Understanding the Physiological and Biochemical Mechanisms of Graft-incompatibility

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Why is graft-incompatibility between pear and quince a major problem in Italy?

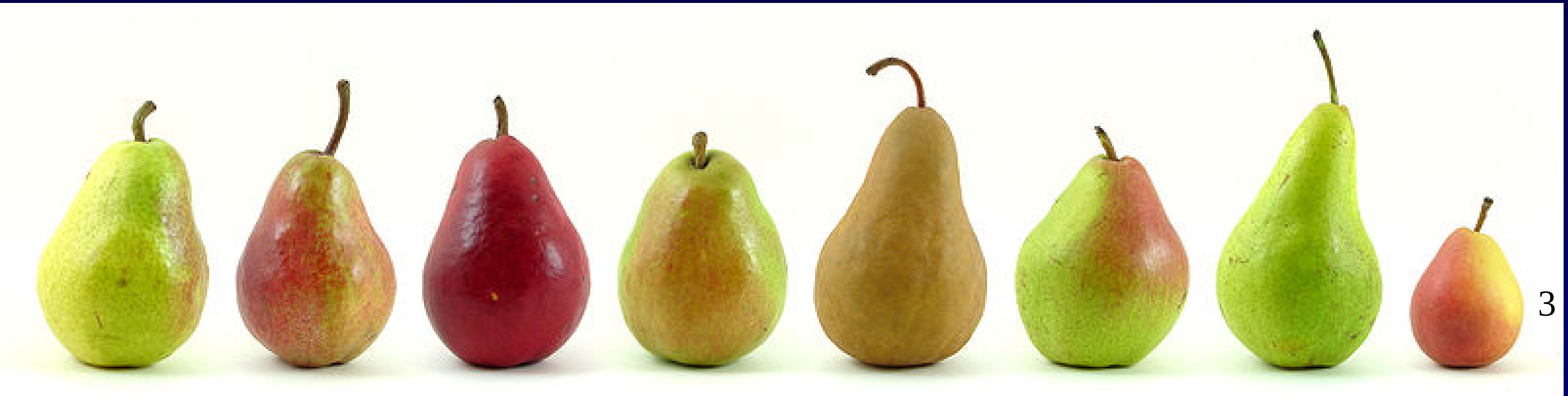
94% of orchards in Italy are grafted on quince

Why do growers still use quince, despite graft-incompatibility?

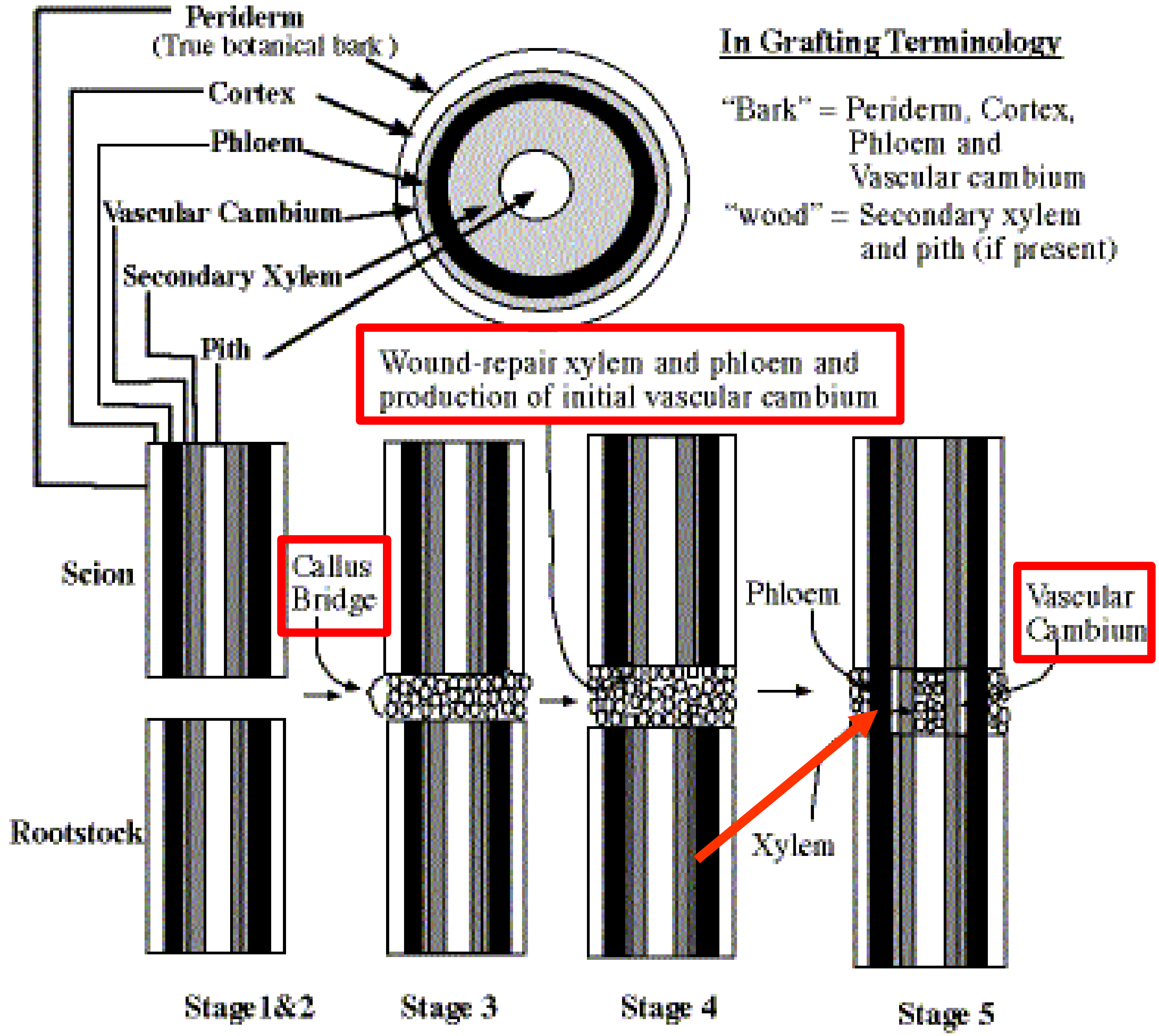
- **Dwarfing (HDP and UHDP)**
- **Early bearing**
- **Easy to propagate**

Pear cultivars classification on the basis of graft-incompatibility level with quince

Compatible	Intermediate	Incompatible
Beurré Hardy	Abbé Fetel	Coscia
Doyenne du Comice	Conference	Clapp’s Favorite
Generale Leclerc	Bartlett (William)	Dr. Guyot
Passe Crassane	Packham's Triumph	Beurré Bosc(Kaiser)
	Max Red Bartlett	



Graft histogenesis



- **Initial phases of graft are the same in compatible or incompatible combinations, because they are a reaction to the wound.**

- **A good vascular tissue formation occurs only in the compatible combinations.**

Graft Incompatibility

Scaramuzzi (1955): inability of some grafted plants to function as a single and unique plant.

Moore and Walker (1981): physiological incompatibility between tissues of the two members that leads to the failure of the graft.

Feucht (1988): phenomenon of premature tree senescence caused by physiological and biochemical dysfunctions.

Classification of graft-incompatibility:

- Translocated
- Delayed
- Localized

Translocated graft-incompatibility

- The agent factor is a substance transported from one graft member to the other as a toxin.
- The use of interstock graft can not overcame the incompatibility.
- Example: peach cv “Hale’s Early” grafted on “Myrobolan B” plum roots (weak union and distorted tissues).

Delayed graft-incompatibility

- After several years of growth, symptoms appear.
- This incompatibility is frequently due to the presence of disease, i.e. virus, phytoplasma, introduced by grafting.
- Incompatibility becomes evident when some mechanical stresses occur.

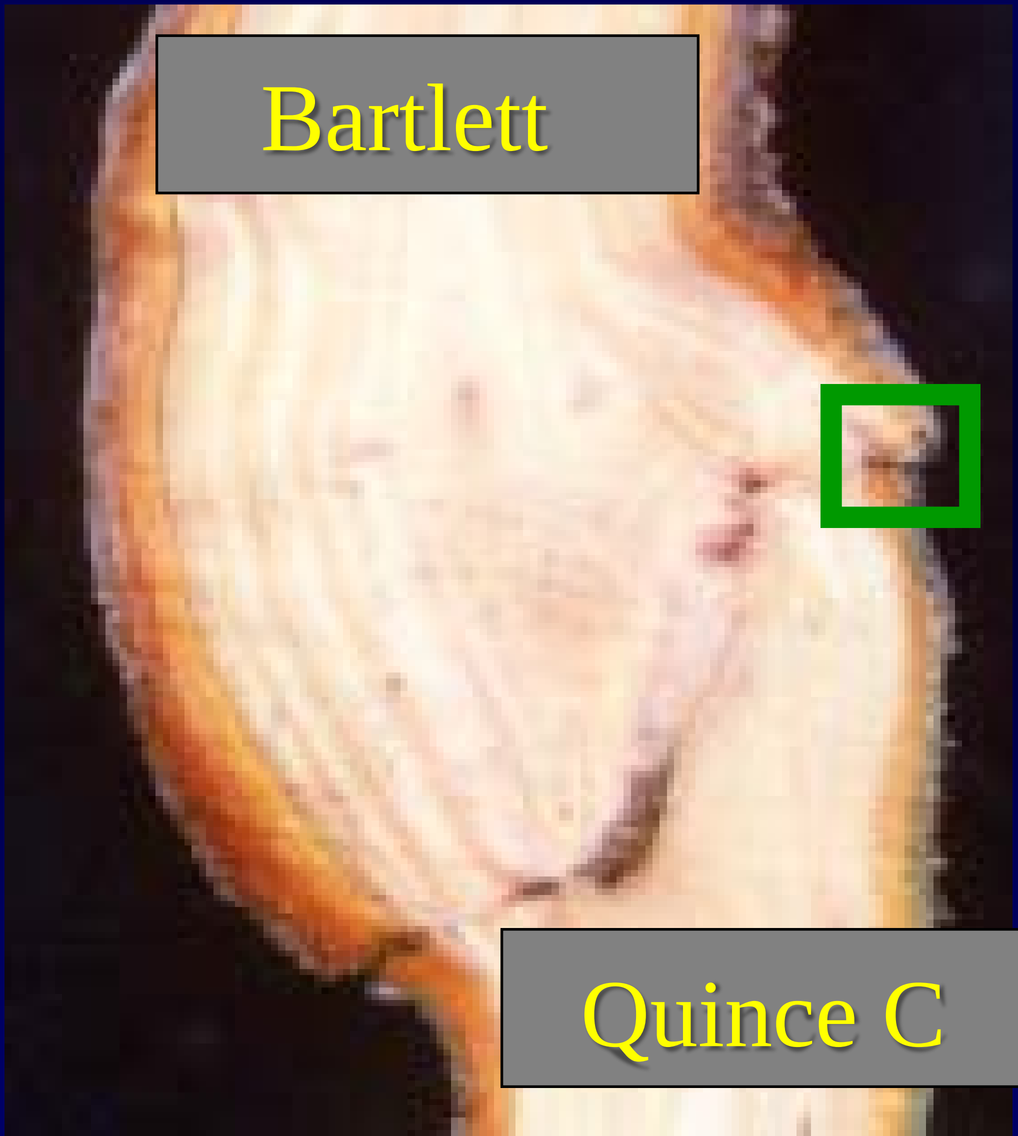
Quick decline

Abbé Fétel/ BA29 -Phytoplasma symptoms

Localized graft-incompatibility

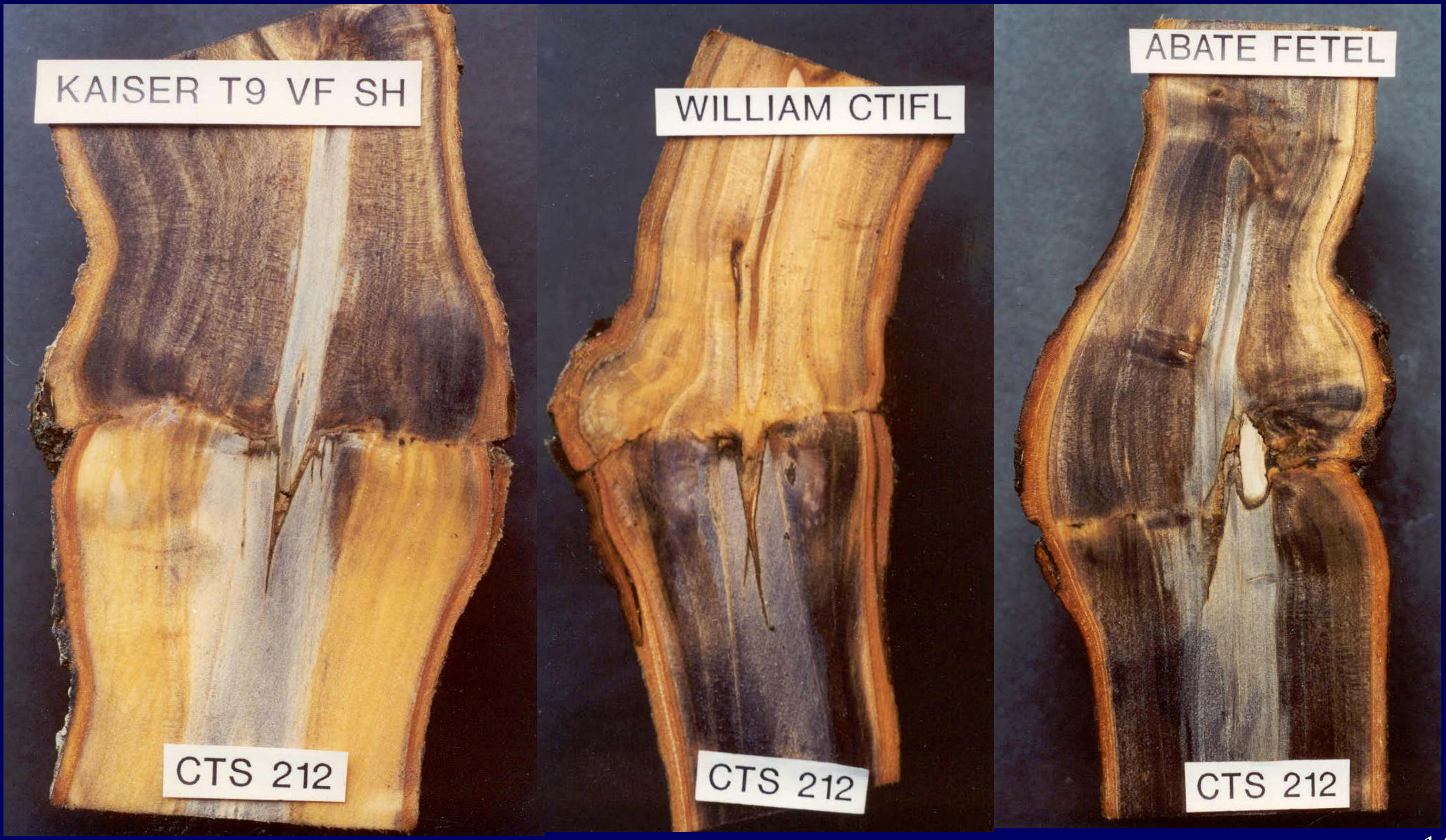
- Physical contact between the two bionts are required.
- Other specific symptoms are necrosis vascular discontinuity, starch accumulation, break of the graft-union.
- Use of mutually compatible interstock overcomes the problem.
- An example: Beurré Bosc (Kaiser) grafted on quince → Beurré Hardy as interstock.

EXAMPLE OF PEAR-QUINCE GRAFT-INCOMPATIBILITY

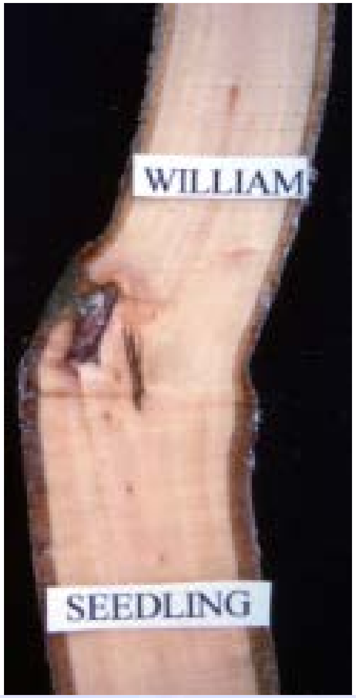


Necrosis enlargement

Starch accumulation



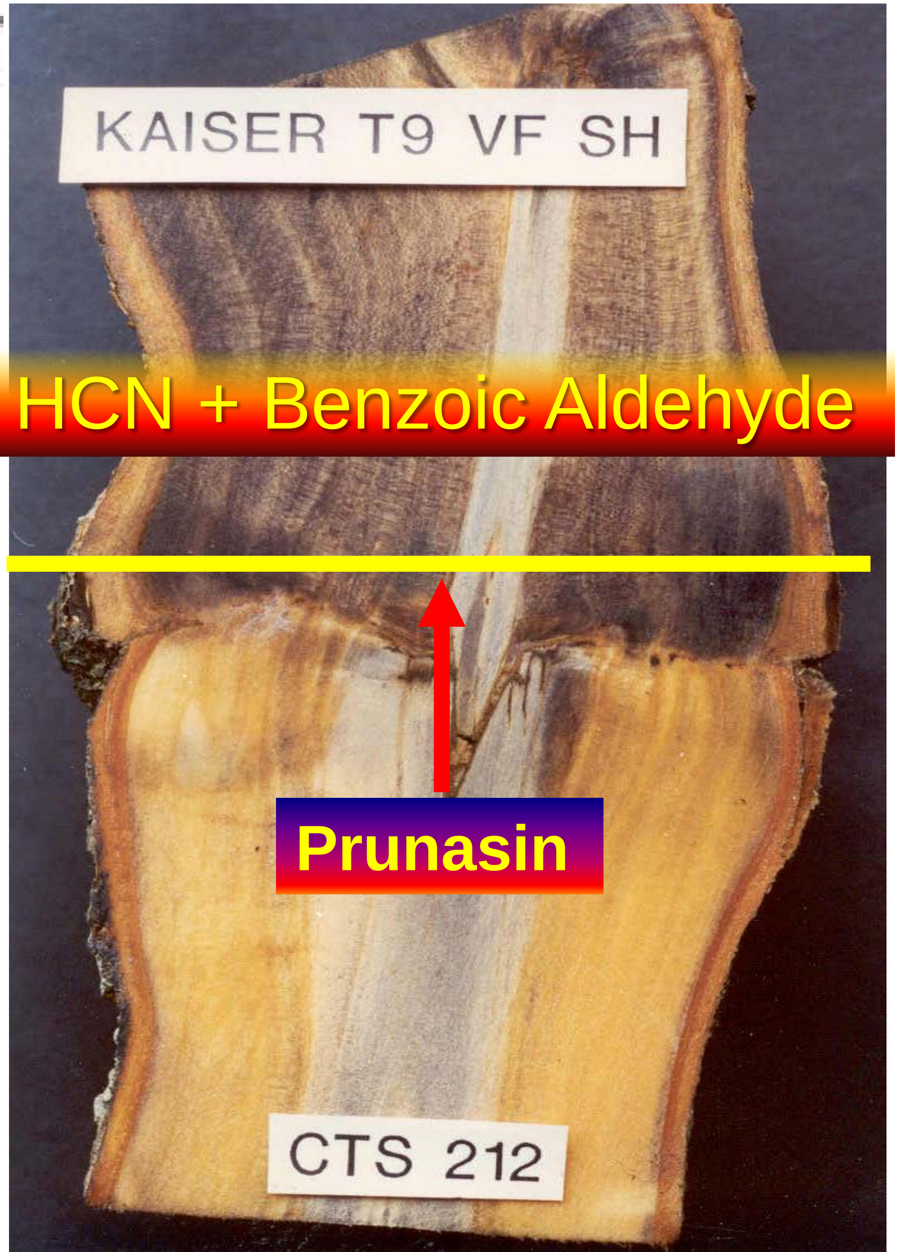
Graft-incompatibility



Gur's model **of graft-incompatibility between pear and quince**

Pear

Quince

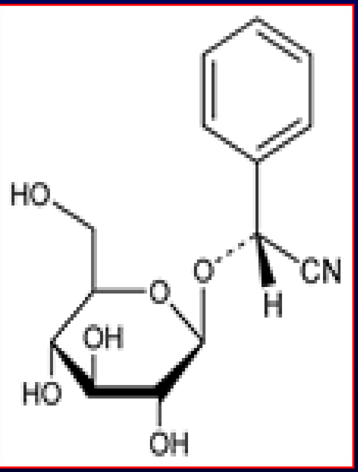


ß-glucosidase

Mandelonitrile lyase

Gur et al., 1968

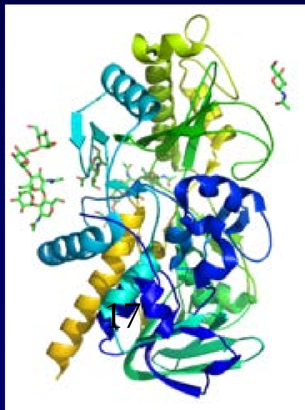
In enzymology, a prunasin beta-glucosidase (EC 3.2.1.118) is an enzyme that catalyzes the chemical reaction:



(R)-prunasin + H_2O $\rightleftharpoons$ 2 D-glucose + mandelonitrile

The mandelonitrile lyase (EC 4.1.2.10) is an enzyme that catalyzes the chemical reaction:

Mandelonitrile $\rightleftharpoons$ cyanide + benzaldehyde

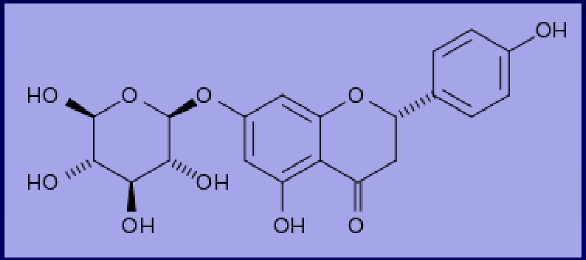


Objections to the Gur's model:

- Cyanogenic glycosides are accumulated and compartmentalised in cell vacuoles and are used as nitrogen reserve.
- There was no evidence of prunasin transportation from quince to pear.
- There was no presence of prunasin in callus.
- Cyanogenic glycosides are not present in pear.
- ß-glucosidase and Mandelonitrile lyase are present also in the quince (detox system?)

Phenol involvement in Graft Incompatibility

Treutter and Feucht (1988) found an accumulation of prunin (*flavanon*) above the graft union in incompatible combinations of *Prunus avium* and *Prunus cerasus*, probably due to histo-anatomical disorders (e.g. changed cellular differentiation).

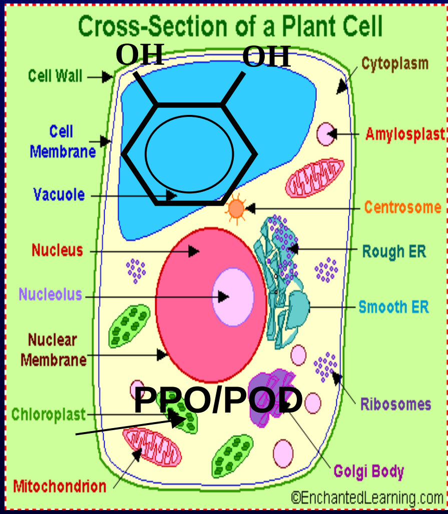


Phenol accumulation & graft-incompatibility

The different composition in polyphenols is known to interfere with the plasmalemma by changing the structure and the orientation of cell-wall microtubules and a corresponding modification of wall cell permeability.

