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Riboflavin induces resistance against *Botrytis cinerea* in bean, but not in tomato, by priming for a hydrogen peroxide-fueled resistance response

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ABSTRACT

Riboflavin (vitamin B2) can act as an activator of plant resistance against biotic stress. Here, we demonstrate the effect of exogenous application of riboflavin on bean and tomato resistance against *Botrytis cinerea*. In bean, riboflavin applied at a concentration of 10 up to 1000 μM reduced the number of spreading lesions by approximately 25% compared to control plants. In tomato, however, riboflavin was not able to control the pathogen at any concentration tested. Hydrogen peroxide accumulation was detected in bean leaf discs of riboflavin-treated plants as early as 4 h post inoculation (hpi) while in tomato leaf discs, no hydrogen peroxide accumulation was detected prior to 48 hpi irrespective of riboflavin treatment. Stimulation of lipoxygenase activity was observed in bean, but not in tomato plants treated with riboflavin, while jasmonate could trigger lipoxygenase activity and resistance to *B. cinerea* in both bean and tomato. Riboflavin may prime bean plants for earlier accumulation of hydrogen peroxide and prompt activation of the lipoxygenase pathway when challenged with *B. cinerea*.

1. Introduction

Induced resistance (IR) is a phenomenon whereby plant resistance to pathogens is enhanced after treatment with biotic or abiotic inducing agents. These agents enhance the plant's basal level of resistance by directly activating defence responses or priming the plant for a faster and stronger defence response after pathogen challenge [1,2]. Besides several well-studied abiotic inducers [3], vitamins appear as potential organic compounds to be used as elicitors of IR in plants.

Effective control of diverse pathogens has been achieved with foliar spray of different vitamins alone or in combination with other compounds, i.e., riboflavin [4–9], thiamine [10–12], a mixture of riboflavin and methionine [13–16], niacin [7], and menadione sodium bisulphite (MSB) [7,17]. With respect to riboflavin (vitamin B_2), it has been shown that foliar application triggers resistance not only in dicot plants such as Arabidopsis and tobacco against several oomycete, fungal, bacterial and viral pathogens [6,44] but also in monocots like rice and pearl millet against *Rhizoctonia* spp. and *Sclerospora graminicola,* respectively [7,9]. Moreover, riboflavin has been shown to suppress disease caused by both foliar pathogens, such as *Hyaloperonospora arabidopsidis* in Arabidopsis and *Alternaria alternata* in tobacco [6], and the soil-borne microbes *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomia phaseolina* in chickpea [5]. In addition, application of riboflavin may enhance tolerance to abiotic stress, as was recently demonstrated in *Hibiscus sabdariffa* seedlings [18].

Riboflavin is biosynthesized by plants and many microorganisms. It is the precursor of the flavin cofactors flavin mononucleotide and flavin adenine dinucleotide, which are involved in several different physiological processes in plants [19]. The role of riboflavin in plant defence reactions is poorly understood since only a limited number of plant-pathosystems have been studied. In Arabidopsis, riboflavin-IR against the bacterium *Pseudomonas syringae* pv. *tomato* has been associated with priming for enhanced hydrogen peroxide (H_2O_2) generation, cell death and callose deposition, and was found to act independently of the classic defence signalling pathways involving the hormones salicylic acid (SA), jasmonates (JA) and ethylene (ET) [4]. However, riboflavin-IR against rice sheath blight seems to be JA-dependent [20,21].

To expand our understanding of the mechanisms involved in the riboflavin-IR, we investigated the ability of exogenously administered riboflavin to induce resistance in bean and tomato plants against the fungus *Botrytis cinerea* Pers. ex Fr., causal agent of the economically important grey mould disease. Furthermore, we explored the function of H_2O_2 accumulation and activation of the JA pathway in the onset and maintenance of riboflavin-inducible resistance in the latter plant-pathosystems.

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2. Materials and methods

2.1. Pathogen and inoculum preparation

Botrytis cinerea Pers. ex Fr. isolate R16 [22] was grown on Potato Dextrose Agar medium (PDA) at 22 °C under UV/dark (12 h/12 h) for 10 days. Spores were washed from the plates with distilled water containing 0.01% (v/v) Tween 20 and filtered. The conidial suspension was centrifuged for 10 min at 10,000*g*, the pellet re-suspended in distilled water and the conidia concentration determined with a haemocytometer. The final conidial suspensions were adjusted to 10^5 conidia ml^{-1} and contained 0.01 M glucose and 6.7 mM KH_2PO_4 and 0.02 M glucose and 13.4 mM KH_2PO_4 for bean and tomato inoculations, respectively. To synchronize conidia germination the suspensions were incubated for 2 h at 22 °C before being used [23–25].

2.2. Plant material and inoculation method

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker) were grown for five weeks and bean plants (*Phaseolus vulgaris* L. cv. Prelude Royal) for two weeks in potting soil (Potgrond Structural; Snebbout, Kaprijke, Belgium) under greenhouse conditions (at 24 ± 2 °C with 16/8 h light/dark photoperiod and 70% humidity).

