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IDENTIFYING *Fusarium avenaceum* AND *Fusarium culmorum* IN SELECTED LEGUMES AND CEREALS WITH THE PCR METHOD

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ABSTRACT

The paper showed the applicability of the PCR method to an early identification of *Fusarium avenaceum* and *Fusarium culmorum* in infected tissues of selected crops. The polymerisation chain reaction used species-specific SCAR primers. There was observed a discrepancy between the size of multiplied DNA of *Fusarium avenaceum* fragment and that of Fa-U17f 5', Fa-U17r earlier described by authors of primers. The present

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research product size for *F. avenaceum* was 950 bp, while for *F. culmorum* - 472 bp which confirmed the reports by other authors.

Key words: Fusarium avenaceum, Fusarium culmorum, DNA, PCR, SCAR primers

INTRODUCTION

Traditional methods of pathogen identification involve culture growth by seed or plant samples placement onto agar or moistened filter paper, however an extensively long incubation period, time-consuming thorough microscopic observation of spores, no possibility of detection for a low inoculum in the material tested and indispensable analysis by highly qualified experts remain major drawbacks of that method [5]. Serologic methods are also frequently futile due to a lack of specialised anti-gene for a given fungal species [2]. All that makes the application of molecular methods based on PCR, which guarantee fast and precise identification of fungus species in the infected plant sample tested, more and more common. The PCR development has, in turn, helped a development of new markers, including SCAR (Sequence Characterised Amplified Regions).

Species-specific molecular markers can be obtained from sequence polymorphism analysis for ITS of nuclear rDNA [4]. SCAR starters have been currently developed for: *Fusarium avenaceum, F. culmorum, F. graminearum, F. poae, F. crookwellense, F. torulosum, F. venenatum, F. tricinctum, F. sambucinum, Microdochium nivale* var. *majus, M. nivale* var. *nivale, Pseudocercosporella herpotrichoides* (W-type, R-type), *Rhizoctonia cerealis, Pyrenophora teres* [1]. The technique incorporates about 20 nucleotide primers, which ensures a specific amplification and a thorough diagnosis of the pathogen species [4].

The aim of the present paper was to evaluate the applicability of SCAR primers to identify *F. avenaceum* and *F. culmorum* in pea, faba bean, yellow lupin, winter wheat, winter triticale and winter rye. The research included the cultivars most common in the north-eastern Poland showing a varied resistance to *Fusarium.* The fungi used to infect plants are pathogens which penetrate the conducting tissue, only. Hyphae, spores and toxins produced by fusarium disease complex move with the ascending water current and reach higher plant organs, which makes their detection in cotyledon and leaf tissues possible.

MATERIAL AND METHODS

The present research draws on the results of two infection experimental series conducted by the Department of the Diagnosis and Plant Pathophysiology of the Warmia and Masuria University in Olsztyn.

A series: Identifying *F. avenaceum* and *F. culmorum* with the PCR method in selected legumes following the application of soil inoculum and fungal spore suspension. Experimental factors:

a. Legume species and cultivars – 'Jaspis' pea cultivar, 'Teo' yellow lupin cultivar, 'Martin' faba bean cultivar,
b. Fungal species – *F. avenaceum and F. culmorum.*

Variant 1 experiment was carried out in pots filled with brown soil. Upper and lower inocula were applied (slant with PDA medium overgrown with *F. avenaceum* and *F. culmorum* mycelium*)*. Variant 2 experiment seeds were soaked in fungal spore suspension and sown into

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pots 3-5 cm deep (pea), 2-3 cm (yellow lupin) and 6-8 cm (faba bean). Further vegetation took place at 24 °C and 12-h day/night light regime and optimum oxygen availability.

Cotyledon and leaf samples for DNA isolation were sampled 4, 10, 12, 16, 20, 25, 31, 34, 40 days after inoculation. The isolated DNA was used for identifying *F. avenaceum* and *F. culmorum* in reaction with the PCR and SCAR primers specific for fungal pathogens studied.

B series: Detection of *F. avenaceum* and *F. culmorum* with PCR method in selected legumes and cereals following fungal spore suspension.
Experimental factors:

a. Legume species and cultivars – 'Jaspis' pea cultivar, 'Teo' yellow lupin cultivar, 'Martin' faba bean cultivar, 'Roma' winter wheat cultivar, 'Fidelio' winter triticale cultivar, 'Nawid' winter rye cultivar.
b. Fungal species – *F. avenaceum, F. culmorum.*

Soaked seeds were placed onto 19-cm Petri dishes with sterile moistened filter paper. After germination the seeds were poured with 10^6 concentration fungal spore suspension. A further growth took place at 24 °C and 12-h day/night light regime and optimum oxygen availability. Cotyledons, epicotyl sheaths and leaves of the cultivars studied were sampled for DNA isolation 7, 9, 12, 14, 20 and 24 days after infection. *F. avenaceum* and *F. culmorum* were identified with the PCR method with specific SCAR primers.

Culturing and preparing inoculum for infection of plants

F. avenaceum and *F. culmorum* pathogenic fungi used to infect plant and to isolate DNA were obtained from naturally infected pea, yellow lupin, faba bean, winter wheat, winter triticale and winter rye tissues. Homogenous and purified fungal isolates were transferred onto Petri dishes with PDA and stored for 12 h with no exposure to light and 12 h exposed to ultraviolet light at 20 - 23 °C. The fungal cultures were used to infect plants and to isolate plant and fungal DNA.

DNA isolation with DNA zol Reagent

Epicotyl sheaths, cotyledons and leaves were rinsed 3 times with sterile water, then with 70% ethyl alcohol for 5 minutes and in 1% sodium hypochlorite and, finally, 3 times with sterile water. The disinfected plant material was crushed in sterile porcelain mortar in liquid nitrogen. When DNA was isolated from a homogenous culture grown on PDA, the mycelia were scraped with scalpel and crushed in mortar in liquid nitrogen. After crushing 1 mL of DNA zol Reagent (Gibco BRL), the material was transferred to 1.5-mL Eppendorf tube. After 10 minute-lysis, material was centrifuged (14000 r.p.m. for 10 min.). The supernatant was placed into the sterile tube, 500 µL of ethyl alcohol was added and mixed, while the residuum was removed. After a repeated centrifugation (14000 r.p.m. for 5 min.) and the alcohol removal, another 1000 µL of ethyl alcohol was added to the residue. Following vortexing and centrifugation (14000 r.p.m. for 5 min.), alcohol was again removed from the sample and residue (DNA) was left at the bottom for 30-40 min. in open tubes to dry out. The mixture was suspended in 100 µL of TE buffer, vortexed and centrifuged (14000 r.p.m. for 5 min.), the supernatant (DNA) was sampled and put into the sterile Eppendorf tube and the residue was removed. The DNA samples were used as a matrix in PCR.

