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TESTING OF INOCULATION METHODS AND SUSCEPTIBILITY TESTING OF PERSPECTIVE CABBAGE BREEDING LINES (*BRASSICA OLERACEA* CONVAR. *CAPITATA*) TO THE BLACK ROT DISEASE CAUSED BY *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS*

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Abstract

PEŇÁZOVÁ ELIŠKA, KOPTA TOMÁŠ, JURICA MILOŠ, PEČENKA JAKUB, EICHMEIER ALEŠ, POKLUDA ROBERT. 2018. Testing of Inoculation Methods and Susceptibility Testing of Perspective Cabbage Breeding Lines (*Brassica Oleracea* convar. *Capitata*) to the Black Rot Disease Caused by *Xanthomonas Campestris* pv. *Campestris*. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 66(1): 0139–0148.

The susceptibility of twenty-four cabbage breeding lines to *Xanthomonas campestris* pv. *campestris* was evaluated. The selection of appropriate inoculation method was done on 4 cabbage cultivars ('Cerox', 'Sintex', 'Sonja' and 'Avak'). One month old plants were infected by 5 inoculation methods (spraying, injection by syringe, multiple pricking, carborundum abrasion and scissor clipping method). Four different bacterial isolates of Xcc (WHRI 3811, 3971A, 1279A; SU) and their mixture were evaluated for the aggressiveness on 'Cerox' and 'Sonja' cultivars. On the basis of obtained results, breeding lines of head cabbage were inoculated by mixture of all tested isolates using multiple pricking method. The disease severity of inoculated seedlings proved high susceptibility of young plants to the Xcc infection. The disease incidence determined 75 and 105 days after sowing showed changes for 16 of tested lines and indicated that resistance testing should be observed until mature stage. The study revealed five breeding lines (DP25, T1, IT10, Kalibos and Avak1) with disease incidence lower than 20 % as perspective sources of resistance for further breeding.

Keywords: Xcc resistance, artificial inoculation, cabbage, symptom expression, PCR

INTRODUCTION

Xanthomonas campestris pv. *campestris* (Pammel) Dowson (Xcc) is a causal agent of black rot disease on a wide range of Brassicaceae plants including vegetable crops, ornamental crucifers as well as weeds (Williams 1980; Schaad and Dianese 1981). The disease is described through the world and is considered to be one of the most destructive diseases of crucifers. The sources of infection are infected seeds, soil and plant debris, splashes and aerosols dispersed from infected fields, weeds and farm machineries. It could be also transmitted by insects (Dzhalilov and Tiwari 1995).

Xcc is gram-negative proteobacterium that enters plant vascular system mainly by the hydathodes and wounds. It causes V-shaped chlorotic to necrotic lesions at leaf margins, leaf tissue necrosis and vein blackening (Cook *et al.,* 1952). However, symptoms of black rot may differ among different cruciferous vegetables. Also the severity of Xcc symptoms and

disease aggressiveness vary between different strains of this bacterium (Alvarez 2000; Alberto 2015).

The race structure of Xcc was firstly presented by Kamoun *et al.* (1992) describing 5 races. From this arrangement, only 3 races were retained (1, 2 and 4) where the race 1 was subdivided into 3 different races. Considering the isolates with narrow host range, finally the Xcc strains have been sorted into 9 physiological races. The races 1 and 4 appear to be predominant worldwide (Vicente *et al.* 2001; Vicente and Holub 2013).

Despite the efforts to obtain resistance in crucifer crops, the black rot is remaining to be the threat of cruciferous cultivation. The available sources of resistance are limited and often race-specific (Ignatov *et al.*, 1998; Tonu *et al.*, 2013). For the species *B. oleracea* (genome CC), resistance for predominant races is rare or only partial. Known sources of black rot resistance occur in A and B genomes of brassica species (Bain 1952; Westman *et al.*, 1999). The interspecific hybridization has been used to transfer resistance but the reduction or loss of this resistance in backcross breeding was reported (van Zijll de Jong *et al.*, 2010).

Successful breeding for disease resistance requires efficient screening techniques and evaluation of large numbers of genotypes. The artificial inoculation technique has to be rapid and able to produce sufficiently high levels of infection to differentiate susceptible and resistant genotypes (Salaheddin *et al.*, 2005). For the genus *Xanthomonas*, two methods are used to inoculate plants, by hydathodes and by artificial wounds. Infection through hydatodes contains suspension spraying or immersing of undamaged plants. Inoculation by wounds could be done via injection infiltration method, carborundum abrasion method, needle or pin pricking method, clipping method or sandpaper method (Topp *et al.*, 1993; Salaheddin *et al.*, 2005; Maji and Nath 2015).

