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A Special Issue devoted to
Smart and Resilient Agriculture

Guest Editors
M. Ashrafuzzaman, Md. Masudul Karim and Md. Arif Sakil

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Pertanika Journal of Tropical Agricultural Science
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PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

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Preface

The rapid evolution of technological innovation, coupled with the increasing necessity for sustainable resource management, has created both profound challenges and transformative opportunities for global agricultural systems. In an era marked by climate variability and growing food security demands, agriculture must extend beyond traditional production methods by integrating precision technologies, bio-innovations, and advanced agro-processing. Fostering research and interdisciplinary collaboration is therefore essential to support agricultural practices that are resilient, sustainable, and future-ready.

It is an honour to present this Special Issue of the Pertanika Journal of Tropical and Agricultural Science (PJTAS), entitled "Advancing Sustainable Agriculture: Integrating Precision Technologies, Bio-Innovations, and Agro-Processing", under the theme "Smart and Resilient Agriculture". This collection brings together cutting-edge research and comprehensive studies that contribute significantly to advancing sustainable agricultural practices and food systems.

The contributions featured in this special issue address critical dimensions of modern agricultural science. Research on precision technologies and smart farming highlights the integration of digital tools, data-driven approaches, and automated systems to optimise input efficiency, crop monitoring, and yield prediction. Studies on bio-innovations explore sustainable crop protection, soil health management, and biological approaches to enhance crop resilience to biotic and abiotic stressors. Furthermore, articles on agro-processing and post-harvest technology emphasise the importance of value addition, waste reduction, and quality preservation in creating robust agri-food supply chains.

Collectively, these contributions demonstrate that achieving long-term sustainability in agriculture requires a holistic approach—combining technological precision, biological solutions, and efficient post-harvest processing. Such integrated strategies are key to building agricultural systems that are both environmentally sustainable and resilient against global challenges.

We sincerely appreciate all the authors for their valuable research contributions, the peer reviewers for their rigorous and constructive evaluations, and the Editorial Team of PJTAS for their unwavering support and dedication throughout

the preparation of this publication. We hope that this Special Issue serves as a valuable resource for researchers, practitioners, and policymakers, inspiring further innovation and collaboration toward smart and resilient agricultural systems.

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Review Article

Sensor-Based Smart Irrigation Management for Rice Production: A Systematic Review

S. M. M. Shahriar Tonmoy[1,2], Md. Kamal Uddin[1]*, Susilawati Kasim[1], Syaharudin Zaibon[1], Md. Asib Biswas[1,2], Ahmad Suhaizi Mat Su[3], Nur Azuan Husin[4], and Yulkifli[5]

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ABSTRACT

Despite advances in sensor-based smart irrigation, existing research often examines sensing, Internet of Things (IoT), and artificial intelligence (AI) approaches separately, with limited synthesis of their combined roles in precision water management for rice production. This review addresses this gap by systematically evaluating soil moisture sensors, IoT-enabled irrigation, and AI approaches to improve irrigation precision, water use efficiency, and rice productivity. A structured major database search identified 210 records, with 80 studies meeting the inclusion criteria. The studies were grouped into four themes: soil moisture sensors and calibration, IoT and automated irrigation, sensor-based water management, rice production and machine learning and AI-based decision making. Soil moisture sensors, including resistive, capacitive, Time Domain Reflectometry, Frequency Domain Reflectometry and neutron probe sensors, provide different capabilities for real-time monitoring. The performance of all those sensors is influenced by soil conditions, calibration procedures and sensor placement. IoT systems enabled continuous monitoring, automated irrigation and cloud-based decision support, while AI support soil moisture forecasting and

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predictive scheduling. Quantitative evidence indicates that sensor-based smart irrigation reduces water use, improves water use efficiency, and maintains or enhances rice yield. However, sensor reliability, calibration, implementation cost, infrastructure, maintenance and scalability remain important barriers to adaptive irrigation under diverse rice-growing conditions. Future research should address gaps in affordable, validated systems that integrate sensing, IoT, and AI technologies for precise and sustainable rice water management.

Keywords: Internet of Things (IoT), irrigation, moisture content, rice, soil moisture sensor, water use efficiency, yield

INTRODUCTION

Rice (*Oryza sativa* L.) is a vital cereal crop worldwide, serving as a staple food for over half the global population, with Asia producing 90% of the total global yield (Fukagawa & Ziska, 2019). It is highly water-dependent and consumes approximately 24-30% freshwater resources in the world (Nandan et al., 2020). The amount of fresh water that is available for human use is less than 0.01% of the total amount of water on Earth, highlighting its scarcity despite being crucial for sustaining biodiversity (Potharaju & Aruna, 2024). Rapid population growth has increased water scarcity across many regions. Agriculture consumes nearly 70% of global freshwater withdrawals, while 15-35% of irrigation water use remains unsustainable, highlighting the need for improved water use efficiency (Ingrao et al., 2023).

Overwatering, nutrient leaching, and water wastage might result from using conventional irrigation techniques. With the growing need to conserve water resources and improve crop yields, precision irrigation technologies have gained prominence in rice farming (Loconsole et al., 2025). Smart farming allows farmers to remotely monitor the plants, including harvesting and crop yields, as well as automation of the sensors and machinery in order to optimise the quantity and quality of product (O'Grady et al., 2017). Local rural economies are greatly impacted by irrigation since it influences crop output and encourages economic growth. Soil type significantly influences water holding capacity and crop productivity, with clay loam soils having higher field capacity than sandy soils (Ma et al., 2016).

Organic matter enhances soil structure and moisture retention, especially in sandy and loamy soils, improving crop resilience under water stress (Cachipuendo et. al., 2025). Ensuring the soil has a suitable amount of moisture in critical stages for maintaining good crop growth because of varying requirements for soil moisture content at different periods and its impact on crop yields (Nangia et. al., 2018). According to several studies, during the mid-to-late grain filling stage, moderate soil drying enhances the activity of key enzymes involved in the synthesis of starch, significantly increasing the grain-filling rate and grain weight, thereby improving rice yield (Wang et al., 2019). Evidence suggests that after drought, rehydration can recover the metabolism of rice source and sink organs, facilitating

normal growth and development (Lawas et al., 2019). A proven technique for controlling agricultural water requirements during the growing season is irrigation scheduling. Various irrigation scheduling techniques, including evapotranspiration (ET), soil moisture sensing and artificial intelligence (AI)-based methods have been previously demonstrated (Kelley et al., 2023). Today's agricultural technology has changed the way farming is carried out, and the Internet of Things has revolutionised traditional methods (Quy et al., 2022).

Internet of Things (IoT) technologies in farm management can provide real-time monitoring of soil moisture, temperature and other critical environmental variables, allowing more precise and automated irrigation practices. Using IoT and microcontroller devices with solar panels, a smart irrigation system for rice cultivation was developed. It can automate irrigation by minimising physical labour through proper monitoring (Binayao et al., 2024). This Internet of Things (IoT) information helps farmers in rice fields by monitoring them remotely. Among these, soil moisture sensors provide real-time data on soil water availability, which forms the foundation of water management decision-making systems (Placidi et al., 2020). In order to guarantee ideal levels of moisture for crops and avoid excess irrigation, using IT-based soil moisture sensing irrigation scheduling helps to maintain proper moisture content in the plant's root zone (Bryant et al., 2023). Therefore, when compared to traditional techniques of irrigation, sensor-based irrigation scheduling improved the efficiency of water consumption. IoT-based irrigation using soil moisture sensors enables precise water application, significantly enhancing water use efficiency and crop yields in diverse cropping systems (Gopal et al., 2025). Despite the fact that a number of studies on soil sensors have been conducted, users of soil sensors continue to face many difficulties because of diverse concepts, types and production requirements, in addition to the characteristics of soil moisture sensors.

Even with the increasing application of soil moisture sensors and IoT technologies in agricultural water management, several research gaps remain. Previous review articles have discussed soil moisture sensing technologies, smart irrigation systems and IoT-based agricultural applications. However, most have focused on general irrigation practices, individual sensor technology or broad precision agriculture applications. Limited attention has been given to the integration of recent advances in soil moisture sensing, sensor calibration, field capacity-based irrigation threshold, IoT-enabled irrigation control, artificial intelligence (AI) and water use efficiency specifically for rice production. Furthermore, previous reviews have generally provided descriptive overviews with limited critical comparison of sensing performance, calibration requirements, irrigation scheduling strategies and the practical challenges associated with sensor deployment under diverse rice growing conditions. Rapid developments in low-cost sensors, wireless communication, edge computing and AI-driven decision support systems have also generated substantial recent evidence that needs systematic and critical analysis.

Therefore, the present review focuses especially on sensor-based smart irrigation management for rice production. It provides a comprehensive synthesis of soil moisture sensing technologies, sensor calibration, field capacity threshold, IoT integration, AI-assisted irrigation scheduling, water use efficiency, sensor deployment, yield improvement and emerging research direction. In contrast to earlier reviews, this study integrates recent literature published during 2015-2026, with particular emphasis on rice-specific smart irrigation applications and a critical comparison of sensor technologies, AI and IoT-driven precision irrigation strategies. The principal contribution of the reviews is to establish a holistic framework that links soil moisture sensing, calibration, automated irrigation control, efficient water management, and sustainable rice production. Also, identifying technological and practical research priorities for improving irrigation efficiency under increasing climate variability and water scarcity. This review further examines the fundamental working principles of major types of soil moisture sensors and their applications in rice irrigation management to assess their integration with Internet of Things (IoT) technologies and automation systems. Also identify the current challenges, research gap and future opportunities for smart irrigation management.

LITERATURE SEARCH AND SELECTION CRITERIA

The literature search was carried out comprehensively following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) to make the process of literature selection transparent and reproducible. Five scientific databases (Scopus, Web of Science, ScienceDirect, IEEE Xplore and SpringerLink) were used to identify relevant publications published between 2015 and 2026. The reference lists of the selected articles were manually searched for additional relevant publications to make sure that the topic was covered in as comprehensive a way as possible. Literature search was done using keywords, machine learning, sensor calibration, precision irrigation, soil moisture monitoring, irrigation scheduling, smart irrigation, soil moisture sensor, Internet of Things (IoT), rice, water use efficiency and combinations of these with Boolean operators (AND, OR). Records were regularly assessed for relevance during the review process because the literature search was carried out manually in phases. Table 1 provides a summary of the inclusion and exclusion criteria utilised in the selection of the literature.

Potentially relevant articles were assessed using abstracts and titles, and after removal of duplicate publications, full-text evaluation was performed. The studies were chosen because they are relevant to soil moisture sensing technologies, smart irrigation systems, irrigation scheduling, Internet of Things (IoT) applications, artificial intelligence, sensor calibration and water use efficiency in rice production. Lastly, 80 articles were selected to participate in the qualitative synthesis, which was then grouped into thematic categories such as soil moisture sensing technologies, calibration and sensor performance, irrigation systems with IoT support, irrigation scheduling, water use efficiency, irrigation decision support using AI, and future research directions. The process of study identification,

screening, eligibility assessment and final inclusion is summarised following the PRISMA 2020 framework in Figure 1.

Table 1
Criteria for inclusion and exclusion in the selection of literature

Criterion	Inclusion Criteria	Exclusion Criteria
Publication period	From 2015 to 2026	Published prior to 2015
Language	English	Publications written in languages other than English
Publication type	Selected conference proceedings and peer-reviewed journal articles	Newsletters, theses, patents, editorials and short communications
Study focus	Water use efficiency, rice production, IoT, AI, smart irrigation, precision agriculture, soil moisture sensors and irrigation scheduling	Research unrelated to soil moisture monitoring or irrigation technologies
Crop	The main focus is rice; specific studies on other crops are utilised to compare technologies.	Other crop studies without relevance to rice irrigation technology
Accessibility	Articles in full text	Publications with only abstracts
Scientific relevance	Experimental investigations, field trials, greenhouse studies, modelling studies and review papers relevant to smart irrigation	Research with insufficient technical details or applicability

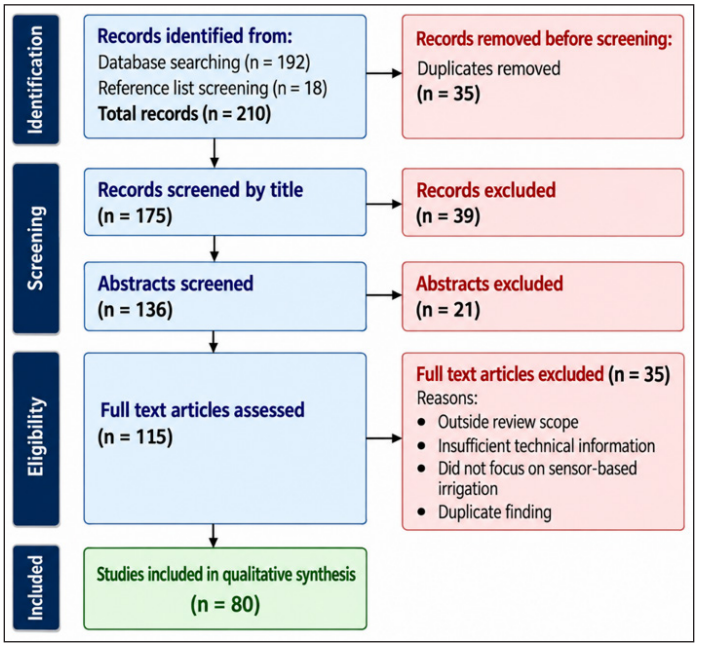

Figure 1. PRISMA 2020 flow diagram showing how studies were identified via database screening in this systematic review

The bibliographic distribution of selected articles by year of publication is shown in Figure 2. The overall trend of the number of publications is an increasing one, especially after 2021, reaching the highest in 2024 (14 publications), followed by 2025 (13 publications) and 2023 (13 publications). The rising publications reflect the growing research interest in sensor-based smart irrigation, IoT-based agricultural systems, and data-driven water management for rice production.

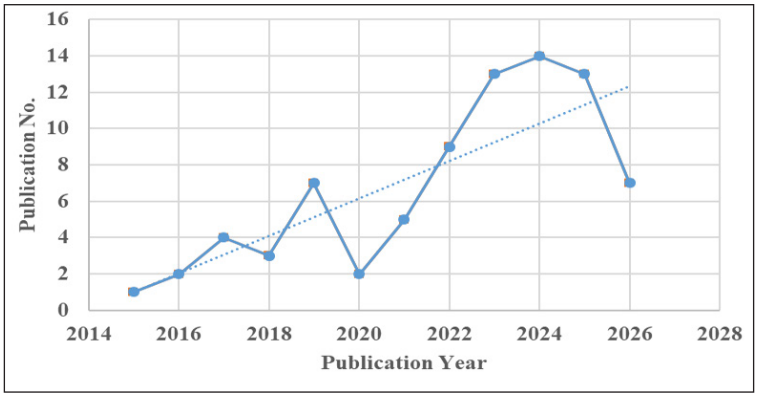

Figure 2. Annual publication trend of the studies included in the systematic review (2015-2026)

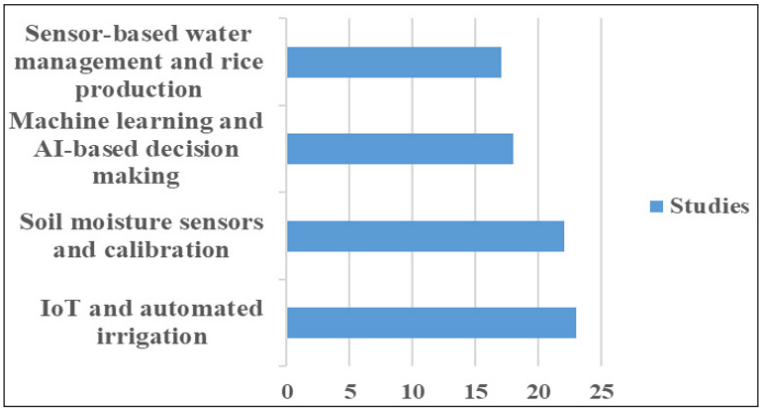

Figure 3. Distribution of the 80 chosen studies among the primary research themes

The selected studies are also grouped into four main research themes, defined by their principal research focus (Figure 3). The largest thematic category was IoT and automated irrigation, with 23 studies, followed by soil moisture sensors and calibration (22 studies), machine learning and AI-based decision-making (18 studies), and sensor-based water management and rice production (17 studies). This thematic distribution illustrates the high focus on irrigation using the IoT and the sensing of soil moisture, as well as growing integration of AI-based irrigation approaches in rice water management.

OVERVIEW OF SOIL MOISTURE SENSING TECHNOLOGIES

Comparative Analysis of Different Types of Soil Moisture Sensors

The soil moisture sensor is a critical part of smart irrigation systems, aiding in the effective irrigation scheduling and water resource management. These sensors are based on different principles and thus have different levels of accuracy, response time, cost and applicability to agricultural uses (Mahmud et al., 2026). A comparative outline of the main categories of soil moisture sensors is given in Table 2, which summarises the working principle, main advantages, limitations, applications and references for them.

Overall, sensor accuracy is highly dependent on soil texture, bulk density, salinity, temperature, sensor configuration, and calibration method. Capacitive, TDR and FDR sensors are generally more accurate than low-cost resistive sensors and neutron probes, which need accurate calibration. However, site-specific calibration is still relevant, especially in the case of dielectric sensors, since there may be inconsistencies in the calibration relationships from one soil to another.

To further illustrate the practical characteristics of the mentioned five sensor categories, representative commercial models are presented in Figure 4. These include (a) resistive sensors (SparkFun Soil Moisture Sensor), (b) capacitive sensors (DFRobot Gravity Capacitive Soil Moisture Sensor), (c) TDR sensors (TDR-150), (d) FDR sensors (BND-MS10) and (e) neutron probe sensors (Schematic hydroprobe). These demonstrate the different sensor configurations used for soil-moisture monitoring in agricultural and smart irrigation applications.

