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Review paper

IS THERE A NEED AND A PLACE FOR REAL-TIME PCR IN DETECTION OF *ERWINIA AMYLOVORA*?

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Molecular-biology DNA based methods for the detection of the fire-blight pathogen have been developed early; Real-Time PCR has been available since 2004. While the overall advantages of Real-Time PCR are frequently stated, this review attempts to assess the usefulness of the available methods in practice and to point out critical steps in its use and development and to identify their potential role in the future.

Key words: *Erwinia amylovora*, fire blight, Real-Time PCR, diagnostics, quantification.

INTRODUCTION

Erwinia amylovora (Burrill 1882) Winslow et al. 1920 causes fire blight, a devastating disease that continues to spread to new geographical areas (van der Zwet, 2006). Detection of *E. amylovora* in symptomatic samples is usually straightforward as bacteria are present in large numbers and grow well on artificial media. Reliable diagnosis, however, can be difficult when bacteria are hindered in their growth on artificial media, or the population size is low due to latent infections. *E. amylovora* populations are also low in dormant cankers in winter and spring, when plant inspectors and farmers control their plants and do cutting and pruning. Detection of latent or symptomless infection is recognized as an important means of its spread to pathogen-free areas through infected budwood or trees (Bonn & van der Zwet, 2000).

The European and Mediterranean Plant Protection Organization (EPPO) published a revised diagnostic protocol, for *E. amylovora* detection in 2004 (EPPO, 2004). The protocol is a guideline compilation of methods that can be adapted according to needs and desired accuracy. Since its publication, new methods became available or were further developed for detection of *E. amylovora*. Among these, molecular methods predominate and include both classical PCRs (Kokosková and Mráz, 2005; Obradović et al., 2007) and Real-Time PCRs (Salm and Geider, 2004; De Bellis et al., 2007; Lehman et al., 2008; Mohammadi et al., 2009; Pirc et al., 2009; Svircev et al., 2009).

REAL-TIME PCR ASSAYS FOR DETECTION AND IDENTIFICATION OF *ERWINIA AMYLOVORA*

Target DNA sequences and available Real-Time PCR assays

Real-time PCR systems that were developed for detection of *E. amylovora* were developed with different aims and authors have thus concentrated on different characteristics to make them fit for their purposes (Table 1). Real-Time PCR is commonly recognized as a highly specific test due to design of primers and probes and the chemistry involved. Among more than twenty different available chemistries that differ in the way that PCR products are generated and detected (see e.g. Buh Gašparič et al., 2008), TaqMan chemistry remains a method of choice also in fire blight detection.

First Real-Time PCRs developed, including the first Real-Time PCR for detection of *E. amylovora* (Salm & Geider, 2004), have often employed SybrGreen chemistry. In case of SybrGreen chemistry an interchelating dye is included in thereaction mixture that specifically binds to dsDNA (its minor groove) and emits fluorescence during binding. The system was popular due to its low costs, since no labelled oligonucleotides are required. Basically any PCR assay can be run with SybrGreen and the product accumulation then be measured by a real-time PCR instrument. The downside of SybrGreen chemistry and the reason for its current lesser use is that any dsDNA product as well as primer-dimers will generate fluorescence and the reaction itself is thus not necessarily specific: From the signal generated we get no information on the DNA sequence of the amplified product or its size. To derive as much information as from classical PCR Real-Time PCR employing SybrGreen should be followed by additional step in which melting curve of generated product is determined (melting curve analysis). The shape of the melting curve and the determined melting temperature depend on

Table 1 - Real-Time PCR systems developed for detection of *E. amylovora* and associated studies with basic characteristics (target DNA sequence, primer and probes sequence).
Tabela 1 - Real-Time PCR sistemi razvijeni za detekciju *E. amylovora* sa osnovnim karakteristikama (ispitivana DNK sekvenca, prajmer i DNK probe).

Reference	Target	Chemistry used	Primers and probes (5' -> 3')[a]	Main objective of the study
Salm and Geider, 2004	pEA29	SybrGreen TaqMan	P29TF CACTGATGGTGCCGTTG P29TR CGCCAGGATAGTCGCATA P29TM FAM-TACCTCCGCAGCCGTCATGG-TAMRA	detection and identification of Ea in symptomatic and asymptomatic samples
De Bellis et al., 2007[b]	pEA29	Scorpion	E3 HEG-AAGACATCCGGCTTCTGAAA E4 GGATTACGGGATGACAAGA PB FAM-TATCTCTTGATTTTTTCAGG ON CCTGAAAAAATCAAGAGATA-MR	detection and identification of epiphytic populations of Ea in washings of flowers and leaves
Lehman et al., 2008	chromosomal levansucrase gene	TaqMan	Ea-lscF CGCTAACAGCAGATCGCA Ea-lscR AAATACGCGCACGACCAT Ea-lscP CY5-CTGATAATCCGCAATTCCAGGATG-IAbRQ	assesing competition between Ea (Ea-lsc) and *E. pyrifoliae* (Ep-hrpw) on pear blossoms: duplex Real-Time PCR
	chromosomal hrpW gene		Ep-hrpwF CGCTAACCCGACTGTGCT Ep-hrpwR TGAAGGTTTGCCCTTTGC Ep-hrpwP FAM-ATGACACCATCATCGTAAAGGCGG-BHQ1	
Mohammadi et al., 2009[c]	amsK gene	SybrGreen TaqMan	AMSK14819 AACGAGTTGCTGCTACC AMSK14948c CATCGCGTAGCTTAAGG AMSK14840Cy5 Cy5-AGCCGTCTGTGGCAGCACAA-BHQ2	screening for Ea strains lacking pEA29 plasmid
	pEA29		see Salm and Geider, 2004	
Pirc et al., 2009	chromosomal amsC gene	TaqMan	Ams116F TCCCACATACTGTGAATCATCCA Ams189R GGGTATTTGCGCTAATTTTATTCG Ams141T FAM-CCAGAATCTGGCCCGCGTATACCG-TAMRA	detection and identification of Ea in symptomatic and asymptomatic samples
	chromosomal ITS region 'optional sequence'		ITS15F TGAGTAATGAGCGAGCTAAGTGAAG ITS93R CGCAATGCTCATGGACTCAA ITS43T FAM-AGGCGTCAGCGCGCAGCAAC-TAMRA	
	pEA29		see Salm and Geider, 2004[d]	
Svircev et al., 2009	chromosomal	TaqMan	see Lehman et al., 2008	

the size of the PCR product, its concentration, nucleotide base composition and to some extent on its nucleotide sequence.

The principle of TaqMan probes relies on the on the 5′>3′ nuclease activity of Taq polymerase. When polymerase upon elongation of primer encounter annealed probe, this is cleaved and the two labels attached to either ends of the probe dissociate in solution. Due to their physical properties (FRET) their fluorescence changes when the distance between them increases resulting in i.e. increase in reporter dye FAM fluorescence and decrease in quencher dye TAMRA fluorescence. Using TaqMan chemistry primers and probes are designed in such a way that the extent of target sequence not complementary to them is minimized, which is reflected in much shorter final products than in classical PCR, typicaly from 70 to 110 bp. The signal is generated only when both primers and a probe, covering most of the target sequence, anneal to it. The choice of the target sequence is therefor crucial for the specificity of the test. Regardless of the chemistry employed, fluorescence generated is measured by real-time PCR instrument and accompanying softwares which also provide data normalisation and some automatic options of analysis. These however may not be optimal and should be checked manually. The result is a calculated cycle in which the generated fluorescence significantly rises above the background fluorescence (a positive signal, often labeled as Ct or Cp).

Targets for conventional PCR methods for detection of *E. amylovora* have historically included plasmid pEA29 or chromosomal DNA. Due to several copies of plasmid DNA per cells as opposed to usually one copy of chromosomal DNA targets, plasmid based system were often more sensitive and thus a preferred method. The plasmid present in vast majority of *E. amylovora* strains also seems to be specific to *E. amylovora* while in PCRs targeting chromosomal targets, unspecific or cross-reactions were sometimes reported in addition to lower sensitivity (Bereswill et al., 1995; Maes et al., 1996; Llop et al., 2000; Rosello et al., 2002). Not surprisingly, the first Real-Time PCR developed for *E. amylovora* was thus designed to detect a target sequence on the pEA29 plasmid (Salm and Geider, 2004). Similarly, De Bellis et al. (2007) have chosen pEA29 as a target in their proof-of-principle paper combining Scorpion primers with nested PCR.

Potential existance of *E. amylovora* strains without pEA29 plasmid has been recognized before, they have only been found in nature fairly recently and while retaining pathogenicity seem to be rare (Llop et al., 2006; Mohammadi et al., 2009). While the biological significance of strains lacking pEA29, or any plasmid, is not yet resolved, methods based on detection of chromosomal DNA rather than plasmid DNA are more suitable when detection of all strains is desired. Real-Time PCR systems developed later employed chromosomal targets, either those previously used in classical PCR or newly chosen. The *ams* region of the *E.*

amylovora chromosome is involved in the synthesis of the capsular polysaccharide amylovoran which seems to be unique to *E. amylovora* (Bugert & Geider, 1995; Bereswill et al., 1995) and is strongly associated with its multiplication in plants and its virulence (Steinberger & Beer, 1988; Menggad & Laurent, 1998). As such it has been used as a target sequence in several classical PCR and later Real-Time PCR methods (amsK in Mohammadi et al., 2009; amsC in Pirc et al., 2009). Sequences for the production of levansucrose, another exopolysaccharide contributing to *E. amylovora* virulence, has been chosen as a target sequence by Lehman et al. (2008). 16S-23S ribosomal intergenic spacer regions (ITS) have been useful in the detection of many bacterial species (Li & DeBoer, 1995; Pastrik et al., 2002). By analyzing ITS regions in *E. amylovora* several copies of rRNA operons were found, with a 139 bp named 'optional sequence' present only in *E.* strains from fruit trees and not in the isolate from *Rubus* spp. (McGhee et al., 2002); this 'optional sequence' was consequently used in Real-Time PCR developed by Pirc et al. (2009).

As in other reasearch fields, early papers report the use of both SybrGreen and TaqMan chemistries for detection purposes (Salm and Geider, 2004). Also a Real-Time PCR system based on the use of Scorpion bi-probes was developed but has not shown advantages over other systems particularly with respect to sensitivity. The authors have overcome this shortocoming by combining the Real-Time PCR assay with a previous nested step (De Bellis et al., 2007). Despite successfully increasing sensitivity, the transferring of reaction products from nested to the real-time PCR step increases the possibility of contaminations, and thus method has not been widely accepted. All other assays were developed with TaqMan probes and advances in technology development are included such as availability of more efficient quencher molecules (Mohammadi et al., 2009; Svircev et al., 2009) that increase the signal to noise ratio (Marras et al., 2002).

Improved formulations of reaction mixtures have become available that allow for faster amplification and temperature cycling and thus shorten the whole amplification and processing time. Real-Time PCR targeting amsC gene has been adapted to such reaction mixture and used with a faster cycling protocol on a portable Real-Time PCR instrument (SmartCycler, Cepheid; Pirc M., pers. comm.).

Testing for specificity

The first step in guarantiing specificity is choosing appropriate DNA target sequence. Oligonucleotide primers and probes should are then usually designed using suitable design software. Cheking primer's and probe's sequences, as well as the whole PCR product against sequences deposited in nucleotide databanks such as NCBI (Genbank, National Center for Biotechnology Information, Bethseda,

MD) is an easy step that can prevent many unnecessary experiments. The conclusive results however are still generated through empirical testing. Specificity has been tested for all designed Real-Time PCR assays (Tab. 1) although the degree of testing differs between laboratories and research projects. Commonly tested strains used by most authors include, in addition to a selection of known *E. amylovora* strains, *Erwinia pyrifoliae* and *Erwinia* spp. isolated in Japan. These bacteria are the most likely candidates to give reactions in tests designed for *E. amylovora* due to their intermediate taxonomical status and genetic similarity (Won-Sik *et al*., 2001; Maxson-Stein et al., 2003; Geider et al., 2009). Indeed, their intermediate status has been supported by real-time PCR analysis targeting ITS and hrpW sequences. The ITS Real-Time PCR gave a positive reaction in more than 200 *E. amylovora* strains isolated from different hosts (Pirc et al., 2009), indicating that this sequence is widely present in the target pathogen; and while no unspecific amplifications were observed a clear positive signal was generated with *Erwinia* spp. strains from Japan (Hokkaido) but not with *E. pyrifoliae*. Real-Time PCR analysis of hrpW, originally designed to detect *E. pyrifoliae* however, also detected *Erwinia* spp. from Japan while no amplification was observed with *E. amylovora* (Lehman et al., 2008). As in both cases the signals are generated because of specific amplification of similar/identical sequences, these tests have potential for detection of a broader range of pathogens when applicable. Several genome sequencing projects have been completed or are in progress; and it is expected that a comparitve genomic analysis of these species will eventually lead to clarification of the relationships among these strains (e.g. Smits et al., 2010).

Other closely related bacteria or other bacteria expected in the same environment have been tested at least by some authors in determining specificity: *Erwinia* spp. isolated in Australia, *Erwinia* sp. isolated from necrotic pear blossoms in Spain (Roselló et al., 2006), *Erwinia billingiae*, *Erwinia tasmmaniensis*, *Erwinia persicina*, *Pectobacterium atrosepticum*, *Dickeya chrysanthemi*, different *Pseudomonas* spp. and *Pantoea agglomerans* (including biocontrol agent strains). In most of these cases the number of available strains was limited and the current results on specificity are only as reliable as the available isolates are representative of the species diversity. Pirc et al. (2009), focusing on development of Real-Time PCRs for diagnostic purposes, have included additional uncharacterized strains isolated from necrotic tissues of fire blight hosts and DNA extracted from the same hosts and associated microflora.

Plant material and DNA extraction

Erwinia amylovora, although genetically a very homogenous species, can infect a vast array of plant hosts (Momol and Aldwinckle, 2000). Of these, pome fruit trees are among the most economically important, but other hosts such as *Crataegus*, *Cotoneaster*, *Photinia davidiana, Sorbus spp.* have been found especially important for the spread of *E. amylovora* under European conditions and their new planting restricted or prohibited (Gianetti et al., 2004; Duffy et al., 2005). Fire blight symptoms can develop on any part of the plant with most typical being the sheperd's crook of twigs, necrosis of blossoms and leaves, ooze and cankers. While sometimes a method is applied to one type of sample only, for a diagnostic laboratory a detection method should be able to detect *E. amylovora* in all different plant species, symptoms and ideally, also in asymptomatic material. In case of Real-Time PCRs critical step to address is the use of DNA extraction methods that sucessfully remove inhibitory substances of PCR amplification.

