Infection Process of Olive Fruits by *Colletotrichum acutatum* and the Protective Role of the Cuticle and Epidermis

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Abstract

Colletotrichum acutatum is a serious concern for the Portuguese oliviculture and food industry, due to both olive oil yield and quality decrease. While the organism's phytopathogenic potential has been well documented, the pathogen adhesion and colonization process in olives remain poorly understood. This paper reports experiments conducted on *C. acutatum*-susceptible, *C. acutatum*-moderately resistant and *C. acutatum*-resistant olive fruits during infection process, in two consecutively years, to identify the physical differences related to the host cuticle promoting resistance and susceptibility to *C. acutatum*. Cuticle thickness, perimeter and area of epidermal cells of 'Galega' (susceptible), 'Cobrançosa' (moderate-resistant) and 'Picual' (resistant) fruits were measured. Here, we also describe the colonization process of olive fruits by *C. acutatum.* To achieve this goal olive fruits

from 'Galega', 'Cobrançosa' and 'Picual' were inoculated in a field trial with an aqueous suspension of *C. acutatum* ($1x10^6$ spores ml^{-1}) under suitable humidity and temperature conditions. Light, fluorescent and scanning electron microscopy were used to view olive fruit sections. Significant differences were observed among the parameters studied: cuticle thickness, perimeter and area of olive fruit epidermal cells. The *C. acutatum*-resistant ('Picual') fruits showed highest mean values for each parameter in both years. Pathogen ultrastructures, such as spores, germ tube, and appressoria were clearly observed using different microscopy techniques. Acervuli sporulation was observed only 192 hours after inoculation in *C. acutatum*-susceptible ('Galega') fruits.

Keywords: *C. acutatum*, Infection stages, Olive fruit cuticle

1. Introduction

The *Colletotrichum acutatum* and *C. gloeosporioides* caused anthracnose in a wide range of hosts found mainly in temperate, subtropical and tropical areas (Martín and García-Figueres, 1999; Moral *et al.,* 2009). Talhinhas *et al.,* (2005) with molecular and phenotypic assays reported that the occurrence of *C. acutatum* in Portuguese olive orchards was higher (>97%) than *C. gloeosporioides* (<3%). The *C. acutatum* J. H. Simmonds is one of the most pathogenic species, which causes anthracnose and blight in agriculturally important hosts including many dicotyledonous plants such as strawberry, apple, citrus, and stone fruits (Horowitz *et al.,* 2002; Peres *et al.,* 2005).

In national terms the prevalent olive cultivar is 'Galega' that gives to olive oil a good specificity when compared with olive oils from others cultivars. However, this cultivar have a sanitary problem that is the susceptibly to olive anthracnose caused by the *C. acutatum*, named in Portugal by 'gafa'. The olive anthracnose disease was firstly reported in Portugal by Almeida (1899). This disease is very aggressive and is one of the main constraints affecting both the olive production and oil quality (Talhinhas *et al.,* 2003, 2005, 2007; Carvalho *et al.,* 2008). Although the disease is distributed along all Mediterranean Basin it is in Portugal that it has a great expression, mainly due to the Atlantic influence (higher moisture during autumn).

There are reports that cultivars resistance is based on an array of defensive mechanisms. These mechanisms could be classified broadly into 'passive' or pre-existing mechanisms (e.g., cell wall, fruit cuticle, needles, and trichomes, among others) that prevent or attenuate the pathogen invasion, while others, such as signal transduction and gene expression are 'active' or inducible by host-pathogen (Mithöfer *et al.,* 2009). In fact, the cuticle is the first site of contact between olive fruit and *C. acutatum* and therefore is thought to play a significant role in plant-pathogen interactions. In several cases, products of cutin hydrolysis induce defense responses in plants (Shah and Chaturvedi, 2009). However, the physiological role of cuticle characteristics in olive and *C. acutatum* interaction are poorly understood compared to colonization process. Since the epidermal surface is the first contact point between plants and pathogens, understanding the differences related to the cuticle characteristics may give indications of its involvement in plant-pathogen interaction (Kerstiens, 1996).

The early stages of infection by *Colletotrichum* spp. are very similar between species, as they all involve a series of processes including the spore adhesion to the fruit cuticle, germination, production of adhesive appressoria, crucial for cuticle penetration, growth and fruit colonization (Prusky *et al.,* 2000; Wharton and Diéguez-Uribeondo, 2004; Arroyo *et al.,* 2005; Diéguez-Uribeondo *et al.,* 2005; Gomes *et al.,* 2007; Mota-Capitão *et al.,* 2008; Gomes *et al.,* 2009). Thus the objectives of this study were to: (i) determine resistance aspects related to the fruit cuticle and epidermis (thickness, perimeter and area) on susceptible and resistant olive fruits; and (ii) monitor the infection and colonization of the olive fruits by the *C. acutatum* using light, fluorescent and scanning electron microscopy.

2. Materials and methods

2.1 Plant and fungal material

Fruits from adult olive tree cultivars 'Galega' (susceptible, highly infected with olive orchards completely destroyed), 'Cobrançosa' (moderately-resistant), and 'Picual' (resistant, inhibits fungus development in the fruit and tree vigour not affected) were used. The study of the anthracnose disease began in September of 2006 up to November of 2007. The olive fruits were obtained from a certified olive orchard, at the National Plant Breeding Station at Elvas, Portugal. *C. acutatum* isolates were collected from diseased olive fruit of cultivar Picual, in the Alentejo region of Southern Portugal. Monoconidial isolate, obtained from olive 'Picual', which has an high level of resistance in natural infection, was used. In this way the probability of the isolate used being aggressive would be higher. These isolates were identified by PTA-Elisa test using antibodies specific for *Colletotrichum acutatum* (Adgen, U.K.) (Carvalho *et al.,* 2003). Monoconidial isolate was used for inoculation experiments. The

isolate was cultured on potato dextrose agar (PDA) for 8 days at 22ºC under a 12 hour photoperiod. Inoculum was prepared by flooding dishes with sterile distilled water, scraping the surface gently with a glass rod, and filtering the resulting suspension through sterile cheesecloth. Spore concentration was adjusted to $1x10^6$ spores ml^{-1} in sterile water for inoculation. Two branches (approximately with 100 fruits) in each olive tree were inoculated with a spore suspension to evaluate the symptoms. Control plants were inoculated with sterile water suspension. After inoculation, plants were enclosed in plastic bags, to create high humidity conditions in order to encourage spore germination and development. In order to study the olive fruits infection process by *C. acutatum*, fruits were sampled at 0, 24, 48, 72 and 192 hours after inoculation (hai), and symptoms appearance were recorded on a daily basis. Fruit samples at 0 h after inoculation and with similar date, were used for cuticle and epidermis characteristics analysis. All experiments were conducted in 6 olive trees per cultivar (3 inoculated and 3 for control) during two consecutively years.

