

## DISEASE SYMPTOMS AND MINERAL NUTRITION IN *ARABIDOPSIS THALIANA* IN RESPONSE TO *VERTICILLIUM LONGISPORUM* VL43 INFECTION

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### SUMMARY

*Verticillium longisporum* is one of the most important pathogens of Brassicaceae. The goal of the present study was to characterize the development of *V. longisporum* (strain VL43) stress symptoms caused to *Arabidopsis thaliana* in relation to plant nutrition. Significant growth inhibition became apparent between 2 and 3 weeks after VL43 inoculation but was not accompanied by reduced leaf water content or wilting. The leaves of VL43-infected plants showed reduced chlorophyll content (-20%) but only marginal effects on photosynthetic electron transport of PS II (-2%) compared with controls. VL43-infection did not have negative effects on nutrient element concentration in leaf tissues during a five-week observation period. Measurements of fungal nutrient content in simulated xylem medium suggested that nutrient intake by VL43 was negligible compared to that of plant tissues. Therefore, growth retardation induced by VL43 infection is not a consequence of nutrient depletion.

**Key words:** Brassicaceae, chlorosis, nutrients, photosynthesis, soil pathogen, stunting.

### INTRODUCTION

*Verticillium* species are soil-borne fungi with worldwide distribution, causing vascular diseases in many plant species including a vast range of economically important crops and trees. *V. dahliae* is probably the most frequent and widespread pathogen. However, *V. longisporum*, which occurs predominately in crucifers (Zeise and Tiedemann, 2002), is one of the most important disease agents of oilseed rape in Europe, whereas *V. dahliae* is not, or little pathogenic to *Brassica napus* (Karapapa *et al.*, 1997; Eynck *et al.*, 2007). *V. longisporum* has probably evolved by parasexual hybridization between a strain of *V. albo-atrum* and a strain of *V.*

*dahliae* (Karapapa *et al.*, 1997). Typical disease symptoms of *Verticillium* infections are wilting, stunting, chlorosis and premature senescence (Fradin and Thomma, 2006).

All crucifer isolates of *Verticillium* produce thick-walled melanised microsclerotia which can survive for more than a decade in the soil. Their germination is stimulated by root exudates following which the fungus penetrates host roots and spreads systemically in the xylem (Fradin and Thomma, 2006). Until the very late stages of infection, *Verticillium* species remain strictly xylem-localized. Spreading in the xylem results in partial vessel occlusion by deposits secreted by neighbouring parenchyma cells (Benhamou, 1995). These obstructions may block transport through the vessels, thus delaying the fungus movement. Since obstructions can be expected to affect water and nutrient transport, disease symptoms such as wilting, stunting and chlorosis have been suggested to occur as the consequence of nutrient limitations or drought stress due to reduced water transport in the xylem (Johansson *et al.*, 2006). In fact, young, still symptomless leaves of potato plants infected by *Verticillium dahliae* displayed lower carbon assimilation than non-infected plants as the result of stomatal limitations (Bowden *et al.*, 1990). However, the effect was age-dependent and affected only some leaf areas (Saeed *et al.*, 1999). Data on plant nutritional status during *Verticillium* sp. infections are scarce (Karagiannidis *et al.*, 2002; Floerl *et al.*, 2008). To understand disease symptoms, which may lead to massive yield loss, it is important to know whether this pathogen compromises vital functions such as plant mineral nutrition and photosynthesis.

The goal of the present study was to provide a comprehensive characterization of the development of *Verticillium*-induced stress symptoms during the growth of *Arabidopsis* and to find out whether these symptoms were caused by effects on nutrition and photosynthesis. For this purpose, growth reduction, chlorosis, photosynthetic electron transport, and changes in macro- and micro-nutrients were measured in *Arabidopsis thaliana* (ecotype Col-0) infected with *V. longisporum* strain VL43 from *Brassica napus* (Zeise and Tiedemann, 2002). Since VL43 present in leaves also sequesters nutrients, we determined optimum fungal nutrition in a synthetic

medium and used these data to assess the upper limit of nutrients incorporated by the pathogen.

## MATERIALS AND METHODS

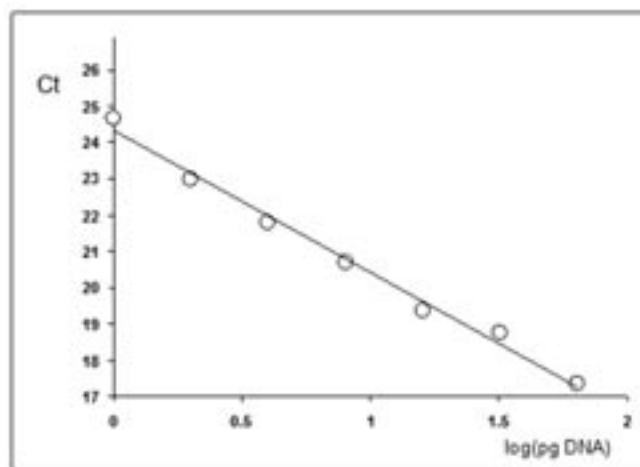
***Arabidopsis* growth conditions.** Seeds of *Arabidopsis thaliana* (ecotype Columbia-0) were sown on MS-medium (Murashige and Skoog, 1962) and stored for 3 days at 4°C, then transferred to 20°C, 60 % relative humidity, and a light intensity of 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation with an 8 h light/16 h dark cycle. After four weeks, seedlings were used for infection.

