Factors to Improve Detection of *Alfalfa mosaic virus* in Soybean

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Abstract

Infection of plants with *Alfalfa mosaic virus* is known to be associated with symptom and virus concentration remission, a phenomenon known as "recovery." However, for the routine detection of AMV in the infected plants, this phenomena is problematic. Indeed in soybean, detection of AMV has been problematic and inconsistent results have been obtained from tissues collected at different growth stage of the infected plants. In order to determine optimum sampling procedure for analysis by ELISA, three soybean cultivars and five biologically distinct strains of AMV were utilized in this study. Each cultivar was rub-inoculated with each of the strains and all the trifoliolate leaf samples of the infected plants were analyzed by ELISA at 30, 60, and 90 days postinoculation. Results suggested that for all virus strains and cultivars tested, tissue samples should consist of pooled fully developed trifoliolate leaflets that are positioned at the upper parts of the plants. Additionally, symptoms of AMV infection were dependent upon soybean cultivar and virus strain and varied from symptomatic to asymptomatic. This makes it problematic to conclude a soybean plant is AMV infected based upon symptomatology alone.

Introduction

Incidence of *Alfalfa mosaic virus* (AMV) in field-grown soybeans [*Glycine max* (L.) Merrill] in the United States has recently been increasing (2,4,13). This is believed to be due, in part, to the introduction of the soybean aphid (*Aphis glycines*) (2,9,15). Efforts to detect AMV in infected hosts have historically been problematic mainly due to fluctuations in virus concentration, decrease in specific infectivity in tissues of infected plants, and symptom remission as plants age (3,5,6,11,14). However, the experimental plants in most of the studies conducted involved tobacco and alfalfa. In a recent study by Mueller and Grau (13), the authors pointed out the decline in AMV-positive plants that occurred in soybean field plots sampled late in the season may result in spuriously low incidence data. This makes the ability to conduct surveys, examine distribution of the virus in soybean fields, and evaluate soybean genotypes for AMV resistance difficult. Further, the apparent symptom variation caused by AMV infection, which is cultivar- and virus-strain-dependent, makes field evaluation of AMV incidence solely based on symptom expression unreliable (10).

Therefore, this work was conducted to demonstrate variation in symptoms induced by AMV in soybean and to identify the optimum sampling protocol by which the virus can be detected reliably by immunoassays. ELISA (enzyme-linked immunosorbent assay) is the most common serological assay being used for large-scale screening of plants for viruses. Since AMV is biologically highly variable with strains causing different symptoms in the same host (7), five AMV strains and three soybean cultivars were utilized in this study. The AMV strains used are well studied with their biological and antigenic properties known (7). In general, AMV strains have shown limited antigenic differences and
antiserum raised against one cross-reacts strongly against other biologically distinct strains of the virus (7). Soybean cultivars Lee68, Colfax, and Williams82 were utilized in this study mainly because of availability of Soybean mosaic virus (SMV) free indexed seeds in our laboratory. SMV is seed-borne in soybean. It should be noted that currently no source of broad resistance to AMV is known in soybean.

Interaction Between Different Soybean Cultivars and AMV Strains and Determination of Optimum Tissue Sampling for Detection of AMV by ELISA

Experiments were conducted to explore the symptoms that occur when various soybean cultivars are inoculated with different AMV strains and to identify the optimum sampling protocol for detection of AMV relative to the growth of the infected soybean plants. In these experiments four plants of each of the soybean cultivars Colfax, Williams82, and Lee68 were rub-inoculated each with well-characterized biologically distinct AMV strains H4, N20, S30, S40, and W1 (ATCC type strains PV845 to 849, respectively). These AMV strains were originally isolated from lucerne in South Australia where homologous polyclonal antibodies against each of the viruses were prepared and their antigenic properties studied (7). For inoculation, each of the carborundum-dusted (600 mesh) unifoliolate leaves of two-week-old seedlings was inoculated with infectious sap extract in 0.01 M phosphate buffer, pH 7.1 as described by Malapi et al (12). The inoculated plants were maintained in a growth chamber operating at 25°C with a photoperiod of 16 h. The AMV was assayed in soybean trifoliolate leaflet positions 1 to 17 with the higher numbers representing the youngest apical leaflets at successive nodes using antigen coated indirect ELISA (12). Thus, leaflets at position 1 were the oldest and leaflets at positions with higher numbers were younger. For these experiments, leaflets at each node that were present at the time of sampling were sampled 30, 60, and 90 days postinoculation (dpi). The samples at 30 dpi consisted of the middle leaflet of each of the existing trifoliolate leaf while the left and then right leaflet of each of the existing trifoliolate leaves were sampled at 60 and 90 dpi, respectively.

For the antigen coated indirect ELISA, leaflets were extracted in 0.05 M sodium carbonate, pH 9.6 (1:4; w/v), and extracts were briefly clarified by centrifugation for 1 min at 5000 rpm prior to loading into duplicate plate wells. All the incubation steps were done at 25°C, except that of blocking that was done at 4°C. After incubation of samples for 1.5 h, plates were rinsed with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.85 % NaCl and 0.05% Tween 20 (PBS-Tween), and subsequently blocked with PBS containing 5% non-fat dried milk for overnight. Following rinsing, polyclonal antibodies against AMV [provided by J. W. Randles of Waite Campus, The University of Adelaide, South Australia (7)] diluted 1:2000 in PBS-Tween containing 5% non-fat dried milk was added and the plates were incubated for 1.5 h. After another rinse, affinity purified alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) diluted 1:2000 in PBS-Tween containing 5% non-fat dried milk was added as the probing antibody. After 1.5 h incubation and rinsing, the substrate ρ-nitro phenolphosphate in diethanolamine substrate solution, pH 9.8, was added. ELISA plates were read with a Microplate Reader Model 680 (Bio-Rad, Hercules, CA).