(Errea, 1998)

Phenols in graft-incompatibility



- In graft incompatibility, the phenols may be exported from the vacuoles to the cytoplasmic matrix where they are oxidated by *PPO* and *POD* to quinones. The quinones polymerise and became toxic (Feucht and Treutter 1989).

- Moreover, they may form irreversible linkages with proteins or other macromolecules and precipitate with necrosis formation (Feucht and Treutter 1989).

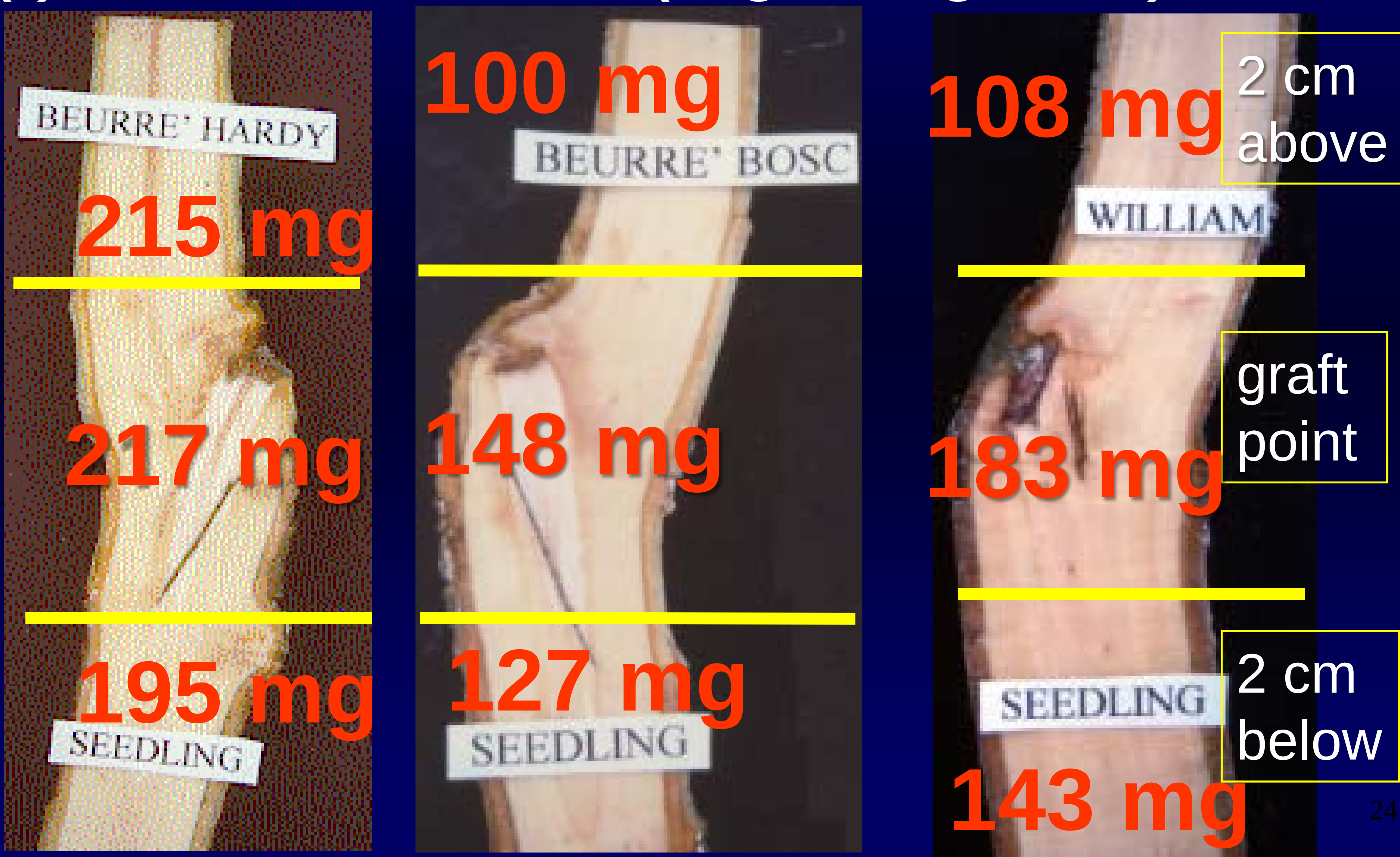
Polyphenols found in the phloem of the different genotypes

Compound	Genotype
naringenina	William e Franco
+catechina	William, Franco e EMC
-epicatechina	William, Franco e EMC
quercitrina	William e Franco
isoquercitrina	EMC
procianidina B1	William
procianidina B2	EMC

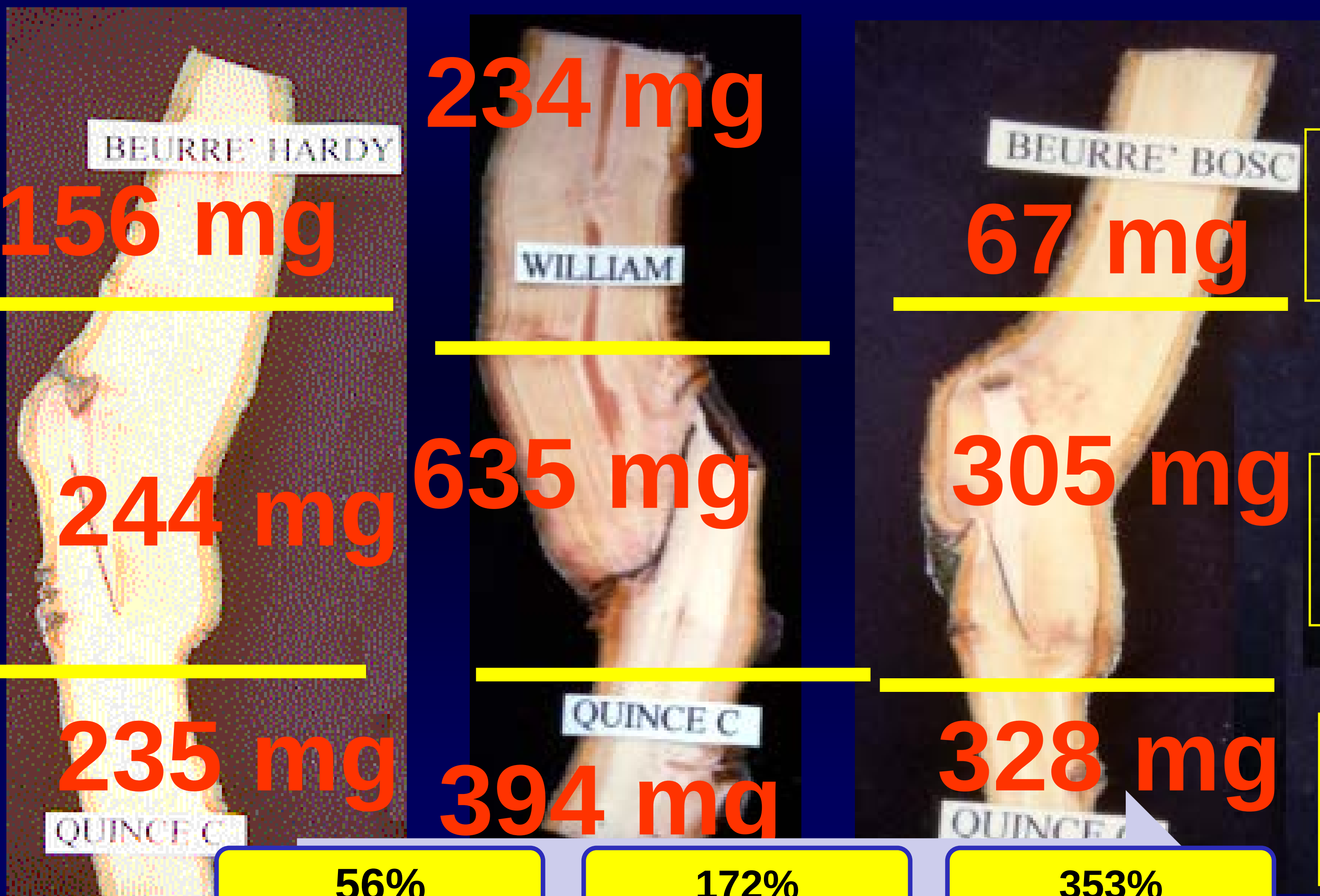
Polyphenol in the phloem

	Combinations	Oligomeric fraction (%)
	William autoradicato	0.78
	Franco	0.95
	Cotogno EMC	1.21
incompatible	W/EMC 2 cm above	1.8
incompatible	W/EMC Grafting point	2.9
incompatible	W/EMC 2 cm below	1.4
compatible	W/Franco 2 cm above	0.9
compatible	W/Franco Grafting point	1.4
compatible	W/Franco 2 cm below	1.1

PEAR-SEEDLING COMBINATION: (-)-EPICATECHIN (mg/100g FW)



PEAR-QUINCE COMBINATION: (-)-EPICATECHIN (mg/100g FW)



2 cm above

graft point

2 cm below

56%

172%

353%

Phenols in pear-quince combinations

- Polyphenols accumulation at the graft point in incompatible combinations with quince was mainly due to (-)-epicatechin.

- This flavan-3-ol increased its concentration *at the graft union* compared with 2 cm above graft according to graft incompatibility levels with quince:

  Beurré Hardy (+56%) ⇒ compatible

  Bartlett (+172%) ⇒ intermediate

  Beurré Bosc (+353%) ⇒ incompatible

Cell-cell recognition

Little information is available about cell-cell recognition, and none of the several theories posited to date has established the existence of a recognition protein between the cells of the two genotypes in the graft union tissues.

Yeoman and Brown (1976)

Proposed a model based on the formation of a cell-cell recognition protein complex for the scion-stock combination: its absence is supposed to demonstrate an incompatible graft.

Moore (1981-1984)

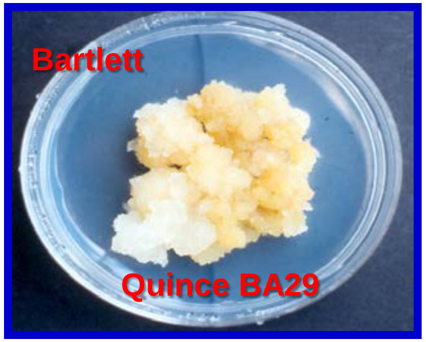
Using "in vitro" callus models, he showed that contact is not necessary for the onset of graft incompatibility.

How to study graft-incompatibility?

- ***In vivo (polyphenols role)***
- ***In vitro (new models to simplify the system and reduce interactions with the environment)***

Models for studying biochemical and physiological aspects of graft-incompatibility

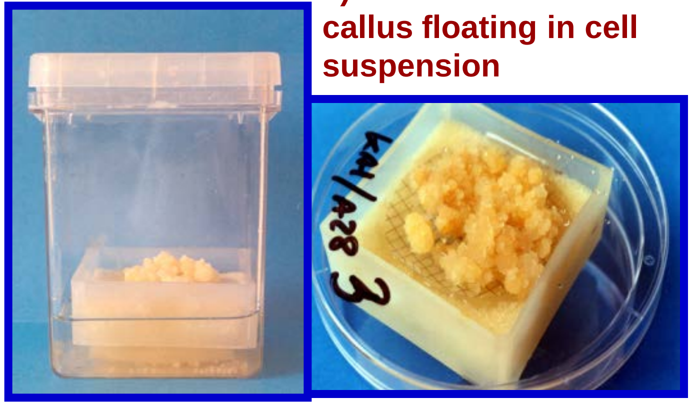
a) Micrografting *in vitro*



c) Co-culture of callus in the same Petri dishes

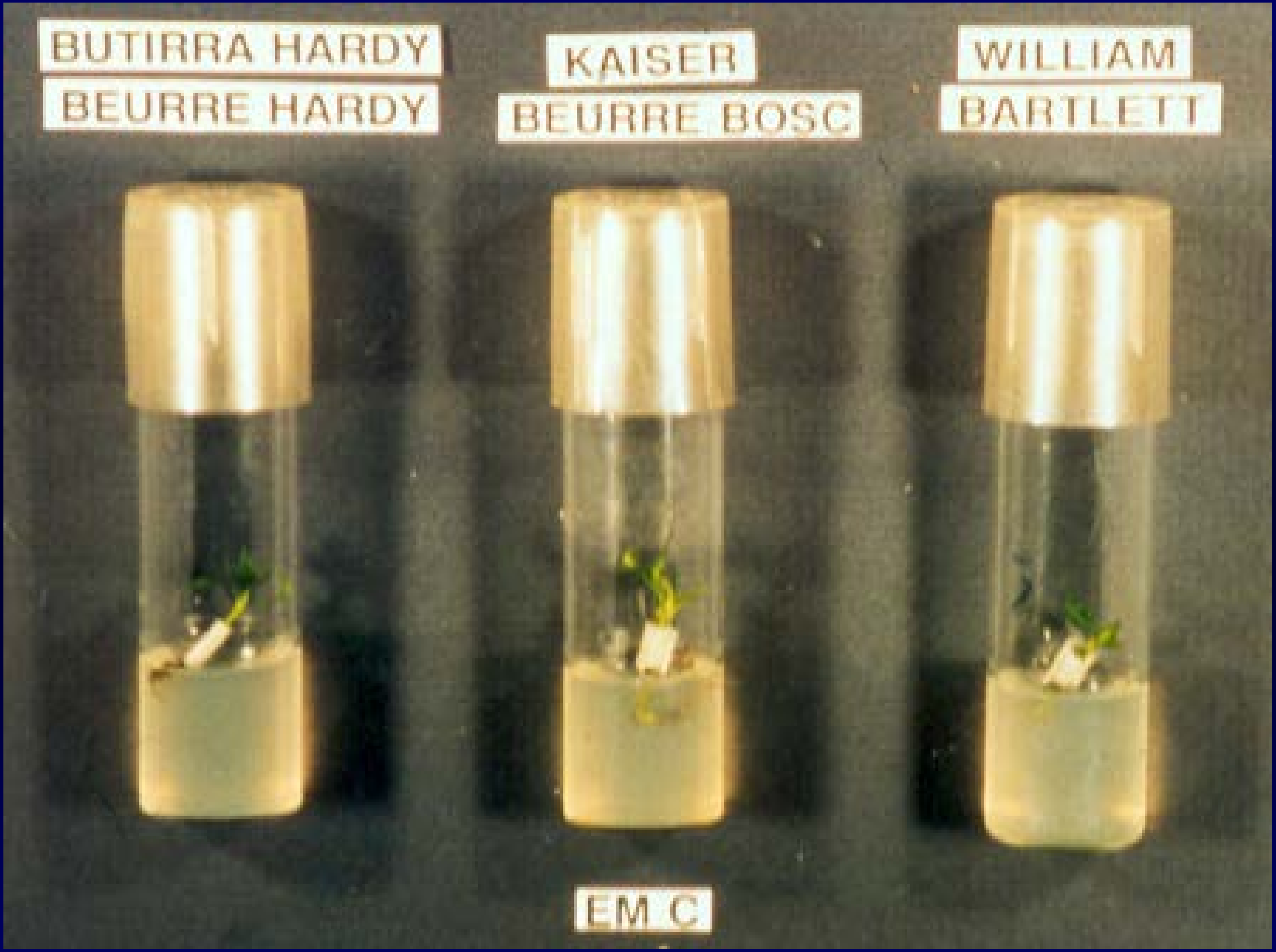
b) Graft of in vitro shoot on acclimating plants

e) Co-culture of callus floating in cell suspension

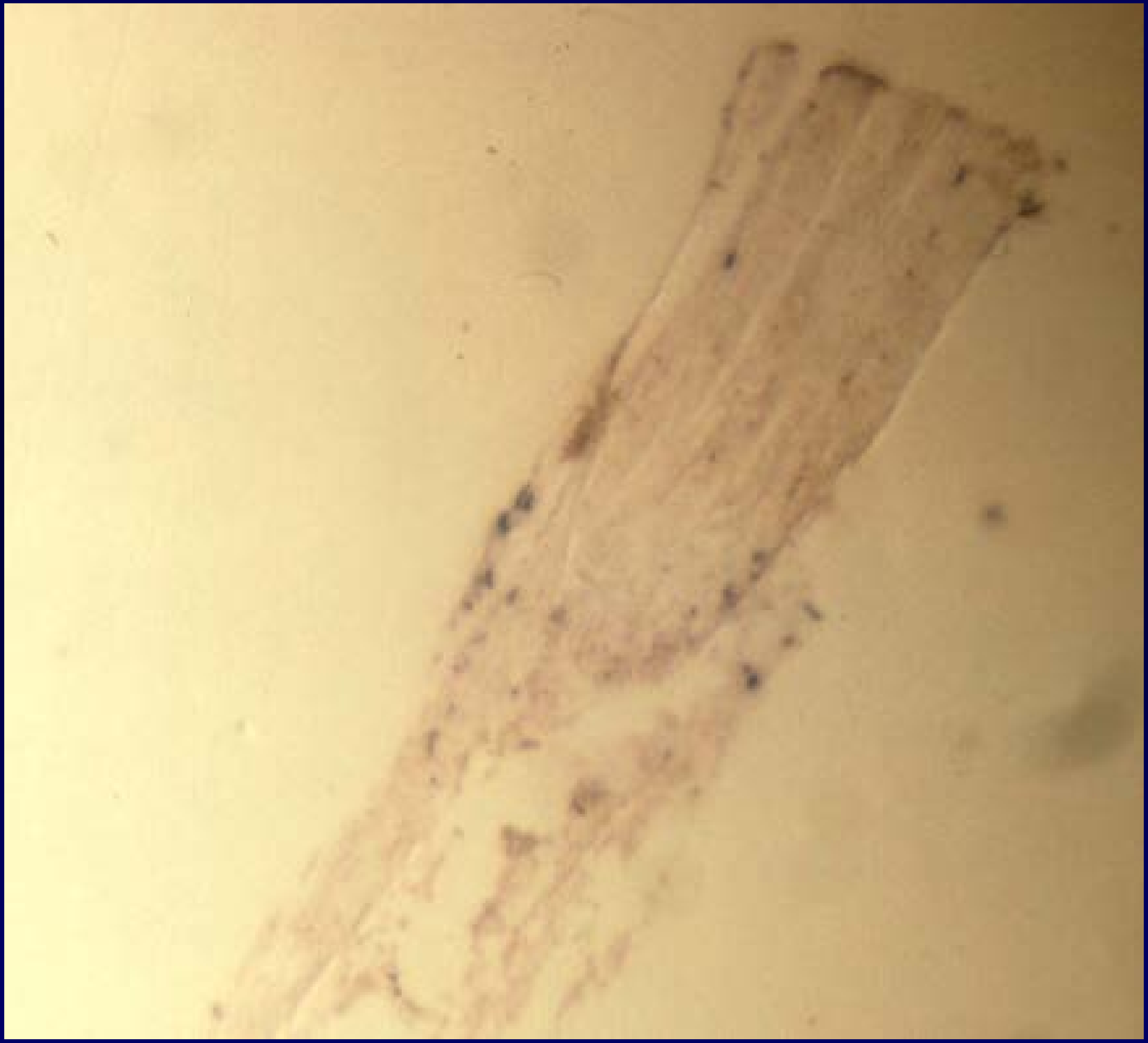


d) Co-culture of cell suspensions

A) *In vitro* Micrografts

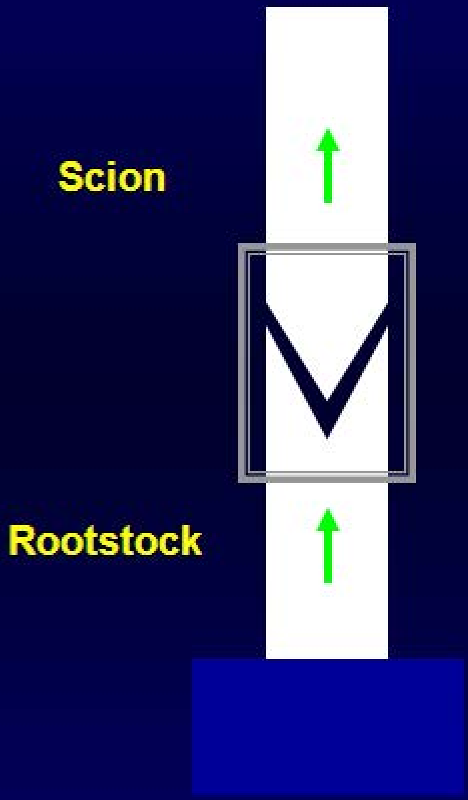

BUTIRRA HARDY
BEURRE HARDY
KAISER
BEURRE BOSC
WILLIAM
BARTLETT
EM C

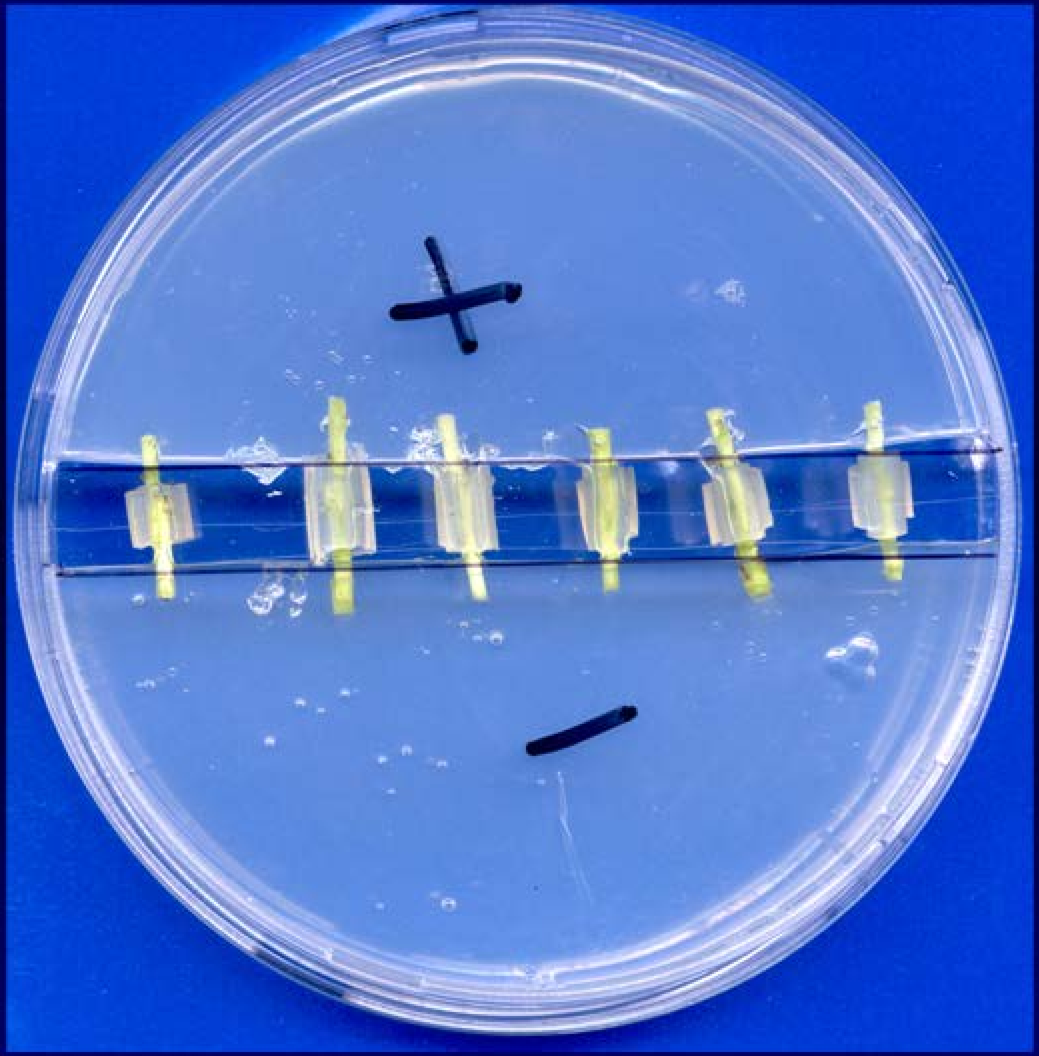
Tissue printing **to determine presence of enzyme (beta glucosidase)**



