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EVALUATION OF DIFFERENT METHODS OF DNA EXTRACTION FOR DETECTION OF BACTERIUM *Xanthomonas campestris* pv. *Campestris* IN CABBAGE LEAVES

Eliška Peňázová, Aleš Eichmeier, Jana Čechová, Miroslav Baránek, Robert Pokluda

Mendel University in Brno, Czech Republic

Abstract. The bacterium *Xanthomonas campestris* pathovar *campestris* (*Xcc*) as the causal agent of black rot of cruciferous plants causes considerable losses in agricultural yield all over the world. The control of black rot is difficult as well as the determination of *Xcc* on the basis of morphological parameters or by pathogenicity testing. Ten different possibilities for extraction bacterial DNA followed by PCR detection method were tested to optimize PCR protocol. On the basis of ISTA validated method, three sets of primers UBP 1052F-BACR, DLH 120-125 and ZUP 2309-2310 were used. The results of measured concentration and quality of DNA and efficacy for PCR amplification were compared. Finally, three approaches for DNA extraction within *Xanthomonas campestris* pv. *campestris* detection protocol were recommended – commercial kits used for isolation from tissues by Macherey-Nagel and MO BIO and kit intended for microbial cultures by MO BIO.

Key words: black rot, *Brassicaceae*, specific PCR

INTRODUCTION

Black rot of crucifers is a worldwide problem of economic importance. It is characterized by blackened vascular tissues and foliar V-shaped chlorotic to necrotic lesions at the marginal part of leaves. As the disease progress, parenchyma cells surrounding vessels in the main stem turn black. Plants stop growing, become wilted and finally rot [Alvarez 2001].

Corresponding author: Eliška Peňázová, Department of Vegetable Growing and Floriculture, Mendel University in Brno, Faculty of Horticulture, Valtická 337, 691 44 Lednice, Czech Republic, e-mail: xpenazo1@node.mendelu.cz

The organism responsible for this disease, *Xanthomonas campestris* pv. *campestris* (*Xcc*), is an aerobic, gram-negative bacterium with a single polar flagellum. As a microbial culture on nutrient agar, the bacterium produces circular, yellowish colonies with mucous surface. The common strains of *Xcc* are mostly vascular inhabitants that invade plants mainly through hydatodes and wounds [Williams 2007].

Xcc is known to be composed by genetically, serologically and pathologically diverse groups of strains [Bila et al. 2013]. Methods for detection of this bacterium in seeds recommended by the International Seed Testing Association (ISTA) are based on plating of seed extracts onto selective media (FS, mCS20ABN) and non-selective YDC (Yeast extract-dextrose-$CaCO_3$ medium) with subsequent examination of the presence of typical *Xcc* colonies by comparison with the known positive control. The final confirmation of identity of the extracted bacteria is based on pathogenicity test on seedlings of *Brassica* genotype, which is susceptible to *Xcc*. Since 2013, the detection of *Xcc* was extended for DNA markers generated by PCR (polymerase chain reaction) technique as an alternative to the pathogenicity test [ISTA 2013]. This addition could be the reaction to the problem of identification of *Xcc* strains from closely related pathovars of *Xanthomonas campestris*. It is not possible to distinguish individual pathovars on the basis of morphological and biochemical characteristics and it is often difficult by pathogenicity testing as well [Bila et al. 2013].

Compared to conventional diagnostic methods, there are many advantages of using PCR. Primarily it is highly sensitive, relatively simple, and fast detection which does not require the cultivation of examined organisms. PCR is considered as a routine technique and recommended in most protocols developed by the European Union and the European and Mediterranean Plant Protection Organisation – EPPO [Palacio-Bielsa et al. 2009].

This work compares 10 different procedures of DNA extraction from cabbage leaves naturally infected by *Xcc*. Each procedure was evaluated on the basis of concentration and quality of extracted DNA and efficacy of PCR amplification using 3 pairs of primers. Obtained results significantly contribute to the optimization of PCR protocol for effective detection of *Xanthomonas campestris* pv. *campestris* pathogen.

