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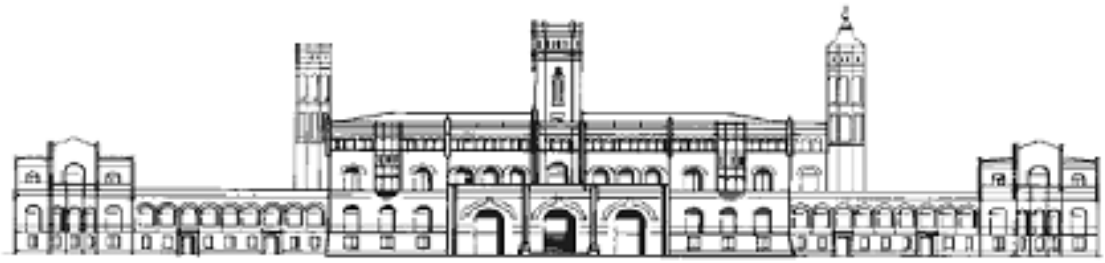

Characterisation of the melano protein of *Venturia inaequalis* and its impact on plant pathogenesis

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades
Doktor der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation
von

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2011

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Tag der Promotion: 16. Februar 2011

Dedicated to

My God (Allah)

Summary

Apple scab caused by the fungal pathogen *Venturia inaequalis* (Cke.) is one of the most harmful diseases for apple trees. A functional role during the process of infection of the host plant *Malus doemstica* by *V. inaequalis* is assessed for the extracellular melano protein that is secreted by the pathogen. Hignett and Kirkham, (1967); Hignett, (1973); Hignett *et al.*, (1984) give the initial overview about the dark colored melano protein and its possible function during the infection. Further characterization by Singh *et al.*, (2005) have shown that during growth in liquid culture, *V. inaequalis* secretes the 36 kD melano protein to the medium and to the host plant into the apoplast during the process of infection. This protein belongs to the group of proteins and has the nature to bind iron.

Since marginal knowledge of the function of melano proteins during the process of infection to the host plant and its role in plant pathogenesis is present, further characterization is required. In the present work the role and function of the melano protein from *V. inaequalis* on molecular level is studied in detail. On the basis of known peptide sequences the corresponding open reading frame (ORF) of the melano protein from *V. inaequalis* was amplified, sequenced and analyzed. The melano protein is encoded by an ORF of 981 bp. In addition, DNA were extracted from *V. inaequalis* with newly developed method and compared with the established CTAB method. Upstream region sequence was derived from the newly developed SSSBT-PCR method (-851 bp length) and analyzed. A class II intron was predicted at the region of 528:591bp. In total DNA a fragment of 2118 bp was obtained.

The deduced amino acid sequence of the melano protein shares similarity to 1,3-glucanase (glycol_hydro_17 superfamily) and the protease to retropepsin_like_LTR_1 (aspergillo-pepsin_like family). Both predicted enzyme activities could be confirmed by enzymatic tests of the overexpressed recombinant melano protein from *E. coli.* Moreover, theses investigations revealed that the proteolytic as well as the glucanolytic enzyme activity of the melanoprotein is inhibited strongly by PMSF (Phenylmethylsulfonylfluorid). Furthermore, it could be demonstrated that the protease function of the melano protein is influenced by different ions ($CaCl_2$, $MgCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $FeCl_3$, $ZnCl_2$). The protease activity was enhanced by the presence of 2 mM Co^{2+} and shows high temperature stability with an optimum at 90°C. All other tested ions reduced the proteolytic activity drastically.

In addition glucanase activity was confirmed with thin layer chromatography. The optimum temperature for glucanase was 60 °C and for protease was 90 °C. The pH optimum for the glucanase and protease was 6.0, and protease activity pH optimum widened (6.0-8.0 pH) in presence of 2 mM Co^{2+}. The protease units in mg were determined for the recombinant melano protein in relation to trypsin protease units (40 U/mg) in protease activity (367 U/mg). Glucanase activity for recombinant melano protein was determined by newly developed Congo red spectrophotometry method and compared to others methods on different substrate sources.

Also the assembly and activity of the recombinant melano protein in presence of the natural pigment melanin and in combination with EDTA or Co^{2+} were studied. The addition of melanin to the melano protein leads to the formation of oligomers with apparent molecular masses of 72, 136, 162, 186, 222 and 265 kD and in presence of Co^{2+} larger oligomers of 209, 536, 973, 1490, 2946, 5126, 7057 kD were formed.

Based on the glucanase and protease activity the growth of gram positive and gram negative bacteria is inhibited by the presence of the recombinant melano protein and offers the application as a potential antibacterial agent. Plant surface proteins were investigated using different newly developed methods (Replica-staining with bromophenol blue, silver staining, Replica-Far-Western-Blot). The Far Western blot technique showed that the melano protein is predominately present on the cuticle membrane is targeting besides other proteins to a plant chitnase. The chitinase activity for the surface protein from *M. domestica* cv. Remo was confirmed with SDS-chitin PAGE. These partners are implying a broad impact of the melano protein to the host physiology during the process of infection. Moreover it was shown that the plant proteins can be affected by melano proteins proteolytic activity (newly developed assay on PVDF membrane) in all parts of the host plant.

The Interaction of the plant proteins of the surface, apoplast and the protoplast of different tissues cells with the recombinant melano protein reflects that the protein is scavenger for all the plant local proteins. The effect of the apoplastic fluid from *ex vitro* and *in vitro M. domestica* cvs. Elstar and Remo on the germination of *V. inaequalis* no. 36 conidia and the enzyme activities of the melano protein (glucanase and protease) of the recombinant melano protein showed that the plant can reduce these activities by the release of its own inhibitors.

In general it can be concluded that the melano protein from *V. inaequalis* with its bifunctional enzyme activities plays a sophisticated role in the process of plant infection by fungal pathogens.

Keywords: apoplastic fluid, bifunctional enzyme, glucanase, *Malus domestica*, plant-fungal protein interactions, protease, melanoprotein, surface proteins, *Venturia inaequalis*

Zusammenfassung

Apfelschorf ist eine der schädlichsten Krankheiten an Apfelbäumen und wird durch das pilzliche Pathogen *Venturia inaequalis* (Cke.) hervorgerufen. Eine funktionelle Rolle während der Infektion der Wirtspflanze *Malus domestica* spielt das extrazellulläre Melanoprotein, dass vom Pathogen sekretiert wird. Hignett und Kirkham, (1967); Hignett, (1973);. Hignett *et al*., (1984) gaben einen ersten Überblick über das schwarzgefärbte Melanoprotein und seine mögliche Funktion bei der Infektion. Die weitere Charakterisierung von Singh *et al*., (2005) hat gezeigt, dass *V. inaequalis* während des Wachstums in Flüssigmedium ein 36 kD Protein in das Medium sezerniert. Dieses Protein gehört zur Gruppe der Melanoproteine, kann Eisen binden und wird während des Prozesses der Infektion in den Apoplasten von *M. domestica* abgegeben.

Da bislang nur wenige Kenntnisse über die Funktion des Melanoproteins während der Infektion der Wirtspflanze und dessen Bedeutung für die Pathosgenese vorliegen, ist eine weitergehende Charakterisierung erforderlich. In der vorliegenden Arbeit wird auf molekularer Ebene die Rolle und Funktion des Melanoproteins von *V. inaequalis* detailiert untersucht. Auf der Grundlage von aus der Literatur bekannten Peptidsequenzen wurde der offene Leserahmen (ORF) des Melanoproteins von *V. inaequalis* amplifiziert, sequenziert und analysiert. Das Melanoprotein wird durch einen ORF von 981 bp kodiert. Die Gesamt-DNA von *V. inaequalis* wurde mit einer neuartigen Methode extrahiert und mit der etablierten CTAB-Methode verglichen. Die Sequenz der Upstream-Region wurde mittels neuartigem SSSBT-PCR-Verfahren abgeleitet (-851 bp) und analysiert. Ein Klasse-II Intron wurde in der Region von 528:591 bp vorhergesagt. Es wurde dabei ein Gesamt-DNA-Fragment von 2118 bp gewonnen.

Die abgeleitete Aminosäuresequenz des Melanoprotein zeigte große Homogenität mit der ß-1,3-Glucanase (glycol_hydro_17 Superfamilie) und einer Protease des retropepsin_like_LTR_1 Typs (Aspergillose pepsin_like Familie). Beide vorhergesagten Enzymaktivitäten konnten durch enzymatische Tests mit dem überexprimierten rekombinanten Melanoprotein aus *E. coli* bestätigt werden. Außerdem ergaben diese Versuche, dass die proteolytische sowie die glucanolytische Enzymaktivität des Melanoprotein durch PMSF (Phenylmethylsulfonyl fluorid) gehemmt werden.

Darüber hinaus konnte gezeigt werden, dass die Protease-Funktion des Melanoprotein durch verschiedene Ionen ($CaCl_2$, $MgCl_2$, $CuCl_2$ $CoCl_2$, $MnCl_2$, $FeCl_3$, $ZnCl_2$) beeinflusst wird. Eine

Konzentration von 2 mM Co^{2+} verstärkte die Protease-Aktivität des Melanoproteins. Alle weiteren getesteten Ionen verminderten die proteolytische Aktivität erheblich.

Weiterhin konnte die Glucanaseaktivität durch Dünnschichtchromatographie nachgewiesen werden. Die optimale Temperatur für die Glucanase-Aktivität betrug 60 °C und für die Protease-Aktivität 90 °C. Das pH-Optimum für die Glucanase- und Protease-Aktivität betrug 6,0 das Protease-Aktivitäts pH-Optimum erweiterte sich in Gegenwart von 2 mM Co^{2+} auf 6,0-8,0 pH. Die proteolytische Aktivität für das rekombinante Melanoprotein wurde ermittelt (367 U/mg). Die Glucanaseaktivität für das rekombinante Protein wurde mittels neuartiger Kongorot-Spektrophotometrie bestimmt und mit den Ergebnissen etablierter Methoden mit verschiedenen Substraten verglichen.

Auch die Aggregation und die Aktivität des rekombinanten Melanoproteins in Anwesenheit des natürlichen Pigmentes Melanin und in Kombination mit EDTA oder Co^{2+} wurde untersucht. Die Zugabe von Melanin zum Melanoprotein führte zur Bildung von Oligomeren mit apparenten molekularen Massen von 72, 136, 162, 186, 222 und 265 kD und in Gegenwart von Co^{2+} wurden größere Oligomere von 209, 536, 973, 1490, 2946, 5126, 7057 kD gebildet.

Basierend auf der Glucanase- und Protease-Aktivität konnte eine Hemmung des Wachstums von Gram-positiven und Gram-negativen Bakterien in Gegenwart des rekombinanten Melanoproteins festgestellt werden. Pflanzliche Oberflächenproteine wurden mittels verschiedener, neu entwickelter Methoden gewonnen (Replica-Färbung mit Bromophenol Blau, Silber-Färbung, Replica-Far-Western-Blot). Durch die Far Western-Blot-Technik konnte gezeigte werden, dass das Melanoprotein überwiegend an die Kutikula-Membran und dort bevorzugt neben anderen Proteinen an eine pflanzliche Chitinase bindet. Es konnte zusätzlich eine Chitinase-Aktivität für Oberflächenproteine des resistenten *M. domestica* cv. Remo durch SDS-PAGE identifiziert werden. Dabei wurde ein großer Einfluss der Glucanase-/Protease-Aktivität des Melanoproteins auf die Wirts-Physiologie während des Infektionsprozesses nachgewiesen. Darüber hinaus konnte gezeigt werden, dass die pflanzlichen Proteine in allen Teilen der Wirtspflanze durch die proteolytische Aktivität des Melanoproteins abgebaut werden (neu entwickelter Test auf PVDF-Membran).

Die Interaktion von Proteinen der Pflanze von der Oberfläche, des Apoplasten und des Protoplasten sowie verschiedener Zellengewebe mit dem rekombinanten Melanoprotein zeigte, dass das Melanoprotein alle Proteine der Pflanze degradieren kann. Die Wirkung der Apoplastenwaschflüssigkeit aus *ex vitro* und *in vitro M. domestica* cvs. Elstar und Remo auf die

Konidienkeimung und die Enzymaktivitäten des rekombinanten Melanoproteins (Glucanase und Protease) von *V. inaequalis* (No. 36) zeigte, dass die Pflanze durch die Abgabe von Inhibitoren sowohl die Enzymaktivitäten als auch die Keimungsrate der Konidien reduzieren kann.
Im Rahmen dieser Arbeit konnte festgestellt werden, dass das Melanoprotein von *V. inaequalis* mit seiner bifunktionellen Enzymaktivität eine komplexe und bedeutende Rolle bei der Infektion der Wirtspflanze spielt.

Stichworte: Apoplastenwaschflüssigkeit, bifunktionales Enzym, Glucanase, *Malus domestica*, Pflanze-Pilz-Protein-Interaktionen, Protease, Melanoprotein, Oberflächenproteine, *Venturia inaequalis*

LIST OF ABBREVIATIONS

µg	Microgram
µl	MicroLiter
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurin
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate (X-phosphate) 4-toluidine salt
bp	Base pair
BPB	Bromophenol blue
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
cDNA	Complementary deoxyribonucleic acid
CM	Catecol melanin
cm	Centimeter
CTAB	Hexadecyltrimethylammonium bromide
cv.	Cultivar
cvs.	Cultivars
Cz	Czapeck`s
DHNM	1,8-dihydroxynaphthalene
DIG	Digoxigenin
DMF	N,N-Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotriphosphate
DOPAM	3,4-dihydroxyphenylalanine melanin
DTT	Dithiothreitol
ESI-Q-TOF	Electron spray ionisation quadrupole time of flight
GDHBM	γ-glutaminyl-3, 4-dihydroxy-benzene
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazine ethansulfonate
IPTG	Isopropyl-ß-D-thiogalacto pyranosid
IWF	Intercellular washing fluid

kB	Kilobase pair
kD	KiloDalton
LB medium	Lauri Bertani medium
M	Molar
M9 medium	minimal medium contains 9 salts
mA	Miliamper
MARS	Melanized appressorial ring structure
MCS	Multiple cloning site
MES	methionine-enkephalin-like substance
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino) propane sulfonic acid
MP	Melano protein
mRNA	Messenger RNA
MS	Mass specroscopy
NBT	Nitro blue -tetrazolium
ng	Nanogram
ns-LTP	Nonspecific lipid transfer protein
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PCI	Phenol chloroform isoamylalcohol
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PMSF	Phenylmethylsulfonylfluorid
PRs	Pathogenesis-related proteins
PVDF	Polyvinylidenefluoride
RNA	Ribonucleic acid
RNase	Ribonuclease

rpm	Round per minute
RT	Room temperature
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
sec	Second
SSSBT-PCR	Specific Single Strand Bridge and T-Primer PCR
TAE	Tris-Acetate-EDTA buffer
Taq	DNA polymerase from *Thermus aquaticus*
TEMED	*N,N,N',N'*- tetramethylethylenediamine
TLC	Thin layer chromatography
UV	Ultra violet
V	Volt
W	Watt
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
µM	Micromolar
CysPI	Cysteine protease inhibitor

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1. INTRODUCTION

1.1*Venturia inaequalis* and Apple Scab Disease

Agrios in 2005 recorded that, *Venturia inaequalis,* a facultative biotrophically growing ascomycetes, causes apple scab which is the most important disease in all apple growing regions with high spring and summer rainfall. Without any control, the disease can damage 70% or more of the total fruit value. Likewise, MacHardy (1996) said that, the apple tree (*Malus domestica*) is one of the most widely grown fruits in the world and it is susceptible to several fungal and bacterial diseases, among which scab is the most severe and economically important. There is major economic loss by apple scab due to the reduction in fruit quality of scabbed fruits, also he recorded the affect of the disease on the apple tree, like, severe infection of leaves which will cause defoliation and subsequently reduction in tree vigor, which may restrict or prevent the fruit bud formation for the next year. Komjanc *et al.,* (1999) suggested that the cultivars have different degrees of susceptibility, and none is immune against *V. inaequalis* infection. Apple scab exists worldwide; it is more severe in areas with cool, moist spring and summers, whereas in regions with semiarid conditions, scab lesions may be so few as to be undetected most years Agrios (2005). The first report of apple scab was from Sweden in 1819, and it was nearly 15 years before a second report, from Germany was published (MacHardy 1996).

1.2 Classification of *V. inaequalis*

Subdivion: Ascomycotina

Class: Loculoascomycetes

Order: Pleosporales

Family: Venturiaceae

Genus: *Venturia inaequalis*

1.3 Class Morphology

The class characters were described by MacHardy (1996). Loculoascomycetes fungi have bitunicate asci, and the ascocarp is an ascostroma. In the Pleosporales, the ascocarp is a perithecium or pseudothecium that contains cylinderical asci with the presence of pseudoparaphyses. Including the genus *Venturia* into the family Venturiaceae is based on

pseudothecium and ascospore characteristics. This fungus attacks exclusively members of the genus *Malus*, cultivated varieties of apple as well as crab apple.

1.4 Life Cycle

Disease cycle of apple scab caused by *V. inaequalis* (Figure 1) which as described by Agrios (2005) starts with over wintering immature pseudothecia in the dead leaves on the ground of apple orchards. In late winter and spring, pseudothecia become mature followed by ascospores discharge as a primary inoculum, may continue for 3 to 5 weeks after petal fall.

During this period, buds start to open, initiating vulnerable sites for the primary infection. Ascospores germinate and cause infection when kept wet at temperature range 6 - 26 °C. The fungus grows between the cuticle and the outer cell wall of the epidermal cells. Lesions appear within 8 to 15 days, contributing in conidia production with large quantity.

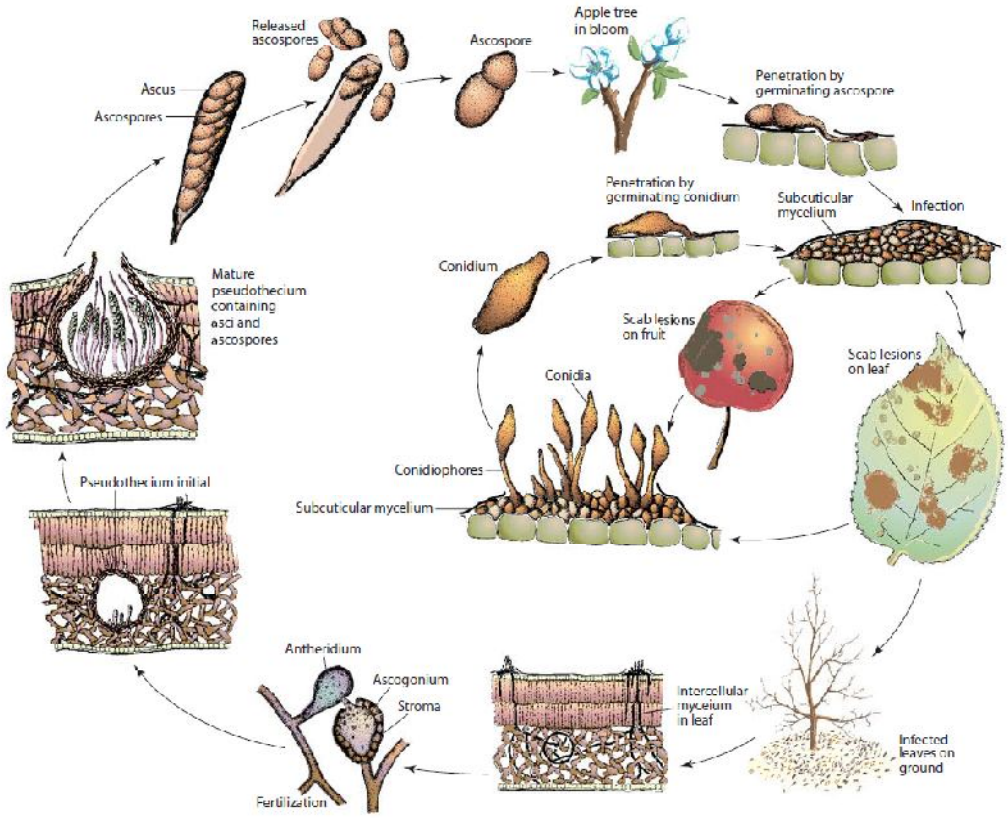


Figure (1): *V. inaequalis* life cycle (Agrios 2005).

These conidia continue to cause secondary infections during wet weather throughout the growing season. After infected leaves fall to the ground in autumn, the mycelium invades the interior of the leaf tissues forming pseudothecium, which maintain the fungus through the winter.

1.5 Melano protein of *V. inaequalis*

During growth of *V. inaequalis* in liquid medium (Singh et al., 2005) as well as during infection in the plant a black coloured melano protein is released by the fungus the importance of this protein during the process of infection is described in detail as well as the role of the melanin in general in other fungi.

1.5.1 Melano protein:

Melano protein when developed contains a covering of protein known as melano protein, forming a granule. Melano protein produced by *V. inaequalis* anchored into the cell wall, since it has importance in pathogencity of *V. inaequalis* to *Malus domestica*. There are many studies was done on the fungal melano protein by many scientists, like Jacobson (2000), he made the study on the pathogenic role of the fungal melanin and made some analysis for the confirmation of the structure, physical, and chemical analysis, but there is no one made characterization for the protein associated to melanin, whereas Singh *et al.,* (2005) did partial characterization for that protein.

Langfelder *et al.,* (2003) mentioned that, many fungal genera and species have been known for their ability to produce the melanin. Predominantly these have been dihydroxyphenylalanine (DOPA)-melanin and dihydroxynaphthalene (DHN)-melanin. The biochemical and genetical analysis of the biosynthesis pathways have led to the identification of the genes and corresponding enzymes of the pathways.

Nosanchuk and Casadevall (2006) reviewed that melanins are negatively charged, hydrophobic pigments of high molecular weight and are produced by wide range of the biological systems including many of fungal species, bacterial species, and helminthes and mammalian.

1.5.2 Melanin:

Natural pigment found in the cortex made of molecules that are capable of reflecting colour for fungi, bacteria, animal hairs. Any of several organic compounds, dark biological pigments that give coloration (Shades of yellow to brown) to skin, hair, feathers, scales, eyes, and some internal tissues called melanin. Melanin classified into two classes according to colour black/brown and yellow/red, and they called Eumelanin and Pheomelanin, respectively (http://www.interhair. com).

1.5.3 Melanocytes:

Cells present in the hair bulb that manufacture melanin (http://www.interhair.com).

1.5.4 Melanosomes:

The melanin filled granules are commonly known as melanosomes. It moves up into the cells forming the cortex fibre. In no set pattern or amount, the melanosomes remain scattered throughout the cortex (http://www.interhair.com).

1.5.5 Melanoma:

Melanoma may arise from cells that produce it. The amount in the skin depends on both genetic and environmental factors. Melanin is produced from the aminoacid tyrosine; albinos lack the enzyme that catalyzes that reaction but its role there is not understood (http://www.interhair .com).

1.5.6 Melanism:

Melanism refers to the deposition of melanin in the tissues of living organism. The chemistry of the process depends on the metabolism of the amino acid tyrosine (http://www.interhair.com).

1.5.7 Albinism:

The other face of melanism is albinism, in which the melanin is lack, because the organism cells lack tyrosinase activity (http://www.interhair.com).

1.6 Cytology of Melanin Synthesis in mamalian

Fitzpatrick *et al.,* (1981) and Niebauer (1968) recorded that, melanin synthesis in animals has been studied in great detail. One of the main differences between animal and fungal melanins is the cellular pattern of polymer deposition. In animals, melanins are synthesized only in melanocytes cells in the warm-blooded vertebrates and melanophores in the cold-blooded vertebrates (Busacker and Chavin, 1981). Within these cells melanin is restricted to a specialized organelle that is referred to as a premelanosome, melanosome, and melanin granule prior to, during, and following melanin deposition, respectively. The main difference between the animal and fungal melanins, are melanosomes and granules within the cytoplasm of specialized cells whereas fungal melanins occur either in cell walls or secreted extracellular.

1.7.1 Type of fungal melanins according to localization

1.7.1.1 Cell-wall bound melanin

The hyphal, conidial, and sclerotial walls of melanized fungi were showed (Figure 2); they appear to have two distinct layers (Wheeler *et al*., 1979):

1-An inner layer that is electron translucent,

2- An outer layer that contains and may be covered by electron-dense granules.

In some cases, chlamydospores produce a secondary inner wall that may be closely appressed to the primary wall, forming three distinct layers. Chlamydospores of *Thielaviopsis* spp. contain electron-dense granules in the outer part of the secondary wall as well as in both layers of the primary walls (Wheeler *et al*., 1979). Whereas chlamydospores of *Verticillium* spp. contain electron-dense granules in the outer layer of the primary cell wall (Griffiths *et al.,* 1982). *Verticillium* (Wheeler *et al.,* 1976) has a porous febrillar matrix (Figure 2) extends outward from the cell wall. This matrix often is two-to-five times as wide as the cell wall. Large electron-dense granules (up to 100 nm diameter) are normally scattered randomly in the matrix (Pechak et *al.* 1979). Hegnauer *et al.,* (1985) and Kozlova *et al.,* (1981) have been reported absence of melanin from fungal cell(s) cytoplasm, resembling premelanosomes structures in animals. A good suggestion were introduced by Bunning *et al.,* (1984) and Griffiths, (1982) that the melanin precursors may be secreted from the cytoplasm to the cell wall where they are oxidized to melanin.

1.7.1.2 Extra-cellular Melanin

Melanin granules in the febrilar matrix are sometimes called extracellular melanin (Gadd 1980; Lingappa *et al.,* 1963, and Ruban 1969). Extra-cellular melanin synthesized as a part of cell walls. These appear to be derived by two mechanisms (Wheeler *et al.* 1978):

(a) Secretion of phenol oxidases into the external environment, to oxidize phenolic compounds of various origins.

(b) Secretion of phenols into the external environment, where they are auto oxidised or by oxidases latterly released from the fungus (often during autolysis). In shake cultures, extracellular melanin formed by auto-oxidation may adhere to the fungal cell wall (or matrix) surface, giving a false impression of wall-bound melanin.

Secretion of phenols by fungi may not discolour media for a few weeks, but after several months the medium around certain fungi becomes darkly pigmented, especially at pH >7.0.

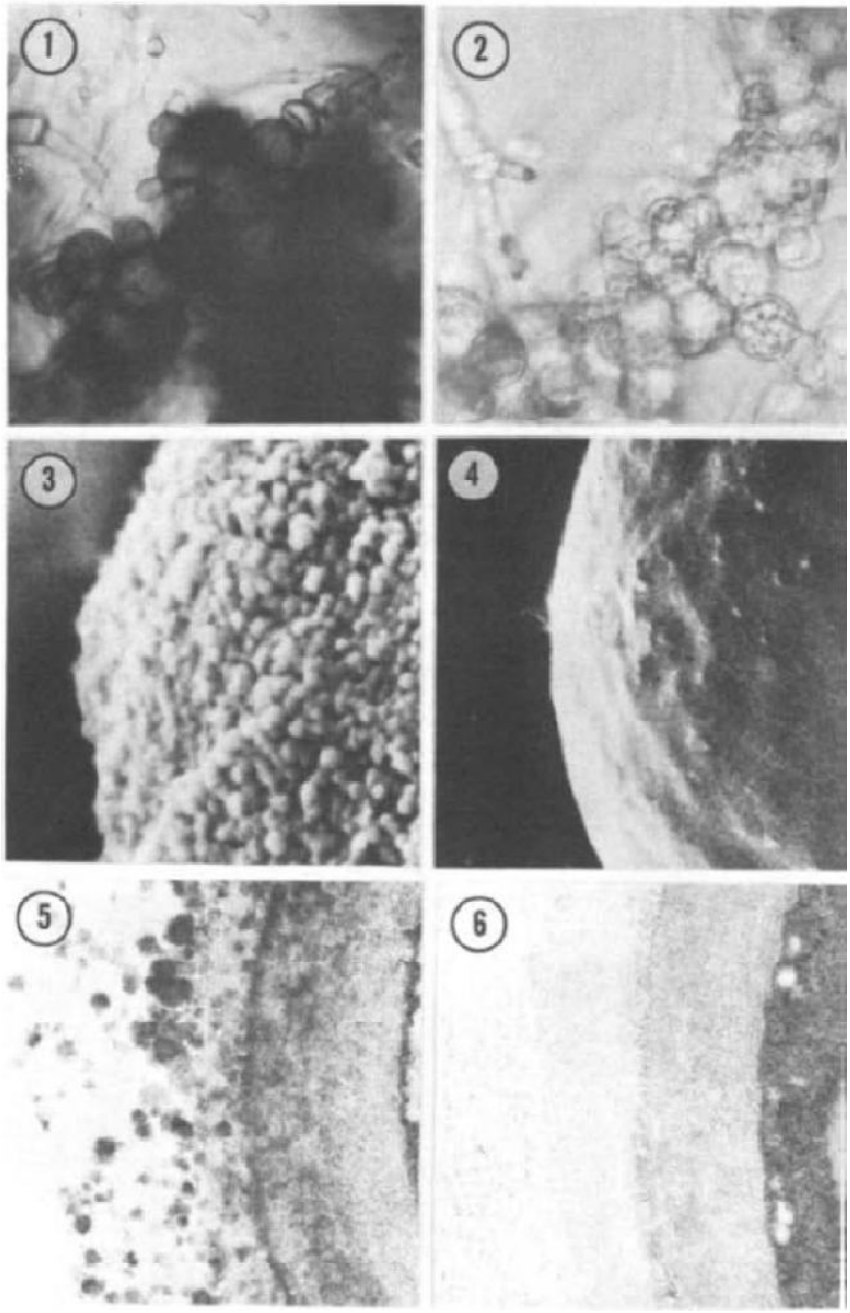


Figure (2): Light-(1&2: Magnification x 750), scanning-(3&4: Magnification x16,400), and transmission electron-(5&6: Magnification x48,000) microscopic pictures of melanin deposition in microsclerotial cell walls of *Verticillium dahliae*. Cells containing melanin (1, 3, and 5) are compared to albino cells (2, 4, and 6) to show patterns of melanin deposition (Bell, 1986).

1.7.2 Fungal melanin's types according to the synthesis and composition

Many of the fungal species and other microorganisms synthesize melanin from tyrosine via 3,4-dihydroxyphenylalanine (DOPA melanin), 1,8-dihydroxynaphthalene (DHN melanin), γ-glutaminyl-3, 4-dihydroxy-benzene (GDHB melanin), Catecol melanin in culture media or heterogynous melanin (associated or binds to enzymes, carbohydrate or lipids). Squid melanin (Piattelli *et al.*, 1965) was analyzed (Figure 3). The structure was deduced through chromatographic and electrophoretic analysis of reductive and oxidative degradation products (Nicolaus, 1968).

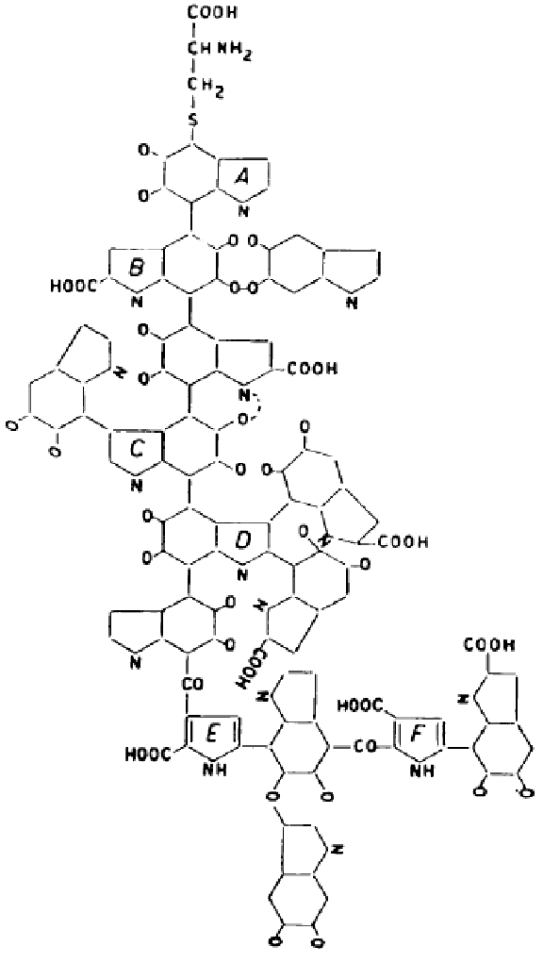


Figure (3): Structure of squid melanin proposed by Nicolaus *et al.*, (1968). Rings are aromatic. Each pair of O-oxygen substutituents can exist as quinone, hydroquinone, or semiquinone groups.

1.7.3 Melanin properties

1.7.3.1 Solubility

Generally, pure melanins are insoluble in water, aqueous acid, and common organic solvents. Conjugated melanin with protein or carbohydrates or other large compounds may be soluble in water and solvents; melanin from *Aspergillus niger* conidia is an example of this conjugation. Many scientists reported that, pure DOPA and DHN melanins are almost completely insoluble in cold alkali, whereas phaeomelanin is very soluble (Fitzpatrick *et al.*, 1981). Heterogeneous melanins from fungi also have some solubility in dilute alkali (0.1-1.0 M NaOH, NH_4OH) solutions, especially if heated.

1.7.3.2 Light absorbance

The black colour of melanin pigments is due to their absorption of all visible wavelengths. However, the light absorbance decreases as wavelength increase. Often this decrease in absorbance is nearly linear with increasing wavelength. The different melanin have different absorbance in both fungal or precursor origin and melanin in different stages (Ellis *et al.*, 1974). Slopes of absorption spectra of melanins from different fungi (and presumably from their precursors) had lower difference than those of a single melanin in different states of oxidation. UV, Visible light and IR (200-800 nm) used for determination the absorbance characters and structure with more efficiency (Blois, 1971).

1.7.3.3 Metal binding

Zhdanova *et al.*, (1973) studied the radiation absorbance by melanin, after that, Singh *et al.*, (2005) studied the changes absorbance of light by chelated ferric and copper ions to melano protein. The minor elements iron and copper are also bound melanin. Nutrient availability and cation-exchange capacity could be determined by the specific types of fungal melanins that are present in soil. The melano protein of *V. inaequalis* binds many of the transition elements (copper, ferrous and ferric ion; Singh *et al.*, 2005).

1.7.3.3 Importance of melanin

High amount of the melanized fungi present in highly stressed environment, such as the desert (Durrell, 1964), the alpine regions (Mirchink *et al.*, 1972), and the upper biosphere (Imshenetsky *et al.*, 1978). That indicates, melanins may be essential for protection of fungi against UV

irradiation, desiccation, and temperature extremes. Durrell (1964); Uspenskaya *et al*., (1980); and Zhdanova *et al*., (1973) recorded fungi which produce melanized spores are more tolerant to UV light, or solar irradiation (Zhdanova *et al*., 1980) than those with hyaline spores. The dormant microsclerotia germinate after soil rehydrated for 15 days. This observation is important because it indicates that melanins may be essential for soilborne propagules to undergo normal cycles of dormancyin induced by drought (Zhdanova *et al.,* 1980).

Melanins play an important role as antimicrobial action (Poinar, 1969 and Unestam, 1975). Hyaline spores or hyphae in soils are quickly killed and lysed, whereas melanized cells may survive several years (Luther *et al.,* 1980 and Sussman, 1968). Accordingly, most of the fungal biomass found in soils is melanized (Hunt *et al.,* (1983)). A comparable defence role was postulated for melanins in plants (Bell *et al.*, 1976). Occasionally melanins and shunt products have an antibiotic effect against antagonistic organisms. For example, melanins from cell walls of *Phellinus weirii* (Li, 1983), or from the culture media of *Trichophyton rubrurn* (Schönbom *et al*., 1971), actinomycetes (Yakovleva *et al*., 1976), are antibiotic to other organisms in culture assays.

Melanin increases the resistance of the microbial attacking by pathogen or host hydrolytic enzymes. Most antagonists cause lyses of fungal cells by production of chitinase, 3-1, 3- and 3-1, 6-glucanase (Jackson *et al.,* 1976). Generally, the ability of this mixture of enzymes to hydrolyze fungal cell walls is inversely related to the melanin content of the walls (Bloomfield *et al.,* 1967 and Piattelli *et al.,* 1963). Melaninless mutants of *Aspergillus nidulans* (Kuo *et al.,* 1967) and *Cochliobolus satvus* (Old *et al*., 1970) were highly susceptible to digestion by the enzyme mixture. Besides melanin, however, the *A. nidulans* mutant lacked α-(1,3)-glucan, which also resists hydrolysis by the enzyme mixture (Polacheck and Rosenberger, 1977).

A preliminary report indicates that microsclerotia from albino and dehydratase-deficient mutants of *Verticillium dahliae* cannot survive desiccation in soil, whereas wild-type microsclerotia and albino microsclerotia melanized by treatment with scytalone become dormant when desiccated (Bell *et al.,* 1976). The heterogonous melano protein of *V. inaequalis* (hypersensitive response (HR factors)) was very resistant to boiling (Win *et al*., 2003).

However, its mode of action is unclear. Likewise, a melano protein from *V. inaequalis* increases lesion number and size when added to inoculated leaves (Hignett and Kirkham, 1967**;** Hignett,

1979). But two low-virulent mutants contain comparatively large amounts of melanin (Hignett *et al.*, 1984). Hignett and Kirkham (1967) proposed that, melanin of *V. inequalis* binds to the organism's extracellular hydrolytic enzymes and to release them very slowly. Yeast protects itself against leukocyte microbicidal proteins by binding it to cryptococcal melanin leukocyte microbicidal proteins, presumably by binding them before they can reach the plasma membrane of the fungus (Franzot and Doering, 1999).

Certain fungi have hyaline hyphae or yeast-like cells and produce melanized chlamydospores, sclerotia, reproductive structures, and sexual spores and cellular differentiation in these fungi (Chet and Huttermatm 1982 and Hermann *et al.*, 1983) are associated with melanin synthesis and active laccase or tyrosinase appearance.

Growth technique with melanin is used extensively to characterize and identify certain bacteria (Mung and Kondrateva, 1981 and Pavlenko *et al.*, 1982), fungal pathogens of humans (Edberg *et al.*, 1980 and Kwonchung *et al.*, 1981&1983).

Recently, it was reported that melanin is very important for the penetration of the fungus into the plant cuticle (Steiner and Oerke, 2007). During the formation of appressoria produced from conidia and ascospores of *V. inaeqaulis*, a dark brown ring structure was detected at the base of appressoria. The application of melanin biosynthesis inhibitor reduced the infection by 45-80%. The author reported that the appressoria without MARS (Melanized appressorial ring structure) were not able to infect the plant, suggesting that this structure is important in as pathogenicity factor as a cutinase enzymes which purified from *V. inaequalis* by Köller and Parker (1989).

1.7.3.4 Melanin polymerization

Wagh *et al.,* (2000) reported that, the melanosomal proteins in total and the purified ones (spotted on PVDF membrane) were subjected to melanin polymerization microassay, before and after extraction of residual SDS. Wagh *et al.*, (2000) reported that, the presence of SDS minimizes or inhibits the binding of some purified melanosomal proteins.

1. 8 Molecular studies of melano protein of *V. inaequalis*

In the recent studies, *Venturia inaequalis* clone no. 36 secreted melano protein (36 kD) with high amounts during growth in liquid culture. The protein was isolated from the culture medium and purified to homogeneity. Partial sequencing of the protein indicates that, there is no significant similarity between these protein and the other proteins (Singh *et al.,* 2005). Also the data and

protein analysis was showed by Singh *et al.* (2005), indicating that the presence of the melano protein in the apoplastic fluid of the infected leaves of *M. domestica* cv. Elstar. The heterogonous melano protein of *V. inaequalis* (hypersensitive response (HR factors)) was very resistant to boiling (Win *et al*., 2003).

V. inaequalis unlike obligate fungal pathogens, it does not develop haustoria to obtain nutrients, nor does it grow intercellularly in host tissue forming subcuticular stroma. MacHardy (1996) recorded that, the nutrient come into the fungus from cell wall degradation with hydrolysable enzymes; likewise, the extracellular melano protein, which is produced by the fungus itself, causes modification of the plant solute transport mediated possibly by membrane damage. These growth sites represent a part of the apoplast.

1.10 Relationship of melano protein to cell membrane permeability

MacHardy (1996), concluded that melano protein play an important role in disease development, by interaction with plant cell membrane and protein biosynthesis sites. The ability of the melano protein to bind or chealate to metals, hexose and protein make that easy to alter the solute transport through the membrane (damage the membrane). That was measured by the conductivity of the membrane during the stage of infection and they found that the conductivity increase by 2 fold after 22 h before symptoms of the hypersensitive response appeared (Pellizzari *et al.,* 1970).

Nusbam and Keitt (1938) introduce indicator for sharing digestive enzymes between the cuticle and the cell wall, peripheral cytoplasm depletetion which disappeared prior to cell death, but the collapse of individual cell was seen occasionally. The author also found that, the chloroplast lost their integrity, and their desecrate structures, and collapsed into larger disorganized masses. They noted that increasing in vacuolation and disintegration of chloroplasts and cytoplasm occurred when cells died.

Different microscopic symptoms of the infected apple leaves were described and identified by Chevalier (1988). Chevalier (1988, and 1991) detect and confirm the defense response mechanisms of *M. domestica* cv. Florina (V*f* gene) and found a very important cell wall changes after 40 h which is the deposition of a new layer on the cell wall and these layers is identical to that of *M. domestica* cv. Golden Delicious inoculated with *V. inaequalis* (Valsangiacomo *et al*., 1988) The strong infection symptoms could increase the apposition on the epidermal and palisade tissue cells wall.

1.11 Melanin inhibitor

Application of the melanin inhibitor to inhibit DHN melanin of *Pyricularia oryzae*, *Colletotrichum lagenariutn*, and *C. lindemuthianium*, was an indicator on the importance of melanin for penetration of rice, cucumber, and bean by appressoria, respectively. Many inhibitors of melanin synthesis were reported, control the disease when applied as preventative fungicide sprays, and give excellent control of the rice-blast disease caused by *P. oryzae* (Sisler *et al.*, 1985, 1984).

2. OBJECTIVES

This study is focused on the characterisation of the fungal melano protein from *V. inaequalis* and its interaction to proteins of the host plant *Malus domestica* to investigate the elusive function of the melano protein.

4. HYPOTHESIS

The melano protein of *V. inaequalis* is supposed to have a function and an important role in the infection of *M. domestica*

4. MATERIALS&METHODS

4.1 *Venturia inaequalis* (Strain and culture conditions)

The fungus *V. inaequalis* clone no. 36, which was isolated from the leaf of *M. domestica* (cv. Elstar) in Julius Kühn Institute (Dossenheim, Germany) and kindly provided by Dr. Iris Szankowski from Institute of Biological Production Systems and Fruit Science, University Hanover Germany. For propagation cellophane membranes with approximately 8 cm in diameter were soaked in distilled water overnight and autoclaved. Subsequently, PDA medium was prepared and pH value was adjusted to 5.6. After autoclaving, approximately 20 ml of the hot medium was poured in each Petri dish. After solidification, the agar surface was covered with a sterile cellophane membrane. Finally each plate was inoculated with 0.5 ml of conidial suspension and incubated at 18 °C in the dark for one week.

PDA medium:

39.0 g Potato Dextrose Agar (Duchefa, Netherlands) per litter distilled water. pH 5.5

4.1.1 Harvest of conidia

Three cellophane membranes were removed from the PDA plates surface under sterile conditions, and transferred to 250 ml bottle. Afterwards 50 ml sterile distilled water was added to the membranes and shaken for 5 min at 270 rpm. The suspension was filtered through a nylon membrane filter (79 µm pore size) and adjusted to a concentration of 10^5 conidia per ml as determined by Thoma chamber.

4.1.3 Mycelia production in liquid culture

Venturia inaequalis clone 36 replicas grown over the cellophane membrane on the surface of PDA medium (Five Petri dishes) can appealed by sterile forceps and transferred to 50 ml tube under aseptic conditions. Another method for mycelia growth harvest were done from the liquid culture on PDB medium by filtration of 2-4 weeks old mycelia growth with filter paper and the transferred the mycelia into 50 ml sterile tube. The two methods are beneficial for mycelia growth for DNA extraction.

The grown fungus on PDA medium as previously reported on cellophane membrane or on normal PDA medium were transferred to sterile 50 ml tube containing 30 ml of sterile PDB (pH

5.2) medium (under aseptic condition). Then the mycelia were homogenized using homogenizer and divided into 6 conical flasks (250 ml Erlenmeyer flask) containing 50 ml of PDB medium (pH 5.2). The flasks incubated on shaker (125 rpm) and temperature 18 °C with light elimination for 2-4 weeks for mycelia growth amplification.

PDB medium: 24.0 g potato dextrose broth (Duchefa, Netherlands) per liter distilled water.

4.1.4 Propagation of *V. inaeqaulis* on liquid M9-Asp broth medium

V. inaequalis clone 36 were grown in 18 petri dish (5 cm) on cuticle membranes (10 discs/ plate) floating on broth M9 medium containing 10 mM asparagine and 20 mM Mes and adjusted to pH 5.5. Afterwards, 50 µl from the previous harvested conidia were put on the surface of each cuticle (under aseptic condition). The petri dishes were then incubated at 20^{o}C. Afterwards three plates were harvested after 2, 4 and 7 days, cuticle membranes and the fungal conidia were collected from the medium by centrifugation at 10000 xg. The supernatant was further analyzed by SDS and 2D PAGE.

M9 medium:

1) 10X M9-Salt: 60 g $Na_2HPO_4x2H_2O$, 30g KH_2PO_4, 5g NaCl, 10g NH_4Cl and complete to 1000 ml (autoclaved)
2) 20% (w/v) Glucose (sterilized by ultra-filtration)
3) 1M $MgSO_4x7H_2O$ (autoclaved)

M9 mixture for one litre:

100 ml 10x M9-Salt, 25 ml 20% (w/v) Glucose, 1 ml 1M $MgSO_4x7H_2O$, pH (5.5) and complete to one litre
100 mM Mes
100 mM Asparagine
15.0 g Agar for the solid medium

4.1.5 Propagation of the fungus under stress conditions of dark, sucrose-glucose Cz medium

The fungus *V. inaeqaulis* were grown on modified Czapek`s Agar medium containing glucose for growth and sucrose for stress and artificial fruiting induction over than that the medium was divided into 6 arts 100 ml three parts of it were amended with 100, 200 and 500 mM L-Alanine and the other three were amended with 100, 200 and 500 mM Glutamic acid as semi natural nitrogen source. The medium were poured in plates. Then the plates were inoculated with equal volumes of homogenized *V. inaequlais* and incubated in light for growth at 19 ℃ for two weeks. The morphological and microscopic results were recorded. Afterwards the plates were transferred

to dark and cold conditions for two weeks and the morphological and microscopic results were noted. The parameter was the formation of the yellow sterile hyphae, conidia surface, and the ascosporic production.

Modified Czapek`s agar medium: 30.0 g Sucrose, 3.0 g $NaNO_3$, $MgSO_4$ x 7 H_2O 0.5 g, 0.5 g KCl, 0.01 $FeSO_4$ x 7H_2O, 1.0 g K_2HPO_4, 15.0 g Agar, and completed to 1 liter with ddH_2O.

Amino acid stock solution

L-alanine: 1 M
Glutamic: 1 M

4.1.6 Effect of apoplastic fluid extracted from *M. domestica* cvs. Remo and Elstar on the conidial germination of *V. inaequalis*

The effect of the apoplastic fluid on the conidial germination of *V. inaequalis* was done using one week freshly harvested conidia. The conidia were adjusted to 10^4/ml. 1000 conidia were incubated with 0, 1.875, 3.75, 7.5, 15, 22.5, 30 µg total extracted apoplastic protein from *M. domestica* cvs. Remo and Elstar *ex* and *in vitro*, then the volume were adjusted to 200 µl in sterile 1.5 ml microfuge tubes, the tubes were incubated for 24 h at 19 °C. Aliquots of 50 µl were placed on agar slides from each treatment and incubation at 19 °C for 24 h. Afterwards, the germinated conidia on the slides were examined and counted under the microscope. The germinated conidia were expressed as percent (%) and subtracted from 100 to find out the inhibition percent (%).

4.2 Bacterial strains

The bacterial strains which were used in this work were varied (Table 1). All of the bacterial strains were propagated on LB medium. The bacterial strains used in the effect of melano protein on the bacterial growth investigations were transferred to M9-Asp medium after the treatment with the melano protein.

Table (1): Bacterial strains

Strain	Aim
XL1-Blue (*E. coli*) BL21 codon plus (*E. coli*) SG130009 (*E. coli*)	Cloning and Overexpression
Erwinia amylovora *Agrobacterium tumefaciens* LB A 4404 *Bacillus mycoides* *Bacillus subtilius*	Melano protein effects on the bacterial growth

4.3 Molecular biology

4.3.1 DNA isolation from *V. inaequalis*

4.3.1.1 New merthod

Venturia inaequalis was grown for two weeks on PDA medium or PDB medium for mycelia production, that mycelia were harvested and incubated into 96% ethanol (30 ml of ethanol for 3 g of fresh fungus mycelia) for 30 min in room temperature (alcohol used for dehydration). Afterward the alcohol was discarded and the fungus mycelia were dried on sterile filter papers. Ground the pellet with a sterile pestle in liquid nitrogen till the pellet got like the dough was done. The frozen powder of the fungus was transferred to 50 ml tube. 6 ml lysis buffer (60 ^{0}C) was added. Afterward equal volume of phenol (80%, pH 8.0 and equal volume) was added and shaking 2 min. The mixture was centrifuged for 2 min at 10000 xg, and then the upper layer transferred into a new 50 ml tube and mixed with equal volume of 96% ethanol and then the mixture was shaked 2 min at RT and centrifuged for 2 min at 10000 xg, respectively. The precipitated DNA is considered as fraction I. The supernatant transferred into new (tube 50 ml) and equal volume of 100% isopropanol alcohol was mixed for 2 min and centrifuged for 2 min at 10000 xg, respectively. The precipitated DNA in the second time was designed as fraction II. The supernatant was transferred into new tube, added equal volume of 100% acetone, shaked and centrifuged for 2 min, 10000 xg, respectively. The produced pellet was named fraction III.

Extensive purification was done from proteins by dissolving the pellet into 0.5 ml sterile dist. water and precipitates the DNA by 1 ml isopropanol and 100 µl of 3 M Na-Acetate (pH 5.2). Afterward, the sample washed with cold ethanol 70%, followed by suspension of the pellet into 0.8 ml sterile ddH_2O. Finally, the DNA solution was treated with RNAse (0.2%) and incubating at 37 ^{0}C, followed by drying the pellet by vacuum, dissolving it in a 5ml for each fraction volume of water, and quantifying the DNA. The three fraction of the extracted DNA purity were determined on 1% Agarose gel separately (capter 4.3.7). Then fraction were collected together and determined qualitatively on 1% Agarose (capter 4.3.7) and quantitatively (Purity and Yield per gram of fungal mycelia) with spectrophotometer at 260 and 280 nm in comparison of CTAB method.

Lysis solution: 200 mM (pH 7.5) TRIS-HCl**,** 250 mM NaCl**,** 25 mM EDTA and 0.5% SDS**.**

4.3.1.2 CTAB method

Weight of 2 from 4 weeks old culture of *V. inaequalis* no. 36 on PD broth medium were harvested and ground in a pre cooled mortar in the presence of liquid nitrogen. The fine powder was transferred equally into 2 ml microfuge tubes. Preheated 800 µl CTAB buffer were added to each tube mixed and incubated for 30 min at 65°C. Eight hundred µl of CI Mix were gently mixed to avoid genomic DNA sharing. Samples were centrifuged for 10 min at 10000 xg. Afterwards the aqueous phase was transferred into new tube; this step was repeated several times to obtain a clear sample. For DNA precipitation 2/3 volume of a precooled at -20 °C isopropanol was added and gently mixed. Afterwards samples were incubated overnight at 4 °C. Centrifugation for 10 min at 13000 xg was done. Supernatant was removed and pellet was washed by 200 µl WB. The washing buffer was carefully removed and pellet was resuspended in 200 µl TE buffer supplemented with RNase A (final concentration 10 µg/ml). After incubation for 30 min at 37 °C, 100 µl of 7.5 M NH_4-acetate and 750 µl ethanol were added and gently mixed. Samples were centrifuged for 10 min at 13000 xg at RT. Subsequently, the supernatant was completely removed and pellet was resuspended in a suitable volume of sterile distilled water.

CTAB-buffer: 3 % CTAB, 1.4 M NaCl, 0.2 % ß-Mercaptoethanol (Add CTAB and ß-Mercaptoethanol after autoclaving), 20 mM EDTA, 100 mM TRIS-HCl pH 8.0, 1 % PVP-40.
CI Mix: 24 ml chloroform, 1 ml isoamylalcohol.
Wash buffer (WB): 76 % ethanol, 10 mM NH_4-acetate.
TE-buffer: 10 mM TRIS-HCl. pH 8.0, 1 mM EDTA.
RNase: 10 mg/ml stock solution in distilled water.

4.3.2 Isolation of RNA from *V. inaequalis* and *M. domestica* cv. Remo

Three grams of *V. inaequalis* mycelia or/ and of *M. domestica* cv. Remo leaves were ground well in liquid nitrogen in precooled mortar and pestle. The obtained powder was mixed with 15 ml of prewarmed (50 °C) lysis buffer and 15 ml of PCI solution and shaken for 20 min. The mixture was centrifuged for 20 min at 13000 xg. The supernatant was removed and mixed with 15 ml of PCI solution and again centrifuged for 20 min at 13000 xg. Supernatant was mixed with 0.75 % volume of 8 M LiCl and stored overnight at 4 °C. Solution was centrifuged for 20 min at 13000 xg and 4 °C. After removal of the supernatant, pellet was mixed with 5 ml distilled autoclaved water, 500 µL 3 M Na-acetate pH 5.2 and 5 ml of 96% ethanol (-20 °C), and stored for 1 h at –20 °C. After centrifugation for 20 min at 4 °C and 13000 xg, the pellet was washed with 10 ml 70 %

ethanol (-20 °C) and centrifuged again for 20 min. Supernatant was carefully removed and pellet was dried by speed vacuum. Subsequently, the pellet was dissolved in 1 ml autoclaved distilled water and kept at –80 °C until use.

Lysis buffer: 600 mM NaCl, 20 mM EDTA, 4% SDS, 100 mM TRIS-HCl pH 8.0.
PCI: 25 volumes phenol: 24 volume chloroform: 1 volume isoamylalcohol, adjust to pH 4.5 by Na-acetate (100 mM; pH 4.5) stored in dark at 4 °C.

4.3.3 RNA electrophoresis

1.5 % agarose gel was used for total RNA separation. An appropriate amount of agarose was melted in autoclaved water 1 min in a microwave (600 W). After cooling down to ~ 60 °C, 1% MOPS, pH 7.0 and 3.4 % formaldehyde were added. Afterwards, solution was poured into the gel cassette. From each RNA sample, 2 µl were taken and mixed with 18 µl Northern-Mix. The mixture was centrifuged and incubated at 65 °C for 15 min. Samples were chilled in ice, centrifuged and loaded to the gel. Samples were electrophortically separated after addition of 1x MOPS as a running buffer. RNA was visualized in UV light.

