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Research article

CHANGES IN PROTEIN PROFILES OF *BEAUVERIA* SPECIES UNDER *IN VITRO* ABIOTIC STRESS

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ABSTRACT: Exposure of *Beauveria* isolates to a set of abiotic factors such as temperature, organophosphorous pesticide and benzimidazole fungicide lead to the expression of several stress related proteins under unfavorable conditions. Novel protein bands corresponding to low and high molecular weight categories were recorded in the isolates exposed to the three abiotic stress factors. The SDS – PAGE protein profiles in the negative controls differed from positive controls in the absence of some of the protein bands. Selected isolates of *Beauveria* from the present study displayed similar banding pattern in response to exposure to a given stress factor. It is assumed that the synthesis of stress proteins is intended for their survival and adaptation to adverse conditions.
Key words: *Beauveria*, Total soluble proteins, Abiotic stress

INTRODUCTION

The entomopathogenic fungus *Beauveria* is an important biocontrol agent of lepidopteran crop insect pests and is being used as an alternative means to minimize the use of synthetic pesticides owing for a safe and sustainable agriculture. Innundative strategies require favorable environmental conditions for the interaction between host and pathogen in many intricate ways. However, major constraints for commercialization and large scale application of biocontrol agents are the abiotic stress factors prevailing in agriclimates. Therefore, evaluation of fungal pathogens for tolerance to abiotic stimulons is a prerequisite for employing potential isolates in practical biocontrol programs. Compatibility studies of *Beauveria bassiana* isolates with different pesticides and fungicides [1,2,3] and to different temperatures are well documented[4]. Expression of heat shock proteins exposed at high temperatures are reported in *Phycomyces blakesleeanus* [5], compatibility studies involving *Metarhizium anisopliae* with organosphosphorous pesticides[6] resistance to thiabendazole fungicide in *Giberella pulicaris* [7] and Benzimidazole resistance in *Trichoderma virens* [8] are some of the studies reported. Understanding the biotic stress induced by entomopathogen towards host and elucidating the mechanisms involved in infection and pathogenesis of fungus are well documented [9]. But equally important is the understanding biochemical mechanisms involved in the abiotic stress and tolerance to the stress stimuli experienced by the fungal agent itself in the field conditions. Stress responses are of particular importance for the field efficacy of a biocontrol agent as the crops are subjected to fluctuations in temperatures and exposure to the chemicals used for crop protection. In view of immense commercial potential of *Beauveria* as biopesticide, understanding the response of these fungi to the prevailing environmental stress factors is imperative. The present work was under taken to fill the lacunae and for elucidating the biochemical basis for abiotic stress tolerance in *Beauveria* isolates at the protein expression level, using SDS-PAGE analysis. Objective of the study was to analyze the stress induced proteins in fungal isolates exposed to abiotic stress in comparison with positive and negative controls in order to understand the nature of proteins expressed in response to abiotic stress factors. Outcome of the study would enable effective fungal isolate selection based on biochemical markers for abiotic stress tolerance in *Beauveria* species.

MATERIALS AND METHODS

Fungal Cultures

Four isolates of *B.bassiana* used in the present study were our collections from the crop fields of Andhra Pradesh, India. The isolates were maintained on SDAY medium at 25ºC. *B. bassiana* isolate B7 was an USDA-ARS collection (AccNo.2427) and isolate B12 was obtained from EMBRAPA Brazil (Acc No.CG151). B33 was the only isolate belonging to the species *B. brongniartii*, provided by Richard Humber (USDA-ARS AccNo.2660).

Selection of *Beauveria* isolates for the present study

Selection was done basing on *in vitro* compatibility of *Beauveria* isolates for tolerance to temperature, organophosphorous pesticide (dichlorvos) and benzimidazol fungicide (bavistin) [10]. B7 and B19 isolates could be grown at 33ºC and hence selected for studying temperature stress effect. B29 and B33 isolates of *Beauveria*, which showed tolerance to 1x concentration i.e. recommended field dosage of organophosphorous pesticide, were selected for examining pesticide stress response. B12 and B32 isolates, which showed growth at 0.5x i.e. half the recommended field dosage of bavistin amended medium were selected for studying fungicide, induced stress. The isolates denoted with * mark are abiotic stress exposed *Beauveria* isolates. Isolates without * marks were grown at normal conditions and therefore mentioned as positive controls. B8 and B20 were randomly chosen as negative controls since they failed to grow under all abiotic stress conditions.

