Characterization of Monoclonal Antibodies Raised Against Citrus Tristeza Virus in Morocco

M. Zebzami, J. H. Hill, R. A. Van Deusen, and E. B. Nadori

ABSTRACT. Fourteen hybridoma cell lines, which secreted monoclonal antibodies (McAb) to citrus tristeza virus (CTV), were produced by fusion of spleen cells from a BALB-c mouse, immunized with purified CTV from Satsuma mandarin and nonsecretor SP2/0 cells. The McAbs were tested by indirect enzyme-linked immunosorbent assay (ELISA) for detection of five Moroccan CTV isolates that showed a wide variation in symptoms on Mexican lime. This test revealed five reaction patterns where eight McAbs were able to react with all CTV isolates tested. Preliminary results indicated that McAbs 4B1 and 4F3, and 4C1 and 4E5 detected, respectively, 57 and 58 of 59 isolates from an international CTV collection. McAbs 4B1, 4F3 and 4C1 were labeled with alkaline phosphatase and used in simultaneous and consecutive competition assays against each other as well as with McAbs 3DF1 and 3CA5 from Spain, MCA13 from Florida, and 4E5 from Morocco. There was no competition with 3DF1 and MCA13. All other assays revealed some competition. These data suggested the existence of at least two epitopic regions on the Satsuma CTV isolate.

Citrus tristeza virus (CTV), although rarely encountered in Morocco, constitutes a potentially significant threat to citriculture because citrus is grown primarily on sour orange rootstock. Surveys have been conducted to detect and eradicate infected trees from orchards of old introductions (4, 10), and an extensive testing program is in progress to control potential CTV introduction via recent importations of citrus material.

Monoclonal antibodies (McAbs), specific to CTV, have been produced in several laboratories (5, 9, 11, 15). So far however, no individual McAb was found to react with all CTV isolates (3). A broadly reactive probe for CTV detection is needed for an efficient eradication of tristeza, especially in geographic regions where the disease is still not endemic such as North Africa. The objective of this study was to produce McAbs able to react with a large number of CTV isolates. Description of the experimental strategy adopted and characterization of obtained CTV specific McAbs are reported.

MATERIALS AND METHODS

Virus sources. CTV isolates used in this study were isolated from citrus germplasm during previous surveys for CTV (10). The Satsuma isolate used as an immunogen was obtained from Saigon Satsuma (SS) mandarin. An isolate obtained from Meyer lemon (ML) was used for initial screening of primary hybridoma cell lines. Other isolates used were obtained from Tripoli orange (TO), Mautauban citrange (MC), Kino-Kuni (KK) and Pan American (PA) mandarins. All isolates were graft-transmitted to Mexican lime seedlings and maintained under greenhouse conditions. Phenotypic response of Mexican lime to these isolates revealed a wide variation in symptoms.

Source of antibodies. McAbs 3CA5 and 3DF1, and MCA13 were kindly provided by M. Cambra (IVIA, Spain) and S. M. Garnsey (USDA-ARS, U.S.A.), respectively. Polyclonal anti-CTV antibody used in this study was from our previous work (16).

Production of McAbs. Four female BALB/c mice were injected intraperitoneally with 50 μg of purified CTV-SS (1) in PBS (0.05 M sodium phosphate, pH 7.2, containing 0.85% NaCl). Three injections were made at 10-day intervals. Approximately one week after the last injection, blood samples were withdrawn for titer determination. Two mice were selected and received 30 μg purified CTV intravenously. Three days later, one mouse was exsanguinated and the spleen cells were fused by previously described methods (13) with the following modifications: i) exposing extracted spleen cells to 5

μg/ml of immunizing antigen for 3 days prior to cell fusion, ii) fusing spleen cells at a 2:1 ratio with nonsecretor SP2/O myeloma cells using polyethylene glycol (PEG, MW = 1450, Sigma), and iii) decreasing the time of cell exposure to the fusogen PEG to 15 sec.

