2-day Protocol for isolation and transfection of *Brassica napus* protoplasts

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Day 0

- Check if all the medium, reagents, and tools are available
- Book a time slot for the flow hood, centrifuge, and microscope

Medium, reagents, and tools needed:

- Enzymatic solution (enzyme powder can be prepared in stock solution should be made freshly every time)
- 3-week-old seedlings
- Autoclaved razor blades (dried immediately in the oven after autoclaved to avoid rust)
- Sterile tweezers, preferably 2
- Sterile Petri dishes
- Sterile plasmolysis solution (PS)
- Sterile filter paper
- Sterile W5 medium
- Sterile 0.5 M mannitol
- Filter sterilised alginate-solution
- Sterile calcium-agar plates
- Sterile calcium solution
- Empty beaker for easy disposing of liquids
    - Preferentially autoclaved one, at the very least spray with EtOH
- Sterile 1ml pipette tips
- Sterile 10ml and 25ml pipettes (plastic ones, in the storage room, common use)
- Ice box
- Sterile 1.5ml Eppendorf tubes
- Haemocytometer
- Sterile flat spoon, preferably 2

Day 1 (preparation 2 PM to 2:30 PM, cutting 3 PM to 5 PM)

- work without light in flow hood
- Make fresh enzymatic solution
    - cool enzymatic solution in cold water bath before adding BSA, BME, $CaCl_2$, H_2O , Use KOH to adjust pH value to 5.7
    - MARK TUBES WITH UN-STERILE STOCK SOLUTIONS AND PLACE IN LAB FRIDGE
- Cut **30-35 fully** expanded top leaves from 3-4 weeks old seedlings into small strips on wet sterile filter paper (wetted with plasmolysis solution) using autoclaved razor blades, change blade every 5 leaves.

    - Place filter paper in Petri dish
    - Remember to cover seedlings and cut leaves to avoid drying out
- Place leaf strips in Petri dish containing ~20ml plasmolysis solution. Incubate in the dark for 30 minutes
    - Cover with lid when not using
    - Cover with aluminium foil to keep dark after finished cutting
- Remove plasmolysis solution using 10ml pipette and add 10 ml enzymatic solution
- Incubate in the dark at RT (?) with gentle shaking (<50 rpm for 15 h in the growth chamber)
    - Seal Petri dish with parafilm

Day 2 (8 am to 2pm)

- Take out W5 medium, 0.5M mannitol, alginate-solution, calcium-agar plates, and calcium-solution from fridge (some protocol suggests use cold W5 to wash ppt.)
    - Make sure you use sterile solutions
- Turn on centrifuge and set temperature to 21C, 0 acceleration, 0 brake)
- Turn on glass bead steriliser
- work without light in flow hood
- **Add 10ml W5 solution to the Petri dish**, cover with aluminium foil, and return to shaker for 10 min
- Filter Petri dish contents through a 40µm cell strainer placed on a NON-SKIRTED 50ml falcon tube
    - Use 25ml pipette to transfer contents from Petri dish to cell strainer
- **Add 20ml W5 solution** to the Petri dish to rinse residuals. Gently swirl to rinse Petri dish. Filter through same cell strainer.
    - Total volume should now be ~40ml
- Centrifuge at 100g, 21C, brake=0, acc=0, for 10min
    - Total time will be ~16min
- Remove supernatant without interfering the pellets at the bottom
- **Add 10ml W5 solution**, resuspend by gentle shaking, and centrifuge at same settings for 5 min
- Remove supernatant
- **Add 10ml W5 solution**, resuspend by gentle shaking, and centrifuge at same settings for 5 min
- Remove supernatant
- **Add 5ml W5 solution, resuspend the cells** and incubate on ice in the dark (cover with aluminium foil) for 30min**. Intact** protoplasts will precipitate to the bottom of the tube
- Remove supernatant **(as much as possible)**
    - Use pipette and autoclaved (preferably filter) tips
- **Adjust volume to 5 ml by adding W5 solution** (up to **10 ml** depending on size of the pellet) and transfer 100 µl solution to 1.5ml Eppendorf tube for protoplast quantification.
- **Centrifuge at 100 g, 21C, brake=0, acc=0, for 3min**, Remove supernatant

