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Correlations between in vivo resistance to *Fusarium* and in vitro response to fungal elicitors and toxic substances in carnation

M. Buiatti [1], A. Scala [1], P. Bettini [1], G. Nascari [1], R. Morpurgo [1], P. Bogani [1], G. Pellegrini [1], F. Gimelli [2] and R. Venturo [2]

[1] Istituto di Anatomia Comparata, Biologia Generale e Genetica, Università, Firenze, Italy
[2] Istituto Regionale per la Floricoltura, San Remo, Italy

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Summary. With the aim of ascertaining the existance of a correlation between in vivo resistance to *Fusarium oxysporum* f. sp. *dianthi* and in vitro response to fungal elicitors and toxic substances, phenylalanine ammonia-lyase and phytoalexin accumulation, on one hand, and resistance to culture filtrate, on the other, were assayed in "in vitro" cultures of three susceptible and four resistant *Dianthus caryophyllus* cultivars. Cultivars showing varying degrees of resistance in vivo either tolerated higher culture filtrate concentrations ('Niki') or showed high PAL activity and phytoalexin production when treated with *Fusarium* elicitor ('Duca'), or responded positively to both treatments ('Mei-Ling', 'Pulcino'). No such responses were shown in tissue cultures of susceptible cultivars. The differential response to the fungal elicitor seemed to be highly specific as genetic differences between cultivars were not observed in tissue cultures treated with other biotic (*Phytophthora infestans*) and abiotic ($HgCl_2$) elicitors.

Key words: *Dianthus caryophyllus* – *Fusarium oxysporum* – Physiological resistance genetics – Tissue cultures – Phytoalexin metabolism – Toxins

Introduction

Plant resistance to pathogens is certainly due to an array of different defence mechanisms acting at different levels of infection. They may include lack of host-parasite recognition, synthesis of antimicrobial compounds, mechanical arrest of pathogen penetration and invasion, resistance to toxic compounds, etc.

Selection of resistant genotypes has been hampered by inadequate knowledge of physiological mechanisms underlying single gene resistance and, hence, by the lack of suitable, fast and reliable, selection methods. The use of plant tissue cultures both for further investigation of the biochemical basis of defence processes and as an aid in the selection of resistant cultivars has been suggested by several authors (Buiatti and Scala 1984; Chaleff 1981). The main reasons for this choice would be the highly standardized culture conditions, the morphophysiological homogeneity of cultured cells, the high mutation frequency in tissue culture (Earle and Demarly 1982) and the possible use of highly efficient selection techniques (Buiatti 1983).

A necessary condition for a ready use of plant tissue cultures as a tool for the study of host-pathogen interactions and as an aid in resistance breeding is, however, the presence of a correlation between in vitro response to the pathogen or its cellular components, and in vivo behaviour. Two kinds of resistant reactions, namely resistance to toxins or culture filtrates (Behnke 1980; Sacristan 1982; Thanutong et al. 1983; Yoder 1981) and antimicrobial compounds (phytoalexins) accumulation and synthesis (Doke and Furuichi 1982; Kuhn et al. 1984; Scala et al. 1983) can be tested in vitro.

The aim of the present paper was to compare the behaviour in vitro of three Carnation (*Dianthus caryophyllus* L.) cultivars susceptible to *Fusarium oxysporum* Schl. f. sp. *dianthi* (Prill. et Del.) Snyn. et Hans. race 2 (Corrida, Mosè, S. Giorgio) and four showing varying degrees of resistance ('Duca', 'Mei-Ling', 'Niki', 'Pulcino') for both growth on media containing fungal culture filtrate and phytoalexins accumulation after treatment with *Fusarium* cell wall components.

Materials and methods

Biological material

The carnation cultivars used in these experiments were 'Duca', 'Mei-Ling', 'Niki', 'Pulcino', all partially resistant to *F. oxy-*

Abbreviations: FuCWC = cell wall components from *Fusarium oxysporum* f. sp. *dianthi* race 2; PhCWC = cell wall components from *Phytophthora infestans;* PAL = phenylalanine ammonia-lyase (EC 4.3.1.5)

sporum f. sp. *dianthi* race 2, and 'Corrida', 'Mosè', 'S. Giorgio', susceptible to it (Garibaldi 1983). *F. oxysporum* f. sp. *dianthi* race 2 was obtained from A. Garibaldi, Institute of Plant Pathology, Turin, Italy; *Phytophthora infestans* from F. Saccardo, ENEA, Rome, Italy.

