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MORPHO-PHYSIOLOGICAL AND MOLECULAR ANALYSES OF *ALTERNARIA ALTERNATA* ISOLATED FROM SEEDS OF *AMARANTHUS*

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Abstract

The majority of 26 *Alternaria alternata* isolates from seeds of *Amaranthus cruentus, A. paniculatus* and *A. retroflexus* were highly pathogenic to *A. cruentus* and *A. paniculatus* seedlings *in vitro*. The pathogen produced necrotic lesions on stems and leaves, and caused seedling wilt and death. The number of diseased *A. cruentus* and *A. paniculatus* plants differed among the *A. alternata* isolates used. Colonies of *A. alternata* isolates differed in their rate of linear growth and colour. The dendrogram based on the presence or absence of DNA fragments amplified with OPA and OPB primers (RAPD-PCR) suggests moderate heterogeneity within the *A. alternata* population from *Amaranthus*. A slight effect of host plant and geographical location on genetic variation in *A. alternata* was suggested, however.

Key words: *Alternaria alternata, Amaranthus cruentus, A. paniculatus, A. retroflexus,* molecular differentiation, RAPD-PCR

Introduction

The genus *Amaranthus* includes species grown for food (*A. cruentus*) and as ornamentals (*A. paniculatus*), as well as weeds (*A. retroflexus*).

Amaranthus cruentus is grown as a pseudocereal almost worldwide from tropical to warm-temperate regions. It is cultivated because of, for example, easy harvesting, production of seeds used as food grain, high tolerance to arid environments, high nutritional value (16–18% of the most balanced protein, high level of essential amino acids, including lysine, which is essential for good health, and high levels of micronutrients). *Amaranthus* is also a very efficient sequester of carbon, which gives it beneficial significance in present-day CO_2-driven global warming. Its cultivation may contribute to global food security in a changing climate.

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Alternaria alternata is the most common fungus colonizing seeds of all *Amaranthus* species (Noelting et al. 2004). The fungus may contribute to decreased quality of seeds of the cultivated and ornamental species, and to natural biocontrol of *Amaranthus* weeds in fields (Bürki et al. 2001).

Genetic variation in pathogenic, endophytic and saprotrophic *A. alternata* isolates has previously been assessed on the basis of analyses RAPD-PCR (Random Amplified Polymorphic DNA – Polymerase Chain Reaction), RFLPs, DNA hybridization, AFLP, and DNA sequences (Ghorbani et al. 2000, Johnson et al. 2000, Peever et al. 2002, Tigano et al. 2003, Guo et al. 2004). In some works both molecular and morphological features of the pathogen were considered (Pryor and Michailides 2002). Investigating *A. alternata* isolated from various *Amaranthus* spp. plant parts are of vital importance to recognition of pathogenic isolates for biological control of *Amaranthaceae* weeds' population (Ghorbani et al. 2000).

The objective of this study was the evaluation of morphological and genetic variation in *A. alternata* originating from *A. cruentus, A. paniculatus* and *A. retroflexus*, and of its pathogenicity towards *A. cruentus* and *A. paniculatus*.

Materials and methods

The isolates of *A. alternata* originated from three *Amaranthus* species and four locations in south-west of Poland (Table 1). Thirteen isolates originated from *A. cruentus* sampled in Pawłowice and Łosiów, in 2004–2005. Three isolates originated from *A. paniculatus* and ten from *A. retroflexus* sampled in Pawłowice, Łosiów, Biskupin and Swojec, in 2005. Collected seeds were surface-disinfected in sodium hypochlorite (1% available chlorine) for 1 min, rinsed in sterile water 3 × for 10 min and placed on synthetic nutrient agar (SNA; 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 20 g agar, 1 l distilled water). After incubation for 10 days at 20°C in a night light cycle, *A. alternata* isolates growing from seeds were transferred onto potato dextrose agar (PDA; 40 g filtered white potatoes, 20 g agar, 1 l distilled water, pH 7) slopes for preservation, and identified on the basis of their morphology on PDA and SNA using microscopy and available literature (Ellis 1971).

The rate of linear growth of *A. alternata* was studied *in vitro*, on 2% PDA in 9-cm-diameter Petri dishes on three repetitions. The fungi were incubated at 22°C in a night cycle. Radial mycelial growth was determined by periodical measurement of two diameters, at right angles to each other, of the colonies. The first measurement was done 24 h after inoculation and subsequent measurements once a day for seven days. Morphocultural characteristics, including colony colour, were recorded according to the ISCC-NBS Names (Rayner 1970).

