

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**DEVELOPING AN ALTERNATIVE BIOLOGICAL CONTROL METHOD
AGAINST *ASPERGILLUS CARBONARIUS* GROWTH AND ITS OCHRATOXIN
A PRODUCTION ON GRAPE**



M.Sc. THESIS

Güliz KONUŞUR

Department of Food Engineering

Food Engineering Programme

JUNE 2023

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**ÜZÜMDE *ASPERGILLUS CARBONARIUS* GELİŞİMİ VE OKRATOKSİN A
ÜRETİMİNE KARŞI ALTERNATİF BİYOLOJİK KONTROL YÖNTEMİ
GELİŞTİRİLMESİ**

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To my family,



FOREWORD

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ABBREVIATIONS

ANOVA	: Analysis of Variance
BCA	: Biocontrol Agent
CCP	: Critical Control Point
CYA	: Czapek Yeast Extract Agar
DI	: Decay incidence
GRAS	: Generally Recognized As Safe
HPLC	: High Performance Liquid Chromatography
MEA	: Malt Extract Agar
OTA	: Ochratoxin A
OTB	: Ochratoxin B
OTC	: Ochratoxin C
RI	: Radial Inhibition
TSS	: Total soluble solids
TSS	: Total soluble solids
VOC	: Volatile Organic Compound
YEPD	: Yeast Extract-Peptone-Dextrose



SYMBOLS

μg	: microgram
a_w	: water activity
CFU	: colony forming unit
kg	: kilogram
mg	: milligram
mL	: mililiter
ng	: nanogram
ppb	: parts per billion



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DEVELOPING AN ALTERNATIVE BIOLOGICAL CONTROL METHOD AGAINST *ASPERGILLUS CARBONARIUS* GROWTH AND ITS OCHRATOXIN A PRODUCTION ON GRAPE

SUMMARY

Natural toxins known as mycotoxins originate from specific species of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, and *Alternaria*. The major mycotoxins affecting human and animal health include aflatoxins (AFTs), ochratoxins (OTs), trichothecenes (TCTs), fumonisins (FUMs), zearalenone (ZEN), patulin (PAT), citrinin (CT), and ergot alkaloids (EAs). About 100,000 fungi were recently discovered, and more than 500 of these have been reported as potentially toxigenic. Ochratoxin A (OTA) is a particularly concerning mycotoxin produced by *Aspergillus* and *Penicillium* species. OTA is associated with various toxicological effects such as kidney toxicity, mutagenicity, teratogenicity, and immunotoxicity, making it a global concern for human and animal health. The International Agency for Research on Cancer has classified OTA as a possible human carcinogen (Category 2B). The European Food Safety Authority has established a tolerable weekly intake limit for OTA at 100 ng/kg body weight and maximum residue levels for OTA concentration in different food products. OTA can be found in various foods, including cereals, coffee beans, dried fruits, beer, grape juice, wine, nuts, cacao products, and spices.

Besides health concerns, approximately 1.3 billion tonnes of food—or almost 33% of all produced—are wasted yearly. Postharvest illnesses brought on by fungal degradation are the main cause of food waste. Fungi can spread quickly and find an excellent environment, especially in fruit that has been harmed because of including a lot of water, sugar, and nutrients. Several important control points should be considered to minimize the risk of OTA contamination during harvesting. These include maintaining cleanliness and ensuring the proper operation of farming equipment, ensuring the plant harvest has an optimal moisture content, harvesting mature and uncontaminated crops, removing overripe, fermented, and damaged produce, avoiding the mixing of low-quality products with other commodities, and promoting homogeneous and swift drying of the harvest in a clean and controlled environment. If contamination occurs during pre-harvest and harvest conditions, post-harvest management strategies are the best way to contain and manage it. Additionally, efficient detoxification of OTA can be achieved via a range of physical, chemical, enzymatic, and/or microbiological methods.

Chemical control of post-harvest diseases is still the most common method for managing post-harvest deterioration. Field treatment with fungicides is a conventional prevention method. Biological control has gained importance because of growing worries about fungicide residues in fruit, the emergence of infections with resistance biotypes, and the dangers of their continued usage. Some microorganisms considered to be potential biological control agents include yeasts, filamentous fungi, and bacteria. *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Bacillus subtilis*, *Pediococcus*

parvulus, *Streptomyces* sp., and yeast are just a few of the bacterial and fungal species that are successful in OTA reduction. Because of their basic nutritional requirements, capacity to grow on inexpensive media in fermenters, and resistance to a wide range of environmental factors, yeasts are incredibly fascinating organisms. Yeasts have been applied to agriculture because of their GRAS classification.

The yeast *Metschnikowia pulcherrima* is found in the phyllosphere, particularly in flowers and fruits, and is widely spread worldwide. *Metschnikowia pulcherrima* can be employed as a biological control agent because it produces naturally occurring antimicrobial chemicals, including pulcherrimin, an insoluble red pigment with antifungal action. The species was also recognized as a possible biocontrol agent against *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Monilinia*, and *Penicillium* species, as well as foodborne infections of freshly cut apples and fungal grape diseases.

In this study, *Metschnikowia* aff. *pulcherrima* DN-UY was used as the antagonistic yeast isolate, isolated and identified from grape leaves. Its biocontrol capabilities against OTA producer *Aspergillus carbonarius* growth and OTA production and the effect of this biocontrol agent on the quality parameters of grapes were investigated.

Dual culture and disc diffusion assay were used as inoculation methods to determine the antagonistic activity of yeast in two different media. In dual culture analysis, *Aspergillus carbonarius* 57-D1AN and *Aspergillus carbonarius* 39-04X4A were used as mould suspension and *Aspergillus carbonarius* 57-D1AN was used for further investigation due to a higher inhibition ratio. Also *in vitro* studies showed that growth of *A. carbonarius* 57-D1AN nearly inhibited by *Metschnikowia* aff. *pulcherrima* DN-UY. To evaluate the effectiveness of *Metschnikowia* aff. *pulcherrima* DN-UY as a biocontrol agent, both curative and preventive effects of yeast were investigated. preventive treatments were carried out by first applying the antagonistic yeast to grapes and then inoculating the treated fruit with the spore suspension to expose it to the pathogen. Curative treatments were conducted by first applying the spore suspension and then the antagonistic yeast, or vice versa. According to the decay incidence ratio of grapes, preventive treatments showed highest inhibition against the fungal damage at 25⁰ C. There is no significant differences between preventive and curative treatments at 10⁰ C ($p < 0.05$). Also, preventive treated grapes has lowest fungal concentration according to curative and mould treated grapes at the end of the 7 days at 10⁰ C. Also curative treatments showed decreasing effect on fungal concentration according to only mould treated grapes.

Following an investigation into the impact of antagonistic yeast on *A. carbonarius* growth and OTA production, the impact of antagonistic yeast on quality metrics was also investigated. When compared to the natural decay of *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes and untreated grapes, there was no significant differences between them at both 10⁰ C and 25⁰ C ($p < 0.05$). Also, there were no significant effect of yeast application to grapes on weight loss, TTA, brix and firmness values at 10⁰ C. While firmness and TTA value of *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes did not affect the brix value of grapes decreased and the weight loss of grapes increased. To sum up, the application of antagonistic yeasts may be an alternative approach to control *A. carbonarius* growth and its OTA production with a more safe, feasible and green strategy as a result of these *in vitro* and *in vivo* studies.

ÜZÜMDE *ASPERGILLUS CARBONARIUS* GELİŞİMİ VE OKRATOKSİN A ÜRETİMİNE KARŞI ALTERNATİF BİYOLOJİK KONTROL YÖNTEMİ GELİŞTİRİLMESİ

ÖZET

Mikotoksin olarak bilinen doğal toksinler, *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps* ve *Alternaria* gibi belirli türlerden kaynaklanır. İnsan ve hayvan sağlığını etkileyen başlıca mikotoksinler arasında aflatoksinler (AFT), okratoksinler (OT), trikotekinler (TCT), fumonisinler (FUM), zearalenon (ZEN), patulin (PAT), sitrinin (CT) ve ergot alkaloidleri (EAs) bulunur. Günümüzde yaklaşık 100.000 küf keşfedilmiş olup ve bunların 500'ünün potansiyel olarak toksin üreticisi olduğu bildirilmiştir. *Aspergillus* türleri, şu anda 4 alt cins ve 19 bölüm arasında tanınan 339 tür ile yeryüzündeki en çok sayıda ve yaygın olarak dağılmış organizmalardır. *Aspergillus* metabolitleri çeşitli antibiyotikler, antitümöral ve antifungal ajanlar geliştirmek için kullanılmaktadır. Soya sosu, miso, sake (*Aspergillus oryzae* ve *Aspergillus sojae*) ve çeşitli organik asitler ve enzimler (*Aspergillus niger*, *Aspergillus aculeatus*, *Aspergillus carbonarius*) dahil olmak üzere çeşitli *Aspergillus* türleri endüstriyel üretimde yaygın olarak kullanılmaktadır. Ayrıca en önemli endüstriyel ürünler olan amilaz ve sitrik asitleri de üretirler. Öte yandan, hasat öncesi ve sonrası, işleme ve taşıma dahil olmak üzere çeşitli aşamalarda gıda ve yemlere bulaşabilen aspergilli, maalesef tarım ürünlerinin bozulmasının ana nedenlerinden biridir. Okratoksin A (OTA), özellikle *Aspergillus* ve *Penicillium* türleri tarafından üretilen endişe verici bir mikotoksindir. OTA, böbrek toksisitesi, mutajenisite, teratojenisite ve immünotoksisite gibi çeşitli toksikolojik etkilerle ilişkilendirilmiştir ve bu durum hem insan hem de hayvan sağlığı için küresel bir endişe kaynağıdır. Uluslararası Kansere Araştırmaları Ajansı, OTA'yı olası bir insan kanserojeni (Kategori 2B) olarak sınıflandırmıştır. Avrupa Gıda Güvenliği Otoritesi, OTA için tolere edilebilir haftalık alım limitini ve farklı gıda ürünlerinde OTA konsantrasyonu için maksimum kalıntı düzeylerini belirlemiştir. OTA, tahıllar, kahve çekirdekleri, kurutulmuş meyveler, bira, üzüm suyu, şarap, fındık, kakao ürünleri ve baharatlar gibi çeşitli gıdalarda bulunabilir. Sağlık endişelerinin yanı sıra, her yıl yaklaşık 1.3 milyar ton gıda israf edilmektedir. Küfler tarafından tetiklenen hasat sonrası hastalıklar, gıda israfının başlıca nedenidir. Küf ve mikotoksin oluşumundan kaynaklanan gıda israfı sorununu çözmek için küresel çapta çaba sarf edilmektedir. Bu çabalar, daha iyi hasat sonrası yönetim uygulamalarının hayata geçirilmesini, depolama ve taşıma koşullarının iyileştirilmesini, izleme ve kontrol önlemlerinin artırılmasını, ayrıca uygun gıda işleme ve depolama yöntemlerinin öneminin farkındalığının artırılmasını içermektedir. Küf gelişimini azaltarak, mikotoksin kontaminasyonunu önleyerek ve hasat sonrası kayıpları etkin bir şekilde yöneterek, bu faktörlerle ilişkili küresel gıda israfını önemli ölçüde azaltmak ve daha güvenli ve sürdürülebilir gıda sistemleri sağlamak mümkündür. Küfler hızlıca yayılabilir ve özellikle su, şeker ve besin maddeleri içeren

zarar görmüş meyvelerde ideal bir ortam bulabilir. Hasat sırasında OTA kontaminasyonu riskini azaltmak için temizlik ve tarım ekipmanlarının uygun şekilde kullanılması, bitki hasadının optimum nem içeriğinde olması, olgun ve kontaminasyondan uzak hasat yapılması, olgunlaşmış ve fermente olmuş ve zarar görmüş ürünlerin çıkarılması, düşük kaliteli ürünlerin diğer ürünlerle karıştırılmasının önlenmesi, hasadın temiz ve kontrollü bir ortamda homojen ve hızlı bir şekilde kurutulması gibi bazı kritik kontrol noktaları (KKN) bulunmaktadır. Ön hasat ve hasat koşullarında kontaminasyon meydana gelirse, hasat sonrası yönetim stratejileri bunu kontrol altına almak ve yönetmek için en iyi yaklaşımdır. Ayrıca, OTA'nın etkili bir şekilde detoksifikasyonu, çeşitli fiziksel, kimyasal, enzimatik ve/veya mikrobiyolojik yöntemlerle sağlanabilir.

Hasat sonrası hastalıkların kimyasal kontrolü, hasat sonrası bozulmanın yönetilmesi için hala en yaygın yöntemdir. Tarlalarda fungusit uygulaması geleneksel bir önleme yöntemidir. Ürünlerde fungusit kalıntısının kalmasına karşı duyulan endişelerin artması, dirençli biyotiplerle enfeksiyonların ortaya çıkması ve bunların devam eden kullanımının tehlikeleri nedeniyle biyolojik kontrol önem kazanmıştır. Potansiyel biyolojik kontrol ajanları olarak kabul edilen bazı mikroorganizmalar arasında mayalar, filamentöz funguslar ve bakteriler bulunur. *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Bacillus subtilis*, *Pediococcus parvulus*, *Streptomyces* sp. ve mayalar, OTA konsantrasyonunun azaltılmasında başarılı olmaktadır. Temel beslenme gereksinimleri, ucuz ortamlarda fermantasyon yapabilme kapasiteleri ve geniş bir çevresel faktörlere karşı dayanıklılığa sahip olmaları nedeniyle mayalar son derece ilgi çekici organizmalardır. Mayalar, GRAS sınıflandırmaları sayesinde tarımda uygulanmaktadır. Biyokontrol sistemi konukçu hammadde (ürün), patojen ve antagonistik mikroorganizma arasında meydana gelen ve çevresel faktörlere bağlı olan üç yönlü bir etkileşimden oluşmaktadır. Biyokontrol stratejisi ile bozucu türlerin yayılmaları ve çoğalmalarını bu türlerin doğal düşmanlarını kullanarak sınırlandırmak ve kontrol altında tutmak amaçlanır. Biyokontrol mayalarının küf kontaminasyonunu kontrol etmek için kullanılan mekanizmalar arasında besin ve yaşam alanı için rekabet, biyofilm oluşumu, parazitizm, yayılabilir antimikrobiyal bileşiklerin üretimi, antibiyozis, lizozomal enzimlerin üretimi ve antimikrobiyal uçucu organik bileşiklerin üretimi yer almaktadır. *Metschnikowia pulcherrima* mayası çiçeklerde ve meyvelerde özellikle filozferde bulunur ve dünya genelinde yaygın olarak dağılım gösterir. *Metschnikowia pulcherrima*, antifungal etkisi olan çözünmez kırmızı bir pigment olan pulcherrimin de dahil olmak üzere doğal antimikrobiyal kimyasallar üretebilme yeteneği nedeniyle biyolojik bir kontrol ajanı olarak kullanılabilir. Bu özel antimikrobiyal aktivite, *Metschnikowia pulcherrima* tarafından salgılanan pulcherriminin bir öncüsü olan pulcherriminik asit ile ilişki nedeniyle demir (III) iyonlarının çökmesiyle sağlanır ve bu da ortamdaki demirin tükenmesine neden olur. Böylece gelişmek ve çoğalmak için demire bağımlı olan diğer mikroorganizmalar için ortam yaşanmaz hale gelir. *Candida tropicalis* ve *Candida albicans* mayalarının yanı sıra *Brettanomyces/Dekkera*, *Hanseniaspora* ve *Pichia* türleri ve *Penicillium*, *Alternaria* ve *Monilia* türleri pulcherrimin mekanizmasıyla etkili inhibe edilmiştir. Olgun üzümler, yüksek şeker ve asit içeriği nedeniyle birçok *Aspergillus* türü için ideal bir ortam sunar. Üzümlerde OTA bulaşma süreci genellikle küf sporlarının üzüm tanelerinin yüzeyine yapışmasıyla başlar. Uygun koşullar altında, küfler meyveye nüfuz edebilir ve iç dokuyu kolonize ederek OTA üretimine yol açabilir. Siyah *Aspergillus* türleri, üzüm gelişimi sırasında yüksek sıcaklık ve güneş ışığı nedeniyle bağlarda yaygın olarak bulunurlar. Güneşte kurutma sırasında, hasat edilen üzümlerde *A. carbonarius* ve *A. niger*'in gelişmesi sonucunda tipik olarak okratoksin A üretilir.

