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# MULTIPLEX-TOUCHDOWN PCR FOR RAPID SIMULTANEOUS DETECTION OF *Rhizoctonia cerealis* AND *Rhizoctonia solani*

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The aim of the study was to develop rapid and sensitive assay for the simultaneous detection of *R. cerealis* and *R. solani*. Pure cultures of fungi were grown on a potato dextrose agar for 5 days at 28 °C, and mycelium was harvested and used for DNA extraction. Total DNA was extracted using a commercial test-systems. Molecular identification of phytopathogenic fungi was performed using a multiplex-touchdown PCR with further electrophoretic separation of amplification products in agarose gel. The specific sequence characterized amplified region primers RtubR4/RtubF4 for *R. cerealis* and ITS1/GMRS–3 for *R. solani* were tested for their specificity and useability in PCR multiplex capacity. The specificity of the multiplex-touchdown PCR was tested using DNA from wide range of fungal species and non-target DNA from healthy wheat. The used primer pairs provided only specific fragments for *R. cerealis* and *R. solani*. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other species. Coupled to this we have optimized the temperature regime for the multiplex PCR protocol. Taken together, our protocol convincingly demonstrated the simultaneous ability to detect *Rhizoctonia cerealis* and *Rhizoctonia solani* and can be used for the diagnosis of compound *Rhizoctonia* root rot.

***Key words***: phytopathogenic fungi, *Rhizoctonia*, multiplex PCR.

Considerable changes came in the pathogenic complex of grain-crops in Ukraine and outside the country during the last decades [1–3]. Intensive technologies mastering resulted in distorting the ecological balance in agrocoenoses, entailed the origin of hearths and growth of distribution of those disease of wheat, that did not have the special value before. The occurrence of these plant diseases is a result of reducing of soil suppression due to the depletion of microbiota [4, 5]. These diseases include collar rot, Fusarium head blight, winter wheat leaf blotch and root rot. Root rot is a group of cereals diseases that affect roots, root portion of the stem, an underground intestine and planting site. Pathogens with necrotrophic phase in life cycle such as soil-borne necrotrophic basidiomycetes including *Rhizoctonia* are responsible for these plant diseases [6, 7]. The sharp eyespot caused by necrotrophic fungus *Rhizoctonia cerealis* van der Hoeven (teleomorph: *Ceratobasidium cereale* D. Murray & L.L. Burpee) is a stem-base wheat disease that is widely distributed in wheat-growing regions worldwide. In Ukraine, sharp eyespot is spread in the steppe and in the south regions of forest-steppe zone [8]. This disease is able to strike more than 230 types of yearling and perennial plants from 66 families, among those the most valuable agricultural crops such as a potato, wheat, barley, rice and other [9]. Sharp eyespot is associated with yield losses due to interference with nutrient and water uptake caused by stem weakening [10, 11]. In the field, rhizoctonia diseases of wheat are frequently caused by at least two soil-borne pathogens fungi, including *R. cerealis* and *R. solani* [12]. *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris*

[Frank] Donk) is another soil-borne necrotrophic fungus causing several wheat diseases, such as collar rot and wet root rot [13, 14]. The severity of disease caused by these pathogens is increased during the last several decades due to global climatic changes, earlier sowing, the use of fungicides etc. Moderate and severe disease can reduce wheat yield substantially and causes deterioration of its quality [15]. In order, to precisely determine yield losses due to these fungi, correct identification of the causal agents is required. In addition, early, rapid, and specific identification of plant pathogens is essential for effective plant disease management. Specific disease diagnosis is also required for timely and proper control measure.

