On the effect of microgravity on colored fungi

Masterarbeit

Ernst Thür

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Studium Biotechnologie

Universität für Bodenkultur Wien
Department für Biotechnologie

Betreuer: Assoc. Prof. Dr. Katja Sterflinger-Gleixner

Institut für angewandte Mikrobiologie
Geomicrobiology

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Abstract

The CFS-A (Growth and Survival of Colored Fungi in Space) experiment was issued by the National Aeronautics and Space Administration (NASA) to determine the effect of microgravity and cosmic radiation on the growth and survival of coloured fungi species. The analyzed species are relatively common biodegraders. They can directly influence human health and the possible decay of any degradable components in the environment. The analysis procedure outlined in this thesis investigates the effect of microgravity conditions, similar to those found in the earth orbit.
Four different species were analyzed for their genomic and proteomic changes after growth under orbital conditions. The samples were subjected to two different exposure times. A short term experiment consisted of two weeks of growth on the International Space Station, and a subsequent long term experiment with about two months of growth in the ISS. Two established methods were used to create the data for the analysis. Firstly, an AFLP (Amplification of Fragment Length Polymorphism) for the detection of genomic changes in the digestion patterns of DNA was carried out, followed by an extensive 2D-Gel analysis to visualize the changes in size and amino acid composition of cellular proteins.

Zusammenfassung

Das Experiment CFS-A (growth and survival of Colored Fungi in Space), zu Deutsch etwa "Wachstum und Überleben von farbigen Pilzen im Weltraum" wurde von der Amerikanischen Weltraumbehörde (NASA) in Auftrag gegeben, um die Effekte von reduzierter Schwerkraft und kosmischer Strahlung, wie sie im erdnahen Orbit vorzufinden sind, auf das Wachstum und die Überlebensfähigkeit von farbigen Pilzen zu analysieren. Die untersuchen Pilz-spezies sind häufig im menschlichen Umfeld anzutreffen, und haben einen direkten Einfluss auf die Gesundheit von Menschen, sowie auf die Integrität und den Verfall von organischem Material. Vier verschiedene Spezies wurden dafür im Rahmen dieses Experiments den Konditionen auf der Internationalen Raumstation ISS ausgesetzt. Nach Wachstum unter diesen Bedingungen wurden die Pilze auf genomische und proteomische Veränderungen hin untersucht. Zwei verschiedene Wachstums-dauern wurden dabei analysiert, ein "short term" (Kurzzeit) Experiment, bestehend aus einer zweiwöchigen Wachstum- bzw. Expositionssphase in reduzierter Schwerkraft, sowie ein "long term" (Langzeit) Experiment mit einer Wachstums- bzw. Expositionsphase von circa 2 Monaten. Zur Analyse wurden die zwei etablierten Methoden, AFLP (Amplifizierung von Fragment-Längen - Polymorphismen), zur Detektion von genomischen Veränderungen sowie 2D- Gel Analysen, zur Darstellung von Veränderungen in der Zusammensetzung und dem Gewicht von zellulären Proteinen, angewandt.

Contents

Chapter 1

Introduction

The question of the changes caused by exposure to the conditions in orbital stations have been a subject of great interest for different space programs since the 1970s, and have been tackled by many different research groups over the last decades. Most notable for the earlier research on fungi under such conditions are the results of Volz et al. retrieved from the Apollo Microbial Ecology Evaluation Device (MEED) mycology experiments (Volz 1990, Volz et al. 1998). Volz et al. analyzed physiological changes after long term exposure, and found fungi to be immensely defiant to alterations caused by the conditions in space stations.
It has since been postulated that mammals under exposure to microgravity seem to have an altered susceptibility to infectious diseases (Boonyaratanakornkit et al. 2005, Wilson et al. 2007) increasing the interest in changes occurring in the genome, proteome and ultimately the pathogenity of microorganisms.
Different approaches and different microorganisms have already produced a variety of data on the subject. Somehow, fungi have as of yet been a somewhat neglected target in this field of research, since most studies have been conducted on bacteria (Horneck et al 2007, Fang et al, 1997) and yeast (Walther et al, 1996, Sheenan et al. 2007), the available data seem to indicate a direct influence of microgravity on different cellular processes such as protein expression in the respective organisms.
Due to the high cost of space travel, experiments are usually carried out in simulated microgravity, creating results of uncertain accuracy.

Different studies, using DNA microarrays, suggest that simulated microgravity may actually change the virulence of bacteria including *Salmonella sp* (Wilson et al. 2002), *Escherichia coli*, *S. typhimurium* (Chopra et al. 2006).
In an experiment on the influence of simulated microgravity on the yeast *Saccharomyces cerevisiae*, Sheehan et al. showed that the expressions of one third of the normalized and filtered genes were changed, compared to growth under a stable gravitational vector after up to 25 generations. 74% of those genes were not associated with normal stress response suggesting a distinctive low gravity response (Sheehan et al. 2007).
In one remarkable study, Yamazaki et al, proved that simulated microgravity, in a three dimensional clinostat, produced no change in morphology, asexual reproductive capability, or susceptibility to anti fungal agents in strains of the pathogenic fungi *Aspergillus niger* and *Candida albicans* (Yamakazi et al, 2012). The results of a paper published by Gomoiu

et al. (Gomoiu et al. 2013) on the other hand, showed significant changes in morphology of the same samples of *Ulocladium chartarum* used also in this experiment, to some extends contradicting the result of Yamazaki et al. Although it has been shown that gravitropism, the effect of the gravity vector, has a strong influence on the morphology of fungi, little is known about their genomic and proteomic changes resulting from real, orbital microgravity. For further reading, a review paper on the development of the research in microgravity conditions on microorganisms by Nickerson et al. can be highly recommended (Nickerson et al 2006)

According to the official sources (CFS-A, communication with Professor Ioana Gomoiu) the aim of the study of this thesis was as follows: " Objective is to determine the effect of microgravity and cosmic radiation on the growth and survival of coloured fungi species. The proposal "Growth and survival of colored fungi in space " is based on experiments both on the Earth and in Space inside of microcapsules made by experts from the Romanian Institute of Space Science. The fungal species chosen for experiments belong to 6 genera selected as organic material decomposers, possible contaminants of materials destined for interplanetary travel, aggressive biodeteriogens of artworks and wooden buildings. Those, due to their melanin content, are protected against UV rays".
Analysis of the environment inside the International Space Station showed *Aspergillus sp*, *Penicillium sp* and *Cladosporium sp* as the most dominant genera in the fungal population (Novikova et al. 2006), explaining the genera chosen as samples for this experiment. The fourth species, *Basipetospora halophila* is a halophilic xerophile,associated with food spoilage on dried fish (Wheeler et al. 1988), possibly affecting food conservation in space travel.
Two different sets of samples were provided. The fist set, from the so called 'short term' experiment consisted of Flight and Ground samples, where the Flight samples were exposed to the above mentioned conditions, and the Ground samples were used as a reference The second set of samples from the so called ' long term ' experiment consisted of Flight, Ground and reference samples. The reference samples were obtained from the reference strains of a strain collection. The exact growth time in microgravity conditions have not been relayed by the Romanian Institute for Space Science. The experiments carried out by the ACBR Laboratory were merely targeting the partial objective of exposure to microgravity conditions. The cultures obtained from the microgravity experiment were subjected to genomic analysis via AFLP (Amplification of Fragment Length Polymorphisms) to reveal any stable genomic alteration that may have occurred. In a further stage of the experiment, proteomic analysis via 2D gel electrophoresis was carried out, in order to highlight changes in protein expression patterns.

The analysis of the short term experiment was started on the 1st of September 2011. In the ACBR Laboratory, Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna.

The analysis of the long term experiment was started on the 1st of November 2011. In the ACBR Laboratory, Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna.

Chapter 2

Materials and Methods

2.1 Samples

2.1.1 Samples of the short term experiment

For the CFS-A short term experiment, the following species were subjected to analysis:
Aspergillus niger
Ulocladium chartarum
Cladosporium herbarum
Basipetospora halophila

From each species, two different samples were provided as living cultures on agar plates:

Flight	Samples were subjected to microgravity conditions.
Ground	Samples were not subjected to microgravity conditions.

The samples, subjected to microgravity conditions (Flight), were exposed to microgravity for a designated period of time. The first three genera were grown on 2% malt extract agar for 7 days before DNA extraction. The *Basipetospora* colonies had to be cultivated on malt extract agar with an addition of 4% NaCl for 14 days to obtain sufficient biomass.

Aspergillus niger	*Basipetospora halophila*
Ground Sample	Ground Sample
Flight Sample 1	Flight Sample 1
Flight Sample 2	Flight Sample 2
Flight Sample 3	Flight Sample 3, ('Sector' from Flight Sample 2)
	Flight Sample 4
Cladosporium herbarum	*Ulocladium chartarum*
Ground Sample	Ground Sample
Flight Sample 1	Flight Sample 1
Flight Sample 2	Flight Sample 2
Flight Sample 3	Flight Sample 3,

Table 2.1: Table 1: Sample list shot term experiment.

2.1.2 Samples of the long term experiment

From each species, at least three different samples were provided:

Flight	Sample was subjected to microgravity conditions
Ground	Sample was not subjected to microgravity conditions
Reference	Sample of the same strain from a strain collection.

The samples, subjected to microgravity conditions (Flight), were exposed to microgravity for 14 days. The first three genera were grown on 2% malt extract agar for 7 days before DNA extraction. The Basipetospora colonies had to be cultivated on malt extract agar with an addition of 4% NaCl for 14 days to obtain sufficient biomass.
11 Additional ulocladia samples were provided, subjected to different exposure conditions.

Aspergillus niger	*Basipetospora halophila*	*Cladosporium herbarum*	*Ulocladium chartarum*
Ground	Ground	Ground	Ground
Flight	Flight	Flight	Flight
Reference	Reference	Reference	Reference

Additional *Ulocladium chartarum* samples		
Biocontainer	Microcapsule	Nr. Of isolate
1	4	4
1	4	5
2	3	2
2	3	3
2	3	4
2	3	7
3	3	1
3	3	2
3	3	3
3	3	4
3	3	4 BLACK

Table 2.2: Table 2: Sample list long term experiment.

Resulting in a total of 23 Samples. 3 *Aspergillus niger*, 3 *Basipetospora halophila*, 3 *Cladosporium herbarum* and 14 *Ulocladium chartarum* samples.

2.2 Methods

2.2.1 DNA extraction

DNA extraction from the obtained fungi cultures was carried out via a two phase extraction from CTAB buffer versus RotiÂő Phenol Chloroform. Cell mass (1cm2 of mycelium) was added to a sample tube containing glass beads and CTAB buffer. After mechanical cell destruction, using a rybolyser, Roti©Phenol/chloroform was added. DNA remains eluted in the CTAB buffer and was subsequently extracted into Ethanol, precipitated, dried, and eluted in water. This was done according to the SOP provided by the ACBR Laboratory.

Materials	
Glass beads	
Ethanol	96%
Water	(HQ- H2O)
CTAB Buffer:	
Roti©	Phenol/chloroform (Phenol/chlorophorm/Isoamyl alcohol by ROTH)
Reagents	
1,2 g	TRIS- HCl
0,81g	EDTA x 2H2O
8,2g	NaCl
2,0g	CTAB
0,2g	Mercaptoethanol

Table 2.3: Extraction materials and reagents.

2.2.2 RAPD / PCR fingerprinting

DNA Extracts were first identified via RAPD analysis as described by Williams JG et al. (1990), according to Hong et al. (2005) the primers PERF and URP1 were used to determine successful extraction and purity of the extracted DNA. The RAPD PCR was carried out according to an SOP provided by the ACBR Laboratory based on the publication of Hong et al. (2005). The reaction is carried out, using a 50µl volume per strain.

Reagents	Amount
Bidest. H2O	30,3 µl
Taq buffer	5,0µl
MgCl2	6,0µl
dNTPs	1,5µl
BSA	0,8µl
Primer	4,0µl
Taq Polymerase	0,4µl

Table 2.4: RAPD / PCR reagents.

The thermo cycler-Program used was composed in the following temperature/time schedule:

95 °C	4min	
95°C	1min	»
55°C	1min	35 repeats
72°C	1min	«
72°C	8min	

Agarose gel electrophoresis

Detection of the results is carried out via agarose gelelectrophoresis.

Component	
Thermocycler:	PTC 150 Minicycler
Bidest. H2O	sterile
10Taq-Buffer + KCl	(Fermentas, Nr. B38)
MgCl2	25 mM
NTPs	5 mM
BSA	10 mg/ml
Taq- Polymerase	5Uµl (Biotherm Nr. 3137)
Primer	10 pM/µl
Primers used:	
PERF	(5'-ATA TCA TCG AAG CCG C-3')
URP1	(5'-ATC CAA GGT CCG AGA CAA CC-3')

Table 2.5: RAPD / PCR materials.

2.2.3 AFLP genotyping

AFLP Analysis was carried out to determine possible changes in the genomic structure. As shown e.g. by Vos et al. (Vos et al. 1995), AFLP is a method used to determine detailed fingerprints of genomic DNA. The method is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA, resulting in a specific band pattern. The reactions were carried out in four steps: (1) A restriction digest of the DNA followed by ligation with oligonucleotide adapters, (2) A preselective amplification, to purify the desired DNA regions and eventually improve the quality of the analysis, (3) A selective amplification of the adaptor regions and the contained DNA segments and (4) A gel or capillary analysis of the amplified fragments. In this study a 50cm capillary in an ABI 3130 genetic analyser (Applied Biosystems) was used. Detection was enabled by the use of fluorescent NED dye linked to the Eco RI adaptor during selective amplification.

Restriction Ligation

Two Master Mixes are created:
Adaptor Master Mix

Component	Volume for 1 reaction	Volume for 20 reactions
EcoRI Adaptor Pair	1µl	20µl
MseI Adaptor Pair	1µl	20µl

Table 2.6: Adaptor pair equivalents

Enzyme Master Mix

Component	Stock Solution	Final Concentration	Volume (µl) for 50µl
T4 DNA ligase buffer + ATP	10x	1x	5
NaCl	0,5M	0,05M	5
MseI	10U/µl	1U/µl	5
EcoRI	100U/µl	5U/µl	2.5
T4 DNA ligase	400U/µl	5U/µl	0.7
bdH2O			31.8

Table 2.7: Enzyme Master Mix

Component	Volume for 1 reaction
10x T4DNA ligase buffer + ATP	1µl
0,5M NaCl	1µl
1mg/ml BSA	0.5µl
MseI adaptor	1µl
EcoRI adaptor	1µl
Enzyme Master Mix	1µl
dd H2O	

Table 2.8: Ligation Mix

For the final restriction ligation Mix the following components are combined:

Restriction ligation Mix 5.5µl
DNA 5.5µl (50-70ng total DNA)

Incubate at 37°C for 2 hours. Then dilute with 10µl TE buffer. (Can be stored at +4°C for up to one month, or at -20°C for a longer period of time.)