Carnation tissue cultures

Calluses of Carnation were established as described in a previous paper (Gimelli et al. 1984) from petals grown on a Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 1 p.p.m. 2,4-D and 0.1 p.p.m. BAP. Cultures were routinely transferred every month and grown in the dark at 24 ± 1 °C.

Callus growth on toxic medium

F. oxysporum f. sp. *dianthi* race 2 was surface cultured in 100 ml Czapek Dox Broth Difco in 500 cc conical flasks at 24 °C for 21 days. The liquid medium was centrifuged at 20,000 × g for 20 min and the supernatant filtered on a 0.45 μm Millipore membrane. Toxic media were prepared by adding fungal culture filtrates at different concentrations to autoclaved basal medium.

0.2–0.4 g callus pieces of the different cultivars were sown on toxic and control media, allowed to grow for 27 days and then weighed.

Preparation of fungus cell wall components

F. oxysporum f. sp. *dianthi* race 2 (FuCWC) and *P. infestans* (PhCWC) cell wall components were isolated according to the modified method of Garas et al. (1979). Ten g of mycelia, prepared as described above, were powdered at −80 °C in a mortar, blended with 50 ml of 0.05 M acetate buffer, pH 4.5, sonicated three times at 4 °C in a sonicator (Branson Sonifier B-12) at 150 W for 10 min and centrifuged at 20,000 × g for 30 min at 4 °C. The pellet was resuspended in 0.05 M borate buffer, pH 8.8, homogenized in a potter and autoclaved for 10 min. The autoclaved suspension was cooled in an ice bath and centrifuged at 15,000 × g for 30 min at 4 °C. The supernatant was filtered through a 0.45 μm Millipore membrane and assayed for eliciting activity; elicitor concentration was expressed as glucose equivalents measured with the phenol-sulfuric acid method (Hodge and Hofreiter 1962).

Extraction and bioassay of antimicrobial compounds

Seven–ten day-old callus pieces (about 1 g fresh weight) were treated with 100 μl of each of the two fungal elicitors (glucose equivalents 0.7 mg/ml) or with different $HgCl_2$ concentrations. Control callus was treated with distilled water in the same manner. Cultures were then incubated in a growth chamber at 24 °C in the dark and after seven days lyophilized. 0.5 g of lyophilized treated and control callus were extracted separately in 50 ml chloroform : methanol (2 : 1) in 200 cc conical flasks at 30 °C for 48 h with continuous shaking. The extracts were filtered and taken to dryness under reduced pressure at 40 °C with a rotary evaporator. The dry materials were resuspended separately in ethanol (0.1 g lyophilized callus/1 ml ethanol) and their inhibition of *F. oxysporum* f. sp. *dianthi* race 2 germ tube length assayed. For this purpose incubation suspensions on hanging drop glass slides contained 170 μl of Czapek Dox broth, 5 μl of conidia suspension at $2 \cdot 10^5$ spores · ml^{-1} and 10 μl of ethanolic extracts. Slides were incubated in the dark for 12 to 18 h at 24 °C, and then germ tube length was determined.

Determination of phenylalanine ammonia lyase activity

PAL activity was measured as nmoles trans-cinnamic acid $\times min^{-1} \times mg^{-1} \times 10^2$ formed in an incubation mixture using L-phenylalanine as substrate. PAl was extracted at various times after treatment with elicitors by grinding 1 g of callus in a mortar with quartz sand in 2 ml of borate buffer 0.1 M, pH 8.8, centrifuged for 10 min at 15,000 × g at 4 °C and then desalted on Pharmacia columns PD-10. Protein concentration was measured according to the method of Lowry et al. (1951). The incubation mixture contained 0.8 ml of desalted enzyme solution, 0.16 ml of borate buffer 0.2 M, pH 8.8, 0.24 ml of L-phenylalanine, 0.1 M in borate buffer 0.2 M, pH 8.8. The control did not contain L-phenylalanine. The absorbance of the reaction products was assayed at 290 nm.

Results

Growth in the presence of Fusarium culture filtrates

Table 1 shows the increase in fresh weight (in % initial weight) of calluses of susceptible and resistant cultivars grown on media containing varying concentrations of *F. oxysporum* f. sp. *dianthi* race 2 culture filtrate. Callus weight losses can be found when death occurs. It can be seen that susceptible cultivars ('Corrida', 'Mosè', 'S. Giorgio') and the resistant 'Duca' all die at concentrations higher than 20% while 'Mei-Ling', 'Pulcino' and

Table 1. Callus fresh weight incrase (in % of initial weight) after 27 days of culture on media containing different *F. oxysporum* f. sp. *dianthi* race 2 culture filtrate concentrations

