DETERMINATION OF *IN VITRO* ACTIVITY OF PANOMYCOCIN AGAINST *BOTRYTIS CINEREA*

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ABSTRACT

DETERMINATION OF *IN VITRO* ACTIVITY OF PANOMYCOCIN AGAINST *BOTRYTIS CINEREA*

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Grey mold disease is one of these postharvest decays. Especially grapes are infected by *Botrytis cinerea* known as a causal agent of the grey mold disease. Some fungicides are used to control this disease. However, they cause funcide- resistant strains of the pathogens and make hazardous for human health and environmental safety. Therefore, The killer yeasts are considered as promising alternative antifungal agents among other biocontrol agents. *Pichia anomala* which is a killer yeast secretes the K5 type yeast killer protein called Panomycocin. The K5 type yeast killer protein has exo-β-1,3 glucanase activity and is stable at pH's and temperatures appropriate for its biocontrol usage. The goal of the research is to determine the inhibition effect of the K5 type yeast killer protein on *B. cinerea* strains including type strain *B. cinerea* (DSMZ 5145) isolated from Italy and other 5 *B. cinerea* strains isolated from different fields in Tekirdağ. This inhibition activity of the K5 type yeast killer protein was known from broth microdilution assay based on CLSI (Clinical and Laboratory Standards Institue) M38-A and found to be highly effective on *B. cinerea* strains. The difference of the growth inhibition of *B. cinerea* strains in 96-well plates was determined with inverted microscope after 72 hours. The K5 type yeast killer protein with exo-β-1,3 glucanase activity resulted in

physiological and morphological changes in the hyphae by causing coagulation and destruction of the protoplasm. The most effective growth inhibition was observed with 2048µg/ml of the K5 type yeast killer protein. MIC_2 value of the K5 type yeast killer protein was 512µg/ml. The results showed that *P. anomala* secreting the K5 type yeast killer protein would be used as a biocontrol agent for prevention of grey mold disease in grape fields.

Key words: *Botrytis cinerea*, grey mold disease, biocontrol agent, K5 type yeast killer protein, grey mold, MIC_2

ÖZ

PANOMYCOCİN'İN *BOTRYTİS CİNEREA* ÜZERİNDEKİ İNHİBİSYON ETKİSİNİN BELİRLENMESİ

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Kurşuni küf, hasat sonrası hastalıklardan birisidir. Kurşuni küf hastalık yapıcı etmen olarak bilinen *Botrytis cinerea* özellikle, üzümleri infekte eder. Bu hastalığı önlemek için bazı sentetik mantar ilaçları kullanılmıştır. Fakat diğer hastalık yapıcı mantarlara karşı direnç oluşumuna sebep olmuştur ve hem insan sağlına hem de çevre güvenliği için risk olmuşturmaktadır. Buyüzden maya öldürücü proteinler alternative control metod olarak düşünülmektedir. Bir öldürücü maya olan Pichia anomala, Panomycocin adında K5 tip maya öldürücü proteini ormata salar. K5 tip maya öldürücü proteini exo-β-1,3 glucanase aktivitesine sahiptir ve biyolojik kontrol için kullanım şartlarında uygun pH ve sıcaklıkta stabildir. Bu çalışmanın amacı K5 tip maya öldürücü proteininin İtalya'dan izole edilen *B. cinerea* (DSMZ 5145) ile Tekirdağ'dan izole edilen 5 adet *B. cinerea* türleri üzerindeki inhibisyon etkisinin belirlenmesidir. K5 tip maya öldürücü proteininin öldürücü aktivitesi, Klinik ve Laboratuvar Araştırmaları Enstitüsüne (CLSI) M38-A bağlı broth mikrodilüsyon deneyi ile anlaşılmıştır ve *B. cinerea* türleri üzrinde yüksek derecede etkili olduğu kanıtlanmıştır. 96'lı kuyucuklardaki *B. cinerea* türlerinin büyüme inhibisyonlarının farklılığı 72 saat sonra inverted mikroskobu ile belirlenmiştir. K5 tip maya ölürücü proteini exo-β-1,3 glucanase özelliği ile hiflerde fizyolojik ve morfolojik olarak

değişikliklere protoplasmalarının katılaşmasına ve parçalanmasına sebep olmuştur. K5 çeşit öldürücü maya türünün en etkili büyüme inhibisyon etkisi 2048µg/ml miktarında gözlemlenmiştir. %50 büyüme inhibisyonu için etkili K5 tip öldürücü maya proteininin miktarı ise 512µg/ml'dir. Sonuçlar gösteriyor ki K5 tip öldürücü maya proteinini ortama salan *P. anomala* kurşuni küf hastalığının önlenmesi için biyolojik kontrol ajanı olarak kullanılabilir.

Key words: Botrytis cinerea, kurşuni küf hastalığı, biyokontrol ajanı, K5 tip maya öldürücü proteini, MIC_2

To my family

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CHAPTER 1

INTRODUCTION

Fungi are eukaryotic microorganisms. They are found as yeast, molds, or as a combination of both forms in nature .They have the ability to invade all kinds of organisms. They use plants, animals, and humans as substrates [1]. Fungal diseases are important problems particularly in plant pathology. Fruit and vegetables are highly sensitive to microbial infections in the postharvest phase. In this phase they contain high nutrient and water content [2]. Especially mold infection destroys large range of pre and post-harvest fruit and vegetables. It leads to severe losses throughout the world. Moreover, it reduces nutritional value of crops [3].

The vine and grape berries, *Vitis vinifera* species, are also sensitive to fungal infection. Some compounds found in defense system of grapes are effective in post flowering but they decline in maturing. This increases susceptibility to all of the infections [4]. After attack by pathogens the infected plant tissue is overwhelmed and necrotization happens. Besides leaves, inflorescences, clusters and berries are also infected by pathogens. This reduces the yield [5].

Grapes have own their flora which includes yeasts, bacteria and fungi. Yeast and lactic acid bacteria play roles in the transformation of must to wine. Moreover, they are known as special agents of wine fermentation. Other microorganisms act as spoilage agents. These agents contain filamentous fungi which produce mycotoxins and off-flovers. They damage the grapes and quality of wine, so some precautions are needed [6]. If these microorganisms are not got under control, they lead to diseases in grape berries. There are also some factors affecting disease formation on grapes. These are abiotic (e.g. climate, rain, hail), biotic (e.g. insects, birds, phtopathogenic and saprophytic moulds) and viticultural (e.g. fungicides) factors [7]

1.1.Postharvest Diseases of Grapevines

1.1.1 Downy Mildew

It is one of the most important grapevine diseases in worldwide. *Plasmopara viticola* is known as a causal agent of this disease. It attacks leaves and bunches of grapevines (Figure 1.1.). This disease is also considered as a threat for grapevine production. It affects both quantity and quality of crop yields (Figure 1.2.) [8].

Figure 1. 1. Sporulation of Plasmopara viticola on leaf surface [9].

Figure 1. 2. Infection of *Plasmopara viticola* on a mature cluster of grape in summer [9].

1.1.2. Powdery mildew:

This is one of the most devastating and widespread diseases of wine, table and raisin grapes. The causal agent of this disease is *Erysiphe necator*. It can infect all green tissues of a grapevine (Figure 1.3.). After infection levels of the photosynthesis is decreased on leaves and this causes poor maturation of the grape berries (Figure 1.4.). Total impact on the crop includes decreased yields, increased acidity and decreased anthocyanin which is a water-soluble vacuolar pigment and sugar content of mature fruit [10].

Figure 1. 3. *Erysiphe necator* on leaf of grapevine [10].

Figure 1. 4. Powdery mildew infection on grape berries [10].

1.1.3. Phomopsis Cane and leaf spot disease:

Phomopsis viticola infects grapevine (*Vitis vinifera*) resulting in Phomopsis cane and leaf spot disease. Grape-growing regions defined as humid temperature climate are more effective for *Phomopsis viticola* infection. It contaminates all green parts of grapevine, so disease symptoms are seen on leaves as small spots that turn pale green to yellow colors (Figure 1.5.). Moreover, these spots have necrotic centers. Canes of the vines have necrotic-irregular shaped lesions which turn brown to black colors and clusters display rachis necrosis and brown, shriveled berries close to harvest (Figure 1.6.) [11,12].

Figure 1. 5. *Phomopsis viticola* infection on leaves and clusters of the grapevine [11].

Figure 1. 6. Brown to black necrotic-irregular shaped lesions at the spur position [11].

1.1.4. Rotbrenner:

Rotbrenner is a foliar disease. *Pseudopezicula traheiphila* known as a causal agent of rotbrenner infects leaves of the grapevines and their inflorescence. At the beginning, lesions on leaves are yellow on white cultivars and bright red to reddish brown on red and black cultivars (Figure 1.7.). In the center of the lesion a reddish brown necrosis occurs. During bloom *Pseudopezicula traheiphila* may attack inflorescences by leading them to rot and dry out (Figure 1.8). Heavy rainfall and wet periods are favorable conditions for infection and cause severe diseases. Nutrient deficiency and water stress can make grapevines sensitive to infection by *P. traheiphila.* Rotbrenner is spread by water and airborne spores. [13,14].

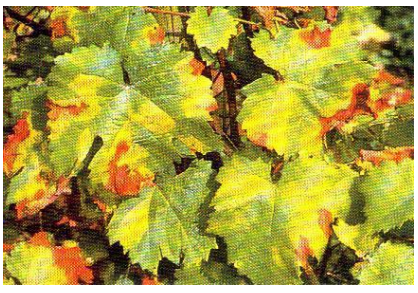

Figure 1. 7. Symptoms of rotbrenner on leaves of grapevines [14].

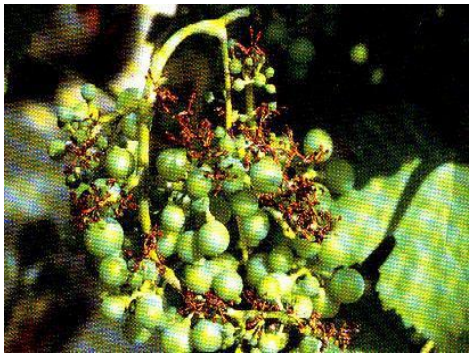

Figure 1. 8. Dried fruit clusters infected by *Pseudopezicula traheiphila* [14].

1.1.5. Grey Mold:

Grey mold disease damages more than 200 plants in their pre- and postharvest stages in worldwide. The important ones are grape, kiwifruit and strawberry. *Botrytis cinerea* (Telemorph: *Botryotinia fuckelina*) is the causal agent of this disease. It infects flowers, fruits, leaves, shoots and soil storage organs (i.e. carrot and sweet potato), so these show that it has a large host range. This fungus is also known to be a true plant pathogen due to the serious pre- and postharvest losses [15,16].

Table 1. 1. Taxonomy of Botrytis cinerea [17]

Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Leotiomycetes
Order	Helotiales
Family	Sclerotiniaceae
Genus	Botryotinia

B. cinerea is an airborne plant pathogen with a necrotrophic lifecycle. It produces cell- wall degrading enzymes, toxins and other low-molecular-weight compounds such as oxalic acid. On many fruits and vegetables Botrytis infection starts by attaching senescent flowers. On the other hand, grape berry infection begins in the center of a cluster and spreads to the entire bunch. However, *B. cinerea* is not primarily a wound pathogen. On the contrary, it can infect wounds and some part of plants where insects or birds damages [17].

In early spring many kinds of symptoms can be observed on buds and young shoots. They turn brown and dry out. Before blooming leaves can be seen large, irregular, reddish brown, necrotic patches which are generally found on the edge of the lamina (Figure 1.10) [17,18]. Moreover, the inflorescences are also infected, which causes the bloom dry out and drop the vine. After this phase, pathogen moves to the aborted berries which are still attached to the clusters of berries and movement of *Botrytis* on the rachis creates small patches that start brown and turn black color. In late summer infected berries are observed. Small brown spots on mature berries show the initiation of the berry infection (Figure 1.9). White grapes will turn into brown while purple grape berries turn into reddish. These parts of the infection can result in high yield losses [19,20].

Figure 1. 9. *Botrytis cinerea* on white grapes [21].

Figure 1. 10. Symptoms of grey mold on leaves of grapevines [21].

1.1.5.1. *Botrytis cinerea* life cycle:

Botrytis cinerea lives as a saprophyte on necrotic, senescent or dead tissues. In winter it is found as a sclerotia which is resistant to hard weather condition on the vines. Generally they germinate in spring season and produce conidia which are spread by both wind and rain to other parts of the plant and other different plants. The conidia is the primary source of inoculum for infection leaves and clusters. When they are found suitable temperatures between 34-86 °F (1-30 °C), they germinate. Presences of free water or humidity supports germination and nutrients found in pollen or leaves also stimulate germination. Thus, temperature and humidity or free water are important for infection. Wound formed by insects, other fungal diseases and birds give advantages for the infection (Figure 1.11.) [19,20].

B. cinerea synthesizes cell wall degrading enzymes for penetration to the hosts [17]. Moreover, there are some compounds including oxalic acid, toxins and reactive oxygen species which play roles in infection of host cells. For example, oxalic acid blocks activation of enzymes which are parts of the plant protective mechanism [22].

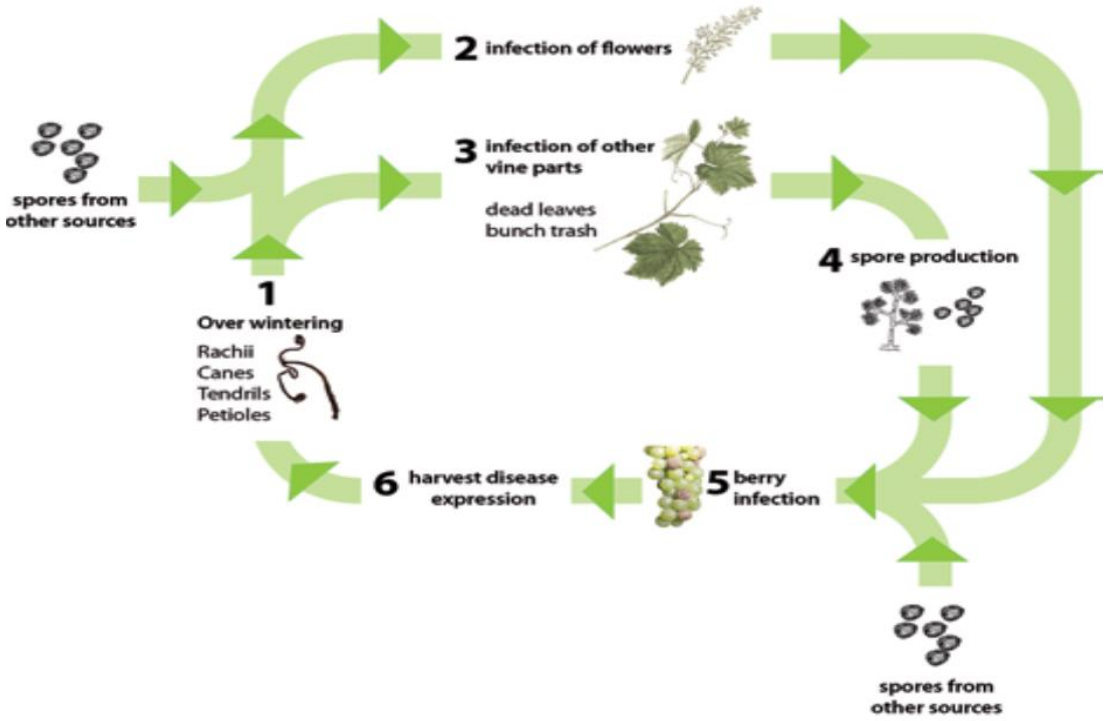

Figure 1. 11. Life cycle of *Botrytis cinerea* [23].

