Procedure of Petri dish test for confirming *Phytophthora infestans* resistance (in *Solanum* genus)

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1 Introduction

The oomycete, *Phytophthora infestans* causes late blight, the most serious potato (*Solanum tuberosum*) disease worldwide. The management of the disease has been estimated to cost $ 3.5 billion annually (Wang et al. 2008). Effective use of resistance germplasm in breeding programs is the only option for reducing the costs and environmental damage caused by the disease. Examples of suitable germplasm include *S. pinnatisectum* (Chen et al. 2003, Ramon and Hanneman 2002) and *S. bulbocastanum* (Chen et al. 2003, Szczerbakowa et al. 2003, Helgeson et al. 1998) from Mexico and, *S. berthaultii* (Rauscher et al. 2006), *S. microdontum* (Sandbrink et al. 2000), *S. mochiquense* (Smilde et al. 2005) and *S. paucissectum* (Villamon et al. 2005) from Andean countries. The wild potato species carry genes that confer resistance to *P. infestans*. Resistance responses are classified as race-specific, race-nonspecific and non-host resistance (Agrios 1997). An essential part of resistance breeding is evidence for resistance trait transfer and following selection. The following are used to provide the required evidence: field test (Shaner and Finney 1977, Fry 1987, Collon and Budding 1988), whole-plant greenhouse assay (Stewart et al. 1983), laboratory test on detached leaves (Lapwood 1961, Vleeshouwers et al. 1999), on leaflets (Malcolmson 1969) or leaf discs (Hodkson 1961). In contrast to laboratory tests, field tests can be performed only once a year during the growing season. This limitation makes laboratory tests more attractive.
The Petri dish test is the laboratory assay on detached leaflets. The test was created as modification of Laboratory assay on detached leaves (Vleeshouwers et al. 1999) for evaluation of somatic hybrids. The results of Petri dish test correlated with Laboratory assay of Vleeshouwers et al. (1999). The Laboratory assay of Vleeshouwers et al. (1999) proved to be a good alternative to the field test.

2 Methodical procedure

The Petri dish test requires sufficient amount of suitable health plant material with good developed leaves without any pests or pathogenes. The age of plants is 6 to 8 weeks. The procedure involves five discrete stages.

- Plant cultivation
- Petri dish preparation
- Inoculum preparation
- Detaching of leaves and experiment initiation
- Evaluation

2.1 Plant cultivation

Five plants per genotype is necessary for Petri dish test (except seedlings, as far as the seedlings will not sow and cloned *in vitro*). As the positive control plants are designed *S. tuberosum* cv. Asterix, Lukava and as negative *S. tuberosum* cv. Blaník, Inovátor, Kuras.
In glasshouse, the plants are planted in mixture of peat, soil and perlite in ratio 3:3:1. The plants is necessary to fasten to rods.

In vitro plants
In vitro plantlets (five per genotype) are cultivated in a culture chamber with a 16/8 h photoperiod (light conditions: fluorescent tubes, daylight, 60 µmol m^{-2} s^{-1}), at 22 °C, on MS medium (Murashige and Skoog 1962). After six weeks, the *in vitro* plantlets are transferred into pots (7 cm) and acclimatized (Polzerová and Greplová 2009). In glasshouse, the plants are kept 1 – 2 weeks covered with transparent breathable containers to rooting. The acclimatization in culture chamber is optimal (16/8 h photoperiod; light conditions: fluorescent tubes, daylight, 60 µmol m^{-2} s^{-1}; 22 °C; 90 % humidity). The rooted plants are transplanted into large vessels (4 l of soil) and standardly cultivated in glasshouse.

