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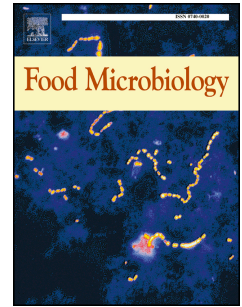
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Isolation and characterisation of the antifungal activity of the cowpea defensin Cp-thionin II

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1        **Isolation and characterisation of the antifungal**  
2        **activity of the cowpea defensin Cp-thionin II**

3  
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## 11 ABSTRACT

12 As a result of the rapidly growing human population, reducing post-harvest crop  
13 losses of cereals due to microbial pests has major importance. Plant defensins have  
14 the potential to fulfil these demands, being highly specific and efficient antimicrobial  
15 agents. Hence, this study aimed to extract and characterise a peptide from cowpea  
16 seeds and investigate its antifungal performance. After extraction and partial  
17 purification, N-terminal sequencing was used to identify the primary peptide in the  
18 extract as cowpea-thionin II. Antifungal activity *in vitro* was found against *Fusarium*  
19 *culmorum* (MIC = 50 µg/mL), but *Aspergillus niger* and *Penecillium expansum*  
20 showed an MIC > 500 µg/mL. The extract was resistant against heat treatment  
21 (100°C, 15 min) but lost its antifungal activity in presence of cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>  
22 and Mg<sup>2+</sup>, respectively). Membrane permeabilization of fungal hyphae was evident at  
23 25 µg/mL, while induction of oxidative stress only had minor contribution to the  
24 antifungal performance. The extract did not induce haemolysis at all concentrations  
25 tested (up to 200 µg/mL). Finally, it was successfully used to protect stored wheat  
26 grains from fungal spoilage (determined via ergosterol content) when applied at 100  
27 µg/mL. In conclusion, the defensin Cp-thionin II showed the potential for future  
28 application as food bio-preservative.

29

## 30 1. INTRODUCTION

31 Satisfying the nutritional demands of the rapidly growing global population has,  
32 during recent decades, turned into an ever increasing challenge. Due to the limited  
33 resources and agricultural area available, research has focused on improved  
34 efficiency in terms of food production and preservation. It was estimated that with the  
35 current amounts of food waste, the food production has to increase by 60 - 110%  
36 until 2050, in order to feed the population worldwide (Ray et al., 2013; Tilman et al.,  
37 2011). The reason behind this rapid increase is not only the growing population, but  
38 also the predicted increase in food consumption per capita (Tilman & Clark, 2014). In  
39 addition, recent studies have demonstrated that the approaches to increase the yield  
40 of agricultural crops, such as cereals, are not sufficient and sustainable to satisfy the  
41 global demands of future generations (Ray et al., 2013). Considering the importance  
42 of cereals for human nutrition over the last centuries (approximately 70 kg per  
43 person per year), this poses a major problem (Albertson et al., 2015). Hence,  
44 research and industry are trying to reduce crop losses and increase the  
45 sustainability.

46 One of the main reasons of food waste is the microbial spoilage of the crops in-field,  
47 as well as post-harvest. According to Freita-Silva et al. (2014), approximately 15% of  
48 the global cereal production is lost due to microbial spoilage. While the reduction of  
49 in-field spoilage is intensely investigated (Mannaa & Kim, 2017), the equally  
50 important microbial protection after harvest and during storage is often overlooked.  
51 However, up to 20% of the harvested cereals worldwide turn into food waste, mainly  
52 as a result of microbial spoilage during storage and downstream processing (Ridolfi  
53 et al., 2018). Microbial contaminants include bacteria, yeasts and filamentous fungi.  
54 For cereals, fungi belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium*  
55 are the most commonly found spoilage organisms (Russo et al., 2017). Growth and  
56 development of these fungi during storage result in grain quality deterioration and, if  
57 not disposed, potential consumer health hazards (Schmidt et al., 2016; Schmidt et  
58 al., 2018; Tournas & Niazi, 2018). In parallel, consumer`s acceptance for  
59 conventional food preservation is decreasing continuously. Consumers demand  
60 "clean-label" products combined with high standards in terms of food safety and  
61 quality (Figiel & Kufel, 2016). Therefore, research has to focus on new, natural

62 approaches to ensure microbial safety, in order to meet consumer`s desire and  
63 nutritional demands.

64 One approach of bio-preservation that recently received a lot of research interest are  
65 plant-derived antimicrobial peptides (AMPs).

66 One such peptide, previously reported as natural antibacterial agent is the cowpea-  
67 thionin II (Cp-thionin II). It can be found in various tissues of the plant with highest  
68 concentrations in the seeds. During germination the peptide concentration was found  
69 to decrease (Franco et al., 2006). This suggests that the peptide is part of the natural  
70 plant defense mechanism, making antifungal activity likely. To the best of the  
71 authors` knowledge, the antifungal activity of natural Cp-thionin II against *F.*  
72 *culmorum*, *A. niger* and *P. expansum* has not been studied previously. However, a  
73 recent study with a synthetic linear analogue of the peptide showed promising results  
74 (Thery & Arendt, 2018).

75 Previous studies have shown the thermal stability of selected plant defensins  
76 (Broekaert et al., 1995; Terras et al., 1992), which increases their potential as food  
77 preservatives. On the other hand, plant defensins often are sensible to the presence  
78 of cations (Vriens et al., 2014), which could be a major drawback for food  
79 applications. Regarding the mode of action of the peptide against fungal hyphae, the  
80 induction of membrane permeabilization and oxidative stress towards the fungal cells  
81 are discussed (Thery & Arendt, 2018). Another important consideration for the  
82 application as preservative is the consumer safety. Although plant defensins are  
83 usually nontoxic towards mammalian cells (Thevissen et al., 2004), depending on  
84 the amount of disulfide bonds and their hydrophobicity and amphipathicity some  
85 toxic effects have been reported (Hollmann et al., 2016; Jenssen et al., 2006).  
86 Therefore, the haemolytic activity against mammalian red blood cells is of further  
87 interest to characterise the peptide. Finally, the application of natural AMPs as food  
88 preservatives was reported by several researchers (Lucera et al., 2012; Rai et al.,  
89 2016; Rydlo et al., 2006). However, the environmental conditions and sample matrix  
90 play an essential role for the efficiency of the peptide. Hence, it is uncertain if the  
91 here investigated application as preservative during cereal storage can be  
92 successful.

93 The results of this study provide important information regarding the potential of  
94 AMPs in general and Cp-thionin II in particular as bio-preservative. Hence, it  
95 increases the knowledge regarding this highly promising approach to naturally  
96 reduce food losses and increase sustainability to satisfy the global nutritional  
97 requirements.

98

## 99 2. MATERIALS AND METHODS

### 100 2.1. Extraction and partial purification of the peptide

101 Extraction of the peptide from commercial organic cowpea (*Vigna unguiculata*) seeds  
102 was based on the method described by Franco et al. (2006), with some  
103 modifications. In brief, organic cowpea seeds were milled to a fine flour, using a  
104 coffee grinder, and extracted with 0.1 M HCl / 0.15 M NaCl buffer (meal : buffer ratio  
105 1:5) under continuous stirring for 4 h at 5°C. Subsequently, the supernatant was  
106 neutralised (using NaOH), filtered (pore size 0.45 µm) and saturated with 60%  
107 ammonium sulphate. The precipitate, formed overnight, was extensively dialysed  
108 against distilled water (2.0 kDa upper cutoff, Sigma-Aldrich), lyophilised and  
109 resuspended in equilibration buffer (0.15 M Tris/HCl buffer, pH 7.0, containing 5 mM  
110 CaCl<sub>2</sub>). The obtained crude extract was applied to anion exchange chromatography,  
111 using a HiTrap<sup>TM</sup> DEAE FF (1 mL) column (GE Healthcare). Chromatography was  
112 carried out using an AKTA protein purification system (GE Healthcare Life Sciences).  
113 The flow rate used was 1.0 mL/min and the eluted fractions (1.0 mL) were collected.  
114 Equilibration buffer (buffer A) was used to elute the non-retained fraction, while  
115 retained proteins were displaced from the column using buffer B (0.15 M Tris/HCl  
116 buffer + 1 M NaCl, pH 7, containing 5 mM CaCl<sub>2</sub>). The non-retained fraction was  
117 applied to cation exchange chromatography, using a HiTrap<sup>TM</sup> SP HP (1 mL) column  
118 (GE Healthcare) with the same conditions. The retained protein fractions were eluted  
119 with a gradient of 0 - 100% buffer B, applied over 40 min, and collected for further  
120 analysis.

121 After further dialysis at room temperature against distilled water and lyophilisation,  
122 the residue was redissolved in distilled water and used as stock solution for further  
123 analysis. The Pierce<sup>TM</sup> BCA Protein Assay Kit (Thermo-Fischer Scientific) was used  
124 according to the supplier's instructions to determine the protein concentration of the

125 solution. The purity of the extract was assessed by SDS gel electrophoresis, using a  
126 Tris/tricine precast gel (Bio-Rad), stained with Coomassie blue G-250. Sample  
127 preparation and electrophoresis of the native and denatured (heated for 7 min in  
128 presence of 9.5 mg dithiotreitol/mL) sample were carried out according to the  
129 supplier`s instructions (Bio-Rad). The band migrating at approximately 7 kDa was  
130 used for N-terminal sequencing by Edman degradation (5 residues), carried out by  
131 LakePharma (Belmont, USA). A BLAST analysis on UniProt protein database was  
132 used to identify the peptide based on the first five amino acids determined by Edman  
133 sequencing.

