SHORT COMMUNICATION

MONOCLONAL ANTIBODIES FOR THE DETECTION OF TAGETES WITCHES' BROOM AGENT

N. Loi[1], P. Ermacora[1], T.A. Chen[2], L. Carraro[1] and R. Osler[1]

[1] *Dipartimento di Biologia Applicata alla Difesa delle Piante, Università degli Studi di Udine, Via delle Scienze 208, I-33100, Udine, Italy*

[2] *Department of Plant Pathology, Rutgers University, New Brunswick, N.J.-08903, USA*

SUMMARY

Tagetes Witches'Broom disease (TWB) was first recorded in Italy in 1958, and in 1972 a phytoplasma was associated with the disease. Infected *Tagetes patulus* plants were used as source of inoculum for phytoplasma transmission to *Catharanthus roseus*. The phytoplasma was also back-transmitted from *C. roseus* to *T. patulus* and *T. erectus* by dodder. Three monoclonal antibodies (Mabs) were obtained against the TWB agent (TWBa); all belonging to subclass IgG1. Using ELISA and DIBA (dot immunobinding assay) it was possible to detect TWBa in experimentally inoculated periwinkle and *Tagetes*, and in naturally infected *Tagetes*. The specificity of the Mabs was also evaluated by ELISA checking 30 phytoplasmas isolated on perwinkle from different herbaceous and woody plants. Serological correlation was observed only between TWBa and a phytoplasma isolated from a yellows-infected grapevine.

RIASSUNTO

PRODUZIONE DI ANTICORPI MONOCLONALI PER LA DIAGNOSI DEGLI SCOPAZZI DEL TAGETE. La malattia denominata scopazzi del tagete (TWB) è stata segnalata in Italia nel 1958, e nel 1972 un fitoplasma è stato associato alla fitopatia. Piante di *Tagetes patulus* infette sono state usate come sorgente d'inoculo per la trasmissione del fitoplasma a piante di *Catharanthus roseus*. Il fitoplasma è stato inoltre trasmesso di ritorno da *C. roseus* a *T. patulus* e a *T. erectus* usando la cuscuta. Sono stati ottenuti 3 anticorpi monoclonali (Mabs) specifici per l'agente di TWB (TWBa), tutti appartengono alla sottoclasse IgG1. I tre Mabs, in ELISA indiretto, in DAS-ELISA e in DIBA (dot-immunobinding assay) hanno permesso di diagnosticare TWBa sia in pervinca e tagete inoculati sperimentalmente che in tageti con infezione naturale. La specificità dei Mabs è stata valutata in ELISA indiretto con 30 fitoplasmi, isolati su pervinca da diverse piante erbacee ed arboree. Correlazione sierologica è stata osservata solo tra TWBa ed un fitoplasma isolato da vite affetta da giallume.

Key words: monoclonal antibodies, phytoplasma, *Tagetes*.

Tagetes witches' broom (TWB), has been recorded in many countries (Sharma *et al.*, 1985; McCoy *et al.*, 1989). The disease was first observed in Italy by Grancini (1958), and in 1972 a phytoplasma was detected in diseased plants using electron microscopy (Amici *et al.,* 1972). Recently, based on the classification proposed by Schneider *et al*. (1993), the TWB agent (TWBa) was placed in cluster I (Firrao, personal communication). TWB-infected *Tagetes patulus* L. plants exhibit characteristic symptoms such as stunting, pale and erect shoots, virescence and phyllody, witches' brooms, leaf reddening and chlorosis.

Serological methods using monoclonal antibodies (Mabs) are known to be specific, rapid and effective in detecting and identifying phytoplasmas (Chen *et al.*, 1989). The production of Mabs reported in this paper, was not chosen for any particular economic importance of *Tagetes* spp. but because the disease is quite common and recurrent in certain areas of northern Italy; the persistence of this phytoplasma suggests the presence of alternative hosts and efficient vectors. Besides Mab production, another aim of the trials was to confirm the pathogenicity of TWBa by transmitting the phytoplasma from naturally infected *Tagetes* to *Catharanthus roseus* L. and then back from periwinkle to healthy plants of *T. patulus* and *T. erectus* L., to induce the typical symptoms of the disease.