2.2 Microscopic assessment

Fruits were collected from Oliviculture Department of the Portuguese National Plant Breeding Station at Elvas and measurements were performed on 30 healthy olive fruit samples of each cultivar during two consecutive years (2006 and 2007). To measure the fruit cuticle thickness, area and perimeter of the epidermis cells, fruit cross sections were prepared for light microscopic examinations. Small pieces of 1 cm^2 taken from the middle part of the fruits of each cultivar were fixed in FAA (70% ethanol: formalin: acetic acid = 90: 5: 5, Johansen, 1940), dehydrated in a series of ethanol solutions (70%, 80%, 90%, 100%, 1 h each) and embedded in paraffin. The blocks were sectioned in a manual rotary microtome (Leica RM 2135; Leica Microsystems, Nussloch, Germany) to obtain 2–8 μm thick sections. Then, the sections were stained with 1% toluidine blue O in 0.1 M phosphate buffer, pH 6.8 for 10 min, using the method of O'Brien and McCully (1981) and observed and photographed using an Olympus IX51 inverted light microscope (Olympus Biosystem, Munich, Germany) equipped with a digital camera (ColorView III; Soft Imaging System GmbH, Münster, Germany) and the image analysis software Olympus cell. Cuticle thickness was measured at 30 different points per sample (approximately in 50 fruits) and the area and perimeter was determined for 30 epidermis cells in 50 fruits. Microscopic and statistical analyses were performed in olive cuticle cross-section, in order to explain the differential behaviour observed when *C. acutatum* attack three olive genotypes. Cuticles were also observed with a scanning electron microscopic (SEM). The fluorescence and optical microscopes were used for *C. acutatum* development observation. The SEM analysis of the fruits was conducted at 10 kV using a Philips/FEI Quanta 400. Fluorescence microscopy used an Axioskop2 MOT microscope fitted with an AxioCam HRC camera (Carl Zeiss MicroImaging Inc.). Olive fruits infection process by *C. acutatum* were analysed by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss, Jena GmbH).

2.3 Statistics

For the analyses of fruit cuticle thickness, area and perimeter of the epidermis cells data it was performed an analysis of variance using the Super ANOVA software (1.11 Abacus Concepts Inc, 1991). Significances of differences were established from a Duncan's Test ($P < 0.05$).

3. Results

3.1 Colletotrichum acutatum-olive interaction

Monitoring olive fruits colonization events by *C. acutatum* and its infection strategies was conducted using three microscopy systems and fruit vibratoming sections. Consistent with previous data (Gomes *et al.,* 2009) sections of olive fruit epidermis revealed no adhesion of spores until 24 hai. In *C. acutatum*-susceptible fruits an electron dense zone with ungerminated spores were observed 48 hai (Figure 1a). In *C. acutatum* -resistant fruits no spores adhesion was observed within 48 hai. Once inside susceptible fruit, the pathogen grew through the mesocarp until it colonized all host cells. The mesocarp of susceptible fruits became densely infected, dehydrated, and finally necrotic lesions and hyphae became visible under SEM (Figure 1b, 1c). Secondary hyphae grew extensively inter- and intracellularly and across cell walls from one cell to the next, killing host cells and dissolving cell walls ahead of the infection (Figure 1b). The end of colonization process is remarkably manifested by host cuticle collapse (Figure 1d). After the necrotrophic phase, the pathogen could be detected, as a mass of mycelium that rapidly colonized surrounding cells culminating with cuticle rupture (Figure 1d). The colonization process of olive fruits by *C. acutatum*, such as spores adhesion and germination on cuticle to form an appressoria were very similar between susceptible and resistant cultivars. However, in *C. acutatum*-resistant fruits interaction, such as 'Picual' this process is quite uncommon.

After pathogen adhesion to the *C. acutatum*-susceptible fruits the spores germinated and developed a germ tube. When germination of spore started one appressoria emerged in an apical position (Figure 2a, 2b). Dark bodies

inside the cytoplasm of ungerminated spores were observed (Figure 2c). These bodies are glycoproteins, lipids and polysaccharides important to protect the spores from desiccation (Jong and Ackerceken, 2009). The acervuli sporulation was observed 192 hai on *C. acutatum*-susceptible fruits (Figure 1d, 3a, 3b). Several acervuli were observed along the epidermis (Figure 3a), which completely disrupt the epidermis structure (Figure 3b).

