Hydrophobins contribute to root colonization and stress responses in the rhizosphere-competent insect pathogenic fungus *Beauveria bassiana*

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Abstract  
The *hyd1/hyd2* hydrophobins are important constituents of the conidial cell wall of the insect pathogenic fungus *Beauveria bassiana*. This fungus can also form intimate associations with several plant species. Here, we show that inactivation of two Class I hydrophobin genes, *hyd1* or *hyd2*, significantly decreases the interaction of *B. bassiana* with bean roots. Curiously, the Δ*hyd1*/Δ*hyd2* double mutant was less impaired in root association than Δ*hyd1* or Δ*hyd2*. Loss of *hyd* genes affected growth rate, conidiation ability and oosporein production. Expression patterns for genes involved in conidiation, cell wall integrity, insect virulence, signal transduction, adhesion, hydrophobicity and oosporein production were screened in the deletion mutants grown in different conditions. Repression of the major MAP-Kinase signal transduction pathways (*Slt2* MAPK pathway) was observed that was more pronounced in the single versus double *hyd* mutants under certain conditions. The Δ*hyd1*/Δ*hyd2* double mutant showed up-regulation of the *Hog1* MAPK and the *Msn2* transcription factor under certain conditions when compared to the wild-type or single *hyd* mutants. The expression of the *bad2* adhesin and the oosporein polyketide synthase 9 gene was severely reduced in all of the mutants. On the other hand, fewer changes were observed in the expression of key conidiation and cell wall integrity genes in *hyd* mutants compared to wild-type. Taken together, the data from this study indicated pleiotropic consequences of deletion of *hyd1* and *hyd2* on signalling and stress pathways as well as the ability of the fungus to form stable associations with plant roots.

INTRODUCTION  
Fungi capable of killing insects are recognised as biological control agents and several commercial products based on these organisms are currently available worldwide [1, 2]. More recently, several fungal species that are categorised as entomopathogens have also been isolated as epiphytic and endophytic from a wide variety of different plants which are termed as endophytic insect pathogenic fungi (EIPF) [3]. EIPF are mainly Ascomycetes and have been reported in various genera including, *Acremonium*, *Metarhizium*, *Beauveria*, *Isaria*, *Cladosporium*, *Lecanicillium* and *Trichoderma* [4, 5]. These findings have opened up new avenues for research and application, particularly the use of these fungi as plant growth promoters, beneficial rhizosphere colonizers and/or as antagonists to plant pathogens. In order to utilise these fungi in an agricultural setting, it is important to know the specific aspects of interaction of EIPF to their plant hosts.

For EIPF, the initial process of adhesion of fungal conidia to insect or plant host surfaces is critical for the successful maintenance of pathogenic (to insects) and/or mutualistic (to plants) relationships [6, 7]. Attachment of pathogenic or symbiotic fungal cells to different biological surfaces often involves both specific and non-specific interactions, either of which may precede the other [8]. Specific interactions are mediated by receptor-ligand-like interactions and are generally reversible occurring either via binding of adhesion moieties within the fungal cell wall to receptors present on the host cell surface or vice versa via fungal receptors that bind to host-cell surface features. Cell surface features, e.g. ionic charge and hydrophobicity, which can be affected by specific proteins, mediate non-specific interactions. Specific adhesion
protein mediated interactions have been demonstrated in *Metarhizium* spp. that expresses two specific adhesins, Mad1 and Mad2 [9, 10]. Loss of the Mad1 via targeted gene inactivation, resulted in loss of binding to plant surfaces and decreased ability of the fungus to form rhizosphere interactions with plant roots and Mad2 deletion mutant was impaired in binding of conidia to the insect surface and displayed reduced virulence [9]. Non-specific interactions can be mediated by various cell surface epitopes including carbohydrates and lipids as well as by proteins. Hydrophobins are amphipathic proteins that can self-assemble at water-air interfaces, and are unique to fungi [11, 12]. These proteins constitute what is known as the ‘rodlet layer’ on fungal spores, due to the characteristic morphology these proteins impart on the fungal cell surface [13, 14]. The importance of hydrophobins during plant pathogenesis has been reported in several fungal species including *Magnaporthe oryzae* and *Clostridium propionicum* [15–18].

