Master Thesis:

Comparison of methods to test antimicrobial activity *in vitro*

With particular emphasis on viscotoxin A3

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Acknowledgement

Initially I would like to express my sincere gratitude to my supervisor Prof. Holger Bohlmann for the continuous support of my master's thesis, for his patience, motivation, enthusiasm and vast knowledge. His guidance helped me to finish this research and write my thesis in a field that had not been my focal point before and to which mostly on his behalf I extended my level of information to a degree of competent familiarity.

Furthermore, I would like to thank my laboratory-colleagues - especially those who offered their support in different parts during the progression of my study. Within this context I would firstly like to express my special thanks to Dr. Stephan Plattner with whom I had next to my supervisor a close collaboration according the experiment. I`m obliged to Dr. Johannes Stadlmann and Dr. Josephine Grass as to Mass Spectrometry and to Dr. Kausar Hussain Shah and Dipl.-Ing. Sabine Daxböck-Horvath for helping me in some relevant parts of this thesis. And of course I am owing thanks to the other laboratory-colleagues for having memorable times together, who although not having a major influence on my thesis had created an atmosphere of well-being with their ultimate kindness.

At the end I would like to thank my parents and my brother for supporting me along all these years of my study far from my native land. Without their positive motivation and encouragements reaching this point would have been impossible.

Thank you all!

Morteza Baselizadeh

Abstract

Viscotoxins are short cationic peptides belonging to thionins type III isolated from European Mistletoe *Viscum album*. These peptides consist of 46 amino acids with a molecular weight of 4828 to 4897 Da and their structure is reported to follow the shape of "L". Viscotoxins are known due to their antimicrobial and cytotoxic activities against several cell lines. Viscotoxin A3 is the most and viscotoxin B is the least potent regarding antimicrobial activity (AMA). AMA of viscotoxin A3 on *Fusarium Solani* and *Phytophthora infestans* has been already tested and a minimum concentration needed to inhibit the growth of the mentioned fungi to the level of 50% (IC_{50}) 7.3-18µg/ml has been reported. In another part of the study by using different methods the concentration needed to reduce the growth to the level of 5%, 48µg/ml has been reported.

The objective of this study was to confirm the published results and test viscotoxin A3 with bacteria and other fungi. Viscotoxins were extracted from *Viscum album* and purified by HPLC and viscotoxin A3 was identified by mass spectrometry MS/MS and other proteomics technics. The microtiter plate (Nunc 96) resazurin based bioassay was tested successfully. In this study the bioassay was used for different fungi and with minor change for bacterial strains. The results were evaluated and determined by measuring absorbance, florescent intensity (FI) and resazurin color change observation. The outcome of the evaluations all suggested that even a high concentration of 300 µl/ml did not have any inhibitory effect on *E. coli*, *P. syringae* and *A. tumefaciens.* Also, no activity was observed against on *F. oxysporum, F. culmorum, V. dahliae,* and *R. solani with* up to 62 µg/ml viscotoxin A3. These results were observed in more than 5 repeats and were all identical in all 3 evaluation methods.

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1 Introduction

Fungal and bacterial infections are two important causes of worldwide economic losses and their negative impacts on human and animal health is not deniable. Strategies, which are involved with developing resistant cultivars by hybrid or molecular breeding approaches to introduce specific resistance genes are time-consuming and limited. Fungicide application for disease control is an alternative method, but only a limited number of drugs are available for treatment of these infections. Rapid multidrug resistance development of bacteria and fungi makes it important to investigate alternative treatments with a minimum negative effect on the environment and human health. Short antimicrobial peptides (AMPs) as a part of the intrinsic defense mechanisms of most organisms have been proposed as a blueprint for the design of novel antimicrobial agents. This project focuses on viscotoxin A3, a short AMP from the thionins family. Antimicrobial activity of viscotoxin A3 against *Fusarium solani* , *Phytophthora infestans* and *Sclerotinia sclerotiorum* has already been reported (Giudici et al., 2004). The main objective of this thesis was to compare different methods for testing the antimicrobial activity of AMPs using viscotoxin A3.

1.1 Antimicrobial peptides

Plants are exposed to several different pathogens during their life period but just in few cases a pathogen dominates the host plant. This is certainly due to different defense mechanisms which plants are able to perform to reduce distribution of damage and fight against exterior invaders. In contrast to the primary plant metabolism, which is responsible for photosynthesis and respiration of the plant, the secondary plant metabolism is involved in plant-animal interactions. Evolution can play a key role in plant defense mechanisms. One part of the plant defense system are AMPs, small, basic, and often cysteine-rich polypeptides isolated from different organisms which possess antimicrobial activity *in vitro* (Bohlmann, 1997). AMPs can be produced constitutively or in response to the presence of a foreign body or in occurrence of an injury (Bohlmann et al., 1998, Vignutelli et al., 1998, Thevissen et al., 1999) in order to restrict the damage caused by another organism and prevent the expansion to other plant parts and recovering of the infected part. The first plant AMP (purothionins) isolated from *Triticum aestivum* were discovered in 1942 by Kent Arnold Balls. They found antimicrobial activity of Purothionins against human pathogens only *in vitro* (Candido et al., 2011).

AMPs are important components of the innate immune system of living organisms of all types. Short peptides are generally between 12 - 50 amino acids long. This cationic host defense peptides can kill gram-positive and gram-negative bacteria, fungi, parasites, and enveloped viruses. Recent research has shown that cationic host defense peptides can also alter the immune response in mammals, for instance, to prevent septic shock (Mookherjee et al., 2009). AMPs are generally believed to kill bacteria through membrane permeabilization and extensive pore-formation (Kolusheva et al., 2000).

Structure activity analysis of peptides revealed that two main requirements for antimicrobial activity are a cationic charge and an induced amphipathic conformation (Powers & Hancock, 2003). AMPs are classified to 3 groups according to their structure and composition: I. Linear peptides II. Cysteine stabilized peptides III. Peptides with unusual composition like glycin or proline-rich peptides, but the majority of plant AMPs belong to the second class (de Caleya RF et al., 1972, Wang, 2004).

The study published in 1995 (Cammue et al., 1995) about 10 kD antimicrobial proteins called *ace*-AMP1 extracted from onion seeds (*Allium cepa* L.), which had inhibitory effect on 12 tested pathogenic fungi at a concentration below 10 µg ml^{-1}. Based on his study *ace*-AMP1 has shown some activity against gram-positive bacteria but not against gram-negative bacteria in mediums with low cationic concentration. This protein has a high homology with plant nonspecific lipid transfer proteins (nsLTPs) and is classified as a member of the nsLTPs family. However, it shares only 76% of the residues that are conserved among all known plant nsLTPs and is unusually rich in arginine, whereas Zm-nsLTP and Tu-nsLTP from radish and maize didn't have this ability (Cammue et al., 1995).

There are several examples of AMPs groups in nature. For instance, it can be pointed out to maganins, peptides isolated from the skin of *Xenopus laevis* with antimicrobial activity which works as a shield for the wet skin of *Xenopus laevis* (Zasloff, 1987). Their mode of action is described with formation of amphipathic helices after binding to the membrane and penetration which leads to their destruction (Jackson et al., 1992). Defensins are another group of antimicrobial peptides (Lehrer et al., 1991). They are 29-34 amino acids long, basic peptides with 3 disulfide bridges to stabilize a triple-stranded ß-sheet structure (Hill et al., 1991). They are produced as preproproteins with an acidic prodomain N-terminal to the defensin (Daher et al., 1988). The mature defensins can be found in azurophil granules *(granulum azurophilum*) of polymorphonucleated neutrophils of different mammals, which ingest and subsequently kill microorganisms. In terms of function they are similar to other peptides with a different arrangement of disulfide bridges, which have been found in bovine neutrophils and named ß-defensins (Selsted et al., 1993). Like other AMPs they target plasma membranes of bacteria and form voltage dependent ion-permeable channels (Kagan et al., 1990). Another group of AMPs, which can be pointed out in this part are mammalian AMPs produced by epithelial cells (Diamond et al., 1993, Jones & Bevins, 1992). Mammals have a second preformed and inducible defense system to protect the epithelial tissues which are potentially exposed to pathogenic microorganisms. For example, cystic fibrosis, is thought to result in salt-inactivation of a ß-defensin in the airway surface fluid of the lung (Goldman et al., 1997). The immune system of insects is highly dependent to AMPs as well (Boman & Hultmark, 1987). For example amoebapores, membrane active antibacterial peptides isolated from the protozoan parasite *Entamoeba histolytica*, seem to function, besides other proteins, in the killing of engulfed bacteria (Leippe et al., 1994). But the last group are plant defensins. They were originally described as thionins but without homology to the classical thionins (Bohlmann, 1994) and have been found in many plant species and are perhaps ubiquitously distributed in higher plants. They are 45-54 amino acids long, basic, and have 8 conserved cysteine residues.

1.2 Thionins

In higher plants a large number of toxic compounds exist whose physiological roles in plants are rarely understood. Thionins are one of these well-defined groups. Toxic effects of these low-molecular-weight polypeptides on bacteria, fungi, yeast and animal and plant cells have been described (Bohlmann & Apel, 1991).The history of the knowledge of thionins reaches back to 1885 when Jago and Jago described for the first time the toxicity of substances isolated from wheat flour against yeast. Later, the responsible polypeptide for the toxicity was named purothionin (Balls et al., 1942). It consists of three different isoforms, basic and cysteine-rich polypeptides with a molecular weight of about 5 kDa like the majority of thionins. In 1992 seed-specific thionins were discovered in wheat and *Aegilops squarrosa*, which in terms of structure had a lack in cysteine residues 2 and 8 (Castagnaro et al., 1992, Castagnaro et al., 1995). In 1987 leaf-specific thionins were found in barley (Bohlmann & Apel, 1987, Gausing, 1987) in addition to those localized in the endosperm. Thionins have been found in different plant tissues from endosperm to leaves of monocotyledonous (Ramshaw, 1982) and various dicotyledonous plants (Samuelsson, 1966, Samuelsson, 1969, Mellstrand & Samuelsson, 1974, Samuelsson & Pettersson, 1977, Thunberg & Samuelsson, 1982, Thunberg, 1983). One of the first species shown to contain large amounts of thionins was the semi parasitic plant mistletoe (*Viscum album*).

In 1949 Winterfeld and Bijl were the first who isolated thionins from mistletoe leaves. Antibacterial function of thionins originally proposed by Fernandez de Caleya, who has investigated antibacterial activity of basic polypeptide, purothionins extracted from *Triticum aestivum* L. flour. In his study he found susceptibility of gram-positive and gram-negative bacteria *Pseudomonas, Xanthomonas, Erwinia, Agrobacterium* and *Corynebacterium* strains to purothionins (Fernandez de Caleya et al., 1972). Thionins are assumed to work as a part of plant defense mechanism after this study, which proved the activity of purothionin present in endosperm of barley against several plant pathogens (Fernandez de Caleya et al., 1972).

Thionins are enriched in cysteine residues and synthesized as larger precursors consisting of a signal peptide, the thionin domain and a C-terminal acidic polypeptide extension (Bohlmann & Apel, 1987, Castagnaro et al., 1992, Gausing, 1987, Hernandez-Lucas et al., 1986, Rodríguez-Palenzuela et al., 1988, Schrader & Apel, 1991). Thionins consist of approximately 20 amino acid leader peptide and another 60 amino acid long trailing acidic peptide (Schrader-Fischer & Apel, 1994), whose cleavage of leader peptide is necessary for toxin activation (Romero et al., 1997).

Thionins sequences show a high degree of conservation and have a very similar three-dimensional structure. Therefore it can be concluded that they have a very close mode of action and functionality and are produced in plants for the common reason. Disparity between different types of thionins lies in their number of disulfide bridges. Based on NMR structures study of thionins their structure consists of an N-terminal short β-strand linked to two antiparallel α-helices connected by a short

random turn motif, followed by another β-strand forming an antiparallel β-sheet and a C-terminal coiled region (Westermann & Craik, 2010).

Two distinct but well characterized groups of plant peptides were originally named α/β thionins and y-thionins. Even though they share the same name, they are distinct in their three-dimensional architectures. The appropriate name, which is proposed for y-thionins is now plant defensins. α/β thionins are classified into 5 groups according to their amino acid homology(Garcia-Olmedo et al., 1989). They considered endosperm thionins and barley leaf thionins as different types. These are now combined into one class together with *Pyrularia* thionin. Based on the number of cysteine-residues and the pattern of disulfid-bridges thionins are now grouped in the following classes (Bohlmann et al., 1994).

- Class I: Thionins with eight cysteines as in purothionins and hordothionins, barley leaf thionins, and *Pyrularia* thionin
- Class II: Without cysteines no. 3 and 6, for instance crambin and viscotoxins from leaves.
- Class III: Without cysteines no. 3 and 6, for instance crambin and viscotoxins from leaves.
- Class IV: Without Cysteine no. 2. New cysteine between no.4 and 5. Thionin 4-1 from *Tulipa* (De Boer, 1994).

Class V is the one, which is less reviewed because it doesn't have any toxic activity. All types are highly homologous concerning the amino acid level (Stec, 2006).

Thionins have small variations in length (45–48 amino acids with 3-4 internal disulfide bonds) and share the same three-dimensional architecture (Stec, 2006) with a high similarity of the distribution of the hydrophobic and hydrophilic residues (Florack & Stiekema, 1994). Thionins type III are known due to their bactericidal, antifungal, insecticidal activities and immunomodulatory function. Their overall shape is like a capital letter L, where the α-helixes define the long arm and the short antiparallel β-sheet the short arm (Romagnoli, 2000). Mode of action in this family is described in (I) membrane disruption by electrostatic interactions through charged residues and (II) DNA binding through the structural helix-turn-helix motif, common in prokaryotic gene regulatory proteins and homeodomain-containing proteins.

It is assumed that thionins are expressed as non-toxic propeptides activated upon attack of pathogenic invaders (Romero et al., 1997, Thevissen et al., 1996). Thionins role in the defense mechanism of plants is described as expression in response to mechanical wounding (Bohlmann et al., 1998) or phytopathogenic fungi (Vignutelli et al., 1998), as accumulation in vulnerable tissues (Orrù et al., 1997, Patey et al., 1976, Gu et al., 1992, Schrader-Fischer & Apel, 1994), as toxicity to bacterial pathogens (Molina et al., 1993, Fernandez de Caleya et al., 1972) and different cell lines (Nakanishi et al., 1979, Kramer et al., 1979), and as improved resistance in transgenic plants expressing different thionins (Epple et al., 1997, Iwai et al., 2002, Carmona et al., 1993), which all prove this important function of thionins. Thionins expression can be in response to external stimuli. For instance, amounts of thionin-specific mRNAs (belonging to thionins type II) present in seedlings of barley grown in darkness are high but this amount declines upon the exposure of barley to light (Reimann-Philipp et al., 1989). Their high accumulation in the endosperm and high cysteine content are two evidences that they may serve as storage proteins as well (Stec, 2006).

There are some variants of thionins, which lack toxicity. The best example for these nontoxic members is crambin. Crambin is a hydrophobic and non-toxic variant of thionins, whose NMR structure is available with high resolution (Jelsch et al., 2000). Crambin is one of the well-studied thionins, whose structure was determined for the first time in the 1980s (Teeter et al., 1981). A lack of toxicity in thionins like crambin is believed to be due to loss of crucial elements of toxicity like Tyr13, which is identified as a key residue for toxicity (Wada et al., 1982) or Lys1 (Stec et al., 2004). Trp8 is also another not-well conserved residue, which is involved in toxicity mechanism (Fracki et al., 1992). In crambin, phenylalanine residue is located on position 13 in the first α-helix instead of tyrosine (Westermann & Craik, 2010).

Thionins toxicity is due to the lysis of the membranes of attacking cells (Fernandez de Caleya et al., 1972, Bohlmann et al., 1988, Carrasco et al., 1981, Oard, 2011). Toxin doesn't have any specific protein receptor on membrane, instead, negatively charged lipids are direct target of toxin (Coulon et al., 2002). They interact with the negatively charged head groups of phospholipids (Hughes et al., 2000, Osorio e Castro & Vernon, 1989) and lead to the loss of membrane integrity via solubilisation of phospholipid (Stec et al., 2004). All these effects can be explained through the Carpet model of membrane action 5 (Stec, 2006). In this model peptides accumulate on membrane surface and change the physical properties of that, which ultimately leads to membrane disruption (Giudici et al., 2003).

The mechanism procedures start by inserting hydrophobic α–helics faces into membrane and forming patches. These patches first cover the part of membranes with toxin and make it rigid, secondly make the membranes at the edges of raft more fluid and facilitate withdrawal of phospholipid from the membrane. During the first process transient ion-change leakage happens, which is recognized as transient ion channel formation. By lysis, massive depolarization occurs and releases components of the cytoplasm, which cause damaging cytoplasmic components like DNA and RNA and finally leads to necrosis or apoptosis (Stec, 2006).

The exact mechanism of membrane permeabilization by thionin is still unknown but the recent study on α-hordothionin, a thionin with broad-range antimicrobial activity showed thionins as a small water selective channel (Oard, 2011). In this study Oard described the formation of water channels by α-hordothionin, similar to aquaporins, , wherein the highly conserved Cys and Tyr residues form the pore wall, leading water molecules to the centre of the phospholipid bilayer, thus causing local membrane disruption. These discontinuous instances of local membrane disruption explain the channel-like activity of thionins (Hughes et al., 2000, Llanos et al., 2006) and membrane disintegration. When thionins bind in a close proximity to each other, local membrane disruptions of each individual peptide can merge and at large concentrations leads to membrane disintegration. The Proposed mechanism in this study can be summarized in two main components 1st insertion of a monomer into the membrane in the outer leaflet and anchoring via binding of the phospholipid-binding site to a phospholipid and 2nd opening of the water-selective pore which delivers water through the α-helix core to the bilayer center that leads to local membrane disruption.

Regardless of the high homology and conserved three-dimensional structure, α and β thionins vary in terms of function and activity (Clore et al., 1986, Teeter et al., 1990, Romagnoli, 2000). In a study about modes of membrane interaction of viscotoxin A3 in 2001 (Coulon et al., 2002) it is mentioned that based on earlier published papers (Florack & Stiekema, 1994, Hughes et al., 2000, Thevissen et

al., 1997, Caaveiro et al., 1997) type I and II thionins are toxic towards gram-positive and gram-negative bacteria and fungi whereas type III and IV thionins do not possess this ability *in vitro*. However, later antifungal activity of viscotoxin A3 which belongs to thionins type III was proven against *Fusarium solani* , *Sclerotinia sclerotiorum* and *Phytophthora infestans* (Giudici et al., 2004), but so far the antibacterial activity of viscotoxins has not been reported.

Alignment of amino acid sequences in the following figure is another evidence to show the high genetic similarity between peptides in the thionins family. The following figure illustrates sequences of thionins from dicotyledonous plants. Yellow, cysteine; cyan, basic amino acids (lysine, arginine); red, acidic amino acids (aspartic acid, glutamic acid); pink, tyrosine; green, phenylalanine and tryptophan.

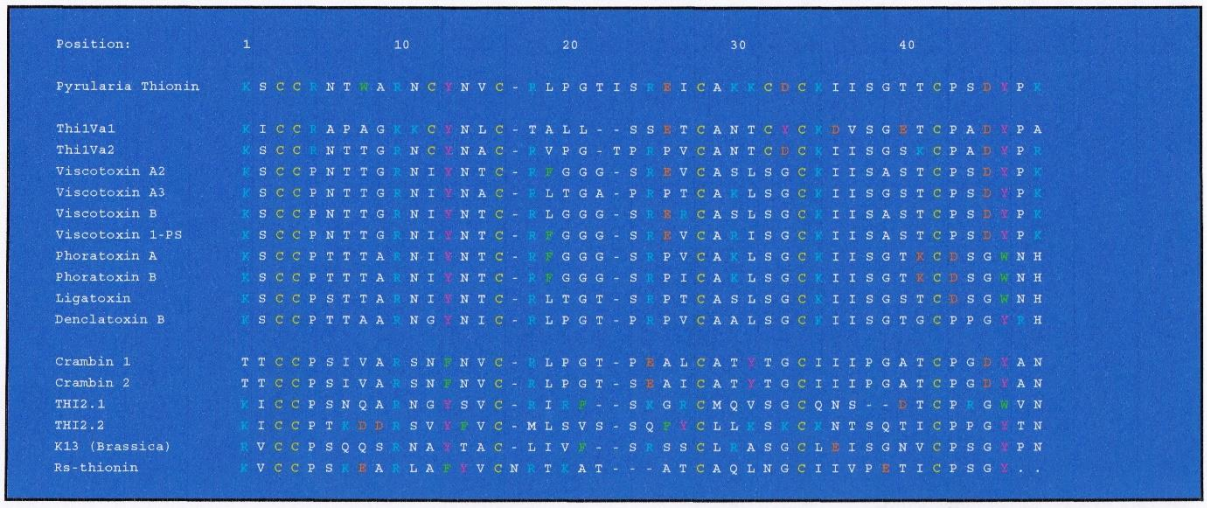


Figure 1 Sequences of Thionins from dicotyledonous plants (Bohlmann, 1997)

1.3 viscotoxins

The European mistletoe *Viscum album* contains numerous small basic proteins with high antimicrobial activity. These peptides were isolated for the first time by Winterfeld and Bijll in 1948 and named as viscotoxin (Samuelsson & Pettersson, 1970). The term viscotoxin derives from the mistletoe family *Viscaceae* and the added toxin refers to the toxicity of these peptides.

