Eur J Plant Pathol (2010) 128:311–316
DOI 10.1007/s10658-010-9665-2

Formation of chlamydospores and microsclerotia in *Alternaria dauci*

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Accepted: 12 July 2010 / Published online: 30 August 2010

Abstract *Alternaria dauci* (Kühn) Groves and Skolko, the cause of leaf blight of carrot, was observed to produce chlamydospores and microsclerotia *in vitro*. Four different isolates produced chlamydospores on potato dextrose agar, V8 agar and Czapek-Dox agar at 10, 18 and 28°C, from neighbouring cells of hyphae of the submerged mycelium, forming long chains. Formation of chlamydospores was completed in a multiple stage process, including swelling of cells, development of secondary septa, rounding up of cells and cell wall thickening. Mature chlamydospores were released from the mycelium mostly in clusters and in short or long chains, and sometimes in pairs. They were globose to ellipsoid, smooth or verrucose, pale brown in colour, and averaged 13.87×11.37 μm, (length × width). Microsclerotia were formed on PDA, by only one isolate, at 28°C first at the edges of the colony, by close contact of hyphae that exhibited extensive branching and intermingling. In their final form microsclerotia were multi-celled, closely attached masses of short and broad dark grey cells, less compact woven at the edges of the microsclerotium. This is the first illustration of chlamydospore and microsclerotia formation in *Alternaria dauci*.

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Keywords Carrot leaf blight · Resting bodies

As Neergaard (1945) stated in his monograph "*Alternaria* themes and variations", characteristic gemmae may be encountered occasionally in most, perhaps all *Alternaria* species. Moreover, Joly (1964) described thick cylindrical cells and anastomoses formed in the submerged mycelium of *Alternaria*. The phenomenon is probably familiar to anyone who has handled significant numbers of aging or refrigerated cultures of *Alternaria* isolates, and is not species-diagnostic. According to the definition of Ainsworth (1971), gemmae are chlamydospores. However, reports of chlamydospores and microsclerotia in *Alternaria* are rare, which, according to Rotem (1994), implies either an insufficient examination of the overseasoning structures or their low frequency in the residual fungal material. Brief reports of chlamydospore formation exist for 11 *Alternaria* species, namely, *A. bursnii* Uppal et al. (Uppal et al. 1938), *A. raphani* Groves & Skolko (Atkinson 1953; Narain and Saksena 1975), *A. porri* (Ellis) Cif. f. sp. *solani* (syn. *A. solani* Sorauer) (Basu 1971; Patterson 1991), *A. brassicae* (Berk.) Sacc. (Tsuneda and Skoropad 1977), *A. tenuissima* (Kunze ex Pers.) Wiltshire (Mohan and Mukerji 1978), *A. carthami* Chowdhury (Narain 1982), *A. longissima* Deighton and MacGarvie (Ellis 1971; 976), *A. chlamydospora* Mouchacca (Ellis 1971, 1976), *A. phragmospora* van Emden (Ellis 1971, 1976) *A. kikuchiana* Tanaka (Tanaka 1933) and

A. alternata (Fries) Kleissler (Amrani and Najim 1985; El Shafie and Webster 1979; Lagopodi and Thanassoulopoulos 1995). At this point it has to be made clear that the taxonomic status of certain of the above mentioned species is unclear at present (Simmons 2007). *A. raphani* is a synonym of *A. japonica* Yoshii, *A. longissima* and *A. phragmospora* have been renamed to *Prathoda longissima* (Deighton and MacGarvie) Simmons, and *Embellisia phragmospora* (van Emden) Simmons, respectively, while *A. kikuchiana* is considered a substitute name for *A. gaisen* Nagano.