Tertiary leaves of tomato plants and primary leaves of bean plants were used for inoculations with *B. cinerea* using the detached leaf method, as described by Audenaert et al. [24]. Ten 5-μl and 10-μl droplets of the conidial suspension were applied on each tomato and bean leaf, respectively. The leaves were placed in plastic trays with high humidity and incubated at 22 °C under dark condition. Disease development was evaluated 3 and 4 days later by recording the number of spreading and non spreading lesions. Each experiment was carried out at least twice with 12 leaves from different plants per treatment.

For leaf disc assays in 24-well plate (VWR, Leuven, Belgium), one 10-μl droplet and two 5-μl droplets were used to inoculate each bean and tomato leaf disc of 1-cm-diameter. Mock inoculation consisted of applying droplets of a solution containing only glucose and KH_2PO_4 in its appropriate concentration for bean and tomato. Each well was filled with 1.5 ml of distilled water or, if indicated, with 1.5 ml of the solution of the chemical treatments [25].

2.3. Riboflavin, JA, catalase and ETYA treatments

Riboflavin (Sigma, Bornem, Belgium) was dissolved in distilled water containing 0.01% (v/v) Tween 20 on the day of application. JA and the lipoxygenase (LOX) inhibitor 5, 8, 11, 14-eicosatetraynoic acids (ETYA) (Sigma, Bornem, Belgium) were dissolved in methanol prior to diluting in water. Tomato and bean plants were sprayed till run-off with 1, 10, 100, and 1000 μM of riboflavin or 10 and 100 μM of JA 5 days before inoculation. Control plants in riboflavin and JA experiments were sprayed with distilled water containing 0.01% Tween 20 or diluted methanol, respectively.

The chemical treatments used in the leaf disc assays consisted of 20 μM ETYA and 1100 units/ml catalase (Sigma–Aldrich). For ETYA assay, bean leaf discs (1 cm in diameter) were punched from different plants and discs were mixed. The leaf discs of the riboflavin-treated plants or control were floated in distilled water or ETYA solution (In the case of ETYA and ETYA + Rib treatment) for 5 h, whereafter the ETYA solution was replaced by distilled water before challenge inoculation with *B. cinerea*. For catalase assay, bean leaf discs treated with riboflavin or untreated (Control) were floated in a solution of catalase or distilled water as control and infected with *B. cinerea*. Disease progress was evaluated at 5 days after inoculation by recording number of spreading and non spreading lesions.

2.4. LOX activity assay

Frozen leaf tissue (100 mg fresh weight) was ground in liquid nitrogen and suspended in 900 μl of sodium phosphate buffer (50 mM, pH 6.5) containing 0.25% v/v Tween 20, 1 mM phenylmethionine sulfofluoride (PMSF), 5 mM mercaptoethanol and 2% w/v polyvinylpolypyrrolidone(PVPP). The homogenate was centrifuged at 15000*g* for 20 min (4 °C). The supernatant was kept on ice and used as enzymatic extract. LOX activity was determined as described by Axelrod et al. [26], using linoleic acid as a substrate. The reaction mixture consisted of 870 μl of sodium phosphate buffer (50 mM, pH 6.5), 100 μl of linoloic acid (10 mM), and 30 μl of enzyme extract. The reaction was conducted at 30 °C and LOX activity was determined by the increase in absorbance at 234 nm for 10 min. In control treatments sodium phosphate buffer (50 mM, pH 6.5) replaced leaf extract. Three determinations were made for each time point and the experiment was repeated twice.

2.5. Histochemical detection of hydrogen peroxide

Leaf discs were inoculated with *B. cinerea* conidial suspension as described above and sampled at 4, 8, 12, 16, 20, 24, 48, and 72 hpi. They were cleared and fixed in 100% ethanol. For each time point, at least six discs originating from different riboflavin-treated plants and control plants were used. Three hours before each sampling time point, inoculated bean and tomato leaf discs were floated in a solution of 1 mg/ml 3, 3′-diaminobenzidine (DAB) (pH 4) under light conditions. H_2O_2 was detected in the leaf tissue after polymerization of the DAB molecule at sites of H_2O_2 accumulation and peroxidase activity, forming a stable brownish colour polymer [25,27].

Fungal structures were stained with ink + vinegar 5%. Leaf discs were mounted in 50% glycerol and examined using fluorescence and bright-field microscopy. An Olympus BX-51 microscope coupled with a Colour View III camera and the software package CELL-F (Olympus Soft Imaging Solutions) was used.

