The combination of *Neosartorya (Aspergillus) fischeri* antifungal proteins with rationally designed γ-core peptide derivatives is effective for plant and crop protection

Liliána Tóth · Péter Poór · Attila Ördög · Györgyi Váradi · Attila Farkas · Csaba Papp · Gábor Bende · Gábor K. Tóth · Gábor Rákhely · Florentine Marx · László Galgóczy

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Abstract Plant pathogenic fungi are responsible for enormous crop losses worldwide. Overcoming this problem is challenging as these fungi can be highly resistant to approved chemical fungicides. There is thus a need to develop and introduce fundamentally new plant and crop protection strategies for sustainable agricultural production. Highly stable extracellular antifungal proteins (AFPs) and their rationally designed peptide derivatives (PDs) constitute feasible options to meet this challenge. In the present study, their potential for topical application to protect plants and crops as combinatorial biofungicides is supported by the investigation of two *Neosartorya (Aspergillus) fischeri* AFPs (NFAP and NFAP2) and their γ-core PDs. Previously, the biofungicidal potential of NFAP, its rationally designed γ-core PD (γNFAP-opt), and NFAP2 was reported. Susceptibility tests in the present study extended the in vitro antifungal spectrum of NFAP2 and its γ-core PD (γNFAP2-opt) to *Botrytis*, *Cladosporium*, and *Fusarium* spp. Besides, in vitro additive or indifferent interactions, and synergism were observed when NFAP or NFAP2 was applied in combination with γNFAP-opt. Except for...
\(\gamma^{\text{NFAP}}\text{-opt}\), the investigated proteins and peptides did not show any toxicity to tomato plant leaves. The application of NFAP in combination with \(\gamma^{\text{NFAP}}\text{-opt}\) effectively inhibited conidial germination, biofilm formation, and hyphal extension of the necrotrophic mold \textit{Botrytis cinerea} on tomato plant leaves. However, the same combination only partially impeded the \textit{B. cinerea}-mediated decay of tomato fruits, but mitigated the symptoms. Our results highlight the feasibility of using the combination of AFP and PD as biofungicide for the fungal infection control in plants and crops.

**Keywords** Neosartorya (Aspergillus) fischeri antifungal proteins · \(\gamma\)-core peptide · Plant pathogenic fungus · Biofungicide · Drug combination · Synergism

**Introduction**

Pre- and post-harvest phytopathogenic fungi cause enormous crop losses worldwide every year and threaten the increase in food supply to the human population despite the intensive agricultural application of chemical fungicides. The development of more efficient, alternative management strategies to control fungal diseases may overcome this problem (Avery et al. 2019). Alternatively, bioactive natural products for plant protection have already been used as biofungicides in sustainable agricultural production systems to reduce the impact of fungal diseases on crops. These natural compounds (e.g., phenolics, flavones, quinones, tannins, terpenes, essential oils, alkaloids, and saponins) act directly as antimicrobial agents and/or indirectly as inducers of plant defense (Gwinn 2018). Natural or rationally designed proteins and peptides from different origins with antifungal activity (van der Weerden et al. 2013) and/or with plant immunostimulatory effects (Campos et al. 2018) are also effective alternatives in an agricultural setting to fight against phytopathogenic and mycotoxicogenic fungi. Primarily, they are expressed as recombinant antifungal proteins/peptides in transgenic plants to confer increased resistance to fungal pathogens (Iqbal et al. 2019). However, different international regulations for genetically modified (GM) plant breeding (Eriksson 2019) and the spread of anti-GM sentiment among the public worldwide (Tagliabue 2018) limit the application of these cultivars. The direct environmental application of antifungal proteins and peptides as topical biofungicides in plant protection has several limitations, such as a narrow antifungal spectrum, potential toxic effects on humans and animals and molecular structures that are easily degradable by extracellular proteases (Jung and Kang 2014). The rational design and the development of new formulations for the application of antimicrobial peptides might overcome these problems (Porto et al. 2012; Ajingi and Jongruja 2020). Solid-phase peptide synthesis based on 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry is becoming more economic nowadays alleviating high production costs (Behrendt et al. 2016). This chemical method could provide an alternative for the industrial-scale production of antifungal proteins and peptides in the future, considering their antimicrobial activity in a low concentration range (\(\mu\text{M}\)) (Hegedüs and Marx 2013). Proof of concept studies previously reported on the economic production of synthetic antifungal peptides with a small chemical footprint, and their cost-effective application as microbicides in an agricultural setting (Rautenbach et al. 2016).