*Beauveria bassiana* is an EIPF in which both insect pathogenic and plant mutualism (potential endophytism) mechanisms can be examined [19–21]. The availability of genetic and genomic resources has led to the characterisation of pathways involved in cuticle degradation and assimilation, secondary metabolite production and regulation, signal transduction and stress responses [22, 23]. However, while the biochemical and genetic mechanisms of insect pathogenesis by *Beauveria* is relatively well understood [24], little is known of the molecular determinants mediating plant interactions. Previous studies have shown that the initial adhesion of *B. bassiana* during insect pathogenesis is facilitated by non-specific hydrophobic interactions mediated, in part, by hydrophobins, although other factors have also been characterised. Two hydrophobin genes, hyd1 and hyd2, which encode for Class I hydrophobins, have been shown to differentially affect fungal cell surface features and virulence. Loss of *hyd1* disrupted rodlet layer formation, decreased spore hydrophobicity, and impaired fungal infection of insects. In contrast, more minor effects were seen for *hyd2* mutants, with little to no effect on insect virulence. The double *Δhyd1/Δhyd2* mutant showed additive effects with a dramatic reduction in virulence [25].

Here we investigated the involvement of *hyd1* and *hyd2* in root colonization using the haricot bean, *Phaseolus vulgaris*, as a model system. Root colonization assays revealed differential impairment of the *Δhyd1, Δhyd2* and *Δhyd1/Δhyd2* mutant in their ability to form plant associations. Unexpectedly, the *Δhyd1/Δhyd2* double mutant was less severely impacted in these assays than the single mutants. Stress and growth response profiling indicated differential effects on growth, conidiation, and expression profiles of genes involved in signal transduction and adhesion. These data indicate a role for hydrophobins in plant association, coupled with complex pleiotropic consequences due to loss of the hydrophobins on signalling pathways.

**METHODS**

**Growth and maintenance of fungal cultures**

*Beauveria bassiana* (ATCC 90517) wild-type (WT), and the *Δhyd1, Δhyd2, Δhyd1/Δhyd2, Δhyd1 : hyd1* and *Δhyd2 : hyd2* strains (Table 1) have been previously reported [25]. Fungal cultures were routinely grown and maintained on Sabouraud dextrose, Czapek-dox, or Potato dextrose agars (SDA, CZA and PDA, Bioshop Canada) as needed. Plates were grown at 27 °C, with PDA routinely used for harvesting of conidia after 10–14 days of growth followed by collecting conidia after flooding of the plates with sterile 0.01 % Triton X-100. Conidia were counted using a haemocytometer and adjusted to indicated concentrations (typically between 10⁶–10⁷ conidia ml⁻¹) as needed.

**Phenotypic analysis**

Fungal vegetative growth rates and conidiation/conidial yields of the WT and hydrophobin (*hyd*) mutants were assessed on PDA and YPDA (yeast extract-peptone-dextrose agar) (Bioshop Canada) in Petri dishes containing 10 ml of media. Five microlitres of fungal conidial suspensions (1 × 10⁷ conidia ml⁻¹ in 0.01 % Triton X-100) were inoculated onto the centre of the agar plate and incubated at 27 °C. Radial growth rates were recorded by measuring colony diameters at 3, 7 and 14 days post-inoculation. Conidial yield was quantified by harvesting conidia from 14 days old PDA plates for each isolate. Briefly, a 0.8 mm diameter agar plug was collected from the centre of the colony and the plugs were vortexed for 2 min in 0.01 % Triton X-100. Conidia were microscopically counted using a haemocytometer. Five replicates were prepared for each isolate and the experiment was repeated twice with independent batches of conidia.

