

# Response of carrot protoplasts and protoplast-derived aggregates to selection using a fungal culture filtrate of *Alternaria radicina*

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**Abstract** Protoplasts isolated from three accessions of cultivated carrot and 5-day-old protoplast-derived aggregates were subjected to selection to identify somaclonal variants with enhanced tolerance to the fungal disease black rot incited by *Alternaria radicina*. Different concentrations [1, 2, 3.5, 5, 10, 20, 35 and 50 % (v/v)] of a fungal culture filtrate (FCF) from 2-week-old liquid cultures of *A. radicina* were used. Protoplasts and aggregates were subjected to short-term selection for a period of 10 days. All FCF concentrations added to the cultures on the day of isolation decreased protoplast survival frequency and plating efficiency, while FCF applied 5 days later inhibited cell divisions in 5–50 % concentrations. The responses of protoplasts to the treatment were genotype dependent. Most R0 plants were regenerated in all accessions from cell lines grown with 1 % FCF, while only a few plants were produced from 2 to 3.5 % FCF-treated cultures of ‘Dolanka’ and the breeding line ‘9304B’, respectively. Nineteen-percent of putative stress-tolerant regenerants were tetraploids, while only 5 % tetraploids were observed in the control. The incidence of unique random amplified polymorphic DNA fragments indicating possible chromosomal rearrangements was low and did not differ among regenerants after selection and those derived from the control. Mobilization of miniature inverted repeat transposable elements was not observed. Some R0 individuals regenerated both from FCF-treated and untreated cultures showed lower susceptibility to *A. radicina* in a

laboratory assay in comparison to control plants grown from seed. Regenerants from FCF-treated cultures showed lower frequency of flowering plants and a higher rate of male sterility. Pollen viability of the putative stress-tolerant regenerants varied over a wide range (6–98 %), independently of in vitro selection conditions. Our data suggest that *A. radicina* FCF may be feasible for the in vitro selection to generate plants with superior phenotypic performance against *A. radicina*.

**Keywords** Black rot · *Daucus carota* · In vitro selection · Petiole assay · Plating efficiency · Pollen viability

## Abbreviations

B5	B5 Gamborg medium (Gamborg et al. 1968)
CPP	Carrot petiole protoplast medium
2,4-D	2,4-Dichlorophenoxyacetic acid
FDA	Fluorescein diacetate
FCF	Fungal culture filtrate
MES	2-(N-Morpholino)ethanesulfonic acid
MITE	Miniature inverted repeat transposable element
MS	Murashige and Skoog medium (1962)
NAA	$\alpha$ -Naphthaleneacetic acid
PEM	Proembryonic mass
PDA	Potato-dextrose agar
PDB	Potato-dextrose broth
TE	Transposable element

## Introduction

Cultivated carrot (*Daucus carota* L. subsp. *sativus* Hoffm.,  $2n = 2x = 18$ ) is one of the most important vegetable crops in the Apiaceae. It is grown worldwide for its storage

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roots (a rich source of  $\alpha$ - and  $\beta$ -carotene, precursors of vitamin A). Carrot roots also contain other compounds such as: disaccharides, phenolics, terpenes, saponins, fatty acids, and proteins (Grzebelus et al. 2011). Black rot, caused by *Alternaria radicina* Meier, Drechsler and E.D. Eddy, a seed-borne fungal pathogen, is one of the most serious diseases found wherever carrots are commercially produced (Pryor et al. 1998). The pathogen attacks carrots in the field, at any stage of growth and development, and also during storage. In the course of vegetation, dry, black, necrotic lesions on leaf petioles, laminae, and root crown can be observed. Large rotten spots might be formed along the roots under post-harvest conditions, eventually leading to total yield loss. *A. radicina* is frequently present on carrot seeds, reducing its quality through decreasing seed germination and causing seedling death. Seeds are the main *A. radicina* source, but the pathogen can attack plants if present in the soil (Pryor et al. 1998). Once introduced, the fungus can survive in soil for years, even in the absence of carrot host plants. The use of fungicides is very often ineffective, and in addition some chemically synthesized fungicides can cause environmental pollution. Therefore, successful control of black rot has been difficult, costly, labor- and resource-intensive, depending on integration of the most appropriate methods for distinct carrot production regions (Tylkowska 1992; Pryor and Gilbertson 2001; Farrar et al. 2004).

Cultivation of varieties resistant to *A. radicina* could overcome this problem. So far, resistant cultivars have not been developed using conventional breeding methods due to the absence of useful sources of resistance genes in cultivated and wild *Daucus* germplasm. Hence, application of biotechnological tools in breeding could be an alternative to develop black rot-tolerant carrot lines. In vitro selection might offer a feasible and cost-effective tool for developing such plants (Rai et al. 2011). That technique can be successfully applied to carrot, which is a model species for plant tissue culture systems and plant regeneration studies (Baranski 2008).

Tissue culture conditions impose severe physiological stress that may be further increased by addition of a selective agent to the medium. It may result in a range of changes in the genome, including polyploidization and chromosomal rearrangements. It was also shown that transposable elements (TEs) can be mobilized in the course of the culture (Kikuchi et al. 2003). In vitro selection exploits that genetic variation, the so-called somaclonal variation (Larkin and Scowcroft 1981), which can result in a range of genetically stable changes, some of them being potentially useful for crop improvement. At the DNA level, the occurrence of these genomic changes can be detected by distinct molecular markers (Gao et al. 2009).

To perform in vitro selection, different explants, such as plant cells (protoplasts and cell suspensions), tissues (callus), or organs (shoots and somatic embryos) are cultured on media supplemented with the selective agents. Exposure of plant explants to selective agents generates selection pressure allowing the survival and growth only of cells with the desired stress-tolerant phenotype. For fungal diseases, a broad range of agents can be used to select resistant lines via in vitro cultures including fungal culture filtrates (FCF), pure phytotoxins, or intact pathogens (Švábová and Lebeda 2005; Rai et al. 2011). These selective agents elicit typical reactions such as the production and accumulation of antimicrobial phytoalexins in the cells (Švábová and Lebeda 2005). A high correlation between in vitro and in vivo resistance to pathogenic fungi was reported (Kumar et al. 2008; Savita et al. 2011), however, resistance to pathotoxins at the cellular level may not always lead to whole plant resistance to the pathogen (Sharma et al. 2010).

Despite the fact that carrot is a model plant for in vitro culture, very few reports describing the in vitro selection for improving its resistance to pathogens can be found (Baranski et al. 1997). To our knowledge, there is no information on applying that technique to select carrot lines with improved levels of resistance to black rot. In the present study, the phytotoxic effect of the fungal culture filtrate from *A. radicina* on protoplasts and protoplast-derived cell aggregates was analyzed. The main objective of our research was to regenerate carrot plants from protoplast cultures under *A. radicina* culture filtrate pressure, and subsequently to obtain and evaluate these regenerants.

