Plant extract induced changes in the proteome of the soilborne pathogenic fungus *Thielaviopsis basicola*.

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Running title: Proteomic changes in *Thielaviopsis basicola.*

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Summary

Thielaviopsis basicola is a hemibiotroph fungus that causes black root rot disease in diverse plants with significant impact on cotton production in Australia. To elucidate how *T. basicola* growth and the proteome are influenced by interactions with natural sources, this fungus was cultured in the presence of root extracts from non-host (wheat, hairy vetch) and susceptible host (cotton, lupin) plants. We found that *T. basicola* growth was significantly favored in the presence of host extracts while hierarchical clustering analysis of two-dimensional electrophoresis protein profiles of *T. basicola* shows a dependency towards the plant rather than the host/non-host status. Analysis by LC-MS/MS of unique and differentially expressed spots and identification using tandem MS, cross-species similarity searching and *de novo* sequencing allowed successful identification of 41 spots. The identified proteins were principally involved in primary metabolism with smaller numbers implicated in other diverse functions. Identification of several "morpho" proteins suggested morphological differences that were further microscopically investigated. Identification of several highly expressed spots suggested that vitamin B6 is important in the *T. basicola* response to components present in hairy vetch extract, and finally, three spots, induced in the presence of lupin extract, may correspond to malic enzyme and be involved in lipid accumulation.

Introduction

The soil-borne fungus *Thielaviopsis basicola* has been recognized as a plant pathogen for over 150 years (1). This fungus causes black root disease in the seedlings of over 137 plant species, representing at least 30 families (2) and has been found in cultivated and virgin soils throughout the world (3, 4). In Australia, it was first recorded as a pathogen of sweet pea in 1930 (5) but since then has been found on various other hosts such as cotton, bean, pea, lettuce and recently soybean (6-8). The distribution and severity of black root rot in cotton have increased since 1990 and it has emerged as a major risk to sustainable cotton production with estimated yield losses of up to 26 % in severely affected crops (9).

It has long been understood that the development of disease symptoms is not solely determined by the pathogen, but by a complex relationship between host and pathogen. Previous studies on the infection process of *T. basicola* have characterized this fungus as a hemibiotroph, an ecological obligate parasite (10, 11). Following recognition, a series of developmental events lead to spore germination and hyphal growth. The precise mechanism by which germination is triggered in *T. basicola* is not known. Germination of spores has been shown to occur in the presence of diverse stimuli including sugars, natural lecithins or their constituents, unsaturated fatty acids or unsaturated triglycerides. However, plant exudates and plant root extracts have been shown to greatly stimulate germination and germ tube elongation (12-14). Variations in hyphal morphology have also been reported previously. Linderman and Toussoun (1968) (15) reported changes in hyphal diameter and branching in soil supplemented with carrot juice as well as increased branching on the surface of host roots. Hood and Shew (1997) (16) reported a

positive correlation between the level of available host root extract and hyphal diameter, length and degree of branching.

This data suggests the possibility of management methods that will impact on spore survival and/or on the molecular pathways from host recognition to infection. To evaluate if common molecules may serve as signals to stimulate/suppress spore germination and fungal growth, we decided to investigate the effect of susceptible host and non-host plant extract on *T. basicola.* It has been shown that cotton root exudates and extract stimulate germination of chlamydospores more than other plants (12, 14), that in severely infested cotton fields the population of *T. basicola* was reduced significantly by a rotation with wheat for three consecutive years (17) and that hairy vetch-amended soils suppress *T. basicola* propagules (18).

The reaction of *T. basicola* to the environment could be assessed by global analysis but the lack of genome sequence information on *T. basicola* is a limiting factor in the global analysis of this fungus. Proteomics has been previously used in the study of plant-pathogen interactions, identifying proteins putatively involved in pathogenicity. Mehta and Rosato (2001) (19) identified differentially expressed proteins of the bacteria *Xanthomonas axonopolis* pv. *Citri* cultivated in the presence of leaf extract from the host plant *Citrus sinensis*. Tahara *et al.* (2003) (20) identified several proteins induced in *Xanthomonas axonopolis* pv. *Passiflorae* by leaf extract of host *Passiflorae edulis*. Mattinen *et al*. (2007) (21) studied the secretome changes of *Pectobacerium atrosepticum* associated with exposure to the host extract and identified known virulence proteins as well as proteins that may be involved in nutrient utilization, while Phalip *et al*. (2005) (22) studied the secretome of the fungus *Fusarium graminearium* grown on *Humulus*

lupulus, L. cell wall and identified many proteins putatively involved in cell wall polysaccharide degradation.

In the present study, we used two-dimensional electrophoresis (2-DE) coupled with MS-MS, *de novo* sequencing and cross species analysis to analyze the soluble protein fraction of *T. basicola* cultured in the presence of susceptible host (cotton and lupin) and non-host (wheat and hairy vetch) extracts. Evaluation of this soluble proteome will most likely reflect the fungus reactivity towards environmental variations and may suggest novel approaches of black root rot control.

Experimental Procedures

Conidial inoculum and culture conditions

T. basicola isolate, BRIP40192, recovered from a cotton field in Narrabri, NSW, Australia was obtained from Dr Jan Dean, Queensland Department of Primary Industries and Fisheries. For conidial production, the isolate was grown on ½ potato dextrose agar (½PDA) (19.5 g potato dextrose agar (Oxoid), 14.5 g Bacto-agar, distilled water 1 liter) at 25ºC for five days. Endoconidia (1 X 10^6 spores/mL of medium) from the surface of a 5-day-old plate were used to inoculate the different Czapek-Dox media supplemented with the root extract and cultured (rotary shaking at 25ºC for 24 hours).

Plant root extract preparation

Cotton (*Gossypium hirsutum*), Lupin SA (*Lupinus angustifolius*), hairy vetch (*Vicia villosa*) and wheat (*Triticum durum*) were surface sterilized and grown for 14 days according to Coumans *et al*. (in press) (23). The seedling roots were rinsed in deionized water and blotted between paper sheets to remove excess water. Root extracts were prepared according to Hood and Shew (1997) (16). Briefly, seedling root pieces were packed into a 5 ml disposable syringe and pressed to exhaustion. The resulting root extract was then sterilized through a 0.2 μm non-pyrogenic syringe filter and stored at -20ºC until use. The extracts were added to liquid Czapek-Dox (Oxoid) medium to a final dilution (1:10 v:v) and an extract-free control was obtained by replacing the root extract with MilliQ water.

Determination of the growth rate of *T. basicola* in root extract

To determine the growth rate of *T. basicola* in the presence of the different root extracts, plates containing either root extract (1:10 v:v dilution) or extract-free medium and 2.2% agar were prepared. A sterilized inoculating needle was used to gently scrape spores from 5 day old ½ PDA plates and stabbed in the centre of the root extract plate. Plates were incubated at 25^{o}C and the colony diameter measured each 2 days. Root extracts were analyzed in triplicate. To determine if the growth rate of *T. basicola* differed between plant root extracts, SPSS software (SPSS Inc. Chicago, USA) was used to perform a one-way ANOVA test. When treatment effects were significant, the Duncan's multiple range test was used to identify treatments that were significantly different to each other.

Protein extraction and quantification

Mycelia were recovered by filtration and washed thoroughly with MilliQ water, dried between paper sheets and stored at -20ºC until further use. Mycelia (100 mg) were extracted with a Mini Beadbeater (4 times, 30 sec, glass beads) in 300 µl of extraction buffer (30% sucrose, 0.1 M Tris-HCl pH 8, 2 mM PMSF, 1% DTT, 100 mM KCl, 5 mM EDTA). An equal volume of phenol saturated with Tris-HCl (pH 8) was then added and the mixture vortexed for 2 min and centrifuged (10,000 X *g*, 5 min). The phenolic phase was removed and re-extracted as described above. Proteins were precipitated from the phenolic phase with five volumes of 0.1 M ammonium acetate in methanol overnight at -20ºC and pelleted by centrifugation (10,000 X *g*, 30 min). The resulting pellet was rinsed twice with ice-cold 0.1 M ammonium acetate in methanol, three times with ice-cold methanol and once with ice-cold acetone/water (80:20, v/v). After air drying, the pellet was dissolved in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.5% IPG buffer pH 4-7 (GE Healthcare Life Sciences, Australia). The total protein

concentration was determined using the 2-D quant kit, purchased from GE Healthcare Life Sciences (Australia).

Two dimensional electrophoresis

250 µg (analytical gel) or 750 µg (preparative gel) of protein extract was loaded onto a rehydrated 18 cm IPG strip pH 4-7 (GE Healthcare Life Science). IEF was carried out on the IPGphor II (GE Healthcare Life Science) at 20 ºC with a current limit of 50 µA/strip to a total volt-hour-product of 34 kVh (analytical gels) or 45 kVh (preparative gels). Before second dimension analysis, the individual strips were equilibrated according to Gorg *et al.* (2000) (24). Second dimension separation was achieved on a PROTEAN II system (Bio-Rad, Australia) with lab cast 1.5 mm SDS polyacrylamide gels (12%) at 10 mA/gel until the bromophenol blue dye front reached the anodic end of the SDS-gel.

Protein visualization, image analysis and data analysis

Proteins were visualized by Blue silver staining (25) for analytical study and by Coomassie blue staining (50% methanol, 0.15% Coomassie blue R-250, 0.75% acetic acid) for preparative 2-D electrophoresis. Stained gels were recorded for image analysis using the Infinity imaging system from Vilber Lourmat and analyzed with PDQuest advanced 2-D analysis software (Bio-Rad). Three biological samples and two technical replicates per biological sample were obtained, grouped and analyzed. Normalization of the gels was performed using the local regression method of the PDQuest software. Statistical evaluation of the expression levels of the protein spots was performed using the SPSS software. Hierarchical clustering (SPSS software) was conducted using the nearest neighbor method with the Pearson correlation used as a measure of similarity. Unique spots and spots showing at least a 2-fold change in their expression level

over all the other root extracts studied and being statistically different to the others as determined by one-way ANOVA ($P < 0.05$) followed by a Duncan's multiple range test (SPSS software), were selected for LC-MS/MS analysis.

