Genetic Analysis of an Emerging Agricultural Pathogen of Turkish Hazelnut

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Abstract

Powdery mildew is a parasite infection among plants. In Turkey, this infection causes decreased product and quality of Turkish hazelnut (*Corylus avellena*) because of the pathogen *Erysiphe corylacearum*. The aim of this project was to understand the genetic structure of the pathogen and establish foundation principles for developing quantitative PCR. To do this, 5 genes found in the fungus but not in plants were evaluated by using PCR. To find suitable conditions for amplification, the temperature and $MgCl_2$ concentration were optimised. Our results showed that 54°C and 0.1 ul - 0.3 ul $MgCl_2$ concentration gave better amplification. Moreover, an effective DNA extraction method was developed and fungus DNA for some of the selected genes was detected.

Keywords: Powdery mildew, *Erysiphe corylacearum,* Turkish hazelnut (*Corylus avellena*)

Introduction

An agricultural pathogen causes powdery mildew. It is a pathogenic fungal infection on the stem, leaf, and fruit of plants (Aghayeva et al., 2018). Infection is spread quickly among plants because fungi provide reproduction via spores. Wind, insects and sometimes humans can cause the spread of spores. Geographic conditions play a significant role in the proliferation of fungi spores (Altın, 2017). Pathogens of powdery mildew spread in a humid and warm environment, which is suitable conditions for fungal reproduction. When a pathogen penetrates the epidermal tissue of a plant, they benefit from the food of host plant (Li, 2012). This causes slow growth in the plants. In addition to this, quality and amount of product starts to decrease. This pathogenic disease is caused by fungi of the genera *Phyllactinia*, *Erysiphe*, *Leveillula*, *Podosphaera* which belong to order of Erysiphales within Ascomycetes (Esser, 2009). Not all plants have this disease, but most of the plant species are affected by powdery mildew. Apple, grapes, cucumbers, peas, grasses, cereal crops, roses and English oak are examples of plants which have powdery mildew disease (Severoğlu, 2012). Beside these species, hazel powdery mildew is not well researched in spite of its economic importance. Hazelnut *(Corylus avellana)* has high economic value in Turkey. Düzce, Sakarya, and Giresun are the most efficient cities for production of hazelnut in Turkey (Altay, 2010). As in other plants that have this disease, the yield in badly affected Turkish hazelnut decreases dramatically. To find a solution for powdery mildew on hazelnut, some genetic analysis has been conducted to illuminate the genetic structure of the pathogen. According to the literature, there are two major types of fungus belong to Erysiphales order that led to powdery mildew on hazelnut. These are *Phyllactinia* and *Erysiphe sp*. The research in the Washington State University has shown that the species *Phyllactinia guttata* causes powdery mildew on hazelnut tree in the USA (Hartney et al., 2005). The recent study has shown that outside this genus, the species *Erysiphe corylacearum* has caused powdery mildew on *Corylus avellana* in Turkey (Sezer et al., 2017*)*.

To understand why *Erysiphe corylacearum* is the reason for powdery mildew on hazelnut, first of all, its life cycle is analyzed. The significant symptom for powdery mildew is a pale, whitish mould surface on the leaf. In growing powdery mildew samples, a cleistothecium structure of sexual stage is also produced. *Erysiphe corylacearum* fungus produces spores in 2 ways. Asexual spores are called conidiospore. They produce via mitosis so all the conidiospores are clones. Sexual spores are called ascospore. Both of them have same primary mission starting with germination on the leaf surface. The germination occurs under favourable conditions, which are moist and warm temperature for the spores (Takamatsu et al., 2015). Having 2 different types of spore is beneficial for *Erysiphe corylacearum*. With the season change, clonal conidiospores don't respond to variable environmental conditions because each new conidiospore has the same genome as their parents (Scholler et al., 2012). They will not be able to demonstrate survival skills in variable weather conditions. On the other hand, ascospores are formed via meiosis so, in harsh winter and drought conditions, ascospores show resistance. Actually, this resistance originates from its genetic makeup. Germinated spores form fibre-like structures called hyphae. Specialized hyphae are called haustoria and the network of hyphae is named mycelium and these 2 structures penetrate and inhibit the growth of host plant by taking its nutrients (Yang et al., 2018).

When the plant's immune system is shown resistance for pathogens, ascocarp which keeps ascospores inside generates recombinant spores at each time. In the developing phase of the disease, ascocarp forms the bubble-shaped cleistothecium structure (Arzanlou, et al., 2018). According to the analysis of the morphology of cleistothecium, its appendages become different from genus to genus (Braun & Cook, 2012). This knowledge shows whether *Erysiphe corylacearum* or another pathogen is causing powdery mildew disease by using morphological diversity.

This distinction also can be made by using the genetic analysis methods. This project was done to increase our knowledge about *Erysiphe corylacearum*. In this way, optimisations were tested to set up the foundation of quantitative PCR method in order to monitor rapidly which fungus species are causing powdery mildew disease on hazelnut tree. It is also desired to contribute to the development of the known sequence by looking at selected genes of *Erysiphe corylacearum*. The selected ADE13, GPI8, GUK1, DIB1 and RNR2 genes played a vital role at genetic analysis steps. However, while some gene products were detected, the data obtained was inconclusive about the nature of these genes in *Erysiphe corylacearum*.

