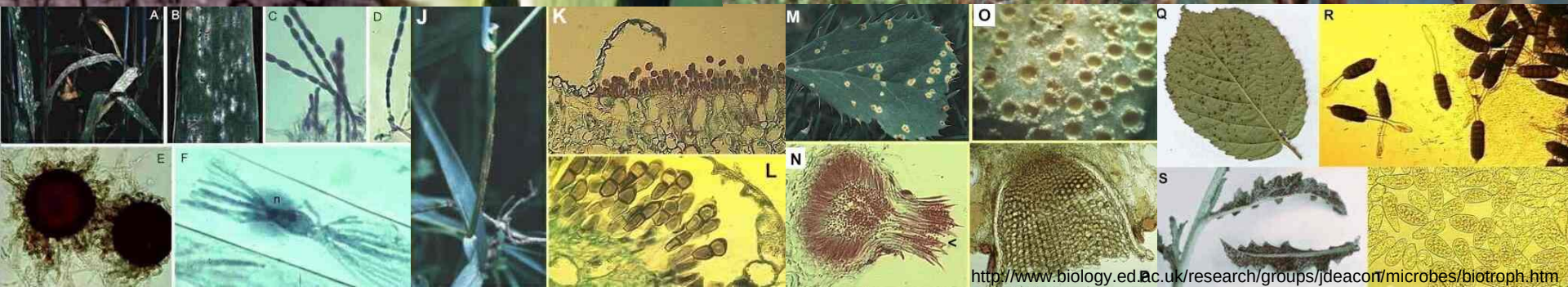
Fungal Secretomes not so secret anymore !

Dr Delphine Vincent

17 Nov 2009

http://www.anbg.gov.au/cpbr/program/sc/evol_bio.html



http://www.biology.ed.ac.uk/research/groups/jdeacon/microbes/biotroph.htm

Introduction

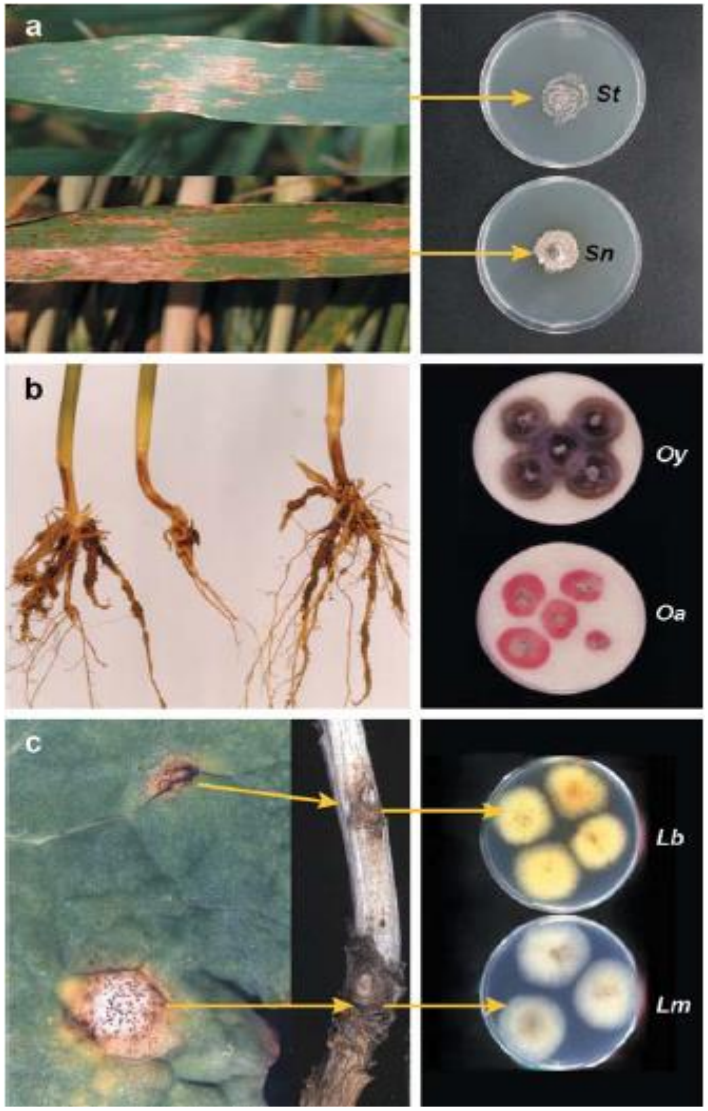
Introduction: fungal secretomics

Plant-pathogen interaction

***Septoria* / wheat leaves**

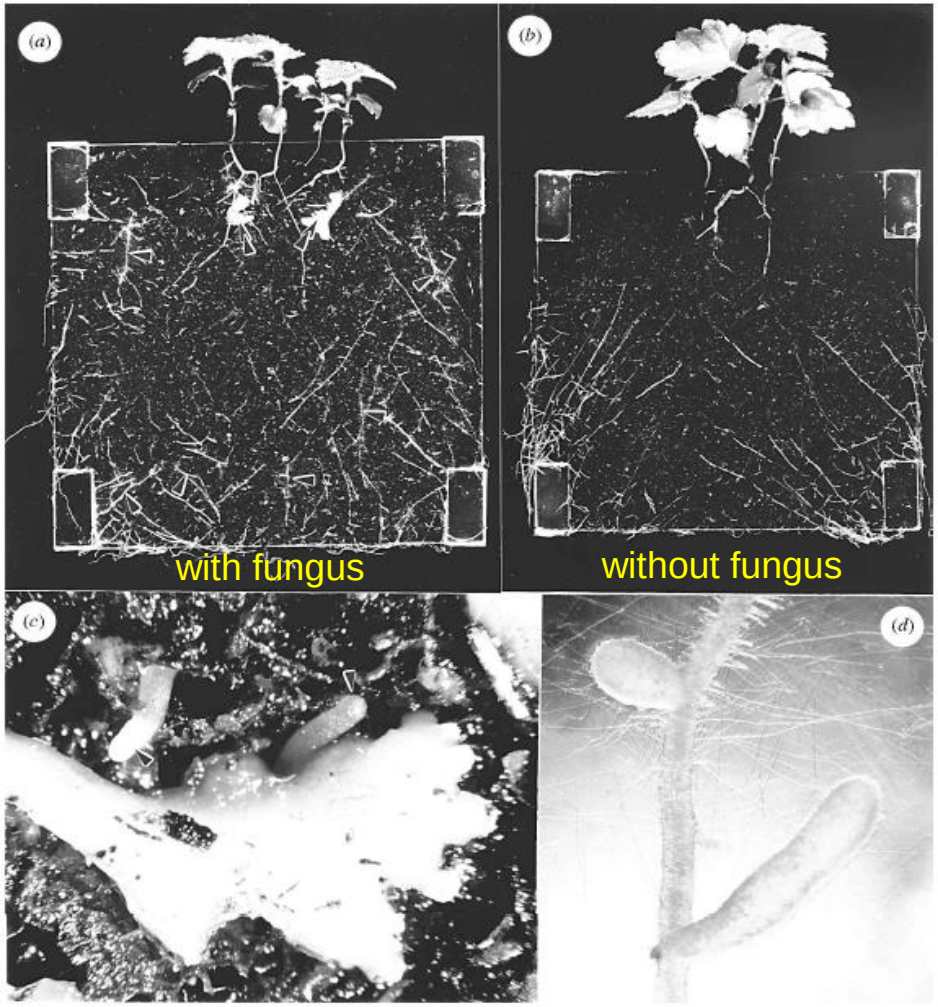
***Oculimacula* / wheat roots**

***Leptosphaeria* / rapessed leaves & roots**



Fitt *et al*., 2006
Annu Rev Phytopathol 44: 163-82

Plant-symbiont interaction

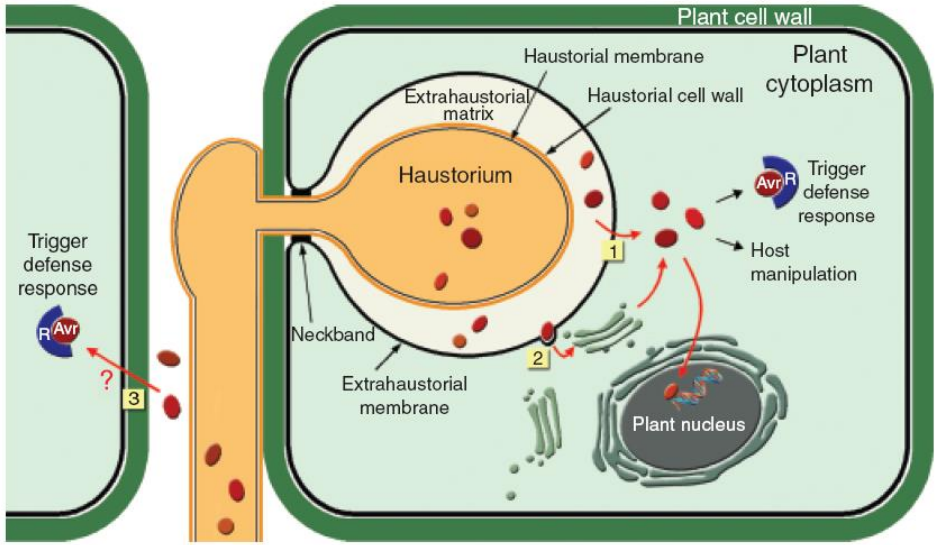


***Cryptothallus* / *Betula* roots**

Read *et al*., 2000
Phil Trans R Soc Lond B 355: 815-831

Introduction: fungal secretomics

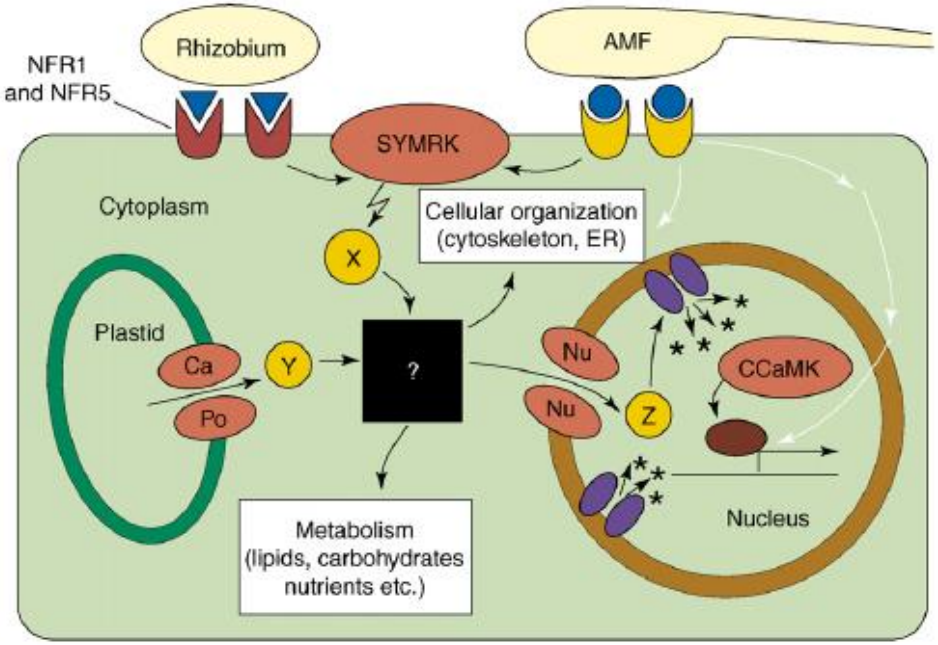
Plant-pathogen interaction



Catanzariti *et al*., 2007
FEMS Microlbiol Lett 269: 181-9

Many avirulence (avr) genes encode small secreted proteins.
Laugé & De Wit, 1998 Fung Genet Biol 24: 285-97

Plant-symbiont interaction



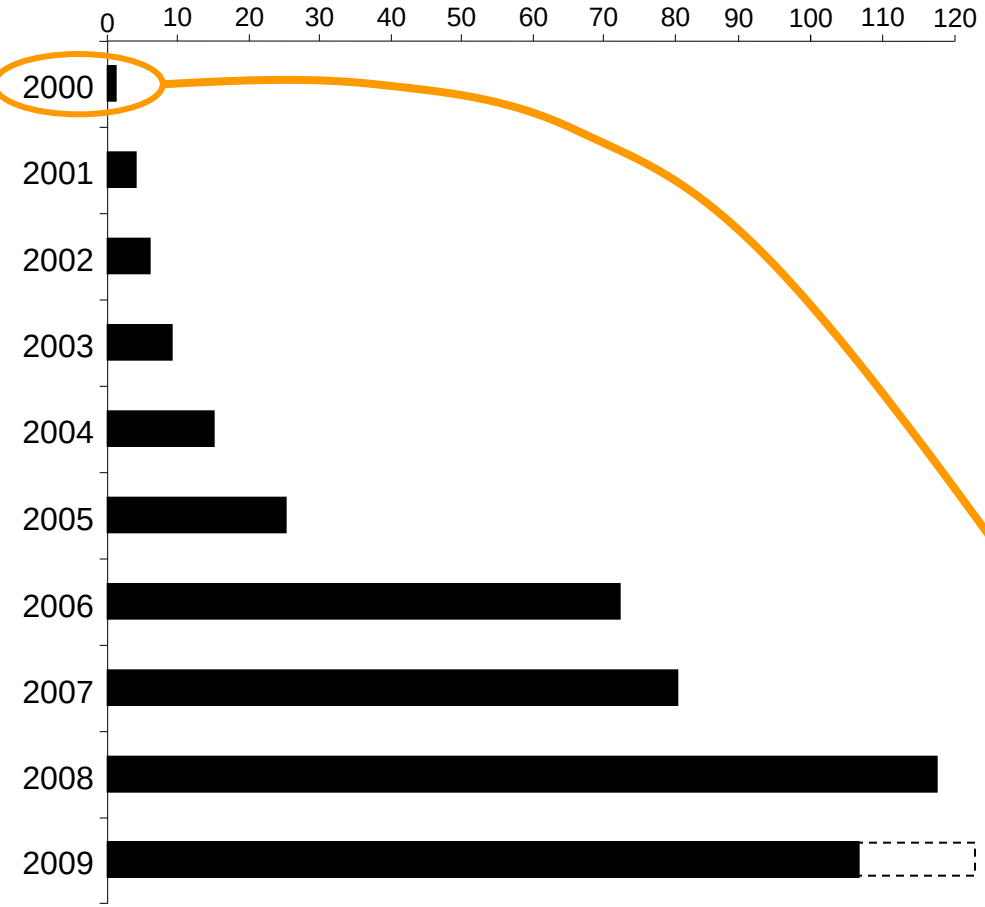
Reinhardt, 2007
Curr Opin Plant Biol 10:98-105

Numerous genes encoding small secreted proteins are induced during symbiosis in *Laccaria bicolor*.
Martin *et al.*, 2008 Nature 452: 88-92

Plant-fungus interactions are triggered by secreted proteins.

Introduction: fungal secretomics

Secretomics, a growing interest.



Number of publications *per* year.

Searched in PubMed.

Let's focus on fungi…

The EMBO Journal 2004, **23,** 4

Secretome

MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Sept. 2000, p. 515–547 Vol. 64, No. 3
1092-2172/00/$04.00+0

Signal Peptide-Dependent Protein Transport in *Bacillus subtilis*: a Genome-Based Survey of the Secretome

HAROLD TJALSMA, ALBERT BOLHUIS,† JAN D. H. JONGBLOED, SIERD BRON,* AND JAN MAARTEN VAN DIJL‡
Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, 9750 AA Haren, The Netherlands

Introduction: fungal secretomics

List of published methods for fungal secretomics (updated on Nov. 2009)

authors	year	species	filtration	desalting	concentration	lyophilization	protein extraction	protein analysis
Zorn *et al.*	2005	*Pleurotus sapidus*	0.22µm	dialysis	no	no	MeOH/chloroform	2-DE (pH3-6)
Phalip *et al.*	2005	*Fusarium graminearum*	no	no	Vivacell 10kD	no	Bio-Rad kit	2-DE (pH3-10NL)
Seidl *et al.*	2005	*Hypocrea atroviridis*	0.22µm	no	Amicon 3kD	no	TCA/acetone	2-DE (pH4-7)
Belen Suarez *et al.*	2005	*Trichoderma harzianum*	0.45µm	dialysis	Minitan 10kD	yes	TCA/DTT/acetone	2-DE (pH4-7)
Medina *et al.*	2005	*Aspergillus flavus*	8µm	no	no	yes	TCA	2-DE (pH4-7)
Yajima & Kav	2006	*Sclerotinia sclerotiorum*				yes	Bio-Rad kit	2-DE (pH4-7)
Oda *et al.*	2006	*Aspergillus oryzae*				no	ammomium sulfate	2-DE (pH4-7)
Kim *et al.*	2007	*Aspergillus fumigatus*				no	resuspension	2-DE (pH4-7)
Giddey *et al.*	2007	*Trycophyton sp.*				no	TCA/acetone	2-DE (pH4-7)
Ravalson *et al.*	2008	*Phanerochaete chrysosporiu*				no	sodium acetate	2-DE (pH3-5.6)
Tseng *et al.*	2008	*Trichoderma harzianum*				no	ammonium sulfate	2-DE (pH4-7)
Tan *et al.*	2009	*Staganospora nodorum*				no	TCA/acetone	2-DE (pH3-10)
Kim *et al.*	2009	*Magnaporthe grisea*	vacuum	no	no	no	phenol/ammonium acetate	2-DE (pH4-7)
Cao *et al.*	2009	*Pyrenophora tritici-repentis*	0.22µm	dialysis	no	yes	Bio-Rad B resuspension	2-DE (pH4-7)
Fragner *et al.*	2009	*Coprinopsis cinerea*	yes	no	centrifugation	yes	TCA/H2O Tris/acetone	2-DE (pH3-10)
Wymelenberg *et al.*	2005	*Phanerochaete chrysosporium*	yes	no	Amicon 10kD	no	acetone	SDS-PAGE shotgun
Paper *et al.*	2007	*Fusarium graminearum*	22µm	PD column	CBIN 900 cartidge	yes	TCA	SDS-PAGE shotgun
Swaim *et al.*	2008	*Kluyveromyces lactis*	no	no	vacuum	no	TCA	2-D/LC shotgun
Shah *et al.*	2008	*Botrytis cinerea*	no	no	no	yes	resuspension	LC shotgun
Guais *et al.*	2008	*Penicillium funiculosum*	no	dialysis	no	no	phenol/ammonium acetate	2-DE + 1-D/SCX sg
Alvim *et al.*	2009	*Moniliophtora perniciosa*	0.45µm	no	no	yes	Phosphate B resuspension	SDS-PAGE no MS
Tsang *et al.*	2009	*Aspergillus niger*	no	no	Amicon 15kD	no	acetone	LC-MS/MS
Shah *et al.*	2009	*Botrytis cinerea*	yes	HiPrep26/10	no	yes	Laemmli B resuspension	SDS-PAGE shotgun
Wymelenberg *et al.*	2009	*Phenerochaete chrysosporium*	yes	no	Amicon 10kD	no	acetone	SDS-PAGE shotgun
Martinez *et al.*	2009	*Postia placenta*	yes	no	Amicon 10kD	no	acetone	SDS-PAGE shotgun
Nagendran *et al.*	2009	*Amanita bisporigera*	yes	no	evaporation	yes	citrate B resuspension	SDS-PAGE shotgun

Fungal secretomics

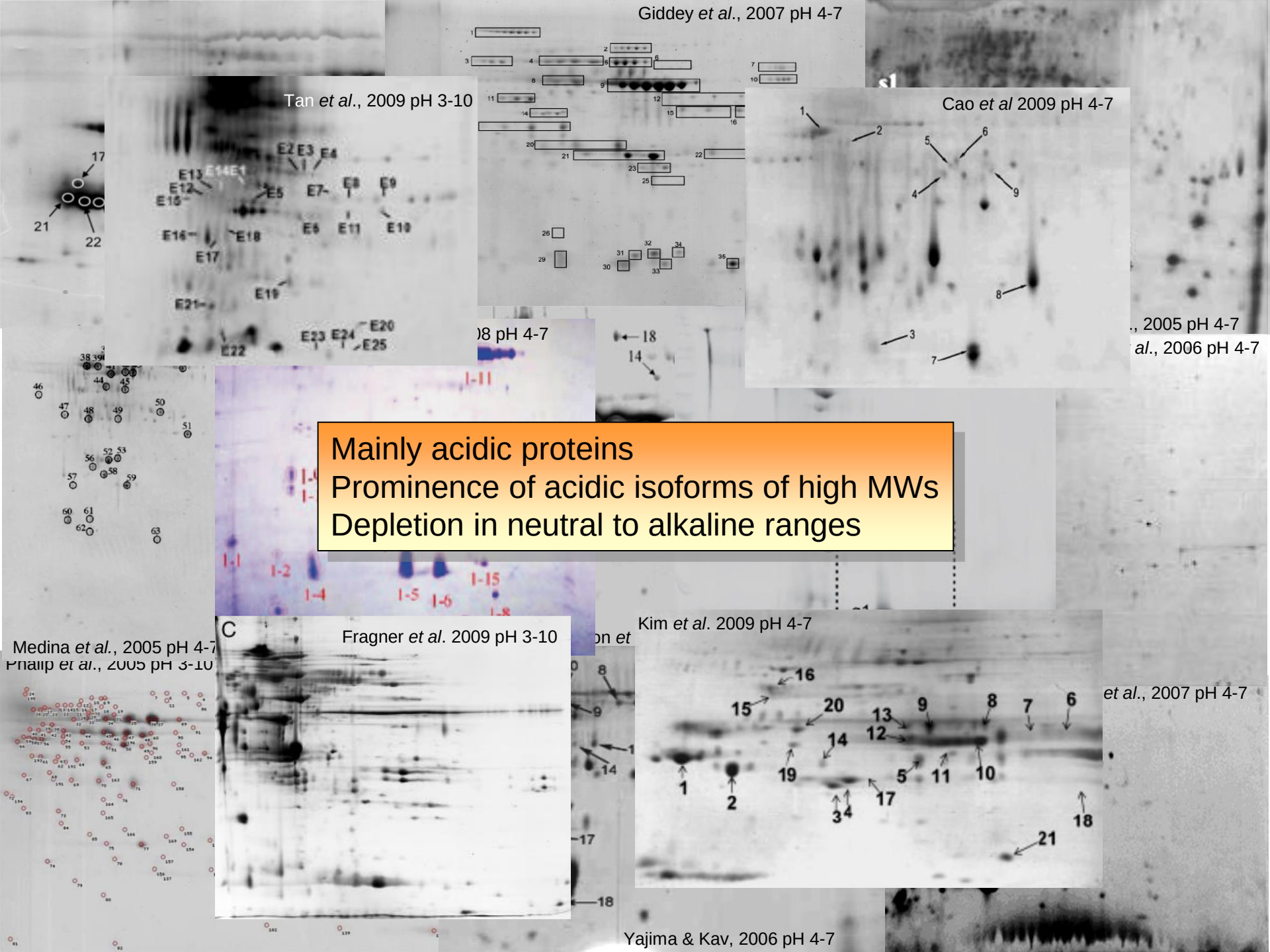
26 publications:

→ 15 2-DE

→ 9 shotgun

→ 1 1-DE (no MS)

→ 1 multiple techniques


Giddey *et al*., 2007 pH 4-7
Tan *et al*., 2009 pH 3-10
Cao *et al* 2009 pH 4-7
., 2005 pH 4-7
al., 2006 pH 4-7
Mainly acidic proteins
Prominence of acidic isoforms of high MWs
Depletion in neutral to alkaline ranges
Medina *et al*., 2005 pH 4-7
Fragner *et al*. 2009 pH 3-10
Kim *et al*. 2009 pH 4-7
et al., 2007 pH 4-7
Yajima & Kav, 2006 pH 4-7

Description of the project

Title: A genome-wide survey of secreted fungal proteins (SFPs) as effectors of pathogenicity and symbiosis in plant-associated fungi.

Objectives: To identify as many SFPs as possible in *in vitro*-grown fungi using proteomic techniques and characterize their pathogenic/symbiotic involvement *in planta*.

