Procedure of protoplast electrofusion
(in *Solanum* genus)

A part of the output V002 within the project NAZV QF4108 “Use of protoplast fusion technique in breeding of economic important crops of *Brassica*, *Cucumis* and *Solanum* genus” applied and verified in the solution of
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1 Introduction

The effective development of new varieties with desirable traits requires a combination of conventional breeding approaches and modern technologies such as *in vitro* cultures, involving production of dihaploid lines, protoplast fusions and use of genetic transformation. Modern plant biotechnologies are a necessary part of plant breeding for resistance to biotic and abiotic stresses. They contribute to an enlargement of variety spectrum and to decrease inputs for profitable yields. Breeding (sexual hybridization) of economic important crops for resistance to biotic and abiotic agents is often interfered with many complications as sexual incompatibility of parental genotypes, unsuitable EBN ratio (Endosperm Balance Number, Carputo et al. 1997) or small success in conventional sexual hybridization. Problems with resistance transfer in classical approaches resulted in development of unconventional approaches. Interspecific or intergeneric protoplast fusions offer an alternative way of gene transfer (Millam et al. 1995). Since there are no barriers to protoplast fusion, hitherto incompatible and therefore reproductively isolated species, can be brought together at the protoplast level. Following fusion, heterocaryons, containing nuclei of two species in common cytoplasm regenerate a new cell wall, enter division and nuclear fusion results in the formation of a somatic hybrid cell. Like the normal plant cell, the somatic hybrid cell is totipotent and therefore capable of developing via embryogenesis or organogenesis, into whole (hybrid) plants. *In vitro* cultured plants are suitable source of protoplasts because the problematic surface sterilization is not required. The cells of leaf mesophyll, young hypocotyls and/or calli are generally suitable for protoplast isolation. Somatic hybridisation therefore, provides a method for producing new hybrids between not only sexually incompatible species combinations, but provides a way for genetically modifying vegetatively propagated crops, sterile or subfertile species and those individuals with naturally long life cycle. Protoplast fusions and regeneration also enables interspecific and intraspecific transfer of extranuclear genetic elements such as mitochondrial DNA (cytoplasmic male sterility), chloroplasts and cytoplasm. An application of somatic fusion requires not only plant regeneration from protoplasts, but also a successful integration of derived regenerants into a breeding programme. A somatic hybrid should be capable of backcrossing with a cultivated crop for the development of a new variety. The technique of somatic hybridization has been already successfully used in the world for the series of cultivated plants incl. *Solanum* genus and transfer of resistance or new qualitative traits was confirmed (Butenko et al. 1982, Austin et al. 1985, Pehu et al. 1989, Pehu et al. 1990, Schilde-Rentschler et al. 1993, Thieme et al.

1997, Helgeson and Haberlach 1999). In the framework of the project solution, procedure of protoplast isolation, protoplast electrofusion and culture of fusion products was elaborated.

2 Methodical procedure

The protoplast fusion by electric field requires sufficient amount of suitable plant material for protoplast isolation and their culture after electrofusion for obtaining plant regeneration. The procedure involves four discreet stages.

- Culture of donor plants
- Protoplast isolation
- Verification of protoplast viability and protoplast fusion
- Culture of fusion products

2.1 Culture of donor plants

In vitro plantlets are grown in a culture chamber with a 16 h/8 h light/dark photoperiod (light conditions: fluorescent tubes, daylight, 60 µmol m^{-2} s^{-1}), at 22 °C. Plantlets are cultivated on hormone-free MS medium (Murashige and Skoog 1962).

Fig. 1 *In vitro* cultured plantlets of *Solanum* genus for protoplast isolation

Plants for protoplast isolations (from 10 to 20 plantlets from each parental genotype) are propagated on SH hormone free medium (Schenk and Hildebrand 1972) supplemented with $AgNO_3$ and Alar 85 (succinic acid dimethylhydrazide) for 4 - 6 weeks (Fig. 1). Plants are cultured at 10 °C in darkness for 24 hours one day before the protoplast isolation; these stress conditions enable integration of cell cycle and this is positively reflected in subsequent protoplast regeneration.

