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Natural elicitors of plant defense response in strawberry[1]

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Abstract. Natural substances such as elicitors of plant defense response, has become a promising option for effective management of plant diseases and are a prerequisite for sustainable and ecological agriculture. Recognition of diverse elicitors, effectors and modulators by specific and nonspecific plant receptors activates signalling cascades eventually leading to gene expression and defense responses. Elicited plants or "primed plants" display either faster, stronger, or both activation of the various cellular defense responses that are induced following attack by either pathogens or insects or in response to abiotic stress.

The aim of this work has been to develop new elicitor compounds from natural vegetal substrates, and predict its effectiveness in the field by using robust molecular techniques and appropriate biomarkers of plant defense. Thus, *Fragaria x ananassa* Duch. cv. Chandler cell suspension cultures, and real-time, quantitative PCR analysis (RTqPCR) were utilized to test the elicitor capability of new natural elicitors. This experimental approach was applied to analyze the pattern of gene expression of specific strawberry genes already known to be good markers of SA- and JA-dependent signalling pathways, which were used as defense induced biomarkers in plants. In addition, determination of total phenols and antioxidant activity, and HPLC-DAD-MS analysis of polyphenolic compounds was performed in these cell suspension samples. Susceptibility to *C. acutatum* of strawberry plants treated with the new elicitor was performed in greenhouse under controlled conditions.

In collaboration with AGROMETODOS, S.A., we have produced new natural compounds that can act as elicitors. We have compared the elicitor activity of a new compound (EH) with the commercial product BROTOMAX®. The new product EH and BROTOMAX® activate plant defense related genes in cell suspension cultures. Also, both products induce polyphenol production in this assay. The new product EH and BROTOMAX® increased the strawberry (*Fragaria x ananassa* Duch. cv. Camarosa) and olive (*Olea europaea* L. cv. Picual) resistance against *Colletotrichum acutatum* and *Fusicladium oleaginum* plant infections, respectively.

We can conclude that new natural bioelicitors can be produced by low-cost procedures based on fermentation of natural substrates derived from plants. The use of suitable, sensitive, appropriate and robust molecular biomarkers also provide an excellent molecular tool to predict the effectiveness in the field of natural compounds with potential eliciting ability, which may be conveniently exploited as ecological bioinducers of plant defense response in strawberry and in many other important crops.

Keywords: Elicitor, bioinducers, plant defense, genetic biomarker, plant resistance, strawberry, olive plant

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1. Introduction

Plant pathogens are responsible for considerable losses in agricultural production and are mainly controlled by application of agrochemicals and by resistance breeding. Studies with different plant–pathogen systems have shown that plants can activate distinct defense pathways involving different regulators, depending on the type of parasites [1]. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), are important signalling molecules in plant defense in response to biotic stresses. ET and JA-dependent defense responses seem to be activated by necrotrophic pathogens, whereas the SA-dependent response is mainly triggered by biotrophic pathogens [2]. These three signal molecules play important roles in induced plant resistance.

Successful plant defense relies on a fast and specific response to pathogens during plant-pathogen interaction. Recognition of diverse elicitors, effectors and modulators by specific and nonspecific plant receptors activates signalling cascades eventually leading to gene expression and defense responses. Thus, over the last years an increasing number of studies have been carried out to analyse the ability of a wide array of natural and synthetic compounds to induce plant defense, hence named "plant defense activators" or "plant elicitors" [3–6]. Elicited plants or "primed plants" display either faster, stronger, or both activation of the various cellular defense responses that are induced following attack by either pathogens or insects or in response to abiotic stress. "Priming" is the phenomenon that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells [7–9]. The effect of "priming" includes accumulation of phytoalexins, increased gene expression and accumulation of defense-related proteins and response oxidative stress [10]. These parameters are useful molecular tools as they can be exploited as biomarkers of plant resistance induction for the screening of new defense-eliciting compounds.