Botrytis bunch rot causes economic losses of yield in table grapes and wine production in many regions around the world [24]. According to the Turkish Statistical Institute vine cultivation stood at around 4.25 million tons in 2011 [25].

Currently, in order to prevent formation of grey mold disease dicarboximides including vinclozolin, iprodine and sulfur dioxide as synthetic fungicides are used against *B. cinerea* [26].

Use of chemical fungicides has certainly decreased the infection of the fungal diseases, but at the same time it causes fungicide-resistant strains of the pathogens. Moreover, this type control of the pathogens are hazardous for human health and environmental safety. Thus, interest of the alternative methods known as biological control to the chemical control of *B. cinerea* has been increasing [27, 28].

1.2. Biological Control

Biological control methods are used in order to prevent the diseases. Bacteria, fungi and yeasts are used as biological agents to control *Botrytis* bunch rot. Antibiotics and lytic enzymes produced by microorganisms inhibit germination and lyse germ tube of *Botrytis* [28].

Beneficial (non-pathogenic) bacterial strains which belong to *Pseudomonadaceae*, *Bacillaceae*, *Enterobacteriaceae* and *Actinobacteria* can block grey mold disease on grapes caused by *Botrytis cinerea* [29]. *Bacillus circulans* found on the chrysanthemum leaves inhibit the spores of *Botrytis cinerea* with pectinolytic activity by causing morphological changes in mycelium. Moreover, they trigger production of phtoalexins as a part of the host defense mechanism [30].

Fungi are used as a mean of biocontrol too. Mycoparasites can parasitize other fungi, as well. Both *Trchoderma harzianum* and *Pythium radiosum* are mycoparasites. They have antagonistic activity, so they are effective in controlling the plant pathogens including *B. cinerea*. They loop around the host hyphae or overgrow on other fungal colonies in agar. [31]. When one of the isolates of these species grows together with *B. cinerea*, it enters in to the mycelium of *B. cinerea* and damages extensively [32, 33].

Some microorganisms, except yeasts synthesize allergenic spores and mycotoxins. Because of these risk factors alternative biocontrol agents are required to prevent grey mold disease [27].

1.3. Yeasts as biocontrol agents

Among other biocontrol agents, yeasts are found to be more effective. They provide many effective ways for prevention of fungal diseases. If simple nutritional requirements are met for them and they can grow rapidly. Moreover, they colonize plat surfaces or wounds for long time, which limits pathogen colonization. These

features make yeasts preferable biocontrol agents. Yeasts produce cell wall degrading enzymes, antifungal metabolites and also they increase host resistance against pathogens. Especially nutritional competition formed between yeast and pathogens is powerful way to prevent the grey mold [27,34].

Pichia membranifaciens, a yeast species, produces β-1,3-glucanase which is a cell wall degrading enzyme. This enzyme hydrolases β-glucans found in cell of *B. cinerea* [28].

Areobasidium pullulans, *Metschnikowia pulcherrima*, *Pichia guilliermondii* and *Saccharomyces cerevisiae* have been shown to inhibit *B. cinerea* [27].

Some yeast strains which have killer activity show significant effect on pathogenic fungi. They prevent fungal growth and kill them with killer toxins [35].

1.4. Killer toxins of yeasts:

Various types of organisms including bacteria, insects, vertebrates, invertebrates and plants produce powerful antifungal proteins. Under optimal conditions some yeasts secrete polypeptide toxins into the medium. They are called killer yeast and their toxins are called killer proteins or killer toxins. The secreted proteins inhibit either the growth of the same yeast species or different species [36-38]. Moreover, killer toxins produced by certain yeast strains have the growth inhibitory effect on gram positive pathogenic bacteria and fungal plant pathogens [35,39]. Synthesis of these toxins provides a benefit to the killer strains in competition with the sensitive cells, especially in availability of nutrients in the environment [40,41]. Generally they are glycosylated and the secretion of them depends on proper pH and temperature in the medium [42, 43].

The first yeast killer toxin was isolated from *Saccharomyces cerevisiae* by Bevan and Makower in 1963 [44]. The other genera are *Candia*, *Crytococcus*,

Debaryomyces, *Hansenula*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Torulopsis*, *Trichosperon*, *Ustilago*, *Williopsis* and *Zygowilliopsis* [45-50].

Killer proteins are classified into 11 different types based on the killing spectra and the cross reactivity in immune responses (K1-K11) [40, 51].

1.4.1. Structure, Processing and Secretion of the killer toxins

Killer yeasts and their toxins have been used as a model system to understand the mechanisms of regulation of eukaryotic polypeptide processing, secretion and receptor binding in fundamental researches. There is similarity between killer system and mammalian hormones and neuropeptides according to their synthesis and action processes [52].

Secretion steps of K1 and K28 which are toxins of *S. cerevisiae* are fully understood. Despite different amino acid designs and mode of action, synthesis, processing and secretion of these two killer proteins show homologies. They are encoded by double stranded RNA (dsRNA) viruses and form two subunits called as α and β. After translation as preprotoxin, they undergo post-translational modifications in the endoplasmic reticulum and the Golgi complex. This process continues until mature α/β heterodimeric protein toxins are secreted [44].

When the preprotoxins enter the endoplasmic reticulum with hydrophobic signal peptides found in the N-terminal region, a signal peptidase splits the hydrophobic sequence and glycosylation of γ subunit occurs. Kex2p that is a product of KEX2 gene cleaves the proregion and take out the intramolecular γ sequence in Golgi complex. On the other hand, Kex1p that is a product of KEX1 gene, removes the dipeptide region found on the C terminal of the α subunit. Afterwords, mature toxin is carried a secretory vesicles and secreted out of the cell. The products of SEC genes play roles in secretion process [44,53-55].

Killer yeast cells are protected against their own killer toxins. Unprocessed killer toxin precursor is enough to confer protective immunity, it may be because of an immunity sequence to the toxin receptors of the precursor cell. Therefore, the killer protein is blocked from binding to the precursor cell [44], but the detailed information about immunity is still unknown.

1.4.2. Mode of action of the Yeast killer proteins

Yeast killer proteins use cell wall components as receptors to exhibit killing activity in two steps, but killing mechanisms of these killer proteins are different. The first step of the killing activity is the binding of the killer toxin to the cell wall receptor. This step requires energy and it is also fast. The second step includes toxin-location to the cytoplasmic membrane and interaction with some compounds of the cell wall known as secondary membrane receptors [44].

Mannoproteins, β-glucans backbone including linear or branched β-1,3 and β-1,6 glucans and chitin form yeast cell wall (Figure 1.14). It has been shown that these cell wall components act as both primary binding sites and cell wall receptors for killer proteins [56-58].

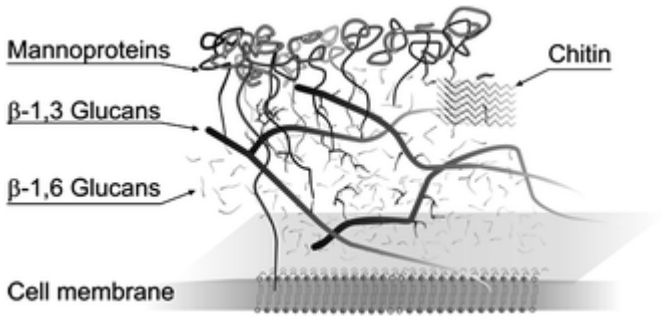

Figure 1. 12. Yeast cell wall structure [56].

Killer toxins secreted by *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Pichia membranifacience* and *Saccharomyces cerevisiae* (K1, K2) bind to primary reseptors named β-1,6-glucan residues known as primary receptors on the yeast cell wall while killer toxins secreted by *Saccharomyces cerevisiae* (K28), *Schwanniomyces occidentalis* and *Zygosaccharomyces bailii* bind to mannoproteins. Other killer toxins secreted by Pichia acacia and Kluyveromyces lactis bind to chitin as receptors [37].

After binding to receptors on cell wall, killer toxins kill sensitive cells in different killing processes.

K28 type killer toxin produced by *Saccharomyces cerevisiae* exerts killing activity during cell division. It leads to arrest of the sensitive cells at G2 phase of cell division which causes the daughter cells lacking DNA molecules because K28 killer toxin prevents DNA to transfer to the toxin exposed sensitive cells [59].

When yeast cells in S phase of the cell cycle are exposed to killer toxins secreted by *Kluyveromyces lactis* , they do not divide in spite of completion of their cell cycles. Therefore, the size of these cells increase since their metabolic activities go on and these yeast cells are arrested in G1 phase. [60]

Some killer toxins cause the formation of ion-permeable channels which are non-selective on sensitive microbial cells. This leads to leakage of K^+ and ATP and sudden cell death. These type of killer toxins are produced by *Pichia kluyveri* [61].

K5 type killer proteins secreted by *Pichia anomala* inhibits the growth of sensitive microbial cells by degrading the main cell wall components called β-1,3- glucans which causes cell burst [62]. On the other hand, killer proteins produced by *Hansenula mrakii* form pores by blocking β 1,3-glucan synthase which play a role in synthesis of β-1,3-glucans on the fungal cell walls. Thus, cell materials leak and cell dies [44,63].

1.4.3. Genetic basis of killer toxins

The whole genome sequence of *Saccharomyces cerevisiae* was the first eukaryotic genome to be sequenced in *April*, 1996. Size ranging of 16 chromosomes found in haploid genome is from 200 to 2,200 kb and total genome size is approximately 13,000 kb [53,64].

Yeast strains contain 2µm-plasmid and mitochondrial DNA as two additional sources. About 5% of total DNA is the 2µm-plasmid. About 10% of total DNA is mitochondrial. About 85% of total DNA is in chromosomes [64]. Yeasts have also five non-homologous species of dsRNA, named as L-A, L-BC, T, W and M which show non-Mendelian inheritance [53,54].

The genome map of the killer proteins are composed of either dsRNA or a linear ds DNA plasmid or a chromosome [43].

Table 1. 2. Features of Yeast Genome [53]

Genome size	12,5 Mb + rDNA
Single copy DNA	12,0 mB
Length of genetic map	>4300
Number of chromosomes	16
Organelle DNA	Mitochondrial, 75 kb
Extrachromosomal elements	2-micron DNA and double stranded RNA

1.4.3.1. dsRNA Encoded Yeast Killer Proteins

Double stranded RNA viruses are responsible for killing activity. They are found in the cytoplasm and surrounded by a protein coat, so these dsRNA's are named as yeast viruses. M dsRNA have a killer toxin production function and immunity. There are three types of M dsRNA viruses including M1, M2 and M3. They are responsible for production of K1, K2 and K3 killer toxins [54].

L-A helper virus is three times larger than M dsRNA. It plays the role in the encapsidation and replication of M dsRNA [53].

1.4.3.2 Linear dsDNA Encoded Yeast Killer Proteins

Linear ds DNA plasmids also encode yeast killer proteins. They are found in various yeast species such as *Debaryomyces, Wingea, Kluyveromyces* and *Saccharomyces*. pGKL1 and pGKL2 are well known ds DNA plasmids. They are found in *Kluyveromyces lactis*. pGKL1 plasmid encodes killer toxins of *K. lactis* which are 180 kD. pGKL2 is responsible for the replication of pGKL1 [65-67].

1.4.3.3. Chromosomally Encoded Yeast Killer Proteins

Such yeast strains as *Williopsis*, *Pichia*, *Candida*, *Debaryomyces* and *Torulopsis* do not produce the killer toxins encoded by neither dsRNA viruses nor ds DNA plasmids. Their killer toxins are encoded by genes on chromosomes [68]. Killer toxins found in *Saccharomyces cerevisiae* known as killer strain are encoded by two different genes which are on chromosome V and Chromosome IX [69].
The genetic basis for the expression of the killer toxins by different killer yeast species is shown in Table 1.3.

Table 1. 3. Genetic basis of killer toxins [44]

Killer yeasts	Genetic basis
Saccharomyces cerevisiae	dsRNA
Hansenula uvarum	dsRNA
Zygosaccharomyces bailii	dsRNA
Ustilago maydis	dsRNA
Kluyveromyces lactis	Linear ds DNA plasmid
Pichia acaciae	Linear ds DNA Plasmid
Pichia inositovora	Linear ds DNA Plasmid
Pichia kluyveri	Chromosomal
Pichia farinosa	Chromosomal
Pichia anomala	Chromosomal
Williopsis mrakii	Chromosomal

1.4.4. Application of Yeast Killer Proteins

Killer yeast strains and their proteins are studied and suggested for different applications. These killer proteins can be used as antifungal preparations to prevent fungal diseases of human and plants and they have a wide killing spectrum on sensitive cells. Moreover, they are also used in biotechnological processes including fermentation in order to fight against the contaminating sensitive cell, and recombinant DNA technology [58].

1.4.4.1. Biotyping of Pathogenic Yeasts

Killer toxins produced by *Saccharomyces*, *Pichia*, *Kluyveromyces* and *Candida* play roles in applications of the biotyping of the pathogenic yeasts including *Candida albicans* and *Cryptococcus* which are sensitive or resistance to killer toxins. These

methods are also used in some applications for the classifications of actinomycetes [70,71].

1.4.4.2. Secretion vectors

The killing function of the toxins can also be applied in the production of foreign proteins in yeast strains. Several heterologous genes encoding biotechnologically important enzymes have been expressed with killer plasmids called as vector. cDNA clones of the killer toxin can construct a secretion vector which contains signal region of the toxin. The yeast expression plasmid designed with *Cellulomonas fimi* DNA fragments was used for the production of carboxymethylcellulase [72]. On this point linear plasmids (pGKL1 and pGKL2) found in *K. lactis* provide some properties including high copy number and extreme stability when contrasted with circular plasmids. Their localization in cytoplasm makes them independent from nuclear control in the way of replication and transcription. The leader sequence of *Kluyveromyces lactis* was fused with h-TPA (human tissue plasminogen activator) cDNA for the achievement of the level of expression of h-TPA in yeast [73].

