Detection of Leaf Blotches – Causal Agents in Barley Leaves and Grains

Jozef GUBIŠ, Martina HUDCOVICOVÁ, Lenka KLČOVÁ, Viera ČERVENÁ, Katarína BOJNANSKÁ and Ján KRAIC

Division of Applied Genetics and Breeding, Research Institute of Plant Production, Piešťany, Slovak Republic

Abstract: The objective of this paper was to adapt PCR-based detection method for *R. secalis* and *P. teres* DNA isolated from pathogens and also from artificially infected juvenile leaves and seeds using pathogen-specific primers. It has been proven that primers specific to *P. teres* and *R. secalis* can reliably diagnose pathogen DNA as well as its presence in the mixture with barley DNA. Two primers set for detection of *R. secalis* were compared. The intensity of the corresponding DNA band after amplification with primer pair RS1–RS3 was higher than that amplified with RS8–RS9. The primer set RS1–RS3 was also used to detect *R. secalis* in barley seeds. DNA from infected seeds was isolated by two ways – according to the method of Dellaporta *et al*. (1983) or by the Adgen DNA Extraction System. The DNA extracted using the Adgen kit showed higher quality, however the amplification of the pathogen DNA was accomplished in both cases.

Keywords: *Pyrenophora teres*; *Rhynchosporium secalis,* DNA; PCR; pathogen diagnostics

Leaf blotch of barley (*Hordeum vulgare* L.) caused by the imperfect fungus *Rhynchosporium secalis* (Oudem.) J. J. Davis and *Pyrenophora teres* (Drechs.), respectively, belongs to the most important diseases of barley in many parts of the world. Both pathogens cause considerable reductions in yield and quality of mature grain. Yield losses from 10% to 40% have been reported (Brown *et al.* 1993; Sanina 1994). The detection of both pathogens is essential since both these pathogens are seed-borne. The seed-borne inoculum of *Rhynchosporium secalis* is important, especially in the dispersal of novel races as well as in the long-range dispersal of the pathogen (Salamati *et al.* 2000). Although seed transmission of the pathogen was shown to vary depending on the environmental conditions (Skoropad 1959; Kay & Owen 1973), coleoptile infection may play an important role in scald epidemics in the field, especially during humid periods. *Pyrenophora teres*, however, can be either seed- or debris-borne and while seed treatment can help to reduce early infection by net blotch, foliar fungicides can also be very effective if the plants become infected with infested plant residues later.

Conventional methods of identifying fungal plant pathogens rely on the evaluation of visual symptoms and/or the isolation, cultivation, and laboratory identification of the pathogen. The accuracy and reliability of these methods depend largely on the experience and skills of the person making the diagnosis. Diagnosis requiring cultivation is time-consuming and impractical when prompt results are required (McCartney *et al.* 2003). Therefore, it is desirable to develop a reliable, rapid and sensitive diagnostic method for detecting early infection by this pathogen.

The most sensitive tests for pathogens based on pathogen DNA detection in plants using polymerase

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chain reaction (PCR). The PCR assay is the only approach that allows the detection of extremely small quantities of specific DNA. PCR is highly sensitive and reproducible for the amplification of diagnostic molecular markers, and could easily be used for identification and detection if species-specific primers are available. Although molecular methods have been widely used for detection, identification and phylogenetic study of the various phytopathogenic fungi, attempts to use molecular approaches to detect seed infection have received attention only in recent years (Yao *et al.* 1990; Taylor 1993; Huff *et al.* 1994; Reeves 1999; Parry & Nicholson 1996; Smith *et al.* 1996; Doohan *et al.* 1998).

The aim of this study was to test the presence of pathogens *Rhynchosporium secalis* and *Pyrenophora teres* in artificially infected juvenile leaves and seeds of barley using PCR and pathogen-specific primers.

MATERIAL AND METHODS

Seedlings of different barley cultivars (listed in Figures 2 and 4) were inoculated by spraying of spores of *P. teres* and *R. secalis*, respectively, collected from primary leaves of barley in six localities of the Slovak Republic – Košice, Borovce, Rimavská Sobota, Bodorová, Spišské Vlachy, and Spišská Belá. Seedlings were placed in water-saturated trays and covered with polyethylene bags immediately after inoculation. Seedlings for the testing of resistance to *P. teres* and *R. secalis* were grown in controlled environment (20°C, continuous illumination). Leaf samples consisting of fresh tissue, each weighing 1.5 g, were taken for testing four, seven and eleven days after inoculation (for *P. teres*) and fourteen and twenty-one days (for *R. secalis*), respectively.