Pigmentation or oosporein production was assessed by inoculation of fungal conidia (1 × 10⁷ conidia ml⁻¹) into 15 ml YPD broth and grown with agitation (120 r.p.m.) at 27 °C for 4 days. Conidial germination was measured on PDA plates as follows; 50 µl aliquots of a 1 × 10⁴ conidia ml⁻¹ suspension were spread on PDA plates and incubated at 27 °C. The percentage of germinated conidia was measured by

<table>
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<th>Fungal isolate</th>
<th>Selection marker</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td><em>B. bassiana</em> ATCC 90517</td>
<td>–</td>
<td>Wild-type (WT)</td>
<td>[25]</td>
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<tr>
<td><em>B. bassiana Δhyd1</em></td>
<td>bar*</td>
<td>Δhyd1 mutant</td>
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<td><em>B. bassiana Δhyd2</em></td>
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<td>Δhyd2 mutant</td>
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<td><em>B. bassiana Δhyd1/Δhyd2</em></td>
<td>bar, sur†</td>
<td>Δhyd1/Δhyd2 double mutant</td>
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<td><em>B. bassiana Δhyd1</em></td>
<td>bar, sur</td>
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<td><em>B. bassiana Δhyd2</em></td>
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*Bar selective marker confers resistance to phosphonothrin.
†Sur selective marker confers sulfonilyurea resistance.*
microscopic analysis of at least 100 conidia/replicate. A conidium was considered germinated when the germ tube was at least the length of the diameter of the conidial cell (>3–4 µm). Five replicates were prepared for each fungal isolate.

**Bioconversion analysis**

Orsellinic acid (OA) (Thermo Fisher) was added to YPD broth at four different concentrations (10, 1, 0.1 and 0.01 mg ml\(^{-1}\)) as a precursor molecule in order to analyse oosporein production in WT and hyd mutants. Aliquots (100 µl) of conidial suspensions (1 × 10\(^6\) conidia ml\(^{-1}\)) of WT and mutant strains were inoculated into 100 ml of YPD broth containing OA and cultures were incubated at 27 °C for 4 days with agitation (120 r.p.m.).

**Penetrant germ tube formation and production of ROS**

Penetrant germ tube formation was assayed by inoculating 10 µl of 1 × 10\(^6\) conidia ml\(^{-1}\) onto peeled onion epidermis. After 30 h, the onion epidermis was stained with lactophenol cotton blue and germ tube formation was observed under microscope. The generation of reactive oxygen species (ROS) was visualised on mutant strains using Nitro blue tetrazolium (NBT) assay as previously reported [26].

**Root colonization assays: c.f.u. and semi-quantitative PCR**

Root colonization assays were performed using *Phaseolus vulgaris* (haricot bean, cultivar soldier) obtained from OSC Seeds, Ontario, Canada. The seeds were surface sterilized with 4 % sodium hypochlorite solution (NaClO) three times with sterile distilled H\(_2\)O to remove residual hydrogen peroxide. Seeds were then kept overnight at 4 °C for synchronisation of growth before planting and fungal inoculation. Seeds were placed in sterile vermiculite and kept at 25 °C for a photoperiod of 16 h a day for 3–4 days until germination. The 3–4 days old germinated seedlings were then planted in pots containing sterile vermiculite (Ther-O-Rock East). Five replicates of each treatment were individually planted in each pot for each fungal strain tested. Conidial suspensions for the WT and hyd mutants were prepared from 14 days PDA plates as described above. Fungal inoculation was performed using a soil drench method with modification to the conidial concentration [27]. Briefly, conidia were harvested in 0.01 % Triton X-100 and the concentration of the conidial suspensions was adjusted to 1 × 10\(^7\) conidia ml\(^{-1}\). Aliquots (5 ml) of the conidial suspensions were evenly poured over the vermiculite surface of each pot. Control bean seedlings were treated with sterile Triton X-100. All pots were kept in a greenhouse at 25 °C during the day and 18 °C during the night with photoperiod of 16:8 h light:dark cycle and a relative humidity between 62–80 %.

Plants were watered daily with sterile distilled H\(_2\)O. Bean roots were collected from 3 and 7 d old plants and then washed with sterile H\(_2\)O to remove the vermiculite attached to the roots. The roots were weighed and cut into ~0.2–0.5 mm pieces and homogenised (Biospec Products) for 2 min [28]. The root homogenate were assayed for fungal recovery of colony forming units (c.f.u.) on modified CTC agar [29] (YPDA supplemented with 0.5 g l\(^{-1}\) chloramphenicol, 0.004 g l\(^{-1}\) thiabendazole and 0.5 g l\(^{-1}\) cycloheximide) and c.f.u. values were calculated as c.f.u. 100 mg\(^{-1}\) of root weight.

