



Reactive oxygen species and NADPH oxidase-encoding genes underly the plant growth and developmental responses to *Trichoderma*

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Received: 15 September 2022 / Accepted: 21 February 2023 / Published online: 6 March 2023
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Abstract

The modulation of plant growth and development through reactive oxygen species (ROS) is a hallmark during the interactions with microorganisms, but how fungi and their molecules influence endogenous ROS production in the root remains unknown. In this report, we correlated the biostimulant effect of *Trichoderma atroviride* with *Arabidopsis* root development via ROS signaling. *T. atroviride* enhanced ROS accumulation in primary root tips, lateral root primordia, and emerged lateral roots as revealed by total ROS imaging through the fluorescent probe H2DCF-DA and NBT detection. Acidification of the substrate and emission of the volatile organic compound 6-pentyl-2H-pyran-2-one appear to be major factors by which the fungus triggers ROS accumulation. Besides, the disruption of plant NADPH oxidases, also known as respiratory burst oxidase homologs (RBOHs) including ROBHA, RBOHD, but mainly RBOHE, impaired root and shoot fresh weight and the root branching enhanced by the fungus in vitro. *RbohE* mutant plants displayed poor lateral root proliferation and lower superoxide levels than wild-type seedlings in both primary and lateral roots, indicating a role for this enzyme for *T. atroviride*-induced root branching. These data shed light on the roles of ROS as messengers for plant growth and root architectural changes during the plant-*Trichoderma* interaction.

Keywords *Trichoderma atroviride* · *Arabidopsis thaliana* · Reactive oxygen species · Plant biomass · Root development

Introduction

Plant roots are inhabited by a myriad of soil microbes due to the attraction exerted by sugars and other exudates (Nath et al. 2016). Some of these microbes, which mostly belong to bacteria and fungi, can colonize the rhizosphere or live inside plant tissues, resulting in enhanced host growth or improved protection against stress and diseases (Hassani et al. 2018; Pascale et al. 2020). Fungal species belonging to the *Trichoderma* genus effectively spread into the roots and behave as probiotic organisms that promote root branching

and nutrient absorption, along with their widely recognized biocontrol properties (Villalobos-Escobedo et al. 2020; Esparza-Reynoso et al. 2021).

Studies with *T. atroviride*-*Arabidopsis* interaction indicated that root exudation of sucrose changes fungal metabolism, thus decreasing the expression of genes encoding degradative enzymes, whereas the release of auxins extends the branching capacity of roots for better soil exploration (Villalobos-Escobedo et al. 2020; Alfiky and Weisskopf 2021). During this chemical dialog, the production of ROS by NADPH oxidases in *T. atroviride* determines its ability to perceive plants and to assimilate simple forms of sugars (Villalobos-Escobedo et al. 2020). On the other hand, ROS production in the plant host increases by biotic and abiotic stresses and occurs as an early response to *Trichoderma* inoculation, activating afterward an antioxidant mechanism that confers tolerance to the oxidative stress imposed by pathogens or environmental factors (Choudhary et al. 2020; Nogueira-Lopez et al. 2018; Huang et al. 2019; Nawrocka et al. 2019; Alfiky and Weisskopf 2021).

In plants, the NADPH oxidases (NOXs) also known as respiratory burst oxidase homologs (RBOHs) are the main

Handling Editor: April H Hastwell

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enzymes that catalyze the apoplastic reduction of molecular oxygen (O_2) into the superoxide anion ($O_2^{\cdot-}$), which in turn is spontaneously or enzymatically dismutated into the more stable form hydrogen peroxide (H_2O_2) (Hu et al. 2020). RBOHs encompass a group of membrane-bound enzymes with homology to the mammalian phagocyte gp91phox (NOX2). These enzymes are directly activated in response to the rapid influx of Ca^{2+} or intracellular phosphorylation events by protein kinases (Chapman et al. 2019; Lee et al. 2020). The *Arabidopsis* genome encodes ten *Rboh* genes potentially involved in plant resilience toward environmental stress as well as developmental responses (Chapman et al. 2019). RBOHD and RBOHF play an important role in ROS production and are required for proper lateral root development (Otulak-Kozieł et al. 2020). The corresponding genes show comparable expression patterns in the developing lateral root primordia (Orman-Ligeza et al. 2016; Chapman et al. 2019), but their function and/or regulation as a response to probiotic fungi that enhance root branching has not been assessed.

In this report, we show that *Trichoderma atroviride* induces the accumulation of ROS at several stages of root development, which could be attributed to the signaling exerted by fungal acidification of the medium and to the release of its major volatile 6-pentyl-2H-pyran-2-one (6-PP). Consistently, comparison of the growth and biomass production in WT *Arabidopsis* seedlings and mutants affected in genes encoding ROBHA, RBOHD, and RBOHE enzymes indicates the importance of these RBOHs for root branching and biomass production elicited by the fungus. Detection of superoxide anion in *rbohE* mutant, which fails to mount an effective response to the fungal phytostimulation, further established the link between ROS levels with root branching patterns, and hence the whole capacity of plants for soil exploration.

Materials and methods

Plant material and growth conditions

A. thaliana ecotype Columbia (Col-0) was used as a wild-type (WT) plant throughout the study. The mutant lines *RbohA* (SALK_047391), *RbohD* (SALK_044865), and *RbohE* (SALK_030395) were obtained from the Salk Institute for Biological Studies (La Jolla, California, USA). Seeds were surface disinfected using 95% (v/v) ethanol and 20% (v/v) bleach for 5 and 7 min, respectively, followed by five washes with distilled, sterilized water. The seeds were stratified for two days at 4°C, and grown on Petri dishes containing 0.2× Murashige & Skoog (MS) basal salt mixture (M524, PhytoTech Labs), 0.6% (w/v) sucrose 1% (w/v) agar micropropagation grade (A111, PhytoTech Labs), and

pH 7.0, 5.5, or 4.5. Petri dishes were placed vertically (at an angle of 65°) in a plant growth chamber (Percival AR-95L) at 22°C under continuous light conditions ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$) and photoperiod (16 h of light/8 h of darkness).

Fungal growth and inoculum preparation

Trichoderma atroviride strain IMI206040 used to perform plant–fungus interaction assays was kindly provided by Dr. Alfredo Herrera-Estrella (CINVESTAV-Irapuato). Four days after germination, seedlings were inoculated with a 1×10^6 conidia at 5 cm from the root tip and incubated for additional four days to evaluate the plant responses to *Trichoderma*. The fungal growth trial and spore harvest were performed according to Pelagio-Flores et al. (2017).

6-PP treatments

Arabidopsis seedlings were germinated and grown in each Petri dish containing 0.2× MS medium supplemented with micromolar concentrations (0, 75, and 150 μM) of 6-PP (Sigma-Aldrich), prepared according to Garnica-Vergara et al. (2016). The seeds were sown to one side of the plate, placing 10 individuals in a row, and at least three plates were included for each treatment. Ten days after germination, the determination of the dose-response effect of 6-PP in plant growth related to ROS accumulation was performed.

Analysis of plant traits

The length of primary roots was measured with a ruler, while the lateral root length was measured using the IMAGEJ software (<http://rsbweb.nih.gov/ij/>). The quantitation of total lateral roots was determined by counting all mature roots that emerged from the primary root using a stereomicroscope (Leica MZ6). Lateral root density was scored as the lateral root number per centimeter of primary root, and was calculated by dividing the number of lateral roots by the primary root length for each seedling. Fresh weights of shoots or roots were determined using an analytical scale. Petri dish images were taken using a digital camera (Nikon D5600, Japan).

