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REVIEW

Botryosphaeria species associated with diseases of grapevines in Portugal

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Summary. Although *Botryosphaeria* species are known to cause cankers and dieback in many different woody hosts, their importance in grapevines has been largely ignored. Indeed, they are more often regarded as saprophytes or weak pathogens. In the work presented here the species of *Botryosphaeria* associated with wood and trunk diseases of grapevines in Portugal were determined. Three species, namely, *B. obtusa*, *B. parva*, and *B. lutea*, were regularly associated with trunk dieback, wood necrosis, brown wood streaking, cane bleaching or incomplete grafts. *Botryosphaeria parva* was the most common and widely distributed species. *Botryosphaeria dothidea* and *B. stevensii* were less common; the former was found on bleached canes and on necrotic tissues at the graft union while the latter was isolated from necrotic buds, and occasionally from brown streaks in the wood. These data indicate that *B. parva* is associated with many of the symptoms normally linked with infection by other fungi in the grapevine decline syndrome. To stimulate further research on this genus, descriptions of the species associated with grapevines and an identification key are provided.

Key words: *Diplodia, Fusicoccum*, *Vitis vinifera*, wood disease.

Introduction

Species of *Botryosphaeria* Ces. & De Not. are saprophytic, parasitic or endophytic on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts (Barr, 1972). Some are known to cause cankers, dieback and other diseases (e.g. Maas and Uecker, 1984; Rumbos, 1987; Michailides, 1991; Smith *et al*., 1994).

Various *Botryosphaeria* species have been reported from grapevines. Often they are regarded as saprophytes, but some have been shown to be pathogens. For example, Lehoczky (1974) found *Botryosphaeria stevensii* Shoemaker to be the cause of a disease he named "black dead arm". Millholland (1991) considered *B. dothidea* (Moug. : Fr.) Ces. & De Not. to be an important pathogen causing a rot of ripening berries of muscadine grapes, while Kuo *et al*. (1989) found that *B. dothidea* (as *Botryosphaeria ribis* Gross. & Duggar) causes a grape cluster rot in Taiwan. Filho *et al*. (1995) reported *B. dothidea* causing a trunk canker of grapevines in Brazil. More recently, Phillips (1998) showed that *B. dothidea* is the cause of excoriose of Portuguese grapevines and some strains were virulent causing a stem dieback. The strains of *B. dothidea* were later (Phillips *et al.*, 2002) shown to

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be a complex of species that included *B. dothidea*, *B. parva* Pennycook & Samuels and *B. lutea* A.J.L. Phillips. Although the most virulent strains were of *B. parva*, weakly virulent strains of that species were predominant.

Pathogenicity of *Botryosphaeria obtusa* (Schw.) Shoemaker has long been the subject of controversy. Chamberlain *et al.* (1964) reported the fungus on lesions on trunks, canes and tendrils of vines affected by dead-arm. Radman & Nadazdin (1981) found two species of *Sphaeropsis* infecting the bark of grapevines in Herzegovina, Yugoslavia. In Italy, Bisiach and Minervini (1985) and Rovesti and Montermini (1987) found *B. obtusa* associated with xylem necrosis. There are also reports of it causing vine stock decline in Hungary (Horváth and Schweighardt, 1991) and infectious drying of grapes in the Ukraine (Kozar' *et al.*, 1990). In most of these reports, pathogenicity was not proved. The isolates tested by Phillips (1998) were weakly pathogenic and were considered to be secondary parasites or saprophytes. However, Larignon and Dubos (2001) found *B. obtusa* associated with symptoms of black dead arm in Bordeaux vineyards. They found that *B. dothidea* also caused the same symptoms, and furthermore, dark lesions developed on 1-year-old canes artificially inoculated with both species.

Not only is there some controversy regarding the pathogenicity of *Botryosphaeria* species, taxonomy of the genus *Botryosphaeria* has also been the subject of some confusion. As a consequence, accurate identification to species level can be difficult, which further confounds the subject of pathogenicity. The teleomorphs are uncommon in nature, they do not form in artificial cultures, and there is insufficient diversity among teleomorph features to allow clear differentiation at species level. For these reasons, taxonomy and identification of *Botryosphaeria* species is based mainly on anamorphic characters.

When they were first described, the anamorph genera of *Botryosphaeria* species were not clearly defined, and this resulted in a multiplicity of names applied to similar fungi. Considerable progress, chiefly in the works of Crous and Palm (1999) and Denman *et al.* (2000), has been made to resolve these problems and to stabilize the anamorph names. Many of the problems and their solutions have been summarised by Phillips (2000). Briefly, of the seven generic names most often applied to anamorphs of *Botryosphaeria* species, only three (*Diplodia* Fr. apud Mont., *Fusicoccum* Corda and *Sphaeropsis* Sacc.) need to be considered.

The genus *Sphaeropsis* has been the subject of controversy because the distinction between it and *Diplodia* has never been clear (Shoemaker, 1964). As defined by Sutton (1980), percurrent proliferation in conidiogenous cells has been regarded as more typical of *Sphaeropsis* than of *Diplodia*. However, percurrent proliferation is also found in strains of *Diplodia*, which suggests that these two genera are indistinguishable. Septation of conidia has also been used to separate these two genera. According to Sutton's (1980) concepts, conidia of *Sphaeropsis* are aseptate but they become euseptate prior to germination, while in *Diplodia* the conidia become euseptate as they mature. The concept of aging and maturity are not well defined, thus making the feature of septation an unreliable criterion for species separation. Age and state of maturity also affect conidium pigmentation. Mature conidia of *Sphaeropsis* are invariably opaque brown, but conidia of *Diplodia* can remain hyaline even when mature. However, in Sutton's (1980) account of the history of *Diplodia mutila* Fr. apud Mont. (the type species of the genus) he recalls that when Stevens (1933) examined slides from Montagne's exsiccata, he found pycnidia with a mixture of hyaline and one-celled brown conidia. When Shoemaker (1964) described the teleomorph of *D. mutila*, he stated that conidia remain hyaline even after discharge from the pycnidium, and that brown, one-septate conidia are rarely found. In contrast to this, however, conidia of specimens that Laundon (1973) considered to be the anamorph of *B. stevensii* were hyaline, but they gradually became dark coloured and 1-septate. Therefore, *D. mutila* can be regarded as having hyaline conidia that become brown with age. Thus, it becomes difficult to distinguish *Sphaeropsis* from *Diplodia* and the two genera can be considered synonymous, with the older name of *Diplodia* taking preference (Denman *et al.*, 2000).

Denman *et al.* (2000) placed *Botryosphaeria* anamorphs into two groups based on conidium colour. Accordingly, species with hyaline conidia were placed in *Fusicoccum* while those with dark conidia fall within *Diplodia*. However, as stated above, the type species of *Diplodia* (*D. mutila*) has hyaline conidia that darken with age. In some collections, it seems that darkening is delayed, or possibly never occurs. Thus, *Diplodia* cannot strictly be con-

sidered a genus with dark spores. Although Denman *et al*. (2000) amended the concept of *Diplodia* to include species with conidia of variable colour, their proposal to place hyaline-spored species in *Fusicoccum* would mean that some collections of *B. stevensii* would have *Fusicoccum* anamorphs. Moreover, conidia in some species of *Fusicoccum* become darker with age. Therefore, conidium colouration is a difficult character to apply in practice.