Pre-selective Amplification

Pre-selective amplification Mix:

Component	Volume for 1 reaction
EcoRI- preselective primer (core sequence)	0,25µl
MseI- preselective primer (core sequence)	0,25µl
Amplification core mix	7,5µl
DNA (restriction ligated)	2,0µl

Table 2.9: Pre-selective amplification Mix

The corresponding PCR cycle is programmed as follows.

72°C	2min	
94°C	20sec	«
56°C	30sec	19 repeats
72°C	2min	»
8°C	–	

The pre-selective amplification product (10µl) should be diluted with 10µl TE buffer.
Selective Amplification

Component	Volume for 1 reaction
dye labeled EcoRI Primer	0,5µl
MseI- Primer	0,5µl
Amplification core mix	7,5µl
DNA (preselective amp)	1,5µl

Table 2.10: Selective amplification Mix

PCR Cycle for selective amplification.

94°C	2min	
94°C	20sec	«
66°C	30sec (reduced by 1°C per cycle)	9 repeats
72°C	2min	»
94°C	20sec	«
56°C	30sec	19 repeats
72°C	2min	»
60°C	30minutes	
8°C	–	

Fragment Analysis

Component	Volume for 1 reaction
Deionized formamide	12µl
GeneScan-500 ROX size standard	0.4µl
DNA Sample	1.2µl

Table 2.11: Fragment analysis Mix

After adding the sample DNA to the Master Mix aliquots, incubate for 3 minutes at 95°C on a thermal cycler. Loading of the resulting samples onto the AB3130 Genemapper is done according to Applied Biosystem Protocol.

Materials

General	
MseI Adaptor	20µM
EcoRI Adaptor	2µM
NaCl	0,5M
10x T4 DNA ligase buffer + ATP	(0,5M Tris-HCl, pH 7.8, 0.1M MgCl2, 0.1M DTT, 0.25mg/ml BSA)
EcoRI restriction endonuclease,	500 U
MseI restriction endonuclease,	100U
T4 DNA ligase,	100Units
BSA,	10 mg/ml
EcoRI presel. primer (core seq) ,	10µM
MseI presel. primer (core seq) ,	10µM
EcoRI selective primer	
MseI selective primer	
Amplification Core Mix	(buffer, nucleotides, Mg ions , AmpliTaq DNA Polymerase)
TE buffer	(20mM TRIS-HCl . 0,1 mM EDTA, pH8,0)
HiDi formamide	
GeneScan- 500 (ROX) size standard	
Nuclease free distilled water	

Thermal Cycler	
AB3130	genetic analyser

Restriction, Ligation	
EcoRI restriction endonuclease	5U µl^1
MseI restriction endonuclease	1U µl^1
T4 DNA Ligase	5U µl^1

Adaptor pairs
EcoRI- F (5‘ -CTC GTA GAC TGC GTA CC-3‘)
EcoRI- R (5‘ -AAT TGG TAC GCA GTC TAC-3)
MseI- F (5‘ -GAC GAT GAG TCC TGA G-3‘)
MseI- R (5‘ -TAC TCA GGA CTC AT -3‘)

Preselective Amplification
Eco RI preselective Primer (5‘ -GAC TGC GTA CCA ATT C- 3‘)
MseI preselective Primer (5‘ -GAT GAG TCC TGA GTA A- 3‘)

Selective Amplification
Eco RI selective Primer (5‘NED - GAC TGC GTA CCA ATT CC- 3‘)
MseI selective Primer (5‘GAT GAG TCC TGA GTA ACA- 3‘)

Fragment analysis
GenScan- 500 [ROX] size standard
Sequencer: AB 3130 genetic analyser (Applied Biosystems)

2.2.4 Protein 2D gel electrophoresis

The necessary biomass for the protein pattern analysis was created by cultivation of the strains in 2% Malt extract broth. The incubation was carried out at room temperature. Growth times were adjusted to meet the necessary quantities of 400 mg fresh biomass per sample. Following the harvest, the biomass was washed with 0.9% sodium chloride and stored at -80°C until protein extraction.

Species	Growth duration to harvest
Aspergillus niger	168 +- 2 hours
Ulocladium chartarum	168 +- 3 hours
Cladosporium herbarum	240 +- 2 hours
Basipetospora halophila	504 +- 3 hours

Table 2.12: Growth time of the different Fungi species

Protein extraction

Cell disruption was performed by a mechanical method using a lysis buffer and shear force obtained by glass beads and a rotary shaker. The precipitation method was based on phenol precipitation and involved the following steps (Isola et al. 2011): 3 ml of TRIS-buffered phenol solution of pH 8.0 (Sigma-Aldrich, Steinheim, Germany) were added to every individual sample after the mechanical disruption. Centrifugation at 7830g for 10 minutes at 4°C (phenol is volatile) was performed, in order to seperate the organic phenol phase from the aquatic phase. The phenolic phase was transferred to a new, weighted tube. 5 volumes of 0.1 M ammonium acetate were added to the phenol phase, and after inversion precipitation was carried out at -20°C over night. The protein pellet was retained via centrifugation at 7830g for 30 minutes at 4°C. This procedure was followed by a washing step with methanol and another washing step with cold acetone (80% v/v). Then the protein pellet was dried and resolved in Modified Sample Buffer (MSB) according to the final pellet weight as described by Chandler and colleages (Chandler et al. 2008) The protein concentrations were determined using the BioRad protein assay (BioRad Lab., Hartfordshire, USA). The standardisation curve was created using bovine serum albumin (Thermo Scientific, Rockford, IL, USA) in concentrations ranging from 0.8 µg/ml to 100 µg/ml

2-D gel electrophoresis

For each obtained sample two technical replicates were created. The 13 cm pH gradient strips (IPG TM DryStrip 3-10 NL, GE Healthcare Bio- Sciences AB, Uppsala, Sweden) were rehydrated in 255 µl buffer for 16 hours at room temperature and loaded with 20 µg of protein, eluated in rehydration buffer [8 M urea, 2% (w/v) CHAPS, 10 mM dithiotreitol (DTT), 0.1% bromophenol blue and 0,5% (v/v) Servalyte]. Isoeletric focusing was carried out according to manufacturer instructions at 20°C and a total of 14 kVh, using a Protean IEF cell system (Bio-Rad Hartfordshire, USA). The strips were re-equilibrated for 15 min under gentle shaking in 2 ml equilibration solution [50 mM Tris-HCl pH = 8.4, 6 M urea,

30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS)], with 2% (w/v) DTT and for subsequent coloring, again for 15 min in 2 ml equilibration solution [50 mM Tris-HCl pH = 6.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS], with 2,5% (w/v) iodoacetamide (IAA) and trace of bromophenol blue (Bjellqvist et al. 1993)]. To obtain the second dimenstion, a 10% (w/v) SDS polyacrylamide gel electrophoresis (SDS-PAGE, 14 cm * 14 cm) with running buffer [24 mM Tris pH = 8.3, 192 mM glycine, 0.1% (w/v) SDS] was used. For the electrophoretic run 160 V and variable mA were applied using the Perfect Blue Twin Gel System (PeqLab GmbH, Erlangen, Germany). The chamber was cooled to about 8°C (type CBN 8-30, Heto, Birkerod, Denmark) using a water cooling system.

Analysis of 2-D gels

The protein spots were visualized, using a high sensitive mass spectrometric compatible silver staining technique(Shevchenko et al. 1996). The gels were fixed in 50% (v/v) methanol and 5% (v/v) acetic acid for 20 minutes, then washed in 50% (v/v) methanol for 10 minutes and rinsed with MilliQ water (Millipore, MA, USA) overnight at +4°C. Subsequently, the 2-D gels were sensitized for staining, by applying a 0.02% (w/v) sodium thiosulfate solution for 1 minute and then incubated in 0.1% (w/v) silver nitrate solution for 20 min at +4°C. While rinsing twice with MilliQ water for 1 minute each time after incubation. The gel development was carried out by the incubation in a 0.04% (v/v) formalin and 2% (w/v) sodium carbonate solution until the desired intensity of staining was achieved. The gels were washed with a 5% (v/v) acetic acid. Stained gels were scanned with a Scanner in TIFF 16 bit format. Image Master 2D Platinum version 5.0 (Amersham Biosciences, Swiss Institute of Bioinformatics, Geneva, Switzerland) was used for spot-matching and image evaluation. All washing solutions used were prepared in MilliQ water (Millipore, MA, USA).

Chapter 3

Results

3.1 AFLP results

3.1.1 Short term experiment

The results of the AFLP analysis are documented as characteristic band patterns, as shown in Fig. 3.1 and further analysed for identical and different bands (referred to as homology value) as shown in Table 3.1.

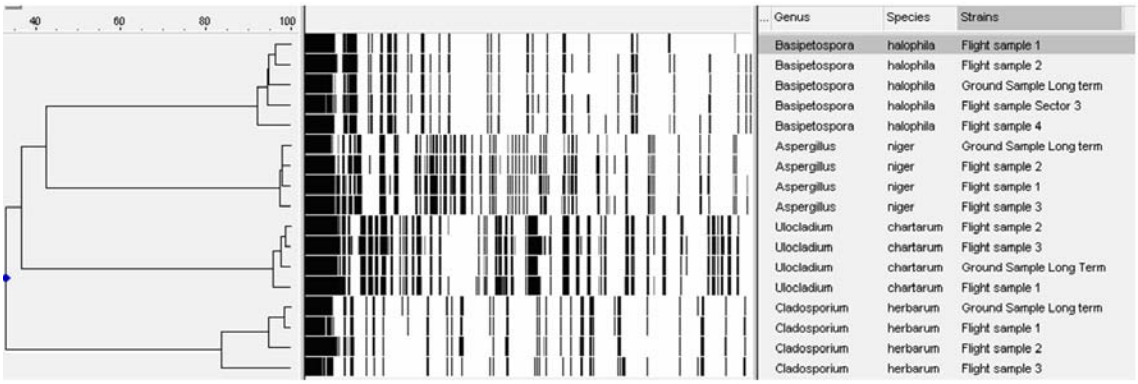

Figure 3.1: Comparison of AFLP Data short term experiment

The correlating homology values from Figure 3.1 are presented in Table 3.1. Table 3.1 shows the correlating homology of the referred samples to the ground sample (100%). The homology value is derived from the overlap of the detected fingerprints. Where 100% represents total overlap, and therefore identical samples.
Due to a lack of comparable data, a homology rating of below 96% will be considered relevant for the long term experiment, expecting the colonies to have grown for a longer time independently and therefore acquired a lower base homology than the samples used in the short term experiment. Significant aberrations are highlighted in yellow. A discussion of the results is presented in chapter 4.

Aspergillus niger		*Basipetospora halophila*	
Sample	% homology	Sample	% homology
Ground	100,0	Ground	100,0
Flight Sample 1	98,0	Flight Sample 1	93,9
Flight Sample 2	98,1	Flight Sample 2	96,1
Flight Sample 3	97,5	Flight Sample 3 Sector from 2	94,0
		Flight Sample 4	94,5
Cladosporium herbarum		*Ulocladium chartarum*	
Sample	% homology	Sample	% homology
Ground	100,0	Ground	100,0
Flight Sample 1	98,3	Flight Sample 1	96,3
Flight Sample 2	95,2	Flight Sample 2	97,9
Flight Sample 3	83,5	Flight Sample 3	97,7

Table 3.1: Table 1: Comparison of AFLP Data short term experiment

3.1.2 Long term experiment

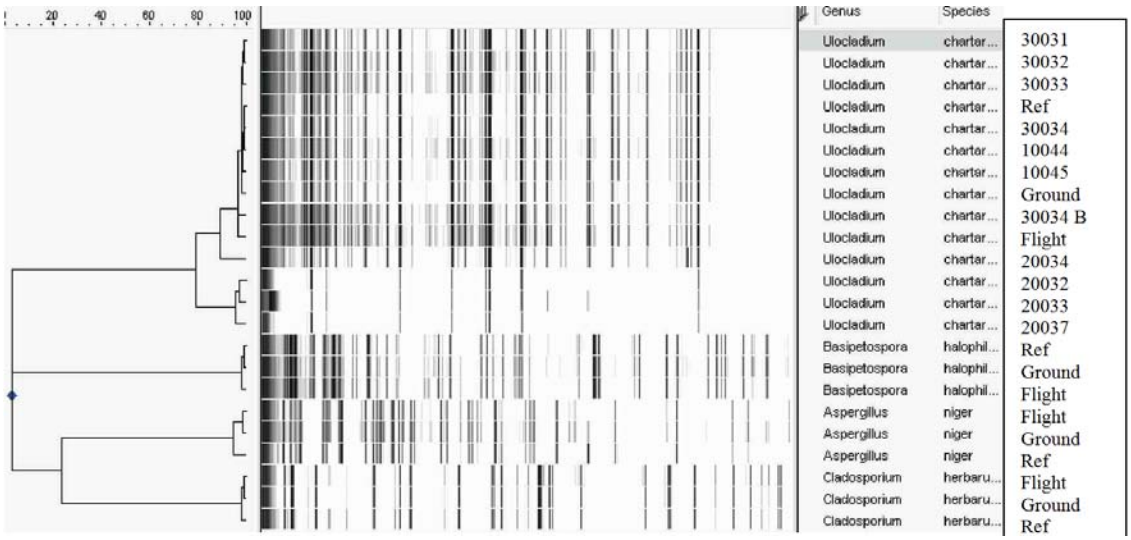

Figure 3.2: Comparison of AFLP Data long term experiment

Figure 3.2. shows the homologies of the different samples, the correlating homology values are presented in table 3.2. Column 1 portrays the correlating homology of the referred samples to the Ground sample (100%). The data in Column 2 compares the same samples to the correlating Reference strain. Due to a lack of comparable data a homology rating of below 98 % will be considered significant (85% of the data points are above or at this level). All of the 2-003-X samples (Biocontainer 2, Microcapsule 3) show a lowered homology towards the ground sample.

