P. ISSN: 0971-8184 II E. ISSN: 0976-1926
DOI: 10.61949/0976-1926.2025.v38i02.05

Indian Journal of Plant Genetic Resources

RESEARCH ARTCILE

Utility of Isozyme Markers for Understanding Genetic Relatedness in Karanja (*Derris indica* L.,)

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Abstract

In present study, we analyzed genetic diversity in Karanja (*Derris indica*) using isozyme markers of four enzymes namely, peroxidase, polyphenol oxidase, esterase, and superoxide dismutase, which collectively revealed 19 isoforms. Polyphenol oxidase exhibited highest number of isozymes (6), followed by peroxidase (5), superoxide dismutase (4), and esterase (4). Polyphenol oxidase exhibited the highest polymorphism (83.33%), while superoxide dismutase showed the lowest (50%). The isozyme polymorphic index, polymorphism information content (PIC) and marker index for isozyme markers was 2.0, 0.42 and 28.81 respectively. The highest polymorphic index and marker index was governed by polyphenol oxidase isozyme, while the esterase isozyme had the highest PIC value. Structure analysis and principal coordinate analysis revealed two main clusters in the karanja germplasm. Additionally, we identified unique as well as presence of specific isozyme marker loci of polyphenol oxidase, peroxidase and esterase isozymes that differentiate the genotypes NAUK-2, NAUK-5, NAUK-6, NAUK-14, NAUK-15, NAUK-16, NAUK-17, NAUK-21, NAUK-26, NAUK-28, and NAUK-30 from other karanja genotypes.

Keywords: Diversity, enzymes, fingerprinting, isozymes, Karanja.

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Received: 09/10/2024 **Revised:** 31/03/2025

Accepted: 30/04/2025

How to cite this article: Hake, A.A. Jha, S.M., Jha, S.K., Mahatma, M.K. (2025). Utility of Isozyme Markers for Understanding Genetic Relatedness in Karanja (*Derris indica* L.,). *Indian J. Plant Genet. Resour*. 38(2), 168-174.
DOI: 10.61949/0976-1926.2025.v38i02.05

Introduction

Karanja (*Derris indica* L., 2n = 22) is a drought-resistant, semi-deciduous leguminous tree in the Fabaceae family and Papilionaceae subfamily (Meera *et al.,* 2003). It is native to humid, subtropical areas of southeast Asia, Australia, and East Fiji (Satyavati *et al.,* 1987). It has gained commercial significance due to its high oil content (30–40%), which presents potential as an alternative fuel source (Nagaraj and Mukta, 2004) as well as it is used for lamp oil, soap making, and as a lubricant (Kesari *et al.,* 2010).

Assessing genetic diversity is a fundamental step in any crop improvement program. Diversity studies help breeders to assess trait variability and select parents for hybridization programs. Traditionally, identifying elite individuals based on agronomic traits has been challenging due to limited morphological markers. Biochemical markers have emerged as effective tools for understanding biosystematics, biogeography, and phylogenetic relationships. Earlier Hake *et al*. (2017; 2018) explored enzyme assays and metabolite profiling in karanja accessions, but these assays alone are inadequate for assessing genetic diversity. Also, karanja specific DNA markers are not available in the public markers database. Owing to this, in the current study, we employed isozymes, a peptide-based marker, which are multiple molecular forms of enzymes that share a common catalytic activity that aid in germplasm classification, genetic segregation monitoring, and phylogenetic relationship determination in plants. Polymorphic

differences at the amino acid level enable the detection of singular peptide polymorphisms as biochemical markers. Variations in isozymes provide insights into population structures, as zymographic patterns reflect specific gene systems (Shahi *et al.,* 1969). Earlier, genetic diversity using isozymes was studied in various crops such as kiwifruit (Shirkot *et al*., 2001), Shirkot *Pinus gerardiana* (Sharma *et al*., 2004), etc. Without sufficient genetic variability in economically important traits, efforts to improve forest trees will likely fail. Selecting superior genotypes from available germplasm is crucial for future breeding efforts. Therefore, the first step in any tree improvement program is to evaluate the amount, causes, and nature of variation present in the target species. This study aims to analyze genetic diversity and fingerprinting in karanja genotypes using isozyme markers.

Materials and Methods

Planting Material

Karanja germplasms were collected throughout Gujarat and established at the Navsari Agricultural University germplasm bank (Supplementary Table 1). Leaf samples from the progeny of candidate plus trees (CPTs) were selected for experimentation.