Üzümler 0,80 veya daha düşük bir su aktivitesine kadar kuruyana kadar, siyah aspergilli gelişmeye ve okratoksin A üretmeye devam eder.

Bu çalışmada, üzüm yapraklarından izole edilen ve *Metschnikowia* aff. *pulcherrima* DN-UY mayası, antagonistik maya izolatu olarak kullanılmıştır. Bu biyolojik kontrol ajanının *Aspergillus carbonarius*'un OTA üretimi ve büyümesine karşı olan etkisi ile üzümdeki kalite parametrelerine etkisi araştırılmıştır.

İkili kültür ve disk difüzyon testi, iki farklı ortamda mayanın antagonistik aktivitesini belirlemek için kullanılan inokülasyon yöntemlerindendir. İkili kültür analizinde *Aspergillus carbonarius* 57-D1AN ($71,43 \pm 1,75$) ve *Aspergillus carbonarius* 39-04X4A ($68,57 \pm 2,02$) kültür süspansiyonları olarak kullanılmış ve daha yüksek inhibisyon oranına sahip olduğu için *Aspergillus carbonarius* 57-D1AN ileri araştırmalar için kullanılmıştır. Ayrıca 3 farklı sıcaklık (20°C , 25°C , ve 30°C) ve 2 farklı su aktivitesinde (0,97 ve 0,99) yapılan disk difüzyon analizi sonuçları *A. carbonarius* 57-D1AN gelişiminin *Metschnikowia* aff. *pulcherrima* DN-UY tarafından neredeyse tamamen engellendiğini göstermiştir. *Metschnikowia* aff. *pulcherrima* DN-UY'nin üzümler üzerinde bir biyolojik kontrol ajanı olarak etkinliğini değerlendirmek için hem iyileştirici hem de koruyucu etkileri araştırılmıştır. Koruyucu etkiyi incelemek için, öncelikle antagonistik maya üzüme uygulanmıştır ve ardından işlem görmüş meyveyi spor süspansiyonuyla enfekte ederek patojene maruz bırakılmıştır. İyileştirici etkiyi incelemek için ise üzümler öncelikle spor süspansiyonuyla enfekte edilip sonrasında antagonistik maya uygulaması gerçekleştirilmiştir. Üzüm çürüme oranına göre, koruyucu uygulama 25°C 'de fungal hasara karşı en yüksek inhibisyonu göstermiştir. 10°C 'de koruyucu ve iyileştirici uygulamalar arasında önemli farklılıklar bulunmamaktadır ($p < 0.05$). Ayrıca, koruyucu uygulama gören üzümler, 7 günlük sürenin sonunda 10°C 'de iyileştirici ve sadece küf aşıl原因 üzümlere göre en düşük fungal konsantrasyona sahiptir. Ayrıca iyileştirici uygulama, sadece küf aşıl原因 gören üzümlere göre fungal konsantrasyon üzerinde azaltıcı etkiye sahiptir.

Antagonistik mayanın *A. carbonarius*'un gelişimi ve OTA üretimi üzerindeki etkisine yönelik bir araştırmanın ardından, antagonistik mayanın üzümün kalite parametreleri üzerindeki etkisi de incelenmiştir. *Metschnikowia* aff. *pulcherrima* DN-UY ile uygulama yapılan üzümlerin ve işlem görmemiş üzümlerin doğal bozulmaları karşılaştırıldığında, 10°C ve 25°C 'de olmak üzere her iki sıcaklık için de anlamlı farklılıklar bulunmamıştır ($p < 0.05$). 14 günlük raf ömrü süresinde 10°C 'de üzümlerin pH'larında yapılan kontrollerde anlamlı bir fark görülmemiştir. Ancak *Metschnikowia* aff. *pulcherrima* DN-UY uygulanmış üzümlerde, üzümlerin pH'ı 7 gün sonra düşmüştür. Buna karşılık üzümlerin TTA ve brix değerlerinde önemli bir değişiklik belirlenmemiştir. 25°C 'de ise hem işlem görmemiş hem de *Metschnikowia* aff. *pulcherrima* DN-UY uygulanmış üzümlerde 14 günün sonunda üzümlerin pH'ında düşme görülmüştür. Ancak 14 günün sonunda, hem işlem görmemiş hem de *Metschnikowia* aff. *pulcherrima* DN-UY uygulanmış üzümlerin pH değerleri arasında önemli bir fark yoktur. Üzümlerin titre edilebilir asitliği *Metschnikowia* aff. *pulcherrima* DN-UY uygulanmış üzümlerde değişmezken, maya uygulanmamış üzümlerde artmaktadır. 14 günlük raf ömrü boyunca işlem görmemiş üzümlerin brix değeri değişmezken, *Metschnikowia* aff. *pulcherrima* DN-UY uygulanan üzümlerin brix değerlerinde azalma tespit edilmiştir. Özetlemek gerekirse, yapılan *in vitro* ve *in vivo* çalışmaların sonucu *A. carbonarius* gelişmesini ve OTA üretimini kontrol etmek için *Metschnikowia* aff. *pulcherrima* DN-UY kullanılabileceğini göstermiştir. Böylece

daha güvenli, uygulanabilir ve çevre dostu bir strateji olarak alternatif bir yaklaşım olabilir.



1. INTRODUCTION

Mycotoxins are low molecular weight (<1000 Dalton) compounds that are mainly produced during secondary metabolism by moulds of the genus *Aspergillus*, *Penicillium*, and *Fusarium* (Janik et al, 2020). Due to their ability to produce mycotoxin, these species significantly impact food safety (Kapetanakou et al, 2012). Ochratoxin A (OTA) produced by *Aspergillus* spp. (*A. ochraceus*, *A. carbonarius*, *A. niger*) and *Penicillium* spp. (*P. verrucosum*, *P. nordicum*, and *P. viridicatum*) is one of the most significant mycotoxins of global concern for human and animal health due to its involvement in a wide range of toxicological consequences, such as kidney toxicity, mutagenicity, teratogenicity, and immunotoxicity (Janik et al, 2020; Ponsone et al, 2011). The International Agency for Research on Cancer (IARC) has rated this mycotoxin as a possible human carcinogen (Category 2B) (1993). The tolerable weekly intake of OTA is 100 ng/kg body weight, according to the European Food Safety Authority (EFSA) (2006). Many foods, such as cereals (oats, maize, wheat, and barley), cereal goods, coffee beans, dried fruits, beer, grape juice, wine, nuts, and cacao products, and spices, are sources of OTA. According to the regulation of European Commission (EC) No 1881/2006 and subsequent amendment, maximum residue levels (MRLs) of OTA concentration are 10 µg/kg in dried vine fruit and soluble coffee, 5 µg/kg in unprocessed cereal products and roasted coffee, and 2 µg/kg in wine and grape juice respectively. *A. carbonarius* is the primary OTA producer in grape juice, wine and wine-dried fruits (Arfoui et al, 2019; Janik et al, 2020).

According to Food and Agricultural Organization (FAO) (2014) almost 1.3 billion tons of food, or nearly 33% of all produced, is wasted annually. Most food waste is caused by postharvest diseases brought on by fungal deterioration. Fruits have a high water, sugar, and nutrient content. Thus fungi can easily spread and find an ideal environment, especially in fruit that has been damaged (Oztekin and Karbancioglu-Guler, 2021). Pre-harvest methods should be used first to prevent or reduce fungal contamination in the field. Crops must be protected against damage from mechanical processes or insects. Mycotoxin-producing moulds can colonize affected parts of

plants. The conventional prevention method is field treatment with fungicides. Still, alternative methods are developed because of the public's demand for decreased pesticides in food & the environment and the development of resistant fungi growth (Ahmed et al, 2015; Oztekin and Karbancioglu-Guler, 2021). The two main post-harvest stages where OTA can be prevented are storage and processing. Detoxification of OTA is a potential alternative strategy in case the adopted techniques (monitoring and controlling of storage & environmental condition) find out to be insufficient.

Physical, chemical, and (micro) biological OTA detoxification techniques are specifically divided into categories with the goal of reducing or eliminating OTA by destruction, modification, or absorption (Kapetanakou et al, 2012; Yousefi et al 2021). Researchers have become increasingly interested in using biological methods to reduce OTA in food. They have been looking at bacteria, yeasts, and non-toxic fungi for their potential to inhibit the growth of ochratoxigenic fungi as well as to reduce OTA through binding or degradation to levels permitted by legislation (2–10 µg/L) (Kapetanakou et al, 2012).

The ability of yeasts and bacteria (mainly lactic acid bacteria-LAB) to grow and survive in food products under a variety of environmental conditions, either as a part of the native microflora or added as a starter or protective cultures, makes them promising potential biocontrol agents of fungal growth or OTA occurrence (Dalie et al, 2010). Many yeast species can grow quickly in bioreactors on various substrates and rapidly colonize fruit surfaces, have basic nutritional needs, are adapted to both colonize wounds and dry surfaces, and are safe for human health. Unlike many filamentous fungi or bacterial antagonists, yeasts do not produce allergenic substances or secondary metabolites. Because of these unique properties, yeasts stand out, and their GRAS (Generally Recognized As Safe) status provides usage in agricultural applications. (Fabro et al, 2018; Liu et al, 2013; Naga Padma et al, 2011). Due to their GRAS status, yeasts have been used in agricultural applications. In general, antagonistic yeasts can be used as biofungicides to reduce or eliminate the need for chemicals as environmentally friendly substitutes for traditional fungicides (Zhang et al, 2016).

Due to its capacity to create naturally occurring antimicrobial chemicals, such as pulcherrimin, an insoluble red pigment with antifungal action, *Metschnikowia* sp. can be a biological control agent (Morata et al, 2019).

1.1 Purpose of Thesis

This study aimed to examine the effect of *Metschnikowia pulcherrima* isolated from grape leaves against *A. carbonarius*, the OTA producer in grapes, by in vitro and in vivo studies. In addition, the applicability of yeast, which has been observed to be effective in in vitro studies, in grapes and grape juice was supported by additional physiochemical and sensory analysis.

1.2 Literature Review

1.2.1 *Aspergillus* spp.

Among all fungi, *Aspergillus* species are the most numerous and widely distributed organisms on earth, with 339 species currently recognised among 4 subgenera and 19 sections. They are not very selective in terms of abiotic growth conditions. For example, they can grow in a wide temperature range (6-55 °C) and at relatively low humidity levels (Krijgheld et al, 2013; Perrone and Gallo, 2017). *Aspergillus* metabolites have been used to develop various antibiotics, antitumoral, and antifungal agents. Several *Aspergillus* species are widely used in industrial manufacturing including soy sauce, miso, sake (*A. oryzae* and *A. sojae*) as well as several organic acids and enzymes (*A. niger*, *A. aculeatus*, *A. carbonarius*). Also, aspergilli produce amylase and citric acids, which are the most significant industrial products. On the other hand, aspergilli, which can infect foods and feeds at several phases, including pre- and postharvest, processing, and handling, is regrettably one of the main causes of agricultural products deteriorating. Additionally, mainly *A. niger*, *A. flavus*, and *A. fumigatus* species are responsible for several diseases that affect both animals and humans, including mycotoxicosis, noninvasive and invasive infections in immunocompromised patients, and hypersensitive reactions (such as asthma and allergic alveolitis) in consequence of exposure to fungal fragments (Bennett and Klich, 1992; Varga et al, 2004).

The black aspergilli are a widespread type of food rotting fungus that are found all over the world. A few species are engaged in animal or human mycoses, and some are employed often in the biotechnology sector. A number of taxonomic schemes have been put out since these fungus are among the more challenging groupings to categorise and identify. 27 species were approved for the *Aspergillus* section *Nigri* in

the most recent revision of the genus. Only a few strains of several of these species, some of which are uncommon or recently described, have been researched. As a result, little is known about where these species are found (Jurjevic et al, 2012; Samson et al, 2014; Varga et al, 2011).

The most prevalent species (like *A. niger*, which is included in this section) are distinguished morphologically by the conidial head's black or dark-colored colour. This is why the colonies that these fungus typically develop are black or dark brown. However, depending on the culture media, the age of the culture, the quantity of sporulation, or the generation of sclerotia, among other parameters, the colour of the colonies varies not only between species but also within the same strain. As a result, some species can produce colonies that are purple-brown, reddish-brown, dark greenish, yellow, or orange depending on these variables (Samson et al, 2007).

Several pathogenic and saprophytic species of *Aspergilli* produce toxic secondary metabolites called as mycotoxin on host tissue and plant products. Aflatoxins (AFTs), ochratoxins (OTA), patulin (PAT), citrinin (CIT), aflatrem (AT), secalonic acids (SA), cyclopiazonic acid (CPA), terrein (TR), sterigmatocystin (ST), and gliotoxin (GT) are only a few of the deadly biotoxins produced by *Aspergillus* species, along with other distinctive compounds (Raduly et al, 2020). Most important mycotoxins produced by *Aspergillus* are aflatoxins, ochratoxins and patulin. Foods grown in humid tropics and subtropics, such as cereals (maize, sorghum, pearl millet, rice, and wheat), oilseeds (peanut, soybean, sunflower, and cotton), spices (chilies, black pepper, turmeric, coriander, and ginger), nuts (almond, Brazil nut, pistachio, walnut, and coconut), and milk are the main sources of these toxins (Reddy et al, 2010). At Table 1.1, mycotoxins produced by *Aspergillus* species was summarized.