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Polymerase chain reaction (PCR)

DNA samples isolated from the plant tissue and from pure fungal culture were amplified with specific SCAR primers. To detect *F. avenaceum*, a pair of primers: Fa-U17f 5'CAAGCATTGTCGCCACTCTC3' and Fa-U17r 5'GTTTGGCTCTACCGGGACTG3' was used [6], while to detect *F. culmorum* - OPT18F470 5'GATGCCAGACCAAGACGAAG3' and OPT18R4705'GATGCCAGACGCACTAAGAT3' [4]. The following reagents were used in the reaction: Tfl 20x (1.25 µL) buffer, magnesium ions - $MgCl_2$ (4 µL), dNTPs free nucleotides (2 µL), primers (1 µL each), PCR Enhancer (3 µL), Tfl DNA polymerase (0.2 U, Epicentre), deionised water (11.25 µL) and matrix DNA (1 µL); the reaction volume per tube amounted to 25 µL. The samples were exposed to serial changes in temperature in Gene Amp 2400 (Perkin Elmer) thermocycler under specific thermal profiles (Table 1).

Table. 1. PCR reaction conditions, time and thermal profiles

Cycle number	PCR reaction stages	*Fusarium avenaceum*	*Fusarium culmorum*
1 cycle	Initial denaturation	94 °C – 2 min.	94 °C – 2 min.
30 cycles	Actual denaturation	94 °C – 30 sec.	94 °C – 1 min.
	Primer annealing	64 °C – 20 sec.	59 °C – 1 min.
	DNA extension	72 °C – 45 sec.	72 °C – 2 min.
1 cycle	Final DNA extension	72 °C – 10 min.	72 °C – 10 min.

To evaluate the amplified PCR product size, there was carried out an electrophoresis in 1.5 % agarose gel in 50V for 2h against mass standard M1 (pU19/Mspl) and M 100-1000.

RESULTS AND DISCUSSION

The PCR was carried out with SCAR specific primers, while DNA isolated from plant tissue constituted the matrix. Series 1 experiment identified *F. avenaceum* in the 6-day cotyledon tissue obtained from faba bean and pea seeds germinated in soil infected with agar slant overgrown with mycelium (Photo 1). The next identification of *F. avenaceum* was observed in 25-day leaf tissue of pea infected with agar slant overgrown with mycelium. Pea plants researched reached the leaf-formation phase. Amplified product scored 950 bp (Photo 2), however Turner et al. [6] recorded 345 bp for PCR with the same SCAR primers.

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Photo 1. PCR products from cotyledon plant tissue infected with *F. avenaceum* on the 6th day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Pea infected by seed soaking**
5. **Pea infected with slant**
6. **Yellow lupin infected by seed soaking**
7. **Yellow lupin infected with slant**
8. **M 100-1000 standard**
9. **Faba bean control**
10. **Pea control**
11. **Yellow lupin control**
12. **Blank**

1 2 3 4 5 6 7 8 9 10 11 12

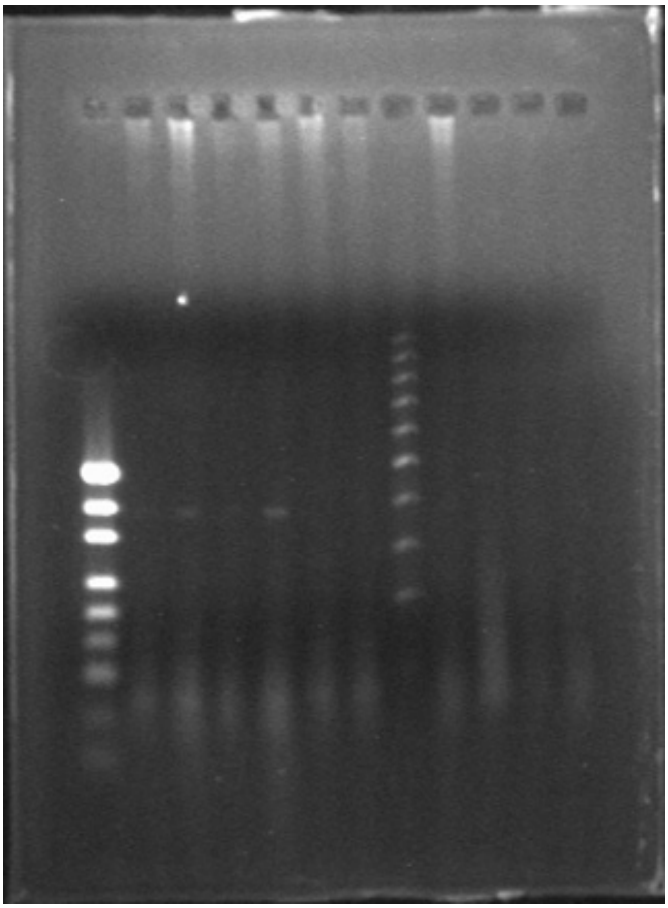

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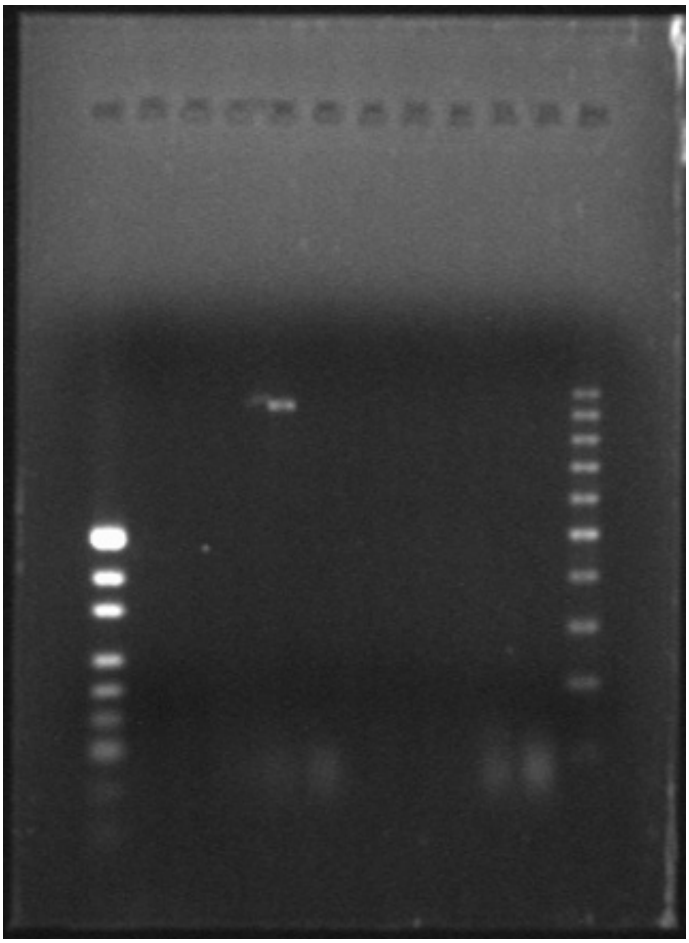
Photo 2. PCR products from cotyledon plant tissue infected with *F. avenaceum* on the 25th day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Pea infected by seed soaking**
5. **Pea infected with slant**
6. **Yellow lupin infected by seed soaking**
7. **Yellow lupin infected with slant**
8. **Faba bean control**
9. **Pea control**
10. **Yellow lupin control**
11. **Blank**
12. **M 100-1000 standard**

