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Spore inoculum size impacts substrate degradation and sporulation but not the secretome during colonization of whole yellow pea (*Pisum sativum*) by *Aspergillus oryzae*

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ABSTRACT

Aspergillus oryzae is used in solid-state fermentation (SSF) to produce plant-based foods. To this end, the substrate is inoculated with spores of this fungus. So far, the effect of inoculum size on SSF with *A. oryzae* has primarily focused on the production of specific enzymes. Therefore, the aim of this study was to examine the impact of inoculum size on the full secretome, combined with enzyme activity assays, assessment of colonization, substrate degradation, and sporulation. To this end, *A. oryzae* was grown for 7 days on whole yellow pea (*Pisum sativum*). Fluorescence microscopy with a GFP-expressing *A. oryzae* strain showed that peas had been colonized externally and internally, irrespective of inoculum size. Yet, the highest inoculum size resulted in a stronger pea biomass reduction when compared to the lowest inoculum size. By contrast, sporulation decreased with increasing inoculum size. Notably, proteomics revealed no effect of inoculum size on the protein profiles of aqueous extracts of the colonized peas. Amylases and proteases were the most abundant secreted proteins, which was consistent with their high activity in the aqueous extracts. Proteomics also identified β-1,3-glucanases and chitinases, indicating hyphal lysis. Indeed, 10–19% of the fungal proteins detected in the aqueous extracts lacked signal peptides. These data contribute to our understanding of colonization of substrates by *A. oryzae* and may be used to optimize SSF with this food grade fungus.

1. Introduction

The expanding world population together with the improved socio-economics of developing countries is expected to result in an increased demand for animal-based protein. However, meat production is associated with detrimental effects on the environment, unfavorable resource use, human health risks, and animal welfare issues (Cassidy et al., 2013; Chulayo and Muchenje, 2015; FAO, 2024; Foley et al., 2011; Godfray et al., 2018; He et al., 2020; Mekonnen and Hoekstra, 2012). Due to this, plant-based meat alternatives (PBMAs) have received increasing interest (He et al., 2020).

Pea (*Pisum sativum*) is an attractive crop to create PBMAs. It is one of the most widespread and economically important legumes globally (FAOSTAT, 2023; Ljuština and Mikić, 2010). Their production has a low water (1.979 m^3 ton^{-1}) and carbon (0.58 kg CO_2 Eq kg^{-1}) footprint (Mekonnen and Hoekstra, 2011; Petersson et al., 2021). At the same time, peas improve soil-health by fixing nitrogen. This reduces the need for fertilizer input and enhances the yield of crops when used in crop rotation (MacWilliam et al., 2018; Tulbek et al., 2017). A major part of peas (76% in Europe) is used as animal feed (FAOSTAT, 2022). Hence, a

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huge environmental gain could be achieved when legumes are directly used for human consumption (Svanes et al., 2022; Tidåker et al., 2021; van Loon et al., 2023).

Yellow peas are a genetic variety of *P. sativum*. They represent a great source of starch (32.5–57.5%), dietary fiber (14.8%), vitamins, and minerals, while being low in fat (0.8–4.6%) (Asen et al., 2023; García Arteaga et al., 2021; Millar et al., 2019; Moussou et al., 2019; Shen et al., 2016). In addition, protein content is similar to that of meat, ranging from 20.0 to 27.2% (Asen et al., 2023; García Arteaga et al., 2021; Millar et al., 2019; Moussou et al., 2019; Tzitzikas et al., 2006). However, pea proteins are limited in the amino acids methionine, cysteine and tryptophan, affecting the nutritional quality (Gorissen et al., 2018; Iqbal et al., 2006; Leterme et al., 1990). In addition, protein digestibility is hampered by the structure of the storage proteins and the presence of proteins in indigestible matrices such as cellulose and hemicellulose (Carbonaro et al., 2012, 2015; Deshpande and Damodaran, 1989; Noel et al., 2024; Park et al., 2010). Furthermore, antinutrients (fructo-oligosaccharides, galacto-oligosaccharides, phytic acid, tannins, lectins, saponins, amylase inhibitors, and chymotrypsin and trypsin inhibitors) interfere with the digestibility of pea and may result in digestive distress, such as cramping and a bloated feeling (Lenhart and Chey, 2017; Vidal-Valverde et al., 2003; Wu et al., 2023).

A clean-label approach to improve the bioavailability of plant nutrients is solid-state fermentation (SSF). *Aspergillus oryzae* is a decomposer of organic substrates in nature and is known for the secretion of large amounts of hydrolytic enzymes (e.g. amylases, cellulases, xylanases, pectinases and proteases) that target complex plant carbohydrates and proteins (Daba et al., 2021; Machida et al., 2005; Mu et al., 2024). Consequently, this fungus is widely used in biotechnology and the food industry (Daba et al., 2021). *A. oryzae* has been used in SSF for millennia to produce foods and beverages like shoyu (soy sauce), miso (soybean paste) and sake (rice wine). The fungus is considered generally regarded as safe by the Food and Drug Administration (FDA), which is supported by the World Health Organization (WHO) (Machida, 2002). SSF using *A. oryzae* has been shown to decrease antinutrient content, to increase protein content, and to improve amino acid composition and flavor of soy and fava bean flour (Chen et al., 2013; Gautheron et al., 2024).

SSF with *A. oryzae* is started by inoculating the substrate with spores of this fungus. Production of enzymes is generally higher with increasing inoculum size until an optimum is reached, after which enzyme production is reduced (Battaglino et al., 1991; Melnichuk et al., 2020; Sandhya et al., 2005; Shankar and Mulimani, 2007). However, these studies only assessed the production of selected enzymes. Therefore, the aim of this study was to assess the effect of inoculum size on the secretome, combined with enzyme activity assays, assessment of colonization, substrate degradation, and sporulation. It is shown that the secretome of *A. oryzae* is not affected by inoculum size after 7 days of SSF of whole yellow pea. Results do show that a high inoculum size results in more efficient pea biomass conversion and in reduced sporulation. These results contribute to our understanding of the biology of SSF, which may be used to improve food production with *A. oryzae*.

2. Material and methods

2.1. Strains and culture conditions

A. oryzae strains RIB40 (IBT 28103; Technical University of Denmark) and its derivative RB#140.1 that expresses GFP from the *A. niger glaA* promoter (Bleichrodt et al., 2012) were used in this study. Conidia of *A. oryzae* were harvested with Milli-Q water from 7-day-old cultures that had been inoculated by homogenous spreading of spores on potato dextrose agar (PDA) and that had been grown at 30 °C in the dark. The spores were filtered over glass wool to remove hyphae and counted with a hemocytometer.

A. oryzae was routinely grown on whole yellow peas (with hull) (*P. sativum* L. var. Ingrid, Pure Dansk, Denmark, www.puredansk.dk) in Petri dishes (12 × 12 cm for proteomics and 5.4 cm in diameter for the other experiments). To this end, the peas were soaked overnight in sterile Milli-Q water (1: 2 (w/v) pea: water), resulting in the take-up of approximately one volume of water. Peas were drained and sterilized. Several sterilization methods were explored with the aim to minimally impact the integrity of the peas (Supplemental Text 1). Autoclaving for 20 min at 121 °C was the only method resulting in sterile peas (Supplemental Table 1). Therefore, autoclaved peas were used in subsequent experiments. The sterilized peas were distributed in the Petri dishes in a single, confluent layer (approximately 44 g and 8 g dry peas per square (12 × 12 cm) and round (5.4 cm in diameter) Petri dish, respectively), and evenly inoculated with 500 μL spore suspension containing 2.4 (SP I), 2.4 10^2 (SP II) and 2.4 10^5 (SP III) spores g^{-1} dry weight of pea. Petri dishes were incubated for a maximum of 12 days at 30 °C in the dark.

2.2. Assessment of colonization and spore production

Colonization of peas by *A. oryzae* was monitored by making color scans ($n = 6$) (600 dpi, 24-bits) of the top and bottom of the cultures with a desktop scanner (Epson Perfection V370 Photo, www.epson.nl) (Vos et al., 2021). Top scans were used for descriptive assessment of sporulation and sclerotia formation, while bottom scans were used to describe fungal colonization and pigmentation. To this end, bottom scans were converted into 8-bits images and a surface area of 9.5 cm^2 (i.e. 810 × 810 pixels) was analyzed in ImageJ to determine the mean gray value (FIJI version v1. 53t, www.imagej.net/software/fiji). Colonization of the peas was also analyzed using fluorescence microscopy ($n = 3$). To this end, cross-sections of RB#140.1-colonized peas were imaged with a Leica MZ10F stereomicroscope (www.leica.com) equipped with a pE-300^{white} LED light source, a GFP LP filter, and an Euromex sCMEX-20 digital camera (www.euromex.com). Finally, fungal colonization was assessed by dry weight loss (n = 3) of the pea substrate.

Spore production was determined (n = 6) by suspending the colonized peas in 20 mL 0.9% (w/v) NaCl, 0.02% (v/v) Tween-80 (ST), vigorously shaking for 30 s, filtering over Miracloth (Millipore, www.merckmillipore.com), and centrifuging at 4816 g for 10 min. Spores were resuspended in 2 mL ST and counted with a hemocytometer.

2.3. Substrate transformation analysis

After 7 days of growth, the colonized matrix in the Petri dish (n = 6) was cut in three pieces, mixed with a volume of Milli-Q water equaling the fresh weight of the peas at $t = 0$, and rotated at 10 rpm for 30 min at 4 °C. The liquid phase was centrifuged at 4816 *g* for 10 min at 4 °C to remove fungal and pea material. The resulting supernatant was aliquoted in Eppendorf tubes and stored at −20 °C until further use for enzyme activity assays and SDS-PAGE. For proteomics (n = 3), a single strip (1 × 12 cm) was cut from the center of the matrix, and a volume of Milli-Q water equal to the weight of the strip at the moment of harvesting was added to prepare the extracts.

