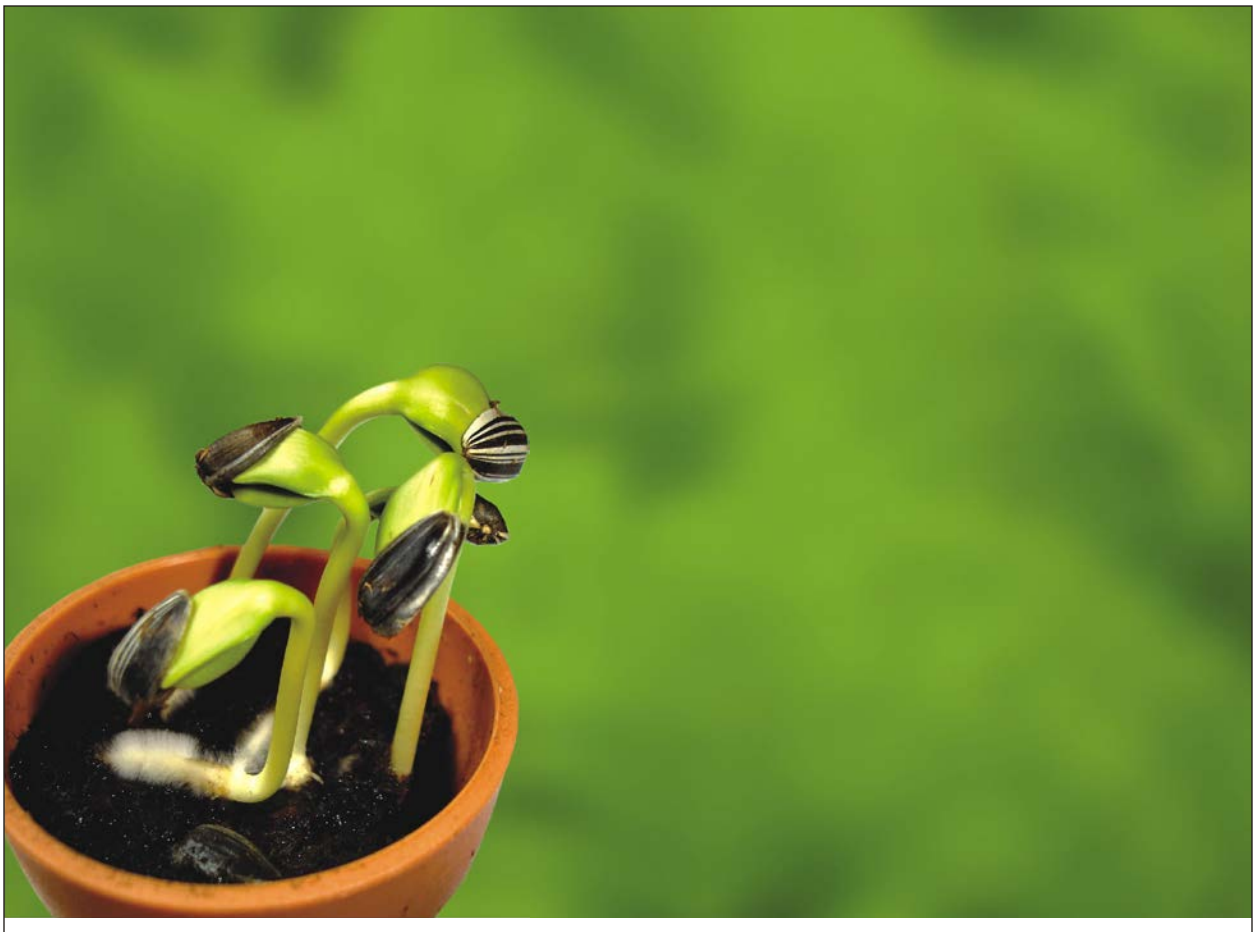

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Isozyme Analysis of Njavara, A Traditional Medicinal Rice Cultivar of Kerala, India Based on Electrophoretic Patterns of Alcohol Dehydrogenase