**Fungal culture.** Stock cultures of *V. longisporum* were stored in potato dextrose medium (PDB, Scharlau, Spain) with 20% glycerol at -80°C. For sporulation, the cultures were grown on potato dextrose agar at 22°C for 4 weeks, then transferred to 250 ml liquid potato dextrose medium (Scharlau, Spain) with 0.2 mg ml<sup>-1</sup> streptomycin sulphate (Sigma, Germany) as described by Fahleson *et al.* (2003). The culture was incubated at 22°C with horizontal shaking (80 rpm) for 14 days in darkness. The fungal suspension was filtered and centrifuged for 10 min at 900 g. The pellet was diluted to 2x10<sup>6</sup> conidia/ml and used for inoculation.

**Infection of *Arabidopsis* with *V. longisporum* and assessment of disease symptoms.** Four-week-old *Arabidopsis* plants were removed from the agar and their root tips were cut. During planting, the roots were placed into a small hole in the soil and were drenched with 10 ml of *V. longisporum* suspension containing 2x10<sup>6</sup> conidia/ml. Control plants were treated in the same way and mock-inoculated with sterile water. The plants (40 per treatment and experiment) were grown under the same conditions specific above, were watered with tap water and fertilized twice a week with Wuxal (Aglucon, Germany).

Consequences of infection were monitored measuring the leaf area development of the plants. For this purpose, the rosettes of all plants were photographed regularly (Digital-Camera: Casio QV R52) for 35 days post inoculation and the projected area of each rosette was recorded using *Image J* (<http://rsb.info.nih.gov/ij/index.html>). During the experimental period whole rosettes were harvested regularly (n=6 plants per sampling date) and stored at -80°C for further analysis.

**Quantification of *Verticillium longisporum* DNA in plant tissue.** Leaves were ground in liquid nitrogen to a fine powder and DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany). The amount of fungal DNA was determined by real-time PCR with the *Verticillium* sp.-specific primers 5'-CAGCGAAACGC-GATATGTAG-3' and 5'-GGCTTGTAGGGGGTT-TAGA-3' (Eynck *et al.*, 2007). Calibration curves were



**Fig. 1.** Real-time PCR assay for *Verticillium longisporum*. Calibration curves were constructed by plotting threshold cycle values (Ct) against the amount of template DNA as described (Eynck *et al.*, 2007).

constructed with purified fungal DNA dissolved in a solution of plant DNA extracted from non-infected plants (matrix standards, Fig. 1).

**Determination of specific DNA content of *V. longisporum* mycelium.** *V. longisporum* was grown in simulated xylem sap (Neumann and Dobinson, 2003) for up to 28 days at 22°C without shaking. Mycelium was harvested by filtration, rinsed briefly with tap water and freeze-dried. DNA was extracted using the CTAB protocol (Brandfass and Karlovsky, 2006). The DNA preparation obtained was diluted 100- to 500-fold and the content of *V. longisporum* DNA was determined by real-time PCR (Eynck *et al.*, 2007) using preparations of pure fungal DNA as standards. To assess the completeness of fungal DNA extraction, the pellet obtained after centrifugation of mycelium lysate and the interlayer formed after chloroform/isopropanol extraction were re-extracted with buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and checked for DNA presence by agarose electrophoresis. No detectable DNA was found, showing that the extraction of DNA from fungal mycelium was complete.

**Chlorophyll fluorescence.** Chlorophyll fluorescence was measured in ambient light (120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetic active radiation, 20°C, 60% relative air humidity) and on dark-adapted leaves using the Mini PAM system (Walz, Germany). The quantum yield of photochemistry was calculated as described by Maxwell and Johnson (2000):

$$\phi = (F_m - F_o) / F_m$$

with  $F_m$  and  $F_o$  referring to maximum and background fluorescence, respectively, in darkness and with

$$\phi_{(acute)} = (F'_m - F'_l) / F'_m$$

with  $F'_m$  and  $F'_l$  referring to maximum and background fluorescence in the light, respectively. Initially 40 plants

per treatment were measured. At each date six plants were removed for biochemical analysis, resulting in decreasing “n” during the time course of the experiment. At the final date n = 6 plants were measured per treatment.