ROS visualization in roots

The production of intracellular ROS was assayed using the oxidation-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA). H2DCF-DA is readily membrane-permeant and is trapped within cells by binding the chloromethyl group to cellular thiols. It becomes fluorescent when oxidized by hydrogen peroxide and downstream free radical products of hydrogen peroxide. *Arabidopsis* seedlings were incubated in 10 μM of H2DCF-DA

(Invitrogen™) in Trizma® hydrochloride buffer solution at 10 mM (pH 7.4) for 60 min at room temperature and in darkness. The roots were rinsed and mounted with fresh buffer solution on microscope slides for fluorescent imaging measurements using a confocal laser scanning microscope (Olympus FV1200). The 2',7'-dichlorofluorescein (DCF) fluorescence was detected through excitation and emission wavelength of 485 nm and 500–535. Fluorescence from at least 8 treated seedlings was measured by calculating the green pixels in a determined area of each image using IMAGEJ software (<http://rsbweb.nih.gov/ij/>). The means of the relative fluorescence of each treatment were normalized according to the pixel values from the control condition.

Histochemical in situ detection of superoxide anion

Intracellular superoxide anion was detected using nitroblue-tetrazolium (NBT), which forms an insoluble dark blue formazan precipitate after reduction by superoxide. *Arabidopsis* seedlings co-cultivated with *Trichoderma* were incubated in 10 mM sodium phosphate buffer (pH 7.8) with 10 mM NaN₃ and 1 mg mL⁻¹ NBT (Sigma-Aldrich) and kept at room temperature (26°C) and darkness for 5 min. The reaction was stopped by removing the NBT staining solution and washing roots twice in distilled water. The seedlings were mounted on glass slides to visualize intracellular color change through Nomarski's differential interference contrast microscopy. For each treatment, at least 8 plants were analyzed. Quantification of the NBT staining of roots was assessed by measuring the pixel intensity of comparable areas by ImageJ software. The means of the relative NBT staining were normalized in a similar way to fluorescence.

Data analysis

The data were analyzed through univariate and multivariate analyses followed by Tukey's post hoc tests using STATISTICA 10.0 program (Dell StatSoft, Austin, Texas, USA). All experiments were repeated three times. Different letters were used to indicate means that differ significantly ($P < 0.05$).

Results

T. atroviride increases ROS accumulation within *Arabidopsis* roots

Root colonization by *Trichoderma* triggers ROS production, which accounts for plant protection against pathogens (Saranakumar et al. 2016; Chen et al. 2019). To visualize ROS in the root before physical contact between the plant and the fungus, we used the fluorescent cell-permeable probe H2DCF-DA and detected the corresponding fluorescence

in root tissues through confocal microscopy. *Arabidopsis* seedlings were inoculated with *T. atroviride* 4 days after germination at 5 cm below the root tip and allowed to grow for 4 days. *Trichoderma*-mediated ROS accumulation increased in the cells surrounding lateral root primordia during their development (Fig. 1(a, b)) and emergence from the primary root (Fig. 1(c, d)), in primary root tips (Fig. 1(e, f)), and in mature lateral roots (Fig. 1(g, h)). Quantification of the fluorescence indicated that the surrounding layers of the primordia had around a 50% increase in ROS detection in inoculated seedlings, being much more evident in the primary root tip and mature lateral roots, which reached roughly 2.5 and 3-fold higher fluorescence, respectively, under fungal cocultivation when compared to axenically grown seedlings (Fig. 1(i–l)). These results clearly showed that *Trichoderma* triggers ROS accumulation on the root as an early response before root colonization.

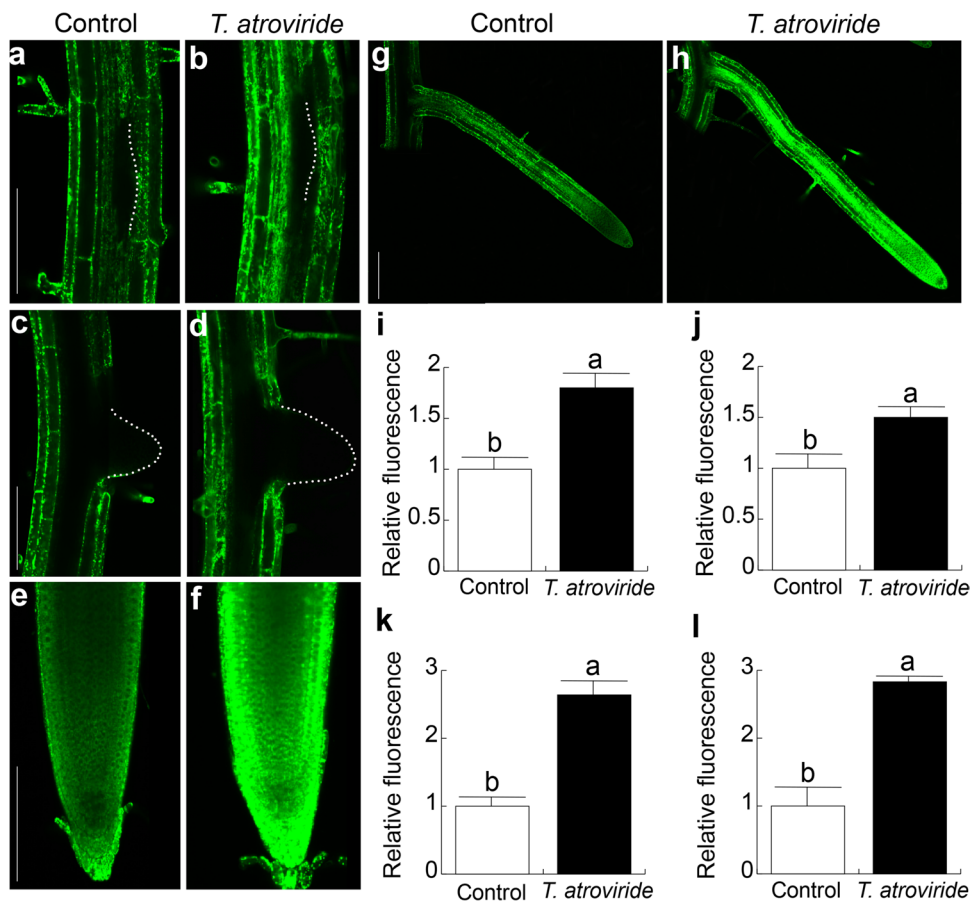
Low pH induces ROS accumulation in roots

Two major factors from the fungus influence root architecture in *Arabidopsis*-*T. atroviride* interactions, acidification of the growth medium, and emission of 6-PP (Garnica-Vergara et al. 2016; Pelagio-Flores et al. 2017). To assess whether any of these factors may cause ROS overaccumulation within the root, we detected the ROS using H2DCF-DA and confocal microscopy on lateral root primordia and primary root tips of *Arabidopsis* seedlings grown on 0.2× MS agar medium adjusted to pH 4.5, 5.5, and 7.0. The more acidic pH (5.5 and 4.5) induced ROS accumulation in cell layers bordering the lateral root primordia (Fig. 2(a–c, g)), as well as at the meristematic zone of the primary root tip, including the stem cell niche and root cap (Fig. 2(d–f, h)). In mature lateral roots, acidity increased ROS accumulation, which was comparable to the pattern already observed in the primary root, suggesting that increases in ROS production occur in root branches (Fig. 3(a–d)). These data indicate that rhizosphere acidification by *T. atroviride* enhances ROS levels within the *Arabidopsis* root system.

