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AtCube: Performing pathogen-root infection tests on *Arabidopsis thaliana* in a completely controlled way

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ABSTRACT

Arabidopsis thaliana represents the main model plant for the experimental analysis of plant genetics and developmental biology of photoautotrophic organisms, due to characteristics such as size, life cycle, fecundity, genetics and its easy experimental manipulation. In turn, *A. thaliana* is used as a model plant in plant-microorganism interaction studies, being of great importance in the knowledge of the form of infection of numerous plant pathogens. The present work is based on the development of an *A. thaliana* plant growth system in Phytatray II boxes and culture substrate, trying to establish a system that is free of contamination, completely controlled, self-sufficient, reproducible and standardized, called AtCube. Through the use of different necrotrophic and biotrophic/hemibiotrophic root-pathogens, bacteria, fungi and oomycetes, and the comparison with a conventional system of growth in plots, various results are analyzed on the effects of pathogens on plants and the advantages and differences of the AtCube system. This work makes it possible to highlight that the AtCube system represents a methodology that allows obtaining results similar to other systems but with important advantages regarding its standardization, rigor and reproducibility with respect to studies with *A. thaliana* and root pathogens. Furthermore, it could represent an equally efficient system in studies with other plant species and the application of foliar pathogens and/or beneficial microorganisms.

1. Introduction

Crop pathogens and pests cause serious economic and food security losses by reducing the yield and quality of agricultural production [1]. Plant diseases can have great economic, social and ecological importance both locally and globally. In this sense, in Ireland in the 1840s, one of the most historically relevant plant diseases developed, the potato late blight, which caused the migration and death of a large number of people. Nowadays, plant diseases are one of the key obstacles in the goal of feeding a constantly growing world population [2]. Worldwide, yield losses caused by pests and diseases are estimated to average 21.5% in wheat, 30.0% in rice, 22.6% in maize, 17.2% in potato, and 21.4% in soybean; these crops account for half of the global human calorie intake [1,3].

Arabidopsis thaliana, in addition to being the quintessential plant science model plant, it has been widely used as a host model to study plant-pathogen interactions. Already in 1993 Mauch-Mani and Slusarenko published a review in which they compiled all the characteristics that made this plant the perfect candidate for this type of study [4]. The molecular biology and genetics of *A. thaliana* are so well defined, and viruses, bacteria and fungi that infect it are representative pathogens of economically important plants [4]. A good example of this use is found in the *Arabidopsis-Pseudomonas* interaction, thanks to whose studies it was possible to decipher host resistance and pathogen virulence, extrapolated to other plants [5]. Even the use of *A. thaliana* in this regard has resulted in the re-classification of plant pathogenic fungi as necrotrophs or biotrophs [6]. In this sense, Agrawal in 2018 compiled all the examples of the use of *A. thaliana* as a model host to plant-pathogen interactions, highlighting the importance of these studies for present and future research. This study will help in understanding how pathogens and plants have evolved to this day, in order to develop better control strategies focused on the virulence of the pathogen or the activation of plant defenses [7].

Furthermore, it is important to highlight that the appearance of the plant-disease does not depend only on the plant-pathogen interaction, but also on the environment. The disease will appear only with the union

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of a virulent pathogen, a susceptible plant host, and the proper environmental conditions [2].

The objective of this study was to establish a controlled, replicable and reliable methodology for studies of *A. thaliana* root-infection with different microorganisms.

2. Materials and methods

2.1. Biological material

The *A. thaliana* ecotype Col-0 was the plants used in this study. *Arabidopsis* seeds were surface-sterilized by vigorous sequential shaking in 70% ethanol and 5% sodium hypochlorite solutions for 10 min each and then washed thoroughly four times in sterile distilled water, as previously described [8].

Different root-pathogens were facilitated by the Regional Diagnostic Center of the Regional Government of Castilla y León (Salamanca, Spain). Fungi *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, and bacteria *Pectobacterium carotovorum* were used as necrotrophs, and fungi *Fusarium oxysporum* f. sp. *conglutinans*, *Leptosphaeria maculans*, and oomycete *Pythium irregular*e were used as biotrophs and hemibiotrophs. Fungi were grown on PDA medium (Sigma-Aldrich, St. Louis, MO, USA) and bacteria in LB solid medium (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Plant growth systems

2.2.1. Plots

Following the methodology previously described by Ref. [8], surface-sterilized seeds were plated on Murashige and Skoog (MS) (Duchefa, Haarlem, The Netherlands) solid medium (agar 1%) with sucrose (1%) and plates maintained in a growth chamber at 22 °C, 40% relative humidity (RH) and a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1}, for 7 days. Seedlings were individually transferred to 0.2 L-pots, containing a mixture of peat/vermiculite (3:1) sterilized by autoclave (twice, 24 h apart), maintained again in a growth chamber.

