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Avirulent strain of *Colletotrichum* induces a systemic resistance in strawberry

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Abstract Strawberry plants exposed to an avirulent isolate of *Colletotrichum fragariae* acquired strong resistance against a virulent strain of *C. acutatum*. Biochemical, morphological and molecular markers indicated that the strong defence response was associated with an oxidative burst and a transient accumulation of salicylic acid (SA). A maximum accumulation of H_2O_2 and O_2^- was observed 8 h after inoculation (hai), callose was detected 48 hai, and a peak of SA was observed 48 hai. Biochemical and phytopathogenic analyses carried out in non-treated tissues revealed that the defence response was systemic and remained fully active 60 days after the first inoculation. Experiments also showed that the resistance acquired by mother plants after the inoculation with the avirulent isolate could be passed to daughter plants through runners. Further characterization of the induced systemic resistance showed that the resistance was not only effective against a virulent strain of *C. acutatum* but also against *Botrytis cinerea*.

Keywords Anthracnose · *Colletotrichum acutatum* · *Colletotrichum fragariae* · Defence · *Fragaria* × *ananassa* · Oxidative burst · Systemic acquired resistance

Abbreviations

dai	Days after inoculation
DSR	Disease severity rating
hai	Hours after inoculation
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance

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Introduction

It is well documented that some plants previously infected with a pathogen become resistant to further infection, suggesting that plants can activate an efficient defence mechanism. Induced resistance to viruses in virus-infected plants has earlier been observed in (Ross 1961a, b; Kuc 1982), and plant protection mediated by attenuated viruses was successfully used to control disease in several agricultural systems making plants more

resistant to subsequent pathogen attacks (Fulton 1986; Kuc 2001). Similar phenomena were also observed in other plant species involving bacteria and fungi, although associated to different molecular mechanisms (Ryals et al. 1994; 1996; Kuc 2001; Métraux 2001; Shoresh et al. 2005). The defensive responses induced in these cases have received different names (e.g. SAR, as for systemic acquired resistance or ISR, as for induced systemic resistance) depending mainly on the microorganism involved (Métraux 2001). The role and participation of phytohormones such as salicylic, and jasmonic acids, and ethylene in these defence mechanisms have been demonstrated and extensively discussed by Métraux (2001). Induction of defence responses mediated by avirulent pathogenic fungi has also been described. Kuc and Richmond (1977) reported a plant protection effect in cucumber mediated by *Colletotrichum lagenarium*, and Manandhar et al. (1998) reported suppression of rice blast when rice was pre-inoculated with a non-rice pathogen, *Bipolaris sorokiniana*, or an avirulent rice pathogen, *Pyricularia oryzae*. Shishido et al. (2005) used the strain Fo-B2 of *Fusarium oxysporum* as a biological control agent against the disease Fusarium wilt on tomato, and Shetty et al. (2003) induced resistance on wheat with a non-pathogenic strain of *Septoria tritici*. In the latter case, a positive correlation between the increment of hydrogen peroxide and the restriction of the pathogen growth was observed (Shetty et al. 2003).

Anthracnose of strawberry (*Fragaria* × *ananassa* Duch.), also called blackspot, or crown rot, is one of the diseases that affect this crop most negatively (Freeman and Katan 1997). Three species of *Colletotrichum* are known to cause anthracnose in strawberry: *C. acutatum* J.H. Simmonds, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz. and Sacc. in Penz. (teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) (Smith 1986; Smith and Black 1990; Howard et al. 1992; Adaskaveg and Hartin 1997; Nam et al. 2006). *C. dematium* (Pers.) Grove (Beraha and Wright 1973) and species of *Gloeosporium* were also initially included as anthracnose agents, but they were later determined to be *C. acutatum* (Howard et al. 1992). These pathogens can attack crowns, leaves (petioles and leaflets), peduncles, pedicels, fruits, flowers, buds, runners and roots (Howard et al. 1992; Freeman and Katan 1997).

We reported earlier that an avirulent isolate of *C. fragariae* was able to induce a defence response against anthracnose caused by a virulent isolate of *C. acutatum* in strawberry plants, and that the defence response required at least 96 h between the first inoculation with the avirulent isolate and the virulent pathogen to allow the plants to acquire a full protection (Salazar et al. 2007).

However, since the treatments were performed on the whole plant, no information about the nature of the response elicited was provided.

Hence we were interested to test the hypothesis that the defence response induced by the avirulent isolate F7 of *C. fragariae* was of the type SAR (systemic acquired resistance). The latter was carried out by investigating whether the defence response fulfilled the conditions required to be considered of the type SAR, namely that: i) it should induce a systemic resistance, ii) the acquired resistance should be persistent over time, and iii) the plant should be protected against other pathogens (Ross 1961a, b).

In this work we wanted to present experimental evidence that plants of strawberry pre-inoculated with an avirulent isolate of *C. fragariae* (F7) induced a fast localized defence response that became systemic after 96 h of the initial inoculation.

Materials and methods

Fungal cultures

Isolates of *C. acutatum* (M11), *C. fragariae* (F7) and *Botrytis cinerea* (B1) used in this work correspond to local isolates characterized in our laboratory, and were obtained from infected plants of the cultivars Pájaro (*C. acutatum* and *B. cinerea*) and Chandler (*C. fragariae*). Isolates were single-spore propagated to obtain pure cultures on PDA medium (potato-dextrose-agar) supplemented with streptomycin (300 μg ml^{-1}) and maintained in PDA slants at 4 °C. Liquid cultures were carried out in PD (potato dextrose) medium.