The objective of this study was: i) to determinate the most appropriate inoculation method; ii) to determinate the Xcc isolate for the testing of response of cabbage cultivars to black rot disease and iii) to evaluate the susceptibility/resistance of selected breeding lines to Xcc infection.

MATERIALS AND METHODS

Bacterial strains and culture preparation

Bacterial strains used in the study were obtained from culture collection of Horticultural Research International Warwick (WHRI) and from naturally infected cabbage head. The WHRI isolates were chosen according to the described pathogenicity. Three cultures were used, the 3811 and 3971A (race 1) and 1279A (race 4). As a representative of natural isolate, culture isolated from the field conditions in Svijanský Újezd, Czech Republic (SU) was included.

Strains were cultivated on Petri dishes with MPA (meat peptone agar) and King's B medium, transferred into liquid nutrient broths and grown up to the final concentrations of 10^8 CFU.ml^{-1}.

Plant material

The testing of inoculation methods was performed on 4 hybrid cultivars of cabbage. Two of them ('Cerox' and 'Sintex') are presented as highly resistant to Xcc. As the susceptible cultivars, 'Sonja' and 'Avak' were used in this study. From these, 'Cerox' and 'Sonja' were subsequently used for the study of aggressiveness of isolates. All plants were grown in greenhouse conditions.

The resistance testing contained breeding lines of head cabbage obtained from Moravoseed company as genotypes with potential for resistance breeding. They all represent inbred material except of no. 42 (hybrid material). Individual lines were marked by code numbers regarding the ongoing breeding processes of this breeding and seed company. For 24 selected lines, the level of susceptibility was evaluated. Both white and red cultivars were included. Plants were grown in the same conditions as mentioned above.

Inoculation method

Different inoculation methods were applied for the selection of appropriate method for evaluation of cultivars susceptibility/resistance to the black rot disease; 1) pressurized spraying, 2) injection by sterile syringe, 3) multiple pricking method, 4) carborundum abrasion method and 5) scissors clipping method. Except of spraying method where whole plant was exposed to the bacterial suspension, two fully developed leaves per plant were inoculated. For all methods, the bacterial suspension (isolate 3811) with concentration of 10^8 CFU.ml^{-1} was used. One-month old seedlings were inoculated, 18 plants for each inoculation technique.

Before the pressure spraying method, the seedling trays with plants were places to the humid cold condition to open hydathodes and stomata. The spraying of inoculum was performed by hand atomizer Bosch PFS 55. The bacterial suspension was applied on lower surface of leaves. Sprayed plants were covered by polyethylene bag to increase humidity and placed into the climabox with 28 °C and 16/8 h photoperiod. After 48 h, they were replaced to the greenhouse conditions. The inoculation by syringe was done by injecting suspension into the midrib and veins of the lower surface of the leaves, approximately 0.1 ml to each plant. For the multiple pricking, the wooden sticks were used according to the recommendation of ISTA (International Seed Testing Association). Leaves were perforated at once by 6 sticks placed in plastic holder. The carborundum abrasion was done by 800-mesh powder followed by spreading of inoculum by soaked cotton swab. After 5 min, the leaf surface was washed by distilled water.

The last method was represented by the clipping of leaves tips by sterile scissors dipped in bacterial suspension. All plants were maintained under greenhouse condition and observed for disease symptoms development. After two weeks (42 days after sowing DAS), visual symptoms were evaluated to find out the efficiency of tested methods. Eight selected plants from each variant were planted in pots and monitored for symptom expression for next month.

Aggressiveness of Xcc isolates

Xcc isolates were evaluated for the aggressiveness on two cabbage cultivars – 'Cerox' and 'Sonja'. All isolates were used separately and also in a mixed bacterial suspension. Based on recommendation of ISTA and previous results, the multiple pricking method was used. The inoculation of each treatment was performed on 8 plants of both cultivars at one-month old seedlings. Visual symptoms were evaluated after two weeks.

