PHOSPHATASE POLYMORPHISM BETWEEN MEMBERS OF THE CLOSELY RELATED MICROORGANISMS

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ABSTRACT

Piriformospora indica and *Sebacina vermifera* belonging to same taxonomic group show similar morphology, functions and isozyme patterns. The acid phosphatase from these fungi showed the pH and temperature optima of 5.3 and 40°C respectively. K_m for p-NPP (monoester) was 0.35mM. However, they show distinct genetic variation based on the random amplified polymorphic DNA (RAPD) analysis. Out of seven, six primers gave scorable and reproducible DNA products (bands) suitable for establishment of genetic diversity. The RAPD data confirmed that even among these two species of *Sebacinales* belonging to same morpho-zymographical groups, the level of variation was substantially high according to RAPD.

Key words: Acid Phosphatase, ELF 97 Endogenous Phosphatase, Fast Garnet GBC Staining, Random Amplified Polymorphic DNA

INTRODUCTION

Piriformospora indica and *Sebacina vermifera* (Fig 1) from *Sebacinales* are documented to function as bio-fertilizer, bioregulator, bioprotector and supplement to the health of the plant and the soil (Verma *et al.*, 1998: Varma *et al.*, 1999: Malla *et al.* 2005, Malla *et al.* 2007). More recently, they are documented to act as agent for biological hardening of tissue culture-raised plants. Despite their enormous potential, their biotechnological applications could not be exploited to the level they deserve. The axenic cultivability of these fungi provided ample opportunity to study the comparative Proteomic and Genomic relationship (Malla and Varma 2007, Malla 2009) and to establish variability in between these two fungi (Weiß *et al.*, 2004, Malla and Varma, 2005, Malla *et al*, 2004, 2006, 2007).

Phosphatases are the enzymes of wide specificity which cleave phosphate ester bonds and this play an important role in the hydrolysis of polyphosphates and organic phosphates. Acid and alkaline phosphatases (ACPase and ALPase) are the two forms of intra cellular phosphatase active at acidic & alkaline condition respectively. ACPase was found to be mainly involved in uptake of P by the fungal mycelia and ALPase is linked with its assimilation (Fries *et al.* 1998).

MATERIALS AND METHODS

Piriformospora indica

The fungus produces characteristic pear shaped chlamydospore (Verma *et al* 1998) and found to mimic the capabilities of typical AM fungus. The properties of the fungus *P. indica*, have been patented (European Patent Office, Muenchen, Germany. Patent No. 97121440.8-2105, Nov. 1998). The culture has been deposited at Braunsweich, Germany (DMS No. 11827) and 18s rDNA fragment is deposited at EMBL under the accession number AF 014929 USA. The fungus has potency to grow axenically on number of complex and synthetic media (Hill & Kaefer 2001). Hyphae are thin walled and of different diameters ranging from 0.7-3.5µm. Hypha are mostly coenocytic and septa are laid infrequently and contain more than one nucleus.

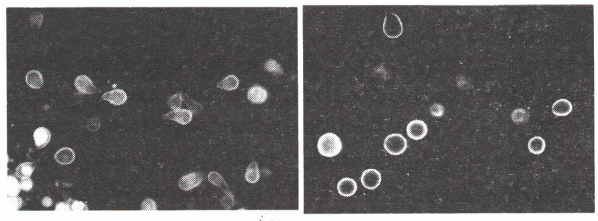


Fig.1. Autofluorescence shown by P. indica (Left) and *S. vermifera* (Right) chlamydospores seen under Leica microscope (model, 020-518.500) using I3 filter with excitations range 450-490 λ at 400x.

Chlamydospores are formed from thin walled vesicle at the tips of the hyphae. The chlamydospores appeared singly or in clusters and were distinctive due to their pear shaped appearance. The dolipores are very prominent, with a multilayered cross wall (Verma *et al* 1998).

Polymorphism study

Enzyme Assay (Straker & Mitchell 1986)

The biomass was homogenized using extraction buffer. ALPase and ACPase activities of aliquots were

determined spectrophotometrically using *P*-nitro phenyl phosphate monoster (Sigma Chemical Co.) as a substrate.

