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DETECTION OF *PENICLLIUM OXALICUM* IN GRAPES WITH A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY

*Elisabeth I. Vogt, Veronika M. Kupfer, Julia D. Bechtner, Lisa M. Frisch, Rudi F. Vogel, Ludwig Niessen**

Address(es):
Technische Universität München, Lehrstuhl für Technische Mikrobiologie, 85354 Freising-Weihenstephan, Germany.

*Corresponding author: niessen@wzw.tum.de

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ABSTRACT

P. oxalicum is known as plant pathogen, mycotoxin producer and was suggested to be involved in the induction of champagne gushing. Early detection of the fungus enforces decision making in the wine industry as to how grapes will be further processed. A loop-mediated isothermal amplification (LAMP) assay for *P. oxalicum* was established with a detection limit of 100 pg genomic DNA per reaction. The assay was highly specific for *P. oxalicum* DNA. The assay was applied for the detection of the fungus on artificially infected grapes using surface washings as target for the LAMP reaction. Screening of grape samples from different European countries revealed the (rare) presence of *P. oxalicum* on grapes harvested in Italy and Portugal. We suggest the developed LAMP assay as a promising new tool for the rapid, robust, highly specific and sensitive as well as cost efficient detection of *P. oxalicum* on grapes used in the wine and champagne industry.

Keywords: LAMP, *Penicillium oxalicum*, Grape, Isothermal amplification, Detection

INTRODUCTION

Penicillium oxalicum is an ubiquitous fungus occurring in different habitats like e.g. soil, corn, tomatoes, cucumber or grapes (**Currie and Thom, 1915; Mislivec and Tuite, 1970a; Jarvis *et al.*, 1990; O'Neill *et al.*, 1991; Sage *et al.*, 2002; Serra *et al.*, 2006; Kwon *et al.*, 2008; Umemoto *et al.*, 2009**). Due to its ability to grow in a wide temperature range of 8-37 °C with an optimal growth temperature between 25-30 °C (**Mislivec and Tuite, 1970b; O'Neill *et al.*, 1991**) it is common in temperate, subtropical or tropical regions. The fungus can act as pathogen in previously wounded plants (**Koehler and Holbert, 1930; O'Neill *et al.*, 1991; Kwon *et al.*, 2008**) and produces the mycotoxin secalonic acid (**Steyn, 1970; Ehrlich *et al.*, 1982**). Moreover, the fungus is used as biocontrol agent against the wilt pathogen *Fusarium oxysporum* (**Cal *et al.*, 1995**). Recent studies in our lab revealed also a linkage between *P. oxalicum* isolated from grapes and the induction of gushing in carbonated water as a model system for sparkling wine (**Vogt *et al.*, 2017**). Therefore, this fungus might be of tremendous relevance for the sparkling wine industry in the future. Detection and identification of *P. oxalicum* is routinely conducted macroscopically and microscopically (**Mislivec and Tuite, 1970a; Kwon *et al.*, 2008**). The methods are highly time-consuming and require a specialist mycological knowledge. Amplification and sequencing of the β-tubulin gene via PCR is another approach to identify *Penicillium* species (**Samson *et al.*, 2004**). However, despite the fact that now deep insight into the micromorphology and fungal taxonomy is necessary, PCR and the interpretation of sequencing results are still time-consuming, involve sophisticated laboratory equipment, and require elaborate sample preparation. Therefore, we assume that an alternative method for rapid detection of the fungus would be highly appreciated by grape and wine producers, which can be applied easily on-site.

Notomi *et al.* (2000) developed an alternative DNA-based technique called loop-mediated isothermal amplification (LAMP) for such applications. In this method the large fragment of the *Bst* DNA polymerase, originally isolated from *Geobacillus stearothermophilus*, is used for DNA amplification under isothermal conditions. Lacking 5´-3´exonuclease activity, this truncated DNA polymerase exhibits strand displacement activity during primer-initiated DNA polymerization. Consisting of three steps – an initial step to generate a primary LAMP product, cycling amplification, and elongation – the LAMP reaction needs a set of four specific primers in addition to the particular DNA polymerase. Two inner primers (FIP, BIP) and two outer primers (F3, B3) with six binding sites recognize distinct target sequences resulting in a highly specific amplification reaction. Two additional primers (loop primers; LF, LB) can be added optionally to the LAMP reaction to increase DNA amplification and therefore reaction speed (**Nagamine *et al.*, 2002**). Instead of using agarose gel electrophoresis (AGE) for product detection, positive LAMP assays can be easily detected in-tube e.g. by using indicator dyes such as calcein (**Tomita *et al.*, 2008**) or pH-sensitive indicators (**Tanner *et al.*, 2015**). Several applications for LAMP assays for the detection and identification of fungi have been recently described (see the review of **Niessen (2015)**). So far only LAMP assays for detection of *P. marneffei* and *P. nordicum* have been established to detect species within *Penicillum* (**Sun *et al.*, 2010; Ferrara *et al.*, 2015**). Therefore, the aim of the current study was the development and application of a LAMP assay for the detection of *P. oxalicum* on grapes. Besides its applicability to the identification of pure fungal cultures and conidia, also samples of artificially and naturally contaminated grapes should be analyzed after simple and rapid sample preparation. Finally, the developed assay should be applied to assess the distribution and frequency of *P. oxalicum* in European grapes of the 2016 harvest.