    - Keep the tube on ice when quantifying!
- Load 16 µl protoplast solution on hemocytometer. Use filter PH1 and 20x magnification on microscope.
- Count 5 big squares (16 small square) and calculate the mean. Use Protoplast counting template (excel_file) to get the correct volume ASAP.
- Resuspend the protoplasts in a calculated amount (depends on pellet size/based on microscopy counting) to reach a concentration of 1350,000pp/ml (!Density is a critical index to optimize)
- **RNP Transfection**
    - 200 ul (270,000 pp) incubate with 20µl of RNP complex
    - 220 ul PEG 40 for 6 min in 2 ml Eppendorf tube. W5 terminates the reaction.
    - 200 ul M I was used to redissolve the pp

- **Add 1:1** (200 µl) **volume** of filter-sterilised **alginate-solution (2.8% (w/v) sodium alginate, 0.4M mannitol) into the 600 ul ppt** in a new 15 ml tube and mix gently
- **Pipette 400 ul of the suspension as blobs** (droplets) onto calcium-agar plates (20mM $CaCl_2$, 0.4M mannitol, 1% agar) to produce alginate disks (5 blobs/PD). **Incubate at RT for 30min**
- Add ~2ml calcium-solution (50mM $CaCl_2$, 0.4M mannitol) onto each blob and **incubate for 1hour at RT** to complete polymerisation
- Transfer the blobs using a sterile flat spoon to 6-well plates with 3ml M1 medium (w/2,4-D+NAA)
- Cover with aluminium foil at RT for 24hours
- After 24hours, replace foil with fiber cloth and place under light
    - Fold fiber cloth twice (! Light intensity needs to be optimized or the plates should not be overlapped)

Protoplast culture

1. M1 medium for 4 days (MI is with 2,4-D 0,5 and NAA0,5) Auxins Cell dedifferentiation
2. MII medium: change every 5 days (MII is with TDZ 1,1 and 2,4-D 0,05) MIIV2(TDZ 2.2 and NAA0.5) MIIV3(TDZ2.2NAA0.1) TDZ-Cytokinins
   for 3 to 4 weeks
3. MII medium in bigger petri dish (time period to be decided)
4. Regeneration medium: MIV

Use pipette to suck up old medium, then replace with fresh medium. Should always be ~2ml medium per well. Be aware of cross-contamination.

Medium

MI medium (1l)

Nitsch medium incl. vitamins	2.18g
Mannitol	100g
Glucose	10g
Sucrose	10g
Casein	100mg
2,4-D	0.5mg
NAA	0.5mg

pH=5.7
Filter sterilise solution (use of vacuum pump is fast).

MII medium (1l)

Nitsch medium incl. vitamins	2.18g
Mannitol	100g
Glucose	10g
Sucrose	10g
Casein	100mg
TDZ	1.1mg
2,4-D	0.05mg

V2 TDZ 2.2 mg; NAA 0.5 mg
V3 TDZ 2.2 mg; NAA 0.1 mg
pH=5.7
Filter sterilise solution (use of vacuum pump is fast).

M3 medium (1l)

MS (with MES)	4.91g
mannitol	50 g
Glucose (K)	10 g
Sucrose (K or in lab)	10 g
TDZ	2.2 mg

NAA	0.5 mg or 0.1 mg
AgNO3	0.5 mg

pH=5.7

Filter sterilise solution (use of vacuum pump is fast).