## Materials and methods

### Plant material

Three male fertile accessions of carrot were used as donors for protoplast isolation: two Polish open-pollinated varieties ‘Dolanka’ and ‘Amsterdamska’ and the breeding line ‘9304B’ (kindly provided by P.W. Simon, USDA, USA). All of them were susceptible to black rot. Protoplasts were isolated from in vitro grown plantlets derived from seeds. The procedure and conditions of seed disinfection, germination, and seedling maintenance were as described by Grzebelus et al. (2012). Briefly, seeds were incubated in a water bath at 40 °C, then transferred to a 0.2 % (v/v) solution of fungicide ‘Bravo’ (Syngenta, USA) and finally immersed in a 20 % (w/v) solution of chloramin T (sodium N-chlorotoluene-4-sulphonamide) (30 min each). After washing in sterile water and air-drying, the seeds were germinated in 9 × 1.5 cm Petri dishes on solid Murashige and Skoog (MS) medium with vitamins (Murashige and

Skoog 1962) supplemented with 30 g l<sup>-1</sup> sucrose and 6.5 g l<sup>-1</sup> plant agar (Biocorp, Poland) at 26 ± 2 °C in the dark. After about 7 days of culture, the seedlings were transferred to glass jars containing regeneration medium (R) composed of MS macro- and micro-elements, 0.1 mg l<sup>-1</sup> thiamine HCl, 0.1 mg l<sup>-1</sup> piridoxine HCl, 0.5 mg l<sup>-1</sup> nicotinic acid, 3.0 mg l<sup>-1</sup> glycine, 100 mg l<sup>-1</sup> myo-inozytol, 20 g l<sup>-1</sup> sucrose, and 2.5 g l<sup>-1</sup> phytigel (Sigma-Aldrich, St. Louis, MO, USA). Cultures were kept in a climate room at 26 ± 2 °C under a 16-h photoperiod and light intensity of 55 μmol m<sup>-2</sup> s<sup>-1</sup>.

#### Isolation and maintenance of *A. radicina*

*Alternaria radicina* was isolated from naturally infected roots of carrot. After surface disinfection in 70 % ethanol, root discs with infected tissue were excised and cultured for 2 weeks on potato-dextrose agar (PDA, Duchefa Biochemie, Haarlem, The Netherlands) at 26 ± 2 °C in the dark. After microscopic verification, single spore isolates were multiplied on PDA in 9 × 1.5 cm Petri dishes at 26 ± 2 °C in the dark. Stock isolates of *A. radicina* were maintained at 8 ± 2 °C and renewed periodically.

#### Preparation of the fungal culture filtrates from *A. radicina*

FCF was produced from *A. radicina* isolate KGHN3. One mycelial disc (approx. 0.5 cm<sup>2</sup>) of a 2-week-old fungal culture was transferred to 100-ml Erlenmeyer flasks with 40 ml of potato-dextrose broth (PDB, Duchefa Biochemie). The flasks were placed on a gyratory shaker (150 rpm) and incubated at 26 ± 2 °C in the dark for 2 weeks. The mycelium and spores were then filtered from the liquid cultures and the filtrate was passed through a glass fiber prefilter (1.0 μm, Millipore, Billerica, MA, USA). The purified filtrate was adjusted to pH 5.6 and filter-sterilized through syringe disposable membrane filters (0.22 μm, Millipore) and added to the protoplast culture medium as a selective agent.

#### Protoplast isolation and culture

Protoplasts were isolated from leaves of 4-week-old in vitro grown plantlets. All steps of protoplast isolation and culture were performed according to the established protocol of Grzebelus et al. (2012). Briefly, leaves were cut into fine pieces in a glass Petri dish in the presence of preplasmolysis solution (0.5 M mannitol), incubated for 1 h at 26 ± 2 °C in the dark, and then digested overnight in the enzyme solution [1 % (w/v) cellulase Onozuka R-10 (Duchefa Biochemie), 0.1 % (w/v) pectolyase Y-23 (Duchefa Biochemie), 20 mM 2-(N-Morpholino)ethanesulfonic acid

(MES, Sigma-Aldrich), 5 mM CaCl<sub>2</sub>, and 0.6 M mannitol (Sigma-Aldrich), pH 5.6, filter-sterilized (0.22 μm, Millipore)] on a gyratory shaker (30 rpm). The released protoplasts were purified from the undigested tissue by filtration through a 80-μm nylon sieve (Millipore), and then from the enzyme solution by centrifugation at 100× g for 5 min. The protoplast pellet was resuspended in the solution of 0.5 M sucrose with 1 mM MES, and overlaid with W5 medium containing 154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5 mM KCl, and 5 mM glucose, pH 5.6 (Menczel et al. 1981). After centrifugation at 145× g for 10 min, the viable protoplasts were collected in the interphase band, transferred to a fresh tube and washed twice by centrifugation at 100× g for 5 min in W5 solution and carrot petiole protoplast medium (CPP), respectively. CPP culture medium consisted of macro-, micro-elements and organic acids according to Kao and Michayluk (1975), vitamins according to Gamborg B5 medium (Gamborg et al. 1968), 74 g l<sup>-1</sup> glucose, 250 mg l<sup>-1</sup> casein enzymatic hydrolysate (Sigma-Aldrich), 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.9 μM zeatin (pH 5.6, filter-sterilized).

The working density was adjusted to 8 × 10<sup>5</sup> protoplasts ml<sup>-1</sup> via a Fuchs Rosenthal hemocytometer chamber. Before the culture, protoplasts were embedded in the alginate matrix. A modified procedure of the thin calcium alginate layer technique (Grzebelus et al. 2012) was used. Equal volumes of protoplasts resuspended in the CPP medium and 2.8 % (w/v) alginic acid sodium salt (Sigma-Aldrich) in 0.4 M mannitol solution were mixed carefully to obtain a final density of 4 × 10<sup>5</sup> ml<sup>-1</sup> of protoplasts in the culture. Polymerization of the alginate matrix took place after spreading of the protoplasts-alginate mixture (in 300-μl aliquots) onto 1 % (w/v) agar (Biocorp) containing 20 mM CaCl<sub>2</sub> and 0.4 M mannitol in 6 × 1.5 cm Petri dishes. Solid sheets of alginate with embedded protoplasts formed after a 1 h incubation at 26 ± 2 °C. The layers were then carefully transferred from the agar plates to 4 ml of liquid CPP culture medium in 6 × 1.5 cm Petri dishes. Protoplast cultures were incubated in the dark at 26 ± 2 °C for about 2 months.

#### Protoplast selection

The selection medium was prepared by adding filter-sterilized volumes of FCF and autoclaved double-distilled water to the double-strength CPP medium to provide concentrations of the selective agent in the culture at 1, 2, 3.5, 5, 10, 20, 35, or 50 % (v/v). Protoplasts were treated with the filtrate in the first (type A experiments) or in the fifth (type B experiments) day of culture. In the control combination, FCF was not applied. After 10 days of selection (i.e., in 10 and 15-day-old cultures for type A and B experiments, respectively), the culture medium with FCF

was removed and replaced by the fresh medium without the FCF. Each selection experimental set was established from protoplasts isolated from a single plant.

To assess the effect of FCF on protoplast growth, cell viability and the ability to form aggregates were defined. Protoplast viability was analyzed after fluorescein diacetate (FDA) staining as described by Anthony et al. (1999) with modification of Grzebelus et al. (2012). In viable cells, the apple-green fluorescence was observed as the result of elimination of acetate groups from FDA by functional esterases present in the intact membrane of active cells. Fluorescein accumulation in the cells was visualized under an Axiovert S100 microscope (Carl Zeiss, Göttingen, Germany) equipped with an excitation-emission filter set at  $\lambda = 485\text{--}515$  nm. The viability of protoplasts was estimated immediately after isolation (1 h) and in the 24th and 48th hour of culture for type A experiments, and only in the first hour of culture for type B experiments, and was expressed as the number of cells with apple-green fluorescence per the total number of observed cells ( $\times 100$ ). Plating efficiency was estimated 10 days after FCF application, i.e., on the 10th day of culture for type A experiments and on the 5th and 15th day of culture for type B experiments, and was expressed as the number of cell aggregates per the total number of plated protoplasts ( $\times 100$ ).