MATERIAL AND METHODS

Microorganisms and growth conditions

Microorganisms used in this study are listed in Table 1. For long term preservation fungal cultures were cryo-conserved as described in **Niessen and Vogel (2010)**. As working cultures fungi were grown on malt extract agar (MEA, 3 % (w/v) malt extract, 0.3 % (w/v) soy peptone, pH 5.2) at ambient temperature. Conidial suspensions were produced by harvesting conidia from MEA plate cultures by suspension in 1-4 ml sterile water before counting total conidial numbers in a hemocytometer (Thoma type, 0.1 mm chamber depth).

DNA preparation

For preparation of fungal DNA fungi were grown in ME broth for 5-7 d at ambient temperature. Mycelia were filtered (folded filters, grade: 3 hw, Munktell, Bärenstein, Germany), washed twice with sterile tap water and finally dried on a filter disc (grade: 3 hw, Munktell, Bärenstein, Germany). The peqGOLD Fungal Mini Kit (PEQLAB, Erlangen, Germany) was used for isolation of genomic DNA according to the manufacturer with slight modifications for the disruption of fungal cells. Cell lysis was achieved by addition of lysis buffer (PL1), 0.5 g sterile sea sand (Merck, Darmstadt, Germany) and 0.1 g glass beads (Ø 1.25-1.65 mm; Carl Roth, Karlsruhe, Germany) followed by severe shaking in a

FastPrep®-24 homogenizer (MP Biomedicals; 45 sec, 24*2, 5.5 m/s). Bacterial DNA was prepared using the QIAGEN Genomic DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. After quantification of DNA in a Nano-Drop 1000 spectrophotometer (PEQLAB, Erlangen, Germany) the DNA concentration was adjusted to ~20 ng/µl with sterile PCR grade water, if not otherwise stated.

DNA amplification

The loop-mediated isothermal amplification (LAMP) method was used for *in vitro* enzymatic DNA amplification. For indirect in-tube detection of amplified DNA during the reaction either calcein or neutral red were used as indicators (**Tomita *et al.*, 2008; Tanner *et al.*, 2015**). The mastermix with calcein as indicator was prepared as described in **Denschlag *et al.* (2012)** but with primers specific for the coding gene of PDE_07106 of *Penicillium oxalicum* (1.6 µM FIP-RET21-ID1 5´-TCACCGCAGTTGACGGGTCCCCTTGCACAC TCGTCGTGAC-3´, 1.6 µM BIP-RET21-ID1 5´-CCTCAGGCTGGAGCGGTCAATCTGGCGGCTCTT GTTGTTGA-3´, 0.2 µM F3-RET21-ID1 5´-CTGGACCTTTGGCATCTACC-3´, 0.2 µM B3-RET21-ID1 5´-TGTCGGTGTAAGCAGGGTAG-3´, 0.8 µM LF-RET21-ID4 5´-TGGACTGGGAGGCCTTTTGG-3´, 0.8 µM LB-RET21-ID4 5´-GTCCCGGCAATGGCTTCACC-3´, Eurofins MWG Operon, Ebersberg, Germany). In case of neutral red as indicator the mastermix was modified by using only 1.9 µl 10x LAMP buffer and 1.5 µl 3 mM aqueous neutral red instead of calcein per reaction. Mastermixes were distributed into 200 µl Multiply®-µStrip Pro 8-strip PCR tubes (Sarstedt, Nümbrecht, Germany) in a separate room with a separate set of pipettes, before 5 µl of sample DNA solution were added with another set of pipettes in another room. Sterile, deionized water was applied as negative control and DNA solution of *P. oxalicum* TMW 4.2539 (~100 ng/µl) as positive control. Sterile filter tips (peqGOLD Safeguard™ filter tips, PEQLAB, Erlangen, Germany) were used for all liquid handling throughout the study. After incubation at 63 °C for 60 min in a Mastercycler®Gradient thermal cycler (Eppendorf, Hamburg, Germany) or a water bath the reaction tubes were visually checked for a color change (in case of neutral red) or for the occurrence of green fluorescence under a 365 nm UV lamp (in case of calcein). Results were documented with a hand-held digital camera.

