Isozyme analysis of VAM spores help in taxonomic enumeration

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Isolates of two species of *Glomus* (*G. deserticola, G. fasciculatum*) and *Scutellispora* (*S. gilmorei, S. calospora*) were recovered from rhizospheric soil of plants growing in arid and semiarid regions of western Indian subcontinent. They have been characterized by subjecting spore extracts to polyacrylamide gel electrophoresis and selective enzyme staining. The banding patterns of malate dehydrogenase have been studied for these isolates to distinguish at intra-generic and intra-specific levels. Attempts were also made to study the difference in banding patterns of the isolates growing under environmentally different conditions. Data reveal that isoenzymic studies are useful for the differentiation and identification of VAMF genera and species. It was found that the species growing in environmentally different conditions gave almost the same banding patterns. It is proposed that isozymic studies should be included in the description of new taxa to show how the banding pattern of different enzymes of newly identified species is related to the banding pattern of the existing genera and other known species.

New species of vesicular and arbuscular mycorrhizal (VAM) fungi are being proposed by such frequency that it is becoming increasingly difficult for researchers in the subject to be sure of the identity of the endophytes which they or others are using. Since VAM fungi cannot be grown in pure culture and exhibit almost no host specificity, they have been described and classified almost exclusively on visual characteristics of their soil-borne resting spores. At the generic level the principal characters used are the formation and morphology of spores (whether they are azygospores or chlamydospores), their mode of germination and sporocarp morphology (where applicable). At the species level the main diagnostic feature used is the appearance of the spores, for example their colour, contents, wall thickness and ornamentation and type of hyphal attachment[1,2]. The use of murographs has been suggested as a means of standardizing descriptions for identification and classification[3]. The anatomy of infection has been proposed as an additional means of identification[4,5], but this has not been used routinely by researchers/taxonomists. Immunological methods have also been tested for identification of VAM fungi[6-9].

The application of electrophoretic technique in mycorrhizal studies enabled the separation of fungal proteins in *Eucalyptus, Pisolithus*, ectomycorrhizas[10] and the detection of fungi and plant specific acid and alkaline phosphatase isozymes within VAM roots[11]. Isozyme techniques have featured in taxonomic and population genetic studies of VAM[12,13]. The methodology has also been further extended to enable the identification of different fungal symbionts in endo[12,14] and ecto-mycorrhizas[15]. The isozymic patterns of malate dehydrogenase (MDH) which is essentially an enzyme of TCA cycle can be used for the identification and differentiation of many species/genera of VAM fungi[12,13,16]. In the present investigation the identification and differentiation in the isozymic mobilities of different VAM species selected from extreme desert and semiarid conditions were studied.

Methods

VAM sample preparation

Spores of different VAM species (*Glomus deserticola, G. fasciculatum, Scutellispora gilmorei* and *S. calospora*) were isolated from rhizospheric soils of plants (*Cenchrus ciliaris, C. biflorus*) growing in the extreme arid environment of Thar desert of western India, Jodhpur, Rajasthan and semi-arid environment of Delhi following the wet sieving and decanting method and sucrose centrifugation method[17,18]. These species have been identified by external morphology and studying the murographs of the spores[2]; the spores were multiplied in autoclaved soil on host plant *Eleusine coracana* and *Zea mays* using earthen pots. The conditions of host growth were simulated as found in arid and semiarid conditions. From each isolate 15 spores were mounted in polyvinyl alcohol-lactophenol (PVL) for observation in compound microscope. The remaining spores were collected in self-devised glass homogenizers (2.5 × 0.5 cm) in 5 μl of buffered sucrose (150 g l^{-1}) containing bromophenol tracking dye (20 mg l^{-1}). The buffer contained 20 mM Tris HCl, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 10 mM β mercaptoethanol and 0.1 mM EDTA, pH 8.0, at 4°C, after washing them several times with sterile deionized water. Equal volume of each isolate spore has been taken for individual experiment. The spores were crushed using a glass piston and a further 10 μl of buffered sucrose

containing 0.6 g l^{-1} Triton X-100 was added to the sample. The samples were prepared rapidly and maintained at 4°C between crushing and loading onto the gel[19].