If it is accepted that conidium colouration is an unreliable character, the main distinction between *Fusicoccum* and *Diplodia* is that in the former the conidia are thin-walled, while in the latter they are thick-walled. In addition, conidia of *Fusicoccum* spp. are usually less than 10 µm wide, while in *Diplodia* spp. they are normally greater than 10 µm wide. Furthermore, percurrent proliferation is often regarded as common in *Fusicoccum* but infrequent in *Diplodia* (Denman *et al.*, 2000). These are minor and somewhat subjective differences and it may be possible to unite the two genera under the older name of *Fusicoccum*, thus reaching the logical state where only a single anamorph genus is associated with the teleomorph genus *Botryosphaeria*. This, however, is not possible at present and in this paper the two genera are retained with the distinction that in *Fusicoccum* the conidia are hyaline, thin-walled and less than 10 µm wide, while in *Diplodia* they are hyaline or coloured, thick-walled and greater than 10 µm wide. Furthermore, phylogenic analysis of ITS sequence data (Jacobs and Rehner, 1998; Phillips *et al.*, 2002) suggests that the difference between *Fusicoccum* and *Diplodia* is clear, but the difference between *Diplodia* and *Sphaeropsis* is indistinct. Therefore, in the following accounts, *Sphaeropsis* will be considered a synonym of *Diplodia*, but *Fusicoccum* will be retained as a distinct genus.

The research reported in this paper was aimed at clarifying the taxonomy of various species of *Botryosphaeria* that occur on grapevines. Isolates from a variety of disease symptoms are described and a key to the species is provided.

Materials and methods

Isolations

Fungi associated with various disease symptoms were isolated by transferring small (ca. 1 mm^3) pieces of tissue to plates of potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA). Isolations were also made from fruit-bodies when these were seen on the host.

Preliminary identifications were made from squash mounts of fruiting structures mounted in lactophenol. Fruit-bodies on the host tissues were either sectioned by hand or soaked in 1% gum arabic before they were sectioned with a freezing microtome and mounted in lactophenol. Conidiomata and ascomata were measured under the ×40 objective. All other measurements were made with the ×100 oil immersion lens.

Cultures were prepared from single ascospores or single conidia germinating on PDA. Cultures were maintained on PDA slopes at 15°C and stored in sterile water. For sporulation, they were grown on oatmeal agar (OA) at 23°C with 12 h of light per day from mixed U.V. and daylight fluorescent tubes. Oatmeal agar was prepared by boiling 20 g of oatmeal in 1 litre of water for 10 mins. This was then strained through a double layer of cheesecloth, 20 g of agar was dissolved in the filtrate and the medium was sterilized by autoclaving at 121°C for 15 min.

Results

Symptoms and isolation

Between 1996 and 1999 isolations were made from five types of symptom (Fig. 1, Table 1). Trunk

Table 1. *Botryosphaeria* species associated with disease symptoms in grapevines. Values are the numbers of samples with which the fungi were associated.

	B. dothidea	*B. parva*	*B. lutea*	*B. obtusa*	*B. stevensii*	Total No. samples
Trunk dieback	0	10	0	0	0	10
Wood streaking	0	40	0	10	5	55
Bud necrosis	0	2	0	0	12	14
Graft failure	1	15	0	4	0	2
Cane bleaching	6	128	108	12	0	254

dieback was characterised by a dark brown discolouration of the wood that appeared to spread from large pruning wounds down the trunk (Fig. 1a). Only *B. parva* was isolated from this symptom. Often, but not exclusively, associated with this symptom was a dark brown streaking of the wood, which in cross section had the appearance of black spots. This was similar to the symptom found in trunks and arms of vines affected by Petri disease and esca from which *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams is frequently isolated. However, the symptom considered here differed in that the "black goo" (i.e., black exudation from transversally cut vessels) associated with *P. chlamydospora* infections was not seen, and the black spots tended to be more diffuse (Fig. 1b). In these cases, *P. chlamydospora* could not be isolated and only *B. parva*, or sometimes *B. obtusa* and *B. stevensii*, were isolated from this symptom.

In vines grafted onto rootstocks infected with *P. chlamydospora*, the graft union was frequently seen to have failed, or was of poor quality (Fig. 1c). Isolations from the necrotic tissue at the graft union yielded mainly *B. parva* but also included *B. obtusa* and, on one occasion, *B. dothidea*. *Botryosphaeria parva* was also isolated from the brown streaks in the wood of the rootstock and scion of vines with poorly formed grafts. However, in these cases it was less frequent than *P. chlamydospora*. In some young vines on poor quality graft unions, the wood of the lower parts of the scion was extensively necrotic (Fig. 1d). In most of these the main fungi isolated were *B. parva* and *B. obtusa*.

Bud necrosis was sometimes encountered and this was characterised by a bud that had failed to burst. Black fruit bodies were embedded in the necrotic tissue surrounding the dead bud. Isolations from the fruit bodies and from dead bud tissues yielded mainly *B. stevensii* and sometimes *B. parva*.

The fifth type of symptom was the typical cane bleaching commonly seen during the winter months on dormant canes, and normally associated with excoriose or Phomopsis cane blight. Mainly *B. parva* and *B. lutea* occurred on this symptom, but *B. obtusa* was sometimes encountered.

Descriptions of the fungi

General characteristics of the genera

Botryosphaeria Ces. & De Not., Comm. Soc. Crittog. Ital. 1: 211 (1863); emend. Sacc., Michelia 1: 42 (1877).

Synonymy listed in Denman *et al.* (2000).

Pseudothecia either unilocular or multilocular and often united with conidiomata on a common basal stroma, embedded in the host tissue or erumpent. Pseudoparaphyses are prevalent in the centrum of immature pseudothecia, but they usually disappear with maturity. Asci are bitunicate, stalked or sessile, clavate. Ascospores hyaline, more or less biseriate, unicellular, thin-walled, smooth, varying from ovoid to fusoid to ellipsoid, widest in the middle. Ascospores sometimes become brown and 1–2 septate with age, and in some species they become slightly verruculose after discharge.

Type: *Botryosphaeria dothidea* (Moug. : Fr.) Ces. & De Not.

Fusicoccum Corda, in Sturm, Deutschland Flora 2: 111 (1829).

= *Macrophomopsis* Petr., Ann. Mycol. 22: 108 (1924).

Conidiomata variable from solitary pycnidia to multilocular and eustromatic structures; walls composed of dark brown *textura angularis*, becoming hyaline towards the inner layer. Ostioles indistinct to well defined, round or irregular. Paraphyses present or lacking. Conidiophores hyaline, cylindrical, branched at the base, smooth, 0–1 septate. Conidiogenous cells enteroblastic, first-formed conidium holoblastic, integrated, hyaline, smooth, cylindrical, determinate or proliferating percurrently with 1–2 indistinct percurrent proliferations, or proliferating at the same level resulting in typical phialides (*sensu* Sutton, 1980) with periclinal thickenings. Conidia hyaline, sometimes becoming olivaceous or darker with age, thin-walled, smooth, aseptate with shapes varying from elliptical to fusiform or clavate, finely guttulate, apex subobtuse to obtuse, base conspicuously truncate with a minute marginal basal frill.

Type: *Fusicoccum aesculi* Corda.

Diplodia Fr. apud Mont., Ann. Sci. Nat. Bot., Sér. 2, 1: 302 (1834).

= *Sphaeropsis* Sacc., *nom. cons.*, Michelia 2: 105 (1880).

= *Dothiorella* Sacc., Michelia 2: 5 (1880).
= *Macrophoma* (Sacc.) Berl & Vogl., Atti. Soc. Venet.-Trent. Sci. Nat. 10: 4 (1886), and Sacc., Syll. Fung. Addit. 1–4: 306 (1886).
≡ *Phoma* Westend. subgen. *Macrophoma* Sacc., Syll. Fung. 3: 65 (1884).
= *Lasiodiplodia* Ell. & Everh., Bot. Gaz. 21: 92 (1896).