In field conditions, root rot type identification in a correct and timely manner is quite problematic. Visual diagnostics is complicated because of the fact that all pathogens are necrotrophic, and therefore the main symptom that they cause is necrosis. In addition, visible symptoms show up at advanced stage of the disease that is why providing the timeliness of realization of treatments is problematic. Molecular diagnostic technologies based on PCR have been developed for rapid and precise detection of numerous phytopathogenic fungi. However, these methodologies are developed for detecting a single pathogen including those for the identification of *R. cerealis* [15] and *R. solani* [16]. As mentioned above, under field conditions, Rhizoctonia diseases occur primarily as mixed infections. Therefore, the multistep PCR methodology seems to be too time- and labor-consuming, as well as too expensive for practical use. Multiplex PCR technology is more advanced. This approach can simultaneously amplify several primers, thereby decreasing the detection cost and overcoming the drawbacks of single PCR detection. This methodological approach is successfully used for the simultaneous detection of several phytopathogenic fungi that cause compound infections [17]. This study aimed to develop a rapid and sensitive assay for the simultaneous detection of *R. cerealis* and *R. solani*. For this purpose we used touchdown PCR. Touchdown PCR provides a simple and rapid means to optimize PCRs, increasing specificity and sensitivity, without the necessity for optimizations and/or the redesigning of primers [18].

## Materials and Methods

All isolates used in the study are presented in Table 1. Isolates were obtained from the collection of the Laboratory of Mycology and Phytopathology, All-Russian Institute for Plant Protection.

Pure culture of all isolates have been growing on a potato dextrose agar (PDA) for 5 days at 28 °C, and mycelium was harvested and used for genome extraction. The total fungal genomic DNA was extracted using the Agrosorb NK kit (LLC Agrogen Novo, Ukraine). The quantity and purity of extracted DNA were measured using a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, USA). Purity DNA preparations have expected at A260/A280 ratio and DNA concentration is estimated by measuring the absorbance at 260 nm.

For assaying primer specificity, DNA was extracted from healthy plants of wheat (negative control) and from other fungal species (*Alternaria solani, Fusarium culmorum, F. graminearum, F. oxysporum, F. poae, F. cerealis, F. sporotrichioides, F. verticilloides, F. proliferatum, F. avenaceum, F. solani, F. subglutinans, F. tricinctum, F. langsethiae*).

To confirm the correct identification of the *Rhizoctonia* isolates the PCR was performed using the specific Sequence Characterized Amplified Region (SCAR) primers RtubR4/RtubF4 for *R. cerealis* and ITS1/GMRS–3 for *R. solani* (Table 2) [19, 20]. PCR reactions were carried out on a T100™ Thermal Cycler (Bio-Rad Laboratories Ltd., USA) under thermal cycling parameters as follows: initial denaturation at 95 °C for 7 min; then, 39 cycles of denaturation at 94 °C for 30 s, primer annealing at 56–65 °C for 30 s and primer extension at 72 °C for 45 s; finally, final primer extension at 72 °C for 5 minutes. The reaction was conducted in a 10-µl reaction mixture consisting of 3.4 µl ddH2O, 1,7 µl 5x PCR-buffer blue 15 µM $Mg^{2+}$ (AmpliSens, Russia), 0.3 µl 5x PCR-buffer 0 µM $Mg^{2+}$ (AmpliSens, Russia), 1 µl dNTP (1.76 µM of each nucleotide), 1 µl primer mix (0.25 µM of each primer), 0.1 µl Taq polymerase (5 U) (Thermo Scientific, USA), 2.5 µl template DNA. Sterile distilled water was used as a negative control to test for the presence of contamination in PCR reagents.

PCR amplicons were electrophoresed on 2.0% agarose gel stained with ethidium bromide and visualized by UV light (260 nm) and photographed using Bio-Rad Gel DocTM XR+ gel documentation system (Bio-Rad Laboratories Ltd., USA).

*Table 1.* **Isolates of R.cerealis, R.solani and other fungal species used in the study**

