Effect of Frequency of Subculturing of different Isolates of *Beauveria bassiana Vuillemin* on their Biological Properties

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Abstract

An experiment was conducted to find the effect of repeated subculturing on biological properties *viz.,* radial growth, sporulation and conidial viability of four strains (Bb-13, Bb-11, Bb-5A and Bb-N) and two local isolates (Bb-L-1 and Bb-L-2) of *Beauveria bassiana* Vuillemin on synthetic media. The results of the experiment showed no significant reduction of the biological properties during the initial 1st to 7th subculturing but showed significant negative effects on the biological properties after 7th subculturing. Molecular characterization of the strains using PCR based RAPD indicated considerable variability among the isolates.

1. Introduction

The success of entomopathogenic fungi in pest management depends on the use of highly virulent, stable and target specific strain. Unfortunately sometimes a potent strain after inclusion in the commercial formulation show poor performance under field conditions in due course of time which is most often attributed to the non-maintenance of the strain in laboratory conditions. The commercial strains generally undergo several conidium - conidium cycles during mass production. Some reports indicate a loss of pathogenicity in isolates of *Beauveria bassiana, Metarhizium anisopliae, Nomuraea rileyi, Paecilomyces farinosus* and *Verticillium lecanii* due to repeated subculturing of the strains under synthetic media (Kawakami, 1960; Schaerffenberg, 1964; Nagaich, 1973; Fargues and Roberts, 1983; Morrow *et al,* 1989). This could be due to loss in certain important biological properties of the fungi i.e., sporulation and production of viable conidia. Progressive attenuation of growth, sporulation and there by virulence was reported in most of the fungi with successive transfer to artificial medium, but the virility was regained after passage through the insect host.

The objective of the current study therefore was to assess effects of repeated *in vitro* passage on the biological properties of the different strains of *B.bassiana* so as to select a stable strain for commercial formulation.

2. Materials and Methods

2.1. Culture conditions and growth study

The present experiment was conducted in the Departmet of Entomology and AICRP on Biological control of crop pests and weeds, College of Agriculture, Rajendranagar, Hyderabad during 2004-07. Three isolates of *Beauveria bassiana* viz., Bb-13, Bb-11 and Bb-5A of Project Directorate of Biological Control (PDBC), Bangalore and a commercial isolate coded as Bb-N were obtained. All the four isolates were host passaged through *Spodoptera litura* and the fungus was recovered from the dead cadavers following single spore isolation procedure on Sabourauds dextrose agar medium with yeast extract (SDAY). Field infected larvae of *S. litura* showing the symptoms of *B. bassiana* infection with white muscardine growth over the body surface were collected from *rabi* groundnut and castor fields. Single spore isolation method was followed for sub culturing the growing fungus on fresh

plates of SDAY medium regularly till the pure cultures were obtained. The so obtained culture was then host passaged and pure culture was recovered which was used for the experimental purpose. The two local isolates obtained from two different localities were named as *B. bassiana* Local-1 (Bb-L-1) and *B. bassiana* Local-2 (Bb-L-2). The six isolates of *B. bassiana* were then studied for the impact of frequency of subculturing on biological properties of the fungal strain.

The pure isolates so obtained were subcultured regularly after every 10 days. The process of subculturing was done upto15 times and effect of subculturing on biological properties such as radial growth, conidia per unit area and viability of conidia, of the strain was studied from selected subcultures i.e. 1st, 3rd, 5th, 7th, 10th, 13th and 15th subcultures.

Circular discs of 10 mm diameter were cut from the vigorously grown cultures of different isolates using a sterile cork borer and were placed in the middle of each petriplate containing SDAY. The petriplates were incubated at 25 ± 1°C. The radial growth of the fungus was measured using a measuring scale after 14 days. Similarly conidia per unit area were calculated by cutting the circular discs of 10 mm diameter randomly from the two weeks old uniformly grown culture plates. Each disc was placed in a test tube containing 10 ml of distilled water. The spores present in the disc were allowed to disperse uniformly in the water by rotating the test tube on a vortex for one minute. The suspension was serially diluted and the spores were counted with the help of an improved Neubaur Haemocytometer under a compound microscope at 40 x magnification and number of spores present per ml was calculated (Aneja, 1996).