Further studies documented the safe applicability of recombinant small, cysteine-rich, cationic antifungal proteins of ascomycetous origins (AFPs) as biofungicides in plants and crops to fight infection with phytopathogenic filamentous fungi (Vila et al. 2001; Moreno et al. 2003, 2006; Theis et al. 2005; Barna et al. 2008; Leiter et al. 2017; Garrigues et al. 2018). Only recently, we provided information about the biofungicidal potential and safe agricultural application of AFPs, and their rationally designed peptide derivatives (PDs). The \textit{Penicillium chrysogenum} antifungal protein (PAF) effectively inhibited the growth of numerous pre- and post-harvest pathogenic fungi in vitro and showed no toxic effects on mammalian cells and plant seedlings (Tóth et al. 2020a). Furthermore, its topical application protected tomato plant leaves against \textit{Botrytis cinerea} infection (Tóth et al. 2020a). Similar observations regarding the antifungal efficacy and potential agricultural applicability were reported for the \textit{Neosartorya (Aspergillus) fischeri} antifungal protein (NFAP) and its PD (\(\gamma^{\text{NFAP}}\text{-opt}\)), which was designed based on the evolutionary conserved antimicrobial \(\gamma\)-core motif of NFAP (Tóth et al. 2020b). NFAP and \(\gamma^{\text{NFAP}}\text{-opt}\) inhibited the development of decay lesions on tomato fruits caused.
The combination of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins... by *Cladosporium herbarum*, proving their feasibility as biopreservative agents in agriculture (Tóth et al. 2020b). The NFAP related *N. (A.) fischeri* antifungal protein 2 (NFAP2; Tóth et al. 2016) was detected to be moderately active in vitro against the post-harvest fungi *Penicillium digitatum* and *Penicillium italicum*, and to control *P. digitatum* infection of citrus fruit (Gandía et al. 2021).

In the present study, we extended the antifungal spectrum of NFAP2 and its rationally designed γ-core PD (γNFAP2-opt) to more pre- and post-harvest plant pathogenic fungi. Additionally, we analyzed the nature of the in vitro interaction of different combinations of NFAP, NFAP2, and their respective γ-core PDs and determined their growth inhibition potential against selected plant pathogenic fungal strains. The results let hypothesize that a combination of *Neosartorya* AFP and PD, showing a synergistic interaction, can be safely administered to protect plants and crops from fungal infection, which has never been tested before. Our assumption was evidenced by the combined treatment of tomato plants infected with the necrotrrophic fungal pathogen *B. cinerea* with NFAP and γNFAP-opt. In this biocontrol experiment, lower effective concentrations of NFAP and γNFAP-opt, when applied in combination, were sufficient to achieve the same protective effect as at higher concentrations in single use. Therefore, our study suggests that the combination of AFPs with PDs is a cost-effective biocontrol strategy and might also limit resistance development.

**Materials and methods**

**Strains and media**

Pre- and post-harvest plant pathogenic fungal strains used in the antifungal susceptibility tests are listed in Table 1. They were maintained on potato dextrose.

### Table 1 Minimal inhibitory concentrations (µg ml⁻¹) of *Neosartorya* antifungal proteins and peptide derivatives against plant pathogenic filamentous fungi (in alphabetic order)

| Isolate | NFAP* | γNFAP-opt* | NFAP2 | γNFAP2-opt | Origin of isolate |
|---------|-------|------------|-------|------------|------------------|
| *Aspergillus flavus* SZMC 3014 | 100 | > 200 | > 200 | > 200 | *Triticum aestivum/Hungary* |
| *Aspergillus flavus* SZMC 12618 | 100 | > 200 | > 200 | > 200 | *Triticum aestivum/Hungary* |
| *Aspergillus* flavus SZMC 20745** | 12.5 | > 200 | > 200 | > 200 | *Zea mays/Hungary* |
| *Aspergillus flavus* SZMC 20755** | 25 | > 200 | > 200 | > 200 | *Zea mays/Hungary* |
| *Aspergillus niger* SZMC 0145 | 50 | > 200 | > 200 | > 200 | *Fruits/Hungary* |
| *Aspergillus niger* SZMC 2759 | 50 | > 200 | > 200 | > 200 | *Raisin/Hungary* |
| *Aspergillus* velutusiae SZMC 21821 | 25 | > 200 | > 200 | > 200 | *Allium cepa/Hungary* |
| *Aspergillus* velutusiae SZMC 21832 | 12.5 | > 200 | > 200 | > 200 | *Allium cepa/Hungary* |
| *Botrytis cinerea* SZMC 21472** | 6.25 | 200 | 50 | 200 | *Rubus idaeus/Hungary* |
| *Botrytis cinerea* SZMC 21474 | 50 | 50 | 12.5 | 12.5 | *Fragaria × ananassa/Hungary* |
| *Botrytis cinerea* NCAIM F.00751 | 50 | 50 | 12.5 | 12.5 | *Hungary* |
| *Botrytis pseudocinerea* SZMC 21470 | 100 | 100 | 12.5 | 50 | *Brassica napus/Hungary* |
| *Botrytis pseudocinerea* SZMC 21471 | 100 | 100 | 12.5 | 200 | *Brassica napus/Hungary* |
| *Cladosporium herbarum* FSU 1148 | 100 | 12.5 | 12.5 | 100 | n.d |
| *Cladosporium herbarum* FSU 969 | 100 | 12.5 | 12.5 | 100 | n.d |
| *Fusarium boothii* CBS 110250 | 25 | 50 | > 200 | > 200 | *Zea mays/South Africa* |
| *Fusarium graminearum* SZMC 6236J | 25 | 50 | > 200 | > 200 | *Vegetables/Hungary* |
| *Fusarium oxysporum* SZMC 6237J | 25 | 50 | 50 | > 200 | *Vegetables/Hungary* |
| *Fusarium solani* CBS 115659 | 50 | 12.5 | > 200 | 50 | *Solanum tuberosum/Germany* |
| *Fusarium solani* CBS 119996 | 100 | 50 | > 200 | 200 | *Daucus carota/The Netherlands* |

n.d. data not available

*According to Tóth et al. (2020b)*

**MIC determination for NFAP and γNFAP-opt in this study**
agar (Sigma–Aldrich, St. Louis, MO, USA) slants at 4 °C. Antifungal susceptibility tests, treatments for plant toxicity, and biocontrol assays were performed in tenfold diluted potato dextrose broth (0.1×PDB; Sigma–Aldrich).