After fusion, cells were diluted to 5×10^5 SP2/O cells/ml in selective HAT medium and cultured in 96-well plates (Corning). Growing hybridomas were weaned from aminopterin 10 days post fusion. Primary hybridoma cell lines were screened for CTV-specific antibody when cell lines expanded to cover at least 25% of the well surface. Wells of Immulon 1 (Dynatech Laboratories) microtiter plates were coated with CTV-infected and healthy control sap extracts prepared (1:5, w/v) in carbonate buffer (0.05 M sodium carbonate, pH 9.6). After rinsing with PBS-Tween (PBS containing 0.05% Tween 20) and blocking with blocking buffer (1% bovine serum albumin prepared in PBS-Tween), undiluted culture fluids were added to wells of antigen-coated plates and incubated overnight at 4 C. Bound hybridoma immunoglobulins were detected with alkaline phosphatase-conjugated rabbit anti-mouse IgG (whole molecule, Sigma) followed by addition of substrate (p-nitrophenylphosphate; 0.75 mg/ml in 10% diethanolamine, pH 9.8). All primary hybridomas which tested positive were expanded and frozen.

Selection of McAbs. Culture medium from confluent cultures of all positive cell-lines was tested for ability to detect five CTV isolates selected for diversity of symptom expression on Mexican lime. Double antibody sandwich indirect (DASI)-ELISA was used as described (2). Wells of microtiter plates were coated with 1 μg/ml of polyclonal anti-CTV antibody in carbonate buffer. Plant tissue containing antigen and uninfected tissue, used as a negative control, were extracted in PBS containing 0.2% diethyldithiocarbamate. Medium that had supported growth of SP2/O cells was used as an additional negative control. After addition of culture medium, bound immunoglobulins were detected as described previously. Some hybridoma cell lines were selected for cloning using the calculation cloning procedure (13). Clones were expanded and injected into mice for production of ascitic fluid.

Purification of McAbs. McAbs were purified from ascitic fluid by affinity chromatography on an affi-gel protein A MAPS column (Bio-Rad) according to manufacturer's instructions. Concentration of IgG was determined spectrophotometrically using an extinction coefficient of 1.4 $cm^2\ mg^{-1}$ at 280 nm.

Immunoglobulin subclass determination. Antibody class and subclass of McAbs 4B1, 4C1 and 4F3 were determined by ELISA using each of the following class- and subclass-specific rabbit anti-mouse immunoglobulins for coating of the solid phase: IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM (Zymed).

Characterization of CTV epitopes. The nature of the viral epitope recognized by each McAb was determined, by ELISA, with a modification of the procedure described by Unfer (12). Purified CTV-SS in carbonate buffer, was heated for 10 min at 40 C, 50 C, 60 C, 80 C, 100 C and 121 C followed by quick cooling in an ice bath. Treated antigen was used to coat wells in microtiter plates and antigen reactivity was tested using McAbs 4B1, 4C1 and 4F3 conjugated with alkaline phosphatase by a one-step glutaraldehyde procedure (8) followed by addition of substrate.

Competition ELISA. In order to define relationships among epitopes of CTV and to characterize the specific immuno-reactivity of different antibodies to the antigen, simultaneous and consecutive competition ELISA was performed using a modification of the procedure described by Kubanek *et al.* (7). McAbs 4B1, 4C1 and 4F3, conjugated with alkaline phosphatase (8), were used at optimal concentrations as determined by the maximum P/N (6). Wells of microtiter plates were coated with 1 μg/ml polyclonal anti-CTV antibodies followed by addition of virus antigen (SS isolate) in sap extract.

In consecutive assays, unlabeled McAbs (4B1, 4C1, 4E5, 4F3, 3CA5, 3DF1 and MCA13) were added to wells,

at serial dilutions of $2^{1-n} \times 0.5$ μg/ml, where n = 0 to 9, and plates were incubated for 1 hr at 37 C. After washing, labeled antibody was added and incubated for 3 hr prior to addition of substrate. Simultaneous assays were conducted in a similar manner except that equal volumes of labeled antibody were added to serial dilutions of unlabeled McAbs and the mixture added to wells of the microtiter plate. After incubation for 3 hr at 37 C, substrate was added and reaction products were recorded at 405 nm using a Dynatech Minireader MR 250 (Dynatech Laboratories). Labeled antibody added to wells containing no competing antibody was considered as having an absorbance of 100% and was used to calculate the competition of each unlabeled McAb.

RESULTS

Production of primary hybridoma cell lines. Approximately 150 wells contained cells which were screened for CTV-specific antibodies. Of these, 20 were positive with sap from infected tissue but not with healthy control in the initial screening and 14 primary hybridoma cell lines continued to produce CTV-specific antibody after three transfers.