Resistance of breeding lines

Twenty-four cabbage lines were sown for the resistance test and maintained under the greenhouse condition for one month where the inoculation was performed. As inoculum, the mixed suspension of all tested isolates in concentration of 10^8 CFU.ml^{-1} was used. Two weeks after inoculation, presented symptoms were evaluated. Six symptomatic plants and 3 control plants of each line were subsequently transplanted into pots and kept out into isolated cold frame. The number of selected plants were established according to the requirement for a balanced set of plants (well developed, with typical shape and free of pests) from all lines. To protect the seedlings from insect damage, the frame was covered with mosquito net. The watering was ensured by drip irrigation. The development of symptoms was recorded twice in one-month intervals (75 and 105 DAS; days after sowing) and the disease incidence (the frequency of plant infection) was evaluated. The last evaluation of Xcc presence was carried out by PCR molecular technique.

Disease assessment

Symptom expression was recorded by visual assessment of disease severity and disease incidence on inoculated leaves or plants. Disease incidence is defined here as the percentage of diseased plants per breeding line and severity as the degree of diseased leaf/plant. For the evaluation of disease severity, the 5-degree scale of infection was followed (Tab. I). The individual degrees recognize rates of infection in percentage of affected surface of the individual leaf (methods no. 2, 3, 4, 5) or whole plant (method no. 1). The median value of disease severity was calculated from average degree of infection per plant.

Evaluations of Xcc presence were confirmed by molecular approach. DNA from tested samples was isolated by NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). The standard PCR targeting the hrpF (hypersensitivity reaction and pathogenicity) gene was used. The reaction was performed as described by Eichmeier *et al.* (2015) using primer pair DLH 120–125 (619 bp, Berg *et al.* 2005). The PCR was done primarily for determination of Xcc spreading in plants.

Statistical analysis

Obtained data were analysed by Statistica CZ software (StatSoft, Prague, Czech Republic). The data were subjected to analysis by the Kruskal-Wallis Test. Statistical differences ($\alpha = 0.05$) were determined according to Multiple Comparisons Test.

RESULTS

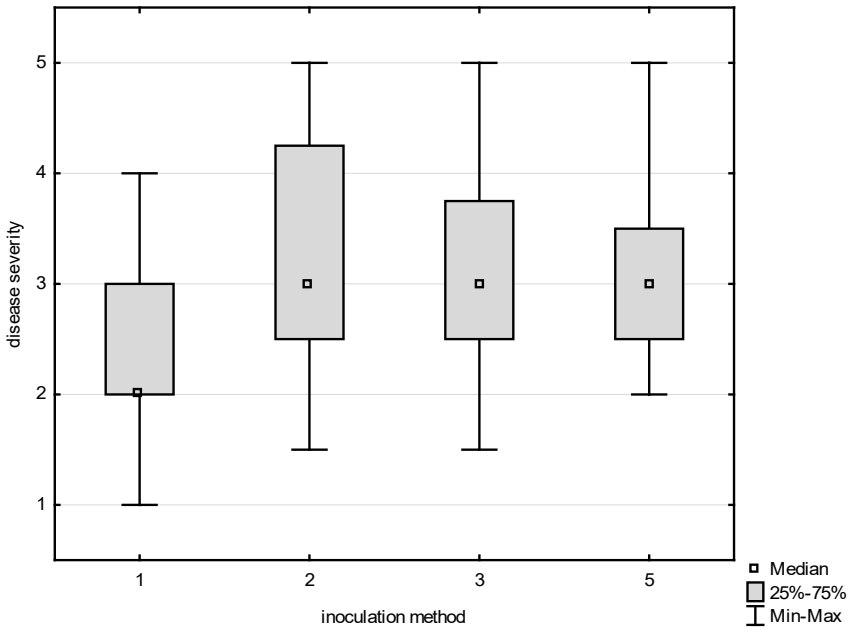
Inoculation method

All inoculation methods proved exploitability of artificial infection with Xcc and led to the expression of symptoms. Presented symptoms ranged within the entire scale. Methods were compared by the total reaction of all cultivars to the infection (Fig. 1). The carborundum abrasion method (no. 4) could not be evaluated because of significant damage of leaves by this method. The pressurized spraying (no. 1) showed the statistical difference from other inoculation methods and led to the lowest symptoms. Otherwise it is a useful tool for non-invasive inoculation by hydathodes because the symptom expression was obtained for all tested cultivars. In case of methods using inoculation by wounds, statistical differences were not found. The inoculation by injection (no. 2) recorded commonly higher disease severity than multiple pricking (no. 3) and scissor clipping (no. 5). Regarding the aspects of inoculating time, equipment and clarity between symptoms caused by pathogen and by plant reaction to wound, the multiple pricking method proved the highest exploitability. Based on these results, the spraying (non-invasive method) and multiple pricking (invasive method) were considered to be suitable for resistance testing.