M4 medium (1l) solid

MS (with MES)	4.91g
mannitol	10 g
Glucose (K)	10 g
TDZ	1.1mg or 2.2 mg or BAP 5.0 mg
NAA	0 mg or 0.1 mg
AgNO3	0.5 mg
pH=5.7	

Gelrite 2.5 g

Solution needed for single isolation (~20 plants/~40 leaves)

Plasmolysis solution	25ml
Enzymatic solution	10ml
W5	60-65ml
0.5M Mannitol	30-50ml
Alginate solution	Depend on your experiment
Calcium solution	Depend on your experiment 10ml per Petri dish
Calcium-agar plates	Depend on your experiment
MI medium	Depend on your experiment 10ml per 6-well plate

Solutions protocols

Enzymatic solution, 25ml (enough for two batches) (prepare fresh every time)

Mix the following reagents in a 50ml Falcon tube

Reagent	**Amount per 25ml**	**Final concentration**
0.5M Mannitol	20ml	0.4M
0.2M MES (pH=5.7)	1.25ml	10mM
Macerozyme R10	0.15g	0.6% (w/v)
Cellulase R-10	0.375g	1.5% (w/v)

Heat tube in 55°C water bath for 10min. Cool to RT (can place in cold water bath to speed up cooling).

MARK TUBES WITH UN-STERILE STOCK SOLUTIONS AND PLACE IN LAB FRIDGE

Add the following reagents to the same 50ml Falcon tube

Reagent	**Amount per 25ml**	**Final concentration**
BSA	25mg	0.1% (w/v)
1M $CaCl_2$*$2H_2O$	25µl	1mM
B-mercaptoethanol	1.75µl	1mM
Milli-Q H_2O	To 25ml	

Adjust pH to 5.7

Filter sterilise

Plasmolysis solution, 1l (can be stored at RT for up to 3 months)

0.4M Mannitol = 72.88g per 1l H_2O

Filter sterilise

W5 solution, 1l (can be stored at RT for up to 3 months)

If using liquid stocks:

Reagent	**Amount per 1l**
NaCl	9g
1M $CaCl_2$*$2H_2O$	125ml
2M KCl	2.5ml
0.2M MES (pH=5.7)	10ml
Milli-Q H_2O	To 1l

Adjust pH to 5.7

Filter sterilise

OR, if using powders:

Reagent	**Amount per 1l**
NaCl	9g
$CaCl_2$*$2H_2O$	18.4g
KCl	372.5mg
MES (pH=5.7)	426mg

Milli-Q H_2O	To 1l

Adjust pH to 5.7
Filter sterilise

25 or 40% PEG-Ca^{2+} 10 ml (prepare fresh every time)

Reagent	**Amount per 100ml**
PEG (polyethylene glycol) 4000	2.5 or 4g
Mannitol	0.91g
$CaCl_2$*$2H_2O$	1ml (1M)
Milli-Q H_2O	To 10ml

Filter sterilise

MMG solution 10 ml (prepare fresh every time)

Reagent	**Amount per 10ml**
Mannitol	0.91g
$Mgcl_2$	75µl 2M $Mgcl_2$*$6H_2O$
MES	200µl 0.2M MES
Milli-Q H_2O	To 10ml

Filter sterilise

0.2M MES, 25ml (can be stored at RT for up to 3 months)
1.07g MES per 25ml H_2O (mw=213.2)
Adjust pH to 5.7
Filter sterilise

0.5M Mannitol, 1l (can be stored at RT for up to 3 months)
91.1g Mannitol per 1l H_2O (mw=182.2)
Filter sterilise

*1M $CaCl_2$*$2H_2O$, 75ml (can be stored at RT for up to 3 months)*
11.03g $CaCl_2$*$2H_2O$ per 75ml H_2O (mw=147)
Filter sterilise

Alginate, 100ml

Reagent	**Amount per 100ml**
Sodium alginate	2.6g
0.4M Mannitol	To 100ml

It’s a bit tricky to dissolve the sodium alginate. Dissolve Sodium alginate in small amount of 0.4M Mannitol, stir with spoon or glass rod until totally dissolved. Adjust volume to 100ml with 0.4M Mannitol. Mix well.
Filter sterilise (slow process, change filter every 10 ml). It’s OK to not sterilise stock solution, just sterilise before use.

Calcium-agar plates, 1l

Reagent	**Amount per 1000ml**
Phyto agar	10g
Mannitol	72.88g
$CaCl_2*2H_2O$	2.94g

Autoclave and pour into Petri dishes. Store at 4°C in sterile room.

Calcium solution, 500ml

Reagent	**Amount per 500ml**
$CaCl_2*2H_2O$	3.68g
Mannitol	36.45g