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Growth substrates and caleosin-mediated functions affect conidial virulence in the insect pathogenic fungus *Beauveria bassiana*

Almudena Ortiz-Urquiza[1], Yanhua Fan[2], Timothy Garrett[3], and Nemat O. Keyhani[1]*

[1]Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611

[2]Biotechnology Research Center, Southwest University, Beibei, Chongqing, China

[3]Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, FL 32610

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*Corresponding author:

Nemat O. Keyhani
University of Florida
Dept. of Microbiology and Cell Science
Bldg. 981, Museum Rd.
Gainesville, FL 32611
E-mail: keyhani@ufl.edu
Tel: (352) 392-2488

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ABSTRACT

The entomopathogenic fungus, *Beauveria bassiana*, is a microbial biological control agent capable of infecting a wide range of insect hosts. Conidia (spores) initiate infection via adhesion, growth, and penetration of the insect cuticle, whose outmost layer is rich in lipids. Conidial virulence was investigated in *B. bassiana* wild type and caleosin mutants *(ΔBbcal1*), the latter a protein involved in lipid storage and turnover. Topical insect bioassays revealed that conidia of the wild type strain showed up to 40-fold differences in mean lethal dose (LD_{50}) values depending upon the growth substrate. The most virulent conidia were harvested from potato dextrose agar (PDA) containing oleic acid, and the least potent those derived from Sabouraud dextrose-yeast extract agar (SDAY). However, with the exception of SDAY and Czapek-dox agar derived conidia, in which values were reduced, mean lethal times to kill (LT_{50}) were essentially unaffected. In topical bioassays, the *ΔBbcal1* mutant displayed LD_{50} values 5-40 fold higher than the wild type depending upon the growth substrate, with *ΔBbcal1* conidia derived from SDAY unable to effectively penetrate the host cuticle. The *ΔBbcal1* mutant also showed concomitant dramatic increases in LT_{50} values from an average of ~4.5 for wild type to >8.5 d for the mutant. In contrast, intrahemocoel injection bioassays that bypass cuticle penetration events, revealed only minor effects on virulence for either wild type or *ΔBbcal1* conidia. These data highlight the importance of caleosin-dependent lipid mobilization and/or signaling in cuticle penetration events but suggest their dispensability for immune evasion and within-host growth.

INTRODUCTION

The Ascomycete fungus, *Beauveria bassiana* is among a group of insect pathogens that have been commercialized as biological control agents, especially within the framework of more environmentally friendly alternatives to chemical pesticides and as part of Integrated Pest Management practices (Glare *et al.*, 2012; Lacey *et al.*, 2015). As a broad host range pathogen, *B. bassiana* conidia (spores) can initiate infection essentially anywhere on the insect surface and does not require any specialized route of entry (Ortiz-Urquiza & Keyhani, 2013; Ortiz-Urquiza & Keyhani, 2016). The insect epicuticle, comprised of lipids that include abundant amounts of long chain hydrocarbons, fatty acids, and wax esters, is the first barrier to infection, and the fungus has evolved mechanisms for adhesion, germination on the scant nutrients available, and subsequent penetration of the host exoskeleton (Charnley, 2003; Holder & Keyhani, 2005; Jarrold *et al.*, 2007; Zhang *et al.*, 2011). Insects actively resist infection at this cuticular level, beginning with the epi-cuticule or waxy layer, via production of compounds toxic to microbes, e.g. certain fatty acids, quinones, and formic/acetic acid (Golebiowski *et al.*, 2011; Toledo *et al.*, 2011; Tragust *et al.*, 2013). Insect behavioral modifications aimed towards eliminating the pathogen and/or mitigating its infectivity can include heat seeking, burrowing, and grooming (de Crecy *et al.*, 2009; Roy *et al.*, 2006; Yanagawa & Shimizu, 2007). Such factors as well as insect chemical defenses can lead to an evolutionary arms race between the host and pathogen (Pedrini *et al.*, 2015).

Conidia are the infectious agents most commonly used in pest control formulations, and important knowledge has been gained in our understanding of factors important for conidial viability and application (Faria *et al.*, 2012; Jin *et al.*, 2013; Qin *et al.*, 2014), although other cell types have also been shown to be virulent and of potential commercial use (Holder *et al.*, 2007;

Mascarin *et al.*, 2015). *B. bassiana* biochemical pathways that can utilize fatty acids, aliphatic and methyl branched alkanes, and glycerides as substrates, target insect cuticular lipids (Crespo *et al.*, 2000; Lecuona *et al.*, 1997; Pedrini *et al.*, 2006; Pedrini *et al.*, 2007). These systems include hydrocarbon oxidative pathways containing a set of cytochrome P450 enzymes implicated in lipid assimilation (Pedrini *et al.*, 2010; Pedrini *et al.*, 2013; Zhang *et al.*, 2012). Long chain alkanes, common constituents of the insect epicuticle are degraded by *B. bassiana* to free fatty acids, acylglycerols, and phospholipids, although important aspects of this process, including how lipids are transported into cells and the biochemical mechanisms of lipid storage and mobilization remain poorly understood (Crespo *et al.*, 2008; Pedrini *et al.*, 2013). In addition, it is known that culture conditions can affect virulence and that growth on insect derived alkanes can increase the virulence of conidia as compared to those harvested from standard glucose containing mycological media (Crespo *et al.*, 2002).

Lipid droplets (LDs) are cellular organelles that act as means for lipid storage, impacting metabolism, energy homeostasis, and development (Murphy, 2012; Welte, 2015). LDs consist of a phospholipid monolayer, embedded with various proteins that surround a lipid core chiefly consisting of triacylglyerols (TAGs). Caleosins, first described in plants, are LD-associated proteins containing EF-hand calcium-binding motifs (Naested *et al.*, 2000). Some caleosins are capable of binding heme and have been shown to display peroxygenase activity, implicating these proteins in lipid-mediated signaling, e.g. stress response (Hanano *et al.*, 2006; Partridge & Murphy, 2009). Caleosins are widely distributed in plants, typically found as gene families, and are also found in algae and fungi, but not in animals. While more extensively studied plants, several reports have examined the functions of caleosins in fungi. In *Aspergillus flavus*, a caleosin-like gene, designated *AfPGX*, was shown to exhibit peroxygenase activity and to be