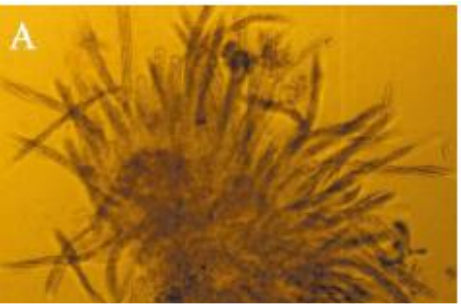
Fungi of interest: 1- *Laccaria bicolor*, tree root symbionte 

2- *Leptosphaeria maculans*, rapeseed pathogen 

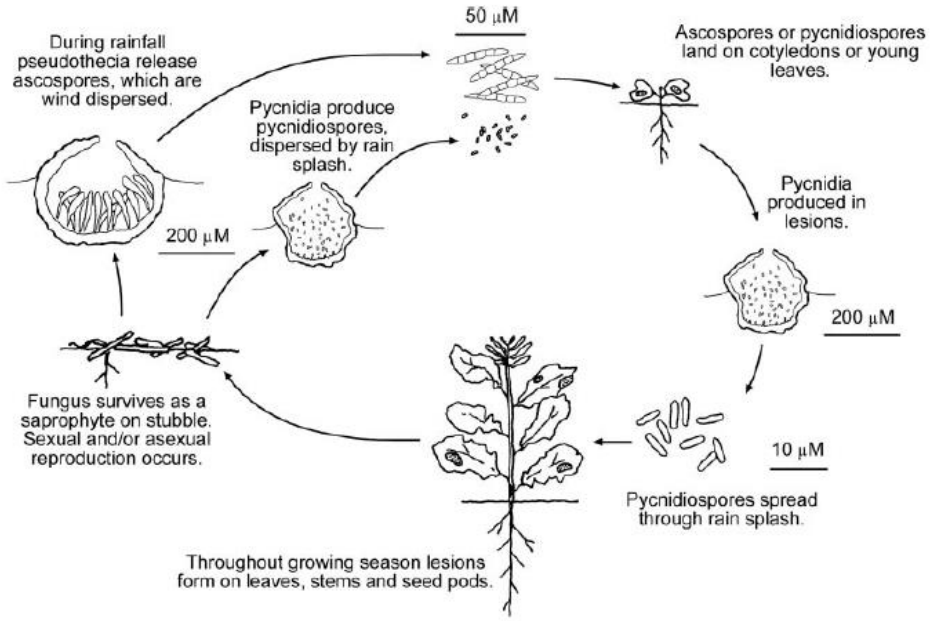
3- *Magnaporthe grisea*, rice pathogen 

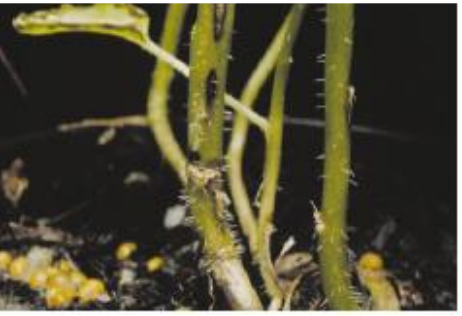
Partners: 1- Marc-Henri Lebrun, CNRS-BayerCropScience Lyon, France (*M. grisea*)
2- Thierry Rouxel, INRA Versailles, France (*L. maculans*)
3- Francis Martin, INRA Nancy, France (*L. bicolor*)
4- Christophe Plomion, INRA Bordeaux, France (Proteomics)

Description of the project



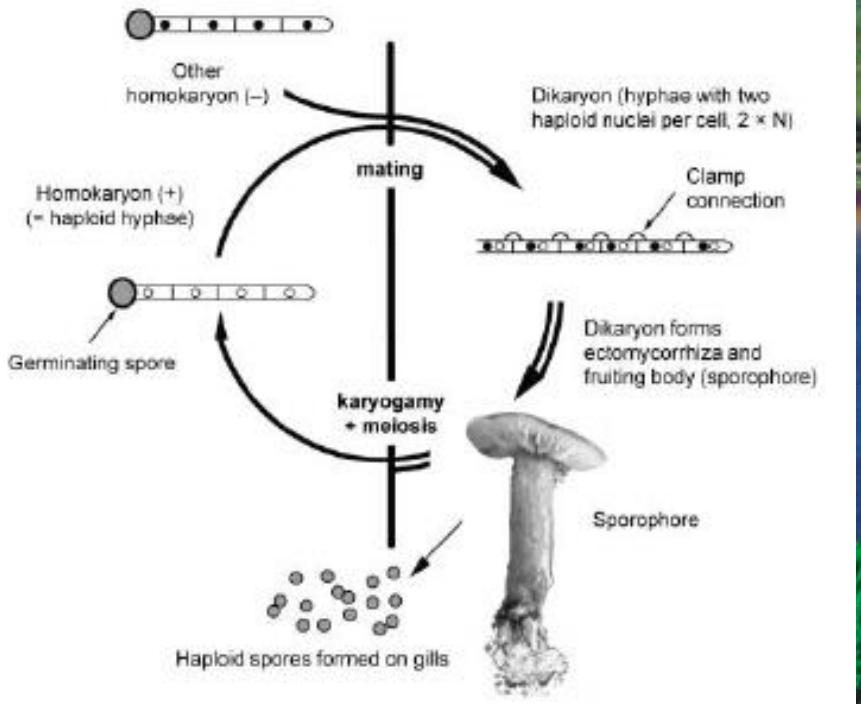
Leptosphaeria maculans

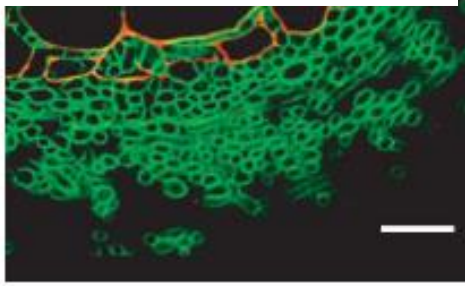




Howlett *et al.*, 2001
Fung Genet Biol 33: 1-14

Laccaria bicolor



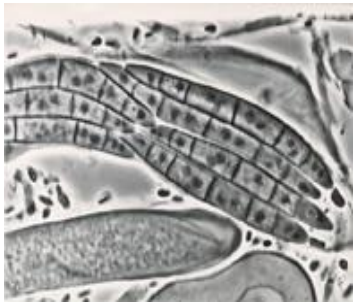


Martin & Selosse, 2008
New Phytol 180: 296-310

1 -

Optimization of 2-D patterns

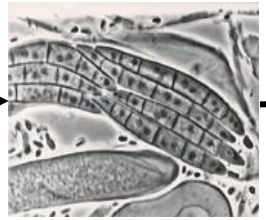
Identifications of secreted proteins from Leptosphaeria maculans

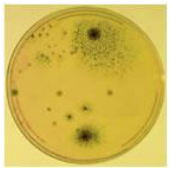


Materials & Methods

Materials & Methods

Culture of *Leptosphaeria maculans*, rapeseed pathogen





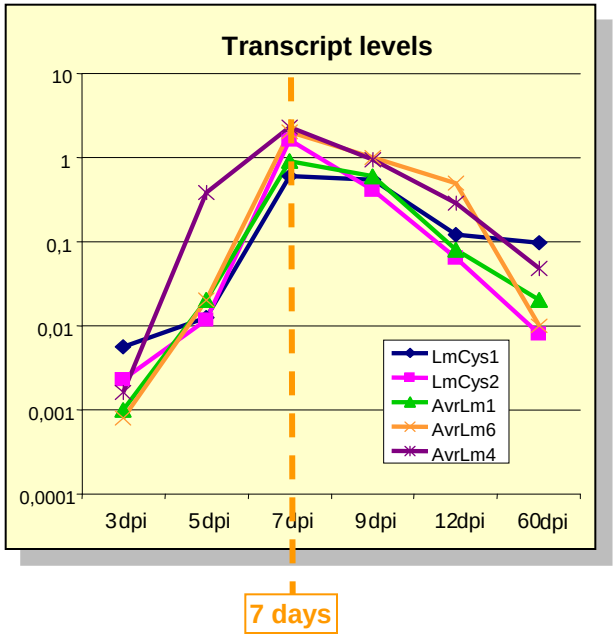
Fries medium
5g/L Yeast Extract
30g/L sucrose
5g/L tartrate NH4
1g/L KH2PO4
0.5g/L MgSO4 7H2O
0.13g/L CaCl2
0.1g/L NaCl
1g/L NH4NO3

Striring for
7 days

mycelium

secretome (medium)

Filtration 0.22µm
Dialysis (H2O)
0.05% PVP
1mM PMSF



Materials & Methods

Protein recovery

Phenol/Ammonium acetate

Solubilization (S)
- 1.4 M sucrose
- 50 mM EDTA
- 1 M Tris-HCl (pH 8)
- 0.1 M KCl
- 2% 2-ME
- 1 mM PMSF

Extraction
- in phenol-Tris (pH 7.5)

Reextraction
- in (S)

Precipitation
- 0.1 M ammonium acetate
- in MeOH

Washing x4
- 100% MeOH and acetone

Drying

Resuspension
- 7.5 M urea
- 2 M thiourea
- 10mM DTT
- 4% CHAPS
- 1% pic
- 1% CA pH 4-7
- 1% CA pH 3-10

Hurkman & Tanaka, 1986

Phenol/Ether

Solubilization (S)
- 1.4 M sucrose
- 50 mM EDTA
- 1 M Tris-HCl (pH 8)
- 0.1 M KCl
- 2% 2-ME
- 1 mM PMSF

Extraction
- in phenol-Tris (pH 7.5)

Reextraction
- in (S)

Precipitation
- in Diethyl ether

Washing
- 100% Diethyl ether

Drying

Resuspension
- 7.5 M urea
- 2 M thiourea
- 10mM DTT
- 4% CHAPS
- 1% pic
- 1% CA pH 4-7
- 1% CA pH 3-10

Sauvé *et al.*, 1995

TCA/Acetone

Precipitation
- 10% TCA
- 0.07% 2-ME
- in acetone

Washing x3
- 0.07% 2-ME
- in acetone

Drying

Resuspension
- 7.5 M urea
- 2 M thiourea
- 10mM DTT
- 4% CHAPS
- 1% pic
- 1% CA pH 4-7
- 1% CA pH 3-10

Damerval *et al.* 1986

Liquid-Phase IEF

Rotofor Cell (Bio-Rad)
large focusing chamber
(50 mL, 20 fractions)

Lyophilization

Ultrafiltration (UF)

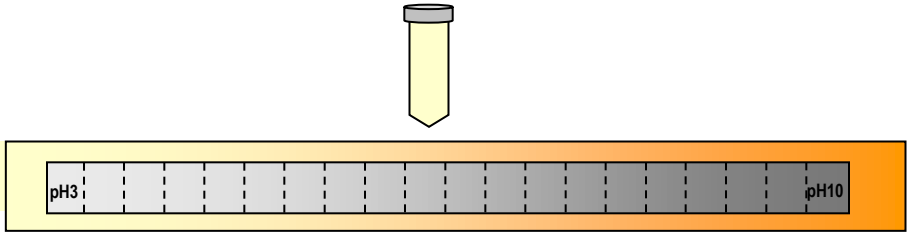
Amicon (Millipore)
15 mL 5kD MWCO

Materials & Methods

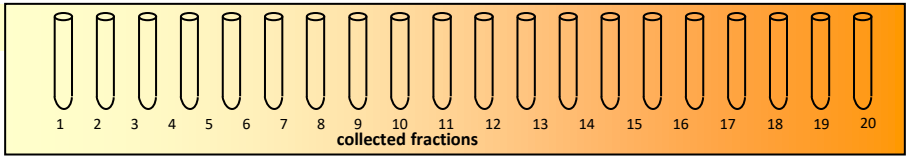
Flowchart of liquid-Phase IEF procedure

dialyzed secretome sample

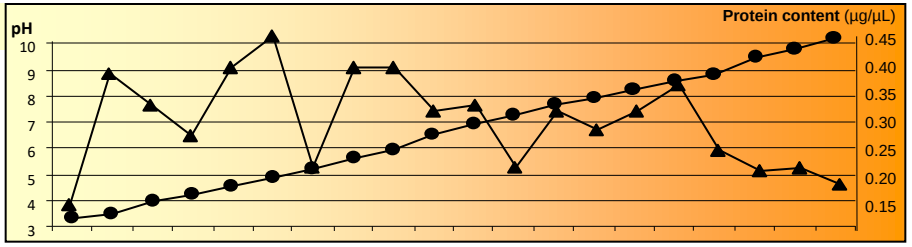
liquid-phase IEF
large rotofor chamber (50 mL)



collection of 20 individual fractions



pH measurement / protein assay

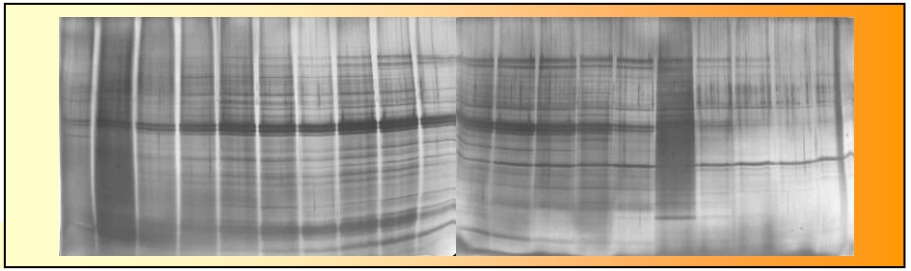


dialysis

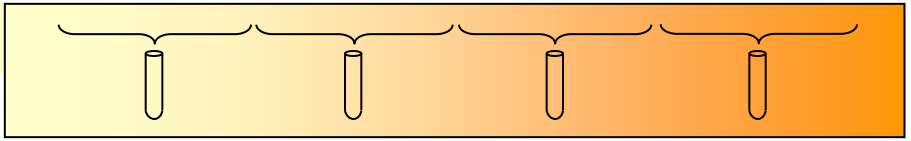
lyophilization

resolubilization

1-DE



fractions pooled according to the pH gradient covered by IPG strips



2-DE using NR or MR pH gradient

Materials & Methods

Protein separation and analyses

Protein assay

to estimate protein concentration

2D Quant Kit (Amersham)

1-DE

to verify protein sample quality

homecast minigel (10 x 8 cm)

4% acrylamide stacking gel

10% acrylamide resolving gel

$AgNO_3$ staining

2-DE

to separate proteins

IPG ReadyStrip 24 cm (BR, NR) or 11 cm (MR)

homecast 2-D gels (24 x 20 cm)

11% acrylamide running gel

$AgNO_3$ staining

Image analyses

to assess the number of spots for each method

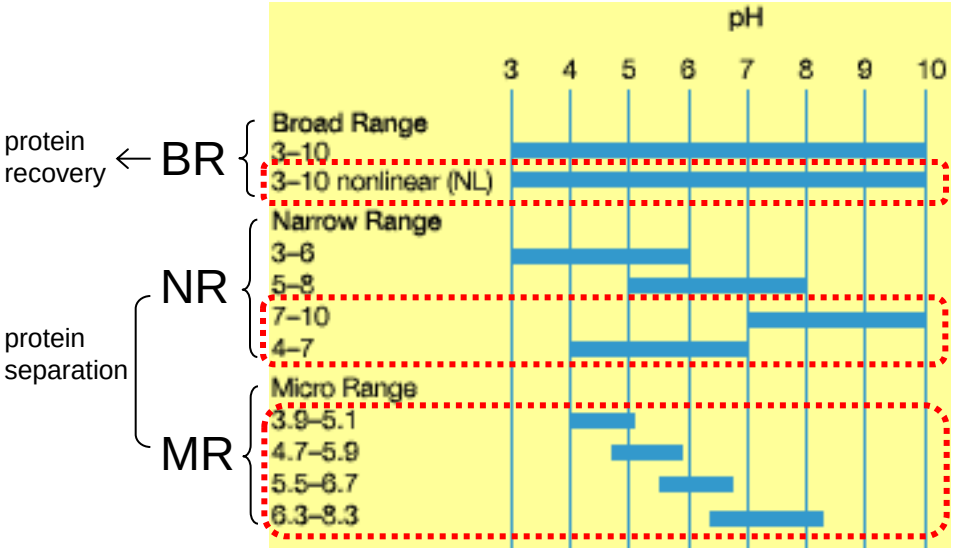
Progenesis PG240 (Nonlinear)

MS analyses (data not shown)

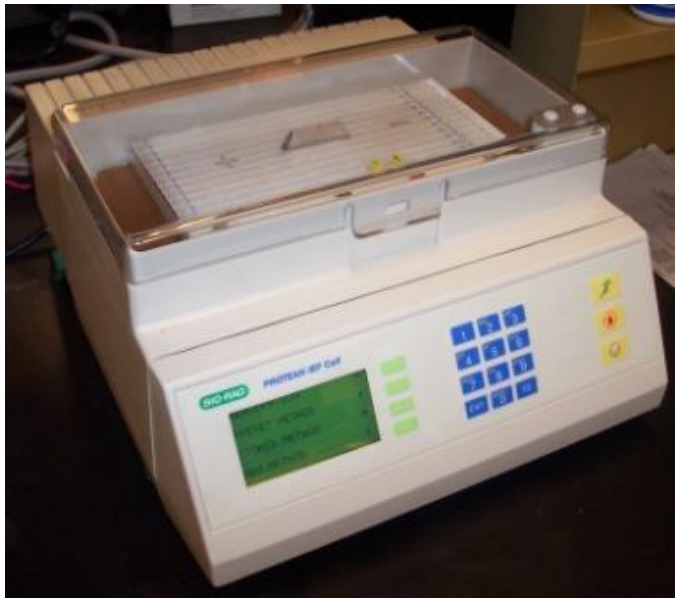
to verify gain in spot resolution

nLC-ESI-MS/MS (Thermo LCQ)

IPG Strips



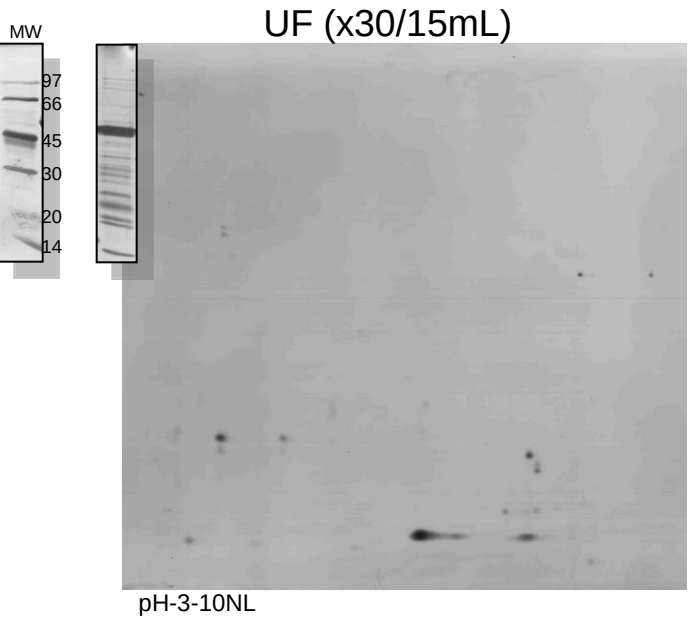
IPG-IEF



Maximization of secreted protein recovery

Maximization of secreted protein recovery

Electrophoretic patterns



complex 1-D profile

poor 2-D pattern
64 spots

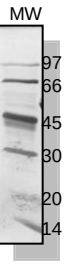
extensive protein losses during 2-DE

Ultrafiltration (UF)

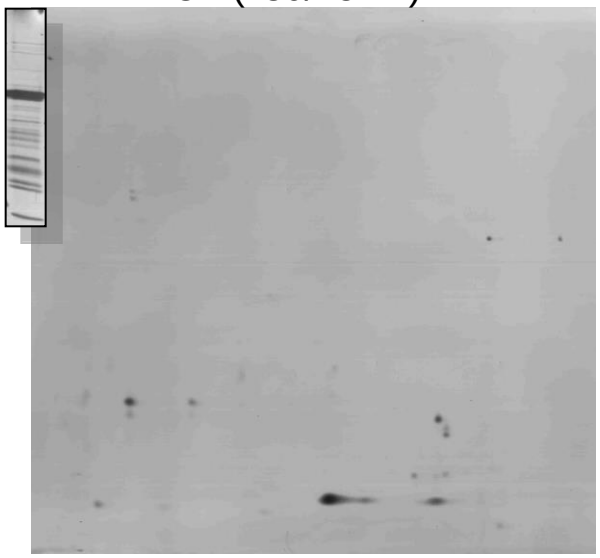
Amicon (Millipore)
15 mL 5kD MWCO

Maximization of secreted protein recovery

Electrophoretic patterns

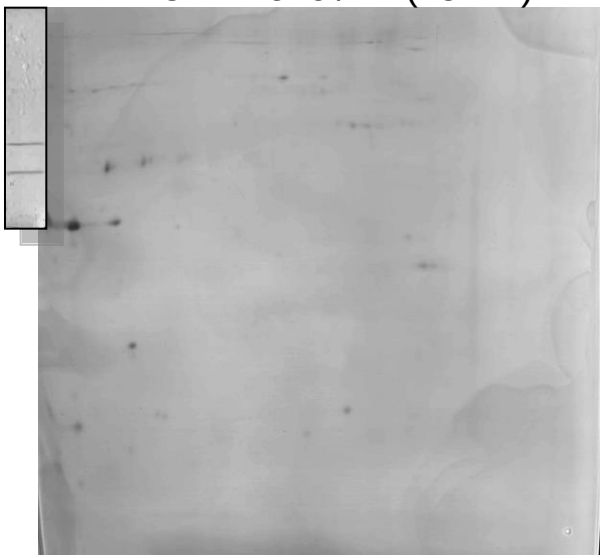


UF (x30/15mL)



pH-3-10NL

UF-Phenol/AA (15 mL)



pH-3-10NL

poor 1-D profile

poor 2-D pattern
180 spots

unsuitable protocol

Ultrafiltration (UF)

Amicon (Millipore)
15 mL 5kD MWCO

Phenol/Ammonium acetate

Solubilization (S)

1.4 M	sucrose
50 mM	EDTA
1 M	Tris-HCl (pH 8)
0.1 M	KCl
2%	2-ME
1 mM	PMSF

Extraction

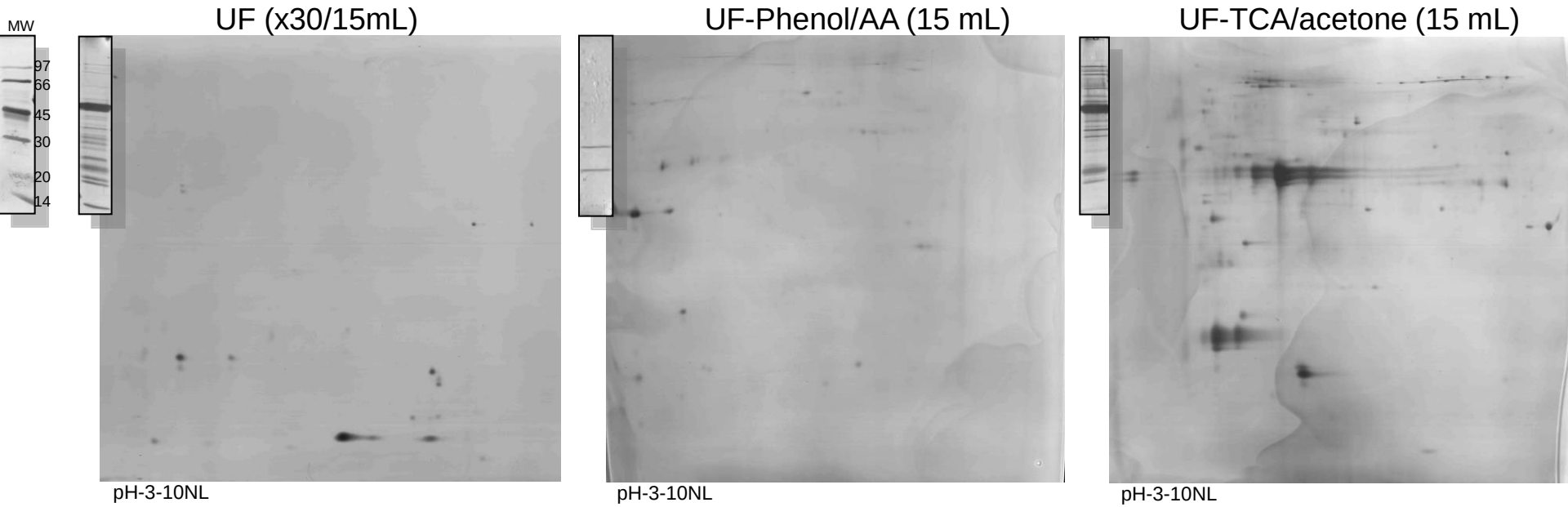
in phenol-Tris (pH 7.5)

Precipitation

0.1 M ammonium acetate
in MeOH

Maximization of secreted protein recovery

Electrophoretic patterns



Ultrafiltration (UF)

Amicon (Millipore)
15 mL 5kD MWCO

TCA/acetone

Precipitation
10% TCA
0.07% 2-ME
in acetone

Washing x3
0.07% 2-ME
in acetone

Drying

1-D profile enriched in high MW bands

improved 2-D pattern
266 spots

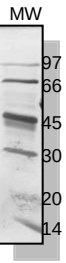
prominent high MW acidic proteins

depletion in low MW basic proteins (TCA)