#### Plant regeneration and acclimatization

After 2 months of culture in the dark at  $26 \pm 2$  °C (including the selection period), both proembryonic masses (PEM) and somatic embryos emerged from the alginate matrix in FCF-treated and control combinations, were regenerated into plants (R0) and were acclimatized to ex vitro conditions according to Grzebelus et al. (2012). Briefly, after depolymerization of Ca-alginate layers in 20 mM sodium citrate solution containing 0.2 M mannitol, released PEM and embryos were washed by centrifugation (5 min at  $100\times g$ ) in the CPPD medium containing 0.5  $\mu\text{M}$  NAA and 0.9  $\mu\text{M}$  zeatin, pH 5.6 (Dirks et al. 1996), plated in glass jars on the solidified R medium, and finally placed in a climate room at  $26 \pm 2$  °C under a 16-h photoperiod and light intensity of  $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Rooted plants were transplanted to the moss-coconut fiber substrate (Ceres International, Poland) and acclimatized to ex vitro conditions by regularly reducing the humidity in a climate chamber at  $20 \pm 1$  °C under a 16-h photoperiod and light intensity of  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

#### Molecular and cytogenetic analysis of R0 plants

##### *Cytogenetic analyses*

Cytometric analysis of acclimatized plants was applied to determine the ploidy level. Measurement of the relative

nuclear DNA content was performed on Partec CA II (Partec, Münster, Germany) following a protocol described by Kielkowska and Adamus (2010). Additionally, mitotic chromosome spreads from root tips were prepared to verify any abnormal numbers of chromosomes (Nowicka et al. 2012). Briefly, root tips were collected from ex vitro grown plants, treated with 2 mM 8-hydroxyquinoline for 3–4 h at  $26 \pm 2$  °C and then fixed in freshly prepared methanol – glacial acetic acid (3:1) solution. Isolated meristems were macerated in an enzyme mixture consisting of 4 % (w/v) cellulase Onozuka R10 (Serva Electrophoresis, Heidelberg, Germany) and 2 % (w/v) pectolyase Y23 (Duchefa Biochemie), in distilled water, pH 4.8 for 45 min at 37 °C, and then carefully washed in distilled water, fixed again, spread on microscopic slides, and chopped with fine forceps. Chromosome number was scored under a phase-contrast microscope (Axiovert S100, Carl Zeiss, Göttingen, Germany).

##### *Random amplified polymorphic DNA*

DNA amplifications were carried out using 12 arbitrary 10-mer primers (Eurofins MWG Operon), viz. OPA01, OPA02, OPA03, OPA09, OPA10, OPA13, OPA15, OPA16, OPB04, OPB05, OPF06, and OPN09. Polymerase chain reaction was performed in 10  $\mu\text{l}$  containing 10 ng of DNA, 1  $\mu\text{M}$  of primers, 0.25 mM of dNTPs (Thermo Scientific, Massachusetts, USA), 0.5 U of Dream Taq polymerase (Thermo Scientific) and 1x Dream Taq buffer. The cycle parameters were: 94 °C for 1 min, followed by 40 cycles of 30 s at 93 °C, 1 min at 42 °C, 90 s at 68 °C, and the final extension time of 5 min at 72 °C. Products were analyzed by electrophoresis in 1 % agarose gels (Prona, Hispanagar, Burgos, Spain) supplemented with 10  $\mu\text{l}$  of 5 % ethidium bromide solution per 100 ml of the gel.

##### *Miniature inverted repeat transposable elements transposon display*

DNA digestion, ligation to adaptors, pre-selective, and selective amplification were performed according to Grzebelus et al. (2007) with modifications. Approximately 200 ng of DNA was digested in 10  $\mu\text{l}$  with 5 U NdeI (5'-CA<sup>^</sup>TATG-3') (Thermo Scientific) for 3 h at 37 °C. After fragmentation, thermal inactivation of the enzyme was conducted at 65 °C for 20 min. A volume of 2.5  $\mu\text{l}$  of the completely digested DNA was ligated to splinkerette-like adaptors modified from Devon et al. (1995). The ligation mixture (10  $\mu\text{l}$ ) was incubated overnight at 4 °C with 1  $\mu\text{M}$  of adaptors, 0.625 U of T4 DNA ligase (Thermo Scientific) and 1x T4 DNA ligase buffer. Adaptors used for ligation were prepared as follows: 40  $\mu\text{M}$  SplAI (5'-CGA ATC GTA ACC GTT CGT ACG AGA ATG TCC TCT CCA

ACG AGC CAA GG-3'), 40  $\mu$ M SplA2 (5'-TAC CTT GGC TCG TTT TTT TTT GCA AAA A-3') and 1x adaptor annealing buffer (0.1 M Tris pH 7.5, 1 M NaCl, 0.01 M EDTA). The adaptor mixture was incubated for 10 min at 65 °C and slowly cooled down to 24 °C. Pre-selective amplification was set up using the adaptor-specific primer tdP1 (5'-CGA ATC GTA ACC GTT CGT ACG AGA A-3') and one of the MITE-specific primers (KRAKtd1: 5'-GCT GCT TTT HGA TTT ACC AAA CAR TTT-3', DcSto1D1M-5'-CCA CTT CAC CCA CTT TTC CT-3', or DchATtd1: 5'-TTT CAG AAG TTC GGT CTT CGG T-3'). Ligated samples were five-fold diluted and 1  $\mu$ l was used for pre-selective amplification, prepared in 10  $\mu$ l with 1  $\mu$ M of MITE-specific primer, 1  $\mu$ M of tdP1, 0.25 mM of dNTPs (Thermo Scientific), 0.5 U of Dream Taq polymerase (Thermo Scientific) and 1x Dream Taq buffer. The amplification profile was as follows: 94 °C for 1 min, 20 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 2 min, and 68 °C for 5 min. For selective amplification, nested primers were used [KRAKtd2: 5'-GAT TTA CCA AAC ART TTT TCA CCT GHT-3', DcSto1TD2M: 5'-TAA ACA ATT GGG TGG GAC GG-3', or DchATT2M: 5'-ACC GAA CCG AAT GCT CA-3' and TdMseI (NNN): 5'-TCC AAC GAG CCA AGG TAA-NNN-3'. For *Krak*, NNN stands for ACT; AGT; CAC; CTG; GTA; TGA; TGT, for *DcSto*, NN stands for AC; AG; GA, TdMseI (NN) for *DchAT*, NN stands for AC; AG; CA; CT; GA; GT; TC; TG]. Diluted pre-amplification (1:50) was used as a template for amplification set up in 10  $\mu$ l containing 2  $\mu$ l of diluted pre-amplified DNA, 1  $\mu$ M of nested MITE-specific primer, 0.5  $\mu$ M of TdMseI, 0.25 mM of dNTPs (Thermo Scientific), 0.5 U of Dream Taq polymerase (Thermo Scientific), and 1x Dream Taq buffer. Amplification was carried out starting using touch-down conditions in the first five cycles: 94 °C (1 min), 5 cycles of 94 °C (30 s), 60 °C (30 s) decreased by 1 °C per cycle, 68 °C (2 min) followed by 35 cycles of 94 °C (30 s), 55 °C (30 s), 68 °C (2 min), and 68 °C (5 min). Products were separated for 4 h on agarose gels supplemented with 10  $\mu$ l of 5 % ethidium bromide solution per 100 ml of the gel.