The pathogenicity of *A. alternata* on *A. cruentus* and *A. paniculatus* was tested in 2005. Seeds of *A. cruentus* and *A. paniculatus* used in the pathogenicity test were collected in Pawłowice, Łosiów and Biskupin, respectively. Seeds were surface-disinfected in sodium hypochlorite (1% available chlorine) for 1 min, rinsed in sterile

Table 1

Alternaria alternata isolates tested

Original host	Place of origin	Isolate	Year of isolation
Amaranthus cruentus	Pawłowice 51°10' N, 17°12' E	CP1/05	2005
		CP2/05	2005
		CP4/05	2005
		CP5/05	2005
	Łosiów 50°51' N, 17°28' E	CL1/04	2004
		CL1/05	2005
		CL2/04	2004
		CL3/05	2005
		CL4/05	2005
		CL5/04	2004
		CL7/04	2004
		CL9/04	2004
		CL10/04	2004
Amaranthus paniculatus	Biskupin 51°06' N, 17°02' E	PB1/05	2005
		PB6/05	2005
		PB7/05	2005
Amaranthus retroflexus	Pawłowice 51°10' N, 17°12' E	RP2/05	2005
		RP3/05	2005
		RP4/05	2005
	Łosiów 50°51' N, 17°28' E	RL1/05	2005
		RL2/05	2005
		RL3/05	2005
		RR4/05	2005
	Swojec 51°06' N, 17°02' E	RS2/05	2005
		RS3/05	2005
		RS6/05	2005

water 3 × for 10 min, spread on to two–three sheets of sterile, moist filter paper placed in Petri dishes. The seeds germinated within 48 h. Four seedlings of *Amaranthus*, 1 cm long, were placed evenly-spaced on two–three sheets of sterile, moist filter paper in 9-cm-diameter Petri dishes. Each seedling was inoculated with a 5-mm-diameter PDA disc cut from the edge of a 7–10-day-old *A. alternata* colony and placed (with mycelium on the agar surface) 2–3 mm from the shoot of the seedling. Control seedlings were inoculated with 5-mm-diameter discs of sterile PDA. Disease symptoms, recorded after four days, were necrotic lesions on hypocotyls and leaves, wilting and death.

Genetic variation among isolates of *A. alternata* (listed in Table 1) was studied with RAPD-PCR. A single-spore culture was prepared for each isolate. DNA was extracted from seven-day-old cultures grown in liquid SNA with DNeasy Plant Mini Kit (Qiagen, GmbH, Hilden, Germany, Cat. no 69104) following the manufacturer's instructions. A DNA quality check was made on 1% agarose gel. DNA concentration was determined by spectrophotometry. A final concentration of

DNA in TE of 20 ng/μl was prepared. DNA was stored in –20°C. Two pairs of 10-mer primers: OPA (OPA1–OPA15) and OPB (OPB1–OPB15) (Operon Technologies, Alameda, CA) were initially used for RAPD-PCR with five isolates of *A. alternata* (CL2/04, RL3/05, PB6/05, CL3/05, RP3/05). Primers were used singly, so as to choose the most suitable ones for subsequent experiments. The maximum amplification and the best genetic polymorphism was obtained with OPA5, OPA9, OPA10, OPB1, OPB4 (Table 2) and these five primers were used for RAPD-PCR with other *A. alternata* isolates.

Table 2

Primers used to study genetic variation among *Alternaria alternata* isolates

Primer	Sequence
OPA5	5'-AGGGGTCTTG-3'
OPA9	5'-GGGTAACGCC-3'
OPA10	5'-GTGATCGCAG-3'
OPB1	5'-GTTTCGCTCC-3'
OPB4	5'-GGACTGGAGT-3'

Each PCR mixture of 25 μl consisted of 2.5 μl buffer contained 50 mM EDTA and 100 mM Tris (TE), pH 8, 3 μl (2.5 mM) $MgCl_2$, 3.5 μl (0.2 mM) dNTP, 2 μl primer, 0.4 μl Taq (5 U/μl platinum), 2 μl DNA and 11.6 μl dd H_2O (Peever et al. 1999).