2.2.2. AtCube

The methodology named AtCube is represented step by step in Fig. S1. After obtaining *A. thaliana* seedlings how described in the previous section, seedlings were individually deposited inside Phytatray II boxes (Sigma, St. Louis, MO, USA) (six plants per box), prepared as follows. In each Phytatray II box 100 mL of a mixture of peat/vermiculite (3:1), sterilized by autoclave (twice, 24 h apart), was deposited in the bottom. Subsequently, the culture substrate was irrigated with 15 mL of sterile water. During the life of the plants, the boxes were kept inside a growth chamber at 22 °C, 40% RH and a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1}, always in sterile conditions.

In both systems, 18 plants were planted per biological replica and condition.

2.3. Pathogens inoculation

In *A. thaliana* three weeks old plants (one week on plate and two weeks on growth substrate) root inoculation of pathogens was carried out. All data resulting from infections were taken 18 d.p.i. For all root-infections, 1 mL of sterile water was added with a determined concentration of pathogen inoculum, as described by other authors with some modifications.

To *R. solani*, *S. sclerotiorum* and *P. irregulare*, all the mycelium formed in three PDA Petri dishes was collected, subsequently, 30 mL of sterile distilled water was added in a Falcon tube along with the mycelium and 0.5 g of Ballotini Glass Balls 0.15–0.25 mm diameter and others 0.5 g of 1 mm diameter (Potters, Saint-Pourçain-sur-Sioule, France), and vigorously shaking for 20 min. The resulting mycelium suspension was adjusted for inoculation to the absorbance of 0,17 per mL at 520 nm. To *F. oxysporum* and *L. maculans* a spore solution diluted to a 1×10^6 per mL concentration was used [9,10]. To *P. carotovorum*, each plant was inoculated with 3×10^6 cfu mL^{-1} [11]. After infection, plots and Phytatray II boxes were kept inside the growth chamber with the conditions described yet.

2.4. Plant biomass quantification

The quantification of the vegetable biomass of *A. thaliana* aerial part was carried out using two different methodologies previously described for *Marchantia polymorpha* by Ref. [12]. Three-weeks old non-inoculated plants and 18 d.p.i. pathogens-roots-inoculated plants were photographed in the inside Phytatray II boxes, and the images obtained were analyzed for the quantification of the percentage of existing plant thanks to the software MulticolorEngine (TinEye, Toronto, Ontario, Canada) (https://labs.tineye.com/color/). Later, dry weight of the aerial part of each plant was obtained, keeping the fresh plant for 48 h at 65 °C. In all cases, data were taken from 18 plants.

2.5. Roots vitality test

In order to determine the radicular viability of *A. thaliana*, roots from six plants per each culture-system and pathogen-inoculation were pooled and root pools from three independent assays were considered, using the methodology previously described by Ref. [13]. Reduction of triphenyltetrazolium chloride (TTC) by tissue to the red-colored insoluble triphenylformazan (TF) is directly linked to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC to TF [14]. From the root pools 100 mg were transferred to 1 mL of 1% TTC in triplicate and incubated for 48 h at 37 °C. After incubation, 100 mg of Ballotini Glass Balls 0.15–0.25 mm diameter and others 100 mg of 1 mm diameter were added to each sample in 1,5 mL Eppendorf tubes, shaking vigorously by a pulse of 20 s in Silamat S6 (Ivoclar Vivadent, Madrid, Spain). After centrifuging the samples for 5 min at 10, 000 rpm, the supernatant was removed, and 1 mL of isopropanol was added per tube. The samples were again agitated in Silamat and centrifuged in the same way, and the supernatant was used to quantify its absorbance at 620 nm, being an indirect measure of the vitality of the roots.

In the case of the analysis of three weeks-old *Arabidopsis* plants without pathogen-inoculation, pools of the aerial part were also used, exactly the same as the root ones.

2.6. Indirect quantification of ROS in roots

The indirect quantification of ROS in roots was carried out by measuring electrolyte leakage similar to the method used by Ref. [12], which really measures cellular oxidative damage related to the production of ROS. From the root pools previously formed 100 mg of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water for 6 h at room temperature. The conductivity of the water was measured using a Crison™ Conductimeter GLP31 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the roots (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total ions present in the roots (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100], normalizing to 1 the value obtained in plants grown in plots.

In the case of the analysis of three weeks-old *Arabidopsis* plants without pathogen-inoculation, pools of the aerial part were also used, exactly the same as the root ones.

2.7. Tissue-pathogen growth

In order to measure the amount of pathogen in the roots of *A. thaliana*, roots from six plants per each pathogen-inoculation were

pooled and root pools from three independent assays were considered. All root material was washed with sterile distilled water until there was no remaining substrate, immediately frozen with liquid nitrogen, and pulverized with a mortar.