Direct analysis of artificially/naturally contaminated grapes

In case of artificial contamination, table grapes from a commercial supermarket were surface sterilized in 70 % ethanol for 5 min followed by sodium hypochlorite (1 % active chlorine) for 30 s with two washing steps with deionized water after each treatment. The infection of grapes was conducted by punching with a disposable needle (Sterican® Ø 0.60x80 mm, B. Braun, Melsungen, Germany) previously dipped into a conidial suspension (~10^8 conidia per ml). Grapes stung with a disposable needle dipped in deionized water were used as negative control. Infected grapes were individually incubated at ambient temperature in sterile 50 ml reaction tubes (Sarstedt, Nümbrecht, Germany) until assessment. For the LAMP analysis 1.5 ml of sterile tap water supplemented with 1 % (v/v) Tween 20 (GERBU, Heidelberg, Germany) was added to an artificially infected grape or a grape sample (3-6 grapes of a bunch randomly picked) from a vineyard. After manual shaking for 1 min, the supernatant was transferred to a 2 ml reaction tube (Sarstedt, Nümbrecht, Germany) and centrifuged for 5 min with 10,000 x *g* at ambient temperature. Two washing steps with sterile tap water were applied to remove the Tween 20 before the resulting pellet was resuspended in 300 µl sterile deionized water. After addition of glass beads (0.1 g Ø 0.5 mm, Scientific industries, New York USA; 0.3 g Ø 1.25-1.65 mm, Carl Roth, Karlsruhe, Germany) samples were shaken severely for 10 min on a vortex mixer. Subsequently, 5µl of supernatant were added to a LAMP mastermix and incubated as described previously.

RESULTS AND DISCUSSION

Design of LAMP primers

For the loop-mediated isothermal amplification of *P. oxalicum* DNA six primers were designed using the Primer Explorer V.4 software tool available on the Eiken Genome site (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). The gene coding for protein PDE_07106 (GenBank accession no. EPS32147.1) of *P. oxalicum* had a length of 438 bp. The nucleotide sequence was used to generate five primer sets for LAMP. From these the primer set RET21 ID1 was selected for external as well as internal primers. A set of loop primers termed RET21 ID4 was designed using the loop-primer design function of the Primer Explorer V.4 with the primer information file generated for primer set RET21 ID1. The specificity of the designed primer set was examined by *in silico* testing with the nucleotide Blast search tool on the NCBI database revealing no significant hits (alignment score ≤ 40) to species other than *P. oxalicum* as to October 2016. The positioning and orientation of the used LAMP primers and their complementarity to their target DNA are displayed in Fig. 1.

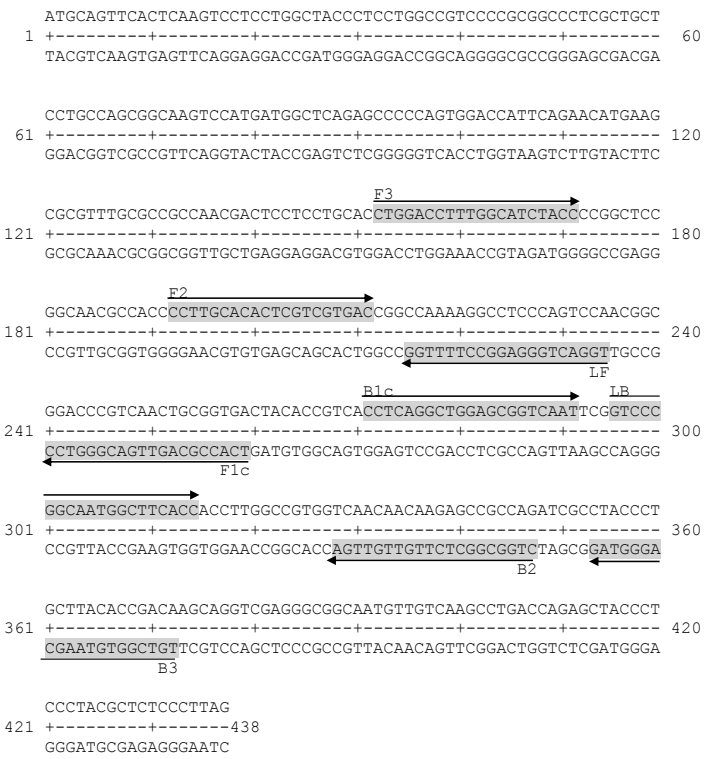

Figure 1 Double-stranded nucleotide sequence of the gene coding for protein PDE_07106 in *P. oxalicum*. Binding sites of the LAMP primers used in this study are marked in grey. Orientation of primers is indicated by arrows.

Optimization, sensitivity and specificity of the LAMP assay

The LAMP assay for *P. oxalicum* was optimized starting with the conditions described by **Denschlag *et al.* (2012)** except that neutral red was used as indicator instead of calcein (**Tanner *et al.*, 2015**) and primers were those designed in this study. The optimal reaction temperature was determined using 100 ng per reaction of genomic DNA of *P. oxalicum* TMW 4.2539 as template. Tubes were incubated in a thermal cycler at temperatures ranging from 55.0-75.4 °C for 1 h. A color change from faint orange to pink indicating a positive reaction could be seen in LAMP assays incubated at 55.0-68.5 °C (Fig. 2). Higher temperatures (≥ 71.0 °C) inhibited the LAMP assay completely. Most intense pink coloration occurred in a temperature range between 58.3-65.9 °C. Accordingly, 63 °C was chosen as the standard temperature for isothermal incubation in all further experiments.

