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Sampling and PCR method for detecting pathogenic *Fusarium oxysporum* strains in onion harvest

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Significance and Impact of the Study: Fusarium basal rot causes serious problems in onion production. To minimize post-harvest losses, a simple protocol based on FTA™ technology and a dual PCR test with *Fusarium oxysporum* species-specific and pathogenicity-specific primers was developed. By testing pooled onion samples using this method, latent infections with *F. oxysporum* can be screened from a representative sample of the harvest. This screening method could be a useful tool to manage the post-harvest losses caused by latent infections with *F. oxysporum* and, with modification of the PCR protocol, with other *Fusarium* species pathogenic to onion.

Keywords
Allium cepa, FTA™ card, Fusarium basal rot, *Fusarium oxysporum*, pathogen screening, PCR, post-harvest.

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Abstract

Fusarium basal rot is a worldwide disease problem in onions, and causes substantial losses in onion production, both during the growing season and in the storage. To minimize the post-harvest losses, a protocol for screening of latent infections with pathogenic *Fusarium oxysporum* strains from harvested onions was developed. This protocol is based on a dual PCR test with primers specific for the fungal species and new *SIX3* primers specific for the onion-pathogenic *F. oxysporum* strains. A pooled sample containing pieces from 50 harvested symptomless onions was prepared for the dual PCR using microwave disruption of the filamentous *Fusarium* fungi and Whatman FTA™ filter paper matrix technology, or as a reference protocol, by extracting DNA with a commercial kit. The two sample preparation protocols gave consistent results with the tested onion samples. Detection limit of the dual PCR protocol was 100 pg of *F. oxysporum* DNA, in a mixture with onion DNA, when the FTA card was applied. The new protocol reported here is simple and sensitive enough for routine testing, enabling the detection of latent infections in harvest lots even at the infection levels under 10%.

Introduction

Fusarium basal rot (FBR) is a serious problem in onion production worldwide. Especially, this disease is a threat to the organic onion production, in which a chemical treatment of onion sets to control fungal pathogens is not applicable. The rot starts from the roots and basal plate, and then spreads upwards inside the bulb and gradually spoils it (Galvan *et al.* 2008; Carrieri *et al.* 2013; Sasaki *et al.* 2015a). The pathogenic *Fusarium oxysporum* f. sp. *cepae* and *Fusarium proliferatum* are among the *Fusarium* sp. that have been reported to cause FBR everywhere where onion is grown (Bayraktar and Dolar 2011; Taylor *et al.* 2013, 2016; Sasaki *et al.* 2015a,b). In Finland, FBR is mostly caused by pathogenic strains of *F. oxysporum* f. sp. *cepae* and *F. proliferatum*, and to a smaller extent by *Fusarium redolens* (Haapalainen *et al.* 2016). The primary source of the pathogenic *Fusarium* isolates found from onion in Finland is the imported onion sets (Haapalainen *et al.* 2016).

After harvest, symptomatic onions can be detected and discarded before taking the onion crop into a cold storage. However, latent infections with pathogenic *Fusarium* species remain unnoticed. For the storage period of 2–8 months, onions are often kept at 0–2°C, and at this temperature *Fusarium* grows slowly. However, during the delivery chain from the storage to the retailers, the onions are subjected to ambient temperatures, and finally are presented to the consumers at temperatures >20°C. Then, the conditions are favourable for *Fusarium* growth and the infected onions become symptomatic and unsuitable to be used as food. To minimize the post-harvest losses, tools for screening of latent *Fusarium* infections in the onion lots prior to storage are needed.

In a previous study, *Fusarium* strains were isolated from onion sets and mature bulbs and tested for pathogenicity on onion seedlings (Haapalainen *et al.* 2016). While all the *F. proliferatum* strains tested were more or less virulent, the majority of the *F. oxysporum* isolates were non-pathogenic. Yet, the most aggressive pathogens were *F. oxysporum* strains, and all the isolates from symptomatic mature bulbs were pathogenic (Haapalainen *et al.* 2016). In the storage, the number of onions infected with *F. proliferatum* was found to be small compared to the number of onions infected with *F. oxysporum* (A. Hannukkala, unpubl. results). Since both pathogenic and non-pathogenic *F. oxysporum* strains are found associated with onions, detecting *F. oxysporum* merely at the species level does not give sufficient information of the risk of disease. Thus, the screening method should be specific for the pathogenic strains. Although the *F. oxysporum* strains show large variation in colony morphology, a test based on morphological features would be ineffectual, since these features showed no correlation to virulence (Haapalainen *et al.* 2016). Instead, most of the highly virulent strains were found to be genetically closely related (Sasaki *et al.* 2015a; Haapalainen *et al.* 2016), and thus a DNA-based method would be applicable for detection of latent infections with these strains. The onion-pathogenic strains have also been shown to carry additional pathogenicity-related genes, called *SIX* genes (for secreted in xylem), encoding small secreted proteins that are supposed to function as virulence factors (Sasaki *et al.* 2015a; Taylor *et al.* 2016). The *SIX3* gene was detected in all the *F. oxysporum* strains pathogenic to onion (*Allium cepa*), and could thus be used for detection of these strains (Sasaki *et al.* 2015a,b).

Screening symptomless onion lots for latent *Fusarium* infections poses many challenges. The sample preparation method has to be optimized to obtain representative pooled samples for screening the pathogen from the crop. The pooling step, in which the potentially infected onions are combined with healthy onions, will cause dilution of the pathogen in the sample. Thus, the detection method needs to be sensitive enough, while still retaining specificity.

Various molecular methods based on polymerase chain reaction (PCR) have been used to diagnose plant diseases (Lau and Botella 2017). As alternatives to traditional PCR-based methods, several isothermal amplification techniques have recently been developed (Villari *et al.* 2013; Ayukawa *et al.* 2016; Yu *et al.* 2019). These new methods are useful especially for the rapid field testing since the methods do not need electric equipment. However, testing on the field is not possible when a representative sample from the onion crop is desired. Then, it is necessary to have facilities to handle large amounts of onions and to prepare pooled samples for testing. Although most molecular approaches can be very effective with pure microbial cultures or a single plant sample, the sensitivity can be reduced when they are applied to pooled samples of symptomless plants. To achieve the sensitivity required for testing pooled samples, a conventional PCR test was chosen as detection method in this study. Finally, to be applicable for routine testing of onion lots, the screening protocol also needs to be cost-effective and simple.

