

The Endophytic Fungus *Cyanoderrella asteris* Influences Growth of the Nonnatural Host Plant *Arabidopsis thaliana*

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Cyanoderrella asteris is a fungal endophyte from *Aster tataricus*, a perennial plant from the northern part of Asia. Here, we demonstrated an interaction of *C. asteris* with *Arabidopsis thaliana*, Chinese cabbage, rapeseed, tomato, maize, or sunflower resulting in different phenotypes such as shorter main roots, massive lateral root growth, higher leaf and root biomass, and increased anthocyanin levels. In a variety of cocultivation assays, it was shown that these altered phenotypes are caused by fungal CO₂, volatile organic compounds, and soluble compounds, notably astins. Astins A, C, and G induced plant growth when they were individually included in the medium. In return, *A. thaliana* stimulates the fungal astin C production during cocultivation. Taken together, our results indicate a bilateral interaction between the fungus and the plant. A stress response in plants is induced by fungal metabolites while plant stress hormones induced astin C production of the fungus. Interestingly, our results not only show unidirectional influence of the fungus on the plant but also vice versa. The plant is able to influence growth and secondary metabolite production in the endophyte, even when

both organisms do not live in close contact, suggesting the involvement of volatile compounds.

Keywords: *Arabidopsis thaliana*, astins, *Cyanoderrella asteris*, endophyte, fungus–plant interactions, plant–microbe-interaction, secondary metabolism, volatiles

The fungal endophyte *Cyanoderrella asteris* (Stictidaceae) was isolated from the inflorescence axis of the tartaric aster *Aster tataricus* (Asteraceae) (Jahn et al. 2017). This plant originates from the northern part of Asia (Siberia, Mongolia, north of Japan, and China) and is known for its use in the traditional Chinese medicine because of its antibacterial, antiviral, antiulcer, and diuretic activities (Shao et al. 1997a, b; Shirota et al. 1997). During the 1990s, Morita and colleagues discovered a new substance group in *A. tataricus*, called astins (Itokawa et al. 1994; Morita et al. 1993a, b, 1994, 1995).

Astins are chlorinated, cyclic pentapeptides of proteinogenic (proline and serine) and nonproteinogenic (*allo*-threonine, α -amino-butyric acid, and β -phenylalanine) amino acids (Fig. 1). More than 20 different astins are currently known (Morita et al. 1993b; Xu et al. 2013), and nearly all of them are differentially chlorinated at the proline residue (Pro¹). Only four of them are dichlorinated, which is important for the antitumor activity of astin A to C (astin K is also dichlorinated but the antitumor activity was yet not shown) (Xu et al. 2013).

The fungus *C. asteris* produces astins in culture and, thus, we assumed that the endophyte is the actual organism capable of synthesizing these interesting and valuable metabolites. Interestingly, not all astins mentioned above could be detected in *in vitro* cultures of *C. asteris* (Schafhauser et al. 2019). We found only astins C, F, and G in *C. asteris* cultures, while the two most prominent other ones, astins A and B, were only found in the plant *A. tataricus*. This observation suggests that the plant takes up and accumulates the fungal astins, most likely after their secretion by *C. asteris*, and is probably able to modify them. To understand astin production and regulation, we studied the interaction between the plant *A. tataricus* and the fungal endophyte *C. asteris* in more detail. The endophyte lives within the host plant and, thus, it was hypothesized that fungal astin biosynthesis in culture

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Conflict of interest: P. Jacques is a co-founder of Lipofabrik and Lipofabrik Belgium and a member of the scientific advisory board of both companies.



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can be stimulated and altered by the action of different plant hormones. Phytohormones such as auxins and cytokinins but also stress hormones such as salicylic acid play an important role in the crosstalk between the host plant and the endophyte (Ali et al. 2017). Auxins and cytokinins are shown to be involved in many plant–microorganism interactions of both phytopathogens (Ludwig-Müller et al. 2017; Reineke et al. 2008) as well as plant-beneficial bacteria (Contesto et al. 2010; Dobbelaere et al. 1999; Kochar et al. 2011; Ortiz-Castro et al. 2008) and fungi (Contreras-Cornejo et al. 2009; Pons et al. 2020; Salas-Marina et al. 2011).

We searched for a feasible plant–endophyte test system, because *A. tataricus* is impracticable due to its winter dormancy and large size. In addition to different Compositae species such as sunflower, we also tested Chinese cabbage, rapeseed, tomato, maize, and *Arabidopsis thaliana*. We decided to use *A. thaliana* for the plant–fungus interaction studies because *A. thaliana* is a well-studied model plant for plant–pathogen and plant–beneficial microbe studies (Jäschke et al. 2010; Peškan-Berghöfer et al. 2004; Proença et al. 2019). For example, the root-colonizing endophytic fungus *Serendipita indica* (formerly *Piriformospora indica*) (Verma et al. 1998) promotes the growth of *A. thaliana* by improving biomass, chlorophyll content, and lateral root density (Abdelaziz et al. 2017) via modulation of phytohormone levels of auxins and cytokinins (Vadassery et al. 2008; Xu et al. 2018). The plant-growth-promoting bacterium *Bacillus megaterium* UMCV1 promotes growth of *A. thaliana* by increasing lateral root growth and root hair elongation (López-Bucio et al. 2007) through influencing the cytokinin signaling pathway (Ortiz-Castro et al. 2008). *C. asteris* produces indole-3-acetic acid, the main auxin in plants, over a tryptophan-dependent and -independent pathway in in vitro cultures and can influence the root phenotype of *A. thaliana* through the additional auxin (Jahn et al. 2021). Despite the influence of fungal auxins, the root phenotype of *A. thaliana* cannot be explained by auxins alone.

In addition to higher molecular weight compounds involved in plant growth promotion, bacteria and fungi also produce small molecular weight volatile organic compounds (VOCs) that stimulate plant growth even over longer distances. Ryu et al. (2003) showed that different *Bacillus* strains induced plant growth and biomass production in *A. thaliana*, although both organisms were physically separated. In the identical experimental setup with *Escherichia coli* DH5 α , these stimulating effects were not

observed. Acetoin was tested and shown to be a key VOC in promoting plant growth (Ryu et al. 2003). In addition to VOCs, inorganic volatile compounds (e.g., carbon dioxide [CO₂] from fungal respiration) may also influence plant growth. Particularly in closed experimental systems, only little or no gas exchange can take place between the interior of the growth chamber or closed cultivation vessel, such as a Petri dish, and the environmental atmosphere; subsequently emitted organic and inorganic volatile compounds can accumulate to unnaturally high concentrations. It was convincingly shown that CO₂ produced by microbes stimulates photosynthesis, growth, and development of the plant during cocultivation (Kai and Piechulla 2009; Van der Kooij et al. 1999), and the effect of CO₂ on plants has to be taken into account when cocultivation assays are performed.

Here, we show the promotion of *C. asteris* on the growth and development of *A. thaliana*. We use different cocultivation assays to show the influence of soluble and volatile fungal compounds on *A. thaliana*. Not only did the fungal partner *C. asteris* affect the plant partner but also *A. thaliana* influenced the fungal growth and metabolism. Therefore, our interaction studies with *A. thaliana* suggest a contribution of soluble astins, VOCs, and CO₂ from *C. asteris*.

RESULTS

Selection of the plant species for fungus–plant cocultivations.

The natural host plant of *C. asteris* is *Aster tataricus*. However, its availability and growth in the laboratory or greenhouse has several limitations. To find a feasible host, sunflower, Chinese cabbage, rapeseed, tomato, and *Arabidopsis thaliana* were cocultivated with *C. asteris*. All plant species showed the same altered growth phenotype in these experiments when they were inoculated with *C. asteris* (Supplementary Fig. S2). Because most plant species grew too large for sterile in vitro cocultivation, the following experiments were performed with *A. thaliana*.

Development of a medium for cocultivation of *A. thaliana* and *C. asteris*.

To find alternatives for studying the interaction of the tartaric aster endophyte *C. asteris* and a plant host, we have established and optimized cocultivation conditions. The endophyte and the plant do not grow well on the same culture medium. Smaller plants such as *A. thaliana* grow well in Petri dishes with various tissue culture media, preferably Murashige and Skoog (MS) medium (Murashige and Skoog 1962), while *C. asteris* grows well on malt extract (MEA) medium (Schafhauser et al. 2019). Different media combinations were tested. *A. thaliana* developed reduced rosette diameter, shorter main roots, and less leaf material in Petri dishes without any MS medium compared with growth on medium containing MS salts and vitamins (Supplementary Table S1). The fungus was able to grow well on half-strength MS medium when it was supplemented with the components that constitute the previously established (Schafhauser et al. 2019) MEA medium (MEAlow). Therefore, a cocultivation medium composed of half-strength MS without sucrose plus MEAlow (1/2 MS/MEAlow) was used for the experiments described in this research.

Cocultivation of *C. asteris* and *A. thaliana* in a closed system.

A. thaliana was cocultivated with *C. asteris* in nonseparated Petri dishes (diameter of 9 cm) sealed with Parafilm. Different distances (1.5, 3.0, and 4.5 cm) between the conidia (inoculation point) and the hypocotyl of the plants were tested. After 35 days, *A. thaliana* developed an altered root phenotype when cocultivated with *C. asteris* that consisted of shorter roots but

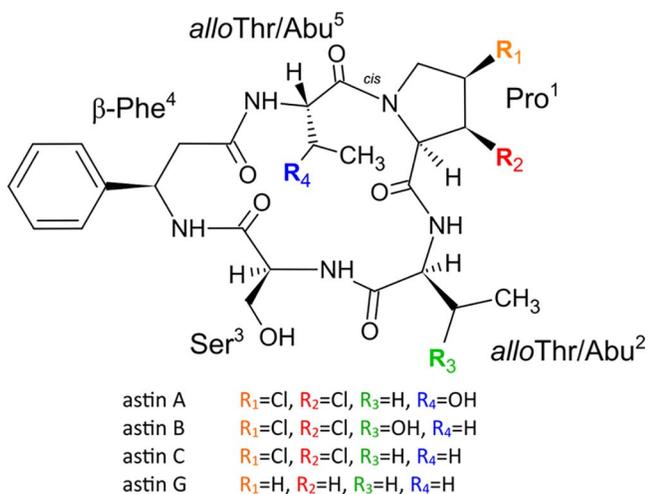


Fig. 1. Basic structure of astins from the plant *Aster tataricus* and the fungal endophyte *Cyanodermea asteris*. The cyclic pentapeptides contain proteinogenic (proline and serine) and nonproteinogenic (allo-threonine, α -aminobutyric acid, and β -phenylalanine) amino acids. The Pro residue can be chlorinated.

produced a massive number of lateral roots (Fig. 2A and C). Furthermore, rosettes as well as roots had a larger biomass (Fig. 2B and D) and flowering occurred earlier than in the plants of noninoculated controls (Fig. 2E). The phenotype was also very similar between *A. thaliana* and *C. asteris* plants when inoculated at a distance of 4.5 and 7.5 cm in squared Petri dishes (12 by 12 cm) (Supplementary Fig. S3). With a larger distance between plants and fungi, the main root length development was more similar to the control (Fig. 2A; Supplementary Fig. S3A). *C. asteris* increased the biomass production of *A. thaliana* at a 7.5-cm distance compared with the noninoculated controls (Supplementary Fig. S3B).