MATERIAL AND METHODS

Source of material and sample preparation. The bacterial isolate used in this study came from naturally infected plants of cabbage *Brassica oleracea* var. *capitata* (L.) cv. Avak from a field in Svijanský Újezd (Liberec region, 50°35'14.13"N, 15°2'37.42"E). Infected leaves were stored at -80°C and symptomatic parts of leaf blades with veins were homogenized to obtain representative samples of a weight 20 g. These samples were used for all protocols of DNA extraction from plant tissues for DNA characterization (samples 1–18).

The microbial culture was also used as a source of DNA. It was obtained by cultivation of the extract of homogenized sample on MPA (meat-peptone agar). After 48 h the resulting colonies were mixed with 0.85% saline and final suspension was used for DNA extraction (samples 19, 20).

For the positive control, the isolate CCM 22 from the Czech Collection of Microorganism (Masaryk University, Brno) was used.

Extraction of DNA. Generally two different approaches were used for DNA extraction from samples containing *Xanthomonas campestris* pv. *campestris* – first by the use of commercial extraction kits, second by solution-based methods (tab. 1). All extractions were performed in duplicates.

The evaluation of commercial kits included products of three companies – QIAGEN (Hilden, Germany), Macherey-Nagel (Düren, Germany) and MO BIO Laboratories, Inc. (Carlsbad, CA, USA). For each of these companies, two types of isolation kits were tested – kit for plant cells, tissues and seeds and kit for animal tissues or cultured cells. Moreover, one kit designed to isolate genomic DNA from microorganisms was added.

The solution-based methods were represented by modified protocols of Ahrens and Seeműller [1992] and Bernatzky and Tanksley [1986]. Moreover, fast protocol based on the thermal and chemical decomposition of plant material by temperature 105°C and mixture of Trizma® Base (Sigma-Aldrich) and Nonidet™ P-40 (Sigma-Aldrich) was also used as described in Klimyuk et al. [1993].

Table 1. Table of used commercial kits and protocols

Sample no.	Recommended input material	Producer/Used protocol
1, 2	plant cells, tissues and seeds	QIAGEN
3, 4	animal tissues or cultured cells	QIAGEN
5, 6	plant cells, tissues and seeds	Macherey-Nagel
7, 8	animal tissues or cultured cells	Macherey-Nagel
9, 10	plant cells, tissues and seeds	MO BIO
11, 12	animal tissues or cultured cells	MO BIO
13, 14	plant tissues and seeds	Ahrens and Seeműller [1992]
15, 16	plant tissues and seeds	Bernatzky and Tanksley [1986]
17, 18	plant tissues and seeds	Klimyuk et al. [1993]
19, 20	microorganism culture	MO BIO

Evaluation of DNA quality and quantity. In order to evaluate the concentration of extracted DNA, the fluorometric measurement was used. PicoGreen stock solution (Molecular Probes, Eugene) was diluted 1 : 200 as per the manufacturer's protocol in Tris-EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH 7.5). One µl of each sample of DNA was mixed together with 49 µl of 1 × TE buffer and 50 µl of diluted PicoGreen solution. The measurement was performed at fluorometer (Turner BioSystems Modulus), with channel Blue (P/N 9200-042), excitation wavelength was 460 nm and emission wavelength 520 nm. The quality of extracted DNA was evaluated based on three parameters: concentration and quality of DNA and suitability for PCR amplification, as intensity, repeatibility and length of amplicons, together with clearness of amplified spectra.

The quality of isolated DNA was controlled by electrophoresis, in 1% agarose gel with 1 × TAE, coloured by GelRed (Biotium, Hayward, USA). Together with analysed samples it was also separated λ-DNA with known concentration.

