Biological control of strawberry grey mold disease caused by Botrytis cinerea *mediated by* Colletotrichum acutatum *extracts*

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Biological control of strawberry grey mold disease caused by *Botrytis cinerea* mediated by *Colletotrichum acutatum* extracts

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Abstract Despite the negative impact on human, animal and environmental health, synthetic fungicides are the most common agrochemicals used to control *Botrytis cinerea,* the causal agent of grey mold disease. Strawberry plants are very susceptible to many pathogens, especially the necrotrophic fungus *B. cinerea.* In this work, we show that two fungal extracts obtained from a local isolate of *Colletotrichum acutatum* (M11) can protect strawberry plants against grey mold. Fungal culture filtrate (CF), and the axenic semi-purified culture filtrate (ACF) induce local and systemic acquired resistance against *B. cinerea*, and reduce fungal virulence. These results suggest that CF and ACF can be used as effective ingredients of bioproducts to control grey mold in strawberry crop. We also show that the elicitor peptide flg22 is effective to confer strawberry plants local and systemic protection against *B. cinerea* but only when applied 24 h prior to the infection.

Keywords Fungal extracts · Strawberry · *Colletotrichum acutatum* · *Botrytis cinerea* · Local acquired resistance (LAR) · Systemic acquired resistance (SAR)

Abbreviations

FEs	Fungal extracts
CF	Culture filtrate
ACF	Axenic culture filtrate
SAR	Systemic acquired resistance
LAR	Local acquired resistance
hpti	Hours prior to infection
dpi	Days post-infection
hpt	Hours post-treatment
FW	Fresh weight

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is a fine-fruit crop of high economic value worldwide, being of great importance in Tucumán (Argentina) and

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occupying a key role in the production nationwide (Kirschbaum et al. 2017). However, most strawberry cultivars are highly susceptible to many phytopathogens, including bacteria, viruses, nematodes, and especially to fungi. The necrotrophic fungus *Botrytis cinerea*, the causal agent of the grey mold disease, is one of the most harmful fungus that seems to have no host specificity (Elad et al. 2016), and is responsible of large economic losses during strawberry pre- and post-harvest (Salazar et al. 2007; Dean et al. 2012). Currently, synthetic fungicides are used to control the pathogen, even though many of them were restricted due to their negative impact on the environment, and human and animal health (United Nations Development Program, UNDP's) being included in the Fungicide Resistance Action Committee (FRAC) (Petrasch et al. 2019). This scenario urges to find phytosanitary strategies with lesser or even no impact on the environment, human and animal health. In this context, the use of biocontrol agents to protect plants against diseases through the activation of plant defenses emerges as attractive alternatives.

Elicitors like the fungal-derived protein AsES (Hael-Conrad et al. 2015, 2018) and the polymer chitosan (Mazaro et al. 2008), are examples of pure elicitors that have been well characterized, and are known to induce defense responses protecting plants against *B. cinerea*. However, the process to obtain a pure elicitor usually demands a considerable amount of effort and money. For this reason, the use of crude or semi-purified fractions of microbial extracts is gaining acceptance among the commercial distributors and users. Some successful examples are the algae extracts that proved to be effective against *B. cinerea* during strawberry post-harvest (Craigie 2011), and the fungal-derived biostimulant PSP1 that was reported to be effective against monocot and dicot crop diseases (Chalfoun et al. 2018a, b). It was also reported that a soft mechanical stimulation can also provide strawberries plants protection against *B. cinerea* (Tomas-Grau et al. 2018).

Recently we have reported that a cell extract obtained from the local isolate M11 of the pathogenic fungus *Colletotrichum acutatum* caused the suppression of the oxidative burst making strawberry plants more sensitive to that pathogen (Tomas-Grau et al. 2019). Taking into account that it has been reported that the oxidative burst sensitizes plants toward *B. cinerea* (Govrin and Levine 2000; Segal and Wilson 2017), we hypothesize that the fungal extract (FE) of the hemibiotroph *C. acutatum* M11 confers protection against the necrotrophic pathogen *B. cinerea.* In line with this hypothesis, and the necessity to find eco-friendly strategies alternative to the toxic and contaminating agrochemicals, the main objective of the study was to explore the possibility to use crude and semi-purified M11-derived FEs to induce strawberry defense responses and protect against *B. cinerea*. Here, we report that M11-derived FEs can induce in strawberry plants a local and systemic defense response, accompanied by ethylene (ET) production and the up-regulation of ET-related genes rendering plants resistant towards *B. cinerea.*

Material and methods

Plant material

Fragaria × *ananassa* Duch. cv. Pájaro plants were obtained from the BGA (Active Strawberry Germplasm) at Universidad Nacional de Tucumán (Argentina). Plants were in vitro propagated, rusticated into pots with a sterilized humus:perlite mix (2:1), and maintained under controlled conditions: 28 °C, 70% relative humidity (RH), and a L:D 16:8 photoperiod (350 µmol photons m^{-2} s^{-1}). 12–15 weeks-old plants were used in all the assays.

Fungal extracts preparation

Fungal extracts (FEs) were prepared from the culture of the local isolate M11 of *C. acutatum.* Briefly, a liquid static culture of M11 grown in potato dextrose broth (PDB) for ten days was gauze-filtrated constituting the extract CF (as for Culture Filtrate). The latter contains conidia and cell debris. CF was then filtered through 0.21 µm pore-size membrane and the Axenic Culture Filtrate obtained constituted the ACF extract. Autoclaved (121 °C, 1 atm, 20 min) samples of CF and ACF were used to test their thermal stability.

