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Evaluation of various methods for the detection of barley yellow dwarf virus by the tissue-blot immunoassay and its use for virus detection in cereals inoculated at different growth stages

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Abstract. Various modifications of the tissue-blot immunoassay (TBIA) for the detection of barley yellow dwarf virus (BYDV, luteovirus) were compared. Similar results were obtained by using three different labelled molecules; goat anti-rabbit antibodies conjugated to alkaline phosphatase, protein A conjugated with alkaline phosphatase and goat anti-rabbit antibodies conjugated with colloidal gold. Blocking the nitrocellulose membrane with polyvinyl alcohol for 1 min was effective and allowed the procedure to be shortened by one hour. TBIA was sensitive enough to detect BYDV in old dry tissue wich had been soaked in water for 1 h.

BYDV was monitored by TBIA in wheat, oat and barley after inoculation at heading, flowering and grain filling growth stages. The later the inoculation date, the greater the chance of detecting the virus in stem bases rather than in the upper part of the stem. The later the inoculation the less virus moved, from the inoculated tiller to other tillers of the same plant.

Introduction

Since the development of the enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses [Clark and Adams, 1977], many variations have been introduced to improve sensitivity and specificity or to simplify the procedure [Van Regenmortel, 1982]. The recent development of a tissue-blot immunoassay (TBIA) was a further improvement which permitted sensitive detection of several plant viruses and most importantly eliminated the need for sample extraction [Lin et al., 1990; Hsu and Lawson, 1991].

In TBIA, a secondary labelled antibody is used, the most common being the goat anti-rabbit antibodies conjugated to alkaline phosphatase. This procedure has been shown to be a practical method for detecting barley yellow dwarf virus (BYDV, luteovirus) [Makkouk et al., 1994]. Other labelled molecules such as protein A conjugated with alkaline phosphatase [Zagula et al., 1990] and antibodies conjugated with colloidal gold [Hsu, 1984] were also reported for detecting different antigens.

In this study, the effectiveness of three different labelled molecules for the detection of BYDV by TBIA was compared. The detectability of BYDV in different cereals inoculated at different growth stages and at different intervals after inoculation was investigated.

Materials and methods

Plant materials

Six cereal cultivars were used: susceptible wheat *Triticum aestivum* cv. Katepwa, tolerant wheat *T. aestivum* cv. Maringa, susceptible oat *Avena sativa* cv. Scott, tolerant oat *A. sativa* cv. C.I. 9311, susceptible barley *Hordeum vulgare* cv. Abee and tolerant *H. vulgare* cv. barley 8081 BQCB-10.

Virus inoculation

The virus isolate used was from Quebec and had been identified earlier by W.F. Rochow, Cornell University, New York, as a PAV serotype of BYDV. A Quebec colony of the aphid *Rhopalosiphum padi* was reared in a growth chamber and used for virus acquisition and inoculation. Plants to be inoculated were grown in 15 × 15 cm pots (3 pots with 3 plants each per treatment) and maintained in a growth room with a 16 h day length. Temperature was maintained at 22 °C during the light period and 18 °C in the dark. Each plant was allowed to produce three tillers.

Cereal plants were inoculated at three different growth stages. The first group was inoculated during heading (Zadoks growth stage (GS) 40) and the second group at flowering (GS 53). In these two groups only one tiller (out of three) was inoculated on each plant using a mini-cage. The third group was inoculated during grain filling (GS 71), and all tillers were inoculated. BYDV inoculation was done by placing 10–15 aphids per tiller; aphids were killed 48 h later by an insecticide spray (Pirimor).

Tissue-Blot Immunoassay (TBIA)

The procedure followed for the tissue-blot immunoassay was essentially as described [Lin et al., 1990; Hsu and Lawson, 1991]. Three treatments were compared for blocking the nitrocellulose membranes after printing the stem sections, 1) 1% bovine serum albumin (BSA) + 3% non fat dry milk in phosphate buffered saline, pH 7.4 containing 0.05% tween 20 (PBS-T); 2) 5% goat normal serum in PBS-T; and 3) 1 μg/ml polyvinyl alcohol (PVA) (Sigma P-8136) in PBS-T.