Inoculum and inoculation

Fungal isolates were grown on PDA for 10 days under continuous fluorescent light (150 $\mu molm^{-2}s^{-1}$) at 28°C to induce conidia formation (Smith and Black 1990). The culture surface was gently scraped with a Pasteur pipette to remove conidia and suspended in distilled sterile water. The conidial suspensions obtained were

filtered through sterile gauze to remove mycelial debris under axenic conditions. Suspensions of both *Colletotrichum* species were then diluted with sterile distilled water (containing two drops of Tween 20 per liter) to a final concentration of 1.5×10^6 conidia ml^{-1} and applied to plants as a spray to run-off (Smith and Black 1990). When the isolate B1 of *B. cinerea* was used for infection experiments, conidia were suspended in melted (45 °C) PDA medium (1.5 % agar) at a final concentration of 1.5×10^6 conidia ml^{-1}, and the inoculum consisted of a 0.5 cm diameter disk obtained from plates prepared with the conidial suspension. The inoculation was performed by depositing a disk containing the conidial suspension on the adaxial side of the three leaflets. Immediately after inoculation, plants were placed for 48 h in infection chambers at 100 % relative humidity (RH); but whereas plants infected with *C. acutatum* were maintained in the dark at 28 °C, plants infected with *B. cinerea* were maintained under continuous white fluorescent light (50 $\mu molm^{-2}s^{-1}$) at 20°C. Then all plants were returned to a growth chamber at 70 % RH, 28 °C and a light (white fluorescent, 350 $\mu molm^{-2}s^{-1}$) cycle of 16 $hday^{-1}$.

Planting material

Plants of strawberry cv. Pájaro were used in the experiments. Plants were obtained from the strawberry BGA (Banco de Germoplasma Activo at University of Tucumán, Tucumán, Argentina), planted in 8-cm pots with sterilized (1 h at 120 °C) substrate (humus:perlite, 2:1), and maintained in a greenhouse for 6 weeks or more to ensure that they were free of anthracnose. Runners of plants that did not show evidence of disease development were used for in vitro multiplication. Healthy in vitro multiplied plants rooted in sterile humus:perlite substrate (2:1), and containing three or four young leaves were used for the experiments (Salazar et al. 2007). Plantlets were grown from 14 to 16 weeks, watered every other day with 50 ml of distilled water and maintained in growth cabinets at 28°C, 70 % RH and a light cycle of 16 $hday^{-1}$ (350 $\mu molm^{-2}s^{-1}$).

Pathogenicity tests

Susceptibility was evaluated by spraying four plants of cv. Pájaro to run-off with a conidial suspension of the isolates F7 or M11. Control experiments consisted of water treated plants. Plants were placed in the infection chamber for 48 h and then transferred to a growth cabinet. Disease Severity Rating (DSR) was assessed according to Delp and Milholland (1980) using the following scale: 1, healthy petiole without lesions; 2, petiole with lesions <3 mm; 3, petiole with lesions from 3 to 10 mm; 4, petiole with lesions from 10 to 20 mm and girdling of petiole; 5 entirely necrotic petiole and dead plant (Delp and Milholland 1980). For the evaluation of DSR the experimental design was randomized with eight plants for each experimental unit; four plants were used for the inoculation and four were not inoculated (control). The DSR was evaluated 50 dai (days after inoculation). When the virulent isolate B1 of *B. cinerea* was used, the pathogenicity test consisted on the evaluation of the necrotic lesion extension according to Vellicce et al. (2006) using the following scale: 1, healthy leaflet without lesion; 2, leaflet with a necrotic lesion of <3 mm diameter; 3, leaflet with a necrotic lesion from 3 to 5 mm; 4, leaflet with a necrotic lesion from 5 to 10 mm and 5, leaflet with a necrotic lesion of more than 10 mm diameter or entirely necrotized; in this case the DSR was evaluated 10 dai. In this case the pathogenicity test was carried out with eight plants for each experimental unit (four inoculated and four non-inoculated) as mentioned above. Pathogenicity tests were repeated three times. At the end of experiments, fungi were isolated from crowns and leaves of plants that developed the diseases and compared with the species used in the inoculation to check the identity of pathogens. Identification was carried out by microscopic morphological characterization and molecular (PCR) analysis.

Induced systemic resistance

Experiments were carried out following the procedure described above except that plants received a double inoculation, first with the avirulent isolate F7 of *C. fragariae* and then, with the virulent isolate M11 of *C. acutatum* (1.5×10^6 conidia ml^{-1}) in the cases of resistance persistence and resistance translocation experiments, or with the isolate B1 of *B. cinerea*. In experiments conducted to evaluate the systemic character of the defence response, the first inoculation was carried out with a suspension (1.5×10^6 conidia ml^{-1}) of the isolate F7 on a single isolated leaf (to run-off), and after 96 h the whole canopy was inoculated by spraying

(to run-off) with the virulent isolate M11 (1.5×10^6 conidia ml^{-1}). To avoid the unintentional inoculation of the rest of the canopy the leaf was isolated from the rest of the plant with a plastic screen. The DSR evaluation was carried out in a randomized experiment with eight plants for each experimental unit; four that were inoculated on a single leaf with F7 and four non-inoculated control plants. Mean DSR values were evaluated from three independent experiments.

