

Serological Characterization of Alfalfa Mosaic Virus in Alfalfa (*Medicago sativa*) in Some Regions of Iran

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ABSTRACT

A survey was carried out in five provinces of Iran (Kerman, Sistan and Baluchestan, Hormozgan, Khorasan and Yazd) for the presence of Alfalfa mosaic virus (AMV) serotypes in alfalfa during 2002 to 2003. The number of samples collected was 250, representing the diversity and geographical distribution of AMV in these areas. Diagnosis was carried out using polyclonal (PAbs) and monoclonal (MAbs) antibodies. A total of 110 symptomatic leaf samples gave a positive reaction in ELISA with polyclonal antibodies. Twelve out of 20 MAbs reacted with all samples tested and were considered as non-differentiating MAbs. Only the MAbs-12, 13, 15, 21, 22 and 24 gave a clear differential reaction and were used for identifying AMV serotypes. Two MAbs (1 and 2) did not react with AMV positive samples. Serological relatedness among AMV samples was studied by indicating the existence of six serotypes of AMV strains in the surveyed areas.

Keywords: AMV, Monoclonal antibodies, Serotype, Strain.

INTRODUCTION

Alfalfa mosaic virus (AMV) is present in most alfalfa (*Medicago sativa* L.) fields. As the age of the stands increases, the incidence of infected plants increases; up to 80% infection has been found in four year-old fields (Gibbs, 1962; Muller, 1965). AMV is one of the most biologically variable plant viruses and numerous natural variants with different pathogen city have been reported (Paliwal, 1982; Walter *et al.*, 1987; Hiruki and Miczynski, 1987; Hajimorad and Francki, 1988).

There are different factors which distinguish virus strains or isolates of AMV. Krall (1975) reported differences between 11 strains on the basis of the chemical properties of the coat protein. Some of these strains were AMV-S (Gibbs and Tinsley, 1961), AMV 425 (Hagedorn and Hanson, 1963), 15/64 and VRU (Hull, 1970), the alfalfa yellow spot mosaic strain (YSMV) (Zaumeier,

1963), AA-1 (Lizuka and Lida, 1969) and A and P (Tremaine and Stace-Smith, 1969). An investigation has showed that, while the last strains (A and P) produced different symptoms on tobacco (*Nicotiana tabacum* L. Haranova), bean (*Phaseolus vulgaris* L.) and cucumber (*Cucumis sativus* L.) plants, there were no differences between their amino acids (Tremaine and Stace-Smith, 1969).

The effects of AMV on yields of different crops were reported. In white clover, AMV has reduced the leaf and stolon dry weight, primary and secondary stolon length, nodes in primary and secondary stolons, nodulation, and leaves per plant (Gibson *et al.*, 1980). Reduction in yield, protein and the degree of nodulation and winter survival of alfalfa infected with AMV was also reported (Edwardson and Christie, 1997). The yield of potatoes infected with AMV showing calico symptoms is reduced by about 20%. Infection with the Alberta (Canada) isolates (A-

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515, severe strain) of AMV reduced the yield of forage and the regeneration potential of several alfalfa cultivars commonly grown there (Miczynski and Hiruki, 1987). Monoclonal antibodies (MAbs) have been shown to be useful tools for analysing the serological properties of plant viruses and virus strains differing in host range, symptomatology, vector transmission or geographical origin (D'Arcy *et al.*, 1989; Swanson *et al.*, 1992). Moreover, antigenic variability among different isolates of a virus which could not be differentiated with polyclonal antibodies was revealed by MAbs (Adam *et al.*, 1991; Cancino *et al.*, 1995).

Differentiation and antigenic characterisation of five AMV strains (H4, N20, S30, S40 and W1) were determined using monoclonal antibodies (Hajimorad *et al.*, 1990). Capsid proteins of two strains of AMV (T6 and 425) were compared using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Kudela and Gallo, 1996). The differences between length and number of nucleotides of RNA3 of AMV strains S, A and Y (van der Vossen, *et al.*, 1993) and S, L and M (Langereis *et al.*, 1986) were determined after sequencing. The objectives of this research were to identify serotypes of AMV and to determine the occurrence of these serotypes in southeastern and central parts of Iran. A preliminary report has been published (Massumi, 2004).