	Concentration of culture filtrates					
Carnation cvs.	Control	10%	20%	30%	40%	50%
'Pulcino'	+338.5	+182.7	+190.9	+45.8	−46.1	−13.8
'Mei-Ling'	+150.0	+ 52.2	+ 33.3	+13.3	−12.5	−31.5
'Niki'	+ 76.5	+ 56.0	+ 44.0	+61.5	0	−37.5
'Duca'	+ 63.9	+ 34.8	− 15.8	− 4.0	−26.0	− 6.9
'S. Giorgio'	+128.6	+131.8	+ 13.8	−20.0	−36.4	−51.8
'Corrida'	+325.0	+345.0	+160.0	− 0.3	− 3.6	−16.7
'Mosè'	+165.5	+160.0	+125.0	− 9.1	− 4.5	− 9.3

'Niki' still grow in the presence of 30% culture filtrate. The last cultivar, moreover, is only slightly inhibited at this concentration and, although not growing, is still alive after a month of permanence on a medium containing 40% toxic filtrate.

Phytoalexin production after treatment with fungal elicitors

Treatment with FuCWC induced browning of callus tissue in some cases (Fig. 1); this effect was particularly evident in the case of 'Mei-Ling', 'Pulcino' and 'Duca'.

Table 2 shows the production of antimicrobial compounds of calluses from susceptible and resistant cultivars treated with elicitors from *F. oxysporum* f. sp. *dianthi* race 2 and *P. infestans,* a fungus non-pathogenic for carnation plants. As mentioned in the "Materials and methods", phytoalexin accumulation was measured as % inhibition of germ tubes of *Fusarium* germinating conidia. As shown by the table, extracts of callus treated with FuCWC induce significant inhibition only in the cases of 'Pulcino', 'Mei-Ling', 'Duca'. No inhibitory effect can be observed in the cases of 'Corrida', 'S. Giorgio', 'Mosè' and 'Niki'. Callus treatments with the elicitor from the non-pathogenic *P. infestans* induces phytoalexin production only in the case of 'Mei-Ling', the effect being just above the significance limits in 'Niki'.

Phenylalanine ammonia-lyase induction by fungal elicitors

PAL is known to be a key enzyme in the biosynthesis of phenolic compounds with antimicrobial activity. Moreover, its activity has been shown to be increased by treatment with biotic and abiotic elicitors (Ebel et al. 1976; Kuhn et al. 1984; Lawton et al. 1983; Loschke et al. 1981).

Hence, in the hypothesis of the induction by fungal elicitors of all enzymes involved in the synthesis of phytoalexins, PAL activity should be more easily induced in tissues of those cultivars which have been shown to produce high amounts of antimicrobial compounds.

Table 3 shows PAL activities measured in callus extracts at different times after treatment with elicitors from pathogenic (*Fusarium*) and non-pathogenic (*Phytophthora*) fungi. Increases in enzyme activity were found in all combinations tested. However, *P. infestans* cell wall components had a low effect of the same intensity on all cultivars and PAL activities in this case were at the same levels at all times after treatment with the only notable exception of 'Mei-Ling' at 44 h. FuCWC behaved as PhCWC in the cases of 'Corrida' and 'S. Giorgio', induced slight increases in 'Niki' and 'Mosè' and a very high PAL activity in 'Pulcino', 'Mei-

Table 2. Inhibition of *F. oxysporum* f. sp. *dianthi* race 2 germinating conidia by ethanolic extracts of callus treated for 7 days with *Fusarium* (FuCWC) or *Phytophthora* (PhCWC) cell wall components. Each value is the mean of at least 300 conidia germ tubes in two experiments

Carnation cvs.	Treatment	Germ tube length ± SE (μm)	Inhibition (% of control)
Pulcino	Control	110.4±6.1	0
	FuCWC	60.2±3.8***	45.5
	PhCWC	124.1±5.6	−12.4
Mei-Ling	Control	185.0±6.6	0
	FuCWC	139.1±7.0***	24.8
	PhCWC	96.3±8.9***	47.9
Niki	Control	77.5±4.7	0
	FuCWC	68.6±5.2	11.5
	PhCWC	59.2±3.8*	23.6
Duca	Control	122.2±6.6	0
	FuCWC	96.3±5.2*	21.2
	PhCWC	126.9±4.7	− 3.8
S. Giorgio	Control	32.4±3.3	0
	FuCWC	54.5±3.3	−68.2
	PhCWC	52.2±3.3	−61.1
Corrida	Control	136.8±8.0	0
	FuCWC	136.8±7.6	0
	PhCWC	131.6±7.5	3.8
Mosè	Control	80.8±5.6	0
	FuCWC	68.6±3.8	15.1
	PhCWC	71.9±4.7	11.0