Conidiomata pycnidial, ostiolate, formed in uni- or multiloculate stromata, comprising thin-walled pycnidia to large erumpent pustules containing up to 20 pycnidial locules, each with a prominent ostiole, immersed or erumpent, separate or aggregated. Paraphyses present or lacking. Conidiophores (when present) hyaline, simple, occasionally septate, rarely branched, cylindrical, arising from the inner layers of the pycnidial cavity. Conidiogenous cells holoblastic, hyaline, cylindrical, determinate or proliferating percurrently with one or two indistinct or distinct annellations. Conidia variable in colour, ornamentation and septation; initially hyaline, thick-walled, smooth or granular, aseptate, often with a central guttule, becoming 1-euseptate in some cases; mature conidia are hyaline or light to dark brown with melanin often deposited on the inner surface of the outer wall which becomes irregularly verruculose or with longitudinal striations. A mixture of hyaline and dark conidia can be present in the same pycnidium.
Type: *Diplodia mutila* Fr. apud Mont.

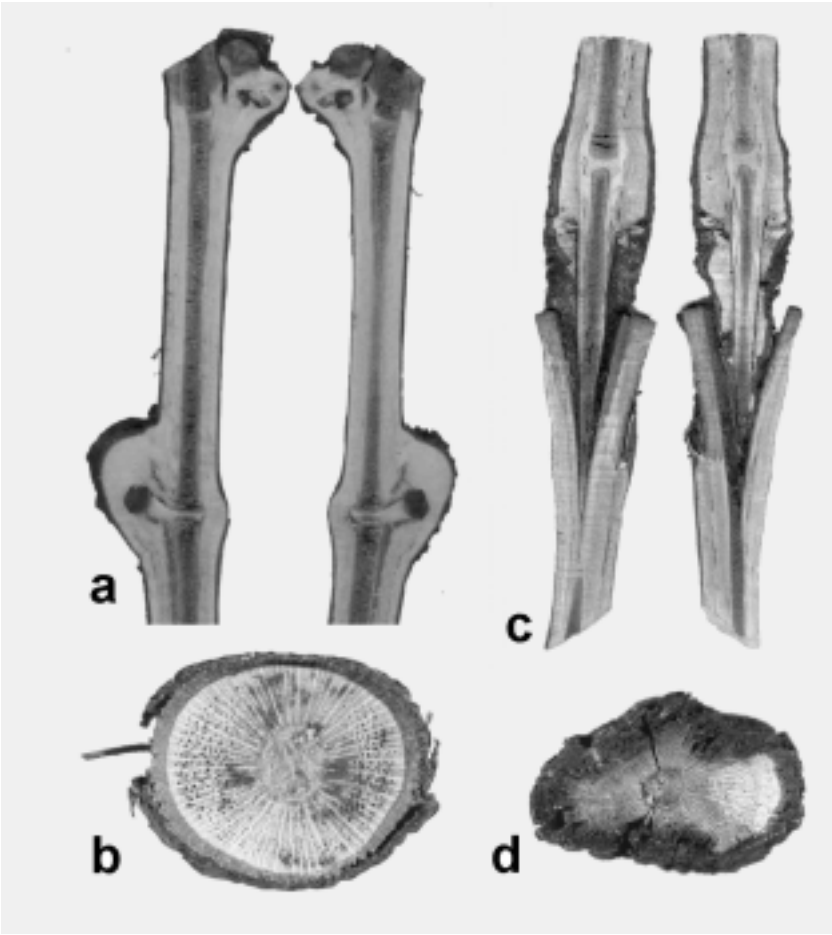

Fig. 1. Symptoms associated with infection by *Botryosphaeria* species. a. Trunk dieback with dark discolouration spreading from a large pruning wound at the tip. b. Cross section of a trunk with brown patches in the xylem. c. Graft failure with necrosis at the graft union. d. Wood necrosis in the lower part of a scion grafted onto a rootstock infected with *Phaeomoniella chlamydospora*.

A key to the species of *Botryosphaeria* recorded on grapevines is provided here. Since this genus has not been studied extensively on this host, it is likely that further species will be added to this list. Therefore, care should be taken in making the final identification and it is essential that the fungus to be identified is checked with the description.

Key to the *Botryosphaeria* species recorded on grapevines based on characters of the anamorph in culture

1. Conidial wall dark opaque brown, internally ornamented 2
1. Conidia hyaline, smooth 3
   2. Conidia mostly 1-septate ..... *B. stevensii*
   2. Conidia mostly aseptate .....*B. obtusa*
3. Conidia thick-walled and more than 10 µm wide ..... *B. stevensii*
3. Conidia thin-walled, less than 10 µm wide 4
   4. Transient yellow pigment formed after 3–4 days in PDA cultures, conidiomata on OA mainly unilocular pycnidial..... *B. lutea*
   4. No yellow pigment, conidiomata on OA mostly multilocular 5
5. Conidia 20–28 µm long, with length/width ratio of 4.3–5.2 ..... *B. dothidea*
5. Conidia 14–18 µm long, with length/width ratio of 3.2–3.9 ..... *B. parva*

***Botryosphaeria dothidea* (Moug. : Fr.) Ces. & De Not.** Comm. Soc. Crittog. Ital. 1: 215 (1863) Fig. 2

Anamorph: *Fusicoccum aesculi* Corda in Sturm, Deutschland flora 2: 111 (1829).

Characteristics in vivo. Ascomata and conidiomata common on all the woody tissues. Ascomata initially immersed, either separate or grouped in complex multilocular stromata, becoming erumpent through the epidermis and opening through a well-developed ostiole, individual locules 180–250 µm in diameter, wall composed of dark, thick-walled *textura angularis*, becoming paler and thinner walled towards the interior, contents conspicuously white when dry, opening through a periphysate ostiole. Asci bitunicate, clavate, stipitate 84–176×16–24 mm. Ascospores ellipsoid to broadly fusoid, widest in the middle or upper third of the ascospore, hyaline, smooth, thin-walled, unicellular, tapering to the obtuse base and apex, multiguttulate, eight spores in each ascus, irregularly biseriate (15–)18.0–25.5(–28)×(6–)7.5–12.0(–14) µm. Pseudoparaphyses hyaline, septate, branched, 2–3.5 µm wide.

Conidiomata often develop on the same stroma as the ascomata, mostly individual but occasionally aggregated and confluent in a single stroma, externally black, the wall composed of thick-walled *textura angularis* becoming progressively paler and thinner walled towards the inner layers. Conidiophores hyaline, cylindrical, smooth, branched at the base, 0–1 septate, 14–24×2–3 µm, entire locule lined with conidiophores. Conidiogenous cells initially holoblastic, becoming enteroblastic, integrated, hyaline, smooth, cylindrical producing one or more conidia apically, often proliferating percurrently to produce conidia at successively higher levels on annellate conidiogenous cells, or proliferating at the same level resulting in periclinal thickenings. Conidia hyaline, thin-walled, smooth, fusiform to fusiform-elliptical, straight, apex subobtuse, base truncate bearing a minute marginal

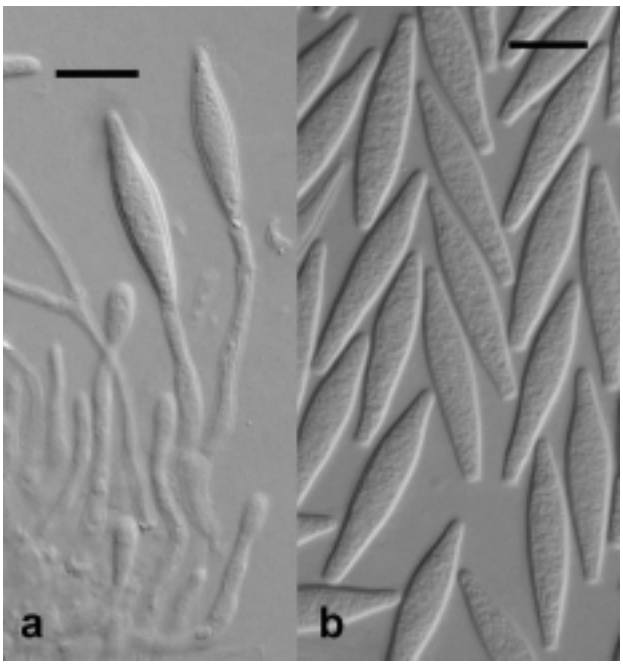

Fig. 2. Anamorph of *Botryosphaeria dothidea*. a. Conidia developing on holoblastic conidiogenous cells. b. Conidia. Scale bars = 10 µm.

frill, 19.5–27(–30)×4.5–6 µm, mean and standard deviation of 50 conidia = 23.5±2.1×5.8±0.5 µm and length/width ratio of 4.1.

