A new in vitro device reveals a specific influence of Arabidopsis nitrogen nutrition on its susceptibility to Alternaria brassicicola at the seedling stage

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Abstract

**Background:** Seedling growth is an early phase of plant development highly susceptible to environmental factors such as soil nitrogen (N) availability or presence of seed-borne pathogens. Whereas N plays a central role in plant-pathogen interactions, its role has never been studied during this early phase for the interaction between *Arabidopsis thaliana* and *Alternaria brassicicola*, a seed-transmitted necrotrophic fungus. The aim of the present work was to develop an *in vitro* device allowing to study the impact of the fungus on *A. thaliana* seedling growth, while modulating N nutrition.

**Results:** The developed device consists of square plates placed vertically and filled with nutrient agar medium allowing modulation of N conditions. Seeds are inoculated after sowing by depositing a droplet of conidial suspension. A specific semi-automated image analysis pipeline based on the Ilastik software was developed to quantify the impact of the fungus on seedling aerial development, calculating an index accounting for every aspect of fungal impact, namely seedling death, necrosis and developmental delay. The device also permits to monitor root elongation. The interest of the device was then confirmed by characterising how N media composition (0.1 and 5 mM of nitrate (NO$_3^-$), 5 mM of ammonium (NH$_4^+$)) affects the impact of the fungus on three *A. thaliana* ecotypes. Seedling development was strongly and negatively affected by the fungus. However, seedlings grown with 5 mM NO$_3^-$ were less susceptible than those grown with NH$_4^+$ or 0.1 mM NO$_3^-$, which differed from what was observed with adult plants (rosette stage).

**Conclusions:** The developed device allows accurate determination of seedling growth characteristics (both on aerial and root parts) and symptoms. Altogether, the device could be used to study the impact of plant nutrition on susceptibility of various genotypes to fungi at the seedling stage.

**Background**

Rapid and homogeneous seedling growth is crucial for successful crop establishment and yield. However, this early stage of plant development is highly susceptible to environmental conditions including variations of nitrogen (N) availability in the soil [1–2], as well as development of diseases generally caused by seed- and soil-borne plant pathogens [3–4].

Nitrogen plays a crucial role driving plant development, growth and yield. Nitrate (NO$_3^-$), the main form and source of N absorbed by seedlings and young plants in temperate regions, also acts as a major signal molecule modulating plant metabolism, growth and plant defence responses [5]. Nitrogen uptake and metabolism are also central to plant defence mechanisms as they provide the material for defence compounds and allow N mobilisation away from pathogens [6]. For instance, the phytoalexin camalexin, which has been shown to have an effect in the *Arabidopsis thaliana* - *Alternaria brassicicola* interaction [7], is derived from the tryptophan amino acid [8]. Amino acids, including methionine, phenylalanine and tryptophan, are also the basis of phytoanticipins such as glucosinolates in plants of the *Brassicaceae* family [9] which, once broken down into isothiocyanates and nitriles, may be toxic to fungi, bacteria and
insects [10]. Another important molecule involved in plant defence mechanisms and derived from \( \text{NO}_3^- \), is nitric oxide (NO). NO is rapidly accumulated during biotic stress conditions and plays a role in phytoalexin accumulation [11] and salicylic acid biosynthesis [12], a major pathway for plant resistance to infection leading for instance to hypersensitive response [13]. Nitrogen is not only vital for the plants, but also for the fungal pathogens, the latter being entirely dependent on plant N sources, such as \( \text{NO}_3^- \), ammonium (\( \text{NH}_4^+ \)) and free amino acids, for their growth [14]. This makes the host N metabolism a direct factor affecting fungal growth, by modulating the N sources available for the invader.

The role of N on pathogenicity is unclear. Indeed, a large supply of exogenous N improves plant nutrition and defences but can also promote disease development, by increasing the availability of N compounds assimilable by the pathogen [15–16]. It has been shown that high N fertilisation could promote the disease [17–18] or increase host resistance [19], which would suggest that the role of N in the plant-pathogen interaction is complex and pathosystem specific. In addition to the overall availability of N, its form also plays an important role in plant-pathogen interaction. Studies have shown that \( \text{NH}_4^+ \) nutrition could increase disease severity compared to \( \text{NO}_3^- \) [20–21], while the contrary was observed in other pathosystems [22], notably \( A. \text{thaliana} - A. \text{brassicicola} \) [23].

Until now, inorganic N fertilisers and conventional agrochemicals, such as fungicides, have been widely used for many years to protect seeds and seedlings against various stresses in the seedbed and to increase seedling establishment. However, the agro-ecological transition requires a substantial reduction of N input and seed treatment with fungicides. These practices would however promote the presence of seed-borne pathogens such as necrotrophic fungi on both seeds and seedlings. Moreover, N fertilisers do not only affect seedling growth but can also have different effects on disease development, shifting the balance in favour of the host or of the pathogen as reported at other stages of plant development [6, 17]. In this context and surprisingly, the impact of N fertilisation on the interaction between plants and pathogens has been little studied at the seedling stage and requires more investigation.