2.3.1. Amylase, cellulase and pectinase activity

Amylase, cellulase, and pectinase activities were measured with the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). All activities were measured in phosphate buffer (0.1 M NaH_2PO_4), pH 6, supplemented with 0.5 g L^{-1} bovine serum albumin (BSA) and 3 mM NaN_3. For the amylase activity assay, 50 μL undiluted extract (non-inoculated pea) or 250-fold diluted extracts (inoculated peas) were mixed with 50 μL 5 g L^{-1} starch (Agro-Organics, www.agro-organics.co.za), water serving as a control. The mixtures were incubated at 50 °C for 2 h, followed by the addition of 100 μL DNS (44 mM 3,5-dinitrosalicylic acid, 1.4 M potassium sodium tartrate and 0.4 M NaOH) and incubation at 100 °C for 5 min. Samples were cooled on ice for 25 min in the dark, after which 100 μL was transferred to a 96-wells flat bottom plate (Greiner Bio-one, Cellstar 655,180, www.gbo.com) to measure the A_{540} in a Synergy HT

Microplate Reader (BioTek, www.agilent.com). A glucose standard curve was used to quantify the amount (mM) of sugar that was released in the assay during 2 h.

Cellulase and pectinase activity were measured in a similar way, replacing starch with a 7 mm diameter disk of Whatman No.1 filter paper (Ghose, 1987) or 0.5 g L^{-1} apple pectin (Sigma-Aldrich; www.sigmaaldrich.com) (Ceci and Lozano, 1998), respectively. Since undiluted extracts were used for the cellulase and pectinase activity assays, these activities were also corrected for blanks containing extract in the absence of substrate. For cellulase activity, the incubation period at 50 °C was extended to 24 h. For cellulase and pectinase activity, glucose and D-galacturonic acid (Sigma-Aldrich) standard curves were used, respectively.

2.3.2. Xylanase activity

Endo-xylanase activity was determined using the Xylanase Assay Kit (XylX6 Method; Mangan et al., 2017) (Megazyme, www.megazyme.com). Aqueous extracts were diluted 2-fold with phosphate buffer containing BSA and NaN_3 (see above) and pre-incubated at 40 °C for 3 min. Next, 50 μL of the aqueous extract was mixed with 50 μL XylX6 substrate (also preincubated at 40 °C) and incubated at 40 °C for 10 min. At the end of the incubation step, 100 μL stopping reagent (165 mM Tris buffer, pH 10) was added and 100 μL of the mixture was transferred to a 96-wells flat bottom plate (Greiner Bio-one, Cellstar 655,180). The A_{400} was measured in a Synergy HT Microplate Reader (BioTek) against Milli-Q water. Reagent blanks, which received stopping solution before addition of the aqueous extracts, were used as a negative control, while *Trichoderma* sp. *endo*-xylanase (supplied by the manufacturer) served as positive control. The xylanase activity unit was expressed as the amount of enzyme required to release 1 μmol 4-nitrophenol per minute from the XylX6 substrate.

2.3.3. Protease activity

Proteolytic activity was determined by the azocasein assay (Langner et al., 1973). Azocasein was taken up in ethanol (330 mg mL^{-1}) (Sigma-Aldrich), after which 100 mM NaH_2PO_4, pH 6, 0.02% (w/v) NaN_3 was added to obtain a final concentration of 10 mg mL^{-1} substrate. A volume of 50 μL azocasein suspension was mixed with 50 μL 0–3 fold diluted aqueous extract and incubated for 1 h at 37 °C at 200 rpm. The reaction was stopped by adding 100 μL cold (4 °C) 12% (w/v) trichloroacetic acid (TCA). After 20 min incubation at room temperature (RT), undigested azocasein was pelleted at 4816 g for 5 min. A volume of 100 μL was transferred to a 96-wells flat bottom plate (Corning Incorporated Costar, www.corning.com) and mixed with 100 μL 1 M NaOH. The A_{440} was recorded using the Synergy HT Microplate Reader (Biotek). Blanks were prepared in a similar way with 12% TCA added before the addition of the aqueous extract. A dilution curve of fully degraded azocasein was used as a reference. To this end, a final amount of 1% SDS was added to 10 mg mL^{-1} azocasein (see above), after which the mixture was incubated at 100 °C for 30 min. This was followed by adding 1 volume of 0.1 mg mL^{-1} proteinase K (Thermo Fisher Scientific, www.thermofisher.com) and incubating at 37 °C in the dark at 200 rpm for 48 h. Addition of 12% TCA, incubation at RT, and centrifugation were performed as described above. A dilution series of the supernatant was prepared with Milli-Q water and the A_{440} was measured after addition of one volume 1 M NaOH (see above). Part of the azocasein adhered to the tube. This amount was quantified and included in the calibration curve by dissolving in ethanol and 12% TCA (1: 1 ratio), preparing a dilution series in Milli-Q water and measuring the A_{440} after addition of one volume 1 M NaOH. The unit of activity was defined as the amount of protease that degrades 1 nmol of azocasein min^{-1}.

2.3.4. SDS-PAGE

Culture media were mixed in a 1: 1 ratio with 2 x sample buffer (20% glycerol, 4% SDS, 125 mM Tris pH 6.8, 0.02% bromophenol blue, 5% β-mercaptoethanol) and separated using 12.5% SDS-polyacrylamide gels and TGS buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) (Laemmli, 1970). Proteins were stained with 0.2% Coomassie blue in 40% methanol and 10% acetic acid and destained in the same solution without dye. PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Cat#26616) was used as a marker.

2.3.5. Proteomic analysis

2.3.5.1. Sample preparation. Protein concentration of aqueous extracts was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Thermo Fisher Scientific) (Smith et al., 1985). Proteins were reduced and alkylated in freshly prepared lysis buffer containing 1% sodium deoxycholate (SDC), 10 mM Tris(2-carboxyethyl) phosphinehydrochloride (TCEP) as a reducing agent, 40 mM chloroacetamide (CAA) as alkylating agent, and 100 mM TRIS, pH 8.0, supplemented with EDTA-free protease inhibitor (cOmplete Mini, Roche Diagnostics) (Post et al., 2017). The denatured, reduced, and alkylated samples were heated at 95 °C for 5 min, sonicated with a Bioruptor Plus (Diagenode, www.diagenode.com) for 15 min (50% duty with 15 cycles), and diluted in SDC lysis buffer to a final protein concentration of 1 μg $μL^{-1}$. Next, a sequential Lys-C trypsin digestion was done using enzyme: protein ratios of 1: 75. Lys-C (Wako, Japan) digestion was done for 4 h at 37 °C, followed by trypsin (Sigma-Aldrich) digestion overnight at 37 °C. SDC in the samples was precipitated with 2% (final concentration) formic acid (FA), followed by centrifugation at 20,000 *g* for 20 min at 4 °C.

Digested peptides were purified using reverse-phase Sep-Pak 1 cc (50 mg) tC18 cartridges (Waters, Ireland) (Southan, 1987). Columns were activated with 100% acetonitrile (ACN) and equilibrated with 0.1% FA (loading buffer). Samples were diluted to pH < 3 with loading buffer and applied to the column with one additional reload of the column flowthrough. The tC18 cartridge was washed once with loading buffer, after which peptides were eluted twice with 300 μL 80% ACN in 0.1% FA. The eluates were combined and dried by vacuum centrifugation and stored at −80 °C until subjected to nLC-MS analysis.

2.3.5.2. LC-MS/MS analyses. Peptides were reconstituted in 2% FA and analyzed in triplicate on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 UHPLC system (Thermo Fisher Scientific). Solvent A was 0.1% FA in water, and Solvent B was 0.1% FA in 80% ACN: 20% water. Peptides were first trapped on a μ-precolumn (C18 PepMap100, 5 μm, 100 Å, 5 mm × 300 μm; Thermo Fisher Scientific) in 3% Solvent B and then separated at 40 °C on an in-house-packed ReproSil-Pur 120 Å C18-AQ analytical column (2.4 μm, 75 μm × 50 cm; bulk media Dr. Maisch) at a flow rate of 300 nL min^{-1}. For proteome analysis, the gradient of Solvent B was 3–9% in 1 min, 9–35% in 65 min, 35–55% in 5 min, 55–99% in 3 min, 99% for 5 min, and re-equilibrated at 3% for 10 min.

Eluting peptides were online-injected into the mass spectrometer for data-dependent acquisition. The electrospray voltage was set at 2000 V and the ion transfer tube was set at 275 °C with an RF lens voltage of 40%. Full MS scans were acquired at 60,000 resolution over *m/z* 375–1600 with a 'Standard' AGC target. Multiply charged precursor ions ($m/z \geq 120$) were selected for fragmentation by higher-energy collisional dissociation (HCD, 28% NCE), with 1.4 m/z isolation window, resolution of 15,000, and dynamic exclusion of 14 s.

2.3.5.3. Raw data processing. MS data were acquired with Thermo Fisher Scientific Xcalibur (version 4.6.67.17). Raw files were processed in FragPipe (v22.0) with the integrated MSFragger search engine (Kong et al., 2017; Teo et al., 2021) (v4.1). Data was searched against the protein sequences derived from the genome of *A. oryzae* RIB40, as described below, and against the *P. sativum* UniProt database (downloaded July 2023, containing 64,176 entries) with decoys and common contaminants appended. Standard parameter settings were used with

semi-tryptic enzyme specificity, up to two missed cleavages, and precursor and fragment mass tolerances of 20 ppm. Variable modifications included methionine oxidation and protein N-terminal acetylation. Label-free quantification ('Run MS1 quant') (Yu et al., 2021) required ≥2 ions per protein, with match-between-runs enabled (top runs = 2). Peptide-spectrum matches (PSMs) were filtered to a 1% false discovery rate (FDR) at the PSM, peptide, and protein levels using Philosopher (da Veiga Leprevost et al., 2020) and ProteinProphet (Nesvizhskii et al., 2003). Quantitative and identification results were exported for downstream analysis.

