ISOZYME MARKERS FOR VIRULENCE IN THE ENTOMOPATHOGENIC FUNGAL ISOLATES OF *BEAUVERIA* SPECIES AGAINST *SPODOPTERA LITURA*

Padmini Palem P.C[1*] and Padmaja. V[2]

[1]Department of Biotechnology, Dr. L. B. PG College, Visakhapatnam, India.
[2]Department of Botany, Andhra University, Visakhapatnam, India.
Padmini49@gmail.com

ABSTRACT Twenty nine pathotypes of *Beauveria bassiana* (Bals.) Vuill and one of *Beauveria brongniartii* (Sacc.) petch were characterized according to virulence towards second instar larval stage of *Spodoptera litura* and isozyme variations. Biological differences were found among the isolates. LT50 values ranged from 4.2 to 9.6 days based on which the isolates were characterized as virulent and less virulent. Electrophoretic polymorphisms of esterase and acid phosphatase were performed to differentiate the isolates. Cluster analysis revealed a demarcation among the virulent and less virulent isolates of *B. bassiana* from that of *B. brongniartii* species.

Key Words: *B. bassiana*, *B. brongniartii*, *S. litura*, LT50, Esterase, Acid Phosphatase, cluster analysis.

INTRODUCTION

Productivity of crops grown for human consumption is at risk due to the incidence of pests. *Spodoptera litura* is regarded as a major destructive polyphagous pest of subtropical and tropical agricultural fields. Utilization of biological agents as a part of integrated pest management strategy could reduce the dependence on chemical control in view of their ecofriendly nature. *Beauveria bassiana* is a known natural enemy of a number of insect pests of crop plants (Amer et al 2008) and can cause epizootic death in various insects with host specificity thus having an important role in IMP. A unique fingerprint is required for individual genotypes prior to their release at field level in biocontrol programs which will enable monitoring of the environmental impact of a particular isolate following its use as mycopesticide.

Biochemical markers offer an independent means of characterising genotypes of *Beauveria* spp. for selecting pathogenic isolates for use in the biocontrol program. According to St. Leger et al (1992) considerable amount of genetic variations and host range are present in *B. bassiana* despite its asexual mode of reproduction and therefore Isozyme studies allow rapid and inexpensive analysis of large number of isolates. The advantage of using enzymatic proteins is the high specificity of migratory bands, since the electrophoretic variants of each enzyme can be directly equated with alleles of a single locus. Esterases by their significant electrophoretic polymorphisms are reliable biochemical markers for studying population genetics and phylogeny (Goullet and Picarad 1995). Variations in esterase banding patterns were observed among the different geographical populations of *B. bassiana* isolated from *Sitona* weevils (Poprowski et al 1988). Isozyme patterns have been studied in several species of the genus *Tolypocladium* by Riba et al (1986) who reported highly significant differences between the species. Similar isozyme banding patterns were observed in *M. anisopliae* isolated from the same host and similar banding pattern in *B. bassiana* were also observed irrespective of their origin by Leucona et al (1996). This study was conducted to determine the virulence potential and investigate variations among and within 30 isolates of endemic as well as exotic collection of *Beauveria* spp with the objective to develop genetic markers for virulence.

MATERIALS AND METHODS

Source of *Beauveria* cultures

Twenty four endemic isolates of *Beauveria bassiana* were isolated from diseased larvae from various agricultural fields of Andhra Pradesh, India. Six isolates which includes, one *B. Brongniartii* species were procured from USDA - ARS, ITCC and EMBRAPA. (Table - 1). Pure cultures were established and maintained in SDAY (Sabouraud's dextrose agar yeast) medium.