3. Results

3.1. Riboflavin induces resistance against B. cinerea in bean, but not in tomato

To investigate the ability of riboflavin to induce resistance against *B. cinerea* in bean and tomato plants, different concentrations of the vitamin (1, 10, 100 and 1000 μM) were sprayed on plants 5 days before inoculation. Disease progress was evaluated 4 and 3 days post inoculation (dpi) in bean and tomato, respectively, by recording number of spreading and non spreading lesions (Table 1). In bean, riboflavin applied at a concentration of 10, 100 or 1000 μM reduced the number of spreading lesions by approximately 25% compared to control leaves (Table 1). No dose response in the range of 10 to 1000 μM was observed, while a 1 μM concentration showed no protective effect against *B. cinerea*, indicating that a minimum concentration of riboflavin, somewhere between 1 and 10 μM, is necessary to properly activate bean defences and stop the pathogen. In tomato, however, foliar application of riboflavin was not able to control the pathogen at any concentration tested (Table 1). These data strongly suggest that riboflavin can effectively induce resistance against *B. cinerea* in bean but not in tomato. Thus, control of *B. cinerea* by riboflavin seems to be plant dependent. Bean and tomato plants sprayed with different riboflavin concentrations, from 1 μM to 10 mM, developed no macroscopically visible symptoms of phytotoxicity at 5 and 15 days after treatment. Treated plants did not exhibit any necrosis or other morphological abnormalities compared with untreated plants (not shown).

Table 1
Effect of foliar application of different concentrations of riboflavin on *B. cinerea* infection in bean and tomato in three different experiments. Within each column, data followed by the same letter are not significantly different according to binary logistic regression ($P = 0.05$).

Riboflavin concentration (μM)	Spreading lesions (%)			
	Exp. 1	Exp. 2	Exp. 3	Mean
Bean				
Ctrl (0)	81.1 a	96.7 a	96.9 a	91.6 a
Rib 1	ND	98.3 a	95.8 a	97.1 a
Rib 10	ND	77.5 b	65.8 b	71.6 b
Rib 100	55.5 b	82.5 b	71.7 b	69.9 b
Rib 1000	54.4 b	83.3 b	70.8 b	69.5 b
Tomato				
Ctrl (0)	92.5 a	84.2 a	98.3 a	91.7 a
Rib 1	ND	85.8 a	99.2 a	92.5 a
Rib 10	ND	87.3 a	96.3 a	91.8 a
Rib 100	95.8 a	88.3 a	100 a	94.7 a
Rib 1000	94.2 a	86.7 a	98.3 a	93.1 a

ND: not determined.

To investigate why riboflavin is active in bean but not in tomato, we hypothesized that either riboflavin is not eliciting defense responses in tomato, or that the defense response triggered by riboflavin is not effective against *B. cinerea* in tomato. Considering the apparent intimacy between H_2O_2 accumulation, activation of the jasmonate pathway and establishment of riboflavin-induced resistance in other plant species [38,44], we focused our research on the expression of the latter defense responses in bean and tomato.

3.2. Rapid H_2O_2 accumulation occurs in epidermal cells of riboflavin-treated bean leaves after inoculation with *B. cinerea*

To explore the putative involvement of H_2O_2 generation, bean and tomato plants were sprayed until run-off with a 100 μM riboflavin solution. Five days later, leaf discs were punched from leaves of different plants, placed floating on distilled water and inoculated with a *B. cinerea* conidial suspension. To visualize H_2O_2 generation in situ, inoculated leaf discs were transferred to a solution containing 1 mg/ml DAB and incubated for a further 3 h until sampling. In these DAB assays, reddish-brown precipitates are deposited at the sites of H_2O_2 accumulation [27]. Accumulation of H_2O_2 was macroscopically detected in bean leaf discs of riboflavin-treated plants, faint at 12 hpi and strong and clear after 16 hpi, but it was delayed in discs of control plants until 48 hpi (Fig. 1B). At 48 and 72 hpi DAB staining was observed in riboflavin-treated and control leaf discs. In tomato leaf discs treated or not with riboflavin H_2O_2, accumulation was not detected before 48 hpi (Fig. 1A). At later time points, successful fungal ingress resulted in intense DAB staining irrespective of the treatment. Importantly, adding catalase, a well-known H_2O_2 quencher, to the DAB solution abolished staining, confirming the specificity of the staining. Moreover, in none of the experiments performed, DAB accumulation could be observed on mock-inoculated bean or tomato plants. These results indicate that riboflavin may prime bean, but not tomato, for earlier accumulation of hydrogen peroxide upon challenge with *B. cinerea*.

Microscopic observations revealed that H_2O_2 accumulation started as early as 4 hpi in bean leaves. At 4 hpi, DAB staining developed in small regions of the anticlinal wall of epidermal cells and later, at 8, 12, 16 and 20 hpi, extended in the epidermis, mainly in anticlinal, but also in outer periclinal cell walls (Fig. 2A). Apparent presence of DAB precipitation inside epidermal cells of riboflavin-treated bean plants was also observed at 20 hpi. Moreover, at all time points investigated, the frequency of epidermal cells showing accumulation was considerably higher in riboflavin-treated than in control leaf discs (Fig. 2B).

