ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 65 24 Number 1, 2017
https://doi.org/10.11118/actaun201765010205

DETECTING PATHOGENS OF VERTICILLIUM WILT IN WINTER OILSEED RAPE USING ELISA AND PCR – COMPARISON OF THE TWO METHODS AND WITH VISUAL STAND EVALUATION

Tomáš Spitzer[1], Pavel Matušinsky[1]

[1]Agrotest fyto, Ltd, Kromeriz, Czech Republic

Abstract

SPITZER TOMÁŠ, MATUŠINSKY PAVEL. 2017. Detecting Pathogens of Verticillium Wilt in Winter Oilseed Rape Using ELISA and PCR – Comparison of the Two Methods and With Visual Stand Evaluation. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 65(1): 0205–0210.

During 2013–2015, oilseed rape samples from various locations in the Czech Republic were analysed for the presence of Verticillium wilt. Samples were evaluated in the lab using ELISA and PCR as well as by visual evaluation during the samples collection. A comparison of detection match in individual methods also was made. ELISA and PCR matched in detecting Verticillium wilt in 60 % of cases. For practical use, a higher match rate would be necessary in cases where samples were to be analysed in various laboratories using only one of these techniques. The possibility was demonstrated to use kits as well as primers not targeted specifically to just *Verticillium longisporum* but recording both main species on oilseed rape (*V. longisporum* and *V. dahliae*). The match rates of lab analyses with visual evaluation of stand infection were surprisingly high at 62 % for ELISA and 77 % for PCR. All three stand evaluation methods matched in 56 % of cases.

Keywords: *Verticillium longisporum*, *Verticillium dahliae*

INTRODUCTION

Verticillium wilt is a disease responsible for yield losses in oilseed rape (*Brassica napus*) in coastal areas of northern Europe. Along with a rising proportion of oilseed rape in crop rotations, this disease is expected to gain in importance also in the Czech Republic. Besides oilseed rape, Verticillium wilt attacks a large number of other host species (e.g. sunflower, potato, cotton, olive).

According to Zhou *et al*. (2006), Verticillium wilt of oilseed rape is caused primarily by *Verticillium longisporum* and has become a serious problem in northern Europe. Two main *Verticillium* species are detected on oilseed rape: *V. longisporum* and *V. dahliae*. These differ in their interaction with oilseed rape. *V. longisporum* favours *B. napus* as host.

The impact of *V. longisporum* on single-plant and whole-plot yield of oilseed rape was previously studied in field experiments with natural infection or artificial soil infestation. Levels reached with artificial soil infestation were 54.3 % and yield losses of single plants accounted for 20 % to more than 80 %. A previous study indicated significant yield damage potential of *V. longisporum* in areas with infestation density above 60 % (Dunker *et al*., 2008).

Monitoring during 2006–2008 at 66 locations in the Czech Republic had demonstrated the presence of *V. longisporum* on oilseed rape plants at rates of 12–41 % (Spitzer *et al*., 2008). Bokor *et al*. (2014) found that it was one of the two most widely spread pathogens among winter oilseed rape in Slovakia.

It is not easy reliably to detect *V. longisporum* on oilseed rape plants due to the pathogen's life cycle, and even mere visual determination of the disease agent is greatly influenced by the evaluator's experience. It would therefore be beneficial to introduce reliable analytical methods for determining the pathogen through such tests as

ELISA or PCR. These methods have already been used in winter oilseed rape. For example, PCR was used to determine sources of resistance to *V. longisporum* in China (Yu *et al*., 2015), to determine and identify individual *Verticillium* species including three lineages of the diploid hybrid *V. longisporum* (Inderbitzin *et al*., 2013), and for a similar purpose but in soil samples (Debode *et al*., 2011). Fortnagel and Schlosser (1996) developed an ELISA test to detect *Verticillium* species on the basis of antibodies obtained from chickens. In France, ELISA tests were evaluated as one of the possibilities for monitoring oilseed rape seed quality (Wagner *et al*., 2012). Gao *et al*. (2012) developed a method based on indirect competitive ELISA for quick, reliable, and sensitive determination of cadmium levels in water, soil, and oilseed rape plants

Both analytical methods were used to determine *Xanthomonas campestris* pv. *campestris* on winter oilseed rape in Serbia (Popović *et al*., 2014). Pathogen species was determined from collected infected plant samples using ELISA and then similarity with GenBank samples collected from kale, cabbage, and broccoli was determined using PCR.