Treatment with elicitors to protect crops against microbial pathogens and pest is still in the early stages of use as a new control method. However, great advantages of using elicitor treatments in crops can be expected:

a) The control of disease effects caused by bacteria, fungi, and pests reduces losses in horticultural crops.

b) Elicitors as bioinducers reduce environmental hazards and provide ecological benefits being less toxic to other organisms than pesticides.

c) Elicitors can be applied as protective compounds by means of methodologies currently being used for agrochemicals.

d) Elicitor-treated plants might constitute an alternative to GM plants for large-scale agriculture.

In collaboration with AGROMETODOS, S.A., we have developed a new elicitor compound (EH) from natural vegetal substrates, which has showed good capability to induce plant defense related genes in strawberry cell suspension cultures and to increase field resistance of strawberry plants after *Colletotrichum acutatum* infection. Moreover, the proved elicitor ability of a commercial compounds used in agriculture, such as BROTOMAX®, as well as the synergistic action of both products have been analysed using molecular technology of gene expression.

2. Material and methods

2.1. Elicitors

New natural bioelicitors were produced by low-cost procedures based on fermentation of natural substrates derived from plants. Elicitor EH, one of these new compounds has been tested according to molecular methods presented in this paper. BROTOMAX®, a commercial compound known to increase resistance in field assays in many plants was used as positive control in these experiments.

2.2. Antifungal activity assays of elicitor EH and BROTOMAX®

The phytopathogenic fungi *Colletotrichum acutatum* CECT 20120 and *Botryotinia fuckeliana* CECT 2100 were used as inhibitory indicators in the study. The strains were maintained on potato dextrose agar (PDA).

The antifungal activity was tested by the agar dilution method. In short, 50 ml of 0.1, 0.2, 2 and 4% (w/v) dilutions of BROTOMAX® or elicitor EH were mixed separately with 50 ml of 2x PDA medium to obtain final concentrations of 0.05, 0.1, 1 and 2% of each product. 20 ml of these mixtures were poured onto sterile Petri dishes and inoculated

by placing 0.5 cm diameter discs of the fungal cultures to be tested in the center of the dish, and incubated at 28°C for 9 days. 1x PDA without leaf extract was used as control. The linear growth of each fungus was measured 3, 6 and 9 days after inoculation.

2.3. Plant material and experimental assays

Callus induction and cell suspension culture. Both, *Fragaria x ananassa* Duch. cv. Chandler callus and cell suspensions used in this work were obtained as previously described [11].

Elicitor assays. 200 ml aliquots of 5-days-old cultures were used per assay. Control (water) and elicitors were added (0.1% of BROTOMAX® or 0.05% of elicitor EH) to these cultures and 50 ml samples collected every 2 h for 0 to 8 h. Cells were filtered through Whatman filter paper, immediately frozen in liquid nitrogen and stored at −80°C.

2.4. Total RNA extraction and cDNA synthesis

Total RNA from strawberry fruits and vegetative tissues, as well as from cell suspension cultures, was isolated according to Manning [12], treated with DNaseI (Invitrogen) to remove the residual contaminating DNA, further purified with the RNeasy MinElute Cleanup Kit (Qiagen) and the integrity checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). First-strand cDNA synthesis was carried out from 1 μg of purified total RNA by using the iScript cDNA Synthesis Kit (Bio-Rad) according the manufacturer´s conditions.

2.5. Real-time PCR

RT reactions were diluted 5-fold with nuclease-free water prior to use in the qPCR step. RTqPCR runs were performed on MyiQ and iCycler Real-Time PCR Systems (Bio-Rad). 2 μl template cDNA were added per 20 μl PCR reaction mixture containing 0.4 μM of specific primer pairs and 10 μl of 2x SsoAdvanced™ SYBR® Green Supermix (Bio-Rad). The protocol was as follows: an initial step of 95°C for 1 min for enzyme activation/DNA denaturation, followed by 40 cycles of 95°C for 15 s, 65°C for 15 s and 72°C for 15 s, and a final standard dissociation protocol to obtain the melting profiles. Data were acquired by means of the MyiQ v1.004 and iCycler v3.1 software (Bio-Rad).

