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ORIGINAL ARTICLE

Isozyme Analysis on Different Varieties of Sugarcane

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Isozymic and protein diversity among five sugarcane varieties viz., Co 6304, Co 85019, Co 8371, Co 89003 and Co 91010 were studied to understand the varietal interrelationship and to identify the biochemical marker for the disease resistance and stress tolerance. The standard technique of vertical gel electrophoresis PAGE was employed for size separation of isozymes. The gel was stained with different staining solutions for different isozyme systems *viz.* peroxidase, esterase, acid phosphatase, alkaline phosphatase and proteins. Rf values of the banding profiles, similarity index and variation between the varieties were analysed. Among the four enzyme systems, peroxidase profile reveals the difference between the disease resistant / susceptible and abiotic stress tolerant / non tolerant varieties. The two isoperoxidase bands with Rf values 0.62 and 0.66 showed their presence in disease resistant and abiotic tolerant varieties. The presence of two marker bands (0.62, 0.66) of resistant and stress tolerant varieties suggest that the variety Co 6304 may also be resistant to smut, wilt and moderately resistant to red rot and tolerant to drought.

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India is a major centre of origin and diversity of crop and medicinal plants. It holds an extraordinary significance among the top gene-rich countries of the world relating to its abundantly rich land race diversity in medicinal, agricultural and horticultural crops and their wild relatives. Sugarcane (*Saccharum officinarum* L.) is the main source of sugar in the world. The major sugarcane cultivated countries are India, Cuba, Brazil, Mexico, Pakistan, China, Philippines, South Africa, Australia and Thailand. It holds a prominent position as a crop in India and occupies about 1.8 % of the total cropped area in the country. The rapid progress in the sugar industry sector is largely due to release of high yielding, early maturing and promising cultivars by sugarcane breeders. However, the total productivity of the sugarcane is declined due to fungal attack, increase in stress conditions and other environmental factors associated with sugarcane agriculture (Virupakshi *et al.,* 2002). Hence to overcome this problem, identifying better clones of disease resistant variety is an important task

(Hemaprabha and Sree Rangasamy, 2001). The modern molecular approaches have been adopted to map the sugarcane genome, in order to select better cross combinations to develop popular hybrids. Isoenzyme markers have been successfully used in several crop improvement programmes (Siva and Krishnamurthy, 2005; Geethalakshmi *et al.,* 2005; Anbazhagan *et al.,* 2009) and proven to be reliable phylogenetic markers in plant breeding and genetic studies due to its consistency in their expression irrespective of environmental factors (Kuc, 1990).

In sugarcane, isozyme analysis was first carried out on 1969 (Heinz, 1969). The previous reports revealed variation among the relatives of sugarcane for peroxidases and other isozymes (Glaszmann, 1989; Eksomtramage, 1992; Waldron and Glasziou, 1971; Manjunatha *et al.,* 2003). Isozymes play a vital role in plant defense mechanism against diseases. These enzymes are ubiquitous in nature and their activity has been reported in wide range of plants. They are mainly involved in the regulation of metabolic pathway in diseased and injured plant tissues (Dhanya *et al.,* 2006). Some reports are available on isozyme variation studies among the smut resistant and susceptible sugarcane varieties but there is no information on smut resistant and susceptible sugarcane varieties like Co 6304, Co 85019, Co 8371, Co 89003 and Co 91010. In this study an attempt has been made to analyse the isozymic and protein diversity of five sugarcane varieties differing for reaction to smut for understanding the varietal interrelationship and to identify the biochemical marker for the disease resistance and stress tolerance.

MATERIALS AND METHODS

The experimental materials consisted of sugarcane Co 6304, Co 85019, Co 8371, Co 89003 and Co 91010 which were collected from Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India. For peroxidase analysis, the young leaves were homogenized in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 10 min. For esterase, the young leaves were collected and ground with pre-chilled isolation buffer (0.1 M phosphate buffer at pH 9.2) and centrifuged at 12,000 rpm for 10 min. For acid and alkaline phosphatase, the young leaves were harvested and homogenized in a mortar and pestle with citrate buffer and centrifuged at 20,000 rpm for 10 min. For total protein, the young leaves were ground with 0.2 M phosphate buffer (pH 7.2) and centrifuged at 10,000 rpm at 10 min. The supernatant was subjected to electrophoresis as described by Sadasivam and Manickam (1992) on PAGE. The staining solution was prepared as described by Sadasivam and Manickam (1992) for the detection of isozymes and total proteins on the gels. After the electrophoresis, the gels were incubated in the staining solution for a few minutes under the dark until the clear bands appeared. The gels were fixed with 7% acetic acid solution for 30 min; the gels were washed with distilled water and photographed using the gel documentation system, Vilber Lourmat. The pairing affinity was calculated between the cultivars accordingly. The cladogram was constructed based on the similarity indices of individual and combined isozymes and proteins using NTSYS.

