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Defensive Role of Antioxidant Enzymes Peroxidase, Esterase and Catalase in *Beauveria* Species under Abiotic Stress Stimuli

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Abstract: *Beauveria* isolates which manifested endurance to a set of abiotic stress stimuli were selected for understanding the changes in antioxidant enzymatic profiles under *in vitro* conditions. Three antioxidant enzymes i.e., peroxidase, esterase and catalase profiles were evaluated by native PAGE method. Stress induced alterations in expression of isozymes were recorded in selected isolates when compared with corresponding isozyme profiles of positive and negative controls. Results of the present study furnish evidence for defensive role of above mentioned enzymes against deleterious effects of stress induced by abiotic stimuli in entomopathogenic fungal isolates of *Beauveria*.

Keywords: *Beauveria,* abiotic stress, peroxidase, esterase and catalase.

INTRODUCTION

Beauveria species are naturally occurring mitosporic fungi having a wide arthropod host range. They are ubiquitous insect pathogenic fungi that exist as saprophytes in soil and often cause epizootics, wiping out insect pest populations on crops. *Beauveriabassiana* is one of the six registered entomopathogenic fungi presently being used[1]. Both biology and physiology of entomopathogenic fungi depends on environmental conditions such as temperature, relative humidity, pesticide residues in soil and also other various abiotic conditions. Commercial crop protection strategies invariably employ chemical pesticides and fungicides. Heat together with solar UV radiation, are the inevitable factors prevailing in climates that affect conidial

germination. Charles *et al*.[2] recorded altered isozyme profiles for catalase, peroxidase, and glutathione reductase and superoxide dismutase in the spores of near-UV resistant strains of *M.anisopliae* compared with those of less resistant strain. One of the imperative factor that could be as significant as virulence for successful use of biocontrol agent is tolerance to high temperatures. Abiotic stress conditions prevailing in agriclimates are the seasonal variations in temperature, and chemical residues in the form of pesticides and fungicides. Compatibility of entomopathogenic fungi with synthetic chemical inputs used for crop protection is also crucial if these fungi are to be successfully utilized for crop pest control. Although, some insecticides may be harmful, fungicides have devastating effect on entomopathogenic fungi[3]. Therefore, selection of promising isolates showing tolerance to abiotic stress factors would result in successful biocontrol programmers. Several laboratory studies have demonstrated *in vitro* inhibition of *B.bassiana* growth by pesticides[4-6]. Agrochemicals may antagonize the efficacy and potential insecticidal activity of *Beauveria species* and may disrupt its natural epizootics. Therefore, utilization of entomopathogenic fungi in agro-ecosystems is limited because of undesirable interference with agrochemicals[7].

Effective use of entomopathogenic fungi depends on the survival of infective propagules in presence of xenobiotics or abiotic stress conditions. There have been many reports regarding response of fungi to different abiotic stress factors.Angelova*etal*.[8] observed that filamentous fungi protect themselves from oxidative injury, caused by paraquat and hydrogen peroxide by enzymatic and non-enzymatic defense systems. A direct consequence of heat shock treatment was suggested to be the development of thermo tolerance acquisition or the ability to withstand lethal temperatures. It is assumed that heat shock and other stress treatments; resulting in thermo tolerance in *N.crassa* cells, stimulate the induction of peroxidase at a high level[9]. A better understanding of the dynamics involved in transmission of entomopathogenic fungi in nature requires quantitative investigations into how interactions of abiotic stress factors affect the biocontrol agent at field level. Entomopathogenic fungi live in uncongenial environments that do not provide optimal growth conditions. They have to cope with environmental changes and other growth restrictive stress conditions. For developing durable strains tolerant to abiotic stress, understanding about mechanisms of tolerance in biochemical terms is necessary. In an attempt to select tolerant isolates of *Beauveria* for mycopesticide development, we have evaluated thirty local and exotic collections of the entomopathogen for tolerance to the three abiotic factors viz., temperature (33ºC), fungicide (Benzimidazole) and organophosphorous pesticide (Dichlorvos)[10]. The present investigation is an effort to understand mechanisms of abiotic stress tolerance in selected isolates through antioxidant isozyme profiles. Peroxidase, esterase and catalase expression patterns were studied in the tolerant isolates grown at normal culture conditions under stress conditions and in the sensitive isolates (negative controls) which failed to grow under abiotic stress conditions.