The conidia were harvested from the uniformly grown culture plates with the help of a fine brush into sterile distilled water and filtered through double layered muslin cloth. Approximately 500 µl of uniformly suspended spore solution was placed in the cavities of a cavity slide containing 100 µl of SDY medium. The slides were placed in a petriplate containing a moistened filter paper at its bottom and were incubated at a temperature of 25 ± 1°C and R.H. of 95 percent The slides were observed after 24 hours under microscope and number of conidia germinated and total number of conidia visible in any of the focused region was recorded.

The percent conidial viability was calculated using the formula-

$$G = \frac{N}{T} \times 100$$

Where G = Percent spore germination.
N = Number of spores germinated
T = Total number of spores observed.

Five replications with a sample size of 10 were fixed for all the treatments studied and the data was analyzed following completely Randomized Design.

2.2. DNA isolation from B. bassiana

The flask containing sterilized broth were inoculated with the fungal strains in aseptic conditions in the inoculation chamber. The flasks were later shifted to a shaker where the fungus was allowed to grow for 10 days at a set temperature of 25°C. After sufficient growth of the fungus, the flasks were removed from the shaker and the fungal biomass was filtered through a muslin cloth which was used for DNA extraction. The fungal material obtained was ground to a fine powder in the presence of liquid

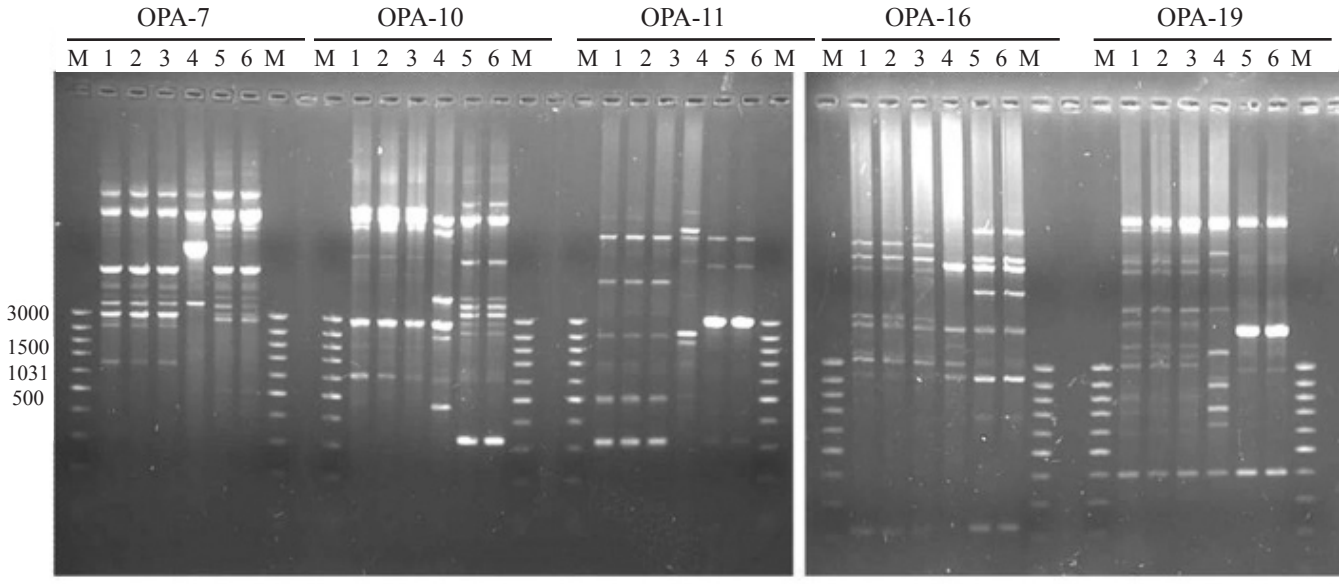

Plate 1: RAPD banding patterns of *B. bassiana* derived using primers (OPA-7, OPA-10, OPA-11, OPA-16 and OPA-19)