1.4.4.3. Brewing Strains

Molding of the product by some yeasts including *Candida*, *Hanseniaspora*, *Kloeckera* and *Pichia* can be prevented by the introduction of the killer traits into fermentation strains. In mating of the brewing yeast strains killing character can be introduced by cytoduction [74]. Moreover, the fermentation ability of the brewing strains are not affected by introduction of the killer feature to these strains. For example, introduction of killer character to the strains of *S. cereviase* provides a potential competitive advantage over other sensitive strains of *S. cereviae* and other killer strains in wine fermentation [75].

1.4.4.4. Food Preservation

Some pathogenic agents including molds, allergenic spores and mycotoxins damage foods and so their nutritional values decrease. Killer yeasts are used as biocontrol agents against plant pathogens in the postharvest storage of fruits and vegetables. Killer toxins produced by *Pichia anomala* were effective on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* which cause fungal infections in storage of wheat. This killer strain was also successful on 17 other food pathogenic fungal strains [76]. *Botrytis cinerea* which infects citrus, apples and grapes was also sensitive to the killer protein of *Pichia anomala* [77-79].

1.4.4.5. Medical Use

Human fungal infections have boosted in immunocompromised patients especially because of chemotherapy, immunosuppressive drugs, HIV and other immunosuppressive diseases. Mycoses are hazardous for HIV-1 infected patients. These kinds of infections can be mortal to the immunosuppressed patients [80-84].
There are two problems observed in the treatment of fungal infections by using different types of antifungal agents including amphotericin B, fluconazole, ketoconazole, itraconazole. However, these antifungal agents cause side effects on the host cells because of being non-selective to the fungal cells. Liver problem is one of these side effects formed by the antifungal agents [85]. Second problem is the resistance formation towards to the antifungal agents due to long time usage. Thus, alternative drugs are required to prevent these problems. These drugs should be selective agents targeting to the components of the fungal cell walls and yeasts and harmless to their host cells due to lack cell wall components [62]. Yeast killer toxins as alternative antifungal agents are used against the fungal agents since they have wide spectrum activity. Cell wall components including mannoproteins, β-glucan backbone and chitin serve as receptors and also act as primary binding site for these killer toxins [44, 86]. Moreover, killer yeast strains are successful to inhibit the growth of gram-positive pathogenic bacteria including *Streptococcus pyogenes, Bacillus subtilis, Sarcina lutea,* and *Staphylococcus aureus* [43].

1.5. K5 Type Yeast Killer Protein As Antimicrobial Agent

According to the Youg and Yagiu classification [62], K5 type yeast killer protein is secreted by *Pichia anomala* NCYC 434.

Pichia anomala is a fungi part of the phylum; Ascomycetes, class; Hemiascomycetes, order; Saccharomycestales, family; Saccharomycetaceae, genera; Pichia (*Hansenula*), species; *anomala* . It is found in different environments and isolated from fruit and plant materials, cereal grain, maize silage and some products containing high sugar. It is also isolated from wine and has been reported to be non-saccharomyces wine yeast [87].

Among other Pichia species, *Pichia anomala* NCYC 434 has been used for different applications [34, 88, 89].

Pichia anomala NCYC 434 is cultivated in special growth mediums in order to produce K5 type yeast killer protein. The presence of the β-glucan stimulates secretion of this killer protein and amount of the protein raises in correlation with the amount of the β-glucan and the type of its linkage. The amount of glucose is also important to maintain the highest level of toxin production [90].

K5 type yeast killer protein was previously purified and characterized in our laboratory. It is a glycosylated protein with a molecular mass of 49 kDa and a pI value of 3.7. According to temperature and pH stability testing of the killer protein the optimum pH value is 4.5 and about 70% of the activity continues even at pH 2.5, but at pH values above 6.5 the toxin is inactivated (Figure 1.15.). The optimum temperature value is 25 ℃ for the toxin activity. While at 37℃ it looses 10 % of the killer activity, at 100℃ it looses 50 % (Figure 1. 16.) [62].

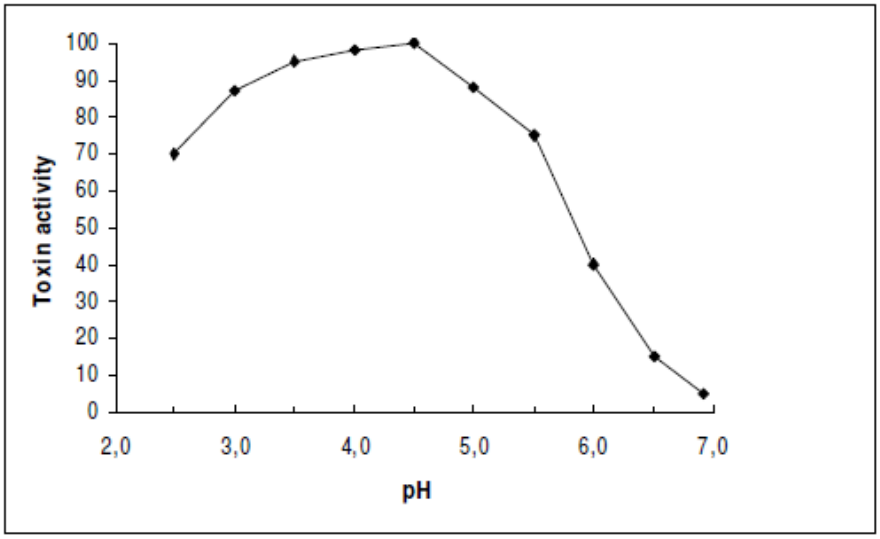

Figure 1. 13. pH stability of the K5 type yeast killer protein [62].

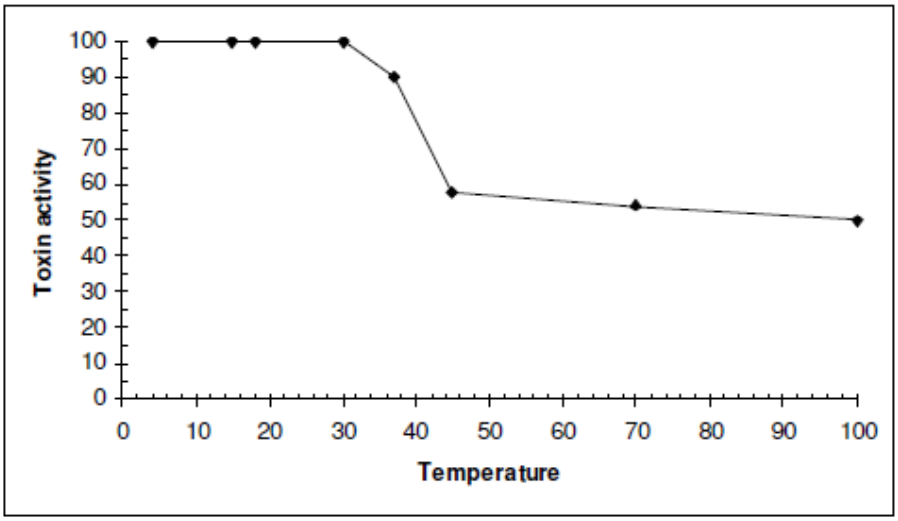

Figure 1. 14.Temperature Stability of the K5 Type Yeast Killer Protein [62].

When K5 type yeast killer protein is compared with other killer proteins based on internal amino acid sequences, it shows 100 % homology with the exo-β-1,3-

glucanase of *P. anomala* strain K which is a glycoprotein of 45.7 kDa with a pI of 4.7. [62].

Mode of action of K5 type yeast killer toxin was researched by Izgü et. al. This killer toxin is absorbed by laminarin formed by β-1,3-glucans. This shows that the toxin applies its killing effect by hydrolyzing β-1,3-glucan components of the cell wall of the sensitive fungal cells and results in loss of cell wall rigidity which causes cell death because of osmotic pressure. Other researches about its mode of action show that K5 type yeast killer protein use hydrolytic activity on the β-1,3-glucans in an exo-like fashion [90].

Specific activity of the K5 type yeast killer protein on laminarin is 120 U/mg and the Michaelis constants K_m and V_{max} are 0.25 mg/ml and 370 μmol/min/mg respectively [90].

The enzymatic activity of the K5 type killer toxin is affected by metal ions. Hg^{+2} inhibits its enzymatic activity while other metal ions including Ba^{+2}, Ni^{+2}, Cr^{+2}, Zn^{+2} and Pb^{+2}increases this activity [90].

Exo-β-1,3-glucanase activity of the K5 type yeast killer protein is known as a highly selective antifungal agent. Hydrolyzing activity of it is characteristic to the fungal cell wall. Moreover, it does not cause side effects in host cells. At pH values between 3-5.5 and temperatures up to 37 ℃ the activity of this protein is highly stabile so it has been used for different purposes in medical and industrial biotechnology [90].

Inhibitory effect of this killer protein was tested either by spotting *P. anomala* NCYC 434 cell suspension or the crude protein preparation on the plates inoculated with the test strains [35, 76, 91,]. However, we observed that the pure killer protein is required for the exact antifungal spectrum of K5 type killer protein [90].

Researches in our laboratory show us the pure K5 type yeast killer protein has wide range antifungal spectrum against human pathogenic fungal strains containing

Candida species and *Dermathophtes* which result in either systemic or superficial fungal infection.

The K5 type yeast killer protein was determined to be effective to inhibit all of the tested 26 strains of *Candida* species within the MIC_2, MIC_0 and *MFC* ranges of 0.25-4, 0.5-8, 1-16 μg/ml respectively [92].

The K5 type yeast killer protein was found to be powerful to inhibit all of the tested 9 human pathogenic dermatophytes strains within the MIC_2 and MIC_0 ranges of 0.25-2, 1-8 μg/ml respectively [93].

The K5 type yeast killer protein was found to effective to inhibit all of the tested 2 strains of *Penicillium* species within the MIC_2 2 and 1μg/ml and MIC_0 16 μg/ml [79].

The K5 type yeast killer protein exerted its cytotoxic effect within 2 hours and kills whole sensitive cells at time periods between 10-36 hours when tested at MFC.

We have isolated, characterized and purified the K5 type yeast killer protein in our laboratory. This killer protein was recommended as a novel antifungal agent. Antifungal susceptibility tests of this killer protein should be applied for use of this protein on human pathogenic fungal strains in medicine as an antifungal agent with an appropriate formulation.

In this study we aim to determine the antifungal spectrum of the pure K5 type yeast killer protein on fungal pathogen, *Botrytis cinerea*, causing grey mold disease on grapes especially in the postharvest period. Therefore, we would direct a primary research about efficiency of this novel yeast killer protein as a biocontrol agent against plant pathogens.

CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

2.1.1. Fungal Strains

Pichia anomala (NCYC 434, K5) which secretes the K5 type yeast killer protein and *Saccharomyces cerevisiae* (NCYC 1006) which is sensitive yeast strain to the killer toxins were obtained from National Collection of Yeast Cultures, Norwich, U.K. *Botrytis cinerea* strains were taken from *Vitis vinifera* in Tekirdağ. They were obtained from Department of Plant Protection, Faculty of Agriculture, Namık Kemal University. . All of *B. cinerea* isolates tested in susceptibility assay to K5 type yeast killer protein are listed in Table 2.1.

Table 2. 1. *Botrytis cinerea* isolates tested in susceptibility assay to K5 tpe yeast killer protein

Isolate	Isolate number	Location
Botrytis cinerea	*DSMZ 5145*	Italy
Botrytis cinerea	4d	Tekirdağ (Merkez)
Botrytis cinerea	11b	Tekirdağ
Botrytis cinerea	15c	Tekirdağ/Yazır Köyü
Botrytis cinerea	19a	Şarköy/Çengelli Köyü
Botrytis cinerea	25a	Şarköy/Çengelli Köyü
Botrytis cinerea	57a	Şarköy/ Kirazlı Köyü

DSMZ: Institute of German Collection of Microorganisms and cell cultures

2.1.2. Culture Media

Pichia anomala (NCYC 434) which produce the K5 type yeast killer protein and *Saccharomyces cerevisiae* (NCYC 1006) which is sensitive strain to the killer toxin was grew in YEPD medium including. In order to synthesis of the K5 type yeast killer protein in cells of *Pichia anomala* YEPD medium was buffered at pH 4.5 with acetic acid and 5% glycerol was also added in YEPD medium. The aim of the usage of the buffer at pH 4.5 was to determine the killer activity of the K5 type yeast killer protein.

Botrytis cinerea isolates were cultivated in potato dextrose agar (PDA) consisting of 4% potato extract, 2% dextrose and 1.5% agar plates. Synthetic RPMI 1640 medium dissolved in pH 4.5 100 mM Na_2HPO_4 -citric acid buffer containing 100mM Na_2SO_4

was prepared for the broth microdilution antifungal susceptibility testing. Sabouraud medium (SDA) which compose of 1% Bacto-peptone and 2% dextrose along with 2% bacto-agar dissolved in pH 5.5 100mM Na_2HPO_4 and 100mM Na_2SO_4 citric acid buffer at 5.6 was used for agar diffusion assays in order to determine susceptibility of *Botrytis cinerea* to *Pichia anomala* strain.

2.1.3. Chemicals

The chemicals and the suppliers used in all methods were indicated in the Appendix A.

2.1.4. Buffers

Buffers and other solutions used in all methods were listed in Appendix B.

2.2. Methods

2.2.1. Sterilizations

The growth medium for the yeast cells and fungal cells were disinfected at 121℃ for 15 minutes on liquid cycle. For sterilization of RPMI 1640 medium 0.45 µm and 0.22 µm (Sartorius, AG, Germany) filters are used respectively. In order to be used in the chromatographic purification steps buffers were filtered through 0.45 µm cellulose acetate filters (Sartorius, AG, Germany). All parts of filter device (Sartorius, AG, Germany) were sterilized before usage of them.

2.2.2. Maintenance of the Fungal Cultures

Freeze-dried strains of *Pichia anomala* (NCYC 434) and *Saccharomyces cerevisiae* (NCYC 1006) in glass ampules were opened aseptically and 0.5 ml of YEPD medium was added in order to dissolve the dried culture by using pasteur pipette

sterilized before. Dissolved yeast cells were inoculated onto petri dishes including YEPD medium and incubated at 25℃ until the yeast colonies were formed [94].
The fungal plant pathogen, *Botrytis cinerea*, was isolated from grapevines (*Vitis vinifera*) in Tekirdağ and inoculated onto petri dish containing PDA medium at pH 5.5 and incubated at 25℃ for 5 to 7 days. All Botrytis strains were kept at 4℃ for a maximum of 3 weeks. Before antifungal susceptibility tests Botrytis strains were subcultured to PDA plates to increase spore formation.