134

## 135 2.2. Circular Dichroism (CD)

136 The analysis of the secondary structure of the extracted peptide was carried out  
137 using circular dichroism (CD) spectroscopy according to the method described by Liu  
138 et al. (2008). In brief, the extract was diluted in deionized water or 20 mM sodium  
139 dodecyl sulphate (SDS) to a final protein concentration of 1 mg/mL. CD  
140 measurements were performed using a Chirascan CD Spectrometer (Applied  
141 Photophysics), at 27°C within a wavelength range of 180-260 nm. Each solution was  
142 measured in triplicate and the solvent CD was subtracted from the sample CD.

143

## 144 2.3. Fungal strains

145 Three different species of filamentous fungi commonly found on cereal products,  
146 namely *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum*, were  
147 investigated. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21 and *P.*  
148 *expansum* FST 4.22 originated from the culture collection of School of Food and  
149 Nutritional Sciences, University College Cork (Cork, Ireland).

150

## 151 2.4. Antifungal activity assay

152 The antifungal activity of the cowpea extract was determined by following  
153 germination and growth of fungal conidia in a microtiter plate assay, as described by  
154 Van Der Weerden et al. (2008). Briefly, fungal conidia were collected from colonies



155 grown for 72 h on potato dextrose agar (PDA) (Sigma Aldrich) at 25°C and diluted to  
156 a final concentration of  $10^4$  spores/mL in half strength potato dextrose broth ( $\frac{1}{2}$   
157 PDB), using a haemocytometer. Filter sterilised extracts (20  $\mu$ L) and fungal spore  
158 suspension (180  $\mu$ L) were combined in the wells of a 96-well microtiter plate. Final  
159 peptide concentrations in the mixture were ranging from 500  $\mu$ g/mL to 6  $\mu$ g/mL.  
160 Fungal growth was followed over 96 h at 25°C by measurement of the optical density  
161 (OD) at 620 nm (Multiscan TM, Thermo Scientific). Addition of 20  $\mu$ L of 0.1% acetic  
162 acid or sterile distilled water to the fungal spore suspensions were used as negative  
163 and positive control, respectively.

164 Additionally, the inhibition of fungal growth was controlled on PDA plates. Fungal  
165 spore suspension was added to warm  $\frac{1}{2}$  PDA to a final concentration of  $10^4$   
166 spores/mL and poured into a sterile petri dish (20 mL). After solidification, 4 wells  
167 were cut into the agar and filled with 50  $\mu$ L of peptide solution, containing 0 – 200  
168  $\mu$ g/mL. A well containing 50  $\mu$ L of 0.1% acetic acid was prepared similarly as  
169 negative control. The extract was allowed to diffuse into the agar and the plates  
170 subsequently incubated for 3 d at 25°C. Fungal growth inhibition was evaluated by  
171 measuring the halo around the wells.

172

173 2.5. Determination of the minimal inhibitory concentration (MIC) and half maximal  
174 inhibitory concentration ( $IC_{50}$ )

175 After 96 h of incubation at 25°C, the MIC was determined as the lowest  
176 concentration of peptide that completely inhibited fungal growth. The concentration  
177 required to inhibit the fungal development by 50% ( $IC_{50}$ ) was determined by non-  
178 linear regression, using the software graph PRISM (GraphPad Software, Inc., La  
179 Jolla, CA) with the microplate reader data.

180

181 2.6. Thermal stability

182 In order to study the thermal stability of the extract, the peptide solution was heated  
183 at 100°C for 15 min. After cooling to room temperature (30 min), the antifungal  
184 activity of the extract at MIC was determined against *F. culmorum* in a 96-well  
185 microtiter plate assay, as described in section 2.3.

186

## 187 2.7. Effect of cations on the antifungal activity

188 The influence of various cations on the antifungal performance of the cowpea extract  
189 was investigated in an antifungal assay, performed in different salt solutions as  
190 described by Terras et al. (1992). *F. culmorum* spores ( $10^4$  spores/mL) were  
191 inoculated in  $\frac{1}{2}$  PDB, containing 100 mM NaCl, 50 mM KCl, 5 mM  $\text{CaCl}_2$  or 5 mM  
192  $\text{MgCl}_2$ , respectively. After addition of the peptide solution (50  $\mu\text{g/mL}$ ), fungal growth  
193 was followed in a microtiter plate assay, as described in section 2.3.

194

## 195 2.8. Membrane permeabilization assay

196 A membrane permeabilization assay on *F. culmorum* hyphae to characterise the  
197 mode of action of the peptide extract was carried out based on the method described  
198 by Van Der Weerden et al. (2008). Briefly, fungal hyphae were grown overnight at  
199 25°C in  $\frac{1}{2}$  PDB from a suspension of  $10^4$  conidia/mL. Following centrifugation (10  
200 min, 5,000 g), the hyphae were washed twice with and resuspended in synthetic  
201 fungal medium (SFM; prepared as described by Rodriguez et al. (2003)). The extract  
202 was added to a final peptide concentration of 100, 50 (MIC), 25 and 12.5  $\mu\text{g/mL}$ ,  
203 respectively. Solutions of hyphae without peptide or with 1% Triton X-100 (Sigma-  
204 Aldrich) were used as negative and positive controls, respectively. After incubation  
205 for 2 h at 25°C, the fluorophor propidium iodide was added to a final concentration of  
206 0.5% and the mixture was incubated for 10 min at room temperature in the dark.  
207 Subsequently, fluorescence of fungal hyphae was measured using a  
208 fluospectrophotometer (Varioscan<sup>®</sup> LUX reader) with excitation and emission  
209 wavelengths of 488 nm and 538 nm, respectively or examined using confocal laser  
210 scanning microscopy (CLSM) (Olympus) (excitation wavelength 460 -490 nm).

211

## 212 2.9. Induction of reactive oxygen species (ROS)

213 The measurement of ROS was carried out based on the method of Van Der  
214 Weerden et al. (2008) with some modifications. *F. culmorum* hyphae (grown as  
215 described above) were treated with water or cowpea extract (containing various

216 concentrations of peptide) for 12 h before incubation with dihydrorhodamine 123  
217 (Sigma-Aldrich) (10  $\mu\text{g}/\text{mL}$ ) for 2 h. After extensive washing with 0.6 M KCl  
218 fluorescence of the hyphae was visualised using a fluorescence microscope  
219 (Olympus) (excitation wavelength 460 – 490 nm) and measured using a  
220 fluospectrophotometer with excitation and emission wavelengths of 488 nm and 538  
221 nm, respectively. Hyphae treated with water and  $\text{H}_2\text{O}_2$  (1% w/v) were analysed as  
222 negative and positive controls, respectively.

223

#### 224 2.10. Haemolysis assay

225 The peptide solution was studied for its ability to induce haemoglobin release from  
226 fresh defibrinated sheep erythrocytes as described previously by Lavery et al.  
227 (2010). Fresh sheep red blood cells (Thermo Fischer Scientific) were washed three  
228 times with equal volumes of phosphate buffered saline, pH 7.4 (PBS). After  
229 centrifugation for 15 min at 900 g, the erythrocytes were resuspended in PBS to a  
230 final concentration of 4% (v/v). In a 96-well microtiter plate, 20  $\mu\text{L}$  of peptide solution  
231 (different concentrations in PBS) and 80  $\mu\text{L}$  of the erythrocyte suspension were  
232 combined and incubated for 1 h at 37°C. Subsequently, the suspension was  
233 centrifuged (10 min, 1,000 g) and the supernatant was transferred to a new microtiter  
234 plate. The release of haemoglobin was measured spectrophotometrically at 405 nm.  
235 Erythrocytes treated with 0.1% Triton X-100 in PBS and PBS alone were treated  
236 similarly as positive and negative controls, respectively. The percentage of  
237 haemolysis was calculated as published by Lavery et al. (2010).

$$\% \text{ Haemolysis} = \frac{(\text{Abs}_{405} \text{ peptide treatment}) - (\text{Abs}_{405} \text{ PBS})}{(\text{Abs}_{405} \text{ 0.1\% triton X} - 100) - (\text{Abs}_{405} \text{ PBS})}$$

238 The release of haemoglobin was determined for six replicates.

239

#### 240 2.11. Wheat grain spoilage protection

241 Wheat grains, supplied by Doves Farm Food Ltd. (Hungerford, UK) were disinfected  
242 according to the method described by Oliveira et al. (2012). Briefly, 300 g of grains  
243 were disinfected in 2 L 10% (w/v) hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) solution for 10 min with  
244 continuous stirring. Subsequently, the grains were washed for 5 min in 4 L distilled

245 water. This procedure was repeated once, but with only 5 min of disinfection.  
246 Immediately, the grains were moved to sterile plastic boxes and dried at room  
247 temperature for 24 h under vertical sterile laminar flow. Finally, the grains were  
248 exposed to ultraviolet light (10 min) and collected aseptically for further use.

249 For preparation of contaminated wheat, disinfected grains were mixed with 2% (v/w)  
250 spore suspension of *F. culmorum* ( $10^4$  spores/mL). After 10 days of incubation at  
251 25°C, complete fungal proliferation of the grains was visible and the grains were  
252 defined as 100% infected.