Culture Conditions

For abiotic stress evaluation, fungal cultures were raised by inoculating 2 ml of conidial suspensions at 10^8/ml concentration in SD broth (4% dextrose, 1% peptone and 1% yeast). Temperature stress experiments were conducted in an incubator shaker at 33ºC. For pesticide and fungicide stress experiments, the test isolates were inoculated in pesticide/fungicide amended medium and incubated at 25ºC. Dichlorvos at 1x concentration (1.3ml/lit) and Bavistin at 0.5x concentration (1gm/lit) were used in the amendment of medium. Isolates, which were selected as negative and positive controls, were included for incubation at 25ºC in SD broth without pesticide/ fungicide. All the isolates under study were incubated in an orbital shaker for 5 days at 200rpm. On completion of 5 days, the mycelia were harvested and washed with double distilled water twice to remove traces of culture medium.

Extraction of Total Soluble Mycelial Proteins

Mycelium was ground to a fine powder using liquid nitrogen in mortar and pestle, transferred to eppendorf centrifugation tubes after adding protein extraction buffer (0.1M Phosphate buffer pH 7.2). The homogenate was centrifuged at 10,000 rpm for 20 min at 4ºC in superspin (R-V/FM-Plastocrafts) cooling centrifuge. The supernatant containing the soluble proteins was transferred to sterilized eppendorf tubes and stored at -30ºC for further usage.

SDS-PAGE

The total soluble protein profiles of the isolates subjected to above mentioned abiotic stress as well as negative and positive controls were analyzed using SDS Polyacrylamide gel electrophoresis. Protein samples were treated with loading buffer (0.125M Tris pH6.8, 20% glycerol, 2% SDS and 14.4mM β-mercaptoethanol) for 3 minutes in a boiling water bath at 100°C. The samples were cooled to ambient temperature and 70µl of each protein samples were loaded on Tris-glycine gels (5% for stacking gel and 15% for resolving gel). For performing electrophoresis, Biotech vertical gel electrophoresis unit was applied with 50/100 volts of current for stacking and separating gels respectively. Gels were stained with 0.25% coomassie blue (0.25gms G250, 10ml acetic acid, 45ml methanol and 45 ml double distilled water) and visualized in the same solution excluding G250. Gels were photographed and scored for protein bands using Vilbert Lourmat Gel documentation system.

RESULTS AND DISCUSSION

RESULTS

Protein profiles of negative controls (B8 and B20)

The two isolates which were set as negative controls produced protein bands of MWs ranging from 95.16 to 29.13 KDa. Similar bands of 66.2, 56.8, 45.74, 43.73 and 40.23 KDa MWs (molecular weights) were recorded in both the fungal isolates indicating activity of apparently similar proteins in response to the three abiotic stress conditions and thereby they might have failed to grow under the given stress (Fig. 1). Specific bands with MWs of 95.16, 80.54, 53.30, 35.91, 31.99 and 29.13 KDa were expressed in B8 whereas B20 expressed unique bands of 84.72, 49.50 and 33.92 KDa (Tab. 1)

Protein profiles in *Beauveria* isolates under temperature stress

Isolates exposed to temperature stress, induced several stress proteins which were prominent in both test isolates. Specific bands of 118, 110, 82, 72, 65, 62, 55 and 51 KDa were scored by B7* (Fig. 2). Whereas, protein bands of 114, 105, 102, 96, 91, 88, 80 and 54 KDa were observed in B19* (Tab. 2). Altogether 23 temperature stress induced proteins were observed in both the isolates. Out of which, 13 were scored in B7* with MWs ranging from 118 to 45 KDa when compared to control. Eleven temperature stress induced proteins were synthesized by B19* with MWs ranging from 114 to 54 KDa. Unique bands with MWs 71, 53 and 50 KDa could be observed in the positive controls (B7 and B19) owing to genetic similarity of the isolates which could be related with tolerance to temperature stress.

Protein profiles in *Beauveria* isolates induced by pesticide stress

Synthesis of pesticide stress induced proteins in isolates exposed to organophosphorous pesticide stress was evident. These proteins had molecular weights in the range of 155.8 to 27.81 KDa (Fig. 3). A total of 11 pesticide induced proteins were synthesized in the test fungal isolates when compared to their corresponding controls. Four isolate specific protein bands with MWs 135.9, 123.1, 76.57 and 36.33 KDa were synthesized in B29* isolate. B33*also produced specific bands with MWs 144.4, and 31.99 27.81 KDa. (Tab. 3). Positive controls showed two common bands with MWs 42.22 and 37.32. B29 produced 9 bands where as B29* produced 12 bands out of which 3 bands of MWs 54.81, 47.59 and 30.85 KDa were common. The most important feature was that the protein bands in the pesticide treated isolates were intensely stained with coomassie blue, when compared to the controls. This indicates over expression of proteins under stress conditions in the stress exposed isolates. Contrarily, intensities of protein bands in both the negative controls were light when compared to rest of the isolates.