2.2.3. Agar diffusion Assay

Antifungal activity of *Pichia anomala* (NCYC 434) on fungal pathogen causing grey mold disease on grapes was determined by agar diffusion assay performed on SDA plates including 1% Bacto-peptone, 2% dextrose and 2% Bacto-agar adjusted with HCL at pH 5.6.

The spore suspension of *B. cinerea* were prepared as described previously and adjusted to the concentration 4 $x10^5$ by microscopic enumeration technique with a hemocytometer (Neubauer Improved Chamber, Brand, Germany).

Before spore inoculation, SDA plates were dried in incubator at 40 minutes at 30℃ in order to absorb the spores easily. One ml of the spore suspension of each strain was inoculated on SDA plates and disseminated with spreader. SDA plates absorbed the spore suspension for 1 hour.

A loop full of *Pichia anomala* was suspended in 1ml of steril water and 50 µl was spread on the middle of each SDA plate including the spore suspension and incubated at 25℃ for 5 days. After incubation, the antifungal activity was determined by observing a zone between *Pichia anomala* and the spore suspension of *B. cinerea*. Experiments were replicated at least three times.

2.2.4. Production and Concentration of the K5 Type Yeast Killer Toxin

The killer protein was produced and concentrated according to the studies reported previously by Izgü and Altınbay [62]. *Pichia anomala* (NCYC 434) cells were inoculated into 10 ml of YEPD medium and incubated for 6 hours at 25℃. Two ml of yeast cell suspension was inoculated into 100 ml of the same medium. After the incubation of the yeast cells at 25℃ for 16 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA), 40 ml of cell suspension was transferred to 4L of YEPD medium including 5% of glycerol buffered to pH 4.5 with acetic acid and incubated until stationary phase at 18℃ for 36 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). In order to separate the culture medium centrifugation (KR 22i, Jouan, France) was applied at 9500 rpm for 10 min at 4℃ and supernatant was filtered 0.45µm and 0.22 µm cellulose acetate membranes (Sartorius, AG, Germany) respectively.

2.2.5. Ultrafiltration of the K5 type yeast killer toxin

Concentration of the cell free culture medium including the K5 type yeast killer protein is done by using 30kDa molecular cut-off ultrafilter (Satorius, Vivaflow 50/200,AG, Germany). At the end of the ultrafiltration concentrated cell free culture is called crude protein (Figure 2.1.) [62].

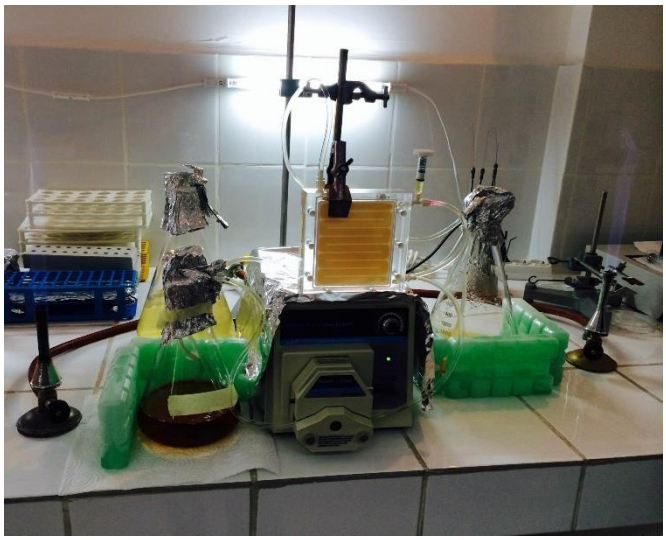

Figure 2. 1. 30kDa molecular cut-off ultrafilter set up unit for concentration of the K5 type yeast killer protein.

2.2.6. Purification of the Killer Toxin

Crude protein obtained from ultrafiltration was submitted to gel permeation chromatography by using FPLC system (Biocad 700E Perspective Biosystems) containing an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan) at 280 nm at 20℃ [62].

2.2.6.1. Gel-Permeation Chromatography

After ultrafiltration crude protein was submitted to gel-permeation chromatography by using a TSK G2000 SW (7,5 mmD/300mmL TosoHaas, Japan) column (Figure 2.2.). Before injection of the sample the column was balanced with 100mM Na_2PO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 at a flow rate of 1ml/min. The equilibration was performed until the base line was stable. 90 µl of the sample was injected into the column and elution was done with the same buffer at a flow rate of 1 ml/min.

Figure 2. 2. FPLC for Gel permeation chromatography for purification of the K5 type yeast killer protein.

At the end of the gel-permeation chromatography active fraction, killer protein, was concentrated and buffer exchanged with the same buffer but the salt by using 5kDa molecular cut-off ultrafilters (Vivaspin VS2021, Sartorius, AG, Germany).

30 µl purified killer protein was spotted to YEPD agar plates at pH 4.5 inoculated with killer toxin sensitive cells, *S. cerevisiae* (NCYC 1006), for the determination of the killer activity of the killer protein [62].

2.2.7. Determination of Killer Toxin Activity

Agar diffusion assay was used to determine the toxin activity of killer protein at different stages of the study as described by Brown et al. [95]. 1ml of *Saccharomyces cerevisiae* (NCYC 1006) cells found in sterile water adjusted at 10^5 was inoculated in 25 ml of liquid YEPD agar at pH 4.5 and poured into petri dishes. 30 µl of protein sample were spotted on the petri dishes and incubated at 25℃. In order to understand the killer activity of the protein sample the clear zone of the growth inhibition of the seeded killer toxin sensitive cells was measured after 48 hours of incubation. The clear zone of 10 mm in diameter was defined as 1 arbitrary unit (AU). This test is used at the end of the studies including filtration, ultrafiltration and purification assays.

2.2.8. SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel in a discontinuous buffer system developed by Ornstein and Davis (1964) [96] was used to determine the molecular weight of the pure killer protein by using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA). Stacking gel called was layered on top of the separating (running) gel. Different buffers were used to make each gel layer and the tank buffer was different from the gel buffers. When electrophoresis was initiated, the ions and the protein began to migrate in stacking gel until they reached to the separating gel.

In order to prove purity of the killer protein, protein sample concentrated with gel permeation chromatographic step was exposed to electrophoresis on 1.5mm thick polyacrylamide gel in a discontinuous buffer system using a SE 600 vertical slab gel electrophoresis (Hoefer, USA).

Separating (running) gel was made and filled in the electrophoresis unit and covered with 50 µl of the saturated n-butanol to prevent contact with air and left over night. When the polymerization step was ended up, n-butanol was washed with both sterile water and stacking buffer respectively, then stacking gel was filled and a comb was inserted on the separating gel solution and left to polymerize for 2 hours. In this step there must be no bubbles below of the combs. Component of the running and stacking gel solutions are found in Table 2.2. and Table 2.3 respectively.

Protein samples were mixed with 125 mM Tris-Cl at pH 6.8. They were heated at 100℃ for 5 minutes for denaturation of protein samples. Sample buffer includes 20% v/v glycerol, 4% (v/w) SDS, 0.02% (v/w) bromophenol blue at pH6.8. 10 % 2-β-mercaptoethanol was also added to the sample buffer for the denaturing SDS PAGE. For one side 40 µl of the protein marker solution including 5µl of protein markers which have specific molecular weight, 20 µl of the treatment buffer and 15 µl of the stacking buffer and for another side 60µl of the protein sample solution including 30 µl of the pure protein sample and 30 µl of the treatment buffer were loaded on the gel at the end of the polymerization of the stacking gel (Figure 2.3.).

Electrophoresis was made at 15 mA/ 1.5 mm gel (Power supply PP4000, Biometra, Germany) at 15℃ using a circulating water bath (Heto Holten, Denmark).

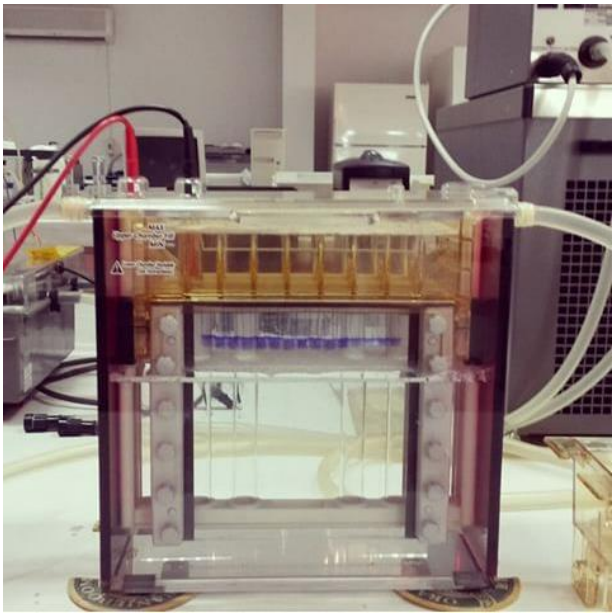

Figure 2. 3. SDS gel electrophoresis (The SE 600) fully assembled.

Table 2. 2. Running Gel recipes for 1.5 mm thick gel

	12.5% Gel
Monomer Solution	25 ml
4X Running Gel Buffer	15 ml
10% SDS	0.6 ml
ddH_2O	19.1 ml
10% Ammonium Persulfate	300 µl
TEMED	20 µl

*Ammonium Persulfate and TEMED were put in after deaeration.

Table 2. 3. Stacking Gel Solution

Gel thickness	1.5 mm
Monomer Solution	2.66 ml
4X Stacking Gel Buffer	5.0 ml
10% SDS	0.2 ml
ddH_2O	12.0 ml
10% Ammonium Persulfate	100 µl
TEMED	10 µl

*Ammonium Persulfate and TEMED were put in after deaeration.

2.2.9. Protein Detection in SDS Gel: Coomassie Brillant Blue

Coomassie Brillant Blue staining method was used to detect the protein bands on the gel. The gel was placed in 100 ml of Staining solution composed of 0.025%

Coomassie Brilliant Blue R 250, 40% methanol and 7% acetic acid overnigt. After staining solution, the gel was placed in 100 ml of Destain 1 solution including 40% methanol, 7% acetic acid and incubated for 30 minutes with gentle shaking. After Destain I solution, the gel was put into 100 ml of Destain II solution containing 7% acetic acid, 5%methanol and incubated overnight. After Destain II, protein sample were visualized as blue bands on a clear background [97].

2.2.10. Measuring Protein Concentration

The K5 type yeast killer protein concentration was measured with nanodrop (Termo Scientific 2000c, UV-Vis Spectrophotometer).

2.2.11. Antifungal Susceptibility Assays

Before antifungal susceptibility testing, *Botrytis cinerea* strains were subcultured on PDA plates and incubated at 25˚C because fresh, mature *Botrytis cinerea* spores were required (1 to 2 week old). In order to obtain spore suspensions, *Botrytis cinerea* cultures plates were flooded with 6 ml of the sterile distilled water including 0.05% Tween 80 (Merck) and waited for ten minutes. Spores were collected with 1000 µl pipette and transferring into a sterile 50 ml falcon tube. Collected spore suspension was filtered with a pore diameter of 11µm (Millipore NY1104700, Madrid, Spain). In this step, hyphae was separated from spore suspension. Hyphae free spore suspension was vortexed for 15 seconds (Heidolph, Germany). Concentrated spore suspension was defined by microscopic enumeration with a cell-counting hemocytometer (Neubauer Improved Chamber, Brand, Germany) and the colonies were counted.

2.2.11.1. Broth Microdilution Assay

Minimum inhibition concentrations (MICs) of the K5 type yeast killer protein for plant pathogenic fungal strains were defined by broth microdilution technique

depending on the guidelines suggested by the Clinical and Laboratory Standards Institute (CLSI) in document M-38-A [98] by using 96-well plates were used.
Two-fold dilution series of the K5 type yeast killer protein ranging from 64 to 2048 µg/ml were prepared in 100mM Na_2PO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 . RPMI 1640 (Sigma Aldrich, 095K8310) concentration was prepared in 50 ml of 100mM Na_2PO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 at pH 4.5 for susceptibility testing as a medium (2x RPMI 1640).

Fungal suspension consisting of *B. cinerea* spores were prepared for each fungal strains by using hemocytometer counting technique and diluted serially at 50 fold in RPMI 1640 medium at pH 4.5. Spore size was seen easily and reliable by using hemocytometer. The inoculum suspension were adjusted to the concentration of 4 $x10^5$ to 50 $x10^5$ spore/ml. Then, the suspensions were diluted 50 fold with 2 concentrated RPMI 1640 medium to take inoculum concentration 4 x 10^4- 10x 10^4 spores/ml.

100 µl of two-fold protein dilution ranging 64 to 1024 µg/ml including100mM Na_2HPO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 were inoculated to each well of 96-well plates, except the growth control and sterility control. 100 µl of spore suspension in RPMI 1640 medium buffered with 100mM Na_2HPO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 were inoculated on the K5 type yeast killer protein found in each well, except sterility control and incubated at 25℃ for 72 hours. This was done for each *B. cinerea* strain. There were 100 µl of 100mM Na_2HPO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 and 100 µl of RPMI 1640 buffered with 100mM Na_2HPO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 in sterility control. On the other hand, 100 µl of the spore and RPMI 1640 mix and 100µl of the buffer solution were found in growth control well.

The growth in each well was compared with growth control well by inverted routine microscope (Nikon Eclipse TS 100, MODEL H-III, Japan). The growth in each well was also seen visually (Figure 2.4.).

Minimum inhibitory concentration (MIC) was described as the lowest concentration of the K5 type yeast killer protein where wells were without any visible growth or 100% decline in the growth. Inhibitory concentration (IC_{50}) was showed the 50% of the growth inhibition of the K5 type yeast killer protein [99].
Two replicates were prepared in all experiments for each treatment and were repeated at least three times for each *B. cinerea* spore suspension.

Under inverted routine microscope the growth in wells including different concentration of the K5 type yeast killer protein were observed and compared to the growth control.

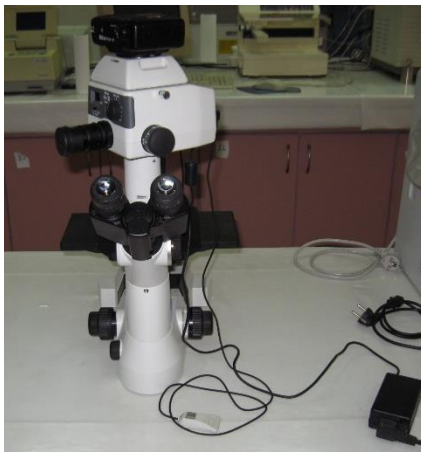

Figure 2. 4. Inverted microscope (Nikon Eclipse TS 100, MODEL H-III, Japan).