Enzyme extraction and Isozyme assays

For isozyme assays, crude extracts of antioxidant (peroxidase, esterase, and superoxide dismutase) and phenolic enzymes (polyphenol oxidase) were prepared according to Hake *et al*. (2017) with protein concentration assessed via the Lowry method (Lowry *et al.,* 1951). Native polyacrylamide gel electrophoresis (native PAGE) was performed with 100 µg protein of each genotype, using a 10% resolving gel and 4% stacking gel at 140 V and 4°C. Once the tracking dye reached the gel's bottom, the gels were stained to analyze enzyme isoforms.

Superoxide dismutase (SOD) isoforms were detected by staining after electrophoresis, following Beauchamp and Fridovich (1971). Gels were incubated in 0.24 mM NBT for 20 minutes, followed by a 20-minute incubation in a sodium phosphate buffer (pH 7.8) with 28 mM riboflavin and 28 mM N, N, N, N tetramethylethylenediamine (TEMED) in darkness, then exposed to light until SOD activity bands appeared as colorless bands on a purple background. Peroxidase (POX) isozymes were visualized by incubating the gel in 100 ml acetate buffer (0.020 M, pH 4.2) containing 100 µL of 30% H_2O_2 and 0.05 % benzidine for 5 minutes till bands appeared (Sadasivam and Manickam, 1996). Esterase isoforms were made visible by incubating gel in a solution of sodium 1.4 g dihydrogen phosphate, 0.55 g disodium hydrogen phosphate, 0.1 g fast blue RR salt, and 15 mg α-naphthyl acetate (dissolved in chilled acetone) in 100 mL millipore water (Sadasivam and Manickam, 1996). Gels were shaken until bands appeared, then immersed in a stop solution of Methanol:Millipore water:Acetic acid:Ethanol (10:10:2:1) before scanning. Polyphenol oxidase isoforms were made visible by incubating in 0.1% p-phenylenediamine in 0.1 M potassium phosphate buffer (pH 7.0) and for 30 minutes, followed by the addition of catechol (10 mM) in the same buffer led to discrete dark brown protein bands (Jayaraman *et al.,*1987). After washing with distilled water, the gels were photographed.

Data analysis and population structure analysis

Isozymes of different enzymes were scored as 1 (present) and 0 (absent), and these scores were used for diversity analysis, including polymorphic information content (PIC), marker polymorphic index, and marker index (MI). For comparing the efficiency of primers, PIC was calculated using the formula $PIC = 1- \sum Pi^2$, where Pi is the frequency of the i^{th} isozyme at a given locus. The sum total of all PIC values for a given isozyme determines the marker polymorphic index. The marker index (MI) was used to estimate the overall utility of each marker. It is the product of the polymorphism percentage and PIC (Kesari *et al*., 2010). Genetic structure was assessed using model-based and distance-based approaches. The model-based approach, implemented in Structure v2.3.4 (Pritchard et al. 2000), estimated the number of subpopulations (K). We ran the structure with admixture and correlated allele frequency parameters, employing a burn-in of 100000 iterations followed by 100000 Markov Chain Monte Carlo MCMC replicates. Ten independent runs were performed for each K value, ranging from 1 to 10. The optimal K was determined by plotting the mean log posterior probability of the data (L(K)) against K and a true number of subpopulations was identified using the maximal value of L(K). Additionally, the ΔK statistic (Evanno et al. 2005), calculated using Structure Harvester (Earl 2012), indicates the optimal K value. Principal coordinate analysis (PCoA) followed by cluster analysis was carried out using DARwin 6.0.14 (Perrier and Jacquemoud-Collet, 2006). Jaccard's coefficient of similarity generated pairwise similarity matrices by using the SIMQUAL format of NTSYSpc 2.0 (Rohlf, 1998).