In the above mentioned *Alternaria* or *Alternaria*-related species, chlamydospores are formed in a variety of manners and under various conditions on natural substrata and in culture. Chlamydospores formed by abnormal swelling of mycelial cells in intercallary or terminal chains have been observed in most cases, namely in *A. phragmospora* (Ellis 1971, 1976), *A. chlamydospora*, (Ellis 1971, 1976), *A. raphani* (Narain and Saksena 1975; Ellis 1971, 1976), *A. solani* (Basu 1971; Patterson 1991) *A. bursnii* (Uppal et al. 1938), *A. kikuchiana* (Tanaka 1933), and *A. alternata* (Lagopodi and Thanassoulopoulos 1995). However, formation of chlamydospores within conidial cells or in an extension of the conidium beak has also been observed, as in the cases of *A. brassicae* (Tsuneda and Skoropad, 1977), *A. solani* (Patterson 1991), *A. tenuissima* (Mohan and Mukerji 1978), *A. carthami* (Narain 1982) and *A. alternata* (Tanaka 1933). Most of the above reports refer to structures formed in cultures, while *A. solani* produced chlamydospores in infected tomato tissues (Basu 1971; Patterson 1991), and *A. brassicae* from conidia placed in natural soil, at room temperature (Tsuneda and Skoropad 1977). Chlamydospores of *A. alternata* have also been observed in nature on sunflower debris (Lagopodi and Thanassoulopoulos 1995).

On the other hand, microsclerotia have been reported in only few *Alternaria* species, namely *A. padwickii* (Ganguly) Ellis (1971, 1976), which is a synonym of *Trichoconiella padwickii* (Ganguly) Jain (Simmons 2007), *A. brassicae* (Tsuneda and Skoropad 1977), *A. alternata* (Amrani and Najim 1985), and *A. argyranthemi* Simmons and Hill (Simmons 2007), while in *A. smyrnii* (Grouan) Simmons multicellular microsclerotia give rise to megaconidiophores (Simmons 2007). In the case of *A. brassicae*, microsclerotia are formed from conidia, while in *A. alternata, A. argyranthemi, A. smyrnii,* and presumably in *A. padwickii,* they are formed by proliferation and aggregation of hyphal cells. However, the hyphal structures described in the above studies, whether as microsclerotia, or chlamydospores, resemble one another significantly regarding both appearance and formation mode, but they presumably differ in size.

Except for the above, there is one brief report on chlamydospore and microsclerotia formation by *A. dauci* (Kuhn) Groves and Skolko (Rukshenaite-Beretskene 1968). In this report it was mentioned that this fungus produces chlamydospores by thickening of mycelial cells and microsclerotia in dried cultures. However, this report gives no details, illustrations or measurements for either chlamydospores or microsclerotia formation and appearance. In the present work, a detailed description of chlamydospore and microsclerotia formation by mycelial cells in *A. dauci* is given, based on progressive observations *in vitro*.

Table 1 Formation of chlamydospores and microsclerotia by four isolates of *Alternaria dauci* on water agar (*WA*), potato dextrose agar (*PDA*), V8 agar (*V8A*) and Czapek-Dox agar (*CDA*), incubated at 10, 18 and 28°C

		Isolates							
		CBS 101592		1A		1B		1C	
		chl[a]	msc	chl	msc	chl	msc	chl	msc
WA	10°C	–[b]	–	–	–	–	–	–	–
	18°C	–	–	–	–	–	–	–	–
	28°C	–	–	–	–	–	–	–	–
PDA	10°C	+	–	+	–	+	–	++	–
	18°C	+	–	+	–	+	–	++	–
	28°C	++	+	+	–	++	–	++	–
V8A	10°C	+	–	+	–	+	–	+	–
	18°C	+	–	+	–	+	–	+	–
	28°C	++	–	+	–	+	–	+	–
CDA	10°C	+	–	+	–	+++	–	+++	–
	18°C	++	–	++	–	++	–	++	–
	28°C	++	–	++	–	+++	–	+++	–

[a] *chl* chlamydospores, *msc* microsclerotia

[b] Three replicates-Petri dish cultures were used per combination of isolate-growth medium- temperature The experiment was repeated twice. Each result comes from the observation of six plates.

