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Deciphering the relationships among enzymatic systems and virulence of *Beauveria bassiana*: A Review

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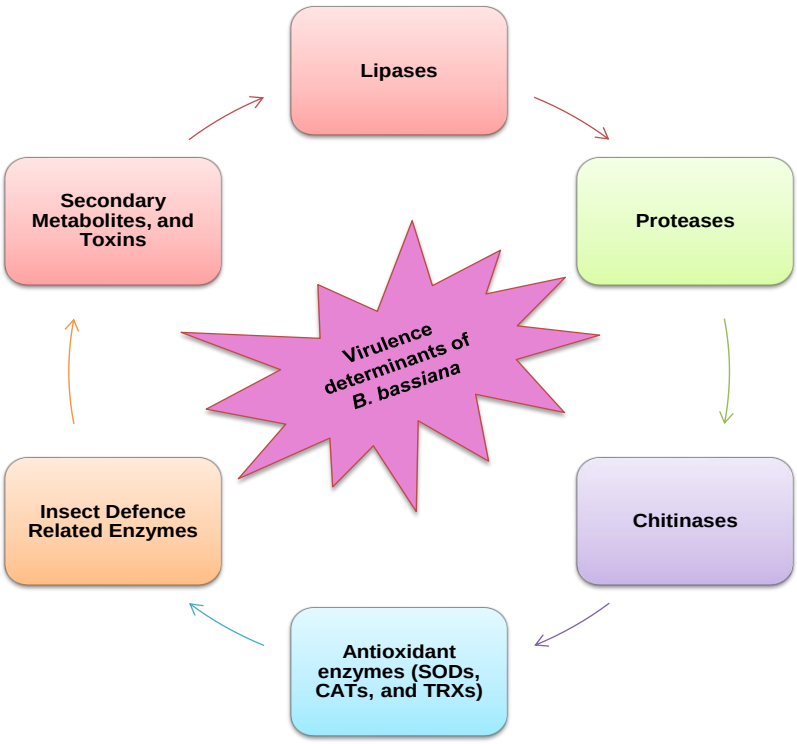
Abstract: Intensive crop production and extensive use of harmful synthetic chemical pesticides create numerous socio-economic problems worldwide. Therefore, sustainable solutions are needed for insect pest control, such as biological control agents. The fungal insect pathogen *Beauveria bassiana* has shown considerable potential as a biological control agent against a broad range of insects. The insights into virulence mechanism of *B. bassiana* is essential to show the robustness of its use. *B. bassiana* has several determinants of virulence, including the production of cuticle-degrading enzymes (CDEs), such as proteases, chitinases, and lipases. CDEs are essential in the infection process as they hydrolyze the significant components of the insect's cuticle. Moreover, *B. bassiana* has evolved effective antioxidant mechanisms that include enzyme families that act as ROS scavengers, e.g., superoxide dismutases, catalases, peroxidases, and thioredoxins. In *B. bassiana*, the number of CDEs and antioxidant enzymes characterized in recent years. The enzymatic activities are crucial for the biological control potential and significantly advanced our understanding of the infection mechanism of *B. bassiana*. This review focuses on the progress detailed in the studies of these enzymes and provides an overview of enzymatic activities and their contributions to virulence.



Keywords: *Beauveria bassiana*, cuticle degrading enzymes, entomopathogenic fungi, pathogenesis, virulence.

Introduction

Insect pests cause severe damage to crops, and an 18 to 20 % reduction of the annual global crop yield recorded by pests in the last decade (Oerke and Dehne 2004). Eight percent loss of the significant crops was estimated in Brazil instead of chemical control measures (Oliveria et al., 2014). Though chemical pesticides are widely applied in agriculture since many decades but environmental and health concerns about the applications of chemical insecticides to reduce large-scale insect pest infestations have led to renewed interest in the development of microbial agents as an alternative control of crop pests (Joshi et al.,2019; Dhawan 2015; Oliveira et al., 2014). Various microbial agents,

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such as *Bacillus thuringiensis* and *Beauveria bassiana*, are currently used as biological control agents for different insect pests to reduce the crop loss (Dhawan et al., 2018; Kaur et al., 2017).