Material and Methods

1. Morphological Observation of Powdery Mildew Disease

Collected dry powdery mildew hazelnut leaves from the Black Sea region of Turkey were examined under the dissection microscope. To observe the morphological structure of asexual and also sexual spores light microscope was used. Their pictures were taken under different magnification rates. A scale bar was added to pictures for calibration. Fresh healthy and unhealthy hazelnut leaves cultivated at SUNUM in Sabanci University were examined by naked eye and microscopically. Healthy and powdery mildew leaves were grown in the agarose to easily reach the fungi DNA and make DNA extractions. 1.2 % agar gel (Green, 2014) was prepared by following the protocol for 4 petri dishes. Two healthy hazelnut leaves were washed with bleach inside the petri dish at the fume hood. The healthy and powdery mildew hazelnut leaves rubbed against each other. Two healthy leaves were placed into petri dishes. A powdery mildew leaf was rubbed against the agar gel. Another powdery mildew leaf was directly placed into the agar gel.

2. DNA Extraction

To obtain fungus DNA from the hazelnut leaves, DNA extraction was carried out for fresh and dry leaves. Two different kits were applied. Young fresh hazelnut leaves were collected from SUNUM. Leaves were classified according to density of powdery mildew on the leaf surface such as high, low and none. The first, Plant/Seed DNA kit was used (Zymo Research, Irvine, CA, USA). By following the protocol, this kit was applied to 3 collection tubes that were contained nearly 700 mg high, low and none powdery mildew leaves. The second kit which was the DNA Purification Nucleospin Plant II (Macherey Nagel, Düren, Germany) was used,then. In the first application of the second kit, collected six fresh hazelnut leaves were divided into 2 groups which were high, low and none, again. By following plant protocol of DNA Purification Nucleospin Plant II (Macherey Nagel, Düren, Germany), steps were applied for first three samples which were 500 mg. For second group, genomic DNA of fungi protocol of same kit was applied.

The main difference between them, 100 ul chloroform was added to second group. In addition to fresh leaves protocols, for dry leaves, some revisions were made at second kit. Ten dry hazelnut leaves from Sakarya, Cumayeri and Akçakoca were used. Leaves were weighed approximately 40 mg. Liquid nitrogen was taken and samples were placed inside it. A 5 mm steel bead was added to each test tube. A TissueLyser II (Qiagen, Hilden, Germany) device was used for physical destruction at 30 rpm for 20 seconds. 600 ul PL1 Buffer and 15 ul RNAase were added. Ten samples were placed to 65°C heater for 45 minutes. 1500 ul was added for PC buffer, 700 ul sample was transferred new collection tube three times. Samples were held 11,000 g centrifuge at 1 min. 400 ul PW1 buffer was added to collection tubes.Respectively, 700 and 200 ul PW2 buffer was added. 25 ul of 65°C of PE buffer was loaded. Collection tubes were incubated in 65°C heater at 5 min twice. NanoDrop 2000C spectrophotometer was used to check DNA concentration. To visualise samples of DNA extraction, 1% agarose gel was prepared for electrophoresis process. 50 grams agarose was weighted for 50 ml TBE. This mixture was placed to microwave for 1.30 min to mix ingredients. 1 ul GelRed (Nucleic Acid Gel Stain) was added to agarose solution. 0.5x TBE buffer was used. 5 ul samples and 1 ul loading dye were placed into parafilm. After mixing them, they were loaded to well, respectively. 1 ul ladder was also placed to well. During 20 minutes, samples were run in gel electrophoresis at 100 voltage. After that time, samples were put in a Gel Doc EZ Imager (Bio-Rad, California, United States) to visualise samples by using Image Lab program.

3. PCR and Nested PCR Amplification

Before setting up PCR, primers were designed. To design primers, genes which exist in the kingdom of fungus but not in plants were searched for using the NCBI gene database (https://www.ncbi.nlm.nih.gov). Criteria for choosing genes was not to see any living thing of the plant species in the taxonomic groups section on the NCBI website. Because the sequences of the selected genes are unknown for *Erysiphe corylacerum, Erysiphe necator* was used for comparison in the EnsemblFungi BLAST server (https://fungi.ensembl.org/Multi/Tools/Blast?db=core). Five genes which had few hits were selected from this program. Few hits were preferred to work the specific region. To obtain primer sequences, the Primer3web (version 4.1.0) program (http://primer3.ut.ee) was used. Some adjustments, which were 0 hairpin value, > 40% GC content, 24 bp primer length and 60°C melting temperature were arranged. Thus, forward and reverse primers were designed from each gene for PCR process.

For PCR amplification, 20 ul Master mix was used for each sample by adding 2 ul Taq Buffer, 2 ul from 2 mM dNTP mix , 0.5 ul forward and reverse primers, 0.1 ul Maximo Taq DNA polymerase and to complete 20 ul, necessary amount nuclease-free water was added. 1 ul DNA was separately added to PCR tubes. PCR amplification was carried out by following conditions: 95°C at 5 minutes for beginning denaturation, 34 cycles of 15 s at 95°C, 25 s at 60°C, 45 s at 72°C in the Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). For taken samples from PCR, electrophoresis was applied with 1.2 % agarose gel at 25 minutes.

In addition to this PCR method for fresh leaves, nested PCR was used to check the presence of fungus DNA at the 10 dry hazelnut leaves from the Black Sea region. The nested PCR was applied with primers PM3, TW14 and NL1, which amplify part of the ribosomal RNA gene in the fungus. By using 2 ul 10x reaction Buffer, 0.4 ul of 10 mM dNTP, 0.4 ul of 10 mM forward and reverse primers, 0.1 ul Maximo Taq polymerase and 1 ul genomic DNA, master mix was prepared for this gene. The process occurred at this conditions; 95 °C at 5 min for denaturation; 16 cycles of 15 s at 95 °C, 25 s at 60 °C, 45 s at 72 °C and 9 cycles of 15 s at 95 °C, 25 s at 52°C, 45 s at 72 °C. The first product of nested PCR was examined at the 1% agarose gel electrophoresis. For the second step of nested PCR, same conditions of first step were applied. Only 2 ul template DNA was used and instead of 52°C, 55°C was arranged for annealing temperature. Separately, another part of the rRNA gene was amplified at the 58 °C annealing temperature by using primers PMITS1 and PMITS2 (Cunnington et al., 2003). Both samples were run on a 1% agarose gel for 25 minutes.