Characteristics in culture. On PDA, cultures initially white with abundant aerial mycelium, gradually becoming grey to dark grey. The reverse side of the colonies at first white, but after 2–3 days becoming dark green to olive green from the centre. This colouration gradually spreads to the edge and becomes darker from the centre until the entire underside of the colony is black. Conidiomata multilocular and eustromatic opening through several non-papillate pores, and oozing spores after 8 d at 25°C on OA, with up to 500 conidiomata per plate. Conidia fusiform to fusiform elliptical with a subobtuse apex and a truncate base bearing a minute basal frill, (18–)21–28.5(–30)×(3.5–)4–4.5(–6) µm, mean and standard deviation of 50 conidia = 24.7±1.9×4.4±0.4 µm. Length/width ratios were in the range of 3.8–6.3 with an average of 5.3±0.6. Conidia not becoming septate or darker with age. Microconidia rarely seen.

Notes. The synonymies proposed by von Arx and Müller (1954) were based on an examination of herbarium material of the teleomorph, and thus indicate morphological similarity amongst ascogenous forms only. Since teleomorph features in *Botryosphaeria* vary little between species, these synonymies cannot be regarded as the final verdict on species in this complex. Thus, *B. dothidea* is considered to be a complex of species that are being resolved based on morphological features of the anamorph (Phillips, 2000a) or on molecular data (Jacobs and Rehner, 1998; Denman *et al.*, 2000; Phillips *et al.*, 2002).

The name *Botryosphaeria ribis* has often been used for *Botryosphaeria* isolates with characters similar to *B. dothidea*. Grossenbacher and Duggar (1911) created the name *B. ribis* to accommodate a species of *Botryosphaeria* occurring on currants in the USA. Since that time it has been the subject of much discussion. Although some researchers regard *B. dothidea* and *B. ribis* as two separate species (e.g. Punithalingam and Holliday, 1973; Rumbos, 1987; Ramos *et al.*, 1991) others regard them as synonyms (e.g. Witcher and Clayton, 1963; Maas and Uecker, 1984; Michailides, 1991). Thus far it has not been possible to separate the two species on the morphology of the ascomycete or anamorph state. Since *B. dothidea* has date priority over *B. ribis*, the synonymy proposed by von Arx and Müller (1954) is followed here.

Botryosphaeria parva Pennycook & Samuels, Mycotaxon 24: 455 (1985). Fig. 3
Anamorph: *Fusicoccum parvum* Pennycook & Samuels, Mycotaxon 24: 455 (1985).

Characteristics in vivo. Ascomata morphologically indistinguishable from those of *B. dothidea*. Asci as found in *B. dothidea*, 75–145×17–20 µm. Ascospores broadly ellipsoid to fusoid, widest in the middle to upper third, (15–)18–27(–29.5)×(6–)8–11 µm, hyaline, smooth, thin-walled, biseriate. Pseudoparaphyses hyaline, septate, branched, 2–3.5 µm wide.

Conidiomata externally indistinguishable from ascomata, uni- or multilocular, individual or formed on the same stromata as the ascomata, globose and non-papillate to pyriform with a short, acute pa-

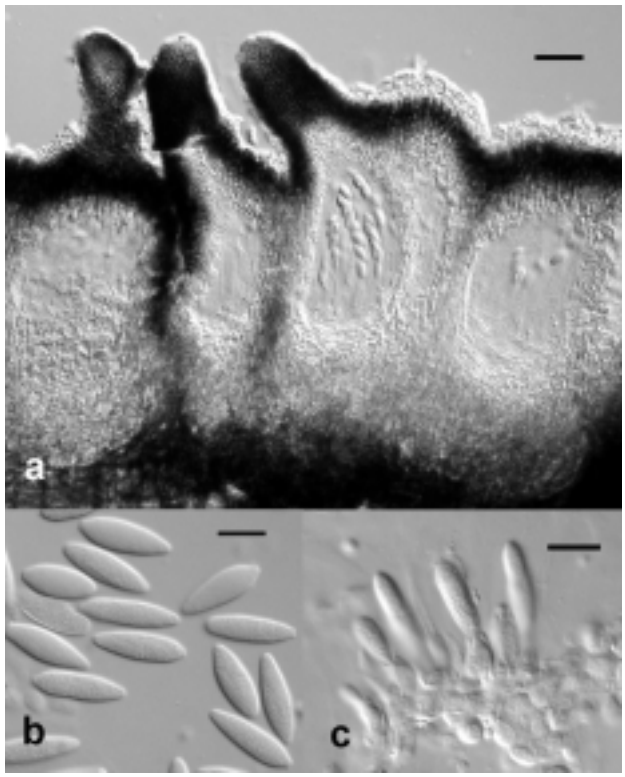

Fig. 3. *Botryosphaeria parva*. a. Section through a multilocular ascoma. b. Conidia. c. Conidia produced from enteroblastic conidiogenous cells with well-developed periclinal thickenings. Scale bars in a = 50 µm; in b and c = 10 µm.

pilla. Conidiophores and conidiogenous cells arising from the inner wall of the locule, and the entire locule is lined with conidiophores, opening through a non-periphysate ostiole. Conidiophores and conidiogenous cells as found in *B. dothidea*. Conidia ellipsoid with subobtuse apex and truncate base, smooth, thin-walled, hyaline, unicellular, (13.5–)15–22.5(–28.5)×4–7.5 µm mean and standard deviation of 300 conidia = 18.9×5.5 µm. Length/width ratio of 2.4–4.5, with mean of 3.5.

Characteristics in culture. Colonies on PDA scarcely different from those of *B. dothidea*. Conidiomata on OA were multilocular, non-papillate, and conidia started to ooze from their ostioles after 13–15 days of incubation at 23°C. Between 35 and 50 conidiomata were formed on each plate of OA. Conidiophores and conidiogenous cells as found in *B. dothidea*. Conidia hyaline, guttulate, thin-walled, non-septate, smooth, fusiform to ellipsoidal with a subobtuse apex and truncate or rounded base, often with a minute basal frill. They were widest in the middle or upper third with dimensions of (12–)15–20(–24)×(4–)4.5–6(–7.5) µm; mean±standard deviation of 320 conidia = 17.2±1.6×5.6±0.6 µm. Length/width ratios were (1.8–)2.5–4(–5), and the mean±standard deviation of 320 conidia = 3.2±0.4. Older conidia becoming olivaceous or light brown with age and sometimes developing a septum before germination. Microconidia, which were seen in some isolates, were hyaline, smooth, rod-shaped and truncate at either end, 3–5×1–1.5 µm.

Notes. Although this species is very similar to *B. dothidea*, it can be distinguished by the shorter, and more ellipsoidal conidia. In cultures on OA it produces larger and fewer multilocular conidiomata than *B. dothidea*. The main feature that distinguishes *B. parva* from *B. lutea* is the absence of a yellow pigment in culture.