The quantification of quantity of the pathogen in roots was performed by the quantification of pathogen DNA by quantitative PCR (qPCR), as previously described to roots microorganisms [15]. The DNA was extracted from *A. thaliana* roots of pathogen-inoculated plants. A mix was prepared in a 10-μL volume using 5 μL of Brilliant SYBR Green QPCR Master Mix (Roche, Penzberg, Germany), 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. The endogenous genes of pathogens and *A. thaliana* used for the quantification are represented in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), programmed for 40 cycles under the following conditions: denaturation, 95 °C for 15 s; annealing, 60 °C for 1 min; extension, 72 °C for 1 min. Each PCR was performed in triplicate by using the DNA extracted from three root pools of six plants each-one for each pathogen-infection. Cycle threshold values served to calculate the amount of pathogen DNA using standard curves. The values of pathogen DNA were referred to the amount of *A. thaliana* DNA in every corresponding sample.

2.8. Roots-gene expression

In order to analyze the expression of different selected genes in *A. thaliana* roots, the methodology previously described by Ref. [8] was carried out. From the root pools formed RNA was extracted with the TRI reagent (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain), and then used for reverse transcription with an oligo(dT) primer with the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Inc., Tokyo, Japan), following the manufacturer's protocol. Gene expression was analyzed by RT-qPCR, using an ABI PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). All PCR reactions were performed in triplicate in a total volume of 10 μL for 40 cycles under the following conditions: denaturation, 95 °C, 30 s; annealing, 60 °C, 1 min; extension, 72 °C, 1 min. Threshold cycles (CT) were determined using the 7000 SDS System Software (Applied Biosystems, Foster City, CA, USA), and CT values were calculated using the *A. thaliana Actin* gene (AGI: AT3G18780) as an endogenous control. The primers used are given in Table 1. Data are expressed using $2^{-\Delta\Delta CT}$ method [16].

In the case of the analysis of the stress-related genes (*RBOHD*, *BI-1* and *SAG12*) in three weeks-old *Arabidopsis* plants without pathogen-inoculation, pools of the aerial part were also used, exactly the same as the root ones.

2.9. Statistical analysis

The statistical analysis of the data was carried out with the Statistix 8.0 software. One-way ANOVA using Student's t-test was used for comparison of means at $P < 0.05$; significant differences are denoted using an asterisk.

3. Results

3.1. Plant status differences in AtCube vs. plots

Visually (Fig. 1a and b) differences were observed in the growth of the aerial part of *A. thaliana* plants in both systems (plots and AtCube). These differences were reflected in a significantly lower production of aerial biomass in plants grown in AtCube compared to those grown in plots, both in percentage of existing plant (Fig. 1c) and in dry weight (Fig. 1d).

The analysis of cellular vitality and indirect accumulation of ROS in the tissues of *A. thaliana* reported how, neither in roots nor in leaves, there are significant differences between plants grown in plots and in AtCube system (Fig. 2). Similarly, no significant differences were reported in the expression of genes related to cell stress (*RBOHD*, *BI-1* and *SAG12*) in both types of organs (roots and leaves) when the plants were grown in plots or in AtCube system (Fig. 3).

Table 1
Oligonucleotides used in this work.

Code	Gene	Sequence (5′-3′)	Use	Reference
Act-At-F	*Actin*	CTCCCGCTATGTATGTCGCC	Endogenous *A. thaliana* gene	[8]
Act-At-R		TTGGCACAGTGTGAGACACAC		
Act-Rs-F	*Actin*	TAGAATGCCTAAGACGGGAA	Endogenous *R. solani* gene	[25]
Act-Rs-R		CCGCAGTAAGTCGTCATTGC		
Tub-Ss-F	*β-Tubulin*	TTGGATTTGCTCCTTTGAC CAG	Endogenous *S. sclerotiorum* gene	[26]
Tub-Ss-R		AGCGGCCATCATGTTCTTAGG		
Tub-Fo-F	*β-Tubulin*	TTCCCCCGTCTCCACTTCTTCATG	Endogenous *F. oxysporum* gene	[27]
Tub-Fo-R		GACGAGATCGTTCATGTTGAACTC		
ITS2-Pi-F	*Internal transcribed spacer 2*	AGCGGCGGGTGCTGTTGCAG	Endogenous *P. irregulare* gene	[28]
ITS2-Pi-R		GCTGCGTTCTTCATCGATGC		
Pel-Pc-F	*Pectate lyase*	TTACCGGACGCCGAGCTGTGGCGT	Endogenous *P. carotovorum* gene	[29]
Pel-Pc-R		CAGGAAGATGTCGTTATCGCGAGT		
Act-Lm-F	*Actin*	AAGAGCGGTGATTTCCTTCT	Endogenous *L. maculans* gene	[30]
Act-Lm-R		AGTGCGATGTCGATGTCAG		
RBOHD-At-F	*Respiratory Burst Oxidase Homolog D*	TCCACAAGGTTATTGCAAGCG	ROS production-related gene in *A. thaliana*	[31]
RBOHD-At-R		CTGCTCCGTGCTTTCAGATCAA		
BI-1-At-F	*Bax inhibitor-1*	GGACTGCTTTCATCTGGCTTGT	Apoptosis-related gene in *A. thaliana*	[32]
BI-1-At-R		GATAGATGCAGAGCCACCAAAGA		
SAG12-At-F	*Senescence-associated gene 12*	GCTTTGCCGGTTTCTGTTG	Senescence-related gene in *A. thaliana*	[33]
SAG12-At-R		GTTTCCCTTTCTTTATTTGTGTTG		
ICS1-At-F	*Isochorismate synthase 1*	GATCTAGCTAACGAGAACGG	Synthesis gene of SA in *A. thaliana*	[8]
ICS1-At-R		CATTAAACTCAACCTGAGGGAC		
PR-1-At-F	*Pathogenesis-related protein 1*	GGCTAACTACAACTACGCTG	Response gene of SA in *A. thaliana*	[8]
PR-1-At-R		GGCTTCTCGTTCACATAATTC		
LOX1-At-F	*Lipoxygenase 1*	GTAAGCTCTGATGTTACTGATTC	Synthesis gene of JA in *A. thaliana*	[8]
LOX1-At-R		CTGCGGTTAACGACGTGATTG		
VSP2-At-F	*Vegetative storage protein 2*	GTTAGGGACCGGAGCATCAA	Response gene of JA in *A. thaliana*	[8]
VSP2-At-R		TCAATCCCGAGCTCTATGATGTT		