critical for normal growth and development, as well as impacting aflatoxin accumulation (Hanano *et al.*, 2015). In *A. flavus*, deletion of the *AfPGX* gene resulted in severe phenotypes with greatly reduced growth and little to no conidiation apparent. In contrast, targeted gene knockout of the caleosin gene in *B. bassiana* (Δ*Bbcal1*) resulted in little to no effects on vegetative growth and only small effects on conidiation (Fan *et al.*, 2015). Impairment of spore dispersal was noted, apparently due to clumping of the conidia, and a moderate effect was seen with respect to the mean lethal time to kill (LT_{50}) larvae of the greater wax moth, *Galleria mellonella* using topical bioassays, although only a single growth substrate was examined, i.e. the standard mycological media potato dextrose agar (PDA). Here, we sought to expand upon these results to: (1) probe the effect of lipid growth substrates on conidial virulence in terms of both the mean lethal dose to kill hosts (LD_{50}) and LT_{50} values, and (2) determine whether caleosin-dependent reduction in virulence occurred mainly at the pre-penetration/penetration stage and/or further downstream, i.e. during hemocoel proliferation and immune evasion. Our data show that growth substrates have significant effects on wild type virulence, particularly in topical assays. In addition, the contribution of the caleosin to virulence was greater during pre-penetration/penetration events; with more moderate effects seen once the insect cuticle was breached. These results reveal a critical role for caleosin-mediated lipid mobilization and/or signaling events during the initial phases of fungal infection.

METHODS

Fungal strains and culture conditions. The wild-type strain *B. bassiana* ATCC 90517 and a caleosin targeted gene knockout strain (Fan *et al.*, 2015), were routinely grown on potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) and/or Czapek-Dox agar (CZA)

supplemented or modified as indicated. For growth on lipid substrates, PDA was supplemented with 0.25% oleic acid, 0.5% glyceride trioleate (triolein), 0.5% olive oil, or 0.2% hexadecane (C_{16}) prepared in hexane at a concentration of 10%, and added to the media immediately prior to pouring of the plates. For conidial production, agar plates were incubated at 26°C for 21 days and aerial conidia were harvested by flooding the plate with sterile distilled H_2O containing 0.02% Tween-80. Conidial suspensions were filtered through a single layer of Miracloth and final spore concentrations were determined by direct count using a hemocytometer and adjusted to the indicated spore suspension concentrations.

Lipid analyses. Fungal conidia were harvested from various growth conditions including: CZA, SDAY, PDA, and PDA supplemented with either 0.25% oleic acid, 0.5% glyceride trioleate, 0.5% olive oil, or 0.2% alkane (prepared in hexane at a concentration of 10%). All the plates were cultured at 26°C for 30 d before harvesting of conidia. The conidia were harvested in sterilized H_2O and 10^8-10^9 conidia were used for lipid profiles analysis. Lipids were extracted using the Folch method (Folch et al. 1957). Briefly, 30 µL of a 10 µg/mL solution of dilaurylphosphatidylcholine (internal standard) was added, then 1 mL of 2:1 chloroform:methanol containing 100 mg/L of butylated hydroxytoluene (BHT) was added to each sample and mixed. The samples were centrifuged at 10,000xg and the supernatant was transferred to a new tube. Next, 200 µL of 0.9% NaCl was added to induce phase separation. After mixing and gentle centrifugation (1000xg), the chloroform layer was removed and transferred to a clean tube. The extraction process was repeated once on the pellet and the chloroform layers were combined. The combined mixture was dried under a gentle stream of nitrogen. The dried samples were then reconstituted with 300 µL of isopropanol and 2 uL was injected for LC-HRMS analysis.

LC-HRMS analysis was performed on a Thermo Q Exactive with Dionex 3000UHPLC and autosampler. The mass spectrometer was operated in positive heated electrospray ionization mode with the following conditions: 3.5 kV, 300C probe temperature, 30 arb sheath gas, 5 arb aux gas, 1.0 ion sweep gas, s-lens of 35, and 320C heated capillary temperature. Spectra were collected from 200-1200 at 35,000 mass resolution and mass accuracy was 5 ppm or better and tandem mass spectra were collected using data dependent scanning (top 5). Separation was achieved on an Waters BEH C18 50x2.1mm, 1.7µm column with mobile phase A as 60/40 Acetonitrile/water with 0.1% formic acid and 10 mM ammonium formate and mobile phase B was 90/8/2 isopropanol/acetonitrile/water with 0.1% formic acid and 10 mM ammonium formate under gradient elution conditions as previously (Ulmer *et al.*, 2015). Data processing was performed with MZmine 2.20 for peak alignment and feature selection. An in house R built script was used to identify lipids based on tandem mass spectra and reference to known fragmentation pathways (manuscript in preparation).

Insect bioassays. Fungal strains were bioassayed using the greater wax moth *Galleria mellonella* (Pet Solutions, Beavercreek, OH, USA) as the insect host. The larvae were treated topically by dipping for 15 s in solutions of 10^5, 10^6, 10^7, $5x10^7$ and 10^8 conidia/ml harvested in sterile distilled H_2O with 0.02% tween 80. Excess liquid on the insect bodies was removed by placement on a dry paper towel. Control larvae were treated with sterile dH_2O. Mortality was recorded every 24 h and the median lethal dose and mortality time ($LD_{\underline{50}}$ and LT_{50}) was determined by Probit analysis. Each treatment consisted of three replicates with at least 25 insects each, and the entire experiment was repeated three times with different batches of fungal conidia. Additionally, conidia from both WT and *ΔBbCal1* strains were injected into *G. mellonella* larvae. Each larva was injected with 800 conidia using a 1 ml syringe coupled to a

programmable syringe pump (World Precision Instruments, Sarasota FL). Three replicates with 20 insects each were used for every treatment and the whole experiment was repeated three times with different batches of conidia.

RESULTS

Growth substrates and caleosin functioning affect virulence in topical insect bioassays

Wild type and a targeted gene knockout of the single identified caleosin gene in *B. bassiana* (Δ*Bbcal1*, (Fan *et al.*, 2015)), were grown on a variety of substrates including (1) standard complex mycological media (SDAY and PDA), (2) minimal mycological media containing sucrose as the carbon source (CZA), and (3) PDA supplemented with various lipids including hexadecane (C_{16}), oleic acid, triolein, and olive oil. No obvious differences were noted in growth rate on the various media and conidia were harvested after 21 d of growth as detailed in the Methods section. In order to calculate LD_{50} values, five different conidial concentrations namely; 10^5, 10^6, 10^7, 5 x 10^7, and 10^8 cells/ml were used in insect bioassays. *G. mellonella* larvae were treated topically with fungal cell suspensions as detailed in the Methods section. Wild type cells harvested from the standard mycological media- PDA and CZA, displayed similar LD_{50} (at 9 d) values of ~ 1.5 x 10^6 conidia/ml (Table 1), with an ~10-fold decrease in virulence (i.e. 10-fold higher LD_{50} value) seen for wild type conidia isolated from SDAY ($LD_{50}^{Wt\text{-}SDAY}$ = 15.4 x 10^6 conidia/ml). The Δ*Bbcal1* mutant fared worse, with LD_{50} values 25-40-fold higher than wild type when derived from PDA and CZA media. *ΔBbcal1* conidia isolated from SDAY were the least virulent of the conditions tested, being reduced to 77.1 x 10^6 conidia/ml, 5-fold lower than wild type cells isolated from SDAY.