Aspergillus niger			
Compared to Ground Sample	% correlation	Compared to Reference Sample	% correlation
Ground	100	Ground 94,4	
Flight	98,7	Flight 95	
Reference	94,4	Reference 100	
Basipetospora halophila			
Compared to Ground Sample	% correlation	Compared to Reference Sample	% correlation
Ground	100	Ground	99,4
Flight	98,5	Flight	98,1
Reference	99	Reference	100
Cladosporium herbarum			
Compared to Ground Sample	% correlation	Compared to Reference Sample	% correlation
Ground	100	Ground	98,7
Flight	99,4	Flight	97,6
Reference	98,7	Reference	100
Ulocladium chartarum			
Compared to Ground Sample	% correlation	Compared to Reference Sample	% correlation
Ground	100,0	Ground	98,3
3-003-1	98,5	3-003-1	98,4
3-003-2	97,9	3-003-2	98,7
3-003-3	98,4	3-003-3	97,9
Reference	98,3	Reference	100,0
3-003-4	98,4	3-003-4	99,4
1-004-4	98,4	1-004-4	99,0
1-004-5	98,5	1-004-5	98,6
3-003-4 BLACK	96,1	3-003-4 BLACK	98,0
flight	94,6	Flight	97,1
2-003-4	91,2	2-003-4	90,5
2-003-2	86,1	2-003-2	80,4
2-003-3	86,8	2-003-3	81,2
2-003-7	81,9	2-003-7	75,4

Table 3.2: Comparison of AFLP Data long term experiment

3.2 2D Protein profiles

3.2.1 Short Term

Aspergillus niger

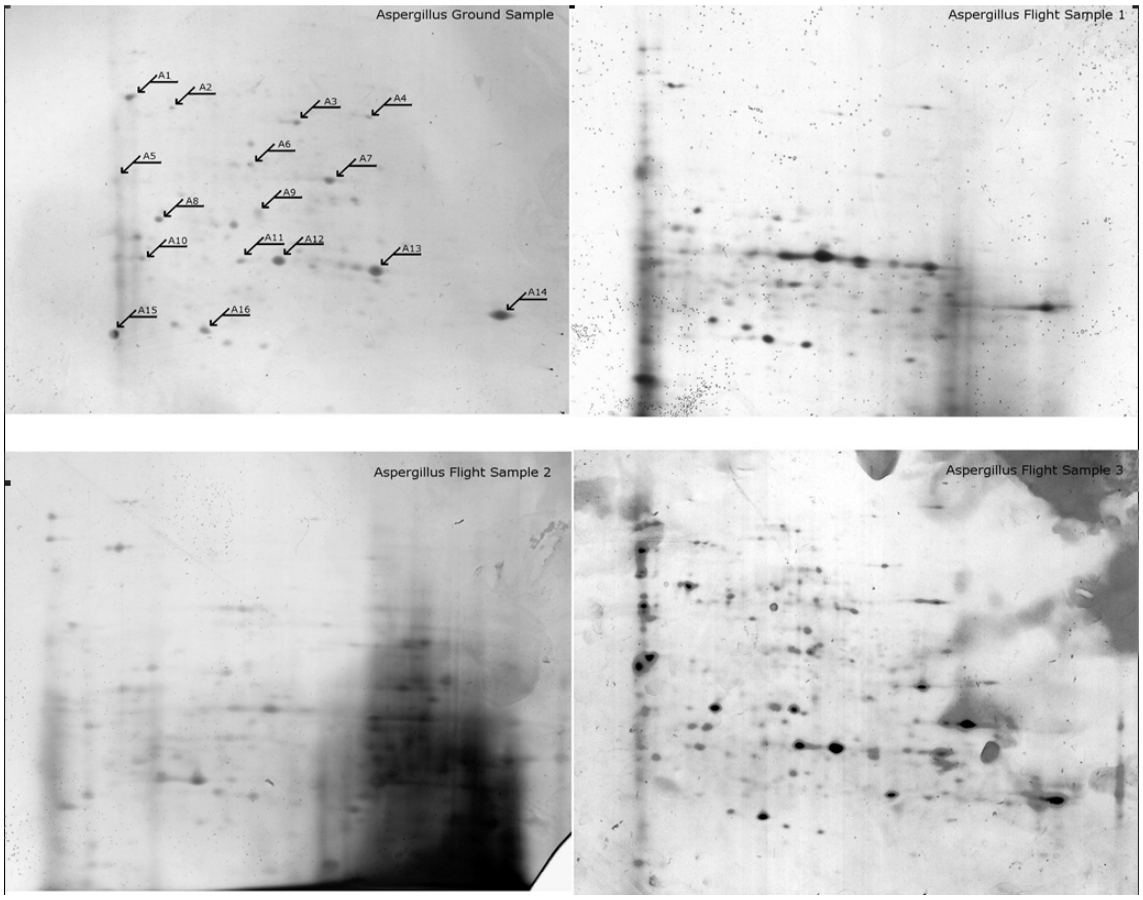

Figure 3.3: 2D gels of *Aspergillus niger* ground and flight samples

A1	Landmark Protein*
A2	Weakly expressed in FS2, otherwise high similarity
A3	Protein showing high similarity
A4	Protein showing high similarity
A5	Protein showing relatively high similarity
A6	Protein showing high similarity, lowered expression in FS 1
A7	Landmark Protein*
A8	Protein showing high similarity, lowered expression in FS 3
A9	Protein showing high similarity
A10	Protein showing high similarity, lowered expression in FS 2
A11	Protein showing high similarity varying slightly in intensity
A12	Protein showing high similarity
A13	Weakly expressed in FS2 and 3, high positioning similarity
A14	Landmark Protein*
A15	Landmark Protein*
A16	Protein showing high similarity, very low expression in FS 2

Table 3.3: *Aspergillus niger* identification Proteins for the short term experiment

*Landmark Proteins are used by the software to evaluate the true position of the overall pattern.

The spots detected on the gels, which represent proteins (or protein clusters) were compared with the associated Ground sample according to their %volume and area. The results are portrayed in the following scatter plots. The slope of the regression line indicates the mean similarity of the spots of the Ground vs. Flight gels. An average of 200 to 300 spots per gel were detected. A representative few, including the documented 16 sample proteins were compared.

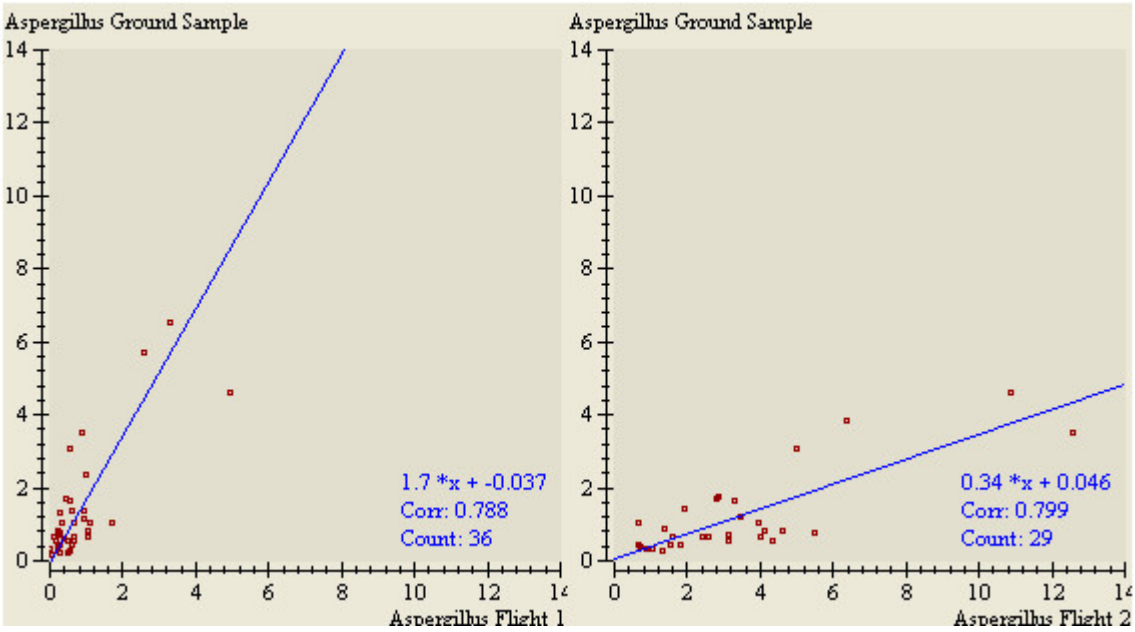

Figure 3.4: Scatter plot of *Aspergillus niger* Ground vs. Flight samples. Comparison by spot %volume

Acceptable correlation factors of near 0.8 indicate acceptable gel comparability. Due to increased sample quantity additional pairs were evaluated.

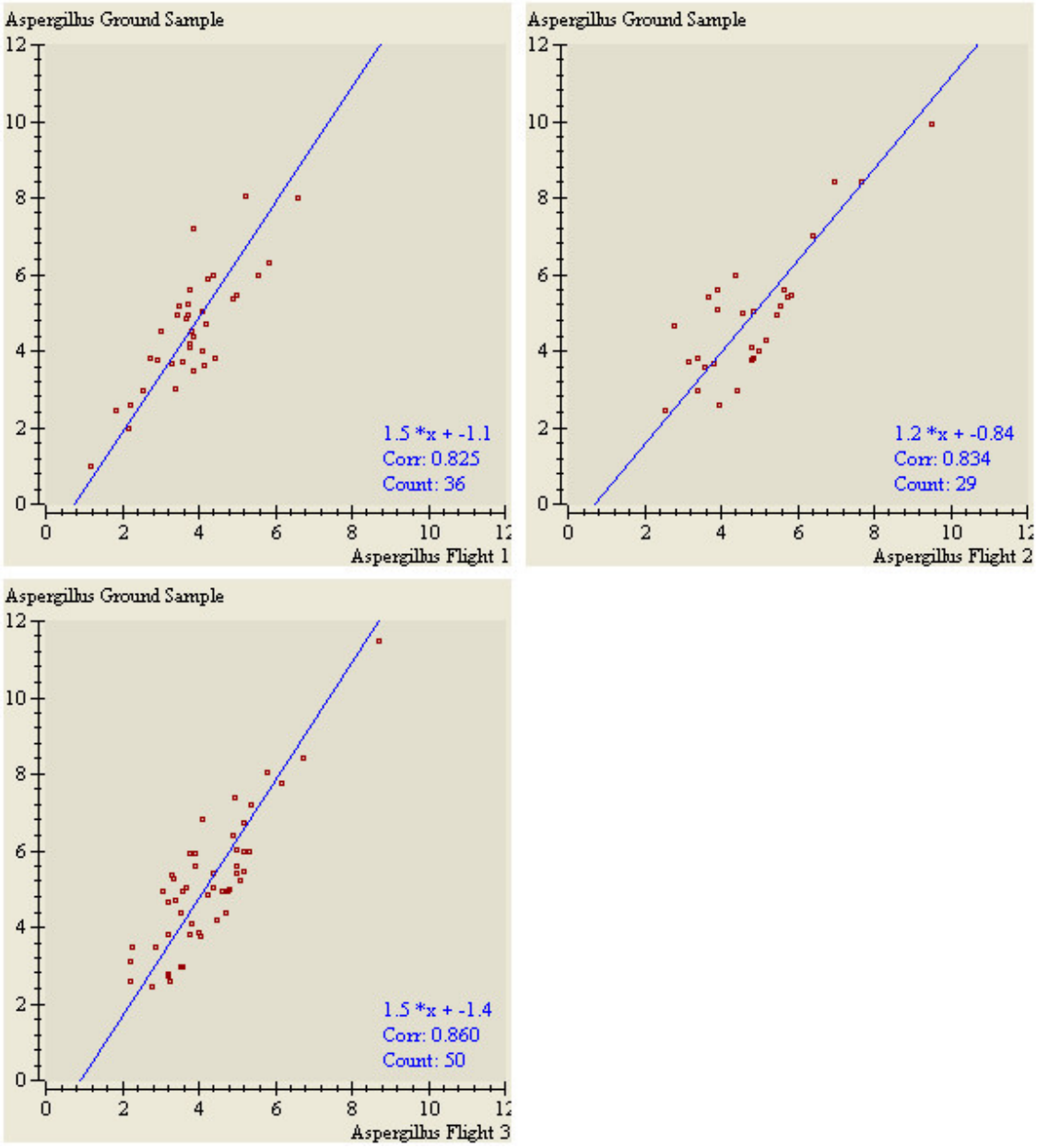

Figure 3.5: Scatter plot of *Aspergillus niger* Ground vs. Flight samples. Comparison by spot area.

The obtained correlation factors indicate acceptable gel comparability. No indication of permanent proteomic changes could be derived from the above analysis. Values obtained are well within the margin of error defined by the detection method.

Basipetospora halophila

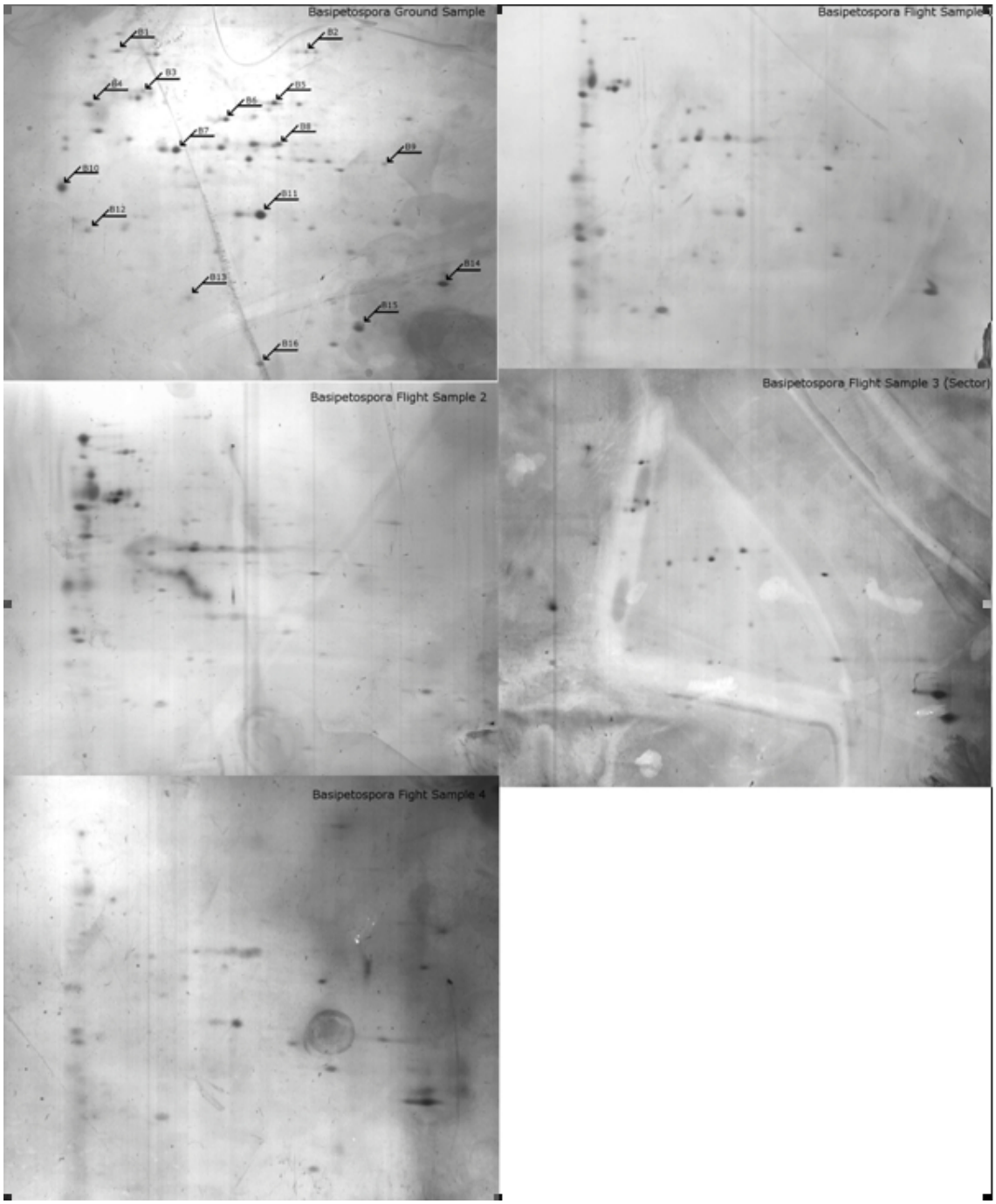

Figure 3.6: 2D Gels of *Basipetospora halophila* ground and flight samples.