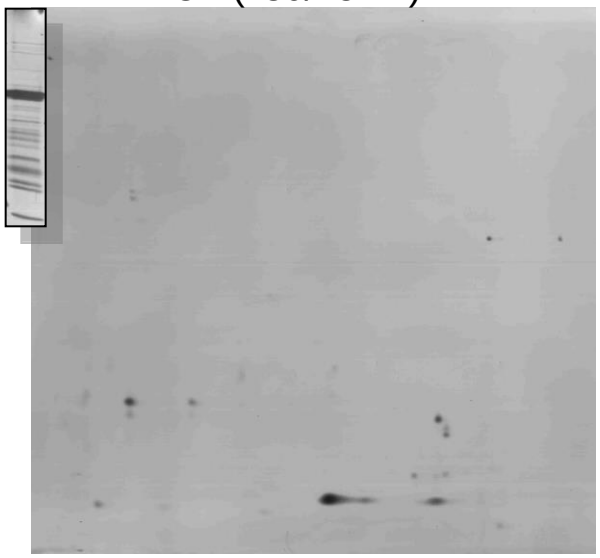
PTMs assumed

Maximization of secreted protein recovery

Electrophoretic patterns

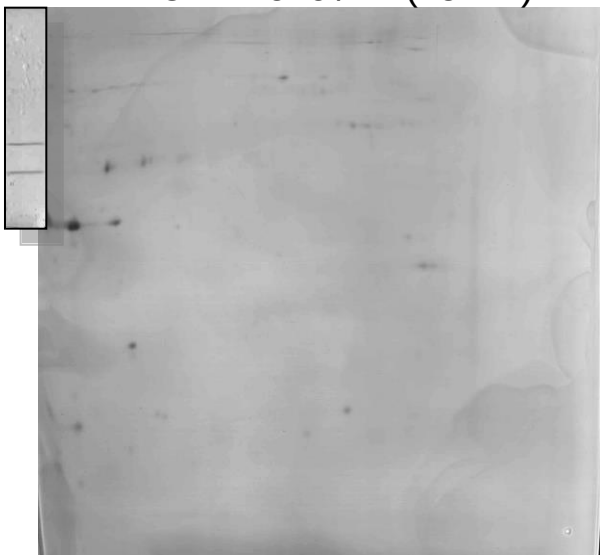


UF (x30/15mL)



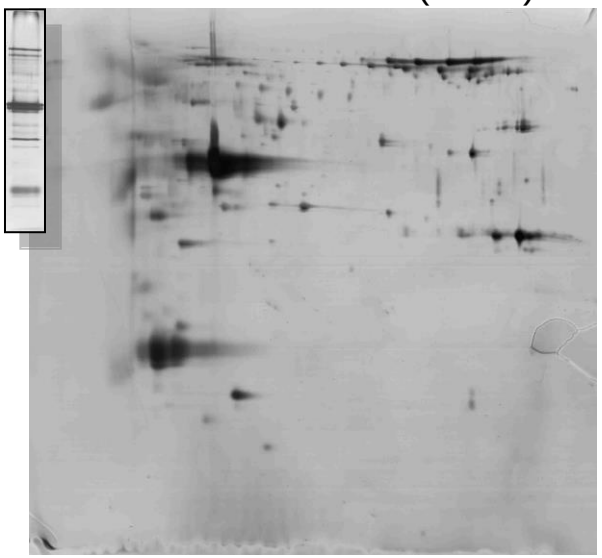
pH-3-10NL

UF-Phenol/AA (15 mL)



pH-3-10NL

noUF-TCA/acetone (40mL)



pH3-10NL

TCA/acetone

Precipitation
10% TCA
0.07% 2-ME
in acetone

Washing x3
0.07% 2-ME
in acetone

Drying

similar 1-D profile

improved 2-D pattern
449 spots

similar trends
prominent high MW acidic proteins
depletion in low MW basic proteins
PTMs assumed

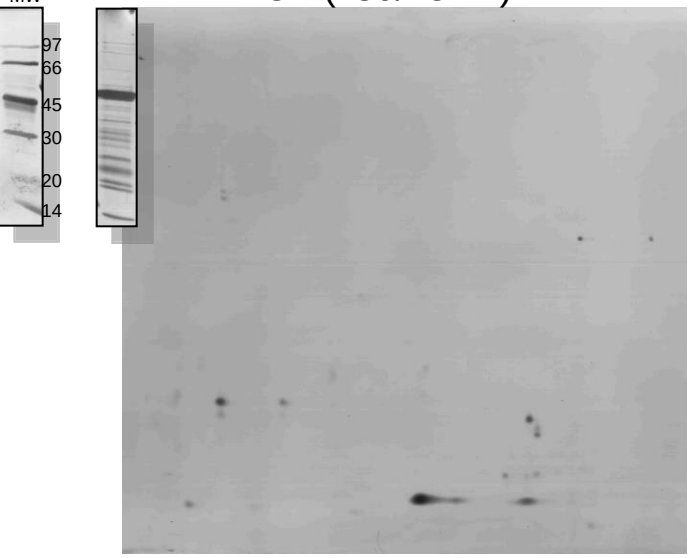
not many new spots

abundant proteins become more prominent

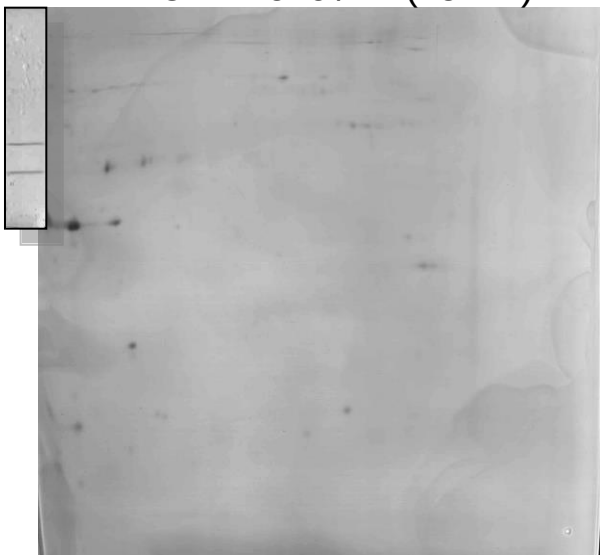
Maximization of secreted protein recovery

Electrophoretic patterns

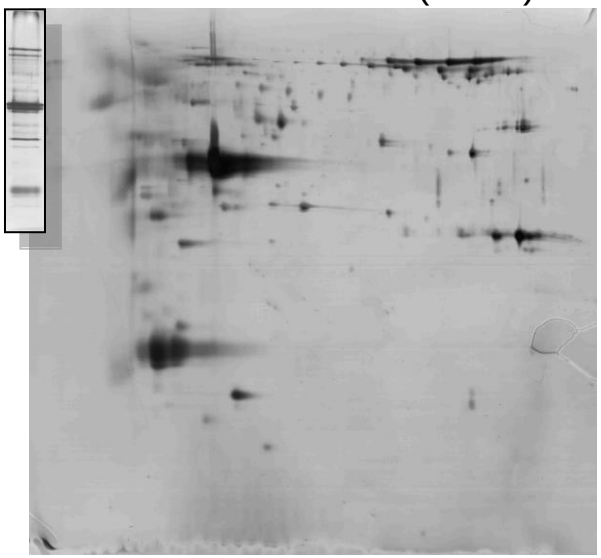
UF (x30/15mL)



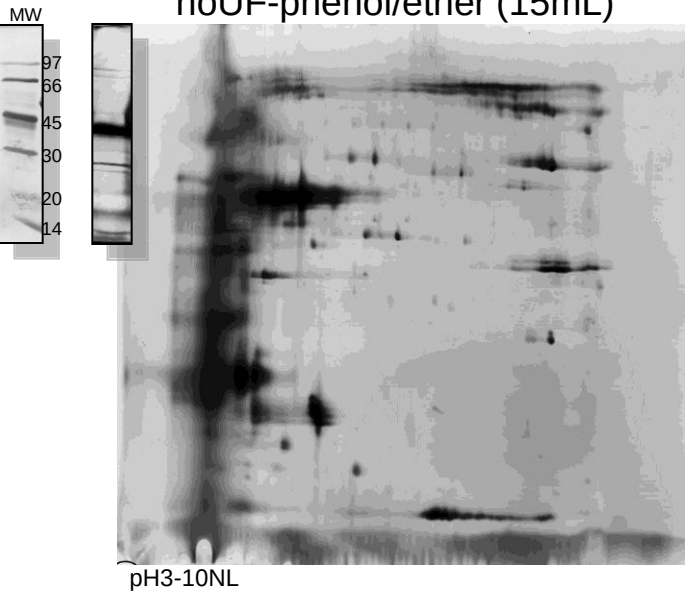
UF-Phenol/AA (15 mL)



noUF-TCA/acetone (40mL)



noUF-phenol/ether (15mL)



1-D profile lacking high MW bands

Low quality 2-D pattern
341 spots

similar trends
prominent high MW acidic proteins
depletion in low MW basic proteins
PTMs assumed

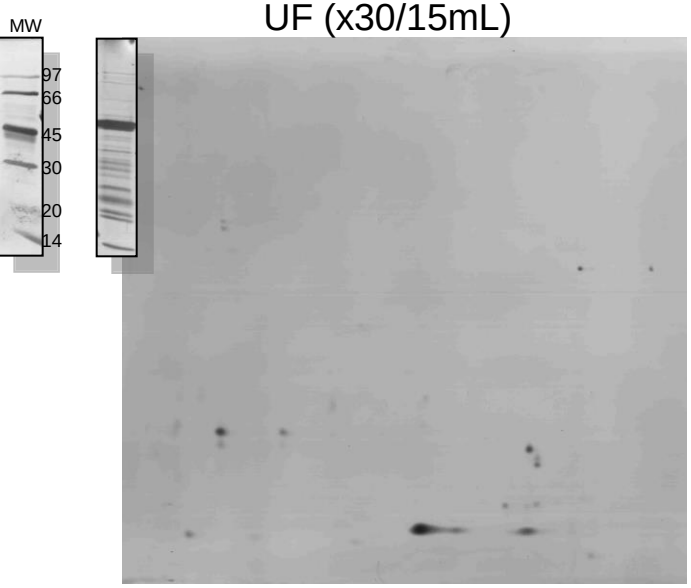
Few new acidic spots of low MW

abundant proteins still very prominent and more recalcitrant to IEF

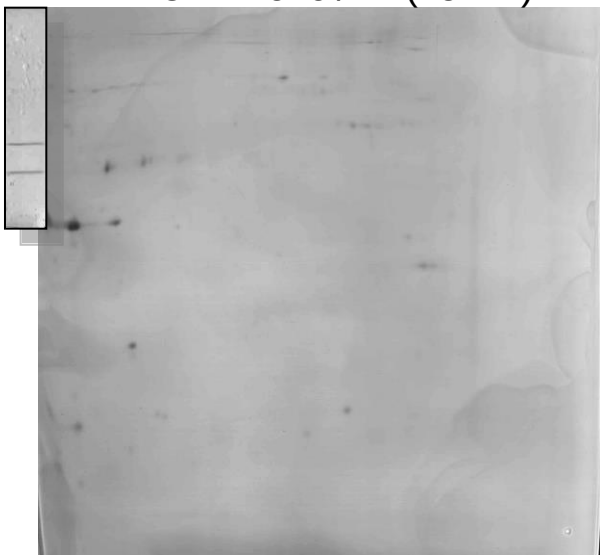
Maximization of secreted protein recovery

Electrophoretic patterns

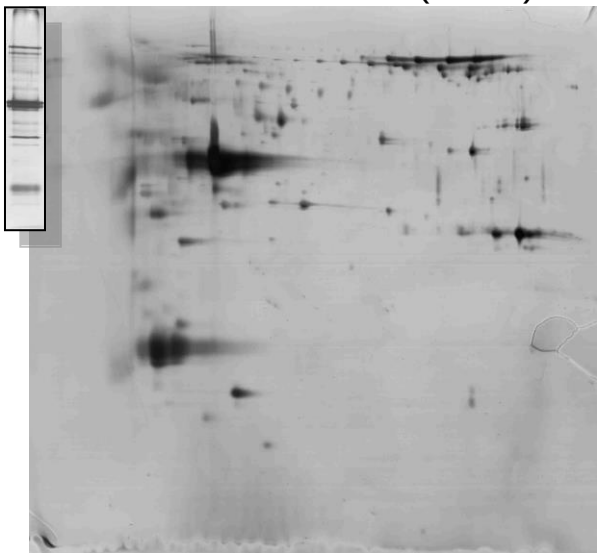
UF (x30/15mL)



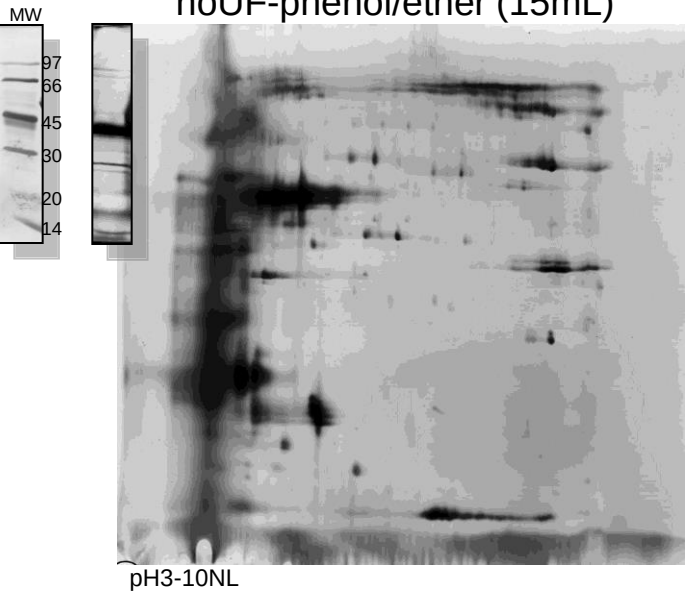
UF-Phenol/AA (15 mL)



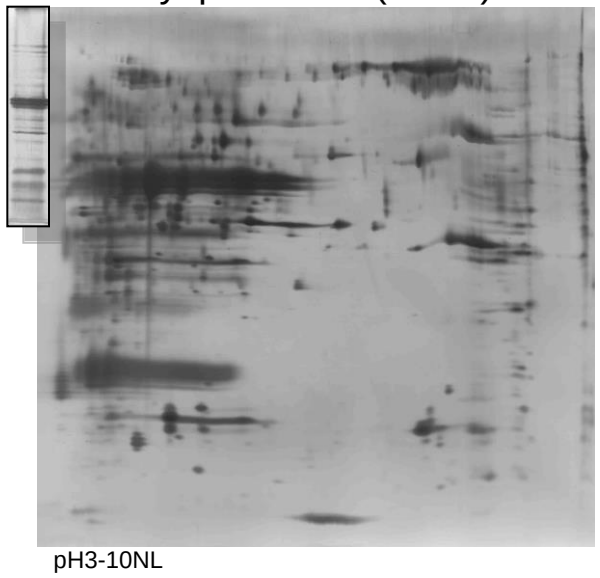
noUF-TCA/acetone (40mL)



noUF-phenol/ether (15mL)



Lyophilization (15mL)



1-D profile enriched in low MW bands

complex 2-D pattern
852 spots

new spots indicating that precipitation methods cause too much protein loss

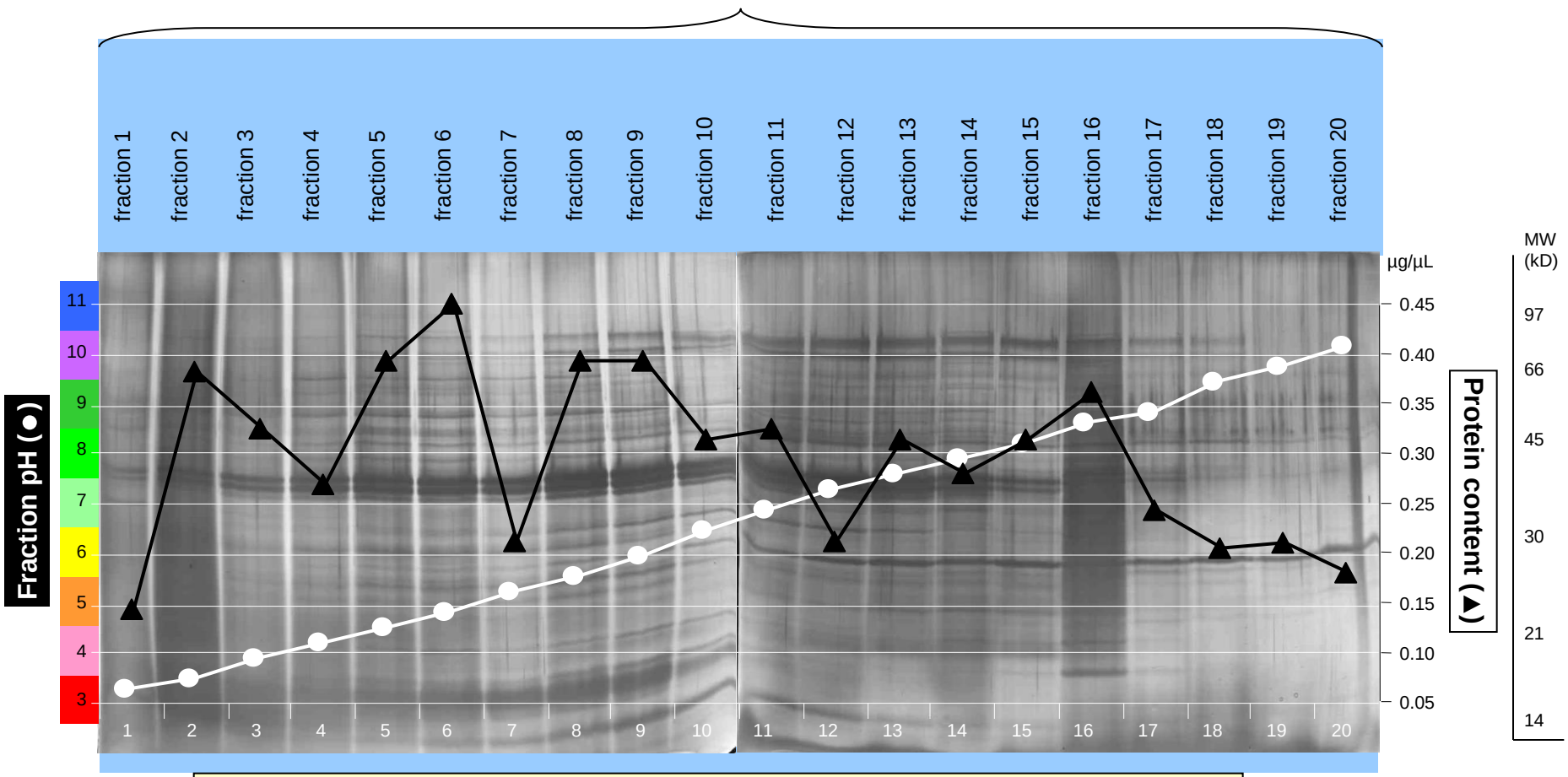
still depletion in low MW basic proteins

Maximization of secreted protein recovery

Pre-fractionation step

Rotofor Cell (Bio-Rad)
large focusing chamber
(50 mL, 20 fractions)

Liquid-phase IEF/SDS-PAGE prior to BR IPG-IEF

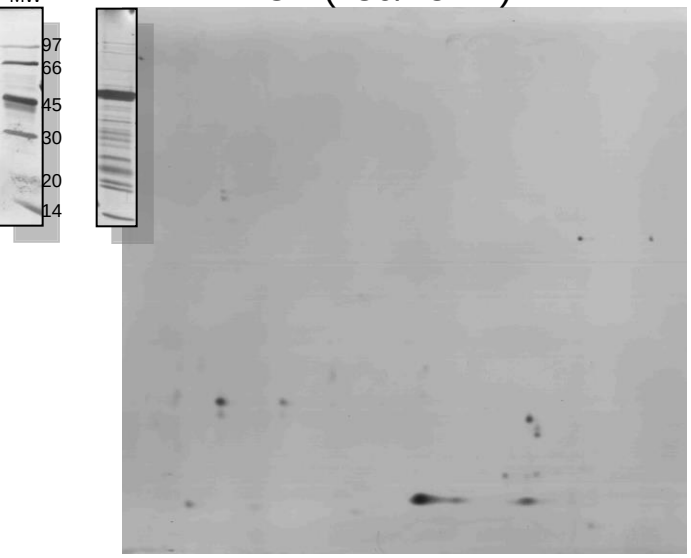


Fraction profiles similar to another (except basic ones) with an increase in band intensity for the fractions bearing close to neutral pH values.

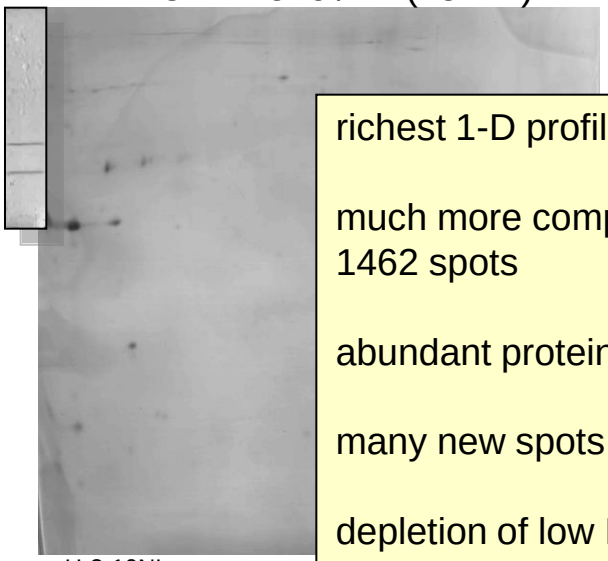
Maximization of secreted protein recovery

Electrophoretic patterns

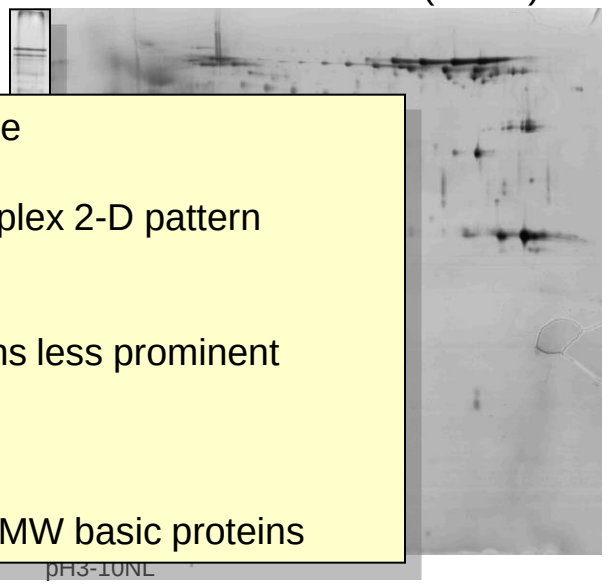
UF (x30/15mL)



UF-Phenol/AA (15 mL)



noUF-TCA/acetone (40mL)



richest 1-D profile

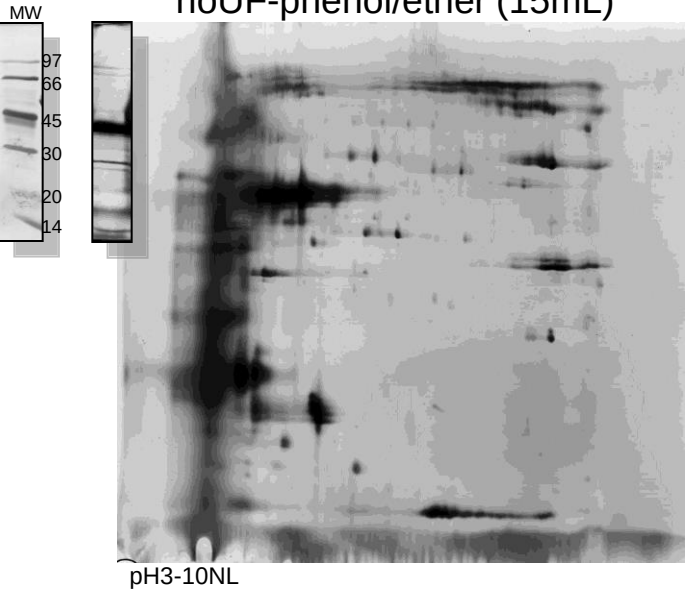
much more complex 2-D pattern
1462 spots

abundant proteins less prominent

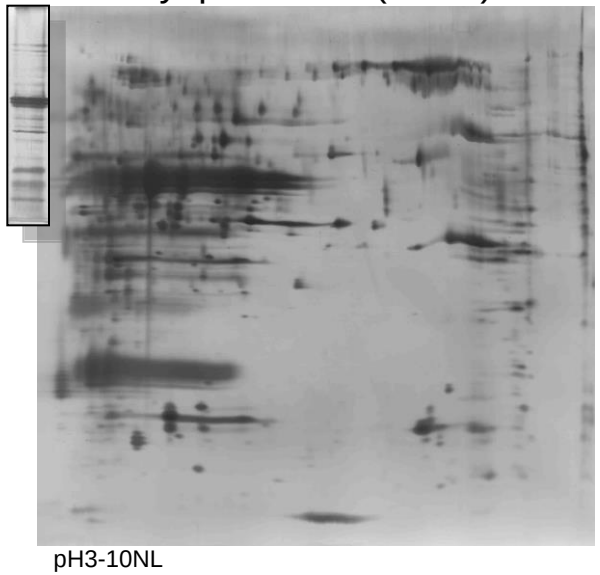
many new spots

depletion of low MW basic proteins

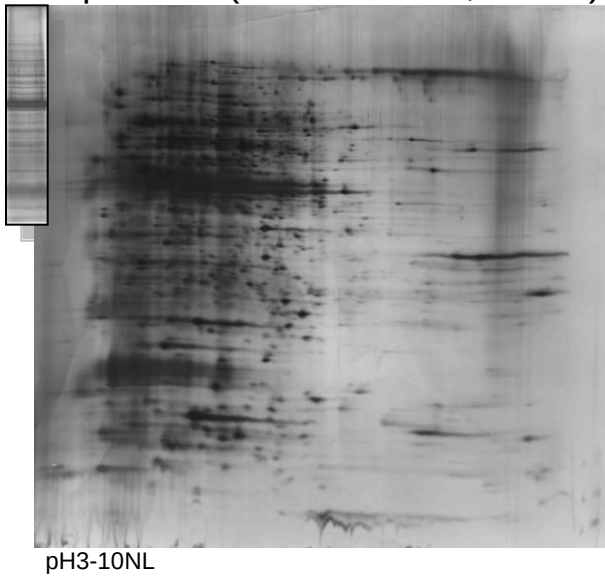
noUF-phenol/ether (15mL)



Lyophilization (15mL)



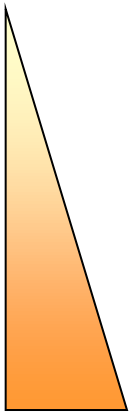
Liq-Ph IEF (fractions 1-20, 30 mL)



Maximization of secreted protein recovery

Quantitative assessment of the gain in protein recovery

extraction method	sample volume	pH range	number of spots
UF (x 30)	15 mL	3-10NL	64
UF-phenol/ammonium acetate	15 mL	3-10NL	180
UF-TCA/acetone	15 mL	3-10NL	266
noUF-TCA/acetone	40 mL	3-10NL	449
noUF-Phenol/Ether	15 mL	3-10NL	341
lyophilization	15 mL	3-10NL	852
liquid-phase IEF	30 mL	3-10NL	1462



A prefractionation step using liquid-phase IEF prior to 2-DE limits the prominence of the extremely abundant proteins (acidic high MW in particular) thus unraveling less abundant proteins (alkaline).

