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Monitoring of pathogenic and non-pathogenic *Fusarium oxysporum* strains during tomato plant infection

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Summary

Monitoring of pathogenic strains of *Fusarium oxysporum* (*Fox*), which cause wilt and rots on agricultural and ornamental plants, is important for predicting disease outbreaks. Since both pathogenic and non-pathogenic strains of *Fox* are ubiquitous and are able to colonize plant roots, detection of *Fox* DNA in plant material is not the ultimate proof of an ongoing infection which would cause damage to the plant. We followed the colonization of tomato plants by strains *Fox* f. sp. *radicis-lycopersici* ZUM2407 (a tomato foot and root rot pathogen), *Fox* f. sp. *radicis-cucumerinum* V03-2g (a cucumber root rot pathogen) and *Fox* Fo47 (a well-known non-pathogenic biocontrol strain). We determined fungal DNA concentrations in tomato plantlets by quantitative PCR (qPCR) with primers complementary to the intergenic spacer region (IGS) of these three *Fox* strains. Two weeks after inoculation of tomato seedlings with these *Fox* strains, the DNA concentration of *Forl* ZUM2407 was five times higher than that of the non-compatible pathogen *Forc* V03-2g and 10 times higher than that of Fo47. In 3-week-old plantlets the concentration of *Forl* ZUM2407 DNA was at least 10 times higher than those of the other strains. The fungal DNA concentration, as determined by qPCR, appeared to be in good agreement with data of the score of visible symptoms of tomato foot and root rot obtained 3 weeks after inoculation of tomato with *Forl* ZUM2407. Our results show that targeting of the multicopy ribosomal operon results in a highly sensitive qPCR reaction for the detection of *Fox* DNA. Since formae speciales of *Fox* cannot be distinguished by comparison of ribosomal operons, detection of *Fox* DNA is not evidence of plant infection by a compatible pathogen. Nevertheless, the observed difference in levels of plant colonization between pathogenic and non-pathogenic strains strongly suggests that a concentration of *Fox* DNA in plant material above the threshold level of 0.005% is due to proliferation of pathogenic *Fox*.

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Introduction

Fusarium oxysporum (Fox) is a well-known pathogen of agricultural and ornamental crops (Nelson *et al.*, 1981). Phytopathogenic strains of *Fox* are responsible for yield loss of many economically important crops worldwide. For example, wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) and foot and root rot of tomato caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*) have been reported in at least 32 countries (Jones *et al.*, 1991). These diseases occur both in greenhouse and field and result in significant crop losses (Hahn, 2002; Cai *et al.*, 2003). Root and stem rot of cucumber, caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (*Forc*), also significantly reduce yield in greenhouses in many countries (Vakalounakis *et al.*, 2004). Besides yield decreases, many *Fusarium sp.* strains produce toxins which can accumulate in the end products and therefore may become dangerous for human and animal health (Pitt, 2000).

Fox is a cosmopolitan species whose representatives can survive as saprophytes in soil (Burgess, 1981). Due to their ability to utilize a large variety of nutrients, both pathogenic and non-pathogenic *Fox* strains can colonize the rhizospheres of various plants and, moreover, enter into the endophytic stage (Garret, 1970). Some non-pathogenic strains of *Fox* have been shown to control tomato foot and root rot (TFRR) caused by *Forl* (Olivain and Alabouvette, 1999; Bolwerk *et al.*, 2005). Moreover 'Bio*Fox* C', a product based on a non-pathogenic strain of *Fox*, is used for the protection of basil, carnation, cyclamen and tomato against pathogenic *Fox* and *Fusarium moniliforme* (Jones and Burges, 1998).

Monitoring of plant pathogens is crucial for disease management. Early detection, identification and quantification of the infestation level can help to choose appropriate defence measures. Monitoring of a

phytopathogenic microorganism can be done indirectly by following disease symptoms appearing on the plants or by analysing volatiles excreted during pathogen multiplication (Prithiviraj *et al*., 2004). Direct approaches, such a dilution plating of infested plant or soil on selective media (Vujanovic *et al*., 2002), detection of fungal spores in plant material (Hahn, 2002), immunological and molecular detection of the causal agent of the disease, give more precise information about the pathogens (Paulitz, 2000).