2.3.5.4. Data analysis and availability. Data were analyzed using Perseus software (Tyanova and Cox, 2018; Tyanova et al., 2016) version 2.1.4.0. MaxLFQ intensities of proteins were $\log_2$ transformed. Proteins quantified in two out of three replicates in at least one experimental condition were retained for further analysis, after imputation based on normal distribution (width = 0.3, downshift = 1.8). No stringent cut of three out of three replicates was imposed because of possible gaps in identification using data dependent acquisition (DDA) method due to variation in top N precursor selection by the MS instrument. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD070642 (Perez-Riverol et al., 2019).

2.3.5.5. Bioinformatic analysis. For annotation of the *A. oryzae* proteins, the latest version of the *A. oryzae* RIB40 genome (GCF_000184455.2) was downloaded from the NCBI GenBank database (28 May 2025) and reannotated using AUGUSTUS v3.5.0 with the pretrained *A. oryzae* model (Nachtweide and Stanke, 2019) (Supplemental Table 2). Functional annotation (Superfamily, CDD, Panther, Pfam, SMART, ProSite-Patterns) was performed with InterPro v102.0 (Blum et al., 2025). Signal peptides for secretion were predicted with SignalP_EUK and PHOBIUS (Käll et al., 2004; Teufel et al., 2022). To map our protein predictions to existing gene numbers, protein accessions, and names and descriptions, BLASTp searches were performed against a custom UniProtKB database consisting of only *A. oryzae* RIB40 protein sequences, using an e-value threshold of 1e-10 and retaining only the best hit per query (lowest e-value, highest bitscore). Additionally, the predicted proteins were annotated with eggNOG-mapper (v2.1.12) based on eggNOG orthology data to increase functional annotation resolution and facilitate the identification of genes (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019). Gene names and descriptions were assigned, when possible, from the UniProtKB database. If missing, or BLAST identity was below 50%, the eggNOG name and description was used. Numeric suffixes were appended when multiple predictions presented the same gene name. General molecular functions were assigned based on Gene Ontology terms and keywords from the InterPro signature descriptions (Aleksander et al., 2023) (Supplemental Table 2).

Proteases were identified by BLAST v2.16.0 against a species-filtered, non-redundant MEROPS library keeping the best hit per query (e-value <1e−10, identity >30%, bit score > 50) (Rawlings et al., 2018). Integration of coincident MEROPS and InterPro predicted proteases was created as a cross-checked prediction. The predicted proteases were further classified based on the mode of action (dipeptidase or *endo-* or *exo*-protease) and mechanistic class (i.e. aspartic acid, cysteine, glutamic acid, metallo-, serine, and threonine proteases). Both the catalytic activity and the mechanistic class were inferred from the annotated Gene Ontology terms and InterPro descriptions of the hits. Subsequently, the annotations were refined by comparison to MEROPS family annotations of protease mechanism and specificity (Supplemental Table 3).

Carbohydrate-active enzymes (CAZymes) were called with CUPP on the isoform-excluded predicted proteome (Barrett et al., 2023). Following the previously described pipeline (Barrett et al., 2022), each CAZy family was combined with its reported Enzyme Commission (EC) numbers to form a Function: Family label (EC: Family) and these were mapped to a manually curated target polysaccharide list using bond information derived from CAZac (Lombard et al., 2025) (Glycoside Hydrolases, Carbohydrate Esterases, Polysaccharide Lyases, and Auxiliary Activities were included, Glycosyl Transferases were excluded as deemed biosynthetic). Entries lacking an EC were assigned a GENERAL: Family label and mapped when the whole family showed a single known substrate or a single activity; the family is widely known to be mostly active on a specific polysaccharide; if two activities co-occurred, both were retained (dash-separated); otherwise, the substrate was set to Unknown. The CAZymes were then cross-referenced to this Function: Family Substrate table to annotate putative polysaccharide targets. If a protein could be assigned to multiple putative polysaccharide targets, the target substrate with the highest CUPP score was retained (Supplemental Table 3).

Lipases were predicted from the annotated Gene Ontology terms ("lipase activity" GO:0016298) and InterPro descriptions (including "lipase" or "sterol"; excluding "binding", "carrier", "biosynthesis", "biosynthetic", "transport") (Supplemental Table 3). Similarly, phytases and tannases were predicted using Gene Ontology terms ("3-phytase activity" GO:0016158, "4-phytase activity" GO:0008707, "5-phytase activity" GO0050533, or "tannase activity" GO:0050318) and the InterPro signature description (including "phytase", "acid phosphatase", or "tannase") (Supplemental Table 3).

The annotation of the top 25 most abundant proteins with a predicted signal sequence for secretion of each spore inoculum condition were manually checked and curated.

2.4. Statistical analysis

Statistical analysis was performed in R (R version 4.3.1, RStudio version 2023.09.1, Posit Software, PBC). One-way ANOVA with Tukey's HSD post-hoc test was used to analyze gray value, dry weight loss, spore counts and enzyme activities ($p \leq 0.05$). Spore count data were $\log_{10}$-transformed before statistical analysis. To check for potential differences between experimental conditions in the proteomic data, ANOVA and two-sided unpaired Student's *t*-tests were performed, with false discovery rate (FDR)–corrected *p*-values (q-values) calculated from 250 randomizations and considered significant if they were ≤ 0.05.

3. Results

3.1. Substrate sterilization and integrity

Different surface and heat treatments were tested to assess substrate sterilization (Supplemental Text 1). Autoclaving was the only treatment resulting in effective sterilization (Supplemental Table 1). All other treatments resulted in bacterial growth, suggesting these microorganisms were protected by the pea matrix and/or had formed heat resistant spores. Therefore, autoclaving was used as sterilization method in all further experiments. It should be noted that starch was stained by iodine in all conditions, even after soaking overnight at RT, except for non-soaked peas. Soaking therefore impacts the integrity of the pea. Also, starch particles moved from the cotyledon to the pea hull during all heat treatments at ≥80 °C (Supplemental Text 1, Supplemental Fig. 1), suggesting that these high temperatures further affect pea integrity.

3.2. A. oryzae colonization of whole yellow pea

Growth of *A. oryzae* on peas was monitored for a maximum of 12 days after inoculation with 2.4×10^5 spores g^{-1} dry weight of pea (Fig. 1). The peas were almost completely colonized after 3 days (Fig. 1A), coinciding with a sharp increase in the whiteness of the sample during the first few days of incubation (Fig. 1B). Whiteness increased further until day 4, after which the gray value stabilized and eventually decreased at day 9 and 12. This decline is explained by the production of

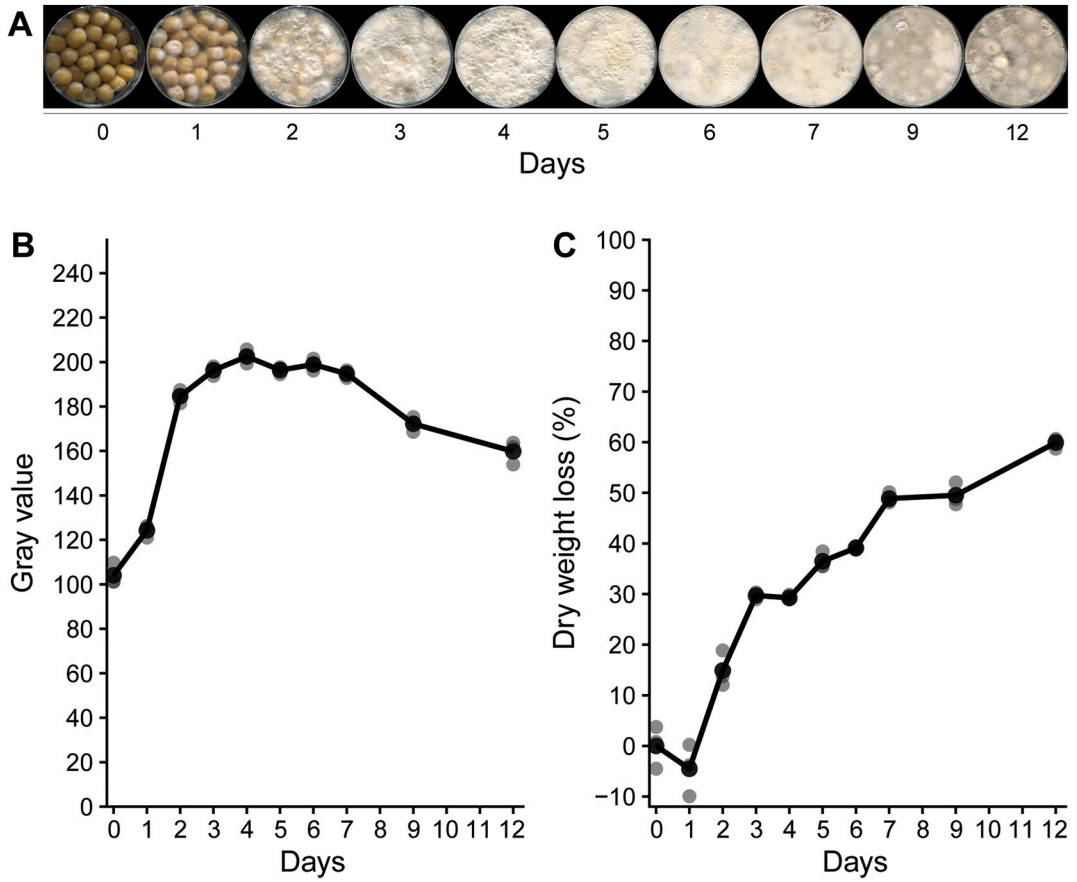


Fig. 1. Colonization of pea by *A. oryzae* RIB40 using 2.4 10^5 spores g^{-1} dry weight pea as inoculum. Top scans of the pea-fungal matrix (A), gray value of the bottom scans (B) and dry weight loss (%) (C) during a 12-day fermentation period. Black dots (B and C) represent sample means, whereas gray dots represent actual data points (n = 3).

green-brown conidia, the formation of dark brown sclerotia and the discoloration of the matrix from white to yellowish due to pigment production. The onset of sporulation of *A. oryzae* RIB40 already occurred at day 2 (Fig. 1A), whereas sclerotia appeared from day 3 onwards. Dry weight loss of the matrix increased up to day 12 with a loss of approximately 60% of the initial weight (Fig. 1C).

