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Serological Aspects of Phytoplasma Associated with Bermuda grass White Leaf (BGWL) Disease

Roya Biabani khankahdani[1] and Sasan Ghasemi[2]

[1]Plant Protection Dept., College of Agriculture, Islamic Azad University, Shiraz Branch, Iran, Roya_biabani@yahoo.com

[2]Plant Protection Dept., College of Agriculture, Islamic Azad University, Arsanjan Branch, Iran, ghasemi@iaua.ac.ir

Abstract. Bermuda grass white leaf (BGWL) is among the destructive phytoplasmal disease of bermuda grass (*Cynodon dactylon*) which is known to occur in Asia, Africa, Cuba and Italy. It also was observed in several provinces of Iran. Affected grasses exhibited whitening of leaves, proliferation of auxiliary shoots, bushy growing habit, small leaves, shortened stolons, stunting and death of the plants. In order to effectively assess and manage the risk to agricultural crops posed by this phytoplasma, it is necessary to establish whether there is relationship between BGWL and other phytoplasmas such as sugarcane white leaf. Different methods like light microscopic techniques, fluorescent microscopic techniques, and DAPI stain are used for detecting the pathogen, but most of these techniques are indirect and non-specific that can detect any other phytoplasmas. Immunoassays are used for diagnosing disease, identifying and quantifying microorganisms as highly specific techniques. Leaf samples with and without (BGWL) symptoms were collected from Bermuda grass which were cultivated and maintained in green house. BGWL phytoplasma was purified from of infected tissue which is suitable for use as immunogens for polyclonal antibody production, and as antigens for Serological test. Antiserum was raised by injection of partially purified BGWL phytoplasma into rabbit. This antiserum exhibited specificity for its homologous phytoplasma antigens in Plate-Trapped Antigen Enzyme-Linked Immunosorbent Assay (PTA-ELISA), Dot Immunoblotting Assay (DIBA) and Tissue Print Immuno Assay (TPIA). In PTA-ELISA all BGWL samples showed higher values than healthy samples and also with other tested phytoplasmas that showing symptoms such as witches' broom in almond and lime, yellowing in periwinkle and leaf whitening in sugarcane and there is no cross-reaction between this antiserum and tested phytoplasams disease. DIBA was highly specific and rapid in that purple-coloured dots were visualized on the nitrocellulose membrane for BGWL sample and not found for others. In TPIA all samples from bermuda grass white leaf were positive to BGWL polyclonal antibody. The non-infected bermuda grass and other used phytoplasmas disease were negative to this antibody. As a result no cross-reactions were observed in reciprocal tests between this antiserum and other tested phytoplasmas including almond witches' broom, lime witches' broom, periwinkle yellowing and sugarcane white leaf.

Key words:Phytoplasma, bermuda grass, white leaf disease,serology

1. Introduction

Bermuda grass white leaf (BGWL) is among the destructive phytoplasmal disease of bermuda grass (*Cynodon dactylon*), first reported in Taiwan (Chen *et al.,* 1972) which is known to occur in Asian countries (Chen *et al.*, 1972; Zahoor *et al*., 1995; Viswanathan, 1997; Sdoodee *et al*., 1999; Jung *et al*., 2003; Rao *et al*., 2007), Africa (Dafalla & Cousin, 1988) , Cuba (Arocha *et al*., 2005) and Italy (Marcone *et al*., 1997). It also was observed in several provinces of Iran. Typical symptoms such as whitening of leaves, proliferation of auxiliary shoots, bushy growing habit, small leaves, shortened stolons, stunting and death of the plants are exhibited by affected grass.

Phytoplasmas are pleomorphic and fragile organisms and have not been isolated and cultivated yet in vitro which is the major impediment limiting research on phytoplasma disease (McCoy *et al*.1989). Different methods are used for detecting the pathogen like light microscopic techniques using Giemsa and Dienes' stain, fluorescent microscopic techniques employing aniline blue and Hoechst 33258, and DAPI stain. But

most of these techniques are indirect and non-specific techniques that can detect any other phytoplasmas (Thomas and Balasundaran, 2001).

Immunoassays are used as highly specific techniques for diagnosis of disease, identification and quantification of micro organisms. Phytoplasma are purified from diseased plants using the techniques such as differential centrifugation method, celite pad filtration and percoll density gradient centrifugation to raise polyclonal antibodies. These antisera exhibited considerable activity and specificity for the organisms against which they were prepared by immunological techniques assay (Clark *et al*., 1983). In order to effectively assess and manage the risk to agricultural crops posed by this phytoplasma, it is necessary to establish whether there is relationship between BGWL and other phytoplasmas such as sugarcane white leaf.