The reaction of individual cultivars to the infection showed the highest susceptibility to Xcc for cv. Sonja (Fig. 2). The disease severity was

I: *Table of 5-degree scale*

Degree of infection	1	2	3	4	5
Affected surface	0%	<25%	25–50%	50–75%	>75%



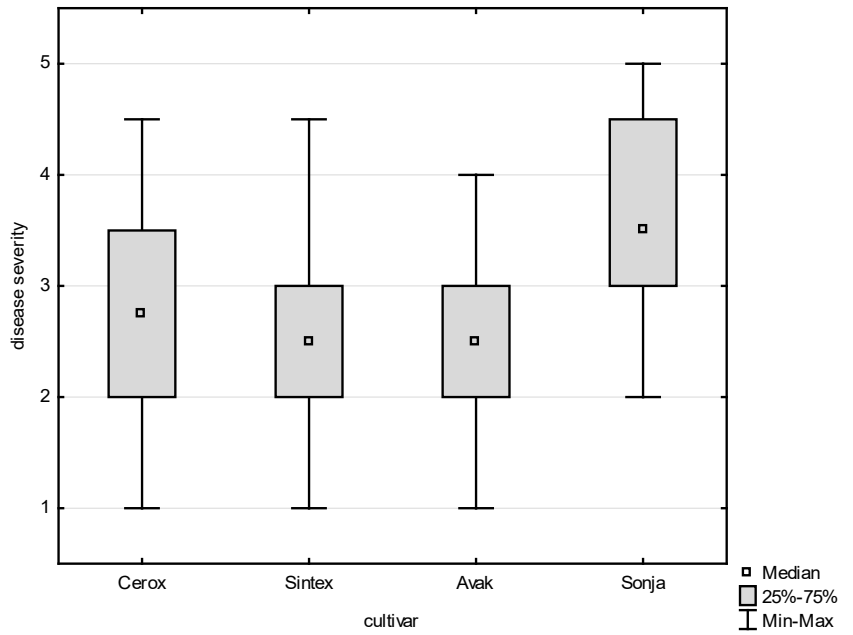
1: *Disease severity on cabbage cultivars caused by artificial inoculations of Xcc – grouped by inoculation methods (KW: H (3, N = 384) = 101.1477 p = 0.000): 1-spraying, 2-injection, 3-multiple pricking, 5-scissor clipping; graph designed by Statistica CZ software*

statistically higher compared to the rest of cultivars. Other differences were not significant.

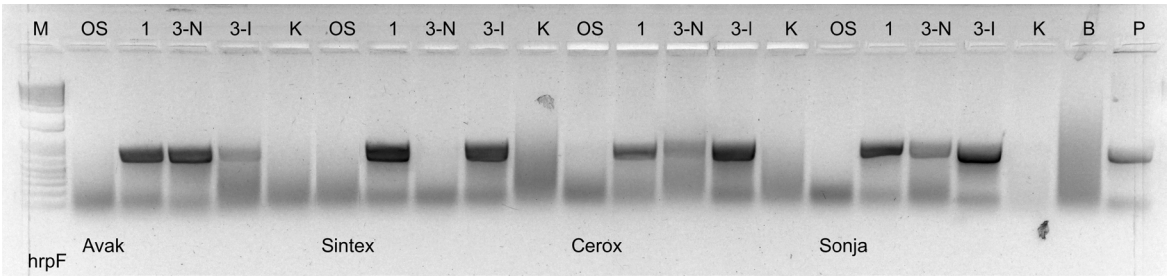
To evaluate the spreading of Xcc through plant tissues, PCR was used. Samples of inoculated and new developed leaves from methods recommended for resistance testing (no. 1 and 3) were subjected to the molecular testing targeting the hrpF gene. The presence of Xcc was confirmed in all inoculated samples and also in new leaves except of cv. 'Sintex' (Fig. 3). The evaluation of seeds was also included for elimination of possibility of seed infection.