The fungal volatile 6-pentyl-2H-pyran-2-one induces ROS accumulation in roots

Before direct contact with fungal hyphae, plants perceive the organic volatile compounds emitted by *Trichoderma atroviride* from which 6-PP is a major constituent (Garnica-Vergara et al. 2016; Carillo et al. 2020). Therefore, we hypothesized that 6-PP might be another factor for ROS accumulation in roots regarding its reported effects in both cell division and elongation (Garnica-Vergara et al. 2016). Supplementation of 75 and 150 μM 6-PP to the medium increased the detection of total ROS in primary root meristem and root elongation zones (Fig. 4(a–c)). Besides, quantification of

Fig. 1 Detection of total reactive oxygen species (ROS) in roots of *Arabidopsis* seedlings inoculated with *T. atroviride*. Intracellular ROS fluorescence using the probe H2DCF-DA and confocal microscopy. Representative micrographs of endogenous ROS levels around lateral root primordia (a, b), lateral roots leaving the parent root (c, d), primary root tips (e, f), and mature lateral roots (g, h). The graphs (i–l) show the mean fluorescence intensities for 8 independent seedlings that were imaged. Relative fluorescence quantitation around lateral root primordia (i), lateral roots leaving the parent root (j), primary root tips (k), and mature lateral roots (l). Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μm . The experiment was repeated three times with comparable results



fluorescence confirms that 6-PP provokes ROS accumulation within the root meristem, which is dependent on the concentration of fungal compound supplied to the growth medium (Fig. 4(d)). Interestingly, in plants grown under 150 μM 6-PP, reduction in the width of the root tip and shortening of the meristematic and elongation zone were observed (Fig. 4(c)). Thus, we propose that the inhibitory effect of 150 μM of 6-PP in primary root growth is caused by ROS overproduction, affecting cell division and expansion processes.

***T. atroviride* induces superoxide anion production in roots**

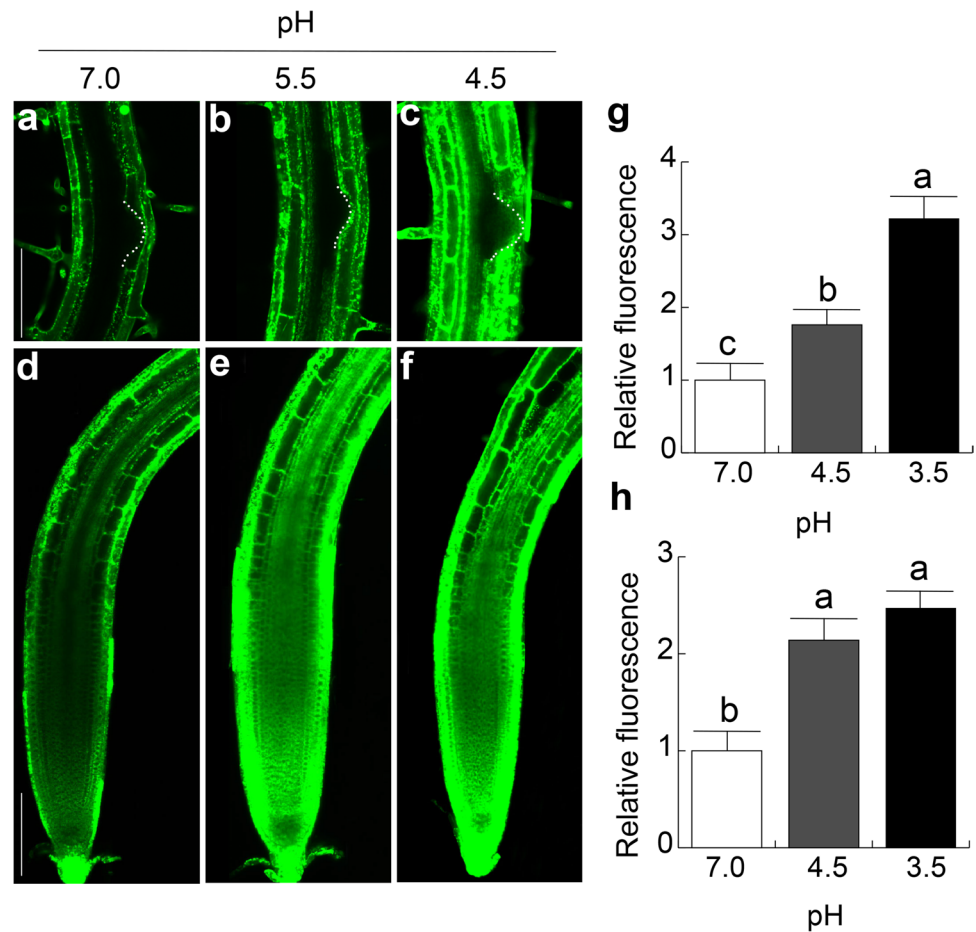
The above results showed that *Trichoderma atroviride* increases ROS production before root colonization, possibly due to the effect of the perception of volatiles. Since the superoxide anion accounts for the formation of all major ROS species, namely hydrogen peroxide, singlet oxygen, and hydroxyl radical, we next analyzed its distribution in roots of *T. atroviride* inoculated and non-inoculated seedlings using NBT, which forms a water-insoluble blue formazan precipitate upon reaction with superoxide. The accumulation of superoxide, detected by NBT staining, was observed at the tip of the primary and lateral roots of un-inoculated plants

(Fig. 5(a–d)). Noteworthy, *Trichoderma*-inoculation led to a stronger NBT signal detection in the lateral roots, and the primary root, respectively (Fig. 5(e, f)). Thus, *T. atroviride* inoculation causes a superoxide-dependent burst that may act in early signaling events influencing root growth processes.

NADPH oxidases RBOHA, RBOHD, and RBOHE are required for *Trichoderma*-induced plant growth promotion

NADPH oxidases, also known as RBOHs in plants, are the main enzymes that catalyze the production of superoxide in the apoplast, which is rapidly dismutated to hydrogen peroxide (Hu et al. 2020). RBOH-dependent ROS production regulates a wide range of biological processes including plant development and stress responses. To investigate the role of NADPH oxidase-generated ROS in *Trichoderma*-plant interaction, we compared the biostimulant effect of *T. atroviride* in wild-type *Arabidopsis* seedlings (Col-0) and mutants defective in *RBOH* genes (*RbohA*, *RbohD*, and *RbohE*). The wild-type (WT) and mutant seedlings were grown for 4 days after germination and inoculated with *T. atroviride* at 5 cm from the root tip.