Protein identification and database search

Unique and differentially expressed spots were manually excised from preparative gels and destained with 200 µl of 50 mM NH_4HCO_3 in CH_3CN until the Coomassie blue disappeared. Proteins in the gel pieces were reduced for 1 h with 100 µl of 20 mM DTT/20 mM NH_4HCO_3 at 37°C and alkylated (30 min, 37°C) by adding 20 µl of 200 mM iodoacetamide. After three washes (10 min, 200 µl of CH_3CN) the gels pieces were dried under vacuum (SpeedVac; Thermo, Australia) for 5 min. Digestion of the proteins was carried out in 25 mM NH_4HCO_3 containing 5 ng/µl of trypsin (Promega, Australia) for 14 h at 37°C. After digestion, the gel pieces were incubated in 40 µl of 0.1% formic acid (10 min) and then in 100 µl CH_3CN (10 min). Supernatant containing the tryptic peptides was removed and dried under vacuum. Finally, the peptides were redissolved in 10 µl of 0.1% heptafluorobutyric acid.

Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, Milford MA). Samples (1-5 µl) were concentrated and desalted onto a micro C18 precolumn (500 µm x 2 mm, Michrom Bioresources, Auburn, CA) with H_2O:CH_3CN (98:2, 0.05 % HFBA) at 15 ul/min. After a 4 min wash the pre-column was automatically switched (Valco 10 port valve, Houston, TX) into line with a fritless nano column (75 µm x ~12 cm) containing Magic C18 (~10cm, 200Å, Michrom) manufactured according to Gatlin *et al.* (1998) (26). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1 % formic acid) to H_2O:CH_3CN (55:45, 0.1 % formic acid) at ~300 nl/min over 30 min. The precolumn was

connected via a fused silica capillary (10 cm, 25 μ) to a low volume tee (Upchurch Scientific) where HV (2400 V) was applied and the column tip positioned ~ 1 cm from the Z-spray inlet of an QTof Ultima API hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTof operated in data dependent acquisition mode (DDA). A Tof MS survey scan was acquired (m/z 350-1700, 1 s) and the 2 largest multiply charged ions (counts > 20) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2 s (m/z 50-2000).

The peak lists were generated by MassLynx (version 4.0 SP1, Micromass) using the Mass Measure program. Protein identification was achieved by combining spectrum quality scoring obtained from a conventional database search using the program Mascot (version 2.1 or 2.2, Matrix Science, London, England) with automated *de novo* sequencing on unassigned high quality spectra using the PEAKS Studio 4.5 (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). This included a SPIDER similarity search and a Mascot search on the PEAKS *de novo* sequence obtained ("PEAKS In Chorus analysis"). The search parameters used were as follow: Precursor and product ion tolerances ± 0.25 and 0.2 Da respectively; Met(O) and Cys-carboxyamidomethyl were specified as variable modification, enzyme specificity was trypsin, one missed cleavage was possible and the $NCBI_{non\ redundant\ protein\ (nr)}$ database (Jan 2008) was searched. For the SPIDER similarity search these additional settings were specified: non-gapped homology match, leucine equals isoleucine, and lysine equals glutamine.

Fluorescent staining of intracellular lipids.

Nile red (Sigma N-3013) was dissolved at a concentration of 0.01 mg/ml in acetone. Lipid staining was carried out on unfixed cells. Fifty µL of root extract (1:10, v:v dilution) or extract-free medium and 3 µL of an endoconidia suspension (5 endoconidia/µL) were applied to autoclaved concave glass microscope slides. After 24 h of incubation at 25ºC in 100% humidity, fungi were stained by addition of 2 µL of the dye solution for a minimum of 15 min (27). Fluorescence microscopy was performed using a Nikon Ti-E motorized inverted microscope with a Plan Apochromat, VC60x WI, 1.2 NA objective, and samples were illuminated using a 450-490 nm bandpass excitation filter. A 505 nm dichroic mirror and a 520 nm long pass barrier filter were used on the emission side. Digital color pictures were obtained with a Digital Sight DS-Ri1 camera (Nikon) and imaged using the NIS-Elements software AR 3.0 (Nikon). Exposure time was 300 ms for cotton and lupin but had to be increased to 600 ms for the wheat, Czapek-Dox and hairy vetch samples because of their low fluorescence intensity. No photobleaching was observed during the acquisition time and a constant gain was maintained for all samples.

Determination of the hyphal morphology.

Determination of the hyphal morphology was carried out as described by Hood and Shew (1997) (16). Briefly, 50 µL of root extract (1:10, v:v dilution) or extract-free medium and 3 µL of an endoconidia suspension (5 endoconidia/µL) were applied to autoclaved concave glass microscope slides. After 24 h of incubation at 25ºC in 100% humidity, slides were examined using a Nikon Ti-E motorized inverted microscope with a Plan Apochromat, 10x, 0.45 NA objective under brightfield illumination. Five germinated endoconidia were randomly chosen for determination of total hyphal length, number of secondary and tertiary branches, septa diameter and apical compartment length. Images of germinated endoconidia were captured through a

Digital Sight DS-Ri1 camera (Nikon) and imaged using the NIS-Elements software AR 3.0 (Nikon). Z stacks of the samples were obtained with a Z-step of 4 μM. The number of slices was variable between samples. Distance measurements were made in 3 dimensions using the slice view function of the NIS-Elements software. The data were subsequently exported and statistical analysis (ANOVA, Duncan's multiple range test) performed using SPSS software.

Results

Influence of non-host and susceptible host plant extract on *T. basicola* growth

The effect of non-host (wheat, hairy vetch) and susceptible host (cotton, lupin) plant root extract on *T. basicola* growth was determined. Growth was evaluated by measuring the diameter of *T. basicola* colony on plates containing root extract (Fig 1). After 8 days of incubation, the colony diameters on non-host and susceptible host plant extracts were significantly different ($P < 0.05$). Moreover, colony morphology was also quite different (Fig 2). *T. basicola* grown on Czapek-Dox medium produced only a few hyphae (data not shown), Czapek-Dox medium supplemented with wheat and cotton root extract produced a colony with gray to olive green appearance and the colony surface on cotton extract presented with more white mycelia as compared with wheat, while in the presence of hairy-vetch root extract, the colony pigmentation of *T. basicola* was found to be light brown. *T. basicola* grown in the presence of lupin root extract had a fluffy appearance, with cottony mycelium on the surface of the media.

Proteome characterization of *T. basicola* grown in the presence of non-host and susceptible host root extract

To assess the influence of non-host and host plant extract on the proteome of *T. basicola*, mycelium recovered from 24 h culture filtrates of *T. basicola* grown in Czapek-Dox medium supplemented with non-host root extract, susceptible host root extract or MilliQ control were extracted and analyzed by 2-DE. An average of 750 protein spots ranging from 20 to 150 kDa in the 4-7 pH were detected by Blue silver staining (25). While similar overall protein pattern were observed, remarkable quantitative and qualitative differences were also seen between the different treatments (Fig 3). These changes were characterized using three separate cultures and

two technical replicates per protein extract were compared and quantified using PDQuest software. Gels were of high quality with reproducible protein patterns between sample replicates (intra-assay CV% = 16.35) and between independent experiments (inter-assay CV% = 31.17). Hierarchical clustering analysis on the overall protein pattern shows that the proteome of *T. basicola* doesn't allow differentiation between host and non-host plant extract (Fig 4). This finding is further supported by the observation that only one protein spot is differentially expressed between non-host and susceptible host root extract. We therefore chose to characterize the proteomic response of *T. basicola* grown in the presence of a particular root extract and first identified protein spots unique to *T. basicola* and which reproducibly appeared in all 2-DE gels. Eleven protein spots with a distinctive electrophoretic mobility were observed in 2-DE gels from hairy vetch supplemented medium (Table 1, Fig 3C). We also found protein spots that were detected in 2-DE gels of all treatments except for hairy-vetch and lupin supplemented medium, which had 7 and 15 matchless protein spots respectively (Table 1). To further compare the influence of the different root extracts on the *T. basicola* proteome, we searched for protein spots statistically different with at least a 2-fold change over the other root extracts analyzed and found 65 protein spots matching these criteria (Table 1, Fig 3). Major differences where principally found in gels derived from hairy-vetch (48 protein spots) and lupin (13 protein spots) root extract.

Identification of differentially expressed protein spots.

The genome of *T. basicola* is unsequenced and to date there is no proteome database available. Protein identification is therefore challenging and depends exclusively on sequence similarity searching to identify peptides that are identical or highly similar in both *T. basicola* and known homologous proteins from closely related species. Recent studies have proposed a

standard workflow to identify proteins from unsequenced organisms. Usually, these combine MS/MS ion searches, *de novo* sequencing and BLAST sequence-similarity searching (28-30). Using these approaches, we excised fifty differentially expressed protein spots from preparative 2-DE gels, and analysed them by LC-MS/MS and searched all MS/MS spectra against the NCBInr database using the MASCOT MS/MS ion search engine. Peptides with an ion score statistically significant at the $P < 0.05$ level were accepted as extensively homologous, based on MASCOT criteria. Such significant hits for individual tryptic peptides were commonly found in multiple species. We therefore recorded only the most significant hits of these individual tryptic peptides and confirmed protein homology by sequence alignment using the computer program ClustalW. Of these aligned proteins, 65% have at least 70% sequence similarity and peptide sequences belonging to proteins with less than 35% homology were discarded. These peptide sequences as well as their individual MASCOT ion scores, protein and species of origin are summarized in Table 2 and shown in greater detail in the supplemental data. For further characterization, the MASCOT homology MS/MS spectra as well as spectra not identified in the first round database search were interrogated using PEAKS *de novo* analysis software (31). The software computes the best amino acid sequences from all possible amino acid combinations that most accurately match the MS/MS spectrum. This is presented as a confidence score for the entire peptide sequence as well as a positional confidence score for each amino acid. These computed peptide sequences were then searched against the NCBInr database using PEAKS database search, the similarity search software SPIDER (32), MASCOT and these results summarized using the In Chorus program. The results of these searches are presented in the supplemental data. This strategy allowed confident assignment of peptide sequences to forty one protein spots, the majority of these peptide sequences being of fungal origin. In twenty protein

spots, we found peptide sequences belonging to more than one protein. Based on their putative function we found that most of the identified proteins had a role in primary metabolism while the others were implicated in diverse functions including genetic information processing, cytoskeleton organization, ligand binding and stress response (Table 2).