Results and Discussion

In the current study, four isozyme systems (polyphenol oxidase, peroxidase, superoxide dismutase, and esterase) expressions were analyzed using native PAGE to assess diversity among thirty genotypes. The number of isozymes expressed by PPO, POX, SOD, and EST varied from 4 to 6 (Supplementary Figures 1, 2, 3 and 4). A total of 6 monomorphic isoforms (PPO-6, POX-4, POX-5, SOD-1, SOD-2, and EST-2) were detected among 30 genotypes. The polymorphic index for PPO, POX, SOD, and EST was 2.79, 1.97, 1.36, and 1.91, respectively, and the mean percentage of polymorphic isozymes was 67.08%. Phylogenetic analysis

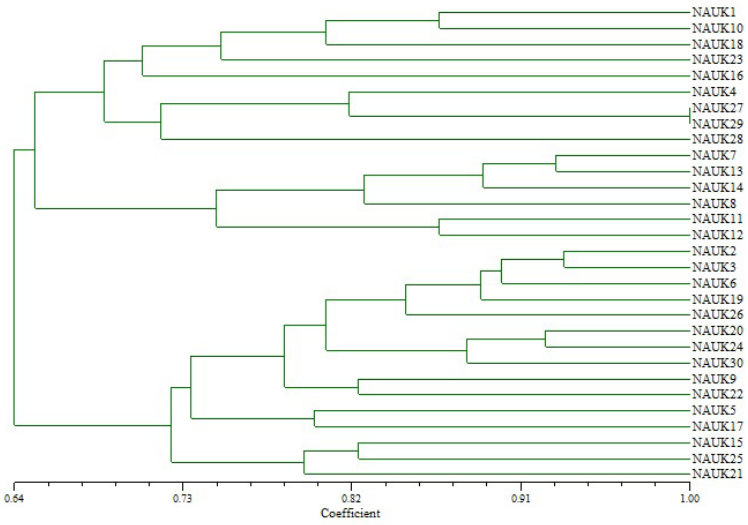

Fig. 1: Phenogram based on Jaccard's similarity index of isozyme data depicting genetic variability in karanja accessions

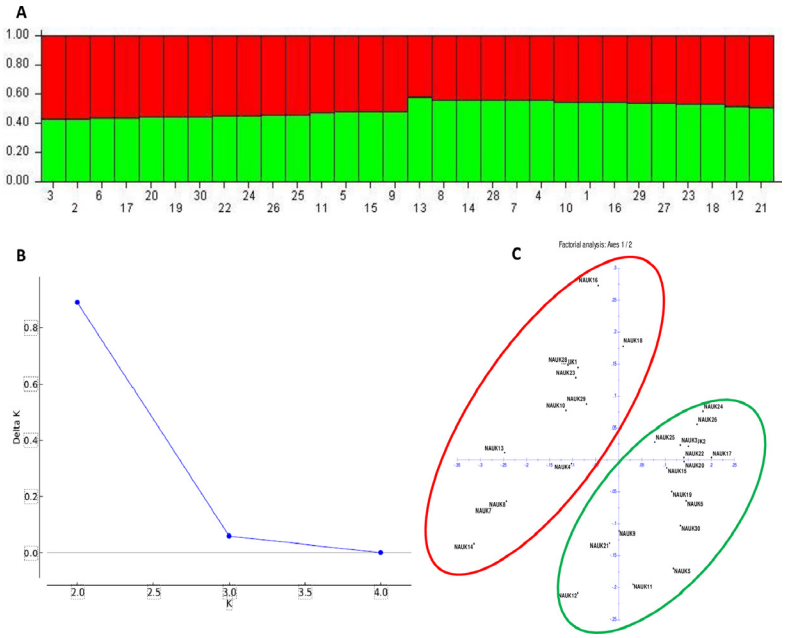

Fig. 2: Population structure of karanja germplasm. A Structure plot of karanja population at K = 2. B Estimation of delta K value (delta K =2) using Evanno's method. c Principal coordinate an

Table 1: Genetic diversity in karanja based on expression data of different isozymes

Sr. No.	*Name of isozyme*	*Total No. of isozymes expressed*	*Number of Polymorphic isozymes*	*Number of Monomorphic isozymes*	*Polymorphism (%)*	*Polymorphism information content*	*Isozyme Polymorphic Index*	*Marker Index*
1	Polyphenol oxidase	6	5	1	83.33	0.46	2.79	38.81
2	Peroxidase	5	3	2	60.00	0.39	1.97	23.59
3	Superoxide dismutase	4	2	2	50.00	0.34	1.36	16.97
4	Esterase	4	3	1	75.00	0.48	1.91	35.90
Total		19	13	6				
Average		4.75	3.25	1.50	67.08	0.42	2.00	28.82

based on NTSYS pc 2.0 revealed a high level of genetic separation within the population (Figure 1 & Table 1). The coefficient values ranged from 0.44 (NAUK-14 & NAUK-17) to 1.00 (NAUK-27 & NAUK-29). The resulting dendrogram showed differentiation into two main clusters, each with fifteen genotypes and nine sub-clusters. Structure analysis revealed two main clusters followed by three sub-clusters, while principal coordinate analysis also revealed two main clusters (Figure 2).