TABLE-1 : HOST AND ORIGIN OF *BEAUVERIA* ISOLATES

Isolate number	Accession number	Geographical origin	Host
B6	USDA ARS	France	*Helicoverpa virescens*
B7	USDA ARS	Indonesia	*Nilaparvata lugens*
B8	ITCC	India	*Un known*
B12	EMBRAPA	Brazil	*Spodoptera frugiperda*
B13	-	Guntur	*Helicoverpa armigera*
B14	-	Sathinapalli, Guntur dist AP, India	*Helicoverpa armigera*
B15	-	Sathinapalli, Guntur dist AP, India	*Helicoverpa armigera*
B16	-	Muthayapalem, Guntur dist AP, India	*Helicoverpa armigera*
B18	-	Sathinapalli, Guntur dist AP, India	*Helicoverpa armigera*
B19	-	Muthayapalem, Guntur dist AP, India	*Helicoverpa armigera*
B20	-	Muthayapalem, Guntur dist AP, India.	*Helicoverpa armigera*
B22	-	Madugula, Guntur dist AP, India	*Helicoverpa armigera*
B23	-	Amaravathi, Guntur dist, AP, India	*Helicoverpa armigera*
B24	-	Peddakorapadu, Guntur dist, AP, India	*Helicoverpa armigera*
B25	-	Muthayapalem, Guntur dist, AP, India	*Helicoverpa armigera*
B26	-	Sathinapalli, Guntur dist, AP, India	*Helicoverpa armigera*
B27	-	Sathinapalli, Guntur dist, AP, India	*Helicoverpa armigera*
B28	-	Sathinapalli, Guntur dist, AP, India	*Helicoverpa armigera*
B29	-	Warangal, AP, India	*Spodoptera litura*
B30	-	Warangal, AP, India	*Helicoverpa armigera*
B31	-	Warangal, AP, India	*Spodoptera litura*
B32	-	Warangal, AP, India	*Spodoptera litura*
B33*	USDA ARS	India	*Coleoptera*
B35	EMBRAPA	Brazil	*Anthonomes grandis*
B37	-	Guntur dist, AP, India	*Helicoverpa armigera*
B38	-	Guntur dist, AP, India	*Helicoverpa armigera*
B39	-	Guntur dist, AP, India	*Helicoverpa armigera*
B40	-	Guntur dist, AP, India	*Helicoverpa armigera*
B41	-	Guntur dist, AP, India	*Helicoverpa armigera*
B42	-	Guntur dist, AP, India	*Helicoverpa armigera*

* *Beauveria brogniartii*

Fungal cultures for Bioassays

Conidia needed for bioassays were obtained from cultures grown on SDAY at 26°C for 15 days. Conidia were harvested by scraping the surface of the plate lightly after flooding with sterile distilled water containing 0.1% Tween 80 and the concentration was adjusted to $2x10^8$ conidia /ml.

Insect cultures and Bioassays

The culture of *S. litura* was raised from field collected larvae and maintained under laboratory conditions. Egg patches laid on the surface of castor leaves by adult females were incubated in a growth chamber separately in sterilized containers and allowed to hatch and develop to 2nd instar stage. Fifteen larvae for each *Beauveria* isolate were topically inoculated with 50µl of conidial suspension at a concentration of $2x10^8$ conidia /ml. Three replicates for each isolate were maintained along with controls. The treated larvae were placed in separate containers provided with castor leaves as diet. Mortality data was recorded at every 24 hour intervals in terms of larvae dead per isolate till pupation.

Esterase and Acid Phosphatase Isozyme analysis

Mycelia needed for biochemical studies were obtained by inoculating 50 ml SD broth in 150 ml flasks with a final conidial suspension of 10^6 conidia/ml. Flasks were incubated on a rotary shaker maintained at 26°C on 150 rpm. After 5 days, the mycelia were separated from the broth and the mycelial mat was used for esterase and acid phosphatases isozyme extraction. All operations were carried out at 4ºC. Isozyme samples were ran on native PAGE mini gels and the electrophoresis was performed. Extraction and staining protocols were performed as per methods described by Sadasivam and Manickam (1991) with slight modification.

Data analysis

For bioassays, Probit analysis (Finney 1962) was used for obtaining the LT50 values, regression, slope, fiducial limits and chi-square. Relative mobilities of the isozyme bands and the numerical analysis were carried out with a Vilbert Lourmat gel documentation system using photocapt computer software. Resolving front (Rf) of each isozyme band was calculated using the anodally moving band of the isolate. The data was organized into a binary matrix, coded as 0 for band absent and 1 for band present for generating similarity matrix. An UPGMA dendrogram and cluster analysis was performed from this similarity matrix using XL stat software.