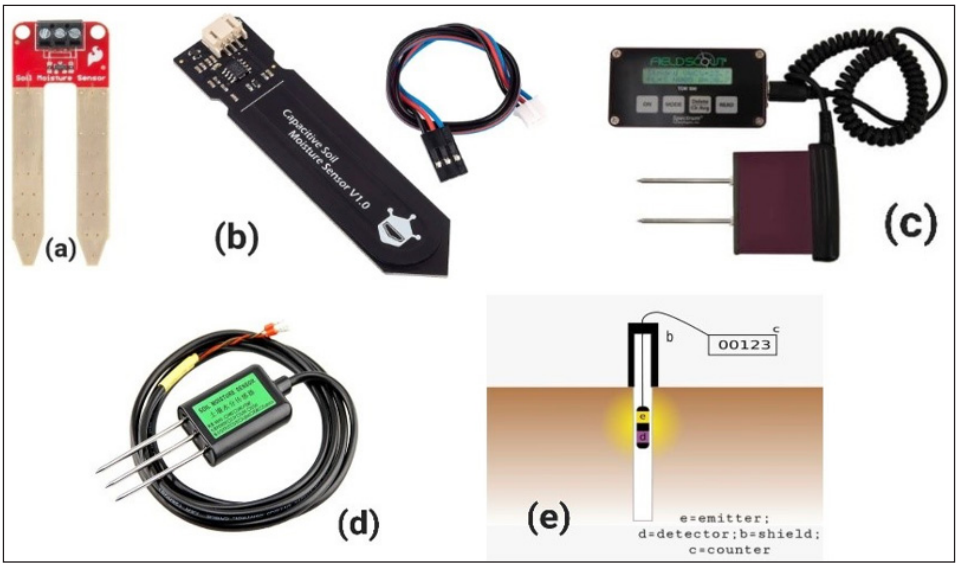

Figure 4. Representative models of five soil moisture sensor types: (a) resistive; (b) capacitive; (c) time domain reflectometry (TDR); (d) frequency domain reflectometry (FDR); and (e) neutron probe sensors

Table 2
Comparison of soil moisture sensor types used in a smart irrigation system

Sensor Type	Working Principle	Advantages	Limitations	Measurement Accuracy/ Error Range*	Typical Application	Key References
Resistive Sensor	Measure the electrical resistance between two electrodes inserted in the soil	Low cost, simple design, easy installation, suitable for basic irrigation monitoring	Sensitive to soil salinity, temperature and electrode corrosion, reducing long-term measurement accuracy	≈4-12.5% VWC error	Small-scale irrigation, educational project	Nandi & Shrestha, 2024
Capacitive Sensor	Estimates the moisture of soil by measuring the changes in soil dielectric constants caused by varying water content	Provide real-time data measurements, low maintenance, minimal calibration requirements, and less affected by salinity than resistive sensors	Accuracy may vary with soil texture and bulk density; periodic calibration is recommended for different soil types	≈1-8.5% VWC error	Precision irrigation, IoT-based smart farming, greenhouse and field monitoring	Escriba et al., 2020; Mahmud et al., 2026; Nandi & Shrestha, 2024
Time Domain Reflectometry (TDR)	Uses electromagnetic pulse travel time to estimate soil moisture	High accuracy, reliable long-term performance, continuous monitoring at multiple soil depths, less affected by salinity	High equipment cost, complex calibration, requires technical expertise for installation and data interpretation	≈2-4% VWC error under calibrated conditions	Scientific research, precision agriculture, and soil water dynamics studies	Zhu et al., 2019; Datta & Taghvaeian, 2023
Frequency Domain Reflectometry (FDR)	Measures changes in resonant frequency or capacitance associated with the soil dielectric constant	Rapid responses, continuous monitoring, lower cost than TDR, suitable for automation	Overestimate moisture in high saline soils; accuracy depends on conductivity and calibration	≈1-4% VWC error under calibrated conditions	Smart irrigation systems, commercial agriculture, and real-time moisture monitoring	Qi et al., 2024; Zhu et al., 2019; Qin et al., 2021; Datta & Taghvaeian, 2023

Table 2 *(continued)*

Sensor Type	Working Principle	Advantages	Limitations	Measurement Accuracy/ Error Range*	Typical Application	Key References
Neutron Probe Sensor	Determine the water content of soil by measuring the scattering of fast neutrons by hydrogen atoms present in soil water	Very high accuracy, suitable for long-term assessment, capable of measuring moisture at multiple soil depths	Expensive, required licensed radioactive sources with safety procedures, not suitable for routine field application	<1% VWC RMSE under well-calibrated conditions	Agricultural research, hydrological studies, and soil water balance investigation	Channuie et al., 2017

Note. Measurement accuracy/error range is an approximate value reported in recent literature and may vary with sensor model, soil texture, bulk density, salinity, temperature, installation condition and calibration method. VWC = Volumetric Water Content

Calibration and Accuracy of Soil Moisture Sensor for Reliable Irrigation Scheduling

Irrigation scheduling in the context of soil moisture estimates is particularly important, as in puddled paddy and direct-seeded. Sensors' accuracy will vary with soil texture, bulk density, temperature, salinity, sensor type and calibration method. These effects can be particularly important in clay-rich soils commonly associated with rice production (Awais et al., 2026; Datta & Taghvaeian, 2023; Zhu et al., 2019). Recent evaluations of TDR and FDR-type sensors have demonstrated that measurement errors can differ substantially across sensor models and soil textures, highlighting the importance of evaluating sensors under representative soil conditions (Zhu et al., 2019). For example, Zhu et al. (2019) also reported RMSE values ranging from approximately 0.014 to 0.041 $m^3\ m^{-3}$ among different TDR/FDR-type sensors and soil textures, demonstrating that sensor performance is strongly soil-dependent.

High precision is often required for irrigation scheduling, and site-specific calibration is therefore recommended, particularly in fine-textured or clay-rich soils (Awais et al., 2026). The calibration of dielectric sensors based on local soil texture and salinity is important for obtaining reliable measurements from sensors such as the ThetaProbe, HydraProbe, and TDR (Mane et al., 2025). A systematic review by Datta and Taghvaeian (2023) reported that the average RMSE under factory calibration was approximately three times higher than that obtained following site-specific calibration, emphasising the importance of calibration for irrigation applications. The general sensing workflow for obtaining reliable soil moisture estimates is illustrated in Figure 5.

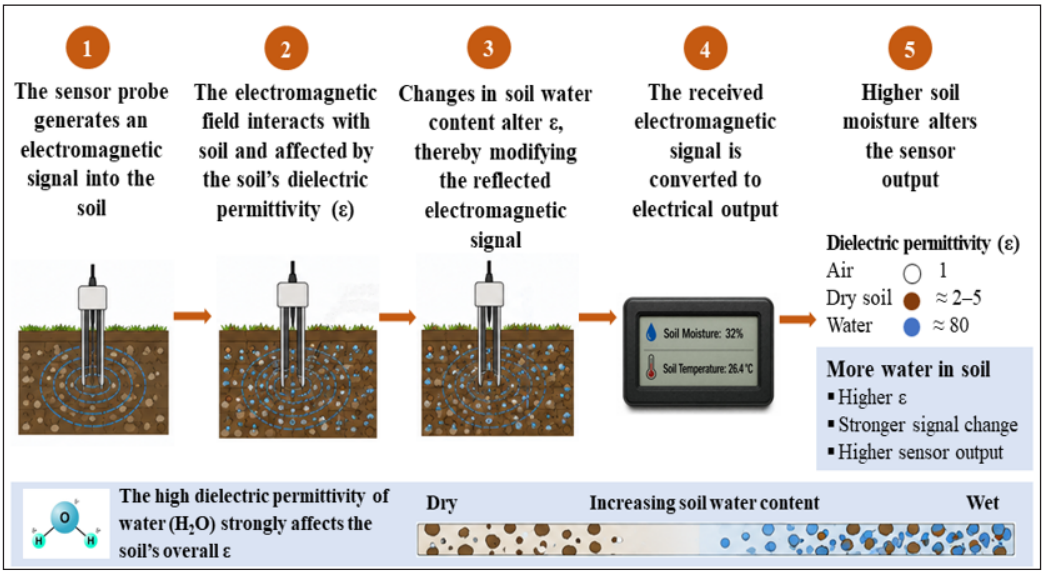

Figure 5. Working principle of dielectric soil moisture sensors for reliable irrigation scheduling

Generalised calibration curves may be inaccurate from one location to another, and soil moisture sensors based on dielectric properties should be calibrated according to soil type and relevant environmental conditions, including temperature and salinity (Mane et al., 2024). Recent studies provide quantitative evidence of this effect. For example, Qin et al. (2021) reported RMSE values of approximately 0.89-1.04% VWC under laboratory conditions and 1.44-1.93% VWC under field conditions for an FDR sensor, demonstrating that accuracy can change between controlled and field environments. Similarly, empirical in-situ recalibration of FDR sensors has been shown to substantially improve soil water content estimation compared with manufacturer calibration, particularly across heterogeneous soil profiles (Qin et al., 2021). A general calibration process for obtaining accurate soil moisture estimates is shown in Figure 6

The two-phase calibration approach can further improve sensor performance by establishing a site-specific relationship between dielectric permittivity and soil water content. Domínguez-Niño et al. (2019) reported an RMSE of 1.03 vol.% following two-phase calibration compared with 5.33 vol.% for factory calibration, demonstrating the substantial improvement achievable through soil-specific calibration. Similar findings have been reported for other dielectric sensors, where calibration performance differed according to soil texture and bulk density (Domínguez-Niño et al., 2019; Zhu et al., 2019). Therefore, the use of factory calibration alone may introduce substantial uncertainty when sensors are transferred to soils that differ from the conditions under which the original calibration was developed.

Sensor performance may also gradually deteriorate due to sensor drift, environmental exposure, and changes in soil conditions, making periodic verification and recalibration

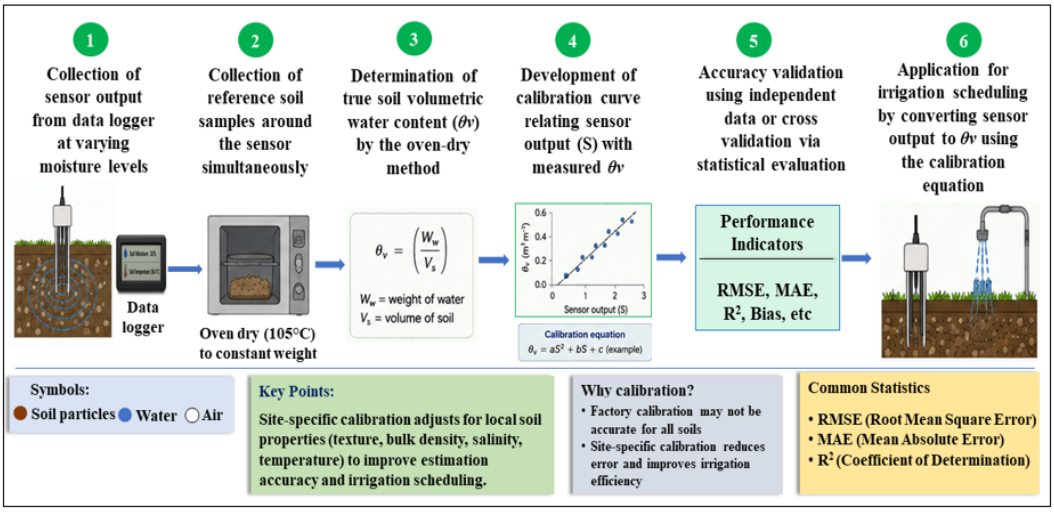

Figure 6. Process workflow for site-specific soil moisture sensor calibration and irrigation scheduling

important for maintaining measurement reliability. Probabilistic drift-correction techniques have been shown to reduce mean square error (MSE) and improve the reliability of long-term sensor observations (Domínguez-Niño et al., 2019). For water-sensitive crops, even relatively small measurement errors can influence irrigation timing and the amount of water applied; therefore, calibration uncertainty should be considered when developing soil-moisture-based irrigation thresholds (Chowdhury et al., 2022). Overall, the available evidence indicates that soil-specific calibration, periodic sensor verification and appropriate correction for environmental effects are essential for obtaining reliable soil moisture estimates and improving irrigation scheduling and water use efficiency.

Sensor Placement and Deployment Strategies for Accurate Soil Moisture Monitoring

Sensors can be used in controlled pot trials to gain a more accurate idea of the impact of varying irrigation levels on rice growth. Sensors are deployed in different field capacity zones in field applications to provide information for irrigation decision-making (Vadivu et al., 2023). The accuracy of moisture estimation can be improved significantly by the optimal placement of the sensors. A study with 42 sensors at different depths demonstrated that optimally placed sensors significantly improve the accuracy of moisture field estimation using an extended Kalman filter, reducing the average root mean square error (RMSE) by approximately 30% compared with random placement (Orouskhani et al., 2023). In addition, watermark tensiometers and TDR-type probes were more accurate in alternating wet-dry conditions than simpler and cheaper devices such as SM150 or PR2 sensors in clay soils (Mahmud et al., 2026).

INTEGRATION OF IOT TECHNOLOGY IN SMART IRRIGATION SYSTEMS

IoT Implementation in Agriculture

The Internet of Things (IoT) has transformed the agriculture industry by allowing for the monitoring of agriculture in real-time and data-driven decisions for automation. In agriculture, IoT solutions cover a wide range of application areas, such as wireless sensor networks (WSNs), GPS, remote sensing, cloud platforms and data analytics, all in pursuit of improving productivity and resource efficiency (Wolfert et al., 2017). Recent findings indicate that precision agriculture with the support of cloud-connected IoT technology can enhance water management by using real-time soil moisture data for automated irrigation systems (Baladevaguru et al., 2025). A smart irrigation method has been developed by implementing IoT sensors and actuators, which enable efficient irrigation scheduling based on evapotranspiration and crop demands (Ahmed et al., 2018). Furthermore, AI-powered platforms are crucial for monitoring as well as forecasting yield, which are all critical functions of IoT (Obaideen et al., 2022). Notably, cloud-based IoT architectures such as

ThingsBoard and FIWARE offer scalable and secure data management in smart farming (Hitiris et al., 2026). For system setup, ThingsBoard is an open-source IoT platform that aggregates and visualises data to optimise irrigation and fertilisation and alerts/notifies farmers when conditions require attention. The increasing adoption of edge computing and the introduction of new technologies like LoRa and NB-IoT have also made real-time responsiveness and energy efficiency more efficient in agricultural IoT systems (Manono et al., 2026). Further, IoT has been applied along with a machine learning approach for forecasting of diseases and intelligent decision support systems (Nawaz & Babar, 2025). There is also a growing emphasis on IoT applications in smallholder farming contexts as a result of the availability of low-cost microcontroller-based mobile integration systems (Kaushik, 2023). The climate variability and sustainability challenges have made IoT a potential solution for resilient and smart agricultural systems (Sarkar et al., 2024). These innovations demonstrate the transformative power of IoT applications in contemporary agriculture. A detailed smart sensor system architecture in precision farming is illustrated in Figure 7, which describes how several sensor nodes that gather information on soil moisture, ambient temperature, and nutrient levels communicate with a central processing hub using reliable wireless communication protocols. A data-driven approach to farm management is made possible by this system architecture, which enables accurate resource application and automated irrigation modifications to increase crop yields, reduce waste, and assure long-term environmental sustainability.

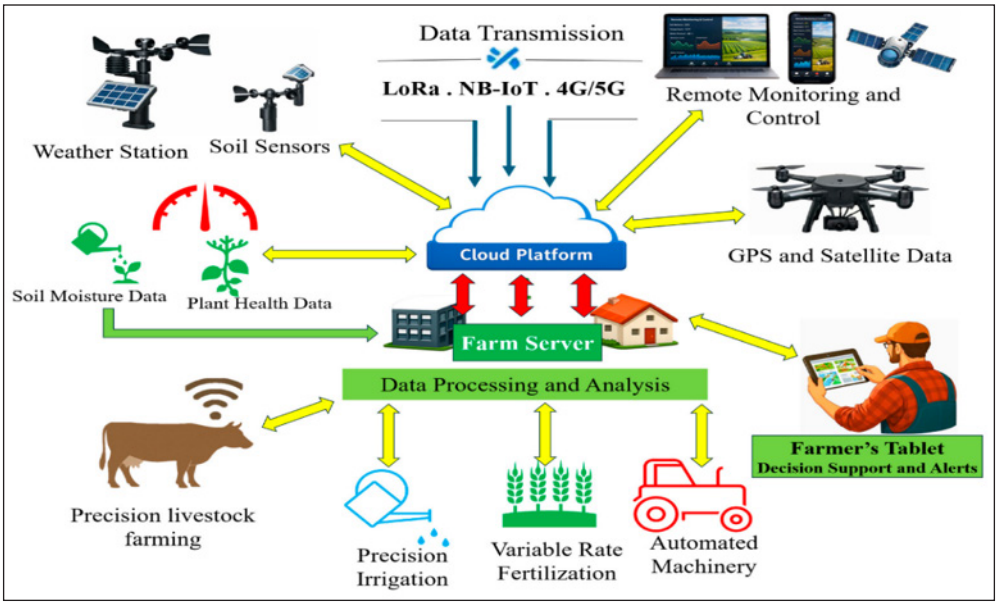

Figure 7. Precision farming with smart sensor architecture (Manono et al., 2026)

Architecture and Components of IoT-Based Smart Irrigation System

A typical smart irrigation system using IoT technology consists of several components, including soil moisture sensors, microcontrollers (such as Arduino or ESP32), wireless communication modules (Wi-Fi, LoRa, etc.), cloud platforms (ThingSpeak, Blynk, Arduino IoT Cloud), and irrigation actuators. The system integrates a soil moisture sensor with a user-defined irrigation threshold to automate irrigation and improve water use efficiency (Gopal et al., 2025). In recent years, rice farming has started using IoT sensors such as soil moisture sensors (SMC) (sometimes capacitive type, with low-cost microcontroller like ESP8266 or Arduino) and communication technologies like Wi-Fi, LoRa communication and a mobile-cloud platform (Mahmud et al., 2026). Optimised irrigation scheduling and automated alerts in applications, achieving water savings up to 30 % and an increase in upland rice yield by 15-20 % (Sutomo et al., 2025).