Interestingly, in the presence of hairy vetch root extract four uniquely expressed or over-expressed *T. basicola* protein spots (spots 1, 5, 18, 39) are putatively involved in pyridoxine/pyridoxal 5-phosphate biosynthesis and four over-expressed protein spots involved in amino acid metabolism (spots 4, 12, 14, 19) each possess a pyridoxal phosphate binding site (Table 2). In the remaining hairy vetch over-expressed protein spots, three belong to the pentose phosphate pathway (spots 4, 15, 19) and one is involved in pentose glucuronate interconversions (spot 19). The lupin root extract induced protein spots are either putatively involved in pyruvate metabolism (spots 33, 34, 35, 37) or in the utilization of pyruvate (33, 35, 36) (Table 2).

Substantiation of protein expression changes

Three protein spots, over-expressed in the presence of lupin root extract contained peptides assigned to hypothetical proteins containing a conserved domain belonging to malic enzyme (spots 33, 35, 37) (Table 2). Malic enzyme has an important and well recognized role in *de novo* lipid biosynthesis through the supply of NADPH (33-36). To determine whether lipid bodies were present in the fungi, *T. basicola* cultured in Czapek-Dox medium (24 h) supplemented with root extract, was stained with Nile red and observed by fluorescence microscopy (Fig 5). Cytoplasmic lipid bodies were found under all conditions studied but with differences in distribution and fluorescence intensity. It was also obvious that *T. basicola* cultured in the presence of lupin extract exhibited numerous highly fluorescent bodies throughout the hyphae

while in Czapek, wheat and hairy vetch extract, lipid bodies tend to cluster at the hyphal tip. While additional quantitative experiments are needed to confirm a significant difference in total lipid concentration, our qualitative observations provide a possible explanation for the over-expression of spots 33, 35, 37 (Table 2) identified as putative malic enzyme, in the presence of lupin root extract.

Protein spots 2, 8, 14, 16, 27, 34, 37 contained peptides assigned to proteins involved in cytoskeleton organization. To assess if the differential expression of these protein spots reflected differences in the patterns of mycelia development, *T. basicola* endoconidia were cultured on microscopic glass slides in Czapek-Dox medium (24 hr) supplemented with root extract. Significant differences were observed between the total hyphal lengths and degree of branching achieved in the presence of host root extract, with *T. basicola* cultured in the presence of lupin root extract with higher total hyphal length and degree of branching (Fig 6 a, b). Variations in the hyphal growth unit (37) were also observed with endoconidia cultured in the presence of host root extract being significantly lower when compared to non-host root extract (Fig 6 c). Septa diameter was significantly increased in the presence of hairy-vetch and lupin root extract (Fig 6 d). The distance between septa for *T. basicola* cultured in the presence of hairy vetch root extract was significantly lower compared to control (Fig 6 e) while no significant differences were observed in the remaining plant extracts.

Discussion

T. basicola has been characterized as a hemibiotroph, which means that to assure its survival this fungus has a mandatory close association with its host. Host infection can be separated into different steps including spore germination, proliferation and sporulation (38). While it is generally assumed that during spore germination and the penetration phase, fungi are completely dependent on nutrients derived from internal stores, the nutritional environment of the rhizosphere is an important factor in triggering spore germination as dormant propagules are stimulated by soluble and volatile components present in seeds and root exudates (39). Therefore, survival of the fungus is a function of its ability to utilize and adapt to the nutrients available in the plant vicinity and within the plant tissue. In the current study, we analyzed the influence of non-host and susceptible host plant root extracts on *T. basicola* growth rate, colony morphology and proteome. Colony diameter was significantly greater in the presence of susceptible host plant extracts. This is in accord with a previous study which showed that cotton root exudates allowed the greatest spore germination when compared to other crops (14). Differences in colony morphology were also observed. Previous studies have described the occurrence of colony variation arising during growth in pure culture principally on rich media such as potato dextrose agar and V8 agar (3, 40) as well as morphological variation among isolates originating from different sources (3, 41, 42). To date only one study by Rawlings (1940) (43), illustrated the influence of different media on growth rate and colony morphology of *T. basicola.* Our observations are in agreement with this published work and suggest that *T. basicola* alters its metabolism in response to the nutrient availablility.

Remaining questions now are what kind of nutrients supplied by the plant host are important for fungal survival and growth and what kind of metabolic adjustments *T. basicola* makes to facilitate its adaptation to a particular plant. In an attempt to provide an answer to these two fundamental questions, 2-DE maps of *T. basicola* cultured in the presence of non-host and susceptible host plant root extracts were analyzed. Based on a hierarchical clustering analysis of the overall protein pattern, we propose that nutrients present in plant root extracts are plant dependent rather than dependent on host/non-host as the *T. basicola* proteome cultured in cotton root extract is more closely related to the *T. basicola* proteome cultured in wheat extract, a non-host, than lupin, a host. To further characterize the metabolic adjustment made by *T. basicola* to a particular plant, we focused on major changes (proteins unique to a particular root extract) and proteins with at least a 2-fold statistically different change over other root extracts. In total, 87 protein spots corresponding to these criteria were identified. Fifty differentially expressed protein spots were analyzed by LC-MS/MS together with cross-species analysis of MS/MS spectra and *de novo* sequencing. Putative protein identifications were obtained for 41 protein spots. Unambiguous functional assignment as well as interpretation of results proved to be challenging, because (1) many peptides were assigned to hypothetical proteins and therefore we could only rely on putative conserved domains to speculate on their possible functions, (2) several protein spots contained peptide sequences assigned to more than one protein making the assessment of the up- and down regulation of a particular protein difficult, and (3) the lack of genome information combined with the probability that about 20 % of the protein spots in an pH 4-7 differentially expressed protein (44).

These important issues not withstanding, we observed that peptide sequences from four spots (spots 1, 5, 18, 39), over-expressed in the presence of hairy vetch root extract, identified hypothetical proteins highly similar to the pyridoxine biosynthesis protein PDX1 and that four additional spots (spots 4, 12, 14, 19) contained peptides belonging to putative proteins containing a pyridoxal phosphate binding site. The protein PDX1 is highly conserved between different organisms, and is a key enzyme in vitamin B_6 *de novo* biosynthesis. Only micro-organisms and plants have the ability to synthesize vitamin B_6 through either of two possible pathways; (1) deoxyxylulose 5-phosphate (DXP)-dependent and (2) DXP-independent. The DXP-dependent pathway is found in *Escherichia coli* and other proteobacteria while the DXP-independent pathway predominantes in archaea, most bacteria, fungi and plants. In the DXP-independent pathway, PDX1 jointly with PDX2 function as amidotransferases in which PDX2 produces ammonia from glutamine and PDX1 combines ammonia with intermediates of glycolysis (glyceraldehyde 3-phosphate) and the pentose phosphate pathway (ribose 5-phosphate) to form pyridoxal 5-phosphate, the biologically active form of vitamin B6 (45). It is worth pointing out that three other protein spots (4, 15, 19) over-expressed in the presence of hairy vetch extract contained peptides belonging to proteins involved in the pentose phosphate pathway. Vitamin B6 is an essential metabolite in all organisms and is required for more than 100 enzymatic reactions, mainly involved in amino acid metabolism. Lack of this vitamin is therefore lethal (46). In *Saccharomyces cerevisiae*, SNZ1 (homologous to PDX1) is induced late in stationary phase as well as in auxotrophic mutants in response to limitation of adenine, uracil and tryptophan (47, 48) and in *Bacillus subtilis*, *PdxS* the counterpart of PDX1 has been found to be an oxidative stress-inducible protein (49). Why vitamin B_6 *de novo* biosynthesis is increased in the presence of hairy vetch root extract is unclear and will need further consideration.

Peptide sequences from five *T. basicola* protein spots over-expressed in the presence of lupin root extract were associated with pyruvate metabolism. Three of these protein spots contained peptides assigned to hypothetical proteins containing a conserved domain belonging to malic enzyme (spots 33, 35, 37). Spot 34 may correspond to a putative phosphoenolpyruvate carboxykinase, however MASCOT/ Peaks also identified a chaperone as another possible protein identification. Spots 33, 35, 36 possess one or two peptides common to putative pyruvate decarboxylase. Malic enzyme (NADP-ME) [malate dehydrogenase (decarboxylating) ($NADP^+$) (EC 1.1.1.40)] catalyses the reaction: L-malate + $NADP^+ \rightarrow$ pyruvate + CO^2 + NADPH and has been found in a range of fungi. Its function in pyruvate metabolism is well known (50-52) as well as its vital role in *de novo* lipid biosynthesis through the supply of NADPH (33-36). *T. basicola* accumulates lipid bodies in the cytoplasm of endoconidia and chlamydospores (16, 53). In our current work we have used fluorescence microscopy to confirm the presence of lipid bodies in the hyphae and suggest an increase in their prevalence in the presence of lupin root extract.

