FINAL PROJECT REPORT

APPLICATION OF CYSTEINE-RICH ANTIFUNGAL PROTEINS SECRETED BY *NEOSARTORYA FISCHERI* NRRL 181 AS BIOPESTICIDES AND CROP PRESERVATIVES

Institutions (in time-order of contract modifications):
University of Szeged, Faculty of Science and Informatics, Department of Microbiology
01.12.2016. – 31.12.2017. (PD 120808)
Biological Research Centre, Institution of Plant Biology
01.01.2018. – 30.11.2018. (PD 131340)
University of Szeged, Faculty of Science and Informatics, Department of Biotechnology
01.12.2018. – 30.11.2019. (PD 135248)
Project leader: László Galgóczy
Beginning of the project: 01. 12. 2016. - End of the project: 30. 11. 2019.
Project duration in month: 36

The main aim of the project was to prove the biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins (NFAP, NFBP, NFAP2), their rationally designed γ-core variants, and γ-core peptide derivatives for the agriculture as safely applicable plant and crop protective biomolecules.

Background of the study: Because of enormous crop losses worldwide due to pesticide-resistant plant pathogenic pre- and postharvest fungi, there is an increasing demand for the development of novel antifungal strategies in agriculture. Application of alternative fungicides, such as antifungal proteins and peptides, provides a promising basis to overcome this problem; however, several factors limit their direct agricultural application, such as the high cost of production, narrow antifungal spectrum and detrimental effects to plant development and human/animal health.

Considering these challenges, the main aims of the project were the followings:

(1) Heterologous expression of *Neosartorya* antifungal proteins, and their rationally designed γ-core variants
(2) Synthesis of rationally designed *Neosartorya* antifungal protein γ-core peptide derivatives
(3) Investigating the antifungal potential of *Neosartorya* antifungal proteins and γ-core peptide derivatives
(4) Investigating the cytotoxic potential of *Neosartorya* antifungal proteins and γ-core peptide derivatives
(5) Proving the applicability of *Neosartorya* antifungal proteins and γ-core peptide derivatives as plant and crop protective biomolecules

Based on the NKFIH regulations, these aims in the PD project are tightly connected with that of the parallel and related ANN 131341 project. The results of both projects were adopted and utilized *vice versa*.

During the project period, we achieved the results discussed below:

(1) Heterologous expression of *Neosartorya* antifungal proteins, and their rationally designed γ-core variants
Before the beginning of the project, we already reported that a *Penicillium chrysogenum*-based expression system is a potential tool to produce ascomycetous cysteine-rich antifungal proteins and their rationally designed variants for functional and structural analyses. Furthermore, this *P. chrysogenum*-based system

for bulk production of correctly folded and functional NFAP was already available at the beginning of the project from this work (Sonderegger et al, 2016). Considering these results, in the present project we adopted this system for bulk recombinant NFAP2 and NFBP production. The average yield of recombinant NFAP2 was 40-times higher than in the native producer. Analyses by mass spectrometry, reversed-phase high performance liquid chromatography and electronic circular dichroism (ECD) spectroscopy revealed that the recombinant NFAP2 was correctly processed and folded. The antifungal efficacy of recombinant NFAP2 was comparable to the native protein. The bulk NFBP production was failed with the *P. chrysogenum*-, and the alternatively applied *Pichia pastoris*-based expression systems due to the structural instability of recombinant NFBP. Taking into consideration the unstable protein structure, we omitted NFBP in the project.

These results were published in the following peer-reviewed paper

Tóth L, Váradi G, Borics A, Batta G, Kele Z, Vendrinszky Á, Tóth R, Ficze H, Tóth GK, Vágvölgyi C, Marx F, **Galgóczy L**. Anti-candidal activity and functional mapping of recombinant and synthetic *Neosartorya fischeri* antifungal protein 2 (NFAP2). FRONTIERS IN MICROBIOLOGY 9: Paper 393. (2018), DOI: 10.3389/fmicb.2018.00393. IF2018: 4.259 (Q1 Microbiology)

and conference proceedings.

Tóth L, Tóth R, Borics A, Váradi G, Kele Z, Fekete L, Vágvölgyi C, Marx F, **Galgóczy L**. *Penicillium chrysogenum*-alapú heterológ expressziós rendszerben termelt rekombináns *Neosartorya fischeri* antifungális protein 2 (NFAP2) jellemzése. MIKOLÓGIAI KÖZLEMÉNYEK-CLUSIANA 56:(1) pp. 24-26. (2017) VI. Magyar Mikológiai Konferencia. Conference place and time: Szeged, Hungary: 03.07.2017-05.07.2017.

Tóth L, Tóth R, Fekete L, Kele Z, Vágvölgyi C, Marx F, **Galgóczy L**. Bulk production of the anti-yeast protein NFAP2 in *Penicillium chrysogenum* In: [Department of Public Health Faculty of Medicine University of Szeged] (ed.) 19th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and abstracts. 65 p. Conference place and time: Szeged, Hungary, 09.06.2017.-10.06.2017. Szeged: University of Szeged, Faculty of Medicine, 2017. p. 54.

The generated recombinant NFAP and NFAP2 producing *P. chrysogenum* strains were utilized in the joint ANN 131341 project as follows.