A. alliaceus, *A. carbonarius*, *A. ochraceus*, *A. steynii*, and *A. westerdijkiae* are the main *Aspergillus* species that create OTA in food and feed. *A. ochraceus*, a significant OTA producer, can grow in the temperature range of 8 to 40 °C (with an ideal growth temperature of 24 to 31 °C), in the pH range of 3 to 10, and at high water activity (a_w) values of 0.95-0.99. Additionally, it should be noted that *A. ochraceus* has the ability to produce other toxic metabolites in addition to OTA, including penicillic acid, viomellein, vioxanthin, and xanthomegnin. *A. carbonarius*, which is frequently found in tropic and subtropical foods such fresh fruits, dried wine fruits, and some vegetables, is another significant generator of OTA. The ideal a_w for spore germination is 0.83 and

the ideal temperature for *A. carbonarius* growth is 32–35 C. This species appears to be very resistant to high temperatures and favours somewhat lower water activity (Duarte et al, 2010; Fernandez-Cruz et al, 2010). Detailed optimal growth conditions, and commodities affected of OTA-producing fungi is given at Table 1.2.

Table 1.1 Some important mycotoxins produced by *Aspergillus* species.

Mycotoxins	Agricultural product	Species	References
Aflatoxins	Peanut, corn, cotton	<i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. nomius</i>	Varga et al, 2004
	Brazil nuts	<i>A. flavus</i> , <i>A. Parasiticus</i> , <i>A. nomius</i>	Calderari et al, 2013
	Cocoa	<i>A. flavus</i> , <i>A. Parasiticus</i> , <i>A. nomius</i>	Copetti et al, 2011
Ochratoxins	Dried fruits	<i>A. flavus</i>	Iamanaka et al, 2007
	Grape, wine	<i>A. niger</i> , <i>A. carbonarius</i>	Varga et al, 2004
	Coffee, spices	<i>A. ochraceus</i> , <i>A. niger</i> , <i>A. carbonarius</i>	Taniwaki et al, 2003
	Cocoa	<i>A. westerdijkiae</i> , <i>A.</i> <i>Ochraceus</i> , <i>A. Melleus</i> , <i>A. Carbonarius</i> , <i>A. niger</i>	Copetti et al, 2013
	Figs	<i>A. alliaceus</i>	Varga et al, 2004
	Peanuts	<i>A. niger</i>	Oyedele et al, 2017
	Tree nuts (almonds, pistachios, walnuts, hazelnuts)	<i>A. carbonarius</i>	Palumbo et al, 2015
	Dried fruits	<i>A. ochraceus</i> , <i>A.</i> <i>carbonarius</i> , <i>A. niger</i>	Cabanes et al, 2017
Patulin	Cereals, malt	<i>A. clavatus</i>	Varga et al, 2004

Table 1.2 Optimal growth conditions, and commodities affected of OTA-producing fungi (Bui-Klimke and Wu, 2015).

OTA-producing species	Opt. Temperature Range (min-max) ° C	Opt. Water activity	Commodities affected
<i>A. ochraceus</i>	24-31 (8–40)	0.95–0.99	Smoked and salted dried fish, dried beans, soya beans, chickpeas, rapeseed, pepper, dried fruit, and sesame seeds, nuts, cereals rice, barley, maize, wheat, flour, and bran, coffee beans
<i>A. carbonarius</i>	32-35 (N/A-40)	0.83	Grapes and grape products, including table grapes, wines, and dried vine fruits
<i>A. niger</i>	35–37 (6-47)	0.77	Nuts, apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes, melons, onions, garlic, and yams

Furthermore, it has been observed that aflatoxin B₁, which is produced by *Aspergillus flavus*, is either absent or present at very low levels when high levels of OTA are present, and vice versa. This suggests that *Aspergillus* species compete with one another for toxin production or absorption in the gastrointestinal tract (GIT) (Zain, 2011).

Due to their high sugar and acid content, mature grapes offer excellent substrates for many *Aspergillus* species. The black aspergilli are a common habitant of vineyards due to the high heat and sunlight during grape development. During sun drying, ochratoxin A is produced as a result of the growth of *A. carbonarius* and *A. niger*, which is typical in harvested grapes. Till the grapes dry down to a water activity of 0.80 or less, the black Aspergilli will keep growing and producing ochratoxin A (Hocking et al, 2003; Pitt et al, 2012). The main species responsible for the production of ochratoxin A in grapes and grape products is *A. carbonarius*. Isolates of *A. niger* and *A. japonicas* and other uniseriate black aspergilli do not produce any of this toxin (Ferranti et al, 2018).

1.2.2 Ochratoxin A

The chemical name of OTA is L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl)carbonyl]-(R)-isocoumarin. It is a pentaketide from the dihyrdocoumarin family linked to β -phenylalanine via a peptide bond. It is a weak organic acid that is soluble in polar organic solvents (chloroform, alcohols) under neutral and acidic conditions. It is soluble in aqueous sodium bicarbonate solution at alkaline pH. The crystalline form of OTA ranges from white to colorless, and this toxin glows green in an acidic environment and blue in an alkaline one. Temperatures between 15 and 40 °C and water activities between 0.77 and 0.99 are ideal for the growth of OTA-producing fungi (Janik et al, 2020; Khoury and Atoui, 2010). Ochratoxins are a class of mycotoxins that are structurally related to isocoumarin derivatives and are largely produced by a few species of *Aspergillus* and *Penicillium* (Wang et al, 2022). There are three essential types: ochratoxin A, B, and C. The three types differ in that ochratoxin B (OTB) is a non-chlorinated form of ochratoxin A (OTA) and ochratoxin C (OTC) is an ethyl ester of OTA (Table 1.3) (Heussner and Bingle, 2015). Chemical structure of these toxins shown at Figure 1.1. In comparison to ochratoxin B (OTB) and ochratoxin C (OTC), OTA is more common

and more toxic (Giancarlo et al, 2011). In addition, Ochratoxin β is the non-amino portion of OTB, whereas Ochratoxin α is the non-amino component of OTA and OTC (Khoury and Atoui,2010).

Table 1.3 Overview of main ochratoxin forms (Heussner and Bingle, 2015).

Name	OTA	OTB	OTC
CAS Number	303-47-9	4825-86-9	4685-85-4
Molecular Formula	C ₂₀ H ₁₈ ClNO ₆	C ₂₀ H ₁₉ NO ₆	C ₂₂ H ₂₂ ClNO ₆
Molar Mass (g·mol ⁻¹)	403.8	369.4	431.9

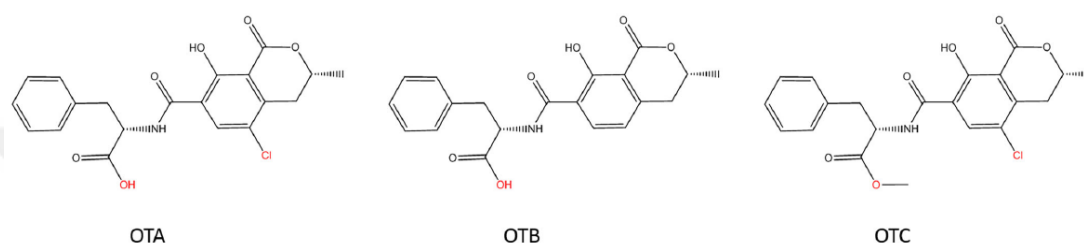


Figure 1.1 Chemical structures of OTA OTB and OTC (X. Li et al, 2021).

OTA production capacity is currently confirmed in two *Penicillium* species, *P. verrucosum* and *P. nordicum*. *P. verrucosum* grows most suitable below 30 °C and is typically found in cool climate locations like North America and Europe. This kind of slow-growing fungus is more frequently detected in cereals and products derived from grains when harvesting circumstances are cool and humid (Cabanes et al, 2010).

OTA can enter the bodies of humans and animals through different routes, including skin contact, inhalation, and consumption of contaminated food. However, numerous studies have highlighted that dietary intake is the primary pathway for OTA exposure in both humans and animals due to its presence in a wide range of food and feed sources. Once ingested, OTA is absorbed from the stomach and the jejunum in the digestive system. Within the circulatory system, OTA has a strong affinity for binding with albumin, a protein found in blood. In fact, approximately 99.8% of OTA in the human circulatory system is known to be bound to albumin (Hassan et al, 2022; Schrenk et al, 2020).

Foods with plant origins such as cereals and cereal goods, fresh and dried fruits, wine, juice, nuts, medicinal herbs, and spices have all been shown to contain OTA contamination (Wang et al, 2022). Among them, cereals, wine and grape juice were found to be the main contributors to OTA dietary exposure for European consumers

(European Food Safety Authority, 2020). After cereals, wine was shown to be the second-largest contributor of OTA exposure in the diet of people in Europe. For certain vulnerable consumer groups, such as children, grape juice and dried vine fruit played a substantial role in OTA exposure. Several investigations have revealed that the primary cause of OTA in wine or dried vine fruits from the world's major viticultural regions is *Aspergillus carbonarius*. (Bragulat et al, 2019). Table 1.4 shows that examples of food which contains OTA. Agricultural products may be contaminated during processing, storage, or transportation or in the field during harvest. While grapes are harmed in the field, products like coffee, cocoa, cereal, and dried fruits are typically affected during storage. OTA mostly forms after harvesting and is influenced by factors like temperature, water activity, kind, and seed integrity. In the presence of biotic components including lactose, urea, and amino acids, OTA production is enhanced. Sucrose and glucose, on the other hand, inhibit its synthesis (Abbas et al, 2009; Bhat et al, 2009).

Table 1.4 Occurrence of ochratoxin A in food.

Product	Positive samples	Range (ng L ⁻¹)	Mean levels (ng L ⁻¹) ± S.D	References
Grape juice (Brazil)	14/48 (29%)	21.2–100	38±24	Rosa et al, 2004
Red Wine (Brazil)	3/10 (30%)	28.3–42.4	35.36±7.05	Rosa et al, 2004
Red Wine (Australia)	49/344 (14%)	50–620	NS	Hocking et al, 2003
White Wine (Australia)	41/257 (16%)	50–500	NS	Hocking et al, 2003
Dry red Wine (China)	NS	<0.1–5.65 (µg/L)	NS	Zhang et al, 2013
Dry white Wine (China)	NS	<0.1–0.07 (µg/L)	NS	Zhang et al, 2013
Dried fruits (China)	NS	0.2–8.8	NS	Wei et al, 2017
Dried fruits (Brazil)	NS	0.1–30	NS	Iamanaka et al, 2005
Dried vine Fruits (China)	NS	<0.07–12.8333	NS	Zhang et al, 2014
Grapes (Portugal)	NS	0.008–1.6	NS	Serra et al, 2006
Dried black fruits (Argentina)	18/31 (58%)	1.5–2.25	1.50±0.48	Chulze et al, 2006
Dried white fruits (Argentina)	4/19(21%)	1.4–2.0	1.71±0.40	Chulze et al, 2006
Dried figs (Turkey)	NS	<0.1–35.1	NS	Senyuva et al, 2005
Baby food (Canada)	NS	0.06–2.4	NS	Lombaert et al, 2003
Cocoa products (Italy)	NS	0.22–0.77	NS	Tafari et al, 2004
Breakfast cereals (France)	NS	0.4–8.8	NS	Molinie et al, 2005

*NS: Not stated

Several quantitative analytical techniques for OTA determination have been published such as GC (Gas chromatography) based methods, LC (Liquid chromatography) based methods, FLD (Fluorescence detector), MS (Mass spectrometry), LC-MS (Liquid chromatography-Mass spectrometry) detection (Li et al, 2021). OTA cannot be eliminated in feed and food raw materials and toxin still remain at finished product by industrial processing of food because of stability of OTA at high temperature. To reduce toxin concentration, higher than 250°C temperature and several exposure minutes are required. Addition to partially degrade at 250°C temperature, it can endure sterilisation for three hours at 121 °C (Boudra et al, 1995; Zhang et al, 2016).

A variety of cyanogenic consequences in animals are caused by OTA, which is created during crop storage. The immune system is harmed as well as the kidneys, which are the toxin's main targets. There is no proof that humans' renal organs have been harmed, despite the fact that there is considerable evidence of renal organ toxicity and renal organ cancer in animals exposed to OTA. Nonetheless, the effects on the kidneys are undeniable. Additionally, OTA has been shown to be teratogenic, carcinogenic, genotoxic, immunotoxic, neurotoxic, hepatotoxic, and immunotoxic in both people and animals (Kozak et al, 2019; Reddy et al, 2019).

OTA induces oxidative stress and disrupts cellular processes at the cellular level. It inhibits mitochondrial respiration, leading to disturbances in calcium balance, peroxidation of cell membranes, and interference with oxidative phosphorylation. Furthermore, at the molecular level, OTA's phenylalanine component affects the phenylalanine-related pathway, impairing enzyme activity and inhibiting protein synthesis, contributing to its toxicity. In terms of teratogenic effects, OTA's ability to cross the placenta is responsible for adverse outcomes such as brain damage and malformation of the central nervous system (Li et al, 2022; Soleas et al, 2001).

Humans are exposed to OTA through a variety of pathways, including dietary, dermal contact, and by inhalation. OTA is a human Group 2B carcinogen, according to the International Agency for Research on Cancer (IARC) (IARC, 1993). OTA has also been found in the kidneys and body fluids of both humans and animals. OTA is absorbed in the stomach, which is important for enterohepatic circulation. Additionally, OTA inhibits the function of the kidney's epithelial cells by reducing the kidney's capacity to absorb the filtrate and negatively affecting the glomerular filtration rate (Navale et al, 2021). Balkan Endemic Nephropathy (BEN), a progressive

condition that causes permanent kidney failure in humans, is caused by OTA. Furthermore, because of its great affinity for human serum macromolecules, it has a serum half-life that is prolonged, delaying the time it takes for the body to eliminate it (Bui-Klimke and Wu, 2015). According to a recent risk assessment on OTA, the substance proved negative in high-specificity genotoxicity assays, and its levels of OTA-DNA adducts were modest and not characteristic of genotoxic carcinogens (Haighton et al, 2012).

1.2.3 Control strategies against OTA

The majority of losses of fresh fruits and vegetables at the postharvest, distribution, and consumption levels are attributed to postharvest fungal infections. According to the Food and Agriculture Organization, the average global loss in industrialized Asia, South East Asia, Africa, and Latin America is about 38%, compared to a loss of roughly 29% in Europe, North America, and Oceania (FAO, 2011). A total of 1.5 million tons of table grapes were produced in Europe in 2017, yet 30 to 40 percent of those postharvest grapes are lost each year as a result of improper treatment and a lack of effective measures to stop rot and senescence (Lappa et al, 2018).