Botrytis cinerea culture and phytopathogenic assays

The strain BMM of *B. cinerea* (kindly provided by Brigitte Mauch-Mani, University of Neuchâtel,

Switzerland) was grown on PDA (potato dextrose broth with 1% agar) for two weeks at 22 °C and a L:D 16:8 photoperiod (200 µmol m^{-2} s^{-1}). Preparation of conidial suspension, and infection procedure in strawberry plants were performed as previously described (Hael-Conrad et al. 2015; Tomas-Grau et al. 2018). Strawberry plants were treated by spray to run-off with CF or ACF at the second totally expanded leaf. As controls, plants were treated with water, PDB, flg22 or with the commercial fungicide Switch® (Cprodnil 37.5% p/p and Fludoxonil 25% p/p, Syngenta, USA; 8 mg l^{-1}). Plants were then kept in trays, covered with a transparent plastic film to keep high RH (100%), and maintained at 28 °C, and a L:D 16:8 photoperiod (350 µmol photons m^{-2} s^{-1}) during 24, 48 or 120 h prior to infection (hpti, induction times). Then, a group of five plants was removed and 6 µl droplets containing *B. cinerea* conidia suspension (5×10^4 conidia ml^{-1}) were applied at the middle of the adaxial side of each pre-treated leaflets for Local Acquired Resistance (LAR) evaluation (n = 5). In another group of five plants droplets of conidial suspension were similarly applied onto the leaflets of the third totally expanded not-treated leaf for Systemic Acquired Resistance (SAR) evaluation (n = 5). Infected plants were then transferred to stress chambers for 48 h at 20–22 °C, 100% RH and darkness, to promote the infection. Thereafter, plants were moved to other chambers at 22 °C, 70% RH, and dim light (70 µmol photons m^{-2} s^{-1}). Grey mold symptoms were evaluated at 2, 5, 7, 10 or 12 days post-infection (dpi), depending on the assay. Images of infected leaves were taken and the lesion area was measured (cm^2) using the software Image J version 1.44 (NIH). Five plants were also used as controls.

Effect of *B. cinerea* growth on ACF-treated leaves

Strawberry plants were treated by spray to run-off with ACF and 24 h post-treatment (hpt) infected with *B. cinerea.* 6 µl droplets containing conidia suspension (5×10^4 conidia ml^{-1}) were applied at the middle of the adaxial side of the leaflet next to the main vein, and evaluated 12 dpi. Mycelium growth was observed on leaf surface, and in free-hand cross-sections of foliar tissue under bright field (BF) and UV light (329 nm) using a fluorescence microscope (BX51 U-LH 100HG, Olympus, Germany). Images were taken with a digital camera attached to the microscope. Observations were performed in three independent assays.

Effect of ACF on morphological and anatomical leaves changes

Leaves of ACF- or PDB-treated strawberry plants were examined 12 dpt. For microscopic observation free-hand cross-sections of the middle portion of the central leaflet were performed. Vascular bundles of central leaflets vein were observed under BF, blue light (U-MWB2) and UV light (329 nm). For the observation of collenchyma associated to vascular bundles, samples were prepared according to Tomas-Grau et al. (2019) and incubated for 2 min in an aqueous solution of Astral Blue dye 0.05%. Samples were mounted on slides with 30% glycerol and examined with a fluorescence microscope (BX51 U-LH 100HG, Olympus, Germany). 15 tissue sections obtained from three treated plants were examined (n = 45).

Effect of ACF on *B. cinerea* germination

Conidia germination suspended in water, PDB or ACF was evaluated in vitro using 96-microtiter plates. For the assays wells were prepared with 190 µl of ACF or controls (Water or PDB) + 10 µl of 1×10^6 conidia ml^{-1}. Plates were incubated at 20 °C and in darkness. Conidia germination and mycelia development were visualized 48 hpt. Photos were taken with a digital camera (DP72, Olympus, Japan) attached to an invert light microscope (IX51, Olympus, Japan). Five wells per treatment were analyzed, and three independent assays were performed (n = 5).

Ethylene measurements

Ethylene (ET) concentration was measured from the head space of vials containing a detached leaf previously treated *in planta* with ACF or PDB. Samples were taken at 12, 24 and 48 hpt and maintained in sealed vials for 24 h. 1 cm^3 of gas was taken and injected in an Agilent 6890 gas chromatographic system (Agilent Technologies, Santa Clara, California, USA) equipped with a 30 m × 0.53 mm alumina column. Injector, column, FID temperature, and carrier gas (N_2) flow rate conditions were 120, 80, 240 °C, and 0.50 ml s^{-1}, respectively. ET

quantification was carried out using a calibration curve made with standard ET of known concentration. Results were expressed as nmol ET g^{-1} leaf fresh weight (FW). Ten plants were evaluated by treatment (n = 10), and the assay was repeated three independent times.

RNA extraction and qPCR analysis

Total RNA was obtained from the youngest totally expanded leaf of strawberry plants treated with ACF, or PDB as control (n = 3). The three leaflets of each treated plant were harvested 48 hpt, pooled, immediately frozen and ground in liquid nitrogen. Total RNA was extracted from 75 mg of frozen tissue using RNAqueous-4PCR kit, according to manufacturer instructions (Ambion). Genomic DNA was eliminated with DNase I (Ambion) for 30 min at 37 °C. RNA purity and quality was analyzed spectrophotometrically at 230, 260 and 280 nm with a Beckman DU7500 spectrophotometer (Fullerton, California, USA). RNA was reverse-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer instructions. qPCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) in a 7500 Real-Time PCR System (Applied Biosystems, USA). The assay was designed with three biological replicates and two technical replicates. qPCR was used to evaluate the expression of the genes *FaPR1, FaChi2-2, FaCAT, FaPRX, FaGLS5, FaCHI23, FaOGBG5, FaERS1, FaETR1, FaERF1* and *FaPDF1.2*. PCR primer designations, corresponding gene origins, sequences, efficiencies and R^2 are shown in Supplementary Table S1. Amplification efficiencies and Ct values were determined for each gene and condition, with the slope of a linear regression model using the LinRegPCR software that takes into account fluorescence in the exponential phase of amplification of each real-time PCR reaction (Remakers et al. 2003). The expression ratio was calculated with fgStatistic software (Di Rienzo 2009) using *FaEF-1α* as reference gene (Amil-Ruiz et al. 2011), based on previously published algorithms (Pfaffl 2001). Gene expressions are reported as the ratio between treated and control plants exposed to the same conditions.

Statistical analysis

Lesion size data, satisfying conditions of normality and homoscedasticity after appropriate transformation ($\log_{10}(x + 1)$) to homogenize the variance, were analyzed by two-way ANOVA considering the treatments and induction times as factors, and the interaction between them. The means were separated at the 0.05 level of significance by a Tukey test. Means of ET production were analyzed by bilateral t-test at the 0.05 level of significance. Statistical analyses for these data were carried out using the software InfoStat version 2013 (Di Rienzo et al. 2013). For q-PCR, data analysis was carried out using the software fgStatistic (Di Rienzo 2009). All data were obtained from at least three independent assays, and expressed as mean ± SE.

