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RESEARCH PAPERS

Olive leachates affect germination of *Colletotrichum godetiae* conidia and the development of appressoria

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Summary. The effects of nutrients and microorganisms from olive carpospheres on germination of conidia and the development of appressoria of *Colletotrichum godetiae* were investigated. The final germination ratio was the result of a dynamic equilibrium between the positive action of nutrients and the negative competition of indigenous microorganisms. In contrast, formation of appressoria was greatly increased by microorganisms and reduced by nutrients. Removal of the microbial fraction from olive leachates rich in natural nutrients amplified the vegetative phase after conidium germination, resulting in increased germtube length, and delayed and reduced production of appressoria. Three exogenous nutrients (sucrose, asparagine and glycine) increased germination of conidia and reduced the formation of appressoria. These results provide evidence that nutrients and microorganisms of the olive carposphere play important roles in the infection processes of *C. godetiae*. Since appressoria are necessary for successful host infection, microorganisms may favour the penetration of *C. godetiae* by stimulating the production of appressoria, reducing saprophyitic pre-infectional mycelial growth and reducing duration of the critical moist period required for host penetration.

Key words: olive anthracnose, microbial fraction, nutrients, penetration, pre-infection process.

Introduction

Olive anthracnose is the most damaging disease of olive fruit worldwide severely affecting both fruit yield and quality of oil (Cacciola *et al*., 2012). Two complexes of species showing high phenotypic and genotypic diversity, *Colletotrichum gloeosporioides sensu lato* (*s.l.*) and *C. acutatum s.l.*, have been associated with olive anthracnose (Moral *et al*., 2008; Cacciola *et al*., 2011; Talhinhas *et al*., 2011). In particular, among taxa of the *C. acutatum* complex, *C. acutatum sensu stricto* (s.s.) and five new species defined as *C. simmondsii, C. fioriniae, C. godetiae* (syn. *C. clavatum*), *C. nymphaeae*, and *C. rhombiforme* have been reported as causal agents of olive anthracnose (Shivas and Tan, 2009; Faedda *et al*., 2011; Damm *et al*., 2012). Furthermore, six different species (*C. aenigma*, *C. gloeosporioides* s.s., *C. kahawae*, *C. queenslandicum*, *C. siamense* and *C. theobromicola*) of the *C. gloeosporioides* complex, and a species (*C. karstii)* of the *C. boninense* complex were associated to the disease (Schena *et al*., 2014). However, currently available data indicate *C. acutatum s.l.* species as the most important causal agents of olive anthracnose worldwide (Cacciola *et al*., 2012). Among them, *C. godetiae*, formerly identified as *C. acutatum* group B or A4, is one of the most common in the Mediterranean basin and the prevalent species in Southern Italy (Agosteo *et al*., 2002; Sreenivasaprasad and Talhinhas, 2005; Talhinhas *et al*., 2009, 2011; Cacciola *et al*., 2012; Mosca *et al*., 2014).

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Although no specific information is available about the life style of *C. godetiae*, this pathogen is expected to be a hemibiotroph like most *Colletotrichum* species (Peres *et al*., 2005; Gomes *et al*., 2009). Early events in the *Colletotrichum* spp. infection process are the recognition of host surface followed by conidium germination and appressoria formation, which are the first steps in host cuticle penetration. Appressoria are thick-walled, melanised infection structures that adhere to host surfaces achieving penetration. Moreover, the appressoria of *Colletotrichum* may act as short-term survival structures, since they possess the capacity to endure conditions of high light intensity, desiccation or antagonism by other microorganisms that can be lethal to germ tubes (Emmet and Parberry, 1975; Fernando *et al*., 1994).

Conidium germination, germ tube elongation and formation of appressoria are different steps of the pre-infection processes for plant anthracnose inciting fungi, which are stimulated or inhibited in response to appropriate physical (surface hardness, topography, hydrophobicity) and chemical exogenous factors. Both the type and amounts of inorganic and organic compounds play major roles in determining the pre-penetration steps of the infection process by *Colletotrichum* spp. (Podila *et al*., 1993; Manandhar *et al*., 1995). These substances can be exuded from internal plant tissues and leached by rain from leaves and fruit surfaces.

