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Characterization of *Cryphonectria parasitica* From Native Chestnut Trees in Kaz Mountain (Mount Ida)

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Abstract: *Cryphonectria parasitica* is a causal agent of chestnut blight worldwide and it dramatically affects natural chestnut trees in Kaz Mountain (Mount Ida). Different biological control approaches have been proposed to constrain the disease. The pathogen has mating system with two alternate idiomorphs which may increase pathogen genetic variability due the recombination events. In this study, *C. parasitica* isolates were diagnosed with partial sequencing of *Translation Elongation Factor-1α* (*Ef-1α*) gene, tested for their pathogenicity, evaluated for their mating types, and the isolates of *Trichoderma* sp. which were isolated from the same lesion where some isolates of *C. parasitica* were obtained were investigated for antagonistic effect on *C. parasitica*. It was not detected any mutation in sequenced part of *Ef-1α* gene. All the isolates were confirmed as pathogen in *in vitro* assays by inoculation to apple fruit. Only one mating type (Mat1-1) was detected for some of the isolates. *Trichoderma* sp. isolates were suppressed *in vitro* growth rate of *C. parasitica.* Both *C. parasitica* and *Trichoderma* sp. caused necrotic symptoms on the fruits once they were inoculated on apple, but, *Trichoderma* sp. did not affect lesion development caused by *C. parasitica*. Overall results present that wild chestnut trees in Kaz Mountain are under threat of chestnut blight pathogens having similar aggressiveness and genetic identity and, isolates of *Trichoderma* sp. obtained in this study should be assessed by *in vivo* experiments for their biocontrol ability against to *C. parasitica*.

Keywords: Chestnut blight, *Ef-1α* gene, pathogenicity, mating type

Kaz (Ida) Dağı'nın Yerel Kestane Ağaçlarındaki *Cryphonectria parasitica*'nın Karakterizasyonu

Öz: *Cryphonectria parasitica* tüm dünyada kestane yanıklığı etmenidir ve Kaz (Ida) Dağı'nda doğal olarak yetişen kestane ağaçlarını da ciddi bir şekilde etkilemektedir. Hastalığı sınırlandırmak için farklı biyolojik kontrol yaklaşımları önerilmiştir. Bu patojen iki alternatif idiomorf ile kontrol edilen eşleme (çiftleşme) sistemine sahiptir ki, bu durum, rekombinasyon yolu ile patojen genetik çeşitliliğini artırabilir. Bu çalışmada, *C. parasitica* izolatlarının translasyon uzama faktörü-1α [*Translation Elongation Factor-1α* (*Ef-1α*)] geninin kısmi baz dizilim verileri üzerinden tanımlaması yapılmış, patojeniteleri test edilmiş, eşleşme tipleri değerlendirilmiş ve bazı *C. parasitica* izolatları ile aynı lezyondan izole edilmiş *Trichoderma* sp. izolatlarının *C. parasitica* izolatlarına antagonistik etkisi araştırılmıştır. *Ef-1α* geninin sekanslanan kısımlarında herhangi bir mutasyona rastlanmamıştır. Elma üzerine yapılan *in vitro* incelemelerde tüm izolatların patojenik olduğu doğrulanmıştır. İzolatlar arasında bir eşleşme tipi (Mat1-1) belirlenmiştir. *Trichoderma* sp. izolatları *in vitro* büyüme oranlarına göre *C. parasitica* izolatınlarını baskılamıştır. Elma üzerine inoküle edildiklerinde ise hem *C. parasitica* hem de *Trichoderma* sp. meyvede nekrotik simptomlara sebep olmuştur, ancak, *Trichoderma* sp., *C. parasitica*'nın lezyon gelişimini etkilememiştir. Tüm sonuçlar göstermektedir ki; Kaz Dağı'ndaki kestane ağaçları, benzer genetik ve patojenik karakterdeki kestane yanıklık patojen izolatlarının tehditi altındadır ve bu çalışmada elde edilen *Trichoderma* sp. izolatlarının *C. parasitica*'ya karşı biyokontrol kabiliyetleri *in vivo* denemelerle de incelenmelidir.

