Antifungal activity of alpha-sarcin against *Penicillium digitatum*: proposal of a new role for fungal ribotoxins

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ABSTRACT

Among the putative defense proteins that occur in fungi, one of the best studied is α-sarcin, produced by the mold *Aspergillus giganteus*. This protein is the most significant member of the ribotoxin family, extracellular rRNA ribonucleases that display cytotoxic activity towards animal cells. Ribotoxins are rRNA endonucleases that catalyse the hydrolysis of the phosphodiester bond between G4325 and A4326 from the rat 28S rRNA. The results of several experimental approaches have led to propose ribotoxins as insecticidal agents. In this work, we report that α-sarcin displays a strong antifungal activity against *Penicillium digitatum*, being able to enter into the cytosol where it inactivates the ribosomes, thus killing the cells and arresting the growth of the fungus. This is the first time that a ribotoxin has been found to display antifungal activity. Therefore this protein could play, besides the already proposed insecticidal function, a role in nature as an antifungal agent.

The establishment, colonization and survival of fungi in their environment rely upon their ability to tackle the competition with other organisms that depend on the same resources and the attack by fungal grazers such as micro-, meso-, and macrofaunal elements in soil ecosystems [1]. For these purposes fungi are able to produce secondary metabolites and secretion proteins as antiviral, antibacterial, antifungal and insecticidal agents [1]. Examples of such secondary metabolites are the mycotoxins [2] and antibiotics [3], and examples of such proteins are proteases [4], defensin-like peptides [5] and antifungal proteins [6]. These chemicals exert a high impact on the microbial ecology of the environment [1].

The most familiar and economically important molds, *Aspergillus* and *Penicillium*, are also the dominant soil fungi [7, 8]. *Aspergillus* predominates in warm areas while *Penicillium* is abundant in temperate and cold climates. In any case, they must compete for the same resources and they produce chemicals such as penicillins (*Penicillium*) or aflatoxins (*Aspergillus*) for this purpose. It has been reported that *Aspergillus giganteus* produces two secreted proteins with a putative defensive role: the defensin-like protein AFP and the ribotoxin α-sarcin [9]. AFP (antifungal protein) is active against filamentous fungi but inactive against mammalian cells, plants, yeast or bacteria [10-12]. By contrast, it has been reported that α-sarcin is toxic to animal cells but does not display antimicrobial or antifungal activity [13] because, in spite of being able to inactivate any type of ribosomes [14], it is unable to cross some types of plasma membranes. α-sarcin is an extracellular RNase reported in 1965 as a new antitumor agent [13, 15]. This protein is the most significant member of the ribotoxin family and its mechanism of action has been known since 1983 [16]. Fungal ribotoxins are extracellular rRNA ribonucleases that display cytotoxic activity towards animal cells [17, 18]. They are rRNA endonucleases (EC 3.1.27.10) that catalyse the cleavage of the phosphodiester bond on the 3' side of the

G4325 residue from the rat 28S rRNA. This nucleotide is located in the Sarcin Ricin Loop (SRL) that is involved in the binding of the EFG or EF-2 elongation factors to the ribosome in prokaryotes and eukaryotes respectively [19, 20]. The rRNA endonuclease activity releases a 460 nt-fragment (α-fragment) at the 3' end of the 28S RNA that is diagnostic for the ribotoxin action. SRL is also target for ribosome-inactivating proteins (RIPs), enzymes produced mainly by plants [21] that inactivate ribosomes due to their specific N-glycosylase (EC 3.2.2.22) activity. Ribotoxins are not the only RNases secreted by fungi being RNase T1 the best known representative of a large family of ribonucleases produced by fungi, mostly *Aspergillus* and *Penicillium* species [17, 22]. However, ribotoxins stand out among them because of their cytotoxic characteristics towards animal cells [18]. Although a specific receptor for α-sarcin in human cultured cells has not been found, the toxin internalizes via endocytosis involving acidic endosomes and the Golgi, cleaves specifically the 28S rRNA, promotes caspase activation and kills cells via apoptosis [23].