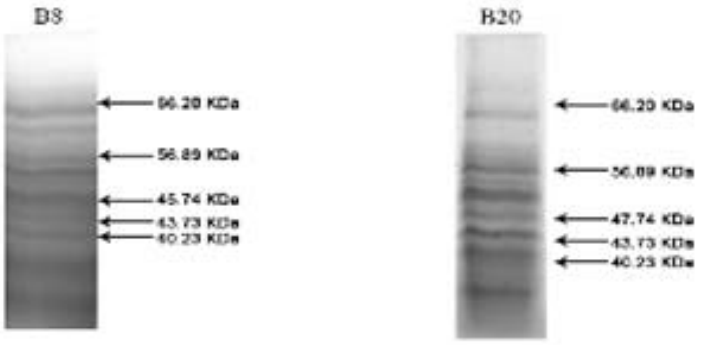

Fig 1. Protein profiles in negative controls (B8 and B20)

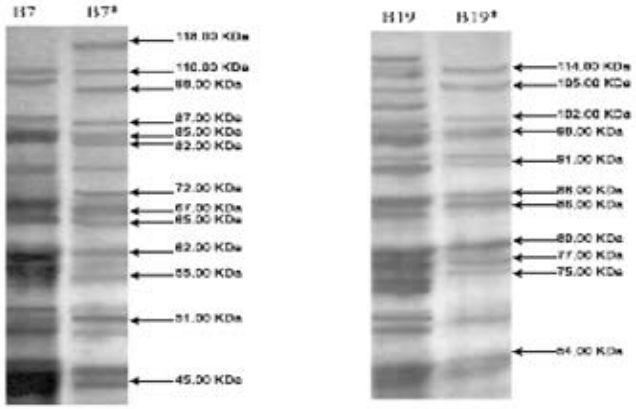

Fig 2. Protein profiles in positive controls (B7 & B19) and temperature stress exposed isolates (B7*&B19*) of *Beauveria*

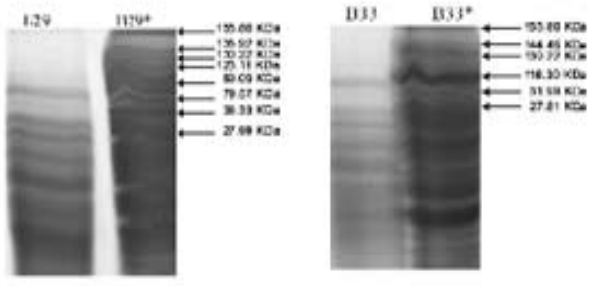

Fig 3. Protein profiles in positive controls (B29&B33) and pesticide stress induced isolates (B29*&B33*) of *Beauveria*

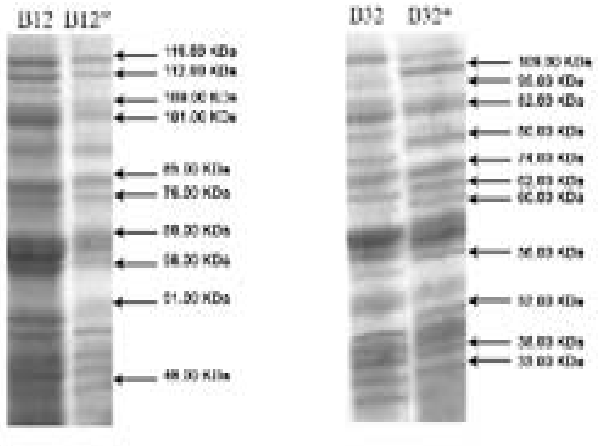

Fig 4. Protein profiles in positive controls (B12&B32) and fungicide stress induced isolates (B12*&B32) of *Beauveria*

Table -1 Protein profiles in negative controls which are sensitive to abiotic stresses