The increase in root biomass was mainly due to massive production of lateral roots (Fig. 2C and D). The plants with the closest distance to *C. asteris* had the lowest biomass in all treatments (1.5 cm) (Supplementary Fig. S3). Rosettes and roots with a larger distance of 3.0 and 4.5 cm had similar biomasses of rosettes and roots. Effects on the main root length were the same as those observed for root biomass. The further away the fungus was, the better the main root grew but the fewer lateral roots were formed (Fig. 2C). Once the roots and the fungus were in direct contact, the roots did not grow over the mycelium

of *C. asteris* (Fig. 2C). The plants treated with *C. asteris* developed faster than the controls (Fig. 2E). All plants with a distance of 4.5 cm flowered 35 days after sowing, while the other plants growing closer to *C. asteris* flowered faster than the control but not as fast as the ones growing at a distance of 4.5 cm to the fungal mycelium (Supplementary Fig. S3C).

The altered phenotypes of *A. thaliana* in the cocultivations are proposed to be due to volatiles or soluble substances of *C. asteris*. In the following sections, three possible fungal compound classes (CO₂, VOCs, and astins) that could be responsible for generating the abovedescribed phenotypes will be addressed.

Fungal CO₂ promotes plant growth of *A. thaliana*.

Plants were cocultivated with *C. asteris* in a closed system (sealed with Parafilm) to show the effect of low molecular weight volatile substances. These cocultivation experiments were performed in tripartite Petri dishes (Fig. 3; Supplementary Figs. S5 and S6). One of these low molecular weight substances was presumed to be the inorganic volatile CO₂ released from the fungus. The volatile CO₂ was trapped by barium hydroxide [Ba(OH)₂] to evaluate the growth effect.

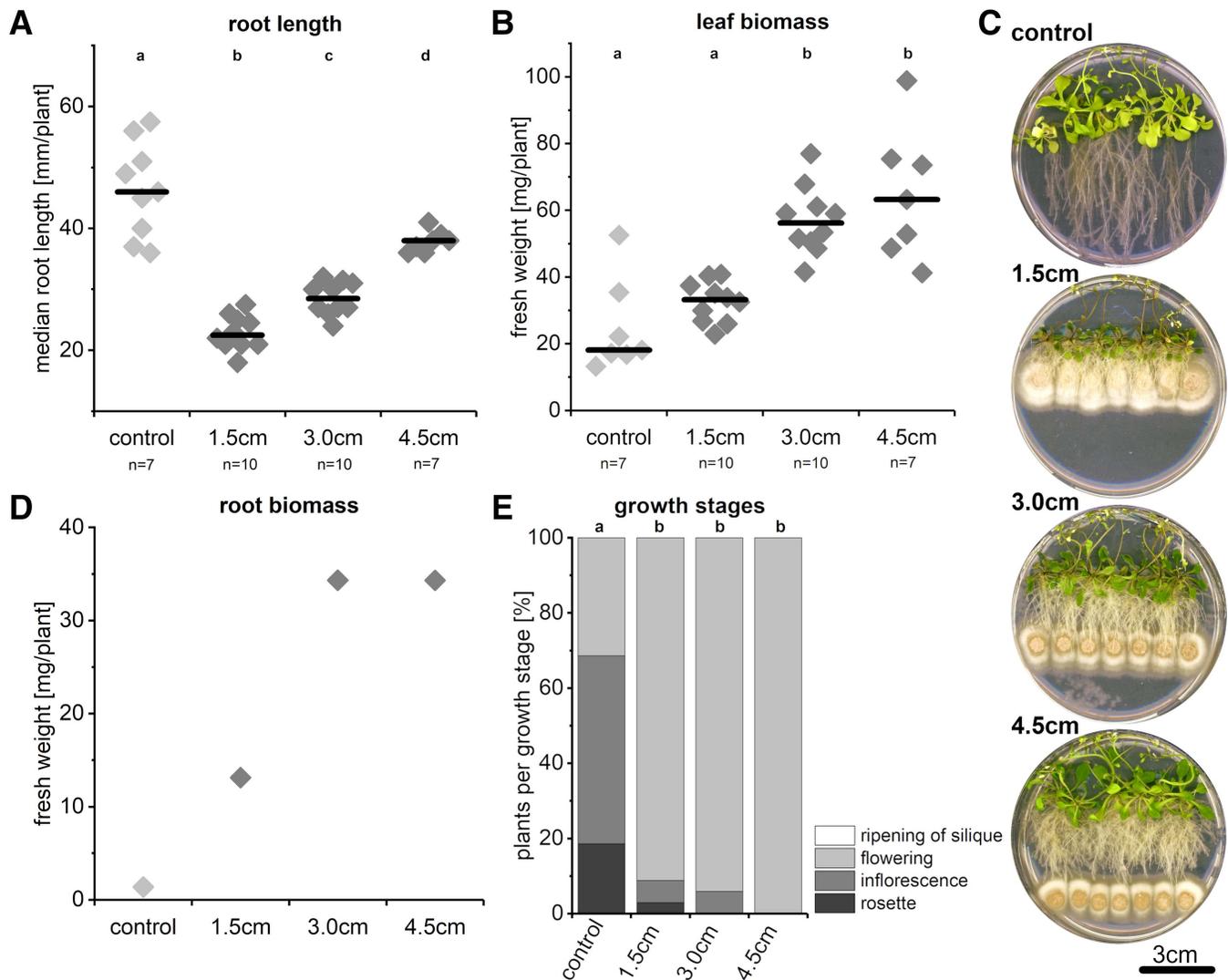


Fig. 2. Cocultivation of *Arabidopsis thaliana* with *Cyanodermella asteris* in 9-cm plates without partitions and sealed with Parafilm. Cocultivation was on half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEA_{low}) medium under long-day conditions for 35 days. *C. asteris* was inoculated at a distance of 1.5, 3.0, and 4.5 cm from *A. thaliana*. Growth of *A. thaliana* is shown. **A**, Main root length ($P < 0.001$, $\eta_p^2 = 0.924$); **B**, leaf biomass ($P < 0.001$, $\eta_p^2 = 0.805$); and **C**, pictures of cocultivation of *A. thaliana* and *C. asteris*. **D**, Root biomass of noninoculated and inoculated plants. **E**, Plant development of *A. thaliana* cocultivated with *C. asteris* ($P < 0.001$, $\eta^2 = 0.448$). Significant differences are labeled with lower case letters (a, b, c) and bar indicates median.

Plants growing in tripartite Petri dishes with *C. asteris* produced more leaf and root biomass than noninoculated plants, regardless of whether or not Ba(OH)₂ was present (Fig. 3B; Supplementary Fig. S5). The addition of Ba(OH)₂ decreased the root biomass in control and *C. asteris*-treated plants compared with plants without Ba(OH)₂. In the control experiments, where plants were incubated with Ba(OH)₂ and without cocultivation with the fungus, leaf and root biomass decreased massively. The plants developed very poorly (e.g., no new leaf development was observed after 14 days of cultivation, inflorescence was decelerated, flowering and ripening of siliques occurred, and plants died after 28 days of cultivation) (Fig. 3A; Supplementary Fig. S6). Due to the weak growth, plant material of all Petri dishes was pooled for weighting. In cocultivation experiments of plants with *C. asteris* in the presence of Ba(OH)₂, *C. asteris* could compensate the CO₂ complexation of Ba(OH)₂. This was indicated by a decreased root biomass of *A. thaliana* cocultivated with *C. asteris* and Ba(OH)₂, which was, however, still higher than in plants without any treatment (Fig. 3B).

If the fungal inoculum was less dense (only two spots, each with 10⁵ or 10³ spores), then *A. thaliana* developed a phenotype similar to the control without *C. asteris* under the Ba(OH)₂ condition (data not shown).

In summary, these experiments clearly indicate that fungal CO₂ of *C. asteris* promoted plant growth of *A. thaliana*, which was documented by the increased leaf and root biomass as well as various parameters of plant development (Fig. 3; Supplementary Figs. S5 and S6). To eliminate or minimize the CO₂ effect in the following fungus–plant interaction studies, we used Petri dishes which were closed by a MaiMed tape that allows diffusion of small molecules (MaiMed 2011). It was hypothesized that, in these experiments, low molecular weight organic and inorganic volatiles could diffuse between the Petri dishes and the environment. The principal idea was tested with bipartite Petri dishes containing Ba(OH)₂ in one compartment and medium plus plant or fungus in the other compartment. The Petri dishes were sealed either with Parafilm, MaiMed tape, or nothing and the amount of barium carbonate (BaCO₃) was determined after 28 days, representing the amount of CO₂ complexed by Ba(OH)₂ (Fig. 4). The BaCO₃ levels were higher in setups where the fungus was growing, indicating that CO₂ was generated by respiration of the

fungus. The BaCO₃ level in Petri dishes with *A. thaliana* or without organisms reached approximately the same amount regardless of whether the Petri dishes were unsealed or sealed with MaiMed tape or Parafilm. In Petri dishes with *A. thaliana* or without an organism, sealing with Parafilm led to a decreased BaCO₃ level.

The results supported the idea that the MaiMed tape can be used to mimic an open system. Therefore, Petri dishes were taped with MaiMed tape in all further experiments to keep the Petri dishes sterile and the medium moist over a long incubation time. In the cocultivation experiment, *A. thaliana* plants inoculated with *C. asteris* showed less root growth but an increased root biomass (Fig. 5; Supplementary Figs. S4 and S5). However, *C. asteris* no longer influenced the plant reproductive development, seen also in a similar leaf biomass (Supplementary Fig. S6).

