A COMPARATIVE STUDY OF VIRAL INFECTIVITY, ACCUMULATION AND SYMPTOMS INDUCED BY BROAD BEAN WILT VIRUS 1 ISOLATES

C. Carpino[1,2], L. Elvira-González[1,3], L. Rubio[1], E. Peri[2], S. Davino[2] and L. Galipienso[1,3].

[1] *Instituto Valenciano de Investigaciones Agrarias (IVIA), 46113 Moncada, Valencia, Spain*

[2] *Department of Agricultural, Food and Forest Sciences, University of Palermo, 90128 Palermo, Italy*

[3] *Departamento de Biotecnología, Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural, Universitat Politècnica de València (UPV), 46022 Valencia, Spain*

Corresponding author: L. Galipienso

e-mail address: galipienso_lui@gva.es

Fax number: (+34) 96 342 40 01

Running title**:** Broad bean wilt virus 1 biological characterization

SUMMARY

Broad bean wilt virus 1 (BBWV-1, genus *Fabavirus*, family *Secoviridae*) is a bipartite positive-sense single-stranded RNA virus distributed worldwide infecting many herbaceous species. Until now, scarce information regarding biological properties of BBWV-1 isolates is available. This work shows a comparative study on virus infectivity (proportion of infected plants over inoculated plants), virus accumulation and symptoms induced by four genetically different BBWV-1 isolates (Ben, B41/99, NSRV and PV0548) which were mechanically inoculated on several herbaceous hosts. The four BBWV-1 isolates infected broad bean, tomato, pepper and *Nicotiana benthamiana* plants, whereas none of them infected cucumber, common bean and melon. Infectivity ranged from 40 to 60% in tomato and from 75% to 95% in pepper, whereas it was 100% in broad bean and *N. benthamiana* for the four BBWV-1 isolates. Symptoms showed differences depending on the host plant, the viral isolate and the infection time. Virus accumulation was determined in broad bean and pepper plants and showed differences among host species and BBWV-1 isolates. No association between plant symptom manifestation and viral titre was observed.

Keywords: BBWV-1, *Secoviridae, Fabavirus*, infectivity, symptomatology, RT-qPCR.

INTRODUCTION

Broad bean wilt virus 1 (BBWV-1) belongs to the genus *Fabavirus* of the family *Secoviridae*, which also contains the genera *Cheravirus*, *Sadwavirus*, *Sequivirus*, *Torradovirus*, *Waikavirus*, *Comovirus* and *Nepovirus* (10th report of the International Committee on Taxonomy of Viruses, ICTV, https://talk.ictvonline.org/, Thompson *et al.*, 2017). The genus *Fabavirus* contains seven virus species: BBWV-1, *Broad bean wilt virus 2* (BBWV-2), *Lamium mosaic virus*, *Gentian mosaic virus, Grapevine fabavirus*, *Prunus virus F*, and *Cucurbit mild mosaic virus*.

The taxonomy of genus *Fabavirus* has been confusing for years since viral isolates of the same virus species were called with different names depending on the host species or the country where they were detected. Indeed, BBWV was first described in infected broad bean (*Vicia faba*) plants in Australia (Stubbs, 1947) but later on it was identified in other host species in other countries being classified as different virus species such as parsley virus 3 (PVS3) (Frowd and Tomlinson, 1970), nasturtium ringspot virus (NRSV) (Boccardo and Conti, 1973) and petunia ringspot virus (PeRSV) (Rubio-Huertos, 1968; Frowd and Tomlinson, 1972; Sahambi *et al*., 1973; Doel, 1975). Further studies demonstrated that all these viruses were different viral isolates of BBWV (Frowd and Tomlinson, 1970; Sahambi *et al*., 1973; Doel, 1975). Serological studies and nucleotide sequence analysis showed that BBWV isolates could be separated in two different species, BBWV-1 and BBWV-2 (Uyemoto and Provvidenti, 1974; Kobayashi *et al*., 1999; Kobayashi *et al*., 2003).

