Examination of Biomaterial Samples Obtained from Coffee and Tangerine Plants at Nepal's Plantations Affected by Phytopathogens and Determination of The Efficiency of Their Suppression by The Biofungicide

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ABSTRACT

Infestation of plants with phytopathogenic infections brings great harm to agriculture. This is a global problem. Entire regions lose their business of growing coffee, date palms, various fruits and vegetables, as well as grain crops, fruit trees, shrubs and flowers. These phenomena are especially manifested in places where chemical pesticides, insecticides and herbicides are used in large amounts, where soils are depleted and where there is a lack of moisture. First of all, the weakened plants are susceptible to various diseases due to reduced immunity. This work examines biomaterial samples from infected coffee and tangerine plants taken from Nepal's plantations. Examination was conducted in the BIOIN-NOVO LTD laboratory. The isolated pathogens were purified, their biomass was built up, and molecular genetic examination was performed, the genus and species of the phytopathogens were determined. A collection of biofungicides based on the fungi of Trichoderma genus available at the company was screened, revealing the possibility of efficient suppression of phytopathogens by various strains. An experimental sample of a complex biopreparation was obtained from the most active strains using biotechnology techniques in order to test its efficiency under the conditions of these plants plantations.

Key words: Biopreparations, phytopathogen, plants, plant protection, rot

INTRODUCTION

Contamination of plants with phytopathogenic infections causes damage to agriculture in different countries, amounting to billions of dollars. The direct damage in the form of a lost crop is more than 25 %. Harvest laid for long-term storage becomes unusable due to rotting caused by phytopathogenic fungi.

Pathogens are also a danger to human and animal health. Commercial risk and losses are borne by companies - producers of vegetables, fruits, nuts and coffee. For countries with a cold climate and harsh winter, the maximum loss accounts for the winter storage of the crop. For countries with warm and humid climates, damage is observed during the period of plant cultivation and transportation of crops to consumers over long distances. The main reason for these phenomena is that farmers do not carry out biological protection of plants in the process of their cultivation, as well as the processing of seed and soil before planting. In addition to direct crop losses, it is very important to lose product quality and its safety for health. Thus, in fruits affected by phytopathogenic fungi, mycotoxins accumulate: zearalenone and its derivatives, aflatoxins: B1, B2, G1, ochratoxin, patulin. Company LTD BIOIN-NOVO for many years engaged in the development and production of various biological products. All bio-preparations are developed on the basis of use of microorganisms which extensive collections are available in the company.

MATERIALS AND METHODS

Selection of objects

During the period from February to September 2018, studies of tangerine and coffee pathogens from Nepal were carried out. Coffee Plantation: Eco Friendly Agro Organic Concept Pvt. Ltd, Palungtaar Municipality 9, Dhuwakot, Gorkha District State 4 Nepal; Tangerine Plantation: Gorkha distinct, Sahid Lakhanthapa village, Ghairung.1 phytopathogen was isolated from the object obtained from coffee, and 3 pathogenic fungi were isolated from the tangerines. A collection of fungi of the Trichoderma genus from 64 different strains was scanned in order to identify the most active options for fighting these infections.

Nutrient medium and method of analysis

To isolate the pathogens, agarized Czapek's nutrient medium of the following composition (g/l) was used: sucrose-30.0; $NaNO_3$ -3.0; KH_2PO_4- 1.0; $MgSO_4$ .7 H_2O; KCI-0.5; $FeSO_4$ .7H_2O-0.01; agar-agar-15.0. The objects were grown at T=24–26°C for 5–7 days in a germinator. Microscopic examination of the cultures was carried out with a Micromed microscope at 10x40 magnifications using a squashed drop method and at 10x90 with immersion. To obtain biomass for molecular genetic expertise, the producers were grown for 2-3 days.

DNA was isolated, and sequence analysis was performed. The method of molecular identification was based on Sanger sequencing of ITS1/2 fragment of a ribosomal genes cluster. Regions ITS1 and ITS2 flanking the 5.8S rDNA gene

show significant nucleotide divergence at the cross-species level. Comparison of the sequences obtained with databases (NCBI, ExTaxon, etc.) makes it possible to determine the species of a sample with high probability, so the method is acknowledged as the "gold standard" in identifying the species of fungi. The biological activity was determined using the method of "counter seeding", the time of complete absorption, growth rate and spore production were analyzed.