Fig. 2 Effect of acidic pH on ROS accumulation in *Arabidopsis* lateral root primordia and primary root meristems. *Arabidopsis* seedlings were germinated and grown for 8 days on agar plates containing MS 0.2× medium with pH adjusted to 7, 5.5, and 4.5. Representative micrographs of the detection of endogenous ROS levels around lateral root primordia revealed by H2DCF-DA (a–c), and primary root tips (d–f). The graphs show the means of relative fluorescence levels around lateral root primordia (g), and from the meristematic zone (h) of 8 seedlings ± SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 100 μm. The experiment was repeated three times with comparable results



After 4 days of co-cultivation *in vitro*, the WT seedlings inoculated with *T. atroviride* showed a clear phyto-stimulation when compared to un-inoculated plants, roughly reaching a 2- to 2.5-fold increase in shoot and total fresh weight, which significantly decreased in each *RbohA*, *RbohD*, and *RbohE* mutant (Fig. 6(a–j)). Consistently, the primary root growth was slightly affected by co-cultivation with the fungus, whereas the number and length of lateral roots increased by 4-fold and 3-fold, respectively, compared to axenically grown seedlings. This response correlated with root biomass accumulation (Fig. 7(a–d)). In contrast, the increased root branching and root biomass production elicited by *T. atroviride* diminished in the *Rboh* mutants. In particular, the *RbohE* mutant showed an impressive reduction of the lateral root number, lateral root length, and root fresh weight when compared to the WT in presence of *T. atroviride* (Fig. 7(b–d)). Taken together, these results suggest that RBOH-mediated ROS production determines the *Trichoderma*-induced lateral root formation and biomass production.

Loss of function of RBOHE compromises the ability of *T. atroviride* to increase superoxide accumulation in *Arabidopsis* roots

RBOH genes show an overlapping expression pattern in different sites of the root, and their corresponding enzymatic activity regulates primary root growth and lateral root formation (Mase and Tsukagoshi, 2021). To further evaluate the role of RBOH-dependent ROS synthesis in growth promotion by *T. atroviride* in *Arabidopsis*, we used NBT staining to detect superoxide anion in roots of wild-type plants and *rbohE* mutant, which was among the *rboh* mutants the less responsive to the *Trichoderma*-mediated plant growth promotion. We observed that fungal inoculation increased superoxide levels in the primary roots of wild-type seedlings (Fig. 8(a–b)), and the overall superoxide amount in the whole root was about 60% compared to non-inoculated plants (Fig. 8(e)). However, the superoxide levels in *rbohE* mutant roots were lesser than wild-type roots with or without *Trichoderma* presence (Fig. 8(c–d)).

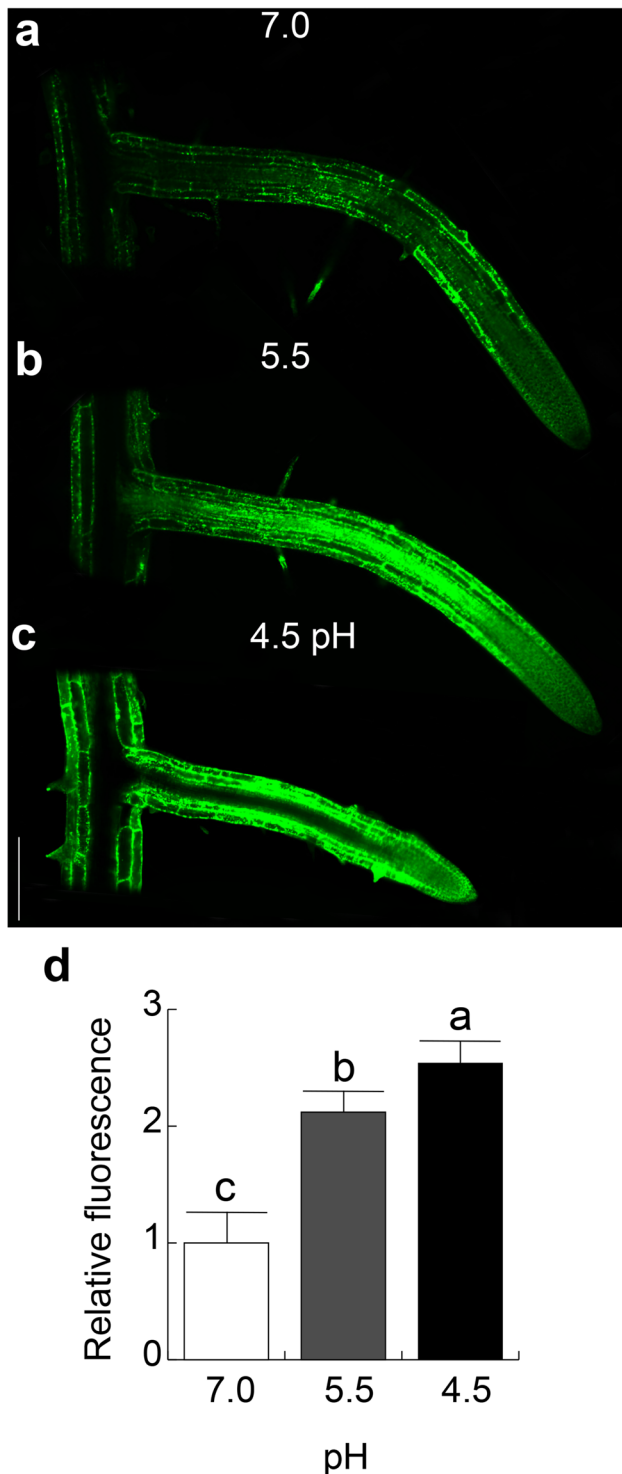


Fig. 3 Effect of acidic pH on ROS production in lateral root meristems. Representative micrographs of the detection of endogenous ROS levels in mature lateral roots (a–d). The graph shows the means of relative fluorescence from meristematic zone for lateral roots at comparable developmental stages, from 8 independent seedlings \pm SD. Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μ m. The experiment was repeated three times with comparable results

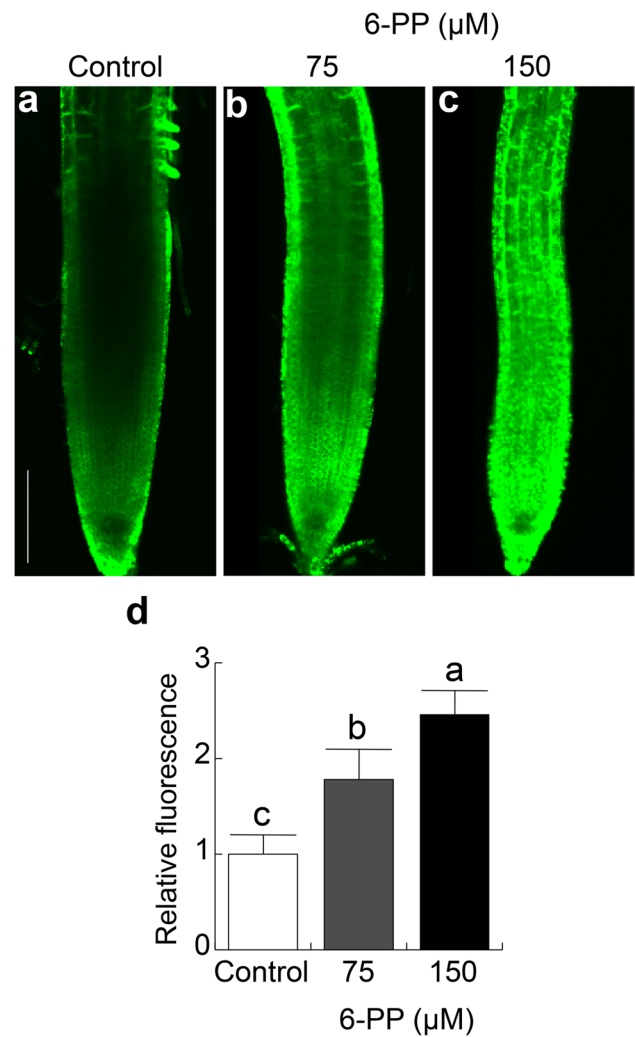


Fig. 4 6-PP enhances ROS accumulation in meristems of *Arabidopsis* primary roots. Seedlings were germinated and grown on 0.2 \times MS medium supplemented with 0, 75, and 150 μ M 6-PP. Ten days after germination, detection of endogenous ROS levels was performed using H₂DCF-DA. Representative micrographs show ROS accumulation in root meristems (a–c). The graph (d) represents the mean values of the relative fluorescence from 8 seedling root meristems \pm SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 100 μ m. The experiment was repeated three times with comparable results