Fungal colonization on roots was also quantified using semi-quantitative PCR. Samples of the bean roots harvested at 7 days post-inoculation were washed, weighed and then ground in liquid nitrogen using a mortar and pestle. Total DNA was extracted using CTAB (cetyl trimethylammonium bromide) method. The plant roots after harvest were washed in sterile distilled water and fresh root weights were recorded. The roots were then grounded in liquid nitrogen using a mortar and pestle. Five volumes of preheated (65 °C) CTAB buffer was added to every 1 vol (1 g) of root. Samples were then incubated at 65 °C for 30 min and subsequently treated with RNaseA (Thermo Fisher). The supernatant was collected by centrifugation and phase separated in chloroform:isoamyl alcohol. The DNA pellet was collected after precipitating each sample with isopropanol. Finally, the DNA pellet was washed in 70 % ethanol and nuclease free water was then added to each sample after air drying. The concentration of the DNA sample was adjusted to 10 ng µl\(^{-1}\) and used as the template for PCR (MyiQ, BioRad).

DNA extracted from the WT strain was used to construct a standard with concentrations ranging from 10 ng to 0.1 ng for quantification of the DNA present in the experimental samples. The primers used for quantification were Bb ITSF (5’GAACCTACCTATYGTTGCTTC) and Bb ITSFR (5’AT YCGAGGTCAACGTTCAG) [30].

**Gene expression analysis and semi-quantitative RT-PCR**

*B. bassiana* WT and the hyd mutants were grown on PDA for 14 days and conidia were collected from the agar plates using 0.01 % Triton X-100. Conidial concentrations were adjusted to 1 × 10\(^7\) conidia ml\(^{-1}\) and 2 ml of the conidial suspension was used to inoculate 200 ml of YPD broth. Cultures were then incubated with agitation (120 r.p.m.) at 27 °C. Fungal mycelia were collected after 4 days by vacuum filtration and were equally divided (~0.25 g of mycelia) and used as the inoculum into separate flasks containing 15 ml of medium. Eleven different culture growth conditions were examined; (1) distilled water, (2–4) three concentrations (v/v) of bean root exudate (1, 10, 100 %), (5) 1 % (w/v) chitin, (6) 1 % (w/v) tomato stem, (7) 1 % (w/v) glucose, (8) 1 % (w/v) trehalose, (9) 1 % (w/v) raffinose, (10) 1 % (w/v) cellulose and (11) YPD. Cultures were then incubated with agitation (120 r.p.m.) at 27 °C and the fungal mycelia were harvested by vacuum filtration 12 h after inoculation. Cultures corresponding to an additional three stress conditions (with a H\(_2\)O control) were inoculated as above, but harvested after 6 h of incubation. These conditions included
cultures supplemented with (a) 300 µg ml⁻¹ Congo red, (b) 20 mM H₂O₂ and (c) 500 mM NaCl.

Bean root exudate (BRE) was prepared after bean seeds were sterilised as described above. The sterile seeds were then germinated on water agar (1 %) for 4 d and then transferred to a flask containing sterile distilled H₂O. Approximately 25 seedlings were used for 500 ml of sterile distilled H₂O. Seedling cultures were incubated at room temperature with agitation (100 r.p.m.). The bean root exudate was collected after 4 days and the sterility of the exudate were confirmed by plating aliquots onto PDA plates.

The fungal mycelia from each growth condition were harvested by vacuum filtration and the total RNA from each sample was extracted using TRI-reagent (Sigma) [10]. The extracted RNA was then treated with RQ1 RNase-free DNase (Promega) and the RNA concentration was determined spectrophotometrically using Qubit (Invitrogen). cDNA for each sample was generated using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Thermofisher Scientific) with 4 µg of total RNA in a total volume of 40 µl, following manufacturer’s instructions. The transcript levels of several genes (Table S1, available in the online version of this article) were assessed via semi-quantitative PCR (My iQ, Bio-Rad) using 2× PCR master mix (Norgen) and the 18S rRNA as the reference gene. RT-PCR reaction mixtures contained 10 µl 2× PCR master mix, 2 µl cDNA, 1 µl of each of forward and reverse primers (2.5 µM) and made the reaction to 20 µl with nuclease-free water. PCR cycling conditions were as follows: 95 °C (2 min), 30 cycles of 95 °C (30 s), 52–60 °C (45 s), 72 °C (1 min), and final extension at 72 °C (3 min). Aliquots (5 µl) of the RT-PCR reaction product were run on 2% agarose gel electrophoresis (30 min at 80V) for quantitation. Images of the GelRed (Biotium) stained agarose gels were acquired with Gel Doc EZ Imaging System (Bio-Rad). The quantification of intensity of bands was performed using Image Lab (Bio-Rad) software. The ratio of band intensity of the corresponding gene of interest to the band intensity of reference gene (18 s) was calculated to normalise the variations in concentrations in each sample. The relative expression was quantified after normalisation and the mean standard error of for all conditions were calculated [31]. Experiments were performed using three biological replicates and primer sequences used to evaluate gene expression patterns are shown in Table S1.