Results

Fungal extracts-induced local and systemic protection against *B. cinerea*

Since the activation of the innate immunity requires time, the defense induction capacity of each FEs (e.g. CF, ACF) was evaluated when applied 24, 48 and 120 h prior to the infection (hpti) with *B. cinerea*. The two-way ANOVA showed differences between treatments and days post-infection indicating that both factors significantly affect the lesion area caused by the fungus when measured seven days post-infection (dpi) (treatments: $F_{5, 147} = 622.57$, $p < 0.0001$; induction times: $F_{2, 147} = 65.81$, $p < 0.0001$). Additionally, the interaction between them also showed a significant impact in lesion area when evaluated locally ($F_{10, 147} = 105.14$, $p < 0.0001$), and systemically ($F_{10, 151} = 48.05$, $p < 0.0001$).

When evaluating LAR, results showed that Switch® and ACF maintained the capacity to protect strawberry plants against the pathogen at 7 dpi as compared to the controls (e.g. water and PDB) at each induction time evaluated (Switch®: 0.25 cm^2 ± 0.19 at 24 hpti, 0.52 cm^2 ± 0.25 at 48 and 120 hpti; ACF: 0.02 cm^2 ± 0.01 at 24 hpti, 2.81 cm^2 ± 1.06 at 48 hpti and 2.57 cm^2 ± 0.87 at 120 hpti). Regarding CF, it improved its capacity to protect plants when applied 48 hpti (3.37 cm^2 ± 1.28) or 120 hpti (0.73 cm^2 ± 0.23) in comparison to the controls (Fig. 1a). In

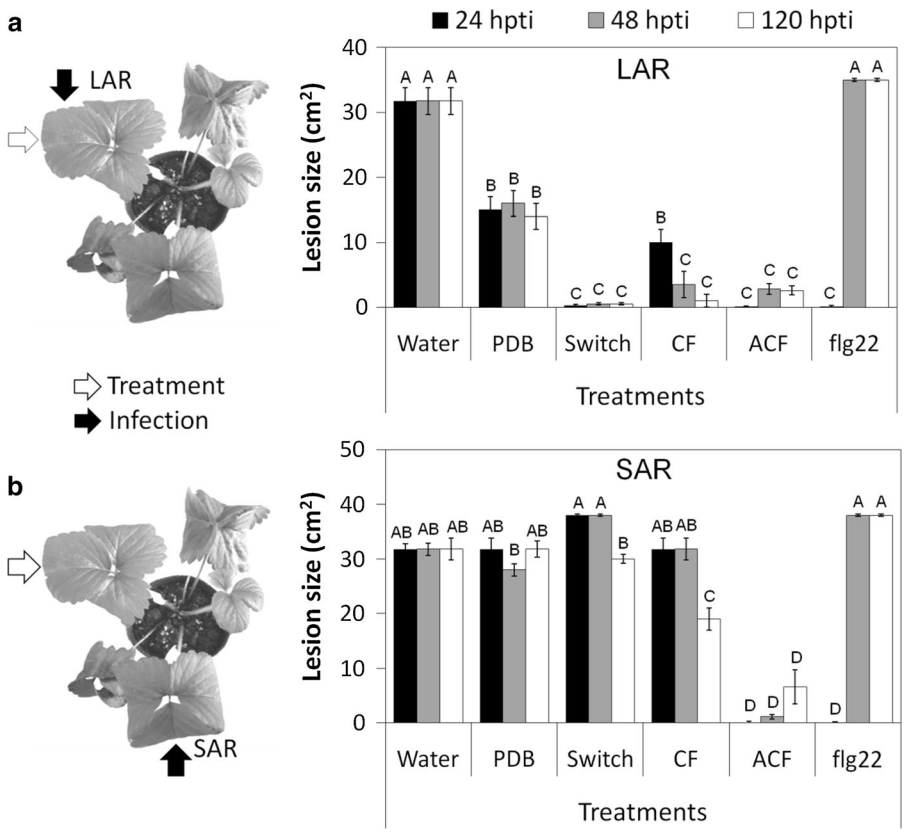

Fig. 1 Effect of FEs on strawberry plants infected with *Botrytis cinerea*. CF, ACF, flg22 or controls (water, PDB and Switch®) were applied 24, 48 or 120 hpti (hours prior to infection) with *B. cinerea,* and lesions area were measured 7 dpi (days post-infection) for local acquired resistance (LAR) (**a**), and systemic acquired resistance (SAR) evaluations (**b**). Bars represent mean values ± SE (n = 5). Different letters represent statistically significant differences among all means at all times and treatments performed ($p < 0.05$). Three independent assays were performed with similar results

contrast, flg22 lost completely its capacity to induce a local protection when time elapsed between induction treatment and infection was more than 24 hpti (0.04 $cm^2 \pm 0.04$ at 24 hpti, and 35.00 $cm^2 \pm 0.02$ at 48 hpti and 120 hpti), comparing to controls at each induction time (Fig. 1a). PBD showed slight local capacity to control the progress of the disease at all the induction times evaluated (14.55 $cm^2 \pm 0.71$ at 24 hpti, 15.21 $cm^2 \pm 0.51$ at 48 hpti and 13.45 $cm^2 \pm 0.93$ at 120 hpti), as compared to water-treated plants (31.79 $cm^2 \pm 1.18$ for all induction times).

When evaluating SAR in distal leaves, ACF induced high protection 24 hpti (0.08 $cm^2 \pm 0.06$) and 48 hpti (1.13 $cm^2 \pm 0.42$) and maintained its capacity to control the disease progress even at 120 hpti (6.58 $cm^2 \pm 3.12$) as compared to the controls at each induction time (Fig. 1b). In contrast, CF showed no systemic protection 24 and 48 hpti (31.78 $cm^2 \pm 1.55$) but slightly improved 120 hpti (17.89 $cm^2 \pm 0.87$) as compared to water-treated plants (31.78 $cm^2 \pm 1.13$ for all induction times) (Fig. 1b). Concerning the effect of flg22 on SAR induction, the behavior was similar to LAR, that is, a strong protective effect was observed only at 24 hpti (0.00 $cm^2 \pm 0.00$), and no effect was detected at 48 or 120 hpti (38.00 $cm^2 \pm 0.05$) (Fig. 1b).

