European Journal of Plant Pathology (2005) 113:343–355
DOI 10.1007/s10658-005-7966-7

Analysis of accumulation patterns of *Barley yellow dwarf virus-PAV* (BYDV-PAV) in two resistant wheat lines

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Accepted 16 May 2005

Key words: AUCPC, *Luteovirus*, *Poaceae*, semi-quantitative ELISA, *TC14*, viral exposure, *Zhong ZH*

Abstract

Barley yellow dwarf (BYD) is one of the main viral diseases of small-grain cereals. This disease, reported on numerous plant species of the *Poaceae* family, is caused by a complex of eight viral species including the species *Barley Yellow Dwarf Virus-PAV* (BYDV-PAV), frequently found in western Europe. Resistance sources against BYDV-PAV are scarce and only identified in perennial *Triticineae*. Some BYDV-resistant wheat lines have been obtained by introgressing these resistances into bread wheat germplasms. Genetic and biological characterization of the resulting lines has been undertaken. However, little information on the resistant behaviour of these lines during the early stages of the infection process is available. To evaluate the resistance of two genetically distinct resistant lines (*Zhong ZH* and *TC14*), 1740 young plantlets, belonging to susceptible reference hosts (barley cv. *Express* and wheat cv. *Sunstar*), *Zhong ZH* or *TC14* wheat lines, were inoculated in controlled conditions with French BYDV-PAV isolates. The infection process was monitored during the first 21 days after inoculation (DAI) using a semi-quantitative ELISA. A standardized protocol including five successive samplings of leaves from all inoculated plants and the collection of plant roots at the end of the monitored period was carried out. This protocol enabled an assessment of the infection percentage and the evolution of the viral load in plants from the 7th DAI to the 21st DAI. Statistical analyses of the BYDV infection kinetics using raw ELISA data, a model of the time-dependent variation of the percentage of infected plants and the area under concentration progress curves (AUCPC) demonstrated that *Zhong ZH* and *TC14* lines (1) reduce the development rate of the BYD disease during the first days of infection, (2) decrease the infection efficiency of BYDV-PAV isolates, in the leaves, from 98.7% for susceptible plant genotypes to 81.9% and 71.7% for *Zhong ZH* and *TC14*, respectively, (3) reduce the virus load in the leaves of infected plants and (4) are not spared from BYDV infection, as 95.1% of *Zhong ZH* and 90.2% of *TC14* inoculated plants accumulated viral particles in roots and/or in leaves at 21 DAI. These results confirm the BYDV-partial resistant behaviour of both *Zhong ZH* and *TC14* lines. The development rate of the disease was the single parameter that allowed the distinction between the two resistant sources present in the tested lines.

Introduction

Barley/cereal yellow dwarf viruses (B/CYDVs, family *Luteoviridae*) are composed of a complex of eight virus species able to infect all members of the family *Poacea*e. These viruses induce the barley yellow dwarf disease (BYDD), one of the most important viral diseases in small-grain cereals

(D'Arcy, 1995). The B/CYDV complex includes members of the genera *Polerovirus* (CYDV-RPV and -RMV), and *Luteovirus* (BYDV-MAV, -PAS and -PAV), and unassigned viral species (BYDV-SGV, -RMV and -GPV) of the family *Luteoviridae* (Van Regenmortel, 1999; Mayo, 2002). The BYDV-PAV is the most common B/CYDV species and is widespread in western Europe (Signoret and Maroquin, 1990; Henry et al., 1993; Lister and Ranieri, 1995). B/CYDVs are transmitted by aphids in a persistent manner (Gildow and Rochow, 1980). This transmission process requires that the aphid spends a 24–72 h acquisition access period (AAP) on infected leaves to efficiently acquire viral particles and a 24–120 h inoculation access period (IAP) on a healthy plant to allow efficient inoculation (Sadeghi et al., 1997a). After inoculation, B/CYDV particles migrate from the inoculated leaf to the roots by cell-to-cell and/or long-distance movements (Carrington et al., 1996). In the roots, active viral replication and accumulation occur, which constitute the early stages of the B/CYDVs systemic infection process of susceptible plant genotypes (Sadeghi et al., 2000). The main symptoms of BYDD consist of dwarfing, yellowing and physiological alterations (Bruehl, 1961; Bayon et al., 1982; Beuve et al., 1999; Ayala et al., 2001, 2002). The extent of such symptoms depends on numerous biotic and abiotic factors such as plant species and cultivar (Bruehl, 1961; Gruntzig and Fuchs, 2000), synergism with other pathogens (Collin et al., 1997), hydric and thermal stress (Grafton et al., 1982; Monneveux et al., 1991), and the age of the inoculated plant (Smith and Sward, 1982). Consequently, BYDD induces yield losses ranging from 5% to 80% with an average of 30% (Lister and Ranieri, 1995; Perry et al., 2000).