Viscotoxins are one of the well known members of the thionin family, which differ from others due to their cytotoxic activity toward different cell types including fungal and also their cytotoxic effects towards tumor cells. They exist in mistletoe leaves, seeds and stems and their antifungal activity has been described as inducing membrane permeabilization and spore death in phytopathogenic fungi (Giudici et al., 2004).

These small cationic peptides with a molecular weight of 4828 to 4897 Da, consist of 46 amino acid residues with a net positive charge (+5 or +6) and three strictly conserved disulfide bridges in order to stabilize their three-dimensional structures (Coulon et al., 2003). viscotoxins with approximately 5

kDa size belong to thionins family type III and their homology to other thionins family is restricted to six cysteine in conserved positions (Schrader-Fischer & Apel, 1993) and one aromatic residue at the position 13, and an arginic at the position 10 (Giudici et al., 2006). Viscotoxins have a very stable structure, which is difficult to destroy and is resistant to heat degradation (Olson & Samuelsson, 1970, Kuttan et al., 1988, Park et al., 1999).

Viscotoxins, used in this experiment, were extracted from *V. album.* Mistletoe is an obligate hemi-parasitic plant, which attaches, grows and penetrates within the branches by a structure called the haustorium and starts to absorb nutrients from the host plant. Mistletoe has a very wide host range, in most cases it just reduces tree-growth but in severe conditions infestation can kill the host. Close relatives of *V. album* produce similar thionins variants, like phoratoxin from mistletoe *Phoradendron tomentosum* (Mellstrand & Samuelsson, 1974), ligatoxin from the mistletoe *Phoradendron liga* (Thunberg & Samuelsson, 1982) and denclatoxin from the mistletoe *Dendrophtora clavata* (Samuelsson & Pettersson, 1977). The similarity of all these thionins lies at six cysteine residues at identical positions. In contrast, thionins from monocotyledonous plant species contain eight cysteine residues (Bohlmann & Apel, 1991, Garcia-Olmedo et al., 1989). Also there are variants of thionins in *V. album* with eight cysteine residues which are distantly related in their amino acid sequences with viscotoxin (Schrader-Fischer & Apel, 1993).

The mistletoe has attracted special interest in medicine throughout the centuries and their treatment for cancer was introduced in 1920. These days mistletoe extracts are the most frequently prescribed unconventional cancer therapy in central European countries. These extracts are complex multi-component mixtures, containing various biologically active substances such as glycoproteins, in particular the mistletoe lectins I, II and III, polypeptides (e.g. viscotoxins), peptides, amino acids, and oligo- and polysaccharides. Numerous preclinical studies have reported their immunostimulatory, cytotoxic and pro-apoptotic effects. In animal models, it can be said that mistletoe extract has a direct anti-tumor effect (Bar-Sela, 2011). Mistletoe lectins have been identified as the main active components and show cytotoxic effects as well as immunomodulatory activity (Podlech et al., 2012). Lectins main effect works directly via abrupt damage to the tumor cells, predominantly due to inhibition of protein synthesis and apoptosis, and indirectly via stimulation of immunological processes (Wiedłocha et al., 1991, Zarkovic et al., 2001). Comparison between extracts of different mistletoes show that cytotoxicity may differ depending on their host plant and environmental factors like temperature, season and region (Hülsen & Mechelke, 1982).

Viscotoxins are about 100 times less toxic than lectins on human tumor cells (Olson & Samuelsson, 1970). But in preparation of tea or medical decoction in anticancer therapy lectins are denatured and lose their toxicity but viscotoxins and alkaloids due to their heat resistance and stable structure remain as a major cytotoxic component in these oncological treatments of tumors (Khwaja et al., 1986). Viscotoxins are less well studied than lectins but their toxic effects towards mammals have been investigated for the first time in 1966 (Rosell & Samuelsson, 1966, Lankisch & Vogt, 1971, Andersson & Johannsson, 1973, Carrasco et al., 1981). It is known that a cytotoxic effect of viscotoxins could be established, caused by rapid lysis of the cell membrane like other thionins. In addition, viscotoxins enhance the activity of cytotoxic T-cells and granulocytes (respiratory burst, phagocytosis), therefore they can significantly better destroy bacteria (and presumably also tumor cells), inhibit RNA-, DNA and protein synthesis and enhance NK-cell-mediated cytotoxicity against

tumor cells (Schaller et al., 1996, Bar-Sela, 2011). Among the important aspects of viscotoxins in the medical field is its effect on improvement of stimulated granulocyte phagocytosis activity, which was also seen in *in vitro* studies. This improvement in granulocyte function is very important for patients under active oncology-treatment and may be one of the causes of improvement concerning side effects of oncology-treatment seen with mistletoe therapy (Stein et al., 1999, Bar-Sela, 2011). The shape of the viscotoxins is comparable to a capital "L", which is reported for hordothionin-α, crambin, purothionin-α and phoratoxin A as well (Han et al., 1996). The two amphipathic helices run in opposite directions and form the long arm of the letter L, while the short arm is formed by the short antiparallel β-sheet (Romagnoli, 2000). Structure–activity analysis of a wide range of peptides reveals that a cationic charge and an induced amphipathic conformation are two main requirements for antimicrobial activity (Powers & Hancock, 2003).

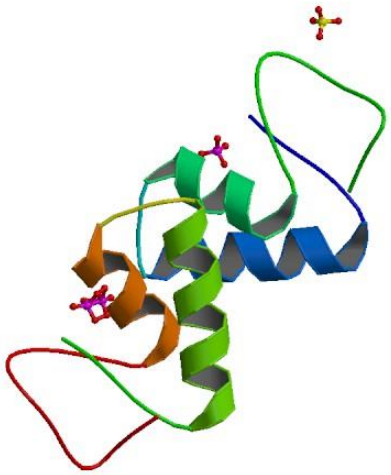


Figure 2 viscotoxin A3 3D structure, (Debreczeni et al., 2003, PDB code 10KH)

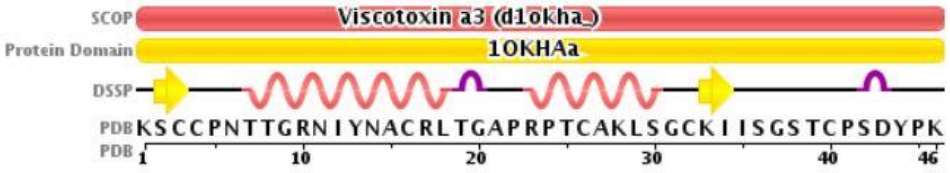


Figure 3 DSSP Secondary structure of viscotoxin A3, PDB code 10KH, it consist of 43% helical (2 helices; 20 residues) Alpha Helix 1 and Alpha Helix 2 and 8% beta sheet (2 strands; 4 residues) Beta Strand 1 and Beta Strand 2, (Debreczeni et al., 2003, PDB code 10KH).

The sequence of viscotoxins is highly conserved and identical among different isoforms(Stec, 2006). Seven types of viscotoxin are described: A1,A2,A3,B,C1,1-PS and U-Ps (Orrù, S,1997). Among all known viscotoxins, A3 has the most and B has the least cytotoxic activity (Schaler G, 1996 ; Büssing A,1999). Viscotoxins are accumulated in high concentrations only in the generative short shoots, leaves and young stems. Viscotoxin A2 and A3 are the predominant isoform of viscotoxin in the young organs whereas viscotoxin B was the main viscotoxin in the old stems. Viscotoxin concentration in mistletoe is seasonal and reaches the maximum concentration in June (20 mg/g dw) and the minimum concentration occurs in the second year before fall (<1 mg/g dw) (C. Jäggy & Schaller, 2007, Urech et al., 2011). Viscotoxin isoforms variations in leaves are less in the first and

second year of growth and with the end of growth in May, degradation of viscotoxin A1, A2, and A3 starts but viscotoxin B remains untouched and increases until the fall of leaves (Urech et al., 2011). To better understand the likelihood degree of different viscotoxins, a FASTA sequence of all known viscotoxins was downloaded from PDB protein database and a phylogeny tree of viscotoxins was created by Phylodraw software. Simply by looking at the phylogram tree we can realize that the highest likelihood in protein sequence is between viscotoxin A3 and viscotoxin A1 with a 95% likelihood degree.

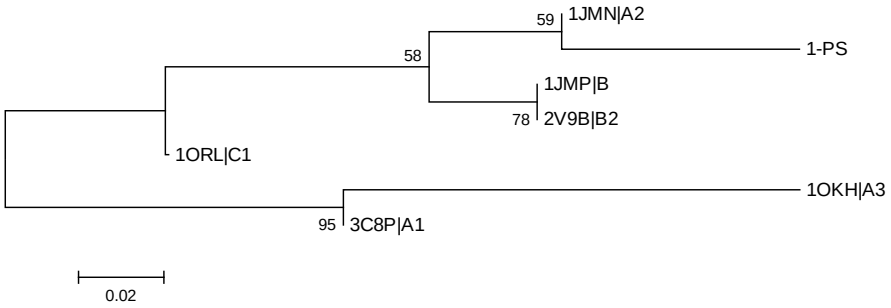


Figure 4 Maximum Likelihood and Bootstrap method used for drawing this Phylogram tree.

Viscotoxins A2, A3 and B, are the most abundant viscotoxin isoforms derived from mistletoe. In spite of their high amino-acid sequence similarity, they differ in their *in vitro* cytotoxic potency towards tumor cells. Interaction of these three viscotoxins was studied with model membranes. Besides viscotoxin B which had less activity, viscotoxin A3 and viscotoxin A2 interact in a similar way, but viscotoxin A2 has a weaker hydrophobic character, which leads to different affinity towards membranes (Coulon et al., 2002). The cellular toxicity of viscotoxin A3 probably is due to its ability to interact with the plasma membrane and especially with the model membranes containing the acidic phospholipid phosphatidylserine (Coulon et al., 2002). Comparison of the amino acid sequence and three-dimensional structure of viscotoxin A3 and B, two viscotoxins, which possess the most and the least antimicrobial activity respectively are illustrated in the following figure.

Figure 5 Amino Acid Sequence and three dimensional structure comparison between viscotoxin A3 and viscotoxin B, Numbers shown on the picture illustrate the points of difference between these two peptides, their exact position is shown via the alignment on top (Giudici et al., 2003)

Fungicidal activity of viscotoxin A3 and viscotoxin B against *F.solani*, *P.infestans* and *S.sclerotiorum* was reported in 2004 (Giudici et al., 2004). In their experiment *F. solani* and *S. sclerotiorum* spores were treated for 30 min at 25°C with 1.5 or 3 µM (7.5–18.75 µg ml^{-1}) viscotoxin A3 and distributed on a plate with PDA medium. The results after 3 days of incubation showed that the percentage of colony forming units was reduced to approximately 5% compared to the untreated one as control. Besides this, they found out that 10 µM viscotoxin A3 or viscotoxin B both are able to inhibit the growth of all mentioned fungi completely (Table 1). The study proved that viscotoxin A3 possesses antifungal activity affecting both spore germination and hyphal growth of phytopathogenic fungi.

Table 1 IC_{50} (µM) table for viscotoxinA3 and viscotoxin B

	viscotoxin A3	viscotoxin B
F. solani	1.5	3.5
S. sclerotiorum	3	3.75

Another study was done with the aim to find out the target cells and mechanism of action of viscotoxin A3 (Giudici et al., 2006). *Fusarium Solani* spores were used as model membrane and the study revealed that viscotoxin A3 mechanism is in induction of an ion-channel like activity, generation of H_2O_2 concentration and increase in cytoplasmic free Ca^{2+}. They also found out that Ca^{2+} is involved in viscotoxin A3 induced spore death as well as augmentation of H_2O_2 concentration.

The study (Giudici et al., 2006) was done to observe leakage in lipid composition of natural and artificially made liposome composition of *F. solani* spores. In presence of viscotoxin A3 natural lipids extracted form *F. solani* and artificial liposomes resembled *F. solani* spores plasma membrane showed, that leakage is lipid composition dependent and observed more in natural one. Liposomes from natural spores' lipid had 100% and 65% leakage with 10 µM viscotoxin A3 and viscotoxin B respectively. Based on this study, they proved that viscotoxin A3 has the capacity to destabilize and disrupt membranes at low lipid/protein ratios.

Viscotoxin A3 can enter and accumulate inside cells. Labeled viscotoxin A3 with Texas red, was monitored by confocal microscopy and they observed that this AMP can enter and accumulate inside *F. solani* cells. Study to understand whether viscotoxin A3 can modify the permeability of spores' membrane was done by using Sytox green, which only enters damaged cells to evaluate the membrane integrity under microscope. After incubation of viscotoxin A3 and spores in case that Sytox green penetrates inside the cell, couples with nucleic acid and becomes observable by fluorescent emission. Only after 5 min of incubation viscotoxin A3 entered inside the cells and after 30 min 70% of spores were filled with Sytox green.This study proved that membrane damage increased by extension of the incubation time. It seems that like other members of the thionins family viscotoxin A3 forms ion channels or pores in cell membranes. Triggering of the channel formation requires a membrane potential different from zero. The activity mostly begins when the membrane is subjected to a positive voltage.

Antifungal activity of viscotoxin is involved with the generation of reactive oxygen species as well. Spores produce H_2O_2 in the presence of 10 µM viscotoxin A3 after 8 hours of incubation (Giudici et al., 2004). H_2O_2 production is related to incubation time and viscotoxin concentration and there is a direct correlation between H_2O_2 production and the death of spores.

Cytosolic Ca^{2+} has an important role in cell signaling and is able to regulate a wide range of physiological functions in diverse organisms (Berridge et al., 2000). Increasing concentrations and the incubation time of viscotoxin A3, increase free cytosolic Ca^{2+}, which may be one of the indirect used mechanisms of viscotoxin A3. To show the relation between cytosolic Ca^{2+} and cell death, the same treatment of spores and viscotoxin A3 was undertaken and Ca^{2+} chelator Bapta-AM added to reduce free cytosolic Ca^{2+} and check whether there is a difference. They observed that H_2O_2 production and spore death stopped and in other words viscotoxin A3 didn't show any antifungal activity (Giudici et al., 2006).

Viscotoxin A3 is also able to induce Ca^{2+} internal increase (Giudici et al., 2004) similar to plant defensins DmAMP1 and RsAFP2 (Osborn et al., 1995, Terras et al., 1992), whose antifungal mechanism is involved in inducing an array of relatively rapid responses in fungal cells, including increased potassium efflux and calcium uptake, which finally leads to membrane-permeabilization (Thevissen et al., 1996, Thevissen et al., 1999).

2 Material and methods

2.1 Viscotoxin preparation

2.1.1 viscotoxin extraction

The viscotoxin extraction was mostly done by Stephan Plattner and I only joined as an assistance in the last steps. For extraction viscotoxin A3 procedures followed:

1. 300 g of mistletoe leaves grinded in 2% acetic acid and stirred over night
2. The sample was centrifuged at 4500 rpm
3. After centrifugation samples were heated to 80°C for 30 min
4. The outcome was centrifuged again but with 12000 rpm
5. 50 mM Tris was added and pH adjusted to 8.5 with NaOH
6. Outcome mixture from step 5 centrifuged at 1200g for 30 min
7. Sample loaded on strong anion exchange EMD TMAE Medcap (M)
8. Flow through dialyzed against 20 mM acetate buffer pH 5.2
9. Centrifuged at 1200g for 30 min
10. Supernatant from step 9 loaded on cation exchange column HIGH PREP SP FF and bound proteins were eluted with a linear gradient of 0 to 100% 1M Nacl
11. Fractions containing viscotoxins were determined by TRICINE SDS PAGE

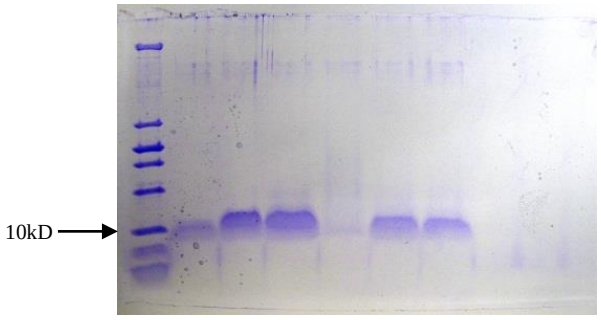


Figure 6 loaded proteins on SDS page gel, as we know viscotoxin size is 5 kDa and 5 sample lines loaded on gel located in between 5 and 10 kDa ladder line. This increases the probability that extracted proteins are to be viscotoxin.

12. Following fractions were pooled, loaded and separated on Reversed Phase Chromatography column SOURCE 15 RPC 6.4/100.

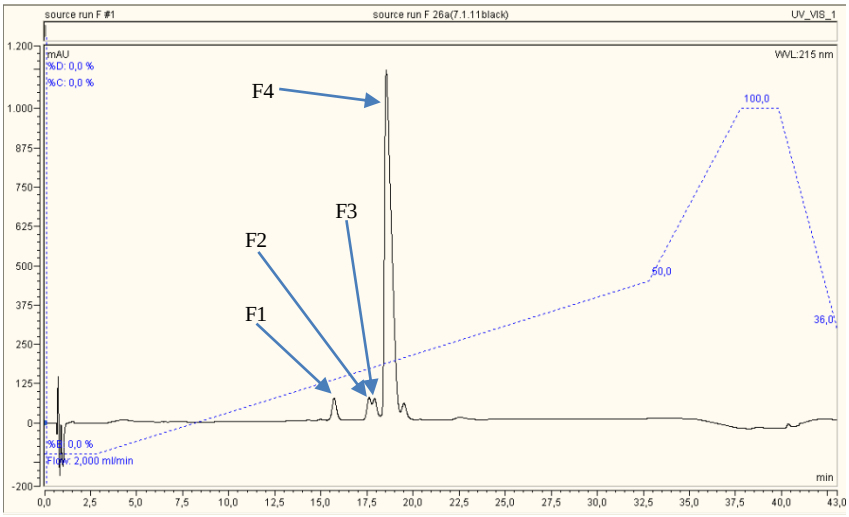


Figure 7 HPLC results for viscotoxin Flow 2ml/min. We measured the concentration of all samples from 15_2-19_4 belong to peak F1,F2,F3,F4. Peak F4 had the highest conecntraion of peptide with 1025.71µg and 736.64µg at 18_3 and 18_4.

13. The 4 peaks were measured offline on an ESI IONTRAP (Electrospray Ion Trap Mass Spectrometry) and identified by Dr. Johannes Stadlmann and Dr. Josephine Grass.

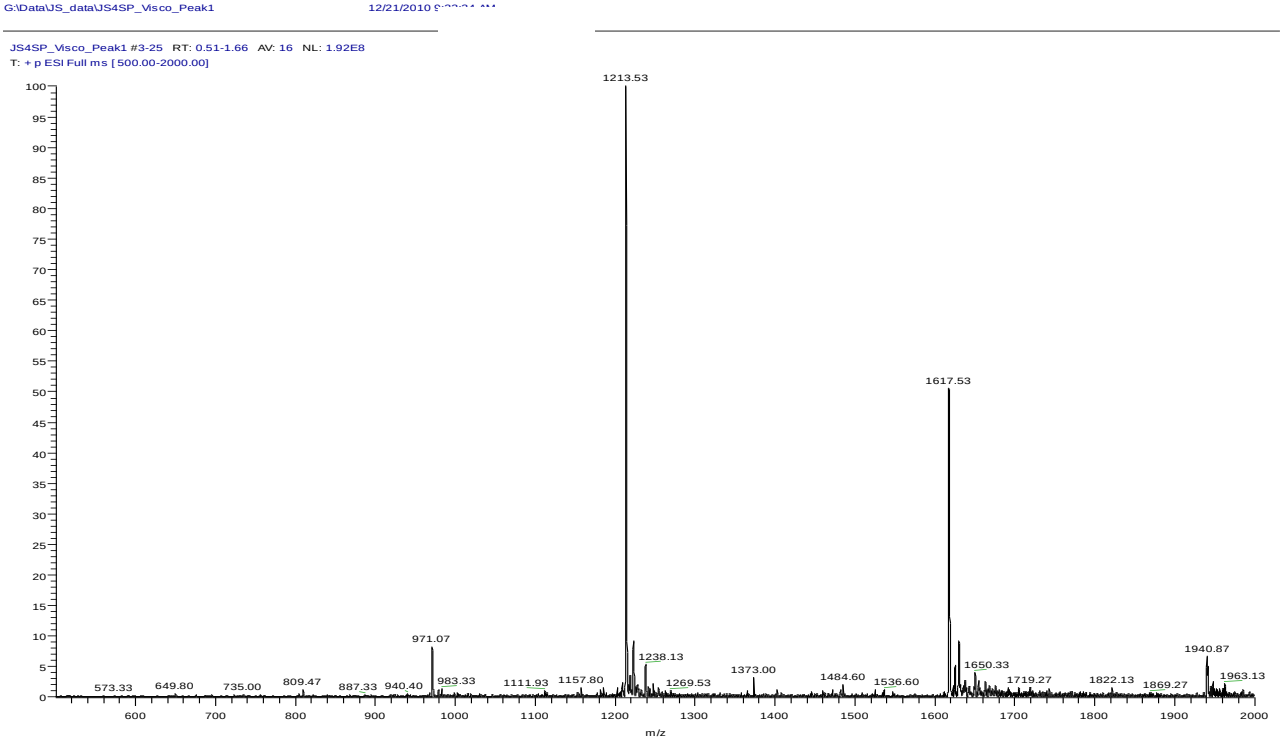


Figure 8 Peak F1, Ion Trap result belongs to viscotoxin B which had the least concentration among all extracted viscotoxins in this Run (Run F) Swissprot database was used to determine the name of peptide.