Phosphoenolpyruvate carboxykinase (PEPCK) is one of the key enzymes of gluconeogenesis which converts oxaloacetate to phosphoenolpyruvate. Filamentous fungi are able to grow on a great diversity of compounds that feed the TCA cycle and therefore require gluconeogenesis (54). These include sources of acetyl-coA (ethanol, acetate, fatty acids), 2-oxoglutarate (amino acids) and sources of both succinate and acetyl-coA (aromatic acids and fatty acids). In 1981, Kelly and Hynes (55) reported an induction of the activity of PEPCK and NADP-ME in the presence of L-proline in *Aspergillus nidulans*. While induction of both

enzymes by L-proline was observed in the presence and absence of sucrose, the induction was greater in the absence of sucrose. Addition of NH_4Cl to the medium suppressed NADP-ME activity. Regulation studies of the *acuF* gene (encoding for PEPCK) in *Aspergillus nidulans* confirmed the induction by proline of this gene in the presence and absence of glucose (56, 57) while mutation of the *acuK* or *acuM* encoding the NADP-malic enzyme show the relation with PEPCK. Indeed, mutation of these genes not only affects growth on carbon sources requiring gluconeogenesis but also results in loss of induction of the *acuF* gene by carbon sources metabolized via the TCA cycle (57).

Several protein spots contained peptide sequences assigned to proteins involved in cytoskeleton organization. These correspond to protein spots differentially expressed in the presence of hairy vetch (spots 2, 8, 14, 16, 27) and lupin root extract (spots 34, 37). Protein spot 2, over-expressed, and protein spot 27, under-expressed contained peptide sequences belonging to putative septin proteins similar to *Saccharomyces cerevisiae* CDC10 and CDC3, respectively. Spot 2 also contained two peptides assigned to the actin-like protein, centractin. Peptide sequences belonging to actin were identified in spot 14 and 37, in spots 8 and 34 subunits of the chaperonin containing T-complex protein 1 (CCT) were identified and finally a hypothetical protein highly similar to the *Saccharomyces cerevisiae* protein RVS161 was identified in spot 16. Interestingly, homologues of some of these proteins in *Saccharomyces cerevisiae* have been shown to interact (58, 59). Septins comprise a family of highly conserved proteins (CDC3, 10, 11, 12 and SEP7/SHS1) known to play an important role in morphogenesis. In *Saccharomyces cerevisiae*, they were first discovered for their role in cytokinesis and septum formation and their ability to assemble into 10 nm filaments was characterized. These filaments formed a ring on the inner surface of the plasma membrane at the mother-daughter neck (60). Subsequently septins

have been implicated in many other cellular processes (for review (61)). Deletion analyses have shown that CDC3 is essential while deletion of CDC10 leads to morphological defects (62, 63). Moreover, in the filamentous fungus *Aspergillus nidulans*, an ortholog of CDC3, AspB has been found to be involved in cellular division and conidiophore development and to be an early marker for branch formation (64). In *Candida albicans*, mutation of CDC10 has been shown to affect hyphal growth and morphology, to be defective in forming chlamydospores due to their inability to undergo septation and to seriously affect virulence capability of this fungus probably through its scaffold role (63, 65-67). CCT has a unique structural composition with superimposed rings constituted by eight different homologous subunits (for review (68)) and was originally discovered for its essential role in assisting the folding of cytoskeletal proteins such as actin, actin-related protein (centractin) and tubulin (for review (69)). Recently this complex has also been shown to interact with the septin proteins, but rather than assisting in their folding it is believe to perform a regulatory role instead (58). In *Saccharomyces cerevisiae*, protein RVS161 in association with RVS167 is known to play an important role in the actin cytokeleton and cell wall organization as well as other cellular process (70). Recently, through tandem affinity purification and mass spectrometry analysis, a possible interaction between RVS161 and CDC10 has been suggested (59).

Here we show differences in *T. basicola* colony morphology when grown in root extracts from host and non-host plants, and corresponding differences in expression of "morpho" proteins by 2-DE. To assess if the identification of these "morpho" proteins may contribute to differences in hyphal morphology, we carried out a microscopic study of *T. basicola* endoconidia cultured in the presence of the different plant root extracts. Morphological hyphal differences were observed

and two important observations need to be emphasized. First, in accordance with Linderman and Toussoun (1968) (15) who reported increased branching of hyphae of *T. basicola* on the host surface, we found a strong positive influence of host root extract on total hyphal length and degree of branching. Second, a significant decrease in length of the apical compartment was observed in the presence of hairy vetch root extract. This decrease may be explained by the over-expression of protein spots 2, 8, 14, 16 as cytokinesis (septation) is initiated through the formation of a transient actin ring at the division site (71). As mentioned previously CDC10 mutants are unable to undergo septation (66). Moreover, this observation may also reflect a stress response, as it has been shown that nutrient limitation influences nuclear mitosis and septation (72).

In summary, our research has demonstrated that non-host and susceptible host root extracts influence growth, colony and hyphal morphology and the proteome of *T. basicola*. Analysis of 2-DE maps of *T. basicola*, revealed that plant root extract influence depends more on the plant than host/non-host status. Using a combination of MASCOT MS/MS ion search, *de novo* sequencing and sequence similarity searches, we assigned peptide sequences to 41 differentially expressed protein spots. Based on homology and identification of putative conserved domains in the homologous proteins identified, we found that vitamin B6 is important in the *T. basicola* response to hairy vetch extract. We also report a possible influence of compounds present in lupin root extract on lipid accumulation and/or an influence on pyruvate metabolism through the activity of malic enzyme and phosphoenolpyruvate carboxykinase and finally we identified putative “morpho” proteins which could account for the morphological differences observed. To conclude, this is the first study to offer insight into the molecular changes occurring in *T.*

basicola when in contact with host and non-host plant material. This work lays the foundations for further studies that will aim to validate and detail the exact role of the putative proteins identified here, as well as their importance in the activities that rule this host-pathogen interaction. Acquisition of this knowledge may provide a rational basis for the development of new control strategies.

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Figure legends

Fig 1. Growth rate assessment of *T. basicola*: *T. basicola* was grown at 25°C on Czapek-Dox/agar medium and Czapek-Dox/agar medium supplemented with non-host (wheat and hairy vetch) and susceptible host (cotton and lupin) root extract (1:10 v:v dilution) and the colony diameter recorded. Columns labeled with the same letters do not differ significantly at $P<0.05$ by Duncan's test. Bars represent standard errors of the mean from 3 combined replicates.

Fig 2. Colonies morphology of *T. basicola* in the presence of non-host and susceptible host root extracts. *T. basicola* was grown for 8 days at 25°C on Czapek-Dox/agar medium (data not shown) and Czapek-Dox/agar medium supplemented with non-host (wheat and hairy vetch) and susceptible host (cotton and lupin) root extract to a final dilution of 10^{-1}.

Fig 3. 2-DE protein profiles of *T. basicola* culture in **(A)** Czapek-Dox control and Czapek-Dox supplemented with **(B**) wheat, **(C)** hairy vetch, **(D)** cotton and **(E)** lupin root extract. Spots highlighted with ○ are matchless in the other conditions studied, □ are over-expressed, ⬠ are under-expressed and △ show the spots with 2-fold expression change between both non-host and susceptible host root extract. Spot numbered return identification after MS/MS analysis.

Fig 4. **Classification based on the proteome of *T. basicola*** culture in Czapek-Dox control and Czapek-Dox supplemented with **(B**) wheat, **(C)** hairy vetch, **(D)** cotton and **(E)** lupin root extract. Hierachical clustering was performed using the nearest neighbor method with the Pearson correlation as similarity measure.

Fig 5. Microscopic photographs of Nile red fluorescence of *T. basicola*. *T. basicola* was grown for 24 h at 25°C in **(A)** Czapek-Dox and Czapek-Dox supplemented with non-host **(B)** wheat and **(C)** hairy vetch and susceptible host **(D)** cotton and **(E)** lupin root extract (1:10 v:v dilution) and stained with Nile red (0.4 µg/ml).

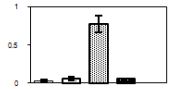
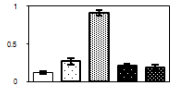
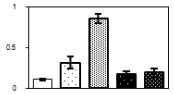
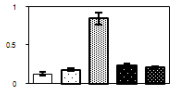
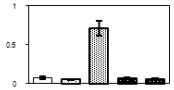
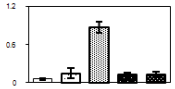
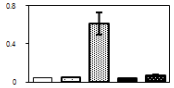
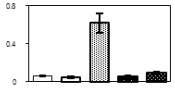
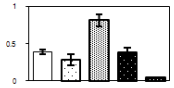
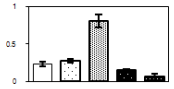
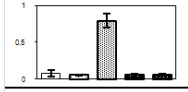
Fig 6. Quantification of hyphal morphology of *T. basicola*. *T. basicola* was grown for 24 h at 25^{o}C in Czapek-Dox and Czapek-Dox supplemented with non-host (wheat and hairy vetch) and susceptible host (cotton and lupin) root extract (1:10 v:v dilution). **(A)** total hyphal length, **(B)** degree of branching, **(C)** hyphal growth unit, **(D)** septa diameter, **(E)** distance between septa. Columns labeled with the same letters do not differ significantly at $P < 0.05$ by Duncan's test. Bars represent standard errors of the mean from 5 germinated endoconidia.

Table 1. Summary of the qualitative and quantitative changes of *T. basicola* cultured in Czapek-Dox supplemented with non-host (wheat and hairy vetch) and susceptible host (cotton and lupin) root extract.