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Abstract: A set of seven Njavara accessions collected from different parts of Kerala State, India along with two local check varieties was studied to demonstrate the utility of alcohol dehydrogenase as a marker for grouping Njavara ecotypes and to study the variation in electrophoretic patterns of alcohol dehydrogenase extracted from samples of 30 days old leaf and five days old germinated seeds. Only five bands were resolved for leaf samples. Two bands ADH-3 and ADH-4 were common for all the genotypes analyzed. Germinated seed sample expressed more alcohol dehydrogenase banding pattern. Nine bands were resolved for germinated seed samples. The Njavara ecotypes showed two unique bands viz., ADH-4 and ADH-8. The pooled data for Jaccard's similarity coefficient values revealed that the similarity coefficient values of Njavara ecotypes with check varieties were low. At similarity coefficient 0.33 the dendrogram was divided into two clusters, one large cluster with Njavara ecotypes and one small cluster with two check varieties. Based on alcohol dehydrogenase data, three major groups were identified amongst Njavara ecotypes. The above study inferred that the isozyme analysis has offered a rapid and reliable method for the estimation of variability/similarity between different accessions that could be utilized for the protection of our biodiversity and also by the breeders for further improvement of this unique medicinal rice cultivar.

Key words: Isozyme, alcohol dehydrogenase, medicinal rice, genetic variability

INTRODUCTION

Njavara is an extra short duration rice cultivar in the genus *Oryza* indigenous to Kerala (Elsy *et al*., 1992). The preliminary evaluation of morphological characters revealed existence of genetic variations in different ecotypes of this unique medicinal cultivar. Detailed characterization of the cultivar revealed the level of diversity existing within the cultivar and established an index of genetic similarities among different ecotypes (Sreejayan and Thomas, 2003). Isozymes and proteins are the commonly studied biochemical markers. Isozymes are multiple forms of an enzyme that catalyze the same biochemical reaction but differ in their kinetic properties. They have distinctly different amino acid composition or sequence and may occur in the same species, in the same tissue, or even in the same cell (Nelson and Cox,

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2008). Rice geneticists had used electrophoretic techniques to add isozyme loci to the linkage groups as additional gene markers (Reyes *et al*., 1998) and to determine the genetic divergence among cultivars and their wild relatives (Thanh *et al*., 2006). Many useful applications in the study of isozymes included study of genetically defined variation, general variation, multilocus analysis etc. (Mukherjee and Dutta, 2008). The isozyme analysis has been used widely in the evolutionary and ecological studies and has also been used for identification of cultivars (Gupta *et al*., 2008; Kumar *et al*., 2008). The use of isoenzymes as markers for identifying cultivars or genotypes is recommended as a supplement for molecular techniques due to their low input cost (Sharma and Maloo, 2006). Here, we demonstrate the utility of alcohol dehydrogenase as a marker for grouping Njavara ecotypes.

In the present study, isozyme analysis with alcohol dehydrogenase (ADH), an enzyme of glycolytic pathway, was conducted with seven Njavara ecotypes and two check varieties (Ptb 10 and Karavella) using the protein isolated from samples of 30 DAS old leaf and five days old germinated seeds.

MATERIALS AND METHODS

Seed Material and Germination

The study was carried out during 2001-2004 using seven Njavara ecotypes viz., four from the National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Vellanikkara and three collected from different locations of Kerala along with Ptb-10 and Karavella as check variety formed the material for this study. The details of the genotypes under study are shown in Table 1.

Rice seeds were placed in Perti plates lined with two layers of Whatman filter paper for 24 h at 30±1°C in an incubator for germination. Germinated seeds were pressed with blotting paper to remove water and used for extraction of enzyme.

Protein Extraction and Electrophoresis

For extraction of alcohol dehydrogenase, 500 mg of the sample was taken and homogenized in a pre-cooled mortar, along with 0.5 M Tris-HCl buffer (pH 7.4) containing 5 mM 2-mercaptoethanol. In general, 0.1 mL of buffer was used per seed. The samples were ground at 4°C by keeping and pestle in an ice tray. The homogenized samples were centrifuged at 10,000 g for 30 min in a Kubota high speed centrifuge at 5°C. After centrifugation, the supernatant was collected in eppendorf tubes, labeled and used for running the gel. Fresh samples were used for the assay though enzyme extracts can be stored at sub-zero temperature for one day. The extract (100 μg protein) along with tracer dye