B1	Protein showing reduced similarity, lowered expression in FS 4
B2	Protein showing high similarity, reduced expression in FS 3
B3	Protein showing high similarity
B4	Protein showing high similarity, increased expression in FS2, reduced in FS 3
B5	Protein showing acceptable similarity, not detected in FS3
B6	Protein showing reduced similarity, not detected in FS 2 and 3
B7	Landmark Protein*
B8	Protein showing high similarity
B9	Protein showing high similarity, increased expression in FS3 and 4
B10	Landmark Protein*
B11	Landmark Protein*
B12	Protein showing relatively high similarity, reduced expression in FS3
B13	Protein showing high similarity, except FS 4, altered IEF point
B14	Protein showing high similarity
B15	Landmark Protein*
B16	Protein showing high similarity

Table 3.4: *Basipetospora halophila* identification Proteins, short term experiment

*Landmark Proteins are used by the software to evaluate the true position of the overall Pattern

The spots detected on the gels, which represent proteins (or protein clusters) were compared with the associated Ground sample according to their %volume and area. The results are portrayed in the following scatter plots. The slope of the regression line indicates the mean similarity of the spots of the Ground vs. Flight gels.

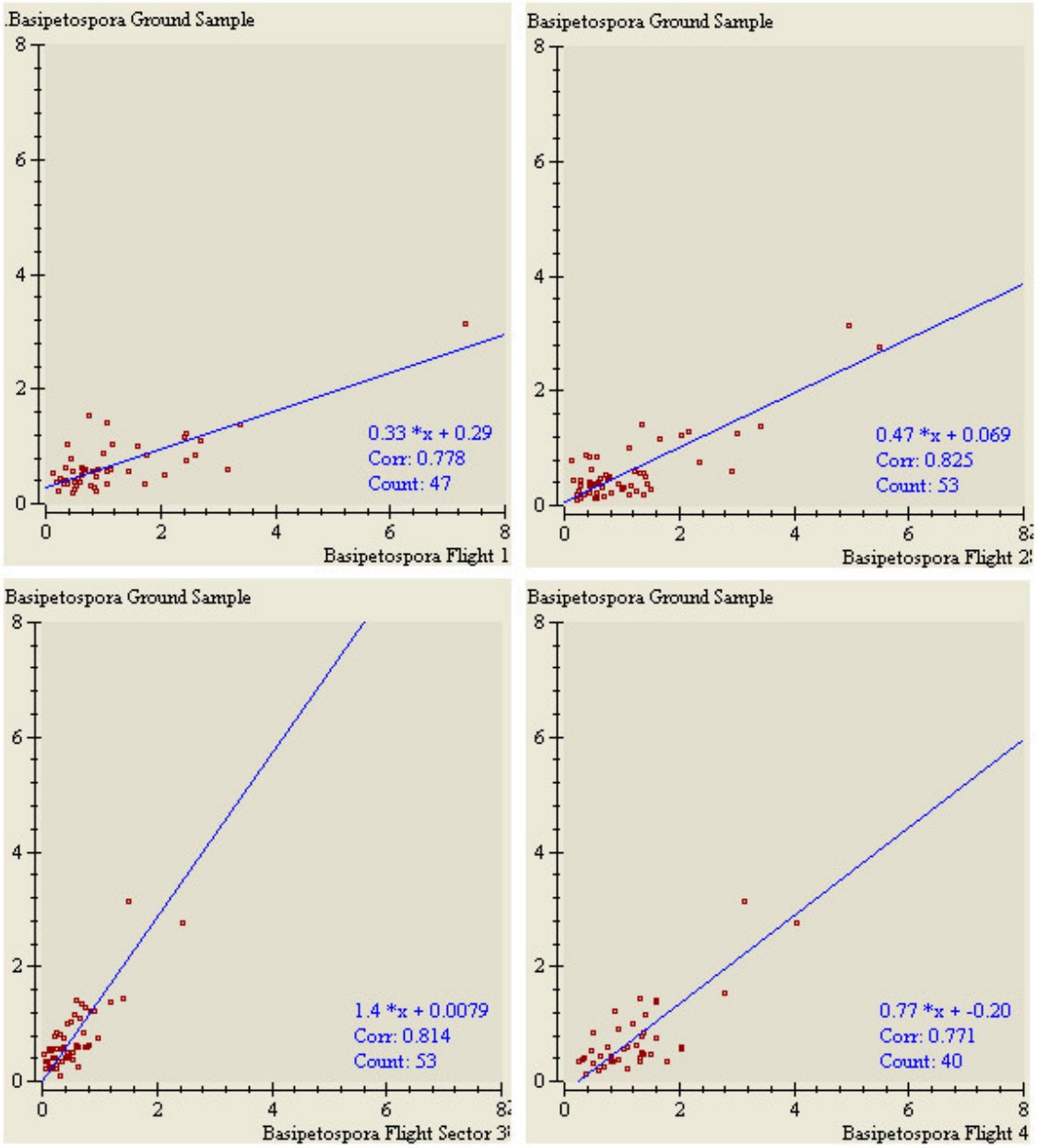

Figure 3.7: Scatter Plot of *Basipetospora halophila* Ground vs. Flight samples. Comparison by %volume

The lowered correlation factor of FS4 and FS1 indicate the presence of aberrations. Coinciding well with the findings of the AFLP analysis.

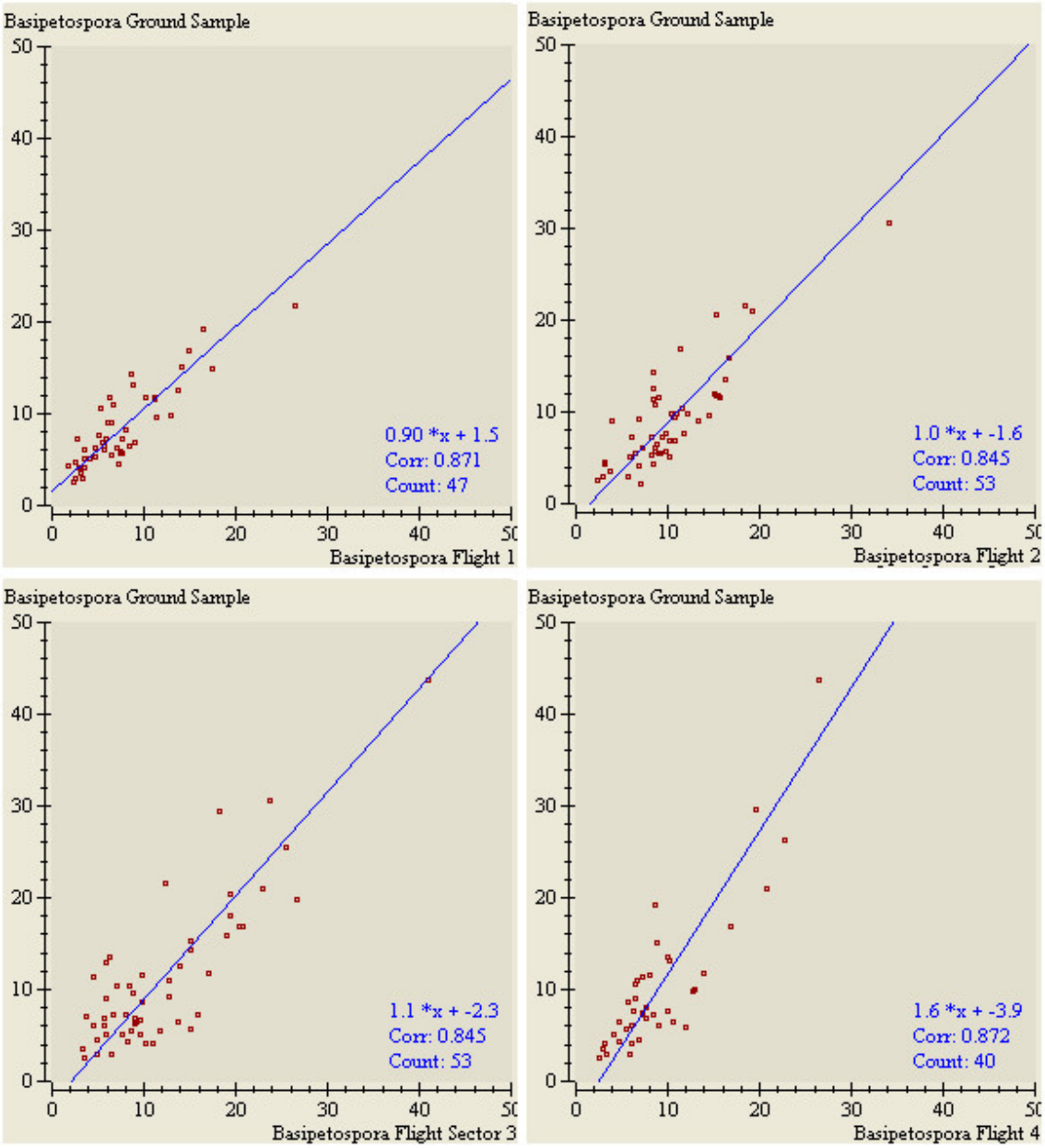

Figure 3.8: Scatter Plot of *Basipetospora halophila* Ground vs. Flight samples. Comparison byarea

No indication of permanent proteomic changes could be derived from the above analysis. Values obtained are, although of lower precision, still within the margin of error given by the detection method.

Cladosporium herbarum

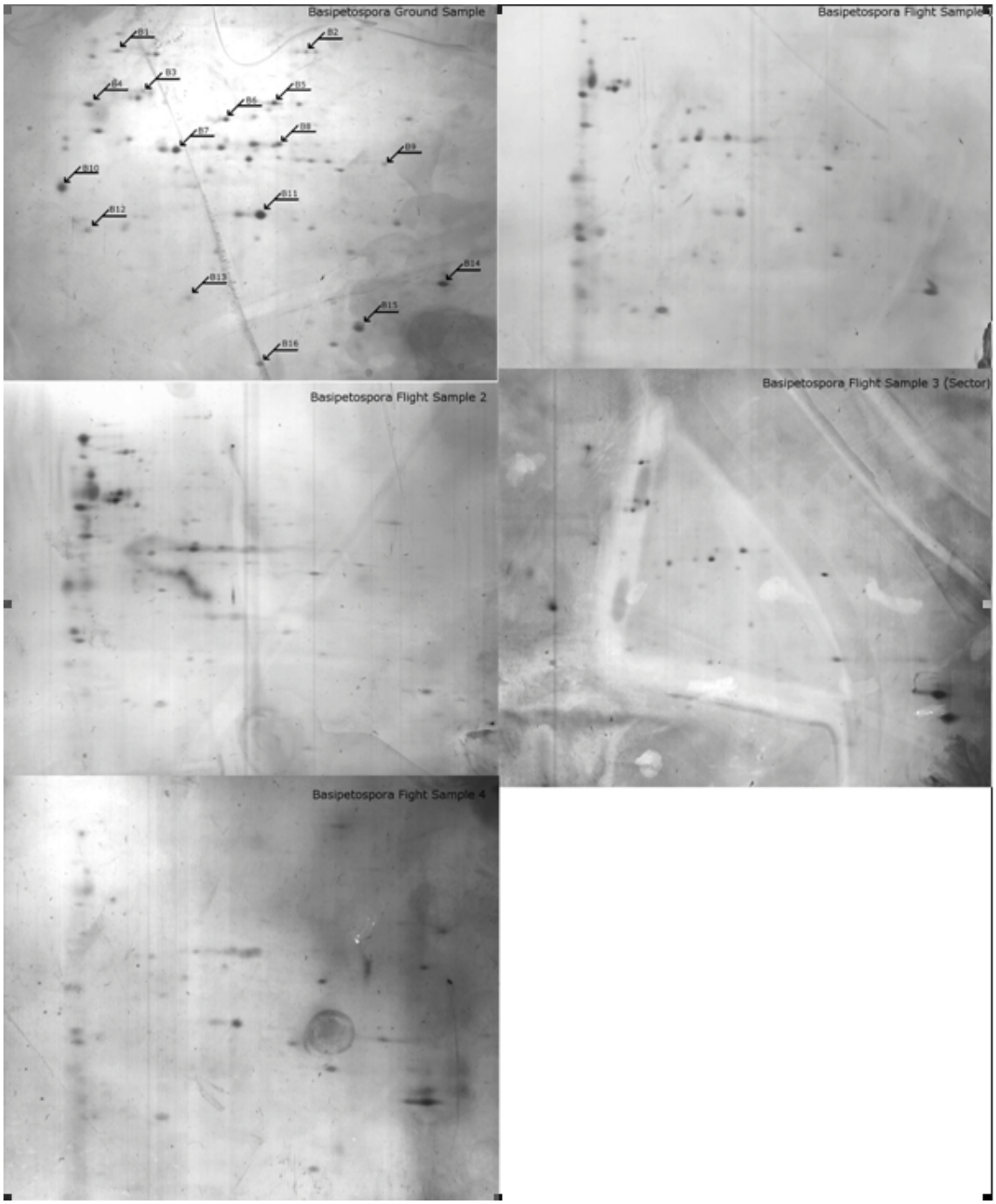

Figure 3.9: 2D Gels of *Cladosporium herbarum* ground and flight samples.

