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BIOCHEMICAL BASIS OF ABIOTIC STRESS TOLERANCE IN NATIVE ISOLATES OF *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN FROM KERALA

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ABSTRACT

A study on the screening of *Beauveria bassiana* (Balsamo) Vuillemin native isolates for abiotic stress tolerance was carried out at the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, Thrissur, Kerala during 2019-2023. The growth and biochemical parameters of the three native isolates of *B. bassiana* (BTL1: OP271760, BTL2: OP290199 and PKDE: OP292066) were studied under different abiotic stress conditions viz., temperature, water stress, acidity and salinity. The results revealed that the highest temperature tolerance (40°C) was displayed by the *B. bassiana* isolate PKDE. It also survived at high water stress (45% polyethylene glycol), acidic (pH2) and saline (1.5 M) conditions. The analysis of biochemical parameters in stress tolerant isolate revealed that the greatest levels of trehalose (2.033± 0.025, 2.043± 0.006 mg/ min/ g of mycelia), catalase (0.0072± 0.007, 0.0032± 0.003 EU/ min/ mg protein) and peroxidase (0.0602± 0.005, 0.0175± 0.017 EU/ min/ mg tissue weight) were observed after exposure to high temperature and water stress, respectively. This shows that exposure to abioticstress and biochemical parameters are closely related and can be used as determinants for evaluating the potential of biocontrol agents.

Key words: Entomopathogenic fungi, *Beauveria bassiana* native isolates, stress tolerance, biochemical parameters, trehalose, enzymes, catalase, peroxidase, protein profiling, heat shock protein

Beauveria bassiana (Balsamo) Vuillemin is an insect pathogenic fungus capable of suppressing a wide range of economically significant pests (Booth et al., 2000; Goettel et al., 2000). It affects several insect orders, including Lepidoptera, Hemiptera, Coleoptera, Hymenoptera, Homoptera, and Orthoptera (Li et al., 2001). The efficacy of *B. bassiana* in the biological control of agricultural pests is mainly hampered by adverse environmental factors such as, high temperature, low humidity and UV radiation (Vidal et al., 2007). The optimal growth temperature of *B. bassiana* has been reported to be between 25˚C and 28˚C (Parker et al., 2003). However, it has been demonstrated that some isolates of *B. bassiana* could grow at a higher temperature of 30˚C, but couldn't survive at 34°C (Hiromori et al., 2004). Matawele et al. (1994) showed that low-water activity and UV tolerant mutants of *B. bassiana* were more virulent than unaltered wild-type strains over a wider relative humidity range. Hence, the identification of abiotic stress tolerant *B. bassiana* isolates represents a possible solution to overcome the poor performance of *B. bassiana* under unfavourable climatic conditions (Alali et al., 2019). To surmount this limitation, development of multiple stress tolerant isolates is a viable strategy. This demands mass screening, isolation and identification of abiotic stress tolerant native isolates of Entomopthogenic fungi (EPF).

Varying ranges of temperature, salt concentration, PEG concentration (water stress), and pH of the growing media can be used to create abiotic stress conditions for in vitro evaluation. Water restrictors or osmotic inducers such as polyethylene glycol (PEG) are commonly used additives in media to induce water stress (Karvembu et al., 2021). In stressed eukaryotic cells, sugars and low-molecule-compatible solutes like trehalose or polyols may accumulate to maintain intracellular homeostasis for membrane integrity and protein stability (Keller et al., 1982). Enzymes play an important role in several species of EPF to cope with various stress conditions. Antioxidant enzymes such as superoxide dismutase, catalase, peroxidase, and glutathione reductase are considered to be the first line of defence in filamentous fungi in response to oxidative stress (Angelova et al., 2005). Reduction in catalase and peroxidase activities in mutant isolates of *Metarhizium acridum* increased susceptibility to oxidative stress when compared to wild-type stains (Li et al., 2017). Abiotic stress tolerant fungal candidates selected

through screening and analysing the biochemical parameters are of greater potential for selecting desired candidates for utilisation in bio control (Rangel et al., 2008). Tolerance to environmental fluctuations is a prerequisite for developing ecologically competent and virulent commercial biopesticides against crop pests. Hence the present study was focussed to screen the abiotic stress tolerant isolates of *B. bassiana* and also to elucidate the biochemical mechanisms of stress tolerance.