Another important factor greatly influencing early infection processes by *Colletotrichum* spp. are microorganisms. In particular, bacteria are by far the most abundant inhabitants of the olive phyllosphere (Ercolani, 1991), and were effective in controlling *Colletotrichum* sp. on olive drupes, probably through production of unknown antimycotic substances (Balestra *et al*., 1997). However, other evidence indicates that phylloplane bacteria may favour the infection process by promoting formation of appressoria (Lenne and Parbery, 1976; Blackeman and Parbery, 1977; Koomen and Jeffries, 1993). Differentiation of appressoria has been reported to occur on inductive surfaces when apical growth of germ tubes is inhibited (Perfect *et al*., 1999; Apoga *et al*., 2004). Fernando *et al.* (1994) observed increased numbers of appressoria and increased disease severity on velvetleaf when *C. coccodes* was co-inoculated with phylloplane bacteria.

The aim of the present study was to investigate the effect of nutrients and microorganisms from olive leachates on germination of conidia, elongation of germ tubes and development of appressoria of *C. godetiae* in order to determine the roles of these factors in the infection process.

Materials and methods

Olive leachate and nutrients

Olive leachates for different experiments were prepared using different olive stocks. In order to standardize the procedure all leachates were prepared with olive drupes cv. Ottobratica, collected from an organic farm of the Gioia Tauro plain (Southern Italy). Ripe olives were uniformly black drupes collected in mid-November, while "breaker" olives were in the initial phase of colour turning from green to black and were harvested at the beginning of October. In all experiments, five olives were stirred in 10 mL of sterile distilled water for 2 min at 250 rpm. The leachates were filtered through a 0.22 μm cellulose acetate/nitrate membrane filter (Millipore, Bedford, USA), to obtain sterile solutions without altering their chemical composition. Alternatively, solutions were sterilized by autoclaving at 120°C for 20 min (Table 1).

Exogenous nutrients to be tested, and their concentrations were decided according to previous reports (Manandhar *et al*., 1995; Mahuku and Goodwin, 1998). Substances were obtained from Sigma-Aldrich, and were diluted with sterile water to obtain stock solutions of the desired concentration of 0.1 or 10 mM for sucrose, 0.02 M for asparagine, and 0.02 M for glycine. Stock solutions were sterilised at 120°C for 20 min and stored at 5°C.

Conidium suspensions of *Colletotrichum godetiae* and cell suspensions of *Bacillus subtilis*

An isolate (OL10 = IMI 398854) of *C. godetiae*, previously characterised using biochemical (electrophoretic patterns of isozymes) and molecular analyses (Agosteo *et al*., 1997; Faedda *et al*., 2011) was utilised in Experiments 1 and 2 (Table 1). Since production of appressoria may vary for different strains, a second isolate of *C. godetiae* (GEA5) was utilised in Experiment 3, in order to investigate the effect of olive leachates on a different target (Table 1). Both isolates were obtained from olive drupes with typical symptoms of anthracnose, and were identi-

Table 1. Summary of the trials conducted to evaluate the effects of non-sterile (NSL) and sterile (SL) olive leachates on conidium germination of different isolates of *Colletotrichum godetiae*. The effects of three exogenous nutrients (sucrose, aspargine and glycine) and the addition of *Bacillus subtilis* cells to sterile olive leachates were also investigated.

Exp. No. and isolate[a]	Treatments[b]	Parameter measurement timing (h)	Measured parameters
Exp. 1 (OL10)	· NSL (ripe olives) · SL (ripe olives; filtration) · Sucrose (0.05 mM) · Sucrose (5 mM) · Aspargine · Glycine · Sterile distilled water	6 12 24	a) Germination of conidia b) Type of germination - Conidia producing 2 germ tubes - Conidia producing appressoria - Conidia producing conidia c) Length of germ tubes forming appressoria* d) Germ tubes growth rate (μm h^{-1})** e) Average time for appressoria formation
Exp. 2 (OL10)	· NSL (ripe olives) · NSL (breaker olives) · SL (ripe olive; filtration) · SL (breaker olives; filtration) · Sterile distilled water	6 12	a) Germination of conidia b) Conidia producing appressoria
Exp. 3 (GEA5)	· NSL (ripe olives) · SL (ripe olives; filtration) · SL (ripe olives; autoclaving) · SL (ripe olives; filtration) + *B. subtilis*	24	a) Conidia producing appressoria