The objectives of this study are to compare the results from analysing winter oilseed rape samples for presence of the fungal pathogen *V. longisporum* using ELISA and PCR, to compare these with the overall visual stand evaluation in the field, and to assess these methods' suitability for practical determination of the pathogen under actual growing conditions.

MATERIALS AND METHODS

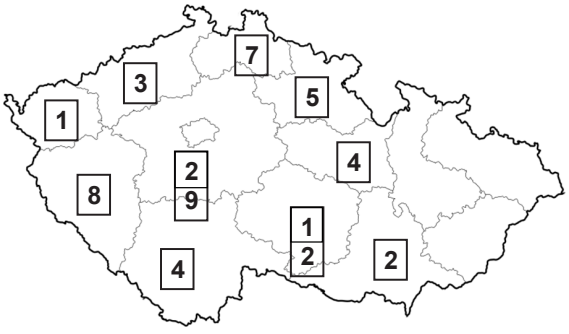
Plant samples were collected immediately after harvest from various fields sown with winter oilseed rape in the Czech Republic during 2013–2015. Each year, 15 samples of stem remains were collected from stubble at each location, and a 20 cm section above the root crown was taken from each (Dunker *et al*., 2008). The locations were not identical each year but differed according to current winter oilseed rape cultivation in selected agricultural enterprises. A total of 28 samples were analysed in 2013, 26 in 2014, and 22 in 2015 (Fig. 1). Each sample was ground in a Pulverisette 19 laboratory mill (Fritsch) on a 1 mm screen and analysed by ELISA. The biomass pulp was subsequently ground into a fine powder using a Mixer Mill MM 301 (Retsch) and then subjected to further DNA isolation before PCR.

ELISA was carried out using a Microplate Reader MRX II spectrophotometer with a Verticillium Complete Kit 480 (BIOREBA). A new kit was acquired for each experimental season. The resulting measured optical densities of individual samples determined by ELISA were compared with positive and negative controls and as appropriate for the individual kits used. Samples were then classified into three categories: positive (with optical density approaching the positive control), negative (with optical density approaching the negative control), and weakly positive (with optical density around the central value between the positive and negative controls). Tab. I presents

I: *Optical density values for positive and negative controls and classification according to measured optical density values*

	2015		2014		2013
positive control	2.965	positive control	2.478	positive control	3.075
negative control	0.148	negative control	0.116	negative control	0.175

Sample	Range	Sample	Range	Sample	Range
positive	2.156–3.398	positive	1.731–3.420	positive	1.997–3.060
weakly positive	1.294–1.824	weakly positive	1.022–1.550	weakly positive	1.227–1.913
negative	0.335–0.899	negative	0.372–0.793	negative	0.459–0.977



1: *A total amount of samples analysed in 2013–2015 from different parts of Czech Republic.*

the optical density values of these three stated categories.

DNA was extracted before PCR using a DNeasy Plant Mini Kit (Qiagen). To identify Verticillium wilt in oilseed rape samples published primers (DB19 5′-CGGTGACATAATACTGAGAG-3′, DB22 5′-GACGATGCGGATTGAACGAA-3′, first used by Carder *et al*., 1994). PCR conditions according to Mercado-Blanco *et al*. (2003) were used with denaturation at 94 °C for 4 min followed by 30 cycles of 1 min of annealing at 58 °C, extension for 1 min at 72 °C, denaturation for 1 min at 94 °C, and final extension step of 6 min. Each reaction contained, in addition to samples of extracted oilseed rape DNA, a negative control (water) and a positive control of purified DNA from *V. longisporum* mycelia. Aliquots (10 µl) of amplification products were electrophoresed through 1.7 % agarose gels prepared using TAE buffer and dyed with ethidium bromide. As for the ELISA test, the resulting bands were classified into three categories according to band colour and intensity. The results are presented in Tab. I.

