Journal für Kulturpflanzen, 69 (9). S. 277–290, 2017, ISSN 1867-0911, DOI: 10.1399/JfK.2017.09.01 Verlag Eugen Ulmer KG, Stuttgart

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Characterisation of *Alternaria radicina* isolates and assessment of resistance in carrot (*Daucus carota* L.)

Charakterisierung von *Alternaria radicina* Isolaten und Resistenzbewertung von Möhren (*Daucus carota* L.)

Abstract

Alternaria radicina is a fungal pathogen that causes the black rot disease of carrot. Four *Alternaria* spp. isolates associated with black rot symptoms collected from carrot (*Daucus carota*) and parsley (*Petroselinum crispum*) were characterised and differentiated in relation to the closely related *Alternaria* species *A. radicina*, *A. carotiincultae* and *A. petroselini* belonging to the *Alternaria* sect. *Radicina*. The *Alternaria* isolates were differentiated for their growth rates and colony margins. A typical feature of *A. radicina* isolates is the production of high amounts of yellow pigments on acidified potato dextrose agar (APDA). Furthermore, sporulation intensity and conidia morphology were determined to classify the potential new *A. radicina* isolates. The pathogenicity of the *Alternaria* isolates was determined by bioassays with detached leaves of four Apiaceae species. Different carrot cultivars and one wild relative were used to estimate aggressiveness of the isolates. The disease symptoms were quantified in bioassays using a digital image analysis system (LemnaTec). Additionally, a DAS-ELISA with polyclonal antibodies was used to detect the development of fungal pathogens. As result of the morphological and molecular characteristics as well as the pathogenicity assay, three isolates were verified as *A. radicina* and one as *A. petroselini*. Finally, two isolates with different aggressiveness were used to screen a set of 14 carrot genotypes for resistance to *A. radicina*.

Key words: *Alternaria radicina*, *A. carotiincultae*, *A. petroselini*, bioassay, carrot, conidia, pathogenicity, resistance, ELISA, PCR

Zusammenfassung

Alternaria radicina ist der Erreger der weltweit auftretenden Schwarzfäule an der Möhrenwurzel. Von Möhren- (*Daucus carota*) bzw. Petersilienblättern (*Petroselinum crispum*) wurden vier *Alternaria* spp. Isolate gewonnen und in Relation zu den *Alternaria* Arten *A. radicina*, *A. carotiincultae* und *A. petroselini* der *Alternaria* Sektion *Radicina*, charakterisiert und differenziert. Untersucht wurden Koloniewachstumskriterien, morphologische Merkmale und die Intensität der Sporulation. Typisch für *A. radicina* Isolate ist die Bildung gelber Pigmente auf angesäuertem Kartoffeldextroseagar (APDA). Die Pathogenität der *Alternaria* Isolate wurde durch Bioassays von Blattsegmenten eines Differenzial-Wirtspflanzensortiments bestehend aus vier Arten aus der Familie Apiaceae untersucht. Verschiedene Möhrensorten und eine Wildform wurden genutzt, um die Aggressivität der Pathogene zu charakterisieren. Die Krankheitssymptome wurden durch digitale Bildanalyse (Digital image analysis system, DIAS, LemnaTec, Deutschland) quantifiziert. Weiterhin konnte mit Hilfe des DAS-ELISA die Erregervermehrung im gesamten pflanzlichen Gewebe erfasst werden. Drei Isolate konnten als *A. radicina* identifiziert werden und

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Accepted
22 May 2017

eins als *A. petroselini*. Zwei *A. radicina* Isolate unterschiedlicher Aggressivität wurden genutzt, um die Resistenz eines 14 Genotypen umfassenden Möhrensortiments zu bewerten.

Stichwörter: *Alternaria radicina*, *A. carotiincultae*, *A. petroselini*, Bioassays, Möhre, Konidien, Pathogenität, Resistenz, ELISA, PCR

1 Introduction

Carrot (*Daucus carota* L. ssp. *sativus* Hoffm.) is one of the most popular vegetables. Nearly 37 million tons are produced annually from over 1.2 million ha worldwide. China, Russia and the United States are the major producers. In Germany carrots are cultivated on approximately 10.000 ha. The organic carrot production in Germany amounts to about 17% (AMI, 2016).

Stable yields and a high quality are important requirements for an economic production. However, diseases caused by fungal pathogens, among them different *Alternaria* species, attack carrots and can reduce quality and yield (Farrar et al., 2004). Black rot disease of carrot was first described in northern European countries in 1888 (Rostrup, 1888) and is now a major problem worldwide. *Alternaria radicina* Meier, Drechsler and Eddy is a seedborne pathogen and attacks all parts of carrot as well as leaves of parsley (Farrar et al., 2004). Significant yield losses and quality reduction occur during the field growing season and cold storage (Farrar et al., 2004). Chemical crop protection and a hot water treatment of seeds are practiced techniques but are sometimes ineffective (Rau et al., 2006). Improvement of the resistance to *A. radicina* by plant breeding is the most effective way to control this disease. All active resistance breeding approaches are based on the application of the pathogen on plants or plant tissue (bioassays). Elementary requirement is the relevance and pathogenicity of the applicated pathogen isolates. Therefore a recurrent observation of the pathogens in the field production and the isolation of current actual strains for breeding approaches are necessary. Furthermore, the isolates must be described taxonomically exactly, should be genetically uniform, which can be realised by using monoconidial lines, and must show sufficient aggressiveness (Miedaner, 2011). For a potential assessment of plant resistance it turned out that it is indispensable to characterise the obtained isolates not only regarding the cultural, morphological or molecular characters but also concerning the pathogenicity and aggressiveness.

The genus *Alternaria* is assigned to the *Fungi Imperfecti* and related to the family of Dematiaceae (Thomma, 2003). The life cycle of these species is saprophytic but several species are also pathogenic for a specific host range (Thomma, 2003).

A. radicina is closely related to *A. petroselini* (Neerg.) Simmons and *A. carotiincultae* Simmons. For this reason these and further species are summarised to the *Alternaria* sect. *Radicina* (Woudenberg et al., 2013). Conidia of *A. radicina* are short ellipsoid or ovoid. Conidiophores are simple or branched and generate solitary apical conidia or rarely clumps of single conidia (Simmons, 2007). Characteristic for *A. radicina* is the high level of yellow pigments on acidified potato dextrose agar (APDA) as a result of the toxin radicinin production (Pryor and Gilbertson, 2002). *A. radicina* is also able to produce the toxic metabolites radicinol and *epi*-radicinol in small quantities (Solfrizzo et al., 2004; Tylkowska et al., 2008). Radicinin generated by *A. radicina* is pertinent to the pathogenicity of *A. radicina* on carrot and could be detected on naturally infested carrots (Solfrizzo et al., 2004).

Another *Alternaria* pathogen described for carrot is *A. carotiincultae* (Simmons, 2007). Conidia of this fungus are either long ovoid or ellipsoid. The conidiophores have short secondary side branches which generate small conidia. Solitary conidia are the usual ones for that species. However, chain formation involving two or three conidia can be found (Simmons, 2007). *A. petroselini* is pathogenic on parsley (*Petroselinum crispum* L.) and the conidia exhibit commonly an ovoid-subsphaeroid character. Conidiophores have lateral branches and produce solitary conidia or clumps of them (Simmons, 2007). *A. petroselini* is also able to produce yellow pigments at lower level (Pryor and Gilbertson, 2002).

In this study cultural, morphological, genotypic and pathogenic differences between four putative *A. radicina* isolates were examined in relation to three reference isolates of the *Alternaria* sect. *Radicina* – *A. radicina*, *A. carotiincultae* and *A. petroselini.* For the identification of the new *Alternaria* spp. isolates, strategies and criteria published by Pryor and Gilbertson (2001, 2002), Simmons (2007), Park et al. (2008), Bulajić et al. (2009) and Trivedi and Hampton (2010) were used. Additionally, a digital image analysis assisted associated symptom classification (DIAS) and a DAS-ELISA established in the Julius Kühn-Institut, Federal Research Centre for Cultivated Plants (JKI) were first applied. Identified *A. radicina* isolates of different aggressiveness classes were verified for application within a laboratory resistance test.

2 Materials and Methods

2.1 Fungal pathogens and plant material

Four *Alternaria* spp. isolates associated with black rot symptoms were collected from carrot and parsley in field. Three reference isolates for *A. radicina*, *A. carotiincultae* and *A. petroselini* (Table 1) from the fungal collection of the Centraalbureau voor Schimmelcultures (CBS, The Netherlands) as well as from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) were used as standards for the discrimination of the new isolates. Additionally *A. alternata, A. dauci, A. brassicae* and *A. brassicicola* from the JKI collection were used for analysis of the genetic distance (Table 1).

