# Purification and Serology of a Moroccan Isolate of Barley Yellow Dwarf Virus

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**Abstract**

**El Yamani, M., and Hill, J.H. 1990. Purification and serology of a Moroccan isolate of barley yellow dwarf virus. Arab J.Pl. Prot. 8(1): 44 - 41.**

A Moroccan PAV-Like isolate of barley yellow dwarf virus was purified in Morocco from symptomatic oat plants (*Avena sativa* L.«Clintland«64») previously inoculated with viruliferous *Rhopalosiphum padi* L. The technique included clarification with chloroform, precipitation with polyethylene glycol, and centrifugation through sucrose density gradient columns. The purified virus preparation had an $A_{260}/A_{280}$ of 1.62 and an ultraviolet absorption spectrum characteristic of nucleoproteins. Virus yield averaged 1.45mg/kg fresh weight of tissue. The virus preparation was used to raise antibodies in rabbits. Antisera had a homologous titer as high as 1/3000 in indirect enzyme-linked immunosorbent assay.

**Keyword:** luteovirus, Morocco.

## Introduction

Barley yellow dwarf virus (BYDV), the type member of the luteovirus group, encompasses several strains of isometric phloem-limited, aphid-borne, and non-sap-transmissible viruses (12,14). The first attempts to purify BYDV resulted in very low yields from infected plant tissue (15, 17). Recently, however, improved purification techniques have increased virus yield substantially (3, 7, 11, 13). The use of macerating enzymes to improve virus yield was described for some luteoviruses (19). Additionally, several variations of the enzyme-linked immunosorbent assay (ELISA) (2, 4, 8, 9) have provided a quantitative serological method for monitoring yields of BYDV. In most cases, ELISA correlates with vector specificity for differentiating among BYDV strains (18), allowing its use as a routine technique for virus identification and assay.

In Mrocco, BYDV was identified by using aphid transmission. It has since been demonstrated to be important in all the cereal-growing areas in Morocco (5, 6, 10). The objective of the present study was to purify a Moroccan isolate of BYDV in Morroco in order to raise specific antiserum for diagnostic purposes.

## Materials and Methods

**Virus origin and purification.** The virus isolate used in this study originated from Beni Mellal and reacted as a PAV-like isolate (6). Virus was purified from symptomatic oat plants (*Avena sativa* L. «Clintland» «64»), which had been inoculated at the 2-leaf stage with viruliferous *Rhopalosiphum padi* L. Oats were grown in a steam-sterilized soil mix. Plants were grown in growth chambers at 15 - 20°C with a 16-hr photoperiod. In general, the purification technique used was as described by Hammond et al. (7).

Inoculated plants were harvested when symptoms developed after inoculation and stored at -80°C until used. Frozen plant material was ground in 0.5M sodium phosphate buffer, pH 6.0, (1:3; w/v) containing 0.5% sodium sulfite and 0.01 M diethyldithiocarbamic acid (DIECA). The product was filtered through cheesecloth, a 2:1 mixture of chloroform:n-amyl alcohol was added dropwise to one fifth volume, and the mixture was blended for 30 sec. at low speed. After incubation for 60 min at 4°C, the mixture was centrifuged at 7,000 rpm for 10min in a Beckman JA14 rotor. Sodium chloride and polyethylene glycol (mol. wt.= 6,000) were added to the supernatant to 0.25 and 10%, respectively, with stirring for 90min at 4°C. Virus was collected by centrifugation at 7,000rpm for 20min as above, and the pellets were resuspended at 4°C overnight in 10ml of 0.1M sodium phosphate, pH 7.0 containing 0.01% $NaN_3$. After centrifugation at 10,000 rpm for 30min in the Beckman JA20 rotor, preparations were layered on a 7-ml cushion of 30% sucrose in 0.1M sodium phosphate, pH 7.0, in Beckman 30 rotor tubes. After centrifugation for 4hr at 27,000 rpm, pellets were resuspended in 1 ml each of 0.1M sodium phosphate buffer, pH 7.0, at 4°C. The preparation was centrifuged at 10,000 rpm for 10 min (Beckman JA20 rotor), and fractions of 0.5 to 1ml each were layered on a 7.5% to 30% (1) linear sucrose density gradient prepared in 0.1M sodium phosphate, pH 7.0. The preparation was centrifuged for 120 min in a SW41 rotor at 30,000 rpm and fractionated with a density gradient fractionator (ISCO Model 640, ISCO, Inc., Lincoln, NE) coupled to an absorbance/fluorescence detector (ISCO Model UA-5). Recovered virus was diluted with four volumes of 0.1 M sodium phosphate, pH 7.0, and concentrated by centrifugation at 27,000 rpm for 4hr in Beckman 30 rotor. Virus was resuspended in 0.1 M sodium phosphate, pH 7.0, and centrifuged at 10,000 rpm for 30 min in the Beckman JA20 rotor. Virus concentration was determined spectrophotometrically with an assumed extinction coefficient of $E^{0.1\%}_{260} = 6.0$(13). The virus preparations were stored at -26°C in the presence of 0.01% $NaN_3$(16).