T. patulus plants with TWB symptoms were collected in Udine (Italy); the DAPI technique (Seemüller, 1976) and electron microscopy were used to verify the presence of phytoplasmas. The disease was maintained by graft propagation (top-grafting) to healthy *Tagetes* plants. Survival of grafts was about 20% but the transmission rate for successful grafts was 100%. TWBa was also transmit-

Corresponding author: R. Osler
Fax: +39.0432.558501
E-mail: osler@pldef.uniud.it

ted to *C. roseus* by dodder (*Cuscuta campestris* Younk) using the 'indirect bridge' technique (Carraro *et al.*, 1991) and then propagated from *C. roseus* to *C. roseus* by grafting. TWBa transmission by dodder from infected *Tagetes* to *C. roseus* gave positive results in 2 cases out of 20; all the periwinkles inoculated by grafting from *C. roseus* developed symptoms. Back transmission tests from *C. roseus* to 30 healthy *Tagetes* (15 *T. patulus* and 15 *T. erectus*) were also performed using *C. campestris*. Two of 15 *T. patulus* developed typical TWB symptoms but *T. erectus* did not show any clear symptom.

Infected periwinkles showing virescence, phyllody, leaf yellowing and witches' brooms were used as source of antigen for Mab production. The purification, immunization, and fusion procedures used were described by Chen *et al.* (1993). A total of 1486 hybridomas were obtained from two fusions and the supernatants were screened for TWBa antibodies using indirect ELISA, and the biotin-avidin system (Jiang *et al.*, 1989). The material for plate coating consisted of healthy and diseased *C. roseus* partially purified as described by Jiang *et al.* (1989), with a final protein concentration of 0.01 mg ml^{-1} (BIO-RAD protein assay, USA) equivalent to about 0.25 g of leaf midribs per ml of coating buffer. Six supernatants gave in indirect ELISA, in the first screening, optical density values that discriminate diseased from healthy plants (Fig. 1). The hybridomas 7H8 and 4E7 were unstable and did not sustain specific TWBa antibody production. The other four hybridomas were subcultured and submitted to limiting dilution to obtain single-cell colonies; from them, three monoclonal hybridoma cell lines producing TWBa-specific Mabs were obtained, grown and stored in liquid nitrogen: 7A4/2D5, 8B2/1C3, and 12C8/3B2. The Mabs belonged to the subclass IgG1 (Isotyping Kit, Pierce Chemical Co.). Ascitic fluid was produced only from the 7A4/2D5 clone; the titre was 10,000 times greater than in the cell culture medium, and a dilution of $1x10^{-5}$ was suitable for indirect ELISA.

The specificity of the three Mabs was tested by indirect ELISA using both healthy and TWB-diseased *Tagetes* and periwinkle plants. Also dot-immunobinding assay (DIBA) (Hammond and Jordan, 1990) was performed using ascitic fluid 7A4/2D5 at a concentration of 1 µg ml^{-1} and the same antigens used for ELISA in aliquots of 100 µl spotted on polyvinyldifluoride membranes (Bio-Rad, USA) at concentrations varying from 0.036 to $7.2x10^{-5}$ µg $µl^{-1}$, in Tris buffered saline pH 7.5. The three Mabs were found to be specific for TWBa whether present in diseased periwinkle or in *Tagetes* using indirect ELISA (Table 2). For DIBA, positive results were obtained for *Tagetes* down to a concentration of $1.4x10^{-4}$ mg ml^{-1}, and for periwinkle to $2.8x10^{-4}$ mg ml^{-1}.