MATERIALS AND METHODS

Collection of AMV Samples

Alfalfa samples were collected during 2002 to 2003 in 18 regions of the southeastern and central parts of Iran (Kerman, Sistan and Baluchestan, Hormozgan, Khorasan and Yazd) and tested for the presence of AMV serotypes. In each field, five to six plants were inspected and samples from those plants showing virus-like symptoms were collected. Each sample consisted of the youngest fully developed leaf from plants exhibiting leaf symptoms such as mosaic,

mottle, vein banding and yellowing. Of a total of 250 AMV symptomatic leaf samples, 70 samples were from 15 fields in Kerman Province (Shahrbabak, Zarand, Bam and Jiroft), 45 samples from seven fields in Yazd Province (Mehriz and Yazd), 40 samples from five fields in Khorasan Province (Tabas and Ferdows), 60 sample from nine fields in Sistan and Bluchestan Province (Kash, Zable and Zahedan) and 35 samples from five fields in Hormozgan Province (Rodan and Hajiabad) were collected. Leaf samples of AMV were collected for the serological tests. Young leaves from some symptomatic plants were placed in plastic bags and examined for the presence of AMV serotypes.

Source of Antibodies

Twenty (MAb-1, 2, 6, 7, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 24, 25, 26, 29 and 33) of the 33 monoclonal antibodies raised to particles of AMV described previously (Massumi *et al.*, 2005) were selected for this study. Also the polyclonal antisera 137-2A and 137-5B raised against intact AMV (kindly provided from the IACR-Rothamsted antiserum collection) were used.

Identification of the Serotypes by ELISA

For mono and polyclonal antibodies, the trapped antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and plate-trapped antigen (PTA)-ELISA was used respectively. For the PTA-ELISA tests, samples were ground in coating buffer (0.06 M Na₂CO₃ and 0.14 M NaHCO₃, pH 9.6) at a ratio of 1:10 (w/v), added to ELISA plate wells (Nunc polysorb). The AMV antiserum was diluted 1:1,000 (v/v) in 0.2 M Tris-HCl buffer and pH 7.2, was added to the wells. For TAS-ELISA, the plates were first coated with 100 µl of polyclonal antibodies (PAb), diluted 1:1000 in carbonate buffer (0.06 M Na₂CO₃ and 0.14 M NaHCO₃, pH 9.6). Wells were washed three times with phos-

phate buffered saline (PBS)-Tween (0.05% Tween 20 in 3 mM KCl, 3 mM NaN₃, 8 mM Na₂HPO₄, 1 mM NaH₂PO₄, and 0.13 M NaCl) and then 200 µl of PBS-Tween containing %2 skimmed milk added to ELISA plate wells. After washing, 100 µl of antigen consisted of tissue ground 1:10 (w/v) in extraction buffer (1:10 wt/vol) (PBS-Tween and 2% polyvinylpyrrolidone [PVP] pH 7.4) and stained through a cheesecloth. After washing, the antigen step was followed by 100 µl of each monoclonal antibodies diluted in 0.2 M Tris-HCL and 0.15 M NaCl (pH 7.2). Bound antibodies were detected by goat-anti-mouse IgG or IgM, or goat-anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) diluted 1:2000 in Tris-HCL buffer. For each step, the plates were incubated at 37°C for two hours. Enzyme reactions were developed 30-60 minutes after the addition of 100 µl of substrate (*P*-nitro phenyl phosphate, disodium [Sigma] at 1 mg/ml in 9.7% diethanolamine buffer, pH 9.8). Absorbance values were determined (A_{405nm}) on a Biotek automated microplate reader, model EL 800 (Bio-Tek Instruments Inc., Winooski, VT).

RESULTS

Surveys were conducted in 41 alfalfa fields distributed over 18 regions in Iran where alfalfa is grown. Of the 250 samples collected from three different cultivars of alfalfa (*Medicago sativa* cvs. Yazdi, Bami and Nikshahri), 110 were positive for AMV in PTA-ELISA. When all the MAbs were used in TAS-ELISA for identification of serotypes, the vast majority (70) of the samples reacted with all MAbs. Even when low AMV concentrations were present in field samples (e.g. ZAH1 in Table 1) as indicated by the low PTA-ELISA readings, reaction with different MAbs revealed the presence or absence of epitopes following substrate incubation periods of only 30 minutes.

Twelve of the 20 MAbs reacted with all 110 positive samples and were considered as

non-differentiation MAbs. Only six MAb-12, 13, 15, 21, 22 and 24 were useful for discrimination of AMV serotypes (Table 1). Two of the MAbs (MAb-1 and 2) failed to react with any sample (data not shown). These six MAbs gave clear differentiation reaction with a total of 40 AMV samples. Eleven samples from Tabas, Jifrot, Hajiabad and Zahedan 1, three samples from Rudan and Zabole 2 and nine samples from Mehriz and Yazd failed to react with one, two and three of MAbs, respectively (Table 1). According to these results the AMV samples can be subdivided into six serotypes and designated as A₁ to A₆.