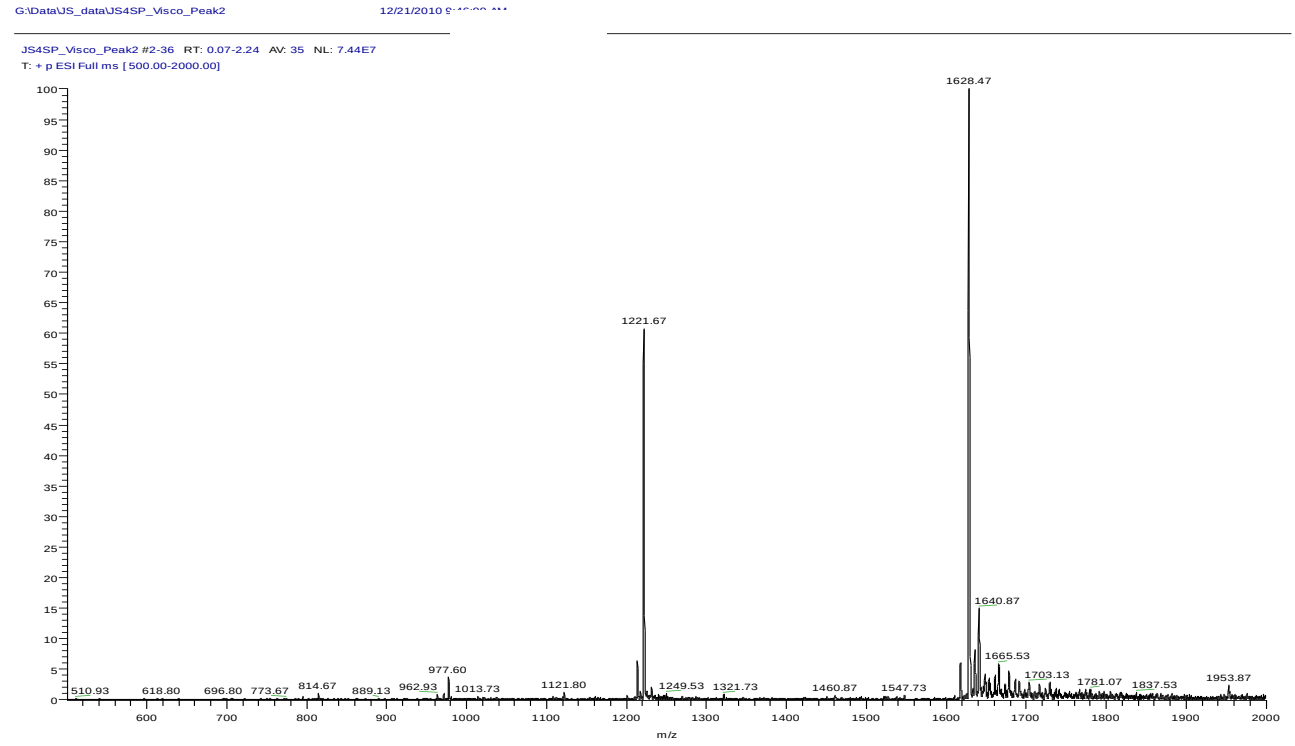


Figure 9 Peak F2, Ion Trap results show that the samples from this peak match with viscotoxin Ps.

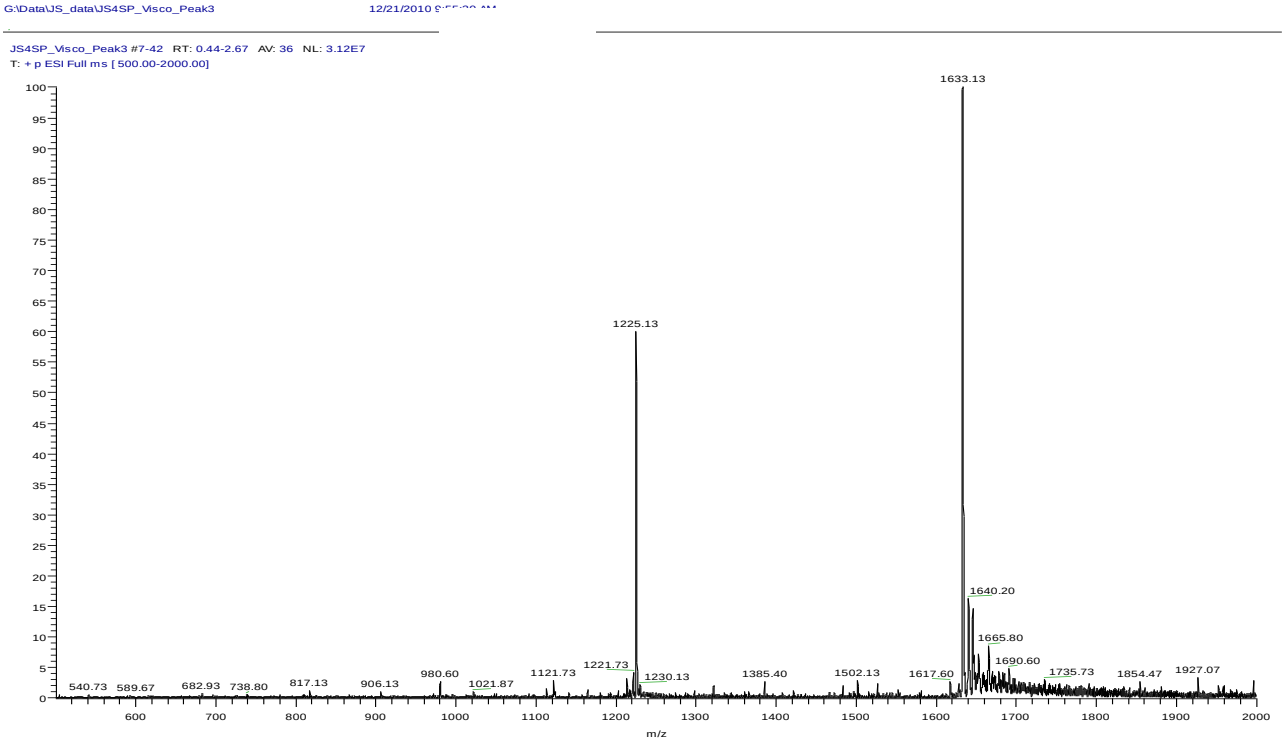


Figure 10 Peak F3 which had an overlap with peak F2 belongs to viscotoxin A1.

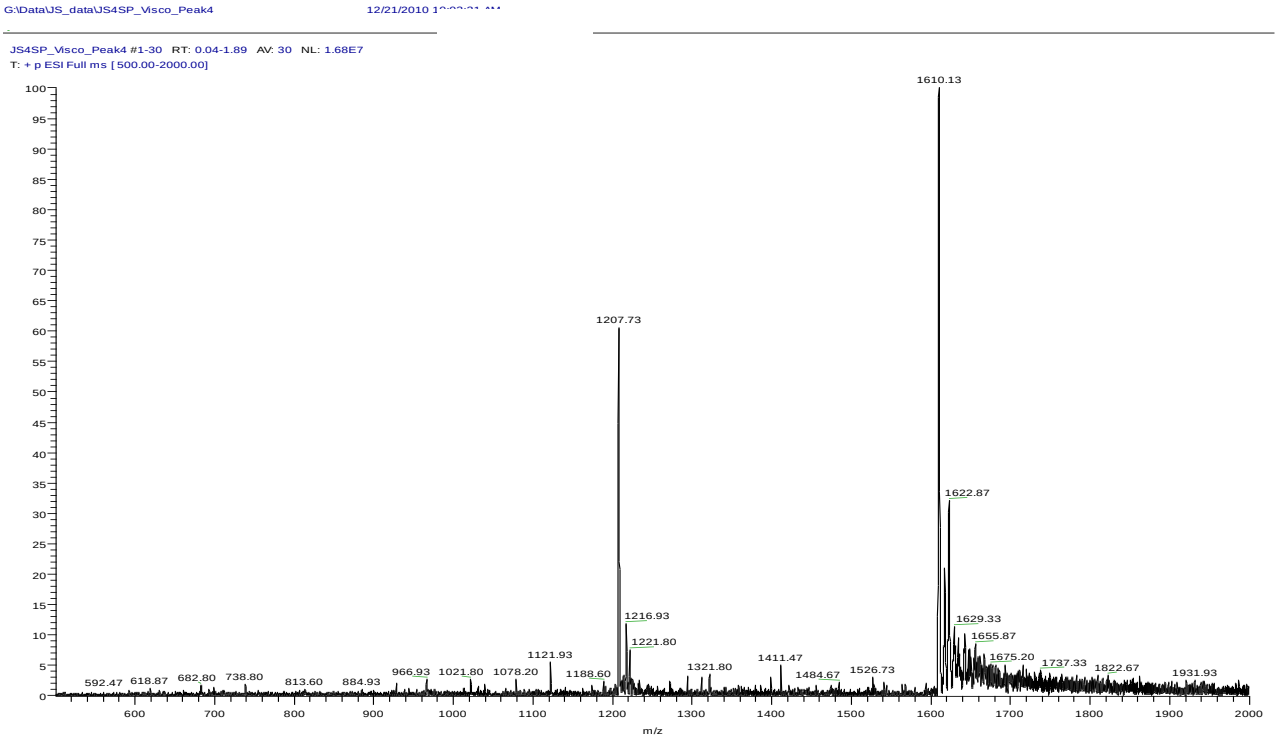


Figure 11 Ion Trap results of Peak F4, which belongs to viscotoxin A3 or A2.

14. From this masses (figure 11) it was impossible to determine the exact name of the current peptide, due to a very small difference between two viscotoxins (only one Dalton). Therefore trypsin digestion and MS/MS were done and it was found that the current sample is A3.

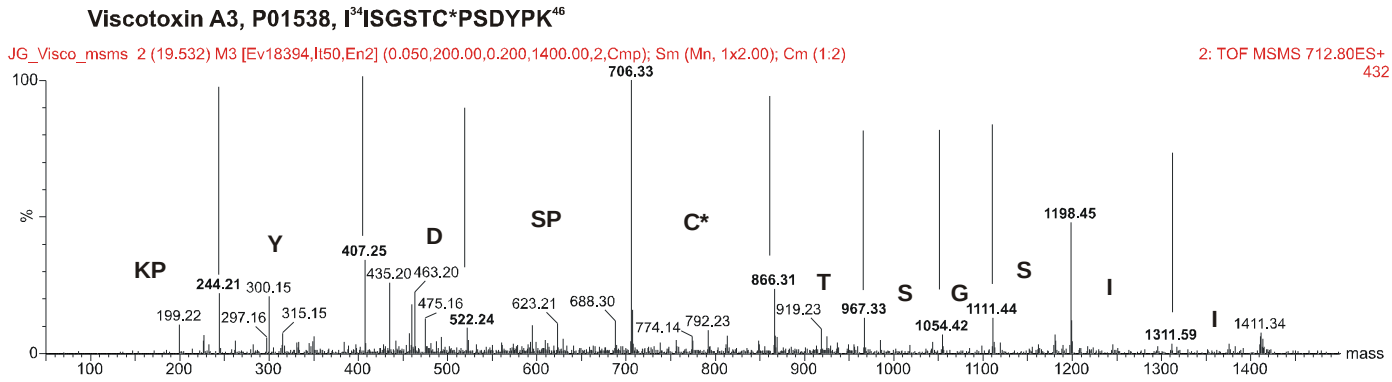


Figure 12 Time-of-flight mass spectrometry (TOF) MS/MS results related to viscotoxin A3.

Table 2 viscotoxin A3 mass found Data , Code P01538

position	Sequence	mass calculated	mass found	RT
1-10	KSCCPNTTGR	1180.5197	590.7	9
11-17	NIYNACR	910.4199	910.4	15
18-28	LTGAPRPTCAK	1171.6252	583.3	9
18-33	LTGAPRPTCAKLSGCK	1716.8883	859	9
29-46	LSGCKIISGSTCPSDYPK	1969.9357	985.4	21.8
34-46	IISGSTCPSDYPK	1424.6726	712.9	19.6

15. The mass difference between A2 and A3 is only 1 Dalton therefore it was impossible to identify the peak number 4.
16. Samples from peak number 4 were reduced and carboxymethylated with jodacetamid, digested with trypsin and the gained peptides analyzed on MS spectrometry ESI and identified as A3.

2.1.2 viscotoxin concentration

To measure the concentration of viscotoxin, the BCA protein Assay kit from the Thermo Scientific Company was used. This kit uses BSA (Bovine serum albumin) as a standard. The major question in this part was, that since the viscotoxin is a very small 5 kDa peptide, which protein should be used as a standard. I thought for the means of comparison it is better to choose a small protein, which is closer to viscotoxin in terms of size. The first samples were tested with aprotinin as a standard, which is closer to viscotoxin than BSA in terms of size and molecular mass. In consequence the results with aprotinin were higher than BSA and it always showed all viscotoxin samples more concentrated.

Table 3 viscotoxin Concentration results, Aprotinin used as a standard reference.

Content	Well Col	Well Row	Linear regression fit based on Blank corrected (562)	Blank corrected raw data (562)	Raw Data (562)	Standards
Standard S1	1	A	2010.5	0.6329	0.7262	2000
Standard S2	2	A	1459.8	0.4671	0.5604	1500
Standard S3	3	A	990.2	0.3257	0.419	1000
Standard S4	4	A	765.3	0.258	0.3513	750
Standard S5	5	A	565.0	0.1977	0.291	500
Standard S6	6	A	281.4	0.1123	0.2056	250
Standard S7	7	A	114.6	0.0621	0.1554	125
Standard S8	8	A	-36.8	0.0165	0.1098	25
Blank B	9	A	-	-	0.0933	0
viscotoxin A3	10	A	1237.0	0.4	0.4933	-

Table 4 BCA Assay kit results measuring concentration of viscotoxin A3 using BSA as a reference.

Content	Well Col	Well Row	Linear regression fit based on Blank corrected (562) µg/ml	Blank corrected raw data (562)	Raw Data (562)	Standards(µg/ml)
Standard S1	1	B	1941.8	2.0363	2.1267	2000
Standard S2	2	B	1523.5	1.6183	1.7087	1500
Standard S3	3	B	1026.5	1.1217	1.2121	1000
Standard S4	4	B	780.2	0.8756	0.966	750
Standard S5	5	B	561.9	0.6574	0.7478	500
Standard S6	6	B	269.4	0.3652	0.4556	250
Standard S7	7	B	112.4	0.2083	0.2987	125
Standard S8	8	B	-65.8	0.0302	0.1206	25
Blank B	9	B	-	-	0.0904	0
viscotoxin A3	10	A	315.9	0.4116	0.502	-

Figure 13 Picture from the BCA assay to measure concentration of viscotoxin A3 (A 10) with both BSA (Row B 1-9) and Aprotinin (Row A 1-9) as a reference.

The result of the assay was almost 4 times higher if aprotinin had been used as reference. Finally to have better results comparison BSA used as a reference since in the paper which studied mechanism of action of viscotoxin A3 and B (Giudici et al., 2003) also BSA was used to determine concentration of viscotoxin by methods described in following papers (Böttcher et al., 1961, Edelhoch, 1967) . If you are interested to know about the concentration of protein with Aprotinin as

a reference, multiplying the mentioned concentration in the result section quantified by BSA as reference to 3.19 would be the answer.

2.2 Bacteria

To measure antimicrobial activity of viscotoxin A3 a cultivation of fresh bacteria strains for each bioassay is needed. But first, it is necessary to find out the CFU (colony forming unit) number related to each strain. Following strains were used in this experiment:

1. *Agrobacterium tumefaciens* (GV3101)
2. *Escherichia coli* (DH10)
3. *Pseudomonas syringae*

2.2.1 Bacteria Preparation

2.2.1.1 Making of Bacterial culture in liquid media

To perform this step the following material and equipment are required:

1. Bacterial strain
2. Icebox
3. Sterile/autoclaved glass tubes with metal cab
4. Autoclaved medium (check table 5)
5. Thin sterile plastic spoon for bacteria transfer
6. Laminar hood
7. Incubator equipped with internal shaker

Sterile glass tubes are filled with 5 ml liquid media related to each bacterium based on Table 5 under laminar in sterile condition. All bacterial strains have to be kept in the -80°C freezer. As consequence an ice box is required to take bacterial tubes from this -80°C temperature to avoid defrostation. Small plastic sterile spoons were used to transfer bacteria to medium in the glass tube. A very small amount of frosted strain transferd with a special spoon into the liquid medium (a few µl) is enough for a successful inoculation. The original strains should go back immediately to -80°C after use. Entire steps must be done in sterile condition under laminar. Tube cabs are closed under laminar and kept in the shaker inside the incubator with a related temperature as it is mentioned in Table 5. The recipes of each medium are completely described in the “ 2.2.2 Mediums for Bacteria “section.

Table 5 list of bacteria used in experiment

	Incubation Temperature	Strain	Related Medium
E. coli	37°c	DH10	LB
P. syringae	26°c	--	King's B
A. tumefaciens	26°c	GV3101	YEB

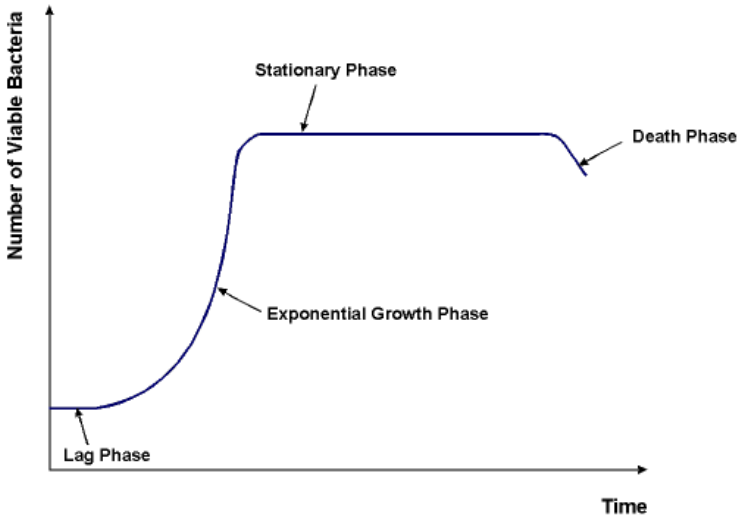


Figure 14 Different Phase in Bacterial growth (Tullmin, 2001)

As it is illustrated in figure 14 bacterial growth has four different phases: 1.Lag Phase 2.Exponential Growth Phase 3.Stationary Phase 4.Death Phase.

1. During the lag phase, bacteria begin the adaptation to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide.
2. Exponential phase is a period during which cell doubling will occur and the number of new bacteria starts to increase dramatically with a constant rate in the present population. The actual growth rate depends on the growth conditions, which affect the frequency of cell division. Under controlled conditions, cyanobacteria can double their population four times a day. Exponential growth continues as far as sufficient nutrients are available for growing bacteria otherwise doubling will stop and the stationary phase will start.
3. During the stationary phase, the growth rate slows as a result of nutrient depletion. In this phase the bacterial growth rate is equal to bacterial death rate.
4. At the death phase, bacteria run out of nutrients and die.

To have a successful bioassay bacteria should be in the exponential growth phase. The best time to use the bacteria is in the middle of the exponential phase. Otherwise cultivation of fresh bacteria in new liquid medium prior to the bioassay start is required. Using the dead bacteria or bacteria, which is already in a stationary phase may not lead to accurate results in bioassay.

Some bacteria have a long lag phase and therefore it takes an extended time to enter into the exponential phase. In this case it is necessary to start incubation in the right time. *E. coli* for instance has a very short lag phase and the suspension is ready to be used after 3-4 hours incubation. For *Agrobacterium* and *Pseudomonas* sometimes it takes more than 7 hours. Therefore two important factors that have to be considered for bacterial cultivation are: 1st to prepare and use a fresh suspension for each new bioassay and 2nd to avoid overnight incubation for the bacteria with a long lag phase. It is better to start incubation in the early morning and use it in the evening.

