Inhibition of agronomically relevant fungal phytopathogens by tyrocidines, cyclic antimicrobial peptides isolated from *Bacillus aneurinolyticus*

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The tyrocidines, a complex of analogous cyclic decapeptides produced by *Bacillus aneurinolyticus*, exhibited noteworthy activity against a range of phytopathogenic fungi, including *Fusarium verticillioides*, *Fusarium solani* and *Botrytis cinerea*. The activity of the tyrocidine peptide complex (Trc mixture) and purified tyrocidines exhibited minimum inhibition concentrations below 13 μg ml⁻¹ (~10 μM) and was significantly more potent than that of the commercial imidazole fungicide, bifonazole. Although the tyrocidines’ activity was negatively influenced by the presence of Ca²⁺, it remained unaffected by the presence of Mg²⁺, Na⁺ and K⁺. Microscopic analysis revealed significant impact on the morphology of *F. solani* and *Bot. cinerea* including retarded germination and hyperbranching of hyphae. Studies with membrane-impermeable dyes, SYTOX green and propidium iodide suggested that the main mode of action of tyrocidines involves the disruption of fungal membrane integrity. Because of the tyrocidines’ broad spectrum and potent antifungal activity, possible multiple targets reducing the risk of overt resistance and general salt tolerance, they are promising candidates that warrant further investigation as bio-fungicides.

INTRODUCTION

With approximately one billion undernourished individuals on earth and a global population of seven billion people that is still growing, global food security is under threat. Microbial pathogens, including fungi, augment this predicament by causing a loss of up to 16% (Chakraborty & Newton, 2011) in the annual global food production. Furthermore, some fungal species secrete mycotoxins, which can be toxic, as well as mutagenic with long-term exposure, when ingested by humans and animals (Bennett & Klich, 2003; Smith et al., 1995).

Conventional chemical fungicides are used to control fungal pathogens (Knight et al., 1997; Muñoz et al., 2007; Narayanasamy, 2005; Spadaro & Gullino, 2004). However, there is increasing evidence of their detrimental effect on the environment and human health (Janisiewicz & Korsten, 2002; Klich et al., 1994). In addition, a number of fungal strains have developed resistance against conventional fungicides (Diáz et al., 2002; Spadaro & Gullino, 2004). Therefore, alternative, eco-friendly fungicides with a low risk for inducing resistance need to be developed (Janisiewicz & Korsten, 2002; Narayanasamy, 2005).

Antimicrobial peptides (AMPs), with their broad range of activity and rapid antimicrobial action (Hancock & Sahl, 2006), can be considered as potential alternatives to fungicides (Keymanesh et al., 2009; Muñoz et al., 2007; Park et al., 2009; Rydlo et al., 2006; Zasloff, 2002). Unfortunately, the antimicrobial activity of AMPs has been shown to be negatively influenced by the presence of cations such as Ca²⁺, Mg²⁺, K⁺ and Na⁺ (Hancock & Sahl, 2006; Lehrer et al., 1988; Park et al., 2009) that are prevalent in the biosphere, and agricultural and food products. Consequently, to be considered useful for agricultural application, an AMP must be active in the presence of these cations at biologically relevant concentrations.

The bacterium *Bacillus aneurinolyticus* produces a group of analogous peptides, namely the tyrocidines, as part of its secondary metabolite complex tyrothricin (Dubos, 1939; Tang et al., 1992). The tyrocidines are part of a cyclic decapetide family with conserved sequences forming β-sheets (Kuo & Gibbons, 1979; Ruttenberg et al., 1965, 1966), only varying in one to three amino acid residues.

**Abbreviations:** AMP, antimicrobial peptide; GS, gramicidin S; ITS, internal transcribed spacer; Trc mixture, tyrocidine peptide complex; UPLC, ultraperformance liquid chromatography.

Supplementary methods, eight supplementary figures and five supplementary tables are available with the online version of this paper.

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[refer to Tang et al. (1992) for the primary structures of 28 natural tyrocidines]. Following their discovery, it was illustrated that tyrocidines have significant antibacterial (Dubos & Hotchkiss, 1941; Leussa & Rautenbach, 2014; Spathelf & Rautenbach, 2009) and anti-malarial (Rautenbach et al., 2007) activity. However, to date, only a few studies have been conducted on the antifungal activity of tyrothricin (the tyrocidine–gramicidin metabolite complex of Bac. aeneurinolyticus) and tyrocidines. Tyrothricin was shown to be active against Candida albicans (Kretschmar et al., 1996) and one report showed the activity of tyrocidines against Neurospora crassa (Mach & Slayman, 1966). Our group recently reported the synergistic activity of the tyrocidines with amphotericin B and caspofungin against Can. albicans biofilms (Troskie et al., 2014). The potential of tyrocidines to inhibit fungal phytopathogens and subsequently to act as a potential bio-fungicide thus remains unexplored. The significant activity illustrated by the tyrocidines against Fusarium solani Collection (ATCC) (Manassas, VA, USA).

Purification of the tyrocidines from commercial tyrothricin. The tyrocidine peptide complex (Trc mixture) was isolated from a potential bio-fungicide thus remains unexplored. The significant activity illustrated by the tyrocidines against Fusarium solani Collection (ATCC) (Manassas, VA, USA).