Barley seeds of the susceptible cultivar Dukos were artificially inoculated by isolates of *P. teres* (from Borovce region) and *R. secalis* (isolate RS 32-2). Single-spore isolates of *R. secalis* used in this study, RS 32-2 and RS 4004, were obtained from Dr. S. Salamati (Kvithamar Research Centre, Stjørdal, Norway). Cultures of *P. teres* were maintained on modified Czapek-Dox agar and cultures of *R. secalis* on potato dextrose agar at 20°C.

Total fungal genomic DNA was extracted from cultures grown on agar plates. The fungal mycelium was scraped off and DNA extracted according to the method of Dellaporta *et al.* (1983). DNA from barley leaves was isolated using the same method. The total DNA from barley seeds was isolated using the Adgen DNA Extraction System (Adgen Ltd.) from one gram of powder from ground seeds.

PCR analyses for the diagnosing of pathogen-specific DNA sequences were done using PCR primers (Table 1) derived from ITS regions of rDNA of *R. secalis* (Lee *et al.* 2001) and from AFLP marker in *P. teres* (Williams *et al.* 2001).

The PCR analysis of *P. teres* had the following optimal reaction mixture: 1 x PCR buffer (20 mmol/l Tris-HCl pH 8.4, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$), 0.5 μmol/l of each forward and reverse primer, 0.2 mmol/l dNTP, 0.8 units of *Taq* DNA polymerase, and 25 ng DNA. Amplifications were performed in total volume of 15 μl using the GeneAmp® PCR System 9700 (Applied Biosystems) with the following amplification conditions: initial denaturation at 94°C for 1 min, 10 cycles at 94°C for 30 s, 65°C for 30 s reducing 1°C per cycles, 72°C for 1 min, 24 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for

Table 1. Sequences of specific primers for detecting *Pyrenophora teres* and *Rhynchosporium secalis*

Target species	Primer name	Sequence (5′–3′)	Product length
Rhynchosporium secalis	RS1	AAGAAGCCTGGTTCAGACCTCC	371 bp
	RS3	ACCGCCACTGATTTTAGGGG	
	RS8	TTGTTTTTAGTGATGTCTGAG	264 bp
	RS9	AGGCACCGCCACTGATTTTAGGG	
P. teres f. *teres*	PTT-F	CTCTGGCGAACCGTTC	378 bp
	PTT-R	ATGATGGAAAAGTAATTTGTA	
P. teres f. *maculata*	PTM-F	TGCTGAAGCGTAAGTTTC	411 bp
	PTM-R	ATGATGGAAAAGTAATTTGTG	

1 min, and extra extension at 72°C for 5 min. The PCR analysis of *R. secalis* was carried out according to Lee *et al*. (2001). Electrophoretic detection of PCR products was performed in 1.4% agarose gels.

RESULTS

The functionality of two primer pairs (RS8-RS9 and RS1-RS3) was detected in the course of detection of genomic DNA from two isolates of *R. secalis* in barley leaves possessing scald symptoms. Both the specific primer pairs correctly amplified diagnostic bands from leaves infected with *R. secalis* (Figure 1). The intensity of the corresponding 371 base pair DNA band after amplification with primer pair RS1-RS3 was higher than that of the band amplified with RS8-RS9 primer pair (264 bp). Therefore primer pair RS1-RS3 was used for further experiments to detect *R. secalis* in barley. Figure 2 shows DNA diagnosing of this pathogen in a set of differential barley cultivars 14 and 21 days, respectively, after artificial infection of juvenile leaves with *R. secalis*. Although this set of barley differentials included susceptible (Lenka, Viktor, Terno), medium resistant (Harabin, Ming, CI 2330, Tifang, CI 739, CI 4976, CI 4922, CI 2750, CI 7584, Norbert, CI 4207, K 29192) and resistant cultivars (CI 5791, CI 9819, CI 9820, CI 9825, CI 1615, CI 1179, CI 9776, CI 5809; CI 9440, CI 9768, K 20019), the presence of the relevant DNA band of pathogen was detected also in resistant cultivars. This implies that even if there were no visual symptoms of the pathogen on leaves, its detection by DNA is already possible. No amplification in samples of barleys CI 739 and Terno occurred after 14 days from inoculation probably due to lower develop-