To determine whether a bacterial suspension is ready to use, the optical density (OD) of bacteria suspension has to be measured. The spectrometer compares the optical density of fresh medium (Blank) with medium inoculated for a determined time with bacteria to find out the different density between two samples. The highest OD in the exponential phase is approximately equal to 1.5, which leads bacterial strains to the death phase due to lack of nutrition and the high density of bacteria. Therefore all ODs between 0.5-0.6 were considered as a suitable OD to use for bioassay.

2.2.1.2 Colony formation in media with agar (CFU)

Colony forming unit (CFU) is a value to measure viable bacterial quantities. The advantage of this method is in counting only viable cells.

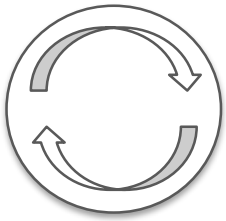


Figure 15 Spreading pattern

Cultivated bacteria in sterile glass tubes are used to make a serial dilution. The serial dilution of 1:10 prepared from each sample to the next one is illustrated in Figure 16. Dilutions are made from the stock suspension with liquid medium related to each bacterium instead of water. Optical density of each diluted tube is measured separately. Optical densities less than 0.005 are not reliable for calculation therefore as it will be described in the following parts all OD values less than 0,005 were not used in calculation steps. From each tube of diluted bacteria 100 µl is transferred and placed in a related plate with agar medium mentioned in Table 5. Samples are distributed gently over the plate like a pattern illustrated in Figure 15. The spreading must continue till the agar absorbs the sample and the agar surface looks dry. All steps are done under laminar in sterile condition. Petri dish lids are closed and sealed with Parafilm and kept under related temperature as it is mentioned in Table 5.

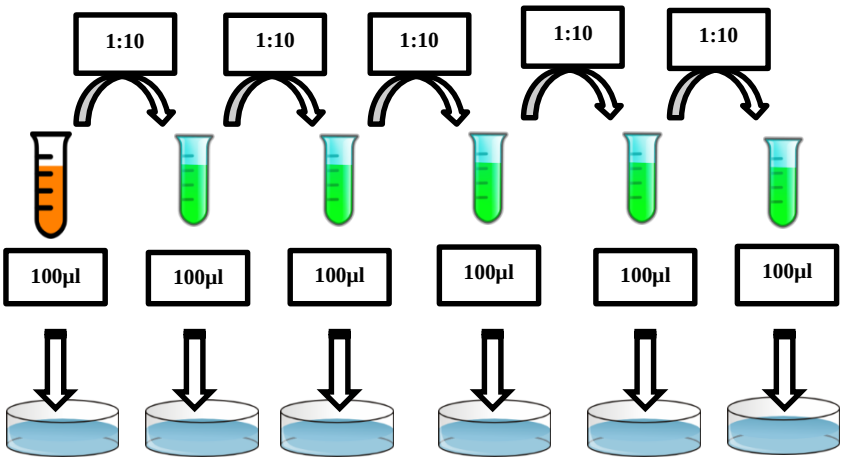


Figure 16 this figure illustrates the used method to make a serial dilution from bacterial stock suspension

Depending on the used strain it may take two or three days for the formation of colonies on the agar surface. After 3 days colonies of bacteria are easily observable as seen in this figure. In this step the number of colonies are counted and used in the CFU formula, which is explained in the calculation part.

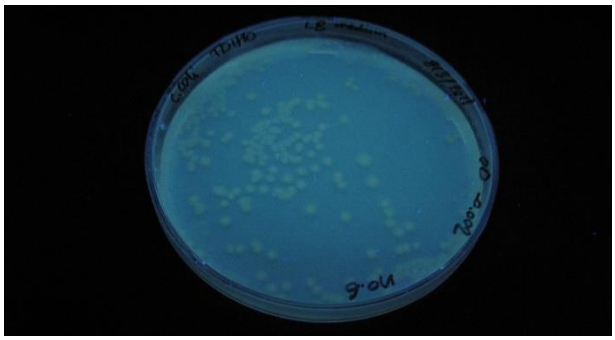


Figure 17 *E. coli* colonies formed on agar plate (Picture taken under UV light)

2.2.2 Mediums for Bacteria

Different mediums were deployed to grow the used bacterial strains in this experiment. These mediums can be prepared as liquid or solid medium based on our needs and the only difference between liquid and solid medium lies in the presence of 1.5 % agar in the solid one. Liquid medium was used for making bacterial suspension, which was used for the final bioassay and for determination of CFU, which is described in the CFU section. Agar mediums were used just once for the calculation of the colony forming unit, whose procedures are explained in the part of calculation and determination of final bacterial concentration.

2.2.2.1 Mediums for Cultivation

2.2.2.1.1 Kings B medium

This medium is generally used for growing *Pseudomonas* strains. All elements except glycerol are mixed with 100 or 200 ml distillated water and when they are completely solved, glycerol is added to the prepared solution. After the glycerol is mixed in the solution, the total volume is brought to 1 liter. pH is adjusted to 6.55 and it is autoclaved. When the medium is ready, all plates are poured with 35 ml (approx.) medium under laminar in aseptic condition.

Table 6 Materials for making 1liter Kings B medium

Peptone	20,0g
di-potassium hydrogen phosphate or $k_2HPo_4.3H_2O$	1,5g or 1,96g respectively
Magnesium sulfate heptahydrate	1,5g
Agar	15gr
Glycerol 95%	10ml

2.2.2.1.2 LB Medium

LB (Luria broth) medium is used for *E. coli*. To prepare 1 liter LB with agar following materials mentioned in the subsequent table are needed. Mix all chemical substances in half a liter of water, then bring the total volume to 1 liter, adjust pH to 6.55 and autoclave the bottle. After autoclaving all plates are poured with 35 ml (approx.) medium under laminar in aseptic condition.

Table 7 Materials for making 1 liter LB medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15g

2.2.2.1.3 YEB medium

For making 1 liter YEB medium for *Agrobacterium* following materials from the subsequent table are needed. All materials are taken and mixed with half a liter of distillated water. When all materials are dissolved the total volume is brought to 1 liter with distillated water. The medium`s pH is adjusted to 6.5 and autoclaved. After autoclaving all plates are poured with 35ml (approx.) medium under laminar in aseptic condition.

Table 8 Materials for making 1 liter YEB solid medium

Trypton	5 g
Yeast extract	1g
Pepton	5g
Sacharose	5g
MgCl2	0.4g
Agar	15g

2.2.2.2 Medium for Bioassay Minimal medium MM for Bacteria

The medium, which was used in the final part for the preparation of the micro titer plate in bioassay is different from mediums, which have been used for the cultivation of bacteria and has a very simple recipe. For making 1 liter of minimal medium for bacteria following materials are mixed with distillated water and autoclaved:

- 1% Tryptone,
- 0.5% LGT agarose

2.2.3 Calculation and determination of final concentration of bacteria

After 3 days of incubation the number of forming colonies in each Petri dish was counted and the CFU value was calculated based on following formula:

$$CFU = \frac{\text{No. of Colonies X dilution of plate}}{\text{Volume of culture on plate}}$$

Table 9 *E. coli* colonies counted after 3 days

Plate No.	**Dilution**	**OD**	**Number of Colonies on plate**
Stock OD	No Dilution	0.872	Uncountable
1st diluted from stock(10)	1:10	0.131*	Uncountable
2nd diluted from 1st (10^2)	1:100	0.015	Uncountable
3rd diluted from 2nd (10^3)	1:1000	0.002	Uncountable
4th diluted from 3rd (10^4)	1:10000	0.000	Uncountable
5th diluted from 4th (10^5)	1:100000	-0.002	231*
6th diluted from 5th (10^6)	1:1000000	-0.002	36

*this value used for calculation of *E. coli* CFU

Table 10 *A. tumefaciens* colonies counted after 3 days

Plate No.	Dilution	OD	Number of Colonies on Plate
Stock	No Dilution	1.340	uncountable
1st diluted from stock (10)	1:10	0.253*	uncountable
2nd diluted from 1st (10^3)	1:1000	0.006	uncountable
3rd diluted from 2nd (10^5)	1:100000	0.003	376*
4th diluted from 3rd (10^6)	1:1000000	0.000	55
5th diluted from 4th (10^7)	1:10000000	0.000	3

*this value used for calculation of *A. tumefaciens* CFU

Table 11 *P. syringae* colonies counted after 3 days

Plate No.	Dilution	OD	Number of colonies on plate
Stock	No Dilution	0.984	uncountable
1st diluted from stock (10)	1:10	0.169*	uncountable
2nd diluted from 1st (10^2)	1:100	0.020	uncountable
3rd diluted from 2nd (10^3)	1:1000	0.001	Difficult to count
4th diluted from 3rd (10^4)	1:10000	0.000	Difficult to count
5th diluted from 4th (10^5)	1:100000	0.000	289*
6th Diluted from 5th (10^6)	1:1000000	0.000	21
7th Diluted from 6th (10^7)	1:10000000	0.000	1

*****this value used for calculation of *P. syringae* CFU

Example to calculate CFU for *E. coli*

No. Of colonies = 231
Dilution of plate = 100,000(5 times 1:10 dilutions to reach 231 colonies)
Volume on plate = 0.100 ml

$CFU = \frac{231 \text{ X } 100{,}000}{0.100}$ = 231,000,000

The CFU of the first suspension diluted from stock solution was calculated and this value with related OD was used as a source in the following formula to calculate CFUs of all other bacteria suspension made for bioassays for bacteria in this experiment without passing all last steps. Bacterial CFU needed for using in this bioassay is equal to 10^5 CFU/ml.

$$\text{CFU for } P.Syringae = \frac{2{,}890{,}000 \text{ x your current OD}}{0{,}169}$$

$$\text{CFU for } E.Coli = \frac{2{,}310{,}000 \text{ x your current OD}}{0{,}131}$$

$$\text{CFU for } Agrobacterium = \frac{3{,}760{,}000 \text{ x your current OD}}{0{,}253}$$

2.3 Fungi

Following fungi were used in this bioassay and antimicrobial acidity of viscotoxin was tested against them.

- *Aspergillus nidulans (a)*
- *Fusarium solani(b)*
- *Fusarium oxysporum (d)* ascomycete fungus pathogen known for Fusarium wilt common vascular wilt fungal disease.
- *Verticillium dahliae (g)* making leaves curly and discoloured.
- *Fusarium culmorum (c)* fungal pathogen known as an agent responsible for foot rot, ear blight, stalk rot, root rot and other diseases of cereals, grasses, and a wide variety of monocots and dicots.
- *Rhizoctonia solani (e)* soil-borne pathogen known as a cause of collar rot, root rot, damping off and wire stem diseases.
- *Thielaviopsis basicola (f)* known for root rot in pea and storage black rot in carrot.

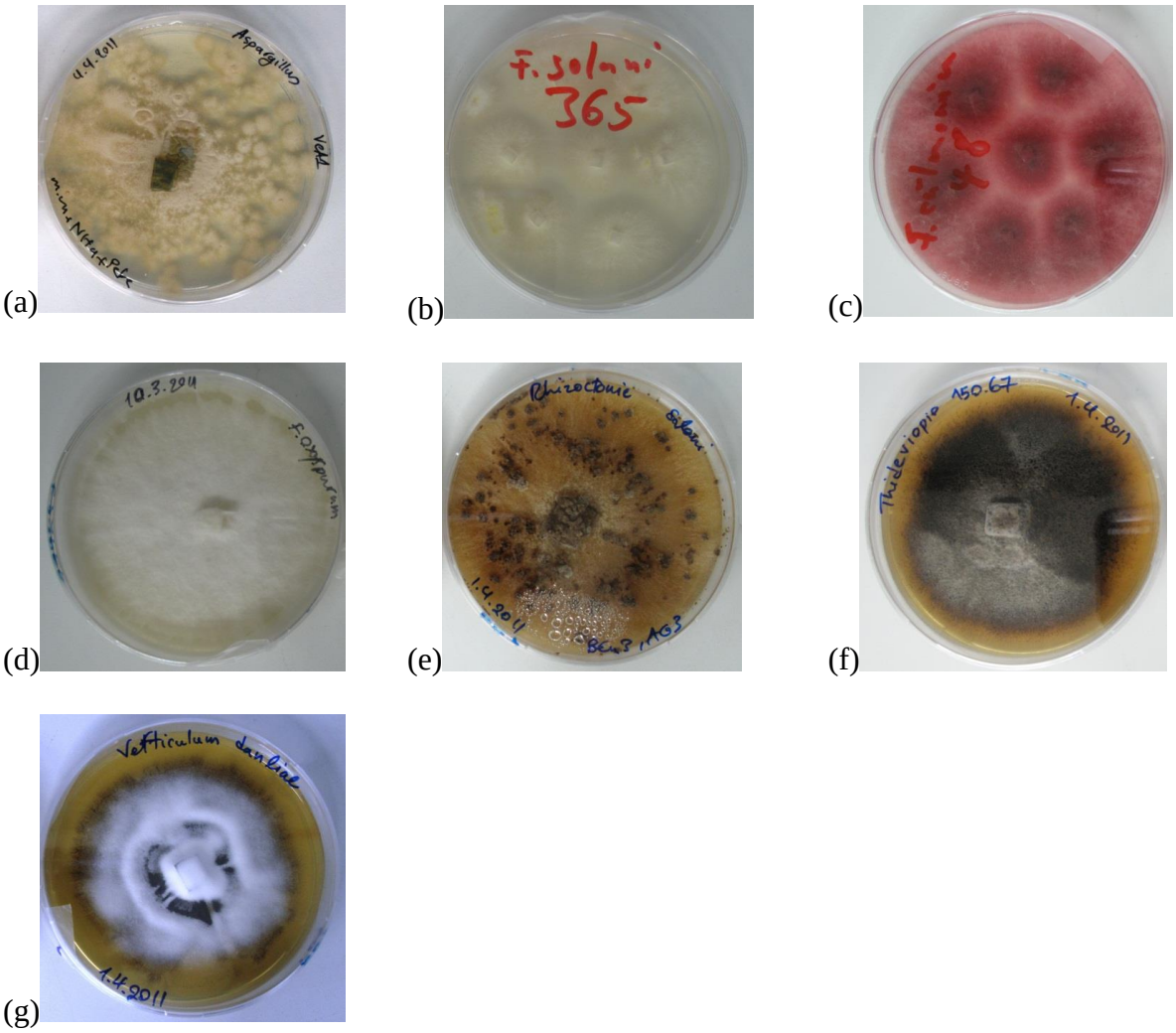


Figure 18 Fungi cultivation on PDA medium

2.3.1 Fungal mediums

Two different mediums were needed for this bioassay (1) medium used for fungus cultivation and (2) medium to use in bioassay.

2.3.1.1 Medium for fungus cultivation

2.3.1.1.1 Potato dextrose agar (PDA)

In this experiment PDA medium was the only used medium for cultivation of fungus. For making the PDA medium, 31 gram potato dextrose agar is mixed with 800 ml distillated water and autoclaved. PH adjustment for this medium is not necessary. All plates were poured with approximately 35 ml PDA medium under aseptic conditions and sealed with paraffin when the medium was enough cooled down and solid.

2.3.1.2 Medium for Bioassay (Minimal medium MM)

To measure antimicrobial activity of viscotoxin A3 in bioassay a liquid medium to provide minimum nutrients for growth of spores is needed. This kind of mediums are basically called minimal mediums. After trying the bioassay several times with Czapek, PDA, and RPMI following medium found as the best choice for this experiment. The protocol to prepare minimal medium (MM) for fungi in this experiment derived from a study, which used Resazurin microtiter plate based bioassay to test antimicrobial activity of 9 selected short peptides with the pathogenic fungus *Aspergillus nidulans* (Mania et al., 2010). In order to prepare MM the following procedures were used:

I. Improved preparation of Hutners Trace element (TE) solution, three versions

A. Procedure for Hutners TE with full solubility at room temperature and during storage.

To prepare 100 ml of 500x TE solution make solutions 1 and 2 and mix them together, pH should be readjusted to 6.5. Bring the final volume to 100 ml with distillated water and store at 4-8°C. The solution color is initially green, but turns to purple upon storage.

1. Dissolve the listed salts in 40 ml of distilled water in the order indicated:

$FeSO_4 \cdot 7H_2O$ (Ferrous sulphate)	0.5 g
EDTA	5.0 g

2. Dissolve the listed salts in 40 ml of distilled water in the order indicated:

$ZnSO_4 \cdot 7H_2O$ (Zinc sulphate)	2.2 g
H_3BO_3 (Boric acid)	1.1 g
$MnCl_2 \cdot 4H_2O$ (Manganous chloride)	0.5 g
$CoCl_2 \cdot 6H_2O$ (Cobaltous chloride)	0.16 g
$CuSO_4 \cdot 5H_2O$ (Cupric sulphate)	0.16 g
$MoNa_2O_{4.2}H_2O$ (Sodium molybdate dihydrate)[1]	0.11 g

B. Modified Hunters TE solution: pH established with tetra-sodium-EDTA

For 100 ml, dissolve the listed salts in 80 ml of distilled water in the indicated order. After addition of Na4EDTA, generally no precipitation is observed. Bring the final volume to 100 ml with distilled water, autoclave, and store at 4-8EC. The unadjusted pH will be about 6.5.

$ZnSO_4 \cdot 7H_2O$ (Zinc sulphate)	2.2 g
H_3BO_3 (Boric acid)	1.1 g
$MnCl_2 \cdot 4H_2O$ (Manganous chloride)	0.5 g
$FeSO_4 \cdot 7H_2O$ (Ferrous sulphate)	0.5 g
$CoCl_2 \cdot 6H_2O$ (Cobaltous chloride)	0.16 g
$CuSO_4 \cdot 5H_2O$ (Cupric sulphate)	0.16 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Ammonium molybdate)	0.11 g
$Na_4EDTA \cdot 4H_2O$ (EDTA, tetrasodium salt)	6.0 g

C. Modified Hutners TE solution with standard EDTA molarity: pH established with mixed sodium-EDTA salts.

Dissolve following salt in 80ml distillated water and bring the total volume to 100ml. The unadjusted pH should be higher than 6.5 (7.0-7.2 depend on temperature).

Na4EDTA ·4H2O (EDTA, tetrasodium salt)	6.5 g
Na2EDTA ·2H2O (EDTA, disodium salt)	0.77 g

II. Improved Preparation of Minimal Medium (MM): Salt stock solutions

[1] Sodium molybdate was used instead of Ammonium molybdate, which was mentioned in the original protocol.

Minimal medium Salts, 2 stock solutions

a. Salt mix A lacking $MgSO_4$ (20x) Stock: use 50 ml for 1 liter MM

For 100 ml, dissolve the listed salts in 80 ml of distilled water in the order indicated. Bring the final volume to 100ml with distillated water and store it at 4-8 °C or autoclave it and keep it at room temperature.

$NaNO_3$ (Sodium nitrate)	12.0 g
KCl (Potassium chloride)	1.04 g
KH_2PO_4 (Potassium phosphate, monobasic)	1.6 g
K_2HPO_4 (Potassium phosphate, dibasic)	2.09 g

b. $MgSO_4$ Solution (200x) Stock: use 5 ml for one liter of MM.

For 10 ml solution, dissolve Magnesium sulphate in 8 ml of distilled water and bring the final volume to 10ml and autoclave and store at 4-8°C.

$MgSO_4 \bullet 7H_2O$ (Magnesium sulphate)	1.04 g

Minimal medium (MM)

To prepare 250ml MM mix the following solution, which is prepared in the last steps with Glucose according the following table. PH adjustment is not necessary. The final pH without adjustment must be equal to 6.6.

Salt mix A lacking $MgSO_4$@ (20x Stock) (II.a)	12,5 ml
$MgSO_4$ Solution (0.4 M, 200x Stock) (II.b)	1,25 ml
Glucose	2.5 g
Trace Element stock Solution (I)	250 µl

Note: 250ml of MM is approximately enough for testing 200 individual samples depending on the total volume used in each well. Therefore making a bigger volume of MM is not necessary.

2.3.2 Fungus preparation

Fungus cultivation has to take place at least a few weeks before starting the first bioassay. Different fungi are cultivated in agar plates with PDA medium. To assure that all fungi are fresh and able to grow *in vitro*, fresh fungi are cultivated in new fresh Petri dishes filled with PDA medium and kept at room temperature in a dark place for at least 7 days. To transfer old fungi to a new Petri dish with medium, cutting and transferring very small parts of medium with fungus from the old medium to new medium is enough for successful inoculation. Since for each bioassay one whole Petri dish has to be used, 5-6 Petri dishes with enough growth are prepared in advance and reserved as an inventory (enough growth means that after few days or weeks after inoculation the whole agar surface in the Petri dish should be covered by mycelium and hyphae otherwise it will not give enough quantity of

spores required for bioassay). The recipe for PDA medium used for fungi cultivation is described in the fungus medium section.

For each bioassay, a fresh suspension of spores has to be made. The prepared suspension can be held for maximum 1 day at a temperature of +4°C for being utilized in another bioassay the next day. To harvest spores, simply 5 ml of autoclaved MM is added to a Petri dish with the desired fungus to test under laminar. A flamed metal bar is used to distribute and mix spores with MM in the Petri dish. Autoclaved Erlenmeyer with milk filter paper on top has to be prepared before starting this part. MM with spores is transferred with a pipet through the filter paper to Erlenmeyer. In consequence only spores pass the filter and the suspension is ready for counting, a step, which is explained in the following section.