MOPS 10x: 200 mM MOPS, 50 mM Na-acetate and 10 mM EDTA, pH 7.0
Northern Mix: 5 ml solution 1 + 1 ml solution 2 + 40 µl ethidiumbromid, 5 mg/ml.
Solution 1: 1x MOPS pH 7, 6.5 % formaldehyde, 50 % formamid.
Solution 2: 0.25 % bromophenol blue, 0.25 % Xylene cyanol, 50 % Glycerol, 1 mM EDTA, pH 8.0

4.3.4 Reverse transcription (RT)

RT-PCR was performed by using peqGold M-MuLV Reverse Transcriptase RNase H-(Peqlab-Biotechnologie GmbH, Germany). 5µl aliquot of total RNA samples were mixed (0.005-0.25 µg/µl total RNA, final concentration) with 1 µl of Oligo (dT) 23 primer (0.5 µg/µl) and 1 µl deoxynucleotide mixture (500 µM each dNTP as a final concentration). Afterwards an appropriate volume of water was added to make the final volume 10 µl. The subsequent steps were carried out in the thermocycler (PTC 200, Biozym, Hessisch Oldendorf, Germany). The reaction mix was incubated initially at 70 °C for 5 min and then chilled on ice. A second reaction mix consisted of 4 µl of 5x buffer for M-MuLV-RT, 1 µl RNAse inhibitor (20u/µl), 1 µl M-MuLV reverse transcriptase and 6 µl of water was added to the first reaction. The first strand of cDNA was built by incubation at 42 °C for 1 h.

4.3.5 Polymerase Chain Reaction (PCR)

PCR were done for amplification of the melano protein fragments with three sets of primers (Table 2), overexpresion of melano protein ORF with modified full length primers (Table 2)and amplification-overexpresion of cysteine protease inhibitor with one two primers(Table 2). In PCR tubes 1µl of cDNA template, 1 µl 2.5 mM dNTP mix, 0.2 unit of Taq polymerase, 5 µl of 10 x complete buffer and 40 µl of sterile ddH_2O. 10 pmol of each primer (1µl of each forward and reverse primer) was added in each tube. The whole reaction was denatured initially for 3 min at 94 °C, thermocycled as following for 36 cycles: denaturation at 94 °C for 30 sec, followed by annealing for 30 sec, and extension at 72 °C for 1 min for amplification-overexpression of the Melano protein and 40 sec for amplification-overexpression of cysteine protease inhibitor. Finally, the reaction extended 3 min more at 72 °C for proofreading and holded at 4 °C till agarose separation. The grey primers (Table 2) were used for genomic sequence of melano protein from the DNA as described to determine the exon-intron in the gene and to Label the DNA by Dig.

Table (2): List of primers used for amplification and overexpression

Name	Primer	Gene or Fragment	Restriction enzyme in the primer	Extension time
Mel#1_ fwd	5'-CACATCTCTCGCTTCTTCTT -3'	Amplification of melano protein gene from DNA (gray background primer) and from RNA (sing all primers)		1 min
Mel#1_ rev	5'-GATTACGGGTTAGCCATTCT-3'			
Mel#2_ fwd	5'-ATGTTCGCAAAACTTCTCGC-3'			
Mel#2_ rev	5'-ACTAGGCTGCGCGGTTGGAT-3'			
Mel#3_ fwd	5'-AACTACGGCTCGACTGATGC-3'			
Mel#3_ rev	5'-CTTATCGAAAAGTTGGTTGC-3'			
O. FW	5'-CCATGGGATGTTCGCAAAACTTCTCGC-3'	Melano protein overexpression	*NcoI*	1 min
L. Rev	5'-AAGCTTGGCTGCGCGGTTGGATCTC-3'		*HindIII*	
CsyPI_ fwd	5'-GGATCCAACATGGCCGCAGTT-3'	Cysteine protease inhibitor amplification and overexpression	*BamHI*	40 sec
CsyPI_ Rev	5'-CTGCAGCCTTGGAGACGAACA-3'		*PstI*	

4.3.6 Determination of quantity and purity of DNA and RNA

DNA and RNA isolations were diluted 1:100 and the absorptions were measured at 230 nm 260 nm and 280 nm. DNA and RNA concentrations were determined by the following formulas;

$$\frac{E260 \times 50 \times \text{dilution factor}}{1000} = \mu g \text{ DNA} / \mu l$$

$$\frac{E260 \times 40 \times \text{dilution factor}}{1000} = \mu g \text{ RNA} / \mu l$$

The quotients E260/E280 and E260/E230 give information about impurities with proteins and polysaccharides, respectively. A quotient between 1.8 and 2 shows a sufficient purity.

4.3.7 Agarose gel electrophoresis

An appropriate amount of agarose (1%) was melted in 1xTAE electrophoresis buffer for 1 min in a microwave (600 W). After cooling down to ~ 50 °C, solution was poured into the gel cassette. 5 µl sample buffer was added to 7 µl of sample and run at 90 V for 30-45 min in an electrophoresis apparatus (BioRad, Munich, Germany) using 1x TAE as buffer system. DNA ladder 100 bp (Gene Ruler, Fermentas, Munich Germany) was used as size standard. Gel was incubated for 30 min in ethidium bromide solution (0.5 µg /ml). Nucleic acids were detected under UV light at 280 and 260 nm.

TAE buffer (50X): 40 mM TRIS, 10 mM Na-acetate, 1 mM EDTA, with acetic acid adjust the pH 7.8.

Sample buffer: BPB, 100 µl xylene cyanol, 400 µl 87% glycerol, 500 µl 1xTAE buffer.

4.3.8 Purification of PCR products for cloning into bacterial host strains

E.Z.N.A. Cycle-Pure Kit (PeQLab-Biotechnologie GmbH) was used to purify the PCR products. PCR products were purified before cloning to remove any contaminants that may affect the cloning step. Four to five volumes of CP buffer were added to total volume of PCR products. Subsequently, 750 µl of the mixture was loaded to the column fitted to a collecting tube and centrifuged at RT for 1 min at 16000 xg. DNA-wash buffer (with 1.5 volumes of ethanol) 750 µl was added to the column and was centrifuged for 1 min at 16000 xg. This step was repeated. In all the centrifugation steps the solution collected in the collecting tubes was discarded. The column was centrifuged at 16000 xg again for 2 min to dryness. The dried column was placed on a sterile reaction tube and 50 µl of sterilized distilled water was added directly on to the

membrane of the column, incubated at RT for 2-3 min and centrifuged at 16000 xg to elute DNA plasmid.

4.3.9 Cloning into bacteria

4.3.9.1 Electrocompetent *E. coli* cells (Table 3)

One ml of overnight culture on LBG medium was inoculated in 250 ml LBG medium, and incubated at 37 °C with shaking. The optical density was measured at 600 nm (0.5 – 0.6). Culture was cooled down in ice for 15 min. After centrifugation for 10 min at 4000 xg, the pellet was resuspended in 5 ml distilled water, and subsequently filled up to 250 ml with distilled water. Centrifugation was done again for 10 min at 4000 xg, and the pellet was resuspended in 250 ml distilled water (this step was repeated once again). Pellet was resuspended in 25 ml 15 % glycerol and centrifugation was done for 30 min at 4000 xg. Finally the pellet was mixed with 1.5 volume of 15 % glycerol, and 100 µl aliquots were stored at – 80 °C.

LBG (per liter): 10 g NaCl, 10 g Tryptone, 5 g Yeast extract, 1 g Glucose pH 7.5.

Table (3): Electrocompetent cells

E. coli strains	Purpose
XL1-blue	Amplification of all vectors
BL21 codon plus	Overexpression of melano protein
SG130009	Overexpression of CysPI

4.3.9.2 Vectors Ligation

The purified PCR was ligated into pGEM-T vector. The ligation was done by adding 1 µl of pGEM-T vector, 5µl of 2x rapid ligation buffer, 3µl of purified PCR product and 1µl of T4 Ligase into PCR tube and incubating the tube for overnight at 4 ^{0}C for normal pGEMTs vectors and 16 °C for 16 h for both pET-32- melano protein and pQE30 vectors.

Ligation buffer: 400 mM TRIS-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8)

4.3.9.3 Transformation of *E. coli* by electroportion

100 µl of electrocompetent cells of *E. coli* strain (Table 4) were mixed with 3 µl ligate in a precooled electroporation cuvettes. Electroporation was done in BTX cell at 1.25 kV. After electroporation, 1000 µl LB medium without antibiotic were added to cell suspension and

incubated for 90 min. eventually, bacterial cells were cultivated on LBA plates containing appropriate antibiotic for selection.

Table (4): *E. coli* strains used in this work for amplification and overexpresion the gene of interest, the plasmids, and antibiotics compatible with the plasmid and the bacterial strains.

Plasmid	*E. coli* strain	Purposes	Antibiotic
pGEMTs-Melano protein	XL1-blue	Amplification for Sequencing	Amp^{100}
pGEMT-CysPI	XL1-blue	Amplification for Sequencing	Amp^{100}
pGEMT-I&II Upstream	XL1-blue	Amplification for Sequencing	Amp^{100}
pET-32 Melano protein	XL1-blue	Amplification for overexpression	Amp^{100}
pQE30-CysPI	XL1-blue	Amplification for overexpression	Amp^{100}
pET-32 Melano protein	BL21 codon plus	Overexpression	Amp^{100} $Chloramp^{34}$
pQE30-CysPI	SG130009	Overexpression	Amp100 $Chloramp^{34}$

LBA: (per Liter): 10 g NaCl, 10 g tryptone, 5 g yeast extract, 20 g agar, pH 7.5.
IPTG: (isopropyl–β-D-thiogalactopyranosid): 1 mM; 2.38 g IPTG in 100 ml deonized water.

4.3.9.4 Screening of the transformed colonies

Several white, as well as blue colonies were picked and subcultured overnight in liquid LB medium in the presence of 100µg/ml ampicillin at 37°C. Afterwards, plasmid was isolated from these cultures by using HB-lysis.

4.3.10 HB-lysis and plasmid preparation

For isolation of the plasmid, 1.5 ml of overnight culture was centrifuged for 2 min at 16000 xg. Pellet was resuspended in 300 µl of buffer I, and lysed by addition of 300 µl of buffer II. The mixture was incubated for 5 min at room temperature, followed by adding 300 µl of buffer III and incubation for 10 min in ice. Afterwards mixture was centrifuged for 10 min at 16000 xg and RT. The supernatant was transferred and recentrifuged for 10 min at 16000 xg. Subsequently, 800 µl were taken from supernatant in a new 1.5 microfuge tubes and mixed with 700 µl isopropanol and centrifuged for 30 min at 16000 xg. Pellet was incubated with 500 µl cold (-20°C) ethanol (70 %) for 2 min. Finally, centrifugation was done for 10 min at 16000 xg in room temperature. Pellet was dried in speed vacuum and resuspended in 50µl-distilled water. Plasmid DNA was dissolved at 50 °C for 5 min, and stored at -20 °C.

Buffer I: 50 mM TRIS-HCl pH 8.0, 10 mM EDTA.
Buffer II: 200 mM NaOH, 1 % SDS.
Buffer III: 2.55 M K-Acetat pH 4.8 (adjust with Acetic acid).

4.3.11 Restriction enzymes digestion

Vectors digestion were done for 10µl aliquots for overnight incubation at 37 ℃ in 1.5 ml microfuge tube containing 3µg DNA plasmid, 1x digestion reaction buffer 0.2 units of the restriction enzyme (Table 5).

Table (5): Vectors digestion, restriction enzymes and digestion buffers

Plasmid	Single digestion	Double digestion	Buffers	Purposes
pGEMTs-Melano protein	*NcoI*	*NcoI* & *SacI*	Yellow Tango buffer	Amplification confirmation
pGEMT-CysPI	*NcoI*	*NcoI* & *SacI*	Yellow Tango buffer	Amplification confirmation
pGEMTs-Melano protein	*NcoI*	*NcoI* & *HindIII*	Yellow Tango buffer	ORF fragment extraction
pGEMT-CysPI	*BamHI*	*BamHI* & *PstI*	Fast digestion buffer	ORF fragment extraction
pGEMT-I-upstream band	*NcoI*	*NcoI* & *HindIII*	Fast digestion buffer	Upstream band
pGEMT-II-upstream band	*NcoI*	*NcoI* & *HindIII*	Fast digestion buffer	Upstream band
pET-32 a (+)		*NcoI* & *HindIII*	Yellow Tango buffer	Overexpression preparation
pQE30		*BamHI* & *PstI*	Fast digestion buffer	Overexpression preparation
pET-32 Melano protein		*NcoI* & *HindIII*	Yellow Tango buffer	Insert confirmation
pQE30-CysPI		*BamHI* & *PstI*	Fast digestion buffer	Insert confirmation

Buffers:
Yellow Tango: 33 mM TRIS-acetate (pH 7.5), 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA
Fast Digestion: Fermentas company (*BamHI* & *PstI*)

4.3.12 Sequencing of nucleic acids

Positive cloned plasmids, which harboring the right fragments were selected for sequencing according to selection protocol of restriction digestion or PCR for each plasmid. The plasmid was sent to a company for sequencing (Sequence Lab, Göttingen, Germany).

4.3.14 Application of Specific Single Strand Bridge and T-Primer PCR (SSSBT-PCR) for the upstream region

4.3.14.1 Primer design and bridge PCR

The first PCR mixture (Table 6) contained the bridge primer (Reverse long primer; Figure 4), which has two complementary positions. The first position is complementary to the genomic DNA and the S-primer (short reverse; Part of the Bridge primer) used in the second PCR, whereas the second position is complementary to the T-primer (Forward) which contains T-overhang for annealing and ligation using T4 ligase. There are free base in the Bridge primer without complementary between the short primer and the T-primer.

Table (6): SSSBT-PCR mixture

Primary PCR (Bridge PCR)		Secondary PCR	
dNTP	1µl (10µmol)	dNTPs	1µl (10µmol)
Taq polymerase	1 units	Taq polymerase	1 units
Bridge primer	1 µl (10 pmol)	Shot primer	1µl (10 pmol)
DNA	3 µl	T-primer	1µl (10 pmol)
H_2O	13.5 µl	H_2O	13.5 µl
PCR buffer (10x)	2.5 µl	PCR buffer (10x)	2.5 µl
$MgCl_2$ (25 mM)	2.5 µl	$MgCl_2$ (25 mM)	2.5 µl

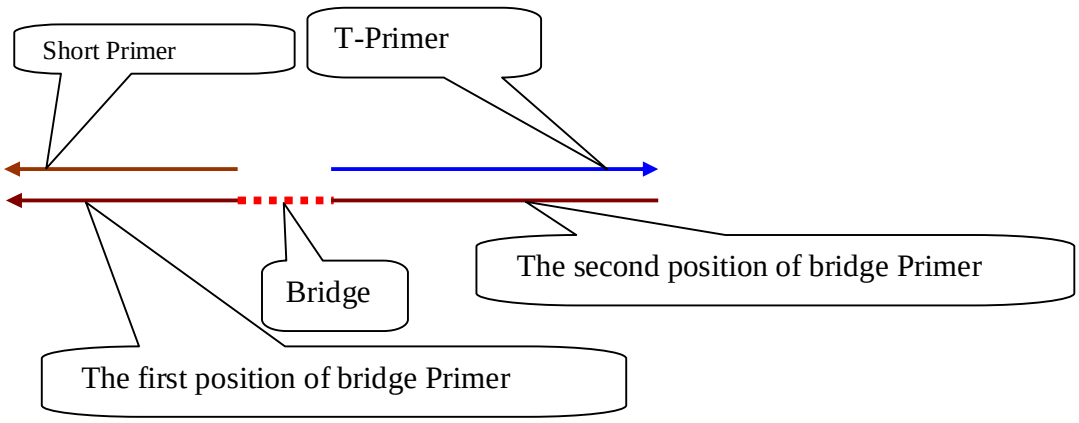


Figure (4): Primers design and the mechanism of working and annealing of SSSBT-PCR.

Bridge Primer: 5'-CCGATACAGAACCATGGAAGCAGCAGGCAA-3'

S- primer: 5'-GGAAGCAGCAGGCAA-3'

T- primer: 5'-GGTTCTGTATCGGT-3'

4.3.14.2 Steps of SSSBT-PCR (Figure 5)

1-Amplification of specific single strand with bridge primer from DNA template

2-Adding of T- primer and let it to anneal

3- Ligation using T4- Ligase for overnight

4- Stop ligation at 65°C

5- Five µl of the assay were used for secondary PCR and linearization (Digestion with restriction enzyme) in the presences of T-primer (5'-GGTTCTGTATCGGT-3') and the S-primer (5'-GGAAGCAGCAGGCAA-3').

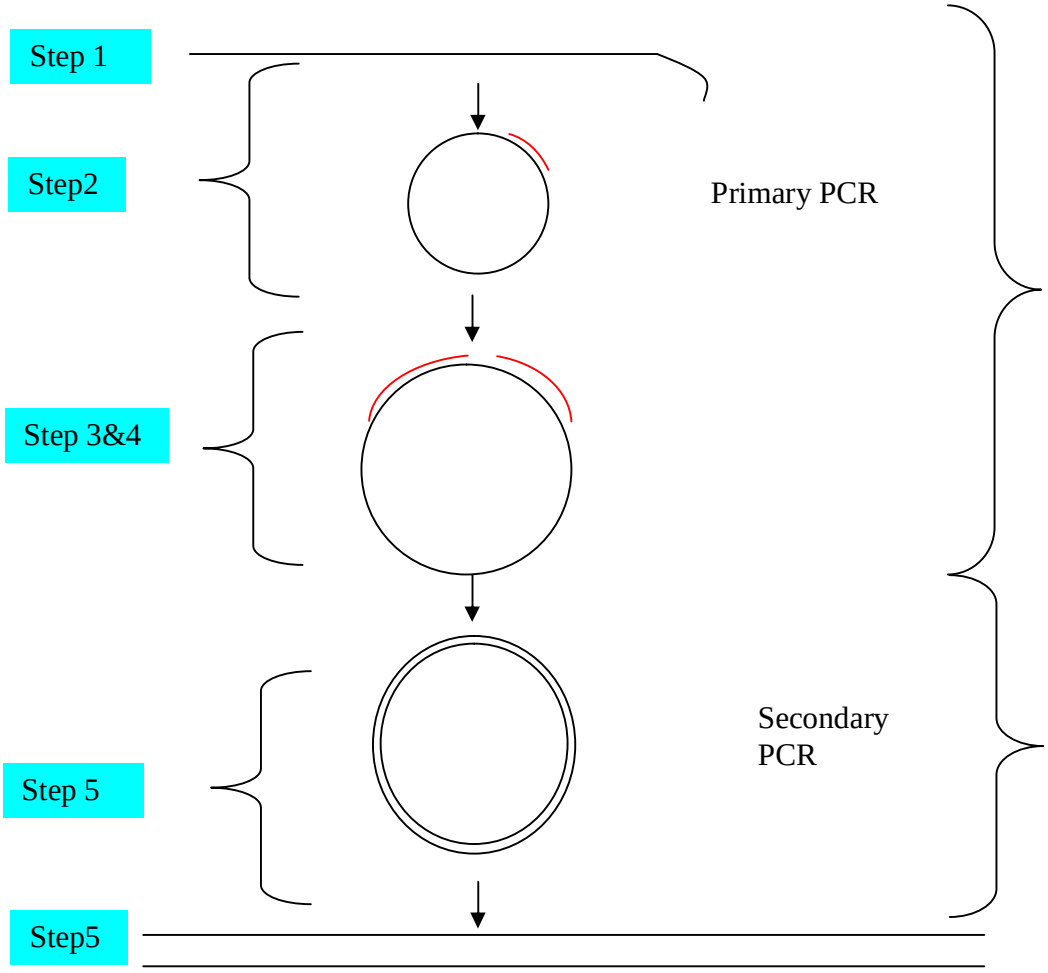


Figure (5): Diagram for SSSBT-PCR (Specific Single Strand Bridge PCR).

4.3.14.3 SSSBT-PCR programs

The detailed of PCR program were designed in Table 7. The produced band then were isolated and purified from agarose and cloned into pGEMT-vector and into XL1 blue for amplification (4.3.9) and sequenced (4.3.10)

Table (7): SSSBT-PCR programs

First PCR program		Second PCR program	
93 °C	3 min	60 °C	30 sec
		72 °C	2 min
93 °C	30 sec	93 °C	30 sec
50 °C	20 sec or 50 sec	60 °C	30 sec
72 °C	30 sec	72 °C	2 min
Replication (30 cycles)			
72 °C	50 sec	72 °C	10 min
4 °C	For ever	4 °C	For ever

4.3.15 Southern blot analysis:

4.3.15.1 Genomic DNA digestion

DNA was extracted from fungal *V. inaequalis* no. 36 mycelia as previously reported and submitted to digestion (section 4.3.1). Around 50 µg extracted genomic DNA were digested with restriction enzymes (*Nco*I, *HindIII*, *BamHI* and *PstI*) in 500 µl assay for 2 h at 37 °C. Afterwards digestion assay was vortexed at low speed, centrifuge for few seconds and incubated overnight at 37 °C.

4.3.15.2 Precipitation of digested DNA

Digested DNA was precipitated by adding 50 µl Na-Acetate (pH 5.2) and 800 µl cooled 96 % ethanol (-20 °C). The mixture was centrifuged for 10 min at 16000 xg. Pellet was washed with 70 % ethanol (-20 °C) at 16000 xg for 5 min. To dry the pellet, tubes were incubated for 20 min at 37 °C to dry. Dried pellet was dissolved in appropriate volume of TE buffer at 4 °C overnight. Dissolved DNA was mixed with DNA loading buffer and loaded in 0.8 % agarose. Gel was left to run overnight at 30 mA for separation (chapter 4.3.7).

DNA loading buffer: BPB, 100 µl xylene cyanol, 400 µl 87% glycerol, 500 µl 1x TAE buffer.

4.3.15.3 DNA transfer to nylon membrane

The separated DNA fragments were denatured by washing two times for 20 min by denaturation solution with gentle agitation. After washing the gel by distilled water, it was neutralized by washing two times for 20 min with the neutralization solution and again washed by distilled water. The blotting cassette was assembled in the following order; 3 mm filter paper, DNA gel, nylon membrane, three layers of 3 mm filter paper, around 10 cm of tissue papers. The cassette was put on one layer filter paper that was submerged in both ends in 20x SSC solution. Above the tissue papers, around 500 g weight was put to facilitate the capillarity transfer overnight at room temperature. Membranes were fixed at 80 °C for 2 h in oven. Membranes were used immediately for hybridization or kept in dark at room temperature for later use.

Denaturation buffer: 1.5 M NaCl, 0.5 M NaOH.

Neutralization buffer: 1 M TRIS-HCl pH 8.0, 2 M NaCl.

20x SSC: 0.3 M Na-citrate pH 7.0, 3 M NaCl.

4.3.15.4 DNA labeling

The gene of the melano protein was amplified as mentioned primer subsequently the product was extracted by phenol/chloroform. Around 1 µg of purified DNA were denatured by boiling in water bath at 95 °C for 10 min and immediately cooled in ice. To the freshly denatured DNA 2 µl of 10x DIG High Prime, 4 µl of 5x decamer nucleotides as a random primer and 1 µl of Klenow enzyme were added. The total volume was adjusted to 15 µl by distilled water. Mixture was incubated for 20 h at 37 °C.

4.3.15.5 Hybridization

Membranes were placed in the hybridization tubes DNA-side directed to the center of the tube and incubated with 20 ml of the prehybridization solution for 2 h in rotor oven at 40 °C. Solution was discarded and membranes were incubated overnight with 20 ml of the hybridization solution DIG labeled probe 3:1 PCR DIG probe and Klenow DIG probe in the prehybridization solution at 42 °C. Labeling process was tested by 1 % Agarose gel; the labeled material should have a larger size than the unlabeled one.

Prehybridization solution (High-SDS-Formamid-solution) 100 ml: 41 ml 100 % deionized formamid, 16.6 ml 30x SSC, 5 ml 1 M Na-phosphate buffer pH 7.0, 20 ml 10 % Blocking solution, 1 ml 10 % N-laurylsarcosine, 16.5 ml 40 % SDS.

Hybridization solution: DIG labeled probe in prehybridization solution

20x SSC: 4.5 M NaCl, 0.45 M Na-citrate.

4.3.15.6 Visualization of the probe-target hybrids

After hybridization, membranes were washed with 100 ml of 2X SSC/0.1 % SDS two times for 5 min at room temperature. Solution was discarded and membranes were again washed two times with 100 ml of 0.5x SSC/0.1% SDS for 15 min at 68 °C. Membranes were washed once for 5 min with 100 ml of washing buffer. To quench the membrane background, membranes were incubated with blocking solution for 30 min at room temperature. After discarding of the blocking solution, membranes were incubated with 50 ml anti-digoxigenin-AP 75 mU/ ml in blocking solution for 30 min. Membranes were equilibrated with 20 ml of the detection buffer for 5 min. Detection of the membranes was done by distribution of 20-60 drops (1-2 ml) of CDPStar diluted 1:100 in detection buffer on a membrane. Membranes were put between two plastic sheets. After removing the excess of detection solution, plastic sheets were sealed avoiding any air bubbles.

Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (adjust with solid NaOH).
Washing buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0. 3 % (v/v), and Tween 20.
Blocking solution: 10x blocking reagent diluted 1:10 in maleic acid buffer.
Detection buffer 1x: 100 mM Tris, 100 mM NaCl, pH 9.5.

4.3.15.7 Autoradiography

Chemiluminescent signals were detected by overlaying Kodak® X-Omat LS film on the membrane for appropriate time at RT. Films were incubated in the developing solution for 1 min and washed by water. Finally membranes were fixed in the fixation solution for 3 min or using color developing method.

4.3.16 Bioinformatics tools

Mentioned bioinformatics tools (Table 8) were used in this study for DNA, ORF, exon intron analysis and biochemical predictions analysis, and structure predictions.

Table (8): Summary of websites used in DNA analysis and melano protein structure prediction

Site	Job
http://www.ncbi.nlm.nih.gov/	Vector contamination detection
DNASTAR Package	For DNA assembly
(http://www.expasy.ch/tools/)	ORF Translation
http://www.ebi.ac.uk/clustalw/.	Multiple alignment tools ClustalW.2
(http://www.expasy.ch/tools/): http://www.cbs.dtu.dk/services/SignalP/	Prediction analysis of physiochemical properties
http://genomics.unibe.ch/cgi-bin/	GPI prediction
http://sulfosite. mbc.nctu.edu.tw/	Tyrosine sulfunation
http://www.cancerresearchuk.org	Tertiary structure prediction
http://www.cbs.dtu.dk/cgi-bin/nph-webface?jobid=netglycate,4C51614D0177CEB9&opt=none	Netglycation sites prediction
http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA	The hydrophobicity prediction
http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html	Chains prediction in the sequence using GOR4
http://disulfind.dsi.unifi.it/process2.php	Disulfide bond prediction

4.4 Biochemical studies

4.4.1 Bacterial cells induction (Overexpression of melano protein and CysPI)

For overexpression BL21 codon plus (pET-32 Melano protein) and SG130009 (pQ30-CysPI) were grown on LB medium separately to an OD600~ 0.6 in Erlenmeyer flask at 37°C (155 rpm). The grown cells were induced by adding IPTG (Thiogalactopyranoside) for 2-3 h. Afterwards, 1 ml of the induced cells were centrifuged at 16000 xg, washed with 50 mM Na-phosphate buffer (pH 7.0), lysis, mixed with protein sample buffer and incubated for 5 min at 95 °C including the control and the uninduced sample. The samples were applied to the SDS system for overexpresion testing.

4.4.2 Extraction of *trx* tag fusion protein for the melano protein

Before crashing (special for melano protein overexpression), the cells were suspended in ice cold resuspention buffer (20 ml) to concentration of OD_{600}. Then incubated in ice for 10 min and centrifuged again for 15 min at 5000xg. The supernatant was decanted and the pellet resuspended in ice cold extraction buffer and incubated on ice for 10 min. Centrifuge for 15 min at 5000 xg, and supernatant analysed as the periplasmic fraction with the Tris-Tricine PAGE (see section 4.4.7.1).

Resuspention buffer: 20 mM TRIS-HCl, 2.5 mM EDTA and 20% sucrose pH 8.0.

Extraction buffer: 20 mM TRIS-HCl, and 2.5 mM EDTA pH 8.0.

4.4.3 French press extract for protein isolation

The collected cells adjusted to 100 µl cell volume per ml (25 ml exposed for crashing with French press to fractionate the cells into cytosolic and inclusion bodies remove the inclusion. The two fractions were separated with centrifugation at 16000 xg for 30 min at 4 °C. The cytosolic is the soluble and the inclusion body is insoluble.

4.4.4 Insoluble and soluble proteins

The insoluble and soluble proteins were suspended in Na-phosphate buffer (50 mM, pH 7.5), then incubated at 37 °C for 10 min, washed, and centrifuged at 16,000 xg. The pellet was washed with 20 ml of Na-phosphate buffer (50mM, pH 7.5), the inclusion bodies were suspended in 8M of urea and incubated at 37 °C for 20 min. All the fractions were saved at -20 °C or directly analyzed with SDS-PAGE to see the expression pattern.

4.4.5 Protein overexpression with large scale for purification

The *E. coli* recombinant plasmid was grown for overnight as a primary inoculum in 10 ml LB broth medium containing ampicillin (100 µg/ml; pH 7.2). The primary inoculum was transfer to 250 ml of the LB medium containing 100 µg/ml ampicillin and grown for 1-2 h till OD 600~ 0.6 then the bacterial culture induced for 1 h with 1 mM IPTG (Thiogalactopyranoside) at 37 °C. Afterwards, the bacterial culture was centrifuged (5000 xg for 20min at 4 °C). The supernatant was discarded and the pellet washed with sterile water under aseptic condition. The pellet was resuspended in sodium phosphate buffer pH 7.0.

4.4.6 SDS PAGE

4.4.6.1 SDS-PAGE as described by Schägger and von Jagow (1987)

Overexpressed protein was analyzed with SDS-polyacrylamide gel, followed by **C**oomassie Brilliant Blue or silvers staining. Control sample should also be analyzed in parallel with the various fractions described below (Table: 9).

Table (9): SDS PAGE electrophoreses.

Component	Reagents and quantity
9% running gel (15 ml)	4.5 ml 30% Acrylamide, 0.8% N,N-bis-acrylamide 5.0 ml 3x Gel buffer pH 8.3 2.3 Glycerol 87%

	3.1 ml H_2O
	7.5 µl TEMED
	75 µl 10% APS
3% stacking gel (6.5 ml)	660 µl 30% Acrylamide, 0.8% N,N-bis-acrylamide
	1.6 ml 3x Gel buffer pH 8.3
	3.8 ml H_2O
	5 µl TEMED
	50 µl 10% APS
Cathode buffer 10X (500 ml)	60.5 g Tris, 89.6 g Tricine and 5 g SDS in H_2O having final volume 500 ml pH 8.3
Anode buffer 10X (500 ml)	121 g Tris in H_2O having final volume 500 ml pH 8.9

4.4.6.2 2D PAGE

The first dimension, isoelectricfocusing (IEF), was performed in an individual IPG gel strips, 3 mm wide (either ready-made Immobiline DryStripsR). Prior to IEF, 10 µl of concentrated melano protein samples from M9-Asp growth medium (See 4.1.4, 10 mg/ ml) was mixed 10 µl of lysis buffer in 1.5 ml tube for 1 h followed by centrifugation for 10 min at 12000 xg at 4 °C. The supernatant was transferred to new 1.5 ml tube and mixed with 200 µl rehydration buffer. IPG dry strip was rehydrated to their original thickness of 0.5 mm by 200 µl of the mixture for 1 h and covered with mineral oil in the rehydration chamber. The rehydrated strip was then placed onto the cooling plate of an isoelectrofocusing chamber and sample cups are placed onto the surface of the gel strips. For better sample entry, a low voltage gradient is applied across the gel for the first hour. The rehydration step takes 11 h (40000 V). Afterwards, the strip equilibrated followed by with 2.5 ml equilibration buffer for 10 min. The equilibration of the strip was repeated for 10 min with 2.5 ml of the equilibration buffer containing 2.5% iodacetamid in rehydration chamber. Then the strip separated on a Tris-Tricine PAGE and stained with silver stain.

Equilibration buffer: 6 M urea, 1.5 M TRIS-HCl pH 8.8, 2% SDS, 1% DTT, urea, 30% glycerol.

Rehydration buffer: 8 M urea, 0.5-2% chaps, 0.2% DTT, small amount of brmophenol and 0.2% ampholytes (3-10 pH)

Lysis buffer: 8 M urea, 4% chaps, 1% DTT, and 0.8% ampholyte (3-10 pH) , small amount of brmophenol and TRIS free base 40 mM.

4.4.7 SDS PAGE staining

4.4.7.3 Coomassie Brilliant Blue staining method

After electrophoresis gel was soaked in fixation solution for 1hr (100 ml), then washed with water, and soaked in staining solution (50 ml) at room temperature for one hour. Afterwards the gel was distained in water, boiling water for 5 min, or covered for overnight in destaining solution with gentle agitation. At the end the gel was documented and stored as a dry sheet in cellophane.

Gel-fixing solution: The final concentrations are 50% (v/v) Ethanol in water with 10% (v/v) Acetic acid.

Staining solution: The final concentration is 0.1% (w/v) Coomassie Brilliant Blue G250, 20% (v/v) Methanol, and 10% (v/v) Acetic acid.

Destaining: The final concentrations are 50% (v/v) Methanol in water with 10% (v/v) Acetic acid.

4.4.7.4 Silver staining

Detection of the proteins on the polyacrylamide gel was done using silver staining (table 10). All steps were performed in room temperature in glass or in plastic containers on a shaker. The volumes of all solution were 60 ml for mini gel 0.75 mm thickness.

Table (10): Procedure for 30-min silver-staining method

Steps	Solution	Time
Fixation	60 ml aceton (Stock A); 1.5 TCA Stock B; 25 µl 37% HCHO	5 min
Rinse	dd H_2O	3x5 sec
Wash	dd H_2O	5 min
Rinse	dd H_2O	3x5 sec
Pretreatment	60 ml aceton (Stock A)	5 min
Pretreatment	100µl $Na_2S_2O_3x5H_2O$ (Stock B) in 60 ml dd H_2O	1 min
Rinse	dd H_2O	3x5 sec
Impregnate	0.8 ml $AgNO_3$ (Stock C); 0.6 ml 37% HCHO; 60 ml dd H_2O	8 min
Rinse	dd H_2O	10-20 sec
Development	1.2 g Na_2CO_3; 25 µl 37% HCHO; 25 µl $Na_2S_2O_3x5H_2O$ Stock B; 60 ml dd H_2O	10-20 sec
Stop	1% Glacial Acetic acid in ddH_2O	30 sec
Rinse	ddH_2O	10 sec

Solution A: 50% aceton in ddH_2O

Stock solution B: 10% $Na_2S_2O_3x5H_2O$ in ddH_2O (shelf-life up to 4 month).

Solution C: 20% $AgNO_3$ in ddH_2O (store in dark; shelf-life up to 4 month)

4.4.8 Protein purification

4.4.8.1 Ni-NTA His•Bind® Resins technology purification

Protein fractionation was done using the Ni-NTA His•Bind® Resins technology for the soluble proteins under native condition. The storage buffer to 1x and mixed with the His-Bind resin by gentle shaking (50 rpm for 10 min). Afterwards 2.5 ml of the suspended resin was added to the column (100 ml). The mixture was packed under gravity flow to 20 ml filled column. 3 volumes (where 1 volume is equivalent to the settled bed volume) of sterile deionized water were used for washing the storage solution buffer (20% ethanol), charge (1x charge buffer) and equilibrate (1x binding buffer) the column, respectively. A flow rate of about 10 column volumes per hour was optimized for efficient purification. Binding buffer was allowed to drain to the top of the column bed and load the column with the prepared extract mixed with the binding buffer. The column was washed with 25 ml (10 volumes) of 1x binding buffer. Afterward it was washed with 15 ml (6 volumes) of 1x washing buffer. Finally, the bounded protein was eluted with 15 ml (6 volumes) of 1x elution buffer (Table 11).

Table (11): Solution used for protein purification by affinity chromatography

8x Binding buffer 40 mM Imidazole 4 M NaCl 160 mM Tris-HCl (pH 7.9)	4x Strip Buffer 400 mM EDTA 2 M NaCl 80 mM Tris-HCl (pH 7.9)
8x Wash Buffer 480 mM Imidazole 4 M NaCl 160 mM Tris-HCl (pH 7.9)	8x Charge buffer 400 mM $NiSO_4$
4x Elution buffer 4 M Imidazole 4 M NaCl 80 mM Tris-HCl (pH 7.9)	

4.4.8.2 Heat treatment purification method

Another strategy for the purification is the denaturation of the proteins after extraction from the cytosol at 65°C for 30 min. At that temperature most of the *E. coli* proteins denatured except the

recombinant protein, followed by the centrifugation to precipitate all the denaturized proteins. The soluble protein pattern and purity were determined using 12% Tris-Tricine PAGE.

4.4.9 Immunoblotting (western blot)

The blot was as described by Towbin *et al*., (1979) and Westermeier (1990). It consist of three successive steps using the mentioned solution (Table 12), Two SDS PAGE gels were run, on stained with Coomassie Brilliant Blue and the other one was transferred to the PVDF membrane (Schleicher & Schull, Dassel, Germany) for one h at 4 ^{0}C using 400 mA, followed by developing as follow: The PVDF membrane was incubated for 0.5 h in blocking buffer, followed by washing 4x 5 min with PBST. Afterwards the membrane was incubated for overnight at 4 °C with the specific melano protein antibody dilution 1:500 with blocking buffer. In the next day the membrane was washed with PBST 4x 5min, then it was incubated with 2nd antibody phosphatase for 1h dilution 1: 20000 with blocking buffer at 4 ^{0}C. The membrane was washed with PBST 4x 5 min, followed by development in 10 ml TMN-buffer containing 66 µl of NBT and 33 µl BCIP. After color detection the reaction was stopped with water.

Table (12): Immunoblotting buffers

Transfer puffer:	5x Laemmli-Buffer 200 ml SDS-Stammlsg 10% (w/v) Methanol 20 % Complete to 1 L dd-H_2O
Ponceau-S staining solution (fresh prepared)	Ponceau-S Staining dd-H_2O
10x PBS pH 7.1	NaCl 1.5 M KCl 27 mM Na_2HPO_4 x$2H_2O$ 87 mM KH_2PO_4 18 mM ddH_2O
10x PBST	10x PBS 0.1% (w/v) TWEEN20 dd-H_2O
TBS-Tween buffer	Tris base HCl pH 7.5 10 mM NaCl 150 mM ddH_2O

TBS-Tween buffer	Tris base HCl pH 7.5 20 mM NaCl 500 mM Tween20 0.5% (w/v)
TBS-Tween buffer	Tris base HCl pH 7.5 20 mM NaCl 500 mM Tween20 0.5% (w/v) Triton -X100 0.2% (w/v)
Blocking Buffer	5% (w/v) Magremilchpulver 1x PBST
TMN-Buffer pH 9.5	10 mM Tris-HCl 10 mM NaCl 5 mM $MgCl_2x6H_2O$ dd-H_2O
BCIP- solution	5-Bromo-4-chloro-3-indolylphosphat 100% Dimethylformamid (50 mg/ml)
NBT-solution	Nitroblue tetrazolium 70% Dimethylformamid (50 mg/ml)

4.4.10 Protein determination, Bradford (1976)

Calibration curve was prepared by using a standard protein sample BSA (Bovine Serum Albumin, Sigma). Then 10 µl from the protein sample was added with 90 µl autoclaved water. 10 µl from the respective medium was mixed with 90 µl distilled water as control. 1 ml of Bradford reagent was added to the sample as well as control and mixed very well. After 3 minutes of incubation on RT the absorbance was measured by spectrophotometer at 595 nm and the protein concentration in the sample was calculated by the aid of calibration curve.

Bradford reagent was prepared by solubilising Coomassie Brilliant blue into ethanol very well in presences of phosphoric acid followed by filtration through filter paper.

Bradford reagent: 100 mg Coomassie Brilliant Blue, 50 ml 96% Ethanol, 100 ml Phosphoric acid 85%, the mixture completed to one liter with ddH_2O.

4.4.11 Heat stability determination of the melano protein

The heat stability for the recombinant melano protein was done for the protein after purification as a percent of activity for equal aliquots of proteins (30 µg) in 1.5 ml tubes using the azocasein as substrate the tubes were preincubated for time intervals (5, 10, 20, 25, 30 and 40 min) in heat block at 70°C.

4.4.12 In gel activity

4.4.12.1 Glucanase

The gel glucanase activity was detected as described by Onsori *et al.,* (2005) in the presence of 0.2% extracted mushroom glucan was added into the separating gel PAGE (10 %) prior to polymerization. For molecular weight determination, the gel was stained with Coomassie Brilliant Blue G250. For glucanase activity, the gel was soaked with gentle shacking in solution 50 mM Sodium phosphate buffer (pH 7.2) containing 25% isopropanol for overnight. Afterwards, the gel was soaked in 50 mM Na-phosphate buffer (pH 7.2) for 90 min to renature the enzyme proteins. Finally, the gel was stained with 1% Congo-red solution for 30 min and distained with 1 M of NaCl solution.

4.4.12.2 Protease

The digestion of BSA (bovine serum albumin) by recombinant melano protein was done in 50 mM Na-phosphate buffer in 1.5 ml microfuge tube at 37 °C for zero and 12 the 24 h in absence and presence of $CoCl_2$. Afterwards the digestion pattern was shown on SDS-PAGE (see Table 9). Afterwards, the gels were stained with Coomassie Brilliant Blue or with silver staining.

4.4.13 Qualitative determinations of protein activity

4.4.13.1 Glucanase

4.4.13.1.1 Glucanase on agarose plate

Activity of the recombinant and the melano protein were determined on the plate in presences of glucan. Therefore the plate was incubated at 37 °C for 3 h and stained with 0.1% Congo-red, followed by distaining with 1 M NaCl. The distaining solution was continuously changed till yellow to white hallow detected around the whole including different tube of the purified recombinant protein of the melano protein of *V. inaequalis*.

4.4.13.1.2 TLC analysis of monosaccharides product referenced to glucose

After hydrolysis of mushroom-glucan with the melano protein from *V. inaequalis*, the products were determined qualitatively by thin layer chromatography (TLC). 5 µl of the enzymatic assay from the tubes were spotted on the front line of the TLC depending on the time including the control (Glucose), and then the sheets were dried. Afterwards, the sample were separated in jars

contain 10 ml of the running solution after 30 min the sheets were dried again and sprayed with 20% H_2SO_4 to ignite the separated sugar in situ the running lane containing glucose as control samples followed by heating of the plate to 75 °C .

Running solution: n-butanol: ethanol: water (50: 30:20)
Visualization solution: 20% H_2SO_4: 25% methanol

4.4.13.2 Protease

4.4.13.2.1 Protease detection on agarose plate

For the protease activity also the protease for both the recombinant protein and the melano protein from *V. inaequalis* were done on agarose plate containing casein incubated at 37 °C for 3 hour. Afterwards the reaction was stopped with 6% TCA to distinguish the proteolytic zones. The whole is related to different pure recombinant protein tubes including the melano protein of *V. inaequalis*.

4.4.13.2.2 Classification of the protease on agarose plate with different technique

Casein solutions were prepared by sequentially blending 150 ml water, 20.4 g Sodium acetate, the pH adjusted to 6.0 and 3.0 g casein on a magnetic mixer and then heating to 40 °C. The gel was prepared by boiling 150 ml water, 20.4 g Na-acetate and 3.0 g agarose until solubilization. The casein solution was added to the hot gel with continuous foam-free agitation. Twenty milliliters of the warm gel were immediately pipetted into Petri dishes (diameter 9 cm). Following solidification, 12 mm holes were bored in the agar with a no. 6 cork porer and 0.20 ml of enzyme solution was added to each hole in the pH 6.0 medium. The plates were incubated without inversion at 32 °C and examined after 12 and 24 h intervals. Total protein isolation from *M. domestica* cvs. Elstar and Remo. From each plant *ex* and *in vitro* 500 mg were taken and grinded in liquid nitrogen to powder and were stored with 5 ml extraction buffer, mixed and incubated at 65 °C for 20 min. Afterwards centrifuged at 12500 xg at RT, the soluble material were transferred into 1.5 ml microfuge tube and saved at -20 °C till using for gel pattern.

4.4.14 Quantitative determination of melano protein activity

4.4.14.1 Quantitative determination of glucanase activity

4.4.14.1.1 Congo red

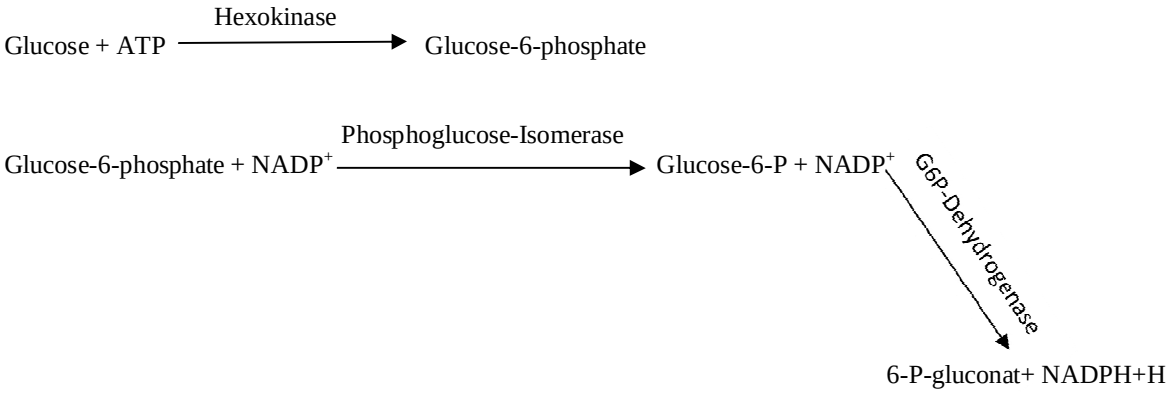
Glucanase

Glucan + H_2O ----------> Glucose + undigested glucan + adsorbed congo red+ acetic acid + soluble congo-red

*Followed by precipitation of undigested materials by centrifugation and monosaccharide determination by three different methods

The reaction was prepared in 1.5 ml microfuge by using protein (30 µg/ml) on glucan (5 mg/ml) for 30 min in 50 mM Na-phosphate buffer pH 6.0 and at 40 °C. Afterwards 20 µl/ ml of congo red (0.1%) was added. The reaction was stopped with 10 µl of 2 M H_2SO_4 and neutralized with 10 µl of 2 M NaOH. 400 µl of 100% acetic acid was added to precipitate glucan followed by 600 µl of 100% acetone. The mixture was centrifuged at 10000 xg for 2-5 min to remove all un-hydrolyzed glucan with some adsorbed congo red. The other hand supernatant contained soluble congo red which was measured at 520 nm on the spectrophotometer. The control for the reaction has the same gradient and treatment except the protein. Every reaction type is controlled for all points even for the pH, the time series, temperature, or kinetics.

4.4.14.1.2 Hexokinase and glucose-6-phosphat-dehydrogenase method



(Demoustier *et al.,* 1989)

Glucose was assayed and standardized by using the mentioned volume concentrations (Table 13). Then the slope factor were calculated and used for sample glucose determination by multiplication of the absorbance to the slope factor.

The experiment were done by adding 100 µl of the glucanase assay (product of hydrolysis assay) for different substrate to 1 ml of the hexokinase and dehydrogenase assay buffer and completed to three ml with sterile ddH_2O. 10 µl of the enzymes mixture were added for each reaction tube and then incubated at 30 °C for 15 min followed by 2 min intervals till stability. Afterwards the assay was measured at 340 °C and the reducing sugar was done. The reducing sugar amount was compared to the Congo red method.

Table (13): Reagent of hexokinase and dehydrogenase assay

Treatment	Water	Reagent 1 (ml)	Glucose solution (µg)	Enzyme mixture (µl)
0	2	1	0	10
2.5 µl Glucose (0.2 µg/µl)	1.9975	1	0.5	10
5 µl Glucose (0.2 µg/µl)	1.995	1	1	10
10 µl Glucose (0.2 µg/µl)	1.99	1	2	10
15 µl Glucose (0.2 µg/µl)	1.985	1	3	10
30 µl Glucose (0.2 µg/µl)	1.97	1	6	10
50 µl Glucose (0.2 µg/µl)	1.95	1	10	10
100 µl Crudlan	1.9	1		10
100 µl Wheat flour	1.9	1		10
100 µl Starch barley	1.9	1		10
100 µl Crudlan + $CoCl_2$	1.9	1		10
100 µl Wheat flour+ $CoCl_2$	1.9	1		10
100 µl Starch barley + $CoCl_2$	1.9	1		10

Assay buffer 100 ml: 7 g Triethanolamin, 242 mg NADP, 577 mg ATP, 260 mg $MgCl_2$ x $6H_2O$, completed to 100 ml with sterile ddH_2O.

Glucose stock solution: 200 µg/ml.

4.4.14.1.2 Determination of the reducing sugar in the polysaccharide (DNS)

The dinitrosalicylic acid (DNS) is the recommended procedure for determination of the reducing sugar in hydrolyzed polysaccharide to monosaccharide; it was done as described by Bailey and Nevalainen (1981; method details described in IUPAC 1987). 300 µl of sample and 600 µl DNS-NaOH and 200 µl of the K-Na-tatarate were mixed, boiled for 5 min, cooled and complete to 2 ml with water, in order to bring the color developed into a readable range. This allowed direct assay of sugar (glucose) concentrations of up to 4 g/1 without the necessity of intuitive sample dilution before the assay. The DNS reaction also was done for the negative control for the hydrolysable polysaccharide. Thus an assay for polymer degrading cellulase activity was obtained which could be demonstrated to be linear. The reactions were measured at 540 nm. The

positive samples were subtracted against the negative controls. The reaction was done for maize tissue, crudulan (wood), flour, starch (containing manose).

Dinitrosalicylic Acid Reagent Solution, 1%: 10 g dinitrosalicylic acid, 0.5 g Na-sulfite, NaOH 10 g, completed with water to 1 liter.
K-Na-Tartrate solution: 40%

4.4.14.2 Protease activity (Colorimetric proteolytic assay using Azo-casein)

Azo-casein consists of casein conjugated to an azo-dye. This serves as a general substrate for proteolytic enzymes. Degradation of the Azocasein liberates free dye into the supernatant that can be quantitatively analyzed. Typical assay procedures using such a substrate have been published.

Protease
Azocasein + H_2O ----------> Colored Reaction Products
Conditions: T = 37°C, pH 8.3, A440 nm

Reagents:

A. 0.50% (w/v) Na-bicarbonate buffer ($NaHCO_3$), pH 8.3 at 37°C (Prepare 100 ml in deionized water using Na-bicarbonate. Adjust to pH 8.3 at 37°C with 1 M HCl.)

B. 2.50% (w/v) Azocasein Solution (Azocasein) (Prepare 15 ml in Reagent A using Azocasein. Gentle heating and stirring may be needed to form a solution.)

C. 5.0% (v/v) Trichloroacetic Acid Solution (TCA)

D. 500 mM NaOH

E. Trypsin Enzyme Solution (Prepare a solution containing 10 mg solid/ml of trypsin in reagent A.) Recombinant melano protein solution 1 mg/ ml dissolved in phosphate buffer pH 6.0)

Procedure:

The experiment of the protease activity was done for trypsin, melano protein and control. For each assay, 2.5 ml of asocasein was added in reaction tube (10 ml) including the control, 1.5 ml of the reaction buffer (A) was added in each tube for enzymes and 2.5 ml for the blank tube. Afterwards, 1 ml of trypsin was added to the reaction tube (40 units /mg; E) and 1 ml for the recombinant melano protein were added to the reaction tube (366.66/mg; E). The reaction tubes were incubated at 37 °C for 30 min. Afterwards 1 ml aliquots were removed from all the tubes including the blank into new tubes. Then 4 ml of reagent C was added for each tube and shacked

and centrifuged at 10000 xg. 1 ml aliquots were removed to a new tubes followed by adding 3 ml of reagent D. Afterwards the color was measured at 440 nm.

Calculation:

DA440nm = A 440 nm Test - A440 nm blank

Calculation was done for both the recombinant protein and trypsin. By comparing both of trypsin and recombinant protein the number of units of recombinant protein was calculated as a ratio of trypsin units.

***** Then the reaction was adjusted to 1 ml.

4.4.15 Temperature, pH changes effect on the glucanase and protease activities

The temperature effects on melano protein activities (gucanase and protease) were done between 20-100 °C to find out the optimum temperature. Each temperature degree had a control. Then both glucanase and protease assay was done as described (4.4.14.1.1, 4.4.14.2, respectively) and incubated in different temperatures. The experiments were repeated under the founded optimum temperature using different pH points (3.0-10.0 pH). The reaction mixtures for the pH changes effects were prepared using a different pH sodium phosphate buffer (3.0-10.0 pH).

4.4.16 Metals and inhibitor effect on melano protein activity

Metals roles in glucanase activity were tested. The metals were incubated 15 min before adding the glucan with 15 µg of protein in each tube using the following concentration 1 mM in the reaction mixtures for each metal ($CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_3$, and $ZnCl_2$) in each reaction tube. The experiment was repeated by using different concentration of metals (0, 1, 2, 3, 4, and 5 mg/ml). It was assayed as mentioned in chapters 4.4.14.1.1 and 4.4.14.2 for gulcanase and protease, respectively. The melano protein activites were assayed again in the same order as mentioned by using 10 mM, 1 mM, 0.3 µM, 100 µM, 100 µM, 100µM and 30 µg of EDTA, PMSF, Aprotinin, Pepstatin, Leupeprin, Chymostatin, Trypsin protease inhibitors and recombinant CysPI, respectively.

4.4.17 Protease Km value determination

Different substrate concentrations (0.5-14 mg of Azocasein and 1-8 mg of glucan) free and in combination with 500 µM or 200 µM of calcium and magnesium chloride were tested

respectively for melano protein protease and glucanase. The metal were incubated with the melano protein 10 min before assaying the reaction and the experimented were done as mentioned in sections 4.4.14.2 and 4.4.14.1.1 for protease and glucanase respectively. V_o, V_{max} and Km values for the melano protein glucanase and protease activates were determined.

4.5 Melanin and melano protein (Recombinant melano protein)

4.5.1 Melanin or combinations of melanin-EDTA and melanin-$CoCl_2$ effect on activity of protease

The primary interaction of melanin (50 µM) with recombinant protein (30 µg, 100 µl) and incubated for 20 min at room temperature against control has the same amount of melanin. On the same experiment (50 µl of ammonia solution 1 %) were mixed with the 30 µg recombinant melano protein as a control. The experiment was assayed as known (4.4.14.2). The mentioned assay was repeated by using different concentration of melanin (10-100 µM), Melanin (100 µM)-EDTA (1-10 mM) and finally melanin (100 µM)-$CoCl_2$ (100-2000 µM).

