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Interactive comment on "Adsorption to soils and biochemical characterization of purified phytases" *by* Maria Marta Caffaro et al.

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Dear Executive Editors Soil Journal

We would like thank you and the anonymous reviewer for the valuable suggestions which helped us to greatly improve our ms. We have followed each one of the suggestions and the detailed responses and changes made to the original manuscript are given below and included in the new manuscript. If additional modifications are required, please let us know.

We are uploading a Word file with all the modifications included.

Regards,

Dr. Gerardo Rubio School of Agriculture - University of Buenos Aires

. . . . . .

1. Comments for editor. The research work carried out under the theme "Adsorption to soils and biochemical characterization of puriïňĄed phytases" is of scientiïňĄc signiïňĄcance and has practical application for release of Pi from native or exogenously added organic P. Though, the study conducted is well organized but certain points need due attention. The Accession no of microbial strains used in the study is missing.

R: Thanks for your comment; in our experiment we used four commercial phytases. Two came from two different batches of A. niger commercially sold under the name "Habio phytases", which were obtained from Sichuan Habio Bioengineering Co.Ltd (Sichuan, China). The other two, came from two strains of E. coli. One is sold under the name "TS Smizyme phytases", obtained from Quimtia EDF (Buenos Aires Argentina) and the other is sold under the name "Ronozyme", obtained from DSM Nutritional Products Argentina S.A. Unfortunately, and as usual for commercial strains, no accession number was provided by the supplier. Anyway, we rearranged the paragraph to provide all available information.

Materials and methods now read as (New ms lines 68-73):

"Two phytases isolated from A. niger and two from E. coli were used in our experiments. In the first case, here named A. niger 1 and 2, phytases came from two different batches of the fungus which are commercially sold under the name "Habio phytases" by Sichuan Habio Bioengineering Co.Ltd (Sichuan, China), In the E.coli group, the first selected enzyme (here called E. coli 1) is sold under the name "TS Smizyme phytase", by Quimtia EDF (Buenos Aires Argentina), and the second (here called E. coli 2) is sold under the name "Ronozyme", by DSM Nutritional Products Argentina S.A."

. . .. 2. The cost incurred on purïïňĄed phytases and their availability needs mention.

R: Enzymes were provided for free by the different companies producing and / or importing enzymes in the country. This information is included in the new version.

Materials and methods now read as (New ms line 73):

“These enzymes are in powder format at a concentration of 5000 U g-1 and was provided free of charge by the companies that produce or import them.”

. . .. 3. A comparative study with crude phytase obtained from wild strains of A. niger and E. coli could have also been conducted alongside.

R: This would be a good option for the next phase of this investigation. Although it was was not the main objective of the present study. Anyway, this comment is highly appreciated and will be taken into account in our next phase.

. . .. 4. Technical Comments for authors Abstract needs some modiïňĄcation indicating the % increase in P release with A. niger over E. coli.

R: Done. Abstract now read as (New ms line 12): “Phytases from A. niger showed a higher capacity to release P, than phytases from E. coli (+13% on average).”

. . .. 5. L-13-14 Please shift substrates pNP, G3phosphate and phytic acid after substrates

R: Done. Acronyms now included throughout the whole ms. Abstract now read as (New ms line 11): “. . .... p-nitrophenyl-phosphate (pNP), glyceraldehyde-3-phosphate (G3Phospahte) and phytic acid.”

. . . 6. L-16 Please write that the order of P release from different substrates by A. niger and E. coli followed this trend (mention the trend).

R. OK. Iif we understood the meaning of the comment, the order is mentioned a couple

of lines above.

Abstract now read as (New ms lines 13-15): “All phytases were active throughout the pH and temperature ranges for optimum crop production. The amount of P that A. niger phytases release at pH that is commonly found in agricultural soils (5.5-7) is as follows: pNP > phytic acid > G3Phosphate, whereas in E. coli phytases the order was pNP / phytic acid > G3phosphate”.

. . . 7. Introduction L-24 Delete appropriate

R: OK. Introduction now read as (New ms line 20): “Most strategies for enhancing P nutrition of agricultural crops aim to maintain soils at the convenient P critical level so that yields. . .”

. . .. 8. There are approximately 38 references in introduction. The no. can be reduced.

R: OK. In the new version, the number of references was reduced to 22.

. . .. 9. L-45-48. The ïňĄrst phytase was discoveredâĂŤ– delete this paragraph.

R: OK, deleted.

