

Epidemiology, histopathology and aetiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal

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Anthracnose is an important disease affecting mature olive fruits, causing significant yield losses, and poor fruit and oil quality. In Portugal, high anthracnose incidence was recorded during 2003–2007 with 41% of 908 orchards surveyed displaying disease symptoms. In another 14% of the orchards, the pathogen was recorded in symptomless plants. Disease severity was on average 36%, frequently reaching 100%. In Portugal, anthracnose is endemic to neglected orchards of susceptible cultivars, but under favourable conditions it can also severely affect less susceptible cultivars. Pathogens were genetically heterogeneous, with *Colletotrichum acutatum* genetic group A2 as the most frequent (80%), followed by group A4 (12%) and group A5 along with *C. gloeosporioides* (3–4%), while groups A3 and A6 of *C. acutatum* were sporadic. Important geographic variations were observed in the frequencies of these populations, accompanied by year-to-year populational shifts. Epidemiology and histopathology studies showed the presence of the pathogens on vegetative organs year-round, particularly on olive leaves and branches, and on weeds. These represent inoculum reservoirs where secondary conidiation occurs, and conidia are then dispersed by spring rains reaching flowers and young fruits or by autumn rains reaching pre-mature fruits. Unripe fruits were colonized without showing symptoms up to penetration of the cuticle, but further colonization and symptom production was completed only as fruits matured. These findings challenge current control practices, particularly the timing of fungicide treatment, and contribute to improved disease management.

Keywords: aetiology, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, epidemiology, histopathology, olive anthracnose

Introduction

Olive (*Olea europaea* ssp. *europaea* var. *europaea*) is an important crop, cultivated in most Mediterranean-climatic regions of the world, covering an area of ca. 9.4×10^6 ha and an overall olive oil production of ca. 2.4×10^9 kg per year (Barranco *et al.*, 2004). Anthracnose caused by *Colletotrichum acutatum* and

C. gloeosporioides is a major disease of olive affecting fruits at maturity, leading to significant yield losses and poor fruit and oil quality. In Portugal the disease has been at epidemic proportions since at least the 19th Century, frequently causing total yield losses (Almeida, 1899; Talhinhos *et al.*, 2005), rivalling olive fruit fly, *Bactrocera oleae*, as the most important phytosanitary problem. This has led farmers to either harvesting the crop early or abandoning production altogether, which in the latter case could contribute to high inoculum levels in the subsequent season. Olive anthracnose is also of major concern in Australia, Italy, Montenegro and Spain, and has been reported from all continents. Varied *C. acutatum* genetic groups (Sreenivasaprasad & Talhinhos, 2005) and *C. gloeosporioides* are known to be associated with olive anthracnose in different countries. In Portugal, *C. acutatum* is dominant (97% of populations) and the genetic group A2 is widely prevalent (Talhinhos *et al.*, 2005). Shifts in pathogen populations and the origin and impor-

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tance of the smaller genetic groups including *C. gloeosporioides* have not yet been investigated.

Olive anthracnose symptoms typically occur on olive fruits at maturity as dark sunken necroses, with abundant production of orange masses of conidia. Although less frequent, symptoms can also be found on branches and leaves, leading to necroses, severe defoliation, death of branches and reduced tree vigour. However, a number of key questions in relation to epidemiological and host interaction processes remain unanswered. For example, it is unclear how and in which form the inoculum is maintained from the end of an anthracnose outbreak in early winter until the symptoms appear the following autumn. Mummified fruit present either on the trees or in the soil and other dead organs are assumed to act as sources of inoculum. The relative importance of other organs, weeds and neighbouring crops to inoculum survival and the pathogenesis cycle has not been fully understood. This is particularly relevant, as examples from other *C. acutatum* pathosystems revealed the presence of inoculum on different symptomless organs, e.g. in peduncles and branches in almond and sour cherry (Förster & Adaskaveg, 1999; Stensvand *et al.*, 2003). In *Colletotrichum* species-host interactions, early steps up to the penetration of plant cuticle are essentially the same, but then the pathogens can follow different infection strategies, namely with or without an initial biotrophic phase or restricted to a biotrophic phase on non-target organs (Diéguez-Uribeondo *et al.*, 2005; Peres *et al.*, 2005; Wharton & Schilder, 2008). *Colletotrichum* species also exhibit diverse host colonization strategies (Perfect *et al.*, 1999), ranging from very short penetration, infection and symptom-development time up to long latent infections. For the olive anthracnose pathosystem, under Mediterranean climatic conditions, the summer is warm and dry, which is generally unfavourable for olive anthracnose dissemination and infection. It has therefore been assumed that the inoculum reaches fruit during autumn, carried by rain drops from reservoirs such as mummified fruit (Trapero & Blanco, 2004). This has led to crop protection strategies centred on autumnal spraying of copper-based fungicides. However, latent olive fruit infection resulting from spring inoculum dispersal has been documented (Mota-Capitão *et al.*, 2008; Moral *et al.*, 2009), resembling the blueberry anthracnose system (Verma *et al.*, 2006, 2007). Nevertheless, the relevance of this phenomenon for olive anthracnose severity in autumn in relation to meteorological conditions and the relative importance of other organs for inoculum overwintering has not been established.

The major objectives of this work were to elucidate key epidemiological and host interaction processes in the olive anthracnose pathosystem along with an improved understanding of spatial and temporal shifts in pathogen populations and anthracnose incidence and severity in Portugal. The genetic and pathological diversity of pathogen populations were characterized and mapped along with disease incidence data across key cropping regions in Portugal. Epidemiological and histopathological stud-

ies were conducted on different olive orchards, cultivars and organs with the aims of understanding the importance of non-target organs for inoculum maintenance, the importance of symptomless fruit infection for disease severity and the effect of agronomical and meteorological conditions in these traits.

Materials and methods

Geography and meteorology

Main olive growing areas of Portugal were surveyed during autumn to record anthracnose incidence and severity and to characterize pathogen populations. Areas with higher olive cultivation were more intensively monitored (1999 olive cultivation data obtained from Statistics-Portugal; <http://www.ine.pt>). In each surveyed locality, besides anthracnose data, topography and agronomic characteristics were recorded, including type of orchard (old and modern) and of olive tree (wild olive, trees in urban areas and cultivated tree within other crops), irrigation and soil mobilization. Such information was also used to select a contrasting set of orchards for more detailed epidemiological studies. Meteorological data from selected weather stations were obtained via the Water Institute of the Ministry for Environment and Spatial Planning, Portugal (<http://snirh.pt>).

Anthracnose incidence and severity

Disease incidence was recorded as the proportion (%) of orchards where symptoms on mature fruits were observed among the 908 orchards surveyed. In addition, the proportion of orchards with inoculum present but without any symptoms was assessed by incubating mature fruits in a wet chamber (100% relative humidity, 22°C) for 1–3 weeks and checking for the appearance of symptoms. Results were recorded per region and per year. Severity was scored as the percentage of fruit with symptoms in each orchard assessed in at least 100 fruit from a minimum of five trees (except for isolated trees).