**Antiserum preparation.** One hundred $\mu$g of purified virus mixed with Freund's complete adjuvant at a ratio of 1:1 was injected intramuscularly into two rabbits at three weekly intervals. Animals were exsanguinated weekly by cardiac puncture 2 weeks following the last injection. Antiserum titer was determined by indirect ELISA.

**Indirect enzyme-linked immunosorbent assay (I-ELISA).** I-ELISA was similar to procedures described elsewhere (4, 9). Wells of Immulon 1 or 2 (Dynatech Laboratories, Alexandria, VA.) microtiter plates were coated with virus antigen (5$\mu$g/ml, 100 $\mu$l/well) in 0.05M carbonate-bicarbonate, pH 9.6, and incubated overnight at 4°C. After plates were washed three times, 4min each, with wash buffer (0.01 M phosphate buffered saline [PBS], pH 7.4, containing 0.85% NaCl, 0.05% Tween 20 and 0.02% KCI), unbound sites in the wells were blocked for 2 hr at room temperature with 400 $\mu$l per well of PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. The plates were washed as just described above and antiserum (in 0.01 M PBS-Tween 20 containing 2%polyvinylpyrrolidone, MW 40,000, and 0.2% BSA) was added (100 $\mu$l/well) in two adjacent wells. After a 4-hr incubation at 37°C, the plates were washed, and alkaline phophatase-conjugated (100 $\mu$l/well) goat-anti-rabbit polyclonal IgG (Sigma Chemical Co., St. Louis, MO, A-8025) diluted 1/500 in PBS (100 $\mu$l/well) was added. The plates were incubated for 4hr at37°C; after the plates were washed, p-nitrophenyl phosphate (1mg/ml in 10% diethanolamine, pH 9.8) was added (100 $\mu$l/well), and plates were incubated for 30 - 60 min at 37°C. Reaction products were measured at 410 nm with a Minireader II (Dynatech Laboratory, Inc.). Healthy plant sap from «Clintland «64» oats was used as a control. Additional controls were preimmune sera from the two rabbits.

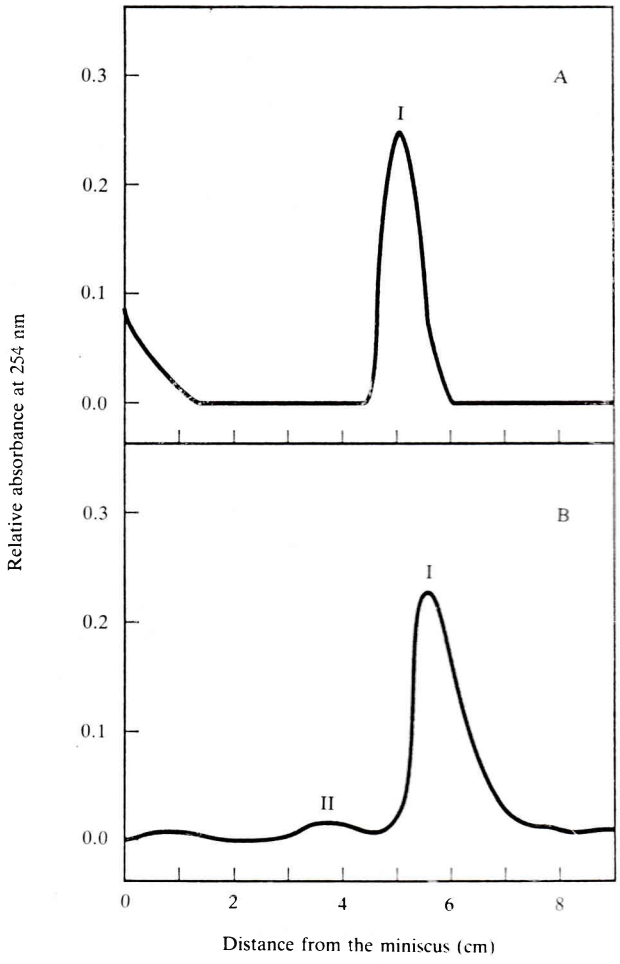


**Figure 1.** Ultraviolet absorption profiles of barley yellow dwarf virus preparations purified from cultivar «Clintland «64» oats and separated on linear 7.5 - 30% sucrose gradients in 0.1 M sodium phosphate, pH 7.0, centrifuged at 30,000 rpm for 3 hr in a Beckman SW41 rotor. Generally a single peak was obtained (A), but occasionally, a second peak was observed (B).