– no chlamydospores or microsclerotia present, + a few, ++ many, +++ abundant

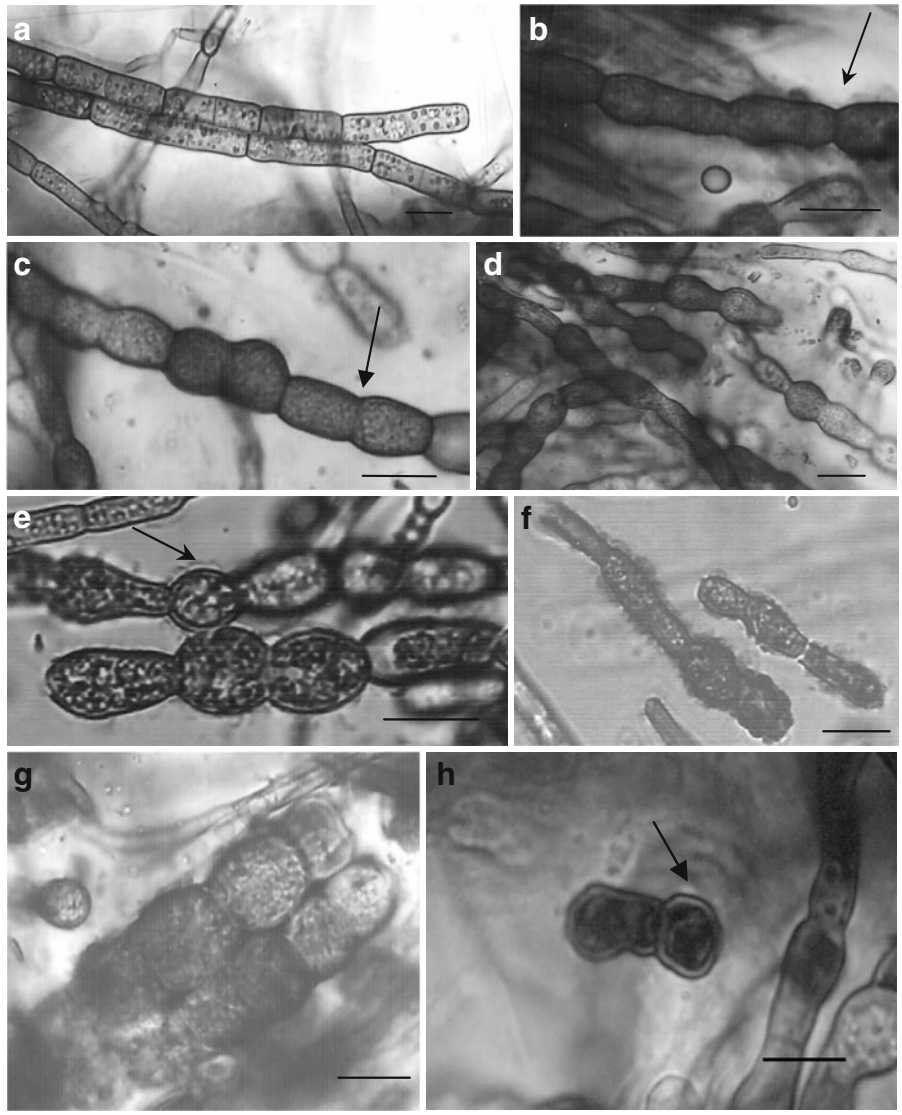

Fig. 1 Stages of chlamydospore formation in the hyphae of the submerged mycelium of *Alternaria dauci.* **a** Non-differentiated hyphae of the submerged mycelium. **b** Swelling of hyphae indicated by constriction at septa (*arrow*). **c** Development of secondary septa (*arrow*). **d** Rounding up of cells. **e** Cell wall thickening (*arrow*). **f** Verrucose chlamydospores released in pairs. **g** Chlamydospores in clusters. **h** Smooth chlamydospores in pairs. Bars on all figures represent 10 μm. Figures a-g are from isolate CBS 101592 grown on PDA, at 28°C, and figure h is from isolate 1C grown on PDA, at 18°C

Four pathogenic isolates of *A. dauci* were used in this study. One isolate designated as CBS 101592 was provided by the Centraal Bureau voor schimmelcultures, The Netherlands, while 3 more isolates designated as 1A, 1B and 1C were kindly offered by Dr. Crystyna Tylkowska, Poznan University of Life, Poland. Cultures were made on 4 different media, potato dextrose agar (PDA), V8 agar (V8A), water agar (WA) and Czapek-Dox agar (CDA), by inoculating 20 ml of each medium in plastic Petri dishes, with a 5 mm disk taken from the edge of actively growing fungal colonies. The dishes were sealed with parafilm to avoid excessive dryness, and the cultures were incubated at three different temperatures 10, 18 and 28°C throughout the experimental period. Observations at all temperatures started 5 days after inoculation. For cultures incubated at 28°C observations were carried out every 2 days during a five week period. Cultures at 18°C were observed at 7-day intervals for 3 months, while those at 10°C, at 10-day intervals for 5 months. Three Petri dishes, comprising of 3 replicates, were used per isolate, for each medium, at each temperature. The experiment was performed twice. Germination was checked by placing the chlamydospores in drops of distilled water in van Tiengem cells at 28°C for 24 h. Resistance of microsclerotia to freezing

was checked by placing them at -20°C for 15 days and subsequently transferring them in new PDA plates.

