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DETECTION OF RATOON STUNTING DISEASE (RSD) BACTERIUM BY ELISA

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ABSTRACT

Ratoon stunting disease (RSD) caused by the bacterium *Clavibactor xyli* subsp. *xyli* display limited symptoms on the diseased stalks and no symptoms are noticed in the root or foliar region. An indirect enzyme linked immunosorbent assay (ELISA) technique was standardised to detect the RSD bacterial infection in sugarcane. Bacterial ooze/diffusates from the infected stalk tissues were used as antigen in the assay. Antiserum dilution of 1:1000 and enzyme conjugate dilution of 1:8000 were found optimum for the detection of the RSD bacterium in ELISA. In general the sugarcane varieties showed variation in the bacterium titer. All the varieties showing apparent disease symptoms have shown positive reaction for ELISA and many clones which were not showing any conspicuous symptoms also proved positive by ELISA. Varieties like Co 421, Co 997, Q 28 and CP 52-68 which are used as indicator hosts for the RSD, showed highest titer for the bacterium. Most of the sugarcane varieties evolved recently have shown negative reaction to the disease as compared to the old varieties. This technique was reliable for the detection of the suspected infection of the bacterium in sugarcane than the visual symptoms expressed by the cultivars, wherein the symptomless carriers might escape the detection and spread the disease.

KEY WORDS : Sugarcane, Ratoon stunting disease, ELISA

Ratoon stunting disease (RSD) was observed first time in India (Chona, 1956) and this disease is present in all sugarcane growing regions in India. Although RSD is present both in plant and ratoon crops, it is a major constraint to the ratoons of sugarcane. Since the disease spreads quickly through the seed material and the infection percentage increases causing a gradual decline in yields. Diagnosis of RSD is based on detection of internal stalk symptoms in sugarcane in the field or upon diagnostic assay using indicator hosts. Internal RSD symptoms have often been unreliable for field diagnosis since they are not produced in all varieties, vary among varieties, and may be ephemeral even in varieties known to produce distinct discolouration symptoms (Steindl, 1961; Ricaud, 1974). Later, biological assays using indicator hosts with a known and more reliable response to RSD have been used for diagnosis. However, these assays take weeks or even months to conduct and sometimes do not work as well in different laboratories. Although electron microscopy has been used to detect the RSD bacterium in host extracts and tissue sections (Worley and Gillaspie, Jr.1975) it is a cumbersome process. Isolation of *C.x.* subsp. *xyli* in axenic culture from plant tissue has also been used for diagnostic purposes; however, isolation usually takes time and laborious which limit the usefulness of this technique for diagnosis. Serological methods have been developed for improved sensitivity and specificity in detection of RSD bacterium in samples from sugarcane (Gillaspie, 1978). The purpose of the study reported herein was to standardise and evaluate the usefulness of an indirect ELISA for diagnosis of RSD in sugarcane.

MATERIALS AND METHODS

Plant materials

Sugarcane varieties with clear internal stalk symptoms and disease free clones were maintained

Table 1. Effect of different loads of antigen and antiserum in detection of the RSD bacterium by ELISA

Antigen load per well	Antiserum load per well*	
	200 µ l	100 µ l
200 µ l	0.207 ± 0.003	0.186 ± 0.004
100 µ l	0.174 ± 0.002	0.167 ± 0.006
50 µ l	0.174 ± 0.005	0.162 ± 0.005

* Mean of 6 replications; Absorbance at 405 nm

Table 2. Variation of antigen from different nodal samples on detection of RSD bacterium by ELISA

Nodal position	Genotype	
	Q 28	Co 421
5th node	0.441 ± 0.012	0.526 ± 0.016
10th node	0.420 ± 0.008	0.504 ± 0.013
15th node	0.416 ± 0.014	0.492 ± 0.19

Mean of 6 replications, Absorbance at 405 nm.
200 µ l of antigen and antiserum used in the test.

at the Plant Pathology Farm, Sugarcane Breeding Institute, Coimbatore. The experiments were conducted during the 1994-1995 and 1995-1996 crop season. For the ELISA tests samples were drawn from 12 months old sugarcane genotypes. For assessing the load of the bacterium in different nodal regions tissue samples from 5th, 10th and 15th nodes were taken from standard susceptible varieties Q 28 and Co 421. Since samples from 5th node recorded higher titre for the antigen, tissues were drawn from 5th node with adjoining internodal tissue in other genotypes for the ELISA. About 5g of nodal and internodal tissues were immersed in 10 ml of phosphate buffered saline (PBS) 0.1M (pH 7.2) containing 0.01M sodium diethyldithiocarbamat (NaDIECA) (Sigma chemical Co., USA) overnight at 4°C. The diffusates centrifuged at 3000 rpm for 15 min. at 4°C and the supernatant collected and used as antigen for ELISA test.