#### Assessment of response of R0 plants to *A. radicina*

Due to an extended plant regeneration period for 'Amsterdamska' and the line 9304B, only 'Dolanka'-derived regenerants were available for evaluation for resistance to black rot. For this purpose, greenhouse-grown R0 plants regenerated from FCF-treated and untreated cultures as well as field-grown control plants were applied to a laboratory petiole assay according to Grzebelus et al. (2003). The isolate KGHN3 of *A. radicina* was used in the experiments. Agar pieces overgrown by *A. radicina*

mycelium were placed on a moistened filter paper in 9 × 1.5 cm Petri dishes. Then, the basal part of the petiole detached from the youngest fully developed carrot leaf per plant was attached to the mycelium. Following suggestions of Baranski et al. (2007), after 8 days of incubation in the dark at 26 ± 2 °C the resistance was assessed by measuring length of the lesion along the petiole.

#### Analysis of pollen viability and seed production from R0 plants

After 4 months of growth in the greenhouse, the potted plants of all accessions were vernalized for 3 months at 10 ± 2 °C to induce flowering. After that period, the plants were transferred back to the greenhouse. Alexander's procedure (1969) based on differential staining of aborted (colored as light-green) and non-aborted grains (colored as magenta-red) was used to evaluate pollen viability. Subsequently, the flowers with receptive stigmas were self-pollinated by hand to produce R1 progeny. Seeds were harvested and counted about 2 months later.

#### Statistical analysis

The experiments were organized in a completely randomized design, with three replications, each treatment being represented by three Petri dishes. For protoplast viability and plating efficiency, counting was carried out in four or five microscopic fields on 100–200 cells per Petri dish. The mean values and standard errors were calculated. The overall effect of treatments was assessed using the analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test in Statistica 9.0 (StatSoft. Inc. 2009). Test for equivalence of two-proportions was used to compute the significance level for the differences between proportions.

## Results

#### Quality and development of carrot protoplasts in CCP medium not containing *A. radicina* FCF

In type A experiments, the level of protoplast viability just after isolation was satisfactory and very similar for all tested accessions ( $P = 0.966$ ), ranging from 72 ('Amsterdamska') to 75 % ('Dolanka', Table 1). In the next 2 days, no decrease in protoplast viability was observed ( $P = 0.932$ ). After cell wall reconstruction, cell division occurred regularly and on the 10th day of culture two-, four-, and multi-cell aggregates were visible under the microscope. At that time the examined accessions responded similarly to the culture conditions ( $P = 0.083$ )

reaching a plating efficiency of 50 % for ‘Amsterdamska’ to 80 % for the line 9304B (Table 1).

In type B experiments, protoplast viability was as high as in the type A experiments yielding from 72 (‘Dolanka’) to 76 % (‘Amsterdamska’ and the line ‘9304B’,  $P = 0.827$ ). Similar to the 10th day, on the 15th day of culture, accessions did not differ in plating efficiency ( $P = 0.581$ ), but the values varied from 68 (line ‘9304B’) to 76 % (‘Dolanka’, Table 1).

#### Effect of FCF on protoplast viability and development

##### *Exposure of protoplasts to FCF at the day of isolation*

Supplementation of CPP medium with the FCF on the first day of culture (type A experiments) reduced protoplast viability on average from 73 to 36 and 27 % after 24 and 48 h of culture, respectively ( $P < 0.001$ ). Cell viability strongly depended on the concentration of FCF in the culture medium ( $P < 0.001$ ). All FCF concentrations lowered the protoplast survival frequency on average from 55 % at 1 % FCF to 39 % at 50 % FCF (Fig. 1a). Such a significant impact of the FCF concentration on protoplast viability was confirmed both after 24 and 48 h of culture ( $P < 0.001$ , Table 2). After 2 days of culture, in the presence of higher concentrations of FCF in the medium (from 20 to 50 %), extremely low levels of viable cells were observed (from 8 to 1 %, respectively). The carrot accessions exhibited slightly different levels of protoplast viability ( $P = 0.003$ ) reaching from  $45.4 \pm 4.5$  % (‘Amsterdamska’) to  $52.9 \pm 4.1$  % (line ‘9304B’).

FCF very strongly affected cell divisions and aggregate formation ( $P < 0.001$ , Fig. 1b) reducing the plating efficiency in comparison to the control. The degree of that reduction was directly related to the concentration of FCF in the culture medium. After 10 days of culture, the plating efficiency decreased from  $67 \pm 6.1$  % in the control to  $24.5 \pm 11.5$  % at 1 % FCF, and to  $0.2 \pm 0.1$  % at 5 % FCF. At higher FCF concentrations in the medium, cell divisions were completely inhibited. Protoplasts isolated

from different accessions responded differently to the applied treatments ( $P < 0.001$ ). In 1 % FCF-treated cultures, a decrease of plating efficiency from 79 to 59 % and from 71 to 15 % was observed for the line ‘9304B’ and ‘Dolanka’, respectively (Table 2). ‘Amsterdamska’ was the most sensitive to FCF and formed aggregates only in the control treatment.

##### *Exposure of protoplasts to FCF on the 5th day of culture*

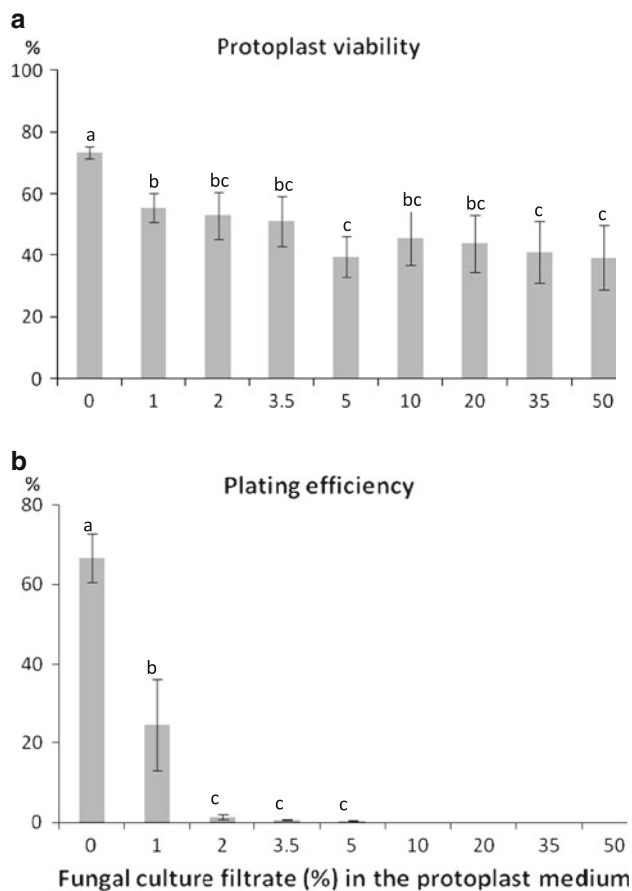
In type B experiments, on the 5th day of culture mainly two- and four-cell aggregates were observed. Multi-cell aggregates were visible only occasionally. At that stage, plating efficiency reached on average 33 % and varied from  $25.1 \pm 1.5$  % for ‘Amsterdamska’ to  $39.4 \pm 2.7$  % for the line ‘9304B’ ( $P < 0.001$ ). At that point, FCF was applied to the culture. Data analysis showed that further cell divisions highly depended on the FCF concentration in the culture medium ( $P < 0.001$ ). Ten days after FCF application in 15-day-old cultures treated only with lower concentrations of FCF (1–3.5 %) plating efficiency was at the same level as in the control (72 %) and varied from 71 to 58 % (Fig. 2). Higher concentrations of FCF inhibited cell divisions, decreasing plating efficiency up to five-fold in 50 % FCF-treated cultures. In 10–50 % FCF-treated cultures, plasmolysis of the cells was observed, explaining the lower number of cell aggregates in comparison to that observed in 5-day-old cultures. Similar to type A experiments, protoplasts isolated from different accessions responded differently to FCF treatments ( $P < 0.001$ ). A significant decrease of aggregate formation was observed in 3.5 and 10 % FCF-treated cultures between the line ‘9304B’ and cultivar ‘Dolanka’ (Table 3), while for ‘Amsterdamska’ in 10–50 % FCF-treated cultures, cells in all aggregates were plasmolysed and new aggregates were not formed.