RESULTS

Bioassays

Bioassays against 2nd instar larvae of *S. litura* revealed variations in virulence among the 30 isolates. Least LT50 values of 4.2, 4.5 and 4.6 days were observed in B19, B30, B25 and B22 isolates (Table – 2). Highest LT50 values of 9.26 followed by 7.28 in B8 and B28 isolates were recorded respectively while rest of the isolates showed a range from 4.8 to 6.9 days. There was no mortality in the controls and therefore no Abbott correction (Abbott 1925) was required. Chi-square test showed no heterogeneity among the insect populations tested and LT50 values were within the fiducial limit range.

Esterase isozyme profiles

A total of 31 bands were recorded with Rf values ranging from 0.050 to 0.490. B35 was the only isolate which did not show any isozyme band. Specific bands were demonstrated in 9 isolates i.e. B8, B12, B14, B18, B22, B26, B28, B32 and B33 (Table -3). Single isozyme bands were observed in B29, B30, B8, B33, B38, B39, B40 and B42 and rest of the isolates showed multiple banding patterns (Fig. 1). A maximum of 6 bands were seen in B24 and B32 while band with Rf value 0.050 was recorded in B18, B22, B24, B26, B27, B28, B37, B38, B39, B40, B41 and B42 isolates. Band with Rf value 0.060 was shown by B7, B13, B14, B31 and B32. Regarding the intensities, bands with Rf 0.400 (B7), 0.416 (B12), 0.15 (B32), 0.26 (B14), 0.43 (B16 and B18), 0.169 (B19), 0.188 & 0.41 (B22) and B32 (0.39) were darkly stained and the zone of isozyme was also very broad. Rests of the bands were light to medium dark in appearance.

Cluster analysis:

The thirty isolates of *Beauveria* were grouped into 5 main clusters (Fig. 3). B27, B28, B37 and B41 were clustered with a similarity matrix of 34%. B15, B25, B19 and B20 showed grouping with a similarity matrix of 52%. While B23, B26, B30 and B31 were paired with a similarity matrix of 66%. On the other hand B7 and B13 were paired with a least similarity matrix of 25%.

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TABLE – 2 : BIOASSAYS OF *BEAUVERIA* ISOLATES AGAINST 2ND INSTAR LARVAL STAGE S. *LITURA*

Isolate number	LT50	Regression (Y)	Slope (b)	Chi square	Fiducial limit
B6	5.91±0.42	2.24+2.34	2.34±0.26	0.045	3.32 to 4.89
B7	6.11±0.31	3.36+2.24	2.24±0.34	0.064	4.66 to 5.63
B8	9.26±0.10	3.51+2.60	2.60±0.21	0.316	5.93 to 7.02
B12	6.65±0.20	2.38+3.02	3.02±0.33	0.217	4.64 to 5.62
B13	6.91±0.54	2.18+3.25	3.25±0.27	0.315	5.26 to 6.30
B14	5.54±0.54	2.43+3.62	3.62±0.24	0.516	4.56 to 5.62
B15	4.52±0.60	2.76+2.10	2.10±0.35	0.372	3.26 to 4.850
B16	6.53±0.31	3.86+3.55	3.55±0.31	0.266	5.29 to 6.88
B18	5.86±0.28	2.01+3.62	3.62±0.34	0.051	5.03 to 6.95
B19	4.20±0.46	3.47+2.65	2.65±0.46	0.532	3.43 to 4.63
B20	6.41±0.33	2.62+3.83	3.83±0.33	0.503	3.36 to 4.86
B22	4.68±0.43	2.80+4.63	4.63±0.38	0.260	5.69 to 6.47
B23	6.91±0.33	2.39+3.86	3.86±0.37	0.656	4.36 to 5.45
B24	5.18±0.28	1.62+3.53	3.53±0.31	0.382	3.80 to 5.60
B25	6.34±0.38	2.32+3.61	3.61±0.25	0.610	4.82 to 5.63
B26	5.58±0.40	2.05+3.68	3.68±0.36	0.536	3.51 to 4.77
B27	6.80±0.78	1.67+3.39	3.39±0.34	0.309	5.98 to7.60
B28	7.83±0.61	2.95+3.01	3.01±0.28	0.825	4.47 to5.46
B29	6.81±0.51	3.36+3.15	3.15±0.34	0.562	4.01 to4.90
B30	4.21±0.26	3.32+2.58	2.58±0.37	0.292	3.65 to 4.96
B31	5.32±0.37	3.58+2.41	2.41±0.31	0.157	5.99 to 6.12
B32	4.81±0.32	2.95+3.01	3.01±0.36	0.305	4.28 to 6.62
B33	6.08±0.28	2.21+4.16	4.16±0.26	0.853	3.51 to 4.91
B35	6.27±0.30	1.36+4.63	4.63±0.25	0.281	5.86 to 7.64
B37	6.45±0.36	3.50+2.40	2.40±0.24	0.556	5.20 to 6.20
B38	5.85±0.39	3.32+3.02	3.02±0.33	0.492	3.52 to 3.91
B39	5.61±0.20	2.86+3.31	3.31±0.24	0.674	4.58 to 5.93
B40	4.81±0.81	3.56+1.05	1.05±0.28	0.610	5.71 to 7.40
B41	5.61±0.28	2.55+3.60	3.60±0.37	0.382	4.28 to 5.63
B42	6.15±0.37	2.13+3.44	3.44±0.26	0.432	4.04 to 4.98

