Oosporein, an abundant metabolite in *Beauveria caledonica*, with a feedback induction mechanism and a role in insect virulence

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ABSTRACT

Oosporein was first identified from the insect pathogen *Beauveria bassiana* >50 y ago. Here, we investigate the insecticidal, anti-feedant and immunomodulation effects of oosporein produced by *Beauveria caledonica* on the forestry pest *Hylobius abietis* and model insect *Galleria mellonella*. We report a novel feedback induction mechanism regulating oosporein production in *B. caledonica;* exogenous oosporein induces the expression of the oosporein cluster, leading to increased abundance of oosporein biosynthetic enzymes, as shown by label-free quantitative proteomics. Oosporein did not have an anti-feedant effect on *H. abietis* adults − on the contrary, insects exposed to oosporein-treated food fed more than those exposed to untreated food only. Injected oosporein did not kill insect larvae but increased susceptibility of *H. abietis* to a subsequent infection. Oosporein did not act as a contact toxin on *H. abietis* adults and *G. mellonella* larvae at the concentrations tested. Therefore, it appears that oosporein promotes infection rather than directly killing insects; this could be mediated both by a reduction in haemocyte numbers and by alterations to the humoral immune system. This work makes a case for future research into the potential use of *B. caledonica* as a biocontrol agent through combinations with oosporein or with enhanced production of oosporein.

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1. Introduction

Secondary metabolites, or natural products, are produced by a range of microorganisms and contribute to their survival in many ways. These include protection from abiotic and biotic stresses, such as UV radiation, desiccation, predation from insects and competition with other microbes, as well as participation in metal homeostasis (Keller, 2015). Fungi have successfully adapted to almost all habitats on earth and a primary explanation for their ability to survive in different environment is their diverse range of natural product pathways. These natural products often exhibit antimicrobial, antifungal, immunosuppressive or cytotoxic effects.

These properties enable them to establish in diverse environments by allowing them to compete for nutrients, deter predators and communicate with other organisms (Quin et al., 2014). The key role natural products play in fungal survival is highlighted by the fact that fungi often have many different secondary metabolite pathways clustered within their genomes. These bioactive products are of considerable interest for, agriculture and pharmaceutical and other industries as they can be harnessed for their antimicrobial, anticancer and antiviral properties (Quin et al., 2014). There is great interest in the development of entomopathogenic fungi (EPF) as biocontrol agents. However, there is concern that the use of EPF as biocontrol agents may increase levels of toxic microbial metabolites in the environment. For a fungal biocontrol agent to be registered in Europe the Directive 91/414/EEC demands data on the potential environmental risks arising from the applied fungus and the metabolites it produces (Strasser et al., 2000a,b). Thus, there is a need for research into the metabolites produced by less well characterized entomopathogenic fungi to ensure their environmental safety and aid their implementation as biocontrol agents.

The genus *Beauveria* contains species that are generally entomopathogenic, these species range from the ubiquitous entomopathogenic fungus *Beauveria bassiana* to rarer species such as *Beauveria pseudobassiana*. The species have been reported as naturally occurring insect pathogens and include *B. bassiana*, *Beauveria brongniartii*, *Beauveria velata*, *Beauveria amorpha*, *Beauveria vermiconia* and *Beauveria caledonica* (Glare et al., 2008). *Beauveria* species are well known for producing a diverse variety of biologically active secondary metabolites including non-peptide pigments and polyketides (oosporein, bassianin and tenellin), nonribosomally synthesized peptides (beauvericin, bassianolides and beauveriolides) and secreted metabolites involved in pathogenesis and virulence (oxalic acid). These metabolites have both potential and realized applications in industry, pharmacology and agriculture (Xiao et al., 2012).

B. caledonica was originally isolated from soil in Scotland and recognized as being both saprotrophic and a significant biological competitor as they are able to infect a wide range of insects and grow as soil saprophytes but also can survive as plant endophytes (Bissett and Widden, 1988; Fomina et al., 2005). The species was later recognized as a naturally occurring pathogen of the introduced pine bark beetles, *Hylastes ater* and *Hylurgus ligniperda*, in New Zealand (Glare et al., 2008) and of the economically significant weevil pest *Hylobius abietis* in the UK and Ireland (Glare et al., 2008; Williams et al., 2013). Thus, *B. caledonica* has potential as a biocontrol agent in forestry for use against coleopteran pests, and has been field-tested against *H. abietis* (Mc Namara et al., 2018a). The available literature regarding *B. caledonica* is sparse compared to the commonly used EPF species *Metarhizium anisopliae* and *B. bassiana*. Mc Namara et al. (2017, 2018b) described the effect of the secretome of *B. caledonica* on the immune response of the wax moth, *Galleria mellonella* and of *H. abietis*.

Considering the wealth of natural products known to be produced by *B. bassiana*, we analyzed the most abundant natural products produced by *B. caledonica*. Culture supernatants were fractionated, and the compounds were screened for insecticidal and immunosuppressive properties. Oosporein was ultimately identified as the most abundantly secreted natural product of *B. caledonica* with immunosuppressive potential. The insecticidal, anti-feedant and immunosuppressive effects of oosporein were investigated using representatives of two insect orders, the forestry pest *H. abietis* (Coleoptera) a natural host of *B. caledonica*, and the model insect *G. mellonella* (Lepidoptera). *G. mellonella* are regularly employed in the study of fungal pathogenesis and biocontrol agents. Use of insects such as Galleria mellonella as models is growing in popularity as it is cheaper, lacks ethical constraints and results are often comparable to mammals and other insects (Kavanagh and Reeves, 2004; Arvanitis et al., 2013). Likewise, *Galleria* may have an application as a model for insects that are difficult and labour intensive to source or that are not sold commercially, in this case *Hylobius*. Including both insects also allows comparison between work by our group on the effect of *B. caledonica* supernatant (which contained oosporein) on *Galleria* and Hylobius immune response (Mc Namara et al., 2017, 2018b).

We also show that oosporein production by *B. caledonica* can be enhanced by spiking this metabolite into culture supernatants. This feedback induction mechanism controlling oosporein production was investigated using quantitative proteomics.

2. Materials and methods

2.1. B. caledonica *strain and growth conditions*

B. caledonica 2c7b isolated from the stump of a felled pine tree (Hortland, Co. Kildare, Ireland), was used in this study.

B. caledonica was cultured in Sabouraud dextrose liquid medium (Oxoid) for 96 h in a shaking incubator at 25 °C, 250 rpm. The culture supernatant was filtered through 0.22 μm syringe filters (Sarstedt). The supernatant was collected and stored at −80 °C.