2.3.3 Calculation and determination of final concentration of spores

In order to determine the concentration of harvested spores counting the number of spores is required. To reach this goal, a fresh spore suspension is prepared and the Thoma Neu counting chamber is used for counting the number of spores. A counting chamber is a measuring instrument made of optical glass used to count cells or other particles in suspensions under a microscope. They can be used for blood analysis or to count bacteria and fungus spores. The one used for this experiment was "Thoma Neu" counting chamber with 0,100 mm depth from Marienfeld Company. In the following figure the structure of the Thoma Neu counting chamber with its exact width and height is illustrated.

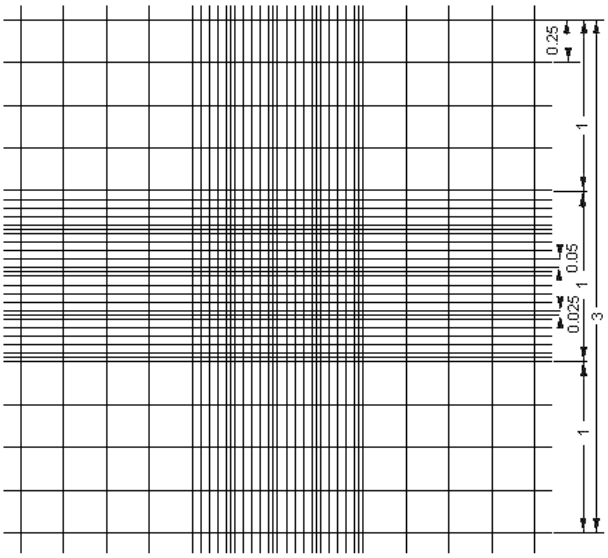


Figure 19 Thoma Neu counting chamber design under microscope picture from Marienfeld company website.

Cover glass and counting chamber are cleaned with 70% ethanol, then the cover glass is gently pushed onto the counting chamber from bottom to top or top to bottom. The cover glass is fragile and too much applied pressure may lead to breaking of the cover glass. The formation of interference

lines between the external support and the cover glass is the sign that the cover glass is positioned correctly. 20µl of the well shaken spore suspension is pipetted between the cover glass and the counting chamber. Due to the existing capillary effect in the gap between the cover glass and the chamber it will suck all the injected suspension in. Overflow heads to the edges of the chamber section therefore it is not that important if the pipetted suspension is a little bit more than required. In the case of observing air bubbles on the liquid, which is located in the grooves, or exactly on top of one of the group squares the chamber must be cleaned again and all feeding steps have to be repeated. When all these steps were done correctly, spores can be counted under the microscope. For counting, a Handheld Tally Counter with 4 digits is used to avoid any confusion during the counting process. Required magnification depends on your ability to recognize spores and grid lines under the microscope. The Thoma Neu counting chamber consists of 16 group squares located in the middle of the glass. Each group square consists of 16 small squares. The number of spores located on 4 group squares was counted as it is illustrated in Figure 20. To ensure that spores, which are to be counted are not counted twice or missed, some certain rules have to be considered.

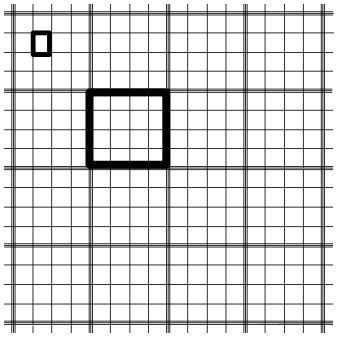


Figure 20 Sample of a small and a group square is marked with black in the picture, the grid lines between group squares can be different based on the counting chamber model. This figure belongs to the Frosenthal counting chamber model from Marienfeld Company.

In this experiment only the observed spores in 4 group squares were counted along the direction, which is illustrated on Figure 21 (counting should start at the top left-hand corner and follow the direction shown by the arrow), and used in the related formula.

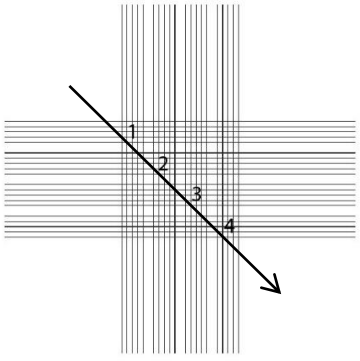


Figure 21 To avoid any counting mistakes only spores, which are in 4 marked group squares have to be counted. The picture is taken from Thoma Neu user manual.

In order to find the current concentration of the stock solution, the number of counted spores in 4 group squares is used in the following formula to earn the final result. If the concentration of stock is too high, due to the high number of spores on the slide it makes it difficult to count the number of spores. In such a case the stock solution can be diluted. The following example shows the way to calculate the number of spores by using the given formula:

$$\frac{\text{Number of Spores}}{\text{Counted area (mm2) x chamber depth (mm)x dilution}} = \text{Spores per 1µl of suspension}$$

Example :

1. Counted spores = 152
2. Counted area:4 group squares is equal to 64 small squares (= 64 x 0.0025[2] mm2) = 64 mm^2
3. Chamber depth 0.1 mm
4. No Dilution (sample is taken from main suspension)

$$\frac{152}{64\text{x}0.0025\text{ mm2 x }0.1\text{mm}} = 9500 \text{ spores per 1µl of suspension}$$

Based on the standards in this experiment the final needed concentration in each well must be equal to $2x10^4$ per milliliter. Therefore the acquired concentration in most cases (if it is higher than the needed one) should be diluted to this amount of spores in each well of the micro titer plate.

3 Resazurin based Bioassay

In bioassays for antimicrobial activity fungal or bacterial growth take place in liquid medium and antimicrobial activity of testing protein in samples is measured spectrophotometrically (Ludwig & Boller, 1990). The resazurin based bioassay works based on the conversion of the redox dye resazurin to a highly fluorescent product resorufin by living cells. Resazurin is a blue, non-fluorescent dye that is converted to pink and fluorescent resorufin in the presence of a respiring organism (O'Brien et al., 2000). When high numbers of cells are incubated with solutions containing resazurin for extended periods of time, a secondary reduction reaction may occur in which the

[2] 0.0025 mm^2 is the size of one small square in Thoma Neu counting chambers

fluorescent resorufin is further reduced to the colorless, non-fluorescent hydroresorufin (O'Brien et al., 2000, Erb, R.E. and Ehlers, M.H., 1950). In these bioassays, fluorescence intensity (FI) and light absorbance are two factors to measure after the incubation time. Even in absence of devices to measure these two factors, viability of living cells or spores can be recognized by observation of the color change. Application of resazurin based assays is mostly used in cell proliferation, evaluation of cytotoxic agents and high-throughput screening for antibiotics and anti-cancer compounds.

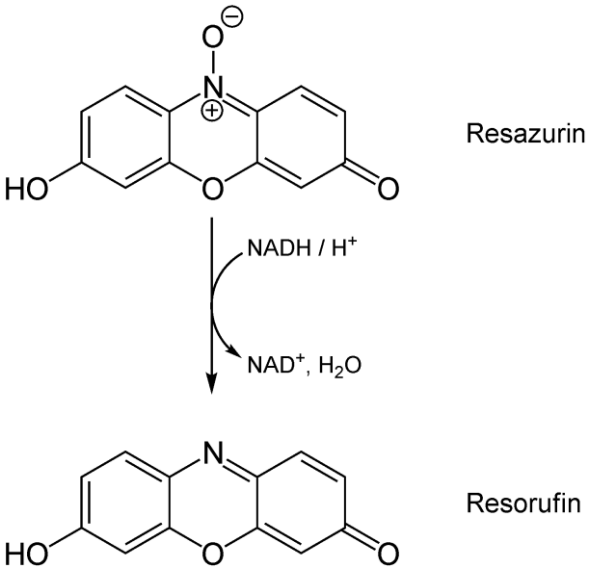


Figure 22 Resazurin converts to Resorufin as result of Deoxygenation of heterocyclic N.

The advantage of using this method lies in the high sensitivity and accuracy of bioassay. In this method a number down to 100 cells can be accurately quantified. Secondly it is very time efficient, for instance 96-well and 384-well microtiter plate can be measured in a few minutes and using several plates allows simultaneous processing of thousands of samples per day. To come to the right conclusion for proving the inhibitory effect of an element in each bioassay the presence of controls are necessary. The activity of AMPs and the percentage of inhibition value are relative to positive and negative controls located on other wells prepared in microtiter plate. Therefore as a first step it would be necessary to identify suitable positive and negative reference controls to use in the bioassay. To set a bioassay the following controls are needed:

A. **Negative controls** are groups where in theory no or the least possible phenomenon is expected. Negative control is used to prove that our selected microorganisms are able to continue their growth in minimal media in absence of our inhibitory factors, which could be viscotoxin, antibiotics or fungicides in this experiment. Negative control in this case is defined as mixture of fungus or bacteria + minimal media. In this mixture we expect that no inhibitory phenomenon takes place and microorganisms show normal growth.

B. **Positive control** is used to assess test validity. Positive (toxic) control is needed to compare the inhibitory effect of an extracted peptide with one of known growth

inhibitors of our selected microorganism. In fact positive control is defined as phenomenon, we would expect to occur. In the following experiment positive control is defined as fungicide or antibiotic + minimal medium + fungus or bacteria. In this combination we expect to see an inhibitory effect of the antibiotic or the fungicide.

In this bioassay 1 antibiotic and 1 fungicide are used as positive control:

I. **Benomyl:** fungicide introduced in 1968 by DuPont and selectively has a toxic effect on some microorganisms, especially on fungi and on invertebrates.

II. **Kanamycin:** is an aminoglycoside antibiotic, which is used to inhibit a variety of bacterial infections.

C. **Blank** is necessary to prove that MM is not contaminated and bacterial or fungal growth in other wells was not due to contamination.

3.1 Preparation of Microtiter plate

3.1.1 Preparation of Resazurin

5mM resazurin stock solution is prepared for this bioassay. The final resazurin concentration needed for all samples is 100 µM. The resazurin solution is prepared by dissolving a 22.9 mg resazurin in 20 ml of sterile distilled water. A vortex mixer is used to ensure that it is well dissolved and we receive a homogenous solution. At the end a syringe filter is used to sterilize the resazurin solution under aseptic condition.

3.1.2 Preparation Benomyl

Benomyl is not water soluble and has to be solved in DMSO. In bioassay the highest needed benomyl concentration for one well ranges between 200-100 µg/ml. Therefore 5mg/ml stock solution of benomyl is prepared. To make a diluted benomyl for bioassay, MM which were described for fungus can be used instead of water. An important point to consider in this part is to not make a stock solution concentration less than the mentioned amount. This helps to minimize the presence of DMSO in the medium as low as possible in bioassay. As it is shown on Table 12, resazurin can be used as a pH indicator. A high concentration of DMSO changes rapidly the pH of a medium in bioassay. This change causes immediate color change from blue to red due to fast conversion of resazurin to resorufin and destroys the final results, because this color change is not related to fungal or bacterial activity.

Table 12 Resazurin as a pH indicator. Following table shows resazurin Color range relative to pH.

pH< 3.8	pH> 6.5
Pink	Blue

3.1.3 Preparation of Kanamycin

10 mg/ml stock solution of kanamycin is prepared and kept in a freezer at a temperature of -20°C. After defrostation, 6 µl of stock solution is mixed with 94 µl of bacteria MM to achieve 100 µl of kanamycin in MM with 600 µg/ml concentration.

3.1.4 Microtiter plate (Nunc 96)

Sterile microtiter plate Nunc 96 with lids was used in this bioassay. The total volume used in each well can vary from 100-200 µl depending on availability of the inventory of peptides. Since I wanted to save our extracted viscotoxin for further studies, the total volume used in this study was 100 µl. The following volumes were dedicated for each well of samples and controls in micro titer plate:

Sample/Positive control

1. 2µl (from 5mM resazurin stock)
2. 50µgr (benomyl, kanamycin or viscotoxin)
3. 48µl (spores or bacteria)
   Total volume: 100µl

Negative Control

1. 2µl (from 5mM resazurin stock)
2. 50µg (minimal medium)
3. 48µl (spores or bacteria)
   Total volume: 100µl

Blank

1. 2µl (from 5mM resazurin stock)
2. 98 µl (minimal medium)
   Total volume: 100µl

Plates were prepared under aseptic conditions. A sterile Nunc 96 well plate was labeled and a volume of 100µL of test material with the highest concentration that the test should start added to the first well of the row (benomyl, kanamycin for rows considered as positive control and viscotoxin A3 for rows dedicated for testing extracted AMP). Then 50 µL of MM was added to other wells except for Blank, which received 98 µl of MM. Serial dilutions were performed using a multichannel pipette except for the two last columns, which were skipped since they were dedicated for negative control

and Blank. The last 50 µl were removed and tips discarded (from column 10). In this step each well must have 50 µL of the test material in serially descending concentrations except the two last wells of each row, which have only 50 µl of minimal medium. 2 µL of 5 mmol resazurin indicator solution was added to each well. Avoid touching tips with medium inside a well.

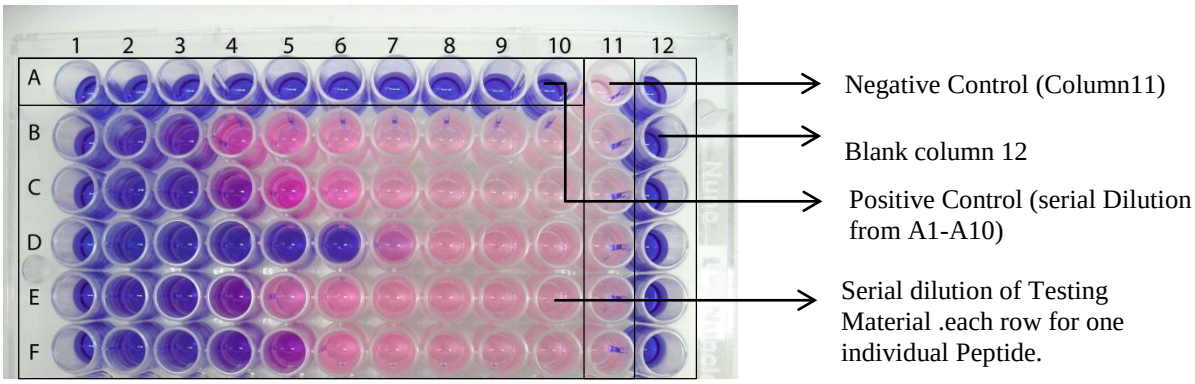


Figure 23 Example to show how controls and testing samples were located in one plate

As a last step, 48 µl fungal suspension was added to achieve $2*10^4$ /ml final spores concentration in each well except for Blank, which contains only 98 µl medium and 2 µl resazurin. In the bioassay, where effect of the protein on bacteria needed to be tested, instead of 48 µl fungal suspension, 48 µL of bacterial suspension was added to reach CFU/ml equal to 10^5 /ml in each well except for Blank. Plates were wrapped loosely with Parafilm to avoid evaporation and unwanted contamination.

The plates were placed in an incubator adjusted to 37°C for *E. coli* and 26°C for *P. syringae* and *A.tumefaciens* for 18–24 h. The incubation time for fungal strains was 24-48 h in the incubator at 26°C till the color change occurred. After the incubation time was finished the color change was assessed visually and pictures were taken.

3.2 Evaluation methods

3.2.1 Observation

Any color changes from purple to pink or colorless indicate fungal or bacterial growth, which means no inhibitory effect occurred in that specific well. The concentrations of the well before the last well, where color change occurred, are considered as a MIC value of the tested AMP for that bacterial or fungal strain. Depending on the used strain the color change must be visible to the naked eye after 24 to 48 h.

3.2.2 **Fluorescence intensity (FI)**

The fluorescence intensity of each plate was measured after 8 hours of incubation with excitation at 530 nm and emission at 590 nm using a FLUOstar Omega plate reader adjusted for 24 to 36 cycles with 30 minutes intervals. If any bacterial or fungal activity take a place in wells, due to conversion of the non-fluorescent redox dye resazurin to a highly fluorescent product resorufin, the florescent of resorufin would be detectable by using mentioned wavelengths. The FI time resolved method which used in this experiment has a peak (highest detected fluorescent) and the FI drops slightly after reaching the peak. Negative controls must show highest FI value because due to bacterial or fungal activity non-fluorescent resazurin converted to highly fluorescent resorufin which is measurable by using mentioned excitation and emission wavelengths. In contrast FI value in blank wells must be low because the resazurin remained untouched and there is no fluorescent for excitation.

3.2.3 Absorbance

Measuring absorbance was done by FLUOstar Omega Spectrometer. Plate covers were removed and 600nm wavelength used to read samples. The following figure shows the spectrum, which was done in the bioassay test for *F. solani*. The maximum difference between two curves is in 600nm wavelength which is used to read the whole plate. Absorbance of the samples found with overlapped or too close curves in all wavelengths between Blank and negative control was not measured due to contamination or lack of bacterial or fungal activity and preparation of that samples repeated again.

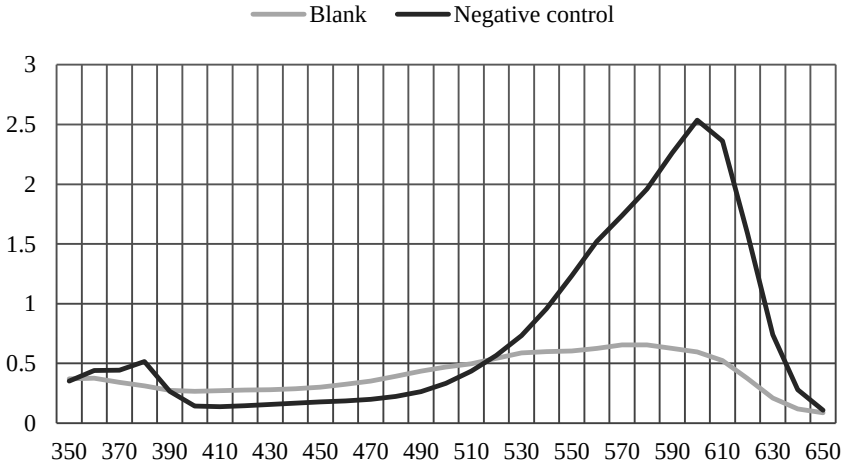


Figure 24 Spectrum 350-650nm between Blank and Negative control.

Reading wavelength was determined and resazurin absorbance of all samples was measured and saved as an Excel file. The aim of resazurin based bioassay is to monitor resazurin reduction in samples. The absorption maximum for resazurin is 605nm and the absorption maximum for resorufin

is 573nm. The color change can be measured by light absorbance at 570 to 600nm. In measuring absorbance negative control must always show the lowest resazurin absorbance because due to high bacterial or fungal activity all resazurin in negative control is reduced and converted to resorufin. In contrast, blank control must have the highest resazurin absorbance because only fresh medium used in these wells and resazurin must remain untouched with the highest absorbance value in compare with other wells. Low resazurin absorbance in Blank indicates contamination in medium and bioassay must be repeated.

4 Results

4.1 viscotoxin A3 results with Bacteria

The optical density (OD) of bacterial stock suspension, which was used to prepare the following microtiter plates was 0.25, 0.73, 0.29 for *P. syringae, E. coli* and *A. tumefaciens* respectivly, which shows that the used strains were in a good exponentional growth period.

After overnight incubation of the bacterial strains with viscotoxin A3, color change was observed as it is shown in Figure 25-27. The following pictures were taken using transmitted light. Kanamycin was used in all samples as a positive control for bacteria. As it is shown in Figure 25 Kanamycin had a very strong inhibitory effect even in the very low concentration of 1,1µg/ml with *P.syringae* (first row). I did not find any antibacterial effect of viscotoxin A3 against this strain (second row).

Figure 25 *P.syringae* after 24 hours incubation.

E. coli was kept for 24 hours at a temperature of 37°C in the incubator. Kanamaycin showed again a very high inhibitory effect in 4.6µg/ml concentration. As it is shown in Figure 26 all wells from B1-B10, which contained serial dilution of viscotoxin A3 turned from blue to a pinkish color, comparable with negative control, which is proof for bacterial growth and the incapability of viscotoxin to inhibit *E. coli* growth.

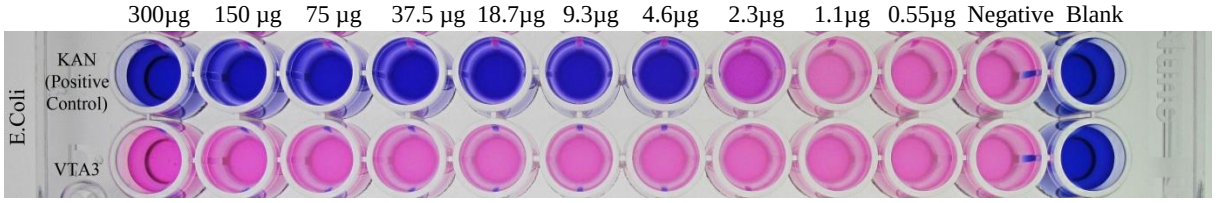


Figure 26 *E.coli* after 24 hours incubation.