25µl of crude enzyme extract was mixed with 100µl of *p*-nitrophenyl phosphate solution and 100µl of buffer of different pH were added. The reaction mixture was incubated in a shaking water bath at 37°C for 30m. The reaction was stopped by adding 1ml of 50 mM NaOH. After filtration through a 0.45µm filter unit (Millex, syringe driven filter unit, Millipore Co. MA 01730. USA), absorbance was measured at 420nm Aamount of nitro phenol released was estimated from a calibration curve (ranging from 0 to 2mmol/ml) of *p*-nitro phenol (spectrophotometric grade Sigma, 104-8).

The activity of extracted crude enzyme was assayed at different temperature (15 - 75°C) under standard assay conditions of pH 5.3, and at different pH (3-10) under standard assay condition at 37°C. After shaking at 120rpm for 30m in water bath shaker absorbance was measured at 420 nm.

Enzyme kinetic studies were performed at 37°C using *p*-nitrophenyl phosphate di-sodium salt at concentration of 100µM-1000µM. The reaction mixture contained 200µl of *p*-nitrophenyl phosphate with 200µl of acetate buffer and 50µl of protein extract.

Two-dimensional PAGE (Gravel & Golaz, 1996)

The enzymes was first separated in a tube cell model 175 (Bio-Rad, Hercules, CA) with glass capillary tube (1.0-1.4mm internal diameter and 210 mm long) with Ampholyte pH 5.0-7.0 and 3.0-10.0 from Bio-Rad. 80µg of the sample were loaded using Hamilton syringe. The experiment was done as described by Gravel & Golaz 1996. Isoelectric Focusing (IEF) carried at 200V constant voltage for 2h, followed by 500V constant voltage for 2h and finally 800V constant voltages for 16h (overnight). Equilibrate the tube gels in equilibration buffer for ½h at room temperature. The protean II chamber (Bio-Rad) employed for second dimension. Tube gels after IEF was transferred on the top of SDS polyacrylamide gel and separated at 90V constant voltage in stacking gel and 120V constant voltage in separating gel. The 2D gel after electro phoresis was stained with silver staining method.

Native Polyacrylamide Gel Electrophoresis (Walker 1994)

Native PAGE was performed in 10% polyacrylamide gel and 4% stacking gel. The gel was run in a cold room maintained at 4°C subjected to 70V stacking gel run and 120V separating gel run.

Enzyme Assay (Walker 1996)

Duplicate samples were run in native gel. Detection of protein for their biological activity was done by staining one set of sample by coomassie to obtain whole protein bands and the other set for phosphatase activity. For the activity assay the gel was equilibrate in 50mM sodium acetate buffer pH 5.3 for 30m at 4°C in cold room. The gel was immersed in solution containing 2mg/ml concentration of the enzyme substrate (*p*-NPP) in shaking water bath till yellow band of acid phoaphatase was developed.

Molecular mass

Molecular mass of polypeptide eluted from the Native Gel was determined by SDS Polyacrylamide Gel Electrophoresis 12% using standard sigma marker (M-4038).

ELF 97 Endogenous Phosphatase Detection (Ingrid & van Aarle 2001)

The culture of *P. indica* grown for 48h in broth was fixed with 3.7% formaldehyde prepared in phosphate buffer saline for 1h at 4°C and permeabilized in 0.2% Tween 20 in PBS for 10m at room temperature. The ELF 97 (Molecular Probes, Kit E-6610) phosphatase substrate (component A) was diluted 20-fold in detection buffer (component B) as already standardized. The diluted substrate was filtered through 0.2 µm pore sized spin filters (E-6606) just before applying to tissue sections. The samples in spin filter was centrifuged in micro centrifuge for 2 minute. Since the reaction occurs very fast the reagent was added while the sample was on the microscope. The Sample was observed using excitation filter and dichroic mirror from emission filter in the Fluorescein set. The filter set provides the appropriate UV excitation and transmits wavelengths greater than 400nm. The development of signal was monitored.

Fast Garnet GBC Staining of Phosphatase Isozymes (Pasteur *et al* 1988)

The sample was prepared as mentioned above in Native PAGE protocol. The PAGE was done at constant current of 70 and then 120V for about 5h. The gel was neutralized in 50mM sodium acetate buffer for 30m in cold room. The gel was then soaked in staining solution containing α-naphthyl acid phosphate in a water bath shaker at 37°C. It was then destained with 1% acetic acid solution till clear bands appear.

