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3-HYDROXYACETOPHENONE IN CARNATIONS IS A PHYTOANTICIPIN ACTIVE AGAINST *FUSARIUM OXYSPORUM* F. SP. *DIANTHI*

PAOLO CURIR, AUGUSTO MARCHESINI,* BRUNO DANIELI†‡ and FRANCESCO MARIANI

Istituto Sperimentale per la Floricoltura, 18038 Sanremo, Italy; *Istituto Sperimentale per la Nutrizione delle Piante, 10100 Torino, Italy; ‡Dipartimento di Chimica Organica e Industriale, Universita' degli Studi di Milano, 20133 Milano, Italy

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Key Word Index—*Dianthus caryophyllus*; Caryophyllaceae; carnation; *Fusarium oxysporum*; resistance; phenols; 3-hydroxyacetophenone; phytoanticipin.

Abstract—Among other constitutive phenols, 3-hydroxyacetophenone has been detected in an uninfected carnation cultivar totally resistant to *Fusarium oxysporum* f. sp. *dianthi*, a fungus known to cause the main disease affecting this plant species. In both *in vivo* and *in vitro* experiments, the isolated compound proved very active in restricting parasite development. Thus, 3-hydroxyacetophenone, as a preformed and constitutive component of the uninfected cultivar, can be considered as a carnation phytoanticipin.

INTRODUCTION

The biochemical studies until now performed on carnation resistance against the fungus, *Fusarium oxysporum* f. sp. *dianthi*, deal with plant response to the pathogen after coming in contact with host tissues. The main response is a fast biosynthesis of resistance compounds, acting as phytoalexins [1–3] and thus possessing the ability of hampering parasite attack [4, 5]. These compounds, identified as anthranilic acid derivatives [6, 7], have been grouped into the category of 'dianthalexins' [8]. Their presence in very small amounts (a few $\mu g\ g^{-1}$ fresh tissue [9]) and concentration have been correlated with the resistance to *F. oxysporum* in several carnation cultivars [10]. Many other non-aminated phenolics are also constituents of carnation [11, 12]; however, they have been rarely considered in previous studies on resistance [13, 14]. Only recently have constitutive phenols been investigated for their possible activity against fungal parasites; the term 'phytoanticipin' was adopted to define those which were able to give plants some protection against pathogens [15]. The present research was carried out on the carnation cv. 'Duca', totally resistant to *F. oxysporum* f. sp. *dianthi* [16, 17], in order to determine the possible role of the constitutive phenols in the resistance to this pathogen.

RESULTS AND DISCUSSION

Extraction of root and stem tissues of carnation cv. Duca gave four main constitutive phenols (**1**–**4**). Compound **1** was recovered from the 0.05 M NaOH phase during the extraction procedure (see Experimental). It showed UV absorption characteristic of acetophenone derivatives and on acetylation gave a monoacetate, thus evidencing the presence of one –OH group in the molecule. Addition of $AlCl_3$ to a methanolic solution of **1** induced a bathochromic shift (50 nm), demonstrating that the –OH function is *meta* to a COMe group. The EI mass spectrum of **1** showed an intense $[M]^+$ at *m/z* 136 accompanied by fragments at *m/z* 121 and 93, due to the loss of methyl and CO groups, respectively. The ^1H NMR spectrum in $CDCl_3$ was structurally diagnostic, containing a singlet at $\delta 2.60$ for a deshielded CH_3CO moiety, a broad singlet at $\delta 6.61$ (exchangeable) for an OH and signals for four aromatic protons at $\delta 7.10$ (*bdd*, $J_1 = 8$ Hz, $J_2 = 3$ Hz), 7.32 (*t*, $J = 8$ Hz), 7.49 (*bd*, $J = 8$ Hz) and 7.53 (*bd*, $J = 3$ Hz). Chemical shifts arguments and splitting patterns led to the identification of compound **1** as 3-hydroxy acetophenone.