Inoculation of *A. thaliana* with dead (= autoclaved) spores or hyphae had no effect on the plants. The inoculated plants

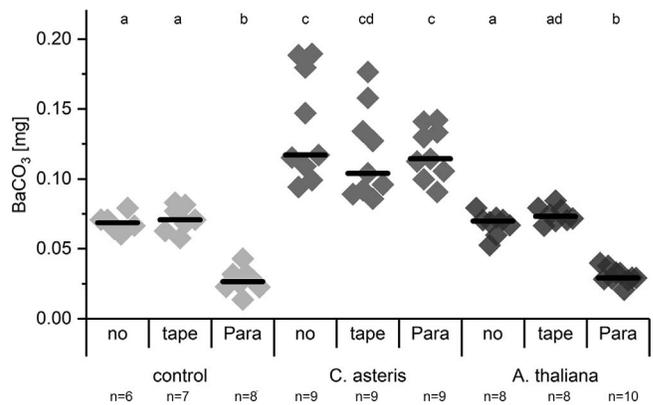


Fig. 4. Accumulation of CO₂ in 9-cm Petri dishes with or without the presence of *Cyanodermella asteris* or *Arabidopsis thaliana*. The Petri dishes without partitions, containing half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEAlow) medium inoculated with *C. asteris*, *A. thaliana*, or without any organisms were closed without tape (no), with MaiMed (tape), or with Parafilm (Para). The BaCO₃ amount of each Petri dish was determined after 28 days ($n = 6$ to 10 , $P < 0.001$, $\eta_p^2 = 0.952$). Significant differences are labeled with lower case letters (a, b, c) and bar indicates median.

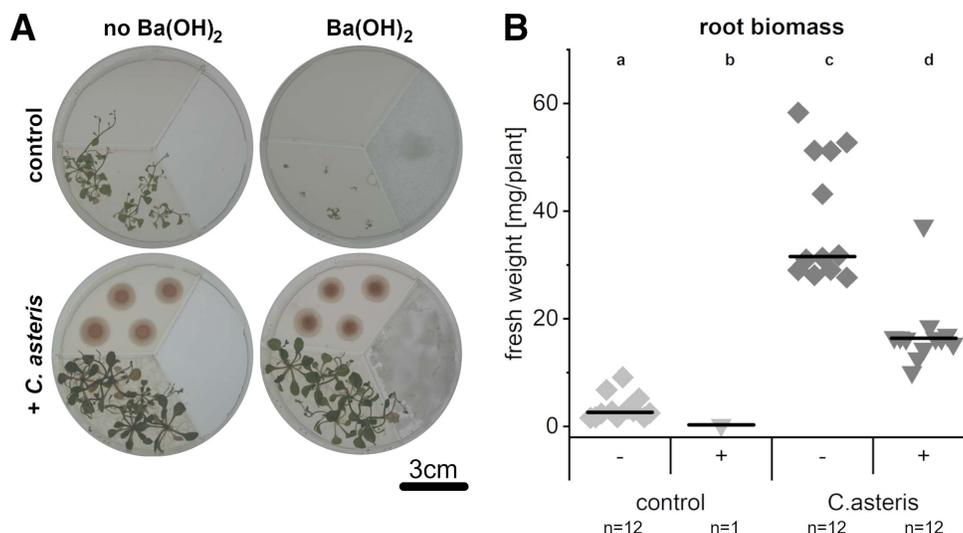


Fig. 3. Cocultivation of *Arabidopsis thaliana* with *Cyanodermella asteris* in 9-cm tripartite plates in the presence or absence of Ba(OH)₂ and sealed with Parafilm. Cocultivation was on half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEAlow) medium under long-day conditions for 35 days. **A**, Growth of *A. thaliana* and *C. asteris* in the presence of Ba(OH)₂. **B**, Root biomass of *A. thaliana* plants noncultivated (control) or cocultivated with *C. asteris* in the absence (–) or presence (+) of Ba(OH)₂ ($P < 0.001$, $\eta_p^2 = 0.921$). Significant differences are labeled with lower case letters (a, b, c, d) and bar indicates median.

developed like the controls without *C. asteris*, indicating that the living fungus is necessary for the growth phenotype reported here (data not shown).

VOCs affect plant growth of *A. thaliana*.

As reported above, part of the growth-promoting effect in separated cocultivation is due to CO₂ released by the fungus; however, the growth effects observed cannot only be assigned to a CO₂ effect. Because VOCs as well as highly molecular-soluble compounds of *C. asteris* might also affect the growth of *A. thaliana*, we examined both hypotheses in cocultivation experiments using bipartite Petri dishes sealed with MaiMed tape.

Growth of *A. thaliana* was enhanced under these conditions (Fig. 6). Plants cocultivated with *C. asteris* produced more roots (Fig. 6A and B) and leaves (Supplementary Fig. S5). The root length (Supplementary Fig. S4) and plant development (Supplementary Fig. S6) of *A. thaliana* were not significantly influenced by the presence of *C. asteris*, as was observed in nonseparated Petri dishes (Fig. 5). The increased biomass of leaves (1.67-fold) and roots (2.88-fold) indicated a direct or indirect (via agar) influence of as-yet-undefined fungal VOCs on plant growth promotion.

C. asteris induces a stress response in *A. thaliana*.

Because *C. asteris* is not a natural endophyte of *A. thaliana*, anthocyanin and chlorophyll levels of plants were determined as general stress indicators. However, such a defense response could also be found in the interaction of a natural endophyte and its host plant (Mandyam et al. 2013; Schulz and Boyle 2005). Cocultivation of *A. thaliana* with the nonnatural endophyte *C. asteris* led to an increased anthocyanin level in plants (Table 1; Supplementary Fig. S7). The anthocyanin content was highly increased (Parafilm: 9.7- to 57.6-fold; MaiMed tape: 3.0-fold) in plants that had direct contact with *C. asteris*, regardless of which sealing was used. Plants that were inoculated 3.0 cm away from the hypocotyl had a higher anthocyanin

content than plants with a distance of 1.5 cm. Because these plants were quickly overgrown by *C. asteris*, they were much smaller and could not develop as much anthocyanin as larger plants. The addition of Ba(OH)₂ did not change the anthocyanin level in *A. thaliana*. Only plants cocultivated with *C. asteris* showed a higher anthocyanin level, which reduced to the control level in the presence of Ba(OH)₂. A separation of plant and fungus on bipartite Petri dishes led to a slight decrease in the anthocyanin level, as long as the Petri dishes were tightly closed with Parafilm. Cocultivated plants without any influence of fungal soluble or volatile compounds (bipartite and MaiMed tape) had anthocyanin levels similar to those of the respective controls without any fungus.

We also measured the chlorophyll content in *A. thaliana* plants cocultivated with *C. asteris* on different Petri dishes (Table 1; Supplementary Fig. S8). *C. asteris* had no strong effect on the chlorophyll content of cocultivated plants under almost all conditions, except when plants were cocultivated with *C. asteris* on bipartite Petri dishes sealed with Parafilm. In this case, a massive decrease in chlorophyll content was observed. If the fungus was in direct contact with *A. thaliana* and in plates sealed with MaiMed tape, the chlorophyll content of the leaves was slightly decreased.

A. thaliana mutants defective in the biotic stress pathways were cocultivated with *C. asteris* in the open system to investigate the possible involvement of stress-associated plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene that could lead to the phenotype caused by *C. asteris*. NPR1 is a key regulator in the systemic acquired resistance in plants induced by SA (Cao et al. 1997), SID2 is an enzyme in SA biosynthesis (Wildermuth et al. 2001) and NahG encodes a bacterial SA hydroxylase, degrading SA (Friedrich et al. 1995). Consequently, all mutants are, to some extent, impaired in their response to SA. The *npr1-1* and *sid2-1* mutants and *NahG* overexpressing plants of *A. thaliana* developed similarly to the control in the presence of *C. asteris* (Supplementary Fig. S9), indicating that *C. asteris* is not using the SA pathway to induce

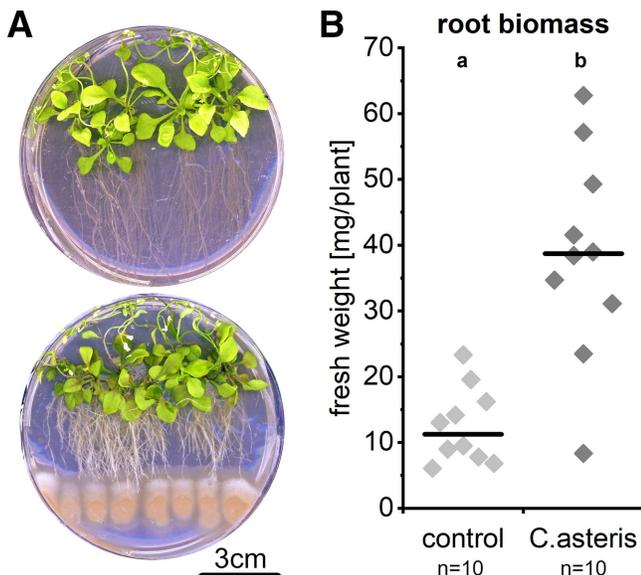


Fig. 5. Cocultivation of *Arabidopsis thaliana* with *Cyanodermella asteris* in 9-cm plates without partitions, sealed with MaiMed. Cocultivation was on half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEA_{low}) medium under long-day conditions for 35 days. *C. asteris* was inoculated at a distance of 4.5 cm from *A. thaliana* seedlings. **A**, Growth of *A. thaliana* without and with *C. asteris* on non-separated plates. **B**, Root biomass of *A. thaliana* without (control) and with *C. asteris* ($P < 0.001$, $\eta^2 = 0.543$). Significant differences are labeled lower case letters (a, b) and bar indicates median.

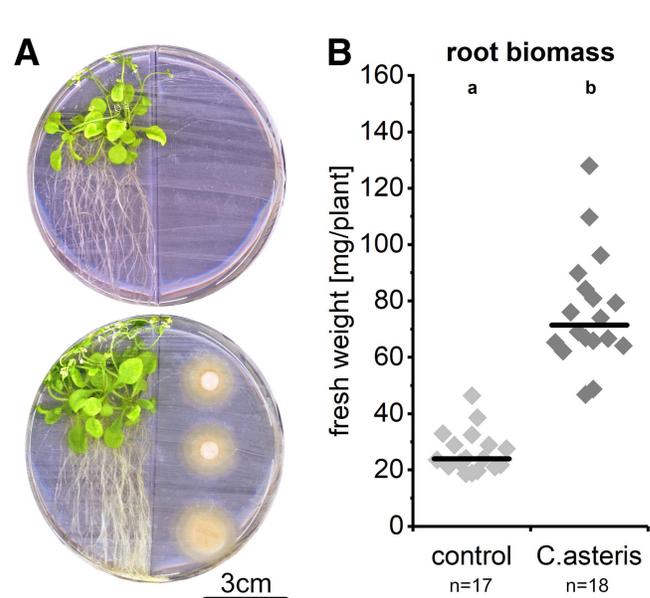


Fig. 6. Cocultivation of *Arabidopsis thaliana* with *Cyanodermella asteris* in 9-cm bipartite plates, sealed with MaiMed. Cocultivation was on half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEA_{low}) medium under long-day conditions for 35 days. **A**, Growth of *A. thaliana* without and with *C. asteris* in cocultivation. **B**, Root biomass produced by *A. thaliana* without (control) and with *C. asteris* ($P < 0.001$, $\eta^2 = 0.723$). Significant differences are labeled with lower case letters (a, b) and bar indicates median.

the altered phenotype of *A. thaliana*. The *jar1-1* mutant, which is defective in the JA response (Staswick et al. 1992), and the *etr1-1* mutant, which cannot perceive the ethylene signal (Gamble et al. 2002), showed also no alterations in the phenotype of *A. thaliana* inoculated with *C. asteris* compared with wild-type plants (Supplementary Fig. S9).