253 Infected and disinfected grains were mixed to samples of 100 g (dry matter),  
254 containing 5% infected kernels. Subsequently, the samples were sprayed with 2%  
255 (v/w) of sterile-filtered extract (protein concentration of 0, 25, 50 and 100 µg/mL,  
256 respectively). Glacial acetic acid was applied similarly as a control. Each sample was  
257 then divided into 6 portions and filled into sterile plastic bags. The bags were sealed,  
258 perforated with two pipette tips, containing a barrier filter to allow gas exchange, and  
259 stored at room temperature. After 0 and 6 weeks of storage, 3 portions of each  
260 sample were collected, milled to a whole grain flour (particle size < 2mm),  
261 homogenised and stored at -20°C until further analysis.

262

## 263 2.12. Determination of Ergosterol

264 The total ergosterol content before and after storage was determined based on the  
265 method of Jedlickova et al. (2008). In brief, 10 g of milled grains were extracted with  
266 50 mL of methanol under constant shaking at room temperature for 30 min. After  
267 centrifugation, 25 mL of the supernatant were transferred into a tube containing 3 g  
268 KOH and shaken until the KOH had fully dissolved. Subsequently, 10 mL of n-  
269 hexane were added and the mixture was incubated for 30 min at 65°C. After cooling  
270 to room temperature, 5 mL distilled water were added and the upper layer collected.  
271 The extraction with n-hexane was repeated 3 times, the combined extracts  
272 evaporated till dry and the residue re-dissolved in 5 mL of methanol before analysis  
273 by HPLC.

274 The RP-HPLC column used was a Nova-Pak C<sub>18</sub> (300 x 3.9mm, 4µm) (Agilent  
275 Technologies). Peak-identity was verified using the UV-spectra recorded by the

276 DAD. The limit of detection (LOD) and the limit of quantification (LOQ) were  
277 determined from the signal/noise (s/n) ratio. The LOD was set for s/n of 3:1 and the  
278 LOQ was set for s/n of 10:1. For calibration, ergosterol standards between 1.0 and  
279 200 µg/mL in methanol were prepared and analysed.

280

### 281 2.13. Statistical analysis

282 Statistical analysis was carried out using Microsoft XLSTAT Version 2015.5.01.  
283 (Adinosoft Inc, New York, USA). Standard deviations were calculated for absorbance  
284 values at each peptide concentration of the extract based on triplicates, unless  
285 otherwise stated. The effect of the various salts and heat treatment on the antifungal  
286 performance of the peptide was analysed with one-way ANOVA followed by a  
287 Tukey-Kramer HSD test to identify differences relative to the control. All cases with  
288  $p < 0.05$  were considered as significant. The same statistical analysis was carried out  
289 to determine individual differences in haemolysis activity for each concentration of  
290 the peptide in comparison to the negative control.

291

## 292 3. RESULTS AND DISCUSSION

### 293 3.1. Extraction, partial purification and identification of the cowpea peptide

294 The purification of the crude extract containing Cp-thionin II, obtained from the  
295 cowpea seeds, was carried out by ion-exchange chromatography using Red-  
296 Sepharose columns. The anion exchange resulted in retention of one fraction which  
297 showed no antifungal activity (data not shown). The unbound fraction was further  
298 purified by cation exchange. After disposal of the unbound fraction, 6 peaks were  
299 eluted, using 1M NaCl solution, and collected. The first 5 peaks showed no  
300 antifungal activity (data not shown) and were not investigated further. The major  
301 peak (Fig. 1, black arrow) was used for further analysis and dialysed against distilled  
302 water, lyophilised and re-dissolved to a final concentration of 10 mg/mL. This  
303 extraction method resulted in a yield of approximately 30 mg peptide per 100 g  
304 cowpea seeds. The purity of the so obtained fraction was analysed by SDS-gel  
305 electrophoresis (Fig. 1). It is visible in Figure 1 (bottom) that the denatured extract  
306 shows an intense band at approximately 6 kDa, as well as 2 bands with lower

307 intensity at approximately 17 and 26 kDa. In contrast, the native sample has no band  
308 migrating at 6 kDa, but therefore a very broad band at 14.5 – 16 kDa, followed by 2  
309 bands at approximately 17 and 26 kDa with very low intensity. This shows that the  
310 main peptide of the extract, when suspended in sample buffer, occurs primarily in di-  
311 and trimers. After denaturation, the dimers were broken into the monomers visible on  
312 the gel. The 2 bands at higher molecular weight show that the purification of the  
313 cowpea peptide was only partial. However, it is also visible that the band migrating at  
314 6 kDa has the highest intensity and hence, contains the peptide primarily responsible  
315 behaviour of the extract during subsequent analysis. In addition, the UniProt  
316 database holds no information regarding a peptide from cowpea seeds with 17 or 26  
317 kDa that exhibits antimicrobial activity. Therefore, the results of the following sections  
318 are primarily attributed to the band migrating at 6 kDa.

319 The N-terminal protein sequencing of the 6 kDa peptide by Edman degradation was  
320 used to identify the extracted peptide based on the first 5 amino acid residues. The  
321 BLAST analysis (UniProt protein database) revealed that the only known peptide  
322 with the N-terminal starting sequence found here is the cowpea-thionin II. Table 1  
323 shows a comparison of antimicrobial peptides previously extracted from cowpea  
324 seeds with the first 5 amino acid residues of the peptide characterised in this study.  
325 Based on the BLAST analysis and comparison of the sequences the extracted  
326 peptide was identified as cowpea-thionin II (Cp-thionin II).

327 In order to obtain further information regarding the structure of the extracted protein,  
328 circular dichroism spectroscopy was performed and the results compared to the  
329 proposed 3D model of Cp-thionin II (Figure 2). The 3D model shows the peptide as  
330 one single subunit containing 3  $\beta$ -sheet and one  $\alpha$ -helical structure. Overall, the  
331 conformation of the peptide is stabilized by 4 disulfide bonds between cysteine  
332 residues, which are displayed in ball-and-stick form. This motif (CS $\alpha$  $\beta$ ) of Cp-thionin  
333 II is typical for native defensins (Almeida et al., 2002). In good correlation with the 3D  
334 model are the CD spectra of the extract. In both solvents (water and 20 mM SDS), a  
335 slightly positive peak was found at ~190 nm, followed by a crossover at ~200 nm and  
336 a minimum at ~210 nm. Furthermore, it is noteworthy that the positive peak is slightly  
337 bigger in water, while in SDS a much bigger negative peak was found. The shape of  
338 both graphs indicates good structured conformers, which is due to the 4 disulfide  
339 bonds stabilising the peptide. Interestingly, They & Arendt (2018) found similar

340 results in SDS but a much less structured conformation in water. The explanation  
341 behind is the lack of disulphide bonds in the synthetic linear analogue studied there.  
342 Furthermore, the peak minima indicate a propensity for helical conformations, as can  
343 be found in the 3D model. In addition, CD spectroscopy revealed a slightly higher  
344 percentage of helical conformation in SDS, combined with a slight reduction of  
345 random coils (data not shown). Overall, the differences between the conformations in  
346 the 2 solvents are marginal, as the disulphide bonds ensure a structured  
347 conformation in both solvents. In addition, the peptide solubility calculator "PepCalc"  
348 indicated good water solubility for Cp-thionin II, allowing potential use in a wide  
349 range of concentrations.

350 As shown by Franco et al. (2006), Cp-thionin II is a peptide consisting of 46 residues,  
351 including 8 cysteines arranged in a typical disulphide bond pattern (C1-C8 / C2-C5 /  
352 C3-C6 / C4-C7) (Lay et al., 2003). It was reported to belong to the super family of  $\gamma$ -  
353 thionins, also known as defensins. The molecular weight determined is with  
354 approximately 6 kDa very close to the previously reported 5.2 kDa for both natural  
355 (Franco et al., 2006) and synthetic peptide (Kraszewska et al., 2016; They & Arendt,  
356 2018). Due to the impurities in the extract and the limited accuracy of the SDS-PAGE  
357 it was not possible to determine the molecular weight more accurately. Additionally,  
358 the extraction method used by Franco et al. (2006) was found to result in monomers  
359 of the defensin only. In contrast, the extraction method applied here also resulted in  
360 di- and trimers. If this has an impact on the antimicrobial performance is unclear, as  
361 the inhibiting effect of residues is not fully understood yet. Franco et al., (2006)  
362 further demonstrated the antibacterial properties of the peptide. However, to the best  
363 of the authors` knowledge and according to the PhytAMP database, no studies  
364 regarding the antifungal performance of the peptide exist. In particular, regarding the  
365 potential application as food bio-preservative, the activity against common food  
366 spoilage fungi is of interest. Also the stability against environmental stress factors  
367 and the potential health risk due to consumption were investigated in this study.

368

### 369 3.2. Antifungal activity of the cowpea extract

370 The extracted peptide Cp-thionin II showed antifungal activity against the spores of  
371 *F. culmorum* but had no significant effect on the growth of *A. niger* and *P. expansum*

372 (Fig. 3). Substantial fungal growth inhibition of *F. culmorum* after 96 h was achieved  
373 for peptide concentrations of 25 µg/mL or higher. Based on the results of the  
374 microtiter plate assay, the IC<sub>50</sub> and MIC were determined as 40 µg/mL and 50 µg/mL,  
375 respectively (Fig. 3A). Peptide concentrations required to inhibit growth of *A. niger*  
376 and *P. expansum* were found to be much higher compared to *F. culmorum*. MIC-  
377 values against both fungi were above the highest concentration investigated here  
378 (500 µg/mL, Fig. 3B and 3C). However, spore germination and fungal growth of *A.*  
379 *niger* after 96 h were reduced for the highest peptide concentration tested (500  
380 µg/mL). Growth inhibition of *P. expansum* over 96 h was found to be not significant  
381 for all concentrations tested. This demonstrates the antifungal activity of Cp-thionin II  
382 against a major food spoilage fungus, despite the relatively low activity against *A.*  
383 *niger* and *P. expansum*. The inhibition of fungal spore germination was further  
384 assessed on PDA plates, which confirmed the results obtained from the microtiter  
385 plate assay (data not shown).