[a] Isolates of *Colletotrichum godetiae* utilized in experiments.
[b] Olive leachates were obtained from ripe (uniformly black) or breaker (initial turning from green to black) olive drupes, and were sterilized by filtration or by autoclaving.
* Parameter recorded only at 24 h.
** Parameter evaluated between 6 and 12 h.

fied by means of morphological features and gene sequencing of internal transcribed spacer (ITS) regions and β-tubulin 2 gene, as described by Schena *et al*. (2014). To prepare a spore suspension, isolates were grown on potato dextrose agar (PDA) for 6 d at 24°C. Conidia were collected by adding sterile distilled water to the dishes and brushing the colony surfaces with a razor blade. Water suspensions of conidia were filtered through a double layer of sterile cheesecloth to remove most of the hyphae, and were diluted to a concentration of 10^5 conidia mL^{-1}.

An isolate of *B. subtilis* obtained from olive fruits was grown overnight at 37°C on Luria-Bertani (LB) agar plates. Cells were harvested with a spatula and directly added to a sterile leachate solution to obtain a final concentration of approx. 2×10^6 cells mL^{-1}. The actual concentration of living *B. subtilis* cells was estimated by plating serial dilutions of the suspension on LB plates.

Experiments

Three different experiments were conducted as outlined in Table 1. Experiments were consequential to each other i.e. later experiments were designed according to results of previous ones. The experiments included the use of sterile and non-sterile leachates obtained from ripe (all experiments) or breaker (experiment 2) olives, and sucrose, asparagine or glycine (Experiment 1) as representative nutrients (Table 1). Furthermore, in Experiment 3, a sterile leachate solution obtained by filtration was amended with cell suspension of *B. subtilis*. In one experiment (Experiment 3), sterile olive leachates were also obtained by autoclaving.

In all of the experiments, 10 μL of *C. godetiae* conidial suspension were added to an equal volume of each tested solution/suspension or sterile distilled water (experimental control), in order to halve the leachate concentration and to have a final concentra-

tion of 5×10^4 conidia mL^{-1} of the pathogen and 0.05 or 5 mM sucrose, 0.01 M asparagine and 0.01 M glycine. Conidial suspensions were incubated at 24°C in the dark for 6, 12, or 24 h according to different experimental schemes (Table 1).

All assays were conducted on sterile microscope hanging-drop slides with 13 mm diam. wells of depth 0.5–0.6 mm. Before use, the slides were washed by immersion in concentrated HCl (37%), thoroughly rinsed with distilled water and then sterilised at 120°C for 20 min. Slides with conidial suspensions were incubated in Petri dishes on a moistened filter paper.

Morphological measurements

Different morphological measurements were performed at 6, 12 and/or 24 h using a light microspore set up for a ×1000 magnification, as summarised in Table 1. Before measurements, the germination process was stopped by adding a drop (5 μL) of lactophenol cotton blue (Sigma-Aldrich).

In Experiments 1 and 2, conidia with clearly visible germ tube initials were considered germinated. Furthermore, those producing appressoria (all experiments) and secondary conidia or two germ tubes (Experiment 1) were specifically recorded (Table 1). The lengths of germ tubes producing appressoria, the germ tube growth rates and the average time needed for the formation of appressoria were estimated during Experiment 1. The germ tube growth rates were determined between 6 and 12 h for all incubation media by analysing actively growing conidia: growth rate = (average length at 12 h – average length at 6 h)/6 h. This parameter was then used to estimate the average time for appressorium formation, assuming that the growth rate was constant during the entire incubation period: average time for appressoria formation = length of germ tubes with appressoria recorded at 24 h/growth ratio.

In all measurements, three sets of 40 conidia each were randomly selected from three different slides and observed. Mean data from the experiments were analyzed for homogeneity of variance with the software package Statistics for Windows (StatSoft), and standard errors of the means (SEM) were calculated.