Standardization of the extract volume

Spores of *G. deserticola* were taken in different numbers (50–400) and run on the gel to standardize the minimum number of spores which gave protein bands good enough for photographic reproduction. A minimum of 300 spores (total vol. 0.2231 mm^3) gave such bands. For the rest of the species the same volume was maintained and the required spore numbers were calculated accordingly (Table 1). The size of the spores taken was an average of 15 spores for further studies.

Electrophoresis

Zonal PAGE using vertical composite gels with different buffer systems (discontinuous system) was done, with 70 × 0.75 × 85 mm size gels (including stacking gel) in a mini gel apparatus of Biometra, Germany[20].

Proteins in spore extracts together with bromophenol blue (BPB) tracking dye were subjected to polyacrylamide gel electrophoresis as described by Rosendahl and Sen[21].

Running conditions

After the gel was loaded, the tank is connected to a power supply (Pharmacia ESP 500/400) and initially 50 V was applied for 2 h, which was increased to 100 V for 5 h. The gel was run for 7 h at 4°C in cold room.

Table 1. *Number of spores required for one experiment of different* isolates

VAM species isolate	Average r of 15 spores (μm)	Average vol. of 1 spore (mm^3)	No. of spores required
G. deserticola (semiarid isolate)	56.2	2.231×10^{-4}	300
S. gilmorei (semiarid isolate)	142.0	1.199×10^{-2}	19
G. fasciculatum (semiarid isolate)	64 6	2.695×10^{-4}	593
G. deserticola (arid isolate)	48.4	4.751×10^{-4}	469
S. gilmorei (arid isolate)	137.0	1.077×10^{-3}	21
G. fasciculatum (arid isolate)	48.0	4.634×10^{-4}	481
S. calospora (Arid isolate)	125.0	8.185×10^{-3}	27

Enzyme staining

The staining solution of MDH is a buffer, at optimum pH, containing artificial substrate, co-factor, and dye. On incubation coloured bands appeared in the gel at position of isozyme activity. Recipe for making 25 ml of staining solution in distilled water for a single gel is: Malic acid = 163 mg, NAD^+ = 5 mg, 3-(3, 4-dimethylthiazole 2-yl) 2 dimethyltetrazolium bromide = 5 mg, phenazine methosulphate = 3 mg.

Malic acid was dissolved in 2.5 ml of 0.5 M Trizma base in 20 ml of distilled water and final pH was adjusted to 8.0 with 1 M KOH. The volume was made up to 25 ml and NAD^+, MTT and PMS were added.

After electrophoresis the gel is removed from the glass cassette and submerged into enzyme staining solution. The gel box was kept in dark at 37° C for one and a half h. Dark blue bands appeared. The gel was photographed immediately after the staining.

Results

The extent of MDH polymorphism and its genetic basis, electrophoretic patterns among spores of different species of VAM were analysed by selective enzyme staining following polyacrylamide gel electrophoresis (PAGE).

Three distinct zones of MDH activity were detected on the gels based on the patterns of electrophoretic variations among the four species of VAM fungi. Zone 1 was very slow moving and close to the cathode (point of origin); zone II was fast-moving compared to zone I and was located relatively closer towards the anode (running front); zone III moved ahead of the zone II and was the fastest moving and located very close to the running front.

Figure 1 shows the zymogram of seven representative isolates showing three discrete zones of MDH activity. All the three zones of activity were different with respect to their electrophoretic mobilities and in the intensity of protein staining. Of the three zones of activity, maximum activity was observed in zone 1 in most of the isolates (five out of seven isolates) investigated, except in *G. deserticola* of semiarid and arid regions where the activity of zone III was maximum (Figure 2).