Kovács R, Holzknecht J, Hargitai Z, Papp C, Farkas A, Borics A, Tóth L, Váradi G, Tóth GK, Kovács I, Dubrac S, Majoros L, Marx F, **Galgóczy L**. *In vivo* applicability of *Neosartorya fischeri* antifungal protein 2 (NFAP2) in treatment of vulvovaginal candidiasis. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY 63: (2) Paper e01777-18. (2019), DOI: 10.1128/AAC.01777-18. IF2018: 4.715 (D1 Pharmacology (medical))

Hajdu D, Huber A, Czajlik A, Tóth L, Kele Z, Kocsubé S, Fizil Á, Marx F, **Galgóczy L**, Batta G. Solution structure and novel insights into phylogeny and mode of action of the *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein (NFAP). INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES 129: pp. 511-522. (2019), DOI: 10.1016/j.ijbiomac.2019.02.016. IF2018: 4.784 (Q1 Medicine (miscellaneous))

During the project period we provided a detailed phylogenetic analysis that proved the presence and conservation of the γ-core motif in all cysteine-rich antifungal protein classes from Eurotiomycetes (including all *Neosartorya* antifungal proteins). First, we reported the important role of the γ-core motif in the biological function of a cysteine-rich antifungal protein: The *Penicillium chrysogenum* antifungal protein PAF variant PAF^{opt}, in which specific amino acids in the γ-core motif were substituted to elevate the positive net charge and the hydrophilicity of the protein showed improved efficacy against *Candida albicans*; or these substitutions influenced the antifungal spectrum of the native protein against filamentous ascomycetes. The amino acid substitutions in the γ-core region did not influence dramatically the secondary and tertiary structure of the protein. PAF^{opt} was produced in the above mentioned *P. chrysogenum*-based heterologous expression system. Based on these results, we emphasized the potential of common γ-core protein motif for the design of short antifungal peptides and as a protein motif in which targeted amino acid substitutions enhance the antimicrobial activity or influence the antifungal spectrum. We adopted and utilized these results to generate γ-core improved variants of NFAP and NFAP2.

These results were published in the following peer-reviewed papers.

Sonderegger C, Váradi G, **Galgóczy L**, Kocsubé S, Posch W, Borics A, Dubrac S, Tóth GK, Wilflingseder D, Marx F. The evolutionary conserved γ-core motif influences the anti-*Candida* activity of the *Penicillium chrysogenum* antifungal protein PAF. FRONTIERS IN MICROBIOLOGY 9: Paper 1655. (2018), DOI: 10.3389/fmicb.2018.01655. IF2018: 4.259 (Q1 Microbiology)

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide Pγ^{opt} in plant protection. MICROBIAL BIOTECHNOLOGY. [Epub ahead of print] (2020), DOI: 10.1111/1751-7915.13559. IF2018: 4.857 (Q1 Applied microbiology and biotechnology)

In NFAP and NFAP2, we changed the slightly hydrophilic negatively charged (NFAP) or neutral (NFAP2) γ-core motif into a *de novo* designed motif (optimized γ-core, the same that we applied also in PAF modification) with increased positive net charge and hydrophilicity (**Table 1**).

TABLE 1. Amino acid sequence and *in silico* predicted physicochemical properties of mature *Neosartorya* antifungal proteins, their γ-core optimized variants (NFAPγ^{opt} and NFAP2γ^{opt}), and the respective γ-core regions.

Protein/ peptide	Number of amino acids	Molecular weight (kDa)	Number of Cys	Number of Lys/Arg/His	Theoretical pI	Estimated charge at pH 7	GRAVY
LEYK**GECFTKDNTC**KYKIDGKTYLAKCPSAANTKCEKDGNKCTYDSYNRKVKCDFRH							
NFAP	57	6.6	6	11/2/1	8.93	+5.0	−1.214
LEYK**GKCKTKKNKC**KYKIDGKTYLAKCPSAANTKCEKDGNKCTYDSYNRKVKCDFRH							
NFAPγ^{opt}	57	6.7	6	15/2/1	9.56	+11.0	−1.402
GECFTKDNTC							
NFAP γ-core	10	1.1	2	1/0/0	4.37	−1.1	−0.840
IATSPYYACNCPNNCKHKKGSGCKYHSGPSDKSKVIS**GKCEWQGGQLNC**IAT							
NFAP2	52	5.6	6	7/0/2	9.01	+5.2	−0.731
IATSPYYACNCPNNCKHKKGSGCKYHSGPSDKSKVIS**GKCKTKKNKC**IAT							
NFAP2γ^{opt}	50	5.4	6	11/0/2	8.83	+10.2	−0.918
GKCEWQGGQLNC							
NFAP2 γ-core	12	1.3	2	1/0/0	5.99	−0.2	−0.933
GKCKTKKNKC							
optimized γ-core	10	1.1	2	5/0/0	9.90	+4.8	−1.910

The γ-core motif is indicated in bold and by the underlined letters. Molecular weight (Mw), isoelectric point (pI) and the grand average of hydropathy (GRAVY) value were calculated by the ExPASy ProtParam tool (Gasteiger et al., 2005). The total net charge at pH = 7.0 was estimated using the Protein Calculator v3.4 server (The Scripps Research Institute; http://protcalc.sourceforge.net/).

The *P. chrysogenum*-based heterologous expression system and chemical synthesis (solid phase peptide synthesis and native chemical ligation of peptide fragments of the full length proteins as described in Váradi et al., 2013) were applied to generate recombinant or synthetic γ-core modified variants of NFAP (NFAPγ^{opt}) and NFAP2 (NFAP2γ^{opt}), respectively (**Table 1**). Both protein variants were degraded during the recombinant production in *P. chrysogenum*, which let us assume that the modification renders the protein structure accessible for extracellular proteases. ECD spectroscopy demonstrated that synthetic NFAPγ^{opt} and NFAP2γ^{opt} had unordered structures compared to the respective wild-type proteins, which showed a β-pleated conformation (**Figure 1**). The γ-core modification improved the antifungal efficacy of synthetic NFAPγ^{opt} against the plant pathogenic fungus *Cladosporium herbarum* FSU 1148 compared the wild-type protein (**Figure 2A**). In contrast, a strong decrease in antifungal activity was observed with NFAP2γ^{opt} against the human pathogen *Candida albicans* ATCC 10231 in comparison to the wild-type NFAP2 (**Figure 2B**). These results suggest a supporting role of the negatively charged or neutral γ-core motif of NFAP and NFAP2, respectively, in correct protein folding.