Anahtar Kelimeler: Kestane yanıklığı, *Ef-1α* geni, patojenisite, eşleşme tipi

1. Introduction

Cryphonectria parasitica is a fungal pathogen causing chestnut blight disease which results serious damages to the trees. The pathogen begins initial infection by entering a wound then starting an infection in the cambium and phloem of the tree, then, causes cankers, dieback, and eventually death of above ground parts (Anagnostakis, 1987). Conidia of *C. parasitica* stay alive in the cambium of dead trees for up to 50 years (Anagnostakis, 1987). The disease affects all over the world where chestnut trees (*Castanea sativa* L.) grow and it has been reported from Marmara and Black Sea regions of Turkey (Akilli et al., 2013). Chestnut trees naturally grow in Kaz Mountain (Mount Ida). It has been observed that chestnut blight disease is commonly seen in the trees in that area which threats against to loss of natural resources and prosperity. Recently, the pathogen of *C. parasitica* was reported from oak trees in Greece and the pathogen was identified based on partial sequence data of the internal transcribed spacer (ITS) region, *translation elongation factor-1a* (*EF-1a*) and *beta-tubulin* genes (Tziros et al., 2015).

Introducing resistant varieties was one of the measurement strategies against to the disease, such as crossing the naturally resistant Asian chestnut diversities with the American chestnut (Bernatzky and Mulcahy, 1992). But, the small number of resistance genes did not provide a successful control of the pathogen (Bernatzky and Mulcahy, 1992). Another strategy was based on biological control. For example, *C. parasitica* has a natural enemy, a fungal virus (mycovirus) named as *Cryphonectria hypovirus* (Day et al., 1977). Different subtypes of the virus have been detected. CHV-1 sub-type I was observed in the Marmara and western Black Sea region, whereas sub-type F2 was detected in the eastern part of the Black Sea region (Akilli et al., 2013). Another biological control strategy against to the disease was suggested by using cryptically parasitizing agent *Trichoderma* species which is an Ascomycete fungus found in soil or on decaying wood and widely used in agricultural practices as a biocontrol agent. It has been used to control chestnut blight pathogen in Turkey (Akilli et al., 2011).

Chestnut blight fungus is one of merely a few fungi which has shown to have a mixed mating system (both outcrossing and selfing happen in the same population) in the field (Marra, 1998; Milgroom et al., 1993). Marra and Milgroom (1999) characterized *Mat* locus which controls mating type based on mating type idiomorphs (Mat1-1 and/or Mat1-2) of *C. parasitica* and developed a PCR-based detection of each mating type. In the Black Sea region, fifty two randomly chosen *C. parasitica* isolates were analyzed for mating types. Both mating types of the pathogen was reported from Turkey (Akilli et al., 2013), Croatio (Krstin et al., 2008) and Slovenia (Krstin et al., 2011). On the other hand, only one mating type (Mat1-1) was reported from southern Balkans (Sotirovski et al., 2004). Distribution of mating types of fungal pathogens is one of the important indicator for population genetic diversity and possible recombination events.

In this study, we aimed to characterize *C. parasitica* obtained from Kaz Mountain by using *EF-1α* sequencing, to identify mating types of the isolates of *C. parasitica*, to compare aggressiveness of the isolates of *C. parasitica* and to compare *in vitro* interactions between isolates of *C. parasitica* with isolates of *Trichoderma* sp.

2. Methodology

Fungal Culturing

Bark samples showing orange color necrotic lesions from two different chestnut trees in Kaz Mountain were obtained on October of 2015. The barks were cut into small pieces including fungal stroma and placed onto potato dextrose agar (PDA). Cultures were incubated at 23 °C under dark conditions for 7-10 days. Cultures were morphologically evaluated. Single spore isolations were performed and cultures grown from a single spore were used. Two of the cultures were colorless with no spore production and a hyphal tip was cut and transferred to new PDA plates for these samples instead of doing single spore isolation. Samples were grouped as group I and II according to the tree origin. All

isolates were stored on filter paper at -20 °C for long term storage.