Evolution of the disease in pre-treated plants

Grey mold evolution in leaves of strawberry plants that were previously induced 24, 48 or 120 hpti with CF or ACF, or treated with water, PDB, Switch® or flg22 as controls, was assessed up to 12 dpi. The two-way ANOVA showed differences between treatments and days post-infection indicating that each factor affects significantly the lesion area caused by the

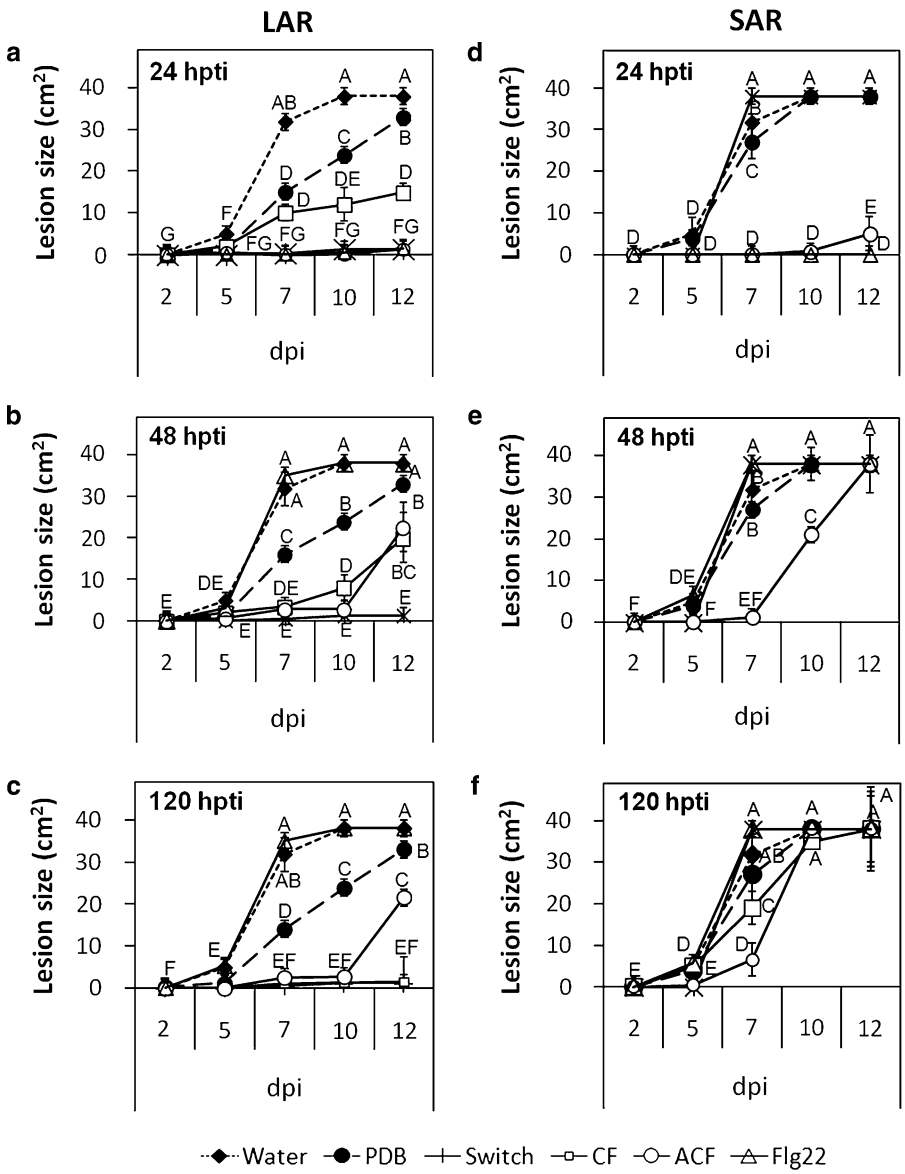

Fig. 2 Evaluation of the disease progress in FEs or controls pre-treated strawberry plants after *Botrytis cinerea* infection. CF, ACF, flg22 or controls (water, PDB and Switch®) were applied 24, 48 or 120 hpti (hours prior to infection) with *B. cinerea,* and lesions area were evaluated at indicated times until 12 dpi (days post infection) for local acquired resistance (LAR) (**a**, **b**, **c**), and systemic acquired resistance (SAR) evaluations (**d**, **e**, **f**) evaluations. Each point represents mean values of lesions area ± SE (n = 5). Different letters represent statistically significant differences among all means at all times and treatments performed ($p < 0.05$). Three independent assays were performed with similar results

fungus. Additionally, the interaction between those factors also showed a significant impact in the lesion area when evaluated either locally (Fig. 2a–c) or systemically (Fig. 2d–f). Statistical parameters are shown in Supplementary Table S2. Regarding CF-induced plants, they exhibited a significant progression of disease symptoms when inducing 24 hpti (1.90 cm^2 ± 0.26 at 5 dpi, 10.13 cm^2 ± 0.78 at 7 dpi, 12.02 cm^2 ± 0.85 at 10 dpi, 14.73 cm^2 ± 0.70 at 12 dpi) (Fig. 2a) and 48 hpti (2.01 cm^2 ± 0.47 at 5 dpi, 3.50 cm^2 ± 0.84 at 7 dpi, 7.93 cm^2 ± 1.60 at 10 dpi, 20.09 cm^2 ± 3.43 at 12 dpi) (Fig. 2b). Nonetheless, no significant symptoms were observed when inducing 120 hpti (0.01 cm^2 ± 0.003 at 5 dpi, 1.00 cm^2 ± 0.23