MATERIALS AND METHODS

The present study was conducted at the College of Agriculture, Kerala Agricultural University Vellanikkara, Thrissur, Kerala during 2019- 2023. Three native isolates of *B. bassiana* (BTL1: OP271760, BTL2: OP290199 and PKDE: OP292066) isolated from the field collected cadavers were used for the study. The influence of abiotic stresses such as temperature, pH, salinity and water stress on the mycelial growth and sporulation of the isolates were evaluated in potato-dextrose broth (PDB) medium under in vitro condition. The temperature tolerance of the isolates was investigated in a BOD (Biochemical Oxygen Demand) incubator at different temperatures of 30°C, 32°C, 34°C, 36°C, 38°C and 40°C. For assessing the pH tolerance, a pH range of 2 - 6 at 0.5 unit intervals was tested. The pH of the medium was adjusted with 0.1 M HCl or 0.1 M NaOH and was set using a pH meter (model: Hanna instruments). The growth medium (PDB) was amended with different concentrations of NaCl (0.5 M, 1 M, 1.5 M and 2 M) for screening salinity tolerance. Potato dextrose medium amended with polyethylene glycol (PEG 6000) at different concentrations (10, 20, 30, 35, 37, 39, 41, 43 and 45%) was used for inducing drought stress. The 5 mm mycelial disc of *B. bassiana* was added to 100 ml potato dextrose broth taken in a glucose bottle and incubated for 10 days, under varying levels of abiotic stress conditions, as described above. The mycelia were then taken out from different treatments and kept in Whatman No. 1 filter paper, separately for 2 h for drainage of excess broth. The mycelial fresh weight in each treatment was recorded separately. The spore count of *B. bassiana* isolates was performed from 10-day-old cultures under different abiotic stress condition. The mycelial mat was taken and ground in a mixer by adding 12 ml of 0.02% Tween 80. The conidial suspension was filtered through two layers of sterilized muslin cloth. The number of spores in the suspension was determined using a haemocytometer (Neubauer improved, Superior Marienfeld, Germany). The calculation was done by following the equation, Number of spores/ml = D x X/ NK

Where, D- Dilution factor, X- No. of spores counted, N- No. of small squares (16x5), K- 2.5×10^{-7}

The *B. bassiana* strain identified as tolerant to both high temperature and drought in the study, based on growth and sporulation parameters were subjected to biochemical analysis. The total protein, catalase, peroxidase and trehalose content in the stress tolerant strain were evaluated. The total protein content of the isolate was estimated as per the method of Lowry et al. (1951). The catalase activity of the isolate was estimated as per the procedure of Sadasivam and Manickam (2008). The peroxidase activity of *B. bassiana* was determined as per the protocol of Mahadevan and Sridhar (1986). The amount of trehalose present in the mycelia of the *B. bassiana* isolate at different temperatures and drought levels was assessed as per anthrone- sulphuric acid colourimetric method of Wang et al. (2013). To check the presence of heat shock protein in stress tolerant *B. bassina* isolate, SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed by following the protocol of Laemmli, (1970). The statistical analyses were carried out by using GRAPES 1.0.0 (General R-shiny based Analysis Platform Empowered by Statistics) software, developed by Kerala Agricultural University. The data were subjected to Analysis of Variance (ANOVA). Multiple comparisons between the treatment means were done with Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Three native isolates of *B. bassiana* were identified from Kerala by cultural, morphological and molecular confirmation. The isolates were named as BTL1, BTL2 (isolated from handsome fungus beetle - Wayanad) and PKDE (isolated from unknown insect specimen-Palakkad). The molecular confirmation of the isolates was carried out and the accession numbers allotted to the isolates were BTL1: OP271760, BTL2: OP290199 and PKDE: OP292066. The Palakkad isolate (PKDE) substantially had the highest mycelial biomass at high temperature (40°C), acidity (pH 2), salinity (1.5 M) and water stress (45% PEG concentration) of the three isolates studied. At 40°C, there was complete inhibition of growth for BTL1 and BTL2 isolates, but the isolate PKDE had a higher mycelial weight of 2.148g (Fig. 1). At 38°C, the isolate PKDE also displayed the highest spore count (4.7×10^7 spores/ml) when compared to the other two isolates (Table 1). Shimazu (2004) noted