Root extract	Unique protein spots	Undetected protein spots[a]	Differentially expressed protein spots[b]	
			Over-expressed	Under-expressed
Wheat	/	/	1	1
Hairy-vetch	11	7	26	22
Cotton	/	/	2	/
Lupin	/	15	6	7

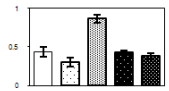
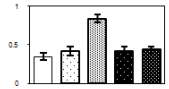
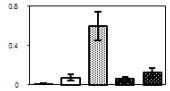
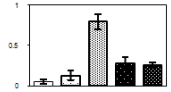
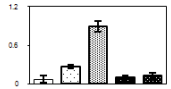
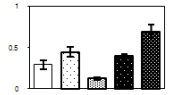
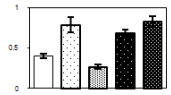
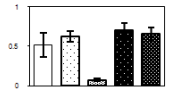
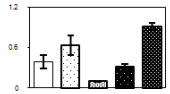
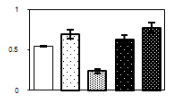
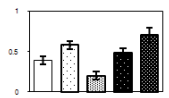
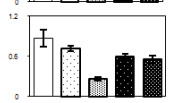
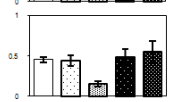
[a] Protein spots not detected in a particular root extract but present in all other treatments.
[b] Protein spots presenting a statistically different 2-fold change over the other root extracts analyzed.

Table 2. List of identified proteins.

Expression pattern [a]	Protein number	Exp. Mol. Mass (KDa)/pI	Protein to which peptide sequence is assigned	Predicted EC number [b]	Molecular function
unique to hairy vetch	1	32.4/5.9	pyridoxine biosynthesis protein		Metabolism of Cofactors and Vitamins (Vitamin B6 metabolism)
unique to hairy vetch	2	43.2/6.1	septin		Cell division/ Cytoskeleton
			actin-like protein (Centractin)		Cytoskeleton organisation and biogenesis
			hypothetical protein (similar to 26S proteasome regulatory particle triple-A ATPase subunit1b)		Genetic Information Processing (Proteasome)
			predicted protein		Genetic Information Processing (RNA binding proteins)
			hypothetical protein		Genetic Information Processing (RNA binding proteins)
unique to hairy vetch	3	43.4/6.1	41 kDa peptidyl-prolyl cis-trans isomerase	5.2.1.8	Genetic Information Processing (Protein folding)
	4	53.2/5.7	6-phosphogluconate dehydrogenase	1.1.1.44	Carbohydrate Metabolism (Pentose phosphate pathway)
			homocysteine synthase CysD	2.5.1.49	Amino acid metabolism (Cysteine, methionine)
	5	33.7/5.4	pyridoxine biosynthesis protein		Metabolism of Cofactors and Vitamins (Vitamin B6 metabolism)
			hypothetical protein MGG_06196		Nitrogen compound metabolic process: CN hydrolase
	6	23.9/5.8	hypothetical protein		Genetic Information Processing (FMN binding)
	7	46.8/5.9	allantoicase	3.5.3.4	Nucleotide Metabolism (Purine metabolism)
	8	67.6/5.5	T-complex protein 1 subunit eta		Genetic Information Processing (Chaperones and folding catalysts)
	9	45.9/6.2	probable FMN oxidoreductase		Genetic Information Processing (FMN binding)
	10	29.3/5.9	uracil phosphoribosyltransferase	2.4.2.9	Nucleotide Metabolism (Pyrimidine metabolism)
			hypothetical protein (similar to catalase (peroxidase I))		
	11	47.2/6.1	phospho-2-dehydro-3-deoxyheptonate aldolase	2.5.1.54	Amino Acid Metabolism (Phenylalanine, tyrosine and tryptophan biosynthesis)
	12	48.2/6.1	pyruvate dehydrogenase E1 component alpha subunit, mitochondrial	1.2.4.1	Carbohydrate Metabolism (Pyruvate and butanoate metabolism)
	13	98.7/5.6	elongation factor 3		Genetic Information Processing (Protein biosynthesis)
	14	49.6/5.3	actin		Cytoskeleton organisation and biogenesis
			hypothetical protein BC1G_06859 (similar to 26S proteasome subunit)		Genetic Information Processing (Proteasome)
			kynurenine aminotransferase	2.6.1.7	Amino Acid Metabolism (Tryptophan metabolism)
	15	37.3/5.2	class II Aldolase and Adducin N-terminal domain protein		
			bifunctional transaldolase/phosoglucose isomerase	5.3.1.9 and 2.2.1.2	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis, Pentose phosphate pathway)

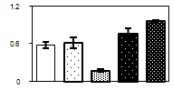
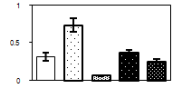
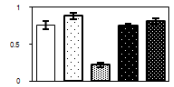
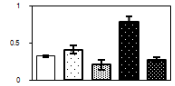
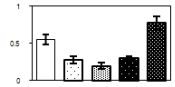
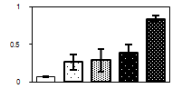
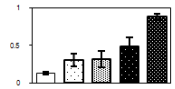
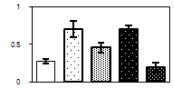
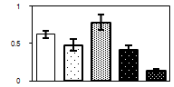
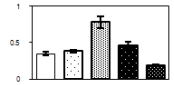
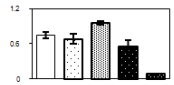
[a] Y axis: normalized expression volume of the spot. X axis: column 1: Czapek, 2: Czapek + wheat, 3: Czapek + hairy-vetch, 4: Czapek + cotton, 5: Czapek +lupin.

[b] http://www.expasy.org/enzyme/

Expression pattern [a]	Protein number	Exp. Mol. Mass (KDa)/pI	Peptide sequence assigned to protein	Predicted EC number [b]	Molecular function
	16	29.1/5.4	hypothetical protein (BAR domain)		Cytoskeleton organisation and biogenesis (Cytoskeletal protein binding)
	17	41.4/5.5	Ketol-acid reductoisomerase, mitochondrial precursor	1.1.1.86	Amino Acid Metabolism (Valine, leucine and isoleucine biosynthesis) Metabolism of Cofactors and Vitamins (Pantothenate and CoA biosynthesis)
			hypothetical protein CHGG_04505		WD40 domain, wide variety of functions
	18	33.1/5.7	pyridoxine biosynthesis protein conserved hypothetical protein		Metabolism of Cofactors and Vitamins (Vitamin B6 metabolism)
	19	63.3/6.1	Glucose-6-phosphate 1-dehydrogenase	1.1.1.49	Carbohydrate Metabolism (Pentose phosphate pathway)
			hypothetical protein (similar to UDP-glucose pyrophosphorylase)	2.7.7.9	Carbohydrate Metabolism (Pentose and glucuronate interconversions)
			threonine dehydratase, mitochondrial precursor	4.3.1.19	Amino Acid Metabolism (Glycine, serine, threonine and valine, leucine and isoleucine biosynthesis)
			hypothetical protein FG09373.1		FAD binding domain
	20	38.2/6.3	glyceraldehyde-3-phosphate dehydrogenase		Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
	21	39.5/4.6	ribosome-associated protein RAP1-like protein		Genetic Information Processing (Protein biosynthesis)
			serine/threonine protein phosphatase PP2A catalytic subunit	3.1.3.16	Environmental Information Processing (iron ion binding)
			hypothetical protein CHGG_04776 (deoxyhypusine hydroxylase)	1.14.99.29	Hypusine synthesis
	22	62.6/5.4	hexokinase	2.7.1.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis, Fructose and mannose metabolism)
	23	91.1/5.2	polyadenylate-binding protein		(Genetic Information Processing) Translation regulation, mRNA binding
	24	66.0/5.8	myo-inositol-phosphate synthase	5.5.1.4	Carbohydrate Metabolism (Inositol phosphate metabolism)
	25	57.2/6.0	hypothetical protein (similar to mannose-1-phosphate guanylyltransferase)	2.7.7.13	Carbohydrate Metabolism (Fructose and mannose metabolism)
	26	63.6/5.3	hexokinase	2.7.1.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis, Fructose and mannose metabolism)
			hypothetical protein FG06055.1 (domain: phosphoglyceromutase)	5.4.2.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
			oxysterol binding protein (Osh7)		Oxysterol-binding protein
	27	54.6/5.1	26S protease regulatory subunit 6B homolog		Genetic Information Processing (Proteasome)
			hypothetical protein NCU02464 (similar to CDC3)		Cell division/ Cytoskeleton
			enolase	4.2.1.11	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
			F1-Atpase	3.6.3.14	Energy Metabolism (ATP synthesis)
	28	74.2/5.7	NADH-ubiquinone oxidoreductase 78 kDa subunit, mitochondrial precursor	1.6.5.3 and 1.6.99.3	Metabolism of Cofactors and Vitamins (Ubiquinone biosynthesis) Energy Metabolism (Oxidative phosphorylation)
			tryptophan synthase	4.2.1.20	Amino Acid Metabolism (Phenylalanine, tyrosine and tryptophan biosynthesis)

[a] Y axis: normalized expression volume of the spot. X axis: column 1: Czapek, 2: Czapek + wheat, 3: Czapek + hairy-vetch, 4: Czapek + cotton, 5: Czapek +lupin.