The primary antibodies used were a 1:1000 dilution of a BYDV antiserum produced in our laboratory against the Cloutier PAV isolate of

BYDV. To detect the primary antibodies, three different labelled molecules were compared: 1) goat anti-rabbit antibodies conjugated to alkaline phosphatase (GAR-AP) (Sigma A-8025); 2) goat anti-rabbit antibodies conjugated with colloidal gold (GAR-G) (Sigma G-7277); and 3) protein A conjugated to alkaline phosphatase (PA-AP) (Sigma P-9650). The nitroblue tetrazolium (NBT)/5-bromo-chloro-3-indolyl phosphate (BCIP) substrate was used with alkaline phosphatase conjugates and the silver enhancer kit (Sigma SE-100) was used to assay for colloidal gold.

Results

Membrane blocking

The three blocking solutions BSA + non-fat milk for 1 h, normal goat serum for 1 h and PVA for 1 min were equally effective in producing a clean background on the nitrocellulose membrane. The last procedure, however, provided a one hour saving in time and was used in all subsequent tests.

The use of GAR-AP as the secondary labelled antibody produced well defined staining of the phloem bundles in the infected tissue and no reaction in the healthy wheat tissue (Fig. 1B and E). Similar results were obtained with oat and barley. BYDV was easily detected in all susceptible cereals tested. When inoculated at the 2–3 leaf stage BYDV was detected in susceptible cultivars 2–3 days after inoculation and then until maturity.

Efficiency of different labelled molecules

When the three labelled molecules GAR-AP, GAR-G and PA-AP were used for BYDV detection, all proved to produce virus-specific staining in the phloem bundles of infected cereals (Fig. 1A, B, C). GAR-AP and PA-AP conjugates gave a satisfactory reaction at dilutions of 1:1000–1:2000. GAR-G was suitable at dilutions of 1:500–1:1000. However, the prepared dilutions of GAR-AP or PA-PA were found to be more stable, as they were reused up to 8–10 times without significant loss in reactivity. The GAR-G conjugate, on the other hand, was used only 1–2 times. Thereafter the reaction was not satisfactory, as the specific reaction was reduced and the non-specific background was increased.

Detection of BYDV in dry stems

For dried stems it was necessary to soak them in water before cutting and blotting. Four soaking periods of 1, 2, 4, and 24 h were compared. The best results were obtained by soaking the stems for 1 h.