In experiments conducted to evaluate the persistence of resistance, the first inoculation was carried out with a suspension (1.5×10^6 conidia ml^{-1}) of the isolate F7 by spraying the whole canopy to run-off, and then at different times (i.e. at 20, 40, 60, 80, 100 and 120 days after the first inoculation) four plants were inoculated at each time with a conidial suspension (1.5×10^6 conidia ml^{-1}) of the virulent isolate M11 of *C. acutatum*.

In experiments aimed to evaluate the translocation of the systemic signal from mother to daughter plants, a "three-plant" system was prepared before inoculating. Each experimental unit consisted of three pots containing separately the mother, the daughter, and the granddaughter plants connected by stolons. Mother plants (the whole canopy) were inoculated (to runoff) with F7 (1.5×10^6 conidia ml^{-1}). After 7 or 21 days the whole canopy of mother, daughter, and granddaughter plants were inoculated by spraying to run-off with a conidial suspension (1.5×10^6 conidia ml^{-1}) of the isolate M11. To avoid the unintentional inoculation of runners, daughter, and granddaughter plants with F7 the mother plants were isolated with a plastic screen. DSRs of mothers and daughters inoculated with M11 were evaluated with eight "three-plants" system per experiment; four were inoculated with F7 and four were not. Mean DSR values were evaluated from three independent experiments. In all cases, after each inoculation, plants were introduced into the infection chamber for 48 h and then moved back to the growth chamber as mentioned above.

Oxidative burst

Hydrogen peroxide was detected according to Thordal-Christensen et al. (1997) using 3,3′-diaminobenzidine (DAB), and superoxide was detected according to Doke (1983) using a superoxide-dependent reduction of nitroblue tetrazolium (NBT). Analyses were performed on leaves of plants that were treated by spraying a single isolated leaf (the three leaflets) with a conidial suspension of the isolate F7 (1.5×10^6 conidia ml^{-1}) or water (as control). At different times from 0 to 96 h after inoculation (hai) ten inoculated (proximal) and ten non-inoculated (distal) leaflets of equally treated plants were excised and analyzed for H_2O_2 and O_2^-. Microscopic observations and photos were obtained with a light microscope (model BXS1, Olympus, Hamburg, Germany).

Callose analysis

Callose deposition was visualized according to Hauck et al. (2003). Plants were treated as for the oxidative burst analysis except that inoculated and non-inoculated leaves were collected 4 or 7 dai. Ten leaflets per treatment and time-point were cleared overnight and dehydrated with 100 % ethanol. Cleared tissues were then transferred to 67 mM K_2HPO_4 (pH 12), and then stained for 1 h at room temperature in 0.01 % aniline blue in 67 mM K_2HPO_4 (pH 12). Stained material was mounted in 30 % glycerol and examined using ultraviolet epifluorescence (microscope model BXS1 equipped with U-LH 100HG reflected fluorescence, Olympus, Hamburg, Germany)

Salicylic acid determination

Salicylic acid (SA) was obtained from phloematic fluid of leaves according to Richardson et al. (1982) and modified in our laboratory. A single leaf (the three leaflets of the youngest recently expanded leaf) or the whole canopy of strawberry plants were sprayed to run-off with a conidial suspension (1.5×10^6 conidia ml^{-1}) of F7, and leaves were detached at different times (0 to 96 hai) to extract the phloematic fluid. In the case of the single-inoculated-leaf experiments, SA was determined in non-treated leaves (other leaves that were not inoculated). Control plants consisted of plants treated with water. Phloematic fluid was collected with micropipette from petioles of fully expanded leaves, pooled and poured in 1 ml of cold acidified (pH 2.5, HCl 0.5 N) ethanol 100 % to precipitate the proteins and other high molecular weight materials (Rasmussen et al. 1991). After removal of the insoluble materials by centrifugation (12,000 × *g*, 15 min) the ethanol extracts were transferred to pre-weighted eppendorf tubes and concentrated to dryness under vacuum using SpeedVac Concentrator (Model SVC 200, Savant Instruments Co., Farmingdale, New

York, USA). The dry weight of the residue was calculated and dissolved in 250 μl of 30 % methanol. SA separation was performed by HPLC (Gilson Inc., Middleton, Wisconsin, USA) using a reverse-phase column C18 (Prodigy 5 ODS-2, Phenomenex, California, USA, *CV*=3.4 ml) equilibrated in 30 % methanol. Runs were carried out with a mobile-phase flow rate of 0.5 ml/min and with a lineal elution gradient of methanol with 0.1 % TFA (0–100 % in 15 min) and then maintained 20 min at 100 %. The SA peak eluted at 100 % methanol and was detected at 280 nm. SA peak was collected and quantified as described previously (Raskin et al. 1989) by fluorometry with λ_{ex}=296 nm and λ_{em}=408 nm (PC1 Photon counting Spectrofluorometer, ISS, Owingen, Germany). Each data point reported corresponds to the average SA content determined from three leaves of five plants per treatment, and is expressed as ng of SA per mg of total dry weight of phloematic fluid per plant.