Real-Time IoT-Based Soil Moisture Monitoring and Decision Support

Real-time data transmission allows farmers to access the field data through mobile applications for irrigation management. The integration with decision support systems (DSS) and machine learning can further improve the predictive capabilities (Vadivu et al., 2023). Emerging technologies in soil moisture monitoring have improved irrigation efficiencies by combining real-time data analytics, cloud computing and wireless sensors (Rajani et al., 2023). They use a variety of hardware platforms and communication technologies to monitor, predict and automatically manage for irrigation. Table 3 lists the main soil moisture monitoring systems based on IoT technology, along with their technology stacks, main results and supporting references.

Table 3
Comparison of recent IoT-based soil moisture monitoring and smart irrigation systems for precision agriculture

System / Study	Tech Stack & Approach	Key Outcomes	Key References
Biswas model + wireless sensors	Sensor network + region-specific modelling	Real-time moisture tracking, $R^2 > 0.97$, error reduction ~15-25%	Zhang et al., 2025
IoT-assisted soil moisture & temperature monitoring system	Raspberry Pi + IoT + wireless sensor network (WSN)	real-time visualisation, RMSE ≈ 3.1%	Rajani et al., 2023
IoT-integrated soil moisture detection & prediction system	ESP8266 IoT nodes + time-series prediction model	High prediction accuracy with a very low RMSE (1.175682×10^{-5})	Setiawan et al., 2022
IoT-driven soil moisture monitoring system	Arduino + Wi-Fi + ThingSpeak cloud alerts	Real-time alerts, cost-effective, and irrigation optimised	Osanaiye et al., 2022
IoT enable Smart Drip Irrigation System (SIS)	Wireless sensor network + ThingSpeak + Android/ web application + Machine learning	Achieved 89% classification accuracy (up to 98% with KNN), reliable automated water management	Ndunagu et al., 2022

APPLICATIONS OF MOISTURE SENSORS IN RICE PRODUCTION

Irrigation Practices and Water Saving Strategies for Sustainable Rice Production

Crop production, resource utilisation, and sustainability are directly affected by irrigation, which is an integral part of modern farming. Alternate wetting and drying (AWD) is a newly developed alternative to continuous flooding of fields that was appreciated in rice farming because it saves water for the crop without reducing yield (Lampayan et al., 2015). In addition, Hendrawati et al. (2025) reviewed and discussed smart irrigation systems that combine IoT and sensor technologies, reporting their increasing use in data-driven irrigation management for all types of irrigation systems. Irrigation scheduling with a soil moisture sensor can reduce water use and increase yield stability as compared to irrigation according to a given schedule, thus providing substantial savings in water (Pushparani et al., 2025). Alternate Wetting and Drying (AWD) is one of the water-saving irrigation methods that is widely promoted as a water-saving option that will reduce water use by ~38 % and improve productivity by ~17 % without a yield penalty (Carrijo et al., 2017). Research has demonstrated that sensor-based irrigation can save up to 30% water without affecting the yields of rice crops (Kishore et al., 2025). Other equipment, such as soil moisture monitoring and sub-surface water level monitoring, is also required, as traditional ultrasonic sensors monitoring above-ground water are not effective under AWD dynamics (Mahmud et al., 2026). However, the study revealed that ultrasonic sensors were not suitable for AWD because of the wave distortion and interference of floating debris in the water, suggesting that the irrigation gate control system be automated using water-level telemetry and soil moisture probes in AWD fields (Pereira et al., 2022). Last but not least, Carrijo et al. (2017) showed that selection of the most suitable irrigation method, based on crop type, soil texture and climate conditions, is important to ensure the best water use efficiency and ensure agricultural production is more resilient to climate variability.

Field Capacity Threshold and Soil Moisture-Based Irrigation Scheduling in Rice Cultivation

Field Capacity (FC) is the moisture-holding capacity of soil after drainage, and it is crucial for smart irrigation because accurate field capacity enhances irrigation efficiency in agriculture (Kan et al., 2025). The knowledge and definition of this threshold are crucial to sustainable water use, particularly in water-intensive crops, such as rice. Soil water balance models are important tools in precision irrigation, and accurate identification of FC threshold levels can make a big difference in these models (Radheshyam et al., 2024). Likewise, Bihon et al. (2024) pointed out that it has a significant impact on hydrological modelling, as different FC values will result in different estimates of evapotranspiration and deep percolation. Furthermore, Awais et al. (2026) pointed out that the threshold values vary depending on soil texture, composition and depth, so calibration needs to be done

on a site-specific basis in precision agriculture applications. Meena et al. (2022) findings showed that maintaining irrigation at around 80-100% of the field capacity threshold is a good practice for enhancing yield and water productivity in the rice and wheat rotations. Mougare et al. (2025) investigated machine learning techniques for estimating threshold capacity levels in field conditions, utilising soil and environmental data to support automated irrigation systems in smart farming. Additionally, Abd El Baki et al. (2024) showed that the adjustment of these thresholds throughout the phenological stage of crops improves the moisture status in the root zone for better nutrient uptake and crop growth.

Water Use Efficiency Optimisation through Smart Irrigation Technologies

With growing water scarcity and climate variability, water use efficiency (WUE) has become a key measure of sustainable agriculture. The goal of water utilisation optimisation strategies is to enhance WUE by matching irrigation with the crop physiological requirements and environment. Recent research has shown that precision irrigation technologies contribute to the optimisation of water use. For instance, Pereira et al. (2022) highlight the advantages of deficit irrigation with regard to the maintenance of crop productivity with lower water inputs. In the case of rice farming that adopts a smart irrigation system incorporating real-time soil moisture conditions and crop performance data, it improves WUE and reduces water usage by 20% (Kulkarni et al., 2025). In a similar way, Agase & Tiwari (2025) reported that the use of soil moisture sensors based on Internet of Things (IoT) in paddy fields resulted in a 30-50% increase in the effectiveness of water use. According to the study, midstream farmers in the Philippines benefited from the alternate wetting and drying approach without reducing the yields, emphasising its potential to alleviate water constraints in rice agriculture (Chandra et al., 2026). Meng et al. (2024) emphasised the implementation and improvement of remote sensing and modelling applications for measuring and optimising water use efficiency in different cropping systems. These Optimised irrigation schedules also helps to save a considerable amount of water and improve water productivity. The findings of Meng et al. (2024) also highlight the importance of applying deep learning algorithms to predict irrigation schedules for maximising WUE. Finally, Gopal et al. (2025) pointed out that the use of weather-based irrigation scheduling and IoT platforms had enhanced water governance and water efficiency at the farm level. To further synthesise the quantitative findings reported in the reviewed studies on improvements in water use, water use efficiency (WUE), crop productivity and sensor/system performance are summarised in Table 4.

The quantitative evidence indicates that sensor-based and IoT-enabled irrigation technologies can improve water management, sensor performance and crop productivity. Reported water savings generally ranged from approximately 20% to 38%, while improvements in water-use efficiency reached 25-50% in selected studies. Yield or

Table 4
Quantitative evidence from previous studies on sensor-based irrigation, soil moisture monitoring and smart irrigation performance

Evidence Category	Quantitative Evidence Reported	Representative Studies
Sensor calibration accuracy	RMSE decreased from 5.33 to 1.03 vol.%	Domínguez-Niño et al., 2019
Sensor placement accuracy	RMSE reduction: ≈30%	Orouskhani et al., 2023
Water saving through AWD/ sensor irrigation	20-38% water reduction; up to 30% in sensor-based systems	Carrijo et al., 2017; Kishore et al., 2025; Kulkarni et al., 2025; Sarkar et al., 2024
WUE improvement	25-50% improvement	Agase & Tiwari, 2025; Munyaradzi et al., 2022
Yield/productivity improvement	15-20% improvement	Carrijo et al., 2017; Sutomo et al., 2025
IoT/model prediction performance	R^2 >0.97; RMSE ≈3.1% and 1.175682×10^{-5}; error reduction ≈15-25%	Zhang et al., 2025; Rajani et al., 2023; Setiawan et al., 2022
Machine-learning classification accuracy	Up to 89-98%	Ndunagu et al., 2022
IoT-enabled irrigation performance	Water saving up to 30%; water use reduction 30%	Sutomo et al., 2025; Chintamaneni et al., 2024

productivity improvements of approximately 15-20% were also reported. Sensor-related studies further demonstrated improvements in measurement and prediction performance, including reductions in RMSE, high R^2 values and machine-learning classification accuracies of up to 98%. However, the magnitude of these improvements varied among studies because of differences in sensor types, calibration procedures, soil characteristics, irrigation strategies, environmental conditions and experimental designs. Therefore, these values represent study-specific findings rather than a pooled statistical effect.

Impact of Sensor-Based Irrigation Scheduling on Water Use Efficiency and Crop Productivity

The adoption of sensor-based irrigation scheduling has proven to be a game-changing method that allows for more precise irrigation timing and water application based on the current soil moisture status. Korlepara et al. (2024) pointed out that integrating soil moisture sensors with a central monitoring system improves decision-making through real-time data to ensure optimal irrigation supply as crop demand. Later, Chintamaneni et al. (2024) showed that the use of soil moisture sensors enabled by the Internet of Things made 30% reduction in the use of water in crops, without adversely affecting yields. Likewise, Munyaradzi et al. (2022) reported that cotton water use efficiency (WUE) was improved by up to 25% with the use of sensor-based irrigation systems indicating their cross-crop

applicability. For rice systems, a low-cost sensor network was employed, and the results showed a 30% reduction in irrigation water use, provided that sensors can work in a paddy environment (Sarkar et al., 2024). Ejofodomi and Ofualagba (2019) emphasised that soil moisture feedback loops should be used for automated irrigation systems to significantly minimise the excess irrigation by utilising real-time data from a moisture sensor. Kaushik (2023) also discovered that the use of ThingsBoard-based IoT platforms greatly improved water use monitoring and control. According to Singh et al. (2024), there were socio-economic benefits of using sensor-based irrigation, such as savings in labour and improved yields. Finally, Kulkarni et al. (2025) discuss how IoT and machine learning enable the prediction of water-yield dynamics in order to support real-time irrigation decision-making.

Relationship Between Water Management, Yield Components, and Rice Productivity

The knowledge of the yield components and their linkages with water use and yield response in rice is essential to improve agricultural productivity in the context of limited water availability. Smart water management, such as deficit and alternate wetting and drying irrigation, has emerged as an effective option for optimising the use of limited water resources without reducing yields (Loconsole et al., 2025). Water application based on soil moisture sensors proved to be an effective way to manage the tillering and flowering stages to improve water productivity, as described by Kumar et al. (2024). Nangia et al. (2018) reported that the timing of irrigation has a positive effect on biomass accumulation and harvest index, which enhances the yield response. Furthermore, the IoT-based monitoring systems have enhanced water use efficiency in real-time, resulting in positive water yield in precision rice cultivation (Agase & Tiwari, 2025). In the meantime, Meng et al. (2024) studied the response in yield to different irrigation amounts in smart farming contexts, revealing that the water-yield curve can be optimised through precision water management, based on real-time information. Habib et al. (2024) showed that there were genotype-environment interactions, meaning that their study rice cultivars had differential responses in terms of yield under different water regimes, which play an important role in rice water-efficient cultivation. These empirical results have been confirmed by more recent modelling studies, which have indicated that the dynamic irrigation scheduling leads to optimum production parameters (Gopal et al., 2025).

FORECASTING AND AI-BASED IRRIGATION MANAGEMENT

Soil Moisture and Crop Water Requirement Forecasting

Moving from a reactive to a predictive approach to irrigation management will require accurate forecasts of soil moisture and crop water demand. Historical observations from sensors can be combined with weather and irrigation data to practice soil moisture forecasting to help in irrigation decision-making. According to a recent systematic

review, there are 60 studies published from 2014-2024, and machine-learning approaches are an important route to predicting soil moisture from sensor, weather and irrigation observations (Ivanova, 2025). Likewise, the ability to estimate water needs, based on data, has increasingly been aided by the observations of IoT technologies, which have provided high-resolution water measurements to predict when, where and how much water should be used in irrigation systems (Togneri et al., 2023). In rice growing, machine-learning models have also been shown to have the potential to predict crop water needs. Nasir et al. (2025) modelled reference evapotranspiration (ETR) and rice irrigation water requirement (IWR) based on climatic variables using Support Vector Regression (SVR) and Random Forest (RF) in the Kerian Irrigation Scheme (KIS), Malaysia. The predictive performance of the SVR model was very good, with R2 of 0.971 and 0.988, respectively, for the testing-phase data for reference evapotranspiration and rice irrigation requirement. AI-based forecasting has also been developed to predict root-zone soil moisture and to schedule and manage the amount of irrigation water, which reflects AI-based potential forecasting techniques for water-saving irrigation scheduling (Gu et al., 2021).

AI and Machine Learning for Irrigation Scheduling

Artificial intelligence (AI) and machine learning can offer the chance to learn nonlinear relationships of soil, weather, crop and irrigation variables, thereby improving irrigation scheduling. According to a review by Umutoni and Samadi (2024), machine-learning (ML) models are increasingly being adopted for irrigation scheduling and crop-water-demand prediction, highlighting data availability, uncertainty quantification and model transferability as key limitations. Similarly, Gao et al. (2023) reviewed the use of ML for irrigation applications, including water-demand forecasting, irrigation decision-making and proposing an intelligent irrigation framework based on machine learning. Neural-network models can be used directly to assist irrigation scheduling by forecasting soil-water conditions and using these forecasts to make irrigation decisions. Gu et al. (2021) created a neural network soil moisture model that can be used for root-zone soil moisture prediction and produce irrigation schedules. Furthermore, for practical agricultural applications, AI can be considered as a technology to support the decision-making process based on sensor observations and agronomic knowledge rather than a technology that replaces traditional irrigation rules.

Predictive and Intelligent Irrigation Management

Forecasting models, IoT sensing and intelligent control are moving irrigation systems into them of predictive and adaptive management in future. Recent studies have shown that intelligent irrigation can utilise soil moisture data that is continuously updated, as well as optimise irrigation decisions, not just based on threshold conditions. A soil moisture

prediction model, which used deep reinforcement learning and a sequence-to-sequence structure, was used to reduce irrigation water by 42.4% with the most optimised reward design while retaining the soil moisture at a field capacity level, as reported by Mukti et al. (2026). Intelligent decision-making for irrigation is also the subject of research using deep learning techniques. According to Yu et al. (2025), a deep learning approach can be used for soil-water prediction, long-term forecasting and irrigation optimisation. Also, hybrid models and edge-cloud architectures can be applied to make real time decision. Another route to adaptive irrigation is through the use of Model Predictive Control (MPC), which permits the use of future soil-water conditions and environmental variables in repeated irrigation decisions. In addition, recent innovations in Malaysia on the application of RNN-LSTM in soil-moisture prediction coupled with real-time smart irrigation management have shown the promise of the integration of sensor networks and AI forecasting in practical agricultural systems (Maniam et al., 2026). These recent developments demonstrate the progression towards predictive and intelligent irrigation management. Table 5 summarises some of the recent uses of AI and machine learning applications in predictive irrigation management.

Table 5
Recent application of AI and machine learning approaches for predictive irrigation management

Study	Crop/ System	Input Variables	AI/ML Approach	Prediction/ Decision Target	Main Outcome
Gu et al., 2021	Agricultural crop	Soil moisture, environmental variables	Neural network	Root-zone soil moisture and irrigation scheduling	Improved irrigation decision
Togneri et al., 2023	Various crops	IoT, weather, soil and crop data	Machine learning (ML)	Crop water requirement	Water-need estimation
Nasir et al., 2025	Rice	Climatic variables	SVR and Random Forest	ETo and rice irrigation requirement	High prediction accuracy
Yu et al., 2025	Various crops	Soil, weather and crop data	Deep learning	Irrigation decision-making	Improved predictive capability
Mukti et al., 2026	Agricultural system	Soil moisture, environmental data	Seq. to Seq. + deep reinforcement learning	Irrigation optimisation	Reduced irrigation water
Maniam et al., 2026	Precision agriculture	IoT-based soil moisture data	RNN-LSTM	Soil moisture prediction	Real-time prediction

With the integration of forecasting models, IoT sensing and intelligent control, irrigation systems are shifting towards predictive and adaptive management. Table 5 highlights

the recent applications of AI and machine learning to forecast soil moisture, crop water requirement estimation, irrigation scheduling and predictive irrigation management. The studies illustrate the transition from traditional machine learning methods, like Random Forest and Support Vector Regression, to deep learning as well as reinforcement learning. Specifically, by combining the data from the sensors provided by IoT devices with AI models, the system can predict irrigation needs in real time and make decisions accordingly. Most of the current studies, however, are crop and site specific, and there is a need for more comprehensive crop models that can take different soil properties, meteorological conditions, crop growth stages and irrigation methods. More research is needed to confirm the use of AI-based predictive irrigation systems across a range of field conditions for rice production and their impacts on water use efficiency and yield.