For the wild type strain, supplementation of PDA media with C_{16}, olive oil, or oleic acid resulted in a 2-4 fold decrease (i.e. increased virulence) in LD_{50}^{Wt} values as compared to PDA.

In contrast, conidia isolated from PDA + triolein displayed an LD_{50}^{Wt} = 3.62 x 10^6 conidia/ml, represented an ~2-fold increase as compared to PDA. Conidia derived from the *ΔBbcal1* mutant, isolated from the same media, i.e. PDA + C16 (LD_{50} = 23.1 x 10^6 conidia/ml), PDA + triolein (LD50 = 18.1 x 10^6), PDA + olive oil (LD_{50} = 11.7 x 10^6), and PDA + oleic acid (15.6 x 10^6) were (2-3 fold) more virulent than mutant conidia harvested from PDA alone, but were still 5-45 fold less effective than wild type cells grown under correspondingly identical conditions.

For *B. bassiana* wild type, with the exception of conidia derived from CZA and SDAY in which increases in the mean lethal time to kill (LT_{50}) was seen, little differences were seen in regards to LT_{50} values between cells grown on PDA, and PDA supplemented with either C_{16}, triolein, olive oil, or oleic acid, with values ranging from 4.31-4.98 d (Fig. 1, Table 1). As compared to PDA, an increase of ~ 1 and a more dramatic 2 d (reflecting decreased virulence) in the LT_{50} was seen for the wild type conidia derived from CZA and SDAY. The *ΔBbcal1* mutant displayed severely reduced LT_{50} values overall, and with the exceptions of conidia from SDAY and PDA + olive oil, requiring 3-4 d longer to kill 50% of infected hosts as compared to corresponding wild type cells. Conidia from PDA + olive oil displayed $LT_{50}^{\Delta Bbcal1\text{-PDA-olive oil}}$ = 6.52 d, which was 2 d more than its corresponding wild type, and *ΔBbcal1* conidia isolated from SDAY were almost avirulent and an accurate LT_{50} value could not be calculated for these cells.

Minor impairment of virulence after intrahemocoel injection

Direct injection of fungal spores into the host hemocoel bypasses penetration events, while maintaining the requirement for hemolymph proliferation and immune evasion. For wild type *B. bassiana* cells, with the exception of conidia harvested from PDA + oleic acid, the mean lethal times to kill (LT_{50}) values were essentially unaffected when comparing cells grown on PDA, CZA, SDAY, PDA + C16, PDA + triolein, and PDA + olive oil using intrahemocoel

injection assays into *G. mellonella* larvae (Table 2, Figure 2). Under these conditions LT_{50}^{Wt} values ranged from 2.42-2.93 d. A moderate decrease (~ 1 d) in the wild type LT_{50} was seen for conidia harvested from PDA + oleic acid (to 3.62 d). In general, small (< 0.5 d for CZA, PDA + triolein, and PDA + oleic acid) to moderate (~ 1 d, PDA, PDA + C16, and PDA + olive oil) increases in LT_{50} values were seen for the *Bbcal1* knockout mutant as compared to their corresponding wild type conidia. *B. bassiana ΔBbcal1* conidia harvested from SDAY were more severely affected, showing a 3 d increase in LT_{50} values as compared to wild type cells.

Regardless of the mode of infection, i.e. for both topical infection and intrahemocoel injection assays, visual inspection of the cadavers revealed alterations in the melanization patterns during infection and death of the insect. Infection of *G. mellonella* larvae by the wild type strain results in a characteristic gradual darkening (melanization) of the insect during the course of the infection, which by the time the infected insect is near death or has died (< 24 h post-mortality) renders the cuticle a brown to dark brown discoloration (Fig. 3). In contrast, at or immediately following death of larvae infected by the *ΔBbcal1* strain only discrete patches of melanization are visible on the insects, and melanization over the entire cuticle as seen for wild type infections does not occur. Within 5-7 d post mortality, a profusion of mycelia and conidiation is seen for both the wild type and *ΔBbcal1* strains on infected cadavers (Fig. 3).

Neutral lipid analysis in *B. bassiana*

Changes in total neutral lipid contents, i.e. diacylglycerol (DAG) and triacylglycerol (TAG) levels in the wild type and *ΔBbcal1* strains were examined in conidia harvested from different growth substrates. Growth substrates included PDA, CZA, SDAY, and PDA supplemented with oleic acid, C16, olive oil, and glycerol trioleate. No significant changes in DAG content was seen between the wild type and *ΔBbcal1* mutant strain in conidia isolated from

231 the various growth substrates with the exception of growth on olive oil, in which the DAG
232 content in the *ΔBbcal1* mutant was significantly higher than the wild type (Fig. 4A, $P < 0.05$).
233 TAG content was much higher (20-30X) than DAG content in the cells examined, however, no
234 significant differences were noted between the wild type and mutant strains in TAG content,
235 although under a number of conditions, i.e. CZA and PDA + oleic acid, a large variation was
236 seen (Fig. 4B).
237

