Journal of Ecobiotechnology 1/1: 024-027, 2009
ISSN 2077-0464
Journal of Ecobiotechnology
http://journal-ecobiotechnology.com/

# Isozyme studies of resistant and susceptible clones of *Saccharum officinarum* L. to fungal diseases

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**Keywords**

Sugarcane clones
Isozyme
Peroxidase
Superoxide dismutase
PAGE

**Abstract**

Sugarcane is one of the important commercial crops of India and it plays an important role in maintaining the economy of the nation. Many diseases of sugarcane lead to loss of net productivity of sugar and by growing resistant varieties the loss can be reduced to considerable amount. Hence, in this study an attempt has been made to identify a resistant clone through isoenzyme analysis. Three resistant (Bo-91, Co-86249, Co-93009) and three susceptible (CoC-671, CoC-86032, CoC-92061) clones of *Saccharum officianarum* L. were selected for the study. In native-PAGE analysis (for peroxidase) distinct bands with Rf value 0.537 and 0.694 were observed in resistant clones, and the susceptible clones lack these bands. An increase in peroxidase activity is also observed in clones that possessed the distinct Rf values 0.537 and 0.694. From this, it can be concluded that the clones possessing distinct Rf values are resistants when compared to other clones as they are having high peroxidase action.

## 1. Introduction

Sugarcane (*Saccharum officinarum* L.) belongs to the family Poaceae, is the major source of sugar in the world. It is an important commercial crop of India. In the world, the major sugarcane cultivated countries are India, Cuba, Brazil, Mexico, Pakistan, China, Philippines, South Africa, Australia and Thailand. In recent years, the productivity of sugar has declined mainly due to the fungal attack in sugarcane crop. Hence, to eradicate this problem, identifying better clones of disease resistant is an important task [1]. The isozymes have been proved to be reliable molecular markers in breeding and genetic studies of plant species [2, 3]. Isoenzyme markers have been successfully used in crop improvement programs mainly due to its consistency in their expression with irrespective of environmental factors [4, 5]. In this investigation, an attempt has been made to study the diversity and role of peroxidases (PRX) and superoxide dismutase (SOD) in resistant and susceptible sugarcane clones and in assessing the utilization of isozyme technique to select desirable clones in sugarcane breeding programme. These enzymes have been proposed to be possibly important in plant defense mechanism against diseases [4]. These enzymes are ubiquitous in nature and their activity has been reported in wide range of plants [6]. They are mainly involved in the regulation of metabolic pathway in diseased and injured plant tissues [7].

## 2. Materials and Methods

### *2.1. Isolation of soluble and insoluble protein*

One gram of fresh leaves was macerated in a mortar with 2 ml of acetone (80 %, v/v) to remove pigments. After centrifugation at 6000 rpm for 10 min, the pellet was mixed by agitation for 1 h with Tris buffer (40 mM, pH 7.8) which contains 350 mM manitol, 2 mM EDTA and PVPP 3% (w/v). Following centrifugation (10,000rpm for 20 min at 4°C), the supernatant was collected as the soluble protein fraction. The pellet was agitated for 1 h with 2 ml of lyse solution (8 M urea, 1 M thiourea, 2% CHAPS and 50 mM DTT). After another centrifugation (10,000g for 20 min at 4°C), the supernatant, *i.e.* the insoluble protein fraction, was kept at 80°C for posterior analysis. Protein concentration was determined by Bradford [8] method using bovine serum albumin as standard.

*2.2. Enzyme extraction and activity assays*

*2.2.1. Extraction*

Sugarcane leaves (1.0 g fresh weight) were homogenized for 5 min with a mortar and pestle in 1.5 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1 mM ascorbic acid. After filtration with cheesecloth, the homogenate was centrifuged at 13,000g for 20 min and the supernatant (crude extract) was used as the source of enzymes. All steps were carried out at 0–4°C. Protein contents of the crude extracts were determined by using Bradford [8] protein assay, with bovine serum albumin as a standard.

*2.2.2. SOD activity*

The activity of superoxide dismutase was determined by adding 50 µl of the crude enzyme extract to a solution containing 13mM L-methionine, 75 µM p-nitro blue tetrazolium chloride (NBT), 100 µM EDTA and 2 µM riboflavin in a 50 mM potassium phosphate buffer (pH 7.8). The reaction took place in assay tubes upon illumination (White fluorescent lamp at 25°C) for 15 min [9]. The blue formazane produced by Nitroblue Tetrazolium (NBT) photoreduction was measured by the absorbance at 620 nm in a spectrophotometer. The control reaction mixture had no enzyme extract. The blank solution had the same complete reaction mixture but was kept in the dark. One SOD unit of activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction in comparison to tubes lacking the plant extract. Activity was expressed as units per mg soluble protein per min (UA°$mg^{-1}$ protein $min^{-1}$).

*2.2.3. Activity of SOD isoforms in native electrophoresis*

Gel activity assays were performed on samples containing equal amounts of protein (40µg) and separated by non-denaturing 12.5% PAGE at 4°C. The gels were placed in a solution of 0.05 M potassium phosphate (pH 7.8), containing 1 mM EDTA, 0.1 mM NBT and 0.05 mM riboflavin, for 15 min. For identification of Mn-SOD and Cu/Zn-SOD activities, the gels were immersed in 5 mM $H_2O_2$ or 2 mM KCN in 0.1 M potassium phosphate (pH 7.8), respectively, for 15 min in the dark. Both $H_2O_2$ and KCN are inhibitors of Cu/Zn-SOD whereas $H_2O_2$ is an inhibitor (but KCN is not) of Fe-SOD. Both $H_2O_2$ and KCN do not inhibit Mn-SOD. Afterwards, all gels were exposed to illumination by a 30 W fluorescent lamp at 25°C until the appearance of the bands [10].