4. Optimisations at PCR Amplification

Optimisations were made for the temperature and $MgCl_2$ concentration in the selected genes which showed proper results in the PCR amplification. For the first group of temperature, Gradient PCR was performed with same protocol for the ADE13 and GPI8 genes by adjusting the temperature to 53°C , 54.6°C , 55.8°C , 59.8°C , 61°C and 63 °C. For second group of temperature, GUK1, RNR2 and DIB1 genes were used at the 52.1°C, 53.3°C, 54.2°C, 56.4°C, 58.5°C and 60°C. For the DNA sample of RNR2, GM3 gene was used because the initial DNA sample of RNR2 was not enough. Control groups were used for both groups. At the $MgCl_2$ optimisation, high powdery mildew leaf sample of ADE13, low powdery mildew leaf sample of GPI8, none powdery mildew leaf sample of RNR2 and high powdery mildew leaf sample of DIB1 were used. The volume of $MgCl_2$ was increased from 0 ul to 0.5 ul by adding 0.1 ul for 6 PCR tubes of each gene. For developing the quantitive PCR, both optimisations were combined with their best value. This process was carried out with ADE13, GPI8 and DIB1 gene. 0.1 ul , 0.2 ul and 0.2 ul $MgCl_2$ have added 3 PCR tubes for each gene at the 54°C, respectively. Last PCR tube was a control group. 50 ul master mix was prepared with the same contents as 20 ul but all volumes multiplied by 2.5; only the DNA template stayed at 1 ul. To make DNA purification after this process, 45 ul samples for PCR purification and 5 ul samples for agarose gel purification were loaded to agarose gel to check the presence of DNA. Gel Doc EZ Imager (Bio-Rad, California, United States) instrument was used to visualize PCR result.

5. PCR Purification

The rest of the samples from the 50 ul of ADE13, GPI8 and DIB1 genes were completed to 100 ul volume by adding water. NucleoSpin Gel and PCR Clean-up (Macherey Nagel, Düren, Germany) were applied by following the protocol of PCR purification.

Results

1. Morphological of Powdery Mildew Pathogens

In healthy leaves near SUNUM, no trace was observed of powdery mildew, rather redness caused by heat stress (Fig. 1a). Distinguishing unhealthy leaves from healthy ones was easy because of whitish marks on the leaf surface at the beginning of disease (Fig. 1b). In the later stages of it, tiny black spots were seen on the surface of hazelnut leaf from a certain distance. When the unhealthy hazelnut leaves were taken, reticular white structures were examined under the microscope (Fig. 1c). These reticular mycelium structures penetrate epidermal openings of the plants. It was observed that the hazelnut leaves began drying because of spreading of these structures on the plant surface. The following process, the dry leaves began to fall. As a result of examination of powdery mildew leaves under the dissection microscope, asexual spores (conidiospores) were seen. Their shape was long, nodular and branched (Fig. 1d). It was observed that long conidiospores merged with each other to form more complex structures. The structure of tiny black points, which is cleistothecium, were rarely seen. The seen cleistothecium was usually close to the vascular system of the plant. At some place of the hazelnut leaf, the mature cleistothecium was black in colour and orange colour was observed for the immature form of them (Fig. 1e). At high magnification ratio, the long, transparent appendages above the cleithoceium were examined. This structure has various shapes and orientations for different types of fungal pathogens. After morphological examination of cleistothecium with other pathogens, it was confirmed that it is *Erysiphe corylacearum* (Braun & Cook, 2012).

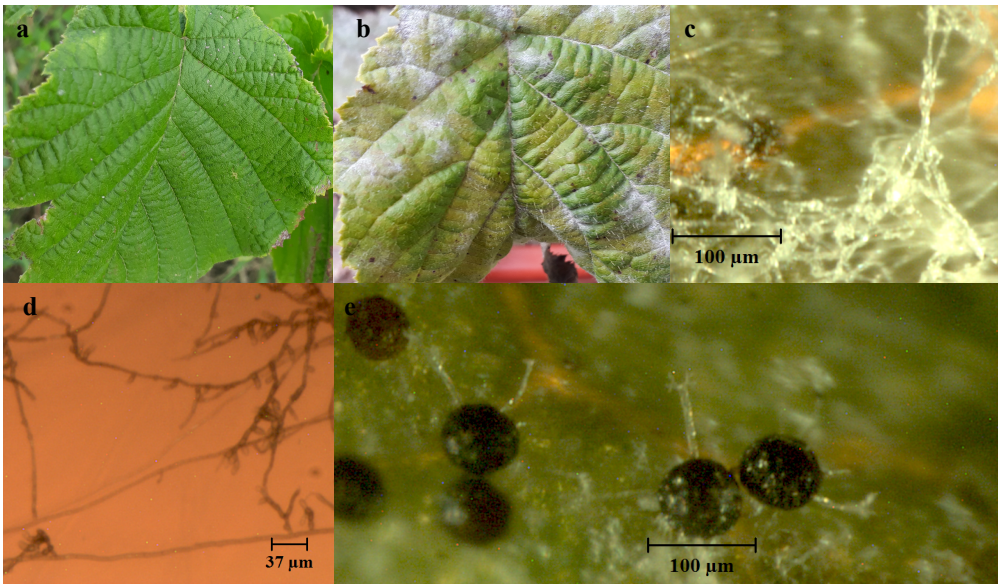

Figure 1: Progression of powdery mildew; a.Healthy leaf , b.Powdery mildew leaf, c.Mycelium structure, d.The asexual spore conidiospore e.The sexual spore of cleistothecium.