DISCUSSION

Monoclonal antibodies were able to differentiate serotypes of AMV on the basis of the presence or absence of one or two antigenic determinants. These minor serological differences were not always apparent or were difficult to interpret by testing with polyclonal antisera. The results demonstrate the utility of using monoclonal antibodies to define serotypes in alfalfa between different isolates of AMV.

A high variability in coat protein was found among AMV field samples when checked against monoclonal antibodies. When a total of 110 AMV samples were tested by TAS-ELISA using 20 AMV-specific MAbs to analyse coat protein variation in AMV, we succeeded in revealing different epitope profiles among positive samples and the AMV was classified into six serotypes. The majority of the field samples reacted with 12 MAbs. This indicates that there is a serological similarity of at least one epitope in the capsid proteins of these field samples. However, about 21% (23/110) of the samples did not react with one and/or the other of the six differentiating MAbs. This difference is in view of the fact that there are at least six or seven epitopes in the capsid protein of these AMV samples. These results also demonstrate the utility of using monoclonal antibodies to define anti-



Table 1. Reaction of selected field samples of AMV from southeast and central parts of Iran with polyclonal and monoclonal antibodies.

Origin ^a (Virus samples)	Cultivars	PAbs	MAB ⁱ 10	MAB 12	MAB 13	MAB 15	MAB 21	MAB 22	MAB 24	Serotypes
SH ^f (1) ^b	<i>Medicago sativa</i> cv. <i>Bami</i>	3 ^h	3	3	3	3	3	3	2	A ₁
ZR1 ^c (2)	<i>Medicago sativa</i> cv. <i>Bami</i>	3	3	3	3	3	3	3	3	
ZR2 ^c (4)	<i>Medicago sativa</i> cv. <i>Bami</i>	3	3	2	3	3	3	3	1	A ₂
BAM ^c (2)	<i>Medicago sativa</i> cv. <i>Bami</i>	3	3	3	3	2	3	3	1	
TAB ^d (1)	<i>Medicago sativa</i> cv. <i>Bami</i>	3	3	3	3	3	3	2	0	A ₃
JIR ^c (4)	<i>Medicago sativa</i> cv. <i>Nikshari</i>	2	3	2	3	3	3	2	0	
HAJI1 ^e (3)	<i>Medicago sativa</i> cv. <i>Nikshahri</i>	3	3	3	3	3	3	2	0	
HAJI2 ^e (2)	<i>Medicago sativa</i> cv. <i>Nikshahri</i>	2	2	2	3	0	1	1	2	A ₄
FERD ^d (3)	<i>Medicago sativa</i> cv. <i>Bami</i>	2	3	2	2	1	1	1	2	
ZAH1 ^f (1)	<i>Medicago sativa</i> cv. <i>Bami</i>	1	2	2	2	0	1	1	2	
ZAH2 ^f (1)	<i>Medicago sativa</i> cv. <i>Yazdi</i>	3	3	2	1	3	3	3	1	A ₅
KHAS1 ^f (1)	<i>Medicago sativa</i> cv. <i>Bami</i>	2	2	2	1	1	1	1	1	
KHAS2 ^f (2)	<i>Medicago sativa</i> cv. <i>Yazdi</i>	3	3	3	2	3	1	2	1	
ZABL1 ^f (1)	<i>Medicago sativa</i> cv. <i>Bami</i>	2	3	2	2	2	1	1	1	A ₆
ZABL2 ^f (1)	<i>Medicago sativa</i> cv. <i>Yazdi</i>	3	3	3	1	2	1	0	0	
MEHR1 ^g (5)	<i>Medicago sativa</i> cv. <i>Yazdi</i>	3	3	3	2	3	0	0	0	
YAZD ^g (4)	<i>Medicago sativa</i> cv. <i>Yazdi</i>	3	3	3	1	3	0	0	0	A ₆
RUDA ^e (2)	<i>Medicago sativa</i> cv. <i>Nikshahri</i>	3	3	3	1	1	0	1	0	

^a SH= Saharbabk; ZR= Zarand; BAM= Bam; TAB= Tabas; JIR= Jiroft; HAJI= Hajiabad; ZAH= Zahedan; KHAS= Khash; ZABL= Zabol; RUDA= Rudan; YAZ= Yazd, and MNA= Minab.

^b Number of samples in each region.

^c: Kerman Province; ^d: South Khorasan Province; ^e: Hormozgan Province; ^f: Sistan and Baluchestan Province, and ^g: Yazd Province.

^h Strength of ELISA reaction, measured as A_{405nm} after a substrate incubation period of 1h, was classed as 3 (>0.8), 2(0.2-0.7), 1(>three times mean value for uninfected control to 0.2) or 0 (<three times mean value for uninfected controls).