Due to its translation inhibitory and apoptotic activities, extensive research has been carried out to investigate the suitability of α-sarcin in experimental therapy. Among the most studied applications of this protein as a therapeutic agent, is its use in the construction of immunotoxins, in which α-sarcin is linked to antibodies allowing its binding and internalization by cancer cells [18]. Moreover, it has been suggested that α-sarcin and other ribotoxins could also be useful as specific tools for the study of human ribosomopathies [18] or, in the field of plant resistance to insects, agents for the design and development of new biopesticides [18, 24].

It has been reported that α-sarcin is active against ribosomes, cultured cells and larvae from insects [25]. This and the fact that other ribotoxins (i.e. restrictocin, HtA and

anisoplin) display insecticidal properties have led to consider α-sarcin as a defense insecticidal agent [18, 24].

In this work, we report that α-sarcin displays a strong antifungal activity against the green mold *Penicillium digitatum*, a necrotrophic postharvest pathogen that colonizes the wounds and grows in the inter- and intra-cellular spaces of the tissues of several edible plants and mushrooms [26, 27].

In order to gain insight into the biological activities of α-sarcin we assayed its effect on ribosomes from *P. digitatum* using an S30 fraction as the source of ribosomes. For comparative purposes the rRNA N-glycosylase activity of the type 1 RIP BE27 on the same ribosomes is also shown (Figure 1). The catalytic activity of BE27 promotes the specific hydrolysis of the N-glycosidic bond of the adenosine residue at position 4324 from the rat 28S rRNA (or its equivalent in sensitive ribosomes from other organisms) [28]. Such depurination releases, upon treatment with acid aniline, an RNA fragment (Endo's fragment) of between 240 and 500 nucleotides (depending on species) from the rRNA of the large subunit, which is only a nucleotide longer than the α-fragment released by α-sarcin. In the case of *P. digitatum*, it has been reported that Endo's fragment displayed a size of 359 nt [29]. As shown (Figure 1), α-sarcin displayed rRNA endonuclease activity on these ribosomes, as indicated by the release of the α-fragment. The released fragment displayed the same size as that of the reported *P. digitatum* Endo's fragment, in accordance with that expected for SRL phosphodiester bond hydrolisis (358 nt; Figure 1). Therefore, *P. digitatum* ribosomes are susceptible of being inactivated by α-sarcin. This ribotoxin might enter into the fungal cells and inactivate the cytosolic ribosomes preventing the propagation of the fungus.

To characterize the antifungal properties of α-sarcin, we carried out experiments to test the effect of this protein on mycelia growth of the mold *P. digitatum*. As illustrated, α-sarcin exerted a strong effect on *P. digitatum* and led to a concentration-dependent inhibition of growth (Figure 2). With conidia as the starting material, 0.05, 0.1, 0.5 and 1 μg mL^{-1} of α-sarcin resulted in 34%, 60%, 94% and 98% growth inhibition, respectively, after 66 h incubation. In addition, the same concentrations of α-sarcin added to mycelia growing for 24 h with conidia as starting material gave after 66 h, exactly the same growth inhibition as above (Supplementary Figure 1), suggesting that α-sarcin has a stronger effect on mycelial growth than on conidial germination. However, a higher α-sarcin concentration (5 μg mL^{-1}) completely inhibited the germination process (data not shown). Therefore, the concentrations that inhibit fungal growth are lower than those reached by α-sarcin when it is produced by *A. giganteus* grown in culture medium, at least 2.25 μg mL^{-1} [9], and lower than those required for toxicity (ranging 0.01-5 μM; 0.168-84 μg mL^{-1}) in human or insect cultured cells [25, 30] or insects [24, 25]. *P. digitatum* cultures were also analyzed by light microscopy, finding hyphal morphology modifications in the samples exposed to the ribotoxin. Whereas control fungus presented regular and homogeneous hyphae, hyper-branching and aborted hyphal branches were observed in the cultures treated with α-sarcin (Figure 2, lower panel). Recently, it has been reported that the ribotoxin restrictocin, from *Aspergillus restrictus*, is active against the yeasts *Pichia pastoris* and *Saccharomyces cerevisiae*, and the filamentous fungus *Zymoseptoria tritici* but at a concentration as high as 20 μM (377 μg mL^{-1}) [31].