Aetiology and pathogen populations

Pathogen isolation was performed from spore masses on fruits with anthracnose symptoms using potato dextrose agar (PDA; Difco) medium amended with an inhibitor of bacterial growth (KCNS 50 mM). Single-spore cultures were prepared and a collection of 530 *Colletotrichum* species isolates associated with olive anthracnose in Portugal was established and stored at the authors' laboratories. These isolates were characterized using molecular and morphological methodologies described previously (Talhinhos *et al.*, 2005) and the pathological and agronomic information related to each isolate was recorded.

For each isolate, DNA was extracted using a rapid freeze-boil protocol (Talhinhos *et al.*, 2008) and the species identity (*C. acutatum* or *C. gloeosporioides*) was established by diagnostic PCR of part of the β -tubulin 2

(*tub2*) gene (Talhinhas *et al.*, 2005). Intra-specific diversity among *C. acutatum* isolates was assessed by PCR-RFLP of the same region of *tub2* and the isolates were assigned to various genetic groups (Sreenivasaprasad & Talhinhas, 2005; Talhinhas *et al.*, 2005). Isolates belonging to diverse *C. acutatum* groups and *C. gloeosporioides* were grown on 9 cm PDA plates at 25°C for 7 days and typical colony characteristics, especially colony colour and growth rate, were recorded (Talhinhas *et al.*, 2005).

Epidemiology

Five orchards were periodically monitored (Fig. 1) over a 2-year period (2006–2007) to investigate the inoculum survival during the period with no disease symptoms (from late winter until beginning of autumn), and to establish disease progression curves. These orchards, selected among a larger network of 14 orchards monitored during 2004–2006 (data not shown), represent different agro-ecological locations in Portugal, as well as different disease incidence and severity scenarios. Among these five orchards, Tomar and Vila Viçosa represent neglected orchards planted with cv. Galega, where anthracnose frequently reaches high severity. The Elvas orchard, with cv. Bical, is a well maintained orchard with low anthracnose severity and where phytosanitary treatments are only rarely conducted. Olive anthracnose is also sporadic at the Rio Maior 1 orchard (cv. Cordovil), regularly treated with copper-based fungicides. Rio Maior 2 represents a set of neglected Galega olive trees, adjacent to the Rio Maior 1 plot, regularly showing high anthracnose severity.

Samples comprised 10–20 pieces each of leaves, dead leaves, year branches (season's growth), previous year branches, 2 or more year-old branches, dead branches, petioles, flowers, fruits, aborted fruits and mummified fruits, as well as perennial weeds and adjacent crops. Each sample was divided into two batches and one was surface disinfected by immersion in 7 g L⁻¹ NaClO for 1 min and rinsing in sterile distilled water, and the other not disinfected, in order to assess the endophytic and epiphytic presence of the pathogen, respectively. As results for both batches were similar, they are presented and discussed together. Samples were then placed in Petri dishes containing PDA supplemented with 50 mM KCNS. The dishes were incubated at 24°C for up to 2 weeks, to allow the identification of *Colletotrichum* species colonies, confirmed by microscopic observation of conidia. In order to quantify the presence of *Colletotrichum* species on samples, the proportion of tissue pieces from which the pathogens could be identified was calculated over the total number of incubated pieces.

Histopathology

Histopathological studies were conducted with *C. acutatum* isolate PT135 (group A2) and *C. gloeosporioides* (isolate PR220) following inoculation of leaves (upper and lower surface), branches, and immature, pre-mature

and mature fruits (Jáen maturation indices 0, 2–3 and 4–6, respectively; Beltrán *et al.*, 2004). Before inoculation, leaves and branches were disinfected for 15 min in 5 g L⁻¹ NaClO, washed in distilled water and air dried. Fruits were washed in distilled water only. Plant parts were inoculated with 5 µL droplets containing 10⁶ conidia mL⁻¹, incubated at room temperature at saturating humidity (in closed bags), and kept in the dark for the first 24 h after inoculation. Leaves, branches and fruits from different cultivars (Bical, Cobrançosa and Galega) were used, according to availability.

Light microscopy

In vivo conidial germination and appressoria formation on the surface of the olive organs were analysed 24 h after inoculation using the varnish mould technique (Silva *et al.*, 1999). This was carried out by laying colourless nail varnish over the inoculated area. About 24 h later, the nail varnish mould was removed with tweezers, stained and mounted with lactophenol cotton blue.

In order to evaluate the colonization process, fragments of fruits, leaves and branches were cut with a freezing microtome at different times after inoculation. The sections (22–27 µm thick) were stained and mounted in lactophenol cotton blue and estimation of hyphal length per infection site was made using a micrometric eyepiece.

Lactophenol cotton blue was found not to be appropriate for staining of mature fruit (as opposed to green fruits, leaves and branches) since it damaged the tissue structure and interfered with the preparation of microscopic slides. Therefore, different techniques to visualize fungal colonization by fluorescence microscopy were tested, using reagents prepared in an aqueous solution, as previously described (Hughes & McCully, 1975). This involved either immersing the sections in an aqueous solution containing 0.1 g L⁻¹ calcofluor White M2R (Sigma) for 20 s, washing and mounting in distilled water, or immersing the sections in 70 mM K₂HPO₄ pH 8.9 with 3 mL L⁻¹ diethanol, washing and mounting in distilled water.

In order to optimize the observation of colonization, small strips (2 × 1 mm) of fruit with or without visible symptoms, were fixed in 25 mL L⁻¹ glutaraldehyde (Merck) in 0.1 M sodium cacodylate buffer (Merck) at pH 7.1. After 2 h, the tissues were washed (3 × 20 min) in sodium cacodylate buffer and post-fixed with 39 mM osmium tetroxide (OsO₄; Merck) in the same buffer for 2 h. Tissues were dehydrated in a graded ethanol series (10–90% at 10% increments) for 10 min each and twice at 100% ethanol for 30 min, embedded in Spurr's resin (TAAB) and polymerized overnight at 70°C. As it was difficult to impregnate plant material with resin, alterations to the described technique were introduced. The dehydration periods in ethanol were increased (30 min at each dehydration point up to 70%; 1 h each at 80% and 90%; two rinses with 100% ethanol for 1 h each) and the embedding in Spurr's resin was more gradual (proportion resin/ethanol: 1:3, 1:2, 1:1, 2:1 and 3:1 for 48 h each), performed at 4°C and with agitation (*ca.* 10 rpm). Semi-thin sections were cut (2 µm), stained with