3.3. Catalase abolishes riboflavin-induced resistance in bean

To verify the importance of H_2O_2 accumulation in riboflavin-induced resistance, leaf discs were punched from different plants treated or not with 100 μM riboflavin and floated on a solution containing catalase (1100 U/ml) or water during *B. cinerea* infection. Catalase did not affect the level of susceptibility of non-treated plants compared to control plants (non-treated plants on water), but clearly restored the susceptibility of riboflavin-treated plants to the level of control plants (Fig. 3C). Macroscopic and microscopic observations of bean leaf discs from riboflavin-treated plants stained with DAB confirmed that catalase suppressed H_2O_2 accumulation at the early time points post *B. cinerea* inoculation (Fig. 3A and B). These results suggest that H_2O_2 is necessary for riboflavin-induced resistance in bean against *B. cinerea*.

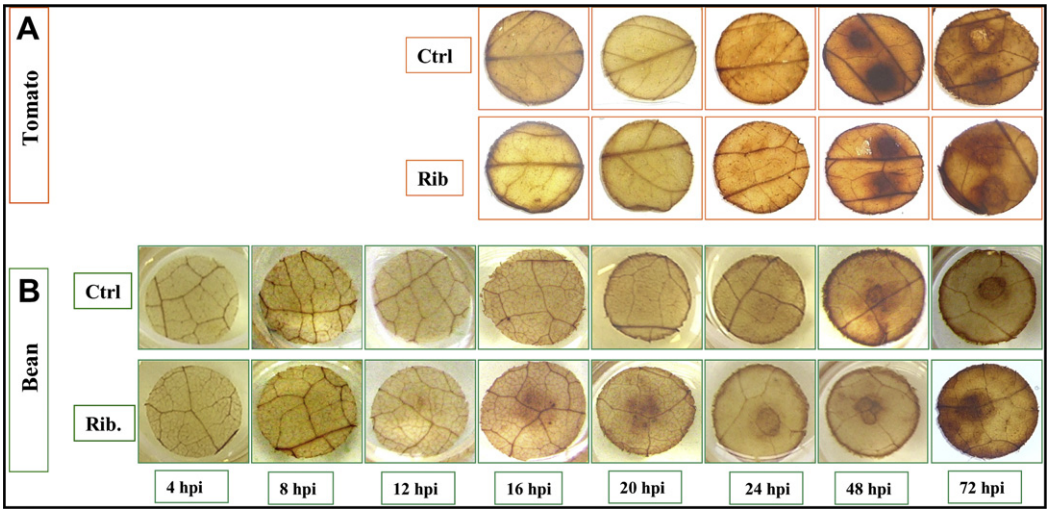

Fig. 1. Macroscopic detection of H_2O_2 accumulation in riboflavin-treated (Rib) and control (ctrl) plants of tomato (A) and bean (B) after inoculation with *B. cinerea*. Leaf discs were punched from different plants 5 days post treatment with riboflavin 100 μM and floated on distilled water. DAB staining of leaf discs inoculated with one 10-μL drop (bean) or two 5-μL drops (tomato) of a conidial suspension was performed at different time points (4, 8, 12, 16, 20, 24, 48, and 72 hpi). One representative disc out of six replicates is shown for each time point. Experiments were repeated with similar results.

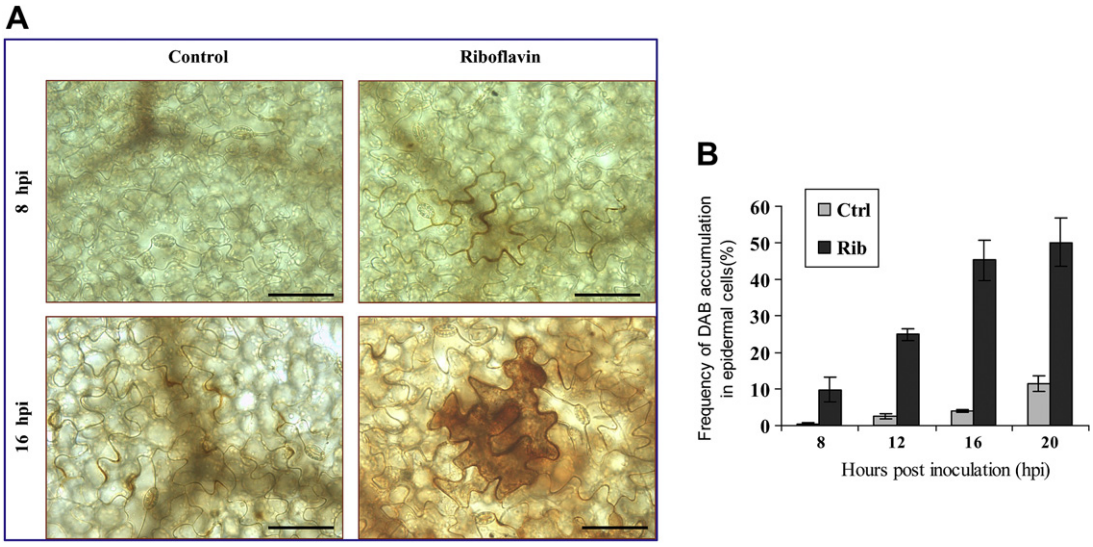

Fig. 2. Effect of riboflavin on H_2O_2 accumulation in bean epidermal cells after inoculation with *B. cinerea*. (A) Microscopic detection of H_2O_2 (DAB staining) in riboflavin-treated and untreated (control) bean plants at 8 and 16 hpi. H_2O_2 accumulation was located in the epidermal cells, mainly in the anticlinal wall. Scale bar = 100 μM. (B) Frequency of DAB in epidermal cells between 8 and 20 hpi. Percentage of epidermal cells showing brown discoloration was determined by examining at least 300 epidermal cells beneath the inoculation drop.