Table 1: Details of the genotypes utilized in the study

		Source	
Genotypes	Accession No.	Village	District
Njavara types			
N1*	NIC 18383-A	Chittoor	Palakkad
N2*	NIC 18430-B	Chittoor	Palakkad
N3*	IC 203771	Tellicherry	Kannur
N4*	IC 203767	Tellicherry	Kannur
N5 (local collection)	-	Kottakkal	Malappuram
N6 (local collection)	-	Alwaye	Alwaye
N7 (local collection)	-	Thrissur	Thrissur

Local check: Ptb-10, Karvella. A short duration traditional variety released from Regional Agricultural Research Station, Pattambi, Kerala, India. A medium duration traditional variety obtained from Regional Agricultural Research Station, Pattambi, Kerala, India. *Njavara ecotypes obtained from NBPGR Regional Station, Vellanikkara, Thrissur, Kerala, India

[Bromophenol blue (1% solution) in 0.125 M Tris HCI buffer (pH 6.8) and 20% glycerol] was loaded onto graded polyacrylamide gel (7.5-10%) and electrophoresis carried out at 4°C. After electrophoresis, the gels were taken out washed, incubated 0.5 M Tris-HCl buffer (pH 7.1) for 5 min at 37°C and then stained in the reaction mixture containing NAD^+-50 mg, NBT-30 mg, PMS-2 mg, Ethanol-3 mL, Tris-HCl buffer (0.5 M, pH 7.1)-15 mL and water-15 mL (Shaw and Koen, 1965).

Nomenclature of the Isozymes

The norms described by Berg and Wijsman (1982) for peroxidase was followed for the nomenclature of the isozymes. The enzyme alcohol dehydrogenase was referred by the abbreviations Adh.

The relative mobility (Rm) of each band was calculated as:

$$Rm = \frac{\text{Distance of band from origin}}{\text{Total distance run}}$$

Based on relative mobility of each band, the isozyme pattern was schematically drawn.

Numbering of Isozymes and Measurement of Similarity

For numbering, all the isozymes of an enzyme in the species studied were pooled. The slowest moving anodal band was numbered 1 (e.g., Adh-l) faster ones were given the subsequent numbers. The gels were scored for computer analysis on the basis of the presence or absence of the amplified products. If a product was present in a genotype, it was designated as 1 and if absent; it was designated as 0. The alcohol dehydrogenase data were analyzed using NTSYS-PC 2.0 (Numerical Taxonomy and Multivariate Analysis System) computer package. The data were used to generate JACCARDS's similarity coefficient for RAPD bands. The JACCARDS's coefficients between each pair of accessions were used to construct a dendrogram using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

RESULTS

ADH Pattern in Germinated Seed and Leaf Samples

Germinated seed sample expressed more alcohol dehydrogenase banding pattern of the nine bands resolved (Fig. 1a, b), the isozyme band ADH-3 (Rm = 0.205) was present only in N1. N6 and N7 shared a high molecular weight band ADH-2 (Rm = 0.19). The Njavara ecotypes showed two unique bands viz., ADH-4 (Rm = 0.282) and ADH-8 (Rm = 0.590). The band ADH-1 (Rm = 0.128) was observed in all genotypes except for N6 and N7. The bands ADH-5 (Rm = 0.359) and ADH-9 (Rm = 0.667) were common for both the check varieties and not present in the Njavara ecotypes. The isozyme band ADH-6 (Rm = 0.462) was shared by N1 and N3. The number of alcohol dehydrogenase bands in leaf samples observed was less compared to the germinated seed samples (Fig. 2). Only five bands were resolved for leaf samples. Two bands ADH-3 and ADH-4 with Rm values 0.310 and 0.429, respectively were common for all the genotypes analysed. ADH-1 (Rm=0.095) was shared by N6 and N7. The isozyme band ADH-2 with Rm value 0.167 was common for N1, Ptb-10 and Karavella. ADH-5 (Rm=0.595) was resolved by N1 alone.