C1	Protein showing high similarity
C2	Protein showing high similarity, missing in FS 1 and FS 2
C3	Protein showing high similarity, changed protein size in FS3
C4	Protein showing acceptable similarity, missing in FS3
C5	Landmark Protein*
C6	Protein showing reduced similarity, changed IEF in FS3, missing in FS1
C7	Protein showing reduced similarity
C8	Protein showing high similarity
C9	Landmark Protein*
C10	Protein showing high similarity
C11	Protein showing high similarity, missing in FS1
C12	Protein showing acceptable similarity
C13	Landmark Protein*
C14	Protein showing high similarity, changed Protein size in FS2
C15	Protein showing reduced similarity, in FS3 spot hidden in artefact
C16	Landmark Protein*

Table 3.5: *Cladosporium herbarum* identification Proteins, short term experiment

*Landmark Proteins are used by the software to evaluate the true position of the overall pattern.

The spots detected on the gels, which represent proteins (or protein cluster) were compared with the associated Ground sample according to their %volume and area. The results are portrayed in the following scatter plots. The slope of the regression line indicates the mean similarity of the spots of the Ground vs. Flight gels.

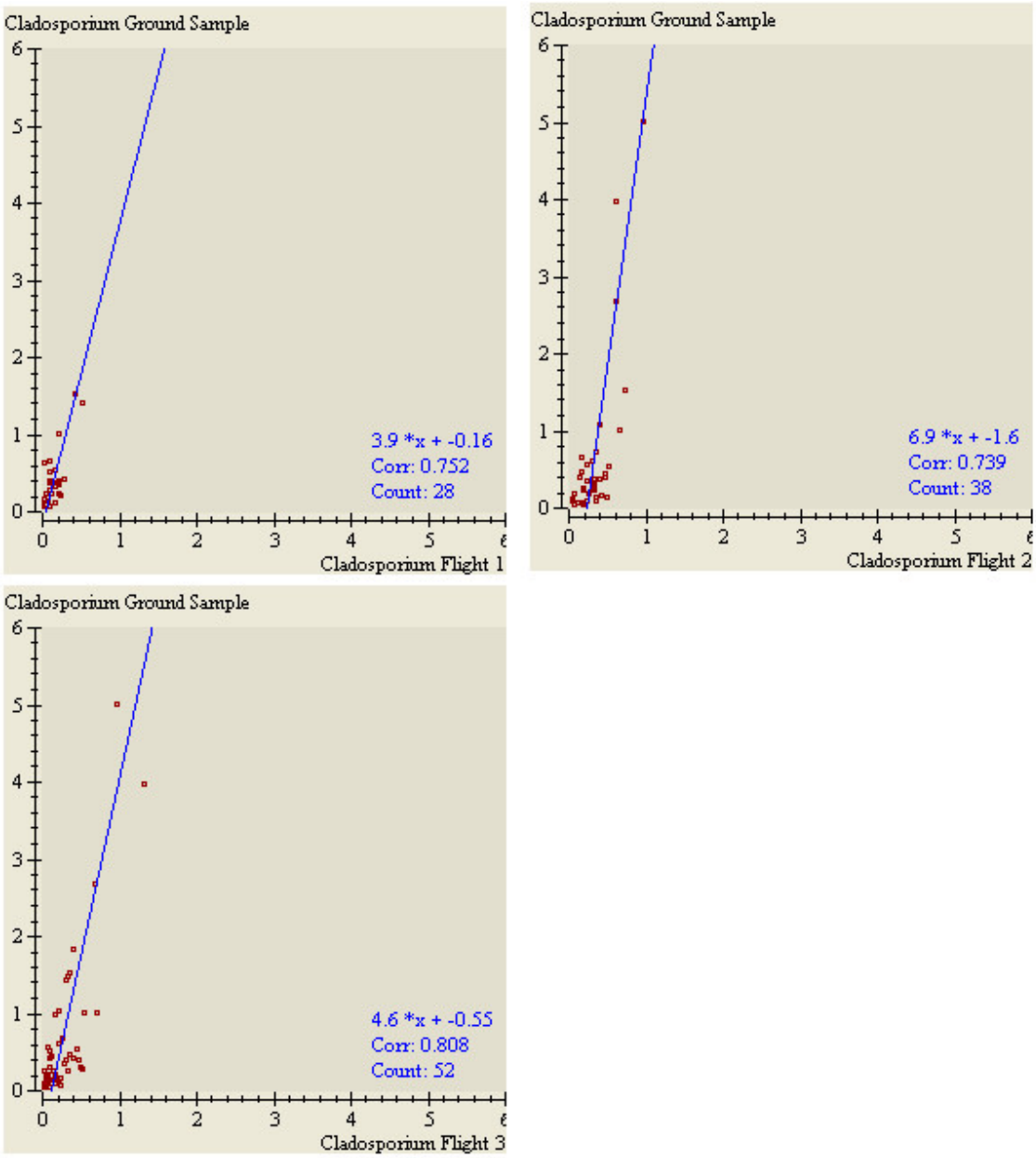

Figure 3.10: Scatter Plot of *Cladosporium herbarum* Ground vs. Flight samples. Comparison by %volume

The obtained correlation factors indicate acceptable Gel comparability. The lowered correlation factor of FS1 and FS2 could derive from weaker protein profile pictures, which is recognized by the software.

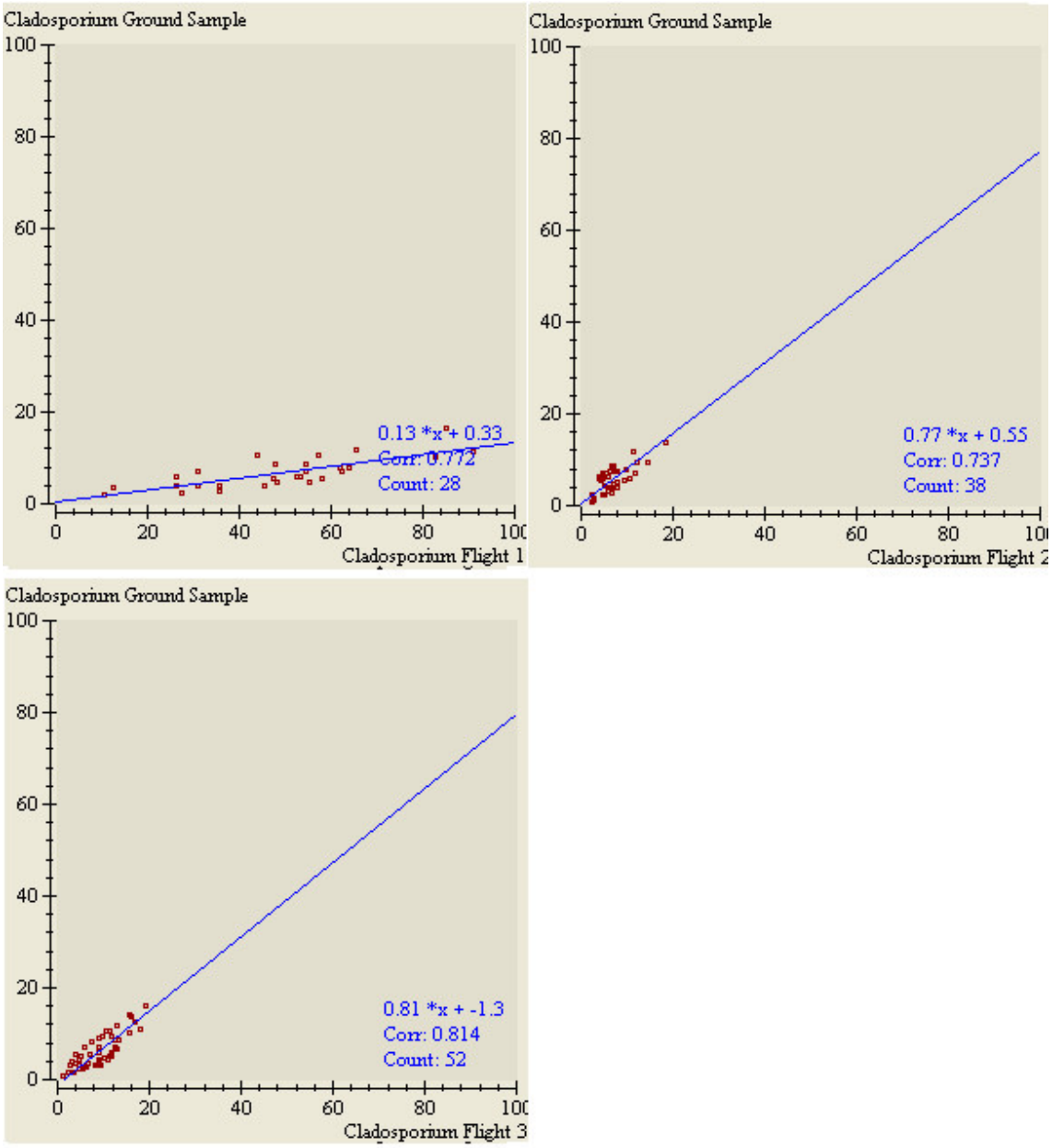

Figure 3.11: Scatter Plot of *Cladosporium herbarum* Ground vs. Flight samples. Comparison by %volume

The obtained correlation factors indicate acceptable Gel comparability. As above, the correlation factors of FS1 and FS2 are lowered because of the weaker gel pictures.

Ulocladium chartarum

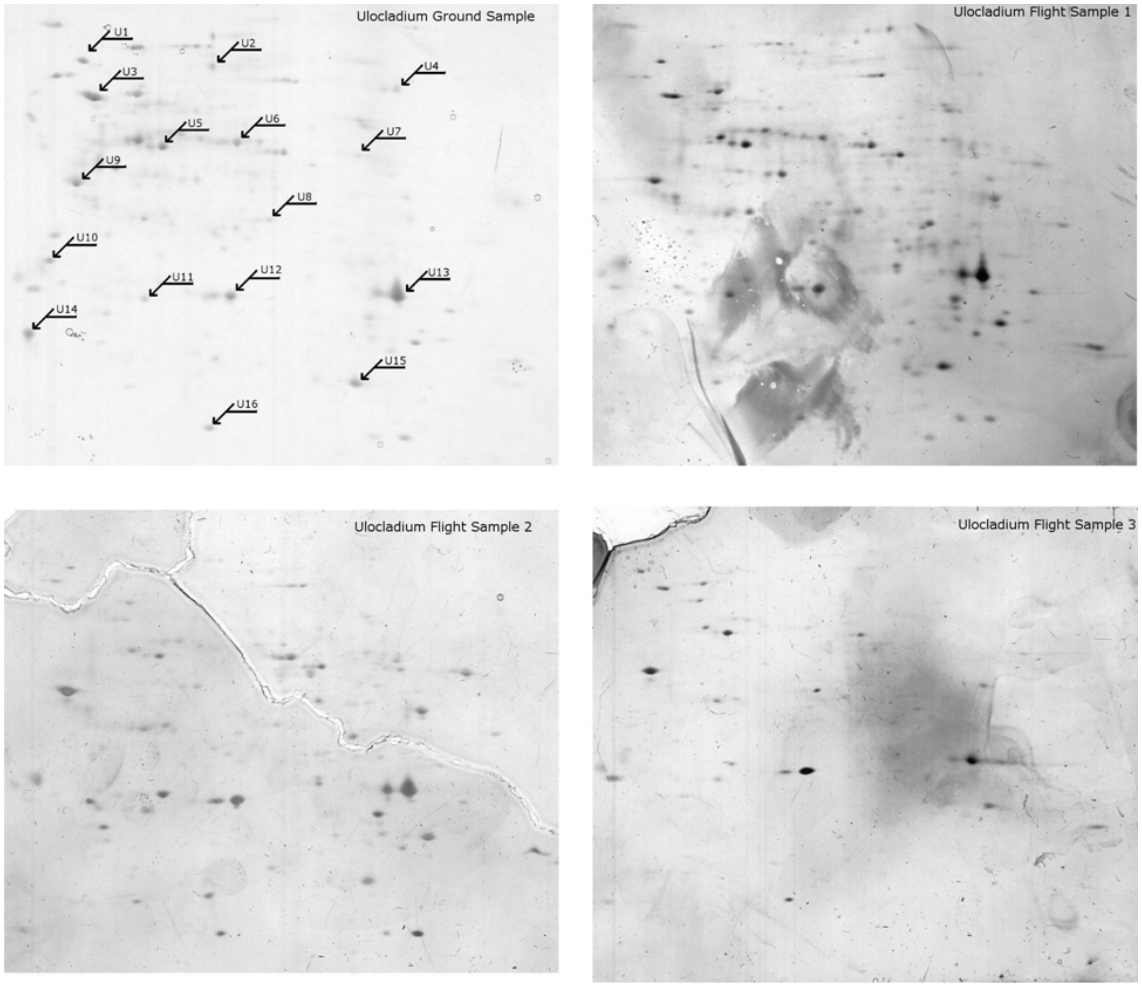

Figure 3.12: 2D Gels of *Ulocladium chartarum* ground and flight samples.

U1	Protein showing high similarity, missing in FS3
U2	Protein showing high similarity,
U3	Landmark Protein*
U4	Protein showing slightly reduced similarity
U5	Protein showing high similarity
U6	Protein showing acceptable similarity, lower expression in FS2 and FS3
U7	Protein showing acceptable similarity
U8	Protein showing high similarity
U9	Protein showing high similarity
U10	Landmark Protein*
U11	Protein showing high similarity, lowered expression in FS 3
U12	Landmark Protein*
U13	Landmark Protein*
U14	Protein showing reduced similarity, missing in FS2
U15	Protein showing high similarity, weakly expressed in FS3
U16	Protein showing acceptable similarity, slightly changed Protein size in FS3

Table 3.6: *Cladosporium herbarum* identification Proteins, short term experiment

*Landmark Proteins are used by the software to evaluate the true position of the overall Pattern

The spots detected on the gels, which represent proteins (or protein clusters) were compared with the associated Ground sample according to their %volume and area. The results are portrayed in the following scatter plots. The slope of the regression line indicates the mean similarity of the spots of the Ground vs. Flight gels.