Primers and PCR amplification. Presented study used three pairs of primers according to the rules of ISTA (International Seed Testing Association) validation. The

universal bacterial primers UBP 1052F and UBP BACR indicate occurrence of bacterial DNA, primers DLH 120 and DLH 125 determine species of *Xanthomonas campestris* and pair of primers ZUP 2309 and ZUP 2310 specifies the pathovar *campestris* (see tab. 2).

Table 2. Primer pairs used in this study

Primer	Sequence (5' to 3')	Length of product	Author
UBP 1052F	GCA TGG TTG TCG TCA GCT CGT	441 bp	Eden et al. [1991]
UBP BACR	TAC GGC TAC CTT GTT ACG ACT T		
DLH 120	CCG TAG CAC TTA GTG CAA TG	619 bp	Berg et al. [2005]
DLH 125	GCA TTT CCA TCG GTC ACG ATT G		
ZUP 2309	AAA TCA GGG GGA TGC GGT GG	370 bp	Rijlaarsdam et al. [2004]
ZUP 2310	TCC GGC CAG GGT CGA TAC AGT G		

The extracted DNA was amplified in total volume of 22 µl. The reaction mixture contained: 17.04 µl of HPLC water, 0.16 µl of dNTPs (25 mM; Promega), 2.5 µl of 10 × ThermoPol Reaction Buffer (New England BioLabs), 0.1 µl of each of primers (100 µM; VBC-Biotech, Austria), 0.1 µl of *Taq* DNA Polymerase (0.5 units; New England BioLabs) and 2 µl of template DNA.

The PCR amplifications were carried out for all pairs of primers as follows: after an initial denaturation by 3 min at 95°C, amplification was performed within 40 cycles of 40 sec denaturation at 95°C, 40 sec of primer annealing at 63°C and 40 sec at 68°C for extension, followed by the final step at 68°C for 5 min.

PCR products were analyzed by electrophoresis in 1.2% agarose gel coloured by GelRed and visualised with a UV transilluminator.

RESULTS

Extraction and evaluation of DNA. The measurements of concentration showed substantial differences between source and kind of kits. A using of kits for plant cells, tissues and seeds by QIAGEN and Macherey-Nagel evinced more homogenous concentration values than kit by MO BIO (tab. 3). In case of kits for animal tissues and cultural cells (tab. 4) low concentration of DNA extracted by QIAGEN was observed. On the other hand, samples 7 and 8 with concentrations 8.11 $ng.\mu l^{-1}$ and 6.15 $ng.\mu l^{-1}$ were evaluated to be applicable for detection.

Table 3. Measured concentrations of DNA ($ng.\mu l^{-1}$) in infected cabbage leaves obtained by kits for plant cells, tissues and seeds

Producer	QIAGEN		Macherey-Nagel		MO BIO	
Sample No.	1	2	5	6	9	10
Concentration	2.84	2.47	2.22	2.58	14.9	5.08

Table 4. Measured concentrations of DNA in infected cabbage leaves (ng.µl^{-1}) obtained by kits for animal tissues and cultured cells

Producer	QIAGEN		Macherey-Nagel		MO BIO	
Sample No.	3	4	7	8	11	12
Concentration	0.10	0.14	8.11	6.15	1.11	3.32

In contrast to commercial kits, the solution-based methods according to Ahrens and Seemuller and Bernatzky and Tankaley yielded higher DNA concentrations (tab. 5). On the other hand, final evaluation can be affected by the quality of extracted samples, because these protocols cannot guarantee the purity of isolated DNA and its successful use in PCR.

Table 5. Measured concentration of DNA [ng.µl^{-1}] in infected cabbage leaves obtained by solution-based methods

Method	Express isolation		Ahrens and Seeműller		Bernatzky and Tanksley	
Sample No.	13	14	15	16	17	18
Concentration	2.57	2.77	26.55	25.23	13.61	10.49

The kit for isolation of genomic DNA from microorganisms achieved satisfactory results with concentrations 4.22 ng.µl^{-1} (sample 19) and 5. 42 ng.µl^{-1} (sample 20).