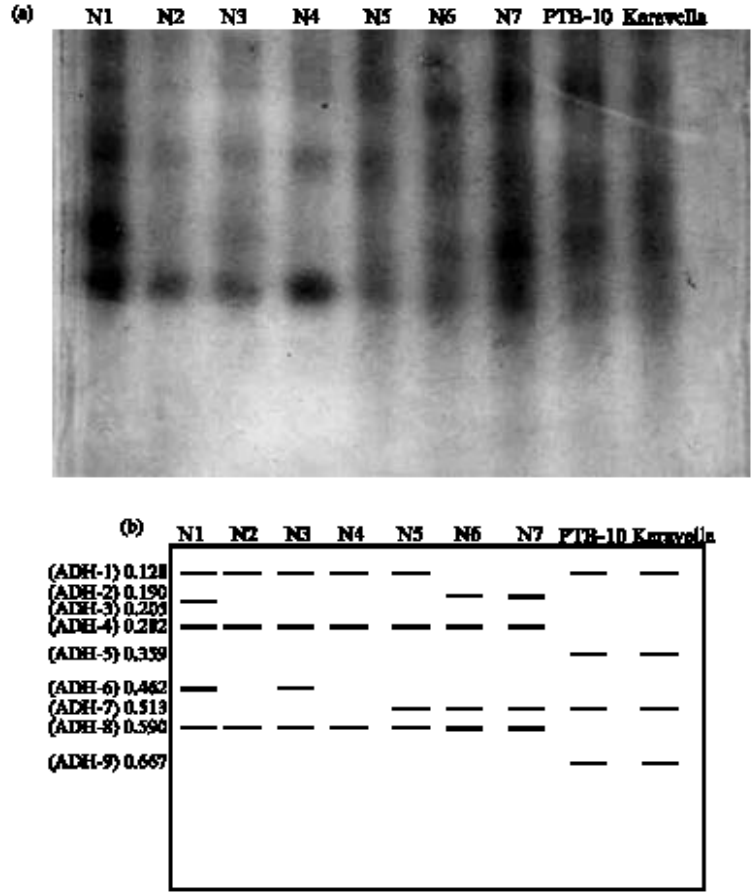

Fig. 1: (a) Alcohol dehydrogenase banding pattern in germinated seeds (5 days after soaking) of Njavara ecotypes and (b) Zymogram of alcohol dehydrogenase in germinated seeds (5 days after soaking) of Njavara ecotypes

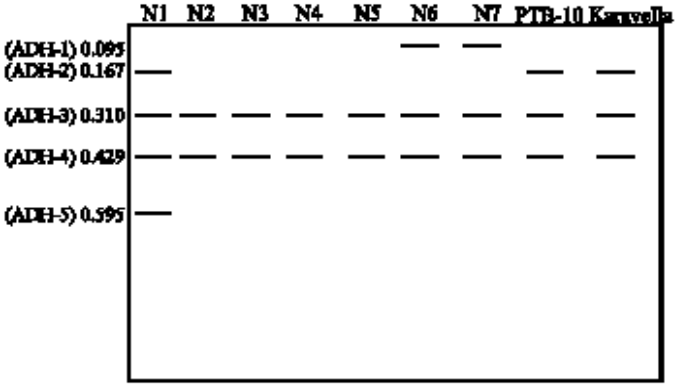

Fig. 2: Zymogram of alcohol dehydrogenase in leaves (30 DAS) of Njavara ecotypes

Table 2: Similarity index among Njavara ecotypes based on alcohol dehydrogenase isozyme pattern in germinated seed

Genotypes	N1	N2	N3	N4	N5	N6	N7	PTB 10	Karavella
N1	1.000								
N2	0.600	1.000							
N3	0.800	0.750	1.000						
N4	0.600	1.000	0.750	1.000					
N5	0.500	0.750	0.600	0.750	1.000				
N6	0.286	0.400	0.333	0.400	0.400	1.000			
N7	0.286	0.400	0.333	0.400	0.400	1.000	1.000		
PTB 10	0.125	0.167	0.143	0.333	0.167	0.143	0.143	1.000	
Karavella	0.125	0.167	0.143	0.333	0.167	0.143	0.143	1.000	1.000

Table 3: Similarity index among Njavara ecotypes based on alcohol dehydrogenase isozyme pattern in leaf