The host range of the isolates was determined by using an assortment of four *Apiaceae* species. Different carrot

Table 1. Origin and isolation of the *Alternaria* spp. isolates

Pathogen	Acronym	Origin		Isolation	
		Country	Collection	Host plant	Tissue
Alternaria spp.	Ar01	Germany	JKI[1]	Parsley	Leaf
Alternaria spp.	Ar04	Germany	JKI	Carrot	Leaf
Alternaria spp.	Ar05	Germany	JKI	Carrot	Leaf
Alternaria spp.	Ar06	Germany	JKI	Parsley	Leaf
A. radicina Meier, Drechsler & Eddy	ArRef	Germany	DSMZ[2]	Carrot	Leaf
A. carotiincultae Simmons	AcRef	USA	CBS[3]	Carrot	Leaf
A. petroselini (Neerg.) Simmons	ApRef	USA	CBS	Parsley	Seed
A. alternata (Fries) Keissler	Aa	Germany	JKI	Carrot	Leaf
A. dauci (Kühn) Groves & Skolko	Ad	Germany	JKI	Carrot	Leaf
A. brassicae (Berk.) Sacc.	Abae	Germany	JKI	Cabbage	Leaf
A. brassicicola (Schwein.) Wiltshire	Abra	Germany	JKI	Radish	Leaf

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cultivars and a wild relative were applied to estimate the isolate aggressiveness and to assess resistance to *A. radicina* (Table 2).

Ten plants of each carrot cultivar were cultivated in 17 cm plastic pots in a standard soil mixture (sand/humus, v/v 3/1) under controlled greenhouse conditions at a

Table 2. Plant material used in this study

Code	Species	Botanical name	Cultivar (cv.) or breeding line (BL)	Source[1]	Application
226	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	BL CR2–6032	Satimex	R
227	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Long Red	WGRU/10246	R
228	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Kokubu Senko Oonaga	MKS	R
229	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Amsterdam 3	Commercial	R
230	Carrot	*Daucus carota* ssp. *commutatus L.*	Wild relative	JKI	A + R
231	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Himuro Fuyugosi Gosun 2	WGRU/11718	A + R
232	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Rotin	Sperling	P + A + R
233	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Texto	Vilmorin	R
234	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Nevis	Bejo	R
235	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Senta	DAU/437	A + R
236	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Presto	Vilmorin	R
237	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	cv. Sapporo Futo	MKS	R
238	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	BL JKI-4	JKI	R
239	Carrot	*Daucus carota* ssp. *sativus* Hoffm.	BL JKI-5	JKI	R
Pa1	Parsley	*Petroselinum crispum* (Mill.) Nym.	BL VP06/33/2	JKI	P
Fe1	Fennel	*Foeniculum vulgare* (L.) Mill.	BL St.NLC	JKI	P
Ce1	Celery	*Apium graveolens var. dulce* L.	BL AP-W 1/10	JKI	P

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temperature range from 20°C to 25°C N/D, a relative humidity of 60–70% and a 16 h photoperiod before application within the resistance tests.

2.2 Cultural and morphological characterisation

To get comparable fungal cultures for the characterisation, monoconidial lines of the isolates were established. For a first discrimination all isolates were propagated in Petri dishes (Ø = 9 cm) on vegetable juice agar (V8-A) (Simmons, 1992), synthetic nutrient deficient agar (SNA) (Nirenberg, 1976) and acidified potato dextrose agar (APDA) (Pryor et al., 1997) for 8 up to 21 days incubation at 22°C in darkness. The experiment was carried out with 6 replications for each isolate and each medium. Colour, structure and margins of the colonies, conidiophores and apical conidia were differentiated after eight days of isolate cultivation. Conidiophores were observed with the binocular "Stemi SV6" (Carl Zeiss AG, Germany) for two characteristics: i) branched or single and ii) flexible or erected. The other colony characteristics were determined by visual examination of the cultivated plates. The growth of the colonies was measured after an eight-day culture period. Ten-day-old cultures on V8-A were used to determine conidia generation intensity. Agar plugs of 0.78 cm^2 were removed from the margin of five petri dishes. The whole culture was carefully scraped from these plugs with a knife and transferred to a reaction tube (Greiner Bio-one GmbH, Germany) with 1 ml aqua dest. The conidia concentration was determined three times using a counting cell chamber (Marienfeld GmbH & Co. KG, Germany, deep = 0.1 mm). After 10-day growth on V8-A, length and width of the conidia were determined using the light microscope "Axio Imager A1" (Carl Zeiss AG, Germany) applying the programme "AxioVision MTB2004" (Carl Zeiss AG, Germany). Conidia were subdivided into four categories with two, three, four or five transepta. About 50 conidia from every transepta category of each isolate were measured. The production of yellow pigments as a result of production of the toxin radicinin, crystals and microsclerotia was determined by visual examination of Petri dishes after 21 days.

2.3 Pathogenicity test

The isolates were cultivated in Petri dishes (Ø = 9 cm) on V8-A for ten days at 22°C in darkness. Subsequently the conidia were washed down from agar with aqua dest. and the concentration was adjusted to 5×10^5 conidia/ml. Each ten leaf segments from 10-week-old carrot, parsley, fennel and celery (Table 2) were inoculated with four drops of 4 µl from the suspension of each isolate. Always two leaf segments of the same plant were incubated in a Petri dish (Ø = 14 cm) on wetted filter paper at 22°C for seven days in the dark. The disease symptoms on leaf segments were analysed 7 dpi using the Digital image analysis (DIAS) and the Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

2.4 Aggressiveness test

An aggressiveness test serves for the classification of the isolates into high and less aggressive classes. The aggressiveness of the isolates was determined with carrot petioles. Four-centimetre pieces were cut from the petioles of 10-week-old carrots. For each isolate a conidia suspension was prepared by flooding the V8-A Petri dishes containing seven-day-old cultures with sterile aqua dest. The conidia concentration was adjusted to 5 x 10^5 conidia/ml. Sixteen petioles collected from four cultivars each of four plants were tested per isolate (Table 2). Each petiole was inoculated with one 4 µl drop of the adjusted conidia suspension. Always four petioles incubated in a Petri dish (Ø = 14 cm) on wet filter paper at 22°C in darkness. The disease symptoms caused by the *Alternaria* spp. were measured 7 days past inoculation (dpi) using the DIAS likewise as described in the pathogenicity test.

2.5 Assessment of resistance

Based on the results of the aggressiveness test, a high and a less aggressive isolate were selected to screen 14 carrot genotypes (Table 2) for their resistance behaviour to *A. radicina*. Detached leaf segments of each genotype were inoculated with four 4 µl drops of a conidia suspension with the concentration of 5 x 10^5 conidia/ml. For each genoytype and isolate eight leaf segments from four plants were tested. Leaf segments were analysed 7 dpi using the DIAS and DAS-ELISA.

2.6 Digital image analysis (DIAS)

The DIAS is an objective method for recording and analysing disease symptoms on plants or on parts of plants. Disease symptoms on leaf segments and petioles were discriminated by the Scanalyzer (LemnaTec, Germany) using the system software SAW Bonit which has to be calibrated to a colour based discrimination of diseased and healthy plant tissue. As result the respective colour grades were recorded (pixel) and transferred to a data base for the statistical analysis.

2.7 Double antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA)

Aditionally to the DIAS in the pathogenicity test and the assessment of resistance a DAS-ELISA was firstly established to quantify the *Alternaria* growth on carrot tissue. The two leaf segments of each Petri dish were crushed using a roller press (MEKU Pollähne GmbH, Germany) and the pooled sap was collected in reaction tubes. The DAS-ELISA was performed according to Clark and Adams (1977). A polyclonal antiserum produced in the JKI was used for *Alternaria* detection. The colour reaction was determined photometrically by measuring the absorbance at 405 nm using the Microplate-Reader MRX II (Dynex Technologies, USA) after 30 minutes. The data were calculated with the software MicroWin 2000 (Ger J.C. Bolt Technical Service, Laboratory Equipment, Germany). The threshold was calculated with the formula: $\bar{x} + 3s$ ($\bar{x}$ = average of the measured data, s = standard deviation of the measured data) (Kegler and Friedt, 1993).

2.8 Molecular analyses

Three monoconidial lines of each isolate were cultivated in Petri dishes (Ø = 9 cm) on V8-A for seven days at 22°C

in the dark. The extraction of fungal genomic DNA has been done by the CTAB-method described by Rogers and Bendich (1985) with the following modifications: mycelium and conidia of each Petri dish were suspended in 700 µl extraction buffer. A mixer mill "MM 300" (RETSCH GmbH, Germany) was used for 3 min at 30 1/s to disrupt the fungal cells with the help of a tungsten bead (Ø = 3 mm; QIAGEN GmbH, Germany). Concentration and purity of total genomic DNA were determined using the "SpectraMax Plus 384 Microplate Reader" (Molecular Devices LLC., USA). According to the results, samples were diluted to an end concentration of 8 ng/µl with TE-buffer.