Aggressiveness of Xcc isolates

Four isolates of the pathogen Xcc and their mixture were evaluated for aggressiveness on two cultivars of white cabbage. The resistant cultivar 'Cerox' showed lower infection rate for all variants than susceptible 'Sonja' (Fig. 4). In case of 'Cerox', individual isolates caused different degrees of



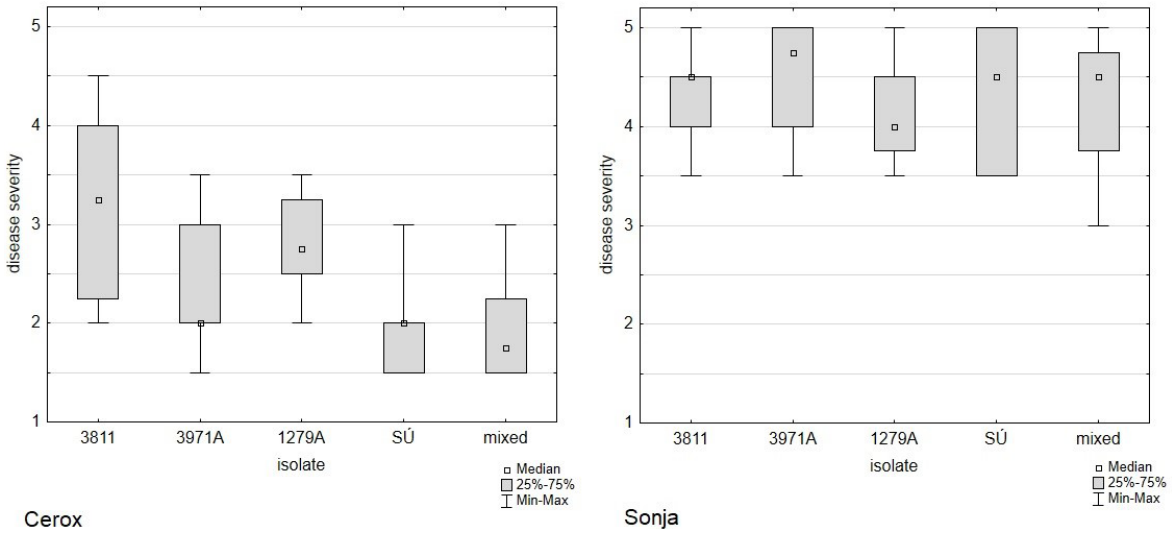
2: *Disease severity on cabbage cultivars caused by artificial inoculations of Xcc - grouped by cabbage cultivars (KW: H (3, N = 384) = 44.70741, p = 0.0000)*



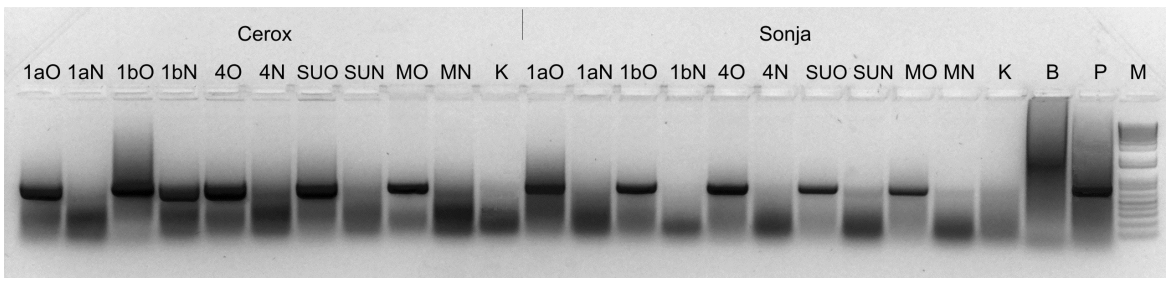
3: *Amplified products from samples inoculated by spraying and multiple pricking method (M-1kB DNA ladder (Thermo Fisher Scientific, Inc., MA, USA), OS-seed, 1-spraying, 3-pricking: N new not inoculated leaf, I inoculated leaf, K – control, B – no template control, P – positive control of Xcc)*

infection. The highest susceptibility was proved for the isolate 3811 which was significantly different than the mixture of all isolates. Other differences were not proved. The reaction of 'Sonja' did not show statistically significant differences between isolates. The reaction to the infection by Xcc race 1 (isolates 3811 and 3971A) differed according to the host cultivar. 'Cerox' was more susceptible to isolate 3811 and 'Sonja' to isolate 3971A. These results pointed on the role of individual isolates that is not identical for whole race.