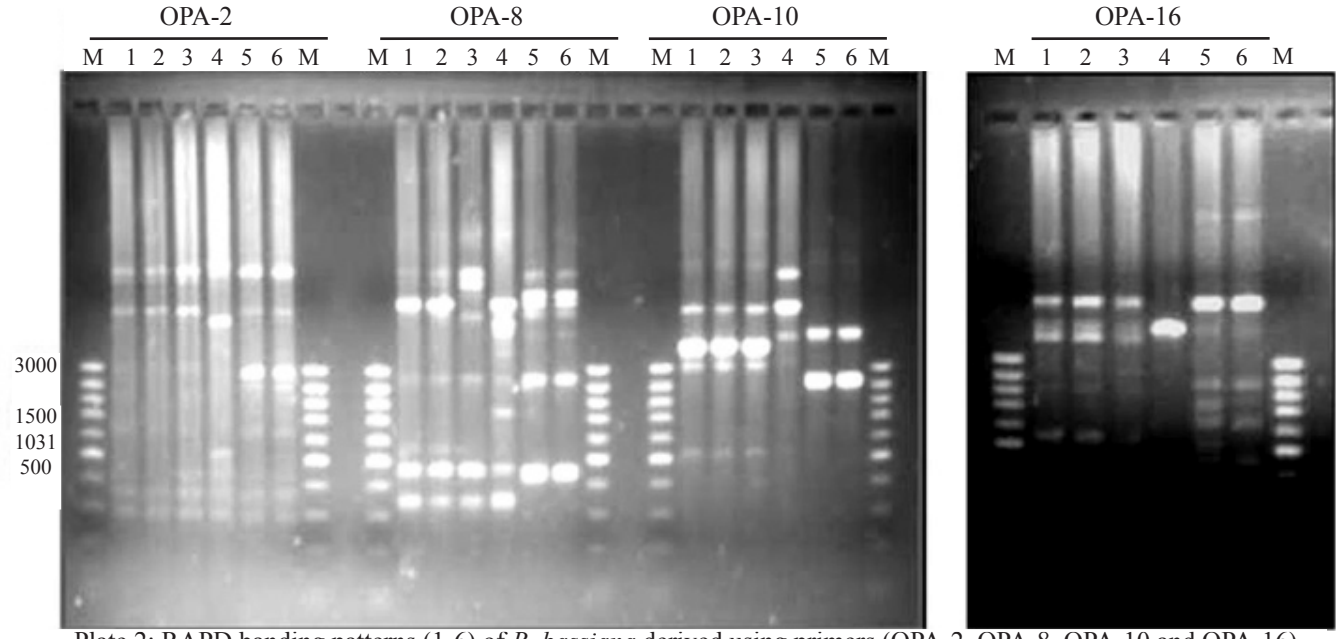

Plate 2: RAPD banding patterns (1-6) of *B. bassiana* derived using primers (OPA-2, OPA-8, OPA-10 and OPA-16)

nitrogen and DNA was extracted using the CTAB (Hexa- decy1 tri- methy1 ammonium bromide of Sigma Chemical Co., St. Louis, USA) method of Lee *et al.,* (1988). The extracted DNA was assessed for quality and quantity running Gel Electrophoresis apparatus (Biorad, USA).

2.3. Molecular characterization of B.bassiana by RAPD

Total forty primers of Operon Kit OPA (A and C) were screened in the present study of which only 9 (OPA-7, OPA-10, OPA-11, OPA-16, OPA-19, OPC-2, OPC-8, OPC-10, and OPC-16.) have shown the reproducibility of DNA bands. All the amplified DNA products were resolved by electrophoresis on agarose gel (1.4%) in TAE (1 X) buffer, stained with ethidium bromide and photographs were taken by using Gel Documentation System.

Gel photographs were scored for the presence '1' or absence '0' of bands of various molecular sizes. Binary matrices consisting of 0's (denoting absence of a given band) and 1's (denoting presence of a given band) were analyzed to obtain Jaccards coefficients among the isolates using NTSYS-pc (version 2.0; Exeter Biological Software, Setauket, NY). Jaccards coefficients were clustered to generate trees using the SAHN clustering program, selecting the unweighed pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc (Rohlf, 1993).

