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Original research article

Suitability of GFP-transformed isolates of the fungal root endophyte *Acremonium strictum* W. Gams for studies on induced *Fusarium*-wilt resistance in flax

Gisela Grunewaldt-Stöcker[1], Nadine Riediger[2] and Christof Dietrich[3]

[1] Institute of Plant Diseases and Plant Protection, University of Hannover, Herrenhäuser Strasse 2, D-30419 Hannover, Germany
[2] Institute of Plant Pathology and Plant Protection, Department of Crop Sciences, University of Göttingen, Grisebachstrasse 6, D-37077 Göttingen, Germany
[3] DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany
Corresponding author: Gisela Grunewaldt-Stöcker, E-mail: grunewaldt@ipp.uni-hannover.de, Phone: +49-511-7623507, Fax: +49-511-7623015

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Abstract: Plant associated endophytic *Acremonium* species are capable of interacting with the host plant and altering its response towards pathogens and pests. Specialized root-colonizing *Acremonium* spp. can achieve biocontrol effects based on induced disease resistance in whole plant assays. However, these fungi are hard to detect and to study in root tissue by classical methods of light microscopy or microbiology. To enable further progress in investigations of induced mechanisms of defence in the plant, better visualization of the endophytic symbiont *Acremonium strictum* was attempted by use of autofluorescent GFP-transformants. Subcultures of three stable transformed *A. strictum*-isolates were used in agar plate assays with sterilized flax seedlings to document root colonization and infection with confocal laser scanning microscopy. In greenhouse tests the fitness of the transformants was proved in comparison to the wild type *A. strictum* for bioprotection against *Fusarium*-wilt. In these trials the endophyte trait to reduce disease incidence and symptom severity was verified in all three transformants as well as in the wild type of *A. strictum*. The suitability of these transformants for further research scopes and microscopy methods are indicated.

Keywords: *Acremonium strictum* W. Gams, fitness, *gfp*-transformants, bioprotection, flax (*Linum usitatissimum* L.).

Introduction

Plant associated *Acremonium* species are numerous, widespread in different host plants and able to survive as facultative saprophytes in soil (Gams 1971). According to Wilson (1995), several *Acremonium* species colonizing either the root or the shoot have been assigned to the endophytic fungi. As non-pathogenic fungi they can invade tissue of living plants and cause unapparent and asymptomatic infection entirely within the tissue. They are capable of interacting with the host plant and altering its response towards pathogens and pests (Bargmann and Schönbeck 1992, Breen 1994, Cook et al. 1991, Raps and Vidal 1998). Most of the research on fungal endophytes has been performed with the clavipitaceous grass endophytes, which were recently separated from the large taxon *Acremonium* and were established as a new form genus *Neotyphodium* (Glenn et al. 1996, Johnson et al. 2003). They are confined to the shoot (Cook et al. 1991) and, in that, to the apoplastic space in leaves and stems (Christensen et al. 2002).

In contrast, there is a lack of knowledge about the infection of soil-borne root-colonizing endophytic *Acremonium* species. In the context of studies on plant endophyte interactions contributing to induced disease resistance in roots and shoots (Grunewaldt-Stöcker and von Alten 2003), it is necessary to improve the visualization of such root endophytes.

In order to better understand the effectiveness of *Acremonium* spp. as biocontrol agents it is indispen-

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sable to follow the infection process of such inconspicuous endophytes within living root tissues. However, the tiny mycelia with hyphae of 1-3µm in diameter grow in the intercellular space of the root cortex, stain poorly and are therefore difficult to detect in non-dissected preparations. Additionally, due to their extremely slow growth in culture and their sensitivity to surface sterilization of root tissue, isolation of these fungi is rather laborious. PCR-based molecular detection was developed only for flax roots (Grunewaldt-Stoecker et al. 1998), but gained little success in bioassays under greenhouse conditions. Further progress could be made by use of green fluorescent protein (GFP*)*-introduction into the fungal endophyte to facilitate observations of fungus-plant-interactions (Bloemberg and Camacho Carvajal 2006).

There have been numerous successful applications of GFP-transformed filamentous fungi (Gold et al. 2001, Lorang et al. 2001). GFP-labelling of fungal plant pathogens like *Ustilago maydis* (Spellig et al. 1996), *Cochliobolus heterostrophus* (Maor et al. 1998), or *Colletotrichum lindemuthianum* (Dumas et al. 1999) enabled new steps to investigate fungal pathogenicity. GFP-markers in isolates of *Leptosphaeria maculans* (Sexton and Howlett 2001) and *Fusarium graminearum* (Skadsen and Hohn 2004) gave rise to specific microscopic detection and observation of fungal development *in planta,* thus avoiding misinterpretation by mistaken fungal identity. Therefore, this molecular tool should also contribute to unequivocal visualization of tiny, inconspicuous root endophytes.

With this approach, we used an isolate of the ubiquitous root colonizer *A. strictum*, which is known to reduce *Fusarium*-wilt in several host plants (Bargmann 1993), for GFP-transformation to improve its visualization *in planta* by confocal laser scanning microscopy (CLSM). In pot trials, under green house conditions, the GFP-transformants were compared to the wild type *A. strictum* and to another antagonistic root endophyte, *A. ochraceum* (Grunewaldt-Stöcker and von Alten 2003), to confirm the retained efficacy to induce resistance and, therefore, their suitability for monitoring bioprotection assays.