CHALLENGES AND FUTURE RESEARCH DIRECTIONS

Smart irrigation using sensors has been shown to achieve substantial water use efficiency and sustainable rice production, but has not yet been well adopted on a large scale due to some technical, economic and operational challenges. There are still significant concerns regarding the accuracy and reliability of the soil moisture sensors, as they are affected by soil texture, organic matter content, temperature, salinity and changing soil field conditions. Hence, there is a need for site-specific calibration to get reliable soil moisture data, especially in heterogeneous rice cultivation areas (Mane et al., 2024; Awais et al., 2026). Long-term exposure to the field can also cause sensor drift, fouling, corrosion and degradation, leading to lower measurement accuracy and higher maintenance needs. Therefore, self-calibrating sensors that automatically correct for drift and increase sensor durability are needed to enable long-term, reliable irrigation management (Domínguez-Niño et al., 2019).

Energy supply, communication reliability and system maintenance are also limitations on the sustainability of IoT irrigation systems. The battery lifetime is a critical problem of wireless sensor networks in rice fields, especially in remote fields where battery replacement/recharging is difficult, in contrast to that of large-scale sensor networks. Low power sensor networks, energy efficient communications, energy auto-powering sensor nodes (e.g. solar) and system autonomy should be undertaken in future research (Manono et al., 2026). Furthermore, in rural areas, network connections may not be reliable, communications may be missed, and network access may be low, which can reduce the value of monitoring tools that can be done in real-time and automatic irrigation in these areas. The use of advanced communication technologies such as LoRa, NB-IoT and edge computing can improve the reliability of data transmission and reduce the need for frequent connections to the cloud (Quy et al., 2022).

Other important issues include the interoperability of IoT-based agri-systems, cybersecurity and privacy management of these systems. Different manufacturers' sensors,

micro controls, communication devices, decision supporting systems, cloud systems and others must be able to communicate with each other, and the same protocols need to be used to ensure seamless data exchanges and system compatibility. Additionally, increasing dependence on cloud-based agricultural platforms raises issues over privacy, data security and illegal access to farm information (Nawaz & Babar, 2025; Obaideen et al., 2022).

Economic viability and farmers' adaptation are still major constraints, especially for smallholder farmers. The initial costs associated with implementing irrigation sensors, communication networks, automation systems and technical support could discourage some farmers from adopting this technology to achieve large water savings and productivity benefits (Quy et al., 2022; Nawaz & Babar, 2025).

So, future research should continue to investigate how to combine advanced digital technologies to create autonomous and scalable irrigation systems. Edge AI can facilitate real-time analysis of sensor data at the farm level to minimise latency and aid in decision-making in areas of poor internet connectivity. AI can be used to optimise the performance of smart irrigation systems, which can be integrated with IoT sensors to forecast soil moisture, predict weather conditions, and model crop water requirements, thereby improving the efficiency of water use in irrigation systems (Kulkarni et al., 2025).

The integration of remote sensing, UAV-assisted monitoring and irrigation scheduling based on satellite data is another promising research direction. UAV-based thermal and multispectral imaging can provide high-resolution data on crop water stress and spatial variability. While satellite observations can be used in conjunction with other meteorological and soil moisture estimation equipment to provide guidance for irrigation management in large areas (Haokip et al., 2025; Meng et al., 2024). Moreover, a multisensory fusion approach that combines soil moisture, soil temperature, weather, crop growth parameters, remote sensing data and machine learning models for irrigation prediction can result in more accurate predictions, especially in environments with fluctuating conditions (Meng et al., 2024; Vadivu et al., 2023).

New possibilities for smart irrigation systems will rely on the development of integrated digital agriculture platforms that aggregate IoT sensors, cloud computing, artificial intelligence, remote sensing and farm management tools. Cloud-based monitoring and decision-support systems can enable real-time visualisation, automatic alerts, and data-driven irrigation decisions (Hitiris et al., 2026). The incorporation of new technologies and sophisticated digital solutions will continue to drive future advancements in intelligent, automatic and sustainable irrigation systems. In Figure 8, a roadmap for future research is presented, indicating major technological directions for smart irrigation management using sensors in rice production.

In general, future research should be aimed at developing low-cost, energy-efficient, self-calibrating AI-enabled sensor networks supported by long-term field validation. These

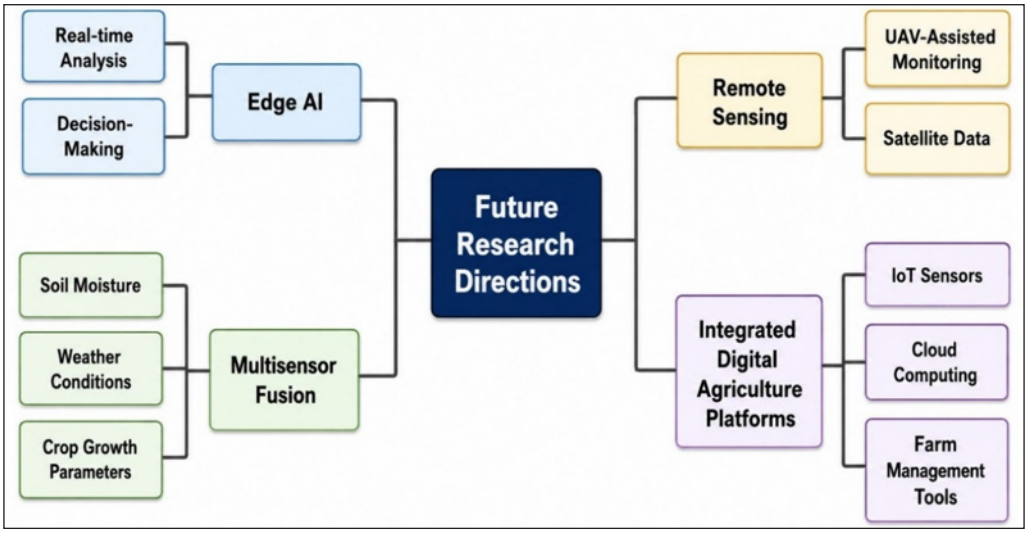

Figure 8. Future research directions for emerging technologies in smart irrigation management for sustainable rice production

innovations will speed up the shift to smart irrigation systems that are sustainable, efficient, resistant to climate change, and economically viable.

CONCLUSION

Sensor-based smart irrigation has become a promising approach for improving irrigation precision, water use efficiency and sustainable rice production. Integration of soil moisture sensors with IoT can enable timely and efficient irrigation management. This systematic review identifies that capacitive, FDR and TDR sensors are particularly suitable for real-time soil moisture monitoring, supported by rapid measurements, IoT integration with proper calibration and placement. However, their effectiveness depends on soil properties, environmental conditions and calibration. Resistive sensors are less suitable for long-term monitoring because salinity, electrode polarisation, and corrosion can affect measurement stability. Neutron probes are reliable but less practical for routine IoT applications because of radioactive sources, specialised operation and regulatory requirements. The reviewed studies further indicate that IoT-enabled irrigation improves monitoring, scheduling, water savings, and water-use efficiency. Additionally, AI and Machine learning support soil moisture forecasting and intelligent irrigation decisions. However, calibration, maintenance, energy use, cybersecurity, adaptation and cost remain key challenges. Overall, future research should focus on low-cost sensors, Edge AI, multi-sensor fusion and energy-efficient IoT networks. Emphasis should also be placed on standardisation, calibration, economic feasibility and farmer-oriented solutions.

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Review Article

In Vitro Culture and Biotechnological Advances in Mango (*Mangifera indica* L.): Progress, Challenges and Future Perspectives

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ABSTRACT

Mango (*Mangifera indica* L.) is one of the world's most economically important tropical fruit crops, yet its genetic improvement through conventional breeding is constrained by a long juvenile phase, high heterozygosity, irregular bearing and complex reproductive biology. Recent advances in plant biotechnology have provided alternative approaches to overcome these limitations, accelerate cultivar improvement, germplasm conservation and the development of superior planting materials. This review critically synthesises current progress in somatic embryogenesis, micropropagation, molecular marker-assisted technologies, genetic transformation and other emerging biotechnological tools for mango improvement. Particular emphasis is placed on explant selection and plant growth regulator combinations for efficient plant regeneration, the application of molecular markers for cultivar identification, genetic diversity assessment and the potential of genetic transformation to enhance disease resistance, postharvest quality and other economically important traits. The major biological factors limiting the large-scale application of these technologies, including genotype dependence, phenolic oxidation, microbial contamination, low regeneration efficiency and limited transformation success, are critically discussed. Finally, future research

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priorities are highlighted, focusing on integrating advanced biotechnological approaches with modern breeding strategies, genomics, genome editing and germplasm conservation to accelerate the development of climate-resilient and high-quality mango cultivars, thus supporting sustainable mango production worldwide.

Keywords: Mango, micropropagation, molecular markers, somatic embryogenesis, transgenic tools

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most popular tropical fruit crops in Asia and worldwide, having an ancient cultivation history dating back 4000 to 6000 years (Yadav et al., 2017). Mango germplasm has dispersed widely across tropical and subtropical regions for its extensive cultivation and domestication. Over the past two decades, global mango-growing regions have expanded significantly, and India is the largest mango producer, producing 26.2 million tons annually from a cultivation area of 2.69 million hectares (https://worldpopulationreview.com/country-rankings/mango-production-by-country) (Figure 1). Indonesia, Brazil, Pakistan, Bangladesh and China are mango-producing countries due to their warm climates with the least frost occurrence, which is ideal for tropical fruit production.

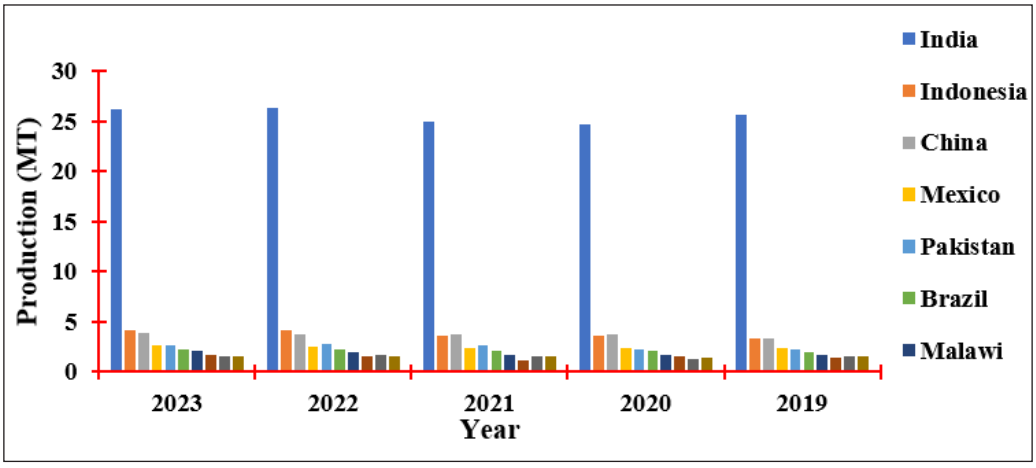

Figure 1. Global mango production report from 2019-2023
Source: WRP (Accessed on 11 April, 2026)

Despite its global importance, mango cultivation faces numerous biological constraints and production challenges like high heterozygosity, long juvenile phase, irregular bearing and inconsistent flowering behaviours. In the past, progress on large-scale commercial micropropagation through somatic embryogenesis has remained difficult due to recalcitrance, severe tissue browning and high genotype dependency (Krishna et al.,

2008). In addition, regeneration using *in vitro* derived explants like cotyledonary nodes is more responsive compared to regeneration protocols from mature elite plants (Conde et al., 2023). Although tropical climates allow year-round production to maintain a constant supply of quality fruits, it remains a challenge for growers and exporters due to natural and biological limitations. Hence, biotechnology offers promising opportunities to overcome some of these limitations. The identification and classification of mango germplasm have advanced significantly due to modern tools like genome sequencing and molecular markers (Faisal et al., 2025). Plant tissue culture techniques, particularly somatic embryogenesis, provide a powerful tool for the development of specific traits in floral biology, fruit quality and overall productivity. Selection and regeneration of elite mango genotypes using plant tissue culture techniques form the basis of breeding improvement programs.

Mango productivity is heavily affected by high levels of cross-pollination. Furthermore, sexual reproduction does not ensure clonal fidelity because many commercially important cultivars are monoembryonic. It has been hypothesised that Florida mango hybrids originated from crosses between Southeast Asian (mostly polyembryonic) and Indian (monoembryonic) mango cultivars, as supported by molecular studies employing isozyme and RAPD markers (Olano et al., 2005). Polyembryonic mango cultivars have been reported to successfully produce somatic embryos (Litz, 2004; Sajana et al., 2022; Shukla et al., 2016). But frequent failure in culture due to the occurrence of contamination is another significant issue when utilising vegetative parts. *Colletotrichum gloeosporioides* isolates from diverse geographic regions are distinguished by their latency and pathogenicity on twigs, leaves, flowers, fruits and petioles of mango plants (Weir et al., 2012). Significant losses of mango yield in South America and Asia have been caused by severe infections with *Colletotrichum spp.* (Li et al., 2019b as cited in Dofuor et al., 2023; Tovar-Pedraza et al., 2020). Besides, *Alternaria alternata* and *Fusarium subglutinans* are among the latent fungal entities that have been documented in mango (Ploetz, 1994). The use of certain antibiotics against microbes to maintain sterile cultures of mango would be made possible by the identification and isolation of endophytic organisms. Most of the commercially grown mango cultivars do not fully satisfy the assertions of modern intensive horticultural systems, which demand dwarfism, early bearing, consistent production, resistance to pests and diseases and improved storage quality. Emerging trends in micropropagation and genetic marker-based technologies have promisingly accelerated mango breeding programs. A number of genes have been explored for genetic transformation in mango, which makes the transgenic mangoes technically more feasible (Pandey et al., 2022).

This review synthesises current *in vitro* methodologies and existing bottlenecks like recalcitrance and phenolic browning, guiding future optimisation of micropropagation protocols, evaluating the transition from juvenile to mature plants, and providing a clear roadmap for establishing reliable clonal regeneration systems in elite cultivars. Also

mapping out advancements in genetic transformation and molecular markers, the review establishes a foundation for integrating biotechnological tools into targeted mango breeding and crop improvement programs. Therefore, future research must focus on optimising explant selection and refining culture protocols along with molecular approaches to make micropropagation work across many different commercial cultivars for the successful application of biotechnological tools in mango improvement programs.

IN VITRO PROPAGATION

The process of developing plant and/or plantlets through a simulated medium under aseptic conditions is known as plant tissue culture. *In vitro* propagation demands cultivating explants such as roots, shoot tips, calli, reproductive organs (pollen, ovules), seeds, embryos or even a single cell from the mother plant on nutrients containing an aseptic medium (Kaya & Huyop, 2020; Vidyagina et al., 2021). Compared to *in vivo,* this technique has the potential to lower costs, while improved formulation and homogeneity result in superior clones and a rapid multiplication system. Mango varieties having positive mutations or gained diversity through somaclonal variation may be screened through *in vitro* selection. Various explants were investigated for *in vitro* regeneration of plantlets using Gamborg's (B5) as well as Murashige and Skoog (MS) media.

IN VITRO PROPAGATION OF MANGO

In vitro propagation is the art and science behind *in vitro* multiplication of plants. The procedure includes a number of steps, including stock maintenance, explant nomination, sterilisation, alteration of the proliferation media, adaptation and development into plantlets (Figure 2). Laboratory-raised seedlings reduce contamination for successful micropropagation of 'Irwin' mango using woody plant medium (WPM) in conjunction with a synergistic combination of zeatin and 6-benzyladenine (BAP) maximised both shoot growth and proliferation (Tetsumura et al., 2016). Numerous investigations have revealed a number of intrinsic concerns related to *in vitro* culture, including explant browning, phenol exudation, medium staining, *in vitro* tissue recalcitrance and extensive systemic contamination. Plantlet regeneration from different explants, such as nucellus tissues and branches, have become successful reported by Sajana et al. (2022) and Zhou et al. (2024), respectively. Despite some constraints, these techniques assure year-round explant availability in contrast to seeds that only offer propagating material at specific seasons of the year. Etiolated shoots exhibit a significant decrease in phenolic exudation and polyphenol oxidase (PPO) activity at earlier stages, as well as phenolic biochemical release at the post-culture phase when *in vitro* cultured. The contamination done by endogenous pathogens is another significant factor that hampers tissue culture of mango when utilising vegetative parts but can be mitigated by pretreatment of the mother plant

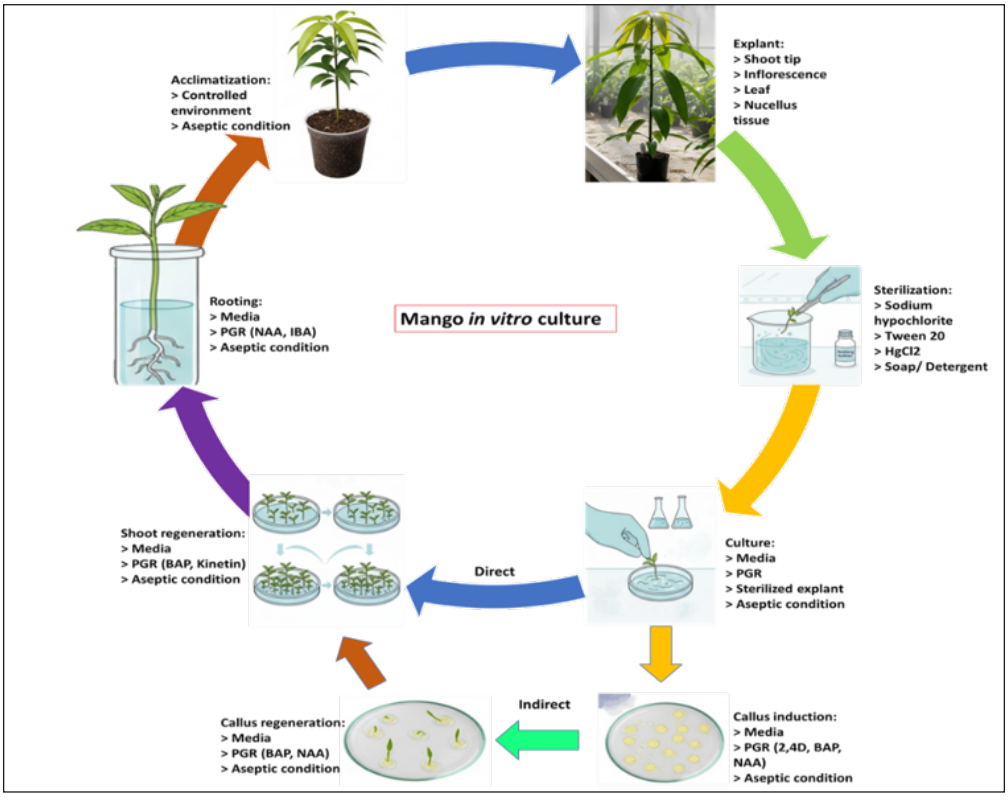

Figure 2. Primary steps of *in vitro* culture of mango

with azoxystrobin and following the preculture sterilisation protocol prescribed by Lee et al. (2025). Successful regeneration in mango tissue culture is also hindered by the physiology and inherent genetics of the donor plants, where nucellar embryos have become more successful compared to mature tissues (Thomas, 1999).