Astins induce growth of *A. thaliana*.

Astins are secondary metabolites from *C. asteris*, known also from its host plant *Aster tataricus*. A biological effect on plants has not been reported yet. To investigate possible biological effects of astins on *Arabidopsis thaliana*, the plants were cultivated on MS medium containing different amounts (10 pM to 10 μM) of astins A, C (both dichlorinated), or G (nonchlorinated) (Fig. 1). As discussed in the introduction, astins A and C differ in a hydroxyl group at the fifth amino acid (*alloThr*⁵ → *Abu*⁵). Astin A can only be found in *Aster tataricus* together with *C. asteris* and not in fungal cultures alone (Schafhauser et al. 2019). Our experiments showed that all three tested astins influenced the growth and development of *Arabidopsis thaliana* during 35 days of cultivation (Fig. 7; Supplementary Figs. S10 and S11).

The plant growth (root length and rosette diameter) of *A. thaliana* was significantly inhibited at 10 μM astin A over the whole period. Lower concentrations of astin A did not suppress root growth (Fig. 7; Supplementary Fig. S10) or rosette growth (Fig. 7; Supplementary Fig. S11) and even induced plant growth below an astin A concentration of 10 μM (Fig. 7). The growth induction of *A. thaliana* by astin A concentrations less than 10 μM was accompanied by faster plant development (e.g., earlier flowering) (Supplementary Fig. S12). *A. thaliana* plants grown with different concentrations of astin C, as determined after 7 days, suppressed root growth at all concentrations (Supplementary Fig. S10). This inhibition turned into a slight growth promotion after 35 days of cultivation (Supplementary Fig. S10). Astin C increased the rosette diameter at all astin C concentrations throughout the 35 days of cultivation (Fig. 7; Supplementary Fig. S11). Astin C plant growth enhancement was similar to that of astin A, and which was accompanied by faster reproductive plant development (Supplementary Fig. S12). The nonchlorinated astin G affected root length and rosette diameter, and flowering started slightly earlier at concentrations higher than 1 nM.

In summary, astins, especially the dichlorinated astins A and C, led to a faster development and, therefore, an earlier flowering of *A. thaliana* compared with the treatment with astin G and with untreated plants. Because *C. asteris* produces only astins C, F, and G (Schafhauser et al. 2019), it is hypothesized that astin C functions as a bioactive soluble compound that influenced the development of *A. thaliana* in in vitro experiments.

A. thaliana induces astin C production of *C. asteris*.

The astin C production of *C. asteris* was investigated in Petri dishes where the fungus had direct contact with the nonnatural host plant *A. thaliana* (Fig. 8). The astin C level of *C. asteris* without plants (control) differed in Petri dishes with different sealings: Parafilm led to a higher astin C production in *C. asteris* than the MaiMed tape. The closer the fungus and *A. thaliana* were on

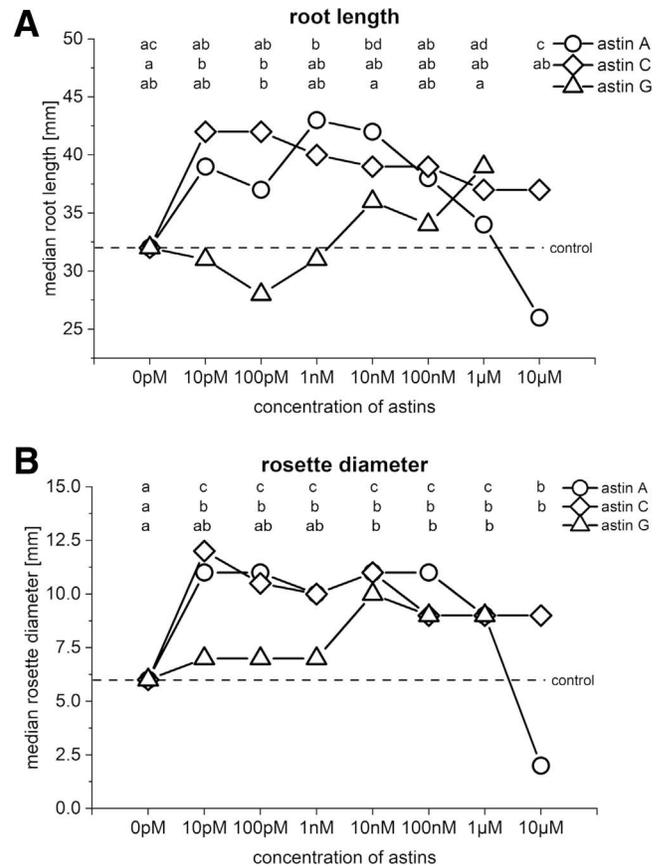


Fig. 7. Growth of main root and rosette of *Arabidopsis thaliana* with different concentrations of astins A, C, or G. Plants of *A. thaliana* grew in 9-cm plates without partitions on half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEAlow) medium including different astins under long-day conditions for 35 days. **A**, Growth of main root ($P = 0.01$) and **B**, rosette growth after 35 days of cultivation with different astins ($P = 0.01$). Control plants grown without any astins are shown in sample 0 pM. Significant differences are labeled with lower case letters (a, b, c).

Table 1. Influence of *Cyanodermella asteris* on the anthocyanin and chlorophyll content in *Arabidopsis thaliana* in cocultivation^a

Experimental settings	Anthocyanins		Chlorophyll			
	P value	Fold	P value	Fold		
Nonseparated	Parafilm	1.5 cm	<0.001	34.5×	0.246	–
		3.0 cm	–	57.6×	–	–
		4.5 cm	–	9.7×	–	–
Bipartite	MM tape	4.5 cm	0.005	3.97×	0.029	<i>0.70×</i>
	Parafilm	4.5 cm	0.030	<i>0.13×</i>	0.007	<i>0.12×</i>
	MM tape	4.5 cm	0.770	1.02×	0.770	0.94×
Tripartite with Ba(OH) ₂	Control	Ba(OH) ₂	0.162	NC	0.298	NC
	+ <i>C. asteris</i>	No Ba(OH) ₂	–	30.47×	–	1.30×
	+ <i>C. asteris</i>	Ba(OH) ₂	–	5.71×	–	1.26×

^a *Arabidopsis thaliana* and *C. asteris* were grown on half-strength Murashige and Skoog plus malt extract without sucrose medium under long-day conditions for 35 days. Plates were sealed with MaiMed tape (MM tape) or Parafilm (Parafilm). P value and upregulation (bold) or downregulation (italics) (relative to respective control; effect of 1 means no regulation) are given for each experiment. NC indicates not calculated because of a very low value of dead plants [control treated with Ba(OH)₂]. Fold = fold induction. The complete dataset is shown in Supplementary Figs. S7 and S8 and Supplementary Tables S2 to S6.

the Petri dishes (1.5 and 3 cm), the more astin C was secreted by the fungus. At a distance of 4.5 cm between *A. thaliana* and the fungus, the fungus produced levels of astin C similar to that in the control. Astin A, only isolated from the host plant of *C. asteris* (*Aster tataricus*) (Schafhauser et al. 2019), was not detected under any of these conditions.

Plant hormones induce fungal growth and astin C production of *C. asteris*.

Because the original habitat of *C. asteris* is within a host plant, we investigated whether plant signals can influence growth and astin production of *C. asteris* in vitro cultures. *C. asteris* was cultivated with different plant hormones. The growth of *C. asteris*, based on dry weight determination and astin C production, was altered by two auxins—1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) (Fig. 9)—while the two stress hormones SA and JA increased astin C production but did not alter the fungal growth (Fig. 10).

The synthetic auxin NAA led to an optimum growth curve, with the maximum at 100 nM NAA. The growth was only inhibited at the two highest NAA concentrations of 100 μ M and 1 mM. The natural auxin IAA did not enhance growth of *C. asteris* but reduced growth at the two highest concentrations (inhibition at 100 μ M and 1 mM). The two stress hormones SA and JA did not influence fungal growth, except for a growth inhibition at high SA concentrations of 100 μ M and 1 mM. Most notable was the increased astin C production at a concentration of 10 μ M JA.

DISCUSSION

The recently isolated novel endophyte *C. asteris* (Jahn et al. 2017) was cocultivated with *Arabidopsis thaliana* to investigate the influence of the fungus on the growth and development of this alternative host plant. *A. thaliana* developed strong phenotypic effects in the presence of *C. asteris*: shorter main roots, massive lateral root growth leading to higher root biomass, and increased leaf biomass (Fig. 2). The term “nonnatural host” we have chosen reflects that an interaction between the two organisms occur: (i) the living fungus is necessary for the interaction, (ii) there is most likely not a classical defense reaction involved, and (iii) the partner plant *A. thaliana* is influenced by fungal compounds in growth and development. Dead spores and hyphae of *C. asteris* had no effect on *A. thaliana*, indicating that the bioactive compounds responsible for the plant phenotype

have to be produced by the fungus. Growth promotion in nonnatural host plants has already been shown for many other fungi such as *S. indica* (Singhal et al. 2017; Xu et al. 2018), *Trichoderma atrovirida*, *T. virens* (Contreras-Cornejo et al. 2009; González-Pérez et al. 2018), *Aspergillus ustus* (Salas-Marina et al. 2011), and *Oidiodendron maius* (Casarrubia et al. 2016).

To determine the relevant bioactive compound or compounds in addition to fungal IAA of *C. asteris* that influenced root growth positively (Jahn et al. 2021), inorganic and organic volatiles as well as soluble astins were investigated in cocultivation experiments. Our results of cocultivation studies of *A. thaliana* and *C. asteris* indicated that plant growth promotion was largely caused by CO₂, but not exclusively (Figs. 3 and 5). Similar observations were already made in other experimental setups, especially in closed systems (Kai and Piechulla 2009). When Petri dishes are sealed (e.g., with Parafilm), fungal organic and inorganic volatiles will be trapped inside the Petri dish. CO₂ that is produced in the cellular respiration can directly be used by the plant in photosynthesis, leading to a higher biomass and stronger plants (Kai and Piechulla 2009; Makino and Mae 1999; Piechulla and Schnitzler 2016). An elevated atmospheric CO₂ level increases shoots as well as roots and altered root architecture in agricultural crops (Madhu and Hatfield 2013). The degree to which extent fungal CO₂ might affect host growth in planta has still to be answered and remains unsolved.