Fungal DNA Isolation

Fungal isolates were cultured in potato dextrose broth (PDB) in a rotary shaker at 150 rpm at room temperature for 5-7 days. Mycelia were harvested by the vacuum filtration and metal beads used for mechanical disruption of tissue. A DNA extraction kit (i-genomic plant DNA extraction kit, Intron Biotechnology) was used for DNA isolation. DNA concentrations were measured by using a spectrophotometer (NanoQuant).

Amplification and Sequencing of *Translation Elongation Factor-1α* Gene

Amplification and partial sequencing of *translation elongation factor-1α* (*Ef-1α*) gene were done for all the isolates obtained. Primers EF986 and EF728 were used (Carbone and Kohn, 1999). Amplification was carried out in 25 µL containing 1 µL DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, primers at 0.2 µM for each, 1x PCR buffer and 1U Taq DNA polymerase. Cycling conditions were 97 °C for 3 min, followed by 35 cycles of 0.5 min at 96 °C, 0.5 min at 56 °C and 1 min at 72 °C, and final extension at 72 °C for 5 min. PCR products were visualized on 1.5% agarose gels, stained with 5 µL/100 mL of nucleic acid staining solution (RedSafe) dye and under a gel documentation system Vilber Lourmat Quantum ST4 1100 (Vilber Lourmat, France). PCR products were sequenced in ABI 3500xL Genetic Analyzer (Applied Biosystems, MedSanTek Lab, Istanbul). After checking and editing DNA sequences with Bioedit v7.0.53 for Windows software (Hall, 1999), each sequence data searched for similarities using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990).

Hyphal Anastomosis

Vegetative compatibility of *C. parasitica* isolates was tested among themselves. Besides *C. parasitica* were placed with *Trichoderma* sp. as dual culture test. Experiments were performed according to Powell (1995) with some modifications. PDA plates were signed to two equal areas and different combinations of the isolates were placed with 4 cm distance. 0.8 cm diameter of fungal mycelia of each 14-day-old isolate was cut and transferred into an area of a PDA plate. The isolates were paired in all possible combinations. Plates were incubated at 23 °C under dark conditions for 7-10 days. Vegetative compatibilities were scored after 14 days incubation which was the day no longer growth of mycelia observed.

Mating Type Analysis

PCR based mating type determination was done according to Marra and Milgroom (1999) and McGuire et al. (2001). Amplification was carried out in 25 µL containing 2 µL DNA, 3 mM $MgCl_2$, 0.2 mM dNTPs, primers at 0.4 µM each, 1x PCR buffer and 1U Taq DNA polymerase. Cycling conditions were optimized as 95 °C for 3 min, followed by 30 cycles of 45 sec at 95 °C, 1.30 min at 58 °C and 4 min at 72 °C, and final extension at 72 °C for 10 min. Amplified DNA fragments were separated by electrophoresis in 1.5% agarose gel including nucleic acid staining solution (5 µL per 100 mL) (RedSafe) for 90 min at volt 90.

Pathogenicity Analysis

Inoculations were performed on apple for pathogenicity tests (Dunn and Boland, 1993). Even though, chestnut is the original host of this pathogen, *in vitro* pathogenicity assays could be done on apple, as well (Dunn and Boland, 1993). Apple cultivar Granny Smith was preferred. All apples were cleaned with distilled water, then, sterilized with a solution of 70% ethanol for 3 minutes, and rinsed with sterile distilled water. One apple was used per each isolate inoculation. Two holes with 1 cm in depth were opened on two different sides of each apple by a cork borer. The same cork borer was used to extract fungal mycelia (0.8 cm in diameter) from 10-day-old colonies in PDA Petri dishes. Cork borer was burned in fire before/after each usage. Samples cut by cork borer were placed into each apple

hole. Each apple was inoculated from two sides with one isolate. The holes of control groups consisted of only PDA media plug. One apple was placed in a different clean plastic box. Experiment was conducted twice. The plastic boxes were placed in a dark incubator at 23 °C for 10 days. Necrotic lesions were measured in cm by ruler on the 7^{th} and 10^{th} days. By using these results, statistical analysis (one-way ANOVA) was performed by using JMP statistical analysis software.

Besides, another pathogenicity experiment was designed to compare lesion developments by *C. parasitica* and *Trichoderma* sp. Two holes were opened on two sides of one apple and one mycelial plug of *C. parasitica* was placed into one hole and one mycelial plug of *Trichoderma* sp. was placed into another hole with four cm distance. Growth of lesions in each side was observed every day and the experiment was ended at day 7.