The evaluation of visual symptoms and the spreading of bacteria to new leaves were verified by PCR (Fig. 5). In case of older inoculated leaves (marked O), the presence of Xcc was confirmed for all bacterial strains in both cultivars. The transmission to the new upper leaves (marked N) in case of 'Cerox' was confirmed for isolate 3971A (1bN), the weak positive reaction was obtained for 1279A (4N). For 'Sonja' cultivar, plants with isolate SU (SUN) and the mixture of isolates (MN) proved weak positive reaction of new leaves to Xcc presence.



4: *Disease severity caused by tested isolates for 'Cerox' and 'Sonja' cultivars (KW: Cerox: H (4, N = 40) = 14.94050, p = 0.0048; Sonja: H (4, N = 38) = 1.932193, p = 0.7482)*



5: *Results of PCR for bacterial spreading from old leaves (O) to the new ones (N) for different Xcc isolates: 1a-3811, 1b-3971A, 4-1279A, M-mixture of isolates, K-control, B-no template control, P-positive control, M-1kB DNA ladder (Thermo Fisher Scientific, MA, USA)*

Resistance of breeding lines

The aim of this study was to select genotypes showing high degree of resistance to the Xcc infection. Twenty-four cabbage breeding lines were evaluated for their susceptibility to black rot disease. Lines were artificially inoculated by multiple pricking method using the mixture of all isolates. In case of seedlings, the susceptibility of lines was variable, with infected leaf area ranging from 0% to 100% (Fig. 6). Nevertheless, only 3 breeding lines contained plants without visible symptoms of infection (no. 36, 38 and 42). The majority of plants exceeded the 3rd degree of infection which means visible symptoms on leaf surface higher than 50%. The high susceptibility of young plants to Xcc infection was proved on 12 lines where 75% of plants showed symptoms of 4th and 5th degree. The highest susceptibility was observed in case of line no. 10 where the infection was statistically higher to lines 11 and 42.

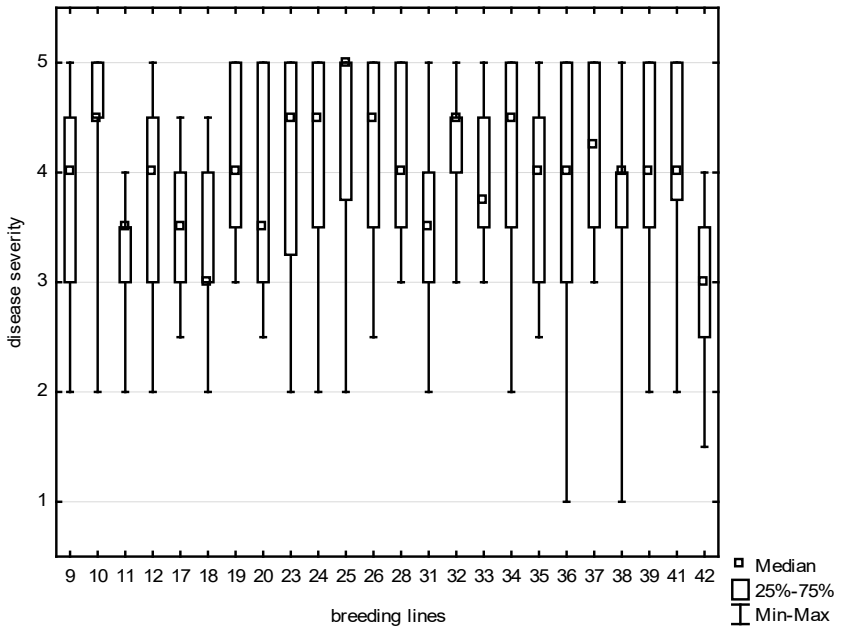
The development of disease symptoms was observed during two following months and the disease incidence of individual lines was evaluated (Tab. II). The first evaluation (75 DAS) showed the disease incidence higher than 50% for 15 lines where 8 of them reached 100%. In the second evaluation (105 DAS), the decrease of the infection was noticed for 11 lines. The increase was observed in 5 lines and 8 lines remained at the same level. The most significant change was observed for line no. 25 of which plants did not show any symptoms at the first observation but after one month, the disease incidence reached 83%.