Real-Time PCR is especially useful for comparing and estimating efficiency of DNA extraction methods as it allows accurate determination of the amount and quality of DNA (de Kok et al., 1998).

Using pure bacterial cultures or ooze, colony PCR is a quick and valuable option as most DNA polymerases in use today require an incubation step at 95°C for 5 to 15 minutes for enzymatic activation during which bacterial cells added directly to the reaction are disrupted and release DNA. Such approach has been used for analysis of pure culturs (Svircev et al., 2009) and a small scale study on enriched plant extracts prepared from asymptomatic plant material. In enriched asymptomatic samples colony Real-Time PCR was shown to be at least as or more efficient than DNA extraction (Pirc et al., 2008). However, since some bacteria present in enriched samples can produce pigments that fluoresce in the same range as reporter dyes used for labelling TaqMan probes, care should be taken in interpretation of negative results as this high level of fluorescence is regarded as background by acquisition software and can mask a real positive signal. In a study conducted on pure bacterial cultures, Mohammadi et al. (2009) as well as previous study by Salm and Geider (2004) the authors have used Tween 20 detergent (0.1- 1 %, with or without heating) to disrupt cells prior to Real-Time PCR reaction. However, this may not be necessary and in fact, too high concentrations of the detergent may have an adverse effect on DNA polymerase stability and PCR amplification.

When the target plant material is expected to contain both, low levels of inhibitors and high levels of bacteria, simple DNA extraction procedures may be sufficient and there is a lesser need for extensive purification of DNA. Lehman et al. (2008) reported using a simple Direct Plant Extraction Buffer (DiPEB) for

DNA extraction from artificially inoculated pear blossoms from which petals and peduncles were removed since it is known that they oxidate quickly and can be a source of potent PCR inhibitors. The same buffer was used by Svircev et al (2009) to isolate *E. amylovora* from bark, stems, leaves, blossoms, anthers and pollen. However, little data is available on its performance in these different samples. The buffer DiPEB originaly developed by Kim et al. (2008) is expected to become commercially available from Agdia Inc. (Elkhart, IN). Stöger et al. (2006) have described a rather straightforward modified protocol of the REDExtract-N-Amp™ Plant polymerase chain reaction kit (Sigma) for detection of *E. amylovora* that has been extensivly tested in combination with classical PCR and has good potential to perform well also in combination with Real-Time PCR.

Several DNA extraction methods based on different principles of DNA extraction (isopropanol precipitation – Llop et al., 1999, silica columns, magnetic beads) were used by Pirc et al. (2009) to isolate DNA from necrotic green tissues containing high levels of inhibitory substances. They report variability in the level of inhibition between different plant hosts and between individual samples of the same host. As observed previously by Maes et al. (1996) the inhibition in a particular sample seems to be more affected by the amount of necrosis and physiological age of tissue than the plant species itself. Of the tested DNA extraction methods, magnetic beads and silica column-based methods were found to be most successful in removing inhibitors leading to increased amplification efficiencies in real-time PCR (Pirc et al., 2009). In addition magnetic-beads based and silica-column based methods are readilly ammenable to automatization and high-throughput analysis (Pirc et al., 2009).

Because of high amount of bacteria usually present in symptomatic fire blight samples positive results can often be obtained through direct addition of plant extract to Real-Time PCR reaction even when it is prepared from necrotic tissues, provided it is diluted at least 1:10 in water and buffer (Salm & Geider, 2004; Pirc et al., 2009). However, since the inhibition seems to be dependent on a particular sample and can not be generalized over plant species or different samples or indeed accurately predicted, sensitivity of such use can vary significantly. Reliable detection of lower concentrations of target bacteria from different plant samples can thus only be.

Determining and reporting sensitivity

The approach to determine sensitivity commonly involves spiking, that is adding target bacteria in a range of concentrations to plant samples or extracts prepared from plant tissues, and then processing them by the chosen methods of DNA extraction and Real-Time PCR. The distribution of positive results at low

concentrations, typically below 10^3 CFUs/mL, follows a Poisson distribution therefore high numbers of replicates are needed to accurately determine sensitivity.

While in most studies performed healthy plant material is used for preparation of spiked plant samples, such an approach may seriously underestimate the influence of inhibition present in natural samples and may be too optimistic in reporting sensitivity. Especially in comparison of different methods, these may be affected by necrotic plant tissues to a different extent and thus their sensitivity and reliability in real-life situations can differ significantly from their performance in healthy tissues.

Studies of Real-Time PCR including necrotic samples report influence of specific sample on sensitivty (Pirc et al., 2009). However, irrespective of the DNA extraction (isopropanol, silica-columns, magnetic beads) and Real-Time PCR system used (targets amsC, ITS or protocol originally developed by Salm and Geider, 2004 with modifications), concentrations at and above 10^3 cells/ml plant extracts (less than 4 cells per reaction) were always detected (Pirc et al., 2009). Detection below this concentration (samples with 100 cells/ml plant extract), was possible however, the probability of detection was affected then by both DNA extraction and Real-Time PCR, specifically target copy number, and ranged from 0.09 to 0.91.

Sensitivity of a test is usually reported as the lowest concentration of the target reliably detected. Sensitivity can be and often is reported in different ways: 8.6 x 10^3 cells from leaf sample and 1.8 x 10^4 cells from bark sample (Salm & Geider, 2004), 3.2 x 104 CFU/mL (De Bellis et al., 2007), 20 CFU/reaction (Lehman et al., 2008), < 500 CFU/reaction (Mohammadi et al., 2008) and 100 CFUs/reaction (Svircev et al., 2009). The meaning of term 'reliable' also varies in the literature and authors rarely provide information on which definition they have used in assessing results. However, when starting amount of the sample and DNA extraction process is taken into account, a general level of detection at 10^3 cells per mL starting amount of plant material emerges. Lower concentrations can be detected however, reliability or probability of such detection is rarely reported.

Increasing sensitivity and testing of latent samples

Sensitivity of Real-Time PCR is affected by many factors including: type of plant material, amount of plant material, concentration steps, DNA target copy number, DNA extraction method, volume of sample analysed in Real-Time PCR, total reaction volume and the number of reactions performed. The reported sensitivity encompasses all this details; changes in any of these factors may improve detection but can also have adverse effect on performance of Real-Time PCR. The influence of modifications thus needs to be empiracally tested.

Enrichment of *E. amylovora* from plant extracts in non-selective and selective media is advised when low concentrations of bacteria are expected, e.g. in symptomatic samples with abundant tissue necrosis, samples treated with pesticides or bactericidal compounds and symptomless samples (EPPO, 2004). Enrichment is expected to increase sensitivity of further screening tests (Llop et al., 2000; EPPO, 2004) and facilitate isolation in pure culture by activating viable but non-culturable cells (Ordax *et al.,* 2006).

Pirc et al. (2009) have used quantification of *E. amylovora* to assess the efficiency of enrichment method by spiking a set of 33 asymptomatic samples prepared from twigs according to EPPO (1992) with low concentrations of *E. amylovora* and quantified them before and after enrichment. From initial concentrations at LOD level (≈10^3 cells/ml) *E. amylovora* cells reliably multiplied in all tested symptomless samples with final concentrations ranging from 10^5 to 10^9 *E. amylovora* cells/ml. Contrary to this prosimising result, isolation of *E. amylovora* in pure culture from enriched samples proved difficult. Despite using five 10-fold dilutions for plating to avoid problems with overgrowth by other bacteria (an increase from three 10-fold dilutions suggested by EPPO, 2004), target bacteria could be isolated in pure culture mainly from samples with at least 10^7 *E. amylovora* cells/mL of enriched extract.

When is a sample positive?

A Real-Time PCR reaction is positive when a signal significantly above background level is detected during the PCR cycling program. The point at which this happens in a particular sample is determined as a theoretical cycle (e.g. Ct or Cp) and this correlates to the starting amount of the target DNA. For a given system, Real-Time PCR assay will provide fairly stable Ct or Cp values for a certain concentration so that after validation of a method is done typical Ct/Cp values are known for a range of concentrations.

For qualitative detection stress is put on determination of the highest treshold cycle that corresponds to the lowest DNA target concentration that can be detected i.e. treshold cycle that represents limit of detection of the assay. Its exact value depends on characteristics of samples, DNA extraction and Real-Time PCR with critical factors being amout of inhibition and amplification efficiency.

A common approach to determine the highest possible Ct is to analyse spiked samples with low concentrations of target DNA or naturally infected samples. This treshold value can then be used as a cut-off value in analyses of further samples. In general, when a treshold cycle is equal or below the cut-off value, corresponding to higher concentrations, it is considered positive, while signals above cut-off treshold cycle are considered negative. However, in certain circu-

mstances e.g. when concentration of DNA target is low and this is coupled with an inhibition level higher than expected and observed during validations, this can lead to positive samples giving Ct values above determined cut-off value

When template DNA concentrations are below an optimal range (usually below 10 cells per reaction), stochastic fluctuations can result in unequal sampling and lead to discrepant results among parallels (Navidi et al., 1992). In the case of high reliability of the selected real-time PCR such signals are interpreted as positive results for the sample provided although they are above the determined cut-off Ct/Cp value.

Due to higher sensitivity of Real-Time PCR there is usually a range of low DNA target concentrations that can only be detected as positive using Real-Time PCR and not by other, less sensitive methods. This can give rise to some difficulties in reporting results and their interpretation. Concept of zero-tolerance for pathogen presence can come in conflict with requirement of diagnostic schemes to have at least two methods with positive results to be able to report it as suspicious or isolate bacteria in pure culture (EPPO, 2004). To some extent this can be resolved by using a combination of Real-Time PCRs with different target sequences to increase the reliability of detection (Pirc et al., 2009).

Guidelines and future steps in development and assessment of Real-Time PCR

The idea of method validations as a reasonably standardised and defined process has in recent years been accepted by diagnostic laboratories in Europe and elsewhere. While the extent of validation and experiments performed certainly depend on the intended use of the method, a need for a good and reliable method is common to research and diagnostic laboratories; while high sensitivity is crucial if a test is to be used as a screening test and specificity is of higher importance when it is to be applied to pure cultures for identification purposes. While most published pathogen detection and identification methods are tested, the extent of testing does vary. Lots of effort has been put into agreeing on a common strategy to validations in the area of plant pathology in the EPPO region. Consequently, basic requirements were published in an EPPO Standard PM 7/84 (EPPO, 2007) and publication of further guidelines is expected.

A promising development is a wider acceptance of 2x2 contingency tables taking into account prevalence and likelihood ratios and reporting on sensitivity of the method as probability of detection rather than only absolute sensitivity and specificity of the method (Lopez et al., 2009).

Quantification and its relevance to fire blight diagnostics and research

Real-Time PCR assays can be used in both quantitative as well as a qualitative way. The threshold value when the signal is recognized correlates to the starting amount of the target. An estimate of concentration is straightforward from the sample's treshold value and its comparison to a general standard curve giving an idea wheter the *E. amylovora* concentration is low or high. This provides additional information on the likelihood of its isolation in pure culture and the possibility of confirming the result with other, less sensitive methods. When normalized to cell or CFU number, quantification can only be accurate when the target copy number is constant and does not vary among isolates. Further important consideration in accuracy of quantification is the similarity of PCR amplification efficiencies between samples and standard curve (Cankar et al., 2006)

In detection of *E. amylovora*, being a quarantine pathogen with zero tolerance levels, quantification is seldom employed in routine detection as any positive result has the same consequences and is interpreted together with other methods used (EPPO, 2004). The results are thus reported as positive, dubious or negative and the threshold value only used in interpretation if necessary. Pirc et al. (2009) have quantified *E. amylovora* concentrations in asymptomatic samples in enrichment showing that subsequent isolation in pure culture requires extremely high concentrations of bacteria (above 10^7 cells/mL).

Quantification however, is more often applied for research purposes. Both Salm and Geider (2004) and De Bellis et al. (2007) have used plasmid based Real-Time PCRs for quantification of *E. amylovora* in artificially inoculated detached flowers and leaves. Lehman et al. (2008) have used duplex Real-Time PCR targeting chromosomal sequences of *E. amylovora* and E. pyrifoliae to study their interaction upon co-inoculation of detached pear blossoms. In this study concentrations of *E. amylovora* of less than 100 cels per blossom lead to fire blight symptoms, indicating that threshold population sizes smaller than 4.7×10^4 CFU/blossom can results in infection of hypanthium. This is in contrast to previous studies indicating that higher populations are required for infections (Thomson et al., 1975; Johnson et al., 1993). As Lehman et al., indicated, however, the observed differences could be a consequence of the different analystical methods used in related studies or different inoculation methods.

An interesting example of real-time PCR use has been recently employed (Holliger et al., 2010) and represents the first attempt of quantification of *E. amylovora* in asymptomatic apple blossoms under natural conditions and early in the season using Real-Time PCR. The study attempts to benefit from high sensitivity of Real-Time PCR and determine concentrations of *E. amylovora* in apple

blossoms under natural conditions in areas where fire blight has been previously observed; the final aim is check the relevance and feasibility of such monitoring for a more evidence support use of control substances during flowering, an epidemiologically critical step in fire blight disease (Holliger et al., 2010).

CONCLUSIONS: WHAT CAN REAL-TIME PCRS OFFER NOW AND IN THE FUTURE?

Real-Time PCR assays have proven to be the most sensitive reliable methods so far available for detection and diagnosis of bacterial and other plant pathogens (Lopez et al.; 2009). Other methods can sometime achieve equal sensitivity as Real-Time PCR e.g. isolation on media and classical PCR, but lack its reliability and repeatability. While sensitivity of isolation on media can sometimes even surpass the sensitivity of Real-Time PCR, its performance is highly affected by the different microflora in plant samples or their chemical composition; also conditions for bacterial survival in plants are not always optimal and can lead to transition of *E. amylovora* into viable but non-culturable state (Ordax et al., 2006). Because of longer products, classical PCR is often more sensitive to inhibition than real-time PCR. Compared to Real-Time PCR nested PCR (Llop et al., 2000) showed higher variations in sensitivity and failed to detect some samples even at higher concentrations (Pirc et al., 2009).