These results were published in the following conference proceedings.

Váradi G, Tóth L, Vendrinszky Á, **Galgóczy L**, Batta G, Borics A, Kele Z, Tóth GK: Chemical synthesis and investigation of the native form and an improved gamma-core analogue of *Neosartorya fischeri* antifungal protein 2 (NFAP2). JOURNAL OF PEPTIDE SCIENCE 24: (Supplemet 144), Suppl. 2, p. 177 (2018) Conference place and time: Dublin, Ireland, 26.08.2018.-31.08.2018.

Tóth L, Váradi Gy, Boros É, Nagy I, Marx F, **Galgóczy L**. Potential role of the evolutionary conserved γ-core motif in the efficacy and structural stability of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins. Acta Microbiol Immunol Hung, 2019, Volume 66, Supplemet 1 p. 204. 18th International Congress of the Hungarian Society for Microbiology. Conference place and time: Budapest, Hungary, 03.07.2019.-05.07.2019.

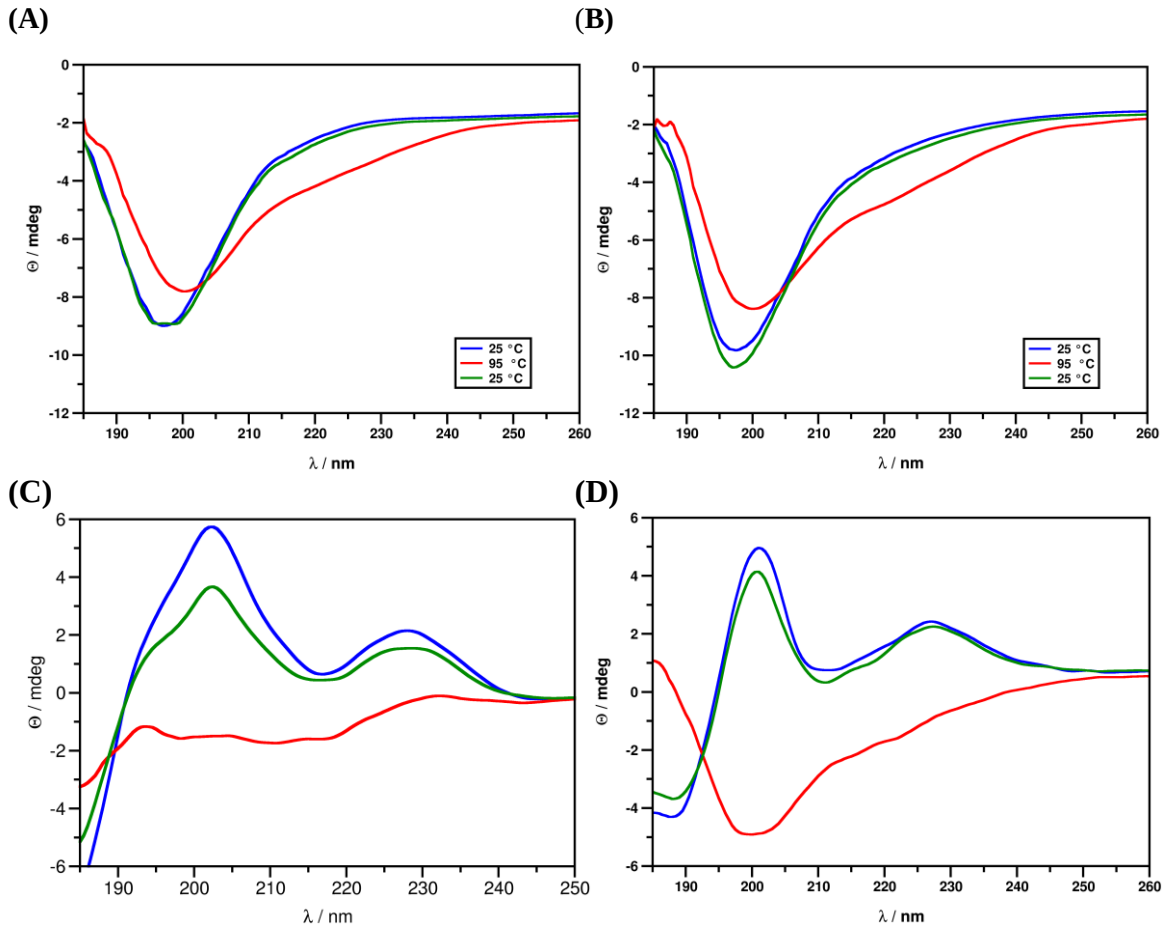

Figure 1. ECD spectra of synthetic NFAPγ^{opt} (**A**), synthetic NFAP2γ^{opt} (**B**), recombinant native NFAP (**C**), and recombinant native NFAP2 (**D**) recorded at 25°C (blue), 95°C (red), and 95°C with immediate cooling to 25°C (green).