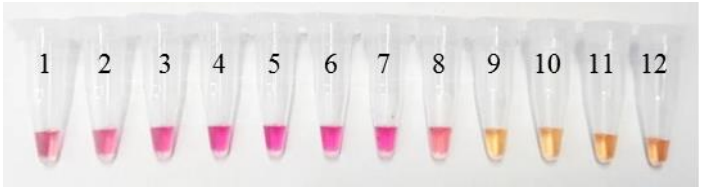

Figure 2 Assessment of LAMP assay reaction temperature. LAMP with purified genomic DNA of *P. oxalicum* TMW 4.2539 incubated at 1 = 55 °C, 2 = 55.3 °C, 3 = 56.5 °C, 4 = 58.3 °C, 5 = 60.6 °C, 6 = 63.2 °C, 7 = 65.9 °C, 8 = 68.5 °C, 9 = 71.0 °C, 10 = 73.1 °C, 11 = 74.6 °C, 12 = 75.4 °C for 1 h. Color change of neutral red from faint orange to pink indicates a positive reaction.

The sensitivity of the developed LAMP assay was assessed by addition of tenfold serial dilutions of genomic DNA of three *P. oxalicum* isolates (TMW 4.2539, TMW 4.2552, TMW 4.2553) ranging from 100 ng to 100 fg, respectively (Fig. 3A). Simultaneously, the incubation time until the color changed was recorded. Results revealed that no color change occurred in reactions with DNA concentrations below 10 pg per reaction or with water added as negative control. A DNA concentration of 100 pg per reaction could be detected consistently, whereas 10 pg per reaction were not amplified in all replicates.

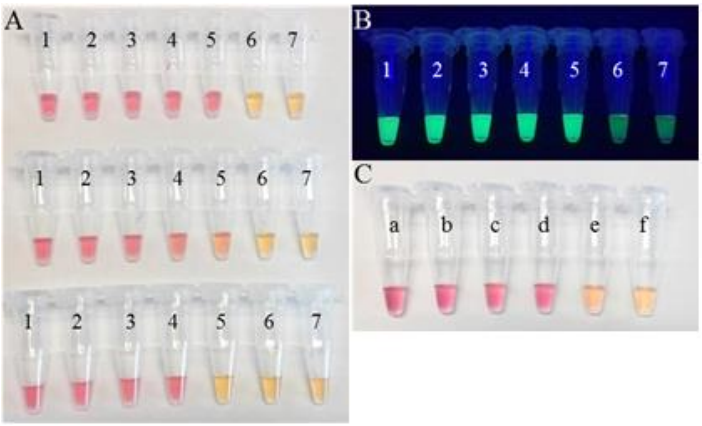

Figure 3 Assessment of LAMP assay sensitivity. (A) Purified genomic DNA of three *P. oxalicum* isolates (TMW 4.2539, TMW 4.2552, TMW 4.2553) was used as template in tenfold serial dilution. Color change of neutral red from faint orange to pink indicates a positive reaction. (B) Purified genomic DNA of *P. oxalicum* TMW 4.2553 was used as template in tenfold serial dilution. Bright green fluorescence of calcein under UV_{365nm} light indicates a positive reaction. (C) Conidial suspension of *P. oxalicum* TMW 4.2539 was directly used as template in tenfold serial dilution. Color change to pink indicates a positive reaction. 1 = 100 ng/reaction (rxn), 2 = 10 ng/rxn, 3 = 1 ng/rxn, 4 = 100 pg/rxn, 5 = 10 pg/rxn, 6 = 1 pg/rxn, 7 = 100 fg/rxn, a = $2.5*10^6$ conidia/rxn, b = $2.5*10^5$ conidia/rxn, c = $2.5*10^4$ conidia/rxn, d = $2.5*10^3$ conidia/rxn, e = $2.5*10^2$ conidia/rxn, f = $2.5*10^1$ conidia/rxn

A positive signal for the set detection limit of 100 pg could be detected within 60 min. Longer incubation time did not improve sensitivity but could lead to false positive reactions. The intensity of a positive signal was not depending on the DNA concentration used. Calcein was tested as another indicator. Here, a positive reaction was represented by a bright green fluorescence under UV light at a wavelength of 365 nm. Under this condition a consistent detection limit was also set to 100 pg per reaction within 60 min incubation time (Fig. 3B). Conidial suspensions of *P. oxalicum* TMW 4.2539 were also analyzed in the LAMP assay with regard to sensitivity. Tenfold dilutions ranging from $2.5*10^6$-$2.5*10^1$ conidia per reaction were used as samples. Results showed that a minimum of $2.5*10^3$ conidia per reaction lead to a positive LAMP reaction with neutral red as indicator (Fig. 3C). Using calcein as indicator, the assay was unsteadily able to detect $5*10^2$ conidia per reaction (data not shown).

Assessment of specificity of the LAMP primer set for the detection of *P. oxalicum* was conducted using genomic DNA (100 pg per reaction) of 77 microorganisms isolated from or associated with grapes or wine (Table 1). Among them were 8 bacterial species, 24 isolates of yeast representing 14 different species as well as 45 isolates of filamentous fungi covering 36 species. All bacterial and yeast DNA was tested negative with the LAMP assay using both indicators. Aside from the DNA of three *P. oxalicum* isolates also the DNA of *P. burgense* TMW 4.2541, *P. raistrickii* TMW 4.2554 and *P. paneum* TMW 4.2542 consistently resulted in a positive reaction. The result of LAMP assays with DNA of *P. coalescens* TMW 4.2537, *P. variabile* TMW 4.2560 and *P. restrictum* TMW 5.2555 were hard to construe as the color change respectively the fluorescence was only slight, the positive signal occurred only with one of the two indicators or results were not readily reproducible.