Cultivation of tomato plants

Tomato plant seeds (Solanum lycopersicum L. cv. Ailsa Craig) were germinated for three days at 27 °C in the dark. Then, the seedlings were transferred to Perlite (bulk density: 90–110 kg m⁻³, particle size: 3–6 mm, moisture content: > 2%, pH = 6.0–7.5; Agrolit Kft., Olaszliszka, Hungary) for 14 days. After that, the plants were grown in a controlled environment applying 200 μmol m⁻² s⁻¹ photon flux density with a L:D 12:12 photoperiod, day/night temperatures of 23/20 °C, and RH of 55–60% for four weeks in hydroponic culture, in accordance with the work of Poór et al. (2011).

Production of proteins and peptide derivatives

Recombinant NFAP and NFAP2 were produced in a P. chrysogenum-based expression system and purified by cation-exchange chromatography, as described previously (Sonderegger et al. 2016; Tóth et al. 2018). To reach maximum protein purity (100%), an additional semipreparative reverse-phase high-performance liquid chromatography step was applied after the cation-exchange chromatography, which was performed as described previously for NFAP2 (Kovács et al. 2019). The peptide γ\text{NFAP}-opt (Tóth et al. 2020b), the peptide spanning the NFAP2 γ-core motif (γ\text{NFAP}2), and its rationally designed variant (γ\text{NFAP}2-opt) were synthesized applying Fmoc chemistry, as described by Sonderegger et al. (2018) (Table 2).

Table 2 Amino acid sequences and in silico predicted physicochemical properties of the investigated Neosartorya antifungal proteins and peptide derivatives

| 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 |
|-----------------|-----------------------|------------------------|---------------|-----------------------|----------------|-------------------------|-------|
| LEYKGECKTDNTCKYKIDKGYLAKCSAACTKEKDGNCTYDYSNRKVKCDFRH | 57 | 6.6 | 6 | 11/2/1 | 8.93 | +5.0 | −1.214 |
| Ac-EYKGKC(-SH)KTKKNC(-SH)K-NH₂ | 14 | 1.7 | 2 | 7/0/0 | 9.84 | +5.8 | −2.264 |
| γ\text{NFAP}-opt* | 14 | 1.6 | 2 | 6/0/0 | 10.05 | +5.8 | −1.079 |
| IATSPYYACNCPNCKKKGSCKYHSGPSDKSISKVSGKEWGGQLNCIAT | 52 | 5.6 | 6 | 7/0/2 | 9.01 | +5.2 | −0.731 |
| Ac-VISKGKC(-SH)EWQGGQLNC(-SH)K-NH₂ | 14 | 1.6 | 2 | 6/0/0 | 10.05 | +5.8 | −1.079 |
| γ\text{NFAP}2 | 16 | 1.8 | 2 | 7/0/2 | 9.01 | +5.2 | −0.731 |
| Ac-VISKGKC(-SH)KTKKNC(-SH)K-NH₂ | 15 | 1.7 | 2 | 7/0/2 | 9.01 | +5.2 | −0.731 |
| γ\text{NFAP}2-opt | 14 | 1.6 | 2 | 6/0/0 | 10.05 | +5.8 | −1.079 |

*According to Tóth et al. (2020b)
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(Pillai et al. 2005). Susceptibility tests were prepared in three technical replicates and repeated two times.

Plant toxicity assay

AFPs and PDs were dissolved in sterile distilled water and dropped in 10 µl aliquots at five points between the left lateral veins of the abaxial leaf epidermis from fully expanded leaves of the second leaf level of tomato plants. The following concentrations were applied: 12.5 µg ml⁻¹ NFAP, 400 µg ml⁻¹ γNFAP-opt, 100 µg ml⁻¹ NFAP2, and 400 µg ml⁻¹ γNFAP2-opt. The plants were then kept in a humid (60%) plant growth chamber for three weeks at 23 °C under photoperiodic day–night simulation (12/12 h with or without illumination at 1200 lx). After this treatment, the leaves were detached and Evan’s blue staining was applied to visualize the necrotic zone around the treatment points, as reported by Tóth et al. (2020b). The stained leaves were photographed with a Canon EOS 700D camera (Tokyo, Japan). Two branches each with five leaves of two plants for each treatment were used in one experiment. Plant toxicity assays were repeated two times.

Scanning electron microscopy (SEM)

SEM experiments were performed on tomato plant leaves. In this set-up, *B. cinerea* SZMC 21472 conidia (10⁷ conidia ml⁻¹) were mixed with NFAP (6.25 µg ml⁻¹ and 1.56 µg ml⁻¹) or γNFAP-opt (200 µg ml⁻¹ and 6.25 µg ml⁻¹). In combinatorial assays, NFAP and γNFAP-opt were applied together at the concentration of 1.56 µg ml⁻¹ and 6.25 µg ml⁻¹, respectively. Ten microliters of this suspension were spotted on three points between the lateral veins of the abaxial leaf epidermis. Leaves treated with conidial suspension without protein/peptide were used as infection controls. Untreated leaves served as uninfected controls. The leaves were placed in Petri dishes containing three sterilized filter papers (0113A00009 qualitative filter paper; Filters Fioroni, Ingré, France) wetted with sterile ddH₂O. These Petri dishes were kept in a humid (60%) plant growth chamber at 23 °C under photoperiodic day–night simulation (12/12 h with or without illumination at 1200 lx). After incubation for four days, the infected leaf zones around the conidial spots were clipped out, and fixed with 2.5% (v/v) glutaraldehyde and 0.05 M cacodylate buffer (pH=7.2) in phosphate buffered saline (PBS; pH=7.4) overnight at 4 °C. Then, they were washed twice with PBS and dehydrated with a graded ethanol series [30%, 50%, 70%, 80% (v/v)] for 2 h each at room temperature, and stored in 100% (v/v) ethanol overnight at 4 °C. The discs were dried with a Quorum K850 critical-point dryer (Quorum Technologies, Laughton, UK), followed by 12 nm gold coating, and observed under a JEOL JSM-7100F/LV field emission scanning electron microscope (JEOL Ltd., Tokyo, Japan). Three leaves for each treatment were analyzed and the experiment was repeated two times.