2. DNA Extraction

When the first kit was used for DNA extraction from the powdery mildew leaves, no DNA was observed on the agarose gel. The application of the second kit for fresh leaves regardless of whether there was a chloroform or not in the samples, DNA was observed only for high powdery mildew samples (Fig. 2). It also showed that when chloroform was added, the DNA was degraded and broken into small pieces. In addition, higher DNA yield could not be obtained when chloroform was present in samples.

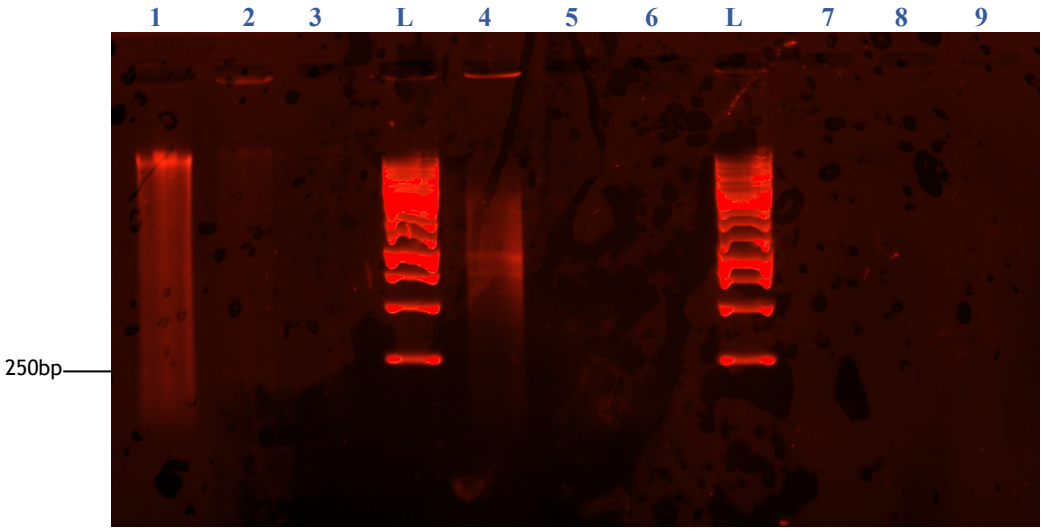

Figure 2: DNA extraction results from fresh hazelnut leaves. Bands 1, 4, 7: High powdery mildew leaf, Bands 2, 5, 8: Low powdery mildew leaf, Bands 3, 6, 9: None powdery mildew leaf. L: Ladder of molecular size (1 kb).

However, when the DNA of dry leaves from the Black Sea region was examined at the gel electrophoresis after DNA extraction, it can be said that the samples had higher bright bands. The DNA extraction from dry leaves was more successful than fresh leaves. Also, this inference was supported by nucleic acid value. The highest nucleic acid concentration belonged the control group. To guarantee DNA extraction purity, it is expected that the DNA absorption ratio A260/280 should be close to 1.80. Sample from Cumayeri region was close to value of 1.8 (Table 1). An emission value of 260/230 is also expected to be 2 but this ratio was not observed for any samples. Even though nucleic acid concentration of the dry sample was high, the valid 260 nm - 280 nm wavelength for DNA was not observed. Instead of this range, the wavelength was seen between 210 nm-220 nm.

Table 1: Nucleic acid concentrations and absorbance value of dry hazelnut leaves which collected from different area and time. **C**: Cumayeri, **S:** Sakarya, **A:** Akçakoca, **PM-:** Control group.

#	Name of Sample	N.acid(ng/ul)	A260(abs)	A280(abs)	260/280	260/230
1	09.05.18- C, P.M-	74.7	1.494	0.841	1.78	0.15
2	09.05.18- A, P.M-	52.8	1.056	0.658	1.60	0.08
3	06.06.18- A	47.6	0.952	0.682	1.40	0.11
4	09.05.18- A	39.8	0.795	0.444	1.79	0.12
5	06.08.18- C	33.0	0.661	0.470	1.41	0.05
6	09.05.18- C	23.8	0.476	0.293	1.62	0.04
7	24.05.18- A	48.7	0.975	0.484	2.02	0.11
8	24.05.18- C	27.2	0.543	0.300	1.81	0.05
9	24.05.18- S	24.1	0.481	0.290	1.66	0.06
10	11.06.18- S	31.6	0.633	0.372	1.70	0.05

By using selected 5 genes which are ADE13, GUK1, GPI8, RNR2 and DIB1 at the appropriate programs, forward and reverse primer sequence for each gene was obtained (Table 2).

Table 2: Forward and reverse primer sequences of each selected gene is shown. Other parameters also indicated.

	Name of Gene	Start	Length	Tm	GC%	Hairpin	Sequence (5'-3')
	Forward Primer	42	24	58.95	33.33	0	AACATCGCGATTTTGGTTCAATTA
GPI8	Reverse Primer	201	24	59.02	41.67	0	GCTATAAACAGTTCCAGGAAAAGC
	Product Size:733						
	Forward Primer	242	24	58.97	37.50	0	CAATGCGTTGTGAAAGAATCTGTA
ADE13	Reverse Primer	541	24	59.07	41.67	0	CTTGTCGAGATGCACCTTTAGATA
	Product Size: 729						
	Forward Primer	59	24	59.01	37.50	0	TACTTACGCGTTATCCATCTGTTT
GUK1	Reverse Primer	302	24	59.04	41.67	0	TCCATCTCTATATCAAGAACCGGA
	Product Size:386						
	Forward Primer	703	24	58.78	41.67	0	CATTTGAAGCACAGACCATCTAAG
RNR2	Reverse Primer	943	24	58.85	37.50	0	CTTCCCAGCCAAAGAGATATTTTC
	Product Size:241						
	Forward Primer	65	24	59.01	41.67	0	AAGAAGAGCGACTAGTTGTAATCC
DIB1	Reverse Primer	220	24	59.00	33.33	0	ACATTTGTTTGAAATCTGGCACTT
	Product Size:414						