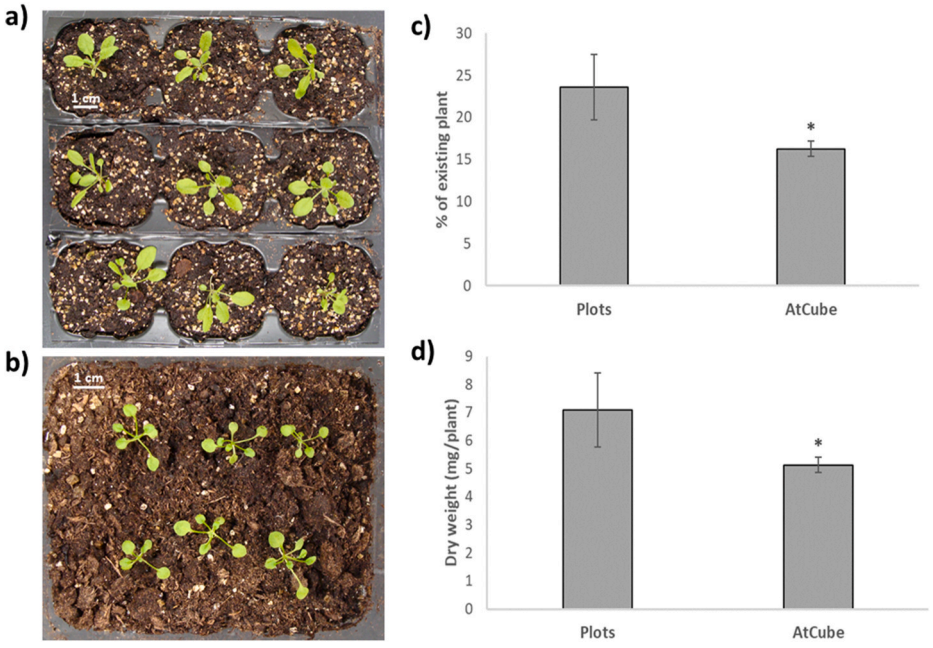

Fig. 1. Growth differences in *A. thaliana* plants in plots (a) vs. AtCube (b). Aerial-plant biomass analyzed by visual quantity of the plant (c) and the dry weight (in mg) (d). Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, aerial part from six plants were used. One-way analysis of variance (ANOVA), followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

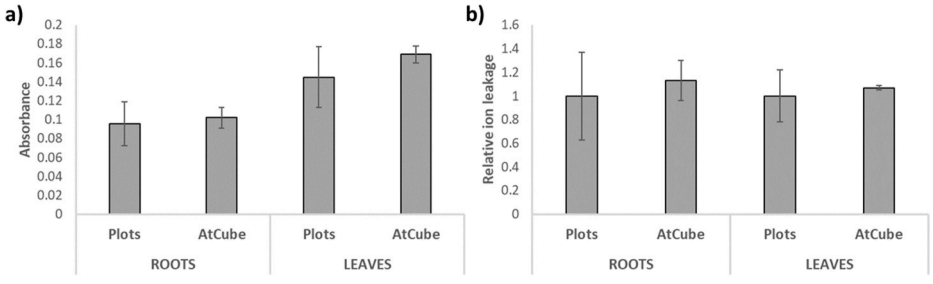

Fig. 2. Tissue vitality by TTC test (a) and indirect quantification of ROS (b) in tissues of *A. thaliana* in plots vs. AtCube. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, aerial part and roots from six plants were used. In indirect quantification of ROS, the values of plants in plots were normalized to 1. One-way analysis of variance (ANOVA), followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