CHAPTER 3

RESULTS

3.1. Agar diffusion Assay

The inhibition of different *B. cinerea* isolates by *P. anomala* was determined as explained in material and methods. There was a clear growth inhibition zone between *B. cinerea* and *P. anomala* on SDA medium because the K5 type yeast killer toxin secreted by *P. anomala* (NCYC 434) degraded the β 1,3 glucan residues in cell wall of mycelium (Figure 3.1 to 3.7). The degree of inhibition was quite different among the *B. cinerea* isolates. Thus, the strongest retardation was observed in *B. cinerea* strain (DSMZ 5145) (Figure 3.1).

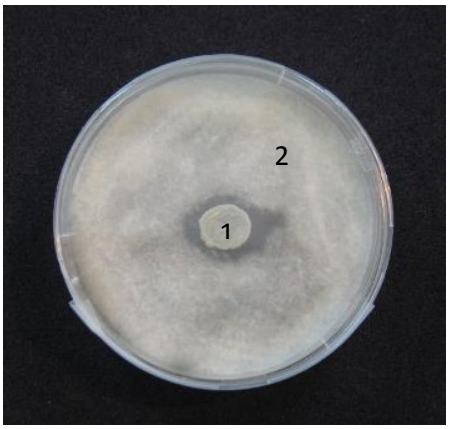

Figure 3. 1. Inhibition of *B. cinerea* strains (DSMZ 5145) with *Pichia anomala* (NCYC 434) 50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at25℃ for 5 days 1) *Pichia anomala* cells 2) Mylcelium *of B. cinerea* spores.

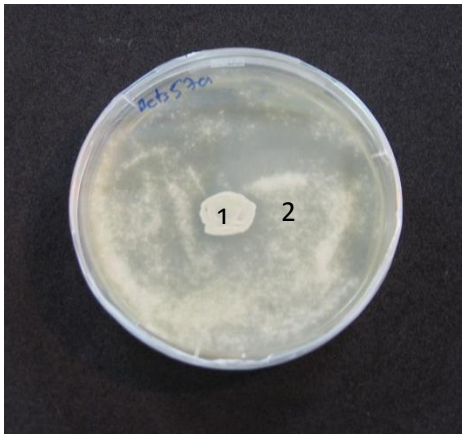

Figure 3. 2. Inhibition of *B. cinerea* strains (57a) with *Pichia anomala* (NCYC 434)50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at 25℃ for 5 days 1) *Pichia anomala* cells 2) Mycelium of *B. cinerea* spores.

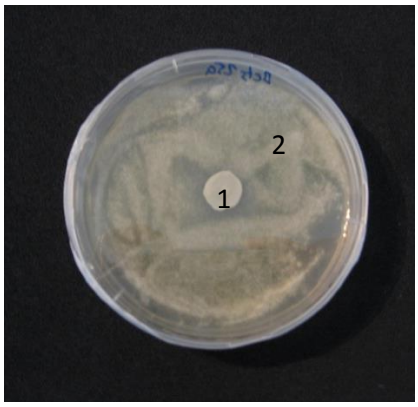

Figure 3. 3. Inhibition of B. cinerea strains (25a) with *Pichia anomala* (NCYC 434)50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at 25℃ for 5 days 1) *Pichia anomala* cells 2) Mycelium of *B. cinerea* spores.

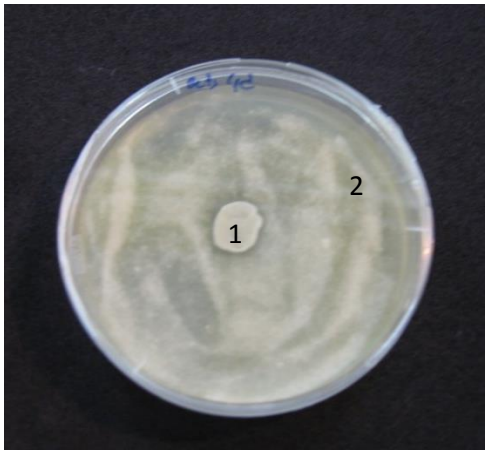

Figure 3. 4. Inhibition of *B. cinerea* strains (4d) with *Pichia anomala* (NCYC 434) 50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at 25℃ for 5 days 1) *Pichia anomala* cells 2) Mycelium of *B. cinerea* spores.

Figure 3. 5. Inhibition of *B. cinerea* strains (11b) with *Pichia anomala* (NCYC 434)50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at 25℃ for 5 days 1) *Pichia anomala* cells 2) Mycelium of *B. cinerea* spores.

Figure 3. 6. Inhibition of *B. cinerea* strains (19a) with *Pichia anomala* (NCYC 434)50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at 25℃ for 5 days 1) *Pichia anomala* cells 2) Mycelium of *B. cinerea* spores.

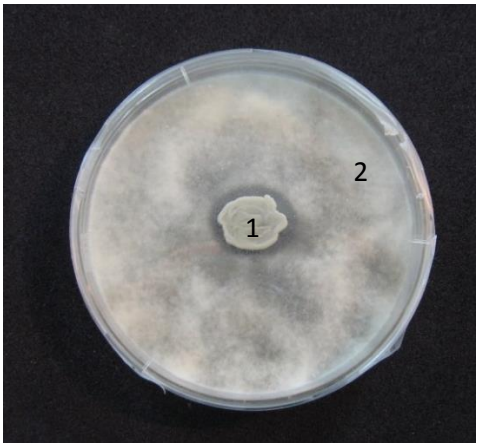

Figure 3. 7. Inhibition of *B. cinerea* strains (15c) with *Pichia anomala* (NCYC 434)50µl of the *Pichia anomala* cells in 1 ml steril water spreaded on *B. cinerea* spores at 25℃ for 5 days 1) *Pichia anomala* cells 2) Mycelium of *B. cinerea* spores.

3.2. Production and concentration of the K5 Type yeast Killer Protein

Temperature and pH value were adjusted to optimum value. *P. anomala* cells were grown in YEPD medium including 5% glycerol to stabilize the killer activity of the protein at pH 4.5 at 25℃. *P. anomala* cell were separated from the culture medium by centrifugation. After centrifugation concentration of cell free culture was done with 0.45μm and 0.22 μm cellulose acetate membrane filters [62].

3.3. Ultrafiltration of the K5 type of killer protein

4 liter of the cell free culture was concentrated to 50 ml with 30 kDa ultrafilter. The protein which was collected in return flask of ultrafilter set up unit was called the crude protein. The crude protein formed of both the K5 type yeast killer proteins and other proteins. The crude protein concentration was measured 20μg/ml.

3.4. Purification of the K5 type yeast killer protein

The crude protein was centrifuged for concentration. The K5 type yeast killer protein was purified by gel-permeation chromatography by using TSK G 2000SW column. In column 90 μl protein sample passed through. This killer protein was collected in 11. fraction between 10 to 20 minutes. Big pick showed us the K5 type yeast killer protein because there were more amount of our killer proteins than this than other proteins. On the other hand, other proteins were observed in small picks (Figure 3.8). After gel permeation chromatography, the the K5 type yeast killer protein was concentrated with 0.22 μl filter in 100mM Na_2PO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4.

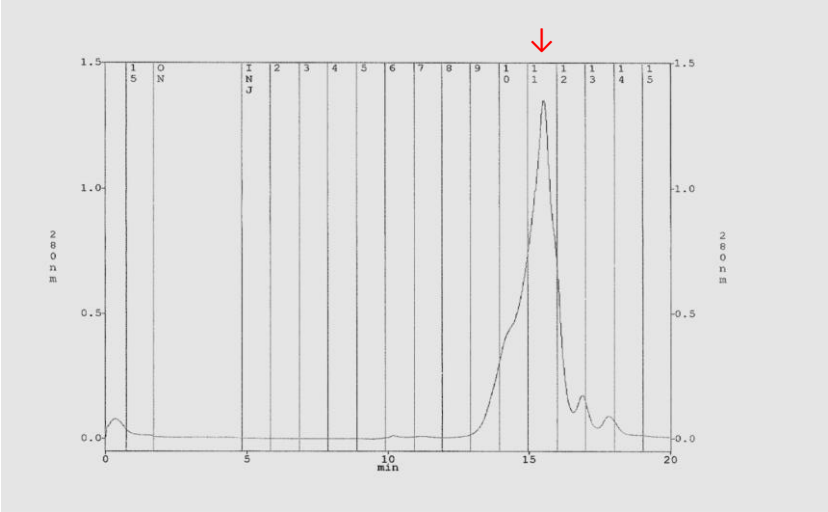

Figure 3. 8. The elution of the K5 type yeast killer protein on a TSK G2000SW Column

Column size: 7.5 mm ID x 30 cm; Protein sample: 90 µl; Elution buffer: 100mM Na_2PO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4; flow rate: 1ml/min; detection 280 nm UV .The K5 type yeast killer protein was indicated by red arrow.

3.5. Protein Concentration

The concentration of the crude protein containing both the K5 type yeast killer protein and other proteins was 20µg/ml. On the other hand, the concentration of the purified K5 type yeast killer protein was 8µg/ml. They were measured with nanodrop (Termo Scientific 2000c, UV-Vis Spectrophotometer).

3.6. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Pure K5 type yeast killer protein was electrophorated on a SDS-PAGE gel in a discontinuous buffer system in order to determine its purity. SDS-PAGE gel was stained with Coomassie Brillant Blue staining method. Molecular mass of the K5 type yeast killer protein was determined with SDS-PAGE gel and learned to be 49 kDa (Figure 3.11).

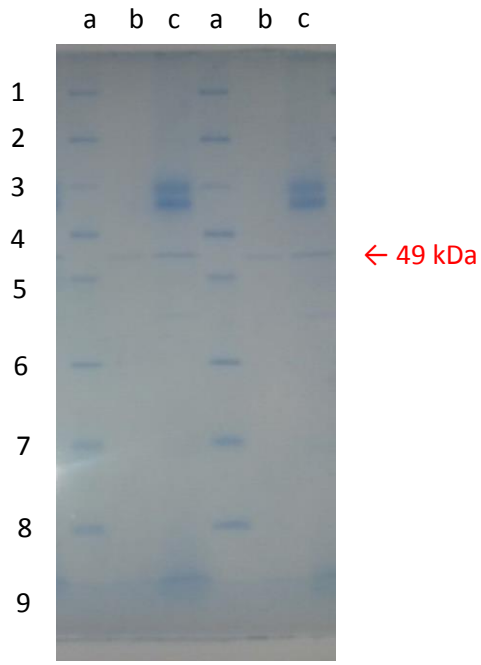

Figure 3. 9. The K5 type yeast killer protein shown in SDS-PAGE geL

a) 40 µl of the protein marker solution contained 5µl of protein markers which have specific molecular weight, 20 µl of the treatment buffer and 15 µl of the stacking buffer b) 60µl of the protein sample solution included 30 µl of the pure protein sample and 30 µl of the treatment bufferc) 60µl of the protein sample solution included 30 µl of the crude protein sample and 30 µl of the treatment buffer .The K5 type yeast killer protein was observed between number 4 and number 5 of the SDS protein markers depending on molekular mass (Da) with red arrow.

1) α_2 –macroglobulin (340.000-170.000) 2) β-galactosidase (116.400) 3) fructose-6-phosphate kinase (85.200) 4) glutamate dehydrogenase (55.600) 5) aldolase (39.200) 6) triose phosphate isomerase (26.600) 7) trypsin inhibitor (20.100) 8) lysozyme (14.300) 9) aprotinin (6.500).

3.7. Determination of killer activity of the K5 type yeast killer protein

Killer activity was tested with agar diffusion assay in both before and after the purification step. Thirty µl of the purified killer protein was spotted on YEPD medium at (pH 4.5) containing the killer toxin sensitive *S. cerevisiae* (NCYC 1006) cells and incubated at 25℃ for 48 hours. The same protocol was performed with the crude protein as well. The K5 type yeast killer protein both crude and purified sample formed clear growth inhibition zone (22 mm) at the middle of the YEPD including the toxin sensitive *S. cerevisiae* (NCYC 1006) cells (Figure 3.10 and Figure 3.11) respectively.

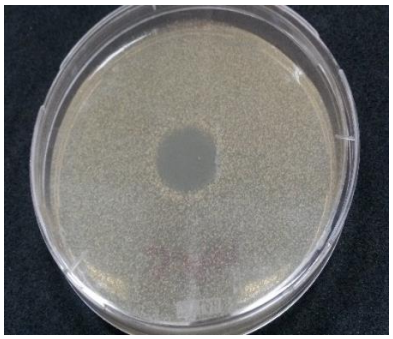

Figure 3. 10. Killer activity of the crude K5 type yeast killer protein determined by Agar Diffusion Assay. 30µl of the crude protein was spotted on the killer toxin sensitive *S. cerevisiae* (NCYC 1006) cells.

Figure 3. 11. Killer activity of the purified K5 type yeast killer protein determined by Agar Diffusion Assay.30µl of the purified killer protein was spotted on the killer toxin sensitive *S. cerevisiae* (NCYC 1006) cells.

3.8. Antifungal Susceptibility Assays

Antifungal activity of the K5 type yeast killer protein on plant pathogen was tested by broth microdilution assays.

3.8.1. Broth Microdilution Assay

According to CLSI (Clinical and Laboratory Standards Institute) M38-A (Reference method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi methodology) susceptibility of spore isolates to pure K5 type yeast killer protein in different concentration ranging from 64 to 2048 µg/ml was studied.

Minimum inhibitory concentrations (MICs) was determined visually by comparing the growth of the spores in each protein concentration of the K5 type yeast killer protein with growth control where the K5 type yeast killer protein was not found.
After incubation at 25℃ for 72 hours growth control wells for all *B. cinerea* strains were visually highly clouded. Although the growth at the protein concentration of

2048µg/ml was not clear, some inhibition of the cell growth could be observed (Figure 3.12 and Figure 3.13).

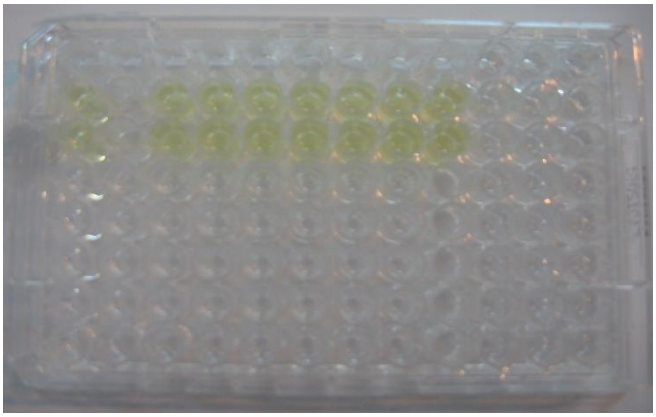

Figure 3. 12. The susceptibility of *Botrytis cinerea* to the K5 type yeast killer protein on a Microtiter plate.

64 to 2048µg/ml of this killer protein were prepared in 100mM Na_2PO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 .The spore suspensions were diluted 50 fold with RPMI 1640 medium to take inoculum concentration 4 x 10^4- 10x 10^4 spores/ml.