Additionally, the sample collector's opinion based on visual stand evaluation as to the rate of infection by *V. longisporum* was recorded. Sample collectors assessed a stand as being in one of two categories: healthy or infected.

RESULTS

Tab. II presents the results by individual year of sample analysis using ELISA and PCR along with the stand condition evaluation. To assess whether or not ELISA and PCR analyses matched for individual samples, a situation was considered to be a good match when both methods provided an identical evaluation (positive–positive, negative–negative, and weakly positive–weakly positive). Weakly positive–positive evaluations were weak matches.

In 2013

In 2013, of the total 28 samples analysed with ELISA and PCR, 19 were evaluated as weak or good matches and 9 were not matched between the two methods' evaluations. Within these 9 non-matches, ELISA evaluated 7 samples as positive and 2 as negative, while PCR had the reverse. ELISA had a weak or good match with the visual infection evaluation in 18 cases and 10 non-matches. PCR had a weak or good match with the visual infection evaluation in 21 cases and 7 non-matches. All three stand evaluation methods had weak or good matches in 15 cases and 13 non-matches.

In 2014

In 2014, of the total 26 samples analysed with ELISA and PCR, 21 were evaluated as weak or good matches and 5 were not matched between the two methods' evaluations. Within these 5 non-matches, ELISA evaluated 5 samples as positive, while PCR had the reverse. ELISA had a weak or good match with the visual infection evaluation in 14 cases and 12 non-matches. PCR had a weak or good match with the visual infection evaluation in 18 cases and 8 non-matches. All three stand evaluation methods had weak or good matches in 13 cases and 13 non-matches.

In 2015

In 2015, of the total 22 samples analysed with ELISA and PCR, 15 were evaluated as weak or good matches and 7 were not matched between the two methods' evaluations. Within these 7 non-matches, ELISA evaluated 6 samples as positive and 1 as negative, while PCR had the reverse. ELISA had a weak or good match with the visual infection evaluation in 15 cases and 7 non-matches. PCR had a weak or good match with the visual infection evaluation in 19 cases and 3 non-matches. All three stand evaluation methods had weak or good matches in 14 cases and 6 non-matches.

DISCUSSION

The performed analyses and visual evaluation of oilseed rape stands yielded some interesting findings. If the match between the two analytical methods, ELISA and PCR, is expressed in percentage terms, they agreed on the presence or non-presence of *V. longisporum* in the samples as a 3-year average in 60 % of cases (2013: 68 % of test results matching, 2014: 80 %, and 2015: 33 %). There are no known publications in the available literature that deal with a comparable detection of fungal pathogens using ELISA and PCR. In oilseed rape, these methods are mostly used separately to detect viruses (Breitenmoser *et al*., 2011; Yu *et al*., 2015), evaluate seed quality (Wagner *et al*., 2012), assess phylogenetic relationships within *Verticillium* species (Pantou *et al*. 2005), and determine fungicide residues in oilseed rape leaves as part of monitoring fungicide activity and current concentrations in relation to the timing and effectiveness of treatments against *Leptosphaeria maculans* and *Pyrenopeziza brassicae* (Coules and Rossall, 2003). The match rate of 60 % between the methods in analysing *V. longisporum* is not poor, but for practical use a higher match rate would be necessary in cases where samples were to be analysed in various laboratories using only one of these techniques.