This experimental approach was used to analyse the pattern of gene expression of specific strawberry genes *Fachi4.2* (JA1), *Fapr10.4* (JA2), *Fawrky1* (SJ3), and *Fawrky70.1* (SA4), already known to be good markers of SA- and JA-dependent signalling pathways [10], which were used as defense induced biomarkers in plants.

2.6. Determination of total phenols and antioxidant activity

Sample preparation. Treated strawberry cell cultures (lyophilized material) were weighed (0.2 g) and homogenized in 10 ml of 80% aqueous methanol containing 1% formic acid by bead beating for 45 min on a vortex followed by sonication in an ultrasonic bath for 15 min. The homogenates were then centrifuged at 11000 g for 10 min and supernatants analysed for total phenols and antioxidant activity. For HPLC-DAD-MS analysis supernatants were concentrated in a rotary evaporator at 37°C. The aqueous residue was made up to 2 ml with aqueous formic acid (1%) and purified by solid phase extraction using EVOLUTE™ ABN™ SPE cartridges (Biotage). Samples were filtered through 0.45 μm nylon filters prior to HPLC-MS analysis.

Determination of total phenols and antioxidant activity. Spectrophotometric measurements were performed on a Shimadzu 160A UV–Vis spectrophotometer. Phenolic compounds were determined following the Folin-Ciocalteu's procedure [13]. Results were expressed as gallic acid equivalent (GAE, μg/ml)/g dry weight of material, and calculated as means ± standard deviation (SD) ($n = 3$). The TEAC/ABTS assay [14] was used to measure antioxidant activity. Results were expressed as Trolox equivalent (TE, μmol)/g dry weight of extracts, and calculated as means ± standard deviation (SD) ($n = 3$).

2.7. HPLC-DAD-MS analysis of polyphenolic compounds

HPLC-DAD-MS analyses of cell culture extracts were performed on an Agilent 1200 HPLC Series system equipped with a UV-Vis photodiode array, and quadrupole mass spectrometer detectors and equipped with electrospray

ionization (ES) and atmospheric pressure chemical ionization (APCI). Components were separated using a 2 μm ACE Excel C18-PFP column (150 × 4.6 mm I.D.) and a binary gradient as mobile phase prepared from methanol/formic acid (98:2, v/v) and water/formic acid (98:2, v/v). Elution started with a linear gradient, beginning at 10 % and ending at 90 % methanol/formic acid for 30 min; isocratic elution followed for the next 10 min. The flow rate was 0.4 ml/min and chromatograms were recorded at 280, 320, 370, and 520 nm. For mass spectrometric analyses the ionization mode was MM-ES+APCI and operating conditions were optimized in order to achieve maximum sensitivity values: drying gas flow, 10 l/s; nebulizer pressure, 40 psig; drying gas temperature 350°C; vapour temperature, 250°C; capillary voltage, 4000 V; corona current, 4 μA; and charging voltage, 2000 V. The instrument was operated in the negative ion mode. Chromatographic data were processed using ChemStation software from Agilent Technologies (Waldbronn, Germany). Polyphenol characterization in strawberry cell suspension cultures was carried out by means of their UV spectra, molecular weight, MS fragments and, whenever possible, chromatographic comparison with authentic standards.

2.8. Plant inoculation experiments

Plant inoculation experiments were performed using 20 randomized blocks of 10 plants from strawberry (*Fragaria x ananassa* Duch. cv. Camarosa, 6 weeks old) or olive plants (*Olea europaea* L. cv. Picual, 1 year old) maintained in green house under healthy conditions. Each treatment included five replications. Plants were inoculated by spraying onto leaves a suspension of 10^4 conidia/ml of *Colletotrichum acutatum* for strawberry as described by Casado-Diaz et al. [15] and 10^5 conidia/ml of *Fusicladium oleaginum* for olive plants. The plants were maintained in a controlled growth room (18–22°C, 99% RH) for one or two days for strawberries and olive, respectively, and then transferred into a greenhouse (25 ± 5°C). Strawberry plants were treated with the elicitors (BROTOMAX® 0.5 % and EH 0.05 %) 1, 3, 7 and 14 days before inoculation (DBI) with *C. acutatum* and olive plants were treated with de same elicitors 7, 13, and 25 DBI with *F. oleaginum*. The disease severity on plant was rated on 14 and 56 days after pathogen inoculation for strawberries and olive plants, respectively. The disease severity index (DSI) for strawberries plants was calculated using a 1–8 scale based on crown tissue response (1 = healthy plant to 8 = dead plant, plus 0.5 when the pathogen was also isolated from root tissues). For olive plants DSI was defined as the percentage of diseased leaves. The disease control value was calculated using the following formula: disease control value (DCV %) = ((A-B)/A) × 100, where A is the DSI caused by pathogen inoculation alone and B is the DSI after treatments.