Acid phosphatase isozyme profiles

A total of 17 bands with Rf values ranging from 0.050 to 0.280 were recorded in all the *Beauveria* isolates except B35 and B42 which did not show the band (Table - 4). B6, B7, B8, B13, B15, B16, B18, B19, B20 and B22 isolates produced single bands while rest of the isolates produced multiple bands. Isolates which showed only 2 bands were B12, B14, B24, B26, B27 and B41 (Fig. 2).

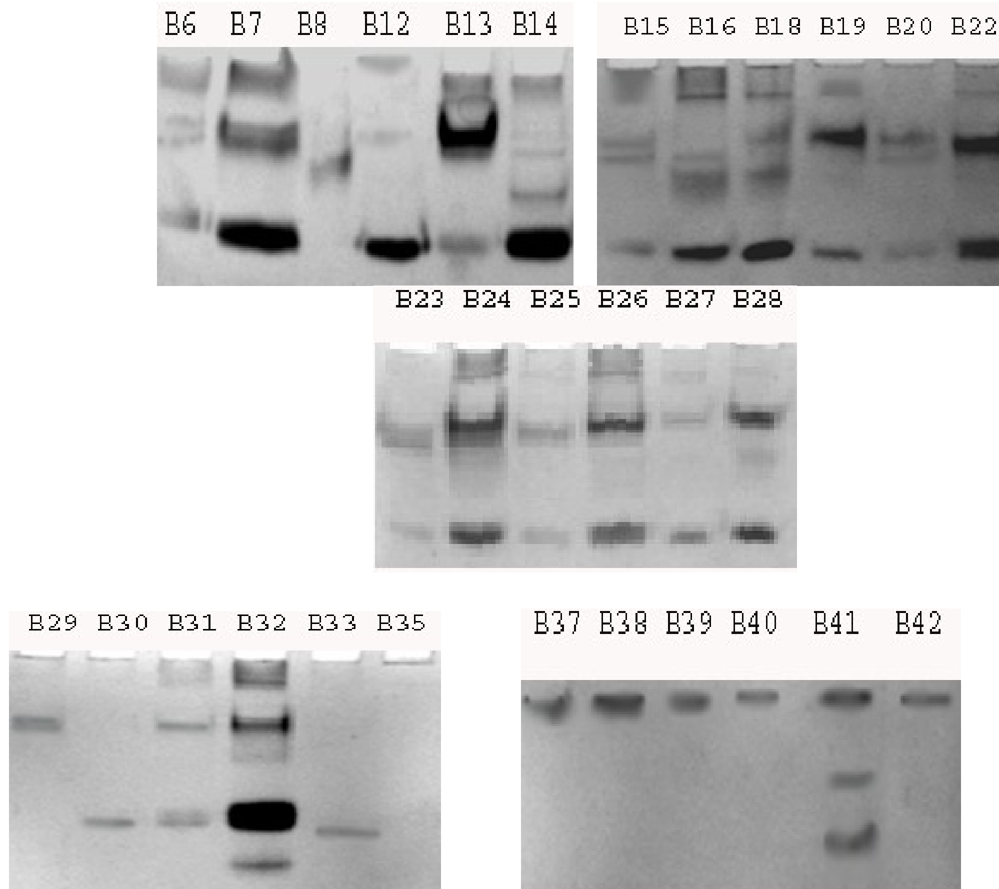
TABLE – 3 : ESTERASE ISOZYME BANDING PATTERN IN *BEAUVERIA* ISOLATES

Band number	Rf value	B6	B7	B8	B12	B13	B14	B15	B16	B18	B19	B20	B22	B23	B24	B25	B26	B27	B28	B29	B30	B31	B32	B33	B35	B37	B38	B39	B40	B41	B42
1	0.050									+			+		+		+	+	+							+	+	+	+	+	+
2	0.060		+			+	+															+	+								
3	0.070							+	+		+																				
4	0.080	+			+																										
5	0.110																						+								
6	0.120																+														
7	0.130						+																								
8	0.150	+	+			+									+			+	+	+			+								
9	0.160						+																								
10	0.169							+		+	+	+																			
11	0.170													+			+					+									
12	0.180	+	+		+	+																									
13	0.188												+																		
14	0.190																									+				+	
15	0.200							+	+			+			+	+															
16	0.210													+			+														
17	0.220														+								+								
18	0.240																		+												
19	0.250			+																											
20	0.260						+			+																					
21	0.280									+																					
22	0.300	+							+																					+	
23	0.380					+									+																
24	0.390		+																		+	+	+								
25	0.400						+							+																	
26	0.410							+					+		+	+	+	+	+												
27	0.416				+																										
28	0.430								+	+	+	+																			
39	0.440																							+							