238 **DISCUSSION**

239 Media composition, i.e. the growth substrates from which fungal spores are isolated, is
240 known to influence virulence of fungal insect pathogens (Kim *et al.*, 2014; Maldonado-Blanco *et*
241 *al.*, 2014; Pelizza *et al.*, 2011), with complex relationships between various spore parameters,
242 e.g. stress response, germination rate and cuticle degrading enzyme activities have been reported
243 (Mascarin *et al.*, 2013; Rosas-Garcia *et al.*, 2014). Conidia derived from media containing lower
244 carbon/nitrogen ratios, including those derived from insect passage but subsequently grown on
245 different synthetic media, were found to display lower LT_{50} values (i.e. were more virulence)
246 (Safavi *et al.*, 2007). However, it has also been reported that *B. bassiana* conidia isolated directly
247 from insect cadavers were less virulent that those harvested from rice or synthetic media and the
248 method of application was found to influence virulence (Santoro *et al.*, 2007). A comparison of
249 *B. bassiana* grown on colloidal chitin, insect (*Sphenarium purpurascens*) cuticle, wheat bran, or
250 Sabouraud-dextrose agar (SDA), revealed similar LT_{50} values for all conidia against adults of the
251 mealworm beetle (*Tenebrio molitor*), but differential mortality against *T. molitor* larvae
252 (Rodriguez-Gomez *et al.*, 2009). Similarly, small effects were reported for the entomopathogenic
253 fungus *Metarhizium anisopliae* when conidia were isolated from media containing various

carbon and nitrogen ratios and only moderate correlations were seen between protease and lipase activities and virulence when tested against larvae of the diamondback moth, (*Plutella xylosta*) (Wu *et al.*, 2010). Stress conditions have also been shown to affect *M. anisopliae* virulence, with the highest mortality reported for conidia grown on minimal media containing lactose (~ = CZA) (Rangel *et al.*, 2008). *B. bassiana* conidia grown on C_{16} as the sole carbon source displayed decreased LT_{50} values (increased virulence) when tested against the bean weevil (*Acanthoscelides obtectus*) as compared to cells isolated from glucose grown agar (Crespo *et al.*, 2002), and inducible pathways for assimilation of long chain hydrocarbons that are prevalent on the insect epicuticle have been reported (Pedrini *et al.*, 2010; Zhang *et al.*, 2012). For the most part, however, relatively small effects have been reported and only the LT_{50} parameter examined.

Our data show that for *B. bassiana* wild type, grown on standard PDA and CZA mycological media produces conidia that have lower LD_{50} values (15-fold more infective) when tested using topical bioassays, as compared to the carbon/nitrogen rich media, SDAY. Conidia isolated from PDA were more efficacious than those derived from either CZA or SDAY, with the latter showing a dramatic ~2 d shift in LT_{50}. These data are in general agreement with previous reports (see above) indicating that production on more minimal media results in more virulent spores. Amongst the mid-to long-chain alkanes, C_{16} is known to be one of the preferred carbon sources for *B. bassiana*, and oleic acid can be used as an energy source that can feed directly into lipid droplet formation pathways (Pedrini *et al.*, 2010; Pedrini *et al.*, 2013). Olive oil consists of TAGs and small amounts of free saturated (palmitic; 13% and stearic; 1.5%) and unsaturated fatty acids (oleic; 70%, linoleic; 15%, palmitoleic; 0.3-3.5%, and α-linolenic; 0.5%), and these minor constituents may act to induce other aspects of fatty acid metabolism. Supplementation of PDA with C_{16}, olive oil, or oleic acid increased the infectivity of conidia 2-4

277 fold but had little effect on the efficacy of the conidia. However, conidia isolated from PDA
278 containing triolein (TAG, glycerol trioleate) were 2-fold less infective than those isolated on
279 PDA alone, although equally efficacious. These data imply that the components of olive oil, i.e.
280 free fatty acids, mixture of TAGs, and/or other compounds, or the combined constituents result
281 in the production of more virulent conidia.

282 In addition to the effects on virulence, a distinct alteration in host melanization that
283 occurs during the infection process was noted. During wild type infections, whether topical or
284 via artificial intrahemocoel injection of the fungal conidia, a gradual darkening of the insect
285 cuticle occurs up to and after mortality of the insect. During the last stages of infection, as the
286 insect is dying, internal fungal hypha penetrate outwards, growing as mycelia and sporulating on
287 the cadaver within 5-6 d post-mortality (Ortiz-Urquiza & Keyhani, 2013; Ortiz-Urquiza &
288 Keyhani, 2016). This host melanization is typically considered to be part of the host defense
289 response, however, in the *ΔBbcal1* mutant, which is impaired in virulence, host melanization
290 also appears to be dramatically reduced, with only small, localized melanized patches visible on
291 infected host during the time of death. The darkening of the host cuticle may also be linked to the
292 production to fungal secondary metabolites. This raises an intriguing alternative hypothesis that
293 this melanization response during the late stages of infection is actively induced by the fungus
294 rather than acting as a defense response or a lack of production of critical late stage fungal
295 metabolites occurs in the caleosin mutant. Although speculative, this may help fungal infection
296 in several ways including by diverting resources away from other defense responses and/or
297 minimizing potential competition by other microbes as the insect dies.

298 Our data strongly support the idea of growth substrate “priming” of conidia. This priming
299 may entail several processes that can include (pre-) induction of pathways in conidia via

accumulation of (1) gene transcripts and/or proteins (e.g. enzyme, transporters, and regulators) as determined by the original growth substrate that would allow for utilization of similar carbon and nitrogen sources more rapidly, (2) metabolites and energy stores that can act as stress response modulators and rapid sources of energy, and/or (3) factors that directly affect host interactions, e.g. cuticle degrading enzymes, secondary metabolites and toxins, compounds needed for adhesion, more rapid germination, and penetration of insect cuticle. As expected, the neutral lipids seen in the fungal conidia were mainly composed of TAGs (10-30-fold higher as compared to DAGs), however little difference was seen between either TAG or DAG content between the wild type and *ΔBbcal1* mutant. This is in contrast to significant changes seen in phospholipid, ceramide, and even ergosterol levels in the caleosin mutant as compared to the wild type (Fan *et al.*, 2015). The only significant difference between the wild type and *ΔBbcal1* mutant was seen in total DAG content when grown on PDA containing olive oil, intriguingly these conditions also result in the formation of copious amounts of lipid droplets in the mutant fungal cells (Fan *et al.*, 2015).