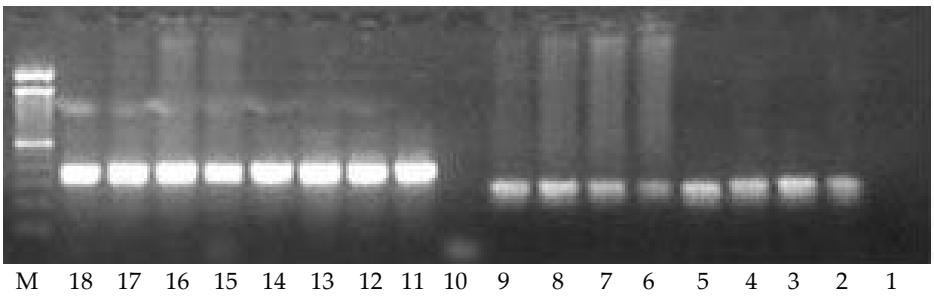


Figure 1. Agarose gel of PCR-amplified products using primer pairs RS8-RS9 (lanes 1–9) and RS1-RS3 (lanes 10–18), respectively. Lanes: 1 – H_2O (negative control); 2 – *R. secalis* 4004 (0.1 μg/μl); 3 – *R. secalis* 4004 (0.025 μg/μl); 4 – *R. secalis* 32-2 (0.1 μg/μl); 5 – *R. secalis* 32-2 (0.025 μg/μl); 6 – CI 1615; 7 – CI 9768; 8 – K 29129; 9 – Lenka; 10 – H_2O (negative control); 11 – *R. secalis* 4004 (0.1 μg/μl); 12 – *R. secalis* 4004 (0.025 μg/μl); 13 – *R. secalis* 32-2 (0.1 μg/μl); 14 – *R. secalis* 32-2 (0.025 μg/μl); 15 – CI 1615; 16 – CI 9768; 17 – K 29129; 18 – Lenka; M – 100 bp DNA ladder

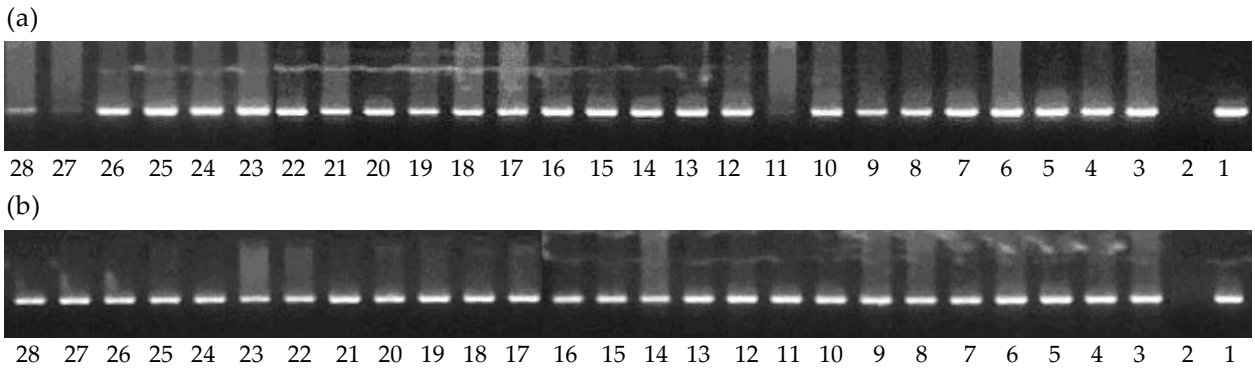


Figure 2. Detection of pathogen DNA in barley leaves artificially infected with *Rhynchosporium secalis* after 14 (a) and 21 days (b) of inoculation. Lanes: 1 – *R. secalis* 4004 (0.025 μg/μl); 2 – H_2O (negative control); 3 – Harabin; 4 – Ming; 5 – CI 2330; 6 – Tifang; 7 – CI 5791; 8 – CI 9819; 9 – CI 9820; 10 – CI 9825; 11 – CI 739; 12 – CI 4976; 13 – CI 4922; 14 – CI 2750; 15 – CI 7584; 16 – CI 1615; 17 – CI 1179; 18 – CI 9776; 19 – CI 5809; 20 – CI 9440; 21 – CI 9768; 22 – Norbert; 23 – K 20019; 24 – CI 4207; 25 – K 29192; 26 – Lenka; 27 – Terno; 28 – Viktor