ELISA

Direct antigen coating indirect ELISA was performed as reported previously for the detection of sugarcane bacilliform virus (Viswanathan *et al.*, 1996). The bacterial diffusates (200 µl) from different varieties were loaded to microtitre plates (Tarson Laboratories, Calcutta) and incubated at 4°C for 18 h. Later contents in the wells were emptied and washed with PBS-Tween (PBST) 3 times at 2 min, intervals. After washing, the plates were loaded with 200 µl of polyclonal antisera raised against RSD bacterium (Supplied by J.C. Comstock, HSPA, Hawaii, USA) diluted to 1:1000 in PBSTPO (PBST, 2% Polyvinyl pyrrolidone ; 0.2% ovalbumin) and incubated at 37°C for 3 h. Antirabbit goat r globulins conjugated with alkaline phosphatase (Sigma chemicals, USA) diluted to 1:8000 were loaded to the wells (200 µl) after washing the plates with PBST and incubated for 3 h at 37°C. After incubation and washing, the enzyme substrate *para* nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8 (1 mg/ml) was added to the wells (200 l) and colour development was monitored at room temperature. After one h colour development was read in a Biotek ELISA reader, Model EL 311s (Biotek Instruments, USA) at 405 nm.

RESULTS AND DISCUSSION

Results of the experiments clearly established that indirect ELISA was suitable in the detection of RSD bacterium in sugarcane. Tests with different loads of antigen and antiserum revealed that not much variation could be observed between the treatments (Table 1). Similarly samples from different nodes of an infected plant showed less varition in absorbance. However, in both varieties (Q 28 and Co 421) basal nodal samples had higher titer than the top younger nodes (Table 2). Based on these results, bacterial diffusates from 5th nodes of certain varieties were screened for bacterial infection. Results showed variation in absorbance values among genotypes when 200 µl of antigen, antiserum (1:1000), enzyme conjugate (1:8000) and substrate (1mg/ml) were used. In the test genotypes showing clear symptoms of RSD *viz.*, Co 421, Co 617, Co 1305, NCo 310, Q 28 and CP 52-68 had higher absorbance values. However other genotypes like Co 213, Co 281, Co 312, Co 356 and Co 453 which are not exhibiting any symptoms at the time of testing, also showed higher titer for the bacterial infection (Table 3).

Several methods like using indicator plants, microscopic detection, isolation of bacterium in axenic cultures were recommended for the detection of the RSD bacterium. These techniques are not reliable on certain situations or cumbersome process as in electron microscopic methods. The

Table 3. Screening of sugarcane genotypes for the suspected RSD infection by ELISA

Genotype	Year or release	Absorbance at 405 nm
Co 213	1918	0.213
Co 281	1924	0.295
Co 312	1928	0.224
Co 356	1931	0.214
Co 419	1933	0.283
Co 421	1934	0.634
Co 453	1938	0.253
Co 617	1944	0.396
Co 997	1953	0.378
Co 1148	1955	0.219
Co 1158	1956	0.124
Co 1305	1959	0.342
CoJ 64	1971	0.248
CoC 671	1975	0.110
CoC 86062	1986	0.057
Co 7704	1983	0.061
Co 8021	1986	0.072
NCo 310*		0.262
Q 28*		0.436
CP 44-101*		0.241
CP 52-68*		0.388
Control		0.043

Mean of 6 replications.
* Genotypes of foreign origin which are used as RSD indicator varieties received before 1975.

bacterium is slow growing in culture (Davis and Dean, 1984). Efficiency of serological techniques in the detection of RSD over microscopic techniques is known (Harrison and Davis, 1990). The present study standardise the ELISA test for the local RSD bacterial infection. Normally an ELISA test is considered positive when suspected sample has atleast 3 times higher values in absorbance to the healthy control. Here all the foreign hybrids and Indian hybrids evolved in 1980 and before has more than 2 to 3 times to the control samples (from first clonal seedling). Sugarcane varieties evolved later showed less titer or negative to the bacterium.

