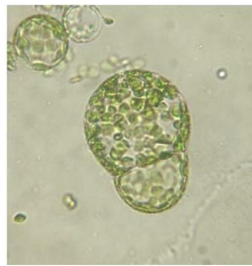
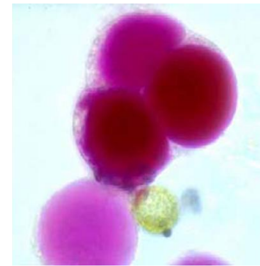
Instructor:

Eric BONCOMPAGNI

Master 1 - BP04 - Introduction Biotechnology – 2013

Plant Biotechnology: Practical 2 – Protoplast isolation and fusion





Laboratory practical and groups:

Because of lack of space in the lab we will do 2 groups. Make sure you know which group is yours.

Group 1: Monday 30 December, 8-12h

Group 2: Tuesday 31 December, 8-12h

Lab Assignments:
You should keep track of all protocols, material and methods that will be use; write down results obtained in the lab and be able to explain them.

General Lab Safety rules:
There are appropriate methods and precautions that must be taken in any biological laboratory. This includes procedures for safe handling and storage of hazardous chemicals and biological materials. You are expected to learn, understand and comply with all safety procedures as listed below:

1. Never eat or drink in the lab. Do not bring food or drinks into the lab.
2. You must wear a lab coat for protection.
3. Dispose of all materials and chemicals correctly under the hood.
4. Label all solutions and chemicals clearly.
5. Report spills and broken glass immediately to your instructor.
6. Clean up your work stations, wipe your lab bench/hood and wash your hands before leaving the lab room.

Protoplast isolation and fusion

Practical objectives:

Protoplasts are biotechnological tools used for genetic transformation or somatic hybridization. However, the poor efficiency of regeneration from these simplified systems makes this application limited to some plant species.

The **plant cell wall** is made of cellulose fibers forming with hemicelluloses a network which is embedded in the pectin matrix. The adjacent cells are linked by the middle lamella mainly composed of pectin (Figure 1).

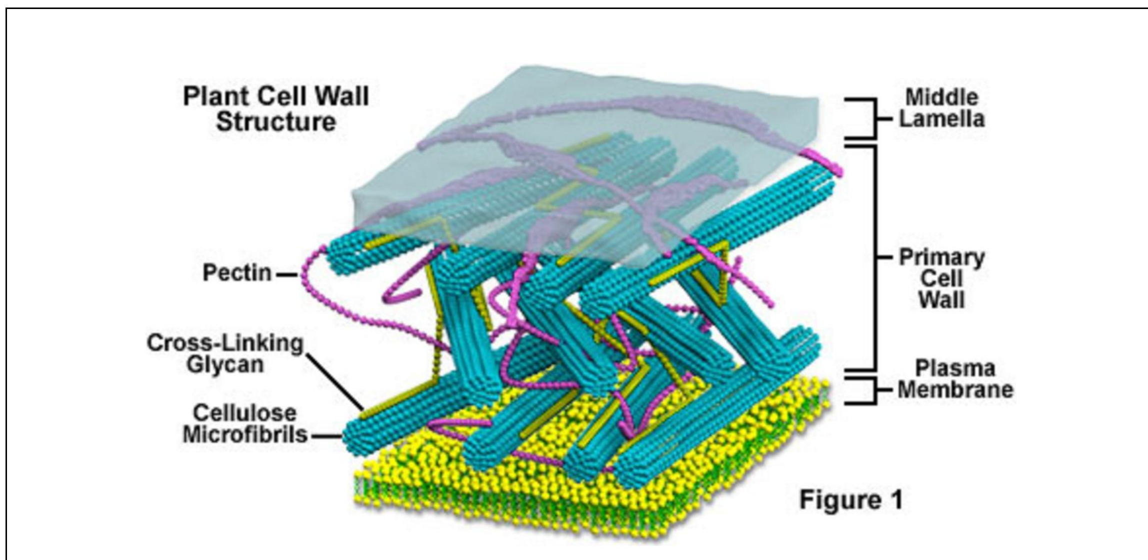


Figure 1

From plant tissues, protoplasts are isolated after **enzymatic digestion** of the cell wall, by using cellulase and pectinase or hemicellulase. After cell wall degradation, the resulting cells remain surrounded by the plasma membrane, which provides a weak protection against mechanic or **osmotic stresses**.

For preventing plasmalemma osmotic lysis, the medium used for protoplasts preparation and culture are enriched in sugars, which maintain a slightly hyperosmotic environment. The medium is supplemented with mannitol (0.9M), a sugar-alcohol not involved in plant cell metabolism.

The different protocols proposed for isolating protoplasts present a first step of **cell wall degradation using enzymes** (pectinase and cellulase) in optimal conditions of temperature and pH. The digestion is stopped by washing the preparation of protoplasts

with a solution free of enzymes. The resulting isolated protoplasts are observed and the viability evaluated by using the Evan's blue staining.

Plant material: Lamb's lettuce and Red cabbage or maybe other plants depending upon availability on the local market.

Preparation of solutions:

- **Prepared solution will be provided to you at the beginning of the practical.**

Buffer and washing solutions

The buffer and washing solutions are made by mixing solutions A (Tris 25 mM, mannitol 0.9 M) and B (MES 25 mM, Mannitol 0.9 M) until getting the correct pH (5.5 or 7.5). Then, the enzymes are added to the buffer to get the digestion solutions.