Plant and crop protection assays

Plant and crop protection assays were performed on detached *B. cinerea* SZMC 21472-infected tomato plant leaves and on tomato fruits, respectively, in accordance with the procedures reported by Tóth et al. (2020a, b). The protective effect of NFAP (6.25 µg ml⁻¹ and 1.56 µg ml⁻¹) and γNFAP-opt (200 µg ml⁻¹ and 6.25 µg ml⁻¹) was tested. In combinatorial assays, NFAP and γNFAP-opt were applied together at the concentration of 1.56 µg ml⁻¹ and 6.25 µg ml⁻¹, respectively. The incidence of the infection was calculated in percentage. Statistical analyses were performed using the Statistics Kingdom online platform to calculate Levene’s, one-way ANOVA, and Tukey’s HSD post-hoc tests (Statistics Kingdom 2021). Plant protection assays were repeated three times involving three technical replicates, while the crop protection assays were repeated two times involving three technical replicates.

Results

In vitro antifungal potential of *N. fischeri* AFPs and their PDs against pre- and post-harvest plant pathogenic fungi

In the present study, we investigated the antifungal activity of NFAP2 and its rationally designed PD γNFAP2-opt. For comparative purposes, the data of this study are summarized with the data reported by Tóth et al. (2020b) in Table 1. NFAP2 showed high growth inhibitory efficacy against *Botrytis* (MIC = 12.5–50 µg ml⁻¹) and *Cladosporium* isolates (MIC = 12.5 µg ml⁻¹). However, none of the tested...
Aspergilli and fusaria were susceptible in the investigated protein concentration range. The PD spanning the C-terminal γ-core motif of NFAP2 (γ NFAP2 in Table 2), had no antifungal activity (data not shown). However, the rationally designed PD γ NFAP2-opt (Table 2) was more positively charged and hydrophilic and inhibited the growth of Botrytis, Cladosporium, and Fusarium isolates at MICs ranging between 12.5 and 200 µg ml⁻¹.

Toxicity of N. fischeri AFPs and their PDs on plant leaves

The potential toxic effects of antifungal active N. fischeri AFPs and PDs were investigated on intact tomato plant leaves at concentrations twofold higher than the respective MIC detected in vitro against the necrotrophic fungal pathogen B. cinerea SZMC 21472. The applied Evan’s blue staining (Vijayara-ghavareddy et al. 2017), which is an appropriate method to monitor necrotic areas at the treatment points, did not indicate any plant cell killing ability of NFAP, NFAP2, and γ NFAP-opt (Fig. 1). However, the area where γ NFAP2-opt was applied stained blue was indicative for plant cell cytotoxicity (Fig. 1). Therefore, this PD was excluded from further experiments.

Antifungal activity of N. fischeri AFPs and their PDs in combination

The checkerboard titration method was applied to reveal the nature of the interaction when Neosartorya AFPs (NFAP and NFAP2) were combined with each other or with γ NFAP-opt, against Aspergillus flavus SZMC 20745, B. cinerea SZMC 21472, Cladosporium herbarum FSU 1148, and Fusarium oxysporum SZMC 6237J (Table 3). Based on the FICI values, the NFAP+γ NFAP-opt combination showed a synergistic interaction against B. cinerea SZMC 21472 (FICI=0.28) and C. herbarum FSU 1148 (FICI=0.31), respectively. Other combinations of AFPs and γ NFAP-opt resulted in additive or indifferent interactions on these two plant pathogens (FICI=1.25–1.50). The same additive or indifferent interaction was found with NFAP2+γ NFAP-opt on F. oxysporum SZMC 6237J. Notably, no MICs could be determined with A. flavus SZMC 20745 upon exposure to the combinations NFAP+NFAP2, NFAP+γ NFAP-opt,

**Fig. 1** Evan’s blue staining of tomato plant leaves to monitor cytotoxic effects of Neosartorya fischeri NRRL 181 anti-fungal proteins and their peptide derivatives. The leaves were treated with 10 µl aliquots of NFAP (12.5 µg ml⁻¹) (e), NFAP2 (100 µg ml⁻¹) (d), γ NFAP-opt (400 µg ml⁻¹) (e), and γ NFAP2-opt (400 µg ml⁻¹) (f) and the appearance of necrotic areas was compared with that of control leaves left untreated (a) or treated with 10 µl of 0.1×PDB (b). Blue-colored zones (marked by black arrows) indicate cell death at the treatment points. Scale bars represent 1 cm. (Color figure online)
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Biocontrol efficacy of NFAP, \( \gamma \text{NFAP}-\text{opt} \) and their synergistic combination