RESULTS

B. bassiana hyd1 and hyd2 affect growth rate, conidiation and pigment production

Subtle differences in colony morphology were observed between the WT and the Δhyd1, Δhyd2 and Δhyd1/Δhyd2 mutants when grown on PDA (Fig. 1a). All strains formed white/off-white colonies. The Δhyd2 and Δhyd1/Δhyd2 mutants produced fluffier colonies than the WT or Δhyd1 strains. Disruption of hydrophobins, however, affected both growth and conidiation on PDA (Fig. 1b, c). Decreased growth was observed for the hyd mutants at 14 days (ANOVA, P<0.001). Conidial yield was also decreased (2–4 fold) for the hyd mutants compared to WT (ANOVA, P<0.01). The complemented mutant strains showed partial restoration of growth and complete restoration of conidiation. No differences in the conidial germination rates were found between the WT and mutant strains, with all reaching >90 % germination after 24 h of growth (Fig. 1d). In liquid culture, the loss of oosporein production (red pigment) was evident for the hyd mutants (Fig. 1e). The loss of oosporein production could not be restored in hyd mutants even after adding orsellinic acid, the major precursor for oosporein production, to the growth media.

No differences in penetrant germ tube formation were noticed between the WT, single and double hyd mutants (Fig. 2) on onion epidermis. ROS production was examined using an in situ semi-quantitative reactive oxygen species dye-based assay (nitro blue tetrazolium) which showed hyphae derived from the hyd mutants (Δhyd1, Δhyd2 and Δhyd1/Δhyd2 strains) and extensive blue staining particularly at hyphal tips, with little visible staining observed in the WT parental strain (Fig. 3).

Loss of hyd1 and hyd2 genes decreases the association of B. bassiana with bean roots

In order to test whether hydrophobins are important in mediating plant root interactions, haricot bean (P. vulgaris) root colonization assays were performed as detailed in the Methods section. Recovery of B. bassiana fungal colony forming units (c.f.u) from the roots of treated plants revealed a significant decrease in plant root association abilities for the hyd mutants as compared to the WT (Fig. 4a). For the WT, a high concentration (~20 000 c.f.u. 100 mg⁻¹ root) was seen 3 days post-inoculation of the soil that decreased by ~50 % by 7 days post-treatment. In contrast, a dramatic decrease of recoverable c.f.u. was seen for both the Δhyd1 and Δhyd2 mutants at both 3 and 7 days post-treatment as compared to the WT; two-way ANOVA, WT v/s Δhyd1 (P<0.001), WT v/s Δhyd2 (P<0.001), for both the 3 and 7 days time points. Recoverable c.f.u. for the Δhyd1 mutant was ~4000 100 mg⁻¹ root at 3 days and ~2500 c.f.u. 100 mg⁻¹ root at 7 days, and even lower for the Δhyd2 mutant, ~2000 c.f.u. at 3 and 7 days. Surprisingly, the Δhyd1/Δhyd2 mutant was not as impaired as the single hyd mutants in terms of plant root association. At 3 days post-inoculation, ~10 000 c.f.u. 100 mg⁻¹ root was detected for the Δhyd1/Δhyd2 strain (WT v/s Δhyd1/Δhyd2, P<0.007). However, the Δhyd1/Δhyd2 mutant showed similar levels of root plant association as the WT at 7 days.