Recent advances in plant tissue culture emphasise that overcoming recalcitrance requires the precise optimisation of plant growth regulators (PGRs), particularly synergistic coalescence of cytokinin and auxins, including kinetin and 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA), respectively, to trigger organogenesis (Foo et al., 2018; Quiroz et al., 2025). Maintaining hormonal balance is vital for success, but the integration of antioxidants and adsorbents like polyvinylpyrrolidone (PVP) is equally critical to neutralise phenolic oxidation and prevent subsequent tissue necrosis (Hesami et al., 2025; Quiroz et al., 2025). Modern protocols increasingly combine these chemical additives with environmental controls such as dark incubation and cold pretreatment to manage the oxidative stress that frequently hinders the regeneration of complex genotypes (Hesami et al., 2025).

EMBRYOGENIC CULTURES

Embryogenic cultures are plant tissue cultures capable of developing into complete plants through the process of somatic embryogenesis, serving as a vital tool for plant regeneration, genetic transformation, and mass propagation. For instance, Khair et al. (2016) reported successful development of a somatic embryogenesis protocol from nucellar tissue of the 'Baramasi' mango variety by using specific hormone combinations, most notably 2,4-dichlorophenoxyacetic acid (2,4-D) for induction and a balanced mixture of gibberellic acid (GA_3), kinetin and IAA for successful plantlet germination. Somatic embryogenesis was found from nucellar tissue of the variety 'Ratnagiri' with the prescribed use of 2,4-D and thidiazuron (TDZ) reported by Malabadi et al. (2011). Sajana et al. (2022) stated that fruits collected 30 to 50 days after pollination are preferable for callus induction from the nucellar tissues of polyembryonic varieties 'Olour' and 'Vellaikolumban' with 2,4-D and GA_3. Nucellar tissues from younger fruits were employed as the initial explant by Flórez-Ramos et al. (2007) and the expected result was obtained with cv. 'Keitt', which had 48% embryogenic culture induction. MS media added with PGRs like N^6 (2-isopentenyl) adenine (2iP); 2,4-D was a successful inducer of embryogenic callus from nucellar tissue for the cultivar 'Chaunsa', reported by Solangi et al. (2023). According to Thomas (1999) pro and globular-shaped embryos were observed 14- 21 days after the formation of embryonic calli on the establishment medium and transferred to the expression medium. Growth regulators, such as cytokinin, can be used mostly to accelerate this process. According to Sajana et al. (2022), the application of abscisic acid (ABA) has a role in embryo maturation. Litz (2005) also recommended lowering the amount of sucrose in the maturation medium from 4-6% to provide good results. Moreover, a high sugar concentration has also been found to cause a delay in the somatic embryo elongation of mangoes at maturity. Ethylene may have an effect on regeneration systems, such as organogenesis and somatic embryogenesis, reported by Neves et al. (2021).

SOMATIC EMBRYOGENESIS

The use of plant growth regulators (PGRs) is essential to guide nucellar tissue through the distinct morphological stages of somatic embryogenesis, with the combination of 2,4-D and GA_3 successfully inducing and proliferating globular embryos, while subsequent treatments with IAA, ABA and zeatin facilitated the transition through heart-shaped stages to final maturation and successful germination reported by Sajana et al. (2022) (Table 1). Jana et al. (1994) successfully leveraged the naturally disease-free properties of nucellar tissue from three mango varieties of India, 'Mundan', 'Alphonso' and 'Baneshan' to rapidly generate morphologically sound somatic embryos within a 75 to 80-day period. In other words, efficient somatic embryogenesis in monoembryonic cultivars allows the preservation of clonal genotypes and the elimination of pathogens, as well as being extensively researched

across polyembryonic varieties to mitigate disease and environmental stress (Pateña et al., 2002, as cited in Zuazo & Pleguezuelo, 2009). Culturing early embryos on a medium containing 5 mg/L kinetin for four weeks successfully produced plantlets *via* direct somatic embryogenesis with minimal tissue browning (Xiao et al., 2004). However, the effective recuperation of mango plantlets has also been hampered by issues with early germination and increasing necrosis. In general, there are some major stages to the protocols used: a) nucellus-induced embryogenic culture induction, b) embryogenic culture maintenance, c) the morphologically regular embryo maturation and development and d) the germination of somatic embryos into fully grown plantlets. DeWald et al. (1989) found that embryogenic cultures performed more effectively in suspension compared to solid media. Furthermore, compared to both MS and WPM, B5 basal modified medium was good for maintaining cultures as well as producing somatic embryos properly. Coconut water boosted somatic embryo development in 'Chaunsa' mango; biochemicals like casein hydrolysate and yeast extract were ineffective, and 'Anwar Rataul' failed to respond due to excessive phenolic interference (Usman et al., 2005).

ORGANOGENESIS

Organogenesis through *in vitro* culture is the developmental process where plant organs such as shoots, roots or flowers are regenerated from cultured cells, tissues or organs under controlled aseptic conditions. For instance, Zhou et al. (2024) successfully established a stable, genetically trustworthy regeneration protocol for cv. 'Keitt' using young shoots in WPM containing 1 mg/L 6-BA and 0.1 mg/L NAA for high-frequency shoot induction (73.63%) and Douglas-fir cotyledon revised medium (DCR) with 3 mg/L IBA for effective (66.13%) rooting (Table 1). Early investigations into mango organogenesis found that while kinetin and NAA could induce roots from cotyledon-derived callus, shoot growth remained elusive, with further studies noting that half-strength semi-solid MS media improved explant survival by reducing phenolic exudation (Rao et al., 1981; Singh et al., 1999). Conde et al. (2023) observed that axillary shoots were found from cotyledonary nodes using different mango varieties as explants. Precise application of zeatin (5 mg/L) plays a role in embryo germination, produces shoots and initiates root induction, as observed by Sajana et al. (2022). A promising protocol for embryogenic callus to organ formation, such as shoot, root and leaf formation reported by Khair et al. (2016) *via* somatic embryogenesis and the germination process using different media and PGRs. But every transitional phase requires different cultural conditions for promising output. As Mishra et al. (2010) reported, the culture conditions for somatic embryo germination and plantlet formation were 16h photoperiod with 40 mmol m^2s^{-1} quantum flux and 55% relative humidity at 25±2 °C.

PLANTLET DEVELOPMENT

The alteration of somatic embryos into viable plantlets is a complex process requiring specific media, environmental conditions and rooting treatments. Somatic embryos must be shifted to a liquid to semi-solid medium, typically half-strength B5 macro-salts with 1.0 mg/L GA_3 and maintained at 25 °C temperature under a 16 h photoperiod with soft white fluorescent light at 40-60 µmol $m^{-2}s^{-1}$ for successful transformation (Ara et al., 2000b; Litz, 2003). Alteration percentage can be further sped up by increasing the intensity of light to 160 µmol $m^{-2}s^{-1}$ in an environment facilitated with 2% CO_2 (Litz, 2003). The somatic embryos can be transferred to soil after full development into seedlings, though achieving high-quality rooting remains a challenge. While treatment with IBA in the dark can induce rooting in up to 90% of micro cuttings, it often results in stunted root elongation and poor overall growth even when utilising optimal potting media such as fine vermiculite (Tetsumura et al., 2016). A combination of 60% coconut fibre, 30% commercial substrate and 10% litonite provides the best output for adaptation of *in vitro* rooted mango shoots with the facility of a growth chamber having 16/8h light/dark cycle, 60% relative humidity, 45-50 µmol $m^{-2}s^{-1}$ light intensity, along with a temperature of 26±1 °C for plantlet rearing reported by Conde et al. (2023).

AUTHENTICATION OF CULTIVARS USING MOLECULAR TECHNIQUES

Cultivar identification can be accomplished by using molecular markers, including DNA and isozyme markers (Figure 3). Marker-assisted selection (MAS) is another tool to accelerate the breeding program. Genetic marker technologies, including Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), Single Nucleotide Polymorphism (SNP), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), isozymes and DNA-based markers, have been extensively utilised to map mango progeny (Table 2). For identification of cultivars and to analyse the genetic diversity of genotypes, many researchers used different types of molecular marker-assisted biotechnological tools based on different monogenic and polygenic traits (Abdelsalam et al., 2018; Jena & Chand, 2021; Kalal et al., 2023; Sherman et al., 2015). Despite the efficiency of these molecular tools for studying traits, researchers frequently face challenges such as low polymorphism and the presence of PCR inhibitors like polysaccharides and phenolics (Ramírez, 2013). To overcome these limitations, specialised extraction protocols have been developed to isolate quality genomic DNA from complex mango tissues, including healthy and malformed floral structures (Gomathi et al., 2005; Ramírez, 2013).

Table 1
Embryogenic and organogenic accomplishments of some mango cultivars

	Genotype	Explant	Media with PGRs	Culture Conditions	Corresponding Effects	References
1.	Keitt	Shoot tips	WPM (1 mg/L 6-BA, 0.1 mg/L NAA)	At 25±2 ºC temperature with 16h photoperiod and 33.6 $molm^{-2}s^{-1}$ light intensity	Adventitious shoot induction	Zhou et al., 2024
2.	Irwin, Keitt (monoembryonic), Ataulfo, Sabre and Gomera-4 (polyembryonic)	Mature seeds (4 months after full bloom)	Half strength of MS, 0.5 mg/L GA_3, 100 mg/L cysteine, 3% sucrose, 0.7% agar)	Cultured in the dark at 25±1 °C for three weeks	Good germination rate	Conde et al., 2023
3.	Keitt	Cotyledonary nodes from *in vitro* germinated seeds	Germination medium (GM); 0, 0.5 and 1 mg/L GA followed by MI2 medium, 3.0 mg/L kinetin and 1.0 mg/L IAA. MI2 medium, 3.0 mg/L kinetin or 1.0, 2.0 and 3.0 mg/L TDZ	Cool-white fluorescent bulbs with 16h photoperiod at 45 µmol m^{-2} s^{-1} light intensity (wavelength 400-700 nm) and incubated at 25±1 °C temperature	Shoot development and regeneration	Conde et al., 2023
4.	Saroli, Langra and Chaunsa	Nucellus tissue	Modified MS and B5 (9.83 µM 2iP and 4.52 µM 2,4-D)	Incubated in complete darkness at 24° C temperature	Callogenesis	Solangi et al., 2023
5.	Vellaikolumban and Olour	Nucellus tissue	Rugini olive medium (RO), 5 mg/L 2,4-D and GA_3, 6% sucrose	Usually maintained at 25±2° C in the dark during the initial callus induction stage, followed by a 16h light with an intensity of 40-45 µmol $m^{-2}s^{-1}$	Embryogenic calli development	Sajana et al., 2022
6.	Anwar Rataul	Shoot tips	MS, 1 mg/L BAP MS media with 3 mg/L NAA, 1 mg/L IAA	At a room temperature of 25±2 °C with a 16/8h of photoperiod	Shoot induction Root induction	Qureshi et al., 2021
7.	Sabre and 13-1	Nucellus	B5 or MS media, 0.5 mg/L 2, 4-D, 1.0 mg/L thiamine HCl, 400 mg/L L-glutamine, 150 mg/L ascorbic acid, 100 mg/L myo-inositol, 6% sucrose, 100 mg/L citric acid, 1.5 g/L activated charcoal	8 weeks in a growth chamber at 27±2 ºC with a photoperiod of 1500 cdm^{-2} from soft fluorescent lamps for 16/8 h per day	Somatic embryogenesis	Shehata, 2021

Table 1 *(Continued)*

	Genotype	Explant	Media with PGRs	Culture Conditions	Corresponding Effects	References
8.	Ubá and Carlota	Immature embryo	Half strength of MS with 0.5 mg/L GA_3, 100 mg/L cysteine and 30 g/L sucrose with 2.2 g/L phytagel	30 µmol $m^{-2}s^{-1}$ photon flux density at 27±1 °C and 16h of photoperiod (for embryo development) 22 µmol µmol $m^{-2}s^{-1}$ photon flux density at 22±1 °C temperature and 12h of photoperiod (for plantlet)	Somatic embryogenesis and plantlet development	Souza et al., 2020
9.	Alphonso, Carabao and Turpentine	Nucellus	Modified MS and B5 media (1 mg/L 2, 4-D alone or in combination with 0.25 mg/L BAP)	Incubated at 25±2 °C, maintaining 60% relative humidity in dark conditions	Somatic embryogenesis	Shukla et al., 2016
10.	Ratnagiri	Nucellus tissue	MS, 2.27 µM TDZ and 4.52 µM 2,4-D	At 55-60% relative humidity and 25±3 °C temperature under soft white fluorescent (100 µmol $m^{-2}s^{-1}$)	Embryogenic callus induction	Malabadi et al., 2011
11.	Irwin	Young shoots	Different strengths of MS, Gamborg (B5) and WPM (10 µM zeatin)	At 28 °C temperature under 16h of photoperiod	Shoot proliferation	Sakota et al., 2010
12.	Ambalavi	Nucellus tissue	Modified basal medium containing (0.25 mg/L BAP, 0.5 mg/L 2iP and 1 mg/L NAA)	At 70±5% relative humidity and 27±1 ºC temperature with a 15h of photoperiod having a quantum flux of 25 µmol $m^{-2}s^{-1}$	Plantlet regeneration through somatic embryogenesis	Chaturvedi et al., 2004
13.	Amrapali	Nucellus tissue	Modified MS medium (half macro, full micro salts and organics) with 6% sucrose, 0.57 mM ascorbic acid, 2.74 mM L-glutamine, 4.5 mM 2,4-D and 0.8% agar	In darkness at 25±2 ºC temperature	Plantlet regeneration through somatic embryogenesis	Ara et al., 2000a

Note. CW = coconut water, CH= casein hydrolysate

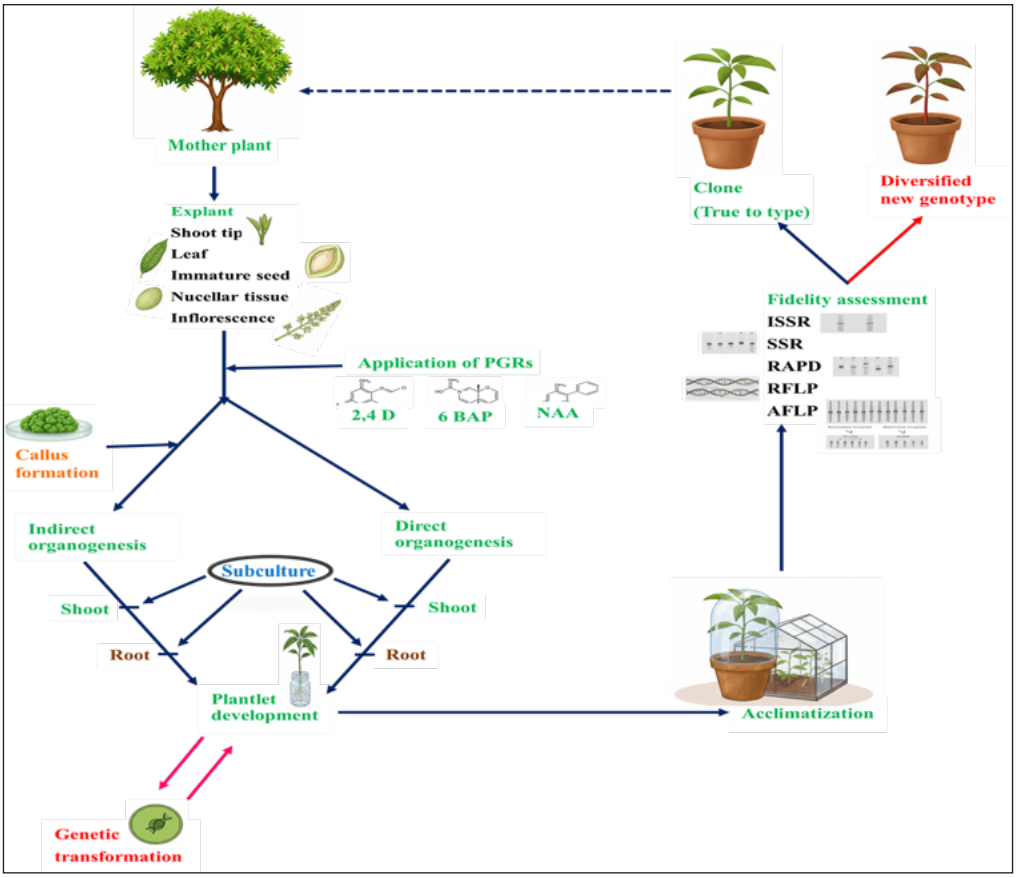

Figure 3. Advancement of *in vitro* culture and fidelity assessment by molecular markers

Table 2
A summary of key molecular tools in in vitro culture and mango breeding purposes