Based on the synergistic activity of NFAP and \( \gamma \text{NFAP}-\text{opt} \) on *B. cinerea* and of NFAP2 and \( \gamma \text{NFAP}-\text{opt} \) on *C. herbarum* in vitro (Table 3), we hypothesized that a combination of specific AFPs and PDs should have a remarkable biocontrol activity and allow the reduction of the antifungal effective dosage of both compounds to reach the same protection against fungal infection as the single application at their individual MICs. To provide a proof of principle, we tested this assumption in biocontrol assays and characterized the antifungal efficacy of the combination of NFAP and \( \gamma \text{NFAP}-\text{opt} \) on tomato plants against the infection with *B. cinerea*, the most common necrotrophic pathogen of above-ground parts of this plant (Nambeesan et al. 2012).

Detached tomato plant leaves were infected with *B. cinerea* SZMC 21472 conidia and treated with NFAP or \( \gamma \text{NFAP}-\text{opt} \) at their MIC (6.25 µg ml\(^{-1}\) and 200 µg ml\(^{-1}\), respectively), NFAP or \( \gamma \text{NFAP}-\text{opt} \) at

Table 3  Checkerboard titration results of *Neosartorya* antifungal proteins and \( \gamma \text{NFAP}-\text{opt} \) peptide against pre- and post-harvest pathogenic fungi based on the fractional inhibitory concentration index (FICI) values

|                  | *Aspergillus flavus* SZMC 20745 | *Botrytis cinerea* SZMC 21472 | *Cladosporium herbarum* FSU 1148 | *Fusarium oxysporum* SZMC 6237J |
|------------------|---------------------------------|-------------------------------|----------------------------------|--------------------------------|
| **NFAP + NFAP2** |                                 |                               |                                  |                                 |
| NFAP (MIC)       | 12.5                            | 6.25                          | 100                              | 25                              |
| NFAP2 (MIC)      | > 200                           | 50                            | 12.5                             | 50                              |
| NFAP (MIC\text{comb}) | > 25                       | 6.25                          | 100                              | > 50                            |
| NFAP2 (MIC\text{comb}) | > 200                     | 12.5                          | 3.125                            | > 50                            |
| FICI             | –                               | 1.25                          | 1.25                             | –                               |
| Type of the interaction | \(^*\) Additive or indifference | Additive or indifference | – \(^*\)                          |
| **NFAP + \( \gamma \text{NFAP-opt} \)** |                                 |                               |                                  |                                 |
| NFAP (MIC)       | 12.5                            | 6.25                          | 100                              | 25                              |
| \( \gamma \text{NFAP-opt} \) (MIC) | > 200                        | 200                            | 12.5                             | 50                              |
| NFAP (MIC\text{comb}) | > 25                       | 1.56                          | 100                              | > 25                            |
| \( \gamma \text{NFAP-opt} \) (MIC\text{comb}) | > 200                     | 6.25                          | 6.25                             | > 50                            |
| FICI             | –                               | 0.28                          | 1.50                             | –                               |
| Type of the interaction | \(^*\) Synergy               | Additive or indifference       | Additive or indifference          | \(^*\)                           |
| **NFAP2 + \( \gamma \text{NFAP-opt} \)** |                                 |                               |                                  |                                 |
| NFAP2 (MIC)      | > 200                           | 50                            | 12.5                             | 50                              |
| \( \gamma \text{NFAP-opt} \) (MIC) | > 200                        | 200                            | 12.5                             | 50                              |
| NFAP2 (MIC\text{comb}) | > 200                      | 12.5                          | 0.78                             | 50                              |
| \( \gamma \text{NFAP-opt} \) (MIC\text{comb}) | > 200                     | 200                            | 3.125                            | 12.5                            |
| FICI             | –                               | 1.25                          | 0.31                             | 1.25                            |
| Type of the interaction | – Additive or indifference    | Synergy                       | Additive or indifference          |                                  |

MIC and MIC\text{comb} MIC of antifungal protein/peptide when applied alone and in combination, respectively. Type of the interaction: 0.5 ≤ FICI ≤ 4.0: additive or indifference, FICI < 0.5: synergy, FICI > 4.0: antagonism (Pillai et al. 2005)