The prototypes of liquid biofungicide were accumulated using the most active strains identified in the process of analyzing the results of "counter seeding". Each strain was cultivated separately using complex culture media containing three different hydrolyzates of protein raw materials. The cultivation time amounted to 2 days at T=24-26°C. The state of the biomass and the formation of chlamydospores were monitored by the results of microscopic examination. After 48 hours, the cultivation process was completed and the components of the commercial form were added to the culture liquid: a stabilizer, a wetting agent, an adhesive, and a thickener.

RESULTS AND DISCUSSION

The samples of phytopathogens were subjected to both visual and microbiological analysis. Seeding samples onto selective culture media made it possible to detect infection with pathogenic fungi of tangerine and coffee plants. Phytopathogens were identified and then purified, Fig. 1-4. From tangerine, 3 major pathogens were identified, from coffee - 1 pathogen.

As a result of molecular genetic analysis, it was found that tangerine was affected by Aspergillus niger and Fusarium solani, widely known plant pathogens, and coffee was affected by *Cunninghamella echinulata* mucoral fungus. This is a species of mucosal zygomycete fungi, the most well-known species of the *Cunninghamella* genus. It is the heterothallic (diclinous) species. Colonies bred on potato-dextrose agar are whitish, becoming yellowish and smoky-gray as they mature. *Aseptated sporangiophores* (often referred to as conidiophores, since they carry monosporous sporangiola), 16-22 µm thick, dichotomously or corymbiformly branched; each branch ends with a wide claviform or almost spherical knob up to 50 µm in diameter (lateral - up to 30 µm). Sporangiola, spherical or nearly spherical, are formed on very short sterigmoid shoots on these knobs, aculeolate (occasionally smooth), 10-14 µm in diameter. Table 1 shows the results of molecular genetic analysis of coffee and tangerine pathogens. Chlamydospores are formed in the substrate mycelium; they are rare, with various shapes.

Table 1. Results of molecular genetic analysis of coffee and Mandarin pathogens

№ sample's	Sequence	Species affiliation
№ 2 - isolated from Mandarin	GTCACCTGGAAAGAATGGTTGGAAAACGTCGGCAGGCG CCGGCCAATCCTACAGAGCATGTGA CAAAKCCCCATACGCTCGAGGATCGGACGCGGTGCCGCC GCTGCCTTTCGGGCCCGTCCCCCCG GAGAGGGGGACGGCGACCCAACACACAAGCCGGGCTTG AGGGCAGCAATGACGCTCGGACAG GCATGCCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTC AAAGACTCGATGATTCACTGAATTC TGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCA TCGATGCCGGAACCAAGAGATCCAT TGTTGAAAGTTTTAACTGATTGCATTCAATCAACTCAGA CTGCACGCTTTCARACAGTGTTCGTG TTGGGGTCTCCGGCGG	Aspergillus niger
№3- Mandarin,	TCTCCGTTGGTGTACCAGCGGAGGGATCATTACCGAGTT ATTCAACTCMTCAACCCTGTGAACT TACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGTG AAACGGGCCGCCCCCGCCAGAGGA CCSTTAACTCTGTTTCTATAATGTTTCTTCTGAGTAAAAC AAGCAAATAAATTAAAACTTTCAWC AACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGC GAAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG CGCCCGCCAGTATTCTGGCGGGCATG CCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCC TGGCGTTGGGGATCGGCGGAGCGCCC CTCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGTC CCGCCGCAGCTTCCATCGCGTAGTAG CTAACACCTCGCGACTGGAGAGCGGCGCGGCCACGCCGT AAACACCCAACTCTTCTGAAGTGAC CTCGAATCAGGTGAGGC	Fusarium solani
№ 4- Mandarin	CTCCGTTGGTGACCAGCGGAGGGatCATTACCGAGTTATT CAACTCaTCaaCCCTGTGAACTTACCT AAACGTTGCTTCGGCGGGAATAGACGGCCCCGTGAAAC GGGCCGCCCCCGCCAGAGGACCCTTA ACTCTGTTTCTATAATGTTTCTTCTGAGTAAAACAAGCAA ATAAATTAAAACTTTCAACAACGGA TCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATG CGATAAGTAATGTGAATTGCAGAAT TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG CCAGTATTCTGGCGGGCATGCCTGTT CGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGT TGGGGATCGGCGGAGCGCCCCTCGT GGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGCC GCAGCTTCCATCGCGTAGTAGCTAA CACCTCGCGACTGGAGAGCGGCGCGGCCACGCCGTAAA ACACCCAACTCTTCTGAAGTTGACCT	Fusarium solani

	CGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATC	
№ 5- - isolated from coffee	GTGTACCTGCGGAGGWCATTAACTATTTGTGGGGGAAGT ATTCTATTCGAATCTTTCACCATTAA TTCATCCATAATGTGGGTCAAACCACATGCGCAATGTTT TTTTTAAAGGGTTAACTTTCGGGTTAC TACTCTTTATTTATTATAATATGGCCTAAAAAACCATATT ATTAATTTTTTTATACTAAATTTACT AATAAACGATTGACCATAATTTATGGTTGTTTTAAAAAT ATATTAATTTATATAAAAACAACTTT CAGCAATGGATCTCTCGGCTTTCGTATCGATGAAGAACG CAGCAAATCGCGATATTTAATGTGAT CTGCCTATAGTGAATCATCAAATCTTTGAACGCATCTTGC ACCCTATGGTATTCCGTAGGGTACAT CTGTTTCAGTACCATTCAAACATCTCCCTCAATCCTTTTT TTTTTTTTAAAAAAGA	Cunninghamella echinulata

Zygospores are 30-80 µm in diameter, spherical or somewhat flattened, warted. Suspensors are usually almost equal, 20-25 µm long and 10-20 µm in diameter, smooth, and uncoloured.

The results of the analysis of phytopathogenic activity of NIKFAN, F biopreparation using the method of “counter seeding” against these pathogens are presented in Figures 5, 6. Within 5 days, the biofungicide occupies almost the entire surface of the cup, absorbing the pathogen completely.

Biological activity of samples of NIKFAN, F biopreparation was studied. The biopreparation contained a complex of *Trichoderma asperellum* genus fungi, strains No. 16, 20, 27, 32, 47. The biopreparation showed high biological efficiency in suppressing infection. Specifically, complete absorption of the pathogen culture took 5 days.

Figure 1. Infected leaves of coffee

Figure 2. Pathogen extracted from coffee

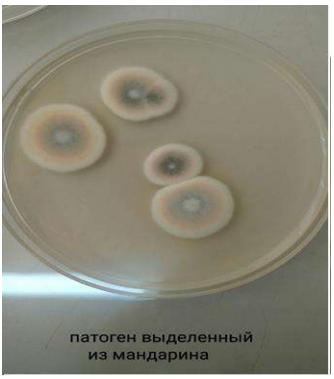

Figure 3. Pathogen extracted from tangerine

Figure 4..Pathogen extracted from tangerine

Figure 5. Effect of fungicide against pathogen in coffee;

Figure 6. Effect of fungicide against pathogen in tangerine

CONCLUSION

As a result of the study performed, pathogens of two plant species were analyzed: coffee and tangerine. Sources of diseases of fungal nature were identified. Molecular genetic examination of the pathogens was carried out, as a result of which the species and genera of these infections were determined. A collection of biofungicide available at the company was screened, and the most efficient producers were identified. An experimental composition of a complex biofungicide was developed that efficiently inhibits the growth and progression of infection on coffee and tangerine. Experimental samples of biofungicide were accumulated. As a result of this work, we may recommend testing experimental samples of NIKFAN, F biofungicide under the conditions of natural growth of coffee and tangerine plants on plantations in order to determine its biological and commercial efficiency.

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