Based on the gray value profile, it was decided to study the influence of inoculum size (2.4, 2.4 10^2, and 2.4 10^5 spores g^{-1} dry weight pea) on *A. oryzae* colonization after 7 days of growth. Complete external colonization was observed in all cases (Fig. 2A). GFP fluorescence of strain RB#140.1 showed that the pea had also been completely colonized internally, irrespective of inoculum size (Fig. 3). Areas with high fluorescence intensity were found at sites of sclerotia formation, whereas heavy sporulation in between the peas showed up as dark patches in the fluorescence images.

Dry weight loss of the pea-fungal matrix was higher with SP III than with SP I (Fig. 2C), showing a higher colonization degree at high spore inoculum size. By contrast, sclerotia (Fig. 3) and conidia (Fig. 2D) production were higher at low spore inoculum than at high spore inoculum.

3.3. Effect of inoculum size on substrate transformation

3.3.1. Proteomic analysis

SDS-PAGE of an aqueous extract of non-inoculated peas showed a complex protein profile (Fig. 4A). Notably, the pea proteins were largely replaced by an abundant fungal protein band at 55 kDa after 7 days of culturing in all three spore inoculum conditions (2.4 (SP I), 2.4 10^2 (SP II), and 2.4 10^5 (SP III) spores g^{-1} dry weight pea). Proteomic analysis was performed on aqueous extracts of colonized peas resulting from the SP I, SP II, and SP III inoculation conditions. Proteins were classified into *A. oryzae* secreted proteins (with a predicted signal peptide for secretion), *A. oryzae* non-secreted proteins (without a predicted signal peptide for secretion), and *P. sativum* proteins. A total of 20 ± 13 pea proteins were identified in SP I extracts, while the SP II and SP III conditions resulted in 25 ± 15 and 11 ± 4 proteins, respectively. On the other hand, the SP I, SP II and SP III samples contained 214 ± 12, 204 ± 26, and 193 ± 28 secreted *A. oryzae* proteins, respectively, while the number of non-secreted *A. oryzae* proteins were 274 ± 156, 340 ± 166, and 200 ± 46 (Fig. 5A, Supplemental Table 4). Statistical analysis showed no effect of inoculum size on the number of proteins identified in each of the groups.

The contribution of the *A. oryzae* secreted and non-secreted proteins and the *P. sativum* proteins to the total protein intensity was determined across the three inoculum conditions (Fig. 5B, Supplemental Table 4). Secreted *A. oryzae* proteins dominated the proteome signal, reaching 68–87%, 73–87%, and 81–89% of the total intensity signal in SP I, SP II, and SP III, respectively. Non-secreted *A. oryzae* proteins exhibited lower overall signal intensities ranging between 12–19%, 12–16%, and 10–18%, respectively. The *P. sativum* proteins contributed only marginally to the total signal (0.1–13%, 0.1–11%, and 0.1–7%, respectively). Overall, these findings indicate that protein abundance patterns remained consistent across the inoculation sizes with secreted fungal proteins representing the dominant portion of the proteome.

A heatmap was generated of the secreted proteins (i.e. the proteins with a predicted signal peptide) (Fig. 5C). The clustering revealed that the overall secretion profiles were highly similar across the spore inoculum conditions, with most proteins showing similar abundance between conditions ($p > 0.05$). This visualization highlights the global

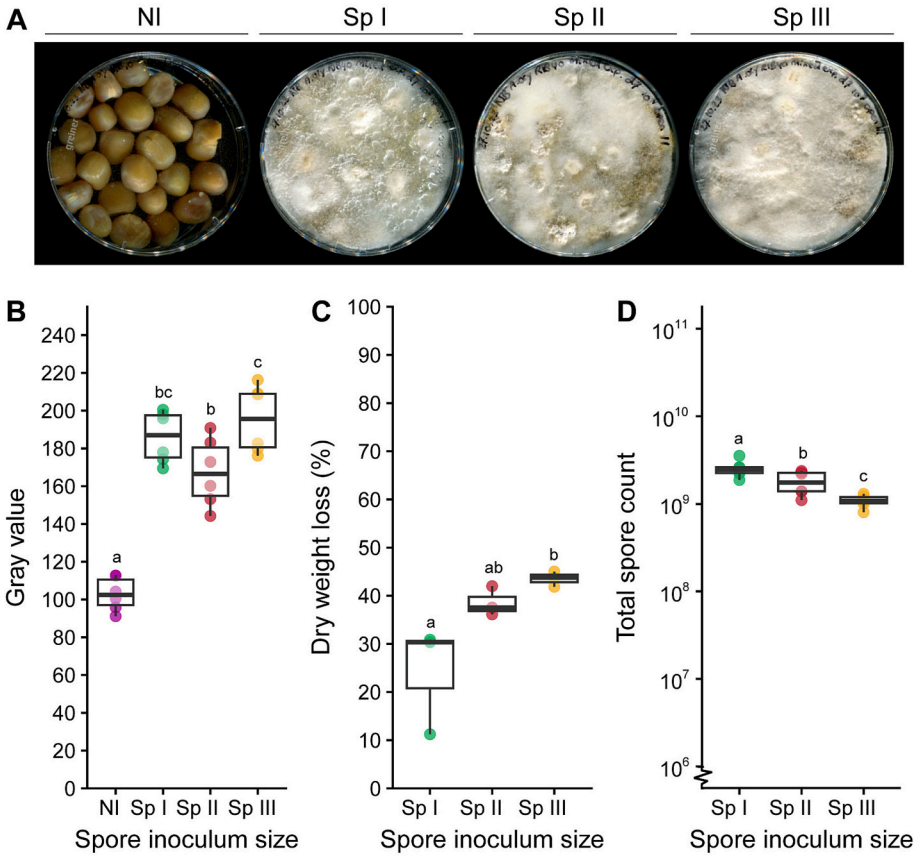


Fig. 2. Gray value, dry weight loss, and spore production of 7-day-old *A. oryzae* RIB40 fermented peas that had been inoculated with 2.4 spores g^{-1} dry weight pea (SP I), 2.4 10^2 spores g^{-1} dry weight pea (SP II), and 2.4 10^5 spores g^{-1} dry weight pea (SP III), non-inoculated peas (NI) serving as a control. Representative top scans (A), gray value of bottom scans (n = 6) (B), dry weight loss (%) (*n* = 3) (C) and total spore count (n = 6) (D) of the pea-fungal matrices. Different letters indicate statistical differences between groups ($p \leq 0.05$). Colored dots behind the box plots represent actual data points.

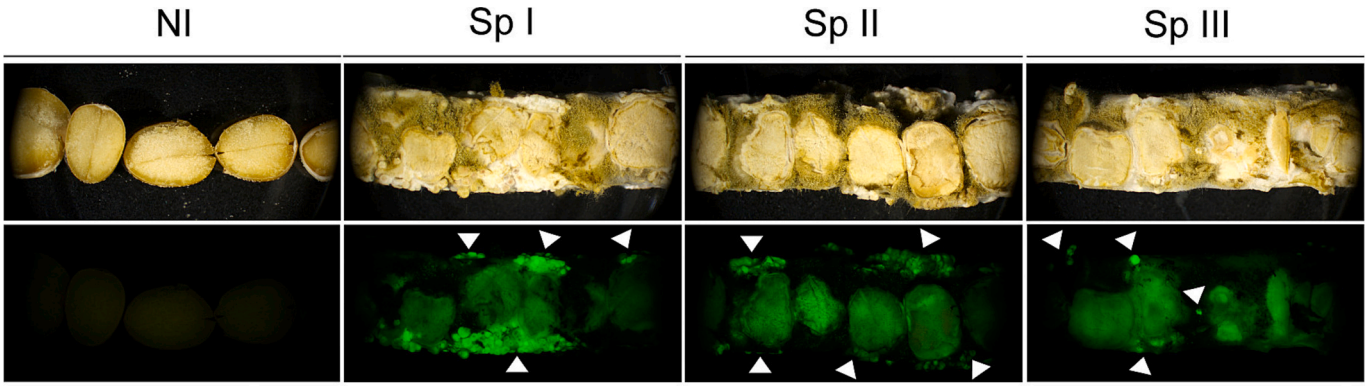


Fig. 3. Localization of *A. oryzae* RB#140.1 in 7-day-old fermented peas inoculated with 2.4 spores g^{-1} dry weight pea (SP I), 2.4 10^2 spores g^{-1} dry weight pea (SP II), and 2.4 10^5 spores g^{-1} dry weight pea (SP III), non-inoculated peas (NI) serving as a control, as depicted by representative bright field pictures (top) and GFP fluorescence of cross-sections (bottom). White arrowheads indicate regions with sclerotia formation.

consistency of the secretome and the functional distribution (CAZymes, lipases, phytases, proteases, tannases, or unannotated if they could not be assigned to any of these classes) of the secreted proteins between the SP I, SP II, and SP III conditions. CAZymes were the most represented group of detected proteins with a predicted signal peptide (SP I: 43.9%, SP II: 41.3% and SP III: 46.9%), followed by unannotated proteins (SP I: 35.9%, SP II: 38.7% and SP III: 33.3%), proteases (SP I: 12.6%, SP II: 12.9% and SP III: 13.0%), lipases (SP I: 6.3%, SP II: 5.3% and SP III: 5.3%), phytases (SP I: 0.9%, SP II: 1.3% and SP III: 1.0%), and tannases (SP I: 0.4%, Sp II: 0.4% and SP III: 0.5%) based on the assumption that a protein within a spore condition is present when it was detected in at least two out of three replicates (Supplemental Table 5). The largest realized genomic potential was found for the proteases with 45.7% of the predicted proteases in the genome identified in the secretome of *A. oryzae* RIB40 grown on peas in at least two of the three replicates in at least one of the three spore inoculum conditions (Fig. 5D). This class was followed by the CAZymes (37.9%), phytases (33.3%), lipases (29.6%), and tannases (20%).