RNA isolation and evaluation of gene expression

Total RNA from leaves of strawberry inoculated with F7 or treated with distilled water (as control) was obtained 48 hai according to Iandolino et al. (2004). Retrotranscription reactions were carried out using 5 μg of DNase-treated total RNA, following SuperScript II RT manufacturer (Invitrogen). To evaluate the relative level of expression of strawberry genes a semi-quantitative RT-PCR method was used (Tian et al. 2007). The expression of *Fapr-1* (AB462752), *Faetr1* (AJ297511), *Fachi2-1* (AF147091) and *Fachi2-2* (AF320111) genes was investigated as molecular markers of the salicylic (*pr1*) and ethylene (*etr1*, *chi2-1* and *chi2-2*) pathways. The gene *Fagapdh*1 (AF421144) was used as internal control in PCR reactions. Specific primers used were: *Fapr-1* sense (5′-TGCTAATTCACATTATGGCG-3′) and antisense (5′-GTTAGAGTTGTAATTATAG TAGG-3′), *Fachi2-1* sense (5′-TCGTCACTTGCA ACTCCTAA-3′) and antisense (5′-GGACTT CTGATTTTCACAGTCT-3′), *Fachi2-2* sense (5′-CAAGTCAGATAACAATGGAGAC-3′) and antisense (5′-TTGTAACAGTCCAAGTTGTCC-3′), *Faetr1* sense (5′-CTTGTCATGGATGATAATGG-3′) and antisense (5′-ACACTCCTCATTTTATCAAC-3′), and *gapdh*1 sense (5′-CTACAGCAACACA GAAAACAG-3′) and antisense (5′-AACTAA GTGCTAATCCAGCC-3′). PCR parameters were: initial, 7 min at 94 °C; a variable number of cycles of 45 s at 94 °C, 1 min at the annealing temperatures shown below, 1.5 min at 72 °C; and 10 min at 72 °C as a final extension. The number of cycles used for each gene was adjusted to obtain the specific band amplified at the exponential phase of the PCR reaction. Annealing temperatures for *Fapr-1*, *Fachi2-1*, *Fachi2-2*, *Faetr1* and *gapdh1* genes were 57, 55, 55, 49, and 52 °C, respectively. Amplified bands were visualized with agarose gel (2 %) ethidium bromide-stained (10 $\mu g ml^{-1}$) and photographed under 340 nm with a digital camera. Band intensity was quantified using the software Total Lab Quant (Nonlinear Dynamics Ltd., Newcastle, UK). Relative expression of genes studied was calculated as the difference between the band intensity of infected leaves and control leaves, referred to the latter. To ensure the absence of genomic DNA in each cDNA sample; *gapdh1* primer sequences were designed to enclose an intronic region. Plants treated with distilled water were used as controls.

Statistical analyses

DSR data of repeated experiments were statistically analyzed by the variance analysis using a Mixed Proportional Odds Model (GLM), and the software InfoStat ver. 2012 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Differences between means were evaluated by LSD Fisher test (P value≤ 0.05). To model the longitudinal data, the structure of the residual covariance matrix and the heteroscedasticity in time were considered.

Penalized likelihood (AIC and BIC) was analyzed to choose the best criterion that described the data.

Results

Phytopathogenicity tests

Strawberry plants cv. Pájaro inoculated with the virulent isolate of *C. acutatum* M11 and the avirulent isolate of *C. fragariae* F7, yielded different results depending on whether the avirulent isolate F7 was applied previously or simultaneously with the M11 (Fig. 1). When whole plants were inoculated with F7 96 h prior to the inoculation with M11, no symptoms developed (F7 + M11, *DSR*=1.2), whereas when

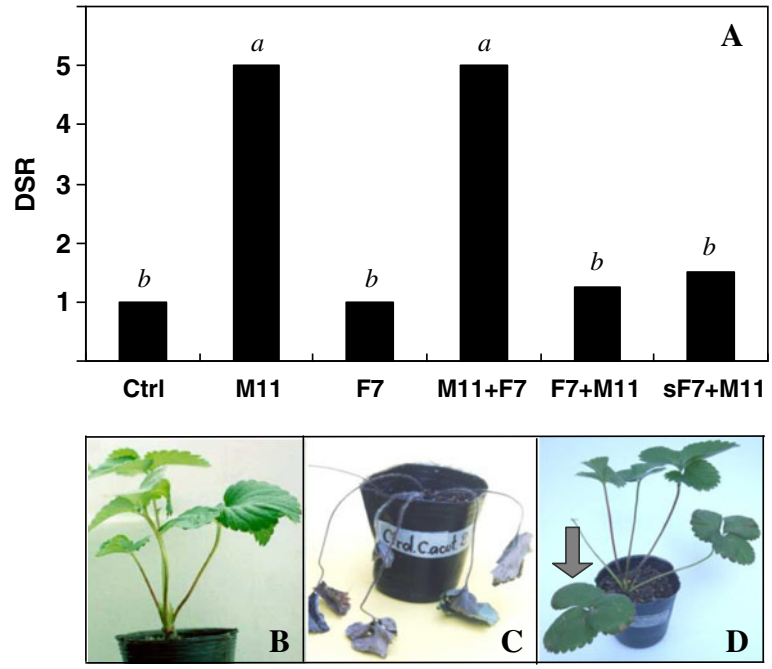

Fig. 1 Defence response against the virulent isolate M11 of *Colletotrichum acutatum* exhibited by the strawberry cv. Pájaro when the whole plant or a single leaf (all leaflets) were pre-inoculated with the avirulent isolate F7 of *Colletotrichum fragariae*. **a** Disease severity rating (DSR) of strawberry plants when the whole plant was treated with water (Ctrl), only with M11 (M11), only with F7 (F7), with a mixture of M11 and F7 (M11 + F7), with F7 96 h prior to M11 (F7 + M11), and with F7 in a single leaf followed by treatment of the whole canopy with M11 after 96 h (sF7 + M11). **b** Aspect of a plant treated with F7 50 days after inoculation (dai). **c** Aspect of a plant inoculated with M11 or M11 + F7, 50 dai. **d** Aspect of a plant inoculated with F7 + M11 or sF7 + M11, 50 dai. The arrow indicates the leaf that received the inoculation with F7, 96 h prior to inoculation with M11. DSR are average values of three independent experiments. Experiments included four plants per treatment and were repeated three times (see Materials and Methods). *Bars* labelled with different letters indicate significant differences (LSD Fisher test, $P \leq 0.05$, $n=24$)