1 2 3 4 5 6 7 8 9 10 11 12



In pea infected with *F. culmorum* slant, the pathogen DNA in tissue was identified with successive vegetation days: - 4 (beginning of germination, Photo 3) -16 (full emergence, Photo 4), -20 (Photo 5), -25 (a few pairs of leaves) and 34 (Photo 8). The presence of the amplification product characteristic for *F. culmorum* in the pea control shows a natural infection from seed.

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Photo 3. PCR products from cotyledon plant tissue infected with *F. culmorum* on the 4th day of vegetation

1. **M1 Standard**
2. **Pea infected with slant**
3. **Yellow lupin infected by seed soaking**
4. **Yellow lupin infected with slant**
5. **Pea control**
6. **Yellow lupin control**
7. **Blank**
8. **M 100-1000 standard**

1 2 3 4 5 6 7 8

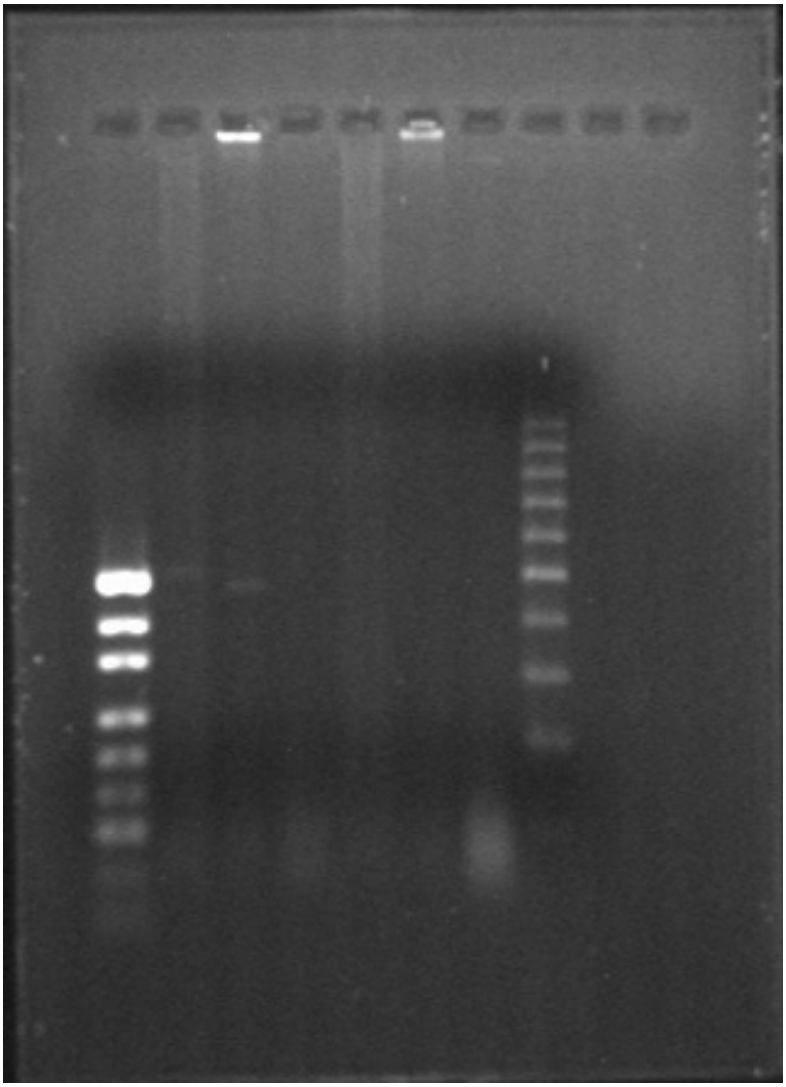

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Photo 4. PCR products from cotyledon and leaf plant tissue infected with *F. culmorum* on the 16th day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Pea infected with slant**
5. **Yellow lupin infected by seed soaking**
6. **Yellow lupin infected with slant**
7. **Faba bean control**
8. **Pea control**
9. **Yellow lupin control**
10. **Blank**
11. **M 100-1000 standard**