The cosmopolitan anamorphic fungus, *B. bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a well-recognized and most exploited biological control agent (BCA). *B. bassiana* is known to infect hundreds of host species belonging to most insect orders, and it is the most appropriate BCA in temperate agriculture regions (Singh and Joshi 2020; Zimmerman, 2007; Glare, 2008). The success of *B. bassiana* as biopesticide not only depends on its high efficacy against insect pests but also on its non-pathogenic behavior towards non-target pests (Thungrabeab and Tongma 2007).

Unlike bacteria or viruses, *B. bassiana* infect insects by direct penetration of the cuticle. However, insect cuticle serves as a physical barrier against fungal attack; it is a structure formed by crystalline chitin nanofibers inside proteins, polyphenols, and lipids matrix (Vincent and Wegst 2004; Sharma et al., 2017). So, *B. bassiana* secretes several extracellular cuticle degrading enzymes (CDEs) such as proteases, chitinases, and lipases to degrade the major constituents of the insect's cuticle and allow hyphal penetration into the insect's cuticle (Dhawan and Joshi 2017). CDEs play pivotal roles in the infection mechanism and considered essential virulence governing factors of *B. bassiana*. CDEs enable *B. bassiana* to coexist with the changing metabolic processes associated with the host's disease (Wang et al. 2005; Cho et al. 2006). In addition to CDEs, *B. bassiana* has evolved several stress-related enzymes such as superoxide dismutases (SODs), catalases (CATs), and thioredoxins (TRXs). These enzymes help in combatting the abiotic stresses such as osmotic stress, U.V. exposure, high/low salt and high/low temperature, et but their role in virulence of *B. bassiana* are still unclear. Antioxidant enzymes determine the shelf life of commercially available *B. bassiana* and determine its efficacy in field applications (Kaur and Joshi 2014).

It is still a mystery about how all these enzymes work together and determine the virulence of *B. bassiana*? The sequence of action of CDEs is still unclear, and in this review, we explore their roles at various steps of the infection in insect pests. The deep insights into the enzymatic machinery of *B. bassiana* will lead us to a better biocontrol approach and sustainable solution to arthropod pests. Hence, the present review focuses on the roles of the various CDEs and stress-related enzymes in the pathogenesis of *B. bassiana*.

Extracellular Cuticle Degrading Enzymes (CDEs)

Lipases

Lipases play fundamental roles in cellular lipid metabolism and participate in the storage, processing, digestion, and transport of lipid substrates (Gupta et al. 2015). Lipases also play significant roles in the host infection process as these enzymes are essential in the initiation of the infection process. These enzymes hydrolyze the ester bonds of triacylglycerols, which leads to the release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol (Keyani 2017).

Moreover, lipases support the adhesion of germinating spores to insect cuticles by increasing hydrophobic interactions between the fungus and the cuticle surface (Santi et al., 2010). Khan et al. (2012) correlated the toxicity of *B. bassiana* with the enzymatic activities, and proposed that the lipases participate more in total virulence or pathogenicity as compared to protease and chitinase while protease was assumed to participate more than chitinase. They evaluated the pathogenicity of *B. bassiana* isolates against *Myzus persicae* and reported variability in the mortality against the target pest and virulent isolates showed marked enzymatic activities as compared to less virulent isolates. Differential secretome of *B. bassiana* induced by the *Rhipicephalus microplus* cuticle was evaluated by the multidimensional protein identification technology. Fifty proteins from a total of 236 proteins were identified exclusively in infection condition, assigned to different aspects of infection such as host adhesion, cuticle penetration and fungal defense, and stress. Several bioinformatic analyses suggested that the highest number of proteins in the differential secretome possesses lipase activity (Santi et al., 2018).

