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Antifungal and Antibacterial Capacity of Extracted Material from non-Glandular and Glandular Leaf Hairs Applied at Physiological Concentrations

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ABSTRACT

Non-glandular hairs are epidermal appendages that have been considered as a potential mechanical barrier for non-specific plant resistance to pathogens. We investigate if the occurrence of mixtures of secondary metabolites in these structures may also provide a potential chemical line of defence of plant surfaces against pathogens. Methanolic extracts from isolated non-glandular leaf hairs of *Olea europaea* and *Quercus ilex* were assessed against a number of phytopathogenic bacteria and fungi at concentrations resembling those of the leaf surface. Moreover, isolated exudates of glandular hairs of *Dittrichia viscosa* and *Cistus creticus*, containing mixtures of secondary metabolites of known antimicrobial activity, were tested. According to the bioassays, the non-glandular hairs materials inhibited the growth of the majority of the bacterial as well as the spore germination and growth of the fungal species tested. The inhibitory effect was plant species- and pathogen species-dependent but in all cases was weaker than that observed using exudates of glandular hairs. The results support the hypothesis that the secondary metabolites contained within non-glandular hair layers may create a chemically adverse environment against the entrance and spreading of pathogens in the leaf interior.

Keywords: bacteria, *Cistus creticus*, *Dittrichia viscosa*, fungi, *Olea europaea*, plant defence, plant-pathogen interactions, *Quercus ilex*, secondary metabolites

INTRODUCTION

The leaf surface, besides its role to protect against abiotic stress factors such as water losses, high densities of UV and visible radiation and temperature extremes, represents a barrier that must be breached before a successful pathogenesis can become established and hence constitutes the first line of defence against pathogens. The physical and chemical characteristics of the leaf surface play an important role in governing the success or failure of microbial growth on, and subsequently within, the leaf (Allen *et al*. 1991). Leaf surface characteristics vary among plant species, cultivars and leaves of the same plant, but also between the adaxial and abaxial leaf surface. Epidermal cells occur in different shapes and are frequently modified to form a variety of cell types including non-glandular hairs, secretory glands, glandular hairs, and stomata. Non-glandular hairs (NGHs) exhibit an extreme variability in size, morphology, and function. Their presence is more prominent in plants thriving in dry habitats and usually more abundant at the first stages of organ development (Karabourniotis *et al.* 1995; Valkama *et al.* 2004).

The occurrence of a NGH layer has been considered as a potential mechanical barrier for non-specific plant resistance to microbial pathogens. Hairs and other related structures act as a passive screen that prevent spores and other microbial structures from reaching the leaf surface or form a water repellent surface preventing the formation of water films on which pathogens might be deposited and germinate or multiply (Johnson 1975; Allen *et al.* 1991; Mmbaga and Staedman 1992; Mmbaga *et al.* 1994; Kortekamp and Zyprian 1999; Kortekamp *et al*. 1999; Brandley *et al.* 2003). However, in some cases the presence of trichomes may facilitate the fungal invasion by providing a physical adhesion point for the hyphae (Calo *et al.* 2006).

NGH layers contain phenolic substances, such as flavonoids, in the cell wall, the cuticle and the soluble fraction of the hairs (Karabourniotis *et al*. 1992; Skaltsa *et al.* 1994; Liakopoulos *et al.* 2006). The occurrence of these compounds has been related to the absorbing capacity of NGHs in the ultraviolet-B (UV-B) region of the spectrum. Thus, NGH layers behave as selective optical filters by altering the quantity and the quality of the radiation penetrating to the internal leaf tissues (Karabourniotis and Bornman 1999). However, the potential antimicrobial activity of the mixture of secondary metabolites of NGH has not been previously tested. Tacking into account that this defensive barrier is superficial, the isolation of hairs is relatively easy and the secondary metabolites located within hairs are solvent-extractable, the application of physiological (realistic) concentrations in the bioassays and thus the estimation of the potential antimicrobial capacity of the pubescent leaf surfaces are possible. In this study, *Olea europaea* and *Quercus ilex* were chosen since their leaves possess dense NGH layers whose chemical composition is known from relevant studies. The plant material prepared from these NGH layers was tested against several widespread phytopathogenic bacterial and fungal species. The application of physiological concentrations of isolated exudates of glandular hairs of *Dittrichia viscosa* and *Cistus creticus* (containing mixtures of secondary metabolites of known antimicrobial activity) against the same pathogens has also been tested.