In order to study the impact of N nutrition on plant-pathogen interaction during seedling development, one would need a protocol allowing symptom severity, fungus growth and seedling development (i.e. both aerial and root parts) to be monitored, in a nutrient controlled sterile environment. Studies on early plant stage interaction with pathogens commonly involve an inoculation on seven to eighteen days old seedlings growing in soil [18, 24–25] or in a Petri dish [26], neither permitting the study of the interaction during germination, early seedling growth nor root development. In order to investigate the interaction as early as possible, inoculation has to take place directly on the seeds, by placing them in contact with dry spores or by immersing them in an inoculum suspension [27–29]. The best option to achieve a sterile and controlled environment allowing to monitor both aerial and root growth seems to be the use of agar media [30]. A protocol combining a seed inoculation with a modular sterile agar medium could therefore be a good solution to study the role of N on the plant-pathogen interaction during seedling development.
In the present work, we developed such a device, focusing on the interaction between the model plant for the *Brassicaceae* family *A. thaliana* and the necrotrophic fungus *A. brassicicola*. *A. thaliana* is a practical and already well studied model for host-pathogen interactions [31], and the interaction with *A. brassicicola*, in particular, has been abundantly used as a model for diseases caused by fungal necrotrophs [32]. This fungus is responsible for the black spot disease on leaves of *Brassicaceae* plants [33], leading to important worldwide economic losses [34]. Infection with *A. brassicicola* is however not limited to the leaves, but this fungus can affect all aerial parts of the plant, such as siliques and seeds in particular. Such a wide infection spectrum constitutes an important mode of conservation and transmission of the fungal inoculum and therefore makes of *A. brassicicola* a major seed-borne disease leading to seedling damping-off [35–37]. The device, allowing both homogenised seed contamination and following seedling aerial and root development by image analysis, was firstly developed on one *A. thaliana* ecotype grown in one N nutrition medium, 5 mM NO$_3^-$.

To validate its interest for screening the impact of the fungus on seedling growth, the protocol was then used to characterise the response to the fungus of three ecotypes with contrasting susceptibilities, on three N media varying in forms and concentration (5 mM NH$_4^+$, 0.1 mM NO$_3^-$ and 5 mM NO$_3^-$). Finally, the results obtained at seedling stage with this developed protocol were compared with behaviours at adult stage (i.e. rosette).

**Results**

**The developed device allows studying how the fungus affects seedling growth**

A new device, allowing an extensive study of the effects of the fungus on seedling growth, was developed (Fig. 1). Seeds of the *A. thaliana* Columbia (Col-0) ecotype, either treated with sterile water (H$_2$O) or inoculated with the Abra43 fungus, were sown in vertical square plates on agar Murashige and Skoog [38] modified medium supplemented with 5 mM NO$_3^-$ (see the Methods section; Fig. 1a). Using this device, seedlings can develop at least to the four-leaf stage, and the impact of the fungus on both seedling aerial and root system development can be monitored, as shown in the figures 1b and 1c with four-leaf stage seedlings treated with H$_2$O and inoculated seedlings affected by the fungus, respectively.

**Choice of seed inoculation method**

Two methods were tested for seed inoculation with *A. brassicicola*: (i) seed immersion in a conidial suspension (10$^3$, 10$^4$ or 10$^5$ conidia per mL) and (ii) deposition of a droplet of conidial suspension (10$^3$ or 10$^4$ conidia per mL) on each individual seed. Results obtained at 3 days after inoculation (DAI) showed that the droplet method of inoculation allowed 100% of the seeds to be contaminated using a suspension of 10$^4$ conidia per mL contrary to the immersion method which allowed a lower and heterogeneous contamination rate, even with 10$^5$ conidia per mL (Fig. 2). In addition, even though the contamination rate is close to 100% with the immersion method of inoculation at 10$^5$ conidia per mL, observations of 50 seeds by scanning electron microscopy revealed that conidia were not homogeneously distributed on the seed surface, with three categories of conidia distribution (Fig. 3), seeds exhibiting either no conidia (Fig.
3b), a few conidia (not illustrated) or a cluster of conidia (Fig. 3c and 3d) on their surface. It is also important to note that symptoms on seedlings were too severe with \(10^5\) conidia per mL preventing the observation of seedlings at 14 DAI (data not shown), which could be explained by the conidial clusters, as illustrated on figures 3c and 3d. On the contrary, none of the 210 observed seeds inoculated with the droplet method with \(10^4\) conidia per mL presented any cluster of conidia on their surface, but only a few scattered conidia (Fig. 3e and 3f). The droplet method of inoculation with a suspension of \(10^4\) conidia per mL was thus chosen as it allowed a more homogeneous seed contamination, as well as a compromise between disease severity and seedling development.