Materials and Methods

Transformation experiments

Acremonium strictum W. Gams, a stock culture from the Institute of Plant Diseases and Plant Protection (IPP), University of Hannover, which was re-identified by W. Gams (CBS Baarn, NL) in the past, was propagated on potato dextrose agar (PDA, Difco Laboratories). Shake cultures were prepared for protoplast preparation and subsequent transformation from PD-broth medium (2.4%) inoculated with 6 mycelium pieces (each of 1 cm in diameter) of *A. strictum* grown on PDA-plates. Incubation was performed in the dark for three to four days with gentle agitation. Protoplasts were prepared according to a modified protocol of Mikkelsen et al. (2003). Mycelium of *A. strictum* was incubated in 250 mL PD-broth and kept on a shaker for three to four days in the dark. After incubation, the mycelium was harvested by filtration through sterile cheese cloth. A solution of 500 mg Glucanex® Lysing Enzyme from *Trichoderma harzianum* (Sigma; Wiebe et al. 1997) and 15 mL Os-medium (1.2 M $MgSO_4$, 10 mM N_2HPO_4, pH 5.8; Yelton et al. 1984) was added to 1 g of washed mycelium and incubated for 45-90 min at 30°C on a shaker. The sample was then filtered trough sterile cheese cloth and washed with 100 mL of 0.6 M $MgSO_4$-solution. The protoplasts in the fluid were centrifuged at 1800 rpm at 4°C and the supernatant was discarded. The protoplast pellet was washed in 5 mL of STC-buffer (1.2 M sorbitol, 10 mM tris-base, 10 mM $CaCl_2$, pH 7.5) and again centrifuged. Finally the protoplasts were re-suspended in STC-buffer and adjusted to 3×10^5 protoplasts mL^{-1}.

A polyethylenglycol (PEG)-mediated method for transformation was modified according to Vollmer and Yanofsky (1986) and Mikkelsen et al. (2003). Eighty µl of protoplasts were gently mixed with 20 µl of a 40% (w/v) PEG-solution (PEG 4000; Sigma), 2 µl of 5 mM spermidine (pH 5.8; Roth), 5 µl heparin (5 mg mL^{-1}; Serva). Delivery of the *gfp*-gene into *A. strictum* was enabled by co-transforming the GFP vector gGFP (Maor et al. 1998) in conjunction with pAN7-1 for selection on hygromycin B (Punt et al. 1987). Four µg of each plasmid DNA (pAN7-1, gGFP) was added. The sample was incubated for 30 min on ice followed by the addition of 900 µl of PEG-solution. After incubation for further 15-20 min at room temperature, 100 µl were plated on CM-medium (Harling et al. 1988) containing hygromycin B (Duchefa) (50-150 µg mL^{-1}). Protoplasts were overlaid with 5 mL of CM-medium (0.8% agar). After incubation at 22°C overnight, the plates were overlaid again and were incubated at 22°C for two to three weeks for the regeneration of transformants. Transformed fluorescent colonies were selected and subcultured on CM-medium with 75 µg mL^{-1} hygromycin B. Cultures of transformed fungi used for biotests were raised in PD-broth or on PDA without antibiotic.

Visualization of Acremonium endophytes

At first the colonization of roots by the GFP-transformants was examined in a semi-sterile biotest-

system. Flax seeds (*Linum usitatissimum* L. cv. Belinka) were sterilized for 15 sec in 70% ethanol, 30 minutes in 1% NaOCl and washed in sterile water, transferred for germination to water-agar plates and incubated at 22°C in a climate chamber under 12h light. When primary roots branched, seedlings were inoculated with droplets of an aqueous conidial suspension from PDA plates or PD-broth cultures (1 x 10^4 conidia mL^{-1}). After a further two weeks of growth, roots were picked and observed microscopically for fluorescence of endophytic fungi.

Epi-fluorescence light microscopy (Axiophot®, Carl Zeiss, Germany) was performed for initial detection of GFP-transformed colonies after regeneration and for first *in situ*-studies on root colonization of subcultured transformants in water agar biotests. Live specimens were observed with a HQ-EGFP filter (AHF Analysetechnik, Germany) optimized for GFP-detection in plant tissues (Dietrich and Maiss 2002). GFP-transformants in flax root tissue were visualized in detail by CLSM using the TCS SP2 (Leica Microsystems, Wetzlar, Germany). GFP was excited with an Argon laser (488 nm), and emitted fluorescence was detected at 510-540 nm.

In order to compare the transformants, flax plantlets on water agar were inoculated with the wild type *A. strictum* (as described above). Furthermore, *A. ochraceum* (Onions and Barron) W. Gams comb. nov. (CBS 951.72, with a subculture internally named Ao5) was used as an additional determined standard strain with distinctive conidia. Root pieces of 2,5 cm length were stained with fuchsin acid (C.I. 42685; 1 g/100 mL) in lacto phenol (Gerlach 1977) in a stepwise rinsing procedure in lacto phenol (5 min), fuchsin acid-lacto phenol (20-30 min) and clearing and mounting in lacto phenol. Samples were analysed in either bright field microscopy with differential interference contrast or in epi-fluorescence microscopy with excitation at 546 nm and emission detection at 580-590 nm.

The presence of *A. strictum* was verified by PCR using *Acremonium* detection primers ACKIL1 5'-GGA TCC GCA TTG GGC GCT-3'and ACKIL2 5'-GGA TCC GCA GGG TCC TAC-3' (data not shown). *A. strictum*-DNA was extracted as described previously (Möller et al. 1992) and used for standard PCR reactions according to the manufacturer's recommendations (Taq-DNA polymerase, Fermentas).

Fitness tests for GFP-transformants

Acremonium root endophytes were tested for infection of flax roots and for their ability to reduce *Fusarium*-wilt in *L. usitatissimum* L. (cv. Belinka) in greenhouse pot trials. *A. strictum* (wild type isolate, see above) and three GFP-transformants derived from this wild type were maintained on PDA at 24°C in the dark. As a second standard strain qualified for bioprotection (Grunewaldt-Stöcker and von Alten 2003), *A. ochraceum* (Ao5, see above) was subcultured in the same way. Aqueous conidial suspensions (5 x 10^6 conidia mL^{-1}) were prepared for inoculation from each of the 10 days old cultures grown in 250 mL of PD-broth in Erlenmeyer-flasks with gentle agitation at 24°C in the dark.