Detailed cocultivation experiments of *A. thaliana* plants with *C. asteris* also showed a higher biomass production in Petri dishes sealed with MaiMed tape, suggesting that, additionally, VOCs may promote growth (Fig. 6). β -Caryophyllene (Minerdi et al. 2011), 3-methyl-1-butanol, 1-decene, and 2-heptylfuran from *Trichoderma* spp. (Lee et al. 2019) are a few examples of VOCs increasing plant growth (Piechulla et al. 2017). In addition to plant growth promotion, VOCs (e.g., isobutyric acid, benzyl aldehyde, or 6-pentyl- α -pyrone) also show antimicrobial activities (Morath et al. 2012) or enhance and prime the plant defense system (Ryu et al. 2003), action modes which are beneficial for plants. *A. thaliana*, for example, showed an increased level of defense genes in the presence of fungal VOCs such as 1-octen-3-ol, *allo*-ocimene, or C6-aldehydes and, thus, can react much faster to pathogen attacks (Kishimoto et al. 2006a, b, 2007). A mixture of VOCs of *Muscodor albus*, an endophytic fungus in the bark of *Cinnamomum zeylanicum* (cinnamon tree) (Worapong et al. 2001), inhibited or killed phytopathogenic bacteria and fungi (Strobel et al. 2001). Additionally, *M. albus* can

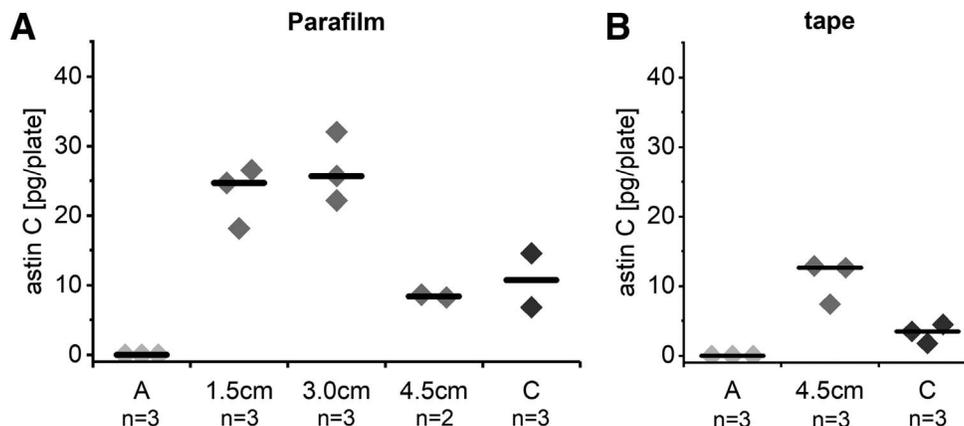


Fig. 8. Astin C production by *Cyanodermedella asteris* in cocultivation with *Arabidopsis thaliana* on 9-cm plates without partitions. Cocultivation was on half-strength Murashige and Skoog plus malt extract without sucrose (1/2 MS/MEA_{low}) medium under long-day conditions for 35 days. Plates were sealed with **A**, Parafilm or **B**, MaiMed. Astin C concentration was measured in the medium of controls (A = only *A. thaliana* and C = only *C. asteris*) and of cocultivation plates where both organisms were placed at different distances (1.5, 3.0, and 4.5 cm). Three plates were pooled for each astin C extraction; bar indicates median.

control phytopathogens such as *Rhizoctonia solani* and *Phytophthora capsici* in soils (Mercier and Manker 2005). It is likely that the plant-associated fungus *C. asteris* is able to influence growth and defense responses in *Aster tataricus* as well as in the nonnatural host plant *Arabidopsis thaliana*. Because the MaiMed tape used in these experiments is only an approach to mimic an open system (because the plates had to be cultivated vertically to analyze the root growth), it has to be taken into account that the CO₂ content could be increased in these systems. Measuring the CO₂ level directly in the cocultivation system would show if there is still a slightly higher CO₂ content in the Petri dishes, which still would contribute to the increased biomass production in *A. thaliana*. The VOCs that additionally cause changes in plant growth need to be identified to investigate their effect during the cocultivation of both partners. Furthermore, it is of interest to learn whether the plant partner might also influence the fungus by producing plant VOCs. Identifying the VOCs responsible for the altered growth phenotype of *A. thaliana* after inoculation with *C. asteris* will be subject of future research.

In addition to small volatile compounds, higher molecular soluble compounds, typically secondary metabolites produced by the endophyte, have to be considered to interact with plant metabolism. Fungi secrete various mixtures of different soluble

compounds and some of them suppress the growth of the (non-natural host) plant (Dovana et al. 2015). One group of such secondary metabolites are fungal phytotoxins that are secreted into the medium and inhibit plant growth or induce a stress response in plants. The endophytic fungus *Edenia gomezpompae* from *Callicarpa acuminata* leaves (Gonzalez et al. 2007), for example, inhibited growth of *Solanum lycopersicum*, *Echinochloa crus-galli*, and *Amaranthus hypochondriacus* by different phytotoxins (e.g., palmarumycin EG₁ and preussomerin EG₄) (Macías-Rubalcava et al. 2014). Such growth inhibition of root length and biomass of *Arabidopsis thaliana* by *Cyandermella asteris* compounds was observed in close-distance cocultivations on nonseparated Petri dishes (Fig. 2). The concentration of the fungal compounds (e.g., such phytotoxins or astins) was presumably highest close to *C. asteris* and decreased at longer distances from *C. asteris*. In addition, *A. thaliana* plants accumulated higher anthocyanin levels when they grew closer to *C. asteris*, whereas this effect was not observed in cocultivations in bipartite Petri dishes (Supplementary Fig. S8; Table 1), indicating the presence of at least one toxic compound in the closer surroundings of the fungal colonies. The biosynthesis of anthocyanins is usually upregulated by environmental stresses such as drought, extreme temperatures, radiation and strong light, nutrient deficiencies, wounding, bacterial and fungal infections, or attack by

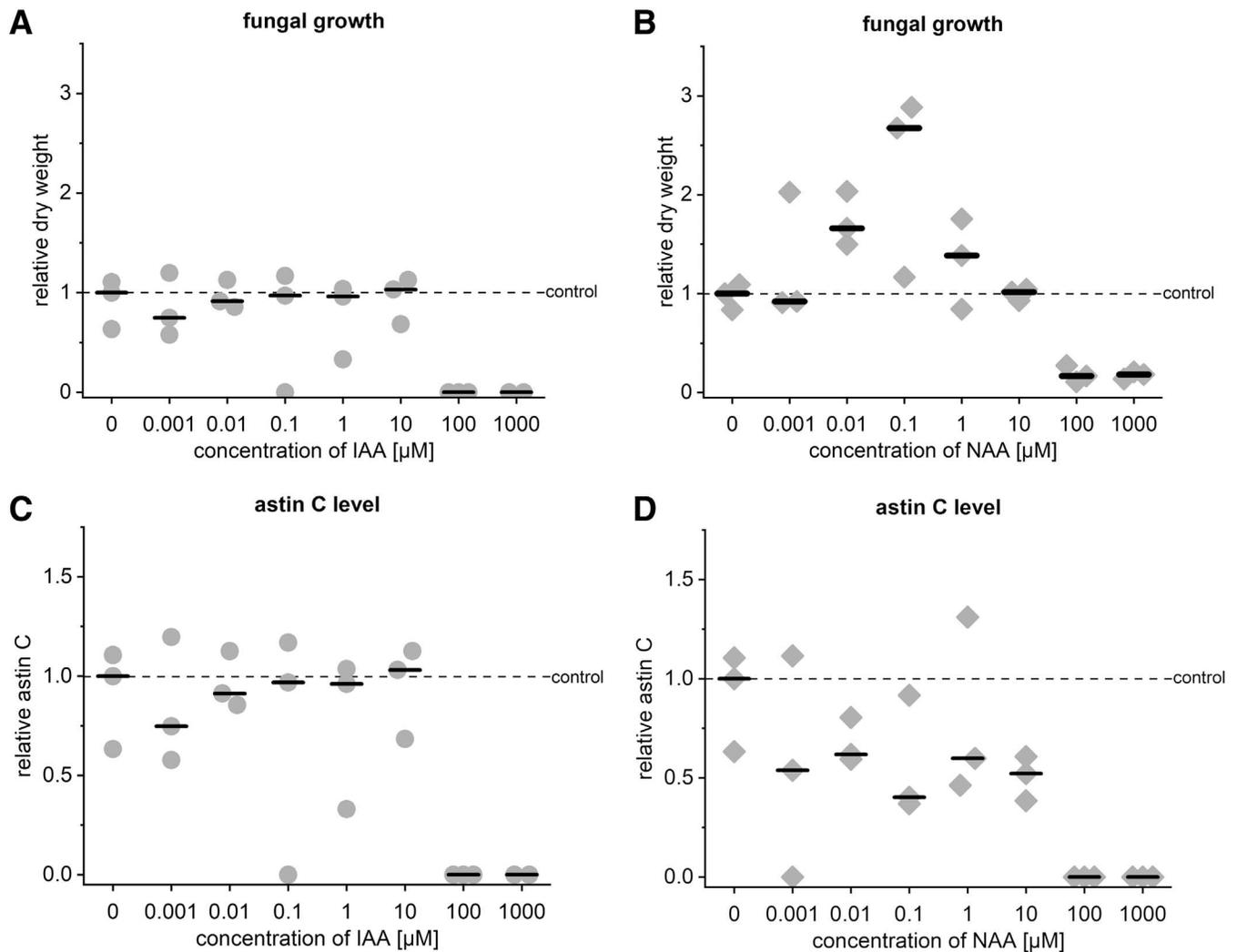


Fig. 9. Relative fungal growth and astin C production of *Cyanoderma asteris* in the presence of two different auxins. *C. asteris* was grown in liquid culture (malt extract without sucrose [MEAlow] including auxins) at 23°C in the dark for 1 week. Fungal growth in the presence of **A**, indole-3-acetic acid (IAA) and **B**, 1-naphthaleneacetic acid (NAA). Astin C production of *C. asteris* in the presence of **C**, IAA and **D**, NAA. Relative values are related to the control without any additives (0 μM). Sample size = 3; bar indicates median.

herbivores (Chalker-Scott 1999; McClure 1979). Because *C. asteris* is not a natural endophyte of *A. thaliana*, it may not be adapted to the fungus and its secreted metabolites. An incompatibility between *A. thaliana* and *C. asteris* was most likely indicated by the strong pigmentation of the plant because the anthocyanin content was higher when *A. thaliana* and *C. asteris* grew within a close distance (Supplementary Fig. S8). In addition to unknown fungi, natural endophytes can also lead to chlorosis or necrosis in *A. thaliana* leaves, especially under stressful conditions such as in vitro cultivation (Junker et al. 2012). Furthermore, plants can also react with direct defense responses to natural endophytes (Mandyam et al. 2013).