The specific primer pair Pa2071 (forward) and Pa2072 (reverse) developed for the detection of *A. radicina* and *A. petroselini* by Pryor and Gilbertson (2001) was used for PCR-analysis of all samples. The PCR was carried out in a volume of 6 µl containing 1.6 µl of fungal genomic DNA (8 ng/µl) and 4.4 µl mastermix (with 1.2 µl 5x MyTaq Reaction Buffer (Bioline, UK), 0.05 µl MyTaq DNA Polymerase (5 U/µl; Bioline, UK), 0.27 µM Pa2071, 0.27 µM Pa2072). The PCR was carried out in the thermal cycler "GeneAmp PCR System 9700" (Applied Biosystems, USA) programmed for the following parameters: 94°C for 1 min, 60°C for 1.5 min and 72°C for 2 min for 40 cycles (Pryor and Gilbertson, 2001). After addition of 2 µl loading-buffer amplification products were loaded on a 1.5% agarose gel in TBE-buffer, size-separated by electrophoresis for two hours at 130 V and visualised by UV illumination after staining in ethidium bromide. For size determination 8 µl of a size ladder (100 bp ladder; Fisher Scientific GmbH, Germany) was loaded.

RAPD analysis (Williams et al., 1990) was performed using the primers OPA08, OPA09, OPA10, OPB10, OPB12, OPB18 and OPC05 (Eurofins Genomics GmbH, Germany). PCR was carried out in a 6 µl reaction mixture containing 1.6 µl of total genomic DNA (8 ng/µl) from the fungus and 4.4 µl mastermix (2.69 µl aqua dest., 1.2 µl 5x MyTaq Reaction Buffer (Bioline, UK), 0.03 µl MyTaq DNA Polymerase (5 u/µl; Bioline, UK), 0.48 µl primer). PCR was carried out also in the thermal cycler "GeneAmp PCR System 9700" programmed for the following parameters: initial denaturation step at 94°C for 2 min; 45 cycles of 94°C for 1 min, 35°C for 1.5 min and 72°C for 2 min and a final incubation step for 10 min at 72°C. The amplification products were separated and visualised as described for the specific PCR products with the specific primer pair. As outgroup total genomic DNA from *A. alternata, A. dauci, A. brassicae* and *A. brassicicola* were used. Clear and strong bands of the amplifications from the three parallel Petri dishes were recorded as 1/0 matrix and analysed using NTSYSpc. 2.1 software package (Exeter Software, USA). Cluster analysis was carried out using SAHN mode.

2.9 Statistical analysis

An ANOVA was performed to clarify significances within each experiment. Subsequently a Tukey-B-Test was performed to determine differences of means ($P < 0.05$) in conidia length and width, growth rates, conidia generation intensity and the data of the analyses with DIAS and DAS-ELISA using the statistic programme SAS Version 9.4 (SAS Institute Inc., USA). Pearson's correlation coefficient was computed for detecting coherences between the two analysing methods in the assessment of resistance.

3 Results

3.1 Cultural and morphological characterisation

Visually determined characters in colony morphology are summarised in Table 3. The grey underlined fields mark differences compared to the *A. radicina* reference (ArRef). Colony margins on APDA differed between the three reference isolates. *A. radicina* (ArRef) had an irregular margin, in contrast *A. carotiincultae* (AcRef) and *A. petroselini* (ApRef) had smooth margins. The isolates Ar01, Ar04 and Ar05 had also irregular margins. Conidiophores of ArRef are erected and far apart from each other on each media, comparable with Ar01, Ar04 and Ar05. The other isolates showed flexible and closely related conidiophores. ArRef, Ar01, Ar04 and Ar05 condiophores generated solitary apical conidia or rarely clumps of single conidia. Apical conidia of AcRef were often branched on V8-A. Colony colour and structure differed usually between the media rather than between the isolates (Table 3).

The sporulation intensity of the isolates on V8-A was presented in Fig. 1. The isolates Ar04 and Ar05 generated significantly the most conidia per ml and Ar01 the fewest. The production of yellow pigments on APDA within 21 days was on a high level in ArRef, Ar01, Ar04 and Ar05 (Fig. 2) but lower in ApRef and Ar06 and failed completely in AcRef (Fig. 3). Dendritic crystals and microsclerotia were not found within the fungal colonies on APDA, SNA and V8-A.

For all isolates, the colony growth 8 dpi on SNA was less compared to the other media. On V8-A and APDA the growth of Ar06 was significantly taller than that of the other putative *A. radcina* isolates and the growth of the reference ArRef was significantly less than that of AcRef and ApRef (Fig. 4).

On V8-A and APDA more differences of the colony growth have been determined. Thus ArRef, Ar01, Ar04 and Ar05 grew slowly and stopped growth 14 dpi without covering the complete Petri dish surface. AcRef covered the dish surface within 14 days and ApRef and Ar06 within 21 days (Fig. 5). The reference isolates AcRef and ApRef grew on V8-A significantly faster than the others. While on APDA isolate Ar06 revealed a growth behaviour comparable to the reference ApRef. Because of the less growth on SNA these differences could not be ascertained.

Conidia measurements for the *Alternaria* spp. isolates were presented in Table 4. The classification of the conidia according to their transverse septa (transsepta T2 –T5) revealed that the isolates with the exception of AcRef and Ar05 had comperatively less conidia with 5 transsepta. Conidia of ApRef appeared in most transsepta categories wider and longer compared with those of references

Table 3. Visually determined characters of the colonies

Pathogen and media	Growth characteristics of eight-day-old cultures			
	colony colour[a]	colony structure[b]	colony margins[c]	conidiophores[d]
		Isolate ArRef		
V8-A	black	fluffy	smooth	1 e.fa.
SNA	br.-og.	ins. med.	smooth	1 e.fa.
APDA	br.-og.	felted	ir	1–2 e.fa.
		Isolate AcRef		
V8-A	black	fluffy	smooth	1–3 f.c.
SNA	black/green	ins. med.	smooth -ir	1 f. fa.
APDA	black	flat – felted	smooth	1–2 f.fa.
		Isolate ApRef		
V8-A	black	fluffy	smooth	1 f.c.
SNA	brown	ins. med.	smooth	1 f.c.
APDA	br.-og.	felted	smooth	1 f.c.
		Isolate Ar01		
V8-A	black	fluffy	ir	1–2 e.fa.
SNA	br.-og	ins. med.[d]	ir	1–2 e.fa.
APDA	br.-og	felted	ir	1–2 e.fa.
		Isolate Ar04		
V8-A	black	fluffy	smooth	1 e.fa.
SNA	br.-og.	ins. med.	ir	1 e.fa.
APDA	br.-gr.	felted	ir	1–2 e.fa.
		Isolate Ar05		
V8-A	black	fluffy	smooth	1 e.fa.
SNA	br.-og.	ins. med.	ir	1 e.fa.
APDA	br.-og.	felted	ir	1–2 e.fa.
		Isolate Ar06		
V8-A	gr. – black	fluffy	smooth	1–3 f.c.
SNA	brown	ins. med.	smooth	1 f.c.
APDA	dg.-og.	flat – felted	smooth	1 f.c.

[a] br. = brown; og. = olive-green; gr. = grey
[b] ins. med. = inside the media
[c] ir. = irregular
[d] e. = erected; f. = flexible; fa. = far apart; c. = close
Grey underlined fields mark differences.

ArRef and AcRef. In the T3 category conidia of ArRef were significantly shorter than condia of AcRef. In the T2 and T3 categories conidia of AcRef and Ar05 were significantly thinner than those of the other isolates. For each of the other categories there were no differences in length of ArRef and AcRef (Table 4).

Morphology of conidia of ArRef corresponded to those reported by Simmons (2007). They were ellipsoid or ovoid. ApRef condia had a similar morphology like ArRef but they were in most cases chubbier than conidia of ArRef. Condia of AcRef were narrow and long with pointed ends (Fig. 6). Conidia of Ar01, Ar04 and Ar05 were similar to conidia of ArRef, whereas Ar06 conidia were similar to ApRef (Fig. 6).

3.2 Pathogenicity test

The host plant bioassays were evaluated with the DIAS 7 dpi. All isolates caused symptoms in varying degrees on the host plants (Fig. 7a). The carrot and fennel showed clearly larger disease symptoms than parsley and celery.