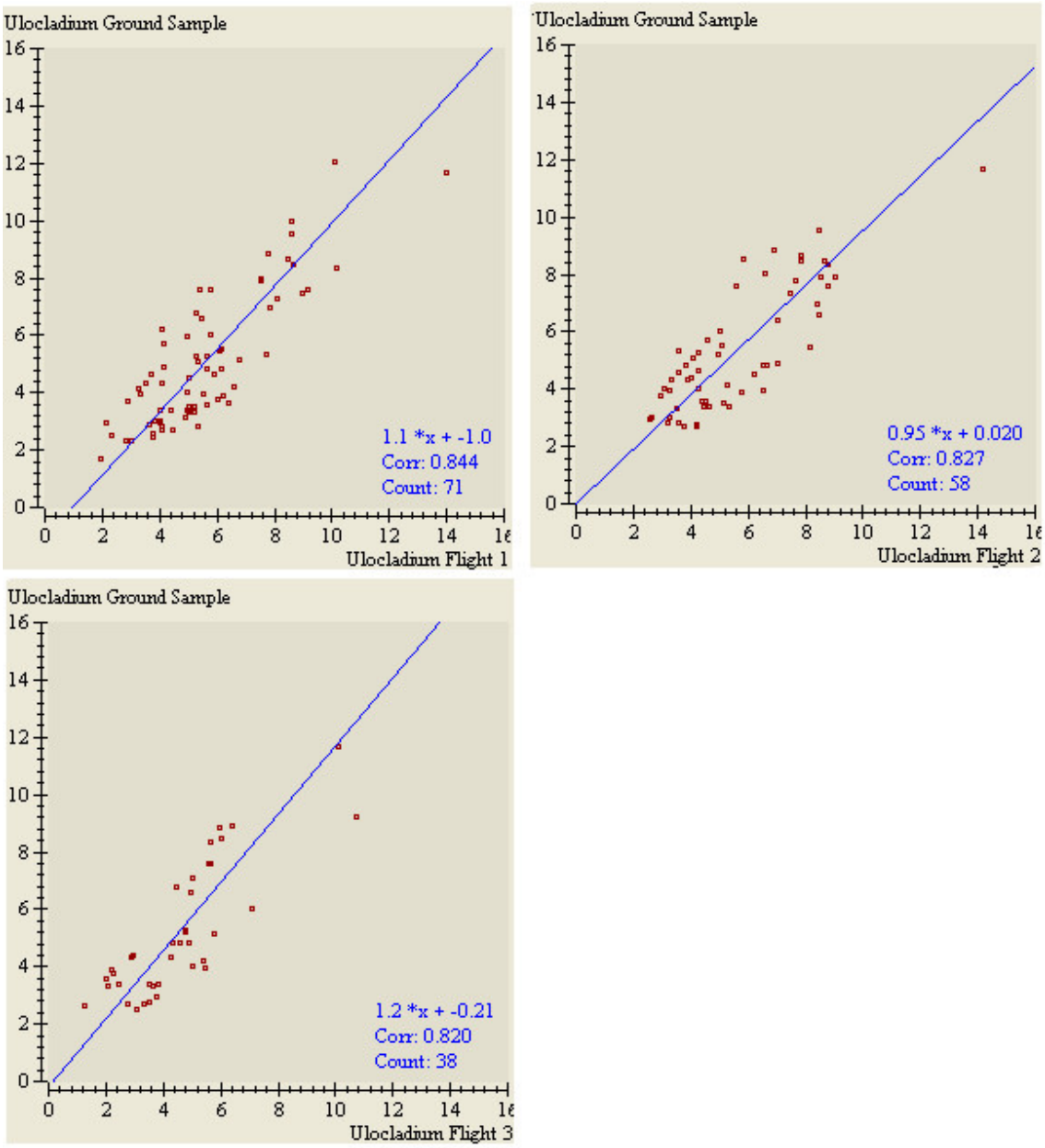

Figure 3.13: Scatter Plot of *Ulocladium chartarum* Ground vs. Flight samples. Comparison by %volume

The obtained correlation factors indicate acceptable gel comparability. Due to few aberrations, the correlation factors are high.

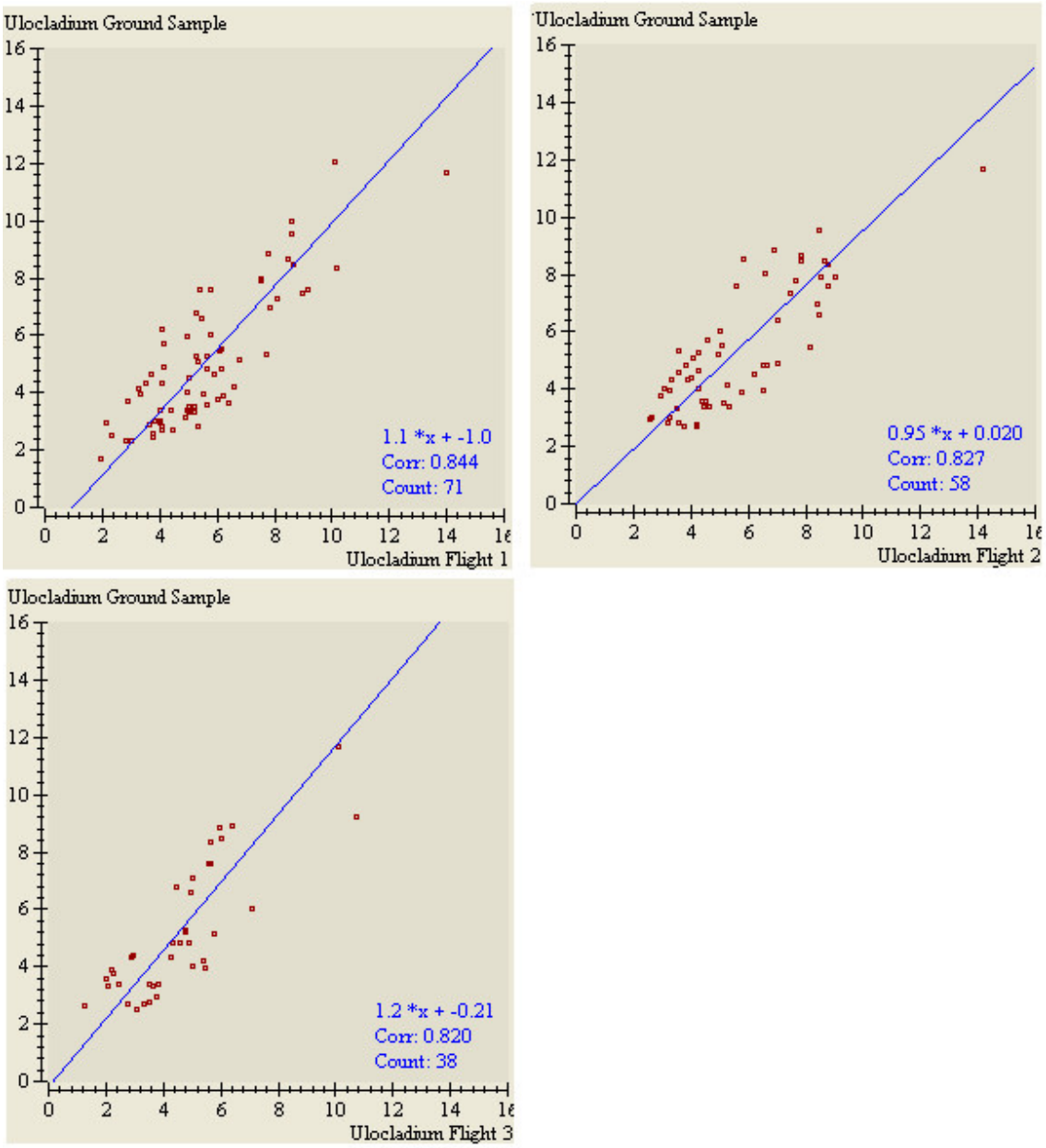

Figure 3.14: Scatter Plot of *Ulocladium chartarum* Ground vs. Flight samples. Comparison by %volume

High correlation values indicate a decent comparability of the gels.

3.2.2 Long Term experiment

***Aspergillus niger* Gound + flight**

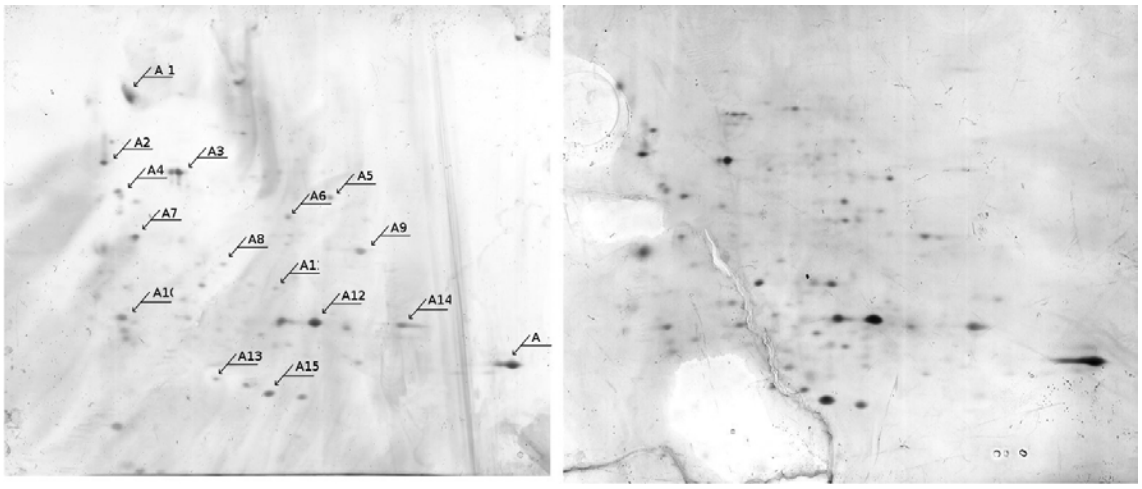

Figure 3.15: 2D Gels of *Aspergillus niger* ground and flight samples.

Selection of Characteristic Proteins and Protein clusters:

A1	Artefakt not a Protein.
A2	Landmark Protein *
A3	Protein showing high similarity
A4	Protein showing reduced similarity (lower molecular weight)
A5	Protein showing high similarity
A6	Protein showing high similarity
A7	Protein showing high similarity
A8	Protein showing high similarity
A9	Protein cluster showing reduced similarity (Protein [right] is underexpressed)
A10	Landmark Protein *
A11	Protein showing reduced similarity (lowered expression, slight change in IEP)
A12	Protein showing high similarity
A13	Protein showing high similarity
A14	Protein cluster showing reduced similarity (change in Protein size and IEP)
A15	Landmark Protein *
A16	Landmark Protein *

Table 3.7: *Aspergillus niger* identification Proteins, short term experiment

* Landmark Proteins are used by the software to evaluate the true Position of the overall Pattern

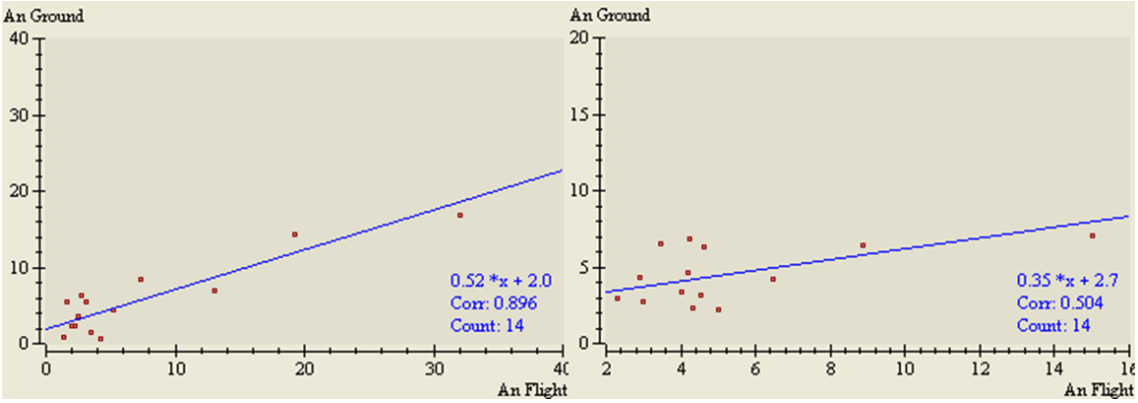

Figure 3.16: Scatter Plot of *Aspergillus niger* Ground vs Flight samples

Analysis: The given correlation value of 0.896 in % volume is relatively high. Two samples were not considered to be matching by the software. There is no reason to assume that the slight change in the protein pattern resulted from the exposure to microgravity.

***Basipetospora halophila* Ground + flight**

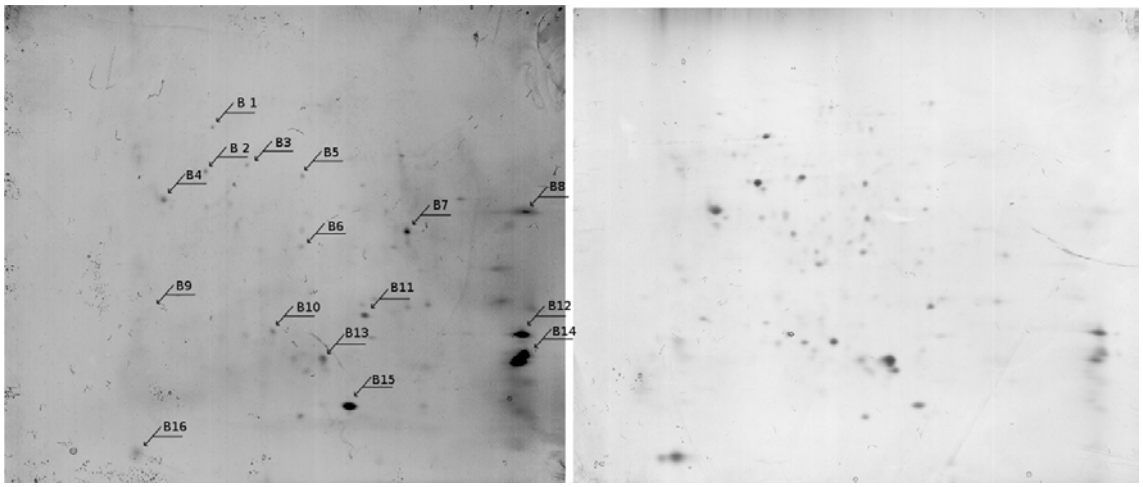

Figure 3.17: Exemplary 2D Gels of *Basipetospora halophila* Ground and Flight samples.