It was shown here that JA and SA as well as ethylene are typical stress hormones involved in the pathogen response (Broekaert et al. 2006; Halim et al. 2006) but were not involved in the development of the altered growth phenotype of *A. thaliana*. Mutants in the SA signaling (*npr1-1*) (Cao et al. 1997) and biosynthetic pathway (*sid2-1*) (Wildermuth et al. 2001) as well as transgenic plants overexpressing bacterial SA hydroxylase (*NahG*) (Friedrich et al. 1995) showed the same phenotype as the wild type after inoculation with *C. asteris* (Supplementary Fig. S9). Similarly, the *jar1-1* mutant, which is defective in the

JA response (Staswick et al. 1992), and the *etr1-1* mutant, which cannot perceive the ethylene signal (Gamble et al. 2002), showed the same altered growth phenotype; namely, shorter roots and more laterals. If other growth-promoting phytohormones such as cytokinins or gibberellins are involved in the interaction between *A. thaliana* and *C. asteris*, is a question currently under investigation.

When the plant and the fungus were separated by a barrier, only VOCs and CO₂ are biologically active; however, fewer stress symptoms were developed (root growth and anthocyanin content). It appears that atmospheric CO₂ (released by the fungus in the in vitro coculture system) primarily reduces plant stress in closed systems. If the cocultivation took place on bipartite Petri dishes with MaiMed tape (to mimic an open system), then the stress symptoms were no longer visible in the interaction of *A. thaliana* and *C. asteris*. This was also reported by Kai and Piechulla (2009), who analyzed the influence of VOCs produced by *B. subtilis* on *A. thaliana* but did not observe growth promotions as Ryu et al. (2003) had. However, the experimental settings were different. Kai and Piechulla (2009) used an open system where the air could circulate with the environment, whereas Ryu et al. (2003) used a closed system where the

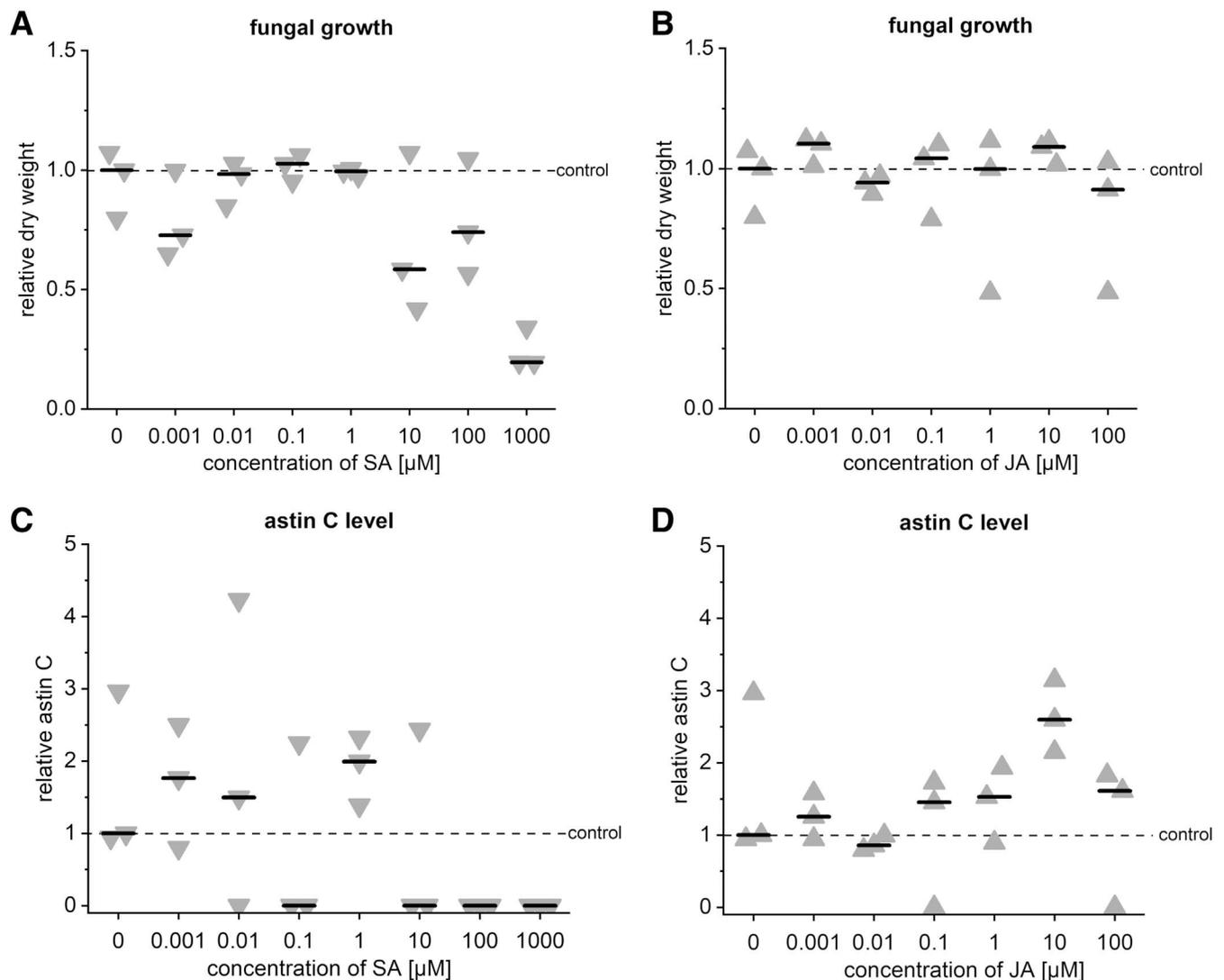


Fig. 10. Fungal growth and astin production of *Cyanodermedella asteris* under different stress hormones. *C. asteris* was grown in a liquid culture (malt extract without sucrose [MEAlow] including salicylic acid [SA] or jasmonic acid [JA]) at 23°C in the dark for 1 week. Fungal growth in the presence of **A**, SA and **B**, JA. Astin C production of *C. asteris* in the presence of **C**, SA and **D**, JA. Relative values are related to the control without any additives (0 μM). Sample size = 3; bar indicates median.

concentrations of VOCs and CO₂ are presumably much higher than in the natural environment.

Due to the low concentrations of VOCs, the structure of the bioactive VOCs could not be determined thus far. In contrast, the dichlorinated astin C produced by *C. asteris* induced plant development of *A. thaliana* (Supplementary Fig. S12). The fungal astin C was less biologically active than astin A, which is only produced by the host plant *Aster tataricus*. The latter is presumably synthesized in plants by hydroxylation from the fungal astin C (Schaffhauser et al. 2019) and might play a major role in the plant–endophyte interaction. It is known that compounds from endophytes can induce plant growth in the nonhost plant *Arabidopsis thaliana*. For example, the class of strobilurines that were primarily isolated from the fungal genus *Strobilurus* (Anke et al. 1977), also found in other fungi (Schramm et al. 1978), are used as fungicides in agriculture, and treated *A. thaliana* plants exhibited growth promotions due to an increased expression of sugar transport genes (Van Dingenen et al. 2017). In addition to the direct way to positively influence the plant host growth, there are also indirect ways to benefit the plant by endophytes; for example, release of siderophores to improve nutrition uptake of iron (Chhabra and Dowling 2017) or release of acid phosphatases to enable uptake of insoluble phosphates (Gill et al. 2016; Resende et al. 2014; Singh et al. 2003).

A third group of soluble compounds, which play an important role in plant–endophyte interactions, are plant hormones. Plant hormones are produced (Contreras-Cornejo et al. 2009; Sirrenberg et al. 2007) or concentrations are modulated (Shahzad et al. 2017) inside the plant by endophytes. *B. amyloliquefaciens* RWL-1, for example, induced the amino acid biosynthesis and the biosynthesis of the stress hormone JA in rice under salinity stress (Shahzad et al. 2017). Plant hormones produced by endophytes are auxins—*Serendipita indica* (Sirrenberg et al. 2007), *T. virens* (Contreras-Cornejo et al. 2009), or *Serratia marcescens* (Shi et al. 2010)—and cytokinins—*Serendipita indica* (Vadassery et al. 2008)—but also gibberellic acid (Bilal et al. 2018) as well as other hormones (Morrison et al. 2015) that play a role in the interaction between plant and endophyte. Here, we showed that plant hormones such as auxins and JA influence the fungal growth and astin production of *C. asteris* (Figs. 9 and 10). Because *C. asteris* synthesizes the natural auxin IAA de novo in in vitro cultures (Jahn et al. 2021), most likely to influence the plant, it is more interesting that the growth of *C. asteris* can be influenced by the more stable auxin NAA in in vitro cultures (Fig. 9). *Saccharomyces cerevisiae* differentiates from a yeast into a filamentous form in the presence of IAA (Prusty et al. 2004), involving an IAA-dependent gene expression in the yeast. These IAA-dependent gene expressions in microorganisms together with the fungal IAA biosynthesis and the auxin-dependent fungal growth in *C. asteris* strengthen the hypothesis that IAA is not only a plant but also a microbial signal.

Particularly interesting is that *C. asteris* produced most of the astin C in close contact with *A. thaliana* in closed experimental systems (Fig. 8). Because *C. asteris* lives inside its host plant *Aster tataricus*, it is essential for the fungus to react to plant signals, especially those that are in its direct surroundings in the plant. For many endophytes, it is even essential for their survival to perceive plant signals, because they may die or become saprophytes when the plant dies. For example, *Serendipita indica* induces gene expression of hydrolytic enzymes and nutrient transporters in barley as soon as nitrogen deficiency occurs in dying plant cells. This switch from endophytic to saprophytic life style is associated with a morphological change to thinner hyphae (Lahrmann et al. 2013). Understanding the communication between *C. asteris* and *A. thaliana* will not only contribute to a better understanding of the *C. asteris*–*Aster tataricus* relationship but also add to our general understanding of plant–endophyte interactions.

What can we learn from our results on the interaction with the endophytic fungus *C. asteris* obtained for the nonnatural host *Arabidopsis thaliana* in the host *Aster tataricus*? In the nonnatural host interaction, the responses ranged from a stress response to growth promotion, depending on which factor was analyzed (Fig. 11). Thus, we hypothesize that *C. asteris* could affect the overall performance of *A. tataricus* by the production of astin C, which is most likely converted into astin A or B by the host (Schaffhauser et al. 2019). Both astin A and C could enhance the growth of *A. thaliana*. Fungal IAA would additionally support plant growth and development. Plant signals such as IAA or SA could be signals to the fungus to sense the environmental stress and maybe activate the metabolism (e.g., astins) or gene expression of compounds, which support *Aster tataricus*. For the non-natural host plant, it was shown that the plant hormones (auxins and stress hormones) influence growth and metabolism (astin C production) of the fungus *C. asteris*.