Fungal decay causes berries to undergo rapid physiological changes, such as weight loss, colour fading, accelerated tissue softening, and reduced shelf life, which significantly reduces the market value of grapes. In addition to these, a fungal infection changes the nutritional value and chemical composition of grapes. More microbial metabolites, degradation of sugars, and acid generation are a few of the changes in the chemical composition. Importantly, since mycotoxins are hazardous to human health, increased production of these compounds raises questions about food safety. (Vazquez-Hernandez et al, 2018; Welke 2019). Many studies have been conducted to find solutions to reduce or eliminate OTA from foods, including chemical, physical, and microbiological studies. However, the limitations of chemical and physical approaches make it difficult for them to be employed successfully for the control of fungi that produce OTA. For instance, sensory qualities like color, taste, and scent could be diminished by physical processes like filtration or adsorption (Zhang et al, 2016; Grizioli et al, 2006).

During harvest, the critical control points (CCPs) include cleanliness and proper operation of farming equipment, optimal moisture content of the plant harvest, mature

and contamination-free harvest, removal of overripe, fermented and damaged produce, avoidance of mixing poor quality products with other commodities, and homogeneous and quick drying of the harvest under clean and controlled environment are important measures to reduce the risk for OTA contamination (Amezqueta et al, 2009; Lopez-Garcia et al, 2008). The ideal time to control OTA is during pre-harvest and harvest conditions. If contamination does occur, post-harvest management strategies are the best approach to contain and manage it. The essential CCPs for the post-harvest phase are suitable packaging materials, such as metallic containers rather than wooden or mud-based ones, and appropriate storage temperatures, such as storing below 20 °C and below 0.70_{aw} at all phases. Additionally, the warehouse needs to be thoroughly cleaned, and the conditions for export storage or transportation need to be maintained with the least amount of transit time. In addition, a variety of physical, chemical, enzymatic, and/or microbiological techniques (described in degradation kinetics) can be used to successfully detoxify OTA (Amezqueta et al, 2009; Bankole et al, 2003; Magan et al, 2003).

The most popular way for managing post-harvest decay is still chemical control of post-harvest diseases, which is applied in the orchard or after harvesting (Spadaro and Droby, 2016; Sharma et al, 2009). Pests are controlled with the use of 6 million tons of pesticides annually; 26% of the market for pesticides was made up of synthetic fungicides (Bernhardt et al, 2017). Sulfur dioxide gas (SO₂) has been used to control fungal rot in table grapes. This has been done either by often fumigating storage rooms or by packaging the grapes in polyethylene-lined boxes with SO₂ generator pads. When levels of SO₂ are high enough, degradation is typically prevented effectively. However, excessive SO₂ can destroy grape berries, leave a off taste, and harm to humans (Lappa et al, 2018). Although growing worries about fungicide residues in fruit, the emergence of infections with resistance biotypes, and the dangers of their continued usage have driven the quest for secure and efficient replacement methods. Among these strategies, biological control based on naturally occurring microorganisms, has been the most studied since the global trend appears to be turning away from the use of fungicides on produce (Sharma et al, 2009; Spadaro and Droby, 2016).

1.2.4 Biocontrol

First at 1919 mentioned the phrase "biological control" to refer to the permanent suppression of insect pests through the use of natural enemies of exotic insects. This word broadly refers to all methods of pest control excluding the use of chemicals. Later, use of microorganisms introduced as biocontrol agents (BCAs) at 1974. They described biocontrol as the reduction in pathogen presence or disease activities by introducing one or more antagonistic organisms (Canito et al, 2021). In order to manage soil and crop infections, several different microbial species are utilized as biological control agents (BCAs). These BCAs have received a great deal of support, maybe as a result of the fact that the target pathogen cannot develop a resistance to them (Asad, 2022). The following are the requirements for choosing an optimal BCA: It must be resistant to pesticides, genetically stable, effective at low concentrations, not rigorous nutritional needs, able to survive in harsh environments, effective against a variety of pathogens and different harvested commodities, non-pathogenic to the host, in a form that can be stored, and compatible with other chemical and physical treatments. A microbial antagonist should also possess an adaptive advantage over particular pathogens (Sharma et al, 2009; Wilson and Wisniewski, 1989).

Bacteria, yeasts, and filamentous fungi are some of the microorganisms that are thought to be potential biological control agents. Numerous bacterial and fungal species, including *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Bacillus subtilis*, *Pediococcus parvulus*, *Streptomyces sp.*, and yeast, have been reported to be effective in OTA breakdown. Numerous environmental *Bacillus* species are widely known for their capacity to create a wide range of antibacterial chemicals, control plant disease, and stimulate plant growth. In addition to their antifungal properties, *Bacillus* bacteria have been shown to be capable of degrading mycotoxins such aflatoxin, deoxynivalenol, fumonisin B₁, zearalenone, and OTA.

Yeasts are very fascinating organisms because of their simple nutritional needs, ability to grow in fermenters on inexpensive media, and ability to endure a variety of environmental conditions. They can also colonize plant surfaces or wounds for extended periods of time in dry environments (Ponsone et al, 2016; Wang et al, 2022). Due to their GRAS status, yeasts have been used in agricultural applications. Table 1.5 displays examples of antagonistic yeasts that were isolated from various sources and are employed in the treatment of postharvest diseases.

Table 1.5 Evaluation of antifungal activity of the yeast with *in vitro* studies.

Yeast strain	Origin	Target fungi	Antagonistic assays	Optimum concentration of biocontrol agent	Inhibition of mycelial growth (%)	References
<i>Aerobasidium pullulans</i>	Grape berries	<i>Aspergillus tubingensis</i>	Dual culture method	- ¹	12.97-95.69	(Pantelides et al, 2015)
<i>Candida catenulata</i>	Fruits and leaves of citrus plants	<i>Penicillium digitatum</i>	Dual culture method	-	<15	(Perez et al, 2017)
<i>Candida tropicalis</i>	Mandarin orange	<i>Colletotrichum musae</i>	Dual culture method	1 × 10 ⁸ cells/mL	31.3	(Zhimo et al, 2017)
<i>Debaryomyces hansenii</i>	Citrus varieties	<i>Penicillium digitatum</i> <i>Penicillium italicum</i>	Dual culture method	-	41 6	(Hammami et al, 2022)
<i>Metschnikowia aff. pulcherrima</i>	Hawthorn	<i>Penicillium expansum</i> <i>Penicillium digitatum</i> <i>Penicillium expansum</i>	Dual culture method	1 × 10 ⁸ cells/mL	84.2 95.3	(Oztekin, Karbancioglu-Guler, 2021)
<i>Pichia fermentans</i>	Grapes	<i>Botrytis cinerea</i>	Dual culture method	1 × 10 ⁶ cells/mL	0-25	(Fernandez-San Millan et al, 2021)
<i>Rhodoturula mucilaginosa</i>	Black olives	<i>Aspergillus carbonarius</i>	Disc diffusion method	1x10 ⁵	75.8	(Tryfinopoulou et al, 2020)
<i>Debaryomyces hansenii</i>	Marine environment	<i>Mucor circinelloides</i> <i>Aspergillus</i> sp. <i>Fusarium proliferatum</i> <i>Fusarium subglutinans</i>	Agar plate inhibition	1 × 10 ⁸ cells/mL	97.2-98.3	(Medina-Córdova et al, 2018)

In general, antagonistic yeasts can be used as biofungicides to reduce or eliminate the need for chemicals as environmentally friendly substitutes for traditional fungicides (Zhang et al, 2016). Developing, registering, and commercializing biocontrol agents to control important postharvest diseases like *Penicillium expansum*, *Penicillium digitatum*, *Penicillium italicum*, *Fusarium sambucinum*, *Rhizopus stolonifer*, and *Botrytis cinerea* have advanced significantly. Various products have advanced to the point of commercialisation. Some of the commercially available biofungicides, based on microorganisms are located at Table 1.6. ShemerTM, based on the yeast *M. fructicola*, have had greater commercial success. ShemerTM was initially approved in Israel for pre- and post-harvest use on a variety of fruits and vegetables, including apricots, citrus fruit, grapes, peaches, peppers, strawberries, and sweet potatoes. Later, Bayer CropScience (Germany) purchased ShemerTM, and Koppert (Netherlands) recently received a sublicense (Spadaro and Droby, 2016).

Table 1.6 Commercially available biofungicides, based on microorganisms, for the control of postharvest diseases (Spadaro and Droby, 2016; Zhang et al, 2020).

Microorganism	Product name	Target pathogens	Fruit	Country
<i>Aureobasidium pullulans</i>	Blossom Protect	<i>Penicillium, Botrytis, Monilinia</i>	Pome fruit	EU (preharvest)
<i>Candida sake</i>	Candifruit	<i>Penicillium, Botrytis, Rhizopus</i>	Pome fruit	Spain
<i>Candida oleophila</i>	Nexy	<i>Botrytis, Penicillium</i>	Pome, banana, citrus	Belgium
<i>Metschnikowia fructicola</i>	Shemer	<i>Botrytis, Penicillium, Rhizopus, Aspergillus</i>	Table grape, strawberry, sweet potato	Netherlands
<i>Metschnikowia fructicola</i>	Noli	<i>Botrytis, Monilinia</i>	Strawberry, blueberry, grape, stone fruit	Netherlands
<i>Pantoea agglomerans</i>	Pantovital	<i>Penicillium, Botrytis, Monilinia</i>	Citrus fruit, pome fruit	Spain
<i>Pseudomonas syringae</i>	Biosave	<i>Penicillium, Botrytis, Mucor</i>	Pome, citrus fruit, cherry, potato, sweet potato	United States
<i>Saccharomyces cerevisiae</i>	Remeo	<i>Botrytis, Erysiphe, Plasmopara</i>	Grape	France

It was discovered that several yeast strains from the genera *Saccharomyces*, *Pichia*, *Metschnikowia*, *Dekkera*, and *Rhodotorula* inhibited the growth of *A. carbonarius* and OTA production (Tryfinopoulou et al, 2020). Despite these incredible accomplishments, single biocontrol product cannot always be able to completely control post-harvest illnesses or to be as effective as some of the well-known synthetic pesticides. Due to growing consumer and farmer demands for food free of chemical residues, they cause worry in the food business. In light of this, there have been numerous reports supporting the use of two or more BCAs in combination to suppress mycotoxins like OTA and postharvest illnesses. These BCA combinations can offer a wider range of control than a single BCA (Droby et al, 2009). Additionally, recent studies demonstrated that culturing the strains in a medium enriched with chemical elicitors might be used to fine-tune the yeasts' biocontrol effectiveness. Trehalose and chitosan were suitable elicitor compounds to improve yeast biocontrol effectiveness (Apaliya et al, 2018).

1.2.5 Antagonistic yeast mechanism of action

The basic mechanism of action of biocontrol yeasts is thought to be a struggle for nutrients and space that all microorganisms engage in with their hosts and other microbes. Competition for nutrients and space, biofilm formation, parasitism, production of diffusible antimicrobial compounds, antibiosis, production of lytic enzymes, and production of antimicrobial volatile organic compounds (VOCs) have all been described as mechanisms for controlling fungal contamination by antagonistic yeasts (Ruiz-Moyano et al, 2020; Freimoser et al, 2019).

BCAs are thought to work primarily through the creation of primary and secondary metabolites, especially when dealing with filamentous fungi. Volatile organic compounds (VOCs), which are one of the metabolites, have a high biocontrol effectiveness. They are tiny molecules (typically <300 Da), with a high vapour pressure and a poor solubility in water. The molecular groups of VOCs, which include hydrocarbons, alcohols, thioalcohols, aldehydes, ketones, thioesters, cyclohexanes, heterocyclic compounds, phenols, and benzene derivatives, exhibit a wide range of antimicrobial activity. Due to their volatility, VOCs are thought of as potential biological fumigants since it enables them to manage postharvest degradation without direct contact with the edible commodities (Csutak et al, 2012; Janisiewicz et al, 2001; Oro et al, 2014; Zhang et al, 2020).

Another specialised and effective technique for competing for space is biofilm formation. Microbial communities known as biofilms can consist of a single species or many species consortia and are able to exist and grow on surfaces. Biofilm production in biocontrol yeasts, particularly in the phyllo- and carposphere (i.e., in wounds), is currently regarded as an essential method of action and has received extensive research (Freimoser et al, 2019).

Mycoparasitism refers to the phenomenon of antagonistic yeasts feeding on fungal pathogens via attaching to the fungal pathogen hyphae and then secreting cell Wall-degrading enzymes to destroy or lyse the fungal structures. Especially in the case of nutritional deficiencies, antagonistic yeasts tend to absorb nutrients from pathogenic cells, leading to the death of these “prey” cells. During mycoparasitism, a variety of enzymes are involved in the degradation of the fungal pathogen cell wall, especially

β -1,3-glucanase (GLU), chitinase (CHT), and proteases, and these secreted enzymes are thought to play an important role in biocontrol (Di Francesco et al, 2016).

Due to its capacity to create naturally occurring antimicrobial compounds, such as pulcherrimin, an insoluble red pigment with antifungal activity, *Metschnikowia pulcherrima* can be used as a biological control agent. This peculiar antimicrobial activity is brought on by the precipitation of iron(III) ions due to the association with pulcherriminic acid, a precursor to pulcherrimin secreted by *Metschnikowia pulcherrima*, which results in the depletion of iron in the medium. By doing this, the atmosphere is rendered inhospitable for other microorganisms that depend on iron for growth. The yeasts *Candida tropicalis* and *Candida albicans*, as well as the species *Brettanomyces/Dekkera*, *Hanseniaspora*, and *Pichia*, and the fungi *Penicillium*, *Alternaria*, and *Monilia* spp. have all been successfully inhibited by pulcherrimin. *S. cerevisiae*, however, doesn't appear to be impacted by this antimicrobial action. As a result, using *M. pulcherrima* as a chosen starter in sequential or combined biotechnologies with *S. cerevisiae* could be very interesting in contemporary enology. The primary antagonistic mechanism of *M. pulcherrima* is its struggle with other organisms for nutrients (Csutak et al, 2012; Janisiewicz et al, 2001; Oro et al, 2014).

The process of fungal infection in grapes, according to numerous studies, consists of four main steps: adhesion of pathogenic fungal/spores to the berry surface; production of infected structures for stable attachment to the host; invasion into host tissue; and successful colonisation and spreading. The pathogenic fungus cause grapes to react by turning on their anti-pathogen mechanisms. The primary defence strategies of grapes include an increase in oxidative burst, the stimulation of defence-related enzymes, increased phytohormone production, and overexpression of proteins related to pathogens (Apaliya et al, 2017; Li et al, 2019).