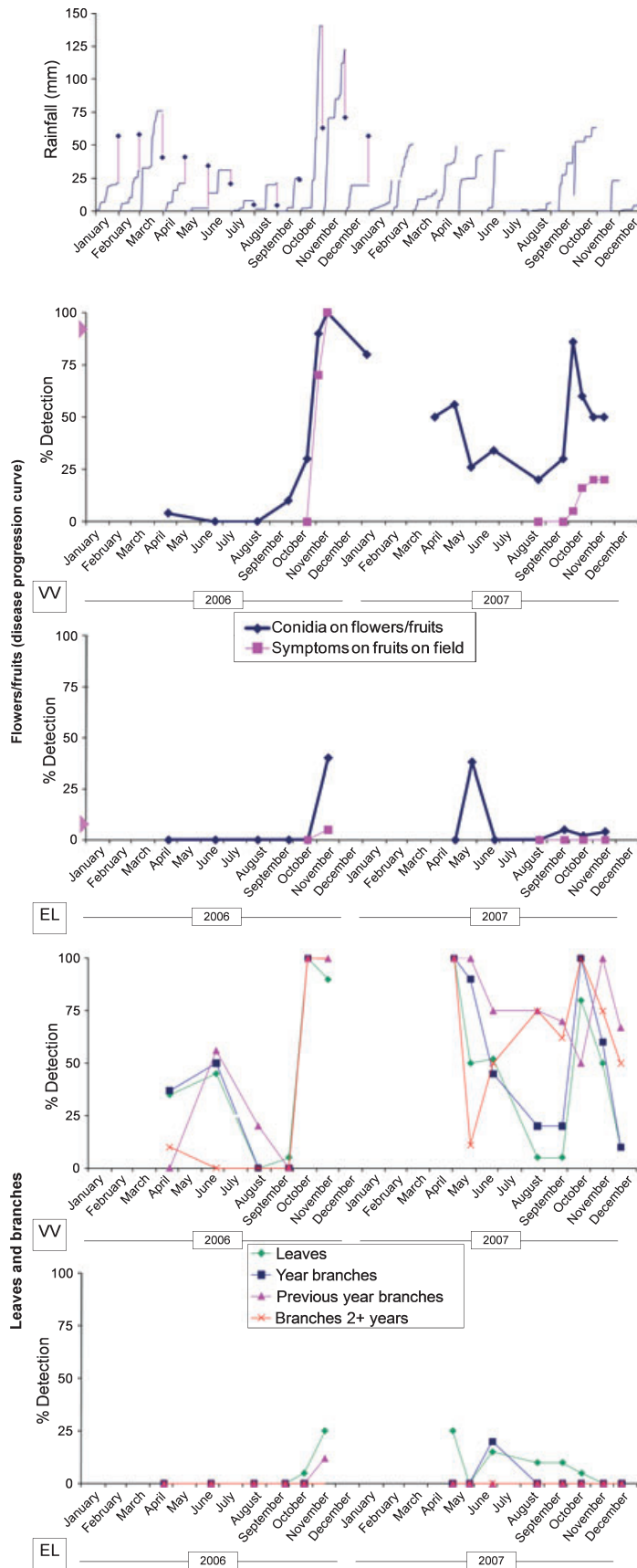


Figure 1 Epidemiological observations including disease progression curve in two contrasting orchards during 2006 and 2007 assessed as the proportion of organs where *Colletotrichum acutatum* was present, either without symptoms, on leaves/branches and on flowers/fruits, or causing symptoms on mature fruits in the field, compared with monthly accumulated rainfall for the city of Elvas (diamonds on the end of each month in 2006 represent 30-year averages); VV – a neglected cv. Galega orchard where anthracnose severity is usually high; EL – a properly cultivated cv. Bical orchard usually with low anthracnose severity; both orchards are located at Elvas region. Anthracnose severity in November 2005 is represented by a triangle on the left of each graph.

10 mL L⁻¹ toluidine blue solution (Merck) in 236 mM Na₂CO₃, and observed directly by light microscopy. All observations were made using light microscopes (Leitz-Dialux 20 and Leica DM-2500) equipped with mercury bulbs HB 100 W, ultraviolet light (excitation 340–380 nm, barrier filter 430 nm).

Scanning electron microscopy

The protocol of Rijo *et al.* (1990) was followed with some modification. Small fragments of different olive organs (from the field and inoculated in laboratory conditions) were fixed in a 30 mL L⁻¹ glutaraldehyde solution in 0.1 M sodium cacodylate buffer pH 7.1 at 5°C, for *ca.* 17 h. The tissues were then fixed in a mixture with equal parts of glutaraldehyde with 79 mM osmium tetroxide in the above buffer for 2 h. They were then washed three times in 0.1 M sodium cacodylate buffer pH 7.1 for 30 min and post-fixed, for 2 h, in 39 mM osmium tetroxide, in the above buffer. The tissues were rinsed with the same buffer (3 × 10 min) and dehydrated in a graded ethanol series (10, 20, 30, 50, 70, 90 and 100%) for 20 min each, once with a mixture (1:1) of 100% ethanol and 100% acetone for 20 min, and kept in 100% acetone. They were then dried using a Polaron Critical Point Dryer. Dried tissues were mounted on aluminum stubs and coated with gold-palladium using a Polaron Sputter Coater and examined using a scanning electron microscope (JEOL, JSM 5410) at 15 kV.

Results

Anthracnose incidence and severity

During 2003–2007 disease incidence was 41%, corresponding to symptoms observed in 375 out of 908 orchards surveyed. Presence of the pathogen without symptoms, confirmed by laboratory incubation of the samples, was recorded in another 14% of the orchards. During the 5 year period, the average disease incidence ranged from a minimum of 26% in 2003 to a maximum of 52% in 2006 (Fig. 2a). In contrast, the proportion of orchards where the pathogen was present but symptoms were absent remained relatively constant at 14–17%. Disease incidence was clearly lower at Trás-os-Montes (7%), contrasting with high incidence in the rest of Portugal (54%). At Trás-os-Montes anthracnose incidence varied between a minimum of 0% in 2004, with 4% of orchards showing symptomless presence of the pathogen, to a maximum of 15% in 2006, with another 8% of orchards with symptomless presence of the pathogen. Within the higher incidence recorded in the rest of Portugal, lower levels of incidence were observed in specific areas, e.g. 34% at Elvas/Campo Maior and 14% at Moura/Serpa.

Disease severity ranged from 0% to 100% (total yield loss), with considerable variation during different years and in various locations (Fig. 2B). Severity values above 50% were commonly registered in most of the country although much less frequently at Trás-os-Montes. Intra-

regional variation was evident, as the severity levels were typically lower at Moura and Serpa as well as Elvas and Campo Maior (18%), compared to the adjacent region (39%). In 2006, disease severity values were invariably high in Portugal excluding Trás-os-Montes (80% average in neglected orchards, 76% in properly cultivated orchards and 60% in wild olives). During the other years, the corresponding values on average were 35%, 24% and 4%, respectively. Average disease severity at Moura/Serpa as well as Elvas/Campo Maior reached 35% in 2006, contrasting with 8% in the remaining years. Similarly, average disease severity was 28% at Trás-os-Montes in 2006 and 0.8% in the remaining years. With high disease severity levels, intense defoliation, occurring as a horizontal band around the medium part of the tree, was frequently recorded.