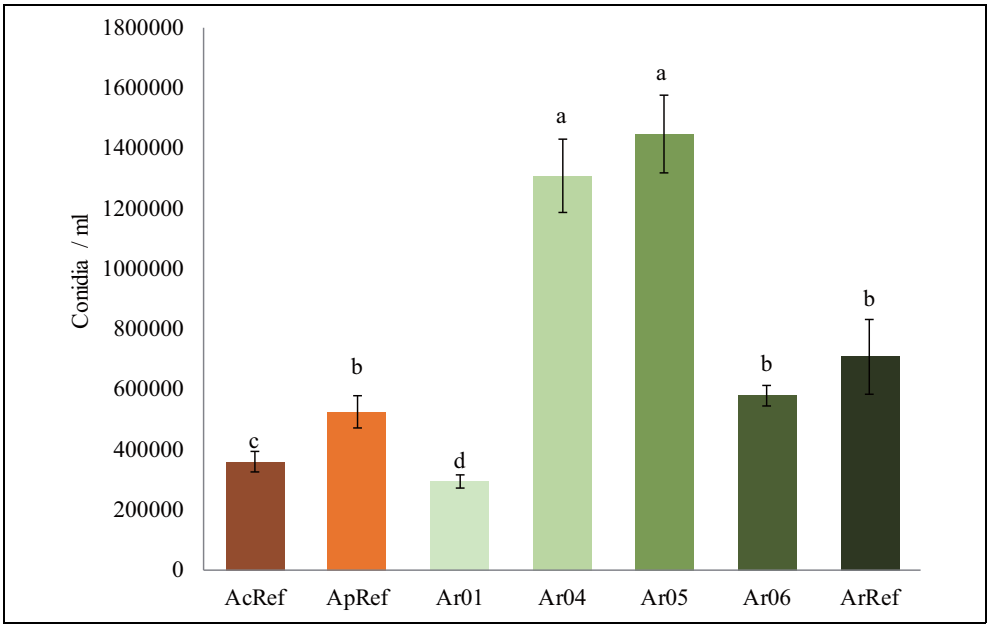

Fig. 1. Conidia generation intensity of 10-day-old cultures on V8-A. (Average of n = 15 censuses, different letters indicated significance, Tukey, P < 0.05).

Fig. 2. Production of yellow pigments of 21-day-old *A. radicina* (ArRef), Ar01, Ar04 and Ar05 colonies (left to right) growing on APDA (acidified potato dextrose agar).

Fig. 3. Low level of yellow pigment production of 26-day-old Ar06 and *A. petroselini* (ApRef) and failure production of *A. carotiincultae* (AcRef) colonies (left to right) growing on APDA (acidified potato dextrose agar).

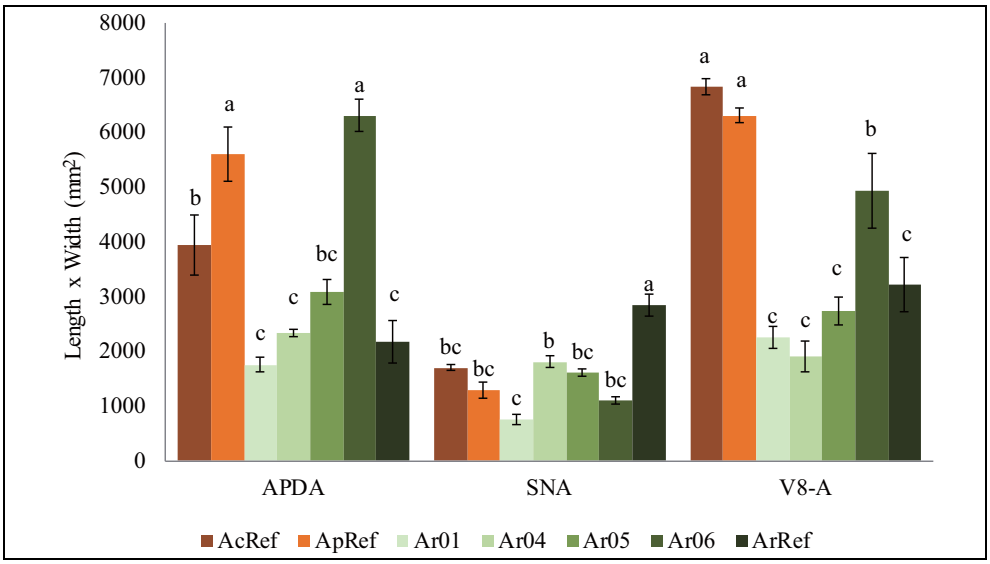

Fig. 4. Colony growth of all *Alternaria* spp. isolates after 8 days on various media. (Values represent the average of six Petri dishes. Different letters indicated significance, Tukey, P < 0.05, subdivided for each media).

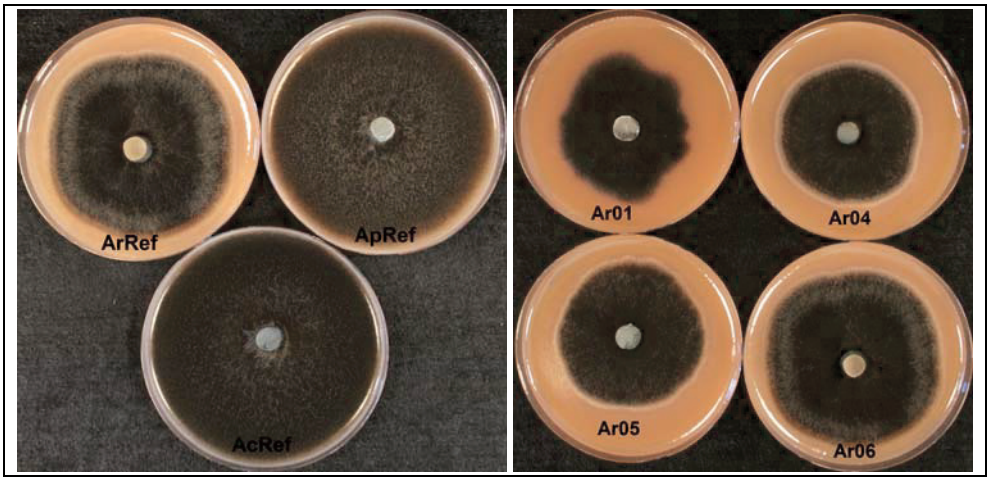

Fig. 5. Colony morphology of all *Alternaria* spp. isolates after 21 days of growth on vegetable juice agar.

Table 4. Average of conidia length and width (µm) of the *Alternaria* spp. isolates grown ten days on V8-A. Different letters indicated significance (Tukey, P < 0.05), n – number of observed conidia

Pathogen	Conidia dimension (µm) separated in the transsepta categories											
	2-septa (T2)			3-septa (T3)			4-septa (T4)			5-septa (T5)		
	n	length	width	n	length	width	n	length	width	n	length	width
Ar01	50	30 ± 3 c	20 ± 3 cd	50	37 ± 5 abc	21 ± 5 bc	50	42 ± 3 c	20 ± 3 b	10	50 ± 8 a	24 ± 8 a
Ar04	50	32 ± 3 cb	21 ± 3 c	50	40 ± 2 a	22 ± 2 ab	50	47 ± 3 ab	23 ± 3 a	20	51 ± 5 a	22 ± 5 ab
Ar05	50	30 ± 3 c	19 ± 3 d	50	36 ± 4 bc	20 ± 4 c	50	45 ± 3 abc	20 ± 3 b	50	52 ± 3 a	19 ± 3 b
Ar06	50	33 ± 4 b	22 ± 4 cb	50	40 ± 3 a	22 ± 3 ab	50	46 ± 3 ab	23 ± 3 a	30	52 ± 2 a	21 ± 2 ab
ArRef	50	31 ± 3 cb	23 ± 3 ab	50	35 ± 3 c	20 ± 3 bc	50	44 ± 3 bc	23 ± 3 a	10	50 ± 2 a	22 ± 2 ab
AcRef	50	32 ± 2 cb	19 ± 2 d	50	38 ± 3 ab	20 ± 3 c	50	44 ± 2 bc	21 ± 2 b	50	52 ± 3 a	21 ± 3 ab
ApRef	50	37 ± 3 a	24 ± 3 a	50	39 ± 2 a	23 ± 2 a	50	48 ± 3 a	24 ± 3 a	10	55 ± 3 a	24 ± 3 a

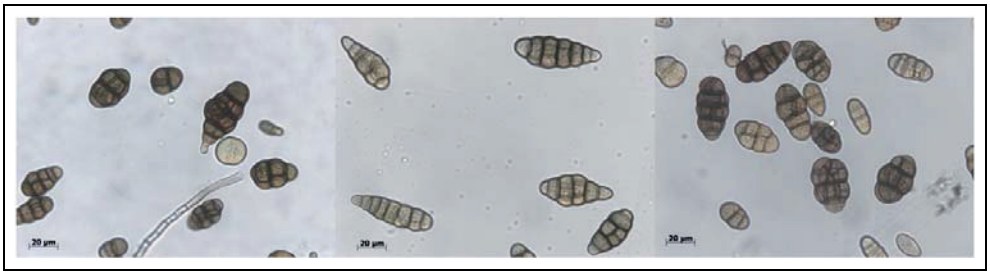

Fig. 6. Conidia morphology of *A. radicina* (ArRef; left), *A. carotiincultae* (AcRef; middle) and *A. petroselini* (ApRef; right).