All isolates used in this study produced chlamydospores in all media except WA, at all temperatures (Table 1). Identical results were obtained in all replicates of each treatment. Production of chlamydospores depended mainly on the medium used and to a lesser extent on the isolate. In some cases temperature affected this process differently, depending on the medium. In particular, all isolates produced chlamydospores more abundantly on CDA. Overall, production of chlamydospores was most pronounced in isolate 1C, and least in isolate 1A. The higher temperatures promoted chlamydospore production by isolate CBS 101592 on PDA, V8A and CDA, by isolate 1A only on CDA, and by isolate 1B only on PDA. In isolates 1B and 1C more chlamydospores were produced at the lowest and at the highest temperature, as compared to the intermediate temperature, on CDA only.

Production of chlamydospores was completed through a multi-stage process. The events described below as successive stages were observed in all combinations of isolate-medium-temperature where chlamydospores were produced. As a first step, differentiation of cells of the submerged mycelium (Fig. 1a) was observed and it was characterized by a slight swelling of cells (Fig. 1b). At the same time, development of secondary septa was observed in these cells (Fig. 1c). In this way more cells were produced on each hypha that underwent differentiation. The cells formed in such a way eventually obtained a round form (Fig. 1d). Later on thickening of cell wall was noticed (Fig. 1e). At the last stage of development, the thallospores formed were sphaerical or ellipsoid, with smooth or verrucose surface (Fig. 1f, h), light brown in colour, and measuring 10.8–16.2×8.1–16.2 μm (average 13.87×11.37 μm, length×width). They were released from bearing hyphae mostly in clusters or in short or long chains, and sometimes in pairs or single (Fig. 1f, g, h). The complete set of alterations described above proceeded on PDA, CDA and V8A and it was faster at higher temperatures. The germinating ability of the produced chlamydospores was confirmed in drops of sterile distilled water, in van Tiengem chambers, at 28°C, where they germinated in 24 h.

Microsclerotia were observed only in isolate CBS 101592 on PDA, at 28°C. They were first observed as mycelial aggregates, in the form of little nods, visible by naked eye, at the edge of the culture, when the mycelium came in contact with the wall of the Petri dish. Microscopic observation of these structures showed extensive branching of hyphae of the aerial mycelium (Fig. 2a) that became darker, and grew towards one another starting to intermingle (Fig. 2b). Eventually structures of dark, multi-celled, closely