Random Amplification of Polymorphic DNA

The fungal DNA was isolated and purified by CTAB protocol of Moller *et al.* (1992). DNA amplification was carried in a total volume of 25 ml containing (ml): 2.5, buffer (10X without $MgCl_2$), 2.5ml, $MgCl_2$, 0.8ml, dNTPs (10 mM), 1.0µl, primer (30 ng/µl), 0.5 µl Taq

polymerase (3U/µl) and DNA concentration ranging from 5-25ml. Random 10 bp Oligonucleotide primers (Operon Technologies Alameda, California) were used to produce amplification. in PTC-200 Thermal Cycler (Techne make, U.K.) The PCR product was observed after electrophoresis in 1.5% agarose gel Using the NTSYS-pc program (Rohlf, 1992). The genetic related ness or similarity was estimated using the Jaccard co-efficient. Clustering of similarity matrices was done by UPGMA (Un-weighted Pair Group method with Arithmetic Mean) and projected by TREE program of NTSYS-pc.

RESULTS

Optimization

Optimum ACPase activity was recorded at pH 5.3 (Fig 2).The activity sharply declined in alkaline pH at 8.8. The result showed measurable activity at temperature 40°C (Fig 3). The activity sharply declined with the increase in temperature. Activity was almost zero above 70°C. The optimum temperature was determined to be 40°C. Fig 4 shows enzyme kinetics performed at 37°C using *p*-NPP in concentration range of 0.05 –1mM under pH 5.3. Km for *p*-NPP (monoester) was 0.35mM. Activities of acid phosphatase from P. indica and S. vermifera sensu were comparatively examined in under identical standard conditions. Optimum values