	Molecular Tools	Key Findings	References
1.	ISSR	Identification as well as genetic diversity in mango	Zhou et al., 2024
2.	SSR, RAPD	Identifying genetic diversity in mango population and efficiency comparison	Hussein et al., 2023
3.	SSR (Including MiIIHR23, MiIIHR17, and LMMA12)	Differentiation of nucellar and zygotic embryos of polyembryonic Olour and Vellaikulamban	Kalal et al., 2023
4.	SSR	Each locus has an average of 3.0 alleles and 2-5 alleles. Each locus had a total of 21 alleles that were identified by 7 polymorphic primers	Arogundade et al., 2022
5.	SCoT, RAPD, CBDP, ISSR, SSR and DAMD	SSRs detected the highest genetic variability	Jena & Chand, 2021

Table 2 *(Continued)*

	Molecular Tools	Key Findings	References
6.	SCoT	244 (72.4%) of the 337 fragments produced by 45 primers expressed polymorphism and 89% of genetic variation was found within populations in 2 clusters, compared to 11% between groups	Zhou et al., 2020
7.	RAPD and ISSR	High polymorphism levels were found using 10 RAPD and ISSR markers; RAPD (91.9%) and ISSR (98.1%) produced 162 and 145 loci, respectively	Ho & Tu, 2019
8.	SSR	10 sets of 3 SSR markers separated 83 genotypes using 46 polymorphic SSR markers. Southeast Asian and Indian types can be distinguished genetically by 3 clusters with Floridian varieties being more similar to the Indian variety	Yamanaka et al., 2019
9.	SSR and EST-SSR	Both morphological characteristics and genetic polymorphism exhibited notable variance, between 0.71% to 100%	Abdelsalam et al., 2018
10.	SSR	The accessions displayed similarity coefficients between (75 to 100) %	Nazish et al., 2017
11.	SSR	387 mango accessions from all over India were reported and classified them into 2 subpopulations (south/west and north/east)	Ravishankar et al., 2015
12.	Subset of 239 SNP	74 Israeli mango accessions were genotyped and surveying its structure and diversity (GWAS preliminary step)	Sherman et al., 2015
13.	SSR	Analysis of the variety of the Australian collection revealed that trinucleotide motifs were more common than dinucleotide motifs and that is consistent with the current findings	Dillon et al., 2014
14.	SSR	Diversification in local germplasm collections	Tsai et al., 2013
15.	RAPD	UPGMA proposed 5 genotypic groups and found 55 polymorphic loci	Souza et al., 2011
16.	RAPD	9 clusters with 75% similarity were constructed and RAPD indicated a (2-31) % range of genetic diversity	Fitmawati et al., 2010
17.	AFLP	Evaluating the genetic relationships and species diversity of mangos	Yamanaka et al., 2006
18.	RFLP	Genetic kinship estimation between poly and monoembryonic cultivars	Ravishankar et al., 2004
19.	Molecular and isozyme markers	Identification of hybridized mangoes	Jintanawongse & Changtragoon, 2000

Note. SCoT = Start Codon Targeted, CBDP = CAAT Box-Derived Polymorphism, DAMD = Directed Amplification of Minisatellite-region DNA

Application of Different Molecular Markers

ISSR amplification is regarded as a rapid, relatively low cost as well as highly repeatable tool for assessing genetic diversity and identifying closely related fruit tree cultivars. Zhou et al. (2024) ensured the genetic fidelity of *in vitro* cultured 'Keitt' mangoes by demonstrating a complete lack of somaclonal variation compared to the mother plant. Beyond stability testing, ISSR primers have proven effective in detecting polymorphisms for variety identification and offer significant potential for enhancing mango breeding through rootstock characterisation and parentage confirmation (Zhou et al., 2024).

The advantages of SSRs, often referred to as microsatellite DNA markers, compared to other markers include their high polymorphism, abundance, co-dominance in inheritance, ease of analysis and transferability. Using SSR markers, Kalal et al. (2023) successfully differentiated zygotic from nucellar embryos in polyembryonic mango genotypes 'Olour'. Characterisation using SSR markers revealed that Indian mango cultivars cluster into two genetically distinct populations that align with their specific geographical origins (Sherman et al., 2015). Genetic diversity and phylogenetic relationships among mango cultivars have also been extensively analysed using RAPDs, RFLPs and microsatellites based biotechnological tools to verify parentage and map genetic structures. Through a comparative analysis of 17 mango cultivars, the cultivar 'Zebda' was identified as the most diverse, with SSR markers demonstrating greater efficiency than RAPD markers in assessing genetic variability and determining cultivar identities (Hussein et al., 2023). High polymorphism levels were found using ten RAPD markers (91.9%) reported by Ho and Tu (2019). Mango cultivars are identified using AFLP data, which is also used to get genetic linkage maps and investigate the genetic relationships between rootstocks and cultivars. Yamanaka et al. (2006) evaluated the genetic relationships and species diversity of mangos by using AFLP markers. Furthermore, the genetic kinship of polyembryonic and monoembryonic cultivars along the Indian coast was examined *via* genomic and chloroplast DNA RFLP analysis, revealing close relationships among certain hybrids despite morphological differences (Ravishankar et al., 2004).

Other Techniques

Polymorphic mango and *Citrus sinensis* may have close kinship according to published genomes, and the sweet orange genome project reported 1.06×10^6 SNPs in the entire genome, with one-third in genic regions (Malik et al., 2012, as cited in Sherman et al., 2015; Xu et al., 2013). DNA fingerprint patterns of specific mango genotypes were examined by Adato et al. (1995) using minisatellite multi-locus probes to analyse variable number tandem repeats (VNTRs), which consist of short nucleotide sequences clustered in tandem across various chromosomes.

GENETIC TRANSFORMATION

Mango improvement initiatives are significantly expedited by biotechnology, which supplements traditional breeding limitations such as long juvenile phases and high heterozygosity (Song et al., 2023). Researchers have primarily relied on *Agrobacterium*-mediated systems and embryogenic cultures to introduce genes associated with delayed fruit ripening, disease resistance, and stress tolerance (Table 3). However, widespread application remains challenging due to strong genotype-dependency and difficulties in regenerating fully developed transgenic plants from targeted tissues. Pandey et al. (2022) reported the amenability of an apical meristem-targeted and tissue culture-independent *planta* transformation strategy to bypass recalcitrant *in vitro* regeneration barriers. A resistance mechanism of red mango skin to abiotic and biotic stresses through transcriptomic analysis was described by Sudheeran et al. (2021).

LIMITATIONS

The *in vitro* propagation of mango (*Mangifera indica* L.) remains a formidable challenge and a highly recalcitrant process plagued by a cascade of physiological and technical barriers. The major constraint is strong phenolic exudation caused by excision, which rapidly browns the culture medium, resulting in tissue necrosis; this problem is especially apparent when using explants from mature plants, which have less morphogenic potential than juvenile tissues (Krishna & Singh, 2007; Petri et al., 2021). Oxidative stress is a persistent issue in microbial contamination, particularly internal pathogens that can survive after surface sterilisation, which has been identified as a major roadblock for specific varieties such as the Harumanis mango (Lee et al., 2025). Furthermore, the success of these cultures is highly sensitive to the initial explant state, as seen in the high oxidation rates (up to 60% within 72 hours) observed in immature embryos (Souza et al., 2020) and the critical dependence on explant age and specific genotype (Krishna & Singh, 2007). A prolonged juvenile phase is another barrier for mango cultivation. Ultimately, these factors culminate in a notoriously slow growth response and low rates of somatic embryo conversion, often resulting in significant attrition during the final acclimatisation phase. Specific protocol development for economically important varieties may solve many of the above-mentioned problems, and biotechnological tools will also play a role in maintaining homogeneity. Sometimes, a coupling approach can also mitigate the constraints of long juvenility, latent pathogenicity and stress tolerance of the variety. Tissue culture techniques, along with the gamma irradiation approach, can reduce the endo-pathogenic problem, provide stress tolerance and balance hormonal effects to shorten the lifespan.

Table 3
Genetic transformation in mango to develop elite traits using transgenic tools

	Variety	Gene/Tool	Goal	Response Activity	Reference
1.	Amrapali	*GFP* and *Hptll* in binary vector *pCAMBIA1302*	Demonstrate the amenability of a tissue culture-independent, apical meristem-targeted *in planta* transformation strategy to bypass recalcitrant *in vitro* regeneration barriers	Preliminary screening identified 65% of the seedlings displaying GFP expression and molecular characterization (PCR, sqRT-PCR) confirmed transgene integration	Pandey et al., 2022
2.	Amrapali	*GUS* (beta-glucuronidase with a catalase intron) and *nptII* (kanamycin resistance) in binary vector *pCAMBIA2301*	Demonstrate the amenability of a tissue culture-independent, apical meristem-targeted *in planta* transformation strategy	Preliminary histochemical assay identified 57.14% of the plants showing GUS expression at the pricked regions; PCR and sqRT-PCR verified transcript accumulation	Pandey et al., 2022
3.	Shelly	Transcriptomic analysis: *WRKY, DREB*	Resistance mechanism of red skin for abiotic and biotic stresses	Induce defense response and resistance of red mango skin against anthracnose (*C. gloeosporioides*)	Sudheeran et al., 2021
4.	Siji	*MieIF* genes	To identify and characterise the eIF gene family	Role in abiotic stress tolerance	Li et al., 2019a
5.	-	*CPLL*, Immunoproteomics	Investigate a protein against a certain allergen	Proteins made cause of allergic reactions	Cardona et al., 2018
6.	Kent	ACC (*ACO*) oxidase and ACC (ACS) synthase	Genes are used in experiments to prolong shelf life and prevent ethylene generation.	Investigating the role of particular hydrolytic enzymes to prolong shelf life.	Dautt-Castro et al., 2015
7.	Zill	Illumina *RNA*-seq technology	Explore the transcriptome sequence	Investigations into protein identification and gene expression of ripening	Wu et al., 2014
8.	-	*CPLL*	Examine peptidome content	Assess the entity of allergens and peptides bestowed with biological activities	Fasoli & Righetti, 2013
9.	Keitt	Proteomic investigation	Reduction of the biosynthesis of the hormone during ripening	Recurrence of biological phenomena related to ripening	Andrade et al., 2012
10.	Vellaikolumban	*β-glucuronidase (GUS)* reporter gene	Viability of gene transfer demonstrated in embryogenic callus.	To identify transgenic plants, the reporter transgene's expression was verified using the *GUS* assay	Samanta et al., 2007

Note. GFP = Green Fluorescent Protein, *hptII* = Hygromycin Phosphotransferase, *CPLL* s= Combinatorial peptide ligand libraries, *DREB* = Dehydration-Responsive Element-Binding protein, *MieIF* = *Mangifera indica* Eukaryotic Initiation Factor, ACC = 1-aminocyclopropane-1-carboxylate, *NptII* = neomycin phosphotransferase II

CONCLUSION AND FUTURE EXPECTATIONS

Significant economic value is attributed to the mango fruit, particularly in tropical regions where productivity is frequently jeopardised by numerous diseases and stresses. The micropropagation of mango is significantly impeded by phenolic browning and contamination during the establishment of aseptic cultures from mature explants. However, the mango-oriented economy could be transformed by biotechnology through the use of precise genetic manipulation to develop desirable varieties. Goals like resistance against endophytic and exophytic pathogens, short juvenile periods, less phenolic exudation and exceptional fruit quality, which have not been accomplished by conventional breeding, may be achieved through these biotechnological approaches. Homogeneous lines can be raised *via* tissue-culture techniques, and the production of stable offspring for various traits is being progressively investigated. Though mango has been successfully transformed using recurrent somatic embryogenesis, a low incidence of effective genotype regeneration into normal plantlets is still observed. In the future, it is expected that research will be focused on increasing the frequency of *in vitro* developed plantlets. Significant genetic loss within the *Mangifera* genus will be mitigated by the essential creation of *ex situ* and *in situ* germplasm collections. Future resistance to anthracnose, stem end rot and powdery mildew is expected to be sourced from other *Mangifera* species to bolster crop security. The challenges posed by both biotic and abiotic stressors will likely be addressed through continued biotechnological advancements. It is anticipated that the role of mango as a significant global crop will be strengthened by these emerging scientific innovations. New protocols for regenerating plants from shoot or nodal segments are expected to be refined to overcome current propagation limits. A more resilient mango industry will be established as genetic transformation techniques are set for commercial use. The preservation of natural biodiversity will be prioritised to ensure that rare genetic traits are not lost. Ultimately, the global contribution of the mango will be expanded as biotechnology is integrated more deeply into agricultural practices.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Characterisation of Hydrochar Produced from Poultry Litter and Eggshell Waste by Hydrothermal Carbonisation for Soil Amendment

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ABSTRACT

Converting poultry litter and eggshell waste into a carbon-rich material represents an effective approach. A hydrothermal carbonisation (HTC) technique was employed to produce hydrochar and assess its characteristics to improve soil fertility and promote plant growth. For hydrochar production, poultry litter and eggshell were combined with distilled water at a solid-to-liquid ratio of 1:3 (w/v). The hydrothermal carbonisation (HTC) temperature of 240 °C was maintained throughout, with residence times of 60, 120, and 180 mins. Hydrochars were characterised for physicochemical analysis, Scanning Electron Microscopy (SEM), Fourier Transform Infrared Ray (FTIR) Spectroscopy, and X-ray diffraction (XRD). Physicochemical properties such as bulk density, moisture content, ash content, total carbon, organic matter, pH, EC, N, P, K, Ca, and Mg were higher at 180 mins compared to 60- and 120-mins, except for yield, which was lower at 180 mins. The recovery of (N, P, K, Ca, Mg) at 180 min was 43.72%, 44.39%, 9.83%, 272.80%, and 43.48%, respectively, exceeding those obtained at 60- and 120-mins. The surface morphology analysis revealed that hydrochars had crispy and flaky surfaces with micro- and mesoporous structures, indicating that lignin and cellulose components were denatured during carbonisation. FTIR and XRD analysis revealed differences in structural stability during HTC operation, due to the breakdown of cellulose and hemicellulose and formation of aromatic

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structures at 180 mins. Based on these characteristics, the hydrochar produced can be used as a soil amendment for enhancing the soil fertility of acidic soil and supporting plant growth as a sustainable and environmentally friendly option.

Keywords: Eggshell, hydrothermal carbonisation, hydrochar, poultry litter, soil amendments

INTRODUCTION

The increased growth of the global population resulted in high worldwide demand for food, livestock feed, and the availability of lean meat that is low in cholesterol, which has sparked the rapid growth of the poultry industry, which contributed to the large amount of waste generated (Richa et al., 2020), which can be recycled into valuable materials. Animal waste from livestock farming, such as poultry litter, has become a particular concern due to its high production volume and the significant environmental and ecological challenges it poses, including greenhouse gas emissions, water contamination, and soil pollution (He et al., 2023). In addition to its environmental impact, its revalorization is also important from a socioeconomic perspective because of its fertilising potential, due to its essential nutrient content for agricultural applications (Moreno Racero et al., 2025). The sustainable management goal of this waste is to transform it into value-added products, such as soil amendments or fertilisers, which can be incorporated into the ecological cycle, while concurrently restoring soil physical and chemical properties (Fu et al., 2022; Moreno-Racero et al., 2025).

Poultry production generates vast quantities of litter, manure, eggshells, feathers, and other residues that may present severe environmental pollution and economic challenges when inadequately managed. Valorisation of these wastes provides a sustainable alternative to conventional disposal by enabling the recovery of nutrients and mineral components for beneficial applications (Zinina et al., 2022). Poultry litter can be utilised as an organic soil amendment, while waste eggshells, which are predominantly composed of calcium carbonate, can be converted into calcium-rich materials for agricultural, environmental, and industrial applications (Awogbemi et al., 2022). Appropriate utilisation of these poultry-derived wastes can reduce environmental pollution, improve nutrient recycling, enhance soil quality, and support sustainable agricultural practices (Thyagarajan et al., 2013). Therefore, converting poultry litter and eggshell waste into value-added products represents a practical approach to waste management and resource recovery within a circular economy framework.

Traditional thermochemical techniques, such as pyrolysis at high and low temperatures, are the main methods used to produce carbon-rich materials that are used for soil amendments from organic waste. Whereas in thermal treatments under limited-oxygen conditions, the first pyrolysis uses dry organic waste at temperatures between 300 °C and

700 °C (Moreno-Racero et al., 2025). The product is called biochar and is used for soil applications. For the biomass materials that are wet, conventional thermal drying is often needed that sometimes economically unfeasible due to the energy required and the time required (Wang et al., 2018).

To overcome the above-mentioned limitation, hydrothermal carbonisation (HTC) has emerged as another thermal treatment for waste biomass, which has recently received increasing attention as it produces a carbon-rich material called hydrochar. Hydrochar could be used as a soil amendment to enhance soil physical and chemical properties and improve plant growth (Huang et al., 2023). It has also been reported to be a cost-effective, carbon-rich material that is environmentally friendly (Wang et al., 2025). Hydrochar, when applied to soil, provides a circular nutrient cycling medium by returning nutrients from low-cost biomass to the earth (Wang et al., 2020). Hydrochar contains a substantial number of major elements such as C, N, P, and other minerals that can increase soil fertility and promote carbon sequestration, as reported by Belete et al. (2021). Hydrochar significantly improves soil physical-chemical properties and microbial communities that are very important in agriculture by directly or indirectly supplying essential nutrients (Wang et al., 2025). The HTC process produces the so-called hydrochar at temperatures between 150 °C and 350 °C in the presence of water, under its autogenous pressure (Cavali et al., 2023).