A total of 303 predicted CAZymes were detected in the secretome at least two of the three replicates in at least one of the three spore

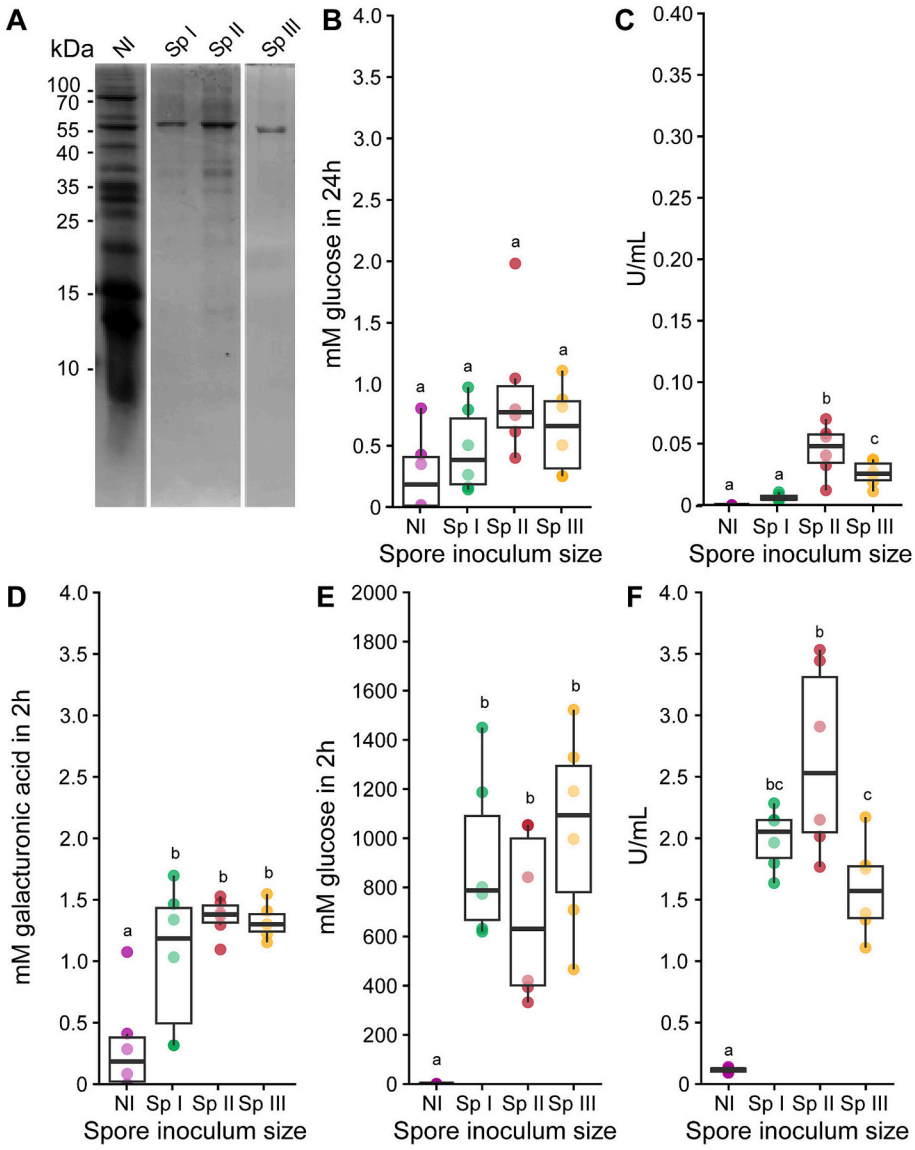


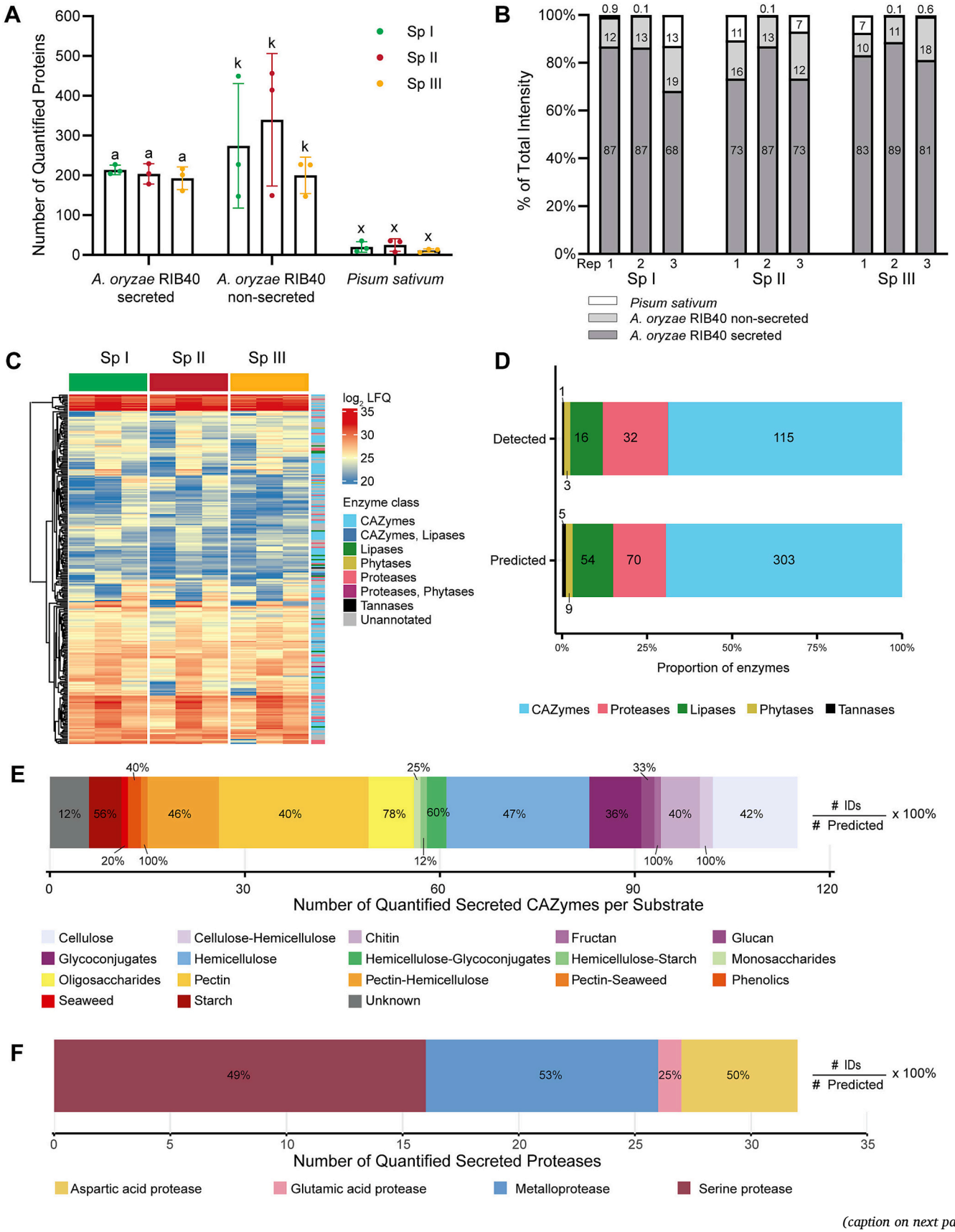
Fig. 4. Protein profile (A) and cellulase (B), xylanase (C), pectinase (D), amylase (E) and protease activity (F) ($n = 6$) of aqueous extracts from 7-day-old pea cultures after inoculation with 2.4 (SP I), 2.4 10^2 (SP II), and 2.4 10^5 (SP III) *A. oryzae* RIB40 spores g^{-1} dry weight substrate. Non-inoculated peas (NI) served as a control. Different letters indicate statistical differences between groups ($p \leq 0.05$). Colored dots behind the box plots represent actual data points.

inoculum conditions (Table 1, Fig. 5E). Among the CAZymes, pectin-degrading enzymes showed the highest diversity with 23 out of the 57 predicted proteins detected. Hemicellulose-active enzymes formed the second largest diverse group (22 out of 47), followed by cellulases (13 out of 31), pectin–hemicellulose active enzymes (11 out of 24), glycoconjugate-degrading enzymes (8 out of 22), oligosaccharide-active enzymes (7 out of 9) and amylases (5 out of 9). Chitinases (6 of 15 predicted) and glucanases (2 of 6 predicted), which target fungal cell-wall components, were also detected and may contribute to autolysis of hyphae or cell wall remodeling. Other CAZyme substrate classes were less diverse and more sparsely represented. Among the secreted proteases, exoproteases were more diverse than the endoproteases with 19 out of 39 predicted proteins and 13 out of 28 proteins detected, respectively (Table 1, Fig. 5F).