The measured concentration of DNA was compared with the quality of extracted samples, as revealed by their separation on the agarose gel (fig. 1). Satisfactory results were obtained for samples 1 and 2 isolated by kit for plant cells, samples 11 and 12 by

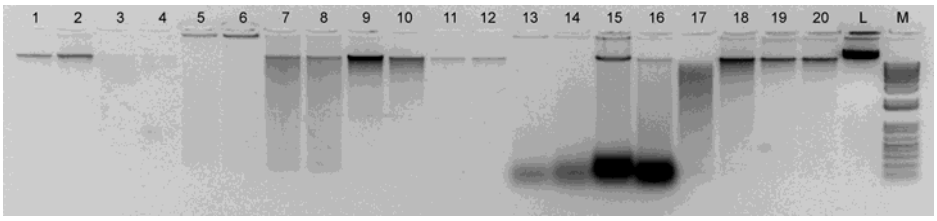

Fig. 1. Quality of extracted DNA (labelling as in the tabs 1 and 2); L – λ; M – 1 Kb Plus DNA Ladder (Invitrogen)

kit for animal tissues and 19 and 20 by kit for microbial cultures. The rest of samples showed considerable fragmentation and absence or too small content of extracted DNA. In case of "fast" isolation (samples 13, 14) and modified protocol according to Ahrens and Seeműller (samples 15, 16) the high amounts of contaminating RNA was proved.

PCR amplification and quality of PCR products. Results for PCR amplifications are shown in Figs 2, 3, 4 and Tab. 6.

Table 6. Results of presence of PCR products used in determination of *Xcc*

Sample no.	UBP 1052F-BACR	DLH 120-125	ZUP 2309-2310	Sample no.	UBP 1052F-BACR	DLH 120-125	ZUP 2309-2310
1	++	+	+	11	++	+	++
2	++	–	+	12	++	++	++
3	++	–	+	13	–	–	+
4	++	–	–	14	–	–	–
5	++	++	++	15	+	–	–
6	–	++	++	16	++	+	++
7	++	++	++	17	++	+	++
8	++	++	++	18	–	–	–
9	++	++	++	19	++	++	++
10	++	++	++	20	++	++	++

Expressed as ++ (positive reaction), + (positive weak reaction), – (no reaction)

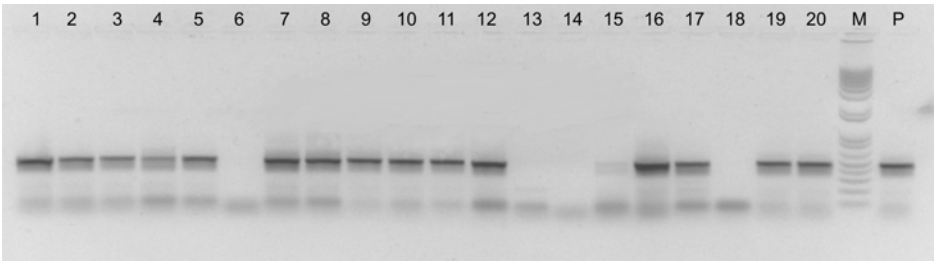

Fig 2. PCR products of 441 bp for universal bacterial primers UBP 1052F and UBP BACR (labelling according to tab. 1 and 2); M – 1 Kb Plus DNA Ladder (Invitrogen); P – positive control