1 2 3 4 5 6 7 8 9 10 11

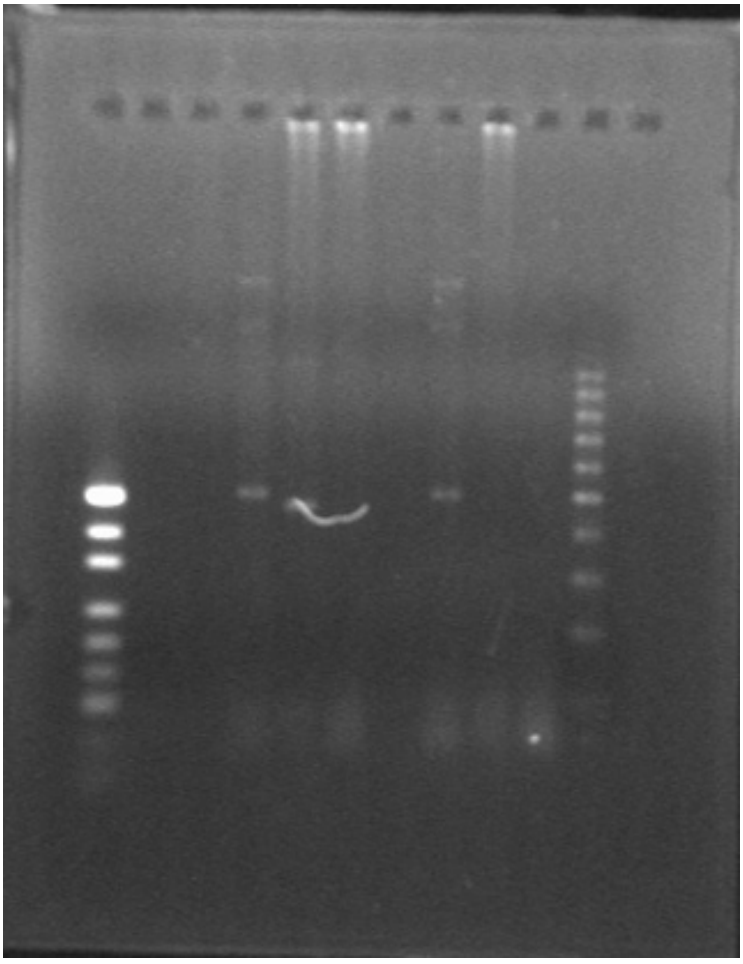

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Photo 5. PCR products from leaf plant tissue infected with *F. culmorum* on the 20th day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Pea infected with slant**
5. **Yellow lupin infected by seed soaking**
6. **Yellow lupin infected with slant**
7. **Blank**
8. **Pea control**
9. **Yellow lupin control**
10. **Faba bean control**
11. **M 100-1000 standard**

1 2 3 4 5 6 7 8 9 10 11

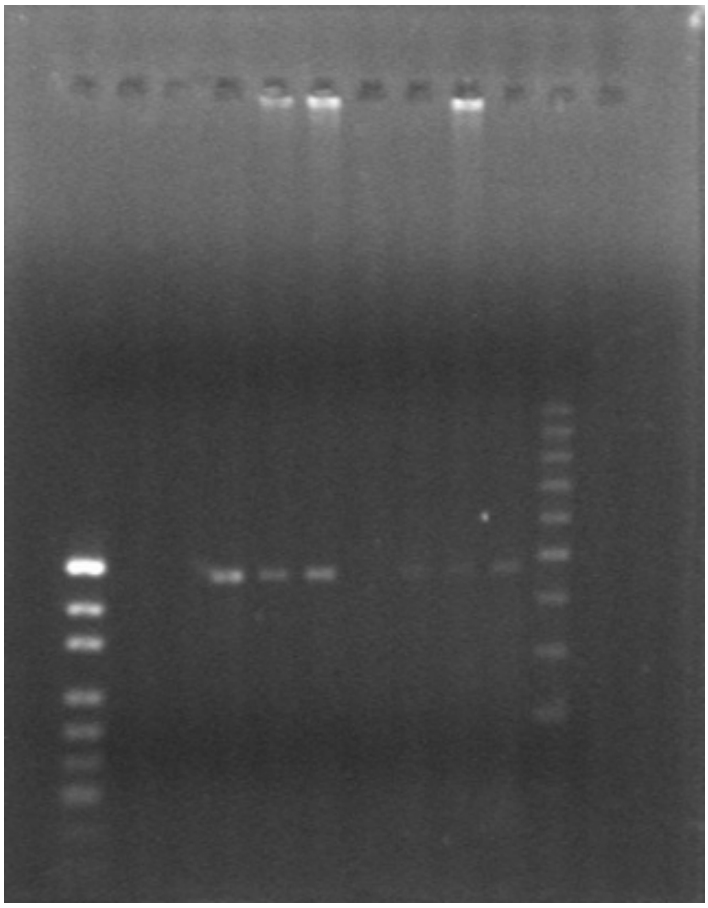

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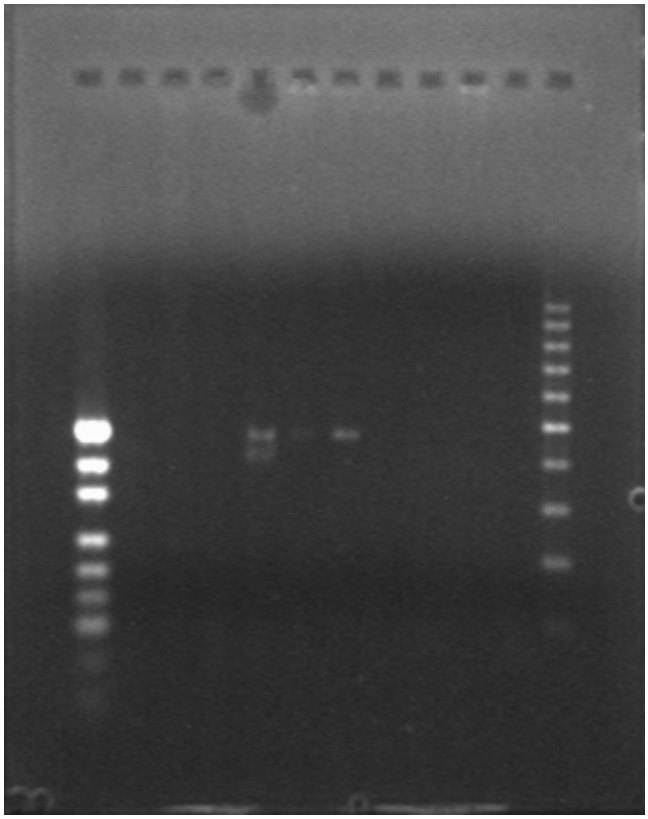
Photo 6. PCR products from leaf plant tissue infected with *F. culmorum* on the 25th day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Yellow lupin infected by seed soaking**
5. **Pea infected with slant**
6. **Yellow lupin infected by seed soaking**
7. **Yellow lupin infected with slant**
8. **Faba bean control**
9. **Pea control**
10. **Yellow lupin control**
11. **Blank**
12. **M 100-1000 standard**

1 2 3 4 5 6 7 8 9 10 11 12



In the tissue of lupin infected by seed soaking, the presence of *F. culmorum* DNA was noted on the successive vegetation days: - 4th (Photo 3), - 16th (Photo 4), - 20th (Photo 5), - 31st (Photo 7), and 34th (Photo 8) day, which coincides with the following phonological phases: beginning of germination, beginning of emergence, full emergence, a few leaf pairs. However in yellow lupin infected with agar slant, the pathogen was identified as late as on the 20th, 25th and 30th day of vegetation over the full emergence phase and at the beginning of leaf formation (Photos 5, 6, and 7). The product size each time it was identified amounted to 472 bp. The results of the present research are confirmed by the data reported by Shilling et al. [4] who, having carried out PCR with the same primers, obtained the product of the corresponding size. In the second series, *F. avenaceum* DNA was identified in pea tissue on the 24th day of vegetation in the leaf-formation phase (Photo 10), while in winter wheat and

winter rye – on the 20th day of vegetation when the plants reached emergence phase – the 3-leaf stage (Photo 9). DNA of *F. avenaceum* was detected neither in yellow lupin nor in triticale. The present product size obtained was also evaluated at about 950 bp. Similarly the DNA from pure culture of *F. avenaceum* was exposed to PCR reaction with fungus-specific SCAR primers. The size of the amplified product was the same (Photo 14). Amplified rDNA of *F. avenaceum* could have been mutated and then fixed, which, in turn, could have resulted in discrepancies in the size of the amplified product (345 bp and 950 bp).