Selection of McAbs. Tests of primary hybridomas against five Moroccan CTV isolates revealed five patterns (Table 1). Eight hybridomas (1D12, 3G8, 4A4, 4B1, 4C1, 4E1, 4E5, 4F3) detected all CTV variants, although apparently with variable affinity; one hybridoma (1E5) detected four of five isolates; hybridomas 1D7 and 2E1 reacted with three and two CTV isolates, respectively; and three hybridomas (2C4, 3B10, 4E8) reacted only with the ML isolate used in the initial screening. On the basis of these results, primary hybridomas 4B1, 4C1, 4E5 and 4F3 were selected for cloning and production of McAbs to be used in CTV diagnostic assays.

Preliminary results have indicated that McAbs 4B1 and 4F3, and 4C1 and 4E5, can detect, respectively 57 and 58 of 59 CTV isolates that, according to previous investigation, exhibit wide serological diversity (M. Cambra and S. M. Garnsey, personal communication).

Purification of IgG from ascitic fluid. The yield of IgG purified from ascitic fluid ranged from 4 to 7 mg of protein per ml.

Isotype of selected antibodies. McAbs 4B1, 4C1 and 4F3 were of IgG2a subclass.

Characterization of CTV epitopes recognized by McAbs 4B1, 4C1 and 4F3. Experiments to test reactivity of heat-treated purified CTV showed that the McAbs did not detect CTV epitopes

TABLE 1
CROSS-REACTION OF MCAB'S OF PRIMARY HYBRIDOMA CELL LINES WITH FIVE CTV ISOLATES IN DASI-ELISA

Primary Hybridoma	CTV-isolates[a]					Healthy control
	ML	KK	PA	MC	TO	
1D7	±[b]	−	+	−	±	−
1D12	+	+	+	±	+	−
1E5	±	±	+	−	+	−
2C4	±	−	−	−	−	−
2E1	+	+	−	−	−	−
3B10	±	−	−	−	−	−
3G8	+	+	±	±	+	−
4A4	±	+	+	+	+	−
4B1	+	+	+	+	+	−
4C1	+	+	+	+	+	−
4E1	±	+	+	+	+	−
4E5	+	+	+	+	+	−
4E8	±	−	−	−	−	−
4F3	+	+	+	+	+	−

[a]Abbreviations of CTV isolates are as defined in the text.
[b]± = weak reaction; − = no reaction; + = strong reaction.

on virions that had been heated to 60 C or above and cooled immediately in ice.

Tentative epitope mapping of CTV by competition ELISA. In order to define the spatial topology of epitopes recognized by McAbs used in this study, simultaneous and consecutive competition ELISA was performed using McAbs 4B1, 4C1 and 4F3 as labeled antibodies and McAbs 4B1, 4C1, 4E5, 4F3, 3CA5, 3DF1 and MCA13 as unlabeled antibodies (Fig. 1). Competition occurred between all pairs of McAbs except those including McAbs 3DF1 and MCA13.

These results suggest the existence of two distinct groups of topographically related epitopes on the SS isolate of CTV. They are presented as a partial epitope map in Fig. 2. The epitopes recognized by competing McAbs, and defined by circles arranged to indicate occurrence of competition by overlapping, belong to a group of related epitopes (region 1). McAb 3DF1, which did not compete with the labeled McAbs used in this study, has been previously reported to compete with McAb 3CA5 (14). This suggests that it, nevertheless, belongs in the same group as well. McAb MCA13 defines a second distinct epitope on CTV (region 2).

DISCUSSION

The objective of this study was to produce McAbs able to react with a large number of CTV isolates. In order to accomplish this purpose, the CTV isolate used for initial screening of primary hybridoma cell lines was different from the isolate used to immunize mice. Further, this antigen preparation was infectious sap, rather than purified virus, to enhance selection of antibodies which would be highly useful for detection of CTV in infected trees. Greater than half of all positive primary hybridomas produced antibodies that reacted with the five Moroccan CTV isolates used for secondary screening.