Compound **2**, recovered in the 0.05 M $NaHCO_3$ phase, showed UV absorption in methanol: 364.2, 323.4, 265.6 and 252.8 nm. Hydrolysis gave glucose and rhamnose in equimolecular amounts and an aglycone having the same UV spectrum as kaempferol. Shifts in the UV spectrum induced by the addition of sodium methoxide, $HCl/AlCl_3$ and sodium acetate indicated that both sugars were linked to position 7 of the aglycone. The negative FAB mass spectrum of **2** displayed a $[M-H]^-$ at *m/z* 593, which showed the consecutive loss (B/E linked scan spectrum) of fragments at *m/z* 146 and 162 due to rhamnose and glucose units, respectively, to afford an ion at *m/z* 285 corresponding to kaempferol. The ^1H NMR spectrum (DMSO-d_6, 80°) contained the signals of an AB system at $\delta 6.40$ and 6.75 for H-6 and H-8 of a 7-substituted flavanol, and an AA'BB' splitting pattern at $\delta 6.95$ and 8.05 for a *p*-OH-substituted phenyl ring.

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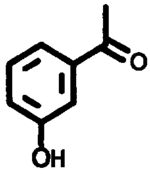
†Author to whom correspondence should be addressed.

A broad singlet at δ5.18 and a doublet at δ5.20 were attributed to the rhamnose and glucose anomeric protons, respectively. Other protons could be identified at δ1.21 (3H, *d*, $J = 6.5$ Hz, C-6‴H$_3$), 3.25 (1H, *t*, $J = 9$ Hz, H-4‴), 3.28 (1H, *t*, $J = 8$ Hz, H-3″ or H-4″) and 3.42 (1H, *dd*, $J = 9$ and 3.5 Hz, H-3‴), whereas the remaining sugars' hydrogens overlapped between δ3.48 and 3.61 (4H) and between δ3.73 and 3.82 (3H). All the spectroscopic data were in agreement with the structure of kaempferol 7-*O*-neohesperidoside (kaempferol 7-*O*-rhamnoglucoside) for **2**. Final proof was achieved by direct comparison with an authentic sample.

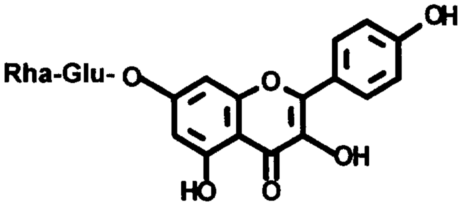
Compound **3** was recovered in part (10%) in the NaHCO$_3$ phase, but mainly (90%) in the NaOH separation phase. Its UV spectrum in methanol showed absorptions at: 349.8, 301 sh, 264.9 sh and 256.0. Rhamnose was recovered after hydrolysis and the UV spectrum of the aglycone was identical to that of the quercetin. Spectral shifts induced by H$_3$BO$_3$, sodium acetate in the presence of H$_3$BO$_3$, and sodium methoxide showed that the –OH group in the 3-position was linked to the sugar. That **3** was quercitrin was further corroborated by the FAB mass spectrum {[M − H]$^-$ at *m*/*z* 447} and ^{1}H NMR (DMSO-d_6, 80°), which allowed the assignment of all the signals of the protons. The rhamnose protons were found at δ5.31 (*bs*, H-1″), 4.03 (*bd*, $J = 4.5$ Hz, H-2″), 3.58 (*dd*, $J_1 = 9$ Hz, $J_2 = 4.5$ Hz, H-3″), 3.34 (*dq*, $J_1 = 9$Hz, $J_2 = 6$ Hz, H-5″), 3.21 (*t*, $J = 9$ Hz, H-4″) and 0.89 (*d*, $J = 6$ Hz, H$_3$-6‴). The H-6 and H-8 of the flavanol aglycone appeared at δ6.24 and 6.41 as an AB system ($J = 2.5$ Hz), while H-2′, H-5′ and H-6′ resonated, respectively, at δ7.35 (*d*, $J = 2$ Hz), 6.92 (*d*, $J = 8$ Hz) and 7.29 (*dd*, $J_1 = 8$ Hz, $J_2 = 2$ Hz). Compound **3** was, therefore, confirmed to be quercitrin (quercetin 3-*O*-rhamnoside).