Band numbers	MW in KDa	B8	B20
1	95.16	+	
2	89.09		
3	84.72		+
4	83.31		
5	80.54	+	
6	76.57		
7	66.20	+	+
8	63.97		
9	60.66		
10	57.42		
11	56.89	+	+
12	54.81		
13	53.30	+	
14	49.50		+
15	47.59		
16	45.74	+	+
17	43.73	+	+
18	42.22		
19	40.23	+	+
20	39.00		+
21	37.32		
22	36.33		
23	35.91	+	
24	35.68		
25	33.92		+
26	33.06		
27	31.99	+	
28	31.78		
29	30.85		
30	29.13	+	

Table–2: Protein profiles in temperature tolerant isolates in relation to the positive controls

Band numbers	MW in KDa	B7	B7*	B19	B19*
1	118.0		+		
2	116.0			+	
3	115.0			+	
4	114.0				+
5	113.0	+	+	+	+
6	111.0	+			
7	110.0		+		
8	107.0	+			
9	106.0			+	
10	105.0				+
12	103.0	+	+	+	
13	102.0				+
14	101.0			+	
15	100.0	+		+	
16	98.00		+		+
17	97.00			+	
18	96.00				+
19	95.00			+	
20	93.00	+			
21	91.00				+
22	88.00				+
23	87.00		+	+	
24	86.00	+			+
25	85.00		+	+	
26	83.00	+		+	
27	82.00		+		
28	80.00				+
29	78.00			+	
30	77.00	+	+		+
31	76.00			+	
32	75.00	+	+		+
33	74.00	+			
34	73.00			+	
35	72.00		+		
36	71.00	+		+	
37	67.00		+	+	
38	66.00	+		+	+
39	65.00		+		
40	64.00	+		+	
41	62.00		+		
42	61.00	+			
43	56.00	+		+	+
44	55.00		+		
45	54.00				+
46	53.00	+		+	
47	52.00				
48	51.00		+		
49	50.00	+		+	
50	47.00				
51	45.00		+	+	
52	44.00				
53	38.00			+	

B7* and B19* subjected to thermal stress at 33º C.
B7 and B19 are the positive controls grown at 25º C.

Table–3: Protein profiles in pesticide tolerant isolates in relation to the positive controls

Band numbers	MW in KDa	B29	B29*	B33	B33*
1	155.8		+		+
2	144.4				+
3	135.9		+		
4	130.2		+		+
5	123.1		+		
6	116.3		+		+
7	95.16				
8	89.09		+	+	+
9	84.72				
10	83.31	+			
11	80.54				
12	76.57		+		
13	66.20				
14	63.97			+	+
15	60.66	+			
16	57.42			+	+
17	56.89				
18	54.81	+	+		
19	53.30				
20	49.50			+	+
21	47.59	+	+		
22	45.74			+	+
23	43.73				
24	42.22	+		+	+
25	40.23				
26	39.00				
27	37.32	+		+	+
28	36.33		+		
29	35.91				
30	35.68	+			
31	33.92			+	
32	33.06				
33	31.99				+
34	31.78				
35	30.85	+	+	+	+
36	29.13	+			
37	27.99		+	+	
38	27.81				+

B29* and B33* subjected to pesticides stress.
B29 and B33 are the positive controls grown in control medium.

Protein profiles in *Beauveria* isolates induced by fungicide stress

The protein profiles of fungicide stress exposed isolates showed the induction of 14 proteins with MWs ranging from 119 to 33 KDa (Fig. 4). Four unique bands of MWs 119, 109, 101, and 85.5 KDa were observed in B12*. In case of B32*, six specific bands were manifested with MWs 80, 62, 60, 52, 38 and 33 KDa (Tab. 4). Synthesis of similar protein bands by the test isolates were observed with MWs 109, 58 and 56 KDa. There were no common bands but specific bands were observed in positive controls.

Table–4: Protein profiles in fungicide tolerant isolates in relation to the positive controls

Band numbers	MW in KDa	B12	B12*	B32	B32*
1	124.0			+	+
2	120.0			+	+
3	119.0		+		
4	116.0	+			
5	115.0	+	+		
6	112.0		+	+	+
7	110.0	+			
8	109.0		+		+
9	106.0	+		+	+
10	104.0			+	
11	101.0		+		
12	100.0	+	+	+	+
13	99.00	+			
14	98.00			+	
15	95.00	+	+		+
16	92.00			+	
17	90.00			+	+
18	88.00	+	+		
19	85.50		+		
20	85.00	+		+	+
21	84.00			+	
22	82.00	+			+
23	80.00				+
24	78.00				
25	77.00				
26	76.50		+	+	+
27	76.00	+			
28	75.00			+	
29	74.00	+	+		+
30	72.00			+	
31	70.00				
32	68.00				
33	67.00			+	+
34	66.00				
35	65.00			+	
36	64.00	+	+		
37	62.00				+
38	61.00	+	+	+	
39	60.00				+
40	58.00		+		+
41	57.00	+	+	+	
42	56.00		+		+
43	54.00	+			
44	52.00				+
45	51.00		+	+	
46	50.00	+			
47	49.00				
48	48.00		+	+	+
49	38.00				+
50	36.00	+			
51	33.00				+