386 In agreement with previous studies, the partially purified cowpea extract showed  
387 substantial antifungal activity. The peptide was previously reported by Franco et al.  
388 (2006) and Kraszewska et al. (2016) for its antibacterial activity against both Gram-  
389 negative and Gram-positive strains. Furthermore, They & Arendt (2018)  
390 demonstrated the antifungal activity of a synthetic, linear analogue of Cp-thionin II  
391 against fungi belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium*. An  
392 explanation for the antifungal performance of the defensin is suggested to be in the  
393 structural similarity with the human beta-defensin 3 (Kraszewska et al., 2016).  
394 However, it was further reported by Kraszewska et al. (2016) that, apart from  
395 structural similarity, the overall net charge of the peptide has a major impact on the  
396 antimicrobial performance. Additionally, other researchers reported the importance of  
397 disulfide bounds, hydrophobicity and amphipaticity for the overall antimicrobial  
398 performance (Hollmann et al., 2016, Jenssen et al., 2006).

399 From an evolutionary point of view, the localisation of the peptide in the seeds  
400 suggests that the peptide is originally produced to protect the germinating seed from  
401 environmental pathogens, such as the fungi studied here (Franco et al., 2006).  
402 Hence, high antimicrobial activity against the tested pathogens is plausible, making  
403 the peptide potentially a promising candidate for bio-protection.



404 Compared to KT43C, the linear analogue of the peptide studied by They & Arendt  
405 (2018), the extract containing the natural peptide showed similar antifungal activity  
406 against *P. expansum* and *A. niger*. However, the MIC against *F. culmorum* was with  
407 50 µg/mL significantly higher than reported for the linear analogue (20 µg/mL). This  
408 discrepancy can be explained by the structural differences between synthetic and  
409 natural peptide. Firstly, as indicated by SDS electrophoresis, the natural peptide  
410 occurs not only as monomer but also as di- and trimer. As a consequence double the  
411 peptide concentration (in µg/mL) results in a similar amount of active molecules  
412 available. In addition, the peptide was only partially purified in this study and  
413 impurities in form of other peptides are visible on the SDS gel. As a result, the true  
414 concentration of Cp-thionin II would be lower than the protein concentration  
415 determined for the extract, explaining the higher concentrations required for fungal  
416 inhibition.

417

### 418 3.3. Effect of heat and cations on the antifungal activity of the cowpea extract

419 In order to gain further information regarding the properties of the cowpea extract  
420 and to estimate its potential for food applications the resistance against  
421 environmental stress factors, such as heat and ionic strength, was investigated.  
422 Therefore, the impact of these stress factors on the antifungal activity against *F.*  
423 *culmorum* was studied. Figure 4 shows the fungal growth curves over 96 h,  
424 incubated with cowpea extract, containing 50 µg/mL (MIC) of protein, after heat  
425 treatment (100°C, 15 min) and in presence of various cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>).  
426 No differences in spore germination inhibition were evident between the heated and  
427 unheated peptide solutions. Both treatments resulted in total inhibition of germination  
428 (10<sup>4</sup> conidia/mL) over 96 h in ½ PDB, while the controls (no peptide, heated and  
429 unheated) resulted in normal fungal development.

430 In the presence of cations, the extract lost most of its antifungal activity against *F.*  
431 *culmorum* when applied at its MIC (50 µg/mL). The monovalent cations Na<sup>+</sup> (100  
432 mM) and K<sup>+</sup> (50 mM) caused a complete loss of antifungal activity. Likewise, the  
433 divalent cations Ca<sup>2+</sup> or Mg<sup>2+</sup> (both at 5 mM) were also found to reduce the  
434 antifungal activity of Cp-thionin II substantially.

435 The antifungal activity remained unaffected by heat treatment, demonstrating the  
436 thermal resistance of Cp-thionin II. The heat resistance of AMPs, including synthetic  
437 Cp-thionin II and its linear analogue was previously demonstrated by Kraszewska et  
438 al. (2016) and They & Arendt (2018), respectively. Different factors influencing the  
439 thermal stability of a peptide are discussed. Cp-thionin II is a small peptide with  
440 flexible order when in aqueous solution (Franco et al., 2006). This attribute  
441 contributes to a higher thermal stability, as the molecules have more freedom of  
442 movement when heated.

443 In agreement with the studies of Kraszewska et al. (2016) and Vriens et al. (2014),  
444 the presence of cations reduced the antifungal activity of Cp-thionin II. The increased  
445 net charge of the peptide further disturbs the hydrophilic/hydrophobic balance of the  
446 peptide, compromising its antifungal activity. As a consequence of the coverage of  
447 the peptide surface, the protein interactions are reduced, inducing a lowered  
448 antimicrobial activity. Furthermore, as a result of the ions binding to the membrane, it  
449 becomes more difficult for the peptide to find receptors on the fungal membrane to  
450 bind and exert its antifungal activity (Wu et al., 2003). Finally, the presence of cations  
451 in the medium can change the overall configuration of the peptide, causing  
452 substantial deviation from its original structure and so explaining the loss in  
453 antifungal performance (Oard & Karki, 2006).

454 For possible future applications, the salt sensitivity of the extract can be a major  
455 drawback, in particular for preservation of food products. However, it has to be  
456 mentioned that the concentration of NaCl (100 mM = 5.8 g/100g), the most  
457 commonly found salt in food products, was higher than in most foods. An  
458 investigation regarding the level of salt tolerated by the peptide could reveal further  
459 details regarding possible food application. Furthermore, the good thermal stability  
460 found in this study would make the extract accessible for a large field of application,  
461 including processed foods.

462

#### 463 3.4. Mode of action

464 In order to identify some characteristics regarding the mode of action of Cp-thionin II  
465 in the extract, a membrane permeabilization assay was performed on *F. culmorum*  
466 hyphae. The permeabilization of the fungal cell membrane was achieved with

467 peptide concentrations of 12.5 µg/mL ( $1/4$  MIC) and higher (Fig. 5). The membrane  
468 permeabilization of the peptide was found in a dose-dependent manner, hence the  
469 increased fluorescence for higher concentrations. In correlation, substantial fungal  
470 growth inhibition was only achieved for peptide concentrations of 25 µg/mL or higher.  
471 Permeabilized hyphae had significant cytoplasmic granulation at higher  
472 concentrations. However, Cp-thionin II induced permeabilization appeared to be  
473 required for inhibition but was not sufficient to cause cell death, as membrane  
474 permeabilization appeared already at concentrations which were insufficient for  
475 fungal growth inhibition. It also has to be considered, that MIC-values for the  
476 inhibition of spore germination and the killing of hyphae can vary.

477 Another inhibitory mechanism is the increased generation of free radicals, usually  
478 from mitochondrial source, which can lead to an excessive level of reactive oxygen  
479 species (ROS), commonly known as oxidative stress. Plant defensins have been  
480 reported to induce an overproduction of ROS as part of their antifungal performance  
481 (Vriens et al., 2014). Substantial production of ROS appeared only at the highest  
482 peptide concentration (100 µg/mL, Figure 6), which is well above the concentration  
483 required for fungal growth inhibition. This suggests that the overproduction of ROS is  
484 not a primary mechanism in the antifungal performance of Cp-thionin II against *F.*  
485 *culmorum*. However, at high concentrations it may be a supportive mechanism  
486 further enhancing the activity of the peptide (Hayes et al., 2013).

487 It is evident that the inhibitory activity of Cp-thionin II against fungal hyphae involves  
488 the permeabilization of the hyphae, leading to leakage and granulation of the  
489 plasma. Interaction of the peptide with intracellular targets, inducing oxidative stress  
490 appears only as a supportive mechanism at high concentrations. However, variations  
491 regarding the mode of action against fungi of a different class, such as *A. niger* or *P.*  
492 *expansum*, are likely (El-Mounadi et al., 2016). Differences in morphology and  
493 cell/wall composition could change the involvement of ROS in the antifungal  
494 performance against different fungi. The results obtained are generally in good  
495 agreement with the findings of They & Arendt (2018) and the values obtained for the  
496 inhibition of fungal spores.

497

498 3.6. Haemolytic activity of the cowpea extract

499 For possible applications as food preservative, it is essential to combine high  
500 antimicrobial activity with consumer safety. Therefore, the cytolytic activity of the  
501 extract against red blood cells of sheep was determined in order to estimate its  
502 haemolytic activity against mammalian cells. The haemolytic activity was evaluated  
503 as the release of haemoglobin after treatment with the extract in a microtiter plate  
504 assay. All concentrations investigated (up to 200 µg/mL) did not lyse the red blood  
505 cells (data not shown).