Characteristic Protein clusters :

B1	Protein showing high similarity considering overall weaker detection
B2	Protein showing high similarity
B3	Protein showing high similarity
B4	Landmark Protein
B5	Landmark Protein
B6	Protein showing relatively high similarity
B7	Protein showing strongly reduced similarity
B8	Protein showing reduced similarity
B9	Protein showing high similarity
B10	Protein showing high similarity
B11	Protein showing high similarity
B12	Protein showing high similarity
B13	Protein showing high similarity
B14	Landmark Protein
B15	Landmark Protein showing over expression in Ground sample
B16	Protein showing high similarity

Table 3.8: *Basipetospora halophila* identification Proteins, short term experiment

Scatter Plot analysis Volume % and Area comparison

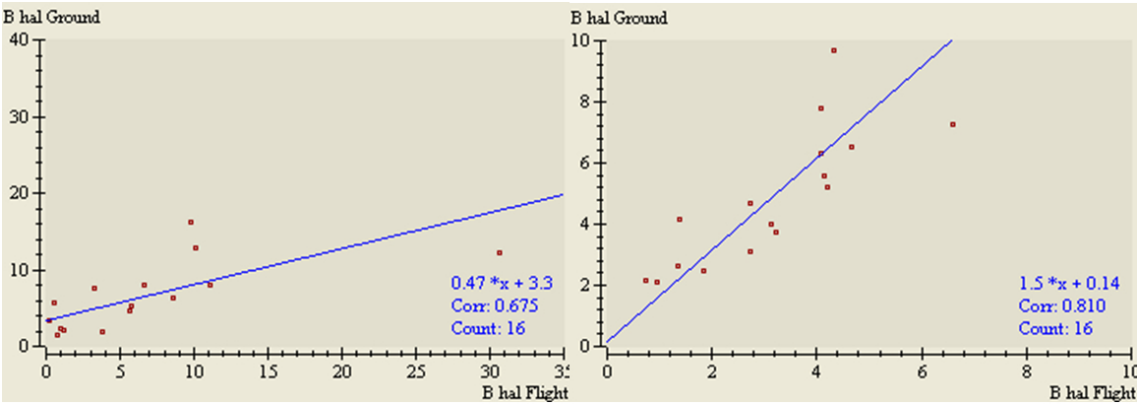

Figure 3.18: Scatter Plot of *Basipetospora halophila* Ground vs Flight samples

The area Comparison delivers a quite high correlation value of 0.810. In the area and the volume% comparison all 16 samples were accepted as pairs.

There is no reason to assume that the slight change in the protein pattern resulted from the exposure to micro-gravity.

***Cladosporium herbarum* Ground + flight**

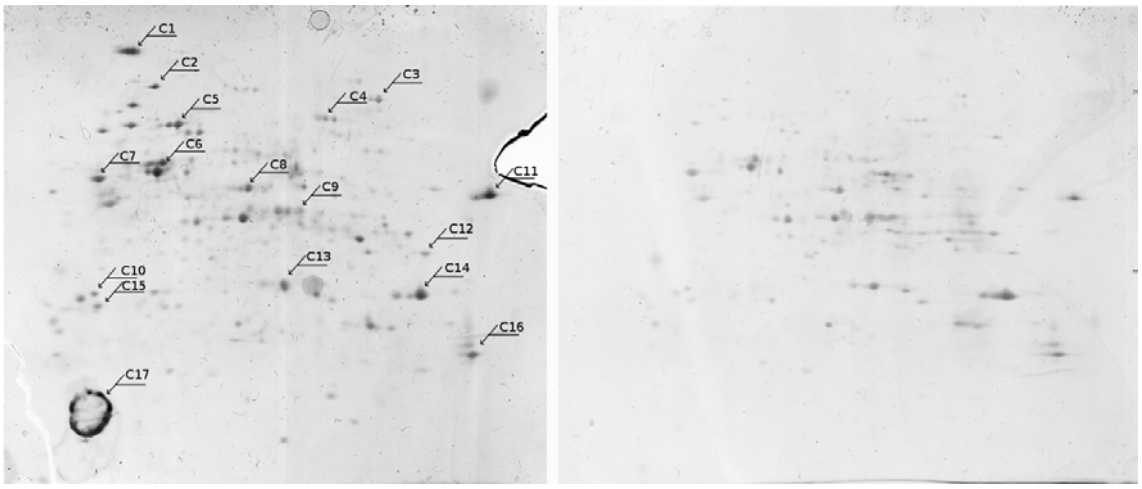

Figure 3.19: Exemplary 2D Gels of *Cladosporium herbarum* Ground and Flight samples.

Characteristic Protein clusters :

C1	Protein showing low similarity (Protein hardly visible in Flight)
C2	Protein showing high similarity
C3	Protein showing high similarity
C4	Protein showing high similarity
C5	Protein showing reduced similarity (Protein relatively underexpressed in G)
C6	Landmark Protein
C7	Landmark Protein
C8	Protein showing high similarity
C9	Protein showing high similarity
C10	Landmark Protein
C11	Protein showing high similarity
C12	Protein showing high similarity
C13	Protein showing high similarity
C14	Landmark Protein
C15	Protein showing high similarity
C16	Protein showing high similarity
C17	Artefact not a Protein

Table 3.9: *Cladosporium herbarum* identification Proteins, short term experiment

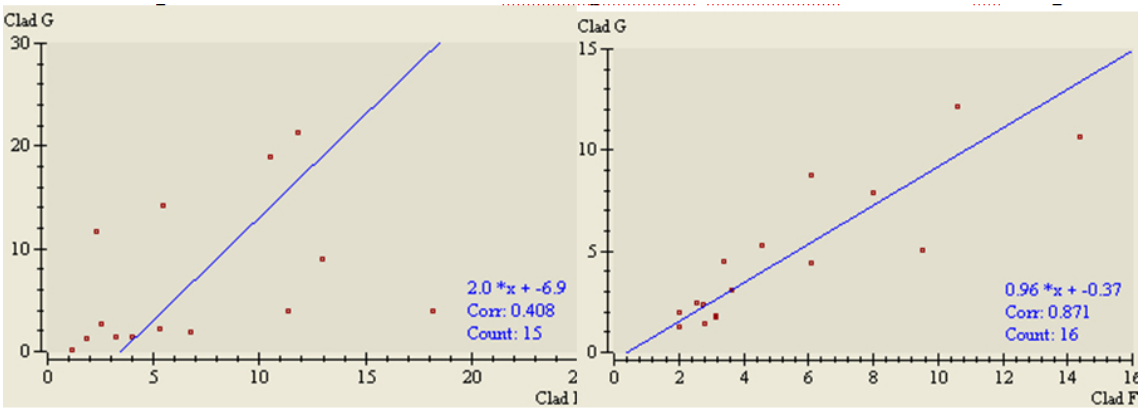

Figure 3.20: Scatter Plot of *Cladosporium herbarum* Ground vs Flight samples

The area Comparison delivers a quite high correlation value of 0.871. In the area comparison all 16 samples were accepted as pairs. There is no reason to assume that the slight change in the protein pattern resulted from the exposure to microgravity.

Ulocladium chartarum Ground + Flight

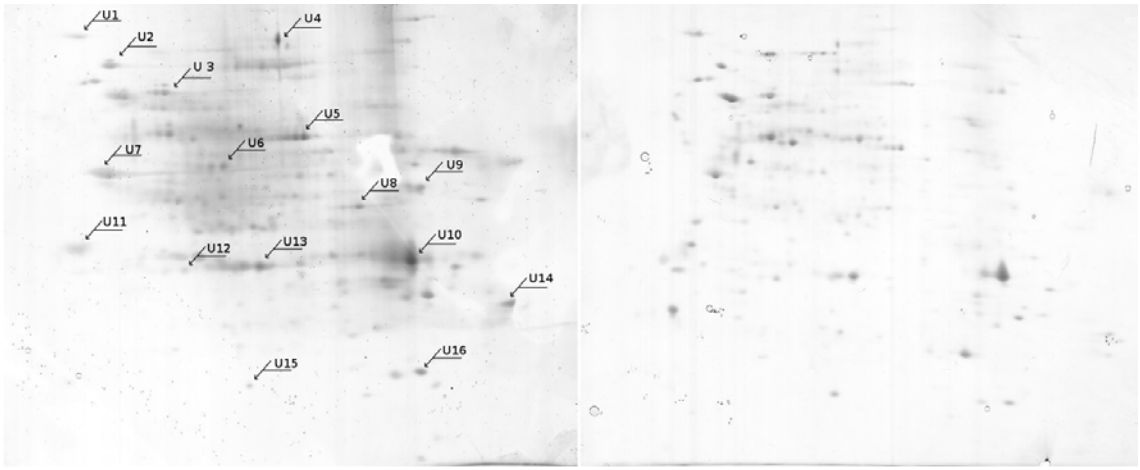

Figure 3.21: Exemplary 2D Gels of *Ulocladium chartarum* Ground and Flight samples.

U1	Protein showing high similarity
U2	Protein showing high similarity
U3	Landmark Protein
U4	Protein showing high similarity
U5	Protein showing high similarity
U6	Protein showing high similarity
U7	Landmark Protein
U8	Protein showing low similarity (barely detectable in Flight)
U9	Protein showing very low similarity
U10	Protein showing high similarity
U11	Protein showing high similarity
U12	Protein showing high similarity
U13	Protein showing high similarity
U14	Protein showing reduced similarity (change in Protein size and IEP)
U15	Landmark Protein
U16	Landmark Protein

Table 3.10: *Ulocladium chartarum* identification Proteins, short term experiment

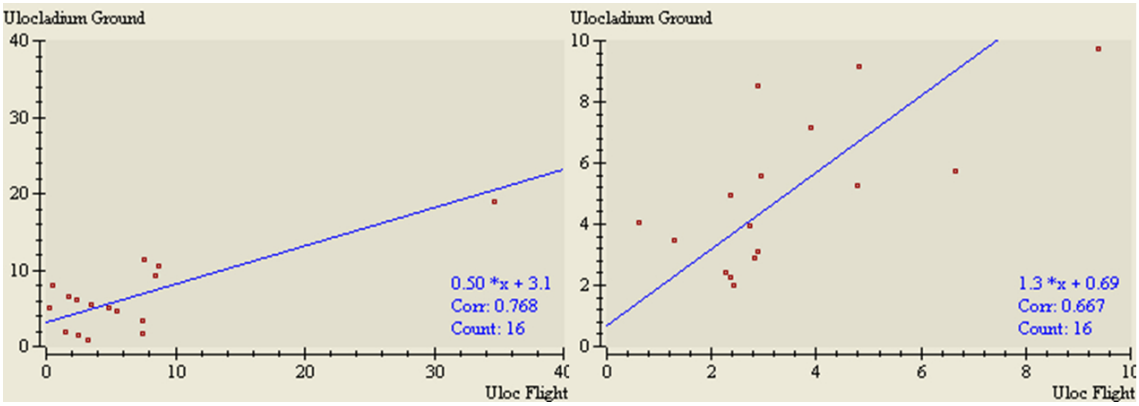

Figure 3.22: Scatter Plot of *Ulocladium chartarum* Ground vs Flight samples

Both, volume % and area analysis show a correlation value of below 0.8, the spot distribution is heterogeneous. Some prominent protein clusters from the Ground sample (in the middle and lower center are missing in the flight sample)

Ulocladium chartarum from Biocontainer

The *Ulocladium* Flight samples from the bio containers each were compared to the Ground sample. Each compared by volume (%) and area. The same sample protein clusters were used as in the *Ulocladium* Ground versus Flight analysis.

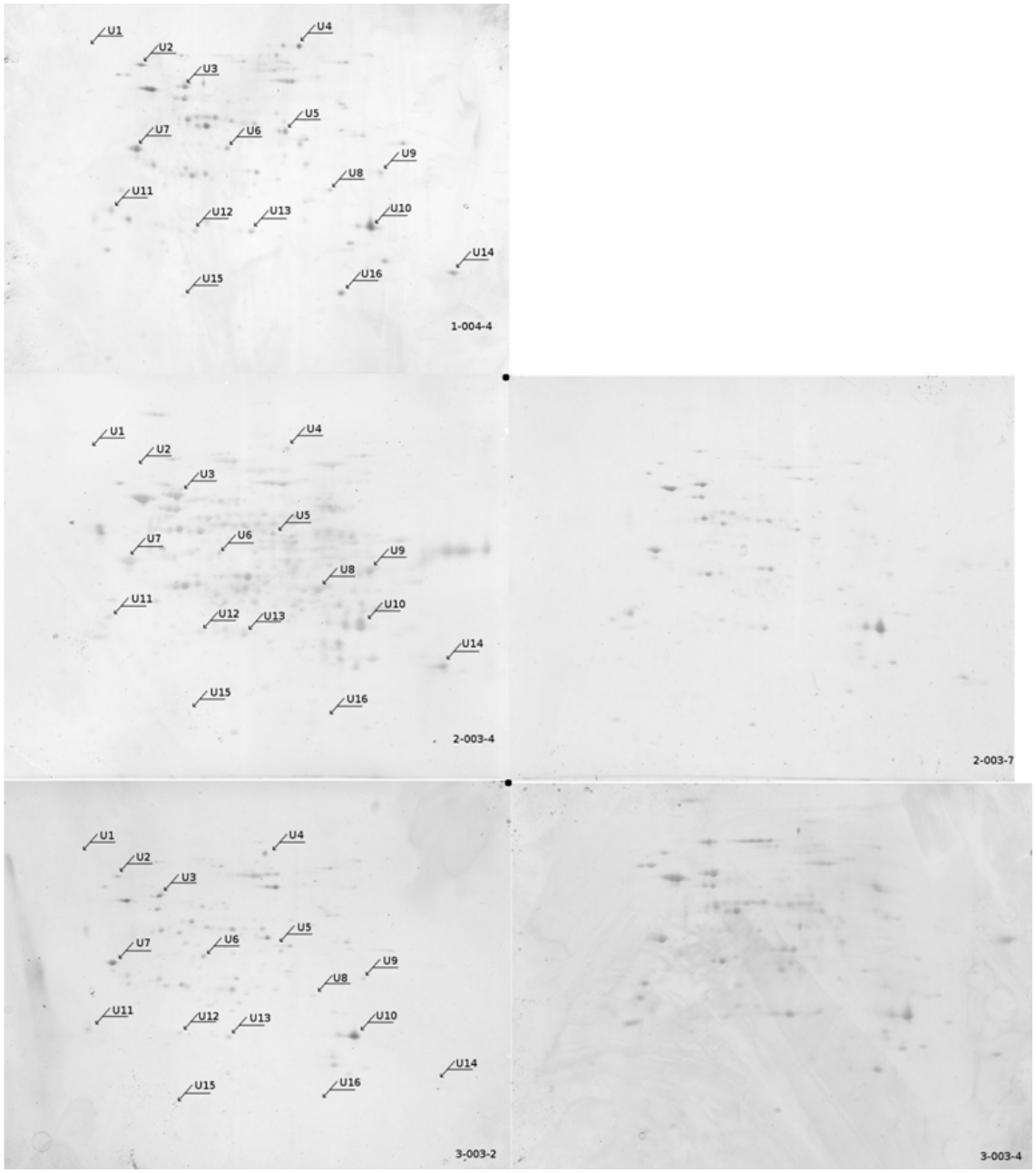

Figure 3.23: 2D Gels example for bio container samples. . 1 of 2 from biocontainer 1, 2 of 4 from bio container 2 and 2 of 5 from bio container 3. Sample proteins marked.