The oilseed rape vascular system is infected by two closely related pathogenic species: *V. dahliae,* localized especially on such lower plant sections as the roots and basal sections of the stem (Johansson, 2006), and *V. longisporum,* which occurs in the entire plant including the leaves. These two fungal species were previously considered to be one (*V. dahliae*), and even currently *V. longisporum* is frequently falsely diagnosed as *V. dahliae*. The two species can be distinguished morphologically based conidia shape and size (Karapapa *et al*., 1997), by polyphenol oxidase activity, and by molecular methods (Steventon *et al*., 2002). Differences at

II: *Verbal classification of individual samples based on analyses and overall visual stand evaluation*

	2015			2014			2013		
Sample	**Evaluation method**								
number	**ELISA**	**PCR**	**Visual**	**ELISA**	**PCR**	**Visual**	**ELISA**	**PCR**	**Visual**
1	weakly positive	positive	infected	positive	positive	infected	positive	positive	infected
2	positive	negative	healthy	positive	weakly positive	infected	positive	positive	infected
3	negative	weakly positive	healthy	weakly positive	weakly positive	infected	positive	positive	infected
4	weakly positive	positive	infected	positive	negative	infected	positive	positive	infected
5	weakly positive	weakly positive	healthy	positive	positive	infected	positive	positive	infected
6	weakly positive	negative	infected	positive	weakly positive	healthy	positive	positive	infected
7	negative	negative	healthy	weakly positive	negative	healthy	positive	positive	infected
8	positive	positive	infected	weakly positive	weakly positive	healthy	positive	positive	infected
9	weakly positive	negative	healthy	negative	negative	healthy	positive	positive	infected
10	negative	negative	healthy	weakly positive	weakly positive	healthy	negative	negative	infected
11	weakly positive	negative	healthy	negative	negative	healthy	weakly positive	negative	infected
12	negative	negative	healthy	weakly positive	positive	healthy	weakly positive	negative	healthy
13	positive	negative	healthy	negative	negative	infected	positive	weakly positive	infected
14	weakly positive	weakly positive	infected	negative	negative	healthy	positive	positive	infected
15	negative	negative	healthy	weakly positive	weakly positive	healthy	negative	weakly positive	infected
16	weakly positive	positive	infected	weakly positive	negative	healthy	positive	negative	healthy
17	negative	negative	healthy	weakly positive	weakly positive	healthy	negative	negative	infected
18	weakly positive	negative	healthy	weakly positive	negative	healthy	negative	negative	healthy
19	negative	negative	healthy	weakly positive	negative	healthy	positive	negative	infected
20	weakly positive	positive	infected	negative	negative	healthy	weakly positive	negative	infected
21	negative	negative	healthy	weakly positive	weakly positive	infected	weakly positive	negative	healthy
22	negative	negative	healthy	positive	positive	infected	positive	positive	infected
23				weakly positive	positive	healthy	weakly positive	negative	healthy
24				negative	negative	healthy	negative	positive	infected
25				negative	negative	healthy	positive	positive	infected
26				negative	negative	healthy	positive	positive	infected
27							negative	negative	infected
28							negative	negative	infected

the level of DNA can be seen in such examples as the presence of a large intron in the SSU-rRNA gene of 839 bp in *V. longisporum* and its absence in *V. dahliae* and *V. albo-atrum* (Karapapa and Typas, 2001). Species-specific primers have been developed to distinguish these two species (Karapapa and Typas, 2001; Steventon *et al*., 2002) and tested in several studies (Johansson *et al*., 2006; Zhou *et al*. 2006). Based on our experience, some primers worked well only with pure fungus mycelium but were not suitable for use in oilseed rape tissues. The method published by Mercado-Blanco *et al*. (2003), originally used to detect defoliating and non-defoliating *V. dahliae* pathotypes in infected olive plants, does not react with oilseed rape DNA and can detect *V. dahliae* DNA. After minor modification, this method can be applied to diagnose *V. dahliae* in oilseed rape. The method does not distinguish between *V. longisporum* and *V. dahliae*.