Reliability of detection of *E. amylovora* is further increased trough a combined use of assays targeting different DNA sequences.This is especially important in cases when other methods are not sensitive enough to detect it and final confirmation in accordance to existing detection schemes (EPPO, 2004) is not possible. Providing a proof of viability of *E. amylovora* in such instances is equally important and can be achieved by quantification before and after sample enrichment as described by Pirc et al. (2009).

Being the most sensitive and reliable method of *E. amylovora* detection, expectations from Real-Time PCR were sometimes unreasonably high with regards to detecting asymptomatic or latent infections. In this field there is a great need to design appropriate plant sampling methodology for the analyses to be relevant. It could well be that the amount of sampling needed for a reliable detection is too high to be practically feasible and ‘scouting’ for fire blight infection, a term used by Kuflik et al. (2008)**,** may be more appropriate than commonly used ‘monitoring’. Even when *E. amylovora* is detected in such samples, the likelihood of confirming a positive Real-Time PCR result in enriched asymptomatic plant samples according to procedure suggested by EPPO (2004) is highly unlikely (Pirc et al., 2009). There are very few mentions of isolating *E. amylovora* from such samples

and to our knowledge they have almost exclusively coincided with occurance of symptoms at the same locations or in the vicinity upon closer inspection. The high-throughput nature of Real-Time PCR could be exploited in further assessment of the sampling strategies and the value of latent testing.

In research, one of interesting Real-Time PCR applications is the resent attempt of quantifying the amount of *E. amylovora* inoculum in apple orchards in areas where fire-blight is already established and integrate the data with disease forecasting models for an evidence supported use of control substances (e.g. streptomycin) (Holliger et al., 2010). The study researches the possibility that there are levels of *E. amylovora* during flowering that under given weather conditions do not result in symptoms and may not warrant the use of control substances.

Another very exiting Real-Time PCR application is gene expression analysis which has been so far mostly overlooked in the case of *E. amylovora*. However, even existing real-time PCRs targeting virulence associated genes can be used as a measure of their expression by isolating RNA instead of DNA and proceding to reverse transcription and Real-Time PCR in either one- or two- step assays and Real-Time PCR assays targeting other interesting genes can be developed.

In conclusion, although the need for a real-time PCR for detection purposes has not been recognized as an urgent one, it has proved to be a most valuable detection method with its place in both diagnostics and research. It is expected that its use as the first or additional screening test for detection of *E. amylovora* will increase in the future. Its application in research of latent infections management, blossom blight and gene expression analyses, will no doubt contribute to the next few years being a very exiting time in fire blight research.

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REFERENCES

Bereswill, S., Bugert, P., Bruchmüller, I., Geider, K. (1995): Identification of the fire blight pathogen, *Erwinia amylovora*, by PCR assays with chromosomal DNA. Applied and Environmental Microbiology, 61(7): 2636 - 2642.

Bonn, W.G., van der Zwet, T. (2000): Distribution and economic importance of fire blight. In: Fire blight the disease and its causative agent, *Erwinia amylovora* by: Vanneste, J.L. (ed). Wallingford, UK CAB International; 2000: pp. 37 - 53.

Bugert, P., Geider, K. (1995): Molecular analysis of the ams operon required for exopolysaccharide synthesis of *Erwinia amylovora*. Molecular Microbiology, 15: 917 - 933.

Buh Gašparič, M., Cankar, K., Žel, J., Gruden, K. (2008): Comparison of different Real-Time PCR chemistries and their suitability for detection and quantification of genetically modified organisms. BMC Biotechnology, 8 (1): 26.

Burrill, T.J. (1882): The Bacteria: an account of their nature and effects, together with a systematic description of the species. Illinois Industrial University 11th Report, 1882, pp. 93-157.

Cankar, K., Štebih, D., Dreo, T., Žel, J., Gruden, K. (2006): Critical points of DNA quantification by Real-Time PCR – effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. BMC Biotechnology, 6 (1): 37.

De Bellis, P., Schena, L., Cariddi, C. (2007): Real-time Scorpion-PCR detection and quantification of *Erwinia amylovora* on pear leaves and flowers. European Journal of Plant Pathology, 118 (1): 11 - 22.

de Kok, J.B., Hendriks, J.C.M., van Solinge, W.W., Willems, H.L., Mensink, E.J., Swinkels, D.W. (1998): Use of Real-Time quantitative PCR to compare DNA isolation methods. Clinical Chemistry, 44 (10): 2201 - 2204.

Duffy, B., Schärer, H., Bünter, M., Klay, A., Holliger, E. (2005): Regulatory measures against *Erwinia amylovora* in Switzerland. EPPO/OEPP Bulletin, 35: 239 - 244.

EPPO (European Plant Protection Organization). (1992): EPPO Standards PM 3/40 *Erwinia amylovora*. Sampling and test methods. EPPO Bulletin, 22: 225 - 231.

EPPO (European Plant Protection Organization). (2004): Diagnostic protocols for regulated pests PM 7/20, *Erwinia amylovora*. EPPO Bulletin, 34: 159 - 171.

EPPO, (European Plant Protection Organization) (2007): Basic requirements for quality management in plant pest diagnosis laboratories. EPPO Bulletin, 37 (3): 580 - 588.

Geider, K., Auling, G., Jakovljević, V., Völksch, B. (2009): A polyphasic approach assigns the pathogenic *Erwinia* strains from diseased pear trees in Japan to *Erwinia pyrifoliae*. Letters in Applied Microbiology, 48 (3):324 - 330.

Gianetti, G., Garofalo, M.C., Morone, C., Scortichini, M. (2004): First report of *Erwinia amylovora* in Piedmont. Informatore Agrario, 60 (5): 62 - 63.

Holliger, E., Braun-Kiewnick, A., Dreo, T., Lehmann, A., Pelludat, C., Schoch, B., Duffy, B., Bünter, M. (2010): Das Feuerbrandjahr 2009. Schweizerische Zeitschrift für Obst- und Weinbau (SZOW) Wädenswil, in press.

Johnson, K., Stockwell, V.O., McLaughlin, R., Sugar, D., Loper, J.E., Roberts, R.G. (1993): Effect of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. Phytopathology, 83: 995 - 1002.

Kim, W., Svircev, A., Stockwell, V., Castle, A. (2008): Direct Real-Time PCR: Towards rapid and cost effective application in fire blight diagnosis. Acta Horticulturae, 793: 519 - 523.

Kokosková, B., Mráz, I. (2005): Reliability of diagnostic techniques for *Erwinia amylovora*, the causative agent of fire blight disease. Folia Microbiologica, (Praha), 50(3): 217 - 221.

Lehman, S.M., Kim, W., Castle, A.J., Svircev, A.M. (2008): Duplex Real-Time polymerase chain reaction reveals competition between *Erwinia amylovora* and *E. pyrifoliae* on pear blossoms. Phytopathology, 98 (6): 673 - 679.

Li, X., De Boer, S.H. (1995): Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. Phytopathology, 85: 837 – 842.

Llop, P., Bonaterra, A., Peñalver, J., López, M.M. (2000): Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. Applied and Environmental Microbiology, 66 (5): 2071 - 2078.

Llop, P., Caruso, P., Cubero, J., Morente, C., López, M.M. (1999): A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. Journal of Microbiological Methods, 37 (1): 23 - 31.

Llop, P., Donat, V., Rodríguez, M., Cabrefiga, J., Ruz, L., Palomo, J.L., Montesinos, E., López, M.M. (2006): An indigenous virulent strain of *Erwinia amylovora* lacking the ubiquitous plasmid pEA29. Phytopathology, 96 (8): 900 - 907.

López, M.M., Llop, P., Olmos, A., Marco-Noales, E., Cambra, M., Bertolini, E. (2009): Are molecular tools solving the challenges posed by detection of plant pathogenic bacteria and viruses? Current Issues in Molecular Biology, 11 (1): 13 - 46.

Maes, M., Garbeva, P., Crepel, C. (1996): Identification and sensitive endophytic detection of the fire blight patgogen *Erwinia amylovora* with 23S ribosomal DNA sequences and the polymerase chain reaction. Plant pathology, 45: 1139 - 1149.

Marras, S.A.E., Kramer, F.R., Tyagi, S. (2002): Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. Nucleic Acids Research, 30 (21): e122.

Maxson-Stein, K., McGhee, G.C., Smith, J.J., Jones, A.L., Sundin, G.W. (2003): Genetic analysis of a pathogenic *Erwinia* sp. isolated from pear in Japan. Phytopathology, 93: 1393 - 1399.

Menggad, M., Laurent, J. (1998): Mutations in ams genes of *Erwinia amylovora* affect the interactions with host plants. European Journal of Plant Pathology, 104 (3): 313 - 322.

Mohammadi, M., Moltmann, E., Zeller, W., Geider, K. (2009): Characterisation of naturally occurring *Erwinia amylovora* strains lacking the common plasmid pEA29 and their detection with real-time PCR. European Journal of Plant Pathology, 124 (2): 293 - 302.

Momol, T.M. and Aldwinckle, H.S. (2000): Genetic diversity and host range of *Erwinia amylovora*. In: Vanneste, J.L. (Ed.), Fire Blight, The Disease and Its Causative Agent, *Erwinia amylovora*. Oxford. London, pp. 55 - 72.

Navidi, W., Arnheim, N., Waterman, M.S. (1992): A multiple-tubes approach for accurate genotyping of very small DNA samples by using PCR: statistical considerations. The American Journal of Human Genetics, 50 (2): 347 - 359.

Obradovic, D., Balaz, J., Kevresan, S. (2007): Detection of *Erwinia amylovora* by novel chromosomal polymerase chain reaction primers. Microbiology, 76 (6): 748 - 756.

Ordax, M., Marco-Noales, E., Lopez, M.M., Biosca, E.G. (2006): Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. Applied and Environmental Microbiology, 72 (5): 3482 - 3488.

Pirc, M., Dreo, T., Ravnikar, M. (2008): Real-Time PCR for testing of symptomless fire blight samples. Acta horticulturae, 793: 533 - 538.

Pirc, M., Ravnikar, M., Tomlinson, J., Dreo, T. (2009): Improved fireblight diagnostics using quantitative Real-Time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathology, 58 (5): 872 - 881.

Rosello, M., Garcia Vidal, S., Tarin, A., Llop, P., Gorris, M.T., Donat, V., Chartier, R., Paulin, J.P., Gardan, L., Peñalver, J., López, M.M. (2002): Characterization of an *Erwinia* sp. isolated from necrotic pear blossoms in Valencia, Spain. Acta Horticulturae, 590: 139 - 142.

Smits, T., Jaenicke, S., Rezzonico, F., Kamber, T., Goesmann, A., Frey, J.E., Duffy, B. (2010): Complete genome sequence of the fire blight pathogen *Erwinia pyrifoliae* DSM 12163T and comparative genomic insights into plant pathogenicity. BMC Genomics, 11 (1): 2.

Stöger, A., Schaffer, J., Ruppitsch, W. (2006): A rapid and sensitive method for direct detection of *Erwinia amylovora* in symptomatic and asymptomatic plant tissues by polymerase chain reaction. Journal of Phytopathology, 154 (7-8): 469 - 473.

Svircev, A.M., Kim, W., Lehman, S.M., Castle, A.J. (2009): *Erwinia amylovora*: modern methods for detection and differentiation. Methods in Molecular Biology, 508: 115 - 129.

Thomson, S., Schroth, M., Moller, W., Reil, W. (1975): Occurrence of fire blight of pears in relation to weather and epiphytic populations of *Erwinia amylovora*. Phytopathology, 65: 353 - 358.

van der Zwet, T. (2006): Present worldwide distribution of fire blight and closely related diseases. Acta Horticulturae, 704: 35.

Winslow, C.E.A., Broadhurst, J., Buchanan, R.E., Krumwiede, J.R.C., Rogers, L.A., Smith, G.H. (1920): The families and the genera of the bacteria. Final report of the Committee of the Society of American Bacteriologists on characterization and classification of bacterial types. Journal of Bacteriology, 5 (S):191 - 229.

Won-Sik, K., Jock, S., Paulin, J.P., Rhim, S.L., Geider, K. (2001): Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. Plant Disease, 85: 1183 - 1188.

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IMA LI POTREBE I KAKVO JE MESTO REAL-TIME PCR U DETEKCIJI *ERWINIA AMYLOVORA*?

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REZIME

Metode molekularno-biološke detekcije prouzrokovača bakterioyne plamenjače su razvijene davno, dok je Real-Time PCR metoda pristupačna od 2004. godine. Dok su prednosti Real-Time PCR metode često navođene, ovaj rad je prilog prikaza primene ove metode u praksi, s kritičkim osvrtom na ključne tačke njenog razvoja i potencijalne uloge u budućnosti.

Ključne reči: *Erwinia amylovora*, bakteriozna plamenjača, Real-Time PCR, identifikacija, kvantifikacija.

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UTICAJ ZEMLJIŠNIH BAKTERIJA NA KLIJANJE SEMENA VILINE KOSICE (*CUSCUTA CAMPESTRIS* YUNCK.) I LUCERKE

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Za ispitivanje uticaja zemljišnih mikroorganizama (PGPR– Planth Growth Promoting Rhizobacteria) na klijanje semena lucerke (*Medicago sativa* L.) i viline kosice (*Cuscuta campestris* Yunck.), koja najčešće parazitira na ovoj gajenoj leguminozi korišćene su bakterijske kulture: *Bacillus licheniformis*, *B. pumilus*, *B. amyloliquefaciens*, *B. megatherium* ZP6 i *A. chroococcum* Ps1. Bakterijske kulture *B. licheniformis*, *B. pumilus* i *B. amyloliquefaciens* su izolovane iz stajnjaka; *B. megatherium* ZP6 iz rizosfere kukuruza; a *A. chroococcum* Ps1 iz rizosfere pšenice i čuvane su u frižideru na temperaturi od 4°C do upotrebe. Seme viline kosice prikupljeno je tokom avgusta meseca 2008. godine sa useva lucerke na području Šapca. Svakodnevno u periodu od devet dana rađeno je brojanje proklijalih semena. Svi tretmani su rađeni u četiri ponavljanja i ceo ogled je ponovljen dva puta.