Cross-reactivity patterns of all primary hybridomas with the five isolates showed five reaction patterns that may correspond to at least five epitopes. Additional epitopes may also be present if reaction intensity is a true reflection of epitopic variability rather than antigen concentration in infectious sap. Although all virus extracts were prepared from Mexican lime plants maintained under optimal environmental conditions, there is no proof that antigen concentration in the sap extracts was equivalent. These preliminary results must be viewed with caution since patterns were obtained with primary hybridomas that had not been cloned.

Antibodies 4B1, 4C1, 4E5 and 4F3 that reacted with all Moroccan CTV isolates tested and a large number of CTV isolates from an international CTV collection may become very useful for routine diagnosis of CTV infection. Furthermore, they seem to have high affinity for CTV-SS relative to McAb 3CA5.

The fact that McAbs 4B1, 4C1 and 4F3 did not react with the CTV-SS previously heated to 60 C, or above, suggests that epitopes recognized by these McAbs are conformation dependent. Quick-cooling of CTV after heating to high temperatures would be expected to keep protein conformation altered.

The competition ELISA results define two distinct groups of epitopic domains. The map is designed only on the basis of limited tests of reciprocal competition for binding sites on the CTV-SS. It is probable that the map does not reflect all the epitopic diversity that exists in this isolate. Additional McAbs will be needed to define other epitopes. Further, other isolates may contain additional epitopic domains as suggested by preliminary selection of primary hybridomas. The data do suggest, however, that at least some of the epitopic regions defined by region 1 may be highly conserved among CTV isolates. McAbs 4B1, 4C1, 4E5, and 4F3, members of the group defined by region 1, detect a very large number of CTV isolates. Further examination of epitopes defined by region 1, as well as additional characterization of other CTV isolates, using additional McAbs, is necessary. This will provide a reli-