B. bassiana contains a broad suite of CYP P450 genes (approximately 83) that act on a wide range of compounds, and only a subset of these enzymes acts on the hydrocarbons (Urlacher et al. 2004). Series of enzymes are required for the various hydrocarbon substrates found on most insect cuticles (Keyhani NO 2017). The substrate

specificity and role of one *B. bassiana* hydrocarbon-acting hydroxylase CYP P450 has been characterized named as Bb*cyp52x1* gene (Zhang et al. 2012). Purified BbCYP52X1 was shown to display terminal hydroxylation activity against fatty acids. Loss of function *B. bassiana* mutants (*Bbcyp52x1*) resulted in reduced virulence against *Galleria mellonella* larvae (Table 1). These mutants showed decreased germination on grasshopper wings as compared to the wild type parent and complemented controls.

In contrast to this, individual targeted gene knockouts of five other (putative) hydrocarbon-specific *B. bassiana* CYP P450s (CYP5337A1, CYP52G11, CYP617N1, CYP53A26, and CYP655C1) did not show any significant decrease in virulence and any significant defects in lipid utilization (Pedrini et al. 2013). Zhou et al., 2018 reported differential expression of lipases encoding genes in *B. bassiana* infecting two different hosts. Cytochrome P450 CYP68N1 was up-regulated to implicate in insect hydrocarbon degradation in *B. bassiana* infecting *Galleria mellonella*, while Cytochrome P450 CYP625A1 was up-regulated in *B. bassiana* infecting *Plutella xylostella*. These studies suggest the differential secretion of lipases against different insect pests. In the future, there is a need for more studies to establish the exact roles of CYP P450 proteins in the virulence of *B. bassiana*.

Proteases

Proteases constitute a large group of hydrolytic enzymes that cleave the peptide bonds of proteins and break them into small peptides and amino acids (Sharma et al. 2006). The production of extracellular proteases is a crucial factor in determining the virulence of *B. bassiana* against the target host. It is believed that after the epicuticle has been broken down by lipases, the invading fungi produces excellent quantities of Pr1 (serine-protease), which degrades the proteinaceous material. The most frequently studied proteolytic enzymes are the subtilisin-like serine-protease Pr1 and trypsin-like protease Pr2. The *Pr1* gene is related to eleven isoforms that have been identified and cloned, including a metalloprotease (St. Leger et al. 1994).

Dhar and Kaur (2010) reported that the protease is one of the most important and earliest enzymes involved in host invasion. Seventeen isolates of *B. bassiana* were evaluated for extracellular protease production, and the highest protease activity was observed during four to six days of culture incubation. Subtilisin type Pr1 and trypsin type Pr2 activities were measured by utilizing different media. Minimal medium supplemented with 1 percent casein showed high protease production, whereas minimal medium supplemented with 2 percent colloidal chitin was also able to induce Pr1 activity. They further reported that 66 KDa protease was commonly observed in all isolates of *B. bassiana*. In the process of enzymatic degradation, the type of chymoelastase serine protease Pr1 served as a crucial cuticle-degrading enzyme because 70% of the cuticle was composed of protein Pr1. Subtilisin type Pr1 has been reported to show a considerable ability to degrade the insect cuticle with a high concentration at the site of the penetration peg or the germ tube (St. Leger et al. 1996). The molecular structure of subtilisin-like protease Pr1 consists of five cysteines forming two disulfide bridges, and the residual cysteine found near the catalytic site consists of Asp39, His69, and Ser224.

Liu et al. (2007) also determined the activities of Pr1 and Pr2 enzymes. They found that proteases are secreted during the first cuticle degradation stage, and they stimulate the signal transduction mechanism by activating protein kinase A (PKA) mediated by AMPc. It validated that the infection of the cuticle initializes the extracellular involvement of protease Pr1 in cuticle penetration. Additionally, protease Pr1 also found as a virulence indicator in several EPF apart from *B. bassiana* (Castellanos-Moguel et al. 2007).