MATERIALS AND METHODS

Selection of *Beauveria* isolates for the present study: Selection was done based 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 were 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.

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

Culture conditions: The selected isolates were used for raising cultures 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 set at temperature 33ºC. For pesticide and fungicide stress experiments, the test isolates were inoculated to pesticide/fungicide amended medium and incubated at 25ºC. Dichlorvos at 1x concentration (1.3 ml/L) and bavistin at 0.5 x concentrations (g/L) were used in the amendment of medium. The isolates, which were selected as negative (sensitive isolates) and positive controls (stress tolerant isolates grown under control conditions), were included for incubation at 25ºC in SD broth without pesticide / fungicide. All the isolates under study were kept in an orbital shaker for 5 days at 200 rpm. On the 5th day, mycelia were harvested and washed with double distilled water twice to remove traces of culture medium.

Preparation of enzyme extract and electrophoresis: One gram of mycelium obtained from the broth cultures were pulverized in liquid nitrogen and then resuspended in 5ml of extraction buffer. For esterases, extraction buffer containing 10 mM sodium phosphate buffer (9.5 pH), 1 mM EDTA, and 1 mM 2-mercaptoethanol was used. On the other hand, 0.1 M phosphate buffer pH 7.0 was used for extraction of peroxidase enzyme and for catalase; phosphate buffer pH 6.7 was used. Homogenate was centrifuged at 10,000 rpm for 20 min at 4ºC and the supernatant was used as enzyme source. Isozymes were separated by native polyacrylamide gel electrophoresis (nPAGE) in a discontinuous buffer system. The stacking gel (5% Acrylamide-bisacrylamide 30:0.8 v/v) comprised of 125 mMTris-HCl, pH 6.8 buffer and separating gel (8% Acrylamide–bisacrylamide 30:0.8v/v) with 3mM Tris-HCl, pH 8.8 as buffer[11] were used. The gels were run at 50V at 4ºC with a Tris-glycine electrode buffer containing 25 mM and 192 mM glycine at pH 8.3. The experiment was repeated thrice to test consistency in extraction schedule as well as electrophoresis.

Visualization of enzyme activity and analysis: Staining of gels were performed by the method described by Sadasivam and Manickam[12] for esterase as well as peroxidase. The substrate for esterase was α-Naphthyl acetate and Benzidine for peroxidase. While for catalase, by the procedure of Anderson *et al.*[11] and substrates for catalase were ferric chloride and potassium ferricyanide. Following electrophoresis, gels were incubated in specific separate enzyme staining solutions. Enzyme activity was assayed for each enzyme system using histochemical stains that produce an insoluble dye where enzyme activity was present. The gels were photographed and scored for isozyme bands using Vilbert Lourmat Gel documentation system. Data generated for each enzyme were recorded in a matrix identifying the presence or absence of a particular band and R_f (resolving front) values were calculated. R_f = distance travelled by the enzyme band in the gel/ distance travelled by the indicator. Isolates that were grown under stress conditions and used in the enzyme studies, were denoted with * mark

RESULTS

1. Peroxidase Isozyme Profiles in the Tolerant and Sensitive Isolates of *Beauveria* Species

A. Temperature stress: Selected isolates of *B.bassiana*, B7* and B19* grown at 33ºC expressed novel isoforms of peroxidase with R_f values of 0.271 and 0.246 respectively **(Fig.1A & Tab. 1A).** On the other hand positive control B7 expressed a single band with R_f value 0.331. While B19 recorded two bands with R_f values 0.337and 0.112.