2.2. Organic extraction of B. caledonica *fungal supernatant, RP-HPLC analysis and fractionation*

Filtered supernatant was organically extracted twice using an equal volume of ethyl acetate. Organic extracts were concentrated by rotary evaporation at 40 °C (Stuart RE300DB). The dried extracts were resuspended in HPLC grade methanol and stored at −20 °C. Organic extracts and neat fungal supernatant were analyzed by RP-HPLC with UV detection (Agilent 1200 system), using a C18 column (Agilent Eclipse XDB-C18, 5 μm, 9.4 × 250 mm) at a flow rate of 2 ml/min. A mobile phase of water (1 % (v/v) trifluoroacetic acid (TFA) in HPLC-grade water) and acetonitrile (0.1 % (v/v) TFA in HPLC-grade acetonitrile) was used under various gradient conditions. Fractionation was carried out under the same mobile phase, gradient and flow rate. Fractions were collected into glass vials (Labquip Limited, Ireland).

2.3. Structural determination of the bioactive fraction 5 from B. caledonica *culture supernatants*

The peak eluting at 16.6 min was fractionated using RP-HPLC until 5 mg of dried metabolite was obtained. Identification of the purified red metabolite of *B. caledonica* was carried out using NMR Spectroscopy, Infrared Spectroscopy, and Mass Spectrometry. All solvents/reagents for NMR and IR analysis were bought commercially and used without further purification. NMR spectra were recorded using a Bruker Ascend 500 spectrometer at 293 K, operating at 500 MHz for the ^{1}H nucleus and 126 MHz for the ^{13}C nucleus. All NMR chemical shifts are reported in ppm and were referenced relative to the relevant deuterated solvent residual peaks or tetramethylsilane (TMS). The following abbreviations were used to explain the observed multiplicities; s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet). High resolution mass spectrometry (HRMS) was performed on an Agilent-LC 1200 Series coupled to a 6210 Agilent Time-Of-Flight (TOF) mass spectrometer equipped with an electrospray source in both positive and negative (ESI+/−) modes. Infrared spectra were obtained as KBr disks in the region 4000–400 cm^{-1} on a Perkin Elmer Spectrum 100 FT-IR spectrophotometer.

2.4. Rapid large-scale purification of oosporein using ion-exchange chromatography

Oosporein was concentrated, purified and chromatographed using Q-Sepharose ion exchange resin. The procedure was automated using an AKTA Purifier 100 system (Amersham Biosciences, UK). Q-Sepharose resin (5 ml) was added to 1 l of *B. caledonica* supernatant. The sample was gently agitated at 4 °C overnight. The culture supernatant containing Q-Sepharose was packed into an XK 16/20 column and washed with 100 ml binding buffer (20 mM Tris−HCL, 10 mM NaCl, pH 8) at a flow rate of 5 ml/min. Fractions were eluted from the column at a flow rate of 2 ml/min with a linear gradient of 1 M NaCl in elution buffer (20 mM Tris−HCL, 1 mM NaCl, pH 8). Fractions containing red/purple pigment were pooled and analyzed by RP-HPLC.

2.5. Spike assay to investigate the influence of oosporein on its own biosynthesis in B. caledonica

A liquid culture (50 ml) of *B. caledonica* was incubated for 24 h after which 0.875 mg of oosporein was added (17.5 μg/ml final). An equal volume of DMSO was added to control cultures. After a total growth period of 96 h, cultures were filtered through miracloth, and the mycelium was dried between layers of tissue and was snap frozen in liquid nitrogen. Mycelium was used for analysis by label-free MS and fungal supernatant was analyzed fusing RP-HPLC. There were four biological replicates per experiment.

2.6. Whole cell lysate preparation and digestion of B. caledonica mycelia for analysis by label free quantification

Mycelium was harvested through miracloth and ground using a pestle and mortar cooled in liquid nitrogen. There were three biological replicates per treatment. Mycelial lysates were prepared in lysis buffer (100 mM Tris—HCl, 50 mM NaCl, 20 mM EDTA, 10 % (v/v) glycerol, 1 mM PMSF, 1 μg/ml pepstatin A, pH 7.5) with grinding, sonication and clarified using centrifugation. The resultant protein lysates were precipitated using trichloroacetic acid/acetone and resuspended in 100 mM Tris—HCl, 6 M urea, 2 M thiourea, pH 8.0. After dithiothreitol reduction and iodoacetamide-mediated alkylation, sequencing grade trypsin combined with ProteaseMax surfactant was added. All peptide mixtures were analyzed via a Thermo Fisher Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 4 % to 35 % B over 2 h, and data were collected using a Top15 method for MS/MS scans. Comparative proteome abundance and data analysis was performed using MaxQuant software (v. 1.3.0.5) (Cox et al., 2009) with Andromeda used for database searching and Perseus used to organize the data (v. 1.4.1.3) as described previously (Dolan et al., 2017). Peptides were matched to the *B. bassiana* D1—5 reference proteome (https://www.uniprot.org/proteomes/UP000030106).

2.7. Determining the effect of oosporein on larval haemocyte densities

For the insect bioassays, *G. mellonella* larvae were injected with 0.125 μg of oosporein (in 20 μl of 5 % (v/v DMSO) through the last proleg. *H. abietis* larvae were injected through their abdominal spiracles. This was the dose of oosporein retrospectively calculated to be in the *B. caledonica* 96 h supernatant treatment previously used in similar bioassays (Mc Namara et al., 2018b; Mc Namara et al., 2017), allowing comparison between the effects of full *B. caledonica* supernatant on the insect immune system versus those of oosporein alone. Control larvae were injected with 20 μl of 5 % DMSO. Haemocyte density was assessed by bleeding five larvae per treatment and then enumerating using a Neubauer haemocytometer. The experiment was performed on three independent occasions.

2.8. Determining the effect of oosporein on yeast cell density in larvae

Larvae were injected through the last proleg or spiracle with 0.125 μg of oosporein (20 μl), a dose previously calculated to have been used for previous bioassays (Mc Namara, 2018b; Mc Namara et al., 2017). Control larvae were injected with DMSO. Larvae were incubated for 24 h at 20 °C. After 24 h larvae were given a second injection, this time with *Candida albicans* (MEN strain, cultured to the stationary phase overnight in YEPD broth at 30 °C and 200 rpm). After incubation for a further 24 or 48 h at 20 °C, three larvae per treatment were homogenized in 3 ml of sterile PBS. After serial dilution in PBS, 100 μl of each sample was spread on YEPD ERY plates (YEPD agar supplemented with 1 mg/l erythromycin). The plates were incubated for 48 h at 30 °C. The yeast cell density was subsequently calculated as the yeast cell density per larva. Experiments were performed on three independent occasions.

2.9. G. mellonella assay to investigate the immunosuppressive properties of oosporein

G. mellonella is a well-tested bioassay-organism, which is highly sensitive to a number of secondary metabolites synthesized by EPF (Abendstein et al., 2001). Larvae were injected through the last proleg or spiracle with 0.125 μg of oosporein (20 μl). Control larvae were injected with DMSO. Ten larvae were injected per treatment. Larvae were then incubated for 24 h at 20 °C after which they were given a second injection with *C. albicans*, as above. Larvae were incubated at 20 °C and mortality was recorded. The experiment was performed on three independent occasions.