The lines 35 (Kalibos) and 42 (Avak1) seem to be suitable for breeding resistance program. Regarding the response of these lines from the inoculation to the 2nd evaluation of adult plants, the decrease of infection is evident. The interesting lines should be also no. 10 (DP25), 31 (T1), 33 (WW) and 38 (IT10) which showed higher infection grades in seedling stage but after two months the disease incidence decreased to 17%. Only line no. 38 obtained symptomless plant in evaluation of seedlings. Including the factor of shedding inoculated leaves (SL*) by seedlings (possible expression of hypersensitivity reaction) the strongest reaction was observed in case of line no. 20. Although 5 plants got rid of inoculated leaves, the line was still 100% infected after one month. Plants of line no. 25 also shed some of inoculated leaves and the 1st evaluation of adult plants was without visible infection but the 2nd evaluation reached disease incidence of 83%. The removal of leaves had a stronger effect for decrease of infection only in case of line no. 38 which showed the infection on 33% of adult plants and subsequently on 17%.

Based on visual evaluation, the most interesting lines could be 35 and 42. In these cases, any symptoms were not shown after two months. The followed molecular testing for elimination of latent infection proved the line 42 free of Xcc infection. The line 35 showed weak positive reaction for Xcc presence (Fig. 7).

DISCUSSION

The various techniques and isolates were tested to obtain the suitable method for cabbage resistance testing to *Xanthomonas campestris* pv. *campestris* infection. The results showed the multiple pricking method as the most suitable inoculation method for Xcc on cabbage cultivars. The development

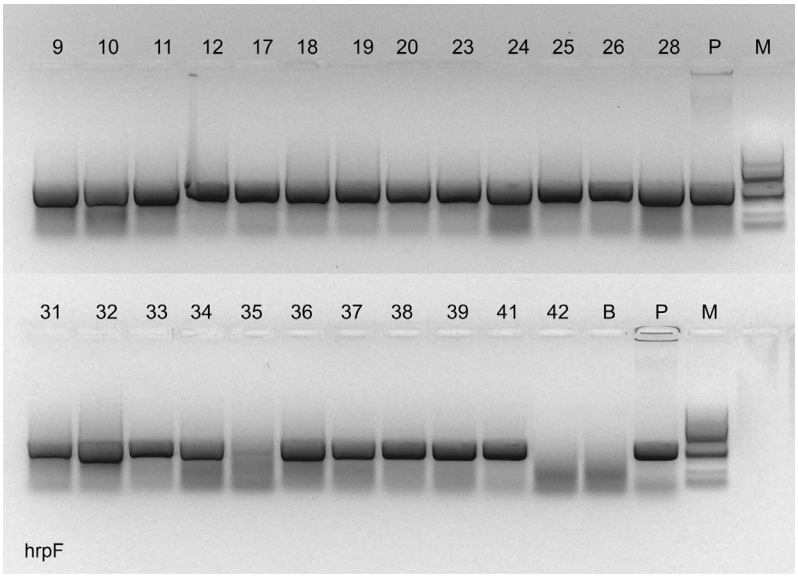


6: *Disease severity on seedlings of different cabbage breeding lines caused by Xcc (KW: H (26, N = 394) = 69.99445, p = 0.0000)*

II: *Disease severity (expressed as number of plants in symptom degrees) and disease incidence (%) of Xcc infection in cabbage breeding lines during the experiment*

lines	seedlings							adult plants	
	number of plants in symptom degrees						median value for disease severity	disease incidence (%)	
	1	2	3	4	5	SL*		75 DAS	105 DAS
9		1	5	5	5	0	4	83.3	66.7
10		1		2	13	0	4.5	66.7	16.7
11		1	5	8		2	3.5	16.7	50.0
12		1	4	5	4	2	4	100.0	100.0
17			6	8	2	0	3.5	100.0	100.0
18		1	7	6	1	1	3	100.0	100.0
19			3	5	7	1	4	33.3	50.0
20			3	4	3	5	3.5	100.0	83.3
23		1	3	1	11	0	4.5	83.3	33.3
24		1	1	5	8	1	4.5	83.3	50.0
25		2	1		9	4	5	0.0	83.3
26			1	6	8	1	4.5	100.0	100.0
28			2	7	7	0	4	100.0	100.0
31		2	5	6	2	1	3.5	50.0	16.7
32			1	4	10	1	4.5	66.7	83.3
33			3	7	5	1	3.75	16.7	16.7
34		1	2	4	9	0	4.5	16.7	33.3
35			4	6	4	2	4	0.0	0.0
36	1		4	3	6	2	4	100.0	50.0
37			2	5	7	2	4.25	66.7	33.3
38	1	1	1	7	3	3	4	33.3	16.7
39		1	2	6	6	1	4	83.3	83.3
41		2	1	6	7	0	4	100.0	60.0
42	1	2	7	6		0	3	16.7	0.0