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Table 1 Microbial phytopathogens used in this study and associated diseases.

Bacteria	
Erwinia amylovora	fireblight of Rosaceae fruit trees
Pseudomonas syringae pv *garcae*	bacterial (or halo) blight of coffee
Pseudomonas syringae subsp. *savastanoi*	olive tree knots
Pseudomonas syringae pv *syringae*	bacterial canker of stone and pome fruit trees
Xanthomonas campestris pv *pelargonii*	bacterial blight of geranium
Fungi	
Geotrichum candidum	post-harvest disease of fruits and vegetables
Nectria haematococca var. *cucurbitae*	foot and root rot of legumes and cucurbits
Ustilago maydis	corn smut

MATERIALS AND METHODS

Chemicals and reagents

Unless mentioned differently, all organic chemicals were of analytical grade and purchased from Sigma-Aldrich, St. Louis, MI, USA. Inorganic salts were of analytical grade and purchased from Merck, Darmstadt, Germany. Organic solvents were of analytical grade and purchased from LabScan, Dublin, Ireland.

Preparation of hair extracts and isolation of exudate materials

Plant material was collected from individuals grown under field conditions at the experimental plantation of the Agricultural University of Athens (*Olea europaea* L.; 37° 58′ N, 23° 42′ E, 37 m a.s.l.) or mount Parnis (*Quercus ilex* L., *Dittrichia viscosa* (L.) W. Greuter and *Cistus creticus* L.; 38° 08′ N, 23° 43′ E, 580 m a.s.l.) during summer. For the preparation of the NGHs of *O. europaea* and *Q. Iles* extracts, hairs were removed from the abaxial surface of mature leaves of the current season by gently rubbing their surface with a razor blade under a stereoscope, tacking care not to include debris from the underlying tissues (Karabourniotis *et al.* 1995). Hairs were extracted in triplicate in 100% methanol, the extracts were evaporated to dryness in a rotary evaporator under nitrogen stream and the dry residues were rediluted in methanol. Exudate materials of glandular hairs were removed from *D. viscosa* and *C. creticus* by immersing twigs of the current season in chloroform for 15 s. The crude chloroformic rinsates were evaporated to dryness as outlined above and finally rediluted in 100% methanol.