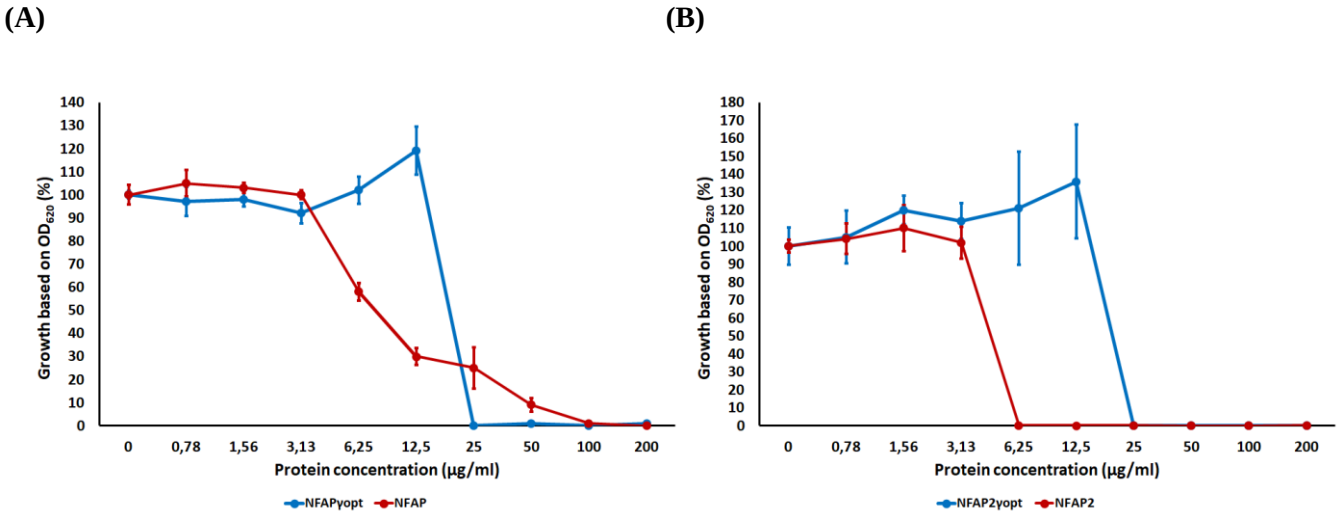

Figure 2. Antifungal activity of synthetic NFAPγ^{opt} and recombinant NFAP against *Cladosporium hebarum* FSU 1148 (**A**); and synthetic NFAP2γ^{opt} and recombinant NFAP2 against *Candida albicans* ATCC 10231 (**B**) in broth microdilution assay. Minimal inhibitory concentration is 25 µg/ml for NFAPγ^{opt}, 100 µg/ml for recombinant NFAP, 25 µg/ml for NFAP2γ^{opt}, and 6.25 µg/ml for recombinant NFAP2. The absorbance (OD_{620}) of untreated control culture was defined as 100% of growth.

(2) Synthesis of rationally designed NFAP, NFAPB and NFAP2 γ-core peptide derivatives

Peptides were synthesized on solid phase applying 9-fluorenylmethyloxycarbonyl chemistry. During the project period, first, we reported the optimization of PAF γ-core peptide derivatives for antifungal efficacy and structural stability. We also reported the antifungal efficacy of two synthetic 14-mer peptides, Pγ and Pγ^{opt}, that span the γ-core motif of wild-type PAF, and the above mentioned γ-core modified PAF^{opt},

respectively. A higher anti-*Candida* efficacy of the more positively charged and more hydrophilic $P\gamma^{opt}$ was proven.

These results were published in the following peer-reviewed paper.

Sonderegger C, Váradi G, **Galgóczy L**, Kocsubé S, Posch W, Borics A, Dubrac S, Tóth GK, Wilflingseder D, Marx F. The evolutionary conserved γ-core motif influences the anti-*Candida* activity of the *Penicillium chrysogenum* antifungal protein PAF. FRONTIERS IN MICROBIOLOGY 9: Paper 1655. (2018), DOI: 10.3389/fmicb.2018.01655. IF2018: 4.259 (Q1 Microbiology)

The rationally designed PAF γ-core peptides were utilized in the joint ANN 131341 project as follows.

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide $P\gamma^{opt}$ in plant protection. MICROBIAL BIOTECHNOLOGY. [Epub ahead of print] (2020), DOI: 10.1111/1751-7915.13559. IF2018: 4.857 (Q1 Applied microbiology and biotechnology)

The peptide derivatives spanning the native γ-core motifs of *Nesoartorya* antifungal proteins (γ^{NFAP}, γ^{NFBP}, γ^{NFAP2} in **Table 2**) were designed based on the findings regarding the stability and antifungal efficacy of the γ-core peptides Pγ and $P\gamma^{opt}$ reported in the above mentioned papers: They contain three additional amino acids at the N-terminus and ends in an extra C-terminal lysine residue. The applied N-terminal acetylation and C-terminal amidation mimicked the propagating native protein backbone, neutral terminals and provided stability against proteolysis. We observed that cyclisation of through the disulphide bridge formation impaired antifungal efficacy; thus, all cysteines in γ-core peptide derivatives possessed free sulfhydryl (–SH) groups. Specific amino acids were substituted in the derivatives of native γ-core motif to create the γ^{NFAP}-opt, γ^{NFBP}-opt γ^{NFAP2}-opt exhibiting an elevated positive net charge and increased hydrophilicity (**Table 2**). ECD spectroscopy indicated that all γ-core peptide derivatives have unordered structure (data not shown). The amino acid sequence and physicochemical properties of the native NFAP, NFBP and NFAP2 γ-core peptide derivatives and their rationally designed variants are summarised in **Table 2**.

TABLE 2. Amino acid sequence and *in silico* predicted physicochemical properties of *Neosartorya* antifungal protein γ-core peptide derivatives and their rationally designed variants.

Protein/ peptide	Number of amino acids	Molecular weight (kDa)	Number of Cys	Number of Lys/Arg/His	Theoretical pI	Estimated charge at pH 7	GRAVY
			Ac-EYKGEC(-SH)FTKDNTC(-SH)K-NH_2				
γ^{NFAP}	14	1.7	2	3/0/0	6.26	−0.1	−1.500
			Ac-EYKGKC(-SH)KTKKNKC(-SH)K-NH_2				
γ^{NFAP}-opt	14	1.7	2	7/0/0	9.84	+5.8	−2.264
			Ac-QSNGNC(-SH)QTNQNQSN-NH_2				
γ^{NFAP}-optChZ	14	1.5	1	0/0/0	5.52	−0.1	−2.264
			Ac-EIKIKC(-SH)KIKKIKC(-SH)K-NH_2				
γ^{NFAP}-optGZ	14	1.7	2	7/0/0	9.93	+5.8	−0.557
			Ac-KC(-SH)DRTGVVEC(-SH)RGGRW-NH_2				
γ^{NFBP}	15	1.7	2	1/3/0	8.96	+1.9	−0.920
			Ac-KC(-SH)KNKKTKC(-SH)KGGRW-NH_2				
γ^{NFBP}-opt	14	1.7	2	6/1/0	10.32	+6.8	−2.057
			Ac-VISGKC(-SH)EWQGGQLNC(-SH)K-NH_2				
γ^{NFAP2}	16	1.8	2	2/0/0	8.02	+0.8	−0.450
			Ac-VISGKC(-SH)KTKKNKC(-SH)K-NH_2				
γ^{NFAP2}-opt	14	1.6	2	6/0/0	10.05	+5.8	−1.079

