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Research article

Limited ventilation causes stress and changes in *Arabidopsis* morphological, physiological and molecular phenotype during *in vitro* growth

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ABSTRACT

A huge number of experiments in plant biology are conducted in sterile, sealed containers, providing environmental stability and full control of factors influencing the plant system. With respect to roots the *in vitro* growth has another benefit – the ease of conducting visual observations when grown in transparent media. Moreover, straightforward measurements of *in vitro* grown root systems make them a sensitive and convenient sensor of multiple stresses which may occur during experiments. In order to optimize root nematode infection tests for *Arabidopsis* mutants with relatively mild phenotypes, two Petri dish sealing techniques were tested (permeable medical adhesive tape and a popular non-permeable plastic film). Using standard experimental settings applied for infection tests, the root architecture, nematode infections, ion leakage, efficiency of photosynthesis, ethylene (ET) production, and CO_2 accumulation were monitored in *Arabidopsis thaliana* Ws-0 wild-type and *lsd1* (lesion stimulating disease 1) plants, which is a conditional dependent programmed cell death mutant.

All tested parameters gave statistically significant differences between the analyzed sealing tapes, indicating the importance of air exchange. This factor is quite obvious but often ignored in experiments performed in Petri dishes. The results clearly indicate that stress is greater in air-tight sealed plates. These observations were supported by the great expression variation of several marker genes associated with reactive oxygen species (ROS), ET, salicylic (SA), and jasmonic acid (JA) biosynthesis and signaling in two-week-old seedlings. These results are discussed in light of the observed changes in the ET and CO_2 concentration. Our results clearly indicate the importance of culture parameters for monitoring of abiotic and biotic stress responses in laboratory conditions, including accurate mutant phenotyping.

1. Introduction

Culturing plants in sterile conditions on growth media and in special growth chambers provides an unusually stable environment, and deprives plants of most classical stresses as compared to field cultivation. However, such plant growth conditions may generate a set of specific stresses for tissue culture including osmotic stress, insufficient nutrient supply/uptake, and others (Carman, 1995). Similar to other environmental factors, tissue cultures are also known to induce epigenetic and genetic changes (Filipecki et al. 2005, 2006; Filipecki and Malepszy, 2006).

Due to time and space limitations researchers usually accept disadvantages of tissue culture strategies, especially when good visibility of the morphology and anatomy of the root system is needed (Xu et al., 2013). Such requirements are important when studying interactions between plants and parasitic nematodes (Sijmons et al., 1991), or monitoring physiological parameters of root growth during abiotic stresses (Hawrylak-Nowak et al., 2012; Hu et al., 2016). When interpreting the results of such experiments researchers tend to ignore stress conditions from the experimental set-up which can interfere with the final phenotype of the examined mutant.

One frequently ignored factor that distorts the results of *in vitro* experiments is the composition of gases in the culture container (Huang and Cheng, 2005). Methods to assure proper aeration were studied in the heyday of tissue culture in the context of morphogenesis, micropropagation, and regeneration efficiency (Pua and Gong, 2004). Nowadays there are plenty of commercially available ventilation systems built into containers. These include ventilation cams, on-plate lids, and vented caps or lids etc. Besides some new developments in the design of culture vessels including microfluidic based culture platforms such as RootChip, RootArray, TipChip, and PlantChip (Nezhad, 2014; Aufrecht et al., 2018), the Petri dish is still the basic vessel type in

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plant-nematode interaction research. A few exceptions include culturing roots in multiwell plates (Sijmons et al., 1991) or on glass slides covered by agar medium (O'Callaghan et al., 2018). However, in laboratory protocols the usual recommendations are to aerate plates by opening and closing them or scotching the otherwise hermetic sealing tapes. Skipping such tricks often generates a great variability in the results due to the frequent occurrence of accidental leaks changing the gas proportions.

Because gases such as ethylene influence nematode parasitism (Wubben et al., 2001; Kammerhofer et al., 2015; Hu et al., 2017), we investigated two different methods of sealing Petri dishes during infection tests of *Arabidopsis thaliana* Ws-0 and the *lsd1* mutant with *Heterodera schachtii* larva. We decided to use the gas permeable medical adhesive tape and commonly used air-tight plastic film, which significantly reduces gas exchange. To demonstrate the influence of the tested sealants and to highlight the possible response mechanisms, besides susceptibility to parasitic nematodes we monitored root architecture, ion leakage, efficiency of photosynthesis, ET production, and CO_2 accumulation. These results are supported by quantitative RT-PCR of transcript markers of the redox status, SA, JA, and ET signaling.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of wild-type (wt) *Arabidopsis thaliana* L. Heynh. ecotype Wassilewskija (Ws-0) and the *lsd1* mutant in the Ws-0 background (Dietrich et al., 1997) were used in experiments. Seeds were surface-sterilized in 0.7% NaClO for 5 min and 70% ethanol for 1 min and subsequently rinsed five times in distilled H_2O. Two seeds per Petri dish (90 mm in diameter) were planted on modified KNOP medium supplemented with 2% sucrose (Sijmons et al., 1991) and subsequently grown at a short day regime (8: 16 h light/dark, 22: 20 °C, 70% HR). Light intensity was between 100 and 120 μmol $m^{-2} s^{-1}$. Petri dishes were sealed with either gas permeable medical adhesive tape (3M™ Micropore™) or air-tight plastic film (Parafilm® M).