Microorganisms and growth conditions

Bacterial and fungal species used in this study are presented in **Table 1**. Bacterial strains were *Erwinia amylovora* (Benaki Phytopathological Institute Culture Collection, Greece (BPIC) 916), *Pseudomonas syringae* pv. *garcae* (BPIC1445; (National Collection of Plant Pathogenic Bacteria, United Kingdom) NCPPB512), *P. syringae* subsp. *savastanoi* (BPIC264), *P. syringae* pv. *syringae* (BPIC1) and *Xanthomonas campestris* pv. *pelargonii* (BPIC69). Fungal strains used in this study were *Geotrichum candidum* (BPIC1393), *Nectria haematococca* var. *cucurbitae* and *Ustilago maydis* (Laboratory of Pesticide Science, Agricultural University of Athens). Bacteria were maintained on Nutrient Agar (NA, 13 g nutrient broth L^{-1}, Oxoid Ltd., Basingstoke, Hampshire, UK) Petri dishes at 26°C under a 12-h photoperiod (light provided by fluorescent lamps at a photon flux density of 120 μmol quanta m^{-2} s^{-1}) and subcultured every week (*E. amylovora*) or every month (all other bacteria). *G. candidum* and *N. haematococca* var. *cucurbitae* were maintained on potato dextrose agar (39 g PDA L^{-1}, Merck, Darmstadt, Germany) Petri dishes at 25°C under a photoperiod of 14 h and subcultured every month. *U. maydis* was maintained on Ustilago Complete Agar Medium (UCM) Petri dishes at 30°C in the dark. UCM was prepared as follows: basic medium consisted of (in g (750 ml)$^{-1}$): glucose 10.0; nucleic acids 0.5; casamino acids 3.0; yeast extract 1.0; agar 15.0. The basic medium was mixed with vitamin and inorganic salts solutions at 750: 1: 250 (basic medium: vitamins solution: inorganic salts solution (v/v)). Vitamin solution consisted of (in mg (100 ml)$^{-1}$): *myo*-inositol 200; thiamine 20; calcium pantothenate 20; niacin 20; pyridoxine 10. Inorganic salts solution consisted of (in mg l^{-1}): NH_4NO_3 12000; KH_2PO_4 8000; KCl 4000; Na_2SO_4 2000; $MgSO_4 \cdot 7H_2O$ 1000; $CaCl_2$ 500; $FeSO_4 \cdot 7H_2O$ 4.2; $ZnSO_4 \cdot 7H_2O$ 3.6; $CuSO_4 \cdot 5H_2O$ 3.3; $Na_2MoO_4 \cdot 2H_2O$ 2.4; $MnSO_4 \cdot H_2O$ 2.2. Ustilago minimal agar medium (UMM) used in some of the bioassays was prepared as UCM by omitting nucleic acids, casamino acids, yeast extract, vitamins. Agar was added at 20.0 instead of 15.0 g (750 ml)$^{-1}$.

For the preparation of bacterial bioassays, one-day old cultures were suspended in sterile water and diluted to a concentration of 0.5-2 × 10^4 colony-forming units (cfu) ml^{-1} with reference to the optical density at 560 nm of the original suspension. For the preparation of fungal bioassays (*G. candidum* and *N. haematococca* var. *cucurbitae*), one-week old cultures were suspended in sterile water and spread onto PDA Petri dishes which were cultured for 24 h at 25°C under a photoperiod of 14 h. Mycelial disks were removed using a cork-borer of 4 mm diameter. Alternatively, spore suspensions were prepared in sterile water from one-week old cultures and adjusted to a concentration of 1.5 × 10^6 spores ml^{-1} (*N. haematococca* var. *cucurbitae*) or 5 × 10^6 spores ml^{-1} (*G. candidum*). For the preparation of *U. maydis* assays, sporidia were obtained by growing *U. maydis* in complete liquid medium (UCM) at 30°C in the dark on a rotary shaker at 150 rpm for 48 h (Ziogas *et al.* 2005). Sporidia concentration was adjusted to 0.5 × 10^3 cfu ml^{-1}.

Bioassays

The concentrations of the methanolic solutions of NGH extracts or of glandular hair rinsates were adjusted properly so as to allow the even distribution of the plant material onto the growth medium using the smallest volume of methanol possible (1-5 μL methanol cm^{-2}, depending on the plant material). Methanolic solutions of the plant materials were applied on the surface of freshly prepared growth medium of assay petri dishes at appropriate volumes so as to result in concentrations resembling those of the leaf surface (latent physiological concentrations). Latent physiological concentration for each plant species used was defined as the amount of plant material deposited on the surface of the growth medium which corresponds to the amount of material extracted from NGHs or collected as exudate material from an equal surface area of the leaf. The term 'latent' is used to indicate that the concentration applied corresponds to that present as a whole in the hair layers, although this may not be the effective one encountered by microorganisms *in vivo*. To facilitate these computations, the volume of plant material solution to be applied was determined spectrophotometrically with respect to total phenolic content (Inderjit and Nilsen 2003) and compared to reference values of absorbance per unit of leaf surface for each plant species used. After the application of the plant material, Petri dishes were left open for several minutes into the laminar flow chamber to allow methanol evaporation by the culture medium. In all cases, assay Petri dishes with an equal volume of methanol applied on the growth medium surface were also prepared to serve as controls. The possible dilution of the plant materials applied on the surface of the growth medium due to diffusion of the substances into the agar during the bioassay period was examined by fluorescence microscopy (see bellow). The degree of diffusion of the applied solution of plant material into the growth medium depended on the plant source. After 72 h, NGHs extracts of *O. europaea* and rinsates of *D. viscosa* showed a diffusion of up to 300 μm in depth from the surface of the growth medium whereas NGHs extracts of *Q. ilex* and rinsates of *C. cre-*

