Revision round #1

Decision for round #1 : ***Revision needed***

I have read the manuscript by Godinho et al. and also the reviews from two very competent experts in the field. Overall, we all agree that the manuscript contributes with relevant information to understand how mites select plants with lower levels of Cadmium. On the other hand, there are also a number of suggestions for clarifications and improvements that can considerably strengthen the conclusions of this work. I ask the authors to critically consider these suggestions when preparing a revised version of their manuscript.

My comments

This work reports on a set of well-planned experiments to clarify the individual and collective decision process of spider mites when faced with a selection of heavy-metal (Cadmium) contaminated plants and plants with high competition from interspecific herbivores. Both feeding preferences and reproductive performance on all treatments is analysed.

The question is relevant and has the potential to significantly advance the state-of-the-art. The manuscript is clear, and the experimental and statistical approaches are mostly well explained. One concern is that the actual presence of cadmium on the tested leaf disks should be further clarified, as from L323 it seems that the presence of cadmium on leaves disks of plants grown in the presence of cadmium has not been confirmed. Please clarify, this critical aspect of the experimental design.

R1: Thank you. The presence of cadmium in the tested leaf discs was indeed not verified as it is a destructive method and would not allow further tests of spider mite performance or choice. However, we have previously established that there is a strong correlation between cadmium supplied to the plant and cadmium accumulated in the leaves (Godinho et al. 2018; 2022). Furthermore, we have also previously shown that the decrease in egg laying and offspring production of spider mite females after feeding of leaf disks with cadmium is recapitulated when they feed directly on a nutritive solution with cadmium (Godinho Branquinho and Magalhães 2023). Therefore, we can conclude that cadmium is accumulated on leaves (thus leaf discs) proportionally to the amount supplied to the soil where plants are grown. What we meant in L323 is that cues, such as plant volatiles, which may inform spider mites of the presence of cadmium, may not be produced by the leaf disks. Note also that this choice protocol has been previously used in other contexts but not to discriminate leaf discs with and without cadmium. We changed the sentence to clarify the message (see lines 335-338).

The term “discriminate” on L21 might be confusing because although there was no significant choice on the presence of mites on cadmium and no-cadmium leaves, the

former laid significantly less eggs on cadmium plants, showing that adults are capable of some form of discrimination, please clarify.

R2: Indeed, the results we are mentioning only refer to the position of individual females, not to oviposition, we changed the text accordingly.

The significance of performing these tests with cadmium and not with other heavy metal, should be briefly presented in the introduction (more abundant? more widely distributed? critical effects? ...).

R3: We have added this information to the manuscript (Lines 81-83).

The results that individual mites don't differentiate between plants with and without cadmium, but they do differentiate between the two when in colonies is puzzling and I feel that it requires some further explanation. In particular, the fact that the choice is significant when in colonies, indicates that they have the individual physiological mechanisms to detect the presence of cadmium and that they probably also have an (individual) preference for cadmium free plants. Therefore, the reason that this effect is only statistically detected when in colonies but not on individuals (If I got it right), is probably because the collective behaviour intensifies the signal of individual choice mechanisms. Please clarify this or explain some alternative rationale.

R4: We do not think that the difference in the responses in the two set-ups stem from differences in statistical power because the number of replicates is actually lower when we tested for the collective behaviour (because this set-up is much more space and time consuming). Instead, we argue that individual and collective choice may be based upon different cues. We have modified the text in the manuscript in order to make our rationale clearer (see lines 345-355).

Figure 3 The treatments Cadmium Vs. No-cadmium and competitors Vs, no-competitors are both identified with colour which make it more difficult to combine treatments (e.g. with cadmium and competitors). Consider using a more intuitive and informative way of indicating the treatments, for example with over imposed images or XX labels. Also, the font size is too small and hard to read. (similar issues on Fig. 4).

R5: We changed the identification relative to the presence of competitors to stripes on top of the colour that identifies contamination by cadmium (or not).

Figure 5 Maybe "daughters" is not the best term here, consider replacing with "emergences" or "offspring". Please make the figure self-understandable by adding the meaning of the XX labels 1, 5, 3, 4 into the caption.

R6: In fact, we only measured the number of female offspring per species, as the males of the two species are not easily distinguishable. We opted not to replace "daughters" with "female offspring" on the axis title as it is already too long. We added the meaning of the numbers into the caption.

L306 (also on L377) Consider "… do not significantly avoid…". Although statistical jargon should be avoided in the discussion, the true is that there is arguably some non-

significant trend for a lower use of cadmium leaves, that could have been picked-up if there were more more replicates (?).

R7: **We agree that there may be a tendency to avoid plants with cadmium, although it could not been easily pick up adding more replicates as the replicates in experimental evolution assays are at the population level. We rephrased as suggested.**

L315 "… feeding on tomato…"

R8: done.