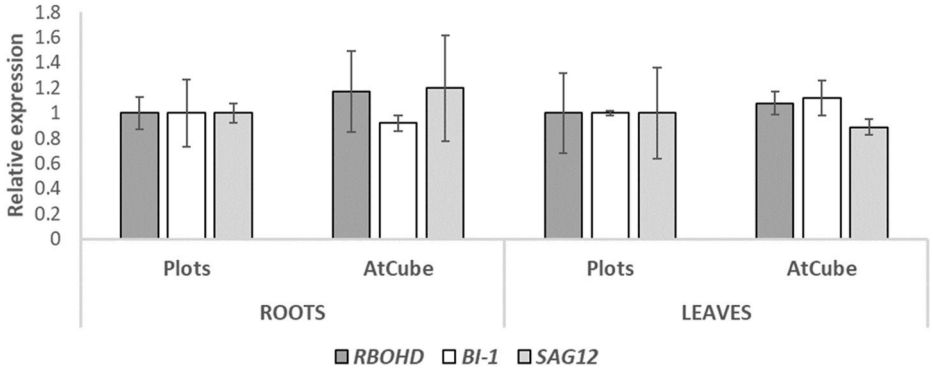

Fig. 3. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some stress-related genes in aerial part and roots of *A. thaliana* grown in plots vs. AtCube. Genes of the respiratory burst oxidase homolog D (*RBOHD*), Bax inhibitor-1 (*BI-1*), and senescence-associated (*SAG12*). Values correspond to relative measurements against plants grown in plots ($2^{-\Delta\Delta Ct} = 1$). The *A. thaliana Actin* gene was used as an internal reference gene. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, aerial part and roots from six plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

3.2. Root-pathogens effect on A. thaliana plants

Eighteen days after root infection with each of the pathogens, the symptoms caused in *A. thaliana* plants grown in plots and AtCube were photographed, without highlighting significant differences between both growth systems (Fig. 4). In analysis of infected plants aerial part biomass, no significant differences were reported in plants grown in AtCube compared to those grown in plots, neither in percentage of existing plant (Fig. 5a) nor in dry weight (Fig. 5b). Similarly, no significant differences were reported in roots with respect to vitality (Fig. 6a) or indirect quantification of stress by the indirect accumulation of ROS (Fig. 6b) between *A. thaliana* plants grown in plots and in AtCube and root-inoculated with the different pathogens.

As far as the quantification of the amount of the pathogen developing in the roots of *A. thaliana* is concerned, no significant differences were reported between the roots of plants grown in plots and in AtCube (Fig. 7).