3. PCR and Optimisation

PCR result for ADE13, GUK1, GPI8, RNR2 and DIB1 was observed, amplification was not seen at the gene of GUK1 because no single bright band was formed (Fig. 3B). Amplification was not showed consistent data with target product size of genes. However, at least, some of samples had better amplification bands. For example, 7th sample of ADE13 and 2nd sample of GPI8 were showed better bands. Also, 5th position of RNR2 and 8th position of DIB1 gene showed significant bands. The unexpected thing was occurred in the RNR2 gene. Even though 5th DNA sample of RNR2 was none powdery mildew leaf, single band was observed at this sample.

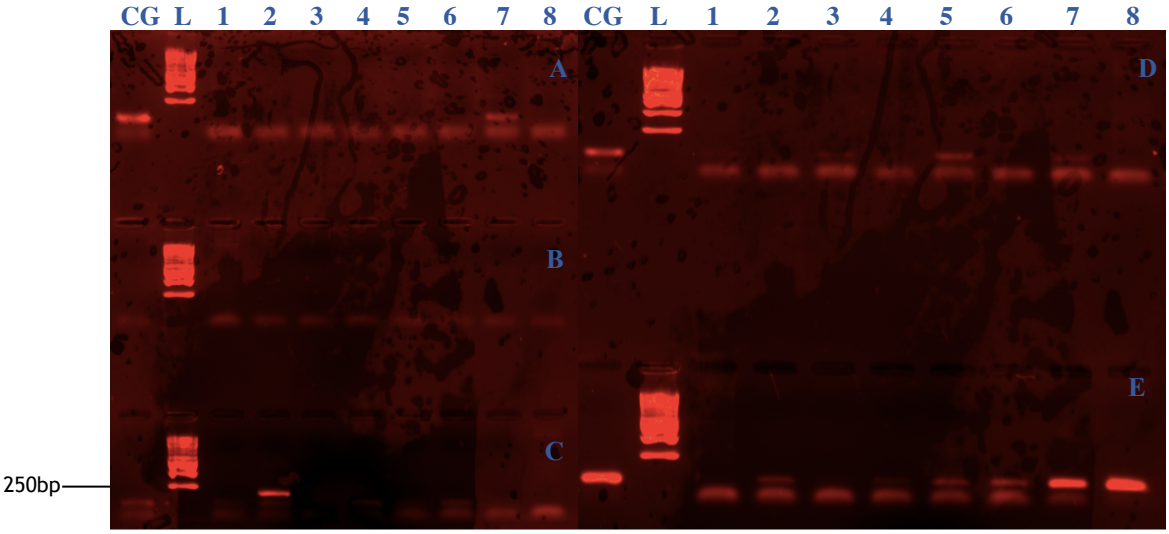

Figure 3: PCR products data of all selected genes. A: ADE13, B: GUK1, C: GPI8, D: RNR2, E: DIB1 genes. CG: Control group, L: Ladder of molecular size (1 kb), 1: High, 2: Low, 3:None, 4: High, 5: None, 6: Low, 7: High, 8: Low Powdery Mildew Leaf.

After taking PCR result, optimisation was applied for the best PCR samples. For first temperature optimisation result for genes of ADE13 and GPI8 at the range from 53°C to 63°C, DNA amplification process was observed successful that previous PCR which had 60°C annealing temperature, especially first 4 order of ADE13 gene was showed a little higher bands from the ladder. It was not easy to say that the visible DNA was belonged to DNA of *Erysiphe coryleceaum* because the product size for GPI8 is 733 bp, but our PCR was shown lower than target bp. For second temperature optimisation result for GUK1, RNR2 and DIB1 genes at the range 52°C to 62 °C, again no amplification for GUK1 was seen. The second and third samples of RNR2 had bright bands which showed existence of DNA (Fig. 4). The ladder was the same line with DIB1 samples. It was shown that DIB1 samples was loaded inside the ladder well by mistake. Product size of DIB1 is 414 bp but samples were still lower than that value. Interestingly, RNR2 only showed band at 60°C. The single band indicated that the annealing temperature can be higher than 60°C. After obtained this data, it observed that 60°C and over was the effective annealing temperature for RNR2 gene in conducted experiments.