α-sarcin, at concentrations of 0.25 μg mL^{-1}, considerably inhibited the growth of *P. digitatum* cultures in liquid medium (Figure 3a) and allowed obtaining appreciable amounts of DNA and RNA. Total RNA was isolated from these cultures and examined

to detect the presence of the RNA fragment which is diagnostic of α-sarcin rRNA endonuclease activity (Figure 3b). The diagnostic fragment of 358 nucleotides was absent in the RNA from control cultures and present in that from cultures grown in the presence of α-sarcin. This suggests that α-sarcin, at a concentration of 0.25 μg mL^{-1}, is able to enter into fungal cells in a manner that allows it to reach the cytosolic ribosomes. This was supported by the fact that cyanine 3 (Cy3)-labelled α-sarcin, which retained full antifungal activity, accumulated in the cytoplasmic space of some cells of *P. digitatum* hyphae (data not shown). Therefore, α-sarcin catalytically inactivates *P. digitatum* ribosomes, killing the cells and arresting the growth of the fungus.

In animal cells, the catalytic activity of α-sarcin on the ribosomes, arrests protein synthesis and induces cell death by apoptosis [23]. We investigated whether the observed toxic effects of α-sarcin on *P. digitatum* were mediated via apoptosis. The DNA obtained from cultures of *P. digitatum* that were grown in the presence of α-sarcin (Figure 3a) was subjected to electrophoresis with the purpose of detecting the presence of oligonucleosomal fragments, which is a hallmark of apoptosis (Figure 3c). In the fungi grown in the presence of the ribotoxin, no internucleosomal cleavage was visible and only a smear of degraded DNA was observed, suggesting that α-sarcin toxicity can be mediated by non-apoptotic mechanisms. By contrast, COLO 320 cells grown in the presence of 30 μg mL^{-1} α-sarcin for 48 h showed the characteristic breakdown of the nuclear DNA into oligonucleosomal fragments (Figure 3c) indicating that α-sarcin intoxication occurs by apoptotic mechanisms in animal cells as has been reported previously [23].

To our knowledge, this study describes, for the first time, a strong antifungal activity exhibited by a ribotoxin and suggests that this protein might play a role as an antifungal agent in nature. One question that deserves attention is why α-sarcin is toxic against *P.*

digitatum considering that this protein is non-toxic to *A. giganteus* [14] and that another ribotoxin, restrictocin, is active against *P. pastoris*, *S. cerevisiae* and *Z. tritici* but at a thousand times higher concentration [31]. In the case of animal cells, the toxicity of α-sarcin arises from the combination of its rRNA endonuclease activity with its ability to cross cell membranes [32]. Several structures have been suggested to be involved in membrane interaction and cytotoxicity to animal cells: an N-terminal β-hairpin [30], an inner β-hairpin [33], and an inner loop [32]. Similar structural motifs have been proposed to be involved in the antifungal activity of some proteins such as plant defensins or ribosome-inactivating proteins [29, 34, 35]. The specificity of binding of such structural motifs to different sphingolipids or rafts containing sphingolipids and ergosterol might be responsible for the disparity in toxicity of α-sarcin against different fungi as has been reported for defensins [36]. Alternatively, differences in the composition, structure and porosity of the different fungal cell walls [37] might be responsible for this disparity in toxicity.

Further work will be directed to study the in vitro and in vivo antifungal potential of α-sarcin and other ribotoxins against different fungi. Taking into account the high sensibility of *Penicillium digitatum* to α-sarcin, this fungus could be a good model for studying the antifungal properties of ribotoxins. The study of the toxicity of α-sarcin mutants against fungal pathogens will clarify the role played by the different structural motifs in the interaction with the fungal plasma membrane.

METHODS

The sources of the chemicals and the methods have been described previously [28, 29, 35]. Particular experimental details are given in the Supporting Information.

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ASSOCIATED CONTENT

Supporting Information Available: This material is available free of charge via the Internet.

