Procedure of *in vitro* mitotic polyploidization

(in *Solanum* genus)

A part of the output V009 within the project NAZV QF4133 „Development of advanced breeding materials with horizontal resistance genes to late blight“

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1 Introduction

The cultivated potato (*Solanum tuberosum* L., 2n=4x=48) is a vegetative propagated plant with tetrasomic inheritance and a high level of heterozygosity (Tai and Xiong 2005). The genetic variability among varieties is limited due to close relationship between cultivars. Wild *Solanum* species are a source of allelic diversity; they possess many valuable traits, incl. resistances to biotic and abiotic stresses (Cardi et al. 1993, Helgeson and Haberlach 1999). *Solanum* represents a model system with a strong sexual isolation mechanism, especially due to "effective" ploidy (EBN – Endosperm Balance Number) (Carputo et al. 1997). Wild diploid (2n = 2x = 24) *Solanum* species with EBN = 1 are sexually isolated from diploid 2EBN species, tetraploid (2n = 4x = 48, 4EBN) and haploid (2n = 2x = 24, 2EBN) *S. tuberosum*. These barriers could be overcome by reduction or increase of ploidy level (Carputo et al. 1997).

Polyploidization - artificial doubling of chromosome number is an approach for ploidy level increase. It could be performed as *in vivo* or *in vitro* application. *In vitro* application is currently preferred. Active substances are directly applied into culture medium (Eeckhaut et al. 2004, de Carvalho et al. 2005) or as aqueous solutions onto nodal segments (Escandón et al. 2006). Colchicine, oryzalin, trifluralin or amiprophos-methyl (Hancock 1997, van Tuyl et al. 1992, Doležel et al. 1994, Hansen and Andersen 1996, Hansen et al. 2000) belong to active substances acting as toxins of mitotic spindle.

The submitted methodics is an innovative procedure utilizing hitherto used *in vitro* methods and application of colchinine and oryzalin is described. Hansen and Andersen (1996) refer that colchicine is generally less effective than several herbicides with antimicrotubular activity (e.g. oryzalin), which express higher affinity to plant tubulin. Moreover, health risk for laboratory staff is reduced during handling with oryzalin. It is always necessary to keep all safety measures according to valid rules.

2 Methodical procedure

The *in vitro* mitotic polyploidization requires sufficient amount of suitable plant material for preparation of nodal segments with dormant axillary bud, application of mitotic spindle poison (colchicine or oryzalin) and culturing of treated nodal segments to achieve plant regeneration. The procedure involves four discreet stages:

- Culture of donor plants
- Preparation of nodal segments and application of mitotic spindle poisons
- Culture of treated nodes
- Evaluation of derived regenerants

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. Plants are propagated in hormone-free MS medium (Murashige and Skoog 1962) for three weeks. At least 20 plantlets are passaged for one application of mitotic poison (Fig. 1).

Fig. 1 Plantlets of *Solanum* genus cultured under *in vitro* conditions for preparation of nodal segments (left dihaploid *S. tuberosum*, right *S. pinnatisectum*)

2.2 Preparation of nodal segments and application of mitotic spindle poisons

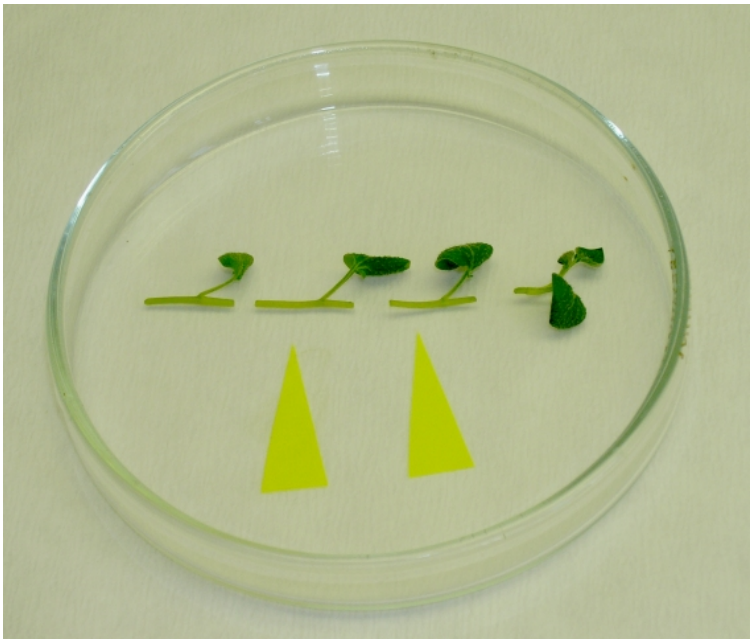
An application of mitotic poisons is performed by a trained person dressed in protective clothing (a whole face filter mask, a long sleeve coat, resistant nitrile gloves, laboratory trousers and laboratory shoes).