that *B. bassiana* spores sustained at high temperatures (36°C) for a longer period than the hypha, indicating that spores had improved stress tolerance. It's interesting to note that the temperature-tolerant isolate identified in this study (PKDE) produced more spores than the other local isolates, which would help in the survival of this isolate at high temperatures. The mycelial weight of isolate PKDE increased steadily as the pH decreased until it reached 4 and then decreased as the pH lowered. Even at extreme acidic conditions (pH 2), the PKDE isolate reported mycelial biomass of 1.936 g, whereas the other two isolates had no growth (Fig. 2). The spore count was also demonstrated that the isolate PKDE is superior to the other isolates (Table 1). As the salinity increased from 0.5 M to 2 M, all the three isolates displayed a noticeable decrease in the mycelial weight. As compared to the other two isolates, the PKDE isolate had the highest mycelial weight at 1M, with a value of 2.874g (Fig. 3). At a higher salt concentration of 2 M, all isolates exhibited a significant reduction in spore count except the isolate PKDE which recorded the highest spore count of 2.7x 10^7 spores/ ml (Table 1). When the medium was amended with 45 per cent concentration of PEG, the highest mycelial weight of 1.208g was observed for the isolate PKDE, followed by BTL2 with 1.060 g mycelial weight (Fig. 4). At the peak water stress condition (45% of PEG concentration), the isolate PKDE recorded the highest sporulation (6.833x10^7 spores/ ml) (Table 1).

These findings are in conformity with the findings of Vidal et al. (1997), who found that entomopathogenic fungal isolates from the Indian subcontinent were more temperature tolerant. According to Fatu et al. (2021), assessment of thermal requirements of different isolates of *Beauveria bassiana* showed that optimum temperature range and the response to higher temperatures varies with isolates. Optimum range for the growth and sporulation of *B. bassiana* lies between 25 to 30°C (McGuire and Northfield, 2020). There was another study by Fargues et al. (1997), where they observed that *B. bassiana* can tolerate temperatures as high as 37°C. Overall, the temperature tolerant isolate (PKDE) had greater fungal biomass than the other native isolates, indicating that it is more adaptable than the other isolates under similar experimental settings to temperature fluctuations. It's crucial to remember that isolates from high-temperature regions like Palakkad (47°C) (PKDE isolate) displayed more temperature tolerance than isolates from low-temperature regions