506 It has been shown that the presence of cholesterol is the primary reason behind the  
507 reduced sensitivity of mammalian cells towards lysis by AMPs. In contrast, fungal  
508 cell membranes contain primarily ergosterol which is more easily targeted by AMPs  
509 (Mason et al., 2007). Furthermore, it has been reported by Thevissen et al. (2004)  
510 that the cytotoxicity of plant defensins is very low. However, it needs to be  
511 mentioned, that natural AMPs are often more cytotoxic compared to their linear  
512 analogues, where the disulfide bridges are removed (Liu et al., 2008). Despite the  
513 use of a natural peptide, containing disulfide bridges, a lack of haemolytic activity  
514 was found for Cp-thionin II, which is in good agreement with the results obtained by  
515 They & Arendt (2018). On the other hand, it has to be considered that the assay had  
516 to be carried out in PBS buffer, which contains different salts. As shown above, the  
517 extract lost its antifungal activity in presence of cations. Hence, a possible  
518 haemolytic activity could be lost as well. Consequently, further studies regarding the  
519 safety of the extract are required before food application can be considered.

520

### 521 3.7. Wheat grain spoilage protection

522 The application of the extract as food preservative was illustrated by its use on  
523 artificially infected (*F. culmorum*) wheat grains prior to storage. The ergosterol  
524 content was measured as a marker of fungal bio-mass, to evaluate the efficiency of  
525 the peptide treatment. The untreated controls showed a substantial increase in  
526 ergosterol during the storage period (Fig. 7). The 5% infected, untreated control, in  
527 particular, had an ergosterol content below detection (<LOD) in week 0 and  $42.3 \pm$   
528  $1.3$  ppm at week 6. In contrast, the acetic acid treatment resulted in total fungal  
529 inhibition with contents < LOD before and after storage. The use of the cowpea  
530 extract reduced the fungal development at all concentrations. The use of solutions

531 with peptide concentrations of 25 µg/mL and 50 µg/mL resulted in  $38.7 \pm 1.4$  ppm  
532 and  $25.0 \pm 0.7$  ppm of ergosterol, respectively. This indicates a significantly reduced  
533 fungal bio-mass compared to the untreated control. The highest concentration tested  
534 (100 µg/mL) resulted in the total inhibition of fungal development during storage  
535 (<LOD after week 6).

536 The extract successfully protected the wheat grains from fungal spoilage during  
537 incubation. The use of other natural AMPs to prevent food spoilage has been  
538 previously reported to be efficient against spoilage fungi (Lucera et al., 2012; Rai et  
539 al., 2016). However the concentrations required for total inhibition of fungal  
540 development were higher than the MIC against *F. culmorum* obtained *in vitro*. This  
541 can be explained by the differences in matrices between the grains and PDB. In  
542 particular, compounds present in the outer layers of the wheat kernels could  
543 compromise the antifungal performance. Furthermore, the fungus was allowed to  
544 proliferate over the kernels before the defensin was applied. This can result  
545 in an increase of the concentration required for inhibition. It also has to be  
546 considered, that the MIC values for inhibition of spore germination and the inhibition  
547 of hyphae can vary significantly. However, the extract containing Cp-thionin II  
548 demonstrated great potential as a bio-preservative during cereal storage, as fungal  
549 development was completely inhibited when applied at 100 µg peptide/mL. Based on  
550 this result further application of the aqueous peptide solution appear promising and  
551 should receive more research interest in the future.

552

#### 553 4. CONCLUSIONS

554 The cowpea extract containing Cp-thionin II showed antifungal activity against *F.*  
555 *culmorum*, a spoilage fungus commonly found on cereals and cereal products. The  
556 peptide showed typical characteristics of plant-derived defensins, including heat  
557 stability and sensitivity towards cations. Based on the characteristics of other plant  
558 defensins, high pH-stability can be assumed as well (Chan & Ng 2013). This  
559 provides a broad range of potential applications for the peptide. Furthermore, it was  
560 found to be safe towards sheep erythrocytes. These results are in good agreement  
561 with previous studies of the natural peptide (Franco et al., 2006) and synthetic linear  
562 analogue (Kraszewska et al., 2016; They & Arendt, 2018) of the cowpea-thionin II.

563 The application to protect fungal contaminated wheat grains during storage was  
564 successful, as fungal development could be completely inhibited using a solution  
565 with a peptide concentration of 100  $\mu\text{g}/\text{mL}$ . This result suggests that further  
566 applications of the aqueous peptide solution should be explored in future research.  
567 However, despite the highly promising results of this study extensive further research  
568 is required. Improvement of the extraction efficiency and purity of the extract are of  
569 high importance. Also a deeper understanding of the mode of action to increase the  
570 antifungal performance is required. The efficiency against other food pathogens  
571 would be of major interest to fully understand the potential and limits of Cp-thionin II.  
572 On the other hand, the interactions with other food constituents (e.g. proteolytic  
573 enzymes) need to be considered before application as bio-preservative.  
574 Furthermore, the safety of the defensin towards the consumers has to be  
575 investigated for both, acute and long term toxicities.

576 In conclusion, despite the need for further extensive investigation, the extract  
577 containing Cp-thionin II showed great potential for the possible application as bio-  
578 preservative. As such it could be a very important tool to reduce food waste and  
579 increase sustainability. Hence, it can become a valuable contribution to satisfy the  
580 global nutritional requirements of future generations.

581

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588

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## 744 FIGURE CAPTIONS

745 Figure 1: Top left: Chromatogram obtained from anion exchange column (HiTrap™  
746 DEAE FF) with un-retained fraction (eluted with equilibration buffer containing 0.1 M  
747 HCl and 0.15 M NaCl) transferred to cation exchange column (HiTrap™ SP HP) (top  
748 right). The retained proteins were eluted with a mixture of equilibration buffer without  
749 (A) and with (B) 1 M NaCl added, using a gradient from 0 – 100% B over 40 minutes.  
750 The fraction used for further investigation is marked with a black arrow. Bottom: SDS  
751 gel electrophoresis of the fraction obtained from cation exchange chromatography.  
752 1;3 – denatured sample, 2;4 – native sample, 5 – ladder.

753 Figure 2: A) Swiss model P84920 for the predicted 3D structure of the peptide  
754 cowpea-thionin II. The disulfide bonds between the cysteine residues are shown in  
755 ball-and-stick form. B) Circular dichroism (CD) spectrum obtained for the cowpea  
756 extract in water (broken line) and 20 mM SDS (solid line).

757 Figure 3: Fungal growth curves in presence of the cowpea extract, containing  
758 various concentrations of Cp-thionin II (in µg/mL) against A) *Fusarium culmorum*  
759 FST 4.05, B) *Aspergillus niger* FST 4.22, C) *Penicillium expansum* FST 4.21. All  
760 values for optical density (OD) are mean values of three independent replicates ±  
761 standard deviation.

762 Figure 4: Heat and cation sensitivity of antifungal activity of the cowpea extract (50  
763 µg/mL). Fungal growth curves of *Fusarium culmorum* after heat treatment (15  
764 minutes at 100°C) (A) and in presence of various salts (B – E) are mean values of  
765 three independent replicates ± standard deviation.

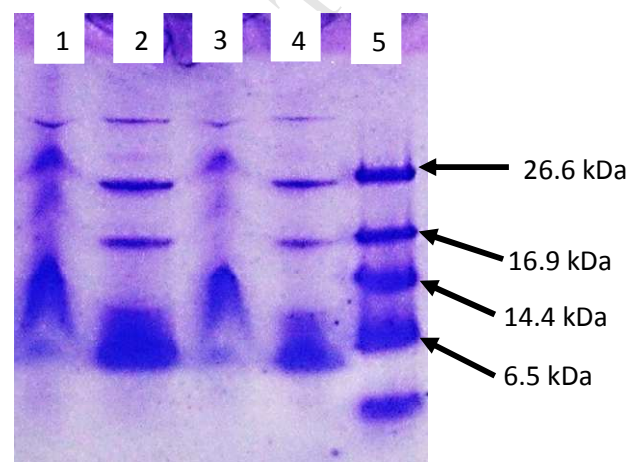
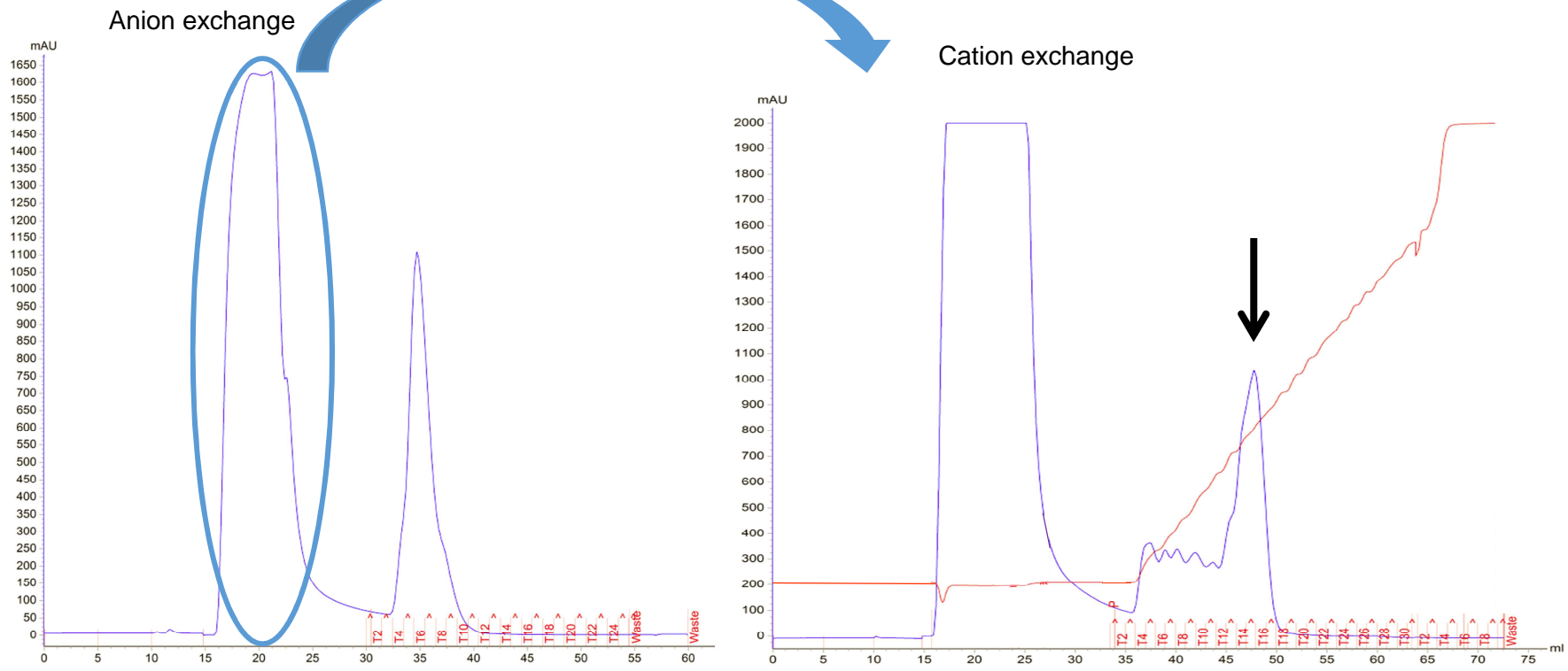
766 Figure 5: Membrane permeabilization assay. Top: After 18 hours of growth, *F.*  
767 *culmorum* hyphae were treated for 2 hours with cowpea extract (0; 12.5; 25; 50 and  
768 100 µg peptide/mL). Permeabilization of the fungal membrane was determined by  
769 fluorescence with propidium iodid (excitation 488 nm, emission 538 nm) and  
770 correlated to the percentage of inhibition. Values are the mean of three independent  
771 replicates. Bottom: *F. culmorum* hyphae were observed with a confocal laser  
772 scanning microscope, with an excitation wavelength 460 – 490 nm. Left: no peptide;  
773 middle: 25 µg/mL; right: 100 µg/mL.