Figure 1: Peroxidase Isozyme Profiles in the Tolerant and Sensitive Isolates of *Beauveria* Species - **B8 and B20 are negative controls, B7, B19, B29, B33, B12 and B32 are positive controls, B7*, B19*, B29*, B33*, B12*and B32* are exposed to stress conditions. The arrows represent the peroxidase isoform patterns.**

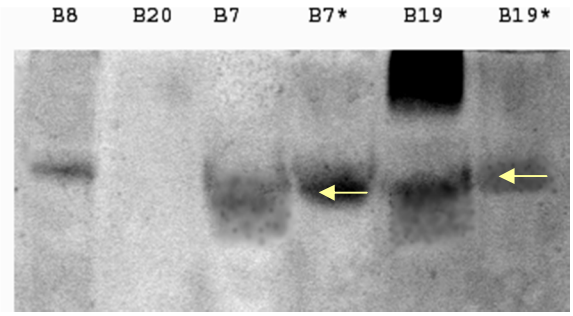

Fig. 1A: Temperature stress

Table-1: Peroxidase isozyme banding profiles in isolates of *Beauveria*

Table-1A: Temperature Stress

Band no.	Rf values	B8	B20	B7	B7*	B19	B19*
1	0.112					+	
2	0.225	+					
3	0.246						+
4	0.271				+		
5	0.331			+			
6	0.337					+	

B.Pesticide stress: Three novel isoforms were recorded in the isolate B29* with R_f values 0.215, 0.273 and 0.405, whereas the corresponding controls expressed three bands with Rf values 0.235, 0.360 and 0.418 indicating activity of lower molecular weight isoform in the positive controls. *B.brongniartii* isolate B33* on the contrary, expressed two isoforms of peroxidase with R_f values 0.209 and 0.389 compared to the three isoforms having R_f values 0.209, 0.280 and 0.469 in the controls **(Fig.1B & Tab. 1B).**

C.Fungicide stress: Bavistin treatment of *B.bassiana* resulted in the expression of novel isoforms of peroxidase in both the selected isolates, B12* and B32* **(Fig.1C).**R_f values of the positive control B12 were 0.234, 0.467, whereas the values in the test isolates were 0.212 and 0.402. Isolate B32 on the other hand displayed three isoforms with R_f values 0.231, 0.283 and 0.477 under control conditions, while under stress conditions; three isoforms of peroxidase bands with R_f values 0.202, 0.243 and 0.442 were recorded. Negative control B8 showed a single band while B20 failed to express the enzyme. **(Fig. 1A &Tab.1C).**

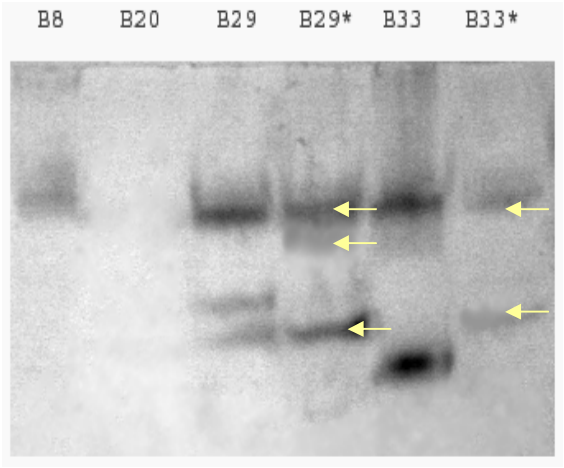

Fig.1B: Pesticide stress

Table -1B: Pesticide stress

Band no.	R_f values	B8	B20	B29	B29*	B33	B33*
1	0.209					+	+
2	0.215				+		
3	0.220	+					
4	0.235			+			
5	0.273				+		
6	0.280					+	
7	0.360			+			
8	0.389						+
9	0.405				+		
10	0.418			+			
11	0.469					+	

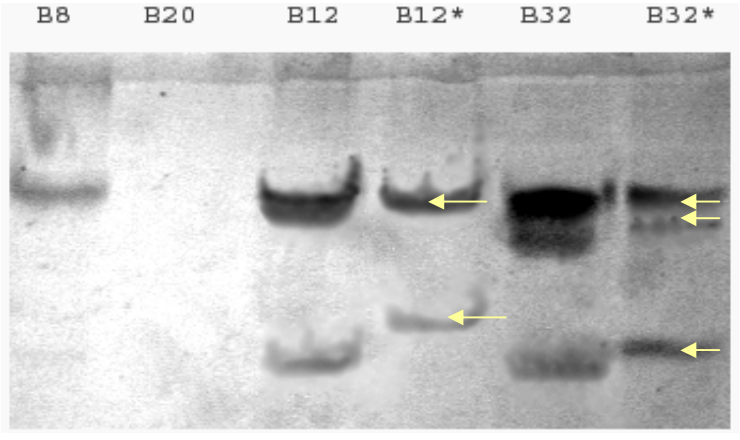

Fig.1C: Fungicide stress.