2.2 Protoplast isolation

The whole process of protoplast isolation is performed under sterile conditions.

Detach fully expanded leaves (from 10 plantlets, 0.5 – 1 g). Cut them into small pieces with a sharp scalpel in one Petri dish (Ø = 5 cm) containing 5 – 6 ml of enzymatic solution (Bříza and Machová 1991), pack the Petri dish with Parafilm.

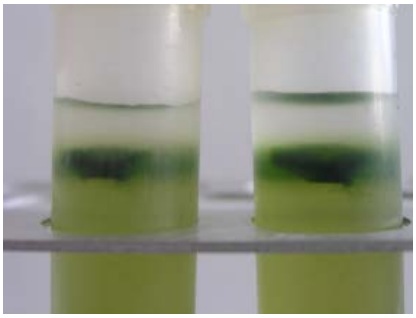
Incubate Petri dishes overnight in dark (14 – 16 h) in a thermostat at 25 °C. After this period, investigate the degree of plant tissue digestion under microscope, if it is necessary (depending on genotype) place Petri dishes onto a shaker for about 15 minutes.

Transfer enzymatic solution with the plant tissue by a disposable serological pipette with open end onto a small sieve (75 µm pore size) placed on Petri dish (Ø = 5 cm). Rinse the sieve with a sterile solution of 0.5 M sucrose while gentle stirring. By this step released protoplasts are separated from plant tissue debris. Divide protoplast suspension derived in this way into centrifugation tubes (8 ml) and overlay with 1 ml of W5 solution (Menczel et al. 1981).

Centrifuge (700 rpm, 12 min).

Carefully take away floating protoplasts forming a ring on the interface of applied solutions (Fig. 2) by a Pasteur pipette and put into a new centrifugation tube, add required amount of W5 (5 ml) and resuspend.

Fig. 2 Undamaged protoplasts on the interface of sucrose and W5 solution



Centrifuge (500 rpm, 6 min).

Remove the supernatant with a Pasteur pipette, add W5 (2 – 5 ml) and resuspend the pellet.

Centrifuge (500 rpm, 6 min) and remove the supernatant with a Pasteur pipette.

Add culture medium SW_{11} (Bříza and Machová 1991) to obtain required density 6×10^5/ml (see 2.3.2) or continue according to the protocol of protoplast fusion by electric field.

2.3 Verification of protoplast viability, density adjustment and protoplast fusion

2.3.1 Evaluation of protoplast viability prior to fusion

Mix equal volumes (20 µl) of diluted FDA solution and protoplast suspension on a slide and form microscope preparation. After 4-5 minutes, examine under a fluorescence microscope. Determine protoplast viability (count protoplasts in selected visual field without UV light and then green fluorescing protoplasts in UV light (green fluorescing protoplasts are metabolically active):

% protoplast viability = no. of green fluorescing ppts/total no. ppts x 100

When viability is higher than 50 %, investigated suspension is usable for fusion.

2.3.2 Determination of protoplast density using a Bürker chamber

Adjustment of protoplast concentration prior to fusion (prior to culture) is done under sterile conditions.

Add adequate amount (2 – 4 ml) of pre-fusion solution (Schilde-Rentschler and Ninnemann 1988) to isolated protoplasts with a Pasteur pipette, resuspend (protoplasts can stay in this solution up to 2 h in a refrigerator at 6 – 7 °C) and count protoplast using a Bürker chamber (haemocytometer).

Count protoplasts in five triple-lined squares (i.e. a square of 1 mm side), in the left and above all cells touching the middle line are counted, in the right and below these ones are not counted. Determine an average number of protoplasts from five squares - **"X".** Cell concentration in 1 ml is: **"X" × 10^4** (1 square represents volume of 0.1 mm^3, i.e. 10^{-4} cm^3 [1 cm^3 = 1 ml]).
Total yield = $\mathbf{V_{suspension} \times "X" \times 10^4}$
Dilution factor $\mathbf{F_d}$ **= required density / "X" × 10^4**
Suspension volume at required density = $\mathbf{V_{suspension} / F_d}$
If needed, concentration is adjusted for fusions (see 2.3.3) or culture of protoplasts.