The last bacterial strain *A. tumefaciens* was also checked visually after 24 hours (Figure 27). The inhibitory effect of Kanamaycin for this strain was 4.6 µg/ml like the previous sample and again no inhibitory effect was observed for viscotoxin A3.

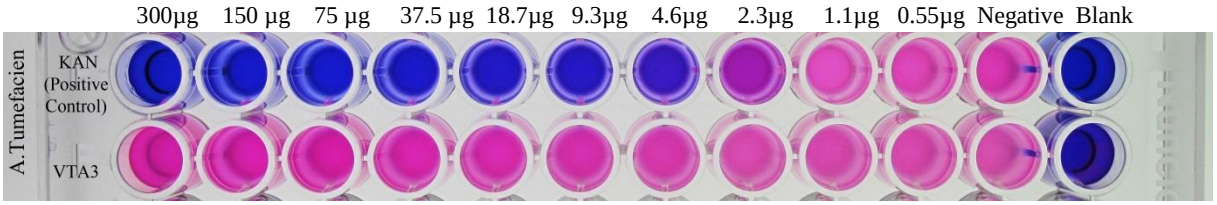


Figure 27 *A. tumefaciens* after 24 hours incubation.

However the results of the bioassay was clear only by color change observation of the microtiter plate, the absorbance of each well was measured by spectrometer as well.

Absorbance of all wells was measured by FLUOstar omega spectrometer. The spectrum of 350-650nm was made between blank and control wells for each bacteria strain separately. the wavelength of 600nm was a for reading the other wells from this bacterial strain.

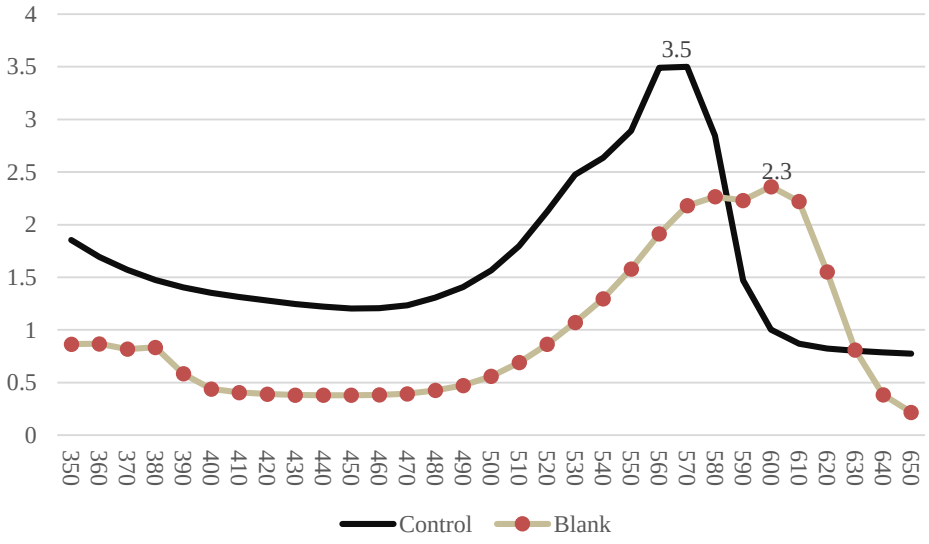


Figure 28 Spectrum 350-650nm between Control and Blank wells for *P. syringae*.

As illustrated in the two following Figures, the same measurement was done for *E. coli* and *A. tumefaciens.* In both cases the wavelength of 600 nm was chosen for reading their samples.

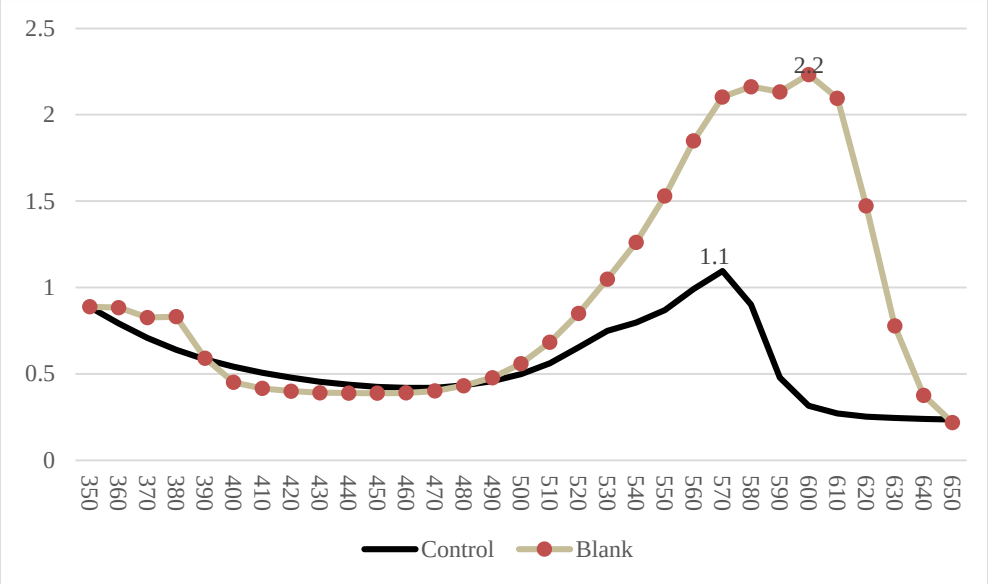


Figure 29 Spectrum 350-650nm between Control and Blank wells for *E.coli.*

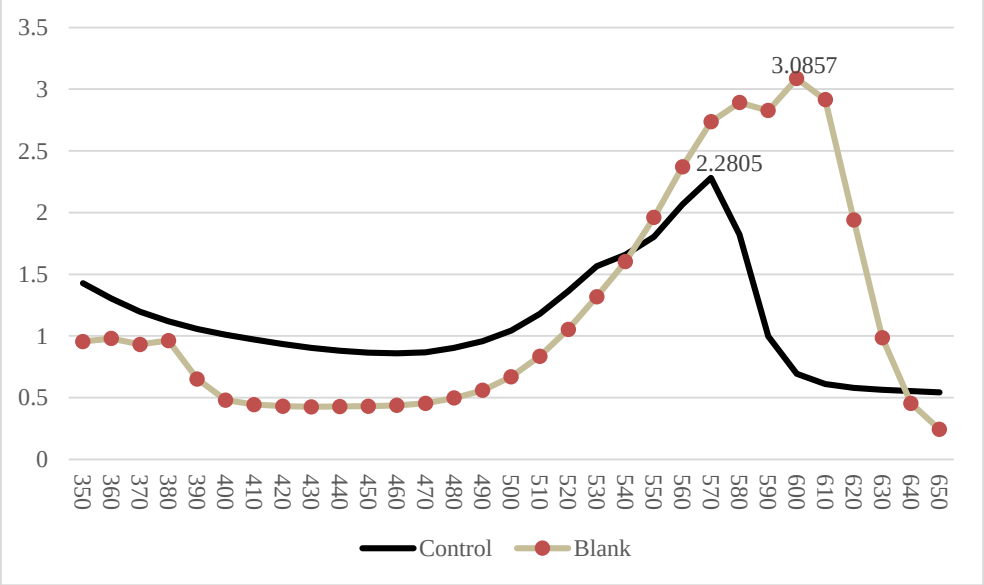
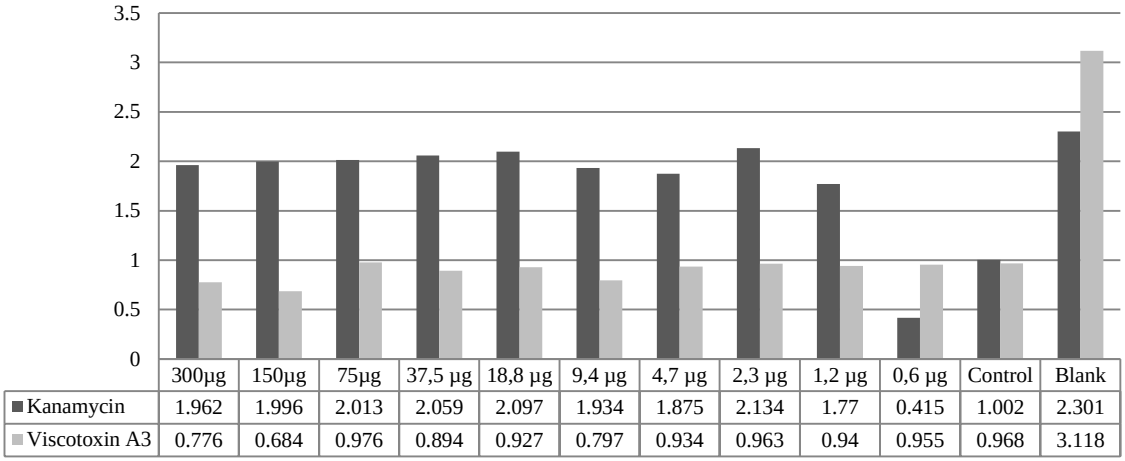


Figure 30 Spectrum 350-650nm between Control and Blank wells for *A.tumefaciens*.

Following the preceding information absorbance of all samples was measured by FLUOstar Omega Spectrometer. A higher absorbance in the following Figures means higher detected resazurin in that specific well, which proves that bacterial activity inhibited or not occurred. As it is easily observable in the following Figure, all bars related to viscotoxin from 300-0.6µg concentration are approximately in the same height as the negative control absorbance value. The resazurin absorbance value for all samples treated with viscotoxin was between 0.7-0.9 which is close to negative control well value which is approximately equal to 1 and was only poured with 10^5 CFU/ml of fresh bacteria without any antibiotic. A strong minimum inhibitory effect of the positive control with kanamycin, which was equal to 1.2 µg/ml is a good proof that the bioassay ran well in this experiment and

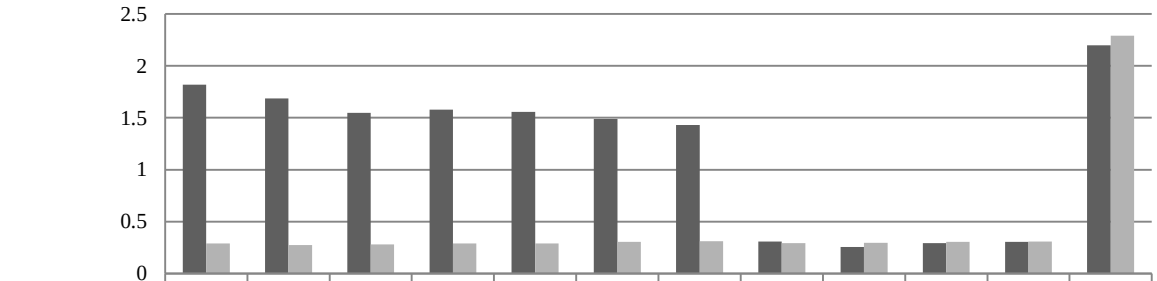
viscotoxin is incapable to inhibit bacterial growth. The following data is raw data and it is not blank corrected.



	300µg	150µg	75µg	37,5 µg	18,8 µg	9,4 µg	4,7 µg	2,3 µg	1,2 µg	0,6 µg	Control	Blank
Kanamycin	1.962	1.996	2.013	2.059	2.097	1.934	1.875	2.134	1.77	0.415	1.002	2.301
Viscotoxin A3	0.776	0.684	0.976	0.894	0.927	0.797	0.934	0.963	0.94	0.955	0.968	3.118

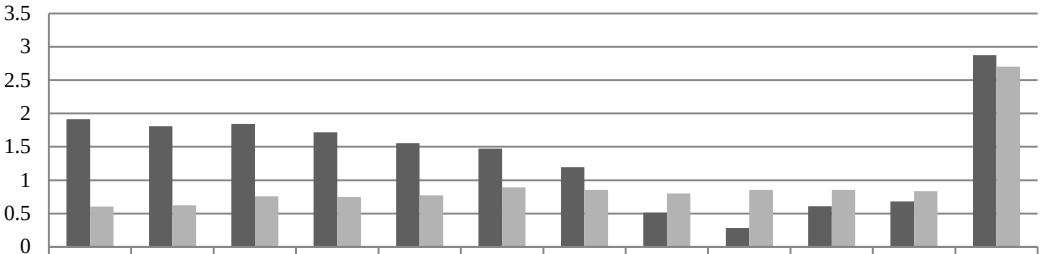
Figure 31 Resazurin absorbance in *P.syringae* after 24 hours.

Very close results were obtained for the two other bacteria strains as well. As it is shown in Figure 32 the resazurin absorbance value of the well with highest concentration of viscotoxin (300µg) is 0.28, which is so close to the Negative control with absorbance equal to 0.3 for *E. coli*. This means that due to bacterial activity all resazurin in the wells, which contained viscotoxin was converted to resofurin. The same results only with different absorbance values were observed in *A. tumfaciens* as it showed in Figure 33.



	300µg	150µg	75µg	37,5 µg	18,8 µg	9,4 µg	4,7 µg	2,3 µg	1,2 µg	0,6 µg	Control	Blank
Kanamycin	1.819	1.686	1.549	1.578	1.556	1.489	1.43	0.308	0.257	0.292	0.305	2.197
Viscotoxin A3	0.289	0.273	0.281	0.29	0.289	0.304	0.312	0.292	0.295	0.305	0.307	2.291

Figure 32 Resazurin absorbance in *E.coli* after 24 hours



	300µg	150µg	75µg	37,5 µg	18,8 µg	9,4 µg	4,7 µg	2,3 µg	1,2 µg	0,6 µg	Control	Blank
kanamycin	1.916	1.81	1.842	1.718	1.556	1.473	1.193	0.511	0.281	0.608	0.681	2.874
Viscotoxin A3	0.603	0.622	0.76	0.748	0.77	0.893	0.856	0.8	0.853	0.853	0.834	2.700

Figure 33 Resazurin absorbance in *A.tumefaciens* after 24 hours

In the following Table the MIC summary of the used antibiotic as positive control and viscotoxin A3 is shown.

Table 13 Minimum inhibitory effect (MIC µg/ml) of viscotoxin A3 tested on 3 different bacteria. None means no inhibitory effect observed

	P. syringae	*E. coli*	*A. tumefaciens*
Kanamycin	1,1	4,6	4,6
viscotoxin	None	None	None

4.2 viscotoxin A3 results with Fungi

Before beginning of the bioassay, the test to select the best medium was done and finally minimal medium described in following paper used (Mania et al., 2010). In the following Figure the result of the comparison test is shown. The major problem with other liquid mediums was that they were in favour of fungi therefore excessive fungal growth, which wasn't suitable for this bioassay occurred. Due to these reasons and to follow the exact pattern explained for bioassay to test *Aspergillus nidulans*, MM was used in my bioassay as well.

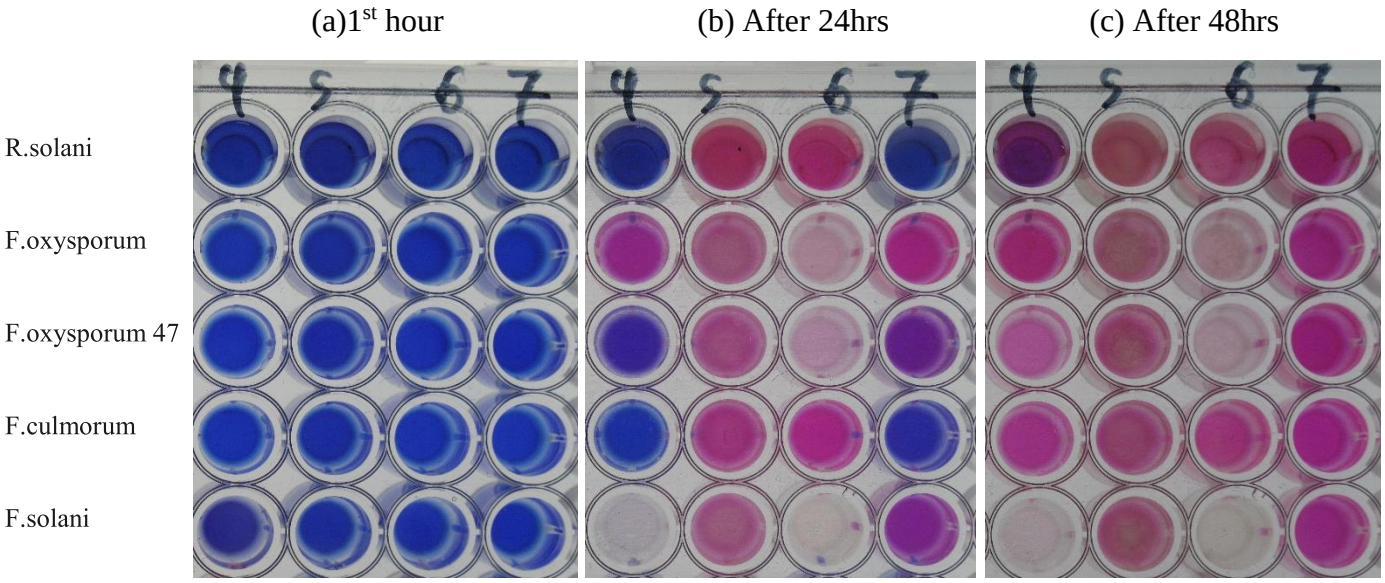


Figure 34 Medium comparison, 4 different mediums, Minimal medium(Column no.4) PDB(Column no.5) CZAPEK(Column no.6) and RPMI(Column no.7) tested with 5 fungi *R.solani* (1st row) *F.oxysporum* (1st row) *F.oxysporum 47*(3rd row) *F.culmorum* (4th row) *F.solani* (5th row). One can see the results up to 48 hours, Resazurin and fungi concentration used in this test is exactly as explained in Material and methods.

Antimicrobial activity of viscotoxin A3 against *F. solani* and *Sclerotinia sclerotiorum* has been tested and the results have been published in 2004. Based on this published research the required concentration of viscotoxin A3 to inhibit the growth of *F. solani* and *S. sclerotiorum* to a 50% (IC_{50}) was between 1.5 and 3.75 µM (Giudici et al., 2004). Therefore, to have a quick comparison with presented results in this paper and to become sure that our extracted peptide is also capable to have the same or a close inhibitory effect the first test was accomplished with *F. solani* and the following results were obtained. Benomyl in this bioassay was used as positive control with serial dilution from 200 µg to 0.37 µg (first row). The concentration of viscotoxin A3 needed to completely inhibit *F.solani* growth was 31 µg/ml (6.4µM), which is slightly higher than the published result. The reason that the initial concentration of Benomyl and viscotoxin in this test were different is due to our decision to use BSA instead of aprotinin as a reference to quantify our peptides concentration.

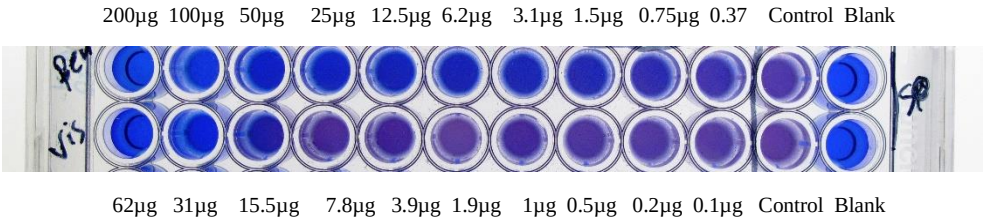
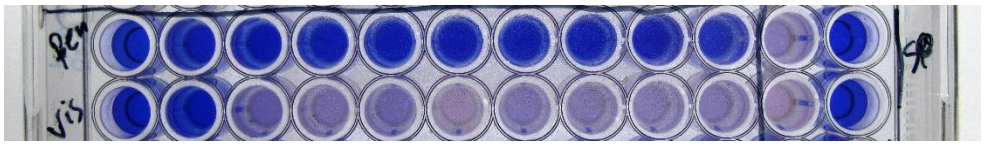


Figure 35 Picture is taken after 24 hours incubation

200µg 100µg 50µg 25µg 12.5µg 6.2µg 3.1µg 1.5µg 0.75µg 0.37 Control Blank



62µg 31µg 15.5µg 7.8µg 3.9µg 1.9µg 1µg 0.5µg 0.2µg 0.1µg Control Blank

Figure 36 Picture is taken after 72 hours incubation

The following pictures show the generation of hyphae in the well with the least and the highest concentration of viscotoxin A3.