In addition to soluble compounds, volatiles play a major role in the interaction between plant and fungus. The presence of CO₂ led to increased plant growth and earlier plant development in the coculture systems. However, the most challenging question remains to be answered: namely, whether and how much of the internally (from fungal respiration) released CO₂ contributes to plant development and growth. This knowledge would be of particular interest, because the endophyte lives within the plant. Furthermore, as-yet-uninvestigated VOCs of the fungus seem to also induce plant growth. The identification of the putative volatile compounds of the fungus will be a subject of future research. In summary, we provided the first evidence of the role of volatile and nonvolatile metabolites involved in the fascinating *Arabidopsis thaliana*–*Cyanoderma asteris* (plant–endophyte) interaction.

MATERIALS AND METHODS

Chemicals.

If not otherwise indicated, all chemicals were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Fisher Scientific GmbH (Schwerte, Germany), Merck KGaA (Darmstadt, Germany), or Duchefa Biochemie (Haarlem, The Netherlands). Astins were extracted from fungal cultures of *C. asteris* according to Schaffhauser et al. (2019).

Biological materials.

The fungus *C. asteris* originated from our own culture (deposited under the accession number DSM 100826 at DSMZ, Braunschweig, Germany), isolated from *Aster tataricus* in 2013 (Jahn et al. 2017) and maintained on MEAlow medium.

The *Arabidopsis thaliana* ecotype Columbia (Col-0) used in the Petri dish assays was obtained from Nottingham Arabidopsis Stock Centre (NASC, Loughborough, U.K.). Mutants of *A. thaliana* were obtained from different sources: *sid2-1* and *npr1-1* from Regina Mencia (Universidad Nacional del Litoral, Santa Fe, Argentina), *NahG* from Sabine Rosahl (Institute of Plant Biochemistry Halle, Germany), and *jar1-1* as well as *etr1-1* from NASC (Nottingham, UK).

Seeds of sunflower (*Helianthus annuus*) and tomato (*Solanum lycopersicum*) were purchased from Hornbach (Bornheim, Germany). Seeds of rapeseed (*Brassica napus* ‘Jumbo’), Chinese cabbage (*Brassica rapa* ‘Cantonner Witkrop’), and maize (*Zea mays*) were obtained from the Sächsisches Landesamt für Umwelt, Landwirtschaft und Geologie (Nossen, Germany).

Development of the cocultivation medium.

Different media combinations were tested for the interaction Petri dish assay. *A. thaliana* was used as plant partner. Fungal (MEAlow: malt extract at 10 g/liter, D-glucose at 10 g/liter,

peptone at 1 g/liter, and Hutner trace elements [Hutner et al. 1950] at 1 ml/liter; pH 6.1) and plant (half- and full-strength MS [Murashige and Skoog 1962], including vitamins) media with or without sucrose were combined 1:1 (Supplementary Table S1). The medium with the best performance of both interaction partners was chosen for further experiments.

Selection of a nonnatural host plant partner.

Different plants were tested for their ability as plant partner in the cocultivation assays. In addition to sunflower as a member of the family Asteraceae, we also tested (model) plants such as *A. thaliana*, tomato, rapeseed, Chinese cabbage, and maize. All of these plants were tested on the cocultivation medium, which was best suited for *A. thaliana* and *C. asteris*. Depending on the size of the plants, we used either square Petri dishes (12 by 12 cm in size) or translucent plastic vessels (9 by 10 by 7 cm in size). Seeds were sterilized as follows: 2 min in 70% ethanol with 0.1% triton X-100, followed by 10 min (sunflower, rapeseed, Chinese cabbage, and maize) or 25 min (tomato) in 1.2% sodium hypochlorite with 0.1% Triton X-100, and finished by washing seeds four to five times with sterile water. Sterile plants were inoculated with 10 μ l of *C. asteris* homogenate either below their roots (4.5 cm away from the roots in square Petri dishes) or

close to their roots (plastic vessels) within 1 week after sowing. The homogenate of *C. asteris* was from a 1-week-old culture, which was harvested by centrifugation (5,000 relative centrifugal force [rcf], 10 min, room temperature). The pellet was resuspended in fresh MEALow medium (10 ml per 1 ml of pellet) and homogenated. The Petri dishes were sealed with Parafilm (Bemis, Neenah, WI, U.S.A.) and the translucent plastic vessels were closed with a lid. The plants were cultivated in the greenhouse under long-day conditions (23°C for 14 h under light, 18°C for 8 h in the dark; light intensities between 100 and 150 μ mol s⁻¹ m⁻²) for three (Petri dishes) or four (plastic vessels) weeks. The plants best suited for in vitro assays according to their growth were chosen.

Cocultivation Petri dish assays.

All experimental setups were performed as shown in Supplementary Fig. S1. The experimental details for the different types of plates and sealings used will be separately described below.

Cocultivation in nonseparated Petri dishes. Prior to cocultivation experiments, *A. thaliana* seeds were soaked for 1 min in 70% ethanol including 0.1% Triton X-100 followed by 1 min in 1.2% sodium hypochlorite including 0.1% Triton X-100. The seeds were washed four times with sterile water to remove the chemical compounds. Seven sterile seeds were sown on nonseparated Petri dishes containing the chosen medium. After 1 week, the plates were inoculated with *C. asteris* (seven inocula) at different distances from the seedlings: 1.5, 3, and 4.5 cm (on round Petri dishes, 9 cm in diameter) as well as 1.5, 4.5, and 7.5 cm (on square Petri dishes, 12 by 12 cm in size) away from each seed. Each fungal inoculum contained the same amount of conidia (each inoculum with 10⁵). The conidia were harvested under sterile conditions from a 1-week-old culture of *C. asteris* (cultivation in MEALow at 23°C, 130 rpm in the dark) and collected after filtration over gauze (mesh size: 20 μ m) by centrifugation (5,000 rcf, 4°C, and 10 min). The conidia were resolved in a defined volume of MEALow medium for application to *A. thaliana* plates. The Petri dishes were sealed with Parafilm or MaiMed tape (MaiMed GmbH, Neuenkirchen, Germany) and cultivated under long-day conditions (23°C for 14 h under light, 18°C for 8 h in the dark; light intensities between 90 and 120 μ mol s⁻¹ m⁻²) in an upright position for 5 weeks (versatile plant growth chamber MLR-351H; Sanyo, Osaka, Japan). The phenotype of rosette growth, root length, and growth stages (Boyes et al. 2001) of *A. thaliana* was recorded weekly. After 35 days of cultivation, rosettes and roots were harvested separately, and fresh biomass and dry weight of both were determined per Petri dish. Each experiment on round Petri dishes (9 cm in diameter) was performed with 10 Petri dishes. Experiments on square Petri dishes (12 by 12 cm in size) were done in triplicate and were not statistically analyzed. The results were reproducible between individual experiments. If the amount of fresh weight was below the detection limit of the scale, plant parts or organs of two or more Petri dishes were pooled for weighing. The freeze-dried rosettes were further used for chlorophyll and anthocyanin measurements.

Cocultivation of *A. thaliana* mutants in nonseparated Petri dishes. Different mutants and overexpressing lines of *A. thaliana* (*jar1-1*, *etr1-1*, *sid2-1*, *npr1-1*, and *NahG*) were inoculated with 10⁵ conidia of *C. asteris* in the same manner as the wild-type Col-0 (discussed above). The Petri dishes were sealed with MaiMed tape and cultivated under long-day conditions for 5 weeks.

Cocultivation in nonseparated Petri dishes with autoclaved fungus. *A. thaliana* Col-0 was grown as described above but supplemented with dead spores or dead hyphae. The spores or hyphae of *C. asteris* were propagated as described above for living materials but, after resuspension in MEALow medium, they were autoclaved for 20 min at 121°C and 1.96 bar. The resulting

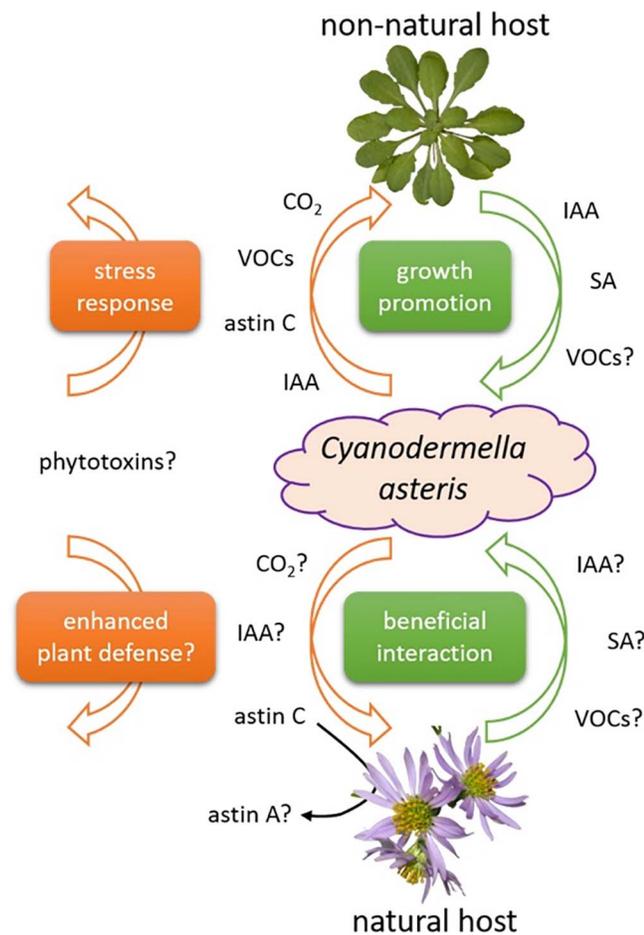


Fig. 11. Possible model of the interaction between the endophyte *Cyanoderrella asteris* and the nonnatural host plant *Arabidopsis thaliana* as well as with its natural host plant *Aster tataricus*. *C. asteris* produces different volatile organic compounds (VOCs) and soluble compounds (including astin C and indole-3-acetic acid [IAA]) to support the nonnatural host plant *Arabidopsis thaliana*. In return, *A. thaliana* can use phytohormones (including IAA and salicylic acid [SA]) to induce fungal growth. The host plant *Aster tataricus* can additionally modify astin C into astin A or B, which show a higher bioactivity.

dead spores (5 µl with 10⁵ conidia) or dead hyphae (5 µl of homogenate) were applied 7 days after sowing 1.5 cm as well as 4.5 cm below the seedlings of *A. thaliana*.