3. Results and Discussion

Fungal Isolates and Identification Based on *Translation Elongation Factor-1α*

As total 15 isolates of *C. parasitica* were obtained, the 5 from group I (coded as I-14, I-7, I-36, I-20, I-15) and the 10 from group II (coded as II-30, II-24, II-18, II-35, II-17, II-19, II-23, II-22, II-16, II-33). Besides, two morphologically different isolates were isolated from lesion I and these isolates were described as *Trichoderma* according to the *Ef-1α* sequencing and coded as I-31 and I-13. *Trichoderma* isolates grew faster in comparison to *C. parasitica*, probably, because it did not use its own energy for sporulation. We did not able to observe spores of *Trichoderma* isolates. Since *Trichoderma* samples were isolated from chestnut blight lesions and presented colorless colonies with no spore production, it might be confused with hypovirulent strain of *C. parasitica.* But, molecular approach prevented misidentification of these isolates. *Ef-1α* region of the fungal samples were sequenced for species level identification. Each isolate resulted ~400 bp amplicon. Alignment of partial *Ef-1α* sequences (about 300 bp) for the fifteen isolates were monomorphic and showed 100% similarity with *C. parasitica* deposited in NCBI-GenBank (GenBank accession number KC879194.1). Sequencing data for the two other isolates (I-31 and I-13) were matched with *T. atrobrunneum* (for example KJ665382.1, GenBank accession number), *T. harzianum* (for example EF488113.1, GenBank accession number). But, *Trichoderma* isolates were not clearly distinguished at the species level based on this data. *Trichoderma* spp. is known as biological control agent for many plant diseases and species complex in *Trichoderma* spp. have been resolved by using sequencing data of *Ef-1α*, *calmodulin*, *actin* and ITS (Chaverri et al., 2015). *T. atrobrunneum* was also reported as one of the new species from *T. harzianum* species complex (Chaverri et al., 2015). Thus, *Trichoderma* samples obtained in this study which might belong to *T. harzianum* species complex based on *Ef-1α* data, but, it is required more phylogenetic information to define species delimit. Since *Trichoderma* samples were isolated from the same lesion area where isolates of *C. parasitica* were obtained, these two species could present together in the host.

Mating Types of *C. parasitica*

Only mating type 1 was detected among *C. parasitica* isolates (II-18, I-14, I-15, II-19, I-36, I-35, II-33). These isolates gave about 2000 bp amplification with Mat1-1 specific primers and did not give any amplification with Mat1-2 specific primers. The rest of the isolates did not give any amplification either Mat1-1 or MAT1-2 isolates. About mating type primers specific to *C. parasitica,* references provide two different sets of primers with minor modifications (Marra and Milgroom, 1999; McGuire et al. (2001). Both sets were tested and all possible optimizations were applied. But, besides amplification with Mat1-1 primers for certain isolates, Mat1-2 primer pairs did not work at all. Chestnut blight pathogens are mostly known as a clonal population structure worldwide due to founder effect (Milgroom et al., 2008). Besides, both mating types were reported for fifty-two isolates of *C. parasitica* from Marmara and Black Sea regions of Turkey and

ratio of Mat1-1/Mat1-2 was 1.79 (Akilli et al., 2013). Both mating types with 1:1 ratio was also reported for chestnut blight pathogen from Slovenia and Crotia (Krstin et al., 2008; Krstin et al., 2011). But, only one mating type (Mat1-1) was found in from southern Balkans and reported with low diversity for the population (Sotirovski et al., 2004). *Ef-1α* sequences also showed one identical sequences for all isolates. Additionally, isolates were obtained from just two lesion sources. Since all isolates were confirmed as *C. parasitica*, the problem might be because Mat1-2 primers are not universal primers or the rest of the isolates carry some mutations on Mat1-2 primer binding sites. Mating types for *Trichoderma* genus has been studied for *T. reesesi* species which is highly popular in biotechnological applications (Seidl et al., 2009). But, it has not been found any information about mating type locus and specific primers for determination of mating types for *T. harzianum* species complex. Since many different species of *Trichoderma* sp. have been reported as a biocontrol agent in many different studies, characterization of mating types for *Trichoderma* species could be useful in population genetic studies. On the other hand, sexual development may affect power of biocontrol traits in progeny from different mating types of *Trichoderma* spp.