Seedlings
The potato seeds are sowed in rows into tray. After four weeks, the sprouted plantlets (2 – 3 cm) are transplanted into pots (10 cm). The good rooted plants are transplanted into large vessels (4 l of soil) and standardly cultivated in glasshouse.
For clonning, seeds are sowed to *in vitro* conditions. The whole process is performed under sterile conditions. The seeds are sterilised in 2% solution of Chloramin BS (Bochemie, CZ) with soaking agent for 15 minutes. This solution is poured through sieve. The seeds are washed with sterile distilled water. The seeds are dipped in 70% ethanol for 1 minute. Ethanol is poured through sieve. The seeds are properly washed with sterile distilled water. The seeds are put on surface of MS medium in tubes. The cultivation – see *In vitro* plants.

Tubers
Five tubers per genotype are planted in vessels (4 l of soil) and standardly cultivated in glasshouse.

2.2 Petri dish preparation
The Petri dishes (10 cm) are covered with cellulose wool saturated with 38 ml of water (immediately before beginning of test).

2.3 Inoculum preparation
P. infestans isolates are ordered at specialized institution. *P. infestans* can be maintained according protokol by Vleeshouwers a kol. (1999). Fresh sample of sporangiospores is plated on rye agar medium supplemented with 20 g/l sucrose (Caten and Jinks 1968) and incubated aat 18 °C in the dark. After a few days, a plug of mycelium is transfered to a fresh agar plate. One week later the plate is covered with mycelium. Cold water (4°C) is added to the sporulating mycelium. The mycelium dip is done by gentle shaking with Petri dish. After 10 minutes, the sporangiospore suspension is pipetted into sterile flasks (100 ml) and incubated at 4°C. After 1 – 2 hours, zoospores are separated from the sporangiospores by filtration through a nylon sieve (50 µm). The concentration is adjusted to 5×10^4 zoospores ml^{-1} for inoculation.

2.3.1 Determination of zoospore density using a Bürker chamber
Count zoospores in five triple-lined squares (i.e. a square of 1 mm side), in the left and above all zoospores touching the middle line are counted, in the right and below these ones are not counted. Determine an average number of zoospores from five squares - **"X".**
Zoospore 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 = $\mathbf{V_{suspension}}$ **× "X" ×** $\mathbf{10^4}$
Dilution factor $\mathbf{F_d}$ **= required density / "X" ×** $\mathbf{10^4}$

Suspension volume at required density = $\mathbf{V_{suspension}}$ /$\mathbf{F_d}$

2.4 Detaching of leaves and experiment initiation

Five plants are used for the Petri dish test. One fully developed leaf (the third to fifth from the top) per plant was used. In case of seedlings (as far as they will not sow and cloned *in vitro*), five leaves per plant are detached.
The surface of the wool was zig zag streamed with 2 ml inoculum suspension. Five wet leaflets detached from one compound leaf were put abaxial site down onto prepared Petri dishes (Fig. Experiment arrangement of Petri dish test). On the first day, the Petri dishes were covered but on the following days they were opened. Petri dishes were cultivated at 20 °C, relative humidity 70 - 80 %, 16/8 h photoperiod and light intensity 15 µmol m^{-2} s^{-1}.

2. 5 Evaluation

Lesions are measured three times, on days three, four and five after inoculation using calliper. The largest length and width of each lesion are measured and the area is calculated: A = ¼ × π × length × width. More than one lesion can be found on one leaflet; in this case, the area A is sum of areas of all lesions per leaflet. The area A is square rooted to obtain the radius of the lesion. The values of the lesion growth rate (LGR; mm den^{-1}) and the infection efficiency (IE; %) are necessary for statistical evaluation. The radii are estimated by linear regression over time to obtain LGR. The infection efficiency (IE) was calculated as percentage of successful inoculation, i.e. percentage of leaflets with the growing lesion to the total number of leaflets per leave or per plant (growing lesions are lesions with an area larger than 16 mm^2 at least at one time point). The statistical evaluation is analysed with ANOVA and Tukey method.