4.5.2 Binding capacity of the melanin to recombinant protein

The binding capacity of melanin to the recombinant protein determined by dialysis of the melanin-protein mixture to determined the binding capacity, 100 µl of melanin (100 µM) were pipetted in 1.5 ml microfuge in presence 0, 10, 20, 30, 50, 60, 70, and 80 µg protein were added in the reaction tubes respectively. Binding was done in presence of 50 mM Na-phosphate buffer (pH 6.0) and incubated for 30 min, then dialyzed in dialysis bag (exclusion size 16 kDa) in 50 mM Na-phosphate buffer for overnight. Afterwards, the absorbance of melanin was done at 400 nm, and then it was calculated as percentage from the control and subtracted from one hundred and plotted against the protein concentration used for binding. The binding assay was repeated using different concentrations of EDTA (1-7 mM) in combination with 100 µM melanin. In another experiment for binding, the EDTA was replaced with $CoCl_2$ (100, 200, 300, 500, 1000, 2000 µM) in combination with melanin (100 µM). In another experiment the extraction of the unbounded melanin were done by 50 % aceton or 50 % DMF followed by determination of the bounded melanin as percent (%) in presence of 1 mM $CoCl_2$ or 1 mM EDTA in comparison of the control.

4.6 Effect of the recombinant melano protein bacterial growth

In this experiment, four species were selected, two of them is gram positive bacteria *(Bacillus subtilis* and *Bacillus mycoides*) and other two are gram negative bacteria (*Agrobacterium tumefaciens* LB A 4404 and *Erwinia amylovora*) the experiment were done with 10^8 bacterial cells were in 1.5 ml tube which preincubated with 50 µg heat purified protein for 24 h at 28 °C. Afterwards 10^7 cells were transferred to 5 ml minimal medium (M9, Table 14) and incubated for 8 h at 28 °C. Finally, the optical density was measured for each species. Each species also have a control without protein also measured. The experiment was repeated using 1 mM $CoCl_2$. The inhibitory effect was subtracted from the control and plotted as percentage. The optical density was measured at 600 nm.

Table (14): Minimal medium for bacterial growth

10 x Minimal medium 9 (M9)	Additional additives
340 mM Na_2HPO_4 x $2H_2O$	200 mM Hepes pH 7.0
220 mM KH_2PO_4	20% Glucose
86 mM NaCl	1 M $MgCl_2$
187 mM NH_4Cl	100 mM Asparagin
	10 ml 2.5 mg/ml Thiamin
Completed to 1 L sterile ddH_2O	dissolved sterile ddH2O and filtrated
For 1 x M9 medium (1 L), 100 ml of 10 x M9 medium, 5 ml 200 mM Hepes pH 7.0, 1.25 ml 20% Glucose, 2.5 ml Thiamin and 5 ml 100 mM Asparagin were mixed and filled with sterile ddH_2O to 50 ml.	

4.6.2 Inhibitory effects of the apoplastic fluid on recombinant melano protein activities (Elstar and Remo)

Inhibitory effects of *M. domestica* cvs. Remo and Elstar on the melano protein glucanase and protease activity were done. 30 µg (100 µl) of the apoplastic fluid from each cultivar (Elstar and Remo) were incubated 15 min with 15 µg of recombinant proteins protein in each tube (three replicate for each cultivar). The incubated and interacted melano protein was assayed for glucanase and protease as described previously (4.4.14.1.1 and 4.4.14.2, respectively)

4.7 Plant apoplastic fluid proteins effect on trypsin activity

The aim of this study was to find out a similarity or difference between the recombinant melano protein and the trypsin protease response to the *M. domestica* cvs. Elstar and Remo, respectively. The study was done for extracted apoplastic fluid proteins from *M. domestica* cvs. Remo and

Elstar (30 µg/ml) as effectors to trypsin protease (30 µg/ml). The protease activity of apoplastic fluid was done as a control. The assay was repeated as mentioned in section 4.4.14.2.

4.8 Cuticle of *M. domestica* cvs. Remo and Elstar

4.8.1 Permeability of the cuticle

Permeability of the cuticle was done for both the two cultivars (Remo and Elstar) leaves when incubated for overnight with aliquots of 50 µl drop of Toluidine blue (0.05%) on the upper surface of the leaves for 2 h and then washed with water (Bessire *et al.*, 2007).

4.8.2 Replica preparation

Replica making was done by painting of the nail varnish on the upper leaf surface of *M. domestica* cvs. Elstar and Remo. Afterwards they were kept in room temperature till dryness, then peeled and used for the next experiments.

4.8.3 Replica staining

4.8.3.1 Silver staining

Detection of the proteins localization on the cuticle upper surface of *M. domestica* cvs. Remo and Elstar were done using the impression silver staining method (Table 15).

Table (15): Procedure for 15-min silver-staining method

Steps	Solution	Time
Rinse	dd H_2O	3x5 sec
Impregnate	0.8 ml $AgNO_3$ (Stock B); 0.6 ml 37% HCHO; 60 ml dd H_2O	8 min
Rinse	dd H_2O	
Development	1.2 g Na_2CO_3; 25 µl 37% HCHO; 25 µl $Na_2S_2O_3x5H_2O$ Stock A; 60 ml dd H_2O	10-20 sec
Stop	1% Glacial acetic acid in ddH_2O	30 sec
Rinse	ddH_2O	10 sec

All steps were performed in room temperature in microfuge tubes 1.5 or 2 ml.

The volumes of all solution were 1 ml for one replica 0.4 cm^2 area.

[A] Stock solutions; 10% $Na_2S_2O_3x5H_2O$ in ddH_2O (shelf-life up to 4 month).

[B] Stock solutions; 20% $AgNO_3$ in ddH_2O (store in dark; shelf-life up to 4 month);

A brown precipitate may appear upon contact of the gel with the developer solution.

4.8.3.2 Bromophenol blue staining

In 1.5 ml microfuge tube the cuticle membrane which is removed with the impression methods were mixed with acidified (acetic acid) and 25 µ1 1x protein loading buffer contain bromophenol blue and DTT to detect the localization of the proteins or mainly for fluorescent development under UV and that really give red florescent. The aim of the stain is to find a staining for the fixed protein on the membranous replica.

Loading buffer: 50 mM TRIS-HCl pH 6.8, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue, 2 % acetic acid

4.9 Plant section preparation

4.9.1 Tissue fixation and embedding in paraplast plus for in situ hybridization

For fixation, 0.4 cm discs from *M. domestica* cvs. Remo and Elstar leaves were collected from *in vitro* culture. The collected tissues were immediately immersed freshly prepared FAA (50% ethanol, 10% formaldehyde, and 5% acetic acid) which is kept on ice in 1.5 ml microfuge tubes. Fixation solution was infiltrated into the tissue by placing the open tube into a small beaker filled with ice and place the beaker into a vacuum chamber and moderate pressure was applied for 15 minutes and then the pressure was slowly released. This process was repeated three more times until after the tissue sank.

The next step is the impeding process which started by formaldehyde for overnight (Fill the tube to the top with fresh, cold FAA), followed by sealed the tube and transferred to a rocking platform in a 4° C incubator or temperature-controlled room. Afterwards, under the fume hood the tissue were washed in 50% ethanol and replaced with fresh 50% ethanol and incubate at 4 °C on a rocking platform for 1-2 h. After incubation in 50% ethanol the tissue going on the dehydration steps which are incubation in increasing concentrations of ethanol: 70%, 85%, 90%, 100%, respectively (1-2 hours at 4 °C on a rocking platform for each incubation step). Always fill the tube to the top. The tissue could be stored in 70% ethanol at 4° C for overnight (or longer) if necessary. The ethanol serial dilutions were replaced with fresh 100% ethanol and left for overnight at 4° C on a rocking platform followed with incubation for 1 h at room temperature for the tubes. The incubated discs in 100% ethanol were incubated in descending alcohol serial (100%, 95%, 85%, 70% and 60%, 1 h each). The 60 % alcohol was replaced with 50% ethanol /50% Histo-clear solution and incubated at room temperature for 2 h. Histo-clear solution (50 %)

was replaced with 100% Histo-clear and incubated at room temperature for 2 h. Then solution was replaced with just enough Histo-clear solution (100%) to cover the tissue. The Histoclear was replaced with melted (60 °C) paraplast plus chips and incubated at a 60 °C waxes and left for overnight. The paraplast plus in the tubes were replaced for 5 times for five following days one time per day at 60 °C. On the sixth day, four molds were prepared. Peel-A-Way molds were used, followed by molds labeling as to the contents (species, tissue type, orientation). The sample block were prepared by filling a mold with fresh molten paraplast plus and the leaves discs were inserted into the mold and placed in a flat box filled with about 0.5 cm depth of room temperature water (Allowing the molds to cool slowly at room temperature), then stored it at 4 °C before using it for the cutting.

4.9.2 Plant sections cuttings

The cuttings were done using the microtome, before cutting the block were stored for 30 min at least to make sure on the hardiness during the cutting process to avoid the malformation of the sections (10 µm thick).

4.9.3 Plant section staining methods

After cuttings and film fixation were done, the films on the surface of the slides were stained with acetified bromophenol blue (10% acetic acid) which loaded directly on the surface of the sections and the slide were heated on hot plate (75 °C) for 5-10 min. Afterwards the slides were sided down two remove the paraplast (paraffin resins) and washed with distal water) to clean out the sections. Slides left for 20 min to dry and examined directly under the microscope under the visible or UV light. Slides were mounted or freshly prepared.

Advantages of this method

- Time saving (fast methods (new))
- Bromophenol blue using for staining of the plant tissue cutting
- Give good results
- Can used for coloration of the plant tissue under the bright field (visible light) (new)
- Could used for detection of metals under UV for florescence under the microscopic because bromophenol blue can chelate to calcium and give green-yellow to red florescence (new).

4.9.4 Deparaplast step for immune-bloating

For de-waxing, the slides were immersed in 100% Histoclear (under fume hood, just long enough to remove wax), followed with 1:1 alcohol: Histoclear (under fume hood), 20 min. Then the slides were immersed in descending serial of alcohol concentrations (100%, 95%, 80%, 70%, and 50%). Afterwards was washed with distilled water 2-3 times.

4.9.5 Slides treatment with proteinase-K to reduce the local alkaline phosphatase

The slide were flooded with proteinase k solution (0.1 mg/ml Proteinase K, in 1x PBS buffer for 20 min (at room temperature) to reduce the local phosphatase inside the section to prevent or minimize the side reaction. Then the slides were washed with distilled water four times and air dried.

4.9.6 Protein isolation and SDS Page

4.9.6.1 Total protein isolation From *M. domestica* cv. Remo and Elstar

Appropriate weight from plant material homogenates with nitrogen were taken in 50 ml Sarstedt-tube with 5 ml extraction buffer, then were vortex together and incubated about 20 min at 65 °C and cooled down for short time, followed by centrifugation for 10 min at 13000 xg. The supernatant were transferred to a new tube and saved till used at -20 °C.

Extraction buffer: 55 mM Na_2CO_3, 2 % SDS (W/V), 2 mM EDTA, 1 mM PMSF, 3 mM ε-amino caproic acid, 12 % glycerol (V/V), 56 mM DTT, 0.1 % BPB (W/V)

4.9.6.2 Characterization the surface (Cuticle Replica) protein

4.9.6.2.1 Isolation of the surface proteins

The proteins in replica were isolated using 50 µl 8 M urea for 10 discs (0.4 cm) of impressions, were mixed with 20 µl protein loading buffer 5x and 30 µl distilled water, followed by incubation with checking at 65 °C for 15 min. Afterwards the replica were remove and the extract was saved at – 20 °C for the needed analysis.

4.9.6.2.2 Chitinase activity isolated from replica of *M. domestica* cv. Elstar and Remo

Tris-Tricine gel with 10% separation containing 1% Glycolchitin as substrate and 3% stacking gel was prepared. Two samples contained the extracted protein from cuticle of *M. domestica* cv. Elstar and Remo, respectively were loaded in the gel and separated electrophoretically. Gel was

renaturated by incubation overnight in 100 ml of renaturation buffer at 37 °C with gentle shacking (40 rpm). Gel was washed two times with TRIS-HCl, pH 9.0. The gel was stained with freshly prepared staining solution for 2 min and washed immediately by distilled water. Finally gel was examined via UV light.

Sample buffer: 5 ml H_2O, 2.5 ml 0.5 M TRIS-HCl pH 6.8, 2.5 ml 10 % SDS and 1.5 g sucrose

Run buffer 10x: 2.5 % glycin, 6 % TRIS base, fill up to 1 litre with distilled water.

Run buffer 1x: 100 ml of 10x buffer, 10 ml 10 % SDS, fill up to 1 litre with distilled water.

Renaturation buffer: 5.96 g Hepes (free acid), 5 ml Triton X100, pH 7.0 (with 3 M KOH), fill up to 500 ml with distilled water and stored at 4 °C.

Staining solution: 200 ml 0.5 M TRIS-HCl pH 9.0, 3-4 grains of Fluorescent Brilliant.

(pipette) of Merckoglas or Permount.

4.9.6.2.3 Identification of the polypeptide fragments by Electron Spray Ionization Quadruple Time of Flight (ESI Q-ToF) mass spectrometry:

4.9.6.2.3.1 Band cutting from the gel

The electrophoretically separated bands of proteins obtained from the plant cuticle and apoplastic fluid of *M. domestica* cv. Remo. The protein content of the bands was digested and extracted from the gel according to Jensen *et al.,* (1998) as described below:

4.9.6.2.3.2 Washing and destaining

The excised gel pieces were put in reaction tubes and washed two times for 15 min with shaking with double volume of deionized water/acetonitrile 1:1 (v/v). Washing solution was discarded and gel pieces were incubated for 5 min in one volume acetonitrile at RT: Acetonitrile solution was replaced by one volume of 100 mM NH_4-bicarbonate (pH 9.0) and tubes were mixed and incubated for 5 min at RT. After addition of one volume acetonitrile to NH_4-bicarbonate (pH 9.0), tubes were mixed and again incubated for 15 min at RT with shaking. The whole liquid was removed and gel pieces were dried in the speed vacuum. In gel digestion The dried gel pieces were gradually quenched by adding 10 µl trypsin (10 ng/µl in 50 mM NH_4-bicarbonate) and incubated in ice for 10 min. Quenching step was repeated several times until saturation of gel pieces with trypsin solution. The excess of trypsin solution was discarded and gel pieces were soaked in excess of 25 mM NH_4-bicarbonate solution. The samples were incubated overnight at 37 °C.

4.9.6.2.3.3 Extraction of peptide fragments from the gel

After overnight digestion, samples were shortly centrifuged and the supernatant was taken in separate reaction tubes. Afterwards gel pieces were sonicated by using ultrasonic for 2 min and the resultant liquid was collected in the same reaction tubes. Gel pieces were incubated for 20 min in one volume 25 mM NH_4-bicarbonate with shaking. After addition of one volume acetonitrile and incubation for 15 min under shaking, the mixture was sonicated again for 2 min. The supernatant was collected in the same reaction tubes mentioned above. Gel pieces were incubated two times for 15 min in 5 % (v/v) formic acid/ acetonitrile (1:1) and the liquid was collected in the same tubes. The whole collected supernatant was centrifuged and concentrated by speed vacuum at 60 °C to make final volume 10-15 µl. These concentrated solutions of polypeptide fragments were ready for sequencing and stored at -20 °C.

4.9.6.2.3.4 Sequencing

Sequencing was carried out on a quadruple time of flight hybrid mass spectrometer (Q-ToF Micromass) in positive ion mode. In ESI Q-ToF mass spectrometry the fragments of the trypsin digested polypeptide were ionized by electron spray. At this stage each of the fragments showed its specific peak on the basis of its mass. Out of many fragments only few fragments were randomly selected for argon treatment for further fragmentation and then allowed to fly through a electromagnetic field. Depending on the time of the flight, individual amino acids were identified and finally the amino acid sequences of the trypsin digested polypeptide fragments were obtained. Amino acid sequences were then compared to known protein by homology search by using the program MS BLAST Search (EMBL, Heidelberg, Germany).

4.9.6.3 Isolation of the intercellular washing fluid (IWF) or apoplastic fluid

The isolation of IWF or apoplastic fluids from *in vitro* and *ex vitro* culture of the *M. domestica* cvs. Elstar and Remo were done using the infiltration/centrifugation technique (Hogue and Asselin, 1987) with slight modifications. The leaves were cut from the trees by a razor blade and its weight was determined. The harvested leaves were submerged in PBS buffer or water in a plastic box, and covered with a sieve. Infiltration with PBS buffer was done for 4 min. Leaves were dried with tissue papers and inserted into homemade holder as illustrated in Figure 6. Centrifugation was done for 5 min at 100 xg in an HS-4 rotor (Sorvall) to remove the excess of liquid. Subsequently, the IWF

samples were collected by centrifugation step at 4 °C for 20 min at 700 xg. The obtained apoplastic washing fluid was stored at –20 °C.

PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4.

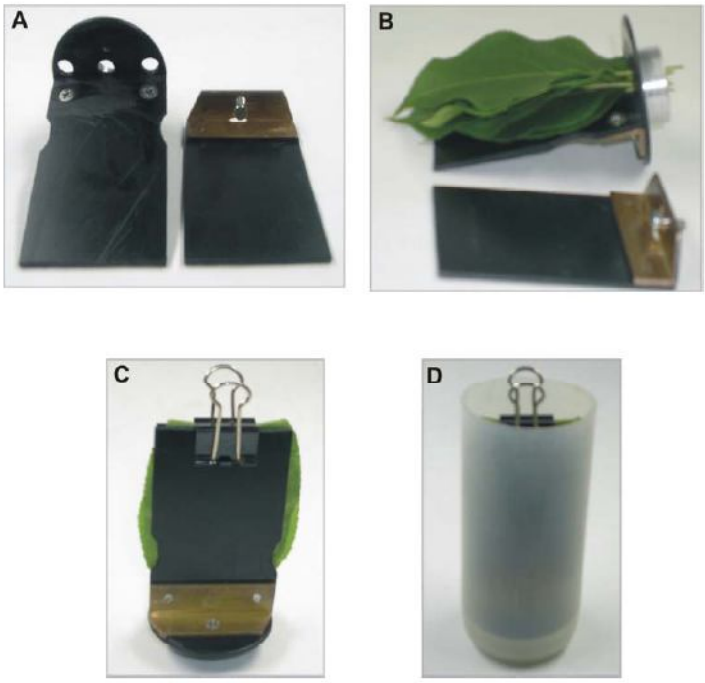


Figure (6): Homemade holder for apoplastic fluid isolation from leaves; A: Holder without plant leaves; B: holder with plant leaves before fixing; C: Fixed plant leaves within the holder; D: Holder with plant leaves, ready for centrifugation (Koutb 2006).

4.10.1 Western-bloat-like technique to monitor the steps of infection

4.10.1.1 Replica technique blotting (Western-blot-like)

Impression of the upper leaf surface of *M. domestica* cvs. Remo and Elstar after and before infection with *V. inaequalis* were done by painting of the upper leaf surface with nail varnish and left it to dry. Afterwards the membranous laminas were removed from the upper surface of the plant containing some proteins. These proteins were blotted directly on the membrane replica because it is ready fixed. Replicas were incubated in 1.5 ml microfuge tubes in blocking buffer. Afterwards, they were incubated with melano protein antibody (1:500) to detect the presence of melano protein in the infected *M. domestica* cvs. Elstar and Remo. The membranes were washed 2 x 10 min with TBS-Tween-Triton and 1 x 10 min with TBS buffer. Replicas were incubated with the alkaline phosphatase (second antibody antigoat; 1:20000) for 1h, followed with the

replica washing with TBS-Tween20-Triton (2 x 10 min) and finally with TBS buffer (1 x 10 min). TBS was removed and replaced by TMN buffer (1 ml) for color detection containing of BCIP (3.3 µl/ml) and NPT (6.6 µl/ml) the steps going on as western blot for melano protein detection the method were done for the resistant cultivar Remo and susceptible Elstar. Finally, the reactions stopped by washing with ddH_2O. The replicas were examined under microscope on slide surface.

4.10.1.2 Localization of the melano protein into the infected tissue by using Local Western Blot

The treated sections of the infected plants were submitted to protein digestion for 10 min (10 mg/ml) with proteinase K to reduce phosphatase in the sections of *M. domestica* cvs. Remo and Elstar. Afterwards they were incubated for 1 h in PBS buffer containing 0.05% Tween 20 (PBST) and 3% skimmed milk powder to block nonspecific protein-binding sites. The slides were washed with PBST four times (15 min intervals, pH 7.0). The blocked sections were washed very intensively 4 x 5 min with PBST, and then incubated 2 h with diluted specific melano protein antibody (1: 250 in blocking buffer). After washing with PBST buffer, the sections were incubated for 1 h with an anti-goat IgG-alkaline phosphatase conjugate diluted 1:20000 in blocking buffer and washed with PBS (three washes, 5 min each). The interacted local proteins was detected colormetrically and developed in TMN-buffer (pH 9.0) containing NBT (6.6 µL/ml; 0.5%) and BCIP (3.3 µL/ml; 0.5%) and allowed to develop for 5 min to 1 h. the reaction stopped with section washing with ddH_2O. Finally the sections were fixed with glass covered, sealed and documented.

4.10.2 Proteins interaction (Far Western Blot)

4.10.2.1 Interaction on PVDF membrane

4.10.2.1.1 Interaction of surface protein from replica of proteins with the recombinant melano protein

The Blot was developed to determine a specific interactive protein(s) in the isolated surface protein(s) from the replica of *in vitro M. domestica* cvs. Remo and Elstar. The proteins were investigated with SDS-PAGE (12 % Tris-Tricine) for three gels one of them were stained with Coomassie Brilliant Blue and the other two gels were submitted to protein electro- transport to PVDF* membrane (Schleicher & Schull, Dassel, Germany) with electrophoresis for 1 h at 4 °C.

Afterwards, the membranes were incubated in blocking buffer (5% nonlipid milk) for 0.5 h, washed 3x 5 min with TBSTT buffer (20 mM Tris ; 0.05% Tween 20; 0.2% Triton X100; pH 7.5) followed by 2 x 5 min with TBS buffer pH 7.5. One of the membranes was incubated in blocking buffer containing recombinant melano protein (1:100) for three hour, then washed 1 x 5 min with TBSTT (pH: 7.5) and 2 x 5 min with TBS (pH: 7.5). This gel was blotted to detect if there are interactive proteins with the melano protein in surface of the plant, Afterward the two membranes were incubated for overnight in blocking buffer containing the specific antibody of the melano protein (1:500). In the next day the; membranes were washed 2 x 5 min with TBSTT and 2 x 5min with TBS. Then membranes were incubated in the blocking buffer containing the second antibody with the ratio 1:20000 for 1 h at room temperature. Afterwards, membranes were washed 2 x 5 min with TBS. Finally the reaction was detected in TMN-buffer (pH 9) containing (6.6 µl/ 1 ml; 0.5%) NBT and (3.3 µl /1 ml; 0.5%) BCIP. Afterwards, the reaction on PVDF membrane was stopped with washing with water and then documented.

4.10.2.1.2 Interaction of apoplastic proteins

The extracted proteins from the apoplastic fluid were blotted for interactivity with the recombinant melano protein on PVDF membrane to determine the interactive proteins in the apoplastic fluid. The interactivity experiment was repeated as described above (See chapter 4.10.2.1.1) for the apoplastic fluid isolated from *in vitro M. domestica* cvs. Remo and Elstar.

4.10.2.1.3 Total proteins interaction

Protease activity was done as an interaction between recombinant melano protein and the total plant proteins of *M. domestica* cvs. Elstar and Remo as substrate proteins on SDS PAGE. After the SDS PAGE for protein separation, transferring of these proteins to PVDF membrane and interacting of these proteins to the melano protein, the latter was detected as done for the apoplastic fluid by Far Western Blot. Afterwards, blotting of the melano protease activity was detected as the decrease in plant protein bands intensities with the time and that detected by staining with both PonceauS solution and melano protein antibody. The time table of the interaction was done using the antibody staining method to determine the band strength with the time. The interaction of the recombinant protein to the plant total protein was repeated on PVDF membrane in presences of 1 mM of PMSF and mixture of $MgCl_2$ and $CaCl_2$ to characterize the inhibitory effect on PVDF membrane.

4.10.2.1.4 Bacterial protein interaction

1 ml from liquid culture of the mentioned bacteria was centrifuged and the pellets were suspended in 200 µl 8 M urea solutions and then were sonicated for 30 sec. 20 µl of each bacterium were mixed with 5 µl of the 5X sample buffer and boiled for 5 min at 95 °C and then loaded in 12% of Tris-Tricine gel. Two gels were made for the bacterial protein separation. Afterwards, one gel stained with Coomassie Brilliant Blue and the other one submitted to the electrotransporter for proteins transfer to PVDF membrane. The interaction with the recombinant melano protein was done as described in chapter 4.10.2.1.1.

4.10.2.2 Local interaction of the plant proteins with the recombinant melano protein

4.10.2.2.1 Replica protein interaction (Far-Western-like)

The removed replicas from the *in vitro* plants (*M. domestica* cvs. Remo and Elstar) were submitted to the interactions to the melano protein to confirm presence of surface protein and to detect of interactive proteins on the surface of the uninfected plants. They were incubated in 1.5 ml microfuge tubes with and without blocking buffer. The blocked and non blocked replicas were incubated with 1 ml (1:100) of the recombinant melano protein produced in *E. coli* for 2 h and then washed 2 x 10 min TBS-Tween-Triton and 1 x 10 min with TBS buffer. Afterwards, they were incubated with melano protein antibody (1:500) to detect the presence of melano protein in the infected *M. domestica* cvs. Elstar and Remo. The membranes were washed 2 x 10 min with TBS-Tween-Triton and 1 x 10 min with TBS buffer. Replicas were incubated with the alkaline phosphatase (Second antibody anti-goat; 1:20000) for 1h, followed with the replica washing 2 x 10 min with TBS-Tween-Triton. Finally with TBS buffer (1 x 10 min). TBS was removed and replaced by TMN buffer (1ml) for color detection containing of BCIP (3.3 µl/ml; 0.5%) and NPT (6.6 µl/ml; 0.5%) the steps going on as Western blot for melano protein detection the method were done for the resistant cultivar Remo and susceptible Elstar. Finally, the reactions stopped by washing with ddH_2O. The replicas were examined under microscope on slide surface.

4.10.2.2.2 Melano protein interaction to local plant proteins in the Section-Far-Western-blotting-like

After sectioning and phosphatase reduction with proteinase K, the sections of the uninfected plants of *M. domestica* cvs. Remo and Elstar were incubated for 1 h in PBS buffer plus 0.05% Tween 20 (PBST) containing 3% skimmed milk powder to block nonspecific protein-binding

sites. The slides were washed with PBST four times (15 min intervals, pH 7.0), followed by incubation with the melano protein mixed with the blocking buffer (1:200; 400 µl) for 3 hours. Afterward the section were washed four times with PBST (15 min intervals, pH 7.0), then the sections were incubated 2 h with diluted melano protein antibody (1:250 in blocking buffer; 400 µl). After washing with PBST buffer, the sections were incubated for 1 h with an anti-got IgG-alkaline phosphatase conjugate diluted 1:20000 (400 µl) in blocking buffer, washed with PBS (three washes, 5 min each), and equilibrated in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; and 50 mM $MgCl_2$). Color reaction was developed in TMN-buffer (pH 9.0) containing NBT (6.6 µL/ ml; 0.5%) and 0.5% of BCIP (3.3 µL/1 ml; 0.5%) and allowed to develop for 5 min to 1 h. After washing with deionized water, a drop of mounting medium (Histovitrex; Carlo Erba, Milan, Italy) was added to the slides. The control section bloated only against the melano protein antibody and the phosphatase.

Mounting medium

The slides were washed with PBST (4 x 5 min) followed by the washing with distilled water, followed by dehydration the tissue with serial of ethanol 30%, 60%, 80%, 95%, and 100% (two times) followed by 50%/50% Histoclear/Ethanol (2 min) then by 100% Histoclear. Finally, pipettes the slides were drip dried followed by adding 2 to 4 drops (from wide end of glass Pasteur

4.11 Agarose-acrylamide gel

There two types of agarose-acrylamide gel according to the size will separated from the protein were prepared. The gel mixture used for melanin-recombinant protein polymer (3.75% acrylamide-0.7% agarose containing 10 % glycerol, Table 16) or polymer interacted with cobalt (1.5% acrylamide-0.7% agarose, Table 15) ion separation was uniform. Afterwards the running polymer was transferred elctrophoretically to PVDF membrane and detected the bigger formed band due to the polymerization with the antibody of the melano protein. The buffer or the gel contain lower amount of SDS to avoid the effect on the hydrophobic polymerization interaction. The higher gel concentration was used for the smaller polymer and the lower one without glycerol used for the interacted polymer with cobalt ions to detect the higher polymer molecular size.

Table (16): Agarose-acrylamide gels composition used in separation of polymerized protein-melanin.

Component	Reagents and quantity
3.75% Acrylamide-0.7% agarose (20 ml)	140 mg (0.7 %) agarose boiled and cooled to 55 °C with stirring 9.1 ml H_2O 2.5 ml 30% Acrylamide, 0.8% N,N-bis-acrylamide 6.0 ml Tris-HCl pH 8.0 2.3 ml Glycerol 10 µl SDS 10% 7.5 µl TEMED 75 µl 10% APS
1.5% Acrylamide-0.7% Agarose (20 ml)	140 mg (0.7 %) Agarose boiled and cooled to 55 °C with stirring 11.4 ml H_2O 1 ml 30% Acrylamide, 0.8% N,N-bis-acrylamide 6.0 ml Tris-HCl pH 8.0 10 µl SDS 10% 7.5 µl TEMED 75 µl 10% APS
Cathode buffer 10X (500 ml)	60.5 g Tris, 89.6 g Tricine and 0.5 g SDS in H_2O having final volume 500 ml pH 8.25
Anode buffer 10X (500 ml)	121 g Tris in H_2O having final volume 500 ml pH 8.9

5. RESULTS

5.1 Total Protein in the Media of M9- ASP and Water on the Surface of the cuticle

5.1.1 SDS PAGE

The produced melano protein by *V. inaequalis* one M9-Asp medium and water culture was submitted to the SDS PAGE, there are more than 12 bands; the most prominant band has a molecular size of 36 kD (Figure 7 A). Also the time course of the protein production on floated cuticle discs on the surface of broth M9 medium and water was plotted (Figure 7 B).

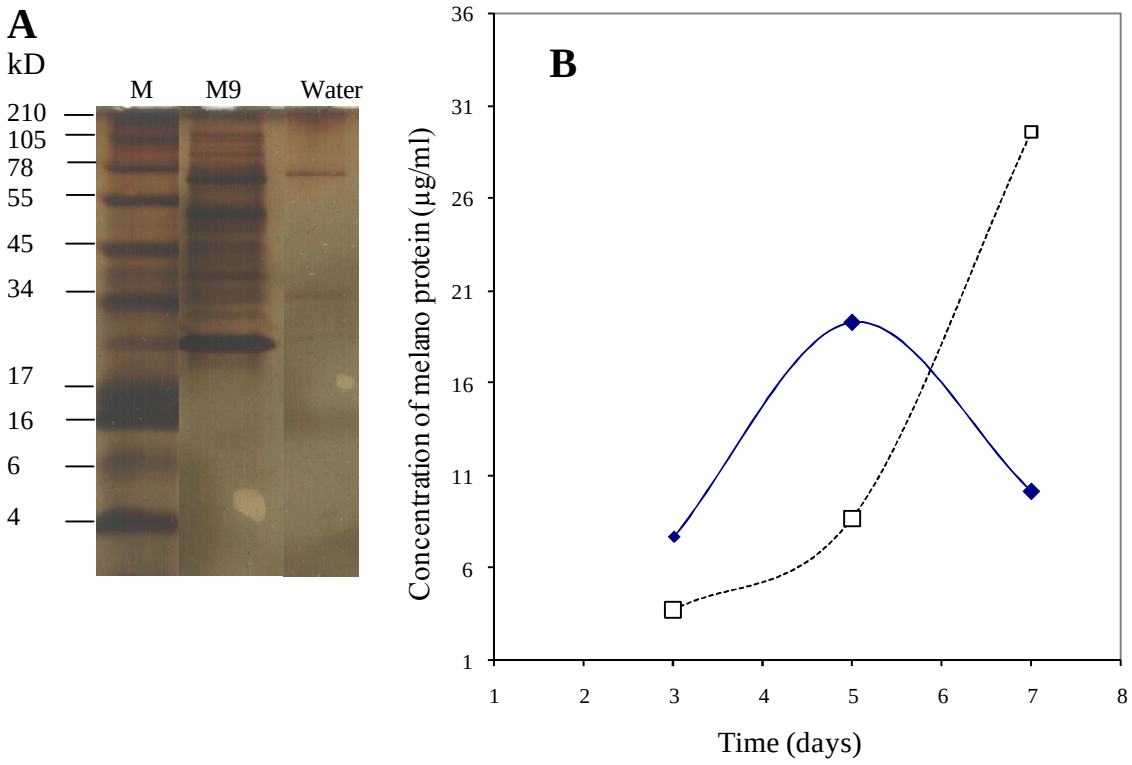


Figure (7): Total secreted protein (B) was measured of grown *V. inaequalis* on M9-Asp and water culture media at 20 ºC. Dashed line signed for M9-Asp connected line signed for water culture medium. Protein concentration was determined by Bradford (1976) method. A: 9% Tris-Tricine-PAGE for *V. inaequalis* extracellular proteins separation obtained from M9-Asp medium and water culture media at 20 ºC on the surface of isolated cuticle for *M. domestica*.

The separation of the extracellular fluid from *V. inaequalis* on 2D gel revealed a single proteins spot at on isolectric point of 5.2 having on appearant molecular mass of 36 kD (Figure 8)

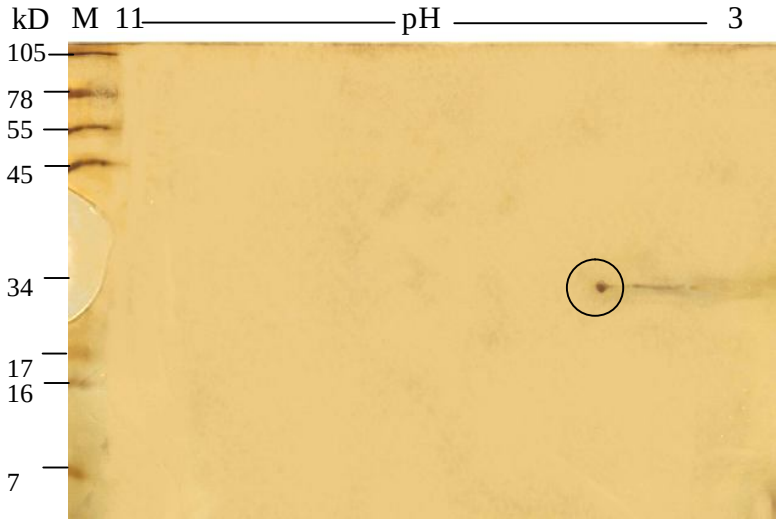


Figure (8): 2D PAGE for *V. inaerqualis* melano protein. It was the dominant protein secreted by the fungus in the medium and has MW of 36 kD.

The incubation of the purified melano protein of *V. inaequalis* in presence of the substrate glucan (distributed in agarose) showed (Figure 9) after staining with congo red the decrease of glucan polymers around the samples containing the melano protein implying the presence of a glucanase activity.

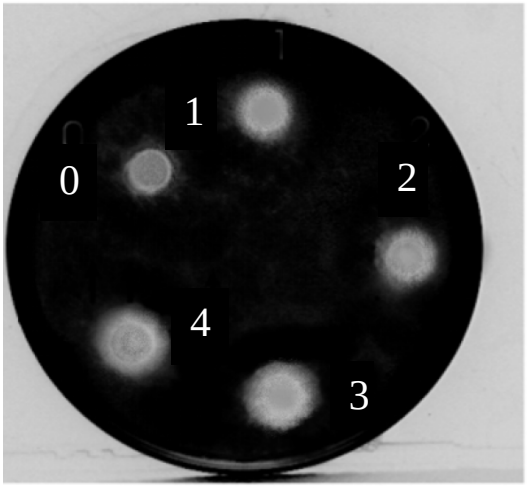


Figure (9): Determination of glucanase activity of the isolated melano protein from PDB medium of the grown fungus *V. inaequalis* on 2% agarose plate containing 0.5% glucan (crud glucan extracted from Mushroom). Hole zero was control filled with PDB medium as a control, hole 1 filled with 3 µg from standard glucanase, hole 2 was filled 5 µg of melano protein, and holes 3 and 4 were filled with 10 µg and 15µg of melano protein from *V. inaequalis*, respectively. The assay incubated at 37 ℃ for overnight and stained by Congo red stain for 30 min and destained with 1 M NaCl.

5.1.2 Determination of the glucanase activity on TLC for the crude melano protein

The hydrolyzed glucan product was investigated on TLC plates (Figure 10) for both the positive control (A) and the melano protein (B) control. The time interval hydrolysis series were spotted on the frontline. After dryness the sample were submitted to the running on the TLC. It is clear that, the intensity of the glucose product increase with the time.

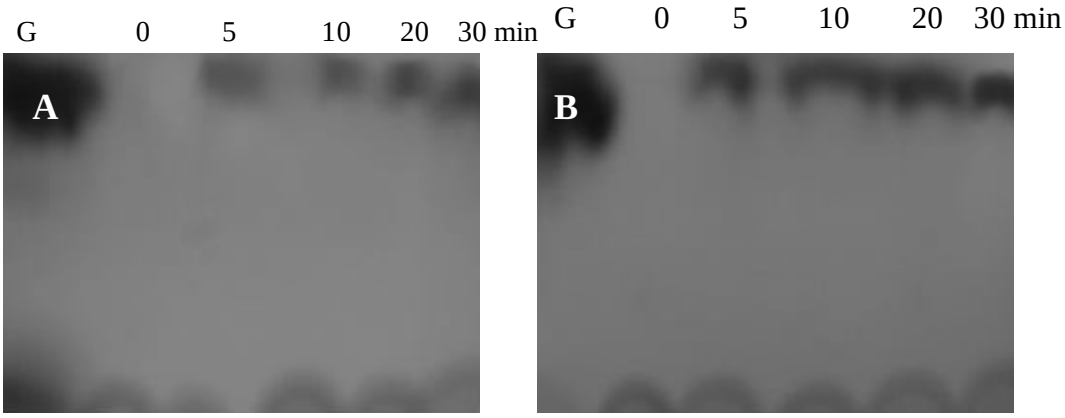


Figure (10): Thin layer chromatography of the hydrolyzed glucan using standard glucanase (glucanex: A, 0.2µg/µl) and the melano protein (B, 0.2 µg/µl) purified from *V. inaequalis* with time intervals 0, 5, 10, 20, and 30 min. Glucose (G) was used as reference for the produced monosaccharide. The protein concentration was measured by Bradford method (1976).

5.2 Isolation of cDNA of *V. inaequalis* melano protein by RT-PCR

5.2.1 Isolation of total RNA from

Agarose gel (Figure 11) shows total RNA separation extracted from of *V. inaequalis* mycelia, which was grown for seven days on PDA medium. Beside ribosomal RNA bands also mRNA could be observed indicated as smear on RNA gel.

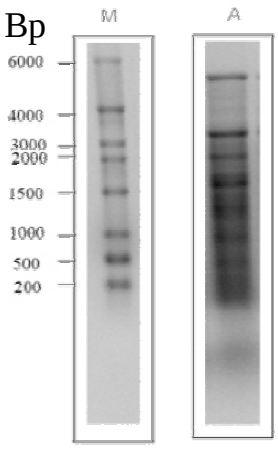


Figure (11): 1.5% Agarose gel of total RNA isolated from the mycelia of *V. inaequalis*. Each lane was loaded with 5 µg RNA. M is high molecular size marker.

5.2.2 Cloning and sequencing

The RT-PCR for the isolated RNA from the *V. inaequalis* were done by using three sets of primers. The PCR produce three bands (see Fig. 12, lanes: 1, 2, and 3) having a size of 750 bp, 1000 bp, and 1100 bp (Figure 12), respectively.

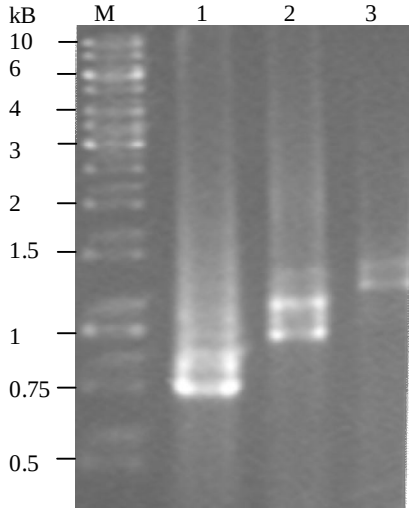


Figure (12): 1% Agarose gel for the RT-PCR product with three sets of primer pairs, internal (lane 1), full-length (lane 2) and outer primer (lane 3) primers, the items present in samples 1, 2, and 3 have a molecular size 0.750, 1, and 1.2 kB, respectively. M is the marker (1 kB; 1.5 µg was loaded), 5 µl were loaded per lane from the PCR product.

The PCR products were cloned into the pGEMT vectors. After isolation of the corresponding plasmids and restriction digestion fragments with a size of 700, 1000, and 1100 bp were obtained, sequenced, and assembled.

5.2.3 Melano protein cDNA sequence and Ms Hits finding

Melano protein cDNA sequence was obtained by sequences assembly integrated fragments into pGEMT vectors (Figure 13). The *V. inaeqialis* melano protein cDNA of the obtained sequence has 981 bp length. The translated cDNA of the melano protein has 327 amino acid and predicted molecular size of 34.051 kD. Amino acid sequence that showed homology to peptide sequences of the melano protein from Singh *et al.*, (2005) and peptide sequence of the *AVR* protein from Win *et al.*, (2003).

```
atgttcgcaaaacttctcgccctggcagcattgcctgctgcttccaacgcatttggagtc
 M  F  A  K  L  L  A  L  A  A  L  P  A  A  S  N  A  F  G  V
ctcaagggtttcaactacggctcgactgatgcctctggagttgtcaaggaccaggcccgt
```

```
 L  K  G  F  N  Y  G  S  T  D  A  S  G  V  V  K  D  Q  A  R
ttcgagcaagaattcagcaccgctcagaacttggttggaaccagtggattcaacagtgcc
 F  E  Q  E  F  S  T  A  Q  N  L  V  G  T  S  G  F  N  S  A
agactatacacaacaatccaaggaggaaccacgaatagcccaatctcagcaattccagct
 R  L  Y  T  T  I  Q  G  G  T  T  N  S  P  I  S  A  I  P  A
gccatcagcacccagacctctctccttctcggaatctggacatccgcaggccaagccatt
 A  I  S  T  Q  T  S  L  L  L  G  I  W  T  S  A  G  Q  A  I
gtggacaacgagattgcggcattgaaggctgctatcagtcagtacggcacagctttcact
 V  D  N  E  I  A  A  L  K  A  A  I  S  Q  Y  G  T  A  F  T
gacctgattgttgccgtttctgttggttctgaggatctttaccgtaactctggatаccca
 D  L  I  V  A  V  S  V  G  S  E  D  L  Y  R  N  S  G  Y  P
ggagcttcagaccctggtccaggtgccaatcctgatgttcttgccaactacattggacaa
 G  A  S  D  P  G  P  G  A  N  P  D  V  L  A  N  Y  I  G  Q
gtcaaggcggccattgctggcacatctgcggagggacgactggtcggacatgtggatacc
 V  K  A  A  I  A  G  T  S  A  E  G  R  L  V  G  H  V  D  T
tggactgccttcgtcaacagctccaacaacgcactcatctccgccgccgacttcttgggt
 W  T  A  F  V  N  S  S  N  N  A  L  I  S  A  A  D  F  L  G
gttgatgcctacccatactacgagtctgccaacggaaacgacatcagtaacgcagccaac
 V  D  A  Y  P  Y  Y  E  S  A  N  G  N  D  I  S  N  A  A  N
ttgttcgcaagcgcatactcccaggtcgttgctgtggcacaaggaaaaccagtctgggtt
 L  F  A  S  A  Y  S  Q  V  V  A  V  A  Q  G  K  P  V  W  V
accgaggctggatggccagtttctggaccaacagtggcacaagctgtagcttcccctgaa
 T  E  A  G  W  P  V  S  G  P  T  V  A  Q  A  V  A  S  P  E
aatgcccgctcattctggacctccgttggctgcaaccaacttttcgataagatcaatgtc
 N  A  R  S  F  W  T  S  V  G  C  N  Q  L  F  D  K  I  N  V
tggtggttccaattggatgactaccccacttcacccaaccctgcgttcggtgttataggc
 W  W  F  Q  L  D  D  Y  P  T  S  P  N  P  A  F  G  V  I  G
acagccttttccaccactcctctgttcgatctttcctgccctgctaccacgaagtctcgt
 T  A  F  S  T  T  P  L  F  D  L  S  C  P  A  T  T  K  S  R
Aggagatccaaccgcgcagcc
 R  R  S  N  R  A  A
```

Figure (13): Nucleotide sequence of the melano protein cDNA of *V. inaequalis* including the amino acid sequence. The gray backgrounds litters showed homology to peptides from Singh *et al.,* (2005) and underline homologous peptide of *AVR* from Win *et al.,* (2003).

5.2.4 Homology search of the protein sequence

The obtained melano protein sequence shares similarity to glycol_hydro_17 superfamily (E value = 6-24, Figure 14). The protein contains similar sequence to the conserved active site motif of *Saccharomyces cerevisiae* glucosidase [LI**V**M]-x-[LI**V**MFY**W**A](3)-[S**T**AG]-**E**-[ST**A**]-**G**-**W**-**P**-[STN]-x-[SA**G**Q] (sequences of the protein are in bold), with conserved glutamic acid residue in the active site region (PROSITE (Hofmann *et al.*, 1999)).

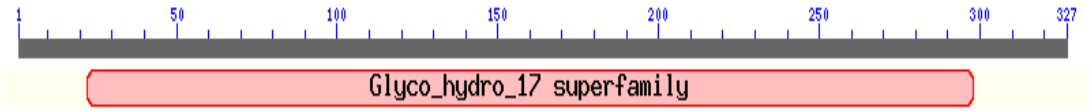


Figure (14): Graphic of the melano protein homology domain with the most similar super family (Glyco_hydro_17).

5.2.5 Multiple sequence alignment of the melano protein amino acid sequence of *Venturia inaequalis*

The relationship between *V. inaequalis* melano protein sequence and the amino acids of the other similar protein for different species was founded through determination of the distance between the species based on the homology search. The phylogenetic tree was built by using multiple alignment program ClustalW (Figure 15). From this analysis it can be concluded that the vast majority of these species is from fungi and one species is from plant and all of them are glucanase.

```
V. inaequalis       -----MFAKLLALAALPAASNAFGVLKGFNYGSTDASGVVKDQARFEQEFSTAQNLVGTS 55
M. domestica        -----MFAKLLALAALPAASNAFGVLKGFNYGSTDASGVVKDQARFEQEFSTAQNLVGTS 55
S. sclerotiorum     ----MLSKTLFAIVASLSTASAV--HQGFNYGATNADGSYRVQSDFQAAFEAANSLVGTS 54
P. tritici          ---MRISSTLFATTALLSSANAA--IKGFNYGAQFNNNQAKQQVDFEYEFNAAKQLPGTN 55
A. flavus           ----MQLTHLLAFALSLATSEAA--YKGFNYGATKSDGSVKSQSDFESEFSTAKNLVGTS 54
A. niger           MLSKMQLAQLAAFAMTLATSEAA--YQGFNYGNKFSDESSKFQADFEAEFKAAKNLVGTS 58
A. nidulans         ----MQLTQLLALALSLATSEAA--YKGFNYGDKKPDGSSKYQADFASEFETAQNLVGAP 54
A. terreus          ---MFTKTQILALALSIASAEAV--SKGFNYGANKPDGTLKVQADFEAEFRTAKNLETTS 55
P. chrysogenum      ----MRSAQFLALAMAVATSDAI--SQGFNYGALKVDGSLKTQADFETEFATAKNLVGTD 54
P. nodorum          -----MWAKAASILALASTAVAQ--SKGFNYGATNADGSCRGYNDFVRFFNEAKSLPGAS 53
G. graminicola      ---MKASISLA-ALTALAATAEAQNWLGFNSGATKDDRSAKFKADFEAEFKTAQNLVGAP 56
G. zeae             ---MKVSSAAV-AVGALAMGAEAKNYLGFNSGATLANREAKFKADFQAEFETAQNLKTSP 56
N. crassa           ---MRATELLAGAAALLASGVSAEAYLGFNSGNTLPSREAKFKKDWVQEFTTAQNLKNSP 57
                                  :         *** *    .   :    :   *  *:.*  :

V. inaequalis       -GFNSARLYTTIQGGTTN-SPISAIPAAISTQTSLLLGIWTSAGQAIVDNEIAALKAAIS 113
M. domestica        -GFNSARLYTTIQGGTTN-SPISAIPAAISTQTSLLLGIWTSAGQAIVDNEIAALKAAIS 113
S. sclerotiorum     -GFTSARLYTMLQGSTTG--PTSAIPAAIASDTTLLLGLWASGGAAGFAAEVNALKTAIT 111
P. tritici          -GWTSARLYTMIQHGTQN-SIISAIEAAINTKTTLLLGLWCSAGQNGVNNEITALKAAIA 113
A. flavus           -GFTSARLYTMIQGGTTN-SPISAIPAAIAENTSLLLGLWASGG--GMDNELAALRSAIS 110
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A. niger           -GFTSARLYTMIQAYSTS-DVIEAIPAAIAQDTSLLLGLWASGG--GMDNEITALKTAIS 114
A. nidulans        -GFTSARLYTMIQAGTAN-DPISAIPAAIAQNTSLLLGLWASGN--NMNNELTALKAAIS 110
A. terreus         -GFNSARLYTMIQG-TGS-TPISAIPAAIAEETTLLLGLWASGG--NMDNEIAALKAAIN 110
P. chrysogenum     NAFTSARLYTMIQGGTTN-GPIEAIPAAIKEKTTLLLGLWASGG--DMSNEIAALTSAIN 111
P. nodorum         -GFTTARLYTSIQCGTAS-DPISAFQAAIDTDTKVLVGLWASAGRGAYENELNALIRGAR 111
G. graminicola     GNFNAVRLYTNIQAYSTD-DPIQAFEAAIETQTHILLGVWASGTD-NIDKEISALKKAVT 114
G. zeae            GDFNAVRLYTNIQAYSQD-DPIEAFEAAIDTKTQILLGVWTSGTD-NIDNEISALKKAVE 114
N. crassa          GVFNAVRLYTNIQAYSQTSEPIEAFEAAIETNTKILLGVWASGTN-TIEPEIKALQNGIA 116
                     :.:.**** :*  :      .*: ***  .* :*:*:* *.       *: **  .
V. inaequalis      QYGTAFTDLIVAVSVGSEDLYRNSGYPGASDPGPGANPDVLANYIGQVKAAIAGTSAEGR 173
M. domestica       QYGTAFTDLIVAVSVGSEDLYRNSGYPGASDPGPGANPDVLANYIGQVKAAIAGTSAEGR 173
S. sclerotiorum    QYGSAFTSRVVGISVGSEDLYRNSPTGIAAKAGYGANPADLVDYIKQVRDAISGTALSGA 171
P. tritici         KYGTRFTDLVVGISVGSEDLYRVTPTGIDNNSGPGAQPQELVKYIQQTRAAIQGTPLQGK 173
A. flavus          QYGDSFAKLVVGISVGSEDLYRASSEGEKVNAGIGIGPDDLVSFIKEVRSIISGTALSSV 170
A. niger           QYGEELGKLVVGISVGSEDLYRNSVEGAEADAGVGVNPDELVEYIKEVRSVIAGTALADV 174
A. nidulans        QYGEDLSKLVVGISVGSEDLYRNSVLGQKVNAGVGVDPHVLASYIEEVRSTISGTPLSGA 170
A. terreus         QYGDEFAKLVVGISVGSEDLYRNSEIGVQANAGIGIEPEELVSYIQRVREAIAGTALSGA 170
P. chrysogenum     TYGEDFTNLVVGISVGSEDLYRVSETGVKANAGLGVGPTELVDYINQVRSAISGTSLSGA 171
P. nodorum         ELGSAFTDRIIGISVGSEDLYRSSPQGVANNAGPGATGPEIEGYIGWMRDWIRGSALESK 171
G. graminicola     QYGTKLTDLIIGASIGSEDLYRNSVTGVTNKSGVGADPETIVGFINDFKTAFKDTPLSKV 174
G. zeae            KYGSKLTDLVIGVSVGSEDLYRNSVTGVKNKGGVGVQPDALVDFIDDFRSAFKGTPIAKV 174
N. crassa          KYGKKLTDLIIGASIGSEDLYRVSVTGIQNKSGVGAGPAELVKFIADWKKAFQGTAIANV 176
                     *  : . ::. *:******* :      . * *     :  :*   :  : .:.
V. inaequalis      LVGHVDTWTAFVNSSNNALISAADFLGVDAYPYYESANGNDISNAANLFASAYSQVVAVA 233
M. domestica       LIGHVDTWTAFVNSSNNALISAADFLGVDAYPYYESANGNDISNAANLFASAYSQVVAVA 233
S. sclerotiorum    SIGHVDTWTAWVNGSNNAVAEALDWVGMDAYPYFQDSEANGVSEGKSLFNAALAATQSAT 231
P. tritici         PIGHVDTWTVYVNASNNAVIDAVDFIGMDAYPYFQTTMNNNVGSGSQLFFDAYHQTVAAA 233
A. flavus          PIGHVDTWTAWTNGSNSAVIDAVDWLGFDGYPYFQSSIS------------------AA 211
A. niger           SIGHVDTWDSWTNSSNSAVVEAVDWLGFDGYPFFQSSMANSIDNAKTLFEESVAKTKAVA 234
A. nidulans        PLGHVDTWNDWVNGSNAAVIDAVDWVGFDGYPYFQNTMANSIDDAKALFNEAVAKTKSAA 230
A. terreus         PIGHVDTWNAWTNGSNAAVAEAVDWLGFDGYPFFQNTMQNSIDDAKALFDESVQKTKAVA 230
P. chrysogenum     PIGHVDTWNSWTNGSNSAVIEAVDWLGFDGYPYYENTDPNSIDDAKALFDKGVEKTKAVA 231
P. nodorum         PIGHVDTFTAWVLPENRGVANTVNWLGHNSFPYFETTRPNAIEQARENFYAGLGQTEAVS 231
G. graminicola     SIGHVDTWDVWGNATNKPVLDAIDWVGVDEYPYYENGKGNSIENAGSLFDKAYAATVATA 234
G. zeae            PLGHVDTWDVWGNVTNKPVLDAIDFIGVDEYPYYENGKGNSIDNAAKLFNKAYDATVATS 234
N. crassa          PIGHVDTWDAWTNGTNKPVIDAVDWVGVDEYPYYENGKGNNIENSGYLFDRAYDAIEGAV 236
                    :*****:  :    *  : .: :::* : :*:::                       .
V. inaequalis      QGKPVWVTEAGWPVSGPTVAQAVASPENARSFWTSVGCNQLFDKINVWWFQLDDYPTSPN 293
M. x domestica     QGKPVWVTEAGWPVSGPTVAQAVASPENARSFWTSVGCNQLFDKINVWWFQLDDYPTSPN 293
S. sclerotiorum    -GKTVWVTETGWPVTGATAGDGIPSAANAKTYWDDVGCPNFG-KINMYWYTLQDQNAAGS 289
P. tritici         KGKPVWVTETGWPVTGQTLNQGVASANNAKIFWDDVTCQLVKDNVNLWYYILQDVQYG-N 292
A. flavus          G------------SATPVGSAVP----------------------------SSSAAV 228
A. niger           GDKEVWITETGWPVSGDSQGDAVASIANAKTFWDEVGCPLFG-NVNTWWYILQDASPTTP 293
A. nidulans        GNKEVWITETGWPVSGKTENLAVASIPNAKRFWDEVGCPLFD-NTNTWWYTLQDAFGASV 289
A. terreus         GNKEVWITETGWPVSGDSQNLAIASVENAKQFWDEVGCPLFD-NVNTWWYILQDASGSSV 289
P. chrysogenum     GGKEVWITETGWPVTGPTENLAVANTANAKQYWDEVACPLLG-NTNTWWYILEDAGTTAP 290
P. nodorum         GGKPVWVTETGWPHKGPVSGSAVASLENSRRYYSEVGCSLFG-TRNVFWYTLKDANSAQT 290
G. graminicola     GGKPVWVTETGWPVTGENWDQAVPSPENAKIYWDEVGCRKLFNKVPTFWYNLRDSNPDNT 294
G. zeae            GGKPVWVTETGWPYKGPDWDEAVPSVKNAQKYWQDVGCKSLFNKVPTFWYNLRDSNPDNK 294
N. crassa          GGKPIWVTETGWPYVGQTWDQAAATIKNQQYYWQEVGCRKLFGKVPTFWYNLRDSNPDNE 296
                                 .     . .
V. inaequalis      --PAFGVI--GTAFSTTPLFDLSCPATTKSRRRSNRAA---------------------- 327
M. domestica       --PAFGVI--GTAFSTTPLFDLSCPATTKSRRRSNRAA---------------------- 327
S. sclerotiorum    VTPSFGIV--GSTLSTTPLFDLSCSNTTSSSSSTASASSKPTS-----------ALASSL 336
P. tritici         PVPSFGIKPAGDLMQVSPLFDLSCPK---------------------------------- 318
A. flavus          -NP----------------------SSSGIVSSAAPS-TTPG------------FTVG-K 251
A. niger           -NPSFGIV--GSTLSTTPLFDLSCKNSTTSSSSAVVS-AAAS------------SAAGSK 337
A. nidulans        PNPSFGIV--GSTLTTQPLFDLSCSKSNTTSSSAIASPTSTA------------AAAGGV 335
A. terreus         PNPSFGIV--GNTLSTTPLFDLSCSASSKKNSSSASASASGS------------SAQSTG 335
P. chrysogenum     ---SFGVT--GSSTDTTPLYDLSCKASTSSSSAAASSSTSASGAGVAEKSGFVSSATSAS 345
P. nodorum         -DLSFALT---PLENNTPYFDLKC----------------------------------- 310
G. graminicola     --MKFAIT---ENLSTTPQFNLTCP----------------------------TH---- 316
```

```
G. zeae            --MKFAIT---ENLSTTPLFDLSCDKVDDETSSSAESKTKTS-----------THASKA 337
N. crassa          --MKFAIT---DNLSTTPHFDLTCP---------------K-----------TFKTTP 323

V. inaequalis       ------------------------------------------------------------
M. domestica        ------------------------------------------------------------
S. sclerotiorum    ASAATGGSVASTGSGLSPSGMGAGISSSAVSG-------------AAAPTGTGSAG--- 379
P. tritici         ------------------------------------------------------------
A. flavus          GFRPSNSSAAAYYSSASASG---SAYPKFTK-----------------TASGSSATS--- 288
A. niger           AVGSSQASSGAAAWATSASG---SAKPTFTVGRPGVNGTVFGNGTYPLRPSGSASAR--- 391
A. nidulans        AGGSTGSASGSSTGTGSSSG---SGSNSNTGAASGAVG------AADRETSGTSGSA--- 383
A. terreus         FVSTTKPAASPSGSSGLGHGGSLGSSGSFSGGHYAGVGSSSVIASPSATPSGSAVPG--- 392
P. chrysogenum     PTGSAGSDASSSDSGASSSG-SGSGSTSGSGSGSGSGSASSPAGSSSVRPTSAAVTSGVR 404
P. nodorum         ------------------------------------------------------------
G. graminicola     ------------------------------------------------------------
G. zeae            TGDSHSTGTFVTATASATSGSSDSEDSTATG---------------TGSSSKPTSGSGS 381
N. crassa          -G---------------SSSSASATSSATG---------------TGVSSPS------ 345

V. inaequalis      ------------------------------------------------------------
M. x domestica     ------------------------------------------------------------
S. sclerotiorum    -SSSGGSSNGTYTASTLR-------------SASGSSATG-------TSTSGSSGSGSS 417
P. tritici         ------------------------------------------------------------
A. flavus          -TSAG---SSSDSSSTN--------------SGKSSSDSS-------STNSGASASSS- 321
A. niger           -PSAGAISSGSGSSSSG--------------SGSSGSTGT-------SATSGQSSSSG- 427
A. nidulans        -NTNGTSGSGSGSNSTSGHGSNVTVPTRPTSVSNVSPSKSSSALFTGAATSMGASPSSVG 442
A. terreus         -SSSGPGSSSGSASGSS--------------SGFGSGAAA-------DSTSGTSTSGD- 428
P. chrysogenum     PSTKPKSKSASASASASG-------------AASSSASGSSATATGGSNSGSGSSASGSG 451
P. nodorum         ------------------------------------------------------------
G. graminicola     ------------------------------------------------------------
G. zeae            GSSSESGSSGSGDASESG-------------SSGSGSSGSS--------GSGSSSESGSK 420
N. crassa          -------------SMESG-------------SSGSGSSGSA--------SSGTASSSEPA 371

V. inaequalis       -------------------------------------------
M. domestica        -------------------------------------------
S. sclerotiorum     S------GSSGSSSSPSSSTGGAASLTGSVALALGAVFAVAAAL 455
P. tritici          -------------------------------------------
A. flavus           -------SILATG--------GASSVSGSVFGALVAVFAFVATL 350
A. niger            -------SSAAAGSSSPAAFSGASTLSGSLFGAVVAVFMTLAAL 464
A. nidulans         NVGPSKSSGAASPSSTTMFTGAATSVSAPVVHVVLLALMMVIAA 486
A. terreus          -------STSSTSATPADFTGAGSRLSGSIFGAAMLVAALAVAL 465
P. chrysogenum      S-GSGSSSSDSASASPSLPVSSATRVAGSAAGALVAALALAFTF 494
P. nodorum          -------------------------------------------
G. graminicola      -------------------------------------------
G. zeae             SEAGSSSETGAAAAETPSTVNGAAGLTFSAAALAFFAMLAL--- 461
N. crassa           TVTAPAETAVATGSATSVKGVSAAAVAGMTLLVGVFAML----- 410
```

Figure (15): Multiple alignment of the amino acid sequence of the melano protein sequence of *V. inaequalis* (clone 36) with plant and fungal species which have high similarities. Asterisk means conservative residue; colon means conservative with small changes. *M. x domestica* EG631228; *Aspergillus flavus* NRRL3357; *Aspergillus niger* CBS 513.88; *Aspergillus nidulans* FGSC A4; *Aspergillus terreus* NIH2624; *Penicillium chrysogenum* Wisconsin 54-255; *Sclerotinia sclerotiorum* 1980; *Glomerella graminicola* partial; *Gibberella zeae* PH-1; *Neurospora crassa* OR74A; *Pyrenophora tritici*-repentis Pt-1C-BFP; *Phaeosphaeria nodorum* SN15

The phylogenetic tree of different plant and fungal species based on the amino acid sequence of melano protein showed that these species are classified into three major groups. The first group classified into two subgroups includes *V. inaequalis* (clone 36) and *M. x domestica* EG631228

where they are identical together and these two are related to *P. nodorum, G. graminicola, N. crassa* OR74A, *P. nodorum* SN15 and *G. zeae* PH-1. The mentioned subgroub is related to *A. nidulans* FGSC A4, *A. flavus* NRRL3357, *A. niger* CBS 513.88, *A. terrues* NIH2624 and *P. chrysogenum* Wisconsin 54-255. The second group includes *S. sclerotiorum* 1980. The last group also includes *P. tritici*-repentis Pt-1C-BFP (Figure 16).