B12* and B32* subjected to fungicide stress.

B12 and B32 are the positive controls grown in control medium.

DISCUSSION

We report protein profiles of *B.bassiana* and *B.brongniartii* species induced by the three abiotic stress factors and analyzed in comparison with the positive and negative controls for the first time. Under temperature, pesticide and fungicide stress conditions some of the proteins that were expressed in controls appear to be missing and some proteins manifested in response to the altered ambience. This could probably be due to modifications of some of the proteins in accordance with the changed abiotic conditions. In case of temperature induced proteins, our results indicated the synthesis of HSPs (heat shock proteins) with MWs ranging from 118KDa to 45KDa at 33ºC. Reports suggests that at 40ºC, the thermotolerant fungi could synthesize HSPs mainly with medium and high molecular weights. It was also observed that there was synthesis of low molecular weight HSPs at 50ºC and the suppression of high molecular weight HSPs [11]. Synthesis of sixteen HSPs with molecular weights ranging from 90.2KDa to 22KDa in *B.bassiana* isolate GK2016 were observed when exposed to a temperature of 45ºC for 1 hr [12]. It is likely that the genotypic response to a given temperature vary depending upon the isolate used and hence the difference in the type of HSPs produced.

HSP synthesis in *Fusarium oxysporum* after 10 mins of heat shock treatment at 40ºC [13] and synthesis of HSPs which peaked at 60 min after heat shock treatment at 45ºC in *Neurospora* [14] and synthesis of HSP 100 in *Phycomyces blakesleeanus* when exposed to heat shock of 30 mins at 34ºC and 42ºC were observed [5]. In our study, *Beauveria* isolates were exposed to a temperature stress at 33ºC for a constant period of 5 days so as to study the temperature effect on protein synthesis in order to understand the basis for *in vitro* temperature tolerance. In accordance to our findings, stress protein response persisted during heat stress in mussels where HSP 70 and 60 remained high over 8 weeks of temperature stress [15]. However, kinetics of stress protein induction and persistence has been found to vary with the stressor involved. Induction of stress proteins are generally slower but persist for longer, during exposure to chemical and thermal stress.

Protein profiles induced by pesticide and fungicide stress displayed wide range of variations in molecular weights ranging from 119KDa to 33KDa. Accumulation of oxidatively modified proteins in some of the filamentous fungi when exposed to Paraquat were recorded [16] and according to observation made elsewhere [17] a large number of stress genes might have organized themselves together in a cluster in the form of a stress gene super family which act and function in harmony as per the needs of the organism. Though the nature of stress inducers are different in terms of their physical and chemical nature, the biological reactions they induce may be similar. That is to say that the stress genes can be induced by altogether different and varied nature of stress inducers or stressors. Stress induced proteins probably increase resistance to potentially deleterious chemicals by stabilizing or renaturing cell proteins, and some proteins synthesized in the oxidative stress response degrade the toxic oxygen radicals generated in reactions between compounds that induce the stress and cellular components [18]. A deliberate introduction of minutest amount of stress might sensitize the host, altering it to switch on its "stress genes" thus rendering protection against stressors.

We observed that some of the selected isolates of *B. bassiana* and *B. brongniartii* responded to unfavorable conditions through rapid expression of stress induced proteins. However, response to all the three stress factors apparently generated some high molecular weight proteins which were absent in the corresponding positive controls. Some of the novel protein bands that appeared under stress conditions might correspond to over expressed version of some of the bands that were present in positive controls. While few other bands which were prominently stained dark and those of high molecular weight suggest induction of same under stress conditions. Investigating protein profiles in isolates exposed to various abiotic stress factors enables in ascertaining the genetic relationship among isolates, since the mycelia proteins are the products of a number of genes present in respective genomes, and hence their analysis helps in revealing genetic relationship and similar response and tolerance to abiotic stress.

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