3.4. Riboflavin-IR against B. cinerea in bean is associated with priming for enhanced attacker-induced LOX activity

It is known that JA is involved in the basal resistance of *Arabidopsis* and tomato to *B. cinerea* [28,29]. In addition, increased endogenous JA levels in *Arabidopsis* enhanced resistance to this pathogen [30]. To explore the possible interplay between riboflavin-induced and JA-induced systemic signalling pathways in bean and tomato, we first investigated the ability of JA to induce resistance against *B. cinerea* in tomato and bean. To this purpose, JA was sprayed on plants till run-off at 10 and 100 μM, five days before inoculation. Both concentrations of JA clearly protected bean and tomato against grey mould disease (Fig. 4). Next, LOX activity was determined in bean and tomato leaves treated with JA (100 μM) or riboflavin (100 μM) and inoculated with *B. cinerea*. Lipoxygenase activity is the first reaction of the so-called LOX pathway which leads to the production of JA and other oxylipins implicated in plant defence responses [31]. At various time points post inoculation (0, 6, 12, 18, 24, 48, and 72 h) samples were taken from bean or tomato leaves treated or not with JA or riboflavin. LOX activity was found to be significantly higher at 12 and 18 hpi in JA-supplied leaves. A clear peak of activity was observed at 24 hpi in both bean and tomato (Fig. 5 A and B). In bean, treatment with riboflavin led to a similar response as that observed following JA application (Fig. 5C). However, tomato plants treated with riboflavin showed no enhanced activity of the enzyme after *Botrytis* inoculation (Fig. 5D). These results clearly demonstrate that the LOX pathway is promptly activated in riboflavin-treated bean plants challenged with *B. cinerea*. To clarify the involvement of the LOX pathway in riboflavin-induced resistance, bean leaf discs were floated for 5 h on a solution containing the LOX inhibitor 5, 8, 11, 14-eicosatetraynoic acid (ETYA) before inoculation. Disease evaluation at 5 days post *B. cinerea* inoculation showed that treatment with ETYA restored the susceptibility of riboflavin-treated plants to the level of control plants (Fig. 6). Taken together, these results suggest that the LOX pathway is involved in riboflavin-induced resistance in bean against *B. cinerea*.

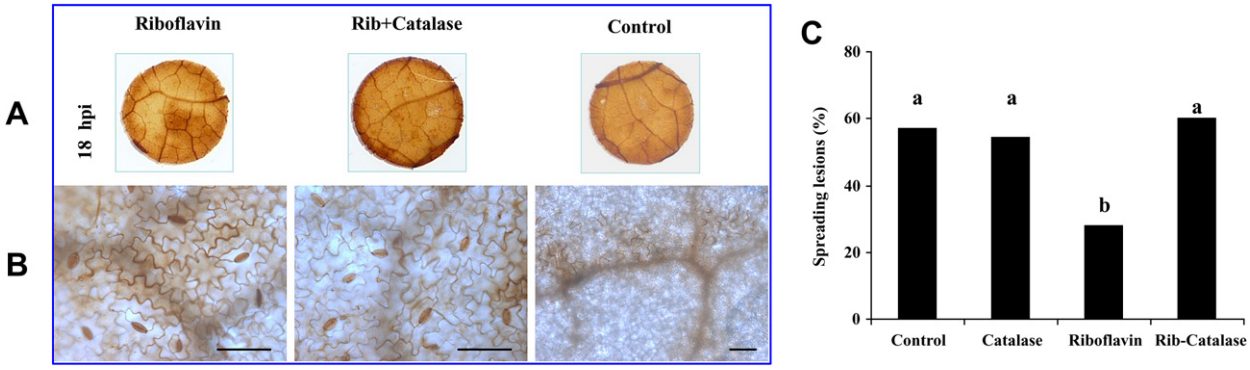

Fig. 3. Effect of catalase on riboflavin-induced resistance against *B. cinerea*. Leaf discs from plants treated or not with riboflavin 100 μM were floated on catalase 1100 U/ml or water and inoculated with one 10-μl droplet of *B. cinerea* conidial suspension. At 18 hpi leaf discs were sampled for DAB staining. Catalase application resulted in a fainter macroscopic (A) and microscopic (B) detection of H_2O_2 in riboflavin-treated plants compared to water treatment (control). For each treatment, at least 6 discs were examined. The experiment was repeated with similar results. Scale bar = 100 μM. (C) Effect of catalase on grey mould symptoms in bean previously treated with riboflavin or untreated (control). Disease progress was evaluated at 5 days after inoculation by recording number of spreading and non spreading lesions. Different letters on the bars indicate a significant difference according to binary logistic regression ($P < 0.05$).