Na osnovu dobijenih rezultata i statističke obrade podataka, generalno se može konstatovati da su svi ispitivani tretmani: MO_1 – *B. licheniformis*, MO_2 – *B. pumilus*, MO_3 – *B. amyloliquefaciens*, MO_4 – *B. megatherium* ZP6 i MO_5 – *A. chroococcum* Ps1 imali sličan efekat na klijanje semena viline kosice i lucerke. Bakterijske kulture različitih vrsta roda *Bacillus* su ispoljile različit inhibitorni efekat na klijanje semena viline kosice, dok su uglavnom potpuno (100%) inhibirali klijanje semena lucerke. Suprotno tome, bakterijska kultura sa *A. chroococcum* Ps1 je stimulativno delovala na klijanje semena obe vrste.

Ključne reči: *Cuscuta campestris*, lucerka, zemljišni mikroorganizmi, PGPR.

UVOD

Parazitske cvetnice čine oko 1% opisanih skrivenosemenica u svetu koje parazitiraju na oko 4 000 drugih biljnih vrsta (Nickrent i sar. 1998). Parazitske cvetnice kao što su vrste roda *Cuscuta* Yunck. (viline kosice) i *Orobanche* L. (volovod) kvantitativno ne zauzimaju značajnu brojnost u odnosu na ukupnu korovsku floru Srbije koja broji 1009 vrsta (Kojić i Vrbničanin, 1998). Rod *Cuscuta* Yunck. obuhvata preko 100 vrsta, rasprostranjenih u umerenim i toplijim krajevima zemljine kugle. U flori Srbije zastupljeno je samo 10 vrsta ovog roda (Kojić i Vrbničanin, 2000). Raširenost i kvantitativnu zastupljenost vrsta roda *Cuscuta* L. na antropogenim staništima (različiti tipovi useva i ruderalna staništa urbanih i ruralnih područja) Srbije istraživali su Vrbničanin i sar. (2008c) i pri tome konstatovali njeno prisustvo na 25% ocenjenih površina kvadranata 10x10 km na UTM (Univerzalna Transverzna Merkatorova mapa) mreži.

Vilina kosica (*Cuscuta* spp.) kao obligatni parazit dovodi do narušavanja opšteg stanja biljke, odnosno njihovog fitnesa. Biljke napadnute vilinom kosicom postaju slabe, njihova bujnost opada i daju veoma male prinose (Koskela i sar., 2001, cit. Fathoulla i Duhoky, 2008), i ukoliko se vilina kosica ne odstrani sa domaćina najčešće dolazi do njegove potpune destrukcije. Vilina kosica pravi posebno veliki problem ako se u velikoj infestaciji javi na tek zasnovanom lucerištu ili deteliništu. Najčešće u takvim situacijama usevi se moraju preoravati, odnosno prekida se eksploatacija višegodišnjih useva. Takođe, problemi sa vilinom kosicom se javljaju pri proizvodnji rasada povrtarskih biljaka, kao i u plasteničkoj proizvodnji, a neretko domaćini ove parazitske cvetnice su i brojne korovske vrste kao što su: *Polygonum aviculare, Convolvulus arvensis, Chenopodium album, Amaranthus retroflexus* itd. (Rančić i Božić, 2004). Dakle, navedene karakteristike parazitskih cvetnica govore u prilog aktuelizaciji ovog problema u poslednje vreme, kao i pronalaženju adekvatnih mera za njihovo suzbijanje.

Uticaj mikroorganizama (PGPR – Planth Growth Promoting Rhizobacteria) na klijanje semena i rast klijanaca gajenih biljaka je bio predmet proučavanja većeg broja istraživača (Bhat i Alagawadi, 1998; Egambediyeva, 2007), dok su efekti mikroorganizama na klijanje semena korovskih biljaka (posebno parazitskih korova) sporadično proučavani (Miche i sar., 2000; Vrbničanin i sar., 2008a,b). Poznavanje efekata delovanja bakterijskih kultura (PGPR) na klijanje semena i rast klijanaca korova, bilo da su ona stimulativnog ili inhibitornog karaktera, može da se iskoristi u globalnom sistemu borbe protiv korova, odnosno konceptu integralne zaštite bilja.

Cilj u ovom radu je bio da se ispita efekat različitih bakterijskih kultura (*Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus megatherium* ZP6 i *Azotobacter chroococcum* Ps1 na klijanje semena viline ko-

sice (*Cuscuta campestris* Yunck.) i lucerke (*Medicago sativa* L.) koja je najčešći domaćin ove parazitske cvetnice.

MATERIJAL I METODE

Bakterijske kulture *Bacillus licheniformis* (MO_1), *B. pumilus* (MO_2) i *B. amyloliquefaciens* (MO_3) su izolovane iz stajnjaka; *B. megatherium* ZP6 (MO_4) iz rizosfere kukuruza; i *Azotobacter chroococcum* Ps1 (i MO_5) iz rizosfere pšenice i čuvane su u frižideru na temperaturi od 4°C do upotrebe. Seme viline kosice prikupljeno je tokom avgusta meseca 2008. godine na području Šapca, prečišćeno i čuvano u laboratoriji na sobnoj temperaturi 20-25˚C. Semena obe biljne vrste su pre postavljanja u Petri posude, dezinfikovana rastvorom varikine i destilovane vode u odnosu (1:1) u trajanju od 10 minuta, a nakon toga tri puta ispirano destilovanom vodom, kako bi se na ovaj način otklonilo eventualno prisustvo drugih mikroorganizama. Po 20 dezinfikovanih i pripremljenih semena obe biljne vrste je preneto u Petri posude gde je dodato po 5ml inokuluma koncentracije 10^8 ml^{-1} bakterijskih ćelija i ostavljeno u uslovima mraka u inkubatoru (Binder CE) na temperaturu od 25°C. Svakodnevno u periodu od devet dana rađeno je prebrojavanje proklijalih semena. Destilovana voda je korišćena u kontrolnim varijanta. Svi tretmani su rađeni u četiri ponavljanja, a ceo ogled je ponovljen dva puta.

Dobijeni rezultati su obrađeni analizom varijanse (ANOVA) i LSD-testom. Stopa klijavosti semena je izračunata po formuli koju je definisao Maguire (1962):

$$M=n_1/t_1+n_2/t_2...+n_x/t_x$$

gde je n_1, n_2, ...n_x broj klijalih semena u vremenima t_1, t_2...t_x iskazanim u danima.

REZULTATI I DISKUSIJA

Rezultati istraživanja Vrbničanin i sar. (2008a) ukazuju na pozitivan efekat zemljišnih bakterija (*A. chroococcum* Ps1, *B. megatherium* ZP6 i *B. circulans*), kao i njihovih kombinacija na klijanje semena korovskih vrsta *Iva xanthifolia* Nutt., *Amaranthus retroflexus* L. i *Sorghum halepense* L.(Pers.). Takođe i rezultati drugih istraživača ukazuju na stimulativno delovanje navedenih bakterijskih kultura na klijanje semena i rast klijanaca kako gajenih tako i korovskih biljaka (Gutiérrez-Mañero i sar., 2001; Carrillo-Castañeda i sar., 2002; Ryu i sar., 2003). Shishido i sar. (1996) ukazuju na pozitivan efekat bakterijske kulture *Bacillus*

na rast klijanaca bora i smreke. Egamberdiyeva (2007) potvrđuje stimulativno delovanja PGPR na rast biljaka i usvajanje azota kod kukuruza na dva različita tipa zemljišta. Uticaj PGPR na klijanje i rast klijanaca kod biljaka je najčešće regulisano fitohormonima (giberilinima, auksinima), vitaminima B grupe, gasovitim supstancama itd. (Revillas i sar., 2000; Gutiérrez-Mañero i sar., 2001; Ping i Boland, 2004). Međutim, kakav će njihov uticaj biti (stimulativan, neutralan, inhibitoran) zavisi i od drugih faktora kao što su npr. uslovi čuvanja semena pre izlaganja njihovom delovanju. Tako su dva izolata *Bacillus licheniformis* ispoljile pozitivan efekat na klijanje semena *Onopordon acanthium*, *Datura stramonium* i *Abutilon theophrasti* kada su semena čuvana u laboratoriji na sobnoj temperaturi, dok su negativan efekat ispoljile u slučaju prethodnog izlaganja semena *D. stramonium* i *A. theophrasti* temperaturi od 4˚C (Vrbničanin i sar., 2008b). Nasuprot očekivanjima i rezultatima prethodnih istraživanja (Shishido i sar., 1996; Gutierrez-Manero i sar., 2001 Egamberdiyeva, 2007), u ovom istraživanju tretmani bakterijske kulture *Bacillus* ispoljili su različit inhibitorni efekat na klijanje semena viline kosice: MO_3– *B. amyloliquefaciens* (27,1%), MO_4– *B. megatherium* ZP6 (37,5%), MO_1– *B. licheniformis* i MO_2– *B. pumilus* (100%) (Tabela 1), dok su isti tretmani manje-više potpuno inhibirali (100%) klijanje semena lucerke.

Suprotno rezultatima koji su dobijeni za rod *Bacillus*, bakterijska kultura *A. chroococcum* Ps1 (MO_5 tretman) je stimulativno delovala na klijanje semena viline kosice (35,42%) i lucerke (8,82%) (Tabela 1 i 2). Ovi rezultati su u saglasnosti sa rezultatima Martinez-Toledo i sar. (1998), Farah i sar. (2008) i Vrbničanin i sar. (2008a) gde je takođe potvrđeno stimulativno delovanje ove bakterijske kulture na klijanje semena i rast klijanaca različitih biljnih vrsta (gajenih i korovskih).

Analiza rezultata koji se odnose na uticaj različitih bakterijskih kultura na klijanje semena viline kosice i lucerke pokazala je da su stope klijanja kod viline kosice (0.00 do 2.84%), kao i u većini tretmana kod lucerke (0.00% kod tretmana MO_1, MO_2, MO_3 i MO_4) bile veoma niske ili jednake nuli, dok je samo u tretmanu MO_5 stopa klijanja bila znatno veća u odnosu na kontrolu (35.57%) (Tabela 3). Dobijeni rezultati su u suprotnosti sa rezultatima koji su dobijeni u slučaju ispitivanja uticaja *A. chroococcum* Ps1 i *B. megatherium* ZP6 na klijavost *I. xanthifolia*, *A. retroflexus* i *S. halepense* (Vrbničanin i sar., 2008a), pri čemu su pored stimulativnog efekta utvrđene i znatno veće stope klijanja (3.85 do 20.01%) nego za većinu tretmana u ovim istraživanjima. Naime, samo je stopa klijanja u tretmanu MO_5 (*A. chroococcum* Ps 1) bila znatno veća nego u pomenutom istraživanju. Osim toga, Rueda-Puente i sar. (2007) ukazuju da delovanje PGPR na klijanje semena i rast klijanaca može da zavisi i od faktora, kao što su salinitet i različiti temperaturni režimi zemljišta, gde su se ovi stresni faktori značajno odrazili na stope klijanja semena *Salicornia bigelovii*. Izračunate

Tabela 1 - Uticaj PGPR (MO_1 - *Bacillus licheniformis*; MO_2 - *B. pumilus*; MO_3 - *B. amyloliquefaciens*; MO_4 - *B. megatherium* ZP6; MO_5 - *A. chroococcum* Ps1) na dinamiku klijanja semena *C. campestris*.

Table 1 - Effect of Planth Growth Promoting Rhizobacteria (MO_1 - *Bacillus licheniformis*; MO_2 - *B. pumilus*; MO_3 - *B. amyloliquefaciens*; MO_4 - *B. megatherium* ZP6; MO_5 - *A. chroococcum* Ps1) on dinamics of seed germination of *C. campestris*.

Tretmani	Procenat klijalih semena nakon dana – Percentage of germinated seeds after days								
Treatments	1	2	3	4	5	6	7	8	9
H_2O	0.00±0.00	4.75±1.50	6.25±1.71	7.75±1.89	9.25±0.50	10.50±1.73	10.75±0.96	10.50±1.91	10.50±1.91
MO_1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MO_2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MO_3	0.00±0.00	0.00±0.00	1.25±0.50	3.75 ±1.79	5.00 ±3.07	5.00 ±3.07	5.00 ±3.07	8.75±6.31	8.75±6.31
MO_4	0.00±0.00	1.25±0.50	2.50±1.00	3.75±1.79	3.75±1.79	6.25±3.50	7.50±3.57	7.50±3.57	7.50±3.57
MO_5	0.00±0.00	0.00±0.00	3.75±0.79	10.00±7.07	10.00±7.07	12.50±8.66	13.75±9.46	16.25±4.79	16.25±4.79

Tabela 2 - Uticaj PGPR (MO_1 - *Bacillus licheniformis*; MO_2 - *B. pumilus*; MO_3 - *B. amyloliquefaciens*; MO_4 - *B. megatherium* ZP6; MO_5 - *A. chroococcum* Ps1) na dinamiku klijanja semena *M. sativa*.

Table 2 - Effect of Planth Growth Promoting Rhizobacteria (MO_1 - *Bacillus licheniformis*; MO_2 - *B. pumilus*; MO_3 - *B. amyloliquefaciens*; MO_4 - *B. megatherium* ZP6; MO_5 - *A. chroococcum* Ps1) on dinamics of seed germination of *M. sativa*.

Tretmani Treatments	Procenat klijalih semena nakon dana – Percentage of germinated seeds after days								
	1	2	3	4	5	6	7	8	9
H_2O	44.17±11.14	58.34±13.66	64.17±13.84	70.84±12.81	75.00±8.37	83.34±6.05	83.34±6.05	85.00±5.48	85.00±5.48
MO_1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MO_2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MO_3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MO_4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MO_5	41.67±5.17	52.50±10.84	70.00±12.65	79.17±4.92	84.17±4.92	90.84±4.92	90.84±4.92	92.50±2.74	92.50±2.74

Tabela 3 - Stopa klijanja semena *C. campestris* i *M. sativa* (%).

Table 3 - Rate of seed germination of *C. campestris* and *M. sativa* (%).