2.2. Analysis of root architecture traits

Root apical growth is best evaluated when seedlings are grown vertically. Two plants per Petri dish were grown on KNOP medium and sealed with either permeable tape (3M™ Micropore™) or air-tight (Parafilm® M). The main root tip of seven-day-old seedlings was marked on the back of the Petri dish with a dot. Seedlings were grown in a vertical position for 5 days. Then, the growth length was determined by photographic analysis. After two more days, the number of lateral roots were counted and the root hairs were analyzed using the Leica (M5C) stereo microscope with appropriate digital camera (Leica DFC 425).

2.3. Determination of ethylene content

The analysis of ET concentration was performed on two-week-old plants using a gas chromatograph (HP5890 GC series II with a FID detector and a stainless steel column (6 fit × 1.8 in. × 2.1 mm)). Five *A. thaliana* plants were grown on KNOP medium plates with a rubber plug and sealed with permeable tape (3M™ Micropore™) or were sealed air-tight (Parafilm® M) (Supplementary Photo 1). After two weeks ET accumulation was measured. Measurements were made using two biological replicates. The determination of ET concentration was calculated from the standard curve created on the basis of two standards with known ET concentration (1 ppm and 3 ppm). The chromatogram analysis was done in UNI chrom software. (http://www.unichrom.com/unichrome.shtml). The results were analyzed by a *t*-test ($p < 0.05$).

2.4. Relative ion leakage analysis

Cell death was quantified by ion leakage from whole rosettes and root systems growing on mesh (50 μm). Two-week-old wt and mutant plants growing in two different sealing conditions were harvested and transferred to sterile 50 ml Falcon tubes filled with 35 ml of Milli-Q ultrapure water (Merck Millipore, Darmstadt, Germany). The relative electrolyte leakage was measured with a conductance meter (WTW, INOLAB Cond Level 1, Weilheim, Germany) and calculated as the ratio between the value obtained after 1 h of incubation, and the total leakage was evaluated after freezing the samples. The experiment was repeated three times. The results were analyzed by a *t*-test ($p < 0.05$).

2.5. Quantitative RT-PCR (qRT-PCR)

RNA was isolated using the Universal RNA Purification Kit (Eurx, Gdansk, Poland) according to the manufacturer's protocol with on-column digestion of DNA. RNA yield and purity were estimated on the NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA). Total RNA (1 μg) was reverse transcribed using (N) 6 random hexamer primers and conditions described in the *QuantiTect* Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was performed in triplicate using the primers described in Supplementary Table 1 using the QuantiTect SYBR Green PCR Kit (Qiagen) with the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Niasto, CA, USA). Real-time PCR cycling conditions were 5 min denaturation at 95 °C, followed by 40 cycles of amplification (15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C). The relative expression levels were estimated by using actin 2 as a reference according to the $^{\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). The significance of differences from the control was revealed by REST (Pfaffl et al., 2002). Following PCR, product melting curves were generated to ensure the purity of product formation. The list of primers used in qRT-PCR are included in the supplementary materials (Supplementary Table 1).

2.6. Nematode assay

Heterodera schachtii Schmidt cysts were harvested from *in vitro* stock cultures on mustard (*Sinapis alba cv. Albatros*) roots growing on KNOP medium (0.2%). The addition of 3 mM $ZnCl_2$ stimulated the hatching of the juveniles. Six to 7 d later, J2s (*H. schachtii* juveniles) were collected and sterilized in 0.05% $HgCl_2$ for 5 min and immediately washed five times in distilled H_2O. Fourteen-day-old *Arabidopsis* plants were inoculated with 80–100 J2s under sterile conditions. Inoculated plates were kept in the dark for 24 h, and subsequently transferred into a growth chamber under 8:16 h light:dark conditions. Two plants were used in one Petri dish (sealed by air-tight tape or permeable tape) and the experiments were repeated three times with 10 plants per genotype in one replicate. The numbers of males and females per plant were counted at 14 dpi and the data were analyzed by a *t*-test ($p < 0.05$) or single-factor ANOVA ($p < 0.05$). In the case of ANOVA, if the F-statistic was greater than F-critical, Fisher's least-significant difference (LSD) test was applied.

2.7. Syncytium and female size measurement

The sizes of syncytia and the associated female nematodes were measured at 14 dpi. For each line, 50 syncytia associated with females were randomly selected and photographed by a Leica (M5C) stereoscopic microscope with appropriate digital camera (Leica DFC 425). The syncytia and females were outlined using the Leica software (LAS V3.8). The individual measurements were used to calculate the average size of syncytium and females. The data were further statistically analyzed using single-factor ANOVA ($p < 0.05$) and Fisher's LSD test *post hoc* analysis.

2.8. Chlorophyll a fluorescence measurement

Chlorophyll a fluorescence was determined using a pulse amplitude-modulated FluorCam 800 MF PSI device (Brno, Czech Republic) on whole *A. thaliana* rosettes. Prior to measurements, the plants were dark-adapted for 30 min to calculate the initial fluorescence (Fo) and the maximum fluorescence (Fm). The maximum quantum efficiency of PSII – Fv/Fm = (Fm–Fo)/Fm, non-photochemical quenching – NPQ = (Fm–Fm′)/Fm′, photochemical quenching – q_p = (Fm′–Ft)/(Fm′–F0′), and the operating quantum efficiency of PSII known as PSII quantum yield – ΦPSII = (Fm′ − Fs)/Fm′ were determined as described previously (Baker, 2008), and the plant vitality index R_{fd} was calculated by the FluorCam 7.0 software. Data were further statistically analyzed using single-factor ANOVA ($p < 0.05$) and Fisher's LSD test *post hoc* analysis.