On celery there were found no or extremely weak symptoms only. On carrot ApRef, Ar01 and Ar06 showed significantly lower disease symptoms than the isolates ArRef, AcRef and Ar04. On fennel ApRef and Ar06 induced the most distinct disease symptoms. The infestation of parsley was low overall, only isolate Ar06 revealed distinct disease symptoms (Fig. 7a).

The average of the ELISA values for detecting the fungal pathogens in the leaf tissue were diagrammed in Fig. 7b. The values determined from the infected host plant species carrot, fennel and parsley which exceeded the calculated threshold of 0.10 suggest pathogenicity for the particular isolate on the host plant species. Thus in celery only isolates Ar06 and ApRef exceeded this threshold. On parsley all isolates could be detected, with the isolates Ar04 and ArRef having the highest extinction values. On fennel the isolates Ar04 and ArRef showed the highest extinction values, while ApRef infected leaf segments had the lowest one. The carrot leaf segments inoculated with Ar06 and ApRef had the lowest values in contrast to the infection with Ar01, Ar04, ArRef and AcRef.

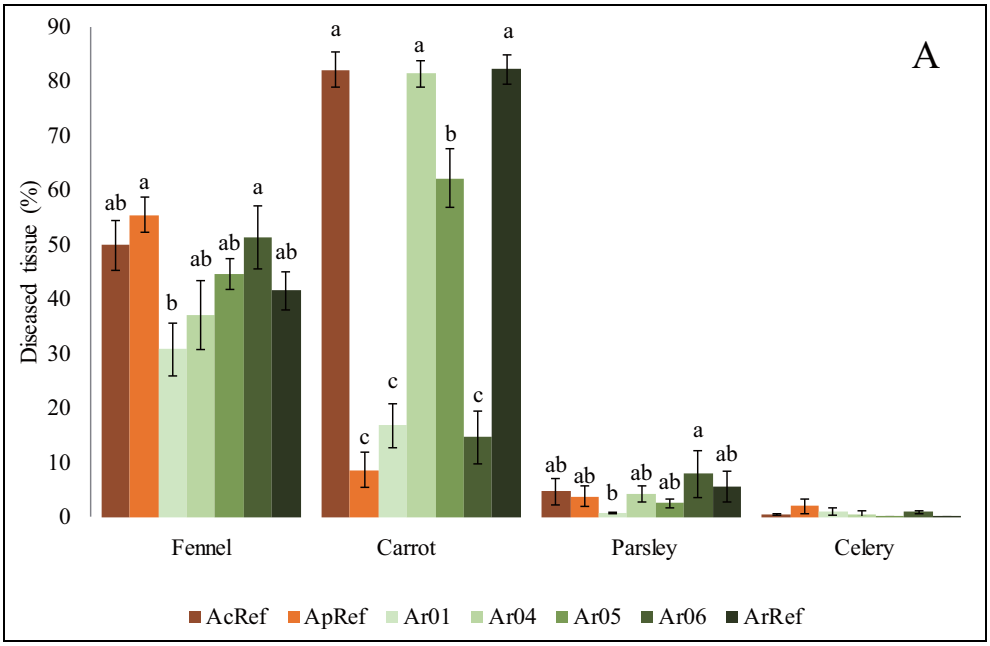

Fig. 7a. Pathogenicity test on different Apiaceae species with seven *Alternaria* isolates 7 dpi: (A) DIAS estimated diseased leaf tissue (%) and (B) DAS-ELISA absorbence values (A_{405nm}). (Different letters indicated significance, Tukey, $P < 0.05$, mean n = 10).

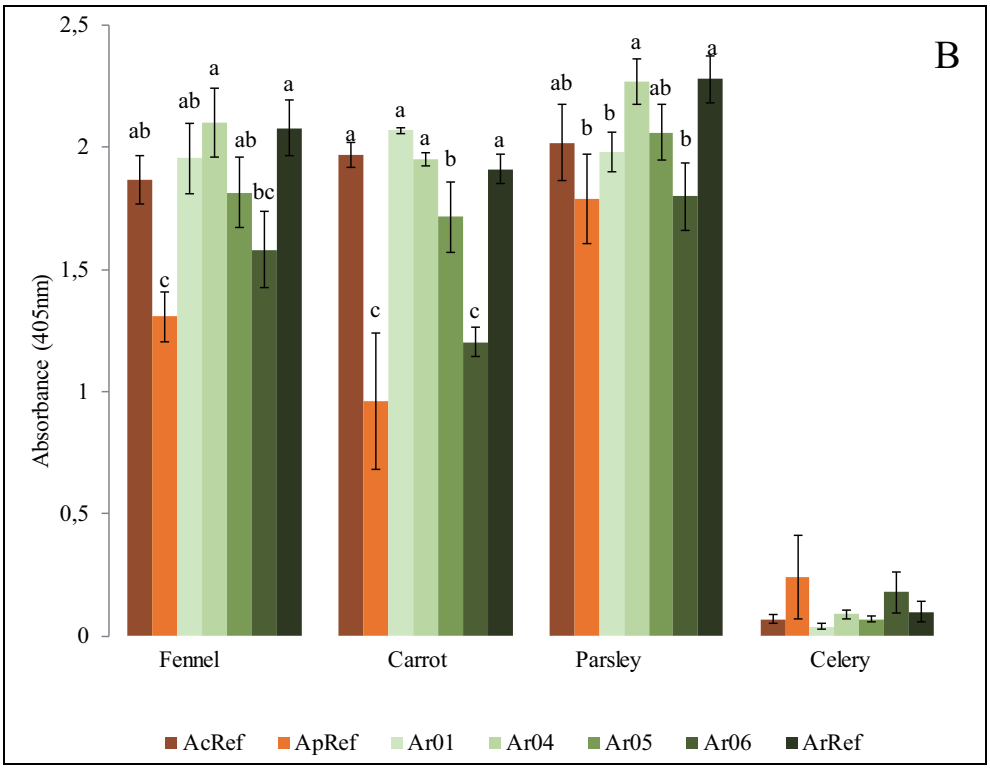

Fig. 7b. Pathogenicity test on different Apiaceae species with seven *Alternaria* isolates 7 dpi: (A) DIAS estimated diseased leaf tissue (%) and (B) DAS-ELISA absorbence values (A_{405nm}). (Different letters indicated significance, Tukey, $P < 0.05$, mean n = 10).

3.3 Aggressiveness test

The tested isolates were pathogenic on the four carrot cultivars but differed in their aggressiveness in the petioles assay (Fig. 8). ApRef belongs for every cultivar to the weakest aggressive isolates. Even the isolates Ar01 and Ar06 are, excepting the cv. Rotin, weaker aggressive than the other ones. Ar05 showed in the most cases a higher aggressiveness than the other putative *A. radicina* isolates.