Periods of contact of 10, 20, 30 and 40 s between the stem cut surface

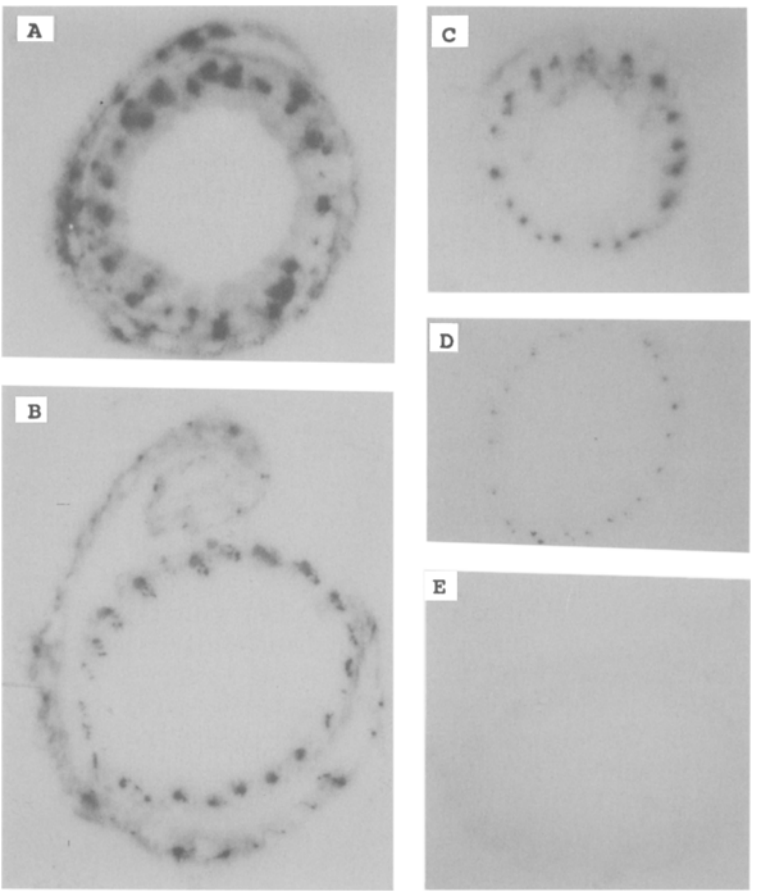

Fig. 1. Tissue-blot immunoassay of stem sections of the wheat cultivar Katepwa inoculated with barley yellow dwarf virus (BYDV) (A, B, C, D) as compared to that of uninoculated stem section (E). The secondary labelled molecules used were: protein A conjugated to alkaline phosphatase (A), goat anti-rabbit antibodies conjugated to alkaline phosphatase (B) and goat anti-rabbit antibodies conjugated with colloidal gold (C). BYDV detection in a dry stem section (using the same system as in B) is shown in D.

and the membrane were tested. The best results were obtained with 30 or 40 s. Therefore, for routine testing, dry stems were soaked in water for 1 h, cut by a single edge razor blade and pressed on the membrane for around 30 s (Fig. 1D). With this approach, BYDV was detected in wheat spike peduncles of infected plants which had been stored at room temperature for 5 years.

Non-developed tissue blots of infected tissue on the nitrocellulose membrane were fairly stable. Keeping the blots at room temperature for one month did not reduce the strength of the reaction.

Effect of incubation time on BYDV detection

Inoculation at the Boot/Heading Stage (GS 40)

In wheat, BYDV was detected by the tissue-blot immunoassay at 20, 40 and 60 days after inoculation. Even only one of three tillers per plant was inoculated in this experiment, BYDV was detected in all three tillers of the susceptible wheat Katepwa 40 and 60 days after inoculation and two out of three (2/3) and 3/3 in the tolerant wheat Maringa at 40 and 60 days after inoculation, respectively (Fig. 2).