**Chlorophyll concentration.** Frozen leaf material was milled in liquid nitrogen and extracted in 80% acetone. After centrifugation (10,000 g, 10 min) the pigment concentration of the supernatant was determined spectrophotometrically (Beckman DU 640) at 646 nm and 663 nm. The chlorophyll concentration was determined as the sum of chlorophyll a and b (Lichtenthaler and Wellburn, 1983).

**Water content and nutrient elements.** Leaves were harvested, weighed and dried at 60°C. The water content was determined as (fresh mass–dry mass)<sub>100</sub>/fresh mass. In addition to plant tissues, freeze-dried fungal tissues obtained after growth in simulated xylem medium (see above) were used for nutrient analyses. The dry material was milled to a fine powder (MM2 Retsch, Germany). Aliquots of the powder were extracted with HNO<sub>3</sub> (Heinrichs *et al.*, 1986) and subjected to inductively coupled optical emission spectroscopy (Spectroflame, Spectro Analytical Instruments, Germany). For carbon and nitrogen, leaf powder was weighed into tin capsules and measured in a CNS-elemental analyser (Carlo Erba Instruments, Italy).

To assess nutrient partitioning between plant and *V. longisporum* in infected leaves, we calculated the amount of an element ( $E_f$ ) per fungal DNA<sub>f</sub> for pure fungal cultures:

$$E_f/\text{DNA}_f \text{ (mg } \mu\text{g}^{-1}) = (\text{mg of } E_f/\text{g of fungal dry mass})/(\mu\text{g of DNA}_f/\text{g of fungal dry mass}).$$

This calculation was conducted at each harvest date, yielding three mean values for each element. These data were used to calculate the relative fraction of E associated with the fungus in infected leaves:

$$\text{Fraction of } E_f \text{ of total E in infected leaves (\%)} = \frac{[(E_f/\text{DNA}_f) \times \text{DNA}_f \text{ in plant} \times 100]}{(\text{mg of total E/g of leaf dry mass})}.$$

Element and fungal DNA amounts in *Arabidopsis* were used for the harvest dates 21, 28, 35 days post inoculation yielding, with three values for  $E_f/\text{DNA}_f$ , a total of nine estimates per element, which were presented as box plots to show the range covered by these assessments.

**Osmotic pressure.** Aliquots of dry leaf powder were extracted with distilled water. After centrifugation at 900g for 25 min the osmolality (osmol kg<sup>-1</sup>) of the supernatant was determined in a Cryo-osmometer (Osmomat 030, Ganotec, Germany). The osmotic pressure (MPa) was calculated as:

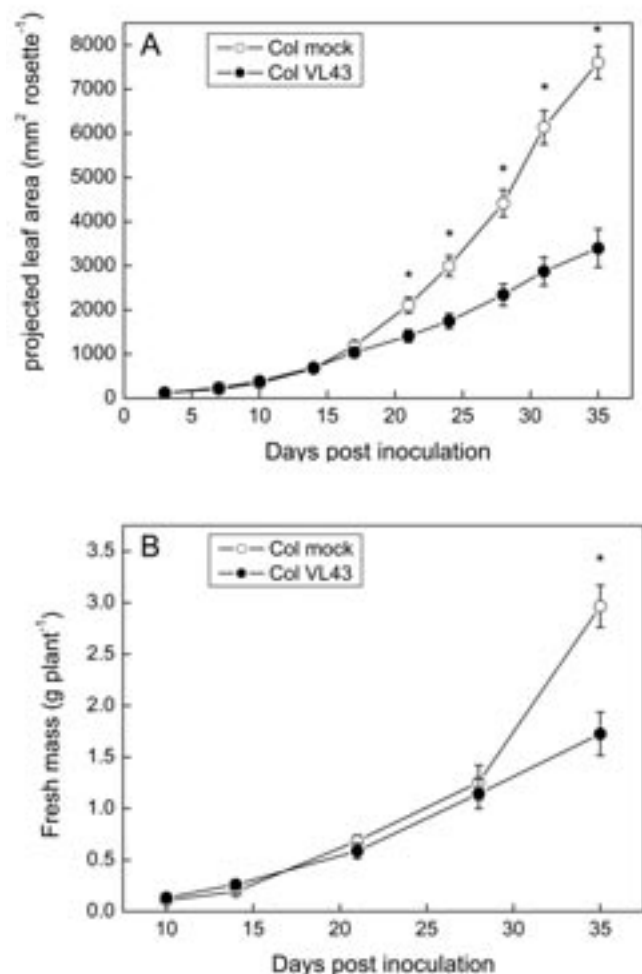
$$\text{osmolality}_{(\text{supernatant})} \times \text{dilution factor} \times R \times T$$

with  $R = 0.00831 \text{ (L}\times\text{MPa} \times \text{mol}^{-1}\text{K}^{-1})$  and  $T = 295 \text{ K}$  (absolute temperature).