Table 1 Microorganisms used in this study isolated from or associated with grapes (see references) and their reaction in the LAMP assay with different indicators. + = green fluorescence under UV_{365nm} (calcein), pink (neutral red), - = no fluorescence under UV_{365nm} (calcein), faint orange (neutral red)

	Strain No	Clone ID	Substrate	Results in LAMP assay	
				calcein	neutral red
Bacteria					
Lactobacillus backii	Not stated	TMW[a] 1.2004	brewery[1]	-	-
Lb. brevis	Not stated	TMW 1.1370	honey fermentation[1]	-	-
Lb. lindneri	Not stated	TMW 1.1433	brewery[1]	-	-
Lb. paracasei subsp. paracasei	F19	TMW 1.1434	human colon[1]	-	-
Lb. paracollinoides	DSM[b] 20197	TMW 1.1979	beer[1]	-	-
Lb. plantarum	TMW 1.277	TMW 1.277	palmwine[1]	-	-
Pediococcus clausenii	Not stated	TMW 2.60	brewery[1]	-	-
Pc. damnosus	Not stated	TMW 2.1641	brewery[1]	-	-
Yeasts					
Aureobasidium pullulans	TMW 3.0393	TMW 3.0393	grape leaf	-	-
Au. pullulans	TMW 3.0400	TMW 3.0400	grape leaf	-	-
Bulleromyces albus	TMW 3.0524	TMW 3.0524	grape leaf	-	-
Candida lambica	TMW 3.0645	TMW 3.0645	bird cherry blossom[2]	-	-
C. lambica	TMW 3.0646	TMW 3.0646	bird cherry blossom[2]	-	-
C. pulcherrima	TMW 3.0643	TMW 3.0643	bird cherry blossom[2]	-	-
C. pulcherrima	TMW 3.0644	TMW 3.0644	bird cherry blossom[2]	-	-
C. sake	TMW 3.0710	TMW 3.0710	white grape juice	-	-
Cryptococcus heimayensis	TMW 3.0525	TMW 3.0525	grape leaf	-	-
Cr. heimaeyensis	TMW 3.0526	TMW 3.0526	grape leaf	-	-
Cr. hungaricus	TMW 3.0528	TMW 3.0528	grape leaf	-	-
Cr. tephrensis	TMW 3.397	TMW 3.397	grape leaf	-	-
Cr. victoriae	TMW 3.0389	TMW 3.0389	grape leaf	-	-
Cr. victoriae	TMW 3.0390	TMW 3.0390	grape leaf	-	-
Cr. victoriae	TMW 3.0398	TMW 3.0398	grape leaf	-	-
Cr. victoriae	TMW 3.0563	TMW 3.0563	grapes	-	-
Cr. victoriae	TMW 3.0564	TMW 3.0564	grapes	-	-
Cr. wieringae	TMW 3.0391	TMW 3.0391	grape leaf	-	-
Hanseniaspora uvarum	BLQ[c] H6	TMW 3.0700	grapes	-	-
Saccharomyces cerevisiae	TMW 3.0709	TMW 3.0709	red grape juice	-	-
Saccharomycodes ludwigii	CBS[d] 820	TMW 3.0020	grape must	-	-