The BBWV-1 genome is composed of two positive single-stranded RNAs molecules which are separately encapsidated into virions with icosahedral morphology. These genomic RNAs encode polyproteins which are processed by proteolytic cleavage into the final functional peptides. RNA1 (~ 5.8 kb) encodes proteins involved in viral replication and polyprotein maturation: a protease cofactor (PRO-CO), a helicase

(HEL), a viral genome-linked protein (VPg), a protease (PRO) and an RNA-dependent RNA polymerase (POL). RNA2 (~ 3.4 kb) encodes for proteins necessary for virion formation and movement: a movement protein (MP), a large coat protein (LCP) and a small coat protein (SCP) (Sanfaçon, 2015).

BBWV-1 is transmitted in a non-persistent manner by more than 20 aphid species and infects important horticultural and ornamental crops worldwide. Information about the biological properties of BBWV-1 isolates, such as host range or induced plant symptoms, is very scarce and unclear. BBWV-1 was reported infecting pepper (*Capsicum annuum*) (Rubio *et al*., 2002; Svoboda and Leisova-Svobodova, 2013), *Vicia faba* (Ferriol *et al.,* 2014) and weed species (Mehle *et al*., 2008). However, economically important horticultural crops such as tomato (*Solanum lycopersicum*), melon (*Cucumis melo*), and cucumber (*Cucumis sativus*) are reported to be BBWV hosts, but in these cases, the studies did not differentiate between BBWV-1 and BBWV-2 (Lisa *et al*., 1986; Edwardson and Christie, 1991; Blancard, 2012; Moury and Verdin, 2012). Nowadays, specific and sensitive BBWV-1 detection methods are available and the genetic variability of the virus has been studied (Ferrer *et al*., 2008; Ferriol *et al*., 2011; Ferriol *et al.*, 2014; Panno *et al*., 2014; Ferriol *et al*., 2015). A high level of genetic diversity was reported among BBWV-1 isolates from different hosts and geographic origins which clustered in seven main groups (Ferriol *et al*., 2014). However, the relationship between genetic and biological variation has not been studied yet. For this purpose, four BBWV-1 isolates (Ben, PV0548, B41/99 and NSRV) from different phylogenetic clusters were evaluated for symptoms, infectivity and viral accumulation in several herbaceous hosts.

MATERIALS AND METHODS

BBWV-1 isolates and plant material. BBWV-1 isolates Ben, PV0548, B41/99 and NSRV (Table 1) were studied to analyse the differences in the infectivity and in the induced symptomatology. These isolates were selected since they were obtained from different plant species collected in distinct geographical regions and they clustered in different phylogenetic clades (Ferriol *et al*., 2014): isolate Ben from Spain, and isolate PV0548 from Syria, representatives of Clade I, were included to study the biological and accumulation differences between viral isolates belonging to the same phylogenetic clade but collected in different countries. For the other phylogenetic clades, we have included isolates NSRV (Clade III) and B41/99 (Clade IV) from Italy and Bulgaria, respectively, since they were available in our laboratory.

BBWV-1 isolates Ben, PV0548, B41/99 and NSRV, were multiplied and maintained in broad bean plants. To exclude possible mixed infections with other viruses, BBWV-1-infected broad bean plants were analysed for the most common viruses infecting broad bean. The absence of bean common mosaic virus, bean common necrosis virus, bean yellow mosaic virus and broad bean V virus infection was tested by RT-PCR with degenerate generic primers of potyviruses (Hu *et al*., 2010). The absence of alfalfa mosaic virus, cucumber mosaic virus and tomato spotted wilt virus was assessed by DAS-ELISA using specific antibodies (Loewe, Germany). The absence of BBWV-2 was evaluated by molecular hybridization with a specific digoxigenin-labelled riboprobe (Ferriol *et al*. 2015).