The wilt pathogen *Fusarium oxysporum* Schlechtend. ex Fr. f. sp. *lini* (Bolley) W. C. Snyder and H. N. Hansen (isolate F. o. lini, IPP collection) was maintained on biomalt extract agar. Inoculum was subcultured for pot trials in a shake culture of 2% biomalt extract for 10 days at 24°C in the dark. All fungal isolates are kept as reference cultures under liquid nitrogen in the IPP collection at Hannover.

Flax seeds were sown into a mixture of sand (33% v/v) and commercial peat substratum (Fruhstorfer Erde Typ P, Flormaris Inc.) in plastic pots (12 cm diameter, 650 mL volume) and kept under greenhouse conditions (22/26°C night/day temperature, additional light: Phillips SGR 102/400, 140 µE s^{-1} m^{-2} for 16h). Plants were fertilized twice a week with Wuxal Top N (N:P:K = 12:4:6; 0.3% solution, 40 mL per pot). With unfolding of the first true leaves (14 days after sowing) the endophyte-isolates were applied as conidial suspensions with 15 mL per pot onto the substrate surface around the seedling stems. Four days later, the wilt pathogen was inoculated as conidial suspension (1 x 10^6 microconidia mL^{-1} and 40 mL per pot).

The fitness experiment was performed in two subsequent repetitions, A and B. Six replicates of the following variants were raised: untreated control; plants treated with the wild type of *A. strictum* (A.s.), with the GFP-transformants T1, T2-1 and T2-3[#1], with *A. ochraceum*-isolate Ao5[#1] as a second standard strain; control plants inoculated with *F. oxysporum* (F); plants inoculated with endophyte and pathogen (A.s.+ F, T1 + F, T2-1 + F, T2-3 + F, Ao5 + F). Each treatment included 6 replications, in a total of 90 plants.

To describe the efficacy of the endophytes to influence the disease development in flax, wilt symptoms were assessed daily, starting 15 days after *Fusarium* inoculation. Symptoms were classified from 0 (healthy) to 4 (heavily wilted, total yellowing, > 60% leaf loss) with a modified rating-scheme related to the *Verticillium* index of Bell (1969). Disease severity was calculated from frequencies of plants in wilting classes as Wilting Index (*WI)* according to the formula

[#1] evaluated only in repetition B

$$WI = \frac{\sum_{j=0}^{4}(n_j \cdot j)}{n} \quad ; \quad n = \sum_{j=0}^{4} n_j$$

in which n = number of assessed plants and n_j = number of plants in rating class j.

To control the spread of the pathogen in healthy appearing host plants, re-isolations were performed on *F. oxysporum*-selective agar plates (Komada 1975). Pieces of 2 cm length from surface sterilized stem bases of all symptom free plants (rating class 0) per inoculated treatment were prepared at the end of the experiment (23 days after *Fusarium* inoculation in repetition A, 27 days in repetition B, respectively). The final incidence of hyphal growth was assessed after 72 h of incubation at 24°C in the dark and verified microscopically.

Detection of GFP-transformants

Root samples were randomly taken during a period of 10 days after endophyte inoculation (five root segments of 2.5 cm length per transformant and day) and were microscopically analysed for fungal structures of GFP-transformants according to the epi-fluorescence methods described above. The samples originated from plants without *Fusarium* inoculation serving as controls free from disease.

Analysis of growth promotion

Flax plants from all treatments without *Fusarium* inoculation (control plants) were harvested for shoot fresh weight assessment 52 days after sowing, with five replications per treatment and 15 plants per pot.

Statistics

Data from flax wilt assessments, presented as frequencies of plants in disease classes, were tested using two-sided exact permutation tests based on Wilcoxen-Mann-Whitney scores (StatXact for Windows, Version 6; Cytel Software Corporation, MA, USA). A multiplicity adjustment for the all-pair comparisons was performed using standard Bonferroni correction; the overall significance level was set to $\alpha = 0.05$.

Results

Transformation of gfp-reporter gene:

The protocol of Mikkelsen et al. (2003) for protoplast preparations in filamentous fungi was modified for *A. strictum* in several parts. Most important for the successful establishment of regenerative protoplasts was the application of Glucanex® lysing enzyme. Moreover, the regenerative power of *A. strictum* protoplasts could only be maintained at a lower concentration of the selective antibiotic than cited above. In two independent transformation experiments a total of 13 colonies could be raised on the selection agar plates; four of them showed GFP-fluorescence. Three fluorescent transformants were stable in subculture for more than ten passages and were used in later trials: T1 derived from transformation experiment 1 with a selection level at 150 µg mL^{-1} hygromycin B, and T2-1 and T2-3 from transformation experiment 2 with a selection level at 50 µg mL^{-1} hygromycin B.

Compared to the wild type of *A. strictum*, the mycelia of all three GFP-transformants were more vacuolated on PDA plates without antibiotic as well as on the selective CM agar plates with 75 µg mL^{-1} hygromycin B. Occasionally length of coniophores was reduced (Fig. 1). Colony morphology and the typical cream to rose colour of the wild type did not differ notably after transformation. Hyphal growth on PDA was slightly, but significantly, slower for T2-3 and for T1 (colony growth reduced to 2/3 and to 5/6 in 18 days, respectively) than for the wild type (data not shown). However, sporulation was abundant in all cases.