2.9. CO_2 measurements during in vitro plant growth

CO_2 was measured with a CO_2 transmitter (Vaisala CARBOCAP® Carbon Dioxide Transmitter GMD20/D (Helsinki, Finland)) during *in vitro* plant growth. After two weeks of *Arabidopsis* growth, plates sealed by air-tight or permeable tape were opened and ventilated. Subsequently, Petri dishes were closed with specially designed lids containing a port for CO_2 detection (Vaisala CARBOCAP®, Helsinki, Finland) (Supplementary photos 2 and 3). The CO_2 concentration was detected three days after closing (72 h). Measurements were made in five biological replications, each containing two Petri dishes with two *Arabidopsis* plants. Data were statistically analyzed using a *t*-test ($p < 0.05$).

3. Results

3.1. The sealing tape influences Arabidopsis thaliana WT and mutant phenotype

The choice of Petri dish sealing tape significantly influenced several parameters of root morphology. Using the *Arabidopsis* ecotype Ws-0 and *lsd1*(Ws-0) mutant we observed that the main root was at least 20% longer when wt and mutant seedlings were grown on aerated plates compared to air-tight sealed plates (Fig. 1A). The most striking observation was made with respect to the lateral root number, which was two times greater at the two-week-old seedling stage when permeable tape was used instead of air-tight sealing tape (Fig. 1B). Moreover, this parameter distinguished wt and mutant roots in aerated conditions only. Differences were also observed in root hair length and density between the wt and the *lsd1* mutant (Fig. 1C and D). These two parameters allowed distinction of the mutant and wt irrespective of ventilation.

3.2. Ethylene production of Arabidopsis seedlings grown on petri dishes

One explanation of the observed morphological changes could be ET accumulation. To test whether two-week-old seedlings can produce physiologically significant amounts of ET we measured the ET levels with gas chromatography. Indeed, the amounts of ET measured were very small, reaching almost 1.4 ng/μl produced by seedlings in plates which were sealed with air-tight tape. Plates sealed by the porous tape contained very small or undetectable amounts of this gaseous hormone, which implied that air exchange occurred during *Arabidopsis* growth (Fig. 2).

3.3. The effect of culture conditions on photosynthetic performance

Frequently used and non-invasive tests of plant general physiological condition are based on chlorophyll *a* fluorescence. To determinate how aeration influences photosystem II (PSII) photochemistry we monitored several parameters such as: maximum quantum efficiency of PSII (F_v/F_m), non-photochemical quenching (NPQ), photochemical fluorescence quenching (q_P), PSII quantum yield (ΦPSII), PSII quantum yield in light-adapted leaves (F_v'/F_m'), and plant vitality (R_{fd}) in short day grown two-week-old seedlings (Baker, 2008).

Ventilation during the *in vitro* growth of *Arabidopsis* seedlings showed greater efficiency of photosystem II (PSII) which was shown by changes in F_v'/F_m', PSII yield, and R_{fd} both in the case of the mutant and wt, while F_v/F_m did not change irrespective of the genotype and culture conditions (Fig. 3 A-C, E). Moreover ΦPSII, q_P, and R_{fd} distinguished the wt from the *lsd1* mutant in ventilated plates (Fig. 3C; E-F), whereas air-tight sealing precluded these distinctions. Only changes in NPQ revealed statistically significant differences between the wt and *lsd1* mutant in hermetic conditions (Fig. 3D). Together, these results indicate that aeration provided by permeable sealing tape gives better conditions for seedling growth and mutant screening.

A: maximum quantum efficiency of PSII photochemistry (Fv/Fm); B: PSII quantum yield in light-adapted leaves (Fv'/Fm'); C: PSII quantum yield (ΦPSII); D: non-photochemical quenching (NPQ); E: photochemical fluorescence quenching (qP); F: plant vitality (Rfd). Data represent the means (± SEM) from three independent experiments, each containing 5 plants per genotype. Statistical analysis was performed by using One Way Analysis of Variance. Fisher's Least Significant Difference (LSD) test was used for post hoc analysis. Asterisk: red – difference between control plants due to aeration; pale blue - difference between lsd1 mutant plants due to aeration, black - difference between control plants and the lsd1 mutant due to the plate sealing technique ($p < 0.05$).

3.4. Ion leakage indicates more stress in air-tight containers

Relative ion leakage is commonly used as an indicator of stress induced membrane permeability. The stress-induced electrolyte leakage is usually accompanied by the accumulation of reactive oxygen species (ROS) and often results in programmed cell death (PCD) when stress becomes severe (Bajii et al., 2002; Burdiak et al., 2015).

Our experiments revealed that aeration influences plant physiological conditions. The relative ion leakage of *Arabidopsis* rosettes exhibited no changes in wt plants, whereas clear differences were exhibited in the *lsd1* mutant (Fig. 4A; C). Roots are a more sensitive indicator of stress *in vitro*, showing more than two-fold greater relative ion leakage when grown on plates without aeration compared to those sealed by permeable tape (Fig. 4B; D).

Relative ion leakage in Ws-0 and *lsd1*(Ws-0) mutant leaves (A and C, respectively) and roots (B and D, respectively). Data represent the means (± SEM) from three independent experiments, with each consisting of 5 Petri dishes with 3 plants per genotype. Statistical analysis was performed by using a *t*-test ($p < 0.05$).