In order to determine host range, virus infectivity, induced symptomatology and virus accumulation, each BBWV-1 isolate was mechanically inoculated in ten plants of the following herbaceous species: i) BBWV-1 natural hosts: broad bean var. 'Reina Mora' (Fito) and pepper var. 'Manolo' (Fito) and ii) BBWV-1 experimental hosts: *Nicotiana benthamiana*, tomato var. 'Marmande' (Fito), common bean (*Phaseolus*

vulgaris) var. 'Helda' (Mascarell), cucumber var. 'Granada' (Fito), and melon var. 'Pinonet' (Mascarell). All plants were inoculated at two true leaf stage and the experiment was repeated twice. Mechanical inoculation was performed by homogenization of 1 g of BBWV-1- infected broad bean plant tissues in the inoculation buffer (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, pH 7.2). The homogenized extracts were rub-inoculated using carborundum. Prior to inoculation, virus inoculum concentration was estimated by quantitative RT-PCR (RT-qPCR) and adjusted to 3 x 10^7 BBWV-1 RNA1 copies per ng of total RNA. As a negative control, plants were mock inoculated (only with inoculation buffer). Plants were maintained in a growth chamber under conditions of 16 h of light at 24°C and 8 h of darkness at 20°C. Symptomatology of BBWV-1 infected plants was recorded at 16, 32 and 40 dpi.

RNA extraction, molecular hybridization and real-time RT-PCR. For molecular hybridization assays and virus quantitation, 0.1 g of apical non-inoculated leaves were ground in a power homogenizer TissueLyser (Qiagen, Germany) with liquid nitrogen. Total RNA was extracted using a phenol:chloroform:isoamyl alcohol standard protocol followed by ethanol precipitation as previously described (Ferriol *et al*., 2011) and resuspended in 40 µl of RNase free water. The concentration of total RNAs was measured with UV–vis Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and adjusted to 10 ng/µl.

Virus infectivity (proportion of infected plants over inoculated plants) was assessed at 16 days post-inoculation (dpi) by molecular hybridisation with an antisense 332 bp riboprobe designed in the 5'-UTR conserved region of RNA1 and RNA2 (Ferrer *et al*., 2008). Total RNAs obtained from apical leaves of BBWV-1 inoculated plants were denatured in a 50% formamide solution at 95ºC for 10 min and then applied onto

the nylon membranes (Roche Diagnostics). Viral RNAs were fixed by UV light exposure (250 mJ) in a cross-linking oven. Conventional hybridisation was performed by incubating the nylon membranes in 10 ml of ULTRAhyb buffer (Ambion, Thermo Fisher Scientific) at 68 ºC for 1-2 h (prehybridisation) and at 68 ºC overnight in 10 ml of the ULTRAhyb buffer containing 20 ng of anti-sense BBWV-1 riboprobe (hybridisation). The membranes were washed twice with 2xSSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS at room temperature, and twice with 0.1xSSC and 0.1% SDS at 68 ºC. Then, the membranes were equilibrated with maleic buffer (100 mM maleic acid, 150 mM NaCl, 0.3% (v/v) Tween 20, pH 7.5) and blocked with blocking buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5 and 2% (w/v) blocking reagent (Roche Diagnostics). Antidigoxigenin-alkaline phosphatase antibodies (Roche Diagnostics) were subsequently added at a concentration of 150 U/ml in blocking buffer and the membranes were incubated for 30 min with this solution. Finally, the membranes were washed twice with maleic buffer and equilibrated with 100 mM Tris/HCl, 100 mM NaCl, pH 9.5 for 2 min. The hybridisation reaction was visualized with the chemiluminescent substrate CDP-star (Roche Diagnostics) after exposition to an X-Ray film at 37 ºC for 20-40 min.