Thakur et al. (2005) identified three different proteases (isoenzymes) in different *B. bassiana* isolates by protease zymography. They reported the variability in the protease activity among the different strains of *B. bassiana*. The variability in the secretion of proteases leads to differences in the virulence of fungal isolates. Recently, Mancillas-Paredes et al. 2019 compared the production of protease in inducing and non-inducing medium and reported the significantly higher production of protease in induction medium and suggested the positive correlation of protease with the virulence of *B. bassiana* against *Zabrotes subfasciatus*.

New strategies such as genetic engineering and recombination of proteases genes have improved the virulence of EPF (Fang et al. 2009). Genetic modification of *B. bassiana* has done through the co-transformation of scorpion *aaIT* and *Metarhizium pr1A* genes. The binary transformants did not show improved insecticidal activity as

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compared to the *aaIT* single transgenic isolates. Hence, it was evident that the PR1A can degrade AAIT when both are present in culture media or insect hemolymph, and provided a reasonable explanation for their non-synergistic effect. These studies conclude that the virulence enhancement seen in binary transformants is mainly due to the secretion of PR1A, which could accelerate cuticle penetration and activate hemolymph prophenoloxidase to melanize the insect body (St. Leger et al. 1992).

In another study, cDNA of the protease gene *CDEP2* isolated from *B. bassiana*. It is combined with the *cry1Ac* gene of *Bacillus thuringiensis*, while the expression of the gene was regulated by the native promoter of the fungus. This experiment improved the insecticidal activity against the *Helicoverpa armigera* larvae (Xia et al. 2009). Overexpression of protease increases the virulence of fungal isolate against the target pest. Hence, proteases can be considered as an essential factor that governs the virulence of *B. bassiana* (Zhang et al. 2008; Fan et al. 2010).

Chitinases

Insect's cuticle is primarily composed of chitin, an unbranched polymer of N-acetyl glucosamine monomers that are linked by β-1,4 glycosidic bonds and chitinases catalyze the hydrolysis of the β-1,4 linkages and resulting in the release of short-chain chitooligomers or monomers. Chitinases are members of glycoside hydrolase (G.H.) families 18 & 19 and widely distributed in fungi, insects, and vertebrates, and do not share similar sequences (Li 2006).

Recent studies have shown that *B. bassiana* produces an extensive amount of endo-chitinases and exo-chitinases, and their activities are positively correlated with the virulence against the insect pests (Sanchez-Perez et al. 2016; Dhawan and Joshi 2017). Endo-chitinases (hydrolyze the β-1,4-glycosidic bond inside the chitin molecule) and exo-chitinases (hydrolyze N-acetylglucosamine oligomers formed during the action of endo-chitinases). The combined action of endo- and exo-chitinases is required for the complete degradation of insect chitin during the infection process.

Several findings state a positive correlation between virulence and chitinase activities of *B. bassiana*. Kim et al. (2010) reported that the higher levels of extracellular chitinases in *B. bassiana* are responsible for virulence towards the aphid *Aphis gossypii*. Matias-Montesinos et al. (2011) found that the higher virulence of the *B. bassiana* mutant was due to the increased production of chitinase. The virulence of the mutant was significantly higher from the wild type. Pelizza et al. (2012) also reported that the fungal isolates of *B. bassiana* with the highest levels of chitinase activities were more pathogenic against *Tropida criscollaris*.

Further, Firouzbakht et al. (2015) compared two different isolates of *B. bassiana* against *Andrallus spinidens* and reported that the chitinase activity is significantly higher in the virulent isolate with lesser LC50 value as compared to less virulent isolate of *B. bassiana*. Perinotto et al. (2014) found that the most virulent strain of EPF against *Rhipicephalus microplus* recorded the maximum chitinase activity. Recently, Alali et al., (2019) isolated thermotolerant strains of *B. bassiana* and reported that the strains with the lowest LD50 and LT50 values possessed significantly higher values of chitinase activity. This suggests a positive correlation of chitinase activity with the pathogenicity and virulence of *B. bassiana*.