In each zone of MDH activity, one prominent, intensely stained or two bands of differentially stained intensity were observed (Figures 1 and 2). Two bands were observed in zone II of *G. deserticola* (arid isolate) and *Scutellispora gilmorei* semiarid isolate and zone I of *G. fasciculatum*. It was observed in *G. deserticola* (arid isolate) and *S. gilmorei* (semiarid isolate) that the intensely stained band was a fast moving one (F) whereas, the faint, less intensely stained band was slow moving (S), while in *G. fasciculatum* (semiarid isolate) the

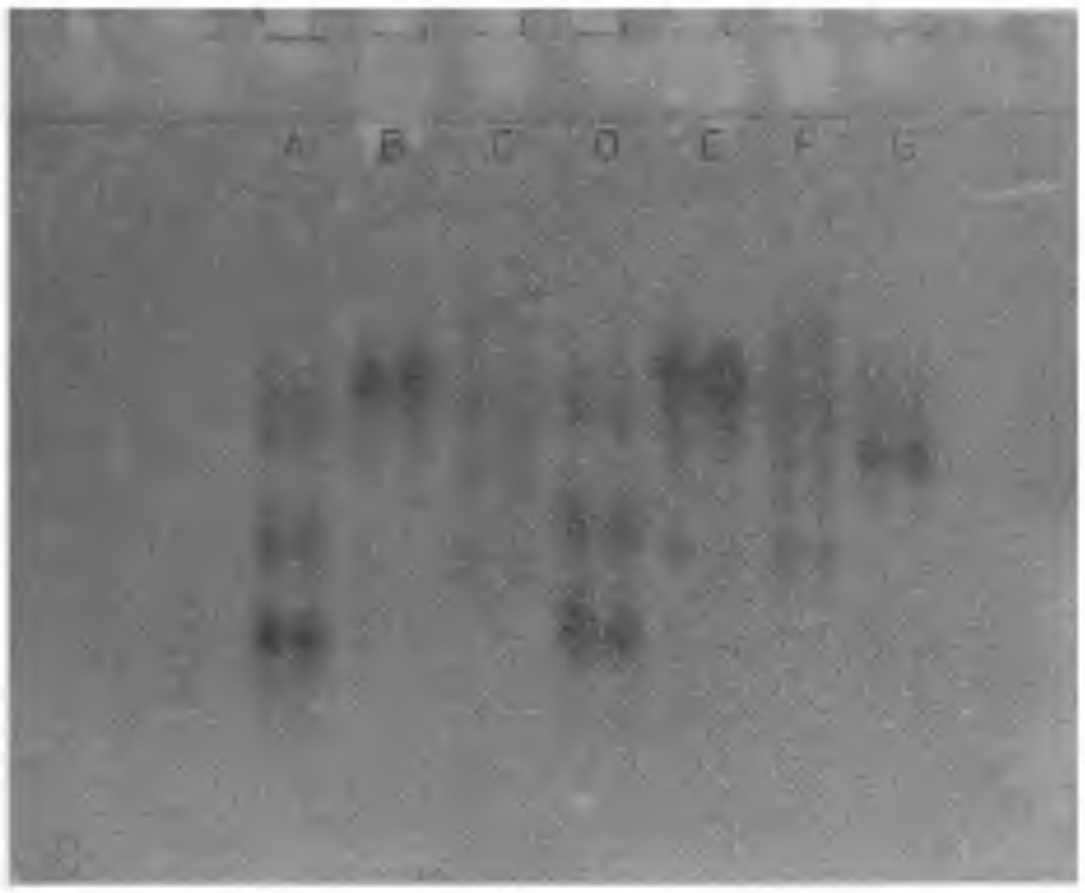

Figure 1. Banding pattern of different VAM isolates after MDH staining A, *Glomus desertıcola* (semiarid isolate), B, *Scutellıspora gilmorei* (semiarid isolate); C, *G. fasciculatum* (semiarid isolate); D. *G. desertıcola* (arid isolate), E, *S gilmorei* (arid isolate), F, *G. fasciculatum* (arid isolate), G, *S. calospora* (arid isolate).