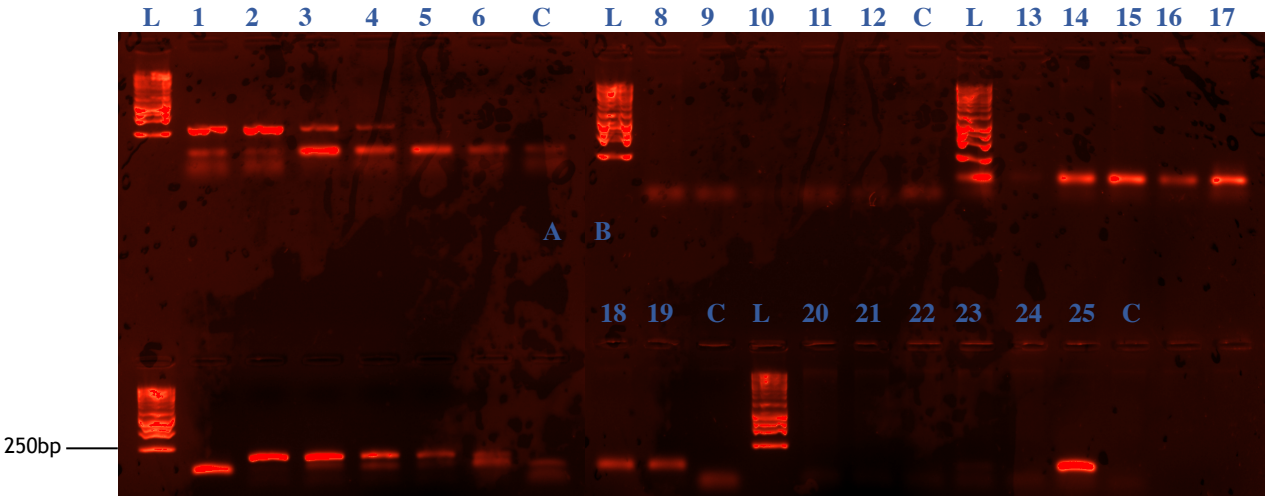

Figure 4: Temperature optimisation for PCR products. A: First order belong to high powdery mildew sample of ADE13, second order belong to low powdery mildew sample of GPI8. Temperature changes from 1 to 6; 53°C, 54.6°C, 55.8°C, 59.8°C, 61°C, 63°C, respectively. B: From 8 to 12 and 17 belong high powdery mildew sample of GUK1, From 13 to 16 and 18,19 belong low powdery mildew sample of DIB1, From 20 to 25 belong to none powdery mildew sample of RNR2. Temperature changes from 20 to 25; 52°C, 53.3°C, 54.2°C, 56.4°C, 58.5°C, 60°C, respectively. L: Ladder of molecular size (1 kb). C: Control group.

At the change of $MgCl_2$ concentration at PCR, some genes were not affected such as GPI8. However, second, and third samples of ADE13 and RNR2 genes bands were observed clearly (Fig. 5). In addition to this, first band of ADE13 was shown same type of band with other 2 samples. Whereas, it was a control group, so magnesium concentration was not shown obvious impact on the ADE13 gene. Second sample of RNR2 might have more intense DNA sample than other samples of genes. Unlike the samples of GPI8, DIB1 and RNR2 genes, the most bands of ADE13 gene were middle of the first and second ladder band. The change of magnesium ratio showed a great difference in the fifth and especially in the third PCR samples of the DIB1 gene. Even though desired basepair was not obtained, concentration of 0 ul and 0.2 ul $MgCl_2$ were successful for ADE13 gene at the PCR. Generally, the high PCR amplification for DNA was taken from 0.1 ul - 0.3 ul magnesium concentration.

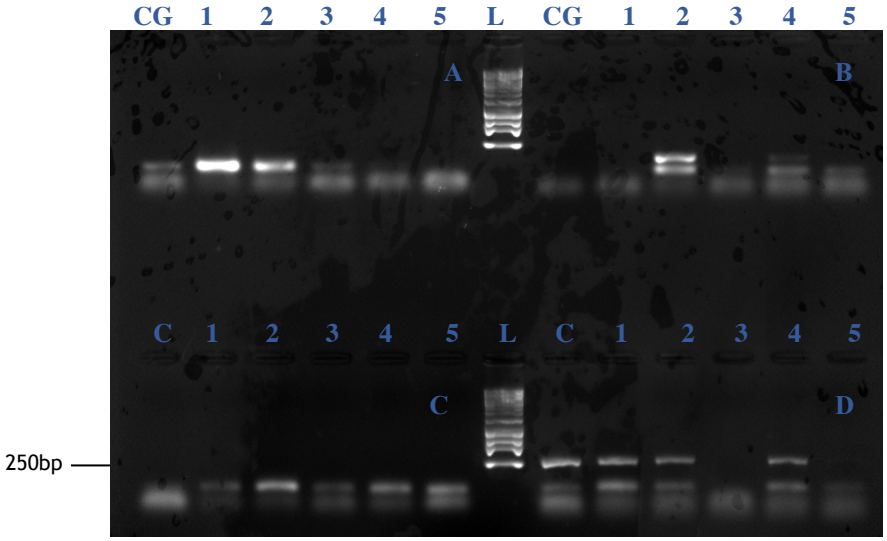

Figure 5: $MgCl_2$ concentration, A. RNR2 gene, B. DIB1 gene, C. GPI8 gene, D. ADE13 gene. From 1 to 5 $MgCl_2$ concentration is 0.1 ul, 0.2 ul, 0.3 ul, 0.4 ul, 0.5 ul. CG: Control group L: Ladder of molecular size (1 kb).

Combination of the average best value of temperature and $MgCl_2$ concentration for the ADE13, GPI8 and DIB1 genes indicated that DNA of plant or fungi was not amplified because all samples were below the ladder (Fig. 6). As in many sample, primers bound each other instead of DNA and consisted of dimer structure.