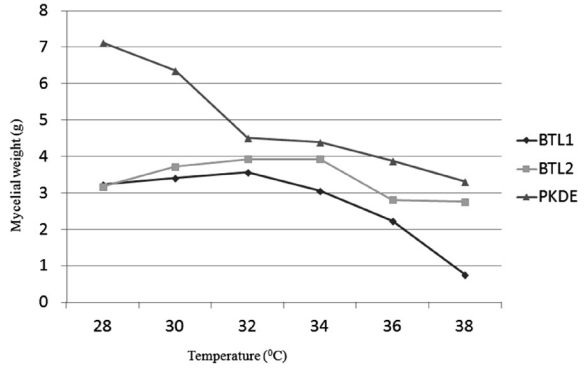

Fig. 1. Effect of temperature on growth of *B. bassiana* isolates

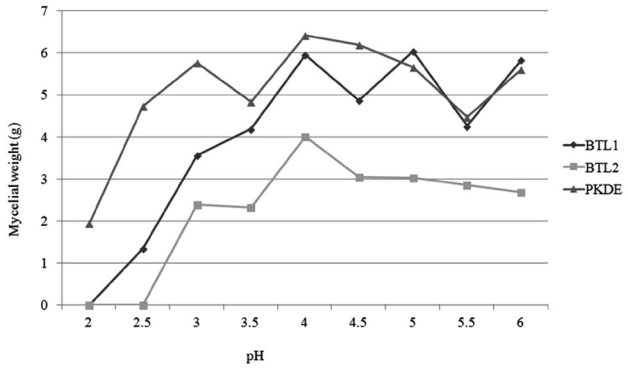

Fig. 2. Effect of acidity on growth of *B. bassiana* isolates

Table 1. Spore count of native isolates of *B. bassiana* after exposure to abiotic stress conditions

Isolates	Spore count at temperature stress (*10^7 spores/ ml)		Spore count at acidic stress (*10^7spores/ ml)		Spore count at saline stress (*10^7spores/ ml)		Spore count at water stress (*10^7spores/ ml)	
	Control (28± 2°C)	38°C	Control	pH 3	Control	2M	Control	PEG 45%
BTL1	4.073^{bc}	0.140^{c}	4.00^{b}	2.467^{c}	4.067^{c}	0.137^{e}	3.733^{c}	2.167^{d}
BTL2	5.587^{b}	3.700^{ab}	6.500^{ab}	4.633^{b}	6.400^{b}	0.220^{e}	5.300^{b}	3.967^{c}
PKDE	7.767^{a}	4.700^{a}	8.633^{a}	6.733^{ab}	8.800^{a}	2.700^{d}	7.767^{a}	6.833^{b}

Since the P-value in ANOVA table is < 0.05, there is a significant difference between at least a pair of treatments; treatments with same letters not significantly different

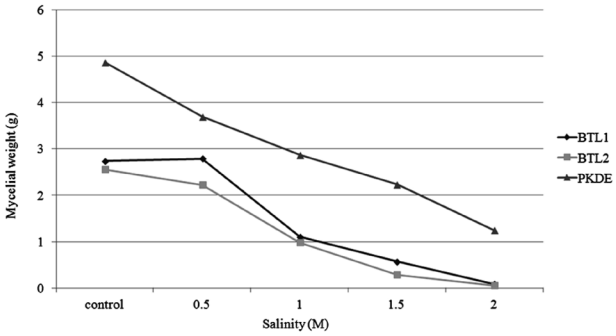

Fig. 3. Effect of salinity on growth of *B. bassiana* isolates

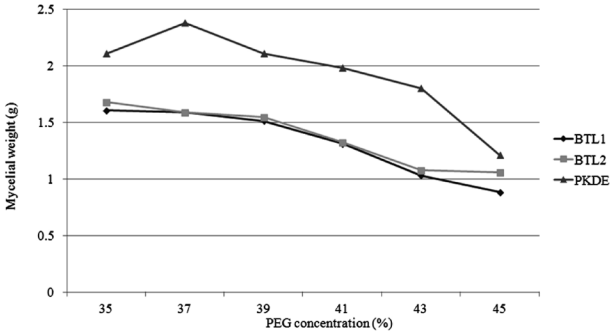

Fig. 4. Effect of water stress on growth of *B. bassiana* isolates

like Wayanad (isolates BTL1 and BTL2), where the temperature ranges from 25 to 27°C. Similar to this, local *B. bassiana* isolates from Syria's subtropical region showed greater thermotolerance than isolates from locations with lower temperatures (Alali et al., 2019).

