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Advanced detection methods for control of plant bacterial pathogens

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Executive Summary:

The aim of the present project is to develop specific and sensitive PCR-based techniques for routine detection of two plant pathogenic bacteria, *Xanthomonas campestris* pv. *campestris* (Xcc) and *Erwinia carotovora* subsp. *carotovora* (Ecc). During the last two years our main efforts were directed towards the establishment of a bacterial collection, particularly in Kazakhstan, and development of suitable specific primers for the various PCR procedures in Israel. The third year was devoted for development the procedure for detecting Ecc in cabbage seeds and determining the sensitivity of this method. In case of Xcc specific primers were developed for the PCR procedure. During the last year we have extensively tested the PCR procedure for detecting Xcc in cabbage seeds. High correlation between pathogenicity of the tested Xcc strains and the PCR detection procedure was established.

The developed procedures are being transferred to Kazakhstan. Dr. Nazira Ajtkhozhina visited Israel during 2000 for characterizing the Kazakh strains and adopting the PCR procedures for routine use in Kazakhstan. However, an additional visit is required for solving problems encountered by the Kazakh scientists and for concluding the project. This visit will also be utilized for transferring chemicals and small equipment that were purchased in Israel to Kazakhstan. During this year we shall also complete the transfer of funds to Kazakhstan.

Research objectives for the last year

A. Continue the development of a PCR detection procedure for Xcc.

B. Detection of Xcc in cabbage seeds by PCR.

C. Continue isolation and characterization of Xcc and Ecc from Kazakhstan and Xcc from Israel.

Research Accomplishments

A. Development of a detection procedure based on PCR for Xcc

During the second year the RAPD procedure was utilized to generate a specific fragment for Xcc. A fragment of 0.46 kb was cloned and sequenced. Two specific primers were generated in order to perform a specific PCR reaction. The primers were examined with DNA isolated from different strains of Xcc and with different strains of *X. campestris*. Positive results were obtained only with Xcc and not with other strains of *X. campestris* or non-pathogenic isolates. In the last year additional isolates from our collection were subjected to PCR reaction to verify and strengthen the specificity of the primers. Of the twenty isolates that were examined 5 were positive in pathogenicity tests and in PCR. Of the non-pathogenic strains, three produced a weak band with the same size as the pathogen. This band will be further characterized in order to determine whether it has the same sequence as the pathogen.

The threshold of the PCR reaction was determined by adding different concentrations of Xcc to extract of cabbage seeds. Non-inoculated seeds (3.7g) were suspended in 15 ml PBS buffer/Tween solution. The suspension was shaken for 15 min and then different concentration of Xcc (between 10^8 to 10^2) were added to the extract. One ml of each concentration was centrifuged and the pellet re-suspended in 500 µl water. Fifty microliters were plated on NSCAA medium for counting the CFU and 10 µl were taken for PCR. Results presented in Fig. 1 demonstrated that about 2 bacterial cells could be detected by this procedure.

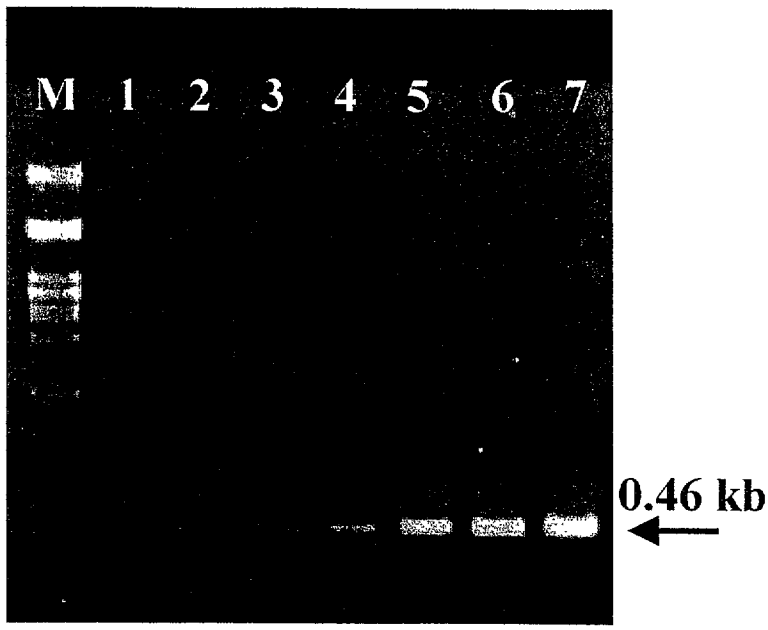

Fig. 1: PCR reaction carried out with different cell concentrations of Xcc
M- molecular markers; Lane numbers 1 to 7 represent different cell concentrations in the range of $2x10^2 - 2x10^8$ CFU/ml.

B. Detection of Xcc in cabbage seeds by PCR

In the last year we have examined the use of the PCR procedure for detecting Xcc in cabbage seeds.