2.5.1 Example of evaluation

The model situation is recorded in following example of *P. infestans* resistance evaluation.
No lesion or maximally one lesion is founded on leaflet.

	number of leave	leaflet	3rd day after inoculation				4th day after inoculation				5th day after inoculation								
			length (mm)	width (mm)	A(mm2)	radius (mm)	length (mm)	width (mm)	A(mm2)	radius (mm)	length (mm)	width (mm)	A(mm2)	radius (mm)	**LGR (mm day^{-1})**	**Ø LGR leave**	**IE leave**	Ø LGR genotype	Ø IE genotype
Karin	1	a	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**	**2,6**	**40%**	2,4	44%
		b	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		c	2,0	3,0	4,7	2,2	15,0	15,0	176,7	13,3	19,0	17,0	253,7	15,9	**4,8**				
		d	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		e	9,0	7,0	49,5	7,0	21,0	17,0	280,4	16,7	45,0	25,0	883,6	29,7	**8,3**				
	2	a	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**	**2,1**	**40%**		
		b	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		c	1,0	1,0	0,8	0,9	5,0	4,0	15,7	4,0	10,0	6,0	47,1	6,9	**2,1**				
		d	11,0	15,0	129,6	11,4	22,0	32,0	552,9	23,5	53,0	26,0	1082,3	32,9	**8,4**				
		e	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
	3	a	10,0	10,0	78,5	8,9	19,0	11,0	164,1	12,8	24,0	20,0	377,0	19,4	**4,5**	**1,7**	**40%**		
		b	15,0	10,0	117,8	10,9	19,0	16,0	238,8	15,5	28,0	17,0	373,8	19,3	**4,0**				
		c	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		d	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		e	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
	4	a	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**	**2,9**	**60%**		
		b	5,0	3,0	11,8	3,4	16,0	11,0	138,2	11,8	23,0	13,0	234,8	15,3	**4,3**				
		c	10,0	8,0	62,8	7,9	25,0	17,0	333,8	18,3	32,0	17,0	427,3	20,7	**5,1**				
		d	13,0	10,0	102,1	10,1	16,0	14,0	175,9	13,3	29,0	20,0	455,5	21,3	**4,9**				
		e	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
	5	a	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**	**2,8**	**40%**		
		b	4,0	3,0	9,4	3,1	15,0	11,0	129,6	11,4	25,0	16,0	314,2	17,7	**5,2**				
		c	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		d	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,0**				
		e	7,0	4,0	22,0	4,7	25,0	14,0	274,9	16,6	45,0	25,0	883,6	29,7	**8,9**				

2.6 Used solutions and media – their preparation

● Murashige & Skoog medium

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

Dissolve MS and sucrose in 450 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.

● Chloramin BS 2% solution

Chloramin BS	2 g
Soaking agent	100 µl

Dissolve Chloramin in 80 ml of distilled water, pippete soaking agent and make up to final volume (100 ml).

● Rye agar medium (Caten a Jinks 1968)

rye	60 g
sucrose	20 g
agar	15 g

Soak rye grain in 500 ml of distilled water for 36 hour in room temperature (or gently boil for 1 hour). Pour of the supernatant (I) and retain it for next using. Macerate and extracte the swollen grains for 3 hours at 50 ºC in 300 ml of distilled water. Filter them through dense cloth. Retain supernatant (II), discard sediment. Add agar to supernatant I and microwave or boil in a water bath. Add sucrose to supernatant II. Mix both solutions and make up to final volume 1000 ml. Autoclave it at 121 °C for 15 min. Diospense the rye medium to sterile Petri dishes and pack with Parafilm.