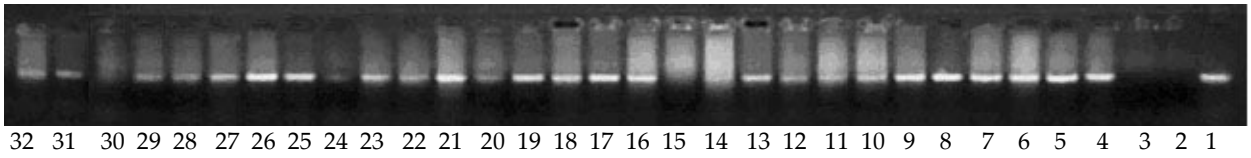


Figure 3. Detection of pathogen DNA in barley leaves artificially infected with *Rhynchosporium secalis* from the regions Spišská Belá (lanes 4–23), Spišské Vlachy (lanes 24–28) and Bodorová (lanes 29–32). Lanes: 1 – *R. secalis* 4004 (0.025 μg/μl); 2 – H_2O (negative control); 3 – Luxor (negative control); 4 – Harabin; 5 – Ming; 6 – CI 2330; 7 – Tifang; 8 – CI 5791; 9 – CI 9819; 10 – CI 9825; 11 – CI 739; 12 – CI 4976; 13 – CI 4922; 14 – CI 2450; 15 – CI 7584; 16 – CI 9776; 17 – CI 5809; 18 – Norbert; 19 – K 8755; 20 – K 29192; 21 – Lenka; 22 – CI 5401; 23 – Viktor; 24 – Tifang; 25 – CI 1179; 26 – CI 2450; 27 – CI 9776; 28 – CI 5401; 29 – CI 2450; 30 – CI 7584; 31 – CI 1179; 32 – CI 9776

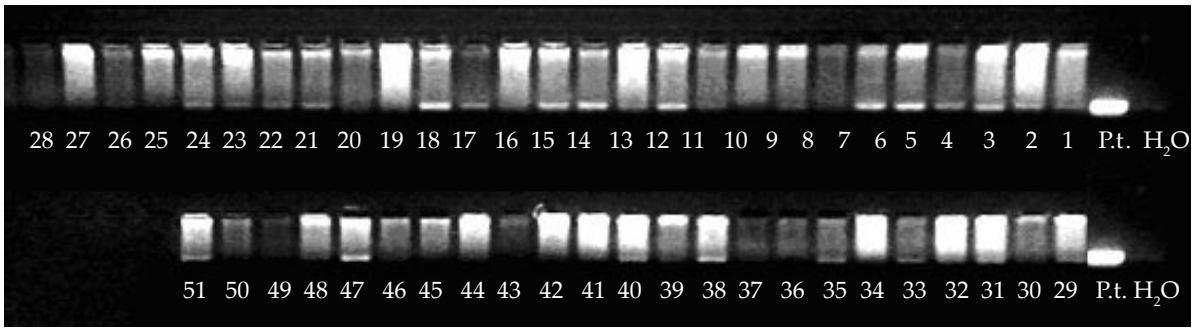


Figure 4. Detection of pathogen DNA in barley leaves artificially infected with *Pyrenophora teres* after 4 (lanes 1–17), 7 (lanes 18–34) and 11 days (lanes 35–51) of inoculation. Lanes: H_2O (negative control); P. t. (*P. teres* positive control); 1, 18, 35 – CI 2750; 2, 19, 36 – CI 4929; 3, 20, 37 – K 8755; 4, 21, 38 – K 20019; 5, 22, 39 – CI 4207; 6, 23, 40 – CI 739; 7, 24, 41 – CI 4407-1; 8, 25, 42 – K 29192; 9, 26, 43 – CI 9825; 10, 27, 44 – CI 5791; 11, 28, 45 – CI 9820; 12, 29, 46– CI 9819; 13, 30, 47 – Lenka; 14, 31, 48 – CI 4922; 15, 32, 49 – CI 5401; 16, 33, 50 – CI 7584; 17, 34, 51 – Dukos

ment of the pathogen. The responses of a set of barley differentials infected with *R. secalis* from all three localities – Spišská Belá, Spišské Vlachy, and Bodorová were studied in the following experiment (Figure 3). In this case the presence of the pathogen DNA in a mixture of was also diagnosed.

