

Dear Reviewer,

We have read your recommendations and comments again and are sincerely grateful to you. Your comments allowed us to review our work, see the flaws in it and fix them. We agree with your comments and have completely changed the manuscript, trying to focus on your views.

1

As indicated in the previous review rounds, the description provided by the authors in Section 4.3 remains imprecise. In lines 462–468, the authors indicated that the experiment included four treatment variants:

“Four variants of the experiment were used (Figure 7):

1 – control – plants grown under control conditions from seeds soaked in distilled water;

2 – nanopriming – plants grown from seeds soaked with Au-NPs ($20 \mu\text{g mL}^{-1}$, 24 h) under control conditions;

3 – CA – plants grown under control conditions (from seeds soaked in distilled water) and then adapted at 9°C for 5 days;

4 – nanopriming + CA – plants grown from seeds treated with Au-NPs ($20 \mu\text{g mL}^{-1}$, 24 h) under control conditions and then adapted at 9°C for 5 days.”

However, in lines 486–487, the authors further indicate that: *“The height of the tomato shoots was recorded at 21 days of growth (before LT exposure) and 26 days (after LT exposure).”*

This means that until day 21, only two treatment variants were present — control and nanopriming. The other two variants, CA and nanopriming + CA, appeared only later, once the plants were exposed to cold stress. Comparing the values of parameters resulted from application of four treatment variants is methodologically incorrect, because the data were collected at different time points — on day 21 for the first two variants and on day 26 for the latter two.

The experiment is two-factor experiment, where one factor is the type of treatment and the other is exposure to cold stress. Such an experimental design determines what the treatment variants should be included. Moreover, values of parameters tested should be measured at the same time point. Only then obtained results can be compared.

Response. We agree with your comment – there are not 4, but 2 experience treatments in the manuscript (control and nanopriming). We have made changes to the Methodology, Results, and Discussion. We have completely changed all the figures and tables in accordance with your comments. Thank you for your help with the work.

Reviewer response:

The fact that the authors implemented this change only at the editorial level does not contribute to an actual improvement in the scientific value of the manuscript.

I understand that the authors have now separated the measurement points by temperature (I would additionally recommend indicating also the day of growth, to clearly indicate the time passed between

both measurement terms). However, the crucial question remains: what clear conclusions can be drawn from this experiment? I continue to believe that the experimental design is fundamentally flawed. Plants naturally grow and develop over time, and therefore, the values of measured parameters are expected to change in subsequent growth days. In this context, I refer specifically to the fact that measurements were performed at different time points: day 21 of growth for plants maintained at 22 °C, and day 26 for plants kept at 9 °C. Consequently, the observed differences reflect not only the effect of temperature, but also the effect of time.

Taking shoot weight as an example, one might incorrectly conclude that lower temperature resulted in higher shoot weight. This interpretation would be misleading, as the obtained values result from two overlapping effects—time of plant growth and temperature conditions. Under such experimental design, it is not possible to determine which of these two factors contributed more strongly to the obtained result. This limitation could be resolved if additional variants of treatment were included—i.e., if a set of plants remained under optimal temperature conditions and were measured simultaneously with those shifted to cold conditions. Only such a comparative approach would allow for a reliable separation of the effects of time and temperature, and thus enable a valid interpretation of treatment impact.

Regardless of how the authors restructure the table or analyze the results, it is impossible to unambiguously determine to what extent the observed differences are due to treatment effects and to what extent they simply reflect time-dependent growth.

1a.

In their accompanying e-mail, the authors provided a list of publications in which, according to them, a similar experimental setup was used. I kindly ask the authors to indicate the specific sections or lines in each of those papers where details of the experimental design are described, so that this claim can be verified.

Response.

Dear Reviewer,

at your request, we provide the information. However, we sincerely apologize for not being able to properly understand the essence of your recommendation earlier. We admit that you are right and have tried our best to meet your wishes.