LD formation has been linked to virulence in a number of fungi including via regulation of cellular DAG in the rice blast fungus, *Magnoporthe oryzae* (Abu Sadat *et al.*, 2014), by the fat storage-inducing transmembrane protein 2 (FIT2) in *Candida parapsilosis*, and through the activity of a glycerol-3-phosphate acetyltransferase that contributes to TAG biosynthesis in *M. roberstsii* (formerly *M. anisopliae*) (Gao *et al.*, 2013), where lipid metabolism has also been linked to autophagy (Duan *et al.*, 2013). In the plant fungal pathogen *Colletotrichum orbiculare*, LDs appear to accumulate and then disappear during appressorial maturation, the latter specialized fungal infection structures used to penetrate host tissues (Asakura *et al.*, 2012), and a perilipin (Plin1 homolog), a major protein constituent of LDs, has been implicated in LD

maintenance, appressorial turgor pressure, and virulence in *M. robertsii*, (Wang & Leger, 2007). These data suggest the importance of lipid mobilization in infection by certain fungi. A *B. bassiana* caleosin (Bbcal1), another protein constituent of LDs has recently been characterized (Fan *et al.*, 2015). Targeted gene inactivation of *Bbcal1* did not significantly affect normal growth and germination or stress response, however, altered cellular phospholipid profiles were noted and changes intracellular vesicle-like structures, that may have represented distorted LDs, vacuoles, and/or endoplasmic reticulum elements, were seen. In addition, a decrease in the LT_{50} was seen in topical insect bioassays from conidia harvested from PDA plates. Here we have extended the analysis of the virulence deficiency of Δ*Bbcal1* mutants to examine both infectivity and efficacy, and in particular, to determine whether impairment occurred during pre/penetration events or post-penetration, the latter during growth in the insect hemocoel and requiring competent immune evasion. Conidia of the Δ*Bbcal1* mutant derived from PDA showed a >20-fold decrease in infectivity as compared to wild type and a dramatic loss (4 d) of efficacy in topical bioassays. On CZA, infectivity was even lower (~ 40-fold compared to wild type CZA conidia), and efficacy was also 4 d lower than the wild type counterpart. Δ*Bbcal1* conidia harvested from SDAY were the least infectious of all conditions tested, although were only 5-fold lower than SDAY-wild type conidia. However, the efficacy of the Δ*Bbcal1*^{SDAY} conidia was so low that an accurate LD_{50} could not be calculated. These data indicate that caleosin functioning is critical for both infectivity and efficacy of *B. bassiana* infection of *G. mellonella* larvae. Addition of C16, olive oil, of oleic acid to PDA improved the infectivity of the resultant *ΔBbcal1* conidia, however LD_{50} values were still 20-44 fold higher than their wild type counterparts. The only exception in the trends observed was seen using PDA + triolein, in which conidia fared worse (~2-fold) in terms of infectivity than those isolated from PDA for the wild

346 type, whereas they fared better (~2-fold) for the Δ*Bbcal1* mutant. However, overall, the
347 Δ*Bbcal1*^{triolien} conidia were 5-fold less infective than their wild type counterparts. These data
348 imply that loss of caleosin functioning impacts the ameliorating effects of growth on the various
349 lipid substrates in terms of infectivity. The efficacy of the Δ*Bbcal1* mutants grown in the
350 presence of various lipids was also significantly affected; ~3-4 d increase for PDA + C16,
351 triolein, and oleic acid, but only an ~ 2 d increase for PDA + olive oil.

352 In conclusion, our data illustrate two key points concerning insect virulence mediated by
353 the entomopathogenic fungus, *B. bassiana*. The first is the growth substrate dependence on the
354 virulence of resultant conidia, with the major effect seen with respect (1) to topical infection with
355 in general minor effects seen once the cuticle has been breached, and (2) to infectivity and only
356 minor effects seen with respect to efficacy. Growth substrates that included lipids commonly
357 found on insect cuticles generally increased (topical) infectivity, and the C/N rich media, SDAY,
358 resulted in spores with lower infectivity and efficacy. These data are potentially useful in
359 production strategies for the biological control agent. The inability to properly produce an/or
360 regulate lipid droplet formation and turnover via a caleosin dependent pathway, significantly
361 decreased both infectivity and efficacy, with the major effect seen in topical assays. These latter
362 data support a model in which lipid mobilization is critical for pre- and/or penetration events, but
363 less important for subsequent proliferation within the hemocoel and immune evasion. Important
364 questions that remain include determining the molecular contributions and functioning of the
365 caleosin within the context of LDs, interacting proteins, and their regulation in fungi.

366

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Table 1. Calculated LD_{50} and LT_{50} values derived from topical infection of *B. bassiana* wild type and Δ*Bbcal1* conidia harvested from different substrate media using *G. mellonella* larval insect bioassays

	LD_{50} (x10^6 conidia/ml)		LT_{50} (days)[2]	
Growth substrate[1]	WT	*ΔBbCal1*	WT	*ΔBbCal1*
PDA	1.52 ± 0.20	36.43 ± 4.71	4.61 ± 0.13	8.69 ± 0.35
CZ	1.34 ± 0.22	51.90 ± 3,71	5.4 ± 0.38	8.75 ± 0.37
SDAY	15.41 ± 2.33	77.07 ± 1.30	6.59 ± 0.7	-
PDA-C_{16}	0.83 ± 0.11	23.12 ± 1.80	4.8 ± 0.20	8.66 ± 0.51
PDA + triolein	3.62 ± 0.14	18.08 ± 0.21	4.31 ± 0.11	7.9 ± 0.32
PDA + olive oil	0.49 ± 0.07	11.74 ± 0.79	4.48 ± 0.07	6.52 ± 0.23
PDA + oleic acid	0.35 ± 0.04	15.57 ± 1.57	4.98 ± 0.15	8.00 ± 0.30

Values indicate Mean ± SE

[1]Conidia were harvested from indicated agar media.

[2]Calculated using 5 x 10^7 conidia/ml.

Table 2. Calculated LT_{50} values derived from intrahemocoel injection of *B. bassiana* wild type and Δ*Bbcal1* conidia into *G. mellonella* larvae

	LT_{50} (days)[1]	
Growth substrate	WT	*ΔBbCal1*
PDA	2.67 ± 0.10	3.32 ± 0.33
CZ	2.93 ± 0.28	2.97 ± 0.34
SDAY	2.99 ± 0.10	5.97 ± 0.45
PDA + C_{16}	2.51 ± 0.16	3.46 ± 0.22
PDA + triolein	2.42 ± 0.11	3.02 ± 0.08
PDA + olive oil	2.57 ± 0.14	3.47 ± 0.12
PDA + oleic acid	3.62 ± 0.17	3.67 ± 0.21

[1]LT_{50} calculated using 800 conidia/larval injection. Values indicate Mean ± SE

614

615 **FIGURE LEGENDS**

616 **Fig. 1. Topical insect bioassays.** Larvae of the Greater waxmoth, *G. mellonella*, were topically
617 treated with conidia derived from *B. bassiana* wild type (blue lines, filled symbols) or the
618 Δ*Bbcal1* mutant (red lines, open symbols). (A) Infections were initiated using conidia harvested
619 from PDA (squares), CZA (triangles, dashed lines), and SDAY (circles, dotted lines). (B)
620 Infections were initiated using conidia harvested from PDA + C16 (diamonds) and PDA + oleic
621 acid (circles, dashed lines). (C) Infections were initiated using conidia harvested from PDA +
622 olive oil (squares) and PDA + triolein (circles, dotted lines). Mock treated controls for each
623 graph are included (●). Data are shown using a cell concentration of 5 x 10^7 conidia/ml. The
624 percentage mortality ± SE over the indicated time course is presented.