Supplementary Figures 1−2

Materials and Methods

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Legends of the figures

Figure 1. rRNA endonuclease activity of α-sarcin on *Penicillium digitatum* ribosomes. Left: rRNA endonuclease activity was assayed as indicated under Methods. Each lane contained 3 μg of RNA isolated from either untreated (C) or treated ribosomes from *P. digitatum* (α-S). For comparative purposes, *P. digitatum* RNA depurinated by the type 1 RIP BE27 is also included in the assay. The arrow indicate the RNA fragments released as a consequence of either the endonuclease activity of α-sarcin or the N-glycosylase action of BE27 upon acid aniline treatment (+). Right: Sarcin Ricin Loop of the large rRNA from *Penicillium*. The sequence from *Penicillium solitum* (**JN642222**) was downloaded from the NCBI sequence database (http://www.ncbi.nlm.nih.gov/nucleotide/). The large rRNA 3' end from *Penicillium* was determined by the alignment with the large rRNA from *Saccharomyces cerevisiae* (accession number **J01355**). The adenine released by the RIP action (boldfaced), the site of splitting by either α-sarcin or the acid aniline (arrows) and the size of the fragment generated by α-sarcin are also indicated.

Figure 2. Antifungal activity of α-sarcin against *P. digitatum*. Upper panel: Antifungal activity of α-sarcin against *P. digitatum* was measured in a microtiter plate bioassay. Conidia of *P. digitatum* were grown at 26 ºC in PDB medium in the presence of

different concentrations of α-sarcin. Fungal growth was measured as an increase in absorbance at 620 nm. The curves represent buffer control (●), 0.05 μg mL^{-1} α-sarcin (○), 0.1 μg mL^{-1} α-sarcin (■), 0.5 μg mL^{-1} α-sarcin (□) and 1 μg mL^{-1} α-sarcin (▲). The mean results ± S.D. of three experiments performed in triplicate are reported. Data were analysed by ANOVA test (confidence range 95%; * $p < 0.1$ versus control, ** $p < 0.01$ versus control, *** $p < 0.001$ versus control, **** $p < 0.0001$ versus control). Lower panel: Morphological changes of *P. digitatum* mycelium exposed to α-sarcin. *P. digitatum* mycelium was grown in the absence (control) or in the presence of 0.5 μg mL^{-1} α-sarcin. After 60 h incubation, samples were visualized using light microscopy at 200x magnification.

Figure 3. Antifungal and rRNA endonuclease activity of α-sarcin against *P. digitatum*. a: *P. digitatum* was grown in PDB in the absence (control) or in the presence of 0.25 μg mL^{-1} α-sarcin for 4 days. Then the mycelium was extensively washed with sterile water and harvested to extract the RNA and the DNA. Representative photographs of two wells are shown. b: rRNA endonuclease activity was assayed as indicated under Methods. Each lane contained 3 μg of RNA isolated from either untreated (control) or α-sarcin treated cultures from *P. digitatum*. The arrow indicates the RNA fragment released as a consequence of α-sarcin action. Numbers indicate the size of the standards (M) in nucleotides. c: The DNA was isolated from either *P. digitatum* or COLO 320 cells as indicated in Methods and 2 μg was electrophoresed. The numbers indicate the corresponding size of the standards (λDNA HindIII/EcoRI) in bp.