Tissue printing to determine presence of beta glucosidase

In vitro micrografts





Bosc/Bosc Bosc/MC
BH/BH BH/MC

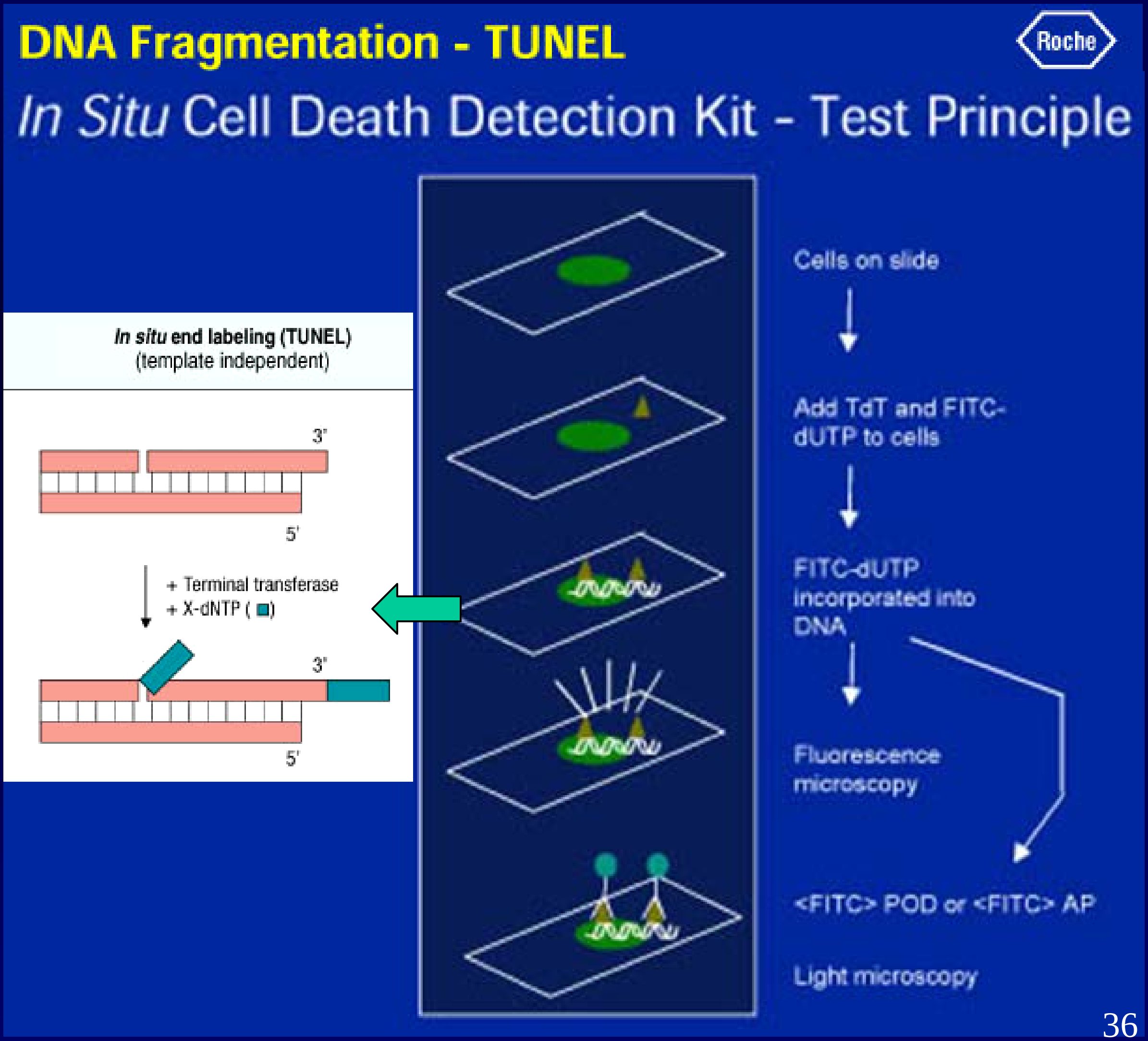
Espen et al., 2002 and 2005

The In Situ Cell Death Detection Kit [Roche Diagnostics, Gmbh] *has been designed as a precise, fast, and simple non radioactive technique to detect and quantify apoptotic cell death at the single-cell level in tissues; based on the* ***TUNEL*** **method** (TdT-mediated dUTP terminal nick-end labeling to detect PCD-characteristic DNA fragmentation)

During apoptosis, DNAse activity not only generates double-stranded, low-molecular-weight DNA fragments (mono- and oligonucleosomes), but also introduces strand breaks ("nicks") into the high-molecular-weight DNA.

These processes can be identified by labeling the free 3'-OH termini with terminal transferase (TdT), which attaches labeled nucleotides to all 3´OH-ends (TUNEL reaction; TdT-mediated dUTP nick end labeling).

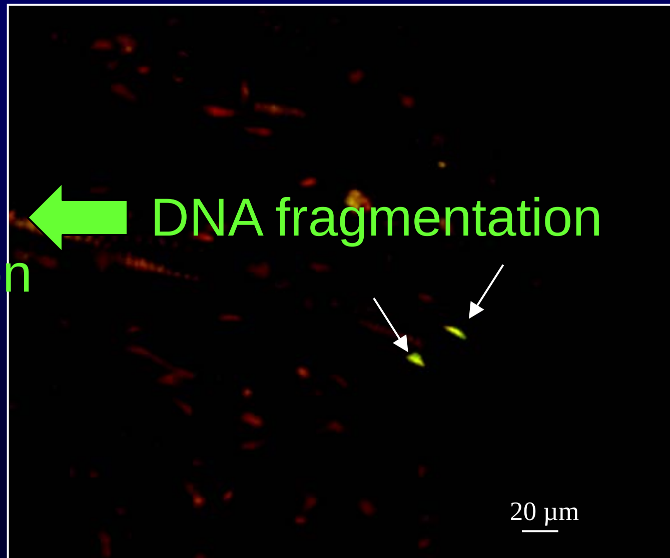
Labeling with fluorescein may also be followed by immunohistochemical detection using anti-fluorescein-specific antibodies that are conjugated to POD or AP.

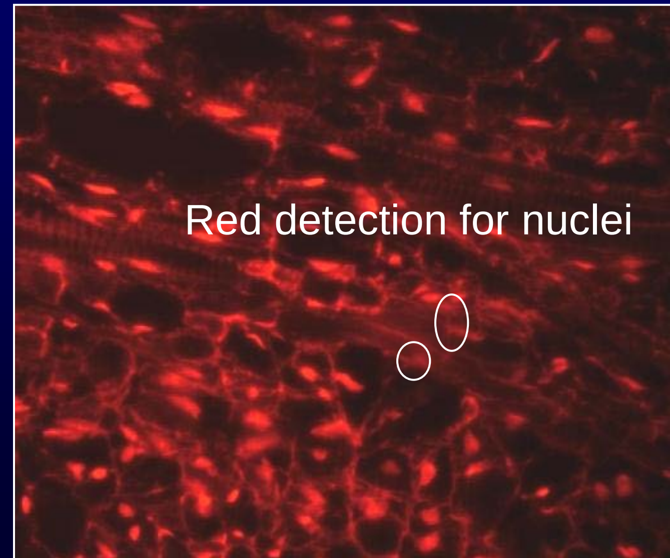


In situ TUNEL assay:

TdT-mediated dUTP terminal nick-end labeling to detect PCD-characteristic DNA fragmentation

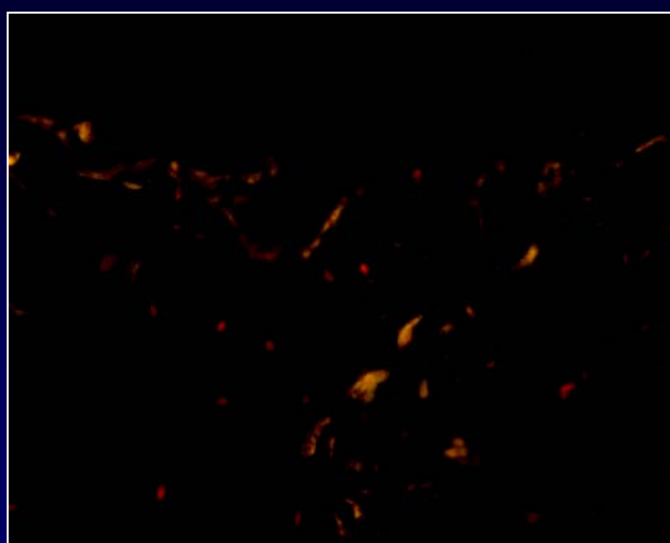
TE differentiation

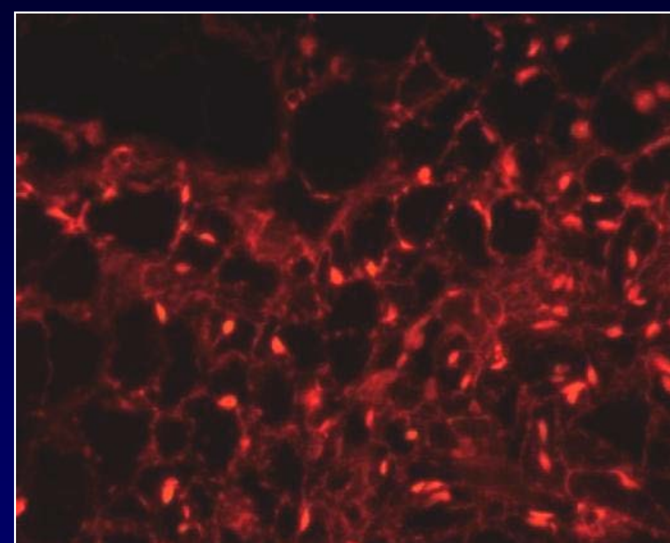




10 DAG

Bosc/Bosc: compatible

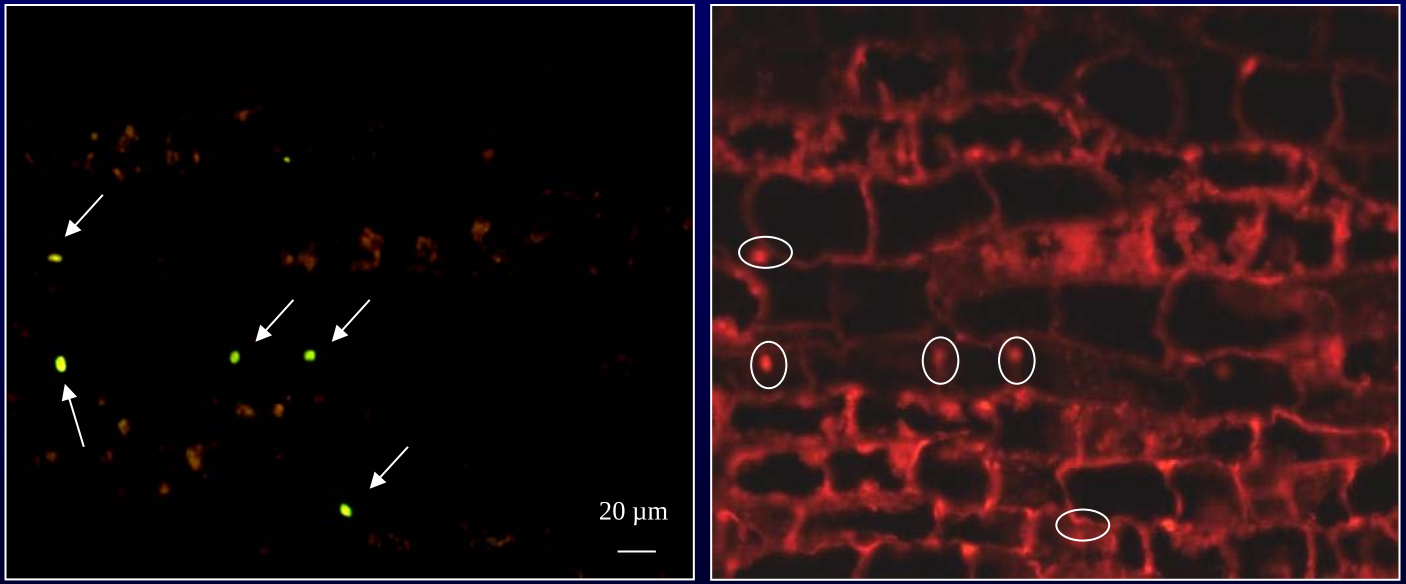




Espen et al, 2002

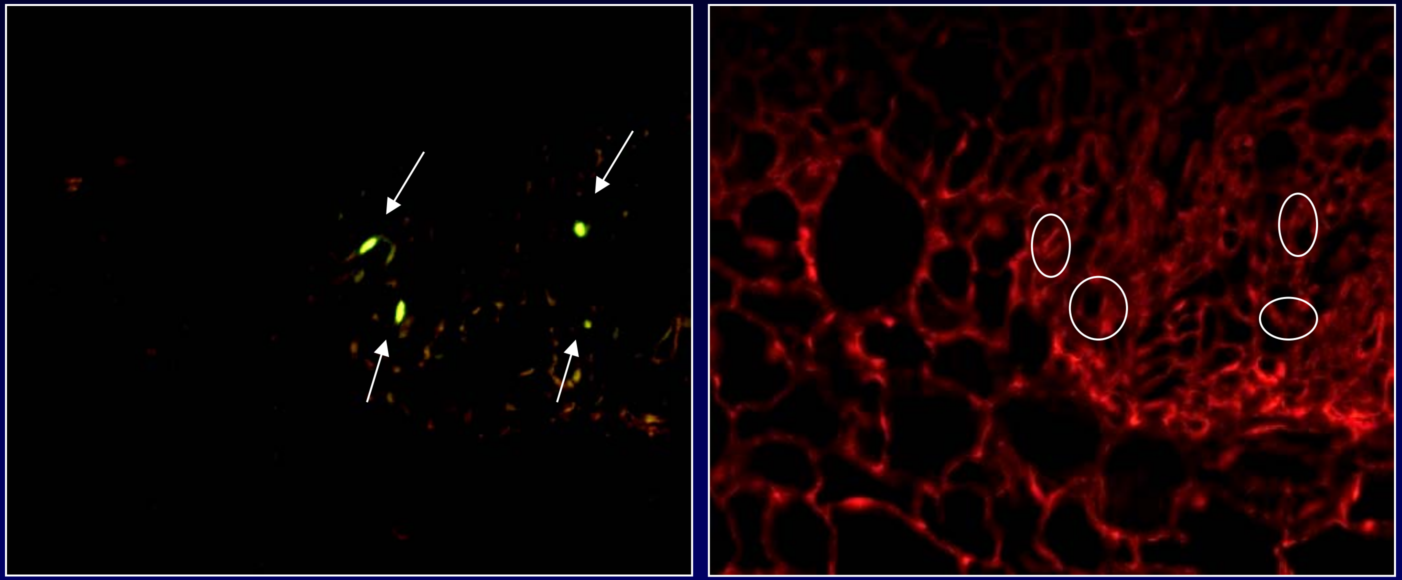
Bosc/MC: incompatible

In situ TUNEL assay



10 DAG

BH/BH: compatible



Espen et al, 2002

BH/MC: compatible

b) Graft of in vitro shoot on acclimating plants

Grafting phases

A) Rootstock preparation.

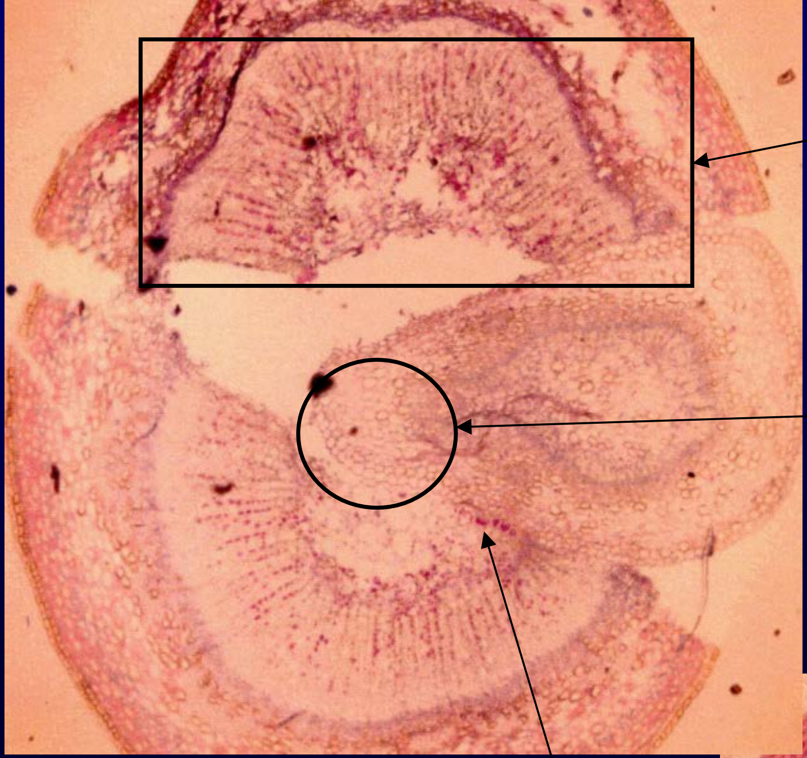
B) In vitro shoot.

C) Grafted plant

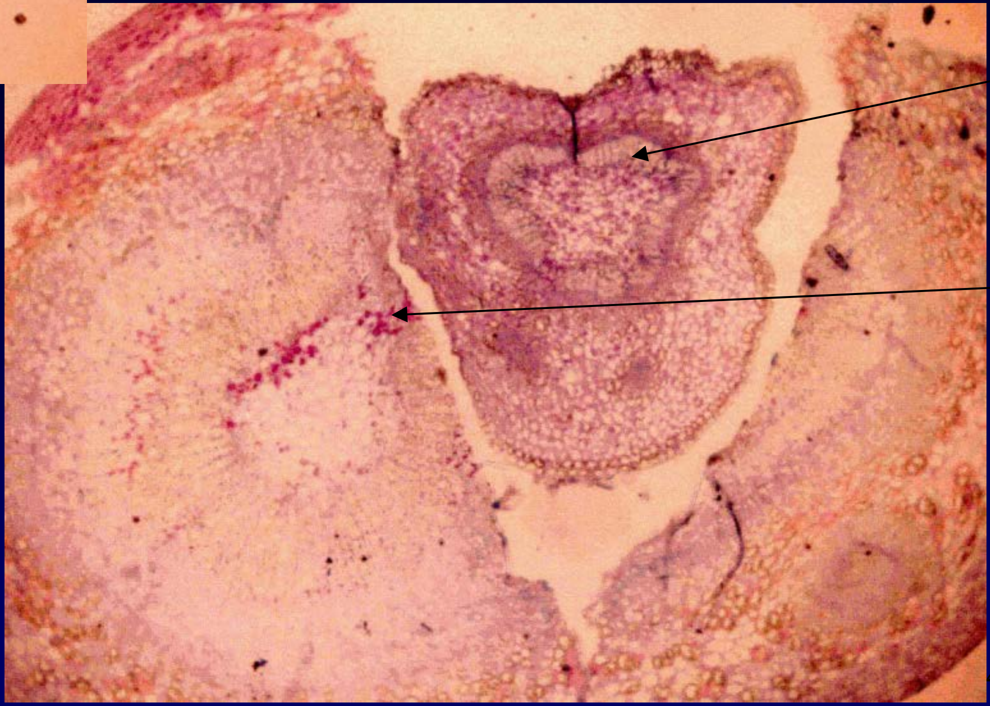
D) Protection to avoid shoot drought.

K/OHF after 30 days

BH/OHF after 30 days



BH/MC after 30 days



K/MC after 30 days

c) Co-culture of callus in the same Petri dishes

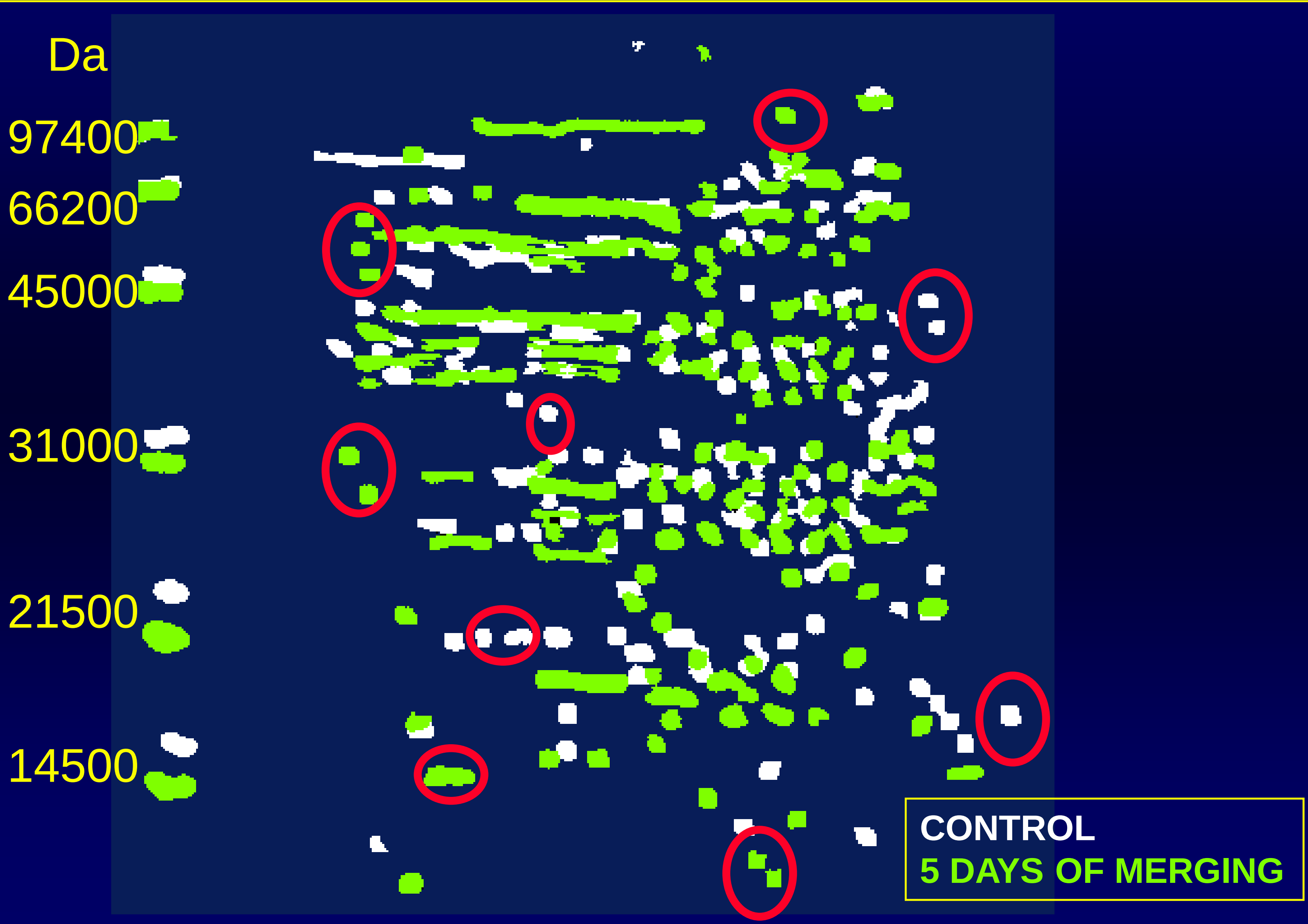
Example 1

Bartlett

Quince BA29

Musacchi *et al.*, 1996

PROTEINS COMPARISON OF BEURRE' HARDY AND QUINCE C CALLUS CONTROL (NEVER MERGED) AND AFTER 5 DAYS OF MERGING



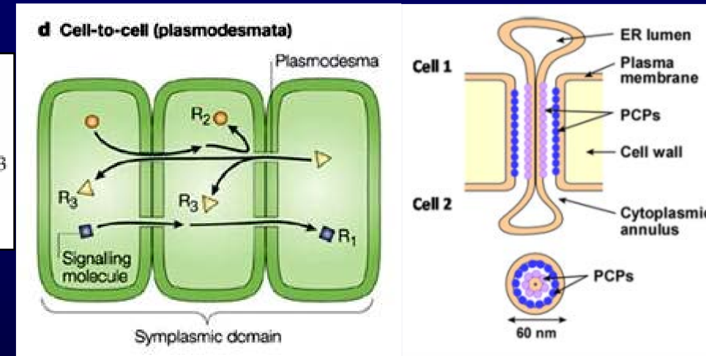
c) Co-culture of callus in the same Petri dishes

Example 2

Cell-to-cell transport through plasmodesmata in tree callus cultures

ANA PINA,[1,2] PILAR ERREA,[1] ALEXANDER SCHULZ[2] and HELLE J. MARTENS[2,3]

Tree Physiology 29, 809–818 2009



Starting point:

- for a successful grafting, the establishment of symplastic contacts in graft interface facilitates compounds transfer between 2 bionts.
- Plasmodesmata mediate the cell-to-cell communication route in the plant kingdom

Hypothesis:

- Localized incompatibility (in some *Prunus* grafts) could be related to insufficient plasmodesmata coupling at an early stage of development within one of the partners. Idea to be verified through bioimaging methods.