2. Material and Methods

Leaf samples with and without (BGWL) symptoms were collected from bermuda grass which were cultivated and maintained in green house. BGWL phytoplasma was purified from 40gr of infected tissue using a slightly modification in Saeed *et. al*. (1993) method (Biabani *et al*. 2009). This purified preparation was suitable for use as immunogenic for polyclonal antibody production, and as antigens for Serological tests. Antiserum was raised by injection of partially purified BGWL phytoplasma into rabbit. This antiserum exhibited specificity for its homologous phytoplasma antigen in immunological assays.

Plate-Trapped Antigen Enzyme-Linked Immunosorbent Assay (PTA-ELISA) was employed to detect the pathogen. The method of Mowat and Dawson (1987) with slightly modification was followed to calculate the optimum titer of the antiserum. The antigen from BGWL as well as almond and lime with whiche's broom, periwinkle with yellowing and sugercane with white leaf symptoms were coated directly on to plate and they were incubated with BGWl antibody. The plate was read at 405 nm after substrate addition. For Dot Immunoblotting Assay (DIBA), the method of Saito and Hibi (1985) was used. The antigens of BGWL, SCWL, LWB, AWB and PY were spotted on the nitrocellulose membrane and treated by raised antiserum against BGWL. Tissue Print Immuno Assay(TPIA) was done following the protocol of Huth (1999).The nitrocellulose membrane was probed by pressing newly cut surface of leaf ,with a firm but gentle force, from infected bermuda grass, sugarcane, almond, lime and periwinkle as antigens. The nitro cellulose membranes were then incubated with BGWL antiserum.

3. Results and Discussion

The dilution of 1:1500 was found to be the best titer of raised antiserum against BGWL phytoplasma (data not shown). Determining a titer of 1500 in this test indicates the high quality of product. PTA-ELISA techniques employed antiserum at 1 :1000 dilution which were highly sensitive and the values between the purified healthy and infected antigens and also with other tested phytoplasmas that showing symptoms such as witches' broom in almond and lime ,yellowing in periwinkle and leaf whitening in sugarcane were very distinguishable. As it can be seen in table (1), all the BGWL samples showed higher values than others and there is no cross-reaction betweenthis antisera and tested phytoplasams disease.

Table 1.Evaluation of the raised antiserum of bermuda grass white leaf with infected and healthy Samples

Antigen	Absorbance at 405 nm at 1000 dilutions of Antiserum									
dilution	b_1	b_1	1	1	2	2	3	3	4	4
25	(0.311)	(0.373)	(0.153)	(0.156)	(0.139)	(0.142)	(0.107)	(0.122)	(0.120)	(0.137)
	5	5	H	H	6	6	7	7	8	8
25	0.031	0.023	0.045	0.039	0.057	0.070	0.050	0.053	0.055	0.066
	9	9	10	10	H	H	11	11	12	12
25	0.504	0.553	0.477	0.497	0.014	0.026	0.451	0.511	0.103	0.077
	13	13	14	14	I	I	b_1	b_1	H	H
25	0.018	0.021	0.000	0.011	0.546	0.539	0.302	0.310	0.022	0.014

Blank(b1), sugar cane with flagging leaf symptoms(1,3,4,13), sugar cane with whitening(2), sugar cane with vein clearing(5), periwinkle yellowing(6), almond witches' broom(7), lime witches' broom (8), bermuda grass with whitening(9,10,11),sugar cane with chlorosis and yellowing(12), bermuda grass without symptoms(14),positive control of bermuda grass white leaf(I) and healthy bermuda grass(H).

DIBA was highly specific and rapid in that purple-coloured dots were visualized on the nitrocellulose membrane for BGWL sample. The purple-coloured dots were not found for healthy bermuda-grass and other phytoplasma infected plants include SCWL, LWB, AWB and PY (Table2).

The technique of Tissue Print Immuno Assay on nitrocellulose membranes can be readily applied of plant phytoplasmas. In TPIA all samples from bermuda grass white leaf were positive to BGWL polyclonal antibody and detected as diseased. The non-infected bermuda grass and other used phytoplasmas disease include sugarcane white leaf, lime witches' broom, almond witches' broom and periwinkle yellowing were negative to this antibody (Fig1).

Table2. DIBA test results using the raised antiserum of bermuda grass white leaf. Healthy bermuda grass, bermuda grass white leaf (1, 2, 3), sugarcane white leaf (with yellowing, whitening and flagging symptoms), almond witches' broom, lime witches' broom, periwinkle yellowing respectively.