The study on the expression pattern of polyphenol oxidase revealed the expression of six isoforms (PPO-1, PPO-2, PPO-3, PPO-4, PPO-5, and PPO-6) in Karanja leaves (Supplementary Figure 1). The number of PPO isoforms varied from 3 to 6 among 30 genotypes. PPO-6 was found in all genotypes, indicating a common function. In the NAUK-30 genotype, only two PPO isozymes loci, PPO-2 and PPO-6 found, while in NAUK-2, all six PPO isozymes loci were identified. Also, PPO-3, PPO-4, and PPO-6 isozymes were identified in NAUK-16, while PPO-3, PPO-4, PPO-5, and PPO-6 loci were identified in NAUK-26. Likewise, the presence of isozyme loci, PPO-1, PPO-2, PPO-4, PPO-5, and PPO-6 determines NAUK-6. Similarly, the expression of isozyme loci, PPO-1, PPO-2, PPO-4, and PPO-6 determines NAUK-17. The identification of a specific combination of these isoform loci of PPO differentiated NAUK-2, NAUK-6, NAUK-16, NAUK-17, NAUK-26, and NAU-30 from other genotypes. The polymorphic index calculated for PPO was 2.79, which was much more compared to the analysis by Parthasarathy *et al.,* (2011) for differentiating the genotypes. Multiple forms of polyphenol oxidase have been isolated from a wide variety of sources, including mushrooms, broad beans, potato, melanoma, etc. (Wong *et al.,* 1971). The number of peroxidase (POX) isoforms varied from three to five among 30 genotypes (Supplementary Figure 2). Out of the five POX isozymes, POX-4 and POX-5 were identified in all thirty genotypes. All five POX isozymes were detected in NAUK-8 and NAUK-16. The peroxidase isoforms, POX-3, POX-4, and POX-5 were identified in NAUK-5, while POX-1, POX-4, and POX-5 were identified in NAUK-14, and POX-1, POX-3, POX-4, and POX-5 were identified in NAUK-21. Previous studies observed five POX isoforms in oilseed rape under drought-stress conditions (Abedi and Pakniyat 2010). The number of SOD isoforms varied from two to four, with SOD-3 and SOD-4 being prominent in all genotypes (Supplementary Figure 3). Similarly, varied isoforms of SOD were detected in 36 accessions of eight species of Trifolium (Lange and Maria, 2000). Two to four isozymes of esterase (EST) with different densities were detected in all genotypes (Supplementary Figure 4). Isozyme EST-2 was common among thirty genotypes. The expression of EST-1, EST-2, and EST-4 collectively determined the marker for NAUK-28 for differentiation, while the expression of EST-2 and EST-3 collectively identified NAUK-15. Similar findings were observed in different tropical and subtropical herbaceous legume species, indicating that different selections had different esterase isozyme patterns. For instance, Lange and Maria (2000) identified twelve esterase isoforms in four cultivars of *Trifolium subterraneum*.

Isozyme markers SOD-3, SOD-4, EST-2, PPO-6, POX-4, and POX-5 were predominantly identified in all thirty genotypes, suggesting a common cellular function in Karanja. In higher plants, the number of isozymes may be extremely high. For instance, up to 40 genes corresponding to iso-peroxidases for each plant and several other isoforms can be generated by post-transcriptional and post-translational modifications (de *et al.,* , 1999). The highest polymorphic percentage, polymorphic index, and marker index value were exhibited by the PPO isozyme, suggesting it is a highly informative marker among thirty genotypes of Karanja, while the highest PIC value was revealed by esterase isozyme, indicating its high ability in the differentiation of Karanja genotypes. In this study, we have identified polyphenol oxidase, peroxidase and esterase isozymes that could be used for diversity studies and fingerprinting of genotypes. In the current study, unique as well as presence of specific isozyme marker loci has been identified specific Karanja genotypes namely, NAUK-2, NAUK-5, NAUK-6, NAUK-14, NAUK-15, NAUK-16, NAUK-17, NAUK-21, NAUK-26, NAUK-28, and NAUK-30, allowing for the differentiation of these genotypes from others. Furthermore, molecular markers could be integrated with other isozyme markers for the fingerprinting of other genotypes.