L316 "…than mites…"

R9: done.

L380 The potential mechanisms enabling "collective choice" that are not present in "individual choice" should be further explored (if possible, by including known examples from other groups – if available).

R10: Unfortunately, there are, to our knowledge, no previous studies assessing collective choice (or avoidance) of metal accumulating plants.

*by **Ruben Heleno**, 28 Jan 2024 13:16*
Manuscript: **https://doi.org/10.1101/2023.08.17.553707**
version: 3

Review by anonymous reviewer 1, 15 Jan 2024 16:55

I reviewed the manuscript titled "Spider mites collectively avoid plants with cadmium irrespective of their frequency or the presence of competitors" by Godinho et al.

The manuscript is in line with previous research line that the group has followed, to study the eco/physiological implications of cadmium accumulation in tomato plants, and the consequences on herbivorous mites.

This paper focus on the effect of cadmium-treated plants on the ambulatory dispersion of Tetranychus urticae, together with the presence or absence of the competitor species T. evansi.

Authors showed that meanwhile single mites do not made a selection between leaf discs coming from plants treated or non-treated with cadmium, the oviposition is affected negatively. On the other hand, when authors performed a choice assay using 200 mites, they found a clear preference for untreated plants compared with the treated plants, independently of the presence/absence of competitors mites. Authors concluded that cadmium accumulation by plants could enhance the herbivorous mite competition in heavy-metal free patches.

Introduction and discussion are well written and are adequate with the aim of the manuscript. Experimental setup and data analysis are appropriated for the research question. In my opinion the manuscript will improve with some text modification that clarify Material and Methods, and Results sections.

R11: Thank you for the nice assessment and useful feedback.

The use of the term "treatment" to describe the "layout" or different experiments performed in the manuscript is confusing. Authors should find another term to describe this factor.

R12: By treatment we mean a set of plants to which mites were exposed to. We believe the use of the term is correct because spider mites are exposed to the set of plants simultaneously. Note that we only use this term to refer to this. For example, we never use it to refer to the presence of cadmium on a plant. Hence, we believe that there is no ambiguity and that it is correct to use this term.

I have some concerns on data presentation and statistical analysis.

- Related to data representation: Figures should be self-explanatory. Authors may represent with letters and/or asterisks the results of the statistical analysis in all plots. Authors should also provide enough information in the Figure legends related to the plot, including statistical analysis represented in each figure.

R13: This information was added to the plots and their respective legend. The exception is for panel 1 of figure 3 as the statistical comparison is with a mock treatment (with even distribution among plants) that is not represented in the figure.

- Authors have to explain in more detail on statistical analyses related to collective choice experiment. Authors should explain how they handle the "missing" mites, i.e. mites that did not make a choice or were not find back on the plants.

R14: We clarified the statistical analyses performed for the collective choice experiment (see lines 195-211). The missing mites were not considered in the analyses. This is now explained with more detail in the manuscript (lines 196-198).

- Figure 1C. Figure C follows a different layout related to cadmium treatment and make this plot very confusing. Authors should follow the same layout (color code) in all the plots. Did the authors move the cadmium-free plants to another setup to monitor the offspring? Authors should explain better the experimental procedure of this setup

R15: We apologize for the confusion. In figure 1C only treatments 1 and 5 are depicted, both having no plants with cadmium (that is why all of them are white). This is now clarified in the legend. We have also been more specific about the conditions in which we measure performance.

- Line 202. According to material and methods, authors used one concentration of cadmium to treat the plants, so authors are not testing differences in "cadmium concentration". Authors should find another term to describe this comparison.

R16: We changed to "To test if the frequency of plants with cadmium affected spider mite choice" (lines 205-207).

In this sense, authors did not show the output of this analysis in Results section. Furthermore, in my opinion the comparison between these two treatments will not give any extra value to the main observation of the manuscript i.e. spider mites avoid plants that accumulate cadmium. Uneven comparison can create an artifact. I mean, the comparison in layout 2 (50% control plants 50% cadmium treated plants) will be compared with layout 3 where the distribution of the treatments is uneven (25% control plans, 75% cadmium treated plants). This could create a difference between the two treatments simply because in layout 3 the frequency of mock plants is lower, then it is expected an increase of mite density in clean plants in layout 3 compared to mock plants in layout 2.

R17: The results from the analyses of the impact of frequency of plants with cadmium are detailed in lines 261-264 and in Figure 3. Indeed, it could be that the different number of plants with cadmium created a bias in the choice of spider mites. This was precisely what we wanted to test. Knowing that mites chose plants without cadmium even when they are rare in the environment provides extra information that the 50/50 treatment does not. We clarified this in the manuscript.