Photo 7. PCR products from leaf plant tissue infected with *F. culmorum* on the 31st day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Pea infected by seed soaking**
5. **Pea infected with slant**
6. **Yellow lupin infected by seed soaking**
7. **Yellow lupin infected with slant**
8. **Faba bean control**
9. **Pea control**
10. **Yellow lupin control**
11. **Blank**
12. **M 100-1000 standard**

1 2 3 4 5 6 7 8 9 10 11 12

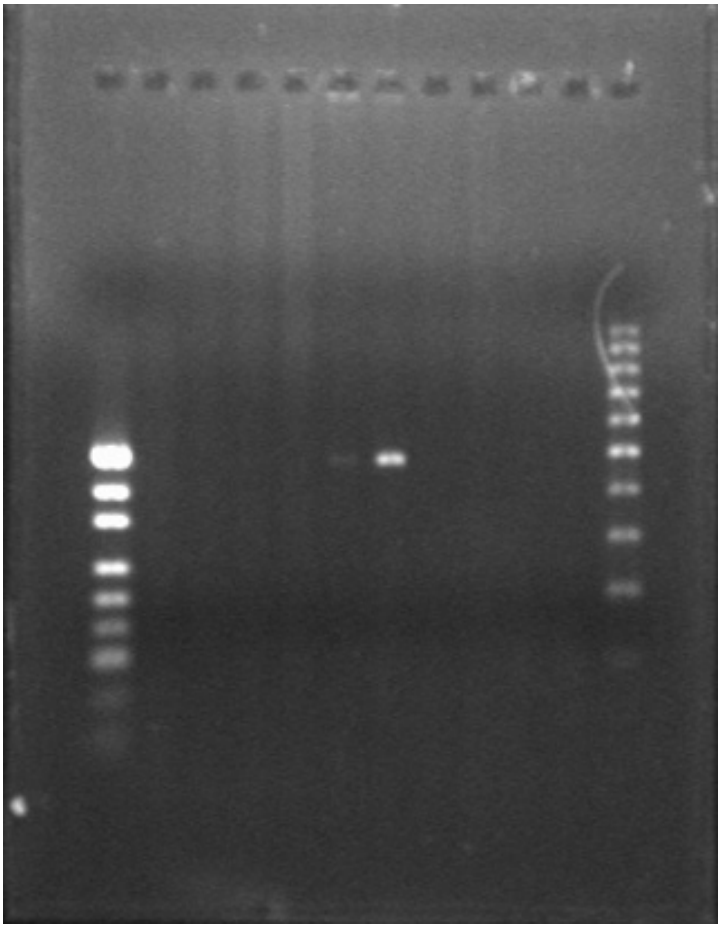


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Photo 8. PCR products from leaf plant tissue infected with *F. culmorum* on the 34th day of vegetation

1. **M1 Standard**
2. **Faba bean infected by seed soaking**
3. **Faba bean infected with slant**
4. **Pea infected by seed soaking**
5. **Pea infected with slant**
6. **Yellow lupin infected by seed soaking**
7. **Yellow lupin infected with slant**
8. **Faba bean control**
9. **Pea control**
10. **Yellow lupin control**
11. **Blank**

1 2 3 4 5 6 7 8 9 10 11 12

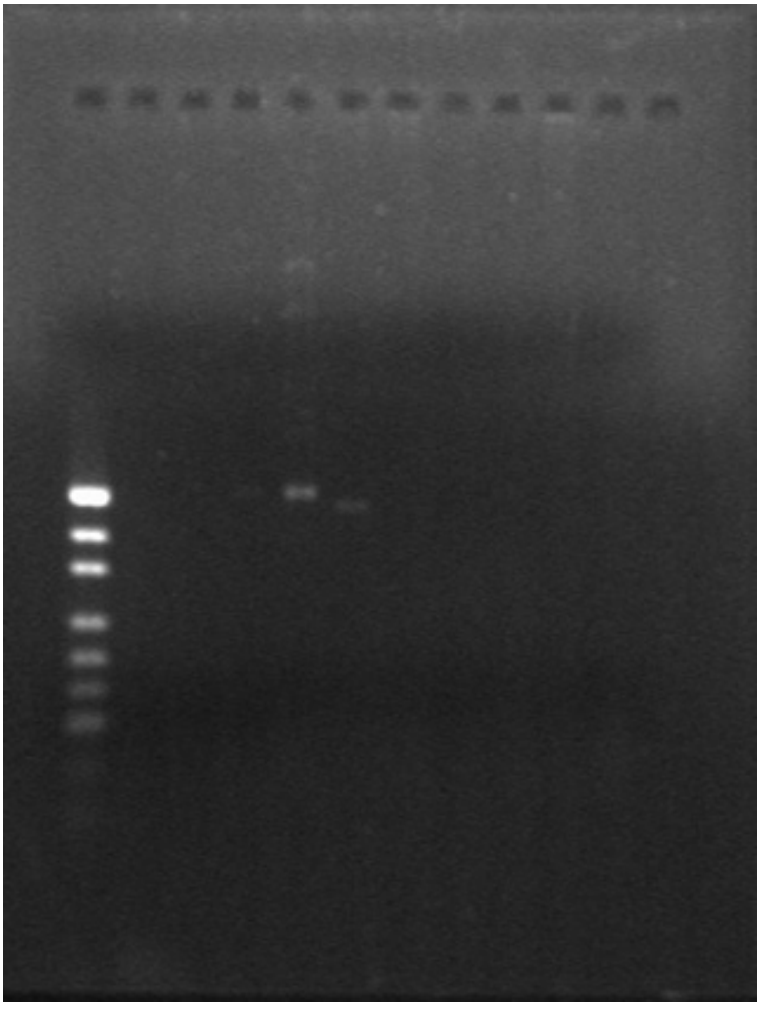

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Photo 9. PCR products from leaf plant tissue infected with *F. avenaceum* on the 20th day of vegetation