ticus showed a lesser diffusion of up to 50 μm from the surface.

Bacterial suspensions were spotted tenfold (replicates) onto NA assay Petri dishes and grown at 26°C under a 12-h photoperiod. During the bioassay period, colony number was recorded every 24 h. Inhibition of colony forming ability was calculated 24 h after the day that colonies were measurable in the controls and expressed as percent (%) of the control. For fungitoxicity assays, mycelial disks (*G. candidum* and *N. haematococca* var. *cucurbitae*) were placed onto PDA or UMM assay Petri dishes (three to five replicates) for studies of mycelial radial growth at 25°C in the dark to prevent sporulation. The inoculum was subtracted during the measurements of the mycelial diameter. Inhibition of mycelial radial growth was calculated 72 h after inoculation, as percent (%) of the control. For *U. maydis*, sporidia suspension was spread onto UCM assay Petri dishes (three replicates) and grown at 30°C under a photoperiod of 16 h. Inhibition of colony forming ability was calculated 72 h after inoculation, as percent (%) of the control. For spore germination assays, spore suspensions of *G. candidum* and *N. haematococca* var. *cucurbitae* were spread onto UMM assay Petri dishes (three replicates) and incubated at 25°C under a 14-h photoperiod. Spore germination ability was measured every 1 h. Spores with visible germ tubes were counted as germinated. Inhibition of spore germination was calculated when the germination in the controls was at 50%, using the following expression:

Inhibition (%) = 100 × [1-(germination (%) in treatment / germination (%) in control)]

Fluorescence microscopy

The diffusion of the plant material into the agar was examined by the fluorescence emitted by the polyphenolic constituents of the applied material. A Zeiss Axiolab fluorescent microscope equipped with a G-365 exciter filter and a FT-395 chromatic beam splitter was used. The induction of polyphenol fluorescence was enhanced by an appropriate fluorescence inducer (Karabourniotis *et al.* 1998). For this, 1 ml of an aqueous solution of 0.6% diphenylboric acid-ethanolamine were applied on the surface of freshly prepared growth medium and the Petri dishes kept closed with shielding parafilm tape for 24 h. This treatment ensured the saturation of the agar volume by the fluorescence inducer. Afterwards, the plant material was applied onto the growth medium surface and cross sections were examined every 12 hrs to assess the degree of material diffusion. Hand-cut transverse sections and isolated trichomes were taken from fresh leaves. Fluorescence yield was increased by immersing samples in a 10% (w/v) KOH solution (Karabourniotis *et al.* 2001). The sections or trichomes were washed with, and subsequently immersed in, distilled water for microscopic observation.

Statistical analysis

Statistically significant differences between means were determined by either a homo- or heteroscedastic *t*-test (two-tailed) according to the similarity of the variances between the data sets.

RESULTS

General characteristics of the NGH layers

The abaxial surface of mature leaves of both *O. europaea* and *Q. ilex* is covered by dense NGH layers at a density of *ca.* 1.5 and 1.8 mg cm^{-2}, respectively (Skaltsa *et al.* 1994; Karabourniotis *et al.* 1995). In case of *O. europaea*, such layers consist of multicellular, peltate NGHs, whereas the epidermis of *Q. ilex* forms multicellular stellate NGHs (Skaltsa *et al.* 1994). In the presence of a suitable fluorescence inducer, hairs emit a bright yellow-green fluorescence under excitation by UV light, indicating the presence of flavonoids and other phenolics (**Fig. 1**; see also Karabourniotis *et al.* 1998). In both *O. europaea* and *Q. ilex*, young leaves are covered on both surfaces by NGH layers of higher density compared to that seen in mature leaves (Karabourniotis *et al.* 1998; see also **Fig. 1**), while hairs are dead at maturity.