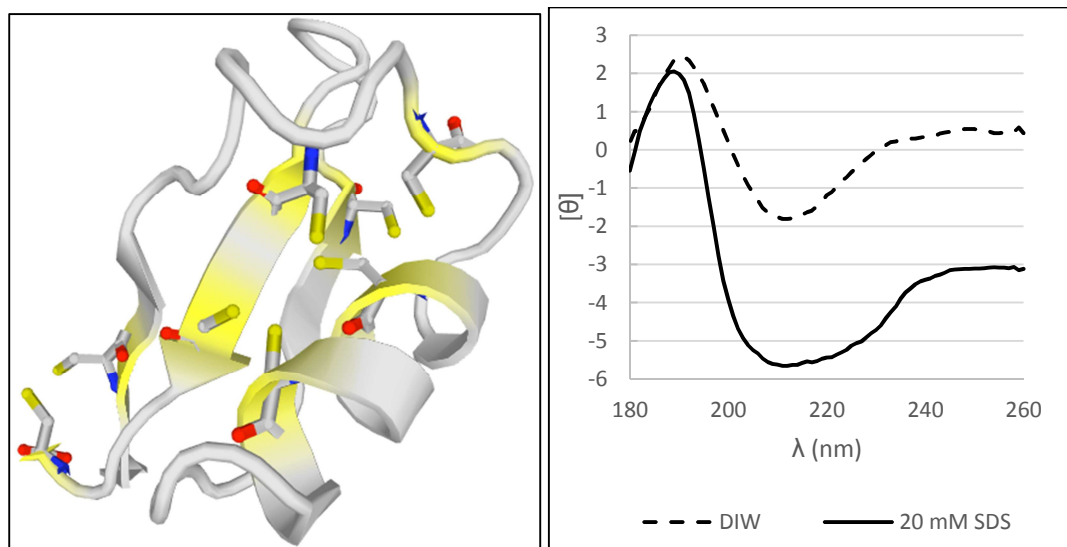
774 Figure 6: Detection of reactive oxygen species (ROS) production. Top: After 24  
775 hours of growth, *F. culmorum* hyphae were treated for 12 hours with cowpea extract  
776 (0; 12.5; 25; 50 and 100  $\mu\text{g}$  peptide/mL). Production of ROS was determined by  
777 fluorescence of DHR 123 (excitation 488 nm, emission 538 nm) and correlated to the  
778 percentage of fungal growth inhibition. Each value is the mean of three independent  
779 replicates. Bottom: *F. culmorum* hyphae were observed using a confocal laser  
780 scanning microscope, with excitation wavelength 460 - 490 nm. Left: no peptide;  
781 right: 100  $\mu\text{g}/\text{mL}$ .

782 Figure 7: Ergosterol contents of the grain samples before (week 0, stripes) and after  
783 storage (week 6, black) with standard deviation. The limit of detection (LOD) was  
784 determined as 0.75 ppm and ergosterol contents determined to <LOD are shown as  
785 "0 ppm" in the chart.

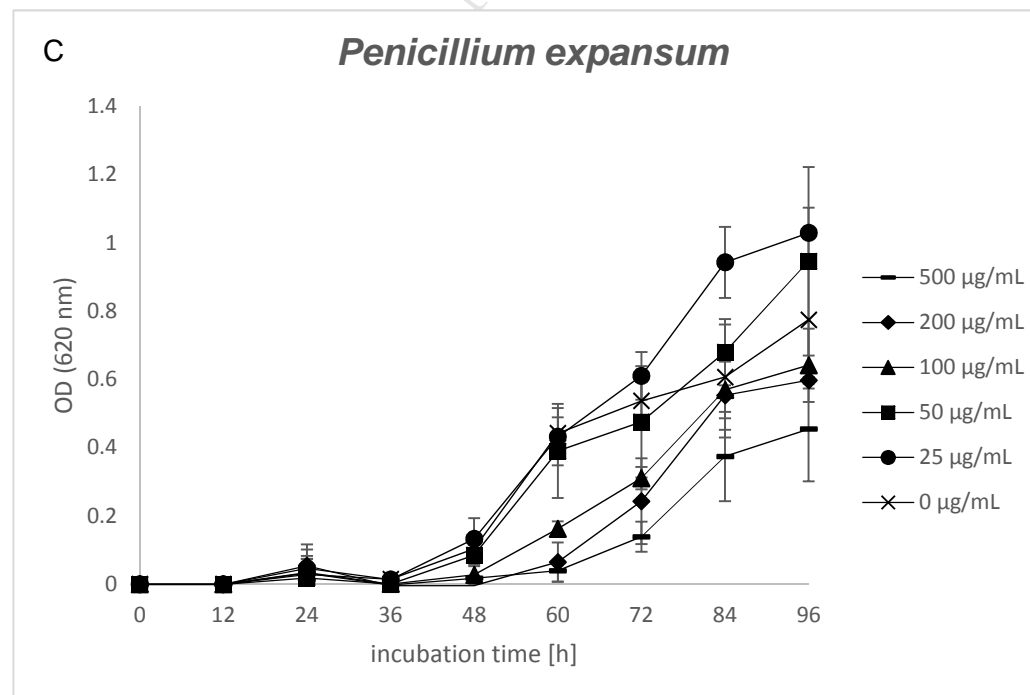
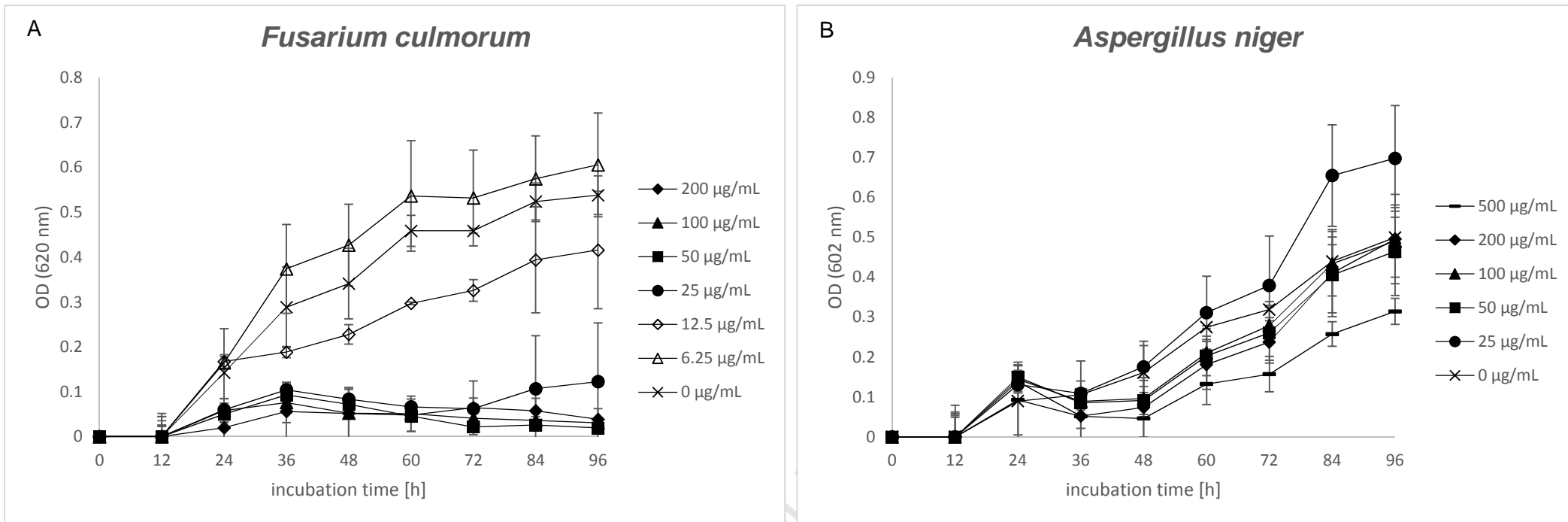
**Table 1: Characteristics of the peptide extracted from cowpea seeds compared with related antimicrobial peptides (amino acids containing a disulfide bond are underlined).**