The inoculated soybean cultivars varied in their responses to infection with the five AMV strains. Williams82 and Lee68 were readily infected with all the AMV strains (Fig. 1A, 1B) based on positive ELISA readings (data not shown). In this work we considered ELISA readings as positive when the mean value at 405 nm exceeded 3x the mean of that of healthy tissues ± SD. Symptoms of AMV infection varied depending upon virus strain and soybean cultivar involved. Williams82 and Lee68 soybean plants inoculated with AMV N20 were shorter than those inoculated with the other strains (Fig. 1A, 1B). Inoculation with AMV N20 resulted in cessation of growth and extreme stunting of Williams82 at 90 dpi (Fig. 1C), and was lethal on Lee68 by 60 dpi (Fig. 1D). The severe phenotype of AMV N20 on soybean is not surprising as this strain has
Fig. 1. Phenotypic response of soybean cultivars Williams82 (A) and Lee68 (B) at 30 days postinoculation (dpi) to five Alfalfa mosaic virus (AMV) strains; and Williams82 (C) at 90 dpi and Lee 68 (D) at 60 dpi to one of the strains. Phenotypic response of soybean cultivar Colfax to three AMV strains at 90, 30, and 60 dpi are shown in (E), (F), and (G), respectively. Plants were rub-inoculated with infectious sap containing viruses and the inoculated plants were maintained in a growth chamber at 25°C with a photoperiod of 16 h. It should be noted that variation in phenotype of disease induced by AMV in soybean precludes reliable detection of the virus solely based on symptomatology.
been shown to be virulent in other hosts as well (7). Colfax exhibited resistance to both AMV H4 and S40 as shown by negative ELISA readings (data not shown). Infection of Colfax with AMV S30 was almost symptomless (Fig. 1E, 1F, 1G). Infected Colfax with AMV S30 showed very mild mosaic by 7 to 10 dpi; however, the symptoms almost disappeared by 30 dpi. This is in contrast to Colfax interactions with AMV strains N20 and W1 where the infected plants remained symptomatic exhibiting mosaic (Fig. 1F, 1G). The variation in symptoms observed in this study further demonstrates the importance of using multiple viral strains as well as soybean cultivars in studies exploring the soybean phenotypic response to AMV. Cultivar variation in response to AMV has been noted previously (1,10). All these observations support the notion that symptoms alone are not reliable parameters for detection and diagnosis of AMV in soybean.

The results of experiments to explore the optimum sampling protocol for all three soybean cultivars showed the expected plant-to-plant variation for the same treatments. Although the complete data set is too extensive to be presented here, the pattern shown by Lee68 (Fig. 2) was consistent and exemplary for all the other strains and cultivars tested in this study. As shown in Fig. 2 (as well as data not shown), the data consistently suggest that for all virus isolates and susceptible cultivars tested, samples should consist of pooled, young fully-developed trifoliolate leaflets that are present and generally harvested at 30 to 60 dpi. Analysis of samples gathered during this time generally resulted in higher ELISA values compared to those for tissues sampled at 90 dpi (see Fig. 2 for comparison). Nevertheless, examination of tissues harvested at 90 dpi, also provided acceptable ELISA readings for all the strains except for AMV S40 in Lee68, which did not infect this soybean cultivar efficiently (Fig. 2B). For soybean producers in the North Central soybean belt that typically plant soybeans during the 30 day period ranging from the end of April to end of May, the data suggest that trifoliolate leaf samples should be gathered for ELISA assay sometimes during late June or late July, at the latest, if AMV is believed to be a concern. By late July, the plants are almost at the stage of post flowering and any AMV infection initiated by that stage of plant growth has no impact on seed transmissibility and minimum effect on yield. Regardless of the time of sampling in the field, in order to increase the probability of virus detection by ELISA, samples should consist of fully developed trifoliolate leaflets gathered from the top two or three nodes of the plant. Samples collected from the older trifoliolate leaves may not be consistently reliable for accurate detection of AMV as previously suggested by the data of Mueller and Grau (13) and confirmed by the results of this study. It is noteworthy, that the cultivar Colfax infected with AMV S30, which was symptomless by 30 dpi and afterward (Fig. 1E, 1F, 1G), remained AMV positive when tested by ELISA at 90 dpi (Fig. 2E). This is important because of the recent report that AMV is seed transmitted in soybean (8). Interestingly, AMV S30 has shown a high rate of seed transmissibility (8%) in Colfax (8). This observation suggests that any conclusions based upon symptom expression alone, regarding susceptibility of Colfax to AMV S30 which has the potential for seed transmission, would be erroneous. Thus, the symptom variation that is dependent upon soybean cultivar and virus strain makes it problematic to conclude that a soybean plant is infected by AMV based upon field symptom exhibition only. Furthermore, it would be erroneous to suggest that seed transmission by AMV could not be a resultant problem if a soybean plant is symptomless.

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Fig. 2. ELISA readings of trifoliolate leaf samples from soybean cultivars Lee68 (A-D) and Colfax (E) rub-inoculated with Alfalfa mosaic virus strains (A) H4, (B) S40, (C) W1, and (D, E) S30 are shown. The inoculated plants were maintained in a growth chamber at 25°C with a photoperiod of 16 h. Leaflets at each node that were present at the time of sampling were sampled 30, 60, and 90 days postinoculation (dpi). The samples at 30 dpi consisted of the middle leaflet of each of the existing trifoliolate leaves while the left and then right leaflet of each of the existing trifoliolate leaves were sampled at 60- and 90-dpi, respectively. Schematic positions of the trifoliolate leaves sampled for analysis by ELISA are depicted at the right side of the panels.
Literature Cited