inoculated simultaneously, F7 could not stop the disease development (M11 + F7, *DSR*=5). Plants inoculated with F7 in a single leaflet could also resist the attack of M11 as those inoculated on the whole canopy (sF7 + M11, Fig. 1a and d). Controls performed with F7 or M11 alone either exhibited an asymptomatic growth (Ctrl and F7, Fig. 1a and b) or plant death (M11, Fig. 1a and c) 50 dai, respectively.

Defence response

With the aim to confirm that the protection effect observed when plants were inoculated in one leaf was due to the activation of a defence response, the accumulation of ROS and callose in proximal and non-treated leaf tissues was investigated in plants inoculated with the isolate F7 of *C. fragariae*. H_2O_2, O_2^- and callose accumulated not only in proximal (Fig. 2b, e and h) but also in non-treated leaves (Fig. 2c, f and i). Although H_2O_2 and O_2^- were analyzed at different times, in Fig. 2 only results obtained 8 hai in proximal and 48 hai in distal leaves are presented, because at those times a maximum accumulation of ROS was detected. On the other hand, since callose required longer time to be clearly visualized, callose was detected 4 dai with F7 in proximal leaves, and 7 dai in distal leaves. Plants treated with water (control) did not exhibit such accumulation in proximal (Fig. 2a, d and g) nor in distal leaves (not shown).

Since results obtained indicated that an SAR-like response was induced, the next step was to investigate whether SA was involved. SA accumulated with time after treatment with F7, but in different manners depending on how the treatment was performed (Fig. 3). When the whole canopy of plants were treated, SA had a clear maximum content 48 hai with F7, after which it declined to the level of untreated plants. There was a slow but continuous increase in SA until 96 hai if single leaves were treated, indicating that the

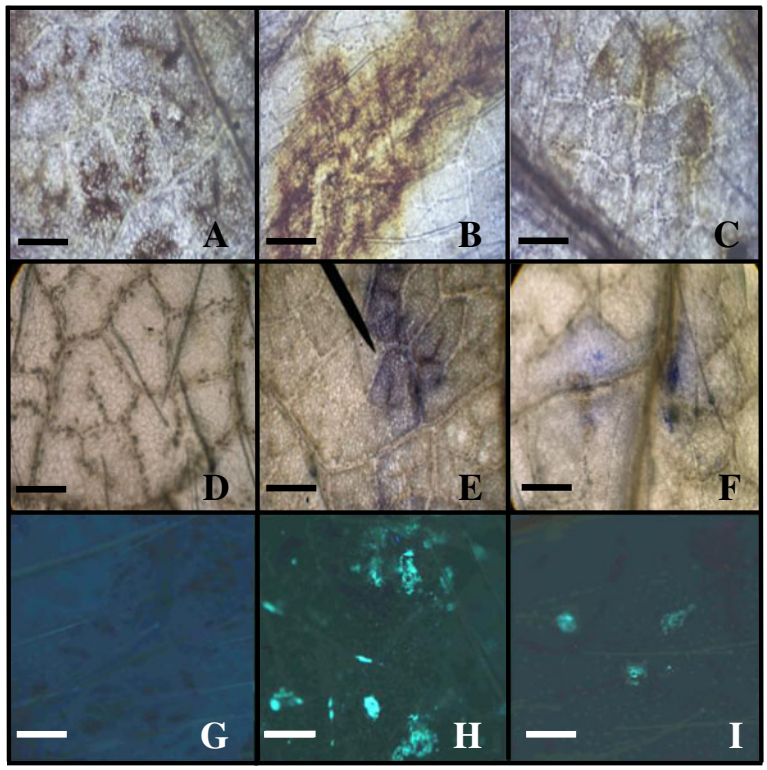

Fig. 2 Systemic defence response induced in strawberry cv. Pájaro by the avirulent isolate F7 of *Colletotrichum fragariae*. A single leaf (all leaflets) was inoculated with the isolate F7 and at different times samples were taken from treated (**b**, **e**, **h**) and untreated (**c**, **f**, **i**) leaves to analyze H_2O_2, O_2^-, and callose deposition. H_2O_2, O_2^-, and callose were visualized by DAB (**a**–**c**), NBT (**d**–**f**), and aniline blue (**g**–**i**), respectively (see Materials and Methods). **b** and **e** show the accumulation of H_2O_2 and O_2^- in treated leaves 8 h after inoculation (hai), respectively; **c** and **f** show the accumulation of H_2O_2 and O_2^- in untreated leaves 48 hai, respectively; **h** and **i** show the callose deposition in treated and untreated leaves 7 dai, respectively. **A**, **d** and **g** correspond to strawberry leaves treated with water (control). Scale bars represent 100 μm. For each treatment H_2O_2, O_2^-, and callose were analyzed in three leaves of three different equally treated plants. Micrographs correspond to one sample of each treatment

defence signal generated in a single leaf could translocate and cause the accumulation of SA in distant tissues. Non-treated plants, exhibited no change over time (Fig. 3).