**Development of an image analysis method to evaluate the impact of the fungus on seedling aerial development**

In order to evaluate the impact of the N supply condition, of the fungus and of their interaction on seedling aerial part growth, a method based on a semi-automated image analysis pipeline was developed associating the Ilastik software [39] with a Python script. The first stage of the process consisted in training a classification random forest model with Ilastik. Twenty-five representative images were selected and annotated by associating pixels to a class. Three classes were defined corresponding to healthy tissues (green), necrotic tissues (brown) and all other objects present in the image that do not correspond to aerial parts of seedlings (Fig. 4a). Once the model trained, it was applied to the other images to identify the healthy (green) and necrotic (brown) pixels of aerial parts of the seedling (leaves, cotyledons and hypocotyl) per square plate (Fig. 4b). Classification results were then subjected to the Python script to evaluate the areas of healthy and necrotic tissues and generate colour-coded final images. The model was validated on a technical standpoint by comparing the spatial overlap between 30 representative seedlings annotated by the Ilastik model or by an expert observer using the ImageJ software, as reported in the Methods section.

To express the results, a healthy area index (HAI) representing the impact of the fungus on seedling aerial growth was developed. It depicts the percentage of healthy area variation between seedlings treated with \(\text{H}_2\text{O (HA}_{\text{H}_2\text{O}}\) and Abra43 (HA_{Abra43}) and integrates both direct and indirect fungal effects (area deficit due to necrotic tissues and seedling growth deficit, respectively).

\[
\text{HAI} = \left( \frac{\text{HA}_{\text{H}_2\text{O}} - \text{HA}_{\text{Abra43}}}{\text{HA}_{\text{H}_2\text{O}}} \right) \times 100
\]

The impact on the fungus was also measured using a visual rating scale (VRS) to quantify the symptom severity on the aerial parts of seedlings. The VRS integrated both colonisation by the mycelium and necrotic area, evaluated on seedlings, and was based on a score ranging from 0 (no symptom) to 5 (100% of surface area colonised and dead seedlings) (Table 1) assigned by an expert observer.

In order to find out if the HAI and the VRS produced similar results regarding the fungal effect on seedlings, the correlation between the two methods was investigated (Fig. 5a). If the results of two methods shared a similar tendency, they did not correlate very well (\(R^2 = 0.67\), exponential function,
Another way to evaluate the fungal impact on seedlings, closer to what is measured with the VRS, would be the necrosis area index (NAI) obtained by dividing the necrosis area by the total area (necrosis + healthy area) of a squared plate. This index correlated better with the VRS ($R^2 = 0.76$, power function, $y=3.4141x^{0.1764}$, Fig. 5b) and presented a very balanced distribution, indicating that it seemed possible to infer the results of VRS from the measures evaluated with the Ilastik model.

It is to note that both HAI and NAI appeared to assess the fungal impact in a more satisfactory way than the VRS. Firstly, VRS being based on a rating given by an observer makes it more qualitative and less objective than methods based on pixel quantification by a software. Secondly, the comparison of the two most correlated methods showed that NAI distribution of values evenly covered the full range of possible values (from 0 to 1) while VRS was only restricted to the 1.5-4 range, the two extremes of the scale being totally unused and not allowing for an adequate representation and discrimination of the fungal impact high variability. Finally, HAI was deemed superior to NAI because it was a more complete representation of the fungal effect on seedlings. Indeed, NAI measured solely the necrosis areas while HAI considered not only the necrosis areas, which are deduced of the measured healthy areas, but also the healthy area deficit caused by the fungal presence, dead seedlings and non-developed organs mostly which are very important aspects of the fungal impact on seedlings. HAI was thus chosen to express the impact of the fungus on seedling growth.

The new device reveals different and specific seedling susceptibility to the fungus depending on nitrogen conditions at the seedling stage

The device was developed using the Col-0 ecotype on a 5 mM of NO$_3^-$ agar medium with seeds treated with either sterilised water or conidia suspension. To confirm whether it enables discriminating susceptibility to the fungus between genotypes and N growth conditions, three ecotypes presenting different susceptibility to the pathogen at adult stage [40] were tested on different media varying in concentration and form of N. The selected ecotypes were Col-0 (resistant), Wassilewskija (Ws; susceptible) and Landsberg erecta (Ler; susceptible) which were grown on media in presence of 5 mM of NO$_3^-$, 0.1 mM of NO$_3^-$ or 5 mM of NH$_4^+$.

Seedling aerial growth is affected by the fungus depending on genotype and nitrogen conditions

The developed image analysis pipeline (Fig. 4) has allowed quantifying the aerial growth of seedlings from seeds treated with H$_2$O by measuring their green area (HA$_{H2O}$; Table 2a). In absence of the fungus, seedling aerial green area was higher under 5 mM NO$_3^-$ nutrition, with an average of 48.7 mm$^2$ per square plate, compared to under the two others N media (18.8 and 15.7 mm$^2$ in average for 5 mM NH$_4^+$ and 0.1 mM NO$_3^-$, respectively) (Table 2a). Significant differences were also found between genotypes, with Ler showing lower green leaf area than Col-0 and Ws under both 5 mM NH$_4^+$ and 0.1 mM NO$_3^-$. Then, using the calculated HAI allowed revealing the effect of N media on susceptibility to the fungus (Table 2b).
Seedlings grown under 5 mM NH$_4^+$ or 0.1 mM NO$_3^-$ were dramatically affected, with a fungal impact amounting for more than 80% of green area deficit for both N conditions and for all genotypes, compared to the H$_2$O treated seedlings (Table 2b). On the contrary, seedlings grown under 5 mM NO$_3^-$ were less susceptible to the fungus (green area reduced by 21% on average). It is also important to note the significant interaction between genotype and N nutrition for HAI, notably explained by the different response of genotypes under 5 mM NO$_3^-$, with both Col-0 and Ws seemingly unaffected in their growth (-4% and +5% of fungal impact on green area, respectively), and by contrast, Ler that was highly impacted (67% reduction of green area). In addition to the different susceptibilities to the fungus revealed depending on N media and genotypes, infected seedlings grown under 5 mM NO$_3^-$ appeared particularly healthy as they were at the same time more developed in control conditions and less affected by the fungus.

