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Effect of natural and synthetic Brassinosteroids on strawberry immune response against *Colletotrichum acutatum*

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Abstract Brassinosteroids (BRs) are steroidal essential compounds for plant growth and development. It was shown that the exogenous applications of BRs induce protection against different pathogens and can give plants tolerance/resistance to different abiotic stresses. The aim of this work was to evaluate the protective effect against the fungal pathogen *Colletotrichum acutatum*, the causal agent of anthracnose disease, on strawberry plants treated with 24-epibrasinolide (EP24) and a formulation based on a brassinosteroid spirostanic analogue DI-31 (BB16). Treatment with both compounds induced a defense response in strawberry plants of the cv. Pájaro against avirulent isolate (M11) of *C. acutatum*, being more effective at the lower concentration of both steroids (0.1 mg l^{-1}), although the analogue BB16 showed a stronger effect than EP24. The evaluation of biochemical defense markers showed that strawberry plants treated with EP24 and BB16 increased the production of H_2O_2, $O_2^{\cdot-}$, NO, calcium oxalate crystals and higher callose and lignin deposition as compared to the control plants. However, stomatal closure was only observed in plants treated with BB16. These results suggests that BB16 and EP24 can be used for the activation of innate immunity in strawberry plants, as a new strategy for crop health protection management, alternative to agrochemicals.

Keywords Brassinosteroids · Defense · Strawberry

Abbreviations

ANOVA	Analysis of variance
BB16	Biobras-16
BRs	Brassinosteroids
DAB	3,3-diaminobenzidine
DAF-FM-DA	4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate
DSR	Disease Severity Ratings
EBL	24-epibrassinolide
EP24	24-epibrasinolide
ET	Ethylene
H2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
IR	Induced resistance
JA	Jasmonic acid
MES	2-(N-morpholino) ethanesulfonic acid

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NBT	Nitro blue tetrazolium
PDA	Potato dextrose agar
ROS	Reactive oxygen species
SA	Salicylic Acid

Introduction

Strawberry anthracnose, caused by fungi of the genus Colletotrichum (Howard et al. 1992), is one of the major diseases that adversely affect the strawberry crop (Freeman and Katan 1997). Three species of Colletotrichum have been identified as the casual agents of the anthracnose disease in strawberry: *C. acutatum*, *C. fragariae* and *C. gloeosporioides* (Smith and Black 1990; Adaskaveg and Hartin 1997; Xiao et al. 2004). A survey carried out in the strawberry crop area of Tucumán (north-west Argentina) revealed the presence of the three species, although 85% of the isolates were identified as *C. acutatum* (Salazar et al. 2007).

In Argentina about 1300 ha of strawberry are grown annually (Kirschbaum et al. 2016; Kirschbaum and Hancock 2000). Strawberry is produced in many provinces of Argentina, although the largest production is concentrated in Tucumán, Santa Fe and Buenos Aires. Fruit activity in Argentina is relevant because it is highly labor-demanding and because 40% of the fruit goes to the industry, with the corresponding added value that this generates to the product (Kirschbaum et al. 2016).

In plants, the presence of steroid hormones is a relatively new discovery, compared with the knowledge of steroid function in animals. In the 70s, lipid extracts with growth promoting activity were isolated from *Brassica napus* pollen and were called "brassins" (Mitchell et al. 1970). Then, it was identified as a steroidal lactone and named brassinolide (BL) (Grove et al. 1979). Thereafter brassinolides and related compounds were generically called brassinosteroids (BRs).

More than twenty years ago, it was reported that exogenous application of brassinosteroids (BRs) protects plants against different pathogens (Vasyukova et al. 1994). It was later demonstrated that the application of a mixture of natural BRs isolated from seeds of *Lychnis viscaria* increased resistance to viral and bacterial pathogens in tomato, cucumber and tobacco. Biochemical analysis of these results showed that BRs elicited the activation of defense mechanisms in treated plants (Roth et al. 2000).

In 2003 the effect of BRs on the induction of resistance to viral, bacterial and fungal diseases in rice and tobacco was demonstrated (Nakashita et al. 2003), suggesting that these compounds are part of the plant immune system. These results served as the basis for numerous subsequent investigations. Xia et al. in 2009, demonstrated that reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber, and then, in 2011, they demonstrated that BRs induce a systemic stress tolerance in *Cucumis sativus*.

It was recently shown that the application of BRs reduces the concentration of the bacterium *Candidatus* Liberibacter asiaticus (the etiologic agent of citrus HLB disease), induces the expression of defense related genes, and other genes involved in the SA, JA/ET and brassinosteroid biosynthesis pathways (Coll 2015; Canales et al. 2016).

Since foliar applications of EP24 induced an oxidative burst accompanied by an increase of the levels of transcripts of genes related to the defense in roots, it was suggested that BRs can generate resistance to biotic stress by inducing the long-distance release of compounds such as reactive oxygen species (ROS), flavonoids or phenolic compounds with anti-stress activity (Ding et al. 2009).

Although several key components of the BRs signaling pathway involved in the regulation of plant growth and development have been identified (Gendron et al. 2012), the mechanism by which these compounds mediate stress tolerance is not fully understood.

Despite the advantages that seem to offer the use of BRs for the induction of tolerance to stress, the high production costs of these compounds due to its low concentrations in plant tissues, and its rapid metabolization after exogenous addition, makes it difficult to be used in agricultural formulations. For this reason, structural and functional analogues of natural BRs are being used as they are cheaper to obtain and exhibit longer half-lives in the plant.

In this research, we used 24-epibrasinolide (EP24) and a formulation based on the synthetic brassinosteroid spirostanic analogue DI-31 (active ingredient of the commercial formulation BIOBRAS 16). This compound has already been successfully tested in the field to increase lettuce production, resulting in a cheap and affordable alternative (Serna et al. 2012). DI-31 is characterized by the presence of a spiroketalic ring instead of the typical BR side chain, and was reported to exhibit an

activity similar to other brassinosteroid analogues such as BB6 and MH5 (Mazorra et al. 2002, 2004; Coll et al. 1995). It was shown that DI-31 counter acts the negative effects of different type of environmental stresses in several crops (Nuñez et al. 2003).

