

DNA based detection of blackleg and soft rot disease causing *Erwinia* strains in seed potatoes.

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ABSTRACT

Erwinia carotovora subsp. *atroseptica* (Eca), *Erwinia carotovora* subsp. *carotovora* (Ecc) and *Erwinia chrysanthemi* (Ech) are the different sub species of *Erwinia* that cause the diseases commonly known as blackleg, aerial stem rot and soft rot on potato. Blackleg and aerial stem rot affect vines during the growing season, whereas soft rot affects tubers in the field and during transit and storage. The three species can cause soft rot under cool and moist conditions. *E. carotovora* subsp. *atroseptica* is the major cause of blackleg, a blackening of the stem base of potato plants, which originates from the mother tuber (Pérombelon and Kelman, 1987). *Erwinia carotovora* subsp. *carotovora* mainly causes aerial stem rot (aerial blackleg), but under high temperatures it has been reported to cause blackleg like symptoms. *E. chrysanthemi* also induces blackleg-like symptoms. Until recently *E. chrysanthemi* had been mainly confined to warmer climates of Europe, Australia and the tropics. To date the species has been known to occur in cool temperate regions including Finland. In Finland *E. chrysanthemi* the strain has been reported for the first time in 2004 and it appears to spread fast in certain parts of country (Degefu, unpublished, Pirhonen, personal communication). Although infested crop residues and rotting tubers are among the important sources of inoculum, latent infections in seed tuber provide the major source of infection in potato production (Hannukkala and Segertedt, 2004). At the seed potato laboratory MTT, Ruukki we are carrying out research and services on PCR (DNA) based detection of latent infection of blackleg and soft rot *Erwinia* strains. The enrichment of the bacteria in semi selective liquid medium prior to PCR (BIO-PCR) is an important initial step for the success in PCR detection extremely low number of the target bacteria from tubers. The different strains appear to differ in their ability to compete with other saprophytes and reach the target detection limit of bacterial population during the enrichment culture of the potato peel extract. Results of prior PCR enrichment of the bacteria, detection limits of the different strains and preliminary data, from the analysis of some seed lots from the high grade area of north Ostrobothnia and Åland regions, on the occurrence of the strains and the new trends of introduction and spread of *E. chrysanthemi* in Finland are presented and discussed. Evaluation of the current status of *Erwinia* diagnostics and areas of future research are highlighted

Key words. *Erwinia carotovora* subsp. *atroseptica*, *Erwinia carotovora* subsp. *carotovora*, *Erwinia chrysanthemi*, Blackleg, soft rot, Polymerase chain reaction, Bio-PCR

INTRODUCTION

An important principle for disease prevention is that when the key sources of inoculum for a give disease are known, effective measures can be implemented to prevent further spread and subsequent disease outbreaks. A range of traditional and modern techniques is available for detection of known bacterial pathogens surviving in seed, plant debris, soil, and water and other vectors. As the majority of bacterial pathogens are transmitted through contaminated seed or propagative materials, detection of pathogens in these plant materials becomes of vital importance to ensure safe and sustainable agriculture. Development in the area is necessary as the threat from introduced diseases is constantly increasing as a result growth in international travel and trade. In addition to phytosanitary issues crop

biosecurity and strategic prevention of deliberate release of crop pests and pathogens is national security priority, which also demands a rapid and efficient diagnostic technology (Schaad et al. 2003). Accurate routine disease detection requires high level of specificity, sensitivity and speed (López et al. 2003).

Potato is subject to pests, diseases and quality problems that increase the cost of production, place the grower at a considerable risk of economic loss, and add environmental quality degradation by virtue of the chemicals used to address the problems. Experiences from North America and Europe indicate that hundreds of billions of dollars are spent on use of pesticides annually to prevent virus, nematodes and late blight problems on potato. There is also risk of withdrawal of some of the most effective chemicals under country's Food Quality Protection Act. This sudden bans of key pesticides usually have great impact on production unless immediate alternatives to these pesticides are made available. Resistance and disease management practices that reduce exposure of the crop to diseases and pests and reduce the need for pesticides are arguably the best alternative options.

