Research

Standard Operating Procedure for Testing Plant Biostimulants from Air Dried Microalgae with Germination Tests within the Nanostructurome Pipeline

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Abstract:
A standard operating procedure (SOP) for testing plant biostimulants from air dried microalgae with germination tests is given. Microalgae-based biostimulants offer a sustainable approach to enhancing plant growth and resilience in response to environmental stressors such as drought and soil degradation. This study presents a standardized protocol for evaluating microalgae-based biostimulants through germination assays. The protocol encompasses sample preparation, seed germination tests, and statistical data analysis to assess biostimulant effectiveness or phytotoxicity. Experimental procedures involve preparing biostimulant solutions from dried microalgae biomass, testing their effects on seed germination and root elongation, and analyzing germination indices. ImageJ software is used for root length measurements, with data subjected to statistical validation. Standard laboratory quality control measures and data management should be followed. This protocol provides a reliable method for assessing the agricultural potential of microalgae-derived biostimulants, facilitating their further development and application in sustainable farming practices.

Keywords: Sustainable agriculture; Microalgae; Plant biostimulant; Germination test

1. Definitions

Biostimulant: A natural substance or microorganism applied to plants or soil to improve nutrient uptake, growth, and resilience to environmental stress.
Microalgae: Microscopic algae, such as Scenedesmus sp., used in this context for biostimulant production.
Biogas Digestate: A nutrient-rich by-product of anaerobic digestion of organic materials from biogas plant.
Germination Index (GI): A measure of seed germination performance and early root growth.
Germination Test: An experimental setup used to assess seed viability, root development, and the effectiveness of growth-promoting treatments.
PPE: personal protective equipment
DM: dry matter

2. Background

Microalgae-based biostimulants present sustainable solutions to contemporary agricultural challenges, including drought, irregular rainfall, and soil degradation (Miranda et al., 2024). These biostimulants enhance seed germination, root development, and nutrient utilization, thereby improving plant resilience to environmental stress (Bulgari et al., 2014; Van Oosten et al., 2017). The efficacy of microalgae as biostimulants is influenced by their growing conditions, with nutrient availability and environmental stresses (Khalid et al., 2024) playing crucial roles in their biochemical composition and effectiveness (Petkov et al., 2009; Alling et al., 2023).
Cultivating microalgae on liquid digestate exemplifies circular economy principles by converting waste into valuable agricultural inputs (Barzee et al., 2022). The liquid fraction of the digestate, obtained after mechanical separation of solid components, is rich in nitrogen, phosphorus, potassium, calcium, sodium, and trace elements. This nutrient-rich medium is cost-effective for cultivating microalgae such as *Scenedesmus* sp. (Resman et al., 2021).

The digestate provides essential nutrients for microalgae growth, supporting the production of secondary metabolites, including amino acids, polysaccharides and other bioactive compounds that are critical for their biostimulatory properties (Hossain Sani et al., 2024). This SOP outlines the testing process for the effectiveness of plant biostimulants using germination tests and data analysis to evaluate their efficacy.

3. Purpose, Scope and Applicability

The purpose of this SOP is to provide a standardized protocol for testing the efficacy of plant biostimulants. This ensures consistent and reliable evaluation of biostimulant performance through germination tests.

This SOP applies to biostimulants produced from microalgae. It encompasses the entire testing process, including sample preparation, germination tests, data collection, and analysis. It is used to determine optimal treatments, concentrations and potential phytotoxicity of developed microalgae biostimulants for further tests on agricultural plants.

It is intended for application in research laboratories, quality control departments, and agricultural product development settings. It is designed for researchers, technicians, and other personnel involved in the development and testing of biostimulants.