Development of real-time PCR (RT-PCR) has provided a powerful tool for pathogen monitoring. RT-PCR technique is highly sensitive for the detection of fungal strains (Zhang *et al*., 2005; Pasquali *et al*., 2006). It allows to detect the pathogen earlier than symptoms of the disease appear on the plants (Pasquali *et al*., 2004). With the use of real-time PCR it is possible to perform a semi-quantification of fungal pathogens such as *F. oxysporum*, *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani* in a single assay (Lievens *et al*., 2005).

Since plants can be colonized by pathogenic and non-pathogenic *Fox* strains, detection of *Fox in planta* is not necessarily evidence of attack of a pathogen. The patterns of tomato root penetration by pathogenic and non-pathogenic *Fox* appear to be quite similar and the differences are mainly quantitative (Olivain and Alabouvette, 1999). In the case of non-pathogenic *Fox*, flax plants appeared to be able to stop invasion of the fungus by building barriers in the cortex, whereas pathogenic strains appeared to avoid the defence system of the host plant (Olivain *et al*., 2003). Microscopical observations showed that, due to the reaction of the plant, the non-pathogenic strain Fo47 is restricted in multiplication in tomato and flax (Olivain and Alabouvette, 1999; Olivain *et al*., 2003).

If differences in proliferation of *Fox* strains *in planta* exist, one should be able to detect them by quantitative PCR. To test this idea we compared colonization of tomato plants by different *Fox* strains: (i) the tomato foot and root rot pathogen *Forl* ZUM2407 strain, (ii) the cucumber root rot pathogen *Forc* V03-2g and (iii) the non-pathogenic biocontrol strain *Fox* Fo47 using qPCR. The results are reported in this paper.

Results

Infection of tomato with Fox strains from different formae speciales

Tomato seeds were inoculated with strains *Forl* ZUM2407, *Forc* V03-2g and non-pathogenic *Fox* Fo47 in concentrations of 10^5 spores per litre of PNS. No difference in rate and level of germination was observed between untreated seeds and seeds inoculated with any of these three *Fox* strains. Tomato plants harvested 1 week after sowing had no symptoms of TFRR. Two- and three-week-old tomato plants sometimes had brownish

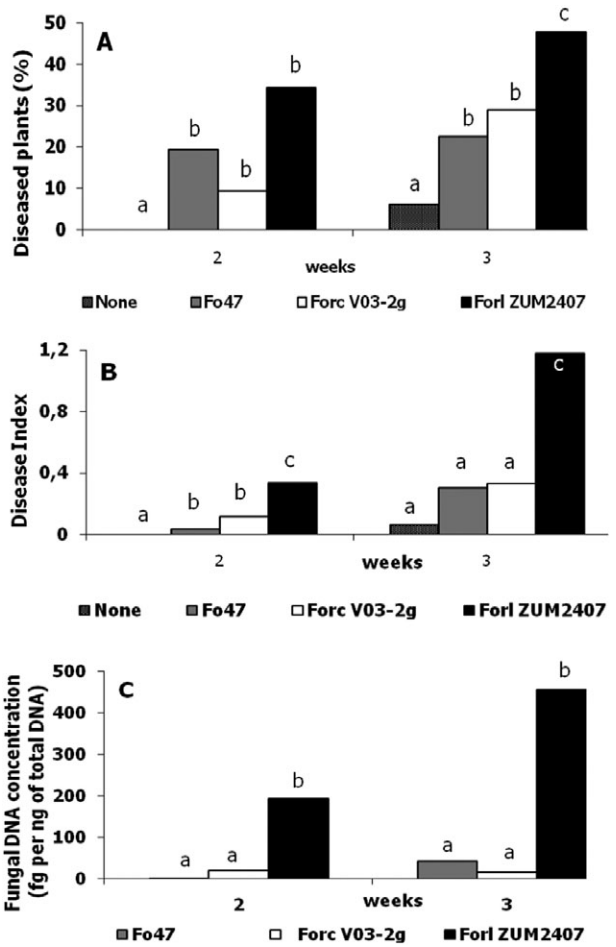

Fig. 1. Quantification of tomato infection by three *Fox* strains based on the results of two independent experiments.
A. Disease level.
B. Disease severity index.
C. Fungal DNA concentration in tomato plantlets. For statistics, a variance analysis followed by Fisher's least-significant-difference test (a = 0.05) was used. Statistically different values are labelled with different letters (a, b and c).