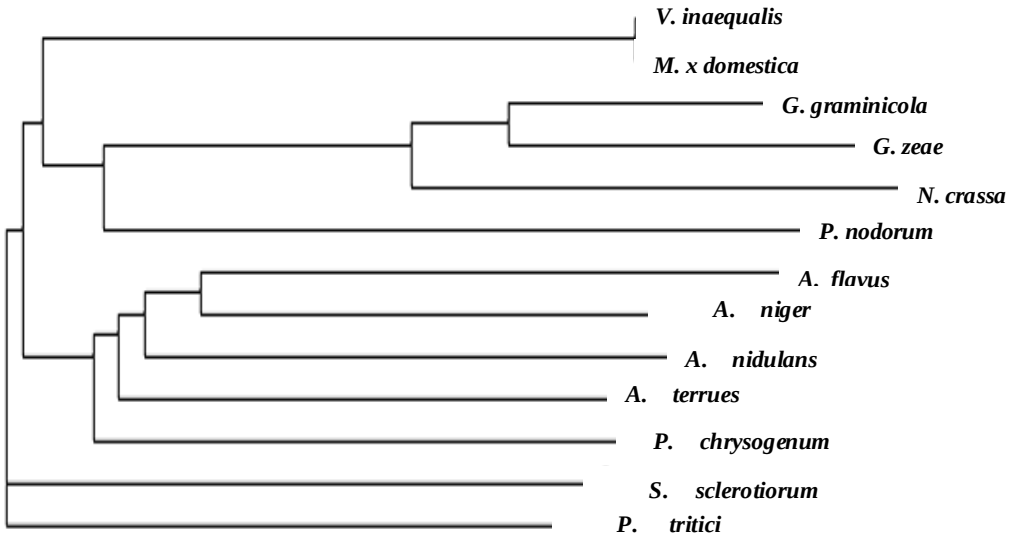

Figure (16): Phylogenetic tree of the amino acid sequences of melano protein of *V. inaequalis* within different plant and fungal species depending on the multiple alignment of the amino acid sequence, using ClustalW method (Phylip).

5.3 Prediction analysis

Some prediction analyses for the melano protein ORF were done to help in this study (Table 17).

Table (17): Functional amino acid sequence prediction for the melano protein from *V. inaequalis*

Function	Position Amino acid residue	Residue	Probability	Bioinformatic tool
Target sequence				
Signal sequence	18-19	MFAKLLALAALPAASNAF	0.8 of 1	SignalP
Putative functional amino acid residues				
Tyrosine sulfo site	288	DD**Y**PTS	0.57	Sulfosite
Phosphorylation site				
Serine site	32	STDA**S**GVVK	0.511	NetPhos 2.0 Server
	76	NSPI**S**AIPA	0.972	

	113	KAAI**S**QYGT	0.839	
	127	IVAV**S**VGSE	0.795	
	137	LYRN**S**GYPG	0.730	
	169	IAGT**S**AEGR	0.565	
	258	QAVA**S**PENA	0.996	
	291	DYPT**S**PNPA	0.615	
	304	GTAF**S**TTPL	0.501	
	319	ATTK**S**RRR**S**	0.833	
	323	SRRR**S**NRAA	0.997	
Threonine site	316	SCPA**TT**KSR	0.521	
	317	CPA**TT**KSRR	0.690	
Tyrosine site	134	SEDL**Y**RNSG	0.991	
	207	AYPY**Y**ESAN	0.842	
	288	QLDD**Y**PTSP	0.759	
Glucanase site	25- 298	[LI**V**M]-x-[LI**V**MFY**W**A](3)-[S**T**AG]-**E**-[ST**A**]-**G**-**W**-**P**-[STN]-x-[SA**G**Q]		PROSITE
Protease site	32-33	SG		Homology to fungal proteases catalytic residues
	55-56	SG		
	137/138	SG		
	248/249	SG		
	159/160	DT		
Metal binding site	156-160	HVD		
Glycosylation	186	**N**SSN	0.5127 of 1	Asn-Xaa-Ser/Thr sequons
Net glycation sites	4	FA**K**LL	0.94	Net glycation tools (Expasy tools)
	22	VL**K**GF	0.834	
	36	VV**K**DQ	0.889	
	236	QG**K**PV	0.906	
	277	LFD**K**IN	0.902 of 1	
Additional analysis related to the structure and the isolectric point				
Disulfide bond	271 - 334	**C**NQLFDKINVWWFQLDDYPTSNP AFGVIGTAFSTTPLFDLS**C**	0.93	Disulfide
Chains in the Melano protein sequence	Alpha helix	LLALAALPAASNAF-VVKDQARFEQEFSTA-ISTQT-QAIVDNEIAALKAAISQ-VLANYIGQVKAAIA-ALISAADF-	27.22%	GOR4 program (Expasy tools)

		ISNAANLFASAYSQVV		
	Extended strand	VLKG-RLYTTI-SLLL-DLIVAV -RLV-TWTAFV-YY-AVA-WVTEA -SFWT-QLFDK-VWWFQL-VIG-RA	18.34%	
	Random coils	MFAK-G-FNYGSTDASG-QNLVGTSGFNSA-QGGTTNSPISAIPAA-GIWTSAG-GTSAEG-GHVD-NSSNN-LGVDAYP-ESANGND-QGKP-GWPVSGPTVAQAVASPENAR-SVGCN-IN-DDYPTSPNPAFG-TAFSTTPLFDLSCPATTKSRRRSN-A	54.43%	
Hydrophobicity	32 hydrophobic residue			Mobyle (Expasy tools)
Isolectric point	4.7			pI/Mw program (Expasy tools)
3D structure				Cancerresearch uk (Expasy tools)

5.4 Analysis of the melanoprotein ORF

The nucleotide sequence length of the ORF is 981 bp and the melano protein sequence is 327 amino acids (Figure 17). The predicted signal peptide (Dashed underline sequence) is for secretion, it shows motives related to different types of enzymes like glucanase, protease The two cysteine amino acids in the protein sequence (271 and 334) could make disulfide bond; the metal binding site (HVD) is present in the dashed box and is overlapped with one active motive for aspartyle protease (D-T). The glucanase active sites were predicted to be between 25 and 298 amino acids sequence. The protease sites is predicted to be aspartyle protease and related to the retroprotease with active motives D-T/S-G (light blue green background) in both N-terminal and C-terminal. The metal binding site is predicted to be closed to the protease active centre (HVD with the red box).

```
1    atgttcgcaaaacttctcgccctggcagcattgcctgctgcttccaacgcatttggagtc
1     M  F  A  K  L  L  A  L  A  A  L  P  A  A  S  N  A  F  G  V
65   ctcaagggtttcaactacggctcgactgatgcctctggagttgtcaaggaccaggcccgt
21    L  K  G  F  N  Y  G  S  T  D  A  S  G  V  V  K  D  Q  A  R
121  ttcgagcaagaattcagcaccgctcagaacttggttggaaccagtggattcaacagtgcc

41    F  E  Q  E  F  S  T  A  Q  N  L  V  G  T  S  G  F  N  S  A
```

```
181 agactatacacaacaatccaaggaggaaccacgaatagcccaatctcagcaattccagct
61   R  L  Y  T  T  I  Q  G  G  T  T  N  S  P  I  S  A  I  P  A
241 gccatcagcacccagacctctctccttctcggaatctggacatccgcaggccaagccatt
81   A  I  S  T  Q  T  S  L  L  L  G  I  W  T  S  A  G  Q  A  I
301 gtggacaacgagattgcggcattgaaggctgctatcagtcagtacggcacagctttcact
101  V  D  N  E  I  A  A  L  K  A  A  I  S  Q  Y  G  T  A  F  T
361 gacctgattgttgccgtttctgttggttctgaggatctttaccgtaactctggatаccca
121  D  L  I  V  A  V  S  V  G  S  E  D  L  Y  R  N  S  G  Y  P
421 ggagcttcagaccctggtccaggtgccaatcctgatgttcttgccaactacattggacaa
141  G  A  S  D  P  G  P  G  A  N  P  D  V  L  A  N  Y  I  G  Q
481 gtcaaggcggccattgctggcacatctgcggagggacgactggtcggacatgtggatacc
161  V  K  A  A  I  A  G  T  S  A  E  G  R  L  V  G  H  V  D  T
541 tggactgccttcgtcaacagctccaacaacgcactcatctccgccgccgacttcttgggt
181  W  T  A  F  V  N  S  S  N  N  A  L  I  S  A  A  D  F  L  G
601 gttgatgcctacccatactacgagtctgccaacggaaacgacatcagtaacgcagccaac
201  V  D  A  Y  P  Y  Y  E  S  A  N  G  N  D  I  S  N  A  A  N
661 ttgttcgcaagcgcatactcccaggtcgttgctgtggcacaaggaaaaccagtctgggtt
221  L  F  A  S  A  Y  S  Q  V  V  A  V  A  Q  G  K  P  V  W  V
721 accgaggctggatggccagtttctggaccaacagtggcacaagctgtagcttcccctgaa
241  T  E  A  G  W  P  V  S  G  P  T  V  A  Q  A  V  A  S  P  E
781 aatgcccgctcattctggacctccgttggctgcaaccaacttttcgataagatcaatgtc
261  N  A  R  S  F  W  T  S  V  G  C  N  Q  L  F  D  K  I  N  V
841 tggtggttccaattggatgactaccccacttcacccaaccctgcgttcggtgttataggc
301  W  W  F  Q  L  D  D  Y  P  T  S  P  N  P  A  F  G  V  I  G
901 acagccttttccaccactcctctgttcgatctttcctgccctgctaccacgaagtctcgt
321  T  A  F  S  T  T  P  L  F  D  L  S  C  P  A  T  T  K  S  R
961 aggagatccaaccgcgcagcc
341  R  R  S  N  R  A  A
```

Figure (17): Melano protein sequence analysis using the EXPASY tools and NCBI database, sequence is 981 bp translated to 327 amino acids, the dashed underline sequence is the predicted signal peptides for secretion. Two cysteine residues present in the protein sequence (271 and 334) could make disulfide bond (red color); the metal binding site is HVD in the red box (177-189) and is overlapped to the active motive for aspartyle protease (D-T). The glucanase activity site is predicted to be between 25 and 298 amino acid. The protease sites is predicted to be aspartyle protease and related to the retroprotease with active motives D-T/S-G in both N-terminal and C-terminal. The SG protease sites singed with the blue background.

5.5 Anaylsis of the complete Melano protein gene

In total DNA fragment of 2118 bps obtained (Figure 18) and summarized in figure 19. Upstream region sequence was derived from the SSSBT-PCR and recorded -851 bps lengths. Promoter prediction for eukaryotic sequences was done and recorded score 0.9-0.94 (using online tools)

and gives rise to the promoter regions (-723: -771, -545: -468 bps). The transcription position (transcription start shown in larger font). The analysis of the nucleic acid sequence indicates a putative start codon at position 852 bp (ATG, signed with black arrow) following by a coding sequence of 527 bps. At position 528 a class II interon could be predicted which ended at position 591 bp. Afterwards a contiguous sequence of 592:1028 bp encoding for the melano protein followed. At position 1029: 1131 bps a stop codon could be predicted.

-851 GATGGAAGCAGCAGGCAATTGCAGAGAATCCAATCTCATAATNTGCGCGCTGCGGTGGTA

-791 CCTTTCGTCGGCTGGAGAACCCACGGTCTATATTGACTGGCAGCTCTTCCGAGTCCATGC

-731 ATCACCAGCTGTGATCAGATCTTCGTCGCAAACATTGGAGAGTATCTAAGCCTTGAGAAT

-671 TGATACTCCGGGGGACATCTATCGTCTGCTCCAAGTGGCTAAACATTTTCCACAGCGACT

-611 GAGATAAGCTGGATACACCACGACCAGAAGTGTCCTCGCGCCCGACTCGTCCATTGAATC

-551 AAAGAATGCTACATCCATTTCGACGAAGACGATGGCATGCAGACACAATAACAGCCGCAC

-491 TGCGGACCGGAATCTTTGGACAAAGCCCTTAGCCAAATGCACCTTCATACAGACGATCAG

-431 GCGTGGAAGAAAGATACGGACGGGAGAGACATTGATGGAAGAAAGAGATGGTGGAAGTGC

-371 GAACGAGACAACGGCATGACTGTTTACGTGGCTCCATATGCAACTCAACGACAATGCCAA

-311 TTTCCCTAGTACGACAAAGACCCCAAGAAGGAAGCGTAGTGTGTTGAAAGCCCCTGCTGG

-251 CACACAAGAAAGGACCCAGACGCTATCCTACCCACACAACCGCCTTCTTCAGGACTCTAG

-191 GCTTGATGCTGTAGTCTGACCCATCCGATTTTCCTATAGCGACAATCGCTGGATATGTCA

-131 CGACCAGGATGTGATTCCCGTCAAGCATATTTGAATCCTCATACAATCTGTGTGCCTCCA

-71 TCATATCCGACGTATGATCGAAGGTTTCTGGGAGCTGCTGAATACCGTACAATAGAATGT

-11 GGCTCTTTTGGATGTTCGCAAAACTTCTCGCCCTGGCAGCATTGCCTGCTGCTTCCAACG

+50 CATTTGGAGTCCTCAAGGGTTTCAACTACGGCTCGACTGATGCCTCTGGAGTTGTCAAGG

+110 ACCAGGCCCGTTTCGAGCAGAATTCAGCACCGCTCAGAACTTGGTTGGAACCAGTGGATT

+170 CAACAGTGCCAGACTATACACAACAATCCAAGGAGGAACCACGAATAGCCCAATCTCAGC

+230 AATTCCAGCTGCCATCAGCACCCAGCCTCTCTCCTTCTCGGAATCTGGACATCCGCAGGC

+290 CAAGCCATTGTGGACAACGAGATTGCGGCATTGAAGGCTGCTATCAGTCAGTACGGCACA

+350 GCTTTCACTGACCTGATTGTTGCCGTTTCTGTGGTTCTGAGGATCTTTACCGTAACTCTG

+410 GATACCCAGGAGCTTCAGACCCTGGTCCAGGTGCCAATCCTGATGTTCTTGCCAACTACA

+470 TTGGACAAGTCAAGGCGGCCATTGCTGGCACATCTGCGGGGGACGACTGGTCGGACAT**GT**

+530 **GGATGTAAGATTTCTTCAACAACCTGATTAATTACTCTTGCCTAATTCTATCTCTAGACC**

+590 **T**GGACTGCCTTCGTCAACAGCTCCAACAACGCACTCATCTCCGCCGCCGACTTCTTGGGT

+650 GTTGATGCCTACCCATACTACGAGTCTGCCAACGGAAACACATCAGTAACGCAGCCAACT

```
+710  TGTTCGCAAGCGCATACTCCCAGGTCGTTGCTGTGGCACAAGGAAAACCAGTCTGGGTTA
+770  CCGAGGCTGGATGGCCAGTTTCTGGACCAACAGTGGCACAAGCTGTGCTTCCCCTGAAAA
+830  TGCCCGCTCATTCTGGACCTCCGTTGGCTGCAACCAACTTTTCGATAAGATCAATGTCTG
+890  GTGGTTCCAATTGGATGACTACCCCACTTCACCCAACCCTGCGTTCGGTGTTAAGGCACA
+950  GCCTTTTCCACCACTCCTCTGTTCGATCTTTCCTGCCCTGCTACCACGAAGTCTCGTAGG
+1010 AGATCCAACCGCGCAGCCTAGTTGCGCHGTCGKCCACAGGAMGCSACGYAGGCGCATCGT
+1070 CRWKCTSAMTGGCCCGTYACMCGGAWGYMTYGGTAAGGAGWTYYGAASYKYGSARMCTCC
+1130 TTTGAAATTGGTATCGCAAAAAGCATTGGATCATGTAACCTGCATTGGATGATGGGATGA
+1190 GACTGGCGTTTACCTATTGCATTCTTGTATCCTAGAATGGCTAACCCGTAATCATTAACT
+1250 CTATTGTTCTAAAAAAAA
```

Figure (18): Complete melano protein gene analysis (2118 bps). The upstream region -851 bps length and the gene including the intron and the downstream region was 1267 the predicted promoter regions were in positions -771:-723 and -517:-468 region (dashed underline). TATA position: -157, -763 (blue-green background), CAAT at the positions: -22,-88,-149,-320, -314, -505, -820 (gray background), CCCCT at the position: -261 (STRE site for stress response, green background), AGGCA at the position: -839 (AceI site for celobiohydrolyase induction in response to cellulose, Box). The intron region was +528: +591 (Double underline). Start codon (black arrow) was in position 1 and the stop codon (big black dot with red center) was +1031 with yellow background.

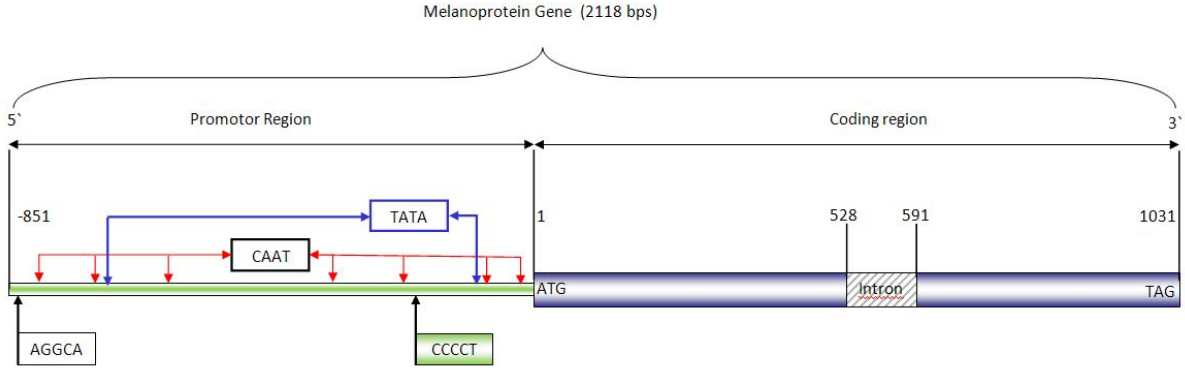

Figure (19): Promoter and melano protein gene structure and analysis diagram.

5.6 Southern blot

The single digestion (Figure 20) gave big DNA size fragments and that is related to methylation. Whereas the double or triple digestion (Figure 21) minimize the DNA fragment what gave the possibility to have more bands to know the copy number of the melano protein gene in genomic DNA of *V. inaequalis* which is five copies at least that what give the possibility of the melano protein to produce in high amount.

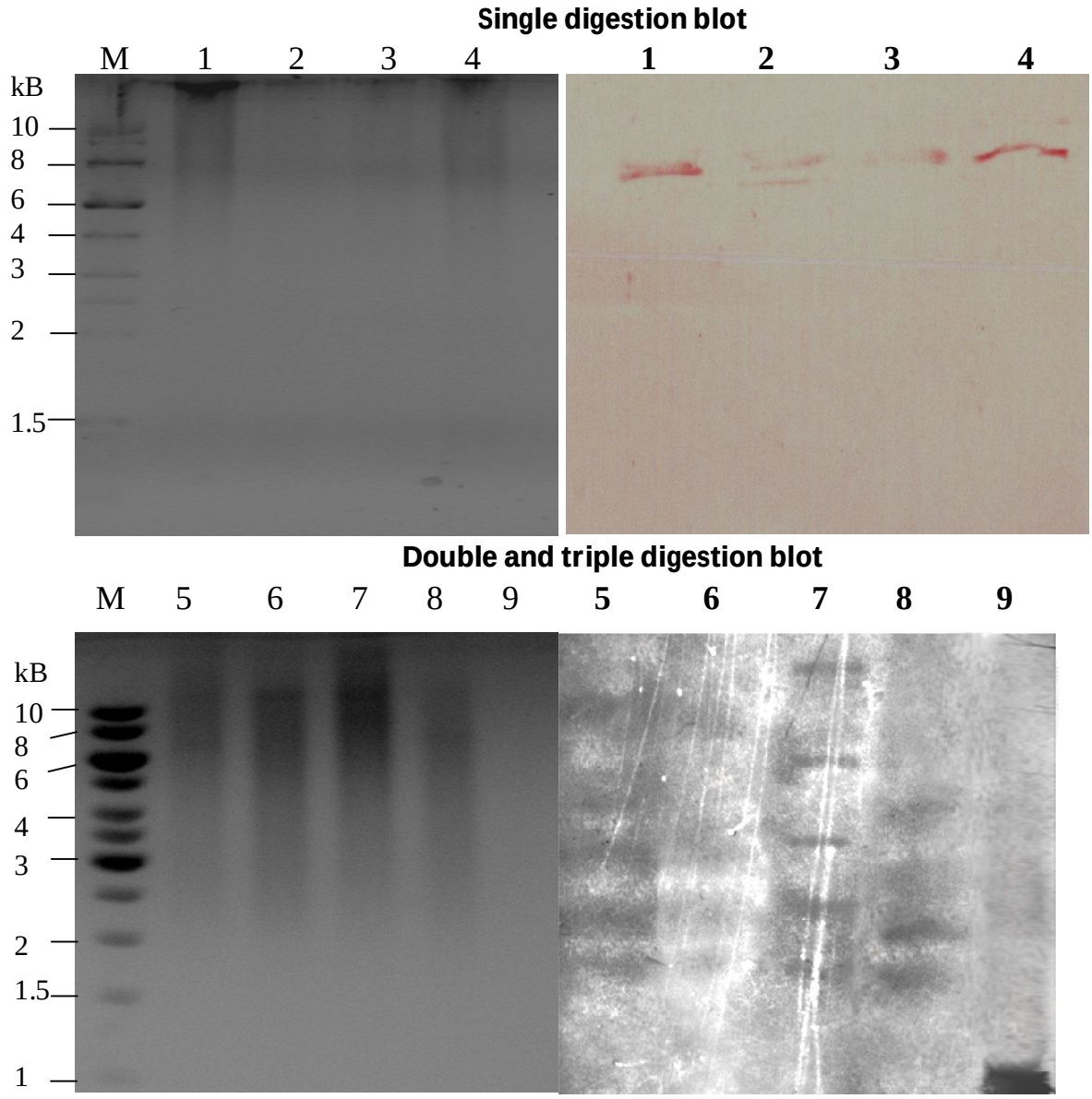

Figure (20): Southern blot analysis for the melano protein and copy number determination (Single digestion (right), Double and triple digestion (Left)) in the genomic DNA of *V. inaequalis.* Single digestion were signed with 1 (*BamHI*), 2 (*HindIII*), 3 (*NcoI*), and 4 (*SacI*). Double and triple digestion were signed with numbers 5 (*NcoI/BamHI*), 6 (*NcoI/HindIII*), 7(*BamHI/HindIII*), 8(*NcoI/BamHI/HindIII*), and 9 (Positive control). The normal numbers signed for digestion and the bold numbers (**1, 2, 3, and 4, 5, 6, 7, 8 and 9**) signed for the blot. The blotting was detected by using alkaline phosphatase.

5.7 Cloning of melano protein ORF into *E. coli*

5.7.1 PCR, cloning into pGEMT and fragment extraction

Melano protein ORF was amplified (PCR) using the full-length primers, as primary step in the ORF analysis and prediction after assembly of the fragments from the sequencing. Afterwards the

full length primers were modified by adding *NcoI* and *HindIII* to the forward and reverse primer, respectively. The modified primer were used for the full length melano protein cDNA PCR and then cloned into pGEMT, electroporated into XL1-blue *E. coli* electrocompetent cells, and grown for plasmid amplification. Afterwards, plasmid was extracted and double digested with *NcoI* and *HindIII* followed by electrophoretically separation on 1% agarose gel. The double digested melano protein ORF were extracted and purified.

5.7.2 pET-32a (+) vector preparation, cloning, double digestion

pET-32a (+) vector was prepared for overexpression by the double digestion with *NcoI&HindIII* and electrophoretically separated on 1% agarose gel. The double digested vector were extracted from agarose gel and purified for cloning. Prepared melano protein fragment and vector were ligated and cloned into XL1-blue as preliminary step for pET32-Melano protein multiplication and verification of the right translation. Afterwards, the right clone were selected and the constructed plasmid were extracted and cloned again into *E. coli* BL21 codon plus. Then bacteria's containing the right plasmid (BL21 codon plus-pET32-Melano protein) was selected.

5.7.3 Verification of the histidine tag by sequencing

For cloning verification the extracted pET32-Melano protein (Figure 21) was sequenced. The melano protein has the right frame overexpression. Additional glycine was added to the sequence to create the right frame, followed by the ORF of the melano protein. The virtual sequence of the translated right frame in pET32-Melano protein gave 52.83 kD this additional sequence include *trx*•Tag, two *his*•Tag at both N and C terminus, and *S*•Tag.

```
atgcatcatcatcatcatagcagcggcctggtgccgcgcggcagcggcatgaaagaa
 M  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  G  M  K  E
accgcggcggcgaaatttgaacgccagcatatggatagcccggatctgggcaccgatgat
 T  A  A  A  K  F  E  R  Q  H  M  D  S  P  D  L  G  T  D  D
gatgataaagcgatgggcatgtttgcgaaactgctggcgctggcggcgctgccggcggcg
 D  D  K  A  M  G  M  F  A  K  L  L  A  L  A  A  L  P  A  A
agcaacgcgtttggcgtgctgaaaggctttaactatggcagcaccgatgcgagcggcgtg
 S  N  A  F  G  V  L  K  G  F  N  Y  G  S  T  D  A  S  G  V
gtgaaagatcaggcgcgctttgaacaggaatttagcaccgcgcagaacctggtgggcacc
 V  K  D  Q  A  R  F  E  Q  E  F  S  T  A  Q  N  L  V  G  T
```

```
agcggctttaacagcgcgcgcctgtataccaccattcagggcggcaccaccaacagcccg
 S  G  F  N  S  A  R  L  Y  T  T  I  Q  G  G  T  T  N  S  P
attagcgcgattccggcggcgattagcacccagaccagcctgctgctgggcatttggacc
 I  S  A  I  P  A  A  I  S  T  Q  T  S  L  L  L  G  I  W  T
agcgcgggccaggcgattgtggataacgaaattgcggcgctgaaagcggcgattagccag
 S  A  G  Q  A  I  V  D  N  E  I  A  A  L  K  A  A  I  S  Q
tatggcaccgcgtttaccgatctgattgtggcggtgagcgtgggcagcgaagatctgtat
 Y  G  T  A  F  T  D  L  I  V  A  V  S  V  G  S  E  D  L  Y
cgcaacagcggctatccgggcgcgagcgatccgggcccgggcgcgaacccggatgtgctg
 R  N  S  G  Y  P  G  A  S  D  P  G  P  G  A  N  P  D  V  L
gcgaactatattggccaggtgaaagcggcgattgcgggcaccagcgcggaaggccgcctg
 A  N  Y  I  G  Q  V  K  A  A  I  A  G  T  S  A  E  G  R  L
gtgggccatgtggatacctggaccgcgtttgtgaacagcagcaacaacgcgctgattagc
 V  G  H  V  D  T  W  T  A  F  V  N  S  S  N  N  A  L  I  S
gcggcggattttctgggcgtggatgcgtatccgtattatgaaagcgcgaacggcaacgat
 A  A  D  F  L  G  V  D  A  Y  P  Y  Y  E  S  A  N  G  N  D
attagcaacgcggcgaacctgtttgcgagcgcgtatagccaggtggtggcggtggcgcag
 I  S  N  A  A  N  L  F  A  S  A  Y  S  Q  V  V  A  V  A  Q
ggcaaaccggtgtgggtgaccgaagcgggctggccggtgagcggcccgaccgtggcgcag
 G  K  P  V  W  V  T  E  A  G  W  P  V  S  G  P  T  V  A  Q
gcggtggcgagcccggaaaacgcgcgcagcttttggaccagcgtgggctgcaaccagctg
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 F  D  K  I  N  V  W  W  F  Q  L  D  D  Y  P  T  S  P  N  P
gcgtttggcgtgattggcaccgcgtttagcaccaccccgctgtttgatctgagctgcccg
 A  F  G  V  I  G  T  A  F  S  T  T  P  L  F  D  L  S  C  P
gcgaccaccaaaagccgccgccgcagcaaccgcgcggcgaaactggcggcggcgctggaa
 A  T  T  K  S  R  R  R  S  N  R  A  A  K  L  A  A  A  L  E
catcatcatcatcatcatagcaccccgctg
 H  H  H  H  H  H  S  T  P stop
```

Figure (21): Verification of the cloning strategy by sequencing and virtual translation of pET32-Melano protein.

5.8 Cell induction and protein analysis

5.8.1 Total protein analysis

Extracted total protein from the negative control (BL21-pET-32a (+)) and non induced positive clone (BL21 codon plus- pET32-Melano protein) and induced positive bacterial clone were extracted and analyzed with Tris-Tricine PAGE (Figure 22). The induced cells (lane 3) give a big band signed with box (50 kD) in comparison to the negative control (lane 1) and the uninduced positive clone (lane 2). The big band between 4-16 kD might be the leader protein sequence is broken by the protease activity of the melano protein.

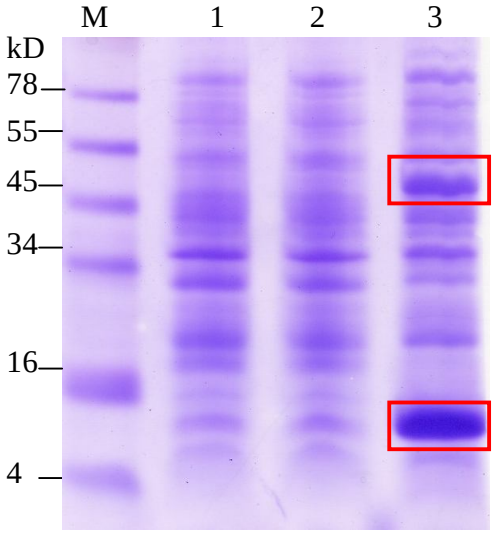


Figure (22): 12% Tris-Tricine PAGE for the total protein to detect the recombinant protein pattern in *E. coli* (BL21 codon plus). Gel includes the negative control (lane 1), and the positive non-induced clone (lane 2), and induced positive clone (lane 3). Each lane loaded with 10 µg protein. M is the marker.

5.8.2 Ni-NTA agarose purification of the recombinant melano protein

The proteins were produced by induction of BL21 codon plus-pET32-Melano protein) on LB medium (at 37 °C; and induced at an optical density of 0.6 with 1 mM of the IPTG for 2-3 hours). The soluble fraction was extracted after induction with French press followed by the purification steps (Figure 23). The extracted protein was loaded (10 µg) in order as following: melano protein from *V. inaequalis*, negative control (BL21-pET-32a (+)), uninduced positive clone (BL21 codon plus- pET32-Melano protein), induced positive clone, flow through, washing twice and purified recombinant melano protein and separated by Tris-Tricine PAGE. As expected the most induced protein is around 53 kD (Figure 23).

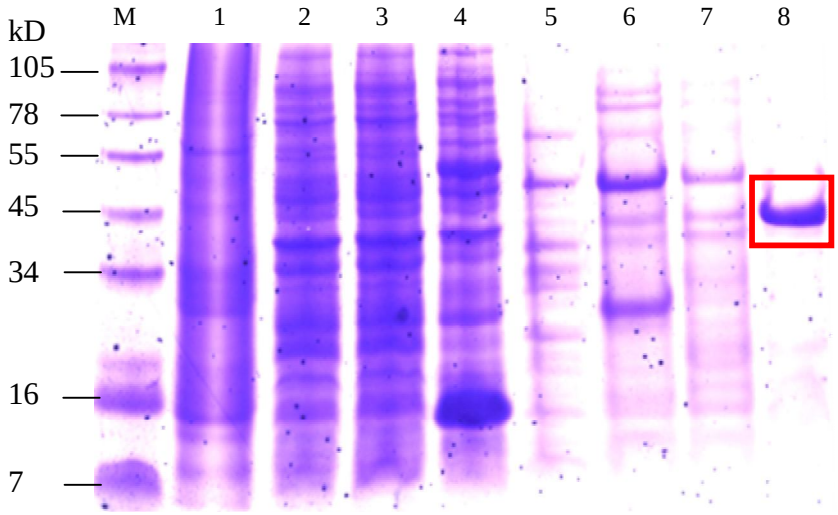


Figure (23): 12% Tris-Tricine PAGE of over expression of melano protein at 37 °C for 3h with 1 mM IPTG. The samples were loaded as following order: 1= crude melano protein from *V. inaequalis*, 2= negative control, 3= uninduced positive clone, 4= induced positive clone, 5= flow through, 6 and 7 = washing and 8 = purified recombinant melano protein. Each lane was loaded with 5 µg protein. M is the marker.

5.8.3 Immunological detection of the purified recombinant melano protein

Tris-Tricine PAGE was done for the proteins samples. Afterwards, western blot (Figure 24) was done for *V. inaequalis* melano protein, negative control (BL21-pET-32a (+), lane 1), and two different storage method of the purified recombinant melano protein from the positive clone (BL21 codon plus- pET32-Melano protein, lanes 2&3), respectively. The partial destroying of the recombinant protein leads to the proteolytic activity investigation.

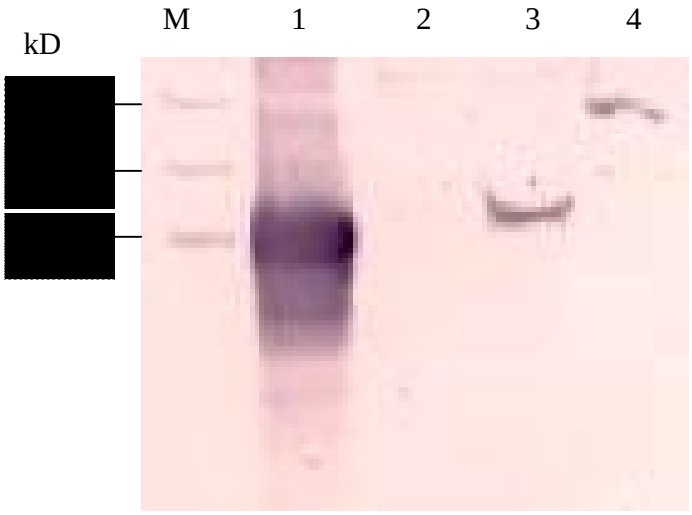


Figure (24): Western blot for the crude melano protein (15 µg, lane 1) from *V. inaequalis*, negative control (lane 2, 10 µg) and the purified melano protein to show the size of the protein if this protein is destroyed after storage for 20 days (lane 3, 10 µg) and number four (10 µg) mixed with glycerin phosphate buffer (5% glycerin (v/v), 50 mM sodium phosphate buffer pH 6.0 and stored for the 20 days. M is the marker. The melano protein antibody was diluted 1: 500.

5.8.4 Heat-treatment of the recombinant melano protein (Tris-Tricine PAGE)

Heat treatment method (70 °C for 30 min, sodium phosphate buffer pH 6.0) was used for purification of the recombinant melano protein (Figure 25). After heat treatment the denaturated protein was precipitated by centrifugation 10 min at 10000 xg. The pellet was the bacterial protein (lane 1) and the soluble pure protein was the purified recombinant melano protein (lane 4). The gel below (see Figure 25) include the purified protein (soluble fraction after heat treatment) of the negative control (lane 2), and the positive non-induced (lane 3). 4-16 kD band in lane 1 could be a breakage product from the preprotein.

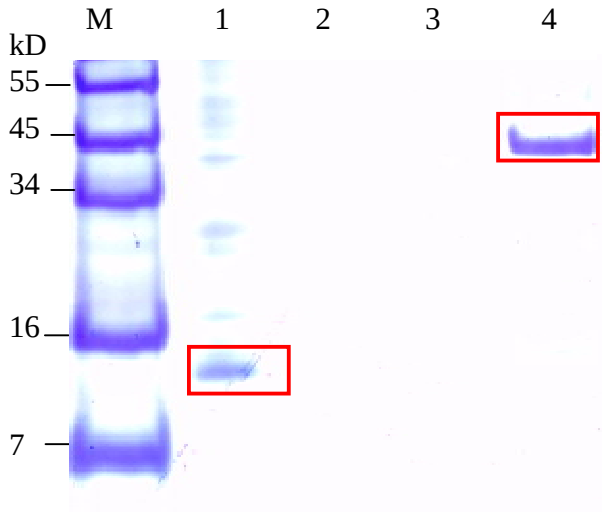


Figure (25): 12% Tris-Tricine PAGE for the total protein to detect the recombinant protein pattern in *E. coli* (BL21 codon plus) after heat-treatment of the soluble fraction at 70 °C for 30 min and denaturation of all the proteins except the recombinant protein. Lanes comtaining proteins were loaded with 15 µg protein. Lane 1: the denaturated proteins after heat treatment, lane 2: the purified protein from the negative control, lane 3: the positive noninduced *E. coli* cells and lane 4: the purified protein with heat treatment from induced positive *E. coli* clone. M is the marker.

5.9 Qualitative and quantitative determination for the melano protein glucanase activity

5.9.1 Agarose plate

Glucanase activity were done for both the purified recombinant protein and the crude melano protein from *V. inaequalis* on agarose plate as a preliminary test giving yellow hallow (Figure 26). The maximum hydrolysis was shown for the extracted melano protein of the fungus *V. inaequalis* (hole 4) followed by the hole 2 and 3 (recombinant melano protein in 15 % glycerol and 5, 6, and 1 in water).

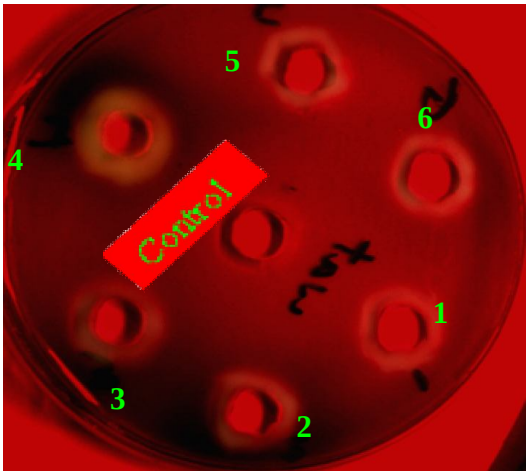


Figure (26): Glucanase activity on agarose plate for different recombinant melano protein fractions (5, 6, and 1 fraction in phosphate buffer, pH 6.0; Fraction 3 and 4 in phosphate buffer and 15 % glycerol, pH 6.0) in addition to the extracted melano protein (4) from the supernatant of *V. ineaqualis* culture (20 µg protein per hole). The plate incubated for four hour at 40 °C and then stained with 0.5 % Congo-red for 30 min, followed by distaining with 1 M sodium chloride.

5.9.2 Glucanase activity gel

Glucanase activity for both *V. inaequalis* melano protein and the recombinant melano protein are investigated with Tris-Tricine PAGE (Figure 27). The expected band for the glucanase activity is signed with arrow, with calculated size of 36 kD for the melano protein and 50 kD for the recombinant melano protein. The active bands appeared in orange color, and the color depends on the pH.

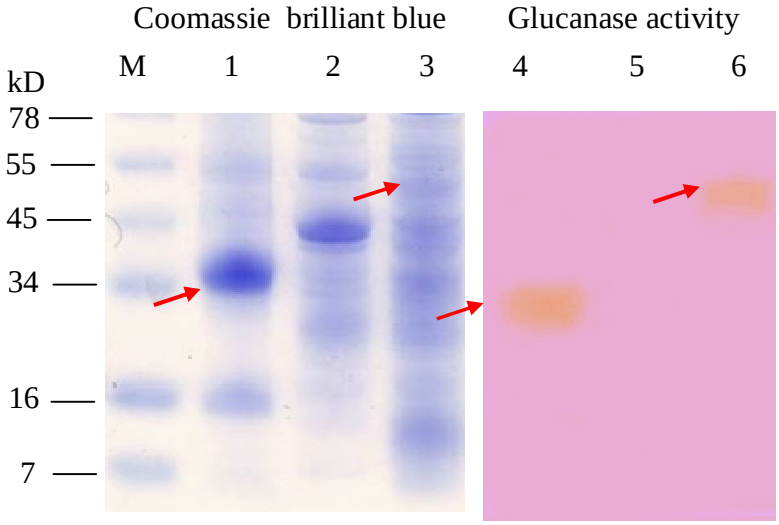


Figure (27): 12% Tris-Tricine PAGE and glucanase activity gel, each lane were loaded with 15 µg protein the expected active band were signed with red arrow in both Coomassie Brilliant Blue stained gel and activity gel (Congo red gel). The tested sample is crude melano protein extracted from the liquid medium of *V. inaequalis*, negative control, positive control and partial purified of recombinant protein. M: marker, Lane 1: Melano protein from *V. inaequalis*, Lane 2: negative control induced, lane 3 induced cells produced recombinant melano protein, lane 4-6 glucanase staining, lane 1-3 Coomassie Brilliant Blue staining.

5.9.3 Effect of pH on the recombinant protein glucanase activity

The glucanase activity (Figure 28) of the recombinant melano protein shows the effect of pH changes. The effect of pH fluctuations were tested from 3.0 to 10.0 pH. Each pH point had a control. The protein was classified as slightly acidic glucanase (pH 6.0).

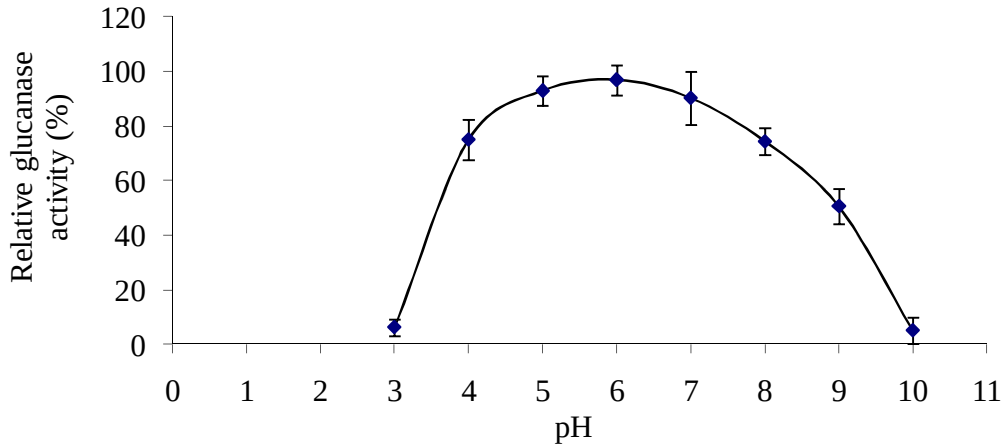


Figure (28): Effect of the pH changes on the glucanase activity of the purified recombinant melano protein protein. Assay was done as described in 4.4.15 section.

5.9.4 Effect of temperature on glucanase activity

The effects of different temperatures (20-100 °C) melano protein glucanase activity were investigated to find out the optimum temperature. Each temperature degree had a control. The effect of temperature on glucanase activity is like the effect of pH give narrow optimum temperature range around 60 °C (Figure 29).

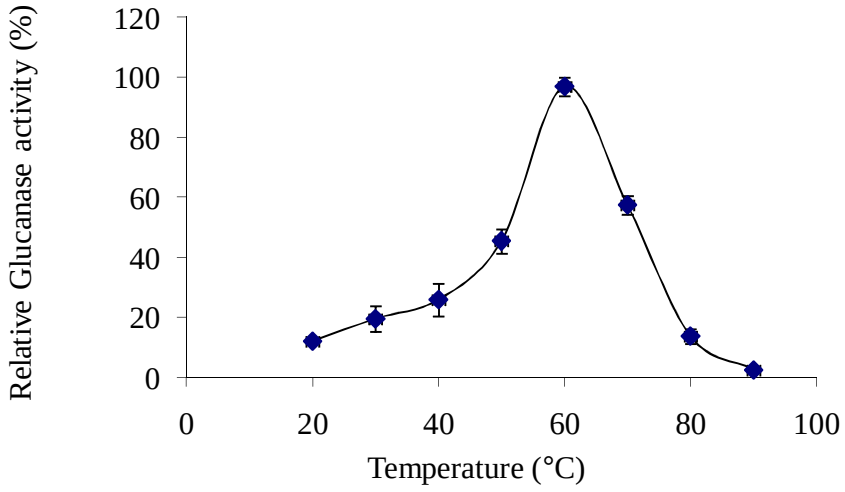


Figure (29): Effect of the temperature changes on the glucanase activity of the purified recombinant melano protein. Assay was done as described in 4.4.15 section.

5.9.5 Glucanase Km value

The glucanase activity was repeated with different substrate concentration from 1 to 8 mg of glucan in presences of 0, 200, and 500 µM calcium and magnesium chloride, respectively for Km values determination. The Km value (Figure 30) for the glucanase enzyme was 0.013 mM, it was

activated by $CaCl_2$ (200, 500 mM; 0.029 and 0.02 mM, respectively) and did not show activation or inhibition by $MgCl_2$ (200, 500 mM; 0.013 and 0.014 mM, respectively).

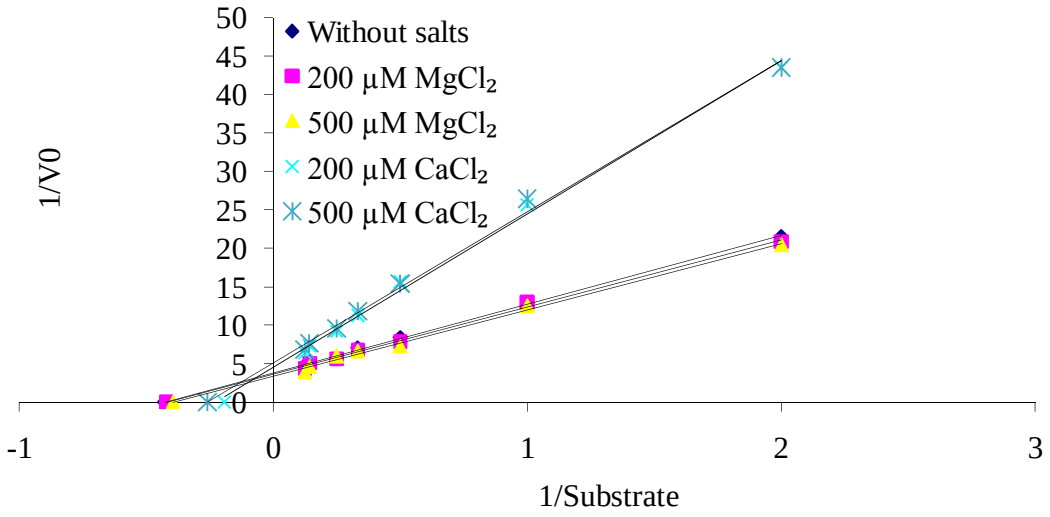


Figure (30): Glucanase Km value and the effect of 200 and 500 mM of $CaCl_2$ and $MgCl_2$ on the Km value. Determination was done as described under 4.4.17.

5.9.6 Effect of some protease inhibitors on glucanase activity

Different protease inhibitor (EDTA, PMSF, Aprotinin, Pepstatin, Leupeprin, Chymostatin) were tested for their effect on glucanase activity. Melano protein incubated with the protease inhibitors 15 min before adding the substrate with 15 µg of protein in each tube using the following concentration 10 mM, 1 mM, 0.3 mM, 0.1 mM, 100 mM and 100 µM, respectively as previously mentioned. Protease inhibitor is clear that, the relative inhibition effect of the recombinant melano protein glucanase activity maximally inhibited by PMSF (67.6%), followed by EDTA (27.7%), Pepstatin (15.3%), Aprotinin (7.3%), Leupeprin (5.5%), and Chymostatin (1.6%), respectively as mentioned above for each concentration (Figure 31).