Cabbage seeds without any chemical treatment were infected with Xcc by suspending them in PBS buffer/Tween20 containing different concentrations of Xcc under vacuum for 30 min. The infected seeds were allowed to dry in a laminar flow hood for 2 hours and stored until used. For detecting Xcc, 1000 seeds (about 3.7g) were suspended in PBS buffer/Tween20 in a ratio of 1:4. The suspension was shaken for 15 min and then 1 ml was centrifuged and the pellet re-suspended in 100 µl of water. Ten-fold serial dilutions were plated on nutrient agar (NA) plates and NSCAA plates. Ten µl were taken for direct PCR. For Bio-PCR, total colonies grown on NA or NSCAA plates were suspended in 5 ml of water and 10 µl were used per reaction. The Xcc level in the inoculated cabbage seeds was determined immediately after drying the seeds and then after 1, 2 and 7 days. Results presented in Table 1 showed that a slightly higher CFU were obtained on NA plates as compared to NSCAA plates. It was possible to observed bacterial growth after a week with low inoculation level of 10^5 CFU/ml but not with 10^4 CFU/ml. Positive results were obtained by direct

PCR with all the other samples. Bio-PCR was less sensitive than direct PCR (Table 1). The latter result can be explained by the difference between the two methods. While Bio-PCR detects only viable cells, direct PCR detects also the dead bacteria.

Table 1: Detection of Xcc in artificially inoculated cabbage seeds by PCR procedure

Xcc concentration	Time after inoculation* (days)			
(CFU/ml)	**0**	**1**	**2**	**7**
NA plates				
10^9	$1.4x10^{10}$**	$1.9x10^7$	$7.2x10^6$	$4.8x10^5$
10^7	$8x10^6$	$6x10^5$	$2.4x10^5$	$2.4x10^4$
10^5	$4x10^4$	$1.2x10^4$	$1x10^4$	$6x10^2$
10^4	$1.2x10^3$	10	-	-
NSCAA plates				
10^9	$9.4x10^9$	$1.4x10^7$	$3.1x10^6$	$1.5x10^5$
10^7	$2.2 x10^5$	$4x10^4$	$1x10^4$	$1.6x10^3$
10^5	$8x10^3$	$7.2x10^3$	$2x10^3$	$2.4x10^2$
10^4	$8x10^2$	-	-	-
Direct PCR				
10^9	+	+	+	+
10^7	+	+	+	+
10^5	+	+	+	+
10^4	+	+	-	-
Bio-PCR				
10^9	+	+	+	+
10^7	+	+	+	+
10^5	+	+	+	-
10^4	+	-	-	-

*Seeds were infiltrated with various concentrations of Xcc cells.

**Number of bacterial cells per g of seeds

The minimal threshold of infested seeds that can be detected by PCR will be determined this year.

C. Continue isolation and characterization of Xcc and Ecc from Kazakhstan and Xcc from Israel

Strains of Xcc and Ecc isolated from different places in Kazakhstan were brought to Israel during the last visit of Dr. Nazira Ajtkhozhina. The strains were re-cultured on selective media (NSCAA and TMB plates for Xcc and CVP for Ecc) and were subjected to PCR reactions. For Xcc we used the primers that were developed by us. Positive results were obtained with 3 isolates. These isolates were subjected to pathogenicity tests on cabbage seedlings as described in the previous reports. The positive strains in PCR were also positive in pathogenicity tests.

Ecc isolates were also characterization by PCR as previously described. None of the strains were positive.

We continue the isolation of Xcc in Israel from seeds of cabbage, broccoli and cauliflower and from broccoli diseased plants. Isolations were carried out on selective media and subjected to biochemical and pathogenicity tests on cabbage seedlings. Out of 11 strains that were examined, only 2 were positive in pathogenicity tests and PCR reaction.

Scientific impact of collaboration

During the last year we continued the communication between the scientists of both countries by frequent e-mail and telephone calls. In Kazakhstan most of the work was directed towards isolation of new strains from different regions and adopting the PCR procedures. Due to the large size of the Kazakhstan it has been important to obtain strains from various distant regions in order to assess the variability of pathogen populations. In Israel efforts were made to develop the PCR procedures for detecting Xcc and Ecc in cabbage seeds. In the next year emphasis will continue for transferring these procedure to Kazakhstan for establishing a diagnostic lab.

Strengthening of Developing Country Institution

Equipment and chemicals were purchased in Israel and sent to Kazakhstan. Classical methods for isolating and detecting the pathogens (e.g. selective media and

pathogenicity tests) are already used in Kazakhstan according to the procedures used in Israel. The PCR procedures were initiated in Kazakhstan.

Future work

We ask for an extension of the project for an additional last year due to late start of the project. We shall complete the practical establishment of the PCR procedure for detecting Xcc in cabbage seeds, In the last year we also intend to solve various problems encountered by the Kazakh scientists with the PCR procedures. For this purpose and for transferring additional chemicals and small equipments a visit of the Kazakh scientists during 2001 is required.