lesion and some of the plants were dead. The results of the disease evaluation in which no differences in the extension of the lesions were taken into account are shown in Fig. 2A. Among the inoculated 2-week-old plants, no statistical difference in disease incidence was evident (Fig. 1A), but the disease was more severe on plants treated with *Forl* ZUM2407 (Fig. 1B). Actually tomato plants inoculated with *Forc* V03-2g and *Fox* Fo47 strains had mainly light lesions. Three weeks post inoculation both disease incidence and severity were statistically higher on tomato plants inoculated with *Forl* ZUM2407 (Fig. 1A and B)

Quantification of fungal DNA in planta

Equal efficacy of templates for DNA fragment amplification is important for comparison of different strains. DNA

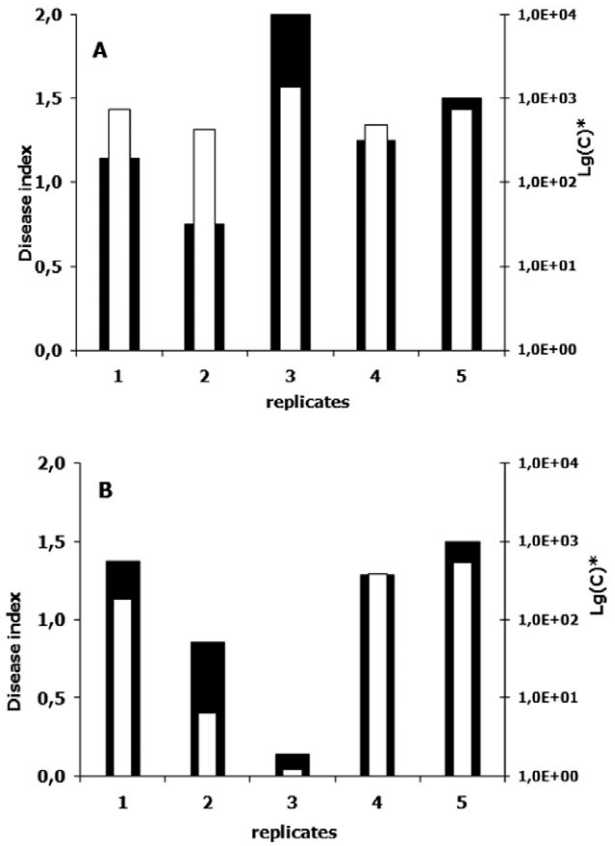

Fig. 2. Comparison of disease severity index and fungal DNA concentration. (A) and (B) represent results of two independent experiments on tomato infection with *Forl* ZUM2407. Plants were scored 3 weeks after inoculation of the seeds with fungal spores. Black bars correspond to disease index (left *y*-axis), white ones show fungal DNA concentration in a logarithmic scale (right *y*-axis). Note that in Lg(C) 'C' is the fungal DNA concentration in fg per ng of plant DNA.

samples isolated from *Forl* ZUM2407, *Forc* V03-2g and *Fox* Fo47 strains were used to check the efficiency of the fragment amplification with primers OMP1049 and OMP1050. No significant difference was observed when DNA dilutions, ranging from 10 ng to 1 fg, from each individual strain were compared using qPCR.

To follow proliferation of *Fox* strains, total DNA was isolated from harvested and assessed groups (replicates) of tomato plants and, subsequently, used for qPCR. Comparisons of the disease severity indexes and DNA quantifications are shown on Fig. 2. In both independent trials the changes in DNA concentrations and disease indexes reveal the same trend. Results of the quantification show that the concentration of *Forl* ZUM2407 DNA is 5–10 times higher than those observed for the other *Fox* strains (Fig. 1C).

Two weeks after the inoculation, the DNA concentrations of the *Forl* ZUM2407 in tomato tissue were 5 and 10 times higher than that of the other *Fox* strains, in the first and second experiment, respectively, whereas no statistical differences were detected between *Forc* V03-2g and *Fox* Fo47 DNA concentration (Fig. 1C).The DNA concentration of strains *Forc* V03-2g and *Fox* Fo47 did not exceed 100 fg per 1 ng of total DNA (Fig. 3).

Discussion

The ribosomal operon is present in 200 copies per haploid *Fox* genome, which offers a high sensitivity for the qPCR reaction. The space between the 18S and 28S rRNA genes of the ribosomal operon is designated as the intergenic spacer region (IGS) region. We used this IGS region as the target for qPCR. It was possible to detect 20 fg of DNA from the three *Fox* strains in 1 ng of tomato plant DNA, using primer pair OMP1049–OMP1050, which amplifies a 150 bp fragment within the IGS. Similar results were obtained when the IGS region was used for the detection of *Fox* f. sp. *vasinfectum* in cotton seedlings (Abd-Elsalam *et al.*, 2006). The lower detection level of 100 fg of pure fungal DNA reported in their article might be explained by the bigger size of the fragment used for amplification (438 bp).