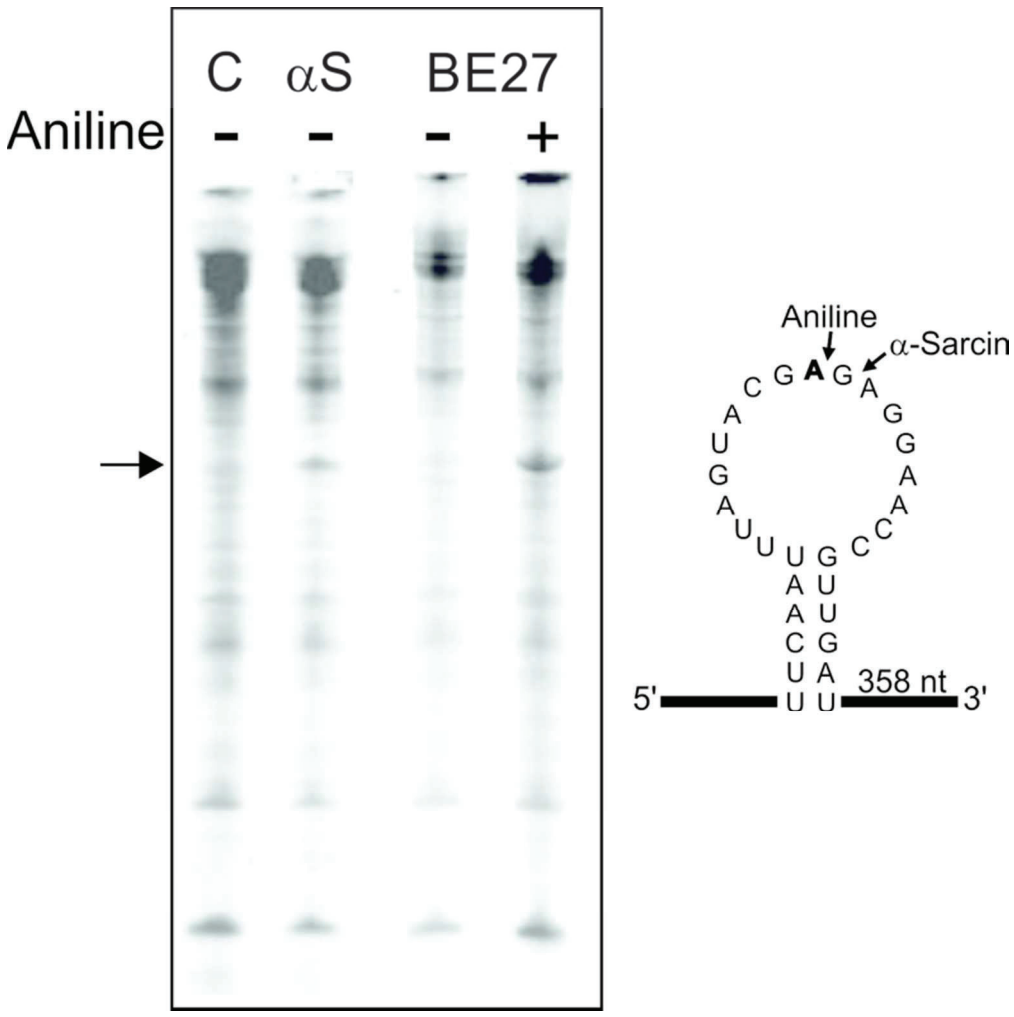

Figure 1. rRNA endonuclease activity of α-sarcin on *Penicillium digitatum* ribosomes.