ⁱ All other MAbs not mentioned in this table but gave a reaction pattern similar to that of MAb-10.

genic relationships between strains of the same virus. A significant percentage of samples (56%) from among symptomatic alfalfa samples did not react with AMV antisera. It seems that other alfalfa viruses may be present in the field-grown alfalfa of Iran, which

could be confirmed using antibodies of other alfalfa viruses and molecular characterization. Other factors may be abiotic agents causing virus like symptoms, as reported by Brown and Graham, 1978.

AMV has one of the widest host ranges

among all plant viruses (Edwardson and Christie, 1997), and also reported to be transmitted by seeds (Hemmati and McLean, 1977), pollen (Frosheiser, 1974) as well as 29 aphids species (Edwardson and Christie (1997)). A1, A2 and A5 serotypes were identified in the alfalfa infected samples collected in Kerman and Sistan and Baluchestan Provinces, respectively. However, A3, A4, and A6 serotypes were found in more than one province. The wide variation in climatic conditions is not found in the alfalfa-producing areas of the five provinces surveyed. Therefore, we can speculate that the wide host range of the virus including weeds, that can harbor different species of aphid vectors and the different method of transmission provide an increased opportunity for natural selection of wide genetic variability within this virus. This may explain why we were able to define six serotypes in only one main host (Alfalfa) of the virus. Superimposed on the variation in host plants and aphid vectors pressures is the long-distance movement of alfalfa seeds and the resulting redistribution of new strains and serotypes.

The benefit of this study is that we have identified the distribution of AMV serotypes in different parts of Iran in advance of the commercial release of AMV-resistant transgenic alfalfa cultivars. Some transgenic lines show resistance, whereas others accumulate fewer viruses than no transformed lines (Hill *et al.*, 1991). Since most transgenic lines have only one gene for resistance, usually the virus coat protein gene (Wilson, 1993), they will undoubtedly exert new selection pressures on the naturally occurring populations of AMV when they are released into commercial production. The panels of MABs which we have described here may be used in the future to study the possibility of changes in AMV serotypes that occur in alfalfa after the release of AMV-resistant transgenic alfalfa cultivars. It is possible that some of the serotypes that are common now will become less prevalent or disappear and that new ones will arise. Halk *et al.* (1984) made parallel tests for production of MABs

against AMV to differentiate AMV serotypes. Based on this result, only Two AMV specific hybridomas which had similar properties were produced. Hajimorade *et al.* (1990) by analysis of a panel of 15 MABs against AMV, revealed the presence of at least three different types of neotopes, three metatopes and one cryptotope.

It is doubtful whether these specific AMV monoclonal antibodies represent the total antigenic repertoire of this virus, but it is interesting that these antibodies have been able to delineate six serotypes of AMV in the alfalfa samples examined in Iran. Production of a further group of monoclonal antibodies to a few selected AMV strains from other AMV hosts should provide antibodies specific to a border range of antigenic determinants among the virus and allow even greater precision in serological analysis.

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شناسائی تعدادی از سروتیپ های ویروس موزائیک یونجه (*Alfalfa mosaic virus*) در گیاه یونجه در مناطقی از ایران

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چکیده

به منظور شناسائی سروتیپ های ویروس موزائیک یونجه نمونه های مشکوک به این ویروس از مزارع یونجه در استانهای کرمان، سیستان و بلوچستان، هرمزگان، یزد و خراسان جنوبی در طی سالهای ۱۳۸۱-۱۳۸۲ جمع آوری گردیدند. نمونه برداری در طول فصل رویشی از بوته های با علائم موزائیک، تاوولی، چین خوردگی و بدشکلی برگ و ساقه ها انجام گردید. تعداد ۲۵۰ نمونه با استفاده از آنتی سرم چند همسانه اختصاصی AMV با آزمون الایزا مورد بررسی قرار گرفتند. ۴۴ درصد (۱۱۰) از نمونه ها به آنتی سرم واکنش مثبت نشان دادند. جهت تعیین سروتیپ های این ویروس، نمونه های مثبت با ۲۰ آنتی بادی تک همسانه ای اختصاصی مورد ارزیابی قرار گرفتند. از بین این آنتی بادی ها، تعداد ۱۲ آنتی بادی بدون هیچ گونه تمایزی کلیه نمونه ها را در واکنش الایزا شناسائی نمودند. اما واکنش ۴۰ نمونه آلوده به ویروس موزائیک یونجه در برابر ۶ نوع آنتی بادی (آنتی بادی هائی به شماره های ۱۲، ۱۳، ۱۵، ۲۱، ۲۲ و ۲۴) متفاوت بود. بر این اساس استرین های بررسی شده این ویروس به ۶ سروتیپ مختلف (A1 تا A6) تفکیک شدند. این اولین گزارش از تنوع سروتیپ های مختلف AMV در ایران است.