Molecular weight (Mw), isoelectric point (pI) and the grand average of hydropathy (GRAVY) value were calculated by the ExPASy ProtParam tool (Gasteiger et al., 2005). The total net charge at pH = 7.0 was estimated using the Protein Calculator v3.4 server (The Scripps Research Institute; http://protcalc.sourceforge.net/).

These results were partially published in the following peer-reviewed paper

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2018: 4.259 (Q1 Microbiology)

and conference proceedings.

Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy L**. Antifungal effect of *de novo* designed peptides according to the y-core motif of *Neosartorya fischeri* NRRL 181 antifungal proteins. In: Abrama, M; Bielen, A; Kifer, D; Vlahovicek, GM; Klaric, MS (ed.) Central European Symposium on Antimicrobial Resistance - Book of Abstracts (2018) p. 111. Conference place and time: Sveti Martin na Muri, Croatia, 19.09.2018.-22.09.2018. (ISBN:978-953-7778-16-3)

Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy, L**. Examination of antifungal activity and mechanism of *de novo* designed y-core peptide motifs from *Neosartorya fischeri* NRRL 181 antifungal proteins. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 66: (S1) pp. 105-106., 2019. A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium. Conference place and time: Eger, Hungary, 17.10.2018.-19.10.2018.

Tóth L, Váradi Gy, Boros É, Ficze H, Nagy I, Marx F, **Galgóczy L**. Agricultural applicability of *Neosartorya fischeri* antifungal protein and *de novo* designed peptide derivatives. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts. 106 p. Conference place and time: Hódmezővásárhely, Hungary, 08.05.2019. Hódmezővásárhely: University of Szeged, Faculty of Agriculture, 2019. pp. 75-76. (ISBN:978-963-306-653-9)

(3) Investigating the antifungal potential of *Neosartorya* antifungal proteins and γ-core peptide derivatives

The antifungal potential of the produced recombinant *Neosartorya* antifungal proteins and synthetic γ-core peptide derivatives were investigated in a broth microdilution susceptibility assay against pre- and postharvest plant pathogenic and mycotoxigenic filamentous fungi (**Table 3**). The respective minimal inhibitory concentrations (MICs) are summarized in the **Table 3**. NFAP inhibited the growth of all tested ascomycetous isolates with various MICs in the investigated concentration range (up to 200 μg/ml). In contrast, NFAP2 was ineffective against aspergilli and fusaria, while *Botrytis* and *Cladosporium* isolates proved to be more susceptible to this protein than to NFAP. The synthetic γ-core peptide derivatives spanning the native γ-core were ineffective at concentrations up to 200 μg/ml, while all rationally designed variants with elevated positive net charge and hydrophilicity inhibited the growth of *Botrytis*, *Cladosporium* and *Fusarium* isolates at various MICs, but were ineffective against aspergilli. All tested mucromycotina fungi (*Gilbertella persicaria*, *Mucor piriformis*, *Rhizopus oryzae*, *Rhizopus stolonifer*) proved to be not susceptible to *Neosartorya* crAFPs and synthetic γ-core peptide derivatives.

Then, we investigated whether the net charge or the hydrophilicity influenced the antifungal activity of the rationally designed γ-core peptide derivatives. Therefore, two different variants of γ^{NFAP}-opt were synthesised. In the γ^{NFAP}-optChZ, amino acid substitutions reduced the net charge from +5.8 to neutral but maintained the GRAVY (**Table 2**). In contrast, the GRAVY was reduced to −0.557, whereas the net charge remained unchanged in the γ^{NFAP}-optGZ variant (**Table 2**). Antifungal susceptibility test indicated the positive net charge, not the hydrophilicity of these γ-core peptides played a major role in antifungal efficacy. ECD spectroscopy demonstrated that the antifungal activity did not require a conformational change of the β-pleated NFAP or the canonically ordered conformation of the synthetic γ-core peptide derivatives.

Based on the results of the *in vitro* susceptibility tests, the most effective antifungal protein, the NFAP and its rationally designed and optimized γ-core peptide derivatives were considered as effective plant and crop protective biocompounds. Therefore, they were selected for toxicity testing.

These results were partially published in the following peer-reviewed paper

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2018: 4.259 (Q1 Microbiology)

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Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy L**, Antifungal effect of *de novo* designed peptides according to the y-core motif of *Neosartorya fischeri* NRRL 181 antifungal proteins. In: Abrama, M; Bielen, A; Kifer, D; Vlahovicek, GM; Klaric, MS (ed.) Central European Symposium on Antimicrobial Resistance - Book of Abstracts (2018) p. 111. Conference place and time: Sveti Martin na Muri, Croatia, 19.09.2018.-22.09.2018. (ISBN:978-953-7778-16-3)

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Tóth L, Váradi Gy, Boros É, Ficze H, Nagy I, Marx F, **Galgóczy L**. Agricultural applicability of *Neosartorya fischeri* antifungal protein and *de novo* designed peptide derivatives. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts.