at 7 dpi, 1.20 cm^2 ± 0.17 at 10 dpi, 1.48 cm^2 ± 0.26 at 12 dpi) (Fig. 2c) compared to controls at this induction time. On the contrary, ACF-induced plants showed significant local protection when inducing 24 hpti (0.010 cm^2 ± 0.004 at 5 dpi, 0.020 cm^2 ± 0.003 at 7 dpi, 0.14 cm^2 ± 0.05 at 10 dpi, 1.39 cm^2 ± 0.31 at 12 dpi) in comparison to controls (Fig. 2a). However, they exhibited an early appearance and rapid progression of disease symptoms when inducing 48 hpti (0.67 cm^2 ± 0.16 at 5 dpi, 2.82 cm^2 ± 0.67 at 7 dpi, 2.83 cm^2 ± 0.67 at 10 dpi, 22.57 cm^2 ± 3.86 at 12 dpi) (Fig. 2b) and 120 hpti (0.44 cm^2 ± 0.11 at 5 dpi, 2.57 cm^2 ± 0.59 at 7 dpi, 2.72 cm^2 ± 0.39 at 10 dpi, 21.48 cm^2 ± 3.75 at 12 dpi) (Fig. 2c). Control experiments showed that Switch® completely prevented the pathogen growth at all the treated times evaluated compared to controls treated with water and PDB, showing smaller lesions at 12 dpi when applied 48 and 120 hpti (1.28 cm^2 ± 0.40) than 24 hpti (1.29 cm^2 ± 0.36). However flg22 was active only when inducing 24 hpti (0.003 cm^2 ± 0.001 at 5 dpi, 0.04 cm^2 ± 0.02 at 7 dpi, 0.39 cm^2 ± 0.16 at 10 dpi, 1.57 cm^2 ± 0.58 at 12 dpi), confirming that the priming effect exerted by flg22 on treated leaves decays 24 h after the induction. Water- and PDB-treated plants exhibited a rapid evolution of the disease regardless the induction times, although the disease symptoms showed by plants treated with PDB evolved slower (Fig. 2a–c).

When evaluating the evolution of disease symptoms in distant not-induced leaves (SAR), results showed that plants were more affected than when plants were locally induced. Plants treated with ACF or flg22 presented a notorious protection effect when induced 24 hpti in comparison to controls water and PDB (Fig. 2d), showing the absence or the presence of very small lesions along the time (ACF: 0.08 cm^{2-} ± 0.05 at 5 dpi, 0.08 cm^2 ± 0.05 at 7 dpi, 0.80 cm^2 ± 0.19 at 10 dpi, 5.09 cm^2 ± 1.23 at 12 dpi; flg22: 0.00 cm^2 ± 0.00 at 5, 7, 10 and 12 dpi). However, the effect was lost at longer induction times, e.g. 48 hpti (ACF: 0.12 cm^2 ± 0.05 at 5 dpi, 1.12 cm^2 ± 0.37 at 7 dpi, 20.92 cm^2 ± 3.48 at 10 dpi, 38.00 cm^2 ± 0.001 at 12 dpi; flg22: 6.63 cm^2 ± 0.36 at 5 dpi and 38.00 cm^2 ± 0.001 at 7, 10 and 12 dpi) (Fig. 2e) and 120 hpti (ACF: 0.49 cm^2 ± 0.14 at 5 dpi, 6.58 cm^2 ± 2.39 at 7 dpi, 22.80 cm^2 ± 4.98 at 10 dpi, 38.00 cm^2 ± 0.001 at 12 dpi; flg22: 5.73 cm^2 ± 0.32 at 5 dpi and 38.00 cm^2 ± 0.001 at 7, 10 and 12 dpi) (Fig. 2f) when compared to controls. Since ACF showed a higher capacity to control the grey mold disease when evaluating LAR (Fig. 2a) and SAR (Fig. 2d) we continued the studies with this extract inducing 24 hpti.

Effect of ACF on *B. cinerea* germination and growth

Experiments were further conducted to evaluate whether ACF exerted a direct antifungal effect on *B. cinerea.* Results showed that conidia could germinate and actively grew on the leaf surface middle portion (Fig. 3a, b), and the lamina portion (Fig. 3d) of ACF-treated plants. When treated tissue was analyzed under UV light, a blue-green fluorescence close to the hyphal growth was observed at the middle portion (Fig. 3e), and the lamina portion (Fig. 3h) of the strawberry leaf, mainly concentrated at the infection site. Vesicle-like structures were detected at the middle and lamina portion of the adaxial surface under bright field (BF) (Fig. 3b, c) and under UV light (Fig. 3f, g). Hyphae growing underneath the leaf cuticles were also observed, suggesting that hyphae were unable to penetrate the epidermal cells (Fig. 3d). In contrast, control not-treated plants infected with *B. cinerea* did not present these features and leaves were completely invaded by fungal hyphae (not shown).

The effect of ACF on the germination and growth of *B. cinerea* conidia was also evaluated in vitro. Results confirmed the observations carried out *in planta* (Fig. 3a–e). ACF exerted no detectable effect on conidia germination and hyphae growth as compared to conidia growth suspended in water or PDB (Supplementary Fig. S1).

Vascular bundles of the median vein were analyzed in histological sections of PDB- or ACF-treated plants, and no significant differences were detected when observations were made under BF (Fig. 4a, b) and UV light (Fig. 4e, f). However, when the histological sections were visualized with a blue U-MWB2 filter, more brilliant, rounded and protruding vascular bundle contours were detected (Fig. 4c, d). The latter indicates that ACF induced a notorious thickening of xylematic beams in plants. When the phloematic and collenchyma tissues were analyzed under UV light (Fig. 4g, h) and BF (Fig. 4i, j), respectively, brighter contours and thicker cell walls were observed indicating that ACF induced a clear cell wall