#### Plant regeneration from FCF-treated protoplasts

In the course of 2 months of culture, steady growth of cell aggregates into microcalli, and the subsequent formation of

**Table 1** Protoplast viability and plating efficiency of donor accessions in the CPP medium without fungal culture filtrate

	Experiment type	Accession	Protoplast viability (% $\pm$ SE)			Plating efficiency <sup>a</sup> (% $\pm$ SE)
			Age of culture (h)			
			1	24	48	
CPP carrot petiole protoplast culture medium, <i>n.a.</i> not applicable	A	Dolanka	74.6 $\pm$ 3.7	77.8 $\pm$ 4.4	71.9 $\pm$ 8.1	70.9 $\pm$ 5.1
		Amsterdamska	71.7 $\pm$ 11.3	68.7 $\pm$ 9.1	64.5 $\pm$ 6.3	49.9 $\pm$ 8.9
		9304B	73.6 $\pm$ 6.8	76.8 $\pm$ 1.8	80.8 $\pm$ 2.4	79.2 $\pm$ 1.2
<sup>a</sup> For type A and B experiments plating efficiency scored after 10 and 15 days of culture, respectively	B	Dolanka	71.4 $\pm$ 1.9	n.a.	n.a.	76.4 $\pm$ 4.4
		Amsterdamska	75.5 $\pm$ 3.4	n.a.	n.a.	71.6 $\pm$ 5.6
		9304B	75.7 $\pm$ 8.5	n.a.	n.a.	67.8 $\pm$ 6.1



**Fig. 1** Effect of the fungal culture filtrate applied on the isolation day (type A exp.) on protoplast viability **a** and plating efficiency **b**, averaged over all accessions. Bars represent standard error. Means denoted with different letters were significantly different ( $P \leq 0.05$ )

proembryonic masses and regeneration into somatic embryos were observed. This growth pattern was characteristic for all accessions on FCF-free medium, as well as in 1 % FCF-treated cultures, and both time points of the selective agent application (Table 4). Somatic embryogenesis occurred in type A and B experiments for 2 % FCF-treated cultures of line ‘9304B’ and in type B experiments for 2 and 3.5 % FCF-treated cultures of ‘Dolanka’. In all other FCF treatments, the development of aggregates was not observed as a result of complete arrest of mitotic divisions following contraction and clustering of the cytoplasm and organelles.

In control cultures, germination of protoplast-derived somatic embryos was very high, and a precise estimation of the numbers was not possible. Thus, the efficiency of somatic embryogenesis was not recorded for FCF-treated nor for untreated cultures. Plants were regenerated from all cultures where PEM and somatic embryos had formed (Table 4). The derived population represented all accessions, but the number of plants from control cultures was intentionally limited. In total, 820 R0 plants were obtained

including 265 control plants and 555 plants from FCF-treated cultures. Within the latter group, regenerants from 1 % FCF-treated cultures were the most frequent, while only a few plants were regenerated from 2 to 3.5 % FCF-treated cultures. In most cases, derived plants appeared normal in growth and shape, however, some individuals with fennel-like leaves were observed.

#### Cytogenetic and molecular characterization of R0 plants

Cytometric analysis was performed on 457 plants regenerated after FCF treatment and on 139 control plants (Table 4). Nineteen percent of regenerants obtained under the stress of exposure to FCF and 5 % of control plants were tetraploids, the remaining regenerants being diploids. More tetraploid plants were produced both from cultures exposed to FCF immediately after protoplast isolation and from those exposed to FCF treatment after 5 days of culture ( $P < 0.05$ ). Chromosomes were counted for 109 plants, of which 83 represented plants regenerated in the presence of *A. radicina* FCF. The results of chromosome counting were consistent with the cytometric data, indicating that no aneuploids were produced in the course of protoplast cultures (data not shown).

RAPD-PCR profiles of the regenerated plants were almost uniform and identical to those obtained for the donor plants. Three to nine additional unique bands per one round of selection were identified in the profiles of some individuals, constituting 0.06–0.19 % of all observed bands, respectively. However, they occurred at a similar rate for plants regenerated from protoplasts treated with FCF and those of the control, suggesting that addition of the selective agent to the culture did not increase the frequency of genomic rearrangements detectable with the RAPD-PCR system. The low incidence of putative rearrangements identified in the regenerated plants could, therefore, be attributed solely to the protoplast culture. None of the investigated MITE families, i.e., *Krak*, *DcSto*, and *Dc-hAT*, were mobilized in the course of protoplast cultures. All transposon display assays produced completely uniform banding patterns, identical to those of the donor plants, regenerants from the control, and regenerants from the cultures treated with the selective agent.

#### Response of R0 plants to *Alternaria radicina*

Eight days after inoculation of detached leaf petioles with *A. radicina*, black lesions typical of black rot were visible. When selective pressure was applied on the first day of culture, both regenerants from FCF-treated and untreated protoplasts displayed lesion progress significantly slower in comparison to the field control. In the case of a later

**Table 2** Mean effect of the fungal culture filtrate (FCF) applied on the isolation day (type A exp.) on protoplast viability and plating efficiency

Concentration of FCF (%)	Protoplast viability (averaged over all accessions) (% ± SE)		
	Age of culture (h)		
	1	24	48
0	73.3 ± 3.6 a	74.4 ± 3.2 a	72.4 ± 4.0 a
1	73.3 ± 3.6 a	52.4 ± 5.2 ab	37.2 ± 9.0 b
2	73.3 ± 3.6 a	33.5 ± 6.2 bc	19.9 ± 3.1 bc
3.5	73.3 ± 3.6 a	28.4 ± 4.8 bc	17.8 ± 6.1 bcd
5	73.3 ± 3.6 a	25.5 ± 3.0 bc	15.7 ± 3.1 cd
10	73.3 ± 3.6 a	21.8 ± 2.7 bc	13.2 ± 3.5 cd
20	73.3 ± 3.6 a	20.0 ± 2.2 bc	8.3 ± 3.1 cd
35	73.3 ± 3.6 a	14.2 ± 0.3 c	2.7 ± 2.0 d
50	73.3 ± 3.6 a	8.8 ± 1.6 c	0.9 ± 0.5 d

	Plating efficiency (after 10 days of culture) (% ± SE)		
	Accession		
	Dolanka	Amsterdamska	9304B
0	70.9 ± 5.1 a	49.9 ± 8.9 a	79.2 ± 1.1 a
1	15.1 ± 11.2 b	0.0 ± 0.0 b	58.6 ± 2.4 b
2	1.0 ± 0.5 b	0.0 ± 0.0 b	3.2 ± 1.2 c
3.5	1.0 ± 0.4 b	0.0 ± 0.0 b	0.9 ± 0.2 c
5	0.7 ± 0.2 b	0.0 ± 0.0 b	0.0 ± 0.0 c
10	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
20	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
35	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
50	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c

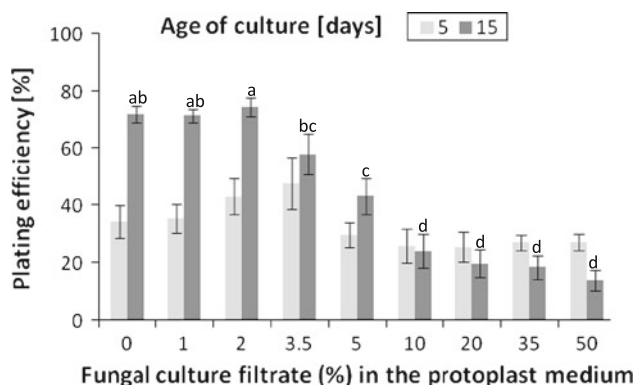
Means within columns followed by different letters were significantly different at  $P \leq 0.05$

application of selective pressure to the culture, only 3.5 % FCF-derived plants exhibited smaller lesions on the petioles than plants from the field control (Table 5). Generally, regenerants obtained from FCF-treated protoplast cultures did not differ significantly from those from untreated cultures with respect to their petiole assay score. However,

within the FCF-derived plants 49–65 % individuals developed lesions smaller than the mean values of the in vitro control.