2.3.3 Adjustment of protoplast concentration prior to fusion and protoplast fusion

The whole procedure of protoplast fusion is performed under sterile conditions.

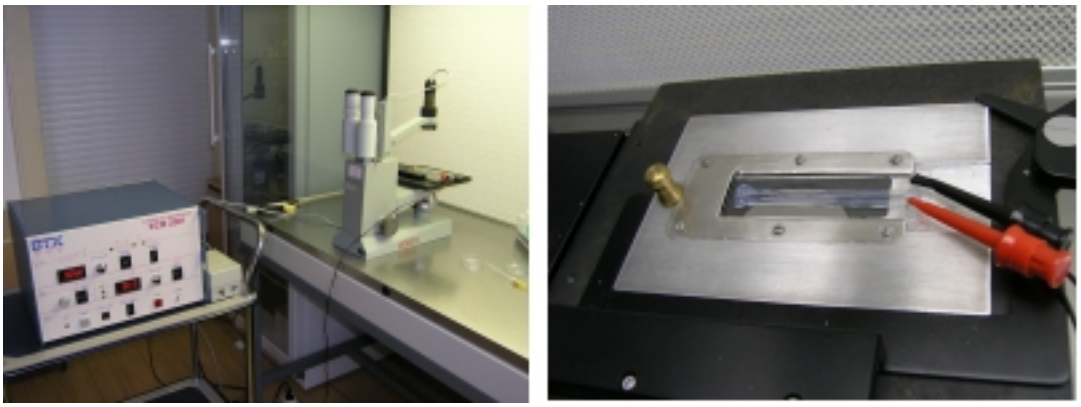
Centrifuge protoplast suspension in pre-fusion solution (500 rpm, 3 min) and remove the supernatant with a Pasteur pipette.

Add a fusion solution (Schilde-Rentschler and Ninneman 1988) with pipette to obtain desired protoplast concentration for fusion 6×10^5/ml and resuspende protoplasts.

Mix protoplast suspensions of two fusion partners in the ratio 1:1.

Protoplast fusion is done using of electroporator (ECM 2001, BTX, Inc., San Diego, CA) and facilities (microscope, holder of fusion chambers, fusion chambers, Fig. 3).
Put microscope into a flow-box, sterilize chambers with 70% alcohol prior to fusion and left to dry out. After drying, transfer 20 – 30 µl protoplast suspension with a Pasteur pipette into an electroporation chamber placing into the holder. Observe the whole process of protoplast fusion under invert microscope.

Fig. 3 Example of fusion experiment arrangement

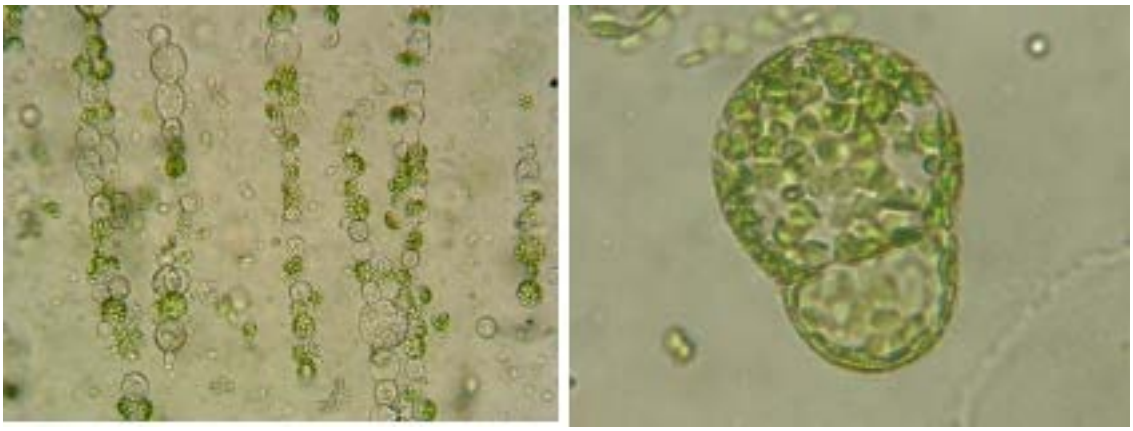


Process of protoplast fusion: protoplasts are aligned by acting of alternate current (AC field), then a pulse of direct current (DC pulse) is applied, which results in fusion (Fig. 4).