625 **Fig. 2. Intrahemocoel injection insect bioassays.** Larvae of the Greater waxmoth, *G.*
626 *mellonella*, were injected with conidia (800 conidia/larvae) derived from *B. bassiana* wild type
627 (blue lines, filled symbols) or the Δ*Bbcal1* mutant (red lines, open symbols). (A) For the wild
628 type, cells derived from PDA, CZA, and SDAY gave essentially the same curves and are
629 represented by a single line (blue squares). For the Δ*Bbcal1* mutant, infections were initiated
630 using conidia harvested from PDA (squares), CZA (triangles, dashed lines), and SDAY (circles,
631 dash-dotted lines). (B) Infections were initiated using conidia harvested from PDA + C16
632 (diamonds) and PDA + oleic acid (circles, dashed lines). (C) Infections were initiated using
633 conidia harvested from PDA + olive oil (squares) and PDA + triolein (circles, dotted lines).
634 Mock treated controls for each graph are included (●). The percentage mortality ± SE over the
635 indicated time course is presented.

636 **Fig. 3. Melanization and fungal growth on *G. mellonella* larvae.** Representative images of *G.*
637 *mellonella* larvae topically infected with wild type and *ΔBbcal1* conidia at or near the onset of
638 mortality (top panels) and 5-6 d post-mortality (bottom panels). Similar results were obtained
639 when *G. mellonella* larvae were assayed via intrahemocoel injection (data not shown).

640 **Fig. 4. Diacylglycerol (DG, A) and triacylglycerol (TG, B) content in wild type and *ΔBbcal1***
641 **conidia.** DG and TG levels were examined in *B. bassiana* wild type and *ΔBbcal1* mutant conidia
642 harvested from PDA, CZA, SDAY, PDA + oleic acid, PDA + C16, PDA + olive oil, and PDA +
643 glycerol trioleate as detailed in the Methods section. Error bars ± SE.

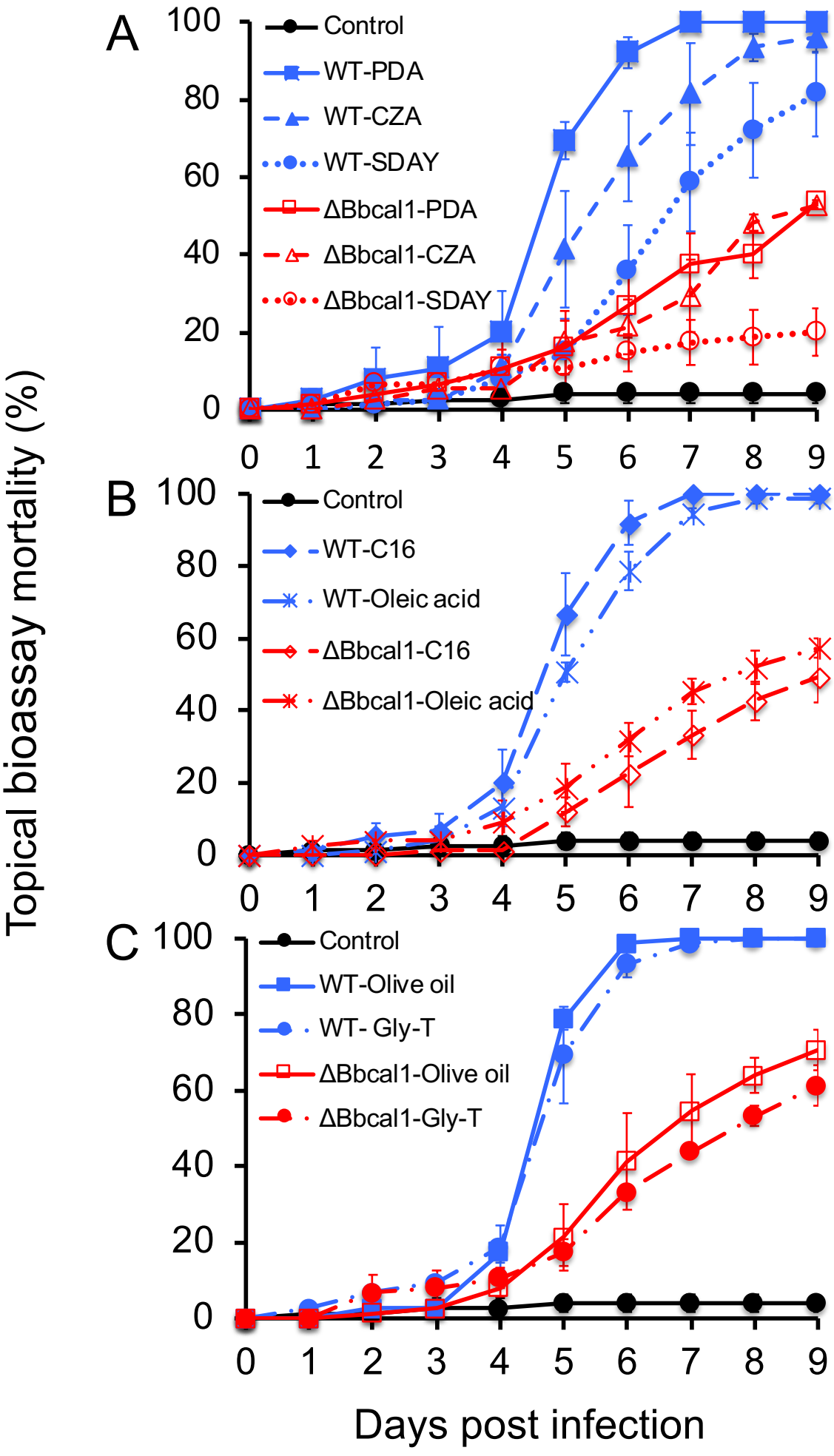
A
B
C
Control
WT-PDA
WT-CZA
WT-SDAY
ΔBbcal1-PDA
ΔBbcal1-CZA
ΔBbcal1-SDAY
WT-C16
WT-Oleic acid
ΔBbcal1-C16
ΔBbcal1-Oleic acid
WT-Olive oil
WT- Gly-T
ΔBbcal1-Olive oil
ΔBbcal1-Gly-T
Topical bioassay mortality (%)
Days post infection
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8 9

