

Response to reviewers

We would like thank the reviewers for their time and comments. We are sure that after this thorough revision the manuscript has been improved. We have made changes in the manuscript addressing all your comments and recommendations. Below you can find the response to your comments in blue, whereas the modifications/additions in the manuscript are marked in red. In very few cases, we considered that the change/modification was perhaps not absolutely necessary. In those cases, we have explained why we did not modify the text.

Reviewer 1

1. Figure 1 and Figure 2. Image content needs to be enriched, such as adding saliency analysis and error lines.

The error lines were added to the two figures and in the case of Figure 2 the statistical analysis was added.

2. When measuring Paib Production, the sampling time needs to be optimized. It is recommended to take samples at a fixed time interval.

The authors agree with the reviewer that sampling time is critical for the quantification of peptaibols. Sampling was performed Monday, Wednesday, and Friday because of laboratory regulations that do not allow entry on weekends. However, prior to the experiments, another fermentation was performed where peptaibols were measured daily and there was not significant difference in the production every 24 hours.

3. Enrich the section of the article on the antimicrobial activity of Paib, including the determination of MIC, MFC and IC_{50} , it is dangerous to directly test antimicrobial activity of Paib at 800 ug.ml^{-1} (Line 200).

We previously determined the MIC of the peptaibol activity against the fungi analyzed. It was determined by measuring the *in vitro* growth of the fungi in PDA media with 200, 400, 800 and 1600 ug.ml^{-1} peptaibols. It was defined that 800 ug.ml^{-1} was the lowest concentration of Paib that prevents the growth of all fungi analyzed. These results are not published. This was briefly included in the text in lines 207-208 and 472-473.

4. Line 109: '*C. gloeosporioides*, *F. oxysporum* and *B. cinerea*' change to '*C. gloeosporioides*, *F. oxysporum* and *B. cinerea*'.

This was modified in the text

5. Line 29: "similai to" should be changed as 'is similar to'.

This was modified in the text

6. Line 43: "consist in" should be changed as 'consist of'.

We think in this case 'in' is more appropriate and have left it unchanged.

7. Line 94. '*T. asperellum*' should be italic.

In line 94 is not *T. asperellum*. On line 97 it was italicized. We confirmed that *T. asperellum* was italicized throughout the document.

8. Line 109. The genus and species name should be italic.

This was modified in the text

9. Line 239: "lower" should be changed as "less than".

Line 239 does not have the word "lower", but we modified this in line 269

10. Figure 2: Typographical error.

This was corrected in comment 1 of reviewer 1

11. Line 303. 'sensing of molecule such as oligopeptides and oligochitosaccharides from the cell membrane of other fungi'. How did author keep the stabilization of components of cell debris in preparing and autoclave the media?

We understand the reviewer's comment. However, the elicitors were autoclaved in order to inactivate the viability of the spores so that they will not affect the fermentation process. It may be that not all elicitor molecules are stable after sterilization but, despite this, a positive response and more Peptaibol production was obtained.

12. Line 470. The concentration of clotrimazole should be added here. Different concentration of fungicides has different effect.

This was modified in the text on line 471. Also, the concentration of clotrimazole used is indicated in the methodology section.

13. Line 478. Unify P_{aib} or $Paib$ in the whole paper.

This was modified in the text. P_{aib} was unified

Reviewer 2

1. In my opinion the introduction is quite short, and the different paragraphs could be more smoothly connected but the essential information are reported.

We have added more information in the introduction section.

2. I noticed the absence of statistical letters in the figures, it would be easier for the readers to have statistical analysis reported graphically (e.g. as letters) in the figures

Statistical analyses were added to the corresponding figures.

Specific comment:

3. Line 24: "Aib" this abbreviation is used here for the first time, it should be written in full and abbreviation between brackets.

This was modified in the text

4. Line 27: "in vitro and in vivo" should be in italics

This was modified in the text

5. Line 109: "the pathogenic fungi *C. gloeosporioides*, *F. oxysporum* and *B. cinerea* " Names of fungi should be in italics.

This was modified in the text

6. Line 282: "...in *T. longibrachiatum* SMF2" If I am not wrong this name is for the first time mentioned in the manuscript so it should be written in full as *Trichoderma longibrachiatum*.

This was modified in the text

7. Figure 2 -> I was wondering why the statistical differences that are described in the text (line 310-312 and 321-322) are not graphically represented in Figure 2.

Statistical analyses were added in Figure 2.

8. Line 337: Space missing between sentences "... (Paib Database [52]). These..."

This was modified in the text

9. Similarly to Figure 2 also in Figure 3 -> the significant differences described in line 345-347 are not graphically represented in Figure 3.