- Basic parameters of electric field

AC	5 V/chamber with 0.5 mm electrode distance	2 – 18s
DC pulse	10 V/chamber with 0.5 mm electrode distance	80 µs
AC	30 V/chamber with 3.2 mm electrode distance	2 – 18s
DC pulse	60 V/chamber with 3.2 mm electrode distance	80 µs

Fig. 4 Example of protoplast fusion (left: application of AC field, right: application of DC pulse)



After fusion, transfer protoplasts from five chambers with a Pasteur pipette into one Petri dish (Ø = 3.5 cm) and add 1 ml of culture medium (liquid SW_{11}, Bříza and Machová 1991) to protoplast suspension. Pack Petri dishes with parafilm, transfer into the thermostat and

culture in dark at 25 °C. Further culture is done according to the culture protocol for protoplast fusion products.

2.4 Culture of protoplast fusion products

The whole process of fusion product culture is performed under sterile conditions.

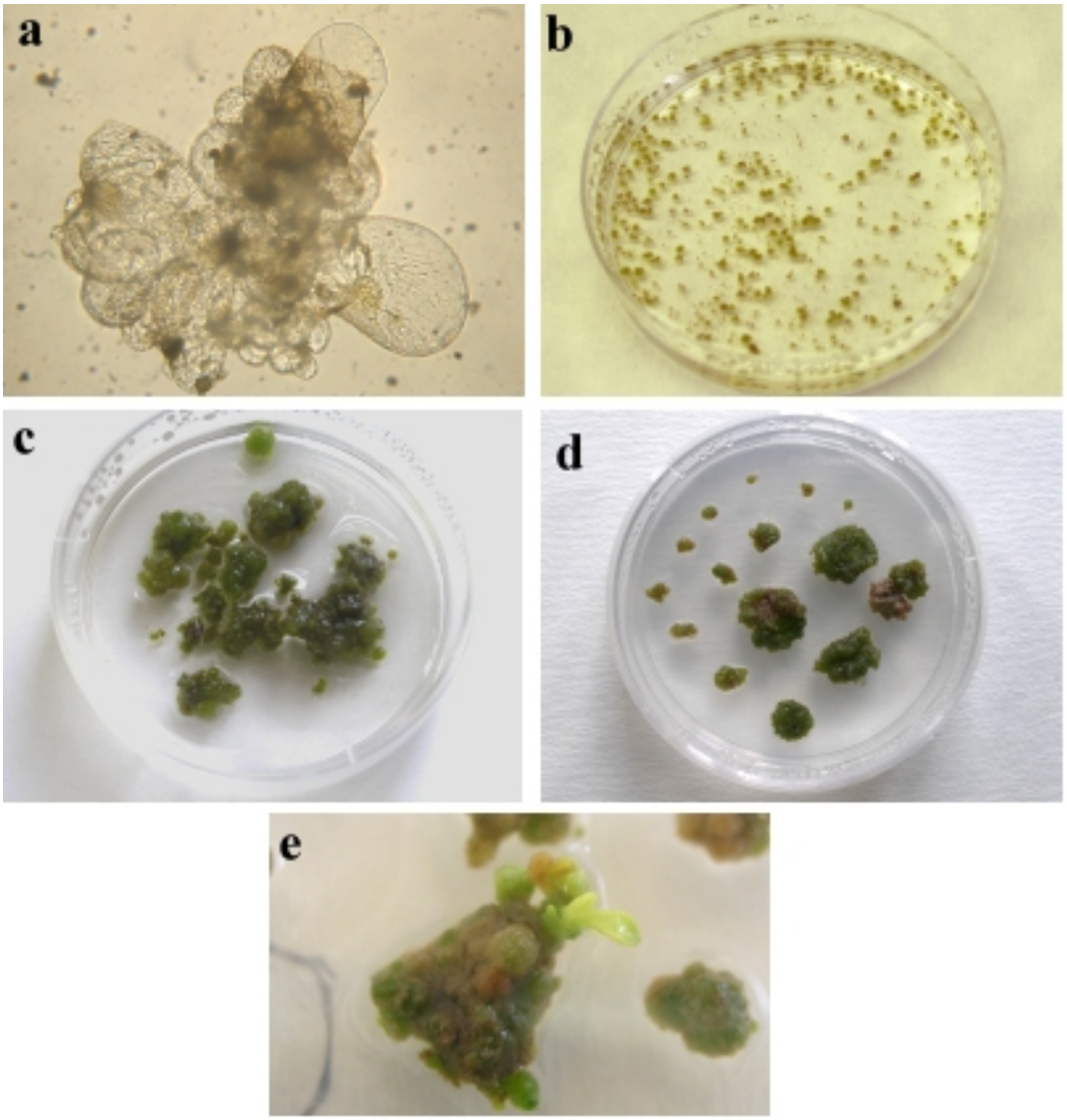
The fist cell division usually occurs 2 – 3 days after fusion (you can observe it under microscope). After cell wall formation, add liquid SW_{11} medium with decreasing level of osmoticum in the interval of 7 – 10 days till the stage of microcalli (i.e. gradually SW_{11} 0.5 M manitol, SW_{11} 0.4 M manitol, SW_{11} 0.3M manitol, Bříza and Machová 1991; Fig. 5 a, b). After cell wall development, regenerating cells could be cultured in the light (16 h/8 h photoperiod; light conditions: fluorescent tubes, daylight, 150 µmol m^{-2} s^{-1}; 22 °C).