Sporobolomyces roseus	TMW 3.0394	TMW 3.0394	grape leaf	-	-
S. roseus	TMW 3.0395	TMW 3.0395	grape leaf	-	-
Trichosporon spp.	TMW 3.0473	TMW 3.0473	grapes	-	-
Filamentous fungi					
Aspergillus aculeatus	TMW 4.2390	TMW 4.2390	soil debris[4]	-	-
A. carbonarius	M324	TMW 4.1512	coffee[1,3,4,5]	-	-
A. flavus	TMW 4.1829	TMW 4.1829	nutmeg[3,5,5]	-	-
A. fumigatus	CBS 113.55	TMW 4.0623	human lung[3,4,5]	-	-
A. japonicus	CBS 114.51	TMW 4.1627	-[3,5]	-	-
A. japonicus	TMW 4.1776	TMW 4.1776	oil tanker[3,5]	-	-
A. niger	CBS 101.698	TMW 4.1068	mesocarp finga - coffee bean[1,3]	-	-
A. parasiticus	CBS 126.62	TMW 4.1768	peanut[3]	-	-
A. ustus	TMW 4.1365	TMW 4.1365	-[4,5]	-	-
P. adametzioides	TMW 4.2529	MUM[e] 14.26	grapes	-	-
P. aurantiogriseum	TMW 4.2536	MUM 14.24	grapes	-	-
P. bilaii	TMW 4.2528	MUM 14.25	grapes	-	-
P. brevicompactum	TMW 4.2545	MUM 14.27	grapes	-	-
P. burgense	TMW 4.2541	MUM 14.44	red grape must	+	+
P. citrinum	TMW 4.2547	MUM 14.29	grapes	-	-
P. coalescens	TMW 4.2537	MUM 14.37	grapes	-/+	-/+
P. crustosum	TMW 4.2530	MUM 14.31	red grape must	-	-
P. crustosum	TMW 4.2548	MUM 14.30	grapes	-	-
P. echinulatum	TMW 4.2549	MUM 14.32	grapes	-	-
P. glabrum	TMW 4.2531	MUM 14.33	grapes	-	-
P. griseofulvum	TMW 4.2532	MUM 14.34	grapes	-	-
P. janczewskii	TMW 4.2550	MUM 14.35	grapes	-	-
P. minioluteum	TMW 4.2533	MUM 14.36	grapes	-	-
P. novae-zelandiae	TMW 4.2551	MUM 14.39	grapes	-	-
P. olsonii	TMW 4.2538	MUM 14.40	grapes	-	-
P. oxalicum	TMW 4.2539	MUM 14.41	grapes	+	+
P. oxalicum	TMW 4.2552	MUM 14.42	grape stalk	+	+
P. oxalicum	TMW 4.2553	MUM 14.43	grapes	+	+
P. paneum	TMW 4.2542	MUM 14.47	grapes	+	+
P. purpurogenum	TMW 4.2540	MUM 02.55	grapes	-	-
P. raistrickii	TMW 4.2554	MUM 14.45	grapes	+	+
P. restrictum	TMW 4.2555	MUM 14.46	grapes	-	-/+
P. sclerotiorum	TMW 4.2535	MUM 03.39	grapes	-	-
P. sclerotiorum	TMW 4.2556	MUM 14.48	grapes	-	-
P. simplicissimum	TMW 4.2557	MUM 14.49	grapes	-	-
P. spinulosum	TMW 4.2543	MUM 14.51	grapes	-	-
P. spinulosum	TMW 4.2558	MUM 14.50	grape must	-	-
P. thomii	TMW 4.2559	MUM 14.52	grapes	-	-
P. vagum	TMW 4.2534	MUM 14.38	grapes	-	-
P. variabile	TMW 4.2560	MUM 14.53	white grape must	+	-
P. variabile	TMW 4.2561	MUM 14.54	red grape must	-	-
P. verrucosum	TMW 4.2546	MUM 14.28	green grapes	-	-
P. verrucosum	TMW 4.2585	ITEM[f] 3716	white table grapes	-	-
P. verrucosum	TMW 4.2586	ITEM 3717	white table grapes	-	-
P. waksmanii	TMW 4.2544	MUM 14.55	grapes	-	-

[a] TMW = Technische Mikrobiologie Weihenstephan, Freising, DE; [b] DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, DE; [c] BLQ = Forschungsinstitut für Brau- und Lebensmittelqualität, Freising, DE; [d] CBS = Centraalbureau voor Schummelcultures, Utrecht, NL; [e] MUM = Micoteca da Universidade do Minho, Braga, PT; [f] ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, IT[1] = **Barata *et al.* (2012), [2] = Jolly *et al.* (2003), [3] = Bau *et al.* (2005), [4] = Sage *et al.* (2002), [5] = Serra *et al.* (2006)**

Application of the LAMP assay to artificially contaminated grapes

In order to evaluate the applicability of the LAMP assay for the detection of *P. oxalicum*, individual artificially contaminated grapes were examined over a 22 d period in daily intervals using calcein as indicator dye (Table 2). Grapes were analyzed after simple mechanical pre-treatment as described previously. Positive LAMP signals for *P. oxalicum* were detected at day two and following days post infection in grapes and in the positive control with pure genomic DNA of *P. oxalicum* TMW 4.2539. Uninfected grapes or grapes infected with other filamentous fungi as well as the negative control with water instead of DNA did not show any positive signal in the LAMP assay.

Table 2 Results of LAMP assay with artificially infected grapes within an incubation time of 22 d. + = green fluorescence under UV_{365nm}, - = no fluorescence under UV_{365nm}; TMW 4.2539, TMW 4.2552, TMW 4.2553 = *P. oxalicum*, TMW 4.1068 = *A. niger*, TMW 4.2531 = *P. glabrum*, not infected = grapes "infected" with deionized water, pos. co. = genomic DNA of *P. oxalicum* TMW 4.2539 (100 ng/rxn) as template, neg. co. = deionized water as template

	TMW 4.2539	TMW 4.2552	TMW 4.2553	TMW 4.1068	TMW 4.2531	not infected	pos. co.	neg. co.
day 0	-	-	-	-	-	-	+	-
day 1	-	+	-	-	-	-	+	-
day 2	+	+	+	-	-	-	+	-
day 3	+	+	+	-	-	-	+	-
day 4	+	+	+	-	-	-	+	-
day 5	+	+	+	-	-	-	+	-
day 6	+	+	+	-	-	-	+	-
day 9	+	+	+	-	-	-	+	-
day 12	+	+	+	-	-	-	+	-
day 15	+	+	+	-	-	-	+	-
day 19	+	+	+	-	-	-	+	-
day 22	+	+	+	-	-	-	+	-

Detection of *P. oxalicum* on grape samples of different origin

61 grape samples (each consisting of 2-3 bunches of grapes) from different European countries (Germany, Austria, Italy, Spain and Portugal) were tested for the presence of *P. oxalicum* with the developed LAMP assay and neutral red as indicator (supplementary material Table S1). Positive reactions indicating the presence of *P. oxalicum* was found in one grape sample from Portugal (Fig. 4) and in two different samples of Italian grapes. Samples collected from vineyards in Spain, Austria, and Germany showed no positive LAMP results indicating they were virtually free from infection by *P. oxalicum*.