However, the effect of BRs on the induction of a strawberry defense response has not yet been reported. Therefore, the aim of this work was to evaluate the potential induction of defense against a fungal pathogen responsible of the anthracnose in strawberry plants treated with BB16 and EP24. In addition, biochemical and physiological markers of the defense response such as H_2O_2, $O_2^{\cdot-}$, NO, callose, lignin and stomatal closure in strawberry plants were investigated to verify the activation of defense mechanisms.

Materials and methods

Plant material

Strawberry plants (*Fragaria ananassa*) of the cv. Pájaro were provided by the strawberry BGA (Strawberry Active Germplasm Bank at Universidad Nacional de Tucumán). Healthy plantlets were obtained from in vitro cultures in MS medium (Sigma), rooted in pots with sterilized substrate (humus and perlite, 2:1), and maintained at 28 °C, 70% relative humidity (RH), with a light cycle of 16 h (white fluorescent, 350 μmol photons m^{-2} s^{-1}).

Fungal cultures

The fungal pathogen M11 of *C. acutatum* isolated and characterized in our laboratory was used in this paper (Salazar et al. 2007). The isolate was grown on potato dextrose agar (PDA) for 10 days under continuous light at 28 °C to induce conidial formation (Smith and Black 1990).

Induced resistance (IR) experiments against anthracnose disease

IR assays were performed as described by Salazar et al. (2007). Infection experiments were carried out with plants of the cv. Pájaro that received a double spray treatment until run-off on their aerial parts. The primary treatment was the application of BB16 or EP24 (water in control plants), and 3 days later the secondary treatment that consisted in the challenge inoculation with the infective conidial suspension of M11 (1.5 x 10^6 conidia ml^{-1}). Immediately after the second treatment plants were moved to a infection chamber (100% RH, 28 °C, in the dark) for 48 h and then were returned to the growth chamber (70% RH, 28 °C, light cycle of 16 h day^{-1}) where they remained for 30 days. Susceptibility to anthracnose disease was evaluated by Disease Severity Ratings (DSR) according to Delp and Milholland (1980). DSR were assessed on petioles using the following scale: 1, healthy petiole without lesions; 2, petiole with lesions <3 mm in length; 3, petiole with lesions from 3 to 10 mm; 4, petiole with lesions from 10 to 20 mm and girdling of petiole; and 5, entirely necrotic petiole and dead plant. DSR was evaluated at 30 dpi and corresponded to the most severe lesion observed in any of the three leaves evaluated per plant. The experimental design was a randomized complete block incorporating ten plants per IR experiment (for each BRs-treatment) and five plants corresponding to control treatment. Experiments were repeated three times to diminish the dispersion of DSR values.

Antifungal activity of BB16 and EP24

BRs were first tested for their ability to inhibit the growth of *C. acutatum* isolate M11 in vitro. Assays were performed in sterile Petri dishes containing PDA medium prepared with 5 ml of an upper layer of conidia suspended in soft agar (0.7%; 1.5 x 10^6 conidia ml^{-1}). M11 growth inhibition was tested at different concentrations of BB16 and EP24 (e.g. 0.05, 0.1, 1, 2, 10 mg l^{-1}). Fifty microliter of all extracts was loaded into wells made on previously prepared plates. Plates were incubated at 28 °C for 2 days, and the antifungal activity of extracts was assessed by the presence of inhibitory halos. The commercial fungicide Switch (Cyprodinil 37.5% + Fludioxonil 25%, Syngenta) at 0.8 mg ml^{-1} and plates without any inhibitor were used as positive and negative controls, respectively. The experiments were carried out in triplicate.

Plant cell suspension

Suspension of mesophyll cells were obtained from young recently expanded leaves of 8 week old strawberry plants. Leaf were homogenized in a potter with 10 ml of W5 modified buffer (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$, and 0.5 M sucrose, 2 mM MES,

pH 5.6) (Ramulu et al. 1995; Fujikawa et al. 2014), filtered through gauze, and then separated by centrifugation (500 × g for 5 minutes at room temperature). Cells were then washed twice with a sucrose solution (0.5 M) and resuspended in W5 modified buffer at 10^6 cells ml^{-1}. The cells were counted in a Newbauer chamber using a light microscope (Leica DM, U.S.A.). The cell suspension was kept in the dark at room temperature for three hours until use.

Intracellular nitrogen oxide determination

The intracellular NO accumulation was analyzed in cell suspensions by fluorometry according to Planchet and Kaiser (2006), modified in our laboratory. Aliquots of 1500 μl of cell suspensions (10^6 cells ml^{-1}) in W5 buffer were kept in the dark during 3 hours at room temperature. Then, suspensions were incubated with the fluorescent probe DAF-FM-DA (1 μM) during 15 minutes in the dark. Then the cells were washed once with W5 buffer and treated with BRs (0.1 or 1 mg l^{-1}). The control consisted in cells treated with distilled water. Fluorescence was measured 10 minutes post treatment at λex = 495 nm and λem = 525 nm with a Photon Counting spectrofluorometer (ISS-APC1, Owingen, Germany). Measurements were made with five technical replicates and experiments were carried out three times.

Oxidative burst

Accumulation of hydrogen peroxide was detected by a peroxidase-dependent in situ histochemical staining procedure using 3,3-diaminobenzidine according to Thordal-Christensen et al. (1997) and superoxide ion using a superoxide-dependent reduction of nitro blue tetrazolium (NBT) according to Doke (1983). Analyses were performed on plant leaves sprayed with brassinosteroids (e.g. BB16 or EP24) at a final concentration of 0.1 mg l^{-1}, and excised at 2, 4 and 6 hours post-treatment (hpt). Leaves were then incubated in a solution of 0.1% (w/v) DAB, 10 mM MES (pH 3.8) (for hydrogen peroxide detection) or in 0.1% (w/v) NBT, 10 mM sodium azide and 10 mM potassium phosphate (pH 7.8) without the addition of NADPH (for the detection of superoxide). Microscopic observations and photos were obtained with an Olympus BH-2 microscope. Water was used as control and specificity of the DAB staining was verified by adding 10 mM ascorbic acid. Five plants were used per treatment, and two leaves excised at the times mentioned above. Experiments were repeated three times.