Microcalli are formed 3 – 4 days after fusion. When the calli are visible, remove liquid SW_{11} medium by pipette and add the liquid Shepard C medium (Shepard and Totten 1977). Repeat replacement of this medium twice in 7 – 10 days.

Subsequently, sterilely transfer calli to clean Petri dishes. Cultivate a part of calli in liquid Shepard D medium (Shepard and Totten 1977). Change the medium in 7 – 10 days till shoot regeneration. Cultivate the second part of calli in agar-solidified Shepard D medium. Transfer the calli onto fresh medium in 14 days till shoot regeneration (Fig. 5 c, d).

First shoots are formed 4 – 12 weeks after culture initiation in Shepard D medium (Fig. 5 e). Cut off regenerated shoots and left them rooting in MS medium (Murashige and Skoog 1962). Cultivate the shoots under the same conditions as donor plants for protoplast isolation. Multiply the plantlets into pairs for subsequent testing of hybridity.

Fig. 5 Fusion product regeneration; a, b – microcalli in liquid SW_{11} medium, c – calli in liquid Shepard D medium, d – calli in agar-solidified Shepard D medium, e – shoot regeneration on calli prior to cutting)



3 Used solutions and media – their preparation

3.1

- **Murashige & Skoog medium**

MS (Duchefa Biochemie)	4.4 g
sucrose	30 g
agar	8 g

Dissolve MS and sucrose in 490 ml of distilled water. Adjust pH to 5.6 – 5.8 and add distilled water to obtain volume of 500 ml. Dissolve agar in 400 ml of distilled water and microwave or boil in a water bath, make up to final volume (500 ml). Mix both solutions

and dispense in culture vessels (to 1 – 2 cm height) and cover with lids. Autoclave them at 121 °C for 20 min.

- **Schenk & Hildebrand medium**

SH medium (Duchefa Biochemie)	3.2 g
Vitamins SH (Duchefa Biochemie)	1.01 g
sucrose	15 g
$AgNO_3$	0.003 g
Alar	0.0015 g
Agar	8 g

Dissolve all substances except agar in 490 ml of distilled water. Adjust pH to 5.6 – 5.8 and add distilled water to obtain volume of 500 ml. Dissolve agar in 400 ml of distilled water and microwave or boil in a water bath, make up to final volume (500 ml). Mix both solutions and dispense in culture vessels (to 1 – 2 cm height) and cover with lids. Autoklave them at 114 °C for 20 min.