*The presence of fungus also impacts primary root elongation depending on genotype and nitrogen conditions*

Primary root length was measured at both 8 and 14 DAI by image analysis with the Image J software (Table S1a and Table S1b). At 8 DAI, root length highly differed between genotypes (G effect), N media (N effect) as well as inoculation modalities (I effect). All interactions between these factors were also significant, with the G x N effect the most significant (Table S1a). Ler presented the shortest roots compared to Col-0 and Ws. Regarding N effect, roots were the shortest under 5 mM NH$_4^+$ (7 mm in average) and the longest under 5 mM NO$_3^-$ (13.8 mm in average). Finally, when considering all the data, the presence of the fungus negatively affected root elongation (-12.8% in average). This was mainly due to the NH$_4^+$ condition where the reduction was the strongest for all the genotypes (-38.2% in average). For the NO$_3^-$ conditions, various genotype behaviours were observed in accordance with the significant interactions revealed (Table S1a), and this is more visible when results were expressed as an index of response of root length to the presence vs the absence of the fungus (Fig. 6). Indeed, root length was highly affected under both nitrate conditions for Ler, but not affected for Ws. Regarding Col-0, root length decreased in the presence of the fungus under 5 mM but increased under 0.1 mM NO$_3^-$ . This result suggested that, in some nutritive conditions, the presence of the fungus could have no effect or even enhance root elongation early after inoculation (Fig. 6).

After 14 days, the same main effects were found (G, N and I effects; Table S1b). Ler was still the genotype presenting the shortest roots (16.2 mm compared to Col-0 and Ws, 22.1 and 24.4, respectively). In addition, roots were still globally the shortest under 5 mM NH$_4^+$ (10.5 mm) and the longest under 5 mM NO$_3^-$ (32.4 mm), with the differences between media being amplified compared to 8 DAI (+50% growth for 5 mM NH$_4^+$, +65% for 0.1 mM NO$_3^-$ and +135% for 5 mM NO$_3^-$). Results also indicated that root elongation was highly affected by the fungus between 8 and 14 DAI (-38.3% at 14 DAI on average for all genotypes and N conditions compared to -12.8% after 8 DAI). This was more or less marked depending on the N medium (Fig. 6), the impact being stronger for seedlings grown on NH$_4^+$ (-62%), than for those
grown on NO$_3^-$ (-38% and -26% for 5 mM and 0.1 mM, respectively). Considering the combined effects, only the G x N and N x I interactions were still significant, while the G x I and G x N x I interactions were not significant anymore, which could be explained by the amplified main effect flattening the subtler differences.

The new device reveals a stage specific effect of nitrogen condition on susceptibility to the fungus on seedlings, compared to adult plants

To go further, findings using the developed device at the seedling stage were compared to those observed on adult plants (rosette stage) grown on a hydroponic system with the three studied N conditions (5 mM NH$_4^+$, 0.1 mM NO$_3^-$ or 5 mM NO$_3^-$). Leaf necrosis area was determined at 7 DAI. Significant differences between genotypes and between N conditions were revealed (Table 3). The genotype Col-0 was on average significantly less susceptible than Ler and Ws genotypes (mean necrosis area, 0.76 mm$^2$, 1.51 mm$^2$ and 1.43 mm$^2$, respectively) under almost all N conditions (except for the comparison with Ws under 5 mM NO$_3^-$). This difference of susceptibility to the pathogen between Col-0 and Ler was also found at the seedling stage, whereas there was no difference at the seedling stage between Col-0 and Ws.

When comparing N conditions, plants grown on NH$_4^+$ were significantly less susceptible than those grown on NO$_3^-$ (mean necrosis area, 0.71 mm$^2$, 1.51 mm$^2$, and 1.42 mm$^2$ for 5 mM NH$_4^+$, 0.1 mM NO$_3^-$ and 5 mM NO$_3^-$, respectively). This difference was also found when considering the genotypes separately, except for Ws under 5 mM NO$_3^-$ condition. This result contrasted very strongly with that obtained at the seedling stage, where seedlings grown under 5 mM NH$_4^+$ and 0.1 mM NO$_3^-$ were much more susceptible than those grown under 5 mM NO$_3^-$.