Control methods against BYDD are mainly based on vector control (insecticide treatments). However, breeding resistant or tolerant plants is a method both less costly and less environmentally damaging. As defined by Cooper and Jones (1983). Tolerance refers to situations where the virus is able to multiply in plants without symptoms and resistance corresponds to a reduced viral multiplication and/or accumulation. Only low levels of B/CYDV resistance and some sources of tolerance (e.g. *Bdv1* gene in wheat) have been found in annual *Triticineae* (e.g. *Triticum*, *Aegilops*) (Singh et al., 1993). B/CYDV resistance has not been found in wheat although thousands of accessions have been tested (Qian et al., 1993; Li et al., 1998; Francki et al., 2001). The only sources of B/CYDV resistance identified are from perennial *Triticineae* (e.g. *Thinopyrum*, sp., *Lophopyrum,* sp.) (Comeau and Plourde, 1987; Larkin et al., 1990; Xu et al., 1994). Transfers of these resistance sources have been initiated into bread wheat genotypes and different wheat lines carrying alien resistance genes are now available. Among them, *P29*, a substitution line (Sharma et al., 1995), *Zhong ZH*, a ditelosomic addition line (Barloy et al., 2003), *TC14*, a translocated line (Banks et al., 1995a) and *OK721154*, a partial amphiploid line (Comeau et al., 1994, Chen et al., 1998) are well documented in the literature.

The genomic and chromosomal constitutions of these lines have been extensively studied (The and Baker, 1970; Forster et al., 1987; Brettell et al., 1988; Banks et al., 1995b; Larkin et al., 1995a; Chen et al., 1998; Anderson et al., 1998; Crasta et al., 2000; Tang et al., 2000). The *P29* and *TC14* BYDV-resistant lines carry alien genes originated from the homoeology group 7 *Th. intermedium* chromosome. However, molecular marker analysis has confirmed that the *Th. intermedium* chromosome in *P29* belongs to a different genome 7 wheatgrass chromosome than the one present in the L1 addition line (Cauderon, 1966), used to produce the translocated *TC14* line (Anderson et al., 1998). The BYDV resistance from *Zhong ZH* is supported by a *Th. intermedium* chromosome belonging to group 2 (Larkin et al., 1995a; Tang et al., 2000). As yet, the number and the group(s) of chromosomes involved in the resistance of the partial amphiploid *OK7211542* line are unknown. However, contrary to the previously described BYDV-resistant lines P29 and *TC14*, the alien genome of *OK7211542* is derived from *Th. ponticum* (Chen et al., 1998). The limited number of available resistant wheat lines and the varied origins of the selected resistance genes have justified investigations of performance of these materials in B/CYDV resistance response, in field and/or in greenhouses (Chen et al., 1997; Anderson et al., 1998; Ayala et al., 2001; Balaji et al., 2003; Barloy et al., 2003).

The observation of symptoms, tissue-blot immunoassay (TBIA) and/or Enzyme-Linked Immunosorbent Assays (ELISA), provided the first data on the behaviour of these resistant lines.

The titre of BYDV-PAV in leaves of infected plants, generally estimated using raw ELISA data, was shown to be reduced in *P29* (42–52%), in *TC14* (27–55%) and in *Zhong ZH* (not calculated in published papers but estimated to be lower than *TC14*) when compared to susceptible plant genotypes. Moreover, 100% and 30% of inoculated *OK7211542* and *TC14* plants, respectively, escaped infection, illustrating the immune status of the partial amphiploid *OK7211542* line and the particular behaviour of the *TC14* line. However, all these conclusions resulted from qualitative data collected on limited numbers of plants using sampling protocols that did not deal with the heterogeneous distribution of BYDV in infected plants or with the dynamic progression of the BYDV particles in the plants during the first stages of the systemic infection. In a recently published paper, Balaji et al. (2003) developed a quantitative approach to examine the time-dependent BYDV accumulation in the plants from 0 to 14 days after inoculation. Applied to different BYDV-inoculated plants, including the *P29* line, the resulting time patterns of viral accumulation in the plants gave an overview of the first stages of the infection kinetic, and identified, for the *P29* line, periods with no (0–3 days after inoculation (DAI)), low (11–14 DAI), medium (7–11 DAI) or high (3–7 DAI) levels of BYDV-PAV resistance when compared with susceptible controls. According to these results, a time-dependent quantitative approach of the first days/weeks of the BYDV-infection process for the other available resistant lines is necessary to further understand the viral accumulation in these plants.

The current work enabled a precise evaluation of resistance efficiency. A semi-quantitative ELISA protocol was applied to BYDV-inoculated plants belonging to the two genetically distinct *Zhong ZH* and *TC14* resistant lines. The developed procedure enabled an assessment of the time-dependent infection percentage and the progression of the viral load in plants during the first 21 days after inoculation.

Materials and methods

Virus isolates

Four French BYDV-PAV isolates were used in the experiments. BYDV-PAV 13, PAV 4, and PAV 2T were collected on barley in 1985 in Loire-Atlantique, in 1989 in Ille-et-Vilaine, and in 1991 in Yvelines, respectively. BYDV-PAV RG was collected on ryegrass in 1988 in Yvelines. Isolates were separately maintained on the susceptible barley cv. *Express* since their collection. The BYDV-PAV RG, PAV 2T and PAV 13 isolates induce, respectively, very severe, severe and mild symptoms on barley cv. *Plaisant* (Chalhoub et al., 1994). BYDV-PAV 4, the BYDV-PAV reference isolate in this study, is a severe isolate on barley cv. *Express* (Papura et al., 2002).