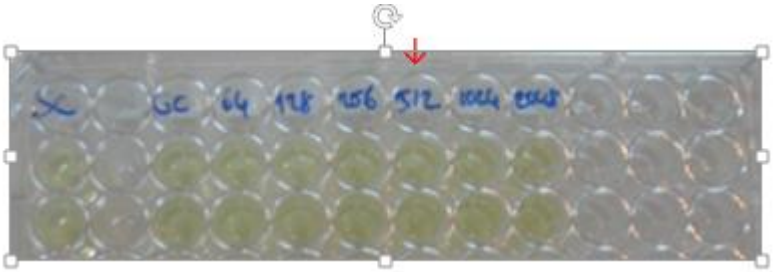

Figure 3. 13. SC: Sterility control, GC: Growth control and the different protein concentration 64µg/ml to 2048µg/ml respectively.

In sterility control of the wells 100µl of 100mM Na_2HPO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 and 100 µl of RPMI 1640 buffered with 100mM Na_2HPO_4-citric acid buffer, at pH 4.5, including 100mM Na_2SO_4 were found. In growth control of thewells 100 µl of the spore and RPMI 1640 mix and 100µl of the buffer solution were found. In other wells both 100 µl of the spore and RPMI 1640 mix and 100µl of the buffer solution and 100 µl of the different protein concentration

64µg/ml to 2048µg/ml respectively. MIC_2 of the K5 type yeast killer protein was 512 µg/ml showed with red arrow.

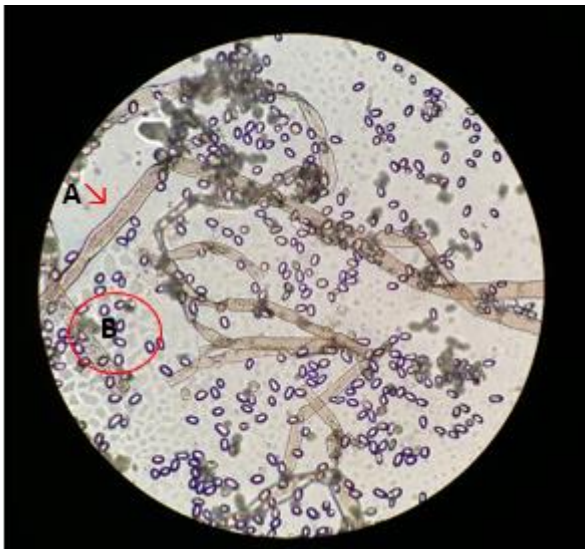

Figure 3. 14. Microscopic examination of *Botrytis cinerea* taken by light microscope Nikon (40X) A) Mycelium of *B. cinerea* B) Spores of *B. cinerea*.

Typically, *B. cinerea* strains are seen with spores and mycelium under light microscope (40X) after incubation at 25℃ for 7 days as given in Figure 3.14. However, mycelial growth was seen as a cottony clump in the wells so the difference between the 96-well plates could not be observed by naked eyes. Thus, in the wells the growth inhibitory activity of the K5 type yeast killer protein was observed with inverted microscope. The images were taken after 72 hours incubation, from each well of the microtiter plates containing K5 type yeast killer protein and growth control wells. The microscopic images from wells containing different protein concentrations were compared with images of the growth control wells with no protein. (Figure 3.15 and 3.21).

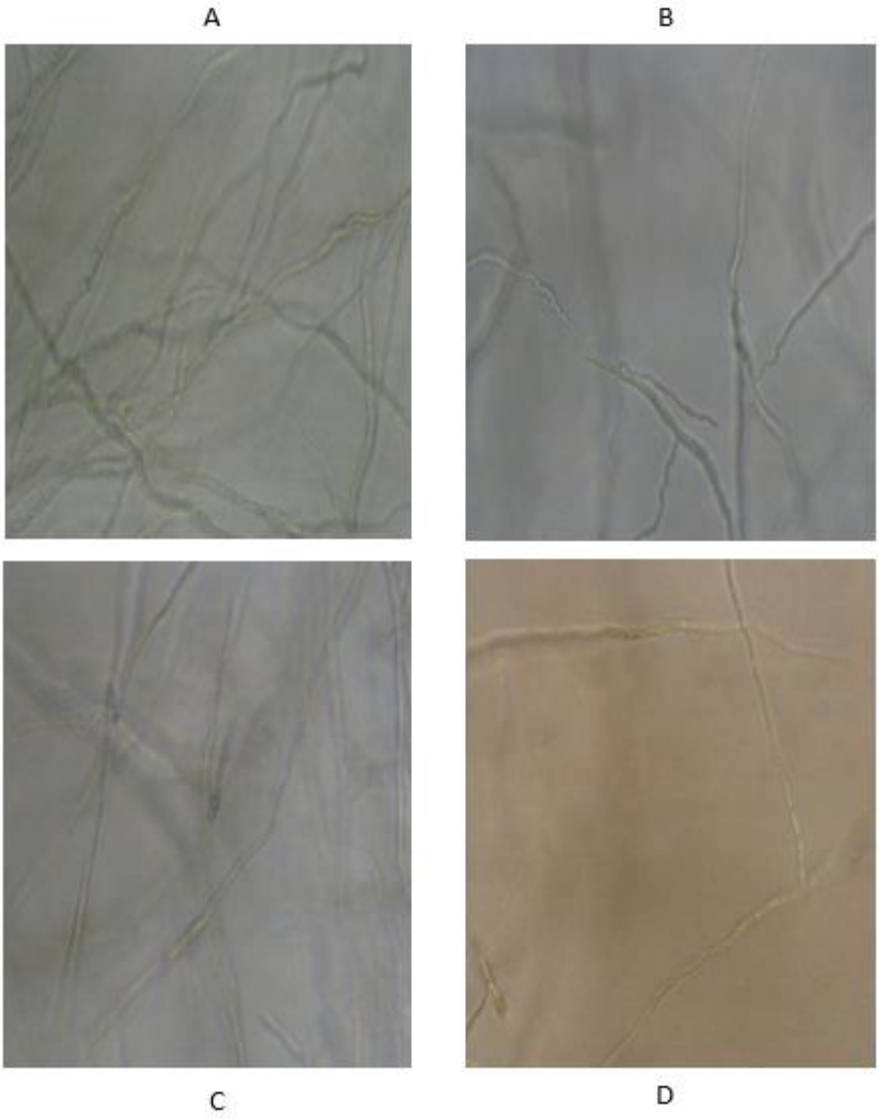

Figure 3. 15. Microscopic examination of the *Botrytis cinerea* strain (DSMZ 5145) taken with inverted microscope (LWD 20X/0.40X)

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 µg/ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 µg/ml of the K5 type yeast killer protein D) Mycelia from culture exposed to 2048 µg/ml of the K5 type yeast killer protein

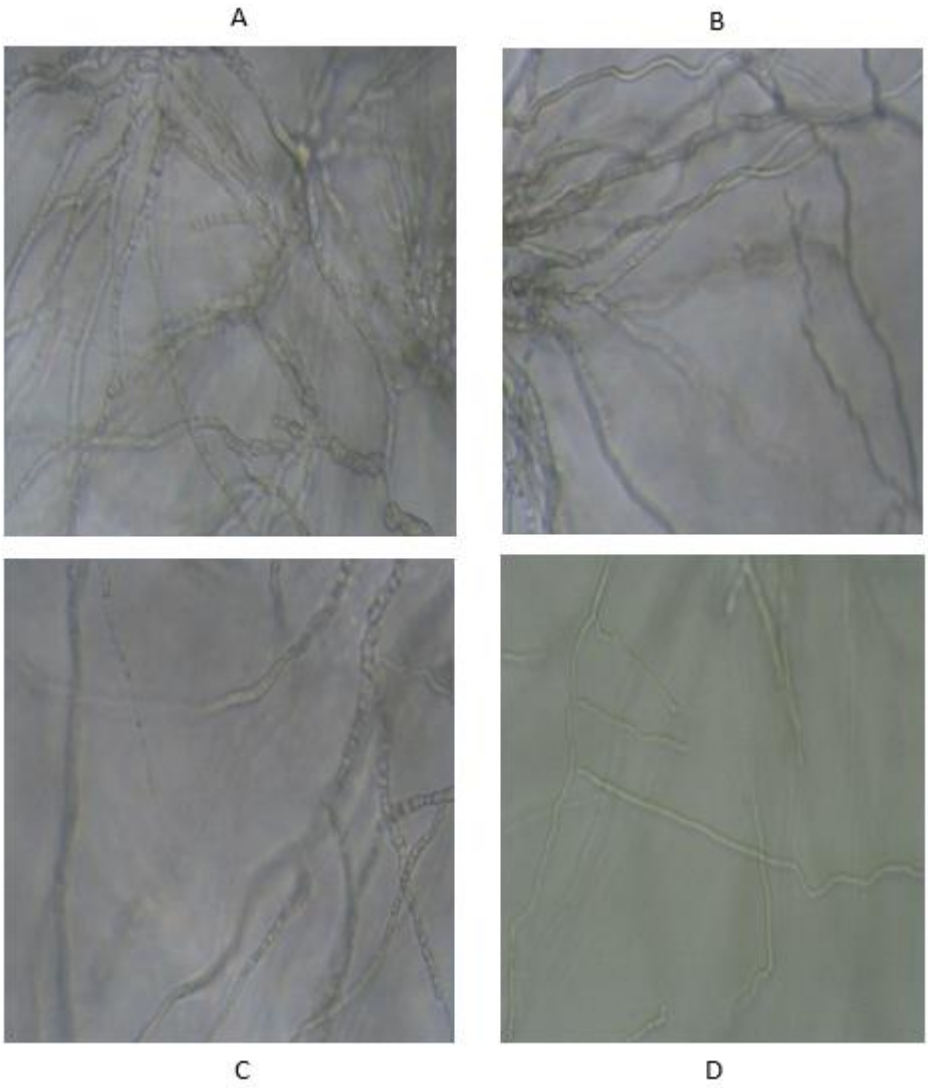

Figure 3. 16. Microscopic examination of *Botrytis cinerea* strain (57a) taken with inverted microscope (LWD 20X/0.40X)

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 µg/ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 µg/ml of the K5 type yeast killer protein D) Mycelia from culture exposed to 2048 µg/ml of the K5 type yeast killer protein

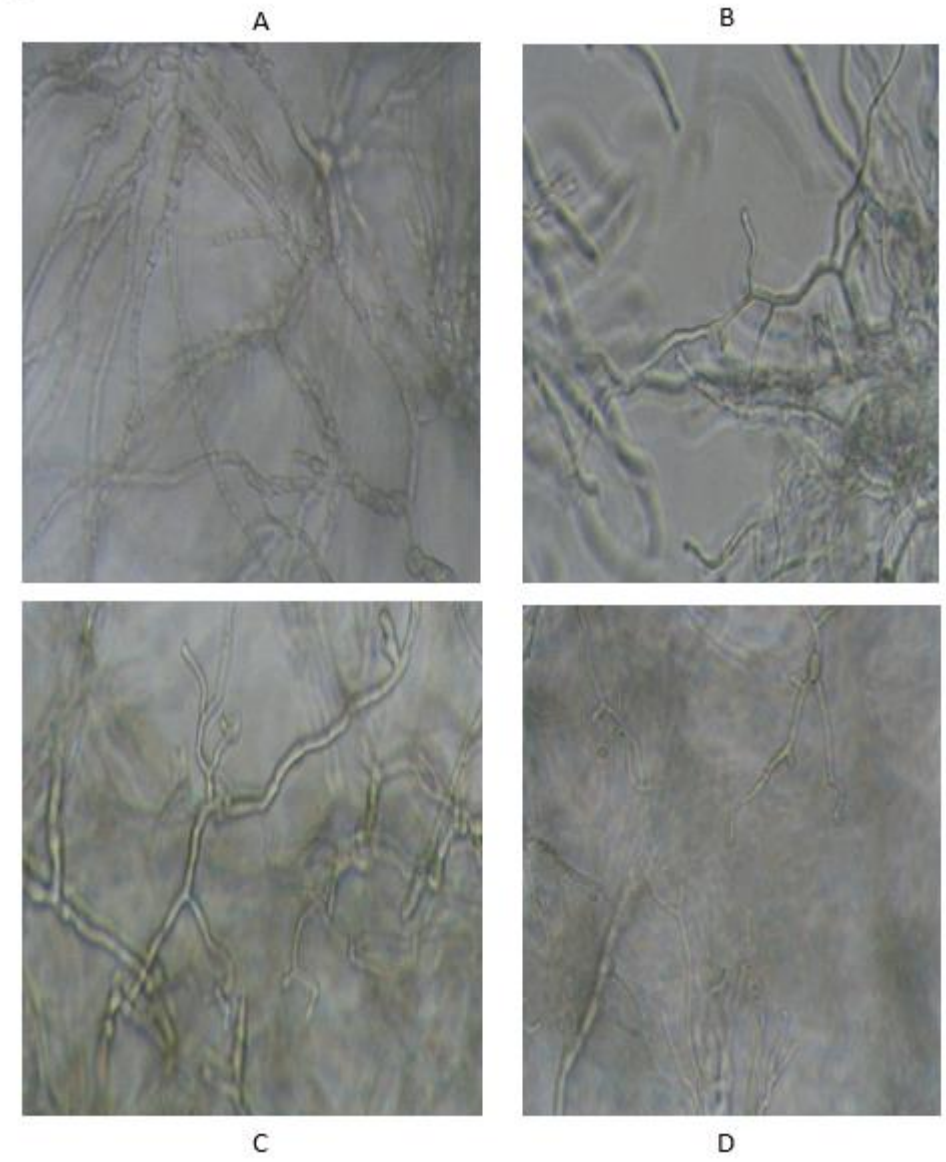

Figure 3. 17. Microscopic examination of *Botrytis cinerea* strain (25a) taken with inverted microscope (LWD 20X/0.40X).