## 3. Results and Discussion

The concentrations of the protein samples isolated from the sugarcane leaf were quantitated by Bradford method (Table 1). The protein samples were concentrated by acetone method and a known concentration of 20µg was used for protein profiling of all the samples by SDS-PAGE analysis [11] (Fig.1). All the sugarcane clones have shown similar protein profiling pattern.

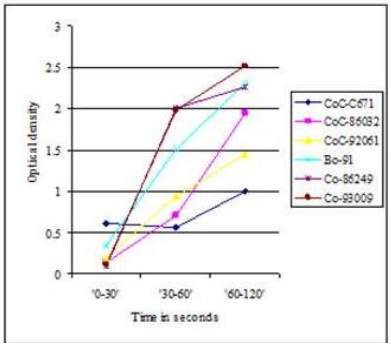
Table 1. The different protein concentrations of sugarcane clones

| S.No. | Clones | Protein (µl) | Water (µl) | Reagent (ml) | OD | Protein Conc. (µg) |
|---|---|---|---|---|---|---|
| 1. | CoC-671 | 100 | 400 | 2 | 0.680 | 11.25 |
| 2. | CoC-86032 | 100 | 400 | 2 | 0.035 | 5.75 |
| 3. | CoC-92061 | 100 | 400 | 2 | 0.028 | 4.5 |
| 4. | Bo-91 | 100 | 400 | 2 | 0.032 | 5.25 |
| 5. | Co-86249 | 100 | 400 | 2 | 0.163 | 20.75 |
| 6. | Co-93009 | 100 | 400 | 2 | 0.134 | 22.75 |
| 7. | Blank | - | 400 | 2 | - | - |

Table. 2. Spectrophotometer assay of peroxidase in different sugarcane clones

| Clones | Absorbance at different time intervals (470nm) | | |
|---|---|---|---|
| | 0-30 seconds | 30-60 seconds | 60-120 seconds |
| CoC-671 | 0.61 | 0.55 | 0.99 |
| CoC-86032 | 0.13 | 0.70 | 1.93 |
| CoC-92061 | 0.19 | 0.94 | 1.44 |
| Bo-91 | 0.34 | 1.50 | 2.30 |
| Co-86249 | 0.09 | 2.00 | 2.26 |
| Co-93009 | 0.11 | 1.97 | 2.52 |

Graph shows OD values of peroxidase in sugarcane clones

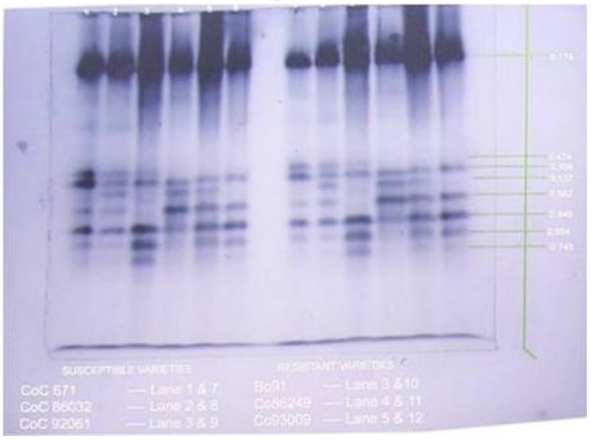



Peroxidase activity by Isozyme analysis of [12] all the three resistant and three susceptible sugarcane clones were carried out in the native-PAGE by using Reddy and Gasber method [13], where the tetramethyl benzidine was used as colouring agent and hydrogen peroxide was used as substrate. The development of blue colour bands after the addition of substrate solution indicated the activity of the peroxidase enzyme (Fig. 2). The development of colour mainly dependent on the gel which was incubated in a solution containing enzyme specific histochemical stain [14] and all the necessary components like substrate, cofactors and an oxidized salt. Enzyme reaction that changes colour when it is reduced to produce a discrete band. The distinct band

at the Rf value of 0.537 and 0.694 were only present in the resistant clones where as the particular size band was not present in all the three susceptible clones. The peroxidase activity has been correlated with resistance in many species [15-17].

Fig. 1. Protein profile


Fig. 2. Isoenzyme analysis of peroxidase




In these interactions, the enzyme is involved in the polymerization of proteins and lignin or suberin precursor in plant cell wall [18, 19] and this constructing a physical barrier that could prevent pathogen entry into cell walls or movement through vessels [20]. The free-radical intermediates produced by peroxidase oxidative activity are toxic to pathogens [21] and this result is then substantiated through spectrophotometric assay of peroxidase. The peroxidase activity in the leaves of resistant and susceptible sugarcane clones exhibited difference in guaicol peroxidation.

The clones with distinct Rf values 0.537 and 0.694 are shown more activity than others. This indicates the high peroxidase action in resistant clones compared to susceptible one. Similar findings have been recorded earlier by Kathiresan and Manjunatha [22, 23]. Isozyme analysis superoxide dismutase [24] showed a little qualitative difference when compare to peroxidase activity.

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