Host plants

Susceptible barley cv. *Express* (Sadeghi et al., 2000), susceptible wheat cv. *Sunstar* (Posadas and Henry, 2002) and resistant wheat lines, *TC14* (Banks et al., 1995b) and *Zhong ZH* (Barloy et al., 2003), were used in the experiments. *TC14* line was obtained from a cross between a resistant translocation line, derived from the L1 line (Cauderon, 1966), and the susceptible wheat cv. *Sunstar* (Banks et al., 1995b). *Zhong ZH* line was derived from the Z6×Mission cross, with Z6 being a disomic addition line (Larkin et al., 1995b). Seeds were individually sown in plastic tubes containing vermiculite and grown in a growth chamber at 20 °C, light: 16 h/dark: 8 h.

Inoculation and sampling

Sets of 10 day-old plants (1–2 leaves stage) per genotype were separately inoculated with either BYDV-PAV 4, 13, 2T or RG. BYDV-PAV 4 was inoculated to *Express* (210 plants), and to *Sunstar*, *Zhong ZH* and *TC14* (230 plants/plant genotype) in nine independent experiments. Additional experiments were performed with BYDV-PAV RG, PAV 13 and PAV 2T isolates. The latter were individually inoculated to *Express* (total number of 40 plants/isolate), and to *Sunstar*, *Zhong ZH* and *TC14* (total number of 80 plants/isolate/plant genotype), in three separate experiments. Each experiment included three non-inoculated plants per plant genotype as healthy controls. Viruliferous aphids were obtained by parthenogenetic reproduction of aphid females on BYDV-infected barley cv. *Express* plants. Three viruliferous third or fourth instar larvae of *Rhopalosiphum padi* RP1

clone (Simon et al., 1991), an efficient BYDV-PAV vector (Sadeghi et al., 1997b), were deposited with a paintbrush at the base of each tested plant. During the BYDV-PAV4 inoculation procedure, 58 sets of three aphids were sampled among the first and last aphids that were transferred on plants in order to test the heterogeneity of their viral load. Each plant was then covered with a micro-perforated cellophane bag for a 5-day IAP. At the end of IAP, plants were sprayed with an insecticide (lambda-cyhalothrine, 1 mg l^{-1}, Karaté® [Syngenta agro]). Each plant was sampled at 7, 11, 14, 18 and 21 DAI by cutting 2.5 cm of the apical part of each leaf longer than 5 cm. For each sampled plant at each sampling day, the 2.5 cm-long collected leaf fragments were pooled. Finally, roots of plants were individually collected at 21 DAI. Immediately after collection, all materials (aphids, leaf fragments and roots) were stored at −20 °C until tested.

Semi-quantitative detection of BYDV-PAV by DAS-ELISA

Symptoms were not monitored as BYDV-PAV induces faint symptoms on young wheat plantlets. Presence of virus in aphids, leaves and roots was assessed using semi-quantitative ELISA tests. The sets of 3 aphids collected during inoculation were ground in microtubes in 100 µl of grinding buffer (PBS–Tween 20 0.05% (v/v)–polyvinylpyrrolidone 2% (w/v)) using plastic pestles. Leaf fragments were ground in microtubes with grinding buffer and a mix of 1 mm glass and 5.6 mm inox balls using a ball mill [Retsch M301] at maximum speed for 45 s. The volume of grinding buffer used was adjusted with the number of leaf fragments according to 100 µl per leaf fragment with a minimum of 200 µl for samples containing only one fragment. Roots were ground in plastic bags in 1 ml of grinding buffer with a rolling press. Standard ranges corresponding to two-times serially diluted fractions were prepared using either a crude sap produced by grinding approximately 1 g of BYDV-infected barley leaves in 1 ml of grinding buffer or a BYDV-purified fraction (4.25 ng μl^{-1}) of viral particles. BYDV particles present in samples were detected by DAS-ELISA using a polyclonal serum raised against BYDV-PAV (IgG *PAV*52, H. Lapierre, INRA, France) and according to previously published protocols (Torrance et al., 1986; Fabre et al., 2003). Standard ranges were included on 96-well microplates (crude sap dilutions on all plates; BYDV-purified diluted fractions on one plate per series) to compensate inter-plate variation of ELISA results and to estimate the virus load of the tested samples. Optical density at 405 nm (OD_{405}) was read using a microplate reader (Titertek Multiscan [MCC]). ELISA tests were considered positive when the OD_{405} reduced by the blank value exceeded three times the value of negative control (healthy plant leaves or roots, or virus-free aphids) with a minimum threshold value of 0.12.

Statistical analyses of the data

Statistical analyses were performed with GLM procedure using SAS software (v8.1, SAS Institute Inc., Cary, NC, USA) and residual normality was checked with the univariate procedure of SAS. Means were compared with Student Newman-Keuls tests using 5% significance thresholds.

Results

Validation of the inoculation procedure

Ten to sixteen aphid sets were put aside during the BYDV-PAV 4 inoculation procedure whilst inoculating the first to the last plant of each tested genotype (Table 1). The 58 tested sets were associated with OD_{405} values ranging from 0 to 1.104 (0.411 on average). Fifty-four sets gave ELISA values above the detection threshold ($OD_{405} > 0.12$) illustrating the presence in 93.1% of the sets of at least one million BYDV particles. This quantity of BYDV particles is required for efficient BYDV detection by ELISA (French, 1995). The sets giving negative results ($OD_{405} < 0.12$) were collected during *Sunstar* (2 sets), *TC14* (1 set) and *Zhong ZH* (1 set) inoculations. In order to test the heterogeneity of the viral load of the aphid sets between the four inoculated plant genotypes, a statistical analysis was performed on raw ELISA values. The ANOVA results did not reveal any significant differences ($F = 2.04$, $P = 0.12$) demonstrating that the inoculum used for the four plant genotypes was homogeneous. The survival of the aphids was individually checked at the end of the IAP.