Evidence of GFP-expression was evaluated by fluorescence microscopy of mycelia from agar plate and broth cultures. The strongest GFP fluorescence was observed in conidia, conidiophores, young

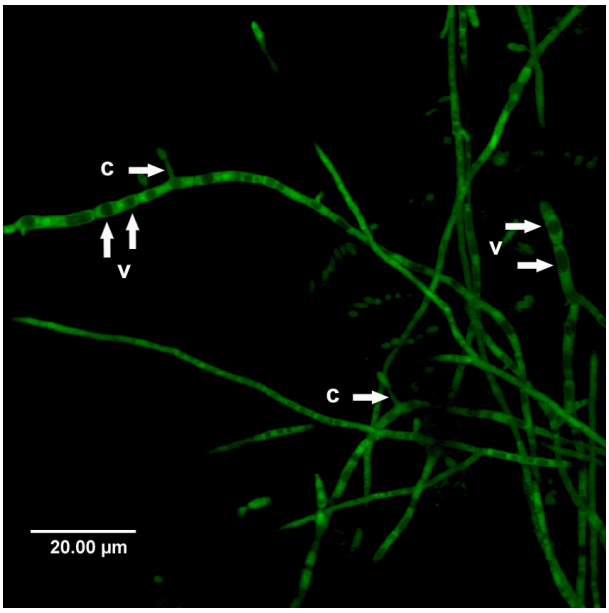

Fig. 1. GFP-transformant T1 of *Acremonium strictum* in liquid culture; vacuolated mycelium (v) with shortened conidiophores (c). GFP-fluorescence visualized in CLSM-technique as maximum projection of a stack of serial sections.

mycelia and hyphal tips. The identity of *A. strictum* transformants T1, T2-1 and T2-3 were verified by PCR.

Visualization of Acremonium root endophytes

Visualization of wild type *Acremonium* spp. in root tissue from semi-sterile flax seedlings grown on water agar was improved by using CLSM after fuchsin acid staining. This fluorochrome stains fungal structures as well as host cytoplasm and cell membrane of flax roots. *A. strictum* and *A. ochraceum* colonized the root surface with delicate hyphae, which mainly followed the host cell walls and developed monophialids for production of abundant conidia. The latter species is distinguishable from *A. strictum* by its typically pointed conidia often released in chains from simple erect conidiophores (Fig. 2).

In several instances for both of the wild type species, penetration of the root tissue was observed at cellular junctions (periclinal/anticlinal) as entry points into the apoplast. However, the precise detection of wild type endophytes was related to the uptake of fluorochrome by the fungal structures and thus restricted to the surface or first cell layer in non-dissected root samples at best.

GFP-transformants were also studied *in planta* first in seedlings on water agar using CLSM. Whereas the non-fluorescent wild type *A. strictum* was only visible after labelling, the native root specimens of plants inoculated with transformed *A. strictum* showed typical green fluorescence, confined to mycelia and conidia on the root surface and within root tissue. The transformants colonized the surfaces of main and lateral roots and spread to the root tips. Sporulation occurred frequently on the root surface after five days (Fig. 3). Detail observations in optical sections revealed intercellular fungal development restricted to the cortex. In several instances, even intracellular infections were detected (Fig. 4A). More rarely, conglomerations of branched, vacuolated hyphae filled the host cell and were connected with the expanding intercellular hyphal net (Fig. 4B). All these results confirmed the maintenance of infectivity in all three GFP-transformants of *A. strictum.*

Fitness tests of GFP-transformants in greenhouse trials

In greenhouse pot trials the fitness of the GFP-transformants to infect flax roots and to induce resistance against *Fusarium*-wilt was confirmed in comparison to the *A. strictum*-wild type and to *A. ochraceum* (isolate Ao5) as a second control strain. In both repetitions A and B of this experiment, the transformants clearly delayed wilt symptoms and reduced disease incidence and severity. The development of disease is demonstrated by means of wilting indices *WI*, calculated from scoring data (Fig. 5A, B). Disease progress in repetition A and B varied despite identical experimental set up. In repetition B it was prolonged and did not reach the high wilting level

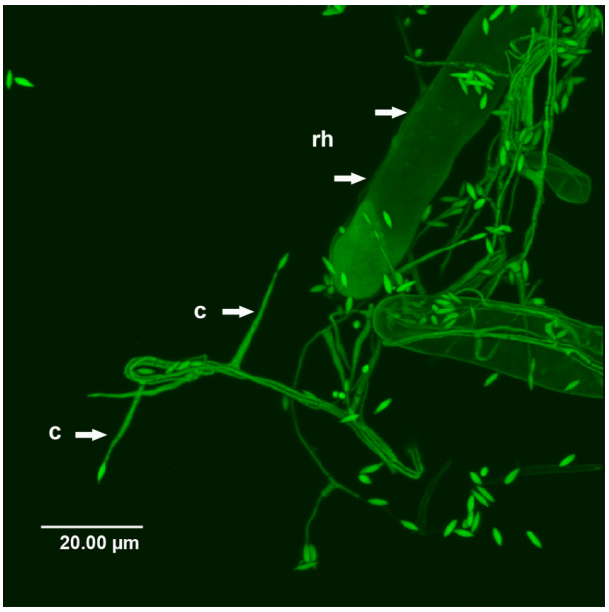

Fig. 2. Mycelium with typical conidiophores (c) of *Acremonium ochraceum* next to root hairs (rh) of *Linum usitatissimum* cv. Belinka in a water agar plate trial; maximum projection in CLSM-technique after fluorochrome staining with fuchsin acid-lactophenole.

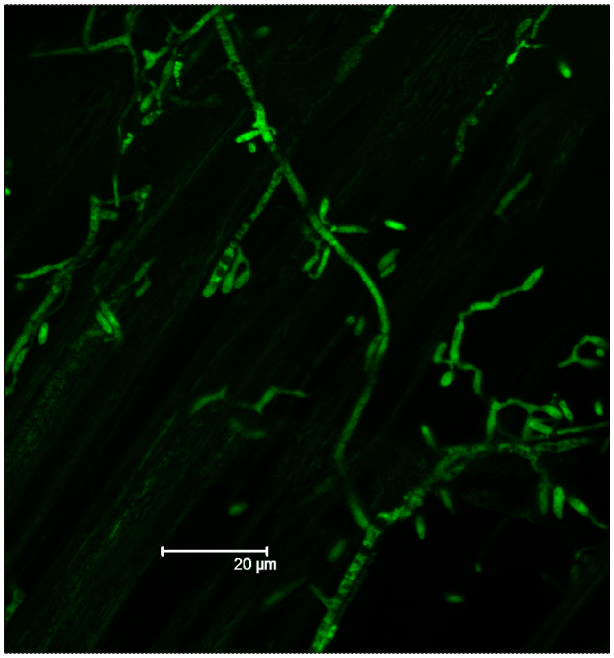

Fig. 3. Infection of axenically grown flax plants 35 days after inoculation with GFP-transformant T1, detected by CLSM-technique; sporulating mycelia on the root surface and hyphal parts in the cortex tissue; maximum projection of a stack of 35 serial GFP-fluorescence-images with total depth of 14 µm.