Discussion

A new device to study plant x pathogen interaction at the seedling stage

A new device allowing the monitoring of seedling growth under modulating N supply as well as the impact of the necrotrophic fungus *A. brassicicola* was developed. It permitted to follow the kinetics of both aerial and root part growth of the seedling, from germination until at least the four-leaf stage, enabling the survey of seedlings at different points in time. It also allowed for easy sampling, the seedlings being well separated and in a clean environment (no soil on the roots for example). This environment, a nutrient controlled agar medium, is another advantage of the device enabling the study of varying nutrition regimes, N forms and concentrations in this work’s case. The fully sterile device suited perfectly the study of biotic interactions at the early stages of plant development, such as the plant-fungal pathogen interaction between *A. thaliana* and *A. brassicicola* featured in the present work and could be adapted for the study of other pathosystems. Inoculation of seedlings is in most studies carried out several days after sowing [18, 24-26], preventing the study of the earlier stages of the interaction. Seed immersion in a conidial suspension is often used to solve this problem [28, 29] but, in our developed
device, the droplet inoculation method implemented allowed for a more homogeneous contamination (Fig. 2). The impact of A. brassicicola on seedling growth was determined for both primary root elongation and seedling aerial development from image analyses. For the aerial development, a specific semi-automated pipeline was developed, based on image analyses with the Ilastik software [39] and a python script (Fig. 4). An index (HAI) was successfully applied to quantify the impact of the fungus on seedling development as it allowed accounting for every aspect of fungal impact, namely seedling death, necrosis and developmental delay. HAI was deemed the best in our study but the pipeline for image analysis is flexible enough to be used for different ways of assessing the fungal effect on plants, depending on the pathosystem or the aim of the research (e.g. necrosis alone). The use of such a method to evaluate the fungal impact on seedlings appears more integrative than what is usually done. Indeed, studies directly inoculating the seeds commonly evaluated the infection percentage [26-27] or the germination rate [28], and not the subsequent seedling growth, while studies inoculating already grown seedlings were restricted to latter stages and evaluated the fungal growth and sporulation [18], often with a visual qualitative scale [24-25].

**Influence of nitrogen nutrition on fungal infection at the seedling stage**

Firstly, N affected aerial development in the absence of the fungus, with seedlings grown under 5 mM NO$_3^-$ being larger than those grown under 0.1 mM NO$_3^-$ and 5 mM NH$_4^+$ (Table 2a). This was expected, because it is well known that NO$_3^-$ is a more advantageous N source than NH$_4^+$. Exclusive NH$_4^+$ nutrition can indeed result in toxicity symptoms in plants, which can lead to important yield losses [41], notably on A. thaliana [42]. However, the conditions in our device were not stressful enough to cause leaf chlorosis, a common symptom of severe NH$_4^+$ toxicity [43]. Furthermore, given the importance of N in plant growth and development, reduced seedling growth under NO$_3^-$ deprivation conditions (0.1 mM) were also expected. The N concentration is so crucial in seedling growth that even in studies where the NO$_3^-$ deficiency was far from as strong (4 mM NO$_3^-$ for the control and 1 mM NO$_3^-$ for the low N condition), deprived A. thaliana seedlings still presented a 30% growth reduction [44]. Ler seedlings grown under 0.1 mM NO$_3^-$ and 5 mM NH$_4^+$ were smaller than those of Col-0 andWs, while no statistical difference between genotypes was highlighted for 5 mM NO$_3^-$: This could be explained by a higher resistance of the two latter genotypes to stresses induced by the two disadvantageous N conditions. Indeed, it has been already shown that Ler seedling aerial growth was more impacted by NO$_3^-$ deficiency than Col-0 and Ws seedlings [44]. Concerning NH$_4^+$, it has been demonstrated that even though A. thaliana was in general sensitive to NH$_4^+$ toxicity, important genotypic differences were present [42], although no study appears to have been carried out on the three genotypes we studied. Regarding primary root growth, the global impact of N nutrition was similar to that found on the aerial part, with the longest primary roots belonging to seedlings grown on 5 mM NO$_3^-$ and the shortest to seedlings grown on 5 mM NH$_4^+$. This is consistent with the NH$_4^+$-induced repression of A. thaliana primary root growth reported in literature [45]. On the other hand, NO$_3^-$ is known for stimulating primary root growth of A. thaliana seedlings [46], while lower
NO$_3^-$ concentrations led to a reduction of fresh root weight [44]. In our study, primary roots of Col-0 and Ws were generally longer than those of Ler, especially in the two NO$_3^-$ conditions. This contrasted with literature, Ws seedling roots being generally shorter than Col-0 and Ler ones [47], and particularly sensitive to low NO$_3^-$ conditions [44]. One explanation could be that in this study of Aceves-García et al. [47], the medium was supplemented with sucrose which is known to induce root growth of *A. thaliana* seedlings [48], to an extent that could vary between genotypes.