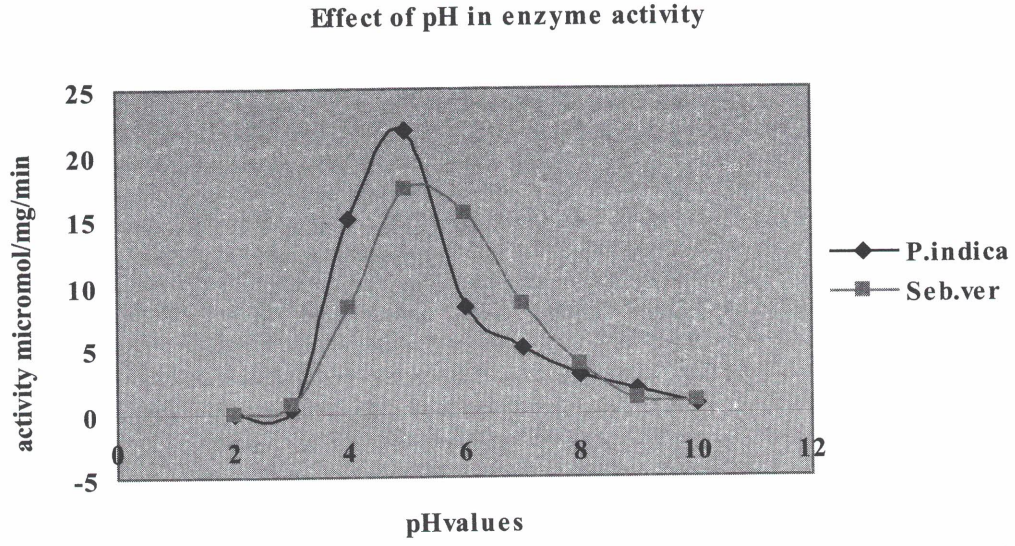


Fig 2: Effect of pH in ACPase activity in *P. indica* and *S. vermifera* sensu

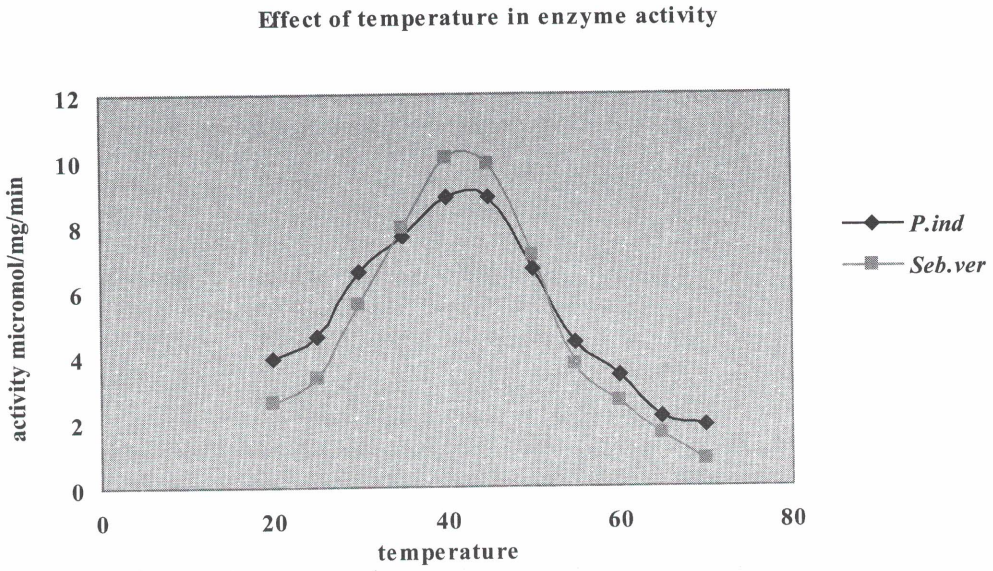


Fig 3: Effect of temperature on ACPase activity in *P. indica* and *S. vermifera* sensu

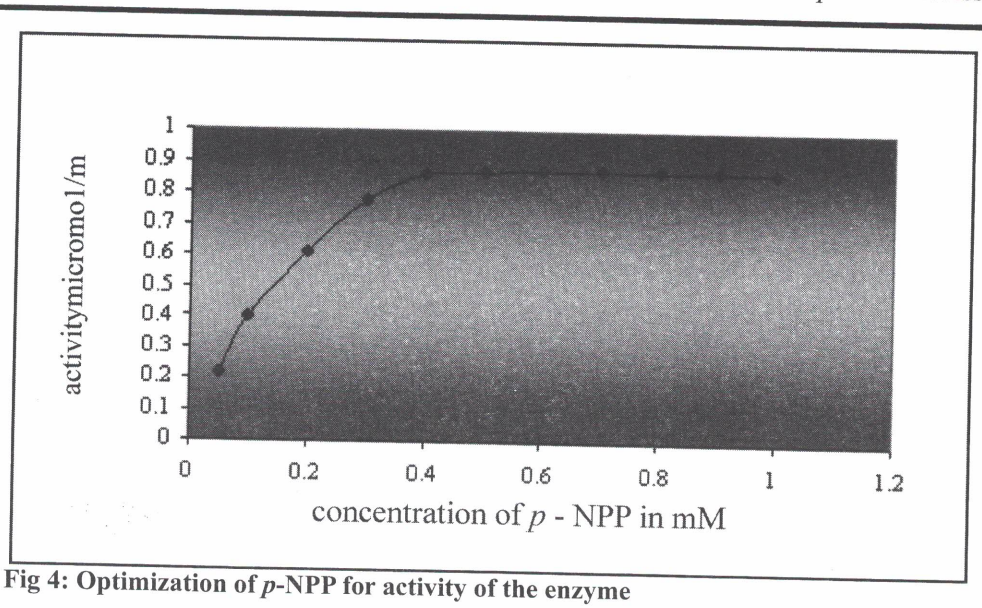


Fig 4: Optimization of *p*-NPP for activity of the enzyme

were almost similar at around 5 pH and temperature of 37°C in both cases.

Two-dimensional Polyacrylamide Gel Electrophoresis

Silver-stained 2- dimensional maps of mycelial proteins of *P. indica* and *S. vermifera* sensu showed similarity in most of the major protein bands. The gel indicated differences in some of the minor proteins (Fig.5).