In order to examine whether the mutants had either persistence or some other issues with survival in the soil, c.f.u. in vermiculite collected surrounding the plant roots (3 and 7 days) was determined (Fig. 4b). Similar levels of c.f.u. were recovered from the WT and mutants at 3 and 7 days, with the exception of higher numbers for the Δhyd2 mutant strain in the vermiculite at 3 days [two-way
ANOVA, Sidak’s multiple comparison test, WT vs Δhyd2 (P<0.003)).

Plant root association was also examined via semi-quantitative PCR amplification of DNA extracted fungus-root associated samples as detailed in the Methods section. These data were consistent with the c.f.u. recovery results in which higher levels of fungal DNA were seen in WT–haricot bean root samples, lower in both the Δhyd1 and Δhyd2 mutants, with greater fungal DNA content seen for the Δhyd1/Δhyd2 mutant as compared to the single mutants, but still lower than WT (Fig. 4c).

**Loss of B. bassiana hyd genes affects the expression of key signal transduction, adhesion and pigment production genes**

Semi-quantitative RT-PCR was used to analyse the expression patterns of 20 genes implicated in virulence, stress response, and cellular signalling. These included genes involved in (a) conidiation; FlbA, FlbB and FlbD, (b) insect
virulence; CDEP1 cuticle degrading protease, Chi1 chitinase, Chi2 endochitinase, and the ChsA2 chitinase, (c) signal transduction; ras1, ras2, ras3, Hog1 MAPK (high-osmolarity glycerol 1 mitogen-activated protein kinase), Slt2 MAPK (mitogen-activated protein kinase), Mkk1 MAPKK (mitogen-activated protein kinase kinase), Bck1 MAPKKK (mitogen-activated protein kinase kinase kinase), and the Msn2 transcription factor, (d) adhesion/cell surface properties; hyd1, hyd2, bad1 (Beauveria adhesin 1, homolog to the Metarhizium adhesin 1 implicated in binding to the insect cuticle), and bad2 (Beauveria adhesin 2, homolog of the Metarhizium adhesin 2, implicated in binding to plant surfaces), and (e) secondary metabolite production; oosporein polyketide synthase (pks9). Gene expression analysis was performed on RNA extracted from the mycelia of the WT and hyd mutant strains grown in YPD broth and then subsequently transferred to media indicated in the Methods section. The expression patterns of FlbA, FlbB, FlbD, Chi1, Chi2, ChiA2, ras genes, hyd1 (except in the respective mutant strains), hyd2 (except in respective mutant strains), and bad1 were similar between the WT and mutant strains in all of the conditions examined (data not shown).

A general pattern in which expression of the signal transduction proteins Slt2, Bck1 and Mkk1 were more highly expressed in the WT > Δhyd1/Δhyd2 mutant > Δhyd1, Δhyd2 was seen when grown in water, bean root exudate (100 %, 1 %), 1 % chitin, and 1 % tomato stem extract. No significant differences in expression of Slt2, Bck1 or Mkk1 were found between the WT and mutant strains in different carbohydrates and under stress (Congo red, H₂O₂ and NaCl), with the exception of the higher Mkk1 expression for Δhyd2, Δhyd1/Δhyd2 for hydrogen peroxide and NaCl (Figs 5a–c and S1a–c). Interestingly, the expression of the Hog1 MAPK was found to be consistently higher for the Δhyd1/Δhyd2 mutant strain when compared to the WT and single mutant strains. Similarly, the expression of the Msn2 transcription factor was higher in the Δhyd1/Δhyd2 when grown in bean root exudate or 1 % tomato stem. However, expression levels of Msn2 were similar in the WT and mutant strains in 1 % chitin, YPD, different carbohydrates and stress conditions (Congo red, H₂O₂ and NaCl) (Figs 5a–c and S1d, e). Expression of the bad2 adhesin as well as the polyketide synthase (pks9) was dramatically reduced in the hyd mutants. Transcript levels of bad2 in WT versus hyd mutants revealed that the expression of bad2 was significantly decreased in the single and double mutant strains. However, the expression of bad2 was found to be elevated in the mutant strains under stress (Congo red, H₂O₂ and NaCl) (Figs 5a–c and S1g, h). The expression pattern of the cuticle degrading protease, CDEP1, was observed to be similar in the Δhyd1, Δhyd1 and Δhyd1/Δhyd2
**DISCUSSION**