It is interesting that when the two methods disagreed on the presence of *V. longisporum* in the samples, ELISA had a considerably higher number of positive results than did PCR. This occurred in all three monitored years. One reason for this phenomenon could be the amount of analysed sample collected from the total amount ground, which differs substantially between the two methods. For ELISA, 1 g of the total ground sample amount is collected for the analysis while for PCR only 50 mg is used for the analysis. In weakly infected samples, ELISA could therefore have had an advantage in a higher probability of its detecting the pathogen than did PCR.

Visual evaluation of stand infection by *V. longisporum* is influenced by all of the negatives associated with subjective evaluation. This evaluation was requested of sample collectors in the field so as to determine how this evaluation differs from analytical lab testing. Averaging the three monitored years, all three methods of evaluating *V. longisporum* infection matched in 56 % of cases. This is a higher percentage than was expected but is nevertheless not a sufficiently good rate such that the individual methods could be used interchangeably. It is interesting that the visual stand evaluation matched with PCR (matching in 77 % of cases in the 3-year average) better than it did with ELISA (62 % in the 3-year average).

CONCLUSION

Comparing analyses for presence of Verticillium wilt in winter oilseed rape using ELISA and PCR results in a match rate between the two methods of 60 %. For practical use, a higher match rate would be necessary in cases where samples were to be analysed in various laboratories using just one of these techniques. The possibility to use kits as well as primers not targeted specifically to only *Verticillium longisporum* but recording both main species on oilseed rape (*V. longisporum* and *V. dahliae*) was demonstrated. The match rates of lab analyses with visual evaluation of stand infection were surprisingly high at 62 % for ELISA and 77 % for PCR. A match among all three stand evaluation methods was found in 56 % of cases.

Acknowledgements

This publication was made possible by institutional support provided for the long-term development of a research organization under Decision of the Ministry of Agriculture of the Czech Republic No. RO1116 from 28 February 2011 and was supported by the Ministry of Agriculture of the Czech Republic, Project No. QJ1310227. The authors would like to thank English Editorial Services, s.r.o. for translation of the manuscript.

REFERENCES

BOKOR, P., BEČKA, D. and TÓTHOVÁ, M. 2014. Occurrence of white rot and verticillium wilt in Winter Rapeseed fields in Slovakia during 2014 [in Slovak]. In: *Proceedings from Prosperous Oil Crops 2014* [in Czech: *Prosperující olejniny*]. Czech University of Life Sciences Prague, 11–12 December 2014. Prague: Czech University of Life Sciences Prague, 82–85.

BREITENMOSER, S., DUBUIS, N., GRILLOT, L., BRODARD, J. and BALMELLI, C. 2011. Viral diseases of oilseed rape in Switzerland [in French: Les viroses du colza en Suisse]. *Recherche Agronomique Suisse*, 2: 388–395.

CARDER, J., MORTON, A., TABRETT, A. M. and BARBARA, D. J. 1994. Detection and differentiation by PCR of subspecific groups within two Verticillium species causing vascular wilts in herbaceous hosts. In: SCHOTS, A., DEWEY, F. M. and OLIVER, R. (eds.), *Modern assays for plant pathogenic fungi*. Oxford: CAB International, 91–97.

COULES, A. and ROSSALL, S. 2003. Disease control and the consequences of timing on the yield of oilseed rape. In: *Proceedings of the BCPC International Congress: Crop Science and Technology*, Volumes 1 and 2.