Zhang Y, He S, Chen G. Metabolomics of chloroplasts combined with photosynthetic properties reveal low-temperature stress responses in sugarcane (*Saccharum officinarum* L.). *Journal of Plant Growth Regulation*. 2025 Feb;44(2):748-64. <https://doi.org/10.1007/s00344-024-11483-9> Page 750, left column, first paragraph

Tang W, Li Z, Xu Z, Sui X, Liang L, Xiao J, Song X, Sun B, Huang Z, Lai Y, Wang C. Transcriptomic and metabolomic analysis reveal the cold tolerance mechanism of common beans under cold stress. *BMC Plant Biology*. 2025 Mar 15;25(1):340. <https://doi.org/10.1186/s12870-025-06333-z> Page 3, left column, second and third paragraphs
- Plant material and cold treatments

Li J, Guo H, Lou Q, Zeng Y, Guo Z, Xu P, Gu Y, Gao S, Xu B, Han S, Su R. Natural variation of indels in the CTB3 promoter confers cold tolerance in japonica rice. *Nature communications*. 2025 Feb 13;16(1):1613.
<https://doi.org/10.1038/s41467-025-56992-7> Page 13, left column, paragraph 5

Liu X, Zhang C, Lamlo SF, Zhao K, Abdelghany AM, Wang X, Zhang F, Yuan R, Han D, Zha B. Genetic Adaptations of Soybean to Cold Stress Reveal Key Insights Through Transcriptomic Analysis. *Biology* 2024, 13, 856 [Internet]. 2024 <https://doi.org/10.3390/biology13110856> Page 4, paragraph 1 - 2.1. Plant Materials

Amin B, Atif MJ, Kandegama WM, Nasar J, Alam P, Fang Z, Cheng Z. Low temperature and high humidity affect dynamics of chlorophyll biosynthesis and secondary metabolites in Cucumber. *BMC plant biology*. 2024 Sep 30;24(1):903. <https://doi.org/10.1186/s12870-024-05615-2> Page 4, left column, paragraph 1

Hu D, Yao Y, Lv Y, You J, Zhang Y, Lv Q, Li J, Hutin S, Xiong H, Zubieta C, Lai X. The OsSRO1c-OsDREB2B complex undergoes protein phase transition to enhance cold tolerance in rice. *Molecular Plant*. 2024 Oct 7;17(10):1520-38. <https://doi.org/10.1016/j.molp.2024.08.006> Page 1533, right column, paragraph 4 - Cold-stress treatments

Yu T, Zhang J, Ma X, Cao S, Li W, Yang G. A multi-omics view of maize's (*Zea mays* L.) response to low temperatures during the seedling stage. *International Journal of Molecular Sciences*. 2024 Nov 15;25(22):12273. <https://doi.org/10.3390/ijms252212273> Page 14, paragraph 2 - 4.1. Plant Materials

Cai P, Lan Y, Gong F, Li C, Xia F, Li Y, Fang C. Comparative physiology and transcriptome response patterns in cold-tolerant and cold-sensitive varieties of *Solanum melongena*. *BMC Plant Biology*. 2024 Apr 9;24(1):256.

Fu X, Feng Y, Zhang Y, Bi H, Ai X. Salicylic acid improves chilling tolerance via CsNPR1–CsICE1 interaction in grafted cucumbers. Horticulture research. 2024 Oct;11(10):uhac231 <https://doi.org/10.1093/hr/uhac231>
Page 11, left column, paragraph 2

Zhao Y, Li S, Wu J, Liu H, Wang P, Xu L. Insights into membrane lipids modification in barley leaves as an adaptation mechanism to cold stress. Plant Growth Regulation. 2024 Jun;103(2):369-88. <https://doi.org/10.1007/s10725-023-01114-w> Page 372, right column, paragraph 1

Ghosh PK, Sultana S, Keya SS, Nihad SA, Shams SN, Hossain MS, Tahiat T, Rahman MA, Rahman MM, Raza A. Ethanol-mediated cold stress tolerance in sorghum seedlings through photosynthetic adaptation, antioxidant defense, and osmoprotectant enhancement. Plant Stress. 2024 Mar 1;11:100401. <https://doi.org/10.1016/j.stress.2024.100401>
Page 2, right column, paragraph 1

Takeuchi K, Ochiai K, Kobayashi M, Kuroda K, Ifuku K. Light-chilling stress causes hyper-accumulation of iron in shoot, exacerbating leaf oxidative damage in cucumber. Plant and Cell Physiology. 2024 Nov;65(11):1873-87. <https://doi.org/10.1093/pcp/pcae111>
Page 1882, left column, paragraph 3 - Chilling treatment

Reviewer response:

I have examined two of the references indicated by the authors. My comments on these papers are provided below. If the authors believe that their assumption is indeed supported by indicated research, I kindly ask them to indicate publications that report two-factor experiments (i.e., with an experimental design comparable to the one used in the present study), paste fragment of these papers and to explain explicitly how these results substantiate their claims.