B. bassiana expresses 20 different chitinases divided into three subgroups: eight appertaining to subgroup A without a chitin-binding domain (ChBD); four appertaining to subgroup B (one ChBD in the extreme C terminal) and eight appertaining to subgroup C having two ChBD sites (Xie et al., 2012). Interestingly, the two endogenous *Beauveria* chitinases (*chit1* and *chit2*) that appear to respond to host cuticles do not contain ChBD often seen in similar enzymes.

However, Fang et al. (2005) reported that the overproduction of *Bbchit1* enhanced the virulence of *B. bassiana* against *Myzus persicae*, as indicated by a significant reduction in LC50 and LT50 of the transformants compared to the values for the wild-type strain. In another study, the virulence of *B. bassiana* improved against silkworm moth *Bombyx mori* with chitinase production from a recombinant *Bbchit1* gene. The recombinant was constructed by fusing the *Bbchit1* gene with the ChBD under the regulation of the promoter. The overexpression of chitinase leads to significant changes in the virulence (Fan et al. 2007). Hence, the Constitutive expression of a mutated *chit1* gene containing a chitin-binding domain proved to be a better approach for creating virulent strains of *B. bassiana*. Moreover, Pinnamaneni et al. (2010) also found that the genetically modified form of *B. bassiana* is more effective in expressing

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pathogenicity as the exochitinase activity was maximum compared to that of crude extract (Table 1). Hence, the production of extracellular chitinases is an essential factor in governing the virulence of *B. bassiana*.

Antioxidant enzymes

In response to fungal attack insect pest also produces free radicals or reactive oxygen species (ROS) that can cause cell damage by oxidizing cell components, such as DNA, protein, and lipids (Figure 1). *B. bassiana* relies upon antioxidant defense systems to scavenge ROS (Zhang and Feng, 2018). Such systems consist mainly of superoxide dismutases (SODs), catalases (CATs) and thioredoxins (TRXs) (Kwok et al. 2004; Kaur et al. 2017). A suite of superoxide dismutases (SODs), catalases (CATs), and thioredoxin (TRXs) proteins have been investigated in *B. bassiana*. These enzymes play several important physiological roles and help in the pathogenesis of *B. bassiana* (Zhang and Feng 2018).

Superoxide dismutases (SODs)

SODs catalyze the conversion of ROS to peroxide, and five different *B. bassiana* SOD genes have been characterized. Some SODs in *B. bassiana* have been proven as essential factors for its biological control potential and tolerance to environmental stresses, such as high temperature, solar U.V. irradiation (Yao et al. 2010), and fungicide application (Zou et al. 2006; Song et al. 2012). Inactivation of the *Sod* genes through RNAi knockdown resulted in decreased tolerance to peroxide, oxidative stress, and UVA/B exposure. However, a small decrease in virulence has been observed for the mutants during insect bioassays (Li et al. 2015). Moreover, a cytosolic manganese-cored SOD (*Bbsod2*) overexpressed in *Beauveria bassiana* led to significant increases in virulence against target insect pests (Xie et al. 2010).

Catalases (CATs)

Catalases (CATs) catalyze the conversion of peroxide to oxygen and water (Figure 1). Five members in the catalase (CATs) family of *B. bassiana* were functionally characterized by one-by-one gene knockout, followed by enzymatic, transcriptional and phenotypic analyses (Wang et al. 2013). These five different *B. bassiana* CAT genes include *CatA* (spore specific enzyme), *CatB* (secreted), *CatC*, (cytoplasmic), *CatD* (secreted or peroxisomal), and *CatP* (peroxisomal). These CATs reported to contribute to stress response and virulence of *B. bassiana* (Wang et al. 2013). A peroxisomal catalase in *B. bassiana* was clued as an enzyme associated with insect hydrocarbon catabolism due to the increase of its activity by the replacement of glucose with an insect-like hydrocarbon in medium (Pedrini et al., 2006). The overexpression of a single catalase (*cat1*) in *Metarhizium anisopliae* could facilitate conidial germination and enhance the fungal virulence against *Plutella xylostella* larvae (Hernandez et al. 2010). These studies indicate that, like SODs, catalases are involved in mediating not only fungal growth and development but also stress tolerance and virulence to determine the biocontrol potential of *B. bassiana* (Feng et al., 1994).