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Identification of isolated fungi. Fungal strains were identified by growing the strains on malt extract agar (Merck, Waddeville, SA) and Czapek yeast agar (30 g sucrose, 5 g yeast extract, 1 g K$_2$HPO$_4$, 0.3 g NaNO$_3$, 0.05g KCl, 50 mg MgSO$_4$, 1 mg FeSO$_4$, 1 mg ZnSO$_4$, 0.5 mg CuSO$_4$, 15 g agar in 1.0 L; reagents from Merck, Waddeville, SA) for 7 days. On the basis of conidiophore morphology, strains were placed into genera. For species identification, DNA was extracted from strains after growing on PDA for seven days, using the ZR Fungal/Bacterial DNA kit (Zymo Research). The ITS1–5.8S–ITS2 rDNA and $\beta$-tubulin gene region was amplified using PCR. Reaction mixtures (25 $\mu$L) consisted of 5 $\mu$L Kapa Readymix (Kapa Biosystems) and 0.25 $\mu$M primers ITS1 and ITS4 (White et al., 1990) for the internal transcribed spacer ITS region and primers Bt2a and Bt2b (Glass & Donaldson, 1995) for the $\beta$-tubulin gene. PCR products were sequenced using a Big Dye terminator cycle sequencing premix (Applied Biosystems) and sequenced with an ABI PRISM 310 automatic sequencer. Sequence contigs were aligned in CLUSTAL X (Thompson et al., 1997) and manually adjusted in Se-Al (Rambaut, 2007) using a dataset of Visagie & Jacobs (2012). The Trichoderma strain was identified using the Trichoderma database (http://nt.ars-grin.gov/taxadesccriptions/keys/TrichodermaIndex.cfm), as well as the online key to species of Trichoderma (TrichoKey) (Druzhinina et al., 2005).

Microdilution assays in broth media. The activity of the Trc mixture against F. solani, F. oxysporum, F. verticillioides, Cyl. liriodendri, Botrytis cinerea, Penicillium digitatum, Penicillium glabrum, Penicillium expansum, Talaromyces mineolutesus, Talaromyces ramulosus and Aspergillus fumigatis. The tyrocidines’ effect on fungal membrane integrity and the influence of the cations Ca$^{2+}$, Mg$^{2+}$, Na$^+$ and K$^+$ on their antifungal activity were also investigated.

METHODS

Purification of the tyrocidines from commercial tyrothricin. The tyrocidine peptide complex (Trc mixture) was isolated from a commercial tyrothricin complex from Bac. aeneurinolyticus (Sigma) using a modified organic extraction method (Hotchkiss & Dubos, 1941). The dry tyrothricin powder was washed three times with ether/acetone (1:1, v/v; Merck, Waddeville, SA). The precipitate, which was collected via centrifugation and dried under vacuum, contained only the tyrocidines, as verified by ES-MS and ultra-performance liquid chromatography (UPLC)-MS and analytical reverse-phase HPLC [HPLC method described by Rautenbach et al. (2007)]. The five major tyrocidines, TrcC$_6$, C$_5$, B$_1$, B$_2$ and A$_1$ (Table 1) were purified from tyrocidin mixtures as described by Rautenbach et al. (2007). Peptides not purified from the commercial tyrothricin complex, TrcA, phenycidine A (PhcA) and tryptocidine C (TpcC), were extracted from the culture broth of Bacillus parabrevis 8185 (Leussa, 2014) (refer to Table S1). Fractions were analysed with TOF-ES-MS, UPLC-MS and preparative HPLC for purity and identity (refer to Table S2, Figs S1, S2 and S3). Also refer to Eyéghi-Bickong (2011) and Spathelf (2010) for more detail on the analysis and purification of the tyrocidines.

Growth and harvesting of fungi. A. fumigatis ATCC 204305 and F. oxysporum ATCC 10913 were from the American Type Culture Collection (ATCC) (Manassas, VA, USA). F. solani STEU 6188, F. verticillioides CK11730, Bot. cinerea CK11731 and Cyl. liriodendri STEU 6170 were provided by the Department Plant Pathology, University of Stellenbosch. Pen. expansum CK11733 and Pen. digitatum CK11734 were isolated from infected tangerines (July 2012, Stellenbosch, South Africa); Tal. mineolutesus CK11736 and Tal. ramulosus CK11735 were isolated from infected nectaries (March 2012, Stellenbosch, South Africa); Pen. glabrum CK11732 and Trichoderma atroviride CK11729 were isolated from wood pellets used in the grape industry (February 2012, Wellington, South Africa). The isolated fungal species and strains were subsequently identified by us and placed in the CJK culture collection at the Department of Microbiology, University of Stellenbosch. All the fungi in this study, excepting Bot. cinerea, were grown at 25 °C on PDA (potato dextrose agarose; Sigma-Aldrich, St Louis, USA) until sporulation (~2 weeks). Spores were harvested with 1 ml 0.1% Tween-analytical water. Bot. cinerea was grown in culture plates on sterile strawberry halves at 23–25 °C until sporulation (~3 weeks). Bot. cinerea spores were harvested dry using vacuum. Subsequent to harvesting, spores were counted using a counting chamber. Standard practices to ensure sterility were followed.

Identification of isolated fungi. Fungal strains were identified by growing the strains on malt extract agar (Merck, Waddeville, SA) and Czapek yeast agar (30 g sucrose, 5 g yeast extract, 1 g K$_2$HPO$_4$, 0.3 g NaNO$_3$, 0.05g KCl, 50 mg MgSO$_4$, 1 mg FeSO$_4$, 1 mg ZnSO$_4$, 0.5 mg CuSO$_4$, 15 g agar in 1.0 L; reagents from Merck, Waddeville, SA) for 7 days. On the basis of conidiophore morphology, strains were placed into genera. For species identification, DNA was extracted from strains after growing on PDA for seven days, using the ZR Fungal/Bacterial DNA kit (Zymo Research). The ITS1–5.8S–ITS2 rDNA and $\beta$-tubulin gene region was amplified using PCR. Reaction mixtures (25 $\mu$L) consisted of 5 $\mu$L Kapa ReadyMix (Kapa Biosystems) and 0.25 $\mu$M primers ITS1 and ITS4 (White et al., 1990) for the internal transcribed spacer ITS region and primers Bt2a and Bt2b (Glass & Donaldson, 1995) for the $\beta$-tubulin gene. PCR products were sequenced using a Big Dye terminator cycle sequencing premix (Applied Biosystems) and sequenced with an ABI PRISM 310 automatic sequencer. Sequence contigs were aligned in CLUSTAL X (Thompson et al., 1997) and manually adjusted in Se-Al (Rambaut, 2007) using a dataset of Visagie & Jacobs (2012). The Trichoderma strain was identified using the Trichoderma database (http://nt.ars-grin.gov/taxadesccriptions/keys/TrichodermaIndex.cfm), as well as the online key to species of Trichoderma (TrichoKey) (Druzhinina et al., 2005).