Fu X, Feng Y, Zhang Y, Bi H, Ai X. Salicylic acid improves chilling tolerance via CsNPR1–CsICE1 interaction in grafted cucumbers. Horticulture research. 2024 Oct;11(10):uhac231 <https://doi.org/10.1093/hr/uhac231>
Page 11, left column, paragraph 2

Chilling and SA treatments All experiments were conducted in an artificial climate chamber to investigate the impact of grafting on cucumber cold tolerance. Two types of cucumber plants were used: the self-rooted (cucumber as rootstock and scion, Cs/Cs) and grafted cucumber plants (with pumpkin as rootstock, Cs/Cm). These plants were treated at 8°C/5°C for 24, 72, and 120 h, with those treated at 25°C/18°C serving as controls. The chilling injury index (CI), reactive oxygen (ROS) accumulation, and cold-responsive genes expression were measured at 72 h.

In my view, the experiment presented in the cited work should be classified as a single-factor design, in which temperature was the only variable under investigation. The comparisons were made exclusively between plants grown under different temperature conditions, which indicates that no second independent factor was introduced or controlled in the experimental setup.

Ghosh PK, Sultana S, Keya SS, Nihad SA, Shams SN, Hossain MS, Tahiat T, Rahman MA, Rahman MM, Raza A. Ethanol-mediated cold stress tolerance in sorghum seedlings through photosynthetic adaptation, antioxidant defense, and osmoprotectant enhancement. Plant Stress. 2024 Mar 1;11:100401. <https://doi.org/10.1016/j.stress.2024.100401>
Page 2, right column, paragraph 1

Overall, the experiment included four treatments: (i) water-pretreated control seedlings (Ctrl), (ii) ethanol-pretreated seedlings (Eth), (iii) water-pretreated cold-stressed seedlings (Cold), and (iv) ethanol-pretreated cold-stressed seedlings (Eth+Cold).

In my view, this experiment should be classified as a two-factor design, with treatment as the first factor and temperature as the second factor. Accordingly, the authors tested four treatment variants.

Overall, in my opinion, the authors' assumption that the proposed experimental design is appropriate has not been sufficiently supported by evidence from other publications.

2.

As for the general comment related to the Results section, the authors continue to report standard errors instead of standard deviations. In my opinion, this approach is not appropriate, as the key aspect in this type of experiment is the variability among the plants subjected to each treatment variant, not the variability of repeated measurements within a single plant (the latter being what the standard error primarily represents). Taking the example of the data on shoot height (expressed in centimetres), a mean value of 7 cm accompanied by an error value of only 0.1 does not reflect the variability among all plants within a given treatment variant. Rather, it indicates that the measurements for individual plants were taken precisely. Therefore, standard deviation would be a more suitable measure to describe the biological variation observed in this experiment.

Response. We have made changes to all the tables and figures, taking into account the standard deviations. We thank for your help in the work, your patience and understanding. We are glad that with your help we were able to improve the manuscript.

Reviewer response:

In my opinion, the dataset presented by the authors still contains inconsistencies. Below I provide a screen of tables for comparison between the previous version of the manuscript (where standard errors were reported) and the current version (where standard deviations are presented). The intention of this comparison is to evaluate whether the values are internally consistent. It seems unlikely that the standard deviation for root dry weight of the 30 examined plants per each variant of treatment (section 4.5.) is equal only to 0.01 for all columns (measurement terms and variants of treatment) Such uniformity across columns is unexpected, particularly considering that the standard deviation reported for shoot height is substantially higher.