Cocultivation on bipartite Petri dishes *A. thaliana* and *C. asteris*. Bipartite Petri dishes containing the cocultivation medium were set up as follows: 1 week after sowing three *A. thaliana* seeds, *C. asteris* was inoculated in the second compartment with three inocula of each 10⁵ conidia and subsequently spread uniformly over the compartment. Conidia were obtained from a 1-week-old *C. asteris* culture (discussed above). The bipartite Petri dishes were sealed either with Parafilm (20 Petri dishes) or MaiMed tape (19 Petri dishes) and cultivated as above. The plants were monitored weekly and harvested after 35 days (discussed above).

Cocultivation on tripartite Petri dishes *A. thaliana* and *C. asteris*. Tripartite Petri dishes containing the cocultivation medium were set up as follows. One compartment contained five *A. thaliana* seeds and, after 1 week, four inocula of each 10⁵ conidia of *C. asteris* were applied to the second compartment. The third compartment contained 7 ml of 0.1 M Ba(OH)₂ for the absorption of CO₂ (Kai and Piechulla 2009). The Petri dishes were sealed with Parafilm (12 Petri dishes) and placed horizontally under long-day conditions (discussed above). The same parameters were determined as above. In addition, the amount of BaCO₃ was determined after harvest (35 days after sowing). The dry weight of BaCO₃ was measured after filtration (folded filters, Ø 150 mm, 84 g/m², grade 1288; Sartorius Stedim Biotech, Göttingen, Germany) and drying at 60°C (incubator Heraeus Function Line T12; Heraeus, Hanau, Germany) for 1 week.

Permeability of sealing. The CO₂ permeability of the different sealings (Parafilm and MaiMed tape) was tested both in the compartment with *C. asteris* and in that with *A. thaliana*. One compartment contained the cocultivation medium, the other 10 ml of 0.1 M Ba(OH)₂ for complexing CO₂. The permeability was tested with *C. asteris* as well as *A. thaliana*. Three inocula of *C. asteris* (10⁵ conidia each inocula) or three seeds of *A. thaliana* were placed on the agar. The control Petri dishes contained no organism. Petri dishes of all three conditions were sealed in three different ways: no sealing (open system) or MaiMed tape or Parafilm (closed system). The amount of BaCO₃ was determined after 28 days. At this time, Petri dishes without any sealing were dried out and could not be used as control. The results of the remaining Petri dishes were statistically analyzed using a Welch analysis of variance (ANOVA) with treatment (organism and sealing) as between-subject factors. A posthoc test was performed to show the differences between the groups and the effect size partial η square (η_p²) was determined.

Extraction and detection of anthocyanins and chlorophylls from plants.

Freeze-dried plant material (rosettes) was ground with pestle and mortar and extracted with either 3 ml of alkaline methanol (pH 8.5) for chlorophylls or 3 ml of acid methanol (pH 1) for anthocyanins. Depending on the biomass, plants of one or more Petri dishes were combined and extracted together. The extract was incubated for 5 min prior to centrifugation (12,000 rcf, 4°C, 10 min). The supernatant was directly used in photometric measurements. The absorbance was measured at 645 and 663 nm for chlorophylls and at 530 and 657 nm for anthocyanins.

The chlorophyll concentration was determined according to formula I to III (Amon 1949):

$$c(\text{chlorophyll } a) [\text{mg/liter}] = 12.7 \times A_{663\text{nm}} - 2.69 \times A_{645\text{nm}} \quad (\text{I})$$

$$c(\text{chlorophyll } b) [\text{mg/liter}] = 22.9 \times A_{645\text{nm}} - 4.68 \times A_{663\text{nm}} \quad (\text{II})$$

$$c(\text{chlorophyll total}) [\text{mg/liter}] = c(\text{chlorophyll } a) + c(\text{chlorophyll } b) \quad (\text{III})$$

The chlorophyll concentrations were calculated according to the extraction volume and the fresh weight (FW) of leaves (formula IV):

$$c(\text{chlorophyll}) [\mu\text{g/g}] = \{c(\text{chlorophyll}) [\text{mg/liter}] \times 1,000 [\mu\text{g/mg}] \times \text{extraction volume} [\text{liter}]\} / \text{FW} [\text{g}] \quad (\text{IV})$$

The anthocyanin concentration was calculated according to Rabino and Mancinelli (1986), which uses a correction factor of 0.25. The correction factor compensates the absorption of chlorophyll and its degradation products at 530 nm (Mancinelli and Schwartz 1984) (formula V).

$$\text{anthocyanin absorption} = A_{530\text{nm}} - 0.25 \times A_{657\text{nm}} \quad (\text{V})$$

An anthocyanin equivalent was defined according to Teng et al. (2005) where the product of formula V with the extraction volume is one unit. The anthocyanin equivalents (unit) were related to the FW of leaves to compare the different treatments (formula VI).

$$\text{anthocyanin content} [\text{unit/g}] = (\text{anthocyanin absorption} \times \text{extraction volume}) / \text{FW} [\text{g}] \quad (\text{VI})$$

Extraction and detection of astins from Petri dishes.

Freeze-dried medium of Petri dishes was ground with pestle and mortar and extracted with 100% methanol for 30 min on a shaker. Afterward, the solution was filtered to remove the solid particles from the solution. The methanol was vacuum dried in a Multivapor P-12 (Büchi, Essen, Germany). The resulting dried components were resolved in 50% methanol and measured by high-performance liquid chromatography (HPLC) mass spectrometry.

The samples were analyzed on a Shimadzu HPLC system (Shimadzu, Canby, OR, U.S.A.) with a ProntoSIL 120-5 C18 AQ 250 × 3 mm (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) and a Chromolith SpeedROD RP-18e 50 to 4.6 mm as precolumn. The astins were separated with an isocratic system (flow 0.7 ml/min) of 40% acetonitrile plus 0.06% formic acid and 60% water plus 0.1% formic acid. The HPLC was coupled to an AB Sciex API 5000 Turbo-Ion-Spray triple quadrupole tandem mass spectrometer equipped with Electrospray Ionization (ESI) Source (AB Sciex, Foster City, CA, U.S.A.). The system was controlled by AB Sciex Analyst software (version 1.5.1). Due to a lack of internal standards for astins, only an estimation of the astin amount by area units was possible. A standard curve (0.1 to 10 ng/ml) for astin C was done to link the arbitrary unit to an astin C concentration.

Growth of *A. thaliana* on different astin concentrations.

A. thaliana was cultivated in Petri dishes containing MS medium (MS including vitamins at 4.4 g/liter, sucrose at 10 g/liter, and phytoagar at 10 g/liter; pH 5.8 ± 0.1) (Murashige and Skoog 1962), including different concentrations of astins A, C, (both dichlorinated), or G (nonchlorinated). Seven plants were sown per Petri dish (10 Petri dishes per treatment). The growth of plants was documented weekly as in the cocultivation system (root length and growth stage). After 35 days of cultivation under long-day conditions, the plants were harvested and the biomass was determined.

Plate medians of root length and leaf biomass of *A. thaliana* treated with different astins were statistically analyzed using a one-way ANOVA (equal sample sizes and variance homogeneity) or Welch-ANOVA (no variance homogeneity), with treatment group (concentration) included as an eight-level (astins A and C) or seven-level (astin G) within-subject factor (*n* = 10). Posthoc tests were performed to specify the differences. The growth stages 35 days after cultivation were analyzed using a Kruskal-Wallis ANOVA (*n* = 62 to 70), with treatment group (concentration) as an eight-level (astins A and C) or seven-level (astin G) within-subject factor. A posthoc test (Dunn's) was performed to show the

differences. Leaf and root biomass were statistically analyzed using a one-way ANOVA or Welch-ANOVA, with concentration included as a within-subjects factor ($n \geq 10$) or with a Kruskal-Wallis ANOVA ($n < 10$). Posthoc tests were performed to show the differences between the concentrations. Effect sizes were calculated for significant differences using η^2 (Lenhard and Lenhard 2016) or η_p^2 .

Growth and astin production of *C. asteris* with phytohormones.

C. asteris was cultivated in liquid MEAlow medium (Schafhauser et al. 2019) with different phytohormones (auxins: NAA and IAA; stress hormones: SA and JA) at different concentrations (10 pM to 1 μ M, dissolved and diluted in methanol or water). IAA is very sensitive to light, temperature, and salty conditions and not stable enough in medium for a long-term experiment (Dunlap et al. 1986; Nissen and Sutter 1990). Therefore, the natural auxin IAA and the synthetic auxin NAA were tested.

Liquid medium (40 ml) was inoculated with 200 μ l of homogenate of *C. asteris* (discussed above). The fungus was cultivated for 1 week on a shaker at 23°C and 130 rpm in the dark. The liquid medium was centrifuged (5,000 rcf, 4°C, 10 min) to remove the mycelium. The supernatant (medium) was extracted with the same volume of ethyl acetate for 30 min on a rotary shaker. The phases were separated by centrifugation (5,000 rcf, 4°C, 10 min) and the upper phase was vacuum evaporated. The resulting sediments were resolved in 1 ml of 50% methanol and measured by HPLC mass spectrometry (discussed above). The pellet (mycelium) was freeze dried and the fungal biomass was determined.

Statistical analyses.

Statistical analyses were generally carried out using OriginPro 2019 version 9.6.0.172 (OriginLab Corporation, Northampton, MA, U.S.A.). Statistically significant differences are given in the figures and respective legends as well as in Supplementary Tables S2 to S10.

For analyses of growth parameters, it has to be considered that the plants within one Petri dish interact with each other through the Petri dish atmosphere. They influence the growth of other plants in the Petri dish. To show a nonindependence of plant interactions statistically, medians of root length, which were measured for each plant, were calculated for each Petri dish separately and used for further statistical analyses. All other growth parameters did not show such a dependence, because they were determined per Petri dish and not per plant. The growth parameters root length, leaf and root biomass, and chlorophyll and anthocyanin content with two groups (control and + *C. asteris*) were statistically analyzed using an unpaired *t* test ($n \geq 10$) or a Mann-Whitney *U* test ($n < 10$). Growth parameters with more than two groups (control; 1.5, 3.0, or 4.5 cm; or control \pm *C. asteris* \pm Ba(OH)₂) were analyzed using a Welch-ANOVA (to allow for a different sample size [*n*]) with treatment as a between-subjects factor ($n \geq 10$) or a Kruskal-Wallis test with treatment as a within-subjects factor for smaller sample sizes ($n < 10$). The growth stages were analyzed using the nonparametric Mann-Whitney *U* test (two groups: control and + *C. asteris*) or the Kruskal-Wallis test (more than two groups: control; 1.5, 3.0, or 4.5 cm; or control \pm *C. asteris* \pm Ba(OH)₂). Variance homogeneity was checked with a Levene's test. Effect sizes were calculated using Cohen's *d* (Lenhard and Lenhard 2016) or η_p^2 .

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