BBWV-1 virus accumulation was estimated by RT-qPCR from total RNA extracts obtained from apical leaves of five broad bean and five pepper plants inoculated with BBWV-1 isolates Ben, PV0548, B41/99 and NSRV at 4, 8, 16, 24 and 32 dpi. Five biological replicates (plants) per each host species and two RT-qPCR technical replicates for each plant were performed. RT-qPCR was carried out in a LightCycler480 (Roche, Switzerland) and each reaction contained 50 ng of total RNA, 1xTaqMan One-Step Master Mix (Roche, Switzerland), 15 U Multiscribe Reverse Transcriptase (Thermo Fisher Scientific), 2 U RNase Inhibitor (Thermo Fisher

Scientific), 0.5 µM of each primer forward and reverse (FabF forward primer and B1R reverse primer, Ferriol *et al*., 2011), and 0.25 µM of a TaqMan minor groove binding (MGB) probe designed in a conserved region of BBWV-1 RNA 1 (Ferriol *et al*., 2011). RT-qPCR conditions comprised reverse transcription at 48°C for 30 min, incubation at 95°C for 10 min, and 45 cycles of 95°C for 2 s, 58°C for 8 s and 60°C for 10 s. As negative controls, RNA total extracts of mock-inoculated and healthy plants were included.

To obtain the calibration curve, an amplicon equivalent to the RT-qPCR product was obtained by conventional RT-PCR using a forward primer that contained the T7-promoter sequence. Then, positive-sense RNA transcripts were synthetized with Megascript T7 Kit (Thermo Fisher Scientific), treated with Turbo RNase-free DNase (Thermo Fisher Scientific) and purified by ethanol precipitation. Ten-fold serial dilutions containing 10^{10}–10^{1} RNA copies of transcripts were prepared and used to obtain a calibration curve with an amplification efficiency of 99% in a range equivalent to 10^{3} - 10^{10} (Ferriol *et al*., 2011).

Statistical analysis. Data on virus titre were statistically analysed by multifactorial ANOVA test with Statgraphics software (Statgraphics Technologies, Inc.). Viral concentration was considered as a dependent variable of dpi and the inoculated isolate. Least Square Difference (LSD) test was used for mean comparisons with a confidence level of 95%. The normal probability plot of the residuals was used to assume the normal distribution of data whereas homoscedasticity was assumed using the Levene's test.

RESULTS AND DISCUSSION

Infectivity and symptomatology of BBWV-1 isolates. Infectivity (measured as the proportion of infected plants over inoculated plants) and symptoms induced by BBWV-1 isolates Ben, B41/99, NSRV and PV0548 was assessed by mechanical inoculation of each isolate into 10 plants of tomato, broad bean, pepper, common bean, cucumber, melon and *N. benthamiana.* As a negative control, the same number of plants of each species was mock inoculated. Virus infection was assessed by molecular hybridization as described in Material and Methods section. The experiment was repeated a second time with almost identical results (Supplementary Table S1). No differences of infectivity among the BBWV-1 isolates were obtained, except in tomato and pepper. The four BBWV-1 isolates infected all inoculated broad bean and *N. benthamiana* plants whereas none of the inoculated plans of cucumber, common bean and melon became infected. In tomato, Ben and B41/99 infected 10 of 20 inoculated plants (50%), NSRV infected 12 of 20 inoculated plants (60%) and PV054 infected 8 of 20 inoculated plants (40%). In pepper, Ben infected 19 of 20 inoculated plants (95%), B41/99 infected 17 of 20 inoculated plants (85%), NSRV infected 16 of 20 inoculated plants (80%) and PV0548 infected 15 of 20 inoculated plants (75%). It is remarkable that none of BBWV-1 isolates used in this work were mechanically transmitted to cucumber, common bean and melon in spite of these species have been reported as natural hosts of BBWV isolates from Germany, Italy, USA and China (Dong *et al*., 2012; Edwardson and Christie, 1991; Lisa *et al.,* 1986). However, those reports did not differentiate between BBWV-1 and BBWV-2 species, hence cucumber, common bean and melon infection could be induced by BBWV-2 isolates or BBWV-1 isolates different from those studied in the present work. In addition, BBWV-1 transmission by

aphids was not assayed in this work, so we cannot definitively discard a natural virus infection of these host species.