1. **M 100-1000 standard**
2. **Pea**
3. **Pea control**
4. **Yellow lupin**
5. **Yellow lupin control**
6. **Winter wheat**
7. **Winter wheat control**
8. **Winter triticale**
9. **Winter triticale control**
10. **Winter rye**
11. **Winter rye control**
12. **Blank**

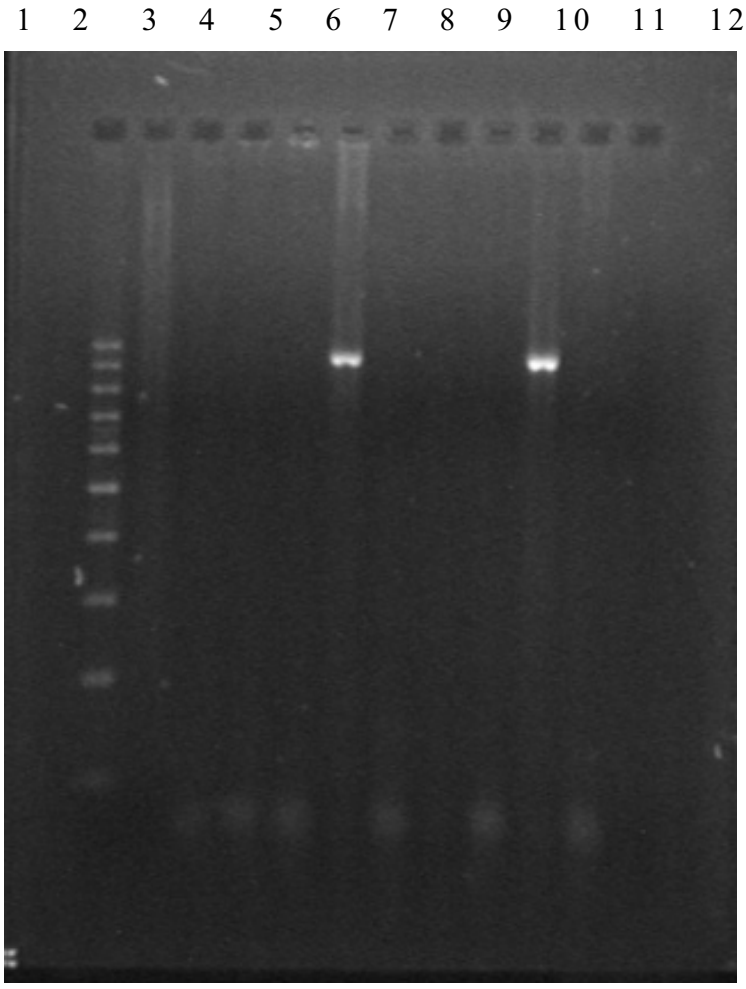

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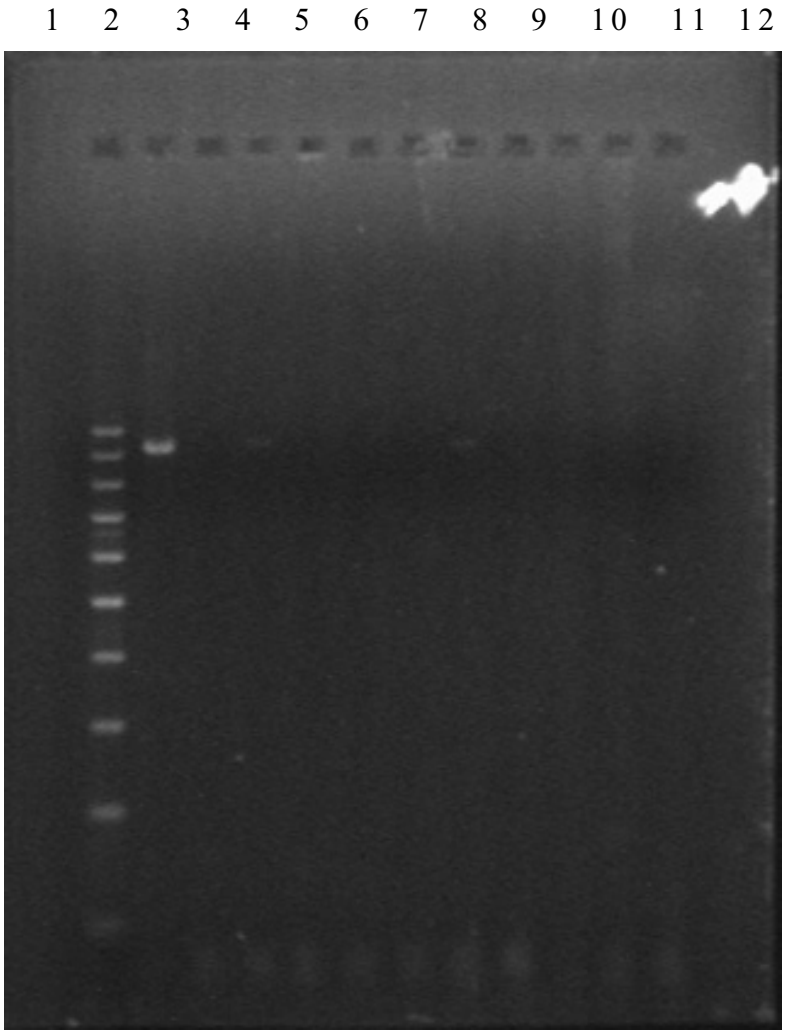
Photo 10. PCR products from leaf plant tissue infected with *F. avenaceum* on the 24th day of vegetation

1. **M 100-1000 standard**
2. **Pea**
3. **Pea control**
4. **Yellow lupin**
5. **Yellow lupin control**
6. **Winter wheat**
7. **Winter wheat control**
8. **Winter triticale**
9. **Winter triticale control**
10. **Winter rye**
11. **Winter rye control**
12. **Blank**



The application of SCAR primers made it possible to identify DNA of *F. culmorum* in the tissue taken from winter rye both on the 14th and 20th day of vegetation in emergence phase, two and three-leaf stage (Photos 11 and 12). In yellow lupin and pea the product was amplified on the 20th day of vegetation, in the initial phase of leaf formation (Photo 12); in pea tissue the product was additionally identified on the 24th day of vegetation, in full leaf-formation phase (Photo 13).