3. Results

3.1. BROTOMAX® and elicitor EH did not show antifungal activity

Under the assayed conditions neither BROTOMAX® nor elicitor EH presented antifungal activity against *Colletotrichum acutatum* or *Botryotinia fuckeliana*. The dose of each product used in these experiments was several times higher than that employed either in cell suspension elicitor assays or in plant inoculation experiments.

3.2. BROTOMAX® and the new product EH activate plant defense related genes in strawberry cell suspension cultures

As shown in Fig. 1 BROTOMAX® significantly up-regulated the expression of two well-known JA-dependent gene biomarkers (JA1 and JA2), and a SA and JA-dependent gene biomarker (SJ3) while the expression of one SA-dependent marker (SA4) remains unchanged or was even slightly reduced. The expression levels reached for each of these defense-related gene markers after treatment with EH were similar or even higher to those found when BROTOMAX® was applied. These results strongly suggest that this new product might act similarly to BROTOMAX® when applied to field crops increasing resistance in plants. Very interestingly, a positive synergy between BROTOMAX® and EH was also detected. Thus, the increase in the expression level for each defense-related gene marker was higher when a mix of both compounds was applied to strawberry cell cultures than when

either product was used alone. All in all, these results indicate that these two compounds should act as good elicitors of plant defense if applied to field-crops.

3.3. *BROTOMAX® and the new product EH induce polyphenol production in strawberry cell suspension cultures*

Total polyphenols (Fig. 2A) and antioxidant activity (Fig. 2B) increased significantly in strawberry cell suspension cultures elicited with either BROTOMAX® or EH. Treatment with EH, showed high capacity to induce both factors, though less than with BROTOMAX®. A lower degree of induction (particularly in the case of the antioxidant activity) was found when SA and JA were applied as control treatments for induction of plant defense. HPLC-DAD and MS studies showed specific increase in the content of catechins, phenolic and chlorogenic acids in strawberry cell cultures