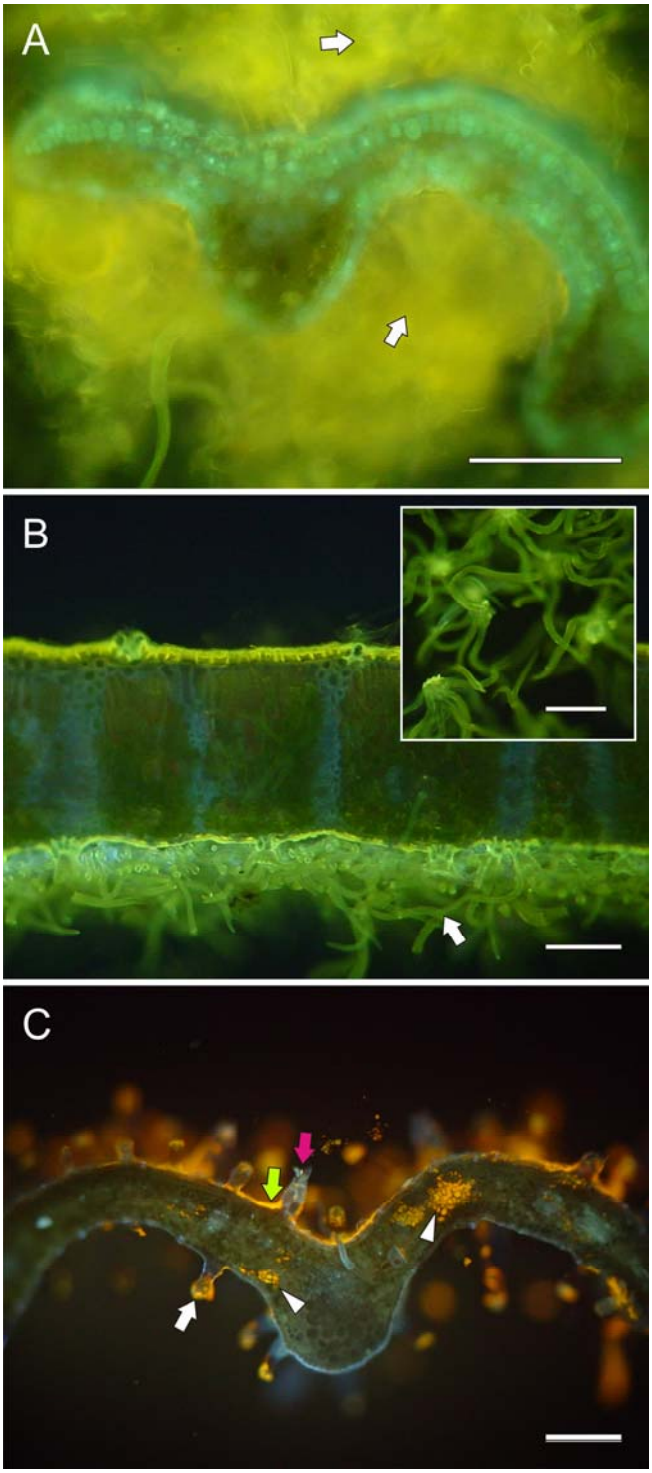

Fig. 1 Epi-fluorescent micrographs of hand-cut transverse sections of leaves of representative plant species used in the present study, showing the different defensive strategies of their leaf surfaces. All micrographs were taken under the same conditions of excitation and fluorescence induction (see Materials and Methods). (**A**) Young, expanding leaf of *Q. ilex*. Stellate non-glandular hairs are abundant in the early stages of leaf development on both surfaces of the lamina (white arrows). (**B**) Mature leaf of *Q. ilex.* As the leaf matures the hairs of the adaxial surface are shed. Insert shows isolated non-glandular hairs from the abaxial leaf surface. (**C**) Young, expanding leaf of *D. viscosa*. Glandular secreting hairs are abundant on both surfaces of the lamina. Bright yellow-orange fluorescence is emitted by the head, indicating the presence of flavonoids (white arrow). The exudates leak out from the head and form a continuous layer covering a significant part of the leaf surface (green arrow). Part of this material stained the mesophyll area during the sectioning procedure (white arrowheads). Magenta arrow shows a glandular hair with broken head. Scale bars 100 μm.

Table 2 The inhibition (% of the control) of colony forming ability of phytopathogenic bacteria by either the methanolic extracts of the non-glandular leaf hairs of *O. europaea* and *Q. ilex*, or the isolated exudate materials of the glandular hairs of *D. viscosa* and *C. creticus*.

Bacterial species	***Pseudomonas syringae*** **pv *syringae*[a,b]**	***Pseudomonas syringae*** **pv *garcae*[a,b]**	***Pseudomonas syringae*** **subsp. *savastanoi*[a,b]**	***Xanthomonas campestris*** **pv *pelargonii*[a,b]**	***Erwinia amylovora*[a,b]**
Plant species					
Olea europaea	73 ± 2 **	9 ± 4	100 ± 0 **	100 **	34 ± 3 **
Quercus ilex	73 ± 3 **	27 ± 5 **	2 ± 7	100 **	-5 ± 3
Dittrichia viscosa	100 **	98 ± 1 **	100 **	100 **	-
Cistus creticus	59 ± 3 **	81 ± 7 **	90 ± 4 **	100 **	-

[a] Plant material was applied at the latent physiological concentration (see Materials and Methods). Values are means ± standard error of the mean.