Because liquid-phase IEF generates discrete fractions according to protein pI, it enables the use of a variety of pH gradients during IEF in order to optimize protein separation conditions.

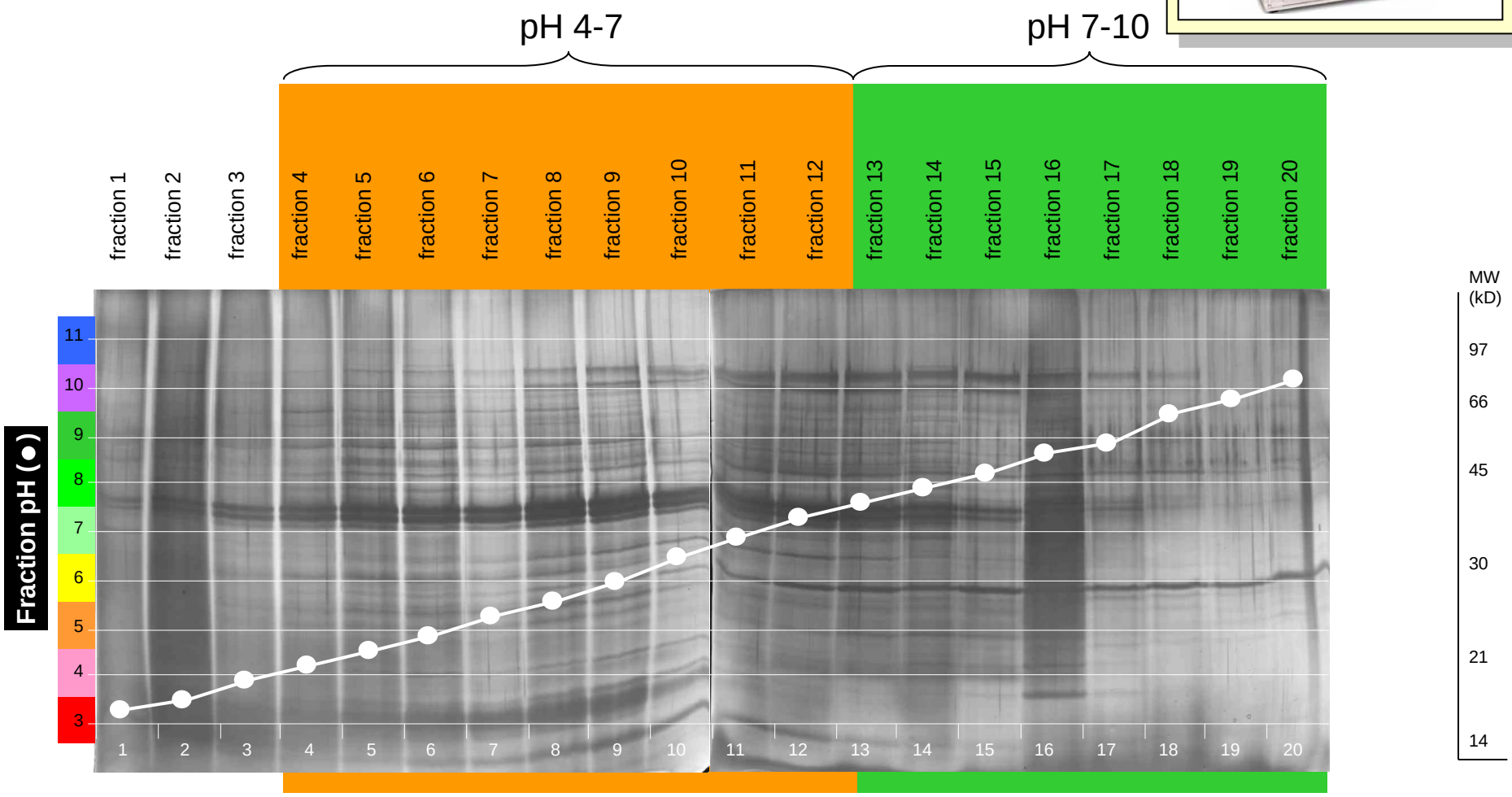
Optimization of spot resolution

Maximization of secreted protein recovery

Pre-fractionation step

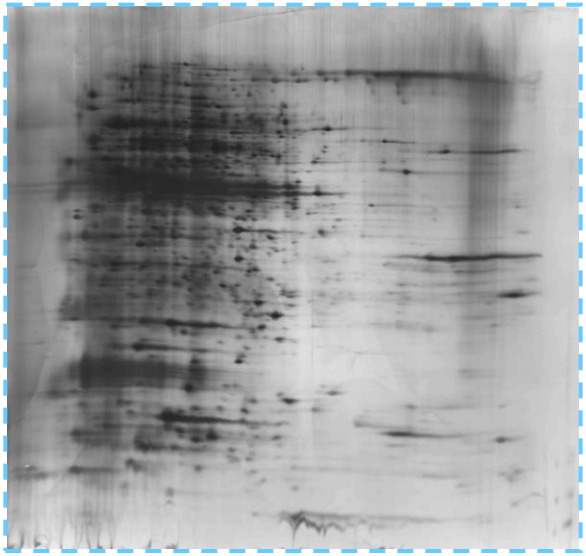
Rotofor Cell (Bio-Rad)
large focusing chamber
(50 mL, 20 fractions)

Liquid-phase IEF/SDS-PAGE prior to NR IPG-IEF



Optimization of spot resolution

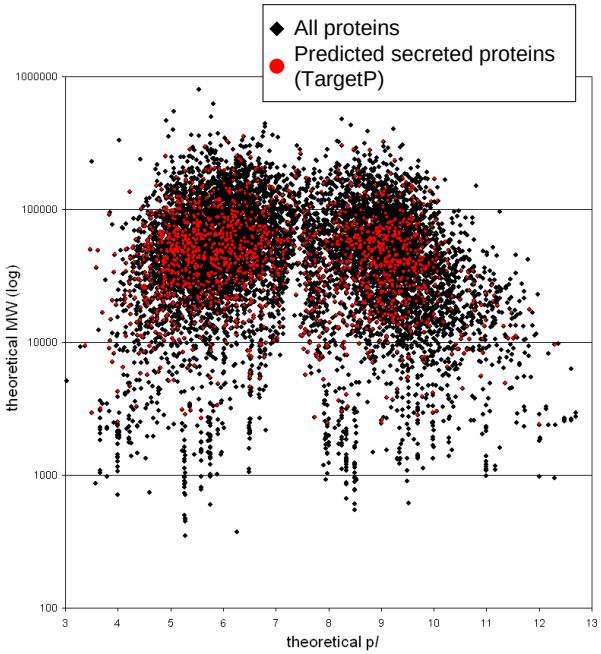
Spot resolution enhanced using NR IPG strips



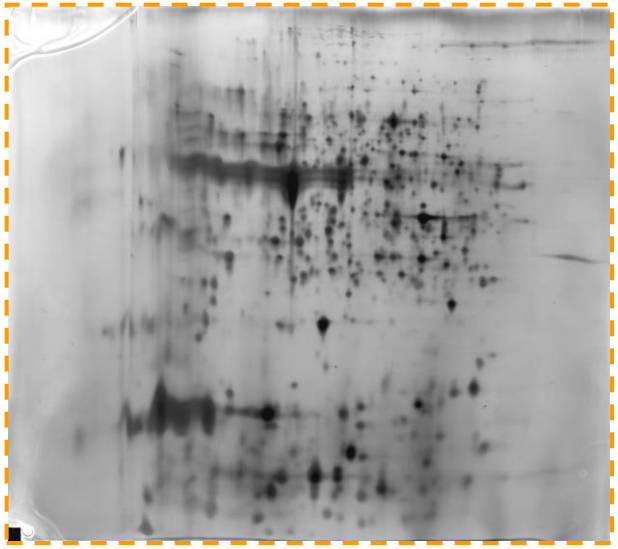
pH3-10NL

Liquid-Phase IEF

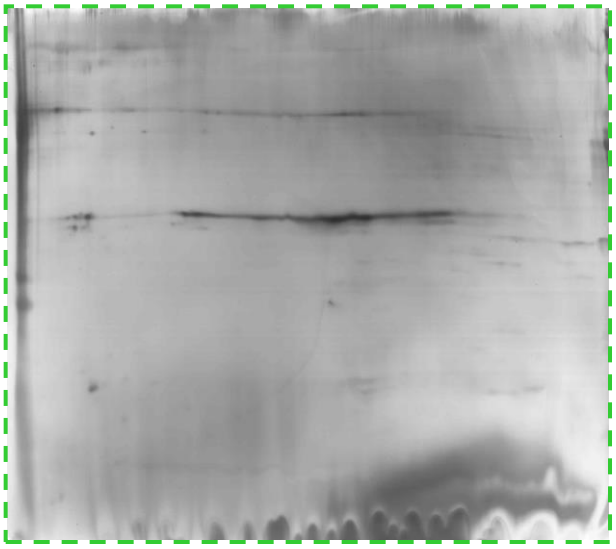
Rotofor Cell (Bio-Rad)
large focusing chamber
(50 mL, 20 fractions)



Liquid-Phase IEF



pH 4-7 (fractions 4-12)

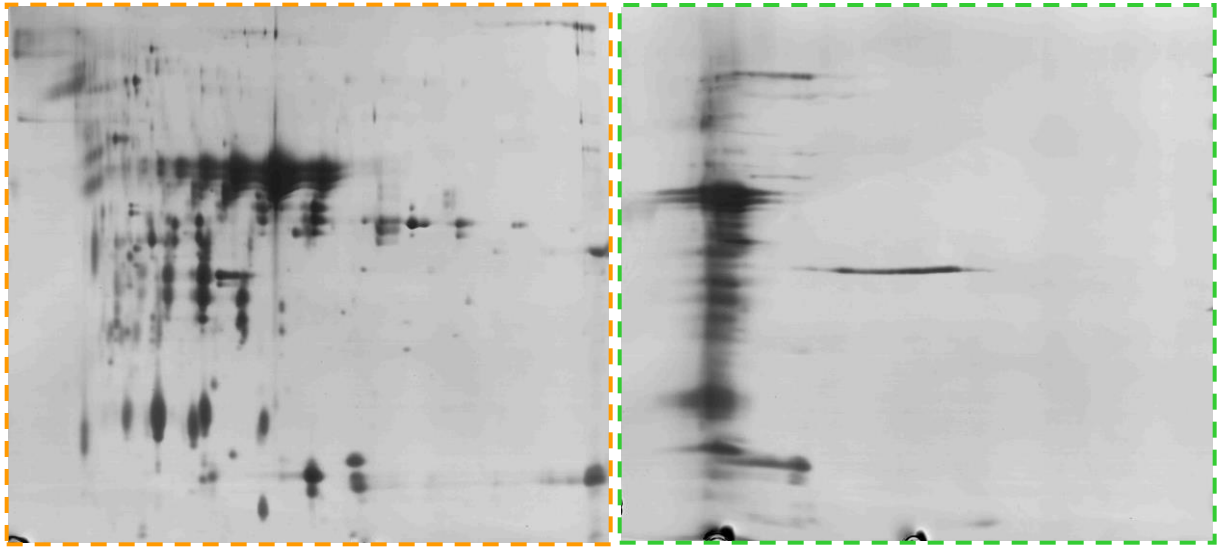


pH 7-10 (fractions 13-20)

Optimization of spot resolution

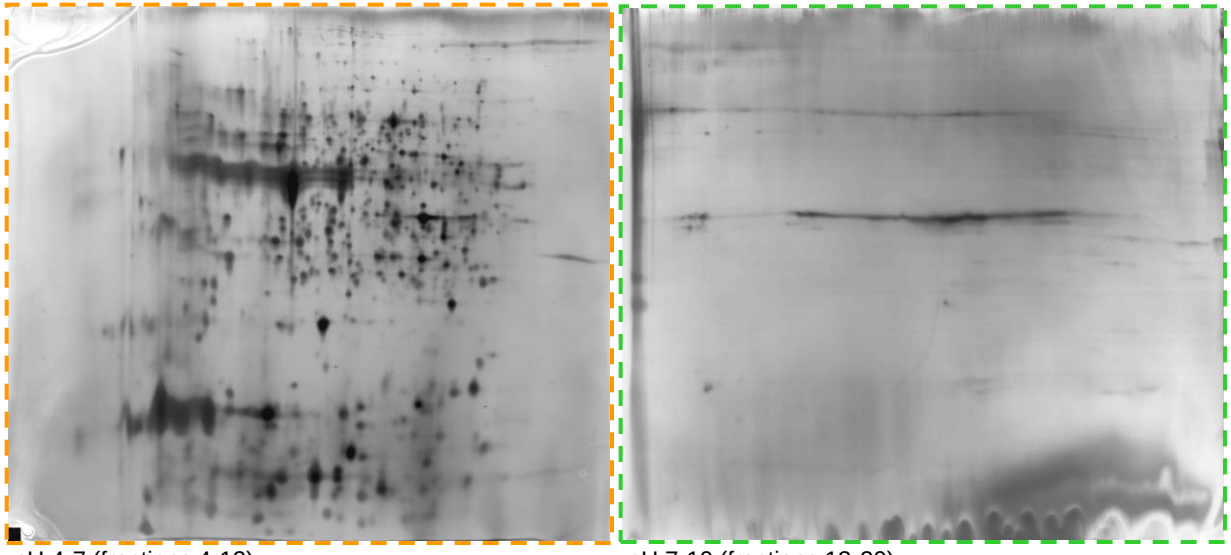
Spot resolution enhanced using NR IPG strips

Lyophilization



pH 4-7 pH 7-10

Liquid-Phase IEF



pH 4-7 (fractions 4-12) pH 7-10 (fractions 13-20)

Much more proteins can be resolved using Rotofor

Huge gain in resolution of acidic spot (pH 4-7)

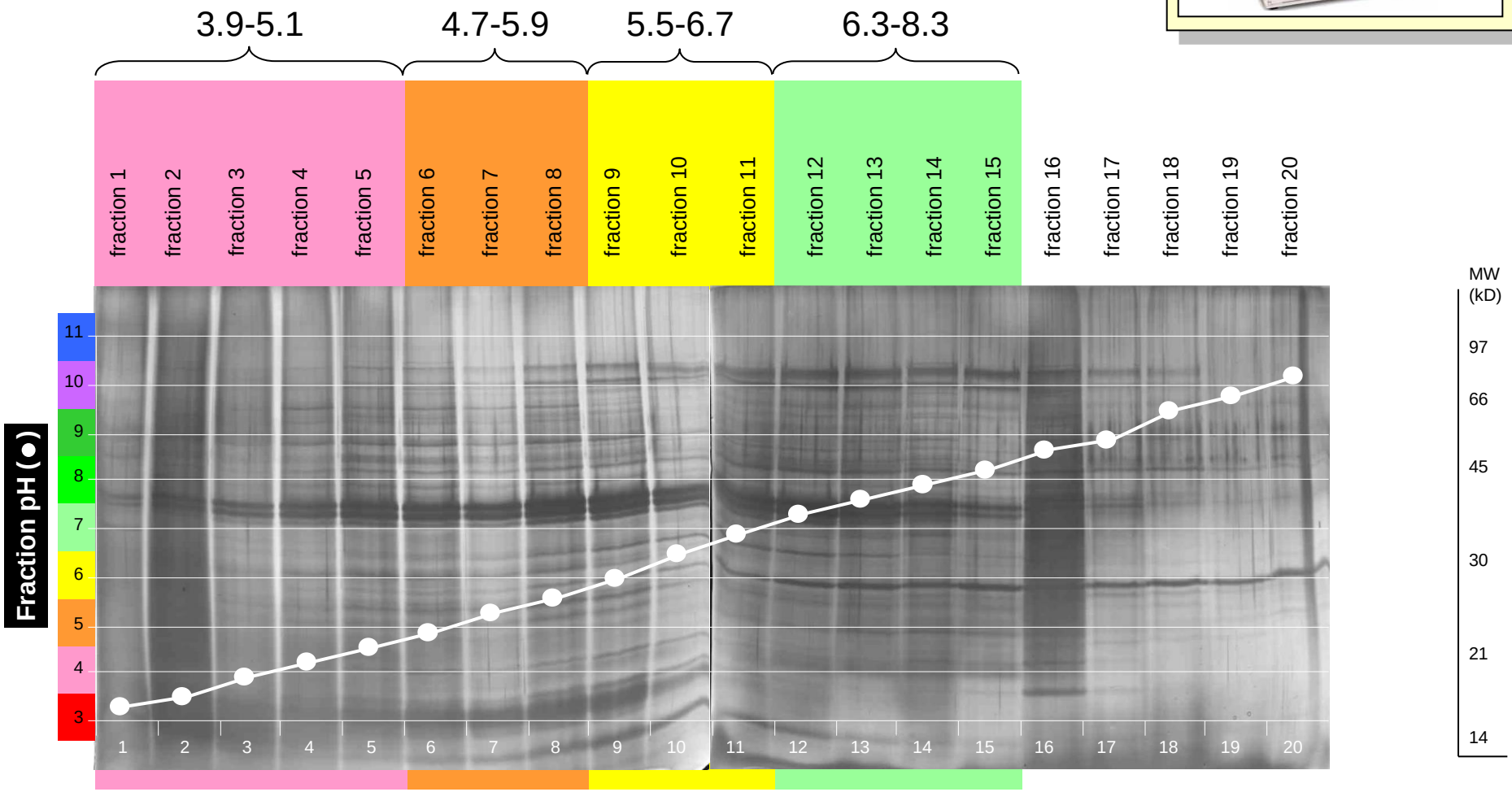
Basic proteins recalcitrant to IEF (pH 7-10). Same observation on mycelial proteins (data not shown)

Maximization of secreted protein recovery

Pre-fractionation step

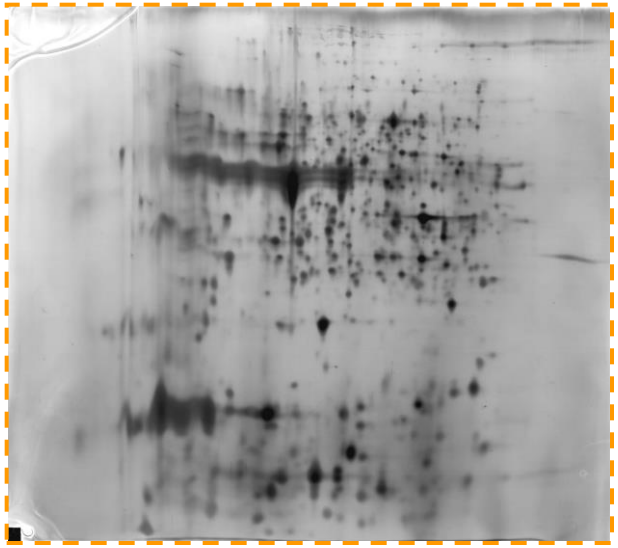
Rotofor Cell (Bio-Rad)
large focusing chamber
(50 mL, 20 fractions)

Liquid-phase IEF/SDS-PAGE prior to MR IPG-IEF



Optimization of spot resolution

Spot resolution enhanced using MR IPG strips

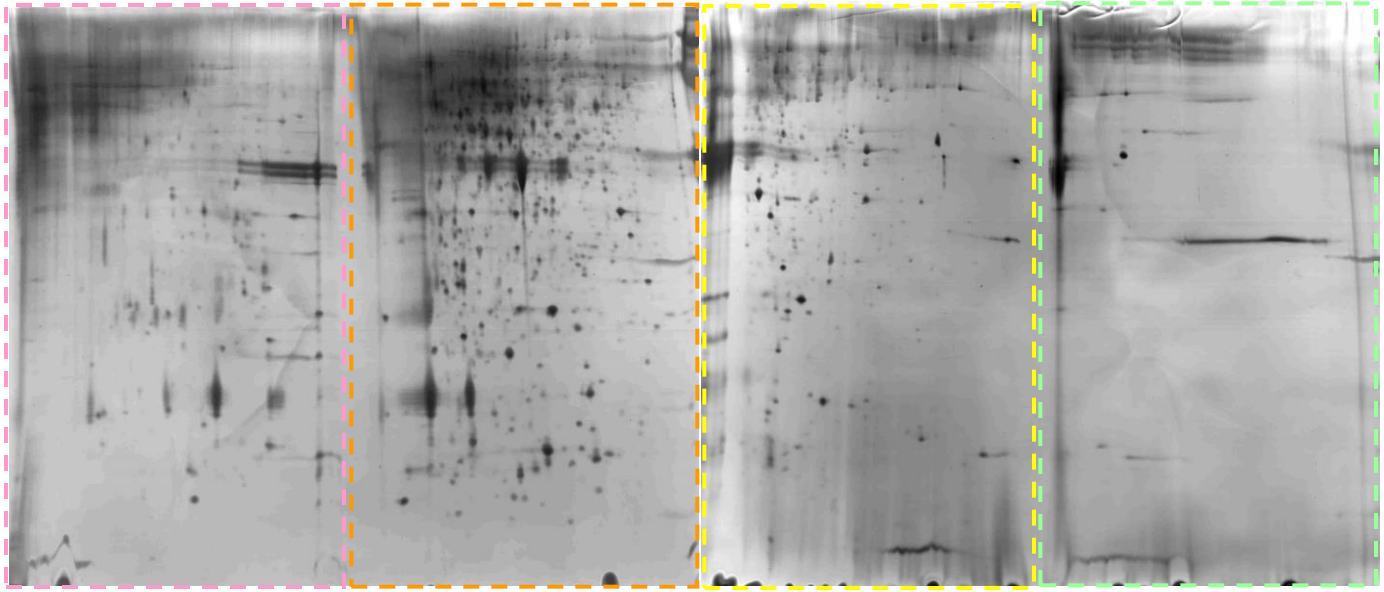


pH 4-7 (fractions 4-12)

Liquid-Phase IEF

Rotofor Cell (Bio-Rad)
large focusing chamber
(50 mL, 20 fractions)

