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Review of the Ph.D. dissertation written by M.Sc. Matteo Marangi entitled
Potential of *Salicornia europaea* in decreasing colonization of plants by human pathogenic microorganisms

The results obtained by the PhD candidate are presented in the dissertation manuscript entitled "Potential of *Salicornia europaea* in decreasing colonization of plants by human pathogenic microorganisms" and in the multi-author scientific publication entitled "Abundance of human pathogenic microorganisms in the halophyte *Salicornia europaea* L.: influence of the chemical composition of shoots and soils", published in 2024 in the Agronomy journal ($IF_{2024} = 3.4$). The main scientific objective of the dissertation was to demonstrate that the chemical composition of plants, as well as the soil in which they grow, influences the efficiency of colonization of *S. europaea* by selected human pathogenic microorganisms (HPMOs). The candidate justifies the importance of this research by the potential risks to consumers posed by plant products consumed without thermal processing.

I begin my evaluation by discussing the scientific article included in the doctoral dissertation, as the thesis repeatedly refers readers to this publication for additional methodological details and results. Reviewing the article at the outset will facilitate a more comprehensive assessment of the research and help ensure that no relevant aspects of the study are overlooked. The presence of potentially pathogenic bacteria in *S. europaea* tissues was demonstrated in plants collected from two locations in France, differing in salinity and environmental history, referred to in the dissertation as "young marsh" and "old marsh". Selective media were used to identify *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *Bacillus cereus* in bulk soil, rhizosphere, and *S. europaea*

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shoots collected from both sites. The tested HPMOs were detected in all the examined ecological niches. Among the tested HPMOs, the highest abundance detected in shoots was observed on the selective medium for *S. enterica* and exceeded that observed in the rhizosphere and bulk soil. This finding demonstrates that the occurrence of HPMOs in plant tissues, including edible tissues, represents a realistic food safety concern.

However, the dissertation does not discuss potential environmental sources of these bacteria, nor does it verify, even with simple confirmatory methods, whether the microorganisms isolated on selective media correspond to the target organisms.

The next stage of the study involved chromatographic analyses of soil and plant extracts to determine the relative abundance of carbohydrates, lignins, and lipids. Thermochemolysis and pyrolysis-gas chromatography coupled with mass spectrometry were employed. The candidate demonstrated no significant differences in carbohydrate content among the tested samples. In contrast, lignin content differed, with rhizosphere soils showing higher levels than plant shoots. Significant differences in lipid content were also observed in bulk soils, with higher values recorded at the young marsh site.

Based on these results, the candidate performed correlation analyses between the abundance of selected bacterial species and soil and plant chemical parameters, including lignin content and pH. Based on these findings, the candidate concluded that high lignin content may limit plant colonization by HPMOs, as lignin content was negatively correlated with the abundance of *S. enterica* in shoots and *E. coli* in soil. Furthermore, the candidate observed that higher soil pH and salinity were associated with lower numbers of pathogenic bacteria, suggesting that these parameters may serve as indicators of environmental risk in future cultivation of *S. europaea* and other crops.

These conclusions, however, are based on a relatively limited number of samples. Therefore, the candidate conducted bioaugmentation experiments using *Salicornia* plants grown in non-saline control soil and soils supplemented with different NaCl concentrations (50, 100, and 200 mM). The soils were inoculated with *E. coli* PCM2057, *S. enterica* PCM2565, and *L. monocytogenes* PCM2191 strains, using 5 ml of bacterial suspension at a density of 1.5×10^8 CFU ml^{-1}. It would be valuable for the candidate to explain the rationale behind the selected inoculum density and discuss its environmental relevance.

Eight-week-old plants (4 weeks after inoculation) were analyzed for plant size and biomass, abundance of culturable endophytic bacteria in roots and shoots, and abundance of inoculated

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HPMOs on selective media. Molecular analyses included qPCR targeting the 16S rRNA gene and HPMO-specific genes, as well as transcriptomic analyses of plant shoots.

An additional component of the study involved interaction analyses between 58 endophytic and rhizospheric bacterial strains previously isolated by Dr. Sonia Szymańska and the tested HPMOs using a bipartite assay. Four strains exhibiting the strongest inhibitory effects through volatile compound production were selected for GC-MS analysis: *Bacillus pumilus* CSR28, *Xanthomonadales* sp. CSE34, *Streptomyces champavatii* CSR4, and *Bacillus pseudomycoides* CSE4. Since the candidate identified previously unclassified strains, it would be worthwhile to explain why the CSE34 strain remained identified only at the order level.

After reading the Materials and Methods section, I would appreciate a clearer description of the candidate's contribution to key aspects of the dissertation, including RNA isolation, ribodepletion, transcriptomic bioinformatic analyses, identification of volatile compounds, and statistical analyses. Clarification of the candidate's direct involvement in these activities would facilitate assessment of both scientific competence and individual contribution to the presented work.

One of the conclusions drawn from the bioaugmentation experiments was that 200 mM NaCl exerted the strongest inhibitory effect on plant growth. At lower salinity (50 mM NaCl), increased plant biomass was observed in treatments inoculated with *S. enterica* and *L. monocytogenes*, which the candidate attributed to pathogen-induced modification of plant responses to salt stress. However, confirmation of this conclusion would require verification of inoculant abundance and metabolic activity in soil throughout the experiment. Without such analyses, it cannot be excluded that differences in bacterial survival contributed to the observed effects. In this context, it would also be useful to know whether minimum inhibitory concentrations (MICs) for NaCl for the tested HPMOs were determined.