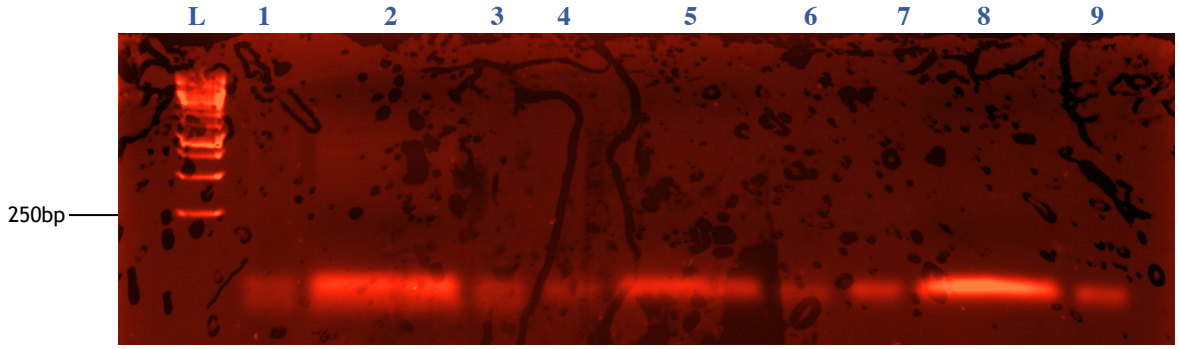

Figure 6: PCR-amplified products at 54°C and 0.1 ul, 0.2 and 0.2 ul $MgCl_2$ concentration, respectively by using ADE13, GPI8 and DIB1 primers on *Erysiphe corylacearum* from Turkish hazelnut. L: Ladder of molecular size (1 kb), Bands 1, 2, 3: Primers of ADE13 and 5 ul, 48 ul samples and control group. Bands 4, 5, 6: Primers of GPI8 and 5 ul, 48 ul samples and control group. Bands 7, 8, 9 : Primers of DIB1 and 5 ul, 48 ul samples and control group.

4. PCR Purification and Nested PCR

Even though desired product size was not taken from ADE13, GPI8, and DIB1 genes as result of optimisations, PCR purification was tried. In this way, the rise in the quality of DNA, especially on the ADE13, was seen (Fig. 7). Band of ADE13 was higher than GPI8 and DIB1. Also, the absorbance of GPI8 and DIB1 gene samples were uncertain.

Last two bands of ADE13 also showed the same type of band with DIB1 and GPI8 genes. Samples were loaded well starting from 5th because of the pipetting mistake.

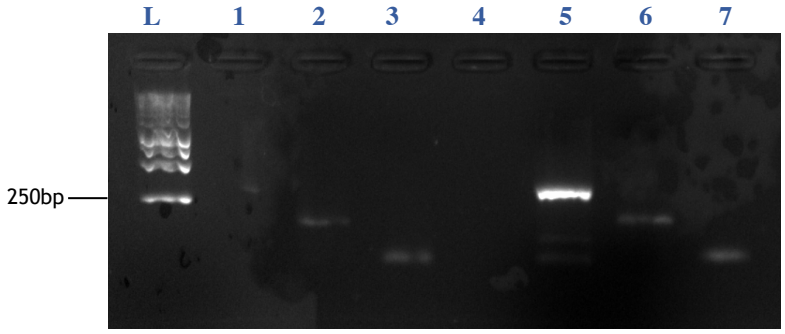

Figure 7: PCR purification 5: ADE13 gene 6: GPI8 gene, 7: DIB1 gene, L: Ladder of molecular size (1 kb).

Instead of application PCR at the fresh leaves, the usage of the nested PCR for dry leaves was provided specific fungus DNA by using known primer which are PM3, TW14, and NL1. Fungus DNA was seen Cumayeri 09.05.18 sample (Fig. 8). This sample was classified as healthy leaf but nested PCR was showed that healthy leaf had fungus DNA. Seen band was close to expected value which is 500-600 bp. Except this, dimer structure which was non-bright band under the ladder was observed at other samples.

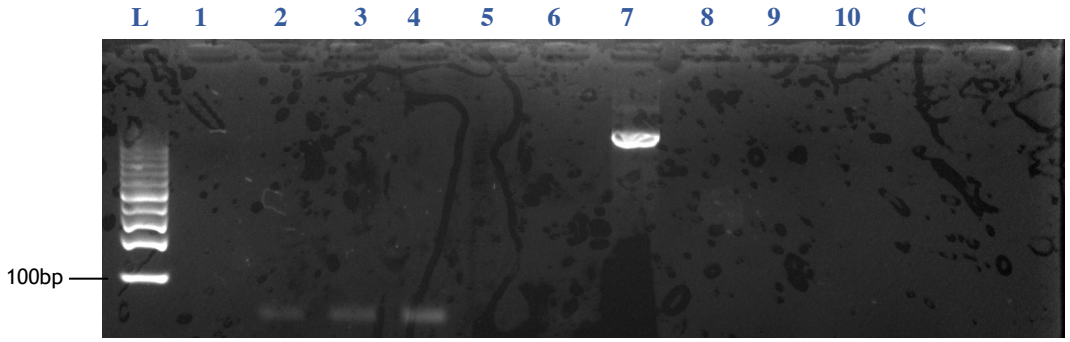

Figure 8: Nested PCR-amplified products by using PM3, NL1 and TW14 primers. L: Ladder of molecular size (100 bp), Band 1: 24.05.18-S, Band 2: 24.05.18- A, Band 3: 24.05.18-C, Band 4: 09.05.18-A, Band 5: 09.05.18-C, Band 6: 09.05.18- A, P.M-, Band 7: 09.05.18-C, P.M-, Band 8: 11.06.18-S, Band 9: 06.06.18- A, Band 10: 06.08.18-C, C: Control group.

In addition to Cumayeri 09.05.18 sample, Cumayeri 24.05.18 sample was shown fungus DNA (Fig 9). Unexpectedly, the band expected to be seen in the unhealthy leaf sample was seen in the control group. The contamination might be caused to see that band in the control group.