Table 1. Characterization of the viruliferous or virus-free status of aphid sets used in the BYDV-PAV4 inoculation procedure

Inoculated host	Number of tested aphid sets	ELISA results (OD_{405})		Aphid sets status	
		Min	Max	Viruliferous	Virus-free
Express	10	0.173	0.705	10	0
Sunstar	16	0.056	0.643	14	2
Zhong ZH	16	0	1.104	15	1
TC14	16	0	0.847	15	1

All test plants were still infested by at least one live aphid, 5 days after inoculation (data not shown), indicating that the aphids had obligatorily fed on test plants during the IAP. Taken together, these data clearly show that aphid sets are likely to inoculate BYDV-PAV 4 in a similarly efficient manner, whatever the plant genotype considered.

Infection kinetics

To study the first stages of the BYDV systemic infection in susceptible and resistant plant genotypes, 1740 plants belonging to four genotypes from two BYDV host species were individually sampled five times from the 7th to the 21st DAI (Figure 1). At the beginning of the infection and/or for the resistant plant genotypes, the OD_{405} values associated with samples were close to the threshold detection limit of ELISA (data not shown). This could induce, between one sampling date and the next one, a shift from infected to healthy status for those plants. To analyse the infection rate of plants at any of the five successive sampling days, each plant was considered infected from the day of the earliest detection of BYDV particles to the end of the experiment. Figure 1a and b illustrate, using these corrected data, the percentage of infection for each inoculated plant and for each isolate, respectively. When host genotypes were considered (Figure 1a), two curve shapes were observed. Curves relating to *Express* and *Sunstar* showed a rapid increase in the percentage of infection (70.9% and 87.1% for *Express* and *Sunstar*, respectively, at 11 DAI) and a plateau close to 100% (98.5% (325/330) and 98.9% (465/470) for *Express* and *Sunstar*, respectively, at 21 DAI) reached at the 14th DAI. The curves relating to *TC14* and *Zhong ZH* showed a more progressive increase in the percentages of infection from the 7th DAI to the 21st DAI to reach only 71.7% and 81.9% at 21 DAI, respectively. This result indicates that 28.3% and 18.1% of inoculated *TC14* and *Zhong ZH* plants were still considered as healthy plants 21 days after inoculation. However, contrary to the susceptible plant genotypes, the infection percentage curves of the resistant lines do not include a plateau in the monitored period of infection. When the same data set was analysed according to the inoculated BYDV isolate (Figure 1b), a single curve pattern was observed. However, a slight difference between the PAV 2T curve and the others could be noticed as all infection percentage associated with BYDV-PAV 2T were higher than those obtained for the other three viral isolates.

In such kinetics, the infection percentage values obtained for each observation is dependent on the previously collected data. This is contradictory to the required hypothesis of independence of variables for classical ANOVA. Consequently, direct comparisons of curves are not strictly reliable. To compare the time-dependent variation of infection percentages, a modelling approach was developed. Simple mathematical models (Gompertz, logistic and monomolecular) were applied to data using the Microsoft® Excel solver function with the least square method. The model based on the monomolecular equation $y = 100-(1-y_0)e^{(-rt)}$ (where y is the percentage of infected plants, r is the rate parameter, t is time in days, and y_0 is y value at $t = 0$) gave the best fits for all curves (Figure 1a and b). The transformation $Y = \mathrm{Ln}(100/(100-y))$ linearised the model in $Y = Rt + Y_0$ equations. Y_0 is linked to the percentage of infection at the beginning of the experiment and R-values (Table 2) correspond to the slopes of the lines and are representative of the disease development rate. ANOVA analysis