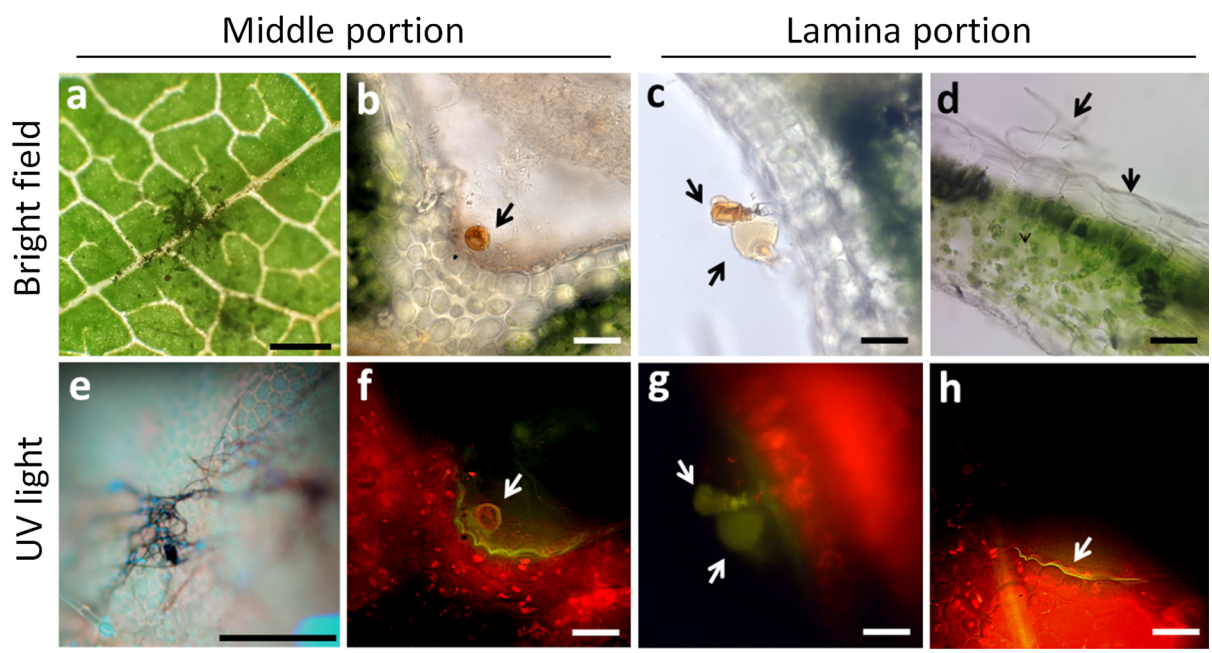

Fig. 3 Microscopic observations of *Botrytis cinerea* growth on ACF-treated leaves. ACF was applied on strawberry leaves 24 hpti, and collected 12 dpi for evaluation. Mycelia growth on the adaxial side of a leaf observed under bright field (**a**) and UV (329 nm) light (**e**). Brownish structures observed under bright field (arrows in **b**, **c**), and UV light (329 nm) (arrows in **f**, **g**, **h**). Hyphae growing underneath cuticle observed under bright field (arrows in **d**). Bar = 500 µm (**a** and **e**), 50 µm (**b**, **c**, **d**, **f**, **g** and **h**). One representative picture was presented for each treatment (n = 45). Two independent assays were performed

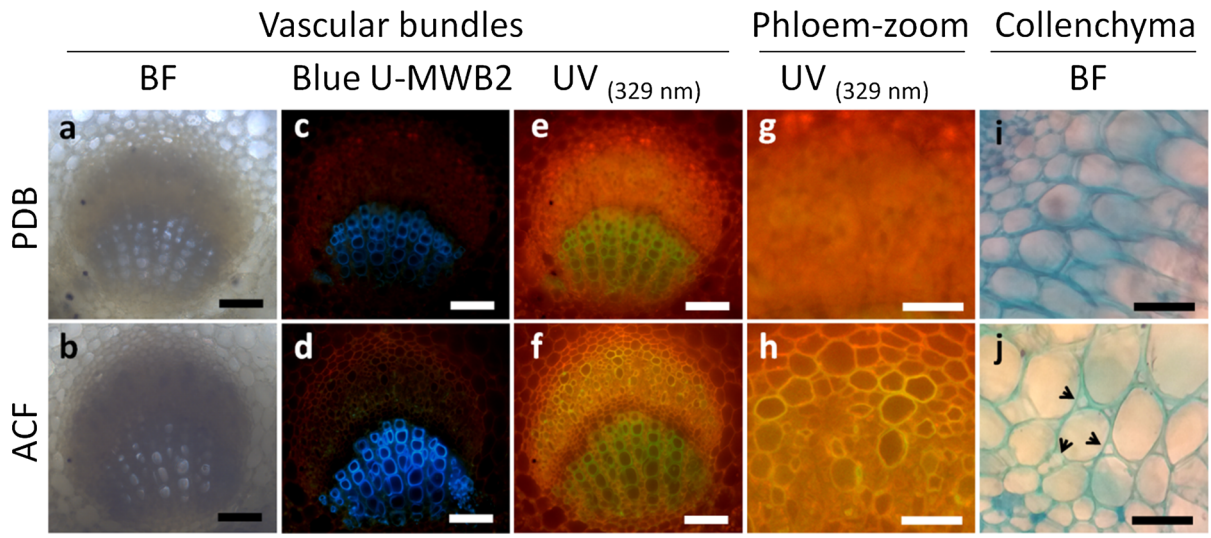

Fig. 4 Morphological and anatomical effect of ACF on strawberry leaves. Cross sections of the leaflet main vein showing vascular bundles were observed under bright field (BF, **a**, **b**), Blue light (**c**, **d**), and UV light (**e**, **f**) (n = 9) in potato dextrose broth (PDB, control) and ACF treated plants. Central leaflet vein sections were observed under UV light of PDB (**g**) and ACF treated plants (**h**). Collenchyma associated to vascular bundles stained with Astral blue were observed under bright field in PDB (**i**) and UV light (329 nm) (**j**) (arrows indicate changes observed at the intercellular space). One representative image was used to illustrate results of each treatment. (n = 45). Bar = 25 µm

reinforcement in comparison to control PDB-treated plants (Fig. 4g, i). The analysis of the collenchyma associated with the median vein exhibited swollen and rounded cells with larger intercellular spaces (Fig. 4j).

Effect of a thermal treatment on ACF capacity to protect plants against *B. cinerea*

With the aim to advance in the characterization of the active agent contained in ACF, phytopathogenic assays were carried out with plants pre-treated with thermal-treated-ACF as indicated in the Materials and Methods section. Results showed that ACF maintained a residual LAR, but SAR was completely lost (Supplementary Fig. S2), suggesting not only that the active agent is sensitive to the temperature but also unable to trigger the systemic signal after the thermal treatment. Supplementary Fig. S2 shows that PDB-pre-treated plants induced a low level of protection, as previously observed in LAR determinations (Figs. 1, 2).