RSD causes severe yield losses to the sugarcane in most of the sugarcane growing countries. RSD caused yield losses ranged from 29 to 60 T/ha in a 3 year cropping (Grasham, 1991). Though losses due to RSD have been encountered in most of the states, severity of the disease noticed mostly from subtropical India (Agnihotri, 1983). At Coimbatore clear RSD symptoms are noticed only in few clones. Even those clones showing clear internal symptoms are not exhibiting any apparent variation in cane weight, height or internodal length. It may be due to continuous irrigation and maintaining optimum soil nutrient status. In this context ELISA found to be a reliable technique to identify RSD suspected clones. Detection of RSD in the seed cane and canes intended for exchange by ELISA would strengthen our quarantine activities at Sugarcane Breeding Institute. Further it is more reliable than the currently used methodologies like indicator hosts and the biochemical tests. Recently use of an indirect dot-blot assay technique on nitrocellulose membrance, for the detection of RSD bacterium in the suspected genotypes showed that it was as sensitive as ELISA (Viswanathan, 1996). The cost of an ELISA test under our laboratory conditions worked about Rs. 75 per assay and in each test atleast 46 samples can be accommodated hence it was found cost effective under Indian conditions also.

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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF SUGARCANE MOSAIC VIRUS

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ABSTRACT

Sugarcane mosaic virus (SCMV) a member of Potyvirus group is an important disease of sugarcane. Currently, the virus is identified based on the foliar symptoms exhibitd by the virus on differential hosts. An indirect-ELISA was standardised to detect the presence of virus in the host very precisely. Antigen dilution of 1:500, antiserum dilution of 1:1000 and enzyme conjugate dilution of 1:8000 were found optimum for the detection of the suspected virus in the host. In highly susceptible varieties, SCMV presence was detected even at a dilution of 1×10^{-5} by ELISA test. Polyclonal antisera developed against SCMV strain-N was found suitable to detect suspected samples of other SCMV strains also. This technique will have greater applications in the routine quaratine work of this Institute.

KEY WORDS : Sugarcane mosaic virus, ELISA, detection, quarantine

Sugarcane mosaic virus (Potyvirus group) is one of the most potentially dangerous diseases of sugarcane and is widely distributed in the sugarcane growing countries. Importance of this disease was recognised in earlier decades after its inadvertent introduction into Argentina with PoJ canes brought in from Java (Artschwager and Brandes, 1958). Only Mauritius and Guyana are known to have SCMV free sugarcane crop (Agnihotri, 1983). Several strains of the SCMV were reported from different countries. In India, strains of A, B, C, D, E, F, H and N were reported to occur (Kondaiah and Nayudu, 1984). Yield reduction effected by SCMV was linked to the strain of SCMV in India (Rishi *et al.*, 1975).

Though much work has been done on strains of SCMV in India, work on detection of SCMV is still based on visual symptoms or symptom expression on differential hosts. Detection of SCMV by improved techniques like serological and nucleic acid probes have been standardised in other countries (Smith, 1996). These techniques are more reliable, less time consuming, efficient and able to detect very low concentrations of the virus present in the suspected samples. Precise detection of SCMV is essential as this virus is of quarantine importance in India and abroad. So an indirect ELISA technique was standardized for the detection of SCMV in sugarcane and the same is reported hereunder.

MATERIALS AND METHODS

Production of antiserum

SCMV strain N was selected for the production of antiserum. Infected sugarcane leaves macerated in 0.01 M phosphate buffer, pH 7.0 containing 0.2% 2-mercaptoethanol at the rate of 9 ml per gm leaf material and the virus was transferred to 10 to 15 days old sorghum seedlings (genotype Rio). Two weeks after inoculation infected leaf materials homogenized in 0.5 M Sodium borate buffer, pH 8.0 containing 0.01M EDTA and 0.2% thioglycollic acid. The homogenate was filtered through muslin cloth and the filtrate was mixed with chloroform and carbon tetra chloride (1:1) mixture to 10% and Triton X-100 to 5%. The mixture was stirred for about 30 min. and centrifuged at 8200 rpm for 10 min. The