The fungus *B. bassiana* is well known as an insect pathogen, however, more recently, their ability to form epiphytic and potentially endophytic symbiotic associations with plants has been recognised [19]. The fungal-plant mutualistic interaction has been shown to include mobilisation of nitrogen from the insect pathogenic activity of the fungus to the plant host, and secretion of plant carbohydrates utilisable by the fungus [32]. Hydrophobins contribute to the hydrophobic nature of many fungal spores or conidia, and in *B. bassiana*, two hydrophobins, *hyd1* and *hyd2* have been shown to differentially contribute to the rodlet layer and insect
However, any role(s) for the *B. bassiana* hydrophobins in mediating plant associations have not been reported. Our data demonstrate that *hyd1* and *hyd2* contribute to the ability of the fungus to form root associations (in contrast *hyd2* was mainly implicated in insect virulence). Root colonization bioassays revealed that for the plant pathogenic fungus, *C. rosea*, a hydrophobin (*Hyd3*) was similarly needed for colonization of *Arabidopsis thaliana* and barley roots [18]. In addition, *Tashyd1*, a Class I hydrophobin gene in *Trichoderma asperellum*, was found to contribute to root colonization. Deletion mutants of *Tashyd1* showed significantly reduced colonization of plant roots when compared to the wild-type [33]. However, our data indicated that the requirement/contribution of hydrophobins may be more complex than originally considered, as plant association was greater for the double Δ*hyd1/Δhyd2* mutant than for each single mutant. This was not due to any generalised loss of survival in the soil since fungal recovery from the surrounding (to the root) vermiculite revealed little to no decreased persistence for any of the mutants, and in fact, survival in the surrounding soil was potentially increased for the *hyd2* mutant. Similarly, a double deletion Δ*Hyd1*Δ*Hyd3* in *C. rosea* resulted in increased root colonization compared to WT or single deletion Δ*Hyd1* or Δ*Hyd3* mutants [18].

Our results indicated that hydrophobins affected radial growth and conidiation in *B. bassiana* but did not affect conidial germination. The partial restoration of growth rate and complete restoration of conidiation in rescued mutants indicated hydrophobins appear to be important for growth and conidiation. Prior studies similarly demonstrated the importance of *hyd* genes in growth and conidiation in other fungi. In *M. brunneum* *hyd1*, *hyd2* or *hyd3* single mutants showed delayed and reduced conidiation compared to the WT parent. The study also showed that the deletion of *hyd1*, *hyd2* or *hyd3* genes did not affect conidial germination or appressoria formation [34]. Likewise, reduced conidiation has been reported for knockouts of either the *MPG1* or *MHP1* hydrophobins in *M. oryzae*, and for the Δ*HFB2* hydrophobin mutant in *T. reesei*. However, loss of specific hydrophobin genes can result in opposing phenotypes in different fungal species. For example, deletion of the *Hyd1* and *Hyd3* genes in *C. rosea* resulted in increased vegetative growth and increased conidiation [18].
Fig. 5. Semi-quantitative RT PCR of selected genes under different conditions. Relative intensity of selected genes for WT and mutant strains in, (a) Water (12 h), Bean root exudate (BRE – 100%, 10 and 1%), 1% chitin, 1% tomato stem extract (TS), (b) Different carbohydrates in water (1% Cellulose, 1% Glucose, 1% Raffinose and 1% Trehalose), (c) YPD and stress conditions (Congo red, H$_2$O$_2$ and NaCl). The image of agarose gel shown is the representative from 3 replicates studied for each condition.
Hcf1 or Hcf6 in another phytopathogenic fungus, *Cladosporium fulvum*, had no significant effects on either growth or conidiation under the conditions examined [35].