The aim of this study was to develop a PCR-based detection protocol that meets all the above-mentioned diagnostic requirements, for screening of a relatively low level of latent infections with pathogenic *F. oxysporum* strains from pooled samples of harvested onions, and that is flexible enough to be modified for practical purposes.

Results and discussion

Sample preparation from pooled onions

The optimal sample size for routine testing of onions, considering the representativeness of the sample, the sensitivity of the test protocol and the cost per sample, was found to be 50 symptomless onions per sample. To define the test sensitivity, *F. oxysporum*-positive and healthy onion material (tested by PCR) were mixed together in different ratios, and the DNA from these mixtures was tested by PCR with the species-specific CLOX1-F/CLOX2-R primers (Mulè *et al.* 2004). The predicted PCR product of 534 bp was reliably detected from all these DNA samples (data not shown), indicating that one *F. oxysporum*-infected onion can be detected from a pooled sample of 50 onions.

As DNA isolation by grinding the plant material into powder in liquid nitrogen and then using a DNA extraction kit is time-consuming and expensive with large amount of samples, we tested a simple and cost-effective protocol for sample preparation: sample homogenization by crushing with a cell press (a marble roll), followed by application on FTA^{TM} card. Harvested onions, 50

symptomless onions per lot, from here on referred to as 'pooled sample', were used as samples for developing the protocol in 2016. Pooled samples from the 2017 harvest were tested using the protocol developed.

First, the efficiency of sample crushing with a cell press was studied using two *F. oxysporum*-positive onion samples (with replicates) homogenized by either grinding in liquid nitrogen or by crushing with a cell press (a marble roll). DNA from the samples was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) prior to PCR with species-specific primers. With the end-point PCR, there was no significant difference in the results of *F. oxysporum* detection when using either of the two homogenization methods, but when the same samples were tested by the real-time PCR, there was variation between the two homogenization methods for one of the samples (data not shown). These results indicate that although crushing the onion tissue with a cell press is sufficient for sample preparation, for routine testing the tissue crushing procedure needs to be standardized using an appropriate equipment, to improve the repeatability of the results.

FTA card has been widely used in testing many different microbial samples, such as viruses, bacteria, yeasts, filamentous fungi and oomycetes, as an easy and rapid method for extracting template DNA for PCR without using organic solvents, commercial kits or special equipment (Ndunguru *et al.* 2005; Borman *et al.* 2010; Grund *et al.* 2010; Chandrashekara *et al.* 2012). In the case of filamentous fungi like *Fusarium*, the fungal cell wall structures need to be disrupted before applying the sample to FTA card, to release enough DNA for successful PCR detection. In this study, a modification of the microwave method described by Suzuki *et al.* (2006) was used with the crushed pooled samples and found to improve the sensitivity of *F. oxysporum* detection. Detection of *F. oxysporum* from single symptomatic onions was successful even without the microwave treatment, whereas from pooled symptomless samples the detection often failed without the treatment (data not shown). Other microwave-based methods used for disruption of filamentous fungi grown on culture plates or of fungal spores have also been reported (Ferreira *et al.* 1996; Borman *et al.* 2006; Pallez *et al.* 2014). In this study, different methods for washing the FTA discs were tested to simplify the protocol, and a single wash with 50 μl of sterile water for 3 min at 50°C was found to perform equally well as the wash protocol recommended by the manufacturer.

Detection of onion-pathogenic *F. oxysporum* strains in the pooled samples

The DNA-based detection method should be able to distinguish the onion-pathogenic *F. oxysporum* strains from the non-pathogenic ones. Sasaki *et al.* (2015a,b) identified the virulence-related gene *SIX3* in the strains of *F. oxysporum* that cause basal rot in onions and thus this gene was chosen as the pathogen marker in this study. New primer pairs specific for the pathogenicity-related *SIX3* gene were designed to obtain primers compatible with the dual hot-start PCR protocol, together with the species-specific CLOX1-F/CLOX2-R primers. The *SIX3*-specific primers previously designed for real-time PCR (Sasaki *et al.* 2015a; Taylor *et al.* 2016) were not compatible with this protocol. Of the new primer pairs, the primer pair SIX3-F5/SIX3-R5 performed best in the dual PCR conditions. The specificity of the new primers was tested on DNA from onion-pathogenic and non-pathogenic *F. oxysporum* isolates, originating from imported onion sets, mature onions, leguminous green manure plants, black radish, barley and also two wild plants regularly growing in the onion fields (Table 1). The *SIX3*-specific primers produced a 311 bp-long pathogenicity-specific product from the pathogenic *F. oxysporum* isolates (Table 1; Fig. 1), but not from the non-pathogenic isolates of *F. oxysporum* (Table 1; Fig. 1). DNA from the other fungal species commonly found on onion did not give any products in the dual PCR (Table 1; Fig. 1), whereas single symptomatic onions and pooled symptomless samples infected with *F. oxysporum* f. sp. *cepae* gave PCR products of the expected sizes, 534 bp and 311 bp with the primer pairs CLOX1-F/CLOX2-R and SIX3-F5/SIX3-R5, respectively (Fig. 2a,b).