4. Health and Safety Warning

When handling biogas digestate (used for algae growth media in our experiments) and microalgae samples, it is crucial to wear appropriate personal protective equipment (PPE), including gloves, lab coats, and safety goggles. This precaution helps prevent skin contact, ingestion, or exposure to possible harmful substances. Proper laboratory ventilation is necessary, especially during material drying. When processing with chemicals e.g. solvents, extractants utilizing fume hoods or local exhaust systems can minimize the inhalation of potentially harmful gasses or particles. Regular inspection and maintenance of ventilation equipment are required to ensure effective operation and safeguard the health of laboratory personnel. Operating centrifuges, microfiltration units, and drying cabinets in accordance with the manufacturer's instructions and laboratory safety guidelines is imperative. Regular inspection, maintenance, and calibration of all equipment help prevent accidents or damage. Training personnel in the safe operation of laboratory equipment and emergency procedures further enhances laboratory safety. Exercise caution when handling chemicals like sodium hypochlorite for seed sterilization and use chemical-resistant gloves and work in well-ventilated areas or fume hoods. It is important to ensure that personnel are aware of the potential hazards of each chemical, including risks of burns, inhalation, and environmental impacts.

5. Cautions

Dispose of biostimulant solutions, germination materials, and other waste must be according to laboratory biohazard protocols. Segregate waste, use designated biohazard containers, and follow safe transport and disposal procedures. Regularly reviewing and updating waste disposal protocols to comply with regulations is essential for maintaining a safe and compliant laboratory environment. After use sterilize reusable glass equipment in an autoclave and disinfect growing plates with 70% ethanol after washing them. Microalgae biomass produced in experiments is strictly for scientific research and is not approved for personal use or any other unauthorized purposes. Ensuring that all personnel understand this restriction is crucial for maintaining the integrity and safety of the research.

6. Personnel Qualifications / Responsibilities

Personnel involved in the testing of plant biostimulants must have a background in laboratory techniques, including experience in handling biological materials and conducting germination tests. They should be familiar with the operation of laboratory equipment such as ventilated areas or fume hoods, autoclave, centrifuges, microfiltration units, and drying cabinets. All safety protocols must be followed, including the use of PPE, proper handling of chemicals, and disposal of waste. Regular safety audits and training personnel in emergency procedures must be conducted. Ensuring that all personnel are thoroughly trained in first aid, emergency procedures, spill response, and safe chemical handling is

vital. Additionally, insufficient training can compromise the accuracy and reliability of experimental results, leading to data integrity issues and potentially invalidating research findings. Laboratory technicians are responsible for preparing samples, conducting germination tests, and recording data accurately. They must ensure that all procedures are followed according to the SOP and maintain a clean and organized workspace. Supervisors oversee the entire testing process, ensuring adherence to the SOP and verifying the integrity of the data collected. They are responsible for training personnel, maintaining equipment, and addressing any issues that arise during the testing process. Training all personnel in the correct use of PPE and emphasizing its importance during sample handling and processing is essential for maintaining a safe laboratory environment. Statistical analysis of the experimental results must be accurate and reliable of the data and provide detailed report on the findings.

7. Materials, Equipment and Supplies

The testing of plant biostimulants derived from algae biomass requires a comprehensive array of materials, equipment, and supplies to ensure accurate and reliable results. Essential materials include microalgae biostimulant samples, which are derived from microalgae species such as *Scenedesmus* sp., at different formulations, testing kits such as Phytotoxkit liquid samples and test plant seeds. These biostimulants are prepared in various concentrations to test their efficacy on plant growth. Various types of seeds can be used in the germination tests specified by the experiment. The choice of seeds can depend on the specific objectives of the study and the plants relevance to agricultural practices. Seeds that are too big for germination test plates used in this SOP are unsuitable for this type of germination tests (e.g. avocado or other stone fruits). Deionized water is used for preparing biostimulant suspensions and for rinsing seeds, as it is free from ions and contaminants that could affect the results of the germination tests. Deionised water is also used in control group.