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 ml of the K5 type yeast killer protein D) Mycelia from culture exposed to the K5 type yeast killer protein

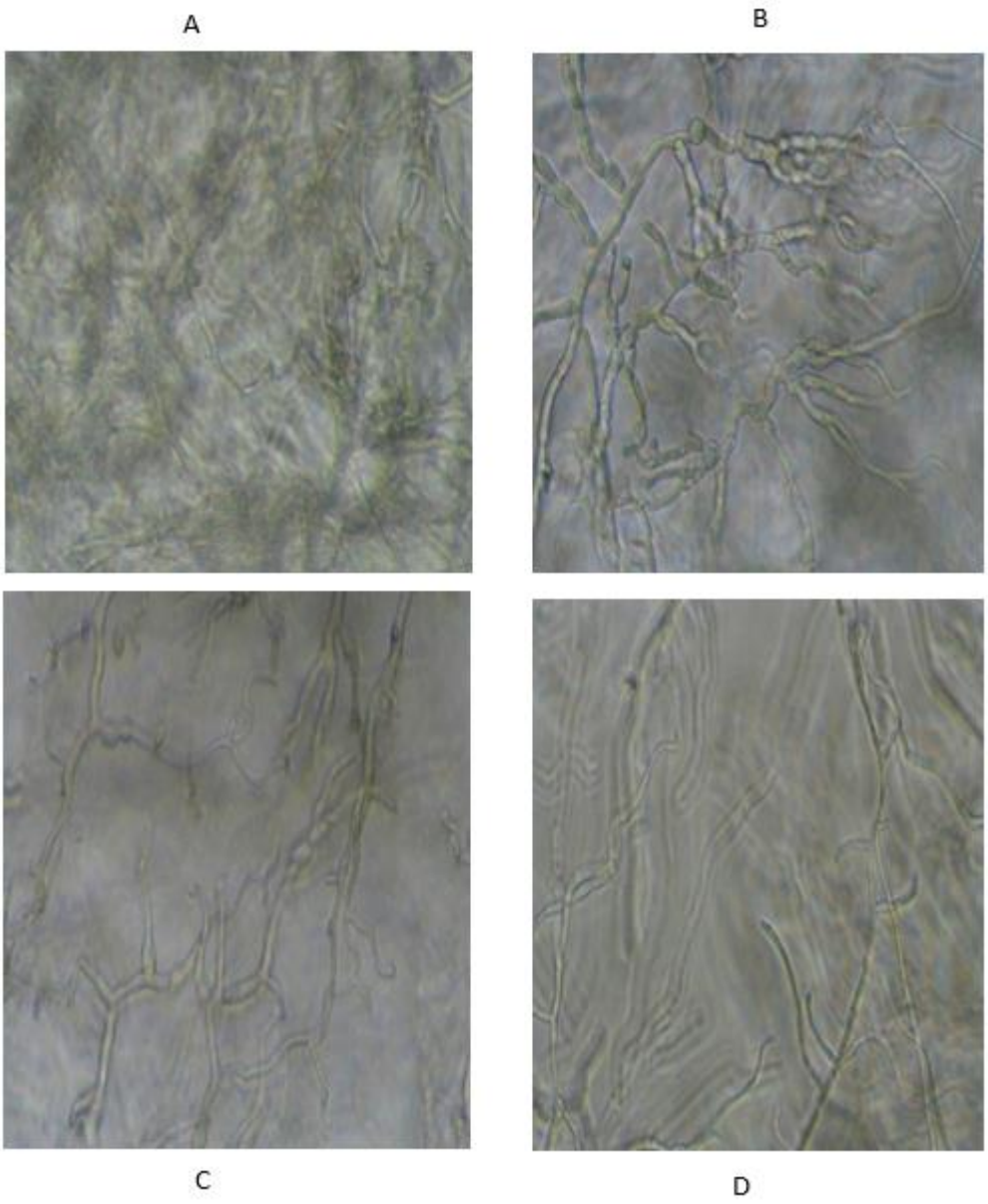

Figure 3. 18. Microscopic examination of *Botrytis cinerea* strain (4d) taken with inverted microscope (LWD 20X/0.40X)

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 µg/ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 µg/ml of the K5 type yeast killer protein D) Mycelia from culture exposed to 2048 µg/ml of the K5 type yeast killer protein

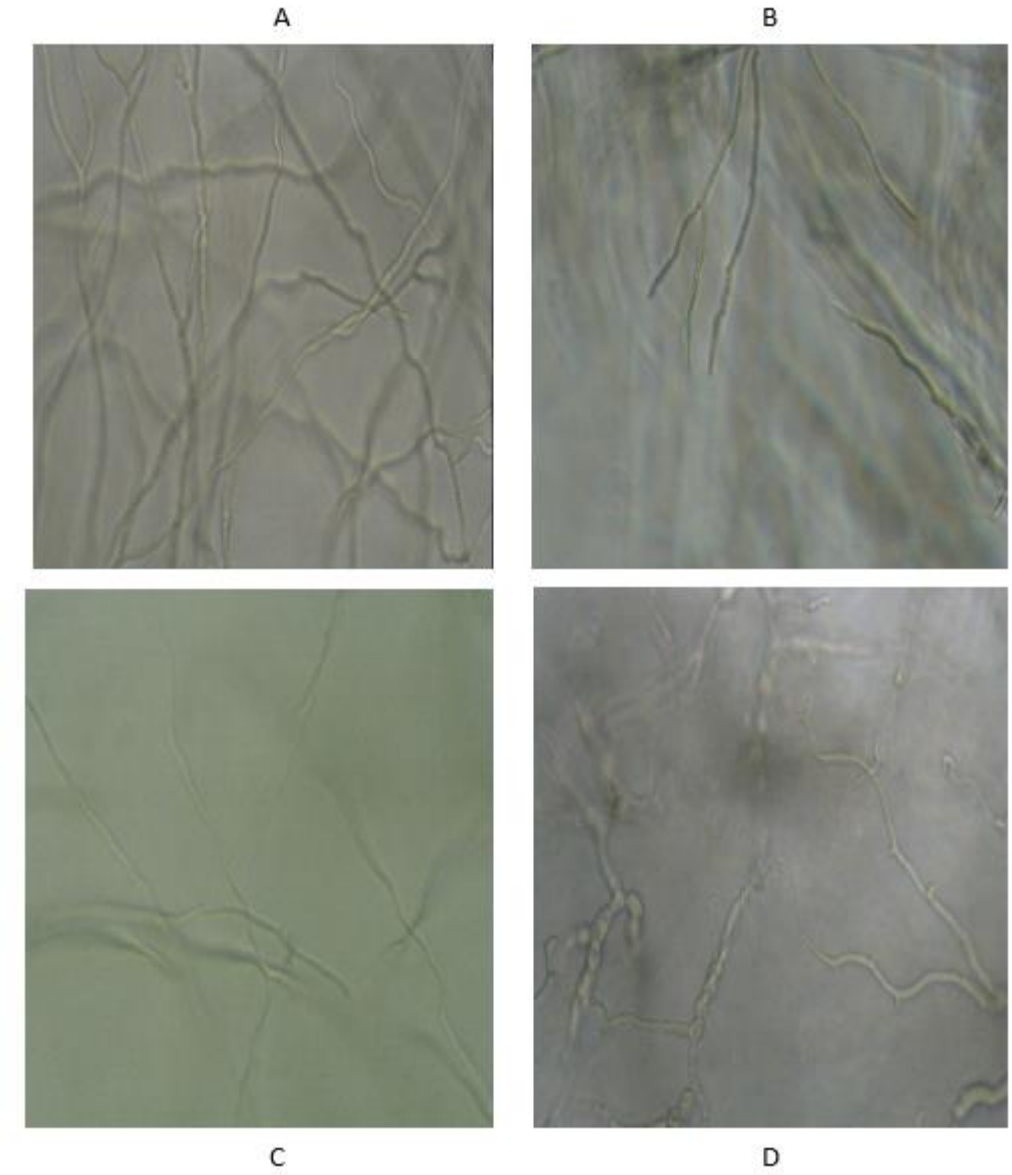

Figure 3. 19. Microscopic examination of *Botrytis cinerea* strain (11b) taken with inverted microscope (LWD 20X/0.40X)

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 µg/ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 µg/ml of the K5 type yeast killer protein D) Mycelia from culture exposed to 2048 µg/ml of the K5 type yeast killer protein.

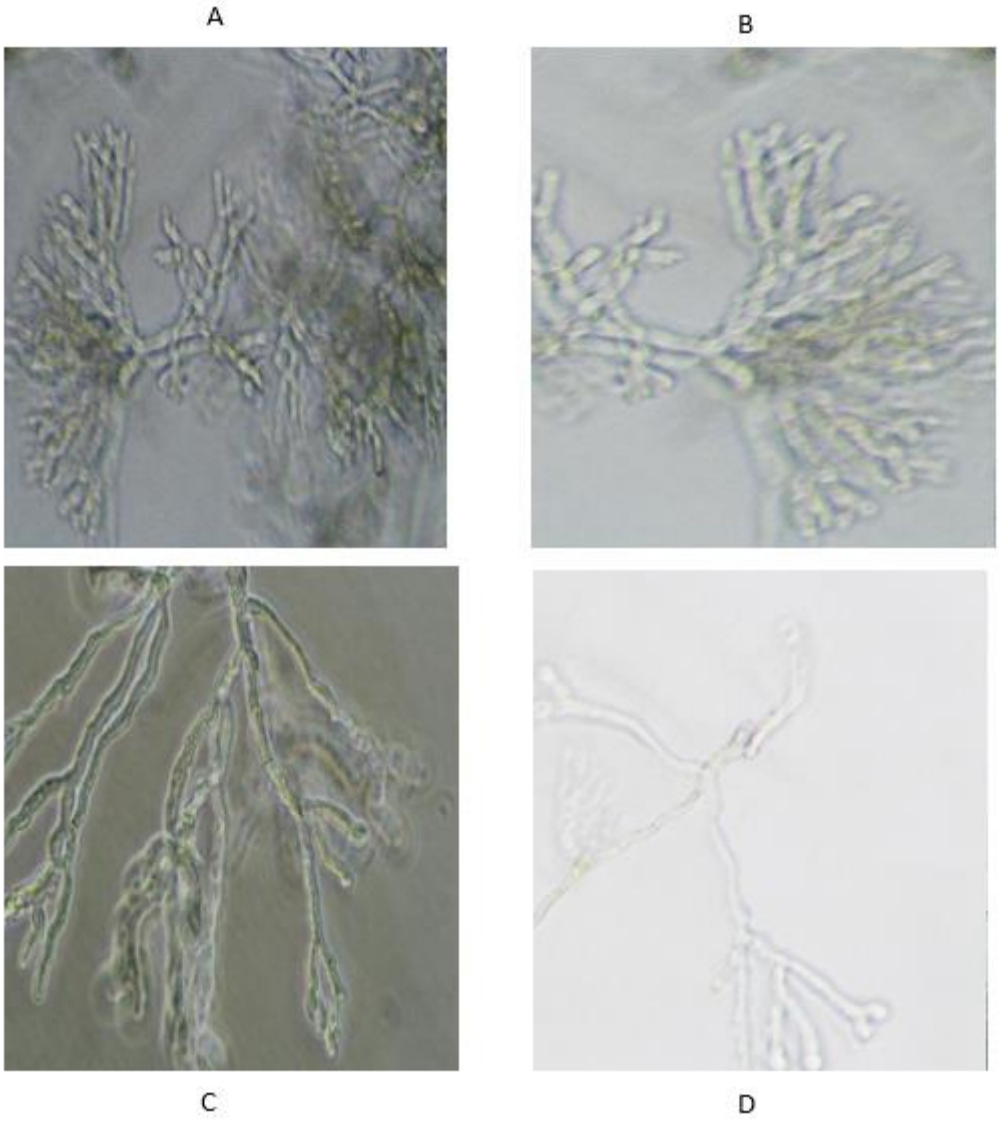

Figure 3. 20. Microscopic examination of *Botrytis cinerea* starin (19a) taken with inverted microscope (LWD 20X/0.40X)

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 µg/ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 µg/ml of the K5 type yeast killer protein D) Mycelia from culture exposed to 2048 µg/ml of the K5 type yeast killer protein

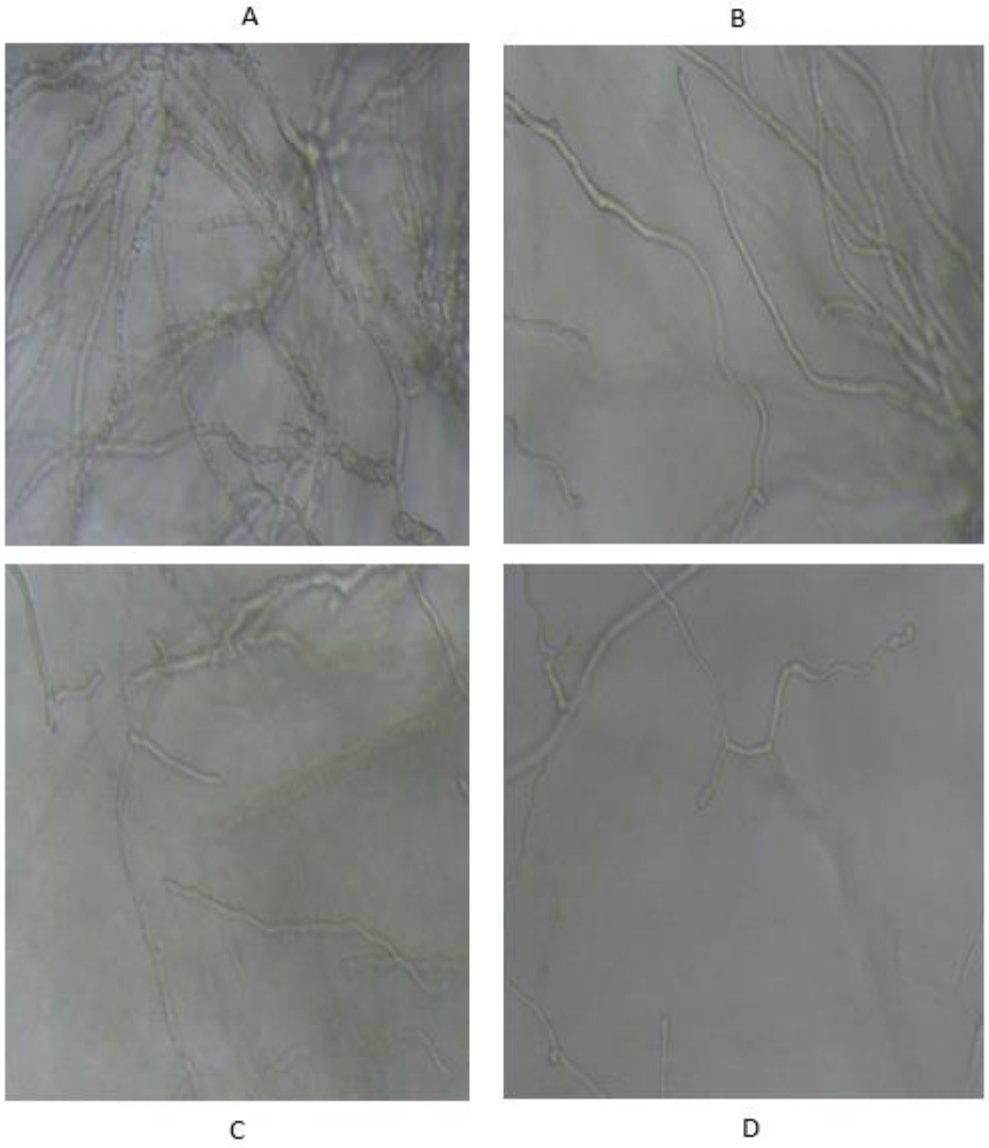

Figure 3. 21. Microscopic examination of *Botrytis cinerea* strain (15c) taken with inverted microscope (LWD 20X/0.40X)

A) Growth control mycelial growth of *B. cinerea* test strain in the absence pure K5 type yeast killer protein B) Mycelia from culture exposed to 64 µg/ml of the K5 type yeast killer protein C) Mycelia from culture exposed to 512 µg/ml of the K5 type yeast killer protein D) Mycelia from culture exposed to 2048 µg/ml of the K5 type yeast killer protein

According to inverted microscope images, growth inhibitory activity increases when the amount of the K5 type yeast killer protein concentration rises. *Botrytis cinerea* strain (DSMZ 5145) called type strain isolated from grape fields in Italy was the most sensitive strain to the K5 type yeast killer protein among the 6 *B. cinerea* strains. On the other hand, 5 *Botrytis* strains (Bcts 4d, Bcts 11b, Bcts 15c, Bcts 19a, Bcts 25a) isolated from grape fields in Tekirdağ were less susceptible to this killer protein. However , in 2048 µg/ml the killer activity of this killer protein on 6 *B. cinerea* strains was significantly. In 64 µg/ml, 128 µg/ml and 256 µg/ml killer protein inhibition activity was not fully on *B.* cinerea strains while in 512 µg/ml , 1024 µg/ml and 2048 µg/ml the killer protein inhibition activity was more effective on *Botrytis* strains. This killer protein reduced the mycelial growth and caused morphological changes in fungal hyphae.