3.2 Cuticle thickness, perimeter and area of epidermal cells

Physical and chemical barriers are now seen as source of signals that influence plant defense (Kerstiens, 1996; Shah and Chaturvedi, 2009). The role of the olive cuticle as a preformed physical barrier against pathogens adhesion, penetration, and how this barrier can be overcome was studied. 50 healthy fruit per cultivar, at the same ripening stage, were measured to evaluate differences on cuticle thickness, perimeter and area of epidermal cells between susceptible and resistant olive cultivars. The microscopic observations related to cuticle thickness ($P < 0.01$), perimeter ($P < 0.01$) and area ($P < 0.01$) of epidermal cells revealed significant differences between *C. acutatum*-susceptible and resistant fruits (Table 1). When cuticle thickness was analysed in fruits from 'Galega', 'Cobrançosa' and 'Picual' during the 2006, the lower mean value was observed in fruits from Galega cultivar (16.08 ± 0.4 µm; Figure 4a) while the highest mean value, for cuticle thickness, was observed in resistant fruits from 'Picual' (23.27 ± 0.3 µm; Figure 4c) (Table 1). 'Cobrançosa' presented an intermediate cuticle thickness (22.03 ± 0.5 µm; Figure 4b). The same trend was observed in the following year (2007), where the cuticle's thickness was between 13.28 ± 0.4 and 18.57 ± 0.5 µm in 'Galega' and 'Picual' fruits, respectively (Table 1). The perimeter of epidermal cells in 2006 ranged from 55.95 ± 2.7 µm to 74.37 ± 1.7 µm among *C. acutatum*-susceptible and resistant fruits, respectively. A similar behavior was observed in the following year (2007; Table 1). 'Cobrançosa' fruits always presented intermediate values of 66.47 ± 3.1 µm in the 2006 year and a slightly higher mean value of 70.80 ± 1.4 µm in the following year (2007; Table 1). Fruits from *C. acutatum*-resistant cultivar showed, in both years, the highest mean value from area of epidermal cells (229.26 ± 9.7 and 311.25 ± 19.6 μm^2) while the susceptible fruits showed the lowest mean value (166.38 ± 14.4 and 148.25 ± 8.6 μm^2) (Table 1).

4. Discussion

Olive anthracnose is caused by *C. acutatum* and *C. gloeosporioides* (Martín and García-Figueres, 1999; Angiolillo *et al.,* 1999; Kimura *et al.,* 2001; Trapero, 2009). The pathotype used for this study was previously isolated and identified as *C. acutatum*, the prevalent species in Alentejo region (Carvalho *et al.,* 2003). *Colletotrichum* species penetrates hosts through natural openings such as stomata, wounds and/or by direct penetration on the plant cuticle (Bailey *et al.,* 1992). Consistent with previous data (Gomes *et al.,* 2009), the interaction between *C. acutatum*-olive fruits occurred only through cuticle by appressoria structure formation. Penetration through stomata or direct penetration by germ tubes or hyphal tips, without appressoria, was not observed in this pathosystem, as it has been reported in bean cultivars (Jerba *et al.,* 2005), and on cowpea leaves (Latunde-Dada *et al.,* 1996). Results from colonization process showed that the severity of infection was greater on the *C. acutatum*-susceptible ('Galega') than on *C. acutatum*-resistant ('Picual') fruits. However, when C. *acutatum* host entry, the early stages of infection are remarkably similar between susceptible and resistant fruits, as they all involves spores adhesion, germination and penetration by appressoria (Figure 1). The appressoria observed in figure 2b is responsible to generate physical force to breach the olive fruit cuticle and outer cell wall. The enzymes needed for initial penetration may result on lesser levels of disease incidence and severity and can be related to lower appressoria number in host surface (Diéguez-Uribeondo *et al.,* 2005; Kubo, 2005). Indeed, we qualitatively detected less germinated spores in cuticle from *C. acutatum*-resistant fruits. In contrast, during the first 48 hai the necrotic lesions in susceptible fruits (from 'Galega') were evident not only at the fungus infection site but were also spread to numerous mesocarp cells. There are several reports with several hosts, such as citrus (Zulfiqar *et al.,* 1996), almond (Diéguez-Uribeondo *et al.,* 2003), strawberry (Leandro *et al.,* 2001), and blueberry (Wharton and Diéguez-Uribeondo, 2004) showing that infection chronology (spores germination and germ tube differentiation) happens within hours (3 to 48 hai) under favourable environmental conditions (Wharton and Diéguez-Uribeondo, 2004). Our observations showed that the acervuli sporulation in olive fruit only occurred after 192 hai on *C. acutatum*-susceptible fruits. Upon fruits' *C. acutatum* colonization, a massive internal proliferation of pathogen hyphae was observed within mesocarp cells and ending with the cuticle's collapse when acervuli sporulated. The acervuli sporulation release spores, which can be dispersed by wind or spread by rain. The symptoms of *C. acutatum* on susceptible fruit were observed when the mycelium breaks through the fruits' cuticle and produces acervuli (Figure 1d, 3a, 3b). Similar results related to the acervuli sporulation, were reported by Wharton and Schilder (2008) on blueberry cultivars 'Jersey' (susceptible) at 108 hai and 'Elliott' (resistant) at 144 hai. Smith *et al.,* (1999) reported that acervuli sporulation on *C. dematium*-cowpea stems pathosystem only became visible approximately 80 hai and on leaves brown lesions

became visible only 100 hai. Latunde-Dada *et al.,* (1996) reported acervuli sporulation in *C. destructivum*-cowpea pathosystem 120 hai. On *C. graminicola-Agrostis palustris* and *C. graminicola-Lolium perenne* pathosystems the acervuli sporulation appeared within 72 and 96 hai, respectively (Khan and Hsiang, 2003).