3. Results and Discussion

In vitro subculturing of different isolates of *B. bassiana* exhibited reduction in the biological properties *viz.,* radial growth, sporulation and conidial viability with increase in the frequency of subculturing (Tables 1-3). The strain Bb-5A exhibited no significant reduction in its biological properties *i.e* radial growth and sporulation up to seventh subculturing but recorded reduced radial growth from 5.60 cm to 5.46 cm and sporulation from 4.98 x 10^7 to 4.90 x 10^7, but during further subculturings the reduction in radial growth and sporulation was drastic. The conidial viability recorded significant reduction from fifth subculturing and the reduction continued with increase in frequency of subculturing. Significant reduction in the biological properties due to subculturing was observed in strain Bb-N which exhibited reduction in radial growth from 4.64 cm to 3.64 cm, conidial concentration from 4.675 x 10^7 conidia per 1cm diameter to 3.542 x 10^7 and conidial viability from 93.16 to 77.68 percent by the end of 15th subculturing.

The strain Bb-13 which exhibited no significant reduction in radial growth up to seventh subculturing recorded 4.44 cm, 4.18 cm and 4.04 cm significant reduction during 10th , 13th and 15th subculturing respectively. The conidial concentration with the pure cultures was 4.97 x 10^7 conidia per 1cm diameter but showed significant reduction from fifth subculturing and by the end of 15th subculturing it recorded 3.84 x 10^7 conidia per 1cm. The conidial viability too showed significant reduction from 91.88 percent with pure cultures to 74.08 percent by the end of 15th subculturing. Similar reduction in biological properties was also observed in strain Bb-11 where the radial growth recorded non significant reduction from 4.54 cm to 4.40 cm till seventh subculturing and there after recorded significant reduction up to 15th subculturing. The conidial concentration recorded significant reduction from 4.78 x 10^7 to 3.586 x 10^7 while the conidial viability was reduced from 93.42 to 70.12 percent.

Table 1: Effect of frequency of subculturing on the radial growth at 14 days interval in different isolates of *Beauveria bassiana*

Isolates	Radial growth (cm)								SEm ±	CD (p=0.01)
	Pure culture	Sc-1	Sc-3	Sc-5	Sc-7	Sc-10	Sc-13	Sc-15		
Bb-5A	5.60 ± 0.09^{a}	5.58 ± 0.05^{a}	5.58 ± 0.07^{a}	5.54 ± 0.05^{a}	5.46 ± 0.05^{a}	5.14 ± 0.02^{b}	4.88 ± 0.06^{c}	4.74 ± 0.05^{c}	0.052	0.150
Bb-N	4.64 ± 0.08^{a}	4.64 ± 0.08^{a}	4.66 ± 0.02^{a}	4.64 ± 0.02^{a}	4.58 ± 0.06^{a}	4.26 ± 0.04^{b}	3.88 ± 0.06^{c}	3.64 ± 0.06^{d}	0.046	0.133
Bb-13	4.88 ± 0.11^{a}	4.86 ± 0.04^{a}	4.86 ± 0.04^{a}	4.84 ± 0.07^{a}	4.72 ± 0.06^{a}	4.44 ± 0.05^{b}	4.18 ± 0.04^{c}	4.04 ± 0.05^{c}	0.050	0.147
Bb-11	4.54 ± 0.09^{a}	4.52 ± 0.04^{a}	4.46 ± 0.05^{a}	4.50 ± 0.05^{a}	4.40 ± 0.05^{a}	4.18 ± 0.04^{b}	3.92 ± 0.06^{c}	3.66 ± 0.04^{d}	0.048	0.141
Bb-L-1	3.90 ± 0.09^{a}	3.86 ± 0.08^{a}	3.88 ± 0.06^{a}	3.88 ± 0.04^{a}	3.80 ± 0.07^{a}	3.48 ± 0.04^{b}	3.22 ± 0.04^{c}	3.04 ± 0.04^{d}	0.055	0.159
Bb-L-2	4.22 ± 0.08^{a}	4.20 ± 0.05^{a}	4.18 ± 0.06^{a}	4.18 ± 0.05^{a}	4.08 ± 0.06^{a}	3.74 ± 0.06^{b}	3.56 ± 0.05^{c}	3.38 ± 0.05^{d}	0.055	0.159