1.2.6 Antagonistic effect of *Metschnikowia pulcherrima*

The yeast *Metschnikowia pulcherrima* is worldwide distributed and frequently isolated from the phyllosphere; in particular from flowers and fruits. The species was also identified as a potential biocontrol organism against foodborne pathogens of freshly cut apples as well as fungal grape diseases because it exhibits strong antagonistic activity against *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Monilinia*, and *Penicillium* species. In addition to yeasts, *M. pulcherrima* inhibits other fungi that

cause fungal diseases in fruits. *M. pulcherrima* is being examined for the development of wines with lower alcohol content, as well as biocontrol applications, the synthesis of the aroma molecule 2-phenylethanol, the biodegradation of the mycotoxin patulin, and the generation of biofuels (Gore-Lloyd et al, 2019). The ovoid-shaped *M. pulcherrima* typically reproduces through budding, although under anaerobic conditions, pseudohyphal development has been seen. *M. pulcherrima* exhibits low to moderate fermentative power, which is typical of the majority of non-*Saccharomyces* species found in grapes (Vicente et al, 2020).

The below Table 1.7 summarizes the antagonistic effect studies of *Metschnikowia pulcherrima* against different microorganisms.

Antagonistic effect (both *in vitro* and *in vivo*) of *Wickerhamomyces anomalus* BS91 and *Metschnikowia pulcherrima* MPR3 species against to *Aspergillus flavus* on pistachio nuts was investigated at the study of Parafati et al (2022). Mycelial growth inhibition induced by yeast cells and by yeast VOCs of *Wickerhamomyces anomalus* BS91 is significantly ($p < 0.05$) higher than that induced by *M. pulcherrima* MPR3, with inhibition values of $62.7 \pm 4.6\%$ & $52.7 \pm 3.1\%$ and $70.3 \pm 1.8\%$ & $33.3 \pm 6.4\%$, respectively. These differences can be originated from their antagonistic mechanism of action. While production of volatile organic compounds (VOCs) and lytic enzymes are the main mechanisms of *W. anomalus*, nutritional competition for iron is the main mechanism of *M. pulcherrima*. Although *M. pulcherrima* MPR3 didn't show best antagonistic performance in comparison to *Wickerhamomyces anomalus* BS91 in the *in vitro* assay, *M. pulcherrima* MPR3 showed antagonistic activity equivalent to the *W. anomalus* BS91 in the *in vivo* assay. Results from *in vivo* investigations highlight the significance of taking into account the tritrophic interaction (yeast-mold-host), which has a significant impact on the final results gained on vegetable matrix.

Table 1.7 Antagonistic effect studies of *Metschnikowia pulcherrima* against different microorganisms.

Yeast Species	Target Pathogens	Medium/Product	References
<i>Metschnikowia pulcherrima</i>	<i>Penicillium expansum</i>	Apple	Janisiewicz et al, (2001)
<i>Metschnikowia pulcherrima</i> , <i>Wickerhamomyces anomalus</i>	<i>Aspergillus flavus</i>	Pistachio nuts	Tejero et al, (2021)
<i>Metschnikowia pulcherrima</i>	<i>Fusarium oxysporum</i> , <i>Fusarium sambucinum</i> , <i>Rhizoctonia solani</i> , <i>Alternaria solani</i> , <i>Alternaria tenuissima</i> , <i>Alternaria alternata</i> , <i>Colletotrichum coccodes</i> , <i>Phoma exigua</i> , <i>Pectobacterium carotovorum</i> , <i>Streptomyces scabiei</i>	Solid media (PDA and TSA) Seed potato	Steglinska et al, (2022)
<i>Metschnikowia pulcherrima</i> , <i>Aureobasidium pullulans</i> (yeast-like fungus)	<i>Aspergillus carbonarius</i>	Wine grape	Curtis et al, (2012)
<i>Metschnikowia</i> sp.	<i>Fusarium oxysporum</i> , <i>Botrytis cinerea</i> , <i>Penicillium digitatum</i> , <i>Penicillium expansum</i> , <i>Alternaria alternata</i> .	Lemon	Oztekin and Karbancioglu-Guler (2018)
<i>Metschnikowia pulcherrima</i>	<i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> , <i>Candida parapsilosis</i> , <i>Candida albicans</i> , <i>Candida krusei</i> , <i>Escherichia coli</i> , <i>Botrytis cinerea</i> , <i>Alternaria alternata</i> , <i>Alternaria tenuissima</i> , <i>Alternaria mali</i> , <i>Pythium spp.</i> , <i>Sclerotium bataticola</i> , <i>Penicillium spp.</i> , <i>P. chrysogenum</i> , <i>Aspergillus ochraceus</i> , <i>Fusarium oxysporum</i> var. <i>lycopersici</i> , <i>F. solani</i> , <i>F. Moniliforme</i> , <i>Rhizoctonia spp</i>	YPG agar Luria-Bertani agar PDA/YPDA	Csutak et al, (2012)

According to study of Steglinska et al (2022), 10 different *M. pulcherrima* strains were tested in terms of antimicrobial activity against to 10 phytopathogens which are *Fusarium oxysporum*, *Fusarium sambucinum*, *Rhizoctonia solani*, *Alternaria solani*, *Alternaria tenuissima*, *Alternaria alternata*, *Colletotrichum coccodes*, *Phoma exigua*, *Pectobacterium carotovorum*, and *Streptomyces scabiei*. Results of *in vitro* analysis is that *Metschnikowia* isolates marked with J2, J3, J6, TK1, and M4 symbols were identified as the most effective in potato biocontrol based on their activity spectrum as

well as the diameter of their inhibition zones. The isolate *M. pulcherrima* TK1 which isolated from strawberry flower showed a 30%–100% reduction in nine phytopathogens except for only *P. carotovorum*. As a result, *M. pulcherrima* TK1 was suggested as a potential biological solution for protecting seed potatoes from phytopathogens.

Curtis et al (2012) conducted laboratory experiments to investigate the impact of temperature and relative humidity (RH) on the activity of three biocontrol agents (*Metschnikowia pulcherrima* LS16 yeast and two strains of *Aureobasidium pullulans* LS30 and AU34-2 yeast-like fungus) against *A. carbonarius* infection and ochratoxin A (OTA) accumulation in wine grape berries. The study involved inoculating the ochratoxigenic *A. carbonarius* strain A1102 into artificially damaged berries that had been treated with the biocontrol agents. The berries were then incubated for 5 days at two RH levels (60% and 100%) and three different temperatures (20°C, 25°C, and 30°C). Results showed that the three biocontrol agents significantly reduced OTA levels in the berries across all tested conditions after 5 days. The biocontrol agents exhibited higher colonization rates on grape berries at 20°C and 25°C compared to 30°C. Additionally, at 20°C and 25°C, higher RH values (100%) appeared to accelerate colonization. These findings underscore the influence of environmental factors on OTA contamination in wine grape berries and demonstrate the effectiveness of biocontrol agents against *A. carbonarius*. They also emphasize the importance of developing biocontrol agents capable of surviving in environments where *A. carbonarius* growth.

In the study of Oztekin and Karbancioglu-Guler (2018), the antifungal mechanism of action was studied by testing eleven different yeast cultures of *Metschnikowia* sp. from six different species against *Fusarium oxysporum*, *Botrytis cinerea*, *Penicillium digitatum*, *Penicillium expansum*, and *Alternaria alternata*. The secretion of lytic enzymes, depletion of iron, and synthesis of volatile organic compounds (VOCs) were identified as factors influencing the antifungal activity. The strongest biocontrol activity against both *P. digitatum* and *P. expansum* on lemons with an increasing shelf-life was shown by *M. aff. fructicola*.

According to study of Csutak et al (2012), three *Metschnikowia pulcherrima* strains isolated from white grapes (strains designated SG1 and SG2) and cherries (strain CPM1) were tested for antagonistic activity against natural and reference yeasts, fungi

and *E. coli*. Inhibitory activity and iron concentration in the media are correlated with the emergence of colonies in a range of colours (from pink to dark pink or with purple margins), which raises the possibility of an iron immobilisation mechanism similar to pulcherrimin production. The yeast strains *Candida tropicalis* OT4 and *Candida albicans* Cc, as well as the fungi *Botrytis cinerea* BCS and BCF1, which were all isolated from natural habitats, showed the best results when tested against the *Metschnikowia pulcherrima* SG2 strain.

1.3 Hypothesis

In this thesis, biocontrol capabilities against OTA producer *Aspergillus carbonarius* growth and OTA production, and effect of this biocontrol agent to quality parameters on grape were investigated.

2. MATERIALS AND METHODS

2.1 Pathogen

Aspergillus carbonarius 57-D1AN and *Aspergillus carbonarius* 39-04X4AN (OTA producer) were isolated and identified by Mutlu-Ingok et al (2020), on slant agar media. Mould culture was grown on Malt Extract Agar (Merck, 70145) at 25°C, and their stock solutions were prepared in 20% (v/v) glycerol and subsequently kept at -80°C for antagonism test.

2.2 Preparation of Yeast and Mould Suspension

Metschnikowia aff. *pulcherrima* DN-UY was used as the antagonistic yeast isolate, which was isolated and identified from grape leaves, and has proven antagonistic properties against *Aspergillus flavus* in another master degree thesis (Dikmetas et al, 2022)

Metschnikowia aff. *pulcherrima* DN-UY was grown on YEPD broth (%1 yeast extract, %2 peptone, %2 glucose) and incubated at 25°C for 48 hours, before the analysis. Yeast concentration was arranged with 0.85% saline solution. *Aspergillus carbonarius* isolates which produce ochratoxin A was grown on MEA (Malt Extract Agar) and incubated at 25°C for 5-7 days. Moreover, spore suspension concentrate was adjusted by Thoma counting method with 0.1% Tween 80 solution.

2.3 *In vitro* Effect of Antagonistic Yeast Against *A. carbonarius* Growth

The antagonistic activity of yeast was investigated in two different media using two different inoculation methods. While the *A. carbonarius* isolate used in the study shows optimum growth at 30°C and 0.97 a_w , it produces OTA at 20 °C and 0.97-0.99 a_w . Therefore, *in vitro* studies were conducted at 20, 25 and 30°C and 0.97-0.99 a_w (Mutlu-Ingok et al, 2020).

2.3.1 Dual culture method

The yeast strains were screened by dual culture technique to select antagonist strains against *Aspergillus carbonarius* 57-D1AN and *Aspergillus carbonarius* 39-04X4A. The concentration of *Metschnikowia* aff. *pulcherrima* DN-UY grown in YEPD broth was adjusted to 1×10^8 cells/mL using 0.85% saline solution. 100 μ L of *Metschnikowia* aff. *pulcherrima* suspension was spread linearly 1 cm beyond the petri dish on MEA by using swab. Sterilized discs were placed 2 cm beyond the yeast. 5 μ L of 1×10^5 spore/mL concentrated spore suspensions of *Aspergillus carbonarius* 57-D1AN and *Aspergillus carbonarius* 39-04X4AN were inoculated into the disc, separately, and the petri dishes were incubated at 25°C for 15 days (Tryfinopoulou et al, 2020). By comparing the radial growth of the mould in the presence of yeast strains (T) with the radial growth of the control during the incubation period (C), the inhibition of radial mycelial growth was calculated. The inhibition percentage calculated using the formula $I \% = (C - T / C) \times 100$. Each yeast strain was tested in three replicates, and the tests were repeated twice. According to dual culture results, further studies were conducted with *Aspergillus carbonarius* 57-D1AN.

2.3.2 Disc diffusion method

The antagonistic activity of yeast was also investigated in Czapek Yeast Extract Agar (CYA) (Table 2.1, Table 2.2 and Table 2.3) by disk diffusion method at different temperatures including 20°C, 25°C and 30°C, and two different water activity values as 0.99 and 0.97 a_w . All plates containing 20 mL of solidified growth medium were surface inoculated by spreading 100 μ L of yeast cell suspension with final concentrations of 10^8 and 10^7 CFU/mL. After solidification, sterilized discs were positioned in the center of the petri dish, 10 μ L of mould suspension (10^6 spore/mL) was inoculated on them, and then the petri dish was allowed to dry before being incubated at different temperatures and water activity levels. Analysis was tested in five replicates, and the tests were repeated twice.

Table 2.1 CYA medium composition.

Czapek Yeast Extract Agar (CYA)	Amount
Dipotassium hydrogen phosphate	1 g
Czapek solution	10 mL
Trace metal solution	1 mL
Yeast extract	5 g
Sucrose	30 g
Agar	15 g
Distilled water	1 L

Table 2.2 Trace metal solution composition.

Trace metal solution	Amount
CuSO ₄ .7H ₂ O	0,5 g
ZnSO ₄ .7H ₂ O	1 g
Distilled water	100 mL

Table 2.3 Czapek solution composition.

Trace metal solution	Amount
NaNO ₃	30 g
KCl	5 g
MgSO ₄ .7H ₂ O	5 g
FeSO ₄ .7H ₂ O	0,1 mL
Distilled water	100 mL

Petri dishes without yeast were used as control samples. To evaluate the growth, the diameter of the developing colonies was measured daily until the petri dish was full. (Ponsone et al, 2011). Each colony's diameter has been measured in order to determine radial inhibition. Equation 2.1 was used to determine the radial inhibition rate (RI):

$$RI (\%) = \frac{C - T}{C} \times 100 \quad (2.1)$$

where C (control) was the average diameter of fungal colonies in the absence of yeast isolates and T (treatment) was the average diameter of fungal colonies in the presence of yeast.

2.4 *In vivo* Effect of Antagonistic Yeast on Grapes

2.4.1 Treatment

A slightly modified method described by de Curtis et al (2017) and Ponsone et al (2017) was used to evaluate the effectiveness of *Metschnikowia aff. pulcherrima* DN-UY as a biocontrol agent. In this study, both curative and preventive effects of yeast

were investigated. Grapes were separated one by one from bunches of grapes and sterilized by dipping in 1% sodium hypochlorite solution for 1 minute and rinsed with sterile water, and then dried in the cabinet until dried (de Curtis et al, 2012).

Curative treatments were conducted by firstly dipping grapes into 1×10^5 spore/mL spore suspension, drying under an air flow cabinet, and leaving for 24 hours to allow the pathogen to infect it, before the antagonistic yeast treatment. Preventive treatments were carried out by first applying the antagonistic yeast to grapes, allowing the fruit to dry for 24 hours at room temperature (25°C), and then inoculating the treated fruit with the spore suspension to expose it to the pathogen (Gonzalez-Estrada et al, 2017; Madbouly et al, 2020). While *Metschnikowia aff. pulcherrima* was sprayed onto the grapes in curative treatments, in preventive treatments *Metschnikowia aff. pulcherrima* was immersed into the 1×10^8 cells/mL suspension. Control treatments were consisted of dipping the fruit into sterile water, before or after inoculation with *Aspergillus carbonarius*. Grapes were placed into disinfected eight plastic boxes containing twenty-four grapes and incubated at two different temperatures (10 and 25°C) at 95% RH, for 7 days (Figure 2.1.). All of the analysis were performed from 3rd, 5th and 7th days. The decayed grapes were counted at days 3, 5 and 7, whereas decay incidence was calculated according to equation 2.2 from Zhu et al (2015)

$$\text{Decay incidence (\%)} = \frac{\text{decayed grapes berries}}{\text{total grape berries}} \quad (2.2)$$

2.4.2 Enumeration of microbiota

10 g of treated grape samples were homogenized in a stomacher devices with 90 mL of peptone water for the purpose of counting the fungi on grapes. Serial dilutions were prepared and then, 0.1 mL from the appropriate dilutions were spread in duplicate on Dichloran Rose Bengal Chloramphenicol agar (Merck: 100466). It was incubated at 25°C for 48-72 hours.