| Species | Isolate code | Year and region of Collection | Host Plant | Source |
|---|---|---|---|---|
| *Rhizoctonia cerealis* | -2 | 2016, Kyiv region, Ukraine | Wheat | - |
| *R. solany* | - | 2015, Kyiv region, Ukraine | Potato | - |
| *Alternaria solani* | P 043-021 | 2006, Primorsky region, Russia | Potato | MF1 |
| *Fusarium culmorum* | G 102100 | 2007, Kirov region, Russia | Barley | MF |
| *F. graminearum* | G 58570 | 2014, Krasnodar region, Russia | Wheat | MF |
| *F. oxysporum* | G 58767 | 2015, Leningrad region, Russia | Cucumber | MF |
| *F. poae* | G 102702 | 2007, Kirov region, Russia | Oats | MF |
| *F. cerealis* | G 58694 | 2003, Heilongjiang province, China | Wheat | MF |
| *F. sporotrichioides* | G 163101 | 2011, Krasnodar region, Russia | Wheat | MF |
| *F. tricinctum* | G 58368 | 2010, Krasnodar region, Russia | Wheat | MF |
| *F. verticillioides* | G 59039 | 2016, Almaty region, Kazakhstan | Corn | MF |
| *F. proliferatum* | G 58380 | 2010, Krasnodar region, Russia | Wheat | MF |
| *F. langsethiae* | G 192201 | 2013, Lipetsk region, Russia | Wheat | MF |
| *F. avenaceum* | G 59019 | 2015, Latvia | Wheat | MF |
| *F. solani* | G 90602 | 2007, Leningrad region, Russia | Barley | MF |
| *F. subglutinans* | G 59045 | 2016, Kostanay region, Kazakhstan | Peas | MF |

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*Table 2.* **Primers used in the study**

| Pathogen | Primer | Sequence (5′–3′) | Direction | Source |
|---|---|---|---|---|
| *R. solani* | GMRS-3 | AGT GGA ACC AAG CAT AAC ACT | Reverse | Johanson et. al., 1998 |
| | ITS1 | TCC GTA GGT GAA CCT GCG G | Forward | |
| *R. cerealis* | RtubR4 | GCT AGT GCG GTC AAT GTA TAG | Reverse | Guo et al., 2012 |
| | RtubF4 | CCT AAA TGA GTC TGG AGT AAG TC | Forward | |

## Results and Discussion

PCR method allows detecting the presence of a certain sequence of pathogenic nucleic acid in samples and due to high sensitivity, determines single copies of its genomes. For today, a PCR-analysis is one of the most widespread technologies of laboratory diagnostics that develops dynamically.

The diagnostic PCR assay involves several critical steps, such as DNA extraction from specimens, PCR amplification, and detection of amplicons. The amount of total DNA in a PCR has a marked effect on the outcome of the analysis procedure. Sample overloading would result in packed DNA in the confined space of the reaction vessel and can lead to false priming and even poor DNA synthesis due to the obstructed diffusion of large *Taq* polymerase molecules [21]. Therefore, the final DNA concentration was adjusted to 2.5 ng/µl by sterile deionized water.

For assaying primer specificity DNA was isolated from different fungal species. As a negative control, the non-target DNA, healthy wheat DNA diluted with distilled water (ddH2O) was used.

The primer pair RtubR4/ RtubF4 provided only a single specific fragment of 138 bp for *R. cerealis*. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other species (Fig. 1, *a*).