[b] Significant differences between treatment and control (no plant material) are denoted by asterisks (** $P < 0.01$).

Table 3 The inhibition (% of the control) of mycelial radial growth (*G. candidum* and *N. haematococca* var. *cucurbitae*) or colony forming ability (*U. maydis*) by either the methanolic extracts of the non-glandular leaf hairs of *O. europaea* and *Q. ilex* or the isolated exudate materials of the glandular hairs of *D. viscosa*.

Fungal species	***Geotrichum candidum*[a,b]**	***Nectria haematococca* var. *cucurbitae*[a,b]**		***Ustilago maydis*[a,b]**
Growth medium	**PDA**	**UMM**	**PDA**	**UCM**
Plant species				
Olea europaea	4 ± 1	53 ± 5 **	23 ± 1 **	34 ± 10 *
Quercus ilex	1 ± 4	52 ± 8	28 ± 2 *	38 ± 21
Dittrichia viscosa	73 ± 4 **	100 **	100 **	100 **

[a] Plant material was applied at the latent physiological concentration (see Materials and Methods). Values are means ± standard error of the mean.
[b] Significant differences between treatment and control (no plant material) are denoted by asterisks (* $P < 0.05$; ** $P < 0.01$).

Table 4 The inhibition (% of the control) of spore germination ability of phytopathogenic fungi by either the methanolic extracts of the non-glandular leaf hairs of *O. europaea* and *Q. ilex* or the isolated exudate materials of the glandular hairs of *D. viscosa*.

Fungal species	***Geotrichum candidum*[a,b]**		***Nectria haematococca* var. *cucurbitae*[a,b]**	
Plant species	**Germination %[c]**	**Inhibition %**	**Germination %[c]**	**Inhibition %**
Olea europaea	37.4 ± 2.9 *	25	26.6 ± 5.6 *	47
Quercus ilex	1.39 ± 1.31 **	97	11.0 ± 1.7 **	78
Dittrichia viscosa	0.68 ± 0.34 **	99	-	-

[a] Plant material was applied at the latent physiological concentration (see Materials and Methods). Values are means ± standard error of the mean.
[b] Significant differences between treatment and control (no plant material) are denoted by asterisks (* $P < 0.05$; ** $P < 0.01$).
[c] Percent of germinated spores of the treatment at 50 % germination in the control (see Materials and Methods).

