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Peptaibol Production and Characterization from *Trichoderma Asperellum* and their Action as Biofungicide

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Introduction

Peptaibols (P_{aib}) are a large family of bioactive peptides composed of 7 to 20 amino acid residues (linear or cyclic) [1,2]. P_{aib} are characterized by the presence of a high proportion of 2-aminoisobutyric acid (Aib), an acetyl or acyl group in the *N*-terminal residue and a *C*-terminal amino alcohol [3,4]. These peptides are assembled by a multienzyme complex called non-ribosomal peptide synthetases (NRPSs), which allows the incorporation of non-proteinogenic amino acids such as Aib [1,5,6]. Their amphipathic nature allows the formation of permanent transmembrane pores that causes the exchange of cytoplasmic material and eventual cell death [2,7].

The bioactivity of P_{aib} against parasites, viruses, bacteria, and pathogenic fungi has previously been reported [1,2]. In addition, its bioactivity has been proved in therapies against cancer, Alzheimer, and some human and animal diseases thanks to their antifungal, antitrypanosomal and anthelmintic activity [2,3,5,7,8].

Trichoderma is one of the most isolated and studied ascomycetes due to its agro-industrial importance as a biocontrol organism and producer of secondary metabolites with biological activity like P_{aib} [9,10]. Some *Trichoderma* strains are currently being used, and even commercialized, as biocontrollers because of their antimicrobial properties [11,12]. However, the whole microorganism is commercialized, not the active compound (P_{aib}). Optimizing the production and isolation of P_{aib} is critical when only the pure active component is required, such as in biomedical applications e.g., the treatment of cancer or Alzheimer [5,8,13]. Likewise, purified P_{aib} could be beneficial in agricultural applications such as biocontrol in post-harvest products, where it is better to apply a treatment free of microorganisms to avoid contamination of the final product.

The present work aimed to produce P_{aib} for their extraction and characterization as a potential biofungicide. The work included the optimization of fungal growth conditions for P_{aib} production. Afterward, mass-spectrometry techniques were applied for the identification and sequencing of P_{aib}. In addition, the biological effect of the biofungicide was evaluated against four phytopathogenic fungi *in vitro* and *in vivo* in tomatoes infected with *Alternaria alternata*. Furthermore, electron microscope images were used to study the effect of P_{aib} on the structure and morphology of the treated fungi.

Results and Discussion

Optimization of *T. asperellum* fermentation for P_{aib} production was carried out to determine the best carbon source, additive amino acid, elicitor and their optimal concentrations. P_{aib} production was optimized in flask by adding sucrose, 2-aminoisobutyric acid, and *Fusarium oxysporum* cell debris. Figure 1 shows the surface response graph obtained with the central composite model where the maximum point of P_{aib} production is located at 2.634 g/L Aib and 0.866 g/L *F. oxysporum* with 30 g/L sucrose.

P_{aib} produced were purified, sequenced and identified by HPLC coupled to mass spectrometry. The mass spectra showed the presence of ions characteristic of trichotoxins with values between 1676 m/z and 1768 m/z. The fragments detected corresponded to ions with m/z 1676, 1691, 1704, 1705, 1718, 1726, 1742 and 1768. Six P_{aib} were identified as trichotoxins of 18 amino acid residues. The general sequence obtained corresponded to Ac-Aib-Gly-Aib-Lxx-Aib-Gln-Aib-Aib-Aib/Ala-Ala-

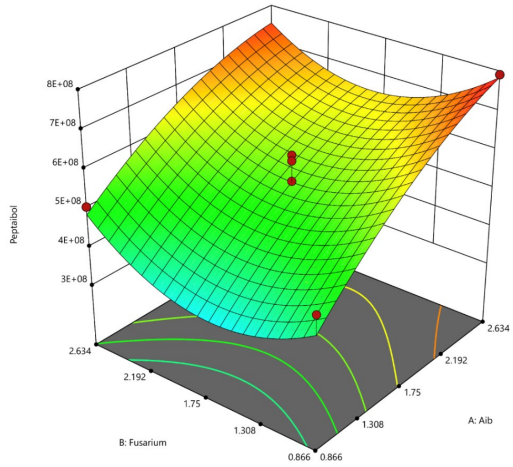

Fig. 1. Surface response graph of the central composite model generated for the optimization of P_{aib} produced in the fermentation of T. asperellum.

Aib/Ala-Aib-Pro-Lxx-Aib-Aib/Vxx-Gln/Glu-Valol, differences are observed in the sequences in the positions 9, 11 and 16 (Table1).