Thermal cycling reactions were performed in a Hybaid-Touchdown Thermal Cycler using the following conditions: initial denaturation at 93°C for 2 min, followed by 44 cycles of 92°C for 1 min, 37°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 8 min. RAPD products were separated by agarose gel electrophoresis (1.8%) with 1X TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8) for 2 h. Molecular size standard: $\phi X174$ DNA *Hae*III digest was included in each gel. Gels were visualized by UV fluorescence. Data documentation was performed using Chemilimager v. 5.5 software (Alpha Innotech Corporation – Bio-Science Kft, Budapest). Polymorphic bands were scored for presence or absence. Agglomerative hierarchical clustering (Ward 1963) was applied to construct a dendrogram using the Statistica Package software. Euclidean distances were transformed according to the formula: 100 × distance between two points / maximal distance. This enabled the interpretation of results as 0–100%.

Analysis of molecular variance (AMOVA) was used to estimate the components of variance attributable to differences among individuals within a population on the basis of the Euclidean distance. Pairwise genetic distance (F_{st}) values, a value of *F* statistic analogs computed from AMOVA, were used to compare genetic distances between any two isolates. To demonstrate the relationship between isolates, a distance matrix generated from AMOVA was used as input to perform a cluster analysis.

Results

Differences in rates of linear growth and colour of the colony were observed among all 26 isolates of *A. alternata* obtained from the *Amaranthus* seeds. The diameter of *A. alternata* colonies, on PDA, after seven days at 22°C in the day/night cycle, was 4.8–6.8 cm. Isolates from *A. retroflexus* grew most quickly (6.0 cm on average) and isolates from *A. cruentus* grew most slowly (3.2 cm in average). The fastest and the slowest growth was observed in isolates PB7/05 from *A. paniculatus* and CP2/05 from *A. cruentus*, respectively. Colonies of *A. alternata* from *A. paniculatus* were light grey, those from *A. cruentus* were usually grey, and those from *A. retroflexus* varied from light grey, through grey, to dark grey (Table 3; Rayner 1970).

Isolates of *A. alternata* pathogenic to *Amaranthus* generally produced necrotic lesions on hypocotyls and leaves of seedlings, and caused them to wilt and die.

Twenty four *A. alternata* isolates were pathogenic to *A. cruentus*. They caused an initial necrosis of the shoot base followed by wilting and death of the seedlings. Four *A. alternata* isolates infected more than 70% of plants. They originated from *A. cruentus* and *A. retroflexus,* mostly from Łosiów (CL3/05, RL2/05, RL3/05, RP2/05). Two isolates (CL3/05, RP2/05) infected only seedlings grown from seeds that had been collected in Pawłowice, and the other two isolates (RL2/05, RL3/05) infected only seedlings grown from seeds collected in Łosiów. The non-pathogenic isolates were collected from *A. retroflexus* seeds (RP4/05, RL4/05, Pawłowice and Łosiów, in 2005, Table 3).

Twenty one *A. alternata* isolates were pathogenic to *A. paniculatus*. One isolate (RS6/05, from *A. retroflexus,* Swojec) infected more than 70% of plants. The non-pathogenic isolates were collected from *A. cruentus* seeds, from Łosiów, in 2004 (Table 3).

There was a large difference between the numbers of plants of *A. cruentus* and *A. paniculatus* infected by *A. alternata*. The percentage of infected plants ranged from 6.25 to 81.25. Generally, infection of *A. cruentus* and *A. paniculatus* plants was not affected by the origin of the *A. alternata* isolates.

Alternaria alternata was reisolated from the inoculated and diseased plants and identified as being identical to the original.

The electrophoretic profiles of the amplification products using primers OPA9, OPB1 and OPB2 yielded a total of 16 (OPA9) or 78 (OPB1, OPB2) markers with molecular weight range between 100 and 900 bp. The electrophoretic profiles of products using primers OPA5 and OPA10 yielded 15 markers with molecular weight range 200–750 and 150–800 bp, respectively. Primers OPB1 and OPB4 produced 17 and 15 DNA fragments with molecular weight range 100–900 and 100–800 bp, respectively. Primer OPB1 amplified high molecular weight DNA fragments more often than primer OPB4.