Results

Experiment 1

All treatments increased the germination of conidia compared with the water controls, at the three assessment times (Table 2). In particular, after 6 h of incubation the greatest proportions of germinated conidia was recorded for those in sterile leachate (98.6%), 5 mM sucrose and asparagine (94.6%), and glycine (94.3%), followed by non-sterile leachate (78.3%) and 0.05 mM sucrose (77.6%). Only 68.3% of the conidia germinated in sterile water. The proportions of germinated conidia increased slightly for all treatments after 12 and 24 h, but these later results largely confirmed those obtained at 6 h (Table 2).

Incubation media modified the morphology of germinated conidia. Sterile leachate increased the differentiation of two germ tubes from individual conidia at 12 and 24 h, and the incidence of conidia producing secondary conidia at 24 h (Table 3). In contrast, sterile leachate almost completely inhibited the formation of appressoria at 6 and 12 h and strongly inhibited appressorium formation at 24 h. Completely different results were obtained with the non-sterile leachate, which gave greater incidence of conidia producing appressoria at all assessment times, and reduced the percentage of conidia producing secondary conidia or two germ tubes at 12 and 24 h.

Table 2. Results of Experiment 1a (Table 1). Mean proportions of germinated *Colletotrichum godetiae* conidia in non-sterile olive leachate, sterile olive leachate, in solutions containing three different nutrient elements, and sterile distilled water (control), after 6, 12 and 24 h of incubation.

Treatment	Germination of conidia (%)[a]		
	6 h	12 h	24 h
Non-sterile leachate	78.3 ± 6.69	85.6 ± 4.33	88.3 ± 3.75
Sterile leachate	98.6 ± 0.28	99.0 ± 0.57	99.3 ± 0.34
Sucrose (0.05 mM)	77.6 ± 0.63	84.6 ± 1.73	87.3 ± 2.48
Sucrose (5 mM)	94,6 ± 3.34	97.0 ± 0.57	97.6 ± 0.75
Aspargine	94.6 ± 0.92	97.3 ± 0.86	97.6 ± 0.92
Glycine	94.3 ± 0.63	95.6 ± 1.32	96.6 ± 1.32
Sterile distilled water	68.3 ± 4.67	77.6 ± 5.77	80.6 ± 3.63

[a] Means ± standard error of the mean (SEM).

Except for sucrose at the lower concentration (0.05 mM), a significant reduction of appressoria was recorded for all tested exogenous nutrients as compared to the water control (Table 3). In contrast, the production of two germ tubes per conidium was increased by most treatments at 12 and 24 h, and there was also a minor influence on production of secondary conidia.

After 24 h of incubation, the lengths of germ tubes from conidia producing appressoria were increased in sterile leachates, but reduced in non-sterile leachates (Table 4). These parameters were not significantly influenced, however, by the tested exogenous nutrients. Both sterile and non-sterile leachates as well as the three tested nutrients caused increased germ tube growth rates as compared to the water controls. Non-sterile leachate gave the shortest length of germ tubes producing appressoria and the greatest growth ratio. The combination of these two parameters resulted in the estimation of reduced time needed for the production of appressoria as compared to the water control and all other incubation media. In contrast, the greatest estimated time for appressorium formation was recorded for the sterile leachate. Similar to the estimated time for appressorium formation, after 6 hours of incubation, 51.6% of germinated conidia in non-sterile leachates, 1.3% in sterile leachate, and 27.3% sterile distilled water produced appressoria (Table 3).

Experiment 2

In Experiment 2, olive leachates from ripe and breaker olives were compared by determining their effects on conidium germination and appressorium formation. After 6 h of incubation, the percentage of germinated conidia was greater in non-sterile leachates from breaker olives than in non-sterile leachates from ripe olives (Table 5). However, an opposite trend was recorded for sterile leachates, i.e. conidium germination was greater in leachates from ripe olives compared to those from breaker olives. Results obtained at 6 h were generally confirmed at 12 h, although differences in the treatments were less.

Non-sterile olive leachates from ripe olives increased the production of appressoria as compared to the water controls after both 6 and 12 h of incubation. In contrast, non-sterile leachates from breaker olives did not modify the production of appressoria. Sterile leachates from both ripe and breaker olives almost completely inhibited the production of appressoria after 6 and 12 h.