2.10. Label free quantification of the effect of oosporein on the G. mellonella hemolymph proteome

Larvae were injected with 0.125 μg of oosporein (20 μl), control larvae were injected with DMSO and incubated for 48 h at 20 °C. Five larvae were bled per treatment into a pre-chilled 1.5 ml centrifuge tube and spun at 1500×g for 5 min at 4 °C. Samples were diluted in PBS and 100 μg of protein was removed and acetone precipitated. The sample was centrifuged at 13 000×g for 10 min. The protein was resuspended in 100 μl of resuspension buffer (6 M urea, 2 M thiourea, 5 mM calcium chloride). Protein samples (75 μg) were reduced with dithiothreitol (200 mM) and alkylated with iodoacetamide (1 M). Samples were digested with sequence grade trypsin (Promega, Ireland) at a trypsin:protein ratio of 1:40, overnight at 37 °C. Quantitative Proteomic Analysis and subsequent data analysis was carried out as described in Mc Namara et al. (2017).

2.11. Bioassay to investigate the role of oosporein as a contact toxin

Oosporein was dissolved in 5 % (v/v) DMSO to final concentrations of 1.25 mg/ml and 125 μg/ml. *H. abietis* adults and *G. mellonella* larvae were dipped in either oosporein or DMSO (control). Insects were incubated for 3 weeks at 20 °C, checked daily and mortalities were recorded. The experiment was performed on three separate occasions with ten insects per treatment on each occasion.

2.12. Choice-bioassay to investigate the effect of oosporein on H. abietis adult feeding

Oosporein was dissolved in 5 % (v/v) DMSO to concentrations of 1.25 mg/ml and 125 μg/ml. Twigs (Scots pine, *Pinus sylvestris*, 2 cm in length) were dipped in either oosporein or DMSO (control). Bioassays were carried out in plastic tubs (6 cm × 9.5 cm) lined with a thin layer of moist moss peat. In each tub there was a choice of two twigs treated with oosporein (1.5 mg/ml or 125 μg/ml) and two control twigs. Control tubs contained four twigs dipped in DMSO; this was used to control for death caused by ingesting oosporein. One adult weevil was placed in each tub. Tubs were placed in a 20 °C room with a 16:8 light regime and were checked daily for feeding, which was recorded using a scoring scale. For the scoring scale the 2 cm twig was visually divided lengthwise into ten sections; when the bark was completely removed from a section it was given a score of one, half removed a score of 0.5 and so on. The twig was turned and scoring repeated to cover the circumference of the twig. Ten insects were tested per treatment and the experiment was repeated on three separate occasions.

2.13. Statistical analysis

Statistical analysis was carried out using Minitab V.16 statistical software and GraphPad Prism V.5. All data were first tested for normality, where data was found not to be normal, the data was either transformed before further analysis was carried out or a non-parametric alternative was used. A one-way ANOVA was used to compare the effects of different RP-HPLC fractions on yeast cell density in larvae. For spike assay data Mann Whitney U tests were carried out to compare treatments. Two-way ANOVAs were carried out with Bonferroni post-tests to investigate alterations in haemocyte densities and yeast cell densities. Mann Whitney Tests were used to compare larval mortality in the absence and presence of *C. albicans*. To assess if oosporein acts as a contact toxin a Kruskal Wallis test was used to assess differences in insect mortality. For the feeding assay a general linearized model (GLM) was used to test for the effect of treatment, experiment number and interaction between treatment and experiment number on feeding score. Replicates were combined, and a one-way ANOVA with Tukey's Multiple Comparison test was used to compare total food eaten in each treatment. Wilcoxon signed rank tests were used to compare the proportion of total food consumed that was treated with oosporein.

3. Results

3.1. Fractionation of B. caledonica supernatant

Five abundant peaks from organic extracts of *B. caledonica* 96 h supernatant were fractionated and collected using preparative RP-HPLC (Fig. 1A). The fractions were injected into *G. mellonella* to investigate if they displayed immunosuppressive potential (Fig. 1B). Pre-treatment with four of the fractions did not increase yeast cell density in *G. mellonella* larvae, nor did they kill the insects. A red pigmented fraction that eluted at approximately 16.6 min caused mortality of some of the test insects (such that immunosuppression could not be assessed) and was selected as a fraction of interest (Fig. 1C). Organic extraction was carried out to concentrate and extract the fraction of interest. This fraction was repeatedly collected, dried and pooled until 5 mg of dried metabolite was obtained and was used to identify the metabolite (Fig. 1D,E).

3.2. Identification of red metabolite from B. caledonica

Identification of the purified red metabolite of *B. caledonica* was carried out using NMR Spectroscopy, Infrared Spectroscopy, and Mass Spectrometry. The unidentified metabolite from *B. caledonica* appeared as an orange/red semi crystalline solid after purification by HPLC. IR and UV data correlated closely with that in literature reports for oosporein (Divekar et al., 1959; Cole et al., 1974; Nagaoka et al., 2004). The ^{1}H NMR spectrum of the solid in DMSO showed one signal as a singlet at 1.81 ppm which represented the terminal methyl protons. The ^{13}C NMR spectrum showed the corresponding signal for the methyl group at 7.6 ppm. In addition, two signals representing quaternary carbons were observed at 112.8 and 107.4 ppm. On addition of excess triethylamine, as previously reported by Love et al. (2009), resolution of the spectrum was achieved with signals resonating at 172.0, 171.2 and also 108.0 and 107.6 ppm. A colour change was also observed on addition of the base, with the orange solution turning a dark violet hue. Colour changes of this description have been previously reported (Seger et al., 2005). Mass spectrometry confirmed the presence of the oosporein anion. Thus the abundant metabolite produced in *B. caledonica* 96 h culture filtrates was identified as oosporein.

3.3. Uncovering a novel feedback induction mechanism regulating oosporein production in B. caledonica

A number of strategies have been developed in order to enhance microbial natural product production. These include the optimisation of growth media, incubation conditions, co-culture and small molecule elicitation. Strategies to maximize the production and secretion of oosporein from *B. caledonica* were investigated. Cultures were grown for 24 h before a sub-inhibitory concentration (17.5 µg/ml) of oosporein was added. Cultures were grown for 96 h in total before they were filtered and analyzed using RP-HPLC (Fig. S2). *B. caledonica* cultures spiked with oosporein produced significantly higher levels of oosporein when compared to the DMSO spiked or media only controls (Fig. 2A). In order to verify that the compound enhancing oosporein production was actually oosporein, both organic extract and purified fractions containing oosporein at the same concentration were spiked into *B. caledonica* cultures (Fig. 2C,D). This increased oosporein production was visible by eye as dark pink pigmented culture (Fig. 2E,F).

3.4. Label free quantitative (LFQ) proteomics of the effect of oosporein on the B. caledonica proteome

Exposure of *B. caledonica* to exogenous oosporein during growth resulted in a very strong induction of oosporein production (Fig. 2 and Fig. S2). To examine this feedback induction mechanism, the proteome of *B. caledonica* with and without oosporein exposure was characterized. A total of 1561 proteins were reproducibly detected in the *B. caledonica* samples using quantitative proteomics (Supplementary File 1 and Fig. S3). Twenty one proteins were deemed to be significantly more abundant upon oosporein treatment compared to a DMSO control. Six proteins were also uniquely detected upon oosporein treatment. Eleven proteins were deemed to be significantly less abundant upon oosporein treatment and eight proteins were uniquely detected in the control (Supplementary File 1). Five proteins (OpS2, Osp4, OpS5, OpS6 and OpS7) encoded by the oosporein cluster (BBA_08179−BBA_08185) were reproducibly detected across all samples.

