

RESPONSE TO REVIEWER

REVIEWER 1

Manuscript ID	microorganisms-4499890
Title	Seasonal Dynamics of 'Candidatus Phytoplasma prunorum' in Selected Prunus Species Revealed by Real-Time PCR

Dear Reviewer,

Thank you for your careful evaluation of the revised manuscript and for your additional comments. We have addressed each comment and revised the manuscript accordingly. Our point-by-point responses, including the locations of the corresponding changes, are provided below.

Point-by-point response

Comment 1: *The main remaining methodological issue is the analysis of the detection rate. The authors correctly use a random intercept for tree identity when analyzing the quantitative titer, but differences in detection rates are assessed using pairwise tests for equality of proportions. At the same time, the same trees were examined multiple times across months and tissues, so individual detection results are also not independent observations. A simple comparison of proportions does not take into account the repeated structure of the data. For a formal analysis of detection probability, a binomial generalized linear mixed model with tree identity as a random effect would be more appropriate. If the authors do not want to carry out additional analysis, the results of pairwise proportion tests should be clearly labeled as descriptive/auxiliary and should not be used for strong statistical conclusions.*

Response 1: We agree with the reviewer and replaced the pairwise tests for equality of proportions with a binomial generalized linear mixed-effects model using a logit link and tree identity as a random intercept. Host group, plant part, sampling month, and the host group × plant part interaction were included as fixed effects; the plant part × month interaction was evaluated by a likelihood-ratio test. Holm-adjusted post-hoc comparisons of estimated marginal detection probabilities were performed using emmeans. The Results now report the overall model tests and the significant within-host comparisons. Figure 1 retains the observed percentages, while its caption clarifies that statistical inference is based on the binomial GLMM. The original proportion tests are no longer reported. These revisions can be found in Section 2.6, Statistical analysis, paragraph 2, beginning “Detection probability was analyzed using a binomial generalized linear mixed-effects model...”; Section 3.1, Overall Detection and Tissue Distribution of ‘Ca. P. prunorum’, paragraph 3, beginning “Monthly observed detection rates are shown in Figure 1...”; and the caption to Figure 1.

Comment 2: *The approach to quantitative analysis of only qPCR-positive samples is now well explained, and the authors rightly point out that the mean values are conditional on detection. Nevertheless, this has an important consequence for biological interpretation, especially for P. domestica, where only 5 out of 96 samples were positive in annual shoots. The average titer among these five positive samples cannot be interpreted as a characteristic of a typical annual shoot of this host group. In the Results, Discussion, and graphs, it is necessary to consistently distinguish between two characteristics: the probability of detection and the titer provided that detection has occurred. The related issue concerns LMM for P. domestica. Given the extremely low frequency of positive annual-shoot samples, the model for the quantitative titer may rely on a very small number of observations and yield unstable estimates for individual month × plant-part combinations. It is necessary to check*

and, preferably, report the number of positive observations that actually entered the relevant models. The interpretation of quantitative differences for groups with very low detection frequency should be carried out with the utmost caution.

Response 2: We agree and have consistently distinguished detection probability from titer conditional on qPCR detection in the Results, Discussion, and figure captions. For *P. domestica*, we now state explicitly that only 5 of 96 annual-shoot samples were positive and that the mean of these five positive samples is not representative of a typical annual shoot of this host group. We also report the numbers of positive root, one-year-old-shoot, and annual-shoot observations entering the host-specific LMMs: 48/142/127 for *P. armeniaca*, 30/49/5 for *P. domestica*, 36/117/116 for *P. persica*, and 47/143/137 for *P. salicina*. Quantitative results involving *P. domestica* annual shoots are identified as exploratory and are interpreted with particular caution. These revisions can be found in Section 2.6, Statistical analysis, paragraph 1, beginning “Statistical analyses were performed in R version 4.6.0...”; Section 3.2, paragraph 4, beginning “In annual shoots, phytoplasma titer differed significantly...”; Section 3.3, paragraphs 1 and 5, the latter beginning “In contrast to the other host groups, *P. domestica* showed...”; the note to Table 3; the captions to Figures 2 and 3; and Section 4, Discussion, paragraphs 1–2.

Comment 3: *The authors indicate the analytical detection limit as 2 log₁₀ cells g⁻¹ phloem, but mark it as “data not shown”. Since the decision to exclude negative results from quantitative analysis is directly based on this limit, it is desirable to present the method for determining LOD/LOQ or the corresponding validation data in the Supplementary Materials. The range of the standard curve up to 10¹ copies μL⁻¹ is not in itself a sufficient description of the analytical LOD.*

Response 3: We agree and have expanded the description of the analytical detection limit. The LOD is now expressed as 16S rDNA target copies per reaction, and the corresponding calculation and validation information are provided in Supplementary Table S1. We also removed the previous “data not shown” wording. These revisions can be found in Section 2.3, DNA extraction and real-time PCR quantification, beginning “Absolute quantification was based on a plasmid standard...”; Section 2.6, Statistical analysis, paragraph 1, beginning “Statistical analyses were performed in R version 4.6.0...”; and Supplementary Table S1.