Biljna vrsta Plant species	Stopa klijanja semena - Rate of seed germination (%)					
	H_2O	MO_1	MO_2	MO_3	MO_4	MO_5
C. campestris	2.84±0.30	0.00±0.00	0.00±0.00	1.19±1.03	1.41±1.30	2.73±1.21
M. sativa	34.66±4.17	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	35.57±2.08

vrednosti stopa klijanja su opadale sa povećanjem koncentracije saliniteta, dok su rasle sa porastom temperature.

Poznavanje interakcija između zemljišnih mikroorganizama (PGPR) i klijanja semena i rasta klijanaca korovskih biljaka može biti od velikog značaja u konceptu bioloških mera kontrole korova, odnosno u sistemu integralnih mera zaštite bilja. Dakle, primenom zemljišnih mikroorganizama koji deluju stimulativno na klijanje semena korovskih biljaka možemo da pospešimo uniformnije klijanje i nicanje korova gde bi narednom agrotehničkom ili hemijskom merom iste uništili i na taj način smanjili potencijalnu zakorovljenost (″seed bank″) takvih površina. S druge strane, PGPR koje deluju inhibitorno na klijanje semena i rast klijanaca korovskih biljaka se opet može prihvatiti kao direktna mera u smanjenju zakorovljenosti određenog useva.

LITERATURA

Bhat, J.M., Alagawadi, A.R. (1998): Seed borne nature of *Azotobacter chroococcum* in chilli (*Capsicum annum*) and its role in seed germination and plant growth. Hindustan Antibiotic bull, 40: 20-30.

Carrillo-Castañeda, G., Juárez Muños, J., Peralta-Videa, J.R., Gomez, E., Tiemannb, K.J., Duarte Gardea, M., Gardea-Torresdey, J.L. (2002): Alfalfa growth promotion by bacteria grown under iron limiting conditions. Advances in Environmental Research, 6: 391-399.

Fathoulla, C.N., Duhoky, M.M.S. (2008): Biological and anatomical study of different *Cuscuta species*. Kurdistan 1st Conference on Biological Sciences. J. Dohuk Univ., 11(1): 22-39.

Egamberdiyeva, D. (2007): The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Applied Soil Ecology, 36: 184-189.

Farah, A., Iqbal, A., Khan, M.S. (2008): Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research, 163: 173-181.

Gutiérrez-Mañero, F.J., Ramos-Solano, B., Probanza, A., Mehouachi, J., Tadeo, F.R., Talon, M. (2001): The planth-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiolically active gibberellins. Physiologia Plantarium, 111: 206-211.

Koskela, T., Salonen, V., Mutikainen, P. (2001): Interaction of a host plant and its holoparasite: effects of previous selection by the parasite. J. of Evo. Bio., 14 (6): 910.

Kojić, M., Vrbničanin, S. (1998): Agrestal, ruderal, grass and aquatic weeds in Serbia. Acta biologica Iugoslavica, series G: Acta herbologica, 7 (1-2): 7-37.

Kojić, M., Vrbničanin, S. (2000): Parazitski korovi – osnovne karakteristike, taksonomija, diverzitet i rasprostranjenje. I deo: Vilina kosica (*Cuscuta* L.). Acta biologica Iugoslavica, series G: Acta herbologica, 9 (1): 21-29.

Maguire, J. (1962): Speed of germination aid in selection and evaluation for seedling emergence and vigour. Crop Science, 2: 176-177.

Martinez-Toledo, M.V., Gonzalez-Lopez, J., de la Rubia T., Moreno, J., Ramos-Cormenzana, A. (1998): Effect of inoculation with *Azotobacter chroococcum* on nitrogenase activity of *Zea mays* roots grown in agricultural soils under aseptic and non-sterile conditions. Biology and Fertility of Soils, 6: 170-173.

Miche, L., Bouillant, M.L., Rohr, R., Salle, G., Bally, R. (2000): Physiological and cytological studies on the inhibition of *Striga* seed germination by the plant growth-promoting bacterium *Azospirillum brasilense*. European Journal of Plant Pathology, 106: 347-351.

Nickrent, D.L., Duff, R.J., Colwell, A.E., Wolfe, A.D., Young, N.D., Steiner, K.E., Depamphilis, C.W. (1998): Molecular phylogenetic and evolutionary studies of parasitic plants. In D. E. Soltis, P. S. and J. J. Doyle (edc.), Molecular systematics of plants II: DNA sequencing, 211-241. Kluwer, Boston, Massachusetts, USA.

Ping, L., Boland, W. (2004): Signals from the underground: bacterial volatiles promote growth in *Arabidopsis*. Trends in Plant Science, 9: 263-266.

Rančić, D., Božić, D. (2004): Uticaj viline kosice (*Cuscuta* sp.) na ptičiji dvornik (*Polygonum aviculare* L.). Acta herbologica, 13 (1): 167-172.

Revillas, J.J., Rodelas, B., Pozo, C., Martinez-Toledo, M.V., Gonzalez-Lopez, J. (2000): Production of B group vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. Journal of Applied Microbiology, 8: 486-493.

Rueda-Puente, E.O., García-Hernández, J.L., Preciado-Rangel, P., Murillo-Amador, B., Tarazón-Herrera, M.A., Flores-Hernández, A., Holguin-Peña, J., Aybar, A.N., Barrón Hoyos, J.M., Weimers, D., Mwandemele, O., Kaya, G., Mayoral, J.L., Troyo-Diéguez, E. (2007): Germination of *Salicornia bigelovii* Ecotypes under Stressing Conditions of Temperature and Salinity and Ameliorative Effects of Plants Growth-promoting Bacteria. Journal Agronomy & Crop Science 193: 167-176.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Pare, P.W., Kloepper, J.W. (2003): Bacterial volatiles promote growth in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America, 100: 4927-4932.

Shishido, M., Massicotte, H.B., Chanway, C.P. (1996): Effect of plant growth promoting *Bacillus* strains on pine and spruce seedling growth and mycorrhizal infection. Annals of Botany, 77: 433-441.

Vrbničanin, S., Jovanović, Lj., Božić, D., Raičević, V., Pavlović, D. (2008a): Germination of *Iva xanthifolia*, *Amaranthus retroflexus* and *Sorghum halepense* under media

with microorganisms. Journal of Plant Diseases and Protection, Special Issue XXI: 297-302.

Vrbničanin, S., Jovanović, Lj., Božić, D., Pavlović, D., Raičević, V. (2008b): Effect Growth –Promoting Bacteria on Germination of *Datura stramonium* L., *Abutilon theophrasti* Medik., *Onopordon acanthium* L. and *Verbascum thapsus* L., 5th International Weed Science Congress, 127.

Vrbničanin, S., Malidža, G., Stefanović, L., Elezović, I., Stanković-Kalezić, R., Jovanović– Radovanov, K., Marisavljević, D., Pavlović, D., Gavrić, M. (2008c): Distribucija nekih ekonomski štetnih, invazivnih i karantinskih korovskih vrsta na području Srbije. II deo: Prostorna distribucija i zastupljenost devet korovskih vrsta na području Srbije. Biljni lekar, XXXVI (6): 408-418.

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EFFECT OF PLANT GROWTH-PROMOTING RHIZOBACTERIA ON THE GERMINATION OF FIELD DODDER (*CUSCUTA CAMPESTRIS* YUNCK.) AND ALFALFA

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SUMMARY

To examine the impact of microorganisms (PGPR-Planth Growth Promoting Rhizobacteria) on seed germination of field dodder (*Cuscuta campestris* Yunck.) and alfalfa (*Medicago sativa* L.) is the most common hosts of this parasite plants were used for bacterial culture: *Bacillus licheniformis*, *B. pumilus*, *B. amyloliquefaciens*, *B. megaterium* and *A. chroococcum Ps* 1 were isolated from the rhizosphere of different plants grown and stored in fridge at a temperature of 4 ° C until use. Seeds collected field dodder during autumn 2008. in the area of Šabac. Every day for a period of nine days is done counting germination seeds. All treatments were done in four repetitions, and the whole experiment was repeated twice.

Based on the results and statistical data processing, in general it can be concluded that all tested treatments (MO_1 – *B. licheniformis*, MO_2 – *B. pumilus*, MO_3 – *B. amyloliquefaciens*, MO_4 – *B. megaterium* and MO_5 – *A. chroococcum Ps* 1) were similar effect on the germination seed of field dodder and alfalfa. Treatments of bacterial culture of *Bacillus* demonstrated different inhibitory effect on seed germination, field dodder while the same treatment of bacterial culture of *Bacillus* are more or less 100% inhibitory alfalfa seed germination. In contrast, the bacterial cultures with *A. chroococcum* has acted in stimulating germination field dodder and alfalfa.

Key words: *Cuscuta campestris*, alfalfa, soil microorganisms, PGPR.

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OSETLJIVOST IZOLATA *CERCOSPORA BETICOLA* PREMA KARBENDAZIMU I FLUTRIAFOLU U SRBIJI

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Ispitivana je osetljivost izolata *Cercospora beticola* (Sacc.), prouzrokovača pegavosti lišća šećerne repe iz 11 lokaliteta u Srbiji prema karbendazimu (grupa benzimidazola) i flutriafolu (grupa triazola DMI) tokom 2007. godine. Uzorkovanje je obavljeno tokom avgusta meseca. Za ispitivane izolate izračunate su srednje efektivne koncentracije (EC_{50}), relativna osetljivost (b) i nivo rezistentnosti (NR) u odnosu na najosetljiviji izolat, po metodi Karaoglanidis i sar. (2000). Dobijeni rezultati su potvrdili već ranije dokazanu rezistetnost izolata *C. beticola* poreklom iz Srbije na karbendazim, ali po prvi put i značajno smanjenu osetljivost izolata poreklom iz Erdevika i Maradika prema flutriafolu.

Ključne reči: *Cercospora beticola,* karbendazim, flutriafol, smanjenja osetljivost.

UVOD

Pegavost lišća koju prouzrokuje *Cercospora beticola* (Sacc.) je najznačajnija bolest koja se javlja na šećernoj repi (Weiland i Koch 2004). Ukoliko se ne vrši adekvatna zaštita, štete mogu značajno smanjiti kvalitet i prinos (25-50%) korena šećerne repe (Byford 1996; Shane i Teng 1992).

Zaštita useva šećerne repe u početku se vršila protektivnim fungicidima kao što su organska jedinjenja kalaja (fentin hidroksid i fentin acetat), ditiokarbamati (maneb, mankozeb). Organo kalajna jedinjenja inhibiraju klijanje spora i delimično respiraciju. Pored protektivne aktivnosti imaju i određenu kurativnu.

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Tokom dugogodišnje primene dokazana je smanjena osetljivost *C. beticola* na fentin hidroksid i fentin acetat (Giannopilitis, 1978; Bugbee, 1995; Weiland, 2000).

Fungicidi sa nespecifičnim dejstvom deluju na više procesa ili mesta u ćeliji i obično imaju protektivnu nesistemičnu aktivnost. Dejstvo ovih fungicida uglavnom je usmereno na sprečavanje kliijanja spora. Ovi fungicidi se zato uglavnom primanjuju pre ostvarenja infekcije i predstavljaju barijeru između biljke i gljive. Rizik za razvoj rezistentnosti prema ovim fungicidima je mali ili ga uopšte nema (Nene i Thapliyal, 1994).

Ranih sedamdesetih godina prošlog veka u primenu se uvode benzimidazoli (benomil i karbendazim) koji su sistemični fungicidi i imaju specifično mesto delovanja. Oni se vezuju za tubulin strukturni protein mikrotubula deobnog vretena koji se sastoji od dve podjedinice α i β čime sprečavaju njihovo izduživanje i formiranje deobnog vretena, što rezultira opstrukcijom deobe ćelija u procesu mitoze. Istraživanja su pokazala da se rezistentni patotipovi na benzimidazole ne javljaju u prirodi bez selekcionog pritiska fungicida, ali se pod njegovim uticajem rezistentna populacija razvija progresivno. Rezistentnost na benzimidazole razvija se brzo i kvalitativnog je tipa, odnosno, zvisi od monogenske mutacije (Damicone, 2006).

Nakon benzimidazola u primenu se uvode i inhibitori sinteze ergosterola (DMI) fungicidi. Oni deluju specifično putem inhibicije citohroma P-450 od koga zavisi aktivnost 14α-demetilaze sterola, odnosno C-14 demetilacija. Rezistentnost na DMI fungicide razvija se postepeno tokom dužeg perioda primene, što u početku otežava razlikovanje rezistentnih i osetljivih populacija. Promene u osetljivosti karakterišu se kao kvantitativne (poligenske) te stoga efikasnost fungicida iz ove grupe zadržava zadovoljavajuću efikasnost duži vremenski period (Karaoglanidis i sar., 2003).

U Grčkoj DMI fungicidi, bitertanol i nuarimol su u primeni od 1979. godine. Upotreba ove dve aktivne materije je stopirana 1990. godine, ali se od 1985. godine u primenu uvodi flutriafol, a 1992. godine još jedna aktivna materija iz ove grupe, difenokonazol. Ovi fungicidi su uglavnom primenjivani u mešavini sa protektivnim fungicidima, manebom i hlorotalonilom. Brown i Waller (1986) su isptitivali efikasnost flutriafola na nekoliko lokaliteta u Francuskoj. Dobijeni rezultati ispoljili su visok nivo efikasnosti, dug period perzistentnosti kao i na eradikativnu aktivnost flutriafola pri suzbijanju *C. beticola*.

Od 1995. godine došlo je do opadanja efikasnosti DMI fungicida u nekim delovima Grčke. Karaoglanidis i sar. (2000) su u laboratorijskim uslovima utvrdili pojavu rezistentnosti *C. beticola* prema DMI fungicidima.

U našim uslovima od 1991. godine, kada je utvrđena rezistentnost *C. beticola* prema benzimidazolima (Gavran, 1991), nema podataka o osetljivosti na

ove fungicide, iako je primena nekih aktivnih materija iz ove grupe i dalje nastavljena. Balaž i sar. (1999) nisu dokazali smanjenu osetljivost izolata *C. beticola* prema flutriafolu testirajući izolate tokom 1994. godine sa područja Vojvodine.

Cilj ovog rada je da se proveri postojeća osetljivost populacija *C. beticola* prema benzimidazolaima i DMI fungicidima u važnijim lokalitetima gajenja šećerne repe u Srbiji.