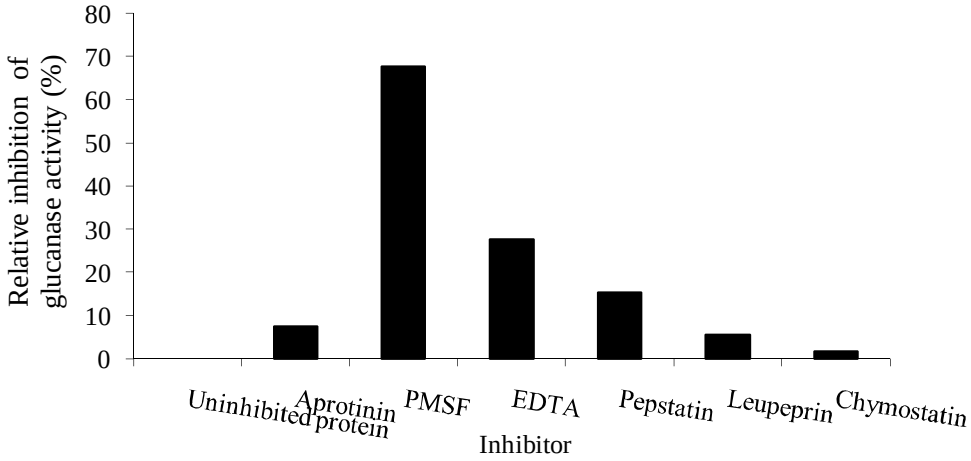


Figure (31): Effect of protease inhibitors (PMSF (1 mM), EDTA (10 mM), Leupeprin (100 mM), Pepstatin (100 mM), Aprotinin (0.3 µM), and Chymostatin (100 mM)) on the purified recombinant melano protein glucanase activity. It was done as described as mentioned in 4.4.16.

5.9.7 Primary effect of some alkali and transition metals on glucanase activity

Metal ions ($CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_3$, and $ZnCl_2$) and its roles in glucanase activity were tested. The metals were incubated 15 min before adding glucan substrate with 15 µg of purified recombinant melano protein in each tube using 1 mM concentration in the reaction mixtures for each metal in each reaction tube. Both the alkali and transition metals (Figure 32**)** were inhibiting glucanase activity giving rise different inhibition ratio with range of 49.2-95.1% except calcium can increase the activity by 30.8%.

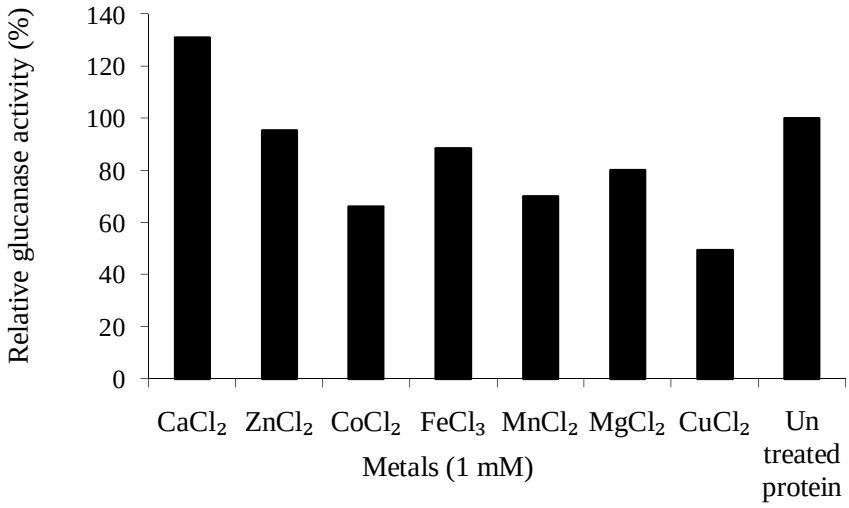


Figure (32): Effect of some metals ions (1 mM; $FeCl_3$, $CoCl_2$, $ZnCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$) on the recombinant protein glucanase activity. Glucanase assay was done as described under 4.4.16.

5.9.8 Effect of different concentration of the metals

Different concentration (1-5 mM) of $CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_3$, and $ZnCl_2$ were incubated 15 min with 15 µg of the purified recombinant melano protein before adding the substrate (6 mg/ml glucan). Glucanase activity decreased gradually or suddenly as the metal concentration increased for metals ($CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$ and $FeCl_3$). On the other hand $ZnCl_2$ and $CaCl_2$ slightly increase glucanase activity (Figure 33).

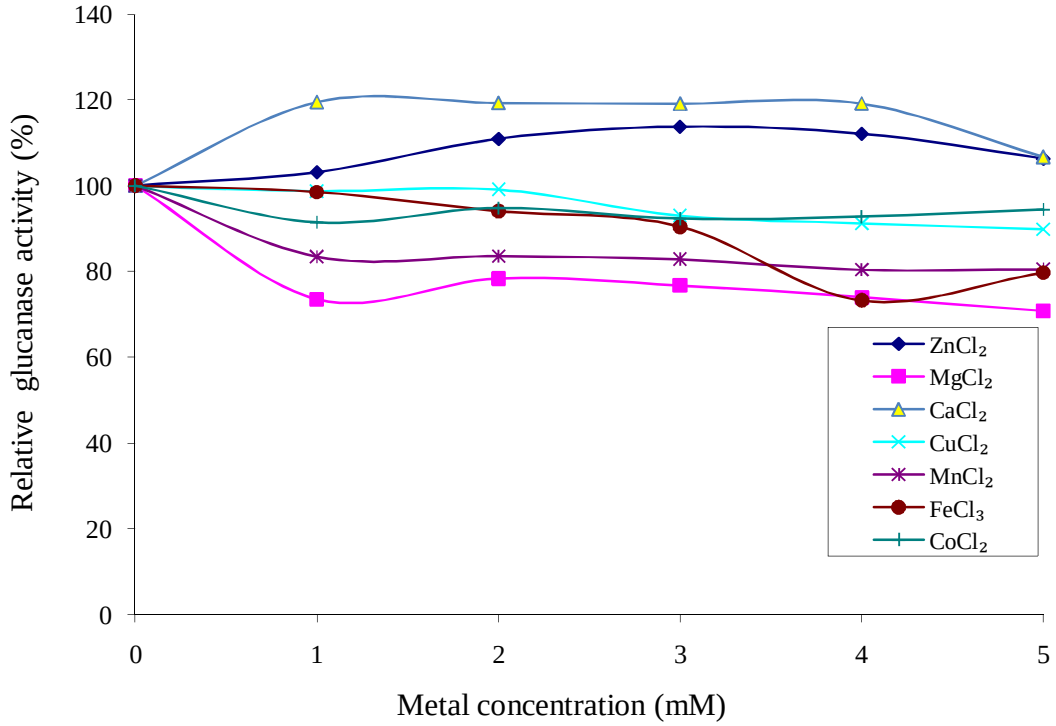


Figure (33): Effect of different concentration of cations and transition metals on the glucanase activity of the purified recombinant melano protein. Assay was done as described in 4.4.16.

5.9.9 Glucanase activity on some different substrates with three method of determination

5.9.9.1 Determination of substrate specificity by detection of products with different method

The protein can hydrolyze the component present in the flour with high amount of released glucose even contain $CoCl_2$ (1 mM) or not, followed by the released amount from the *Zea maize* tissue in untreated and treated with 1 mM $CoCl_2$ followed by the untreated crudlan and treated crudlan with 1 mM $CoCl_2$ (Figure 34)

5. 9.9.2 Congo-red

Congo red method (Figure 35) was developed for glucanase activity determination which recorded similar result to glucose assay method (Figure 34), especially for flour and crudlan. On the other hand *Zea maize* gave small with congo red method in comparsion to the determination with glucose assay method. The barley starch hydrolysis gave a positive results indicating on occurrence of hydrolysis, the results was confermed with DNS method results.

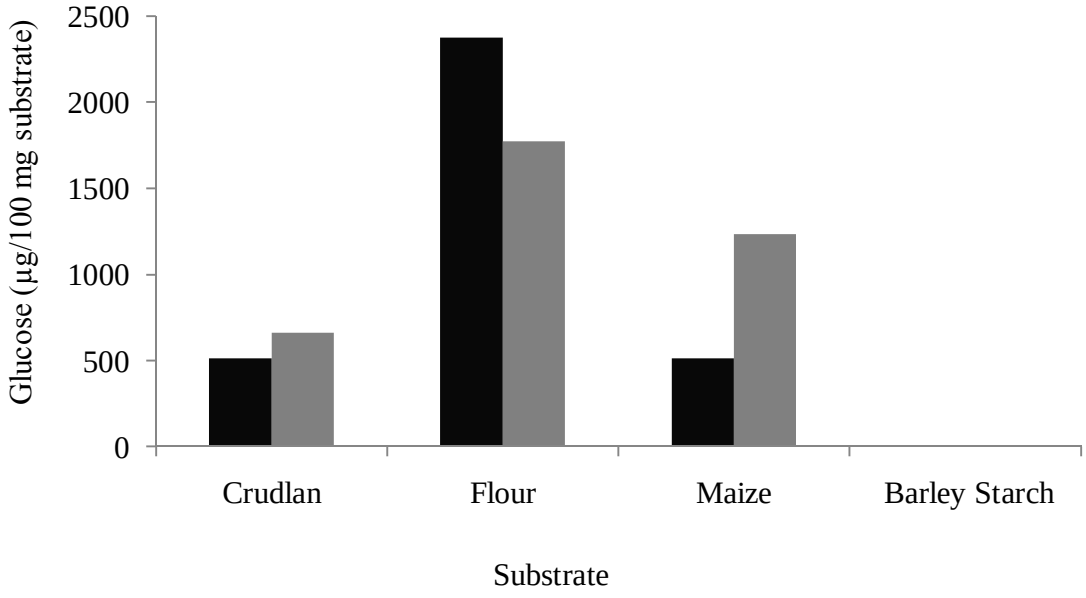


Figure (34): Effect of the recombinant melano protein on the different polysaccharide as substrate and determination of the released glucose with the glucose assay reaction. The black columns are the glucanase activity without cobalt chloride and the gray ones are with 1 mM $CoCl_2$.

5.9.9.3 DNS method

DNS method was used for determination of glucanase ability to hydrolyze polysaccharide into monosaccharide (crudluan, flour, maize tissue, barley starch) and to confirm Congo-red method reality (Figure 36). The determination of the monosaccharide by DNS method gave 74, 230, 107, 41, 81, 46 mM), respectively as mentioned before. Adding of $CoCl_2$ (1 mM) to the reaction mixture of glucanase, increased the resulted monosaccharide for most of the substrate to 84, 321, 101, 41, 86, 164 mM, as mentioned respectively.

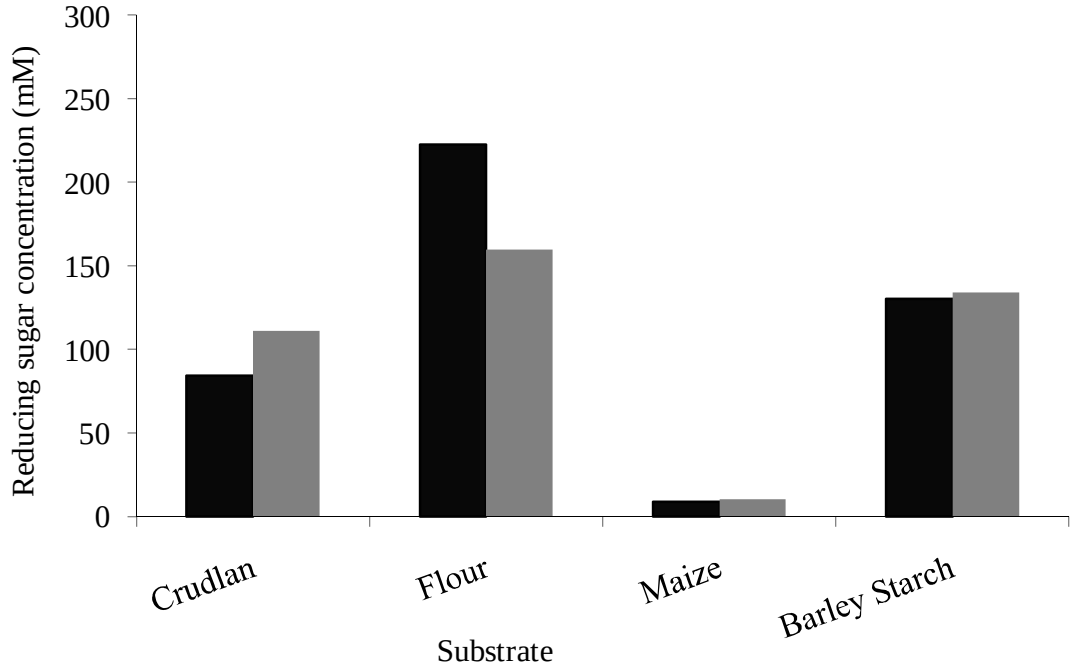


Figure (35): Effect of the protein on the different polysaccharide as substrate and determination of the released glucose with the Congo-red method. The black columns are the glucanase activity without cobalt chloride and the gray ones are with 1 mM $CoCl_2$.

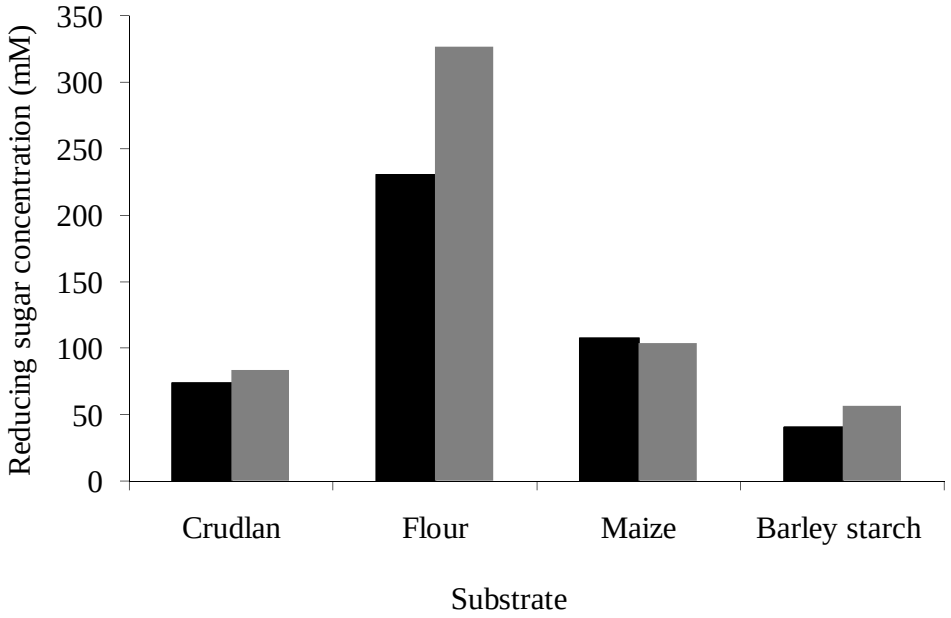


Figure (36): Polysaccharide hydrolysis determination using DNS method for determination of the over expressed melano protein activity. The black columns are the glucanase activity without cobalt chloride and the gray ones are with 1 mM $CoCl_2$.

5.10 Protease activity of the melano protein

5.10.1 Protease activity on agarose plate

Protease activity on agarose plate shows gradient increase as recombinant melano protein amount increases (10, 15, 20, 25, 30, 40 µg) from 1 to 6, respectively. The reaction were done at pH 6.0 at 37 °C (Figure 37) for 3 h and stopped by adding 5% TCA. The previous experiment were repeated but with crude melano protein (10, 20 µg) from *V. inaequalis* shows high-resolution effect of the protein on casein for proteolytic activity for both the recombinant protein (Figure 37 A) and the melano protein (Figure 37 B). The reaction was stopped after three hour incubation at 37 °C using 5% TCA.

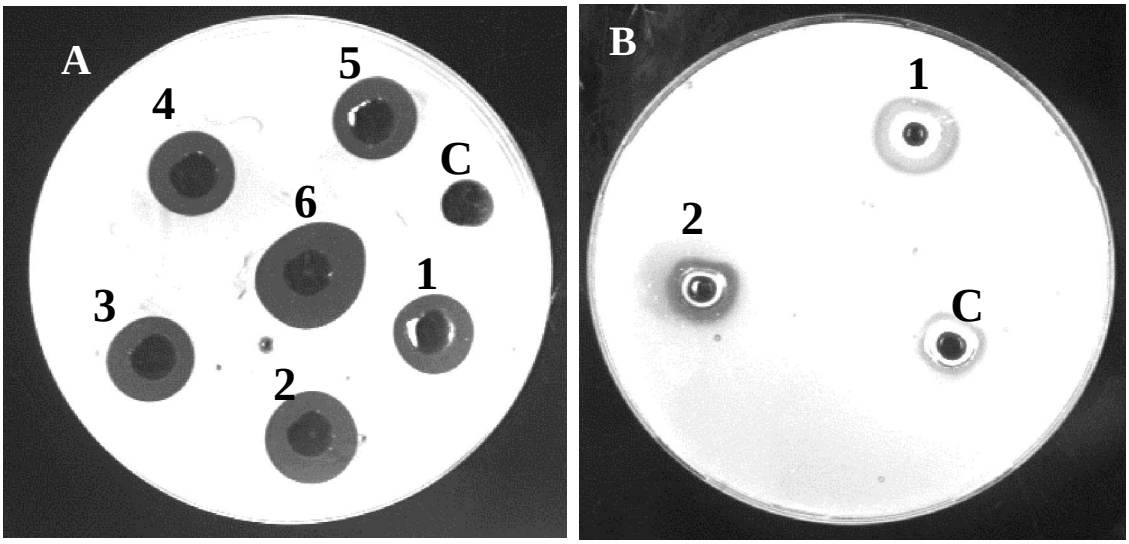


Figure (37): Protease activity on agarose plate containing 1% casein. A: recombinant melano protein from different tubes (1-6, 20 µg), B: melano protein from *V. inaequalis* (1 (10 µg), 2 (20 µg)), C: control buffer (C).

5.10.2 Proteolytic activity on agar-casein (Renin like (Retropepsin_like_LTR_1))

The protolytic activity of the melano protein (Figure 38) classify the protease type of the recombinant melano protein, and find a relation to retropepsin_like_LTR_1 more evidence for hypothesis confirmation was founded by analyzing the cDNA of the melano protein sequence and presence of important motifs of D-T/S-G (See Figure 17). The information was built up on the homology search for these motives which similar to retropepsin_like_LTR_1 or aspergillopepsin_like (Aspartic proteases of fungal origin). The precipitation of the hydrolyzed protein become faster and the clear band disappears if the amount of the protein increase to 150 µg, this was obtained after 12 h. The clear band appears if fewer amount of melano protein applied (100 µg).

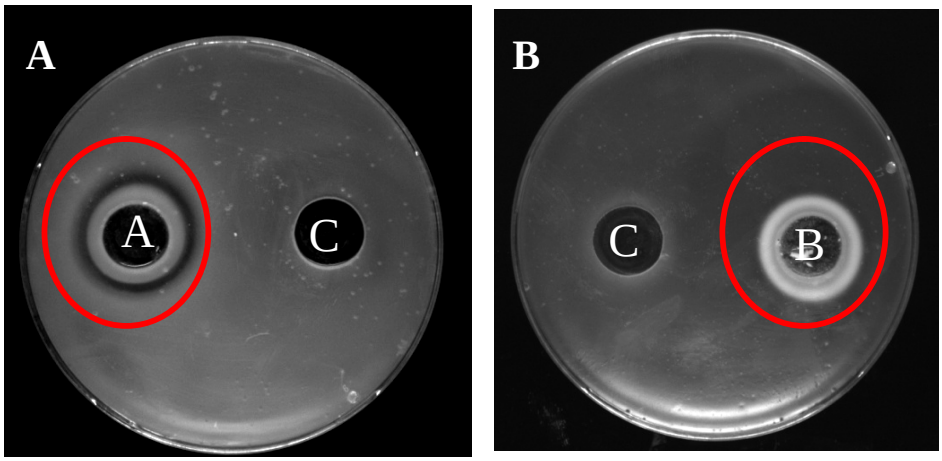


Figure (38): Proteolytic activity on agar casein (3%) plate for the recombinant melano protein showing the activity of the protease on A (100 µg) and B (150 µg) and the control (C).

5.10.3 Temperature effect of protease activity

The effect of temperature on protease activity were tested from 20 °C to 100 °C to find out the optimum temperature in a controlled way. The protease activity has a broad range temperature range (Figure 39), that different from glucanase activity. The optimum activity increased to the temperature degree increased to 90 °C, then decrease. The optimum temperature was recorded at 90 °C. The lower protease activity for the protein was at 20 °C. Cobalt ion (2 mM) increased protease activity of the recombinant melano protein by 50 to 100% more than the control.

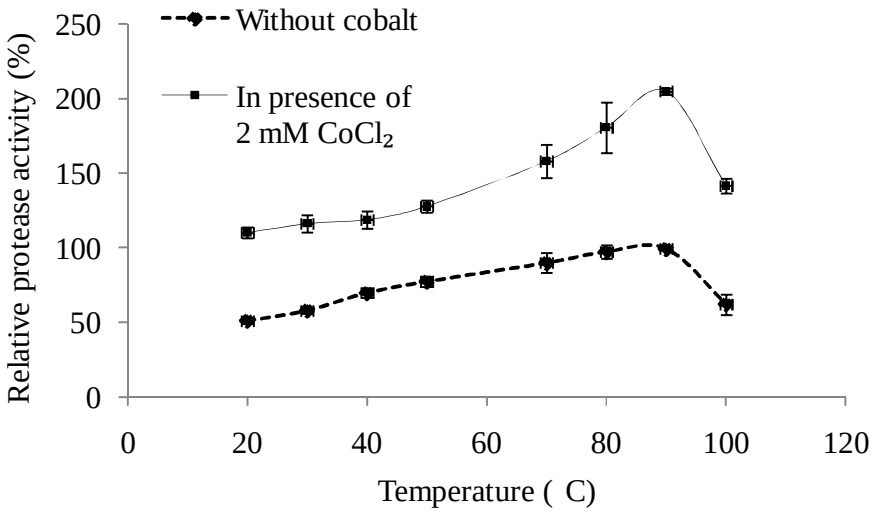


Figure (39): Effect of the temperature on the protease activity of the purified recombinant melano protein. The assay was done with 15 µg protein and was performed as given under 4.4.15.

5.10.4 Effect of pH on the protease activity of the recombinant protein

Different pH point (3.0 - 11.0 pH) was tested for the protease activity of the recombinant melano protein. The protease activity affected with the pH but showed broad range as well as the temperature effect. The optimum pH for protease activity is similar to glucanase activity of the pure recombinant melano protein pH 6.0 (Figure 40). Adding of cobalt to the activity assay shifted the optimum pH in range from pH 6.0 to pH 8.0 with slightly increase with pH 8.0.

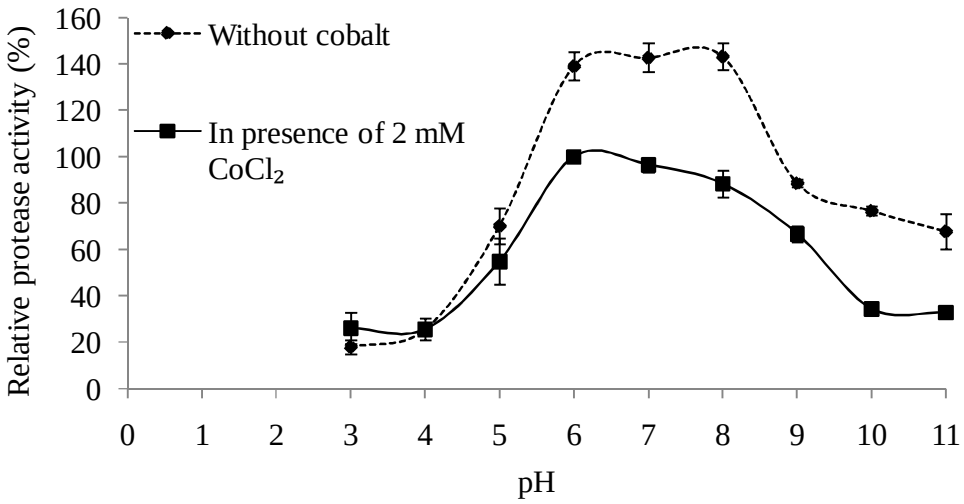


Figure (40): Effect of the pH changes on the protease activity of the purified recombinant melano protein. The assay contained 15 µg of the recombinant melano protein. It was done as described in 4.4.15., in presence and absence of 2 mM $CoCl_2$. The number of replicates is n=3.

5.10.5 Unit determination of the recombinant melano protein in comparison to trypsin units

The relative activity of recombinant melano protein has 8.15 fold higher than trypsin. The protease units/ mg (Figure 41) were determined for the recombinant melano protein (367 U/mg) in comparison to tyrpsin (40 U/mg).

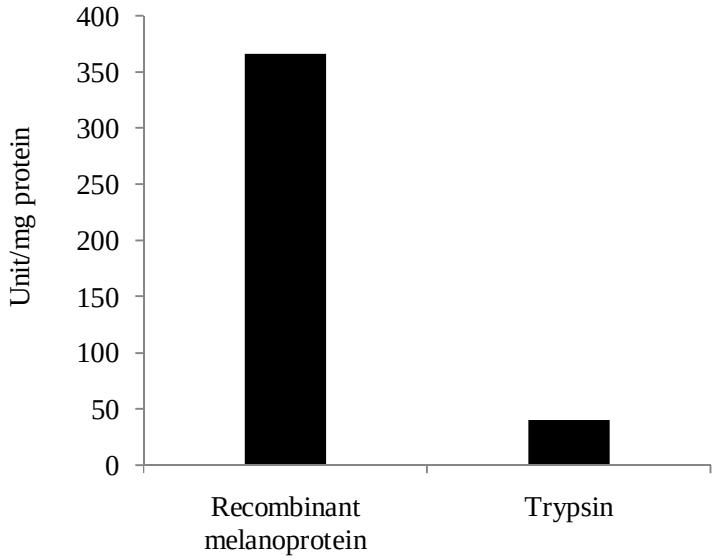


Figure (41): Quantitative unit calculation per mg recombinant protein (367U/mg) in comparison to trypsin protease (40 U/mg). Azocasein was used as substrate for quantitative method. Determination of the activity was done at the optimum temperature for each (90 °C, 37°C, respectively). Assay was done as described under 4.4.14.2.

5.10.6 Protease Km value

Different substrate concentrations were used (0.5-14 mg of azocasein) in presence of 0, 200, and 500 µM of Calcium and Magnesium chloride, respectively. Metal ions were incubated with recombinant melano protein 10 min before substrate adding (Figure 42). The Km value for the protease enzyme was 0.774 mM it was inhibited by $CaCl_2$ (100, 200 mM; 0.623 and 0.563 mM, respectively) and $MgCl_2$ (100, 200 mM; 0.432 and 0.224 mM, respectively).

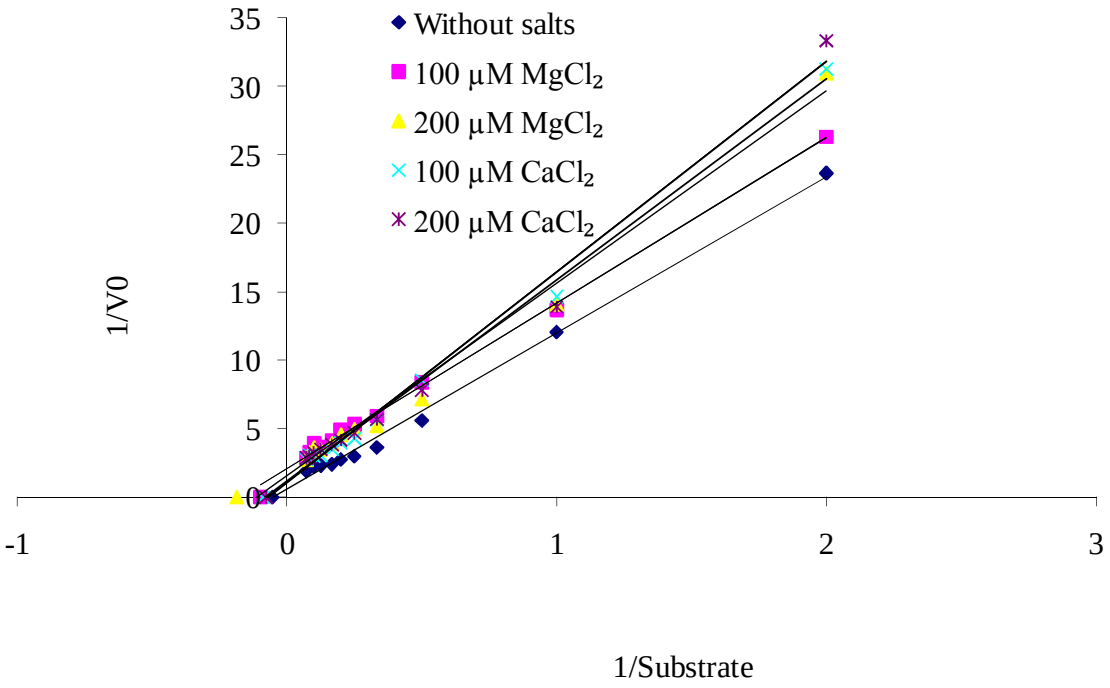


Figure (42): Protease Km value and the effect of 100 and 200 mM of $CaCl_2$ and $MgCl_2$ on the Km value. Determination was done as described under (See section 4.4.17).

5.10.7 Effect of protease inhibitor on the protease activity of the recombined melano protein

The inhibitors (EDTA, PMSF, Aprotinin, Pepstatin, Leupeprin, and Chymostatin) effect was tested on the protease activity to determine the specificity of protease. The inhibitor incubated 15 min before adding the substrate (Azocasein) with 15 µg of protein in each tube using the Different concentrations from 0.3 µM to 100 µM in the reaction mixtures, respectively as mentioned. The effect of inhibitors on the protease activity was clear (Figure 43). The maximum relative inhibition effect was for PMSF (98.2%) and followed by Aproteinin (46.9%), Pepstatin (28.5%), EDTA (4.6%), Leupeprin (20.4%), and Chymostatin (12.4%), respectively.

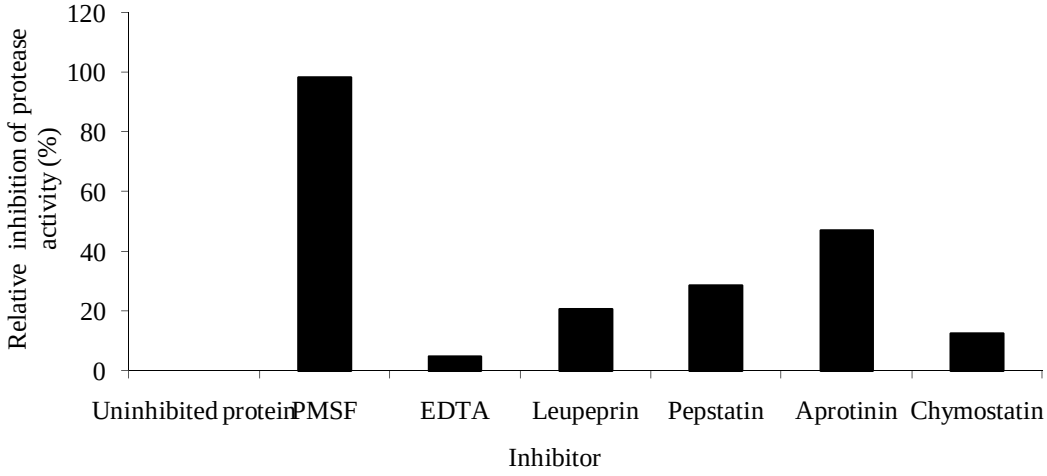


Figure (43): Effect of protease inhibitor (PMSF (1 mM), EDTA (1 mM), Leupeprin (100 mM), Pepstatin (100 mM), Aprotinin (0.3 µM), and Chymostatin (100 mM)) on relative protease activity. The assay was done as described in (See section 4.4.16).

5.10.8 Primary effect of some cations and transition metals on protease activity

1 mM of $CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_3$, and $ZnCl_2$ were used to determine the effect of these ions on protease activity (Figure 44). The relative activity of the melano protein increase in presence of $CoCl_2$ (170.6%) followed by $ZnCl_2$ wich has not effect (103.0%). Whereas $FeCl_3$, $CuCl_2$, $MnCl_2$ inhibit the protease activity in different levels of activity (16.2%, 30.5%, 82.6%, respectively). On the other hand the alkali metals inhibit the activity ($MgCl_2$, $CaCl_2$) with low level of activity (0.1%, 3.1%, respectively).

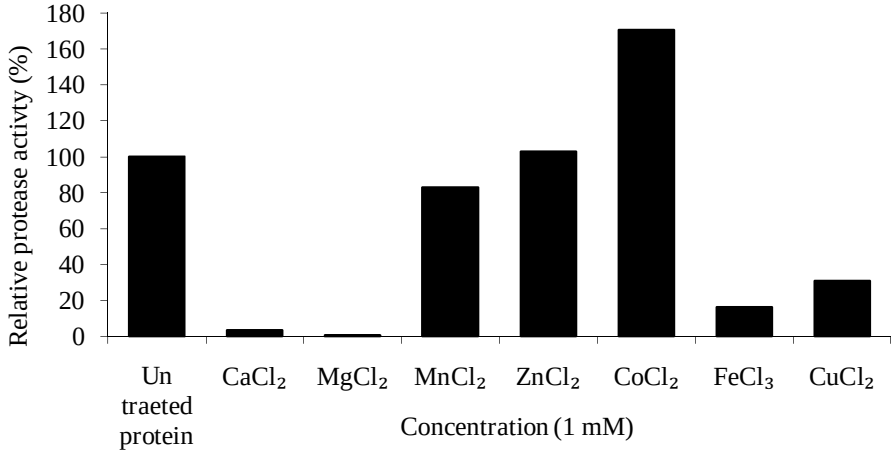


Figure (44): Effect 1 mM of some metals ions ($FeCl_3$, $CoCl_2$, $ZnCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, and $CaCl_2$) on protease activity of the recombinant protein. Assay was done as described in (See section 4.4.16)

5.10.9 Effect of different concentration of the transition and cations metals on protease activity

0-5 mM of $CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_3$, and $ZnCl_2$ were used to study the effect of these metals on melano protein protease activity (Figure 45). 2 mM Cobalt chloride increase the activity to the maximum value and decrease slightly soon. In the same way 4 mM $ZnCl_2$ also increase protease activity. Increase of the transition metals and cations concentration inhibit protease activity as the.

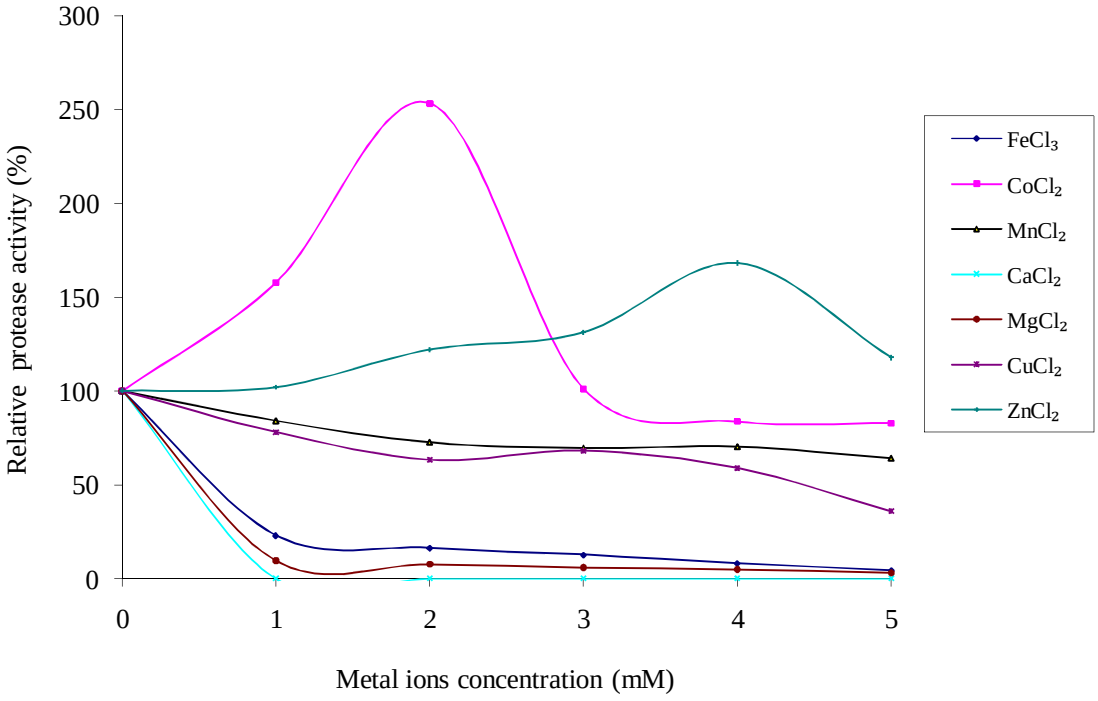


Figure (45): Effect of different concentration of cations and transition metals ions ($FeCl_3$, $CoCl_2$, $ZnCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, and $CaCl_2$) on the protease activity (See section 4.4.16).

5.10.10 Effect of EDTA on protease activity

Protease activity of the recombinant melano protein increased as the metal removed from the substrate (azocasien treated with 2 mM EDTA and dialyzed for overnight against Na_2CO_3: pH 8.3). In Figure 46 the depleted azocazein with EDTA showed high activity more than untreated one.

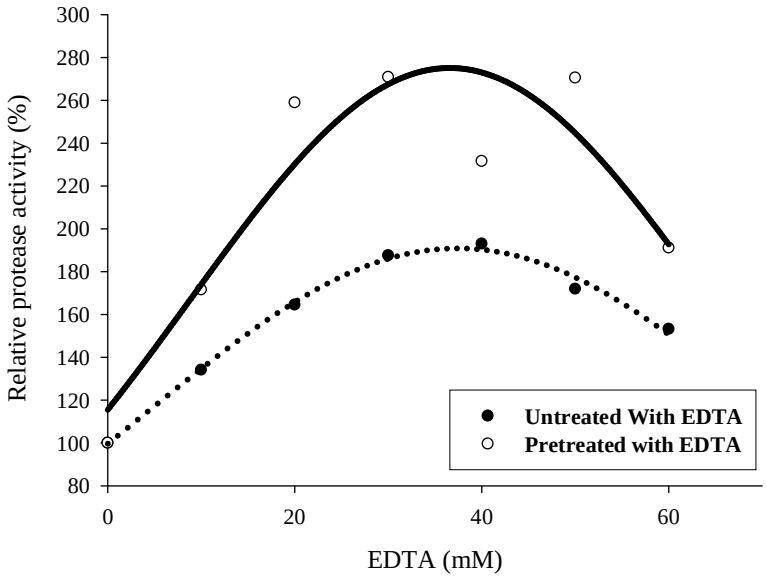


Figure (46): Effect of EDTA on the protease activity using calcium containing azocasein or depleted calcium substrate by treatment with EDTA using (2 mM) and dialysis. Ca^{2+} (4.4.14.2).

5.10.11 Effect of the apoplastic fluids on the protease activity of trypsin protease

Apoplastic fluids of *M. domestica* cvs. Elstar and Remo in general increase the activity of trypsin (Figure 47). That could be due to presence of additional protease in the apoplastic fluid of the selected cultivars. The maximum activity was achived by appling the apoplastic fluid from Remo cultivar (186.4%) followed by Elstar (124.8%).

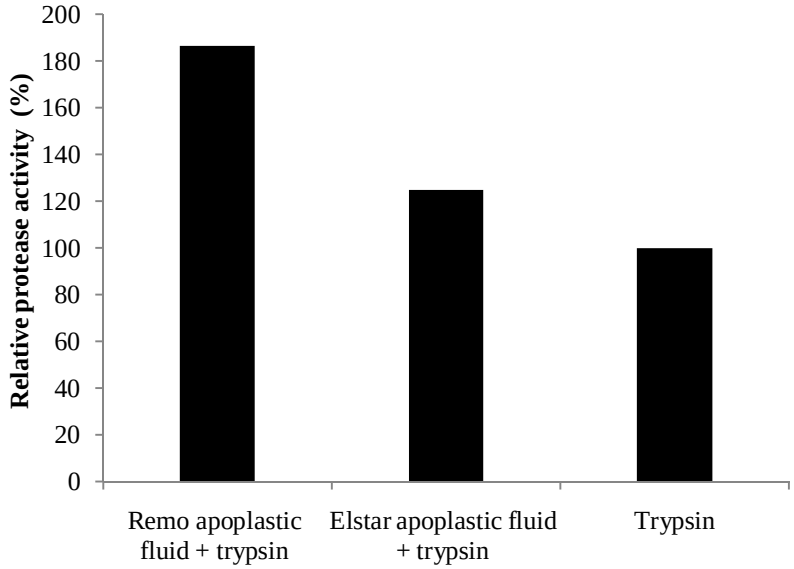


Figure (47): Effect of the apoplastic fluids on the protease activity of the trypsin. Assay was done as described under chapter 4.4.7.

5.10.12 Effect of trypsin inhibitor on the recombinant melano protein protease activity

The protease and glucanase activities of recombinant melano protein were repeated in presence of trypsin protease inhibitor giving 16.7 and 12.2 % relative inhibition, respectively (Figure 48).

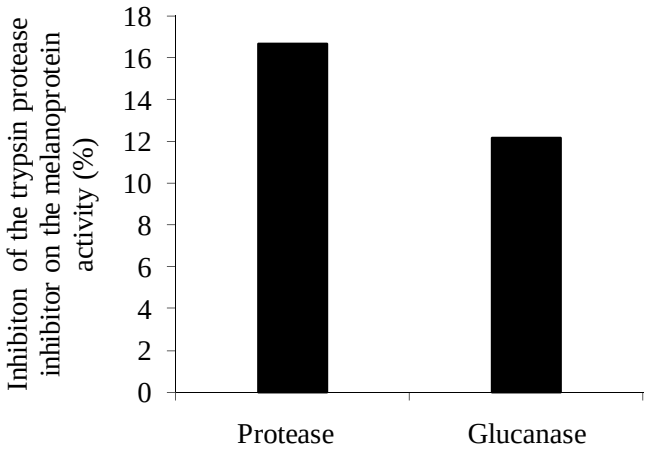


Figure (48): Effect of the trypsin inhibitor on the protease activity of the recombinant protein. Assay was done as described at section 4.4.16).

5.10.13 Thermostability of the melano protein

Before protease activity done and after purification with heat treatment (20 min at 70 °C), the recombinant melano protein was incubated at 70 °C for 0, 5, 10, 15, 20, 25, 30, 35, and 40 min time intervals (Figure 49). Protease activity indicated on that the protein is very stable for long time.

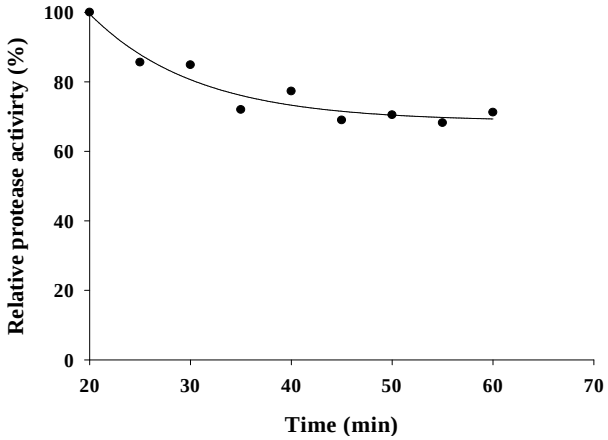


Figure (49): Thermostability of the recombinant melano protein and protease activity at 70 °C for 20, 25, 30, 35, 40, 45, 50, 55, and 60 min preincubation time. Investigated assay was done as described under section 4.4.11.

5.10.13 BSA proteolysis

BSA was used as starting material for digestion with the recombinant melano protein to show the digestion pattern on Tris-Tricine PAGE. The digestion pattern as appear on the SDS PAGE gives strong indicator for digestion (Figure 50). The starting band which signed as blue arrows (78 kD) was used as strain material for 12 and 24 h digestion period at 40 °C. After 12 h the main band still existing and the digestion pattern gave more than 7 main band products with an appearant molecular mass of 47 kD. Whereas prolonged digestion time (24 h) cause complete digestion of the main band (lane 2) and one of the big product band (47 kD) still existing (Figure 50).

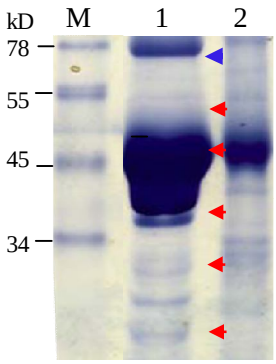


Figure (50): 12% Tris-Tricine PAGE for BSA digestion with the recombinant melano protein. Lane 1: digestion for 12 h, and lane 2: digestion for 24 h, respectively, and M Seeblue marker. 15 µg digested protein were loaded per lane. The digested band is signed in red arrow after 12 h. And the upper main band (before digestion) signed in blue arrows.

5.11 Investigation related to cultivar cuticle

5.11.1 Cuticle permeability

Cuticle permeability of cv. Elstar (Figure 51) could help in the process of infection. This study was done by using toluidine blue to measure permeability. It was clear that toluidine blue invaded cuticle of Elstar and stained the internal tissue, but could not do that for Remo cultivar (Figure 51).

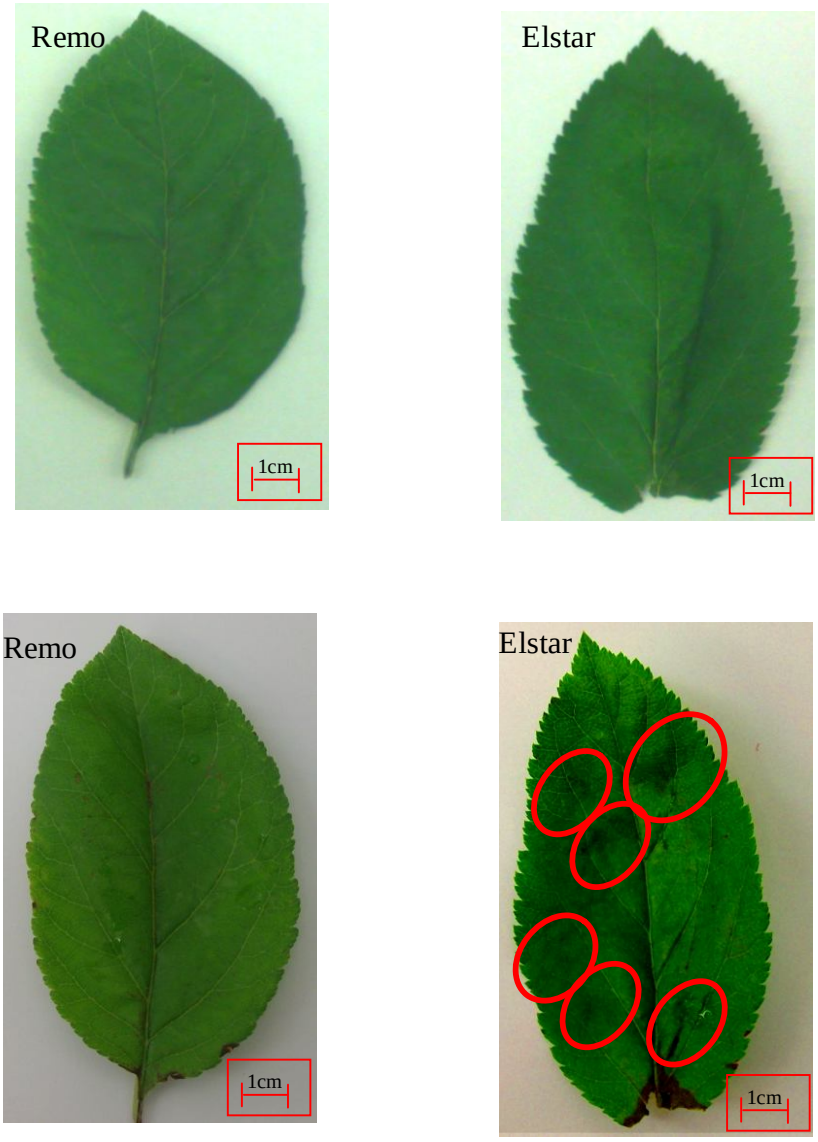


Figure (51): Permeability of cuticle of the upper surface of the leaf using 0.05% of the toluidine blue. The permeability was done as described under section 4.8.1.

5.11.2 Impression silver staining method

The staining method was developed for proteins which bind to the silver ion in poly acrylamide PAGE but in this time the method was modified to use in staining of the membranous film (impression replica) of the surface impression (Figure 52) of the *in vitro* plant leaves (*M. domestica* cvs. Elstar and Remo). The staining method used to detect the presence of proteins or proteins related compounds.

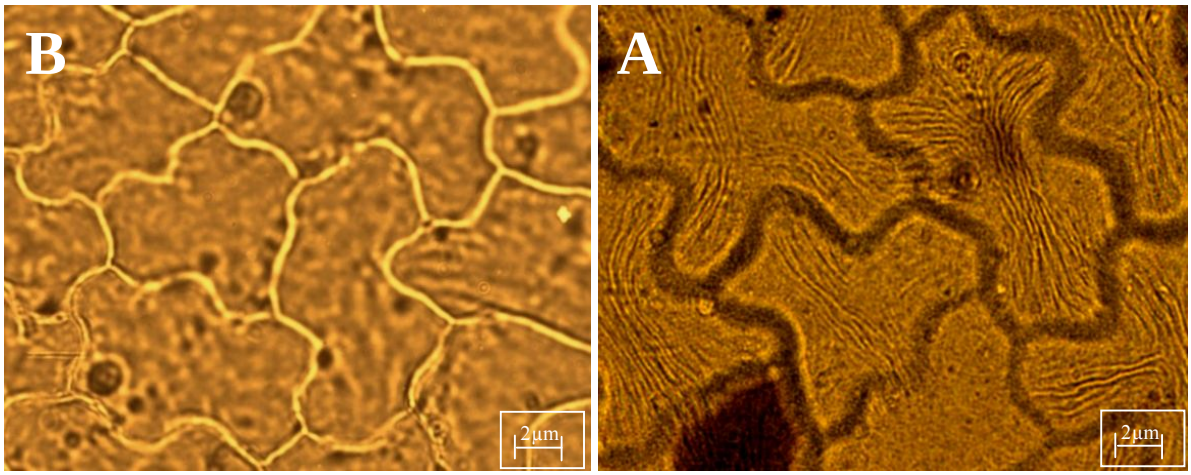


Figure (52): Replica-cuticle staining using silver staining for Remo (A) and Elstar (B) as protein indictor. The staining was done as described under section 4.8.2.

5.11.3 Impression staining using bromophenol blue

In the impressions below (Figure 53), the thickness of the cells wall of Remo was thicker than Elstar cultivar and the protein deposit in the cells wall of Remo is higher than in the cv. Elstar. The protein or protein like compound deposited in the lines of the longitudinal lines of Remo cultivar.

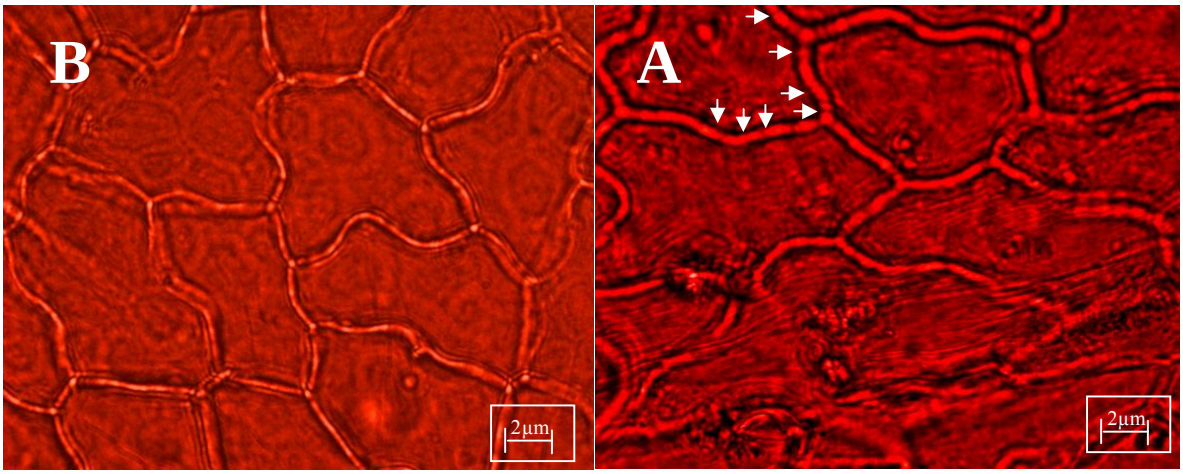


Figure (53): Cuticle-replica staining with acidified bromophenol blue and fluorescence staining under UV light from Remo cuticle replica (A) and Elstar (B) as protein indictor. The white arrow indicates on presences of protein layers stained beside the cell wall of Remo and continues but in Elstar is very week. The staining was done as described under 4.8.3 section.

5.11.4 Cell wall thickness calculation

The cell wall thickness in Elstar (57.4%) *ex vitro* cultivars (fifth leaf from the top) is less than the cell wall thickness in Remo (100%). That means cell wall thickness of cv. Remo could use this advantage as a tool in defense mechanism against infection of *V. inaequalis* (Figure 54).

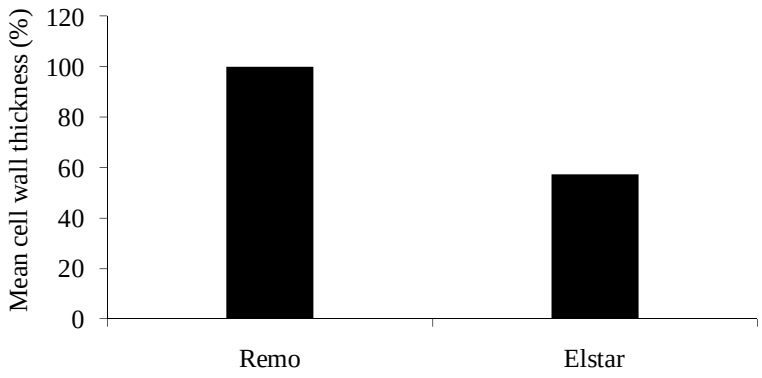


Figure (54): Cell wall thickness of the epidermal cells of *M. domestica* cvs. Remo and Elstar. The thickness calculated as percentage of Remo cultivar.

5.11.5 Impression interaction with the melano protein (plant technique to discover the surface protein which works as receptor or for plant defense)

These harvested proteins by replica were bloted directly for interaction on the replica (Figure 55 E&F).

This method can give an overview for the melano protein secretion position from *V. inaequalis* during infection because the pattern of the melano protein could help in the imagination in the way of infection and give an additional explanation for melano protein of *V. inaequalis* role during the process of infection (Figure 56 C and D). The interactive protein plot gave an overview about the localization of the protein in the cuticle membrane, the way of secretion of the interactive protein and the function of the pattern of localization in defense response (Figure 56).