2.4.3 Ochratoxin A detection

100 mL of solution containing 2% sodium bicarbonate was added to 10 g of sample, which were then homogenized with a strong mixer (Waring Blender) for 3 minutes. The solution was filtered, and 10 mL of filtered extract was cleaned by passing through the OchraTest immunoaffinity column (Romer Lab). After that, 10 mL of 20%

methanol solution passed through column. Finally, 1.5 mL 98% methanol passed through column, respectively and collected.

HPLC was conducted in a Shimadzu HPLC system equipped with an LC-20AT separation module, SIL-20A autosampler, DGU-20A5 degasser, fluorescence detector (excitation, 330 nm; emission, 443 nm), CBM-20A communication bus module, CTO-20A column oven, and pre-packed reversed-phase C18 column. The used mobile phase acetonitrile:water:acetic acid (47:51:2, v/v/v) was pumped at 1.0 mL/min with 100 μ L injected volume. OTA was quantified by comparing the areas of peaks obtained at the same retention time as the standard OTA with the corresponding calibration and expressed as ppb.

2.5 Effect of Yeast on Natural Infection and Quality of Grapes During Storage

2.5.1 Treatment

After the effect of antagonistic yeast to control *A. carbonarius* growth and OTA production, the effect of antagonistic yeast on quality parameters were also investigated. Randomly selected grapes were submerged in sterile deionized water as a control, or in cell suspensions of 1×10^7 CFU/mL as a treatment. The grape berries in the various treatments were then air-dried and stored at 25 °C and 10° C and 95% RH in plastic baskets (Figure 2.2.). After 7 and 14 days of storage, the incidence of natural infection and a fruit quality evaluation were determined. Each treatment consisted of four replications consist of twenty-four berries (Chen et al, 2022). The physicochemical quality parameters were determined by the following methods from Godana et al (2022) studies.

2.5.2 Physico-chemical quality of fresh table grapes

2.5.2.1 Natural decay

The natural decay was determined by subtracting the total amount of unmarketable fruits from the total amount of initial fruits and multiplying the result by 100%.

2.5.2.2 Weight loss

The initial weight of the grape berries was subtracted from their weight at the end of storage, and the weight loss was calculated as a percentage.

2.5.2.3 Fruit firmness

Firmness was measured using a 5 mm diameter flat probe on a texture analyzer (TA Plus, Ametek Lloyd Instruments Limited, UK). The pre-test speed of the probe was 10 mm sec⁻¹ and penetrating distance of 4 mm into the fruit and trigger force of 5g (Gunness et al, 2009). Fruit firmness was registered as the maximum force (N).

2.5.2.4 Total soluble solid

Total soluble solids (TSS) were measured and expressed as a percentage (%) using a portable refractometer (LYT-330, Shanghai Linyu Trading Co., Ltd.).

2.5.2.5 pH and titratable acidity

The pH of the grape samples were measured from the Testo pH-meter. Titratable acidity (TA) was determined by titrating grape solution (10 g pulp sample in 100 mL deionized water) with NaOH solution (0.1 N) until pH 8.1 was reached and were expressed as percentage of tartaric acid in the grapes, from the equation 2.3 and N refers to normality of titrant, V refers to volume of titrant and W refers to mass of sample. Tartaric acid equivalent weight was taken as 75.05 (Takma and Korel, 2017; Sadler and Murphy, 2010).

$$\% \text{ acid (wt / wt)} = \frac{N \times V \times \text{Eq wt}}{W \times 1000} \times 100 \quad (2.3)$$

2.6 Statistical Analysis

All experiments were statistically analyzed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA) with the expression mean $\pm$ standard deviation. Different yeast isolates were compared with a one-way analysis of variance (ANOVA) test. In addition, Tukey's multiple range test was used to assess separations and differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 *In vitro* Effect of Antagonistic Yeast Against *A. carbonarius* Growth

3.1.1 Dual culture method

Antagonistic activity of *Metschnikowia* aff. *pulcherrima* DN-UY against OTA producers *A. carbonarius* 39-04X4AN and *A. carbonarius* 57-D1AN was initially screened using the dual culture test. Mycelial growth inhibition was clearly seen in Figure 3.1. against *A. carbonarius* 39-04X4AN and *A. carbonarius* 57-D1AN.

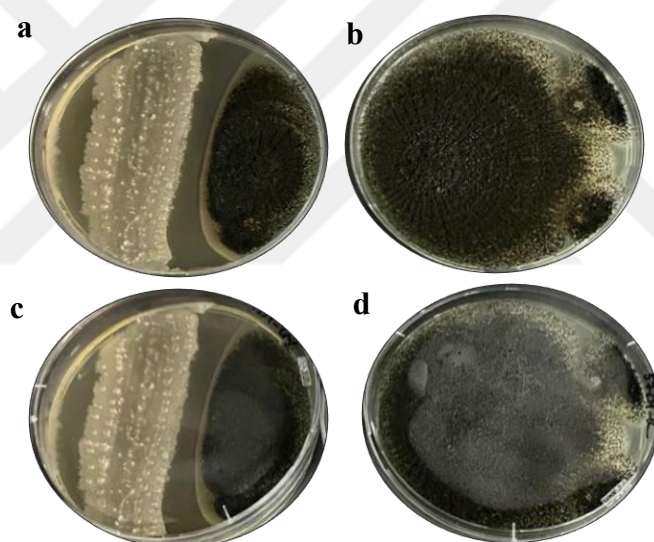


Figure 3.1 *In vitro* test of antagonism of a *Metschnikowia* aff. *pulcherrima* DN-UY against *A. carbonarius* 39-04X4AN (a) and *A. carbonarius* 57-D1AN (c) using the dual culture technique on MEA plates after 12 days of incubation with the control (b and d, respectively).

Inhibition ratio of *Aspergillus carbonarius* 57-D1AN ($71.43 \pm 1.75\%$) was greater than *A. carbonarius* 39-04X4AN ($68.57 \pm 2.02\%$) after 12 days, because of that further studies were conducted with *Aspergillus carbonarius* 57-D1AN.

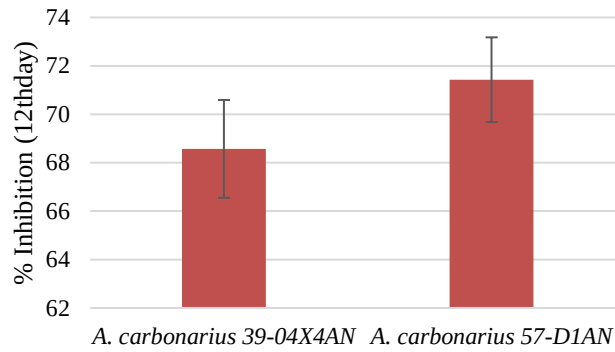


Figure 3.2 Inhibition ratio of *Metschnikowia* aff. *pulcherrima* DN-UY against *A. carbonarius* 39-04X4AN and *A. carbonarius* 57-D1AN after 12 days by *in vitro* test.

Dikmetas (2022) studied with *Metschnikowia* aff. *pulcherrima*, *Metschnikowia* aff. *fructicola*, *Metschnikowia pulcherrima*, *Meyerozyma guilliermondii* and *Moesziomyces bullatus* which were isolated from hawthorn, bean leaves, corn tassel, and hoşkiran. The study showed that all yeasts had an antagonistic effect against *Aspergillus flavus*. Inhibition was clearly obvious in the zone surrounding the yeast colony, as evidenced by the limited fungal mycelium growth and spore production inhibition. Colony diameter was reduced to 36-40% after 9 days of incubation when compared to the untreated control.

In the study of Oztekin and Karbancioglu-Güler (2021), *Metschnikowia* isolates including *M. pulcherima* obtained from different sources were tested on *Fusarium oxysporum*, *Botrytis cinerea*, *Penicillium digitatum*, *Penicillium expansum*, and *Alternaria alternata*. The mycelium growth of *F. oxysporum*, *B. cinerea*, *A. alternata*, *P. digitatum*, and *P. expansum* was inhibited at varied degrees by a total of 11 *Metschnikowia* yeasts. Some yeasts including *M. aff. fructicola*, *M. pulcherrima*, and *M. sinensis*, which have high pulcherrimin production, have totally inhibitory effect against *Penicillium* and *Alternaria*.

3.1.2 Disk diffusion method

In this study, three different temperature (20-25-30⁰ C) and 2 different water activity (0.99, 0.97 a_w) conditions and 10⁷ CFU/mL and 10⁸ CFU/mL yeast concentration was examined. According to results, *Metschnikowia* aff. *pulcherrima* have an antagonistic effect on OTA producer *A. carbonarius* 57-D1AN as seen at Figure 3.3, 3.4 and 3.5, while the control samples have mould growth, there is no mould growth observation at treated petri dishes. All petri dishes were investigated under microscope. So it can

be said that growth of *A. carbonarius* 57-D1AN nearly inhibited by *Metschnikowia* aff. *pulcherrima*.

Table 3.1 *In vitro* test results of antagonism of a *Metschnikowia* aff. *pulcherrima* DN-UY against *A. carbonarius* 57-D1AN.

Temperature ($^{\circ}$ C)	Water Activity (a_w)	Yeast Concentration (cells/mL)	Mould Concentration (spore/mL)	% Inhibition
20	0,97	10^7	10^6	Nearly inhibited
25	0,99	10^8		
30				

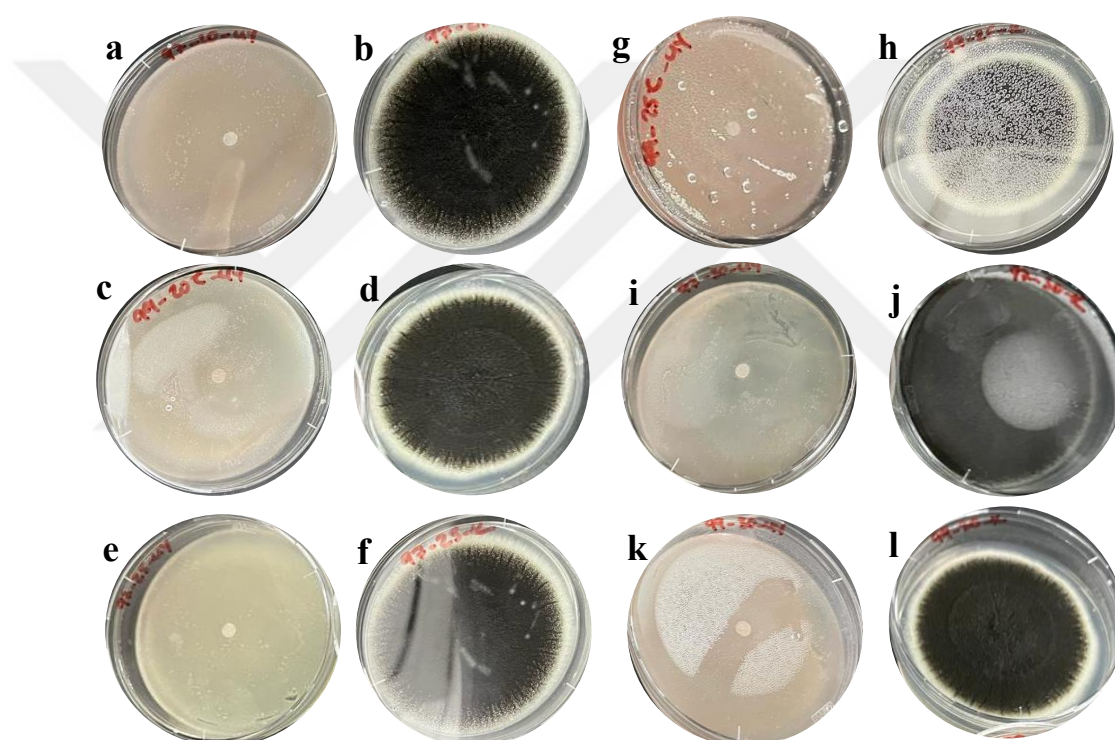


Figure 3.3 *In vitro* test of antagonism of a *Metschnikowia* aff. *pulcherrima* DN-UY (10^7 cells/mL) against *A. carbonarius* 57-D1AN using the disk diffusion technique on CYA plates with 0,97 a_w at 20° C (a) and with 0,99 a_w at 20° C (c), 0,97 a_w at 25° C (e) and with 0,99 a_w at 25° C (g), 0,97 a_w at 30° C (i) and with 0,99 a_w at 30° C (k) after 15 days of incubation with their control (b,d,f,h,j,l respectively).

In the study of Tryfinopoulou (2019), influence of *Saccharomyces cerevisiae* Y33 and *Rhododurula mucilaginosa* Y38 on the growth and ochratoxin A (OTA) production of four *Aspergillus carbonarius* isolates grown either individually or in mixed culture were investigated. Five different temperatures (15, 20, 25, 30, and 35° C) and two different growth media (Czapek Yeast Extract Agar (CYA) and grape juice medium (JA) were used for assaying fungal growth. Results shows that *S. cerevisiae* Y33 have

totally growth reduction on four *A. carbonarius* isolates at 15⁰ C both 10⁴ and 10⁵ concentration. Also at 35⁰ C temperature, total inhibition is shown for *A. carbonarius* F71 as well. Similarly, in this study growth of *A. carbonarius* 57-D1AN nearly inhibited by *Metschnikowia* aff. *pulcherrima*.

In the other study, antagonistic effect of three different strains of *M. pulcherrima* which were isolated from Turkey originated grapes, were studied against to *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma* spp., *Paecilomyces* spp., and *Bipolaris* spp. (Turkel and Ener, 2009). This investigation revealed that while all three strains of *M. pulcherrima* produce the same quantity of the pigment pulcherrimin, there are significant differences between them when it comes to their antimicrobial activity against different microorganisms.