Scottish Exhibition and Conference Centre, 10–12. November 2003. Alton, Hampshire, UK: British Crop Production Council, 659–662.
DEBODE, J., VAN POUCKE, K., FRANÇA, S. C., MAES, M., HÖFTE, M. and HEUNGENS, K. 2011. Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. *Plant Disease*, 95: 1571–1580.
DUNKER, S., KEUNECKE, H., STEINBACH P. and VON TIEDEMANN, A. 2008. Impact of Verticillium longisporum on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology*, 156: 698–707.
FORTNAGEL, B. and SCHLOSSER, E. 1996. Polyclonal antibodies against *Verticillium* dahliae Kleb from oil-seed rape via immunized hens. *Journal of Plant Diseases and Protection*, 103(6): 582–589.
GAO, W., NAN, T., TAN, G., ZHAO, H., WANG, B., LI, Q. X. and MENG, F. 2012. Development of a sensitive monoclonal antibody-based enzyme-linked immunosorbent assay for the analysis of cadmium ions in water, soil and rape samples. *Food and Agricultural Immunology*, 23: 27–39.
INDERBITZIN, P., DAVIS, R. M., BOSTOCK, R. M. and SUBBARAO, K. V. Identification and differentiation of *Verticillium* species and *V. longisporum* lineages by simplex and multiplex PCR assays. *PLoS One*, 8(6): e65990.
JOHANSSON, A. 2006. *Verticillium longisporum, infection, host range, prevalence and plant defence responses*. Licentiate thesis. Uppsala: Swedish University of Agricultural Sciences.
JOHANSSON, A., GOUD, J-K. C. and DIXELIUS, C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. *European Journal of Plant Pathology*, 114: 139–149.
KARAPAPA, V. K, BAINBRIDGE, B. W. and HEALE, J. B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycological Research*, 101: 1281–1294.
KARAPAPA, V. K. and TYPAS, M. A. 2001. Molecular characterization of the host-adapted pathogen *Verticillium longisporum* on the basis of a group-I intron found in the nuclear SSU-rRNA gene. *Current Microbiology*, 42: 217–224.
MERCADO-BLANCO, J., RODRÍGUEZ-JURADO, D. and PARRILLA-ARUAJO, S. 2003. Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. *Plant Disease*, 87: 1487–1494.
PANTOU, M. P., STRUNNIKOVA, O. K., SHAKHNAZAROVA, V. Y., VISHNEVSKAYA, N. A., PAPALOUKA, V. G. and TYPAS, M. A. 2005. Molecular and immunochemical phylogeny of *Verticillium* species. *Mycological Research*, 109: 889:902.
POPOVIĆ, T., BALAŽ, J., IGNJATOV, M., MITROVIĆ, P., GAVRILOVIĆ, V. and JOŠIĆ, D. 2014. Identification and genetic characterisation of *Xanthomonas campestris* pv. campestris as an oilseed rape pathogen in Serbia. *Journal of Plant Pathology*, 96: 553–560.
SPITZER, T., MATUŠINSKY, P., MIKOLÁŠOVÁ, R. and WOLF, G. 2008. Occurrence of Verticillium wilt of oilseed rape in the Czech Republic detected by PCR and ELISA. In: *Proceedings of the 56. Deutsche Pflanzenschutztagung*. Kiel, Germany, 22–25 September 2008. Quedlinburg, Germany: Julius Kühn-Institut.
STEVENTON, L. A., FAHLESON, J., HU, Q. and DIXELIUS, C. 2002. Identification of the causal agent of Verticillium wilt of winter oilseed rape in Sweden, V. longisporum. *Mycological Research*, 106: 570–578.
WAGNER, M.-H., DUCOURNAUA, S., LUCIANIA, A. and LÉCHAPPÉA, J. 2012. From knowledge-based research towards accurate and rapid testing of seed quality in winter rape. *Seed Science Research*, 22(S1): S80–S85.
YU, S., SU, T., CHEN, J., WANG, W., ZHANG, F., YU, Y., ZHANG, D., ZHAO, X. and LU, G. 2015. Real-time PCR as a diagnostic tool for evaluating the resistance of Chinese cabbage cultivars to Verticillium wilt. *European Journal of Plant Pathology*, 143: 543–557.
ZHOU, L., HU, Q., JOHANSSON A. and DIXELIUS C. 2006. *Verticillium longisporum* and V. dahliae: infection and disease in Brassica napus. *Plant Pathology*, 55: 137–144.

Contact information:

RNDr. Tomáš Spitzer PhD: spitzer@vukrom.cz