Antigen \ Antiserum	
Healthy Bermuda grass	
Bermuda grass white leaf 1	
Bermuda grass white leaf 2	
Bermuda grass white leaf 3	
Sugarcane white leaf with yellowing	
Sugarcane white leaf with whitening	
Sugarcane white leaf with flagging	
Almond witches' broom	
Lime witches' broom	
Periwinkle yellowing	

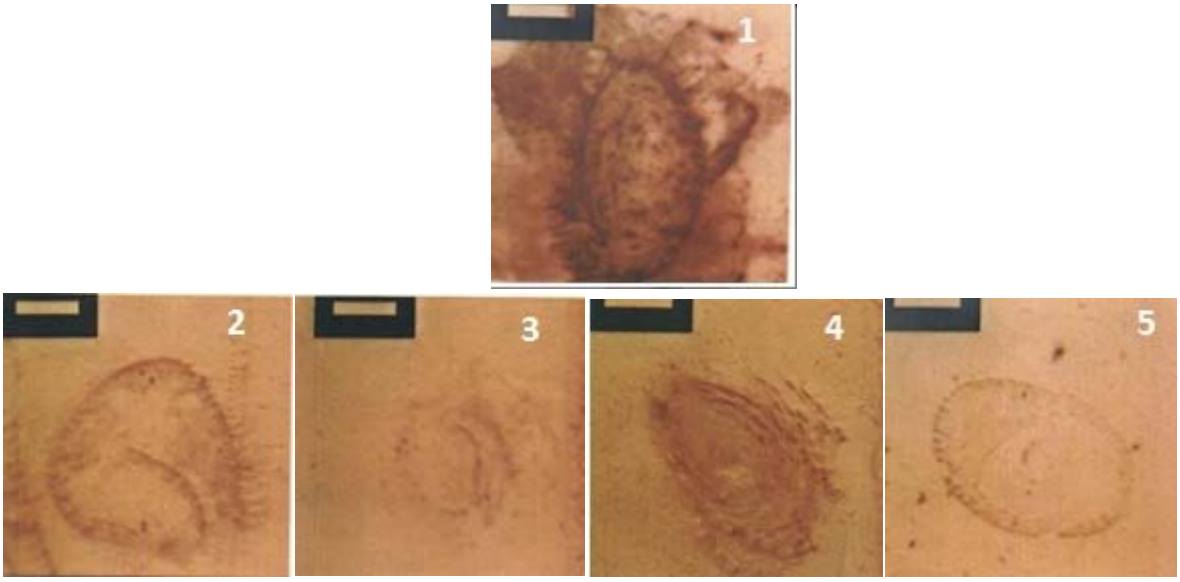

Fig.1.Effect of the raised antiserum of bermuda grass white leaf on samples : Bermuda grass white leaf(1), sugarcane white leaf (2), lime whiche's broom(3), almond whiche's broom (4) and periwinkle yellowing(4).

Phytoplasma are often very difficult to purify as they don't have cell walls, are located in phloem and cannot be cultured in vitro. Therefore, obtaining only partially purified plants extracts is very common which are produce poor quality antisera when injected in to rabbits. Furthermore, the use of polyclonal antisera for the detection of phytoplasma has been relatively unsuccessful because of their high content of antibodies to

plant proteins.This is the mainly due to the difficulty in obtaining highly purified phytoplasma preparation. However, serological assays are very valuable in detection of phytoplasma and in some cases such as electron microscopy, polyclonal antibody acts better than monoclonal antibody(Gomez *et al*.,1996) .In this study, the raised antiserum was used for serological test after cross-absorption with healthy plant material to have very low level of plant proteins .This antiserum has high specificity to detect its homologous phytoplasma and reliably differentiated healthy from infected and other phytoplasma. This is the first report of effectively purification and serological detection of grass phytoplasma in the world. According to the results of the all serological assays used in the present study, there was no serological relationship between BWL and SCWL, LWB, AWB and PY. Positive reactions were obtained only with homologous combinations of antigen and antibody of bermuda grass and no cross reactions were found with any of heterologous combinations and antibody. Likewise,the results demonstrate that detecting phytoplasmas and investigating serological specificity and relationships among phytoplasmas are able to be achieved without the necessity for culturing the organisms in *in vitro* or for obtaining highly purified preparations from plant extracts. Plate-Trapped Antigen Enzyme-Linked Immunosorbent Assay offer a simple diagnosis that is more cost effective and easier to handle. PTA-ELISA was used in this study for detection of phytoplasma as a first time which doesn't need to purify gamaglobulin and with the lowest nonspecific background reactions. Although DIBA and TPIA are less sensitive compared to PTA-ELISA, the large number of samples can be processed within two or three hours rather than a time required for ELISA easily with using low amount of antigen (2ml) and high specifity, so they can be useful to detect phytoplasma. The results of DIBA and TPIA infer that there is no relationship between BWL and SWL, LWB, AWB and PY. As a result no cross-reactions were observed in reciprocal tests between this antiserum and other tested phytoplasmas including almond witches' broom, lime witches' broom, periwinkle yellowing and sugarcane white leaf.

4. References

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