3.5. Effect of oosporein on haemocyte densities of insect larvae

G. mellonella larvae treated with oosporein showed a reduction in haemocyte densities relative to controls at both 24 h ($p < 0.001$) and 48 h ($p < 0.01$) (Fig. 3A). Treatment ($F_{1,15} = 38$, $p < 0.001$) and time ($F_{1,15} = 53.22$, $p < 0.001$) both had a significant effect. *H. abietis* larvae treated with oosporein showed a reduction in haemocyte densities at both 24 h ($p < 0.05$) and 48 h ($p < 0.01$) relative to control larvae (Fig. 3B). Treatment ($F_{1,15} = 16.2$, $p < 0.01$) and time ($F_{1,15} = 32$, $p < 0.001$) both had a significant effect. There was not a significant interaction between treatment and time in either species of insect.

3.6. Effect of oosporein on yeast cell density in insect larvae

In *G. mellonella* larvae injected with oosporein, treatment ($F_{1,11} = 18.64$, $p < 0.01$) and time ($F_{1,11} = 156.6$, $p < 0.001$) showed a significant effect. There was no significant interaction between treatment and incubation time of insects. *G. mellonella* larvae treated with oosporein had an increased yeast cell density ($p < 0.05$) relative to control larvae when the larvae were incubated for 48 h following injection with *C. albicans* (Fig. 3C). In *H. abietis* larvae, treatment ($F_{1,11} = 109.12$, $p < 0.001$) showed a significant effect but incubation time of insects did not. There was a significant interaction between treatment and time ($F_{1,11} = 48.05$, $p < 0.001$). *H. abietis* larvae treatment with oosporein had an increased yeast
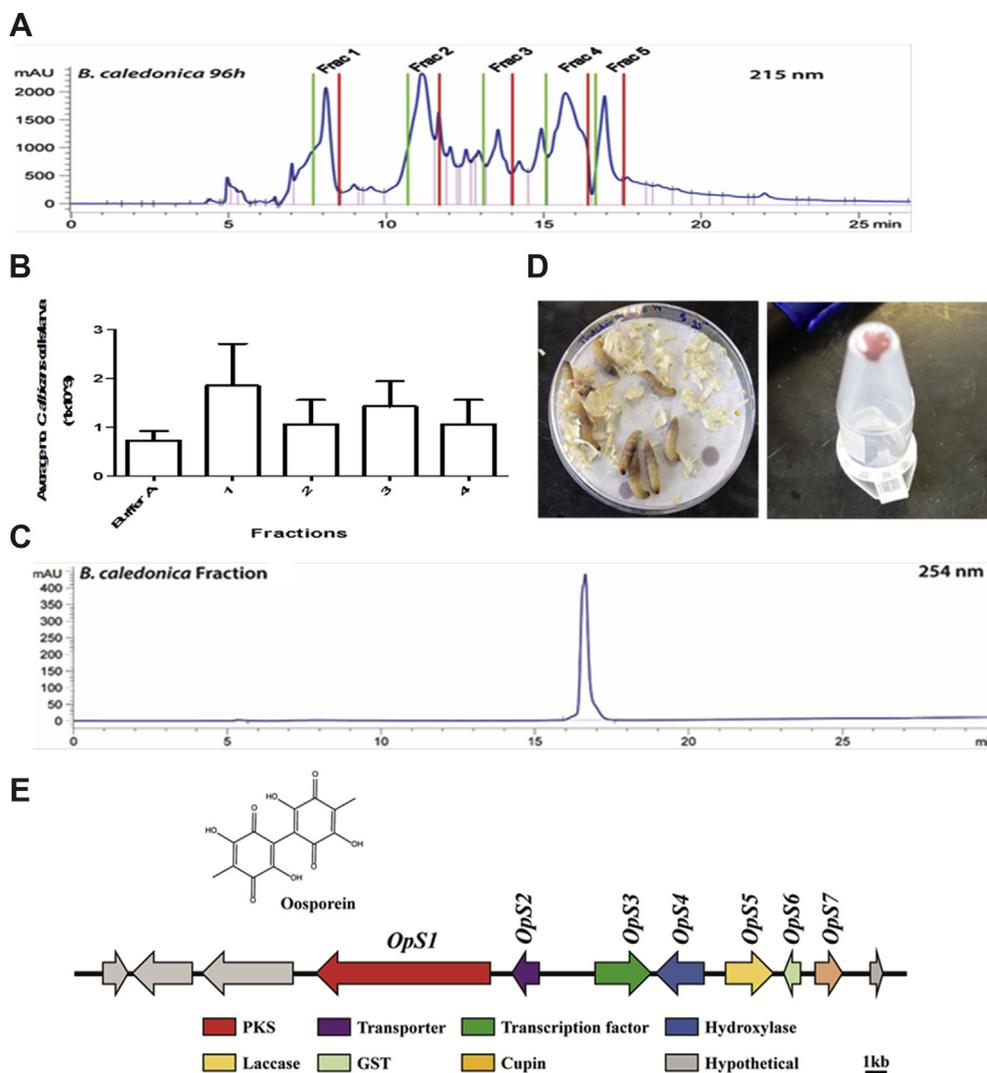
Fig. 1. Identification of oosporein. (A) RP-HPLC profile of organic extracts of *B. caledonica* 96 h culture filtrate at 215 nm indicating where fractions were collected. (B) Yeast cell density in *G. mellonella* larvae pre-treated with RP-HPLC fractions 1–4 of *B. caledonica* 96 h culture. Number of *C. albicans* cells per larva after incubation for 24 h at 20 °C. Fractions were injected 24 h prior to inoculation of larvae with *C. albicans*. RP-HPLC buffer A (0.1 % (v/v) trifluoroacetic acid (TFA) in HPLC-grade water and solvent) was used as a control. One way ANOVA showed that pre-treatment with the fractions tested did not significantly increase yeast cell density in *G. mellonella* larvae. (C) Fraction of interest (fraction 5) from *B. caledonica* 96 h culture filtrate; RP-HPLC profile of immunosuppressive compound. (D) *G. mellonella* larvae injected with fraction of interest (fraction 5). Microcentrifuge tube containing dried metabolite with distinctive red colour. (E) Structure of oosporein and oosporein cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cell density ($p < 0.001$) relative to controls when the larvae were incubated for 24 h following injection with *C. albicans* (Fig. 3D).

3.7. Investigating whether oosporein increases susceptibility of larvae to a subsequent infection

H. abietis larvae treated with oosporein followed by *C. albicans* had a higher mortality compared to larvae treated with oosporein alone or larvae treated with DMSO followed by *C. albicans* ($p < 0.05$) (Fig. 3F). Oosporein increased the susceptibility of *H. abietis* larvae, but not *G. mellonella* larvae, to a subsequent infection (Fig. 3E).