The importance of converting wet biomass, poultry litter (PL) and Eggshell (ES), into hydrochar, instead of biochar, is primarily due to the lower production costs associated with it, reduced energy requirements and the elimination of the need for pre-drying the biomass (Paneque et al., 2019). Soils enriched with hydrochar also have a lower biochemical reaction than soils treated with biochar. The result makes it easier for nutrients that were stabilised during the HTC treatment to be remobilised into forms that plants can use. However, it has been observed that during the HTC process, water-soluble components are released into or removed with the process water (Moreno-Racero et al., 2025). This can lead to the loss of beneficial plant nutrients, such as nitrogen (N), phosphorus (P), and other essential minerals, alongside potentially phytotoxic compounds (He et al., 2023). However, plants can utilise a significant amount of nutrients, as hydrochar has the capacity to retain them.

Poultry litter (PL) and Eggshells (ES) are known to be poultry and food industry waste, consisting of macronutrients, organic materials, and trace minerals. Eggshells, often regarded as waste from kitchens and the food industry, are primarily made up of calcium carbonate, different organic materials, and microminerals, in which hydrochar production from them results in a product rich in calcium content that can boost the fertility of the soil and promote plant growth and nutrient availability. Additionally, its porous nature improves aeration, rendering it an important soil amendment. The use of eggshells not only creates an innovative process for reusing shells but also offers significant benefits for sustainable agriculture and environmental cleanup (Wang et al., 2025). Eggshell is one of the liming materials that is applied for the improvement of soil pH, available P, and exchangeable

cations, and it can also be used to decrease the concentration of heavy metals (Almaroai et al., 2014). High calcium, magnesium and phosphorus content from eggshells makes it reusable as a fertiliser or soil amendment (Bashir & Manusamy, 2015). Mixing of PL and ES to get a carbon-rich material known as hydrochar is important in soil amendments. Treatments of these wastes at higher temperatures and pressures using a water medium, through hydrothermal carbonisation, have resulted in the production of a solid product with high carbon content and improved nutrient retention properties. High porosity, low density, and different surface chemistry are some of the unique characteristics possessed by hydrochar, making effective soil amendment that can enhance soil structure, moisture retention, and improve plant growth (Wang et al., 2025). Hydrochar can be used as a source of energy through combustion, an adsorbent for heavy metals and organic pollutants, and for soil amendments (Mau et al., 2020). It is also reported that hydrochar application enhances nutrient availability and reinforcement of soil-carbon structural stability (Vieira et al., 2026). Therefore, knowledge about the most suitable methods for transforming poultry litter and eggshell waste into thermally treated products appropriate for use as soil amendments to improve crop production is still limited. Considering this scenario and to address these knowledge gaps, this study investigated hydrochar production through hydrothermal carbonisation and evaluated its potential as a soil amendment to enhance soil fertility and promote plant growth.

MATERIALS AND METHODS

Raw Materials

The poultry litter was sourced from D'Khairan poultry farm located at Sungai Besar, Selangor, and eggshells were sourced locally from an open restaurant kitchen, all in Malaysia.

Biomass Preparation

The poultry litter collected were separated from debris, shredded and ground into pieces, while the eggshells (ES) were washed thoroughly with distilled water to remove impurities, crushed by mortar and pestle, oven-dried, and milled using a blender. The biomass was dried at 105 °C for 24 hr and subjected to particles of decreasing diameter openings. Manual agitation using a sieve was used to get enough of the desired particle sizes. The samples were then stored in an airtight plastic container before the hydrochar production.

Production Method

The hydrochar was produced following the method described by Bento et al. (2019) as shown in Figure 1. The ground poultry litter and eggshell powder were mixed at a ratio

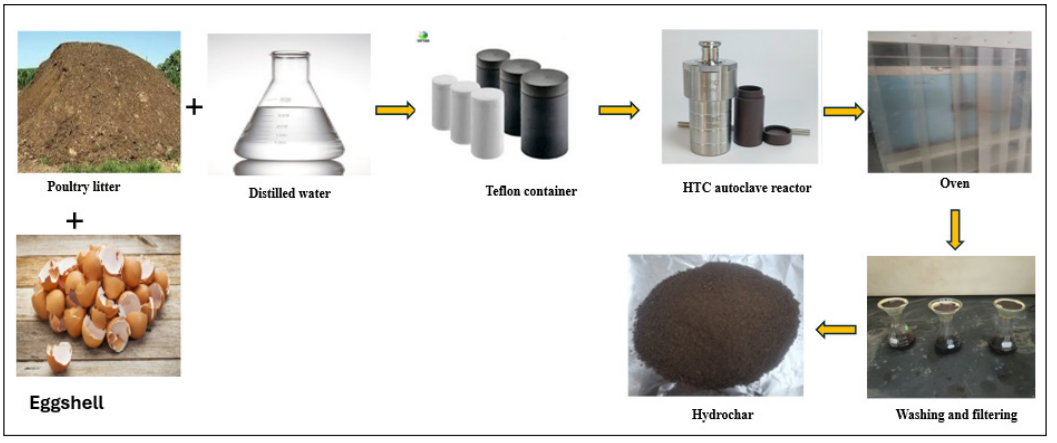

Figure 1. Graphical summary of the multi-step hydrothermal carbonisation process for transforming poultry litter and eggshell into hydrochar

of 50:50 (w/w). After which the resulting mixture was combined with distilled water at a solid-to-liquid ratio of 1:3 (w/v) and transferred into a 100 mL non-stirred Teflon-lined reactor suitable for hydrothermal treatment. The reactor was sealed within a stainless-steel autoclave and subsequently placed in an oven for the hydrothermal carbonisation process. The hydrothermal carbonisation (HTC) temperature of 240 °C was maintained throughout and a reaction time of 60, 120, and 180 mins for the biomass mixtures was employed; it took about an hour of heating time to stabilise the desired heating temperature, and all the experiments were conducted in triplicate to improve the reliability of the data and reduce the effects of random experimental error. After carbonisation, the autoclave reactor was allowed to cool down to room temperature before opening the Teflon container. The carbonised products were separated from the liquid phase using a Whatman filter paper No. 2 to obtain the solid hydrochar. The hydrochar was washed with distilled water during the separation to remove the organic components and subsequently oven-dried at 80 °C for 6 hr. The final dried hydrochar product was stored in an air-tight plastic bag for further analysis

Characterisations Method

Analytical techniques used for the structural composition were Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), and Fourier Transform Infrared Red (FTIR) spectroscopy.

X-Ray Diffraction (XRD)

The X-ray diffraction (XRD) analysis of hydrochar produced at different residence times was conducted using a Rigaku MiniFlex600-C X-ray Diffractometer, Japan. The prepared

powder samples were placed in a sample holder. Thereafter, the sample holder was arranged in the diffractometer operated at 40 kV voltage, and the sample was scanned from 20-100° with a speed of 1° per minute.

Scanning Electron Microscopy (SEM)

Field Emission Scanning Electron Microscopy (FEI Nova Nanosem 230 FESEM, Netherlands) was used to determine the surface morphology of the raw biomass and the resulting hydrochar. The voltage at which the machine was used was accelerated from 0.2 to 30 kV. The high image resolution was achieved by the high-vacuum conditions with the use of an in-lens secondary electron detector, which gave a spatial resolution of about 1.0 nm at 15 kV. Before the analysis, the samples were placed on the aluminium sheets and then coated with a thin conductive layer.

Fourier Transform Infrared (FTIR) Spectroscopy

Determination of the functional group in each sample was done using an FTIR analyser (Bruker; Invenio R; Germany). The wavenumbers for each sample were measured in the range 4000 to 400 cm^{-1} using 32 scans and a resolution of 4 cm^{-1}. Each sample was ground in a mortar and pestle to produce a fine powder. About 1g of the dried sample was taken for analysis.

Physical Properties Determination

The physical properties of the hydrochar, such as bulk density, moisture content, and ash content, were determined according to the standard procedure. Bulk density and ash content were determined following the method described by Pavkov et al. (2022). The ash content was measured on a dry basis, where about 5g of dried hydrochar sample was weighed and ashed in the muffle furnace at 550° C for 4 to 5 hrs until the sample became completely white. The difference in mass before and after the ashing represents the ash content of the hydrochar. Bulk density of the hydrochar was measured using a cylinder with a defined volume, where the dried and ground hydrochar was filled into a glass cylinder (100 cm^3) by gently tapping it against the table. The excess amount on the top of the cylinder was removed with a stirring rod sliding along the top edge of the cylinder, and the sample mass was then measured. Bulk density was measured by dividing the mass of the sample by volume. The moisture content was determined by Peters et al. (2003), in which the samples were oven-dried at 105° C until a constant weight was reached.

Elemental Composition

The chemical composition of the corresponding hydrochars was determined following a standard procedure. pH and EC of the hydrochar samples were determined using the

method by Lee et al. (2013), where 5g of the sample was weighed into a plastic vial and mixed with 50 mL of distilled water. The plastic vial was covered with a lid and shaken for 1 hr, after which it was allowed to settle for another 1 hr before the reading was taken using the pH and EC meters, respectively. Total carbon, hydrogen, nitrogen, and sulphur contents were determined using the CHNS 628 analyser (Leco®, USA) (Dumas method), and other elements (P, K, Ca, and Mg) were determined using the dry ashing method (Cottenie, 1980). The total (P, K, Ca, and Mg) from the hydrochar were determined using the dry ashing method (Cottenie, 1980). 0.5 g of ground hydrochar samples were weighed and put into a crucible, after which they were transferred into a muffle furnace to be ashed at an initial temperature of 300 °C for 1 hr, and then the temperature was subsequently increased to 500 °C for about 4 to 5 hrs. It was then allowed to cool down in the desiccator; the samples were then placed in a fume cupboard, and a few drops of distilled water were added to the ash samples, followed by the addition of 2 mL concentrated HCl, and were then allowed to evaporate to dryness on a hot plate. Subsequently, 10 mL of 20% HNO_3 (200 mL HNO_3 in 1L distilled water) was added to the samples and was then placed in a water bath for I hr. The samples were transferred into a 100 mL volumetric flask and made up to volume with distilled water before filtering into a 100 mL plastic vial using Whatman No. 2 filter paper. The concentration of P was determined using an auto-analyser machine (AA), while K, Ca, and Mg were determined using an Atomic Absorption Spectroscopy machine (AAS). Cation exchange capacity (CEC) was measured by a comprehensive exchange method based on Gillman and Sumpter (1986)

Determination of Nutrient Recovery

The Nutrient recovery into hydrochar was calculated using the Equation 1 below, as described by Devnath et al. (2024)

$$Nutrient\ recovery\ (\%) \frac{Hydrochar\ yield\ \times\ Hydrochar\ nutrient\ content}{Feedstock\ nutrient\ content} \times\ 100 \quad [1]$$

Statistical Analysis

The data were analysed using SAS software (version 9.4), and the effects of different treatments were separated by Duncan's multiple range test, which was carried out to establish significant differences between the means ($p \leq 0.05$).

RESULTS AND DISCUSSION

Characteristics of Poultry Litter and Eggshell Waste Mixtures

Table 1
Selected physico-chemical properties of poultry litter and eggshell waste mixtures used for hydrochar production

Parameters	Value Obtained
Bulk density (g/cm^3)	3.75
Moisture content (%)	40.14
Ash content (%)	58.15
Total Carbon (%)	24.27
Organic Matter (%)	41.85
pH	8.45
Electrical conductivity (mS/cm)	3.38
Nitrogen (%)	0.90
Phosphorous (%)	0.52
Potassium (%)	1.50
Calcium (%)	14.60
Magnesium (%)	0.48
Cation exchange capacity ($Cmol_+$/kg)	6.95

The combined mixture of poultry litter and eggshell used in the study has a bulk density of 3.75g/cm^3 and a moisture content of 40.14%, which indicates its suitability for hydrothermal carbonisation. Feedstock that has a moisture content ranging from 75 to 90% has been reported to be ideal for hydrothermal carbonisation (Hejna et al., 2022). The proximate results of other physical and chemical properties of the raw feedstock used in this study are presented in Table 1. Organic matter, total carbon content and other elements like K and Mg were lower than the reported range from previous studies (Bernal et al., 2024; Maruthamuthu et al., 2024), but all other physical and chemical properties obtained were within the reported range of values. The 0.90% N, 0.52% P, and CEC of 6.95 cmol(+) kg^{-1} indicate its suitability for use as a nutrient-rich material for hydrothermal carbonisation. The nitrogen content is primarily derived from protein residues and other organic compounds present in poultry litter, while phosphorus is contributed by both organic and mineral components. The addition of eggshell introduces calcium-rich mineral phases, mainly in the form of $CaCO_3$, which may affect the mineral composition and cation exchange properties of the feedstock (Awogbemi et al., 2022). The observed CEC value indicates the presence of exchangeable cations and negatively charged functional groups on the material surface, which play an important role in nutrient retention and transformation during HTC (Wang et al., 2018).

The moisture content of the sample, at 40.14%, exceeds the specified range for raw poultry litter, which is 8.5% to 38.8%. The elevated moisture content is beneficial for HTC, as the process uses water as a reaction medium and does not necessitate pre-drying, in contrast to traditional pyrolysis. The pH value of 8.45 signifies that the poultry litter is mildly alkaline. This alkalinity arises from the decomposition of uric acid and the presence of calcium carbonate from dietary supplements (Risberg et al., 2017). This feature indicates that the resultant hydrochar may function as a liming agent, helping in the mitigation of soil acidity and enhancing nutrient accessibility in acidic soils. The electrical conductivity of 3.38 mS/m, falling within the literature range of 1.47-10.05 mS/m as reported by

(Maruthamuthu et al., 2024), indicates the existence of soluble salts and exchangeable cations, including K^+, Ca^{2+}, and Mg^{2+}, which are advantageous for plant growth when administered at suitable levels.

Effects of Residence Time, Product Yield, and its Properties

The effects of different residence times (60, 120, and 180 minutes) on the product yield and other physical properties of hydrochar produced through HTC are presented in Table 2. There were significant differences ($P \leq 0.05$) between the different times.

Table 2
Mean (± s.e.) effects of different processing times (60, 120, and 180 minutes) on the product yield and other properties

Temperature	Time (Mins)	Yield (%)	Bulk density (gcm^{-3})	Moisture content (%)	Ash content (%)	Organic matter (%)	Carbon (%)
240 °C	60	79.31±0.006[a]	0.38±0.014[c]	67.89±0.338[c]	62.89±0.672[c]	29.20±0.333[c]	17.39±0.338[c]
	120	73.97±0.003[b]	0.43±0.021[b]	69.01±0.662[b]	65.13±0.667[b]	36.36±0.028[b]	21.54±0.328[b]
	180	71.00±0.557[c]	0.49±0.012[a]	69.51±0.328[a]	72.28±0.026[a]	38.57±0.105[a]	22.86±0.095[a]

Note. Means with the same letter in the same column are not significantly different by the Duncan multiple range test at $p \leq 0.05$

Yield and Bulk Density

There was a clear inverse relationship noticed between the processing time and product yield. The hydrochar yield decreased significantly from 79.32% at 60 mins to 71.00% at 180 mins, representing a reduction of 8.32% points, equivalent to a 10.49% decrease relative to the initial 60 mins yield Table 2. This decrease indicates longer thermal conversion processes, in which prolonged residence time enhances the devolatilization of light organic compounds and promotes secondary decomposition reactions, resulting in greater mass loss (Wang et al., 2021). A temperature of 240°C combined with a longer residence time of 180 mins result to greater decomposition, fragmentation, and dissolution of organic waste. The observed decrease in hydrochar yield could be attributed to the progressive decomposition of hemicellulose, lignin, and, to a lesser extent, cellulose in the raw material under hydrothermal conditions (Sabry et al., 2024). The reduced hydrochar yield is most likely due to ongoing dehydration during the hydrothermal carbonisation

process at prolonged residence times, and the removal of oxygen and hydrogen from the hydrochar as the hydrochar becomes more coalified (Azaare et al., 2021; Mau et al., 2020). Bulk density showed a significant positive trend, increasing from 0.38 to 0.48 gcm^{-3} that account for 26.32% increase with longer residence time. The increase in bulk density from 0.38 to 0.48 g cm^{-3} as residence time increased corresponds to a 26.32% rise and may indicate gradual structural rearrangement and consolidation of the carbonaceous matrix during hydrothermal treatment. Extended treatment may enhance the decomposition of labile components, condensation of carbonaceous structures, rearrangement of particles, and alterations in interparticle void volume, thereby influencing the packing characteristics of the hydrochar. Similar increases in density with longer hydrothermal treatment have also been reported for biomass-derived carbonaceous materials (Hussain et al., 2024; Marrocchi et al., 2024). This densification is likely because of the loss of volatiles and moisture, resulting in a more consolidated and energy-dense solid product. High bulk density is an important characteristic for solid biofuels, as it improves handling, storage, and transportation efficiency (Chen et al., 2021).

Proximate Composition: Moisture, Ash, Organic Matter, and Carbon

The effect of different residence times on moisture, ash, organic matter and carbon is presented in Table 2, which shows a slight but significant increase over time, rising from 67.89 to 69.51%, corresponding to an absolute increase of 1.62% points and a relative increase of approximately 2.39%. This trend could be explained by the nature of the process (e.g., hydrothermal treatment), where bound water is retained or even reformed within the polymeric matrix of the biomass as hemicellulose and cellulose degrade (Li et al., 2022). The values of the moisture content were also in line with the report of Mau et al. (2020), who found the moisture content from the poultry litter hydrochar to be 74.05%, 69.48%, and 50.88% for 180, 220, and 240 °C hydrothermal carbonisation temperatures. The Ash content value increased from 62.89 to 72.28%, representing an absolute increase of 9.39% point and a relative increase of nearly 14.93% at 180 mins residence time and 12.99% decrease under 60 mins residence time. This study shows a concentration effect; as organic matter volatilizes, the inorganic mineral fraction (ash) becomes a larger proportion of the remaining solid. High ash content can be a setback for thermochemical energy conversion due to slagging issues, but may be beneficial for applications such as soil amendment, where minerals contribute to nutrient supply (Niu et al., 2019). Organic matter and carbon content both increased significantly with processing time. Organic matter rose from 29.20 to 38.57%, which accounted to 32.09% increase for 60 mins to 180 mins residence time, while carbon content increased from 17.39 to 22.86%, which showed about 31.45% increase from 60 mins to 180 mins hydrothermal carbonisation time. This shows that prolonged treatment residence time promotes carbonisation, enhancing the relative abundance of

stable, carbon-rich structures in the solid product. On the other hand, carbon content in hydrochar products increases significantly. Higher carbon content is mainly as the result of the carbonisation process that occurs during HTC. Furthermore, carbon content tends to rise with higher reaction temperatures and significantly changes with prolonged residence times, confirming that both temperature and time are important factors in the carbonisation process (Sabry et al., 2024). This aligns with established biochar production principles, where extended residence time promotes aromatisation and carbon stabilisation (Lehmann & Joseph, 2024).