Plant symptoms induced by the BBWV-1 isolates showed differences depending on the host plant, the viral isolate and the infection time. In broad bean, PV0548-infected plants showed at 16 dpi a mild leaf mosaic and a vein chlorosis that disappeared at 32 dpi, remaining only the mild mosaic (Fig. 1). New leaves showed a slight distortion at 40 dpi. Ben-infected plants showed intense vein chlorosis at 16 and 32 dpi that evolved to mild mosaic and slight leaf distortion in old and new leaves at 40 dpi. B41/99- and NSRV-infected plants showed similar symptoms which consisted in intense vein chlorosis at 16 dpi that increased in severity until 40 dpi. Strong leaf deformation was observed in old and new leaves inoculated with both BBWV-1 isolates but those inoculated with NSRV showed also leaf curling symptoms. In pepper, BBWV-1 isolates induced symptoms that usually started at 10 dpi and remained constant up to 40 dpi, which consisted in mosaic and leaf distortion, whose severity was dependent on the viral isolate (Fig. 2). Ben-infected plants showed a severe mosaic, PV0548- and NSRV-infected plants showed a mild mosaic whereas B41/99-infected plants showed only a slight mosaic that was almost imperceptible. In *N. benthamiana* plants, the four BBWV-1 isolates induced the same symptoms consisting of stunting, chlorosis and severe mosaic (data not shown). In contrast, tomato plants which were infected with BBWV-1 isolates remained asymptomatic like the mock-inoculated controls. It has been reported that BBWV-infected tomato plants show symptoms of mosaic, chlorosis, reduced growth and deformation (Blancard, 2012). However, in that report the BBWV species was not identified and the absence of other virus species in mixed infections was not assessed. So, reported tomato symptoms could be induced by BBWV-2 or by BBWV-1 isolates different from those studied in the present work.

Alternatively, symptoms could be also induced by other virus species which infected the tomato plants in mixed infection with BBWV.

Accumulation of BBWV-1 isolates. To determine if the different severity of plant symptoms induced by PV0548, Ben, B41/99 and NSRV was associated to viral replication, over-time virus accumulation of each BBWV-1 isolate was estimated in the inoculated broad bean and pepper plants by RT-qPCR (Fig. 3). Statistical significance was measured with p-values < 0.05 according to LSD test. PV0548, Ben and B41/99 accumulated more in broad bean than in pepper plants (p-value < 0.05). In contrast, the accumulation of NSRV was similar in both host species (p-value > 0.05) showing the lowest viral accumulation. So, these results are in concordance with those of Ferriol *et al*. (2011) who showed a differential over-time accumulation of isolate Ben in broad bean and *Chenopodium quinoa* plants. However, the accumulation of isolate Ben estimated in our study was different from that reported by Ferriol *et al*. (2011) at the first stage of virus infection (8 and 16 dpi). Thus, BBWV-1 isolate Ben titre increased in our analysis, whereas Ferriol *et al*. (2011) reported a decrease. The final concentration of isolate Ben at 32 dpi was similar in both studies. These differences in the pattern of viral accumulation could be caused by many factors such as the amount of BBWV-1 inoculum source, plant variety, temperature and humidity conditions, or inoculation procedure.