Groups Based on Hyphal Fusion

In compatible pairs, the two mycelia merged completely (Figure 1). Barrages between mycelia or merge of mycelia were also investigated under stereo microscope. Four different groups were distinguished according to the anastomosis (Figure 1). Group I included only three isolates from tree 2 (II-16, II-23 and II-33); group 2 was the largest one including isolates from both tree 1 (I-15, I-36, I-20) and tree 2 (II-19, II-35, II-30, II-24); group 3 included two of the isolate (I-7 and I-14) and the group 4 included just one isolate (II-22) which was not compatible with any other isolates. These observations were made on hyphal fusions among the all possible combinations of the isolates of *C. parasitica.* But, of course, it would be more informative determining vegetative compatible groups based on European vc types (EU type) which are universally used. Six *vic* loci, each locus with two alleles, have been determined to distinguish each vc type (Cortesi and Milgroom, 1998).

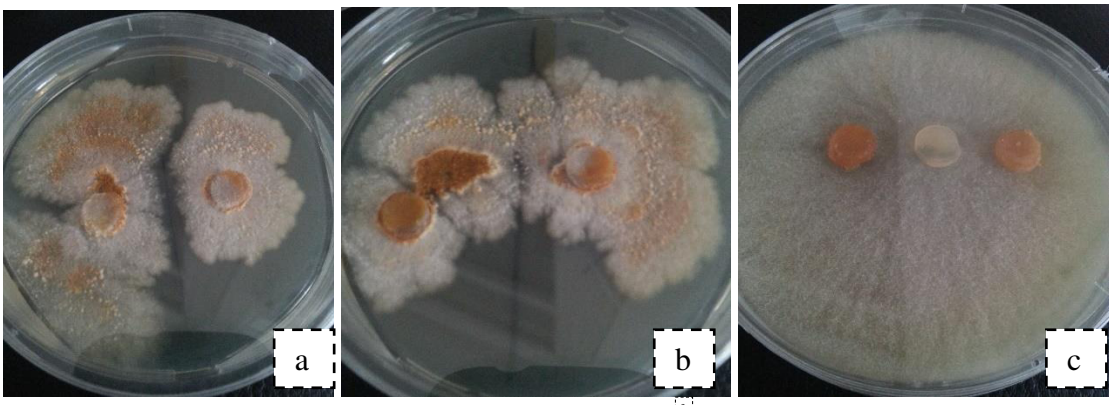

Figure 1. Hyphal anastomosis of pairs of Cryphonectria parasitica isolates as example for (a) different vegetative groups: isolate II-19 and isolate I-14, (b) same vegetative group: isolate I-7 and isolate I-14, and (c) dual test among Cryphonectria parasitica isolates (II-16, II-23) and Trichoderma sp. İsolate

Şekil 1. *Cryphonectria parasitica'nın hifsel anastomoz eşlemelerine örnek olarak (a) farklı vejetatif gruplar: izolat II-19 ve izolat I-14. (b) aynı vejetatif grup: izolat I-7 ve izolat I-14, ve (c) Cryphonectria parasitica izolatları (II-16 ve II-23) ve Trichoderma sp. izolatı arasında ikili test*

In some previous studies from Turkey, isolates of *C. parasitica* from Marmara regions were found as EU-1 vc type (Gurer et al., 2001 a and b; Celiker and Onogur, 2001; Akilli, 2013). Another type (EU-12) was also reported for isolates of *C. parasitica* from Aegean region of Turkey (Celiker and Onogur, 2001). Besides, isolates of *Trichoderma* sp. grew faster compared to isolates of *C. parasitica* and suppressed growth of *C. parasitica* isolates in PDA media (Figure 1). *Trichoderma* sp. isolates prevented growth of wild type *C. parasitica* strains behaving like hypovirulent *C. parasitica* strain. In this study, T*richoderma* sp. was successful in controlling *C. parasitica in vitro* conditions. But, it should be tested by *in vivo* experiments to suggest as a biocontrol agent of *Trichoderma sp.* against to *C. parasitica.*