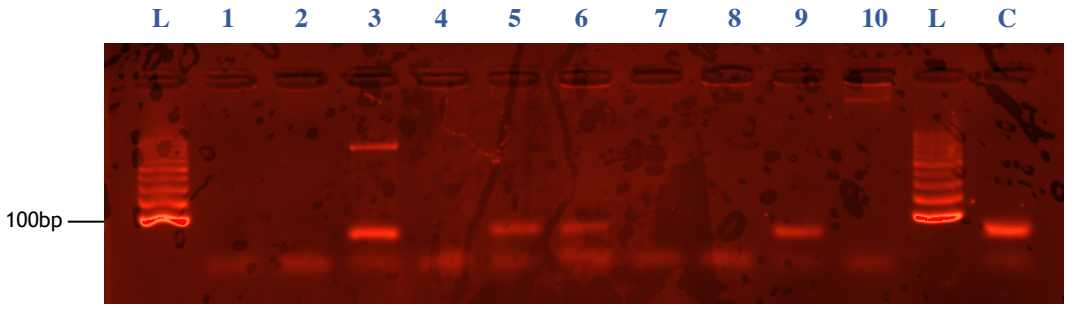

Figure 9: PCR-amplified products by using PMITS1, PMITS2 primers. L: Ladder of molecular size (100 bp), Band 1: 24.05.18-S, Band 2: 24.05.18-A, Band 3: 24.05.18-C, Band 4: 09.05.18-A, Band 5: 09.05.18-C, Band 6: 09.05.18-A, P.M-, Band 7: 09.05.18-C, P.M-, Band 8: 11.06.18-S, Band 9: 06.06.18-A, Band 10: 06.08.18-C, C: Control group.

Discussion and Future Work

It is known that pathogenic fungus of the Erysiphales order cause powdery mildew disease. While *Phyllactinia guttata* causes powdery mildew in Turkey, the recent research has concluded that a new species, *Erysiphe corylacearum,* gives much greater damage to growth of Turkish hazelnut (Sezer et al., 2017). The reason why powdery mildew disease spreads quickly between hazelnuts in the Black Sea is because this region provides a suitable environment, which is warm temperature and humidity for fungus reproduction. Observing most of sexual cleistothecium structure on the vascular system of the plant might be concluded that the extensions of cleistothecium can easily penetrate the vascular system to obtain food and water of the hazelnut tree. According to our observations under the microscope, the appendages shape of cleistothecium belonged to *Erysiphe corylacearum* (Braun & Cook, 2012). Morphological diversity of Erysiphales genus makes it easier to confirm that *Erysiphe corylacearum* causes powdery mildew on Turkish hazelnut. Turkish hazelnut has lot of varieties such as Tombul, Çakıldak, etc. The research is showed that Tombul variety caught the powdery mildew more frequently than Çakıldak, because Tombul is genetically more susceptible to powdery mildew disease. Our observations also supported this research by seeing more powdery mildew at the Tombul surface when comparing cultivated Tombul and Çakıldak hazelnut trees in SUNUM.

For the usage of first DNA extraction kit, the inability to see the DNA sample at the gel electrophoresis may be occurred because of lysis buffer is not adequate to break both cell wall of plant and fungus, chemically. When implementation of first and second kit was compared, for the second kit, the chemically defined protocol changed because chloroform failed to produce the desired data. Normally, chloroform separates fat, proteins and nucleic acids from DNA (Sun, 2010) but not to see any difference between chloroform and non-chloroform conditions, it might conclude that the excess metabolite ratio in the plant and the metabolite ratio in fungus were too extreme for chloroform function. Having two different types of cell wall in our sample caused to apply extra protocols making easy to reach the DNA. Using durable test tubes during application of new protocol is essential, otherwise, time and samples can be lost.

The long-term aim of this project is to uncover whole genomic sequence of *Erysiphe corylacearum*. Some data were wanted to collect about *Erysiphe corylacearum* genome from 5 selected genes, which are ADE13, GPI8, GUK1, DIB1, and RNR2. When the selected genes were used at the PCR and PCR optimisation by changing temperature and $MgCl_2$ concentration, the access of target product size was not seen. Observing most of dimer structures and low bands from the product size , it can be explained that primers was designed from *Erysiphe necator* and these product sizes are consistent with it. For this reason, if our primers match with DNA of *Erysiphe corylacearum*, we shouldn't expect the same value of product size so, its product size can be smaller than *Erysiphe necator* because of mutations and other kind of variations in *Erysiphe corylacearum*. On the other hand, two reasons for not observing DNA bands by using selected genes can be identified. The first reason can be there was a problem with the DNA extraction method. To test these assumptions, usage of primers PM3, TW14, NL1, PMITS1 and PMITS2 known to found at the fungus were used. Observing fungus DNA from 24.05.18-C sample and 09.05.18-C,PM- sample proved that there was not any problem our new DNA extraction protocol. Obtained data was consistent of our NanoDrop 2000C spectrophotometer (Table 1). 09.05.18-C,PM- sample have high nucleic acid concentration. Beside of it, that sample also showed fungus DNA. In this way, we propose an effective DNA extraction method to obtain fungus DNA. Seeing fungus DNA from 09.05.18-C,PM- sample was not expected because it is healthy leaf. It concluded that the mistake was made at the identification or weighing process. Also, the observing fungus DNA at 09.05.18-C,PM- sample by using PM3, TW14 and NL1 primers but it was not observed again by using PMITS1 and PMITS2. Maybe annealing temperature which is 58°C for PMITS1 and PMITS2 wasn't provided the stronger connections between template DNA and primers. We can conclude powdery mildew is widespread in Cumayeri, because fungus DNA was seen in the samples from this region. The second reason can be primers of selected genes which was found at the fungi genome. DNA was not obtained in the desired size, despite the optimisations made for each selected gene along with the data obtained in the PCR. The obtained small size of DNA makes it difficult to say whether the selected genes are present in the fungi, without looking at the sequence results. Between the genes, ADE13 and GPI8 showed better amplification than other genes at the separate optimised conditions. The further studies, the obtained optimisation conditions will be the foundation to complete the quantitative PCR method for detecting *Erysiphe corylacearum* on the Turkish hazelnut. Although the project duration was over, the obtained DNA was sent to sequencing.

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