Proteins present in at least two out of three replicates were ranked by abundance in the different inoculation conditions (Fig. 6, Supplemental Fig. 2, Supplemental Table 6). CAZymes and proteases consistently represented the most dominant secreted proteins (Fig. 6A, Supplemental Fig. 2A, B), with starch-degrading enzymes being the most abundant among the CAZymes (Fig. 6B, Supplemental Fig. 2C, D) and an *endo*-acting serine protease among the proteases (Fig. 6C, Supplemental Fig. 2E, F). The top 25 proteins in the three different spore conditions revealed high similarity. α-Amylase (AmyA/B/C) and alkaline protease 1 (Alp1) were repeatedly identified as the first and second most abundant secreted proteins, followed by glucoamylase B (GlaB) (Table 2). Other enzymes occurring in the top 25 most abundant secreted proteins of all three spore inoculum conditions included the starch-active AA13 lytic polysaccharide monooxygenase (LPMO) and glucoamylase A (GlaA), the proteases neutral protease II (NptB), leucine aminopeptidase A (LapA) and an uncharacterized *exo*-cutting serine carboxypeptidase (AO090102000079); an endo-1,3(4)-β-glucanase (AO090003000825) and the glycoconjugate-active enzyme α-1,2-mannosidase 1B (Mns1B). Unclassified enzymes in the top 25 s included proteins that protect against oxidative stress, such as catalase B (CatB) and superoxide dismutase 4 (SOD4). Glutaminase A (GtaA), which might play a role in taste development, was also found in all top 25 s (Table 2).

3.3.2. *Carbohydrate and protein degrading activity*

Cellulase, xylanase, pectinase, amylase and protease activities were determined in aqueous extracts of the 7-day-old pea cultures that had



(caption on next page)

Fig. 5. Proteomics of aqueous extracts of 7-day-old fermented pea cultures after inoculation with 2.4 (SP I), 2.4 10^2 (SP II), and 2.4 10^5 (SP III) *A. oryzae* RIB40 spores g^{-1} dry weight substrate. (A) Number of *A. oryzae* secreted proteins (with a predicted signal sequence), *A. oryzae* non-secreted proteins (no predicted signal sequence), and *P. sativum* proteins that were present in at least two out of three replicates per spore condition. The number of proteins within each group (i.e. the secreted and non-secreted *A. oryzae* proteins and the *P. sativum* proteins) is not different between the SP I, II, and III samples ($p > 0.05$) as is indicated by the letters a, k and x. (B) Percentage of *A. oryzae* secreted proteins, *A. oryzae* non-secreted proteins, and *P. sativum* proteins of the total protein intensity of each replicate. (C) Heatmap of secreted proteins identified in at least two out of three replicates in any condition. Rows represent proteins clustered by log_2 LFQ intensity, with vertical side bars indicating the enzyme class assigned to each protein. (D) Comparison of the number and distribution of predicted secreted enzymes derived from the *A. oryzae* RIB40 genome and enzymes quantified in at least two out of three replicates in any inoculum condition. Stacked bars show the absolute counts per enzyme class as well as their relative contribution to the secretome. (E) Stacked bar chart showing the number of secreted CAZymes quantified in at least two out of three replicates in any inoculum condition. Numbers within the bars indicate the percentage relative to all genome-predicted CAZymes for the corresponding substrate category. Note that data analysis found five out of nine amylases because all peptides of TAKA α-amylase were assigned to AmyC despite the fact that this protein is near identical to AmyA and AmyB. Substrate categories with no detected members are omitted. (F) Stacked bar chart showing the number of secreted proteases quantified in at least two out of three replicates in any inoculum condition. Bars are stacked by protease type and the length of the bar corresponds to the total number of detected secreted proteases. Numbers inside each segment report the percentage of genome-predicted proteases of that protease type that were detected. Protease families with no detected members are omitted.

Table 1
CAZymes and proteases with a signal sequence for secretion in the secretomes of *A. oryzae* RIB40 in at least two out of three biological replicates in at least one inoculum condition after 7 days of fermentation of whole yellow pea.

	Predicted number IDs	Detected number IDs	% Detected
CAZyme substrate			
Cellulose	31	13	42
Cellulose-hemicellulose	2	2	100
Chitin	15	6	40
Cutin	2	0	0
Fructan	1	1	100
Glucan	6	2	33
Glycoconjugates	22	8	36
Glycoconjugates-chitin	1	0	0
Glycosaminoglycans	1	0	0
Hemicellulose	47	22	47
Hemicellulose-glycoconjugates	5	3	60
Hemicellulose-starch	8	1	12
Monosaccharides	4	1	25
Oligosaccharides	9	7	78
Pectin	57	23	40
Pectin-hemicellulose	24	11	46
Pectin-seaweed	1	1	100
Phenolics	5	2	40
Seaweed	5	1	20
Starch	9	5	56
Unknown	48	6	12
Protease class			
Endoprotease	28	13	46
Aspartic acid protease	10	5	50
Cysteine protease	1	0	0
Glutamic acid protease	4	1	25
Metalloprotease	6	3	50
Serine protease	7	4	57
Exoprotease	39	19	49
Cysteine protease	1	0	0
Metalloprotease	13	7	54
Serine protease	25	12	48
Unknown	3	0	0
Cysteine protease	1	0	0
Serine protease	1	0	0
Threonine protease	1	0	0

been inoculated with the different spore inocula (Fig. 4). Cellulase activity in the extracts was not different from that of the non-inoculated peas (Fig. 4B). This was also the case for xylanase activity of the low spore inoculum sample. The higher spore inoculum sizes did show xylanase activity, although the activity was low (Fig. 4C). Pectinase activity was found in all inoculated samples, but was generally low and close to the non-inoculated control with no differences between spore inoculum sizes (Fig. 4D). By contrast, amylase activity was high, but no effect of inoculum size was found (Fig. 4E).

As described above, pea protein had largely been degraded during fungal colonization (Fig. 4A). In accordance, proteolytic activity could be detected in aqueous extracts of all spore inoculum sizes (Fig. 4F). The intermediate inoculum size showed a higher protease activity than the highest spore inoculum size. The pH of the extracellular medium is often increased when fungi catabolize protein and amino acids (Vylkova, 2017). Indeed, the pH of the aqueous extract of the colonized pea was higher in the case of SP III when compared to the non-inoculated peas (7.28 ± 0.37 and 6.53 ± 0.04, respectively), while the pH of the SP I and SP II extracts was not significantly different from each other and from those of the non-inoculated peas and the SP III extract (6.87 ± 0.23 and 6.92 ± 0.27).

Together, amylase and protease activities were the main enzymatic activities found in the aqueous extracts of the colonized peas, irrespective of inoculation size.

4. Discussion

4.1. Impact of inoculum size on pea degradation, spore production, and secretome

Previously, it has been shown that a high inoculum size in liquid shaken cultures results in small *A. niger* and *A. oryzae* micro-colonies, while microcolonies become larger when the number of spores in the inoculum is reduced (Li et al., 2022; Papagianni and Mattey, 2006). Notably, large micro-colonies of *A. niger* (1.5–3.2 mm) secrete a lower amount and diversity of proteins in liquid shaken cultures when compared to small micro-colonies (± 0.3 mm) (Lyu et al., 2023). This reduced amount of secreted proteins in large micro-colonies is explained by spatial secretion heterogeneity. Secretion in micro-colonies only occurs in an outer shell with a certain width (Tegelaar et al., 2020). Consequently, the whole mycelium will contribute to secretion if the micro-colony radius is smaller than the width of this shell. By contrast, only the outer part of the mycelium will contribute to secretion if the micro-colony radius is higher than the width of the shell. Therefore, cultures with large micro-colonies have a smaller proportion of their fungal biomass contributing to protein secretion than cultures with similar biomass but smaller micro-colonies. Why the large micro-colonies secrete a reduced diversity of proteins is not yet known but this may be due do zonal and hyphal differentiation too (Wösten et al., 2013). We hypothesized that spore inoculum size also impacts colony size in SSF and thereby affects protein secretion and colonization of plant substrates. To test this hypothesis, whole yellow pea was inoculated with inoculum sizes SP I, II and III, which would theoretically result in micro-colonies of about 12.8 mm (SP I), 2.7 mm (SP II), and 0.3 mm (SP III) (Supplemental Text 2). Results showed that a high spore inoculum increased pea biomass degradation but it reduced sporulation. By contrast, the secretomes (both with regard to protein abundance and diversity) and enzyme activities (amylase, cellulase, and pectinase activity and to a lesser extent protease and xylanase activity) were highly