Botryosphaeria lutea A.J.L. Phillips, Sydowia 54: 77 (2001). Fig. 4, 5

Anamorph: *Fusicoccum luteum* Pennycook & Samuels, Mycotaxon 24: 456 (1985).

Characteristics in vivo. Teleomorph indistinguishable from *B. dothidea*. Stromata initially immersed, later becoming erumpent through the host tissue, black, <0.5 mm diam., uni- or multilocular, locules spherical to ovoid, 150–200 µm diam. Pseudothecia and conidiomata often formed in the same stroma. Pseudothecia with a short neck, opening through a nonperiphysate ostiole, wall consisting of 8–12 layers of dark brown to black, thick-walled cells, forming pseudoparenchymatic *textura angularis*, up to 60 µm thick, with 3–4 layers of thin-walled, hyaline cells lining the cavity. Asci bitunicate, cylindrical, to clavate, stipitate, 84–176×16–24 µm, 8-spored, associated with filamentous pseudoparaphyses. Ascospores irregularly biseriate, hyaline, guttulate, smooth, aseptate, oval to broadly fusiform, widest in the middle or upper third of the ascospore, tapering to the obtuse base and apex 18–22.5(–24)×7.5–12 µm. Pseudoparaphyses hyaline, septate, branched, 2–3.5 µm wide. Similar to *B. dothidea* but differing in anamorphic characters formed in culture, of which the formation of a yellow pigment is the most discriminative.

Conidiomata eustromatic, separate or confluent, dark brown to black, uni- or multilocular immersed in the host, sub-peridermal, locules up to 150 µm diam., walls consisting of a dark brown *textura angularis*, becoming smaller, thinner-walled and hyaline towards the conidiogenous region. Ostioles papillate, circular. Conidiomata frequently formed on the same stromata as the ascomata. Conidiophores hyaline, smooth, thin-walled, rarely branched at the base, cylindrical, formed from the cells of the inner locule wall, 8–19×3–4 µm. Conidiogenous cells discrete, integrated, hyaline, smooth, cylindrical, producing the first conidium holoblastically and subsequent conidia enteroblastically, proliferating percurrently with 2–3 indistinct percurrent proliferations, or determinate with typical phialides and periclinal thickening (*sensu* Sutton, 1980), (6–)8–16(–18)×(2.5–)3–4(–4.5) µm. Conidia hyaline, thin-walled, aseptate, smooth, fusiform, widest in the middle or upper third of the conidium, apex subobtuse, base truncate, often with a minute basal frill with size range of (12–)16.5–22.5(–24)×4.5–6(–7.5) µm; mean and standard deviation of 115 conidia = 17.5±2.2×5.9±0.7 µm. Length/width ratios were (1.8–)2.0–3.0(–4.0) with mean and standard deviation of 115 conidia = 2.8±0.5.

Characteristics in culture. Colonies on PDA initially pale to colourless, gradually darkening with age and ultimately becoming grey to dark grey. A distinctive feature is the production of a yellow pigment that diffuses into the agar ahead of the leading edge of the colony. The colour is most intense after 3 days at 25°C, thereafter becoming

violaceous and by 6–7 days the yellow colour can no longer be seen. Finally, the violaceous colour darkens and is obscured by the dense growth of dark mycelium. The yellow pigment is also formed by cultures growing on other media but normally it is less intense than on PDA. This yellow pigment was the main character that Pennycook and Samuels (1985) used to separate *F. luteum* from *F. aesculi* and *F. parva*.

On oatmeal agar conidiomata are unilocular, pycnidial and spores start to ooze from the ostioles within 5–7 days at 23°C. Up to 800 conidiomata are produced by a colony in a 9 cm diameter Petri dish. They are partially immersed in the medium,

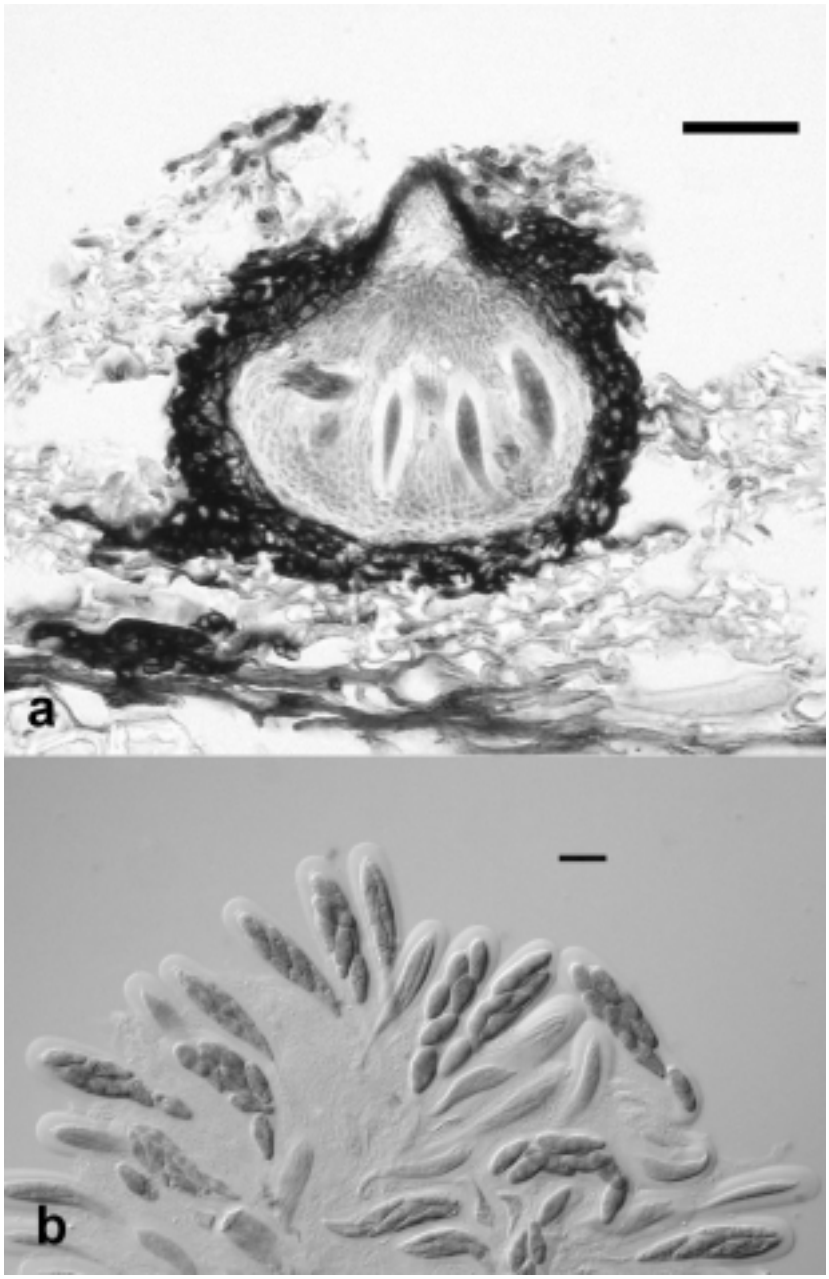

Fig. 4. *Botryosphaeria lutea*. a. Unilocular ascoma. b. Asci with ascospores. Scale bar in a = 50 µm; in b = 20 µm.

globose, usually papillate and covered with olive green appendage-like hyphae. Conidia hyaline, thin-walled, aseptate, fusiform to fusiform elliptical, with a subobtuse apex and a truncate or rounded base often bearing a minute basal frill. Conidia were (15–)18–22.5(–24)×4.5–6(–7.5) µm; mean±standard deviation of 242 conidia = 19.7±1.8×5.6±0.6 µm. Length/width ratios were (2.4–)3.4–3.9(–5.3), and the mean±standard deviation of 242 conidia = 3.6±0.5. Microconidia, which are produced by some isolates are rod-shaped to reniform with either truncate or rounded ends 3–5×1–2 µm.