Inhibitory effect of NGH extracts against phytopathogenic microorganisms

The methanolic extracts of NGHs of *O. europaea* leaves, applied at latent physiological concentations (see Materials and Methods), inhibited colony forming of *Erwinia amylovora*, *Pseudomonas syringae* subsp. *savastanoi*, *P. syringae* pv. *syringae* and *Xanthomonas campestris* pv. *pelargonii* by 34-100% compared to the controls (**Table 2**). The inhibitory effect of the extracts against *P. syringae* pv. *garcae* was negligible (**Table 2**). The methanolic extracts of NGHs of *Q. ilex* leaves generally showed comparable or lower inhibitory effects on the growth of the above bacterial species compared to those of the leaf hairs of *O. europaea* (**Table 2**). Methanolic hair extracts of *Q. ilex* did not significantly affect the growth of *P. syringae* subsp. *savastanoi* and *E. amylovora.* On the other hand, these extracts showed a stronger inhibition against the growth of *P. syringae* pv. *garcae* than did those of *O. europaea* (**Table 2**).

The methanolic extracts of leaf hairs of *O. europaea* inhibited the radial growth of *Geotrichum candidum* and *Nectria haematococca* var. *cucurbitae* on PDA by 4 and 23%, respectively and the colony forming of *Ustilago maydis* by 34% compared to the controls (**Table 3**). The inhibitory effect was pronounced when the mycelial radial growth of *N. haematococca* var. *cucurbitae* was tested using UMM instead of PDA as growth medium (**Table 3**). The UMM was used in the present study as an alternative growth medium of different composition compared to PDA. The methanolic extracts of leaf hairs of *Q. ilex* exhibited a similar inhibitory effect compared to that of *O. europaea* extracts against the growth of the three fungal species tested (**Table 3**). In addition, the methanolic extracts of the leaf hairs of both plants showed an inhibitory effect against spore germination of *G. candidum* and *N. haematococca* var. *cucurbitae* (25-97% inhibition compared to the controls; **Table 4**). After careful examination of the results, the inhibitory effect on spore germination was primarily attributed to the extension of the lag phase (data not shown). All the above results indicate that NGHs contain substances that, when assayed at concentrations that resemble those at the leaf surface, display an inhibitory effect against the growth of a number of phytopathogenic bacteria, as well as the growth and spore germination of certain phytopathogenic fungi.

Inhibitory effect of glandular hair rinsates against phytopathogenic microorganisms

Chloroformic rinsates of the leaves of *D. viscosa* and of *C. creticus* were assessed against the same phytopathogenic bacteria, excluding *E. amylovora*, under the same experimental conditions. The rinsates were applied to the surface of the growth medium in Petri dishes at latent physiological concentrations (see Materials and Methods). A strong inhibitory effect of the chloroformic rinsates of both plants against the growth of all bacteria tested was observed (**Table 2**). In almost all cases, the observed inhibition was the same or stronger than that observed by applying the methanolic hair extracts of the leaves of *O. europaea* and *Q. ilex*. Chloroformic rinsates of the leaves of *D. viscosa* were also assessed against the same phytopathogenic fungi, excluding *N. haematococca* var. *cucurbitae* spores, under the same experimental conditions. Again, in all cases, the observed inhibition was stronger than that observed by applying the methanolic hair extracts of the leaves of *O. europaea* and *Q. ilex* (**Tables 3, 4**).

DISCUSSION

The results of the present study clearly showed that the NGH materials, applied at latent physiological concentrations, inhibited the growth of the majority of the bacterial as well as the spore germination and growth of the fungal species tested. It is worth noticing that, although *P. syringae* subsp. *savastanoi* is pathogenic to olive (**Table 1**), it is extremely sensitive against the methanolic NGH extracts of olive leaves (**Table 2**). This may be explained by the fact that, although this bacterium has a resident phase on the

leaves of olive (Ercolani 1978), the main sites for bacterial invasion are fissures of the bark or leaf abscission scars and not the leaves (Rudolph 1995).