Performance and sensitivity of FTA card-based PCR protocol

In addition to being simple and cost-effective, the new FTA card-based PCR protocol needs to be sensitive enough to detect small amounts of pathogenic *F. oxysporum* in the pooled symptomless onion samples. Therefore, the new protocol was compared to the reference protocol that included sample grinding in liquid nitrogen and use of a commercial DNA extraction kit prior to PCR. Eight pooled symptomless onion samples from the 2017 harvest were first tested with the reference protocol in the dual PCR. Two of the eight samples were shown to have latent infection with pathogenic *F. oxysporum* (data not shown). DNA of these two samples (37 and 38) and an *F. oxysporum*-negative sample (41) were prepared again, starting from frozen chopped onion tissues, and using both of the two methods, the FTA card-based protocol and the reference protocol. End-point PCR results showed that the sensitivity of the developed FTA card-based PCR protocol was satisfactory, although the intensity of the bands was reduced in comparison with the reference protocol (Fig. 2a,b). From all the onion samples tested, a 163 bp

Table 1 Fungal isolates used for testing the dual PCR method

Fungal isolate*	Species	Host	Source of isolation†	Country of origin	Isolate pathogenicity‡	Year of isolation	PCR§	
							CLOX1-F/ CLOX2-R	SIX3-F5/ SIX3-R5
Fox090	*Fusarium oxysporum*	Onion	Symptomless set	The Netherlands	Very pathogenic	2013	+	+
Fox091	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Very pathogenic	2013	+	+
Fox129	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Very pathogenic	2013	+	+
Fox263	*F. oxysporum*	Onion	Injured set	The Netherlands	Very pathogenic	2013	+	+
Fox264	*F. oxysporum*	Onion	Injured set	The Netherlands	Very pathogenic	2013	+	+
Fox220a	*F. oxysporum*	Onion	Diseased mature bulb	Finland	Pathogenic	2013	+	+
Fox245	*F. oxysporum*	Onion	Diseased mature bulb	Finland	Very pathogenic	2013	+	+
Fox260_pc	*F. oxysporum*	Onion	Diseased mature bulb	Finland	Very pathogenic	2013	+	+
Fox261	*F. oxysporum*	Onion	Diseased mature bulb	Finland	Very pathogenic	2013	+	+
Fox262	*F. oxysporum*	Onion	Diseased mature bulb	Finland	Very pathogenic	2013	+	+
FUS16063	*F. oxysporum*	Onion	Diseased mature plant grown in a greenhouse from an imported set	Norway	Very pathogenic	2016	+	+
FUS16164	*F. oxysporum*	Onion	Symptomless mature plant grown in a greenhouse from an imported set	Norway	Mild symptoms	2016	+	–
FUS17039	*F. oxysporum*	Onion	Injured set	France	Nt	2017	+	–
FUS17040	*F. oxysporum*	Onion	Injured set	France	Nt	2017	+	+
FUS17037	*F. oxysporum*	Onion	Symptomless set	France	Nt	2017	+	–
FUS17056	*F. oxysporum*	Onion	Diseased mature plant grown in a greenhouse from an imported set	France	Nt	2017	+	+
Fox125a	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Mild symptoms	2013	+	–
Fox236	*F. oxysporum*	Onion	Symptomless mature bulb	Finland	Non-pathogenic	2013	+	–
Fox015	*F. oxysporum*	Onion	Injured set	The Netherlands	Mild symptoms	2013	+	–
Fox072a	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Mild symptoms	2013	+	–
Fox069	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Mild symptoms	2013	+	–
Fox093a	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	+	–
Fox049	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	+	–
Fox112	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	+	–
Fox017	*F. oxysporum*	Onion	Injured set	The Netherlands	Non-pathogenic	2013	+	–
Fox116a	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	+	–
Fox094	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	+	–
Fox134	*F. oxysporum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	+	–
Fox174a	*F. oxysporum*	Pea	Diseased stem base	Finland	Non-pathogenic	2013	+	–
Fox215f	*F. oxysporum*	Red clover	Symptomless root	Finland	Non-pathogenic	2013	+	–
Fox182	*F. oxysporum*	Broad bean	Diseased root	Finland	Non-pathogenic	2013	+	–
Fox197a	*F. oxysporum*	Black radish	Diseased root	Finland	Non-pathogenic	2013	+	–
Fox205b	*F. oxysporum*	Barley	Diseased stem base	Finland	Non-pathogenic	2013	+	–
Fox212	*F. oxysporum*	Vetch	Diseased root	Finland	Non-pathogenic	2013	+	–
Fox228	*F. oxysporum*	Sow thistle	Symptomless root	Finland	Non-pathogenic	2013	+	–
Fpr057	*F. proliferatum*	Onion	Diseased mature bulb	Finland	Very pathogenic	2013	–	–
Fre076	*F. redolens*	Onion	Injured set	The Netherlands	Very pathogenic	2013	–	–

(Continued)

Table 1 *(Continued)*

Fungal isolate*	Species	Host	Source of isolation†	Country of origin	Isolate pathogenicity‡	Year of isolation	PCR§	
							CLOX1-F/ CLOX2-R	SIX3-F5/ SIX3-R5
Ftr044	*F. tricinctum*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	–	–
Fso002	*F. solani*	Onion	Symptomless set	The Netherlands	Non-pathogenic	2013	–	–
BC_17002	*Botrytis allii*	Onion	Diseased mature bulb	Finland	Nt	2017	–	–

*All the fungal isolates were isolated and provided by A. Hannukkala.

†All the onion sets were obtained for research purposes from lots imported to Finland from the country of origin by a commercial company. Injured and symptomless onion sets were either used directly for fungal isolation or the (symptomless) onion sets were first grown into mature onions in a greenhouse in disease-free peat for later fungal isolation.

‡Pathogenicity as tested on onion seedlings in 2014 (Haapalainen *et al.* 2016), except for the isolates Fox220a, FUS16063 and FUS16164 which were also tested on mature onion bulbs (A. Hannukkala, unpubl. results); Nt, not tested.

§CLOX1-F/CLOX2-R, *F. oxysporum*-specific primer pair; SIX3-5F/SIX3-5R, pathogenicity-specific primer pair; +, detection; −, no detection.

PCR product was obtained with the onion-specific primers AcCOX1F/AcCOX1R (Wang *et al.* 2019), regardless of the infection status of the sample (Fig. 2c), indicating that all the samples contained DNA with good enough quality for PCR. Positive and negative controls included in PCR tests produced expected results in every experiment, indicating that the equipment used and the PCR reactions performed well.