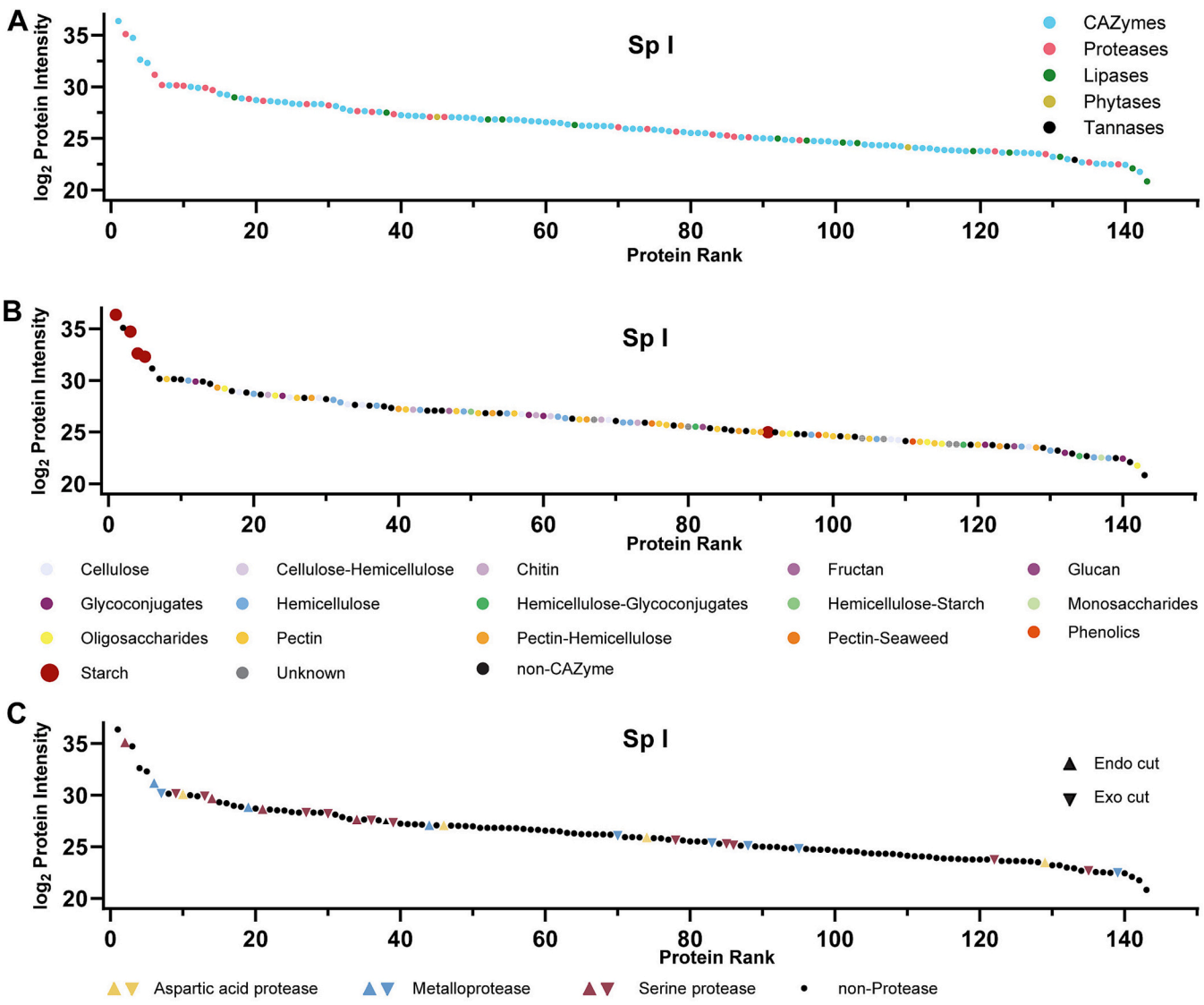


Fig. 6. Ranked plots of *A. oryzae* RIB40 enzymes with a predicted signal sequence for secretion identified in at least two out of three replicates of pea cultures after inoculation with 2.4 spores g^{-1} dry weight substrate (SP I). Proteins are shown as dots ranked by abundance and colored by enzyme class (A), CAZyme substrate category (B), and protease family (C). Triangle orientation in (C) encodes endo- (▲) or *exo*- (▼) cleavage mode.

similar between the SP I, SP II and SP III conditions after 7 days of SSF.

The discrepancy in effect of inoculum size/colony size on protein secretion and diversity between *A. niger* shaken cultures and *A. oryzae* SSF on pea could be explained by fermentation time and substrate complexity. Differences in quantity and diversity of secreted proteins between the inoculum sizes might have existed during the early stages of SSF and may have been missed by sampling after 7 days of colonization. For example, inoculum size did affect phytase production during the first 48 h of *A. niger* fermentation, but this difference disappeared when fungal growth was no longer exponential (Krishna and Nokes, 2001). Such an early effect may also explain why the highest inoculum condition (SP III) showed the highest pea biomass conversion. The composition complexity of whole yellow pea may also result in reduced secretion heterogeneity. Colonies of *A. niger* grown on xylose-containing agar medium show clear zonal differences in protein secretion (Wösten et al., 1991; Krijgsheld et al., 2012), but the entire colony participates in protein secretion when this fungus is grown on sugar beet pulp (Benoit et al., 2015). This does not exclude that heterogeneity in protein secretion is totally absent on this complex substrate. Indeed, expression of protease genes is higher at the colony periphery than in the inner zones when *A. niger* grows on sugar beet pulp (Benoit et al., 2015).

Suppose zonal and hyphal differentiation is absent in SSF, how can we then explain the increased pea biomass reduction and the reduced sporulation in the case of high inoculum size? A higher substrate degradation may be explained by a higher number of micro-colonies resulting from a higher spore inoculum. Decreased sporulation at a high spore inoculum is less easily explained. Inoculation with different numbers of spores may result in different temporal dynamics of the composition of the pea substrate, which, in turn, may impact spore production. Alternatively, reduced spore production at higher inoculum size may be due to accumulation of degradation products of the pea substrate that inhibit sporulation. *A. oryzae* may also release more sporulation inhibitors when fungal biomass builds up more quickly. Together, future experiments should study the temporal dynamics in the composition of the substrate focusing on nutrient availability and the release of sporulation inhibitors.

4.2. *The secretome of A. oryzae after 7 days of SSF*

Proteomic studies of the secretome of *A. oryzae* during SSF have primarily focused on wheat, rice, and soy for koji, sake, miso, and shoyu production (Liang et al., 2009; Melnichuk et al., 2020; Oda et al., 2006;

Table 2

Top 25 detected *A. oryzae* RIB40 proteins with a predicted signal for secretion after 7 days of fermentation on pea. Proteins are ranked from highest to lowest abundance (log_2 MaxLFQ intensities) and ordered according to their presence in all, two out of three, or only one of the spore inoculum conditions (SP I: 2.4, SP II: 2.4 10^2, and SP III: 2.4 10^5 spores g^{-1} substrate).

				SP I		SP II		SP III			
Protein	Gene Number	Enzyme Class	CAZymes Substrate/Protease Class	Rank	Mean log2 MaxLFQ Intensity	Rank	Mean log2 MaxLFQ Intensity	Rank	Mean log2 MaxLFQ Intensity	Protein Name	Description
g3260.t1 g7128.t1 g4223.t1	A0090003001210 A0090120000196 A0090023000944	CAZymes	Starch	1	36,34655	1	36,46725	1	36,81954	AmyA Amy3 AmyII Taa-G3 AmyB Amy1 AmyI Taa-G1 AmyC Amy2 AmyII Taa-G2	Alpha-amylase A; Taka-amylase A
g3127.t1	A0090003001036	Proteases	Endoprotease, Serine protease	2	35,08267	2	35,21634	2	35,12959	Alp1 Alk1 Alp AlpA	Alkaline protease I; Oryzin; Aspergillopeptidase B
g2571.t1	A0090003000321	CAZymes	Starch	3	34,73867	3	34,97450	3	34,83098	GlaB	Glucoamylase B; 1,4-alpha-D-glucan glucohydrolase
g6721.t1	A0090701000246	CAZymes	Starch	4	32,61483	7	31,49640	5	32,65907	AA13	Lytic polysaccharide monooxygenase AA13
g9810.t1	A0090010000746	CAZymes	Starch	5	32,30876	5	32,17341	6	32,45270	GlaA GluB	Glucoamylase A; 1,4-alpha-D-glucan glucohydrolase
g4108.t1	A0090023000804	NA	NA	6	32,21029	4	33,39300	4	33,93197	HsbA	Cell wall mannoprotein 1; Hydrophobic surface binding protein A
g7037.t1	A0090120000068	NA	NA	7	32,19658	6	32,01490	7	31,97144	CatB	Catalase B; Serves to protect cells from the toxic effects of hydrogen peroxide
g4640.t1	A0090026000357	NA	NA	8	31,85186	8	31,32641	8	31,55957	-	Allergen Asp f 15; Ortholog of A. fumigatus aspf13
g8329.t1	A0090038000258	NA	NA	9	31,53609	13	30,53842	23	29,37683	SOD4	Superoxide dismutase
g4922.t1	A0090026000003	NA	NA	10	31,24302	9	31,16064	16	29,90521	CpaO	Beta-cyclopiazonate dehydrogenase involved in the synthesis of the fungal neurotoxin alpha-cyclopiazonic acid (CPA)
g9998.t1	A0090010000493	Proteases	Endoprotease, Metalloprotease	11	31,15272	10	31,06762	10	31,04390	NptB	Neutral protease II; Deuterolysin
g8745.t1	A0090011000119	NA	NA	12	30,96650	11	30,85445	9	31,32121	-	Cell wall mannoprotein 1
g8693.t1	A0090011000052	Proteases	Exoprotease, Metalloprotease	13	30,16040	17	29,97938	12	30,30184	LapA	Leucine aminopeptidase A
g5449.t1	A0090012000708	NA	NA	17	30,02524	12	30,74643	14	30,11442	-	Uncharacterized protein
g2964.t1	A0090030000825	CAZymes	Hemicellulose	18	29,99980	24	29,45443	24	29,35622	-	Endo-1,3(4)-beta-glucanase
g2683.t1	A0090003000476	CAZymes	Glycoconjugates	20	29,90495	21	29,73963	18	29,74739	Mns1B ManA MsdS	Mannosyl-oligosaccharide alpha-1,2-mannosidase 1B
g6095.t1	A0090102000079	Proteases	Exoprotease, Serine protease	21	29,88501	16	30,13191	11	30,34090	-	Serine carboxypeptidase
g7830.t1	A0090020000289	NA	NA	22	29,85778	25	29,41874	21	29,67817	GtaA	Glutaminase A
g8617.t1	A0090138000055	CAZymes	Pectin	14	30,14454	22	29,61006			AbnC	Probable arabinan endo-1,5-alpha-L-arabinosidase C
g5448.t1	A0090012000706	Proteases	Exoprotease, Serine protease	15	30,14337	15	30,16274			-	Serine carboxypeptidase
g7341.t1	A0090120000474	Proteases	Endoprotease, Aspartic acid protease	16	30,09248	23	29,58768			PepA PepO	Aspergillopepsin I; Acid protease A2; Aspergillopepsin O
g6496.t1	A0090701000541	NA	NA	19	29,98287	18	29,91167			-	Mitochondrial division protein 1
g2972.t1	A0090003000833	NA	NA	24	29,59644	14	30,23973			-	Cysteine-rich secreted protein
g9231.t1	A0090011000795-A	Proteases	Endoprotease, Serine protease	23	29,67513			13	30,19531	DPP5	Dipeptidyl-peptidase 5
g978.t1	A0090005001044	NA	NA	25	29,32522			20	29,69192	RntB	Ribonuclease T2
g9731.t1	A0090103000075	NA	NA			19	29,81611	22	29,50023	-	Major facilitator superfamily (MFS) profile domain-containing protein
g3053.t1	A0090003000935	NA	NA			20	29,75640	15	29,92703	-	FG-GAP repeat protein
g7890.t1	A0090020000207	CAZymes	Chitin					17	29,87921	-	Chitinase
g267.t1	A0090090000356	CAZymes	Cellulose					19	29,70626	BglA Bgl1	Probable beta-glucosidase A; Cellobiase A; Gentiobiase A
g2259.t1	A0090010000617	CAZymes	Oligosaccharides					25	29,29480	-	FAD-binding PCMH-type domain-containing protein; Ortholog(s) have a role in asperfuranone biosynthetic process, fumiquinazoline C biosynthetic process