3.8. Label free quantitative (LFQ) of the effect of oosporein on the G. mellonella proteome

Label free proteomics was used to compare the proteome of hemolymph from *G. mellonella* larvae injected with 0.125 μg oosporein to control larvae injected with DMSO. Principal component analysis (PCA) confirmed a difference between the expression profiles of treated and control larvae (Fig. S4). In total, 288 proteins were identified, 282 having two or more peptides. When these proteins were filtered to contain only proteins found in three replicates of at least one group 142 proteins remained; 27 of these proteins were either significantly changed in abundance or exclusively detected in one sample group. Three proteins were found to be absent (below the level of detection) in oosporein-treated larvae and two proteins were found to be absent in control larvae (Supplementary File 1). These proteins were termed 'uniquely detected proteins'. These protein hits were also used in statistical analysis of the total differently expressed sample groups following imputation based on normal distribution. Two proteins were exclusively expressed in oosporein-treated *G. mellonella*, an uncharacterized protein and a DEAD-Box RNA helicase. DEAD-Box RNA helicases are a family of proteins that participate in virtually every aspect of RNA metabolism (Cordin et al., 2006). Three proteins were below the level of detection in larvae treated with
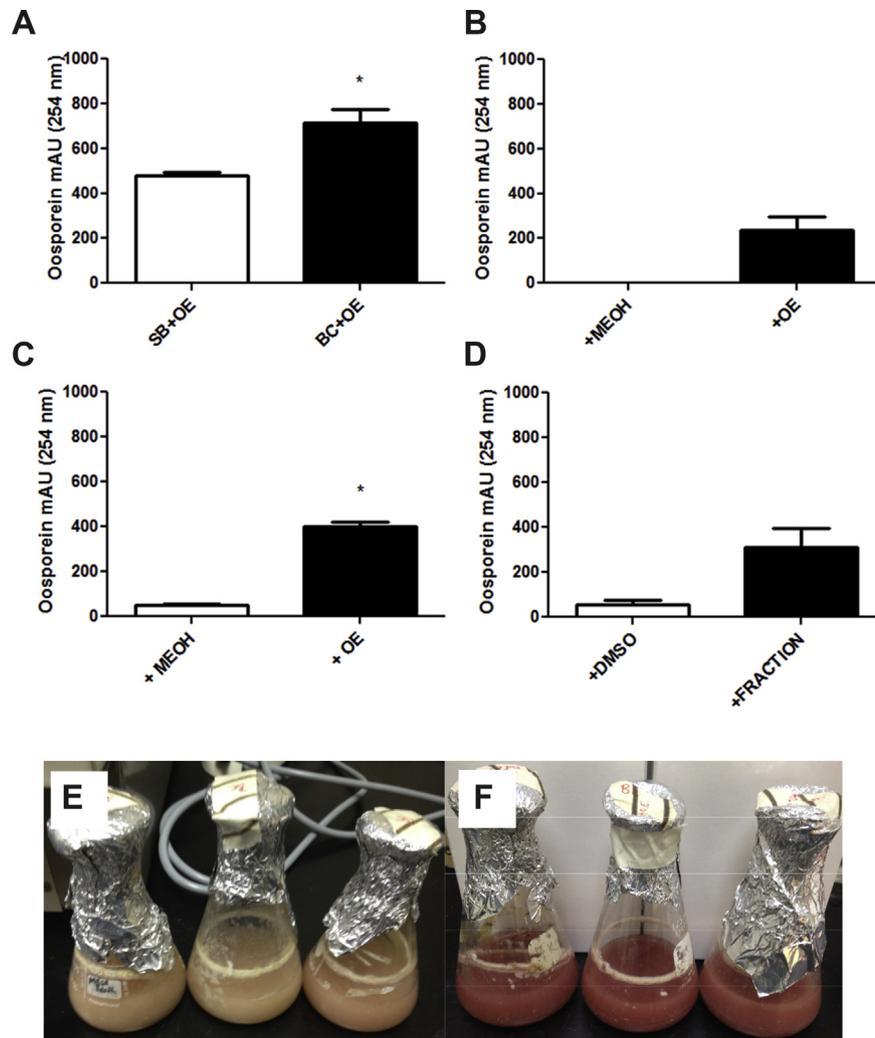
Fig. 2. Spike assays to investigate the effect of exogenously added oosporein on oosporein biosynthesis in *B. caledonica*. *B. caledonica* cultures were grown for 24 h before 875 μg of oosporein was added (17.5 μg/ml final). Cultures were grown for 96 h in total before they were filtered and analyzed using RP-HPLC. Peak surface area is denoted as milli-Arbitrary Units (mAU). Each bioassay was repeated on four occasions. Mann Whitney *U* tests were carried out to compare treatments, *p < 0.05. For B, C, D the average peak surface area for the media control was already subtracted to allow comparison of the amount of oosporein produced by *B. caledonica* cultures without/without the addition of exogenous oosporein. (A) SB + OE, Sabouraud dextrose liquid medium (SB) spiked with total organic extract (OE) containing oosporein; BC + OE, *B. caledonica* cultures (BC) spiked with total organic extract containing oosporein. (B) +MEOH, BC spiked with solvent as a control; +OE, BC spiked with organic extract containing oosporein. (C) +MEOH, BC spiked with solvent (control); +OE, BC spiked with oosporein. (D) +DMSO, BC spiked with solvent (control); +Fraction, BC spiked with purified oosporein (rather than total organic extract). (E) *B. caledonica* cultures spiked with solvent. (F) *B. caledonica* cultures spiked with oosporein.

oosporein: fructose-1,6-bisphosphatase, cathepsin B-like cysteine proteinase and serine protease inhibitor 13 precursor. Cathepsin B-like cysteine proteinase is involved in digestive proteolysis by many insects and such proteins are often targets of plant defensive cystatins (Koiwa et al., 2000). Cysteine peptidases/proteinases may have an important role in digestion of coleopterans such as *Tenebrio molitor* and *Tribolium castaneum* (Morris et al., 2009; Dunse et al., 2010; Perkin et al., 2016). *T. castaneum* cysteine peptidase genes include cathepsin B. Cathepsin B-homolog was expressed in all life stages of *T. castaneum*, although considerably lower in pupae. While these peptidases may not have a catalytic role, their expression patterns suggest that they have biological relevance in *T. castaneum* (Perkin et al., 2016).

The overall proteomic profile can be seen in Fig. S5, in a heat map derived from hierarchical clustering following *Z*-score normalization of LFQ intensities using Perseus software. Proteins with high abundance are represented in red and those with low abundance are represented in blue. In larvae treated with oosporein 19 proteins were significantly (*p* < 0.05) increased in abundance

at a fold change of >1.5 (Supplementary File 1). The defensin-like AMP spodoptericin (Seufi et al., 2011) is increased in abundance in oosporein treated larvae.