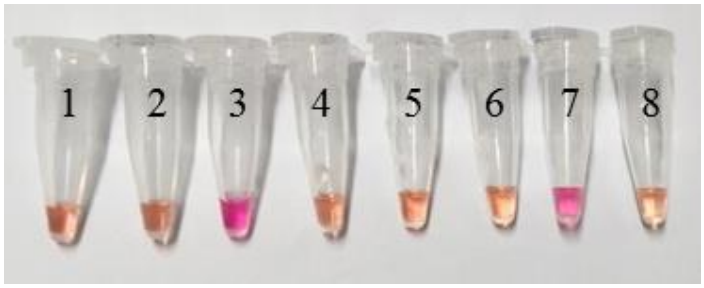

Figure 4 Assessment of some grape samples from Portugal with the developed LAMP assay.
LAMP with crude washing extracts of grape samples from Portugal as template. 1 = variety Cerceal, location Bairrada, bunch 1; 2 = Bical, Bairrada, bunch 2; 3 = Fernão Pires, Bairrada, bunch 2; 4 = Bical, Bairrada, bunch 1; 5 = Trajadura, Vinhos Verde, bunch 1; 6 = Arinto, Bairrada, bunch 3; 7 = positive control; 8 = negative control. Color change of neutral red from faint orange to pink indicates a positive reaction.