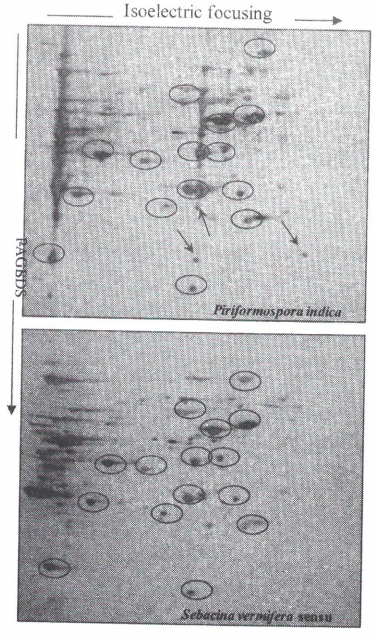


Fig. 5: Silver-stained two- dimensional maps of mycelial protein of *P. indica* and *S. vermifera* sensu loaded with 80µg of protein onto IEF gels. Separation in the horizontal dimension was achieved by isoelectric focusing using carrier ampholyte in the pH range of 3-10 in the presence of 9.2M urea and separation in the vertical dimension by 12% SDS-PAGE. The arrows in *P. indica* represent the bands absent in *S. vermifera* sensu

Native enzyme detection

The yellow band of ACPase of *S. vermifera* sensu was found at equidistance to the band of P. indica in native PAGE shows that the ACPase from both species have similar molecular mass (Fig. 6).

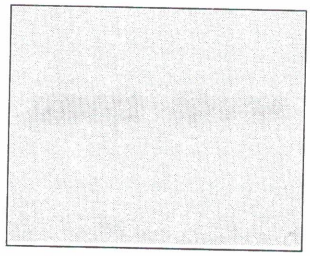


Fig.6: The Yellow colored band formed is due to *p*-nitrophenol, product of enzyme reaction upon substrate. Lane 1 *P. indica* and 2 *S. vermifera*. The banding pattern of the fungus shows precise similarity in between two fungi in their molecular mass and ionic strength

Molecular size

The molecular mass value of ACPase in *P. indica* and *S. vermifera* sensu was identical. The enzyme eluted from 8% native gel was separated in SDS gel. The molecular size of about 66kD was observed in both cases.

ELF 97 Detection of Phosphatase

Using excitation filter and emission filter from the fluorescein set, the activities of ACPase were detected by confocal microscopy. The fluorescent color developed in *P. indica* hyphae with dense production of ELF crystal due to phosphatase activity was comparable with that of control. Since the hyphae was not sectioned, only the activities were prominent but exact position or location of phosphatase was not confirmed. Extracellular phosphatases and alkaline phosphatase activity were not detected by this method. (Fig. not given)

Isozymes

Using α- naphthyl phosphate as substrate, different isoforms of ACPase were obtained by Fast Garnet GBC staining. *P. indica* and *S. vermifera* sensu showed three distinct isoforms of ACPase each, one with higher molecular mass and two other with lower molecular mass. The pattern of isoforms was similar in both the fungi. The Isoforms with higher molecular mass is suspected to be di-meric form of the enzyme and lower is suspected to be mono-meric form of ACPase on the basis of the position they formed in native gel (Fig.7).

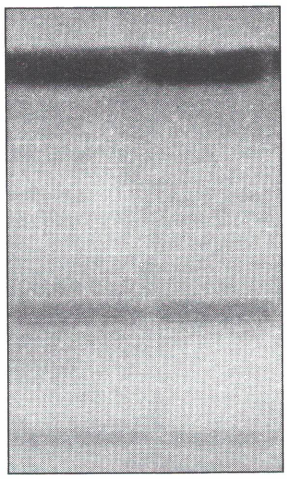


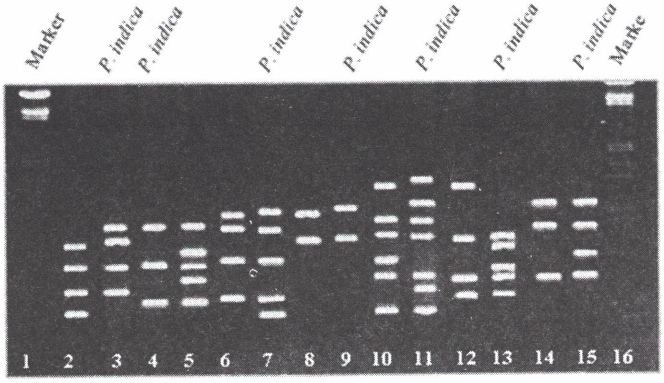
Fig. 7: Native PAGE Zymogram of acid phosphatase isoforms with Fast garnet GBC staining Lane1. *P. indica* Lane 2. S. vermifera sensu. The precisely localized band shows similar molecular mass and ionic strength of isoforms between *P. indica* and *S. vermifera*