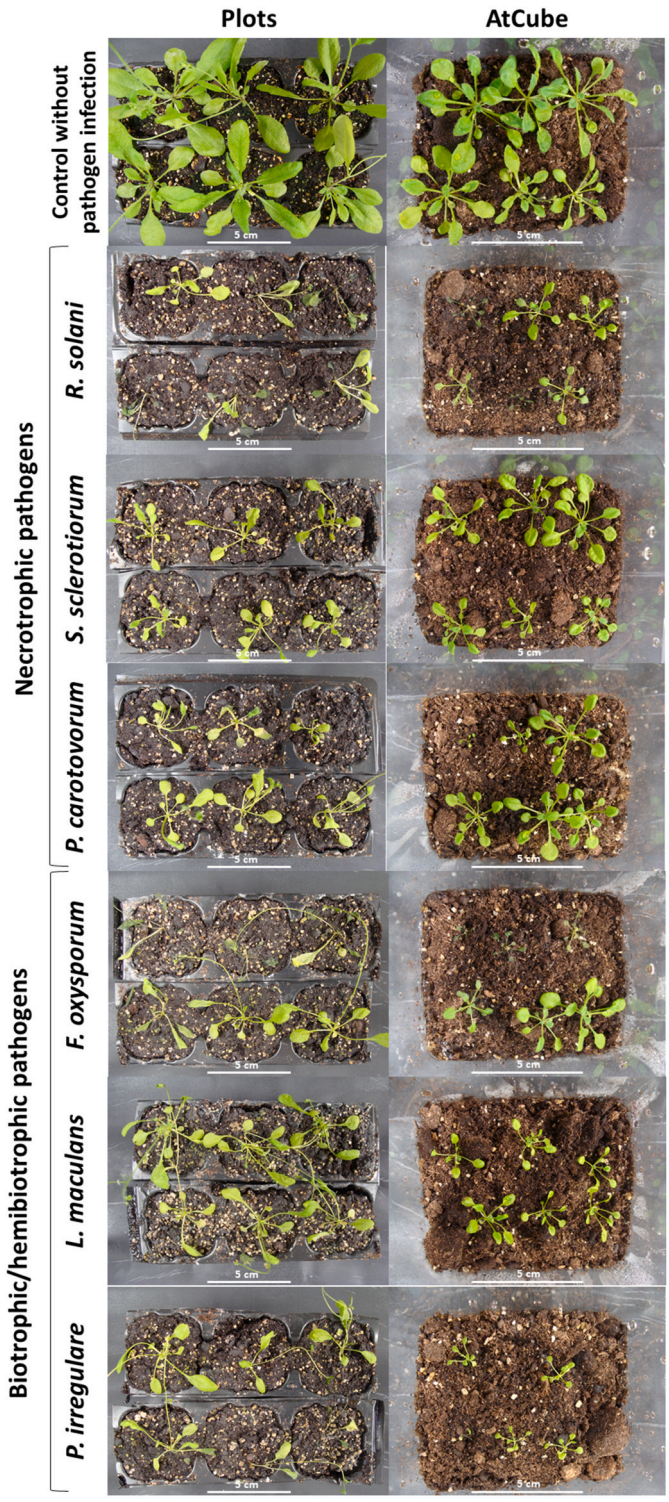

Fig. 4. Visual appearance of *A. thaliana* plants grown in plots vs. AtCube, root-infected with the different pathogens. The photographs were taken on 39-day-old plants, 18 d.p.i.

3.3. Defense gene expression

The analysis of defense gene expression by RT-qPCR (Fig. 8) in *A. thaliana* roots showed how both in plots and AtCube there is a significant increase in the expression of SA-related genes (*ICS1* and *PR-1*) and a significant decrease in the expression of JA-related genes (*LOX1* and *VSP2*) in response to biotrophic and hemibiotrophic pathogens. On the contrary, in response to infection by necrotrophic pathogens, a significant increase in the expression of JA-related genes and a significant decrease in the expression of SA-related genes were reported in both growth systems. The comparison between the gene expression data obtained in each one of the systems for infection with each of the pathogens did not report significant differences (Fig. 8).

4. Discussion

Since Friedrich Laibach in 1943 proposed *A. thaliana* as a model organism for the experimental analysis of plant genetics and developmental biology of photoautotrophic organisms, it has become the most widely used model plant. This is due to characteristics as a small size for ease of growth in large populations, well established classical genetics, ease of crossing, a short generation time, high fecundity, small genome size, and the possibility to perform mutational screens in the laboratory [17]. As a consequence of these characteristics, *A. thaliana* has also been considered as an important model plant in the study of plant-microorganism interactions, both pathogenic and beneficial [7].

The AtCube system proposes a standardized cultivation methodology for *A. thaliana* that allows the inoculation of different root pathogens in a controlled way. With regard to plant growth, we have been able to verify how the biomass production in *A. thaliana* plants is slightly reduced in AtCube compared to its growth in plots, although it is carried out in a more uniform way, greatly reducing the difference between plants. On the contrary, no differences were reported with respect to the expression of stress-related genes (ROS production, senescence and apoptosis) neither in roots nor in leaves, as well as with respect to the vitality of the tissues and the indirect accumulation of ROS, all of them being rigorous forms of stress measurement in plant tissues [12,18,19], also in *A. thaliana* [8].

Furthermore, AtCube represents a closed system where there is only exchange of gases with the outside through the union of the two parts that make it up, which can greatly facilitate the measurement of this gas exchange compared to other cultivation systems, something very difficult to standardize in *A. thaliana* [20]. But something even more important is that AtCube represents a closed system with respect to water. The water present in the cultivation substrate is absorbed by the roots, transpired from the leaves and condensed on the walls of the system, which allows to completely close the water cycle, fully supplying the plant needs and maintaining a suitable humidity for growth and development of pathogens inoculated exogenously. In this sense, the control of the amount of water introduced into AtCube allows the reproducible standardization of studies of plant responses in situations of deficit or excess of water, something of great importance [21].

As far as the root application of each of the selected pathogens (bacteria, fungi and oomycetes; necrotrophs and biotrophs/hemibiotrophs) is concerned, it has been possible to verify the total absence of significant differences between the plots and AtCube. In this sense, the results obtained 18 d.p.i. in both systems were very similar with respect to plant biomass, vitality, ROS accumulation, and colonization by root tissue pathogens. Furthermore, there were no significant differences in the expression of the different defense genes in infected roots in both systems. These results are indicative that the AtCube system is, at least, similar to the plot system in parameters analyzed in this study.

In addition, it is important to note that, together with the absence of differences, AtCube has a series of very relevant advantages compared to the by plots system. AtCube represents a totally sterile system isolated from exogenous contaminants, it does not require any type of care (irrigation, phytosanitary treatments, etc.) during the entire plant development, greater manageability and transport possibilities, easy establishment in growth chambers, or greater safety, since the entire infection process is carried out in laminar flow cabinet and the complete systems can be eliminated using the same methodology used for the Petri dishes. All these characteristics make the AtCube system a methodology that considerably facilitates the standardization and