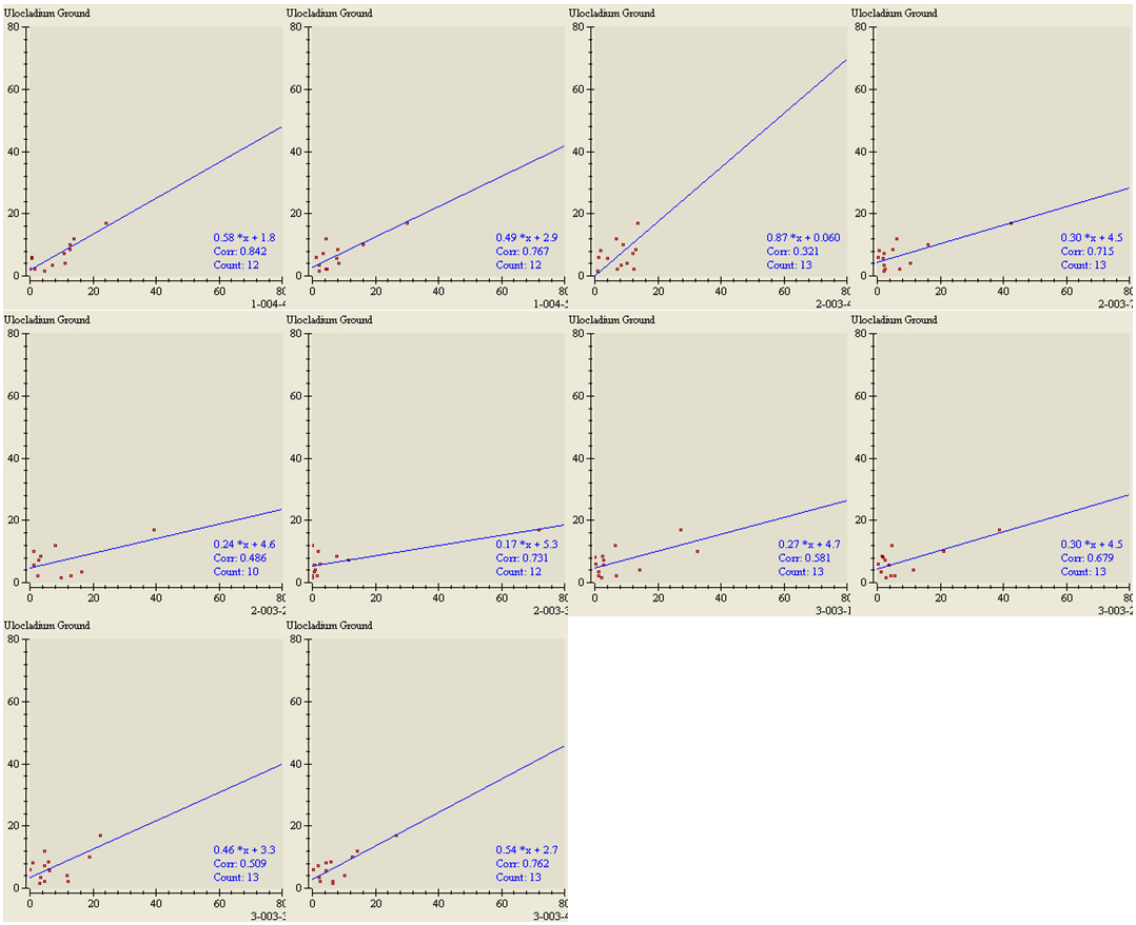

Figure 3.24: Scatter Plot of *Ulocladium chartarum* Ground vs Flight samples from biocontainer. Comparison by %Volume

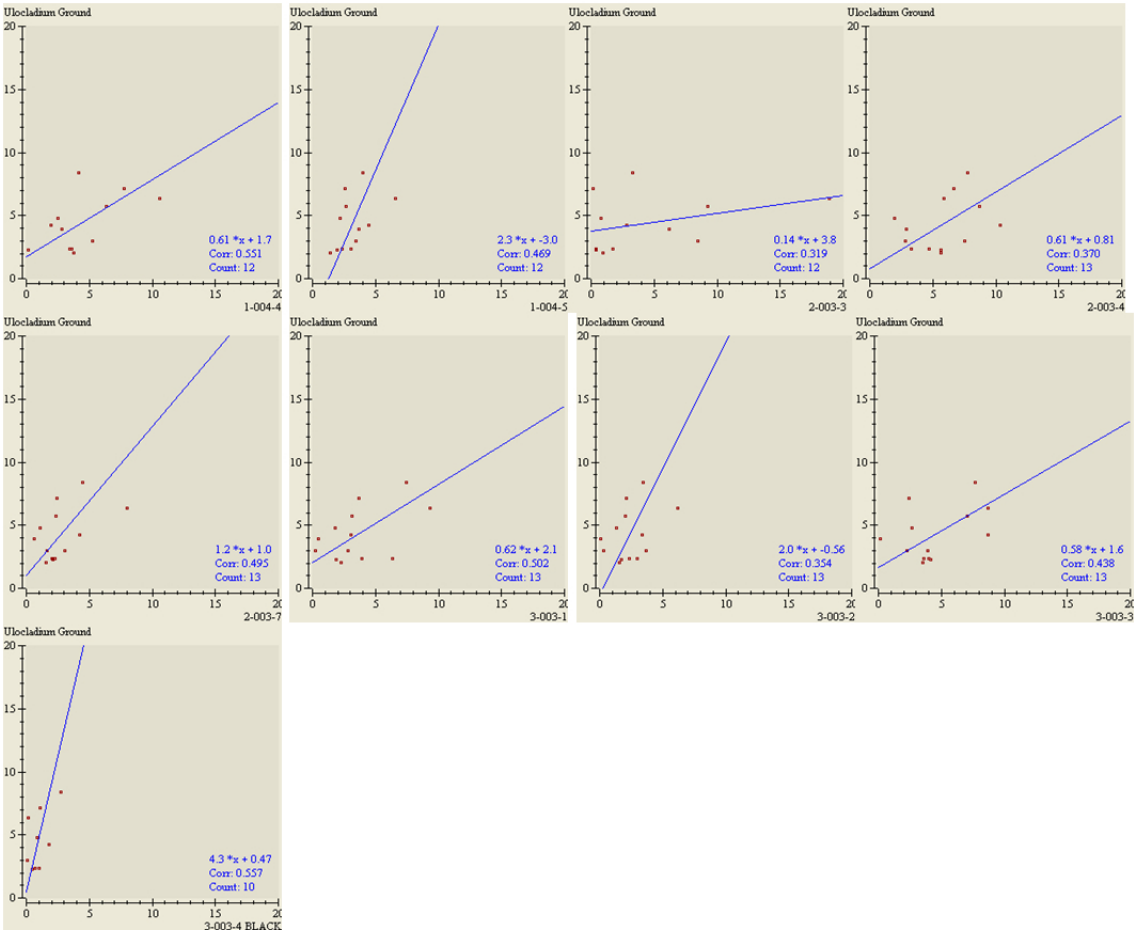

Figure 3.25: Scatter Plot of *Ulocladium chartarum* Ground vs Flight samples from biocontainer. Comparison by area

Analysis

Sample code	Corr. factor volume	Corr. factor area
1-004-4	0,842	0,551
1-004-5	0,767	0,469
2-003-2	0,486	0,149
2-003-3	0,731	0,319
2-003-4	0,321	0,370
2-003-7	0,715	0,495
3-003-1	0,581	0,502
3-003-2	0,679	0,354
3-003-3	0,509	0,438
3-004-4 BLACK	0,762	0,557

Table 3.11: Analysis, correction factors and correction areas for long term samples

Chapter 4

Discussion

4.1 Discussion of short term experiment

After the analysis of the obtained results there seems to be no proof that exposure to micro-gravity conditions did permanently alter the genomic DNA. All samples that show a homology of less than 91% when compared to the Ground samples are obtained from one Bio container and one microcapsule. (Bio container nr. 2 Microcapsule 3).
Cladosporum herbarum samples showed a 99,4% homology between the ground and the flight sample, compared to a 97,6% homology of the flight samples versus the reference strain. Proteomic analysis suggests that the Proteome did not undergo significant long term alteration during exposition to micro-gravity.
Basipetospora halophila samples showed a 98,5% homology between the ground and the flight samples, compared to a 98,1% homology of the flight samples versus the reference strain Proteomic analysis suggests that the Proteome did not undergo significant long term alteration during exposition to micro-gravity.
Aspergillus niger samples showed a 98,7% homology between the ground and the flight samples. The relatively low homogeneity between the ground and flight samples when compared to the reference strain may be due to the wrong strain being used as a reference. Proteomic analysis suggests that the Proteome did not undergo significant long term alteration during exposition to micro-gravity.
Ulocladium herbarum samples showed only a 94,6% homology between the ground and flight samples, which can be considered significant. Considering that the average strain does have a homology of above 98%. This spike is placed in the same homological dimensions as the *A. niger* samples compared to their reference strain. Most *ulocladia* strains are however in the homology range of above 98%. All of the *Ulocladium* samples from Biocontainer 2, Microcapsule 3 show a reduced homology towards the original ground strain. The samples, showing a homology of below 97,9%, were reanalyzed together with the new samples from the CFS-A long term experiment. These analyses was carried out in early December 2011, the obtained results reassured the first measurements.

The 2D gel analysis revealed sufficiently high correlations between the samples, Proteomic analysis suggests that the Proteome did not undergo significant long term alteration during exposition to micro-gravity.

4.2 Discussion of long term experiment

The AFLP Patterns suggest, that the DNA of *Aspergillus niger* and *Ulocladium chartarum* as well as *Cladosporium herbarum* were not affected by long term exposure to micro-gravity. *Aspergillus niger* samples retained a genomic homology of above 96%.
Basipetospora halophila samples showed a reduced homology towards the ground sample: Flight sample 2, showing 96% homology is above the defined border of what is considered a significant deviation. All other *Basipetospora* samples show a reduced homology of around 94%. *Ulocladium chartarum* samples showed a genomic homology of above 96%. In *Cladosporium herbarum* 1 out of 3 samples (Flight sample 1) retained a homology of above 96%. Flight sample 2 still retained 95% homology. Flight sample 3 seems to be a rogue result with a reduced homology of 83% towards the Ground sample. Again, due to the insufficient amount of samples, an in-depth evaluation, or sound statistical analysis could not be performed. The proteomic analysis showed similar homologous results. For *Aspergillus niger* the results show that exposure to micro-gravity conditions did not result in a permanent alteration of the proteome. The same is true for *Basipetospora*, for which the proteomic analysis showed no significant alteration of the protein expression pattern. However, for further clarification and interpretation, the experimental set up including the definition of the long term growth conditions should be taken into consideration, yet they are not available to us. Also, for *Ulocladium chartarum*, the obtained protein pattern was consistent through the short- and long term experiment, suggesting that *Ulocladium chartarum* did not suffer any detectable permanent genomic, or proteomic alteration due to exposure to micro-gravity. For *Cladosporium herbarum* the proteomic pattern results of FS1 and FS2 were relatively similar to the ground sample. FS 3 shows a slightly deviating profile. Since no systematic error can be proven, FS3, again is suggested to be a rogue result. As already mentioned, the technical build up and the precise conditions should be considered for a more detailed analysis. Due to the design of the experiments all results can only be considered exploratory and statistical evaluation can not really be achieved with the given sample size and the rather low comparability of the proteomic and genomic analysis methods. We were not able to give a more detailed interpretation of these results because no information on the conditions in the bio containers (such as eg. temperature, water availability, CO2 and O2 partial pressure etc.) was available for us.

4.3 Conclusion

In a publication of 2013, the group of Ioana Gomoiu showed that for *Ulocladium chartarum* significant changes in morphology occured during their 14 day exposure to microgravity (Gomoiu et al, 2013). Since the same samples were used in this experiment and by the group of Ioana Gomoiu , it similar results should be obtained. Yet, the hypothesis that the exposure to micro-gravity causes genomic and proteomic alterations can not be proven conclusively, and we have to assume that the four species of fungi, analyzed in the experiment, did not suffer any significant alterations during their exposure. Additionally, the results should merely be seen as exploratory. In other words, the possibly expected differences that may have been caused by an exposure to micro-gravity are below the detection limit. The results from the analysis of this experiment seem to support the results of Yamakazi et al. In a paper form 2012, Yamazaki showed that simulated microgravity had no significant effect on the morphology, asexual reproductive capability, or susceptibility to anti fungal agents of *Aspergillus niger* or *Candida albicans* (Yamazaki 2012). Another study, carried out by Makimura et al. on the fungal flora of the MIR space station produced comparable results, and also suggests that the fungi remained largely unaffected by the lack of gravity, and were growing as well as in residential areas on the earth (Makimura et al. 2001).
The relatively high limit of detection in this experiment was caused by several factors. The most prominent is certainly the lack of samples, since merely duplicates were provided and a minimum of five samples would be necessary for even a qualitative result. A more extensive analysis would be necessary to obtain the correct minimum sample size, since not even the expected variances can be discerned from two samples, but as a rough estimate, the necessary amount of replicates should be between 15 to 20 replicates per sample for a quantitative result .
In addition, it should be remarked that the two methods used, naturally, differ greatly in both accuracy and robustness. So consolidating the two sets of results is challenging.
Finally, no data about the growth conditions or the precise time and conditions of exposure were provided by the Romanian Institute for Space Science, making an analysis of the samples even more difficult.
The final analysis has to rest in the hands of the group of Doctor Ioana Gomoiu, who provided the samples to us and has a more in-depth knowledge of the experimental conditions.

4.4 Bibliography

Bibliography

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