Generation of doubled haploid plants through microspore regeneration
Why Fytagoras?

- Over 15 years experience in DH technology
  - Protocol development, Protocol implementation, Consultancy
  - Basic scientific research and scientific publications

- Worked for over 8 years on exclusive basis on DH of vegetable crops for one of Holland's leading breeding companies.

- Development of numerous successful DH protocols and commercial implementation

- Ongoing development of DH protocols for different crops (also ornamentals), for several Dutch and foreign seeds companies

- Great track record in (confidential) contract research and in-company instructions
Why Fytagoras?

- Experience on a broad range of crops, amongst them some Solanaceae.
- We worked on more than 7 ornamental crops, and more than 5 vegetable crops (such as pepper, and eggplant).
- Barley, rice, and tobacco as model crops.
- We developed and implemented an efficient protocol for pepper, where many others failed.
- Establishment of different basic approaches in the development of DH plants, which increases the success rate considerably.
On-going research program (in cooperation with Leiden University) on the fundamentals of microspore regeneration: evolutionary aspects, metabolism aspects, genes and transcriptions factor, cell signaling aspects, which benefits protocol development.

Less trial and error, but instead focus on crucial physiological and cellular processes which are related to the division of microspores, and so the production of a DH plant.

Chemical compounds/hormones only, when we assume that they are relevant for the development of microspores.

Why successful?
Why successful?

- **Systematic approach:**
  - growth conditions
  - single flower treatment
  - energy status of cells
  - stress
  - dedicated treatments

- **Supporting techniques** on cells (upgrading, imaging, cell sorting)
Treatment of microspores with Percoll for upgrading microspores

Percoll gradient in g/ml

Untreated Exp 3

7 days treated Exp 8

>1.10 g/ml

Exp3

Exp8
Visualization of treatment by image analysis

before treatment

crude fraction after treatment

fraction 1 after percoll gradient
Treatment of upgraded cells

Exp8. cultivar "P" number of multicellular structures (MCS)

- ruw: number of MCS without hormones
- fr1, fr2, fr3, fr4, fr5: number of MCS with hormones

Number of MCS:
- ruw
- fr1
- fr2
- fr3
- fr4
- fr5
System for staging development of microspores

- Flower age, and size
- Anther color
- Microspore morphology
- Supporting techniques to use optimal cells
Stages in microspore development

I. Tetrade
   → 4 haploid cells

II. E → M → ML → L → P → B

III. Pollen tube growth
Stages of microspores

- Tetrad
- Microspore vacuolation
- Mitosis
- Pollen maturation

- Lycopersicon esculentum
- Olea europea
- Prunus avium
- Smilax aspera
- Lolium perenne
- Orchids
### Different approach Fytagaras

<table>
<thead>
<tr>
<th>Year</th>
<th>Protocol Development</th>
<th>Research Programme</th>
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<tbody>
<tr>
<td>1992-2000</td>
<td>Better control and visualization of cellular processes</td>
<td>From anthers to microspores</td>
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<tr>
<td>2000-2012</td>
<td>Better control of microspore quality</td>
<td>Strict growth conditions</td>
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<tr>
<td>2012-...</td>
<td>- Direct control of developmental pathways</td>
<td>- Energy status</td>
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<td></td>
<td>- Higher efficiency</td>
<td>- Single flower growth</td>
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<td>- More tests possible</td>
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**From anthers to microspores**
Critical steps in DH technology

- Donor plants; stage of development
- Pretreatment; induction of cell division (many different possibilities)
- Culture; formation of multi-cellular structures; embryos
- Formation of plants
- Implementation of the protocol
- Adaptations of the protocol for all varieties
Different approach Fytagoras

- Formation of DH plants by regeneration of microspores
- Project is divided in 3 steps
- All activities are done at facilities of Fytagoras
- Delivery is a working protocol, or on request DH plants
- Implementation (support) in your laboratory
Different approach Fytagoras

PART 1
DEVELOPMENT OF INITIAL PROTOCOL

PHASE I
- generation of multi-cellular structures
  1. preparation and sterilization of microspores
  2. induction of cell division
  3. formation of multi-cellular structures

PHASE II
- embryo and plant formation

PART 2
OPTIMIZING OF PROTOCOL
APPLICATION TO OTHER VARIETIES
Different approach Fytagoras

PART 1: From microspores to doubled haploid plants

Phase 1 Induction of multi-cellular structures

- Step 1 Selection of plant material, technical aspects concerning the preparation of microspores, determination of developmental stages, and characterization of microspores
- Step 2 Pretreatment and induction of cell division
- Step 3 Cultivation and formation of multi-cellular structures

Phase 2 Embryo and plant formation

PART 2 Optimizing of the procedure and implementation
Growth facilities
Pictures from our laboratory
Tissue culture
Different approach Fytagoras

1. Donorplant

2. Induction of cell division and growth of multicellular structures

3. Formation of plants