Compound **4** was recovered in part (10%) in the 0.05 M sodium acetate separation phase, but mainly (90%) in the NaHCO$_3$ phase. The UV spectrum in methanol showed absorptions at 352.0, 302 sh, 265 sh and 257.2 nm. On hydrolysis, **4** gave rhamnose and an aglycone having the same UV spectrum in methanol as myricetin. A hypsochromic shift of 20 nm induced in the UV methanolic spectrum by AlCl$_3$ after the addition of HCl demonstrated the presence of three adjacent free –OH groups on the B ring. The [M − H]$^-$ of **4** at *m*/*z* 463 indicated the presence of an additional oxygen atom on the molecule with respect to **3**. This oxygen atom was located on the flavanol moiety because of the loss of a fragment of 146 mu due to rhamnose. The ^{1}H NMR spectrum contained the signals of rhamnose at similar chemical shifts as compared to the ones of **3** (δ3.50, 4.04, 3.62, 3.46, 3.22 and 0.92), the signal for H-6 and H-8 at δ6.22 and 6.38, and a singlet at δ6.96 for the two equivalent protons at positions 2′ and 6′. Compound **4** was thus finally identified as myricitrin (myricetin 3-*O*-rhamnoside).

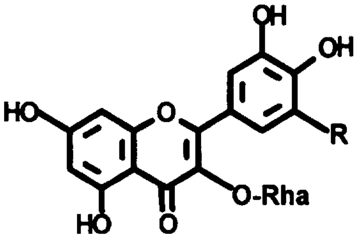
Fluctuations in the concentrations of these four compounds were found to occur in the tissues of different plants within the same cultivar. The range of concentrations in stem tissues (mg g^{-1} dry wt) was 3-hydroxyacetophenone (**1**): 1–3 (i.e. 7.3–22 mM), kaempferol 7-*O*-neohesperidoside (**2**): 3.5–4.0 (i.e. 5.9–6.7 mM), quercitrin (**3**): 4–5 (i.e. 8.9–11.2 mM) and myricitrin (**4**): 0.5–2.0 (i.e. 1.07–4.38 mM). The concentrations found in roots were about two-thirds of stem values.



1



2



	R
3	H
4	OH

When tested *in vitro*, the flavonoid glycosides had only a slight inhibiting effect on *F. oxysporum* growth (Table 1), while 3-hydroxyacetophenone proved to be very effective. Its inhibitory activity towards the fungus was also confirmed by *in vivo* experiments (Table 2). 3-Hydroxyacetophenone is present both in the roots and in the stems in concentrations comparable to those which proved to be effective in our biological experiments. Surprisingly, 3-hydroxyacetophenone appeared to be the main phenol responsible for the constitutive resistance in the studied carnation cultivar. Its absence in the resistant carnation cv. Novada [18] would appear to indicate that 3-hydroxyacetophenone cannot be considered as a ubiquitous effector of the resistance in *Dianthus caryophyllus*. It is clear, however, that the present case is another example [19–21] in which plants are able to develop a passive defence system, concomitant with the one of phytoalexins, based on the presence of constitutive phenols; from this point of view, 3-hydroxyacetophenone must be considered as a phytoanticipin [15].

Table 1. Mycelial growth of *oxysporum* in the presence of phenolics isolated from carnation cv. Duca. The data, expressed as growth percentage in comparison with the control, were arcsin transformed to be statistically analysed using the Student–Newman–Keuls test. Values followed by the same letter are not statistically different at $P = 0.05$.

Phenol	Concentration (mM)	Mycelial growth (%)
3-Hydroxyacetophenone	10	0.38c
	25	0.22b
	50	0.00a
Kaempferol 7-*O*-neohesperidoside	10	0.99h
	25	0.93g
	50	0.79d
Quercitrin	10	0.99h
	25	0.89f
	50	0.84e
Myricitrin	10	0.99h
	25	0.94g
	50	0.89f
Control	—	1.00h

Table 2. Effect of spray treatments with 3-hydroxyacetophenone on the susceptible carnation cv. Manon grown in soil infected with *Fusarium*. The controls are represented both by the untreated samples and by benomyl-treated plants. Percentages of attacked plants were arcsin transformed for statistical analysis. Values followed by the same letters are not statistically different at $P = 0.05$ according to the Student–Newman–Keuls test