Antagonistic effect of five strains of *M. pulcherrima* against five mould strains which are *Alternaria alternata* LOCK409, *Botrytis cinerea* LOCK453, *Penicillium expansum* (syn. *P. glaucum*) LOCK535, *Rhizopus oryzae* LOCK547, and *Verticillium cinnabarinum* LOCK576 were investigated at the study of Pawlikowska et al (2019). *In vitro* studies showed that tested yeast strains have inhibitory effect against various fungal pathogens. The yeasts *W. anomalus* and *D. bruxellensis*, which are frequently isolated as spoilage microbiota in foods and beverages, showed high inhibition.

Antagonistic effect of *M. pulcherrima* can be examined by *in vitro* analysis at lower yeast concentration than 10⁷ cells/mL and so on. According to inhibition ratio, product implementation can be managed.

3.2 In vivo Effect of Antagonistic Yeast on Grapes

3.2.1 Decay incidence (DI%)

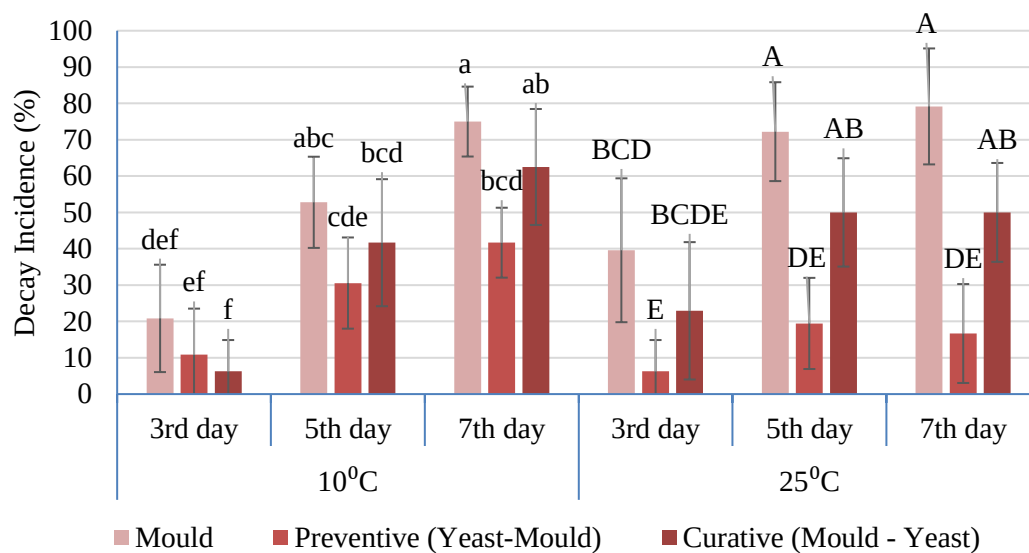
Two different temperature and two different treatments were applied to table grapes. While no significant differences were between preventive and curative treatments at 10⁰ C, *Metschnikowia* aff. *pulcherrima* DN-UY application as a preservative showed the highest significant inhibition against the fungal damage caused by *A. carbonarius* 57-D1AN at 25⁰ C. Also, there were no significant differences between only mould and curative application on grapes at 25⁰ C ($p < 0.05$). Table 3.2 and Table 3.3 shows the treated grapes after 3rd, 5th and 7th days at both 10⁰ C and 25⁰ C.

According to these results, the application of yeast strains as an preventive or curative agent, *M. pulcherima* efficiency was changed. *M. pulcherima* reduced *A. carbonarius* infections by 16.67% (preventive) and 50.00% (curative) from 79.17%, at 7th days of storage at 25° C. However, at 10° C, *A. carbonarius* infections reduced to 41.67% (preventive) and 62.50% (curative) from 75.00%, but there were no significant differences among the non-treated and antagonistic yeast application as curative.

Similar to our results, Abraham et al (2010) reported that *Bacillus* sp. while applied before the the *P. digitatum* infection, reduced to disease incidence 5%, however there were no curative effect on lemons and oranges. The yeasts use all of the nutrients released by a fruit wound once they have taken hold there. In this situation, there were not any nutrients are left to stimulate *Penicillium* spores for germination. However, if the *Penicillium* spores had access to wound nutrients for 3 hours prior to the yeast application, the *Penicillium* stimulation to germinate will have already taken place. Therefore, yeast isolates wouldn't used well in a curative treatment.

In addition, Mewa-Ngongang et al (2021) found lower inhibition with curative treatment than preventive treatment that *Pichia kluyveri* effect on apples. On contrast, there were no significant changes in *Alternaria* inoculation time compared to *M. pulcherrima* RCM2 at 60 and 90 days of evaluation on grapes, and they indicated that the yeast can be utilized as a preventive or curative agent (Stocco et al, 2019).

Moreover, antagonistic yeasts especially showed their effect on the 5th day of storage, because the decay incidence found as stable. All of the previous research used higher temperatures than are typically used in refrigerated chambers for their biocontrol activity experiments. *M. pulcherrima*, on the other hand, appears to be capable of colonizing the surface of fruit stored at temperatures below 4°C (Janisiewicz et al, 2001; Spadaro et al, 2004, 2010). Rodriguez Assaf et al (2020) demonstrated that *M. pulcherima* species was found to be an efficient antagonist in cherries at 1°C, as it inhibited blue mold and brown mold, respectively. According to our result, also *M. pulcherima* was found effective at 10°C.



A, B, C, D, E values within the 25⁰ C temperature with the different superscript upper case are significantly different (p<0.05).

a, b, c, d, e, f values within the 10⁰ C temperature with the different superscript lower case are significantly different (p<0.05).

Figure 3.4 Decay incidence ratio of grapes with preservative and curative treatments at 10⁰ C and 25⁰ C

Table 3.2 Treated grapes with only yeast, only mould, yeast+mould (preventive) and mould+yeast (curative) at 10⁰ C after 3rd, 5th and 7th days.

Temperature (⁰ C)	Day	Yeast	Mould	Preservative (Yeast-Mould)	Curative (Mould-Yeast)
10	3				
	5				
	7				

Table 3.3 Treated grapes with only yeast, only mould, yeast+mould (preventive) and mould+yeast (curative) at 25⁰ C after 3rd, 5th and 7th days.

Temperature (⁰ C)	Day	Yeast	Mould	Preservative (Yeast-Mould)	Curative (Mould-Yeast)
25	3				
	5				
	7				

3.2.2 Enumeration of microbiota

Fungal growth of untreated, preventive treated and curative treated grapes at two different temperatures (10^0 C and 25^0 C) after 3 ,5 and 7 days of incubation were shown Table 3.4.

Table 3.4 Fungal growth (log/mL) of untreated, preventive treated and curative treated grapes.

Day	Fungal count (log/mL)					
	10^0 C			25^0 C		
	Control	Prevent. (Yeast-Mould)	Curative (Mould-Yeast)	Control	Prevent. (Yeast-Mould)	Curative (Mould-Yeast)
3	<2.00 ^{Ba}	<2.00 ^{Ba}	<2.00 ^{Ba}	6.04±0.09 ^{Ca}	<2.00 ^{Ab}	<2.00 ^{Bb}
5	5.45±0.16 ^{Aa}	4.58±0.20 ^{Ab}	<2.00 ^{Bc}	6.20±0.09 ^{Ba}	<2.00 ^{Ac}	5.35±0.11 ^{Ab}
7	5.61±0.11 ^{Aa}	4.35±0.12 ^{Ac}	4.60±0.11 ^{Ab}	7.39±0.07 ^{Aa}	<2.00 ^{Ac}	5.23±0.02 ^{Ab}

^{A, B, C} values within the same column with the different superscript upper case are significantly different (p<0.05).

^{a, b, c} values within the same row of each temperature with the different superscript lower case are significantly different (p<0.05).

For both 10^0 C and 25^0 C, preventive treated grapes had the lowest fungal concentration according to curative applied and non-treated grape samples at the end of the 7 days. But also, curative treated grapes have lower fungal concentration than control grapes. Addition to this, storage temperature and fungal concentration are directly proportional with each other.

3.2.3 Ochratoxin A detection

Ochratoxin A concentration of untreated, preventive treated and curative treated grapes at two different temperatures (10^0 C and 25^0 C) after 3, 5 and 7 days of incubation were shown Figure 3.5.

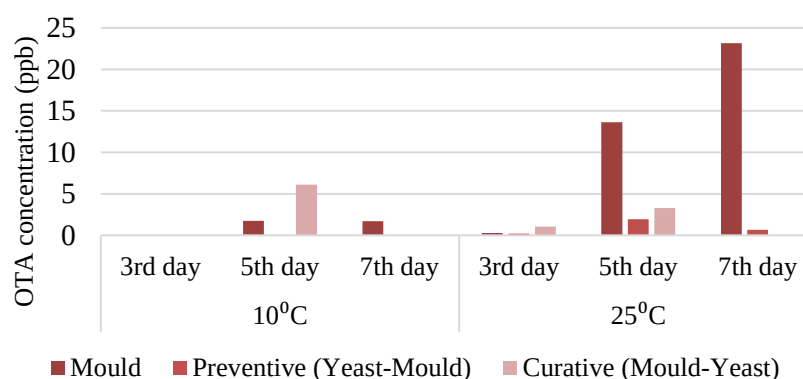


Figure 3.5 OTA concentration.

According to results, preventive treated and curative treated grapes shown lower OTA concentration according to only mould treated grapes. According to 25⁰ C storage temperature, OTA concentration was lower at 10⁰ C.

In the study of Curtis et al (2012), artificially wounded berries were treated with biocontrol agents and then exposed to the ochratoxigenic *A. carbonarius* strain A1102. The berries were incubated for five days at two different levels of relative humidity (RH) - 60% and 100% - and three different temperatures - 20°C, 25°C, and 30°C. At 100% RH, all three biocontrol agents (*M. pulcherrima* LS16, *Aureobasidium pullulans* LS30, and *A. pullulans* AU34-2) demonstrated effective protection of the grape berries at 20°C throughout the five-day incubation period. However, at higher temperatures, significant protection was achieved only until the second day of the experiment: all three strains at 25°C, and only strain LS16 at 30°C. After five days, the three biocontrol agents were successful in significantly reducing the level of ochratoxin A (OTA) in the berries under all tested conditions.

Tryfinopoulou et al (2020) used 67 different yeast isolates and 3 wild isolates of *A. carbonarius* in their study to investigate interaction. OTA analyses were performed on CY broth, agar and also grape berries, and grape juice on days 8 and 15 of incubation at 25 °C. Toxin production was observed in all samples inoculated with *A. carbonarius* F_{mix}, but no consistent pattern was found across different sampling days. Specifically, on day 7, a statistically significant concentration of OTA was detected in natural grapes inoculated with mixed cultures of *A. carbonarius*, compared to control and co-culture with *S. cerevisiae*. This trend was also observed on day 24, but not during intermediate sampling times. This variability could be attributed to the presence of indigenous microbiota, which may have influenced toxin presence. Interestingly, sterilized grape juice showed a different trend, with higher toxin production due to the absence of indigenous microbiota. However, there was a difference in OTA concentration between synthetic growth media and food matrices. For instance, after 15 days of spot inoculation on CYA plates and grape juice samples, F_{mix} culture produced 15,908 ng/g and 5 ng/mL OTA, respectively. These findings align with previous studies on OTA reduction and *Aspergillus* inhibition (Armando et al, 2013; Ponsone et al, 2011), indicating a strong dependence on factors such as yeast species, OTA-producing species, environmental conditions, and the type of study (*in vitro* or *in vivo*).

3.2 Effect of Yeast on Natural Infection and Quality of Grapes During Storage

3.2.1 Physico-chemical quality of fresh table grapes

3.2.1.1 Natural decay

Decay ratio was controlled at different temperature conditions (10⁰ C and 25⁰ C) with both control grapes and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes. The results showed that there were no significant differences in *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes according to untreated grapes in terms of decay ratio ($p<0.05$).

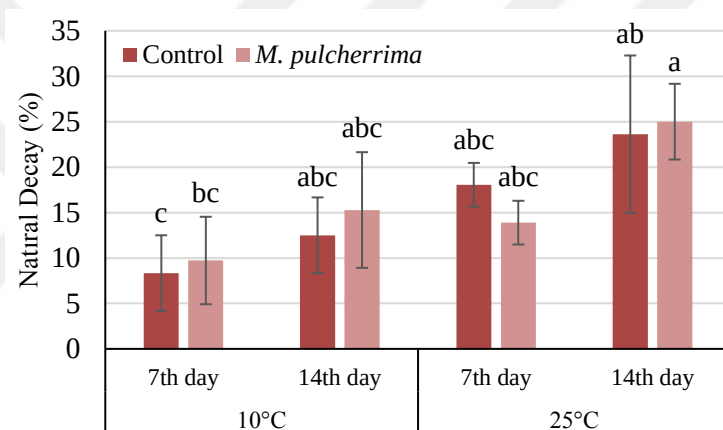


Figure 3.6 Natural decay ratio of both untreated and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes were stored at two different temperatures (10⁰ C and 25⁰ C) after 7 and 14 days of storage.

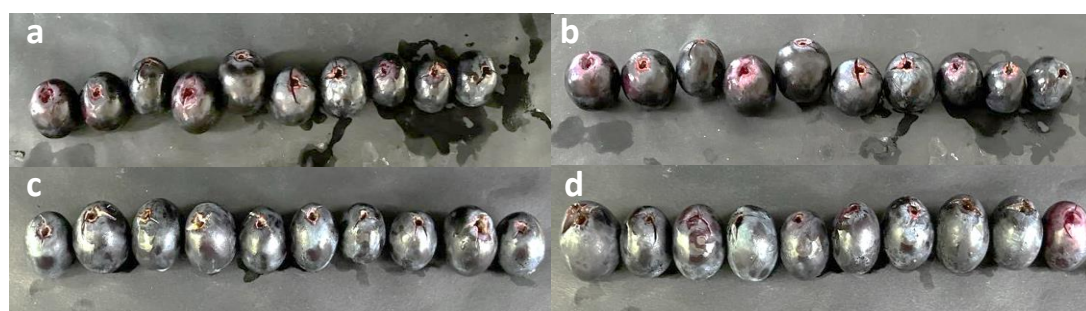


Figure 3.7 Both *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes (a for 7th days, c for 14th days) and untreated grapes (b for 7th days, d for 14th days) at 10⁰ C.

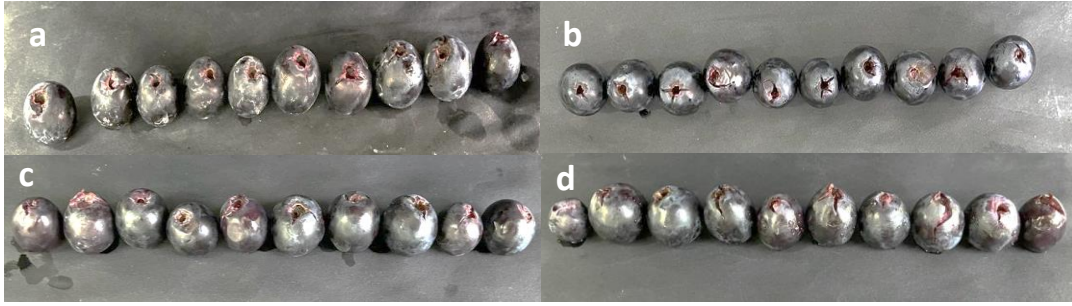


Figure 3.8 Both *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes (a for 7th days, c for 14th days) and untreated grapes (b for 7th days, d for 14th days) at 25^o C.