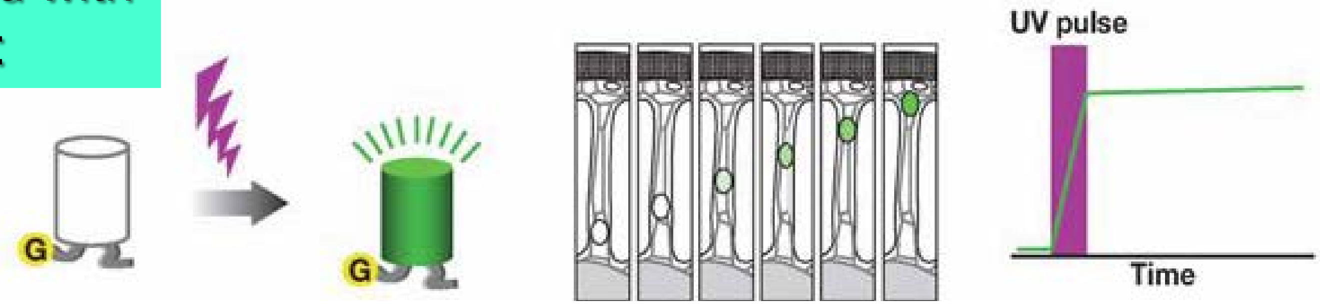
Material:

MO callus= apricot cv. Moniqui
MN callus= plum rootstock Marianna 2624
MO/MO and MN/MN → compatible homografts
MO/MN → incompatible heterograft

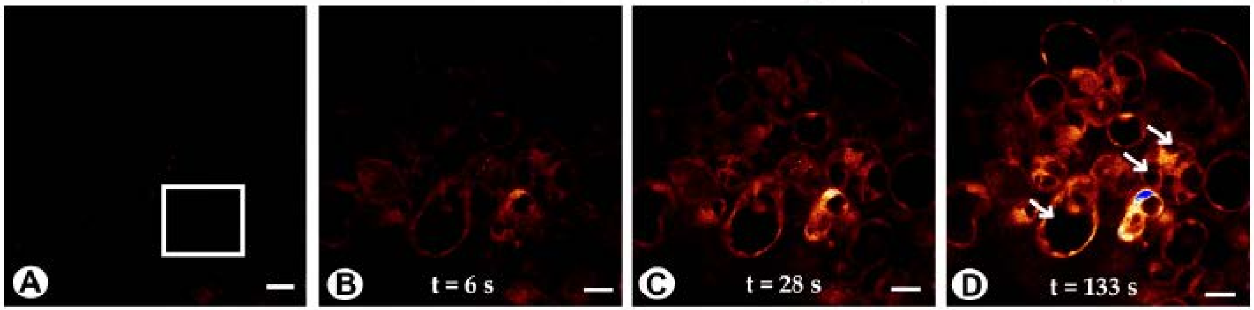
Callus heterograft

Techniques combined with confocal microscopy:

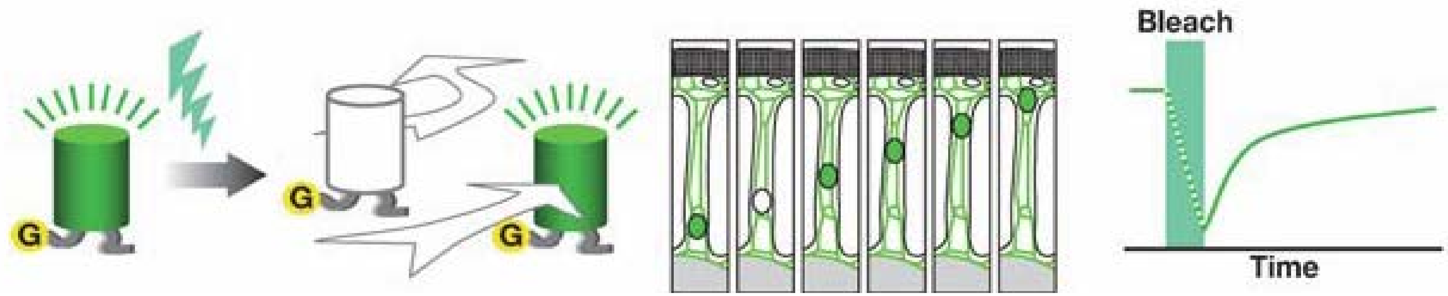
Photoactivation (PAF)



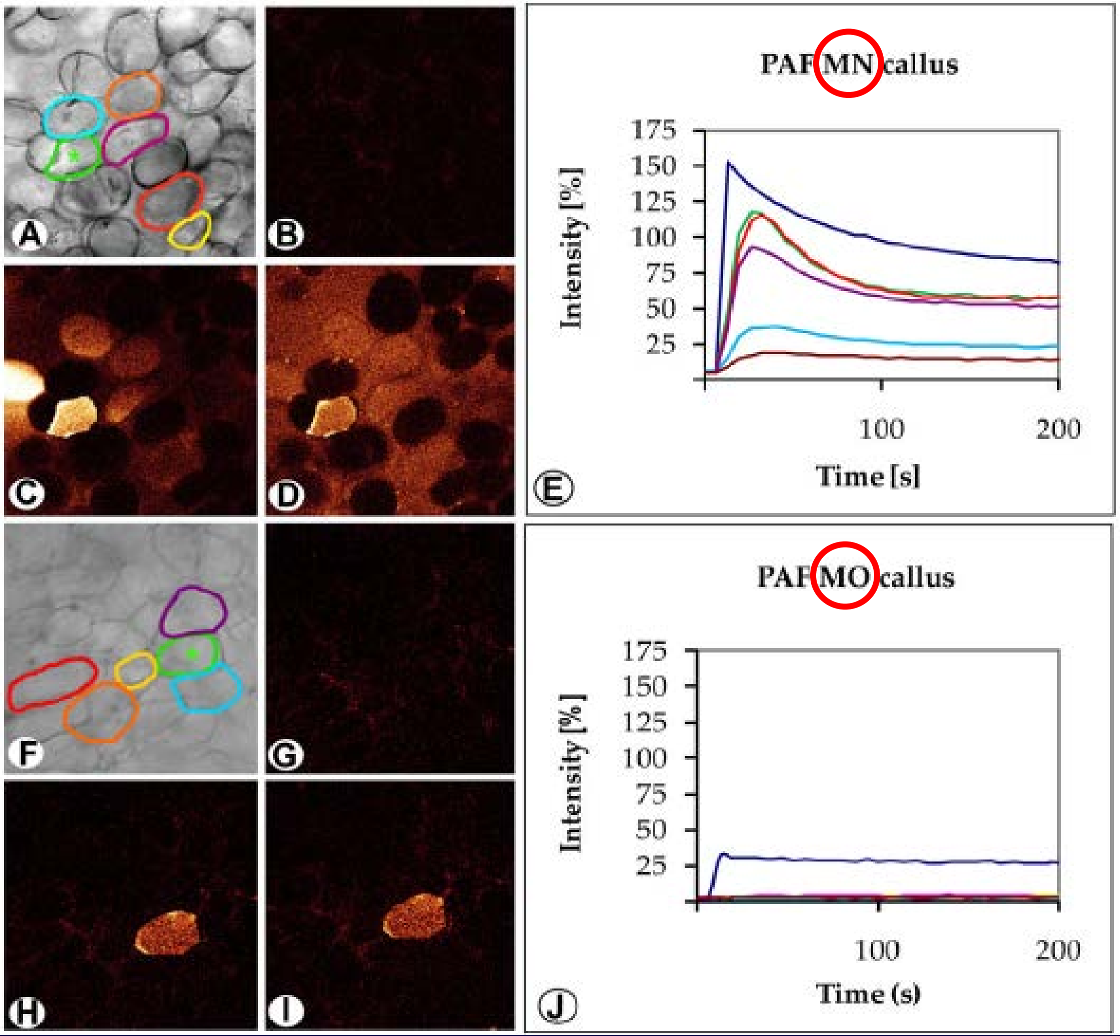
To track the diffusion of released fluorescein via plasmodesmata

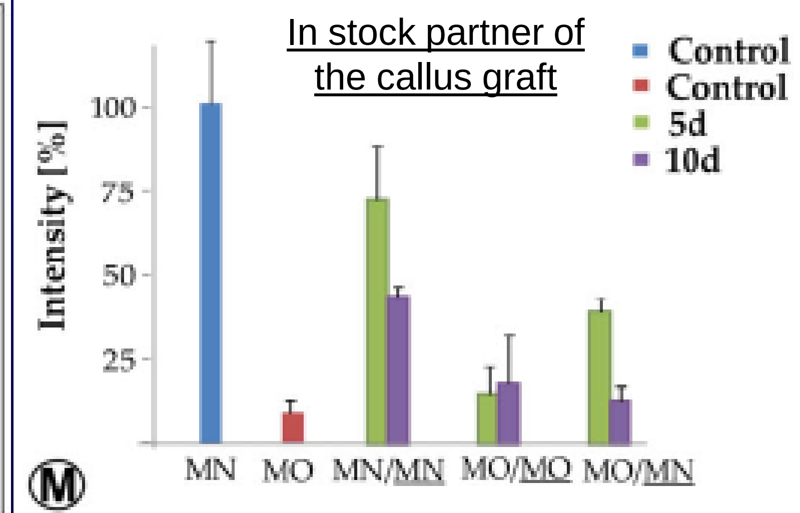


Fluorescence recovery after photobleaching (FRAP)



To quantitatively measure the movements of fluorescently tagged molecules or structure within live cells after photobleaching





FRAP quantitative experiments confirm the results of a poor rate of symplastic communication in the apricot MO cv. as found with PAF. This may affect the passages of nutrients and macromolecules at early stages of developmental between MO and MN in heterograft.

Novel control factor of connectivity that reaches the graft partner and alters its innate rate of communication.

(Pina *et al.*, 2009)

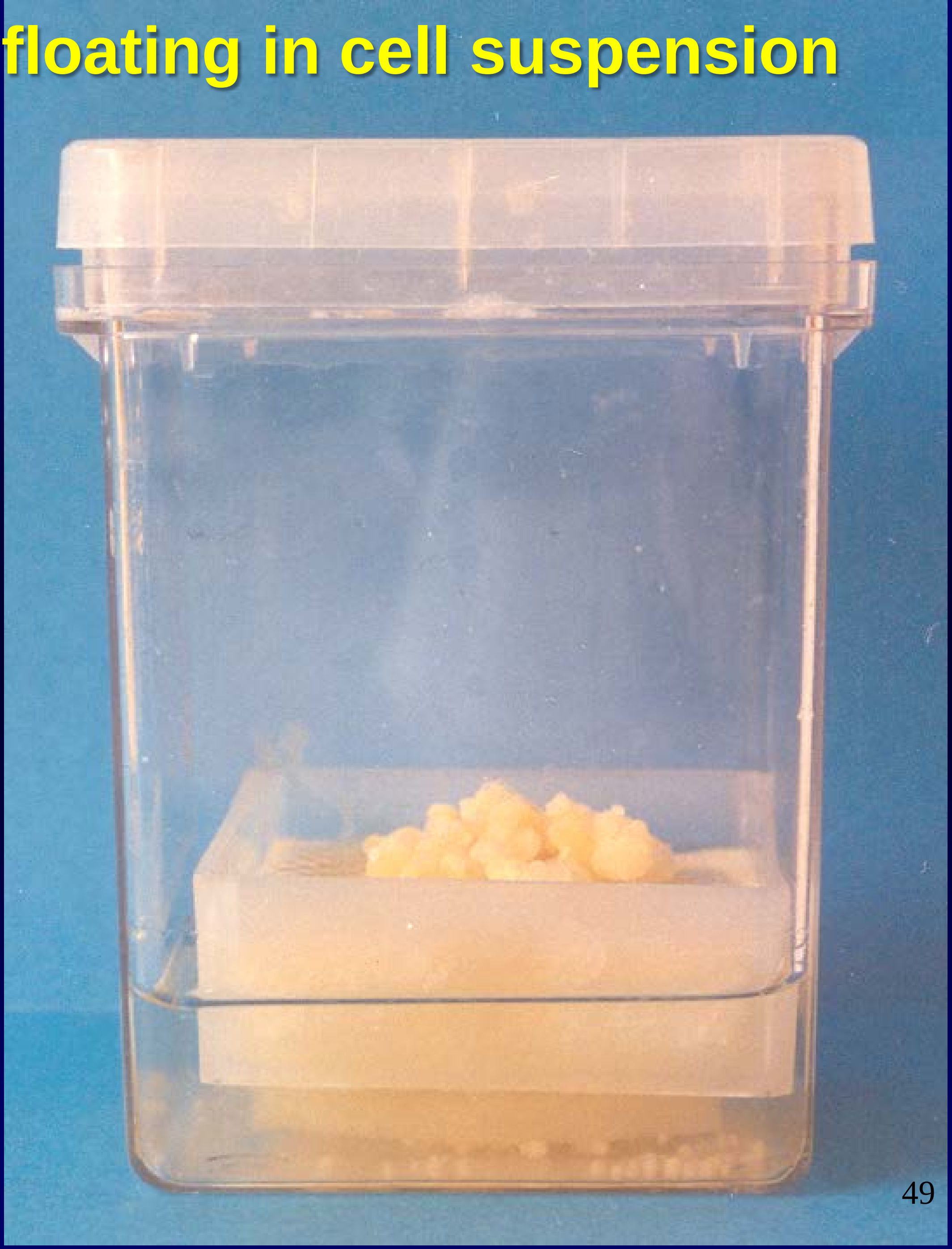
d) co-culture of cell suspensions

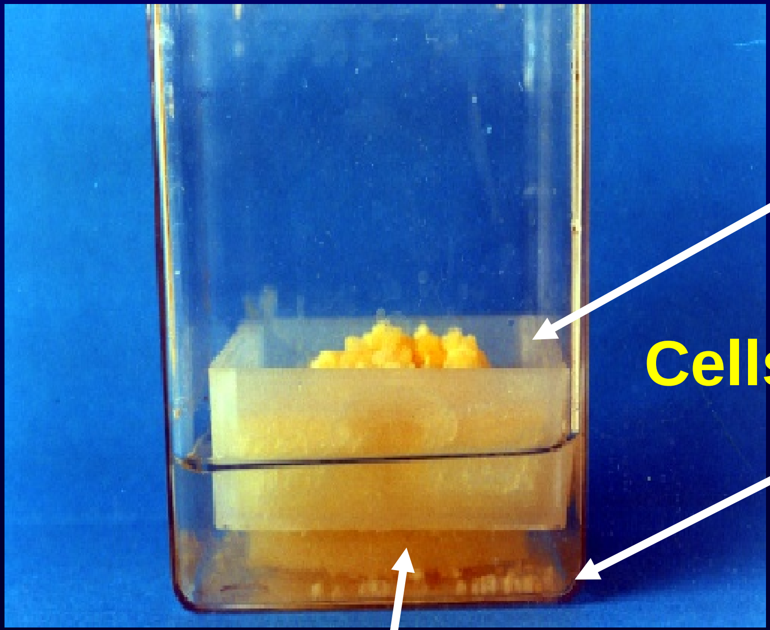
Dark, ~22°C for 14 days at 78 rpm

e) co-culture of callus floating in cell suspension

A "Magenta" and a raft with a low protein-absorption membrane (Durapore, 5 µm pore size, Millipore)

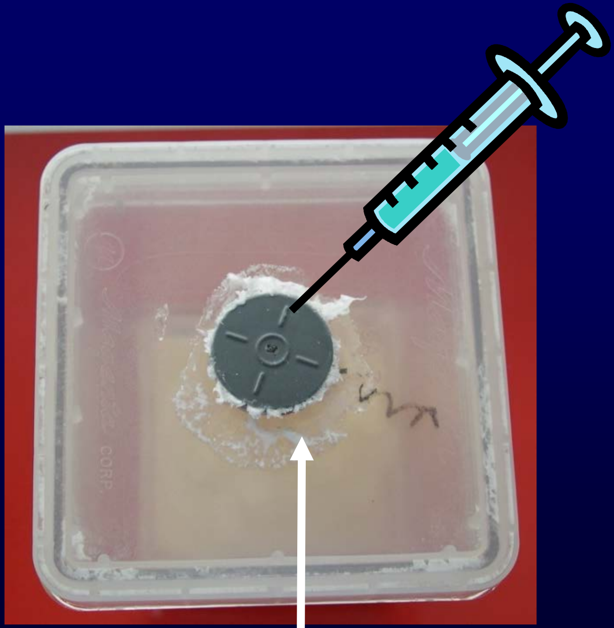
The graft union is simulated by placing the callus over the membrane and the cell-suspension cultures under it, so that the raft, kept in motion by a mechanical stirrer, floats.