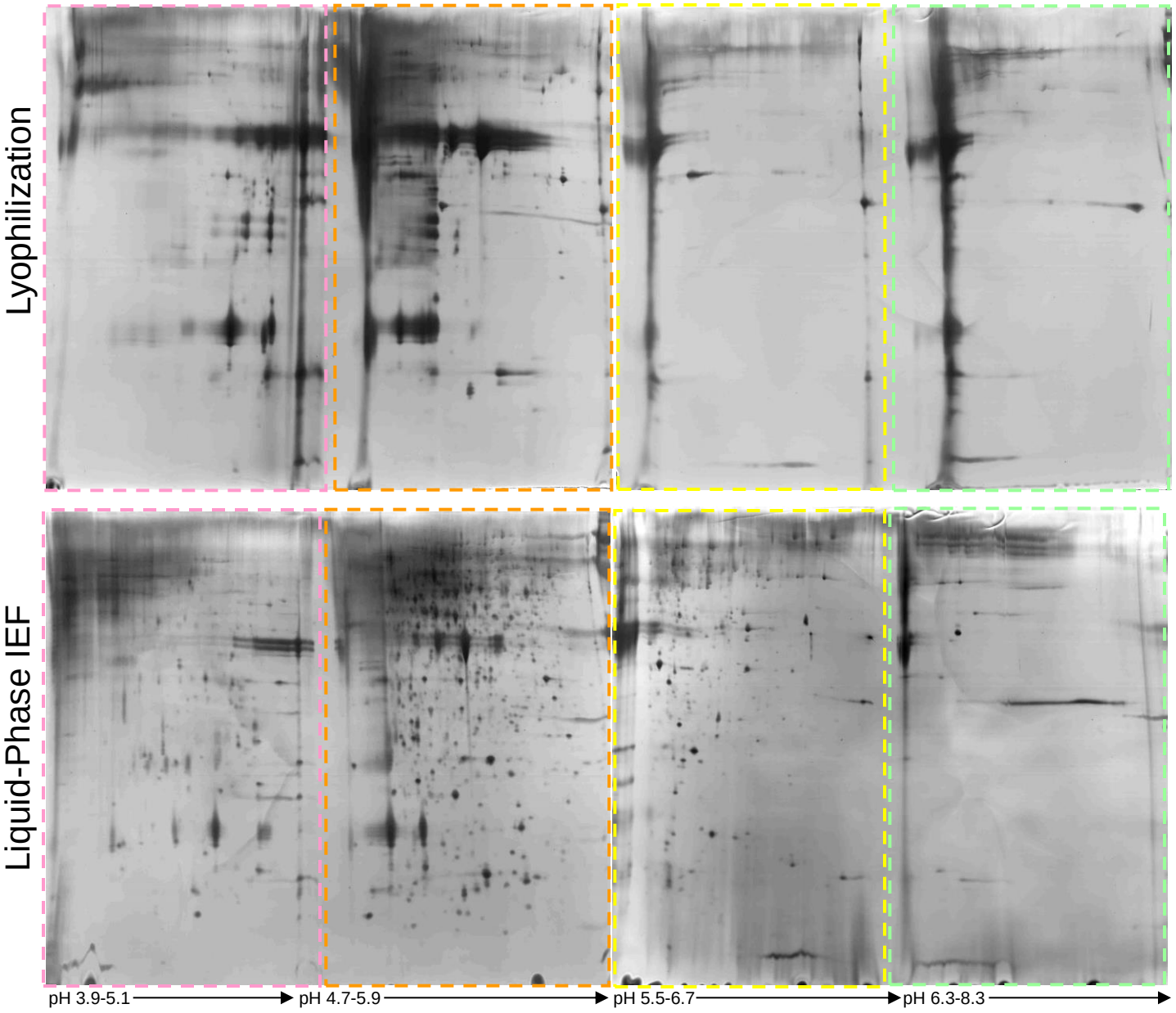
Liquid-Phase IEF



pH 3.9-5.1 → pH 4.7-5.9 → pH 5.5-6.7 → pH 6.3-8.3 →

Optimization of spot resolution

Spot resolution enhanced using MR IPG strips



Gain in spot number and resolution even more evident upon using MR pH ranges.

Optimization of spot resolution

Quantitative assessment of the gain in spot resolution (*L. maculans*)

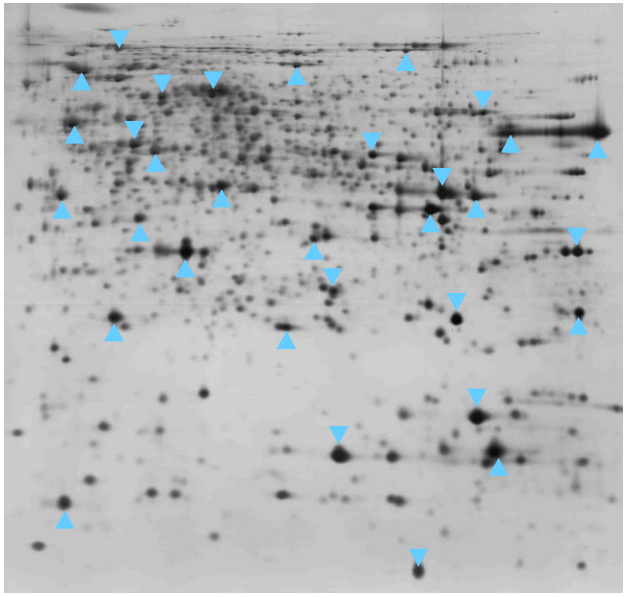
tissue	extraction method	pH range	number of spots	total	global range	length
mycelium						
	TCA/acetone	3-10NL	1862 →	1862	3-10NL	24 cm
		4-7	3632	4493	4-10	48 cm
		7-10	861			
		3.9-5.1/4.7-5.9	2836	4260	3.9-8.3	44 cm
		5.5-6.7/6.3-8.3	1424			
secretome						
	lyophilization	3-10NL	852 →	852	3-10NL	24 cm
		4-7	519	642	4-10	48 cm
		7-10	123			
		3.9-5.1/4.7-5.9	766	986	3.9-8.3	44 cm
		5.5-6.7/6.3-8.3	220			
	liquid-phase IEF	3-10NL	1740 →	1740	3-10NL	24 cm
		4-7	961	1006	4-10	48 cm
		7-10	45			
		3.9-5.1/4.7-5.9	1306	1941	3.9-8.3	44 cm
		5.5-6.7/6.3-8.3	635			

Protein identification

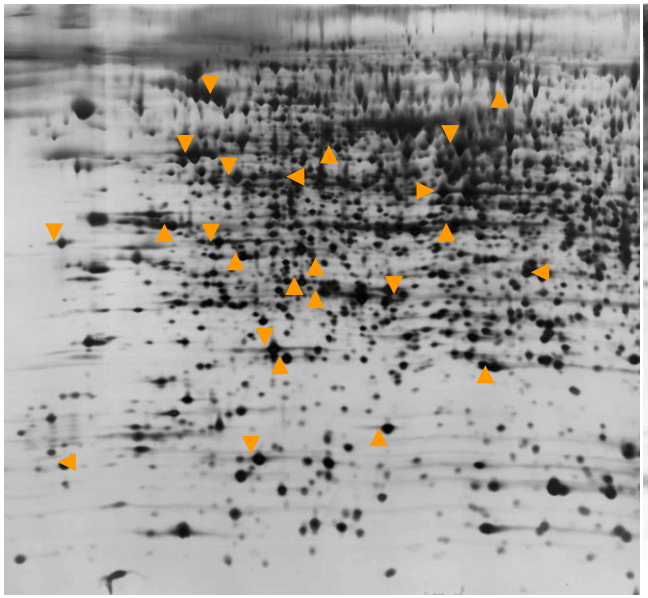
Protein identification

Gain in spot resolution confirmed by MS (secretome of *L. maculans*)

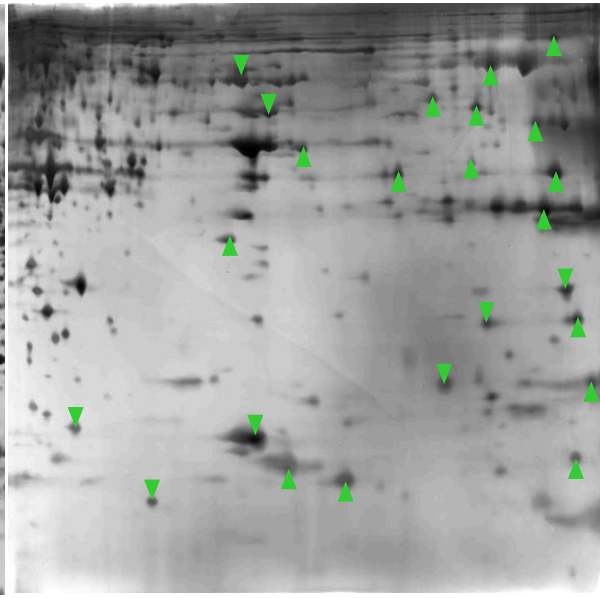
Mycelium



pH3-10NL, 32 spots

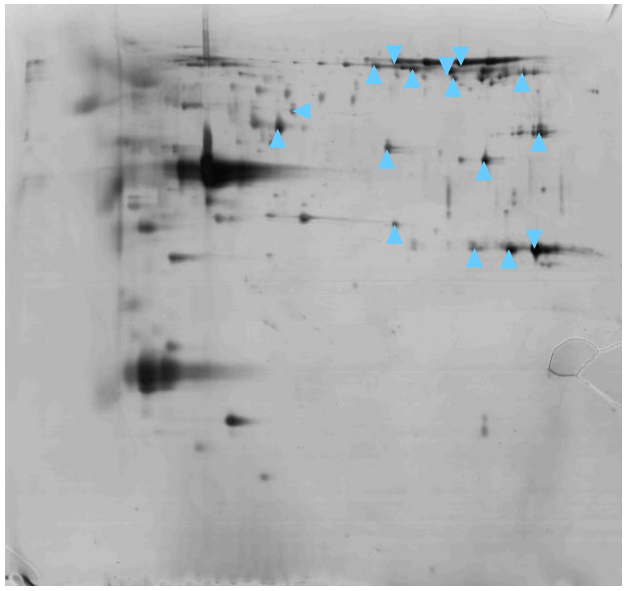


pH4-7, 24 spots

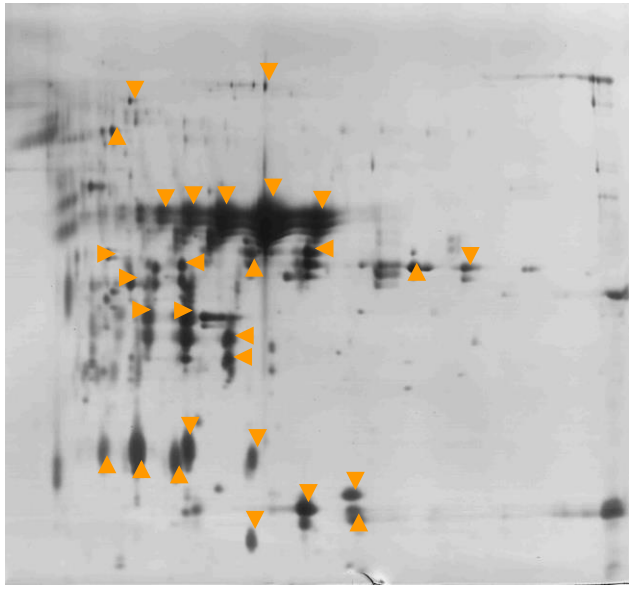


pH7-10, 24 spots

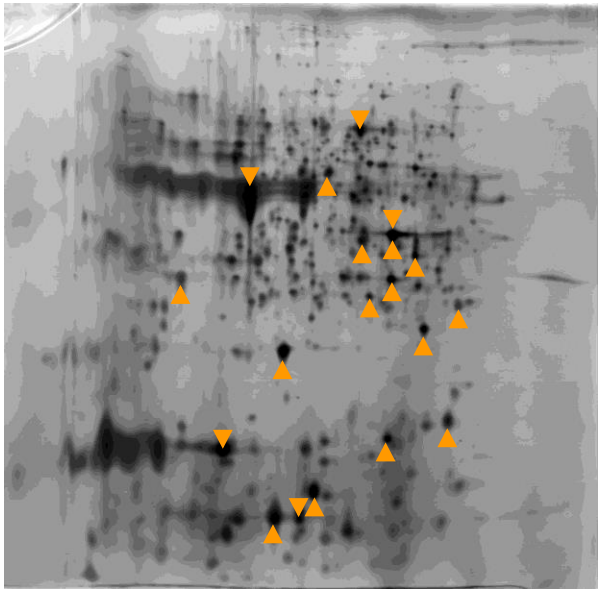
Secretome



pH3-10NL, 16 spots



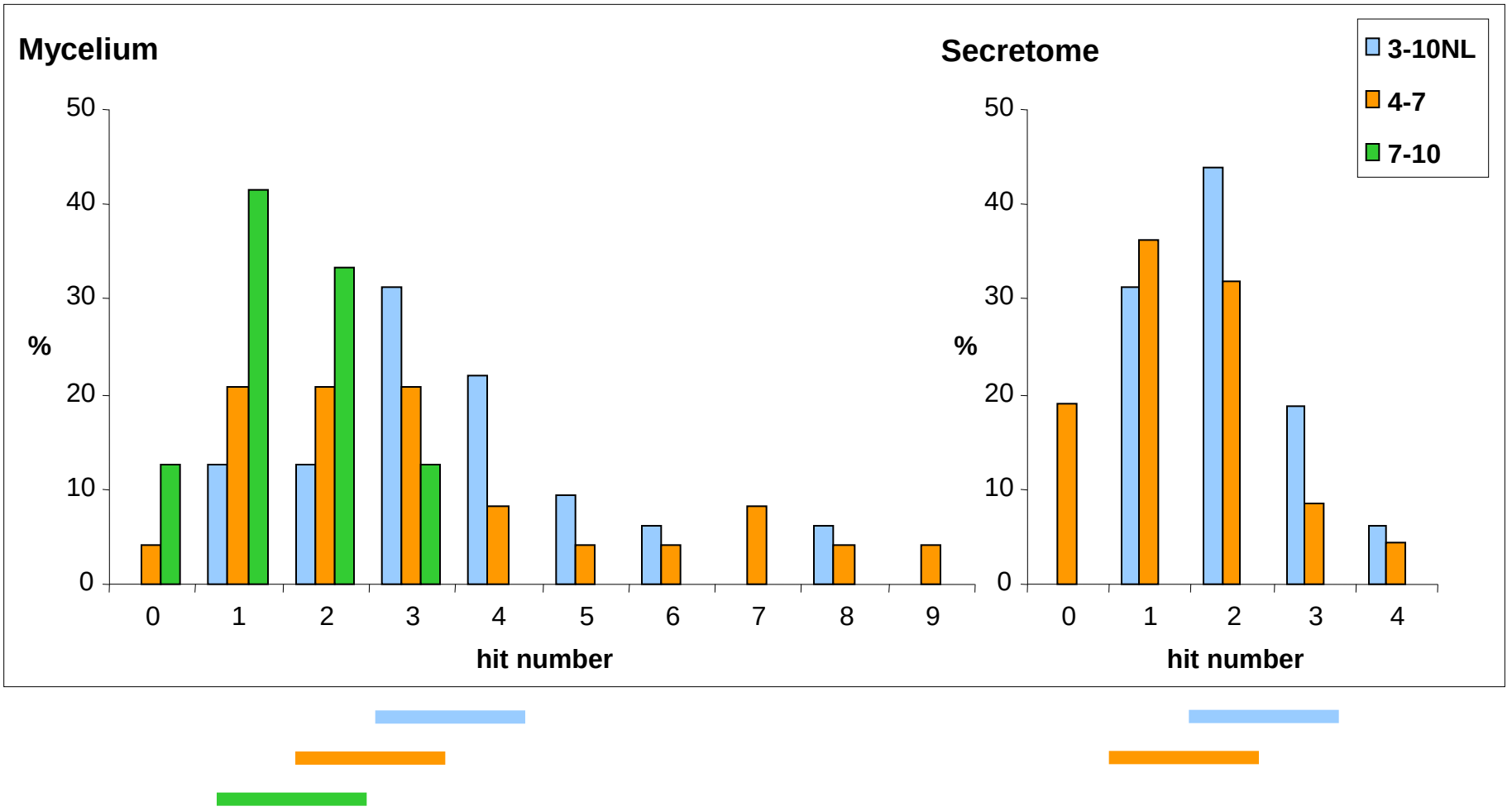
pH4-7, lyophilized, 24 spots



pH4-7, rotofor, 23 spots

Protein identification

Gain in spot resolution confirmed by MS (secretome of *L. maculans*)



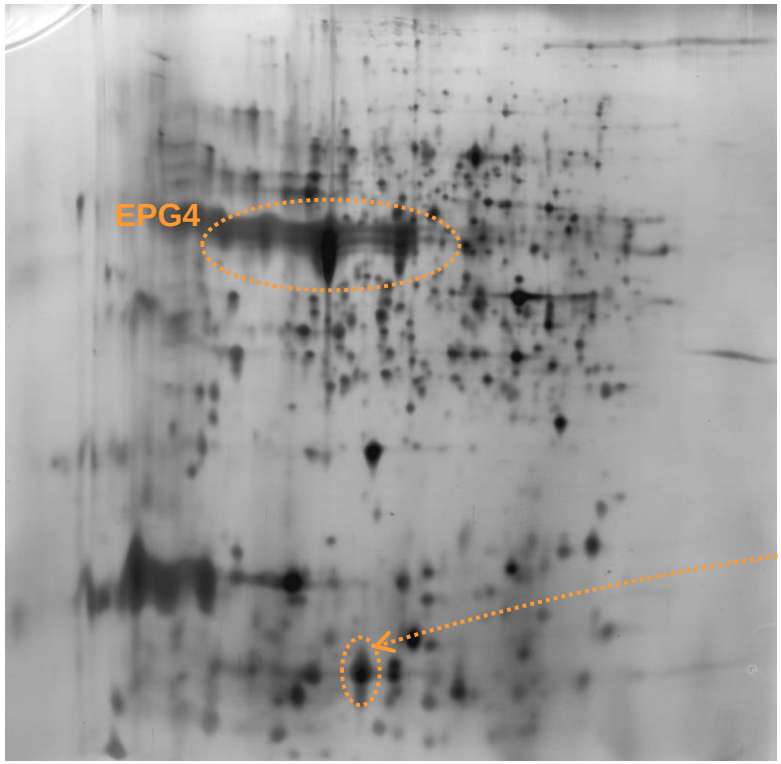
Less proteins are identified within a single spot when narrower pH gradient are used, further demonstrating the enhanced spot resolution also visible on 2-D gels.

Conclusions

- Protein extraction through precipitation methods (TCA or Phenol protocol) caused excessive losses of the secreted proteins.
- Freeze-drying the samples overcame protein losses, yet the extremely abundant proteins proved difficult to resolve and obscured the proteins present in lesser quantities.
- Superior 2-D patterns were obtained after an initial prefractionation step with liquid-phase IEF (Rotofor), which, by reducing the prominence of major proteins, made way for minor proteins and, combined to 2-DE, yielded complex 2-D patterns. This result was confirmed on *Laccaria bicolor* (data not shown).
- Although theoretical pI/MW computations predicted as many acidic proteins as basic ones in *L. maculans* species, the recovery of secreted alkaline proteins was too moderate to produce acceptable 2-D patterns. Same observation on *L. bicolor* (not shown).
- Improved spot resolution was achieved using narrow pH gradient, especially within acidic ranges and confirmed by MS-based protein identification (data not shown).

Perspectives

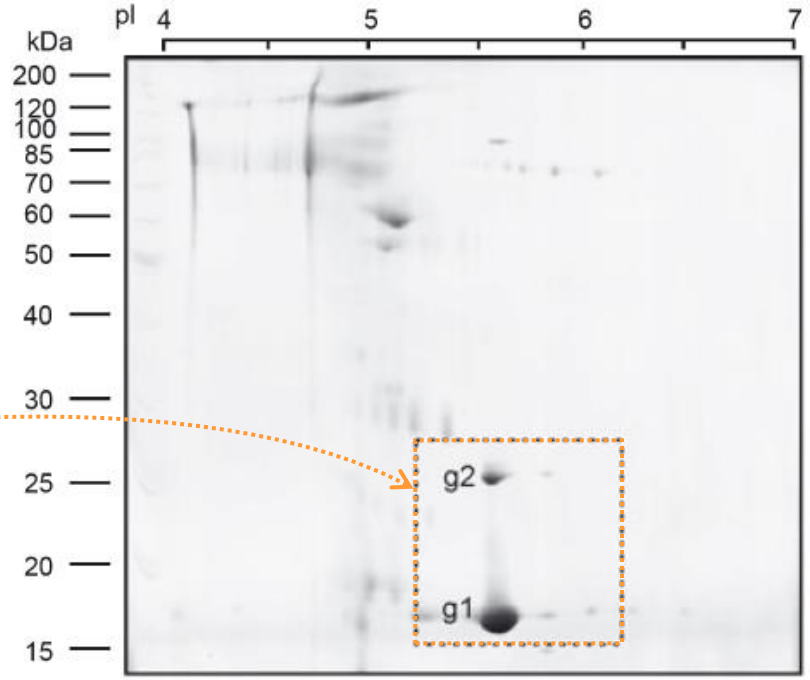
MS results from *L. maculans* secretome are consistent with extracellular proteins and enzymes either protecting the fungus cell wall integrity (*chitin-binding protein, gpi-anchored cell wall organization protein ecm33, gpi-anchored cell wall beta-endoglucanase*) or participating to the degradation of the host cell wall by targeting plant polysaccharides (*pectate lyase a, alpha- and beta-glucosidase, glucoamylase, glycosyl hydrolase, beta-glucanosyltransferase bgt1, endopolygalacturonase 4*). Epl1, assumed to participate to plant pathogenesis and elicitation of plant defense responses (Seidl *et al*., 2006), is also massively secreted. There are many isoforms. Many proteins are of unknown function. Such approach should help identifying new fungal effectors .