During handling it is necessary to keep all safety measures according to valid rules.

The whole process of mitotic polyploidization is performed under sterile conditions.

Use well-growing plants as a source of nodal segments. Take nodal segments from upper part of a plant (1st or 2nd nodal segment under shoot apex, Fig. 2).

Fig. 2 Example of nodal segment preparation



Place the explants immediately into agar-solidified culture MS medium in vessels (180 ml volume, containing 30 ml of medium). Undeveloped axillary buds must be 0.5 - 1 mm above level of solid medium. Culture the explants for 24 hours under similar conditions like donor plants.

Overlay the nodes placed in medium with 15 ml of the working mitotic poison solution. (Fig. 3a, 3b).

Fig. 3a The nodal segment in medium before the mitotic poison application

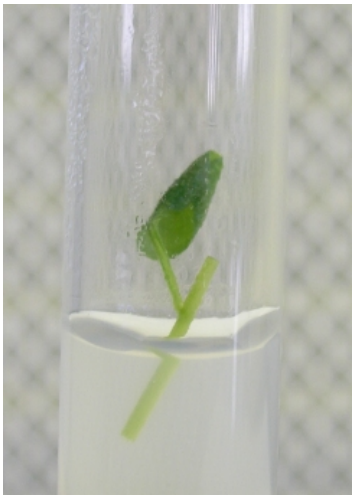


Fig. 3b Example of application of mitotic poison solution to 20 nodes

Oryzalin treated nodes are cultured under standard conditions, while colchicine treated ones are cultured at 22 °C in dark. The recommended duration of oryzalin treatment is 24 or 48 hours (two variants of duration are necessary due to possible diverse response of selected genotypes). The recommended duration of colchicine treatment is 24 hours.

After this period, pour off carefully the mitotic poison solution not to damage agar culture medium.

Rinse nodes: pour sterile distilled water into a culture vessel and after a short time pour off carefully. Repeat the rinsing procedure two more times.

2.3 Culture of treated nodal segments

Transfer treated rinsed nodes with a tweezers from a culture vessel to Petri dish. Cut brownish stem tips and place explants into fresh agar-solidified MS medium. Culture under standard conditions.

In 10 days, transfer explants to fresh MS medium (cut eventual brownish stem tips).

Axillary buds start their development gradually depending on genotype. Culture the explatnts to obtain shoots (0.3 – 0.4 cm). Excise the shoots and transfer them to fresh MS medium.

After rooting, clone plants (2-3 cm) to provide samples for ploidy estimation.

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.

3.2

Solutions of mitotic poisons are prepared by a trained person dressed in protective clothing (a whole face filter mask, a long sleeve coat, resistant nitrile gloves, laboratory trousers and laboratory shoes).

- **Oryzalin stock solution (10 mM)**

Oryzalin	0.0346 g
DMSO	10 ml

Dissolve oryzalin in DMSO (dimethylsulphoxide) in a sterile vessel. DMSO aseptizes itself, i.e. no sterilization by filtration is needed.

- **Oryzalin working solution (25 µM and 30 µM)**

Add sterile distilled water to stock solution achieving a required volume.

25 µM solution: supply 250 µl of stock solution with sterile distilled water to obtain volume of 100 ml

30 µM solution: supply 350 µl of stock solution with sterile distilled water to obtain volume of 100 ml

- **Colchicine working solution (3.5 mM)**

Colchicine 0.1398 g

Dissolve colchicine in small volume of 96% ethanol, add distilled water to obtain volume of 100 ml and sterilize by filtration (microfilter - 0.22 µm).

4 Evaluation of regenerants

Ploidy of regenerants must be verified. There are indirect and direct methods for ploidy determination. Indirect method is "Assessment of chloroplast number in guard cells of stomata" (Frček 1985). Direct methods are "Assessment of chromosome number in root tip cells (Zlesák et al. 2005) or flow cytometry (Doležel et al. 1994). Methods "Assessment of chloroplast number" and "Assessment of chromosome number" are very laborious for *in vitro* plantlets. Most suitable and highly reliable method for ploidy evaluation is the flow cytometry (http://www.ueb.cas.cz/Olomouc/LMCC/Protocols/protocols.html).

4 References

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