Our observation also showed a lack of anthracnose symptom on *C. acutatum*-moderately ('Cobrançosa') and *C. acutatum*-resistant ('Picual') fruits. We believe that this fact can be an indication of host defence responses. In field trial, differences in *C. acutatum* attack in susceptible and resistant cultivars have been reported by Carvalho *et al.,* (2006). In terms of infection time, the cultivar Galega (susceptible) was invaded by *C. acutatum* earlier than the cultivar Picual (resistant). One of the reasons that this may happen maybe due to the fact that 'Galega' ripens earlier than 'Cobrançosa' and 'Picual', therefore it may not escape to *C. acutatum* infection when conducive conditions occur (first rainfall- normally in September). The intersection between fruit ripening and susceptibility has been reported in different pathosystem such as tomato (Cantu *et al.,* 2008) and olive (Moral *et al.,* 2008; Mota-Capitão *et al.,* 2008). The *C. acutatum* colonization process has been studied in the context of olive susceptibility. However, the contribution of ripening associated with cuticle and susceptibility will be extensively evaluated in future work. Several reports bring out that the higher concentration of phenolic compounds in immature olive fruits constitutes an important resistance factor. It might be considered that when maturation occurs earlier in a cultivar, dependent on the genotype, there is a general decrease in phenolic compounds and this fact increase the cultivar susceptibility. Fruit ripening is characterized by processes that modify fruit texture but also by a dramatic increase in susceptibility to necrotrophic pathogens (Cantu *et al.,* 2008). However, the resistance mechanisms in olive anthracnose, described above, need to be further investigated. We observed differences in ripeness in all three cultivars. Galega was the first to be mature, and there were small differences between Cobrançosa and Picual cultivars. In *C. acutatum*-moderately ('Cobrançosa') and *C. acutatum* -resistant ('Picual') cultivars the appressoria formed on immature fruits may remain quiescent until onset of ripening in September. According Guestsky *et al.,* (2007) the greater susceptibility during fruit ripening may be related to the levels of flavonoids that decrease, while the quiescent *Colletotrichum* infections are active. According Barbosa *et al.,* (2006) the dark bodies observed inside the ungerminated spore (Figure 2c) is related to the lipid reserves that represent a crucial role in energy storage, indispensable for spores' germination under suitable conditions. The lipid bodies in ungerminated spores have been reported in *C. graminicola* (Schadeck *et al.,* 1998), *C. lagenarium* (Kimura *et al.,* 2001), and *C. gloeosporioides* (Kuo, 1999). The anthracnose resistance observed in *C. acutatum*-moderately ('Cobrançosa') and *C. acutatum*-resistant ('Picual') fruits can have close analogies with the pathogen dormant phase until fruit ripening. In these cases the pathogen development is restricted within the epidermal layer (Gomes *et al.,* 2009). When physiological changes occur in fruits, such as the maturity stages, the pathogen development may occur (Angiolillo *et al.,* 1999). Another characteristic related to the fruit maturity is the cuticle and exocarp thickness (Manandhar *et al.,* 1995). The differences observed in olive cuticle thickness, perimeter and area of epidermal cells can be positively related to the fruit susceptibility. Our observations also showed significantly differences in cuticle thickness from *C. acutatum*-susceptible and *C. acutatum*-resistant fruits, whose cuticles constitute a physical barrier to pathogen adhesion and development (Figure 3). The epidermal cells perimeter and area in *C. acutatum*-susceptible ('Galega') and *C. acutatum*-resistant ('Picual') fruits were also significantly different (Table 1). The majority of epidermal cells are small and compact with cuticle consisting of cutin and wax. This cuticle effectively protects the plant from water loss and functions as a barrier against pathogen. The results obtained, in terms of cuticle parameters can be important data for understanding the *C. acutatum*-olive interaction. During ripening, many fruit, including olive, disassemble components of the cell wall, with cuticle thickness and degree of cutinisation decreasing significantly from immature to fully ripe fruits, thereby contributing to fruit susceptibility.

Differences related to the cuticle characteristics could explain why some olive cultivars are more severely attacked by *C. acutatum* than others. In previous work the expression of resistance, on olive cultivars, occurred only after pathogen penetrated the physical barriers (Gomes *et al.,* 2008). However, once penetrated, the infection development in 'Picual' (resistant) occurred very slowly, suggesting that fungus can remain in a latent period waiting for favourable environmental conditions. This latent infection can be related, among other factors, to olive fruits ripeness. Moshe *et al.,* (1994) reported that spore from *C. gloeosporioides* and *C. musae* developed mechanisms to use the host's ripening hormone as a signal to initiate spore germination, appressoria formation, and colonization process. Little is known on the different factors that affect resistance with regard to the olive cuticles. This study revealed that the initial phase of interactions between fruit cuticle and *C. acutatum* can be correlated to the nature of the cuticle and epidermal cells (cuticle thickness, perimeter and area of epidermal cells). In fact, an increase in cuticle thickness may provide a mechanical obstacle to pathogens attack. The thinner epidermis cells of 'Galega' also confer lower protection against pathogens. Indeed, dermal tissue

functions to protect the plant from injury and water loss. Bacelar *et al.,* (2004) reported considerable differences among olive genotypes related to the morphological and structural leaf cuticle characteristics against abiotic stress, such as water loss.

This study is the first to report there were significant differences of cuticle parameters in olive fruits that suppress resistance or increase susceptibility to *C. acutatum*, resulting in large economic losses of olive orchards.

References

Almeida, M.J.V. (1899). La gaffa des olives en Portugal. *Bulletin de la Société Mycologique de France*, 15, 90-94.

Angiolillo, A., Mencuccini, M., & Baldoni, L. (1999). Olive genetic diversity assessed using amplified fragment length polymorphisms. *Theoretical* and *Applied Genetics,* 98(3-4), 411-421. http://dx.doi.org/10.1007/s001220051087, http://www.springerlink.com/content/0mth7e1kp97mnm9e/

Arroyo, T.F., Moreno, J., García-Herdugo, G., De los Santos, B., Barrau, C., Porras, M., Blanco, C., & Romero, F. (2005). Ultrastructure of the early stages of *Colletotrichum acutatum* infection of strawberry tissues. *Canadian Journal of Botany,* 83(5), 491-500. http://www.nrcresearchpress.com/doi/abs/10.1139/b05-022

Bacelar, A.E., Correia, M.C., Moutinho-Pereira, M.J., Gonçalves, C.B., Lopes, I.J., & Torres-Pereira, M.G.J. (2004). Sclerophylly and leaf anatomical traits of five field-grown olive cultivars growing under drought conditions. *Tree Physiology*, 24, 233–239. http://treephys.oxfordjournals.org/content/24/2/233.full.pdf

Bailey, J.A., O'Connell, R.J., Pring, R.J., & Nash, C. (1992). Infection strategies of *Colletotrichum* species. In: J.A. Bailey and M.J. Jeger, Editors,*Colletotrichum: biology, pathology and control*, Commonwealth Agricultural Bureau International, Wallingford, Oxon, UK, pp. 88–120.