Intracellular H_2O_2 determination

The accumulation of H_2O_2 was evaluated using the fluorescent probe H_2DCF-DA (Sigma-Aldrich) (Li et al. 2012; Ye et al. 2013). H_2DCF-DA is a fluorescent probe that reacts mainly with intracellular H_2O_2 but only after being transported and deacetylated by cell membrane deacetylases. Cell suspension aliquots of 100 μl (10^6 cells ml^{-1}) were treated with BRs (0.1 mg l^{-1}), and at different times (2, 4 and 6 hpt) 1.5 μl of H_2DCF-DA (10 μM) was added, mixed and incubated at 25 °C in the dark for 15 minutes before measuring. Fluorescence was measured at λex = 485 nm and λem = 525 nm with Photon Counting spectrofluorometer (ISS-APC1, Owingen, Germany). Control experiments consisted in cell suspension treated with water. Measurements were made with 5 technical replicates and experiments were carried out three times

Calcium oxalate accumulation

The accumulation of calcium oxalate crystals was studied in leaves of strawberry plants of cv. Pájaro, treated by spraying BB16 or EP24 at a final concentration of 0.1 mg l^{-1}. The leaves were collected at 4 hpt and the evaluation was performed by observing sections of foliar tissue from three leaflets of three different plants that received the same treatment. The leaves were decolorized by heating in 95% (v/v) ethanol and clarified by immersion in a solution containing lactic acid/glycerol/H_2O (3:3:4) for 24 hours. The calcium oxalate crystals were observed with a polarized light microscope (Zeiss AXIO Lab. A1, Germany). Experiments were repeated three times.

Stomata closure measurement

The induction of stomata closure was studied on leaves of Fragaria ananassa. Plant leaves were treated with BB16 or EP24 (0.1 mg l^{-1}) and collected at 6 hpt. Histological sections of abaxial epidermis were observed under the microscope (Carl Zeiss Axiostar Plus, Germany). The control consisted in plants treated with

water. Stomata apertures were recorded on a digital camera and the width of the aperture was analyzed with a graphic analysis software (ImageJ). Measurements were made on ten central leaflets of three treated plants. Experiments were repeated three times.

Analysis of callose deposition and lignification

To analyze lignin and callose deposition, strawberry plants were sprayed with BB16 or EP24 (0.1 mg l^{-1}) and ten central leaflets collected at 9 days post-treatment (dpt). For the observation of callose deposition, "Method III" (Martin 1959) was used, with modifications. Fragments of the middle portion of the leaflet were treated with KOH 10% until clarification. The material was then washed and stained with 0.05% aniline blue in 0.15 M KH_2PO_4, kept in the dark for 2 hours, mounted in 30% glycerol and examined by fluorescence with an Olympus microscope (model BXS1) equipped with U-LH 100HG reflected fluorescence system.

For the detection of lignin, the phloroglucinol test was used (D'Ambrogio de Argüeso 1986). Cross sections from the middle portion of the leaflets were clarified with HClO 5.25%, washed with distilled water, stained with phloroglucinol, softly flamed in a Bunsen burner and finally two drops of HCl (25%) were added to the preparation. Experiments were repeated three times.

Statistical analyses

The statistical analyses of the data were carried out using the InfoStat software (Di Rienzo et al. 2013). All data were obtained from at least three independent experiments, and expressed as mean ± standard error, where corresponded. The data were also analyzed by one-way variance analysis test (ANOVA), and the means were separated using Tukey's test for $p < 0.05$.

Results

Evaluation of BB16 and EP24 induction of strawberry resistance against anthracnose

In order to study the protective effect of brassinosteroids treatment against M11, an IR test was carried out as explained above. The results showed a slight protection when using EP24 0.1 mg l^{-1} (DSR=3), while EP24 at a concentration of 1 mg l^{-1} showed no effect on anthracnose protection (DSR=5) (Fig. 1). On the other hand, with BB16 both concentrations induced a protective effect against the disease. Despite of not observing significant differences between both concentrations of BB16, phenotypically, it was possible to appreciate a better physiological state of the plants treated with at the concentration of 0.1 mg l^{-1}. Finally, water-treated control plants showed the irreversible progress of the disease, presenting severe necrosis at 10 dpi and plant death before 20 dpi (Fig. 1).

Antimicrobial effect of BRs

We evaluated in vitro the behavior of M11 growing on a PDA plate in the presence of different concentration of BB16 or EP24 (Fig. 2). As positive and negative controls the commercial fungicide Switch (0.8 mg ml^{-1}, Syngenta), and sterile PDB medium were used, respectively. Results presented in Figure 2 show that none of the BRs exhibited inhibitory effect on M11 growth regardless of the concentration tested; whereas the commercial fungicide exhibited a clear inhibition growth hallo around the well.

Effect of BRs on NO production

Nitric oxide production was evaluated by fluorometry on cell suspensions treated with BB16 or EP24 at concentrations of 0.1 mg l^{-1} and 1 mg l^{-1}. Results showed that both BRs caused a rapid accumulation of NO at concentrations of 0.1 mg l^{-1} (Fig. 3). Nevertheless, NO production showed no significant difference on water-treated cells (control) when treated with 1 mg l^{-1} of BB16 or EP24.

ROS accumulation

The production of hydrogen peroxide and superoxide anion in plants exposed to BB16 or EP24 are presented in Fig. 4. Since the best protection effect (Fig. 1) and NO production (Fig. 3) were observed when plants were treated with 0.1 mg l^{-1} we decided to evaluate the oxidative burst at this concentration. In these experiments, leaves were collected at 2, 4 and 6 hpt. Results showed that plants sprayed with BB16 or EP24 produced a