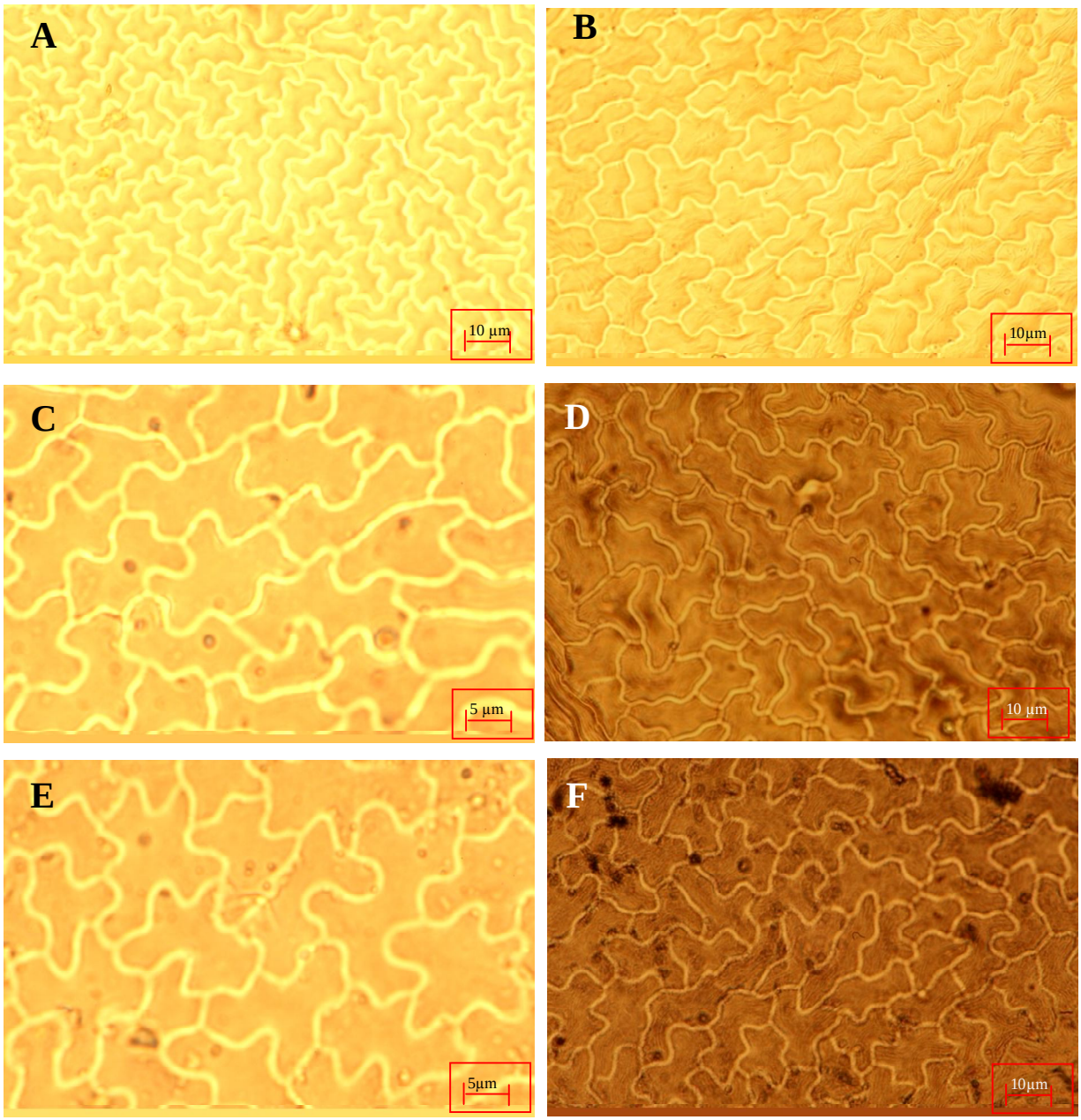


Figure (55): Interaction of the recombinant melano protein and the surface plant protein of the *in vitro* Remo cultivar including A (Elstar) and B (Remo): Control incubated with first antibody of the melano protein, C (Elstar) and D (Remo): Incubated with melano protein after the blocking step, E (Remo) and F (Remo): Incubated directly with the melano protein. The melano protein (100 µg/ml) diluted 1:200 and its antibody 1: 500.

In Figure 56 the infection stages were monitored by the replica system blotting and shows no lesion in cv. Remo (A, B), whereas in cv. Elstar shows large difference and development stages of the lesion (C, D, E, and F, respectively). This method can used for lesion frequency determination by counting number of lesion per cm leaf surface area (F) or used in detection of the fungal establishment (Figure 57 A and B).

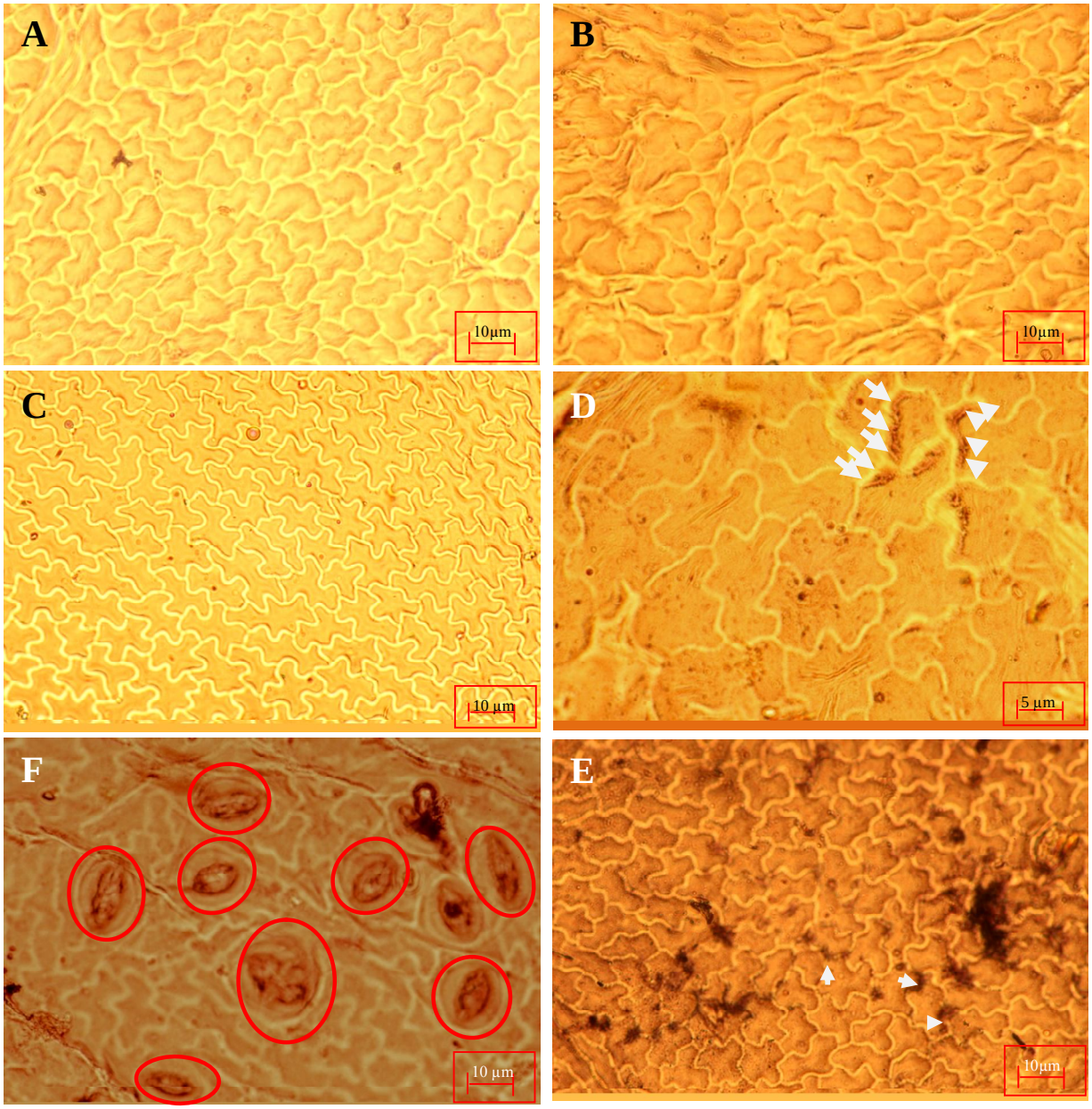


Figure (56): Immune-replica blot for detection of the melano protein of the *V. inaequalis* during the infection for *ex vitro* cvs. Remo and Elstar and mointoring the infected stages of Elstar by using the melano protein antibody. A: Control of cv. Remo, B: Remo replica incubated with the melano protein antibody, C: Control of cv. Elstar, D: Replica of cv. Elstar show the excreted fungal protein beside the interface of the cells wall between the cells in the early stage of infection (white arrow), E: Replica of cv. Elstar show the next stage of infection and the increase in melano protein amount beside the interface of the cells wall (white arrow) and F: Replica of cv. Elstar show the presence of the melano protein around the infection holes or pores after cuticle invasion (red ring). The melano protein (100 µg/ml) diluted 1:200 and its antibody 1: 500.

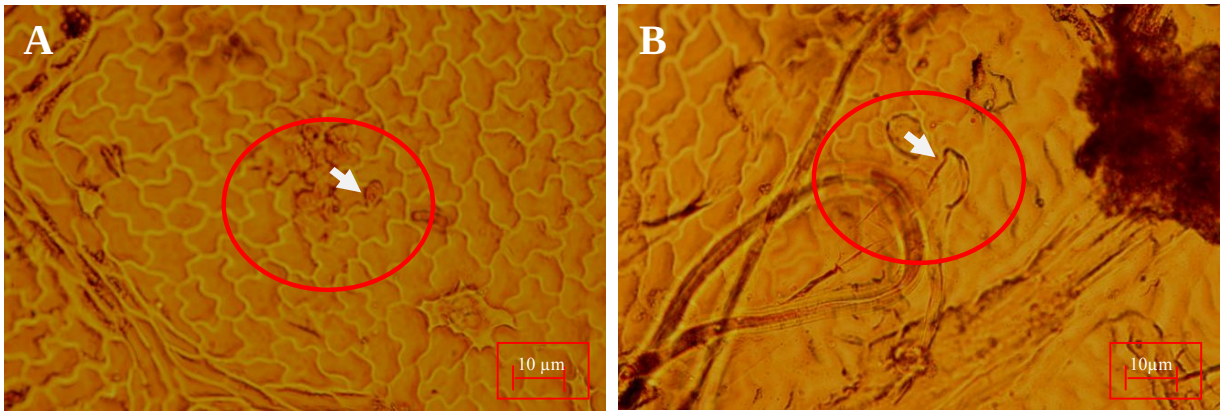


Figure (57) Monitored conidia on the upper surface of the *Malus domestica* cv. Elstar (ring) in the early infection stages (A) and after establishment (B). The melano protein (100 µg/ml) diluted 1:200 and its antibody 1: 500.

5.11.6 Surface protein

5.11.6.1 Isolation and interactivity on PVDF membrane

For further investigations the proteins were isolated from the replica and separated with SDS PAGE and subsequently technique (Figure 58). The data shown include also cuticle protein interference or interaction transferred onto PVDF membranes. For the identification of putative, interactivity melano protein partners were incubated with the recombinant melano protein and stained by the anti melano protein antibody.

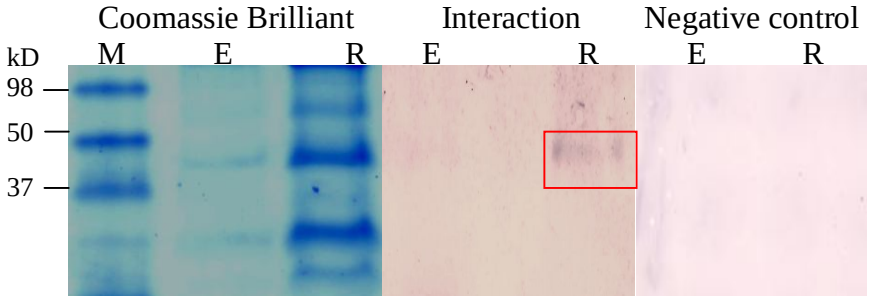


Figure (58): SDS-PAGE for the cuticle protein isolation from cuticle of *M. domestica* cvs. Remo (R) and Elstar (E) and interference of this protein with the recombinant melano protein. Each lane was loaded with the same area of cuticle membranes. The interaction was done in the presence and absence of recombinant melano protein. The melano protein (100 µg/ml) diluted 1:200 and its antibody 1: 500.

5.11.6.2 Cuticle chitinase activity

The extracted protein from the surface of the cuticle of the resistant and susceptible cultivar contains amount of proteins but in the resistant one there are many folds of proteins. As a tool for the defence mechanisms the chitinase activity was tested on SDS PAGE to investigate the defence mechanisms in the Remo and Elstar cultivars. It was founded that Remo contained chitinase activity whereas Etstar has not this activity. That was also supported by the MS analysis of these bands (45 kD, Figure 59).

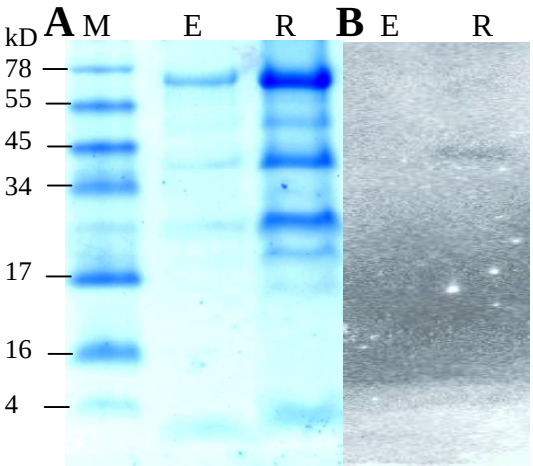


Figure (59): 10% Tris-Tricine PAGE for the cuticle protein chitinase activity from the cuticle of *M. domestica* cv. Remo (R) and Elstar (E). Gel contains 1% chitin. **A**: Coomassie Brilliant Blue, **B**: Chitinase PAGE activity.

5.11.6.3 Mass spectroscopy for cuticle proteins

The ESI Q-TOF mass spectroscopy analysis for the interacted surface plant protein with the melano protein and extracted from the replica revealed that *de novo* sequence of the tryptic fragments from a 48 kD band from the isolated cuticle protein of the resistant apple cv. Remo has significant homolgy to chitinase from *Pyrus pyrifolia,* NADP dependent sorbitol 6-phosphate dehydrogenase from *Amelanchier alnifolia*, and Hypothetical protein from *Vitis vinifera* (Table 18). The chitinase is supported by the SDS activity PAGE.

Table (18): ESI Q-TOF mass spectroscopy of tryptic peptide fragments and identification of the derived peptide sequences from cv. Remo by homology search. § NCBI accession number; Swiss Prot accession number.

Charge	Mass [D]	Derived amino acid sequence	Sequence similarity	Organism	Accession number
2	940.4241	K.DLGMDAYR.F	Hypothetical protein	*Vitis vinifera*	gi/225461189
1	882.4506	R.FSISWSR.I	Hypothetical protein	*Vitis vinifera*	gi/225461189
1	715.3386	R.SFNGFGTTGDVATR.K	Chitinase	*Pyrus pyrifolia*	gi/222139388
1	428.7669	K.AATLGLIR.N	NADP dependent sorbitol 6-phosphate dehydrogenase	*Amelanchier alnifolia*	gi/19310930

5.12 Apoplastic fluid *M. domestica* cvs. Remo and Elstar

5.12.1 Isolation of the apoplastic fluid Tris-Tricine PAGE

The isolated proteins from the apoplastic fluid from *ex* and *in vitro* culture of *M. domestica* cvs. Remo and Elstar were separated on Tris-Tricine PAGE. The available information of these proteins was recorded to have defensive role. It is known from the literature that the apoplastic fluid contains many of the PR proteins likewise cysteine protease (Protease-protease interaction) cysteine protease inhibitors (inhibitory role) to stop the infection by inhibition of hydrolytic enzymes.

5.12.2 Apoplastic fluid interaction with the recombinant melano protein

The interactive protein blot (Figure 60 A) of the apoplastic fluid gives positive signale with the melano protein antibody for both *ex vitro M. domestica* cvs. Remo (lane 1), *in vitro* Remo (lane 2 and 3) and *in vitro* Elstar (lane 4). These results could explain why *M. domestica* cv. Remo cultivar is resistant and *M. domestica* cv. Elstar is susceptible. On the other hand for

the western blot (Figure 60 B) in presence of melano protein of *V. inaequalis* as a positive control, the antibody gave positive interaction only with melano protein (Mel). The interaction pattern between the melano protein and the apoplastic fluid (Figure 60 C) against melano protein antibody gives a positive result.

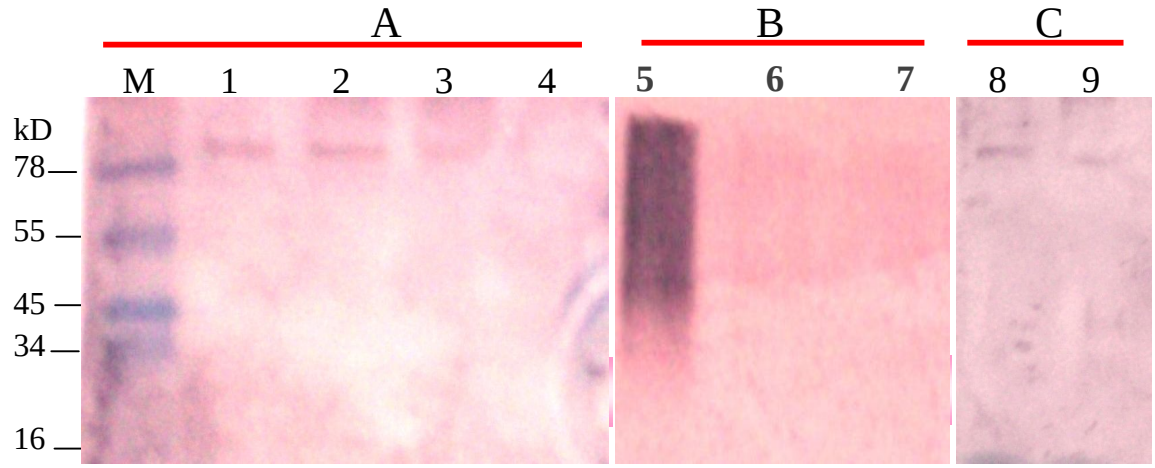


Figure (60): Interaction blots. A: A positive interactive protein blot (modified western blot) for both *ex vitro M. domestica* cv. Remo (lane 1) and *in vitro M. domestica* cv. Remo (lane 2 and 3) and *in vitro M. domestica* cv. Elstar (lane 4); B: A negative interactive protein blot (ordinary western blot) for melano protein (positive control) of *V. inaequalis* (lane 5) and *ex vitro* apoplastic fluid of *M. domestica* cv. Remo (lane 6 and 7); C: A Positive interactive protein blot (modified western blot) for the apoplastic fluid of *M. domestica* cv. Remo (lane 8 and 9). Each lane loaded with 20 µg protein. The antibody (produced in goats) and applied in 1: 500 ratios. M is marker. The interacting melano protein (100 µg/ml; A and C) diluted 1:200.

5.12.3 Inhibiting effect of the apoplastic fluid to the glucanase and protease activity of the recombinant melano protein

The effect of the apoplastic fluid (*M. domestica* cv. Elstar and Remo) on both the glucanase activity (Figure 61 A) and protease activity of the melano protein (Figure 61 B) was done and show strong inhibitory effect on glucanase activity on the other hand they inhance the protease activity. The apoplastic fluid from Elstar and Remo reduced glucanase activity to 54.4% and 22%, respectively. On the other hand, it increased the protease activity to 112.4% for Elstar and 149.6% for Remo with higher impact for Remo.

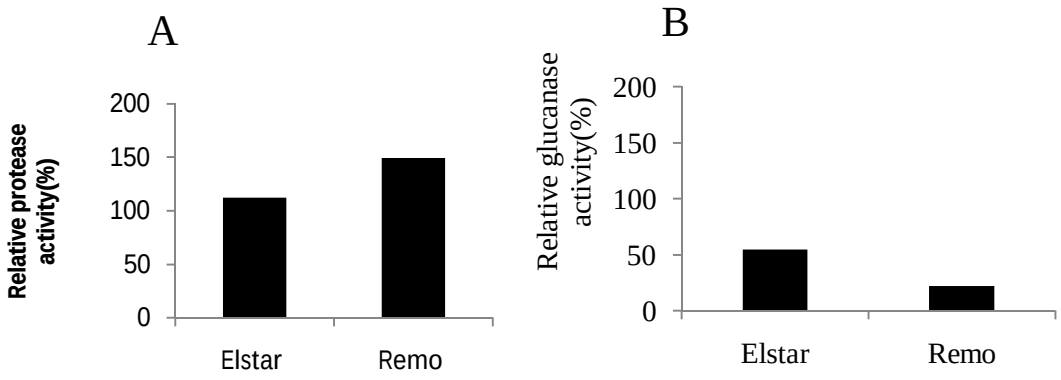


Figure (61): Effect of the apoplastic fluid from *M. domestica* cvs. Elstar and Remo on, A: The glucanase activity, B: The protease activity. The Figure also includes the control for comparison. The determination was done with 15 µg melano protein in presence of 30 µg/ml of apoplastic fluid. Untreated samples were calculated as 100%

5.12.4 Effect of the apoplastic fluid on *V. inaequalis* conidial germination

Effect of the apoplastic fluid (Figure 62) of Remo show high inhibition on the conidial germination either *in vitro* or *ex vitro*. It can be concluded that Remo can do complete inhibition for all the conidia at 15 µg of the apoplastic fluid protein for 156 conidia (control). These means 1 µg protein /µl of the apoplastic fluid were needed for inhibition of 10 conidia germination. This means each conidia needs 100 ng of the apoplastic fluid proteins (The inhibition must not relate to proteins) for germination inhibition. On the other hand Elstar apoplastic fluid cannot inhibit all the conidia till this concentration or till 30 µg proteins this means the effective proteins is not present in high amount. The curve pattern indicate on the inhibition was not coming from one factor. And these supported by the induction of more than one of the PR proteins.

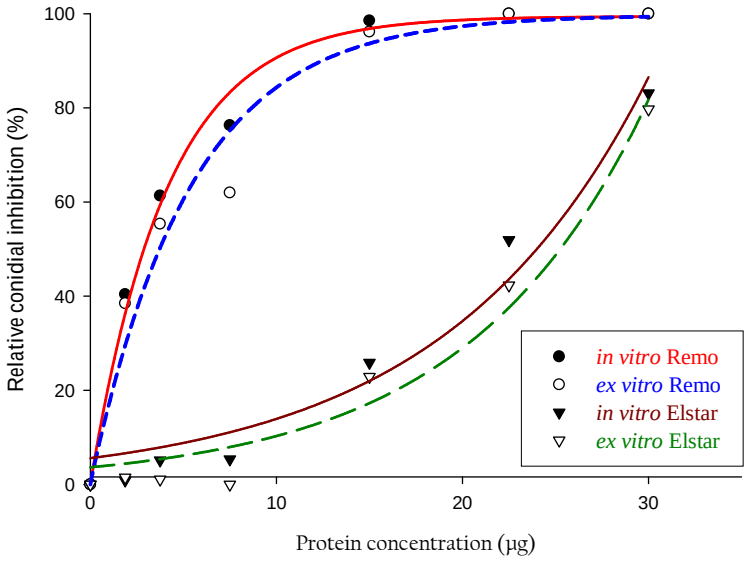


Figure (62): Effect of the apoplastic proteins extracted from *M. domestica* cvs. Remo and Elstar on the conidial germination of *V. inaequalis*. Inhibition assay was done as determined under the microscope given under 4.1.6.

5.13 Interaction of the melano protein with total plant proteins

5.13.1 Protein isolation and interaction

The melano protein interferes with most of the plant protein of both cvs. Remo and Elstar (Far Western Blot). Other proteins were susceptible for attacking by the melano protein as a substrate according to the protease activity. In general, Remo cultivar interacted in high amount than Elstar cultivar that leads us to the hypothesis that Remo protein has general resistance than Elstar proteins for proteolysis (Figure 63). The previous results lead to PVDF-protease activity developments according to the decrease of the band intensity of the interaction and the inhibition test.

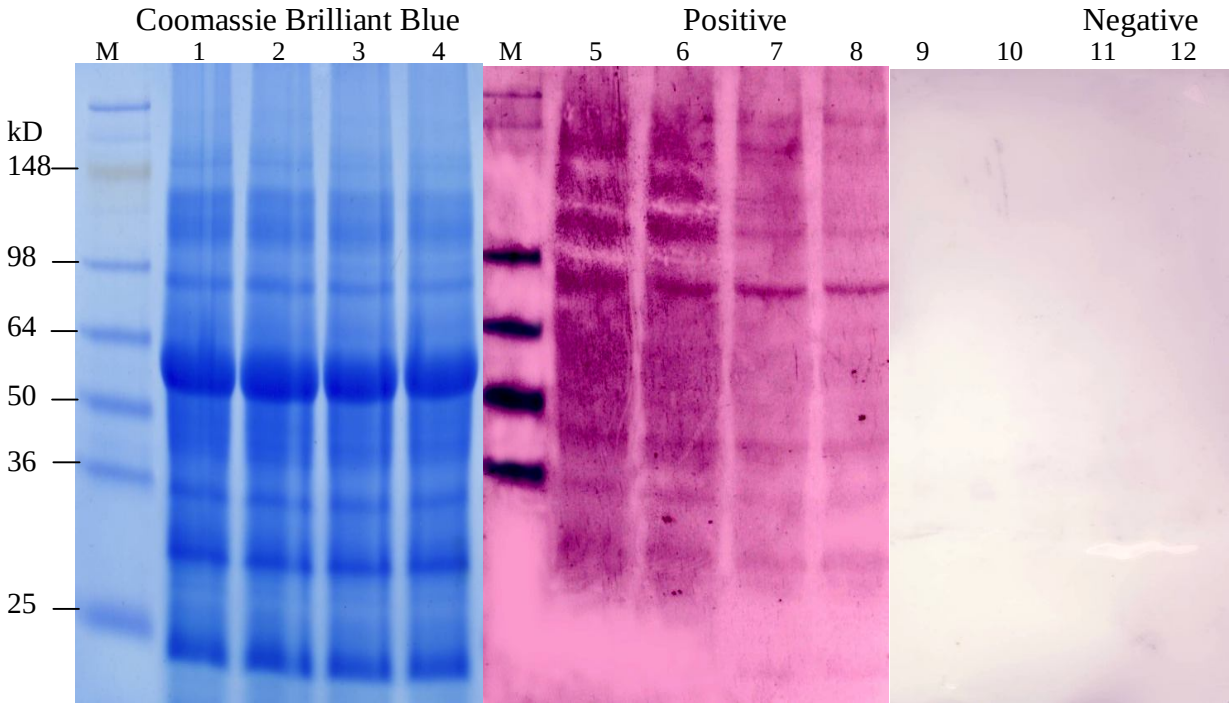


Figure (63): Total protein interference against the recombinant melano protein for total extracted protein from *M. domestica* cvs. Remo and Elstar. Positive is the incubation with the melano protein for 2 h and negative incubated only in blocking buffer gel for interactivity with melano protein. The opposite gel was stained with Coomassie Brilliant Blue (Right) and the middle one was the interactive gel and the left was the negative control. M is the marker. Remo loaded (15 µg protein) in lanes 1, 2, 5, 6, 9 and 10, and Elstar loaded (15 µg protein) in lanes 3, 4, 7, 8, 11 and 12.

5.13.2 Protease activity on PVDF membrane

The protein band intensity in the interaction on PVDF membrane decreased as the incubation time (2, 4, 6, 10, 18 and 28 h) of the membrane with the recombinant melano protein increased. This action might be due to the protease activity of the melano protein which digested the plant proteins (Figure 65) and used it as substrate. Another method (See 4.10.2.1.3) was used for detection of the melano protein protease activity on PVDF membrane

of the separated plant proteins. The method depends on the existence and intensities of the plant proteins bands in comparsion to the untreated one (control, Figure 64) which decreased due to the action of melano protein.

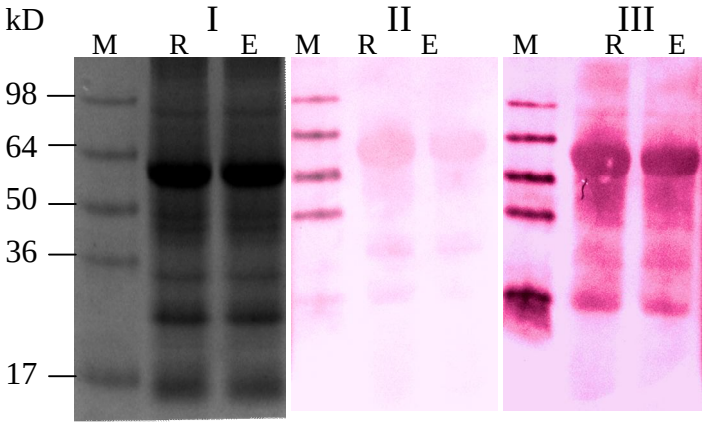


Figure (64): Total protein interference blots against the overexpressed melano protein for total extracted protein from *M. domestica* cvs. Remo (R) and Elstar (E) stained with Coomassie Brilliant Blue (I). The midlle membrane is a positive interacted total plant protein extract with the melano protein 10 h (II) and negative incubated only in blocking buffer (III). The opposite gel was stained with Coomassie Brilliant Blue. M is the marker.

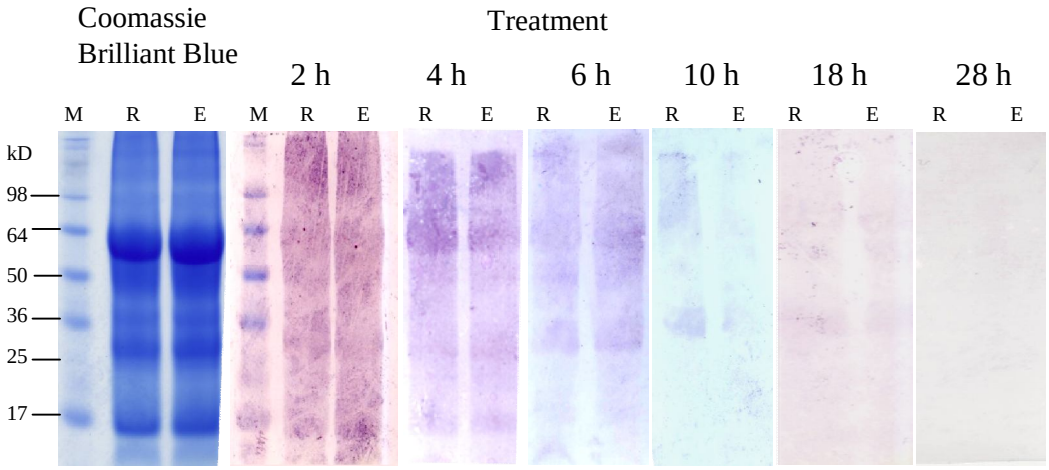


Figure (65): Tris-Tricine PAGE (10%) of *M. domestica* cvs. Remo (R) and Elstar (E). Total proteins of cvs. Remo (R) and Elstar (E) interaction with recombinant melano protein in different incubation time 2, 4, 6, 10, 18 and 28 h, on the other hand the right PAGE for the plant total protein extract stained Coomassie Brilliant Blue. M: Seeblue marker. Protein interaction was done as

5.13.3 Protease activity inhibition on PVDF membrane

Protease activity on PVDF membranes were inhibited with $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) mixture or PMSF (1 mM). All lanes in the positive control has no interaction according to the method of interaction that means there are possibility for the inhibition of the protease activity or enzymatic activity on the nitrocellulose activity and the interactivity on the membrane was confirmed (Figure 66).

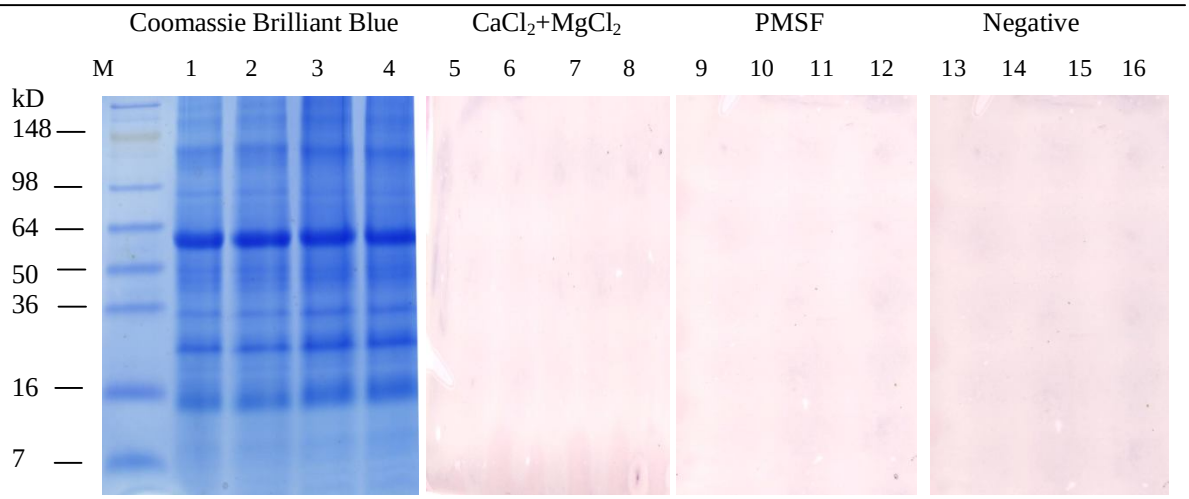


Figure (66): Total protein extract interference blots against the overexpressed melano protein from *M. domestica* cvs. Remo (1, 5, 9, 13 *in vitro* and 2, 6, 10, 14 *ex vitro*) and Elstar (3, 7, 11, 15 *in vitro* and 4, 8, 112, 16 *ex vitro*). Positive is the incubation with the melano protein (melano protein containing a mixture of $CaCl_2$ and $MgCl_2$ (1 mM), 30 min before incubation), PMSF (1 mM), 30 min before incubation), and negative incubated only in blocking buffer and the melano protein antibody. M is the marker.

5.14 Impact of melano protein to bacterial growth

5.14.1 Effect of melano protein on the bacterial vitality using the growth method

It is shown in figure 67 that, the gram positive bacteria inhibited by 99 and 98% as percent, respectively. The gram negative bacteria (*Erwinia amylovora* and *Agrobacterium tumefaciens* LB A4404) inhibited by 14 and 18%, respectively (Figure 67). On the other hand the application of 1 mM of $CoCl_2$ can improve the inhibition for most of the bacterial species. Application of cobalt on the culture of *E. amylophora* was inhibiting both the treatment and the control even with low concentration 200 µM (Figure 67).

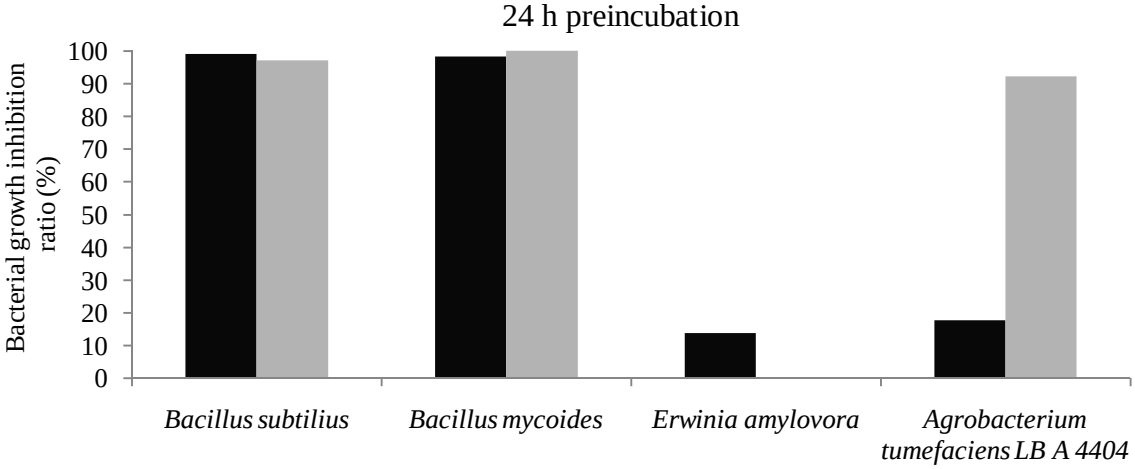


Figure (67): The effect of recombinant melano protein on growth of different gram positive and negative bacterial species (Black) and the effect of melano protein in presence of 1 mM $CoCl_2$ (See 4.6).

5.14.2 Melano protein and bacterial protein interaction

The interaction of the melano protein with the bacterial proteins (*Agrobacterium tumefaciens LB A4404*, *Bacillus mycoides, Bacillus subtilius,* and *Erwinia amylovora,* respectively) after 3 h incubation on the PVDF membrane, if the time extended to 8 h the melano protein destroyed the bacterial proteins completely (Figure 68).

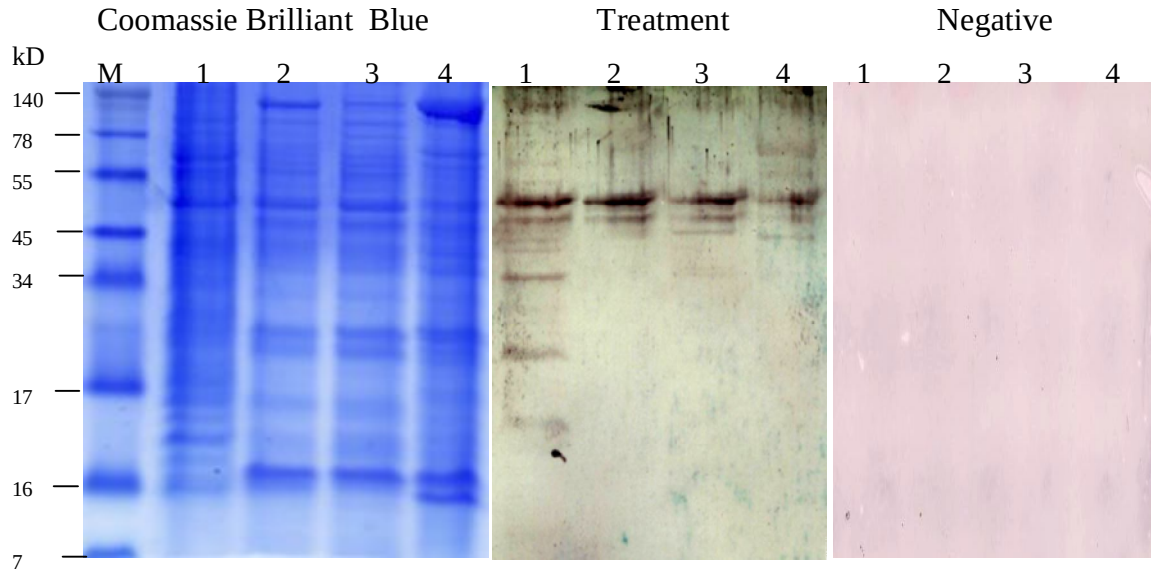


Figure (68): Coomassie Brilliant Blue staining (right side) for the bacterial stain *A. tumefaciens* LB A4404 (lane 1), *B. mycoides* (lane 2), *B. subtilius* (lane 3) and *E. amylovora* (lane 4), respectively. The interaction with the same order for the four species was done. M is the marker. 15 µg protein were loaded in each lane.

5.15 Plant section staining and interaction to the melano protein

5.15.1 Section staining with bromophenol blue

Most of the organic substances make fluorescence as action of π-π transition. when bromophenol blue bind to cations in presence of DDT and acidic condition (acetic acid) it gives fluorescence and when it bind to transition metal in the acidic pH, fluorescence is decreased (Figure 69). The preliminary experiment (Figure 69) lead to develop or using of bromophenol blue in staining of the plant section and to detect the high concentration of the interested metal like calcium, magnesium, and the transition metal under the UV light and visible light, respectively (Figure 69).

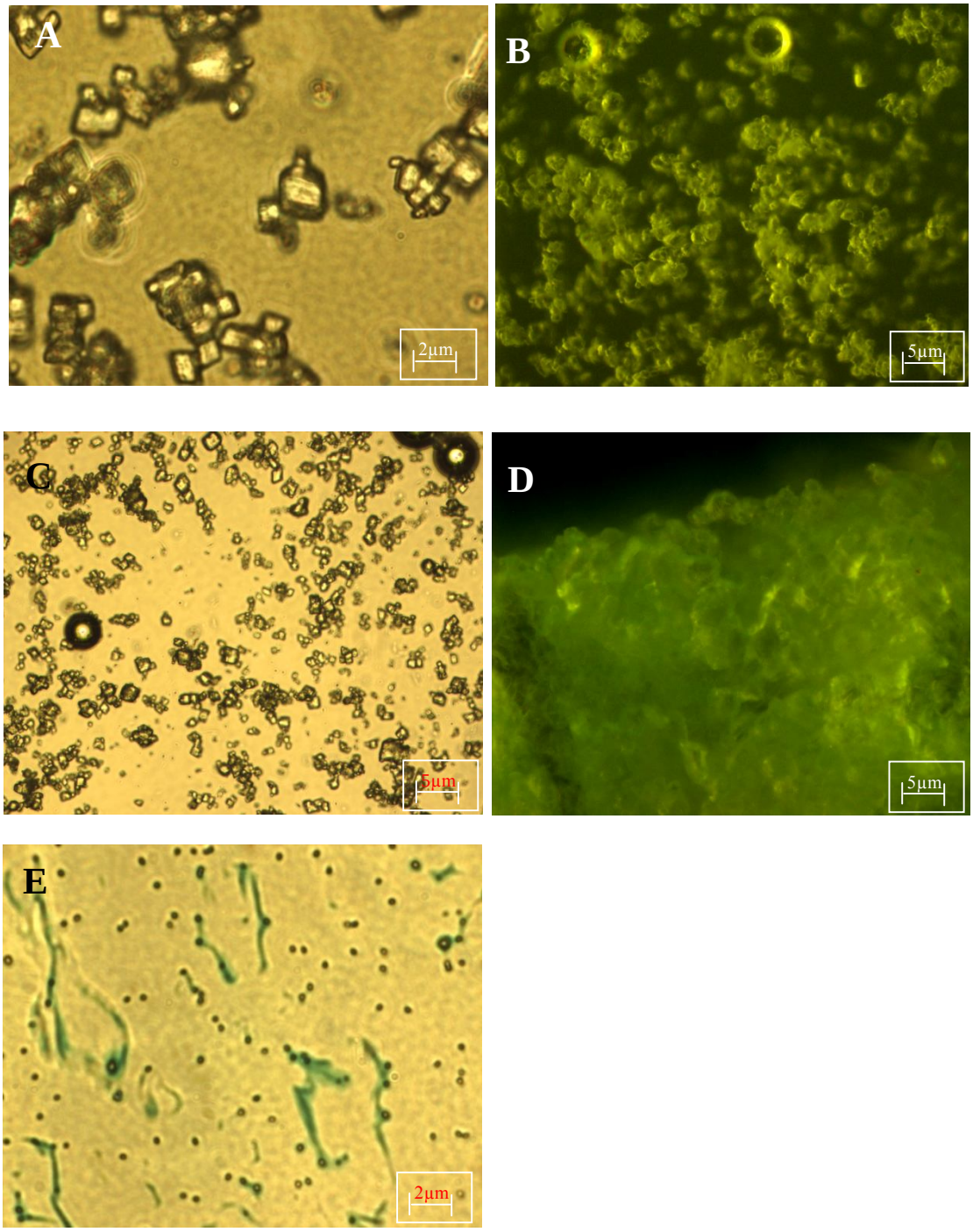


Figure (69): Calcium detection by acetified bromophenol blue. A: Calcium crystal bright field (1000 x magnification), B: Calcium crystal Dar field under UV light (400 x magnification), C: Calcium crystal bright field (400 x magnification), D: Calcium salts under the UV light (400 x magnification), E. cobalt crystal bright Field (400 x magnification).

The intensity increases by shifting of the colour from green to yellow under UV light. That increases of the calcium concentration in the region of cuticle in cv. Remo many folds more than cuticle layer of Elstar (Figure 70).

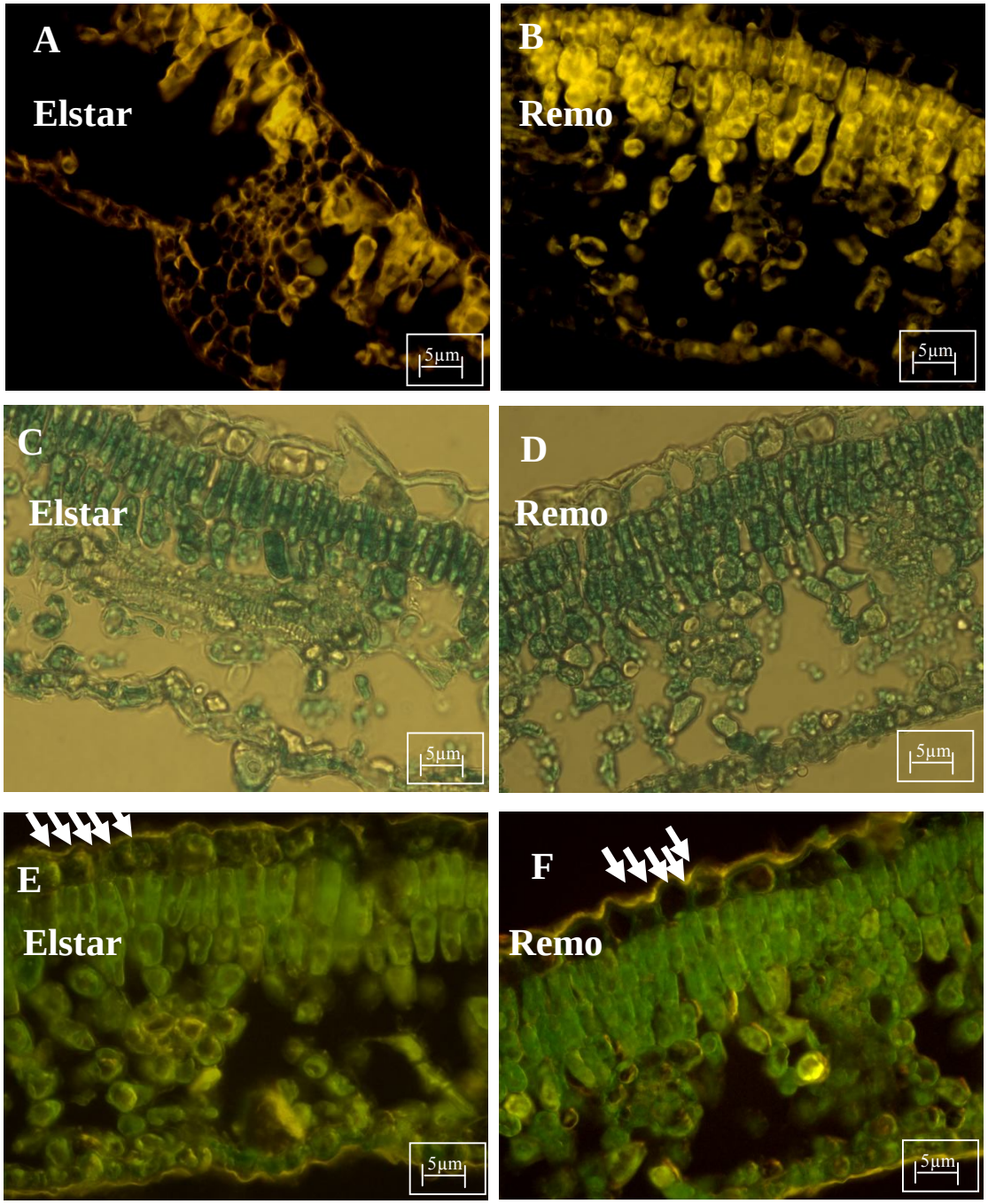
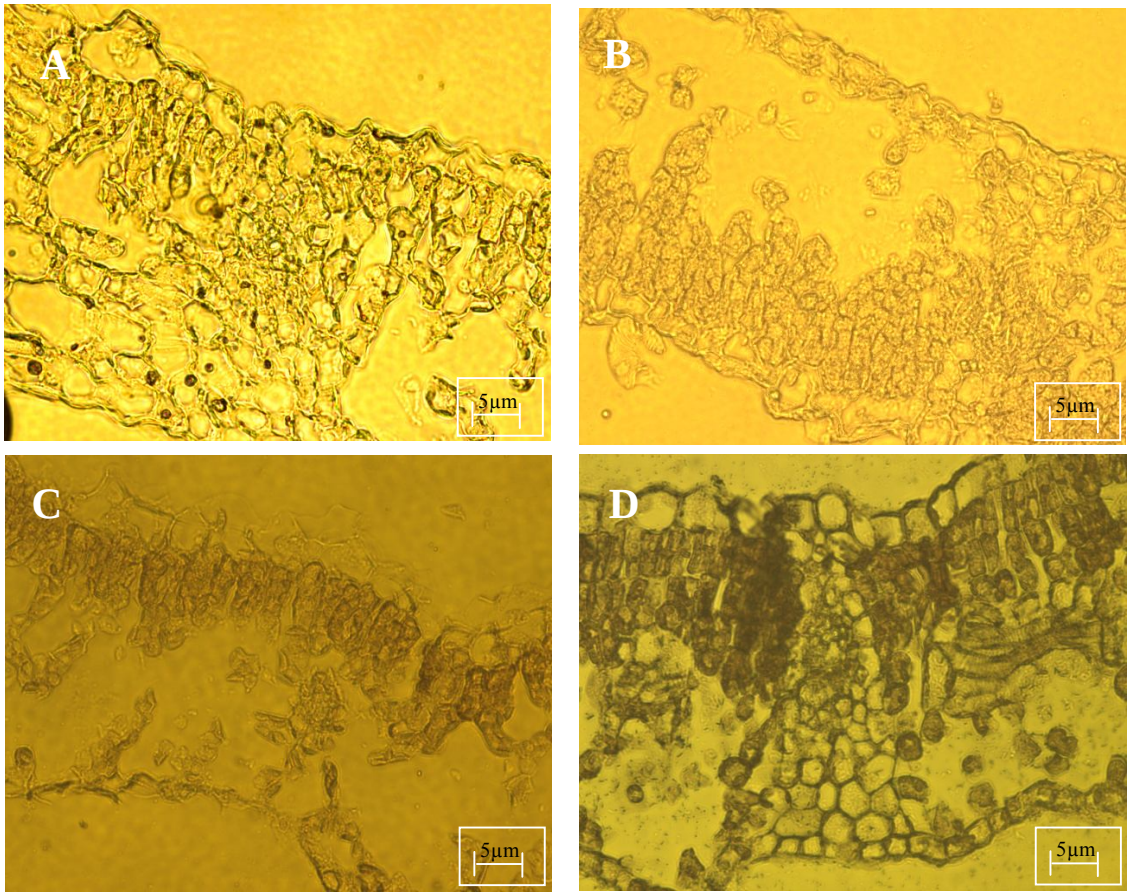


Figure (70): Transverse section for *M. domestica* cvs. Remo and Elstar, A and B. For auto fluorescence under UV (400 x magnification), C and D. The bright field of Elstar and Remo cultivars stained with the bromophenol blue (400 x magnification), and E and F. The same stained section under the ultraviolet fluorescence (400 x magnification). The white arrows give a good indication for the cuticle thickness difference or the cuticle salt content differences between Elstar and Remo cultivars (E and F, respectively).

5.15.2 Detection and localization of plant protein interaction parteners in tissues of *M. domestica* cvs. Remo and Elstar with recombinant melano protein

Sections of *M. domestica* cvs. Elstar and Remo were submitted for interaction (local plant proteins- mleno protein). Figure 71 A&B used as a negative control giving a clear background for leaves sections interaction. As shown the plant proteins have positive interaction (C, D, E, F, G and H, Figure 71) through the different tissues of the leaf with the melano protein. The sections interaction of Remo (Figure 71 D) cultivar has higher interactive protein amount more than Elstar (Figure 71 C) in the apoplast region of the epidermal cells and vascular bundles (Figure 71 E&F). The interaction with the melano protein going on through the palisade tissue cells (Figure 71 H) and their organelles like chloroplasts (Figure 71 G and H)). The apoplastic fluid (Figure 71 G and H) of the epidermal cell gave a strong signal interaction in Remo than Elstar cultivar. The previous records support that, apoplastic fluid proteins importance in defense mechanism and theory of resistance. The defense proteins are constitutive expressed in Remo but in Elstar they are induced. That explains why Remo gave stronger signal than Elstar which was not inducted yet.



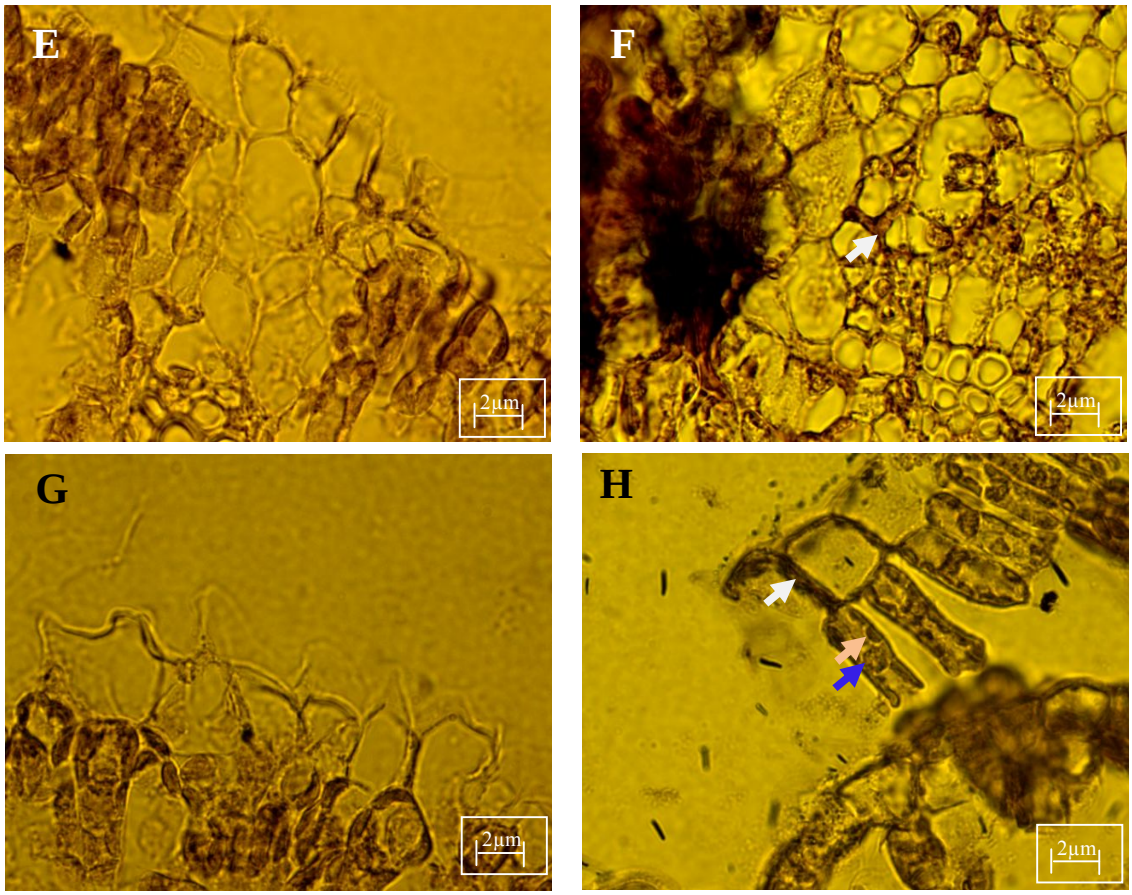


Figure (71): *In situ* interaction of the transverse section of *M. domestica* cvs. Elstar and Remo, A and B. The reaction of the first and the second antibody only (normal hybridization, control: 400 x magnifications). C and D, General view for the interaction of the melano protein and the plant protein *in situ* showing enhancement of Elstar protein degradation (400 X magnification). E and F. The pictures focus on the apoplastic fluid interaction in Elstar and Remo (400 x magnification). G and H. Interaction of the melano protein to the apoplast of the epidermal cells, the chloroplast and the nucleus membrane in the palisade tissue (100 x magnification). The white arrow = apoplastic fluid interaction, the red arrow = chloroplast, the blue arrow = the interaction of melano protein to the nucleus.