. . .. 10. L-74 instead of level write pH and temperature optima.

R: OK. Introduction now read as (New ms line 65): . . . “ the two evaluated phytases differ in their optimum pH and temperature to reach their maximum activity ..”

. . .. 11. Material and methods L-77 A. niger in italics L-79 powder form and not format L-81 Superscript g-1

R. OK, all these editing issues were arranged as suggested.

. . .. 12. L-87 If one g soil was mixed with 20 ml phytase solution how can you take a sub sample of 500 ml. Please check the unit

R: We apologize because it was our mistake. The subsample volume is 500 microliters (not ml). Arranged in the new version.

Materials and methods now read as (New ms line 84): “. . .sub-samples of soil slurry (500 ïA■l) were taken for phytase activity measurements. . .”

. . .. 13. L-92 150 ml or 150 microliter

R: Correct, same as above, it is 150 microliter. Arranged in the new version.

Materials and methods now read as (New ms line 86): “An aliquot (150 ïA■l) of the soil slurry was used to measure the enzyme activity. . .”

. . .. 14. L-105 total protein (Lowry et al)

R. OK. Materials and methods now read as (New ms line 100): “Biochemical characterization of the phytases included: total protein (Lowry et al., 1951)”

. . .. 15. Phytase activity was measured with 3 substrates

R: OK, arranged as suggested

Materials and methods now read as (New ms line 102): “Phytase activity was measured with 3 substrates containing. . .”

. . .. 16. L-119 Blanks for measuring enzyme activity included (i) (ii) (iii)

R: OK. Materials and methods now read as (New ms lines 112- 113): “The activities were tested against three blanks: (i) reaction buffer without enzyme or substrate; (ii) reaction buffer with enzyme without substrate; and (iii) reaction buffer without enzyme with substrate.”

. . .. 17. L123-126 Please rewrite this portion

R: OK. Materials and methods now read as (New ms line 114):

“For the pNP substrate, the enzymatic activity was measured at 412 nm which is the absorbance value of p-nitrophenol (Hayes et al., 1999). The concentration of 3 substrates was determined as the concentration of the whole sample minus the concentration of the reaction blank.”

Please check that the sentence: “Phytase activity with phytic acid and glyceraldehyde-3-phosphate as substrates was measured as P release measured by the 125 Murphy-Riley method (Murphy and Riley, 1962)” was eliminated because this procedure is provided in the previous sentence.

. . .. 18. Please mention the amount of TCA added to stop the reaction

R: OK. Materials and methods now read as (New ms line 91): “Reactions were stopped with an equal volume of 10% TCA (300 ïA■l in soil slurry experiments and 700 ïA■l in soil solution experiments).”

. . .. 19. L-192 Modify the sentence

R: OK. Results now read as (New ms line 180): “All four enzymes were effective in releasing P from phytic acid throughout the entire pH range analyzed”

. . .. 20. L-200 pH 7.8 was detrimental for release of Pi from pNP by A.niger

R: OK, arranged as suggested. Results now read as (New ms line 186): “pH 7.8 was detrimental for release of Pi from pNP by A.niger, probably because the hydrolysis of the substrate

. . .. 21. L-216 Change offered to tested substrates

R: OK. Results now read as (New ms line 202):

“A. niger showed maximum activity at 24 °C (Fig. 3a), releasing 33% of the original P contained in the substrate.”

. . .. 22. Results and discussion Discussion part is totally missing and needs to be written properly

R: OK, results and discussion sections were now written separately so as not to create confusion for the reader.

. . .. 23. No explanation for ïňĄndings is given Conclusion needs to rewritten.

R: OK, conclusion section was reworded accordingly. After reorganizing the ms and split the Results and Discussion section into to separate parts, the conclusion was incuded in the last paragraph of the discussion. In this paragraph the test of hypothesis is specifically considered

Conclusion now read as (New ms lines 281- 295:

“Obtained results partially support our first hypothesis since the selected phytases showed a great ability to release P from different organic P sources, but A. niger 1, 2 and E. coli 1 release more P from pNP than phytic acid while E coli 2 has no preference for any particular substrate. In contrast, our results did not support the second proposed hypothesis, since the retention of phytases by the soil solid phase did not have a clear association with the analyzed soil properties. In this regard, it must be taken into account that the seven selected soils belonged to the Mollisol order. After being added to the soil, tested phytases showed an adsorption to soil solid phase ranging from 20 to 40%. Those phytases that remain in the solution could release Pi from the organic P of the soil, whereas phytases that remain adsorbed to the soil solid phase could be released later. Regarding our third hypothesis, although the evaluated phytases exhibited some differences in their pH and temperature levels to reach their optimum activity, all studied phytases remained active at the optimum soil pH range of the most productive agricultural soils (5-7). In the same line, optimal temperatures for phytase activity were also within the temperature range more suitable for most agricultural crops (20-30°C). Our results suggest that purified phytases may constitute a feasible tool to be used as a complement to P fertilization. In such sense, further experiments should be performed to evaluate the enzyme performance under field conditions to evaluate the ability of phytases to release from organic soil P sources, their interaction with soil microorganisms and to test if crops can capitalize the eventual provision of inorganic P released.”

. . .. 23.Tables and Fig titles need to be precise

R: OK, all titles were rewritten

Tables and figures now read as:

“Table 1. Characteristics of seven representative soils of the Argentina’s Pampa Region used for testing phytases adsorption. Samples were taken at 0- 20 cm, air dried and sieved at 1 mm prior to the analysis.

Table 2. Coefficients of the adjusted Lorentizian-peak functions for phytase activity (see graphs in Fig. 2) at different pH levels with phytic acid, p-nitrophenyl-phosphate and glyceraldehyde-3-phosphate as substrates. The equations were adjusted from the observed results of the release of P from each substrate used. Four purified phytases (two isolated from A. niger and two from E. coli) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted. Different letters correspond to signiïňĄcant differences between treatments (P <0.05, LSD procedure) Coefficient a is the maximum percentage of P released; b is the pH value where the enzyme has maximum activity (a P release peak); c estimates the standard deviation of the distribution and x is the pH value.

Table 3. Coefficients of the adjusted Lorentizian-peak functions for phytase activity (see graphs in Fig. 2) at different temperature levels with phytic acid, p-nitrophenyl-phosphate and glyceraldehyde-3-phosphate as substrates. The equations were adjusted from the observed results of the release of P from each substrate used. Four purified phytases (two isolated from A. niger and two from E. coli) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted. Different letters correspond to signiïňĄcant differences between treatments (P <0.05, LSD procedure) Coefficient a is the maximum percentage of P released; b is the temperature value where the enzyme has maximum activity (a P release peak); c estimates the standard deviation of the distribution and x is the temperature value.

FIGURE 1. Phytase activity distributed in the liquid and solid phases for the phytase soil

adsorption experiment. Four purified phytases (two isolated from A. niger and two from E. coli) were evaluated. Experiments were performed with the seven soils described in Table 1. For A. niger 1 and 2 and E. coli 1 phytases, a unique curve decay (Eq. 2), and exponential increase (Eq. 3) involving the seven soils was fitted because no significant differences (after F tests) were found between them. For E. coli 2, no function could be adjusted because a 37% binding to the soil solid phase was observed at 5 minutes and remained stable throughout the incubation period. Each point represents the average of three observations. Bars represent standard error of the mean.

FIGURE 2. Phytase activity measured at different pH levels with phytic acid, pNP and G3Phosphate as substrates. Four purified phytases (two isolated from A. niger and two from E. coli) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. Each point represents the average of three observations. Bars represent standard error of the mean. Coefficients of each adjusted model are shown in Table 2.

FIGURE 3. Phytase activity measured at different temperature levels with phytic acid, pNP and G3Pphosphate as substrates. Four purified phytases (two isolated from A. niger and two from E. coli) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. Each point represents the average of three observations. Bars represent standard error of the mean. Coefficients of each adjusted model are shown in Table 3.

FIGURE 4. Kinetic parameters for phytic acid, pNP and G3Pphosphate as substrates of purified phytases (two isolated from A. niger and two from E. coli). The activity was determined at different P concentrations (0 to 100 mM) contained in each substrate. Each point represents the average of three observations. Bars represent standard error of the mean. Data were fitted to a Michaelis-Menten curve and the estimated Vmax and Km values obtained by the Lineaweaver-Burk method are shown."

. . .. 24. Table 1 Mg+2 and Ca+2 and not Ca+1 25. Provide space between C total, P

total and P inorganic 26. Fig.1. spelling for enzymatic

R: OK, all these editing issues were arranged as suggested.

Please also note the supplement to this comment:
https://www.soil-discuss.net/soil-2019-50/soil-2019-50-AC1-supplement.pdf

Interactive comment on SOIL Discuss., https://doi.org/10.5194/soil-2019-50, 2019.