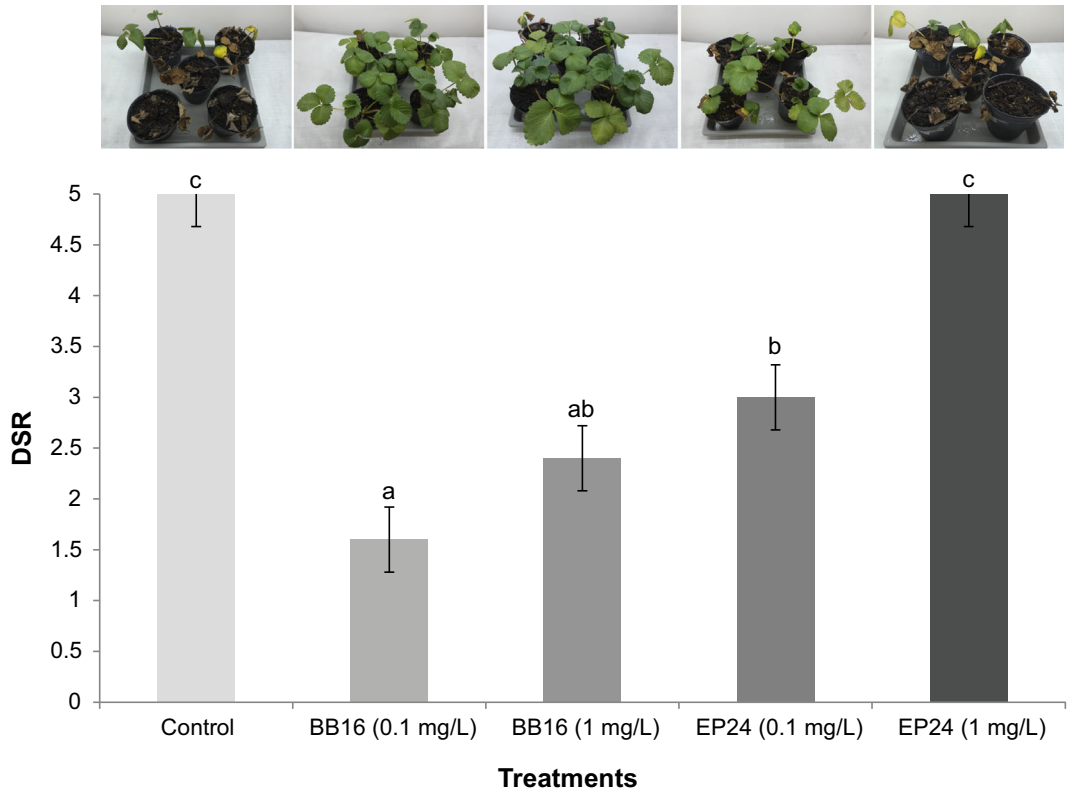

Fig. 1 Protection effect exerted by BB16 and EP24 on strawberry plants of cv. Pájaro against the virulent isolate M11 of *C. acutatum*. DSR values were evaluated at 30 dpi on plants inoculated with the virulent isolate M11 that were previously treated with either BRs or water (Control). DSR values with different letters correspond to statistically different values (Tukey test, $P < 0.05$)

strong and transient accumulation of hydrogen peroxide (Fig. 4a) and superoxide anion (Fig. 4b) within the first hours exhibiting a maximum production of both ROS at 4 hpt.

Since the use of DAB can bring about false positives and incorporate some ambiguous results, the accumulation of H_2O_2 was also evaluated using the intracellular fluorescent probe H_2DCF-DA and a mesophyll cells

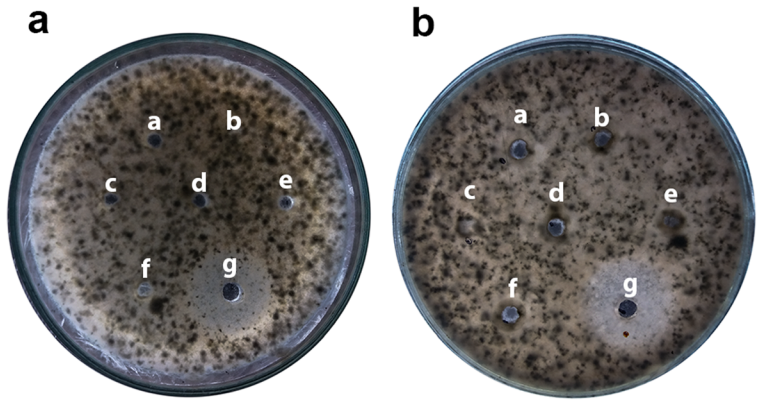

Fig. 2 Antifungal activity of BRs on the growth of the virulent isolate M11 of *C. acutatum*. M11 isolate was spread on a PDA plate and incubated for 48 h in presence of different concentrations of (**a**) BB16 or (**b**) EP24. The concentrations evaluated were a) 0.05 mg l^{-1}, b) 0.1 mg l^{-1}, c) 1 mg l^{-1}, d) 2 mg l^{-1} and e) 10 mg l^{-1}. Controls consisted of commercial fungicide Switch (Cyprodinil 37.5% + Fludioxonil 25%, Syngenta) at 0.8 mg ml^{-1} (g) and sterile PDB medium (f)

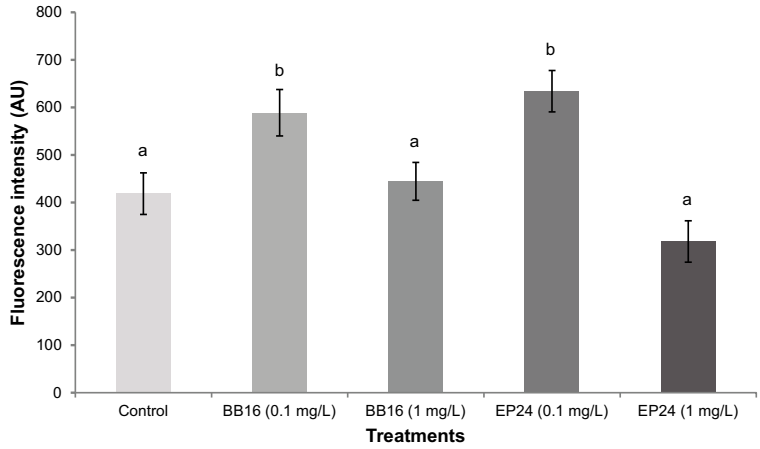

Fig. 3 NO production of strawberry cells evaluated as intracellular fluorescence intensity of DAF-FM-DA (λex = 495 nm; λem = 515 nm) 10 min after treating with distilled water (control) or BRs (0.1 and 1 mg l^{-1}). The values represent five repetitions. Bars represent relative errors. The comparison between two groups was made using Tukey test, $p < 0.05$. Different letters denote significant difference

suspension. The latter allowed us to evaluate with greater precision the temporal production of H_2O_2. Results obtained corroborated the result obtained in histochemical staining observations with DAB (Fig. 5).