Table -1C: Fungicide stress

Band no.	R_f values	B8	B20	B12	B12*	B32	B32*
1	0.199	+					
2	0.202						+
3	0.212				+		
4	0.231					+	
5	0.234			+			
6	0.243						+
7	0.283					+	
8	0.402				+		
9	0.442						+
10	0.467			+			
11	0.477					+	

*** *Beauveria*isolates subjected to the above mentioned stress**

2. Esterase Isozyme Profiles in the Tolerant and Sensitive Isolates of *Beauveria*Species

(A). Temperature stress: Remarkable differences were recorded in the negative and positive controls. The bands in the negative controls i.e. B8 and B20 were very faint when compared to the positive as well as the stress exposed isolates. Novel isoforms of esterase were observed in the selected isolates of *B.bassiana* grown under stress conditions i.e., 33ºC **(Fig.2A & Tab.2A)**. B7* isolate expressed isoforms with R_f values 0.184, 0.341 and 0.560, while B19* expressed four novel isoforms having R_f values 0.026, 0.270, 0.359 and 0.548. The R_f values of esterase isoforms expressed by B7 were 0.300 and 0.574 while in B19 were 0.169, 0.338 and 0.569.

Figure.2: Esterase Isozyme Profiles in the Tolerant and Sensitive Isolates of *Beauveria* Species
B8 and B20 are negative controls, B7, B19, B29, B33, B12 and B32 are positive controls, B7*, B19*, B29*, B33*, B12*and B32* are exposed to stress conditions. The arrows represent the esterase isoform patterns.

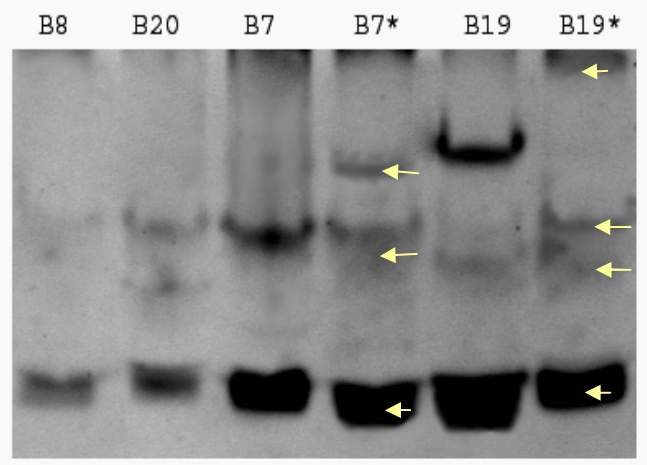

Table-2: Peroxidase isozyme banding profiles in isolates of *Beauveria*

Table-2A: Temperature Stress

Band no.	Rf values	B8	B20	B7	B7*	B19	B19*
1	0.112					+	
2	0.225	+					
3	0.246						+
4	0.271				+		
5	0.331			+			
6	0.337					+	

(B). Pesticide stress: The positive control B29 expressed only one isoform of esterase, which is assumed to be low in molecular weight depending on the R_f value of 0.500. In the test isolate B29*, two isoforms of esterase were expressed having R_f values 0.300 and 0.466 **(Fig.2B & Tab.2B)**. In B33, two isoforms of esterase with R_f 0.027 and 0.476 were depicted while, B33* expressed two isoforms showing R_f values 0.152 and 0.444.