Microdilution assays in broth media. The activity of the Trc mixture against F. solani, F. oxysporum, F. verticillioides, Cyl. liriodendri, Bot. cinerea, Pen. digitatum, Pen. glabrum, Pen. expansum, Tal. mineolutesus, Tal. ramulosus and Asp. fumigatus. The tyrocidines’ effect on fungal membrane integrity and the influence of the cations Ca$^{2+}$, Mg$^{2+}$, Na$^+$ and K$^+$ on their antifungal activity were also investigated.
Table 1. Summary of tyrocidines present in the Trc mixture extracted from the commercial tyrothricin mixture, and the purified peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Abbreviation</th>
<th>Sequence*</th>
<th>Detected monoisotopic M, (theoretical M0)†</th>
<th>Abundance (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenycinidine A§</td>
<td>PhcA</td>
<td>Cyclo-(VOLPFINQF)</td>
<td>1253.6625 (1253.6600)</td>
<td>Not detected</td>
</tr>
<tr>
<td>Tryptocidine A</td>
<td>TpcA</td>
<td>Cyclo-(VOLPFINQW)</td>
<td>1292.6549 (1292.6706)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tyrocidine A</td>
<td>TrcA</td>
<td>Cyclo-(VOLPFINQY)</td>
<td>1269.6515 (1269.6538)</td>
<td>17.6</td>
</tr>
<tr>
<td>Tyrocidine A1</td>
<td>TrcA1</td>
<td>Cyclo-(VKLPFNYQ)</td>
<td>1283.6694 (1283.6703)</td>
<td>13.2</td>
</tr>
<tr>
<td>Tryptocidine B</td>
<td>TpcB</td>
<td>Cyclo-(VOLPWNQW)</td>
<td>1331.6676 (1331.6815)</td>
<td>3.1</td>
</tr>
<tr>
<td>Tyrocidine B</td>
<td>TrcB</td>
<td>Cyclo-(VOLPWNQY)</td>
<td>1308.6632 (1308.6655)</td>
<td>21.3</td>
</tr>
<tr>
<td>Tyrocidine B1</td>
<td>TrcB1</td>
<td>Cyclo-(VKLPPWNQY)</td>
<td>1322.6730 (1322.6812)</td>
<td>15.7</td>
</tr>
<tr>
<td>Tyrocidine C</td>
<td>TrcC</td>
<td>Cyclo-(VOLPWWNQY)</td>
<td>1347.6633 (1347.6764)</td>
<td>14.3</td>
</tr>
<tr>
<td>Tyrocidine C1</td>
<td>TrcC1</td>
<td>Cyclo-(VKLPPWWNQY)</td>
<td>1361.6768 (1361.6921)</td>
<td>10.3</td>
</tr>
<tr>
<td>Tryptocidine C</td>
<td>TpcC</td>
<td>Cyclo-(VOLPWWNQW)</td>
<td>1370.6866 (1370.6924)</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Conventional one-letter abbreviations are used for amino acids, except that O is used for ornithine. Lower case one-letter abbreviations indicate D-amino acid residues. Sequence data were obtained from Tang et al. (1992) and identities were confirmed by our group (Eyéghé-Bickong, 2011; Spathelef, 2010; Vosloo et al., 2013).
†Detected monoisotopic M0 of peptides determined from purified peptides (except for TpcA and TpcB determined in mixture) using high-resolution TOF-ES-MS. These values correlated very well with those in the tyrocidine mixture (refer to Fig. S3 and Table S1).
‡Abundances calculated from the peak areas determined with UPLC linked to high-resolution ES-MS analysis of the Trc mixture and purified peptides (refer to Fig. S2 D and Table S2).
§Named by Vosloo et al. (2013).

1 mg ml\(^{-1}\) and doubling dilution series were made using half-strength PDB. Subsequent to peptide/fungicide addition, the microtitre plates were incubated at 23–25 °C for 48 h. Light dispersion of each well was determined spectrophotometrically at 595 nm using a Bio-Rad microtitre plate reader.

*Microdilution assays on PDA. Micro-gel dilution assays were used to determine the activity of the Trc mixture on agar medium against *F. solani* and *Bot. cinerea*. The method as described by Troskie et al. (2012) was followed. In brief, 96-well microtitre plates containing 70 µl PDA per well were prepared using the reverse pipetting method (Troskie et al., 2012). Of a 1 × 10\(^5\) spore/half-strength PDB solution (PDB/water, 1 : 1, v/v), 20 µl was added to each well. The Trc mixture was prepared as described for the broth assays. Following peptide addition, the microtitre plates were incubated at 23–25 °C for 48 h. The plates were subsequently stained with Coomassie blue (0.063 % Coomassie blue R-250 (Sigma, St Louis, USA), 50 % methanol, 10 % acetic acid; Merck, Wadeville, SA) for 1 h and afterwards destained (12.5 % 2-propanol, 10 % acetic acid; Merck, Wadeville, SA) for 72 h. Results were captured with a Canon 600D camera. Image results were analysed as gels in UN-SCAN-IT gel (version 6.1; Silk Scientific) and the mean pixel count for each well was used to calculate growth inhibition (Troskie et al., 2012).