The developed device highlighted the effect of N nutrition on the interaction between *A. thaliana* seedlings and the necrotrophic fungus *A. brassicicola*. Seedlings subjected to the 0.1 mM NO$_3^-$ and 5 mM NH$_4^+$ conditions were more susceptible to the fungus than those under 5 mM NO$_3^-$ (Table 2b). While the effect of N on plant-pathogen interactions has been extensively studied at the adult stage [49], studies at the seedling stage are very uncommon and most appear to be on months to years-old tree seedlings [50-53], which makes our results at this stage a novelty. The closest study was on the effect of N supply on barley seedlings inoculated with *Erysiphe graminis* f.sp. *hordei* [18], but the latter inoculation date (12 days after sowing) makes it very different from the present work. Generally, the role of N on pathogenicity can depend on both N concentration and N form [15-17]. In our study, higher NO$_3^-$ concentration was linked with less susceptibility, as it is the case of *Botrytis cinerea* on tomato [19], while NH$_4^+$ nutrition was linked with an increase of disease severity compared to NO$_3^-$, something that was also observed in the *Solanum lycopersicum* L. - *Fusarium oxysporum* and *Nicotiana tabacum* - *Pseudomonas syringae* interactions [20-21]. It is important to keep in mind that the effects of N concentration and form are pathosystem specific and can have an opposite effect on plant susceptibility [18, 22, 49]. High N supply commonly enhances susceptibility to biotrophs and hemibiotrophs while reducing that to necrotrophs [22, 54-55] which is consistent with our results. Finally, these results could in part be explicated by an aggravating effect of direct stresses caused by mineral nutrition, low N nutrition possibly weakening the plant in regard to defences against pathogens [55].

The influence of N nutrition on fungal infection was also evaluated at the rosette stage with the same ecotypes and nitrogen media as those studied at the seedling stage. We showed that plants grown on NH$_4^+$ were significantly less susceptible than those grown on NO$_3^-$ (Table 3), which was consistent with a previous study we conducted with one ecotype and two N media [23]. The results obtained at the rosette stage thus contrasted strongly with those obtained at the seedling stage. The device developed to study specifically seedling growth thus allowed to demonstrate that N nutrition modulates the susceptibility of *A. thaliana* to *A. brassicicola* according to the developmental stage of the plant. Susceptibility to pathogens often vary with age, generally in the sense that younger plants are more susceptible, as demonstrated in numerous studies [56-59]. This phenomenon can be linked to leaf maturity [60] and leaf rank, it was for instance demonstrated that the 8$^{th}$ leaf of the *Arabidopsis* rosette was more susceptible to *P. syringae* than the 16$^{th}$ leaf [58]. Epidermal cuticle thickness is an important part of the physical barriers involved in the control of fungal penetration, consequently reducing the incidence of disease [61], and have been shown to increase with leaf age [62] and *A. thaliana* developmental stage [63]. Fungal
tissue penetration is also affected by N nutrition. It has for instance been demonstrated that fungal species such as *Fusarium graminearum*, *F. oxysporum*, or *Magnaporthe oryzae* fail to penetrate cellophane membranes under NH$_4^+$ nutrition, suggesting that the ability of hyphae to pierce cellulosic material is affected by NH$_4^+$ [64]. This could create a synergistic effect between age and NH$_4^+$ nutrition raising greatly the plant physical defences and be an explanation for the different effect of N nutrition between seedlings and rosettes. It should also be noted that early seedling growth relies on both exogenous N supply and endogenous N resources from seed reserve mobilisation. This specificity may also contribute to explain the difference highlighted between the two developmental stages studied. Finally, in rosettes, the genotype Col-0 was on average significantly less susceptible than Ler and Ws, which is consistent with the literature [40]. This difference between Col-0 and Ler was also present at the seedling stage but, in contrast, there was no difference at the seedling stage between Col-0 and Ws, showing that genotype responses to *A. brassicicola* seem dependent on developmental stage.

**Conclusions**

A new sterile device allowing the monitoring of *A. thaliana* seedling growth under modulating N supply, as well as the impact of the necrotrophic fungus *A. brassicicola*, was developed. It enables the survey of seedlings at different points in time, in varying nutrition regimes, N forms and concentrations. A specific semi-automated image analysis pipeline was developed to quantify the impact of the fungus on seedling development. It has allowed calculating an index (HAI) accounting for every aspect of fungal impact, namely seedling death, necrosis and developmental delay, as thus highlighting the influence of N nutrition on fungal infection at the seedling stage. Seedlings subjected to NH$_4^+$ and lower NO$_3^-$ conditions were more susceptible to the fungus than those subjected to higher NO$_3^-$ conditions. The assessment of fungal impact on adult plants revealed that they were significantly less susceptible when grown on NH$_4^+$, compared to NO$_3^-$, which dramatically contrasted with findings at the seedling stage. Thanks to the device developed in this study, it has therefore been possible to highlight that N nutrition modulates *A. thaliana* susceptibility to *A. brassicicola* according to the plant developmental stage. Because primary and secondary metabolism are of critical importance to the effect of N in the plant-pathogen interaction, elucidation of the metabolic bases associated with seedling susceptibility regulated by N supply, as well as the role of endogenous N nutrients from mobilisation of seed reserves, should provide insight into the specific findings observed at the seedling stage.

**Methods**

**Biological material**

Three ecotypes of *A. thaliana* (Col-0, Ler and Ws) were studied. Seeds of each genotype were collected from plants simultaneously grown for 13 weeks under long day conditions (16 hours of light at 21°C, 8 hours of darkness at 19°C) in a growth chamber (IRHS, Angers, France). The *A. brassicicola* wild-type
strain Abra43 was initially isolated from *Raphanus sativus* seeds [7], sequenced recently [65], and was routinely grown and maintained on potato dextrose agar at 24°C.