Table 1. Differences between the sequences of the six trichotoxins of the P_{aib} produced by T. asperellum. Vxx:valine/isovaline.

Trichotoxin	*m/z*	*9*	*11*	*16*
T5D2[1]	1676	Ala	Ala	Aib
1690	1691	Ala	Ala	Vxx
1703A[3]	1704	Aib	Ala	Vxx
A-40[2]	1705	Aib	Aib	Aib
1717A[3]	1718	Aib	Aib	Vxx
A-50 G[1]	1726	Aib	Ala	Vxx

Source: [1][14], [2][15], [3][16]

The biological activity of the P_{aib} extract was evaluated in *vitro* against four phytopathogenic fungi. Antifungal activity assays proved the efficiency of the P_{aib} extract to inhibit the growth of *Colletotrichum gloeosporioides*, *Botrytis cinerea*, *A. alternata* and *F. oxysporum* with a growth inhibition of 92.2, 74.3, 58.4 and 36.2%, respectively (Figure 2). Additionally, the extract completely inhibited the germination of *A. alternata* spores on tomatoes (Figure 3). The tomatoes treated with the extract showed no growth of the fungus, even after 15 days which suggests that the P_{aib} extract is not only effective in the short term but that it can show a continuous inhibitory effect even after two weeks. The incidence of the disease in tomatoes treated with the P_{aib} extract was 0% (same as with clotrimazole), while the untreated fruit (extract without P_{aib}) showed a 92.5% incidence of infection.

SEM results showed how P_{aib} generates damage in the morphology of hyphae and spores of the treated fungi. The images showed noticeable differences between the structures of the fungi treated with P_{aib} and the control (Figure 4). While the control showed hyphae with smooth surfaces and normal conidia, all the images of the treated fungi showed dehydrated hyphae with granules and an evident damage to the fungus wall. These results indicate that the P_{aib} extract could be used as a growth inhibitor against phytopathogenic fungi of agricultural relevance.

Results from this study suggest that environmental soil fungus from Costa Rica may represent an interesting source of known and new P_{aib} and antimicrobial compounds of biotechnological interest. Future studies may incorporate the determination of effective application levels in the field, the validation of proposed treatment against other species and strains and best use strategy on the product.