2006. Vol 273: 4346-59 the FEBS Journal

Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors

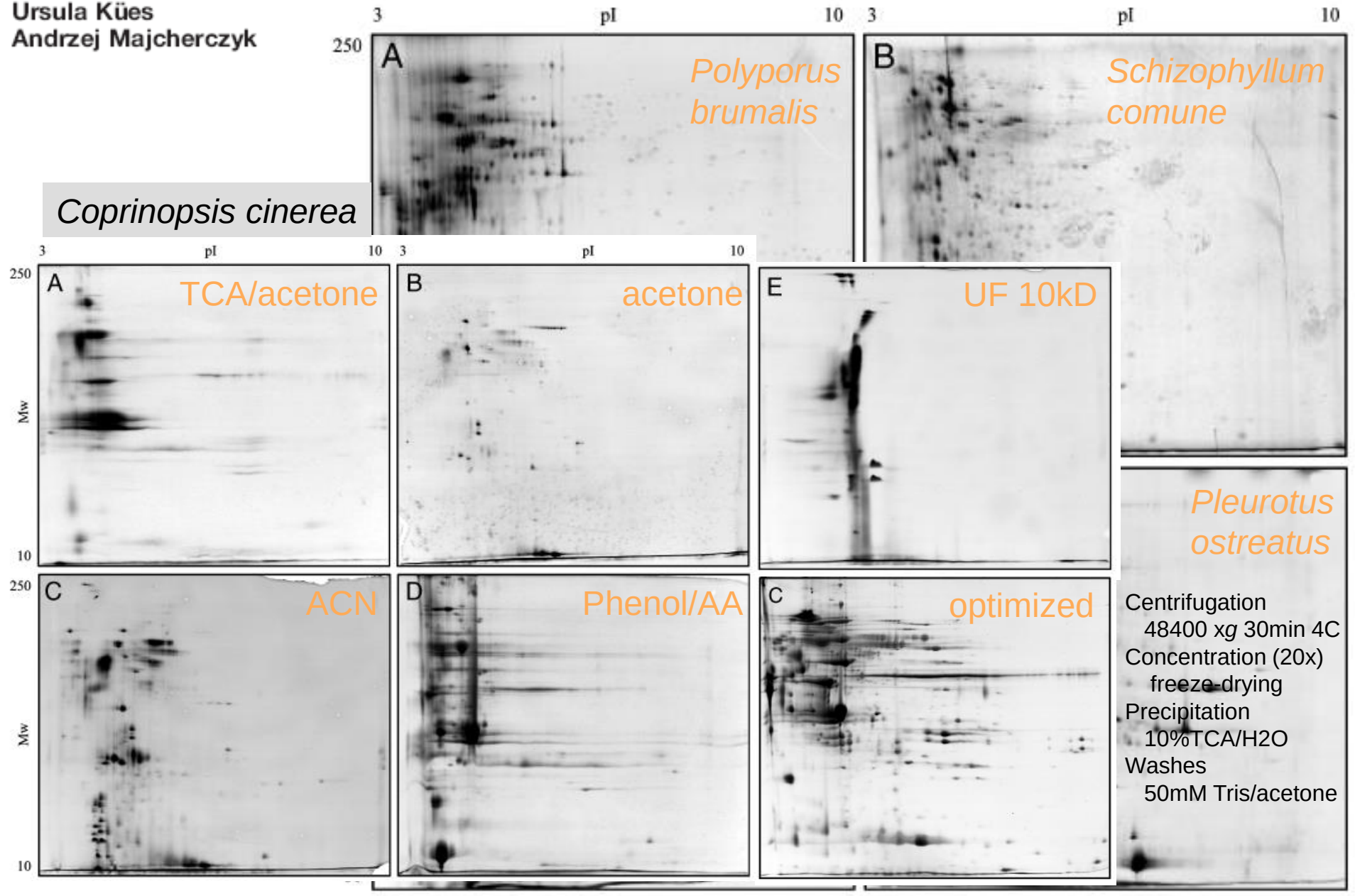
Verena Seidl[1], Martina Marchetti[2], Reingard Schandl[1,2], Günter Allmaier[2] and Christian P. Kubicek[1]



Electrophoresis 2009, *30*, 2431–2441

Dorothea Fragner
Mojtaba Zomorrodi
Ursula Kües
Andrzej Majcherczyk

Research Article



2 -

Increasing protein identification coverage

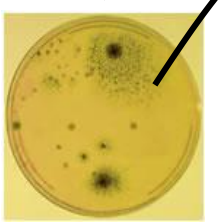
Identifications of secreted proteins from Laccaria bicolor

Materials & Methods

Materials & Methods

Culture of *L. bicolor,* mycorrhizal symbiote

P5L medium
0.5g/L di-NH4 tartrate
1g/L KH2PO4
0.5g/L MgSO4 7H2O
5g/L maltose D+
20g/L glucose D+
1mL/L thiamine-HCl
1mL/L 10% Kanieltra



stiring for 5 weeks

filtering

mycelium

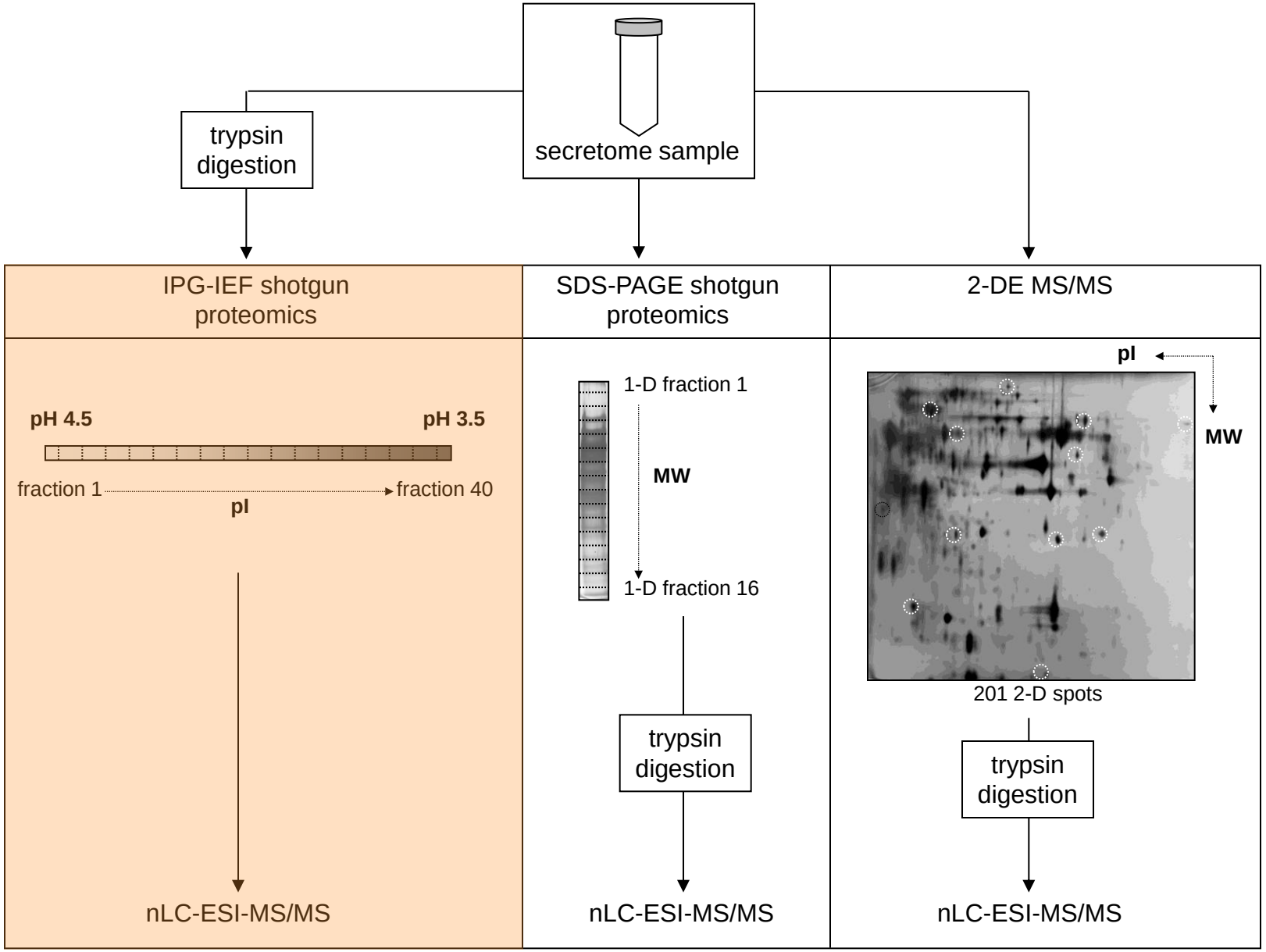
secretome (medium)

Filtration 0.22µm
Dialysis (H2O)
0.05% PVP
1mM PMSF

Lyophylization
Resolublization
in IEF buffer

Materials & Methods

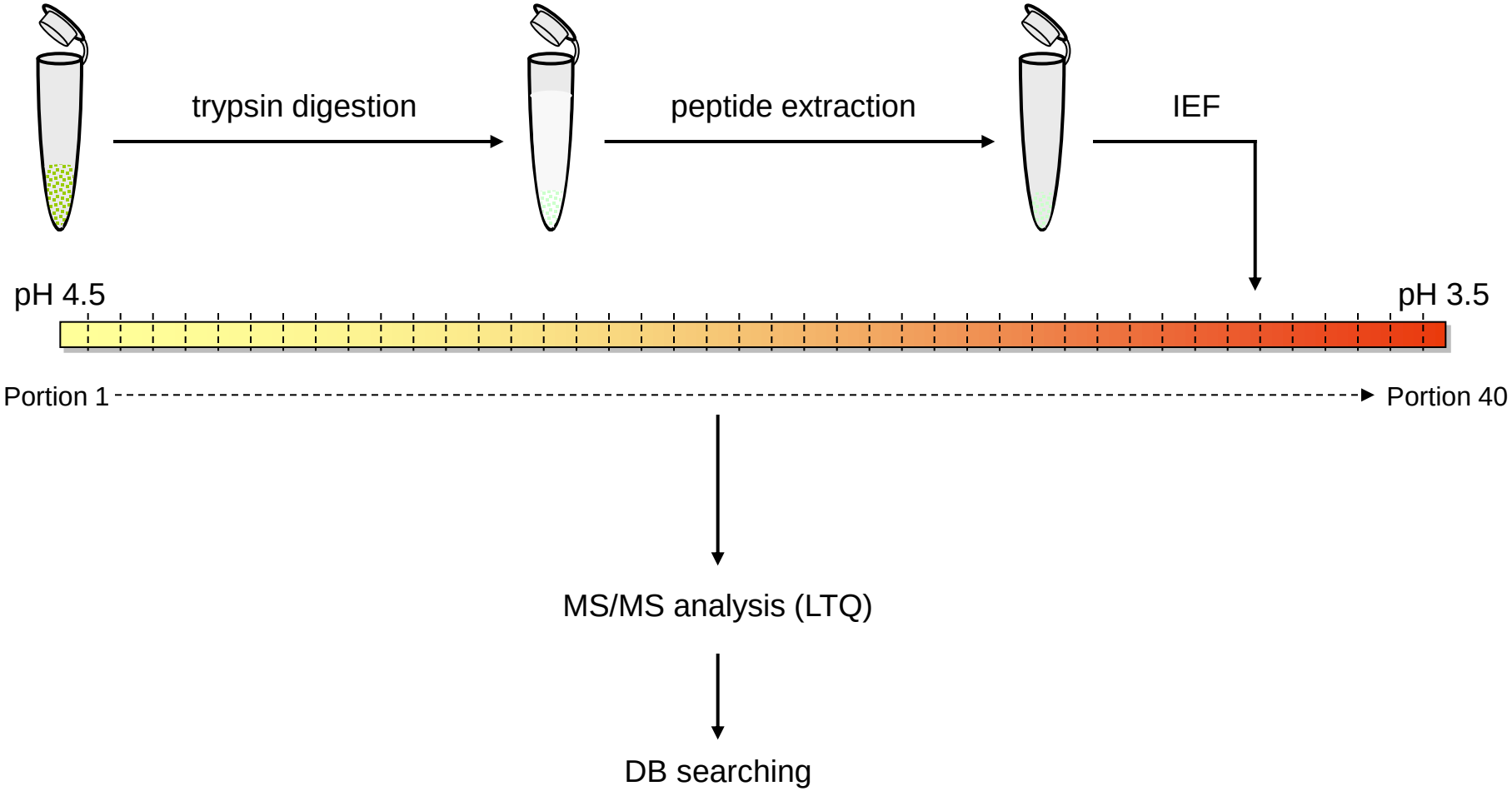
Hunting down secreted proteins!



Hunting down secreted proteins!

IPG shotgun (Essader *et al*. 2005 Proteomics 5: 24-34)

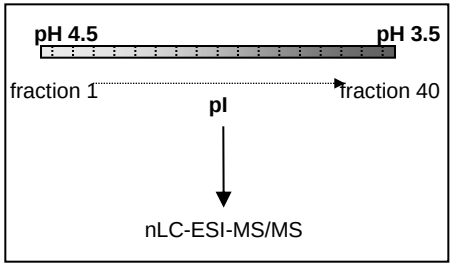
IPG strip pH 3.5-4.5, 18cm, 40 x 4.5 mm-portions



Complementary approaches

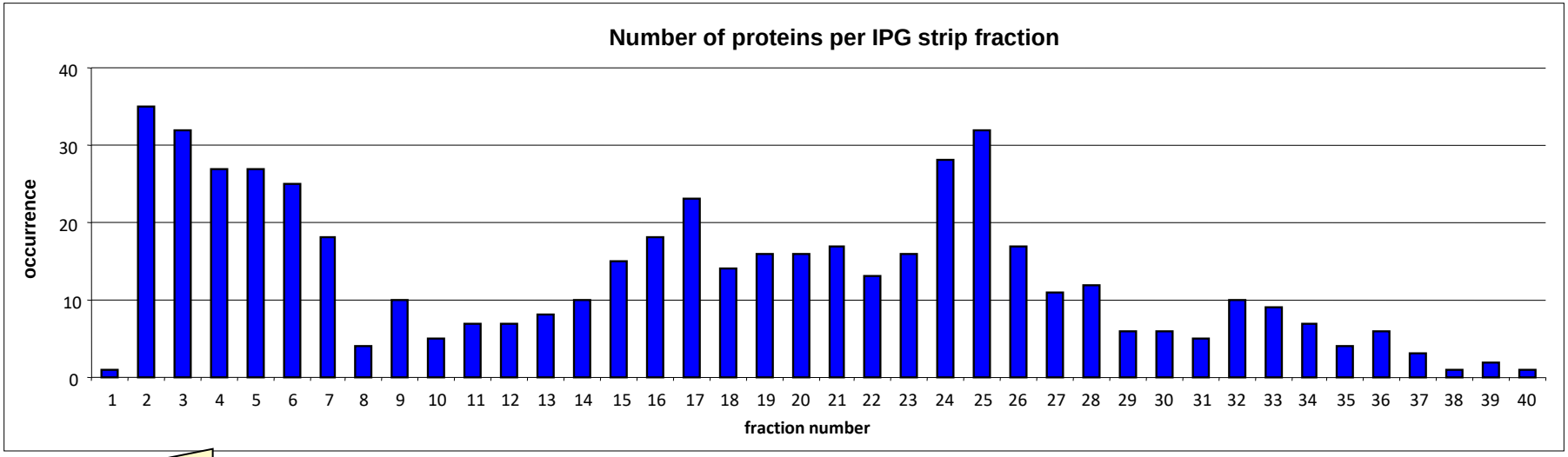
Complementary approaches

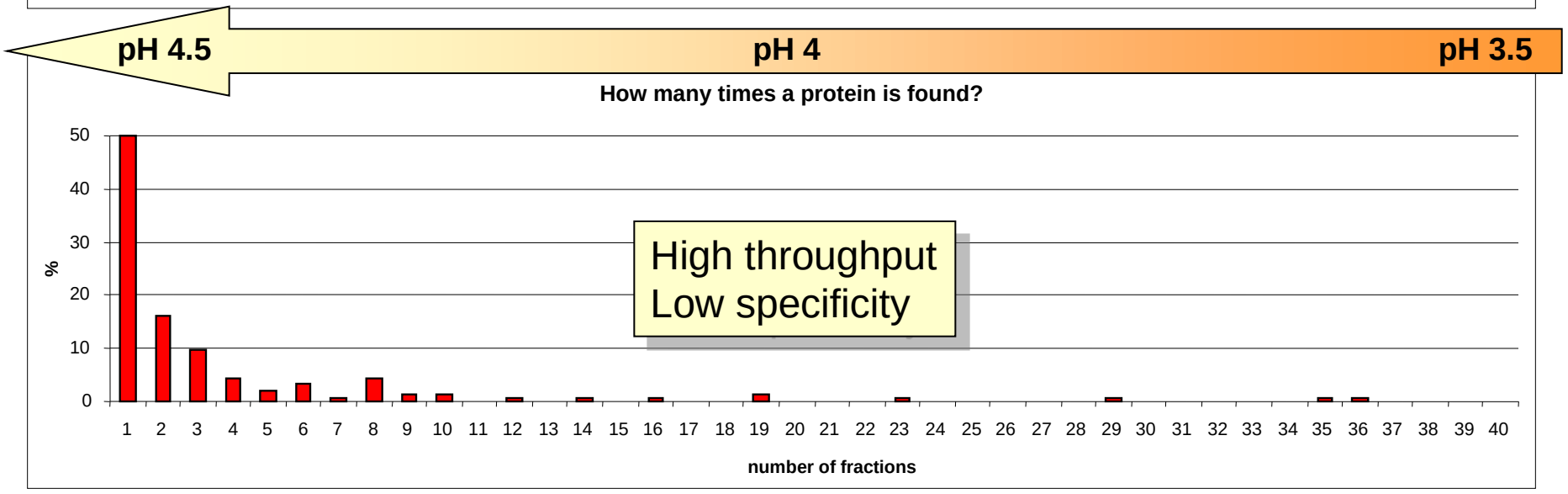
IPG-IEF shotgun proteomics (secretome only)



524 secreted proteins identified in total

→ 142 unique proteins (50% redundancy)



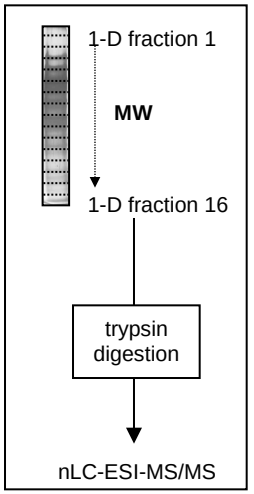


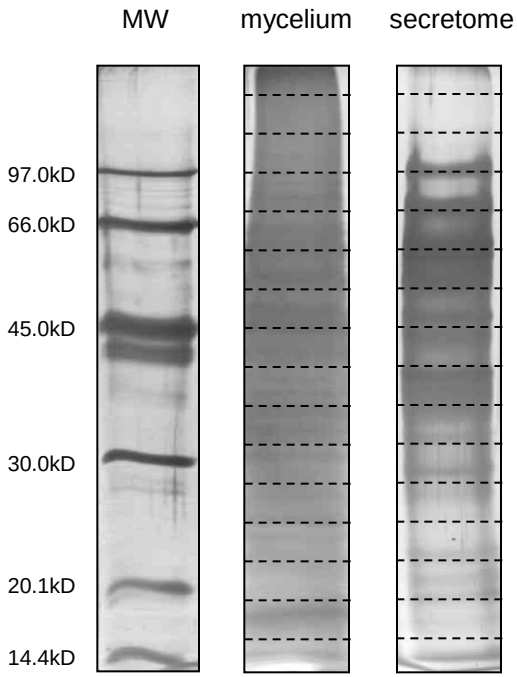
Complementary approaches

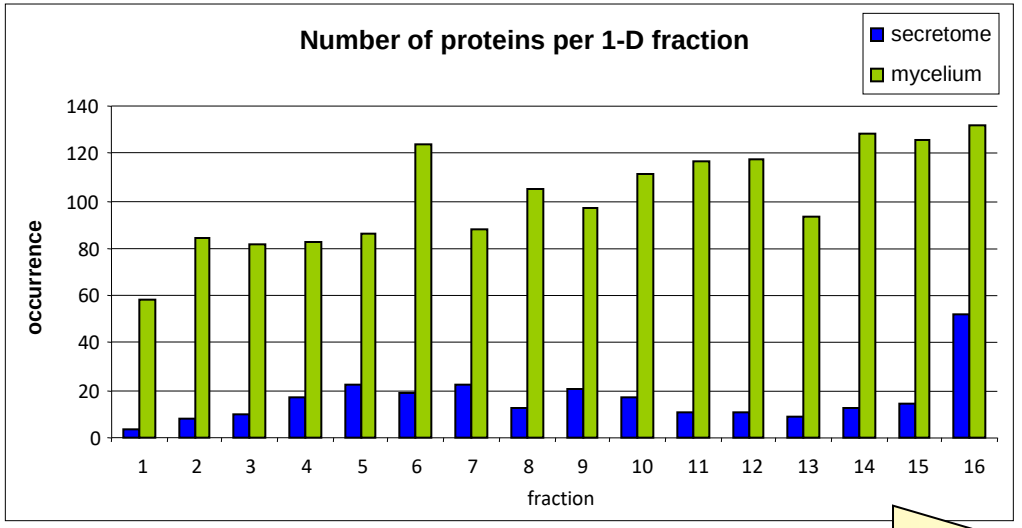
SDS-PAGE shotgun proteomics (secretome and mycelium)

264 secreted proteins identified in total
→ 116 unique proteins (40% redundancy)

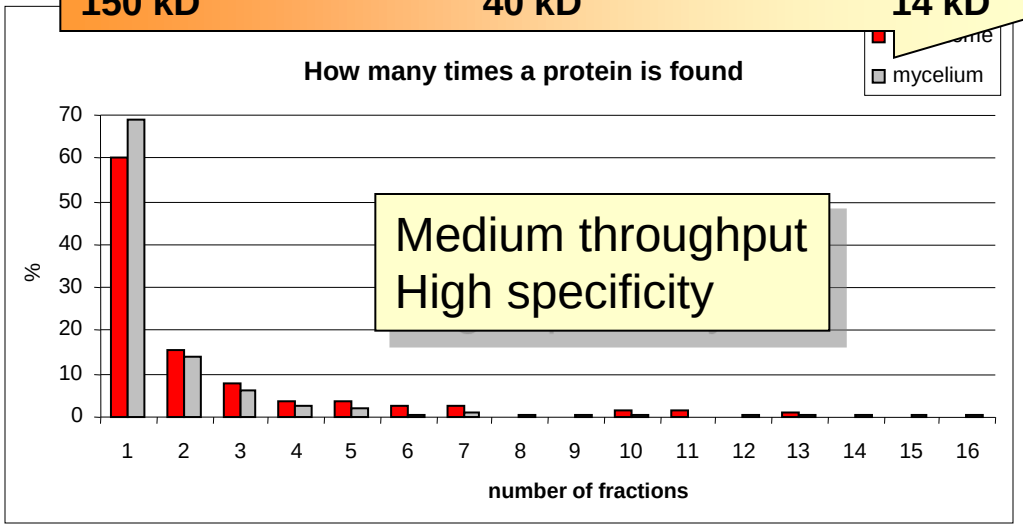
1632 mycelial proteins identified in total
→ 815 unique proteins (31% redundancy)







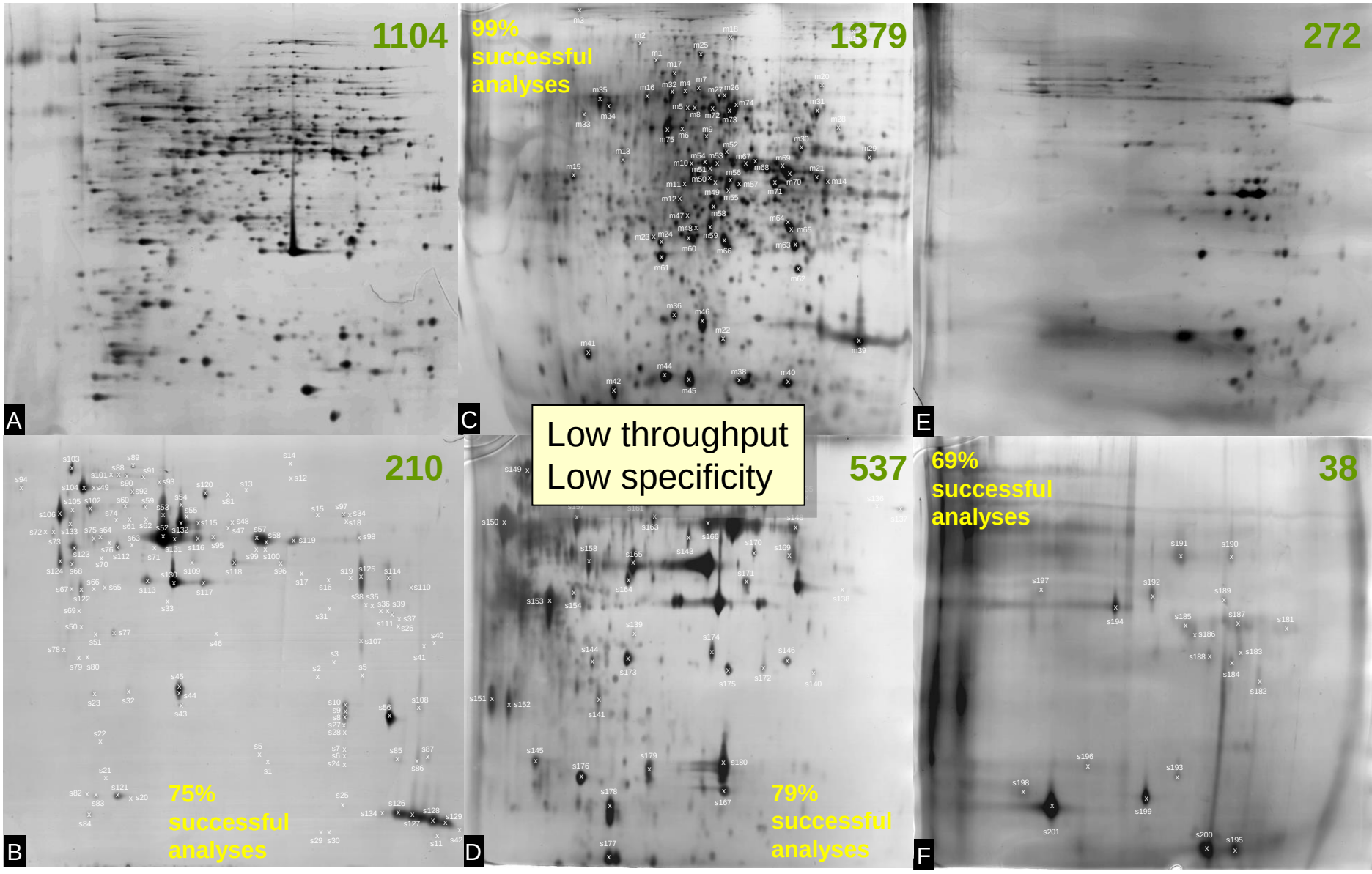
150 kD **40 kD** **14 kD**



Complementary approaches

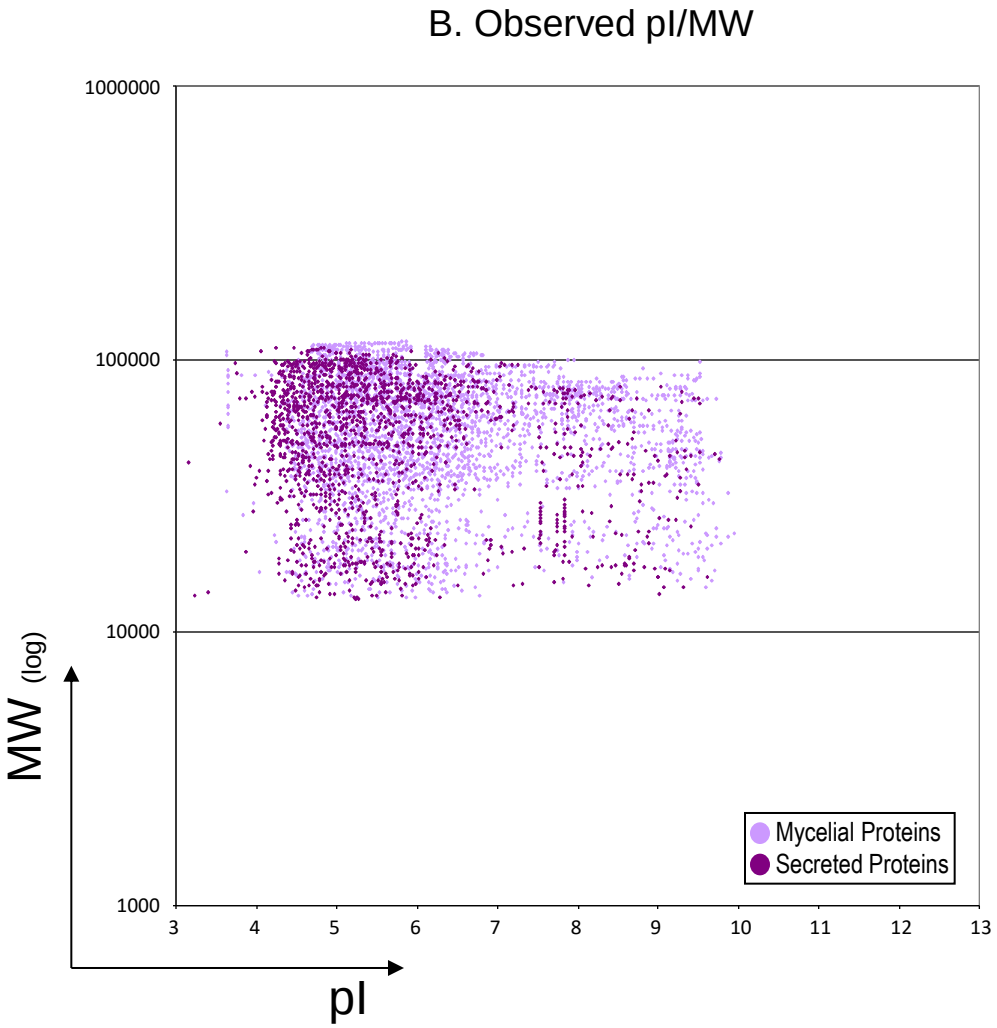
2-DE (mycelium and secretome)