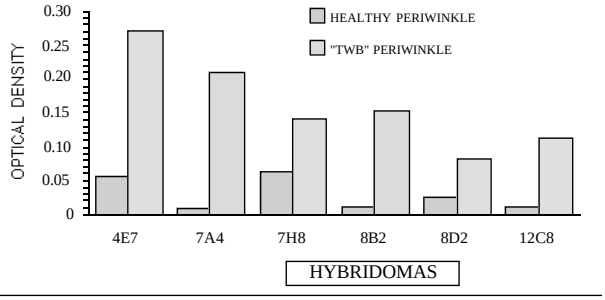

Fig. 1. Optical density values at 405 nm of selected hybridomas after first screening by indirect ELISA, using partially purified material from healthy and TWB-infected periwinkle.

Specificity of the three Mabs was also investigated using indirect ELISA by comparing their reaction with 30 phytoplasmas belonging to different clusters (Schneider *et al.*, 1993) isolated in *C. roseus* from several naturally infected herbaceous and woody plants (Table 1). The Mabs reacted only with an isolate obtained from a 'Chardonnay' grapevine with typical yellows symptoms.

Twenty symptomatic *T. patulus* collected in four different areas of northeastern Italy were also tested by indirect and DAS-ELISA. In DAS-ELISA the coating consisted of purified 7A4/2D5 Mab from ascitic fluid at 7.5 µg ml^{-1} in carbonate buffer 0.5 g of healthy and diseased plants were partially purified and diluted in 5 ml of extraction buffer (phosphate buffered saline, 2% PVP, 0.05%-Tween 20, pH 7.4). The 7A4/2D5 Mab conjugated with alkaline phosphatase (Clark and Adams, 1977) was diluted $1x10^{-3}$ in phosphate buffered saline-Tween 0.05%. Both indirect and DAS-ELISA were able to detect TWBa in all the naturally infected *T. patulus.*

TWBa Mabs were also used to confirm back-transmission of TWBa and indirect and DAS-ELISA were applied to the 30 inoculated *Tagetes*. Positive results were obtained from the two symptomatic *T. patulus* and from six asymptomatic *T. erectus*. The Mabs were able to demonstrate back-transmission of the phytoplasma to *Tagetes* even when symptoms were not expressed.

In the present work three main aims were achieved: the phytoplasma isolated from naturally infected *T. patulus* was shown to be the cause of the disease; three monoclonal antibodies to TWBa were obtained, and these were shown to be highly specific.

The first result was achieved by means of positive back-transmission to *T. patulus* (two became symptomatic) of the phytoplasma previously isolated in *C. roseus.* This result was reinforced by consistent

Table 1. Phytoplasmas isolated on *C. roseus* from naturally infected herbaceous and woody plants and used to investigate the specificity of TWBa Mabs.

Original host	No. of tested phytoplasmas isolates on periwinkle	Cluster[a]
Achillea millefolium L.	1	I
Apium graveolens L.	1	I
Apple	1[b]	V
Apple cv. 'Golden Delicious'	1	V
Aster chinensis L.	1[b]	I
Catharanthus roseus L.	2	I, I
Chrysanthemum leucanthemum L.	1	VI
Crepis biennis L.	1	VI
Daucus carota L.	1	I
Erigeron annuus L.	1	VI
Grapevine cv. 'Chardonnay'	1	VI
Leontodon hispidus L.	1	I
Oxalis acetosella L.	1	I
Peach	1[b]	VI
Prunus salicina Lindl.	5	V, V, V, VI, VI
Ranunculus sp.	1	VI
Silene alba Mill.	1	I
Silene vulgaris Moench	1	I
Taraxacum officinale Weber	1	VI
Trifolium sp.	1	I
Trifolium pratense L.	1	VI
Trifolium repens L.	2	VI, VI
Vaccinium sp.	1[b]	VI
Veronica arvensis L.	1	I

[a] Based on the classification of Schneider *et al.*, 1993.
[b] Kindly provided by Dr. E. Seemüller

detection of phytoplasmas in all the plants belonging to the transmission chain, starting from the original source of inoculum through the *C. roseus* host plants, and concluding with the back-inoculated *T. patulus*.