**Experimental device developed for Arabidopsis seedling growth**

To follow seedling development and fungus-induced symptoms, *A. thaliana* seedlings were grown vertically on agar nutrient medium in square plates (12×12×1.3 cm) filled with 1.2% agar modified MS medium (0.5 mM CaCl$_2$, 0.5 mM MgSO$_4$, 1 mM KH$_2$PO$_4$, 50 µM iron-EDTA and 0.5 mL/L micro-elements buffered with 0.5 g/L MES (2-(N-morpholino)ethanesulfonic acid), pH 5.7) [38], either supplemented with 5 mM NO$_3^-$ (provided as KNO$_3$), 0.1 mM NO$_3^-$ or 5 mM NH$_4^+$ (provided as NH$_4$Cl). Twelve seeds per plate were sown on a plane surface obtained by cutting the medium 3 cm from the top. Before sowing, seeds were stratified for 3 days (in order to break their residual dormancy) and then sterilised by successive immersions, 5 min in ethanol 70°, 15 min in sodium hypochlorite (2.6% active chlorine) and three times 5 min in sterile water. Once sown, seeds were individually inoculated by deposit of a 1 µL droplet of conidia suspension (10$^4$ conidia per mL; Abra43 condition) or of sterile milli-Q water (H$_2$O condition). The plates were then placed for 18 days in a growth chamber under long day conditions (16 h of light at 21°C, 8 h of darkness at 19°C). Three independent experiments were carried out, containing each 3 plates of 12 seedlings per set of conditions.

**Seed observation by scanning electron microscopy**

Seeds inoculated either by immersion (1 h in a 10$^5$ conidia per mL suspension or sterile milli-Q H$_2$O) or by deposit of a 1 µL droplet (10$^4$ conidia per mL suspension or sterile milli-Q H$_2$O) were dried and observed directly by scanning electron microscopy (Phenom™ G2 Pro) in collaboration with the IMAC platform (IMAgerie Cellulaire; SFR 4207 Quasav, Angers, France).

**Image analysis**

Pictures of square plates containing seedlings were taken using a Nikon D5000 digital camera, in the same conditions of lighting and distance. The classification of images parts for the seedling aerial part analyses was performed with the Ilastik software (version 1.3.3), as presented in the global pipeline (Fig. 4). The training dataset was composed of 19 pictures of square plates (3216x2136 px) and 6 pictures of selected seedlings (from 176x328 to 1230x332 px). The training dataset was chosen to best represent all the variations in our data: pictures of seedlings from the three genotypes grown in different N conditions and inoculated with the fungus or not, with either homogeneity or heterogeneity in the picture regarding levels of disease severity or seedling size. These images were annotated by associating pixels to a class. Three classes were defined corresponding to healthy tissues (green), necrotic tissues (brown) and all other objects present in the image that do not correspond to aerial parts of seedlings. These annotations were then used to train Ilastik random forest model classifier. The selected features were the colour/intensity, the edge and the texture and the spatial scales for the convolution 0.70, 1.00, 1.60, and 3.50 for every feature, plus 0.30 for the colour/intensity feature. Once the model trained, it was
applied to the other images to identify the healthy (green) and necrotic (brown) pixels of aerial parts of
the seedling (leaves, cotyledons and hypocotyl) per square plate. Classification results were then
subjected to a Python 3.7 script to evaluate the areas of healthy and necrotic tissues and generate the
colour-coded final images. This script was based mainly on the numpy [66], scikit-image [67] and
matplotlib [68] libraries.

The pipeline was tested on 18 pictures of square plates (3216x2136 px) and 5 pictures of selected
seedlings (from 176x328 to 1230x332 px) to assess the quality of the classification. The evaluation of
the quality of the model was based on the comparison of the areas of 30 representative seedlings
measured by our image analysis pipeline and a ground truth corresponding to annotations performed
manually by an expert observer with the ImageJ software (version 1.53k) [69]. The metric selected to
measure the spatial overlap between those two sets of classified pixels was the classical Dice Sørensen
coefficient (DSC) = 2*|A∩B| / |A|+|B|. The DSC then equals twice the number of elements common to both
sets (|A∩B|) divided by the sum of the number of elements in each set (|A| + |B|). The DSC for our data
was high (0.98), showing that there was no real difference between the model and the manual
annotation.

To assess the impact of variability of annotation by the expert, the random forest model was trained with
the same image dataset with new annotations. The variability found was 1.6% of surface on the test data
set. This was found to be negligible by comparison with the biological variation inside the conditions.

Regarding the root part, ImageJ (version 1.53k) [69] was used to determine the length of primary roots.