*Light microscopy of peptide-challenged spores and hyphae.* For the fungal hyphae studies, half-strength PDB suspensions containing spores of either *Bot. cinerea* or *F. solani* were distributed into the wells of sterile 96-well microtitre plates so that each well held a volume of 90 µl and 2000 spores. The plates were incubated for 16 h at 23–25 °C. The tyrocidines were dissolved and diluted in 15 % ethanol using polypropylene plates. The final Trc mixture concentrations were 9 µg ml\(^{-1}\) (for *F. solani*) and 6 µg ml\(^{-1}\) (for *Bot. cinerea*). TrcA was added to *Bot. cinerea* spores at a concentration of 6.2 µM. Controls received 10 µl of a 15 % ethanol solution (final concentration of 1.5 %). Each well had an end volume of 100 µl. The microscope studies with *Bot. cinerea* and *F. solani* spores were performed using the procedures described for the hyphae, leaving out the 16 h incubation step. Subsequent to the addition of the Trc mixture, events were monitored using a Leica light microscope coupled to a DCM510 digital camera 12 h and 24 h after peptide addition at × 25 magnification.

*Fluorescence microscopy.* Half-strength PDB containing *Bot. cinerea* spores (2.5 × 10\(^4\) spores ml\(^{-1}\)) was added to sterile microtitre plates in order that each well have a volume of 90 µl. The Trc mixture (10 µl), dissolved in 25 % ethanol and diluted in analytical grade water, was subsequently added in order that the final peptide concentration be 3 µg ml\(^{-1}\) (~2.3 µM) and the final ethanol concentration less than 0.3 %. The growth control had a final ethanol concentration of 0.3 %. After incubation at 25 °C for 2 h (spores) or 24 h (hyphae), the cultures were incubated with 0.1 µg propidium iodide ml\(^{-1}\) (Sigma, St Louis, USA) for 15 min prior to image acquisition. Images were captured with a Nikon Eclipse E600 fluorescence microscope, using UV filters, fitted with a ×100 Apochromat objective and a Media Cybernetics CoolSNAP-Pro monochrome cooled CCD camera.

*F. solani* hyphae were grown in half-strength PDB from a starting concentration of 2.0 × 10\(^4\) spores ml\(^{-1}\) for 16 h at 25 °C. Hyphae were incubated at 25 °C for 1 h with 25 µM TrcA, TrcB or TrcC, or 25 µg Trc mixture ml\(^{-1}\), and 1 % Triton X-100 served as lytic control. Subsequent to the 1 h incubation step, the hyphae were incubated for 10 min with 0.5 µM SYTOX green (Lonza, Walkersville, MD, USA; Van der Weerden et al., 2008). SYTOX green uptake was captured with a Carl Zeiss confocal LSM 780 Elyra S1. The laser was set at 488 nm and the filter at 500–676 nm. The collected data were processed using ZEN 2011 software.

*Pre-incubation of peptides with chloride salts.* Procedures were followed as described for the broth microdilution assays with a few
adjustments. Briefly, the Trc mixture, TrcA, TrcB and TrcC were pre-incubated for 60 min in NaCl, KCl, CaCl$_2$ or MgCl$_2$ (salts from Merck, Wadeville, SA)20 at concentrations ranging from 2.0 to 100 mM. Each well received 90 µl of the spore-broth suspension followed by the addition of 10 µl tyrocidine–cation suspension so that the wells had a final concentration of 6 µg ml$^{-1}$ (Trc mixture) or 5 µM (TrcA, B and C) for Bot. cinerea and 18 µg ml$^{-1}$ (Trc mixture) or 12 µM (TrcA, B and C) for F. solani. The 90 µl growth controls received 10 µl of 15 % ethanol modified with chloride salt at the same concentration that was used for the peptide challenge in the assay. The activity control received Trc mixture or pure peptides without the chloride salts. Sterility control consisted of half-strength PDB and final ethanol and chloride concentration of, respectively, 1.5 % and 10 mM. After the addition of tyrocidine–salt mixture, the microtitre plates were incubated at 25 °C for 48 h. Light dispersion of each well was determined using a Bio-Rad microtitre plate reader set at 595 nm.

**Data analysis.** In order to calculate the percentage growth inhibition, the light dispersion at each concentration (mean pixel count for PDA plates) was used as described by Rautenbach et al. (2006). GraphPad Prism 4.03 (GraphPad Software) was used to plot the dose–response curves. Nonlinear regression and sigmoidal curves (with a slope default setting at <7) were fitted for dose–response analysis (Rautenbach et al., 2006). The point halfway between top and bottom (IC$_{50}$) represents the concentration necessary to cause 50 % growth inhibition. The MIC, calculated as the x-value at the intercept between the slope and the top plateau of a full dose–response curve, was denoted as IC$_{max}$ (Rautenbach et al., 2006) to make the distinction with MIC values obtained from visual inspection of a dose–response result.

GraphPad Prism 4.03 was also used for the statistical analysis of data. Analysis included 95 % confidence levels, absolute sum of squares, SEM, ANOVA analyses, Bonferroni’s test and unpaired Student’s t-test.

**RESULTS AND DISCUSSION**

**Antifungal activity of the tyrocidines**

The activity of antifungals has been shown to be influenced by the environment in which an activity test is conducted, i.e. media composition and broth versus agar medium (Troskie et al., 2012). Agar-based methods have also been proposed to aid in the exposure of resistant microorganisms (Pfaller et al., 2001). Therefore, both a broth-based assay (Broekaert et al., 1990; Spathelf & Rautenbach, 2009) and a gel-based assay described by Troskie et al. (2012) were used to evaluate Trc mixture activity against F. solani and Bot. cinerea (Fig. S4).