\(^*\) paradoxical effect
concentrations below the MIC (1.56 µg ml⁻¹ and 6.25 µg ml⁻¹, respectively), the synergistic combination of NFAP (1.56 µg ml⁻¹) and γNFAP-opt (6.25 µg ml⁻¹), and the incidence of infection was compared to the infected, but untreated control. ANOVA showed that there was a significant difference between the incidences of infections at the six different treatments (F₅,₁₂ = 25.58, p < 0.001). In leaves that were infected but left untreated, the appearance of necrotic lesions and intensive Evan’s blue staining around the infection areas indicated the severe destruction of tomato plant leaf tissues by B. cinerea (Bcin in Fig. 2c). The application of NFAP or γNFAP-opt at their MIC (6.25 µg ml⁻¹ and 200 µg ml⁻¹, respectively) protected the leaves against this fungal pathogen: necrotic lesions and intensive blue staining were not observed under these experimental conditions (NFAP(MIC) and γNFAP-opt(MIC) in Fig. 2e and g). The incidence of infection was significantly lower (p < 0.001 according to the Tukey’s HSD post-hoc test) compared to the infected leaves that were left untreated (Fig. 2i). The application of NFAP or γNFAP-opt at concentrations below the MIC was not effective (NFAP(MICcomb) and γNFAP-opt(MICcomb) in Fig. 2i). NFAP mitigated the symptoms of B. cinerea infection at a concentration of 1.56 µg ml⁻¹ by reducing the area of tissue destruction, but could not fully protect the leaves from infection (NFAP(MICcomb) in Fig. 2f). In contrast, 6.25 µg ml⁻¹ γNFAP-opt did not prevent the development of infection: extensive necrotic lesions and blue-colored zones were visible at the points of B. cinerea inoculation (γNFAP-opt(MICcomb) in Fig. 2h). Notably, the synergistic combination of NFAP (1.56 µg ml⁻¹) and γNFAP-opt (6.25 µg ml⁻¹) significantly (p < 0.001 according to the Tukey’s HSD post-hoc test) reduced the invasion of the fungus into the leaf tissue and protected tomato plant leaves against B. cinerea infection (Comb in Fig. 2d and i). According to Tukey’s HSD post-hoc test, there was no significant difference in the efficacy between the protective effects of Comb and NFAP(MIC) (p = 0.988), Comb and γNFAP-opt(MIC) (p = 1.000), NFAP(MIC) and γNFAP-opt(MIC) (p = 0.988). The above results indicated that combined application of NFAP and γNFAP-opt allowed the reduction of their effective dosage to achieve the same protective effect against B. cinerea infection as high concentrations of these compounds in single use.

Next, we visualized the infection of leaves with B. cinerea and the protective effect of NFAP and γNFAP-opt by SEM. The results indicated that without any treatment, the hyphae colonized the leaf surface forming a dense mycelium, which spread out beyond the inoculation area (Bcin in Fig. 3b). The application of NFAP at its MIC (6.25 µg ml⁻¹) and at the lower concentration of 1.56 µg ml⁻¹ reduced the dispersion of the fungal infection from the treatment areas (NFAP(MIC) and NFAP(MICcomb) in Fig. 3d and f, respectively). The same was true for γNFAP-opt when applied at its MIC (200 µg ml⁻¹) (γNFAP-opt(MIC) in Fig. 3e), while at lower concentration (6.25 µg ml⁻¹) this peptide was not effective enough to inhibit the colonization of the leaf surface beyond the treatment area (γNFAP-opt(MICcomb) in Fig. 3g). The synergistic combination of NFAP and γNFAP-opt (1.56 µg ml⁻¹ and 6.25 µg ml⁻¹, respectively) significantly reduced the colonization and hyphal extension of B. cinerea SZMC 21472 on the leaf (Comb in Fig. 3c). The SEM analysis further revealed that B. cinerea SZMC 21472 established a biofilm on the leaves which consisted of several layers of well-developed hyphae (Bcin in Fig. 4b). When applied at their MIC, NFAP (6.25 µg ml⁻¹) and γNFAP-opt (200 µg ml⁻¹) destroyed most of the conidia and germlings, and reduced biofilm formation (NFAP(MIC) and γNFAP-opt(MIC) in Fig. 4d and e, respectively). At concentrations lower than the respective MIC, NFAP (1.56 µg ml⁻¹) and γNFAP-opt (6.25 µg ml⁻¹) did not prevent the germination of conidia and the initiation of biofilm formation (NFAP(MICcomb) and γNFAP-opt(MICcomb) in Fig. 4f and g, respectively). The synergistic combination of NFAP (1.56 µg ml⁻¹) and γNFAP-opt (6.25 µg ml⁻¹), however, remarkably reduced the germination ability of B. cinerea SZMC 21472 conidia, and hampered the formation of a biofilm (Comb in Fig. 4c).

Finally, we tested the biocontrol efficacy of NFAP, γNFAP-opt and their combination in the protection of tomato fruits from fungal infection. None of the treatments applied fully protected tomato fruits from B. cinerea-caused decay. However, the treatment with the MIC of NFAP or γNFAP-opt and their synergistic combination decreased the fungal spread on the fruit surface (data not shown).
The combination of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins…

**Figure 2** Evan’s blue staining of necrotic plant tissue on tomato plant leaves after *Botrytis cinerea* SZMC 21472 infection in comparison with the uninfected control (a). Leaves were treated with 0.1×PDB (b), *B. cinerea* (Bcin) (c), *B. cinerea* + synergistic combination of NFAP and γNFAP_opt (Comb: 1.56 and 6.25 µg ml⁻¹, respectively) (d), *B. cinerea* + MIC of NFAP (NFAP_MIC: 6.25 µg ml⁻¹) (e), *B. cinerea* + 1.56 µg ml⁻¹ NFAP (NFAP_MICcomb) (f), *B. cinerea* + MIC of γNFAP_opt (γNFAP_opt_MIC: 200 µg ml⁻¹) (g), *B. cinerea* + 6.25 µg ml⁻¹ γNFAP_opt (γNFAP_opt_MICcomb) (h). Blue-colored zones indicate cell death at the treatment points. Scale bars represent 1 cm.