## Results

**Virus purification.** Virus preparations usually yielded one visible band (peak I) at about 4 cm from the meniscus of the sucrose density gradient column following centrifugation (Fig. 1 A).

Occasionally (1 of 3 purification attempts), a small or minor peak (peak II, Fig. 1 B) was evident in all the tubes scanned. In further tests, peak II reacted only with antibodies raised against the nonspecific PAV-like strain of BYDV in a direct ELISA described elsewhere (6). Purified virus preparations had an ultraviolet absorption spectrum characteristic of 2 nucleoproteins with an absorption maximum at 260 nm and a minimum at 240 nm (Fig. 2). The $2_{260}/A_{280}$ of the three virus preparations were 1.70, 1.63, and 1.53, which gave a mean value of 1.62. Virus yields ranged from 1.25 to 1.65 mg/kg, with an average of 1.45 mg/kg fresh weight of oat plant tissue.

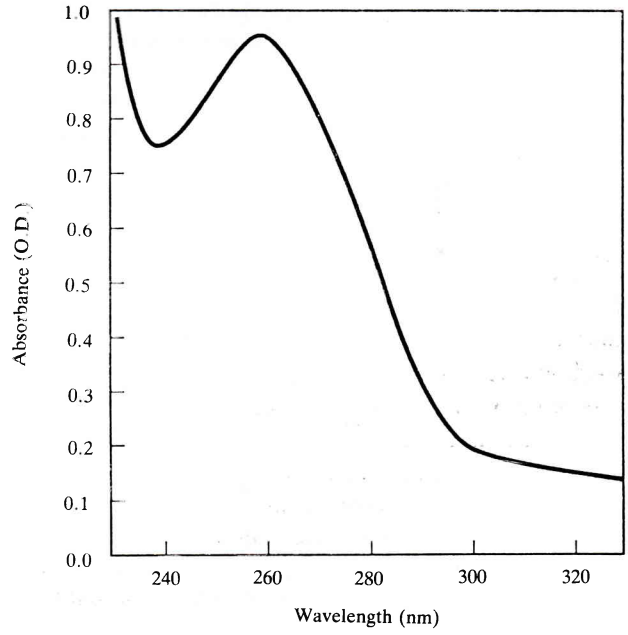


**Figure 2.** Ultraviolet absorption spectrum of a purified preparation of PAV-BYDV obtained from infected «Clintland «64» oat tissue.

**Antiserum preparation.** Table 1 summarizes the characteristics of the antisera produced by the two rabbits 17 and 27 days following the last injection. Only one rabbit survived the first bleeding, which permitted only one second bleeding. Reaction products, as measured by $A_{410}$ in the presence of the virus, were 3 to 4-fold greater than those obtained in the presence of healthy oat sap. On the basis of the criterion that $A_{410}$ values greater than that of healthy sap plus three standard deviation are considered positive (4), it was concluded that the antisera harvested after 17 days had a 1/1000 titer and that harvested after 27 days had at least a 1/3000 titer, which represented the highest dilutions used in these trials. Preimmune sera from both rabbits included in the tests as controls gave low reactions with healthy sap ($A_{410}$ = 0.03and 0.04 O.D.) as well as with pure virus ($A_{410}$= 0.04 and 0.05 O.D.).