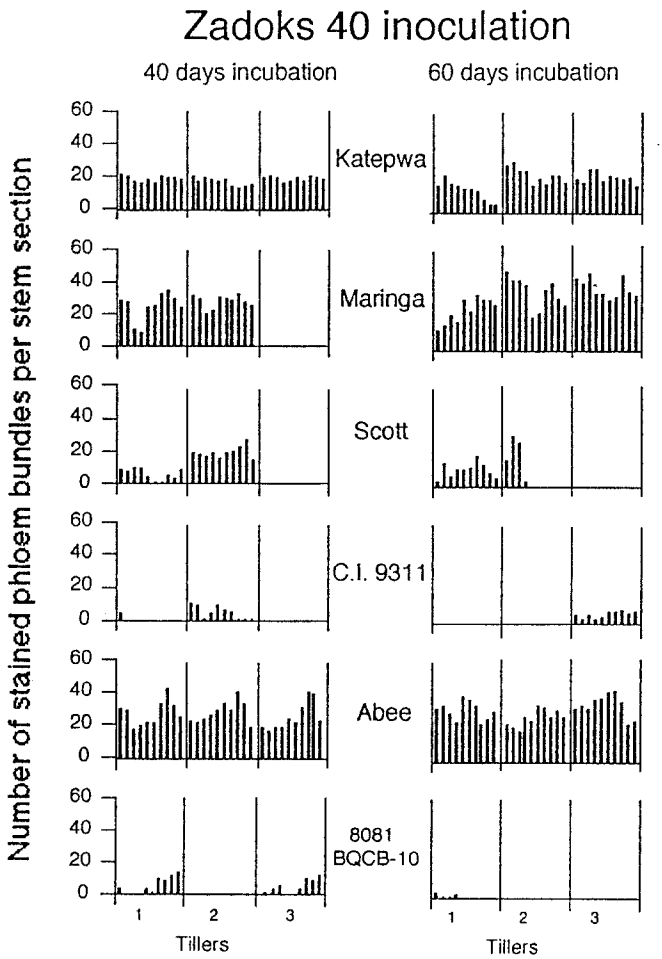

Fig. 2. Numbers of stained phloem bundles in three tillers of the same plant (one of them was inoculated) for the wheat cultivars Katepwa (susceptible) and Maringa (tolerant), oat cultivars Scott (susceptible) and C.I. 9311 (tolerant), and the barley cultivars Abee (Susceptible) and 8081 BQCB-10 (tolerant). Barley yellow dwarf virus (BYDV) was inoculated at Zadoks 40 growth stage and testing for BYDV was made at 40 and 60 days after inoculation. In each tiller, virus presence was determined by the tissue-blot immunoassay at 10 positions along the stem, the leftmost bars represent infected phloem bundles close to the collar and the rightmost close to the spike.

BYDV was detected in oat at 20, 40 and 60 days after inoculation. Virus invasion was more rapidly detected in the susceptible oat Scott than in the tolerant oat C.I. 9311. However, movement of virus from the inoculated tiller to the non-inoculated tillers in the susceptible Scott was slow, as 60 days after inoculation virus had moved only to another tiller of the same plant; the third tiller remained virus-free (Fig. 2). Virus movement was even slower in the tolerant oat C.I. 9311 as virus was almost restricted to the inoculated tiller. Detection of BYDV in the susceptible barley Abee was similar to that in wheat at 40 and 60 days after inoculation, whereas virus invasion in the resistant barley 8081 BQCB-10 was limited and there was little movement from the inoculated to non-inoculated tillers.

Inoculation at flowering (GS 53)
When plants were inoculated at the flowering stage, virus invasion was much slower than in wheat. Inoculated at GS 53, 20 days after inoculation virus was detected only in the stem bases of all three tillers of both susceptible and tolerant wheat genotypes (Fig. 3), but was not detected in the upper part of the stem of any of the three tillers of the susceptible wheat Katepwa and only in one of the three tillers of the tolerant wheat Maringa. 40 days after incubation, virus was detected everywhere in the stem of one tiller of three of both wheat genotypes, suggesting that virus movement out from the inoculated tiller was restricted (Fig. 3).

BYDV was detected 20 days after inoculation in one of the three tillers of the susceptible oat Scott but not in any of the tillers of the tolerant oat C.I. 9311. Virus invasion did not change much when assayed 40 days after inoculation. However, at this later date, more tillers were tested positively when stem bases, rather than stem tops, were assayed (Fig. 3).

Virus invasion in the susceptible barley Abee was significantly more extensive at 20 days than at 40 days after inoculation; this was unexpected. Virus invasion of the resistant barley 8081 BQCB-10 was much less than in Abee when assayed at 20 or 40 days after inoculation and more tillers tested positive at the stem base than in the upper part.

Inoculation at the grain filling stage (GS 71)
When plants were inoculated at the grain filling stage, the virus was detected in the stem bases of susceptible and tolerant wheat genotypes 20 days after inoculation, but not in most stem tops (Fig. 4). The pattern of BYDV detection in the stem basses and tops of both oat and barley was similar to that of wheat. However, the difference in virus spread between tolerant and susceptible genotypes of oat and barley was clearer than in wheat.