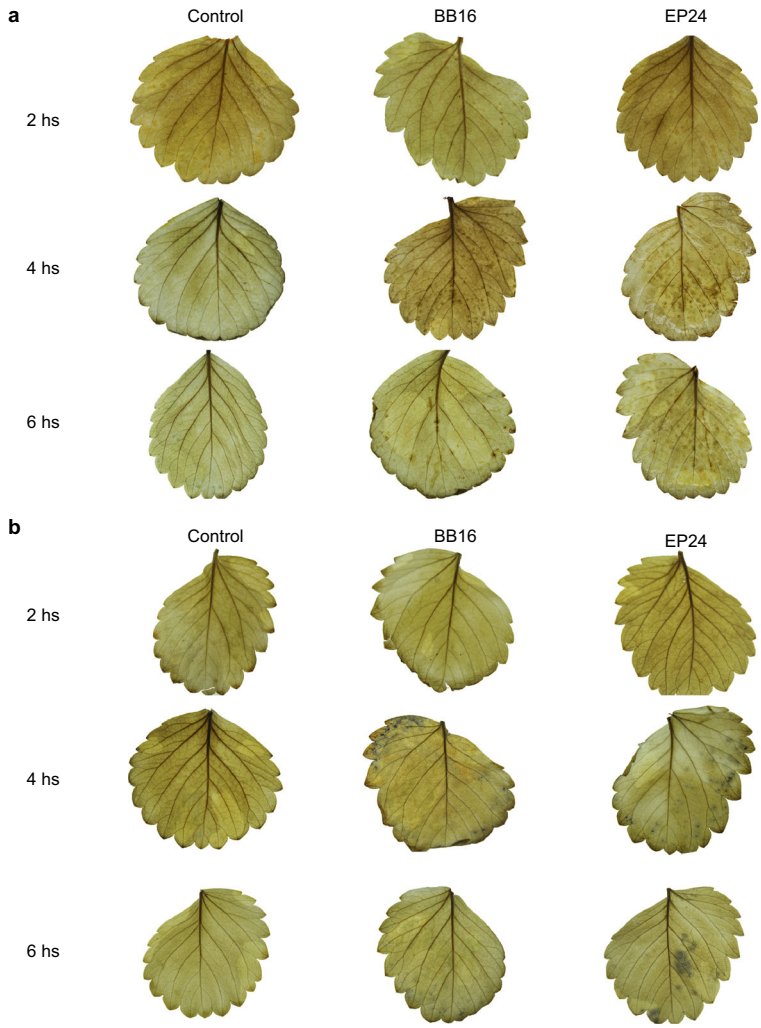

Fig. 4 Hydrogen peroxide (**a**) and superoxide anion (**b**) detection on leaflet tissue of strawberry plants treated with: water (control), BB16 (0.1 mg l^{-1}) and EP24 (0.1 mg l^{-1}) 2, 4 and 6 hpt

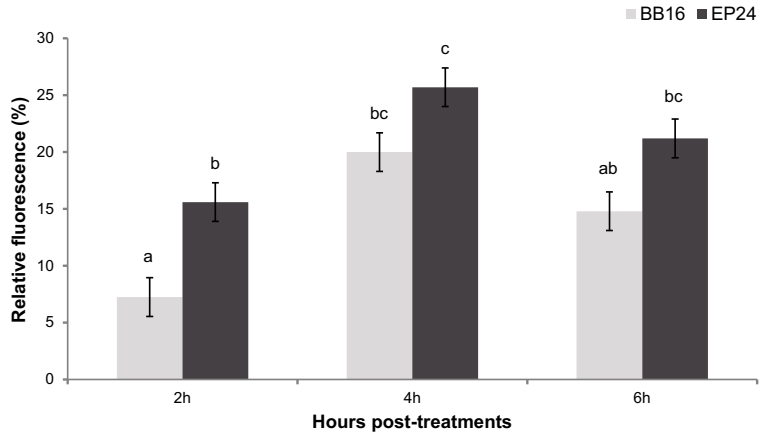

Fig. 5 Intracellular content of H_2O_2 after BB16 or EP24 (0.1 mg l^{-1}) treatment. Fluorescence of H_2DCF-DA was evaluated at λex = 485 nm and λem = 525 nm and is reported as the fluorescence change with respect to untreated cells. Values are means of five independent experiments. Bars represent standard errors (mean values ± SE, $n = 5$)

BB16 induced stomatal closure in strawberry

Effects of BRs on fully opened stomata were studied in *Fragaria ananassa* (Fig. 6). Application of BB16 at 0.1 mg l^{-1} caused stomata closure by 40%. However, application of EP24 at 0.1 mg l^{-1} did not cause any change of stomata aperture.

Accumulation of calcium oxalate crystals

Another anatomical feature observed after the treatment with both BRs was the appearance and accumulation of intracellular calcium oxalate crystals mainly at mesophyll cells. The spray treatment with a solution of BB16 or EP24 at 0.1 mg l^{-1} to strawberry plants caused a significant accumulation of calcium oxalate crystals, which was 170% and 105% higher than in water treated control plants, respectively (Fig. 7).

Lignin and callose accumulation

To analyze lignin and callose deposition (Fig. 8), strawberry plants were sprayed with BB16 or EP24 (0.1 mg ml^{-1}) and leaves collected at 9 dpt. The control samples (treated with water) and both treatments evidenced the presence of lignin in the vascular system xylem, being higher in the treated plants.