3.5. Lack of ventilation strongly influences the stress-related signaling pathways

In order to monitor stress related signaling pathways, the expression level of several marker genes was assessed. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was done for markers of oxidative stress (*APX1, CAT2, OXI1,* and *RRTF*) and senescence (*SAG2*). Moreover, we examined several genes connected to plant hormones such as ET signaling (*EIN2, ERF1*), SA (*PR1*), JA (*PDF1.2, LOX2*), abscisic acid (*ORA47, WRKY33*), auxin (*PIN3*), and gibberellin (*GA3*). The expression level was checked in leaves and roots of wild-type *Arabidopsis thaliana* (Ws-0) plants. Since previous results indicated that properly aerated plants were less stressed, we decided to study gene expression levels where seedlings were sealed with permeable tape (Fig. 5A and B). The strongest effect in the rosette was observed in the case of the *CAT2, RRTF, PIN3, WRKY33, LOX2*, and *ERF1* transcripts. These genes were down-regulated in hermetic plates. Only *PR1* (marker of SA mediated response) was strongly up-regulated in leaves,

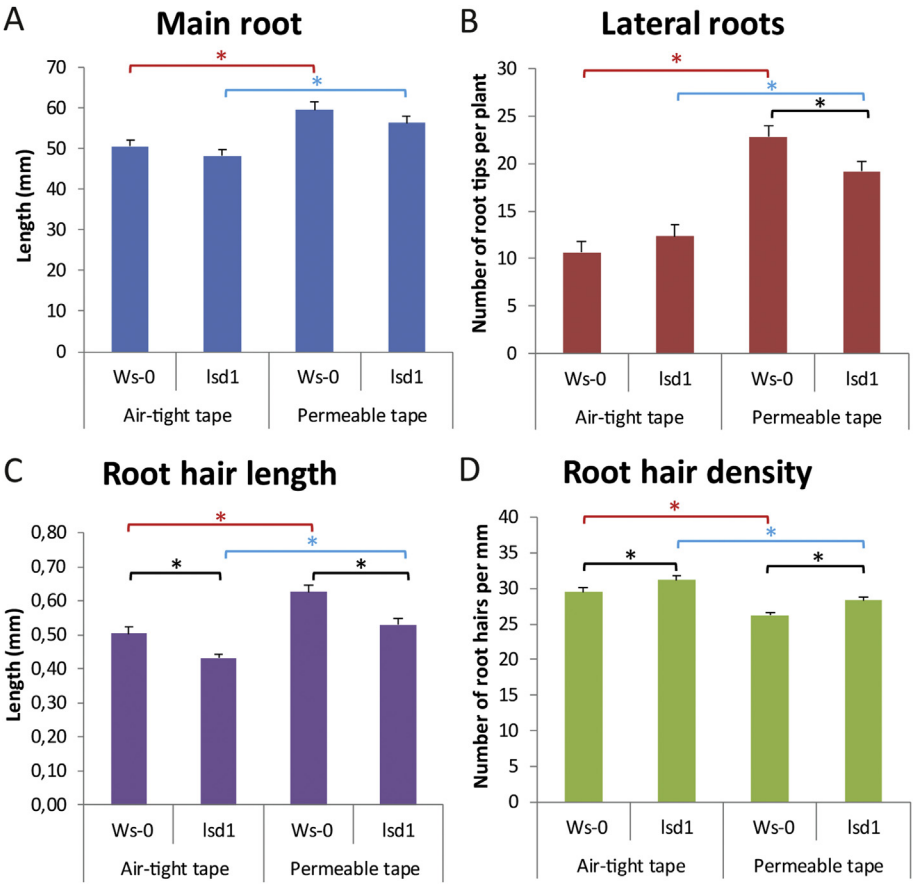

Fig. 1. Sealing type changed root morphology parameters such as: (A) main root length, (B) number of lateral roots, (C) root hair length, and (D) root hair density. Data represent the means (± SEM; standard error of mean) from three independent experiments, each containing 10 plants for each genotype. Data were analyzed using One Way Analysis of Variance. Fisher's Least Significant Difference (LSD) test was used for *post hoc* analysis. Asterisk: **red** – difference between control plants due to aeration; **pale blue** - difference between *lsd1* mutant plants due to aeration, **black** - difference between control plants and the *lsd1* mutant due to sealing technique ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

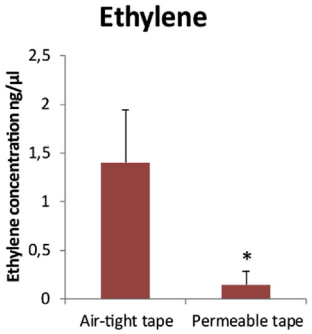

Fig. 2. ET production of two-week-old *Arabidopsis* plants sealed with air-tight tape and permeable tape. Data represent the means (± SEM) from two independent experiments, each containing 10 plates with two plants. Data were analyzed using a *t*-test. Asterisk: significant difference from control plants (Ws-0 air-tight sealed; $p < 0.05$).

however its change was statistically insignificant. Interestingly, the *Arabidopsis* root system was more sensitive to stress conditions during *in vitro* growth. Almost all the investigated genes changed significantly. In the group of ROS-sensitive genes we observed the down-regulation of *CAT2* and up-regulation of *RRTF* and *APX1*. Also, all genes involved in SA, JA, and ET signaling were up-regulated in the absence of proper aeration. The strongest up-regulation was detected for *PDF1.2* (marker of JA; 8.03 $\log_2$FC) and *ERF1* (marker of ET; 7.45 $\log_2$FC). The second most down-regulated root gene was responsible for gibberellin biosynthesis (*GA3*;-1.07 $\log_2$FC). Also, the senescence marker *SAG2* was up-regulated in the belowground plant organs when ventilation was limited. Interestingly, antagonistic trends in the expression level between the aboveground and belowground parts of plants were observed for *RRTF, WKRY33, LOX2*, and *EFR1*. Only *CAT2* was down-regulated in both plant organs. Different expression patterns in the aboveground and belowground plant organs suggest separation of leaf and root regulatory networks, and emphasize the importance of proper sealing techniques during *in vitro* experiments.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of various marker genes associated with phytohormones, ROS, and biotic stress responses were examined in leaves (A) and roots (B) of wild-type *Arabidopsis thalina* (Ws-0) plants sealed by permeable and air-tight tape. The expression levels of target genes were quantified with reference to the expression of *ACT2 (At3g18780)* compared to the control (plants sealed with permeable tape). The relative expression levels are shown as fold changes relative to the copy number of a particular mRNA gene in the control sample. Results are the means (± SE; standard error) from three independent experiments. The asterisks indicate the significant differences from the control as revealed by REST (Pfaffl, 2002) ($p < 0.05$).