We followed three different strains in plant infection/colonization and compared the results of two scoring systems with those obtained with PCR quantification. Results of scores without differentiation of lesion extension did not show a statistically significant difference

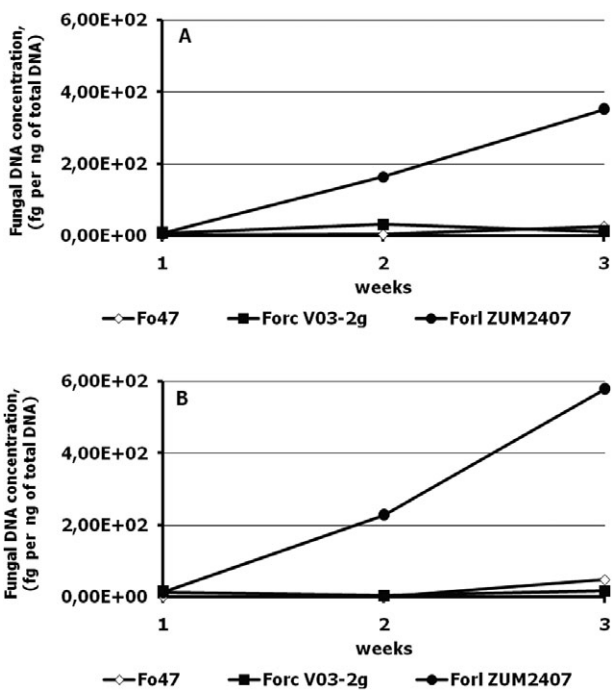

Fig. 3. Changes in fungal DNA concentrations during growth of inoculated tomato plants. (A) and (B) represent two independent experiments.

between *Fox* strains in the second week after inoculation (Fig. 1A). In contrast, when the disease severity is taken into account, a statistically significant difference was observed between *Forl* ZUM2407 and the other two *Fox* strains, since *Fox* Fo47 and *Forc* V03-2g strains did produce only small lesions on tomato plantlets (Fig. 1B).

Fungal DNA concentrations determined by qPCR were in a good agreement with the results of the disease indexes. Replicates containing more plants with larger lesions were given a higher disease index and they also showed a higher DNA concentration of *Forl* strain ZUM2407 (Fig. 2). A similar correlation between biomass of *Alternaria brassicola* and *Botrytis cinerea* was obtained when disease progression of the pathogens on *Arabidopsis thaliana* was quantified (Brouwer *et al*., 2003).

PCR-based methods are very sensitive. Therefore, they can reduce the detection time (Lievens *et al*., 2003). Choice of the target DNA fragment is pivotal for the monitoring. Two types of targets, namely fragments specific to a certain group of *Fox* strains (supposedly *forma specialis*) and orthologous sequences (ribosomal operon, tubulin gene, etc.) can be used for the detection of the fungal strains in various substrates. For example, anonymous fragments generated with RAPD methods were used for the detection of specific pathogenic *Fox* in Paris daisy and basil (Pasquali *et al*., 2004; 2006). The target fragments used for amplification gave a high sensitivity for the detection of specific pathogens of Paris daisy and basil. The authors do not discuss whether all *Fox* that are pathogenic to these crops can be detected using primers specific for these anonymous fragments. Similarly to many formae speciales, such as *Fox*. f. sp. *cubense* and *Fox*. f. sp. *melonis*, *Forl* is comprised of strains which have a polyphyletic evolutionary origin (Jacobson and Gordon, 1990; Kistler, 1997). This means that some of the unrelated *Fox* strains that are pathogenic to the same plant species can miss an anonymous fragment, which is the target for qPCR. Also, vice versa, detection of DNA fragments in the samples cannot fully guarantee that the detected *Fox* is a specific pathogen of the given plant, except when the genes are involved in host-plant infection (Rep *et al*., 2004).

Orthologuous sequences cannot be used for distinguishing heterogenic *formae specialis* or for discrimination of pathogenic *Fox* strains from non-pathogenic ones. On the other hand, multicopy orthologuous sequences are convenient targets for qPCR due to the wide range of strains, which can be detected due to the high sensitivity of the reaction.