The sensitivity of the FTA card-based PCR test was further studied using a 10-fold dilution series of DNA prepared from a mixture of *F. oxysporum* Fox260_pc mycelia and healthy onion tissue. The undiluted DNA preparation contained *F. oxysporum* DNA 4·8 ng $\mu l^{-1}$and onion DNA 10·0 ng μl^{-1}. The dilutions were prepared with a constant amount of healthy onion DNA, and 2 μl of each dilution was applied on FTA card prior to PCR. Detection limit of the dual PCR protocol was approximately 100 pg of *F. oxysporum* DNA, in the mixture with onion DNA (Fig. 3). When the same DNA dilutions were tested by PCR without the FTA card step, the detection limit was found to be lower, approximately 10 pg of *F. oxysporum* DNA (data not shown). A decreased sensitivity of an FTA card-based PCR method in comparison with the method using traditional nucleic acid extraction was previously reported by Grund *et al.* (2010) in their study on *F. graminearum*. Grund *et al.* (2010) speculated that the main constraint for the FTA-card method seemed to be the low concentration of fungal DNA in the sampled plant tissue, and even after good homogenization, the release of nucleic acids on the FTA cards was not optimal with higher fungi, leading to weaker signals of PCR products.

Verifying the sensitivity of the new protocol by real-time PCR

Sensitivity of the new protocol was also verified by real-time PCR by comparing results obtained from the pooled symptomless onion samples with the FTA card-based PCR method to those obtained with the real-time PCR. Eight pooled onion samples from 2017 harvest, prepared by grinding the tissue in liquid nitrogen and extracting DNA by DNeasy Plant Mini Kit protocol, were analysed by real-time PCR using *F. oxysporum*-specific primers

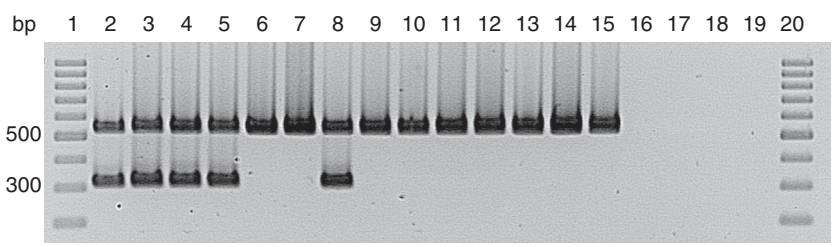

Figure 1 Dual PCR test results for pathogenic and non-pathogenic *Fusarium oxysporum* strains and *F. proliferatum* (Fpr) and *F. redolens* (Fre) strains, with the *F. oxysporum* species-specific primers CLOX1-F/CLOX2-R (product 534 bp) and the pathogenicity-specific primers SIX3-F5/SIX3-5R (product 311 bp). Lanes 1, 20: marker GeneRuler 100 bp DNA Ladder (Fermentas); 2: Fox260_pc; 3: Fox264; 4: Fox245; 5: FUS16063; 6: FUS16164; 7: FUS17037; 8: FUS17056; 9: Fox125a; 10: Fox112; 11: Fox017; 12: Fox116a; 13: Fox174a; 14: Fox197a; 15: Fox228; 16: Fpr057; 17: Fre076; 18: DNA from a *Fusarium*-negative onion; 19: water control.