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Photo 11. PCR products from cotyledon plant tissue infected with *F. cumorum* on the 14th day of vegetation

1. **M 100-1000 standard**
2. **Pea**
3. **Pea control**
4. **Yellow lupin**
5. **Yellow lupin control**
6. **Winter wheat**
7. **Winter wheat control**
8. **Winter triticale**
9. **Winter triticale control**
10. **Winter rye**
11. **Winter rye control**
12. **Blank**

1 2 3 4 5 6 7 8 9 10 11 12

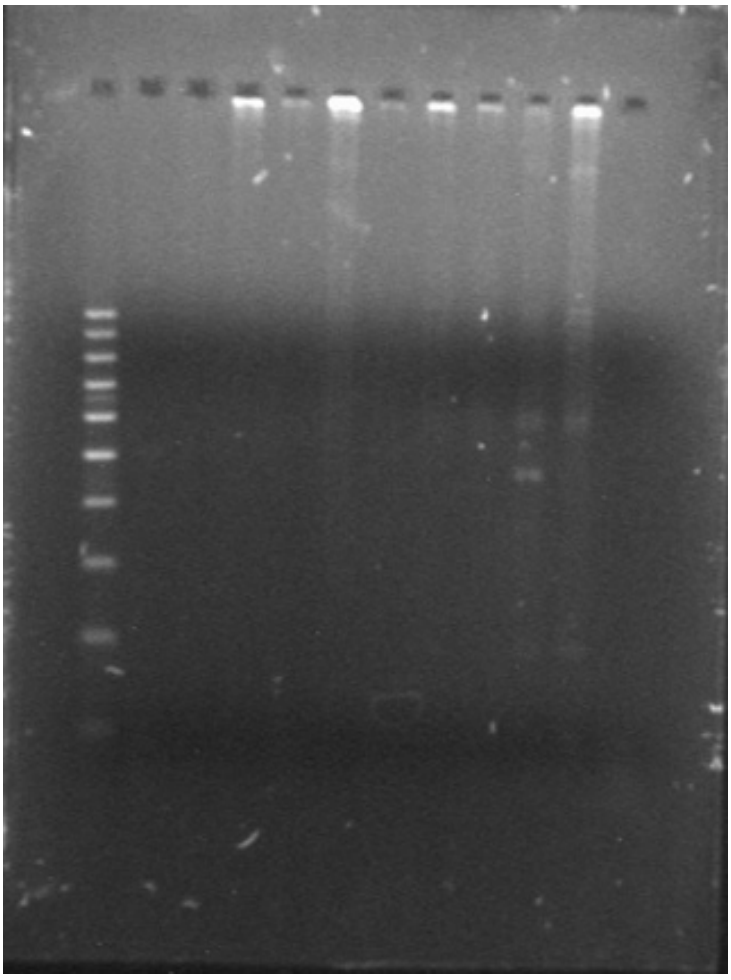

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Photo 12. PCR products from leaf plant tissue infected with *F. culmorum* on the 20th day of vegetation

1. **M 100-1000 standard**
2. **Pea**
3. **Pea control**
4. **Yellow lupin**
5. **Yellow lupin control**
6. **Winter wheat**
7. **Winter wheat control**
8. **Winter triticale**
9. **Winter triticale control**
10. **Winter rye**
11. **Winter rye control**
12. **Blank**

1 2 3 4 5 6 7 8 9 10 11 12

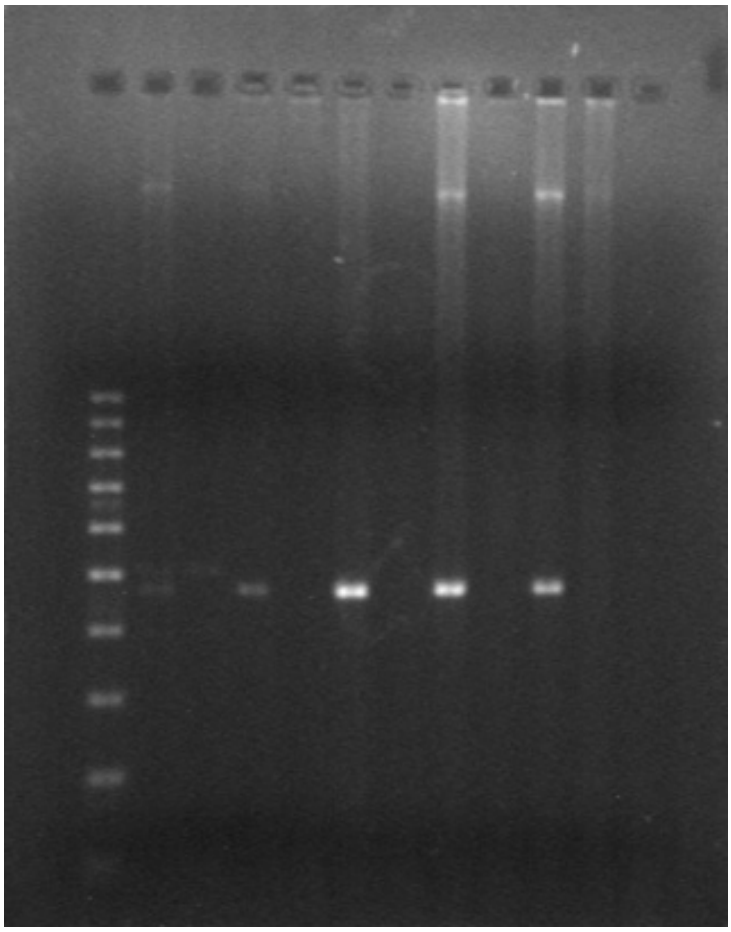

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Photo 13. PCR products from leaf plant tissue infected with *F. culmorum* on the 24th day of vegetation

1. **M 100-1000 standard**
2. **Pea**
3. **Pea control**
4. **Yellow lupin**
5. **Yellow lupin control**
6. **Winter wheat**
7. **Winter wheat control**
8. **Winter triticale**
9. **Winter triticale control**
10. **Winter rye**
11. **Winter rye control**
12. **Blank**