The Trc mixture showed potent activity in the broth media against both target cells and retained its activity on agar media against Bot. cinerea with a slight IC$_{max}$ increase from 4.8 (in PDB) to 7.6 µg ml$^{-1}$ (on PDA). However, tyrocidine activity was significantly reduced against F. solani on PDA medium relative to the PDB medium (Fig. S4). The IC$_{max}$ obtained against F. solani in PDB was 7.1 µg ml$^{-1}$, while on the PDA, even at a concentration of 100 µg ml$^{-1}$, 100 % inhibition could not be reached. There are various possible reasons for this decrease in activity, including the influence of different media compositions on fungal growth (Troskie et al., 2012) and peptide structure and activity being influenced by their environments (Loll et al., 2014; Munyuki et al., 2013; Spathelf, 2010). Except for the scientific question on how the environment influences peptide structures and consequent peptide activity, it is important, with development of formulations for application purposes, to know and understand the activity range and limitations of the relevant peptide in different environments. From these results it was evident that the tyrocidines do have potent antifungal activity, particularly in broth media; therefore, all the subsequent assays were performed in broth media.

Using F. solani and Bot. cinerea as target organisms, the antifungal activity of the Trc mixture was evaluated in terms of the activities of the purified tyrocidines. Furthermore, in order to put the activities of the tyrocidines as a group into perspective, their ability to inhibit F. solani and Bot. cinerea was compared to that of bifonazole, a known antifungal (Fig. 1, also refer to Table 1 and Fig. 2).

The activities of the Trc mixture and purified tyrocidines were significantly higher than that of bifonazole (Fig. 1). The activities of the Trc mixture and the purified tyrocidines were statistically similar ($P>$0.05), which supports the hypothesis that the individual tyrocidines contribute similarly to the antifungal activity in the Trc mixture (Figs 1 and 2). Due to the conserved nature of the tyrocidine structure, it is highly probable that they share a similar major mode of action and target.

![Fig. 1. Comparison of the antifungal activity of Trc mixture, purified tyrocidines and bifonazole against Bot. cinerea and F. solani. The mean of at least five biological repeats (each with three to four technical repeats) with SEM is shown. Significant differences ($***P<0.001$, **$P<0.01$) in activity between bifonazole and all the tyrocidines were found with one-way ANOVA using Bonferroni’s test. The tyrocidines all had similar activity towards the two fungal targets ($P>$0.05).](image-url)
Tyrocidine peptides inhibit fungal phytopathogens

We, therefore, compared the activities of the purified tyrocidines and analogues against Bot. cinerea, the IC\textsubscript{max} for PhcA against TrcC was significantly lower than that of the other tyrocidines and the analogue PhcA (Fig. 2a, also refer to Tables S3 and S4). When the activities of the individual tyrocidines were determined in YTSB (Fig. 2b, also refer to Tables S3 and S5) the phenycidine, PhcA, stood out as the peptide with significantly lower activity (P<0.01) compared with the other tyrocidines and the analogue TpcC.

In terms of sequence, these two analogues differ from some of the tyrocidines in only one residue, namely a Trp\textsuperscript{7} or Phe\textsuperscript{7} instead of the Tyr\textsuperscript{7} of the major tyrocidines. The side chain of Trp contains a bicyclic indole group, which makes it structurally larger than Tyr. Tyr differs only from Phe by an additional OH-group. However, this minor difference makes tyrosine amphipathic, dipolar and ionizable (pK\textsubscript{a}=10.07). Phe and Trp are also less polar amino acids than Tyr (Tyr>Trp>>Phe). The three aromatic amino acids also differ in their hydrophilicities (Phe=2.8, Trp=−0.9, Tyr=−1.3) (Freder, 2006). It could be that the Tyr residue has the optimal chemical properties for target interaction and activity in both the low-salt (PDB) and high-salt (YTSB) media.

Other than a preference for Tyr as the aromatic residue in position 7, no overt activity–structure relationships regarding the tyrocidines’ other structural/chemical parameters could be established. Neither the size of the tyrocidines, their surface area, side chain surface area nor lipophilicity seemed to play a role in the degree of antifungal activity exhibited by the tyrocidines (results not shown). Minor differences in activity are most probably due to minor differences in peptide aggregation (Ruttenberg et al., 1965, 1966), such as different tendencies to form dimers and higher-order structures (Loll et al., 2014; Munyuki et al., 2013), which can potentially influence the initial binding to the fungal target. Furthermore, small differences in amino acid structure can influence the tightness of binding to the targets. For example, tightness of binding of the tyrocidines to membranes could be modulated by the cationic residue (Lys or Orn) and the aromatic amino acid residues (Rautenbach et al., 2007).

However, it must also be kept in mind that the structure–activity relationships of individual tyrocidines may not give an adequate representation of the true activity events. From modelling and biophysical studies it has been proposed by several investigators that the tyrocidines form higher-order structures, possibly dimers, essential for biological activity (Loll et al., 2014; Munyuki et al., 2013; Spathelf, 2010). In this scenario, the structural and chemical characteristics of this higher-order structure and not necessarily that of the individual tyrocidines have to be taken into account when comparing activities. The higher antifungal activity observed for the major tyrocidines implies that the presence of a Tyr\textsuperscript{7} may favour the formation of a higher-order structure with high antifungal activity and/or stability in different environments. The activity results of the tyrocidines in the different media, together with the hypothesis that the environment...
influences the active structure of the tyrocidines and consequently tyrocidine activity, are not only important for subsequent studies into the development of a commercially viable product, but for any study involving the activity of the tyrocidines.

With the purpose of determining the spectrum of antifungal activity of the tyrocidines, the growth inhibition by the Trc mixture was assessed using microbroth dilution assays with *F. solani*, *F. oxysporum*, *F. verticillioides*, *Cyl. liriodendri*, *Bot. cinerea*, *Pen. digitatum*, *Pen. glabrum*, *Pen. expansum*, *Tal. mineoluteus*, *Tal. ramulosus* and *A. fumigatis* as target organisms. With IC$_{50}$ values between 2.0 and 8.9 µg ml$^{-1}$, the Trc mixture exhibited significant inhibitory activity against all of the fungal pathogens relevant to this study. Refer to Table 2 for a summary of inhibitory values in µg ml$^{-1}$ and the corresponding values in µM.