Chantasingh et al. (2013) identified a set of differentially expressed genes in *B. bassiana* in response to *Spodoptera exigua* larvae. Polymerase chain reaction-Suppression subtractive hybridization (PCR-SSH) was used by which they identified differentially expressed genes during the initial aspects of the fungal-insect interaction. Ten fungal genes were identified by PCR-SSH, and these were further confirmed to be up-regulated by semiquantitative RT-PCR. They further characterized the catalase gene (*catE7*), which is implicated in stress resistance and has a role in the pathogenesis of *B. bassiana*. They constructed a transgenic strain of *B. bassiana*. This strain was overexpressing CatE7, and fungal transformant lines with extra *catE7* copies (Bb::BbcatE7) showed two-fold higher catalase activity than the wild type. Bb::BbcatE7 strains. These strains were found to be germinated faster than the wild-type parent and exhibited significantly higher virulence against *S. exigua* larvae. They suggested that responses mediated by catalases play an important role in the fungal-insect infection process, and manipulation of catalase expression can produce more virulent fungal strains for efficient insect control.

Thioredoxins (TRXs)

TRXs are small molecular weight (12 kDa) oxidoreductase enzymes that help in maintaining the redox balance of the cell. TRXs responds to ROS to regulate a wide range of signaling and developmental processes. They act as

potent antioxidants by catalyzing protein reduction via cysteine thiol-disulfide exchange and are essential in many organisms. Six *B. bassiana* TRXs have been named TRXs 1 to 6, and TRX 1-4 located in the cytoplasm of the fungal cells. Whereas TRX5 located in the nuclear membrane, and TRX6 present in the mitochondrial membrane (Zhang et al. 2015). Targeted gene knockouts of any of the TRX genes resulted in varying degrees of reduced virulence.

Insect defense-related enzymes:

The nutrient and water limiting insect epicuticle, when coupled with the secretion of antifungal compounds, act as a potential defense against the microbial attack. (Ortiz-Urquiza and Keyhani 2014). It has long been recognized that certain insect species are resistant to infection by *B. bassiana*, even though other closely related species are susceptible. *Tribolium castaneum* (the red flour beetle) is one such resistant insect species and produces large amounts of benzoquinones that act as defensive compounds against microbial attack. These benzoquinones provide a strong defense against *the B. bassiana* attack. Some wild-type strains of the *B. bassiana* result recorded only 20-25% mortality when tested against *T. castaneum*. However, *B. bassiana* produces a benzoquinone reductase (BqrA) that can detoxify benzoquinones and targeted gene knockouts of the *BqrA* gene reduced mortality to 10%. A strain overexpressing BqrA led to an increase in virulence to 40 to 45%. (Pedrini et al. 2015). Apart from benzoquinone reductase, there are several reports suggested the role of secondary metabolites and toxins such as oosporein, beauvericin, tenellin, and brassinolide in protecting *B. bassiana* against insect's defense (Ortiz-Urquiza et al. 2013; Gibson et al. 2014).

Conclusion

CDEs and antioxidant enzymes are crucially involved in the pathogenesis of *B. bassiana* and these enzymes determine the fungal virulence. The variations among the *B. bassiana* isolates dependent on the secretion and activities of these enzymes. The relationship of enzymes with the virulence can be exploited as a useful tool to select a most virulent fungal isolates of *B. bassiana* against any target insect pest. This knowledge will contribute to improve the efficiency of biocontrol products, which can serve as alternatives to chemical pesticides to prevent crop loss. Moreover, once specific virulence factors are identified, it will be essential to explore the natural variation (in nucleotide sequence and expression of these genes) in the fungal population. It is also highly relevant to anticipate how insects can undergo adaptation to evade the action of fungal virulence factors and thereby develop resistance to the biocontrol agent. Further, CDEs and antioxidant enzymes can be genetically manipulated to create more virulent strains of *B. bassiana*. However, the use of genetically modified organisms has numerous hurdles that limit their applications in biological control.