Expression of *pr1, etr1, chi2-1* and *chi2-2*

Plants inoculated with F7 exhibited an up-regulation of the genes *pr1*, *chi2-1* and *chi2-2*, and a down-regulation of *Faetr1* 48 hai (Fig. 4), suggesting that F7 induced the expression of not only genes associated with the salicylic acid (*pr1*) pathway, but also to the ethylene pathway (*chi2-1*, *chi2-2*). However, the evaluation of the level of expression of *Faetr1* indicated that the ethylene receptor gene (*Faetr1*) was down-regulated with respect to control untreated plants at 48 hai (Fig. 4).

Characterization of the induced SAR

Plants previously treated with F7 kept the defence mechanisms 20 to 40 dai after which they became gradually more susceptible to M11 until 100 to 120 dai when plants became highly susceptible (Fig. 5).

The ability to transmit the resistance to clonal offspring was tested in mother plants inoculated once with the isolate F7 of *C. fragariae*. Mother plants inoculated with F7 could not transmit the resistance to daughter plants 7 dai (Fig. 6c and d), but could transfer the ability to resist the attack of M11 to the first generation of daughter plants after 21 days (Fig. 6e and f). However, evaluation of DSR in granddaughter plants showed that mother plants were unable to transmit the resistance to the second generation of plants at least up to the experimental time evaluated (Fig. 6e and f).

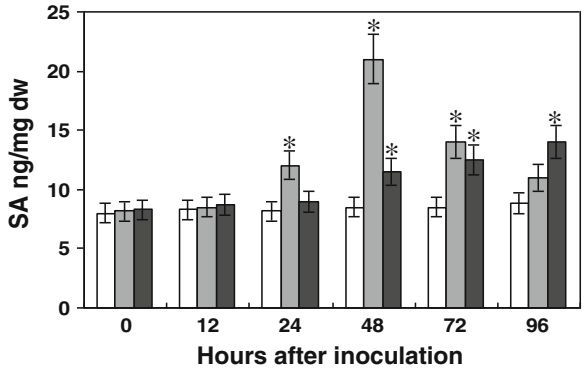

Fig. 3 Change over time in salicylic acid (SA) content in the phloematic fluid of strawberry plants of cv. Pájaro inoculated with the avirulent isolate F7 of *Colletotrichum fragariae* in the canopy (*light grey bars*), and in a single isolated fully expanded leaf (all leaflets) (*dark grey bars*). White bars correspond to water treated leaves (control). Numbers are means from three independent measurements. SA content was determined from three leaves on each of five plants per treatment and time evaluated. Error bars represent standard error of the mean. *Represent values statistically different with respect to the control for each time evaluated ($P \leq 0.05$, $n=90$)

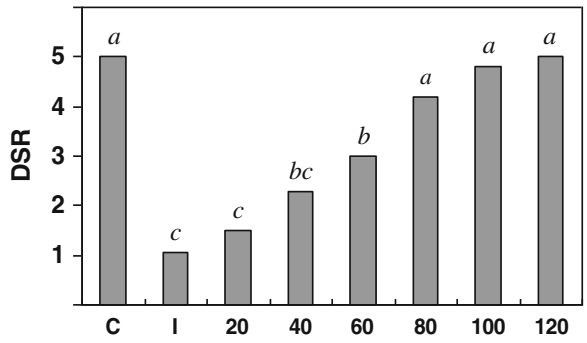

Fig. 5 Persistence of the resistance against the virulent isolate M11 of *Colletotrichum acutatum* of strawberry plants (cv. Pájaro) inoculated with F7 of *C. fragariae*. Defence response was induced by treating the whole canopy of plants with the avirulent isolate F7 only once, and then inoculated with M11, 20, 40, 60, 80, 100 or 120 days after the first treatment. C and I correspond to disease severity ratings (DSR) values of control plants treated only with M11 (compatible interaction) and F7 (incompatible interaction), respectively. Disease symptoms were evaluated 50 day after inoculation with M11. DSR are average values of three independent experiments. Experiments were carried out with four plants per treatment and were repeated three times. *Different letters* indicate significant differences (LSD Fisher test, $P \leq 0.05$, n=32)

Plants pre-treated with F7 exhibited higher tolerance to *B. cinerea* (Fig. 7b) as compared to non treated plants that showed clear symptoms of necrosis (Fig. 7c). Plants that were only inoculated with F7 displayed no symptoms (Fig. 7a).