Sc-Subculture; Figures indicated by same letters are not significantly different from one another as per DMRT

Table 2: Effect of frequency of subculturing on the conidial concentration at 14 days interval in different isolates of *Beauveria bassiana*

Isolates	Conidial concentration per 1 cm diameter x 10^7								SEm ±	CD (p=0.01)
	Pure culture	Sc-1	Sc-3	Sc-5	Sc-7	Sc-10	Sc-13	Sc-15		
Bb-5A	4.98 ± 0.05^{a}	4.94 ± 0.03^{a}	4.91 ± 0.03^{a}	4.88 ± 0.06^{a}	4.90 ± 0.05^{a}	4.27 ± 0.06^{b}	4.03 ± 0.04^{c}	3.86 ± 0.02^{d}	0.044	0.130
Bb-N	4.67 ± 0.06^{a}	4.60 ± 0.05^{a}	4.51 ± 0.04^{a}	4.62 ± 0.06^{a}	4.19 ± 0.04^{b}	3.98 ± 0.09^{c}	3.76 ± 0.04^{d}	3.54 ± 0.05^{e}	0.054	0.160
Bb-13	4.97 ± 0.07^{a}	4.88 ± 0.045^{ab}	4.90 ± 0.04^{a}	4.62 ± 0.05^{c}	4.71± 0.08^{bc}	4.20 ± 0.11^{d}	4.02 ± 0.01^{de}	3.84 ± 0.03^{e}	0.062	0.180
Bb-11	4.78 ± 0.17^{a}	4.61 ± 0.04^{a}	4.29 ± 0.04^{c}	4.41± 0.03^{bc}	4.13 ± 0.05^{d}	3.93 ± 0.05^{e}	3.75 ± 0.05^{f}	3.59 ± 0.05^{g}	0.044	0.129
Bb-L-1	4.04 ± 0.07^{a}	3.95 ± 0.07^{a}	3.64± 0.07^{bc}	3.70 ± 0.07^{b}	3.55 ± 0.03^{c}	3.34 ± 0.03^{d}	3.13 ± 0.02^{e}	2.88 ± 0.02^{f}	0.050	0.146
Bb-L-2	4.16 ± 0.09^{a}	3.98 ± 0.05^{ab}	4.08 ± 0.03^{a}	3.70 ± 0.07^{c}	3.85 ± 0.04^{b}	3.50 ± 0.05^{d}	3.26 ± 0.03^{e}	3.00 ± 0.03^{f}	0.047	0.138

Sc-Subculture; Figures indicated by same letters are not significantly different from one another as per DMRT

Significantly high reduction in biological properties due to subculturings was observed in local isolates Bb-L-1 and Bb-L-2 where the radial growth of isolate Bb-L-1 was reduced from 3.90 cm to 3.04 cm, while the reduction in isolate Bb-L-2 was from 4.22 cm to 3.38 cm. The conidial concentration in the isolates, Bb-L-1 and Bb-L-2 was reduced from 4.04 x 10^7 to 2.88 x 10^7 and 4.16 x 10^7 to 3.00 x 10^7 respectively, but their conidial viability got reduced from 89.64 to 70.06 and 90.21 to 71.40 percent respectively.