3.6. Infection tests of Heterodera schachtii strongly depend on plate aeration

The interaction of plant roots and parasitic nematodes is a very complex process involving developmental and metabolic changes of plant cells as well as sophisticated responses to pathogens (Gheysen and Mitchum, 2011; Ali et al., 2017). To determine the susceptibility level, we counted the females and males which had developed at 14 dpi, and measured the size of the nematode feeding structure and the female associated with it. Our experiments showed a substantial increase in the number of females and males on aerated plates, which indicates more favorable conditions for nematode infection. Petri dishes sealed by permeable tape exhibited an approximate 80% and 30% increase in the number of female nematodes on the wild-type and *lsd1* mutant, respectively, when compared to the same genotype grown in air-tight

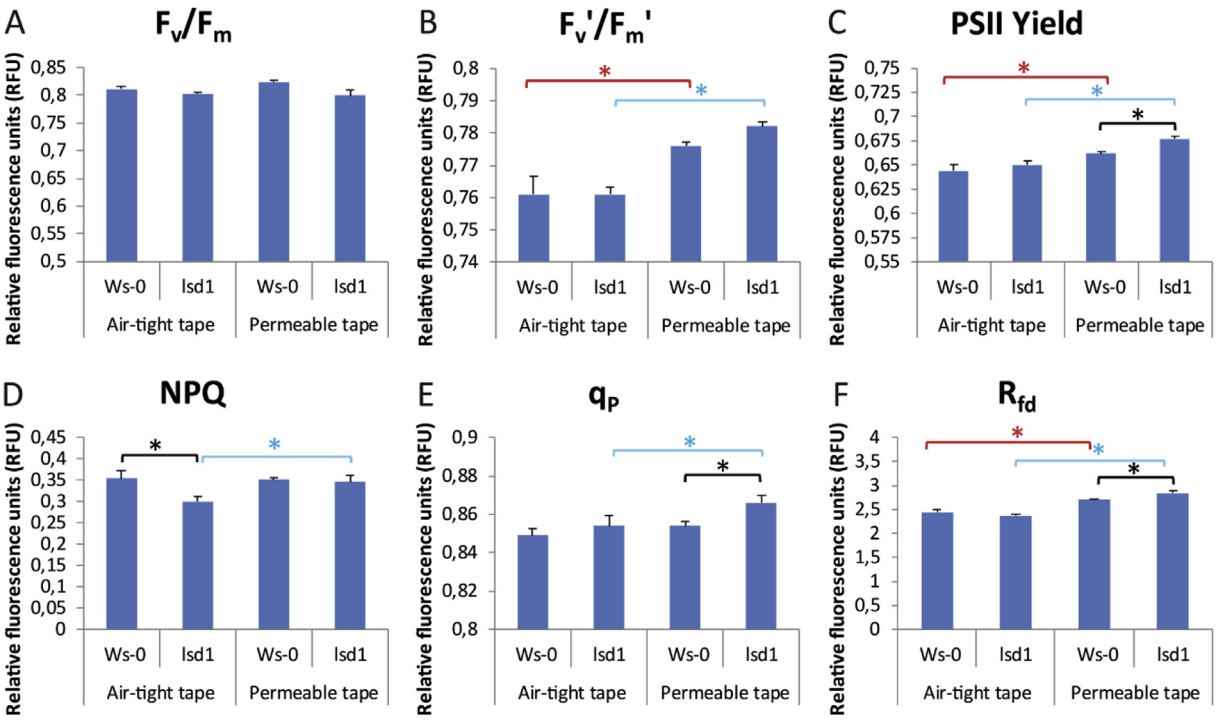

Fig. 3. Chlorophyll a fluorescence in the *lsd1*(Ws-0 background) mutant and wild-type *Arabidopsis* plants.

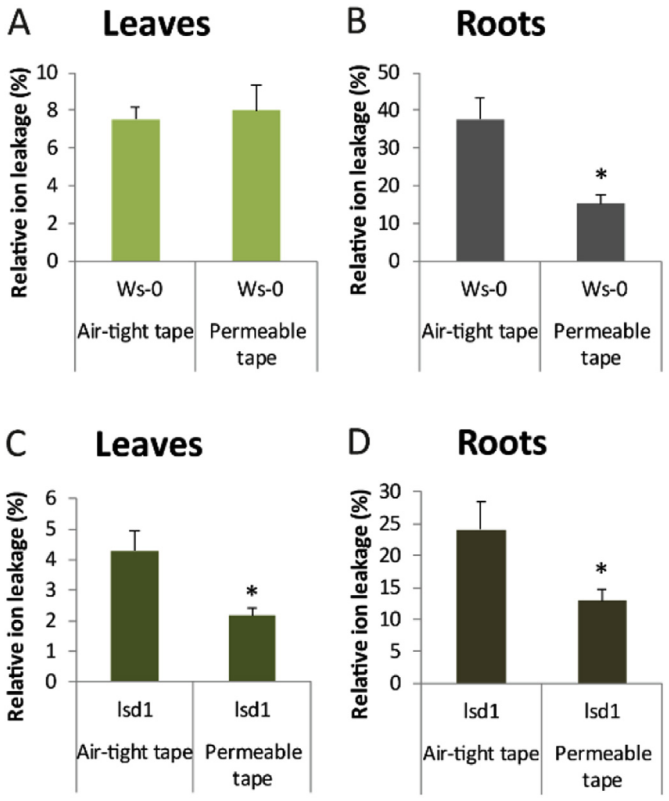

Fig. 4. Relative ion leakage in the *lsd1*(Ws-0) mutant and wild-type (Ws-0) plants.