3.9. Oosporein does not act as a contact toxin

All *G. mellonella* larvae and the majority of *H. abietis* larvae survived for 2 weeks after being dipped in oosporein at a concentration of 1.25 mg/ml and 125 μg/ml (Fig. S6a), with little further mortality occurring at three weeks (Fig. S6b). There was no significant difference in mortality caused by treatment for either insect. Thus, it appears that oosporein does not act as a contact toxin on *H. abietis* adults and *G. mellonella* larvae at the concentrations tested.

3.10. Oosporein encourages feeding by H. abietis adults

Oosporein did not display an anti-feedant effect on *H. abietis*. When given a choice between oosporein-treated and untreated twigs, the weevils ate equal amounts of both foods (Fig. 4). Overall,
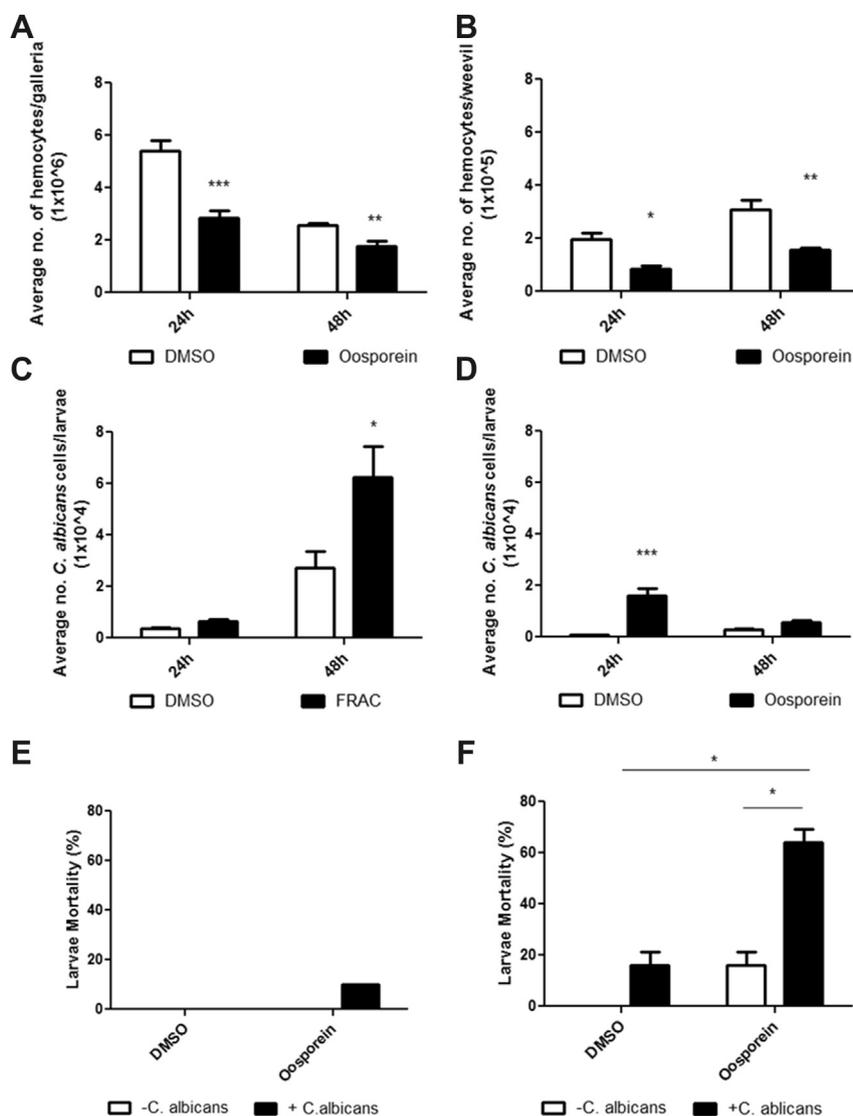
Fig. 3. Immunomodulation bioassays. (A & B) Haemocyte densities in larvae treated with oosporein. Following inoculation with oosporein or DMSO (control), larvae were incubated for 24 h or 48 h at 20 °C before bleeding and enumeration (A) *G. mellonella* larvae. (B) *H. abietis* larvae. (C & D) Yeast cell density in larvae pre-treated with oosporein. Number of *C. albicans* cells per larva after incubation for 24 h and 48 h at 20 °C. Larvae were treated with oosporein 24 h prior to injection with *C. albicans*. (C) *G. mellonella* larvae: treatment with oosporein increased ($p < 0.05$) yeast cell density relative to controls after a 48 h incubation. (D) *H. abietis* larvae: treatment with oosporein increased ($p < 0.05$) yeast cell density relative to controls after a 24 h incubation. Two way ANOVA was carried out with Bonferroni post-tests to compare treatments to relevant controls. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (E & F) Mortality of larvae treated with oosporein alone and in combination with *C. albicans*. Results shown are larval mortality one week after treatment with *C. albicans*. DMSO (5 %) was used as a control. $+C.$ albicans indicates larvae that received a dose of *C. albicans* after 24 h, $-C.$ albicans indicates larvae that did not. Mann Whitney Tests were used to compare larval mortality between treatments, $^*p < 0.05$. (E) *G. mellonella* larvae: there was no significant increase in mortality caused by either oosporein or *C. albicans*. (F) *H. abietis* larvae: There was a significant difference in mortality between larvae treated with oosporein and *C. albicans* compared to those who only received oosporein. There was also a significant difference between larvae treated with DMSO and *C. albicans* compared to larvae treated with oosporein and *C. albicans*.

H. abietis adults fed significantly more in tubs that contained oosporein-treated twigs than in tubs containing only untreated twigs (Fig. 4).

4. Discussion

4.1. Oosporein is an abundant secreted natural product of B. caledonica

A wide range of secondary metabolites displaying bioactivity such as insecticidal properties have been identified from *B. bassiana* including the cyclopeptides beauvericin, bassianolide and beauverolide, the yellow pigment pyridines tenellin and bassiatin and the dibenzoquinone oosporein (Xiao et al., 2012). A comparative genome survey of the core genes involved in secondary metabolite synthesis in *B. bassiana* found that NPRS, PKS and terpene synthases encoding genes were highly conserved in the four insect pathogens tested (*B. bassiana*, *Metarhizium robertsii*, *Metarhizium acridum* and *Cordyceps militaris*) but absent in other fungi, implying that evolution of entomopathogenic fungi may be associated with production of some similar secondary metabolites (Xiao et al., 2012). Oosporein is known to be produced by *Beauveria brogniarti* and *B. bassiana* (Feng et al., 2015). Here we show that oosporein is abundantly produced in the entomopathogenic fungus *B. caledonica*.