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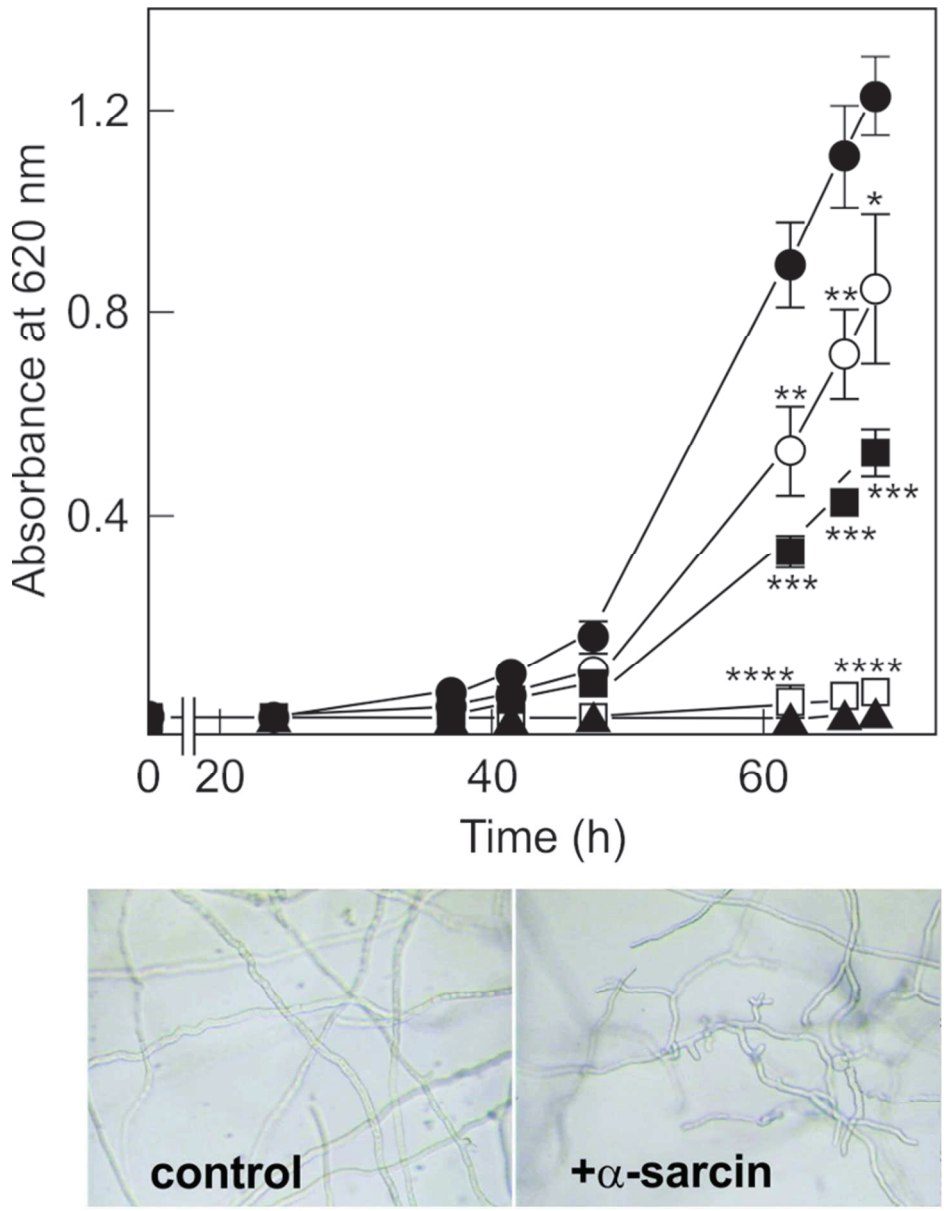

Figure 2. Antifungal activity of α-sarcin against *P. digitatum*.