3.4 Molecular analyses

The application of primer pair Pa2071/Pa2072 resulted in the expected amplification of an approximately 900 bp DNA fragment from total genomic DNA of ArRef, AcRef and the putative *A. radicina* isolates. However, the DNA amplification of Ar06 was relatively weak (Fig. 9). Each Operon primer amplified the total genomic DNA of all isolates and the controls 1–4. The amplified fragments of

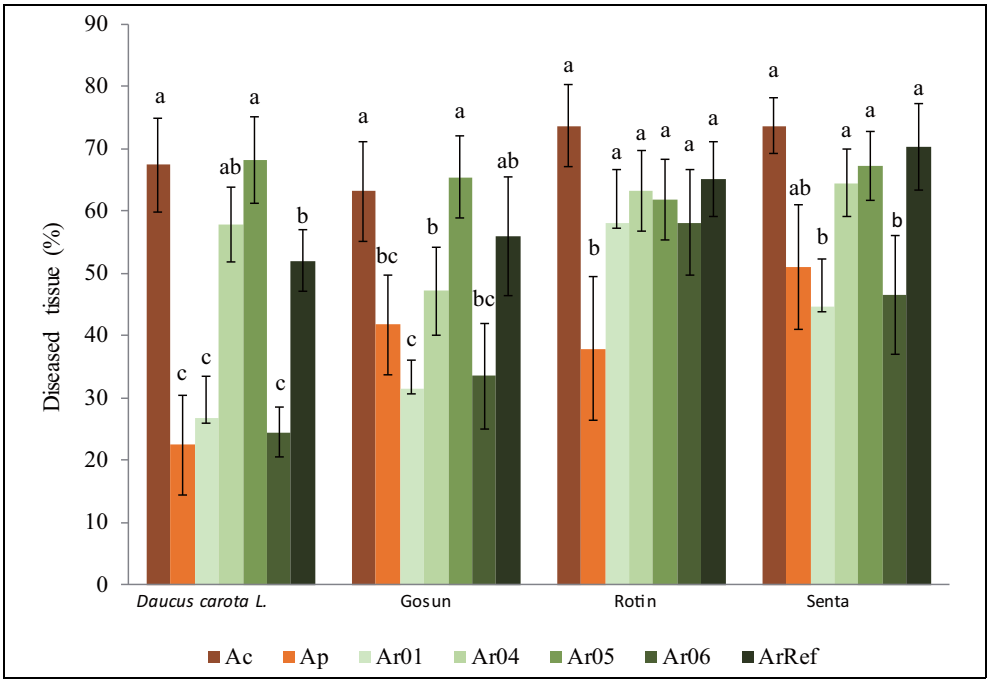

Fig. 8. Aggressiveness test of the *Alternaria* isolates with three carrot cultivars and a wild relative 7 dpi: DIAS estimated diseased petiole tissue (%). (Different letters indicated significance, Tukey, $P < 0.05$, mean n = 16, subdivided for each cultivar).

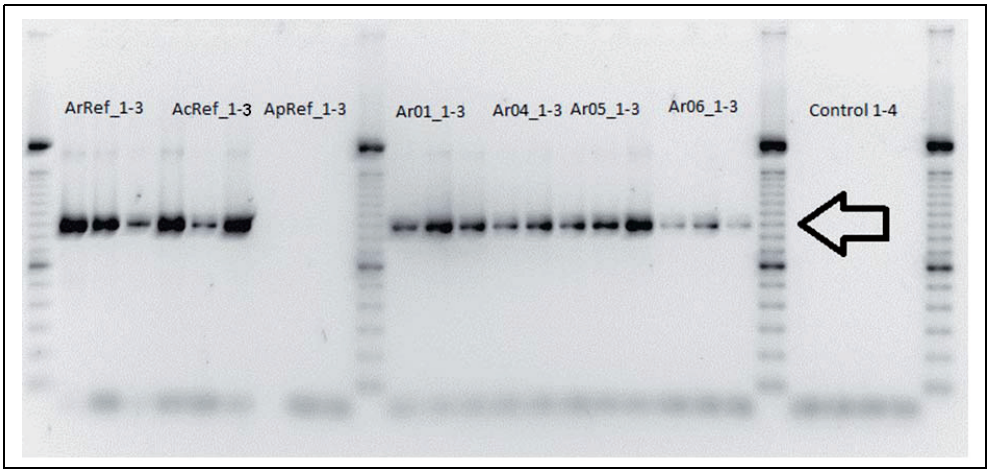

Fig. 9. DNA patterns generated from total genomic DNA of each of the 3 monoconidial lines of *A. radicina* (ArRef), *A. carotiincultae* (AcRef), *A. petroselini* (ApRef) and the putative *A. radicina* isolates (Ar01, Ar04, Ar05, Ar06) with the primer pair Pa2071/Pa2072. Control 1 = *A. alternata*, Control 2 = *A. dauci*, Control 3 = *A. brassicae*, Control 4 = *A. brassicicola* (n = 3). (Size marker: 100 bp ladder).

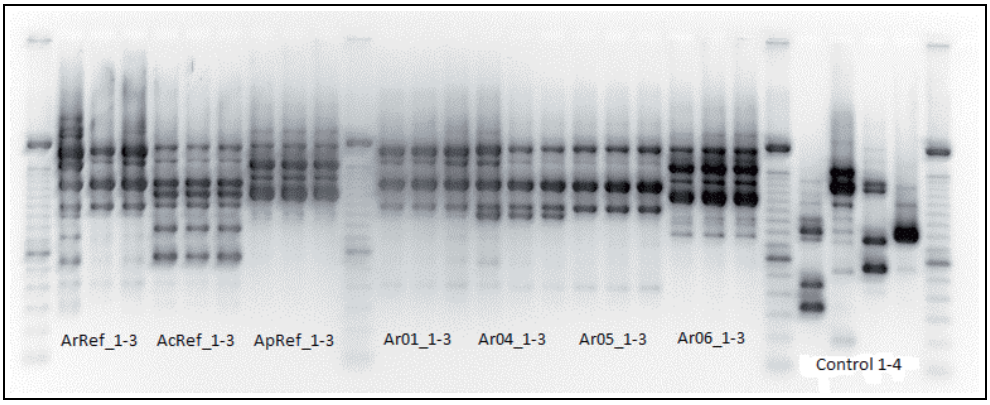

Fig. 10. Random amplified polymorphic DNA patterns from total genomic DNA of each 3 monoconidial lines of *A. radicina* (ArRef), *A. carotiincultae* (AcRef), *A. petroselini* (ApRef) and the putative *A. radicina* isolates (Ar01, Ar04, Ar05, Ar06) with the Operon Primer OPA-10 (GTGATCGCAG). Control 1 = *A. alternata*, Control 2 = *A. dauci*, Control 3 = *A. brassicae*, Control 4 = *A. brassicicola* (n = 3). (Size marker: 100 bp ladder).

Ar01, Ar04 and Ar05 are similar to those of ArRef and Ar06 is similar to ApRef (Fig. 10).

The distance analysis for the seven isolates was performed by RAPD amplification using seven Operon prim-