**Statistical analyses.** Data are shown as means. The number of biological replicates was n = 6, if not indicated differently in the figure legends. Statistical analysis (ANOVA, multiple range test) was performed using Statgraphics (Centurion XV, USA).

## RESULTS

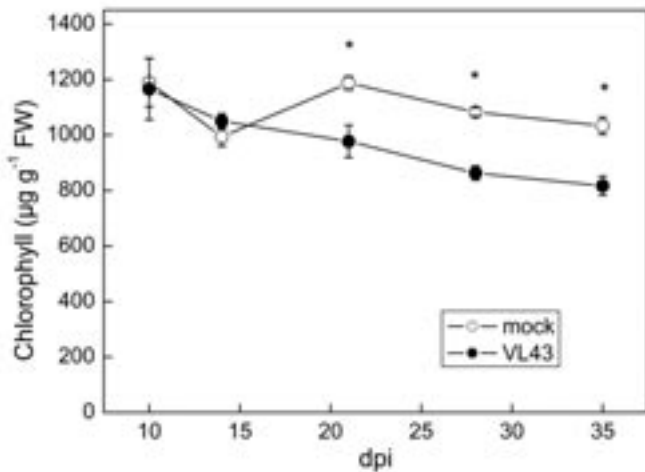
***Verticillium* infection causes growth reduction, chlorosis of leaf tissues, affects PSII activity but not plant water relations.** Infection with *V. longisporum* VL43 caused a drastic reduction in projected rosette area compared with controls (Fig. 2 A,B). The difference in time course between expansion and fresh mass was mainly caused by the fact that the VL43 coloniza-



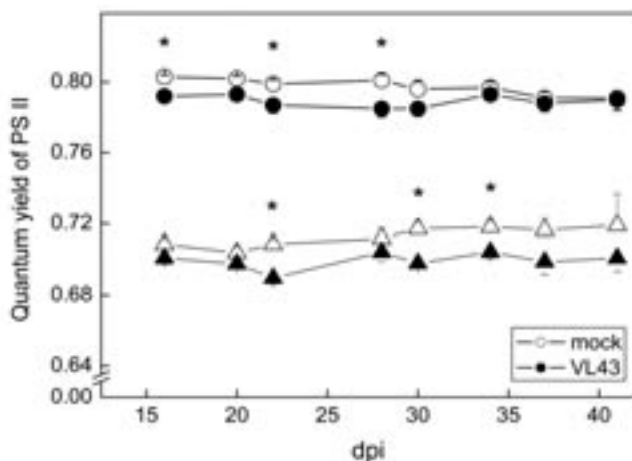
**Fig. 2.** Development of projected rosette area (A) and fresh mass of rosette (B) after infection with *Verticillium longisporum* and of mock-inoculated *Arabidopsis thaliana* plants. The projected leaf area and fresh mass was determined per plant. Data are means (n = 6, ± SE). Stars indicate significant differences between treatments at P ≤ 0.05.

tion initially resulted mainly in stunting of *Arabidopsis* plants because petiole elongation was severely inhibited.

In both controls and VL43-infected plants, the amount of chlorophyll declined with plant age; however, this effect was more pronounced in infected plants (Fig. 3). Overall, on a fresh mass basis infected plants contained 20% less chlorophyll than non-infected plants. Quantum yield of photosystem II ( $\phi$ ) was determined to obtain information on photosystem II activity. Very small but significant reduction in  $\phi$  was found in response to VL43 infection in ambient light (-2%) and in darkness (-1%, Fig. 4). The influence of VL43 infec-



**Fig. 3.** Chlorophyll content in leaves of *Arabidopsis thaliana* after infection with *Verticillium longisporum* and in mock-inoculated controls. Data are means ( $n = 6$ ,  $\pm$  SE). Stars indicate significant differences between treatments at  $P \leq 0.05$ .



**Fig. 4.** Chlorophyll fluorescence of leaves of *Arabidopsis thaliana* after infection with *Verticillium longisporum* and of mock-inoculated controls. The maximum quantum yield of photosystem II was measured on dark-adapted leaves (circles) and the acute quantum yield was measured in ambient light ( $120 \mu\text{mol m}^{-2}\text{s}^{-1}$  of photosynthetic active radiation) (triangles). Stars indicate significant differences between treatments at  $P \leq 0.05$ .

**Table 1.** Water relations in *Arabidopsis thaliana* leaves. Data are means of  $n = 8$  ( $\pm$  SE) plants measured at 25 days post inoculation after infection with *Verticillium longisporum* strain 43.

	Mock-inoculated	VL43-infected
Relative water content (%)	91.7 $\pm$ 0.7	93.3 $\pm$ 0.8**
Osmotic pressure (MPa)	0.893 $\pm$ 0.014	0.843 $\pm$ 0.018*

Significant differences between treatments are marked as \* ( $P \leq 0.05$ ) and \*\* ( $P \leq 0.01$ ).