30	0.490																						+								

TABLE – 4 : ACID PHOSPHATASE ISOZYME BANDING PATTERNS IN *BEAUVERIA* ISOLATES

Band number	Rf value	B6	B7	B8	B12	B13	B14	B15	B16	B18	B19	B20	B22	B23	B24	B25	B26	B27	B28	B29	B30	B31	B32	B33	B35	B37	B38	B39	B40	B41	B42
1	0.050	+													+											+					
2	0.060																		+								+	+	+		
3	0.070																			+		+	+	+							
4	0.080				+									+		+	+	+			+									+	
5	0.100					+			+	+		+																			
6	0.110		+				+																								
7	0.119							+																							
8	0.120			+																											
9	0.130										+		+																		
10	0.150				+																										
11	0.180																	+		+		+									
12	0.190																						+	+							
13	0.200													+	+	+	+		+		+					+	+	+	+		
14	0.210						+																							+	
15	0.250																			+											
16	0.270													+							+										
17	0.280															+			+			+	+	+		+	+	+	+		

Fig 1. Zymograms of esterase activity in *Beauveria* species



Isolate specific bands were produced by B15, B8, B12, and B29 isolates. Regarding band intensities among the isolates, isozyme band with less Rf value in each of the isolate was intensely stained and also showed a large colored zone where that particular isozyme was located except in B41 which recorded darkly stained isozyme bands with Rf values 0.08 and 0.21. Rest of the bands among the isolates with Rf values were more than 0.08 were lightly stained and also the zone of enzyme was narrow when compared to the broad and darkly stained isozymes with least Rf values in each isolate.

Cluster analysis: Isolates could be grouped into 7 main clusters (Fig. 4). B12, B27, B41 were paired with a similarity matrix of 67% and B23, B26 and B24, B37 were grouped with a similarity matrix of 35%. On the other hand B29, B30 and B31 groups showed a similarity matrix of 50%.