The candidate quantified bacterial abundance using both culture-based methods and qPCR targeting the 16S rRNA gene. Higher statistical differences among treatments were detected using qPCR than culture-based approaches. However, it should be emphasized that qPCR-based estimation of bacterial abundance has limitations. Different bacterial species contain varying numbers of 16S rRNA gene copies, and DNA may also originate from dead microorganisms. In addition, the uptake and translocation of extracellular DNA from soil by plants may influence the obtained results. These limitations are not adequately discussed in the dissertation, despite the observation that more 16S rRNA gene copies were detected in shoots than in roots, which contrasts with culture-based results p.3

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and numerous literature reports. The same concerns apply to the analyses of HPMO-specific genes in *S. europaea* shoots and roots. For example, in Appendix III, the number of bacteria is equated with the number of gene copies per ng of DNA, which constitutes an oversimplification.

Undoubtedly, transcriptomic analyses of plant shoots represent one of the most innovative aspects of the dissertation. Although the results were appropriately statistically verified, the dissertation lacks comprehensive lists of differentially expressed genes (DEGs), including both upregulated and downregulated genes. The fold-change thresholds used to define DEGs are not provided, the contrasted groups are not clearly indicated, and the quality of several figures hinders interpretation. Only a limited number of examples of enzymes and mechanisms showing altered transcript abundance are discussed. Given the complexity of the experimental design, the presented results do not fully support broader biological conclusions. I would therefore encourage the candidate to present DEG lists and fold-change thresholds during the doctoral defense.

The final part of the study focused on identifying bacterial strains from Dr. Sonia Szymańska's collection. Most strains had been identified previously, whereas the candidate identified the remaining twelve strains. Subsequently, 58 strains were tested for the production of volatile compounds that inhibit the growth of three HPMOs using a bipartite *in vitro* assay. Based on overlapping inhibitory activity, four strains were selected for analysis of microbial volatile organic compounds (mVOCs). The efficiency of volatile compound production was assessed using Headspace-Solid-Phase Microextraction GC-MS (HS-SPME-GC-MS) based on relative peak areas.

A methodological issue that requires clarification concerns the comparability of inocula used in these experiments. If inoculation was performed with an inoculation loop, differences in inoculum size may have introduced additional variation. Similar concerns apply to the HPMO strains used in the assays.

The candidate identified dimethyl disulfide, hexane, methylcyclopentane, cyclohexane, and 3-methyl-1-butanol as volatile compounds that may inhibit HPMOs growth. Some of these compounds were also produced by HPMOs themselves, which does not exclude their inhibitory activity toward other microorganisms.

In the discussion, as in earlier sections of the dissertation, the reader is frequently referred to the candidate's published paper and other external sources. This approach reduces the dissertation's self-contained nature. The discussion also includes repeated presentation of results and extensive commentary on DNA isolation kits, which appears unnecessary given that only a single kit was used

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in the study. Nevertheless, the discussion of microbial volatile compounds is particularly well developed. Overall, the interpretation of results is appropriate, although it could be expanded in several areas.

In the summary, the candidate states that all hypotheses were verified. The results indicate that colonization of *S. europaea* is correlated with soil and plant chemical composition, particularly salinity, pH, and lignin content. The candidate further suggests that the plant can modulate responses to pathogen attack. According to the transcriptomic data presented, colonization is associated with reduced activity of selected defense mechanisms and increased production of reactive oxygen species (ROS). Such observations require broader discussion, as enhanced ROS production is often accompanied by the activation rather than the suppression of plant defense responses.

In my opinion, the transcriptomic results have been interpreted somewhat superficially and would benefit from further analysis. It would also be valuable to discuss the motility of the tested HPMOs, as it is a key determinant of successful microbial colonization. Regarding the final hypothesis, dimethyl disulfide was identified as a key inhibitory compound; however, the analyses were qualitative rather than quantitative, which limits the strength of this conclusion. Numerous biologically active antimicrobial compounds are produced at very low concentrations, and dominant components are not necessarily those responsible for biological activity.

In summary, the dissertation topic is highly interesting and important from a food safety perspective. Nevertheless, the dissertation does not always form a fully coherent narrative, and the rationale underlying some experimental stages is not always apparent. Several methodological issues, as well as the candidate's contribution to the most advanced analyses, require clarification. Furthermore, the potential of *S. europaea* to limit pathogen colonization was not comprehensively evaluated despite being the central theme of the dissertation and despite the availability of transcriptomic data that could have supported such analyses.

At the same time, the dissertation includes an impressive number of experimental variants in both the bioaugmentation study and inhibition assays. The candidate successfully applied several microbiological and molecular methods, including DNA isolation, molecular bacterial identification, and qPCR analyses. Moreover, part of the results has already been published in a peer-reviewed scientific journal.

Overall, I conclude that the dissertation meets the requirements expected of a doctoral thesis, and several of the issues raised in this review may be effectively clarified during the doctoral defense.

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As a result of my review, I declare that the Ph.D. thesis written by M.Sc. Matteo Marangi meets the criteria pursuant to art. 187 of the Act of 20 July 2018, The Law on Higher Education and Science (Journal of Laws of 2023, item 742, as amended), and request that the Research Discipline Council of Biological Sciences of the Nicolaus Copernicus University in Toruń accept M.Sc. Matteo Marangi for further stages of doctoral proceedings.

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