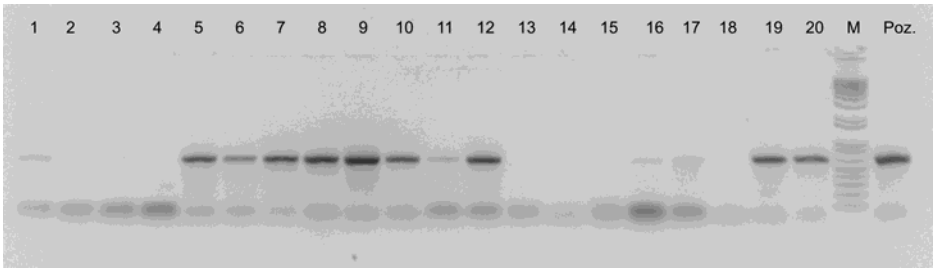

Fig. 3. PCR products of 619 bp for primer pair DLH 120 and DLH 125 detecting presence of *Xanthomonas campestris* (labelling according to tab. 1 and 2); M – 1 Kb Plus DNA Ladder (Invitrogen); Poz. – positive control

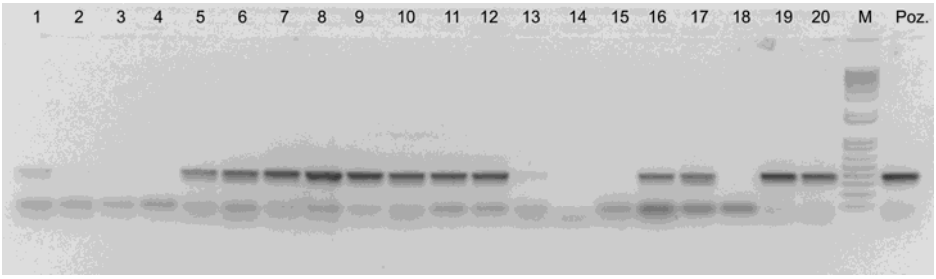

Fig. 4. PCR products of 370 bp or primer pair ZUP 2309 and ZUP 2310 specified to *Xcc* (labeling according to Tab. 1 and 2); M – 1 Kb Plus DNA Ladder (Invitrogen); Poz. – positive control

Some contrary results were obtained. In case of samples 2, 3 and 4, the strong positive reaction was obtained using universal bacterial primers (fig. 2) but for specific primers for the species *X. campestris* (fig. 3) and pathovar *campestris* (fig. 4), very poor or no reaction was observed. The opposite situation was observed in the sample 6, which did not amplify the product for bacterial presence (fig. 2) but with primer pairs DLH 120-125 and ZUP 2309-2310 (fig. 3) did. As well the sample 13 seems to be questionable because of no reaction for universal and species specific primers but positive reaction on the level of pathovar determination.

DISCUSSION

The aim of this work was to optimize the protocol for DNA extraction and determination of plant pathogen bacterium *Xanthomonas campestris* pv. *campestris* from *Brassica* plant samples.

The combination of primers UBP 1052F-BACR, DLH 120-125 and ZUP 2309-2310 used in this study confirmed results of Grimault et al. [2012] and can be recommended as appropriate for validation of *Xcc* on *Brassica* ssp. seeds. In 99.6% of cases, they obtained precise results or get impulse to additional pathogenicity test to confirm the PCR result.

Apart from the commercial kits the suitability of other isolation procedures were studied. The express isolation according to Klimyuk et al. [1993] was included because of low costs of this method. The original protocol was developed for the verification of the success of genetic transformations [Mynarzová 2013]. It used small pieces of leaves from the tested material and required DNA was obtained from marginal cells damaged by high temperature (105°C) and chemical lysis. This protocol was modified by using crushed, homogenized sample to improve the yield of DNA and thereby increasing the possibility of obtaining the bacterial DNA. Nevertheless, the results did not confirm the suitability of this procedure for extraction of bacterial DNA and its use in the tested PCR model.

The modified protocol of Ahrens and Seeműller [1992] is commonly used for the detection of phytoplasmas [Holleinová and Čechová 2012]. Due to the taxonomic relation of phytoplasmas and plant pathogenic bacteria, the modified protocol was tested.

The concentration and quality of extracted DNA were sufficient although a high amount of contaminations by RNA was proved. Concerning to the PCR products, one of tested samples did not determine the pathogen on other than bacterial level.