Table 1 summarizes the data obtained and provides a comparative evaluation for BYDV detection by TBIA if conducted only at the bases or only at the tops of the cereal stems. The data suggest that when inoculation was done at the boot or early heading stage, assessment of number of tillers

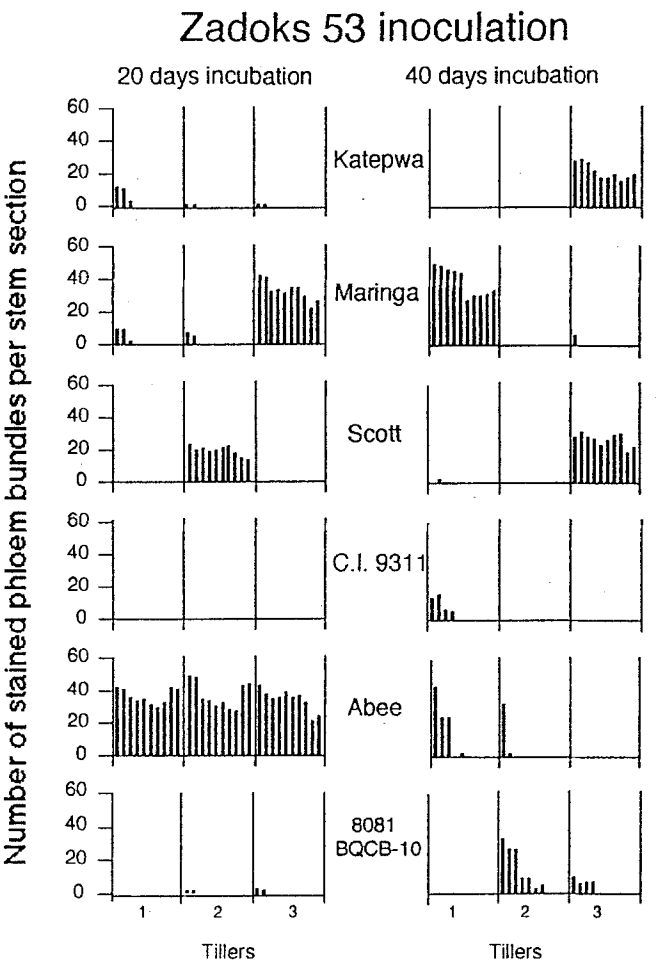

Fig. 3. Number of stained phloem bundles in three tillers of the same plant (one of them was inoculated) for six cereal cultivars (see caption of Fig. 1). Barley yellow dwarf virus (BYDV) was inoculated at Zadoks 53 growth stage and testing for BYDV was made at 20 and 40 days after inoculation. Virus assay was as described in the caption of Fig. 1.

infected were more or less similar, if tests were made from either stem bases or tops. However, when inoculation was done at flowering or grain filling stages, positive virus detection at stem bases was many folds higher than that in stem tops.

Discussion

TBIA proved to be a simple, sensitive and quick method for BYDV detection, as has been recently reported [Makkouk et al., 1994]. The three

Table 1. Tissue blot immunoassay of BYDV from the top and bottom of stems of various cereals inoculated at three different plant growth stages

Germplasm	Incubation period (days)	No. of tillers infected / No. assayed					
		Inoculation* at Zadoks growth stage (GS)					
		GS 40		GS 53		GS 71	
		base	top	base	top	base	top
Wheat							
Katepwa	20	–	–	3/3	0/3	3/3	0/3
	40	3/3	3/3	1/3	1/3	–	–
	60	3/3	3/3	–	–	–	–
Maringa	20	–	–	3/3	1/3	3/3	1/3
	40	2/3	2/3	1/3	1/3	–	–
	60	3/3	3/3	–	–	–	–
Oat							
Scott	20	–	–	1/3	1/3	2/3	0/3
	40	2/3	2/3	2/3	1/3	–	–
	60	2/3	1/3	–	–	–	–
C.I. 9311	20	–	–	0/3	0/3	1/3	0/3
	40	2/3	0/3	1/3	0/3	–	–
	60	1/3	1/3	–	–	–	–
Barley							
Abee	20	–	–	3/3	3/3	3/3	1/3
	40	3/3	3/3	3/3	0/3	–	–
	60	1/3	1/3	–	–	–	–
BQCB-10	20	–	–	2/3	0/3	2/3	2/3
	40	2/3	2/3	2/3	0/3	–	–
	60	1/3	0.3	–	–	–	–
Total		27/36	23/36	22/36	8/36	14/18	2/18

* Inoculation at growth stages Zadoks 40 and 53 were made to one tiller out of three per plant, whereas inoculation at growth stage 71 was made to all three tillers per plant.

different labelled molecules used for BYDV detection in this study were equally successful. However, protein A and goat anti-rabbit conjugates were more economical, as it was possible to reuse the diluted preparation several times without significant loss in sensitivity.