Ethylene production in plants treated with ACF

Since ACF induced protection against the necrotrophic pathogen *B. cinerea* in strawberry plants, we were interested to test whether ACF induced ET production, which is an indicator of the activation of a defense response associated to the ET pathway.

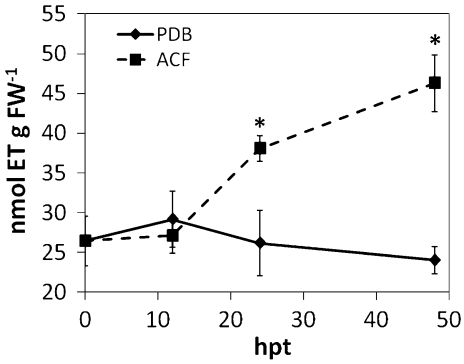

Fig. 5 Effect of ACF on ethylene a production in strawberry plants. Plants were treated with ACF or potato dextrose broth (PDB, control) and ethylene production was quantified at 12, 24 and 48 hpt (hours post treatment). Values correspond to nmol ET g^{-1} leaf fresh weight (FW). Mean values were obtained from ten independent samples. Vertical bars represent SE. Asterisks indicate statistically significant difference between PDB- and ACF-treated plants at each time point evaluated, according to Student's *t* tests ($p < 0.05$)

Results confirmed that ACF induced the production of ET after 12 hpt (38.06 nmol ET gr FW^{-1} ± 1.63) ($t = -3.39$; $df = 7$; $p = 0.0116$), reaching the highest differences at 48 hpt (46.28 nmol ET gr FW^{-1} ± 3.58) ($t = -7.89$; $df = 4$; $p = 0.0014$) (Fig. 5). At the earliest time point evaluated (12 hpt), no significant differences were observed with respect to control plants (27.14 nmol ET gr FW^{-1} ± 2.23) ($t = -1.88$; $df = 10$; $p = 0.0890$).

Gene expression associated to defense response

Evaluation of the expression of genes associated to the defense response indicated that ACF brought about a change of the expression level of genes associated to the activation of ET signaling rather to the salicylic acid (SA). Figure 6 shows that the genes *FaPR1* ($p = 0.1719$), *FaChi2-2* ($p = 0.1227$), *FaCHI23* ($p = 0.3431$) and *FaOGBG* ($p = 0.1829$) that are regulated by SA (Graham et al. 2003; van Loon et al. 2006; Hael-Conrad et al. 2018; Martinez-Zamora et al. 2012) remained unchanged whereas genes associated to the ET signaling pathway *FaETR1* ($p = 0.0171$), *FaERS1* ($p = 0.0427$), and *FaERF1* ($p = 0.0321$) (Hua and Meyerowitz 1998; Elias et al. 2018; Merchante et al. 2013) exhibited an up-regulation with respect to control plants. In the figure, we can observe that the genes associated to ROS scavenging *FaCAT* ($p = 0.2993$) (encoding catalase isozyme 1, Guerrero-Molina et al. 2015) and *FaPRX* ($p = 0.084$) (encoding a class III plant peroxidase, Hael-Conrad et al. 2018) did not change the level of expression confirming previous reports about the ability of *C. acutatum* M11 to suppress the oxidative burst (Tomas-Grau et al. 2019). Evaluation of ROS accumulation confirmed that FEs did not bring about the accumulation of H_2O_2 and $O_2^{\cdot -}$ (results not shown). Also, ACF induced the up-regulation of *FaGLS5* ($p = 0.0289$), a gene coding for an enzyme involved in the synthesis of cell wall components (Glucan synthase-like 5, Hael-Conrad et al. 2018), confirming the effect of ACF on the cell wall reinforcement as observed in the tissue analyses (Fig. 4). In contrast, the expression level of *FaPDF1.2* ($p = 0.1829$) associated to the ET and jasmonic acid (JA) signaling pathway was not affected by ACF.

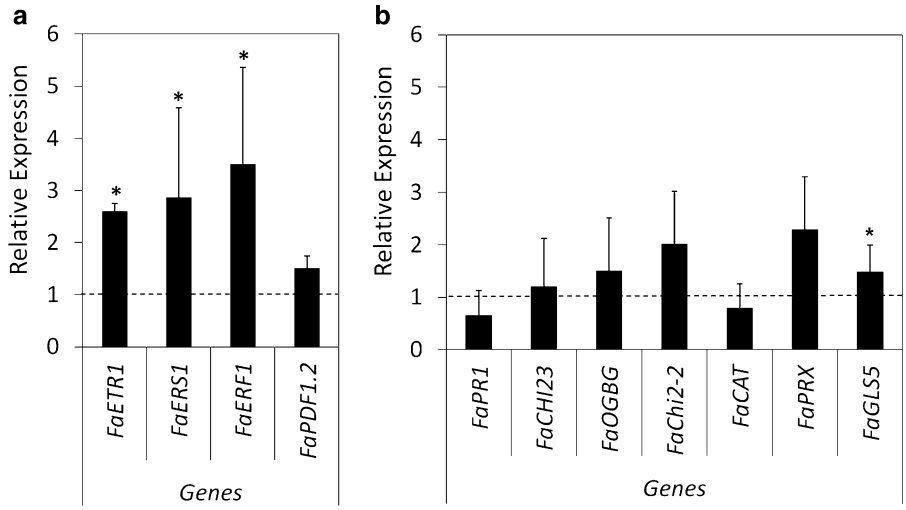

Fig. 6 Effect of ACF on defense-related genes of strawberry plants treated with ACF or PDB. **a** *FaETR1, FaERS1, FaERF1, FaPDF1.2* **b** *FaPR1, FaCHI23, FaOGBG, FaChi2-2, FaCAT, FaPRX* and *FaGLS5* genes were evaluated at 48 hpt. Expression values were normalized with respect to the reference gene (*FaEF-1α*) and reported relative to the plants treated with PDB. Bars represent average values ± SE of three technical replicates per qPCR reaction (n = 3). The horizontal dashed-line (relative expression = 1) represents values of control plants treated with PDB. Values above the dashed-line mean up-regulation, and values below it down-regulation. Asterisks indicate a statistically significant difference gene expression between control and ACF-treated plants, calculated by fgStatistics software ($p < 0.05$)

Discussion

In the current trend towards a sustainable agriculture, biocontrol of fungal diseases is achieving more attention. This promissory biotechnology is based on the use of elicitors of different origin to induce plant defenses. In this study, we provide evidence that the blend of fungal-derived PAMPs, namely CF and ACF, have the capacity to protect strawberry plants against *B. cinerea* inducing LAR and SAR (Fig. 1), and the induction period is crucial for activating an effective defense. Whereas ACF needs a minimum of 24 hpti to induce an optimum LAR, CF requires 48 hpti to yield a similar effect in strawberry plants (Fig. 1a).