Aetiology

A collection of 530 *Colletotrichum* species isolates associated with olive anthracnose across Portugal, obtained over a period of 6 years, was characterized by diagnostic PCR based on the *tub2* gene. This analysis revealed that 20 isolates belonged to *C. gloeosporioides*, while the remaining 510 belonged to *C. acutatum*. PCR-RFLP analysis of the *tub2* fragment of the *C. acutatum* isolates further showed that 423 isolates clustered in group A2, while groups A4 and A5 contained 62 and 19 isolates, respectively, and groups A3 and A6 contained only four and one isolates, respectively (Fig. 2c). *Colletotrichum gloeosporioides* isolates could be clearly distinguished from *C. acutatum* by a faster growth rate (data not shown). Within *C. acutatum*, group A5 isolates could be distinguished as they showed typical salmon colonies, with purple reverse. Colonies of A3 isolates were pink to cream and pink to yellow or cream on reverse. Colonies of A2 and A4 were less distinguishable and their coloration tended to overlap. A2 colonies were mostly olive-green, but sometimes also cream or mouse-coloured, while A4 colonies were predominantly cream, but sometimes also olive-green or yellow-whitish. Presence of abundant orange spore masses frequently masked the colony colours, but their occurrence was irregular across the different *C. acutatum* groups.

Notable variations were observed in terms of the spatial distribution of the pathogen populations. For example, *C. acutatum* group A2 was dominant in most parts of the country, whilst at Trás-os-Montes A2 was less frequent (42%) compared to group A4 (55%). In the Algarve region, four different populations occurred at relatively similar levels: *C. gloeosporioides*, which was sporadic in the rest of the country, was 17%, and *C. acutatum* groups A2, A4 and A5 were 34%, 27% and 20%, respectively. The geographic locations from where the pathogens were collected and the identity of the genetic groups of the isolates, along with regional frequencies of populations represented by these genetic groups, are shown in Figure 2c.

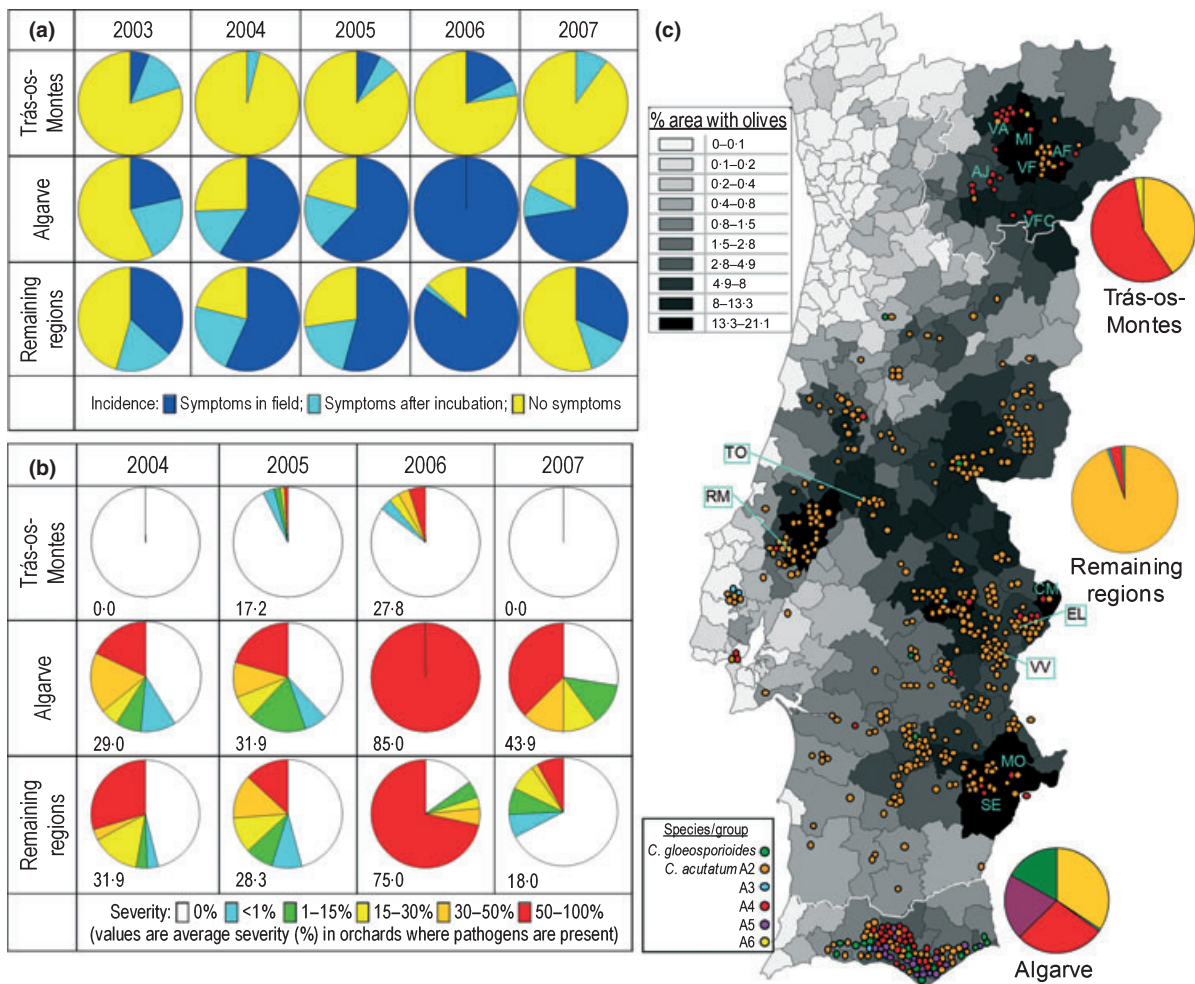


Figure 2 Olive anthracnose incidence, severity and aetiology. (a) Olive anthracnose incidence in the main olive producing regions of Portugal in 2003–2007, showing proportion of orchards where symptoms were present in field, including information on orchards where symptoms were detected only after incubation of fruits, representing a disease potential. (b) Olive anthracnose severity (% of fruits with symptoms per orchard) in 2004–2007 per region and per year. (c) Geographic distribution of olive anthracnose pathogens in Portugal (2002–2007), according to species (*Colletotrichum acutatum* or *C. gloeosporioides*) and the genetic group within *C. acutatum*, including proportions per region (shades of grey in maps indicate proportion of olive cultivation per municipality; locations referred to throughout the text are identified in the map: AF: Alfândega da Fé; AJ: Alijó; CM: Campo Maior; EL: Elvas; MI: Mirandela; MO: Moura; RM: Rio Maior; SE: Serpa; TO: Tomar; VA: Valpaços; VF: Vila Flor; VFC: Vila Nova de Foz Côa; VV: Vila Viçosa).