Fig . 2 Zymograms of Acid Phosphatase activity in *Beauveria* species

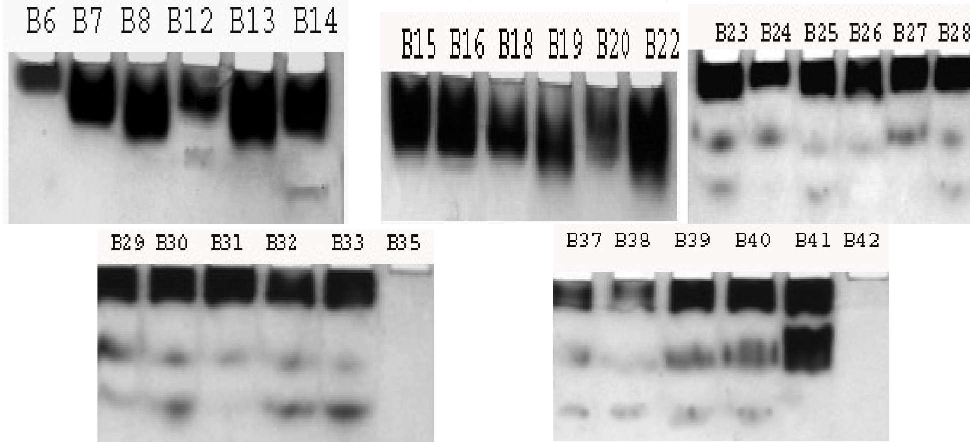


Fig 3. UPGMA dendrogram of esterase isozyme similarities between *Beauveria* species

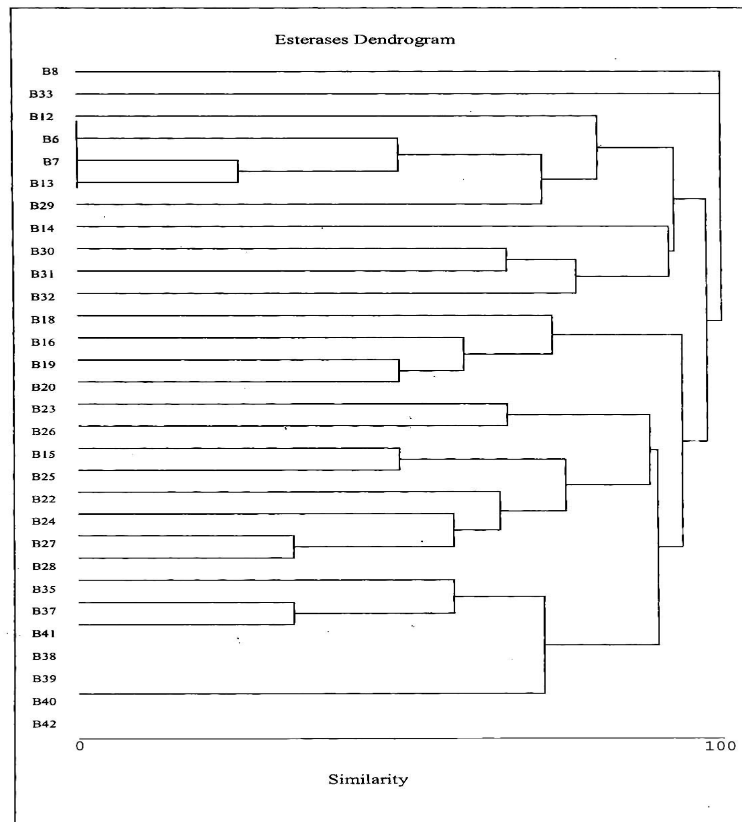
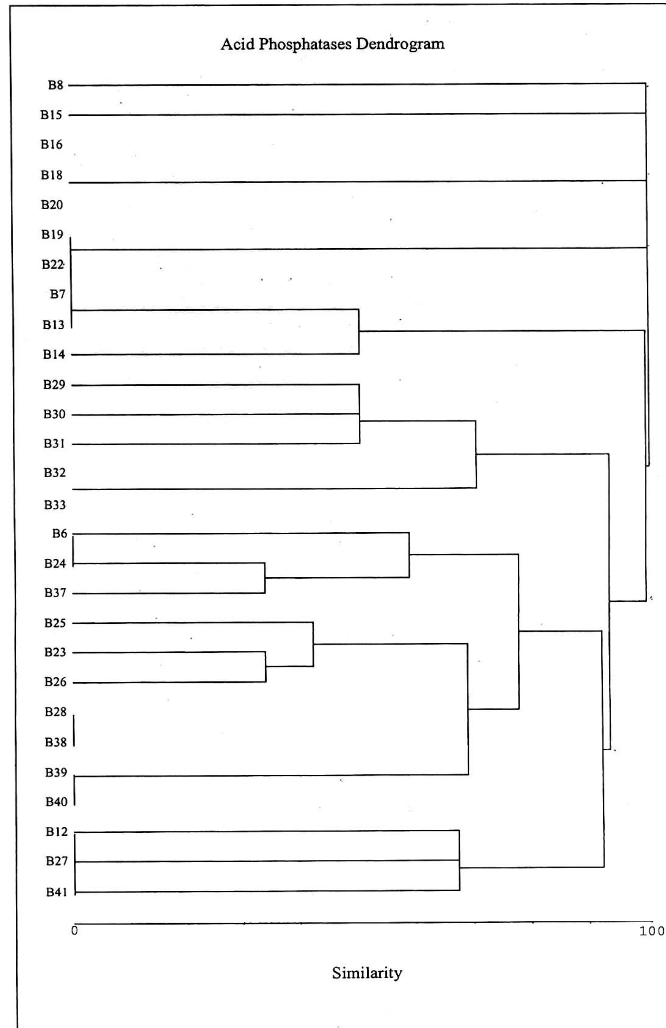