**Experiments at the rosette stage**

Experiments at the rosette stage were carried out according to Barrit et al. [23]. Seeds of three A. thaliana
ecotypes (Col-0, Ler andWs) were sown in pots filled with soil and have been grown in a growth chamber
(8 h light / 16 h darkness, 21°C). After 4 weeks, plants were transferred individually in a hydroponic
system containing modified MS medium supplemented with 5 mM KNO$_3$ for 2 weeks. Afterwards, plants
were placed in a new hydroponic nutrient solution containing either 5 mM NH$_4^+$ (provided as NH$_4$Cl) or
0.1 mM or 5 mM NO$_3^-$ (provided as KNO$_3$), as sole N source for 2 weeks. Nutritive solution was replaced
every week. For plant infection assays, 5 µL drops of A. brassicicola (Abra43) conidia suspension (10$^5$
conidia per mL) were deposited on leaves from eight weeks-old plants. For control plants, 5 µL drops of
H$_2$O were used. Plants were then maintained under saturating humidity (100% relative humidity) for 2
days. Foliar symptoms were quantified at 7 DAI by measuring manually the lesion area surface.

**Statistical analyses**

Statistical analyses were carried out with the R software (version 4.1.2). Parametric tests (ANOVA
followed by Tukey’s HSD test in the case of multiple comparisons, Student’s t test in the case of
additional side-by-side comparisons) were performed when the conditions of normal distribution and
homogeneity of variances were met. Otherwise, nonparametric tests (ANOVA using permutation tests for
multiple comparisons, or Wilcoxon-Mann-Whitney test for additional side by side comparisons) were used to test the significance of the results. Statistically significant differences were denoted by P < 0.05.

**Abbreviations**

DAI, day after inoculation; DSC, Dice Sørensen coefficient; GABA, Gamma-aminobutyric acid; HAI, healthy area index; N, nitrogen; NAI, necrosis area index; PRL, primary root length; SEM, standard error of the mean; VRS, visual rating scale.

**Declarations**

**Ethics approval and consent to participate:**

Not applicable.

**Consent for publication:**

Not applicable.

**Availability of data and materials:**

All data generated or analysed during this study are included in this published article and its supplementary information file. Script source code used during the current study for image analysis is available from the corresponding author on reasonable request.

**Competing interests:**

The authors declare that they have no competing interests.

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**Authors’ contributions:**

TB, CC, EP and BT designed this work. TB and SA performed the experiments. TB, JB and DR developed the semi-automated image analysis pipeline. TB, CC, EP and BT wrote the manuscript. TB, CC, JB, DR, EP and BT interpreted data and revised the manuscript. CC, EP and BT supervised the work. All authors read and approved the final manuscript.
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References


52. Fleischmann F, Raidl S, Osswald WF. Changes in susceptibility of beech (Fagus sylvatica) seedlings towards Phytophthora citricola under the influence of elevated atmospheric CO₂ and nitrogen fertilization. Environ Pollut. 2010;158(4):1051-60.


**Tables**

Tables 1 to 3 are available in the Supplementary Files section

**Figures**
Figure 1

Pictures of 14 days old Col-0 seedlings in vertical square plates filled with MS modified agar medium supplemented with 5 mM KNO₃ (scale bars = 1 cm). (a) Overview of a square plate. (b) Seedlings treated with sterile water. (c) Seedlings inoculated with *A. brassicicola*. 
Figure 2

Seed contamination percentage observed 3 DAI for the immersion and droplet methods at different *A. brassicicola* conidial suspension concentrations (10^3, 10^4 and 10^5 conidia per mL). Values are means of 20 seeds per biological replicate, 8 biological replicates. Error bars indicate standard error of the mean (SEM).
Figure 3

Scanning electron microscopy observations showing the heterogeneity of *A. thaliana* seed contamination with the immersion method compared to the droplet method, white circles highlighting *A. brassicicola* conidia. The white scale bars show 50 μm.

(a) Seed treated with sterile water. (b) Seed inoculated with the immersion method (10⁵ conidia. mL⁻¹) showing no *A. brassicicola* conidia on its surface. (c) Seed inoculated with the immersion method (10⁵ conidia. mL⁻¹) presenting a cluster of conidia on its surface, (d) zoom on the conidia cluster. (e) Seed inoculated with the droplet method (10⁴ conidia. mL⁻¹) presenting 2 conidia on its surface, (f) zoom on the conidia.
Figure 4

Overview of the semi-automated pipeline used to detect and calculate the green and necrotic areas per square plate. The pipeline is divided in two steps: (a) a model training phase on a sample of images and (b) the analysis of every image and computation of the surfaces. In the training phase, blue pixels correspond to green areas, red pixels to necrotic areas and yellow pixels to the background.
Figure 5

Correlation between the Ilastik-based indexes and the visual rating scale (VRS). Each point corresponds to the mean of three plates of 12 seedlings for a set of conditions. (a) VRS as a function of healthy area index, supplemented with the estimated correlation curve and $R^2$ coefficient. (b) VRS as a function of necrosis area index, supplemented with the estimated correlation curve and $R^2$ coefficient.
Figure 6

Primary root length (PRL) variation between H₂O and Abra43 seedlings at 8 and 14 DAI, expressed as a percentage and calculated as follows: 100 * ((PRL_{Abra43} - PRL_{H₂O}) / PRL_{H₂O}). Red lines correspond to 5 mM NH₄⁺, green lines to 0.1 mM NO₃⁻ and blue lines to 5 mM NO₃⁻. Solid lines correspond to Col-0, dashed lines to Ler and dotted lines to Ws. Error bars indicate SEM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- Table1.docx
- Table2.docx
- Table3.docx