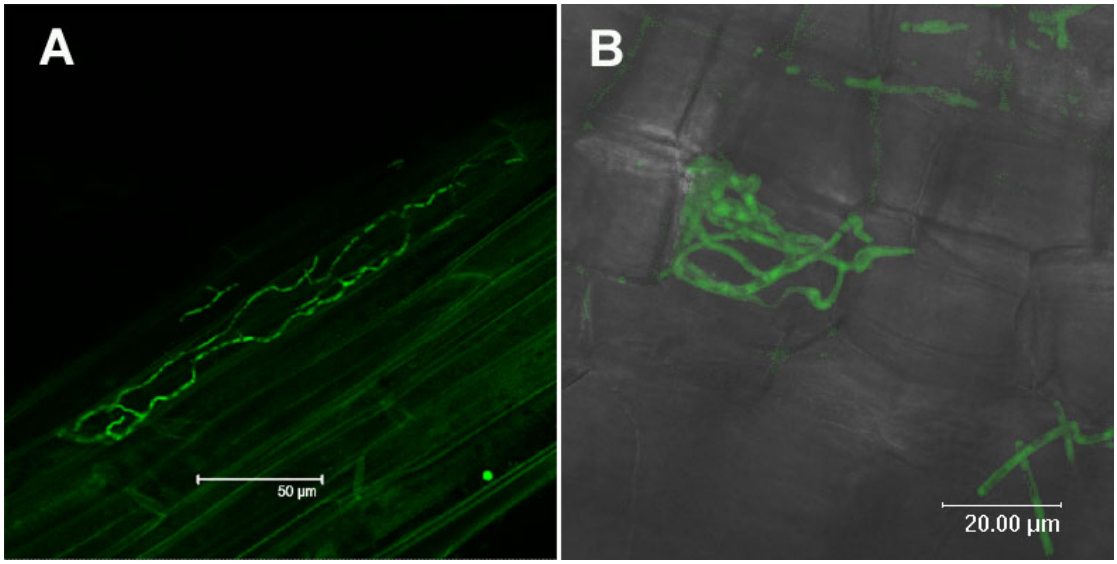

Fig. 4. Visualization of *A. strictum*-transformants in roots of *Linum usitatissimum* cv. Belinka in water agar plate trials with CLSM-technique. A: Intracellular mycelium of GFP-transformant T1 in the cortex cell; maximum projection of a stack of 7 serial GFP-fluorescence-images with total depth of 2.8 µm. B: Fluorescent mycelium of GFP-transformant T2-3 in a cortex cell; conglomeration of branched, vacuolated hyphae connected with the expanding intercellular hyphal net. Overlay of GFP-fluorescence-images and transmission-images in a maximum projection of 47 serial sections with a total depth of 17 µm.

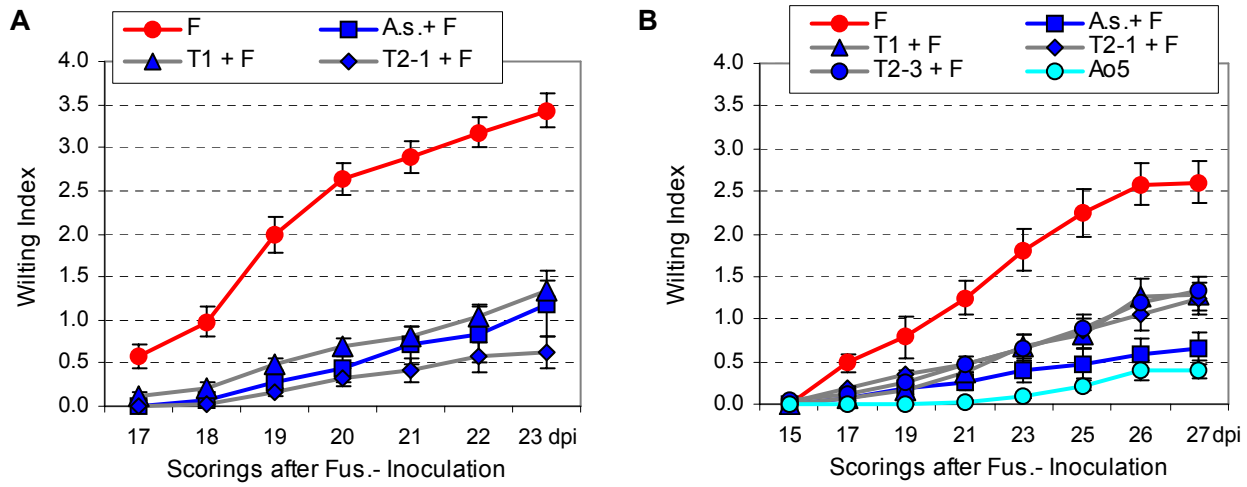

Fig. 5. Reduction of *Fusarium*-wilt in flax plants (cv. Belinka) due to pre-inoculation with *Acremonium* endophytes in the pot experiment, two repetitions (A, B). A: *Acremonium strictum* wild type (A.s.) and GFP-transformants (T1, T2-1), *Fusarium oxysporum* f. sp. *lini* (F); n = 6. B: *A. strictum* wild type (A.s.), GFP-transformants (T1, T2-1, T2-3), and *A. ochraceum* (Ao5), *F. oxysporum* f. sp. *lini* (F). Wilting indices calculated from symptom ratings, shown as mean values with standard errors of n = 6 replications.

of the first trial A, where > 65% of control plants with *Fusarium* infection were mostly dead and dried already after 23 days.