sealed plates (Fig. 6A). When we compared the susceptibility between the wt and *lsd1* mutant in the context of the two sealing techniques, and differences were seen only in plants with better aeration. However, differences in syncytium size were only observed when plants were grown in air-tight conditions (Fig. 6B). These results clearly show that screening conditions such as plate ventilation may mask interesting mutant phenotypes in plant-parasite interaction research.

A: The numbers of females and males were counted at 14 dpi. Bars represent the average number of males and females developed on the Arabidopsis root system. B: Average size of the females and syncytium at 14 dpi. Data represent the means (± SEM) from three independent experiments, each containing 10 plants per genotype. Statistical analysis was performed by using single factor analysis of variance (ANOVA) ($p < 0.05$). The statistical significance was determined by three independent replicates. Asterisk: red – difference between control plants due to aeration; pale blue - difference between lsd1 mutant plants due to aeration, black - difference between control plants and the lsd1 mutant due to the sealing technique ($p < 0.05$).

3.7. Air-tight sealing caused CO_2 accumulation

To show the effect of photorespiration in non-aerated Petri dishes we performed CO_2 concentration measurements. The atmospheric CO_2 level is around 400 ppm (*parts per million*) in the air. Our *in vitro* experiments showed a slight increase in the CO_2 accumulation in ventilated plates (by 40 ppm), whereas air-tight tape caused a rise in CO_2 > 50% up to 600–650 ppm. These differences were seen during the night time (Fig. 7). However, during the day (100–120 µmol $m^{-2} s^{-1}$) after 4 h of light exposure the CO_2 concentration decreased almost two-fold in plates sealed by air-tight tape (Fig. 7). These results illustrate CO_2 usage in plants which were growing on media supplemented with sucrose and changed by light and dark periods. In the case of air-tight plates, photosynthesis activity was reduced due to CO_2 limitation. Plants sealed by permeable tape were not subjected to CO_2 fluctuation, and hence the photosynthetic dynamic was improved.

4. Discussion

4.1. Aeration during in vitro growth modifies plant phenotype

In relation to well established and published protocols, there are many modifications and hints which are omitted in the literature which

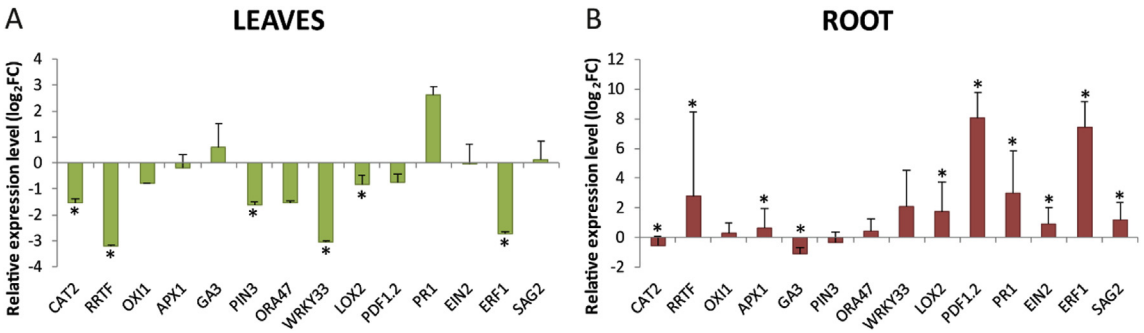

Fig. 5. Gene expression analysis on roots and leaves of *Arabidopsis* plants.

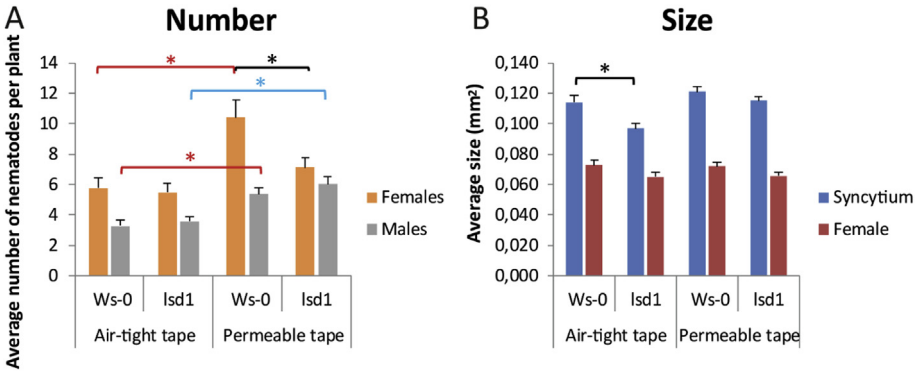

Fig. 6. Nematode infection assay in *lsd1*(Ws-0) mutant and wild-type *Arabidopsis* plants.