DISCUSSION

P. oxalicum is known as an opportunistic plant pathogen and as a mycotoxin producer. Only recently data were published, which indicate that it may also be involved in the induction of gushing in champagne and sparkling wine (**Vogt *et al.*, 2017**). To date, no on-site test is available for the rapid, robust and specific detection of this fungus. In order to improve this situation we have developed, optimized and applied a DNA amplification assay on the basis of the loop-mediated isothermal amplification technology. The primers used in this study target the gene coding for protein PDE_07106 in *P. oxalicum*. A correlation between this protein and the induction of gushing has been suggested in a previous study (**Vogt *et al.*, 2017**). Therefore, the application of the PDE_07106 coding sequence as target for a molecular detection assay appears to be a logical step for screening of samples in regard to a potential gushing inducing activity.
The LAMP assay was functional within a broad temperature range of 55.0-68.5 °C (Fig. 2). **Francois *et al.* (2011)** also reported high robustness of their LAMP reaction to detect typhogenic serotypes of *Salmonella enterica* strains within a temperature interval of 10 °C and a range of 2 pH units. Robustness against variable buffer and incubation conditions is an important feature of assays which should run with low processed samples under on-site and thus variable conditions. For the current assay, an optimal temperature of 63 °C was chosen. This temperature lies within the temperature optimum of the *Bst* DNA polymerase which is between 60-65 °C according to the manufacturer. Even if the most intense coloration of reactions occurred in a broad temperature range of 58.3-65.9 °C, too low incubation temperatures should be avoided since they favor non-specific binding of primers to DNA. Calcein or neutral red were used as alternative indirect indicators for the amplification of DNA during the LAMP reaction. Both dyes follow a different mechanism for the color change as reactions switch from negative to positive. In the case of calcein, the complexation of pyrophosphate anions ($P_4O_7^{4-}$) with manganese cations (Mn^{2+}) leads to the recovery of fluorescence of the previously quenched calcein fluorophore. Without DNA amplification no pyrophosphate is produced leaving manganese to quench the calcein fluorescence. In contrast, neutral red as a pH indicator displays a positive reaction by a pH depending color change from yellow-orange (pH 8) to pink (pH 6.8) which allows differentiation of positive and negative reactions by visible detection under day light conditions. During DNA amplification protons are released causing an acidification of the reaction mixture. In order to allow for such acidification, buffering of the master mix had to be adjusted by lowering the concentration of the MOPS-buffer used in LAMP reactions. With both indicators an in-tube detection via the naked eye was possible directly following the LAMP reaction without any further manipulation needed, except the necessity of a UV lamp for visual calcein fluorescence detection. Most importantly, cross contaminations due to opening of the reaction tube for time-consuming AGE can be avoided. Nonetheless, we employed AGE and subsequent dimidium bromide staining for the confirmation of DNA amplification in LAMP reactions. Results obtained during direct signal detection by AGE were in accordance with results obtained by indirect visual detection either using neutral red or calcein as marker dyes (data not shown). With the developed LAMP assay a minimum of 10 pg of purified genomic *P. oxalicum* DNA per reaction could be detected (Fig. 3). According to the genome size of *P. oxalicum* found in the NCBI database of ~30 Mb (*Penicillium oxalicum* 114-2 genome size: 30.18 Mb) the minimum detected amount of DNA is equivalent to a copy number of ~300. Consequently, a positive reaction requires the presence of around 300 nuclei from mycelial cells or fungal spores. The minimum detected amount for conidia after direct addition to the LAMP master mix before incubation, was only marginally higher. 500-2500 conidia are sufficient to result in a positive LAMP signal. Intensity of color or fluorescence was independent from the initial concentration of target DNA as long as the mentioned detection limits are exceeded.
Proper binding of primers to their six target sequences is one requirement for a functional LAMP assay, coincidentally ensuring high specificity. Therefore, different microorganisms commonly prevailing on grapes or in vineyard environments according to the literature were assessed for cross reactions in the *P. oxalicum* specific LAMP assay (Table 1). Results showed that the LAMP assay for *P. oxalicum* is highly specific for its target species. Nonetheless, one isolate each of *P. burgense*, *P. paneum*, and *P. raistrickii* led to a positive result in the LAMP assay. A comparison of the amino acid sequence of PDE_07106 of *P. oxalicum* with the annotated proteome of the three mentioned species revealed no significant similarities. However, less than 80 proteins for each of these three species have been annotated (status October 2016) so that the occurrence of a cross-reaction with closely related but yet un-annotated proteins could explain the phenomenon. As *P. burgense*, *P. paneum*, and *P. raistrickii* are even more rare in vineyards compared to *P. oxalicum* (**Sage *et al.*, 2002; Serra *et al.*, 2003; Bau *et al.*, 2005; Bejaoui *et al.*, 2006; Serra *et al.*, 2006**), false positive reactions can be supposed to occur only rarely.
P. oxalicum could be detected after at least two days of incubation in artificially infected grapes (Table 2). Consequently, the detection was possible before the development of visually detectable mold symptoms. It has been demonstrated that simple washing of samples and using the crude washing as target in the LAMP assay is sufficient to provide the minimum DNA concentrations needed for detection. This is a maximum simplification of sample processing as compared to other methods which need time-consuming and expensive DNA extraction steps previous to analysis. Such rapid and simple sample processing together with LAMP based detection greatly promotes early assessment of samples for *P. oxalicum* and will therefore be highly beneficial for vine growers, wine and champagne producers because it enables informed decisions about further processing of grapes and grape derived products. Furthermore, the LAMP assay seems not affected by substances originating from grapes as the uninfected grapes led to no positive signal in the reaction within the investigation period of 22 days. A high tolerance against inhibitory substances has previously been reported for LAMP in other studies (**Kaneko *et al.*, 2007; Nixon *et al.*, 2014**). LAMP assays for the detection of another fungal species (*B. cinerea*) on grapes were already described by **Tomlinson *et al.* (2010)** and **Duan *et al.* (2014)**. Albeit these assays require more expensive and cumbersome DNA extraction, a higher sensitivity is reached with purified DNA than with crude extracts.
The developed LAMP assay was used to assess for the presence of *P. oxalicum* in 61 grape samples (each consisting of 2-3 bunches of grapes) harvested in autumn 2016 from vineyards in Austria, Germany, Italy, Portugal and Spain. *P. oxalicum* could be detected only in three of the samples, which had been harvested in Italy (2) and Portugal (1). According to different European mycological studies about the mycobiota on grapes, *P. oxalicum* occurred occasionally in Portugal (**Serra *et al.*, 2003; Serra *et al.*, 2006**), France (**Sage *et al.*, 2002; Bejaoui *et al.*, 2006**), Spain (**Bau *et al.*, 2005**) and Italy (**Lorenzini *et al.*, 2016**). To the best of our knowledge, data concerning the occurrence of *P. oxalicum* in Germany or Austria are not available. Considering an optimal growth temperature of 25-30 °C for pure cultures of the fungus (**Mislivec and Tuite, 1970b; O'Neill *et al.*, 1991**) it is more likely that it will have a preference for Mediterranean regions rather than Middle- or North European regions. Even if no inhibition from uninfected grapes has been detected during the current study, the possibility of false negative results must also be taken into account. **Luo *et al.* (2014)** reported an inhibition of the LAMP reaction due to a crude sample washing from peanuts and other non-grape samples. According to their study a circumvention of this problem was achieved by diluting the samples prior to LAMP analysis. However, an internal control to distinguish true negative from false negative results would be advisable. Unfortunately, so far no convenient internal control for a LAMP assay has been described due to the fact that two different amplification products cannot be differentiated in a LAMP reaction (**Rodríguez *et al.*, 2015**). Besides inhibitors from the food matrix, also mycotoxins, causing mutations in the target DNA and therefore affecting the primer hybridization, could be a problem for DNA amplification methods (**Paterson and Lima, 2014**). Furthermore, it should be considered that LAMP indicates only the presence of fungal DNA and does not give any information about the viability of the detected organism. Nonetheless, the results of the current study showed the great potential of the developed LAMP assay for the detection of *P. oxalicum*.

CONCLUSION

Results of the current study showed the great potential of the developed LAMP assay for the detection of *P. oxalicum*. Besides purified fungal DNA also spore

suspensions or even (artificially) contaminated grape samples could be assessed. The described LAMP assay gives an expedient alternative to known microbiological or PCR methods.

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