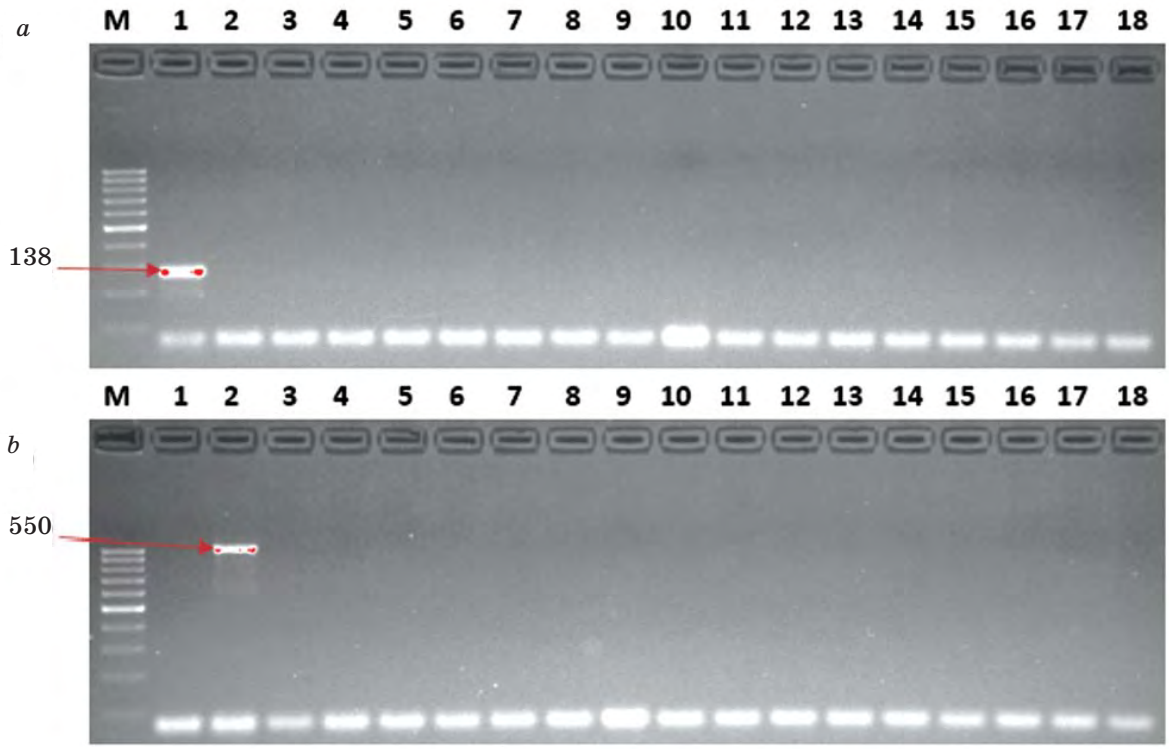


*Fig. 1.* **Agarose gel electrophoresis of the amplification products of target and non-target DNA using species-specific primer sets:**
*a* — RtubR4/RtubF4; ***b*** — ITS1/GMRS–3; M: GeneRuler 50 bp DNA Ladder (IsoGen, Russia).
Lane 1 — *Rhizoctonia cerealis;* lane 2 — *R. solani;* lane 3 — healthy plant; lane 4 — $ddH_2O$; lane 5 — *Alternaria solani;* lane 6 — *Fusarium culmorum;* lane 7 — *F. graminearum;* lane 8 — *F. oxysporum;* lane 9 — *F. poae;* lane 10 — *F. cerealis;* lane 11 — *F. sporotrichioides;* lane 12 — *F. verticilloides;* lane 13 — *F. proliferatum,* lane 14 — *F. avenaceum;* lane 15 — *F. solani;* lane 16 — *F. subglutinans;* lane 17 — *F. tricinctum;* lane 18 — *F. langsethiae*

The PCR reaction with ITS1/GMRS-3 primers confirmed the occurrence of *R. solani*, giving the expected product of amplification 550 bp. Moreover, the specific PCR fragment was amplified from the DNA of *R. solani* isolates, but not from the DNA of other fungal isolates (Fig. 1, *b*).

Multiplex PCR is a valuable tool in many biological studies, but it is a multifaceted procedure that has to be planned and optimised thoroughly to achieve robust and meaningful results [22]. The development of an efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize reaction conditions. For a successful multiplex PCR assay, the relative concentration of the primers, concentration of the PCR buffer, balance between the magnesium chloride and deoxynucleotide concentrations, cycling temperatures, and amount of template DNA and Taq DNA polymerase are important. An optimal combination of annealing temperature and buffer concentration is essential in multiplex PCR to obtain highly specific amplification products [23, 24].

Optimization of the reaction conditions in our experiments included: optimal annealing temperature screening for each pair of primers, determining the optimal composition of the reaction mixture and the number of primers.

Testing different temperature regimes, it was found that oligonucleotide systems respond specifically to DNA from phytopathogenic fungi at the gradient from 59 °C to 62 °C (Fig. 2, *d–g*). In this case, the annealing of the primers in the multiplex reaction did not occur at the temperature gradient from 63 °C to 65 °C (Fig. 2, *a–c*). It also should be noted, that the multiplex system worked at all temperature regimes, but only for the DNA from single pathogen, not for DNA from both fungi.

As the temperature rise, the reaction specificity increases, and, other way around, temperature decrease leads to reaction specificity reduction, but at the same time, increases the PCR products yield. Taking into account, we decided to use the Touchdown PCR method.