- Figure 3. Panel 1. It seems there are strong preference for plants in positions 1 and 4 (bars aprox. 45 and 50 females respectively) meanwhile the other two positions (2 and 3) showed lower densities. Authors should include an explanation on this positional effect and how can affect the output of the assays.

R18: The plants in the figure are ordered by the number of mites found on them, not by their position. We now added this information to the figure legend.

- Figure 5. I understand from Material and Methods and Result section that this Figure represent the offspring of plants free of cadmium in treatment 1; 5; 3 and 4. Authors should describe if the average of Bar 1 corresponds to the average of 4 plants per repetition of the assay, or is the average of a single plant per repetition. In this case authors should explain which plant and in which position they selected to record the offspring specially since there are differences in mite densities depending on the position.

R19: As stated in the materials and methods (lines 167-170), in treatments 1-3, we counted the eggs on all plants, then did the average of the number of eggs on plants with or without cadmium per female found on those plants. For treatments 4-5, we used the leaf with competitors, and one leaf of one plant from the corresponding treatments but without competitors (i.e., with the same proportion of plants with or without cadmium: treatment 1 for 5 and 3 for 4; in the former case the plant was randomly selected). We changed the sentence in Material and Methods to further clarify this in lines 172-178. We have also changed the legend of figure 5.

This Figure also shows that when there are plants treated with cadmium in the setup, the offspring is reduced (independently of the presence/absence of the competitor). Authors should include in the discussion an explanation for this observation. Are mites that end up in cadmium-treated plants moving to cadmium-free plants? This testing could impair the performance/offspring?

R20: This would indeed be interesting, but unfortunately it is not the case: the average number of daughters in the treatments with plants without cadmium (1+5) is not different from that of the treatments with plants with cadmium (3+4; data not shown in the ms). Additionally, the data presented in this figure concerns females that have developed on detached leaves isolated in small boxes. The presence of plants with cadmium in the environment could, therefore, affect the number of eggs laid but not offspring survival. Hence, we prefer not to develop this line of thought.

Other minor points (Authors should review the manuscript to refine the text and correct some typos)

Line 51. Add space between scarce and bracket

R21: done.

Line 193. Correct “general” to “generalized”

R22: Corrected.

Line 226. Specify the posthoc test performed.

R23: Information added.

Line 340. Delete “h”

R24: Deleted.

Line 248. Delete “even”

R25: done.

Review by anonymous reviewer 2, 24 Jan 2024 09:03

Plants can accumulate heavy metals from polluted soils, and herbivores would be affected by the bottom-up effect mediated by heavy metal accumulation. This research showed that aggregation may be an important mechanism through which spider mites avoid contaminated plants. They also indicate that cadmium accumulation in plants is a stronger selective pressure than interspecific competition. This suggests that non-accumulating plants will suffer more from herbivore than accumulating plants in metal polluted environments. It is an interesting and meaningful research. I endorse the work and have a few suggestions for minor improvements, as listed below.

R26: Thank you.

Abstract

The abstract is very long. I do not know if it is asked to show in five paragraphs. I suggest make it shorter and sharper. Then the authors should add some details of data from the results.

R27: We have shortened the abstract, while adding details on the results.

Introduction

This section points look good, but the paragraphs are too many. I suggest that some statements can be combined. L48-59, L67-L84.

R28: we merged these two paragraphs as suggested, as well as the two following paragraphs (see lines 42-53).

Material and Methods

The authors should check and revise the format. For example, 48h should be 48 h.

R29: Format revised.

L 102: the concentration of cadmium solution is 3mM or 2 mM? where did you treated with 3mM?

R30: A cadmium solution of 2 mM was used for the individual choice and performance tests and a solution of 3 mM was used for the collective choice and performance tests. This is stated in lines 122 and 147. In order to account for this difference in cadmium treatment, we estimated the effect size for cadmium. We observed that, despite the differences in cadmium concentration, the effect was similar (lines 225-227).

L 137: these experimental set-ups look good, but it can be mortified by adding border for each section to make clear.

R31: In this case we opted not to modify the figure with borders for each section as it would clutter too much de figure.

Results

Fig.2 Statistical analyses results can be added in the figure to make clear.

R32: We agree, we added the statistics to the figure.

Figures, the replications can be added in the legend, and the data points can be shown on the column. The treatments are not clear in the figure legends, I suggest that add details in the legends.

R33: We added the number of replicates on the legend of the figures and made minor changes to the legend to make them clearer.

Discussion

L 340: “h”?

R34: It was a typo, we corrected it.

Have you checked the accumulation of Cd in tomato leaves? Does the treated ones did accumulate Cd and is that the reason for the results? Dose competition among the 200 individuals happen? Did they fight with each other?

R35: Please refer to our reply #1.