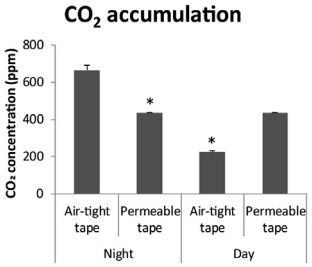

Fig. 7. CO_2 accumulation of two-week-old *Arabidopsis* wild-type plants sealed with air-tight tape and permeable tape in different light conditions. Data represent the means (± SEM) from five independent experiments, each containing 2 plates with two plants. The data were analyzed using a *t*-test. Asterisk: significant difference from control plants (Ws-0 sealed by air-tight tape) ($p < 0.05$).

are known only to the members of a given research group. Such modifications may also be introduced unconsciously. In this article, we present a case study done in sterile conditions with plants grown on agar solidified media which are useful when observations of the root systems are needed. The protocols used do not require specific tissue culture skills and *ipso facto* some obvious details may be ignored. It is well established knowledge that shape, material, volume, and aeration of the culture vessel influences the proliferation and morphogenesis of *in vitro* grown tissues and organs (Filipecki and Malepszy, 2006). Since research on plant-nematode interactions often relies on the *Arabidopsis thaliana* model and the infection tests are carried out in Petri dishes, we tested the influence of tape used for sealing sterile plates on root architecture, physiological parameters, and nematode performance using wild-type and *lsd1* mutant plants. We also used common molecular markers of biotic and abiotic stress to investigate how presumably minor changes in culture conditions influence these parameters.

First, we observed several morphological changes of seedling roots depending on the ventilation system of the Petri dishes. These changes however did not alter the discrimination between the genotypes used. One quite obvious reason for such observations was the possible difference in gas composition in the culture container. Gaseous ET is an especially good candidate to influence the phenotype of young *Arabidopsis* plants due to its well-known crosstalk with many developmental processes and interactions with other hormonal signaling pathways (reviewed in Kumar et al., 1998; Liu et al., 2017; Iqbal et al., 2017). Besides stress inducibility this gas is produced continuously during plant growth and development. Wheeler et al. (2004) monitored ET production over vegetative growth in wheat, soybean, lettuce, potato, and tomato. It reached the greatest level of 2.5 ppb per hour during the first two weeks of growth of lettuce. Unsurprisingly, unfavorable growth conditions causes enhanced accumulation of endogenous ET. This happens not only locally, in stressed organs, but also systemically. Else et al. (1998) showed that during oxygen-deficient conditions roots export via xylem sap sufficient ACC (1-aminocyclopropane-1-carboxylic acid) to raise ET production rates in the shoot to promote epinastic growth. Similarly, flooding increases the production of ACC in leaves (2.5-fold greater than control plants; Else et al., 1998). We don't expect root hypoxia or anoxia in our conditions since the medium is 5–7 mm deep on the plate providing sufficient gas exchange. However, observed fluctuations of CO_2 in sealed plates may indirectly influence ET production causing anatomical, physiological, and molecular alterations in plants. Buer and co-authors discovered that the accumulation of ET increased gravitational effects on root waving (Buer et al., 2000, 2003). Since this may be an offshoot of classical triple response, the question is whether the ET concentration is biologically effective. In our case, five two-week-old *Arabidopsis* seedlings per plate were able to produce an ET concentration which was 10 times greater (1.4 ng/μl) in air-tight conditions than in ventilated conditions. Chang (2016) postulated that even very low concentrations of ET between 0.01 and 1.0 ng/μl may have biological activity, albeit specificity and sensitivity to this hormone is species dependent. Inaba and Nakamura (1986,1988) showed that less than 1 part per million (ppm) of ET was sufficient to trigger the ripening of banana, however for apples only

0.1 ppm caused physiological effects (Thompson, 2010). The inhibition or delay in lateral root formation is the clearest effect which can be ascribed to ET and its interplay with IAA (Negi et al., 2008; Lewis et al., 2011). Additionally, we confirmed the effectivity of the detected ET concentrations via the significant up-regulation of ET signaling molecular markers (*EIN2* and *ERF1*) in seedling roots.

Moreover, ET can be regarded as an integrator of several pathways including developmental, environmental, and pathogen triggered PCD (dPCD - leaf and petal senescence, tracheary elements differentiation, determination of leaf shape, aerenchyma formation; ePCD – low and high temperature stress, UV stress, shading, oxidative stress, hypoxia, shading, nutrient deficiencies, drought and salt stress; pPCD - the hypersensitive response) (Trobacher, 2009; Huysmans et al., 2017 and references therein). Our findings reveal a large degree of overlap between ET-dependent and ROS to induce PCD, which is supported by measurements of ROS marker genes and ion-leakage in roots and leaves of plants sealed by air-tight or permeable tape. Greater ion leakage and activation of either ROS or/and ET sensitive marker genes in leaves and roots of *Arabidopsis* from hermetic conditions are the evidence of ET-dependent PCD (Figs. 4 and 5). Both ET signaling and the molecular regulation of PCD are also important for complex reactions of plant roots to parasitic cyst nematodes (Jones et al., 2013; Matuszkiewicz et al., 2018). These parasites in addition to the induction of stress responses initiate the extensive developmental reprogramming of root cells towards the development of syncytia, which relies on complex hormonal crosstalk. The engagement of ET was proven in many experiments pointing to its role in root localization, syncytium growth, and functioning (Gheysen and Mitchum, 2011). However, relatively low levels of ET detected in our experiments seem to have an opposite effect compared to the previously reported ET overproducing mutants (Goverse et al., 2000). Furthermore, Wubben et al. (2001) found a positive correlation with respect to root hair development and nematode susceptibility. ET overproducing mutants (*eto1-1, eto2*, and *eto3*) which had longer root hairs were more susceptible, however ethylene-insensitive mutants (*etr1-1, ein2-1*, and *ein3-1*) with decreased root hair length were less susceptible. In our results we also observed a similar trend, as plants cultivated in aerated conditions had longer root hairs and hosted more females (Figs. 1 and 6). Moreover, Wubben et al. (2001) used a precursor or biosynthesis inhibitor to modify the ET level. Both ACC (1-aminocyclopropane-1-carboxylic acid; precursor) and AVG (2-aminoethoxyvinylglycine; inhibitor) showed changes in attractiveness and susceptibility. Plants treated with ACC were more attractive during early infection stages and established more females, whereas AVG caused the opposite effect. The complex role of ET in the *H. schachtii* and *Arabidopsis* interaction could be partly explained by the existence of two independent ET signaling pathways postulated by Piya et al. (2018). The first signaling pathway involves the canonical ET signaling pathway which results in suppression of SA-based immunity. Second pathway is dependent on the activity of the ethylene receptor ETR1 and either direct or indirect regulation of the cytokinin response. Our experiments revealed a low dose ET-related fine-tuning mechanism of root performance when challenged with nematode parasites, and highlighted the relevance of minor factors in an experimental set-up.