3 References

Agrios G (1997) Plant pathology, 4th edn. Academic Press. San Diego

Caten CE, Jinks JL (1968) Spontaneous variability of single isolates of *Phytophthora infestans* I. Cultural variation. Canadian Journal of Botany 46: 329-347

Chen Q, Kawchuk LM, Lynch DR, Goettel MS, Fujimoto DK (2003) Identification of late blight, Colarado potato beetle, and blackleg resistance in three mexican and two south american wild 2x (1EBN) *Solanum* species. Am J Pot Res 80(1): 9-19

Collon LT, Budding DJ (1988) Resistance to late blight (*Phytophthora infestans*) in ten wild *Solanum* species. Euphytica Supplement: 77-86

Fry WE (1987) Quantification of general resistance of potato cultivars and fungicide effects for integrated control of potato late blight. Phytopathology 68: 1650-1655

Helgeson JP, Pohlman JD, Austin S, Haberlach GT, Wielgus SM, Ronis D, Zambolim L, Tooley P, McGrath JM, James RV, Stevenson WR (1998) Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor. Appl. Genet. 96: 738-742

Hodkson W (1961) Laboratory testing of the potato for partial resistance to *Phytophthora infestans*. Americal Potato Journal 38: 261-264

Lapwood D (1961) Laboratory assessments of the susceptibility of potato haulm to blight (*Phytophthora infestans*). European Potato Journal 4: 117-127

Malcolmson J (1969) Factors involved in resistance to blight (*Phytophthora infestans* (Mont.) de Bary) in potatoes and assessment of resistance using detached leaves. Annals of Applied Biology 64: 461-468
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497
Polzerová H, Greplová M (2009) Acclimatization of somatic hybrids and plantlets after chromosome doubling in *Solanum* genus. Acta Horticulturae (ISHS) 812: 337-342
Ramon M, Hanneman RE (2002) Introgression of resistance to late blight (*Phytophthora infestans*) from *Solanum pinnatisectum* into *S. tuberosum* using embryo rescue and double pollination. Euphytica 127: 421-435
Rauscher GM, Smart CD, Simko I, Bonierbale M, Mayton H, Greenland A, Fry WE (2006) Characterization and mapping of *Rpi-ber*, a novel potato late blight resistance gene from *Solanum berthaultii*. Theor Appl Genet 112: 674-687
Sandbrink JM, Colon LT, Wolters PJCC, Stiekama WJ (2000) Two related genotypes of *Solanum microdontum* carry different segregating alleles for field resistance to *Phytophthora infestans*. Mol Breed 6: 215-225
Shaner G, Finney RE (1977) The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67: 1051-1056
Smilde WD, Brigneti G, Jagger L, Perkins S, Jones JDG (2005): *Solanum mochiquense* chromosome IX carries a novel late blight resistance gene *Rpi-moc1*. Theor Appl Genet 110: 252-258
Stewart HE, Flavelle PH, McCalmont DC, Wastie RL (1983) Correlation between glasshouse and field test for resistance to foliage blight caused by *Phytophthora infestans*. Potato Research 26: 41-48
Szczerbakowa A, Boltowicz D, Lebecka R, Radomski P, Wielgat B (2005) Characteristics of the interspecific somatic hybrids *Solanum pinnatisectum* (+) *S. tuberosum* H-8105. Acta Physiol Plant (27) 3a: 265-273
Szczerbakova A, Boltowicz D, Wielgat B (2003) Interspecific somatic hybrids *Solanum bulbocastanum* (+) *S. tuberosum* H-8105. Acta Physiol Plant 25/4: 365-373
Villamon FG, Spooner DM, Orrillo M, Mihovilovich E, Perez W, Bonierbale M (2005) Late blight resistance linkages in a novel cross of the wild potato species *Solanum paucissectum* (series *Piurana*). Theor Appl Genet 111: 1201-1214
Vleeshouwers VGAA, van Dooijeweert W, Keizer LCP, Sijpkes L, Govers F, Colon LT (1999) A laboratory assay for *Phytophthora infestans* resistance in various *Solanum* species reflects the fiels situation. European Journal of Planat Pathology 105: 241-250
Wang M, Allefs S, van den Berg RG, Vleeshauwers VGAA, van der Vossen EAG, Vosman B (2008) Allele mining in *Solanum*: conserved homolugues of *Rpi-blb1* are identified in *Solanum stoloniferum*. Theor Appl Genet 116: 933-943

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