Callose deposition in control plants was observed as light blue patches only in the base of the eglandular trichomes, whereas in cells treated with both brassinosteroids, the deposition of callose was observed

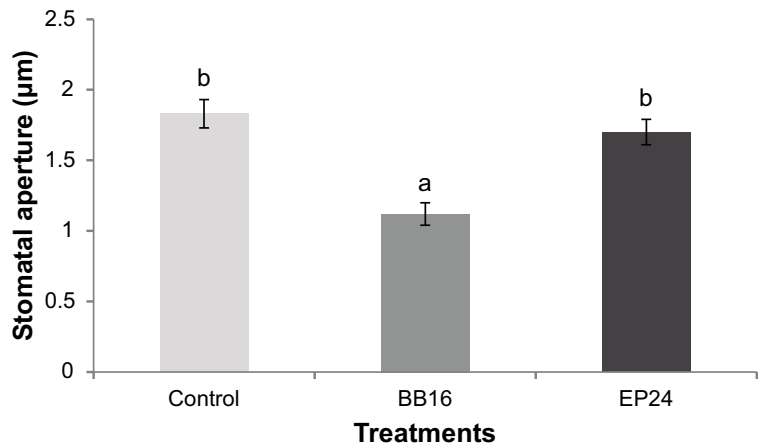

Fig. 6 BR-induced stomata closure in *Fragaria ananassa* plants, treated with BB16 or EP24 (0.1 mg l^{-1}) were collected at 6 hpt. The stomata apertures were observed by light microscopy and the width of the aperture was analyzed with graphical analysis software (ImageJ). The values represent five repetitions. Bars represent relative errors. The comparison between two groups was made using Tukey's test ($p < 0.05$). Different letters denote significant differences

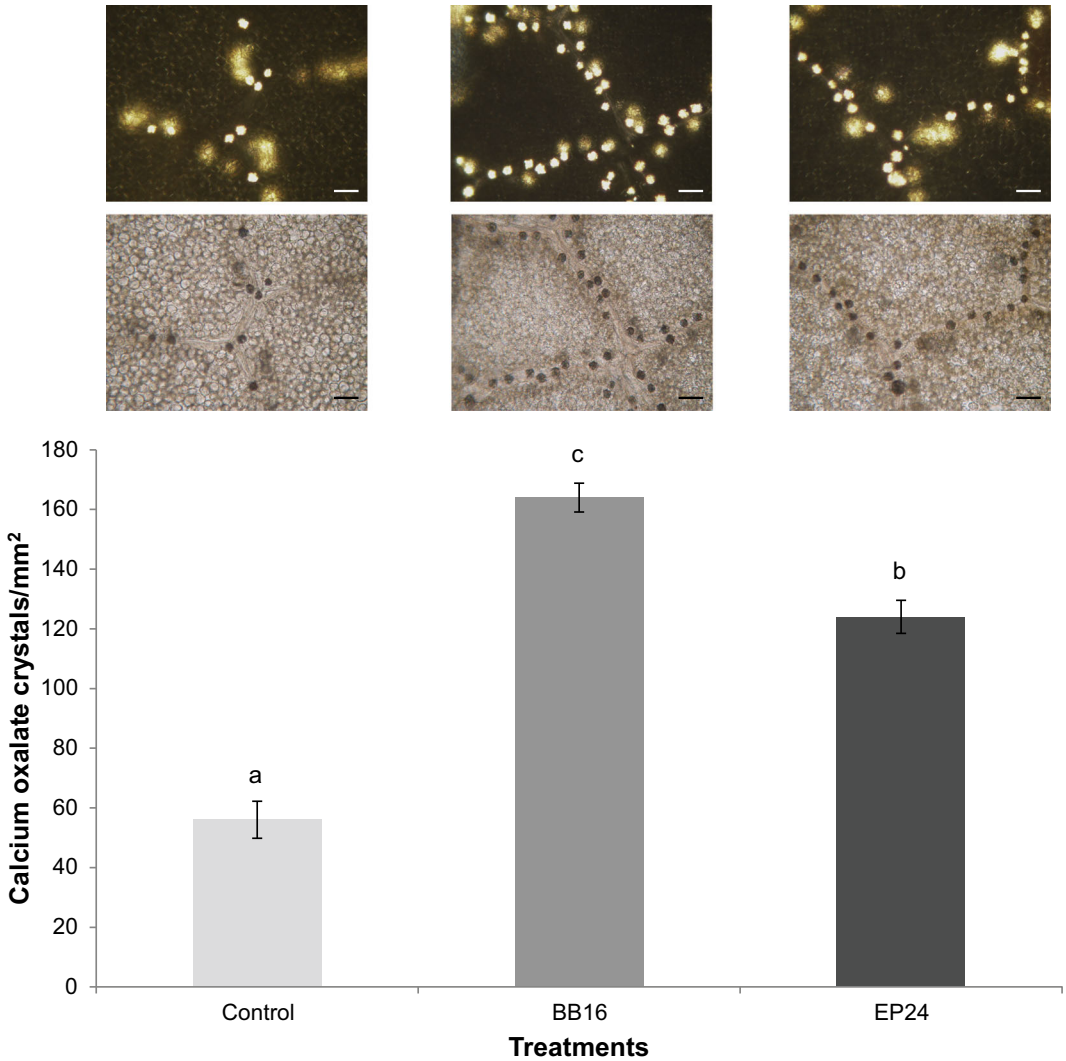

Fig. 7 Density of calcium oxalate crystals induced by BB16 or EP24 (0.1 mg l^{-1}) in strawberry plants of cv. Pájaro. The comparison between two groups was made using Tukey test, $p < 0.05$. Different letters denote significant difference. Representative micrographs showing the accumulation of calcium oxalate crystals (druses) induced by BB16 and EP24 in the parenchyma cells of strawberry plants of cv. Pájaro is presented. Micrographs were taken 4 hpt. Scale bars correspond to 100 μm

not only in the eglandular trichomes, but also at the palisade parenchyma.

Discussion

Phytopathogenic assays revealed that BB16 exhibited a stronger protective effect than EP24 at the concentration of 0.1 mg l^{-1}, whereas at a concentration of 1 mg l^{-1} the effect could also be observed, but to a lesser extent. The protective effect of EP24 was only observed at the concentration of 0.1 mg l^{-1}, but this effect was lower than that observed with BB16. To rule out that the plant protection effect observed was due to antifungal properties of BRs against M11, the behavior of M11 growing in the presence of both BRs was evaluated. It was found that none of the BRs exhibited inhibitory effect on M11 growth regardless of the concentration tested; whereas the commercial fungicide exhibited a clear inhibition growth hallo around the well. These results clearly indicated that BRs do not inhibit the growth of the isolate M11, suggesting rather that the protection effect of BRs against anthracnose was due to a response of Induced Resistance (IR) triggered in the plant.