Table from previous version of manuscript:

Table 2. Effects of nanopriming on the growth and contents of photosynthetic pigments and soluble sugars in tomato leaves

Parameters	Control	Nanopriming	CA	Nanopriming+CA
Shoot height, cm	7.0±0.1 ^b	7.2±0.1 ^b	7.7±0.1 ^a	7.9±0.1 ^a
Dry weight of shoot, g	0.15±0.002 ^c	0.16±0.004 ^b	0.16±0.002 ^b	0.17±0.001 ^a
Chl <i>a+b</i> , mg g ⁻¹ DW	8.2±0.2 ^b	10.4±0.4 ^a	6.7±0.3 ^c	6.8±0.1 ^c
Car, mg g ⁻¹ DW	1.4±0.1 ^b	1.7±0.1 ^a	1.4±0.1 ^b	1.5±0.03 ^b
Glu, mg g ⁻¹ DW	8.8±0.03 ^c	6.0±0.1 ^d	30.0±0.5 ^a	22.0±0.5 ^b
Fru, mg g ⁻¹ DW	14.6±0.5 ^c	6.2±0.3 ^d	78.0±1.0 ^a	63.1±0.5 ^b
Suc, mg g ⁻¹ DW	14.1±0.7 ^c	12.5±0.2 ^c	50.7±1.1 ^a	38.6±2.4 ^b
Sum of sugars, mg g ⁻¹ DW	37.5±0.5 ^c	24.7±0.3 ^d	158.7±1.0 ^a	124.7±1.0 ^b

The table shows the mean values and their standard errors. The means that significantly differ at $P < 0.05$ are denoted by different letters. CA – cold adaptation at 9°C for 5 d, Chl *a+b* – sum of pigments chlorophyll *a+b*, Car – content of carotenoids, Glu – content of glucose, Fru – content of fructose, Suc – content of sucrose, DW – dry weight.

Table from current version of manuscript:

Table 1. Effects of nanopriming on the growth, contents of photosynthetic pigments and soluble sugars in tomato leaves at 22°C and after cold adaptation (9°C for 5 d)

Parameters	At 22°C		At 9°C	
	control	nanopriming	control	nanopriming
Shoot height, cm	6.97±0.51	7.18±0.48	7.70±0.78	7.88±0.66
Dry weight of shoot, g	0.15±0.01	0.16±0.01*	0.16±0.01	0.17±0.01*
Chl <i>a+b</i> , mg g ⁻¹ DW	8.17±0.31	10.37±0.65*	6.70±0.56	6.78±0.26
Car, mg g ⁻¹ DW	1.37±0.10	1.68±0.11*	1.44±0.24	1.49±0.05
Glu, mg g ⁻¹ DW	8.79±0.05	6.04±0.22*	30.03±0.34	21.98±0.92*
Fru, mg g ⁻¹ DW	14.60±0.96	6.26±0.55*	78.02±2.12	63.06±0.93*
Suc, mg g ⁻¹ DW	14.13±1.35	12.49±0.39	50.74±1.96	38.62±4.09*
Sum of sugars, mg g ⁻¹ DW	37.51±0.41	24.71±1.13*	158.69±3.67	124.65±5.86*

The table shows the mean values and their standard deviations. The means that significantly differ from control at $P < 0.05$ are denoted by asterisks. Chl *a+b* – sum of pigments chlorophyll *a+b*, Car – content of carotenoids, Glu – content of glucose, Fru – content of fructose, Suc – content of sucrose, DW – dry weight.

3.

The method of selecting the plants used for subsequent measurements remains unclear to me. The authors state that a total of 30 plants were used for measuring shoot height, and that, depending on the parameter analyzed, various number of plants (biological repetitions) were then selected for further biochemical or physiological analyses (e.g. lines 548-549 - "*To determine the level of relevant gene expression 3 biological and 3 statistical repetitions were used*"; lines 527-528 - "*To determine the contents of sugars 6 biological and 3 statistical repetitions were 527 used*"). In the context of selecting plants from the total of 30 individuals used in the experiment, and considering the different numbers of plants (biological replicates) analyzed for various parameters, I understand that these plants were randomly selected. However, this approach still appears methodologically incorrect, as it negatively affects the representativeness of the samples. A consistent sampling method should be applied across all measured parameters to ensure that the obtained data accurately reflect the variability within each treatment group.

Response.

We thank the Reviewer for his comments. Indeed, plants for biochemical analysis were randomly selected from a general sample. We will take your comments into account and adjust our working methods in future.

We also attach a photo of the examined tomatoes to show that the material is really quite homogeneous.



Reviewer response:

I appreciate the authors' willingness to improve methodological consistency in future experiments, however this does not fully address the core concern raised in reviewer's comment. The photo attached indeed suggests general visual uniformity of the plants, but it does not replace a methodological explanation nor does it confirm biological homogeneity with respect to the measured traits.

We sincerely thank you once again,

Yuliya Venzhik and team