Comment 4: *The absolute quantitative assessment is expressed as “phytoplasma cells g⁻¹ phloem”, although qPCR actually measures the number of copies of the 16S rDNA target relative to the plasmid standard. It is necessary either to justify the transition from copy number to cell number, including the number of copies of the corresponding rRNA operon in the genome of ‘Ca. P. prunorum’, or to use a more rigorous designation such as “target copies g⁻¹ phloem”. This is especially important for an article whose central result is the absolute quantitative assessment.*

Response 4: We agree and have clarified the conversion from 16S rDNA target-copy number to estimated phytoplasma cell number. Because one phytoplasma cell contains two copies of 16S rDNA, the calculated target-copy number was divided by two. The manuscript now provides the calculation formula and defines each term used in the conversion. This revision can be found in Section 2.3, DNA extraction and real-time PCR quantification, immediately after the paragraph beginning “Absolute quantification was based on a plasmid standard...”.

Comment 5: *Multilocus genotyping is now correctly limited by the authors as an additional characteristic of the material, and they rightly refrain from making statistical conclusions about the genotype–titer–symptom relationship. Nevertheless, selecting a single sample with the maximum titer from each tree means that the study does not allow for an assessment of possible intratree or temporal variability of genotypes. It should be briefly noted that the assigned multilocus profile characterizes the detected dominant/amplified profile of the selected sample and does not rule out mixed infection or temporal genotype variation.*

Response 5: We agree and have clarified the limitation of selecting one high-titer sample per tree for multilocus genotyping. The assigned multilocus profile is now described as the dominant profile

amplified in the selected sample and does not exclude within-tree or temporal genotype variation or mixed infection. This revision can be found in Section 2.4, Multilocus genotyping, paragraph 1, beginning “One representative sample per tree was selected for multilocus genotyping...”.

Comment 6: *The authors have significantly improved the interpretation of the presumed seasonal movement of phytoplasma and now explicitly state that the direction of movement was not measured. This is correct. However, the expression “progressive colonization of newly formed shoots” should still be used as an interpretation compatible with the data, rather than as an established process. The design itself represents successive slices of the titer distribution and does not track the movement of a specific phytoplasma population.*

Response 6: We agree and have revised the statement so that progressive colonization of newly formed shoots is presented only as an interpretation compatible with the observed pattern, not as a demonstrated process. We also state explicitly that phytoplasma movement within individual trees was not directly assessed. This revision can be found in Section 4, Discussion, paragraph 3, beginning “*P. armeniaca*, *P. persica* and *P. salicina* showed high overall detection rates...”.

Comment 7: *The discussion of the possible influence of *Cacopsylla pruni* is interesting, but somewhat lengthy relative to the data presented in this work. Since the vector population, feeding sites, and new transmission events have not been studied, it is advisable to shorten this fragment to a brief alternative explanation so as not to dilute the main result of the study.*

Response 7: We agree and have shortened the discussion of *Cacopsylla pruni* to a brief alternative explanation. The revised text states that additional inoculation events could have contributed to detection in developing shoots, while making clear that vector abundance, feeding sites, and transmission events were not monitored and that the data cannot distinguish systemic colonization from additional vector-mediated inoculation. This revision can be found in Section 4, Discussion, paragraph 7, beginning “Additional inoculation events associated with the seasonal activity of *Cacopsylla pruni*...”.

Comment 8: *The practical conclusion about roots as the most stable material for year-round diagnostics within the scope of this experiment is supported by the data. However, it is necessary to maintain the distinction between analytical reliability and practical convenience. Root sampling is more labor-intensive and potentially traumatic, so it is advisable to formulate the recommendation as using root tissue when there is a high risk of false-negative results in above-ground tissues, rather than as a universally preferred material for routine diagnostics. The conclusion in the new version is generally correctly limited to one site and one year.*

Response 8: We agree and have clarified that consistent detection does not make roots universally preferable. Above-ground shoots remain first-line material, whereas roots are described as complementary or confirmatory material when false-negative results in above-ground tissues are likely. We also note that root collection is laborious, invasive, and less readily standardized. These revisions can be found in Section 4, Discussion, paragraph 11, beginning “From a practical perspective, the observed tissue and monthly variation...”; and Section 5, Conclusions, paragraph 1.