Besides ET, CO_2 concentration also change in culture containers. In fact, there is limited knowledge about respiration and photosynthesis in plants grown on sugar supplemented media at growth chamber light intensities. Our observations were in line with dogma of CO_2 consumption. During the night plants emitted CO_2, whereas during the day time plants adsorb CO_2 for photosynthesis (Azcon-Bieto and Osmond, 1983). In tightly sealed plates *Arabidopsis* plants in daylight conditions accumulated CO_2 at twice the lower level than in ventilated plates where the CO_2 concentration was always available at atmospheric concentrations. This may at least partially explain the better main and lateral root development observed with respect to two-week-old seedlings. (Fig. 7). Differences in photosystem II efficiency parameters such as: F_v'/F_m', PSII yield, and R_{fd} (described as the plant vitality parameter - Lichtenthaler, 1996) in air-tight sealed plates reflected CO_2 limitation. The molecular response to a low CO_2 level (100 ppm) was investigated by Li et al. (2014). The authors showed the up-regulation of RRTF and ORA47 in leaves at a reduced CO_2 concentration. In our experimental set-up these genes were down-regulated, possibly due to the presence of sugar in the medium, which can partially compensate for low CO_2 stress. Moreover, the regulation of these genes may depend on overlapping ET accumulation.

While discussing gas composition it's worth mentioning humidity, which may vary depending on the plate ventilation. When the humidity is too high, the rate at which plants draw water from the medium is reduced due to slowed transpiration. This can result in diminished nutrient uptake efficiency (Tibbitts, 1979). Though, this is another possible stress factor in sealed plates, but the duration of our experiments was too short to see any clear phenotypical deficiency symptoms. Yet unvisible deficiencies may contribute to observed changes in marker gene expression. Related to the nutrient availability, the agar medium drying in ventilated plates is unlikely to influence seedling growth and development, because less than 5% of water escapes from ventilated plates during two weeks of seedling growth (unpublished data).

Our results indicate that gas composition in tightly sealed culture containers is a significant stress factor, which in turn could cause greater ion leakage. Greater content of ROS possibly caused peroxidation of the lipids membrane which influenced the membrane fluidity and permeability. This could be the reason for the increased electrolyte leakage from *Arabidopsis* roots during growth in stress conditions. Interestingly, in plants carrying a null mutation of *LSD1*, which is a negative PCD regulator acting via inhibition of ET and ROS imbalance, the reaction of the plant roots to stress (air-tight plates) was slightly different than expected. While stress conditions caused by inappropriate aeration induced significantly greater ion leakage in wt and *lsd1* plants (2.5 and 2-fold increase, respectively, when compared to well aerated containers) the overall relative ion leakage of the stressed plants was lesser in *lsd1* than in wt plants, which indicates that in stressed mutant roots cell death may be induced by alternative pathways. Moreover, the development of lysigenous aerenchyma in roots elevated ET production, which is crucial for its proper formation (Evans, 2003). Muhlenbock et al., (2007) postulated the independent regulation of foliar runaway cell death (RCD) and aerenhyma formation in waterlogged roots. In fact, their observations were done only on hypocotyls and the adjacent parts of the root, where the anatomy largely differs from the basic root architecture. Therefore, the observations presented here and earlier reports (Matuszkiewicz et al., 2018) indicate that dPCD regulation in roots involves LSD1, but in a different manner than that described for leaves and hypocotyls.

5. Conclusion

Understanding the plant's response to abiotic and biotic stresses is often associated with *in vitro* testing. It is very important to create suitable and reproducible conditions for plant growth to be able to observe significant changes. Our results indicate that the ventilation of plants cultivated on Petri dishes affects *Arabidopsis* phenotype. These changes are visible with respect to root morphology, photosynthetic apparatus efficiency, stability of cell membranes, susceptibility to *H. schachtii*, as well as the expression of marker genes related to reactive oxygen species and phytohormones. We indicate that changes in the monitored parameters may depend on the accumulation of ET and CO_2. Therefore, proper selection of sealing technique during *in vitro* experiments is crucial to avoid masking the true phenotype.

CRediT authorship contribution statement

M. Matuszkiewicz: Formal analysis, Writing – original draft. **M.D. Koter:** Writing – original draft. **M. Filipecki:** Writing – original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.003.

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