In our comparative assays over 48 h, the tyrocidines were found to be two- to fourfold more active towards Cyl. liriodendri, Bot. cinerea, Tal. mineoluteus, Tal. ramulosus Pen. digitatum and Pen. expansum than towards A. fumigates, Pen. glabrum, F. solani, F. oxysporum and F. verticillioides. This variance in activity can point to the possibility that the nature of the target cell influences/determines the activity of the tyrocidines. The different fungal strains may present the same tyrocidine target, such as the fungal cell membrane, but differ in their sensitivity due to target concentration. The difference could also be ascribed to the difference in the growth rate of various fungal species, rendering the fungi less or more susceptible to tyrocidine activity (Lewis, 2001). As has been observed for other antifungals (McMaster et al., 2013), we found that the Trc mixture exhibited activity (IC$_{50}$ 7.5 µg ml$^{-1}$ and IC$_{max}$ 9.8 µg ml$^{-1}$) against a strain of *Tri. atroviride*, a fungus that is used in bio-control and seen as biologically relevant metal cations. Accordingly, their activity against Bot. cinerea and F. solani was determined in the presence of 0.2 to 10 mM Ca$^{2+}$, Mg$^{2+}$, Na$^{+}$ and K$^{+}$. The Trc mixture and selected purified tyrocidines proved to be salt tolerant except in the presence of Ca$^{2+}$, which led to a significant loss (P<0.001) of >70% activity against *F. solani* (Fig. 3a). The tyrocidines were slightly more salt tolerant against Bot. cinerea with regard to 5 mM Ca$^{2+}$ (Fig. 3b). Of the three major tyrocidines, TrcB exhibited the highest loss in activity against both target organisms in the presence of 5 mM Ca$^{2+}$, while TrcA performed the best in the presence of calcium. This is probably due to the solubility and aggregation differences (Munyuki et al., 2013; Paradies, 1979; Spathelf, 2010) of the three selected peptides in 5 mM CaCl$_2$. We also confirmed that the tyrocidines had a tendency to aggregate, even under the harsh ES-MS conditions (Fig. S5).

The loss of activity in the presence of increasing concentrations of CaCl$_2$ was compared for Bot. cinerea and *F. solani*. A significant (P<0.01) decrease (∼30%) in activity against Bot. cinerea could be observed at a concentration as

### Salt tolerance of tyrocidine antifungal activity

It has been observed that peptide activity and selectivity are influenced by their environment (Andreu & Rivas, 1998; van’t Hof et al., 2001; Zasloff, 2002). Cations, for example Na$^+$, K$^+$, Mg$^{2+}$ and Ca$^{2+}$, have been illustrated to decrease the activity of the majority of AMPs (Bals et al., 1998; Cociancich et al., 1993; Hancock & Sahl, 2006). The antibacterial activities of the tyrocidines have been shown to be variably affected by the presence of metal cations (Marques et al., 2007; Spathelf, 2010).

Since the incentive of this research was to explore the potential of an antifungal agent, and the agricultural and food processing environments are exposed to salts, the tyrocidines were tested for the ability to retain activity against fungi in the presence of chloride salts containing biologically relevant metal cations. Accordingly, their activity against Bot. cinerea and *F. solani* was determined in the presence of 0.2 to 10 mM Ca$^{2+}$, Mg$^{2+}$, Na$^{+}$ and K$^{+}$. The Trc mixture and selected purified tyrocidines proved to be salt tolerant except in the presence of Ca$^{2+}$, which led to a significant loss (P<0.001) of >70% activity against *F. solani* (Fig. 3a). The tyrocidines were slightly more salt tolerant against Bot. cinerea with regard to 5 mM Ca$^{2+}$ (Fig. 3b). Of the three major tyrocidines, TrcB exhibited the highest loss in activity against both target organisms in the presence of 5 mM Ca$^{2+}$, while TrcA performed the best in the presence of calcium. This is probably due to the solubility and aggregation differences (Munyuki et al., 2013; Paradies, 1979; Spathelf, 2010) of the three selected peptides in 5 mM CaCl$_2$. We also confirmed that the tyrocidines had a tendency to aggregate, even under the harsh ES-MS conditions (Fig. S5).

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### Table 2. Summary of activity parameters obtained for the Trc mixture against selected fungal pathogens

<table>
<thead>
<tr>
<th>Fungal pathogen</th>
<th>IC$_{50}$ ± SEM µg ml$^{-1}$ (µM)*</th>
<th>IC$_{max}$ ± SEM µg ml$^{-1}$ (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus fumigatis ATCC 204305</td>
<td>4.8 ± 0.49 (3.7)</td>
<td>8.6 ± 0.69 (6.6)</td>
</tr>
<tr>
<td>Fusarium solani STEU 6188</td>
<td>4.7 ± 0.4 (3.6)</td>
<td>9.3 ± 1.2 (7.1)</td>
</tr>
<tr>
<td>Fusarium oxysporum ATCC 10913</td>
<td>6.2 ± 0.5 (4.8)</td>
<td>10 ± 1.1 (7.7)</td>
</tr>
<tr>
<td>Fusarium verticillioides CKJ1730</td>
<td>8.2 ± 1.9 (6.3)</td>
<td>12 ± 2.9 (9.2)</td>
</tr>
<tr>
<td>Botrytis cinerea CKJ1731</td>
<td>3.0 ± 0.2 (2.3)</td>
<td>4.8 ± 0.7 (3.7)</td>
</tr>
<tr>
<td>Cylindrocarpon liriodendri STEU 6170</td>
<td>2.0 ± 0.1 (1.5)</td>
<td>2.9 ± 0.1 (2.2)</td>
</tr>
<tr>
<td>Penicillium glabrum CKJ1732</td>
<td>8.9 ± 0.2 (6.8)</td>
<td>11 ± 0.4 (8.4)</td>
</tr>
<tr>
<td>Talaromyces ramulosus CKJ1735</td>
<td>2.7 ± 0.3 (2.1)</td>
<td>3.7 ± 0.4 (2.8)</td>
</tr>
<tr>
<td>Talaromyces mineoluteus CKJ1736</td>
<td>2.5 ± 0.1 (1.9)</td>
<td>3.3 ± 0.2 (2.5)</td>
</tr>
<tr>
<td>Penicillium expansum CKJ1733</td>
<td>3.8 ± 0.3 (2.9)</td>
<td>5.2 ± 0.3 (4.0)</td>
</tr>
<tr>
<td>Penicillium digitatum CKJ1734</td>
<td>2.3 ± 0.1 (1.8)</td>
<td>3.7 ± 0.2 (2.8)</td>
</tr>
</tbody>
</table>