#### Pollen viability and seed production from R0 plants

Populations of 558 plants regenerated from FCF-treated cultures and 200 control plants were vernalized. After 1–2 months of growth in the greenhouse, 75 and 91 %, respectively, started to flower. For the plants obtained from protoplasts treated with FCF immediately after isolation, significantly lower levels of flowering were observed in comparison to the control population ( $P < 0.001$ ). Pollen viability was the most variable trait, both for the regenerants from the FCF-treated cultures and those from the control (Table 6). In both populations, plants producing only sterile pollen grains were identified. In all cases, male sterility was accompanied by the presence of brown anthers. A higher frequency of male sterile plants (37 vs. 20 % of control plants) was recorded only for regenerants derived from protoplasts treated with FCF on the 5th day of culture ( $P = 0.01$ ). For individuals forming flowers with typical yellow anthers, the level of viable pollen grains



**Fig. 2** Effect of the fungal culture filtrate applied on the fifth day of protoplast culture (type B exp.) on plating efficiency, averaged over all accessions. Bars represent standard error. Means for plating efficiency evaluated after 15 days of culture denoted with different letters were significantly different ( $P \leq 0.05$ )



**Table 3** Effect of the fungal culture filtrate (FCF) applied on the fifth day of carrot protoplast culture (type B exp.) on plating efficiency scored at the fifteenth day of culture

Concentration of FCF (%)	Plating efficiency (% ± SE)		
	Accession		
	Dolanka	Amsterdamska	9304B
0	76.4 ± 4.4 a	71.6 ± 5.5 a	67.8 ± 6.1 a
1	70.9 ± 4.4 a	72.7 ± 5.7 a	70.5 ± 5.7 a
2	79.2 ± 4.3 a	77.8 ± 1.7 a	65.0 ± 7.7 a
3.5	64.6 ± 6.2 a	76.2 ± 1.6a	27.1 ± 2.5 b
5	44.5 ± 17.7 ab	47.2 ± 20.8 a	39.2 ± 4.7 ab
10	28.7 ± 4.7 b	0.0 ± 0.0 b	35.2 ± 5.0 b
20	23.0 ± 6.1 b	0.0 ± 0.0 b	29.2 ± 1.6 b
35	25.4 ± 3.3 b	0.0 ± 0.0 b	23.6 ± 0.3 b
50	19.4 ± 5.9 b	0.0 ± 0.0 b	17.3 ± 1.9 b

**Table 4** Plant regeneration from fungal culture filtrate-treated cultures and ploidy analysis of derived regenerants

Accession	Concentration of FCF (%)	FCF applied on the 1st day of culture				FCF applied on the 5th day of culture					
		Regeneration ability <sup>a</sup>	No. of plants		Ploidy		Regeneration ability	No. of plants		Ploidy	
			Regenerated	Analysed	2x	4x		Regenerated	Analysed	2x	4x
Dolanka	0	pem + se	32	31	30	1	pem + se	37	18	18	0
	1	pem + se	112	107	73	34	pem + se	19	19	17	2
	2	pl	–	–	–	–	pem + se	74	67	44	23
	3.5	pl	–	–	–	–	pem + se	10	10	10	0
Amsterdamska	0	pem + se	81	21	20	1	pem + se	14	10	10	0
	1	pem + se	241	166	151	15	pem + se	15	12	11	1
	2	pl	–	–	–	–	pl	–	–	–	–
	3.5	pl	–	–	–	–	pl	–	–	–	–
9304B	0	pem + se	68	41	39	2	pem + se	33	18	15	3
	1	pem + se	49	44	36	8	pem + se	27	25	23	2
	2	pem + se	3	3	1	2	pem + se	5	4	3	1
	3.5	pl	–	–	–	–	pl	–	–	–	–
Total	0	pem + se	181	93	89	4	pem + se	84	46	43	3
	1–3.5	pem + se, pl	405	320	261	59	pem + se, pl	150	137	108	29

FCF fungal culture filtrate

<sup>a</sup> pem + se: proembryonic mass and somatic embryos formed as a result of development of cell aggregates derived in the first 10–15 days of culture, pl: plasmolysis of cells in aggregates, no further development observed

reached on average  $59.9 \pm 1.1$  %. The differences in pollen viability among regenerants derived from different accessions were noted ( $P < 0.001$ ). The highest proportion of viable pollen grains, reaching  $73.1 \pm 1.2$  %, was noted for ‘Amsterdamska’ regenerants while the lowest ( $45.5 \pm 1.7$  %) was observed for ‘Dolanka’ regenerants. That trait did not depend either on the application time of FCF to the culture medium or the concentration of FCF in the culture medium. However, pollen viability varied over a broad range from very low (6–7 %) to very high (91–98 %) both for regenerants derived from FCF-treated and untreated cultures (Table 6).

Seeds were produced from 351 individuals including 251 regenerants from FCF-treated cultures and 100 control

plants (Table 6). The yield of seed varied from very low (0.1 g) to high (5.3 g per plant) reaching on average  $0.8 \pm 0.1$  g and  $0.9 \pm 0.3$  g for regenerants from the FCF-treated cultures and the control plants, respectively.

## Discussion

The present study was the first attempt to assess the potential of using *A. radicina* FCF for in vitro selection against black rot in carrot protoplast cultures. Methods that utilize in vitro selection to induce tolerance to biotic stresses were very rarely used for carrot, which has been recognized as a model species for in vitro culture systems.

**Table 5** Lesion length on detached petioles of ‘Dolanka’-derived R0 plants after inoculation with *Alternaria radicina*

Origin of R0 plants		No. of tested plants	Lesion length		% plants below mean of	
Application of FCF to culture	Concentration of FCF (%)		Range	Average	Field control	In vitro control
On 1st day	Field control	6	22.4–72.0	44.4 ± 8.9 a	–	–
	0	18	16.2–31.5	25.7 ± 0.9 b	100	38.9
	1	26	13.2–33.9	22.6 ± 1.1 b	100	65.4
	Field control	6	22.4–72.0	44.4 ± 8.9 a	–	–
On 5th day	0	9	22.3–44.8	29.8 ± 2.2 ab	88.9	55.6
	1	11	19.7–35.8	31.4 ± 4.3 ab	90.9	54.5
	2	65	19.3–45.5	31.1 ± 0.9 ab	96.9	49.2
	3.5	7	20.9–35.3	27.9 ± 2.2 b	100	57.1

A single petiole was collected from each plant. Plants were treated as replicates

Means within columns followed by *different letters* were significantly different at  $P \leq 0.05$