Callus

Cells suspension

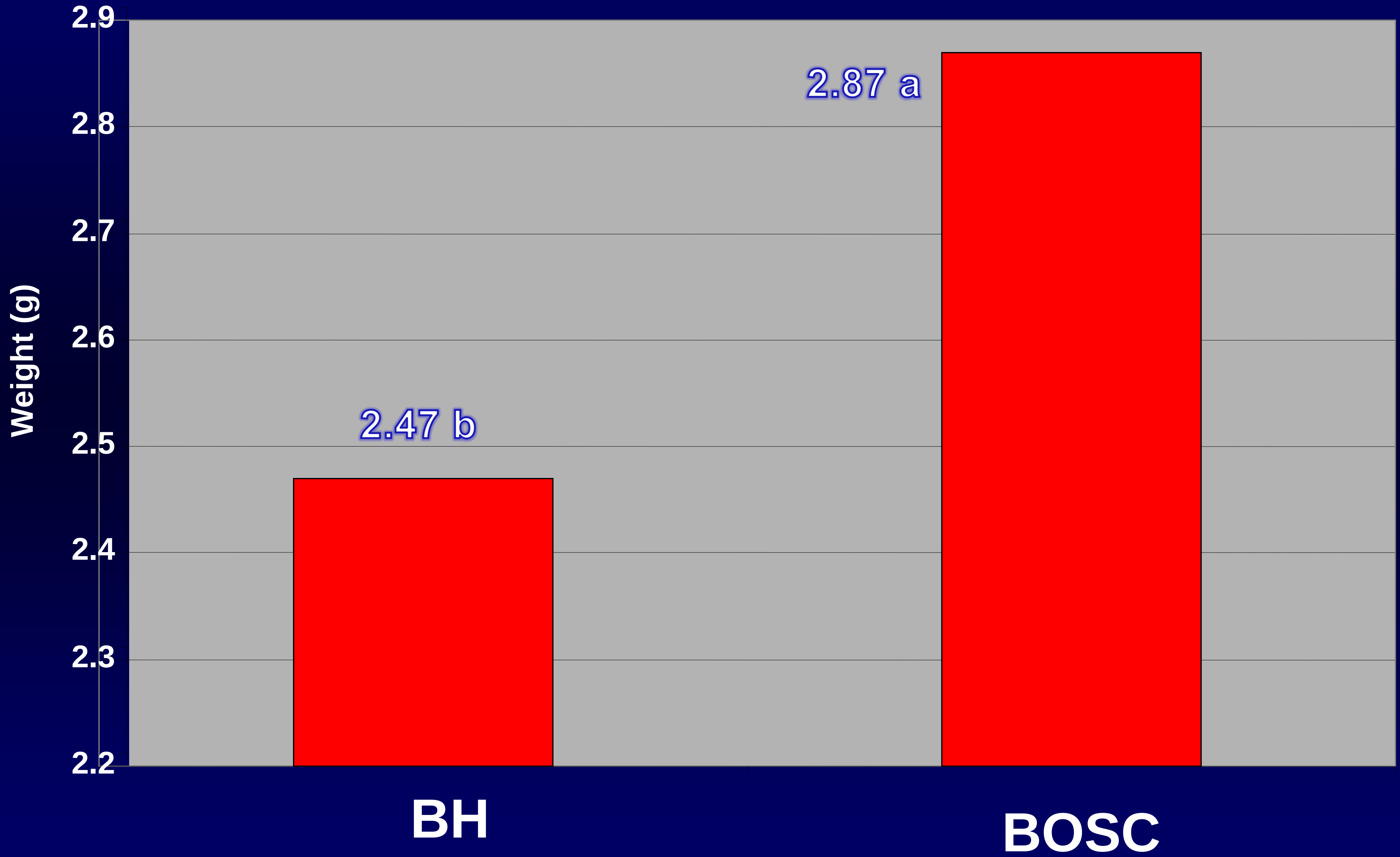


Magenta lid with rubber septum

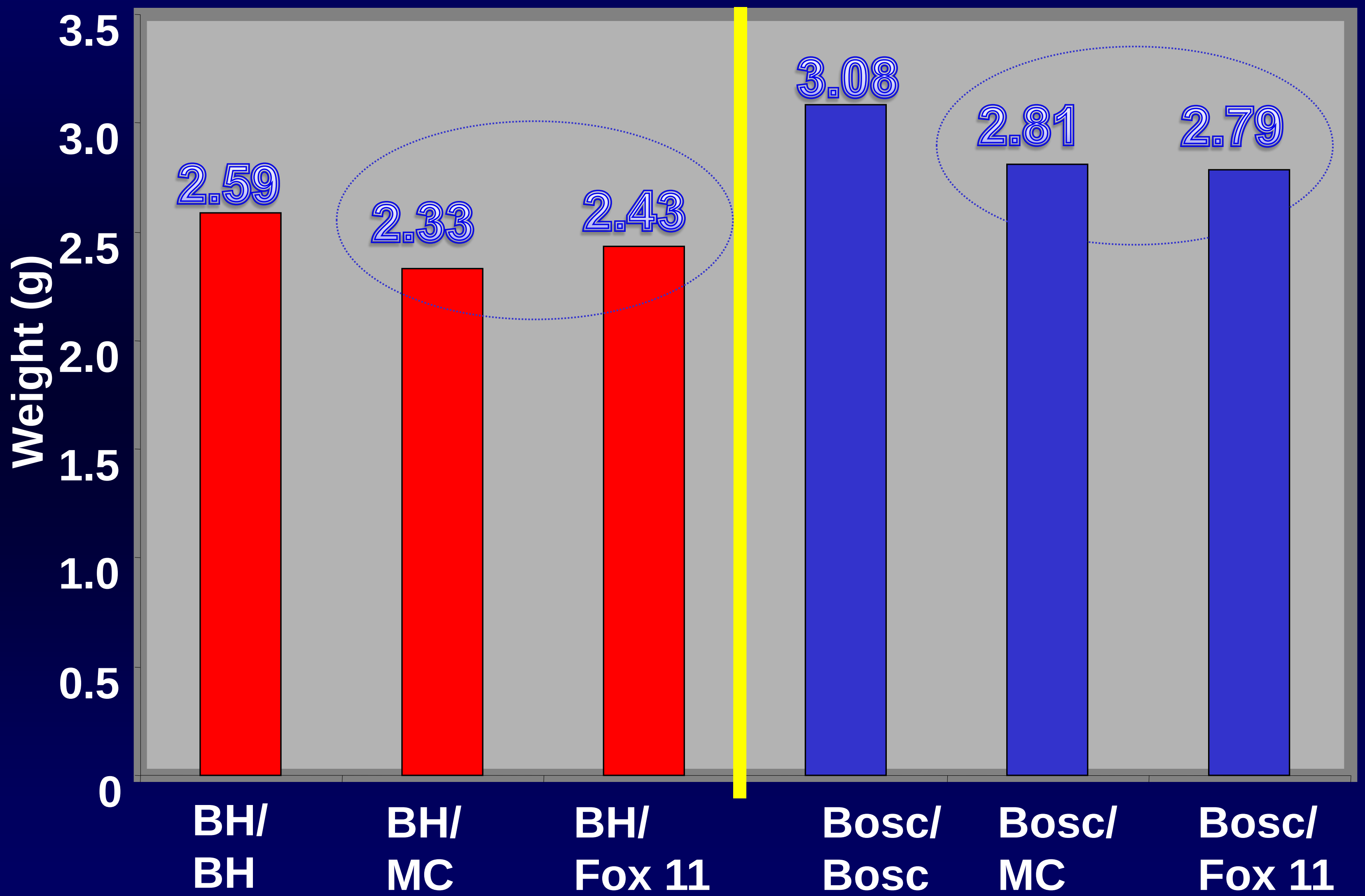
Sponge

Membrane

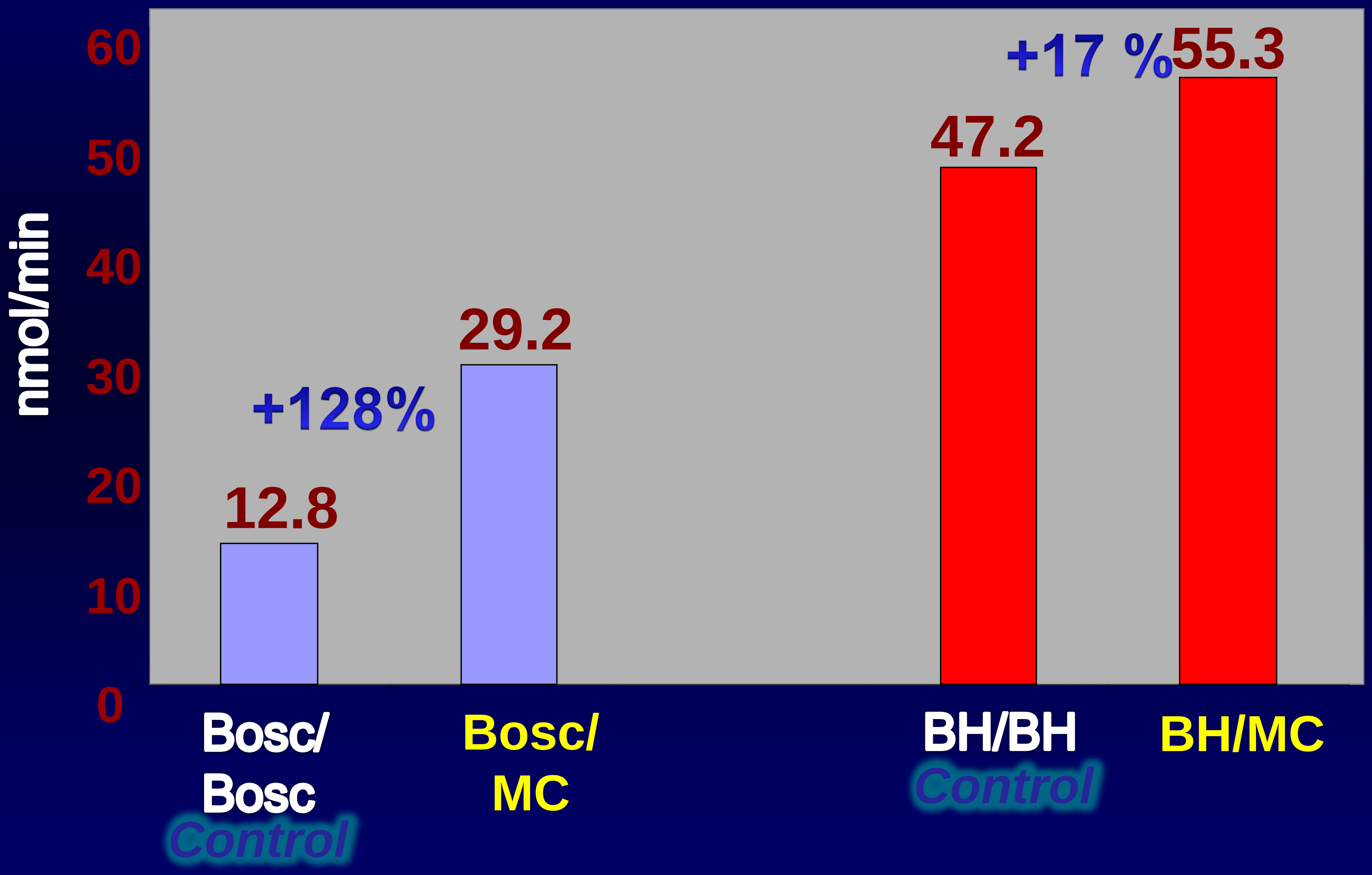
Cultivar callus growth



Callus growth after one week of co-culture

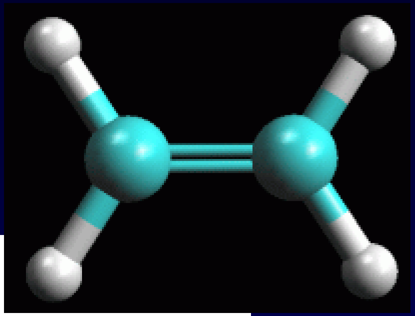


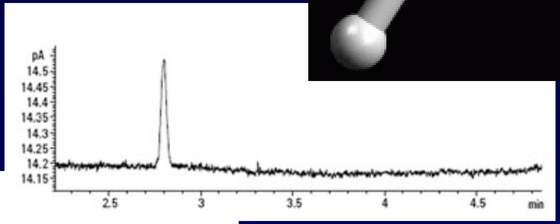
Respiration rate

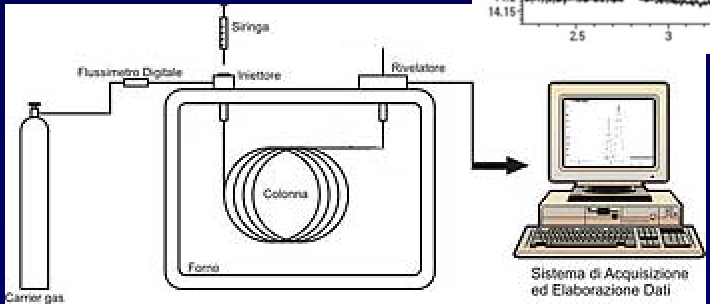


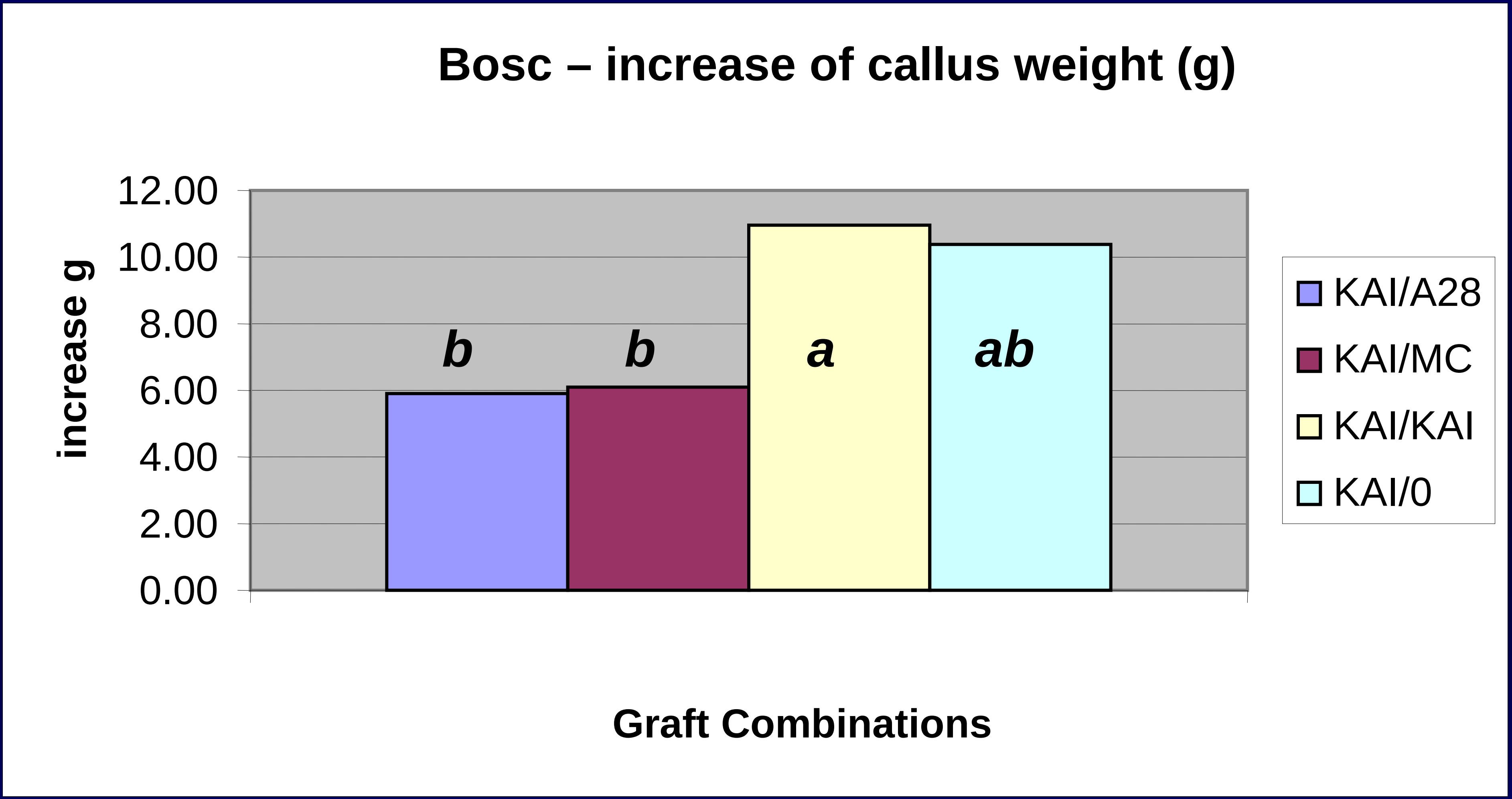
GAS ANALYSIS

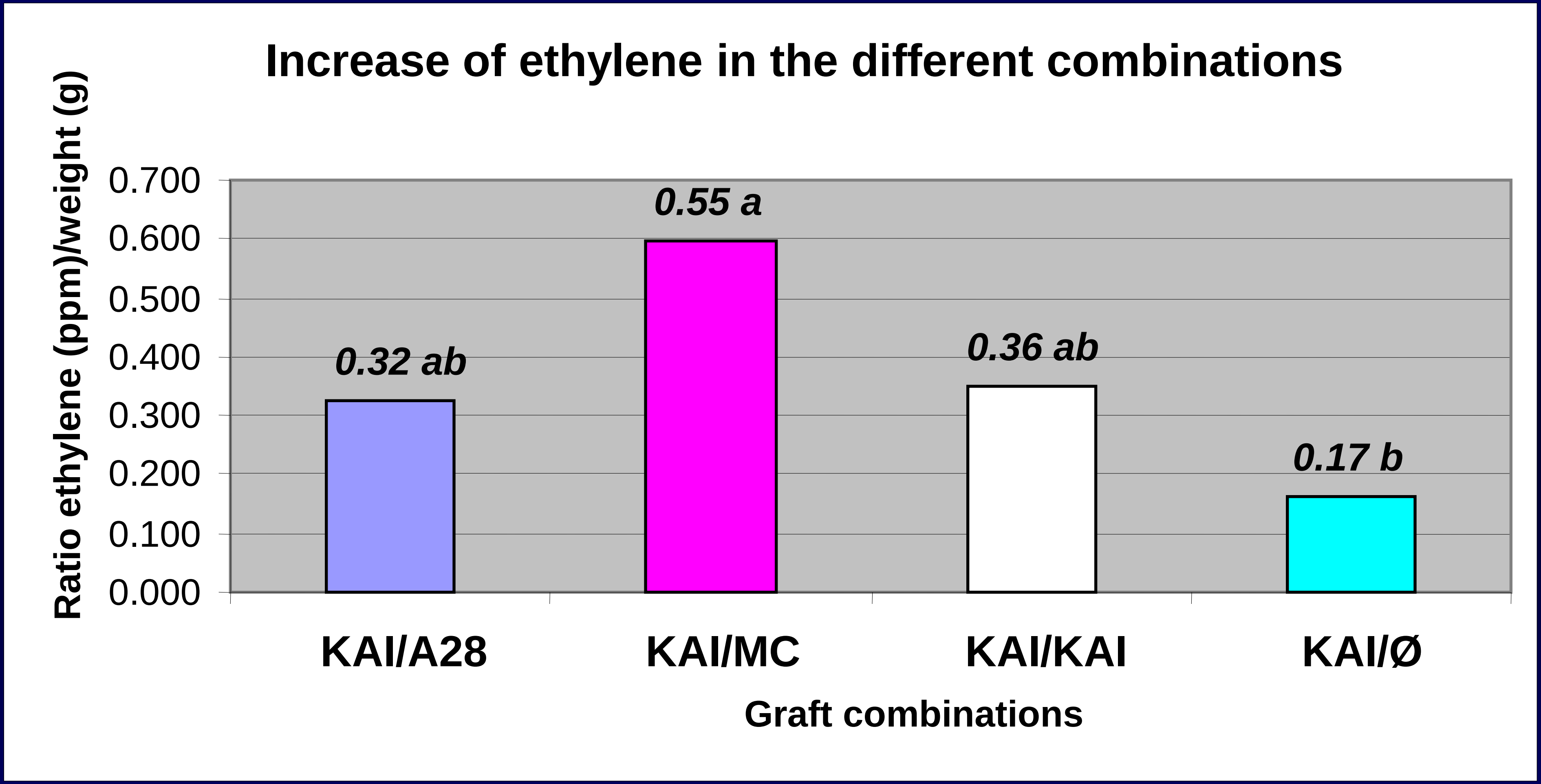
- CO_2
- Ethylene



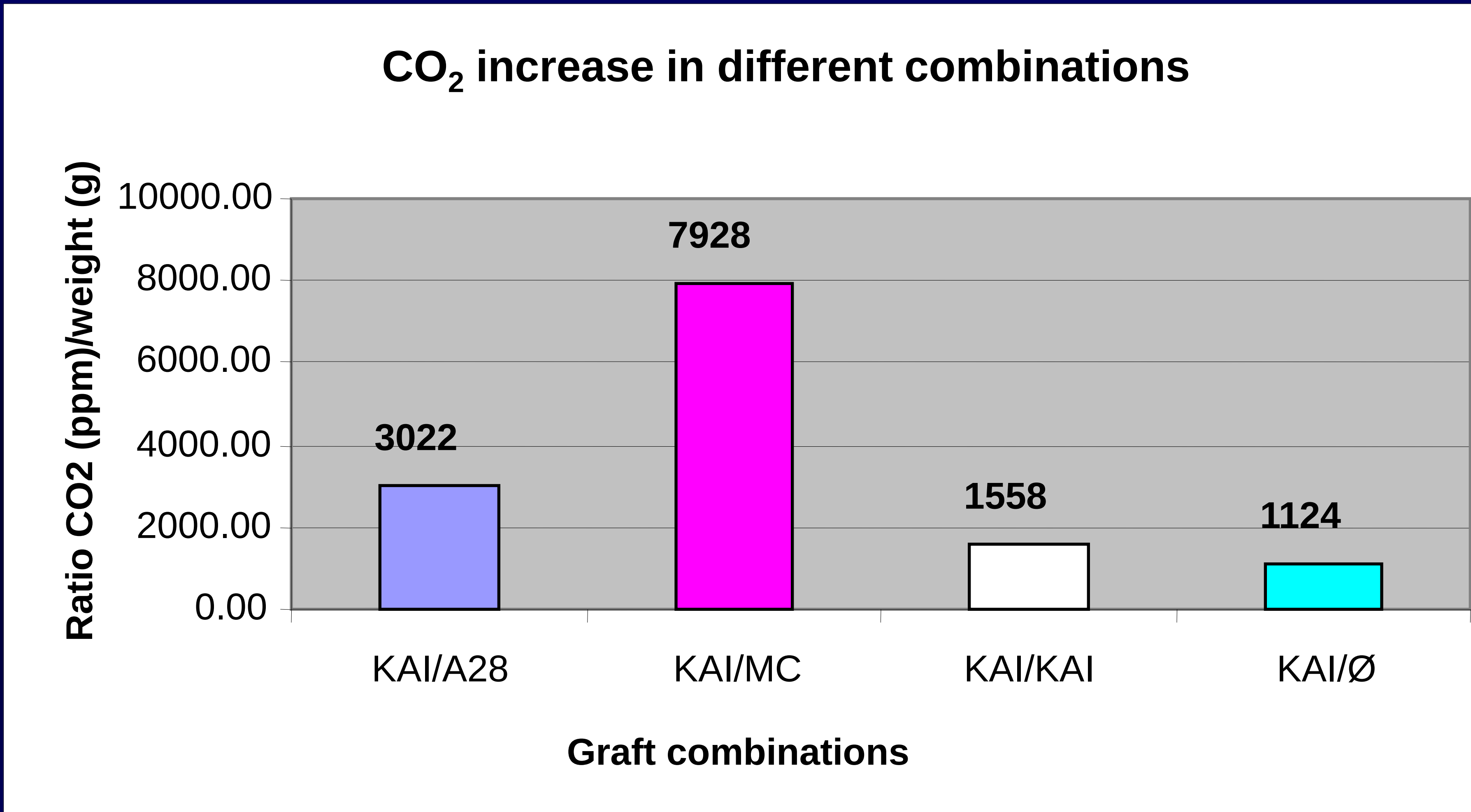





Bosc – increase of callus weight (g)
increase g
12.00
10.00
8.00
6.00
4.00
2.00
0.00
b
b
a
ab
KAI/A28
KAI/MC
KAI/KAI
KAI/0
Graft Combinations



Increase of ethylene in the different combinations
Ratio ethylene (ppm)/weight (g)
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.32 ab
0.55 a
0.36 ab
0.17 b
KAI/A28
KAI/MC
KAI/KAI
KAI/Ø
Graft combinations



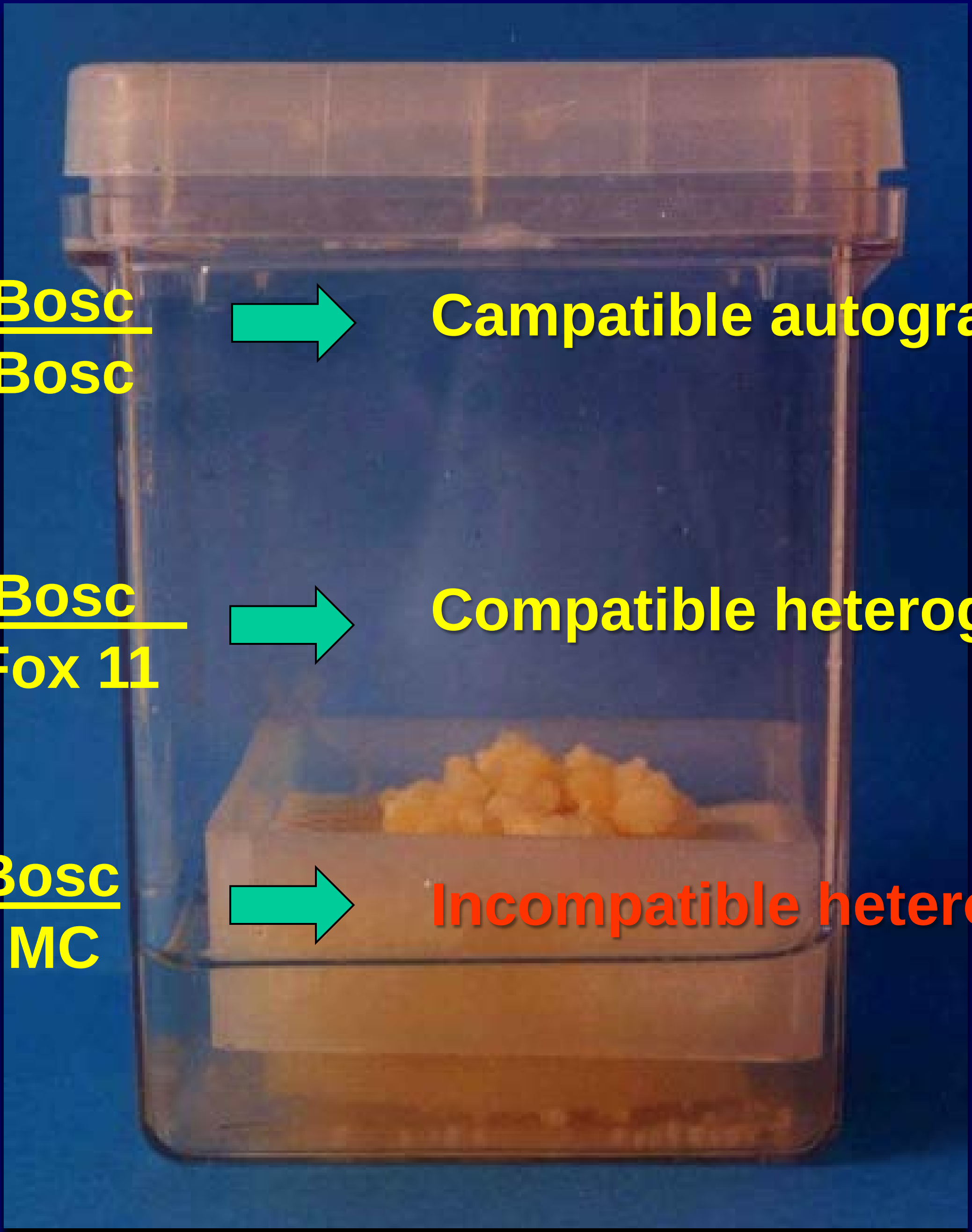
CO2 increase in different combinations
Ratio CO2 (ppm)/weight (g)
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
7928
3022
1558
1124
KAI/A28
KAI/MC
KAI/KAI
KAI/Ø
Graft combinations