Zhang et al., 2012; Zhu et al., 2017), but has also been done on *A. oryzae* fermentation of pea protein isolate (Das et al., 2023). The number of secreted proteins (i.e. with a predicted signal sequence for secretion) identified in the current study (±200) is higher than in the previous studies. This may be due to the relative late stage of fermentation since the previous studies focused on earlier fermentation times (up to 3 days). In the current study, secreted proteins were detected in almost all functional enzyme classes involved in plant-biomass degradation. Proteomics revealed that starch-degrading enzymes and proteases were the most abundantly secreted proteins, resulting in a high overall amylase and protease activity in the enzyme activity assays. This is in line with the fact that starch and protein are the main components of pea seeds (26.9–75.5% and 13.7–30.7%, respectively). Due to the high protease activity, pea proteins were replaced by fungal proteins in the substrate extracts. After 7 days of fermentation, fungal proteins were between 6.7-fold and 990-fold more abundant in the protein extracts when compared to the pea protein. Indeed, SDS-PAGE showed the disappearance of pea proteins after SSF. α-Amylase (AmyA/AmyB/AmyC) was the most abundant *A. oryzae* secreted protein in all spore conditions, followed by alkaline protease 1 (Alp1) and glucoamylase B (GlaB). These enzymes have also been identified as abundantly secreted proteins in other *Aspergillus* SSF studies (Das et al., 2023; Melnichuk et al., 2020; Nekiunaite et al., 2016; Zhang et al., 2012; Zhu et al., 2017), even when grown on starch-poor substrates (Das et al., 2023). Other starch-degrading enzymes belonging to the top 25 most secreted proteins in all spore conditions included glucoamylase A (GlaA) and the AA13 LPMO. The latter is interesting because our study has revealed for the first time this type of enzyme in the secretome during *A. oryzae* SSF. Oxidative LPMO activity renders the substrate more susceptible to hydrolysis by glycoside hydrolases, thereby boosting polysaccharide degradation. The boosting effect of AA13 LPMOs on starch degradation has been well-described in *Aspergillus nidulans* (Haddad et al., 2020; Lo Leggio et al., 2015; Nekiunaite et al., 2016). The *An*AA113B LPMO was found in the top 4 most abundant secreted proteins when the fungus was grown on wheat and maize flour (Nekiunaite et al., 2016). The *A. nidulans* LPMOs are in particular involved in degradation of resistant (i.e. insoluble) starch, which is in line with their increased importance during later stages of growth (Haddad et al., 2020; Nekiunaite et al., 2016). In our current study, Catalase B (CatB) and super oxide dismutase 4 (SOD4) were also highly abundant in all conditions. These proteins may protect the fungus from H_2O_2 production resulting from LPMO activity (Kittl et al., 2012). Catalase and other antioxidant proteins have been identified in previous secretome studies of *A. oryzae* fermentations too (Li et al., 2023; Melnichuk et al., 2020; Oda et al., 2006; Zhang et al., 2012; Zhu et al., 2017). Similarly, catalase B was among the most abundant proteins in the *A. nidulans* secretome when grown on pea, wheat or maize flour (Nekiunaite et al., 2016).

Enzyme activity assays revealed low or absent cellulase, xylanase and pectinase activity. Yet, cellulose, hemicellulose and pectin are the predominant constituents of dietary fiber in peas. Dietary fiber content of peas ranges from 23.2 to 30.7%, with 3.9–8.0% soluble dietary fiber (SDF) and 19.3–23.1% insoluble dietary fiber (IDF) (Wu et al., 2023). SDF in peas consists mainly of pectic polysaccharides and IDF of cellulose, xylans and arabinans (Wu et al., 2023). The dietary fibers are present in the pea hull and in the cell walls inside the cotyledon. Theoretically, these components would be the first carbohydrate sources that the fungus encounters during colonization, implying that pectinases and (hemi)-cellulases are important during initial stages of colonization. However, our preliminary results show that (hemi)-cellulase activities are also low during these early stages. This discrepancy may be explained by affected integrity of the whole yellow pea due to soaking and sterilization before the inoculation, resulting in easy accessibility of starch and other easily digestible compounds like proteins without the need to first degrade the hull.

A substantial amount of proteins (10–19% of protein quantity) without a predicted signal sequence for secretion was identified in the aqueous extracts of the colonized peas, irrespective of inoculum size. This may be explained by non-classical secretion as well as by lysis. The chitinases and β-1,3-glucanases that were identified in the aqueous extracts of the colonized peas would enable lysis by breakdown of the fungal cell wall. Indeed, our preliminary experiments have shown the presence of *N*-acetylglucosamine (the building block of chitin) in homogenized material of the pea fungal matrix, indicative of cell wall degradation. Future studies should reveal whether the number of proteins without a signal sequence for secretion is also relatively high in early stages of pea colonization.

5. Conclusions and perspectives

The impact of inoculum size on the secretome, substrate degradation, and sporulation was studied after 7 days of colonization of whole yellow pea (*P. sativum*) by *A. oryzae*. Starch-degrading enzymes and proteases were the most abundantly secreted proteins, irrespective of the inoculum size. Differences between inoculum size were also not found for other secreted proteins and for enzyme activities in the culture medium. Yet, such differences might have existed during the early stages of SSF. Therefore, future studies should address the effect of spore inoculum size on the secretome over the course of SSF.

Results showed that a high inoculum size results in increased substrate degradation and reduced sporulation. The latter is of interest since sensory attributes such as texture and visual appeal play a huge role in consumer acceptance (Forsberg et al., 2025). The increased substrate degradation and reduced sporulation may be caused by the phenomenon of secretion heterogeneity that has previously been observed in liquid shaken cultures of *A. niger* (Lyu et al., 2023). A high spore inoculum results in small micro-colonies that secrete a high amount and diversity of proteins in liquid shaken cultures. By contrast, a low spore inoculum results in large micro-colonies that secrete a lower amount and diversity of protein. It may also be that secretion heterogeneity does not play a role in the higher pea conversion and the reduced sporulation when a high spore inoculum is used. Possibly, fungal biomass builds up faster during SSF when a higher spore inoculum is used, which would result in a higher rate of pea biomass conversion when compared to cultures inoculated with a lower spore number. Such differences in temporal dynamics of pea conversion would result in differences in nutrient availability ((i.e. relative amounts of poly-, oligo- and monosaccharides, lipids, protein and essential amino acids), which in turn may impact sporulation. Alternatively, temporal dynamics of pea conversion could impact accumulation of sporulation inhibitors. Together, future experiments should study temporal dynamics of substrate composition during SSF focusing on nutrient availability and the release of sporulation inhibitors.

SSF with *A. oryzae* will result in a reduced pea biomass and an increased fungal biomass. Moreover, the fungus preferentially degrades components of the pea substrate. As a consequence, the nutritional composition of the solid state fermented pea changes in time. For instance, it was shown that pea protein degradation was very high after 7 days of SSF. Future studies should assess whether the fungal proteins fully replace pea protein quantity and whether protein digestibility and amino acid composition will be improved by SSF with *A. oryzae*. Our preliminary data show that pea protein digestion is also high during early stages of colonization. A shorter SSF period would thereby not be beneficial per se. In fact, longer SSF might be associated with higher amounts of digestible protein. The same may hold for taste development during fermentation. In the current study, glutaminase A (GtaA) was identified among the top 25 most abundant secreted proteins in all spore conditions, which may be beneficial in a PBMA as it could play an important role in the development of meaty umami flavors (Sakai et al., 2025). It may be that secretion of this enzyme is relatively high during later stages of SSF.

Together, this study advanced the understanding of *A. oryzae* colonization on pea substrate and identified research areas that require

further investigation before *A. oryzae* fermentation of peas can effectively be applied to produce nutritious and protein-rich PBMAs.

CRediT authorship contribution statement

Maaike Braat: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Nienke Braat:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Nadzeya Staliarova:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Tomás Vellozo-Echevarría:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Hania M. Siwek:** Investigation. **Julius F. Kemmer:** Investigation. **Kristian Barrett:** Writing – review & editing, Supervision. **Anne S. Meyer:** Writing – review & editing, Supervision, Funding acquisition. **Kelly E. Stecker:** Writing – review & editing, Supervision. **Han A.B. Wösten:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2026.111853.

Data availability

Data will be made available on request.

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