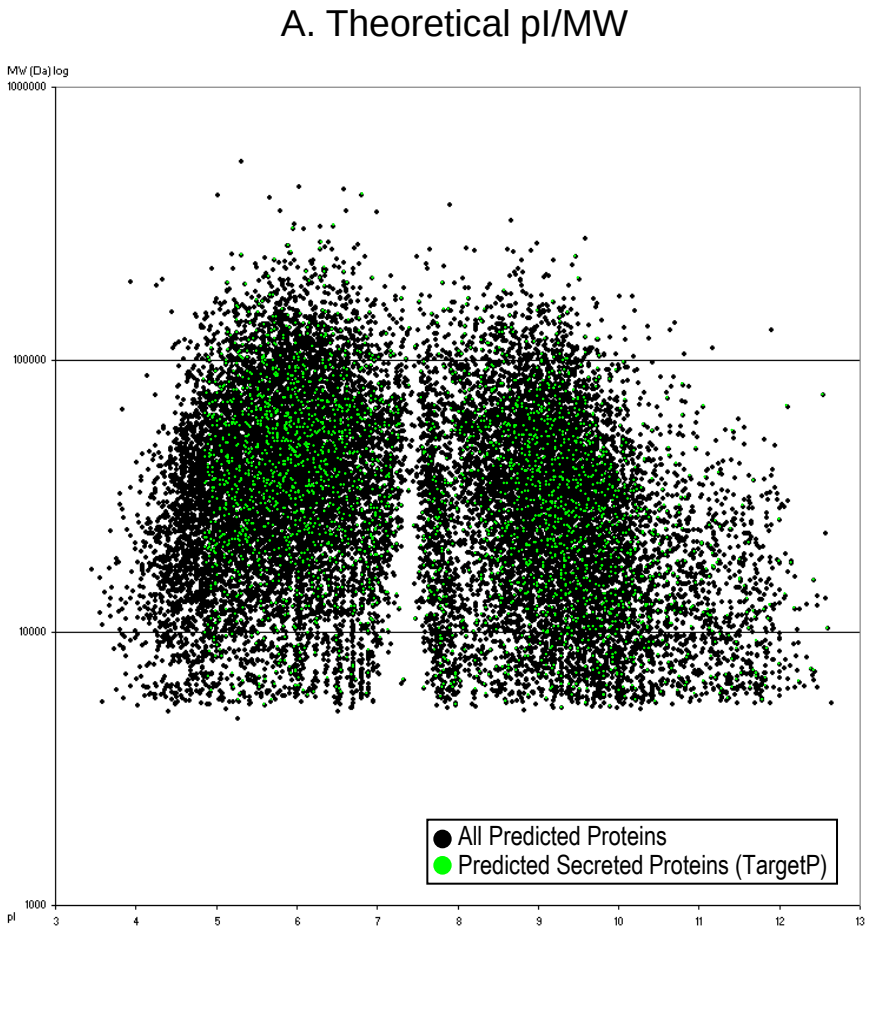
267 secreted proteins identified in total
→ 71 unique proteins (73% redundancy)



Complementary approaches

2-DE



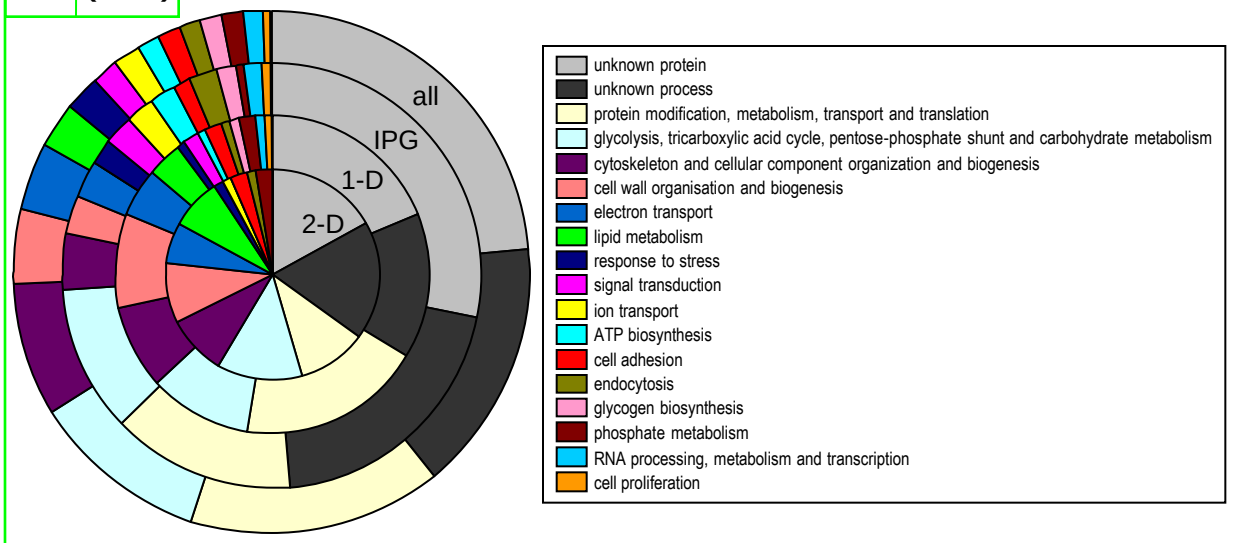


The experimental pI/MW distribution doesn`t fit the theoretical one.

Complementary approaches

Functional comparison of the 3 methods

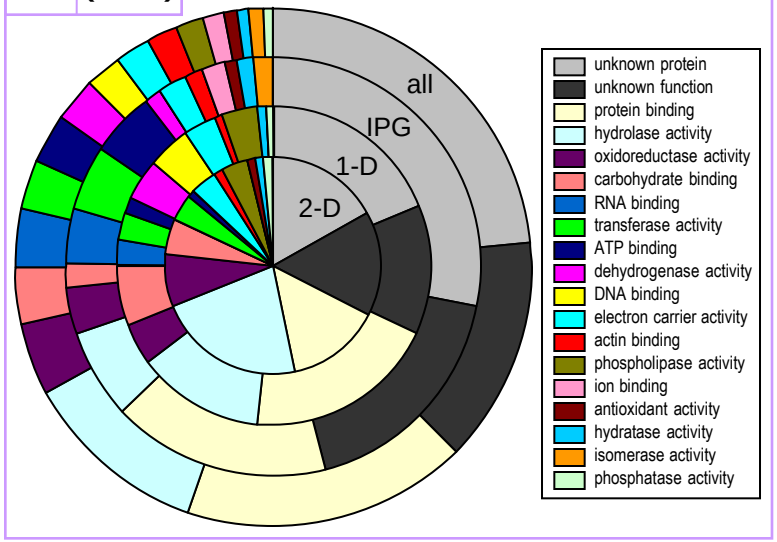
A. (BP)



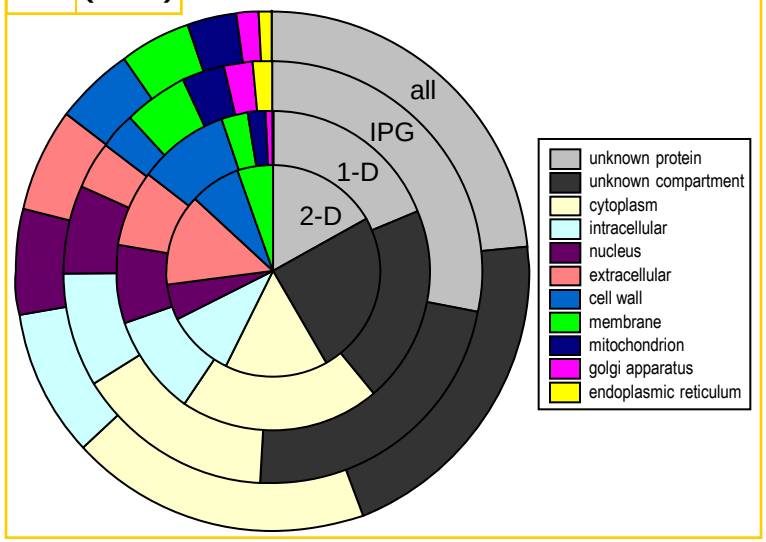
IPG shotgun	142
+ 1-D shotgun	116
+ 2-DE	77
=	224

unique secreted proteins from *L. bicolor* identified across all three methods.

B. (MF)



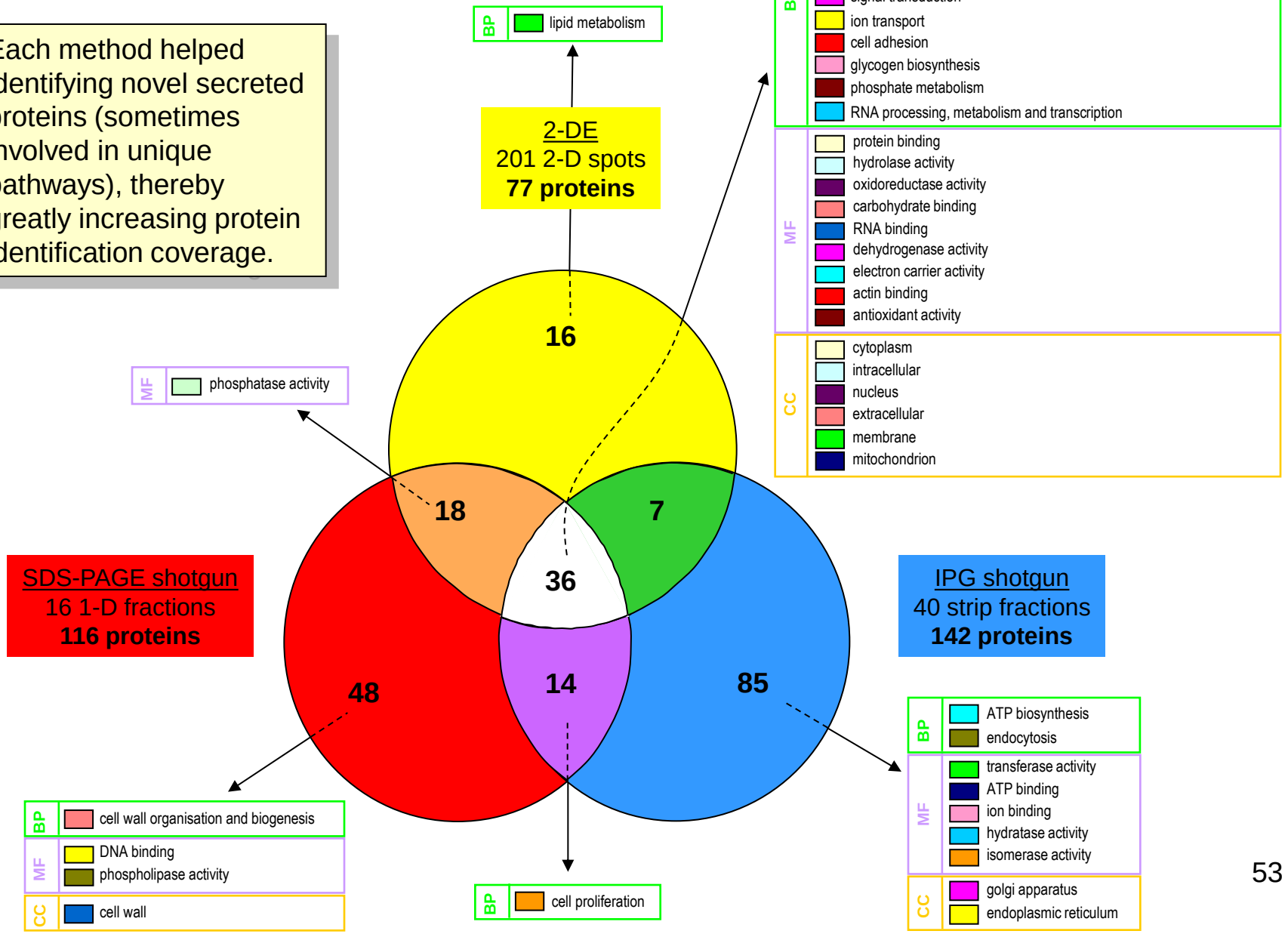
C. (CC)



Complementary approaches

Functional comparison of the 3 methods

Each method helped identifying novel secreted proteins (sometimes involved in unique pathways), thereby greatly increasing protein identification coverage.



Conclusions

- A strategy combining several proteomic techniques greatly increased the coverage of identification of secreted proteins in all 3 fungal species (shown for *L. bicolor* here). Only 11% the proteins were shared between the 3 methods; most of the secreted proteins identified were method-specific (in particular, IPG shotgun (60% specificity)) .

- Out of the 3 methods used, IPG-IEF shotgun provided the highest throughput, and was both time- and cost-efficient. 1-D gel of higher resolution would increase the number of proteins identified. Not all the 2-D spots were excised and analyzed, therefore the number of proteins identified following 2-DE was underestimated. Moreover, a unique functional category was highlighted (lipid metabolism).

- 40% of the identified secreted proteins are either unknown (24%) or their function remains unknown (16%). More biological experiments to decipher *L. bicolor* secretome are needed. Among the known proteins, the prominent functional categories pertain to protein metabolism with many CW remodelling enzymes.

3 -

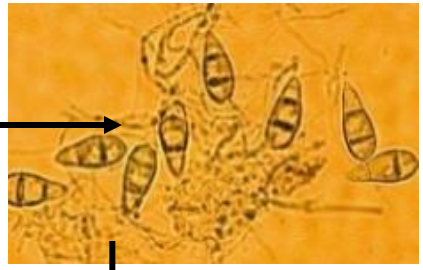
Learning from bioinformatics

Identifications of secreted proteins from Magnaporthe grisea

Materials & Methods

Materials & Methods

Culture of *M. Grisea*, rice pathogen



TNK-YE medium, pH 5.6
- 55mM glucose
- 23mM NaNO3
- 14mM KH2PO4
- 2mM MgSO4 7H2O
- 0.7mM CaCl2 2H2O
- 15µM FeSO4 7H2O
- 2g/L yeast extract
- oligoelements

110 rpm
26°C
3 days

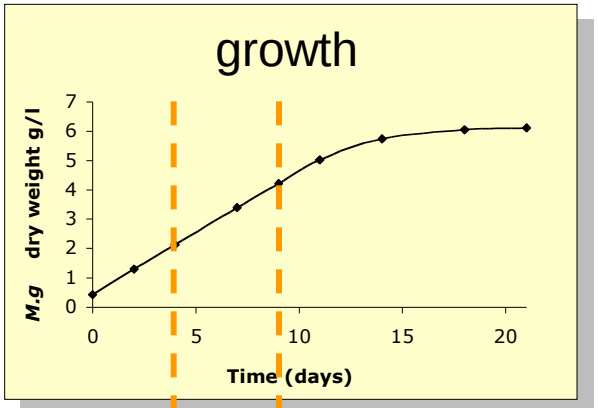
\|20 homogenized mycelium

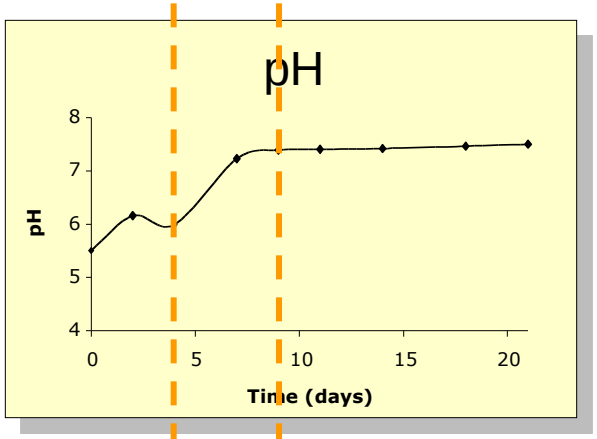
26°C
up to 20 days

filtering

mycelium

secretome (medium)





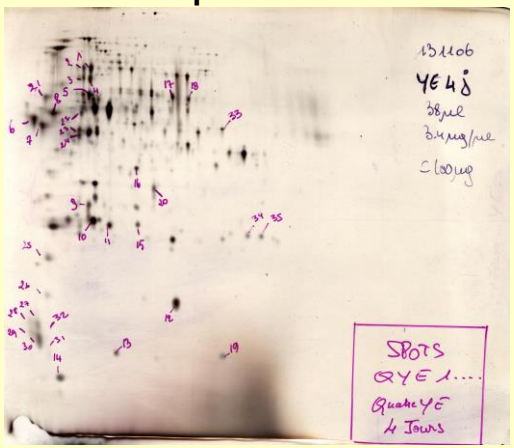
4 days **9 days**

Materials & Methods

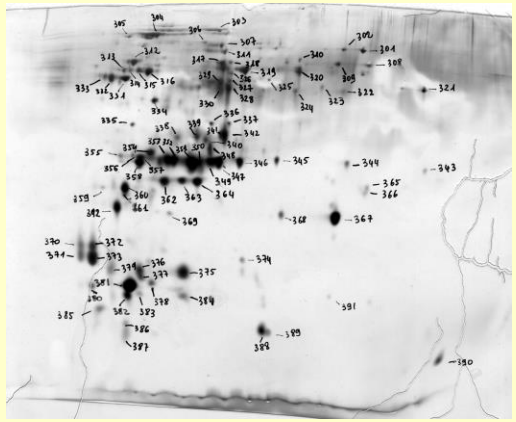
2-DE and manual excision of 410 spots

Mycelium: TCA/acetone precipitation
Secretome: 15mL medium filtered (0.22 µm), dialysed (MWCO 1kD) at 4°C twice for 10h against H2O and freeze-dried
2-DE: 20µg protein load, 3-10NL or 3-7 pH IPG strips, 24 cm, MS compatible nitrate silver stain.

Secretome 4 days
112 spots excised

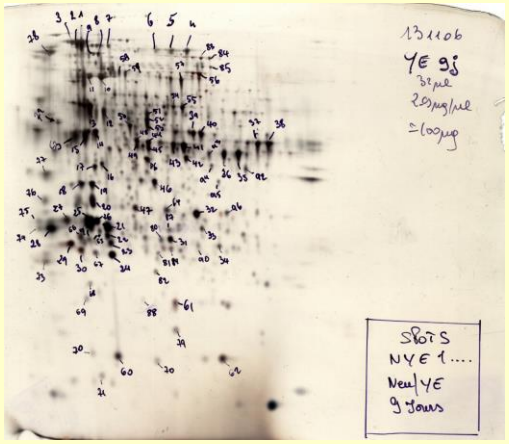


pH 3-10NL

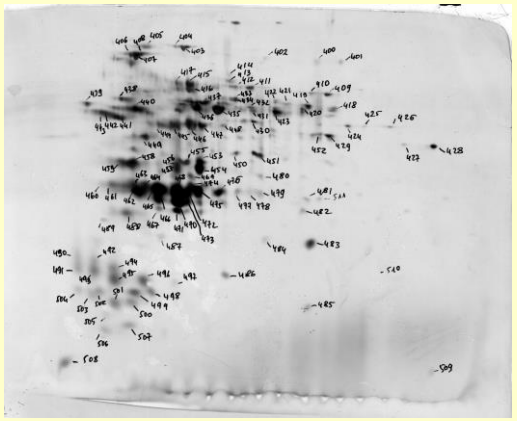


pH 3-7

Secretome 9 days
206 spots excised

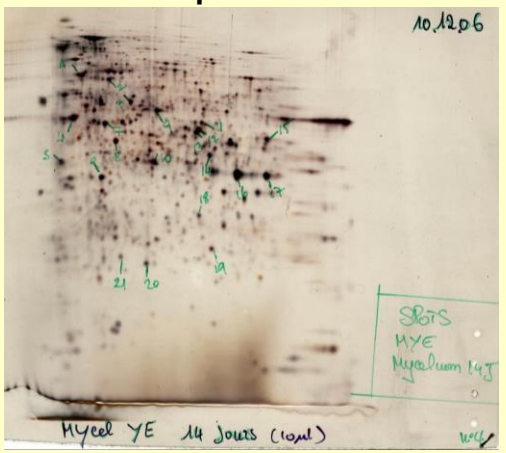


pH 3-10NL

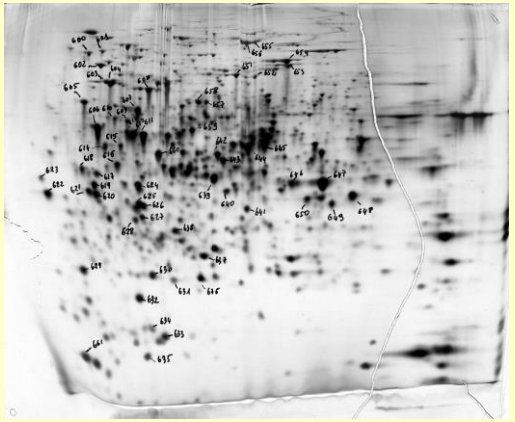


pH 3-7

Mycelium 9 days
80 spots excised



pH 3-10NL



pH 3-7

Materials & Methods

MS analyses

Spots trypsin-digested and analyzed using nLC-ESI-MS/MS
Peptide lists searched against the *M. grisea* protein sequences retreived at the Broad Institute
Manual curation of the hit redondancy and assessment of peptide remanance.

Trends from whole data analysis

Trends from whole data analysis

legend

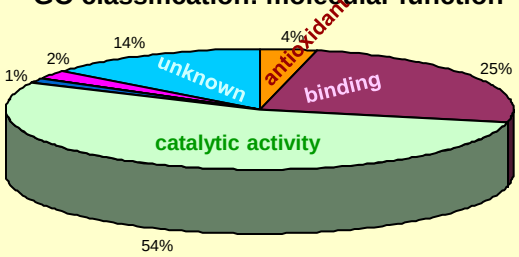
Bioinformatics

The protein sequences were re-annotated using Blast2GO software (www.blast2go.de) against SwissProt or nr databases, and the E.C. numbers and GO terms retrieved as well.