Aside from growth defects, inactivation of *B. bassiana* hyd genes resulted in increased ROS production in growing hyphae, indicating elevated oxidative stress, a finding that could potentially account for the reduced growth and conidiation phenotypes. The finding of increased ROS in the hyd mutants suggests that perturbation in cell wall structure and/or its integrity can result in oxidative stress. Although overall conidial yields were decreased in the mutants, no significant changes in the expression of the FlbA, FlbB, or FlbD conidiation genes were noted under a variety of growth conditions. In contrast, the hyd mutants failed to synthesise oosporein, a 1,4-dibenzoquinone implicated as an antimicrobial factor that suppresses competing microbes on the insect cadaver, allowing the fungus to grow and conidiate on the dead host [36, 37]. In this case, loss of oosporein production correlated with decreased expression of the *pks9* required for its synthesis. Similar observations have been reported for *M. brunneum*, where deletion of *hyd1*, *hyd2* or *hyd3* reduced pigment production compared to the WT [34].

The *Mad1* and *Mad2* (*Metarhizium*) adhesins 1 and 2 are specific adhesins expressed by *M. robertsii* that mediate conidial interactions with insect cuticle and plant surfaces, respectively [9]. Comparative analysis of the genome sequences revealed that BBA_02419 and BBA_02379 are the orthologs of *Mad1* and *Mad2* genes in *Beauveria* and have hence been named *bad1* and *bad2*, respectively [38]. No significant differences in germ tube formation were found between the *B. bassiana* hyd mutant strains in comparison to the WT on onion epidermis; however, *bad2* expression was significantly reduced in the hydrophobin mutant strains compared to the WT. The *bad2* gene was significantly down regulated in deletion mutants compared to WT for all the conditions tested, whereas the expression of *bad1* was unaffected for deletion mutants. This correlation between the *bad2* gene and both hydrophobin genes suggests that the expression of *bad2* is somehow linked to the expression of both *hyd1* and *hyd2*. The low level of *bad2* expression could help account for the decreased root colonization of *hyd* mutants in comparison to WT. However, several possibilities can account for higher colonization levels observed for the Δ*hyd1*/Δ*hyd2* mutant on bean roots compared to the single mutants. Most notably, whereas the single mutant showed increased expression of critical signal transduction pathway genes under a variety of conditions, this decrease was much lower (albeit still not up to WT levels) than Δ*hyd1*/Δ*hyd2* mutant. These data suggest that, in the double mutant, compensatory pathways may be involved due to the cell wall stresses that may result from the loss of both hydrophobins.

Significant progress has been made in the characterisation of signalling pathways and transcription factors that regulate stress responses and virulence in *B. bassiana* [39, 40]. In *B. bassiana*, *Bck1*, *Mkk1* and *Slt2* constitute the MAPK cell wall integrity pathway, which regulates multiple developmental processes [41, 42]. The high-osmolarity glycerol (Hog1) pathway is also critical for stress response and other developmental programs and *B. bassiana* mutants in any of these components display affect growth, development, conidiation, virulence and/or stress susceptibilities [43]. In *B. bassiana* Hog1 regulates a novel mitochondrial oxidative stress response [44]. These pathways are also linked to the hydrophobins, with transcript levels of *hyd1* and *hyd2* down regulated in Δ*hyd1*/Δ*hyd2* strain compared to the WT under all of the growth conditions tested indicating that these pathways are unaffected. Although, deletion of *hyd1* and *hyd2* genes appear to be involved in transcriptional regulation of signal transduction pathways (*Slt2* and *Hog1*), it could not be disregarded that the relative stoichiometry of the MAPK pathways is not solely based on transcript or protein abundance, but may be based on the overall phosphorylation of the MAPK cascade.

In conclusion, our data show that the *hyd1* and *hyd2* hydrophobins in *B. bassiana* contribute to plant root colonization and the rhizosphere competence of this fungus. These cell wall constituents may participate in mediating initial as well as consolidation of associations with the plant (root) surface. In contrast, *hyd1* had little impact on adhesion to insect cuticles but affected virulence, whereas *hyd2* appeared to have a significant role in mediating adhesion to the insect surface but contributed little to pathogenicity [25]. Hydrophobin effects were mediated, at least in part, via changes in the expression of specific MAPK signalling pathways, which correlated with downstream phenotypes including increased ROS production and oxidative stress seen in the mutants. These data indicate that rhizosphere interactions are specific and mediated by discrete pathways in *B. bassiana* and that some of these pathways, but not all, are shared with the ability of the fungus to parasitise insect hosts.

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Conflicts of interest
The authors declare that none of the authors has a conflict of interest regarding this article.

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