Notes. *Botryosphaeria lutea* differs from *B. dothidea* and *B. parva* only in the anamorph in culture. The most distinctive feature is the yellow pigment that diffuses into the agar medium in young cultures. This feature has been noted before, not only by Pennycook and Samuels (1985) who described the species, but also earlier by Witcher and Clayton (1963). However, Witcher and Clayton (1963) did not consider this to be of any great taxonomic significance. Although Pennycook and Samuels (1985) used conidium dimensions to distinguish *F. luteum* from other species of *Botryosphaeria*, the data presented by Phillips *et al*.

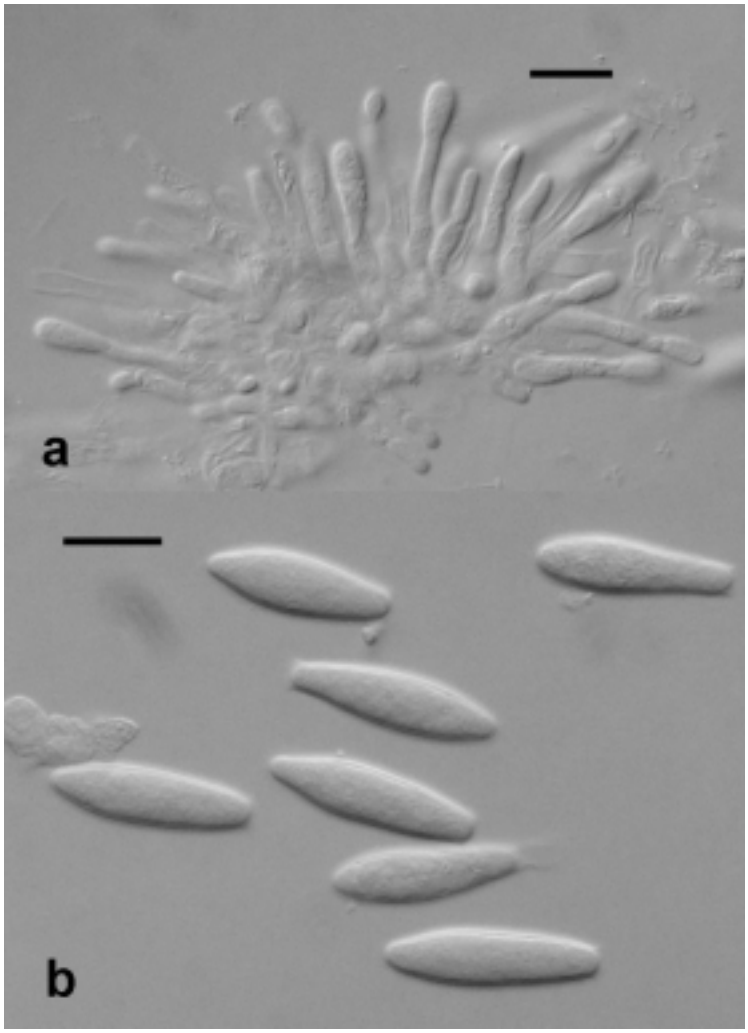

Fig. 5. Anamorph of *Botryosphaeria lutea*. a. Young conidia developing from holoblastic conidiogenous cells. b. Conidia. Scale bars = 10 µm.

(2002) indicate that this is not a reliable character unless the cultural conditions are controlled carefully. Additional features that differentiate *B. lutea* from *B. dothidea* and *B. parva* are the unilocular conidiomata produced on OA. Although the type of conidiomata produced in culture was consistent through many transfers (Phillips *et al*., 2001), and between different isolates, this feature is probably less reliable since it may well be influenced by cultural conditions.

Botryosphaeria obtusa (Schwein.) Shoemaker, Can. J. Bot. 42:1298 (1964). Fig. 6

= *Sphaeria obtusa* Schwein.Trans. Amer. Phil. Soc. II, 4: 220 (1832).

= *Physalospora obtusa* (Schwein.) Cooke, Grevillea 20: 86 (1892).

Anamorph: *Diplodia* sp.

= *Sphaeropsis malorum* Peck, hom. illeg., Ann. Rept. N.Y. State Mus. 34: 36 (1881).

Characteristics in vivo. Conidiomata pycnidial, immersed, separate or aggregated, dark brown, unilocular, thick- or thin-walled, wall formed of dark brown thick-walled *textura angularis*. Ostiole central, single, papillate. Conidiophores absent. Conidiogenous cells holoblastic, discrete, determinate or indeterminate, with one or two percurrent proliferations, long lageniform, hyaline, smooth, formed from cells lining the inner wall of the pycnidium. Conidia oblong, straight, aseptate, broadly rounded at apex, truncate at base, wall brown, internally ornamented, (16–)18–26(–27)×(9–)10–15.0 µm, mean and standard deviation of 56 conidia = 22.2±2.1×11.7±1.8. Length/width ratios were in the range of (1.4–)1.8–2.2(–2.4) with an average of 1.9±0.2.

Characteristics in culture. Colonies on PDA grey-brown with dense aerial mycelium. Conidiomata start to form on OA after 4–5 days at 23°C, small and numerous, scattered over the agar surface, 0.2–1.0 mm diam., conidia start to exude in 7–14-day-old cultures. Conidiophores absent. Conidiogenous cells hyaline, smooth, thin-walled, holoblastic, determinate or indeterminate proliferating percurrently with 2–3 distinct annelations. Conidia cylindric, rounded at both ends, some truncate at base, dark brown when mature, wall smooth on the outside, finely ornamented on the inner surface, mostly (13–)22–26×(9–)10–13(–15) µm, mean and standard deviation of 30 conidia = 22.5±2.3×11.6±1.1. Length/width ratios were in the range of (1.43–)1.6–2.1(–2.3) with a mean and standard deviation of 1.9±0.2. Both hyaline and dark-walled conidia can be seen in the same pycnidium.

Notes. Only the anamorph has been found in Portugal. This fungus is common on diseased, mature grapevine canes and various wood symptoms in several viticultural regions of the world. It is unclear whether it is a pathogen or a saprophyte. Frequently it is found on tissues that have been damaged by physical factors or killed by primary pathogens. This species can be distinguished from other *Botryosphaeria* species by the large, dark brown conidia, and the distinctly annellate conidiogenous cells.

Botryosphaeria stevensii Shoemaker, Can. J. Bot. 42: 1299 (1964). Fig. 7

= *Physalospora malorum* (Peck) Shear, Mycologia 17: 100 (1925).

= *Physalospora mutila* N. Stevens, nom. inval. Mycologia 28: 333 (1936), as *Physalospora mutila* (Fr.) N.E. Stevens comb. nov.

Anamorph: *Diplodia mutila* Fr. apud Montagne, Ann. Sci. Nat. II. 1: 302 (1834).

= *Sphaeria mutila* Fr., Syst. Myc. 2: 424–425 (1823).

= *Sphaeria malorum* Berk., Eng. Fl. 5: 257 (1836).

= *Sphaeropsis malorum* (Berk.) Berk., Outlines Brit. Fung. 316 (1860).