Interestingly, *rbohE* mutants showed shorter meristem and elongation zones than WT roots due possibly to the imbalance of ROS accumulation (Fig. 8(f, g)). Regarding lateral roots, NBT detection unveiled lower levels of superoxide in lateral root tips of the *rbohE* mutant, which also was impaired in the promotion of root branching by *T. atroviride* (Fig. 9(a, b)). These results involve RBOHE for the *Trichoderma*-induced ROS production in roots and the configuration of root system architecture.

Fig. 5 *T. atroviride* induces the accumulation of superoxide anion in the root. Visualization of superoxide was recorded through NBT staining. *Arabidopsis* seedlings were co-cultured for 4 days with or without *T. atroviride*. Micrographs show lateral roots and primary roots of plants stained with NBT (a–d). The graphs illustrate differences in NBT staining intensity present in the elongation zone of lateral roots (e) and primary roots (f). Scale bar: 200 and 100 μm, respectively. The values shown represent the means for root tips imaged from 8 independent seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated three times with comparable results

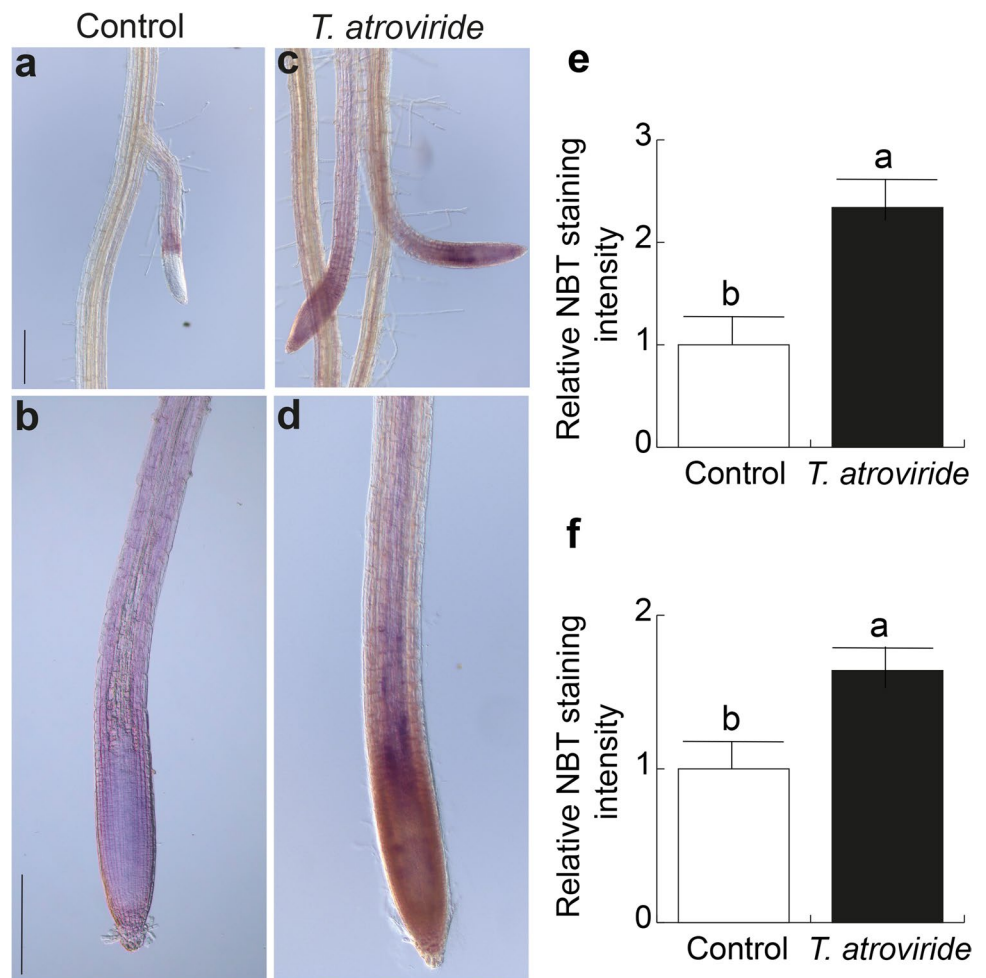


Fig. 6 Effect of *T. atroviride* on biomass production of *Arabidopsis* WT and *rboh* mutants. Four-day-old WT *Arabidopsis* seedlings and mutants lacking the functional isoforms of *RbohA*, *RbohD*, and *RbohE* enzymes were inoculated with *Trichoderma* at 5 cm from the root tip. After 4 days of co-culture, representative photographs of seedlings co-cultivated with *T. atroviride* were taken (a–h), and shoot fresh weight (i), and total fresh weight (j) were recorded. Bars show the means ± SD, among three groups of ten plants each that were weighed on an analytical scale. Different letters indicate statistically significant differences ($P < 0.05$; $n = 15$). Scale bar: 1 cm. The experiment was repeated three times with comparable results