Table 1 Percentage frequency of detection of *Colletotrichum acutatum* in symptomless plants other than olive present in and around the olive orchards during 2006 and 2007

	2006						2007								
Plant	April	June	August	September	October	November	April	May	June	August	September	October	November	December	Orchard
<i>Rubus</i> sp.	0	0	0	0	0	0	50	50	10	0	50	12	0	0	Vila Viçosa
<i>Quercus</i> <i>rotundifolia</i>	0	0	0	0	0	0	0	0	40	10	0	0	0	0	
Peach tree	0	13	0	35	0	50	30	80	20	60	0	0	0	0	Tomar
<i>Rubus</i> sp.	0	0	0	0	33	67	69	20	50	0	0	50	0	0	Rio Maior 2
Barley	0	20	n.a. ^a				n.a.								

^aBarley plants absent from the field from August 2006 onwards.

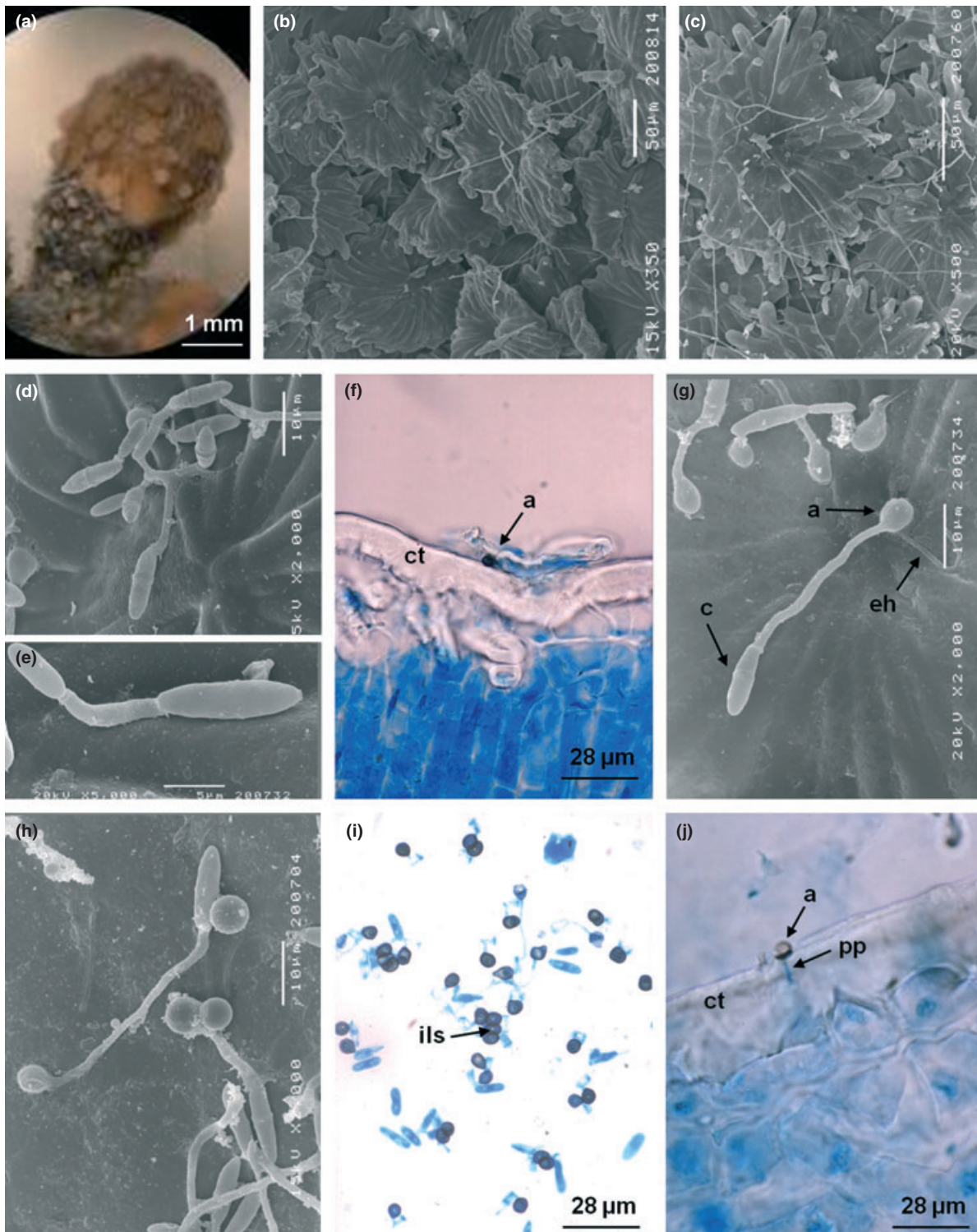


Figure 3

Epidemiology

The results from epidemiological observations including disease progression curves on vegetative and reproduc-

tive organs during 2006 and 2007 at two contrasting orchards, Vila Viçosa (also representing Tomar and Rio Maior 2, where similar results were observed) and Elvas (also representing Rio Maior 1) are summarized in