* Significant at 5%; *** Significant at 1%

Table 3. PAL activity (× fold of control) after different times from treatment with 1) elicitor from *P. infestans;* 2) elicitor from *F. oxysporum* f. sp. *dianthi* race 2

Carnation cvs.		Time (h) after treatment				
		12	24	44	90	160
'Pulcino'	1)	1.94	1.22	3.17	1.84	1.41
	2)	11.89	12.30	14.76	8.80	3.56
'Mei-Liing'	1)	4.77	3.49	11.06	2.48	1.00
	2)	11.13	13.72	10.24	4.74	6.39
'Niki'	1)	2.43	1.46	1.80	0.92	1.21
	2)	2.62	5.01	3.35	1.55	1.74
'Duca'	1)	1.50	2.85	1.64	1.56	1.15
	2)	5.59	14.59	8.71	–	6.82
'S. Giorgio'	1)	1.74	3.10	1.79	2.42	0.51
	2)	3.75	4.17	2.52	4.28	2.42
'Corrida'	1)	–	2.29	2.24	0.67	0.96
	2)	1.66	1.89	3.38	1.68	2.80
'Mosè'	1)	3.09	3.40	1.54	0.96	1.22
	2)	7.31	7.32	7.84	6.81	6.47

– = not determined

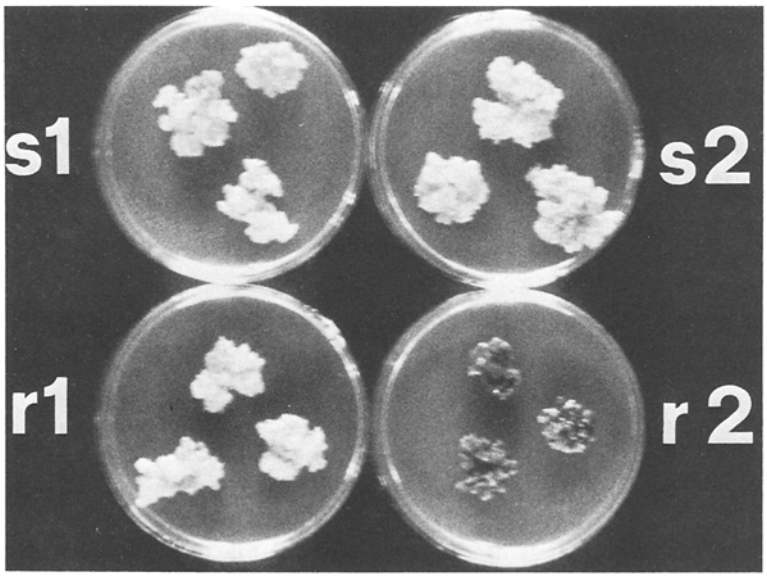

Fig. 1. Differential browning of carnation callus from a susceptible ('Corrida') (*s*) and a resistant ('Mei-Ling') (*r*) cultivar following treatment with FuCWC. *1* = control; *2* = treated

Table 4. Induction of browning and PAL activity in carnation (cv. 'Mei-Ling') callus by total FuCWC and fractions of different molecular weight

Fractions M.W.	Glucose equiv. (mg/ml)	Proteins (mg/ml)	Browning of 'Mei-Ling' callus	PAL activity (fold increase over control)
> 300,000	0.29	0.62	+ + + + +	7.39
10,000–300,000	0	0.02	±	1.49
1,000– 10,000	0	0.08	±	3.51
< 1,000	0.06	0.16	±	1.69
Total FuCWC	0.55	1.32	+ + + + +	8.00

± = no browning; + + + + + = high browning

Ling' and 'Duca'. Moreover, in this case a peak of enzyme activity was found, in the most "inducible" genotypes, between 24 and 44 h after treatment, further suggesting a specific and fast response of tissues from phytoalexin producing cultivars.

Partial characterization of eliciting substances

With the aim of obtaining a partial characterization of the "eliciting cell wall components" of *F. oxysporum* f. sp. *dianthi* race 2, the crude elicitor was fractioned on Amicon Diaflo ultrafiltration membranes with different cut-offs (XM 300, YM 10 and YM 2) and the fractions were tested for induction of browning and PAL activation. The parameters tested (Table 4) showed an activity comparable to that of total extracts in the higher molecular weight fraction (> 300,000 daltons).