In broad bean, the concentration of the four BBWV-1 isolates was similar at 4 dpi (3.43×10^3, 3.47×10^3, 6.34×10^3 and 4.57×10^3 RNA1 copies/ng of total RNA for Ben, B41/99, NSRV and PV0548, respectively). At 8 dpi, concentration of Ben, B41/99 and PV0548 increased rapidly to 1.48×10^7, 5.04×10^6 and 6.68×10^6 copies/ng of total RNA, respectively, whereas NSRV concentration increased only to 1.00×10^5 copies/ng

of total RNA, and this isolate had a lower concentration in all analyzed period than the others. The maximum accumulation peak of the four isolates was at 16 dpi reaching concentrations of 5.94 x 10^7, 8.88 x 10^7, 1.73 x 10^7 and 7.96 x 10^5 copies/ng of total RNA for Ben, B41/99, PV0548 and NSRV, respectively. Then, the virus concentration decreased slightly at 24 and 32 dpi to values of 7.48 x 10^6, 2.44 x 10^7, 5.62 x 10^6 and 1.09 x 10^4 copies/ng of total RNA for Ben, B41/99, PV0548 and NSRV, respectively. In pepper, BBWV-1 isolates showed strong differences between some isolates unlike broad bean. At 4 dpi, isolates Ben and B41/99 had similar titre (1.44 x 10^7 and 2.51 x 10^7 copies/ng of total RNA, respectively) which was higher than those of isolates PV0548 and NSRV (2.49 x 10^6 and 2.27 x 10^3 copies/ng of total RNA, respectively). Virus concentrations of Ben and B41/99 decreased to reach concentrations of 2.49 x 10^5 and 4.28 x 10^5 copies/ng of total RNA at 16 dpi, respectively. In contrast, NSRV viral concentration increased to 1.71 x 10^5 copies/ng of total RNA at 16 dpi. From 16 dpi up to 32 dpi, isolate Ben concentration increased to 2.39 x 10^6 copies/ng of total RNA whereas the concentration of B41/99 increased to 2.34 x 10^6 copies/ng of total RNA at 24 dpi and then decreased to 8.43 x 10^5 copies/ng of total RNA at 32 dpi. NSRV concentration decreased from 16 dpi to 24 dpi reaching values of 3.50 x 10^4 copies/ng of total RNA and increased to reach the maximum value of 3.56 x 10^5 copies/ng of total RNA. PV0548 did not show noticeable changes in virus accumulation values during all quantitation assays. No association between the concentration of PV0548, Ben, B41/99 or NSRV and induced symptoms in broad bean or pepper plants was observed. In broad bean plants, Ben, B41/99 and PV0548 showed similar viral accumulation up to 32 dpi (p-value > 0.05), although these BBWV-1 isolates induced different symptoms. NSRV, accumulated significantly less than Ben or PV0549 (p-value < 0.05) but it induced more severe symptoms than those induced by Ben or PV0549. In pepper, Ben and B41/99

were the isolates inducing the most and the least severe mosaic symptoms, respectively despite both showing similar accumulation up to 32 dpi (p-value > 0.05). In contrast, mosaic symptoms induced by NSRV were more severe than those induced by B41/99 but NSRV accumulation was lower than B41/99 at 4, 8 and 24 dpi (p-value < 0.05).

In some cases, virulence can be associated to the virus accumulation in the plant tissues and in vector-borne viruses, virulence can be also associated with its transmission rate by the vector (Froissart *et al*., 2010). Consequently, virus accumulation increases virulence and virus transmission to another host. Studies performed with *Papaya ringspot virus* strains in zucchini squash, squash and watermelon plants (Pacheco *et al*., 2003) associated viral multiplication and symptom severity. In addition, analysis of different strains of cucumber vein yellowing vi*rus* (CVYV) demonstrated that severe strain CVYV-Jor accumulated earlier than mild strain CVYV-Alm1 (Galipienso *et al*., 2013). However, studies performed with different cucumber mosaic virus strains infecting *Arabidopsis thaliana* plants showed that virulence, measured as plant growth and seed production, was unrelated to virus replication (Pagán *et al*., 2007). Supporting these results, studies performed with prunus necrotic ringspot virus (Moury *et al*., 2001) suggested that virus accumulation and symptom severity were unrelated. In contrast, the results obtained in this work support the hypothesis that BBWV-1 virulence, measured as symptom severity, does not depend on virus replication. So, further studies to identify the BBWV-1 products that are implicated in virulence and plant symptom development are needed.