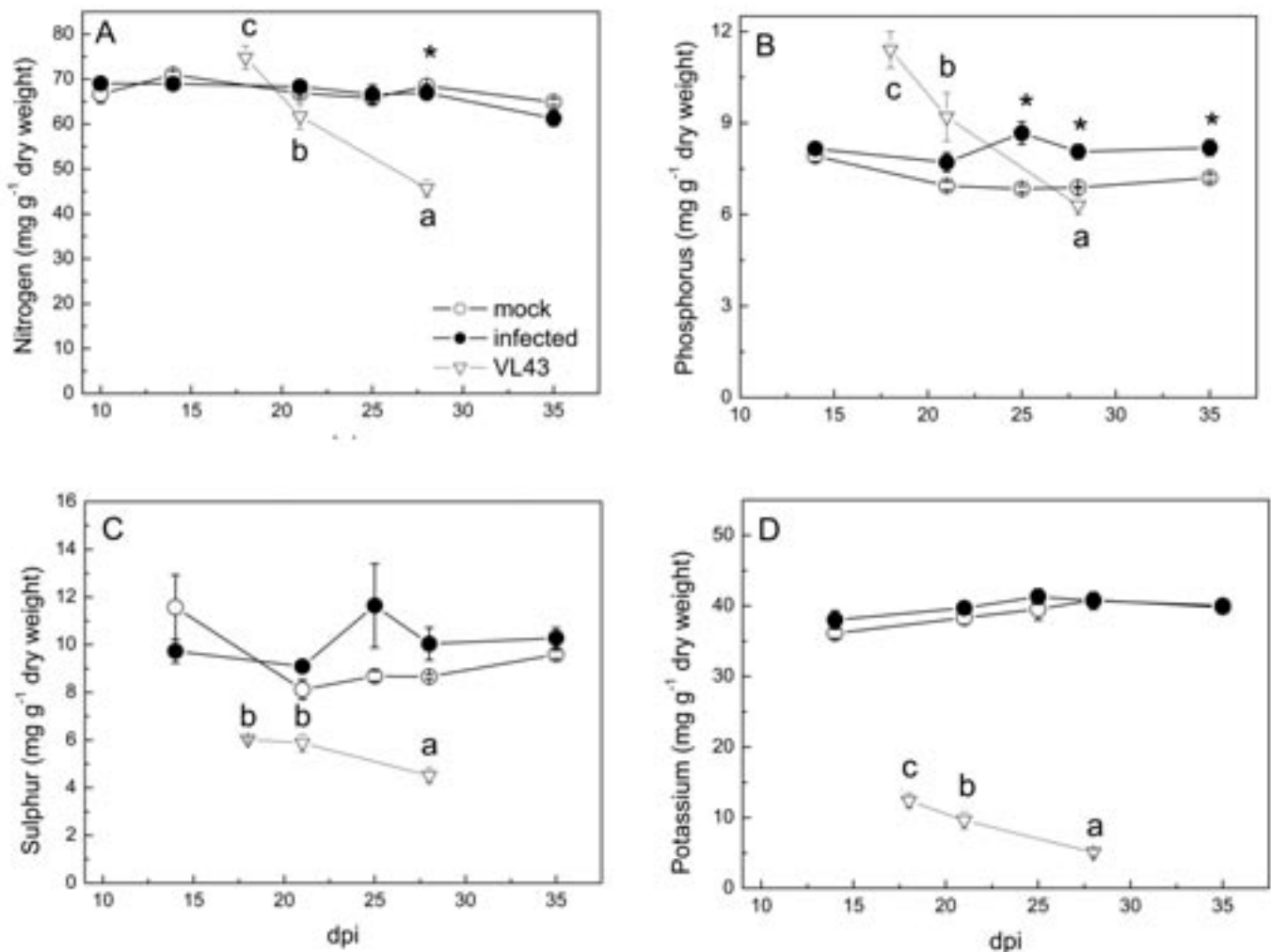
tion on PSII activity was, thus, much lower than that on chlorophyll content. Neither effect was detectable in old plants.

To find out whether the water relations were disturbed, we determined leaf water content and osmotic pressure at 25 days post inoculation. No evidence of drought stress was obtained since VL43-infected plants contained slightly more water and osmotic pressure was lower than in non-infected plants (Table 1).