5.15.3 Vertical section for the surface protein interaction

Sections proteins interactions were done (72). The magnification concentrates more on the cuticle surface to determine the presences of protein on the plant surface of Elstar and Remo cultivars. The cuticle surface interaction of Remo indicated on the presences of some fixed protein on Remo cuticle upper surface and not in Elstar.

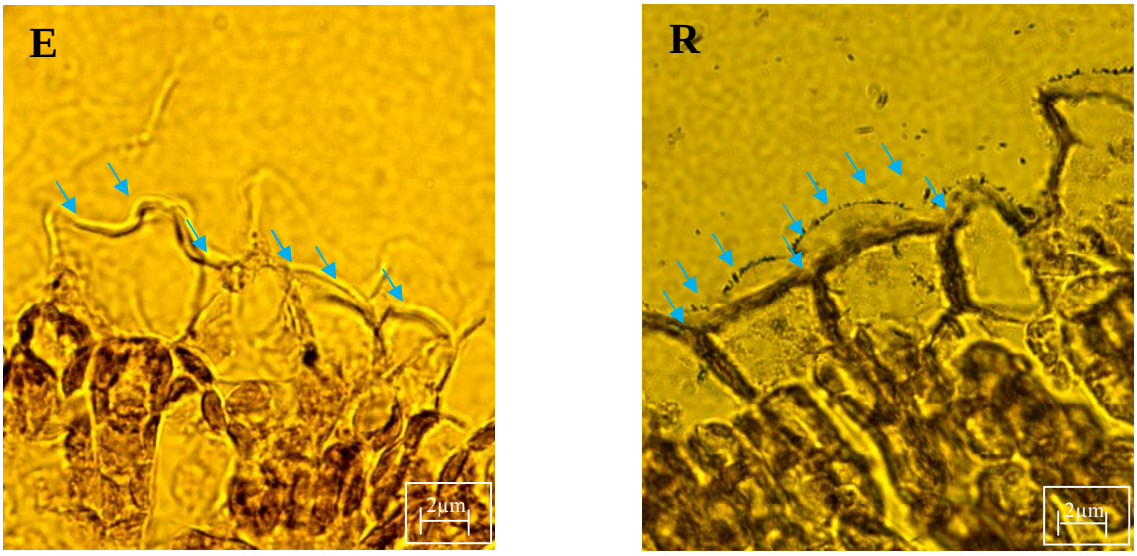


Figure (72): *In situ* interaction of the melano protein to *M. domestica* cvs. Elstar (E) and Remo (R) showing the difference of apoplastic fluid between the epidermal cells and the protein on the surface of the cuticle of Remo, this protein is not present on the surface of Elstar. Also there is no reaction inside the epidermal cells (1000 X magnification). The melano protein was diluted 1:100 and its antibody 1: 500.

5.15.5 Inhibition of melano protein interaction to the local plant proteins

The proteins interaction patterns through the sections of cvs. Remo and Elstar with the recombinant melano protein were done in presences of PMSF protease inhibitor (1 mM) and $CalCl_2$ (1 mM). The protein interaction inhibited completely and that support the importance of calcium in the defense work as one of the calcium increase task in the time of infection around the infection place. The use of the protease inhibitor also could lead to presence of protease inhibitor in the apoplastic fluid used by plant for defense (Figure 73).

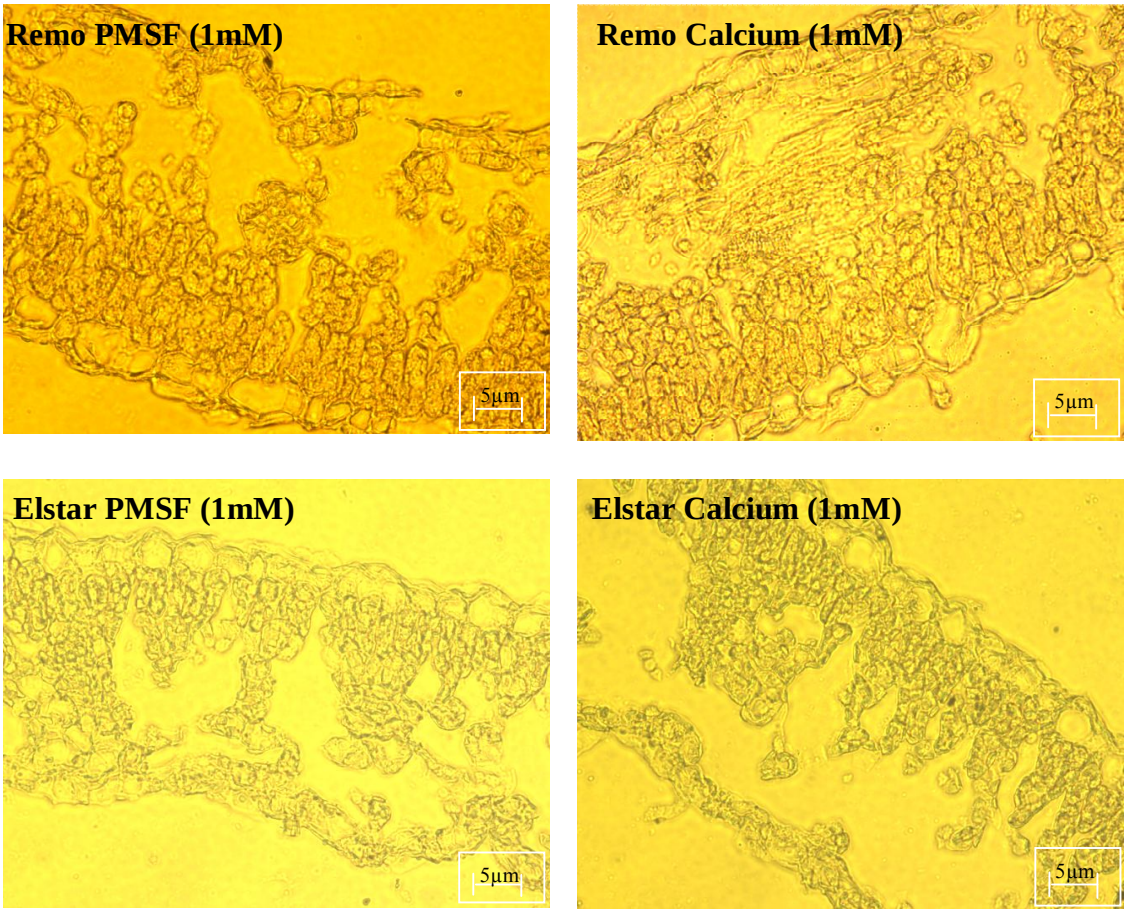


Figure (73): Effect of PMSF and $CaCl_2$ on the interaction of the melano protein to the *M. domestica* cvs. Remo and Elstar proteins. The magnification was 400 x. 1 mM calcium chloride and PMSF was inhibiting the interaction of the melano protein to the melano protein receptors and appears like the control for Remo and Elstar. The melano protein (200 µg/ml) was diluted 1:100 and the melano protein antibody diluted 1:500.

5.15.6 Detection of melano protein effect on the infected *M. domestica* cv. Elstar using the specific antibody

Melano protein of *V. inaequalis* was detected locally inside the infected tissue of *M. domestica* cv. Elstar by using melano protein specific antibody (1:500 dilutions with the blocking buffer). Epidermal cell triggered by the fungal melano protein to death. The cells collapsed gradually and then degraded due to the protease and glucanase melano protein action. As shown the melano protein spread from cell to cell inwards and by diffusion to digest everything in the leaf and used it as a food. At the end the fungal form necrosis by the death of the infected area and become black or brown due to fungal mycelia and melano protein stain (Figure 74).

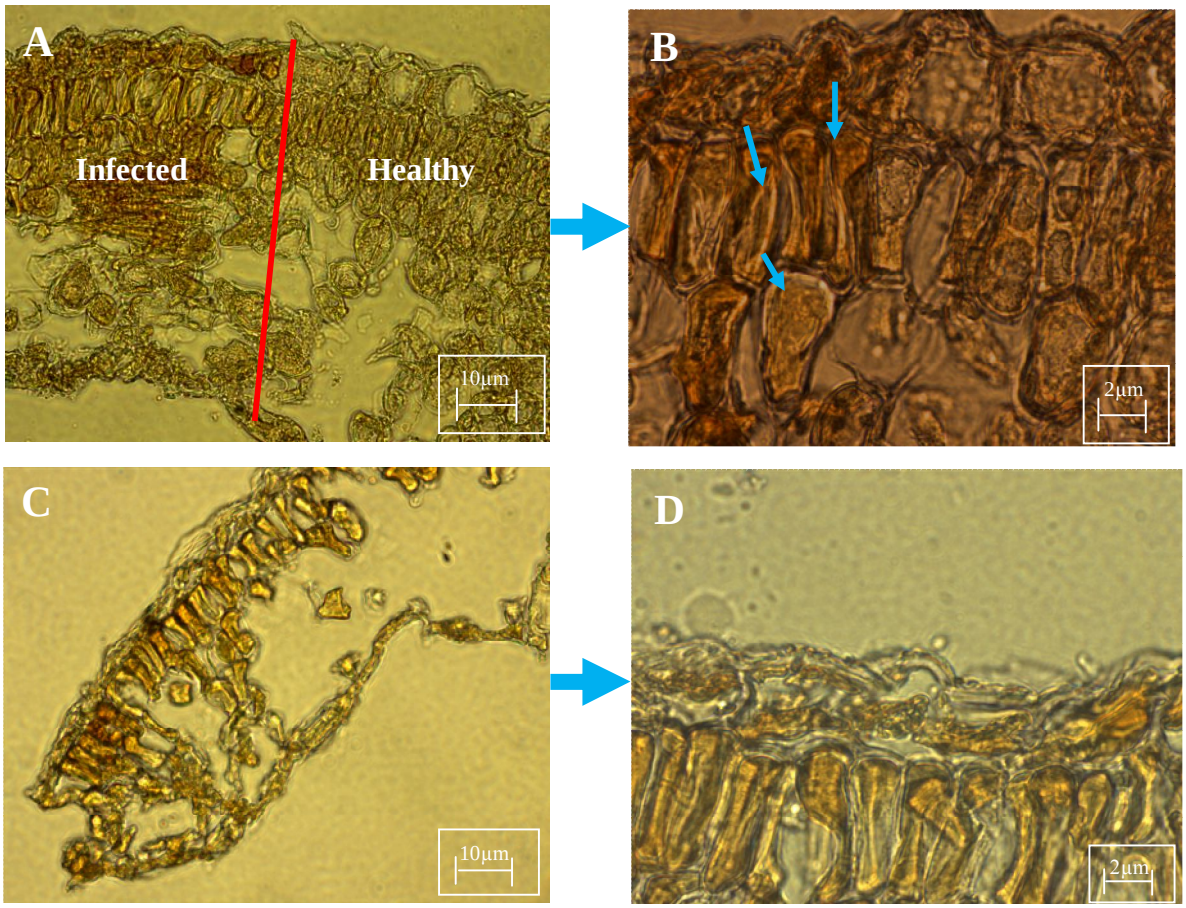


Figure (74): Infected *M. domestica* cv. Elstar by *V. inaeqaulis* containing melano protein damaged the cells and detected by the melano protein antibody. Different infected sections with different magnification to show the effect of melano protein in the hypersensitive pattern. A: Part of leaf show infection spread from infected to healthy signed with arrow and red line border between infected and uninfected, B: Stage of cells invasion with melano protein and debilitation of the cell content, C: Epidermal cells collapsing and debilitation, D: magnified parts of C. The melano protein antibody diluted 1:1000.

5.16 Cysteine protease Inhibitor

5.16.1 Sequence analysis

Cysteine protease inhibitor was identified in the apoplastic fluid of *M. domestica* cv. Remo by Gau *et al*., (2004). It was suggested that may have a role in defense mechanism. So that it is important to study its effect on melano protein protease activity.

The sequence of cysteine protease inhibitor is similar to that of *M. domestica* only there are stop codon (bold) in the middle but it was not know why it has the molecular weight 10.7 kD but the real over expressed one is less than 10 kD (Figure 75).

M A A V G T I R D N Q G S A N S V E T E S L A R Y A V D E H N
K K E N T L L E F V R V L D D K V Q V V S G T M H Y L K I **Stop**
V T D G G K K K V Y E A K V W V K P W E H F K Q V Q E F K L V
S D S **Stop**

Figure (75): Sequence of *M. domestica* cv. Remo cysteine protease inhibitor fragments.

5.16.2 Total protein analysis

As shown in Figure 76, induced cells give a band signed with red box (8 kD) in comparison to the negative control. The gel includes the negative control of the bacteria, which contains only the plasmid (lane 1), and the positive non-induced (lane 2) and the positive clone induced (lane 3). Gel was done from the total protein of the bacterial cells.

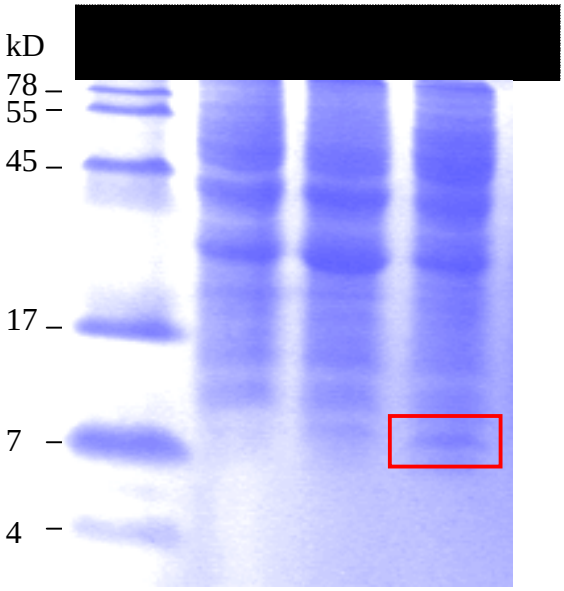


Figure (76): 12% Tris-Tricine for the total protein to detect the recombinant protein (cysteine inhibitor protease) pattern in *E. coli* (SG130009). The gel include the negative control (lane: 1), and the positive non-induced (lane: 2), and the induced positive control (lane: 4), each lane loaded with 15 µg protein. M is the marker.

5.16.3 Effect of recombinant cysteine inhibitor protease on the recombinant melano protein

Protease activity of the recombinant melano protein using Azocasein method was repeated using 15 µg recombinant cysteine protease inhibitor to 15 µg recombinant melano protein. The mixture was incubated for 20 min before adding the substrate (Azocasein). The relative inhibition percentage was 34.5 % giving indicators for incomplete inhibition of the melano protein by CysPI. That mean CysPI is not the main key in defense (Figure 77).

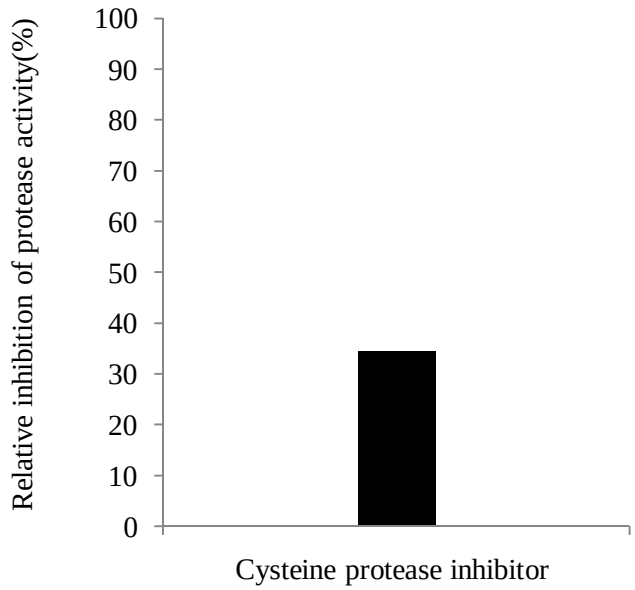


Figure (77): Inhibition of the recombinant melano protein by recombinant cysteine protease inhibitor. Assay was done as given under 4.4.16.

5.17 Melanin binding to the recombinant melano protein

5.17.1 Melanin extraction with organic solvents

The hydrophobic bounded melanin was extracted with 50% DMF or 50 % acetone solvent (%) to find out the real bounded melanin to the recombinant melano protein in µM. The free melanin decreased by adding $CoCl_2$ (1 mM) and increased by adding of EDTA (1 mM). That mean melanin aggregate together (Figure 78) or bounded to the protein with hydrophobic and metal chelation.

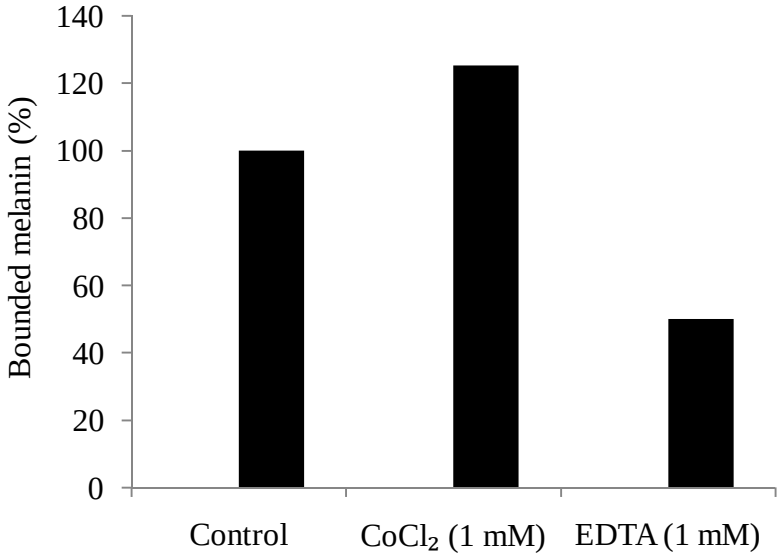


Figure (78): Bounded melanin to recombinant protein. The experiment was done as described under 4.5.2.

5.17.2 Melanin binding to recombinant melano protein and its protease activity

Binding of melanin to the recombinant melano protein increased either recombinant melano protein amount increase (Figure 79 A) or in combination of melanin with cobalt chloride (Figure 79 B), on the other hand the binding of melanin decreased as increasing of EDTA molarity (Figure 79 C).

Melano protein protease activity increases as increasing of melanin amount (Figure 79 D) as well as increase cobalt chloride amount in presence of melanin (Figure 79 E) or increase by increasing of EDTA concentration in presence of melanin (Figure 79 F), respectively.

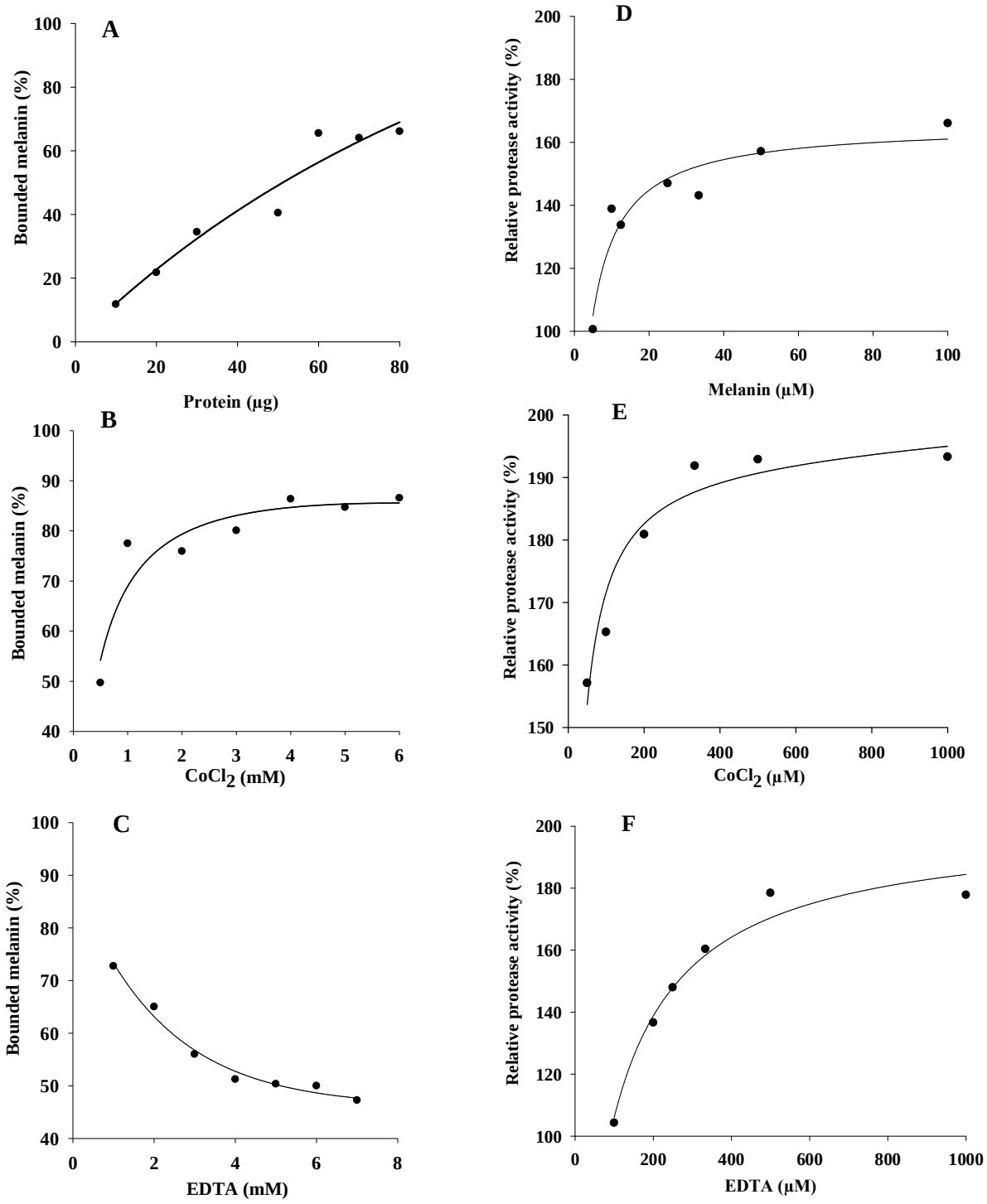


Figure (79): Bounded melanin to the recombinant protein (Left; Assay were done as given under 4.5.2) and activity (Right). A, increase in protein amount binding (µg); B, increase cobalt concentration (100 µM melanin, 80 µg protein); C, increase in EDTA concentration (100 µM melanin, 80 µg protein). D, E, and F: Effect of different concentration melanin on the melano protein protease activity (80 µg protein), either combined with different concentration of cobalt or EDTA (80 µg protein+100 µM melanin), respectively (Right; See 4.5.1).

5.17.3 Role of melanin and cobalt in polymerization of the recombinant melano protein

The assembly of the native melano protein is affected by the presence of melanin and cobalt ions. As shown in Figure 80A presence of melanin caused a polymerization of the melano protein with calculated molecular mass 72, 136, 162, 186, 222 and 265 kD. Combination of $CoCl_2$ (1 mM) and melanin enhance the melano protein polymerization and built high aggregation size (Figure 80 B) with calculated molecular mass 149, 415, 753, and 1368 kD. If the cobalt chloride increase to 2 mM the polymerization will increase to higher masses (209, 536, 973, 1490, 2946, 5126, 7057 kD).

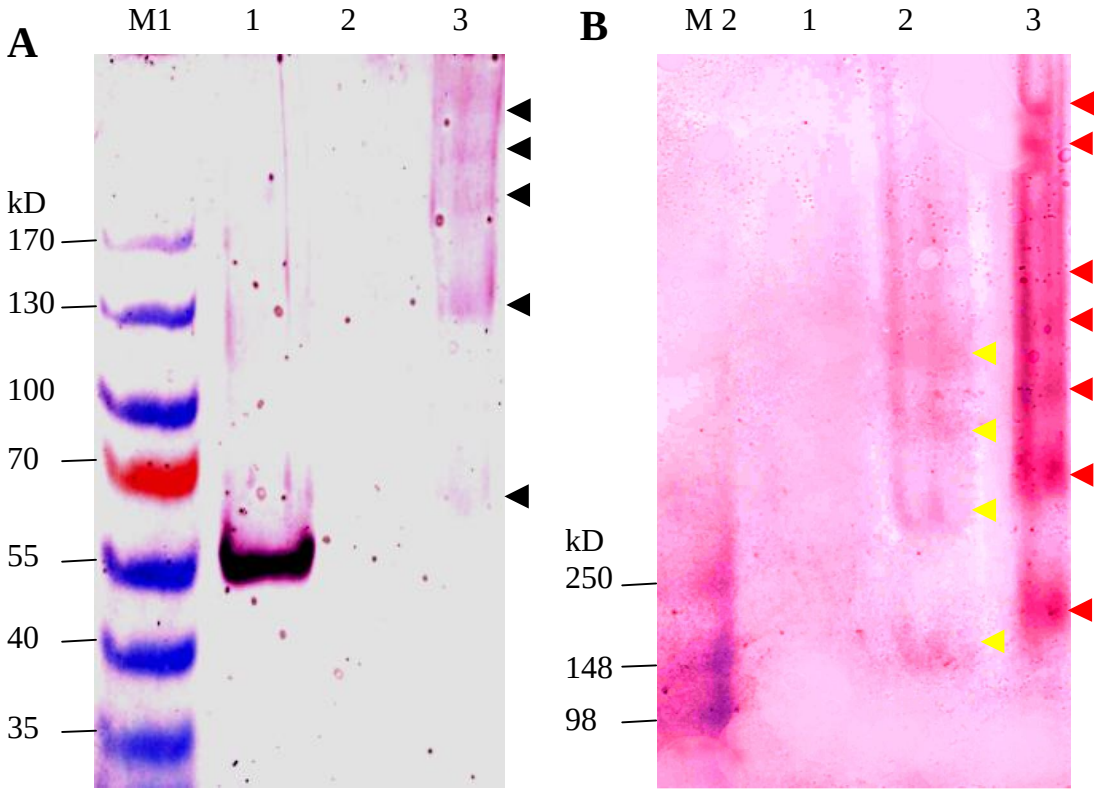


Figure (80): A: Agarose-Acrylamide PAGE containing glycerol (3.75 %: 1 %: 8%) for separation of recombinant protein (protein control), melanin (control) and melanin aggregation around the protein. M1 is marker for A gel (35, 40, 55, 70, 100, 130, 170 kD). Lane 1: the original recombinant melano protein, lane 2: melanin, lane 3: melanin-protein polymerization. B: Agraose-Acrylamide PAGE (1: 1%) is for separation of melanin and $CoCl_2$ and aggregation or/and polymerization mixture (melanin-protein-$CoCl_2$). M2 is marker for B membrane (98, 148, 250 kD). The aggregated bands singed with arrows. Lane 1: the original recombinant melano protein, lane 2: melanin-protein polymerization in presences of 1 mM $CoCl_2$, lane 3: melanin-protein polymerization in presences of 2 mM $CoCl_2$.

5.18 Propagation of *V. inaequalis* on M9 modified Capeckz's medium

The studies aim to simulate the growth of *V. inaequalis* on the leaves after the summer seasons to find possible artificial way to enhance ascospore production. The work here could be used for further studies on perfect stage for this fungus, which may be help in more understanding for the fungal behaviours and disease control

The presence of oligosaccharide and polysaccharide like sucrose, lactose or starch enhance of ascospore production under the optimum conditions of Day/night period, temperature, pH and nitrogen/carbon source ratio in the growth medium. The mentioned condition effects also on the sterile hyphae and conidiospore production.

The grown fungus *V. inaequalis* on PDA medium is producing only conidia and the plate was very black (Figure 81 A). It is noted that the fungus was not stressed, so that cannot produce the sexual stage or at least sterile yellow hyphae because the nutrient is available.

The fungus growth on the modified czapeck`s and M9-Aps medium (pH 5.5) gave rise different results from that of PDA medium. The superficial yellow hyphae and rough conidia were produced as results of stress condition which might associate to dark period, long light period, cold (17 °C) and amino acids amendment (Figures 81 A and 82).
On the other hand the production of the ascospore needs the entire factor to be perfect and need more dark condition with small light intervals and that might be very important for the sex organ production and the sexual reproduction (Figures 81 A and 83).
Total and rough conidia production by grown *V. inaequalis* increased as the amino acid concentration increase on modified Cz medium. Special case on the M9 medium, it gave huge products in the total conidia when the glutamate concentration increased to 500 mM in comparison to L- alanine concentrations. On M9 medium, the rough conidia production was reduced in comparison to the modified Cz medium (Figure 81 C&D).
Increasing of the roughness in the conidial surface expected to increase the infection power by giving bitter stacking to the cuticle surface. The Darkness is very important for the sexual reproduction. Concentration of the amino acid is very important for nutrition and food saving for long time.
The double carbon source medium (sucrose and glucose) is very important as stress and survive tool for asci and ascospore. The entire previous or similar natural factors in the leaf could combine into rough conidia production for secondary infection and then the sexual ascospore for overwintering (Table 19).

Table (19): Reuslts summary of *V. inaequalis* rough conidia, yellow hyphae, and ascospore production results

	Cocentration (mM)	Ascospore	Yellow sterile hyphae	Rough conidia
Glutamate	0		+++	+++
	100	+++	+++	+++
	200	+++	+++	++++
	500	+++	+++	++++
L- Alanine	0		+++	++
	100	+	+++	++
	200	+	+++	++
	500	++	+++	++
Glucose		++++	---	---
Light		---	++++	++++
Dark		++++	disappear with long time incubation	abssiion

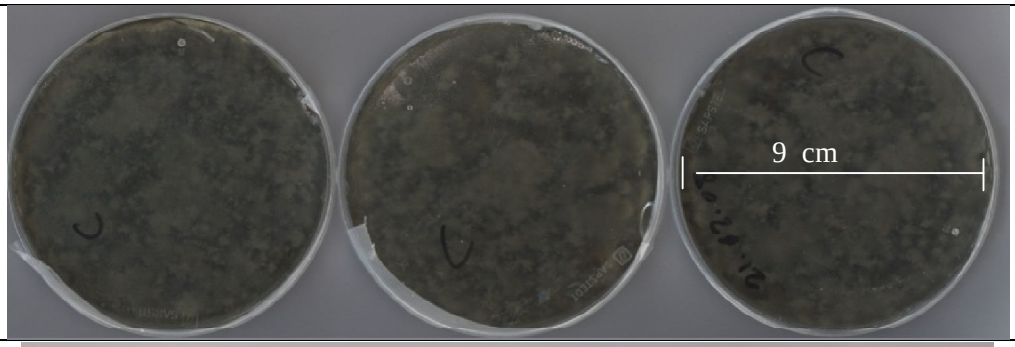
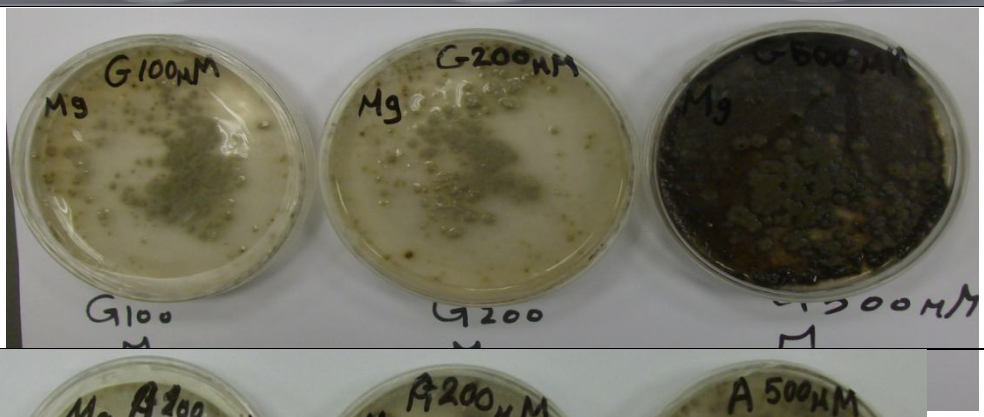
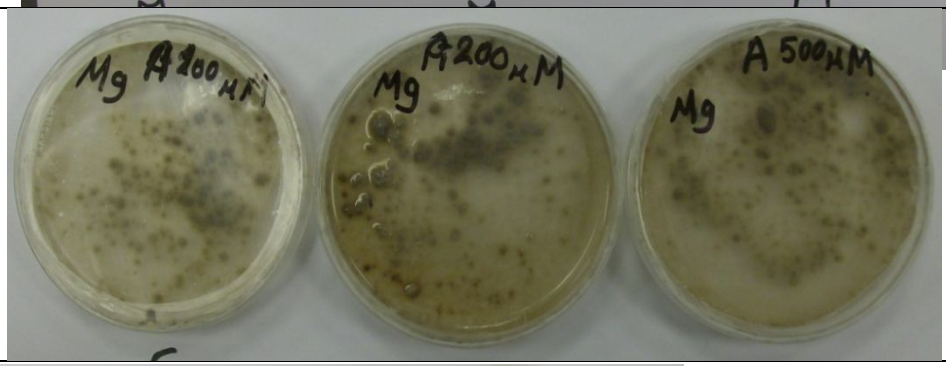
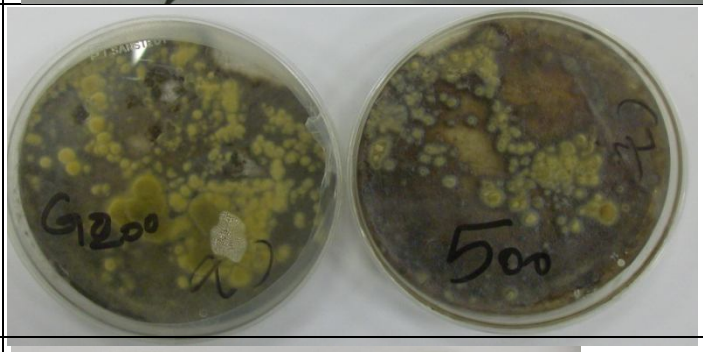
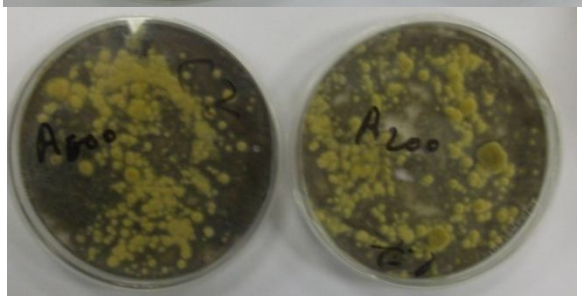
A	
B	
C	
D	
E	

Figure (81): the fungal growth on M9 and Modified Cz medium. **A** refers to = Control PDA, **B**= M9+ Glutamate, **C**= M9+L-Alanine, **D**= Modified Cz+ Glutamate and **E**= Modified Cz+L-Alanine.

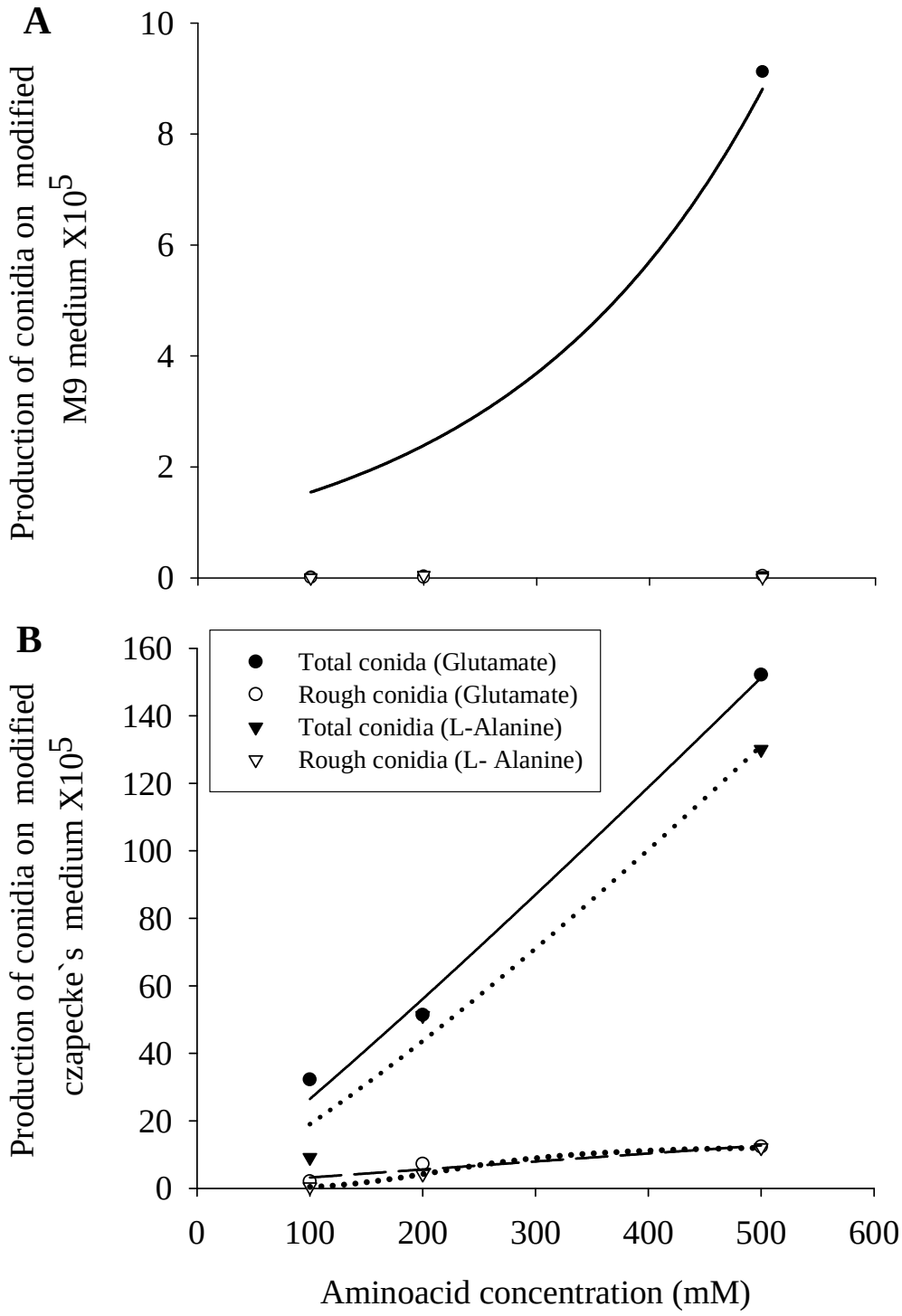


Figure (82): Count of total and rough conidia for the grown fungus *V. inaequalis* on the M9 (B) and modified Cz mediua (A) containing different carbon source and differentNitrogen source.

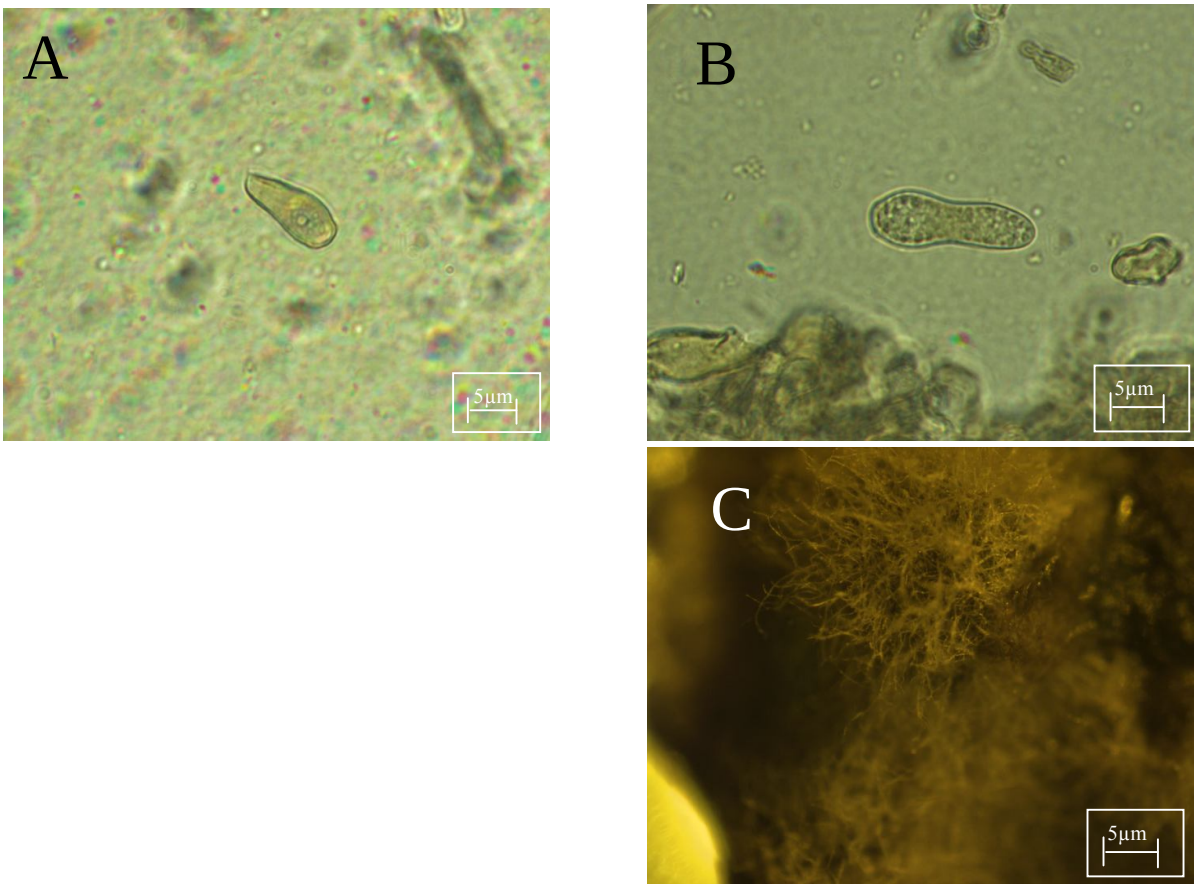


Figure (83): Microscopic examination of propagated *V. inaequalis* on sucrose-glucose medium amended with L-alanine (A) and glutamate amino acids (B) for detection of the stress effect of the sucrose on variation of conidial structure and sterile hyphae (C).

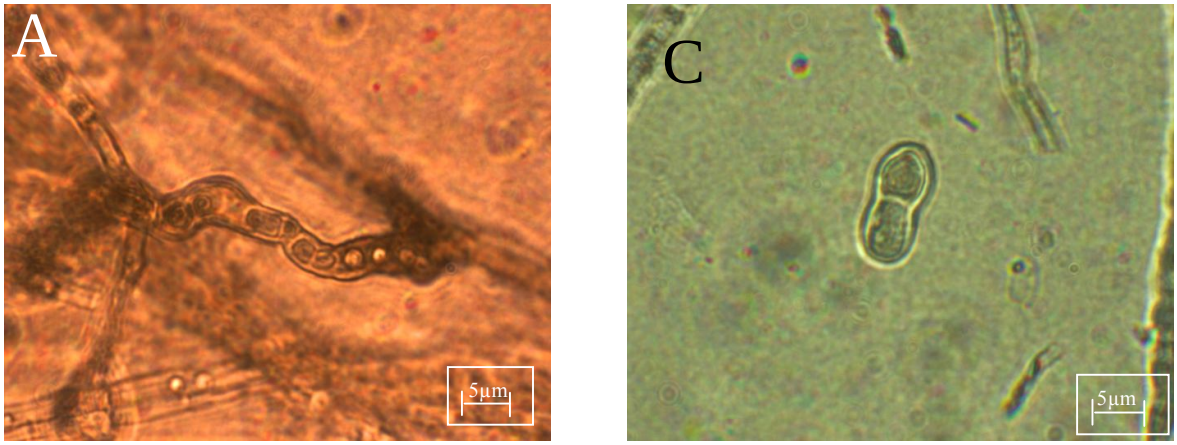


Figure (84): Examination of propagated *V. inaequalis* perfect stage on sucrose-glucose medium amended with glutamate and L-alanine amino acids for detection of the stress effect of the sucrose on the production of the asci (A) and ascospore (B).

5.19 DNA extraction from *Venturia inaequalis*

DAN extraction was a task since the day of looking to heredity, the extraction of the DNA developed from time to time and now become very fast and in advance. The advantages of the needed time for extraction, application, DNA quality, purity, coast, and the safety especially with the pathogenic fungi lead to the developmental work. Good method for DNA extraction from the black fungus *V. inaequalis* is needed to achieve the goals.

5.19.1 Agarose gel

The DNA extracted by the new method were fractionated into three fraction to see if there are difference between the size of the three fraction, the samples were elctrophoretically separated on 1% agarose gel and visualized under the UV light (Figure 85).

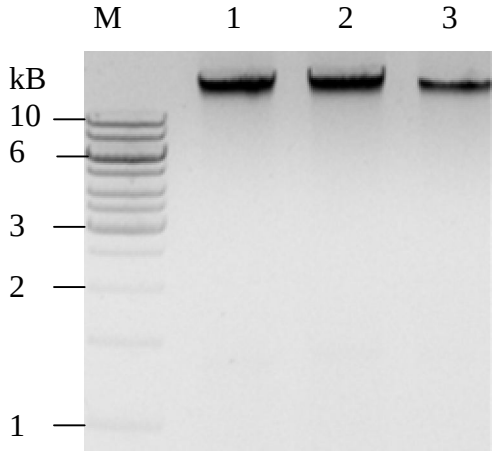


Figure (85): 1% Agarose gel from the extracted DNA from the fungus *V. inaequalis* (F1, F2, F3 three fractions). M is the marker. F1 precpetate by isopropanol, F2: precipitate by ethanol, F3 precpeated by acetone.

5.19.2 DNA Quantification in comparison to CTAB methods

The DNA extracted from *V. inaequalis* by the new method (195±21 µg) is close to be four times more than the CTAB method (55±4 µg. The extracted DNA purity by the new methods has high range (1.96-1.99), whereas, extracted DNA by CTAB method has low purity (1.55-1.41) (Figure 86).

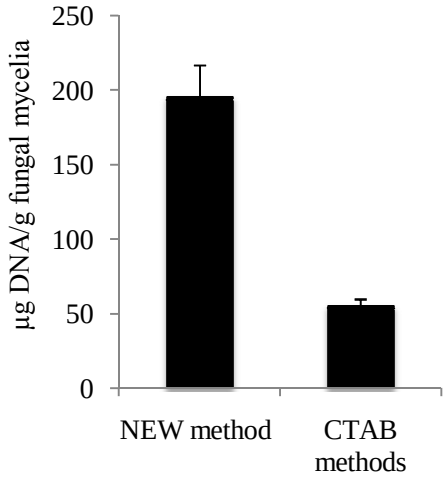


Figure (86): Comparison of *V. inaequalis* extracted DNA by two methods (new method and the CTAB method).

5.19.3 Qualitative analysis of DNA from *V. inaequalis* isolated by different methods

To compare newly developed method for DNA extraction with CTAB method quality, the two samples from the two methods were electrophoretically separated on agarose gel. In general, CTAB method has low quality than the new method. DNA extracted by CTAB method has smearing in but for the new method no smears can be seen. There are residues of destroyed DNA which present in the well of the lanes for CTAB method which is not present for the new method (Figure 87).

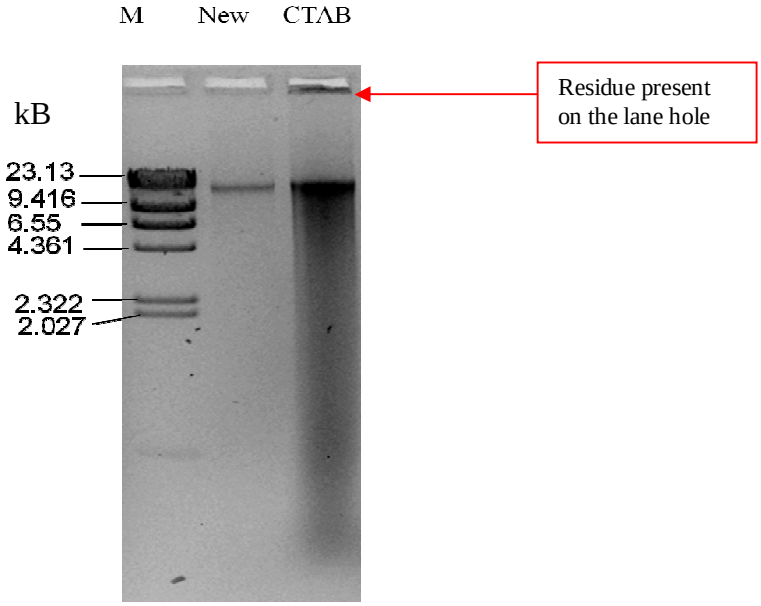


Figure (87): Comparsion of DNA extraction methods. Methods of the DNA isolation are given under 4.3.1.

6. DISCUSSION

6.1 Characterization of the melanoprotein from *V. inaequalis*

The ORF (chapter 5.3.3) of the identified melano protein from *V. inaequalis* shares 100% of an EST clone (EST: EG631228) sequence from *M. domestica*. The latter shows also homology to the recently published EST clone from Win *et al.,* (2003). The EST clone 180212 is partially homologue to a fractional peptide sequence of the AVR protein from *V. inaequalis* (Win *et al*., 2003). This finding implies that both extracellular proteins could be identical. Moreover the intriguing finding was that a gene sequence of the fungal pathogen was also located in the genome of the test plant *Malus domestica*.

Homology search for the protein sequences was mostly similar to the fungal and plant species glucanase (See chapter 5.3.5). The current prediction was in accordance to the Win *et al.* (2003) prediction for glucosidase (chapter 3.3.4) and partial peptide sequence by Singh *et al*. (2005). The current results gave strong evidence that the melano protein is bifunctional (protease and glucanase). The proteolytic active site in the protein was predicted using the homology of the melano protein motives to the active sites in the data base (NCBI) of the fungal protease. The protease active sites (See chapter 5.5) show homology to the retropepsin_like_LTR_1 which include the motifs of Asp-Th/Ser-Gly and is related to the aspergillopepsin_like_protease (Aspartic proteases of fungal origin).

6.2 Bifunction of the recombinant melano protein of *V. inaequalis*

Protease (chapter 5.10) and glucanase (chapters 5.9 and 5.11) activities play a major role in biochemical processes. The role and the function of protease activity of the melano protein is in accordance with evidence found by Nikolov (2000) when he performed screening tests of trypsin protease inhibitor with respect to some phytopathogenic fungi including *V. inaequalis*. The fungus was inhibited by 67-95% during infection of apple after application of protease inhibitor.

Proteins have been extracted from the apoplast of infected and uninfected apple seedlings (Fitzgerald *et al.,* 2001). In this work approximately 200 apoplastic proteins of healthy seedling leaves, which are mainly acidic with low MW (20-40 kD), have been separated by 2D electrophoresis. After infection of the leaves, apoplastic proteins appeared to be degraded to peptides of MW 3-6 kD. Hignett (1973) suggested that, the melano protein binds to metals and apparently pass through cell membranes and it can alter membrane permeability. All of

this previous evidence indicates protease activity, which is supported by the apoplastic protein destruction (Fitzgerald *et al*., 2001). The glucanase activity was shown by Koller *et al.*, (1992) and Valsangiacomo *et al.*, (1992). The high effect of the PMSF on glucanase and protease activities introduced strong evidence of active sites overlapping. The combination of two hydrolytic enzyme activities in one protein offers to the pathogen an opportunity for an effective possibility to overcome the fine defense line of the plant. The bifunctionality of the melano protein is intriguing and offers a new view of pathogen access to host plants.

6.3 Metals and inhibitor effect on protease and glucanase activity of the recombinant melano protein

Generally, most of the investigated metal ions (Ca^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, and Zn^{2+}) were used to inhibit the enzyme activities (glucanase and protease) except of Co^{2+} (chapters 5.9.7-5.9.8, 5.10.8-5.10.9). Cobalt played a major role for the protease activity of the melano protein, enhancing it at concentrations of 1 to 2 mM. On the same way the effect of the protease inhibitor PMSF (see chapters 5.10.7 and 5.9.6) indicates the presence of serine active sites, which followed by effect of Aprotinin, Pepstatin, Leupeptin and Chymostatin. This confirms the presences of serine, aspartily, threonine, leucine, and arginine active centers in both glucanase and protease. The effect of PMSF on both glucanase and protease give strong evidence for overlapping or sharing of active centers. The effect of serine protease inhibitor on glucanase was reported before by Rose *et al.*, (2002) and Damasceno et al., (2008). Blanco-Labra and Iturbe-Chinas (1980) identified a bifunctional α-amlyase/trypsin inhibitor from corn.

The protease activity difference (chapter 5.10.10) on EDTA treated azocasien suggested that Ca^{2+} has an important role on the protease activity. The treated substrate has a low calcium content so the relative percentage activity was larger than that of the untreated protein substrate. The presence of calcium or other metals bounded to the substrate can cause inhibition of the proteolytic activity. Inhibition could be due to shielding effects by the metal binding site in substrate, blocking the enzyme active site, both the substrate and enzyme aggregation, or substrate reconformation to be unfitted to the enzyme structure.

Trypsin protease inhibitor (chapter 5.10.12) was used to determine and confirm specificity of the protease and glucanase activities. PMSF was the strongest protease inhibitor for protease and glucanase activities for the melano protein and followed by the others. Nikolov (2000) reported that using of trypsin protease inhibitor extracted from *Capsicum annium* inhibit the

pathogen and by the way the lesion formation with range 67-95% on host plant. There is no literature about these new enzymatic foundations (Naturally found in one subunit).

6.4 Thermostability of the melano protein enzyme

High thermostability (See chapter 5.10.13) of the melano protein is indicated in this study and in accordance with that of Win *et al.,* (2001). They concluded that, the hypersensitive response-inducing activity factor of *V. inaequalis* is resistant to boiling. All of the previous evidence helped in developing of a new method depending on the denaturation of the other proteins of *E. coli* associated to the melano protein. In this study, glucanase activity was supported by the experimental results (chapter 5.9.4) and has an optimum temperature at 60°C. The high temperature stability can be concluded by the aminoacid sequence of the melano protein. The presence of a putative disulphide bond and a high percentage of aminoacids forming strong intermolecular interaction by hydrogen bonds underline the experimental finding.