The previously unidentified metabolite from *B. caledonica* appeared as an orange/red semi crystalline solid after purification by HPLC. IR and UV data correlated closely with that in literature reports (Divekar et al., 1959; Cole et al., 1974; Nagaoka et al., 2004). Mass spectrometry confirmed the presence of the oosporein anion.
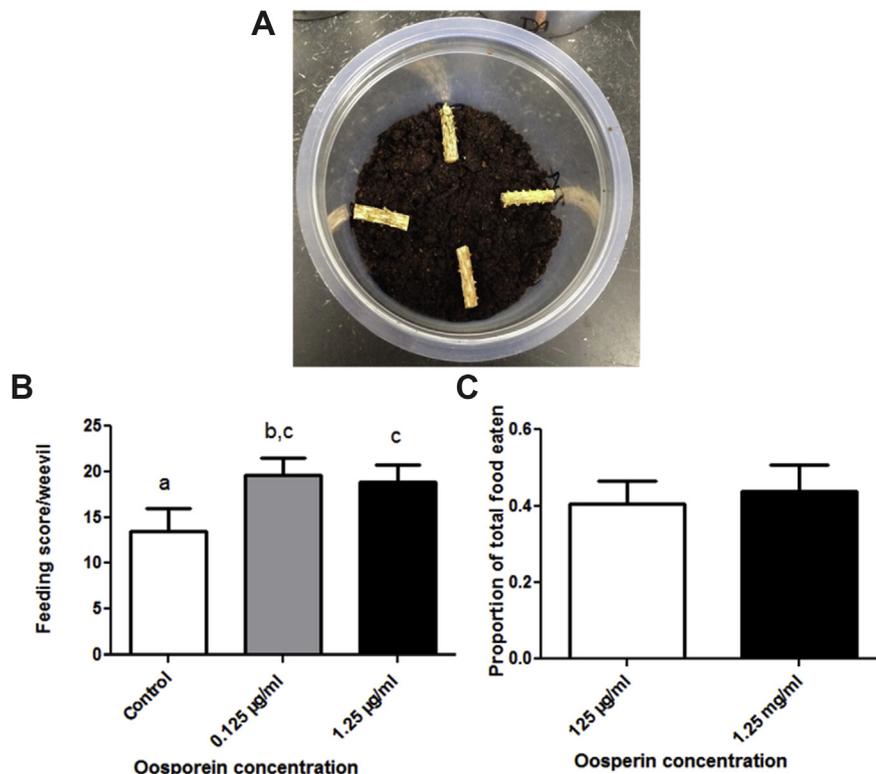
Fig. 4. Choice-bioassay to investigate if oosporein affects feeding of *H. abietis* adults. (A) Bioassay choice test set up. (B) The effect of oosporein on *H. abietis* feeding after one week. (A) Total food eaten per tub (total of four twigs) for each treatment. *H. abietis* adults fed more in tubes that contained oosporein treated twigs ($F_{2,88} = 9.06$, $p < 0.001$). Tukey's Multiple Comparison test showed that tubes containing 1.25 mg/ml and 125 μg/ml oosporein were significantly different from control tubes but not from each other. (C) Oosporein treated food: as a proportion of the total food consumed in tubs containing both untreated and oosporein-treated food. Wilcoxon signed rank tests showed that *H. abietis* adults did not feed significantly more or less on twigs treated with oosporein than control twigs, at either concentration.

Thus the abundant metabolite produced in *B. caledonica* 96 h culture filtrates was identified as oosporein.

Oosporein is linked with avian gout in chickens and turkey (Pegram and Wyatt, 1981), which raises safety concerns for the application of oosporein-producing EPF to control insect pests (Feng et al., 2015).

4.2. The effect of oosporein on its own regulation in B. caledonica

The addition of oosporein to *B. caledonica* cultures significantly increased levels of oosporein relative to a solvent control. There are few examples of 'self-activating' natural product pathways in the literature. Exposure of *Aspergillus fumigatus* to gliotoxin has been shown to induce the expression of genes in the gliotoxin biosynthetic cluster (Cramer et al., 2006). This was also shown on the proteomic level, demonstrating that the pathway can be completely activated by this external signal (Dolan et al., 2014). However, the exact mechanism of this induction remains unclear. In fungi, oosporein is synthesized by a polyketide synthase (PKS) pathway including seven genes for quinone biosynthesis (Feng et al., 2015). The PKS oosporein synthase 1 (OpS1) produces orsellinic acid that is hydroxylated to benzenetriol by the hydroxylase OpS4. The intermediate is oxidized either non-enzymatically to 5,5′-dideoxy-oosporein or enzymatically to benzenetetrol by the putative dioxygenase OpS7. The latter is further dimerized to oosporein by the laccase OpS5. The transcription factor OpS3 regulates intra-pathway gene expression (Feng et al., 2015). A highly conserved OpS3 CGGA binding motif was identified in the promoters of the clustered oosporein genes, except for in OpS5. This motif was also detected in the OpS3 promoter, suggesting the formation of a positive feedback loop for self-activation. In order to

investigate the mechanism underlying the induction of oosporein production by oosporein exposure, the proteomic response of *B. caledonica* to exogenously added oosporein was characterized.

Five proteins (OpS2, Osp4, OpS5, OpS6 and OpS7) encoded by the oosporein cluster (BBA_08179−BBA_08185) were reproducibly detected across all samples, and the abundance of two of these, OpS4 and OpS6, was increased following oosporein treatment. OpS4 is a FAD binding domain hydroxylase which carries out the hydroxylation of orsellinic acid to benzenetriol. The abundance of OpS4 was significantly increased (↑0.746) following exposure to oosporein. This indicates that exogenous oosporein exposure leads to an increased expression of the biosynthetic enzymes required for oosporein production. OpS6 is annotated as a glutathione-s-transferase (GST), which has been proposed to scavenge leaked free radical (6-methyl-1,2,4,5-benzenetetrol) during oosporein biosynthesis. OpS6 was also detected with significantly increased abundance (↑0.973). A *B. bassiana* OpS6 mutant did not produce oosporein, but overexpression of the oosporein pathway specific transcriptional regulator *OpS3* in this mutant ($\Delta OpS6::OpS3$) restored oosporein production (Feng et al., 2015). The abundance of OpS6 may be increased upon oosporein exposure to combat excessive free radical production as oosporein production is increased.

The putative major facilitator superfamily transporter *OpS2* was unchanged in abundance between oosporein and DMSO treatment despite the increased production of oosporein. Deletion of *OpS2* was shown to result in significantly increased oosporein production compared to the wild-type (Feng et al., 2015). This surprising result can be explained in the context of the substrate feedback activation described in this study. We have shown that *B. caledonica* oosporein production is significantly increased when oosporein is spiked into the culture. Spiking in additional oosporein to cultures may result in

saturation of the dedicated oosporein transporter (*OpS2*), leading to an accumulation of intracellular oosporein, and enhanced oosporein biosynthesis. If the signal to decrease the expression of genes encoding oosporein biosynthetic enzymes is regulated by intracellular oosporein concentration, saturating the oosporein efflux machinery would lead to sustained oosporein cluster expression. This would result in the increased accumulation of oosporein in the culture medium. Deletion of the ABC transporter gene *sirA* from the *Leptosphaeria maculans* biosynthetic gene cluster for sirodesmin resulted in elevated sirodesmin biosynthesis (Gardiner et al., 2005).