Buffer solution: Tris-MES 20 mM (pH 5.5) Mannitol 0.9 M

Washing buffer: Tris-MES 20 mM (pH 7.5) Mannitol 0.9 M

Digestion solutions

Solution 1: Macerozyme (0.1%) and Cellulase (1%) in buffer solution

Solution 2: Pectolyase (0.1%) and Caylase (1%) in buffer solution

PEG solution (50ml)

Solution 3 (20 ml): PEG (0.4%) by heating in a water bath.

Solution 4 (20 ml): glucose (5.4%) + $CaNO_3 4H_20$ (0.75%)

Mix the solutions 3 and 4, adjust at pH 7 (KOH 0.1N) and complete to 50 ml.

Evans's blue: 1% in H_2O

1- Protoplasts isolation

- **each group will test only one protocol**

Protocol 1:

- Remove the main vascular bundles from the leaves of lamb's lettuce and cut them into pieces if the leaves are large;
- Transfer immediately these pieces into a Petri dish (or a bottle) containing 15 ml of digestion solution 1;
- Incubate at 26°C in the dark and under gentle shaking (50 rpm);
- After 10, 20 and 30 min of incubation, transfer 20 µL of the sample onto a slide;
- Using a microscope, evaluate the progress on cell wall digestion and observe the resulting protoplast isolation.

Protocol 2:

- Remove the main leaf vascular tissues and the very thick part of the red cabbage leaves before slicing gently their surface every 0.5-1 mm wide using a sharp razor blade;
- Cut them into small pieces and transfer them immediately into a Petri dish (or a bottle) containing 15 ml of digestion solution 2;
- Incubate at 26°C in the dark and under gentle shaking (50 rpm);
- After 10, 20 and 30 min of incubation, transfer 20 µL of the sample onto a slide;
- Using microscope, evaluate the progress of cell wall digestion and observe the resulting protoplast isolation.

2- Washing the isolated protoplasts

- After incubation, remove the digestion solution using a pipette and filter it though a nylon mesh or other filter (75-150 µm) on a funnel.
- Rinse the Petri dishes by 5 ml of washing solution, and add onto the filter.
- Collect the liquid into a conical tube.
- Centrifuge 10 min at 500 rpm. => The protoplasts will be in the pellet.

Never let the pellet without liquid.

- Remove the supernatant with a pipette and discard it.
- Wash the pellet with 5 ml of washing buffer.
- Gently re-suspend the protoplasts in the solution with a Pasteur pipette.
- Centrifuge 10 min at 500 rpm.
- Repeat 2 times this washing operation (steps 3, 4 et 5)
- After the third centrifugation, re-suspend the pellet with 300 µL of washing solution.

3- Evaluation of viability and counting of the protoplasts

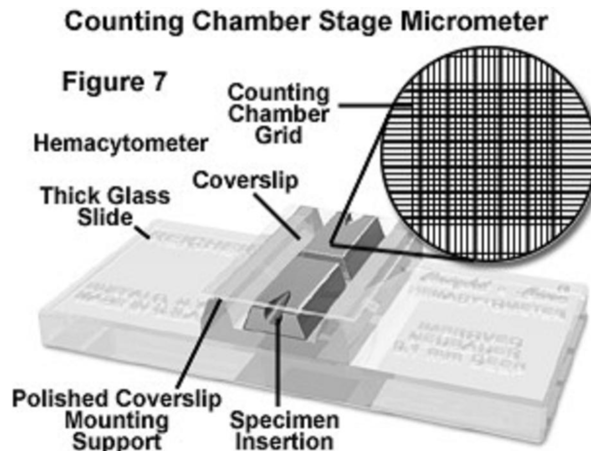
The viability of the protoplasts will be estimated using the **Evan's blue staining**. The Evans blue reports the damages on the cell membranes because it cannot penetrate by itself into the viable cell but accumulates only in protoplast which lost their plasmalemma integrity.

=> **Consequently, the dead protoplasts are blue and the living cells are not stained.**

- The protoplast suspension is gently shacked before transferring 50 µL into an Eppendorf tube;
- Add 5 µL of Evan's blue solution (1% in water) and mix very gently;
- Take 20 µL to spot on a specific **hemacytometer** (Thoma cell or Malassez cell).

- Start microscope observation by using the objective x10, which allows focusing onto an interesting zone of the sample and then use the objective x20 for counting the blue and viable cells on a precise surface.

From the total number of isolated protoplasts and also the blue protoplasts which are counted in 3 rectangles or squares, calculate the mean value of protoplasts and deduced the mean value of living cells.



Malassez cell: 1 rectangle = $0.01 mm^3$, consisting of 20 small squares (4x5)
Thoma cell: 1 square = $0.004 mm^3$, consisting of 16 small squares (4x4)
Calculate the number of protoplasts/ml and from blue protoplast number, deduce the % of living cells.

4- Protoplast fusions

When the PEG-Ca^{2+} solution is added to the suspension, the **aggregation of protoplasts** could be induced and in some cases, the fusion between protoplasts could be observed after association of the plasmalemma.

- On a slide, mix gently a drop of the red cabbage protoplast suspension with a drop of lettuce protoplast suspension with the tip of pipet cone;
- Add a drop of the PEG-Ca^{2+} solution;
- Add a cover slip and observe immediately using microscope.