Yang et al (2021) studied the preventive effect of *M. pulcherrima* against grey spot rot (*Pestalotiopsis vismiae*) on loquat fruit. The application of *M. pulcherrima* E1 resulted in a significant reduction ($p < 0.05$) in decay in loquat fruits compared to untreated fruits. When different concentrations of *M. pulcherrima* E1 cells were applied, the decay incidence decreased as follows: 79.66% at 10^6 cells/mL, 77.75% at 10^7 cells/mL, 43.68% at 10^8 cells/mL, and 31.81% at 10^9 cells/mL, in comparison to untreated fruits. *M. pulcherrima* E1 was found to colonize wounds and the surface of loquat fruits quickly and maintain a stable population. The mechanisms by which *M. pulcherrima* E1 acts against the pathogen include competing with other microorganisms for nutrients (like iron), forming a biofilm, producing volatile organic compounds (VOCs), and increasing the activity of defense-related enzymes in the treated fruits.

3.2.1.2 Weight loss

While weight loss of untreated grapes increases after 14 days at 10^o C, no significant increases for weight loss of *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes ($p < 0.05$) were observed. On the contrary, significant increases in terms of weight loss of *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes were observed compared to untreated grapes at 25^o C .

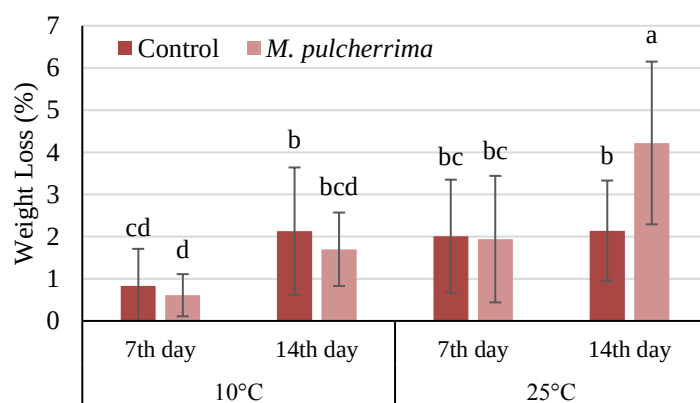


Figure 3.9 Weight loss of both untreated and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes stored at two different temperatures (10^0 C and 25^0 C) after 7 and 14 days of storage.

3.2.1.3 Fruit firmness

The results from the texture analysis were expressed in terms of the stress. According to results, there were no significant differences between untreated and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes at both 10^0 C and 25^0 C ($p < 0.05$).

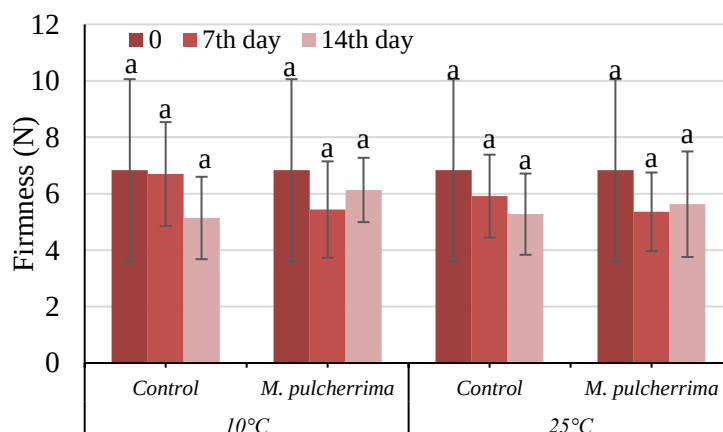


Figure 3.10 Texture analysis results of both untreated and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes at two different temperatures (10^0 C and 25^0 C) after 7 and 14 days of storage.

3.2.1.4 Total soluble solid & pH & titratable acidity

Physicochemical parameters of both untreated and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes stored at two different temperatures (10^0 C and 25^0 C) after 7 and 14 days of storage were shown at Figure 3.13 and Table 3.4.

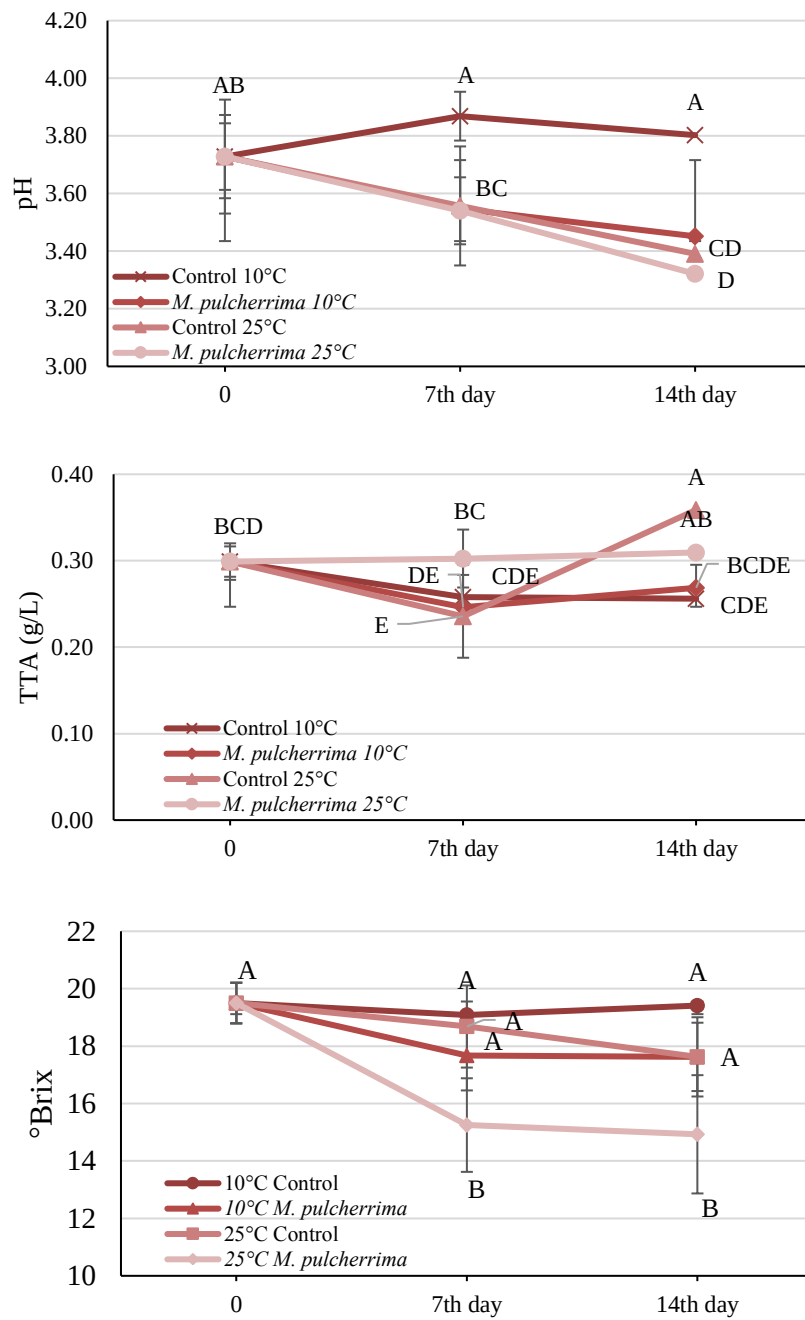


Figure 3.11 Physicochemical parameters (pH, TTA and Brix) both untreated and *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes stored at two different temperatures (10⁰ C and 25⁰ C) after 7 and 14 days of storage.

Table 3.5 Changes in quality parameters of *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes during storage.

Storage Temp. (°C)	Treatment	Storage Time (day)	pH	TTA (g/L) %Tartaric acid	°Brix
10°C	Untreated	7	3.87±0.15 ^A	0.26±0.02 ^A	19.08±1.43 ^{AB}
		14	3.80±0.09 ^A	0.26±0.01 ^A	19.42±1.38 ^A
	<i>Metschnikowia</i> aff. <i>pulcherrima</i> DN-UY treated	7	3.55±0.12 ^B	0.25±0.02 ^A	17.67±1.19 ^C
		14	3.45±0.16 ^B	0.27±0.01 ^A	17.63±0.52 ^{BC}
25°C	Untreated	7	3.56±0.21 ^A	0.24±0.01 ^B	18.68±1.21 ^A
		14	3.39±0.21 ^{AB}	0.36±0.05 ^A	17.63±1.19 ^A
	<i>Metschnikowia</i> aff. <i>pulcherrima</i> DN-UY treated	7	3.54±0.12 ^A	0.30±0.02 ^A	15.25±1.63 ^B
		14	3.32±0.12 ^B	0.31±0.04 ^A	14.93±2.06 ^B

A, B, C values within the same column for each temperature with the different superscript upper case are significantly different (p<0.05).

For 10⁰ C, there were no significant differences on pH during the 14 days shelf life of table grapes and yeast applied grapes. But pH of *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes lower than the control grapes. Also, Brix value shows same trends on grapes. But, there were no significant changes on TTA value of table grapes.

For 25⁰ C, pH of grapes decreased after 14 days on *Metschnikowia* aff. *pulcherrima* DN-UY treated grapes. But at the end of 14 days, no significant differences on final pH between both untreated and treated grapes were observed. While titratable acidity of grapes did not change on yeast treated grapes, it increased at untreated ones. Brix value of untreated grapes did not change during the 14 days shelf life, it decreased when the grapes treated with *Metschnikowia* aff. *pulcherrima* DN-UY.

Depending on our results, *M. pulcherima* found effective to control *A. carbonarius* infection on grape without any change of the grape sample with the physicochemical analysis. The two temperatures were chosen to represent room temperature and cold storage for grapes. Postharvest treatment of fruits and vegetables should retain the quality of the produce in addition to decay control. Natural decay, weight loss, total soluble solids, fruit firmness of table grapes are postharvest quality characteristics that impact their acceptability to wholesale buyers and consumers (Tomala et al, 2020). The TSS of fruits is an important quality criterion that is often calculated by calculating the fruits' Brix degree. It contains the fruits' carbohydrates, organic acids, proteins, lipids, and minerals. As a result, it is an essential quality criterion that influences the nutrition and flavor balance of the fruit (Godana et al, 2022).

The current study investigated the physicochemical quality factors of table grapes such as weight loss and firmness. These two variables have an impact on the texture of the fruit. Fruits with a high weight loss are less turgid, resulting in a softer texture that consumers dislike. Similar to our study, the texture of the product was protected when compare with the control samples at two storage conditions (Godana et al, 2022), however, yeast treated samples weight loss were significantly higher on cold storage conditions. Yeast antagonist application, had no significant effect on weight loss, fruit firmness, pH, TSS and titratable acidity after 7 and 14 days of storage at 20⁰ C. Zhang et al (2008), demonstrated that peaches weight loss, fruit hardness, total soluble solids, ascorbic acid, and titratable acidity were all unaffected by the *Rhodotorula glutinis* application at 20⁰ C. The current study found that mandarin fruits treated with *R. minuta* var. *minuta* had decreased TSS and sugar/acid ratio, while weight loss of Kinnow mandarin fruits increased significantly as storage time increased, in accordance with our results (Bhan et al, 2023). In our present work, there were no significant differences found in all physicochemical properties, except 25⁰ C storage at 14 days. After treatment of grapes with a suspension of *M. pulcherima*, natural disease incidence reduced, but quality of grape was not affected at both temperature, similar to Shen et al (2019) studies on the efficacy of *S. pararoseus* on strawberry

4. CONCLUSIONS AND RECOMMENDATIONS

Ochratoxin A (OTA), a mycotoxin of global concern for human and animal health, is produced by certain species of *Aspergillus* (*A. ochraceus*, *A. carbonarius*, *A. niger*) and *Penicillium* (*P. verrucosum*, *P. nordicum*, and *P. viridicatum*). Its toxicological effects include kidney toxicity, mutagenicity, teratogenicity, and immunotoxicity. The International Agency for Research on Cancer has classified OTA as a possible human carcinogen (Category 2B). Various food sources, including cereals (such as oats, maize, wheat, and barley), cereal products, coffee beans, dried fruits, beer, grape juice, wine, nuts, cacao products, and spices, can be contaminated with OTA. Fungal deterioration, caused by postharvest diseases, is a major contributor to food waste. In response to public demand for reduced pesticide use in food and the environment, as well as the emergence of resistant fungal strains, alternative methods like biological control are being developed. Yeasts and bacteria have been extensively researched as biocontrol agents. Therefore, in the mentioned study, antagonistic yeasts were isolated and identified for the purpose of controlling the growth of *Aspergillus carbonarius* and the production of ochratoxin A.

The antifungal effects of *Metschnikowia* aff. *pulcherrima* DN-UY were examined through *in vitro* analysis, demonstrating its antagonistic properties. Additionally, the efficacy of *Metschnikowia* aff. *pulcherrima* DN-UY as a biocontrol agent was assessed by investigating both its curative and preventive effects. The findings indicated that the preventive treatments exhibited greater effectiveness on grapes compared to the curative treatments, regardless of the temperature conditions. Moreover, the impact of the antagonistic yeast on quality parameters was evaluated. The application of yeast to grapes had no significant effect on physiochemical properties, except for a slight influence on brix value and odor properties at higher temperatures. Therefore, further studies should explore the potential use of yeast treatments in grape-based products. Based on these results, utilizing antagonistic yeasts as an alternative to conventional chemical methods appears promising for controlling the growth of *A. carbonarius* and mitigating its production of OTA.



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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Konuşur, G., Dikmetaş, D.N., Karbancıoğlu-Güler, F.** (2023, May). An Alternative Biological Control Method Against *Aspergillus carbonarius* Growth. 10th International Conference on Raw Materials to Processed Foods (RP-Foods 2023), Turkey.

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

National Articles

- Dikmetaş, D.N., **Konuşur G.**, Mutlu-İngök A., Gülsünoğlu Z., Karbancıoğlu-Güler F., 2019. Antimicrobial and Antioxidant Properties of Hydrosol/Essential Oils Obtained from Orange (*Citrus sinensis*) Peels. *Düzce University Journal of Science and Technology* 7(1): 274-283.

CERTIFICATES

- Food Defence Coordinator / AIB International / Jan'22
- BRC Global Standard for Food Safety - Issue-9 / BRCGS / Nov'22