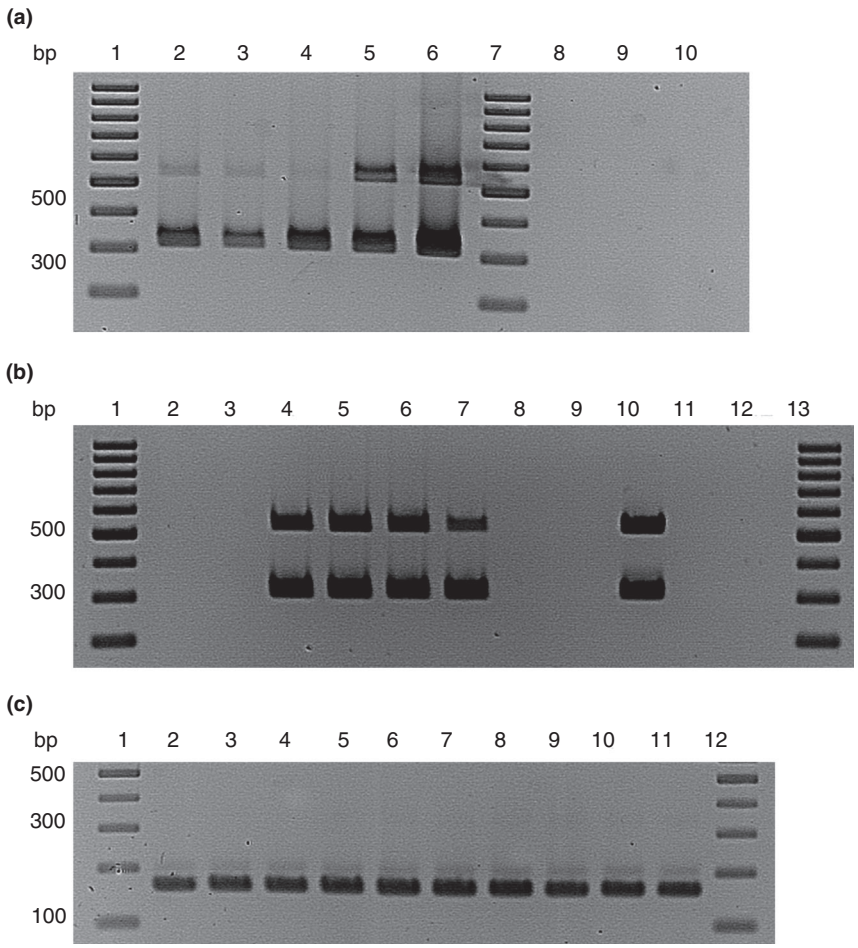

Figure 2 Screening of latent infections with pathogenic *Fusarium oxysporum* strains from harvested symptomless onion lots using a dual PCR method with CLOX1-F/CLOX2-R and SIX3-F5/SIX3-R5 primers. (a) Samples tested using the FTA™ card-based PCR method. Lanes 1, 7: L; 2, 3: sample 37; 4, 5: sample 38; 6: PC; 8: water control; 9, 10: sample 41. (b) The onion samples were ground in liquid nitrogen and DNA was extracted using DNeasy Plant Mini kit (Qiagen) prior to PCR. Lanes 1, 13: L; 2, 3: sample 35; 4, 5: sample 37; 6, 7: sample 38; 8, 9: sample 41; 10: PC; 11: NC; 12: water control. (c) Onion DNA detected with primers AcCOX1-F/AcCOX1-R of the samples prepared either using the FTA™ card-based method: Lane 2: sample 37; 3: sample 38; 4: PC; 5: sample 41, or by extracting DNA using DNeasy Plant Mini kit (Qiagen): Lane 6: sample 35; 7: sample 37; 8: sample 38; 9: sample 41; 10: PC; 11: NC. Lanes 1, 12: L. L: marker GeneRuler 100 bp DNA Ladder (Fermentas); PC: positive control DNA from a *F. oxysporum*-infected symptomatic onion; NC: negative control DNA from a healthy onion.

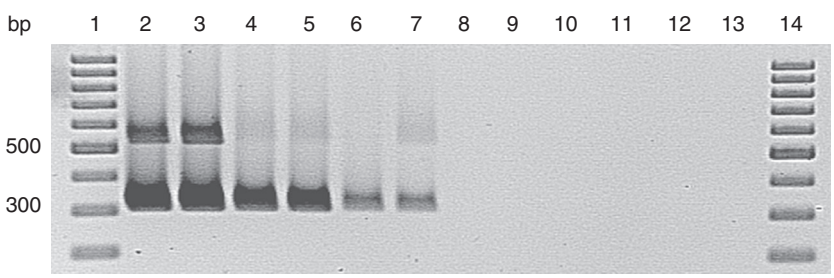

Figure 3 Dilution series of *Fusarium oxysporum* DNA detected by FTA card-based PCR method. Lanes 1, 14: marker GeneRuler 100 bp DNA Ladder (Fermentas); 2, 3: undiluted DNA containing 9·6 ng of *F. oxysporum* DNA; 4, 5: 10^{-1} dilution; 6, 7: 10^{-2} dilution; 8, 9: 10^{-3} dilution; 10, 11: 10^{-4} dilution and 12, 13: 10^{-5} dilution of the DNA.

OMP1049/OMP1050 (Validov *et al.* 2011) and onion-specific primers AcCOX1F/AcCOX1R (Wang *et al.* 2019) for quantification. With the onion COX primers the reaction efficiency E-value was 1·85 and standard curve R^2 = 0·9988, and with the *F. oxysporum* IGS primers the E-value was 1·89 (on onion DNA background) and standard curve R^2 = 0·9977. The most diluted standard, with 0·03 pg of *F. oxysporum* DNA in the reaction, gave a Ct mean value 36·8, which is still positive. The ratio of *F. oxysporum* (Fox) DNA to onion (Ac) DNA in each sample was calculated based on the standard curves as follows: $\text{Fox/Ac} = 10^{((\text{CtFox}-20{\cdot}336)/(-3{\cdot}6247))}/10^{((\text{CtAc}-24{\cdot}596)/(-3{\cdot}7386))}$, in which CtFox and CtAc are the means of the Ct values of three reactions.

Comparison of the results of *F. oxysporum* detection by end-point PCR and real-time PCR shows good agreement between the two PCR methods (Table 2). Two of the eight pooled samples (37 and 38) tested positive for *F. oxysporum* by end-point PCR (Fig. 2a,b; Table 2). This result was confirmed by real-time PCR (Table 2). The pooled samples 34 and 35 appeared negative in the end-point PCR, and in the real-time PCR with species-specific primers only one of the two biological replicates gave a borderline positive result (Ct values >37) for both samples (Table 2). The other pooled samples tested negative for *F. oxysporum* with both end-point PCR and real-time PCR. The results obtained by the FTA card-based PCR method and the PCR with extracted DNA were similar (Fig. 2a,b), and thus the sensitivity of the FTA card-based PCR method was adequate for testing the pooled samples. Considering the samples with a low amount of *F. oxysporum*, due to a low infection level (<5% of the onions infected) or a low colonization level per onion, false positives could be avoided by testing at least two biological replicates, that is, pooled onion samples, per each onion lot.

In addition to a sensitive diagnostic procedure, it is essential to have statistically sound sampling procedures to reveal relevant risk levels for making the decision on when to discard an onion lot from long-term storage. On a farm, the relevant risk level at which such a decision should be made could be close to 10% (A. Hannukkala, pers. comm.). In theory, when the proportion of infected onions in the lot is 10%, the probability that a random sample of 50 onions contains an infected onion is over 99%. This estimate was made based on the binomial distribution.