MATERIJAL I METODE

Prikupljanje uzoraka

Uzorci su prikupljani iz jedanaest lokaliteta (Crvenka, Erdevik 1,2, Bečej 1,2, Padinska Skela, Maradik, Negotin 1,2 i Loznica 1,2). Sa lokaliteta Crvenka, Erdevik, Bečej, Padinska Skela i Maradik uzorci su uzimani tokom avgusta 2007. godine sa polja šećerne repe, a sa lokaliteta Negotin i Loznica iz useva cvekle. Uzimani su mladi listovi sa aktivnim pegama *C. beticola* i pakovani u papirne kese. Zatim je lišće prenošeno u laboratoriju Instituta za zaštitu bilja i životnu sredinu i dalje obrađivano.

Izolacija patogena

Izolacija patogena obavljena je po metodi koju su opisali Karaoglanidis i sar., (2000). Vrhom igle iz jedne pege prenošena je po jedne konidija *C. beticola* u Petri šolju sa KDA podlogom. Inkubacija je vršena dva dana u tami na temperaturi 25ºC. Nakon dva dana kolonije su prenošene na svežu KDA podlogu gde su inkubirane deset dana pod istim uslovima. Kolonije su zatim korišćene za dalja ispitivanja. Ukupno je izolovano po deset izolata iz svih lokaliteta.

Testovi osetljivosti

Fungicidi koji su bili korišćeni za ispitivanje osetljivosti *C. beticola* su karbendazim iz grupe benzimidazola, a flutriafol iz grupe triazola (DMI). Pripremana su razređenja fungicida koja su dodavana u KDA podlogu ohlađenu do 50ºC. Raspon koncentracija za karbendazim bio je 1, 10, 100, 1000, 2000, 4000, 8000 i 12000 μg/ml, dok su koncentracije za flutriafol bile 0.10, 0.25, 0.50, 1.0, 2.0 i 4.0 μg/ml. Koncentracije 8.0 μg/ml i 16.0 μg/ml korišćene su za test najmanje osetljivosti (Karaoglanidis i sar., 2000). Kontrole su bile Petri šolje sa KDA podlogom u koje nije dodavan fungicid već destilovana voda. Uz pomoć bušača isecani su sa ivice kolonije krugovi micelije prečnika 5mm i prenošeni u Petri

šolje sa ili bez fungicida. Nakon sedam dana inkubacije, u tami na 25ºC, meren je porast kolonija oduziman za početni prečnik isečka. Ogledi su postavljeni u četiri ponavljanja.

Obrada podataka

Izračunavana je srednja efektivna koncentracija koja inhibira porast micelije za 50% EC_{50} za sve ispitivane izolate (110). Zatim je EC_{50} najosetljivijeg izolata korišćena za izračunavanje nivoa osetljivosti. Za sve izolate izračunate su vrednosti EC50, relativni pokazatelj nivoa osetljivosti (b) i nivo rezistentnosti.

REZULTATI

Osetljivost izolata prema karbendazimu

Izolati su pokazali veoma nizak nivo osetljivosti prema klarbendazimu. Samo jedan izolat Negotin-2 pokazao je značajnu osetljivost na karbendazim, njegova vrednost EC_{50} bila je 3.4 µg/ml. Za ostale izolate vrednosti EC_{50} su bile 3381.3 ─ 11913.8. Vrednosti EC_{50} su bile ujednačene za sve izolate iz istih lokaliteta. U tabeli 1 i 2, prikazane su vrednosti za izolate sa najmanjom osetljivošću. Koeficijent osetljivosti (b) za najosetljiviji izolat Negotin-2 bio je 3.32. Vrednost EC_{50} za izolat iz Bečeja koji je ispoljio najmanju osetljivost je 11913.8, a koeficijent osetljivosti b= 0.22. Vrednost EC_{50} osetljivog izolata iz Negotina korišćena je za izračunavanje nivoa osetljivosti za karbendazim. Nivoi osetljivosti dobijeni su stavljanjem u odnos EC_{50} određenog izolata sa vrednošću EC_{50} najosetljivijeg izolata (Negotin = 3.4).

Osetljivost izolata prema flutriafolu

U devet lokaliteta od ukupno testiranih 11 dokazana je visoka osetljivost na flutriafol. Izolati poreklom iz Erdevika i Maradika ispoljili su smanjenu osetljivost prema flutriafolu. Najniži nivo osetljivosti na flutriafol pokazao je izolat iz Erdevika EC_{50} = 8.25 µg/ml, a koeficijent osetljivosti b = 0.75. Takođe sanjenu osetljivost imao je i izolat iz Maradika EC_{50} = 7.99 µg/ml, koeficijent osetljivosti b = 0.84. Slične vrednosti ispoljili su i ostali izolati iz ovih lokaliteta. Izolat sa najvećom osetljivošću izolovan sa lokaliteta Crvanke imao je EC_{50} = 0.24, a koeficijent osetljivosti b = 1.61. Za ostale izolate srednja efektivna koncentracija je bila u intervalu 0.25 - 1.39 µg/ml, a koeficijent osetljivosti 0.68 - 1.43.

Tabela 1 - Osetljivost izolata *C. beticola* na karbendazim. [a]

Table 1 - Sensitivity of *C. beticola* isolates to carbendazime. [a]

Br. izolata No of isolates	Lokalitet Locality	EC_{50} [b]	b [c]	NR [d]
1.	Crvenka	4085.5	0.15	1201.6
2.	Erdevik-1	4010.3	0.14	1179.5
3.	Erdevik-2	4129.8	0.22	1214.6
4.	Bečej-1	4899.2	0.21	1440.9
5.	Bečej-2	11913.8	0.22	3504.0
6.	Pad. Skela	4855.4	0.18	1428.0
7.	Maradik	5883.1	0.25	1730.3
8.	Loznica-1	7047.3	0.17	2072.7
9.	Loznica-2	3381.3	0.19	994.5
10.	Negotin-1	9357.5	0.23	2752.2
11.	Negotin-2	3.4	3.32	1.0

a Obračun urađen po metodi koju su primenili Karaoglanidis i sar. (2000) – Calculation done by the method which was applied by Karaoglanidis et al. (2000).
b Srednje efektivne koncentracije – Medium effective concentration.
c Relativna osetljivost – Relative sensitivity.
d Nivo osetljivosti u odnosu na izolat Negotin-2 – Resistance level in relation to isolate Negotin-2.

Tabela 2 - Osetljivost izolata *C. beticola* na flutriafol.

Table 2 - Sensitivity of *C. beticola* isolates to flutriafole.

Br. izolata No of isolates	Lokalitet Locality	EC_{50} [b]	b [c]	NR [d]
1.	Crvenka	0.24	1.61	1.0
2.	Erdevik-1	0.29	1.39	1.2
3.	Erdevik-2	8.25	0.75	34.3
4.	Bečej-1	0.39	1.0	1.6
5.	Bečej-2	0.51	1.36	2.1
6.	Pad.Skela	0.40	1.30	1.6
7.	Maradik	7.99	0.84	33.2
8.	Loznica-1	0.47	0.69	1.9
9.	Loznica-2	0.32	0.68	1.3
10.	Negotin-1	0.25	1.43	1.0
11.	Negotin-2	1.39	1.12	5.7

a Obračun urađen po metodi koju su primenili Karaoglanidis i sar. (2000) – Calculation done by the method which was applied by Karaoglanidis et al. (2000).
b Srednje efektivne koncentracije – Medium effective concentration.
c Relativna osetljivost – Relative sensitivity.
d Nivo osetljivosti u odnosu na izolat Negotin-2 – Resistance level in relation to isolate Negotin-2.

DISKUSIJA

Prva pojava rezistentnosti populacija *C. beticola* prema fungicidima konstatovana je u Grčkoj (Georgopoulos i Dovas, 1973), samo dve godine nakon intenzivne primene benzimidazola zaštiti šećerne repe od prouzrokovača pegavosti lišća. Početni nivo efiaksnosti primene benzimidazola bio je izuzetno visok, da bi naglo došlo do njegovog smanjivanja kada su se koristili više puta u vegetaciji.

Pojava rezistentnosti utvrđena je i u drugim delovima sveta gde se za suzbijanje *C. beticola* koristio benomil. Ruppel i Scott su (1974) su utvrdili pojavu rezistentnosti na benomil u svim regionima gde se gaji šećerna repa u SAD.

Kod nas je benomil počeo intenzivno da se koristi 1971. godine. Već tokom 1974. i 1975. godine na nekoliko lokaliteta konstatovan je slab efekat zaštite na njivama na kojima se za zaštitu koristio benomil i do tri puta uzastopno. Potvrdu da se radi o rezistentnosti dali su Marić i sar., (1976) nakon provere osetljivosti izolata uzetih sa polja šećerne repe tokom 1974-1975 i Gavran (1991) u periodu od 1986-1991. Vrednosti EC_{50} karbendazima za ispitivane izolate *C. beticola* dobijene pre dvadeset godina, ukazuju na činjenicu da su se one kretale u najvećem broju slučajeva do 10 µg/ml, a da su samo pojedinačni izolati u okviru svih ispitivanih lokaliteta ispoljavali EC_{50} prema karbendazimu više hiljada µg/ml. U narednom periodu benomil se koristio u kombinaciji sa organokalajnim jedinjenjima, da bi se smanjio selekcioni pritisak na rezistentne populacije. Ovaj vid zaštite se ispoljio kao neefikasan, jer je rezistentna populacija *C. beticola* i dalje prisutna u svim ispitivanim lokalitetima.

Međutim, neki preparati koji se i danas koriste za zaštitu šećerne repe za suzbijanje *C. beticola* sadrže aktivne materije iz grupe benzimidazola. Dobijeni rezultati ukazuju da se rezistentne populacije održavaju u prirodi zahvaljujući preparatima koji u svom sastavu sadrže benzimidazole.

Kod nas DMI fungicidi su u upotrebi od početka 80-tih godina. Marić i sar., (1981) utvrdili su visok stepen efikasnosti preparata iz grupe triazola, kao što su bitertanol i nuarimol. U narednom periodu zaštita šećerne repe od *C. beticola* zasnivala se na jednom tretmanu u toku godine sa benomilom, a ostala tretiranja su se izvodila sa DMI fungicidima i organokalajnim preparatima (Gavran, 1991). Opsežnijih ispitivanja osetljivosti populacija *C. beticola* prema DMI fungicidima do sada nije bilo. Balaž i sar. (1999) su testirajući osetljivost uzoraka *C. beticola* iz različitih lokaliteta iz Vojvodine prema flutriafolu utvrdili da se njihove EC_{50} kreću ispod 1 µg/ml, a nivo rezistentnosti od 1 do 1.4. Noviji rezultati iz 2008. godine ukazuju da je u nekim lokalitetima utvrđena smanjena osetljivost populacija *C. beticola* prema DMI fungicidima (Trkulja i sar., 2008). U ovom radu navedeni rezultati su potvrđeni i ukazuju na visoke vrednosti EC_{50} flutriafola

prema izolatima poreklom iz Erdevika (8.25 µg/ml) i Maradika (7.00 µg/ml) koji su i potvrđeni rezultatima prikazanim u ovom radu. Izolati iz ova dva lokaliteta su ispoljila visok nivo rezistetnosti 34, odnosno 33, pa bi se po kriterijumima Karaoglanidisa i Thanassoulopoulos-a (2003) mogli svrstati u rezistetne izolate, jer su ispoljili NR preko 10, dok bi se po kriterijumima Leroux-a (1984), ovi izolati svrstali u osetljive (NR<50).

Obzirom da rezistentnost prema DMI fungicidima zavisi od promena na više gena poligenetska rezistentnost, dugotrajna upotreba DMI fungicida menja odnos rezistentnih i osetljivih populacija tako što rezistentna populacija postepeno raste i postaje dominantna. Dalja upotreba DMI fungicida bez primene antirezistentne strategije može indukovati drastično povećanje rezistentnih populacija *C. beticola* na poljima šećerne repe u našoj zemlji. Ovim radom želeli smo da skrenemo pažnju na takve moguće posledice.

LITERATURA

Balaž, F., Stojšin, Vera, Gavran-Starović, Mira. (1999): Osetljivost izolata *Cercospora beticola* (Sacc.) na području Vojvodine prema nekim fungicidima. Pesticidi, 14: 514.

Brown, M. C., Waller, C.D. (1986): The use of flutriafol based fungicides for the control of sugar beet diseases in Europe. In 1986 British Crop Protection Conference. Pests and Diseases. Vol. 3: 1055-1061.

Bugbee, W.M. (1995): *Cercospora beticola* tolerant to triphenyltin hydroxide. J. Sugarbeet Res. 32: 167-174.

Byford, W.J. (1996): A survey of foliar diseases of sugar beet and their control in Europe. In Proceedings of the 59th NRB Congress. Brousseles: International Institute for Beet Research, pp:1-10.

Damicone, J. (2003): Fungicide resistance management OSU (Oklahoma State University) F-7663: 1-6.

Gavran-Starović, Mira. (1991): Rezistentnost *Cercospora beticola* (Sacc.) na benzimidazole. Magistarska teza. Poljoprivredni fakultet, Brograd-Zemun.

Georgopoulos, S.G., Dovas, C. (1973): A serious outbreak of strains of *Cercospora beticola* resistant to benzimidazole fungicides in Northern Greece. Vol. 57, N° 4 Plant Disease Reporter, 321: 324.

Giannopolitis, C.N. (1978): Occurrence of strains of *Cercospora beticola* resistant to triphenyltin fungicides in Greece. Plant Disease Reporter. N° 3, Vol. 62: 205-208.

Karaoglanidis, S,G., Ioannidis, P.M., Thanassoulopoulos, C.C. (2000): Reduced sensitivity of *Cercospora beticola* isolates to sterol-demethylation-inhibiting fungicides. Plant Pathology 49: 567-572.

Karaoglanidis, S,G., Thanassoulopoulos, C.C. (2003): Cross-resistance patterns among sterol biosynthesis inhibiting fungicides (SBIs) in *Cercospora beticola*. European Journal of Plant Pathology, 109: 929-934.

Karaoglanidis, S.G., Menkissoglu-Spiroudi, U., Thanassoulopoulos, C.C. (2003): Sterol composition of DMI-resistant and sensitive field isolates of *Cercospora beticola*. J. Phytopathology 151: 431-435.