(i) Incidence of *B. cinerea* SZMC 21472 infection on the treated leaves in comparison with the untreated controls (Bcin). Bars represent the mean ± SE of developed infection at treatment points (n = 3). a: significant difference (p < 0.001) in comparison with infected, untreated leaves (Bcin). b: significant difference (p < 0.001) in comparison with leaves that were infected and treated with synergistic NFAP and γNFAP_opt combination (Comb). (Color figure online)

**Discussion**

There is an urgent need to develop new antifungal treatment strategies in order to counteract the enormous crop losses due to fungal infection and contamination, and to support the increase in global calorie consumption in the coming decades. In the present study, we further evidenced the potential applicability of AFPs of ascomycetous origin and their rationally designed PDs for protecting plants...
and crops from infection with phytopathogenic fungi.

In our previous studies, we reported that PAF from *P. chrysogenum* and NFAP from *N. fischeri* inhibit the growth of several pre- and post-harvest plant pathogenic fungi in vitro, and they differed in their antifungal spectrum and efficacy (Tóth et al. 2020a, b). We observed that these features are highly dependent on the amino acid composition of the evolutionary conserved γ-core motif (consensus sequence GXC-X[3–9]-C) of the protein, in which X can be any amino acid (Sonderegger et al. 2018).
Increasing the positive charge and hydrophilicity of this motif by amino acid substitutions elevated the antifungal efficacy of PAF against yeasts (Sonderegger et al. 2018), and changed its antifungal spectrum on phytopathogenic molds (Tóth et al. 2020a). These results led us to assume that the antifungal activity of AFPs is at least in part regulated by the γ-core region. This assumption was further supported by the observation that short synthetic peptides spanning the γ-core region of PAF and NFAP are antifungal active and that their efficacy is increased by elevating the positive net charge (Sonderegger et al. 2018; Tóth et al. 2020a, b). NFAP2 is primarily known as an anti-yeast AFP (Tóth et al. 2016, 2018). However, our recent study provided information about its growth inhibitory activity on post-harvest pathogenic Penicillium spp. (Gandía et al. 2021). Here we extended the antifungal spectrum of NFAP2 to other plant pathogenic fungi, such as B. cinerea, B. pseudocinerea, C. herbarum, and F. oxysporum (Table 1). Our results clearly indicate that NFAP2 is not solely a yeast-specific AFP, as we previously supposed. Unsurprisingly, the NFAP2-derived PD, spanning the native γ-core motif (γNFAP2 in Table 2), did not inhibit fungal growth, but its rationally designed variant with elevated positive charge and increased hydrophilicity (γNFAP2-opt in Table 2) showed remarkable antifungal activity (Table 1). These results strengthen our previous observations regarding the features of γ-core PDs of PAF and NFAP, namely, that a high positive net charge improves the antifungal efficacy (Sonderegger et al. 2018; Tóth et al. 2020a, b).

The agricultural application of AFPs and PDs requires their good tolerance in plants and mammals. We already proved that NFAP (Tóth et al. 2020b) and NFAP2 (Kovács et al. 2019) are non-toxic to mammalian cell lines. The rationally designed γ-core PDs with high positive net charge and low hydrophilicity may have adverse effects on the viability of mammalian cells (Tóth et al. 2020b). Evans’ blue staining indicated that NFAP and NFAP2 are non-toxic to plants (Fig. 1), similar to the results obtained for PAF and its γ-core optimized protein variant (Tóth et al. 2020a). This was also true for the highly hydrophilic PD γNFAP-opt. Although the net charge of γNFAP2-opt is similar to that of γNFAP-opt (Table 2), it is less hydrophilic and negatively affects plant cells (Fig. 1). One might speculate that the potential toxicity of short γ-core PDs on plant cells depends on their overall hydrophobicity.

The combinatorial application of antifungal compounds with different modes of action is considered when the infective fungus shows low susceptibility or resistance to one of these molecules, and/or prolonged administration of a single drug at a high dosage is toxic to the host or promotes the development of resistance (Hill and Cowen 2015). In case that there is a synergistic or additive interaction of two antifungal compounds, their co-administration allows a reduction in the effective dosage for successful therapy. It may also shorten the treatment period, decrease the risk of toxic effects in the host, and minimize the potential of the fungus to develop resistance (Belanger et al. 2015). Therefore, we investigated in the present study the in vitro interaction between Neosartorya AFPs and PDs and the efficacy of their combined application for protecting plants and crops against B. cinerea infection. The successful combination of Aspergillus giganteus AFP and the insect-derived antifungal peptide cecropin A against B. cinerea was reported by Moreno et al. (2003), who observed an additive effect of these two compounds in vitro in combinatorial titration assays. In agreement with this finding, an additive effect was detected with the two Neosartorya AFPs (NFAP and NFAP2) (Table 3). However, we proved that NFAP and NFAP2 synergistically interact with the rationally designed PD γNFAP-opt in vitro (Table 3). Similarly to these results, in vitro synergistic interactions between PAF and PDs derived from the P. digitatum antifungal protein B (AfpB) against the post-harvest mold P. digitatum, and between PAF and a rationally designed antifungal hexapeptide (PAF 26) against P. digitatum and Aspergillus niger were documented by Garrigues et al. (2017).

The observed synergistic interaction between NFAP and γNFAP-opt against B. cinerea could result from their different modes of action or cellular targets. NFAP induces apoptosis in Aspergillus fumigatus via a heterotrimeric G-protein signaling pathway (Virágh et al. 2015), or by binding to an intracellular target in Neurospora crassa after its internalization by an energy-dependent uptake mechanism (Hajdu et al. 2019). Annexin V-FITC/propidium iodide staining revealed that NFAP triggers apoptosis that results in necrosis in B. cinerea conidia after a 16 h incubation, whereas γNFAP-opt is a membrane-acting peptide...
that does not induce apoptosis, but readily (4 h incubation) disrupts the outer layers of B. cinerea conidia (Supplementary Fig. S1). The observed synergism between NFAP and $\gamma_{NFAP}$-opt suggests that killing of fungal pathogens by their combination results from different antifungal mechanisms.