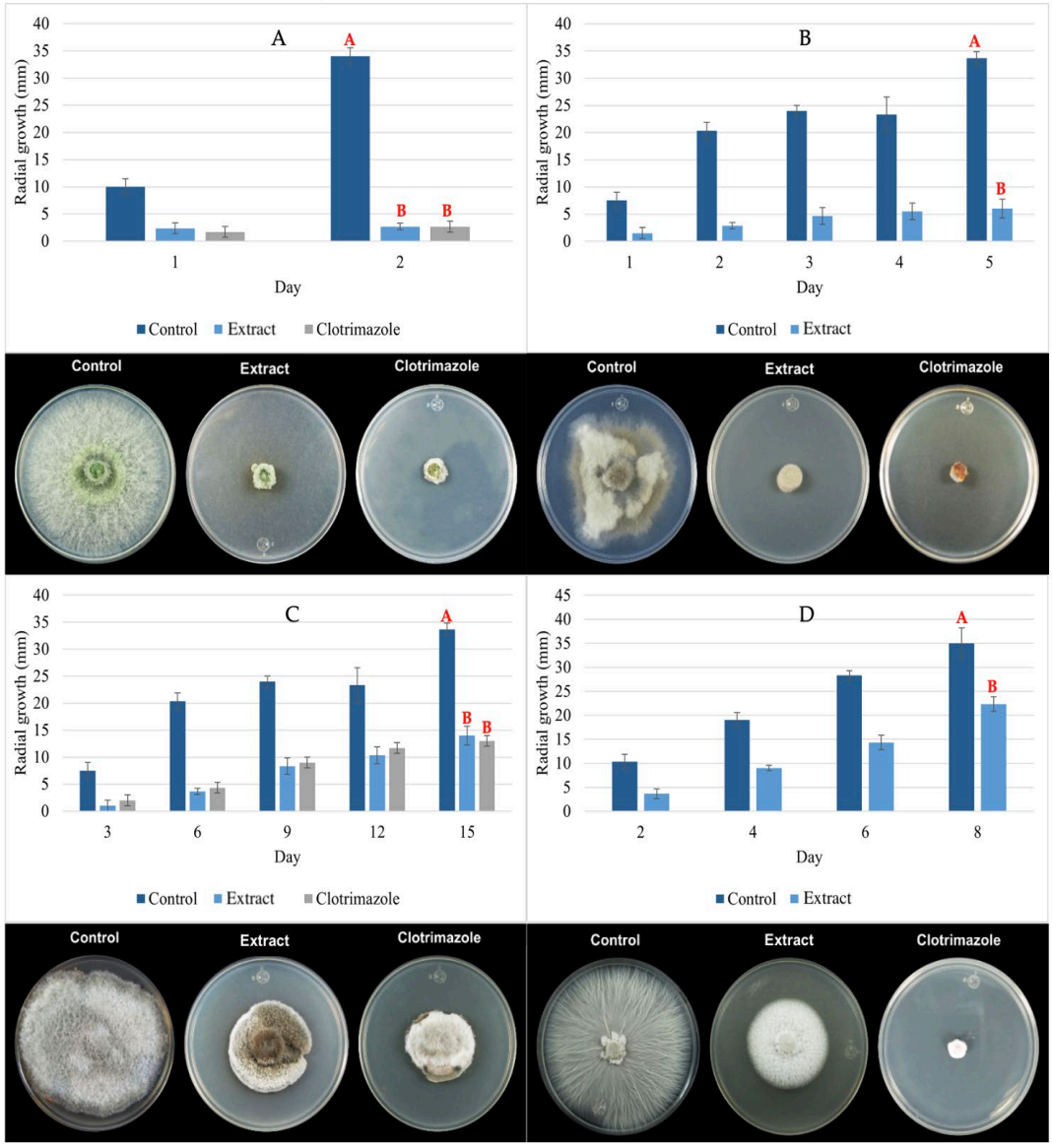

Fig. 2. Inhibition effect of P_{aib} against mycelial growth of (A) C. gloeosporioides, (B) B. cinerea, (C) A. alternata and (D) F. oxysporum on PDA media after treatment with 800 µg mL^{-1} of P_{aib} extract. Different letters represent significant differences between treatments.

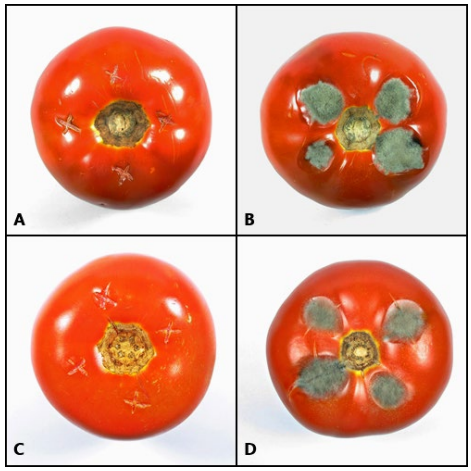

Fig. 3. Effect of P_{aib} on growth of A. alternata in tomatoes 8 days after spore inoculation and application of treatment. (A) extract with P_{aib}, (B) control, (C) clotrimazole, and (D) sterile distilled water.

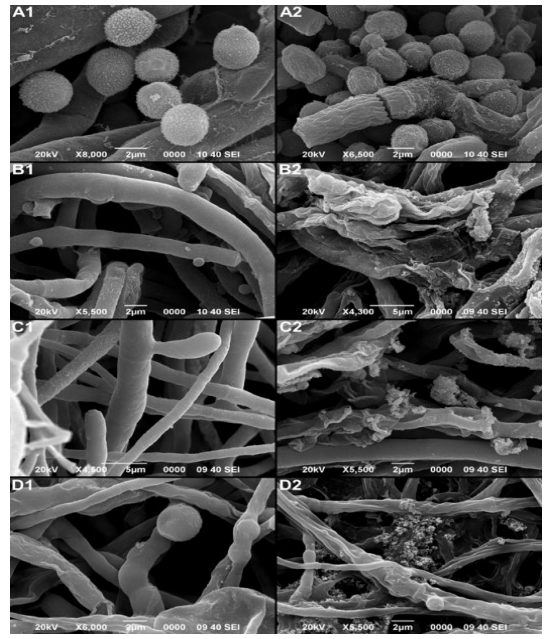

Fig. 4. SEM images showing the effects of P_{aib} over the morphology of untreated fungus (1) and fungi treated with P_{aib} (2). (A)C. gloeosporioides, (B) B. cinerea, (C) A. alternata and(D) F. oxysporum.

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