Barbosa, A.C., Schadeck, R.J.G., Do Carmo, A.E., Graf, L.V., Tomáz, R., De Souza, C.F., Mendes, J., Randi, M.A.F., & Buchi, I.D. (2006). Morphology, lipid body and vacuole dynamics during secondary conidia formation in *Colletotrichum acutatum*: laser scanning confocal analysis. *Canadian Journal of Microbiology*, 52 (2), 117-124. http://www.ncbi.nlm.nih.gov/pubmed/16541147

Carvalho, M.T., Piteira, M.C.C., & Clara, M.I.E. (2003). Identificação de *Colletotrichum acutatum* em *Olea europaea* afectada pela doença da gafa. *Revista de Ciências Agrárias*, III Simpósio Nacional de Olivicultura. pp. 54.

Carvalho, M.T., Simões-Lopes, P., Monteiro da silva, M.J., Pires, S., & Gonçalves, M.J. (2006). The effect of *Colletotrichum* control on getting high quality olive oils. Proceedings, Vol.II, of Second International Seminar: Biotechnology and quality of olive tree products around the Mediterranean basin". pp. 239-242.

Carvalho, M.T., Simões-Lopes, P., & Monteiro da Silva, M.J. (2008). Influence of different olive infection rates of *Colletotrichum acutatum* on some important olive oil chemical parameters. *Acta Horticulturae*, 791, 555-558, http://www.actahort.org/books/791/791_85.htm

Cantu, D., Vicente, A.R., Greve, L.C., Dewey, F.M., Bennett, A.B., Labavitch, J.M., & Powell, A.L.T. (2008). The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. *Proceedings of the National Academy of Sciences*, 105(3), 859–864. http://dx.doi.org/10.1073/pnas.0709813105, http://www.pnas.org/content/105/3/859.abstract

Diéguez-Uribeondo, J., Förster, H., & Adaskaveg, J.E. (2003). Digital image analysis of internal light spots of appressoria of *Colletotrichum acutatum*. *Phytopathology*, 93(8), 923-930. http://www.ncbi.nlm.nih.gov/pubmed/18943858.

Diéguez-Uribeondo, J., Förster, H., Soto-Estrada, A., & Adaskaveg, J.E. (2005). Subcuticular-Intracellular Hemibiotrophic and Intracellular Necrotrophic Development of *Colletotrichum acutatum* on Almond. *Phytopathology*, 95(7), 751-758. http://dx.doi.org/10.1094/PHYTO-95-0751, http://apsjournals.apsnet.org/doi/pdf/10.1094/PHYTO-95-0751

Gomes, S., Prieto P., Martins-Lopes, P., Barradas, T., Martin, A., & Guedes-Pinto, H. (2007). Pathological study of *Colletotrichum acutatum* in *Olea europaea* L. cultivars. Proceedings of 3rd European Meeting of the IOBC/WPRS Working Group Integrated Protection of Olive Crops', pp. 73.

Gomes, S., Bacelar, E., Martins-Lopes, P., Carvalho, T., & Guedes-Pinto, H. (2008). Infection Process of *Colletotrichum acutatum* on *Olea europaea* L. *Proceedings of VI International Symposium on Olive Growing*, pp.163.

Gomes, S., Prieto P., Martins-Lopes, P., Carvalho, T., Martin, A., & Guedes-Pinto, H. (2009). Development of *Colletotrichum acutatum* on Resistant and Susceptible *Olea europaea* L. cultivars: A Microscopic Analysis.

Mycopathologia, 168(4), 203-211. http://dx.doi.org/10.1007/s11046-009-9211-y, http://www.springerlink.com/content/rk848760652185v3/

Guestsky, R., Kobiler, I., Ávila-Quezada, G., & Prusky, D. (2007). Metabolism of epicatechin by laccase of *Colletotrichum gloeosporioides*. Proceedings VI World Avocado Congress (Actas VI Congreso Mundial del Aguacate). ISBN No 978-956-17-0413-8.

Horowitz, S., Freeman, S., & Sharon, A. (2002). Use of green fluorescent protein-transgenic strains to study pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum*. *Ecology and population biology*, 92(7), 743-749, http://www.agri.gov.il/download/files/GFP.Sigal.pdf

Jerba, V.F., Rodella, R.A., & Furtado, E.L. (2005). Relationship between bean leaf structure and the *Glomerella cingulata* f.sp. *phaseoli* preinfection. *Pesquisa agropecuaria brasileira*, 40(3), 217-223. http://dx.doi.org/10.1590/S0100-204X2005000300004, http://www.scielo.br/scielo.php?pid=S0100-204X2005000300004&script=sci_arttext.

Johansen, D.A. (1940). *Plant microtechnique.* (pp 523). New York: McGraw Hill Book.

Jong, M., & Ackerceken, G. (2009). Fungal and oomycete biotrophy. *Annual Plant Reviews*, 34, 77-101, http://dx.doi.org/10.1002/9781444301441.ch4

Kerstiens, G. (1996). Plant Cuticles: an Integrated Functional Approach. BIOS Scientific Publ Oxford, (pp 20-87). Cambridge University Press. ISBN 1 85996 130 4. http://dx.doi.org/10.1046/j.1469-8137.1997.00794-1.x

Khan, A., & Hsiang, T. (2003). The infection process of *Colletotrichum graminicola* and relative aggressiveness on four turfgrass species. *Canadian Journal of Microbiology*, 49, 433-442. http://dx.doi.org/10.1139/W03-059, http://www.uoguelph.ca/~thsiang/pubs/pdf/03cgram_cjm.pdf

Kimura, A., Yoshitaka, Y., Furusawa, I., & Okuno, T. (2001). Peroxisomal metabolic function is required for appressorium-mediated plant infection by *Colletotrichum lagenarium. The Plant Cell,* 13(8), 1945–1957, http://dx.doi.org/10.1105/tpc.13.8.1945, http://www.plantcell.org/content/13/8/1945.abstract.

Kubo, Y. (2005). Studies on mechanisms of appressorial penetration by *Colletotrichum lagenarium*. *Journal of General Plant Pathology*, 71(6), 451–453. http://dx.doi.org/10.1007/s10327-005-0229-9, http://www.springerlink.com/content/f1nj243165661l28/

Kuo, K. (1999). Germination and appressorium formation in *Colletotrichum gloeosporioides*. *Proceedings of the National Science Council. Roc (B),* 23, 126-132.