The effectiveness of the transformed endophytic fungi to induce wilt resistance was estimated by statistical analyses of scoring data in symptom classes. In Fig. 6 the final scoring results of repetition A and B are shown. There was a significant protection from wilt disease regarding incidence and severity in each of the endophyte variants compared to the non-treated *Fusarium* variant ($p = 0.000$). The GFP-transformants proved identical among one another in both trials A and B and were significant different from Ao5 in repetition B. They had similar efficacy as the wild type *A. strictum* in repetition A, while in the second trial B they proved significantly less effective than both wild type endophytes. In all *Acremonium* treatments in repetition A and B the frequencies of heavily wilted plants (class 4) were very low (< 12%), and many plants (42-78%) remained free from any wilt symptoms (class 0).

These symptom-free plants were tested for *Fusarium* infection in re-isolation assays from stem base pieces. Irrespective of the treatments nearly 50% of these plants were free from *F. oxysporum* in the stem. In repetition A of the fitness experiment, GFP-

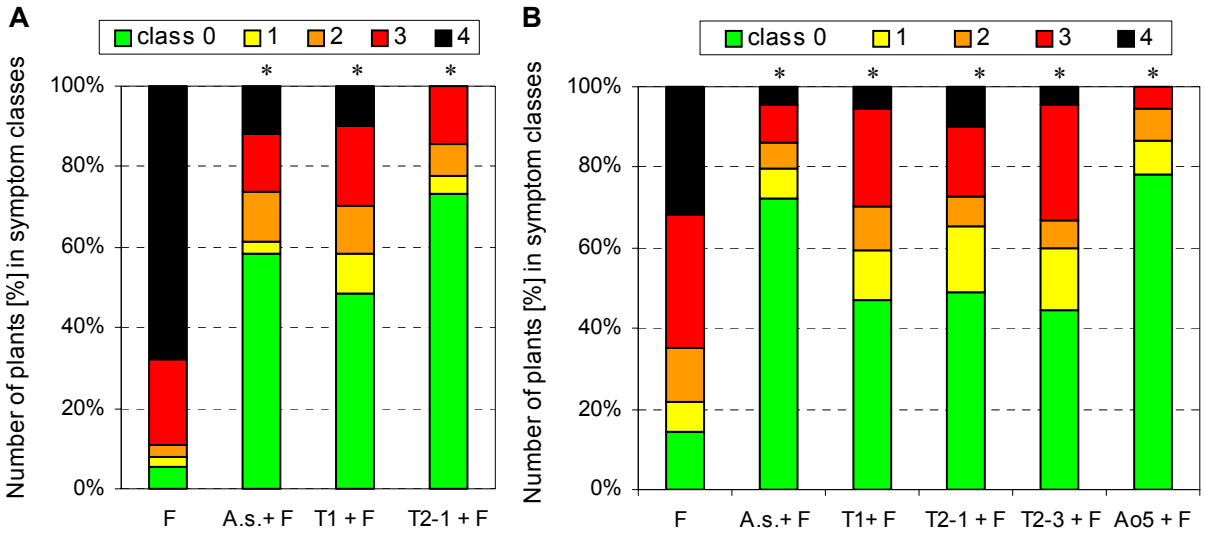

Fig. 6. Visual scoring of wilt symptoms in classes 0 (no symptoms) to 4 (heavily wilted) in the pot experiment A and B, 27 days after inoculation of flax plants (cv. Belinka) with *Fusarium oxysporum* f. sp. *lini* in endophyte-pre-inoculated plants; wild type *Acremonium strictum* (A.s.), GFP-transformants T1, T2-1, T2-3, *A. ochraceum* (Ao5), and control plants with pathogen-inoculation (F); n = 90 plants; * significant different from F (two-sided exact permutation tests) with $P_{adj.} \leq 0.0083$ and $P_{adj.} \leq 0.0033$ for repetition A and B, respectively.

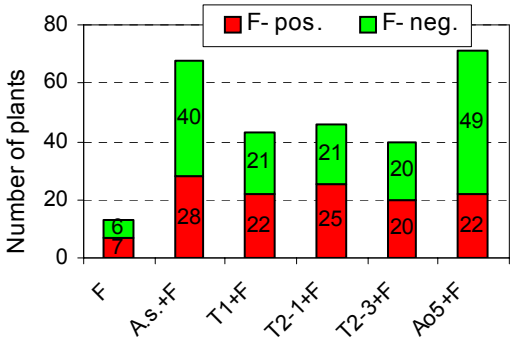

Fig. 7. Presence of *Fusarium oxysporum* in flax plants (cv. Belinka) without symptoms. Re-isolation (F-pos.) from the stem basis of plants scored in class 0 in the pot experiment B, 27 days after inoculation with *F. oxysporum* f. sp. *lini*; wild type *Acremonium strictum* (A.s.), GFP-transformants T1, T2-1, T2-3, *A. ochraceum* (Ao5), and control plants with pathogen-inoculation (F).

transformant T1 yielded 26 *Fusarium*-free plants from a total of 44 tested plants, and in treatment with T2-1 even 42 from 62 tested plants remained healthy. The treatment with *A. strictum*-wild type resulted in 26 *Fusarium*-free plants from a total of 53 plants. These results were confirmed in repetition B, including the two wild type *Acremonium*-species (Fig. 7). The selective re-isolations of the pathogen in several symptom free plants, however, indicated a low and/or late level of infection limited to the roots and stem bases.