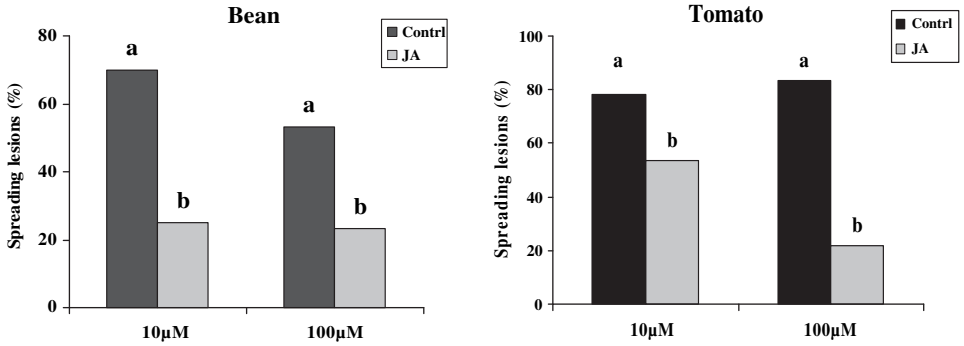

Fig. 4. Effect of exogenous JA application on symptom development of *B. cinerea* in bean and tomato. Plants were sprayed with distilled water containing 0.01% Tween-20 or JA (10 and 100 µM). Five days after treatment detached leaves were inoculated. The number of spreading lesions was evaluated 4 and 3 days post inoculation in bean and tomato, respectively. Each experiment was carried out at least twice. Columns associated with the same letter in each doses are not significantly different according to binary logistic regression ($P < 0.05$).

4. Discussion

Little is known about the molecular mechanisms underpinning riboflavin-IR in agronomically important crops such as vegetables. Here, we demonstrate that exogenous application of riboflavin effectively protects bean but not tomato from attack by the grey mould fungus *B. cinerea*. Moreover, our data reveal that this riboflavin-IR is tightly associated with priming of the host tissue for accelerated activation of a multifaceted cellular defense response, including a timely and highly localized generation of H_2O_2 in the epidermis and enhanced LOX activity upon pathogen attack.

One of the most peculiar events in the early stages of plant-pathogen interactions, H_2O_2 formation has been tightly linked to the establishment of riboflavin-IR in various plant-pathosystems [4,44]. Our results demonstrate that also in the bean-*B. cinerea* interaction, riboflavin induces resistance at least in part by priming host cells for boosted accumulation of H_2O_2 following pathogen infection. In bean plants treated with riboflavin and inoculated with the pathogen, H_2O_2 was detected in the epidermal cells as soon as 4 hpi, similar to what [25] observed in the highly resistant interaction between the abscisic acid-deficient *sitiens* tomato and *B. cinerea*. These authors stated that timely production of H_2O_2 and