Table 3 Minimal inhibitory concentrations (μg/ml) of *Neosartorya* antifungal proteins and their rationally designed γ-core peptide derivatives against pre- and postharvest plant pathogenic and mycotoxigenic filamentous ascomycetes.

Isolate	NFAP	γ^{NFAP}-opt	NFAP2	γ^{NFAP2}-opt	γ^{NFBP}-opt	Origin of isolate
Aspergillus flavus SZMC 3014	100	>200	>200	>200	>200	*Triticum aestivum*/Hungary
Aspergillus flavus SZMC 12618	100	>200	>200	>200	>200	*Triticum aestivum*/Hungary
Aspergillus flavus SZMC 20745	12.5	>200	>200	>200	>200	*Zea mays*/Hungary
Aspergillus flavus SZMC 20755	25	>200	>200	>200	>200	*Zea mays*/Hungary
Aspergillus niger SZMC 0145	50	>200	>200	>200	>200	Fruits/Hungary
Aspergillus niger SZMC 2759	50	>200	>200	>200	>200	Raisin/Hungary
Aspergillus nomius SZMC 22631	25	>200	>200	>200	>200	chees/Hungary
Aspergillus parasiticus SZMC 22727	200	>200	>200	>200	>200	indoor air/Croatia
Aspergillus pseudonomius SZMC 22631	200	>200	>200	>200	>200	*Zea mays*/Serbia
Aspergillus welwitschiae SZMC 21821	25	>200	>200	>200	>200	*Allium cepa*/Hungary
Aspergillus welwitschiae SZMC 21832	12.5	>200	>200	>200	>200	*Allium cepa*/Hungary
Botrytis cinerea SZMC 21474	50	50	12.5	50	25	*Fragaria* × *ananassa*/Hungary
Botrytis cinerea NCAIM F.00751	50	50	12.5	50	25	Hungary
Botrytis pseudocinerea SZMC 21470	100	100	12.5	50	200	*Brassica napus*/Hungary
Botrytis pseudocinerea SZMC 21471	100	100	12.5	50	200	*Brassica napus*/Hungary
Cladosporium herbarum FSU 1148	100	12.5	12.5	100	3.125	n.d.
Cladosporium herbarum FSU 969	100	12.5	12.5	100	3.125	n.d
Fusarium boothi CBS 110250	25	50	>200	>200	12.5	*Zea mays*/South Africa
Fusarium graminearum SZMC 11031	200	>200	>200	>200	>200	*Citrus sinensis*/New Caledonia
Fusarium graminearum SZMC 6236J	25	50	>200	>200	25	Vegetables/Hungary
Fusarium oxysporum SZMC 6237J	25	50	>200	>200	200	Vegetables/Hungary
Fusarium solani CBS 115659	50	12.5	>200	50	6.25	*Solanum tuberosum*/Germany
Fusarium solani CBS 119996	100	50	>200	200	25	*Daucus carota*/The Netherlands
Fusarium verticillioides SZMC 11411	200	>200	>200	>200	>200	*Zea mays*/Hungary

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FSU: Fungal Reference Centre University of Jena, Jena, Germany; SZMC: Szeged Microbiological Collection, University of Szeged, Szeged, Hungary; NCAIM: National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary. n.d.: data not available.

(4) Investigating the cytotoxic potential of *Neosartorya* antifungal proteins and γ-core peptide derivatives

One of the requirements for new fungicides designed for agricultural applications is their harmlessness in the host. As a proof of principle, we tested the cytotoxic potential of NFAP and the two antifungally active γ-core peptide derivatives γ^{NFAP}-opt and γ^{NFAP}-optGZ on human keratinocytes and the colonic epithelial cells. These cell types were in direct contact with them if applied as biofungicides, and the treated agricultural products were considered for human consumption. Keratinocytes are the predominant cell type in the epidermis, whereas colonic epithelial cells play a role in nutrient absorption and the innate and adaptive mucosal immunity. Monocytes were also subjected to toxicity tests. They are important parts of the human body's defence system against infectious organisms and non-self-molecules. NFAP and γ^{NFAP}-opt did not reduce the viability of the human cell lines in the tested concentration range up to their 2 × MIC (**Table 3**). In contrast, a significant reduction in the viability of the keratinocytes exposed to 25 and 12.5 μg/ml γ^{NFAP}-optGZ in comparison with the untreated control was observed. The viability of the other cell lines was not significantly affected by this peptide. The cell membrane disruption ability of NFAP and the antifungally active γ-core peptide derivatives was investigated on erythrocytes. None of the tested proteins and peptides caused haemolysis.

Cytotoxic potential of NFAP and γ-core peptide derivatives on plant was investigated on *Medicago truncatula* seedlings, which is a fast-growing, small legume, easily cultivable on water agar in Petri dishes (Barker et al., 2006), and an appropriate model organism to investigate the harmful effects of antifungal peptides and proteins on the growing plants. Treatment with 2 × MIC NFAP and γ^{NFAP}-opt and γ^{NFAP}-optGZ

did not cause any changes to the plant morphology Furthermore, no significant changes in the primary root length and the number of evolved lateral roots were observed following the treatment period.

Based on the results of the cytotoxicity tests, NFAP and γ^{NFAP}-opt were selected for plant and crop protection assays.

These results were partially published in the following peer-reviewed paper

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2018: 4.259 (Q1 Microbiology)

and conference proceeding.

Tóth L, Váradi Gy, Boros É, Ficze H, Nagy I, Marx F, **Galgóczy L**. Agricultural applicability of *Neosartorya fischeri* antifungal protein and *de novo* designed peptide derivatives. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts. 106 p. Conference place and time: Hódmezővásárhely, Magyarország, 08.05.2019. Hódmezővásárhely: University of Szeged, Faculty of Agriculture, 2019. pp. 75-76. (ISBN:978-963-306-653-9)

The developed methodologies for investigation of cytotoxicity were adopted and utilized in the joint ANN 131341 project as follows.