Experiment 3

The influence of the procedure utilised to sterilize olive leachates and the role of bacteria on the appressorium formation was evaluated in this experiment. Overall the isolate utilised in this experiment (GEA5) produced many fewer appressoria compared with isolate OL10, used Experiments 1 and 2. After 24 h of incubation, 9% of the germinated conidia in water and 42.3% of those in non-sterile leachates produced appressoria (Table 6). The smallest percentage of conidia producing appressoria was recorded in sterile leachate obtained by filtration (3.6 %) followed by sterile leachate obtained by autoclaving (11.0%) and sterile leachates obtained by filtration and amended with *B. subtilis* (13.6%).

Discussion

Experiments conducted in this study with two different isolates of *C. godetiae* have clearly demonstrate that the germination of conidia, in terms of both percentage and mode of germination, is likely to be greatly influenced by the nutrient substances and microorganisms of the olive carposphere. The same trends in measured parameters were detected for conidia of two different isolates, although the isolates differed in capability to produce appressoria after germination.

Olive leachates increased the percentage of conidium germination in both Experiments 1 and 2, conducted with isolate OL10 of *C. godetiae*, compared to germination in sterile distilled water. This effect was more marked with sterile leachates compared to the non-sterile leachates. The early recognition events that take place in host-pathogen interactions are complex phenomena, which are conditioned by host wax surfaces and the quality and quantity of solutes present on plant surfaces. Some of these many compounds may be regarded as nutrients (e.g. sugars, amino acids, inorganic ions) or fungitoxic molecules (e.g. phenols), and have been reported to have a stimulating action on germination and/or appressorium formation (Emmett and Parbery, 1975). In our study, the presence of different nutrients in both sterile and non-sterile olive leachates stimulated the ger-

Table 3. Results of Experiment 1b (Table 1). Mean proportions of germinated conidia of *Colletotrichum godetiae* producing appressoria, secondary (2°) conidia or two germ tubes in non-sterile olive leachate, sterile olive leachate, solutions containing three different nutrient elements and sterile distilled water (control), after 6, 12 and 24 h of incubation.

Treatment	Incidence of germinated conidia (%)[a]								
	6 h			12 h			24 h		
	Two germ tubes	Appressoria	2° Conidia	Two germ tubes	Appressoria	2° Conidia	Two germ tubes	Appressoria	2° Conidia
Non-sterile leachate	2.0 ± 0.0	51.6 ± 5.65	1.0 ± 0.57	1.0 ± 0.57	62.0 ± 6.46	3.0 ± 0.57	28.0 ± 2.65	73.3 ± 4.96	4.0 ± 0.57
Sterile leachate	3.3 ± 0.28	1.3 ± 0.28	0.0 ± 0.0	54.3 ± 4.67	2.6 ± 0.28	2.0 ± 0.57	92.0 ± 1.15	22.6 ± 2.94	57.0 ± 4.72
Sucrose (0.05 mM)	9.0 ± 1.15	25.0 ± 1.96	1.0 ± 0.0	38.0 ± 3.00	40.3 ± 3.69	14.3 ± 1.45	71.3 ± 4.33	56.4 ± 2.48	29.0 ± 3.00
Sucrose (5 mM)	6.6 ± 1.03	18.3 ± 2.02	0.0 ± 0.0	41.0 ± 3.17	34.3 ± 4.90	10.3 ± 1.85	55.6 ± 4.73	42.1 ± 3.75	25.6 ± 2.33
Aspargine	1.3 ± 0.28	12.6 ± 1.38	3.3 ± 0.88	31.6 ± 1.84	22.0 ± 1.96	1.0± 0.40	79.0 ± 3.69	46.9 ± 1.96	34.6± 4.25
Glycine	1.0 ± 0.57	22.6 ± 7.1	0.0 ± 0.0	29.3 ± 2.48	28.6 ± 2.02	1.0 ± 0.40	79.0 ± 3.23	45.3 ± 2.42	25.3 ± 3.17
Sterile distilled water	4.0 ± 0.57	27.3 ± 3.17	0.0 ± 0.0	6.3 ± 0.63	45.6 ± 0.69	9.0 ± 1.22	66.3 ± 2.42	55.0 ± 3.05	31.0 ± 4.35

[a] Means ± standard error of the mean (SEM).