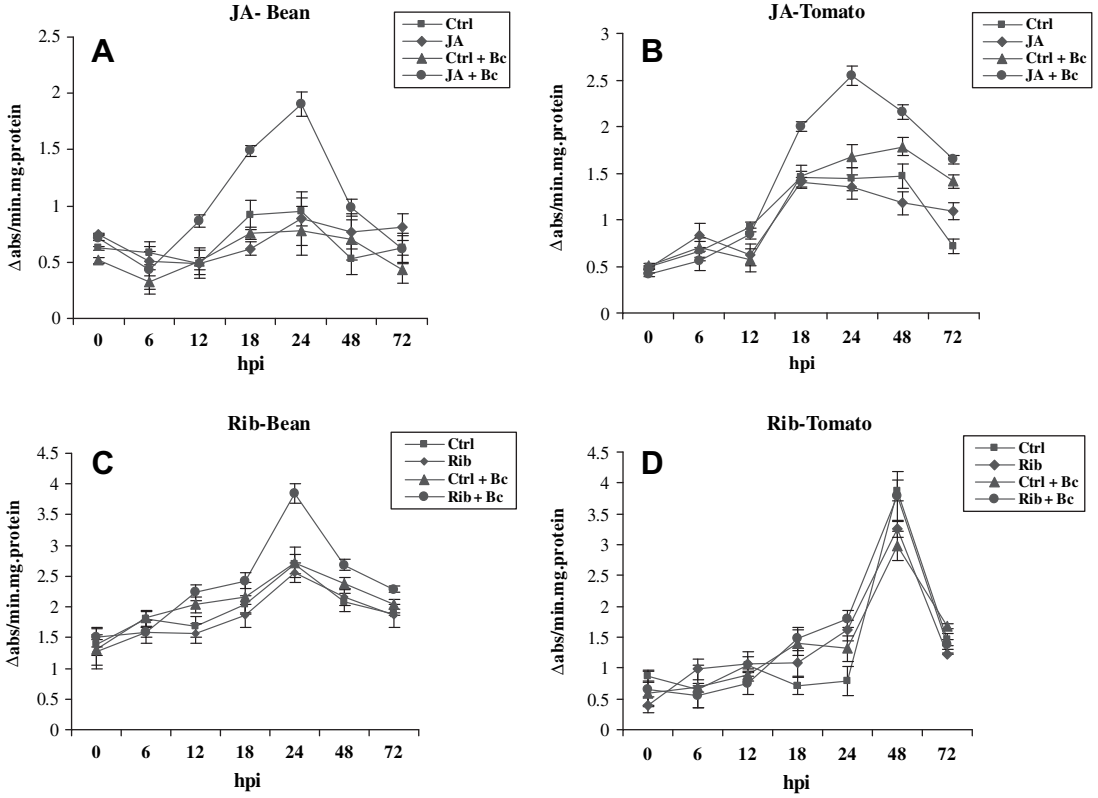

Fig. 5. Influence of JA and riboflavin on lipoxygenase (LOX) activity in bean and tomato leaves inoculated with *B. cinerea*. LOX activity in bean and tomato plants treated with JA (100 µM) (A and B) or riboflavin (100 µM) (C and D) 5 days before inoculation. Reaction was conducted at 30 °C and enzyme activity was determined spectrophotometrically by the increase in absorbance at 234 nm after 10 min. Data are means and standard errors of three measurements performed on leaf material sampled at each time point. Riboflavin assay: Control (Ctrl), Riboflavin (Rib), Control + *Botrytis* (Ctrl + Bc), Riboflavin + *Botrytis* (Rib + Bc). JA assay: Control (Ctrl), Jasmonic acid (JA), Control + *Botrytis* (Ctrl + Bc), Jasmonic acid + *Botrytis* (JA + Bc).

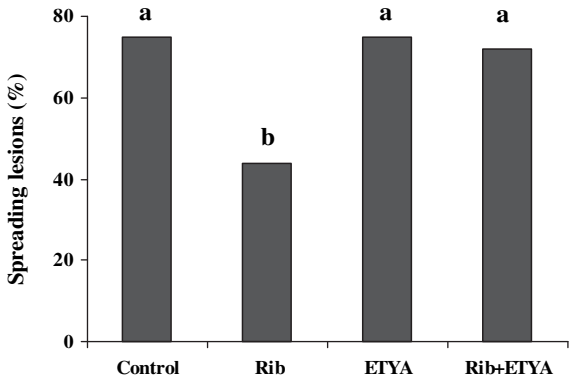

Fig. 6. Effect of LOX inhibitor 5, 8, 11, 14-eicosatetraynoic acid (ETYA) on the infection of *B. cinerea* in bean leaf discs. Discs were punched from different plants treated or not with riboflavin and floated in distilled water or ETYA solution (for 5 h before inoculation with *B. cinerea*). Disease progress was evaluated at five days after inoculation by recording number of spreading and non spreading lesions. Different letters on the bars indicate a significant difference according to binary logistic regression ($P < 0.05$).

resultant prompt activation of H_2O_2-fueled defence responses are highly effective in arresting *B. cinerea*. Interestingly, in contrast to ABA deficiency, riboflavin failed to prime H_2O_2 generation in tomato, resulting in the same level of *Botrytis* susceptibility as observed in control plants. In bean on the other hand, riboflavin was highly effective in enhancing H_2O_2 generation at sites of attempted pathogen entry and inducing disease resistance. Hence, it is not unlikely that riboflavin-IR against *B. cinerea* in bean results from a sensitization of the tissue to express basal defences faster and stronger following pathogen invasion, resulting in an early oxidative burst and associated resistance to *B. cinerea*. In compliance with this concept, there is ample evidence demonstrating the protective effect of early, epidermis-localized H_2O_2 in bean-*B. cinerea* interactions. Most tellingly in this regard, H_2O_2 production occurs faster and much stronger during infection of bean cells with a non-aggressive than with an aggressive strain [32]. Moreover, the effect of catalase in eliminating H_2O_2 formation in riboflavin-treated bean plants and increasing the number of spreading *B. cinerea* lesions clearly demonstrates the key role of this defence response in the riboflavin-IR. In keeping with our results, early attacker-induced H_2O_2 accumulation has also been uncovered as a crucial facet of the resistance mechanism underpinning riboflavin-IR as well as thiamine-IR in *Arabidopsis* against the bacterial pathogen *Pseudomonas syringae* pv. tomato [4,12]. Rapid generation of H_2O_2 at the site of infection serves many functions in plant disease resistance; among others, it drives cell wall modification, regulates gene expression and cross talks with various defence signalling pathways which act in a synchronized manner to fend off pathogen invasion [25,33]. Future studies should be focused on exploring the relative contribution to the riboflavin-IR of each of aforementioned functions.