Induction of PAL and phytoalexin synthesis by mercuric chloride

In the experiments just reported, genetic differences have been found in the response to *Fusarium* elicitor. These differences may be due to differential susceptibility to induction either of the genes coding for key enzymes such as PAL, or for proteins somewhere upstream in the chain of events starting from fungus-plant recognition and leading to the hypersensitivity reaction. If gene(s) coding for PAL are different in this regard in different cultivars a similar response of "inducible" genotypes is to be expected independently of the eliciting substance used. The lack of specificity of PAL induction by PhCWC already suggests that this was not the case. As a further test of the hypothesis, however, callus from a resistant cultivar ('Mei-Ling') and a susceptible one ('Corrida') were treated with varying concentrations of $HgCl_2$, a compound which is known to stimulate phytoalexin production (Moesta and Grisebach 1980; Yoshikawa 1978). The results are reported in Tables 5 and 6. $HgCl_2$ treatments induce increases in PAL activity in both Carnation cultivars. However, no significant difference between the two genotypes is evident in this case as far as phytoalexin production is concerned.

Table 5. PAL actiivity (× fold of control) following $HgCl_2$ treatment. a) 24 h after treatment; b) 65 h after treatment

Carnation cvs.	Treatment			
	Control		$HgCl_2$ 0.5 mM	$HgCl_2$ 5.0 mM
'Mei-Ling'	1.00	a)	2.11	1.11
		b)	2.97	3.81
'Corrida'	1.00	a)	0.94	4.40
		b)	2.07	3.67

Table 6. Germ tube mean (± s.e.) (μm) of *Fusarium* germinating conidia in presence of ethanolic extracts of callus treated for 7 days with various concentrations of $HgCl_2$. Each value is the mean of at least 300 conidia germ tubes in two experiments

Carnation cvs.	Treatment		
	Control	$HgCl_2$ 0.5 mM	$HgCl_2$ 5.0 mM
'Mei-Ling'	54.0±4.7	35.2±2.3**	35.7±3.3*
'Corrida'	53.1±3.8	31.5±2.3***	30.5±1.9***

* Significant at 5%; ** Significant at 1%; *** Significant at 1%

Discussion

Significant correlations between in vivo resistance to pathogens and phytoalexin production following elicitor treatments have been found in several cases (see Keen 1982 for a review). As far as toxin resistance is concerned, although data are lacking on culture filtrate tolerance of existing resistant and susceptible cultivars in several instances resistant plants and lines have been selected on the basis of toxin tolerance in vitro (Buiatti 1983; Hartman et al. 1984; Thanutong et al. 1983). In our experiments, carnation cultivars showing varying degrees of resistance to *F. oxysporum* f. sp. *dianthi* race 2 either tolerated higher culture filtrate concentrations ('Niki') or were strong phytoalexin producers ('Duca') or showed both responses ('Mei-Ling', 'Pulcino'). On the other hand, susceptible genotypes ('Corrida', 'Mosè', 'S. Giorgio') were poor in all respects. In addition, 'Mosè', which showed a relatively high PAL induction, did not produce significant amounts of phytoalexin. Induction of phytoalexin seemed to be rather specific in our case, but the specificity of *Fusarium* elicitor-plant cell interactions was more evident when data concerning PAL induction were taken into account. In this case, 'Mei-Ling', 'Pulcino' and 'Duca' all showed highly specific induction by *Fusarium* cell wall components both when enzyme activities and induction time-course were considered. The correlation between PAL induction and phytoalexin production was also rather strict. Cell wall components from non-pathogenic fungus and anabiotic elicitor also induced both PAL and, in some cases, phytoalexins. However, their behaviour was the same in all cases and the level of induction rather low. This is confirmed by results previously reported on PAL induction by blue light showing a slight not significant effect both in 'Corrida' and 'Pulcino' (Scala et al. 1983).

In our experiments the differential response to specific (*Fusarium*) and aspecific (*Phytophthora*) elicitors, in agreement with the results of Yoshikawa (1978) and Kuhn et al. (1984) and at variance with those of Moesta and Grisebach (1980), suggest that genetic differences may reside in genes coding for proteins involved in the fungus-plant interaction upstream of PAL synthesis. Cell membrane protein components certainly are a good candidate for this function (Keen 1982).

Finally, our data, showing a strict correlation between in vivo and in vitro behaviour, support the use of plant tissue cultures as a tool in breeding for resistance. This may be done both with positive selection for toxin resistance and through the use of negative selection (Chaleff 1981) for high phytoalexin producers. It should be noted in this respect that this could be an entirely new research avenue as no such mutant selected in culture has so far been reported.

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