Blackleg and soft rot are a seed borne disease of potato caused by the soft rot Edwina namely *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. Chrysanthemi*. The three subspecies are responsible for tuber soft rot and are reported to cause blackleg symptoms of varying degree depending on the (Bång, 1989, Pérombelon, 2000). *E. carotovora* subsp. *atroseptica* is the major cause of blackleg, a blackening of the stem base of potato plants, which originates from the mother tuber (Pérombelon and Kelman, 1987). *Erwinia carotovora* subsp. *carotovora* mainly causes aerial stem rot (aerial blackleg), but under high temperatures it has been reported to cause blackleg like symptoms. *E. chrysanthemi* also induces blackleg-like symptoms. While *E. carotovora* subsp. *carotovora* is widespread and has a wide host range besides potato, *E. carotovora* subsp. *atroseptica* is more specifically associated with potato and is prevalent under cold to temperate climates (Hélias et al., 1998). Until recently *E. chrysanthemi* had been confined mainly to warmer climates of Europe, Australia and the tropics. To date the species has been detected from seed potato cool temperate regions including the high-grade area of Finland (Degefu, unpublished) and other sources such as river water (Pirhonen, personal communications)

The economic importance of blackleg is two fold. First it can reduce yield when the disease incidence exceeds 5-10% and second it causes downgrading or rejection of seed potato in the process of certification (Pérombelon, 2000). According to Pérombelon and Hyman (1988) even latent infections of plants can depress potato yield. In addition extensive soft rotting can develop when tubers with blackleg infection are stored. Although infested crop residues and rotting tubers are among the important sources of inoculum, latent infections in seed tuber provide the major source of infection in potato production (Hannukkala and Segertedt, 2004). Under field condition bacteria from rotting mother tubers are transmitted by soil water to the lenticels of progeny tubers (Pérombelon, 1998). Extensive contamination also known to occur during harvest and grading when *Erwinia* from rotting tubers spread to fresh wounds on large number of tubers. Since the use of chemicals is impractical and there are no resistant potato cultivars control of blackleg and soft rot depends primarily on production of healthy seed, which is best checked by detecting and quantifying tuber contamination by the blackleg and soft rot causing *Edwina*. Storing healthy and staying healthy demands vigilant monitoring and risk management.

Detection and diagnostic techniques available for bacteria were based on microscopy, isolation, biochemical characterization, serological techniques, bioassay and pathogenicity tests (Lopez et al, 2003). These are obviously not suitable for routine analysis of large number of samples. Among the major developments in the past two and half decades the Enzyme Linked Immunosorbent assay (ELISA) and the Polymerase Chain Reaction (PCR) is the most significant advance. Currently the detection of phytopathogens has become a very dynamic and evolving world characterised by advances molecular techniques, sequencing, nanotechnology and computer sciences. Automation and electronic data management have become vital to increase the efficiency of for routine analysis of for detection of plant pathogenic bacteria. Effort to develop diagnostic protocols to detect all the 25 potato

pests and diseases listed under EU plant health legislation in a single test is already underway (http://europa.eu.int/comm/research/infocentre/export/success/article_691_en.html). There are strong tendencies of using more integrated approaches (Alvarez, 2004) including the use of biosensors or an electronic nose (Alvarez, 2004, Deisingh, 2004).

Polymerase chain reaction is one of the potential tools for detection plant pathogens. Although further advances have been made in new variants of PCR such as simple multiplexed nested PCR in a single closed tube, cooperative PCR and real-time monitoring of amplicons or quantitative PCR (Lopez et al 2003), these methods have not been adopted for routine detection soft rot *Erwinia* in diagnostic laboratories. PCR has proved to be highly sensitive and more specific than the ELISA method for the detection of soft rot and blackleg in potatoes (De Boer, 1995) and is expected to serve as an important tool in the integrated approach for elucidating the spread of blackleg. However, many things need to be optimised to guarantee its applicability in large-scale routine test.