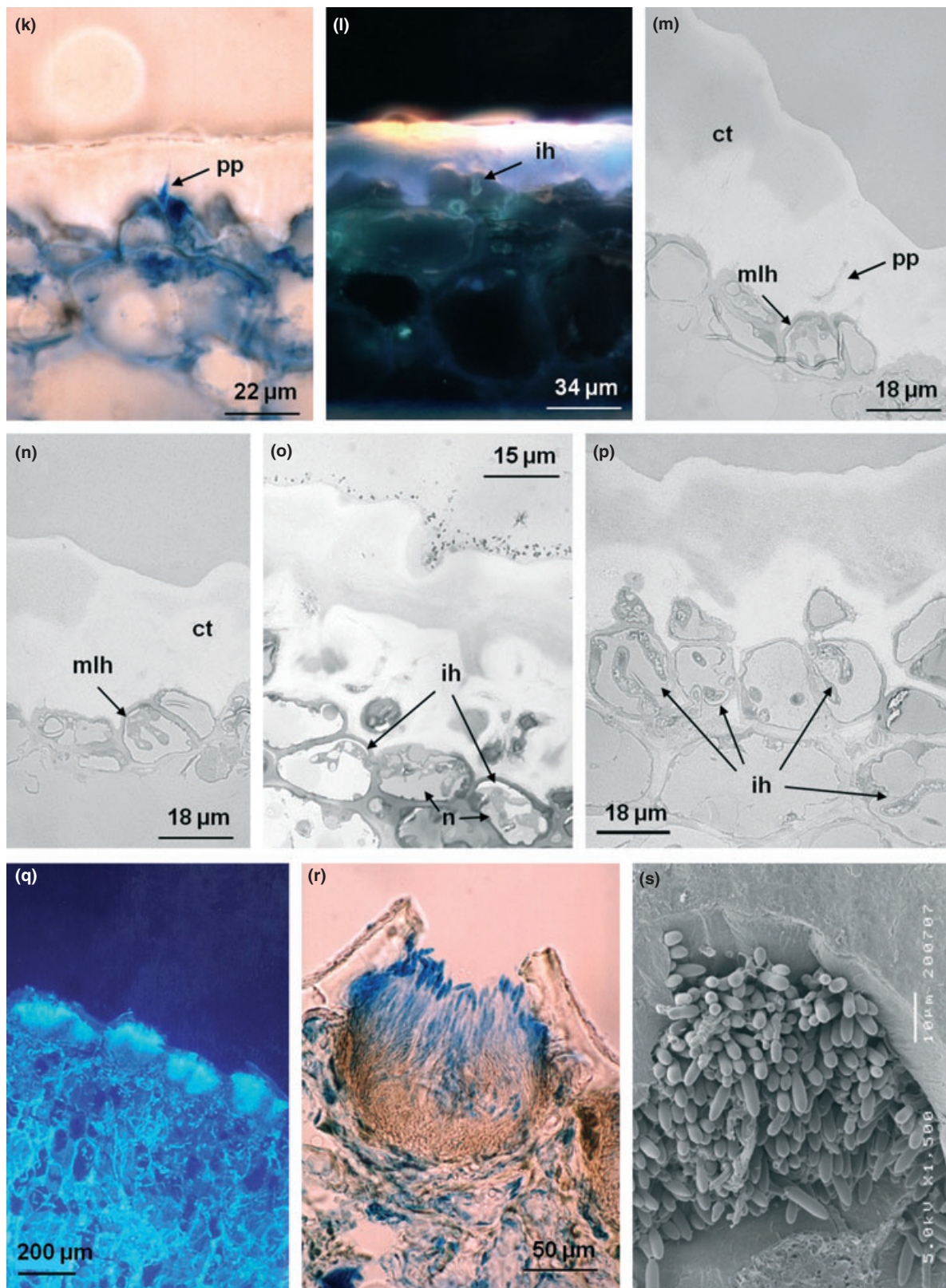


Figure 3 Continued.

Figure 3 Histopathology of *Colletotrichum* species on olives. a, Spore masses of *Colletotrichum acutatum* formed on the surface of a flower bud collected in the field in April 2007 without symptoms and incubated under high humidity. Germination of *Colletotrichum* species conidia (c) on b, olive branch; and c–g, leaf surfaces: c, long germination tubes; d–e, secondary conidiation; f, formation of melanised appressoria (a); g, production of external hyphae (eh) from appressoria. h–k, Initial phases of olive fruit penetration: h, conidia germination and appressoria formation; i, internal light spot (ils) on melanised appressoria; j, penetration peg (pp) formed from appressoria (a) penetrating the fruit cuticle (ct); k, enlargement of the penetration peg when reaching first mesocarp cell. l–p, Colonization of olive fruit, with penetration, invasion and necrosis (n) of the mesocarp cells: l, infection hypha (ih) penetrating the first mesocarp cell; m–n, multilobed hyphae (mlh) in the first mesocarp cell, beneath the penetration peg; o, invasion and necrosis of the first layer of mesocarp cells; p, generalized colonization of hyphae in necrotic cells. q–s, Production of acervuli, disruption of olive fruit cuticle and release of conidia. (c, i, m, n, o, p and q – *C. gloeosporioides*; b, d, e, f, g, h, j, k, l, r and s – *C. acutatum*).

Figure 1. The Vila Viçosa site represents a neglected orchard of cv. Galega where anthracnose severity is usually high (90% in autumn 2005), and Elvas represents a properly cultivated orchard of cv. Bical usually with low anthracnose severity (5% in autumn 2005). These orchards are only 25 km apart, and the meteorological data for Elvas is valid for both orchards (Fig. 1, rainfall panel). High rainfall and prolonged high humidity periods occurred in autumn 2006, while autumn 2007 had below average rainfall and infrequent periods of high humidity. During spring 2006 the pathogen was frequently detected in most organs, particularly those already formed in the previous autumn, in high disease severity orchards represented by Vila Viçosa, but not in low disease severity orchards represented by Elvas. In June 2006, the pathogen presence increased substantially on vegetative organs but also on peduncles of young fruits and sporadically on unripe fruits at Vila Viçosa, but not at Elvas. A substantial reduction in the number of organs where the fungus was present was observed by August. Following the on-set of autumn rain, the fungus was detected with increasing frequency in September. At Vila Viçosa, detection of conidia on fruit increased with autumn rain, with fruit infection following a similar pattern a few days later. The extremely favourable weather conditions observed during autumn 2006 can be related to the pathogen incidence of nearly 100% from the different organs sampled at Vila Viçosa where all fruits showed symptoms, along with severe defoliation and death of branches. The detection of the pathogen was substantially lower at Elvas, although inoculum was detected in 40% of fruits. In 2006, during the periods in which the pathogen was more frequently detected on olive tree organs, it was also identified without symptoms on other plants present in and around the orchards (Table 1), e.g. at Tomar in a peach tree and at Rio Maior 2 in blueberry (*Rubus* sp.) and barley (a non-host crop for olive anthracnose pathogens).

A high level of inoculum was consistently found on various olive tree organs during 2007, reflecting the high disease outbreak in the previous year, and coinciding with frequent rain events during the middle and late spring 2007 (40–50 mm each month in April, May and June). As in 2006, clear differences were observed between the orchards at Vila Viçosa and Elvas, with high and low disease severity, respectively. However, in 2007, the frequency of pathogen detection on organs developed in the previous autumn and during the year was compara-

ble. Among these newly formed organs in 2007, *C. acutatum* was frequently detected on flowers and young fruits, not only at Vila Viçosa, but also at Elvas. In fact, flowers and young fruits were the only organs on which the fungus was found in the orchards with low severity of anthracnose. Microscopic observations of such organs, following incubation under high humidity conditions, showed production of spore masses and acervuli on flowers (Fig. 3a). In addition, the fungus was also frequently present without symptoms in other plants in and around the orchards (Table 1). In 2007, there was a marked decrease in pathogen presence in August, with a subsequent increase in September, following first autumn rains. However, after early rain events in September and early October (up to 100 mm in the region), the autumn was quite dry (<50 mm until the end of the season) and the disease remained undetected at Elvas, although inoculum was present at very low levels. At Vila Viçosa orchard, disease progressed to a 20% severity level, in contrast to 90–100% severity recorded in 2006 and previous years. However, the presence of the pathogen in symptomless fruits was as high as 86% early in October as a result of late September and early October rain events (Fig. 1). This pattern was very similar to autumn 2006. After this, the pathogen detection steadily decreased to 50% in mid-November, as a consequence of scarce rain and infrequent high humidity periods.