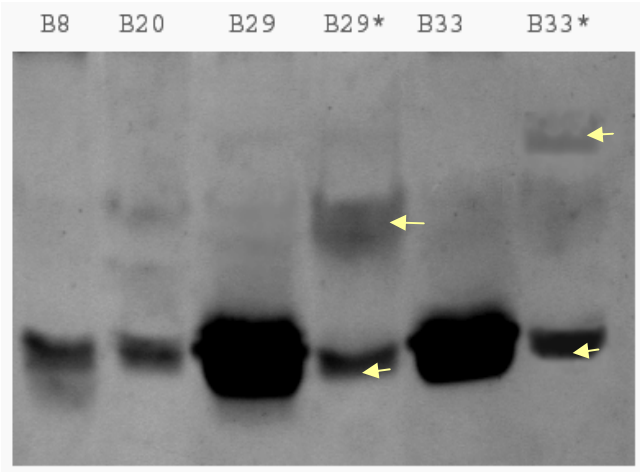

Fig.2B: Pesticide stress

Table-2B: Pesticide stress

Band no.	Rf. Values	B8	B20	B29	B29*	B33	B33*
1	0.027					+	
2	0.152						+
3	0.256		+				
4	0.300				+		
5	0.329		+				
6	0.444						+
7	0.459		+				
8	0.466				+		
9	0.476					+	
10	0.500			+			
11	0.507	+					

*** *Beauveria* isolates subjected to the above mentioned stress**

(C). Fungicide stress: Expression of novel esterase isoforms were observed in both the test isolates. B12* isolate expressed five isoforms of esterase with R_f values 0.812, 0.252, 0.369, 0.420 and 0.520 **(Fig.2C & Tab. 2C)**. While in control only four bands were recorded. Paradoxically the test isolate B32* expressed three novel isoforms with R_f values 0.388, 0.425 and 0.515 compared to only two bands in the corresponding control. Expression of novel esterase isoforms and over expression of the isozyme in both the test isolates under fungicide stress was evident.

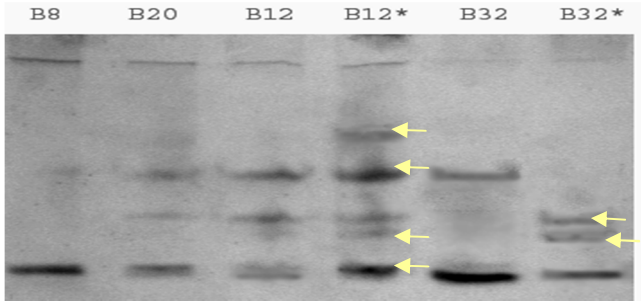

Fig.2C: Fungicide stress

Table-2C: Fungicide stress

Band no.	Rf values	B8	B20	B12	B12*	B32	B32*
1	0.182				+		
2	0.271			+		+	
3	0.274		+				
4	0.282				+		
5	0.366		+				
6	0.369				+		
7	0.379			+			
8	0.388						+
9	0.417			+			
10	0.420				+		
11	0.425						+
12	0.507	+					
13	0.509		+	+			
14	0.515						+
15	0.520				+		
16	0.528					+	

*** *Beauveria* isolates subjected to the above mentioned stress**

3. Catalase Isozyme Profiles in the Tolerant and Sensitive Isolates of *Beauveria*Species

(A). Temperature stress: The temperature stress exposed isolates B7* and B19* expressed two novel isoforms of catalase compared to only one isoform in the control **(Fig.3A &Tab. 3A)**. The R_f values of the isoforms recorded in B7* were 0.309 and 0.436 where as in B19*, 0.287 and 0.395. Intensity of the achromatic zones formed by isoforms was bigger when compared to the control and this indicates pronounced catalase activities in the stress-exposed isolates.

Figure 3: Catalase Isozyme Profiles in the Tolerant and Sensitive Isolates of *Beauveria* Species
B7, B19, B29, B33, B12 and B32 are positive controls, B7*, B19*, B29*, B33*, B12*and B32* are exposed to stress conditions. The arrows represent the catalase isoform patterns.