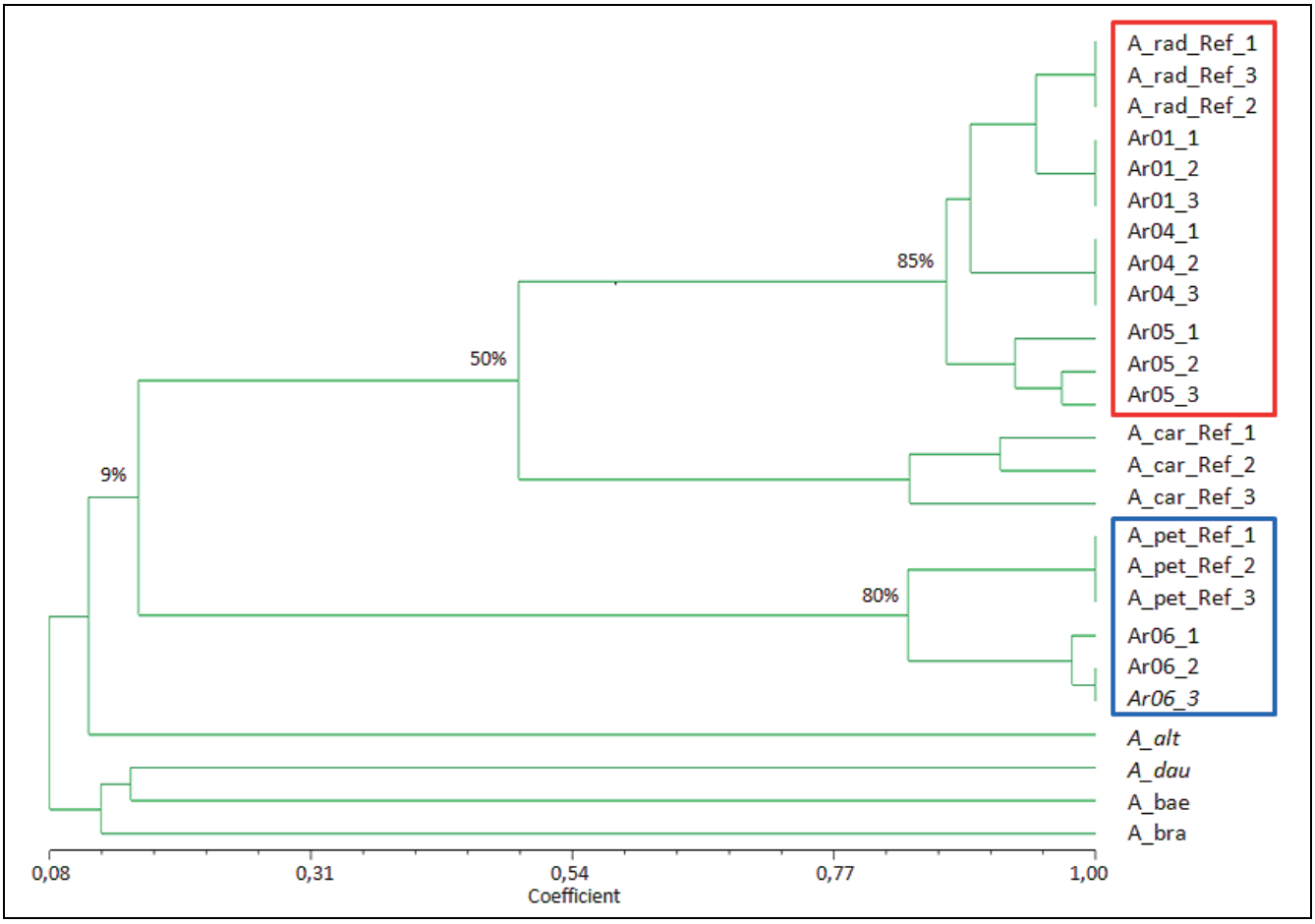

Fig. 11. Dendrogramm showing the genetical distance between the reference isolates and the putative *A. radicina* (Ar) isolates used the SAHN mode of the NTSYSpc 2.2 programme. A_rad_Ref_1-3 = ArRef (*A. radicina* Referenz); A_car_Ref_1-3 = AcRef (*A. carotiincultae*); A_pet_Ref_1-3 = ApRef (*A. petroselini*); A_bra (*A. brassicicola*); A_alt (*A. alternata*); A_bae (*A. brassicae*); A_dau (*A. dauci*) .

ers. 152 polymorphic fragments were estimated. The highest number of 60 polymorphic fragments was found for the primer OPB10 and only 24 polymorphic fragments for the primer OPB12. A dendrogram was created to show the genetic distances between the isolates (Fig. 11).

Two groups of isolates were obviously determined. The first group, framed red, consists of ArRef, Ar01, Ar04 and Ar05. There was a similarity of approximately 85% between ArRef and the 3 isolates. The second group, framed blue, consists of ApRef and Ar06 with a similarity of approximately 80%. AcRef was not associated with the other isolates, there was only a minor similarity of 9% or 50% to ArRef or ApRef, respectively. The *A. carotiincultae* isolates made up an individual cluster, the isolates of *A. alternata, A. dauci, A. brassicae* and *A. brassicicola* used as outgroup.

3.5 Assessment of resistance

The low aggressive *A. radicina* isolate Ar01 and the high one Ar05 were used to assess resistance response of 14 carrot genotypes in a leaf segment test. Disease symptoms were analysed using the DIAS 7 dpi. Disease symptoms revealed on all cultivars infested by both isolates, but as expected Ar05 expressed significantly more distinct symptoms than Ar01. The cv. 237 was hardly affected by the low aggressive isolate Ar01 and also less by the high one Ar05. In contrast the cv. 226 revealed the highest susceptibility to both isolates (Fig. 12a).

The ELISA showed for all genotypes tested with Ar05 higher values than Ar01. The highly aggressive isolate Ar05 could be detected in all tested cultivars as well as in the wild relative 230. In contrast the low aggressive one Ar01 was not detectable in the cultivars 234, 236, 238 and 239. The wild relative 230 revealed the highest absorbance values after inoculation with both isolates. Infection with Ar05 caused higher absorbance values than Ar01 with the exception of cv. 233 (Fig. 12b).

A moderate positive Pearson correlation coefficient (r = 0.404) between disease symptoms and the relative pathogen concentration in the immunoassay was determined for the whole experiment (Fig. 13).

4 Discussion

4.1 Identification and characterisation of Alternaria spp. isolates

Main focus of this study was the identification and characterisation of putative *Alternaria radicina* isolates obtained

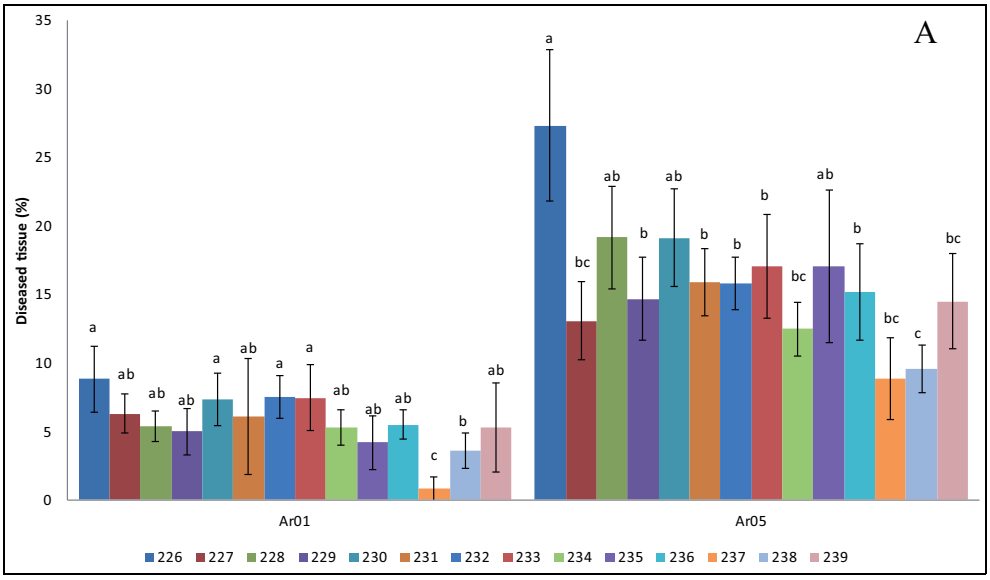

Fig. 12a. Resistance test with 14 carrot cultivars and two isolates: (A) DIAS estimated diseased leaf tissue (%) and (B) DAS-ELISA absorbence values (A_{405nm}). (Different letters indicated significance, Tukey, $P < 0.05$, mean $n = 10$, subdivided for both isolates Ar01 (7 dpi) and Ar05 (5 dpi)).

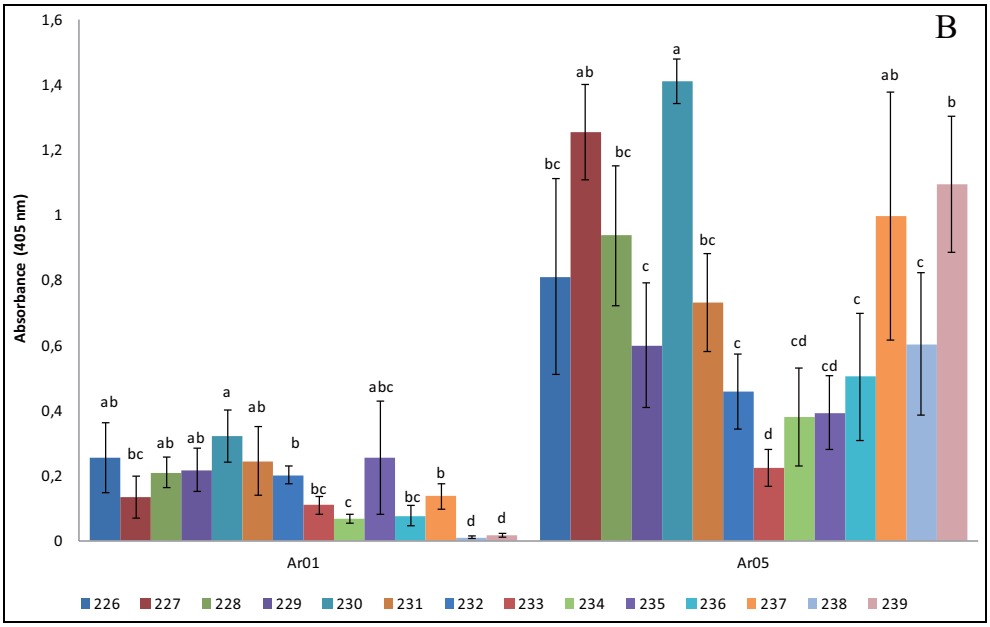

Fig. 12b. Resistance test with 14 carrot cultivars and two isolates: (A) DIAS estimated diseased leaf tissue (%) and (B) DAS-ELISA absorbence values (A_{405nm}). (Different letters indicated significance, Tukey, $P < 0.05$, mean $n = 10$, subdivided for both isolates Ar01 (7 dpi) and Ar05 (5 dpi)).

from carrot and parsley. Identification of *A. radicina* based solely on conidia morphology is insufficient because two taxonomically related species *A. carotiincultae* and *A. petroselini* with similar conidia could appear on carrot too. For these reasons WOUDENBERG et al. (2013) assigned these different *Alternaria* species in the *Alternaria* sect. *Radicina*. Therefore different features such as pigment production, colony structures, pathogenicity on host plants as well as molecular analysis were additionally used to identify *A. radicina* and differentiate this species from *A. carotiincultae* and *A. petroselini* PRYOR and GILBERTSON, 2001, 2002; PARK et al., 2008; BULAJIĆ et al., 2009).