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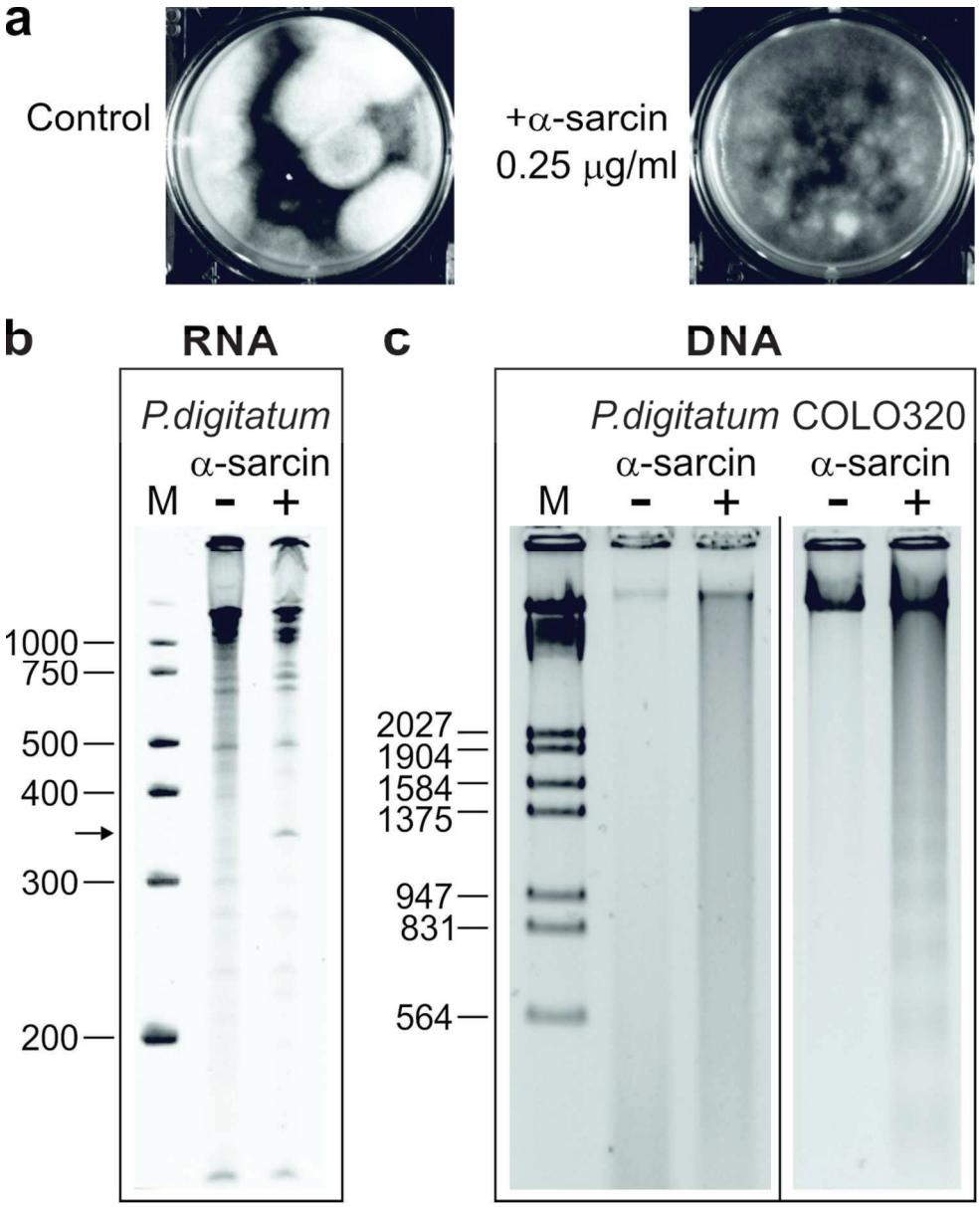

Figure 3. Antifungal and rRNA endonuclease activity of α-sarcin against *P. digitatum*.

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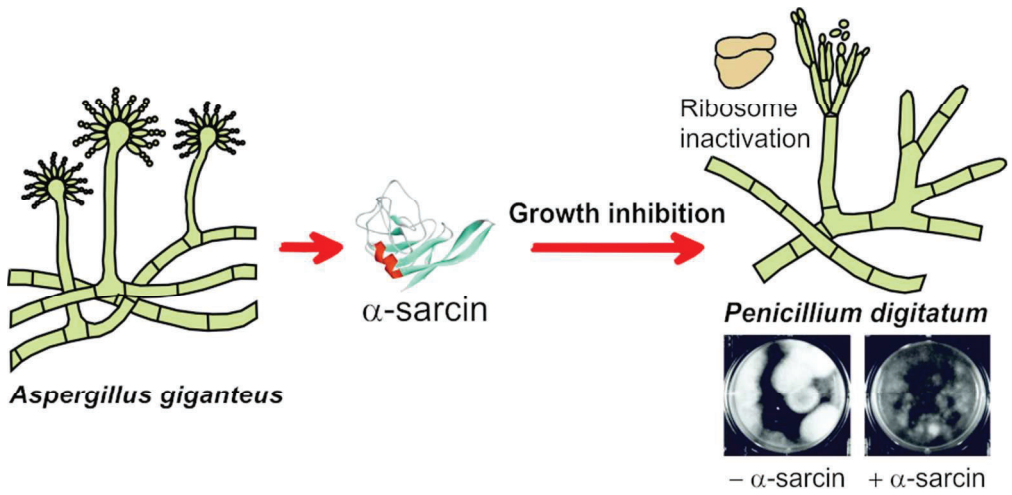

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