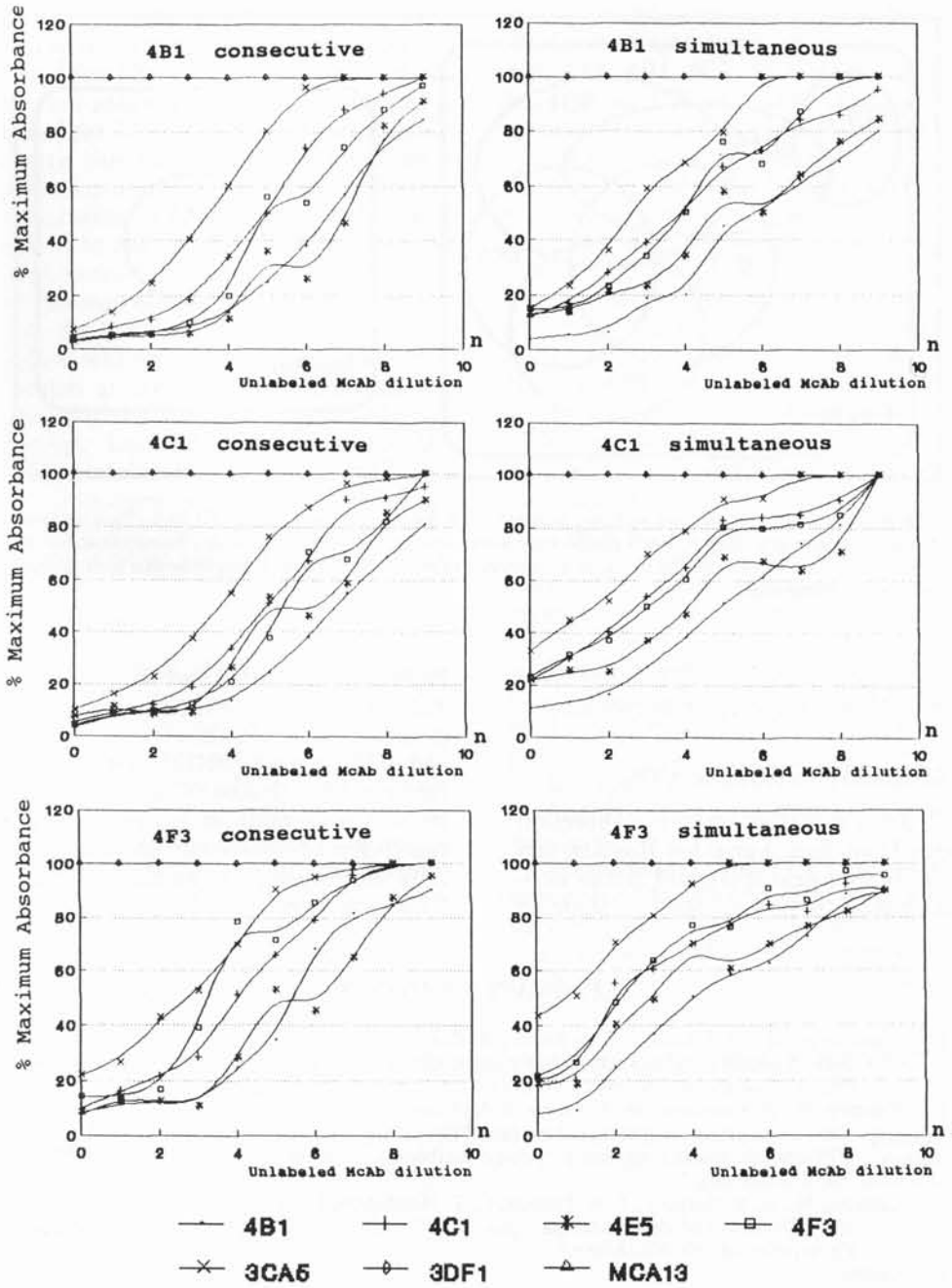

Fig. 1. Consecutive and simultaneous competition ELISA of alkaline-phosphatase labeled anti-CTV McAbs 4B1, 4C1 and 4F3 with unlabeled anti-CTV McAbs 4B1, 4C1, 4E5, 4F3, 3CA5, 3DF1 and MCA13. For the assays, wells of microtiter plates were coated with 1 μg/ml of polyclonal anti-CTV antibodies followed by addition of CTV-SS sap extract. For consecutive assays, 200 μl of serial dilutions of unlabeled McAbs ($2^{1-n} \times 0.5$ μg/ml, n = 0 to 9) were incubated for 1 hr at 37 C; then, 200 μl of labeled antibody was added followed by incubation for 3 hr at 37 C. For simultaneous assays, 100 μl of serial dilutions of unlabeled McAbs was mixed with an equal volume of labeled antibody followed by incubation for 3 hr at 37 C. Labeled antibodies were used at their maximum P/N dilutions.

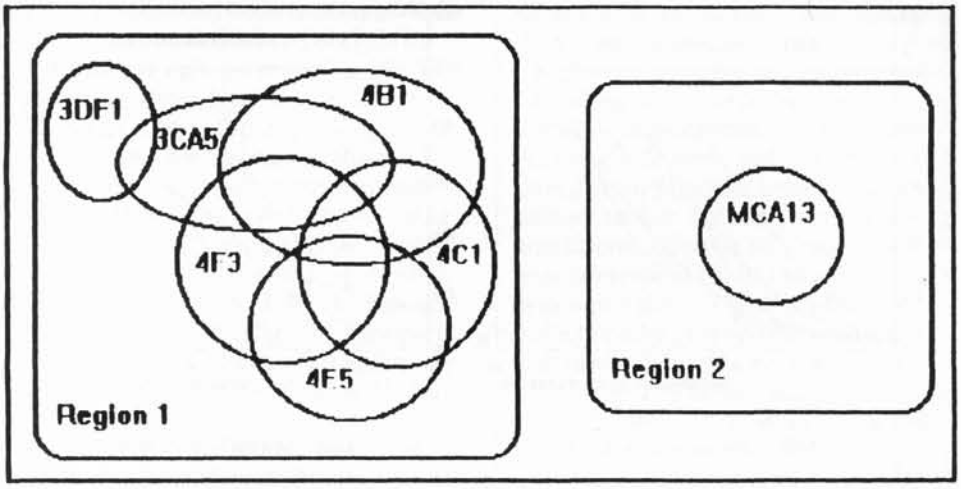

Fig. 2. A tentative partial epitope map of CTV based on competitive ELISA. Two distinct antigenic zones are shown, each circle represents one antigenic determinant recognized by the indicated McAb. Overlappings indicate occurrence of competition which might be due to structual epitopes overlapping.

able epitopic map for CTV that could be exploited for strain differentiation.