Table 2 Comparison of the results of *Fusarium oxysporum* detection by real-time PCR and end-point PCR. Eight pooled samples (from 34 to 41 with two biological replicates a and b) were tested using DNA extracted after grinding the onion tissue in liquid nitrogen

Sample	CtFox*	Standard deviation of CtFox	CtAc*	Standard deviation of CtAc	pg Fox DNA per reaction	DNA ratio Fox/Ac pg μg^{-1}	Fox detection†	
							qPCR	cPCR
34a	38·29	0·660	23·01	0·338	0·011	4·20	+/−	−
34b	-		22·70	0·392			−	−
35a	37·51	0·160	22·46	0·160	0·018	4·89	+/−	−
35b	-		23·96	0·113			−	−
36a	-		23·01	0·171			−	−
36b	-		23·45	0·194			−	−
37a	31·87	0·171	24·58	0·074	0·658	652·46	+	+
37b	31·97	0·108	24·57	0·103	0·616	604·77	+	+
38a	33·63	0·076	22·69	0·059	0·215	66·46	+	+
38b	33·64	0·181	22·74	0·123	0·214	67·97	+	+
39a	-		23·26	0·062			−	−
39b	-		23·68	0·099			−	−
40a	-		22·15	0·088			−	−
40b	-		23·00	0·058			−	−
41a	-		23·59	0·137			−	−
41b	-		23·71	0·070			−	−
PC1‡	26·4	0·071	22·30	0·163	21·234	5173·59	++	+
PC2‡	26·31	0·125	21·87	0·177	22·436	4185·95	++	+
NC§	-		23·50	0·184			−	−

*CtFox and CtAc, real-time PCR threshold cycle values for the target sequences of *F. oxysporum* and *Allium cepa*, respectively, the mean of three measurements.

†qPCR, interpretation of the real-time PCR results; cPCR, interpretation of the end-point PCR results; −, negative; +/−, borderline; +, positive; ++, positive with a high titre.

‡PC, positive control DNA from an *F. oxysporum*-infected symptomatic onion.

§NC, sample of *F. oxysporum*-negative onion DNA.

In conclusion, a specific DNA-based detection method was developed in this study for screening of latent infections with pathogenic *Fusarium oxysporum* strains from samples of harvested onion lots. The virulence-related gene *SIX3* was chosen as the pathogen marker for the DNA-based detection method to distinguish the onion-pathogenic *F. oxysporum* strains from the non-pathogenic ones. The screening protocol developed in this study could be a useful tool to manage the post-harvest losses caused by latent infections with *F. oxysporum*, and it could also be used for testing batches of onion sets. This protocol can also be applied, with modification of the PCR protocol, for screening of latent infections with other *Fusarium* species potentially pathogenic to onion.

Materials and methods

Fusarium oxysporum control samples

The *F. oxysporum* isolates were stored cryo-preserved in 2 ml cryovials amended with 10% dimethyl sulfoxide, in automated liquid nitrogen freezer at −150°C, as part of the culture collection of Natural Resources Institute Finland (Luke), and grown on potato dextrose agar (PDA) for 3 weeks prior to DNA extraction performed as previously described (Haapalainen *et al.* 2016). The *F. oxysporum* isolates used in primer testing are listed in Table 1.

Number of onions per pooled sample

To determine the number of onions that could be combined into one sample, symptomatic onion tissue, verified as *F. oxysporum*-positive by PCR, was mixed with healthy *F. oxysporum*-negative onion tissue in ratios 1 : 5, 1 : 10, 1 : 20, 1 : 30, 1 : 40 and 1 : 50. Five grams of each onion tissue mixture was ground in liquid nitrogen, and 100 mg of the resulting fine powder was used for DNA extraction prior to *F. oxysporum*-specific PCR with CLOX1-F/CLOX2-R primers (Mulè *et al.* 2004).

Samples of harvested onions

Onions were collected in 2016 and 2017 from two farms in eastern Finland, and from two field trials conducted at Luke Mikkeli in South Savo, and Luke Piikkiö in Southwest Finland. In both years, the onion samples were collected at harvest time, in August and September. From each farm, three samples of 50 symptomless onions were randomly collected at three different locations on the onion field. At the Luke research fields, 50 onions per location were randomly collected from protection plots surrounding the trials or experimental plots, and combined into one sample. The onions were stored at room temperature (RT) for 2 weeks, then cut in half, and the onions with any FBR symptoms were discarded. From each symptomless onion, a 1 g piece of tissue was cut just above the basal plate, and the pieces from 50 onions were combined into one sample, from here on referred to as 'pooled sample'. Samples were chopped into smaller pieces using a knife and stored at −80°C.

Sample preparation by FTA card protocol and by DNA isolation

Five gram subsamples were taken from the 50 g pooled samples and prepared using either the new FTA card-based protocol (A) or DNA isolation, the reference protocol (B) For controls, samples were also prepared of a *F. oxysporum*-infected symptomatic onion and a PCR-verified non-infected onion.

A. 5 g of chopped onion tissue was placed into a plastic bag with 7·5 ml TE-buffer (10 mmol l^{-1} Tris–HCl, pH 8·0, 1 mmol l^{-1} EDTA) and crushed using a marble roll and the resulting suspension was used for FTA-card sample preparation.

Microwave treatment described by Suzuki *et al.* (2006) was applied for disruption of filamentous *Fusarium* fungi in the crushed onion samples. The microwave treatment was performed also for a healthy onion tissue and for symptomatic onion tissue infected with *F. oxysporum*, used in the protocol as a negative and a positive control, respectively. Briefly, from each sample, three aliquots of 25 μl were applied into a microplate and treated two times in a microwave oven at 750 W for 30 s, with chilling on ice for 1 min between the treatments. The aliquots of each sample were combined, and four spots per sample (each 5 μl) were applied onto Whatman FTA™ MicroCards (GE Healthcare, Buckinghamshire, UK). FTA cards were dried for 1 h at RT. Sample discs from the dried spots were taken using a Whatman Uni-Core™ 2·00 mm punch (GE Healthcare) and placed into 1·5 ml Eppendorf tubes. The discs were washed once with 50 μl of sterile water for 3 min at 50°C, after which the water was removed from the tubes and the discs were used directly for PCR. Two FTA discs were added per 50 μl PCR reaction.

B. 5 g of chopped onion tissue was ground in liquid nitrogen and 100 mg of the ground tissue was used for DNA extraction using DNeasy Plant Mini Kit (Qiagen) prior to PCR. The DNA samples were eluted in 100 μl of sterile distilled water, and the concentration was measured by NanoDrop 2000C spectrophotometer (Thermo-Scientific, Waltham, MA). The samples were stored at −20°C.