**Verticillium does not have any negative effects on Arabidopsis nutrient relations.** Concentrations of macro- and micro-nutrients in the leaves were measured regularly after VL43 infection of *Arabidopsis*. Among the major nutrient elements P, N, K, and S, no pronounced age-dependent nor VL43-induced decreases were observed (Fig. 5A-D). The C/N ratio of the leaves was also unaffected and amounted 5.4 throughout the whole time course of the experiment (data not shown). In VL43-infected plants the concentration of phosphorus was higher than in non-infected plants (Fig. 5B). Other nutrient elements showed small and inconsistent changes in response to VL43. Calcium was accumulated with increasing age to an about 10% higher concentration than that of controls (Fig. 5E). The magnesium concentration of infected plants was initially lower than that of non-infected plants, but this difference disappeared three weeks post infection (Fig. 5F), when stunting symptoms became pronounced (Fig. 2A). The manganese content fluctuated with age ranging from 90 to  $150 \mu\text{g g}^{-1}$  dry mass (Fig. 5G). Although the time courses of these fluctuations in infected and non-infected plants were different, the amplitudes were similar (Fig. 5G). The iron concentration decreased until 4 weeks post infection. There were no differences between infected and non-infected plants (Fig. 5H).

When the element concentrations in VL43 mycelium grown in xylem sap-simulating medium were determined, the content of N, P, S, K, and Mn decreased during cultivation, whereas Mg increased (Fig. 5). Young fungal mycelium contained higher concentrations of P than *Arabidopsis* leaves (Fig. 5B). The concentrations of most other elements were lower in VL43 than in *Arabidopsis* (Fig. 5).