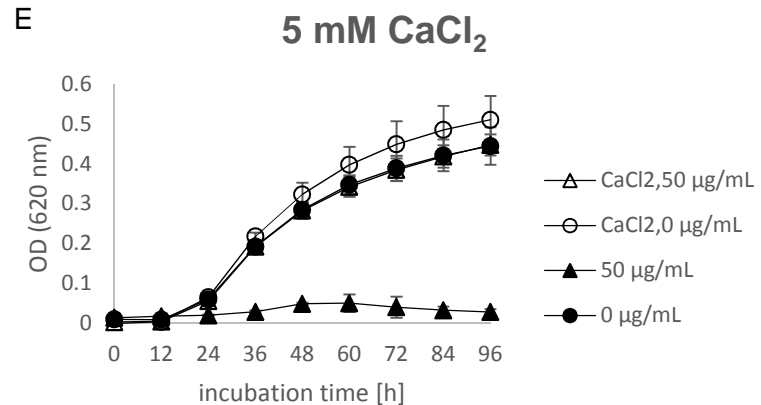
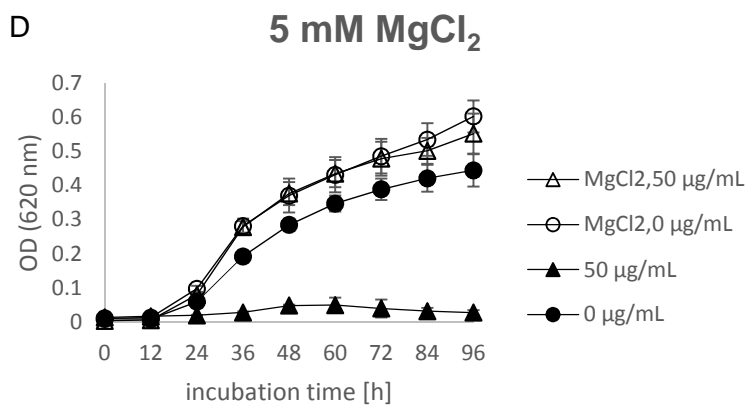
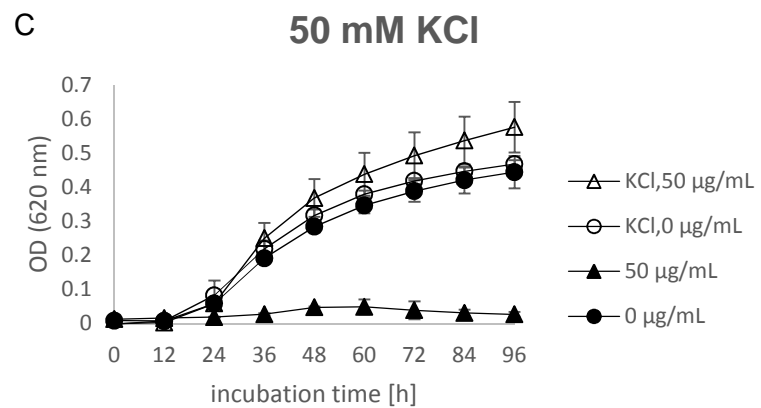
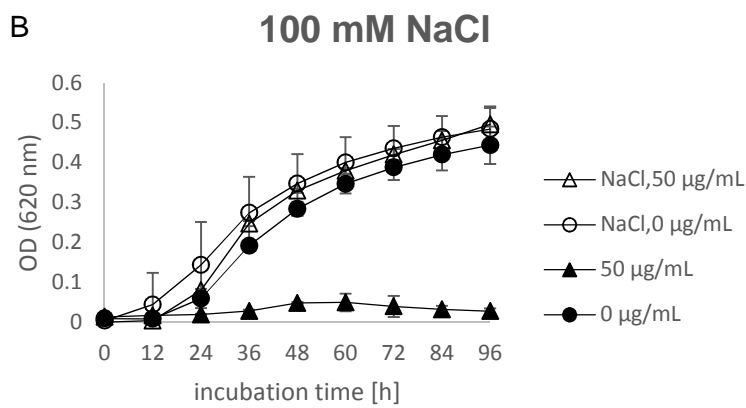
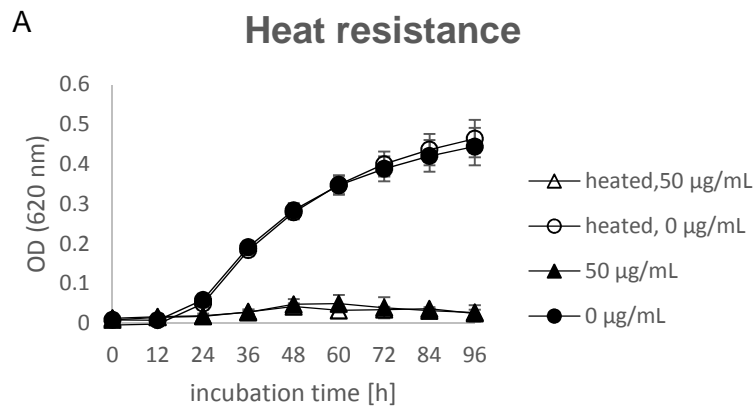
Name	Source	Sequence	Activity	Function	Reference
peptide extracted here	<i>Vigna unguiculata</i>	KTCMT-	<i>F. cumorum</i> <i>A. niger</i> <i>P. expansum</i>		this study
Cp-thionin II	<i>Vigna unguiculata</i>	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMG GKCQGITRRCYCLLNC	Gram-positive <i>S. aureus</i> Gram-negative <i>E. coli</i> , <i>P. syringae</i>	$\gamma$ -tionin	Franco et al. (2006)
Cp-thionin	<i>Vigna unguiculata</i>	RVCESQSGFKGACTGDHNCALVCRNEGFS GGNCRGFRRRCFCTLKC	unknown	Trypsin inhibitor	Melo et al. (2002)
Linear peptide KT43C	synthetic peptide	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMG GKCQGITRRCYCLLNC	<i>F. cumorum</i> <i>A. niger</i> <i>P. expansum</i>		Thery & Arendt (2018)

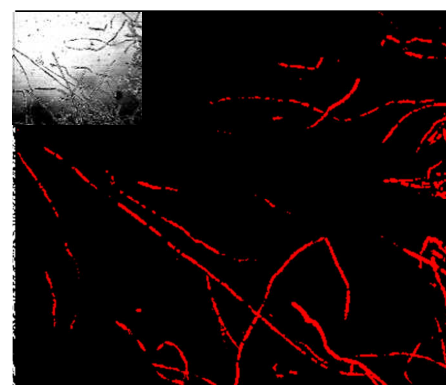
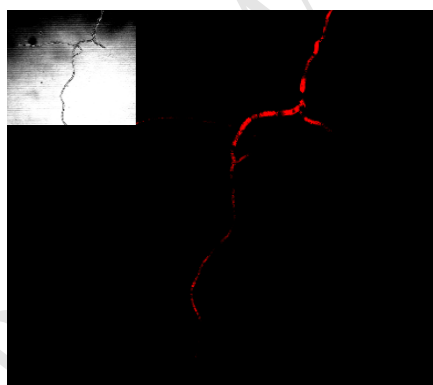
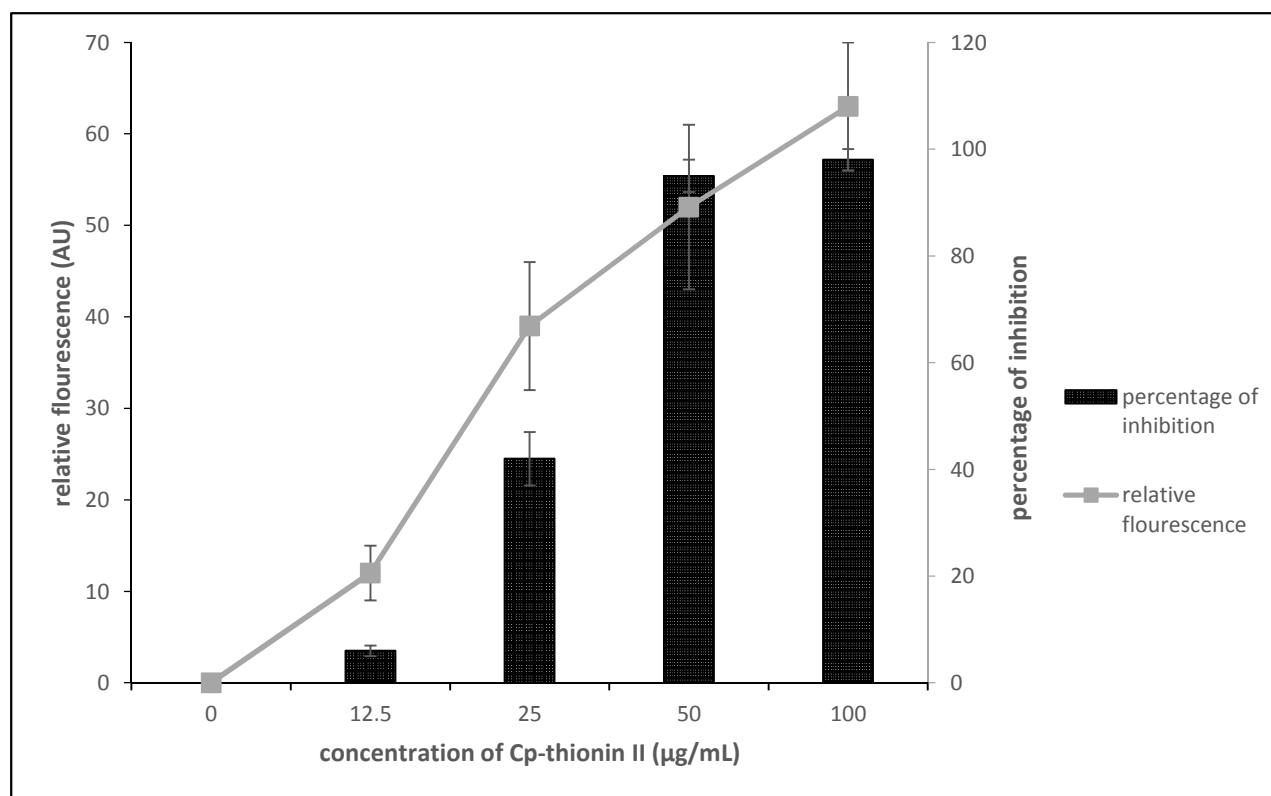