Results of this study revealed that cultural and morphological characters of the *Alternaria* isolates from carrot and parsley could be used in a different manner for their identification and differentiation. Notably the high production of diffusible pigments on acidified potato dextrose agar affected by the production of the toxin radicinin and radicinol is an appropriate trait for the identification of *A. radicina*. However, the taxonomically closely related species *A. carotiincultae* does not produce this yellow pigment (PRYOR and GILBERTSON, 2002; TRIVEDI and HAMPTON, 2010). Based on the high intensity of yellow pigment production isolates Ar01, Ar04 and Ar05 were assigned to the *A. radicina* reference isolate. In contrast the isolate Ar06 and the *A. petroselini* reference represented a group of low pigment production intensity. Additionally, colony margins revealed to be another feature for the differentiation of *A. radicina* from *A. petroselini* and *A. carotiin-*

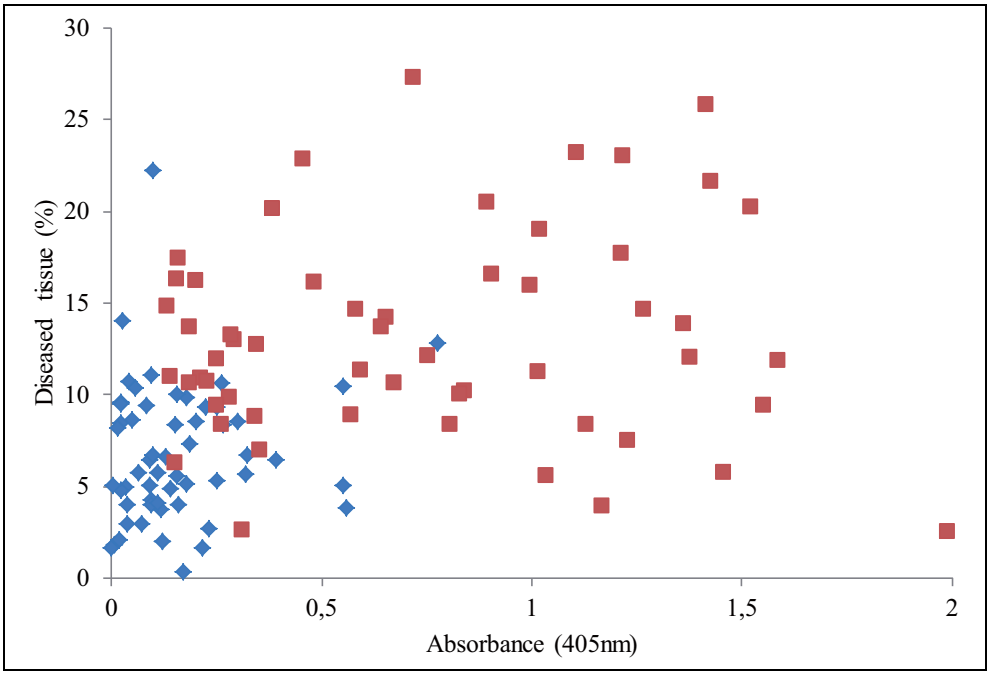

Fig. 13. Correlation between disease symptoms (DIAS) and the relative pathogen concentration in the immunoassay (ELISA) with leaf segments for assessment of resistance using the *A. radicina* isolates Ar01 (blue) and the Ar05 (red) ($P < 0.05$, $y = 4.9965x + 7.704$).

cultae (PRYOR and GILBERTSON, 2002; PARK et al., 2008; TRIVEDI and HAMPTON, 2010). Thus isolates Ar01, Ar04 and Ar05 showed more irregular colony margins like the *A. radicina* reference, and colonies of isolate Ar06 grew with even margins like *A. carotiincultae* or *A. petroselini*. Another typical trait for *A. radicina* colonies is the growth finishing before the edge of a Petri dish was reached. This feature was observed in isolates Ar01, Ar04 and Ar05 according to the *A. radicina* reference. While the type of colony margins and their extent is species specific, colours and structures of the colonies depend on culture media.

Another reliable feature was the assessment of the pathogenicity on different host plants. Different species of family Apiaceae became infected by the *Alternaria* species including the claimed *A. radicina* isolates. On carrot, reference isolates *A. radicina* and *A. carotiincultae* as well as the isolates Ar01, Ar04 and Ar05 were pathogenic.

The unexpected differences appearing in diseased leaf tissue and absorbance values of the respective parsley cultivar could result either from tolerance to symptom development or from high sensitivity of the immunoassay. However, the pathogenicity test in carrots allowed a differentiation of *A. radicina* and *A. carotiincultae* from *A. petroselini*. Comparable results were published by PRYOR and GILBERTSON (2002) and BULAJIĆ et al. (2009).

In contrast to the growth characteristics and determined pathogenicity, measurements of the conidia do not allow drawing clear conclusions for affiliation of isolates to the respective *Alternaria* species. In the majority of cases the phenotype of conidia may be used for a first grouping of isolates. But a differentiation of the taxonomically closely related *A. radicina* and *A. carotiincultae* was difficult as described by BULAJIĆ et al., 2009 and TRIVEDI and HAMPTON (2010).

A clear species differentiation resulted from the molecular analyses. The isolates Ar01, Ar04 and Ar05 were classified as *A. radicina* because of 80% similarity to the *A. radicina* reference, whereas isolate Ar06 is probably *A. petroselini*. The primer pair Pa2071/Pa2072 amplified an expected 900 bp fragment for the ArRef, AcRef and the four new isolates, but not for the ApRef and the *Alternaria* species used as outliers. According to PRYOR and GILBERTSON (2001) and BULAJIĆ et al. (2009) this primer pair amplified a 900 bp DNA fragment for *A. radicina* and *A. petroselini.* However *A. carotiincultae* was not tested. In contrast, no amplification was detected for ApRef in our experiments. A possible reason may be the different sources of the reference isolates. PRYOR and GILBERTSON (2001) used an isolate of the Mycological Service Crawfordsville, whereas we used an isolate of the Centralbureau voor Schimmelcultures. The amplification of the specific fragment for Ar06 may be a clue for a broad genetic variability of *A. petroselini. A. carotiincultae* was first tested with this primer pair and showed amplification.

4.2 Aggressiveness test and assessment of resistance

The aggressiveness test revealed diversity in the determined *A. radicina* isolates ranging from low (Ar01) to high (ArRef, Ar04 and Ar05) aggressiveness. High aggressiveness stood for a large extent in disease symptom development as well as for a fast invasion of plant tissue. This was considered to represent pathogen diversity in the resistance evaluation of carrot genotypes. In the resistance screenings carrot leaves showed just few symptoms by using the low aggressive isolate Ar01 but distinct symptoms with the highly aggressive Ar05. This clearly revealed that the estimated level of susceptibility depends on aggressiveness of the used isolates. Aggressiveness could

be associated with conidia generation intensity of isolates. Thus the less aggressive isolate Ar01 revealed lower conidia generation intensity than the highly aggressive isolate Ar05. Furthermore, aggressiveness could depend on the ability to produce different amounts of toxins required for pathogenesis produced by *A. radicina* (Robeson et al., 1982; Pryor and Gilbertson, 2002). Both characters could determine the vitality of an *A. radicina* isolate. Hence, to screen for durable resistance to *A. radicina*, isolates with a high level of aggressiveness will be of relevance. Overall, there was not found any resistance to *A. radicina* in the tested carrot accessions but differences in their susceptibility to the highly aggressive isolate. Thus cv. 237 was not affected by isolate Ar01 and showed also relatively less symptoms after inoculation with the highly aggressive isolate Ar05. The cvs. 237 and 227 are probably symptom tolerant because only few disease symptoms appeared in relation to the estimated relative pathogen concentration in immunoassay. Therefore analysis of disease symptoms and immunological pathogen detection are necessary for characterisation of the resistance response to *A. radicina* underlined by the moderate positive correlation coefficient.

Results of this study revealed that a precise determination and differentiation of *Alternaria* species on carrot required a combination of cultural, biological as well as molecular methods. Characterisation of aggressiveness of *A. radicina* isolates is a prerequisite for searching for new genetic resistance resources. Resistance to *A. radicina* was not found in carrot until now but genotypes with lower susceptibility. Screenings of a broad spectrum of carrot genotypes in particular in carrot wild relatives could be promising to find resistance to *A. radicina*.

Acknowledgements

We thank Martina Malorny, Elke Zjaba, Martina Fuss and Ursula Apel for the excellent technical assistance.

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