Genotypes	N1	N2	N3	N4	N5	N6	N7	PTB 10	Karavella
N1	1.000								
N2	0.500	1.000							
N3	0.500	1.000	1.000						
N4	0.500	1.000	1.000	1.000					
N5	0.500	1.000	1.000	1.000	1.000				
N6	0.400	0.667	0.667	0.667	0.667	1.000			
N7	0.400	0.667	0.667	0.667	0.667	1.000	1.000		
PTB 10	0.750	0.667	0.667	0.667	0.667	0.500	0.500	1.000	
Karavella	0.750	0.667	0.667	0.667	0.667	0.500	0.500	1.000	1.000

Table 4: Similarity index among Njavara ecotypes based on pooled data of alcohol dehydrogenase isozyme pattern

Genotypes	N1	N2	N3	N4	N5	N6	N7	PTB 10	Karavella
N1	1.000								
N2	0.556	1.000							
N3	0.667	0.833	1.000						
N4	0.556	1.000	0.833	1.000					
N5	0.500	0.833	0.714	0.833	1.000				
N6	0.333	0.500	0.444	0.500	0.625	1.000			
N7	0.333	0.500	0.444	0.500	0.625	1.000	1.000		
PTB 10	0.333	0.333	0.300	0.333	0.444	0.273	0.273	1.000	
Karavella	0.333	0.333	0.300	0.333	0.444	0.273	0.273	1.000	1.000

JACCARDS's Similarity Coefficient

Based on the Similarity Index (SI) values based on ADH banding pattern in germinated seeds, the Njavara ecotypes N2 with N4 showed highest SI of 100% (Table 2) and the same pattern was shown between check varieties Ptb-10 and Karavella. The SI value of 80% was showed by N1 with N3. Among the Njavara ecotypes, N4 showed the highest SI value of 33.3% with the check varieties. The Similarity Index (SI) of the Njavara ecotypes (leaf samples) with check varieties, showed a highest value of 75% for N1 followed by N2, N3, N4 and N5 with 66.7%. The Njavara genotype N6 showed SI value of 100% with N7 (Table 3). The pooled data for Jaccard's similarity coefficient values revealed that genotypes N2 with N4, N6 with N7 and Ptb-10 with Karavella had highest similarity coefficient of 100%. The similarity coefficient values of Njavara ecotypes with check varieties were low (Table 4).

Dendrogram Based on Pooled Data

At 33% similarity the dendrogram was divided into two clusters, one large cluster with Njavara ecotypes and one small cluster with two check varieties (Fig. 3). The cluster with Njavara ecotypes was divided into two at 48% similarity with N1, N2, N3, N4 and N5 in one cluster and N6 and N7 in another cluster. At 57% similarity N1 came in an individual cluster. N2 and N4, N6 and N7 and Ptb-10 and Karavella were clustered separately at similarity coefficient 1.00.

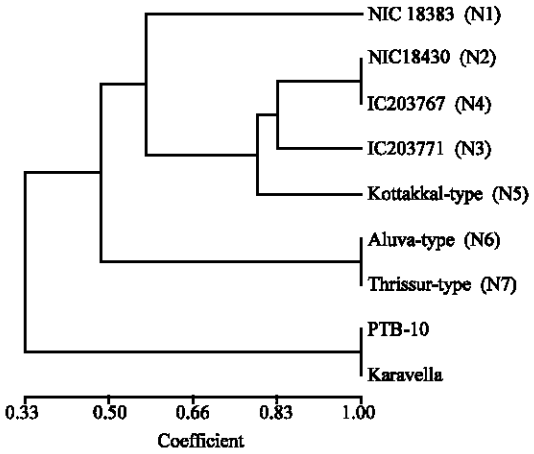

Fig. 3: Dendrogram of Njavara ecotypes from pooled isozyme data using UPGMA clustering