Figure 1

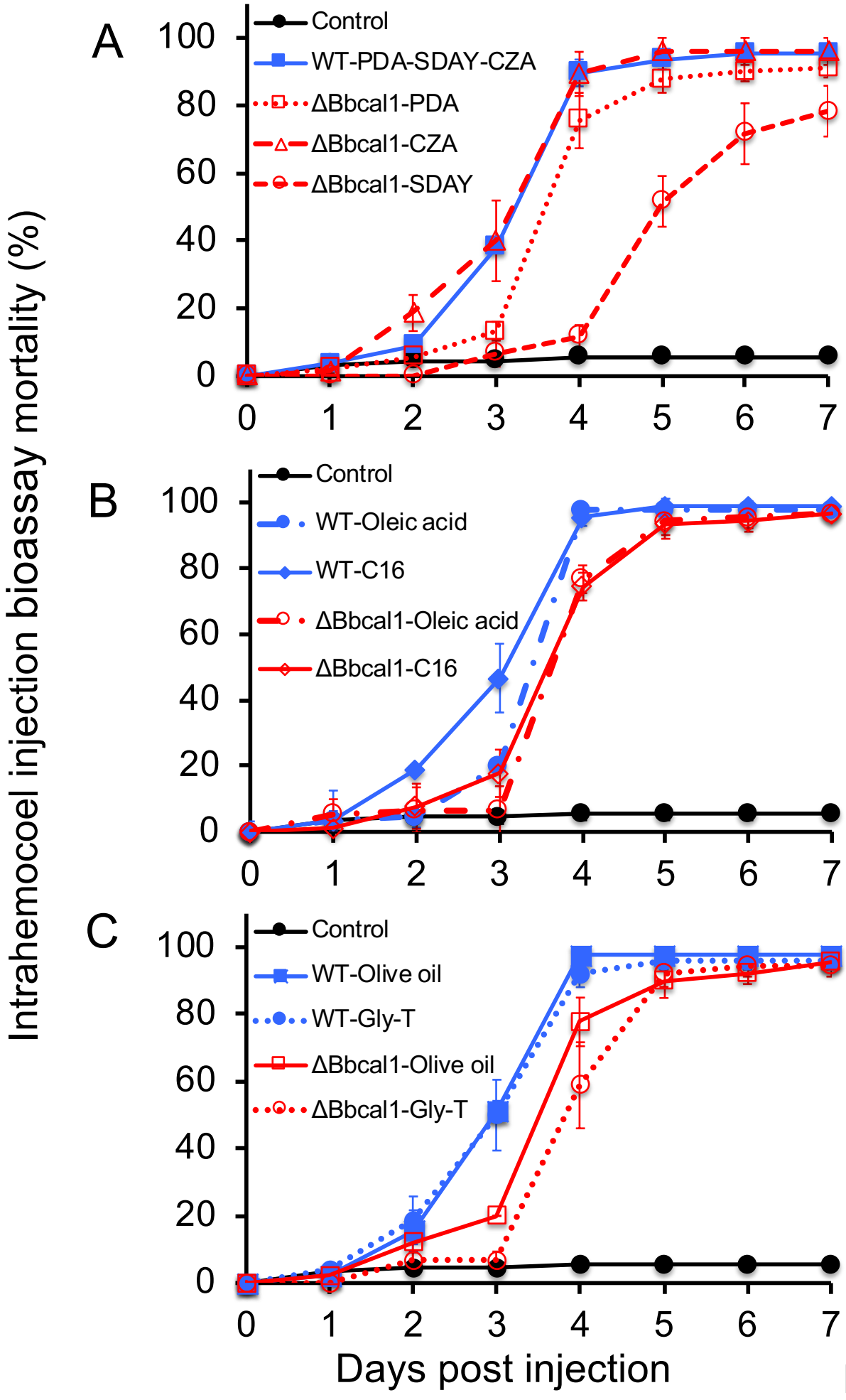
A
B
C
Control
WT-PDA-SDAY-CZA
ΔBbcal1-PDA
ΔBbcal1-CZA
ΔBbcal1-SDAY
WT-Oleic acid
WT-C16
ΔBbcal1-Oleic acid
ΔBbcal1-C16
WT-Olive oil
WT-Gly-T
ΔBbcal1-Olive oil
ΔBbcal1-Gly-T
Intrahemocoel injection bioassay mortality (%)
Days post injection
0
20
40
60
80
100
0
1
2
3
4
5
6
7