In conclusion, the BBWV-1 isolates analysed here, which are genetically very distant, showed different biological behaviour, so virus-induced symptoms varied depending on host species and BBWV-1 isolate. Differences on infectivity among isolates were observed only in tomato and pepper plants. Finally, quantitation of

BBWV-1 isolates in broad bean and pepper plants showed differences in accumulation among virus isolates and host species but no association between viral concentration and severity of induced symptoms was observed.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest

STATEMENT OF HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Table 1. BBWV-1 isolates used in this study.

BBWV-1 isolate	Geographical origin	Collection host	Collection date	Genbank acession
PV0548 (SV-3-88)	Syria	*Vicia faba*	1998	JF440100, JF440076 JF440124, JF440053
Ben	Spain	*Capsicum annuum*	2001	AY781172 AY781171
B41/99	Bulgaria	*Capsicum annuum*	2000	JF44009, JF440068 JF440115, JF440036
NSRV	Italy	*Unknown*	1996	JF440097, JF440074 JF440121, JF440049

411**Figure legends**

412

413**Fig. 1.** Symptoms of vein chlorosis, mosaic, leaf distortion and curling observed at 16,
41432 and 40 dpi in broad bean leaves infected with BBWV-1 isolates Ben, B41/99, NSRV
415and PV0548. At the bottom, symptoms of the new leaves at 40 dpi.

416

417**Fig. 2.** Symptoms of mosaic and distortion leaf observed in pepper plants infected with
418BBWV-1 isolates Ben, B41/99, NSRV and PV0548 at 20 dpi.

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420**Fig. 3.** Graphic representations of viral accumulation over time in pepper and broad
421bean plants inoculated with BBWV-1 isolates PV0548, Ben, B41/99 and NSRV. Five
422biological replicates (plants) per each host species and two RT-qPCR technical
423replicates for each plant were performed. Viral accumulation is represented as logarithm
424of BBWV-1 RNA 1 copies per ng of total RNA. Bars represent the standard error of the
425mean. Letters above the bars indicate significant differences according to a Gamma
426generalised linear model (overall p-value <0.05 using Bonferroni correction).

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436 **Figure 1**

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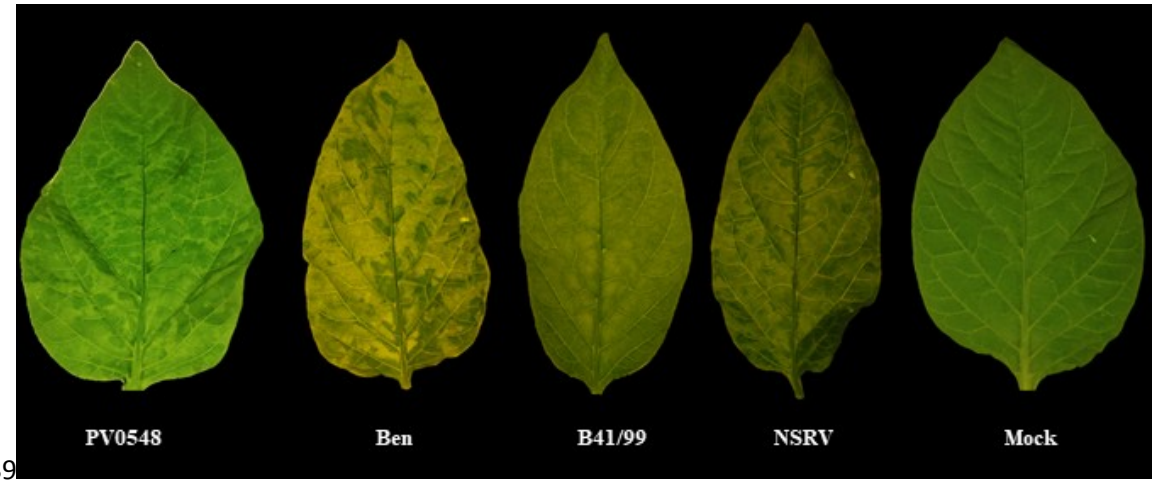
438 **Figure 2**