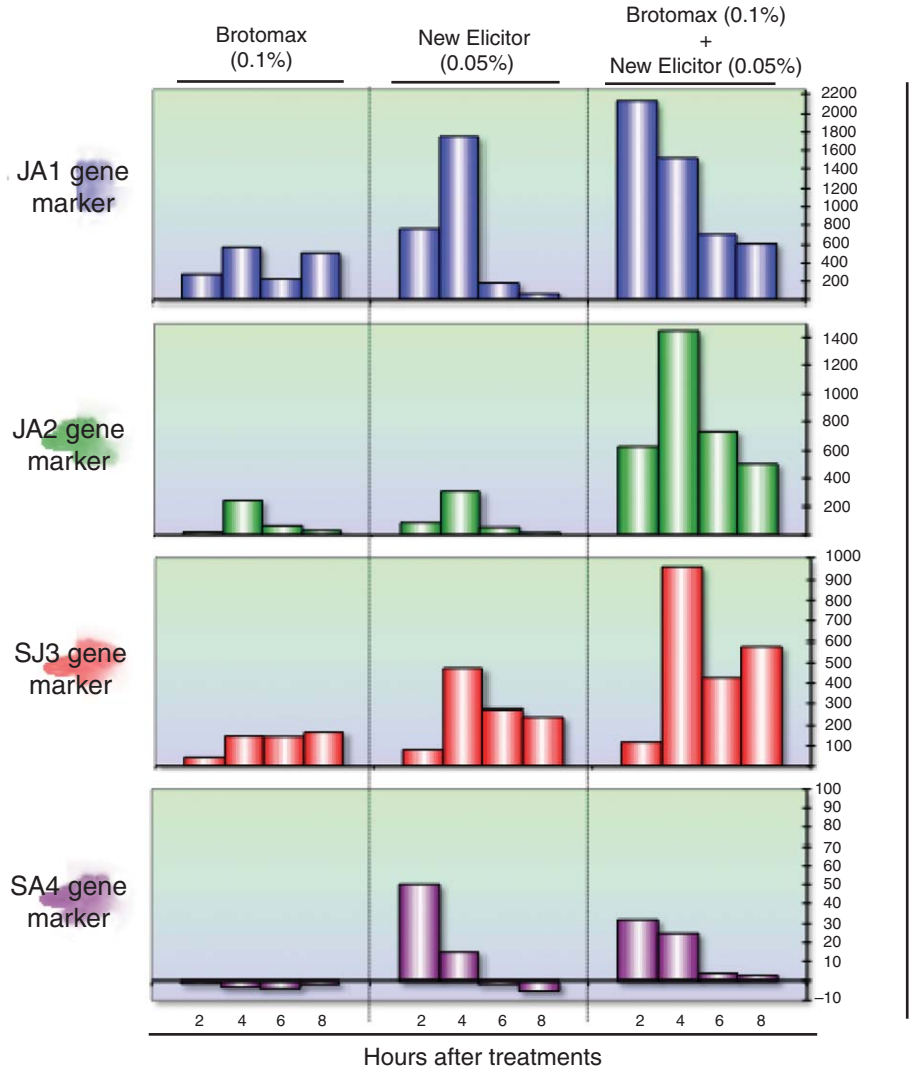

Fig. 1. Relative gene expression of known defense related markers in strawberry suspension cell culture after several treatments with: BROTOMAX® (0.01% w/v), EH (0.05% w/v) or a mix of both BROTOMAX® and EH (0.1% and 0.05% w/v, respectively). Samples were taken 2, 4, 6 and 8 h after treatment. Quantitative RT-PCR analysis was performed on total RNA prepared from these samples to determine transcript levels. Mean expression from three independent determinations is shown. The expression values were normalized using the expression level of the internal standard gene GAPDH and were relative to that at 0 h in every treatment set to one as described in Casado-Díaz et al. [15].

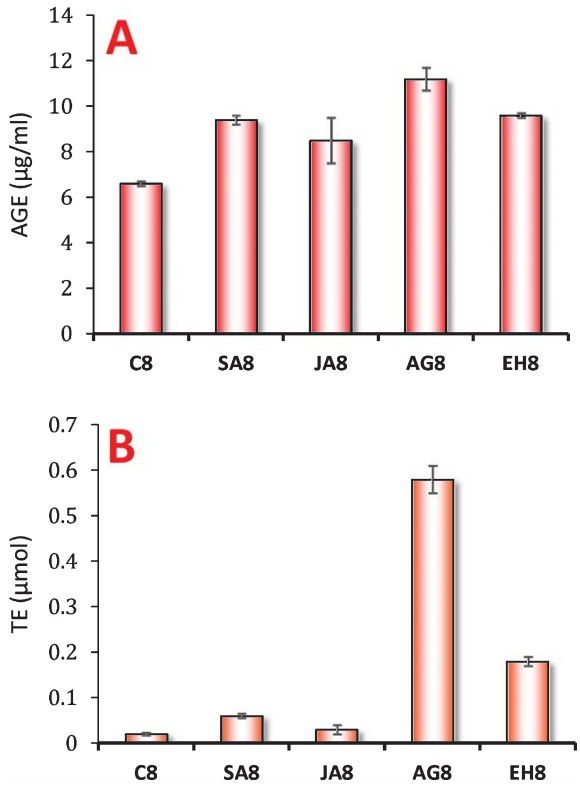

Fig. 2. Total phenolic compounds (A) and antioxidant activity (B) in methanolic extracts of strawberry cell suspension cultures treated with salicylic acid (SA8), methyl jasmonate (JA8), BROTOMAX® (AG8) and EH (EH8). Water control (C8). All cell cultures were assayed for 8 h.