DISCUSSION

The ADH banding pattern and the corresponding similarity indices in germinated seed divided Njavara ecotypes into distinct groups. The isozyme bands, ADH-4 and ADH-8, were observed with Njavara ecotypes alone and hence could be used as a marker to distinguish Njavara from other traditional rice cultivars. A unique band, ADH-3 was present with genotype N1 alone and hence could be distinguished from other Njavara ecotypes. With the identification of these unique bands, it is possible to mark the specific nutritional characters of this medicinal cultivar. It also could be an ideal source of desirable genetic traits to play a role in improving the nutritional qualities of rice. Based on the ADH isozyme pattern in leaf, the isozyme bands ADH-3 and ADH-4 was observed for all the rice genotypes under study. The band ADH-1 was specific for N6 and N7 suggesting their similarity. The isozyme band ADH-5 was specific for the genotype N1 and it could be easily identified. The banding pattern of the check varieties was similar exhibiting three bands ADH-3, 4 and 5. Similarly unique bands for Njavara ecotypes were reported by Reddy (2000) for isozyme analysis of Njavara ecotypes with peroxidase and esterase enzymes and suggesting the use of those isozymic bands as markers for identifying Njavara ecotypes. Bimb *et al.* (2004), also reported unique Adh bands while studying isozyme variation of 24 aromatic and fine rice varieties collected from Nepal. The native-PAGE by Grover and Pental (1992) and Dattarwal *et al.* (1999) of protein extract made from germinating seeds of rice cultivars also revealed five bands of alcohol dehydrogenase.

The dendrogram drawn from the pooled data of alcohol dehydrogenase revealed the similarity index ranging from 0.33 to 1.00. At a similarity coefficient of 0.33 the dendrogram was divided into two clusters, one large cluster with and one small cluster with two check varieties. A dendrogram drawn by Arencibia *et al.* (2001) reported similar results for biochemical genetic characterization of nineteen rice accessions using leaf extracts and PAGE with peroxidase, esterase and polyphenol oxidase isoenzymes that showed the formation of ten groups, with the main similarities in groups IV and V. Out of the biochemical markers tested, alcohol dehydrogenase banding pattern in germinated seed showed maximum polymorphism and was able to classify Njavara ecotypes into distinct groups that is in line with the results reported by Reddy (2000).

Genetic Similarity/diversity within Njavara Population and with other Traditional Cultivars

Based on the ADH isozyme pattern and its corresponding similarity indices with check varieties, Njavara ecotypes exhibited clear difference with the local cultivars under study by forming two separate clusters, one small cluster with two check cultivars and a large cluster with all seven Njavara ecotypes. Similarly the morpho-physiological studies conducted by Elsy *et al.* (1992) exhibited the extra short growth duration of 69 days nature of Njavara types and the nutritional quality analysis of Njavara grains by Menon and Potty (1999) revealed that the content of free amino acids appeared to be the unique characteristic of the cultivar.

The Njavara ecotypes were further classified into four groups viz., N6 and N7 shared a separate cluster that represent the central region of the state, while the large cluster comprising of five represents the North and North-eastern region. This indicates that the five Njavara ecotypes that shared the large cluster may have common parentage. The morphological characterization of the cultivar revealed black and gold and/or gold furrows on straw background color for lemma and palea (glume) color (Sreejayan and Thomas, 2003). The glume color of the ecotypes sharing the small cluster is black whereas, that of the ecotypes sharing the larger cluster is straw/gold color and black furrows/patches on straw background. The three ecotypes sharing single cluster viz., N2, N3 and N4, exhibited glume color of straw while ecotype N1 exhibited black patches on straw background and N5 showed gold colored glume that fell in two separate clusters. The results revealed that the Njavara ecotypes are a composite of distinct morphotypes. The distinction between Njavara types, we observed, did exist but they certainly did not represent all the elements of the actual genetic structure of the Njavara ecotypes.

CONCLUSION

With the detailed characterization of the cultivar, it becomes possible to study the level of diversity existing within the cultivar and to establish an index of genetic similarities among different ecotypes. Along with morphological characterization, molecular characterization and documentation is essential for the protection of this unique cultivar. The importance of such characterization and documentation has to be perceived in the context of extension of Intellectual Property Rights (IPRs) to agricultural sector and the controversies related to our genetic resources. Since, the area under traditional cultivars is reducing at fast pace due to the spread of high yielding and improved varieties, there is every chance that this unique rice cultivar may become extinct in the near future.

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