Pathogenicity Results

All the isolates inoculated to apple caused disease symptoms including necrosis (Figure 2). Lesion sizes from two inoculation sides were 0.17-0.8 cm^2 (average 0.6 cm^2) at day 7 and 0.56-1.9 cm^2 (average 1.36 cm^2) at day 10. The controls did not show any necrotic symptom. Aggressiveness did not show any significant differences among the isolates in the experiments. On the other hand, isolates of *Trichoderma* sp. caused similar symptoms on apples and lesion sizes were 0.6 cm^2 at day 7 and 0.9 cm^2 at day 10. Aggressiveness by considering average lesion sizes was statistically insignificant between these two species. Thus, both *C. parasitica* and *Trichoderma sp.* caused similar necrotic lesions with similar lesion sizes (Figure 2). In a separate experiment, *C. parasitica* and *Trichoderma* sp. were inoculated together on the same apple fruit. Each of the isolate caused symptoms. Lesions developed as similar as each was inoculated solely on apple fruit. Experiment was ended day 7 but continued to observation until day 15. Lesions sizes were 2.2 cm^2 for *C. parasitica* and 2.6 cm^2 for *Trichoderma* sp. Once mycelial discs of *C. parasitica* and *Trichoderma sp.* were placed close to each other on apple, lesions caused by two different species were merged at day 7 and did not stop lesion development of each other. Thus, this experiment indicated that *Trichoderma* sp. could not inhibit disease caused by *C. parasitica* and could not grow faster than *C. parasitica* could on apple. Growth rate of *Trichoderma* sp. was different on the PDA medium and apple fruit.

Figure 2. Disease symptoms on apple caused by Cryphonectria parasitica isolates (a) and C. parasitica and Trichoderma sp. (b)

Şekil 2. *Cryphonectria parasitica izolatları (a) ve (b) C. parasitica ve Trichoderma sp. tarafından neden olan elma üzerindeki hastalık simptomları*

Thus, poverty for biological control between organisms must be tested in *in vivo* experiments due to possible contradictions in artificial and natural environments for pathogen behavior. Few isolates of *Trichoderma* sp. were tested as antagonistic organism against to *C. parasitica in vivo* and found that disease inhibition of the isolates was different and one of these isolates showed the highest inhibition with 68% (Akilli et al., 2011). It seems that *Trichoderma* isolates have diversity to be a good candidate as a biocontrol agent. Thus, besides with *in vitro* and *in vivo* assays, genetic identification is important to characterize isolates of *Trichoderma* sp.

4. Conclusions and Recommendations

Chestnut blight is still one of the important diseases for chestnut trees and it also occurs in wild ecosystems. Even though many attempts were tried to prevent the disease by using hypovirulent strain of the isolates, it did not provide any succeed practically in anywhere. On the other hand, species in *Trichoderma* genus is presented as biocontrol agents in different studies. We isolated *Trichoderma* sp. from the chestnut blight lesions and it also caused similar lesions on apple in comparison to *C. parasitica. Trichoderma* sp. suppressed colony growth of *C. parasitica in vitro*, it caused similar symptoms on apple fruit and did not prevent development of lesions by *C. parasitica* on apples *in vitro* detached fruit experiments. Thus, it is required that, biocontrol ability of these *Trichoderma* isolates to prevent chestnut blight pathogens should be assessed in *in vivo* experiments. On the other hand, it is required detailed phylogenies to resolve species level identifications and which certain species of *Trichoderma* species complex are better candidate for biological control. Moreover, these two species can present same tissue parts of the hosts. We did not detect any hypovirulent strain of *C. parasitica.* This study firstly reports some aspects of chestnut blight pathogens in Kaz Mountain, but includes less number of isolates. The results demonstrated that molecular identification of chestnut blight pathogens, interactions with *Trichoderma* sp. in artificial media and detached fruits *in vitro*, and mating type determination with questioning for Mat1-2 primers. It is required more isolation of the pathogen from chestnut blight lesions from different trees and explore diversity and population genetic structures of the pathogen to develop strategies for disease development.

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