

A critique of the statistical methods and reporting of Co-inoculation with novel nodule-inhabiting bacteria reduces the benefits of legume-rhizobium symbiosis by Kosmopoulos et al.

Kosmopoulos, J. C., Batstone-Doyle, R. T., & Heath, K. D. (2024). Co-inoculation with novel nodule-inhabiting bacteria reduces the benefits of legume-rhizobium symbiosis. *Canadian journal of microbiology*, 70(7), 275–288. <https://doi.org/10.1139/cjm-2023-0209>

For this assignment, I will focus on one of the study's goals where they aim to examine direct and indirect effects of 4 different non-rhizobial endophytes (NREs) on the fitness of *Medicago truncatula* legume and *Sinorhizobium meliloti*. Therefore, I will only discuss the greenhouse and nitrogen addition experiments.

In their methods, the authors explain that for the greenhouse experiment inoculation procedure, they controlled for the number of *Sinorhizobium* cells rather than total number of cells (NRE + *Sinorhizobium*) and their justification followed:

"Given that Sinorhizobium was the plant's only source of fixed N and was thus expected to be a major limiter on plant growth regardless of NRE presence, we opted to control for the number of Sinorhizobium cells across inoculation treatments rather than the total number of cells".

This seems logical and sensible however it poses the question on the reasoning behind inoculating *M.truncatula* with only NREs if it is predicted beforehand that they will have very poor growth. Nonetheless, in attempt to remedy this, the study designed a nitrogen addition experiment:

"...which plants were supplied with moderate amounts of N and were either inoculated with a single NRE or were left uninoculated (control)."

However, this nitrogen supplementation experiment was only for NRE's found to "to significantly impact plant traits" from the previous, greenhouse experiment. More on this later.

The reporting of the results seems unclear. The study reports the difference in EM means value of shoot biomass (mg) and number of nodules. However, the study changes the baseline between co-inoculations (*Sinorhizobium* + NREs) and NRE-only inoculations. For co-inoculations, it was a difference between *Sinorhizobium* only and co-inoculations: Meanwhile, for NRE-only inoculations, they compute the difference NRE-only and inoculated controls:

"To compare among groups, we used the Dunnet-adjusted "trt.vs.ctrl" option to compare the treatment groups to two different controls: for plants uninoculated with rhizobia, treatment groups were considered to be plants inoculated with each of the four NREs individually while the control was uninoculated plants. For plants inoculated with rhizobia, treatment groups were co-inoculated plants (rhizobia + each NRE) while the control was plants inoculated with rhizobia only."

Since the study is using different baselines to compute the EM means difference, this can be misleading and suggest that experiment is designed to highlight 'significant' differences. It's not addressed by the study why the comparison was as described.

I'd also like to draw attention to how the study report the results of NRE-only inoculation:

"Every group of plants singly inoculated with an NRE without N-fixing Sinorhizobium and uninoculated control plants were in observably poor condition upon harvest (shoot mass ranging from ~36 to 46 mg on an average compared to ~166–263 mg for plants inoculated with rhizobia; Table S5), with no clear difference in shoot mass between the NRE-only and uninoculated control groups (Fig. 2A; Table S4)."

It appears to report raw numbers which can be easily understood but expressing it as a percentage or an alternative statistic would be more meaningful. Moreover, and more importantly, it's referring to Figure 2A to report the differences in shoot biomass computed however Figure 2A seems to only report the difference in EM means for co-inoculations and not NRE-only inoculations. The same concern can be observed when reporting differences in nodules number (Figure 2B).

More concerns on Figure 2, the y-axis displays the differences of emmeans of the values in raw units of the response (mg or no. of nodules) by each point that corresponds to each tick point is labelled with the percent difference. The presentation of the plot feels unclear when the labels of the individual points doesn't align with the y-axis.

Moving on to the nitrogen addition experiment. If the study established from the first experiment – the greenhouse experiment – that NRE presence and *Sinorhizobium* absence would limit plant growth, it is curious why the nitrogen addition experiment was not performed on all 4 NREs and only on the two that we found to ‘significantly’ impact the host plant based on the results from the greenhouse experiment. It would be more sensible to perform the experiment on all co-inoculations and potentially report the ‘non-significant’ co-inoculation from the greenhouse experiment in the supplementary material.

Then, I'd like to discuss the language used to report the results. In some instances, the authors use ‘safe’ and cautious language when discussing their results. This includes the use of “no clear impact” or “no clear effect” to describe what would traditionally be labelled as ‘non-significant’ – see excerpts below. This is sensible because the authors are suggesting that the evidence and data collected do not clearly showcase a difference between inoculation groups. Nonetheless, empowering their inference by saying ‘statistically clear’ may have more impact. However, the same sentiment is not shared when reporting differences in emmeans between co-inoculation groups that the authors deemed as ‘significant’ reduction in the host shoot biomass and number of nodules.

This is demonstrated by their verbiage on the greenhouse experiment:

“We found **no clear impact** on shoot mass between plants inoculated with *Sinorhizobium* alone compared to plants co-inoculated with *Sinorhizobium* and either *Paenibacillus* sp. 522 or *Pseudomonas* sp. 702A, whereas plants coinoculated with *Sinorhizobium* plus either *Bacillus* sp. 717A or *Pseudomonas* sp. 733B **grew significantly more poorly compared to *Sinorhizobium*-only plants (P values < 0.05)**, with a 37% and 33% reduction in shoot mass, respectively”

And the nitrogen addition experiment:

“**There was no clear effect on growth** when plants were inoculated with either strain compared to the uninoculated controls ($P > 0.1$, type II ANOVA), suggesting that the NREs 717A and 733B indirectly affected plant fitness in co-inoculations by inhibiting the legume–rhizobium symbiosis. Again, plants grown without supplemental fertilizer (and without *Sinorhizobium*) were in poor condition (Table S6), and no nodules were observed on any plant.”

In their caption of Figure 3, they use the statement, “No evidence that non-rhizobial endophytes (NREs) *Bacillus* sp. 717A and *Pseudomonas* sp. 733B had any direct effects on nitrogen-supplemented plants”. A more appropriate statement may be something along the lines of “No clear statistical evidence....”

Lastly, the study doesn't report the actual P values in their results of the greenhouse experiment and suffices by stating “ $P > 1$, type II ANOVA)” for the nitrogen addition experiment. Reporting the p-values regardless of what the authors infer is important, in addition to that confidence intervals across all treatment comparisons was not reported explicitly. Confidence intervals for the greenhouse experiment are shown on Figure 2.

I believe there are many more shortcomings to discuss from this paper, but for the purposes of this assignment this may suffice.