Characteristics in vivo. Conidiomata pycnidial, separate or aggregated, globose, dark brown to black, immersed, unilocular, thick-walled; outer wall layers thick-walled *textura angularis*, inner layers thin-walled, hyaline; ostiole central, papillate. Conidiophores hyaline, branched, septate, smooth, cylindrical, formed from the inner layers of the pycnidial wall. Conidiogenous cells holoblastic, integrated or discrete, determinate, cylindrical, hyaline, smooth, forming a single, apical conidium. Conidia smooth, unicellular, cylindric with broadly rounded ends, some with a large central guttule, smooth, with a thick glassy wall that normally remains hyaline even after the conidia have been discharged from the pycnidium, (19.5–)20–27(–29)×10–14(–15) µm, average of 50 conidia = 23.1±2.7×11.9±1.5 µm. Length/width ratios were in the range of (1.5–)1.9–2.1(–2.3) with a

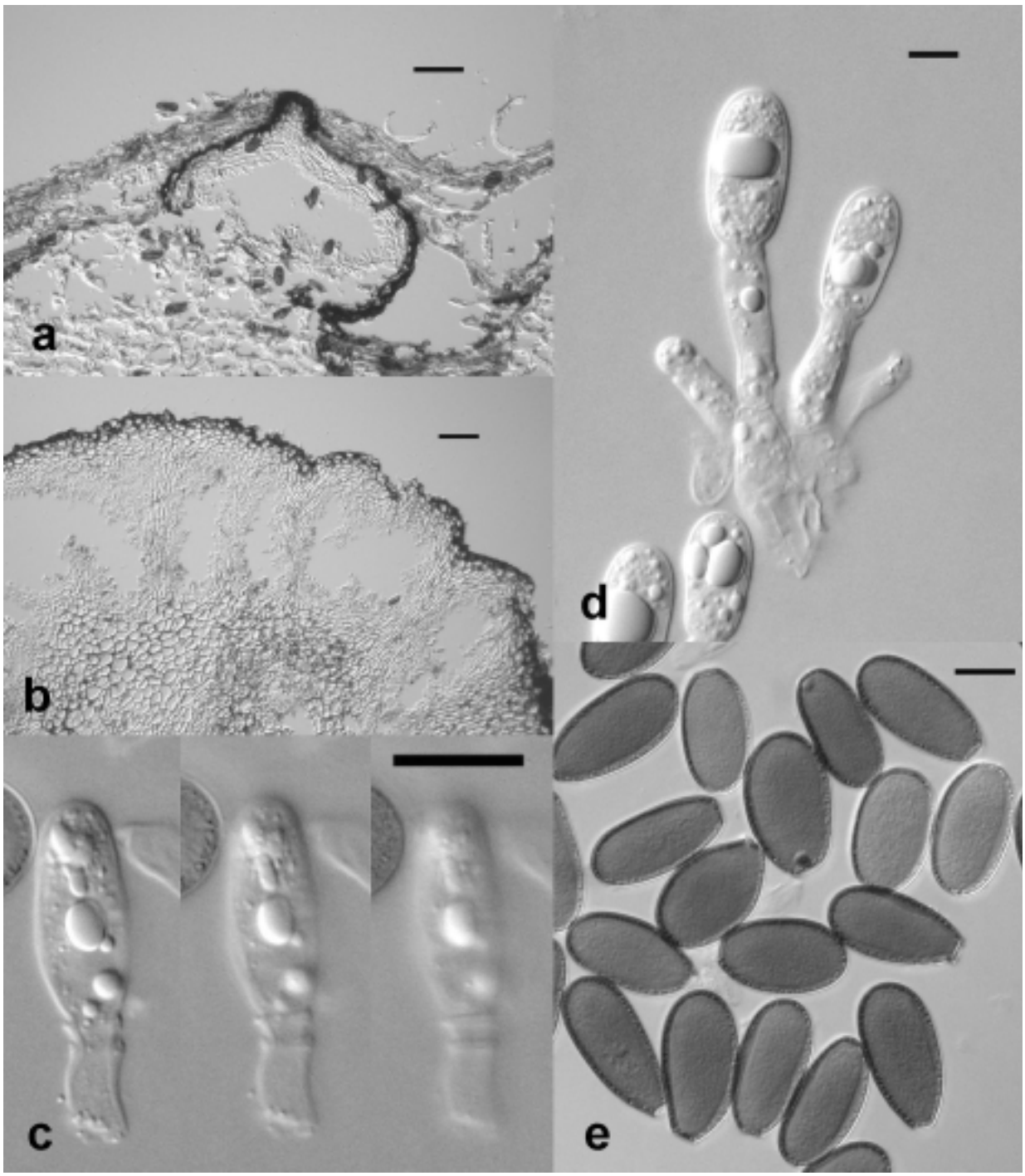

Fig. 6. Anamorph of *Botryosphaeria obtusa*. a. Thin-walled, unilocular pycnidium. b. Part of a thick-walled, multilocular conidioma. c. Three different levels of focus of a conidium developing from a percurrently proliferating conidiogenous cell. Two distinct annellations can be seen on the conidiogenous cell. d. Conidia developing on conidiophores. e. Dark conidia showing smooth outer surface and ornamentation on the inner surface of the wall. Scale bars in a and b = 20 µm; in c and e = 10 µm; in d = 5 µm.