The dendrogram based on the presence or absence of amplified DNA fragments shows the genetic relationships within the *A. alternata* population (Fig. 1). It contains two well-separated clusters with very high (almost 100%) percentage of ge-

Table 3

Pathogenicity and morphology of *Alternaria alternata* isolates

Origin of *A. alternata*	Code of the isolate	Pathogenicity on *A. cruentus*				Pathogenicity on *A. paniculatus*		Colony colour	Colony diameter (cm)
		seeds collected in Pawłowice		seeds collected in Łosiów		seeds collected in Biskupin			
		symptoms	infected plants (%)	symptoms	infected plants (%)	symptoms	infected plants (%)		
A. cruentus	CP1/05	+	46.2	+	68.7	+	56.2	Grey	5.5
	CP2/05	+	42.9	+	56.2	+	29.4	Grey	4.8
	CP4/05	+	12.5	+	60.0	+	29.4	Grey	5.3
	CP5/05	+	13.3	+	40.0	+	68.8	Grey	5.2
	CL1/05	+	66.7	+	50.0	+	35.7	Grey	5.4
	CL3/05	+	73.3	+	46.7	+	20.0	Grey	5.0
	CL4/05	+	46.2	+	15.4	+	30.8	Grey	5.1
A. retroflexus	RP2/05	+	37.5	+	81.2	+	18.8	Light grey	6.2
	RP3/05	+	25.0	+	37.5	+	12.5	Grey	5.2
	RP4/05	–	–	+	50.0	+	37.5	Grey	5.6
	RL1/05	+	53.3	+	30.8	+	53.9	Light grey	6.2
	RL2/05	+	71.4	+	50.0	+	7.1	Light grey	6.0
	RL3/05	+	43.75	+	84.6	+	46.1	Dark grey	6.3
	RL4/05	–	0	+	50.0	+	18.7	Dark grey	5.9
	RS2/05	+	35.7	+	57.1	+	25.0	Light grey	6.3
	RS3/05	+	42.9	+	46.7	+	56.2	Grey	5.9
	RS6/05	+	46.7	+	20.0	+	71.4	Light grey	6.7
A. paniculatus	PB1/05	+	53.3	+	46.7	+	31.2	Light grey	6.5
	PB6/05	+	7.1	+	18.8	+	6.2	Light grey	6.6
	PB7/05	+	25.0	+	26.7	+	46.7	Light grey	6.8
A. cruentus	CL1/04	+	20.8	+	34.8	–	0	Light grey	5.0
	CL2/04	+	16.7	+	22.8	–	0	Grey	5.2
	CL5/04	+	16.7	+	58.3	–	0	Grey	5.1
	CL7/04	+	8.0	+	17.3	–	0	Grey	5.1
	CL9/04	+	20.8	+	29.2	–	0	Grey	5.2
	CL10/04	+	38.0	+	20.8	+	6.7	Grey	5.3

"+" – with symptoms on seedlings, "–" – with no symptoms on seedlings.

netic difference. In both clusters there are two subclusters, with genetic difference between 38 and 52%.

The first cluster does not include *A. alternata* isolates from *A. paniculatus* or isolates from Biskupin. The second cluster includes *A. alternata* isolates from all three

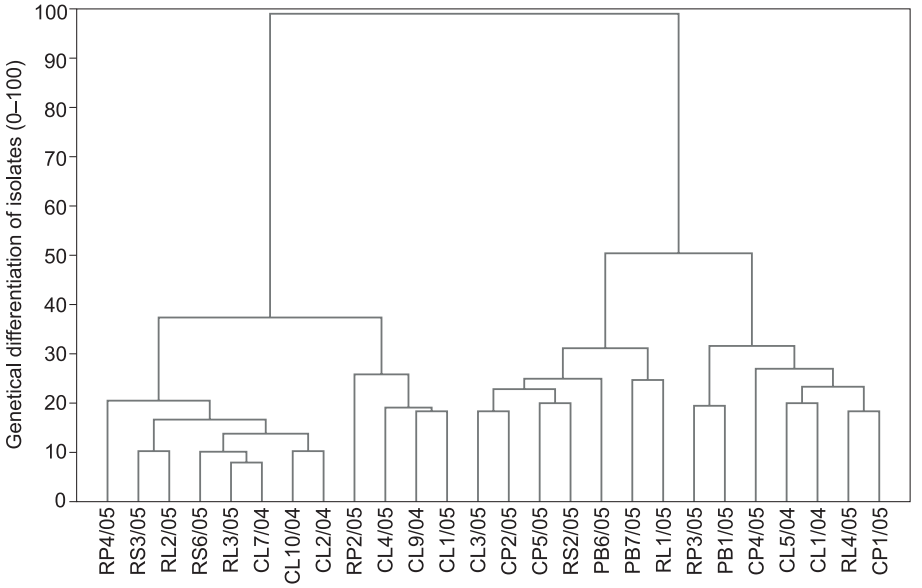

Fig. 1. Relationship among 26 isolates of *Alternaria alternata* originating from *Amaranthus* seeds