Table 4. Results of Experiments 1c, d and e (Table 1). Mean length (μm) of germ tubes of *Colletotricum godetiae* conidia forming appressoria after 24 h of incubation, and estimated mean germ tube growth rates (μm h^{-1}) and average time for the production of appressoria in sterile and non-sterile olive leachate, solutions containing three different nutrients, and sterile distilled water (control).

Treatment	Length of germ tubes (μm) [a]	Germ-tube growth rate (μm h^{-1}) [a]	Average time for appressorium formation (h) [a]
Non sterile leachate	19.9 ± 3.56	5.7 ± 0.7	3.5 ± 0.5
Sterile leachate	59.8 ± 4.20	4.4 ± 0.7	13.6 ± 1.6
Sucrose (0.05 mM)	30.0 ± 2.56	4.7 ± 0.9	6.4 ± 0.4
Sucrose (5 mM)	33.0 ± 2.69	4.9 ± 0.6	6.7 ± 0.9
Aspargine	37.3 ± 3.73	3.8 ± 0.1	9.8 ± 0.8
Glycine	22.5 ± 5.42	4.2 ± 0.4	5.4 ± 0.6
Sterile distilled water	32.6 ± 4.24	3.3 ± 0.5	9.9 ± 0.7

[a] Means ± standard error of the mean (SEM).

Table 5. Results of Experiments 2a and b (Table 1). Mean percentage of germinated *Colletotricum godetiae* conidia and of conidia producing appressoria after 6 and 12 h incubation in sterile (SL) and non-sterile (NSL) olive leachates from ripe and breaker olives, or in sterile distilled water (control).

Treatment	Germinated conidia [a]		Conidia producing appressoria [a]	
	6 h	12 h	6 h	12 h
NSL (ripe olives)	77.1 ± 3.1	85.0 ± 2.1	50.9 ± 1.7	64.1 ± 2.1
NSL (breaker olives)	99.3 ± 3.3	100 ± 0.0	25.6 ± 1.4	54.3 ± 3.6
SL (ripe olives; filtration)	97.5 ± 2.4	98.2 ± 1.1	1.5 ± 0.9	3.0 ±1.9
SL (breaker; filtration)	92.0 ± 1.8	98.6 ± 0.9	0.3 ± 0.1	1.3 ± 0.7
Sterile distilled water	66.0 ± 2.6	77.2 ± 3.1	26.1 ± 2.6	52.8 ± 1.8

[a] Means ± standard error of the mean (SEM).

Table 6. Results of Experiment 3a (Table 1). Mean percentage of *Colletotrichum godetiae* conidia forming appressoria after germination in sterile distilled water, non-sterile olive leachate, sterile olive leachate obtained by filtration or autoclaving, sterile leachate obtained by filtration and amended with cells of *Bacillus subtilis* and sterile distilled water (control), after 24 h of incubation.

Treatments	Conidia producing appressoria (%)[a]
Non-sterile leachate	42.3 ± 1.17
Sterile leachate (filtration)	3.6 ± 0.66
Sterile leachate (autoclaving)	11.0 ± 1.50
Sterile leachate + *B. subtilis*	13.6 ± 1.71
Sterile distilled water	9.0 ± 0.57

[a] Means ± standard error of the mean (SEM).

mination of conidia, but this effect was partially reduced in non-sterile leachates due to the presence of natural competing microorganisms. The non-sterile olive leachate from breaker olives was more effective for increasing conidium germination than the non-sterile leachate from ripe olives, probably because of lower microbial concentrations in the breaker olive leachate. The total microbial population in the carposphere, as well as that associated with other plant organs, is mainly represented by bacteria, and these populations are likely to increase with fruit ripening as more nutrient elements are available (Teixidó *et al.*, 1999; Fürnkranz *et al.*, 2012).

In agreement with these considerations, sterile leachates from ripe olives stimulated germination to a greater extent than those from breaker olives. Ripe fruits are likely to have greater content of nutrients than unripe fruit, and induce effects on germination of conidia, but this was not observed when more nutrients were also associated with rich microbial populations. In other words, nutrients and microorganisms both influenced the germination of conidia. The final germination ratio was the result of a dynamic equilibrium between the positive effects of nutrients the negative competition of indigenous microorganisms. This conclusion was also supported by data obtained with the three different exogenous nutrients utilised in the present study. Sucrose, asparagine and glycine significantly increased the germination of conidia as compared to the water control.