Discussion

The results obtained clearly revealed that the isolate F7 of *C. fragariae* had the capacity to activate a defence response in the strawberry cv. Pájaro, conferring a strong resistance against the virulent isolate M11 of *C. acutatum*. The resistance was acquired not only when plants were inoculated with F7 on the whole canopy, but also when a single leaf was treated. Although the latter constituted strong evidence that a SAR may be involved, biochemical and genetic analyses were carried out to confirm this. Results showed that H_2O_2, O_2^- and callose accumulated in proximal and distal leaf tissues after the treatment but at

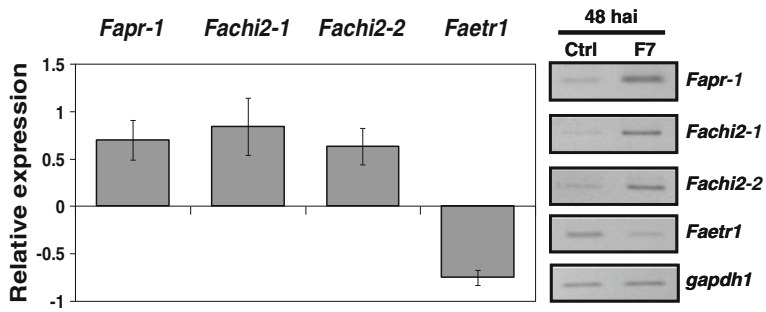

Fig. 4 Expression of the *Fapr-1*, *Fachi2-1*, *Fachi2-2*, *Faetr1* genes in strawberry cv. Pájaro leaves 48 h after inoculation with the avirulent isolate of *Colletotrichum fragariae* isolate F7 or treated with distilled water (Ctrl). Transcript levels were measured by semi-quantitative RT-PCR, visualized in agarose gel and expressed as the relative gene expression change in inoculated leaves respect to water-treated leaves. Relative expression corresponds to average values of three independent experiments. *gapdh1* was used as internal control. Primers and amplification conditions were as described in Materials and Methods. *Error bars* represent standard error of the mean

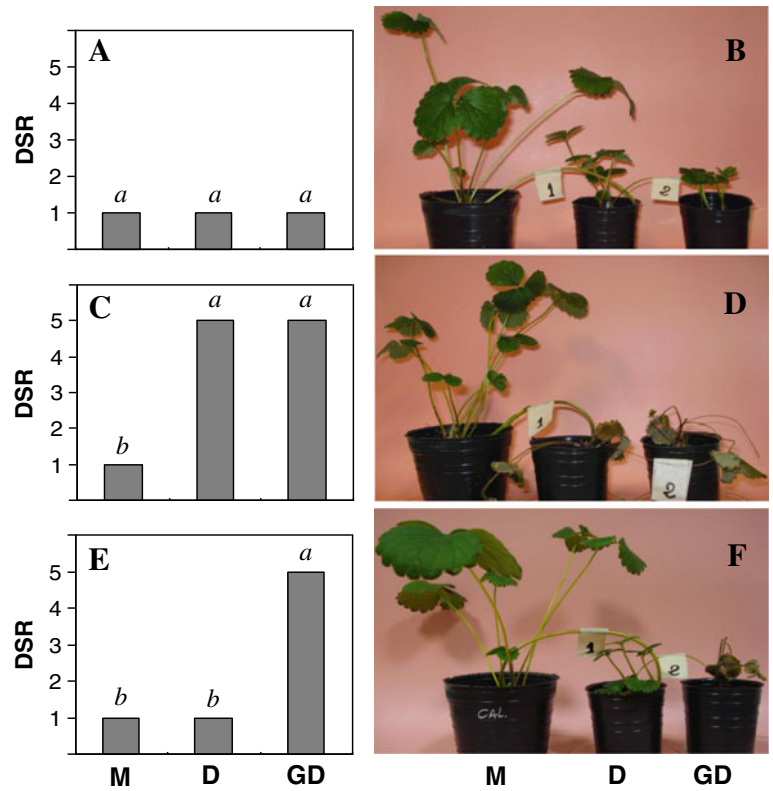

Fig. 6 Translocation from mother to daughter plants of strawberry cv. Pájaro of the defense signal induced by the avirulent isolate F7 of *Colletotrichum fragariae*. Disease severity rating (DSR) values (**a**, **c**, **e**) and symptoms observed (**b**, **d**, **f**) in mother (**m**), daughter (**d**) and granddaughter (**gd**) plants when the mother plants were inoculated with the isolate F7 and then all three plants (mothers, daughters and granddaughters) were treated with water (**a**, **b**) or inoculated with the virulent strain M11 of *C. acutatum* 7 (**c**, **d**) or 21 (**e**, **f**) days after the first inoculation with F7. Disease symptoms were evaluated 50 days after inoculation with M11. Daughter (**d**) and granddaughter (**gd**) plants were not pre-inoculated with F7. Labels attached indicate the first (1) and the second (2) runners. Experiments were carried out with four "three-plants" systems per treatment and were repeated three times. *Different letters* indicate that DSR values are statistically different (LSD Fisher test, $P \leq 0.05$, $n=36$)

different rates. Proximal tissues exhibited a faster accumulation as compared to distal tissues. These results revealed that although F7 could activate a defence response in strawberry plants, it took some time before daughter plants became resistant.

Since the induction of SAR was associated with the salicylic acid pathway and the expression of the *pr1* gene (Métraux 2001), the accumulation of SA and the expression of this gene were investigated in strawberry plants treated with the isolate F7. Also, since we had no insight whether the defence response relied on the activation of the SA or the ethylene pathways, the expression of the genes *Faetr1*, *Fachi2.1* and *Fachi2.2* were also investigated. Whereas the participation of the *etr1* gene in the ethylene pathways is well documented (Guo and Ecker 2004), the association of the *Fachi2.1* and *Fachi2.2* genes with this signalling pathway was investigated in our lab. Genes encoding a basic and an acidic chitinase were earlier reported for *Arabidopsis* by Samac et al. (1990). These authors reported that while the basic chitinase was induced by ethylene, the latter was almost insensitive. Later, Khan and Shih (2004) reported two chitinases in strawberry (*Fachi2.1* and *Fachi2.2*) that were differentially expressed depending on whether strawberry plants were inoculated with *C. acutatum* or *C. fragariae*. These antecedents stimulated us to investigate whether the strawberry chitinase genes *Fachi2.1* and *Fachi2.2* were also regulated by ethylene. Preliminary outcomes indicated that plants treated with ethylene showed a similar expression pattern of *Fachi2.1*, and *Fachi2.2* to plants inoculated with the isolate F7 (Salazar et al. unpublished data).