Acknowledgment

Authors are gratefully acknowledged to NOVORD and Navsari Agricultural University, Navsari, Gujarat (India) for providing financial and technical support during the research study.

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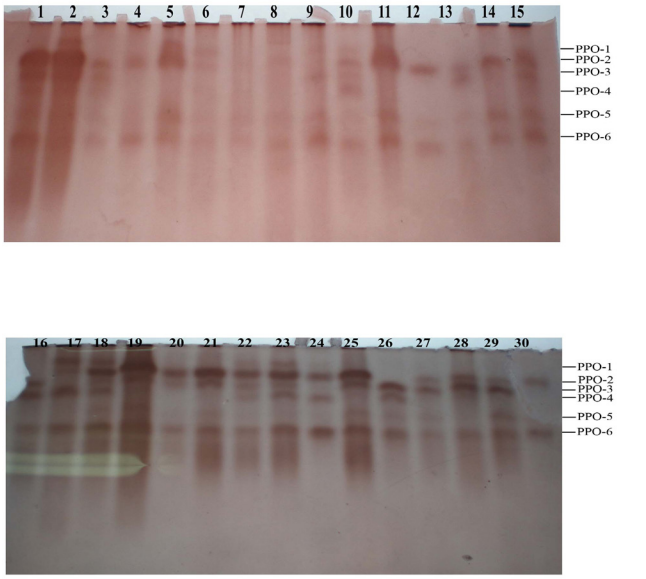
Supplementary Data

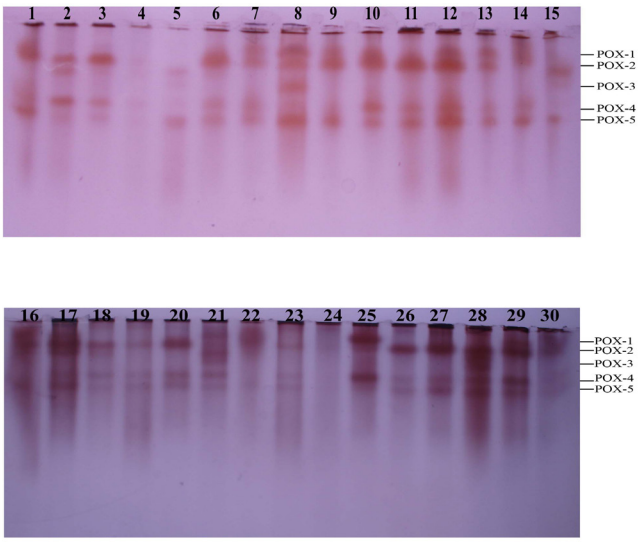
Supplementary Table 1: Karanja accessions collected from different places from Gujarat

Accessions	*Site of collection*	*Longitude n latitude*
NAUK1	Vansda	N- 20.11666', EO-73.15000 n at 794 ft.
NAUK2	Vansda	N- 20.11666', EO-73.15000 n at 794 ft.
NAUK3	Vansda	N- 20.11666', EO-73.15000 n at 794 ft.
NAUK4	Ahwa	N-20.80395', EO-73.63072' n at 624 ft.
NAUK5	Ahwa	N-20.80395', EO-73.63072' n at 624 ft.
NAUK6	Navsari	N-20.96950, EO-72.93094 n at 9 ft.
NAUK7	Navsari	N-20.96950, EO-72.93094 n at 9 ft.
NAUK8	Navsari	N-20.96950, EO-72.93094 n at 9 ft.
NAUK9	Navsari	N-20.96950, EO-72.93094 n at 9 ft.
NAUK10	Navsari	N-20.96950, EO-72.93094 n at 9 ft.
NAUK11	Navsari	N-20.96950, EO-72.93094 n at 9 ft.
NAUK12	Surat	N-21.33538, EO-72.96049 n at 78 ft.
NAUK13	Surat	N-21.33538, EO-72.96049 n at 78 ft.
NAUK14	Surat	N-21.33538, EO-72.96049 n at 78 ft.
NAUK15	Surat	N-21.33538, EO-72.96049 n at 78 ft.
NAUK16	Vasava	N-22.70084, EO-72.82697 n at 104 ft.
NAUK17	Vasava	N-22.70084, EO-72.82697 n at 104 ft.
NAUK18	Bayad	N-23.19940 EO-73.21892' n at 350 ft.
NAUK19	Bayad	N-23.18505' EO-73.21943' n at 386 ft.
NAUK20	Bayad	N-23.16784EO-73.21752' n at 354 ft.
NAUK21	Gandhinagar	N-23.19159', EO-72.61333'n at 240 ft.
NAUK22	Dhahod	N-22.92803', EO-73.9477.' n at 626 ft.
NAUK23	Khado	N-23.388252', EO-72.43951'n at 482 ft.
NAUK24	Khado	N-23.388252', EO-72.43951'n at 482 ft.
NAUK25	Khado	N-23.388252', EO-72.43951'n at 482 ft.
NAUK26	Patan	N-23.31389', EO-72.334187'n at 9 ft.
NAUK27	Banaskantha	N-24.04352', EO-72.78370'n at 678 ft.
NAUK28	Banaskantha	N-24.16646', EO-72.43392' n at 9 ft.
NAUK29	Bharuch	N-22.70083, EO-72.82698'n at 104 ft.
NAUK30	Junagadh	N-21.44198' EO-70.58613' n at 332 ft.