In this study we used the peptide flg22 as a reference of a well known defense induction elicitor (Zipfel et al. 2004), and the commercial synthetic fungicide Switch® used to control *B. cinerea* in fields. When evaluating LAR, results showed that flg22 and Switch® induce maximum protection when applied 24 hpti (Fig. 1). However whereas the former is unable to induce defense responses at 48 and 120 hpti, the latter maintains its activity at all induction time evaluated. The result obtained with flg22 is in line with previous reports denoting the immediate induction of defense in the model plant *A. thaliana* (Boller and Felix 2009). When evaluating SAR, results showed that CF requires 120 hpti to achieve a strong activation, whereas ACF achieves a maximum protection 24 hpti, and keeps activated thereafter (Fig. 2d). These results represent the first report about the use of flg22 to provide protection against *B. cinerea* in strawberry plants, and bring support to a previous report suggesting the existence of an flagellin sensing-2 receptor (FLS2) ortholog gene in *F.* × *ananassa* plants (Guerrero-Molina et al., 2015), previously reported in *Arabidopsis* (Zipfel 2008). Results obtained with Switch® showed that it is unable to induce SAR at all evaluated times (Fig. 2). However, since one of its active ingredients (e.g. Fludioxonil 25% p/p) is supposed to exert a systemic effect (according to the product label), Switch® may require more time to induce SAR.

In an effort to rule out whether ACF exerts any direct effect on the growth and germination of *B. cinerea*, different experiments were performed. The effect on the growth of *B. cinerea* on strawberry ACF-treated leaves was analyzed by microscopic observations under UV light (329 nm). The accumulation of a blue-green fluorescence at the site of infection was observed, due to the accumulation of fluorescent secondary metabolites, as reported by Talamond et al.

(2015). Kårlund et al. (2014) also observed this phenomenon suggesting the accumulation of polyphenolic compounds in response to different stresses. Since those molecules are reported to play an important role in plant resistance against fungi (Mclusky et al. 1999), we may speculate that ACF activates the synthesis of autofluorescent compounds as a physical barrier to prevent *B. cinerea* infection in strawberry plants. The formation of vesicles-like structures similar to druses were also observed, as previously reported in strawberry plants treated with the elicitor AsES (Hael-Conrad et al. 2018). Further microscopic analysis showed that *B. cinerea* hyphae cannot penetrate the cuticle and epidermal cells, and cannot invade inner tissues, but grow on the surface of the cuticle without detaching it (Fig. 3). This effect was previously observed by Salazar et al. (2007) when analyzing the interaction between an avirulent isolate of *C. fragariae* with the cv. Pájaro of *F.* × *ananassa*. In vitro analyses of the effect of ACF on conidia germination and mycelium growth confirmed that ACF has no effect on the pathogen growth, suggesting therefore that the effect observed is caused by a plant reaction when treated with ACF. The modification of anatomical and histological leaf structures observed (Fig. 4) brings further support to the opinion that plants activate the innate immunity.

The increase of ET production in ACF-treated plants clearly indicates that ET defense pathway is activated (Fig. 5). However, since SA pathway may also be activated in response to another type of fungal elicitor, as reported by Hael-Conrad et al. (2015) in *Arabidopsis*, the expression of genes associated to both pathways were analyzed. Results revealed that ACF does not induce the up-regulation of genes regulated by SA, namely *FaPR1*, *FaChi2-2, FaCHI23*, and *FaOGBG* (Graham et al. 2003; van Loon et al. 2006; Hael-Conrad et al. 2018; Martinez-Zamora et al. 2012), suggesting that this signaling pathway does not participate in ACF-induced defense response. However, ACF does induce the up-regulation of the genes *FaETR1*, *FaERS1*, and *FaERF1* (Fig. 6) associated to ET pathway (Hua and Meyerowitz 1998; Elias et al. 2018; Merchante et al. 2013), validating therefore that the ET pathway is activated. This outcome further reveals, and confirms, that a crosstalk, and the mutual regulation between the SA and ET signaling pathways is actually taking place in strawberry plants, as reported in *Arabidopsis* (Pieterse et al. 2012).

Analysis of the expression of genes *FaCAT* and *FaPRX* (Fig. 6) associated to ROS scavenging enzymes confirm previous reports showing that the isolate M11 of *C. acutatum* can suppress the oxidative burst induced by a defense elicitor (Tomas-Grau et al. 2019). In fact no oxidative burst was observed in strawberry plants treated with ACF. Finally, the up-regulation of *FaGLS5* (Fig. 6) which encodes for an enzyme involved in the synthesis of cell wall components further supports the results shown in Fig. 4 in which we show the ACF induces cell wall reinforcement as a plant defense mechanism.

Taking these results together, the hypothesis stated above about the capacity of M11, and its derived extracts, to activate the defense response against a necrotrophic pathogen such as *B. cincera*, and to suppress the defense response against an hemibiotrophic pathogen such as *C. acutatum*, is confirmed. The latter means that the effect of M11 and its derived fungal extracts would depend on the pathogen tested. This outcome casts a great alert on the application of bioproducts containing defense elicitors without a previous evaluation of the pathogenic agents present in the crop area. In this sense, if a pathogen screening is not possible it would be recommendable to use bioproducts containing an elicitor that proved to be effective against both type pathogens (e.g. biotrophic and necrotrophic) as the protein AsES (Chalfoun et al. 2013, 2018a, b; Hael-Conrad et al. 2015, 2018).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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