Germination tests are customized after Zhao et al. (1992) and Maunuksela et al. (2012). Phytotoxkit liquid samples (MicroBioTests Inc.) test plates were used to assess the phytotoxicity of the biostimulants. The Phytotoxkit is a standardized bioassay that measures the effects of substances on seed germination and early plant growth and can be used for toxicity testing, helping to determine the potential toxic effects of the biostimulants on plant growth and ensuring that they are safe for agricultural use. The ISO 18763:2016 soil quality standard outlines the methods for determining the toxic effects of pollutants on germination and early growth of higher plants. Adhering to this standard ensures that the testing procedures are consistent and reliable. Sterile testing plates are used to conduct germination tests. Sterility is crucial to prevent contamination that could affect the results. They provide a controlled environment for observing seed germination and root growth. Filter paper is placed on testing plates to support seed germination. Pipettes or micropipettes are used for accurate measurement and application of biostimulant suspensions. Precision in measuring and applying solutions is essential for obtaining reliable and reproducible results. Measuring cylinders are used for preparing and measuring biostimulant suspensions, ensuring that the correct volumes of solutions are prepared for the experiments. Sterile forceps are used for handling seeds and samples to prevent contamination. Gloves and laboratory safety equipment are essential for personal protection and maintaining a safe laboratory environment. An incubator or controlled temperature environment is used for maintaining consistent conditions during germination tests. The incubator provides a controlled environment with precise temperature and light conditions, ensuring that the germination tests are conducted under optimal conditions. In practice, a temperature-controlled room is also acceptable (25±5°C).

8. Computer Hardware and Software

Needed hardware is a personal computer with installed software, such as photo editing software e.g. ImageJ for analysing root length and germination data, data management and word processing software such as Microsoft Office (Word, Excel). Used data saving and sharing infrastructure can be any hard drive or cloud-based services and communication platforms. For statistical analysis, the suitable programme is needed e.g. in R language (R Commander).

9. Step by Step Procedure for Testing Plant Biostimulants from Microalgae

9.1 Sample Preparation

- Prepare obtained microalgae biomass. It can be used as fresh green biomass or dried at 35°C±2 to air-dryness and subsequently ground into fine powder (fraction size passes 0.2 mm mesh).
- Microalgae biostimulant solution preparation depends on the chosen treatments for germination tests. Mix the powder in deionized water to prepare suspensions with at least three different concentrations such as 1 g DM/L, 5 g DM/L, and 10 g DM/L and control with only dH_2O (this is crucial for statistical evaluation of the results).
- Suspension volume depends on number of repetitions. For one germination test plate (Phytotoxkit liquid samples, MicroBioTests Inc.; Figure 1) 20 mL of biostimulant suspension is needed to fully saturate the absorbing material (foam sheet or absorbing paper towels) of the test plate.

9.2 Germination Test Setup

- Use seeds of selected plant species to account for the plant species variability in sensitivity, the assays are performed with the seeds of three plant species: one monocotyl (proso millet - *Panicum milliaceum*) and two dicotyls (e.g., garden cress – *Lepidium sativum*, cabbage - *Brassica Oleracea* var. *capitata*).
- It is recommended to sterilize seeds by soaking in 1% sodium hypochlorite (NaOCl) solution for 5 minutes, followed by thorough rinsing with deionized water. This depends on plant species. Note: check for possible adverse effect of NaOCl on seeds germination, e.g. garden cress can be susceptible. If NaOCl is not used the chance of mould development on seeds is higher, therefore use more replicates.
- For preparation of growing medium, foam sheet and black filter paper are placed on test plates. Instead of foam sheet folded paper towel can be also used (3 paper towels folded in thirds and then in half so it fits test plate).
- First foam sheet or paper towel is saturated with the first half (10 mL) of the prepared suspension. After that the black filter paper is placed on top.
- Distribute 5-10 seeds (depending on seed size; more seeds when smaller) evenly on each half of test plate on black filter paper. Seeds must be offset towards the top of test plate.
- The remaining 10 mL of suspension per replicate is carefully evenly applied with pipette on black filter paper with seeds.
- Prepare at least three replicates for each treatment concentration and the control.
- Place the prepared test plates for incubation in a growth chamber or incubator vertically at 25±5°C for at least three days (time depending on plant species). It can be placed in dark or under dimmed even lighting. Monitor daily.