In this paper we report the use of BIO-PCR whereby viable cells of *Erwinia* from potato peel extract are enriched in liquid medium and detected in low levels in seed potato without DNA extraction. Besides, some preliminary results optimisations of the procedure to enhance the level of detection sensitivity are presented.

MATERIALS AND METHODS

Bacterial Strains media and culture conditions

E. carotovora subsp. *carotovora* (Ecc 107, Ecc 204), *E. carotovora* subsp. *atroseptica* (Eca 5b, Eca 1039, Eca 440, Eca 2286) and *E. chrysanthemi* (Ech 1A, Ech 04026) test strains kindly provided by Hannukkala and Joutsjoki, MTT, Jokioinen Crop Protection section were used in the study. In addition bacterial cultures to test specificity of PCR amplification were also obtained from over one hundred potato samples obtained from experimental fields and seed potato growers from the North Ostrobothnia and Åland regions. Bacteria were grown on either CVP (crystal violet polypectate) plates or NA (nutrient agar). For routine enrichment of the bacteria liquid sodium polypectate medium (PEM) was used. Bacteria in liquid culture were grown at 27° C shaking at 150 rpm. Bacterial strains were stored in as glycerol stock at – 80° C.

Sample handling and potato peel extract preparation

Tubers were washed individually in a running tap water to remove soil and other debris and approximately 3 mm thick peel strips were removed from single tubers with a hand held peeler. The peel strips (One round from stolon end to stolon end) were furnished to plant juice press machine (Meku Erich Pollähne, GmbH, Germany) corrugated roller system. Peel extract running down the corrugated rolls was collected in a sterile glass test tube. An antioxidant, dithiothreitol (DTT) was added to a final concentration of 0.075% and the extract was placed on ice for about 10 min to allow debris and starch to settle before supernatant was pipetted to the polypectate enrichment medium.

Prior PCR enrichment

One hundred microliter of supernatant of the peel extract was transferred to 15 ml Sartedt tubes containing 5 ml of PEM and incubated at 27 °C for 48 hrs shaking at 150 rpm. The resulting bacterial cells were collected by centrifugation at 7000 rpm for five minutes and washed twice by resuspending in 2 ml sterile ultra pure water. The washed bacterial cells were then resuspended in 250 µl of sterile

ultra pure water and used as PCR template or stored in $-20\text{ }^{\circ}\text{C}$ until analysed which is only for a couple of days.

PCR assay

Primers ECA1f and ECA2r derived from a DNA probe specific to Eca (De Boer and Ward, 1995) and Ec001F and Ec001R specific to Ecc and primers Ade1 and Ade 2 specific to Ech were used to amplify DNA. Dynazyme II DNA polymerase and dNTP mix (Finnzymes, Espoo, Finland) were used in a standard PCR reaction assay which employed a one cycle of 2 min denaturation step at $95\text{ }^{\circ}\text{C}$ and 2 min of annealing temperature according to the primer and 2 min of extension at $72\text{ }^{\circ}\text{C}$ and 34 cycles of 30 seconds denaturation, 1 min annealing and 2 min extension. Test strains served as positive controls.

Amplification products were analysed on 1.4% agarose gel and stained with ethidium bromide. Visualization of the amplification products and documentation of results was carried out using Gel Doc (BioRad) and quantity one (Bio Rad) software program.