The germination ratio of conidia is an important aspect that can have significant impacts on the severity of a disease. However, other events are also necessary for successful infection by hemibiotroph pathogens like *C. godetiae*. Development of appressoria is one of the most important of these (Gomes *et al.*, 2009, 2012). Data obtained in the present study suggest a primary role of natural microbial populations in olive carpospheres in the formation of appressoria. Non-sterile olive leachates greatly increased the formation of appressoria compared to the water controls in all experiments, conducted with two different *C. godetiae* isolates. In contrast, the formation of appressoria was almost completely inhibited when conidia were incubated in olive leachates sterilized by filtration. More precisely, the elimination of the microbial fraction in combination with the high content in nutrients of the fruit leachate amplified the vegetative phase (saprophytic growth) after conidium germination, induced longer germ tubes, greater average time for appressorium formation and reduced production of appressoria. In agreement with these results, minor incidence of appressoria was also recorded when the nutrients sucrose, asparagine and glycine were added to the incubation media. Similarly, reduced production of appressoria has been reported for *C. graminicola* grown in nutritively rich media (Apoga *et al.*, 2004).

When leachates were sterilized by autoclaving greater incidence of appressoria was recorded, compared with leachate sterilized by filtration. In both cases, however, the incidence of appressoria was much less compared to non-sterile leachate. These data confirm that the microbial fraction of leachate is likely to be a major factor inducing appressoria, but particles suspended in the non-sterile leachate and removed during filtration may also play also a role in appressorium formation. It has been suggested that toxic substances, such as the anthranilic acid found in banana, can initiate appressorium formation by *C. musae* (Emmett and Parbery, 1975; Swinburne, 1976). It is also possible that olive leachates contain substances that are toxic to fungi. However, their role in appressorium formation are likely to be of minor relevance considering that putative toxic substances should not be lost during filtration.

Further confirmation of the role of microorganisms in appressorium formation was obtained by the lower number of appressoria differentiated in the sterile leachate as compared to same medium amended with cells of *B. subtilis*. This bacterium is one of the most abundant saprophytic genera in olive phylloplanes, and is a well-known bacterial antagonist (Ercolani, 1978). Furthermore, a few strains of *B. subtilis* are registered and used as biocontrol agents of plant pathogens, including *Colletotrichum* spp. causing anthracnose on leaves and fruits of various crops (Cawoy *et al.*, 2011). Although the amendment of *B. subtilis* to the sterile leachate significantly increased the production of appressoria, the incidence of these structures remained much less compared to the non-sterile leachate, and was similar for conidia incubated in the leachate sterilized by autoclaving. This suggests that microorganisms may not be the only factors playing a role in the formation of appressoria, which are lost/changed during filtration. However, it is important to note that cells of only one bacterium were added to the sterile leachate, while a rich natural microbial population colonizes natural olive carpospheres. Although the olive microbiota was not

evaluated in the present study, very complex indigenous populations are known to colonize the carposphere (Janisiewicz *et al*., 2010; Janisiewicz and Buyer, 2010). Both quality and quantity of nutrients and microorganisms seem to be important. It is possible to suppose a strict relationship between the microbial composition of the infection drop and the quality and quantity of plant exudates that, varying in relation to different plant and environmental condition, could modify the conidia response (Tukey, 1971).

In conclusion, results from the present study provide strong evidence that nutrients and microorganisms of the olive carposphere can play important roles in the infection processes of *C. godetiae*. On one hand, nutrients enhanced the germination of conidia, while on the other, indigenous microorganisms established a hostile environment that led to a highly increased appressorium differentiation, probably related to micobial competition (Lenne and Parbery, 1976). Since appressoria are necessary for successful infection of host tissues (Deising *et al*., 2000), signals that reduce their differentiation may enhance disease control and *vice versa* (Gomes *et al*., 2009). In particular, bacterial species present on host surfaces may accelerate the penetration of *C. godetiae* by stimulating a rapid production of appressoria, and reducing the saprophytic pre-infectional mycelial growth of the pathogen. This will result in reduced duration of the critical moisture period required by the fungus to produce infection in the field (Fernando *et al*., 1994).

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