RESULTS

Peroxidase (PRX)

Two regions of activity (PRX 6 and 7) with four bands were observed in this enzyme system. Two bands were observed in each variety, but with different Rf values. There was no common banding profile in peroxidase enzyme profile among the five varieties. The first band MW-Rf 0.58 (PRX 6^1) was shared by three varieties viz., Co 85019, Co 8371 and Co 89003. The second band MW-Rf 0.62 (PRX 7^1) was common in two varieties Co 6304 and Co 91010. The third band MW-Rf 0.64 (PRX 7^2) was

present in three varieties viz., Co 85019, Co 8371 and Co 89003. The fourth band MW-Rf 0.66 (PRX 7^3) was observed in two varieties Co 6304 and Co 91010 (Table 1: Fig. 1 C and H).

Acid Phosphatase (ACP)

The acid phosphatase enzyme system showed two regions of activity (ACP 7 and 8) with three bands. The variety Co 85019 showed its unique banding profile in region ACP 8^2 with 0.78 MW-Rf value. The band ACP 7^1 was observed in Co 6304 and Co 8371 with MW-Rf 0.70 in the acid phosphatase enzyme system. The band ACP 8^1 was commonly present in three varieties viz., Co 85019, Co 89003 and Co 91010 with MW-Rf 0.71 in the acid phosphatase enzyme system (Table 1: Fig. 1 D and I). Acid Phosphatase system failed to express the common banding profiles among the five different sugarcane varieties.

Esterase (EST)

In the Esterase enzyme system, two regions (EST 7 and 8) of activity with five different bands were obtained. The variety Co 6304 showed its unique banding profile in region EST 8^2 with 0.74 MW-Rf value. The variety Co 91010 displayed its presence only in region EST 7^1 with 0.65 MW-Rf value. The band EST 7^2 was observed in two varieties Co 89003 and Co 91010 with MW-Rf 0.70 in the esterase system. The band EST 8^1 was jointly present in two varieties Co 85019 and Co 8371 with MW-Rf 0.72. The band EST 8^3 was common to two varieties Co 8371 and Co 89003 with MW-Rf 0.78. (Table 1: Fig. 1 E and J).

Alkaline Phosphatase (AKP)

In the Alkaline Phosphatase enzyme system, three regions (AKP 6-8) of activity with seven bands were obtained but no common banding pattern was generated by these five varieties. The variety Co 6304 failed to show its uniqueness in the alkaline phosphatase enzyme system. The variety Co 85019 illustrated its presence only in AKP 8^2 with MW-Rf 0.72. The variety Co 8371 showed its presence only in AKP 7^3 with MW-Rf 0.69. Bands of AKP 8^1 (0.71) and AKP 8^3 (0.75) were found only in Co 89003. AKP 6^1 with MW- Rf 0.59 was jointly present in Co 85019 and Co 91010. AKP 7^1 with MW- Rf 0.63 showed its common presence in Co 8371 and Co 91010. AKP 7^2 with MW- Rf 0.67 was shared by the varieties Co 6304 and Co 85019 (Table 1: Fig. 1 A and F).

Protein (PP)

Two regions (PP 5 and 6) of activity were obtained in the protein system. The variety Co 6304 had distinctive banding profile in two regions PP 5^2 and PP 6^1 with MW-Rf value of 0.47 and 0.51 respectively. The bands PP 5^1 and PP 5^4 were shared by two varieties Co 89003 and Co 91010 with MW-Rf values 0.44 and 0.50 respectively. The varieties Co 85019 and Co 8371 showed its common banding pattern in PP 5^3 and PP 6^2 with MW-Rf values 0.48 and 0.51 respectively.