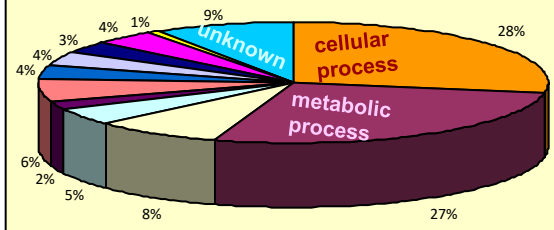
Secretome 4 days

150 excised spots
103 unique proteins (21 enzymes)
12 unknown proteins

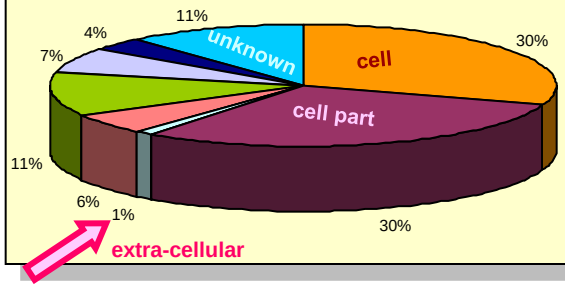
GO classification: molecular function



GO classification: biological process



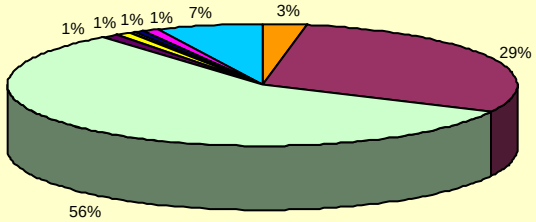
GO classification: cellular component



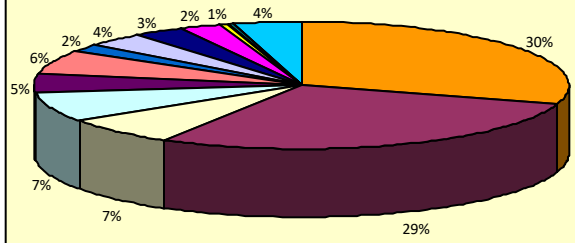
Secretome 9 days

177 excised spots
172 unique proteins (56 enzymes)
12 unknown protein

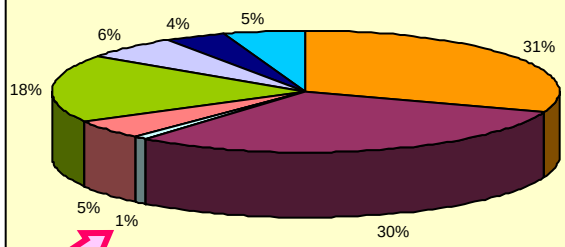
GO classification: molecular function



GO classification: biological process



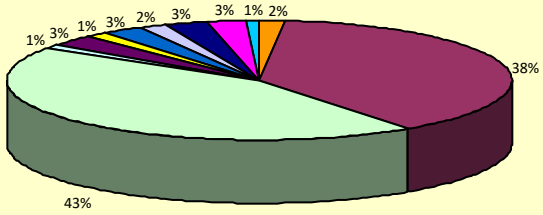
GO classification: cellular component



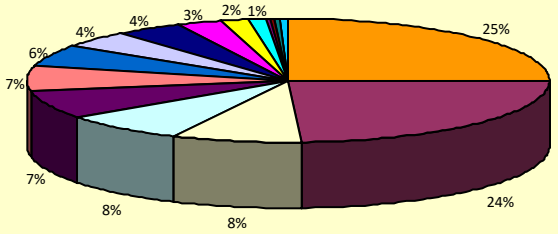
Mycelium 9 days

83 excised spots
227 unique proteins (98 enzymes)
3 unknown proteins

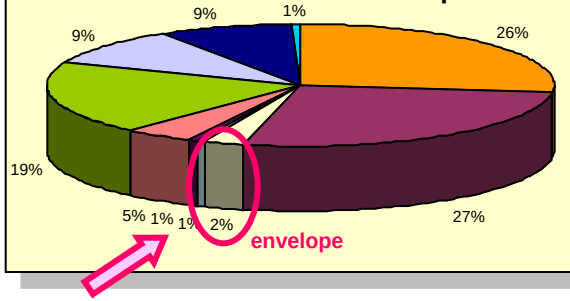
GO classification: molecular function



GO classification: biological process



GO classification: cellular component

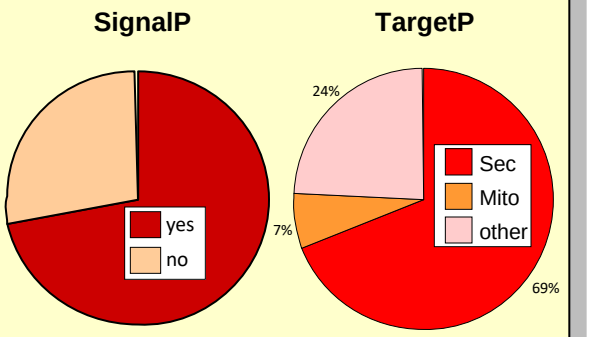


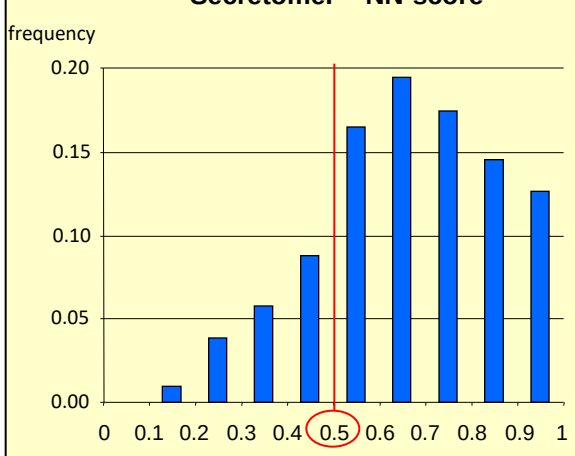
Trends from whole data analysis

Bioinformatics

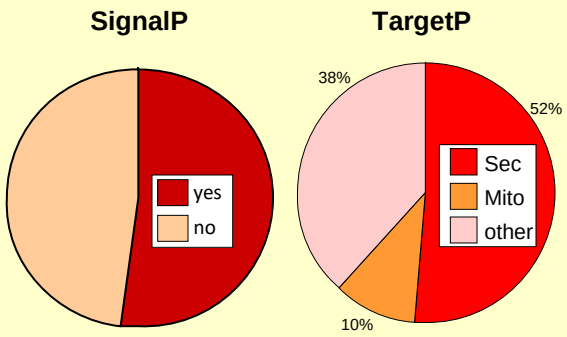
Peptide signals and sub-cellular localization were invastigated using SignalP and TargetP, as well as whether the proteins were secreted or not using SecretomeP (www.cbs.dtu.dk/services).

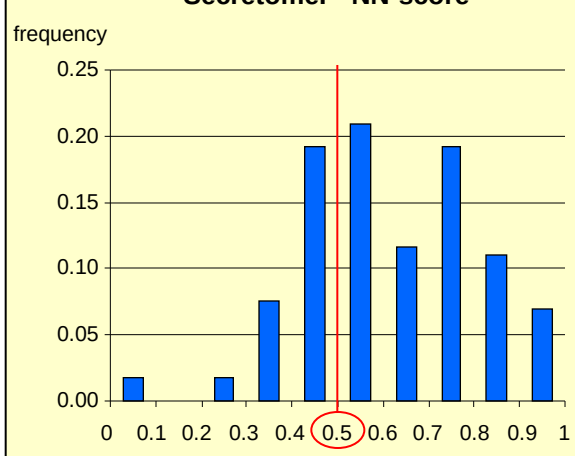
Secretome 4 days



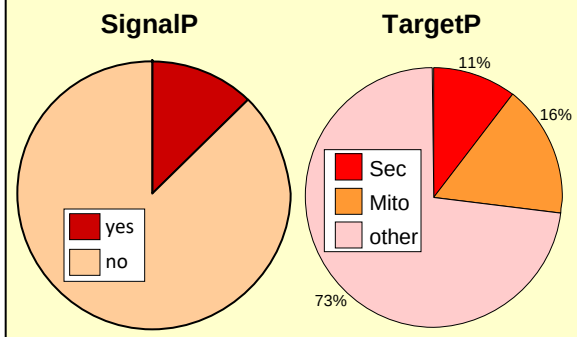


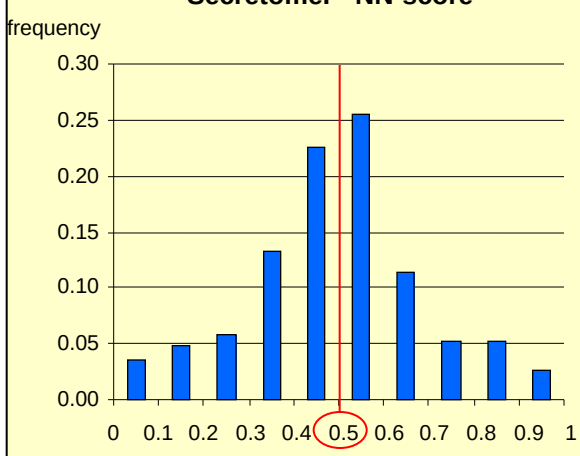
Secretome 9 days





Mycelium 9 days





Trends from whole data analysis

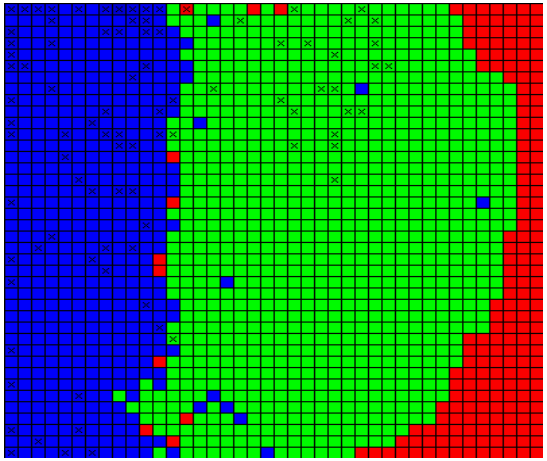
Bioinformatics

Glycosylated phosphatidylinositol (GPI) anchors and mannosylation sites were also searched.

Secretome 4 days

GPI-SOM

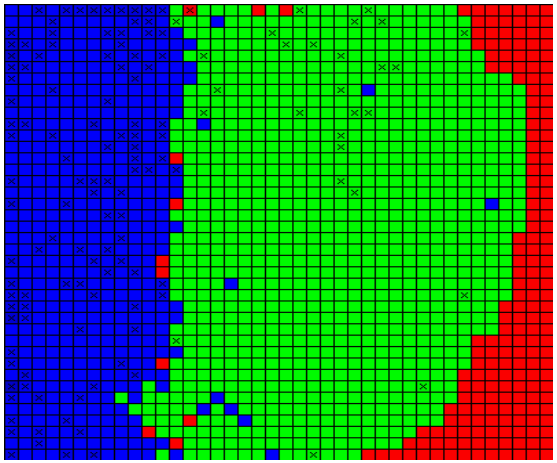
23 GPI-anchored proteins

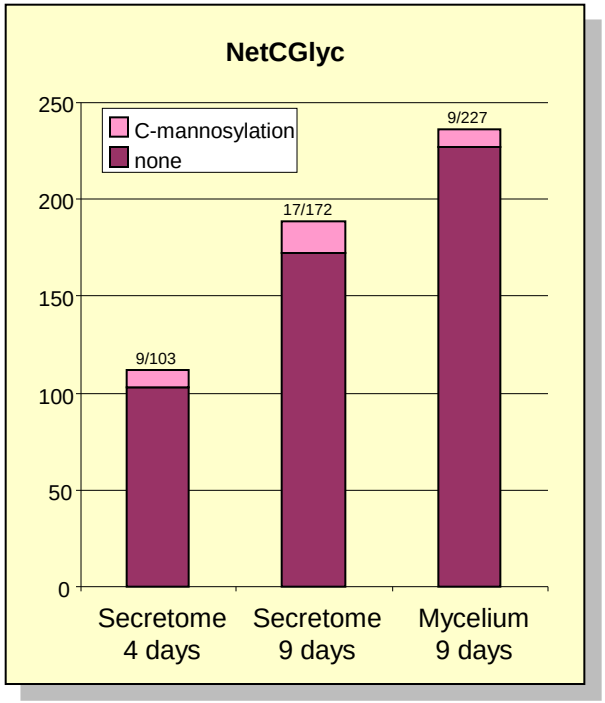


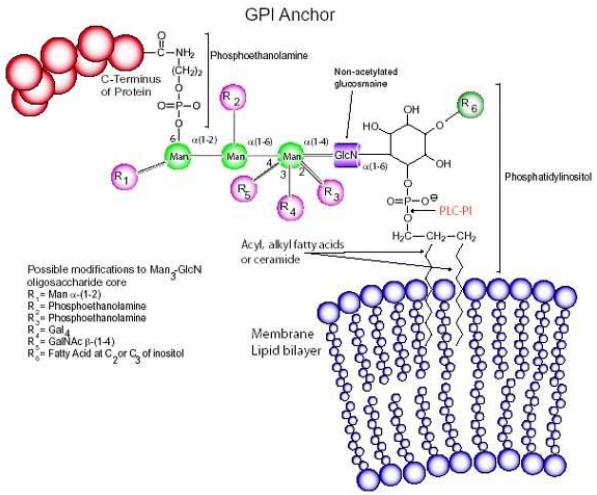
Secretome 9 days

GPI-SOM

22 GPI-anchored proteins







Trends from whole data analysis

Bioinformatics

In-depth analysis. Example: MGG_00052, an excellent candidate

Ex: >MGG_00052 | Magnaporthe grisea predicted protein (225 nt) Broad annotation

MRFSTAIISTVAFGLAAASPLVNRMAGGP
SVIPGKGFFLAVDGDLREMDPENAASNA
WVLSLRRVATGFYAAVIEXRSRDTQNPTF
YINGTLADEQTLKYDIPGAFPLSMTVRAD
REVADGEYVVSFRNSDQDNFVAVAKRAD
GIPVLAPALYGGTWAVCRRDLPTSTGPV
SVMMPRAVGAGGQSAPEGCVTVSFVSMC
TDLAELQPTDGWNHDNVYEIKCVAN

Specific to secretome samples.

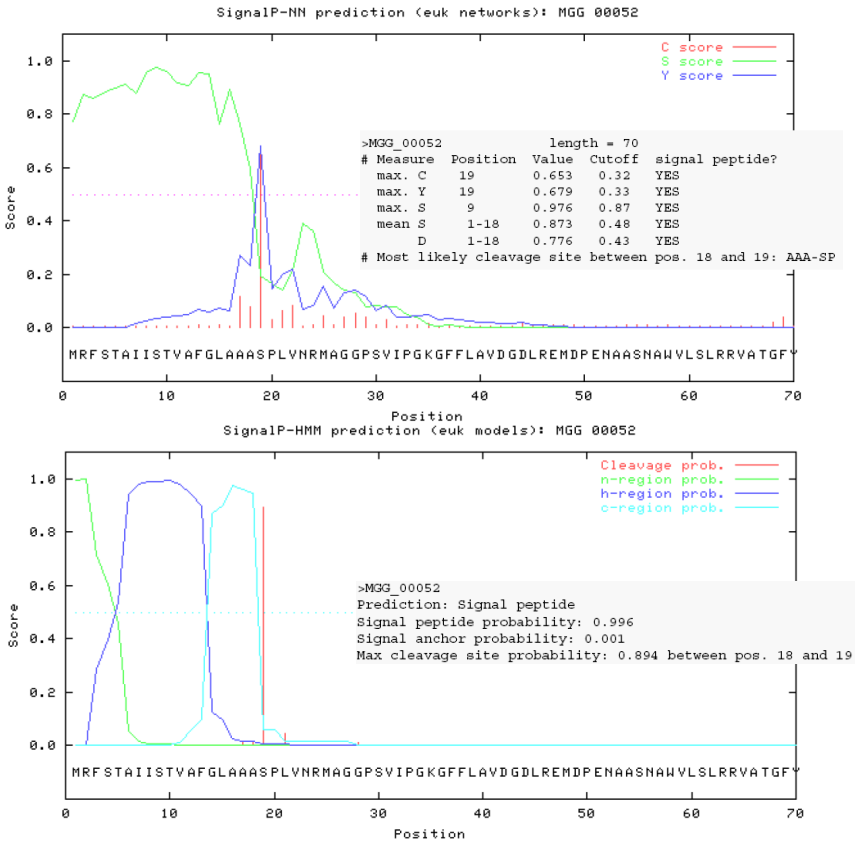
No annotation found in SwissProt or nr.

Peptide signal length: 18 AA.

C-mannosylation site: 158th AA.

Excellent candidate.

SignalP



NetCGlyc

```
##Type Protein
# seqname              source          feature  start    end    score +/-  ?
# ------------------------------------------------------------------------
MGG_00052              netCglyc-1.0b         C-manno     58     58   0.234  .   .
MGG_00052              netCglyc-1.0b         C-manno    158    158   0.550  .   W
MGG_00052              netCglyc-1.0b         C-manno    211    211   0.195  .   .
# ------------------------------------------------------------------------
```

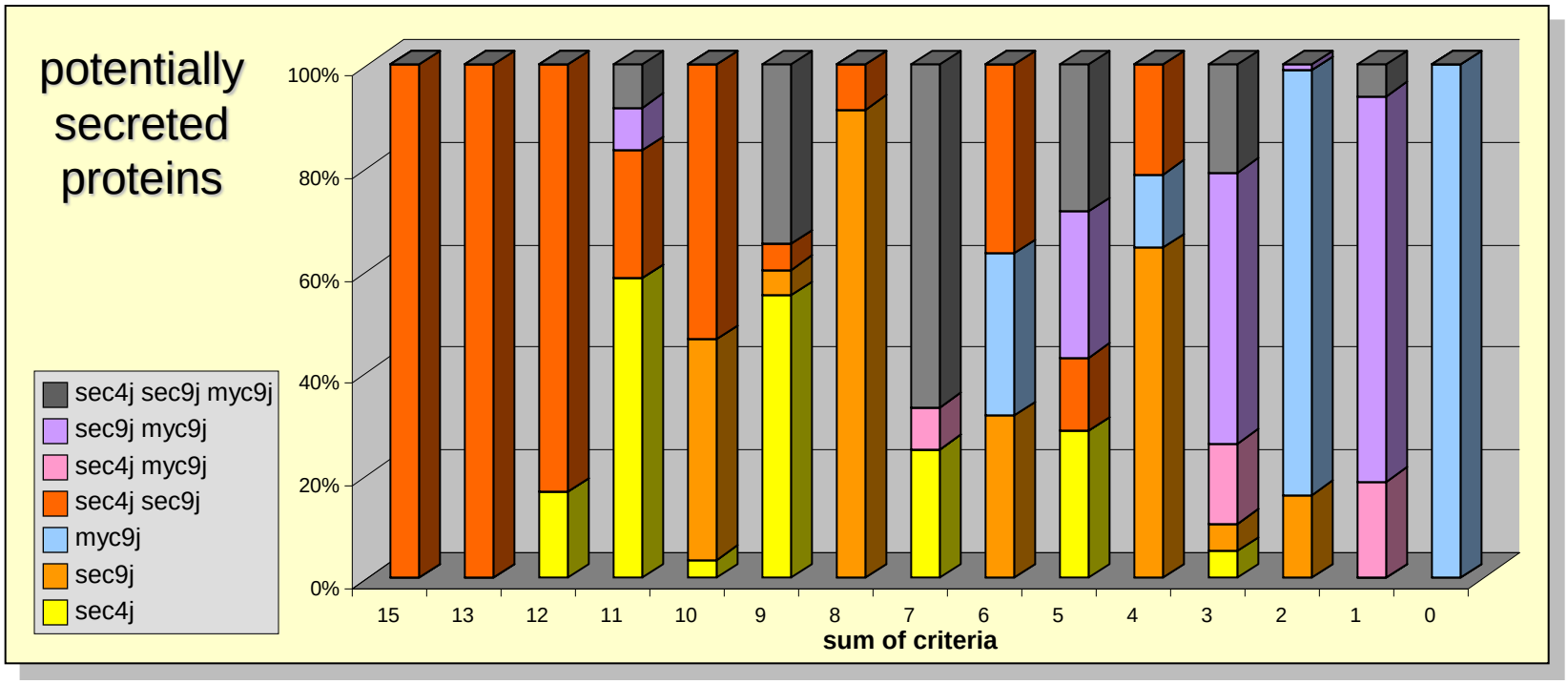
Trends from whole data analysis

Bioinformatics

In-depth analysis: Criteria to use to isolate proteins of interest.

What is known about extra-cellular proteins ?

- they are secreted (obvious but it has to be said !) → TargetP
- they contain a signal peptide → SignalP
- they contain a GPI anchor if they are targeted to the cell wall → GPI-SOM
- they are massively glycosylated (mainly mannosylated) → NetCGly
- they are assumed to be the principal effectors triggering fungus-plant interaction, thus interacting with the host components (polysaccharides, protein…) → experiments



Conclusions

- Despite the lesser number of spots excised from mycelium (80), many more unique protein functions were found (227) than in spots from secretome (ex: secretome days 9, 206 spots excised corresponding to 172 unique functions. Secretome samples are more simple than that of mycelium.

- GO classification in respect to cellular component is very poorly annotated (only 1% of the proteins contained in the liquid medium bore an extracellular localization).

- Most proteins identified in liquid medium were predicted to contain a peptide signal and to be secreted, either through canonical route (TargetP) or non classical secretion mode (yet to be described, and assessed by secretomeP).

- Approximately 20% of the proteins found in liquid medium were predicted to contain GPI anchors, and 10% of them presented potential mannosylation sites.

- *Magnaporthe grisea* culture medium seems to be significantly enriched in secreted proteins (especially after 4 days). More contamination (possibly due to cell lysis) could occur later on (after 9 days) because patterns become more similar to those of mycelium.

- Using a set of criteria based on the known features of secreted proteins can help isolating all the potential proteins of interest (*in silico* analysis).

General Conclusions

Although the growing conditions from one fungal species to another differed, thus resulting in variation in secretion patterns, it would be interesting to compare the secreted proteins identified in each organism. Unique growing conditions suitable for all fungi are currently being tested to allow for a less biased comparison.

Secretion profiles completely changed according to the medium used for *in vitro* culture (observed on *M. grisea* and *L. maculans*; data not show). Multiplying the culture media is a way to increase the coverage of secreted proteins potentially involved in fungus-plant interactions.

Fungal secretomes are not completely deciphered yet but multiplying the tools and optimizing their use will help experimentally both validate *in silico* predictions and unravel novel secreted proteins.

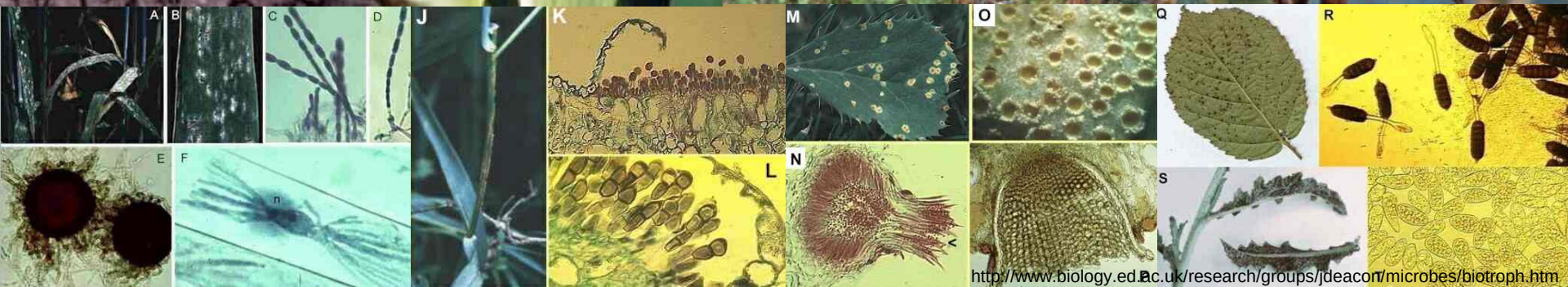
Other proteomic methods, in particular gel-free techniques such as iTRAQ or ICAT, could be used to confirm previously identified secreted proteins as well as identify novel extracellular proteins, and help profiling secretion over time or under different growing conditions.

Eventually, *in planta* experiments must be performed to validate effectors among *in vitro* secreted proteins and unravel the biological processes underlying fungus-plant interactions.

Functional analyses such as protein-protein interactions or protein-sugar interactions must be carried out to identify the targets of fungal effectors.

The adventure continues on *Staganospora nodorum*, pathogen of wheat. Stay tuned...

Thank you for your attention !



http://www.anbg.gov.au/cpbr/program/sc/evol_bio.html

http://www.biology.ed.ac.uk/research/groups/jdeacon/microbes/biotroph.htm