Amaranthus species and all four locations. However, some effect of host-plant and of geographical origin on intraspecific genetic variation in *A. alternata* is suggested by the architecture of the two clusters. The first sub-cluster in first cluster includes a group of four isolates originating from *A. retroflexus* (RL2/05, CP3/05, RS3/05, RS6/05), a group of three isolates originating from *A. cruentus* (CL2/04, CL7/04, CL10/04); five of these isolates came from Łosiów (CL2/04, CL7/04, CL10/04, RL2/05, RL3/05). The second sub-cluster includes three isolates originating from *A. cruentus* grown in Łosiów (CL9/04, CL1/05, CL4/05). The genetic variation among isolates from the same host-plant or location was low and ranged between 8 and 20%.

Only the second cluster includes *A. alternata* isolates originating from *A. paniculatus*. Its first sub-cluster grouped three isolates from *A. cruentus* (CL3/05, CP2/05, CP5/05) and its second sub-cluster grouped another four isolates from *A. cruentus* (CL1/04, CL5/04, CP1/04, CP1/05). The genetic variation between isolates from the same host-plant or location was usually greater than in the first cluster and ranged between 18 and 32%.

Discussion

The *A. alternata* population studied was genetically only moderately heterogeneous. Neighbour-joining clustering based on OPA and OPB primer profiles only partly grouped the isolates according to host-plant and geographical origin.

A few isolates originating from *A. cruentus* were genetically similar to a few isolates originating from *A. retroflexus*. This suggests that *A. retroflexus*, which is a common annual weed worldwide and found in a wide range of habitats, may be a source of *A. alternata* inoculum for *A. cruentus*. This may create a risk situation for *A. cruentus* if cultivated on a larger scale.

The results presented are in agreement with observations of Peever et al. (2000), who also showed that similar pathogenicity was associated with genetic similarity within an *A. alternata* population originating from different citrus host--plants and geographical locations. These findings suggest the ability of *A. alternata* to adapt easily to closely related host-plants.

Alternaria alternata is considered to have potential for biological control of *A. retroflexus*. Ghorbani et al. (2000) and Lawrie et al. (2000, 2002) confirmed its usefulness as a mycoherbicide under specific environmental conditions. The pathogenicity test showed that the majority of *A. alternata* isolates, regardless of original host-plant, can be pathogenic on *A. cruentus* and *A. paniculatus*. This means that the selection of *A. alternata* isolates suitable for potential biological control in *Amaranthus* would be difficult (Blodgett and Swart 2002).

The results presented do not agree with observation of Guo et al. (2004), who found high genetic variation within 112 *A. alternata* isolates originating from *Pinus tabulaeformis* and that *A. alternata* appears to have the potential for relatively quick evolution, which may lead to significant diversification.

In the present case, however, the low genetic variation found in the *A. alternata* population may be a result of high adaptive ability of the fungus and also of the relatively close proximity of the collection sites and host-plants. It may also be partly a result of uniform dispersal by wind of *A. alternata* spores, which may travel hundreds of miles or only a few hundred meters from the same source (Bashan et al. 1991).

Conclusions

1. *Alternaria alternata* is a pathogen of *Amaranthus cruentus* and *A. paniculatus* in Poland with low host-specificity.

2. Isolates from a population of *A. alternata* from seeds of *A. cruentus*, *A. paniculatus* and *A. retroflexus,* from four geographical locations in south-west Poland studied by RAPD-PCR using OPA and OPB primers, were only moderately genetically heterogeneous.

Streszczenie

MORFOFIZJOLOGICZNE I MOLEKULARNE ANALIZY *ALTERNARIA ALTERNATA* Z NASION AMARANTUSA

Dwadzieścia sześć badanych izolatów *Alternaria alternata* izolowanych z nasion *Amaranthus cruentus, A. paniculatus* i *A. retroflexus* infekowało siewki *A. cruentus* i *A. paniculatus*. Objawem porażenia było zbrunatnienie i przewężenie łodyżki, a następnie zamieranie siewki. Kolonie *A. alternata* różniły się od siebie dynamiką wzrostu oraz kolorem. W wyniku przeprowadzonych badań molekularnych z wykorzystaniem metody RAPD-PCR stwierdzono prawdopodobny wpływ lokalizacji i gatunku gospodarza na różnorodność genetyczną badanej populacji *A. alternata*.

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