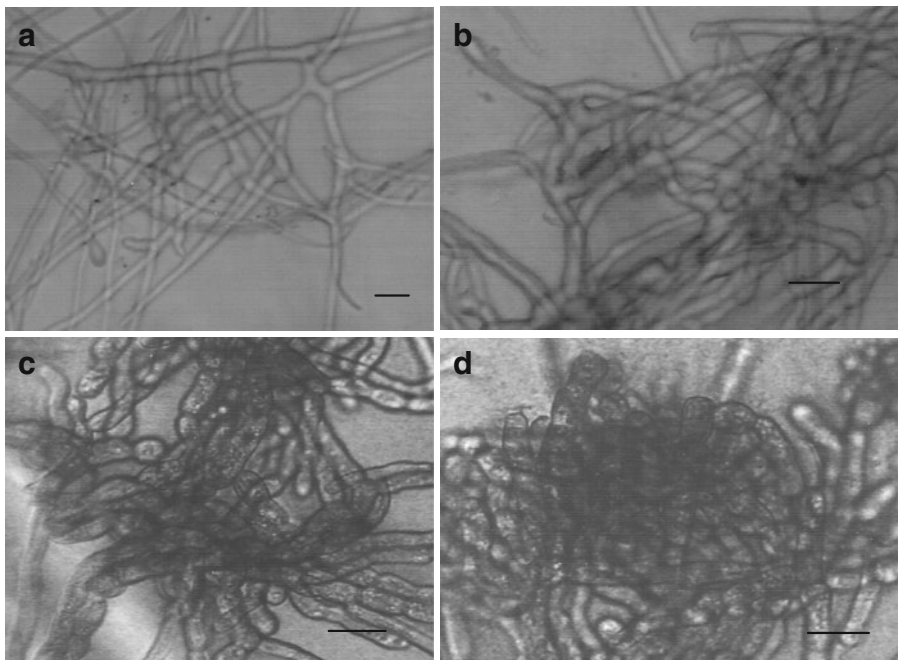

Fig. 2 Stages of microsclerotia formation in *Alternaria dauci*. **a** Extensive branching of hyphae. **b** Hyphae that darken, grow towards one another and intermingle. **c** Formation of closely attached multi-celled mycelial masses by intermingled hyphae. **d** Final form of microsclerotia with loosely arranged hyphae at their edges. Bars on all figures represent 10 μm. All figures are from isolate CBS 101592 grown on PDA, at 28°C

attached masses of short and wide dark grey cells were produced (Fig. 2c). The microsclerotia were easily visible as dark pin-point to pin-head size bodies, ranging 11.30–89.10 μm in diameter, and appeared first at the periphery of the colony. Microscopic observations showed that microslerotia in their final form were composed of compact masses of cells. However, compared to classic sclerotia from other fungi, they were less compact woven, and relatively flat, and they had no clearly defined zones at their edges, where hyphae were more loosely arranged (Fig. 2d). As cultures desiccated, microsclerotia were distributed all over the colony. Exposure of microsclerotia to -20°C for 15 days did not affect their ability to reproduce the fungus, and colonies with a regular growth rate were generated from them. Closely related multi-celled structures were observed in almost all isolates. Especially in isolate 1C, such structures were also visible by naked eye at 18°C, on PDA. However, with the exception of isolate CBS 101592 on PDA, at 28°C, these structures were loosely woven and never resulted in the well defined compact masses of cells described above.

According to Ainsworth's definition (1971) "chlamydospore is a thick-walled intercalary or terminal asexual spore that is created by the rounding up of a cell or cells", whereas microsclerotium is "a firm, frequently rounded, mass of hyphae with or without the addition of host tissue or soil, normally having no spores in or on it". Comparing these definitions to the way the above mycelial structures, produced by *A. dauci,* were formed and functioned it is concluded that the thallospores produced by the differentiation of mycelial cells are chlamydospores, whereas the multi-celled mycelial masses are microsclerotia. Germination of chlamydospores regenerated mycelium and microsclerotia resisted deep freezing. These observations confirm their function as resting bodies and justify the attribution of the terms to them.

According to the results of this study, production of chlamydospores in *A. dauci* seems to be a common phenomenon among various isolates, as previously reported for most *Alternaria* species by Neergaard (1945), but it takes place on rich media and is affected by temperature. On the other hand, production of microsclerotia in this species seems to happen rarely, which explains the existence of one single reference (Rukshenaite-Beretskene 1968). While the growth medium and temperature affected the production of chlamydospores in a variable manner, these two factors dramatically affected the production of microsclerotia.

A. dauci causes leaf blight of carrot, a severe disease especially prevalent in the temperate zone. The pathogen is disseminated in and on seed, and overwinters in soil-borne debris from diseased tissue and on weed hosts, where it produces spores at all temperatures during the growing season (Pryor et al. 2002). The infected seeds, host debris, and infected volunteer carrot plants on which spores are produced are considered to be the main source of inoculum, and control measures are concentrated mainly on seed disinfestations and sprays (Sherf and Macnab 1986).

Formation of chlamydospores and microsclerotia in pathogenic fungi is an interesting phenomenon, since their main function is to preserve inoculum as resting bodies, especially in fungi where the sexual stage is not known or extremely rare. Additional experiments *in vivo* will possibly confirm the formation of chlamydospores and microsclerotia from *A. dauci* in nature as well. Probably, the thick-walled mycelium or chlamydospores and microsclerotia-like mycelial aggregates as observed on agar plates contribute to the survival of this pathogen. This would be an important feature in epidemiological studies and control strategy of the carrot blight disease.

Acknowledgements We are grateful to Dr. Krystyna Tylkowska, Department of Seed Science and Technology, Poznan University of Life, Poznan, Poland, for providing *Alternaria dauci* isolates 1A, 1B and 1C.

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