RNA ANALYSES:

mRNA DIFFERENTIAL DISPLAY

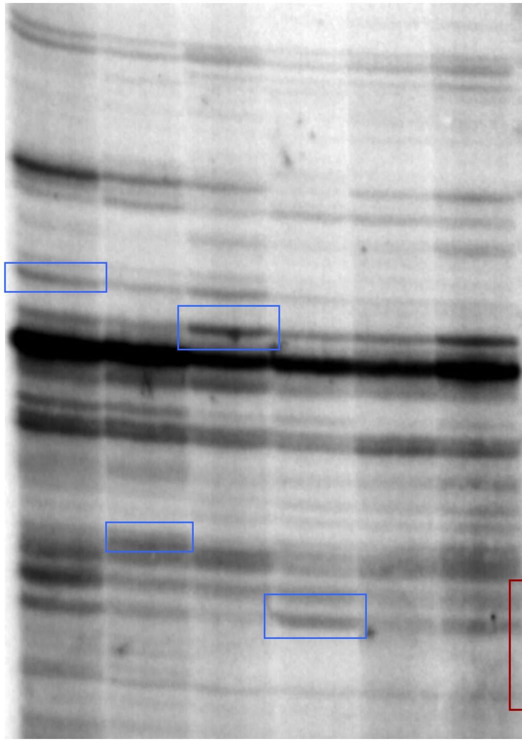
allows direct isolation and cloning of cDNA fragments corresponding to mRNAs differently expressed in the compatible and incompatible combinations *(Pirovano et al., 2002)*

- **RNA extraction**
- **Reverse transcription with 3'-anchored primer ($T_{12}AC$)**
- **PCR amplification with 3'-anchored primer and random decamer**
- **Separation on acrylamide gels and identification of DD-fragments**
- **DNA extraction from excised bands of interest**
- **Re-amplification by PCR with same conditions**
- **Separation on agarose gels**
- **Excision of amplified products and cloning and sequencing**

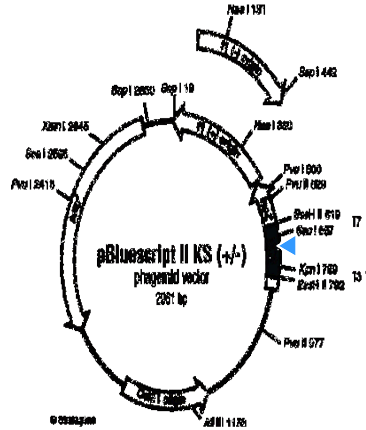

Bosc
Bosc
Campatible autograft
Bosc
Fox 11
Compatible heterograft
Bosc
MC
Incompatible heterograft

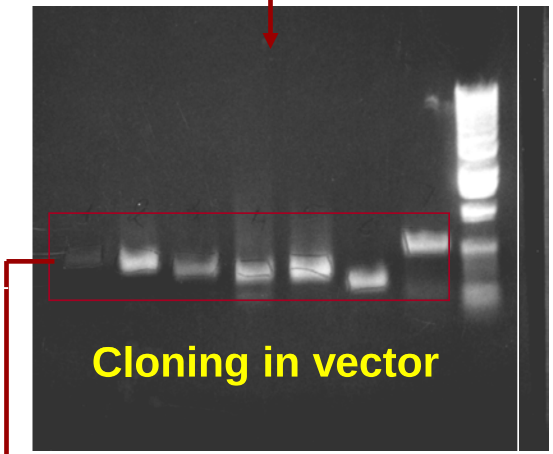
mRNA Differential Display

Reamplification of cDNA fragments



B/B B/E B/F B/B B/E B/F





Reverse Northern Dot-Blot
To screen cDNA fragments really differentially expressed

Northern blot
to study gene expression

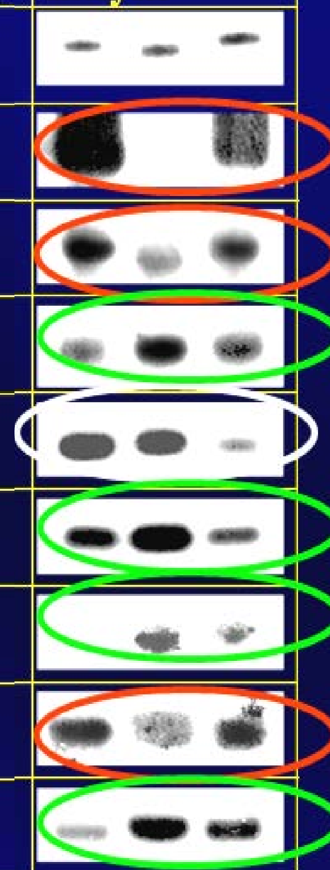
Sequencing cDNAs

Homology research in GenBank and EMBL databases

Primers:
$T_{12}AC$; $T_{12}CA$; $T_{12}AA$; $T_{12}GA$; $T_{12}AG$; $T_{12}CG$

GTGGCCGATG
AGCAGCGAGG
CCTGGGTCAG
GTCGGTTGTC

Clone	Tag size (pb)	Putative identity	Associated sequence	% identity, expect value	Northern analysis
T12CA-7	292	NT3	*Nicotiana tabacum*	39% 5e-10	
T12CA-10	138	Pathogenesis related protein I (SaPRI)	*Santalum album*	100% 4e-53	
T12AG-14	261	Unknown protein	*Arabidopsis thaliana*	60% 9e-15	
T12AG-7	215	Import intermediate associated protein (chloroplast)	*Pisum sativum*	86% 3e-24	
T12AG-10	195	Extracellular dermal glycoprotein (EDGP)	*Daucus carota*	89% 1e-13	
T12AG-14	383	Putative protein translation factor SUI1 homolog (eIF-2A)	*Arabidopsis thaliana*	92% 8e-15	
T12CG-7	302	NADH dehydrogenase (ubiquinone) subunit 1	*A. Thaliana*	94% 2e-20	
T12CG-11	192	Platelet/endothelial cell adhesion molecule	*Homo sapiens*	61% 5e-3	
T12AA-14	113	No significant similarity			



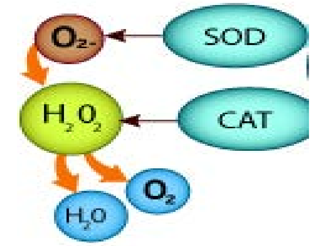
B/B B/MC B/F

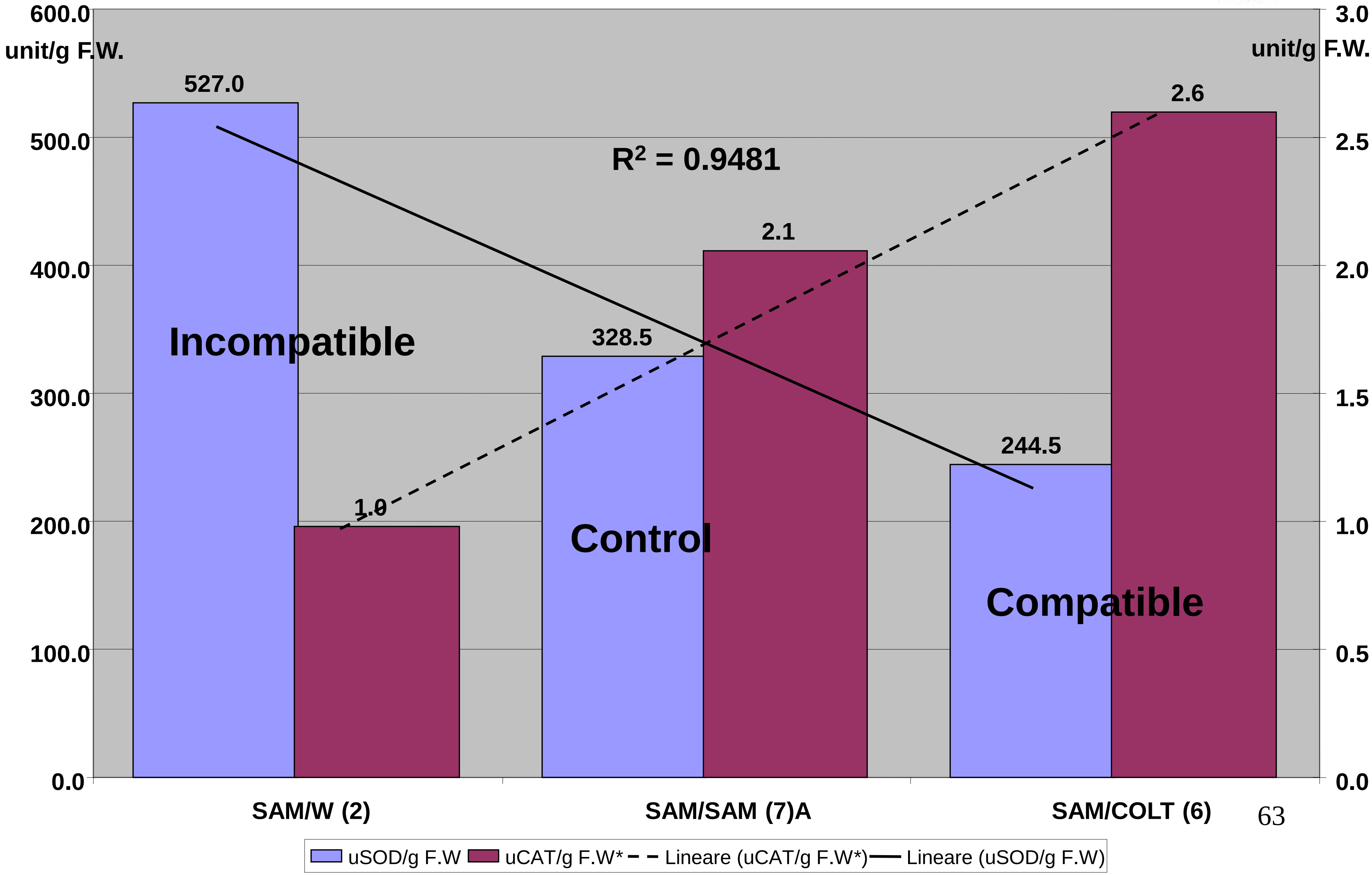
Pirovano et al. 2002

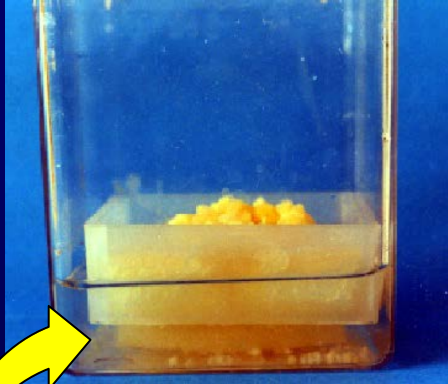
PROTEINS ANALYSES

- Enzyme activity (SOD and CAT)
- SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)
- 2D-PAGE (Two-dimensional gel electrophoresis)

SOD and CAT activities in cherry co-culture





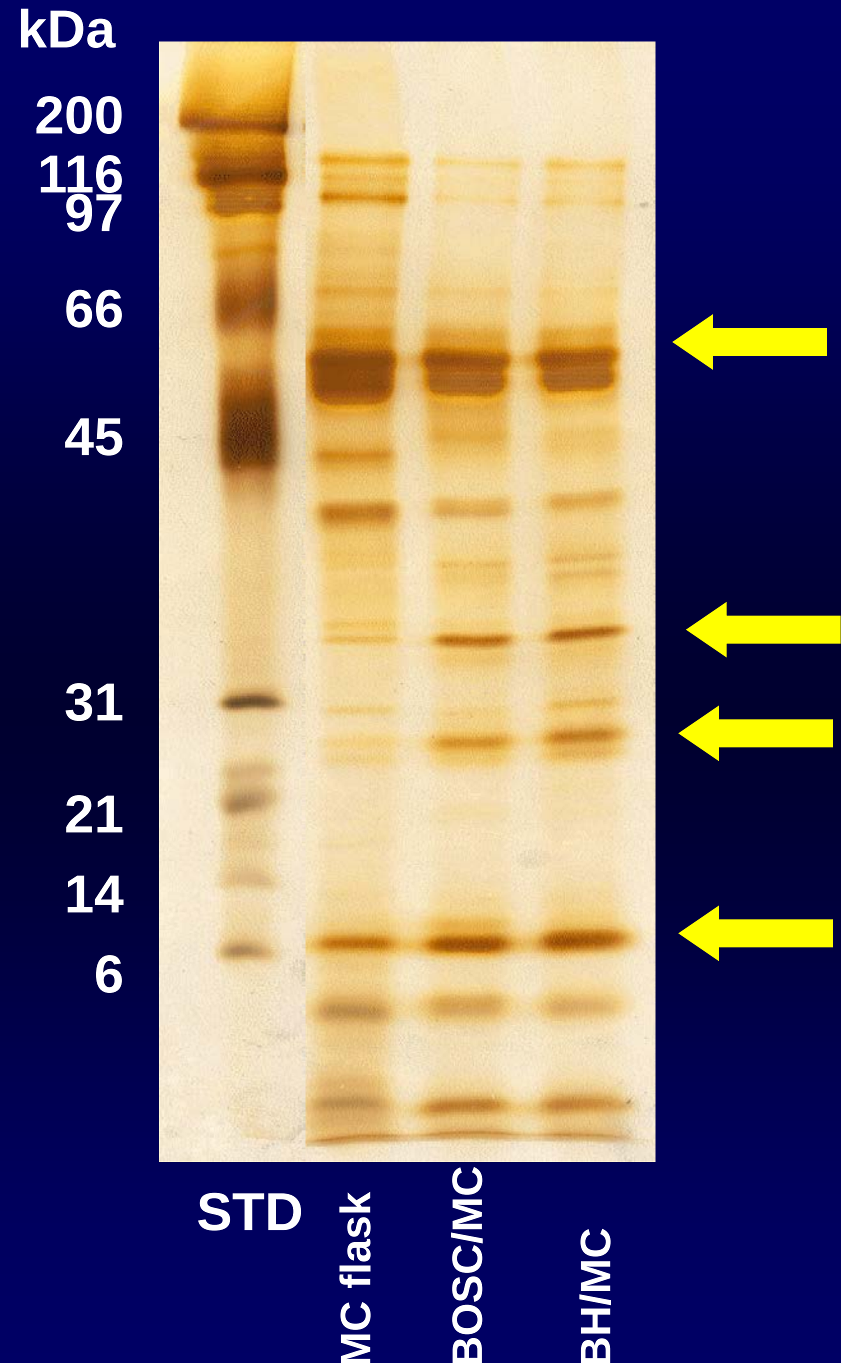


Proteins extracted from the liquid medium

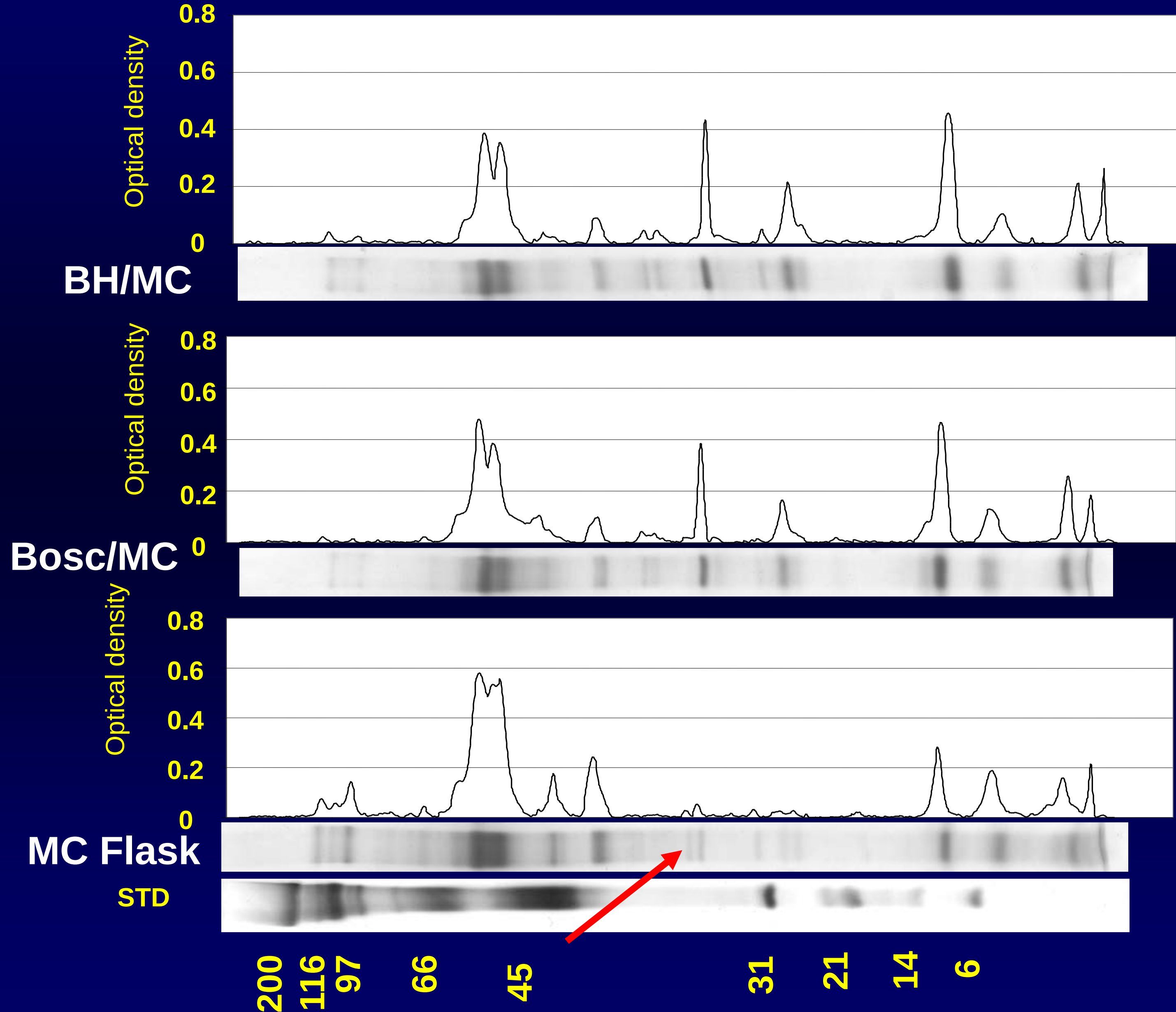
1 - MC flask

2- Bosc/MC

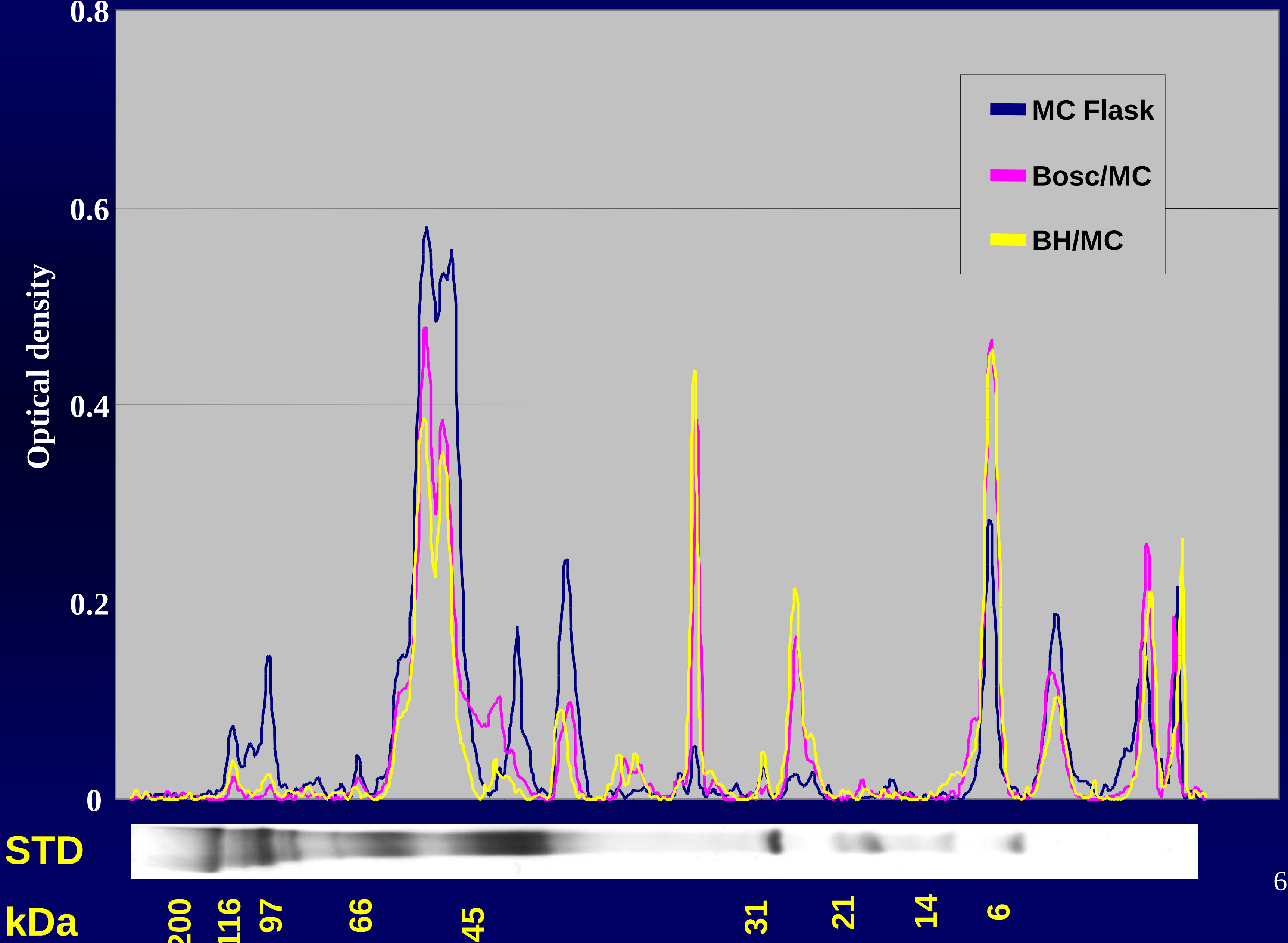
3 - BH/MC



Quince MC: densitometric analysis of proteins extracted from the **liquid medium** of cell suspension alone and in co-culture



Quince MC: densitometric analysis of proteins extracted from the liquid medium of cell suspension