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One of the few reports of the development of carrot calli in the presence of a crude extract was from *Erwinia carotovora* cultures (Baranski et al. 1997). Many such attempts have been reported for major crops including maize, sugarcane, cotton, potato, and rice (reviewed in Rai et al. 2011). To enhance disease tolerance in the presence of selective agents, callus tissue was often used as an explant (as reviewed in Švábová and Lebeda 2005). Protoplasts may be an attractive alternative allowing the use of much larger cell populations at a physiologically uniform stage, as compared to other types of explants, thus, offering the opportunity to develop protocols for efficient selection both in controlled conditions and using less space. Protoplasts or small cell aggregates are usually much more sensitive to pathotoxins than other types of plant material, being very suitable for use in bioassays (Gentile et al. 1992). Another advantage of using protoplasts for in vitro selection, especially after embedding in a gel matrix to prevent cell agglutination, is the adequate exposure of all cells to the selective agent. In such conditions, diffusion of compounds from the medium to the protoplasts is increased (Dovzhenko et al. 1998), and cells escaping from the selection agent are minimized. In contrast, clumps of calli are represented by heterogenic and multi-cell layers where susceptible cells may survive selective pressure by escaping full exposure to the selective agent or by proximal protection of resistant cell lines (Remotti et al. 1997; Mahlanza et al. 2013). However, the number of species with protoplasts capable of reconstituting cell walls and undergoing mitotic divisions resulting in callus formation and normal plant regeneration is still limited. Often, only carefully selected genotypes within the species are capable of producing protoplast-derived plants (Davey et al. 2005). Such recalcitrance of many species, sometimes combined with the complexity of protoplast isolation and culture, is probably the main reason that protoplast cultures are rarely

used for in vitro selection. From that point of view, the carrot should be considered as especially suitable, as fast and highly efficient regeneration protocols for protoplasts isolated from different tissue sources have been developed (Dudits et al. 1977; Dirks et al. 1996; Grzebelus et al. 2012). Exposure of protoplasts to biotic stresses was reported for other species including alfalfa, grape vine, hop, lemon, orange tree, rape, tobacco, and tomato (reviewed in Švábová and Lebeda 2005).

Pathogen culture filtrates and phytotoxins are most commonly used for in vitro selection and regeneration of disease-tolerant plants in many crops (reviewed by Švábová and Lebeda 2005; Rai et al. 2011). Culture filtrates are known to contain a number of phytotoxic compounds, which may play an important role in pathogenicity. Explants from susceptible and tolerant materials cultured in vitro vary in response to these toxic metabolites, thus, making such phytotoxins effective selective agents for in vitro selection strategies towards disease tolerance (Binarova et al. 1990; Remotti et al. 1997; Kumar et al. 2008; Flores et al. 2012). Radicinin, radicinol, and *epi*-radicinol are the main phytotoxins involved in the pathogenicity of *A. radicina* against carrots (Solfrizzo et al. 2004). These were identified in naturally infected carrot roots, but also produced in vitro on carrot discs or in liquid potato-dextrose medium after inoculation with *A. radicina* mycelium (Shakir 1999; Solfrizzo et al. 2004, 2005). Higher levels of these phytotoxins were observed in carrot cultivars more susceptible to *A. radicina* (Solfrizzo et al. 2004). It was reported that the culture filtrate of *A. radicina* caused carrot wilting, leaf burning, necrosis on seedlings, and inhibited root elongation of germinated seeds, thus, confirming its toxicity on the intact plant material (Shakir 1999; Tylkowska et al. 2003). However, to our knowledge it was never previously evaluated at the cellular level. FCF toxicity can be verified with a range of staining assays that

**Table 6** Pollen viability and seed production of R0 plants derived from fungal culture filtrate-treated cultures

Accession	Application of FCF to culture	Concentration of FCF (%)	No. of plants			Pollen viability		Seed production after self-pollination (g)		
			Evaluated	Sterile	Fertile	Range	Mean $\pm$ se	No. of plants	Range	Mean $\pm$ se
Dolanka	On 1st day	0	48	19	29	6.7–93.0	42.0 $\pm$ 4.3	26	0.1–2.3	0.8 $\pm$ 0.1
		1	92	40	52	5.7–82.1	38.6 $\pm$ 3.1	39	0.1–1.9	0.6 $\pm$ 0.1
	On 5th day	0	36	10	26	25.3–74.8	50.9 $\pm$ 3.6	13	0.4–2.1	0.9 $\pm$ 0.2
		1	12	2	10	6.6–75.8	41.8 $\pm$ 6.4	12	0.3–1.8	0.7 $\pm$ 0.1
		2	63	31	32	29.1–80.7	57.5 $\pm$ 2.1	24	0.3–2.0	0.8 $\pm$ 0.1
Amsterdamska	On 1st day	0	30	4	26	29.4–91.4	68.3 $\pm$ 3.1	23	0.4–5.3	2.1 $\pm$ 0.3
		1	128	6	122	33.8–97.5	73.3 $\pm$ 1.4	120	0.3–4.4	1.5 $\pm$ 0.1
	On 5th day	0	12	3	9	53.3–92.6	74.4 $\pm$ 7.1	7	0.1–0.6	0.2 $\pm$ 0.1
		1	12	3	9	68.6–92.7	83.3 $\pm$ 2.9	9	0.1–0.5	0.1 $\pm$ 0.05
		2	12	3	9	68.6–92.7	83.3 $\pm$ 2.9	9	0.1–0.5	0.1 $\pm$ 0.05
9304B	On 1st day	0	31	8	23	17.4–82.7	59.1 $\pm$ 3.3	15	0.1–1.0	0.4 $\pm$ 0.1
		1	30	8	22	7.1–87.5	54.3 $\pm$ 4.3	20	0.1–1.3	0.5 $\pm$ 0.1
		2	3	1	2	55.0–72.3	63.6 $\pm$ 8.7	1	0.5	0.5
	On 5th day	0	24	2	22	23.8–84.0	63.8 $\pm$ 4.0	16	0.1–3.1	1.1 $\pm$ 0.2
		1	26	3	23	25.8–92.6	56.1 $\pm$ 4.0	23	0.1–4.2	1.2 $\pm$ 0.3
		2	2	0	2	70.6–79.6	75.1 $\pm$ 4.5	1	1.5	1.5
Total	On 1st day	0	109	31	78	6.7–91.4	56.5 $\pm$ 7.7	64	0.1–5.3	1.1 $\pm$ 0.5
		1–2	253	55	198	5.7–97.5	57.5 $\pm$ 7.4	180	0.1–4.4	0.8 $\pm$ 0.2
	On 5th day	0	72	15	57	23.8–92.6	63.0 $\pm$ 6.8	36	0.1–3.1	0.7 $\pm$ 0.3
		1–3.5	124	46	78	6.6–92.7	61.9 $\pm$ 6.1	71	0.5–4.2	0.8 $\pm$ 0.2
Total	Total	0	181	46	135	6.7–93.0	59.8 $\pm$ 4.8	100	0.1–5.3	0.9 $\pm$ 0.3
		1–3.5	377	101	276	5.7–97.5	60.1 $\pm$ 4.5	251	0.1–4.4	0.8 $\pm$ 0.1

FCF fungal culture filtrate

detect living cells, such as those based on the use of FDA (Widholm 1972). Survival of cells in the presence of the selective agent may be the major resistance criterion. Exposure of cells to different concentrations of toxins present in the FCF results in death, reduced growth or viability, so that surviving cells can be identified (Griffith and Anderson 1987). In our experiments, the percentage of surviving protoplasts on media containing various concentrations of FCF decreased with an increase of the selective agent concentration, reaching 1 at 50 % FCF after 2 days of culture. A similar tendency in response to different doses of the fungal filtrate after viability staining was documented in protoplast cultures of different *Brassica* accessions (Sjödin and Glimelius 1989), lemon and orange (Gentile et al. 1992), and *Coffea arabica* (Nyange et al. 1997).