RESULTS AND DISCUSSION

The standard PCR assay when tested with suspension containing 10^2 - 10^8 cells ml^{-1} successfully detected a minimum of about 10^4 cells ml^{-1} of the three strains only with slight differences consistent to other finding (Hyman et al., 1997). Direct PCR from peel extract with out prior enrichment either failed or was very inconsistent even using high fidelity Phusion ploymerase suggesting that there is perhaps extremely low number of the bacterial strain in the tuber or substances of potato origin inhibitory to the PCR reaction of potato origin are present in the peel extract as it was often speculated by Hyman and co workers (1997) and other investigators. The BIO-PCR protocol that involves the prior PCR enrichment of the bacteria by growing the bacteria for 48 hours in a polypectate medium (PEM) enhanced the sensitivity of the PCR. An added advantage of the enrichment or in general the BIO-PCR is that although small amplicons from dead bacteria can not be ruled out (Alvarez, 2004), the major bands result from amplification of the DNA from living cells. The sample is also free from inhibitory substances as the cells are thoroughly washed before the PCR. In addition no DNA extraction is needed since the cells will lyse and release the during the initial denaturing step (2 min at $95\text{ }^{\circ}\text{C}$). Other investigators (Hyman et al 1997) used CVP plates as medium for enrichment, we have found that CVP to be inconsistent, less efficient and more expensive as it requires much sodium polypectate. By using polypectate medium we have managed to reduce the amount of sodium polypectate by about 12 times (1.5 g instead of 18 g per litter of the medium) and obtained an good and consistent enrichment of the bacteria.

The three strains appear to differ in their ability to compete among themselves as well as with other saprophytes. Growth of the bacteria (OD660) reduced significantly, compared to the control shake culture, when potato peel extract was added to a culture containing 10^3 bacteria of the pure test strains. Our preliminary result indicated that Eca appear to be less competitive than both Ecc and Ech. The competitive ability of Eca was improved an anaerobic growth condition apparently because of the suppression of aerobic saprophytes in the culture.

The optimised PCR protocol is consistent and successfully detects the three soft rot causing Erwinia strains. We have analysed hundreds of samples. It is evident that seeds could be contaminated by either one or all the three strains of Erwinia. We have documented new record of *E. chrysanthemi* infection in seed potatoes in Finland, also confirmed from report of other labs (Hannukkala and Joutsjoki, Pirhonen , personal communication) a strain, which was confined mostly to warmer tropical regions of the world. This indicates the likely occurrence freeze tolerance strain.

CONCLUSIONS AND PROSPECTS

Pérombelon (2000) emphasized on four important steps in seed-potato health determination or detection of *Erwinia* which need to be evaluated namely (1) sampling (2) tuber tissue preparation for testing (3) quantification of tuber contamination, and (4) interpretation of the results in terms of blackleg risk assessment. Conclusions drawn from ultrasensitive analytical methods that require only microliter of the sample might often misrepresent the real situation unless sampling that represents realistic order of scale is practiced. The key elements in all sampling procedures are random sampling of the seed lot and blending of primary, composite, and submitted samples (Morrison, 1999). The crucial step in the PCR detection process is the handling of the tubers and preparation of peel extract. Extreme care is needed to avoid cross contamination because of the high sensitivity of the BIO-PCR. One of the main challenges in the diagnosis of *Erwinia* in seed potato is to separate the target pathogen from numerous saprophytes that overgrow it in culture media

The conventional PCR can only indicate the presence or absence of bacteria. There has been significant development in real time PCR technology that has an advantage of determining the absolute quantity of the starting target DNA in a tuber. Our goal in this regard is to develop the quantitative *Erwinia* diagnostic technology and generate epidemiological data in relation to initial inoculum density of the pathogen to establish threshold infection and tolerance levels for meaningful prediction and certification programs. This rather challenging task. It is hard to accurately from the number of seed-tuber borne bacteria (Bain and Pérombelon, 1990) since growing conditions, which are not known at the time of tuber analysis can have large role in disease development. However, seeds could be designated as high, moderate or low risk with respect to blackleg infection and the estimation of the risk level could be improved by obtaining data under diverse climatic and weather conditions.

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