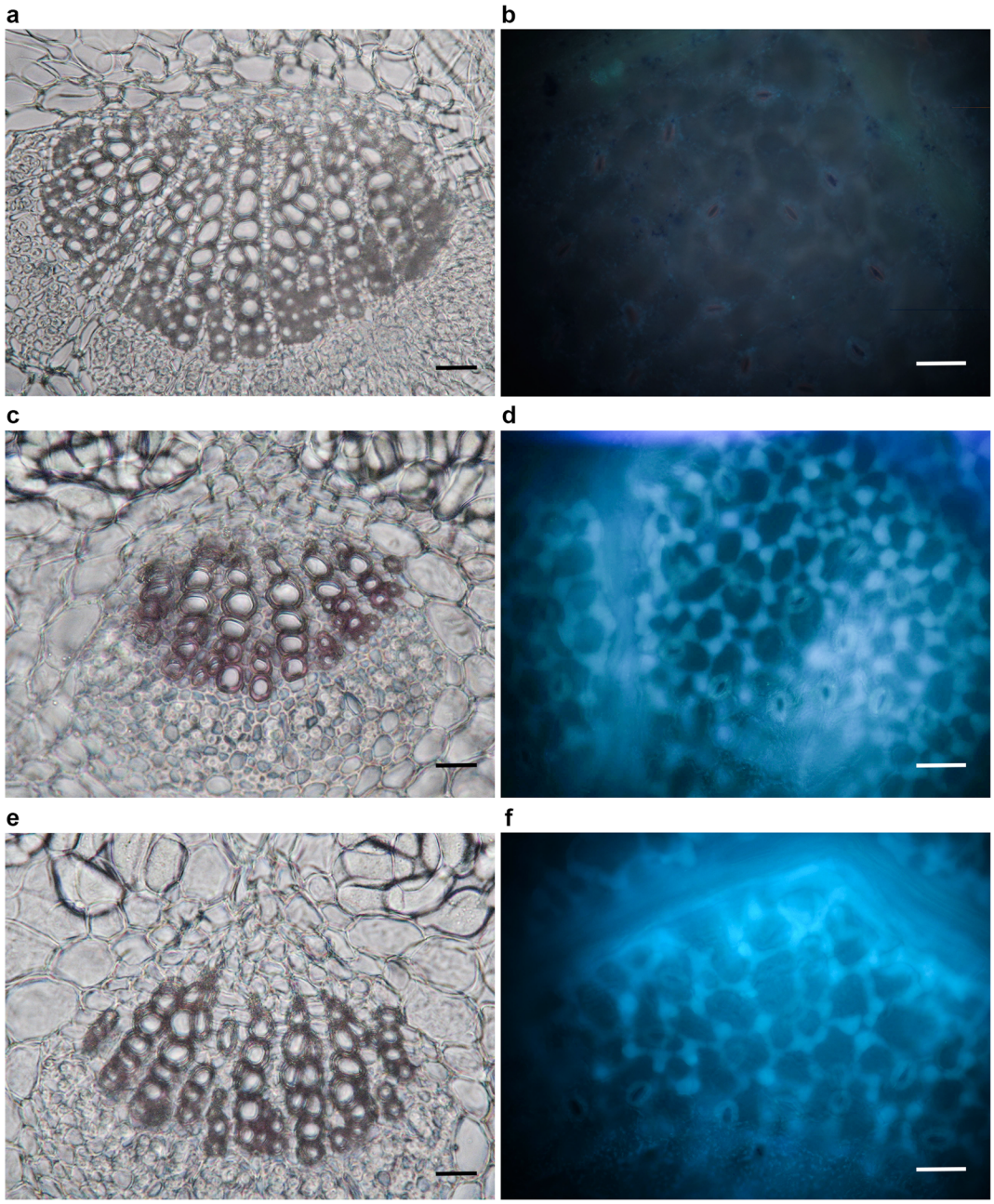

Fig. 8 Accumulation of lignin and callose induced by BB16 or EP24 in strawberry plants of cv. Pájaro. Lignification (**a**, **c** and **e**) and callose deposition (**b**, **d** and **f**) were visualized with phloroglucinol and aniline blue, respectively, in foliar tissue of plants treated with water (**a** and **b**), BB16 (**c** and **d**) and EP24 (**e** and **f**). Micrographs were taken 9 dpt. Each micrograph represents an example of the 20 leaflets analyzed coming from four plants. Scale bars correspond to 30 μm

The effect of brassinosteroids as positive regulators of plant defense against a wide range of fungal, bacterial or viral pathogens has already been studied (Bajguz and Hayat 2009; Nahar et al. 2013; Xia et al. 2009). In tomato, for instance, it is known that BRs induce local resistance against *Cucumber mosaic virus* (CMV) and systemic resistance against Fusarium pathogens (Nie et al. 2013; Xia et al. 2009, 2011). In citrus, the effect of epibrasinolide as a possible control strategy against HLB was studied. Epibrassinolide applied as a foliar

spray to citrus plants infected with the causal agent of HLB, '*Candidatus* Liberibacter asiaticus' exhibited a reduction of bacterial titers and an up-regulation of known defense genes in leaves, such as glutathione peroxidase, chitinase, β-1, 3 - glucanase, allene oxidase synthase, hydroperoxidelyase, and phenylalanine ammonia lyase (Canales et al. 2016).

It is known that in the activation of the defense response are involved several biochemical and molecular events triggered by the interaction between the elicitor and the plant component that act as target (Boller and Felix 2009; Garcia-Brugger et al. 2006; Thakur and Sohal 2013; Zhao et al. 2005). These events include oxidative bursting (ROS accumulation), calcium influx, changes in gene expression leading to increased production of defense compounds and proteins, strengthening of the secondary wall by deposition of callose and lignin, and stomata closure, among others.