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Conflicts of Interest

The authors declare that they have no competing interests.

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Table 1: Enzymes and their encoding genes involved in pathogenesis and physiological processes of *B. bassiana*.

Enzyme	Gene name	Knock-out mutant phenotype	Reference
Chitinase	*Bbchit1*	Overexpression increased infection efficiency	Pinnamaneni et al., 2010
	Chi1, Chi2, ChsA2	N.A.*	Liu *et al.,* 2013
Protease	*Cdep1*	N.A.*	Zhang et al. (2008)
	Pr1, Pr2	N.A.*	Joshi et al. (1995), Dias et al. (2008)
Lipase/Esterase	*Cyp p450s*	N.A.*	Pedrini et al. (2013)
	Bbcyp52x1	Reduced virulence, germination and cuticle breaching	Zhang et al. (2012)
Catalases (CATs)	*CatA-D*, *CatP*	Reduced stress tolerance and decreased virulence	Wang et al., 2013
Superoxide dismutases (SODs)	*Sod1- Sod5*	Reduced stress tolerance and slight decreased virulence	Xie *et al.,* (2012); Xie *et al.,* (2010) and Li et al. (2014)
Thioredoxins (TRXs)	*Trx1- Trx6*	Reduced abiotic stress tolerance, virulence. Decreased germination and conidiation	Zhang et al. (2015)

*N.A. (Not Available)

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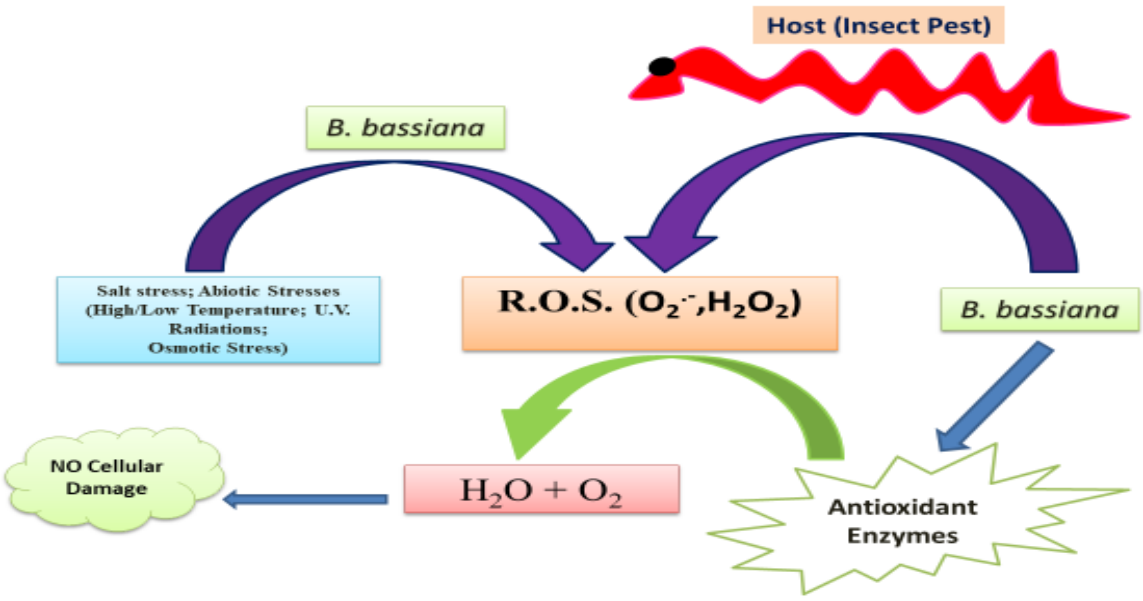

Figure 1: The antioxidant machinery (SODs, CATs, and TRXs) of *B. bassiana* provide protection against abiotic and biotic stresses.