To assess the fraction of elements present in VL43 in

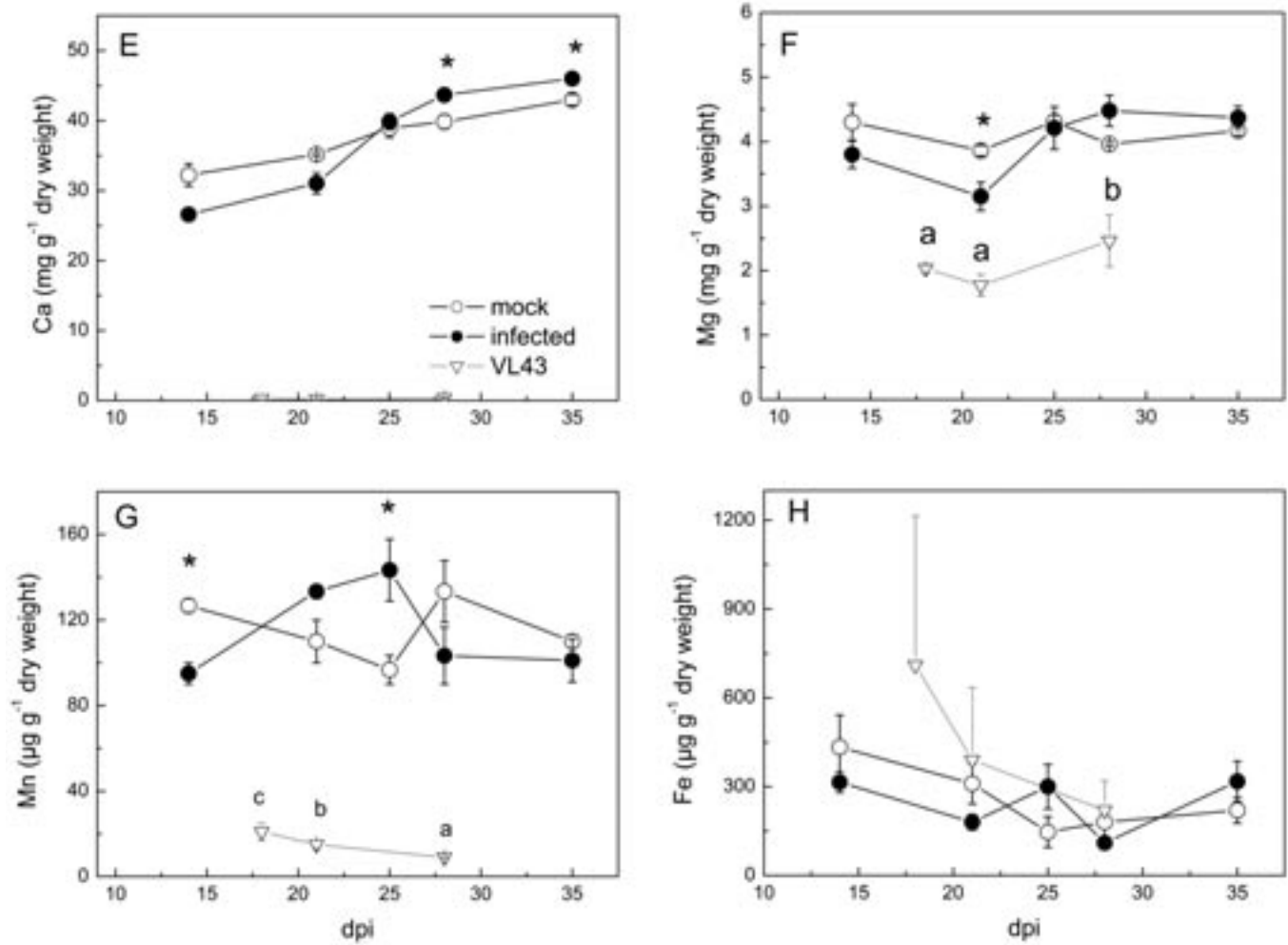


**Fig. 5A-D.** Nitrogen (A), phosphorus (B), sulphur (C) and potassium concentrations (D) in the leaves of *Arabidopsis thaliana* after infection with *Verticillium longisporum* (full circles) and in mock-inoculated controls (open circles) and in fungal tissues grown in pure culture (open triangles). Data are means ( $n = 6$ ,  $\pm$  SE). Stars indicate significant differences between treatments at  $P \leq 0.05$  for plants and letters indicate differences due to increasing culture time in *V. longisporum*.

infected leaves and thus not available for the plant, we determined the amount of fungal DNA in fungal tissues grown in simulated xylem medium as well as in plant leaves (Fig. 6). The amount of fungal biomass in infected plant leaves contributed approximately 0.001% to their dry mass. With this figure and the known nutrient content of fungal mycelium, the fraction of mineral nutrients sequestered by the fungus within plant tissue was estimated (Fig. 7). The mean fractions of nutritional elements present in the fungus in infected leaves were  $0.0008 \pm 0.0003\%$  of the total leaf contents, except for iron whose fractions was slightly higher (0.006%). Even if we assume that fungal nutrient demand *in planta* exceeded the demand of mycelium grown *in vitro* by one or two orders of magnitudes, the effect of fungal consumption of mineral nutrients on the plant would be negligible. Therefore, we found no evidence that fungal infection caused nutrient deficiencies in *Arabidopsis*

## DISCUSSION

In the present study we documented that the growth reduction in *Arabidopsis*, which became very pronounced between two and three weeks post inoculation with *V. longisporum*, was not caused by foliar nutrient deficiencies but was mainly related to decreased elongation of the petioles. This finding does not support the hypothesis that insufficient supply with nitrogen or other major nutrients might have affected plant growth. This agrees with previous findings showing that VL43 infection did not cause nutrient deficiencies in oilseed rape (Floerl *et al.*, 2008). One might argue that in infected leaves the fungus may bind significant amounts of nutrients and that despite high total foliar nutrient concentrations the availability for the plant may be lim-



**Fig. 5E-H.** Calcium (E), magnesium (F), manganese (G) and iron concentrations (H) in the leaves of *Arabidopsis thaliana* after infection with *Verticillium longisporum* (full circles) and in mock-inoculated controls (open circles) and in fungal tissues grown in pure culture (open triangles). Data are means ( $n = 6$ ,  $\pm$  SE). Stars indicate significant differences between treatments at  $P \leq 0.05$  for plants and letters indicate differences due to increasing culture time in *V. longisporum*.

ited. To obtain an estimate for the amount of nutrients sequestered by the fungus in the leaf tissue, we determined the content of mineral elements in fungal mycelium grown in medium with a composition similar to xylem sap. Although culture conditions *in vitro* differ from the situation *in planta*, the data provide a reasonable estimate for the maximum amounts of mineral nutrients withheld off the plant by the fungus.

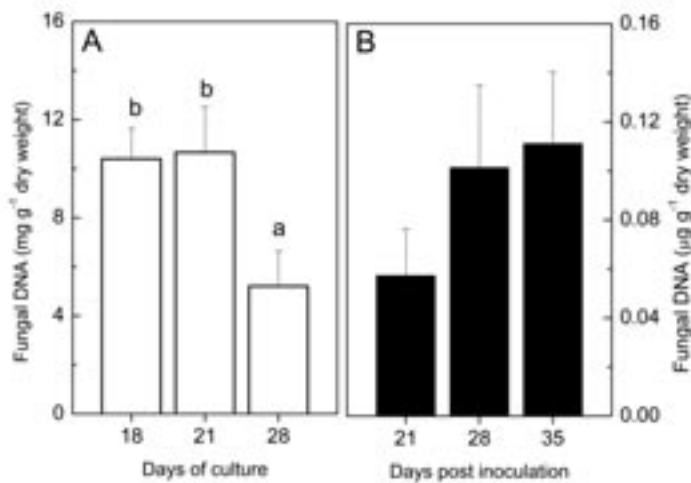
Nitrogen deficiencies have been shown to cause chlorosis in *Arabidopsis* (Hanaoka *et al.*, 2002). Since there was no evidence that fungal proliferation sequestered a significant fraction of nitrogen, N limitation was not the cause of chlorophyll loss observed during *Verticillium* infection.

Potassium acts in plants as a major osmolyte and is critically involved in cell expansion growth (O'Toole *et al.*, 1980). Our data do not support K deficiency as a cause of stunting of VL43-infected plants.