Latunde-Dada, A.O., O'Connell, R.J., Nash, C., Pring, R.J., Lucas, J.A., & Bailey, J.A. (1996). Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum*) from cowpea (*Vigna unguiculata*). *Mycological Research*, 100(9), 1133-1141. http://www.sciencedirect.com/science/article/pii/S0953756296802267.

Leandro, L.F.S., Gleason, M.L., Nutter, F.W., Wegulo, S.N., & Dixon, P.M. (2001). Germination and sporulation of *Colletotrichum acutatum* on symptomless strawberry leaves. *Phytopathology*, 91(7), 659-664. http://www.ncbi.nlm.nih.gov/pubmed/18942995

Manandhar, J.B., Hartman, G.L., & Wang, T.C. (1995). Anthracnose development on pepper fruits inoculated with *Colletotrichum gloeosporioides*. *Plant Disease*, 79(4), 380–383. http://www.mendeley.com/research/anthracnose-development-on-pepper-fruits-inoculated-with-colletotrichum-gloeosporioides/.

Martín, M.P., & García-Figueres, F. (1999). *Colletotrichum acutatum* and *C. gloeosporioides* cause anthracnose on olives. *European Journal of Plant Pathology*, 105(8), 733-741. http://dx.doi.org/10.1023/A:1008785703330, http://www.springerlink.com/content/h6541000x4182841/

Mithöfer A., Boland W., & Maffei, M.E. (2009). Chemical ecology of plant-insect interactions. In J. Parker (Ed.), *Plant Disease Resistance* (pp. 261-291). Chichester: Wiley-Blackwell.

Moral, J., Bouhmidi, K., & Trapero, A. (2008). Influence of fruit maturity, cultivar susceptibility, and inoculation method on infection of olive fruit by *Colletotrichum acutatum*. *Plant Disease*, 92(10), 1421-1426. http://dx.doi.org/10.1094/PDIS-92-10-1421, http://apsjournals.apsnet.org/doi/abs/10.1094/PDIS-92-10-1421

Moral, J., Oliveira, R., & Trapero, A. (2009). Elucidation of the disease cycle of olive anthracnose caused by *Colletotrichum acutatum. Phytopathology*, 99(5), 548-556. http://dx.doi.org/10.1094/PHYTO-99-5-0548, http://apsjournals.apsnet.org/doi/abs/10.1094/PHYTO-99-5-0548

Moshe, A., Flaishman-Pappachan, E., & Kolattukudy. (1994). Timing of fungal invasion using host's ripening hormone as a signal. *Plant Biology*, 91, 6579-6583. http://www.pnas.org/content/91/14/6579.full.pdf

Mota-Capitão, C., Talhinhas, P., Várzea, V., Oliveira, H., & Silva, M. C. (2008). Histopathology of *Colletotrichum* spp. causing olive anthracnose. *Journal Plant Pathology*, 90, S2.232

O'Brien, T.P., & McCully, M.E. (1981). *The Study of Plant Structure*: Principles and selected methods. Termarcarphi Pty Ltd., Melbourne, Australia. pp. 357.

Peres, N.A., Timmer, L.W., Adaskaveg, J.E., & Correll, J.C. (2005). Lifestyles of *Colletotrichum acutatum*. *Plant Disease*, 89(8), 784-796. http://dx.doi.org/10.1094/PD-89-0784, http://apsjournals.apsnet.org/doi/abs/10.1094/PD-89-0784

Prusky, D., Kobiler, I., Ardi, R., Beno-Moalem, D., Yakoby, N., & Keen, N.T. (2000). Resistance mechanisms of subtropical fruits to *Colletotrichum gloeosporioides*. In: Prusky D, Freeman S, Dickman MB. (eds.), *Colletotrichum:* Host Specificity, Pathology, and Host-Pathogen Interaction. *The American Phytopathological Society*. St. Paul MN. 232-244.

Shah, J., & Chaturvedi, R. (2009). Lipid signals in plant-pathogen interactions. *Annual Plant Reviews*, 34, 292-333. http://dx.doi.org/10.1002/9781444301441.ch10

Schadeck, R.J.G., Leite, B., & Buchi, D.F. (1998). Lipid mobilization and acid phosphatase activity in lytic compartments during conidium dormancy and appressorium formation of *Colletotrichum graminicola*. *Cell Structure function*, 23(6), 333-340. http://www.ncbi.nlm.nih.gov/pubmed/10206735

Smith, J.E., Korsten, L., & Aveling, T.A.S. (1999). Infection process of *Colletotrichum dematium* on cowpea stems. *Mycological Research*, 103(2), 230-234. http://www.sciencedirect.com/science/article/pii/S0953756208610941.

Talhinhas, P., Ferreira, P., Neves-Martins, J., Screenivasaprasad, S., & Oliveira, H. (2003). *Colletotrichum acutatum*: principal agente causal da gafa da Oliveira em Portugal, Proceeding of III Simpósio Nacional de Olivicultura, Castelo-Branco, Portugal.

Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J., & Oliveira, H. (2005). Molecular and phenotypic analyses reveal association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive anthracnose. *Applied Environmental Microbiology*, 71(6), 2987-2998. http://dx.doi.org/10.1128/AEM.71.6.2987-2998.2005, http://aem.asm.org/cgi/content/short/71/6/2987

Talhinhas, P., Neves-Martins, J., Sreenivasaprasad, S., & Oliveira, H. (2007). Biology and etiology of olive anthracnose. Proceedings of 3rd European Meeting of the IOBC/WPRS Working Group Integrated Protection of Olive Crops', pp. 71.

Trapero, A. (2009). Genetic resistance: a part of integrated control of olive diseases?. Proceedings of 4th European Meeting of the IOBC/WPRS Working Group Integrated Protection of Olive Crops', pp. 32.

Wharton, P.S., & Diéguez-Uribeondo, J. (2004). The biology of *Colletotrichum acutatum*. *Anales del Jardín Botánico de Madrid*, 61, 3-22.