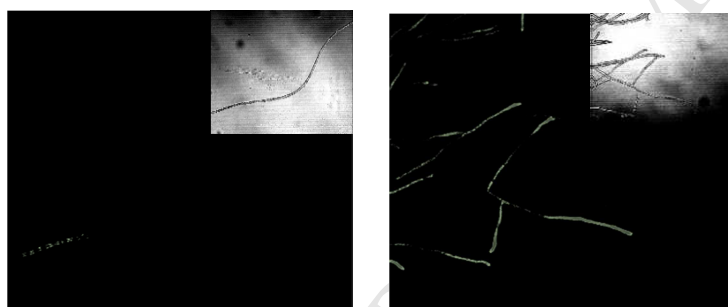
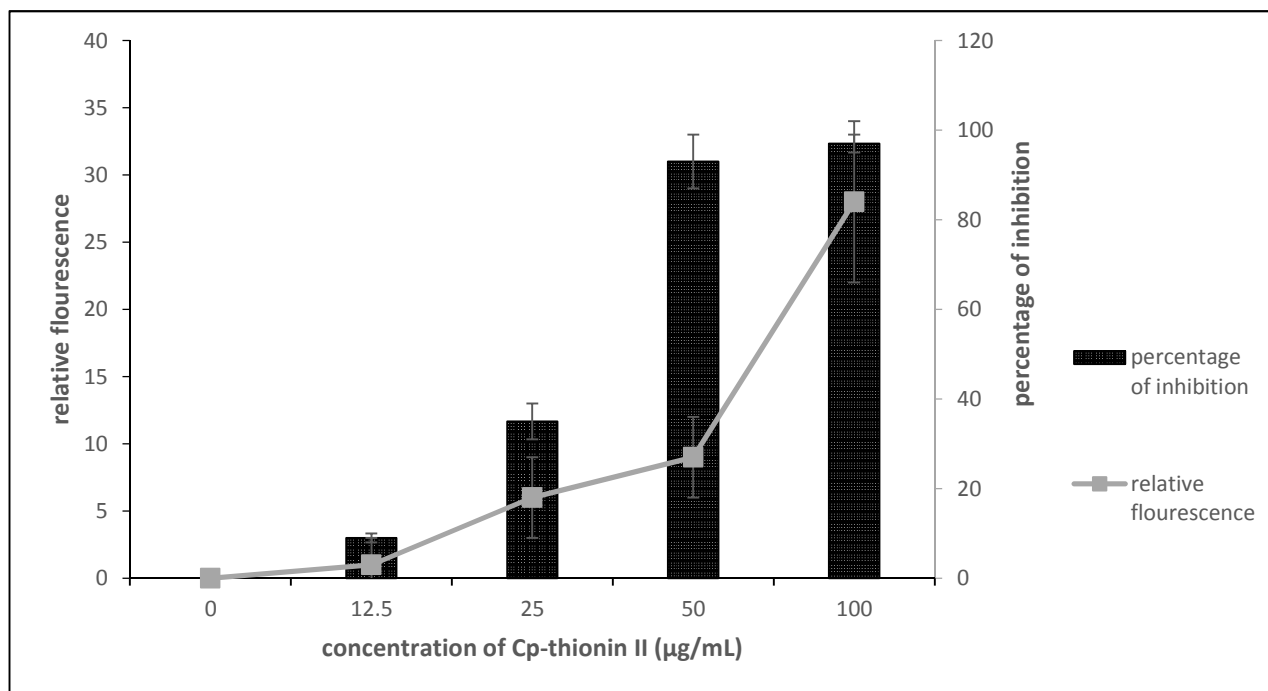


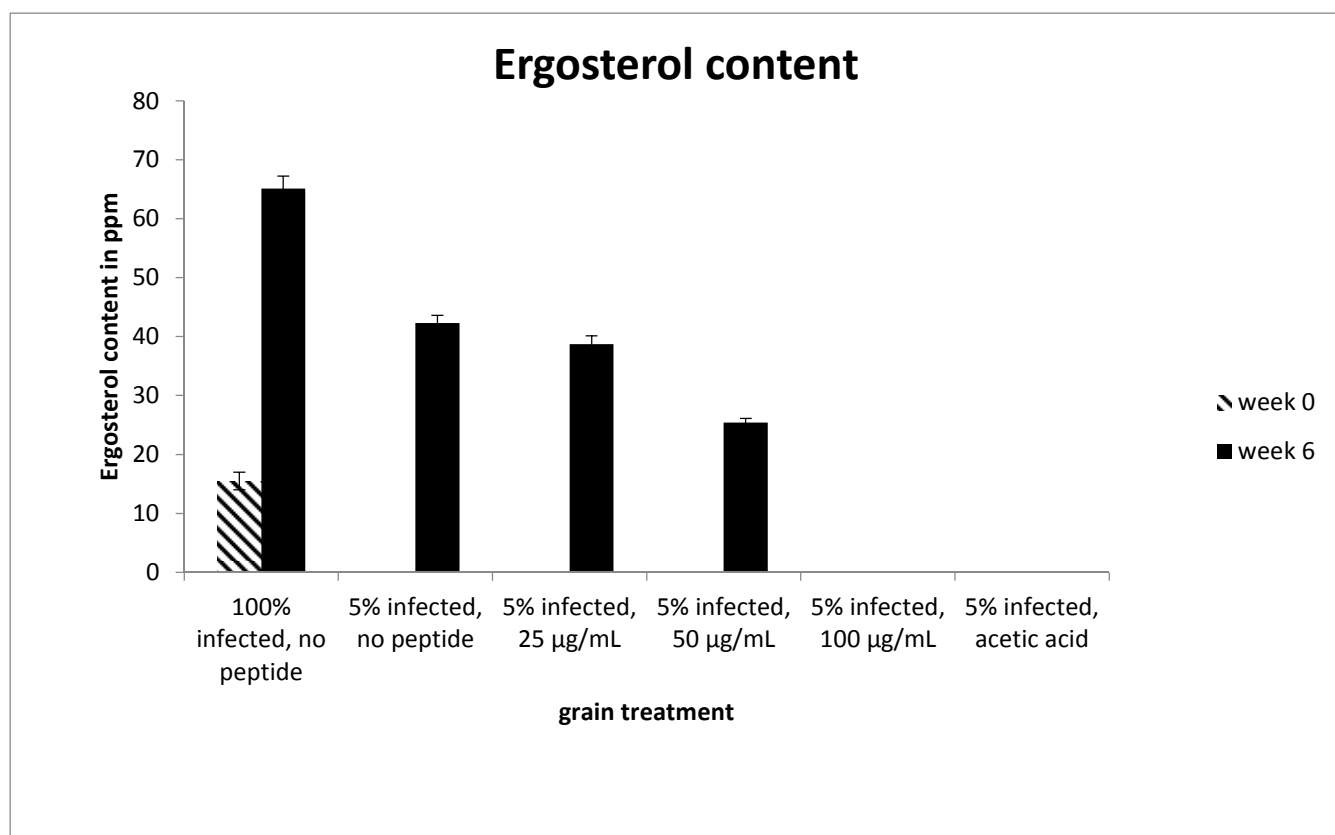












**Highlights:**

- Cowpea-thionin II expressed antifungal activity against *F. culmorum*
- Antifungal activity was lost due to cations but resistant against heat
- Membrane permeabilization was found to contribute to fungal inhibition
- Extract was applied to protect cereal spoilage during storage