To increase the effectiveness of EPF, efforts should be made by concentrating on endemic fungal communities and applied within similar locations with similar climatic circumstances from where it has been previously isolated (McGuire and Northfield, 2020). According to several reports, the pH tolerance range of *B. bassiana* isolates is 5 to 6 (Sanzhimitupova, 1980), 6 to 8.5 (Galani, 1988), as well as 10 and beyond (Shimazu and Sato, 1996). This reveals that depending on different strains the pH tolerance of *B. bassiana* fluctuate in a wider range. This is supporting the results of the present investigation since the three isolates showed a different pattern in mycelial weight with varying pH levels. In *Metarhizium anisopliae*, conidia produced in culture media (PDAY- Potato Dextrose Agar with 1% Yeast) amended with sodium chloride (0.8 M) were more virulent than conidia cultured on PDAY without salt, indicating that conidia grown under osmotic stress have increased virulence (Shah et al., 2005). Similar results were also recorded by Mascarin et al. (2023) where they observed that ionic osmolytes (NaCl/ KCl) when applied at lower concentrations generating an osmotic pressure 2.5-2.7 MPa enhanced the blastospore yield of *B. bassiana* isolates. Similar to inorganic salts, organic acid salts were previously reported in a protective effect on microorganisms against thermal destruction. Stress tolerant fungi produce more compatible solutes especially sugar alcohols viz., glycerol and erythritol which accelerates better performance of enzyme systems (Magan, 1997). The protective effect might result from a reduction in the water activity of microorganism cells by salts, which decreased the heat penetration, thereby leading to enhanced thermotolerance and it was also noted that certain fungi have a minor salt requirement that can accelerate growth (Yuan et al., 2012).

It was also noted that certain fungi have a minor salt requirement that can accelerate growth (Larsen, 1986). From these results, it could be inferred that the slight increase in the mycelial weight of PKDE isolates at 1M salt concentration is due to favourable conditions for the growth of *B. bassina* in the presence of slight saline conditions. The salt accumulated in hyphae and reduced intracellular water activity, assisting with osmotic adjustment under moderate water stress. Nevertheless, excessive amounts of KCl are known to be hazardous since it is not a compatible solute in nonhalophiles and may impair membrane and enzyme structure and function (Yancey et al., 1982). These findings are following observations of the present study since a reduction in mycelial weight of all the fungal isolates was observed at 2 M salinity. Devi et al. (2005) tested 29 isolates of *B. bassiana* for their ability to germinate and grow in temperature and water availability conditions. Germination and growth assays revealed that some isolates were found with >90% growth relative to control in a medium having 30% PEG with water availability (1.33 MPa), nearly equivalent to that in soils which induce permanent wilting point of plants. These findings will aid the selection of isolates for use in hot or dry agricultural climates. Hallsworth and Magan (1996) indicated that there is a possibility for extending this water activity range through the manipulation of growth conditions by adding certain chemicals and boosting the accumulation of certain intracellular biochemicals into conidia, resulting in better environmental fitness of entomopathogens.

The PKDE isolate of *B. bassiana* isolated from

the Palakkad district was shown to be exceptionally resistant to the effects of temperature and drought stress under in vitro conditions. Thus, biochemical characterizations of the isolate were performed to elucidate the relationship between stress tolerance and the level of biochemical produced by the fungi. The enzymes (catalase, peroxidase) sugar (trehalose) as well as total protein content was examined. The findings revealed that when compared to the control, under conditions of high heat and water stress, the protein content elevates. A considerable variation in catalase activity was discovered after exposure to high temperature (40ºC), and at high concentration og PEG (45%) with a values of 0.0072± 0.007 and 0.0032± 0.003 EU/min/mg protein respectively, when compared to control (0.0024± 0.001 EU/ min/ mg protein). Fungi have evolved several defence mechanisms to shield the cells from oxidative damage and the most important one is the production of antioxidant enzymes (Zhang et al., 2016). Catalases are the most important enzymes for turning hydrogen peroxide (H_2O_2) into water and oxygen, which reduces the levels of H_2O_2 inside the cells. The hydroxyl radical, which is the most dangerous reactive oxygen species and can destroy all adjacent biomolecules, is assumed to be the principal cause of the damaging effects of H_2O_2 (Hossen et al., 2023). According to Chakravarty et al. (2022), catalases and peroxidases are the two main types of enzymes involved in the detoxification of H_2O_2 generated by several cellular processes. The catalase overexpressing strain's higher virulence can be explained by different theories. The most simple is that accelerated germination results in enhanced pathogenicity and the mechanism underlying this may be related to the conidia's ability to go through the hyper-oxidant stage of germination more quickly due to enhanced catalase activity (Michan et al., 2003). Furthermore, catalase activity may help remove or eliminate reactive oxygen species and other host-derived toxins that are found on the cuticle surface during the fungal invasion of insect tissues and the hemocoel (Vierstraete et al., 2004). The reduction of insect defence mechanisms like melanization by catalase activity is another possibility, hence the ability of *B. bassiana* to target its insect hosts can be increased by a slight increase in catalase activity (Chantasingh et al., 2013). This information can be utilised to discover strains that produce high levels of catalase or to design improved strains to increase their pathogenicity towards insect pests.