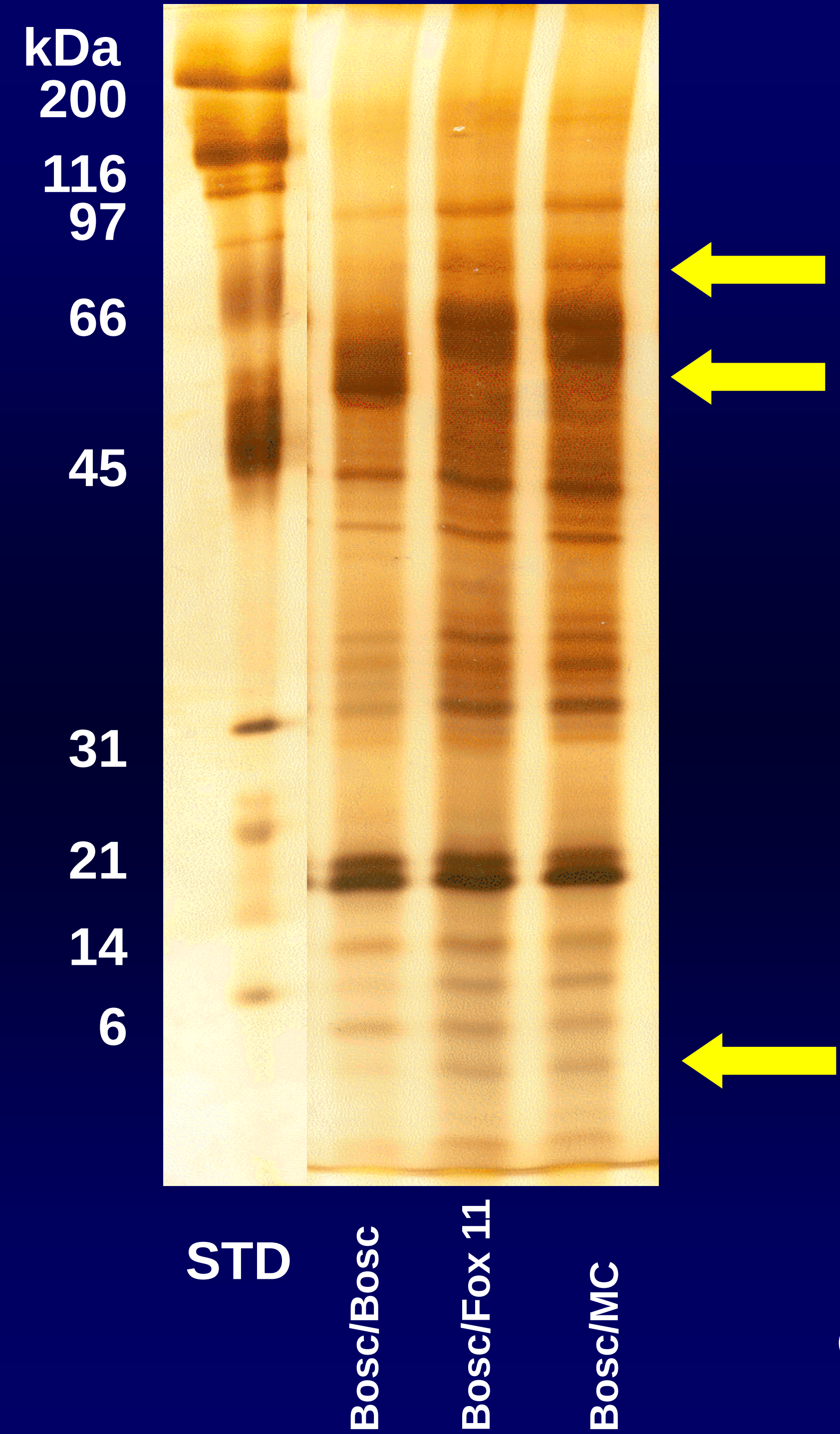


Proteins extracted from callus above the porous membrane

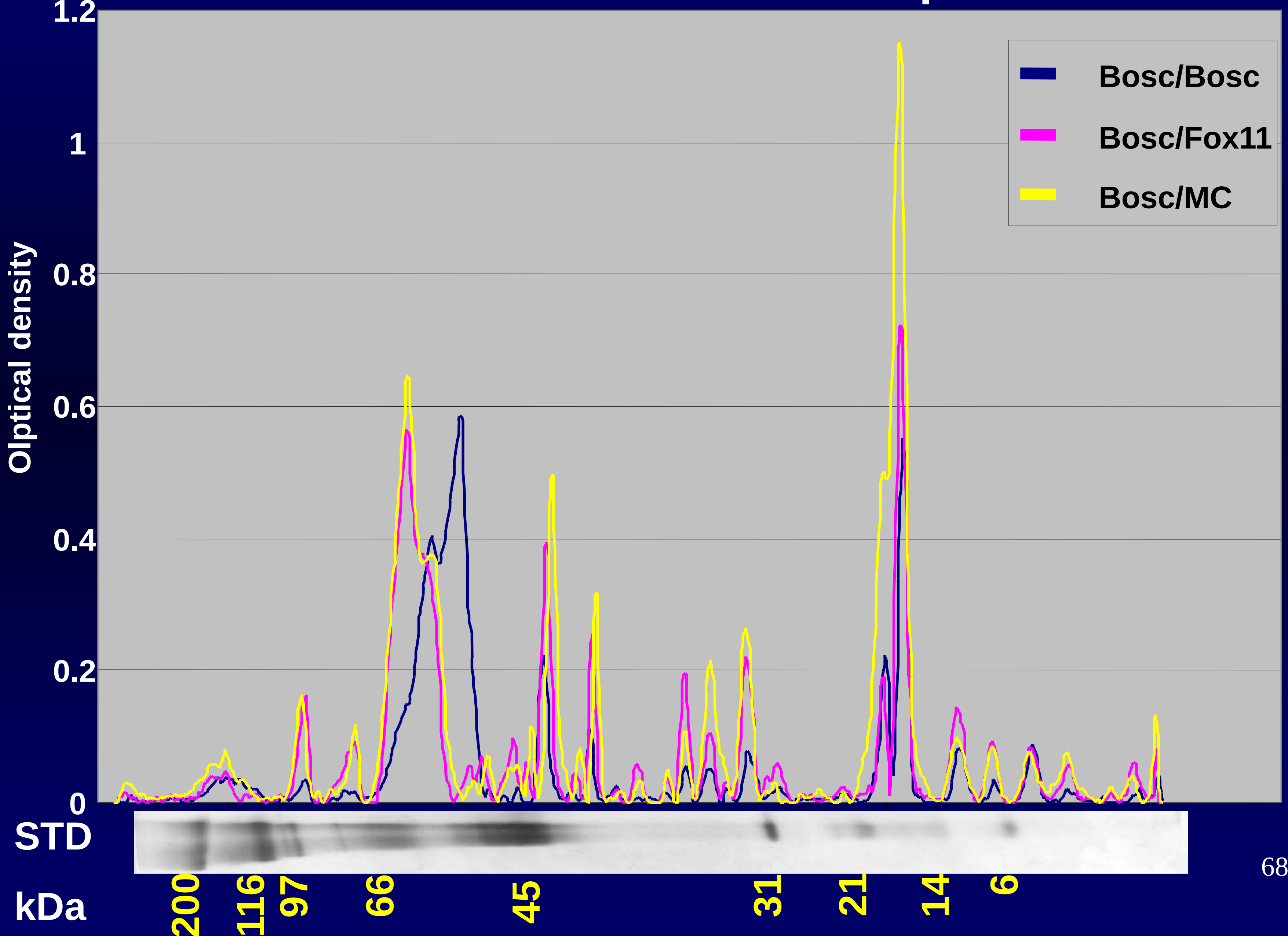
1. Bosc/Bosc

2. Bosc/Fox 11

3. Bosc/MC

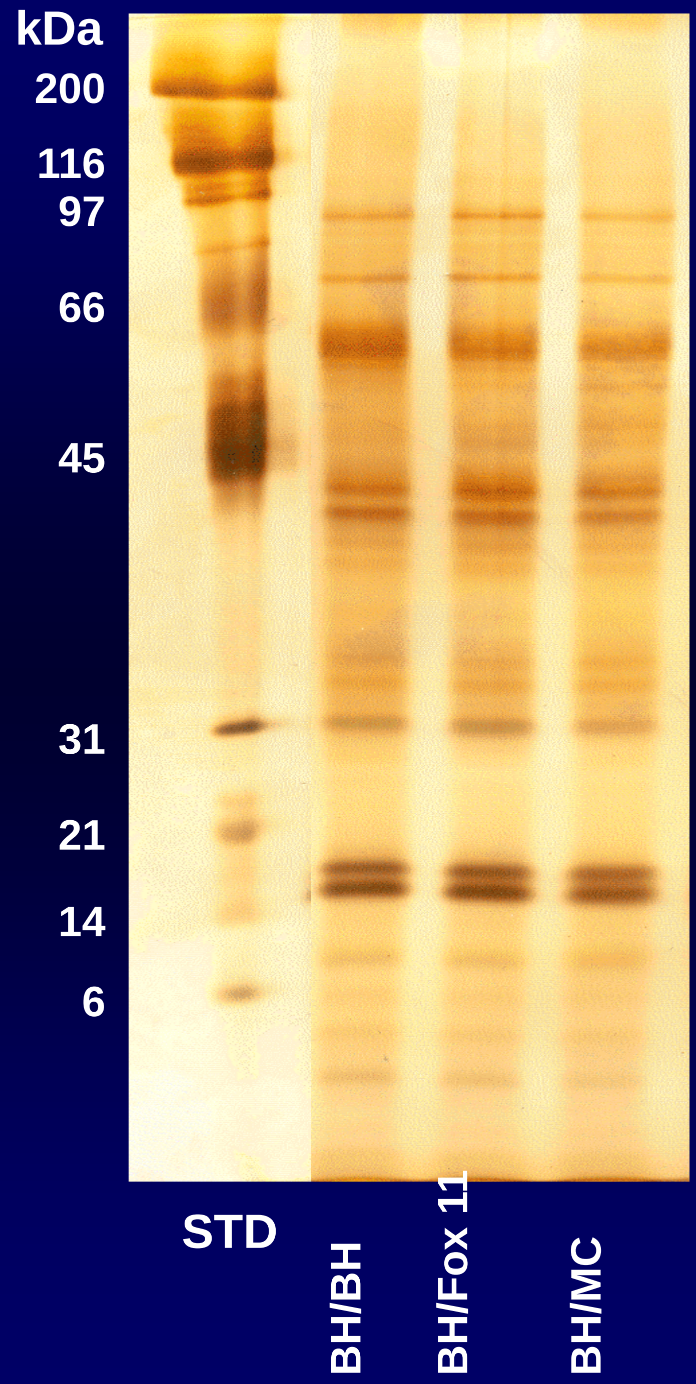


Cv Beurré Bosc: Densitometric analysis of proteins extracted from the callus above the porous membrane

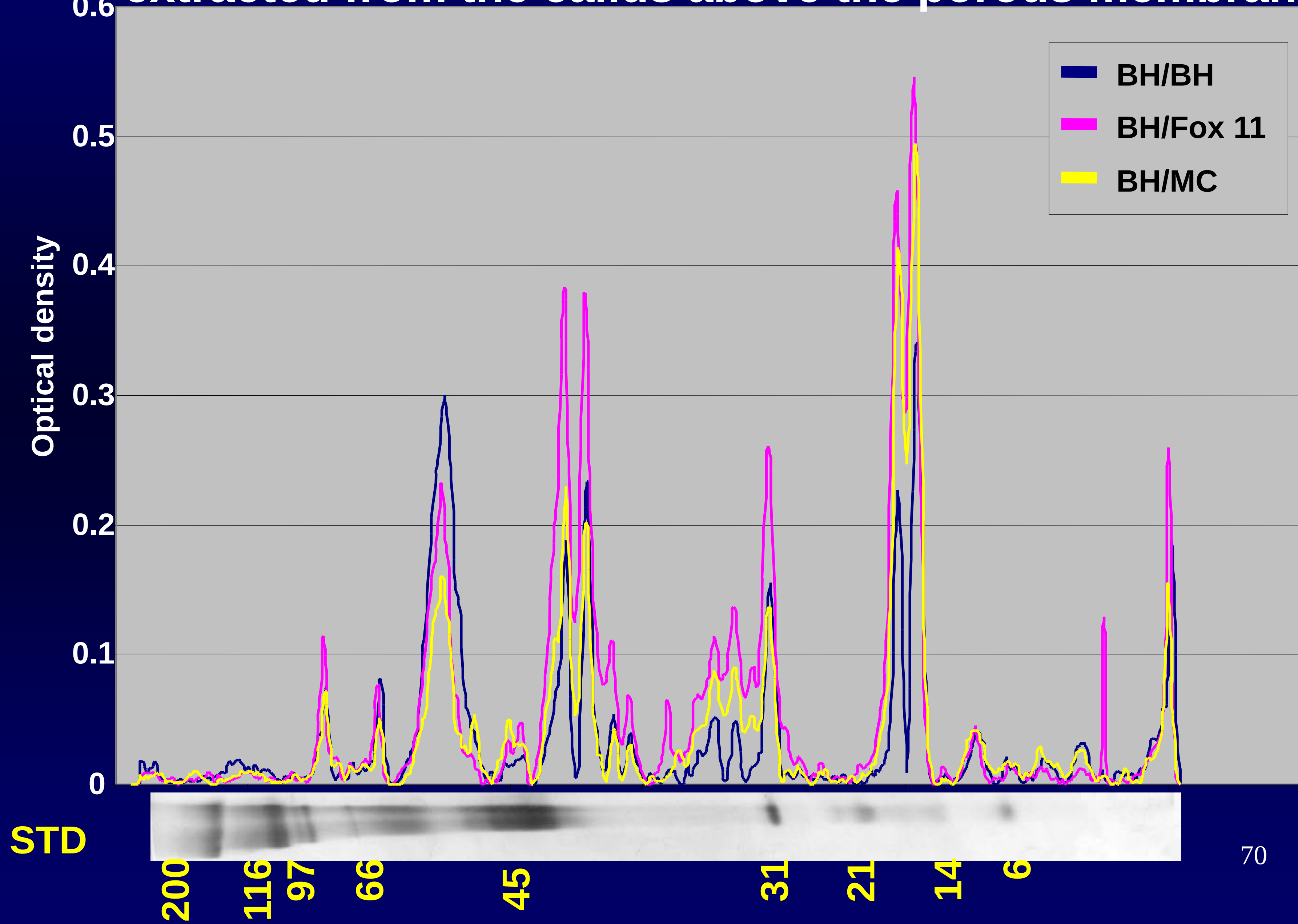


Proteins extracted from callus above the porous membrane

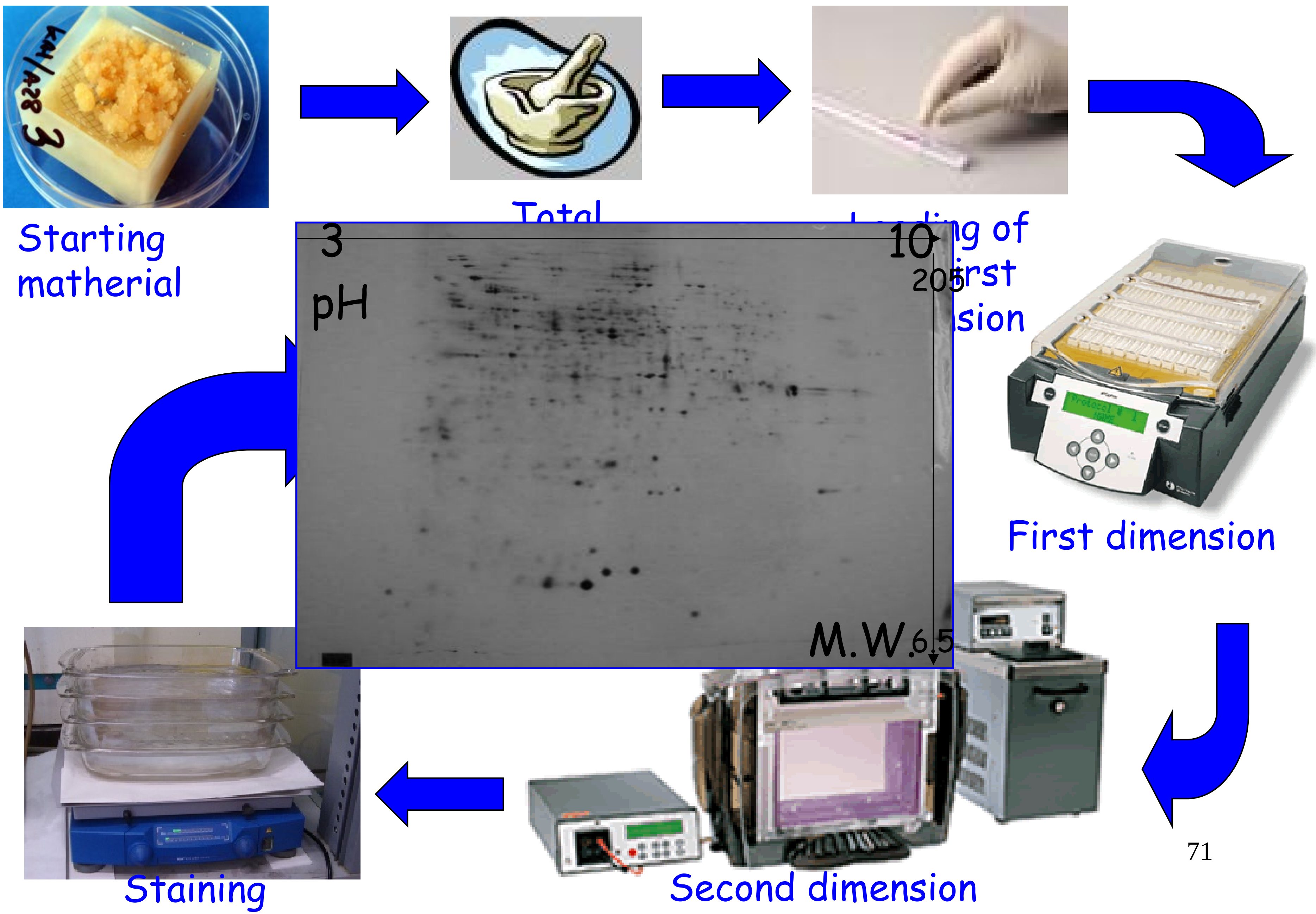
1. BH/BH
2. BH/Fox 11
3. BH/MC



Cv. Beurré Hardy: densitometric analysis of proteins extracted from the callus above the porous membrane

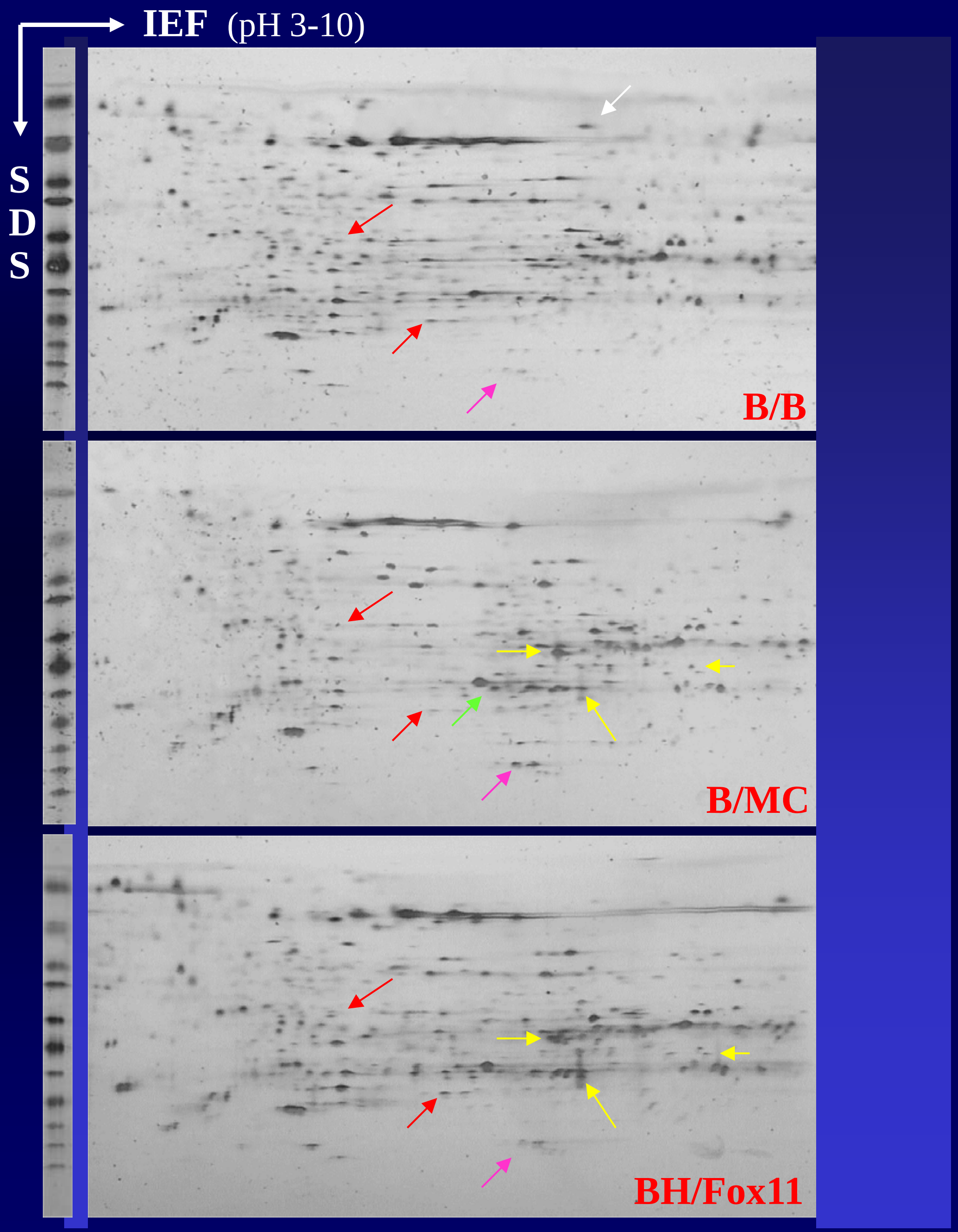


2D-PAGE



Starting
matherial
3
10
pH
205
M.W.
6.5
First dimension
Second dimension
Staining

Protein expression by 2D electrophoresis



only in B/B

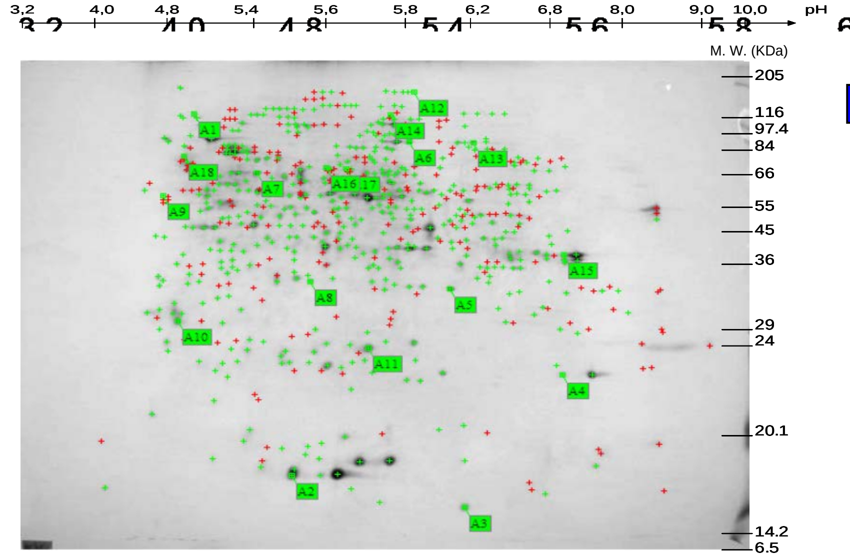
only in B/MC

decrease in B/MC

increase in BMC

only in B/MC and BH/Fox11

Gel comparison between pear combinations



n° spots: 706

n° spots: 802

matches: 476

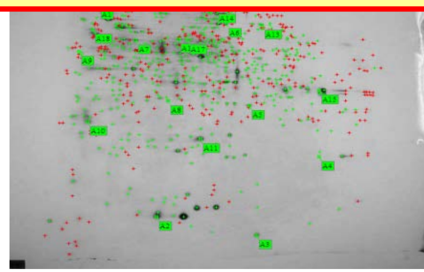
% matches: 63%

n° spots: 857

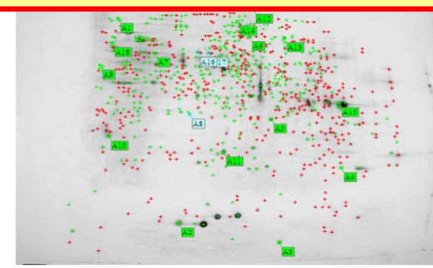
matches: 506

% matches: 65%

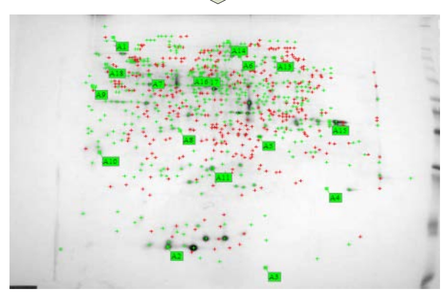
The incompatible combination exhibits the gel with the higher spots number



K/K



K/MC



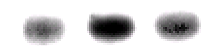
K/A28

n° spots: 774

matches: 425

% matches: 57%

Starting from Pirovano *et al.* (2002) we tried to identify the following proteins in a databank.