The protocol by Bernatzky and Tanksley [1986] for isolation of genomic DNA from tomatoes was successfully used in modifications e.g. for apricots [Krichen et al. 2008, Pilařová et al. 2010], grapes [Gogorcena et al. 1993], carnation [Smulders et al. 2003] or triticale [Divashuk et al. 2012]. In this study, the required DNA was obtained in high concentration. The sample no. 17 was highly fragmented but the pathogen *Xcc* was determined on the bacterial and pathovar level clearly, with species specific primers poorly. For the sample 18, DNA of high quality was extracted but no PCR results were obtained.

The suitability of used primers for the determination of species *X. campestris* was confirmed also by Fargier and Manceau [2007]. In their study, the specificity of primer set of Berg et al. [2005] was proved in all of 47 tested strains of *X. campestris*. For determination of pathovar *campestris*, they cannot confirm the absolute reliability of primers by Rijlaarsdam et al. [2004], neither ZUP 2309-2310 nor ZUP 2311-2312. These primers reached expected results in 38 out of 47 strains of *X. campestris*, including all *Xcc* strains, but also four of five strains of *X. campestris* pv. *incanae* (*Xci*) and one of three strains of *X. campestris* pv. *armoraciae*. Because of high improbability of occurrence of *Xci* on *Brassica* ssp. seeds, the primers were found to be applicable for determination of *Xcc* on the pathovar level in case of vegetable *Brassica* seeds. Compared to our results, universal bacterial primers and primers determining species *X. campestris* were acceptable for the determination although the complete success rate was not obtained. For the detection of pathovar *campestris*, we could confirm the occasional inaccuracy of primer pair ZUP 2309-2310. For objective results other pair of primers for *Xcc* level has to be tested.

CONCLUSION

The results of PCR analyzes cannot confirm suitability for all used extracting methods by for PCR detection of *Xcc* in cabbage leaves. In fact, the convincing results were obtained with three protocols. The DNA extracted by kit for animal tissues and cultured cells by Macherey-Nagel (samples 7 and 8) had positive results for all three sets of primers but the quality of extracted DNA was not optimal. The second method (samples 9 and 10) using commercial kit for the isolation from plant tissues by MO BIO Laboratories Inc. proved suitability in case of PCR products too and it had better quality of extracted DNA then previous method. The best method seems to be the extraction of bacterial DNA from microbial culture (samples 19 and 20). It succeeded in all analyzes but it represents more complicated and longer methods to reach material for DNA extraction.

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OCENA RÓŻNYCH METOD EKSTRAKCJI DNA W CELU WYKRYCIA BAKTERII *Xanthomonas campestris* pv. *campestris* W LIŚCIACH KAPUSTY

Streszczenie. Bakteria *Xanthomonas campestris* pathovar *campestris* (*Xcc*) jako przyczyna czarnej zgnilizny roślin z rodziny krzyżowych powoduje znaczne straty w plonie rolniczym na całym świecie. Zwalczanie czarnej zgnilizny, a także określenie *Xcc* na podstawie parametrów morfologicznych lub za pomocą testów patogeniczności jest trudne. W celu zoptymalizowania protokołu PCR przetestowano dziesięć różnych możliwości ekstrakcji DNA, a następnie metodę PCR. Na podstawie walidowanej metody ISTA zastosowano trzy zestawy primerów, mianowicie UBP 1052F-BACR, DLH 120-125 i ZUP 2309-2310. Porównano wyniki zmierzonego stężenia i jakości DNA oraz skuteczności dla amplifikacji PCR. Zalecono trzy sposoby ekstrakcji DNA w ramach protokołu detekcji *Xanthomonas campestris* pv. *Campestris*: zestaw komercyjny używany do izolacji z tkanek według Macherey-Nagel, MO BIO oraz zestaw dla hodowli mikrobowych według MO BIO.

Słowa kluczowe: czarna zgnilizna, *Brassicaceae*, specyficzne PCR

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