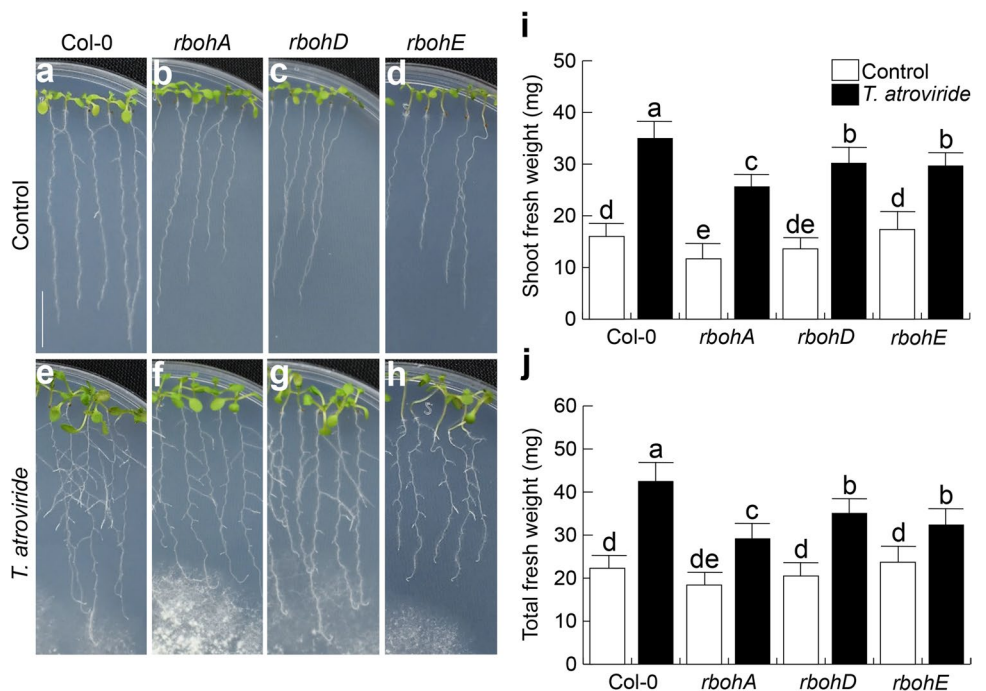
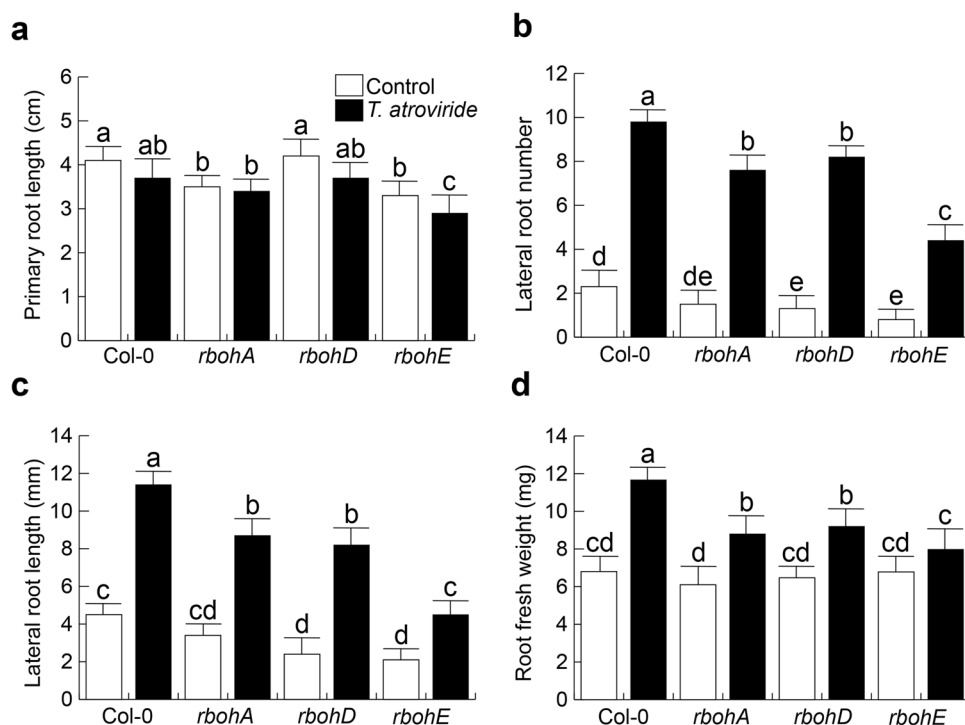


Fig. 7 Effect of *T. atroviride* on the root system architecture of *Arabidopsis* WT and *Rboh* mutants. Four-day-old WT *Arabidopsis* seedlings and mutants lacking functional isoforms of *RbohA*, *RbohD*, and *RbohE* enzymes were inoculated with *Trichoderma* at 5 cm from the root tip and allowed to grow for 4 additional days. Primary root length (a), lateral root number (b), lateral root length (c), and root fresh weight (d) were recorded. The values shown represent the means of 30 seedlings \pm SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated three times with comparable results



Discussion

Trichoderma positively influences plant health and productivity by stimulating growth and development, and suppressing diseases caused by pathogens (Guzmán-Guzmán et al. 2019; Alfiky and Weisskopf 2021). The versatile mechanisms employed by these fungi to promote plant growth include synthesis of phytohormones (mainly IAA-related indoles), solubilization of soil nutrients, increased uptake and translocation of minerals, enhanced tolerance to abiotic stress, improved photosynthesis and sucrose metabolism, and production of secondary metabolites (Guzmán-Guzmán et al. 2019; Ramírez-Valdespino et al. 2019; Khan et al. 2020; Esparza-Reynoso et al. 2021; Harman et al. 2021; Vinale and Sivasithamparam 2020). All these functions rely upon a dedicated plant-fungus communication in which rhizosphere acidification and the emission of the unsaturated lactone 6-PP orchestrate root growth and branching (Garnica-Vergara et al. 2016; Pelagio-Flores et al. 2017; Estrada-Rivera et al. 2019; Guzmán-Guzmán et al. 2019).

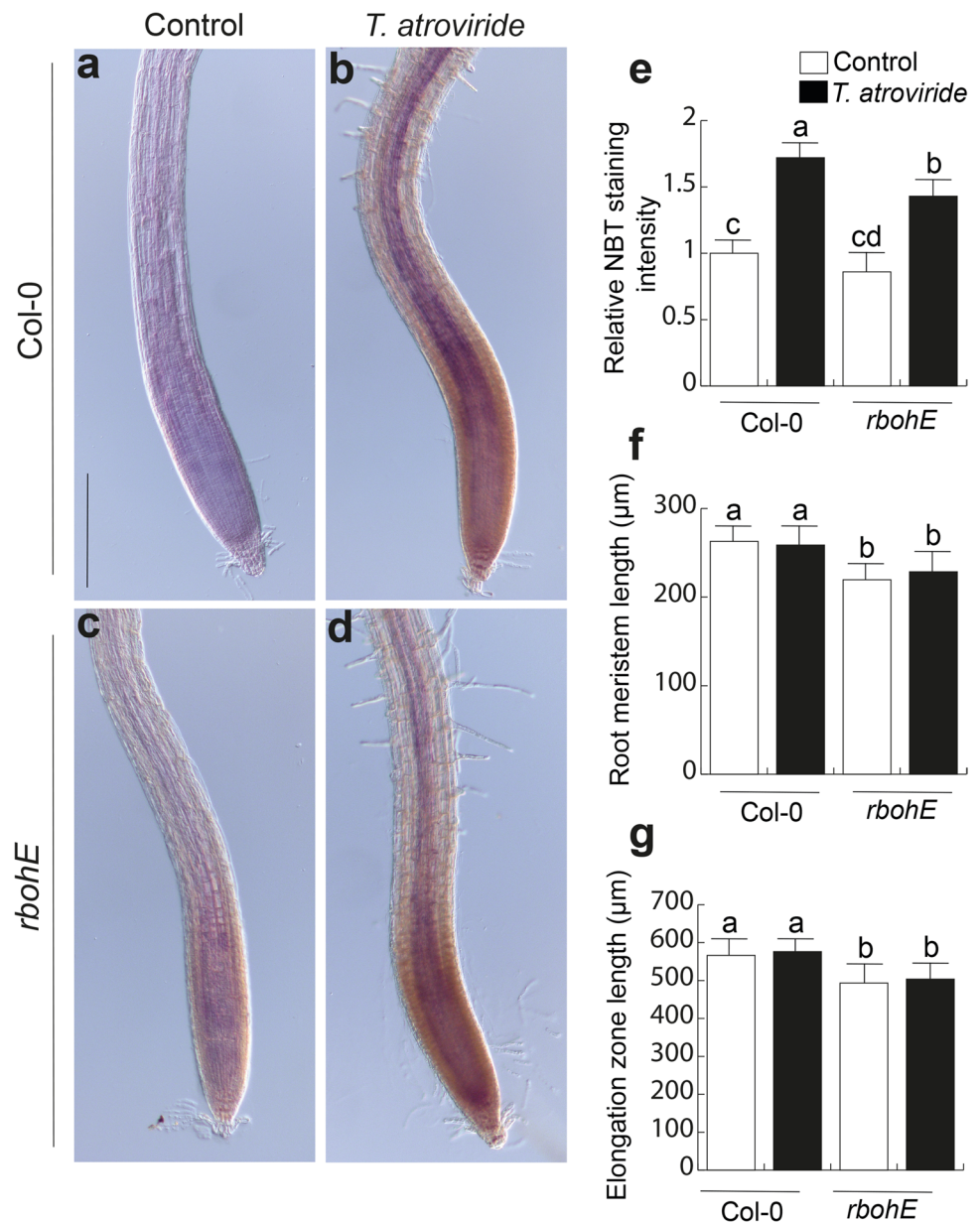
In a recent report, Villalobos-Escobedo et al. (2020) showed the importance of ROS production by fungal NADPH oxidase for the mutual *Arabidopsis*-*T. atroviride* recognition, for which mutation of *NoxR* changed the saprophytic behavior toward the more effective usage of sugars secreted from roots as energetic sources, and this in turn led to a stronger plant defense response. ROS have been considered second messengers in signal transduction pathways; in particular, during plant-microbe interactions, the