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440 **Figure 3**

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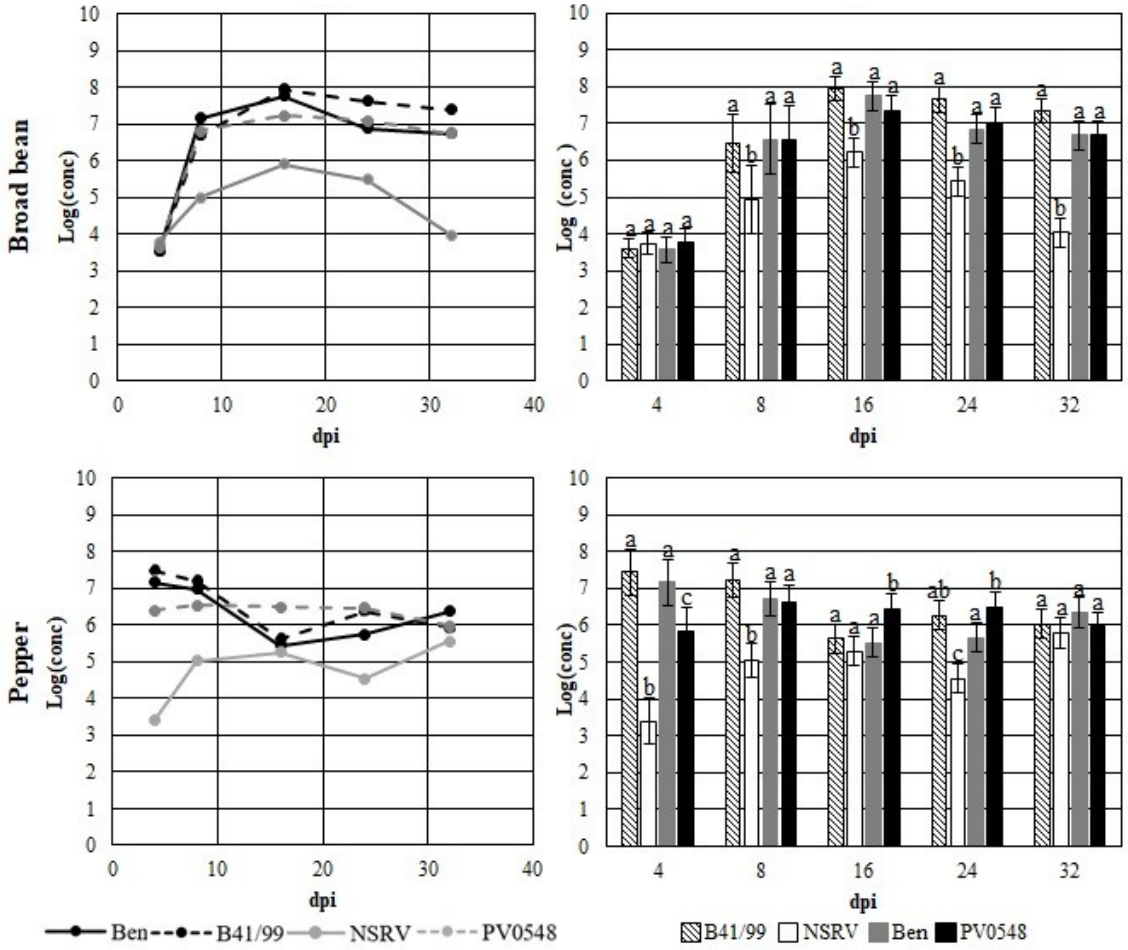

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