A similarity index was calculated based on the isozyme and protein profile of the five different sugarcane varieties, with emphasis on the isoperoxidase, isoesterase, acid phosphatase, alkaline phosphatase and total proteins (Table 2-7) and a cladogram was generated (Fig. 2). Highest percentage (0.2631) of similarity was observed between the varieties Co 85019 and Co 8371, while the highest percentage (100%) of variation was observed between Co 6304 and Co 89003 (Table 2). While Co 89003 is susceptible to smut, wilt and red rot diseases, the varieties Co 91010, Co 8371 and Co 85019 are resistant to smut and red rot diseases (Table – 8; URL, 2008; URL 2009; URL 2011).

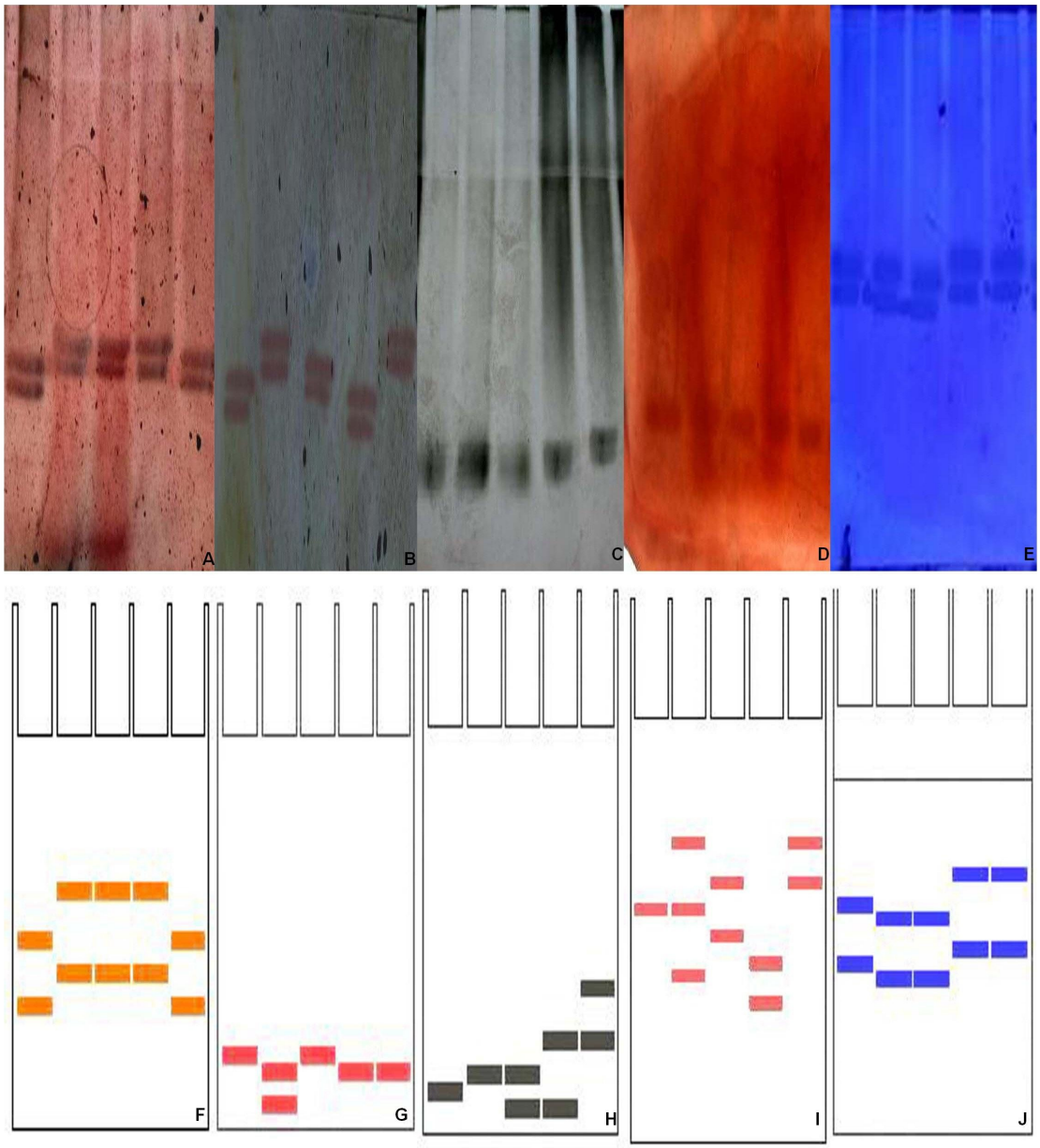

Figure 1. Isozyme Analysis on Different Varieties of Sugarcane

A - Peroxidase banding pattern of selected sugarcane varieties
B - Acid Phosphatase banding pattern of selected sugarcane varieties
C - Esterase banding pattern of selected sugarcane varieties
D - Alkaline Phosphatase banding pattern of selected sugarcane varieties
E - Protein banding pattern of selected sugarcane varieties
F – Zymogram of Peroxidase of selected sugarcane varieties
G - Zymogram of Acid Phosphatase of selected sugarcane varieties
H - Zymogram of Esterase of selected sugarcane varieties
I - Zymogram of Alkaline Phosphatase of selected sugarcane varieties
J - Zymogram of Protein of selected sugarcane varieties
K - Cladogram of selected sugarcane varieties

Table 1: MW- Rf values and banding profile of selected smut resistant sugarcane varieties

MW-RF	Band Positions	Co 6304	Co 85019	Co 8371	Co 89003	Co 91010
PEROXIDASE						
0.58	PRX 6^1	-	+	+	+	-