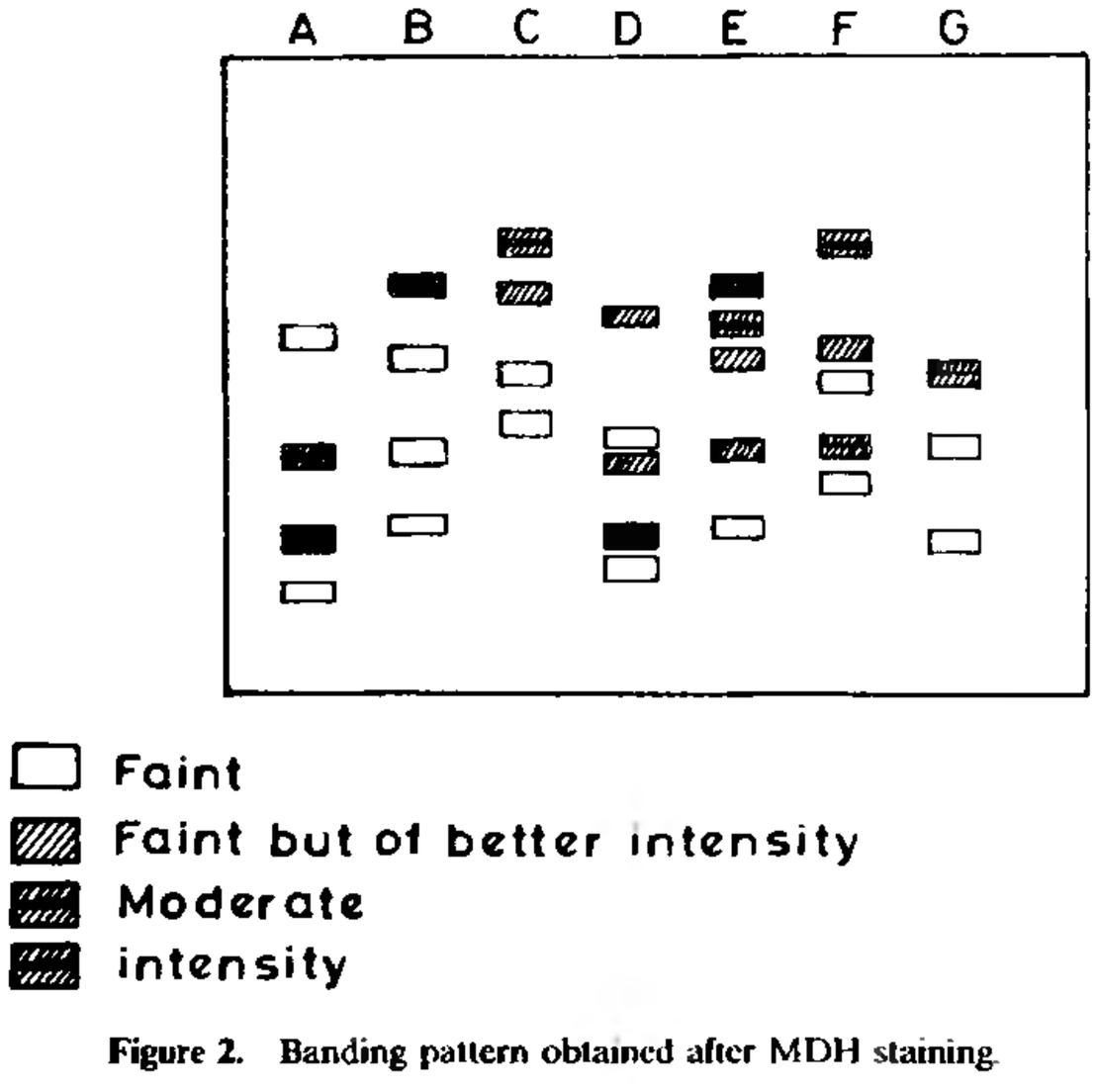

Figure 2. Banding pattern obtained after MDH staining.

intensely stained band was slow moving (S) and near to the cathode (point of origin) and the fast moving (F) band was faint.

Discussion

To be of use in the characterization and identification of VAM fungi, any chemotaxonomic method should be sensitive to differentiate at the species level, since demarcation of genera is fairly easily accomplished using the morphological characteristics of the resting spores. It is also essential that the method should be reproducible, not just between assays, but such that a fungus maintained under a range of conditions, as on different hosts in a variety of soils, should exhibit the same banding patterns[9].