Besides boosting attacker-induced H_2O_2 generation, riboflavin-induced resistance in bean was also found to involve stimulation of LOX activity. Interestingly, treatment of bean with exogenous JA yielded similar levels of protection as observed in riboflavin-amended plants and was likewise associated with enhanced LOX activities. In tomato, however, exogenous application of JA and riboflavin resulted in different outcomes. While JA treatment clearly induced resistance against the pathogen accompanied by higher levels of LOX activity, riboflavin failed to enhance resistance in tomato plants and LOX activity remained at the level of inoculated control plants. Lipoxygenases catalyse the first reactions of the octadecanoid pathway, a multibranched signalling conduit leading to production of JA and other oxylipins implicated in plant defence responses [31,34]. Supporting a role for JA as a positive signal in *Botrytis* defence, LOX has been implicated in induced resistance against *B. cinerea* by non-pathogenic bacteria. For instance, disease caused by *B. cinerea* significantly decreases and LOX activity increases on leaves of tomato following treatment of roots with lipopeptide-overproducing *Bacillus subtilis* mutants as compared to the wild-type [35]. In other studies, stimulation of both LOX and hydroperoxide lyase activities have been associated with reduction of disease caused by *B. cinerea* in tomato and bean plants upon root colonization by *Pseudomonas putida* strain BTP 1 [36,37]. Taken together, these findings clearly indicate that, when properly activated, LOX and the JA pathway can increase resistance to *B. cinerea* in both bean and tomato. Here, we show that riboflavin is able to effectively trigger this pathway in bean, but not in tomato. In addition, we verified the importance of this pathway in the riboflavin-IR by suppressing resistance of inoculated bean plants using ETYA, a chemical inhibitor of LOX activity. These results agree with those obtained in the rice-*Rhizoctonia* plant-pathosystem. When LOX activity was impaired in rice plants, riboflavin lost the ability to induce resistance to the pathogen [38]. In Arabidopsis, however, riboflavin appears to function independently of the octadecanoid pathway given the ability of the JA-insensitive mutant *jar 1* to mount wild-type levels of riboflavin-IR [4].

One outstanding question is why riboflavin activates plant defence and increases disease resistance in bean but not in tomato. Several scenarios can be hypothesized to explain this differential effectiveness. First, we may not completely rule out the ability of riboflavin to induce resistance in tomato. Riboflavin may trigger defences in tomato via other pathways which are not capable to stop *Botrytis*, but may arrest other pathogens. A similar situation was observed for example in pineapple plants where riboflavin was found to trigger resistance against the root nematode *Meloidogyne javanica*, but not against *Rotylenchulus reniformis* [39]. However, such a scenario seems rather unlikely in our case. Mounting evidence indicates that riboflavin-IR involves the LOX pathway [38] (Figs. 5 and 6) and activation of this pathway seems very effective in enhancing resistance against *B. cinerea* not only in bean, but also in tomato [36,37] (Figs. 4 and 5). Therefore, we speculate that tomato may not adequately perceive riboflavin, explaining the lack of priming and associated loss of riboflavin-IR in this plant system. Such lack of perception could be brought about by several factors, such as poor uptake, the relatively high endogenous levels of riboflavin present in tomato and/or the absence or non-expression of an appropriate riboflavin receptor. At approximately 1.2 μg per g fresh weight, tomato leaves contain around twice as much riboflavin as bean leaves [40]. Similar to the situation in rice where high basal SA levels significantly reduce responsiveness to exogenous SA treatment [41], elevated riboflavin levels in tomato may interfere with its ability to induce resistance when exogenously applied. Alternatively, though less parsimoniously, tomato plants may either lack one or more riboflavin receptors or these receptors may not be adequately expressed. Particularly relevant in this regard is the identification of a riboflavin-binding protein in the plasma membrane of *Curcubita pepo* hypocotyls with an apparent dissociation constant for riboflavin in the order of 1 μM, while 10 μM riboflavin was close to binding site saturation [42]. Interestingly, this fits with the lowest concentrations of riboflavin needed to trigger resistance in this study. Originally identified as a PIP1-homologous protein of the aquaporin family, this riboflavin-binding protein is closely related to AQUAPORIN1 of bean, PIP1b of Arabidopsis and NtAQP1 of tobacco [42] and it was suggested that this protein may have a photoreceptor function. The tomato homologue of this protein is PIP1-SOLLC (Q08451), also called tomato ripening-associated membrane protein, a protein that accumulates in roots, ripening fruit and senescing leaves [43]. It remains to be investigated whether riboflavin-binding proteins are involved in the induced resistance response

observed in bean and other plants and whether differences in their expression may explain the inability of riboflavin to trigger resistance in tomato.

In conclusion, the findings denoted in this study clearly demonstrate that riboflavin can effectively induce resistance against *B. cinerea* in bean but not in tomato and support the notion that this riboflavin-IR is tightly associated with priming of the host tissue for earlier accumulation of hydrogen peroxide and prompt activation of the LOX pathway upon pathogen attack.

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