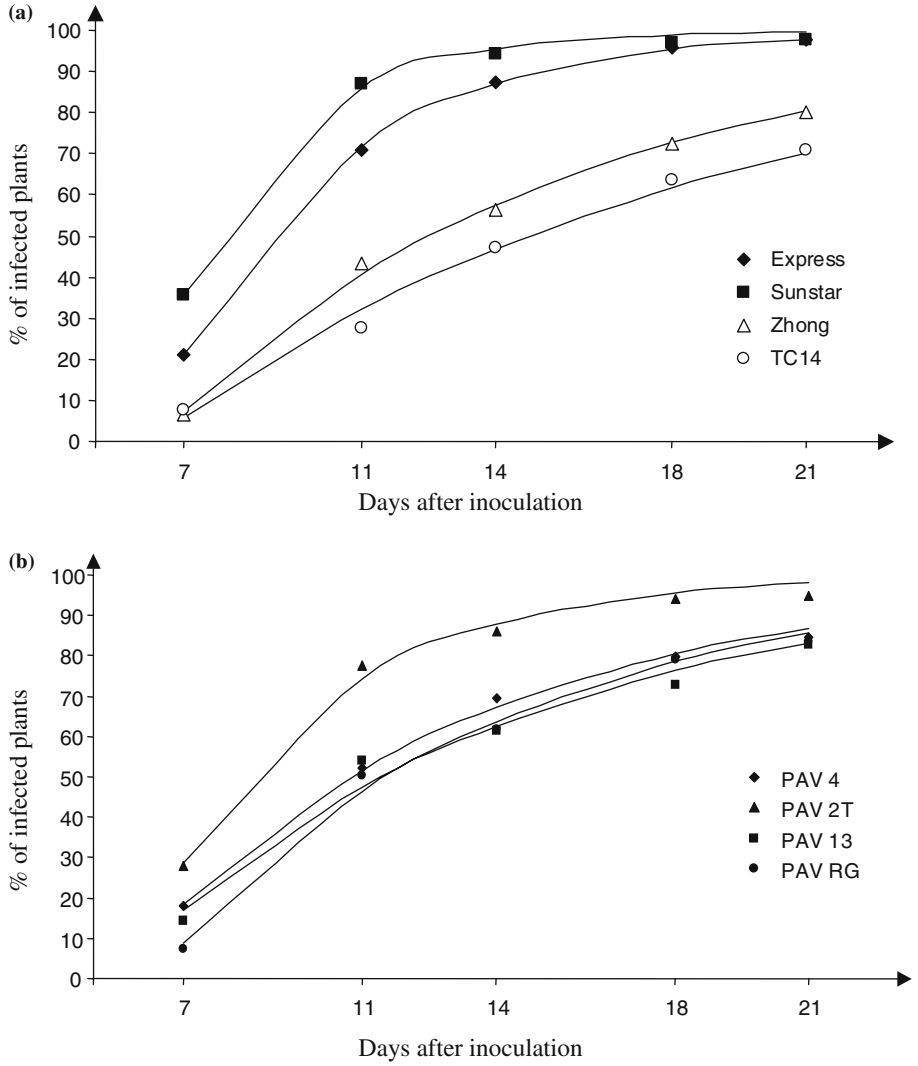

Figure 1. Mean percentages of BYDV-infected plants obtained during the first 21 DAI for each inoculated plant genotype (a) and for each used isolate (b). Symbols represent mean values for percentages of infected plants at each sampling date according to the legends presented in the graphs. Plain lines are theoretical values of the infection curves, according to the monomolecular model.

performed on Y_0 values did not reveal any significant difference between plants or isolates (not illustrated). However, statistical analysis performed with *R*-values showed a significant host effect ($F = 16.61$, $P = 0.0001$) and no virus isolate effect ($F = 1.20$, $P = 0.352$). Moreover, host-virus isolate ($F = 5.44$, $P = 0.0208$) and host-replicate ($F = 6.49$, $P = 0.002$) interaction effects were revealed but they were significantly weaker than the host effect. A SNK test at 5% risk resulted in a ranking of *R*-values into three groups (Table 2) from the highest to the lowest slopes constituted by susceptible plant genotypes (*Express* and *Sunstar*, group a), *Zhong ZH* (group b) and *TC14* (group c).

Detection of BYDV in roots

Based on BYDV detection assays performed using ELISA on leaf fragments, 208 plants (5 *Express*, 5 *Sunstar*, 85 *Zhong ZH* and 113 *TC14*) out of the 1740 inoculated plants were considered as healthy during the 21 day post-inoculation period. The root systems of 59 plants (2 *Express*, 2 *Sunstar*, 26

Table 2. Development rate of the disease obtained for each isolate, host and isolate/host combination according to the monomolecular model

	BYDV-PAV isolate				Mean per host[2]
	2T	4	13	RG	
Inoculated host					
Express	0.29[1]	0.25	0.25	0.38	0.27 (a)
Sunstar	0.25	0.22	0.28	0.29	0.25 (a)
Zhong ZH	0.23	0.15	0.20	0.13	0.17 (b)
TC14	0.15	0.11	0.07	0.11	0.11 (c)
Mean per isolate[2]	0.22 (a)	0.18 (a)	0.19 (a)	0.20 (a)	

[1]*R*-value, which represent the slope of the line $Y = Rt + Y_0$ where $Y = \mathrm{Ln}(100/(100-y))$, y being the infection percentage.
[2]Mean *R*-value were calculated by taking into account the number of tested plants for each isolate/host combination.
(a), (b) and (c) correspond to the groups defined by Student–Newman–Keuls test at the 5% risk with the mean data.

Zhong ZH and 29 *TC14*) were tested for their healthy or infected status (Table 3). The *Express* and *Sunstar* tested roots did not contain viral particles while 73.1% (19/26) and 65.5% (19/29) of *Zhong ZH* and *TC14* roots, respectively, were associated with OD_{405} values above the detection threshold. The detection of virus in aerial parts, and the root infection percentage enabled the calculation of the overall infection efficiency for the four tested plant genotypes: 98.5%, 98.9%, 95.1% and 90.2% for *Express*, *Sunstar*, *Zhong ZH* and *TC14*, respectively. This 90.2–98.9% range in infection efficiency strongly lowers the distinction between BYDV-susceptible and -resistant plant genotypes.