0.62	PRX 7^1	+	-	-	-	+
0.64	PRX 7^2	-	+	+	+	-
0.66	PRX 7^3	+	-	-	-	+
ACID PHOSPHATASE						
0.70	ACP 7^1	+	-	+	-	-
0.71	ACP 8^1	-	+	-	+	+
0.78	ACP 8^2	-	+	-	-	-
ESTERASE						
0.65	EST 7^1	-	-	-	-	+
0.70	EST 7^2	-	-	-	+	+
0.72	EST 8^1	-	+	+	-	-
0.74	EST 8^2	+	-	-	-	-
0.78	EST 8^3	-	-	+	+	-
ALKALINE PHOSPHATASE						
0.59	AKP 6^1	-	+	-	-	+
0.63	AKP 7^1	-	-	+	-	+
0.67	AKP 7^2	+	+	-	-	-
0.69	AKP 7^3	-	-	+	-	-
0.71	AKP 8^1	-	-	-	+	-
0.72	AKP 8^2	-	+	-	-	-
0.75	AKP 8^3	-	-	-	+	-
PROTEIN						
0.44	$PP5^1$	-	-	-	+	+
0.47	$PP5^2$	+	-	-	-	-
0.48	$PP5^3$	-	+	+	-	-
0.50	$PP5^4$	-	-	-	+	+
0.51	$PP6^1$	+	-	-	-	-
0.52	$PP6^2$	-	+	+	-	-

Table 2: Similarity Index of different sugarcane varieties of peroxidase

Varieties	Co 6304	Co 85019	Co 8371	Co 89003	Co 91010
Co 6304	1.00				
Co 85019	0.00	1.00			
Co 8371	0.00	0.50	1.00		
Co 89003	0.00	0.50	0.50	1.00	
Co 91010	0.50	0.00	0.00	0.00	1.00

Table 3: Similarity Index of different sugarcane varieties of acid phosphatase

Varieties	Co 6304	Co 85019	Co 8371	Co 89003	Co 91010
Co 6304	1.00				
Co 85019	0.00	1.00			
Co 8371	0.50	0.00	1.00		
Co 89003	0.00	0.34	0.00	1.00	
Co 91010	0.00	0.34	0.00	0.50	1.00

Table 4: Similarity Index of different sugarcane varieties of esterase

Varieties	Co 6304	Co 85019	Co 8371	Co 89003	Co 91010
Co 6304	1.00				
Co 85019	0.00	1.00			
Co 8371	0.00	0.34	1.00		
Co 89003	0.00	0.00	0.25	1.00	
Co 91010	0.00	0.00	0.00	0.25	1.00

Table 5: Similarity Index of different sugarcane varieties of alkaline phosphatase

Varieties	Co 6304	Co 85019	Co 8371	Co 89003	Co 91010
Co 6304	1.00				
Co 85019	0.25	1.00			
Co 8371	0.00	0.00	1.00		
Co 89003	0.00	0.00	0.00	1.00	
Co 91010	0.00	0.20	0.25	0.00	1.00