Figure 2

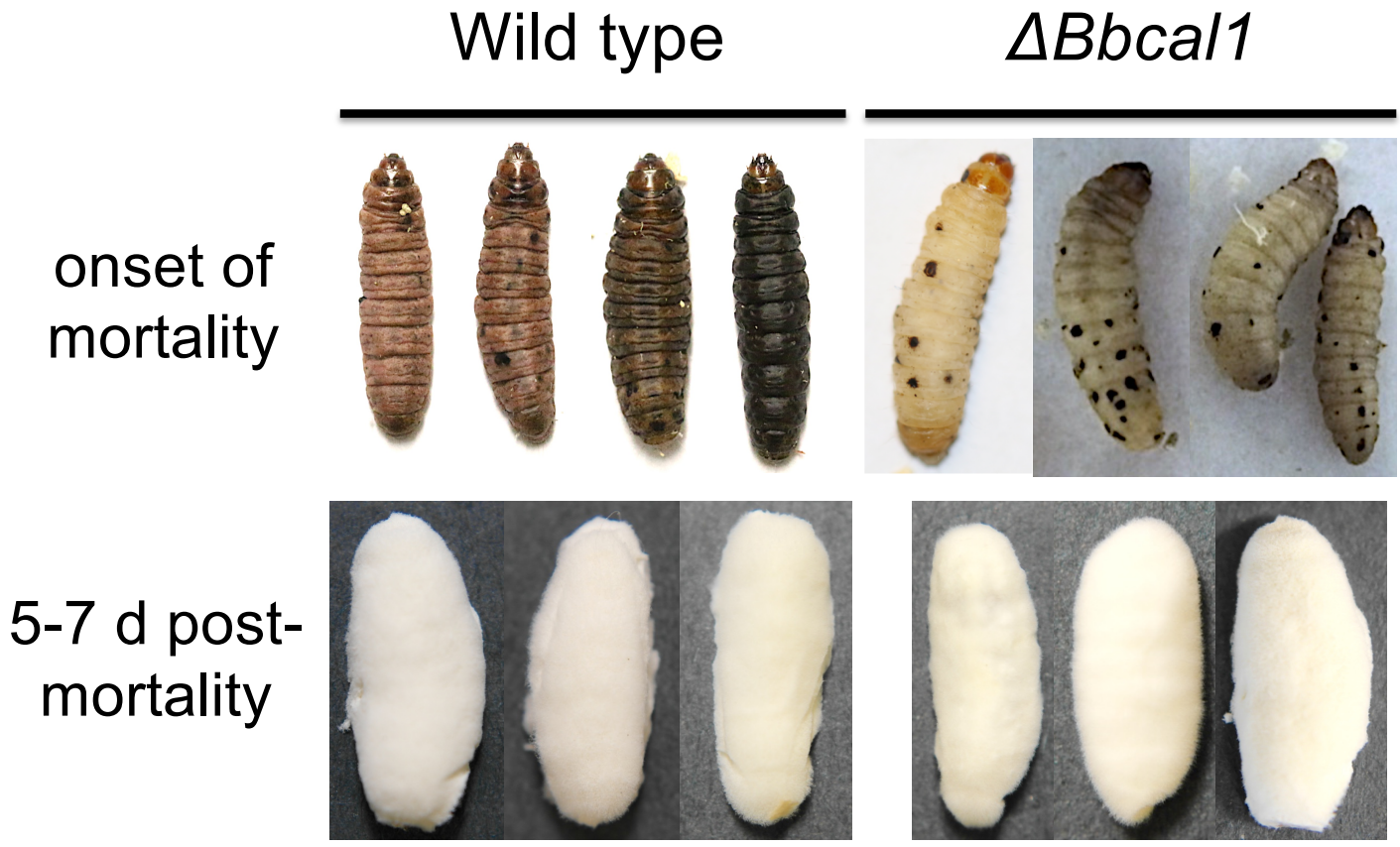

Figure 3

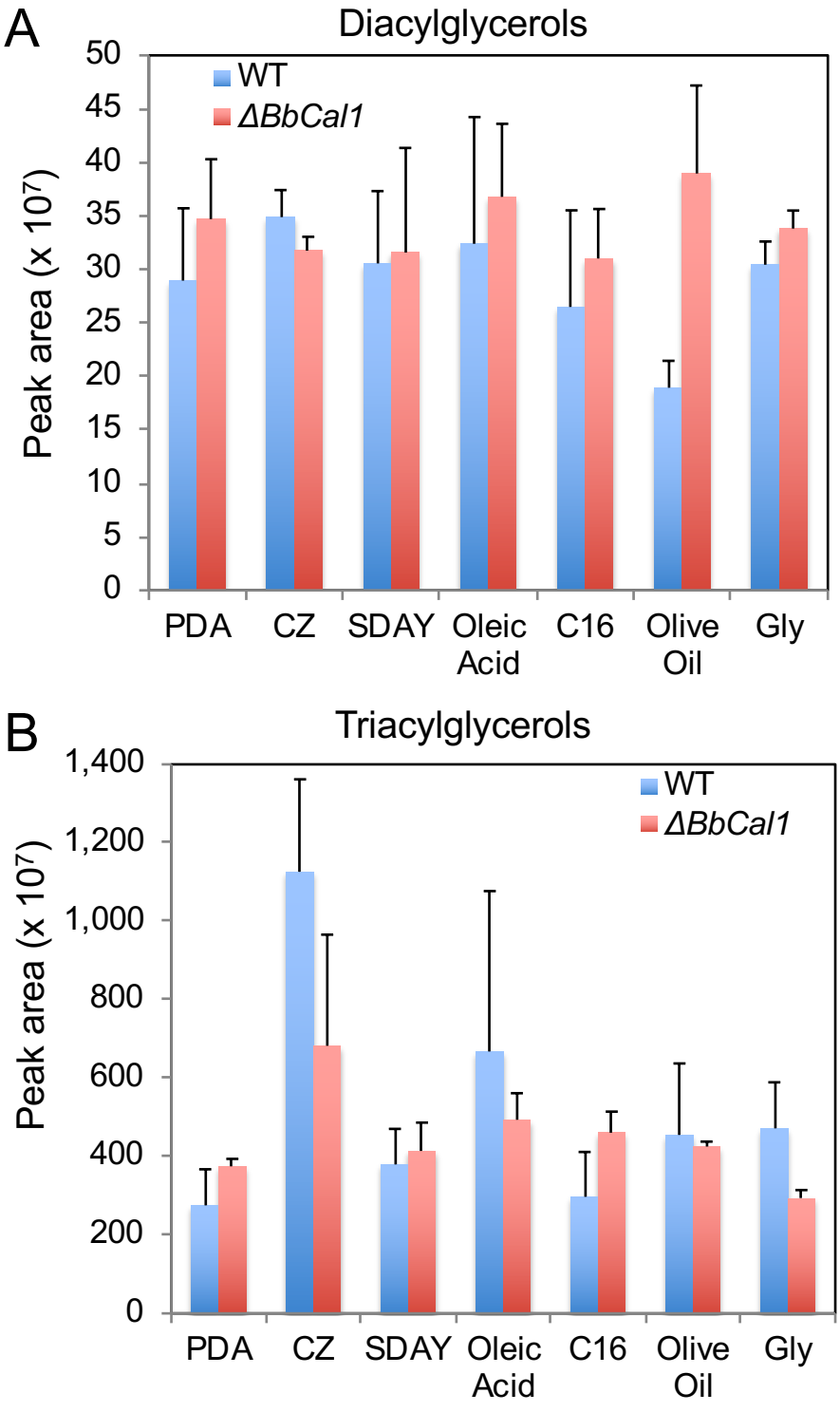
A
Diacylglycerols
WT
ΔBbCal1
Peak area (x 10^7)
50
45
40
35
30
25
20
15
10
5
0
PDA
CZ
SDAY
Oleic Acid
C16
Olive Oil
Gly
B
Triacylglycerols
WT
ΔBbCal1
Peak area (x 10^7)
1,400
1,200
1,000
800
600
400
200
0
PDA
CZ
SDAY
Oleic Acid
C16
Olive Oil
Gly

Figure 4