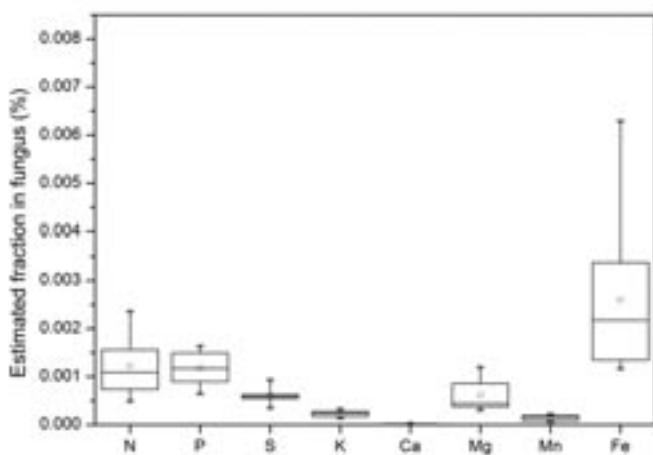
Surprisingly, VL43-infected plants displayed in-

creased phosphorus concentrations. Since this effect was also observed in tomato and eggplants infected with *V. dahliae* (Karagiannidis *et al.*, 2002), it seems that *Verticillium* infection influences the metabolism of phosphorus. Notably, microarray analysis revealed induction of the inorganic phosphate transporter gene *LePT2* in *V. dahliae*-infected tomato roots (Van Esse *et al.*, 2009). Whether this effect is relevant for fungal infection or plant physiology is unknown. However, it has been shown that plants well supplied with nutrients, especially nitrogen and phosphorus, are more susceptible to *V. dahliae* infection than starved plants (Sivaprakasam and Rajagopalan, 1972), whereas iron deficiency increases *Verticillium* infection of tomatoes (Macur *et al.*, 1991). These reports underline the importance of interactions between fungal infection and plant nutritional status, but our data do not support that *Verticillium* infection itself reduces plant nutrition.

It is possible that the observed chlorophyll loss was



**Fig. 6.** Changes of fungal DNA during cultivation in artificial medium (A) and in *Arabidopsis thaliana* leaves (B). Data are means ( $n = 4$  for the fungal cultures and  $n = 6$  for plant tissues,  $\pm$  SE). Significant differences at  $P \leq 0.05$  are indicated by letters.



**Fig. 7.** Assessment of element partitioning in infected leaves of *Arabidopsis thaliana* between *Verticillium longisporum* and plant tissue. All possible combinations of element concentrations per fungal tissue and plant were calculated and are shown as box plots with maximum and minimum values (x) and mean (open square) and median (horizontal line in box).

the result of phytotoxin action. Evidence for the role of phytotoxins in *Verticillium*-plant interactions has been provided by numerous studies (Fradin and Thomma, 2006). For example, Mansoori *et al.* (1995) isolated a heat stable toxin (MW < 1000) from the culture fluid of *V. dahliae*, which caused chlorosis and necrosis of leaf discs. Others reported that a protein-lipopolysaccharide complex produced by *Verticillium* sp. acted as phytotoxin (Fradin and Thomma, 2006). Since the toxins were isolated from culture fluids, it is unclear whether they are also formed in infected plants.

Phytotoxin production by *V. longisporum* has not been reported. Fungal phytotoxins often disrupt the in-

tegrity of photosynthetic electron transport (Kshirsagar *et al.*, 2001). Our data indicate that the loss in chlorophyll had only small impact on the quantum yield of PSII. The slightly stronger decrease of quantum yield in light than in darkness in VL43-infected plants might have been the result of small effects on stomatal opening as reported by others (Bowden *et al.*, 1990; Saeed *et al.*, 1999; Sadras *et al.*, 2000) and was not indicative for direct injury of PSII or CO<sub>2</sub> assimilatory enzymes. In fact, microarray analysis of *V. dahliae* responsive genes in tomato revealed increased transcript abundance of several genes involved in photosynthesis (Robb *et al.*, 2007). These data contrast those of Veronese *et al.* (2003) who showed significant loss in Rubisco transcripts in *Arabidopsis* infected with *V. dahliae*. Although *V. dahliae* is non-pathogenic to several cultivars of oilseed rape (Karapapa *et al.*, 1997; Eynck *et al.*, 2007), it caused significant stunting and chlorosis in *Arabidopsis*. However, the response varied between different ecotypes, cv. Columbia being among the most sensitive ones (Veronese *et al.*, 2003).

In conclusion, our data show that *V. longisporum*, one of the most important pathogens of Brassicaceae, infects also *A. thaliana*. While VL43 caused strong stunting of the stem of oil seed rape (Floerl *et al.*, 2008), it suppresses leaf expansion in *Arabidopsis* which, according to our results, is not caused by nutrient depletion.

## ACKNOWLEDGEMENTS

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