Fig. 4 UPGMA dendrogram of Acid Phosphatase isozyme similarities between *Beauveria* species



DISCUSSION

In view of the potential insecticidal properties of *B. bassiana,* initial selection of a truly aggressive isolate is of utmost importance for the success of a biocontrol program. Variation between isolates collected from different origin of place and host, necessitates the need for specific parameters for their identification. All isolates tested were able to infect *S. litura* larvae in the laboratory irrespective of host origin and place of collection. Therefore the differential susceptibility of larvae to different *Beauveria* isolates can be considered as being due to genetic difference among isolates. Depending on the categorization of isolates for LT50 values, B19, B22, B25 and B30 were more virulent towards 2nd instar *S. litura*. Isolates which depicted LT50 values in the range of 5-6 days were considered as virulent. According to Vitalis et al (2005) LT50 values of the most active isolates of *B. bassiana* for *Tetranychus evansi* varied between 4.6 and 5.8 days.

Denny et al (2005) also observed similar LT50 values for second instar larvae of *Delia radicum* Native PAGE of esterases showed differences in banding patterns among isolates ranging from a single band to multiple bands with a maximum of 6 bands. Results of the esterase and acid phosphatase isozyme analysis revealed distinct clusters for the low virulent isolate B8 and also for B33 a *B. brongniartii* species. B8 is a less virulent isolate with highest LT50 value for the polyphagous pest tested. Reineke and Zebitz (1996) observed a different zymogram pattern of isozymes for *B. brongniartii* isolates virulent to *M. melolontha.* Wu et al (1988) analysed isoesterases from 13 strains of *B. bassiana* isolated from different insect hosts from different geographic areas and found variations among the strains isolated from same host but different locations. On the other hand Alves et al (1984) found no correlation between zymograms for α esterases and the pathogenicity of *M. anisopliae* isolates to larvae of the sugarcane borer *D. saccharalis.* B19 and B22 the high virulent isolates against second instrar larvae of *S. litura* formed a separate cluster based on acid phosphatases isozyme banding pattern. Moreover, the banding pattern for acid phosphatases in all *Beauveria* isolates showed a single major band which corresponded to low Rf values hence high molecular weight though a slight variation in Rf values were recorded. Bridge et al (1993) also reported similar banding pattern for acid phosphatases in *M. ansiopliae.*

In conclusion intracellular esterase and acid phosphatase isozyme characterization revealed differences in the case of less virulent isolates where individual clusters were observed in B8 and B20 and species differentiation could be seen in B33 isolate as it was the only *brongniartii* species. Variable virulence of *Beauveria* spp. associated to *S. litura* correlated with the presence of genetic variations as observed through isozymes patterns.

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