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide $P\gamma^{opt}$ in plant protection. MICROBIAL BIOTECHNOLOGY. [Epub ahead of print] (2020), DOI: 10.1111/1751-7915.13559. IF2018: 4.857 (Q1 Applied microbiology and biotechnology)

(5) Proving the applicability of *Neosartorya* antifungal proteins and γ-core peptide derivatives as plant and crop protective biomolecules

For the plant protection assay, we adopted the pathogenicity test method described by El Oirdi *et al.* (2010; 2011). *Botrytis cinerea* is known as fungal necrotroph of tomato plant leaf tissue (Nambeesan et al., 2012). Considering the promising results from the *in vitro* susceptibility and toxicity tests, the plant protection ability of NFAP and γ^{NFAP}-opt at *in vitro* detected MIC was tested against *B. cinerea* SZMC 21472 infection of tomato plant leaves. To reveal the potential toxic effect of the protein and peptide, uninfected leaves were first treated with NFAP or γ^{NFAP}-opt. A reliable cell viability assay applying Evan's blue staining (Vijayaraghavareddy et al., 2017) was used to monitor the size of the necrotic zones after treatment. This dye can stain only those cells blue around the treatment site, which have a compromised plasma membrane due to a microbial infection or suffer from membrane disruption by the activity of NFAP and γ^{NFAP}-opt. All treatments at *in vitro* MICs were not toxic to the plants because cell death was not indicated by Evan's blue staining (NFAP and γ^{NFAP}-opt in **Figure 3**). The same was true for the 0.1 × potato dextrose broth-treated control (0.1 × PDB in **Figure 3**). This medium was used to establish the infection and to allow conidia germination. The *B. cinerea* infected but untreated leaves exhibited extensive necrotic lesions and blue coloured zones around the infection points indicating cell death in the consequence of an established and extensive fungal infection (Bcin in **Figure 3**). Next, the tomato leaves were infected with *B. cinerea* and treated with NFAP and γ^{NFAP}-opt. The lack of intensive blue coloured zones and necrotic lesions around the inoculation points indicated that NFAP protected tomato plant leaves against *B. cinerea* infection and the invasion of the fungus into the leaf tissue (Bcin + NFAP in **Figure 3**). In contrast, γ^{NFAP}-opt was not able to fully impede fungal infection, but mitigated the symptoms. Slight blue coloured zones appeared at the inoculation points of *B. cinerea* (Bcin + γ^{NFAP}-opt in **Figure 3**).

The ability of NFAP and the antifungally active γ-core peptide derivatives to protect crops was studied on tomato fruits against *C. herbarum* FSU 1148 infection. This fungus is known as a postharvest spoilage agent of fresh fruits and vegetables, including tomatoes under storage conditions, especially when the vegetable surface was damaged (Snowden, 1992). Control treatments with NFAP, γ^{NFAP}-opt and γ^{NFAP}-optGZ did not cause any decay on the surface of the tomato fruits. The same was observed when the fruits were treated with 0.1 × PDB, the medium used for the infection. *C. herbarum* infection, instead, was established within the applied incubation period at the sting points and the deeper tissues. Application of NFAP, γ^{NFAP}-opt and γ^{NFAP}-optGZ at *in vitro* detected MIC inhibited the development of decay. No intensive fungal growth was observed on the surface or in deeper tissues of the tomato fruits.

These latter results were published in the following peer-reviewed paper.

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2018: 4.259 (Q1 Microbiology)

The developed tomato plant protection assay was adopted and utilized in the joint ANN 131341 project as follows.

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide Pγ^{opt} in plant protection. MICROBIAL BIOTECHNOLOGY. [Epub ahead of print] (2020), DOI: 10.1111/1751-7915.13559. IF2018: 4.857 (Q1 Applied microbiology and biotechnology)