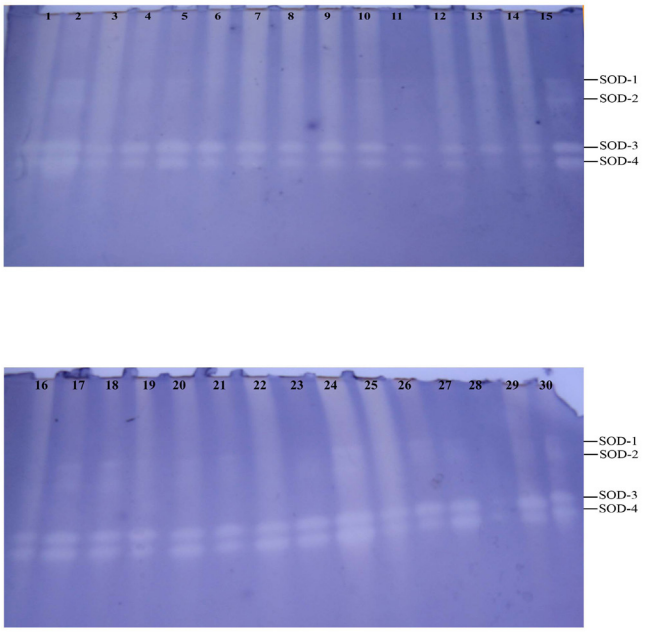
1-30: Accessions of karanja

Supplementary Figure 1: Isoforms of polyphenol oxidase detected among thirty genotypes of Karanja



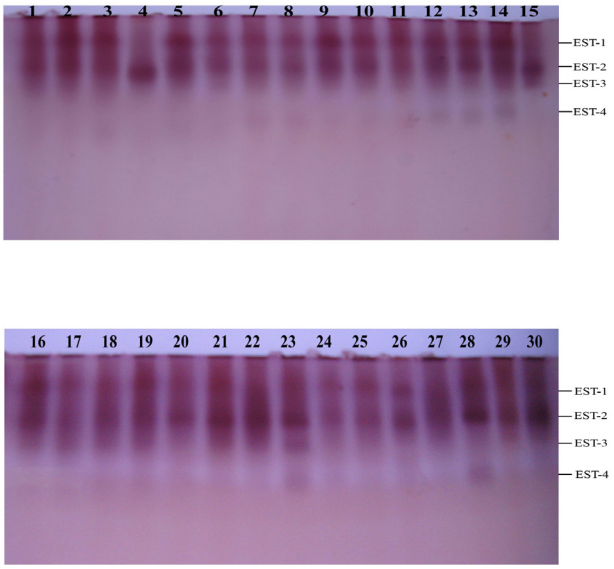
1-30: Accessions of karanja

Supplementary Figure 2: Isoforms of peroxidase detected among thirty genotypes of Karanja



1-30: Accessions of karanja

Supplementary Figure 3: Isoforms of superoxide dismutase detected among thirty genotypes of Karanja



1-30: Accessions of karanja

Supplementary Figure 4: Isoforms of Esterase detected among thirty genotypes of Karanja