Another method used for the quantitative determination of pathogen filtrate toxicity is the evaluation of cell growth expressed by plating efficiency (Breiman and Galun 1981). In the present study, we presented a significant inhibition of mitotic divisions in carrot protoplast cultures following treatment with varying doses of FCF, which may be

correlated with susceptibility to the pathogen. The decrease in plating efficiency was observed for both times of selective agent application. However, a much stronger negative response of cells was observed when the FCF was added on the first day of culture. This can be explained by the fact that cells with reconstructed cell walls forming small protoplast-derived aggregates present in 5-day-old cultures exhibited slightly higher tolerance to the fungal toxin in comparison to the cell wall-free 1-day-old protoplasts. Similar observations were reported in *Brassica* protoplast cultures (Sjödin and Glimelius 1989). This may suggest that the presence of the cell wall constitutes active or passive barriers to the phytotoxic agents (Breiman and Galun 1981). However, higher concentrations of FCF completely inhibited cell growth in both types of experiments. Similar to the findings of Nyange et al. (1997) we observed protoplast death, i.e., contraction and clustering of the cytoplasm and organelles, suggesting that this damage was caused by toxins present in the FCF. Analysis of plating efficiency and ability to regenerate plants from FCF-treated cultures showed the differential response of carrot accessions to the selective agent, likely correlated

with different susceptibility levels to *A. radicina*. The maximum selection pressure that allowed us to recover tolerant cell lines was low and stayed in the range of 1.0–3.5 % FCF depending on the protoplast donor accession and the time of FCF application. Similar low concentrations of *A. alternata* filtrate were appropriate for selection of tobacco protoplasts (Ishida and Kumashiro 1988).

Two in vitro selection variants were proposed: (1) stepwise long-term treatment where cultures were exposed to stress with a gradual increase in concentrations of the selective agent, and (2) shock treatment where cultures were directly subjected to a high concentration of the selective agent (Rai et al. 2011). The first variant helped to avoid escapes and decreased the level of mosaicism through regular elimination of sensitive cells. This was preferred for explants consisting of many cell layers such as plant organs, callus tissue, or suspension cultures. Successful selection with stepwise treatment was reported in the callus tissue of carnation (Thakur et al. 2002), chrysanthemum (Kumar et al. 2008) and citrus (Savita et al. 2011). However, prolonged exposure to toxins can result in reduced regeneration ability and occurrence of undesired mutations. On the contrary, shorter selection cycles may yield more regenerants and healthier plants (Remotti et al. 1997; Sharma et al. 2010). Therefore, in the present study we applied a one-step procedure with a short period of selection pressure (10 days), and single cells or small aggregates were subjected to selection in the liquid medium after embedding protoplasts in the alginate matrix. These conditions ensured adequate infiltration of the FCF to each cell present in the culture. Shorter selection cycles lasting from 2 to 4 days were employed with tobacco protoplasts (Ishida and Kumashiro 1988), while longer (21 days) cycles were used with cell aggregate cultures of *Brassica* accessions (Sjödén and Glimelius 1989).

The in vitro selection techniques are based on the induction of genetic variability in the explants cultured under stress pressure. Somaclonal variation generated in such conditions may be identified on morphological, physiological, molecular, or cytological levels (reviewed by Bairu et al. 2011). In many cases, plants regenerated under biotic stress did not show phenotypic variation and remained morphologically identical to the donor plants (Thakur et al. 2002; Ganesan and Jayabalan 2006; Kumar et al. 2008; Savita et al. 2011), or the frequency of changed plants was low, i.e., 2 % in FCF-challenged embryogenic callus cultures of sugarcane (Mahlanza et al. 2013). Our results are consistent with these observations. Occasional variation in leaf morphology and abnormal development of storage roots in R0 plants (data not shown) are typical negative effects caused by in vitro culture conditions and occur commonly in carrots (Baranski 2008). Here, we also

noted a high incidence of male-sterile plants among ‘Dolanka’ regenerants. Although it has never been reported, we frequently observed the phenomenon in regenerants obtained from ‘Dolanka’ donors, and the cultivar itself is characterized by a high share of partially or fully male-sterile plants. Any stress in the course of in vitro cultures can induce somaclonal variation. Ploidy level or chromosome number variation is a direct and strong evidence of changes in the genetic composition of an organism (Bairu et al. 2011). It has been hypothesized that toxic filtrates used for selection may affect the ploidy of regenerated plants, and that the level of resistance can be simply amplified in polyploid somaclones (Latunde Dada and Lucas 1983; Hartman et al. 1984). Our results, in part, agree with these speculations as more tetraploid plants were obtained from the FCF-treated cultures. Most tetraploids included in the petiole assay exhibited smaller lesions after inoculation with *A. radicina* in comparison to the mean for the control plants (data not shown). This may indicate a lower susceptibility of tetraploids to black rot. Other types of chromosomal mutations, e.g. chromosome breakage and chromosome loss have been documented in plants derived from in vitro cultures (Larkin and Scowcroft 1981; Van den Bulk 1991; Bairu et al. 2011). We determined the chromosome number to identify aneuploids within R0 plants. Our results were consistent with previous observations (Dudits et al. 1976; Palmer and Widholm 1975; Grzebelus et al. 2012), suggesting that the chromosome number was stable in carrot suspensions or protoplast cultures, except for a possible full genome duplication. In addition, the level of rearrangements revealed by RAPD-PCR was very low and FCF treatment did not increase its frequency. In vitro cultures and application of the selective agent did not drive any mobilization events of the three MITE families under investigation, likely because of the short time of the culture. It was reported that TE activation in carrot callus cultures resulting in somaclonal variation required a long-term exposure to culture conditions (Ozeki et al. 1997).

An important aspect of crop improvement via in vitro selection strategy is that traits selected at the cellular level must be expressed in the regenerated plants. Since tolerance showed by single cells may vary from that of the whole plant (Van den Bulk 1991), tests to screen the putative tolerant lines and regenerants to the pathogen in planta are necessary. In the carrot, the best reliable evaluation concerning the level of tolerance to black rot of R0 regenerants should be performed after storage. However, this was not possible, as plants regenerated from in vitro cultures produce abnormal, deformed and forked roots, not suitable for storage. Adaptation of in vitro cultured cells to the selective agent can be epigenetic (Rai et al. 2011), and analysis of R0 plants is not sufficient. Thus, to assess the

level of tolerance to *A. radicina*, a preliminary laboratory assay on petioles was carried out including only a population of regenerants from one donor accession. Some individuals with higher tolerance to black rot were observed, both in the population derived from FCF-treated and untreated cultures. This observation may suggest that a desirable characteristic was an effect of in vitro conditions in general. It is known that results of laboratory tests may not correlate with the plant response in the field (Szczonek et al. 2006), thus, a detailed analysis of resistance to *A. radicina* should be performed on R1 progenies both in the course of vegetation and in storage.

In conclusion, we applied a large-scale selection based on in vitro cultures to develop carrot materials with improved tolerance to black rot. To our knowledge, this is the first report describing the development of plants from *D. carota* protoplasts maintained under the selective pressure of toxins from *A. radicina* FCF. We proved its negative effect on protoplast viability and plating efficiency suggesting its usefulness as an effective selective agent. The analysis of protoplast viability presented here may also be used as a quick assay to determine the phytotoxicity of FCF prepared from different isolates of *A. radicina*. The basic advantage of the established protocol is its simplicity and low cost compared with disease-resistant transgenic plants, which still are not accepted by the consumers (Costa-Font et al. 2008). Application of a long-term selection to the culture might possibly increase the level of tolerance to black rot of treated cells and protoplast-derived plants.

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