3.2

- **Enzymatic solution**

Stock solution:

$CaCl_2.2\ H_2O$	0.735 g
MES	1.952 g
Sucrose	171.15 g
NAA	0.005 g (stock solution 100 mg/100 mg, sterilization by filtration)
ZT	0.002 g (stock solution 50 mg/100 ml, sterilization by filtration)

Dissolve ingredients without growth regulators in 480 ml of distilled water. Adjust pH to 5.6 – 5.8. Autoclave stock solution at 121 °C for 20 min.

After sterilization, add growth regulators and make up to volume 500 ml by sterile distilled water. Dispense in 50 ml aliquots for usage. Solution could be frozen to -20 °C.

(ZT – zeatin; NAA – alpha-naphthalenacetic acid;

MES – 2-(N-morpholino)ethanesulfonic acid)

Preparation of enzymes:

Celulase Onozuka R-10 (Duchefa Biochemie)	1 g (and/or 1 U)

Macerozyme R-10 (Duchefa Biochemie) 0.2 g (and/or 0.2 U)

Dissolve ingredients in distilled water. Adjust pH to 5.6 – 5.8 and add distilled water to obtain volume of 50 ml and filter sterilize. Adjust weight amount according to Units written on the label of individual package of enzyme.

Working enzymatic solution:

Mix 50 ml of enzymes with 50 ml of stock solution (the unexpended rest could be frozen to -20 °C up to 1 month).

- **0.5 M sucrose**

  Sucrose 171.15 g

  dissolve in distilled water to final volume of 1000 ml, autoclave at 121 °C for 20 min and cold-keep at 6 – 7 °C.

- **W5 solution**

  NaCl 9.0 g

  KCl 0.8 g

  $CaCl_2.2\ H_2O$ 18.4 g

  glucose 1.0 g

  glycine 1.0 g

  dissolve in 990 ml of distilled water, adjust pH to 5.8, add distilled water to obtain volume of 1000 ml and autoclave at 121 °C for 20 min, cold-keep at 6 – 7 °C.

3.3

- **FDA solution (fluoresceindiacetate)**

  FDA 0.005 g

  Prepare stock solution by dissolving of weighted amount in 1 ml of acetone. Working solution: add 20 µl of stock solution to 1 ml of culture solution, sucrose or W5.

- **Pre-fusion solution**

  Manitol 7.4 g

  $CaCl_2$.2H2O 0.011 g

dissolve in 90 ml of distilled water, adjust pH to 5.6 and add distilled water to obtain volume of 100 ml. Autoclave at 121 °C for 20 min.

- **Fusion solution**

  Manitol 7.4 g

  dissolve in 90 ml of distilled water, adjust pH to 5.6 and add distilled water to obtain volume of 100 ml. Autoclave at 121 °C for 20 min.

3.4

- **SW_{11} medium**

SW_{11}	**SW_{11}**	**SW_{11} 0.4M**	**SW_{11} 0.3M**	**SW_{11} 0.2M**
KNO_3	950 mg			
$CaCl_2.2\ H_2O$	367.5 mg			
$MgSO_4.\ 2\ H_2O$	185 mg			
KH_2PO_4	85 mg			
NH_4Cl	133 mg			
Myo-inositol	100 mg			
Casein hydrolysate	500 mg			
L-glutamin	100 mg			
Yeast extract	100 mg			
Sucrose (0,01M)	3423 mg			
Mannitol	89.2682 **g**	72.87 **g**	54.65 **g**	36.44 **g**
Thiamine. HCl	10 mg	prepare stock solutions (100 ml)		
Pyridoxine. HCl	1 mg	with concentration of 1 mg/1 ml		
Nicotinic acid	1 mg			
H_3BO_3	3 mg	(and adequate amount, e.g. Thiamine. HCl		
$MnSO_4.\ 4H_2O$	14 mg	pipette 10 ml into the medium)		
$ZnSO_4.\ 7H_2O$	2 mg			
$CuSO_4.5H_2O$	0.025 mg			
KI	0.75 mg			
$Na_2MoO_4.\ 2H_2O$	0.25 mg			
$CoCl_2.\ 6H_2O$	0.025 mg			
$FeSO_4.\ 7H_2O$	13.9 mg			
$Na_2EDTA.2H_2O$ Chelaton III	37.3 mg			
NAA	2 mg*			
2,4 D	0.2 mg			
Zeatin	0.5 mg*			

dissolve in 950 ml of distilled water, adjust pH to 5.6 – 5.8, autoclave at 121 °C for 20 min. Sterilely add Zeatin and NAA and sterilized distilled water to obtain volume of 1000 ml, cold-keep at 6 – 7 °C.