Table 1
Disease control value (DCV %) of strawberry and olive plants infected with *Colletotrichum acutatum* or *Fusicladium oleaginum,* respectively. [a]3 DBI, [b]7 DBI, [c]7 DBI and [d]25 DBI. DBI, days before inoculation

Plant-pathogen interaction	DCV %	
	EH	BROTOMAX®
Strawberry *Colletotrichum acutatum*	42.4[a]	52.4[b]
Olive *Fusicladium oleaginum*	47.5[c]	49.8[d]

treated with a mix of both BROTOMAX® and EH (Fig. 3). However, the content of other polyphenols remained unchanged.

3.4. *EH and BROTOMAX® increased the strawberry (Fragaria x ananassa Duch. cv. Camarosa) and olive (Olea europaea L. cv. Picual) resistance against Colletotrichum acutatum and Fusicladium oleaginum infections, respectively*

Greenhouse experiments of strawberry and olive plants treated either with EH or BROTOMAX® and later infected with *Colletotrichum acutatum* or *Fusicladium oleaginum,* respectively, determined a clear and significant reduction of DCV in all DBIs assayed (results are summarized in Table 1). The effect of a simultaneous application of these two compounds (EH and BROTOMAX®) in both plant-fungal interaction systems remains to be tested.

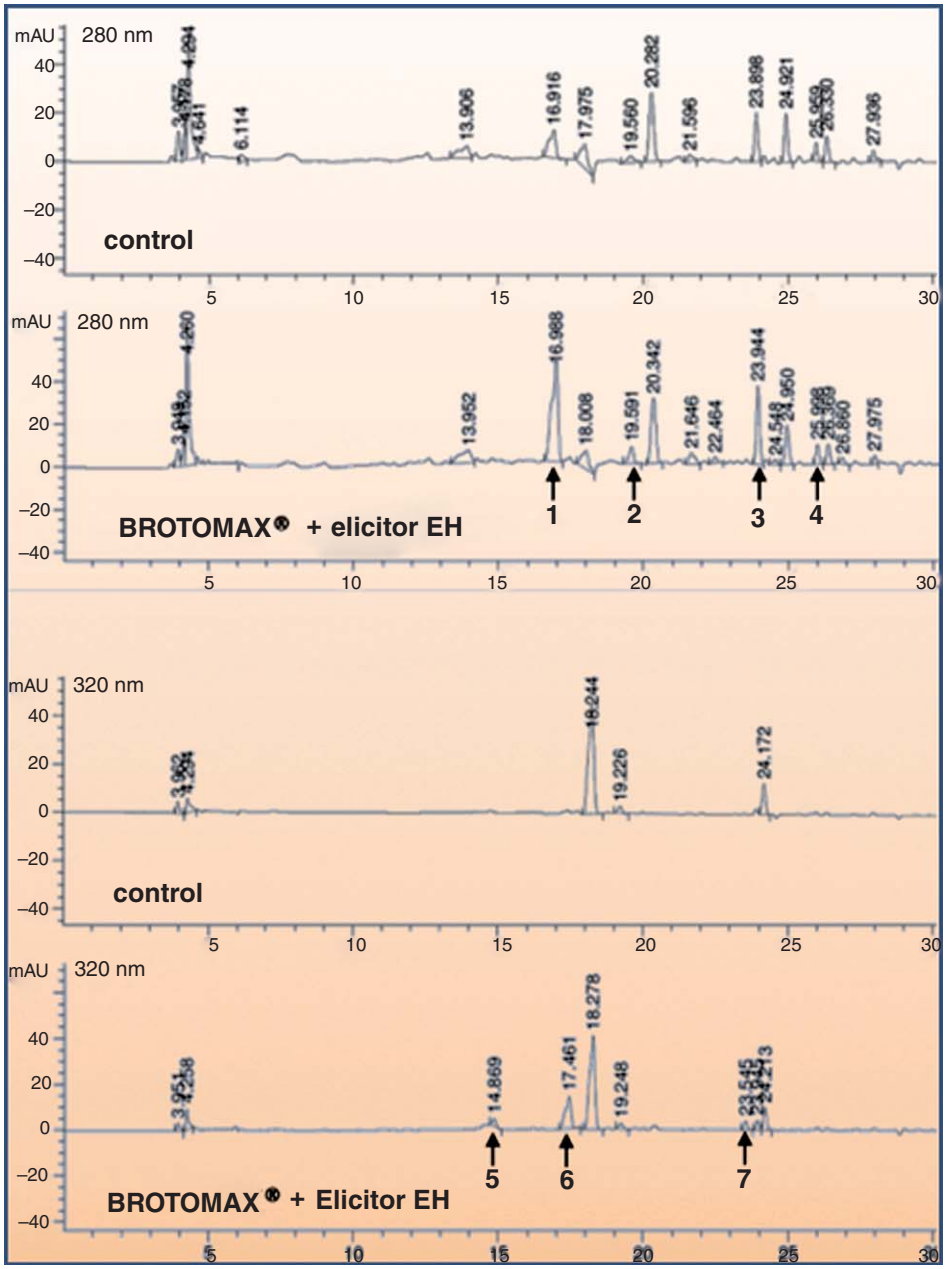

Fig. 3. HPLC-DAD chromatograms at 280 and 320 nm of methanolic extracts of strawberry cell suspension cultures treated with BROTOMAX® plus EH. Control: water. Arrows indicate different polyphenolic compounds identified by MS: 1 (+)-catechin and (−)-epicatechin, 2 phloretin-2' glucoside, 3 sinapoyl quinic acid, 4 benzoic acid, 5 caffeoyl 3-o-glucoside, 6 and 7 coumaroyl 4-o-glucoside.

4. Discussion

Induced resistance against pathogens and pest by active eliciting compounds, employed alone or as part of an integrated management approach, has emerged as a promising tool for fruit rot control while exerting low environmental impact and reducing the need for synthetic fungicides. Particularly promising are biologically active natural products arising from the plant itself or exogenous elicitors (coming from the pathogen or other natural sources),