The fungal DNA concentration, as determined in plants inoculated with different *Fox* strains, correlated with disease severity and showed a statistically significant difference between *Forl* strain ZUM2407 and the other two *Fox* strains (Fig. 1C).

The level of colonization of tomato plants by the three *Fusarium* strains was followed using the fungal DNA concentration in isolated DNA of the plant as a criterion. The DNA concentration of *Forl* strain ZUM2407 has increased from week 1 to week 3, whereas the concentration of DNA from strains *Fox* Fo47 and *Forc* V03-2g hardly changed and never exceeded 100 fg per 1 ng of total DNA (Fig. 3).

According to Olivain and Alabouvette (1999), the difference in colonization level of tomato by pathogenic and non-pathogenic strains of *Fox* is mainly quantitative. Perhaps this difference can be explained by an observation made during flax root colonization by pathogenic and non-pathogenic strains of *Fox.* Strain *Fox* Fo47 triggers formation of barriers, which apparently stop further invasion by the fungus, whereas the pathogenic strain avoids the plant's defence system (Olivain *et al*., 2003). In this scenario non-pathogenic strains are doomed to stay outside the inner root parts and have to compete with rhizosphere microorganisms for the limited amounts of nutrients from root exudates and/or remain restricted in proliferation by defence mechanisms of the host within the root cortex. In contrast, pathogenic strains proliferate on the abundant level of nutrients present in the plant's cortex and root stele.

Our results show that the non-pathogenic *Fox* strain Fo47 and the non-compatible pathogen *Forc* V03-2g could not exceed the level of 40 fg per 1 ng of total DNA, neither in week two nor in week three post inoculation. This concentration might reflect the level of colonization by the non-pathogenic strain and by the non-compatible pathogenic *Fusarium*, and this colonization level apparently is not dangerous for tomato plant health. In contrast, the phytopathogenic strain *Forl* ZUM2407, using nutrients of plant cortex and vascular system, could proliferate considerably. This proliferation resulted in disease symptom development. Differences in fungal biomass of non-pathogenic/non-compatible pathogenic and compatible phytopathogenic strains can be detected using RT-PCR. Therefore, exploitation of a conserved multicopy region, such as an IGS fragment, allows the highly sensitive detection of *Fox* strains and the quantification of *Fox* DNA in plant material. It can be an option for distinguishing the disease progress of *Fox* pathogens from root colonization by non-pathogenic *Fox* strains.

Experimental procedures

Strains and growth conditions

Strains used in this study are listed in Table 1. Strains were kept frozen at −80°C. When needed, cultures of the strains were plated on Czapek-Dox agar (CDA, Difco Laboratories, Detroit, MI, USA) and grown at 28°C for 5–10 days. To avoid bacterial contamination CDA was amended with kanamycin

Table 1. Strains used in the study.

Strain	Host	GenBank Accession No. of the IGS region	Reference/source
Fox f. sp. *radicis-lycopersici* ZUM2407	Tomato	EF437260	Syngenta, The Netherlands
Fox f. sp. *radicis-cucumerinum* V03-2g	Cucumber	EF437279	ARRIAM, Russia[a]
Fox Fo47	None	EF437222	Olivain and Alabouvette (1999)

a. All-Russian Research Institute of Agricultural Microbiology, Saint-Petersburg-Pushkin, Russia.

(Duchefa, Haarlem, the Netherlands) and tetracyclin (Duchefa, Haarlem, the Netherlands) in final concentrations of 50 and 40 μg ml^{-1} respectively.

Plant inoculation with Fox strains and scoring of disease symptoms

To obtain spores, 1 l Erlenmeyer flasks containing 200 ml of Czapek-Dox broth (CDB, Difco Laboratories, Detroit, MI, USA) were inoculated with one-third of a 10-day-old CDA Petri dish culture of the *Fox* strains. The cultures were grown at 28°C for 72 h under aeration (110 r.p.m.). Spores were separated from the mycelium and the agar pieces by filtering of the cultures through miracloth (Omnilabo International BV, Breda, the Netherlands). The microspore concentration was determined using light microscopy. The filtrate was adjusted to concentrations of 10^5 spores per litre of Plant Nutrient Solution (PNS; PPO, Naaldwijk, the Netherlands).