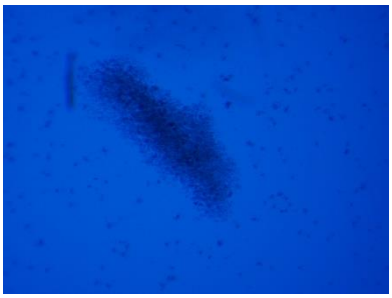
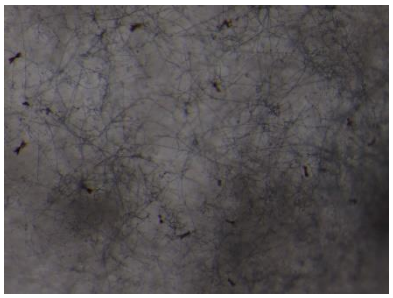
(a)  (b) 

Figure 37 pictures are taken with microscope from wells (a) with highest concentration and (b) with least concentration of viscotoxinA3

Table 14 Absorbance values earned in *F.solani* bioassay absorbance measured with 600nm wavelength, *F.solani* tested with viscotoxin A3 and Benomyl

	200µg	100µg	50µg	25µg	12.5µg	6.3µg	3.1µg	1.6µg	0.8µg	0.4µg	Control	Blank
Benomyl	2.008	1.973	2.232	2.224	2.215	2.227	2.155	2.111	1.938	1.577	0.592	2.551
viscotoxin A3	2.377	2.506	0.802	0.628	0.726	0.453	0.651	0.566	0.554	0.567	0.479	2.496
	63µg	31.5µg	15.8µg	7.9µg	3.9µg	2.0µg	1.0µg	0.5µg	0.2µg	0.1µg	Control	Blank

The bioassay for different fungal strains was repeated several times and in most cases the same results were achieved for viscotoxin A3. The following picture belongs to the bioassay for measuring antifungal activity of viscotoxin A3 against *F. culmorum* (row C, D), *F. oxyspurum* (row E,F) and *Verticillium* (row H,G) after 48 hours of incubation at 26ºC. The control for *F. oxysprum* and *Verticilium* shows a good growth, which means that the right amount of spores were used and the spores were fresh enough to grow and reproduce in minimal medium. MIC for Benomyl used as a positive control was 3.1 µg (row E) and 0.7µg (row H) for *F. oxysprum* and *Verticilium* respectively. However viscotoxin didn't show any inhibitory effect even in the highest concentration of 63 µg1/ml on none of the tested species in this experiment.

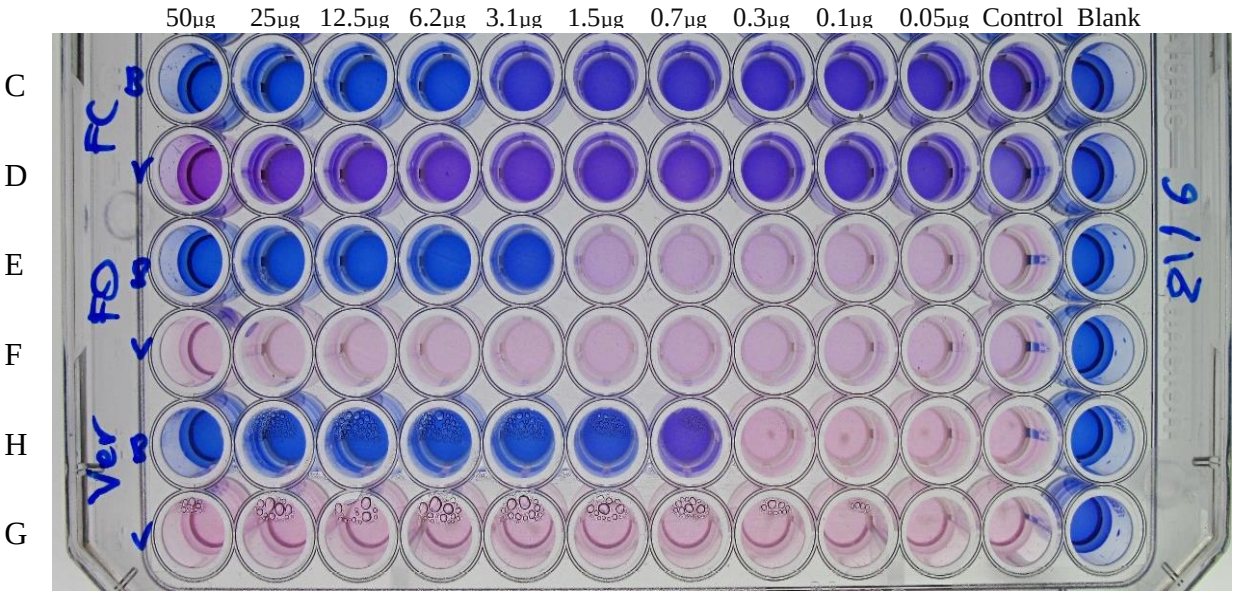


Figure 39 Nunc 96 picture is taken after 48 hours of incubation

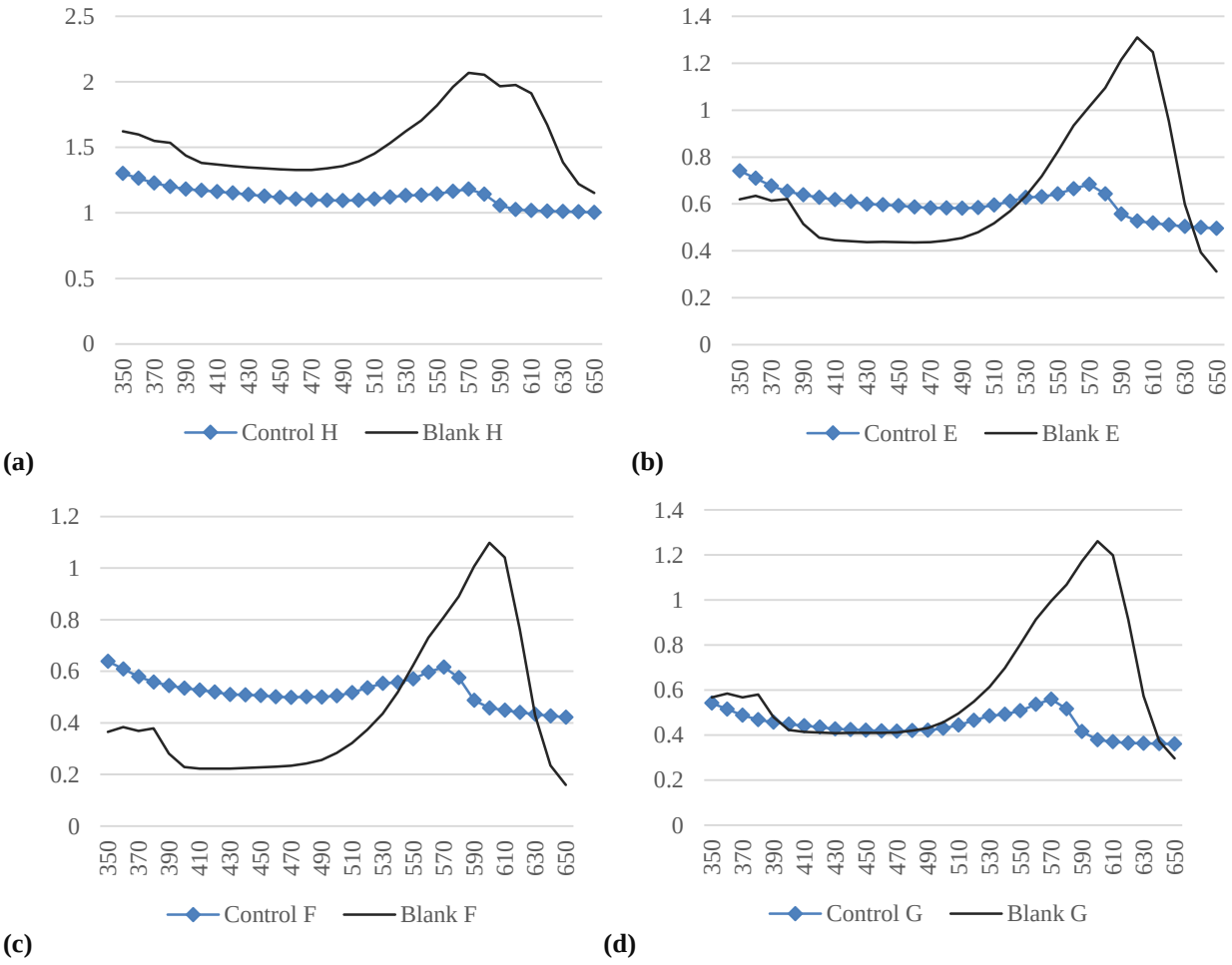


Figure 40 This Figure illustrates the spectrum of 350nm-650nm made between Blank (only medium) and Control (only $2x10^4$) spores wells. The highest difference between two spectrums is on 600nm wavelength therefore this wavelength was used for reading the other samples.

Fluorescence Intensity of all wells was measured with 540 nm Emission and 590 nm Excitation after 48 hours of incubation and the following results were obtained:

Table 15 FI values, Row C,D belongs to *Fusarium culmorum*, Row E,F are with *Fusarium oxysporum* and finally the two last rows, row G and H are tested with *Verticillium dahlia*.

		50 µg	**25 µg**	**12.5 µg**	**6.3 µg**	**3.1 µg**	**1.6 µg**	**0.8 µg**	**0.4 µg**	**0.2 µg**	**0.1 µg**	**Control**	**Blank**
C	Benomyl	44	52	62	92	338	380	386	408	424	418	441	192
D	viscotoxin A3	510	509	486	486	463	462	470	472	442	438	400	160
E	Benomyl	38	39	31	39	138	304	285	289	285	280	273	32
F	viscotoxin A3	281	281	287	283	284	293	303	278	276	279	283	33
G	Benomyl	46	46	44	47	48	122	382	391	378	375	370	46
H	viscotoxin A3	400	400	381	370	368	353	347	368	344	366	336	58

Just by looking at the data and comparing them with the picture it can be concluded that viscotoxinA3 doesn't have any inhibitory effect against these three fungi as well. In the following figure the same table is illustrated as a graph. FI measured for the wells with 50 µg/ml.

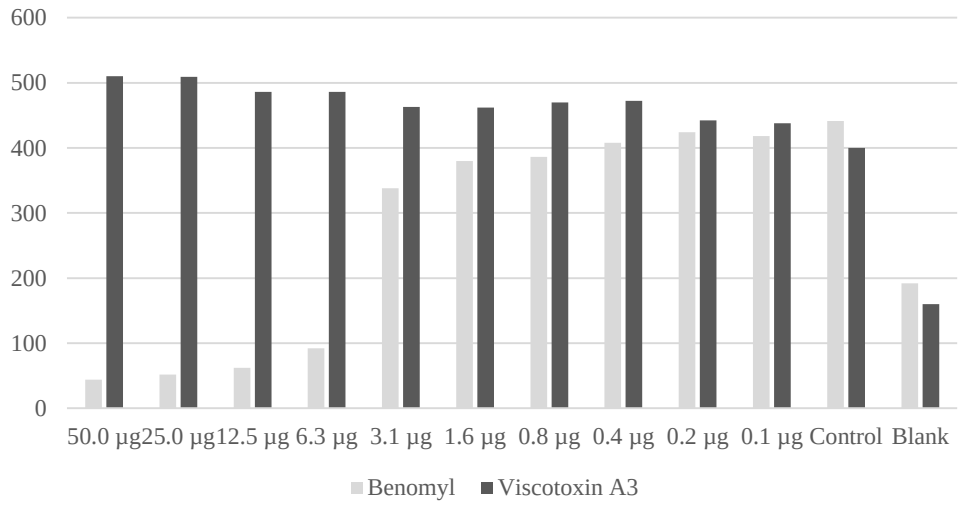


Figure 41 FI results for *Fusarium culmorum*

Shorter bars mean less fluorescence was produced, which indicates a low level of *Fusarium* activity in that specific well. In this case 6.3µg/ml Benomyl completely inhibited *Fusarium* growth, but viscotoxin, which is presented in this graph didn't show any anti-fungal activity.

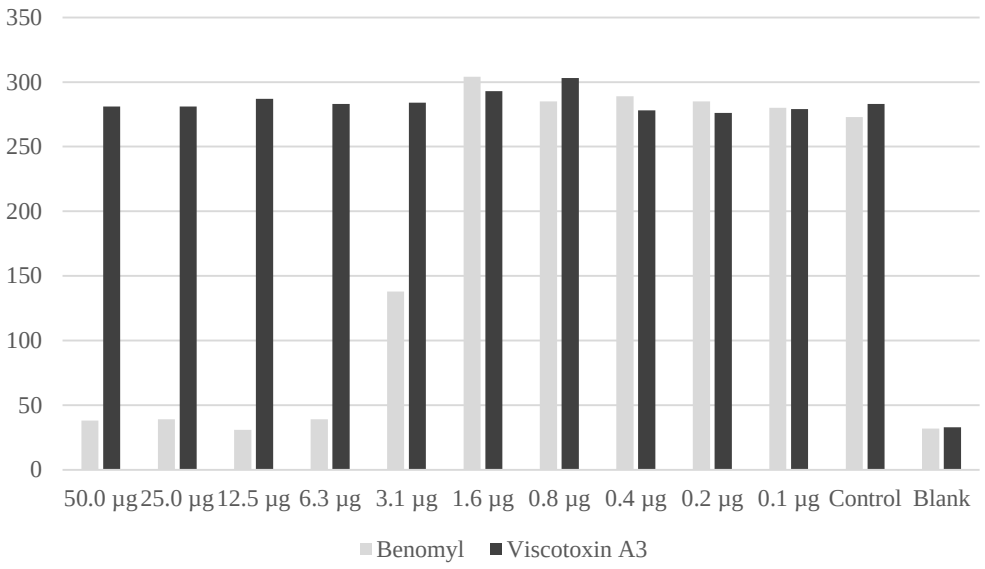


Figure 42 FI results for *Fusarium oxysporum*

As it is illustrated in the last graphs the same results as for *F.culmorum* and V.Dahilae were obtained for *F.oxysporum* and no antimicrobial activity was observed for viscotoxin A3. In Figure 39 a better growth in *F.oxysporum* and *V.dahilae* is indicated compared with *F.culmorum* in Blank or wells with lower concentration of Benomyl and the color change was easier to distinguish.

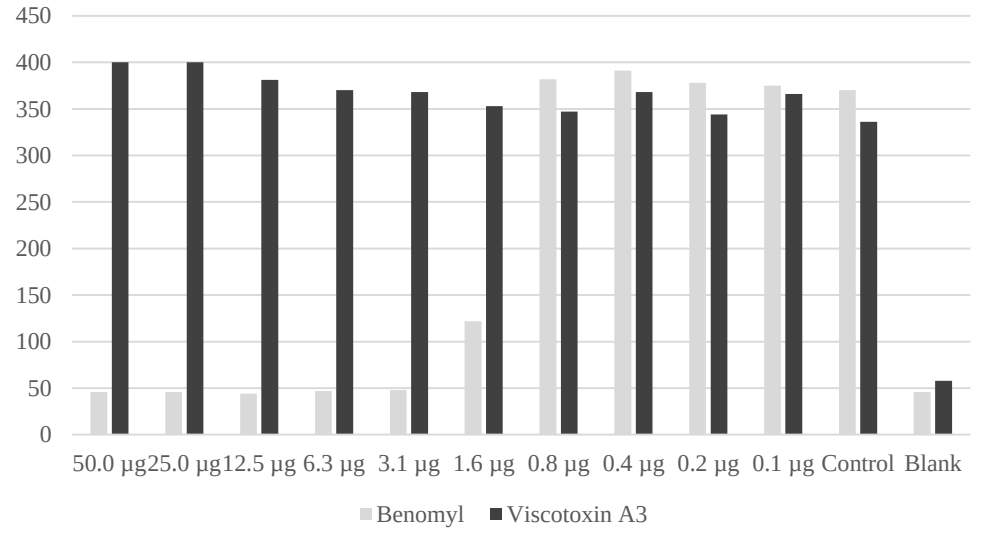


Figure 43 FI results for *Verticilium dahliae*

The results related to *Rhizoctonia solani, Thielaviopsis basicola* and *Aspergillus nidulans* is not shown due to a lack of an adequate number of repeats and more studies on the 3 mentioned fungi is needed.

4.3 Fusion proteins bioassay results

Furthermore, the same bioassay method was used to test antimicrobial activity of some fusion proteins THI 2.1, THI 2.2, THI 2.3, and THI 2.4 from *Arabidopsis thaliana*. These proteins had been made by one of our PHD students Abbas Amjad and were handed to me to test their antibacterial activity. The bioassay worked very well with both fungal and bacterial strains. The results obtained with *Pseudomonas syringae* and *F.solani* are shown in Figure 44-48 as a sample. Thus, the following test ensured us that the methods used for the bioassay in this experiment were functional with both fungi and bacterial models and the possibility that a lack of antimicrobial activity in viscotoxin A3 be due to the wrong method is low.

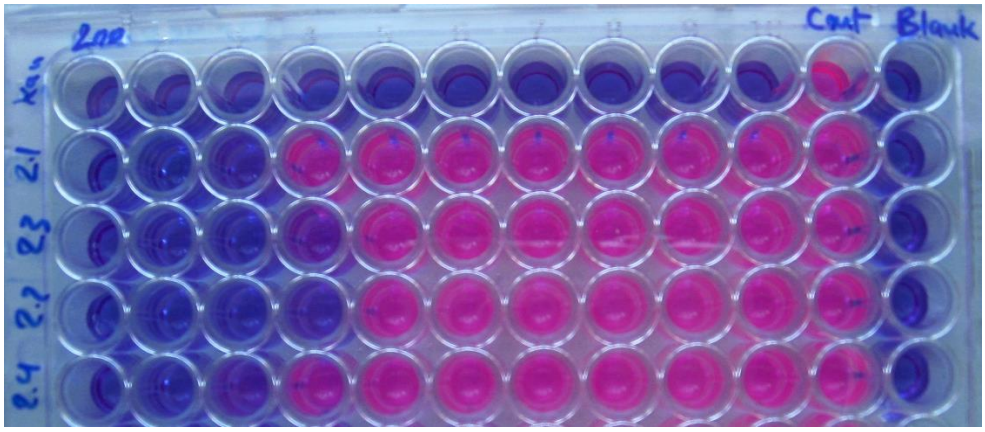


Figure 44 Bioassay to test antibacterial activity of THI 2.1, 2.2, 2.3, 2.4 on *Pseudomonas syringae*, Kanamycin used as Control in this Bioassay (1st row) and stopped bacteria activity compeletly .

Table 16 Resazurin Absorbance measurement at 600nm after 18 hours incubation time

Name	200µg	100µg	50µg	25µg	12.5µg	6.2µg	3.1µg	1.5µg	0.7µg	0.3µg	Control	Blank
Kanamycin	0.809	0.903	0.95	0.927	0.976	0.988	0.971	0.943	0.895	0.87	0.234	1.165
THI 2.1	0.856	1.01	0.85	0.249	0.234	0.263	0.272	0.239	0.256	0.258	0.251	1.011
THI 2.3	0.817	0.952	1.047	0.557	0.258	0.265	0.284	0.234	0.249	0.25	0.249	1.112
THI 2.2	1.044	1.022	1.043	0.737	0.223	0.253	0.276	0.24	0.25	0.235	0.272	1.135
THI 2.4	0.755	0.959	0.724	0.25	0.251	0.229	0.24	0.246	0.228	0.23	0.245	1.138

Table 17 FI measurment after 18 hours incubation time

Name	200µg	100µg	50µg	25µg	12.5µg	6.2µg	3.1µg	1.5µg	0.7µg	0.3µg	control	Blank
Kanamycin	19147	18185	18229	17456	16831	16452	18133	19173	21804	25153	117747	12507
THI 2.1	12398	11801	31438	116827	122380	123318	129294	125802	125728	126151	126990	13436
THI 2.3	17415	16629	15046	66377	119598	123520	129390	123992	127103	124827	125414	12885
THI 2.2	9708	12150	14475	29967	118315	120437	125041	124517	126655	125334	126361	11779
THI 2.4	16289	17504	44448	112624	121092	122392	125630	124880	124475	124586	127368	12165

Table 18 Minimum inhibitory concentration of THI 2.1, THI 2.2, THI 2.3,THI 2.4 on *Pseudomonas syringae*

Peptide name	(MIC)
THI 2.1	50µg/ml
THI 2.3	25µg/ml
THI 2.2	25µg/ml
THI 2.4	50µg/ml

In another example, bioassay for testing two Fusion protein Th2.2 and Th2.4 against *Fusarium solani* was done. All three methods were used to measure the final outcome and as it is illustrated in the following Figures the obtained result from all methods was the same. Resazurin absorbance of the plate was measured after 48 hours and the following results were obtained. In the first 24 hours I couldn't see any significant changes in measured absorbance.