host-produced ROS includes the superoxide anion and H_2O_2 , which accumulate at both colonized sites and distal parts (Contreras-Cornejo et al. 2011; Huang et al. 2019; Nawrocka et al. 2019; Xu et al. 2020; González-López et al. 2021). With this in mind, we visualized ROS in several parts of *Arabidopsis* roots prior to physical contact with a growing colony of *T. atroviride*. According to the H2DCF-DA fluorescence detection, the intracellular ROS levels were higher in the roots of plants inoculated with *T. atroviride* compared with non-inoculated plants, showing a higher ROS accumulation in cell layers bordering the lateral root primordia, as well as the root apex of primary and lateral roots.

ROS accumulation at the root tip could influence the balance between cell proliferation and elongation processes which are the hallmark of the indeterminate growth pattern of healthy roots. Our data are consistent with previous reports, in which exogenous application of H_2O_2 to *Arabidopsis* seedlings stimulates lateral root development (Su et al. 2016; Orman-Ligeza et al. 2016). H_2O_2 also affects the directional transport of auxin through changes in the expression of auxin carriers, mainly PIN transporters, a process also observed in plants exposed to *Trichoderma atroviride* or its main volatile 6-PP (Ivanchenko et al. 2013; Orman-Ligeza et al. 2016; Su et al. 2016; Velada et al. 2020).

The acidification generated by *Trichoderma* induces a redistribution of auxin within the root apex that originates a reorientation of the root growth, previously to growth cessation (Pelagio-Flores et al. 2017), and this deviation from the normal gravity-response also follows ROS generation

Fig. 8 Effect of *T. atroviride* in the RBOH-mediated superoxide anion accumulation in primary roots, root meristem length, and elongation zone length. NBT staining of the roots of wild-type and *rbohE* mutant roots grown axenically or inoculated with *Trichoderma* (a–d). Bars graphs show differences in NBT staining intensity in the meristem of the primary roots (e), measurements of meristem length (f) from the quiescent center to the start of the elongation zone, and elongation zone length (g). The values shown represent the means of 8 seedling roots \pm SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 100 μ m. The experiment was repeated three times with comparable results



(Eljebbawi et al. 2021). Therefore, we hypothesized that *T. atroviride* through acidification of the rhizosphere, and/or emission of 6-PP, may account for ROS overproduction. To test this hypothesis, we analyzed the impact of acidic pH on the architecture of the *Arabidopsis* root system. According to the results obtained, the plants grown at acidic pH (5.5 and 4.5) manifested stronger intracellular ROS accumulation in the whole root tissues and their lateral roots were shorter than those of plants grown in medium with pH 7.0. Such result fits well with previous reports, since exposure to low-pH stress causes root growth and developmental alterations that correlate with excessive accumulation of ROS, such as superoxide anion and H_2O_2 in root tips (Koyama et al. 2001; Zhang et al. 2015; Long et al. 2019; Graças et al.

2020). Lager et al. (2010) indicated that pH sensing by the plant triggers the regulation of gene expression resembling the transcriptional response provoked by auxin or pathogen defense signaling. They also assume that perception of environmental pH may act as an underlying signal to the cellular responses of auxin and pathogens. Under this assumption, we suggest that low pH-dependent accumulation of ROS functions as a downstream component in signal transduction during plant-*Trichoderma* recognition, although deciphering the molecular components of such signaling mechanisms is still pending.

The inhibitory effect of 6-PP on primary root growth at high concentrations was associated with an increased accumulation of ROS in root tips, which was missing in

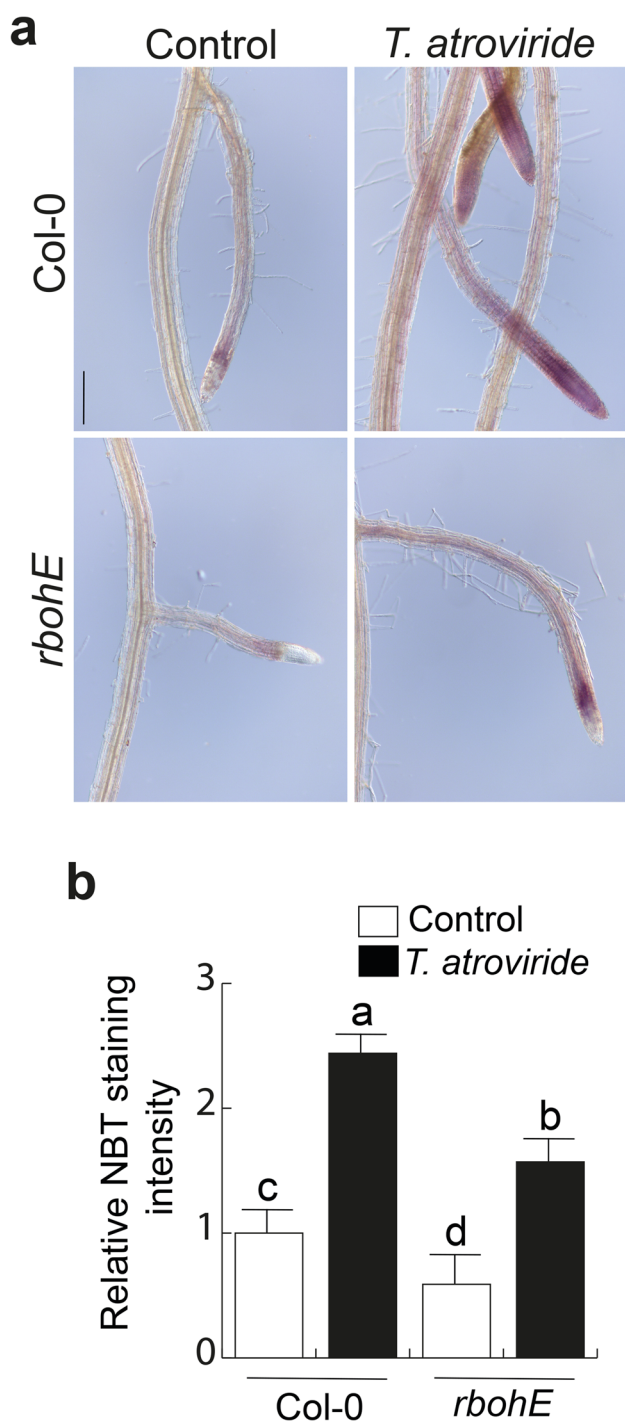


Fig. 9 Effect of *T. atroviride* in the RBOH-mediated superoxide anion accumulation in lateral roots. NBT staining of lateral roots of wild-type and *rbohE* mutants grown axenically or inoculated with *Trichoderma* (**a**). The graph shows differences in NBT staining intensity in lateral root tips (**b**). The values shown represent the means of 8 seedlings \pm SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 200 μ m. The experiment was repeated three times with comparable results