Marić, A., Petrov, Mirjana, Maširević, S. (1976): Pojava tolerantnosti kod *Cercospora beticola* Sacc. prema benomilu u Jugoslaviji i mogućnosti suzbijanja ovog parazita. Zaštita bilja, Vol. XXVII (3-4), N° 137-138: 227-236.

Marić, A., Kosovac, V., Maširević, S., Radulović, R. (1981): Efikasnost nekih fungicida u suzbijanju pegavosti lišća (*C. beticola*) i pepelnice (*E. betae*) šećerne repe u mikro i makro ogledima tokom 1981. godine. Jugoslovensko savetovanje o primeni pesticida. Zbornik radova. Opatija. 175-180.

Nene, Y.L., Thapliyal, P.H. (1994): Fungicides in plant disease control. Third edition.

Ruppel, E.G., Scott, P.R. (1974): Strains of *Cercospora beticola* resistant to benomyl in The U.S.A. Plant Disease Reporter 58: 434-436.

Shane, W.W., Teng, P.S. (1992): Impact of Cercospora Leaf spot on root weight, sugar yield, and purity of Beta vulgaris. Plant Disease. N° 8, Vol. 76: 812-820.

Trkulja, N., Živković, Svetlana, Ivanović, Ž., Dolovac, N., Starović, Mira, Vukša, P. (2008): Osetljivost izolata *Cercospora beticola* (Sacc.) prema flutriafolu u Vojvodini. IX savetovanje o zaštiti bilja, Zlatibor, 24-28 novembar, 2008. Zbornik rezimea, 66-67.

Weiland, J.J. (2000): A survey for the prevalence and distribution of *Cercospora beticola* to triphenyltin hydroxide and mancozeb and resistant to thiophonate methyl in 2000. USDA, Agricultural research service. Northern Crop Science Laboratory.

Weiland, J., Koch, G. (2004): Sugarbeet spot disease (*Cercospora beticola* Sacc.). Molecular Plant Pathology. 5 (3), 157-166

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SENSITIVITY OF *CERCOSPORA BETICOLA* ISOLATES TO CARBENDAZIME AND FLUTRIAFOL IN SERBIA

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SUMMARY

The sensitivity of 110 *Cercospora beticola* (Sacc.) isolates to carbendazime (benzimidazoles) and flutriafol (triazole DMI) was investigated. Isolates on PDA were obtained from diseased sugar leaves collected in 11 localities in Serbia during the August 2007. The medium effective concentration (EC_{50}), the relative sensitivity (b) and the level of resistance (NR) in relation to the most sensitive isolate were calculated the method applied by Karaoglanidis et al. (2000).The obtained results confirmed already resistance of Serbian isolates of *C. beticola* to carbendazime, but for the first time isolates from Erdevik and Maradik showed a significantly decreased sensitivity to flutriafol.

Key words: *Cercospora beticola*, carbendazime, flutriafol, reducing sensitivity .

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ETIOLOŠKA PROUČAVANJA BAKTERIOZNE VLAŽNE TRULEŽI USKLADIŠTENIH GLAVICA KOMORAČA

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U radu su proučene patogene i biohemijske odlike fitopatogenih bakterija izolovanih iz glavica komorača sa simptomima truleži. Proučavani sojevi prouzrokuju trulež glavica komorača, veštački inokulisanih, plodova paprike, kao i kriški mrkve i krompira i pri tome ispoljavaju izraženu pektolitičku aktivnost. Proučavanjem bakterioloških odlika utvrđeno je da su su izolovani sojevi Gramnegativni, ne fluoresciraju na Kingovoj podlozi B, glukozu metabolišu i u aerobnim i anaerobnim uslovima, ne stvaraju levan i oksidazu. Na osnovu navedenih karakteristika zaključeno je da ispitivani sojevi pripadaju rodu *Pectobacterium.*

Proučavani sojevi ne stvaraju fosfatazu, lecitinazu, indol, redukujuće supstance iz saharoze, a na Loganovoj podlozi formiraju karakteristične kolonije ružičaste boje sa tamnim centrom. Na osnovu ovih, ali i rezultata ostalih diferencijalnih testova za vrste roda *Pectobacterium* (metabolizam ugljenih hidrata, razvoj pri 5% NaCl, osetljivost prema eritromicinu), zaključeno je da vlažnu trulež komorača prouzrokuje bakterija *Pectobacterium carotovorum* subsp. c*arotovorum.* Ovo je prvo saopštenje o nalazu ove bakterije kao patogena komorača u Srbiji.

Ključne reči: komorač, *Pectobacterium carotovorum* subsp. *carotovorum,* vlažna trulež, patogenost, bakteriološke odlike.

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UVOD

Komorač (*Foeniculum vulgare* Mill., Apiaceae) je jednogodišnja zeljasta biljka poreklom sa područja Mediterana, gde se najviše i gaji, naročito u Francuskoj i Italiji. U ishrani se koristi lišće komorača, kao i zadebljale lisne drške koje formiraju glavicu. Upotrebljava se kao začinska biljka, a iz semena se mogu dobiti etarska ulja lekovitih svojstava (Đinović, 2000).

U nas se ne gaji u komercijalne svrhe, a i malobrojni pokušaji njegovog gajenja su ostali bezuspešni, usled pojave truleži uskladištenih glavica visokog intenziteta.

Simptomi bolesti glavica se ispoljavaju u vidu truleži tkiva glavice, koje postaje mrke boje i izrazito vlažne konzistencije (sl. 1). Obolelo tkivo je mrke boje, a nešto svetlije nijanse na granici obolelog i zdravog tkiva. Trulež se veoma brzo širi zahvatajući glavicu u celosti, koja postaje praktično neupotrebljiva. Bolest ubrzo zahvata i ostale glavice, naročito ako su mehanički oštećene tokom prikupljanja, transporta i dr.

S obzirom da simptomi vlažne truleži raznih povrtarskih biljaka mogu biti prouzrokovani infekcijom fitopatogenim bakterijma pektolitičkih svojstava u obolelom tkivu (bakterije rodova *Erwinia* i *Pseudomonas*), cilj istraživanja je bio da se identifikuje prouzrokovač ovog oboljenja. Tim pre što fitopatogene bakterije paraziti komorača do sada nisu opisani u nas.

MATERIJAL I METODE

Izolovanje bakterije

Obolele glavice komorača sa simptomima truleži su najpre ispirane pod mlazom tekuće vode u cilju odstranjivanja nečistoća i smanjenja populacije saprofitnih bakterija koje mogu zagaditi hranljivu podlogu i na taj način otežati izolaciju bakterija, stvarnog prouzrokovača truleži.

Izolacija bakterije je vršena standardnim metodom razmaza macerata na mesopeptonsku podlogu (NA) i mesopeptonsku podlogu obogaćenu s 5 % saharoze (NAS), radi provere eventualnog stvaranja levana. Macerat je dobijen gnječenjem biljnih fragmenata sa granice obolelog i zdravog tkiva u 1ml sterilisane vode u keramičkom avanu, takođe prethodno sterilisanom (Klement, 1990; Arsenijević, 1997). Pojedinačne kolinije bakterija su posle 36-48 sati razvoja pri 26^{o}C prihvatane i nanošene na zakošenu mesopeptonsku podlogu obogaćenu s 2% glicerola (NAG), radi kraćeg čuvanja u kolekciji tokom istraživanja.

Provera patogenosti

Patogenost proučavanih izolata proverena je veštačkom inokulacijom glavica komorača (zadebljalih lisnih drški), kriški krompira i mrkve i plodova paprike. (Arsenijević, 1997; Obradović, 1999; Jovanović, 1998a).

Glavice komorača i plodovi paprike inokulisani su ubodom bakteriološkom iglom, pri čemu je na ozleđeno mesto naneta kap suspenzije bakterija koncentracije 10^8 cfu/ml. Kriške krompira i mrkve su inokulisane nanošenjem suspenzije bakterija iste koncentracije u prethodno pripremljene „bunarčiće". Rezultati testova patogenosti su očitavani posle 24 i 48 časova od inokulacije (Jovanović, 1998; Obradović, 1999).

Bakteriološke odlike

U cilju identifikacije sojeva izolovanih iz glavica komorača proučene su biohemijske odlike karakteristične za pektolotičke bakterije rodova *Pectobacterium* i *Pseudomonas*: razlikovanje po gramu, fluorescentnost, metabolizam glukoze (O/F test), stvaranje levana, aktivnost oksidaze. Takođe, proučeni su i diferencijalni testovi za detekciju bakterija roda *Pectobacterium* prouzrokovače vlažne truleži: aktivnost fosfataze, lecitinaze, stvaranje indola i redukujućih supstanci iz saharoze; metabolizam laktoze, maltoze, trehaloze i α metil glukozida; razvoj u podlozi sa 5 % NaCl i pri 37^{O}C; karakteristike razvoja na Logan-ovoj podlozi i osetljivost prema eritromicinu (Lelliott i Stead, 1987; Arsenijević, 1997; de Boer i Kelman 2001; Gardan i sar., 2003).

Kao kontrolni sojevi u istraživanjima su korišćeni C-1 (*P. marginalis*), KFB-07 (*P. c*. subsp. *atrosepticum*), KFB-08 (*P. chrysanthemi*), KFB-085 (*P. c*. subsp. *carotovorum*). Kontrolne sojevi bakterija roda *Pectobacterium* dobijeni su ljubaznošću dr Alekse Obradovića, vandrednog profesora Poljoprivrednog fakulteta u Beogradu, na čemu mu se ovom prilikom najsrdačnije zahvaljujemo.

REZULTATI

Na mesopeptonskoj podlozi (NA) posle 24-48 sati razvoja pri 26^{o}C, uočavaju se brojne kolonije bakterija bele boje. One su sjajne, glatke, blago ispupčene, prečnika 1,5-2 mm. Na pomenutoj hranljivoj podlozi, obogaćenoj saharozom, bakterija ne stvara levan.

Proučavani sojevi ispoljavaju izrazitu pektolitičku aktivnost pri proveri patogenosti. Prvi simptomi vlažne truleži glavica komorača uočavaju se posle 24 sata od inokulacije. Inokulisano tkivo je svetlomrke boje, a daljim razvojem bakterije, trulež se širi i zahvata glavicu u celosti (sl. 2). Potpuna trulež glavice

Sl. 1 - *Pectobacterium carotovorum* subsp. *carotovorum*. Trulež glavica komorača (prirodna infekcija).
Fig. 1 - *Pectobacterium carotovorum* subsp. *carotovorum*. Soft rot og fennel bulbs (natural infection).

Sl. 2 - *Pectobacterium carotovorum* subsp. *carotovorum*. Vlažna trulež inokulisanih glavica komorača.
Fig. 2 - *Pectobacterium carotovorum* subsp. *carotovorum*. Soft rot oh artificial inoculated fenel bulbs.

praćena dezorganizacijom tkiva nastaje 4-5 dana od inokulacije, pri čemu tkivo dobija mrku boju.

Simptomi vlažne truleži inokulisanih kriški krompira i šargarepe se uočava već posle 12-15 časova od inokulacije, a potpuna dezintegracije tkiva posle 24 sata. Trulež inokulisanih plodova paprike se pojavljuje 24 sata posle inokulacije i intenzivno se širi zahvatajući ih u celosti. Obolelo tkivo ploda ne menja boju, njegov epidermis ostaje kompaktan, dok tkivo mezokarpa istruli u potpunosti.

Proučavani izolati su Gram negativni, neflurescirаju na King-ovoj podlozi B, glukozu metabolišu i u aerobnim i anaerobnim uslovima i ne stvaraju levan i oksidazu. Na osnovu ovih odlika zaključeno je da sojevi izolovani iz trulih glavica komorača pripadaju rodu *Pectobacterium*. Rezultati diferencijalnih testova za vrste ovog roda pokazuju da naši sojevi ne stvaraju lecitinazu, indol, fosfatazu. Pri ovim testovima s identično ponašaju i soj KFB 07 (*P. carotovorum* subsp. *atrosepticum*) i KFB-085 *(P. c.* subsp. *carotovorum*), dok kontolni soj KFB 08 (*P. chrysanthemi)* daje pozitivne rezultate (tab.1).

Tabela 1 - Rezultati diferencijalnih biohemijskih testova.
Table 1 - Results of differential biochemical tests.

Testovi Tests	Izolati - Isolates			
	komorač - fenel	KFB-07[a]	KFB-085[b]	KFB-08[c]
Aktivnost- Activity				
Lecitanaza-Lecithinase	- [i]	-	-	+ [h]
Fosfataza- Phosphatase	-	-	-	+
Indol-Indole	-	-	-	+
Metabolizam-Matabolism				
Laktoze-Lactose	+	+	+	-
Trehaloze- Trehalose	-	+	+	-
AMG- AMG [d]	-	+	-	-
Razvoj pri - Growth at				
37ºC	-/+	-	+	+
5%NaCl	+	+	+	-
Eritromicin-Erytromicin	R [f]	R	R	S [g]
RSS – RCC [e]	-	+	-	-

a Kontrolni soj *P. c*. subsp. *atrosepticum* – Check strain of *P. c*. subsp. *atrosepticum*;
b Kontrolni soj *P. c*. subsp. *carotovorum* – Check strain of *P. c*. subsp. *carotovorum*;
c Kontrolni soj *P. chrysanthemi* – Check strain of *P. chrysanthemi*;
d α-metil glukozid – α methyl glucoside;
e redukujuće supstance iz saharoze - RCC – reducing compounds from sucrose;
f rezistenost – resistance;
g osetljivost – sensitivity;
h pozitivan rezultat – positive result;
i negativan rezultat – negative result.

Izolati sa komorača na Loganovoj podlozi formiraju ružičaste kolonije prečnika oko 2 mm sa tamno ružičastim centrom. Ovakve kolonije na ovoj podlozi obrazuje i kontrolni soj *P. c*. subsp. *carotovorum*, i one se značajno razlikuju od soja *P. chrysathemi* koja na Loganovoj podlozi formira tamno crvene krupne kolonije i *P. c*. subsp. *atroseptica* koja na Logan-ovoj podlozi obrazuje sitne kolonije crvenkaste boje.