The NGHs of *O. europaea* contain high concentrations of flavonoids of which quercetin, quercetin 3-*O*-rhamnoside and apigenin 7-*O*-glucoside are the main constituents (Liakopoulos *et al.* 2006). Quercetin from stems and roots of *O. europaea* was one of the most active antimicrobial compounds against two phytopathogenic fungi, *Phytophthora megasperma* and *Cylindrocarpon destructans* (Báidez *et al.* 2006). Acylated kaempferol glycosides are the main constituents of the methanolic extracts of the leaf NGHs of *Q. ilex* (Skaltsa *et al.* 1994). Provided that these compounds are readily extractable by solvents, and thus non-covalently bound (Karabourniotis *et al.* 1998; Liakopoulos *et al.* 2006), they may actively participate in the defence of plant against pathogens as constitutively occurring compounds and preformed inhibitors (Nicholson and Hammerschmidt 1992; Bennett and Wallsgrove 1994; Wallace and Fry 1994). Moreover, flavonoids and other polyphenol compounds from various plant sources are toxic to bacteria and fungi (Wang *et al.* 1989; Dixon 2001).

NGHs are not the only epidermal appendages offering protection to the surface of plants organs. The results of the present study show a strong inhibitory effect of the exudates of glandular hairs of both plants against the majority of pathogens. Glandular secreting trichomes (glandular hairs) produce and store mixtures of secondary metabolites, usually effective against pathogens. In some plant species, the principal role of these structures is to produce high levels of exudate materials (exudates) that form a continuous layer on the surface (Wagner 1991; Nonomura *et al.* 2009, see also the bright yellow-orange fluorescence emitted by the glandular hairs of *D. viscosa* leaves in **Fig. 1**). The exudates usually have the form of a complex, resinous mixture of secondary metabolites, consisting mainly of terpenoids, flavonoid aglycones and phenolic acids, frequently imbedded in a lipophilic matrix (Wollenweber and Dietz 1981). The leaves of *D. viscosa*, an invading ruderal species of the Asteraceae family, bear sessile and stalked glandular hairs, which secrete a resinous mixture of secondary metabolites throughout their life span (**Fig. 1**; see also Nikolakaki and Christodoulakis 2004). These exudates are composed of a number of flavonoid aglycones (Wollenweber *et al.* 1991), along with a great number of terpenoids (Zhao *et al.* 2006). Likewise, the exudates of leaf glandular hairs of *C. creticus*, are composed mainly of terpenoids and flavonoid aglycones (Vogt *et al.* 1987; Demetzos *et al.* 1995). Essential oil of *D. viscosa*, isolated by whole leaf extraction, was found to exhibit fungitoxic activity against soil-borne phytopathogenic fungi (Muller-Riebau *et al.* 1995). Strong antibacterial activity was shown for the essential oil of *C. creticus* (Demetzos *et al.* 1995). However, no attempt was made to apply the plant material at physiological concentrations. In addition, the exudates of *D. viscosa* leaves show strong allelopathic activity (Stephanou and Manetas 1995; Stavrianakou *et al.* 2004).

In conclusion, both types of epidermal appendances either deposit (NGHs) or excrete (GHs) mixtures of secondary metabolites possessing *in vitro* antimicrobial activity at concentrations resembling those of the leaf surface. NGHs represent permanent structures that offer physical protection against biotic or abiotic stress factors, even after the total removal of the defensive compounds due to gradual leaching. On the other hand, exudates secreted by glandular hairs offer better protection to the leaf surfaces against pathogens, but extensive leaching may result in partial or total removal of this protective layer. The efficiency of each defensive strategy may be related to factors such as the leaf life span, climatic conditions, water and nutrient availability and competition of neighbouring species.

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