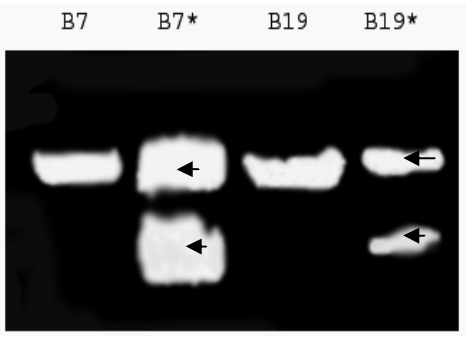

Fig.3A: Temperature stress**,**

Table-3: Catalase isozyme banding profiles in isolates of *Beauveria*

Table- 3A: Temperature stress

Band no.	Rf values	B8	B20	B7	B7*	B19	B19*
1	0.287						+
2	0.300			+			
3	0.307					+	
4	0.309				+		
5	0.395						+
6	0.436				+		

(B). Pesticide stress: The remarkable difference was the absence of catalase activity in B33 while B33* isolate expressed two catalase isoforms with R_f values 0.227 and 0.329 with much intensity in terms of the achromatic zones **(Fig .3B & Tab. 3B)**. Contrarily B29 produced a single isoform of catalase, while B29* showed two isoforms with R_f values 0.256 and 0.364. It is evident from the results that under pesticide stress conditions novel isoforms of catalase were induced and some isoforms were over expressed as indicated by the presence of high molecular weight catalase isoforms.

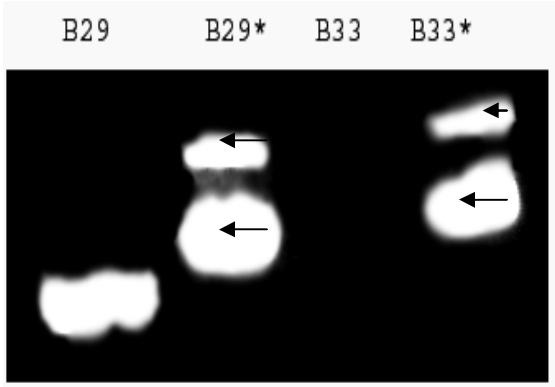

Fig.3B: Pesticide stress

Table- 3B: Pesticide stress

Band no.	Rf values	B8	B20	B29	B29*	B33	B33*
1	0.227						+
2	0.256				+		
3	0.329						+
4	0.364				+		
5	0.427			+			

(C). Fungicide stress: The expressions of catalase isoforms were distinct in the stress-exposed isolates. In B12 isolate only one band with R_f. 0.544 was produced while 0.351 and 0.528 were expressed in B12***(Fig.3C & Tab. 3C)**. On the other hand, in B32 isolate, enzyme band with R_f. 0.392 was recorded while in B32*, two bands with R_f values 0.282 and 0.287 were recorded.

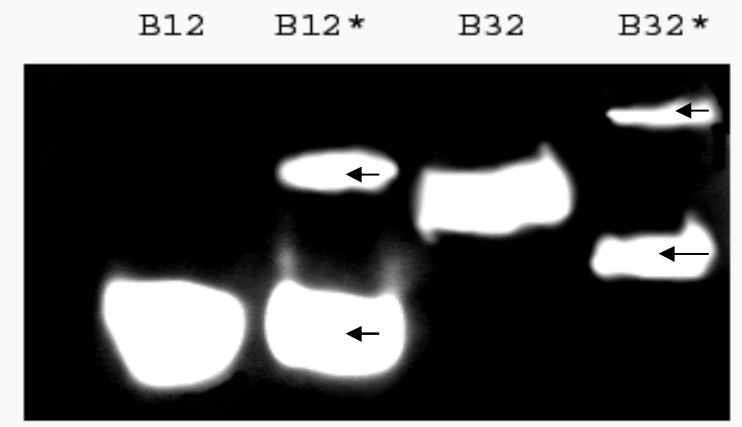

Fig.3C: Fungicide stress

Table-3C: Fungicide stress

Band no.	R_f values	B8	B20	B12	B12*	B32	B32*
1	0.287						+
2	0.351				+		
3	0.392					+	
4	0.430						+
5	0.528				+		
6	0.544			+			