In essence, the GFP-transformants proved to have similar competence to reduce wilt disease as the tested original wild type isolate and to be inferior to *A. ochraceum*. Furthermore, there was no significant indication that either resistance inducing wild types or GFP-transformed *Acremonium* isolates improved plant performance by raising shoot biomass production in the absence of the pathogen (data not shown).

During the pot experiments randomly selected root samples were microscopically checked for colonization and infection by the transformed isolates. However, the detection of fluorescent mycelium of GFP-transformants by CLSM was less than expected. Regularly, but in low frequencies, germinating conidia and hyphal fragments could be confirmed on root surfaces within the first week after inoculation. Sporulating mycelia of GFP-transformants were rarely found (Figs. 8A, B). A prominent hyphal net on the root surface or connected colonization patterns were not detectable.

Discussion

We introduced the *gfp*-gene into *A. strictum* and gained stable transformants with the aim of improving the visualization of *Acremonium* root endophytes *in planta*. The biological properties of the labelled isolates were compared to those of the wild type and were reliably detected due to the specific fluorescence. The trait to induce wilt resistance was also maintained from the wild type. This might open new working fields for investigations on endophyte-host-pathogen interactions *in situ*.

Transformation and protoplast regeneration

Starting from the first report on a GFP-transformed endophytic strain of *Neotyphodium lolii* (Mikkelsen et al. 2001) we chose a protocol with the PEG-based

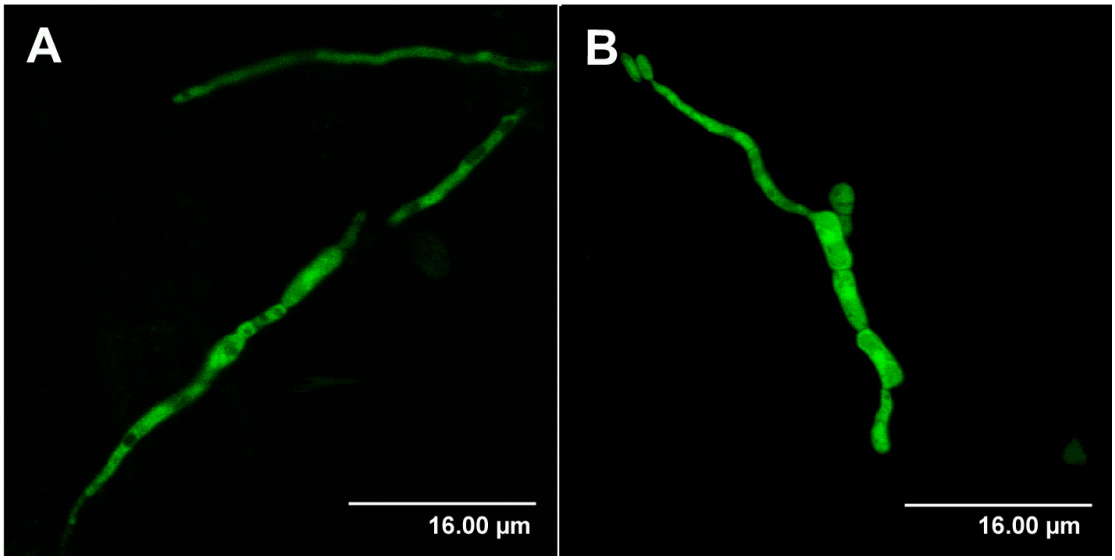

Fig. 8. Visualization of GFP-transformant T1 of *A. strictum* on the root surface of flax plants (cv. Belinka) in pot experiment B; maximum projection of serial sections; GFP-fluorescence in CLSM-technique; (A) hyphal fragments and conidia, (B) mycelium with conidiophore and conidia.

technique for transformation of *A. strictum*. Using some minor modifications of the enzymes for protoplast production and in the application of hygromycin B, we obtained three GFP-transformants of *A. strictum*, which proved to be stable in subculture and bright fluorescent in pure culture as well as in association with flax roots. Thrane et al. (1995) critically mentioned PEG in transformation experiments with protoplasts of *Trichoderma* spp. The authors found irreversible physiological changes in the membrane leading to fewer transformants compared to electroporation treatments. This would explain the comparable low transformation efficiency in our experiments. However, the *A. strictum*-transformants were suitable for use in the studies presented herein.

Visualization of GFP-transformants

Visualization by CLSM documented in detail the spread of GFP within the fungal plasma and revealed pronounced vacuolisation of even young mycelium growing without hygromycin B. In this attribute, the transformants seem to vary from the wild type *A. strictum*. Concurrently, we observed similar effects in GFP-transformed *Verticillium dahliae* after *Agrobacterium*-mediated transfers (Eynck et al. 2007), thus suggesting an alteration due to the GFP-expression. Vacuoles serve as compartments either for storage of resources or even for detoxification purposes. Increased vacuole formation in fungal hyphae could be regarded as a stress response (Bloemberg and Camacho Carvajal 2006). Here, the question of unfavourable side effects of the *gfp*-gene still remains to be addressed in specific experiments. As growth and vitality in subcultures of the three *A. strictum*-transformants were not markedly affected, the transformants were used for biotests with flax.

Visualization in planta

Using the advantages of confocal scanning processes, GFP-transformants were easily localized in axenically raised flax roots. They colonized the surface and could be followed in depth up to the second cortex cell layer *in situ*. However, as the protein expression in the fungal cytoplasm apparently depends on hyphal age, we have not been able to demonstrate the entire endophytic hyphal net by means of maximum projection obtained from stacks of confocal sections

Xiao and Berch (1999) reported on an *A. strictum*-isolate from salal (*Gaultheria shallon*) as an unusual ericoid mycorrhizal fungus (ERMF) due to its endophytic spread in roots. Intracellular structures of GFP-transformants of *A. strictum* in the flax roots (Figs. 4A and 4B) support that interpretation because of similarities to invading and coiling hyphae of ERMF in hair root cells of ericaceous plants. This is the first report on intracellular colonization of flax roots by *Acremonium* root endophytes. However, it is still necessary to confirm the infection of living cells under natural growth conditions to give evidence for a mycorrhiza-like symbiotic association in the non-ericaceous flax plant. In electron microscopy studies Bargmann (1993) observed *A. kiliense* (later re-defined by W. Gams as *A. strictum*, the wild type-isolate described in this paper; Vidal 1996) only in dead cortex cells of axenically raised tomato roots. From *Neotyphodium*-grass endophytes the colonization pattern is known to be restricted to the

apoplastic space in shoot tissue of various differentiation stages (Christensen et al. 2002). On the other hand, the root-restricted *A. strictum* isolates from this study seem to be able to infect the host cells at least occasionally. In any case they are candidates for specialized mycorrhizal fungi with flexible features in different host plant associations.