Arabidopsis mutants defective on the gene encoding ETHYLENE INSENSITIVE 2 (EIN2), and this led to insensitivity to primary root growth stoppage (Garnica-Vergara et al. 2016). The emission of 6-PP largely varies according to environmental factors, growth media, presence of plants, etc. (Garnica-Vergara et al. 2016). In this study 0, 75, and 150 μ M 6-PP were used as the two later concentrations show clear changes in the configuration of the *Arabidopsis* root architecture according to a previous report (Garnica-Vergara et al. 2016), and would help to correlate these changes with endogenous ROS detection. Interestingly, the inhibitory effect of 150 μ M of 6-PP in primary root growth may be caused by ROS overproduction, affecting cell division and expansion processes.

Inhibition of root growth and superoxide anion accumulation in roots are typical effects of ethylene or its precursor ACC (Lv et al. 2018); thereby, we anticipated that 6-PP could regulate primary root elongation via ethylene-dependent ROS homeostasis. Intriguingly, EIN2 is required for the exacerbated oxidative stress and root growth repression caused by plant-pathogen effectors such as bacterial flagellin (flg22) and pyocyanin; however, loss-of-function of EIN2 enhanced the generation of ROS under salinity stress too, indicating the involvement of ethylene/ROS crosstalk in activation of both biotic and abiotic stress, clearly modulated in plants colonized with *Trichoderma* (Mersmann et al. 2010; Lin et al. 2013; Beck et al. 2014; Ortiz-Castro et al. 2014). Besides, *Trichoderma* triggers the specific accumulation of superoxide at the apex of lateral roots and inner tissues that form the stele. This vascular tissue-dominated accumulation of superoxide matches well with the gene expression of NADPH oxidase RBOHF, which can be induced by salinity or ACC treatment, suggesting the possible involvement of this RBOH isoform in *Trichoderma*-mediated oxidative signaling in roots (Jiang et al. 2012, 2013; Chapman et al. 2019). Recently, RBOH-mediated ROS production was involved in important root developmental processes, such as primary root elongation, and lateral root formation (Chapman et al. 2019).

Orman-Ligeza et al. (2016) reported the ROS generation by RBOH enzymes, which facilitates cell wall remodeling of overlying cell layers for the outgrowth and emergence of lateral root primordia. Rboh genes comprise a large, functionally redundant family, which makes very difficult to assign specific functions to particular members. Nevertheless, we decided to evaluate the *rbohA*, *rbohD*, and *rbohE* mutants because the expression patterns of the corresponding genes are in endodermis, cortex, and epidermal cells overlying lateral root primordia (Orman-Ligeza et al. 2016). Moreover, *RbohE* was selected for more detailed analysis because this mutant exhibited a semi-dwarf phenotype and showed less response to the promoting effects of *Trichoderma*. The wild-type (Col-0) seedlings and RBOH-deficient mutants *rbohA*, *rbohD*, and *rbohE* were inoculated with *T. atroviride* for

4 days and their growth and root developmental responses were compared to wild-type seedlings. *T. atroviride* promoted root and shoot biomass production and increased root branching in WT seedlings compared to non-inoculated plants; however, the mutations in *RBOH* genes slightly decreased the growth-promoting activity of *Trichoderma* and root branching. Interestingly, the *RbohE* mutant plants displayed poor lateral root proliferation and lower superoxide levels, indicating that the loss of RBOHE is critical for ROS generation during the plant–fungus interaction.

The evaluation of ROS production in *rbohE* mutant plant also demonstrated that ROS accumulation in lateral roots is mainly dependent on this isoform and this coincided with the specific pattern of *RbohE* expression in the cells overlying/surrounding the lateral root primordia and the mutant phenotype, which exhibits a delayed development of the primordia (Chapman et al. 2019; Eljebbawi et al. 2021). This is consistent with the phenotype of *rbohE* mutants, which exhibited a root meristem shorter than the wild-type due to decreased cell proliferation. On the other hand, RBOHA showed a comparable expression pattern to RBOHE within lateral root primordia, and the maturation zone of the primary root (stele and endodermis) and both were auxin-inducible, suggesting that RBOHA also participates in the initial stages of development of lateral roots (Orman-Ligeza et al. 2016; Chapman et al. 2019). *RBOHD* and *RBOHF* have been reported as regulators of lateral root formation (Otulak-Kozieł et al. 2020; Mase and Tsukagoshi, 2021), which indicates developmental stage-specific functions for each RBOH. In this sense, we cannot exclude possible redundant functions for RBOHA, RBOHD, and RBOHE for the root branching process being stimulated by *Trichoderma* and its metabolites. The possibility is open that their encoding genes could act as downstream target genes of the auxin-dependent growth programs and/or defense response signaling pathway elicited by *Trichoderma* in plants.

Conclusions

Taken together, the data presented in this work add a missing piece in the signal transduction events in the *Arabidopsis*–*T. atroviride* interaction. The notion that ROS are merely toxic molecules changed in recent times owing their function in modulating transcription factors and other regulatory proteins, which led the proposal of the term “oxidative signaling” for the ROS control of plant morphogenesis. Here, we described the dynamic changes in total ROS levels and superoxide anion at several stages of root development, which coincided with root growth and branching patterns stimulated by *T. atroviride*, its acidification of the rhizosphere and emission of its highly bioactive volatile 6-PP. Although the RBOH family includes many members, and

functional redundancy may account for the dynamic ROS production in a tissue-specific manner and in response to abiotic or biotic stimuli, our work uncovered the important function of RBOHE for the phytostimulation and root architectural configuration driven by *Trichoderma* in *Arabidopsis*. Overall, the current data increase our knowledge into how plants interact with a fungal partner, widely applied in agriculture as a biocontrol agent and biostimulant microorganism.

Acknowledgements The authors thank Dr. León Francisco Ruíz Herrera for helping with image acquisition in laser confocal microscopy. SER and AAR are indebted to the Consejo Nacional de Ciencia y Tecnología (CONACyT) for doctoral and MSc fellowship, respectively.

Author contribution SER, AAR, and JLB designed and performed experiments and interpreted data; RPF and AAR provided technical support and analyzed data. SER and JLB wrote the manuscript. All authors revised and approved the submission.

Funding This work was supported by grants from SEP-CONACYT A1-S-34768.

Data availability The data and materials reported in this work are available upon contact with the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

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