Treatment	Attacked plants (%)
3-Hydroxyacetophenone sprayed at 1 mM	0.45a
3-Hydroxyacetophenone sprayed at 5 mM	0.52ab
Benomyl sprayed at 0.5 g l^{-1}	0.63b
Control	0.90c

EXPERIMENTAL

Phenol extraction and identification. One-year-old carrotian plants cv. Duca were obtained from the Albani-Vitalia farm, Sanremo, Italy. Roots were sepd from stems. Phenolics were extracted from root and stem tissues (200 g dry wt) with 10 l MeOH–EtOH (1:1) and sepd from pigments using an already published protocol [22]. The alcoholic crude extract (CE), concd to 1.25 l *in vacuo* at red. pres., was studied by 2D PC using TBA and HOAc as eluents according to ref. [23], and by TLC on silica gel (Kieselgel G 60, Merck) using MeOH–HOAc–H_2O (18:1:1) as eluent. Analysis revealed the presence of 4 main phenols in both stem and root extracts. Aliquots (100 ml) of CE were evapd to dryness, then redissolved in *n*-AmOH and the alcoholic solns extracted with aq. solns of 0.05 M NaOAc, 0.05 M $NaHCO_3$ and 0.05 M NaOH. The aq. frs obtained were then acidified with 5% HCl and re-extracted with *n*-AmOH. The solvent was then evapd to dryness under N_2 and replaced by MeOH. Further CC purification was carried out an Li-Chroprep RP-18 (Merck) (30 × 1 cm) eluting with an A–B linear gradient from 0% B in A to pure B, where A = MeOH and B = *n*-AmOH. Sepd compounds were also analysed after hydrolysis in boiling 1 N HCl for 30 min. To the aq. hydrolysis solns *n*-BuOH was added to separate the aglycones from the sugars. Sugars were identified by TLC on silica gel (Kieselgel G 60, Merck) eluted with EtOH–H_2O–MeOH–HOAc (13:3:3:4) using reference compounds and located with *p*-anisidine phthalate [23]. Both flavonoid glycosides and aglycones were studied spectrophotometrically and UV spectra in MeOH were obtained before and after addition of suitable diagnostic reagents [24]. Final identification of purified phenols was achieved by MS and NMR analyses. Phenol quantitation was spectrophotometrically performed on the basis of the ε value for each identified compound.

Biological tests. For *in vitro* expts, *F. oxysporum* f. sp. *dianthi* race 2 was used [25, 26]. The pathogen was the isolate no. P_2 75, kindly supplied by Prof. A. Garibaldi (DI.VA.P.R.A., Pathology Institute of Turin University, Italy). The fungus was grown on potato dextrose agar (39 g l^{-1}) with or without the phenols to be tested in 3 dosages (10, 25 and 50 mM); every treatment consisted of 36 Petri dishes. Phenols were added to the medium dissolved in Me_2CO and the solvent concn in the medium was maintained at 2%. Petri dishes were inoculated with a 1 cm Φ mycelium disc, kept in culture for 60 days at 22° in darkness and mycelial growth then evaluated by comparing the area of developed mycelium in the presence of phenols with that of the control assumed as 100% of growth.

Phenol **1** was also assayed for its activity in an *in vivo* expt. Rooted cuttings of the carnation cv. 'Manon', susceptible to *F. oxysporum* f. sp. *dianthi* [27] and constitutively lacking in 3-hydroxyacetyophenone (Curir and Marini, pers. commun.) were planted in benches artificially inoculated by mixing 100 g m^{-2} of the pathogen, grown on autoclaved *weath* kernels [28]. The phenol was sprayed every 2 weeks at 1 and 5 mM dissolved in 20 ml EtOH further dild with H_2O to a final alcoholic concn of 5%. Controls consisted of 5% EtOH in H_2O and of the fungicide, benomyl, 0.5 g l^{-1} in H_2O sprayed every 2 weeks. Experiments were arranged in a completely randomized design, where any treatment had 4 repetitions, each one consisting of 60 plants. Results were recorded 5 months later.

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