Random Amplified Polymorphic DNA Analysis

Out of seven 10mer oligonucleotide primers, six primers gave scorable, reproducible DNA products (bands) suitable for establishment of genetic diversity. RAPD patterns of the isolates amplified with primers OPA-10 (5'GTGATCGCAG3') (Lane 2 and 3), OPD-01 (5'ACCGCGAAGG3') (Lane 4 and 5), OPC-06 (5'GAACGGACTC3') (Lane 6 and 7), OPC-10 (5'TGTCTGGGTG3') (Lane 8 and 9), OPC-01 (5'TTCGAGCCAG3') (Lane 10 and 11), OPI-04 (5'CCGCCTAGTC3') (Lane 12 and 13) and OPI-10 (5'ACAACGCGAG3') (Lane 14 and 15). Figure 8A clearly differentiated between *P. indica* and *S. vermifera* sensu. 58 RAPD bands were scored for the presence or absence of bands among the isolates with these seven primers. Most amplicons obtained with 6 primers were polymorphic between *P. indica* and *S. vermifera* sensu. Thus, the amplified band revealed a high degree of variation between these two species of fungus groups.

Statistical analysis of the data was preformed using the NTSYS-pc (Numerical Taxonomy System, Applied Biostatistics) program (Rohlf 1992). The degree of genetic relatedness or similarity was estimated using the Jaccard coefficient. The RAPD bands were scored as present (1) or absent (0) for each DNA sample with the 7 primers. Clustering of similarity matrices was done by UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) and projection by TREE program of NTSYS-pc. The Dendrogram showed 58% similarity between these two fungi (Fig. 8.B).

(A)



(B)

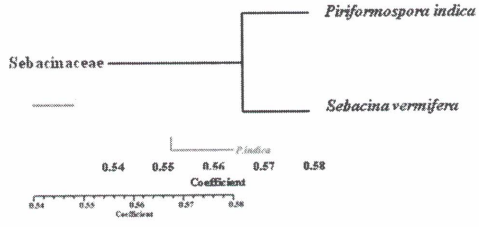


Fig. 8 :(A) Random amplified polymorphic DNA (RAPD) analysis of *Piriformospora indica* and *Sebacina vermifera* sensu. The RAPD analysis of *P. indica* and *S. vermifera* to show genetic veriation between these two fungus. Out of seven primers used for amplification six has given a productive polymorphism. Lane 1 and 16. marker. Lane 2 and 3 Primer OPA10. Lane 4 and 5 OPD01. Lane 6 and 7. OPC06 Lane 8 and 9. OPC10 Lane 10 and 11. OPC 01 Lane 12 and 13 OPI04. Lane 14 and 15 OPI10. No polymorphism was observed when the genomic DNA was amplified with OPC10 Lane 8 and 9.

Fig.8 :(B) Dendrogram showing Phylogeny between *P. indica* and *S. vermifera* sensu. The NTSYS-pc (Numerical Taxonomy System Applied Biostatistics) computer program was used for data analysis.

DISCUSSION

The acid phosphatases in *Piriformospora indica* and *Sebacina vermifera* sensu were similar in their molecular mass (Malla, 2008). The optimum physical condition such as pH, temperature are almost similar in both fungi supporting strong relationship between these two. The protein separated in SDS PAGE and Native PAGE of P.indica and S. vermifera showed their position at précise location. The pattern of phosphatase isozymes in Native PAGE shows the evidences of strong relationship of these fungi. Almost similar observation was noticed in *P.indica* and *S. vermifera* sensu showing closeness of these fungi to each other by applying ELF-97 substrate. Two dimensional map of crude protein of these two fungi showed some differences in minor proteins.

These two fungi showed similar morphology, functions and isozyme patterns. However, they showed distinct genetic variation based on the random amplified polymorphic DNA (RAPD) analysis. An average genetic similarity between both the fungi was 58% can be considered to place in species of same ancestral roof.

The application of different techniques for characterization of ACPase in these two fungi has provided new insights into important aspects of this field. *Piriformospora indica* and *Sebacina vermifera* sensu belonging to same taxonomic group show similar morphology, functions, protein profiles and characterization along with closely related nature of acid phosphatase and their isoforms. However, they showed distinct genetic polymorphism based on the random amplified polymorphic DNA analysis.

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