It is well known that ROS act as secondary messengers in stress and hormonal responses (Apel and Hirt 2004; Kwak et al. 2006). Analysis of $O_2^{\cdot-}$ and H_2O_2 revealed a noticeable accumulation mainly at 4 hpt. Previously, is was reported that strawberry plants (*Fragaria ananassa*) treated ectopically with a fungal defense inducer (AsES) induced a strong oxidative burst with a transient accumulation of $O_2^{\cdot-}$ and H_2O_2 with a maximum accumulation at 4 hpt (Chalfoun et al. 2013). In the case of brassinoesteroids, Xia et al. (2009) demonstrated that treatment with BR in cucumbers improves the activity of the enzyme NADPH oxidase, leading to the accumulation of H_2O_2 at 3 hpt. Our results confirm those observations, demonstrating the importance of ROS in the induction of brassinosteriods-mediated defense response.

The deposition of callose on the cell wall can be observed mainly in roots and leaves in response to the penetration of the cell wall by the pathogen or the perception of Pathogen Associated Molecular Patterns (PAMPs, Galletti et al. 2008, Millet et al. 2010). Strengthening of the cell wall contributes to the innate immune response in strawberry plants challenged with a pathogen (Amil-Ruiz et al. 2011).

In the present study, the accumulation of callose was clearly observed in the cell wall palisade parenchyma (Fig. 8) of strawberry plants treated with both BRs (BB16 and EP24).

In agreement with these results, other authors have also observed the callose deposition in plants of strawberry plants upon treatment with plant growth-promoting bacteria (Tortora et al. 2012) and an avirulent pathogen (Salazar et al. 2007).

Lignification is another mechanism activated during the onset of disease resistance in plants. During defense responses, lignin (a major component of the cell walls of vascular plants) accumulation was shown to occur in a variety of interactions between plants and microorganisms (Vance et al. 1980; Nicholson and Hammerschmidt 1992). It was reported that lignin accumulates in structures called papillae at the sites of penetration attempts of biotrophic fungi and in surrounding areas (Vance et al. 1980; Nicholson and Hammerschmidt 1992; Zeyen et al. 2002), hence it is considered a first-line defense against successful penetration of invasive pathogens.

Results obtained in this paper showed a higher accumulation of lignin at the xylem of the vascular system in strawberry plants treated with brassinosteroids in relation to the control plants. The thickening of the secondary wall of the xylem may have relevance in the defense of the plants, since it could cause a restriction of the access of pathogens to the cells of the xylem parenchyma. Lignified walls in xylem vessels may further provide a barrier to the transfer of materials essential for the virulence of pathogens into plant cells.

The NO, a reactive nitrogen species, plays an important role as a signaling component during plant-pathogen interactions, plant resistance, hypersensitive response (HR) and expression of defense-related genes (Lamattina et al. 2003; Trapet et al. 2014; Baudouin 2011). During defense responses, NO interacts with various upstream and downstream signaling molecules, including mitogen-activated protein kinase (MAPK), reactive oxygen species (ROS), cyclic nucleotides and free Ca^{2+} (Gaupels et al. 2011). Apart from its effective role in plant defense, NO also plays an important role in stomata closure induced by ABA, defense inducers or molecular pathogen associated pathogens (PAMPs) (Garcia-Mata et al. 2003). Results presented in this paper show that BRs cause a rapid accumulation of NO in the cells (Fig. 3). These results agree with numerous reviews on the importance of NO during innate immunity responses in plants (Trapet et al. 2014; Gaupels et al. 2011; Yoshioka et al. 2011; Simontacchi et al. 2013).

Another defense marker analyzed, which also has an important relationship with NO, is the stomata closure. Interestingly, whereas the application of BB16 caused a noticeable stomata closure, EP24 did not cause any

significant difference. Since stomata are the entry gates not only for metabolic gases (e.g. O_2, CO_2 and H_2O) (Hetherington and Woodward 2003), but also for microorganisms (Agurla et al. 2014), stomata closure is essential to prevent the entry of pathogens into leaves tissues, therefore its regulation is essential for the plant innate immune response (Zeng et al. 2010; Melotto et al. 2014). The higher level of stomata closure observed with BB16 (Fig. 6) is coherent with the higher level of plant protection achieved this BB16 compared to EP24 (Fig. 1).

The occurrence of calcium oxalates and the diverse crystal shapes, sizes, prevalence and spatial distribution, have led to a number of hypotheses regarding to their function in plant, namely: calcium regulation, ion balance (e.g. sodium and potassium), plant protection, tissue support (plant rigidity), detoxification (e.g. heavy metals or oxalic acid), and light gathering and reflection (Franceschi 2001; Franceschi and Horner 1980). In this study, we observed that the spray application of BB16 (0.1 mg l^{-1}) and EP24 (0.1 mg l^{-1}) to strawberry plants caused a significant accumulation of calcium oxalate crystals, which was 170% and 105% higher than in water treated control plants, respectively. These results let us speculate that Ca^{2+} signaling, the metabolism of calcium oxalate and crystal formation play important roles on the induction of the defense response in strawberry plants treated with BRs. It has been reported that some plants utilize oxalate to detoxify hazardous metals, such as lead (Yang et al. 2000), aluminum (Ma et al. 2001), strontium (Franceschi and Schueren 1986; Zindler-Frank 1991), and cadmium (Choi et al. 2001), when present in the environment. The latter strongly suggests that the oxalate formation is strongly associated to a defense mechanism against different kind of stresses.

However, more detailed and exhaustive studies should be carried out to elucidate this issue. Likewise, the differential effect observed between BB16 and EP24 on strawberry plants requires a deeper investigation. We may hypothesize nevertheless, that the change of the aliphatic lateral chain comprised between the C21-25 of the EP24 D ring, to the spirostanic rings, and/or the oxygens of the spirostanic ring exposed to the media enhances (or changes) the biding to the BRI1/BAK1 receptors (Wang et al. 2001; Nam and Li 2002). Further studies would be required to unveil the nature of the differential effect observed.

The capacity of brassinosteroids to activate the innate immunity, in addition to its already proven growth promoting effects opens new avenues to use these compounds as an environmentally safe alternative to the toxic agrochemicals, normally used in strawberry crop, with possible application to other cultivated species.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The manuscript has not been published and is not under consideration for publication elsewhere. All authors have approved the manuscript and agree with submission to European Journal of Plant Pathology. The research was conducted in the absence of any commercial relationships that could be considered as a potential conflict of interest.

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