Kinetics of BYDV accumulation in infected plants

The semi-quantitative ELISA protocol that includes standard ranges (infected plants and purified BYDV particles) enabled the transformation of raw OD_{405} values into an estimation of viral concentration in the course of the infection process. Only samples associated with OD_{405} values above 0.12 were considered for viral load estimation (Figure 2). Concentration curves relating to susceptible plant genotypes (*Express* and *Sunstar*) showed an increase of virus titre in infected plants from the 7th DAI to the 11^{th}–14th DAI: 1.52 ng μl^{-1} and 1.20 ng μl^{-1} at 11 DAI for *Express* and *Sunstar*, respectively. These curves presented a pattern close to a plateau from the 14th to the 21st DAI (1.68 ng μl^{-1} and 1.21 ng μl^{-1} at 21 DAI for *Express* and *Sunstar*, respectively). However, the calculated virus load for *Sunstar* at 14 DAI (1.90 ng μl^{-1}) was 60% higher than the 11, 18 and 21 DAI data. None of the observed biological traits (size and number of developed leaves) of the *Sunstar* line could explain this particular behaviour at 14 DAI. The curves obtained

Table 3. Estimation of BYDV infection efficiency based on ELISA results

Inoculated host	BYDV-PAV detection								Infection percentage[c]
	In leaves[a]				In roots[b]				
	Number of tested plants	Infected	Healthy	Infection efficiency	Number of tested plants	Infected	Healthy	Infection efficiency	
Express	330	325	5	98.5%	2	0	2	0%	98.5%
Sunstar	470	465	5	98.9%	2	0	2	0%	98.9%
Zhong ZH	470	385	85	81.9%	26	19	7	73.1%	95.1%
TC14	470	337	113	71.7%	29	19	10	65.6%	90.2%

[a]BYDV detection in leaves was performed on all inoculated plants.
[b]BYDV detection in roots was performed on 59 plants associated with no-BYDV detection in leaves.
[c]The infection percentage results from the synthesis of leaves and roots infection efficiencies.

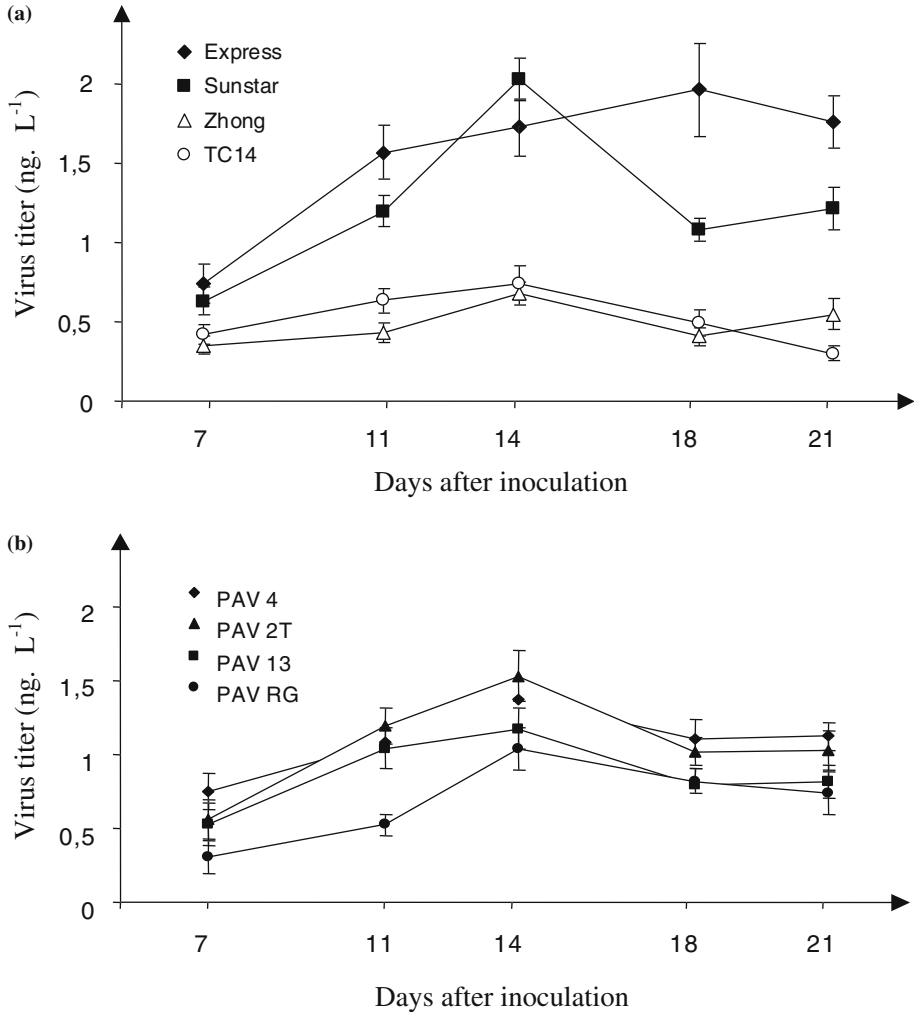

Figure 2. Mean virus concentrations of BYDV-infected plants obtained during the first 21 DAI for each inoculated plant genotype (a) and for each used isolate (b). Symbols represent mean values for virus concentration at each sampling date according to the legends presented in the graphs. Vertical bars represent ±5% confidence interval.

for *TC14* and *Zhong ZH* showed a nearly flat bell shape, with a maximal virus load at 14 DAI with 0.82 ng μl^{-1} and 0.76 ng μl^{-1} for *TC14* and *Zhong ZH*, respectively. The titre of BYDV-PAV in resistant lines was significantly reduced: 33.68% decrease on the 7th DAI and 55.72% decrease on the 14th DAI, when compared with susceptible lines. Concentration curves relating to BYDV isolates (Figure 2b) presented a quite similar time pattern whatever isolate was considered. The viral load increased in infected plants from the 7th DAI to the 14th DAI to reach a plateau close to 1 ng μl^{-1}.