Protein	Species	Homology level	Expression level
NT3	*Nicotiana tabacum*	39%	
Patogenesis Related Protein I (SaPRI)	*Santalum album*	100%	
Import intermediate associated protein	*Pisum sativum*	86%	
Extracellular dermal glycoprotein (EDGP)	*Daucus carota*	89%	
Protein translation factor SUI1 homolog (eIF-2A)	*Arabidopsis thaliana*	92%	
NADH dehydrogenase Subunit I	*Arabidopsis thaliana*	94%	
Platelet endothelial cell adhesion molecule	*Homo sapiens*	61%	

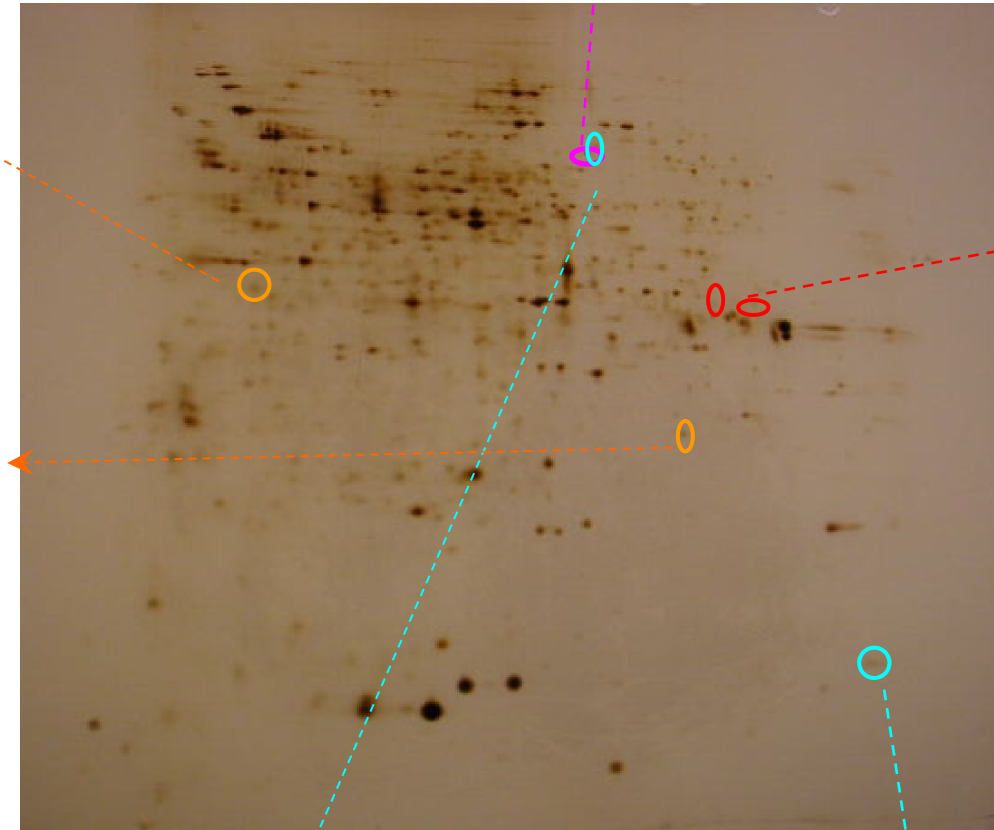
$\frac{K}{K}$ $\frac{K}{MC}$ $\frac{K}{F}$

Protein name	Accession number	Entry name	Molecular weight (KDa)	Theoretical pI
NT3	Q9XEY9	Q9XEY9_TOBAC	70.78	-
hrgpNT3	P13983	EXTN_TOBAC	65.41	10.00
Pathogenesis Related Protein I (SaPRI)	O22479	O22479_9MAGN	15.31	-
Import intermediate associated protein	Q43715	TOC75_PEA	88.27	7.00
Extracellular dermal glycoprotein (EDGP)	Q39688	EP1G_DAUCA	43.55	7.97
Protein translation factor SUI1 homolog (eIF-2A)	Q9FE78	Q9FE78_ARATH	38.80	5.00
Protein translation factor SUI1 homolog (eIF-2A) (subunity β)	Q41969	IF2B_ARATH	30.07	6.79
Putative NADH dehydrogenase Subunit I	Q9M9M9	N7BM_ARATH	18.31	9.26
NADH dehydrogenase Subunit I (75 Kda subunity)	Q9FGI6	NUAM_ARATH	81.50	6.24
NADH dehydrogenase Subunit I (18 Kda subunity)	Q9FLX7	NUFM_ARATH	19.18	4.73
Platelet endothelial cell adhesion molecule	P16284	PECA1_HUMAN	82.53	6.55

Grid references

Import intermediate associated protein

eIF-2A



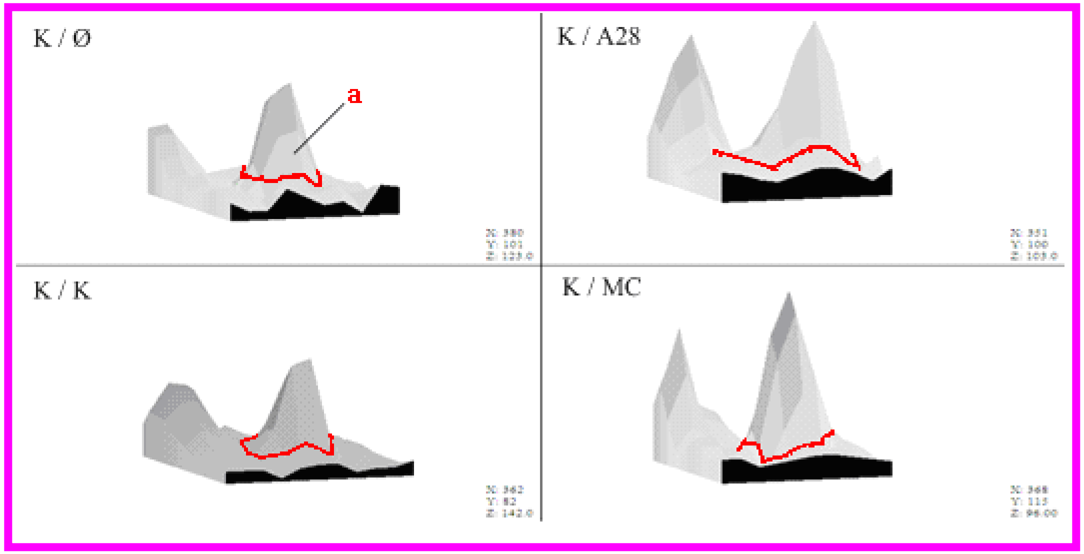
Extracellular dermal glycoprotein (EDGP)

Subunità β dell'eIF-2A

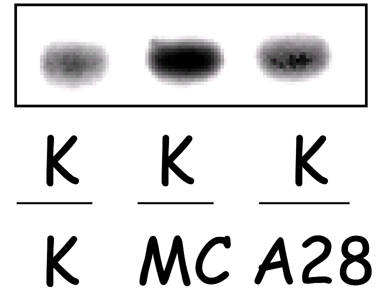
Putative NADH dehydrogenase

Subunità I della NADH dehydrogenase

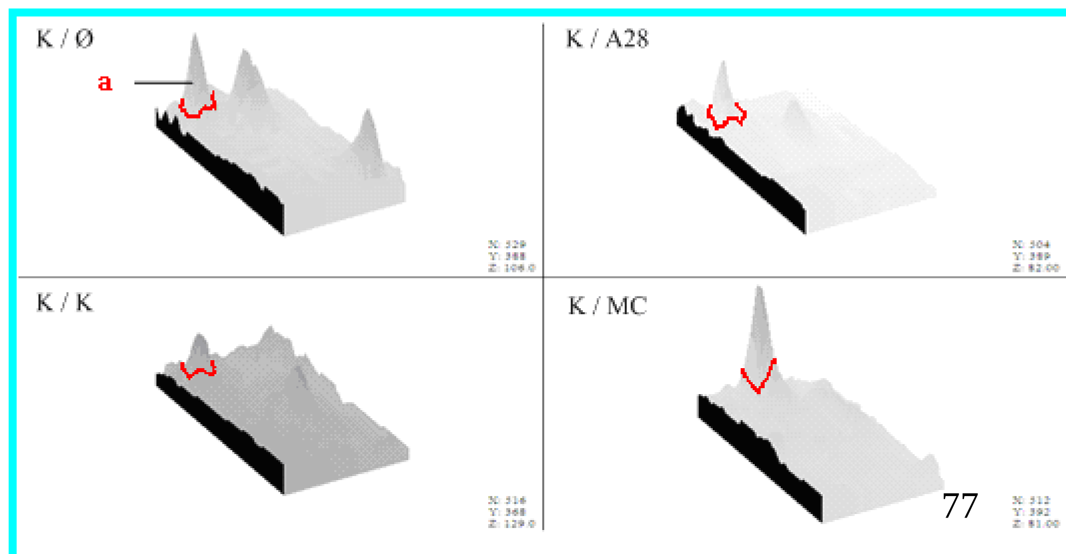
For these proteins, a correlation between transcripts and spot intensities have been found.

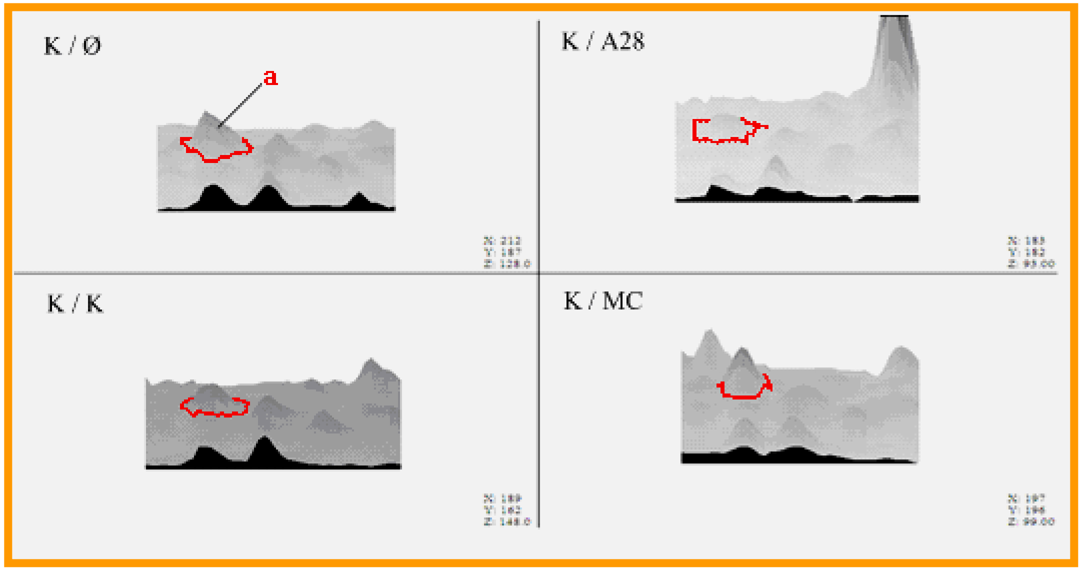


Import intermediate associated protein

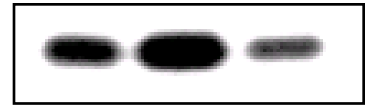


Putative NADH dehydrogenase





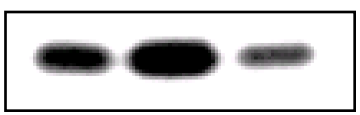
eIF-2A



K K K

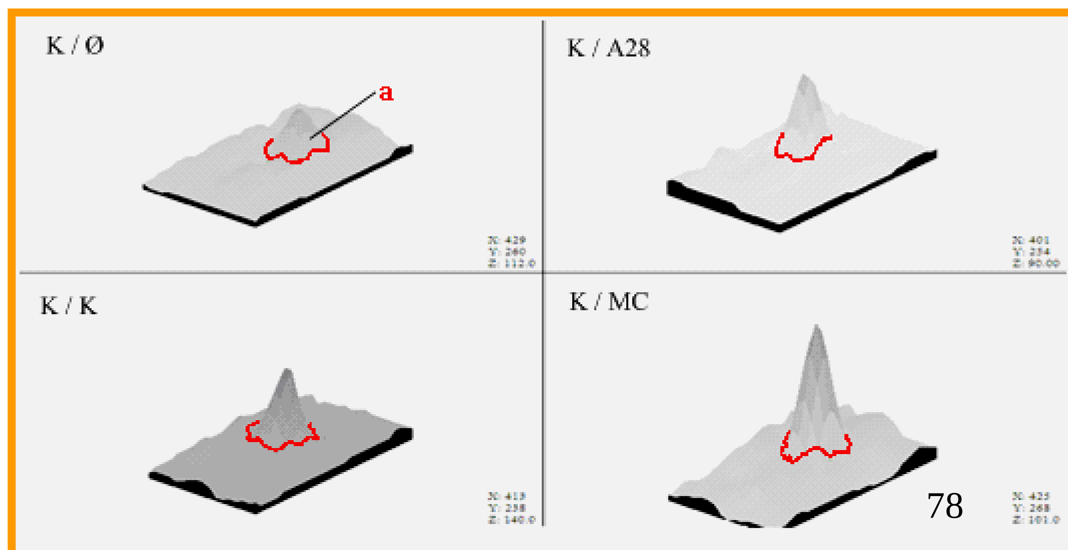
K MC A28

Subunit β of IF-2A

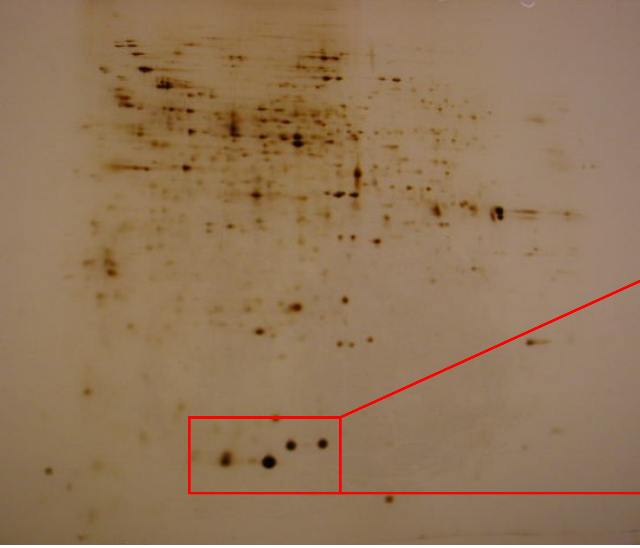


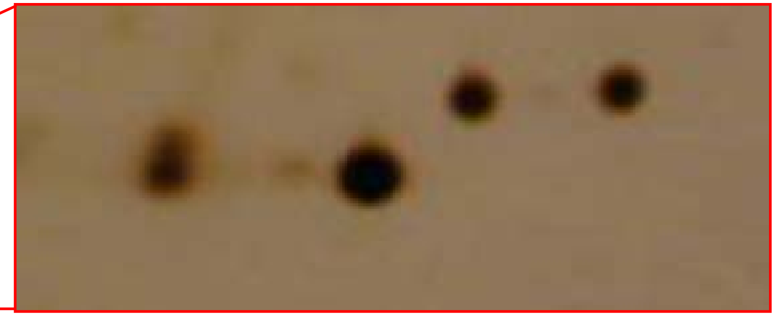
K K K

K MC A28



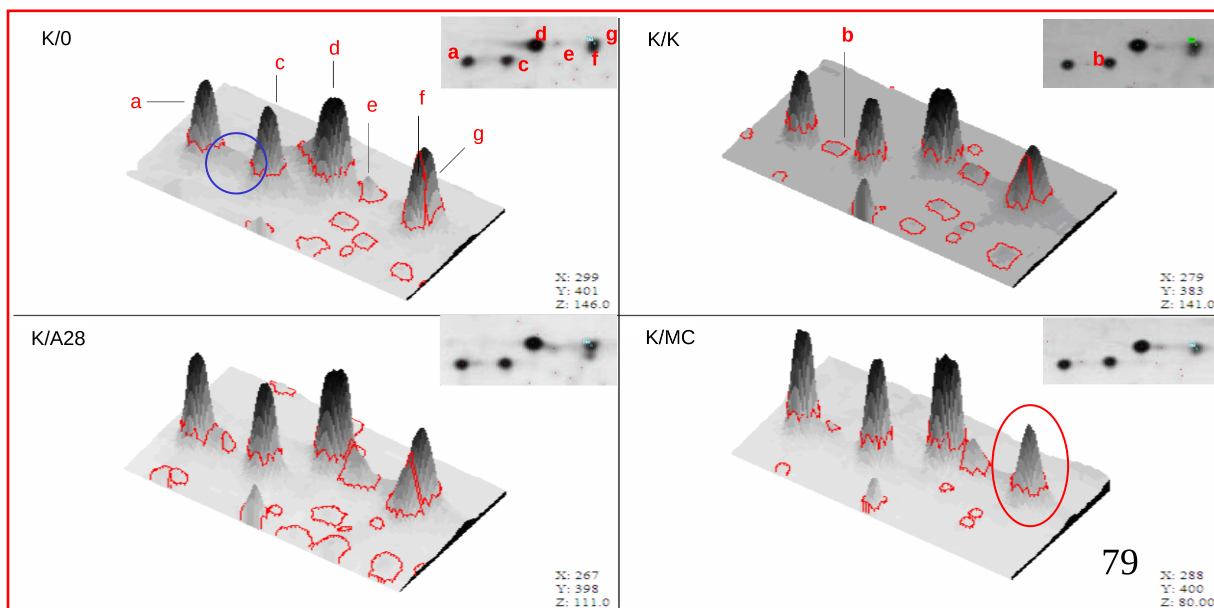
Gel analysis



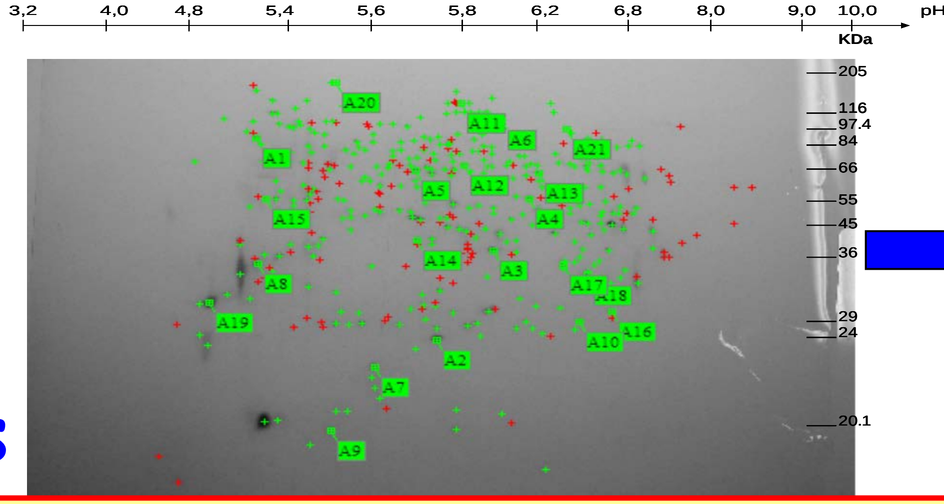


KAISER/KAISER

Details



Gel comparison between cherry combinations



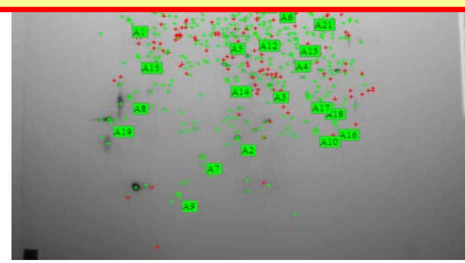
n° spots: 435

n° sp… : 451

match… : 312

% ma… :70%

- The compatible combination exhibits the gel with the highest number of spots.
- The combination with the highest level of homology is the self-graft.
- The values of percent matches decrease with the increase of the graft-incompatibility level.



S/C

n° spots: 471

matches: 326

% matches: 72%

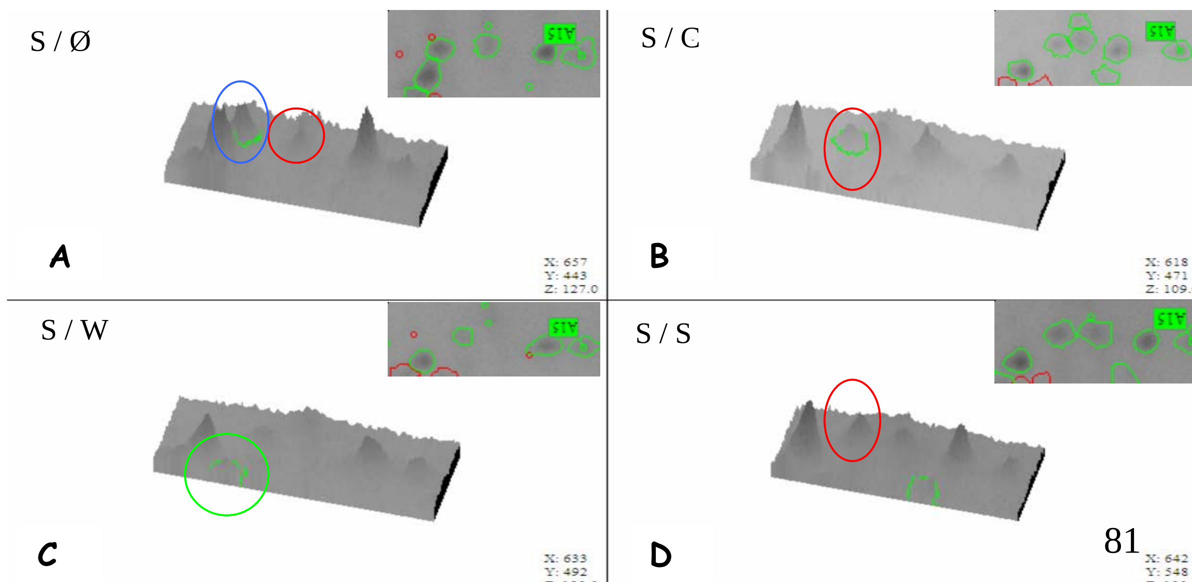
Gel analysis

SAM/SAM

Spot area

Differences in peak intensities

Differences in peak presence/absence



CONCLUSIONS

- **The “*in vitro*” model provides a lot of information about relationship between genotypes and cell-cell recognition**

- **Beurré Bosc showed a higher growth compared to Beurré Hardy**

- **Callus respiration of Bosc seems to be higher in presence of incompatible rootstock quince C.**

- **Proteins changes can be associated with cell-cell recognition mechanism.**

- **Identification of proteins as ‘messengers’ of biological cell-cell recognition between the two genotypes in incompatible graft combination would provide an early screening “marker”.**

Conclusions

- **Gene expression is affected by callus co-culture combination**

- **The effect on gene expression does not require direct tissue contact: *is a mobile solute involved?***

- **Some differentially expressed cDNAs match with nucleotide sequences coding for proteins involved in: cell adhesion (T_{12}CG11), senescence and/or programmed cell death (T_{12}CA-10; T_{12}AG-14), wounding response (T_{12}AG-10) and tracheary element differentiation (T_{12}CG-7).**

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THANK YOU FOR YOUR ATTENTION

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