Primers used in end-point PCR and real-time PCR

The virulence-related gene *SIX3* of *F. oxysporum* f. sp. *cepae* was chosen as the pathogenicity marker in this study. To obtain primers compatible with the hot-start PCR protocol, several primer pairs were designed for the detection of *SIX3*, using the Primer3 tool (Koressaar and Remm 2007). The primer pair SIX3-F5 (5′-CCACTATGCGTTTCCTTCTGC-3′) and SIX3-R5 (5′-AGGTGCGACATCAATGACAGT-3′), amplifying a 311 bp fragment of *SIX3*, was found to perform best in the dual PCR with *F. oxysporum* species-specific primers CLOX1-F (5′-CAGCAAAGCATCAGACCACTATAACTC-3′) and CLOX2-R (5′-CTTGTCAGTAACTGGACGTTGGTACT-3′) (Mulè *et al.* 2004), amplifying a 534 bp fragment of the *calmodulin* gene. The fungal DNA samples used as templates for testing the dual PCR detection method are listed in Table 1. *A. cepa* (onion)-specific primers AcCOX1F (5′-CGTGCTTACTTCACCGCAGCT-3′) and AcCOX1R (5′-TTCCTGTGAGCCCGCCTATGG-3′) (Wang *et al.* 2019) amplifying a 163 bp fragment of the onion mitochondrial gene *cytochrome c oxidase* 1 were used in the PCR amplification control reactions.

In real-time PCR, *F. oxysporum* species-specific primers OMP1049 (5′-TGCGATTTGGACGAGATATGTG-3′) and OMP1050 (5′-ATTTGCCTACCCTGTACCTACC-3′) (Validov *et al.* 2011), amplifying a 110 bp fragment of the ribosomal intergenic spacer region (IGS) were used for detecting *F. oxysporum*. To determine the relative amount of *F. oxysporum* in each sample, reference reactions were run with the *A. cepa* (onion)-specific primers AcCOX1F/AcCOX1R.

End-point PCR

For DNA extracted from the onion samples by DNeasy Plant Mini Kit, 20 ng of DNA was used as the template in a 20 μl PCR reaction. For DNA bound to FTA sample discs, two sample discs were placed in a 50 μl PCR reaction. All the PCR reactions contained primers at 250 nmol l^{-1} and dNTPs at 200 μmol l^{-1} concentration. Phire Hot Start II DNA Polymerase and the 5× reaction buffer were added at the amounts recommended by the manufacturer (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The optimized PCR programme used was as follows: an initial denaturation step at 98°C for 30 s, then 40 amplification cycles with denaturation at 98°C for 10 s, primer annealing at 66°C for 10 s and elongation at 72°C for 15 s, followed by a final elongation step at 72°C for 5 min. PCR amplification was carried out using Biorad S1000 Thermal Cycler (Biorad, Hercules, CA). The size and quality of the PCR products were analysed by electrophoresis on 1·5% agarose gel in TBE buffer. The gels were stained with ethidium bromide and the DNA was visualized under UV-transilluminator (SCIE-PLAS, Cambridge, UK).

Dilution series for sensitivity testing

To determine the sensitivity of the dual PCR protocol and the effect of FTA card to the sensitivity, DNA from a mixture of 1 mg of 1-week-old mycelia of *F. oxysporum* strain Fox260_pc (Haapalainen *et al.* 2016) and 99 mg of healthy onion tissue was extracted by the DNeasy Plant Mini kit protocol (Qiagen). The concentration of Fox260_pc DNA in the DNA stock was 4·8 ng μl^{-1}. Ten-fold dilutions of the DNA stock were prepared with a constant amount of DNA from an uninfected onion. Two microlitres of each dilution was applied onto Whatman FTA^{TM} MicroCards (GE Healthcare) with two replicates and treated as described in *Sample preparation by FTA card protocol and by DNA isolation*. PCR reactions and conditions were as described in *End-point PCR*.

Real-time PCR

Real-time PCR was performed with the DNA from the eight pooled samples from 2017, after grinding the onion tissue in liquid nitrogen and extracting DNA by DNeasy Plant Mini Kit protocol. DNA from uninfected onion tissue was used as an *F. oxysporum*-negative control and DNA from an *F. oxysporum*-infected symptomatic onion served as the *F. oxysporum*-positive control. DNA samples were diluted 1/50 with nuclease-free water, and 5 μl of the diluted sample was used per reaction in the real-time PCR assay. For quantification of *F. oxysporum*, reactions with species-specific primers OMP1049/OMP1050 (Validov *et al.* 2011) were performed in parallel with *A. cepa* (onion)-specific reference reactions with primers AcCOX1F/AcCOX1R (Wang *et al.* 2019). Standard curves for determining the reaction efficiencies were prepared by serial dilutions of DNA from healthy onion, diluted with nuclease-free water and of the mixture of *F. oxysporum* and onion, diluted with healthy onion DNA solution. The real-time PCR equipment, the reaction constituents and the program run were the same and the melting curve analysis was performed similarly as previously described in Wang *et al.* (2019), with the exception that primer annealing temperature 60°C was used in this study. The primer efficiencies (*E*-values) were determined from the standard curves as follows: $E = 10^{(-1k^{-1})}$, where k is the slope of the linear regression Ct *vs* log[C]. Based on the standard curves, the concentrations of onion DNA and *F. oxysporum* DNA in the samples were determined, and the ratio of *F. oxysporum* DNA to onion DNA was calculated.

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Conflict of Interest

No conflict of interest declared.

References

Ayukawa, Y., Komatsu, K., Kashiwa, T., Akai, K., Yamada, M., Teraoka, T. and Arie, T. (2016) Detection and differentiation of *Fusarium oxysporum* f. sp. *lycopersici* race 1 using loop-mediated isothermal amplification with three primer sets. *Lett Appl Microbiol* **63**, 202–209.

Bayraktar, H. and Dolar, F.S. (2011) Molecular identification and genetic diversity of *Fusarium* species associated with onion fields in Turkey. *J Phytopathol* **159**, 28–34.