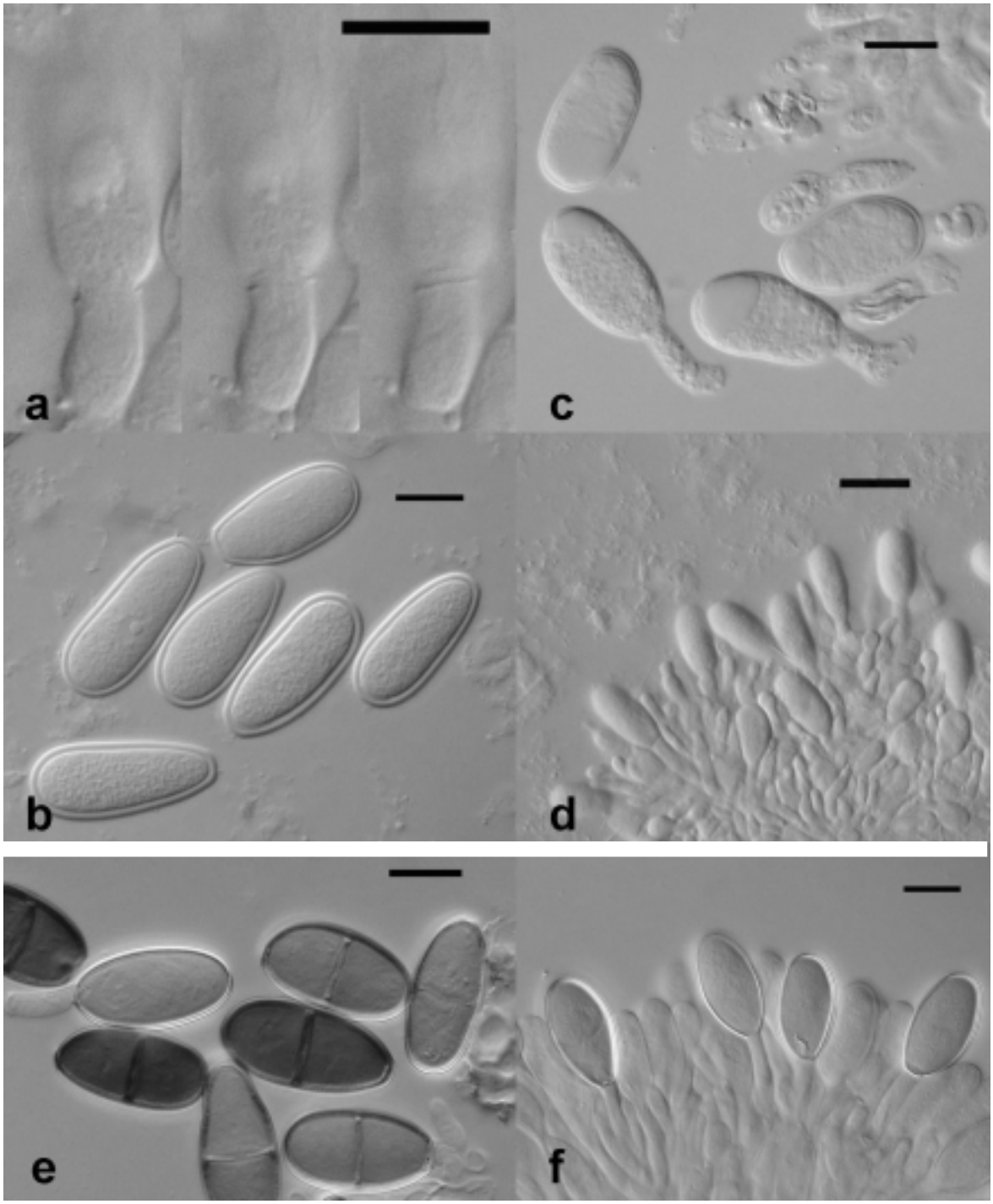

Fig. 7. Anamorph of *Botryosphaeria stevensii*. a. Three different levels of focus of a conidiogenous cell showing two indistinct annellations. b. Conidia. c. Holoblastic conidiogenesis. d. Conidiogenous layer of a pycnidium. e. Mixture of dark-walled, septate conidia and hyaline, aseptate ones. f. Conidia becoming dark-walled while still attached to the conidiogenous cell. Scale bars in a, b, c, e and f = 10 µm; in d = 20 µm.

mean and standard deviation of 1.96±0.2.

Characteristics in culture. Colonies on PDA very dark olivaceous brown with dense aerial mycelium. Conidiomata on OA large, 1–2 mm diameter, relatively few on each plate and formed only towards the centre of the colony, start to form on OA after 7 days at 23°C, not exuding conidia until the cultures are more than 21 days old. Conidiophores absent. Conidiogenous cells smooth, hyaline, thin-walled, holoblastic, determinate or indeterminate proliferating percurrently with one or two indistinct annelations. Conidia cylindric with broadly rounded ends, hyaline, unicellular, wall smooth, (21.5–)23–27(–28.5)3(10.5–)11–12(–13) µm with mean and standard deviation of 25.5±1.9311.5±0.6. Length/width ratios were in the range of (1.8–)2.1–2.4(–2.6). Conidia normally remain hyaline and aseptate even after discharge from the pycnidia. In some isolates, however, the conidia become dark brown and 1-septate even while they remain in the pycnidium.

Notes. Only the anamorph has been found in Portugal and this was at only one locality, namely, Quinta de São Jorge, Montemor-o-Novo. Although *B. stevensii* has been reported to cause black dead arm of grapevines in Hungary (Lehoczky, 1974), such symptoms were not found in the material examined in the present work. It was, however, frequently associated with shoot necroses, and was commonly found around dead buds. The isolates tested by Phillips (1998) were weakly pathogenic.

This species can be distinguished from *B. obtusa* by the conidia, which become dark only some time after discharge from the pycnidium, and the indistinct annelations on the conidiogenous cells. Of these two characters, annellation is the most distinctive since the conidia are reported to become dark-walled in some isolates. Indeed, in some isolates the conidia become dark-walled and septate even before they are discharged from the pycnidium. However, this species has not been critically evaluated and it is possible that it is a complex of species.

Discussion

Five species of *Botryosphaeria* were found associated with several different disease symptoms in Portuguese vineyards. Some of the symptoms, especially brown wood streaking, are strikingly similar to those caused by *Phaeomoniella chlamydospora* (Mugnai *et al.*, 1999) in esca and Petri disease. Three of the species, *B. stevensii*, *B. dothidea*, and *B. obtusa* have previously been reported as the cause of grapevine diseases elsewhere (e.g., Lehoczky, 1974; Phillips, 1998; Larignon and Dubos, 2000, respectively). Although *Botryosphaeria dothidea* has frequently been reported from grapevines, most of the reports did not take into account that this is a complex of species (Pennycook and Samuels, 1985). Thus, the isolates of *B. dothidea* regarded by Phillips (1998) to be pathogenic on grapevines, and to be the cause of excoriose (Phillips, 2000b), were later shown to represent three different species (Phillips *et al.*, 2002). Of these, *B. parva* was the most common, while *B. dothidea* was the least common. However, it is important to point out that in the present work, a survey as such was not carried out and the data represent somewhat random encounters of the diseases described.

The five species of *Botryosphaeria* described here are known not only from grapevines but also from other hosts in different parts of the world. Although they are known to be pathogens, their status on grapevines has not been fully resolved. The frequent association of *B. parva* with the disease symptoms described here, together with proof of its pathogenicity on grapevines (Phillips, 1998), albeit under the name of *B. dothidea*, indicate that its role in wood disease cannot be ignored. What is not clear, however, is how widespread it is in viticultural regions, and how much it contributes to the general decline syndrome.

In view of the differing reports on pathogenicity of *B. obtusa*, it is possible that this is also a complex of species. Therefore, a more detailed examination of isolates with the general characteristics of this species is warranted. The conflicting reports on the pathogenicity of *B. obtusa* are not restricted to grapevines. In North America *B. obtusa* is reported to cause an important leaf spot, canker and fruit rot of apple, while in England and New Zealand it is chiefly a weak secondary pathogen associated with, but not necessarily the primary cause of cankers, leafspots and fruit rots. Despite this difference it seems that they are the same species. It has not been established whether the difference in pathogenicity is due to the presence of more virulent strains in North America, or whether the conditions there are more conducive to dis-

ease. Thus, it is possible that *B. obtusa* is a heterogeneous group of species differing in their pathogenicity, or that within the single species, different strains exist. Since Jacobs and Rehner (1998) showed that isolates conforming morphologically to *B. obtusa* were genetically heterogeneous, a reassessment of this species is warranted.

It is possible that identifications have not always been correct, especially as it is now clear that the species previously known as *B. dothidea* is in fact a complex. Recent advances in molecular techniques, especially analysis of ribosomal RNA genes have helped to resolve a number of taxonomic problems associated with this group. Among the variable regions of rDNA, the internal transcribed spacers (ITS) have been successfully applied to study phylogenies of several fungal genera including *Botryosphaeria* (Jacobs & Rehner, 1998; Denman *et al*., 2000; Phillips *et al*., 2002). However, the names appended to the terminal clades of dendrograms generated by such data are based largely on morphological characters, and these names are linked to the dead, dried herbarium specimens of the types. Furthermore, such molecular techniques are too expensive for use in routine identifications of large numbers of samples. Therefore, although molecular techniques are powerful tools in taxonomy, the descriptions given in this paper are based solely on morphology. Despite the fact that the sexual states of many *Botryosphaeria* species are rare, the exclusive use of teleomorph names avoids the nomenclatural problems of the anamorphs. Therefore, although species are distinguished on anamorphic characters, and anamorphs are largely featured in the above descriptions, it is recommended that teleomorph names alone should be used.

For accurate identifications of *Botryosphaeria* species, it is necessary to grow the fungi in pure culture. Characteristics of conidia formed on the host in nature are more variable than those formed in culture, and certain characters, such as pigment formation, can be seen only in culture. The type of conidiomata formed under standardised conditions is also helpful in distinguishing certain species. For these reasons, the cultural conditions and medium compositions must be carefully controlled. The conditions described in this study proved to be useful and easily standardised.

Although a restricted view of the species found on grapevines has been followed here, this work, together with previous studies on grapevines, has shown that *Botryosphaeria* species should not be regarded only as saprophytes. It is hoped that the data presented here, together with the descriptions of the species, will stimulate greater interest in this genus as potential pathogens of grapevines.

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