In spite of the severe drought that occurred during winter and spring 2004/2005 (ca. 30% of average annual rainfall), *C. acutatum* was present without the appearance of symptoms throughout 2005 in all 14 orchards monitored, mostly in organs such as leaves and branches that were already formed in the previous autumn. The pathogen was detected more frequently in orchards with high disease severity in the previous autumn. Because of the very dry weather conditions during winter and spring 2004/2005, fruit left on the orchard floor remained undecomposed at all 14 orchards surveyed. However, *Colletotrichum* species were never isolated from this fruit at any of the locations used in this study. In the remaining years, fallen fruits were rapidly decomposed, and only seeds were found throughout winter and spring, from which *Colletotrichum* species were never isolated. The pathogen was isolated from mummified fruits remaining on the trees during winter and spring 2004/2005, but in the remaining years these were extremely rare.

Histopathology

Conidial germination and appressorial formation of both *C. acutatum* and *C. gloeosporioides* was observed 24 h after inoculation on the surface of branches, leaves and fruits at different stages of maturity. While germ tubes on fruit surfaces were most frequently short in length, they were long on branches and leaves (Fig. 3b,c), which made quantification of germination difficult. Nonetheless, the extent of conidial germination and appressorial formation on leaves and branches was lower (maximum 11% for conidial germination and 6% for appressorial formation on leaves) compared to fruit surfaces (minimum 60% and 45%, respectively). Very often conidia attached to the abundant trichomes on leaves and branches, rather than to the leaf/branch surface. Secondary conidiation was repeatedly recorded (Fig. 3d,e) on leaves and branches. Appressoria remained non-discharged on the organ surface (Fig. 3f), and neither an internal light spot nor the perforation of the cuticle by a penetration peg was ever observed, but occasionally external hyphae were produced (Fig. 3g). Disease symptoms were not observed on these organs following artificial inoculation.

Upon germination of conidia and formation of melanized appressoria (Fig. 3h), an internal light spot was observed for both *Colletotrichum* species on immature, pre-mature and mature fruits (Fig. 3i). This represents the point of origin of the penetration peg (Fig. 3j), which enlarged when reaching the first mesocarp cell (Fig. 3k). Length of the penetration peg was similar for both *Colletotrichum* species and for different stages of fruit maturation, ranging from 7.5 to 8.1 μm . In immature fruits, the infection process was restricted to the germination of conidia, appressorium formation and cuticle penetration, with no further structures observed (Fig. 3j,k), and no symptoms developed except when senescence/forced maturity occurred. In pre-mature and mature fruits the fungus penetrated subcuticularly (Fig. 3l) and colonized the epiderm and mesocarp tissues intra- and inter-cellularly (Fig. 3m-p). Multilobed hyphae were observed in the first invaded host-cell, beneath the penetration peg (Fig. 3m,n). Host cells became necrotic after invasion (Fig. 3o). The penetration of cuticle on fruits always occurred soon after inoculation, while the time until further fungal colonization and subsequent development of symptoms varied greatly, from over 1 month in immature fruits to 1–9 days in pre-mature and mature fruits. These advanced infection phases corresponded to an intense colonization of hyphae in necrotic fruit cells (Fig. 3p), giving rise to the formation of acervuli which erupted through the cuticle (Fig. 3q-s).

Discussion

Surveys carried out during 2003–2007 demonstrated that anthracnose is a major threat to olive cultivation in Portugal with 41% of the 908 orchards covered displaying disease symptoms and the symptomless presence of the inoculum in another 14% of the orchards. Similarly, olive

anthracnose severity frequently reached high levels including total yield loss, with average severity ranging from 29% to 68%. Olive anthracnose incidence and severity varied both temporally and geographically. Persistent lower incidence and severity values were recorded at Trás-os-Montes region and in the areas of Moura/Serpa and Elvas/Campo Maior, as opposed to the rest of the country where the susceptible cv. Galega is widely cultivated. However, the increase of olive anthracnose incidence and severity at Trás-os-Montes demonstrates the growing importance of this disease in the region, where anthracnose occurred only sporadically until recently. In 2006, disease incidence and severity in general were greater than in the other years surveyed. Meteorologically, autumn 2006 in Portugal was characterized by abundant and precocious rainfall, long periods of high humidity and mild minimum temperatures, which favoured inoculum dissemination, spore germination and fruit infection, thus accelerating the occurrence of symptoms.

Molecular and morphological characterization of 530 *Colletotrichum* species isolates obtained over a period of 6 years clearly revealed *C. acutatum* as the major olive anthracnose pathogen in Portugal (96.2%) with *C. gloeosporioides* present at a low level (3.8%). Within *C. acutatum*, genetic group A2 was dominant (82.9%), with A4, A5, A3 and A6 in decreasing frequencies. In other geographical locations, aetiology of olive anthracnose presents a varied picture. In Spain, *C. acutatum* and *C. gloeosporioides* occur at similar proportions to those found in Portugal (Martín *et al.*, 2002), but *C. acutatum* group A4 appears more frequently than in Portugal (Moral *et al.*, 2008). In Italy and Montenegro *C. acutatum* group A4 can also be common (Talhinhos *et al.*, 2005). In Australia, group A9 of *C. acutatum* was reported as the causal agent (Whitelaw-Weckert *et al.*, 2007), and indirect evidence suggests that *C. acutatum* group A5 isolates are responsible for olive anthracnose in South Africa (Talhinhos *et al.*, 2009). Previously, a specific taxon, *Gloeosporium fructigenum* f. sp. *chromogenum*, was created to accommodate South-African olive anthracnose pathogens with pink/red-coloured cultures (Gorter, 1956). Whilst these genotypes are very common in parts of the southern hemisphere (southern Africa and Oceania), they were never reported from field crops in the northern hemisphere and South America except on olives in Portugal. This demonstrates the high diversity of *C. acutatum* genetic groups associated with olive anthracnose in Portugal. Further, intra-group diversity was also detected by rRNA-ITS nucleotide sequence analysis among *C. acutatum* isolates belonging to group A2 in the Algarve and A4 at Trás-os-Montes (Talhinhos *et al.*, 2009).