Results obtained showed that plants treated with F7 produced: i) the local accumulation of SA within 48

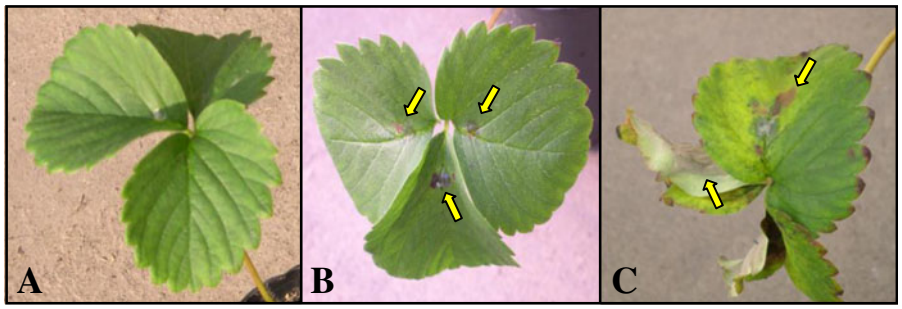

Fig. 7 Effect of the pre-inoculation with the isolate F7 of *Colletotrichum fragariae* of leaves of strawberry cv. Pájaro on the susceptibility to *Botrytis cinerea*. **a** Leaf inoculated with the avirulent isolate F7 of *C. fragariae* but not inoculated with *B. cinerea*. **b** Leaf inoculated with *B. cinerea* 72 h after a pre-inoculation with the avirulent isolate F7. **c** Leaf inoculated with *B. cinerea* but not pre-inoculated with F7. Plants were maintained under controlled conditions (20 °C, 100 % RH, 16 h light day^{-1}) and symptoms were evaluated 10 days after inoculation with *B. cinerea*. Arrows show necrotic tissues developed at the infection places. Experiments were carried out with four plants per treatment and were repeated three times. Photographs correspond to one sample of each treatment

hai, ii) a systemic accumulation of SA, and iii) the up-regulation of *Fapr1*, *Fachi2.1* and *Fachi2.2* genes. Since SAR is associated with the SA and ISR with ethylene/jasmonic acid pathways (Pieterse et al. 2009), these results let us to conclude that the defence response induced by F7 activated both signalling pathways as suggested by the genes evaluated. However, the fact that F7 exerted a down-regulation effect on the *etr1* gene encoding for one of the ethylene receptors (Guo and Ecker 2004), indicated that the ethylene pathway may not be fully activated and let us to speculate that SA exerts a differential regulatory effect over *etr1* with respect to *chi2.1* and *chi2.2* as reported by Pieterse et al. (2009).

Experiments conducted to evaluate the persistence of the acquired resistance showed that plants previously treated with F7 maintained the defence mechanism active for many days, exhibiting a slow increase of susceptibility with time and becoming very susceptible to M11 80 dai. The current experiment clearly showed that regardless of the defence mechanism activated, plants remained resistant during at least 2 months, and when plants received a new treatment after this time, they became resistant again (Salazar et al. unpublished data).

Taking into account that plants could maintain activated by the defensive response over a prolonged period of time, the ability to transfer this character to the vegetative progeny was tested. Results showed that when mother plants were inoculated with F7 only once, the resistance was transferred to the first generation of daughter plants, but it required more than 7 days. Daughter plants acquired a complete resistance (*DSR*=1) 21 days or more after the pre-treatment with F7; nevertheless the resistance signal failed to move to the second generation of plants (granddaughter), at least during the time that the experiments took place. Hence, outcomes obtained so far indicated that the signal involved in the activation of the defence response could move out of the inoculation site activating not only distal tissues of the same plant, but also daughter plants through the runners. Also, the fact that plants treated with F7 acquired enhanced tolerance to *B. cinerea* led us to conclude that the resistance achieved was not restricted to pathogens of the genus *Colleotrichum*, but to other fungal pathogen species as well.

Finally, if we take into account all results obtained, we conclude that the defence response reported in this paper fulfils all requirements necessary to consider it of the SAR type. Interestingly, although outcomes indicated that a salicylic acid mediated SAR response was activated, the expression of the genes *chi2.1* and *chi2.2* associated with the ethylene signal pathway suggested that an ISR may also be involved. This apparent contradictory result made us speculate that the type of response observed in this microbe/plant interaction is of a mixed type, or alternatively that the defence response is accomplished by activating the SA and the ethylene dependent pathways in a time dependent manner as suggested by Pieterse et al. (2009) when analyzing the cross-communication between the defence signalling pathways. Further studies are being conducted to elucidate this issue.

This finding represents the first report of an induced systemic acquired resistance described in strawberry

and let us to envision potential technological uses of the isolate F7 of *C. fragariae* or defence elicitors produced by avirulent isolates as suggested by Chalfoun et al. (2011), opening new avenues on the design of innovative and environmentally safe strategies for the biocontrol of anthracnose in strawberry or other crops (Walters et al. 2005).

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