The strains though recorded reduction in radial growth patterns, during initial subculturings (Sc-1 to Sc-7) but were not significantly different from the pure cultures but, recorded a drastic and significant reduction during later subculturings (Sc-10 to Sc-15). Similarly the strains recorded low but non significant reduction in conidial concentration and conidial viability up to fifth subculturing but recorded significant reduction during later subculturings (Sc-7 to Sc-15). It can be concluded form the results that repeated subculturing on

Table 3: Effect of frequency of subculturing on the conidial viability at 14 days interval in different isolates of *Beauveria bassiana*

Isolates	Conidial viability (%)								SEm ±	CD (p=0.01)
	Pure culture	Sc-1	Sc-3	Sc-5	Sc-7	Sc-10	Sc-13	Sc-15		
Bb-5A	94.65 ± 0.46[a] (76.64 ± 0.59)	92.93 ± 0.24[a] (74.56 ± 0.27)	93.60 ± 0.41[a] (75.34 ± 0.48)	91.06 ± 0.17[b] (72.58 ± 0.173)	88.26 ± 0.27[c] (69.94 ± 0.24)	88.60 ± 0.31[c] (70.24 ± 0.28)	83.42 ± 0.31[d] (65.95 ± 0.24)	81.27 ± 0.26[e] (64.33 ± 0.19)	0.290	0.844
Bb-N	93.16 ± 0.85[a] (74.92 ± 0.97)	92.22 ± 0.42[a] (73.80 ± 0.45)	90.10 ± 0.46[b] (71.65 ± 0.45)	91.18 ± 0.52[ab] (72.72 ± 0.54)	85.06 ± 0.16[c] (67.24 ± 0.13)	83.12 ± 0.35[d] (65.72 ± 0.27)	81.36 ± 0.31[e] (64.40 ± 0.23)	77.68 ± 0.34[f] (61.78 ± 0.23)	0.383	1.114
Bb-13	91.88 ± 0.39[a] (73.44 ± 0.40)	90.24 ± 1.13[a] (71.88 ± 1.10)	88.02 ± 0.51[bc] (69.74 ± .450)	89.68 ± 0.35[ab] (71.24 ± 0.34)	86.32 ± 0.46[c] (68.28 ± 0.38)	88.16 ± 0.50[b] (69.86 ± 0.45)	80.10 ± 0.34[d] (63.48 ± 0.24)	74.08 ±0.46[e] (59.37 ± 0.30)	0.592	1.723
Bb-11	93.42 ± 0.53[a] (75.15 ± 0.60)	92.33 ± 0.30[a] (73.90 ± 0.32)	90.17 ± 0.24[b] (71.70 ± 0.23)	86.56 ± 0.17[c] (68.47 ± 0.14)	83.22 ± 0.47[d] (65.80 ± 0.36)	79.32 ± 0.33[e] (62.93 ± 0.24)	74.05 ± 0.14[f] (59.35 ± 0.09)	70.12 ± 0.21[g] (56.84 ± 0.13)	0.284	0.828
Bb-L-1	89.64 ± 0.68[a] (71.23 ± 0.64)	86.02 ± 0.51[b] (68.03 ± 0.43)	88.24 ± 0.35[a] (69.93 ± 0.32)	84.18 ± 0.35[c] (66.54 ± 0.28)	80.10 ± 0.34[d] (63.48 ± 0.25)	76.36 ± 0.20[e] (60.88 ± 0.14)	72.48 ± 0.22[f] (58.34 ± 0.14)	70.06 ± 0.24[g] (56.80 ± 0.15)	0.332	0.967
Bb-L-2	90.21 ± 0.69[a] (71.78 ± 0.66)	89.16 ± 0.76[a] (70.79 ± 0.71)	86.04 ± 0.33[b] (68.04 ± 0.27)	84.24 ± 0.36[c] (66.59 ± 0.28)	81.04 ± 0.37[d] (64.16 ± 0.27)	78.32 ± 0.37[e] (62.23 ± 0.26)	73.66 ± 0.44[f] (59.10 ± 0.29)	71.40 ± 0.36[g] (57.65 ± 0.23)	0.450	1.311

Sc-Subculture; Figures in parentheses are angular transformed values; Figures indicated by same letters are not significantly different from one another as per DMRT

synthetic media can deteriorate the biological properties of the fungal strains.

Decrease in growth, sporulation and viability was reported in certain entomopathogenic fungi when repeatedly cultured on artificial media (Kawakami, 1960; Evlakhova, 1966; Hall, 1980, Prasad 1989; Dayakar and Kanaujia, 2004). The findings of the present study are in accordance with these earlier reports.