Similarly, artificial infection of juvenile leaves of a set of differential barley cultivars with *P. teres* originating from three regions (Borovce, Košice and Rimavská Sobota) was established. Primer set PTT specifically amplified a band of 378 bp from *P. teres* f. *teres* isolates (*P. teres* f. *maculata* was unidentified within our isolates). The total DNA from infected leaves was isolated 4, 7, and 11 days after inoculation. Also in this case the PCR analysis detected the presence of the pathogen in mixed DNA samples (Figure 4).

The next step was to infect barley seeds of the cultivar Dukos with *P. teres* and *R. secalis* artificially. Four samples of barley seeds infected with *R. secalis*, ranging from 70% to 90% of infected seeds, were analysed (Figure 5). Each extract of DNA from all four samples was amplified as a positive one in comparison with negative control.

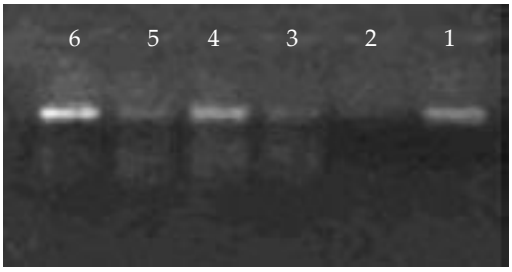
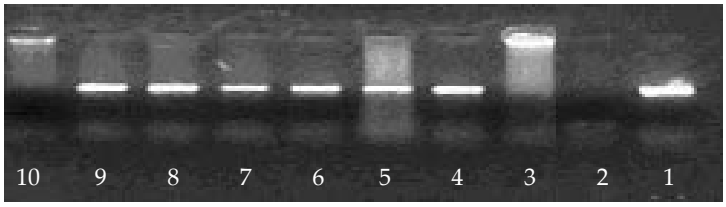


Figure 5. Amplification of *Rhynchosporium secalis* DNA in barley seeds artificially inoculated by an isolate of *R. secalis* (RS 32-2). Total DNA from barley seed was isolated by the method of Dellaporta *et al.* (1993). Lanes: 1 – positive control (*R. secalis* DNA); 2 – negative control (H_2O); 3–6 – infected samples of barley seeds

DNA from seeds infected with *P. teres* was also isolated using the Adgen DNA Extraction System and the amplification quality was compared to DNA extracted by the method according to Dellaporta *et al.* (1983) (Figure 6). Although DNA isolated by



Lanes: 1 – positive control (*P. teres* DNA); 2 – negative control (H_2O); 3, 10 – DNA extract from the uninfected barley cultivar Dukos (negative control); 4–9 – infected samples of barley seeds

Figure 6. Amplification of *Pyrenophora teres* DNA in barley seeds artificially inoculated by an isolate of *P. teres* (from Borovce locality). Total DNA from barley seed was isolated by the method of Dellaporta *et al.* (1983) (lanes 3–6) and by DNA Extraction System (Adgen Ltd.) (lanes 7–10)

the Adgen DNA Extraction System showed higher quality, the amplification of the pathogen DNA was accomplished in both cases.

DISCUSSION

The precise identification of pathogens is important for disease management, plant breeding, epidemiological and other studies. Conventional diagnostic methods for seed health testing include agar plate tests, seedling bioassays and microscopic observations. These methods, however, are time-consuming, labour-intensive and subjective. The technology of nucleic acid test methods is expanding rapidly and the main advantage of these methods is that they are highly specific, sensitive and rapid, with the potential for automation, leading to high throughput (Reeves 1999).