*** *Beauveria*isolates subjected to the above mentioned stress**

DISCUSSION

Tolerance to abiotic conditions such as temperature, pesticide and fungicide at metabolic level require adaptable physiological alterations of the pathogen to varied microenvironments. In the present study, increase in temperature from 25ºC to 33ºC significantly enhanced the expression of peroxidase isozyme. Peroxidase isozyme systems in one of the negative controls (B20) displayed absence of enzyme activity while the positive controls and stress exposed isolates manifested different degrees of expression of this enzyme. The positive control B19 produced two bands while a single band with lower R_f value was recorded in the stress exposed material.Machwe*etal*.[14] reported a 6-fold increase in specific activity of peroxidase in the heat-shocked cells of *N.crassa*. It was also reported that peroxidase was virtually undetectable in non-shocked cells but appeared within 10 minutes of stress treatment. In *Beauveria* system of our study, peroxidase activity was detectable in all the positive controls as well, but the heat-shocked lines appear to be associated with over expression of the enzyme. Anna Marie *etal*.[9] reported that peroxidase activity was of special interest as a correlation between stress treatments leading to the induction of peroxidase and the development of thermo tolerance. In the present study, esterase mediated abiotic stress tolerance was prominent in all the stress exposed isolates. In the temperature-exposed isolates of *Beauveria*, novel esterase bands were expressed. Some of the bands with similar Rf values in the positive controls and temperature exposed isolates differed in intensity.

Similar situation prevailed in case of organophosphorous pesticide and benzimidazol fungicide induced stress in both *B.bassiana* and *B.brongniartii* isolates. Catalase enzyme patterns in the abiotic stress exposed isolates of *Beauveria*, recorded a clear enhanced expression and appearance of novel isoforms. Chary *etal*.[15] while studying the effects of oxidative stress and heat shock regulation of catalase genes in *N.crassa*, reported that superoxide generating compounds, paraquat and menadione, resulted in substantial increase in the specific activity of the Cat-1 enzyme. In our observations, prominent catalase activity was detected in the temperature and fungicide exposed isolates of *Beauveria* after five days of incubation. With respect to pesticide induced stress, both *B.bassiana* and *B. brongniartii* demonstrated shift in the expression of catalase. Blinski*et a*l.[16] also reported catalase induction in *S.cervisiae* upon shifting of cells from 22ºC to 37ºC.

Results regarding the changes in enzyme profiles of catalase in fungicide stress exposed *Beauveria* isolates, revealed the expression of two isoforms instead of one band in the positive controls. Choi *et al*.[17] also observed that, when *Botrytis cinerea* was incubated with vinclozolin (Dicarboxide fungicide), CAT and SOD activities were considerably enhanced at 1h after the fungicide treatment and then decreased to the level of their respective controls. In our findings, catalase activity of the isolates incubated with 0.5X concentration of bavistin for five days did not diminish. Persistent high levels of catalase probably may account for counteracting the reactive oxygen species formed in the organism under stress conditions. Moreover, over expression of catalase isoforms was observed and in contrast to our findings, Choi *et al*.[17] observed slightly reduced catalase activity after 1 h of prolonged treatment with 2 µM vinclozolin in *Botrytis cinerea.*

Stressors can trigger higher order biological responses at the organism level only after initiating certain biochemical and cellular events because organisms often withstand the on slought of stressful conditions by activating specific group of genes, which translate specific proteins and enzymes [18, 19]. According to Ayar[20], organisms have evolved mechanisms for allowing them to cope with a variety of stresses including nutrient limitation, the presence of oxidants in their environment, and damage caused by toxic chemicals or physical agents. When exposed to these stresses, prokaryotic and eukaryotic cells respond rapidly and adopt their

metabolism to the altered environmental conditions. The microbial oxidative stress response is a result of well-orchestrated reactions involving synthesis of many enzymes and small molecules involved in direct detoxification and/or protection of oxidative stress[21].

All the three enzymes studied i.e., Peroxidase, Esterase and Catalase displayed novel enzyme bands and also over expression of some of the isoforms in the selected isolates of *B.bassiana and B.brongniartii* under stress conditions. On the contrary, few isozymes manifested as either faint bands or as low molecular weight (high Rf value) bands in the stress exposed isolates. It is likely that the predominant reactive oxidative radicals produced under prolonged stress conditions resulted in inactivation of some of the isozyme forms and the balance between ROS, the antioxidant enzyme levels determine resistance to a given stress factor. Results of the present study furnish evidence for the defensive role of antioxidant enzymes like catalase, peroxidase and esterase under abiotic stress conditions. This information would be helpful in selecting abiotic stress tolerant isolates of *B.bassiana* for successful integrated pest management as well as biocontrol programs.

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