which induce plant defense responses in plant. Because of their potential to elicit plant defense responses against pathogens, the biological activity of these putative natural bioinducers should be carefully and unmistakably tested. The use of suitable, sensitive, predictable and robust molecular biomarkers is completely necessary for this purpose. In the present paper we report on the production of a new natural elicitor, which is environmentally compatible and appropriate for ecological and sustainable agriculture, and the convenience of suitable defense related biomarkers, which allow us to predict its eliciting ability of plant defense response against fungal pathogens.

Thus, both BROTOMAX® and EH treatments activated the JA-dependent defensive pathways. In addition, EH increased the expression of the SA-dependent gene marker SA4. These results suggest that BROTOMAX® could activate plant defense preferentially through the JA-dependent signalling pathway. Contrastingly, EH was capable of inducing both SA- and JA-dependent defense related pathways. These molecular changes after treatments were also detected by metabolomic analysis, which indicated an increase in the cell content of new molecules with known defensive role (such as polyphenols and lignifying compounds).

As predicted by these molecular analyses, the application of these elicitor compounds either to strawberry or olive plants, in controlled greenhouse experiments, significantly reduced the disease severity index of infections by *C. acutatum* and *F. oleaginum,* respectively. On the other hand, this reduction cannot be attributed to an antifungal activity of the tested compounds against *C. acutatum*, as previously shown in the *in vitro* assays.

5. Conclusions

The use of suitable, sensitive, appropriate and robust molecular biomarkers should provide an excellent molecular tool to predict the effectiveness in the field of natural compounds with potential eliciting ability, which may be conveniently exploited as ecological bioinducers of plant defense response in many important crops.

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