Significant differences were added in Figure 3.

10. Concerning Figures 2 and 3 I think it would be better to be able to determine the statistical differences among the different objects tested just looking at the figures. The figures should be totally self explanatory .

Significant differences were added in Figure2 and 3.

11. Line 465-467: "*The prototype demonstrated to be more effective against C. gloeosporioides with a GH of 92.2%. The GH for B. cinerea, A. alternata and F. oxysporum were 74.29, 58.4 and 36.2%, respectively.*" Growth inhibition values are reported in the text but it is not specified at which time point of the experiment these values correspond. The mycelial growth was assessed at different time points (days after incubation) and it is not clear which data point has been used to calculate GH. Please, clarify.

The GH reported was calculated at the endpoint of each experiment. This is better explained in the text in lines 466-467.

12. Also in Figure 5 the statistical analysis are not reported in the figure but only partially mentioned in the text (Line 468-469).

The statistical analysis was added in Figure 5.

Reviewer 3

Trichoderma asperellum and their Action as Biofungicide IS very good title and interested to readers, thanks. I completely love the figures, look presented and clear.

Reviewer 4

Although there is some value in the research performed, this review cannot recommend the publication of the article in its current state due to the following reasons:

13. No information is included in the article regarding the isolation and taxonomic identification of the *T. asperellum* strains used in the research.

Line 97 details where the strain was obtained: "*Trichoderma asperellum* was isolated from agricultural soil". Regarding its taxonomic identification: *T. asperellum* was identified using ITS, rpb2 and tef1 sequences as previously described by Cai and Druzhinia (2021), this was added in the text (line 95-96)

14. The authors mention in their article the purification of peptaibols, but their purification seems to be limited to the generation of a crude extract from the fungal cultures. In all the analytical studies leading to the determination of the best production conditions a solid phase extraction using C18 reversed phase silica gel has been performed in which, after water washing, the column was eluted with ethanol 96 % v/v. For the determination of biological activities after optimization of the fermentation parameters, what the authors refer as prototype is a simple ethyl acetate extract with any further purification. In both cases the extracts will contain for sure other components whose presence has been completely overlooked. Some of these components might also contribute to the antifungal activity observed and their presence must therefore be accounted and evaluated.

The authors thank and agree with the reviewer's observation regarding the word prototype, we have now used 'Paib extract' instead. A short explanation was included in lines 555-559: "It must be considered that the extract could contain other minor components that contribute to antifungal activity. However, *T. asperellum* is a well-documented antifungal Paib producer [55]. These peptides were identified in the Paib extract, assuring these peptides are present in determined concentrations. The results suggest the antifungal activity of the extract is mainly produced by Paib."

15. In this sense, the analytical work performed seems to be limited to a relative quantification of peptaibols production under the different conditions tested, without a real absolute quantification of the presence of these compounds in the extract to confirm the relevance of the experiments performed when compared to previous works describing the presence of the same compounds in cultures of the same fungus.

In fact, the amount of peptaibols in the prototype was quantified. Line 200 shows the concentration of the peptaibol extract added (139,400 µg mL⁻¹). With this value we calculated the concentration to be used in all the inhibition tests carried out.

16. The biological activity measured for the prototype prepared is somehow predictable based on previous reports. The relevance of the findings in this article, if any, must be highlighted with respect to previous studies. In this sense, a real purification of a peptaibol fraction would have been desired in order to evaluate their real contribution to the antifungal activity observed in the absence of other components of the extracts.

To the best of the authors' knowledge, the article provides with new insights that could contribute to the subject under study. For example, the optimization of a fermentation model to produce P_{aiib} using an elicitor and an inducer simultaneously. In addition, although there are reports of the inhibition generated by peptaibols against pathogenic fungi, all the reports that exist are in vitro, there is no information on the effectiveness of peptaibols to treat an infected fruit, in this work we show a test on tomatoes infected with *A. alternata* and its effect during 15 days, this test is new and gives us information on the possible use of peptaibols in the agricultural field. Finally, the effects of peptaibols on the structure of hyphae and conidia is little known, in this work we show electron microscopy images that give valuable information on the state of the mycelium after being treated with P_{aiib}. There are no results with this specific technique for all the phytopathogenic fungi used in this work.

17. Finally, a more detailed description of the effects observed by SEM would have been desirable. In this sense, please note that the identification of the treated and untreated versions of the fungal cultures in the caption of Figure 7 seems to be wrong.

We agree with the comment. We corrected the detail in figure 6 (SEM images) and added more information in this section. Line 575