In this study, a PCR-based diagnostic assay was used to detect *P. teres* and *R. secalis* in barley leaves and seeds using pathogen-specific primers. PCR primers RS1–RS3 and RS8–RS9 were screened in order to detect several isolates of *R. secalis*, microbes and other pathogens and saprophytes commonly found on barley some years ago and they correctly distinguish the infected samples with *R. secalis* from other organisms (Lee *et al.* 2001). Reliability of these primers to identify *R. secalis* in leaves and seed samples was observed in our experiments too. These primer sets designed from ITS regions of the rDNA of the pathogen showed high specificity for detecting the pathogen in seed. ITS regions have been widely used for developing species-specific probes or for designing primers for detection of pathogens (Beck & Ligon 1995; Goodwin *et al.* 1995; Kageyama *et al.* 1997).

PCR primers PTT-F, PTT-R and PTM-F, PTM-R designed by Williams *et al.* (2001) were used for detection of *P. teres* in plant material of barley. They tested these primer sets for their ability to identify the spot form of the net blotch fungus (*P. teres* f. *maculata*) and the net form of the net blotch fungus (*P. teres* f. *teres*) in barley leaves with blotch symptoms, which cause the lesion types indicated by their disease names, although symptom overlap and similar spore morphology can make the identification difficult. The authors reported that the formae-specific primers correctly amplified diagnostic bands from leaves infected with either isolates, even if DNA extracts from both types of blotch were mixed. Identical results were achieved in our experiments with leaf and seed samples. The detection of *P. teres* was also studied by several authors (Thomas *et al.* 2000, 2001; Bates *et al.* 2001).

The conventional PCR approach has some drawbacks, including laborious optimisation procedures, limited range of quantification and sample variation caused by increased handling. However, the results suggested that this conventional PCR assay would provide an accurate means of detecting the inoculum in plants and predicting levels of leaf and/or seed infection. This method not only offers a reasonable alternative approach to plant material health testing but also it will be a sensitive tool in epidemiological studies of barley diseases.

Recently, real-time quantitative PCR, based on detection of a fluorescent signal produced proportionally during amplification of a PCR product, has provided a rapid and accurate method for determination of specific DNA and RNA sequences levels (Grove 1999; Zhang *et al.* 1999; Taylor *et al.* 2001; Fraaije *et al.* 2001). This technology is relatively expensive, but should offer a better alternative for routine seed and/or leaf health testing in the future.

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Abstrakt

Gubiš J., Hudcovicová M., Klčová L., Červená V., Bojnanská K., Kraic J. (2004): **Detekcia listových škvrnitostí – pôvodcov chorôb listov a semien na jačmeni**. Czech J. Genet. Plant Breed., **40**: 111–117.

Cieľom práce bolo adaptovať PCR detekčnú metódu pre identifikovanie DNA izolovanej z patogénov *R. secalis* a *P. teres* a tiež umelo infikovaných juvenilných listov a semien, s použitím patogén-špecifických primerov. Bolo dokázané, že špecifické primery pre *P. teres* and *R. secalis* dokážu spoľahlivo diagnostikovať prítomnosť DNA patogéna, ako aj jej prítomnosť v zmesi s DNA hostiteľa, teda jačmeňa. Porovnávané boli dve sady primerov pre detekciu *R. secalis*. Po amplifikácii s párom primerov RS1–RS3 bola intenzita príslušného DNA fragmentu vyššia, než po amplifikácii s párom primerov RS8–RS9. Pár primerov RS1–RS3 bol tiež použitý na detekciu *R. secalis* v semenách jačmeňa. DNA infikovaných semien bola izolovaná dvoma spôsobmi – podľa Dellaporta *et al*. (1983) alebo pomocou Adgen DNA extrakčného systému. DNA extrahovaná pomocou kitu Adgen vykazovala vyššiu kvalitu, avšak amplifikácia DNA patogéna prebehla v oboch prípadoch.

Kľúčové slová: *Pyrenophora teres*; *Rhynchosporium secalis*; DNA; PCR; diagnostika patogénov

Corresponding author:

Ing. Jozef Gubiš, Výskumný ústav rastlinnej výroby, Divízia aplikovanej genetiky a šľachtenia,
Bratislavská cesta 122, 921 68 Piešťany, Slovenská republika
tel.: + 421 33 772 23 11, fax: + 421 33 772 63 06, e-mail: gubis@vurv.sk