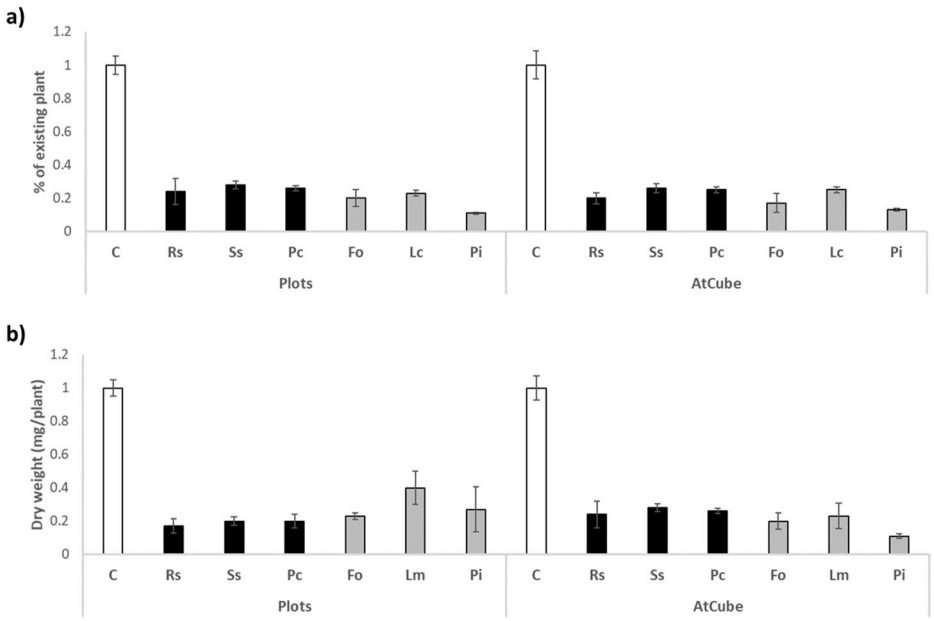

Fig. 5. Plant biomass of 39-day-old *A. thaliana* plants grown in plots vs. AtCube, root-infected with the different pathogens, 18 d.p.i. Aerial-plant biomass analyzed by visual quantity of the plant (a) and the dry weight (in mg) (b). In controls without pathogen infection (C), infection with *R. solani* (Rs), *S. sclerotiorum* (Ss), *F. oxysporum* (Fo), *L. maculans* (Lm), *P. irregularulare* (Pi) and *P. carotovorum* (Pc) the visual quantity of the plant and the dry weight were analyzed. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, aerial part from six plants were used, being the values of the uninoculated plants (C) in plots and AtCube normalized to 1. One-way analysis of variance (ANOVA), followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

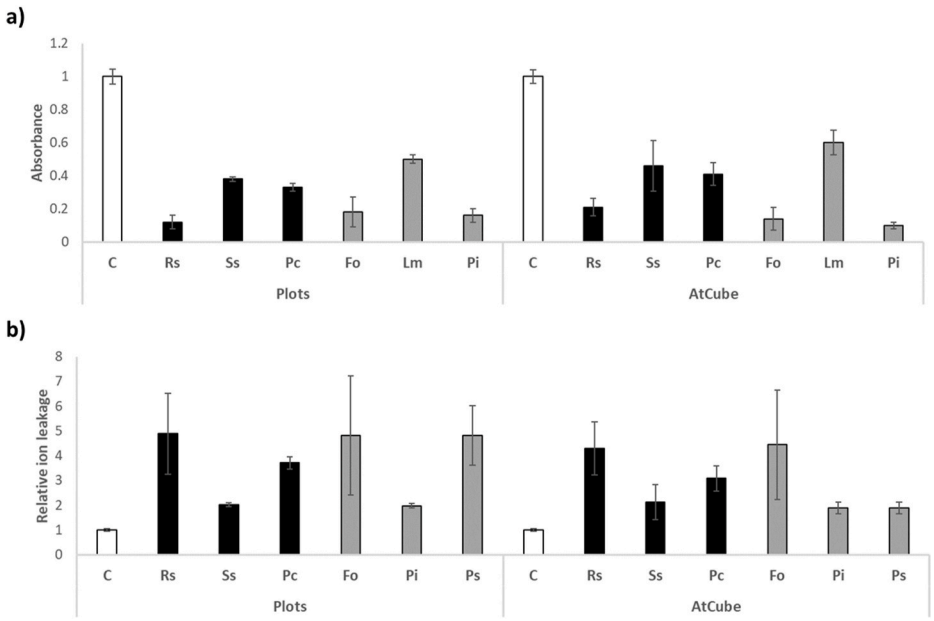

Fig. 6. Roots vitality by TTC test (a) and indirect quantification of ROS (b) in roots of 39-day-old *A. thaliana* plants grown in plots vs. AtCube, root-infected with the different pathogens, 18 d.p.i. In controls without pathogen infection (C), infection with *R. solani* (Rs), *S. sclerotiorum* (Ss), *F. oxysporum* (Fo), *L. maculans* (Lm), *P. irregularulare* (Pi) and *P. carotovorum* (Pc) the absorbance at 620 nm (TTC test) and the relative ion leakage (indirect ROS measurement) were analyzed. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, aerial part from six plants were used, being the values of the uninoculated plants (C) in plots and AtCube normalized to 1. One-way analysis of variance (ANOVA), followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

reproducibility of root infection assays in *A. thaliana*.