ACKNOWLEDGEMENTS

Joint contribution of the Direction des Domaines Agricoles Royaux and the Iowa Agriculture and Home Economics Experiment Station. Journal Paper No. J-15174 and Project No. 2428. The authors express their deep gratitude to M. Cambra (IVIA, Spain) and S. M. Garnsey (USDA, ARS, USA) for providing McAbs 3CA5, 3DF1 and MCA13 and valuable information on reactivity of Moroccan McAbs with CTV serotypes from an international CTV collection.

LITERATURE CITED

1. Bar-Joseph, M., D. J. Gumpf, J. A. Dodds, A. Rosner and I. Ginzberg.
1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75: 195-198.
2. Cambra, M., E. Camarasa, M. T. Gorris, S. M. Garnsey and E. Carbonell.
1988. Comparison of different immunosorbent assays for citrus tristeza virus (CTV) using CTV-specific monoclonal and polyclonal antibodies, p. 38-45. *In:* Proc. 11th Conf. IOCV. IOCV, Riverside.
3. Cambra, M., S. M. Garnsey, T. A. Permar, C. T. Henderson, D. Gumpf, and C. Vela.
1990. Detection of citrus tristeza virus (CTV) with a mixture of monoclonal antibodies. Phytopathology 80: 103 (Abstr.).
4. Cassin, J.
1963. Découverte de 8 cas de tristeza parmi un lot de plants agés de citrus introduits au Maroc. Al Awamia 9: 53-77.
5. Gumpf, D. J., G. Y. Zhen, P. Moreno and J. M. Diaz.
1987. Production and evaluation of specific monoclonal antibodies to citrus tristeza virus strains. Phytophylactica 19: 159-161.
6. Hill, J. H., G. R. Bryant and D. P. Durand.
1981. Detection of plant viruses by using purified IgG in ELISA. J. Virol. Methods 3: 27-35.
7. Kubanek, C. A., J. H. Hill, R. A. Van Deusen and D. P. Durand.
1991. Consecutive and simultaneous competition ELISA: Characterization of epitope relationships or immunoglobulin idiotypes. J. Phytopathol. 133: 99-104.
8. Mackenzie, D. J.
1990. Preparation of antibody-enzyme conjugates, p. 87. *In:* Serological Methods for Detection

and Identification of Viral and Bacterial Plant Pathogens. R. Hampton, E. Ball, and S. De Boer (eds.). APS Press. St. Paul, Minnesota, U.S.A.

9. Tsai, M. C. and H. J. Su.
   1988. Development and characterization of monoclonal antibodies to citrus tristeza virus (CTV) strains in Taiwan, p. 46-50. *In:* Proc. 11th Conf. IOCV. IOCV, Riverside.
10. Nadori, E. B. and M. Zebzami.
    1992. Citrus germplasm blocks control for citrus tristeza virus by enzyme-linked immunosorbent assay in Morocco. *In:* Proc. 7th Intl. Citrus Congress (In press).
11. Permar, T. A., S. M. Garnsey, D. J. Gumpf and R. F. Lee.
    1990. A monoclonal antibody that discriminates strains of citrus tristeza virus. Phytopathology 80: 224-228.
12. Unfer, R. C.
    1985. Production and use of monoclonal antibodies for antigenic characterization of newcastle disease virus. MS Thesis. Iowa State University. Ames, IA, USA.
13. Van Deusen, R. A. and C. A. Whetstone.
    1981. Practical aspects of producing and using anti-viral monoclonal antibodies as diagnostic reagents. Annu. Proc. Amer. Assoc. Vet. Lab. Diag. 24: 211-228.
14. Vela, C., M. Cambra, A. Sanz, and P. Moreno.
    1984. Use of specific monoclonal antibodies for diagnosis of citrus tristeza virus, p. 55-61. *In:* Proc. 10th Conf. IOCV. IOCV, Riverside.
15. Vela, C., M. Cambra, E. Cortes, P. Moreno, J. G. Miguet, C. Perez De San Roman, and A. Sanz.
    1986. Production and characterization of monoclonal antibodies specific for citrus tristeza virus and their use for diagnosis. J. Gen. Virol. 67: 91-96.
16. Zebzami, M.
    1987. Purification et production d'antiserum contre la souche locale du virus de la tristeza des agrumes, p. 6-7. *In:* Rapport de Fin de Stage, Institut National de la Recherche Agronomique, Rabat, Maroc.