*Filter sterilise Zeatin – stock solution 50 mg/100 ml, NAA – stock solution 100 mg/100 ml.

Shepard C and Shepard D medium

	$C_{0.3M}$	$C_{0.2M}$	**$D_{0.2M}$**	
NH_4NO_3			1650 mg	
KNO_3	1900 mg		1900 mg	
$CaCl_2.2H_2O$	440 mg		440 mg	
$MgSO_4.7H_2O$	370 mg		370 mg	
KH_2PO_4	170 mg		170 mg	
Na_2EDTA	37.3 mg		37.3 mg	
$FeSO_4.7H_2O$	27.8 mg		27.8 mg	
H_3BO_3	6.2 mg		6.2 mg	prepare stock solutions (100 ml)
$MnCl_2.4H_2O$	19.8 mg		19.8 mg	with concentration of 1 mg/1 ml
$ZnSO_4.7H_2O$	9.2 mg		9.2 mg	
KI	0.83 mg		0.83 mg	(and adequate amount, e.g. H_3BO_3
$Na_2MoO_4.2H_2O$	0.25 mg		0.25 mg	pippete 6.2 ml into the medium)
$CuSO_4.5H_2O$	0.025 mg		0.025 mg	
$CoSO_4.7H_2O$	0.03 mg		0.03 mg	
Myo-inositol	100 mg		100 mg	
Thiamin. HCl	0.5 mg		0.5 mg	
Glycin	2 mg		2 mg	
Nicotinic acid	5 mg		5 mg	
Pyridoxine. HCl	0.5 mg		0.5 mg	
Folic acid	0.5 mg		0.5 mg	
Biotin	0.05 mg		0.05 mg	
Casein hydrolyzate	1000 mg		1000 mg	
Adenin sulfát	40 mg		40 mg	
NAA	0.05 mg*			
IAA			0.1 mg*	
BAP (6-benzylaminopurin)	0.5 mg*			
Zeatin			0.5 mg*	
Mannitol	54.654 **g**	36.44 **g**	36.44 **g**	
Sucrose 15 mM	5.1345 **g**		5.1345 **g**	
MES	0.976 **g**		0.976 **g**	
Agar (solidified medium)			7 **g**	

dissolve in 950 ml of distilled water, adjust pH to 5.8, autoclave at 121 °C for 20 min. Sterilely add NAA, IAA, zeatin, BAP and/or gibberellic acid (see above) and distilled water to obtain volume of 1000 ml. Cold-kept medium. Prepare Shepard D medium as liquid one or with agar. Dispense the agar-solidified medium in Petri dishes in a flow-box.

*Filter sterilise Zeatin – stock solution 50 mg/100 ml, NAA – stock solution 100 mg/100 ml, BAP – stock solution 50 mg/100 ml.

3.5. Sterility verification of used solutions

Verify each solution prior to use by a bacterial test: 1 ml of tested solution + 1 ml of provocation medium. Culture it in dark at 25 °C. Examine tested solutions in 3 – 4 days (presence of infection is indicated by milk discoloration of the solution).

Provocation medium: Standard I nutrient broth for microbiology (Merck) – 25 g/l

4 Regenerant evaluation

Hybridity of regenerants must be verified. For hybridity evaluation it is suitable to use flow-cytometry as pre-selection method for ploidy estimation (http://www.ueb.cas.cz/Olomouc1/LMCC/protocols/Protocols.html). Potential somatic hybrids must be verified by any of DNA analysis methods e.g. RAPD (Greplová et al. 2007).

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