Molecular markers based on ITS and *tub2* sequences are useful for the differentiation between *C. acutatum* and *C. gloeosporioides* as well as various genetic groups belonging to these species. Morphological characters are frequently insufficient for a reliable distinction between *C. acutatum* and *C. gloeosporioides*. Conidial size and

shape in particular provided little distinctive information (Talhinhas *et al.*, 2005). However, colony characteristics, especially growth rate, enabled distinction between *C. gloeosporioides* and *C. acutatum*, and colony colour was useful for the discrimination of some of the *C. acutatum* populations. Despite the spatial and temporal variations in olive anthracnose levels, the relative proportions of the two species remained constant throughout the survey period. However, *C. acutatum* genetic groups representing various populations displayed important shifts. For example, group A5 identified exclusively in the Algarve (Talhinhas *et al.*, 2005, 2009) showed a steady increase from 9% in 2004 to 34% in 2007. Similarly, at Trás-os-Montes, group A2 became dominant in 2006 (62%) in contrast to other years when A4 was more frequent (*ca.* 60%). Importantly, this change of frequency between A2 and A4 relates to the higher disease severity recorded in this region during 2006, suggesting a build up of the A2 populations under favourable meteorological conditions. In contrast, isolates from the less frequent groups (A3 and A6) were only detected in the absence of symptoms during the years with low disease levels (2002, 2003 and 2007). In fact, isolates from groups A2 and A5 of *C. acutatum* were among the most aggressive in an inoculation experiment of detached fruits from eight olive cultivars, while those from *C. gloeosporioides* were less aggressive, with isolates from group A4 of *C. acutatum* achieving diverse severity scores (unpublished data).

Epidemiology and disease cycles are not fully understood in the olive anthracnose system. Conidia carried by autumn rains to mature fruits were considered as the source of inoculum (Trapero & Blanco, 2004), but the occurrence of latent flower and fruit infections has recently been shown (Moral *et al.*, 2009). Symptomless infection of flowers has also been reported in Australia (Sergeeva *et al.*, 2008a). Besides flowers and young fruits, the present study has clearly demonstrated that inoculum dispersal to vegetative organs, such as leaves and branches, also occurs in the field during spring, and that these observations correlate with meteorological data. Under wet conditions this inoculum causes symptomless infections of a considerable proportion of flowers and young fruits, leading to anthracnose symptoms once the fruits mature. Weather conditions have a huge influence on pathogen epidemiology and disease cycle, as evident from the results in 2006 and 2007. Following a dry spring in 2006, pathogen inoculum on vegetative organs, flowers and young fruits was low even though the disease severity in the previous autumn was very high. Rainy, wet and mild conditions during autumn 2006 led to a comprehensive spread of the pathogen and severe disease. Such high inoculum levels, together with periodic rain events during middle and late spring in 2007, led to high levels of pathogen presence on vegetative organs, flowers and young fruits during summer, resulting in high levels of inoculum on fruits. However, the disease severity levels were low, reflecting the unfavourable weather conditions during autumn 2007. Interestingly, under wet conditions

favourable for inoculum dispersal (e.g. autumn 2006 to spring 2007), the pathogen tends to be present on leaves and year branches more frequently than on older organs, and is more frequently found on older organs than on recent ones when weather conditions are dryer. This further suggests that leaves and branches from the current year of growth (year branches) are important short term inoculum reservoirs, while older branches act as long term inoculum reservoirs.

Within the olive anthracnose system, the roles of mummified fruit as a source of inoculum has historically not been clear (Zachos & Makris, 1963). In the present study, the pathogen was only successfully isolated from mummified fruits present on trees but not from fallen fruits. Conidia released into soil from mummified fruits were thought to rapidly lose their viability (Zachos & Makris, 1963). In other pathosystems, soil temperature and moisture are known to influence the viability of *C. acutatum* conidia present in soil or on buried plant parts (Norman & Strandberg, 1997; Feil *et al.*, 2003). In the field surveys carried out here, mummified fruits were generally very infrequent during spring, either on trees or in soil. These results strongly suggest that mummified fruits on the soil surface are not an important inoculum reservoir for olive anthracnose.

Details of the infection process of *Colletotrichum* species have been studied on various hosts (Perfect *et al.*, 1999; Curry *et al.*, 2002), but little is known about the histopathology of olive anthracnose, particularly the interaction between the pathogens and non-target organs such as leaves, branches and unripe fruits. *Colletotrichum acutatum* and *C. gloeosporioides* are capable of epiphytic survival on the surface of olive leaves and branches as germinated conidia forming appressoria, with frequent secondary conidiation, but without the penetration of the cuticle. Abundance of trichomes prevents direct access of conidia to the surface of the leaves and branches, although penetration of trichomes leading to host colonization and disease symptoms has been reported for other *Colletotrichum* species (Villanueva-Arce *et al.*, 2006). Leaf necrosis and spore masses observed in natural infections could result from epiphytic development of the pathogen under very favourable environmental conditions (Sergeeva *et al.*, 2008b).

On immature, pre-mature and mature fruits, conidia germinated and produced melanized appressoria, where an internal light spot could be observed, corresponding to the penetration pore (Diéguez-Urbeondo *et al.*, 2003), followed by the formation of the penetration peg. No further colonization and no symptoms were observed in immature fruits. However, in pre-mature and mature fruits, multilobed hyphae were observed in the first invaded host-cell, resembling the multiseptate, multilobed primary hyphae associated with hemibiotrophy in other *Colletotrichum* species (O'Connell *et al.*, 2004; Latunde-Dada & Lucas, 2007). While the penetration of cuticle always occurred soon after inoculation, the time until further colonization and symptom development was much shorter in mature fruits than in pre-mature

fruits, coinciding with recent observations (Moral *et al.*, 2009). It is not known exactly which host factors are involved in triggering this process, although pH is an important factor in other *Colletotrichum* pathosystems (Prusky & Yacoby, 2003). Extracts of immature and mature fruits did not show any significant differences in supporting the growth of olive anthracnose pathogens in culture (unpublished data).

Combined epidemiology and histopathology results showed that the pathogenesis cycle of *C. acutatum* causing olive anthracnose closely resembles the fruit hemibiotrophic lifestyle observed in blueberry, apple and peach (Peres *et al.*, 2005; Wharton & Schilder, 2008). This differs from the varied lifestyles of *C. acutatum* on sweet orange (leaf biotroph-flower necrotroph), strawberry (necrotroph in most organs) and almond and key lime (biotroph-necrotroph combination; Peres *et al.*, 2005). However, with the olive anthracnose pathogenesis cycle, leaves play a more important role for inoculum survival, multiplication and dissemination than on other hosts, where branches, buds and twigs are the main overwintering organs.

Spatial and temporal variation in olive anthracnose incidence linked to the shifts in pathogen populations is clearly influenced by agronomic and climatic factors. Under highly favourable meteorological conditions, current disease management practices such as the use of copper-based fungicides and disease-resistant varieties are not fully successful in reducing disease levels. Olive anthracnose pathogens are capable of epiphytic survival on leaves and branches as well as in symptomless flowers and young fruits, which leads to symptom development on mature fruits.

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