The proteomic results suggest that exogenous oosporein can induce the expression of the oosporein cluster, leading to the increased abundance of the oosporein biosynthetic enzymes. This activation may be mediated directly by activation of the oosporein pathway specific transcriptional regulator *OpS3* or by activating a conserved signalling pathway in this organism. BBA_07446, annotated as an arid/bright DNA binding domain-containing protein, was uniquely detected upon oosporein exposure, also BBA_04214, an uncharacterised transcriptional repressor was uniquely detected in the control samples. Two other uncharacterised proteins (BBA_00943 and BBA_01405) were also uniquely detected upon oosporein exposure. The large number of uncharacterised proteins in *Beauveria* spp. highlights the lack of knowledge as to how their natural product pathways are activated or sustained. Two putative peptidases (BBA_04603 and BBA_07828) had the most significant increases in abundance in the oosporein spiked samples compared to the control. BBA_04603 (↑2.43) is a putative ATP-dependent peptidase which is likely a component of the mitochondrial inner membrane protease complex (i-AAA complex). BBA_07828 (↑2.43) is annotated as a M48 peptidase family protein CAAX-box protein processing.

4.3. Oosporein bioactivity towards insects: immunosuppression and feeding

The biological activity of oosporein produced by EPF is not fully understood. Fan et al. (2017) found evidence that it acts as a late stage antimicrobial compound, produced after host death, allowing the fungus to complete its life cycle and sporulate on the host cadaver (Fan et al., 2017). However, oosporein has also been shown to contribute to the evasion of the insect host immune system: strains of *B. bassiana* producing oosporein germinated and escaped from haemocyte encapsulation faster than strains that had lost their ability to produce the toxin (Feng et al., 2015). Here we provide evidence supporting an immunosuppressive function of oosporein. Both *G. mellonella* and *H. abietis* larvae treated with oosporein had significantly increased yeast cell density and a reduction in haemocyte densities relative to control larvae. Moreover, oosporein increased the susceptibility of *H. abietis* larvae to a subsequent yeast infection. However, this was not the case for *G. mellonella* larvae, indicating that mode of action may vary between hosts.

The insect immune system consists of both cellular (haemocyte) and humoral components. LFQ detected changes in the proteome of insect haemolymph treated with oosporein. A number of proteins were significantly altered in abundance, many with immune related functionality. Treatment with oosporein affects the proPO pathway: a serine proteinase inhibitor was below the level of detection in oosporein treated larvae. Insect melanisation is controlled by a serine protease cascade that ultimately activates the enzyme PPO. Inactive PPO is cleaved into active PO by a serine protease PPAE, PPAE also must be activated by other serine proteases (De Gregorio et al., 2002). Proteinase inhibitors can be present constitutively and may also be produced in response to infection or wounds. Serine proteinase inhibitors are reversible inhibitors, key modulators of the proPO cascade and deactivators of

microbe derived proteases (Butt et al., 2016). Thus, the decreased abundance of a serine proteinase inhibitor below the level of detection may be an indication that oosporein is suppressing the immune response of larvae as insects use protease inhibitors as a safety mechanism to regulate PO activation (Butt et al., 2016). The proPO activation cascade is key to the insect immune response and links many humoral and cellular defense responses such as melanisation, healing, cytotoxic reactions, phagocytosis, encapsulation, nodulation and sclerotization (Marmaras and Lampropoulou, 2009). Complementary to these findings, there is evidence that injection of oosporein inhibits PPO activity in *G. mellonella*: it was found to block PPO cleavage, and cleavage of PPO to PO is the hallmark of PPO activation (Feng et al., 2015). These authors also found that oosporein inhibition of PPO activity had a dose dependent trend. Inhibiting PPO activity may contribute to fungal virulence by weakening the immune response and thereby facilitating fungal multiplication within the host (Feng et al., 2015). This proposed weakening of the host immune response leaving the insect more susceptible to infection is reflected in the effect of oosporein on yeast load and insect mortality in our experiments. Amin et al. (2010) found that a red pigmented metabolite (presumably oosporein) applied with *B. bassiana* spores resulted in greatly increase mortality of whitefly *Bemisia tabaci*, and suggested that this synergistic action may be as a result of antibacterial activity of the pigment against the insect's intestinal bacteria, allowing rapid spore germination and proliferation.

Although we tested oosporein without its natural fungal associate, it has previously been shown to contribute to fungal virulence in *G. mellonella* as fungal mycosis of insect cadavers was considerably slower in *B. bassiana* strains that had lost oosporein producing ability (Feng et al., 2015). Feng et al. (2015) showed that oosporein promoted *B. bassiana* infection both by inhibiting PO activity and by downregulating expression of antifungal peptide genes in the insect fat body. Wei et al. (2017) further elucidated the mechanism by which oosporein facilitates fungal infection, showing that it mediates down-regulation of Duox expression in the midgut, which reduces midgut ROS production. Duox-dependent ROS generation plays a major role in gut immunity and the control of gut-associated bacteria.

In support of previous studies reporting limited toxicity of oosporein for insects, oosporein was found not to act as a contact toxin on *H. abietis* adults and *G. mellonella* larvae at the concentrations tested, nor did it cause substantial mortality when injected into larvae of either species. One of the limitations of EPF in biological pest control is that they kill slowly. Inhibition of feeding can ensure plant protection in the interim between deployment of controls and eventual death of the insect. However, previous work has not shown antifeedant activity or oral toxicity against the cockchafer *Melolontha melolontha* or various lepidopteran larvae (Abendstein et al., 2001, 2003). Similarly in our study, oosporein did not exhibit anti-feedant or toxic properties against *H. abietis* adults (the damaging stage of this pest). In fact, insects exposed to oosporein-treated food actually fed more overall than those which were only exposed to control food. The finding that exposure to oosporein stimulated feeding correlates with the increased abundance in a gustatory receptor following oosporein treatment (Supplementary File 1). Perhaps this reflects the energy demands of the immune response to oosporein. Essential immune processes such as the prophenoloxidase cascade are energy dependent and thus can be limited by food resources (González-Santoyo and Córdoba-Aguilar, 2012).

5. Conclusion

Oosporein was the major secondary metabolite produced by the isolate of *B. caledonica* in this study. Consistent with work by Feng

et al. (2015), it appears that oosporein promotes infection rather than directly killing insects. Oosporein extracted from *B. caledonica* cultures exhibits immunosuppressive properties that may have potential in pest control by promoting faster and greater mortality in combination with other treatments. High yields of oosporein would be desirable if it was to be applied in combination with spores as suggested by Amin et al. (2010), and/or for research and use in other industries, and our results indicating that addition of oosporein promotes its own production in culture has potential for optimizing its yields through fermentation. Similarly, it may also point to an avenue for improving strains for biocontrol by increasing production of oosporein. These data shed light on *B. caledonica* as a biocontrol agent, while also providing insight into the production of this potent natural product.

Author contribution

LMN & SKD designed research. LMN & SKD conducted experiments. JMDW & JCS carried out NMR. LMN & SKD analyzed data. TRG provided *Beauveria caledonica* genome. CTG & KK were involved in planning & supervising the work. All authors contributed to writing, read and approved manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.01.004.

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