[b] http://www.expasy.org/enzyme/

Expression pattern [a]	Protein number	Exp. Mol. Mass (KDa)/pI	Peptide sequence assigned to protein	Predicted EC number [b]	Molecular function
	29	72.9/5.9	translation initiation factor eIF3		Genetic Information Processing (Protein biosynthesis)
			transketolase	2.2.1.1	Carbohydrate Metabolism (Pentose phosphate pathway)
	30	65.7/6.0	hypothetical protein (similar to flotillin domain protein)		Insulin signaling pathway
	31	70.9/4.9	importin alpha subunit		Protein import into nucleus
	32	38.9/6.0	succinyl-CoA ligase alpha-chain, mitochondrial precursor	6.2.1.4	Carbohydrate Metabolism (Citrate cycle, propanote metabolism)
			uricase	1.7.3.3	Nucleotide Metabolism (Purine metabolism)
			hypothetical protein MGG_06494		
			glyceraldehyde 3-phosphate dehydrogenase, putative	1.2.1.12	
			60S ribosomal protein L4-A		
	33	72.2/5.4	hypothetical protein (domain: Malic enzyme)	1.1.1.40	Carbohydrate Metabolism (Pyruvate metabolism)
			pyruvate decarboxylase	4.1.1.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
	34	67.3/5.7	T-complex protein 1 subunit gamma		Chaperones and folding catalysts
			phosphoenolpyruvate carboxykinase	4.1.1.49	Carbohydrate Metabolism (Citrate cycle, pyruvate metabolism)
	35	70.9/5.3	hypothetical protein (domain: Malic enzyme)	1.1.1.40	Carbohydrate Metabolism (Pyruvate metabolism)
			molecular chaperones mortalin/PBP74/GRP75		
			pyruvate decarboxylase	4.1.1.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
	36	69.8/5.4	indole-3-pyruvate decarboxylase	4.1.1.74	Amino Acid Metabolism (Tryptophan metabolism)
	37	70.2/5.4	hypothetical protein (domain: Malic enzyme)	1.1.1.40	Carbohydrate Metabolism (Pyruvate metabolism)
			actin		Cytoskeleton organisation and biogenesis
			possible exodeoxyribonuclease V subunit C 125 kD polypeptide	3.1.11.5	Genetic Information Processing (Homologous recombination)
	38	56.2/5.4	Glycerol-3-phosphate dehydrogenase [NAD+]	1.1.1.8	Lipid metabolism (Glycerophospholipid metabolism)
			gamma-glutamyl phosphate reductase	1.2.1.41	(Amino Acid Metabolism) Urea cycle and metabolism of amino groups
			pyruvate decarboxylase	4.1.1.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
			GA12266-PA (domain:ATPase family)		Genetic Information Processing (Proteasome)
			hypothetical protein CHGG_00843 (6-phosphogluconate dehydrogenase)	1.1.1.44	Carbohydrate Metabolism (Pentose phosphate pathway)
			putative phosphoglyceromutase	5.4.2.1	Carbohydrate Metabolism (Glycolysis / Gluconeogenesis)
			hypothetical protein SNOG_07132 (domain: Choline/ethanolamine kinase)		Lipid Metabolism (Glycerophospholipid metabolism)
	39	33.1/5.6	pyridoxine biosynthesis protein		Metabolism of Cofactors and Vitamins (Vitamin B6 metabolism)
			conserved hypothetical protein (domain: S-adenosyl-L-homocysteine hydrolase (AdoHycase))	3.3.1.1	Amino acid Metabolism (Methionine metabolism)
	40	54.4/5.9	NADP-specific glutamate dehydrogenase (NADP-GDH)	1.4.1.4	Amino acid Metabolism (Glutamate metabolism)
					Energy metabolism (Nitrogen metabolism)
	41	32.9/6.0	hypothetical protein (domain: elongation factor 2)		Genetic Information Processing (Protein biosynthesis)
			hypothetical protein (domain:K homology RNA-binding domain)		Genetic Information Processing (RNA binding proteins)

[a] Y axis: normalized expression volume of the spot. X axis: column 1: Czapek, 2: Czapek + wheat, 3: Czapek + hairy-vetch, 4: Czapek + cotton, 5: Czapek +lupin.

[b] http://www.expasy.org/enzyme/

Fig 1.

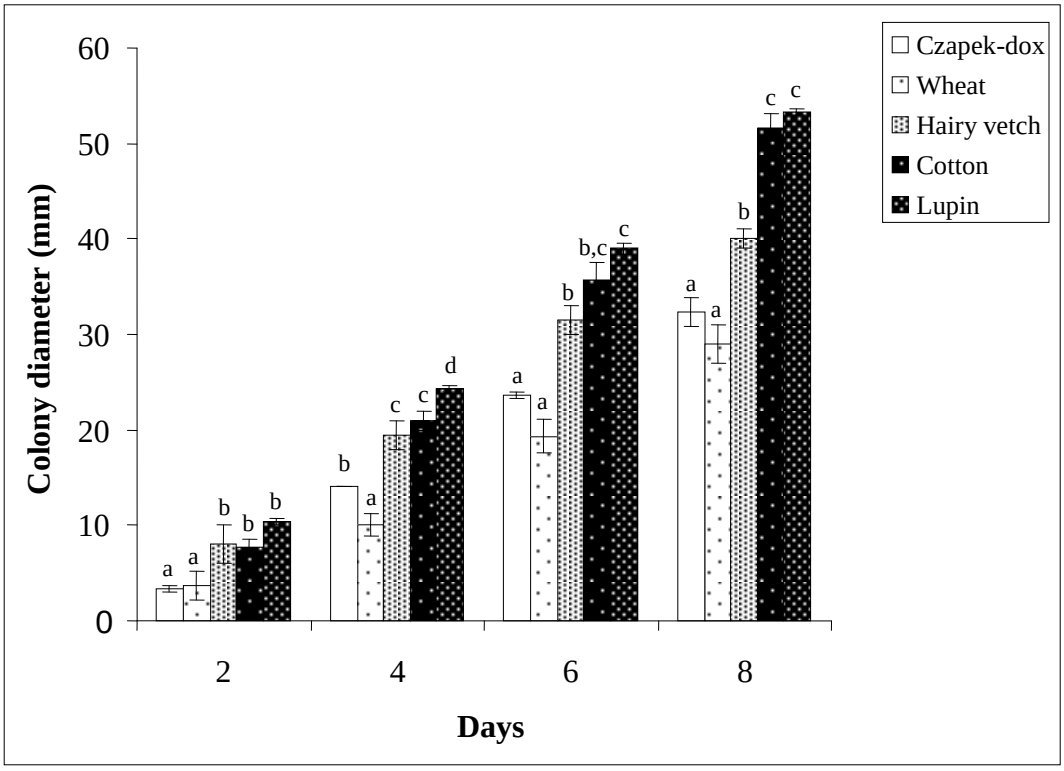

Czapek-dox
Wheat
Hairy vetch
Cotton
Lupin
Colony diameter (mm)
60
50
40
30
20
10
0
2
4
6
8
Days
a a b b b
b a c c d
a a b b,c c
a a b c c

Fig 2.