*The Trc mixture consists of a group of tyrocidines with relative molar mass (M$_r$) ranging from 1200 to 1400. A mean M$_r$ of 1303.7 was calculated from the tyrocidine abundances and used to calculate the approximate inhibition parameters in µM. Each value represents the mean of at least three biological repeats with three to four technical repeats per assay ± SEM.
Tyrocidine peptides inhibit fungal phytopathogens

Influence of tyrocidines on fungal morphology

As indicated by the growth inhibition assay results, the tyrocidines exhibit significant antifungal activity. Light microscopy was used to analyse the morphological effect of tyrocidines on the spores and hyphae of fungi. Alterations in fungal morphology as a result of antifungal peptide activity are used to classify the peptides either as morphogenic, when peptide activity leads to alterations in fungal morphology, or non-morphogenic, when the peptides inhibit fungal growth with no visible morphological changes. Morphogenic antifungal peptide activity can, for instance, result in hyperbranching of fungi (Thevissen et al., 1996).

The Trc mixture, at 9 μg ml⁻¹ (double the IC₅₀ concentration), added to 16-hour-old F. solani cultures had a significant effect on F. solani growth and morphology (Fig. 5). After 12 h, the visual difference between the control (Fig. 5a) and tyrocidine-treated hyphae (Fig. 5b) was pronounced. There was, in comparison with the control, minimal progress in growth, and shorter hyphae and atypical branching were observed (Fig. 5b). The hyphal cells treated with the Trc mixture also appeared thicker/swollen compared with the control. The hyperbranching
was more pronounced after 24 h for hyphae treated with 9 µg ml⁻¹ Trc mixture (Fig. 5c) compared with the control (Fig. 5d). Similar results were obtained for the Trc mixture on Bot. cinerea hyphae (Fig. S6). From the microscopy studies, it is evident that the tyrocidines not only inhibit fungal hyphae growth, but also alter the morphology of the hyphae by inducing hyperbranching.

The effect of the Trc mixture and TrcA on Bot. cinerea spores was visualized 12 and 24 h after peptide addition (Fig. 6). After 12 h of incubation, germination of the Bot. cinerea spores, with long healthy germination tubes, could be observed in the control (Fig. 6b). Germination was evidently retarded in the spores treated with 6 µg ml⁻¹ Trc mixture (Fig. 6a). Germination of individual spores was visible; however, the germination tubes were short compared with those of the control and they appeared swollen. After 24 h of incubation, the tyrocidine-treated spores, in contrast to the control (Fig. 6c), appeared swollen and multiple germination tubes were visible (Fig. 6d). On some of the germination tubes, dichotomous branching could be seen (Fig. 6d). The purified tyrocidine, TrcA, had similar effects on Bot. cinerea spores (Fig. S7). Comparable results were obtained for spores of F. solani (Fig. S8).

The morphology of fungal hyphae and spores is clearly influenced by the tyrocidines and it is evident that these peptides influence hyphal elongation and induce hyperbranching. This is probably due to interruption/interference with the fungal cell's ability to germinate and propagate the normal growth of hyphae. Similar symptoms of hyperbranching have been observed for fungal mutants with alterations in their ability to establish and maintain polar growth (Muñoz et al., 2006). Not every aspect of spore germination, hyphal elongation and lateral branching is known and understood. However, a few factors/regulators have been identified as probable candidates involved in these processes. The establishment of a polar axis, essential

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**Fig. 4.** The dose-dependent influence of CaCl₂ on the activity of (a) 12.5 µg Trc mixture ml⁻¹ and 12 µM (b) TrcA, (c) TrcB and (d) TrcC against F. solani and Bot. cinerea. The mean of a minimum of six repeats with SEM is shown in all graphs. Percentage growth inhibition induced by the chloride-incubated tyrocidines was calculated in terms of the growth of untreated fungi grown in the corresponding concentration of CaCl₂.
for asymmetrical growth during germination (Nesher et al., 2011), is probably regulated by Rho-type GTPases (Nesher et al., 2011) and cAMP signalling (Boyce et al., 2005; Fillinger et al., 2002). Subsequent to establishing the polar site, hyphal elongation is controlled by various factors such as GTPases (Harris, 2008; Nesher et al., 2011), formins and septins (Boyce et al., 2005; Harris, 2008), the Spitzenkorper, the microtubule cytoskeleton (Harris, 2008; Kaiserer et al., 2003), actin polymerization (Kaiserer et al., 2003) and sterol-rich plasma membrane domains (SRDs) (Takeshita et al., 2008, 2012), as well as tip-high cytoplasmic Ca\(^{2+}\) gradients (Silverman-Gavrila & Lew, 2000, 2002, 2003). The cell cycle also seems to have a regulatory influence on branching in some fungi (Binder et al., 2010; Harris, 2008). Retarded germination and hyperbranching as a result of AMP activity can be the result of interference with any one of the above-mentioned processes or their regulators. For example Thevissen et al. (2012) hypothesized that RsAFP2-induced septin mislocalization, which results in altered morphology of Can. albicans, is similarly responsible for the hyperbranching of the filamentous fungus Fusarium culmorum (Terras et al., 1992; Thevissen et al., 2012). The activity of AMP PAF26 on Pen. digitatum results in altered polar growth, hyperbranching and abnormal chitin deposition (Muñoz et al., 2006).