The three Mabs obtained did not react with healthy plants and the reaction with extracts from TWBa-infected plants was high especially if *Tagetes* was used. This could mean that the phytoplasmas are more concentrated in marigold (the original host) than in periwinkle. Thus sometimes the original diseased plant species may be a good source of antigen.

These Mabs were highly specific, and in fact besides TWBa they reacted only with the 'grapevine yellows' (GY) isolate. However, GY and TWB are not identical

Table 2. - Indirect ELISA results using three selected Mabs and both infected and healthy *C. roseus* and *Tagetes* as antigen.

Antigen	Mabs		
	7A4/2D5	12C8/3B2	8B2/1C3
Infected *C. roseus*	0.420	0.155	0.247
Healthy *C. roseus*	0.008	0.005	0.015
Infected *Tagetes*	1.319	0.203	0.335
Healthy *Tagetes*	0.010	0.003	0.003

since no positive ELISA reactions were obtained with TWBa from periwinkle, using GY-Mabs obtained in the USA by Chen *et al.* (1993). Probably the two phytoplasmas have a common antigenic determinant.

Because they appear to be very selective, the TWBa-Mabs will be useful in future research on the vector and also in detecting the presence of presumed host plants.

REFERENCES

Amici A., Grancini P., Osler R., Refatti E., 1972. Alterazioni a probabile eziologia da micoplasmi in varie specie di piante in Italia. *Rivista di Patologia Vegetale* **8**: 21-50.

Carraro L., Osler R., Loi N., Favali M.A., 1991. Transmission characteristics of the clover phyllody agent by dodder. *Journal of Phytopathology* **133**: 15-22.

Chen K.H., Guo J.R., Wu X.Y., Loi N., Carraro L., Guo H.Y., Chen Y.D., Osler R., Pearson R., Chen T.A., 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows disease agent. *Phytopathology* **83**: 915-922.

Chen T.A., Lei J.D., Lin C.P., 1989. Detection and identification of plant and insect mollicutes. In: Whitcomb R.F., Tully J.G. (eds.). The Mycoplasmas, Vol. V, pp. 393-424. Academic Press Inc., San Diego, USA.

Clark M.F., Adams A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**: 475-483.

Grancini P., 1958. Un gruppo di virus delle piante importanti ma poco noti. *Bollettino dell'Agricoltura.* **36-37**: 22.

Hammond J., Jordan R., 1990. DOT-immunobinding assay. In: Hampton R., Ball E., De Boer S. (eds.). Serological methods for detection and identification of viral and bacterial plant pathogens, pp. 237-248. APS Press, Minnesota, USA.

Jiang Y.P., Chen T.A., Chiykowski L.N., Sinha R.C., 1989. Production of monoclonal antibodies to peach eastern X-disease and their use in disease detection. *Canadian Journal of Plant Pathology* **11**: 325-331.

Mc Coy E., Gerard T.N. de Leeuw, Marwitz R., Chen T.A., Cousin M.T., Sinha R.C., Petzold H., Chiykowski L.N., Caudwell A., Chang C.J., Dale J.L., Golino D., Kirkpatrick B., Sugiura M., Whitcomb R.F., Yang I.L., Zhu B.M., Seemüller E., 1989. Plant diseases associated with mycoplasma-like organisms. In: Whitcomb R.F., Tully J.G. (eds.). The Mycoplasmas, Vol. V, pp. 545-640. Academic Press Inc., San Diego, USA.

Schneider B., Ahrens U., Kirkpatrick B.C., Seemüller E., 1993. Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *Journal of General Microbiology* **139**: 519-527.

Seemüller E., 1976. Investigations to demonstrate mycoplasma-like organisms in diseased plants by fluorescence microscopy. *Acta Horticulturae* **67**: 109-112.

Sharma A.K., Misra S., Raychaudhuri S.P., Parameswaran N., 1985. Phyllody of marigold, a disease associated with mycoplasma-like organisms. *International Journal of Tropical Plant Diseases* **3**: 45-49.

Received 28 July 1997
Accepted 10 June 1998