PRELIMINARY DEVELOPMENT OF REAL TIME PCR FOR QUANTIFICATION OF *ERWINIA* SPECIES INFECTING POTATO TUBERS

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INTRODUCTION

Erwinia carotovora subsp. atroseptica (Van Hall) (Eca), *Erwinia carotovora subsp. carotovora* (Dye) (Ecc), *Erwinia chrysanthemi* (Burkholder) (Ech) are pathogenic bacterias of potato crop, causing blackleg of stems in the field and soft rot of tubers in storage. During these last years, an outbreak of soft rot potatoes caused by the 3 *Erwinia* species has been observed in Belgium. Several methods are currently available for the detection of *Erwinia* sp. in potato tubers. These methods include plating on selective media, roting by the "*Erwinia* kit", immunological methods and classical PCR tests. The probability of black leg or soft rot development depends on the population level of *Erwinia* on potato tubers (Perombelon, 2002). So, the development of methods able to quantify the *Erwinia* population on tubers is required. Such quantification may be achieved by the use of real-time PCR. This technique combines the advantages of classical PCR (specificity, sensitivity, rapidity) with unique characteristics (lower limit of detection, no post-PCR step, easier quantification of the target). In this paper, we describe the first steps of the development of a real-time PCR method for the quantification of *Erwinia* population on potato tubers. To our knowledge, this is the first report of the development of a real-time PCR to detect *Erwinia* spp. on potato tubers.

MATERIAL AND METHOD

Sixteen *Erwinia* strains (5 Eca, 5 Ecc and 6 Ech) were used in this work. Other strains belonging to 10 bacterial species, cited hereunder, were also tested. These micro-organisms were cultured on nutrient agar (NA) at 24° C during 48 or 72 hours. Genomic DNA (gDNA) was extracted using the Puregene extraction kit of Gentra system (Minneapolis, USA). The DNAMAN Software was used for sequence alignment. The primer pair SR3F-SR1cR and the corresponding PCR amplification conditions (Toth *et al.*, 1999) were used. The Taq polymerase (Roche Applied Bioscience, Penzberg, Germany) and the qPCR Mastermix Plus (Eurogentec, Liège, Belgium) were used for classical and real-time PCR respectively. The sequencing reactions were made at GATC (Constance, Germany).

RESULTS

PCR amplification and sequencing

The primer pairs amplified the expected 119 bp fragment from all the tested strains of Eca, Ecc and Ech.

Sequencing and probe design

Figure 1. Observation on agarose gel of the 119 bp DNA fragment amplified with the Eca (*E. carotovora atroseptica*), Ecc (*E. carotovora carotovora*) and Ech (*E. chrysantemii*) strains. M: DNA ladder 100 bp plus (Fermentas).

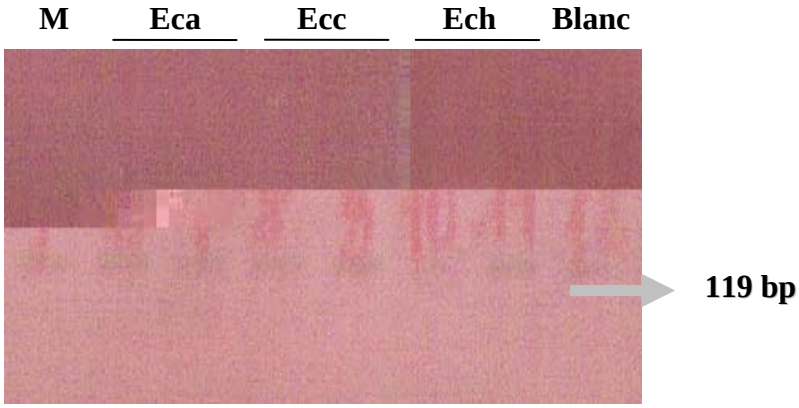
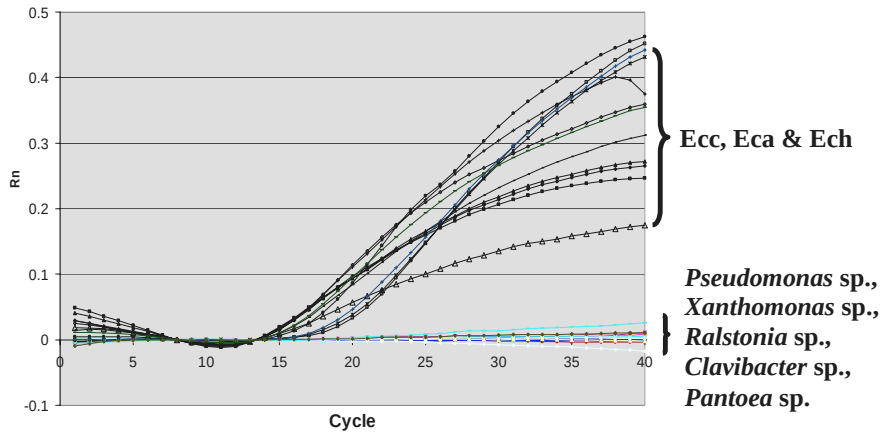


Figure 2. Real-time measurement of emitted fluorescence during amplification cycles with sample containing gDNA from various bacterial species.



Two DNA fragments by subspecies (Figure 1) were cloned and sequenced. The six obtained sequences were aligned with 31 other sequences of *Erwinia* spp. from the public databases. A MGB-probe, polyvalent for all the published sequences of Eca, Ecc and Ech, was selected.

Polyvalence and specificity of the real-time PCR detection

The real-time PCR method succesfully detected the 16 *Erwinia* strains in our collection. Moreover, no fluorescence signal was observed for 10 other bacterial species (*Ralstonia solanacearum*, *Clavibacter michiganensis michiganensis*, *Pseudomonas fluorescent*, *P. marginalisis, P. synrigae*, *Xanthomonas campestris*, *Pantoea agglomerans*, *Burkholderia cepacia*, ...).

Detection on potato tuber

Ten tubers were randomly sampled in commercial potato lots. Among them, 8 presented a latent contamination of *Erwinia* spp. The actual limit of detection of the method, calculated by adding known amounts of *Erwinia* cells to *Erwinia*-free potato tissue, corresponds to 1000 cells per ml of peel extract.

CONCLUSION

A real-time PCR protocol based on the primer pair SR3F-SR1cR and on a newly designed MGB-probe is under development. This protocol specifically detected our strains of Eca, Ecc and Ech. No fluorescence signal was obtained from other bacterial species commonly observed in potato tubers microflora. Furthermore, the limit of detection of the method (1000 cells/g of tuber or/ml of peel extract) was compatible with the thresholds proposed in the litterature for blackleg development (1000 cells/ml of peel extract) or for soft rot development (10^7 cells/g of tuber) (Perombelon, 2000 and 2002). Ongoing research will be focused on the development and the validation of a quantitative real-time PCR protocol.

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