The synergistic activity of NFAP and $\gamma_{NFAP}$-opt administered in combination in vitro and in the biocontrol experiments was clearly detectable. The bioassays evidenced that the combination of reduced dosages of NFAP and $\gamma_{NFAP}$-opt protected tomato plant leaves against B. cinerea infection as effectively as their application alone at their MICs (Figs. 2 and 3). More importantly, this synergistic activity inhibited the ability of B. cinerea to form a biofilm on detached tomato plants leaves (Comb in Fig. 4), which was unambiguously documented by SEM analysis (Bcin in Fig. 4). This parallels previous descriptions of B. cinerea growing in heavily layered extensive hyphal networks embedded in an extracellular polymeric substance matrix on tomato stems (Harding et al. 2010). Biofilm formation of plant pathogenic fungi plays a critical role in the pathogenesis of plant diseases, and underlines the need for developing novel plant disease management strategies (Villa et al. 2017).

Recently, we demonstrated for the first time in fruit protection experiments that combinations of AFPs of different fungal origin (such as P. chrysogenum antifungal protein B from P. chrysogenum, Penicillium expansum antifungal protein A from P. expansum, and NFAP2 from N. fischeri) did not improve the efficacy to protect orange and apple fruits from infection with the postharvest molds P. digitatum and P. expansum compared to single treatments (Gandía et al. 2021). We observed also in the present study that the application of a synergistic combination of NFAP and $\gamma_{NFAP}$-opt did not fully impede the tomato fruit decay. However, it remarkably inhibited the extension of B. cinerea infection on the fruit surface (data not shown).

Taken together, our findings demonstrated that NFAP and $\gamma_{NFAP}$-opt reduced biofilm formation on plant surfaces and crop decay by the phytopathogenic mold B. cinerea when topically applied in combination. The synergistic interaction of this AFP and PD allowed their administration at lower concentrations than their MICs in single dosage. In this study we provided new insights into the biocontrol potential of AFPs and PDs, which promise the development of new protection strategies against phytopathogenic fungi.

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Author contributions CP, GKT, GR, FM, and LG conceived and supervised the study, designed experiments, and edited the manuscript; GV, FM, and LG performed peptide design; GV performed peptide synthesis; LT and GB performed protein preparation, in vitro antifungal susceptibility tests, and analysis of the related data. LT, PP, AO, and GB performed plant toxicity and plant/crop protection bioassays and analyzed the related data; LT and AF performed SEM experiments; and LT, GKT, GR, FM, and LG wrote the manuscript and revised it. All authors read and approved the submitted version of the manuscript.

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Data availability Data and materials are available upon request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest associated with this publication.

Ethical approval There are no ethical concerns regarding the organisms and the topic of this research.

Research involving human and/or animal rights This article does not refer to any studies with human participants or animals (vertebrates) performed by any of the authors.

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Liliána Tóth is a research associate at the at the Department of Biotechnology, Faculty of Science and Informatics, University of Szeged (Hungary). Her research focuses on development of antifungal protein/peptide-based biofungicides and their agricultural applicability.

Péter Poór is an assistant professor at the Department of Plant Biology, Faculty of Science and Informatics, University of Szeged (Hungary). His research focuses on plant stress physiology and plant defence mechanisms regulated by phytohormones.

Attila Ördög is an assistant professor at the Department of Plant Biology, Faculty of Science and Informatics, University of Szeged (Hungary). His research focuses on plant-microbe interactions under different light conditions.

Györgyi Váradi is an associate professor at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged (Hungary). Her research focuses on design and chemical synthesis of antifungal peptides and proteins.

Attila Farkas is a senior research assistant, experience with high-end scanning electron microscopy at Institute of Plant Biology, Biological Research Centre, Eötvös Loránd Research Network, Szeged (Hungary).

Csaba Papp is a research associate at the Department of Microbiology, Faculty of Science and Informatics, University of Szeged (Hungary). His research focuses on potential of human pathogenic fungi for development of resistance mechanisms against conventional antifungal drugs.

Gábor Bende is a PhD student at the Department of Biotechnology, Faculty of Science and Informatics, University of Szeged (Hungary). His research focuses on mode of action antifungal proteins and peptides.

Gábor K. Tóth is a full professor at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged (Hungary) and head of the Biomimetic Systems Research Group of the Hungarian Academy of Sciences. His research focuses on the synthesis of modified polypeptides having anti-microbial or ion-channel blocker properties.

Gábor Rákheley is the head of department of Department of Biotechnology, Faculty of Science and Informatics, University of Szeged (Hungary). His main research focuses on biopesticides including phage therapy, microbial biocontrol and enzymiotics.

Florentine Marx is associate professor at the Institute of Molecular Biology, Biocenter, Medical University of Innsbruck (Austria). Her main research interests lie in antimicrobial proteins and peptides, their structure, mode of action and applicability as novel antimicrobial drugs.

László Galgóczy is an associate professor at the Department of Biotechnology, Faculty of Science and Informatics, University of Szeged (Hungary). His research focuses on antifungal proteins/peptides and their applicability in agriculture and medicine.