Metabolišu maltozu i laktozu, ali ne trehalozu i metil glukozid, i ne stvaraju redukujuće supstance iz saharoze; razvijaju se u podlozi s 5% NaCl ali se odlikuju slabim razvojem pri 37^{o}C i rezistentni su prema eritromicinu (diskovi 15 mikrograma na hranljivoj podlozi (tab. 1).

Na osnovu dobijenih rezultata zaključeno je da vlažnu trulež uskladištenih glavica komorača prouzrokuje fitopatogena bakterija pektolitičkih svojstava *Pectobacterium carotovorum* subsp. *carotovorum*, patogen širokog kruga domaćina koji obuhvata brojne zeljaste biljke različitih familija i rodova.

DISKUSIJA

Fitopatogene bakterije roda *Pectobacterium* obuhvataju Gramnegativne bakterije sa izraženom pektolitičkom aktivnošću, odnosno sposobnosti razgradnje ćelijskih zidova, što za posledicu ima potpunu dezorganizaciju inficiranog tkiva (Arsenijević, 1997). Patogeni su širokog kruga domaćina koji obuhvata brojne zeljaste biljke, a naročito one koje tokom svog razvoja obrazuju organe bogate ugljenim hidratima (krtole, korenove, zadebljale listove , lisne drške i dr.) (Arsenijević, 1997).

Po svojoj rasprostranjenosti se naročito ističe bakterija *Pectobacterium carotovorum* subsp. *carotovorum* koja parazitira veliki broj biljnih vrsta prouzrokujući na njima simptome truleži (Bradbury, 1986; Arsenijević, 1997). U Srbiji je eksperimentalno potvrđena kao patogen krompira, mrkve, paprike, kupusa, karfiola, salate, celera, paštrnaka (Arsenijević i sar., 1994; Obradović, 1996; Obradović, 1999; Jovanović, 1998a, 1998b; Arsenijević i sar., 1996, 1997; Gavrilović i sar., 2001).

Utvrđena je i kao patogen cvetonosnih stabala kupusa, pri proizvodnji semena prouzrokujući trulež cvetnih drški i stabla (Arsenijević i Obradović, 1996). Pored povrtarskih biljaka, poslednjih godina sve češće pojavljuje i kao parazit ukrasnih biljaka (kala, lala i dr.) (Obradović i sar., 2009; Nuh i sar., 2006). Biljke domaćine parazitira u polju, ali i tokom skladištenja naročito u neodgovarajućim uslovima čuvanja. Pri zarazama visokog intenziteta značajno se smanjuje prinos i kvalitet plodova, korena, listova i drugih organa parazitiranih biljaka (Arsenijević, 1997; Obradović, 1996; 1999).

Iako su simptomi bolesti koje prouzrokuju bakterije ovog roda veoma karakteristični, slični se pojavljuju i u slučaju infekcije drugim fitopatogenim agensima (gljivama, bakterijama drugih rodova) pa i abiotskim faktorima. Stoga je neophodno izvršiti izolaciju bakterije i proučiti njene patogene i bakteriološke odlike sa ciljem pouzdane detekcije.

Iz obolelih uzoraka glavica komorača sa lakoćom je izolovan veći broj sojeva *P. c*. subsp. *carotovorum*, korišćenjem mesopeptonske podloge na kojoj se kolonije bakterije uočavaju 2-3 dana posle izolacije. Pogodnost mesopeptonske podloge za izolovanje ove bakterije ističu i drugi autori (Arsenijević i sar., 1996; Obradović, 1996, 1999; Jovanović, 1998).

Proučavani sojevi ne stvaraju fluorescentni pigment na King-ovoj podlozi B, glukozu metabolišu kako u aerobnim tako u anaerobnim uslovima, ne stvaraju levan i oksidazu. Na osnovu ovih odlika isključena je mogućnost da trulež glavica komorača prouzrokuju pektolitičke bakterije roda *Pseudomonas* (*P. marginalis* i *P. viridiflava*) koje se takođe odlikuju širokim krugom domaćina i na parazitiranim biljkama prouzrokuju simptome truleži (Arsenijević, 1997; Obradović, 1999; Gavrilović i sar., 2007).

Na osnovu diferencijalnih biohemijskih testova za bakterije roda *Pectobacterium* ispostavilo se da izolati pripadaju široko rasprostranjenoj vrsti *P. c*. subsp. *carotovorum*. Oni ne stvaraju redukujuće supstance iz saharoze čime se odlikuje *P. c*. subsp. *atroseptica* ali ni indol, fosfatazu i lecitinazu što je karakteristika *P. chrysanthemi*. Takođe, rezultati ostalih difencijalnih testova (metabolizam ugljenih hidrata, izgled kolonija na Loganovoj podlozi, razvoj u podlozi s 5 % NaCl i rezistentnost prema eritromicinu) (tab.1) ukazuju da izolati sa komorača ispoljavaju odlike *P. c*. subsp. *carotovorum* i u punoj su saglasnosti sa literaturnim podacima (Cother i Sivasthimparam, 1983; Lelliot i Stead, 1987; de Boer i Kelman2001; Gardan i sar. 2003) . Izneneđenje je slab razvoj proučavanih izolata pri 37^{O}C i ne korišćenje trehaloze u metaboličkim procesima, što su karakteristike ove bakterije. Međutim, Janse i Spit, (1989) osporavaju pouzdanost nekih diferencijalnih testova a među njima i razvoj pri ovoj temperaturi.

Predstavnici roda *Pectobacterium* parazitiraju veliki broj predstavnika familije Apiaceae, kao patogen komorača navodi se i *Pectobacterium chrysanthem*i takođe prouzrokujući simptome truleži (Farrar i sar., 2002). Pouzdana detekcija bakterija ovog roda je veoma značajna, jer i pored veoma sličnih simptoma koje prouzrokuju na obolelim biljkama, razlikuju se u pogledu ekoloških zahteva što je od značaja za proučavanje njhove epidemiologije i razrada mera suzbijanja. Iako su navedeni diferencijalni testovi pouzdani, za detekciju ovih bakterija se u današnje vreme sa uspehom koriste molekularne metode, što je od velikog značaja za utvrđivanje patogena u reproduktivnom biljnom materijalu, kao i u biljnom tkivu bez vidljivih simptoma bolesti, zemljištu i vodi (Degefu i sar., 2008; Laurila i sar., 2009).

Komorač je novi domaćin bakterije *P. c*. subsp. *carotovorum* u Srbiji, što ukazuje da se bakterija intenzivno širi, nanoseći značajne štete i omugućuje postojanje inokuluma za ostvarenje novih infekcija biljaka domaćina. Bakterija se uspešno održava u zemljištu, biljnim ostacima, mašinama za obradu, pa i uvodi za navodnjavanje, što predstavlja značajan način njenog širenja (Arsenijević, 1997; Laurila i sar.,2009).

Poslednjih godina su izvršene i značajne promene u taksonomiji bakterija ovog roda, pošto su primenom molekularnih metoda utvrđene značajne razlike među sojevima poreklom sa različitih domaćina i lokaliteta. To je rezultiralo uvođenjem novih vrsta pa čak i novih rodova (Gardan i sar., 2003; Samson i sar., 2004). Ova raznolikost zasigurno utiče i na epidemiološke karakteristika ove grupe bakterija, što je od velikog značaja sa stanovišta njhovog suzbijanja.

LITERATURA

Arsenijević, M. (1992): Fitopatogene bakterije. Naučna knjiga, Beograd, 89-90.

Arsenijević, M. (1997): Bakterioze biljaka. S- Print, Novi Sad, 576 pp.

Arsenijević, M., Đurišić, S., Milošević, D. (1994): *Erwinia carotovora* subsp. *atroseptica*, potato pathogen in our country. Zaštita bilja Vol. 45,(3), N° 209: 169-178.

Arsenijević, M., Trkulja, V., Mitrović, P. (1996): Bakteriozna trulež glavica kupusa. Zaštita bilja, Vol. 47 (4), N° 218: 301-311.

Arsenijević, M., Obradović, A. (1996): Occurrence of bacterial wilt and soft rot of seed cabbage plants (*Brassica oleracea* var. *capitata*) in Yugoslavia. J. Phytopathology 144: 315-319.

Arsenijević, M., Trkulja, V., Obradović, A. (1997): Pathogenic and bacteriological characteristics of Yugoslav *Erwinia* soft rot strains originating from pepper and eggplant fruits. Journal of Plant Diseases and Protection, 104 (4): 394-402.

Bradbury, J. F. (1986): Guide to Plant Pathogenic Bacteria. CAB International Mycological Institute, Kew, Surrey, England.

Cother, E.J., Sivasithamparam, K. (1983): *Erwinia* the „*carotovora*“ group. In: Fahy, P.C. and Persley, G.J. (Eds.): Plant Bacterial Diseases, a Dignostic Guide, Academic Press, Sydnez , Australia.

de Boer, S.H, Kelman, A.: (2001) *Erwinia* soft rot. In: Schaad, N.W., Jones J.B., Chun, W (Eds.): Laboratory Guide for Identification of Plant Pathogenic Bacteria. Third Edition. APS Press, St. Paul, Minnesota, pp. 56-73.

Degefu,Y, Virtanen, E., Vairinen, T. (2008): Pre-PCR processes in the molecular detection of blackleg and soft rot *Erwiniae* in seed potatoes. J. Phytopathology, 157: 370- 378.

Đinović, I. (2000): Svet povrća.Reprograf, Beograd, 253-254.

Farrar, J.J. (2002): Soft rot. In: Davis, M.R., Raid R.N (Eds.): Compendium of umbelliferous crop diseases. APS, St Paul, Minnesota, USA, pp.14-15.

Gardan, L., Gouy, C., Christen, R., Samson, R. (2003): Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov and *Pectobacterium wasabie* sp. nov. International Journal of Systematic and Evolutionary Bacteriology, 53: 381-391.

Gavrilović, V., Obradović, A., Arsenijević, M. (2001): Bacterial soft rot of carrot, parsley and celery. In: S.H. de Boer (Ed.) Plant Pathogenic Bacteria, Kluwer Academic Publishers, Netherland, 269-271.

Gavrilović, V., Milijašević, S., Arsenijević, M. (2007): *Pseudomonas marginalis* – prouzrokovač truleži korena celera i paštrnaka. Zaštita bilja, Vol. 58 (1-4), N° 259-262: 5-13.

Ivanović, M., Gašić, K., Gavrilović, V., Obradović, A. (2009): *Pectobacterium carotovorum* subsp. *carotovorum* – prouzrokovač vlažne truleži biljaka kale u Srbiji i Crnoj gori. Pestic. Fitomed, 24 (4), 287-293, Beograd.

Janse, J.D., Spit, B.E. (1989): A note on limutations of identifying soft rot *Erwinias* by temperature tolerances and sensitivity to eritromycin on a pectate medium. J. Phytopathology, 125: 265-268.

Jovanović, O. (1998a): Patogene karakteristike grupe *Erwinia carotovora* različitog porekla. Zaštita bilja, Vol. 49 (1), N° 223: 29-55.

Jovanović, O. (1998b): Biohemijsko-fiziološke karakteristike bakterije grupe »*Erwinia carotovora*«. Zaštita bilja, Vol. 49 (2), N° 224: 169-180.

Klement, Z (1990).**:** Inoculation plant tissues. Canker and dieback disease. In: Methods in Phytobacteriology. (Eds. Z. Klement, K. Rudolph, and D. Sands), 105-106. Akademiai Kiado, Budapest, 1990.

Laurila, J., Hannukkala A., Nykyri, J., Pasanen, M., Helias, V., Garlant, L., Pirhonen, M. (2009): Symptoms and yield reduction caused by *Dickeya* spp. Strains isolated from potato and river water in Finland. European Journal of Plant Pathology, 126: 249-262.

Lelliott, R A., Stead, D.E. (1987): Methods for the diagnosis of bacterial disease of plants. British Society for Plant Pathology. Blackwell Scientific Publications. Oxford, London, Edinburgh. pp. 200.

Nuh, B., Bastas, K.K., Maden, S. Yasar, A. (2006): Bacterial leaf and peduncle soft rot caused by *Pectobacterium carotovorum* on tulips in Konya, Turkey. Phytoparasitica, 34 (3): 279-280.

Obradović, A. (1996): Patogene i biohemijsko fiziološke karakteristike bakterija grupe »*ERWINIA CAROTOVORA*« parazita krompira. Zaštita bilja, Vol. 47 (1), N° 215: 57-70.

Obradović, A. (1999): Identifikacija bakterija rodova *Pseudomonas* i *Erwinia* patogena salate i karfiola. Zaštita bilja , Vol. 50 (1), Nº 227: 9-60, Beograd.

Samson, R., Legendre, J.B. Christen, M.F., Saux, M.F., Achouak, W., Gardan, L. (2005): Transfer of *Pectobacterium chrysantemi* (Burkholder et al. 1953) Brenner et al.1973 and *Brenneria paradisica* to the genus *Dickeya* gen . nov. as *Dickeya chrysanthemi* comb. nov. nnd *Dickeya paradisiaca* comb. nov. and delineaton of four novel species, *Dickeya dadanatii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. International Journal of Systematic and Evolutionary Microbiology, 55 (4): 1415-1427.

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ETIOLOGY OF SOFT ROT ON FENEL BULBS

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SUMMARY

Soft rot bacterial strains were isolated from diseased fennel bulbs. They were characterised on the basis of its pathogenicity morphological, cultural and biochemical properties. All investigated strains caused the soft rot of inoculated fenel bulbs, pepper fruits as well as clices of potato and carrot. Results of bacteriological properties show that soft rot of fennel bulbs were caused dy Gram negative, nonfluorescent, facultative anaerobic, levan and oxidase negative bacteria belonging to the genus *Pectobacterium*. The investigared strains utilised lactose, grew at 5% NaCl and weak growth was recorded on 37°C; they are resistent to erytromicin. Negative results were recorded in indol, phospathase, lecithinase and reducing compounds pf sucrose tests. These results as well as the characteristics growth on Logan differential medium indicated that sof rot of fenel bulbs was caused by *Pectobacterium carotovorum* subsp. *carotovorum*. This is the first report of this bacterium affecting fennel bulbs in Serbia.

Key words: fennel, soft rot, *Pectobacterium carotovorum* subsp. c*arotovorum*, bacteriological properties.

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