Table 6: Similarity Index of different sugarcane varieties of protein

Varieties	Co 6304	Co 85019	Co 8371	Co 89003	Co 91010
Co 6304	1.00				
Co 85019	0.00	1.00			
Co 8371	0.00	0.50	1.00		
Co 89003	0.00	0.00	0.00	1.00	
Co 91010	0.00	0.00	0.00	0.50	1.00

Table 7: Similarity Index table of selected smut resistant sugarcane varieties

Varieties	Co 6304	Co 85019	Co 8371	Co 89003	Co 19010
Co 6304	1.0000				
Co 85019	0.0588	1.0000			
Co 8371	0.0625	0.2631	1.0000		
Co 89003	0.0000	0.1578	0.1666	1.0000	
Co 19010	0.125	0.1052	0.0555	0.2222	1.0000

Table 8: Sugarcane Varieties with their resistant characters

Sugarcane Varieties				
Co 6304	**Co 85019**	**Co 8371**	**Co 89003**	**Co 91010**
?	Moderately Resistant to smut	Resistant to Smut	Susceptible to Smut	Resistant to Smut
?	Resistant to Red Rot	Moderately Resistant to Red Rot	Susceptible to Wilt	Resistant to Insect Pest
?	Tolerant to Drought	Tolerant to Drought and Water Logging	Moderately Susceptible to Red Rot	Moderately susceptible to Red Rot
?				Tolerant to Drought and Water Logging

The close relationship between the variety Co 6304 and the varieties Co 91010, Co 8371 and Co 85019 with the similarity indices 0.059, 0.063 and 0.125 reveals the resistant and tolerant character of the variety Co 6304. The cladogram based on the individual isozyme banding profile of different varieties showed different degrees of relationship. In general, higher degree of variation has been found in isoperoxidase profile when compared with three other isozyme profiles and a protein profile, in which more or less uniform pattern of relationship has been observed (Fig. 2 A-E).

DISCUSSION

The isozyme electrophoretic banding profile insisted multiple bands on different loci with different Rf values and unequal intensities. The results of isozymic analysis on different varieties of sugarcane confirmed their heterozygous nature by producing more complicated banding pattern with multiple expression in the same region (heteromeric or hybrid bands) (Table 1). Electrophoretic banding patterns showed less degree of polymorphism among five different varieties of sugarcane.

The morphological variation, a product of genotype and the environment, is an important parameter, but much diversity, which remains unexpressed morphologically, can be revealed by biochemical methods. Study of isozymic variation is one such important and powerful procedure that has often been employed to unravel the hidden variation. The isozyme forms of this enzyme, being the primary gene products, can be used to deduce gene homology with precision by comparing variation in their expression patterns in different varieties of sugarcane in relation to disease resistance and stress tolerance as evident in the present study. Manjunatha *et al.,* 2003 used isoperoxidase profiles to study the polymorphisms between the different somaclones of sugarcane cultivars developed against biotic and abiotic stress tolerance. SOD isozymes have also been used as biochemical markers for genotype discrimination and for genetic diversity determination in five varieties of sugarcane cultivated in the northern Paranб, Southern Brazil (Orasmo and Machado, 2003). To distinguish the resistant and susceptible clones of *Saccharum officinarum* L. to fungal diseases isoperoxidase analysis has been carried out by Anbazhagan *et al.,* 2009.

In the present study, peroxidase, estearase, acid phosphatase, alkaline phosphatase and protein separation, by electrophoresis, has been carried out to reveal the variation among five different varieties of sugarcane in relation to disease resistance and stress tolerance. Among the four enzyme systems, peroxidase profile reveals the difference between the disease resistant / susceptible and abiotic stress tolerant / non tolerant varieties. The two isoperoxidase bands with Rf values 0.62 and 0.66 showed their presence in disease resistant and abiotic tolerant varieties. For the commonly cultivated variety Co 6304, there is no information on disease resistance and stress tolerance. The presence of two marker bands (0.62, 0.66) of resistant and stress tolerant varieties suggest that the variety Co 6304 may also be resistant to smut, wilt and moderately resistant to red rot and tolerant to drought. Similar to the present study, Anbazhagan *et al.* (2009) distinguished the disease resistant clones using isoperoxidase profile by the presence of two bands with the Rf values 0.537 and 0.694 as marker. From the present study it is concluded that the difference in the profile of isozymes can be used not only to understand the phylogenetic relationships among different varieties of sugarcane but also to identify the disease resistant and stress tolerant varieties of sugarcane.