Wharton, P.S., & Schilder, A.C. (2008). Novel Infection strategies of *Colletotrichum acutatum* on ripe blueberry fruit. *Plant Pathology*, 57(1), 122-134. http://dx.doi.org/10.1111/j.1365-3059.2007.01698.x

Zulfiqar, M., Brlansky, R.H., & Timmer, L.W. (1996). Infection of flower and vegetative tissues of citrus by *Colletotrichum acutatum* and *C. gloeosporioides*. *Mycologia*, 88(1), 121-128. http://www.jstor.org/pss/3760791

Table 1. Measurements of cuticle thickness, perimeter and area of epidermal cells between *C. acutatum*-susceptible, *C. acutatum*-moderately resistant and *C. acutatum*-resistant olive fruits during two years of field trials

	1st year (2006)			**2nd year (2007)**		
	Cuticle thickness (μm)	Perimeter of epidermal cells (μm)	Area of epidermal cells (μm^2)	Cuticle thickness (μm)	Perimeter of epidermal cells (μm)	Area of epidermal cells (μm^2)
'Galega'	16.08± 0.4c	55.95± 2.7 c	166.38±14.4 b	13.28±0.4b	51.90±1.5 b	148.25±8.6 b
'Cobrançosa'	22.03±0.5b	66.47± 3.1 b	213.01± 12.3 a	13.95±0.4b	70.80±1.4 a	281.37±11.1a
'Picual'	23.27± 0.3a	74.37± 1.7 a	229.26± 9.7 a	18.57±0.5a	74.84±2.2 a	311.25±19.6 a

Each value is the mean with standard error (±S.E.). Mean values followed by a different letter for a particular parameter differed significantly ($P < 0.05$).

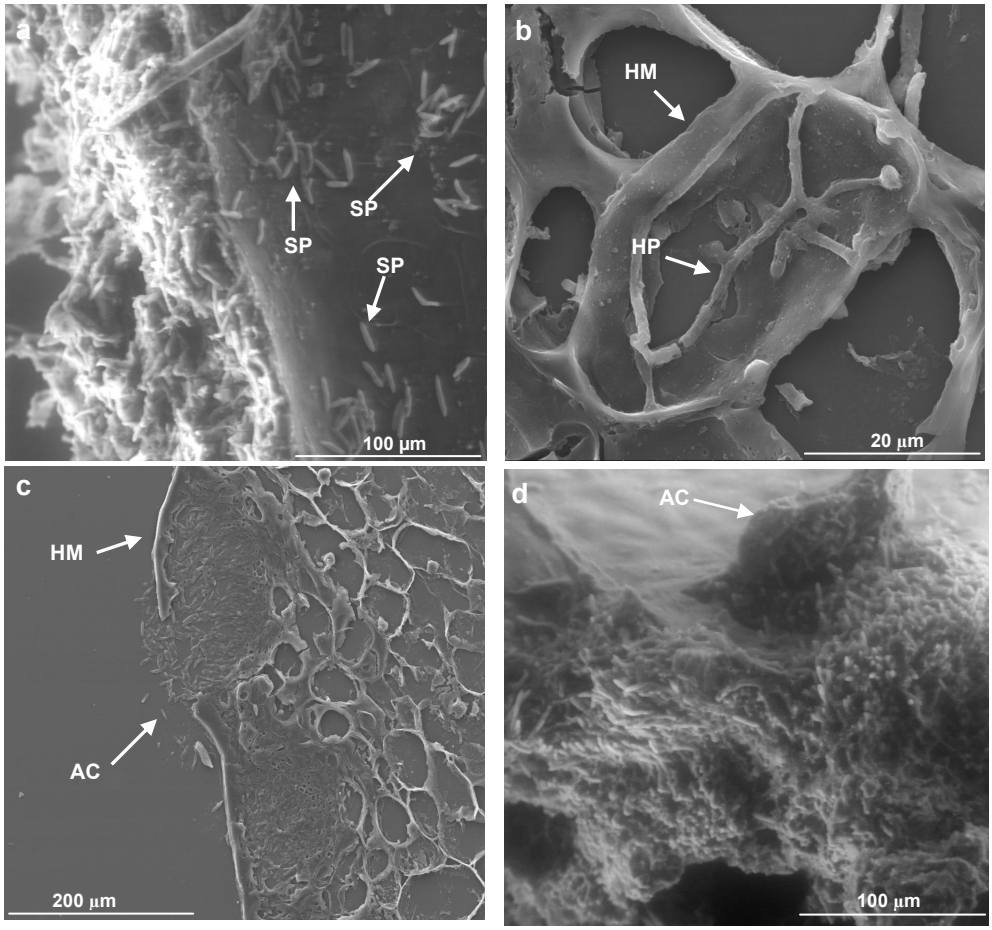

Figure 1. Scanning electron microscopy images showing the time-course of colonization process of susceptible fruits ('Galega') by *Colletotrichum acutatum*. Vibratome olive fruit sections were made to demonstrate olive fruit colonization by *C. acutatum*. **a.** View of the 'Galega' fruit cuticle profusely colonized by *C. acutatum* spores 48 h after inoculation. **b.** Hyphae internally colonizing host cell. **c.** Hyphal growth of *C. acutatum* developed as aggregated and confluent structures that erupted through host cuticle. **d.** View of acervuli sporulation of *C. acutatum* on susceptible olive fruits. SP: spores; HM: host membrane; HP: hyphae; AC: acervuli