In present industrial processes, cellulolytic enzymes are employed in the color extractions of juices, in detergents causing color brightening and softening, in the biostoning of jeans, in the pretreatment of biomass that contains cellulose to improve nutritional quality of forage and in the pretreatment of industrial wastes (Buchert *et al.*, 1997; Niehaus *et al.,* 1999; Puchart *et al.,* 2000; Nakamura *et al*., 2001). In order to attack the native crystalline cellulose, which is water insoluble and occurs as fibers of densely packed structures, however, thermostable cellulases active at high temperature and high pH are required.

The protease activity of the recombinant protein has a temperature optimum at 90°C (chapter 5.10.3) and glucanase at 60 °C (chapter 5.9.4). That result is in accordance with industrial enzymes (proteases), and constitutes more than 65% of the world market (Rao *et al*., 1998). The thermostability is related to many factors. McCoy (2001) studied that with a gene fusion expression system that uses thioredoxin and fusion partner. The author found that the inherent thermal stability of thioredoxin and its susceptibility to quantitative release from the *E. coli* cytoplasm by osmotic shock are useful tools for thioredoxin fusion protein purification.

Kristjansson (1989) mentioned the main industrial processes which use enzymes operate at relatively high temperatures (Table 20). Most of these enzymes are quite thermostable even though they originate from mesophilic bacteria or fungi.

Table (20): Industrial enzymes process at high temperature from mesophilic organisms

Enzyme	Operating temeperature (°C)	Major applications
α-Amylase (bacterial)	90-100	Starch hydrolysis brewing, baking, detergent
Glucoamylase	50-60	Maltodextrin hydrolysis
α-Amylase (fungal)	50-60	Maltose
Pullulanase	50-60	High glucose syrups
Xylose isomerase	45-55	High fructose syrups
Cellulase	45-55	Cellulose hydrolysis
Pectinase	20-50	Clarification of juices/wine
Lactase	30-50	Lactose hydrolysis, food processing
Acid protease	30-50	Food processing
Fungal protease (neutral protease)	40-60	Baking, brewing, food processing
Alkaline protease	40-60	Detergents
Lipases	30-70	Detergents, food processing

6.5 Purification methods for the recombinant melano protein

Many methods for tagged protein purification system Ni-NTA (chapter 5.8), Cu-NTA, Zn-NTA, Co-NTA for His-binding, S-tag *GRS*-tag and *TRX*-tag were used (Novagen protein purification and detection tools). In this study, heat stability of the melano protein enzyme leads to find out another technology than the previous large scale production method. In this study the heat treatment purification study were done by incubation of the bacterial lysate at 70°C for 30 - 40 min (chapter 5.8). Similarly, four enzymes involved in the breakdown of cellulose and hemicellulose were purified 7-19-fold in a single heat-treatment step (70°C) for 30-65 min (Patchett *et al.*, 1989). Le-Bris *et al.,* (2007) purified *AATP* (The *GPCR* α-factor receptor and a methanococcal transporter) protein by heat treatment in one or two steps. Patchett (1989) studied four enzymes involved in the breakdown of cellulose and hemicellulose and purified them 7-19-fold in a single heat-treatment step. Olichon *et al.,* (2007) found that the yield of recombinant *RE3 VHH* antibody increased by heat treatment purification similarly to affinity chromatography. Similar protocols for heat treatment were used in *E. coli* to purify thioredoxin fusion proteins (McCoy, 2001). Heat treatment can simplify the purification protocol of thermotolerant proteins and is an alternative to the common affininty purification process.

6.6 Models

6.6.1 Surface proteins

The surface proteins model (chapter 5.6.2) is based on the results of Pelletier (1962) and Bondarenko, (1972). The interaction of the melano protein to the surface protein of the

resistant cultivar Remo results (chapter 5.6.3) is confirmed by the isolation of these proteins from the replica and finally by the interaction of this protein, which has a chitinase-activity (antifungal). The model gave a very fine view about the protein secretion from the epidermal cells through the cuticle membrane. These proteins may be a complex of proteins aggregated with different functions, which lead to fungal cell and fungal weapon elicitors disturbance and finally cause infection failure. The palisades like protein shape may help these proteins to reach to the membrane of the fungus, to cause permeability and damage the cells which are associated with the chitinase.

6.6.2 3D model proposition for the structure and localization of the surface protein on the surface of *M. domestic*a cv. Remo

Figure (89) shows the surface and the vertical section through the cuticle interactions were collected to illustrate of the presence of cuticle surface proteins.

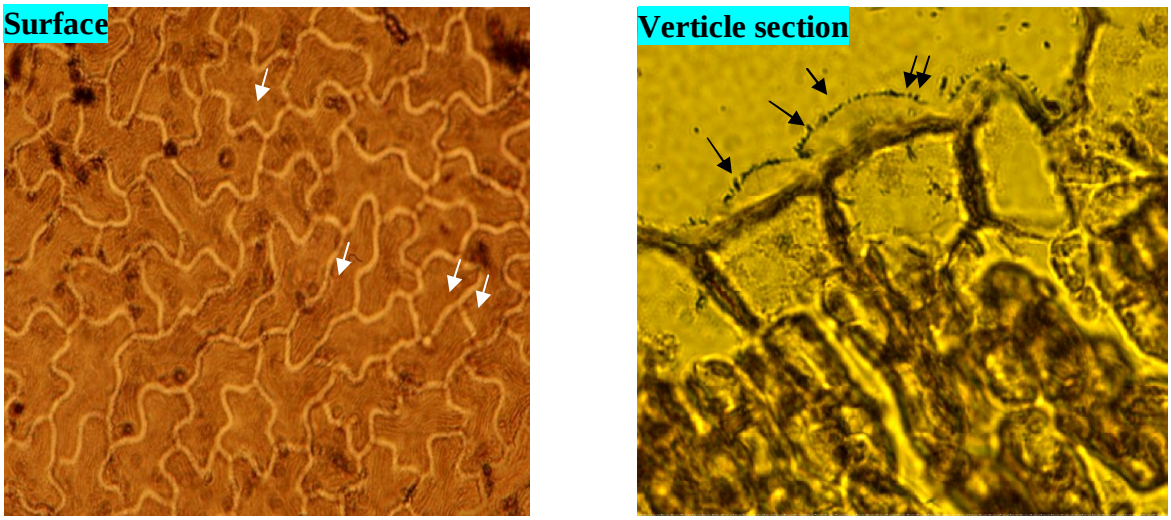


Figure (89): Cuticle surface proteins of *M. domestica* cv. Remo interact to the melano protein of *V. inaequalis*.

From the epidermal surface and vertical section (Figure 89) and confirmed with impression staining (chapters 5.11.2 and 5.11.3) and extraction followed with interaction of the cuticle protein on PVDF membrane (chapters 5.11.4-5.11.6) it can build up the structure model of the cuticle-membrane of the *M. domestica* cv. Remo. The brown to black interacted protein is signed with black arrow. The model depends on the horizontal and the vertical view of the melano protein interaction to the embedded proteins present on the surface of Remo cultivar cuticle (Figure 90).

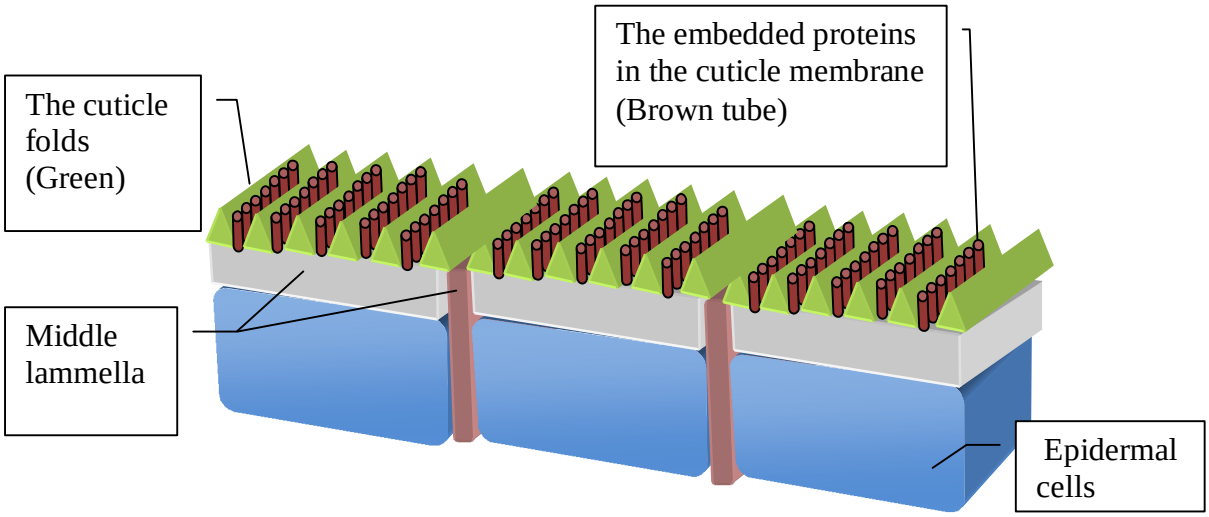


Figure (90): Three dimensional model of the embedded protein in the cuticle of the resistant *M. domestica* cv. Remo

6.6.3 The melanin-protein interactions

The Melanin-protein-model (Figure 91 and 92) illustrates the melanin-protein interaction and provides an overview in relation to the results of this and the previous studies. Specific concentrations of melanin in the melano protein activate the protein complex, while elevated melanin concentrations may inhibit the activity. The melanin is aggregated in two ways; hydrophobic with the hydrophobic parts of the proteins or by metal chelating to it other melanin molecules or to amino acids of the proteins, which may be increase the folding quality. This folding protects the proteins from the oxidative stress coming from the host or the environmental condition which increases the chance for infection and colonization. The aggregation or polymerization system of the melano protein is investigated finally and no comparable literature is presently available.

However, *Pyricularia oryzae*, *Colletotrichum lagenariutn*, and *C. lindemuthianium*, need melanin for penetration of rice, cucumber, and bean by appressoria, respectively. Pure melanin and melano protein have also been implicated in disease. A purified melanin from *Stachybotrys alternans* shows allelopathic action against root growth of wheat, rye, and cress (Svishchu *et al.*, 1970). Melano protein from *V. inaequalis* increases lesion number and size when added to inoculated leaves **(**Hignett, and Kirkham, 1967).

The following functions of melanin were shown in this work; the polymerization (aggregation hydro-phophobically or by chelating with metals) of the protein unit to micelle, micelle to granule and granule to grain formation. Furthermore melanin increased the protease activity, and is used to protect the enzyme activities against the host elicitors or inhibitors (See chapter 5.17). According to this hypothesis, the protein unprotected by melanin might be inhibited by

plant secreted enzyme inhibitors, causing a reduction of fungal virulence. The addition of cobalt to protein solutes increased the protein activity. A similar effect could be observed after the application of melanin. Adding both melanin and cobalt ions increased the activity and polymerization by up to 195 %. According to Svishchu *et al.*, (1970) melanin may bind metals by chelation and thus inactivating important plant metabolytic or defence enzymes or melanin may bind hydrophobically to some enzymes or substrates and therefor prevent enzymatic activities.

6.6.4 Generation of melanin-protein interaction model

Depending on the metal chelating or hydrophobic aggregation, a model was generated for melanin-protein interaction (cp. Figure 91). As shown the brown melanin is aggregated around hydrophobic amino acid sequences in the protein (light green and gray hexagonal shape of amino acids). The brown melanin chelates metals (rose star like Co^{2+}) with coordinating bonds (red lines). Melanin could bind to more than one metallic center because, it has more than 8 electron donor groups and transition metal needs commonly eight coordination bonds. Transition metal could bind to melanin as well as to amino acids (electron donor). The final structure like net work and the protein monomer size should increase due to the additives.

Presence of bounded melanin and metals with proteins leads to more aggregation of the interacted protein to form a micelle (Figure 92). The micelles aggregate to form granules and the granules could aggregate to form grain.

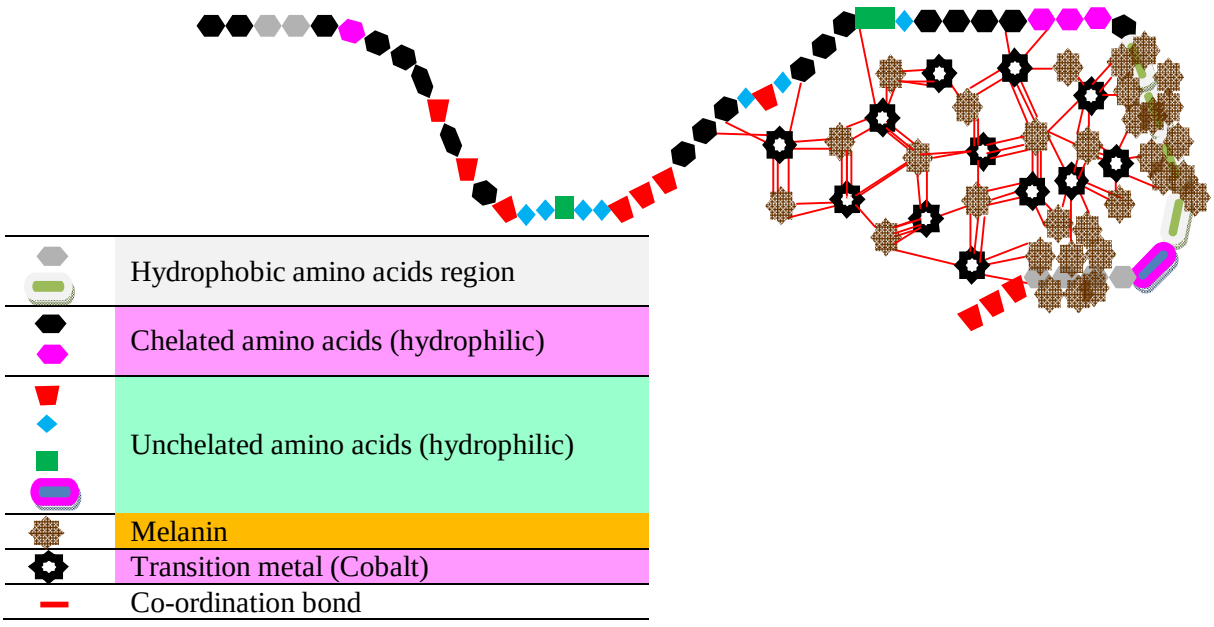


Figure (91): Proposed structure of melanin-protein- metal interaction (protein unit).

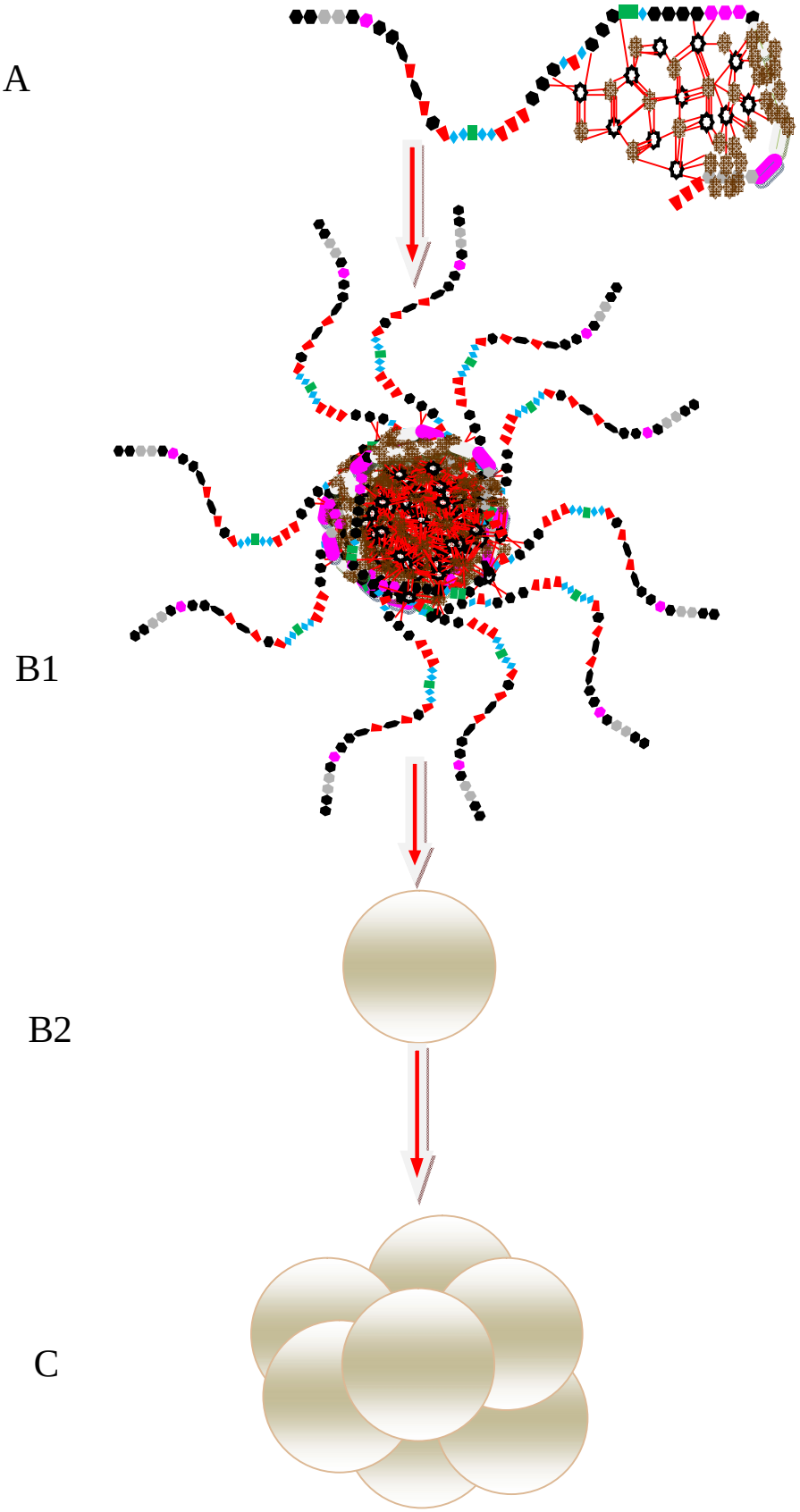


Figure (92): Proposed aggregation of interacted protein-melanin units (A) to form micelle (B1, B2) of melano protein to form melanin granules (C) which may be aggregate more to form grains.

6.6.5 The infection model

The infection models (Figure 93) for the susceptible plant summarize the target points and the fungal strategies in infection in all the resistant and susceptible plants. In the susceptible plants according to the results of this study and other studies (Singh *et al.,* 2005) the glucanase and protease activity of the melano protein diffuse through the plant tissue before the cuticle invasion to prepare the medium in the cells below. The second case is susceptible plant produces some of the antifungal proteins which can delay or partial inhibit the protease and glucanase activity especially of apoplastic and surface proteins. The produced antifungal proteins in the plant of current case may be not sufficient to prevent the infection. The third case is the resistant cultivar which has the full system for defense molecules before infection (proteins on the surface, proteins in the apoplastic fluids, the antifungal protein, minerals like calcium (Shear and Drake, 1971, Selitrennikoff, 2001)). This case is the best one in all of the previous cases and these proteins are constitutively formed. In comparison to the first and second cases, the antifungal tools are inducible. The infection in the first case succeed without competition and in the second case the fungus succeed with high strength of competition and can achieve colonization and production of new generation but in the third and the first cases the fungus failed completely to colonize. The best plant case for the fungus is the second case because it gives the chance for the fungus to achieve infection and spread. Whereas in the other two cases the fungus will probably die.

For fungal growth and live cycle completion, both melano protein flow and the food uptake have to be continuous to and from the infection side tissues, respectively (double direction, Figure 93). The model gives an overview of the targeted plant protein by the melano protein produced by the fungus *V. inaequalis* to establish the infection. In the model drawn below the melano protein can attach to the cell walls, apoplastic fluid membranes and the organelles of the epidermal cells but it takes long time depending on the inhibitor competition in the epidermal cells. The epidermal cells collapse afterwards and are partially digested. Extensive infection steps happen due to the attachment to the layers below by the diffused melano protein (palisade and spongy tissue) and the organelles inside (membranes chloroplast, nucleus, ribosomes, most of the plant cell proteins, mitochondria and vacuoles). The protease activity (high Km value) more than glucanase activity (low Km value) leads to cell death of the infected cells. The extended cell wall hydrolysis explains why the invading of further plant tissue is delayed after the cell death.

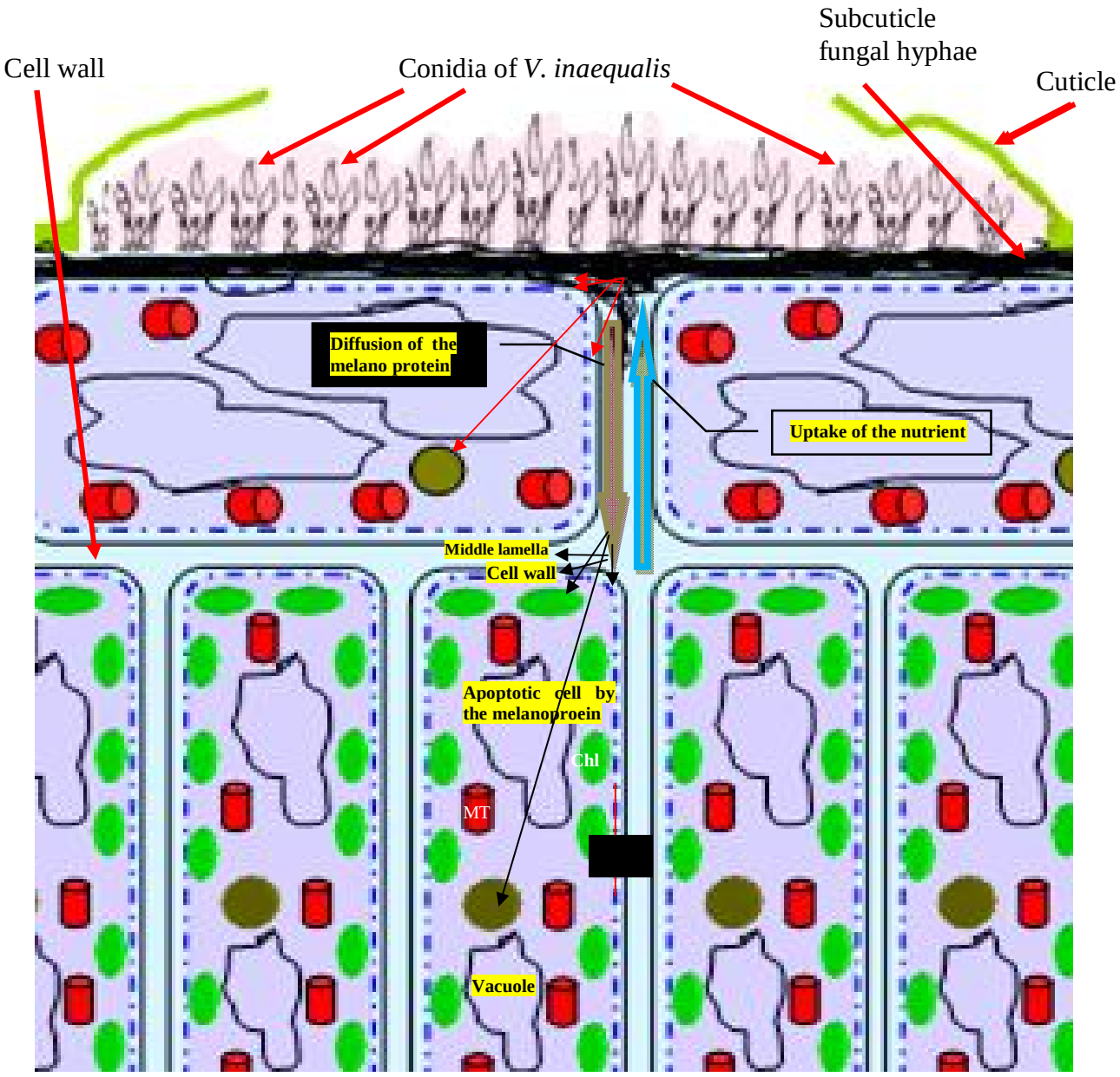


Figure (93): Model for the infection mechanisms and strategy for fungal surviving in the beginning of the seasons which include the attacking of the protein to the cell proteins during the early infection. The fungus can attack the epidermal cells in the beginning to inhibit the defense mechanisms and take up of the nutrients. Red cylinder = Mitochondria, Brown disc = nucleus, Green Ellipsoidal shape = Chloroplast, Rigorous unstright closed line = Vacuoles, Disconnected line = Plasma membrane of the cells, Connected line around the cells = Cell wall, background of the cell (light violet matrix) = Cytoplasm, Light green ruptured line on the top beside the fungal conidia = Cuticle, Inward brown arrow = Inward diffusion of the melano protein, Outward blue arrow = Uptake of the digested food from the dead cells, upper thick black line under the cuticle = Fungal mycelium growth.

6.7 Impact of melano protein on bacterial growth

Growth of gram positive bacteria is inhibited in presence of the melano protein probably due to the presence of murein in the cell walls of the bacteria. The melano protein is presumably capable of hydrolyzing murein. Gram negative bacteria which have high resistance to the protein are not affected, since murein is not present in their surface structure (chapter 5.14.1).

The addition of Co^{2+} activated the protease activity of the recombinant protein and increased the effect of growth inhibition of the tested bacterial species.

Hignett (1973) concluded that the melano protein is secreted by *V. inaequalis* during the infection of *Malus domestica* may interfere with cell wall function and the host metabolism on protein level. Supporting experiments for this hypothesis were obtained before by Hignett and Kirkham (1967) and Kirkham and Hignett (1976) which indicated that plant infection by *V. inaequalis* is closely associated with solute transport and hormone system alteration. Some investigators (Evins and Varner, 1972; Trewavas, 1968; Srivastava and Arglebe, 1968) said that the melano protein affects plant hormones which are involved in the ribosomal polyribosome system, RNA, protein synthesis, and the light reaction of the plants. Due to the known melano protein effect on the ribosomal protein of bacteria and on membrane permeability, the effect of melano protein on the bacterial growth was investigated. The impact of the recombinant melano protein on bacterial proteins on a PVDF membrane was studied.

The antibacterial effect could be explained by a rapid destruction of the murein structure of the gram positive bacteria, caused by the glucanase activity. In addition the second enzyme activity (proteolytic) of the melano protein was also responsible for the suppression of bacterial growth. The fast degradation of bacterial surface structure by the melano protein hydrolytic enzymes activities cannot be compensated by the bacterial membrane synthesis (chapter 5.14.2).

6.8 *Malus domestica* surface protein

In general, the replica technique was used to visualize the surface plant shape and structure of the cells. It is important to investigate the epidermal cell structure and to monitor the expansion rate over a period of time. In the current study, it was used to achieve additional goals.

The discovery of an elevated level of plant surface proteins in the resistant cultivar Remo (See chapters 5.11.5 and 5.11.6.1) in comparison to the susceptible cultivar Elstar could be the reason for the natural resistance of apple varieties against apple scab. This finding solved the enigma of surface resistance which explains the results of previous experiments done by Bondarenko (1972). The exudates on the surface of the resistance apple leaf (exosmosis water soluble substances) were tested on the vitality of conidia of *V. inaequalis* and were claimed to have reduced the germination rate, slowed the growth of the germ tube, and reduced the

frequency of appressorial formation with prolonged leaf moisture; malformation and lysis of conidia and germ tubes were observed. Similar adverse effects were not observed when repeating the experiments with leaves of susceptible cultivars.

Additional evidence for the existence and effect of leaf surface proteins gave an increase in lesions after using a non-ionic detergent (Agral). It was applied to test plants five days before inoculation with *V. inaequalis* (Hignett 1973). The scientist proposed that the membrane damage increased as a result of Agral usage, which reduced the host resistance. Based on the current study this increased membrane damage was caused by the protease activity of the melano protein. The adding of Agral minimized or removed the surface protein from the leaf surface which supplies resistance against the pathogen or has a direct effect on the pathogen. The identification of plant surface proteins with chitinase activity in this work confirms the defensive role of surface proteins. However, it is not known if some of these proteins play an important role in the signal transduction from the external environment to the internal tissues or the apoplast of the plant leaf and if they are responsible for starting specific physiological responses.

6.9 Putative targets of the melano protein in the host plant

The detection of proteins that have a high affinity for the melano protein in the host plant could be a part of the molecular response of the host to the pathogen.

The interacting proteins have a defensive role against the fungal pathogen (chapter 5.16.2, 5.16.3). Some mysteries of surface defense theory for resistant plants (Pelletier, 1962) were revealed in this study. The pathogen-host interaction is described by Flor (1942, 1946, and 1955) as gene-for-gene interaction. This was explained by secreted proteins by the fungal pathogen to invade the host. The host produces resistant interactive protein which can stop or inhibit the action of the pathogen proteins. The proteins secreted by the pathogen are called avirulence proteins while the proteins from the host are called virulence or resistant proteins. However, the pathogen can easily colonize a host plant, which does not have the corresponding resistance gene. Thus, avirulence of a pathogen can only be described in the context of a gene-for-gene relationship in which a suitable resistance gene is present in the host plant. The first *avr* gene was cloned from *Pseudomonas syringae* cv. *glycinea*, the causal agent of bacterial blight of soybean (Staskawicz *et al.*, 1984). More than 40 *avr* genes have been cloned from bacteria mostly from the genera *Pseudomonas* and *Xanthomonas* (Bonas and Lahaye, 2002). The cloning of the first fungal *avr* gene, *avr9*, was reported (Van Kan *et al.*, 1991). The *avr9* gene was cloned from *Cladosporium fulvum*, a fungus that causes tomato

leaf mould. Since then, only nine *avr* genes have been cloned from pathogenic fungi. It appears that, like bacterial *avr* genes, the fungal *avr* genes do not share any similarity except the fact that they are mostly small proteins that are secreted, and the majority contains a high number of cysteine residues.

The mass spectrometry analysis of the candidate protein band was analyzed and gave four sequences. One of this hits showed homology to the PR3 class I chitinase, identified in different species. It is the first time to find such chitinase. A second hit for was related to hypothetical proteins of *Vitis vinifera* and third one it was related to NADP-dependent sorbitol 6-phoaphate dehydrogenase of *Malus x domestica*. This protein might be has a very strong role in oxidative stress against the pathogen.

6.10 Cuticle permeability, protein secretion though the cuticle, and cuticle invasion

The permeability of the cuticle (chapter 5.11.1) reflects the resistance of plant cultivars (*M. domestica*) to the invasion of proteins and subsequent the infection. The Elstar cultivar showed permeability of the cuticle to water soluble stains, on the other hand the cv. Remo did not show any stained regions. The obtained results indicate the first action of infection and hypersensitivity mechanisms by *V. inaequalis.* The melano protein produced by the pathogen can diffuse through the cuticle to the layers below, causing hypersensitive response or competition effect with the PR proteins, PR molecules like calcium and others inhibitors. The porous cuticle simplifies the invasion by the fungal appressorium. The latter just requires a small amount of the MARS (Melanized appressorial ring structure; Steiner and Oerke, 2007) to invade the cuticle what increases the infection efficiency. But a blocked cuticle decreases the infection frequency, which leads to the role of the melano protein in invasion of the cuticle. The importance of the melano protein ring (MARS) in invasion of the plant cuticle was described by Steiner and Oerke (2007). Nusbam and Keitt (1938) introduced evidence for digestive enzymes shared between the cuticle and the cell wall. That reflects the probability of the presence of cutinase which is associated in the grain of the melano protein. This theory of heterogeneity of the fungal melano protein grain may explain the multiple enzymatic functions. The melanin biosynthesis inhibition gave strong evidence of the importance of melanin as supporting substance for proteins function, anti-inhibitor, folding tool or activator factors (Steiner and Oerke, 2007). The impermeable cuticle of the resistance cv. Remo supports the idea that surface proteins are secreted before the cuticle is structured. This differs from the opinion of presence of one or more of the inhibitory substances on the surface of resistant plant leaf due to the exosmosis (Bondarenko, 1972).

The secretion mechanisms of protein molecules to the phylloplane through the leaf surface are unknown. This study revealed the presence of antifungal and other proteins on the upper surface of the leaf of *M. domestica* cv. Remo (chapters 5.11.2, 5.11.3, 5.11.5). Gau *et al.,* (2004) reported that apoplastic proteins containing N-terminal amino acid signals are secreted by the endoplasmic reticulum. Others PR proteins are mainly expressed in the epidermal cells (Broekaert *et al.*, 1997), likewise: defensins (Silverstein *et al.,* 2005; Segura *et al.,* 1998) and a chitinase (Ancillo, 2003). Secretes of the epidermal cells could directly diffuse through the plant cuticle from the apoplast to the phylloplane. On the other hand the hydrophobic character of cuticle overlaying could limit the deposition of these proteins, so that deposition and diffusion might only be possible in proximity of stomata and other epidermal pores. Singh *et al.,* (2004) studied the disposal of proteins through the plant cuticle with cutinases. Some workers (Kerstiens., 2006; Popp, 2005; Riederer, 2006; Schönherr, 2006; Schreiber, 2005) found existing pathways in plant cuticles that enable passage of carbohydrates, salts and other small molecules from underlying areas, but for protein secretion to the phylloplane no pathways through the cuticle are known. There is little evidence for the presence of apoplastic proteins on the leaf surface. Oh *et al.,* (2005) analyzed the *Arabidopsis* secretome and identified a novel lipase that is induced in the apoplast after pathogen infection. Further study is needed to investigate if this and other secreted proteins can move to the phylloplane. Replica interaction protein blot helps in the discovery of these proteins on the upper surface of the plant leaf. It was not reported that the upper surface of leaves of *M. domestica* contain pores, stomata or glandular structures. The new finding in the present work justifies further investigation of the protein diffusion or secretion mechanism through the leaf cuticle. Previously investigated cuticle properties (by the mentioned scientist) and the newly gained data could help in prediction of the pathway of protein transport.

Direct detection of the melano protein position on the replica by using the antibody of the melano protein showed the accumulation of the melano protein at the vertical cell wall between two epidermal cells (Replica-Western blot). According to these experiments it was found that the surface protein is reduced in this region and the fungus invades the cuticle to the layer below mostly from this point. This pattern was recorded in the cv. Elstar but not detected in cv. Remo. These results of Replica-Western blot are supported by Valsangiacomo *et al.,* (1988), who reported that the fungus prefers to invade in between the epidermal cell walls (see chapter 5.11.5) what may be caused the pectinolytic activity (middle lamellae) or the glucanase activity for the hemicelluloses or less abundance for the anti-pathogen proteins in these interface regions (chapters 5.11.2, 5.11.3). More evidence, that this is the preferred

place to invade the cuticle in the border lines between the cells on the cuticle. The replica staining with silver and the melano protein-interaction were not reported for this region and give indicate no staining in this region on the replica. This means there is no proteins or protease inhibitor which could interact with silver or bromophenol blue (chapters 4.6.2-4.6.3). This was supported by the results of the Replica-Western-Blot-like for infection of Elstar cultivar where a marginal deposition of the melano protein was observed (chapter 4.6.5).

6.11 Role of melano protein in the alteration of the plant metabolic activities during infection

If permissible, the fungus starts with epidermal cells to take up the nutrients from the dead cells, including proteins, amino acids, peptides, sugars, minerals and other ingredients especially substances which cannot be synthesized by the fungus. That means, the fungus infects the plant because it needs to obtain essential amino acids or ingredients for growth and living. The infected plant with the fungus has a very week defense mechanisms in comparison to the resistance cultivar Remo, especially outside the cells (apoplastic fluid or cuticle surface).

The glucanase activity of the fungal melano protein helps the fungus to invade the cell wall of the host plant or bacteria as primary step for infection in order to loosening the cell structure. The protease activity of the fungal melano protein is used by the pathogen to destroy plant proteins (Apoplastic fluid, cell membranes and the protoplast proteins, respectively; see chapter 5.13). The use of a bifunctional enzyme (glucanase and protease) could explain so far unidentified mechanisms of the infection and lesion formation.

The recombinant melano protein works as a protease scavenger (chapter 5.15) of plant proteins in the plant, starting from the protein present in the cuticle, apoplastic fluids, cell wall, plasma membrane and cytosolic proteins followed by organelles (mitochondria, plastids, nucleus, chloroplast). The protease activity of melano protein destroys the key enzymes in the biological processes (eg: signaling (messenger-proteins and channels), dark reaction and citric acid cycle or coenzyme A proteins). In fact by breaking down the biological reaction chains the entropy of the cell will increase and cause apoptosis by destroying cell defense mechanisms.

Possible impact of rubisco interfered by the melano protein on plant physiology

The melano protein could destroy rubisco enzymes by its protease activity and stop the carbon dioxide fixation, which leads to the increase of pentoses, hydrogen peroxides and $NADP^+$ molecules will be fully occupied with hydrogen. The light independent reaction will decrease accordingly until it stops when rubisco is completely blocked or destroyed. The light reaction

may be still working to increase the production of oxygen, ATP and NADPH+. But as the building up of new sugar compounds stops there is no possibility for recovering.

The mitochondrial enzymes could also be affected by the melano protein protease activity. The destruction of citric acid cycle enzymes leads to apoptosis and death of the infected cells triggering necrosis. These effects could occur from cell to cell by diffusion of the melano protein from damaged cells to other cells via plasmodesmata or semi-digested cell walls and permeable membranes. Finally the cell character will disappear or cells will be malformed due to the degradation and fluid loss.

The results in this study confirm this effect and are in agreement with previous results of Spotts *et al.,* (1979) and Dancs *et al.,* (1968). Their findings are in agreement with the expectation of cell apoptosis especially in the palisade tissue. They found that the photosynthesis decrease after infection according to the severity of the disease. The reduction of carbon dioxide assimilation was detected after visible symptoms appeared. Scabbed apple leaves were sampled to determine the effect on carotenoids, chlorophylls, CO_2 fixation, protein content and protein synthesis. The author concluded that the infection caused a general inhibition of metabolism (in infected tissue) that due to a suppression of photosynthetic CO_2 fixation and a reduction of the activity of chlorophylls and carotenoids.

In this study it is suggested that, the presence of melanin associated to protein during the infection helps the fungus to protect their protein activities and even enhance it (chapter 5.17). The melanin chelates many metals which are necessary as enzyme activators or melano protein activity inhibitors and in this way protects the melano protein and inhibit some enzyme activities of host proteins.

Melanin could prevent the effect of glucanase and protease inhibitors (PR proteins) of the host and therefore support the pathogen in order to survive in an inhibiting medium. This is indicated by previous results where phenol oxidase was used to destroy melanin and therefor remove the protective cover of the melano protein. This was supported experimentally by the use of melanin inhibitors, which inhibits melanin production (Sisler *et al*., 1985, 1984). According to this, melanin can be regarded as a sheath the protein and functions as a barrier for inhibition of the protein inside.

6.12 Melano protein as avirulence, necrosis and HR factor

The melano protein is one of the avirulence proteins causing hypersensitive response (HR) in *Vm* (virulence gene) plant of *Malus domestica*. It was tested and isolated by Win *et al.,* (2003) and studied further by Singh *et al.,* (2005). Win *et al.,* (2003) found that the *Vm* resistance

response is preserved in detached leaves. The HR and necrotic zones appear 2-3 days after infection in proximity to the infection sites. Chevalier and Lespinasse (1991) recorded that the necrosis is associated with collapse and death of the epidermal and mesophyll tissues. Win *et al.,* (2003) recorded that in the early stage of infection the fungus does not penetrate the epidermal outer cell wall, but the response in the mesophyll may be due to signaling between epidermal and mesophyll layers. A similar accumulation of auto-fluorescent materials after HR has been reported in other patho-systems such as cowpea-cowpea rust fungus (Skalamera and Heath, 1998), rice–rice blast fungus (Koga, 1994), cotton-*Xanthomonas campestris* pv. malvacearum (Smith) Dye (Essenberg *et al.,* 1992) and lettuce-downy mildew (Bennett *et al.,* 2008). The signaling messengers (reactive oxygen intermediates, salicylic acid, jasmonic acid, nitric oxide) of the plant HR during apple scab disease have not been identified (Leach *et al.,* 2001). Wing *et al.,* (2003) suggested that a delay of the HR may reflect that avirulence proteins are not in direct contact with the mesophyll cells immediately upon infiltration. Broggini *et al.,* (2010) studied the genetic mapping of 14 avirulence genes in an EU-B04_1639 progeny of *V. inaequalis* based on microsatellite and AFLP markers, and investigated for inheritance of avirulence traits. The author reported that the clustering of avirulence genes in *V. inaequalis* reflects the clustering of resistance genes in *Malus* and suggests that this pathosystem is a classical example of an ‘‘arms race’’ between host and pathogen.

The hypersensitive response and the death of the cells indicate the importance of the defense mechanism to the plant. It protects other regions from infection and prevents the expansion of the fungal infection site through cutting of the food supply by death of cells. The fungus will die due to the food deficiency. The PR proteins and the PR tools in plants are mostly inducible.

On the other hand sporulating, necrotic areas are still alive for some time and are an important food source for the fungus. The cells can secrete proteins, sugars and salts to resist the fungal infection and reduce the expansion of the pathogen propagules.

Three types of PR response in plants are known. In the first type the infection is assuaged by low amount of PR proteins on the cuticle surface and in the apoplastic fluid. Most PR responses are inducible in this case and the signaling can be delayed according to a long promoter activation time. In the second type of plants, some of the PR proteins and tools are constitutive while others are inducible.

In the third and most resistant type of plants the PR proteins and the PR tools (cuticles, salts and surface proteins) are mostly constitutive. The signaling in this case is very high and very fast. Thus promoters of the PR proteins activation and expression are in the real time.

6.13 Role of melano protein in PCD

The most important role of the melano protein of *V. inaequalis* is to destroy the cell wall, cell wall protein and apoplast proteins around the cells using both protease and glucanase followed by attacking the cells membrane and the cells (scavenger). For the first time in plant pathogenesis research the involvement of the protease-glucanase protein (melano protein) in the infection process by *V. inaequalis* is described. After diffusion of the melano protein into the apoplast fluid of *M. domestica* cv. Elstar the protein attacks and destroys the membrane channels and the secretory systems of the cell membrane. That causes enhanced membrane permeability, wherefore the cell can no longer control the solute movement. Afterwards the physiological pathways are disrupted which causes cells death. Some reports describes the development of treachery elements (TE) in zinnia and it was concluded that the secretion of a TED4 (LTP) inhibit proteasome activity to protect the neighboring cells. The depletion of TED4 protein from the culture medium results in an increased mortality of living cells (Endo *et al.,* 2001). From this results as a defensive role for the non specific lipid transfer protein (nsLTP) was assessed. Koutb (2006) stated that LTP expression prevented PCD and consequently necrotic lesion formation. If the nsLTP can interfere and prevent PCD in the plant cell, it can play a vital role in the life cycle of *V. inaequalis,* particularly after penetration of the cuticle layer. During this critical period LTP can delay PCD until the fungus switch on its own mechanism by releasing of the melano protein into the apoplast. The melano protein has the ability to bind iron and therefore interferes with oxidative stress (Singh *et al.,* 2005). Consequently, LTP can be regarded as a susceptibility factor especially in the case of biotrophic pathogens like *V. inaequalis*. The newly gained results support the direction of PCD by the melano protein of *V. inaequalis* firstly and the plant protease secondarily. The melano protein diffuses through the cuticle before the fungus invades the cuticle to prepare an easy way for the fungal hyphae. This is performed by low pressure into the dead and semi digested plant cells. LTPs, ribosomes, nucleus proteins and enzymes could be hydrolyzed by the melano protein (substrate). This function is performed without mechanical pressure by the fungal hyphae or other factors. During this stage the PCD is triggered by both virulence melano protein protease and plant protease. In this case of apple scab LTPs did not play a role in the defense mechanisms, because the protein concentration could decrease by the protease

activity and the transcription or translation ceased, because the related enzymes were destroyed by melano protease (chapters 5.13, 5.15). The second argument against a role of LTP in plant defence mechanisms is given by current and previous studies for membrane permeability, in which the permeability increased by the melano protein protease activity during infection. The permeability of the membrane could be caused by melano protease which digests the membrane channels proteins (membrane control systems). LTPs involved on membrane lipids or phospholipids carrying and repairing. Depending on these facts LTPs cannot delay the PCD because it is not involved on inhibiting of the melano protein by repairing channels proteins. The LTP could be used as a substrate protein by the melano protein protease. This point is supported by Koutb (2006) and Gau *et al.,* (2004); they found the nsLTP transcription and nsLTP protein level in the apoplastic fluid decreased after infection. This is supported by two findings, one of them is the protease activity of the melano protein and that the plant nsLTP cannot delay the PCD of the cells otherwise the cells life time would increase and the nsLTP still exist. Koutb (2006) ranked nsLTP as a susceptibility factor especially in the case of biotrophic pathogens *V. inaequalis*. The ranked nsLTP protein and the interaction of plant total protein in this study on PVDF membrane support the role of fungal melano protein in PCD of *M. domestica* plant cell.

6.14 Infection-defence theory

V. inaequalis fungus is an enigma in its life cycle, reproduction, infection method, growing beneath the cuticle, necrosis formation, strategy of infection establishment and occupation mechanisms. Also the plant defence mechanism is not clear. The coming discussion introduce a possible point of view for the infection-defence theory depending on the previous and the current work.

6.14.1 Melano protein

For the first time in plant pathology, molecular pathology, patho-physiology, and enzymology the double function of the melano protein (glucanase and protease) and the interaction of the melano protein with the host proteins can solve the enigma of the infection and provide insight into the defence mechanisms and build up a model for infection. It can help in the understanding of the infection mechanisms of *M. domestica* by *V. inaequalis*, necrosis formation in the susceptible plant and the defence mechanisms in the resistant plant. The function of the protein will help to evaluate and rethinking known protein characteristics and basics of molecular science and pathology.

6.14.2 Melanin

Melanin plays many roles in the infection as the protection of the fungal cells against harmful and stress conditions, sexuality maturation ascospore maturation, sclerotia formation and metal binding. It is known from the literature that melanin bind to certain protein or hydrolytic enzymes and form grains, which release the enzymes in slow way to achieve the fungal goals. In this study the important role of melanin in protein polymerization to micelles, granules, and grains was investigated. This structure may help in protecting melano proteins against inhibitors, protease-protease reactions, and metal action by masking over the active sites of the proteins.

Melanin can work alone to inhibit some of the host metalo-protease or metalo-enzymes by chelating metals or by hydrophobic interaction, which leads to enzymes aggregation especially in an acidic medium. Furthermore it can decrease or cease their activity and inhibit the host defence mechanisms.

6.14.3 Defence mechanisms in plant

The plant defence mechanisms (*M. domestica* cv. Remo) depends on the thickness cuticle, some structural proteins (chitinase), a folded cuticle and waxes, sensors, thick cell walls high calcium content and different minerals, proteins in the surface of the plant embedded in the cuticle, proteins in the apoplastic fluid (including the cell wall proteins) that can inhibit the melano protein activity of *V. inaequalis* and prevent the destruction of the cells. The plant can overcome the glucanase by increase amount of waxes or cell wall thickness to prevent disease establishment through slowing down the infection. It can overcome the protease of the melano protein by protease inhibitors, by protease-protease interaction in the apoplastic fluid or by folding of the cuticle to prevent water and conidia attachment to the leaf surface. These defence mechanisms are supported by experimental results of this study and previously published works.

6.14.4 Defensive role of the cuticle proteins of *M. domestica* cvs. Remo and Elstar

The task of this work was to investigate the presence and function of the surface protein of the resistant cv. Remo. One of the surface proteins working against the fungal cell wall was isolated and identified and tested as a chitinase. Another protein was identified as dehydrogenase, which might be involved in oxidative stress reaction against the pathogen. These functions are important tools in defense mechanisms of resistant plants.

7. CONCLUSION

The melano protein of *V. inaequalis* is a mysterious protein and may have a key role in infection of *M. domestica*. The melano protein was determined *in vitro* by the traditional methods on the glucan-agarose plate for glucanase activity and casein-agarose plate for protease and on the SDS PAGE. Both glucanase and protease activities of the melano protein are very important in explanation of the melano protein role in HR developing or infection mechanisms that supported by this finding which are in agreement with previous studies like prediction of the glucanase activity (Win *et al.,* 2003) and the analysis of the digested proteins from the host after infection (Fitzgerald *et al.,* 2001). This protein possesses a very important role in plant cell wall polysaccharide hydrolysis or cell wall destruction by glucanase and protease activity. The bifunction activities (protease and glucanase) are a pioneer record in enzymology and molecular pathology.

The defense proteins discovered on the plant surface have a direct role in fungal cell wall attacks (chitinase activity). This activity is directed to fungal hyphae or the infection pegs as a chitin source which causes weakness for the fungal power and minimize invasion of cuticle. The other discovered protein is as well as the chitinase in direct contact with the mycelia cell wall or closer to the plasma membrane possibly in order to increase the membrane permeability of the fungal cell membrane and to damage the membrane system, especially the H+ ATPase. Chitinase and other proteins are located on the plant surface of the epidermal cell cuticles. The detection methods and isolation of the surface proteins from the resistant cultivar Remo are new. The surface proteins have a strong role in plant defense (Pelletier, 1962 and Bondarenko, 1972). On the other hand the interaction of the surface protein with the melano protein may provide strong evidence of both the avirulence of the melano protein as well as the roles of plant surface proteins in infection-defense mechanisms. The staining method was newly developed by using bromophenol blue which chelating calcium. Its fluorescence advantage provides evidence for the presence of calcium in the cuticle region. The staining method revealed a difference between the resistant (Remo) and susceptible (Elstar) apple cultivars. It was supported by the so far unexplained results of Shear and Drake (1971) for the cuticle stain of the fruit which gave a very strong evidence of calcium accumulation in the fruit cuticle. The interaction of the surface proteins in both the vertical section and the replica allowed the construction of a three dimension model for the established proteins on the surface of the resistant cultivars.

The presences of melanin with the hydrolytic enzymes according to the melanin function increased the resistance of the melano protein units against the oxidative stress or inhibitors. Moreover, melanin can inhibit the host enzyme by aggregating them hydrophobically or by chelating metals required for enzyme metal binding sites. Therefore it increases the hypersensitive response of the host. In this study the melanin caused a polymerization of the recombinant melano protein and the molecular size of aggregated grains increase as metal was added. This result revealed that the interaction of the melanin with the recombinant proteins depends on both hydrophobic and metal chelating properties. The moderate amount of melanin in the melano protein complex enhances the melano protein functions. High amounts of melanin could reduce the function of the melano protein. The melano protein as a component of the grain complex reflects the wide range of possible activities present in the grains. The granules are powerful, multifunctional enzymes which perform eminent tasks in pathogenicity. Furthermore cutinase and other enzymes could also aggregate with the current studied melano proteins and that is indicated by the presence of a black halo around the infection pore of the cuticle, supported by this study and Steiner and Oerke (2007).

The interaction of the melano protein with the susceptible apple cultivar Elstar was detected. The melano protein diffuses very fast into the epidermal cells before the invasion of the fungal infection parts. The fast diffusion of the melano protein causes cells death according to its protease and glucanase activities which induce hypersensitive response (HR). The melano protein protease activity effects the host cells faster than the glucanase activity during the infection according to the Km values. Moreover the fast diffusion reflects the absence or low amount of defense proteins and other defense mechanisms, which function as glucanase or protease inhibitors. The infection triggers metal accumulation at the point of infected area, either constitutively or by induction. The negative and positive interaction of the surface replica proteins in the interaction of *in vitro* resistant and sensitive cultivars (Remo and Elstar), represents the first line of defense against infection, including chitinase and other unidentified proteins present on the surface of the resistant cultivars (First line of defense). The interaction of the apoplastic fluid proteins on PVDF membrane or locally inside the plant leaf section supported the theory of a defensive role of the apoplastic fluid and apoplastic PR proteins (Second line of defense).

This study gave a basic model of the function and importance of the avirulence melano protein for the infection process. Investigations of the melano protein role (protease and glucanase activities) in hypersensitive response were determined correctly. The model of melanin-protein interaction was built based on previous and the present studies. The model of

plant resistance relies on the surface protein and structure, the apoplastic proteins, cations and metal salts. The effects of metals, inhibitors, pH and temperature on the melano protein glucanase and protease activities were studied. Km values for glucanase and protease activity of the melano protein were determined.

Future work with the focus on the characterization of the protein structure and defense proteins of the resistant cv. Remo and Elstar during the real infection time should be performed. Protein role tests on more wide range of microorganisms to find further advantages of the melano protein. The industrial use of the melano protein must be studied in detail. Based on the current study and the studies before, the design of new bio-anti-fungul substances related to factors affecting on the structure and function of the melano protein should be aspired. The bifunction of the melano protein may open the door for biologists to reinvestigate many of the known enzyme function. Therefore additional information of these molecules could be gained, which would lead a better understanding of their features in the biological science and physiology pathways.

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ACKNOWLEDGMENTS

In the name of Allah, the most Gracious, the most merciful.

First I would like to thank our Almighty Allah who helped me to achieve this dream of mine successfully. I remember my dear mother for her unequivocal sacrifices and my father for his struggle to ascertain better education for me. I would like also to thank my little son who is my God´s gift Abdallah and my wife Heba without their love, patient, encouragement and support, this work would never been done. with the same level, I would like to give an unlimited thank to my siblings Nagah, Osman, Amin, Entsar, Enas, Mamdoh, Mohamed, Rasha, Ali and my little brother Ahmed for praying for me everyday to come through this work successfuly.

In deed words fail to express my sincerely grateful and deeply indebtedness to my supervisor PD. Dr. Achim Gau for his continuous guidance, patience, and encouragement during the entire study period. His teaching strategy helped me to build a self confidence beside the knowledge. Really his supervision was a profound inspiration and he was an excellent representative for the German scientists. Therefore I am so indebted to him there is no way to repay it.

My honest thanks and gratitude must go to Prof. Dr. Edgar Maiß for his delightfully cceptance to be the co-referee. Greeting must go for his strong background in the scientific work.

Special thanks must go to Henrik for his help in revesion and correction of the disscusion, Mohamed Hassan Mohamed, Silvia, for their unequivocal support and cooperation during the whole period of study.

My thanks and gratitude must go to Prof. Dr. Peterhäsel Christoph, Prof. Dr. Huchzemeyer Bernhard, Prof. Dr. Papenbrock, Jutta and their groups. I also wish to thank Dr. Iris Szankowski for providing the *in vitro* apple cultures and to Dr. Katia Schulze for providing *Venturia inaequalis* isolate.

I am very grateful to Mrs. Julia Lensing and Mrs. Yvonne Leye and all the people working in the green haus for their excellent technical assistance especially with the cultivation and propagation of the plant materials.

Many thanks for my home institution, Botany department, Faculty of science, Assuit University, Assuit, Egypt for securing my sabbatical leave to complete my Ph.D. study.

I am gratefully appreciating the financial support of this work that was awarded to me by the Egyptian government as a long-term scholarship.

ERKLÄRUNG ZUR DISSERTATION

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

Characterisation of the melano protein of *Venturia inaequalis* and its impact on plant pathogenesis

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.
Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

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Hannover, den 16.0 2.2011

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