The impact of tyrocidines on hyphal morphology, together with the observation that the presence of Ca\(^{2+}\) impedes tyrocidine antifungal activity, indicated the possibility that the tyrocidines interfere with regulator(s) and/or processes(s) involved in generating and maintaining the Ca\(^{2+}\) gradients required for normal hyphal growth. As mentioned, it is hypothesized that one aspect of hyphal elongation takes place through a Ca\(^{2+}\)-regulated process (Jackson & Heath, 1993) and interference of this process by AMPs may, for instance, induce hyperbranching (Spelbrink et al., 2004). Spelbrink et al. (2004) illustrated that the antifungal defensin from Medicago sativa (MsDef1), which inhibits Fusarium graminearum and leads to the hyperbranching phenotype, blocks the mammalian L-type Ca\(^{2+}\) channel. Similar hyperbranching activity was observed for the known Ca\(^{2+}\) channel blocker KP4. Furthermore, the presence of Ca\(^{2+}\) antagonizes the antifungal activity of MsDef1. The investigators proposed that MsDef1 acts through the disruption of a Ca\(^{2+}\) gradient (Spelbrink et al., 2004). Intracellular Ca\(^{2+}\) gradients appear to be essential for the hyphal growth process (Silverman-Gavrila & Lew, 2000). These tip-high cytoplasmic Ca\(^{2+}\) gradients are internally induced and maintained by inositol (1,4,5)-triphosphate (IP\(_3\))-induced Ca\(^{2+}\) release from tip-localized vesicles and subapical removal of Ca\(^{2+}\) (Silverman-Gavrila & Lew, 2002, 2003). Arbitrary fluctuations in Ca\(^{2+}\)
**Fig. 7.** Fluorescence microscopy of *Bot. cinerea* treated with 3 μg Trc mixture ml$^{-1}$ after 2 h of treatment (a) and 24 h of treatment of the germinating spores (b). Cultures were incubated with propidium iodide prior to capturing of images at ×10 magnification. Dense mat-like growth was observed for the control group (c) and no propidium iodide fluorescence could be detected (at ×4 magnification). The top panels are fluorescence images with propidium iodide, while the bottom panels show the phase-contrast images.

**Fig. 8.** Fluorescence microscopy of *F. solani* hyphae incubated with SYTOX green alone (a), or with 25 μg Trc mixture ml$^{-1}$ (b), 25 μM TrcC (c) and 1 % Triton X-100 (d) at ×40 magnification. The top panels are fluorescence images with SYTOX green, while the bottom panels show the phase-contrast images.
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Distribution, which results in localized calcium spikes, may be responsible for initial commencement of tip growth. The stretch-activated phospholipase C, which increases tip-localized IP₃, is then believed to be partly responsible for continued hyphal elongation (Silverman-Gavrila & Lew, 2003).

Rautenbach et al. (2007) illustrated that the tyrocidines hamper the life cycle and development of another organism, namely the malaria-causing protozoa, Plasmodium falciparum. The tyrocidines may possibly have a similar effect on the spores of certain fungal species, interfering with the cell cycle and inducing retarded germination and hyperbranching. However, to fully elucidate how the tyrocidines influence germination, hyphal elongation and induce hyperbranching is a complex future study in which the process(es) and/or regulator(s) targeted by tyrocidine activity must first be identified.

Further investigation into the changes in fungal spores and hyphae shows that at least one mode of action of the tyrocidines is related to cell permeabilization. Fluorescence imaging with the membrane impermeable dye propidium iodide showed that after 2 h, 3 µg Trc mixture ml⁻¹ leads to overt permeabilization of Bot. cinerea spores and propidium iodide uptake (Fig. 7a). Similarly we found pronounced propidium iodide uptake in Bot. cinerea hyphae, specifically in the growth tips after 24 h of treatment with the tyrocidines (Fig. 7b). The permeabilization of fungal hyphae was confirmed by SYTOX green uptake after one hour of treatment of F. solani with the Trc mixture and selected purified major tyrocidines. With the tyrocidine concentration at 25 µM, ~100% release of the dye was obtained for F. solani hyphae, similar to Triton X-100. The SYTOX green results for the Trc mixture and TrcC are shown in Fig. 8(a–d). TrcA and B treatment of F. solani led to similar dye uptake (results not shown). Similar results on the permeabilization activity of the tyrocidines were recently found by Troskie et al. (2014) with Can. albicans biofilm cells as target. They also illustrated the rapid permeabilization and lysis of model phospholipid membranes containing the fungal steroid ergosterol, illustrating that the fungal membrane is one of the tyrocidines’ targets (Troskie et al., 2014).

CONCLUSION

Evidently, the tyrocidines exhibit significant low micromolar antifungal activity against a range of phytopathogens and their activity remains relatively stable in the presence of biological salts. The absence of overt structural–activity relationships implies that the conserved sequence of tyrocidines, NQQV(O/K)LiP, may be important for activity, with the aromatic dipeptide unit, Ff, Wf or Ww, determining the hydrophobicity and putative active dimer formation (Loll et al., 2014; Munyuki et al., 2013; Spathelf, 2010). The tyrocidines’ major mode of action involves the disruption of fungal membrane integrity, but they also have morphogenic activity, indicating a non-membrane target or targets. Because of the tyrocidines’ broad spectrum and potent antifungal activity, general salt tolerance and possible multiple targets reducing the risk of overt resistance, they are promising candidates that warrant further investigation as potential bio-fungicides.

REFERENCES


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