The use of polyvinyl alcohol as a blocking agent instead of BSA + milk or goat normal serum allowed the test period to be shortened by one hour without any loss in sensitivity. The use of PVA generally gave slightly stronger specific reactions, suggesting that virus displacement during the short PVA treatment was less than that with other blocking agents as reported recently by Miranda et al. [1993]. Thus, the test for BYDV can be easily completed within 3 h.

Results of experiments on BYDV detection as influenced by the infection at various growth stages, showed that virus detection by TBIA is

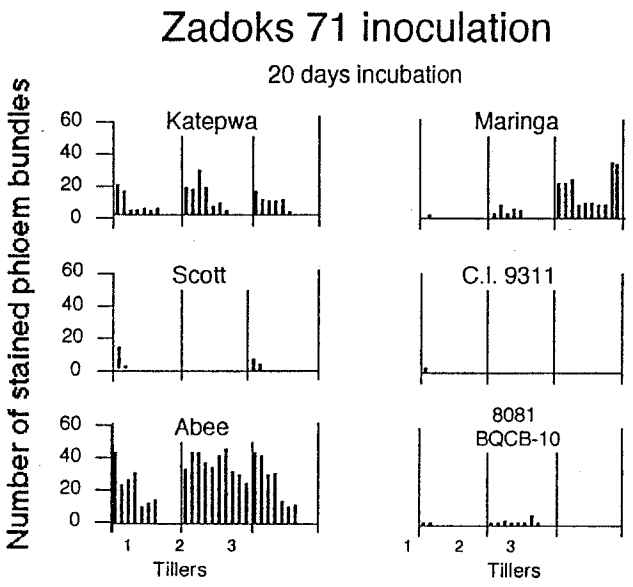

Fig. 4. Number of stained phloem bundles in three tillers of the same plant (all were inoculated) for six cereal cultivars (see caption of Fig. 1). Barley yellow dwarf virus (BYDV) was inoculated at Zadoks 71 growth stage and testing for BYDV was made 20 days after inoculation. Virus assay was as described in the caption of Fig. 1.

possible even when plants were inoculated at a late stage. However, more precise data on the percentage of infected plants can be obtained if the blots were made from basal stem sections rather than from upper stem sections. This is especially important for a successful detection of BYDV in tolerant cereal cultivars. Detection of late BYDV infection is of practical value, as it was previously shown that late inoculation caused significant yield losses in most cereal cultivars [Comeau, 1987].

It was not our intention in this study to provide a comparison between tolerant and susceptible cereal cultivars, but to assess detection patterns as influenced by different plant growth stages and genetic backgrounds of wheat, oat and barley. Nevertheless it was clear from the data obtained that the tolerant wheat cultivar Maringa did not restrict virus spread suggesting that the tolerance of this genotype is not related to virus multiplication or movement. In the cases of tolerant barley and oat cultivars 8081 BQCB-10 and C.I. 9311, however, there was an indication that virus multiplication and/or movement was restricted [Makkouk et al., 1994].

Current studies showed that there was restriction of virus passage from the inoculated tiller to other tillers of the same plant in both resistant and susceptible genotypes. Such a trait is important at the field level, as it reduces virus spread, especially in case of late infections. Thus, in assessing virus infections, one should not assume that all the tillers of one plant are infected if only one of them tested positive for BYDV. However,

for disease survey purposes, collecting a large number of tillers at random from a single field will compensate for the variability within the tillers of each plant.

The quantification of the reaction produced by the tissue-blot immunoassay was done by counting, under the steromicroscope, the number of stained phloem bundles per stem section blot, irrespective of the size of the stained bundle or its colour intensity. More precision and speed can be obtained by using a digital image analysis system [Shine and Comstock, 1993].

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