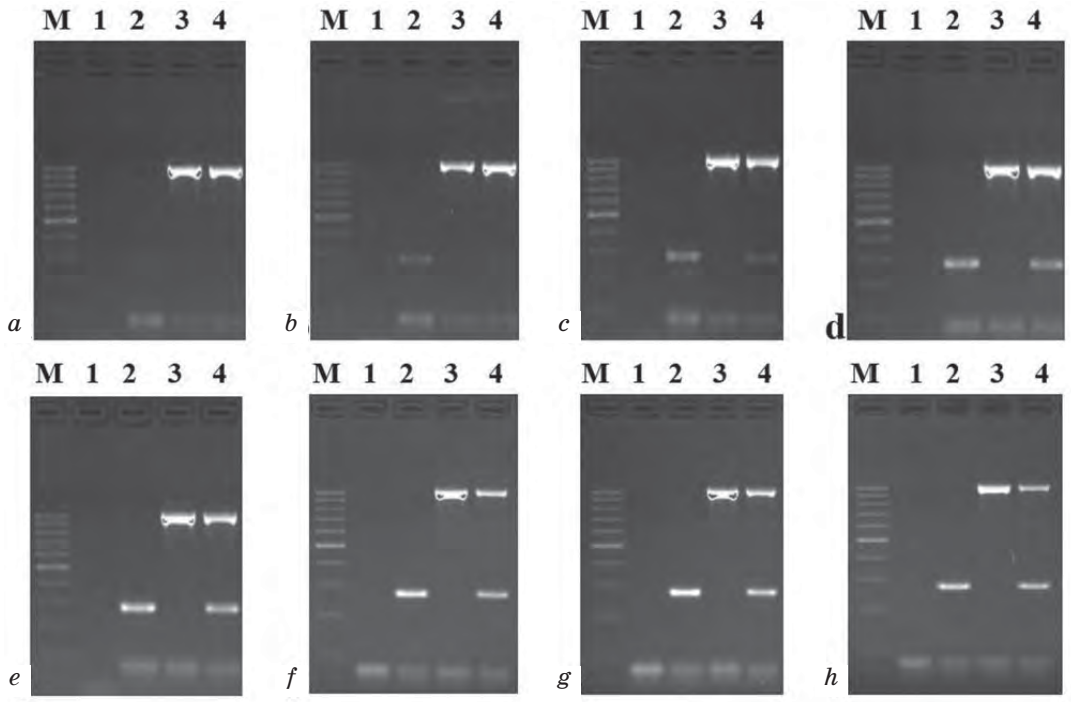


*Fig. 2*. **Effect of different annealing temperature on multiplex PCR detection of *Rhizoctonia* species using species-specific primer sets RtubR4/RtubF4 & ITS1/GMRS–3:**
*a* — annealing temperature 65 °C; *b* — annealing temperature 64 °C; *c* — annealing temperature 63 °C; *d* — annealing temperature 62 °C; *e* — annealing temperature 61 °C; *f* — annealing temperature 60 °C; *g* — annealing temperature 59 °C; *h* — Touchdown (TD) program: the program gradually lowers the hybridization temperature by 1 °C per-cycle increments from 65 °C to 56 °C; M: GeneRuler 50 bp DNA Ladder (IsoGen, Russia). Lane 1 — healthy plant; lane 2 — *Rhizoctonia cerealis;* lane 3 — *R. solani;* lane 4 — DNA mixture of *R. cerealis* & *R. solani*

With this method, the influence of non-specific hybridization of primers on the formation of the product was reduced. Touchdown PCR uses an initial annealing temperature above the projected melting temperature of the primers being used, then progressively transitions to a lower, more permissive annealing temperature over the course of successive cycles. The first cycles in our experiments were carried out at a temperature higher than the annealing temperature, and then the subsequent 10 cycles were performed at the reduced annealing temperature by 1 °C. At that temperature, the oligonucleotide system passed through the band of optimal specificity of the primers to the DNA (Fig. 2, *h*).

Thus, due to the lack of rapid effective methods of detection of causative agents of the diseases affecting the root system, the root portion of the stem and seeds (grain) of wheat, data on their distribution in Ukraine is mostly fragmentary. Based on the inherent difficulties and inaccuracies associated with an attempt to distinguish fungal organisms based on their growth pattern and morphological characteristics, we have optimized multiplex PCR that can detect these fungal pathogens simultaneously.