Preparation of biostimulant suspensions for germination tests and two germination test plates with treated cabbage seeds are shown in **Figure 1.**

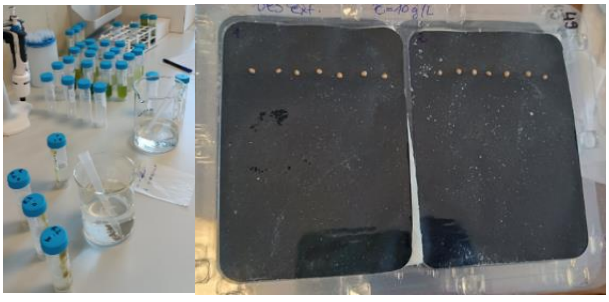

Figure 1. Preparation of different microalgae biostimulant suspensions for germination tests (left) and two germination test plates with cabbage seeds treated with 10 g DM microalgae biostimulant/L (right).

9.3 Observations and Data Collection

- After seeds germinate (in compliance with ISO 18763:2016 root length at least 1 mm) and when the root sprout shoots (approximately 3 days, depending on plant species and temperature), take the digital photograph with the measure (ruler) next to it.
- Measure the length of roots (in compliance with ISO 18763:2016 from seed to root tip) and shoots (in compliance with ISO 18763:2016 from seed to tip) using the ImageJ software or similar tools. Measure is determined with the help of ruler next to the taken photograph of the germinated test plate.
- In ImageJ software determine scale with the help of the ruler on the photograph (new scale for each photograph). This is crucial if you do not have a fixed stand for the camera (if the digital photograph was taken with the camera on the stand, you can determine one scale for all photographs).
- Count number of successfully germinated seeds.
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- Calculate Germination Index (GI):

$$\text{GI}\,[\%] = \frac{(\text{no. germinated seeds} * \text{root lenght})}{(\text{no. germinates seeds in control} * \text{root lenght in control})}\ \text{x}\ 100 \qquad (1)$$

- Log all germination percentages, root lengths, and GI values in a laboratory notebook or spreadsheet.

9.4 Data Analysis

- Analyse the calculated GI using suitable software for statistical evaluation for determining significant differences among biostimulants at 95 % confidence. Compare treated samples against controls using appropriate statistical tests (e.g., t-tests).
- The efficacy of the biostimulant is determined on observed improvements in GI compared to control.

9.5 Quality Control

- Conduct the tests at least in triplicates to ensure consistency and reliability of results.

9.6 Troubleshooting

- Seeds may fail to germinate because of poor seed quality or dormancy, incorrect incubation conditions (temperature, light, or moisture), contamination in the germination medium. Therefore the seeds must be fresh, tested for germination prior to experiments (FAO and ISTA, 2023). Incubation must be verified and conditions adjusted to match species requirements. Preparation of germination materials must be in sterile enviroment.
- Uneven germination across treatments may be because of variability in suspension application or filter paper saturation or uneven distribution of seeds. Even saturation of filter paper before placing seeds must be ensured. Seeds must be placed evenly and maintain uniform spacing.
- Contamination (e.g., mold growth) may result from inadequate sterilization of seeds, tools, or containers, or from high humidity and prolonged incubation periods. Ensure thorough sterilization of all materials and equipment. If mold is detected the test is not valid and should be repeated.
- Inconsistent root growth measurements may be caused by errors in image capture or analysis, or variations in seedling orientation during measurement. Calibrate imaging equipment and use consistent lighting. Align seedlings and make shure the roots are not crossed before capturing images. Validate measurements in ImageJ manually if needed.
- Phytotoxicity may arise from excessive biostimulant concentrations or impurities in the microalgae biomass. Reduce the concentration of the biostimulant ssuspension.
- Double-check of all recorded data (photograps) before ImageJ analyses and calculations; since some photographs can be blured or light reflection may be visible which all distort results.

- Poor ventilation may result from inefficient systems or malfunctioning fume hoods. Inspect and clean ventilation systems regularly.

