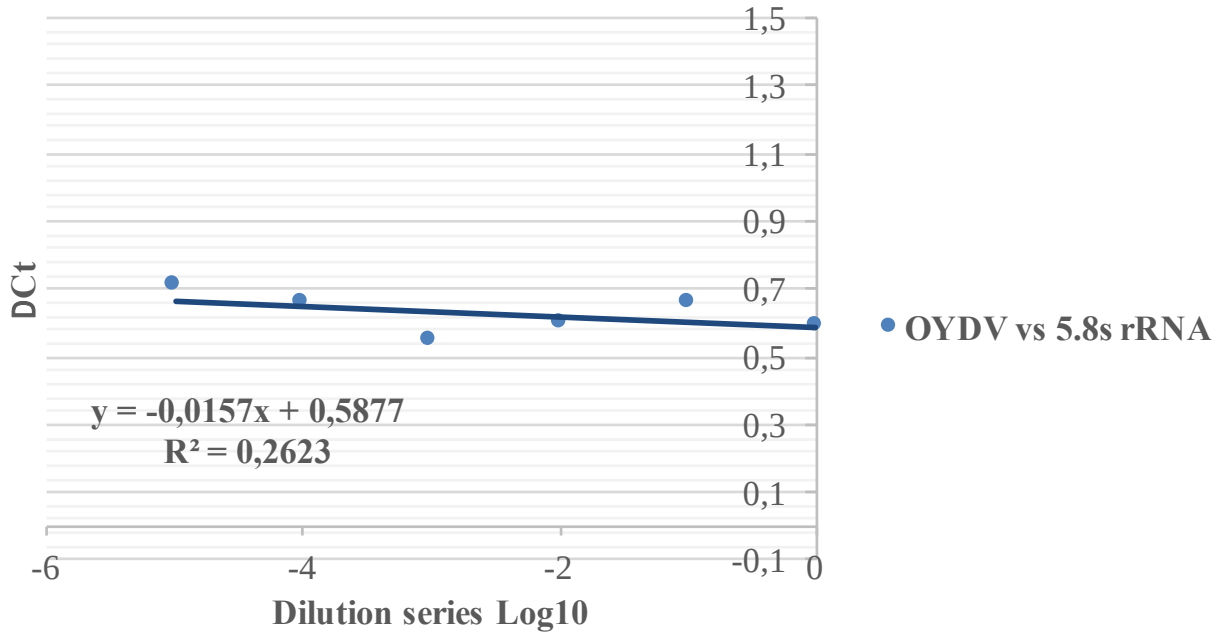

a)
Amplification Plot
0.010951
ΔRn
L
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
C
b)
Amplification Plot
0.013594
ΔRn
L
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
C

Figure 5


1,5
1,3
1,1
0,9
0,7
0,5
0,3
0,1
-0,1
DCt
y = -0,0157x + 0,5877
R² = 0,2623
-6
-4
-2
0
Dilution series Log10
OYDV vs 5.8s rRNA

Annex 1. Expression stability (SV), standard deviation (SD) and coefficient of variance (CV) values of ubiquitin (UBQ), elongation factor (Elf) and 5.8S rRNA reference genes obtained using independently the three softwares (geNorm, NormFinder and BestKeeper) ranked for each group: healthy leaves (LH), *Onion yellow dwarf virus* (OYDV)-infected leaves (LI), healthy bulbs (BH), OYDV-infected bulbs (BI) and all the samples (General Rank).

Rank group LH	GeNorm		NormFinder		BestKeeper		
	Gene	SV	Gene	SV	Gene	SD	CV
1	**5.8S rRNA**	**0.40**	**5.8S rRNA**	**0.09**	**5.8S rRNA**	**0.17**	**4.63**
2	UBQ	0.40	UBQ	0.11	UBQ	0.07	4.73
3	Elf	0.68	Elf	0.21	Elf	2.05	5.16
Rank Group LI							
1	**5.8S rRNA**	**0.40**	**5.8S rRNA**	**0.08**	**5.8S rRNA**	**0.25**	**4.71**
2	UBQ	0.40	UBQ	0.09	UBQ	0.37	4.81
3	Elf	0.69	Elf	0.27	Elf	1.72	4.57
Rank Group BH							
1	**5.8S rRNA**	**0.38**	**5.8S rRNA**	**0.05**	**UBQ**	**0.03**	**3.23**
2	UBQ	0.38	UBQ	0.06	5.8S rRNA	0.12	3.24
3	Elf	0.40	Elf	0.12	Elf	0.87	3.56
Rank Group BI							
1	**5.8S rRNA**	**0.38**	**5.8S rRNA**	**0.05**	**5.8S rRNA**	**0.09**	**3.35**
2	Elf	0.40	Elf	0.06	UBQ	0.12	3.52
3	UBQ	0.40	UBQ	0.06	Elf	0.37	3.63
General Rank							
1	**5.8S rRNA**	**0.39**	**5.8S rRNA**	**0.07**	**5.8S rRNA**	**0.40**	**3.95**
2	UBQ	0.39	UBQ	0.08	UBQ	0.84	4.44
3	Elf	0.54	Elf	0.18	Elf	1.97	4.89