Statistical analysis of these data demands the management of the non-independence of the time-dependent variables. As none of the previously tested simple models fitted all curves, an area under the concentration progress curve (AUCPC) approach was used to compare the BYDV concentration data. AUCPC integrates all hosts, pathogens and environmental effects (Campbell and Madden, 1990; Jeger and Viljanen-Rollinson, 2001) during the interaction period. So AUCPC provides a useful tool to compare resistance levels between different hosts. AUCPC was calculated using the formula derived from the area under disease progress curve (Nutter, 1997; Jeger and Viljanen-Rollinson, 2001): $\sum_{i=1}^{n}\left[\frac{Y_{i-1}+Y_i}{2}\right][D_{i-1}+D_i]$ where Y_i is the virus load of the sampled leaves for

each plant and D_i is the number of DAI at the ith observation. This formula gives an estimation of the area under the curve by calculating trapezoidal surfaces between two points in the kinetics (Table 4). AUCPC scores ranged from 12.20 to 20.54 for *Express* inoculated with −PAV 13 and −PAV 4, respectively, with an AUCPC mean equal to 16.67. Values ranged from 14.41 to 22.74 for *Sunstar* inoculated with −PAV 4 and −PAV 2T, respectively, with a mean equal to 15.73. Resistant plant genotypes were associated with lower AUCPC values ranging from 4.18 to 8.32 for *Zhong ZH* inoculated with −PAV 13 and −PAV 2T, respectively (mean AUCPC = 3.79), and from 3.54 to 10.93 for *TC14* inoculated with −PAV 4 and −PAV 2T, respectively (mean AUCPC = 4.19). Statistical tests showed clear host, replication and isolate effects ($F = 146.1$, $P < 0.0001$; $F = 102.6$, $P < 0.0001$; $F = 38.6$, $P < 0.0001$, respectively). During the 21-day monitored period, the exposures to BYDV of *Zhong ZH* and *TC14* leaves, estimated by AUCPC data, were reduced from 73.36% (*Zhong ZH* vs. *Sunstar*) to 77.26% (*TC14* vs. *Express*) when compared with the susceptible plant genotypes. SNK test at the 5% risk resulted in a ranking of mean AUCPC values associated into three homogeneous groups corresponding to (a) *Sunstar*, (b) *Express*, and (c) *Zhong ZH* and *TC14* (Table 4). Although a clear distinction between resistant and susceptible plant genotypes was denoted, no statistical differences between *Zhong ZH* and *TC14* were observed according to AUCPC values. Analysis of isolate effects allowed a clear distinction to be established between virus isolates. SNK tests ($\alpha = 0.05$) ranked isolates BYDV-PAV 2T, 4, 13 and RG from the higher to the lower AUCPC score. Effects of the host × isolate interaction ($F = 4.42$, $P = 0.0001$) were also detected, but the variances of the host and virus isolate effects were significantly larger than the variances of interactions.

Discussion

The first stages of the BYDV-PAV systemic infection of *Zhong ZH* and *TC14* resistant lines were monitored during the first 21 days following inoculation using the semi-quantitative ELISA procedure. The standardized protocol, using four BYDV isolates and four plant genotypes, enabled the assessment of the time-dependent infection percentage and the progression of the viral load in plants from the first day of BYDV detection until the end of the monitored period. Using the data from the infection kinetics obtained with the range of BYDV isolates, this study demonstrated that *Zhong ZH* and *TC14* lines (1) have an impact on the development rate of the barley yellow dwarf disease, by increasing the time required to reach maximal percentage of systemically infected plants from the 11th DAI in susceptible plant genotypes to the 21st DAI in resistant lines, (2) decrease the infection efficiency observed in leaves at 21 DAI from 98.7% for susceptible plant genotypes to 81.9% and 71.7% for *Zhong ZH* and *TC14*, respectively, (3) reduce in a similar manner (33–55%) the virus load in leaves of infected plants when compared with susceptible plant genotypes, (4) are not spared from BYDV infection as 95.1% of *Zhong ZH* and 90.2% of *TC14* inoculated plants accumulate viral particles in roots and/or in leaves at 21 DAI and (5) have an effect on the movement of the viral particles from root to aerial

Table 4. Area under concentration progress curves (AUCPC) values obtained for each isolate, host and isolate/host combination

	BYDV-PAV isolate				Mean per host[1]
	2T	4	13	RG	
Inoculated host					
Express	12.41	20.54	12.20	7.06	16.67 (a)
Sunstar	22.74	14.41	16.68	13.47	15.73 (b)
Zhong ZH	8.32	4.50	4.18	4.67	4.19 (c)
TC14	10.93	3.54	3.85	4.20	3.79 (c)
Mean per isolate[1]	13.31 (a)	9.65 (a)	8.27 (a)	6.74 (a)	