**Table 1.** Titration of antisera prepared against a Moroccan PAV-like isolate of barley yellow dwarf virus by using the indirect enzyme-linked immunosorbent assay (I-ELISA).

| Time between last injection and exsanguination | Animal used | Antigen used | $A_{410}$[a] Reciprocal of antiserum dilution (x 10) 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 days | Rabbit I | Pure virus (500ng/well) | 0.24 | 0.19 | 0.17 | 0.19 | 0.19 | 0.17 | 0.17 | 0.17 | 0.17 | 0.16 |
| | | Healthy sap | 0.05 | 0.04 | 0.06 | 0.05 | 0.05 | 0.06 | 0.04 | 0.04 | 0.04 | 0.04 |
| | Rabbit II | Pure virus (500ng/well) | 0.33 | 0.30 | 0.25 | 0.28 | 0.23 | 0.28 | 0.25 | 0.25 | 0.20 | 0.19 |
| | | Healthy sap | 0.04 | 0.04 | 0.04 | 0.05 | 0.06 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 |
| | | | Reciprocal of antiserum dilution (x 10) 50 | 100 | 150 | 200 | 250 | 300 | | | | |
| 27 days | Rabbit II | Pure virus (500ng/well) | 0.63 | 0.42 | 0.30 | 0.25 | 0.20 | 0.19 | | | | |
| | | Healthy sap | 0.15 | 0.13 | 0.14 | 0.14 | 0.09 | 0.07 | | | | |

a) $A_{410}$ readings are the mean value of two values per dilution.

## Discussion

A PAV-like isolate of BYDV from Morocco has been successfully purified. The ultraviolet absorption profiles of scanned sucrose density gradient tubes containing preparations of this isolate as well as absorption ratios ($A_{260}/A_{280}$ = 1.53 to 1.70) performed on pure virus during this study were similar to those reported elsewhere for this strain of BYDV. Indeed, absorption ratios of 1.89 and 1.76 were reported for Illinois and Indiana isolates, respectively (3,7), in the United States, 1.71 for a Japanese isolate (11), and 1.71 to 1.79 for a Canadian isolate of the same strain of BYDV (13). The peak II in Fig. 1B could have originated from denaturation of virions, giving empty shells that migrated less rapidly in the sucrose columns compared with entire virus particles. This is consistent with the fact that preparations of peak II reacted positively in direct ELISA with antisera against a PAV-like isolate of BYDV. Our virus yield, which averaged 1.45 mg/kg fresh weight of «Clintland «64» oats, was reasonably high. However, this yield is still low compared with an average of 4.4 mg/kg fresh weight of root tissue obtained by D'Arcy et al. (3). Since ELISA was adapted for plant viruses by Clark and Adams (2), several other forms of this technique, including the indirect ELISA, have been developed. Lommel et al. (9) demonstrated the usefulness of the indirect ELISA for studying carnation viruses. Diaco et al. (4) studied serological relationships among BYDV. They reported that variation in antibody specificity was dependent upon the use of directly labelled or unlabelled antibodies. The present study supports the use of indirect ELISA as a rapid and effective detection technique. Antiserum titers obtained in this study were very encouraging; thus, an antiserum is now available to the most common Moroccan isolate of BYDV (PAV). Consequently, ELISA will make routine diagnosis of this isolate possible in Morocco. This will augment laborious aphid transmission techniques (18). Efforts are continuing to obtain similar results for the other BYDV strains present in the country.

## Acknowledgement

This research was supported in part by US-AID/MIAC project No 608-0136. Journal Paper No. J-13763 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project 2428.

## الملخص

**اليماني محمد، وجون. هـ. هيل. 1990. استخلاص وتنقية فيروس إصفرار وتقزّم الشعير (BYDV) في المغرب، وتحضير مصل مضاد له. مجلة وقاية النبات العربية 8(1): 44 - 41.**

تمّ في المغرب، استخلاص وتنقية فيروس إصفرار وتقزّم الشعير (BYDV) الطراز «PAV» من نبات شوفان (خرطال) *Avena sativa* L. صنف «Clintland 64» سبق تلقيحها بالفيروس بوساطة نوع المنّ *Rhopalosiphum padi* L.. وقد جرت التنقية على عدة مراحل تضمنت استخلاص العصير النباتي بالكلوروفورم، وترسيبه بوساطة بولي إيثلين جليكول، ثم تنقيته وذلك بتثقيله في أنابيب تحوي تراكيز متدرجة من السكروز. كانت نسبة امتصاص مستخلص الفيروس للأشعة فوق البنفسجية عند موجة 260 نانومتراً مقارنة بامتصاصه لها عند موجة 280 نانومتراً تعادل 1.62. وبلغ معدل كمية الفيروس التي تم استخلاصها 1.45 مغ/كغ وزن رطب من نبات الشوفان. هذا وقد تمّ استعمال مستخلص الفيروس لانتاج مصل في الأرانب، واحتفظ المصل بكفاءته في كشف الفيروس باستخدام اختبار «الإليزا» غير المباشر حتى بعد تخفيفه بمعدل 1/3000.

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