* SL Number of plants shedding inoculated leaves before evaluation of presented symptoms



7: *PCR amplicons corresponding to hrpF gene of X. campestris obtained from inoculated plants of tested breeding lines 105 DAS: B-no template control, P-positive control, M-100 bp DNA ladder (New England BioLabs, Herts, United Kingdom)*

of V-shaped symptoms was observed within two weeks which corresponds with the time stated in the ISTA protocol (2015). The use of wooden sticks placed in holder provided uniformity of leaf damage, simplicity and low economic demands for plant inoculation. The pricking technique was also successfully used for Xcc inoculation by Maji and Nath (2015). The inoculation described in that study was done after pricking leaves by pins where the bacterial suspension was atomized on the injured leave surface. This separation of inoculation into two steps differs from the procedure used in our study but led to the similar symptom expression. The injection of inoculum by sterile syringe caused the highest disease severity. This method was also used by Mahmood and Hussain (1993) for screening cotton varieties for reaction to bacterial blight caused by *X. campestris* pv. *malvacearum*. They stated that the use of hypodermic syringe was found to be the best and quicker method as compared to scratching, hand rubbing and spraying. This is in contrast to our results where the time required for inoculation, the necessity of syringe exchanges between different variants and uniformity of caused wounds were found inconvenient for extensive testing. The carborundum abrasion method was rejected for the large injury of inoculated leaves that made the symptom evaluation difficult. On the other hand, the carborundum method is recommended in the study of Maji and Nath (2015). The reason of leave damages could be caused by insufficient particle size (800-mesh) that resulted in a higher force to disrupt leaf tissues. The clipping by scissors was also evaluated as easy and quick method for bacterial inoculation. However, this method showed some doubts regarding the evaluation of symptoms where the rate of expression of plant response for injury was discussed.

The selection of isolate for testing was done with four different isolates and their mixture. For the breeding programs in the Czech Republic, the local natural isolate SU could be included in testing of breeding lines. Unfortunately, the race of this isolate was not determined and therefore the mixture of known races and local isolate was also tested. The use of mixed suspension for resistance screening is generally not preferred but also Griesbach *et al.* (2003) used mixture of Xcc race 1 and 4 for testing of cabbage resistance response. Comparing the reactions of plants to individual isolates and mixture, they stated no indications of an interaction between races in inoculum. The isolate 3811 is the reference strain for Xcc race 1 and is often used in studies of Xcc identification, pathogenicity or diversity (Guy *et al.,* 2013; Jensen *et al.,* 2010; Vicente *et al.* 2001). The isolate 1279A represents the reference strain for Xcc race 4 and is also used in scientific researches (Bila *et al.,* 2013; Chitarra *et al.*, 2002; Taylor *et al.,* 2002). The mixture of all isolates showed lower symptoms than individual isolates in case of resistant 'Cerox' but similar values on susceptible 'Sonja' where reached mostly 4th and 5th degree. The elimination of susceptible lines from breeding lines is a key for the efficiency of breeding process. On the basis of these results, the mixture of isolates was used for resistance testing of breeding lines.

The seedlings of breeding lines showed high susceptibility to the Xcc infection. The median values commonly reached 3rd and higher degrees that confirmed the magnitude of infection for young plants. The infection of 11 lines tends to decrease with leaves shedding and reduction of inoculated leaves in later stages. This could be the indication of stem resistance mentioned by Griesbach *et al.* (2003) and Ignatov *et al.* (1999). This type of resistance protect seedling from massive spreading of bacterial particles into the vascular system of the stem and has a race non-specific character. The opposite is the leaf resistance (race-specific nature) that was not possible to evaluate because of use of mixture suspension.

CONCLUSION

The presented study showed the multiple pricking method as a suitable inoculation method for resistance testing. The inoculation by mixed suspension of 4 different isolates of Xcc led to the symptom expression of black rot and the presence of Xcc was confirmed even in adult plants. Current results showed the lines no. 10 (DP25), 31 (T1), 38 (IT10) and 42 (Avak1) as a perspective source for breeding programs because of showing some attributes of stem resistance. The line 35 (Kalibos) seems to be also interesting in case of non-visible symptoms during older stages. These lines will be further examined to conclude the character of response to Xcc infection and for future exploitation into breeding processes.

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