[1]Mean AUCPC value were calculated by taking into account the number of tested plants for each isolate/host combination. (a), (b), (c) and (d) correspond to the groups defined by Student–Newman–Keuls test at the 5% risk with the mean data.

parts of the plant. The statistical analysis of the time-dependent variables collected from the 1740 BYDV-inoculated plants confirmed the resistant behaviour of both *Zhong ZH* and *TC14* lines. In our experimental conditions, the development rate of the disease (*R*) was the only parameter that allowed the distinction between these two resistant lines. Such results are contradictory to previous published studies. Indeed, *Zhong ZH* and *TC14* lines have been described as lines exhibiting clearly different BYDV-resistant patterns. Based on qualitative ELISA tests performed on the youngest fully expanded leaves at one or two sampling dates, several authors have concluded that (1) the *Zhong ZH* line is more effective at reducing virus multiplication than the *TC14* line (Barloy et al., 2003), (2) 20–30% (Alaya et al., 2001; Barloy et al., 2003) of inoculated *TC14* were not infected while all inoculated *Zhong ZH* were infected (Barloy et al., 2003). Such varied results between the lines were probably due in part to the distinct genetic origins of resistance sources present in *Zhong ZH* (chromosome from *Th. intermedium* group 2) and *TC14* (chromosome from *Th. intermedium* group 7).

The use of a sampling procedure, in this present study, that takes into account (1) the homogeneity of the inoculum, (2) the heterogeneous distribution of virus in plant leaves, (3) the dynamics of the viral infection by sampling all developed leaves of each tested plant five times in the first three weeks of the infection and (4) the presence of viral particles in the plant roots with apparent virus-free leaves, leads to a revision of the previous conclusions about these resistant lines and improves their characterization.

As denoted by Balaji et al. (2003), the differential viral accumulation between resistant and susceptible plant genotypes, generally used as a resistance efficiency indicator, could vary from a 0- to a 14-fold decrease of viral quantities per plant depending on the day after inoculation. Such observation demonstrates that reports based on one or two sampling dates could not be used to accurately evaluate the resistance behaviour of the tested line. The delay between inoculation and detection of viral particles in the plants reflects the time required for BYDV to accumulate in the plant tissues at a level above the detection threshold of the method. Such a delay corresponds, for each plant/virus combination, to the result of additive or synergic effects between the dynamics of viral invasion of the host tissues and viral replication in infected cells. Contrary to most of the parameters previously used for *Zhong ZH* and *TC14* resistance characterisation, the dynamics of the infection process and the exposure of BYDV in plants during the monitored period were both assessed by the use of (1) a monomolecular model that fits the time-dependent percentage of infected plants and (2) the AUCPC. Such a temporal approach allowed us to conclude that during the first 21 days of the BYDV infection, the exposure of virus in resistant lines was reduced by 75% when compared with the BYDV-susceptible control lines. Reduced virus titer has already been studied by some authors and has proven of interest by reducing yield losses (Gray et al., 1994; Alshahwan et al., 1995) or by decreasing the dissemination of the virus by lowering its transmission efficiency from plant to plant (Pereira et al., 1989; Caroll et al., 2002; Sivamani et al., 2002). The exposure concept constitutes a better resistance efficiency indicator than single or double observations during the infection process as it includes recurrent measures of the host response (virus load and infection status) from inoculation to systemic infection.

The escape rates of *Zhong ZH* and *TC14* obtained in this study are in agreement with previous results. As an example, an escape proportion of 20% of inoculated *TC14* plants was denoted and our results are in agreement with this value (Alaya et al., 2001; Barloy et al., 2003). However, the analysis of our results, performed using the whole data set, led to some original conclusions for the *TC14* line. Indeed, more than 2/3 of the defined healthy plants by the ELISA tests performed on leaves accumulated particles in their roots resulting in 90.2% overall infection percentage of the *TC14* line at 21 DAI. Such leaves/roots differential behaviour in virus accumulation has been already described with the BYDV-resistant chromosome 7 substitution line *P29* (Anderson et al., 1998). Moreover, the infection percentage pattern obtained during the monitored period for resistant lines did not include a plateau. As a consequence, the 80% infection obtained at leaf level at 21 DAI would probably increase to reach the global leaves/roots infection percentage later in the infection process. However, *Zhong ZH* and *TC14* lines delay sys-

temic infection. Thus, these results support an impact of these resistance lines on viral movement in the plant, especially long-distance movement from roots to aerial parts.

The use of the exposure of plants to virus, i.e. studying dynamics of viral accumulation through time, gave a better understanding of the resistances. Such knowledge is required for the integration of any resistance source in breeding programmes. The partially resistant behaviour of *Zhong ZH* and *TC14* was confirmed in this study and the involvement of long-distance movement in their resistance mechanism was proposed. However, before using these resistances on a large scale, their potential durability should be tested. Such aspects are currently under investigation in different international laboratories.

Acknowledgements

The authors wish to thank Olivier Valles, Gaëlle Mauvais, Agnès Delaunay and Christine Helloco-Kervarrec for their help in data acquisition, Joseph Jahier for providing seeds and Charles-Antoine Dedryver for critical reading of the manuscript. The authors are especially grateful to Olivier Plantard for his constructive remarks. This work was supported by the French National Institute for Agronomic Research (INRA) and Région Bretagne.

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