10. Data and Records Management

All raw data, observations, and measurements, including germination percentages, root lengths, and Germination Index (GI) calculations, must be systematically recorded in a laboratory notebook or digital spreadsheet. Ensure experimental conditions, such as incubation temperature, light cycles (in hours) and light intensity (bright light, dimed light, in darkness), and biostimulant concentrations, are documented clearly to support reproducibility. Digital data, including images and analysis outputs, must be securely stored on systems with regular backups, using standardized file naming conventions (e.g., experiment name, date, treatment identifier). Apply version control to digital documents to maintain a traceable record of changes, and export image analysis results in formats compatible with statistical software such as R. Validate data regularly by cross-referencing entries with original observations or images to ensure accuracy. Restrict access to raw and processed data to authorized personnel and share data securely using encrypted methods or password-protected files. Archive finalized datasets and reports in designated repositories with metadata descriptions that include study objectives, methods, and data collection dates. All data management activities must comply with institutional policies, legal requirements, and ethical standards while safeguarding confidentiality and intellectual property rights.

11. Waste Management

Dispose of all biostimulant suspensions, germination materials, and other waste according to local biohazard disposal protocols to prevent environmental contamination and health hazards.

12. Related Protocols or SOPs

This SOP includes a combination of optimized known procedures. Related protocols and SOPs include the SOP for Microalgae Cultivation, Harvesting and Biomass Processing, which outlines the procedures for cultivating microalgae in laboratory conditions, harvesting methods such as sedimentation or microfiltration, and drying processes. The SOP for Procedure for Interferometric Light Microscopy of Extracellular Particles can be used to analyze microalgae biomass as microalge exude extracelular compounds.

13. Quality Control and Quality Assurance Section

13.1 Instrument calibration

Instruments calibration (laboratory scale, thermostat of drying cabinet) regularly according to the manufacturer's specifications is essential to maintain the accuracy and reliability of results. Calibration schedules should be documented and strictly followed, with a record of all calibration activities maintained for quality assurance purposes. Any deviations in equipment performance must be identified and corrected promptly to avoid inaccuracies in measurements.

For imaging systems, use a calibration scale (e.g. millimeter graph paper) or ruler to verify accurate measurements in software such as ImageJ. 13.2 Critical processes parameters and checkpoints

Critical process parameters and checkpoints are essential to maintaining the quality and reliability of the testing process. During microalgae biostimulant preparation, it is vital to confirm the exact concentrations of suspensions (e.g., 1 g DM/L, 5 g DM/L) and ensure uniform mixing to avoid variability. Seed sterilization must be conducted using appropriate sterilizing agents, such as sodium hypochlorite, with thorough rinsing to prevent residual chemicals from interfering with germination. In the germination test setup, filter papers must be evenly saturated with the correct volume of biostimulant solution or control water (e.g., 20 mL per test plate), and seeds must be distributed uniformly on test plates to minimize bias. Incubation conditions, including temperature (25±5°C) and light intensity of environment. Light intensity is generally not critical for seed germination in most

plant species. Many seeds germinate best in darkness, while some require light to trigger germination. Light preferences depend on a plant species germination demands (e.g. garden cress, *Lepidium sativum,* is somewhat sensitive to light during germination. While it does not require complete darkness, light can enhance its germination rate compared to total darkness. However, it can still germinate under a wide range of light conditions as long as moisture and temperature are optimal).

Data collection requires careful validation of root length measurements using calibrated imaging systems, with recorded values double-checked for accuracy. Calculations of the Germination Index (GI) should be double-checked to ensure consistency and precision. Adherence to these parameters and checkpoints ensures the accuracy, reproducibility, and credibility of the testing process.

14. Data on Procedures and Samples

Data on procedures and samples are given in **Table 1.**

Table 1. Data on procedures and samples.

Description of the microalgae sample	Dried microalgae biomass
Needed dry microalgae	10 g
Number of plant seeds	120 (10 per grow plate)
Minimal volume of the suspensions (dH_2O) needed	20 mL/grow plate
Time required to obtain results	4 to 5 days (3 days germination + 1 day image analyses)
Manpower	Technician
Minimal estimated cost without manpower	25 €/treatment (min. 3 replications + control)
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15. Conclusions

This SOP describes laboratory procedures for testing the germination stimulation potential of microalgae-based biostimulants.

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