1 2 3 4 5 6 7 8 9 10 11 12

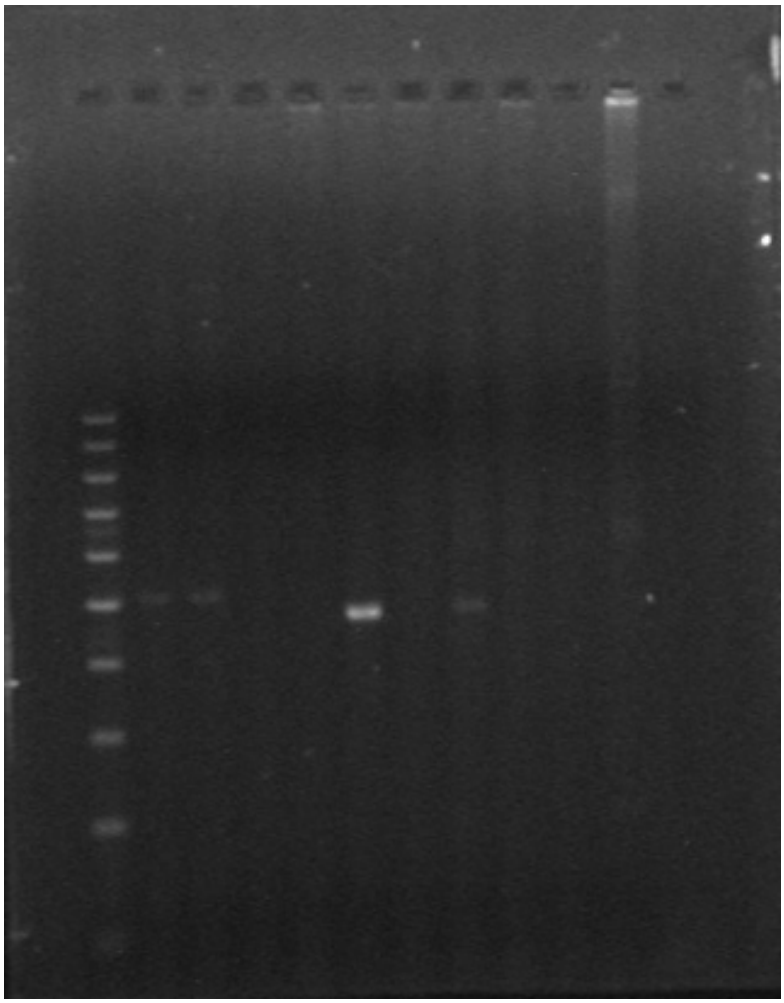

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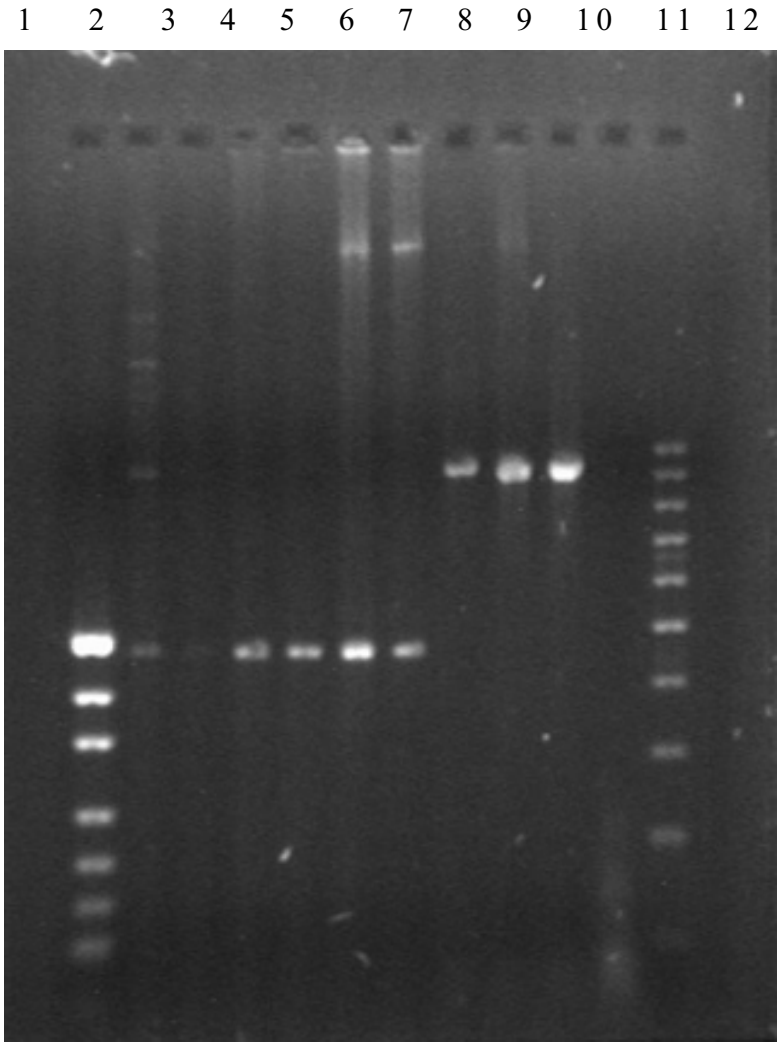
Photo 14. PCR products from plants infected with *F. culmorum* and *F. avenaceum* and from pure fungal cultures

1. **M 1 standard**
2. **Pea infected with *F. culmorum***
3. **Yellow lupin infected with *F. culmorum***
4. ***F. culmorum* culture**
5. **Winter wheat infected with *F. culmorum***
6. **Winter triticale infected with *F. culmorum***
7. **Winter rye infected with *F. culmorum***
8. **Pea infected with *F. avenaceum***
9. **Winter triticale infected with *F. avenaceum***
10. ***F. avenaceum* culture**
11. **Blank**
12. **M 100-1000 standard**



In winter wheat and winter triticale *F. culmorum* was identified on the 20th day of vegetation, in 3-leaf stage and in winter wheat additionally on the 24th day of vegetation (Photos 12 and 13). To confirm the results, the pathogen was isolated from pure marked culture of *F. culmorum* and PCR was carried out with fungus-specific SCAR primers. The product obtained equalled to 472 bp (Photo 14). The negative result obtained for the other

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identification trials could have been due to an inhibitory effect of the PCR e.g. phenols present in plant tissue [3]. For the last few years the resistance of the plant cultivars studied to fusarium disease complex is close to the Cultivar Research Centre standard.

CONCLUSIONS

1. The present results showed the applicability of the PCR method to an early identification of *F. avenaceum* and *F. culmorum* in tissue of some crop species.
2. The application of 5'CAAGCATTGTCGCCACTCTC3' and 5'GTTTGGCTCTACCGGGACTG3' primers to PCR made it possible to identify *F. avenaceum* in faba bean and pea plants on the 6^{th} day after infection (initial seed germination).
3. The application of *F. culmorum*-specific SCAR primers showed amplification product after 14 days of vegetation in winter rye and after 20 days in the other species researched.
4. *F. avenaceum* was identified with the PCR following the plant tissue infection with winter wheat and winter rye spore suspension on the 20^{th} day of vegetation (plant emergence phase). *F. avenaceum,* however, was detected neither in yellow lupin nor in winter triticale.
5. The differences in the size of the *F. avenaceum* product (345 bp and 950 bp) could have been due to a fixed mutation of the rDNA fragment for ITS.
6. The negative result obtained for the other identification trials could have been either due to inhibitors of the PCR common in the plant tissue or due to absence of infection.

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