Borman, A.M., Linton, C.J., Miles, S.-J., Campbell, C.K. and Johnson, E.M. (2006) Ultra-rapid preparation of total genomic DNA from isolates of yeast and mould using Whatman FTA filter paper technology - a reusable DNA archiving system. *Med Mycol* **44**, 389–398.

Borman, A.M., Fraser, M., Linton, C.J., Palmer, M.D. and Johnson, E.M. (2010) An improved protocol for the preparation of total genomic DNA from isolates of yeast and mould using Whatman FTA filter papers. *Mycopathologia* **169**, 445–449.

Carrieri, R., Raimo, F., Pentangelo, A. and Lahoz, E. (2013) *Fusarium proliferatum* and *Fusarium tricinctum* as causal agents of pink rot of onion bulbs and the effect of soil solarization combined with compost amendment in controlling their infections in field. *Crop Prot* **43**, 31–37.

Chandrashekara, K.N., Prasannakumar, M.K., Deepa, M. and Vani, A. (2012) A rapid, sensitive and reliable method for detecting *Ralstonia solanacearum* using FTA (Whatman) card. *J Plant Pathol* **94**, 219–221.

Ferreira, A., Glass, V.B. and Louise, N. (1996) PCR from fungal spores after microwave treatment. *Fungal Genet Rep* **43**(1), 25–26.

Galvan, G.A., Koning-Boucoiran, C.F.S., Koopman, W.J.M., Burger-Meijer, K., González, P.H., Waalwijk, C., Kik, C. and Scholten, O.E. (2008) Genetic variation among *Fusarium* isolates from onion, and resistance to Fusarium basal rot in related *Allium* species. *Eur J Plant Pathol* **121**, 499–512.

Grund, E., Darissa, O. and Adam, G. (2010) Application of FTA® cards to sample microbial plant pathogens for PCR and RT-PCR. *J Phytopathol* **158**, 750–757.

Haapalainen, M., Latvala, S., Kuivainen, E., Qiu, Y., Segerstedt, M. and Hannukkala, A.O. (2016) *Fusarium oxysporum*, *F. proliferatum* and *F. redolens* associated with basal rot of onion in Finland. *Plant Pathol* **65**, 1310–1320.

Koressaar, T. and Remm, M. (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics* **23**, 1289–1291.

Lau, H.Y. and Botella, J.R. (2017) Advanced DNA-based point-of-care diagnostic methods for plant diseases detection. *Front Plant Sci* **8**, 1–14.

Mulè, G., Susca, A., Stea, G. and Moretti, A. (2004) Specific detection of the toxigenic species *Fusarium proliferatum* and *F. oxysporum* from asparagus plants using primers based on calmodulin gene sequences. *FEMS Microbiol Lett* **230**, 235–240.

Ndunguru, J., Taylor, N.J., Yadav, J., Aly, H., Legg, J.P., Aveling, T., Thompson, G. and Fauquet, C.M. (2005) Application of FTA technology for sampling, recovery and molecular characterization of viral pathogens and virus-derived transgenes from plant tissues. *Virol J* **2**, 45.

Pallez, M., Pasquali, M., Bohn, T., Hoffmann, L. and Beyer, M. (2014) Validation of a quick PCR method suitable for direct sequencing: identification of *Fusarium* fungal species and chemotypes for preventive approaches in food safety. *Food Tech Biotech* **52**, 351–358.

Sasaki, K., Nakahara, K., Tanaka, S., Shigyo, M. and Ito, S. (2015a) Genetic and pathogenic variability of *Fusarium oxysporum* f. sp. isolated from onion and Welsh onion in Japan. *Phytopathology* **105**, 525–532.

Sasaki, K., Nakahara, K., Shigyo, M., Tanaka, S. and Ito, S. (2015b) Detection and quantification of onion isolates of *Fusarium oxysporum* f. sp. cepae in onion plant. *J Gen Plant Pathol* **81**, 232–236.

Suzuki, S., Taketani, H., Kusumoto, K.-I. and Kashiwagi, Y. (2006) High-throughput genotyping of filamentous fungus *Aspergillus oryzae* based on colony direct polymerase chain reaction. *J Biosci Bioeng* **102**, 572–574.

Taylor, A., Vagany, V., Barbara, D.J., Thomas, B., Pink, D.A.C., Jones, J.E. and Clarkson, J.P. (2013) Identification of differential resistance to six *Fusarium oxysporum* f. sp. *cepae* isolates in commercial onion cultivars through the development of a rapid seedling assay. *Plant Pathol* **62**, 103–111.

Taylor, A., Vágány, V., Jackson, A.C., Harrison, R.J., Rainoni, A. and Clarkson, J.P. (2016) Identification of pathogenicity-related genes in *Fusarium oxysporum* f. sp. *cepae*. *Mol Plant Pathol* **17**, 1032–1047.

Validov, S.Z., Kamilova, F.D. and Lugtenberg, B.J. (2011) Monitoring of pathogenic and non-pathogenic *Fusarium oxysporum* strains during tomato plant infection. *Microbial Biotech* **4**, 82–88.

Villari, C., Tomlinson, J.A., Battisti, A., Boonham, N., Capretti, P. and Faccoli, M. (2013) Use of loop-mediated isothermal amplifIcation for detection of *Ophiostoma clavatum*, the

primary blue stain fungus associated with *Ips acuminatus*. *Appl Environ Microbiol* **79**, 2527–2533.

Wang, A., Islam, M.N., Johansen, A., Haapalainen, M., Latvala, S. and Edelenbos, M. (2019) Pathogenic *Fusarium oxysporum* f. sp. *cepae* growing inside onion bulbs emits volatile organic compounds that correlate with the extent of infection. *Postharvest Biol Technol* **152**, 19–28.

Yu, J., Shen, D., Dai, T., Lu, X., Xu, H. and Dou, D. (2019) Rapid and equipment-free detection of *Phytophthora capsici* using lateral flow strip-based recombinase polymerase amplification assay. *Lett Appl Microbiol* **69**, 64–70.