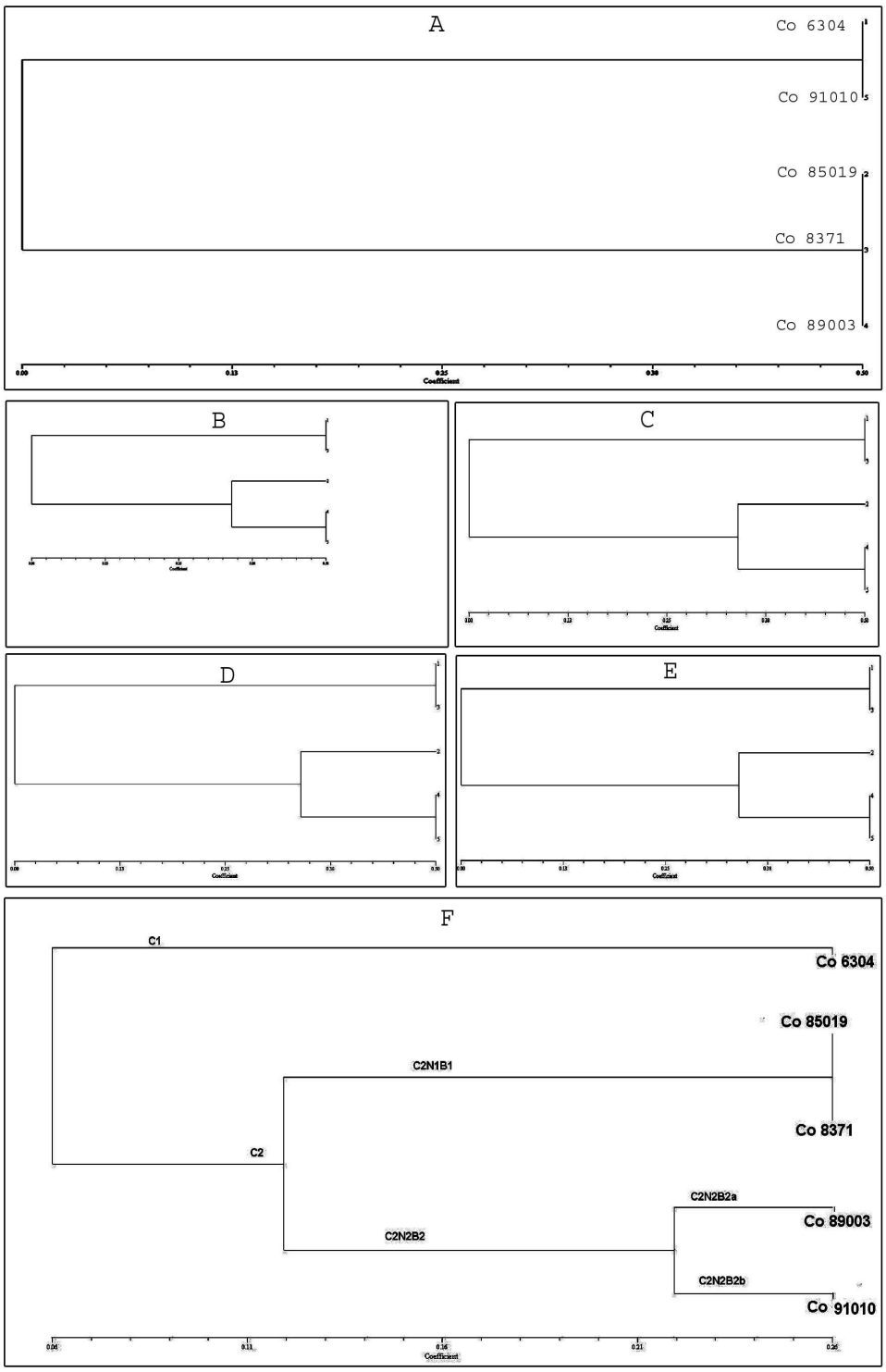

Fig. 2. Cladograms of five sugarcane varieties based on isozymes and prote

A - Isoperoxidase B - Protein C - Esterase
D - Acid Phosphatase E - Alkaline Phosphatase
F - Cladogram based on isozymes and protein

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