As a result of research, the PCR conditions (composition of the reaction mixture, amplification conditions) were optimized for multiplex PCR analysis. It has been established that used combination of primers under given conditions does not show cross-reactivity with other phytopathogenic fungi, and can be used for the diagnosis of *Rhizoctonia* root rot, which will allow detecting of two pathogens at ones and reduce the cost of reagents and timing by half.

Optimized multiplex PCR system for identifying *Rhizoctonia cerealis* and *Rhizoctonia solani* from the plant material successfully tested in Syngenta diagnostic centers and allowed us to conduct qualitative analyzes of pathogens among grain crops.

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**Conflicts of Interest:** Authors declare no conflict of interest.

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## МУЛЬТИПЛЕКСНА ТАЧДАУН ПЛР ДЛЯ ШВИДКОГО ОДНОЧАСНОГО ВИЯВЛЕННЯ *Rhizoctonia cerealis* І *Rhizoctonia solani*

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Метою роботи було розробити швидкий і чутливий метод одночасної ідентифікації *R. cerealis* та *R. solani* упродовж однієї реакції. Чисті культури фітопатогенних грибів культивували 5 днів на картопляно-глюкозному агарі за 28 °С, після чого відбирали міцелій та виділяли ДНК за допомогою комерційних тест-наборів. Молекулярну ідентифікацію проводили за допомогою ПЛР з подальшим електрофоретичним розділенням продуктів ампліфікації в агарозному гелі. Специфічні пари праймерів для ділянки з відомою послідовністю: RtubR4/RtubF4 для *R. cerealis* та ITS1/GMRS-3 для *R. solani* тестували щодо їхньої специфічності та можливості застосування у мультиплексному варіанті ПЛР. Для перевірки специфічності праймерів здійснювали аналіз ПЛР з іншими фітопатогенними грибами та з ДНК, виділеною зі здорових рослин пшениці. Встановлено, що обрані нами пари праймерів продукували єдині специфічні фрагменти *R. cerealis* і *R. solani* та не виявляли перехресної специфічності щодо інших фітопатогенних грибів. За допомогою градієнта температур встановлено оптимальні значення температур відпалу праймерів. Таким чином, розроблений нами протокол мультиплексної ПЛР придатний для одночасної ідентифікації *Rhizoctonia cerealis* та *Rhizoctonia solani* протягом однієї реакції і може бути використаний для діагностики поліетіологічних ризоктоніозів.

***Ключові слова:*** фітопатогенні гриби, ризоктонії, мультиплексна ПЛР.

## МУЛЬТИПЛЕКСНАЯ ТАЧДАУН ПЦР ДЛЯ БЫСТРОГО ОДНОВРЕМЕННОГО ВИЯВЛЕНИЯ *Rhizoctonia cerealis* И *Rhizoctonia solani*

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Целью работы было разработать быстрый и чувствительный метод для одновременной идентификации *R. cerealis* и *R. solani* на протяжении одной реакции. Чистые культуры фитопатогенных грибов культивировали 5 дней на картофельно-глюкозном агаре при 28 °С, после чего отбирали мицелий и выделяли ДНК с помощью коммерческих тест-систем. Молекулярную идентификацию осуществляли с помощью ПЦР с последующим электрофоретическим разделением продуктов амплификации в агарозном геле. Специфические пары праймеров для области с известной последовательностью: RtubR4/RtubF4 для *R. cerealis* и *ITS1/GMRS–3* для *R. solani* тестировали относительно их специфичности и возможности использования в мультиплексном варианте ПЦР. Для проверки специфичности праймеров проводили анализ ПЦР с другими фитопатогенными грибами и с ДНК, выделенной из здоровых растений пшеницы. Установлено, что выбранные нами пары праймеров продуцировали единственные специфичные фрагменты *R. cerealis* и *R. solani* и не проявляли перекрестной специфичности относительно других фитопатогенных грибов. С помощью градиента температур установлены оптимальные значения температур отжига праймеров. Разработанный нами протокол мультиплексной ПЦР пригоден для одновременной идентификации *Rhizoctonia cerealis* и *Rhizoctonia solani* на протяжении одной реакции и может быть использован для диагностики полиэтиологических ризоктониозов.

***Ключевые слова:*** фитопатогенные грибы, ризоктонии, мультиплексная ПЦР.