The dendogram (Figure 1) obtained after RAPD fingerprinting to find out the genetical differences between the strains and to correlate changes in the pathogenicity of the strains due to subculturing of *B. bassiana* strains showed that there was distinct homogenous population within the very diverse species of *B. bassiana*. The strain Bb-5A was found genetically distinct from rest of the five strains. (Fig-1) The superiority of the strain Bb-5A over others in its pathogenicity and also its stability during subculturing which was exhibited by it during the study may be due to its high genetic divergence compared to other strains.

Though all the six strains shared about 43 percent genetic similarity as a whole but the strains Bb-13, Bb-11 and Bb-N were found to be genetically identical. The local isolates Bb-L-1 and Bb-L-2 too exhibited 100 percent genetic similarity, clustering of Bb-13, Bb-11 and Bb-N in a single group with no genetic difference, in spite of significant differences shown by these strains phenotypically in their biological properties may be due to the adoption of a single molecular technique (RAPD) with a limited number of primers, which might have classified these strains broadly into a single group. Clustering of local isolates Bb-L-1 and Bb-L-2 in a single group may be due to the isolation of the two isolates from a similar geographic region. Distinction of *Beauveria* isolates has always been problematic

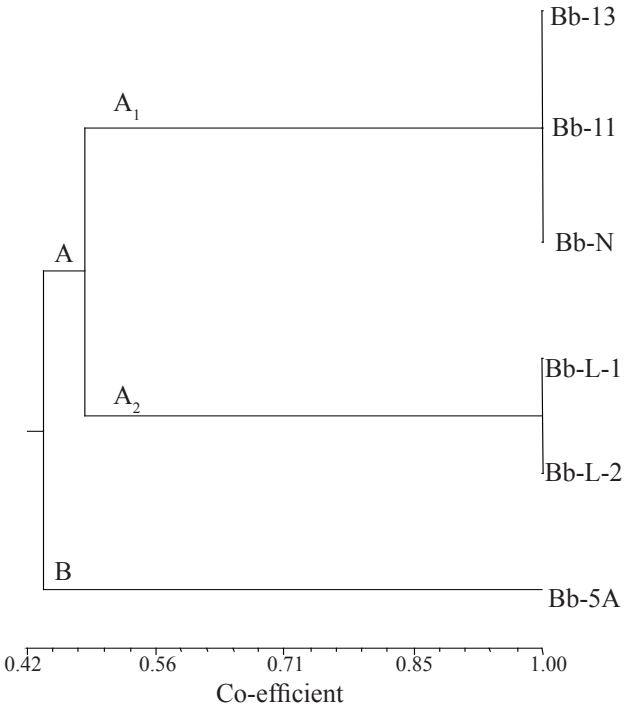

Figure 1: Dendogram based on RAPD banding pattern of B. bassiana strains/ isolates with nine random primers

possibly due to its high genetic diversity, hence different methods should be used for establishing genetic diversity between the isolates which include use of morphological traits, biochemical products, isoenzyme patterns, RAPD, RFLP, DNA probes, rDNA analysis, specific primers and SSCP analysis etc. Use of more than one method will certainly reduce the anomaly and also gives accurate results in identifying the genetic divergence of the strains. In the present study though all the strains exhibited reduction in the biological properties with an increase in subculturing frequency, the reduction was less expressed by strains *viz.,* Bb-5A and Bb-N while it was more in others such as Bb-L-1 and Bb-L-2. The expression of less variability in the biological properties between initial pure culture and final subculture for a given strain can be attributed to its high genetic stability which is further proved in the molecular characterization where the strain Bb-5A was found to be distinct from rest of the strains or local isolates and hence, the strains Bb-5A can be designated as stable and can be used for mass multiplication.

4. Conclusion

The results of the present study indicate that, of the six isolates tested the isolate Bb-5A is more stable as the deterioration of the biological properties of the isolate is comparatively low during repeated sub-culturing on synthetic media and hence it can be best employed for pest management by mass production. However, during commercialization care should be ensured to regain the lost or deteriorated biological properties of the isolate by host passaging it at regular intervals.

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