On the other hand, the AtCube system can not only be considered as a rigorous system in studies with root pathogens in *A. thaliana*, but also in foliar infections and in studies of interaction with beneficial microorganisms, even simultaneously, applying beneficial microorganisms and root pathogens or foliar simultaneously. In this way, completely isolated and standardized assays are facilitated in the induction studies of local and systemic plant defensive responses [22,23]. In addition, the AtCube system can be used with little or no modifications in plant-microorganism interaction studies with other model plants, such as the bryophyte *M. polymorpha*, which is of great importance in understanding the evolution of these interactions [24].

As final conclusions, our work has reported that the AtCube system represents a methodology that allows obtaining results similar to other systems but with important advantages regarding its standardization, rigor and reproducibility with respect to studies with *A. thaliana* and root pathogens. Furthermore, it could represent an equally efficient system in studies with other plant species and the application of foliar pathogens and/or beneficial microorganisms.

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Authors' contributions

JP is the Principal Researcher of the research project. JP thought and designed the work. JP performed the assays and analyzed the results. JP wrote the manuscript and made the corresponding revisions.

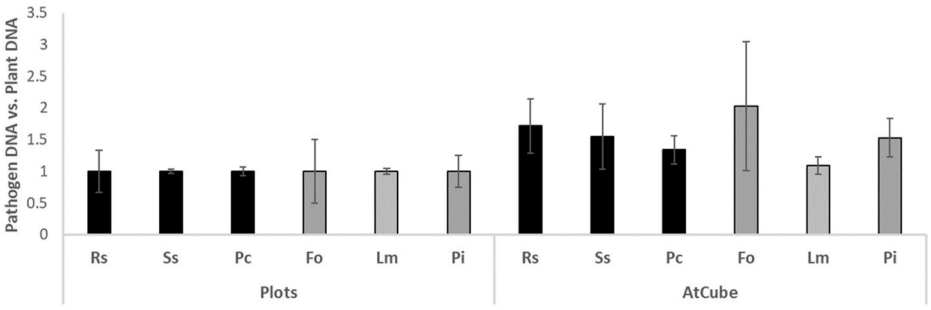

Fig. 7. Analysis of root tissue-pathogen growth of *R. solani* (Rs), *S. sclerotiorum* (Ss), *F. oxysporum* (Fo), *L. maculans* (Lm), *P. irregularulare* (Pi) and *P. carotovorum* (Pc) in *A. thaliana* 39-day-old plants, 18 d.p.i. grown in plots vs. AtCube. To quantify root-pathogen growth, the DNA of the pathogens were quantified by qPCR from radicular samples using the *Actin* gene from *A. thaliana* and *R. solani*, the *β-Tubulin* gene from *S. sclerotiorum* and *F. oxysporum*, the *Internal transcribed spacer 2* gene from *P. irregular*, the *Pectate lyase* gene from *P. carotovorum*, and the *Hipersensitive response protein Z* gene from *P. syringae*. Pathogen DNA/plant DNA ratio was normalized to 1 in the case of *A. thaliana* plants grown in plots and was calculated based on this data for the plants grown in AtCube. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, roots from six plants were used. One-way analysis of variance (ANOVA), followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

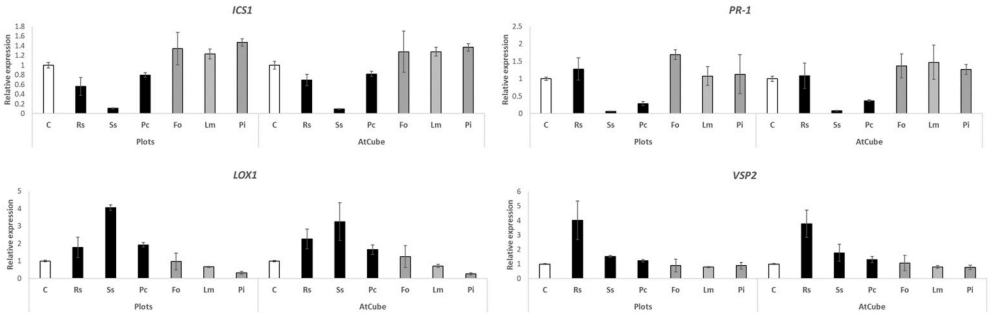

Fig. 8. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in the roots of 39-day-old *A. thaliana* plants grown in plots vs. AtCube, root-infected with the different pathogens, 18 d.p.i. In controls without pathogen infection (C), infection with *R. solani* (Rs), *S. sclerotiorum* (Ss), *F. oxysporum* (Fo), *L. maculans* (Lm), *P. irregularulare* (Pi) and *P. carotovorum* (Pc). Values correspond to relative measurements against plants without infection ($2^{-\Delta\Delta Ct} = 1$). The *A. thaliana Actin* gene was used as an internal reference gene. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, roots from six plants were used. One-way analysis of variance (ANOVA), followed by the Student's t-test was performed. Asterisks denote significant differences at $P \leq 0.05$ between plots and AtCube.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2021.101780.

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