A significantly higher peroxidase activity was reported at 40ºC and 45% PEG and the values for the enzyme activities were 0.0602± 0.005 and 0.0175± 0.017 EU/ min/ g tissue weight respectively than the control (0.0075± 0.005 EU/ min/ g tissue weight). Similarly, Angelova et al. (2005) observed that oxidative stress due to heat shock increased peroxidase activity in *Neurospora crassa*. The results obtained in the present study follow the observations of Palem and Padmaja (2014). They observed that antioxidant enzymes peroxidase, esterase and catalase in *Beauveria* sp under abiotic stress stimuli play an important role in the defence mechanism to overcome adverse situations. Under abiotic stress conditions such as heat, desiccation, freezing, dehydration, nutrient starvation, osmotic or oxidative stress and exposure to toxic chemicals, trehalose plays a crucial defence mechanism that stabilizes proteins and cellular membranes from inactivation and denaturation (Hottiger et al., 1994). Similarly in the present study an increase in the trehalose level was observed when the PKDE isolate exposed to a high temperature of 40ºC (2.033±0.025 mg/ min/ g mycelia) and water stress induced by 45% PEG (2.043± 0.006 mg/ min/ g mycelia) compared to control (1.865± 0.004 mg/ min/ g mycelia). Ocón, et al. (2007) observed how arbuscular mycorrhizal (AM) fungus responded to various conditions of stress (heat shock, osmotic stress, and chemical stress) by altering trehalose turnover and it was noticed that after five hours at 37°C, trehalose levels were ten times greater than in control hyphae grown at 27°C. There is a considerable association between intracellular trehalose accumulation and the shelf life of filamentous fungus conidia. According to Fillinger et al. (2001), conidia of the wild-type strain of *Aspergillus nidulans* were germinated for three hours at 30°C and then subjected to a 50°C heat shock and observed that trehalose is a major stress metabolite in *A. nidulans* and is probably involved in the acquisition of resistance to an array of stress conditions, including heat, oxidative stress and long time storage. To create inocula of higher quality with increased desiccation tolerance and storage life, as well as greater efficacy in the field, it may be necessary to modify the polyol and trehalose concentration of fungal propagules (Hallswortht and Magan, 1994). Yan et al. (2021) investigated the mechanism by which trehalose mediates defence against numerous stresses in reducing high-temperature-induced damage in *Pleurotus ostreatus*. Trehalose helps *P. ostreatus* to cope with high-temperature stress by preventing glycolysis and promoting pentose phosphate pathway activity. From the perspective of intracellular metabolism, this study may offer additional insights into the heat stress defence mechanism of trehalose in edible fungus.