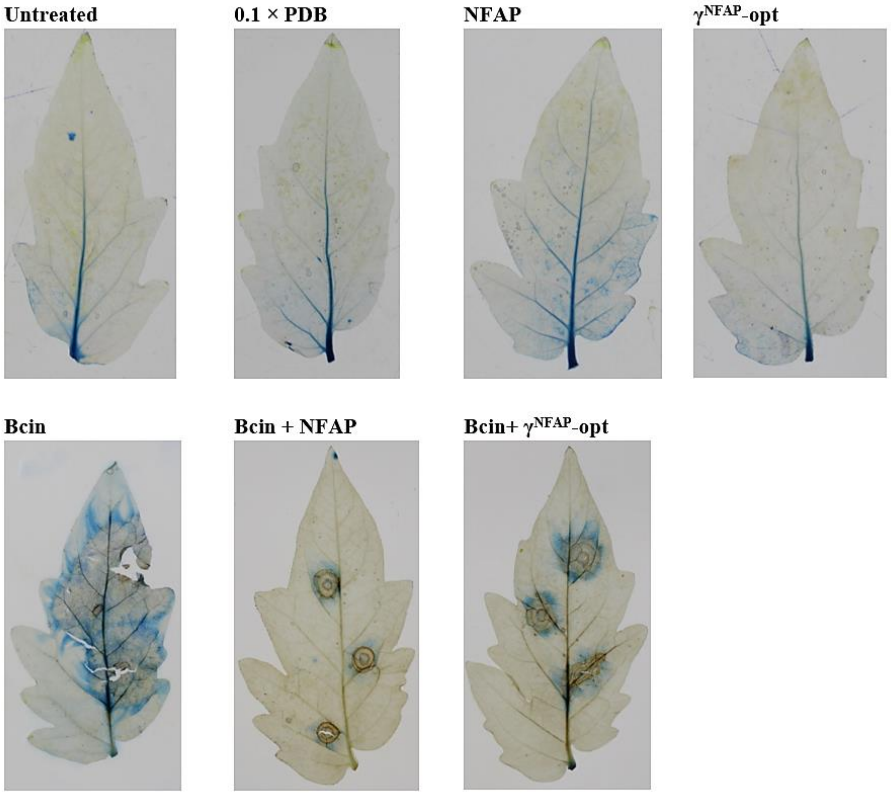

Figure 3. Evan's blue staining of untreated tomato leaves (Untreated), and tomato leaves treated with 0.1 × PDB (0.1 × PDB), 1 × MIC NFAP (NFAP), 1 × MIC γ^{NFAP}-opt (MIC γ^{NFAP}-opt), *Botrytis cinerea* (Bcin), *B. cinerea* + 1 × MIC NFAP (Bcin + NFAP), *B. cinerea* + 1 × MIC γ^{NFAP}-opt (Bcin + γ^{NFAP}-opt). Leaves were kept at 23°C, 60% humidity, and under 12 - 12 hours photoperiodic day-night simulation at 1200 lux for 4 days. Blue coloured zones or necrotic lesions on the leaves indicate cell death and established infection at site of the treatment points with *B. cinerea*.

Conclusions

Taken together, our results demonstrated that NFAP and rationally designed synthetic γ-core peptide derivatives are promising candidates for biopreservation in agriculture and food industry because:

1) **They effectively inhibit the growth of several pre- and postharvest plant pathogenic and mycotoxigenic filamentous fungi.**
2) **They do not show cytotoxic potential on human cell lines and intact plants.**
3) **They are able to inhibit the establishment of fungal infection on plant, and to protect stored crop against decay caused by postharvest moulds.**

However, further studies that focus on their environmental impact and address their pharmacokinetic properties in the human body are essential to push forward their applicability. Additionally, our results provide a proof-of-principle for biotechnological production of protein-based biofungicids and bioactive peptides. A patent application is therefore considered.

PhD thesis partially based on the project results

Liliána Tóth (2018) Isolation and characterization of *Neosartorya fischeri* antifungal protein 2 (NFAP). University of Szeged, PhD School of Biology

MSc theses based on the project results

Roberta Tóth (2017) Heterologous expression of *Neosartorya fischeri* antifungal protein 2 and its gamma-core improved variant in *Penicillium chrysogenum*. University of Szeged, Faculty of Science and Informatics, Department of Microbiology

Hargita Ficze (2019) Potential applications of *Neosartorya fischeri* antifungal protein and its peptide derivatives in the agriculture. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Project collaborators

Present project is tightly connected with the ANN 131341 project based on the regulations for NKFIH Postdoctoral Excellence Programme from 2016. The ANN 131341 is an NKFIH-FWF joint research project in cooperation with **Florentine Marx's group** (Institute of Molecular Biology, Biocenter, Medical University of Innsbruck; Innsbruck, Austria) on the investigation of the potential role of the evolutionary conserved gamma-core motif in the antifungal activity of cysteine-rich antifungal proteins from filamentous ascomycetes. The project was conducted in collaboration with the following national partners. They supported the project with those techniques that are not routinely applied in the host institution laboratories. **Gábor K. Tóth's group** (Department of Medical Chemistry at Faculty of Medicine, University of Szeged; Szeged, Hungary): Electrospray ionization mass spectrometry, reversed-phase high performance liquid chromatography solid-phase peptide and protein synthesis. **Gyula Batta's group** (Department of Organic Chemistry, Faculty of Science and Technology, University of Debrecen; Debrecen, Hungary): Nuclear magnetic resonance spectroscopy. **Attila Borics** (Chemical Biology Group of the Institute of Biochemistry, Biological Research Centre; Szeged, Hungary): Electronic circular dichroism spectroscopy. **László Majoros** and **Renátó Kovács** (Department of Medical Microbiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary): *In vivo* animal model experiments.

References

Barker D, Pfaff T, Moreau D, Groves E, Ruffel S, Lepetit M, et al. (2006) "Growing *M. truncatula*: choice of substrates and growth conditions", in *Medicago truncatula* Handbook. eds. E-P. Journet, and U. Mathesius (Ardmore: The Samuel Roberts Noble Fondation), 1−25.

El Oirdi M, Trapani A, Bouarab, K. (2010) The nature of tobacco resistance against *Botrytis cinerea* depends on the infection structures of the pathogen. Environ Microbiol 12: 239−253.

El Oirdi M, El Rahman TA, Rigano L, El Hadrami A, Rodriguez MC, Daayf F, et al. (2011) *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. Plant Cell 23: 2405−2421.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. (2005) "Protein identification and analysis tools on the ExPASy server", in The Proteomics Protocols Handbook. ed. J.M. Walker. (Totowa, NJ: Humana Press), 571−607.

Nambeesan S, AbuQamar S, Laluk K, Mattoo AK, Mickelbart MV, Ferruzzi MG, et al. (2012) Polyamines attenuate ethylene-mediated defense responses to abrogate resistance to *Botrytis cinerea* in tomato. Plant Physiol 158: 1034−1045.

Snowden A. (1992). "Solanaceous fruit vegetables", in Post-Harvest Diseases and Disorders of Fruits and Vegetables. ed. A. Snowden (London: CRC Press), 53−93.

Sonderegger C, Galgóczy L, Garrigues S, Fizil Á, Borics A, Manzanares P, et al. (2016) A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. Microb Cell Fact 15:192.

Váradi G, Tóth GK, Kele Z, Galgóczy L, Fizil Á, Batta G. (2013) Synthesis of PAF, an antifungal protein from *P. chrysogenum*, by native chemical ligation: native disulfide pattern and fold obtained upon oxidative refolding. Chemistry 19:12684−12692.

Vijayaraghavareddy P, Adhinarayanreddy V, Vemanna RS, Sreeman S., Makarla, U. (2017) Quantification of membrane damage/cell death using Evan's blue staining technique. Bio-protocol 7: e2519.

Szeged, 05 May 2020

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