Our observations have shown that these two requirements are met, using specific enzyme staining following gel electrophoresis. *G. desertıcola*, *G. fasciculatum* and *S. gilmorei* spores recovered from arid and semiarid regions have shown almost the same banding pattern of MDH in different zones of activity, except that some additional allozymes were observed in arid region isolates (Figure 2). In *G. desertıcola* zone II has shown one allozyme while it is absent in isolate from semiarid region. Simlar results were observed with *G. fasciculatum* and *S. gilmorei* in zone I (Figure 2), indicating that the mobility of these enzymes (which is based on the molecular weight and charge) was not very much influenced by the environmental or physiological conditions. It was further observed that desertic isolates of different species had more allozymes and isozymes, which may be a manifestation of adaptation to the suress conditions of the desert. Evidences obtained so far suggest that the degree of variability observed between different isolates of a particular species of VAM fungi in terms of number of isozymes detected was not greater than that observed in other fungi.

Hepper *et al.*[12] studied isolates of *G. clarum*, *G. monosporum* and *G. mosseae* from different geographical regions. These authors found no great increase or decrease in the isozyme numbers of the isolates. In contrast, geographically different isolates of *Cryphonectria cubensis* gave a variety of banding pattern when esterase, hexokinase, phosphoglucomutase and peroxidase were stained[22]. On the other hand, isolates of *Botrytis cinerea* showed rather weak variability and this confirms of EST banding patterns.

The present results confirm with those of Hepper *et al.*[12] that different isolates of *G. clarum* have shown the same banding patterns of MDH which are quite distinct from MDH banding pattern of other species. Rosendahl[13] reported 80 per cent isozymic similarity between two isolates in isozymic patterns although one of the isolates lacked the outer hyaline wall. The study further showed that though *Scutellispora* and *Glomus* are quite distinct from each other in morphological characters, resemblances were seen in their banding patterns. Rosendahl[13] has also reported identical results to our findings.

In the present study VAMF species exhibited considerable intragenic variation in the banding patterns of MDH. The fungi which had identical spore morphology and have been classified as similar species

(arid and semiarid isolates) were separable using specific enzyme staining, although they did exhibit a great degree of homology in the intense and moderate protein bands. Of the total number of bands, 85 per cent were identical in all the three isolates. Besides being useful for taxonomic studies, this technique also provides a sensitive method for investigating the biochemical activities of the resting spores of VAM fungi. Different isozymic patterns in the arid and semiarid isolates suggest a similar phylogenic line but difference in gene pool of both population.

It will be of value if isozymic banding patterns were included in the descriptions of new taxa, showing how they relate to those of known species. The technique is also useful for separating strains of VAM fungi, but a word of caution is important. Lack of similarity in isozyme patterns should not solely be used as a criterion for separating fungi into distinct taxa.

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RESEARCH COMMUNICATIONS

Exploration for gold using termitaria

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Termitaria sampling in the Sakalagunta old working for gold in the Bargur area, Dharmapuri District, Tamil Nadu has shown some significant results. The analytical result of termitaria samples indicates that it can be used as direct indicator for sub-surface gold mineralization. Panning of termitaria samples also shows the presence of fine specks of native gold.

GREEK historian Herodotus (484–425 BC) described how the natives located gold by using ant heaps in desert regions in a locality believed to border the Gobi desert[1]. The hydrological significance of termite mounds is discussed by Varahamihira in his *Brihat Samhita* (507–587 AD)[2]. West[3] writes that the ancient miners in Rhodesia (Zimbabwe) located and worked over 4000 gold mines without digging numerous prospecting pits and trenches. During recent times, several authors have reported on the application of termite mound sampling to mineral exploration in Zimbabwe (Rhodesia), Mozambique, Botswana, India and USSR[4,5].

In the Sakalagunta area, termite mounds are found in abundance in an area of gold mineralization. The height of termite mounds varies from 1 to 2 m and diameter at the base varies from 1 to 2 m. These mounds were studied to find out whether they would serve as indicator for gold mineralization and the results are reported here.

The Sakalagunta old working falls within the Varatanapalli Reserve Forest area (12° 33′ 40″: 78° 19′ 50″–57 L/6), situated about 1.5 km north of Krishnagiri–Madras highway (NH 7) (Figure 1).

The rock types exposed in the study area comprise migmatite, banded iron formation, amphibolite, quartz and pegmatite veins. Amphibolite and banded iron formation occur as enclaves within the migmatite. Quartz and pegmatite veins are seen as intrusive into the migmatite and amphibolite.

The Sakalagunta old working is located on a