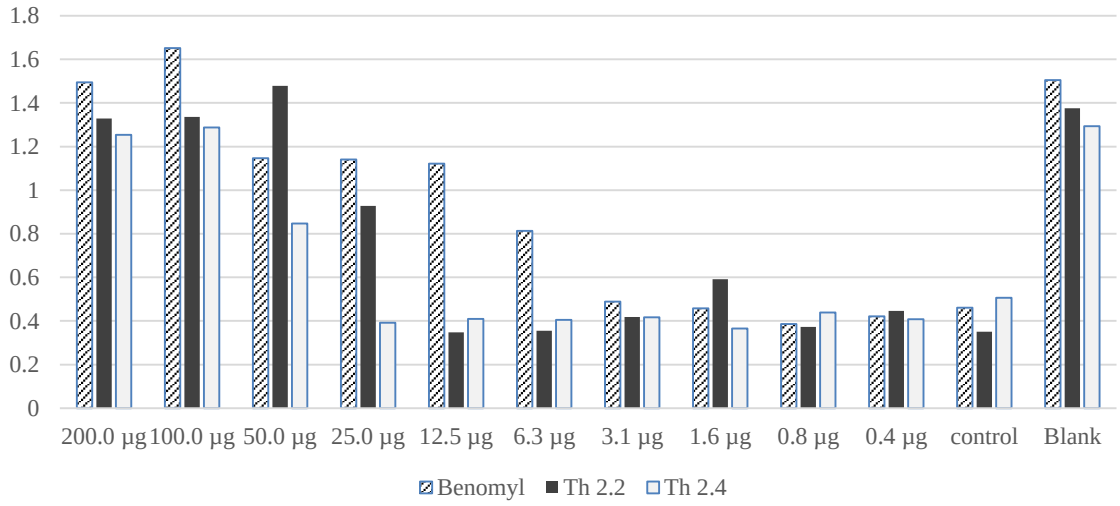


Figure 45 Absorbance results after 42hours. Anti-Microbial activities of two fusion proteins thi2.2 and thi2.4 tested against *Fusarium solani* and absorbance measured after 42 hours with wavelenght 600nm. IC50 based on this figure would be 6.3, 25 ,50µg/ml for Benomyl, th 2.2,th 2.4 respectivly.

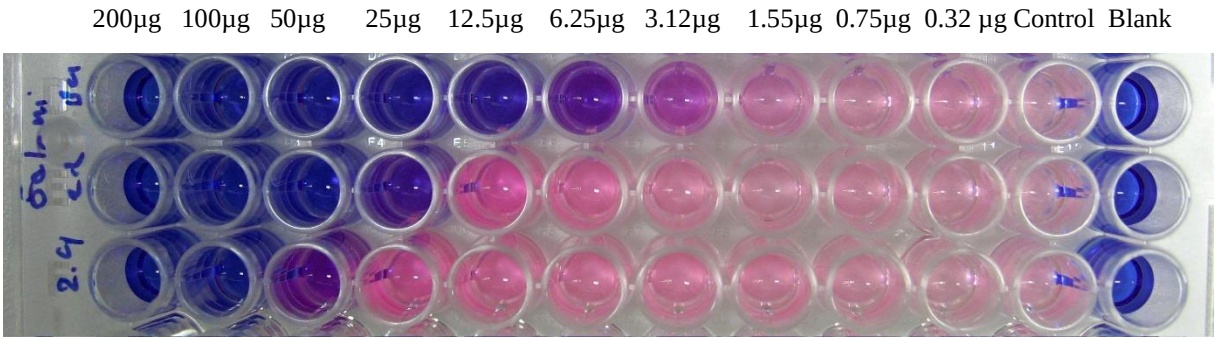


Figure 46 Picture is taken after 48 hours and shows color change from blue to pink in wells, which had less concentration of Benomyl or tested fusion protein THI 2.2 and THI 2.4. It is taken after 48 hours because in the first 24 hours no significant color change could be observed. It shows that minimum inhibitory effect against *F.solani* is 6.25µg , 25µg ,50µg for Ben and THI 2.2 ,THI 2.4 respectively.

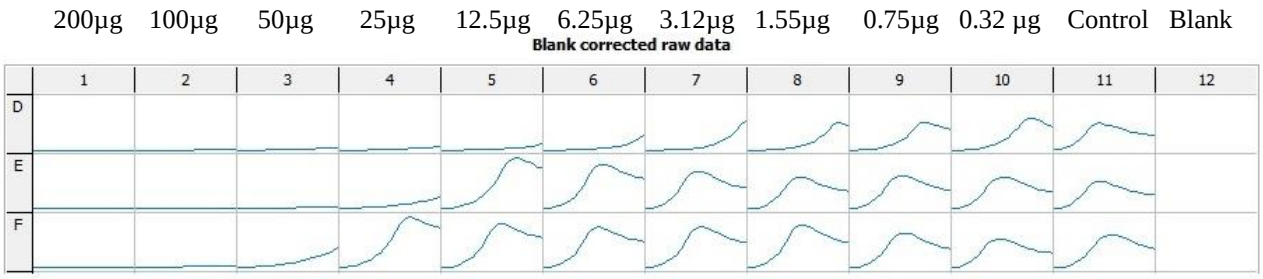


Figure 47 After 4 hours incubation FI was measured once per hour for 21 hours (21cycles with 1 hour intervals) as it is observable the curves started to rise in the first 12 hours

Figure 47 perfectly shows the growth trend of *F.Solani* in different wells. As it is shown in figure 47, in the first three columns, which contain a higher concentration of Benomyl, Th2.2 and Th2.4, no fluorescent production can be detected. But on the other hand in the other wells except Blank (column12), in those which have less concentration of Benomyl, Th2.2, Th2.4 *Fusarium* growth slightly increased and these curves formed. By comparing the obtained results from these 3 methods it can be concluded that all methods gave the same results, which is shown in the following Table:

Table 19 IC_{50} for Benomyl, THI 2.2, THI 2.4 tested with *F.solani*

	IC_{50} (µg/ml)
Ben	6.25
THI 2.2	25
THI 2.4	50

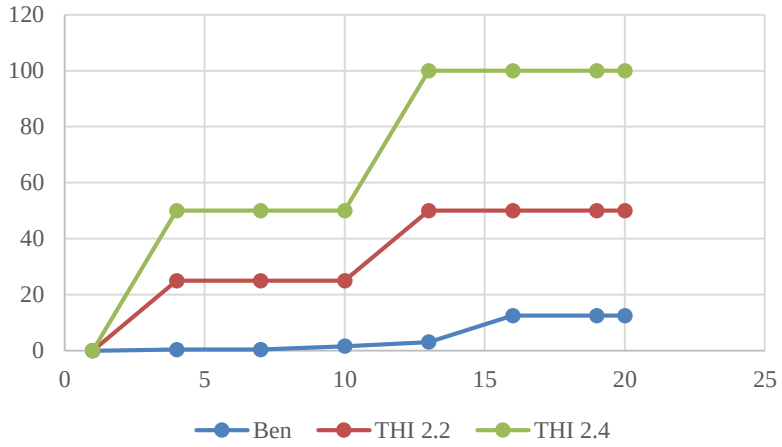


Figure 48 Maximal inhibitory concentration of THI2.2 and THI2.4 on F.Solani. The required concentration to inhibit *F.solani* growth obtained by measuring FI over the time. After 16hours of measurement the values became fixed for all samples. Concentration needed to inhibit *F.solani* growth completely is 12,5µg/ml, 50µg/ml ,100µg/ml for Benomyl , THI 2.2 and THI 2,4 respectively. The results were highly predictable because the FI curves (figure 47) related to mentioned concentrations (D5, E3, F2) were flat from the beginning of measurement. Therefore IC_{50} would be 50% of mentioned concentrations which is exactly comparable with the results which is obtained by visual observation and absorbance measurement after 42 hours.

Therefore based on this experiment it can be stated, that in case of lack of facilities results gained visually by monitoring color change or measuring absorbance are reliable, but need more time than FI measurement.

5 Discussion

In a paper which was published in 2004 the capability of viscotoxin A3 and viscotoxin B to inhibit or control growth of phytopathogenic fungi was tested. Viscotoxin A3 and viscotoxin B were incubated at a final concentration of 10 µM (48.4 µg/ml) with *F. solani* which resulted in a complete blockage of the germination of spores from three different pathogenic fungi. It was also shown that the protein dosage required to inhibit the growth of *F. solani* and *S. sclerotiorum* to a 50% (IC_{50}) is between 1.5 and 3.75 µM, which represents a potent activity (Giudici et al., 2004).

To verify the functionality of our extracted peptide and have a quick comparison between our results and the one, which have been published by Guidici in 2004 the first bioassay in this experiment was done to test antimicrobial activity of viscotoxin A3 with *F. solani*. As it is mentioned in the Results section viscotoxin A3 showed the same functionality, but the needed concentration to inhibit *Fusarium* growth was more than the one, mentioned in this paper. Considering 4842.6 (da) as formula weight of viscotoxin A3 to convert µM unit, which was used in that report to µg/ml indicates that the amount of required peptide to inhibit the growth of *Fusarium solani* to 50% (IC_{50}) in µg/ml should be 7.3 µg/ml for viscotoxin A3, which is almost less than our obtained result for the same fungus, which was around 31 µg/ml. However if we use just their first report with 10 µM (48.4 µg/ml) for full inhibition of *F. solani* as a reference it can be concluded that our peptide possesses the full functionality mentioned in this paper.

The difference maybe derives from different methods used in these bioassays. In that bioassay formed colonies on a PDA medium inoculated with spores of *F. solani* and viscotoxin A3 were counted after 3 days of incubation in this bioassay. For their second assessments method PDB medium described by Regente (Regente & de la Canal, 2000) with some minor modifications was used as a medium to measure the ability of viscotoxins in inhibition of mycelial growth. Optical density was measured at 550 nm wavelength after 48 hours with ELISA plate reader without using resazurin. In terms of considered time for incubation prior to measuring optical density, and concentration of spores both studies were identical. Therefore the only main difference remains between mediums used in these studies and the absence of resazurin, which shouldn't play any role concerning the difference between obtained results. It is known that medium plays an important role in bioassay designed for AMPs. A typical feature of AMPs is that high cation concentrations reduce antimicrobial activity observed *in vitro* (Broekaert et al. 1997). This can be seen for thionins as well. Several studies suggest that electrostatic interaction of thionins with membranes can be inhibited by divalent cations such as calcium and magnesium (Florack & Stiekema, 1994, W., 1942). The activity of viscotoxins was evaluated in the presence of a divalent cation after adding 5mM $CaCl_2$. Antifungal activity of viscotoxin A3 and viscotoxin B was blocked and spores germinated (Giudici et

al., 2004). Minimal medium (MM), used in our experiment, has been tested in a bioassay, which used the same microtiter plate-based screening method and tested successfully nine cationic short AMPs against the model fungus *Aspergillus nidulans*. The Minimal medium used in that study had no negative effects on the growth patterns of the tested *Aspergillus* strains (Mania et al., 2010). In my experiment, after several tests, I chose MM over RPMI, CZAPEK and PDB medium.

It is difficult to describe why viscotoxin A3 didn't show any antibacterial activity in this bioassay. More studies are needed to know the relation between the structure and functionality of AMPs in depth. The following Figure illustrates the mechanism of cationic peptides in killing bacteria.

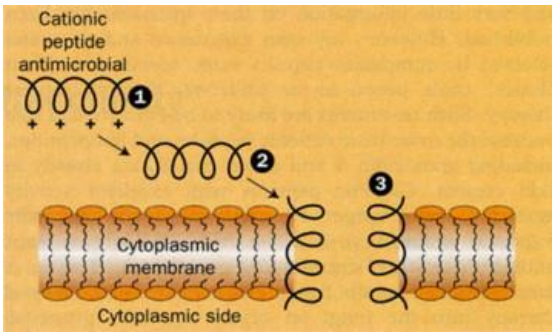


Figure 49 Cationic AMPs killing bacteria (Hancock, 1997)

Based on studies on reported cationic peptides with antibacterial activity, their mechanism is described in binding of positively charged peptides (1) to the external surface of the negatively charged phospholipid bilayer (2) which leads to a localized thinning of the bilayer and under the influence of membrane potential, peptides insert into membrane and form channels (3) leading to leakage of cytoplasmic molecules and cell death (Hancock, 1997).

Between 238 AMPs reported up to 2011 from plants, only 61 peptides among them were with antibacterial activity, of which 57 are cysteine stabilized peptides, six are linear and one is glycine-rich (Hammami et al., 2009). However NMR structure of viscotoxins A3 has been determined (Romagnoli, 2000), among the rest of reported AMPs only for a few a three-dimensional structure has been already elaborated by NMR or crystallography (Daly et al., 1999, Rosengren et al., 2003, Caldwell et al., 1998, Stec et al., 1995, Martins et al., 1996). This lack of information makes it more difficult to find the relation between peptide structure and antibacterial activity.

On the other hand, looking to the available examples among AMPs shows that sometimes even comparing three-dimensional structure and sequence similarity information are insufficient to explain antibacterial activities (Candido et al., 2011). An example to prove this is Cp-thionin. Cp-thionin is a defensin from cowpea seeds, *Vigna unguiculata*, which showed antibacterial activity against gram-positive and gram-negative bacteria like *E. coli*, *Ralstonia solanacearum*, *Rhataybacter* sp., *Erwinia* sp., *Pseudomonas syringae* and *Staphylococcus aureus* (Franco et al., 2006). This peptide shares about 73% of structural identity with VrD1, a defensin from *Vigna radiata*, which has no reported bactericidal activity.

Another example are Cyclotides with 2 subfamilies Möbius and Bracelet. Circulin A and B from bracelet subfamily and Kalata B1 from Möbius, despite their high sequence similarity, have a distinct structure and activities.

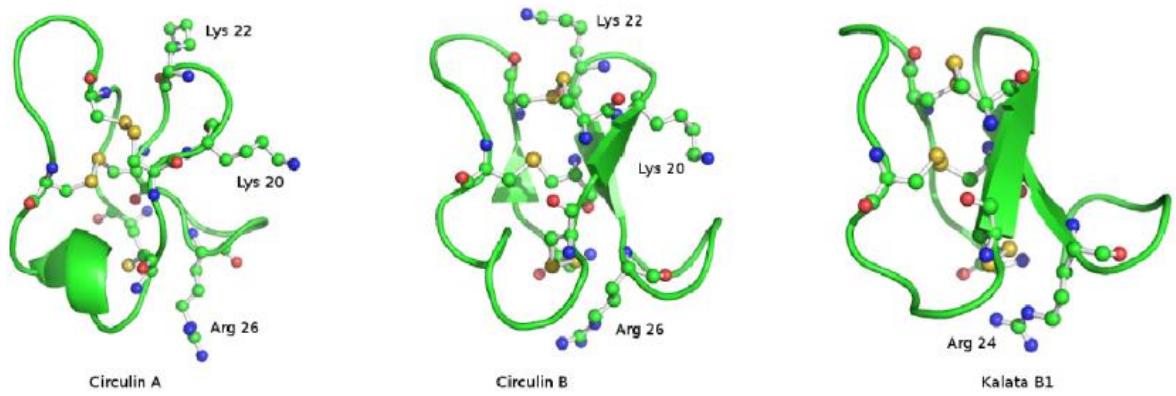


Figure 50 Three dimensional structure of Cycloids (Candido et al., 2011)

Kalata B1 and Circulin A have only activity against gram-positive bacteria, but Ciculin B has activity on both gram-positive and gram-negative. The reason is that even though the sequence similarity is high, the orientation of residues is different, which makes cationicity of Circulin A similar to Kalata B1 (Tam et al., 1999).

Therefore, so far, in most cases explanation of antibacterial activity of peptides relies on amphipathic surface of proteins and their interaction with membranes. MiAMP1 from *Macadamia integrifolia* is a peptide with antibacterial activity, whose structure has already been determined. The structure has a Greek key β-barrel shape and antibacterial activity was described due to its amphipathic surface, which interacts with negative membranes (McManus et al., 1999). But it cannot certainly be concluded that all peptides with amphipathic surface possess antibacterial activity, because all members of plant defensins have an amphipathic surface, which are mostly only antifungal (Candido et al., 2011). For instance, it can be pointed out to viscotoxins for which so far only antifungal activity has been reported. To understand mechanisms of action of peptides identifying the role of each residue in the structure is needed, besides relying on amphipathic surfaces interacting with membranes.

In contrast, antifungal activity of viscotoxin A3 was expected in this experiment more because their activity on *F.solani, S. sclerotiorum* and *P. infestans* has been already reported. Furthermore, based on known information about the structure of viscotoxin A3 and with considering the characteristics, which have been described earlier, that a peptide needs to have to show antifungal activity, I expected to observe some activities. To get accurate results the quality of the peptide is very important. For instance if the protein is folded or possesses a damaged structure it may reduce the functionality of the protein. Since the results earned in this experiment show slight deviations from the test, which had revealed antifungal activity of viscotoxin A3 on *F.Solani* (Giudici et al., 2004), this could have be the reason why I didn't see the expected activity of viscotoxin A3 on fungi used in my experiment. However, this possibility can be excluded. The cytotoxic activity of viscotoxin A3 against HeLa cells has been determined and showed activity comparable to published results (Mohr and Bohlmann, personal communication).

In this experiment minimal medium used for fungi was not favorable for all strains and I could not observe acceptable growth even in controls for *Thielaviopsis basicola, Fusarium graminearum*,

Aspergillus nidulans and *Rhizoctonia solani*. Maybe the problem derives from the used strains, especially in case of *Aspergillus nidulans.* As the medium has been specifically optimized for *A.nidulans* (Mania et al., 2010) maybe it can be concluded, that the problem derived from the strain that I used but for the rest, perhaps, other mediums than minimal medium would be needed to achieve better results.

The last question that is needed to be answered is, which method is faster and more reliable. In this work I found that obtaining the final results with measuring the fluorescence intensity is faster than absorbance and the outcome is the same. In a paper published in 2008 rapid micro plate-based resorufin fluorescence inhibition bioassay was compared with a 24 h micro plate-based yeast growth inhibition bioassay using eight fungicides. In this paper it was mentioned, that the resorufin fluorescence inhibition bioassay was both faster and more sensitive than the growth inhibition bioassay. Inhibitory concentrations obtained just after 30 min of incubation with amphotericin B (AMB) and captan as fungicide. IC50 and IC90 values that were obtained by this method were comparable to previous reported ones (Fai & Grant, 2009). In my experiment only changing a color from blue to red in case of microbial activity took 24 to 48 hours depending on the used bacteria or fungi. In the Giudici study also 48 hours have been reported as an incubation time needed before measuring optical density by ELISA plate reader, which proves the long duration of time, required in their applied method to achieve the final results. By comparing the results obtained from absorbance, fluorescence intensity measurement and color change observation it can be concluded that FI is definitely the faster way to achieve the final result than the two other methods. FI of samples incubated for 20 hours with viscotoxin and *F.solani* spores was measured each 30min for 18 hours. FI of negative control reached the peak (highest FI) in 7 hours. Even by looking at the values achieved in the very first hours the final result were highly predictable. The results were even more impressive for bacteria. FI measurement of bioassay to test *E.coli* with all fusion proteins (THI2.1,2.2,2.3,2.4) started right after preparation of Micro titer plate and continued each 30 minutes for 15 hours (picture is not shown). The results were clearly observable after 8 hours and comparable with absorbance results achieved after 18 hours. This time for color change observation and absorbance measurement for some fungi and bacterial strains was much longer and 48-72 hours was needed to obtain final results. In this study the results from all methods were identical but if there is no time restriction to ensure and make a final decision about the obtained data, one can wait for the results of the two other methods as well.

6 Conclusion

Looking to the results obtained in these experiments it can be concluded that in spite the reported antifungal activity of viscotoxin A3 in 2004 it seems that this peptide doesn’t have any antibacterial potency. As discussed in the last part, antibacterial activity of thionins and AMPs are reported less than their anti-fungal activity and cytotoxicity against tumor cells. Specifically about the tested peptide, viscotoxin A3, its activity against three tested bacterial strains in this experiment so far has not been reported. The tested concentration of viscotoxin A3 against 3 bacterial strains was much higher than the concentration used for fungi. Since the method to reach these results worked for

bioassay done for fusion proteins with the same bacterial strains it can be concluded, that the methods were reliable and viscotoxin A3 doesn't have any inhibitory effect on *E. coli*, *A. tumefaciens* and *P. syringae*. Antifungal activity of viscotoxin A3 on *F.solani* was observed as we expected based on earlier reports (Giudici et al., 2004, Giudici et al., 2003). The minimum concentration needed to inhibit *F.solani* growth in this experiment was 6.4µM, which was close to earlier published results, which employed 1.5µM to reduce *Fusarium* growth to 50% and 5µM to completely abolish the germination of spores of *F.solani*. This was sufficient evidence to rely on and continue the bioassay with other fungal and bacterial strains. Obtained results from all used methods in this experiment suggested that in spite of proved antifungal potency of viscotoxin A3 on *F.solani*, *S.sclerotiorum* and *Phytophthora infestans* this peptide does not possess antifungal activity on other tested strains in this experiment. *F.oxysporum*, *V.dahliae* and *F.culmorum* were three fungi, which were tested successfully in this experiment.

Three different methods were used in this experiment to achieve and determine the final results. Resazurin based bioassay has not been used in other studies to test antimicrobial activity of viscotoxin A3 and is applied for the first time in this experiment. The incubation time needed to observe a clear resazurin color change was relatively long. This time represented 24-48 hours and 48-72 hours for bacteria and fungi respectively. In lack of clear color changes all absorbance values remains very close to each other and difficult to distinguish. In most resazurin based studies FI measurement preferred to absorbance measurement due to higher accuracy and sensitivity. In this study FI values were achieved faster than the resazurin absorbance ones and the obtained results from all different methods were identical. Therefore it can be concluded that achieving results by measuring FI is slightly faster and is as reliable as other methods explained in this study.

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