Tomato (*Solanum lycopersicum L.*) seeds (cv. Carmello) were sown in stonewool plugs (Grodan BV, Roermond, the Netherlands). Plastic trays with the stonewool plugs were soaked in PNS supplemented with *Fox*. Seeds were allowed to germinate for 3 days in the dark at 23–27°C and subsequently the plantlets were grown in a greenhouse at 21–24°C, 70% relative humidity and 16 h daylight.

For each treatment, 144 plants were tested. Forty-eight plants were harvested after 1, 2 and 3 weeks and the disease level was assessed using six replicates of 8 plants each. To evaluate the disease, plants were removed from the stonewool, washed, and the plant roots were examined for TFRR (tomato foot and root rot) symptoms as indicated by browning and lesions. Only roots without any disease symptoms were classified as healthy. The disease level, in this case, was calculated as the percentage of plants with a lesion. Alternatively, the disease was assessed by indexation of the disease severity: healthy plants were given a value of 0, plants with small lesions (< 2 mm) were given a value of 1, plants with developed lesions received a value of 2, and plants with large lesions (rotten foot, vast root rot) a value of 3. The value for dead plants was 4. The disease index (DI) was calculated using following formula:

$$DI = \frac{n_0 \times 0 + n_1 \times 1 + n_2 \times 2 + n_3 \times 3 + n_4 \times 4}{n_0 + n_1 + n_2 + n_3 + n_4}$$

in which n_0, n_1, n_2, n_3 and n_4 are the numbers of plants with indexes of 0, 1, 2, 3 and 4 respectively.

Differences in disease level among treatments were determined by analysis of variance (ANOVA) and mean comparisons were performed by Fisher's least-significant difference test ($a = 0.05$), using SPSS software (SPSS, Chicago, IL, USA). The experiment was performed twice.

Sample collection and DNA isolation

Each replicate containing eight whole plants was pulverized in liquid nitrogen. One gram of ground plant material was mixed with 1 ml of extraction buffer consisting of 2% hexadecatrimethylammonium bromide (CTAB; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) – 100 mM Tris-HCl (pH 8.0) – 1.4 M NaCl – 20 mM EDTA (MP Biochemicals, Amsterdam, the Netherlands). The mixture was incubated at 55°C for 20 min and then centrifuged at 14 500 r.p.m. The supernatant was extracted with one volume of chloroform. The upper phase was transferred to a new Eppendorf tube and the DNA was precipitated by adding 0.6 volume of isopropanol followed by centrifugation. The pellets were dissolved in 500 μl of TE buffer (pH 8.0). To remove RNA from the preparations, RNase (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) was added at a final concentration 2 μg ml^{-1}. DNA preparations were incubated at 60°C for 30 min and subjected to phenol-chloroform extraction. DNA was precipitated by adding 50 μl of 3 M sodium acetate and 350 μl of isopropanol followed by centrifugation at 14 500 r.p.m. for 5 min. DNA pellets were washed twice with 70% ethanol and dried. DNA was dissolved in 50 μl of milliQ water, quantified and adjusted to a concentration of 5 ng μl^{-1}. To isolate the DNA from *Fox* strains, the fungi were cultured for 5 days at 28°C on sterile filter paper placed on plate with CDA. The filter papers with fungal hyphea of the strains were removed from plate and ground in liquid nitrogen; further isolation was performed as described for the isolation of DNA from tomato plants.

Quantitative PCR reaction

Primers OMP1049 (5′-TGCGATTTGGACGAGATATGTG-3′) and OMP1050 (5′-ATTTGCCTACCCTGTACCTACC-3′) for quantitative PCR reaction were designed using Beacon Designer 5.0 (Bio-Rad Laboratories BV, Veenendaal, the Netherlands) on the basis of the IGS sequence of *Forl* ZUM2407 strain. Real-time PCR was performed in Chromo4 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories BV, Veenendaal, the Netherlands) with the following thermal profile: initial DNA denaturation and polymerase activation at 95°C for 10 min, followed by 40 cycles each containing denaturation and annealing steps at 95°C and at 58°C, respectively, both for 15 s. Amplification cycles were followed

by a melting curve built from 50°C to 90°C, with measurements made every 0.2°C. The PCR mixture was prepared using qPCR Core kit for SYBR Green I No ROX (Eurogentec, Seraing, Belgium) according to the recommendations of the manufacturer (see reference number RT-0000-06, at http://www.eurogentec.com). A standard curve for quantification was generated by plotting the log of the concentrations (from 28 fg to 5 ng) of total DNA isolated from *Forl* strain ZUM2407 in the presence of 5 ng of tomato plant DNA.

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