CHAPTER 4

DISCUSSION

Grapes were produced in mainly Aegean, Mediterranean, and Central Anatolian regions in Turkey. Viticulture as an essential part of Turkey's agricultural structure makes a significant contribution to the Turkish economy. Fungal infection form more than 25 % of the major postharvest decays and lead to damage the crop quality and economic loss. Grey mold rot is one of these severe postharvest diseases [25].

Generally in order to prevent these fungal pathological diseases including grey mold rot synthetic fungicides are used. However, alternative control methods are required since these fungicides increase resistance to other fungicides [30-34,100,101].

Yeast killer proteins take attention among the alternative antifungal agents. Especially *Pichia anomala* has been widely studied among the other yeast strains. *P. anomala* known as killer yeast has wide range killing spectrum and powerful antagonistic activity against plant pathogens. Moreover, killer toxin of *P. anomala* called K5 type yeast killer protein has a wide range intergeneric killing spectrum and very high stability among other toxins of the other killer yeasts [35, 102].

This killer protein was used against different types of the fungal pathogens. Recently, antifungal activity of the pure K5 type yeast killer protein was tested on human pathogenic fungal species including *Candida* species, dermatophytes and on plant pathogenic fungal strains *Penicillium digitatum* and *Penicillum italicum* [92, 93,103]. We purified and characterized the K5 type yeast killer protein called Panomycocin from *P. anomala* with chromatographic methods [62, 79].

In this study the susceptible tests of the K5 type yeast killer protein was done on the type strain *B. cinerea* (DSMZ 5145) isolated from Italy and other *B. cinerea* strains including Bcts 4d, Bcts 11b, Bcts 15c, Bcts 19a, Bcts 25a isolated from different fields in Tekirdağ. The reasons to use these strains were both being causal agent of the grey mold disease of the grapevine and characterized as *B. cinerea*. Moreover, there were many studies with these isolates [109-111].

We have determined antifungal activity of *P. anomala* against *B. cinerea* strains which cause grey mold disease with agar diffusion assay. On petri plates when *P. anomala* and *B. cinerea* isolated from grapes (*Vitis vinifera*) grown together, clear inhibition zone was formed between them (Figure 3.1 to 3.7). Moreover, we can say that the presence of the cell wall of *B. cinerea* stimulated the production of the exo-β-1,3 glucanase [28].

When we viewed the results, we saw that *P. anomala* resulted in physiological and morphological changes in the hyphae by causing coagulation and destruction of the protoplasm [30]. Moreover, the secretion of exo-β-1,3 glucanase *by P. anomala* was highly effective for control of germination of the *Botrytis* spores on grapes. On other hand, this enzyme was also applied for prevention of Botrytis inhibition on apples [78]. When we compared with other *Pichia* species, they showed the same inhibitory effect on *B. cinerea* by secreting exo-β-1,3 glucanase [28].

This inhibition activity of the K5 type yeast killer protein was learned from broth microdilution assay depending on CLSI (Clinical and Laboratory Standards Institute) M38-A. When we analyzed the data, the pure K5 type yeast killer protein have significant inhibitory effect on *B. cinerea* strains in 96-well plates (Figure 3.12 and 3.13). However, the difference between the wells containing various concentration of the K5 type yeast killer protein could not be observed by naked eye. Thus, each of the well including spore suspension and the killer protein was investigated for the morphological changes of the mycelium with inverted microscope after 72 hours. We took different microscope images from the 96-well plates (Figure 3.15- Figure 3.21).

We viewed all images of the mycelial growth of *B. cinerea* strains taken from 96-well plates. When we compared to the wells containing the growth control and the wells including both spore suspension and the K5 type yeast killer protein range from 64 μg/ml to 2048 μg/ml, it was obvious that the plant pathogen *B. cinerea* was susceptible to the Panomycocin which have exo β 1,3 glucanase activity.

However, in wells including 64 μg/ml, 128 μg/ml and 256 μg/ml of the killer protein inhibition activity could not be seen on *Botrytis* spores. Instead, the growth of *Botrytis* spores in these three wells was the same with the growth controls. The reason was thought that this killer protein was not enough to exert inhibitory activity on the spores [79]. We also thought that the cell walls of these strains have minor modification of the β-1,3 glucans as seen in Figure 3.15 - Figure 3.21.

On the other hand, increasing of the concentration of the K5 type yeast killer protein was more effective for inhibition of the *Botrytis* spores. The mycelial growth was reduced when the concentration increased from 512 μg/ml to 2048 μg/ml of the K5 type yeast killer protein. Moreover, abnormal morphological changes in fungal hyphae and spores were seen in Figure 3.15 - Figure 3.21. The K5 type yeast killer protein hydrolyzed β-1,3 glucan residues found in the cell wall of sensitive *Botrytis* cells and resulted in loss of cell wall rigidity [30,79]. Thus, changing osmatic pressure resulted in cell death in the wells containing 512 μg/ml, 1024 μg/ml and 2048 μg/ml, respectively.

Minimum inhibitory concentration for 50 % growth inhibition (MIC 2) value of the K5 type yeast killer protein was determined to be 512 μg/ml .Below 512 μg/ml the K5 yeast killer protein was less effective on *B. cinerea* strains and killer activity decreased because of deficiency of the killer protein and minor modified of the β-1,3 glucans in cell wall structure [79].

Above the 512 μg/ml the K5 type yeast killer protein inhibited mycelial growth increasingly. We said that the concentration of the killer protein was important to show antifungal activity on sensitive cells of *B. cinerea* strains [79, 92, 93].

According to these results ,we observed that type strain *B. cinerea* (DSMZ 5145) isolated from fields in Italy was the most sensitive strain to the K5 type yeast killer protein among the 6 *Botrytis* strains in this study because this isolate exposed no synthetic or chemical fungicides. On the other hand, the other 5 *Botrytis* strains (Bcts 4d, Bcts 11b, Bcts 15c, Bcts 19a, Bcts 25a) isolated from different regions in Tekirdağ were less susceptible to the K5 type yeast killer protein. However, the 2048 µg/ml of the K5 type yeast killer protein significantly exerted killer activity on all of these *Botrytis* strains.

When we compared with *B. cinerea* strains including Bcts 25a and Bcts 19a isolated from same regions in Şarköy/Çengelli Köyü in Tekirdağ, the same inhibitory effect of the K5 type yeast killer protein was observed under inverted microscope. The mycelial growth of these two different *B. cinerea* strains in 2048 µg/ml of the K5 type yeast killer protein were smaller than the growth controls without the K5 type yeast killer protein.

When we compared with morphology of the mycelia of *Botrytis* strains, there were difference between them. This difference was depended on some factors including soil types, climate changes, geographic location, humidity, wind, sunshine, rainfall and irrigation. Because of playing role in the quality and composition of the grapes, they affect mycelial growth of fungal plant pathogens like *B. cinerea* [104-106]. Application of the pesticides also affects both the quality and composition of the grapes and the mycelial growth of *B. cinerea* [107, 108]. Thus, changing of the microbial flora of the grapes with these factors caused different mycelial growth in some images of *B. cinerea* strains (Figure 3.14. and Figure 3. 15).

This showed us even if there was difference between two morphological mycelial growth of these two *B. cinerea* strains isolated same field in Tekirdağ, the K5 type yeast protein hydrolyzed β-1,3 glucan residues in cell wall of these *Botrytis* strains (Figure 3.11 and Figure 3. 14). *B. cinerea* strains known as fungal plant pathogens isolated from the grapevines in the same fields in Tekirdağ have different

characteristics, but they show same susceptibility to the K5 type yeast killer protein [109-111].

This study showed us the K5 type yeast killer protein produced by *P. anomala* was the most effective way to control or prevent the grey mold disease caused by *B. cinerea* on grapes. However, isolation and purification of the K5 type yeast killer protein is a time consuming way for biological control methods. Thus, according to these results, *Pichia anomala* synthesized the K5 type yeast killer protein can be used as a biocontrol agent for prevention of grey mold disease in grape fields. This method is more economic than isolation of the K5 type yeast killer protein [112].

CHAPTER 5

CONCLUSION

1. The K5 type yeast killer protein produced by *Pichia anomala* was found to be effective against grey mold disease caused by *Botrytis cinerea.* MIC of the K5 type yeast killer protein for this fungal plant pathogen was 512 µg/ml. *Botrytis cinerea* strains isolated from grape fields in Tekirdağ and type strain *B. cinerea* (DSMZ 5145) isolated from Italy were susceptible to the β-1,3 glucanase activity of the K5 type yeast killer protein.

2. The microscopic images of the 96-well plates showed that the K5 type yeast killer protein was the most effective on type strain *B. cinerea* (DSMZ5145) among the 6 *Botrytis* strains.

3. The other 5 *Botrytis* strains (Bcts 4d, Bcts 11b, Bcts 15c, Bcts 19a, Bcts 25a) isolated from different regions in Tekirdağ were less susceptible to the K5 type yeast killer. Above the 512 µg/ml of the K5 type yeast killer protein inhibited the growth of the sensitive *Botrytis* strains gradually.

4. Both wide antifungal spectrum and selectivity because of strong exo- β-1,3 glucanase activity and high stability in wide range pHs (3-5.5) and temperatures up to 37℃ emphasize the possible use of the K5 type yeast killer protein against *Botrytis cinerea* as a novel antifungal agent.

5. *Pichia anomala* secreted the K5 type yeast killer protein can be used as a biocontrol agent for prevention of grey mold disease in grape fields.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Acetic Acid (Merck, Germany)

Aceton (Merck, Germany)

Acrylamide (Boehringer Mannheim, Germany)

Ammoniumpersulphate (Pharmacia Biotech, Sweden)

Bacto-agar (Difco, USA)

Bacto-peptone (Difco, USA)

Bis-acrylamide (Boehringer-Mannheim, Germany)

Bovine Serum Albumine Fraction V (Boehringer-Mannheim, Germany)

Bromophenol Blue (Sigma, USA)

Butanol (Merck, Germany)

β-mercaptoethanol (Sigma, USA)

Citric Acid (Merck, Germany)

Coomassie Brilliant Blue R-250 (ICN, USA)

Coomassie Brilliant Blue G-250 (ICN, USA)

Dithiothreitol (DTT) (Boehringer Mannheim, Germany)

D-Glucose (Merck, Germany)

Di-sodium Hydrogen Phosphate (Merck, Germany)

Di-potassium Hydrogen Phosphate (Merck, Germany)

Ethanol (Merck, Germany)

Formaldehyde (Riedel-de Haen, Germany)

Glutaraldehyde (Fluka, Switzerland)

Glycerol (Merck, Germany)

Hydrochloric Acid (Merck, Germany)

Malt extract (Difco, USA)

Methanol (Merck, Germany)

PDA (Merck, Germany)

Phosphoric acid (Merck, Germany)

Potassium Dihydrogen Phosphate (Merck, Germany)

RPMI 1640 (Sigma, USA)

Silver Nitrate (Merck, Germany)

Sodium Carbonate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium Dodecyl Sulfate (Merck, Germany)

Sodium Hydroxide (Merck, Germany)

Sodium Sulfate (Merck, Germany)

TEMED (Pharmacia Biotech, Sweden)

Tween 80 (Merck, Germany)

Trichloroacetic Acid (Merck, Germany)

Tris (Merck, Germany)

Yeast extract (Difco, USA)

APPENDIX B

BUFFERS AND SOLUTIONS

Table B.1.Buffers and Solutions

Buffers / Solutions	Composition
1.SDS-PAGE Monomer Solution	30.8% T , 2.7% Cbis
4X Running Gel Buffer	1.5 M Tris-Cl , pH 8.8
4X Stacking Gel Buffer	0.5 M Tris-Cl , pH 6.8
SDS	10%
Initiator	10% Ammonium Persulfate
2X Treatment Buffer	0.125 M Tris-Cl , 4% SDS , 20% Glycerol , 10% β-mercaptoethanol , 0.020% Bromophenol blue , pH 6.8
Tank Buffer	0.025 M Tris , 0.192 M Glycine , 0.1% SDS , pH 8.3.
2. Standard Staning Staining Solution	(0.025 % Coomassie Brilliant blue R 250, 40% methanol, 7% acetic acid)
Destaining Solution I	(40% methanol, 7% acetic acid)
Destaining Solution II	(7% acetic acid, 5% methanol)

Table B.2. Components of RPMI 1640 Medium

RPMI 1640 COMPONENTS	**Conc. (mg/L)**
INORGANIC SALTS:	
Calcium nitrate (Ca(NO3)2 4H2O)	100.00
Potassium chloride (KCl)	400.00
Magnesium sulfate (MgSO4)	48.84
Magnesium sulfate (MgSO4 7H20)	100.00
Sodium chloride (NaCl)	6000.00
Sodium Phosphate (Na2HPO4)	800.00
OTHER COMPONENTS:	
Glucose	2000.00
Glutathione Reduced	1.00
Phenol red	5.00
AMINO ACIDS:	
L-Arginine	200.00
L-Asparagine	50.00
L-Aspartic Acid	20.00
L-Cystine dihydrochloride	65.00
L-Glutamic Acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine hydrochloride	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine disodium, dihydrate	29.00
L-Valine	20.00
VITAMINS:	
Biotin	0.2
D-Ca Pantothenate	0.25
Choline Chloride	3.00
Folic Acid	1.00
i-Inositol	35.00
Niacinamide	1.00
p-Aminobenzoic Acid (PABA)	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20