To confirm the temperature tolerance of PKDE isolate, protein profiling was done to check the presence of heat shock protein. The results revealed some extra bands were observed in PKDE isolate when exposed to temperature stress and the molecular weight of extra bands that appeared were in the range of 11-17 kDa, 48-63 kDa and 100-135 kDa (Fig. 5). It was also observed that the disappearance of some protein bands happens when compared to the protein profile of control without exposure to stress conditions. The protein band present between 20-25 kDa in control was absent in the protein profiles of PKDE isolate when exposed to temperature stress and water stress. One of the protein bands between 100-135 kDa in control was also absent in the isolates when exposed to abiotic stress conditions. To carry out a biological activity under stress, organisms express chaperons or heat-shock proteins (Hsps) (ul Haq et al., 2019). Hsps are involved in several common biological activities, including transcription, translation and posttranslational modifications, protein folding, and the aggregation and disaggregation of proteins and these proteins are produced in reaction to thermal stress as well as other stressful circumstances such oxidative stress, chemical toxicity, salt, high heat, and drought (Zhao et al., 2023). As a result, it is critical to comprehend how Hsps function holistically in fungi in response to stress and other biological situations (Tiwari et al., 2015). Low molecular weight heat shock proteins called small Hsps range in size from 12 to 43 kDa (Jaya et al., 2009) and when fungus cells experience non-lethal heat shock, they develop resilience to temperature extremes and other abiotic stimuli by the production of Hsps (Tereshina, 2005). In this study, the SDS PAGE analysis of the total crude protein from the stress-tolerant *B. bassiana* isolates (PKDE) revealed that there was overexpression of protein under high-temperature stress, with the molecular weight ranging between 35- 48 kDa, 11-17 kDa, 48-63 kDa and 100-135 kDa. These results are following the findings of Welker et al. (2010), Mayer et al. (2012), Seymour and Piper (1999), Hirt et al. (1997) and Boreham and Mitchel (1994). They observed that Hsps having molecular weight 10-100 kDa play some crucial role in the stress tolerance ability of several fungi.

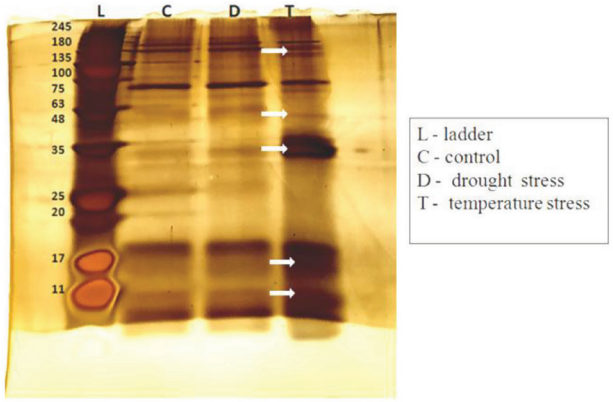

Fig. 5. SDS PAGE protein profile of *B. bassiana* isolate PKDE, lane L: prestained protein ladder, lanc C- control, land D- isolate grown at 45% PEG concentration, lane T- isolate grown at 40 °C temperature, white arrow indicate heat shock protein with molecular weight 35-48kDa, 48-63 kDa and 100-135 kDa

The relationship between conidial protein contents and thermotolerance in fungal biocontrol agents suggests a novel strategy for improving fungal formulations by increasing the contents of thermotolerance-related proteins in conidia. This can be accomplished by looking for fungal isolates with higher thermotolerance or by optimising growth substrate components to produce more thermotolerant conidia (Wang et al., 2020). Further mechanisms of heat stress tolerance are likely to be discovered, providing more avenues for further enhancement of mycoinsecticides. Generally, growth under stress is associated with the production of conidia with increased stress tolerance, but with very low conidial biomass that does not favour commercial production. One way to produce conidia with high-stress tolerance is possible by identifying the genes for increased stress tolerance and upregulation of biochemicals which favour stress tolerance. Such research works are currently under investigation.

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AUTHOR CONTRIBUTION STATEMENT

HB, MC and DKB designed research, NT conducted experiments and wrote the manuscript, DKB analyzed data and corrected the manuscript.

CONFLICT OF INTEREST

No conflict of interest.

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