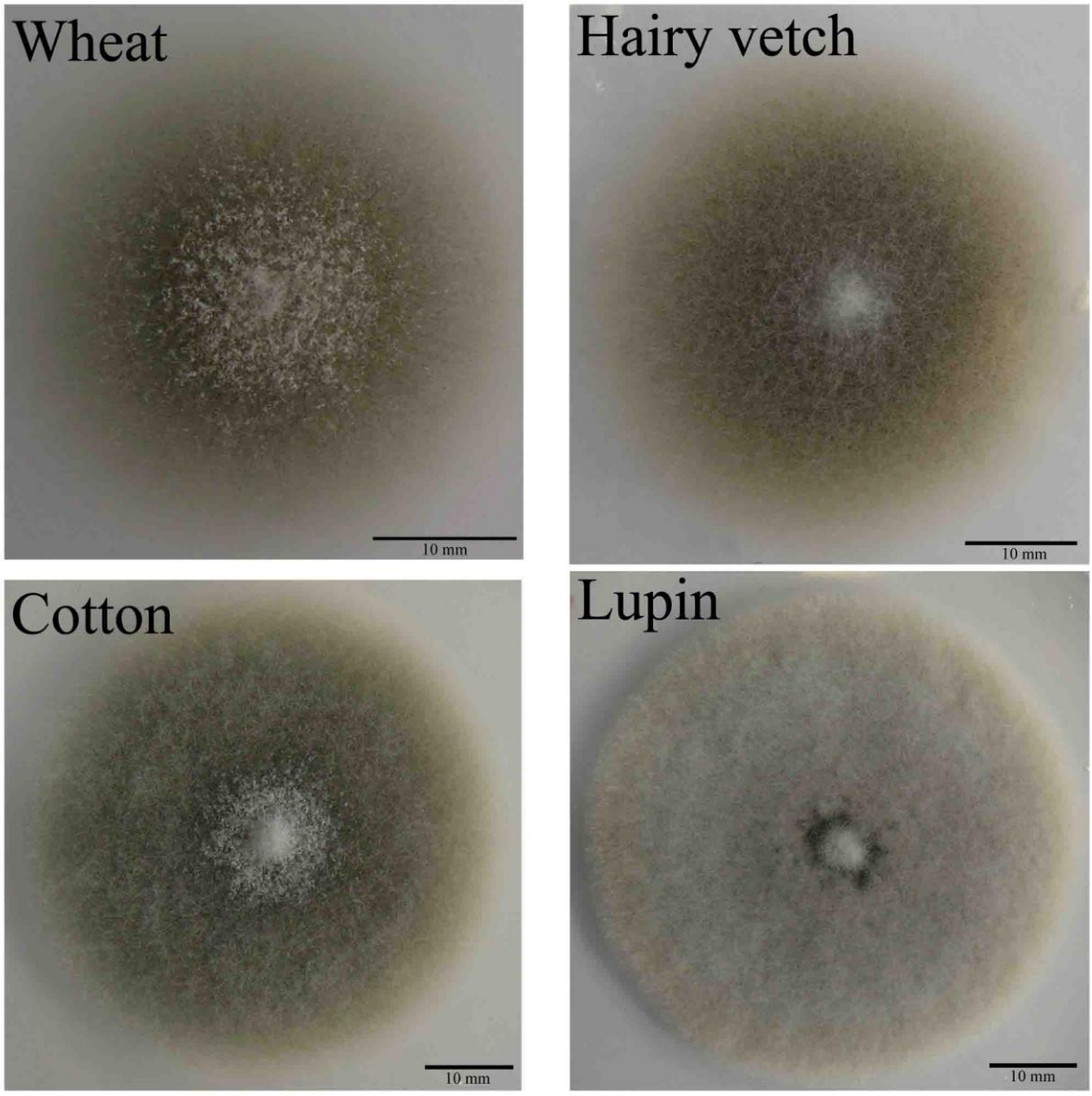


Fig 3AB

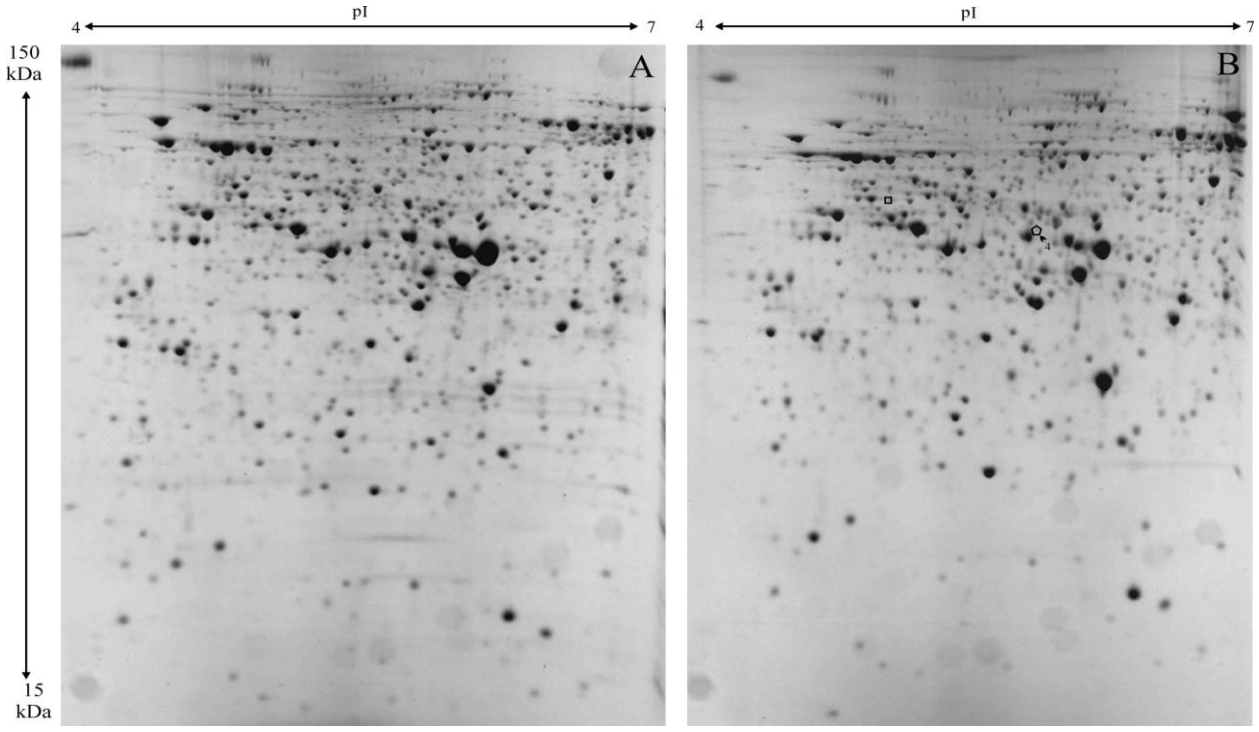


Fig 3CD

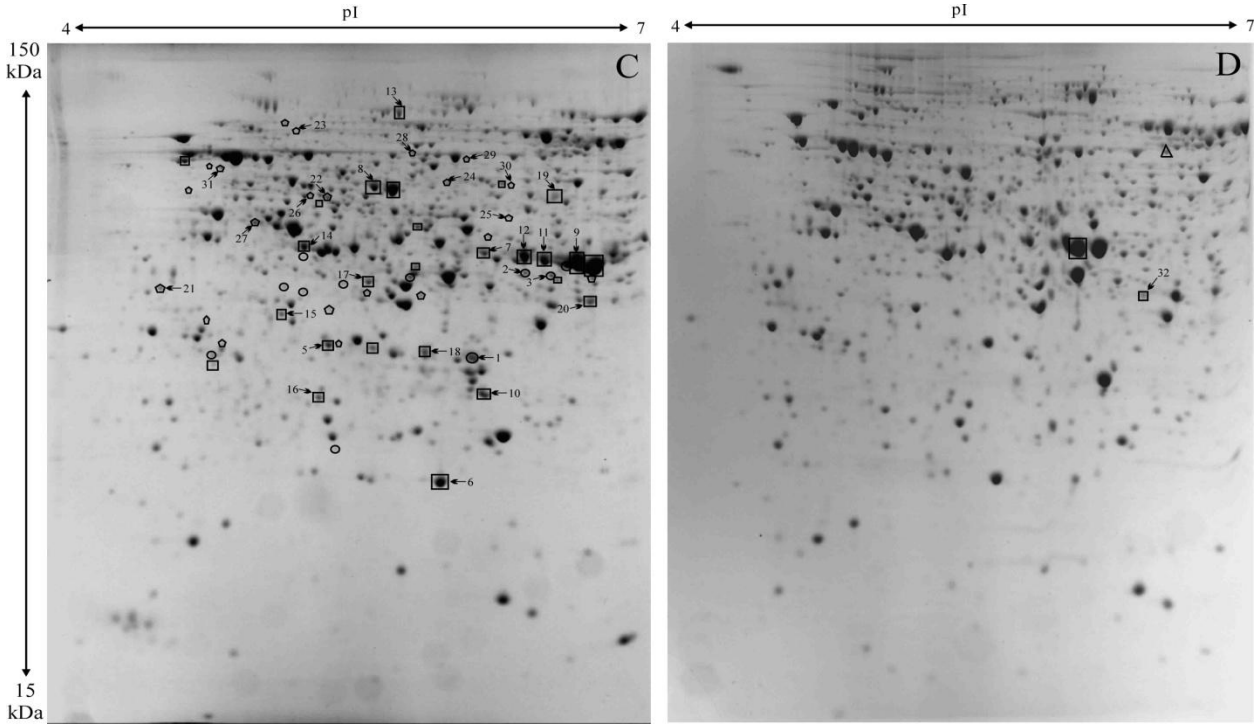


Fig 3E

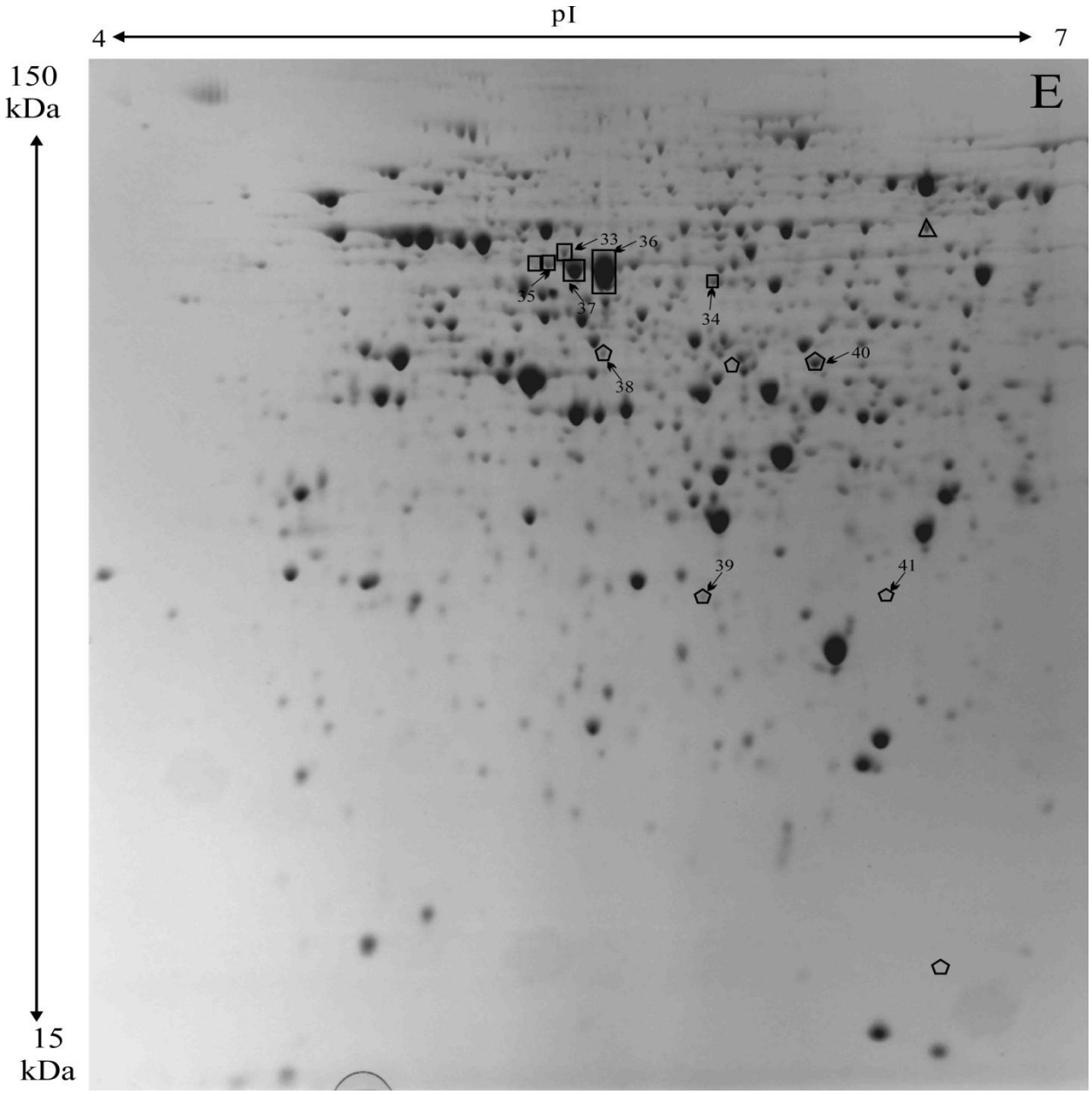


Fig 4

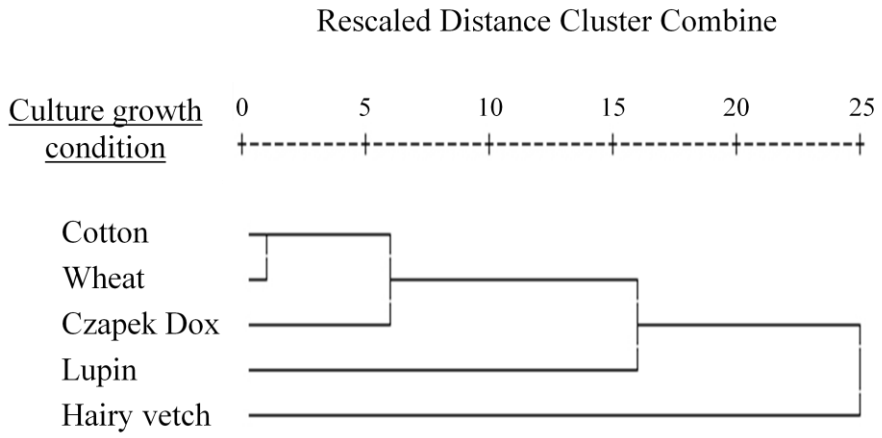

Rescaled Distance Cluster Combine
Culture growth condition
0
5
10
15
20
25
Cotton
Wheat
Czapek Dox
Lupin
Hairy vetch

Fig 5

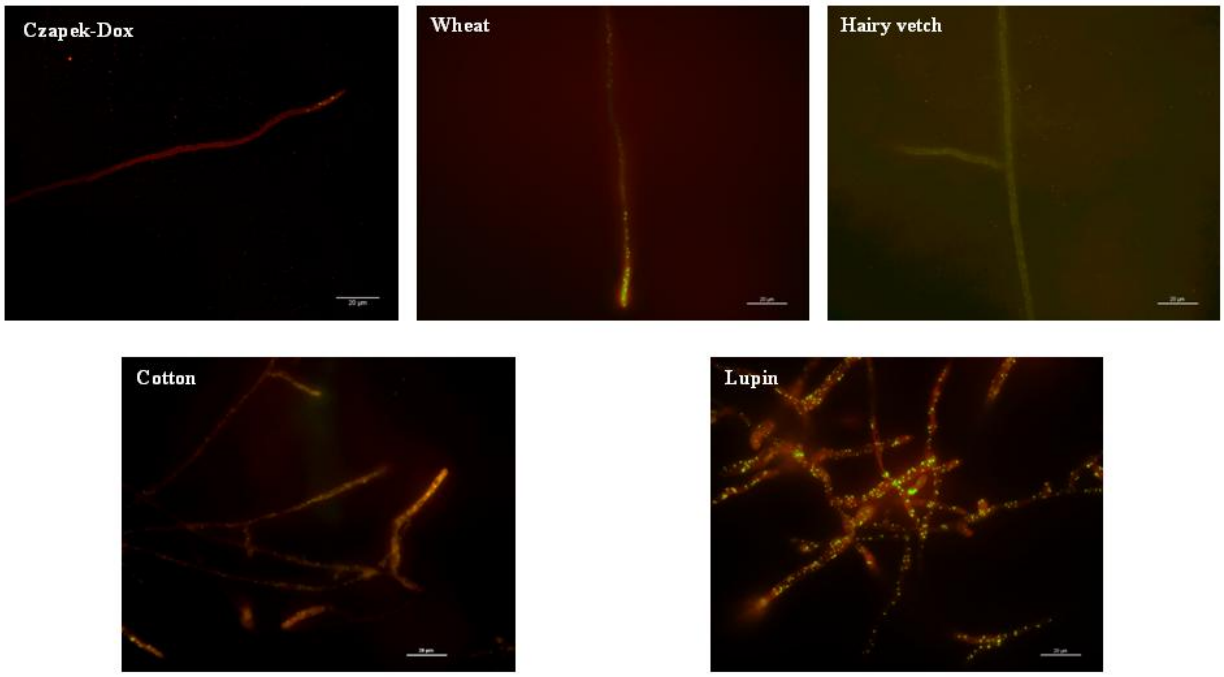
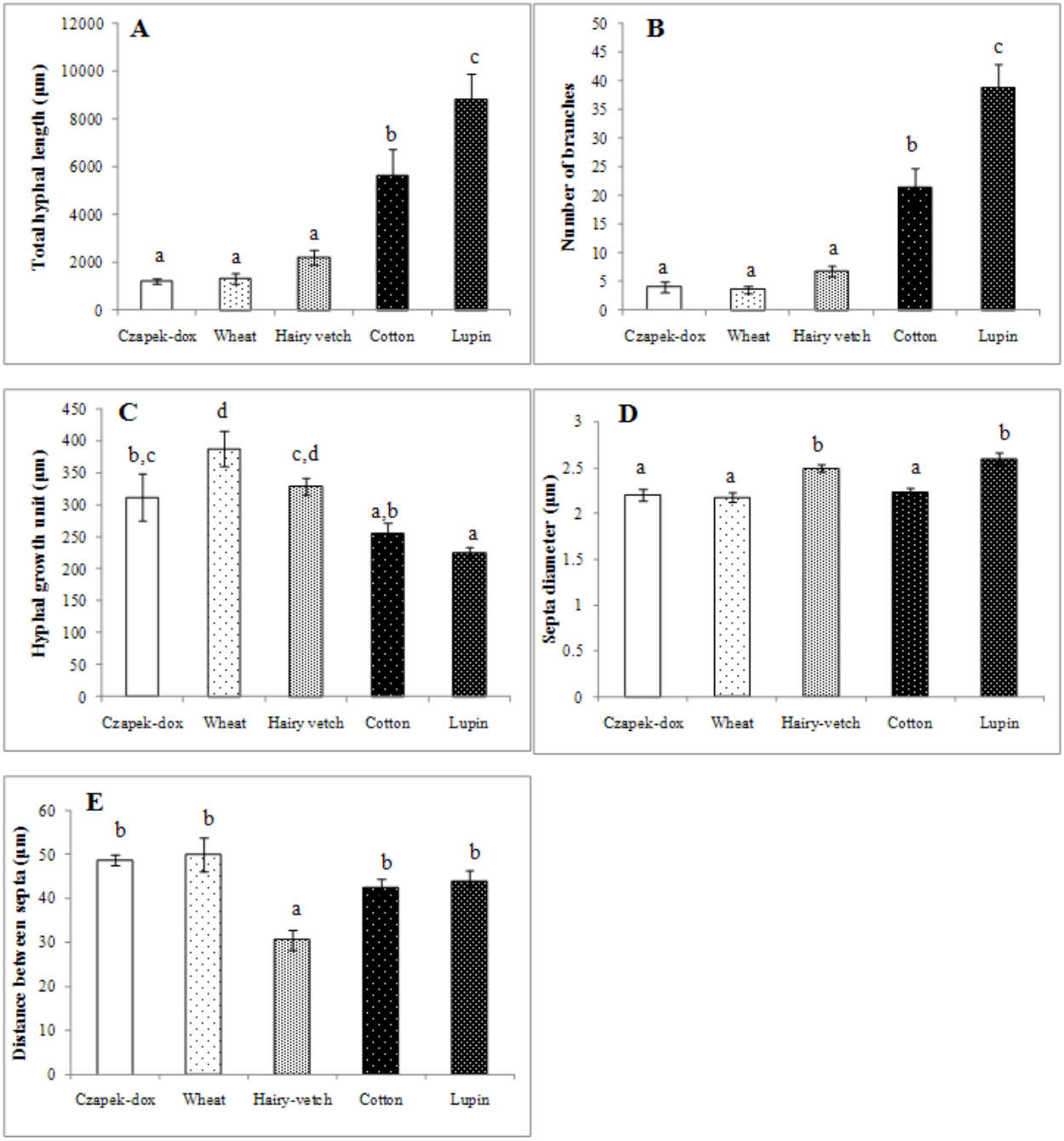

Czapek-Dox
Wheat
Hairy vetch
Cotton
Lupin

Fig 6


A
Total hyphal length (µm)
12000
10000
8000
6000
4000
2000
0
a
a
a
b
c
Czapek-dox
Wheat
Hairy vetch
Cotton
Lupin
B
Number of branches
50
45
40
35
30
25
20
15
10
5
0
a
a
a
b
c
Czapek-dox
Wheat
Hairy vetch
Cotton
Lupin
C
Hyphal growth unit (µm)
450
400
350
300
250
200
150
100
50
0
b,c
d
c,d
a,b
a
Czapek-dox
Wheat
Hairy vetch
Cotton
Lupin
D
Septa diameter (µm)
3
2.5
2
1.5
1
0.5
0
a
a
b
a
b
Czapek-dox
Wheat
Hairy-vetch
Cotton
Lupin
E
Distance between septa (µm)
60
50
40
30
20
10
0
b
b
a
b
b
Czapek-dox
Wheat
Hairy-vetch
Cotton
Lupin