In greenhouse pot trials the GFP-transformants were applied for verification of their fitness including the ability to induce wilt resistance. In randomly checked root samples they could be detected unequivocally, but less frequently during the infection period. One reason for the small amount of visible fluorescent fungal structures colonizing the cortex tissue might be a reduced total amount of labelled protein in the cytoplasm of aging hyphae with increasing vacuolisation. Furthermore diffuse localisation of GFP in the small fungal cytoplasm areas of older hyphae might have hampered the detection in non-dissected root samples. However, one week after inoculation fluorescent conidia of our GFP-transformants were still found in random soil samples and on the root surface, thus indicating a rather adequate rhizosphere for the *Acremonium*-transformants and at least a stable *gfp*-expression.

A low colonization and infection density under greenhouse conditions could be another reason for little yield in visualization of our GFP-transformants. Considering the reduced vigour of the transformants compared to the wild type in agar plate growth tests on PDA, there might be an even greater loss of competitive fitness of the transformants under more challenging conditions in the greenhouse tests. Recovery rates of GFP-transformants from root segments by re-isolation were not assessed, as earlier re-isolation rates for wild type *A. strictum* in flax roots grown in substrate were proven to be very poor (< 0.1%) due to high sensitivity to surface sterilization and to slow growth progress on agar plates (unpublished data). In particular this corroborates results from tomato roots inoculated with *A. kiliense* (later re-defined as *A. strictum*, see above) in pot trials (Bargmann 1993). In general, the isolation of fungal root endophytes is problematic as Hallmann et al. (2006) pointed out in detail.

Fitness of GFP-transformants in pot trials

In several independent greenhouse assays, the antagonistic character of *Acremonium* root endophytes against *Fusarium oxysporum* had been shown (Grunewaldt-Stöcker and von Alten 2003). This feature was also apparent in three GFP-transformants of *A. strictum*. They reached the same level of efficacy as the *A. strictum*-wild type in reducing incidence and severity of the wilt disease in flax. Therewith, the aim of producing an improved isolate for detailed studies on host plant-endophyte-interaction processes *in situ* was reached, even if *A. ochraceum* used as a second standard strain performed significantly better.

Like the wild type, the GFP-transformants of *A. strictum* did not express any shoot growth promoting character in the flax experiments. They share this feature (which was demonstrated for *A. strictum* in tomato plants as well by Raps in 1997) with the wilt resistance inducing *A. ochraceum*. This unspecific trait of *Acremonium* root endophytes is of great advantage for studies of fungal functions in induced host response, because there is no overlay of growth enhancement effects interfering with symptom development.

Conclusions

The GFP-transformants of *A. strictum* provide a new tool for studies of early endophyte colonization stages and localization in the host tissue and of spatiotemporal host-fungus-interactions *in situ* with clearly identifiable fungal structures. They were shown to share interesting features of fitness with other antagonistic root endophytes within the large group of *Acremonium* spp. and therefore might serve as representative root colonizing isolates for studies on induced resistance. Their use for long-term observations on fungal development in soil environment, however, seems to be limited. Visualization of the endophytes for such issues could be improved in further transformation experiments in several ways: e.g. by use of a different promoter or of recently constructed *gfp*-variants with more intensive fluorescence (Lorang et al. 2001, Lagopodi et al. 2002).

As a consequence, for microscopic investigations with the described GFP-transformants the combination of two detection methods is recommended: firstly, GFP-tagging for unequivocal identification and localization of the endophyte structures and secondly, conventional classical staining with e.g. fuchsin acid for development studies in sectioned root specimens from pot or soil trials. Detailed observations of *Acremonium*-flax-interactions with CLSM will be facilitated because photobleaching impairing the GFP-analysis during extended scanning processes does not markedly occur with fuchsin acid-fluorescence. As this fluorochrome stains the host cytoplasm and cell membrane in addition, the orientation within the tissue is easier for the observer. Thus, its use can replace two-channel-scanning, which is necessary for compound overlay images from GFP- and transmission-scannings. Recently, this technical advantage has been shown in the system *Verticillium longisporum* - *Brassica napus* (Eynck et al. 2007).

The small yield of visible fungal structures from soil grown roots and the convincing antagonistic effects of the *Acremonium* isolates in the same fitness tests lead to an essential question: what is the necessary minimal infection frequency or fungal quantity in the roots for induction of resistance? In future studies it will be necessary to apply quantitative molecular methods in a tight conceptual design with the visualization of the endophytes.

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Dr. Gisela Grunewaldt-Stöcker is a plant pathologist with main interests in effects of fungal root endophytes inclusive mycorrhizal fungi for plant health and is specialized on microscopy.

Biologist Nadine Riediger did her Diploma Thesis on molecular studies with the root endophyte *Acremonium strictum* at the university of Hanover. Now she is a Ph.D. student at the university of Göttingen and works on systemic signals in xylem of *Brassica napus* infected with *Verticillium* sp.

Dr. Christof Dietrich is a plant virologist at the German Collection of Microorganisms and Cell Cultures working in the field of mixed infections, with special interests in the use of fluorescent reporters to locate viruses and their gene products in infected plants.