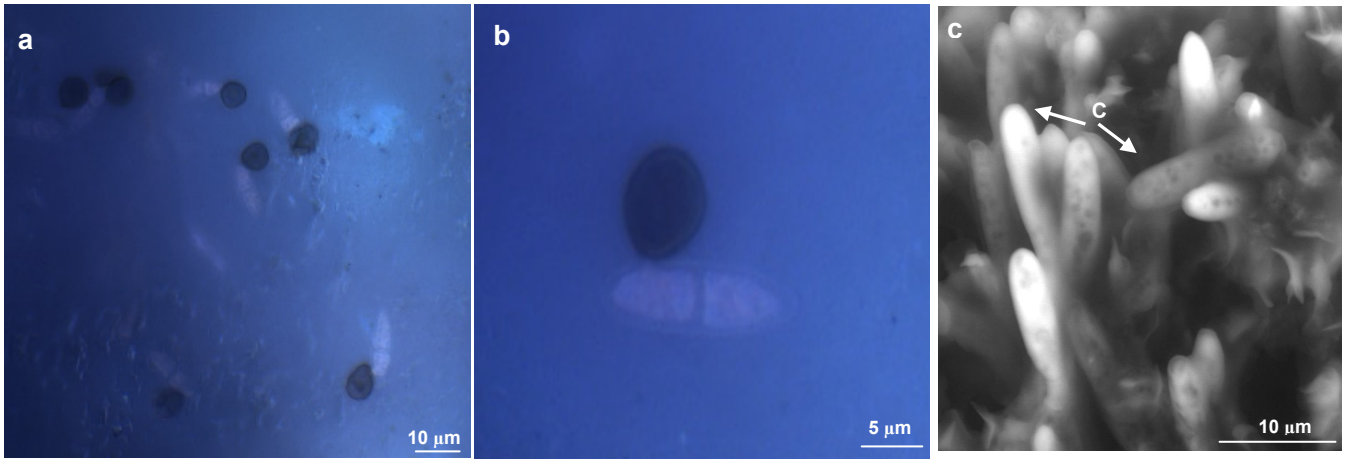

Figure 2. Vibratome longitudinal sections of the inoculated *C. acutatum*-susceptible fruits ('Galega') 48 h after inoculation. **a.** 48 h after inoculation spores adhere to fruit cuticle and germinate to develop a germ tube. **b**. the spore and germ tube germination allow the appressoria formation in an apical position of the germ tube. **c.** View of the internal lipid bodies, glycoproteins and polysaccharides inside immature spores

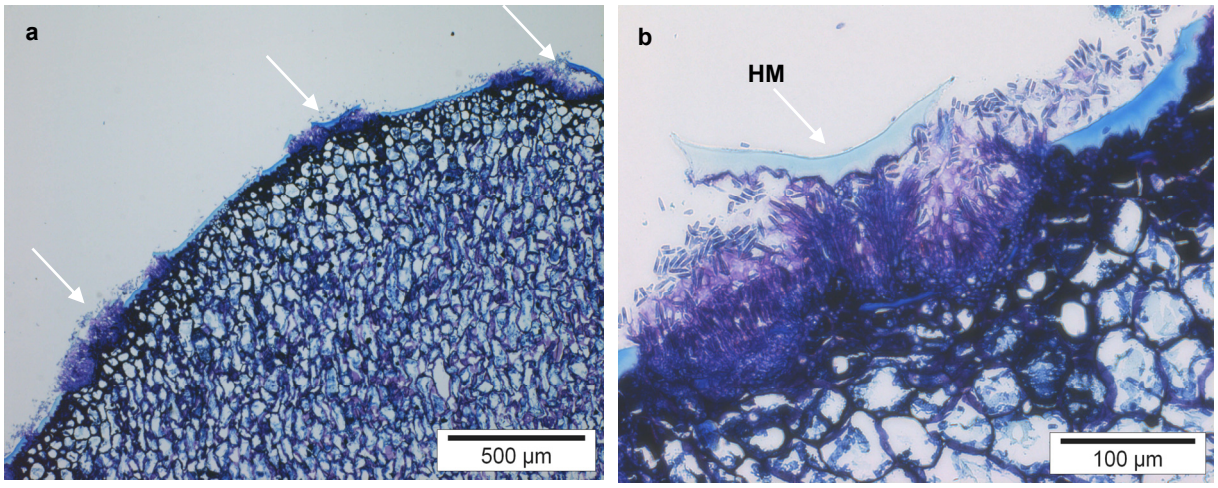

Figure 3. Toluidine blue staining of the inoculated *C. acutatum*-susceptible fruits ('Galega') 192 h after inoculation. **a.** After a generalized infection in olive fruit mesocarp, the host-pathogen interaction became necrotrophic with cuticle breakdown (arrows). **b**. Acervuli sporulation was clear observed in different cuticle regions and hundreds of ascospores break the cuticle in many points (arrows) and erupted.

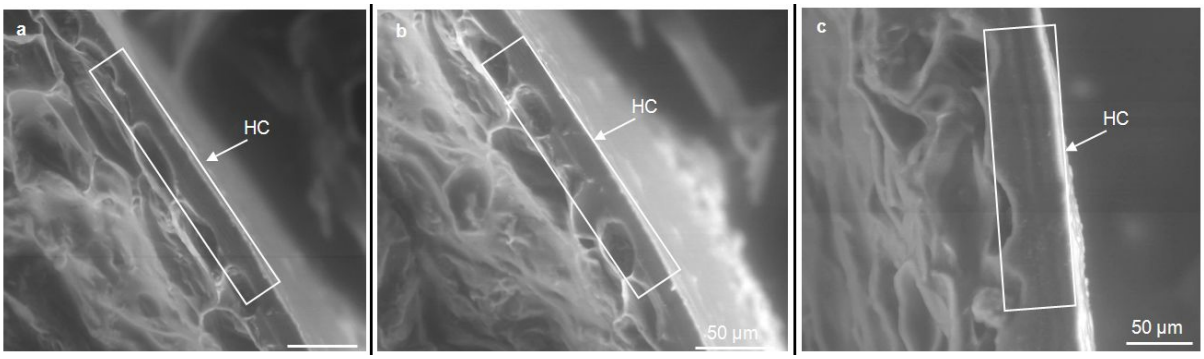

Figure 4. Scanning electron microscopy images showing the cuticles thickness of the (**a**) *C. acutatum*-susceptible ('Galega'), (**b**) *C. acutatum* –moderately ('Cobrançosa') and (**c**) *C. acutatum* -resistant ('Picual') fruits. The images showing that the susceptible fruit ('Galega') had the thinnest cuticle while resistant fruit ('Picual') had the thickest. HC: olive fruit cuticle

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