Potential hydrogen (pH) and Electrical Conductivity (EC)

The pH and EC of the hydrochar are presented in Table 3. There was a significant increase in pH from 8.63 to 8.77 with prolonged processing time, indicating progressive alkalization. The pH value progressively increased with increasing residence time, rising from 8.63 at 60 mins to 8.72 at 120 mins, corresponding to a 1.045 increase. A further increase from 8.72 at 120 mins to 8.77 at 180 mins was observed, representing an additional 0.5% increase. Generally, the value increased from 8.63 at 60 mins to 8.77 at 180 mins, relating to a total relative increase of nearly 1.62%. This is ideal in thermal conversion processes like pyrolysis or hydrothermal carbonisation, in which the alkaline salts (carbonates, oxides) are formed and concentrated as volatilized organic acids (Wang et al., 2021). Electrical conductivity (EC) also increased significantly from 1.64 to 2.69 $mScm^{-1}$, the EC value increased significantly with increasing residence time, from 1.64 at 60 mins to 2.06 at 120 mins, accounting for a 25.61% increase. It then increased further from 2.06 at 120 mins to 2.69 at 180 mins, resulting in an additional 30.58% increase. Overall, the value rose from 1.64 at 60 mins to 2.69 at 180 mins, representing a total relative increase of approximately 64.02%, indicating a substantial rise in soluble ion concentration that could be attributed to the release of mineral elements (K^+, Ca^{2+}, Mg^{2+}, etc.) and the formation of insoluble salts during longer treatment (Xiao & Meng, 2020). Nutrient availability will be elevated with high EC but may also cause a problem of salinity risk if applied to sensitive soils.

Macronutrient Content (N, P, K, Ca, Mg), and Cation Exchange Capacity (CEC)

All the measured macronutrients were significantly increased with the processing time, which shows that the mineral matter is conserved or concentrated because volatile losses are minimised under the specified conditions. Nitrogen (N) increased 0.93 to 1.08, which indicates that the mineral matter is concentrated as organic matter is reduced at any rate. Phosphorus (P) and Potassium (K) also showed increased trends, and they can be explained by the conservation of mineral matter, as volatile losses are minimised in the given conditions. Calcium (Ca) and Magnesium (Mg) rose, as well, which supports the forecast of enriching ash and forming stable mineral phases based on the attractive effect

Table 3
Mean (± s.e.) effects of different residence time (60, 120, and 180 minutes) on the hydrochar chemical properties

Temp.	Time (Mins)	pH	EC (mS/cm)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	CEC $Cmol_+/kg$
240 °C	60	8.63±0.028^c	1.64±0.006^c	0.93±0.011^b	0.78±0.006^b	1.14±0.006^c	13.31±0.028^c	1.40±0.006^b	7.06±0.009^c
	120	8.72±0.380^b	2.06±0.006^b	0.96±0.018^b	0.83±0.012^b	1.34±0.038^b	14.40±0.035^b	1.47±0.009^a	7.56±0.003^b
	180	8.77±0.028^a	2.69±0.006^a	1.08±0.006^a	0.87±0.009^a	1.45±0.012^a	14.54±0.038^a	1.50±0.015^a	7.91±0.012^a

Note. Means with the same letter in the same column are not significantly different by the Duncan multiple range test at $p \leq 0.05$

of thermal treatment, whereby the disappearance of volatile organic matter causes the relative enrichment of non-volatile nutrients in the solid product. N, P, K, Ca, and Mg are quite high, which confirms that it can be used as a slow-release mineral supplement. It is unlikely that the nutrients will be in the unstable forms, and the chances of leaching are reduced compared to synthetic fertilisers (Joseph et al., 2021).

The cation exchange capacity (CEC) of poultry litter-eggshell hydrochar was determined and presented in Table 3. There was a significant increase in CEC with increasing HTC residence time at the processing temperature of 240 °C. The CEC values increased from 6.95 in the raw feedstock to 7.06, 7.56, and 7.91 cmol(+) kg^{-1} after 60, 120 and 180 min of HTC treatment, respectively. It noticed about 13% increase in the CEC after 180 mins indicating that longer HTC improved the ion-retention power of the produced hydrochar by enhancing the availability of exchange sites, increasing the reorganisation of the mineral and facilitating structural redistribution with the carbon matrix. However, prolonged HTC residence time conditions cause the oxygen-containing functional group to be lost due to the dehydration and decarboxylation process. Integration of eggshells as a Ca-rich mineral with poultry litter improved the CEC. The increase in the associated minerals at the exchange sites and strengthened organo-mineral interaction within the hydrochar could be the result of these mineral components. Previous research has shown that HTC affects the properties of poultry-litter-derived hydrochars by modifying nutrient retention, mineral transformation and surface characteristics (Ghanim et al., 2018; Mau et al., 2016). Similarly, carbon stability and mineral content may be enhanced through HTC-induced modifications in manure-derived hydrochars, which can subsequently influence surface charge characteristics (Li et al., 2022). Similar results were reported by Zhang et al. (2015), where CEC increased in hydrochar produced at 260 °C for 1, 6, and 12 hrs respectively. The relatively small 4.63% increase in CEC was observed between 120 and 180 min, showing that the hydrochar was reaching a more stable state, contributing to lower benefits from future increases in residence time. Overall, these results confirm that HTC effectively improved the functional properties of poultry litter-eggshell hydrochar, enhancing its suitability as a nutrient-retaining soil amendment and a potential material for environmental remediation applications.

Nutrient Recovery

The nitrogen (N) content of the hydrochar produced at 240 °C for 180 mins was higher than that of the hydrochars produced at 60 and 120 mins. Additionally, nitrogen recovery from the hydrochar at 180 mins residence time was significantly higher (Figure 2a). Surprisingly, the hydrochar at 180 mins preserved 43.75% of the initial N, compared with lower recovery rates of 38.85% and 37.65% for 120- and 60-mins residence times, respectively. These results confirmed that longer residence time improves N retention during

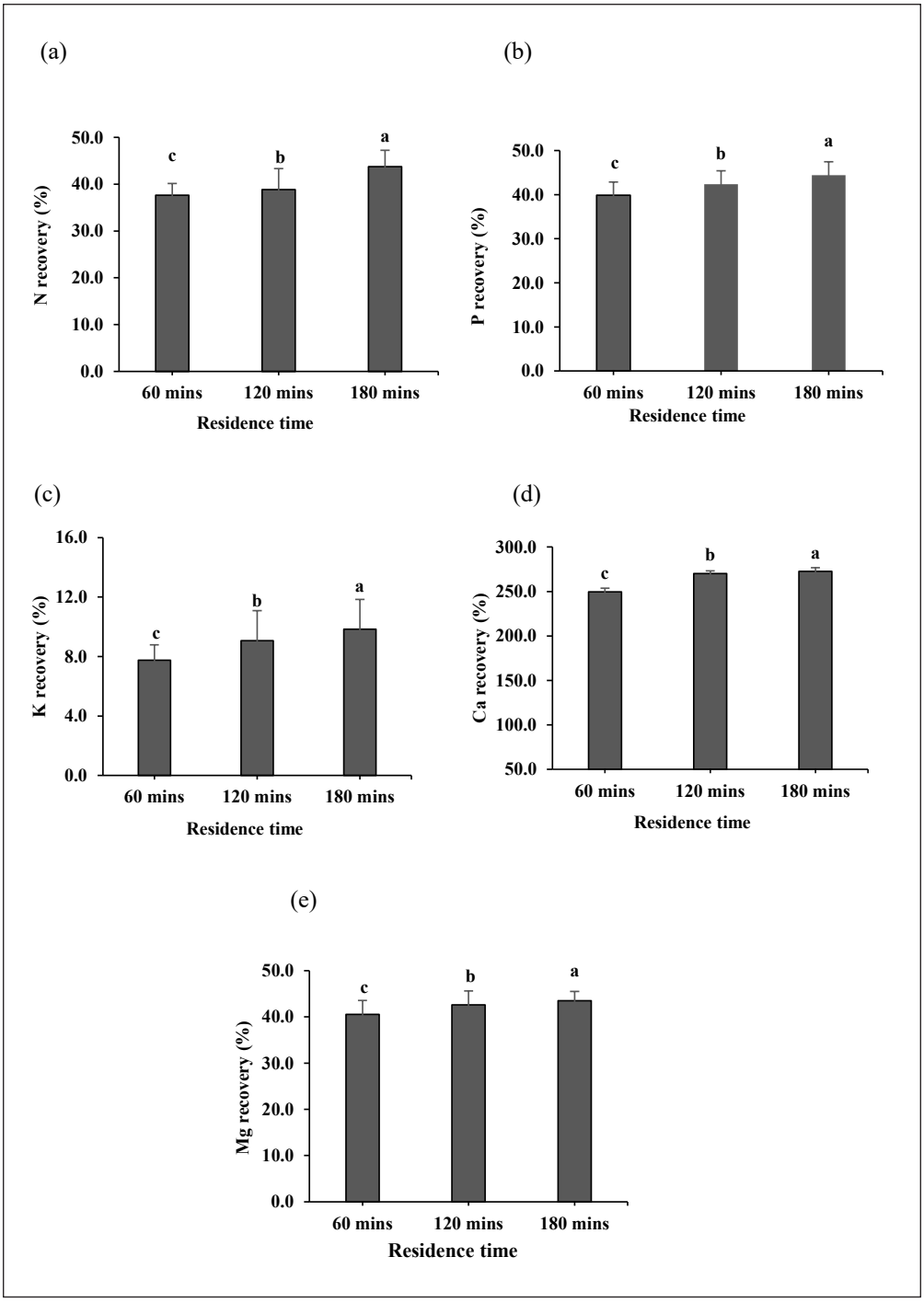

Figure 2. (a) Nitrogen (N), (b) phosphorus (P), (c) potassium (K), Calcium (Ca), and Magnesium (Mg) recovery rates in percentage for hydrochar from poultry litter and eggshell. Different letters above the bars denote statistically significant differences in nutrient recovery among the treatments

the hydrothermal carbonisation process. The increase in nitrogen concentration of the hydrochar with prolonged residence time could be as the result of progressive hydrothermal transformation of the feedstock and nitrogen redistribution carbonisation. As residence time increases, more reactions such as hydrolysis, dehydration and decarboxylation occur, improving the removal of large carbon and oxygen-containing components into liquid and gaseous phases. Therefore, the decrease in the mass of the solid part can lead to a relative enrichment of nitrogen in the remaining hydrochar. Furthermore, increasing the hydrothermal residence time promotes more condensation and polymerisation reactions between nitrogen-containing compounds and carbohydrate-derived intermediates, thereby incorporating nitrogen into more stable solid structures. Longer residence times can also promote the formation and condensation of N-containing heterocyclic structures, thereby increasing the retention of nitrogen in the solid hydrochar (Leng et al., 2021).

In contrast, the recovery rates of P, K, and Mg for hydrochar produced at 240 °C for 60, 120, and 180 mins residence times were slightly below 100%, indicating that most of these nutrients were lost during the production process (Figures 2b, c, and e). Conversely, the Ca recovery rates for different residence times slightly exceeded 100%, indicating that virtually no Ca was lost during the production process (Figure 2d). Residence times of 180, 120, and 60 mins recovered about 272.80, 270.20, and 249.72 of Ca, respectively. It was generally observed from the recovery data that even if the prolonged residence time preserves most of N, P, K, and Mg (>60%), they could conserve almost 100% of the Ca in the poultry litter and eggshell.

X-ray Diffraction Analysis

The X-ray diffraction (XRD) patterns of the poultry litter and eggshell hydrochar produced at 240 °C for 60, 120, and 180 mins residence times were presented in Figure 3 and found to be complex, indicating various peak patterns with some degree of order in short-range, showing an amorphous material. Such characteristics made it difficult to attribute minor peaks to specific minerals, corroborating Da Silva Mendes et al. (2021). Irrespective of the different carbonisation residence times, the hydrochar showed similar peaks and had a wide range of amorphous hump on them, which showed that it had a lot of organic content and little crystalline minerals. There were only a few spectral patterns that had to do with quartz, sylvite, or small amounts of phosphates. The poultry litter-eggshell hydrochar produced showed a different and intense pattern of peaks characteristic of crystalline calcite ($CaCO_3$), particularly the dominant reflection at approximately 29-30° 2θ, alongside several secondary Ca-based mineral peaks. This confirms that eggshell-derived $CaCO_3$ remained stable under hydrothermal conditions and became uniformly incorporated into the hydrochar matrix. The presence of high calcite crystallinity indicates strong liming potential, as $CaCO_3$ showed a high impact for neutralising soil acidity and reducing exchangeable aluminium

in acid soils. The high abundance of calcite at 29-30° 2θ was aligned with the finding of Yang et al. (2023) from the hydrochar produced with sawdust and calcined eggshell.

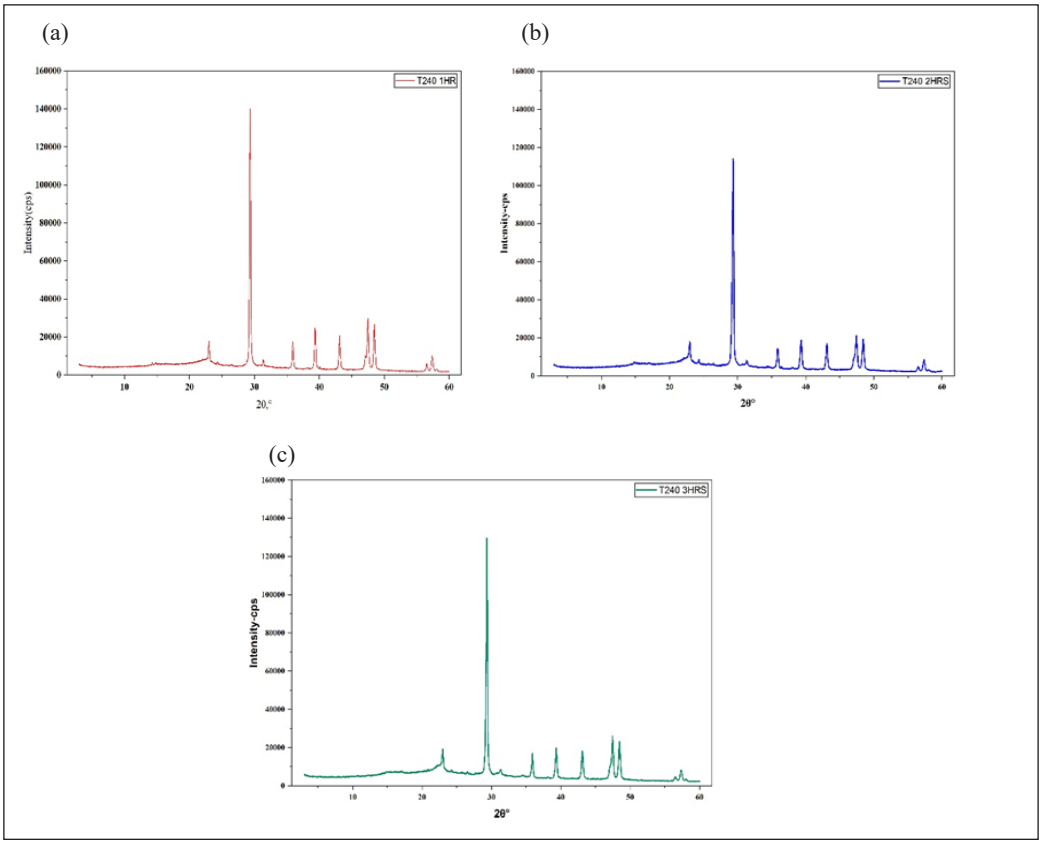

Figure 3. XRD analysis images of a, b, and c poultry litter and eggshell powder hydrochar at 240 °C for 60, 120, and 180 mins, respectively

Morphology Transformations

The colour of the resulting hydrochars was observed based on qualitative visual inspection of their physical appearance. No instrumental colour measurement was performed. The original appearance of poultry litter was a brown powder Figure 4. Under the processing temperature of 240°C at different residence times of 60, 120, and 180 mins respectively, physical changes were observed in the appearance of the hydrochar. The heterogeneous appearance with observed light brown PL particles and pale off-white eggshell fraction was exhibited by the hydrochar at 60 mins residence time. A coarse and particulate surface, related to little thermal activity and minimal structural transformation, was also seen. At 180 mins residence time, the materials became darker, indicating the appearance of organic matter degradation; more homogenised materials were also noticed, and the

eggshell particles became less distinguishable, and the surface texture shows more softened and smoother. The hydrochar looks darker and more uniform, probably because of high thermal decomposition. Eggshell particles are fully integrated into the matrix, and the surface is smoother with finer particles, indicating chemical transformation with longer residence time. The colour changes were the result of dehydration, decarboxylation, and carbonisation reactions occurring during the hydrothermal treatment process. Prolonged residence times also caused the hydrochar to change from light brown to dark brown or black. However, temperature had a more significant effect on the color development than residence time (Yao et al., 2016)

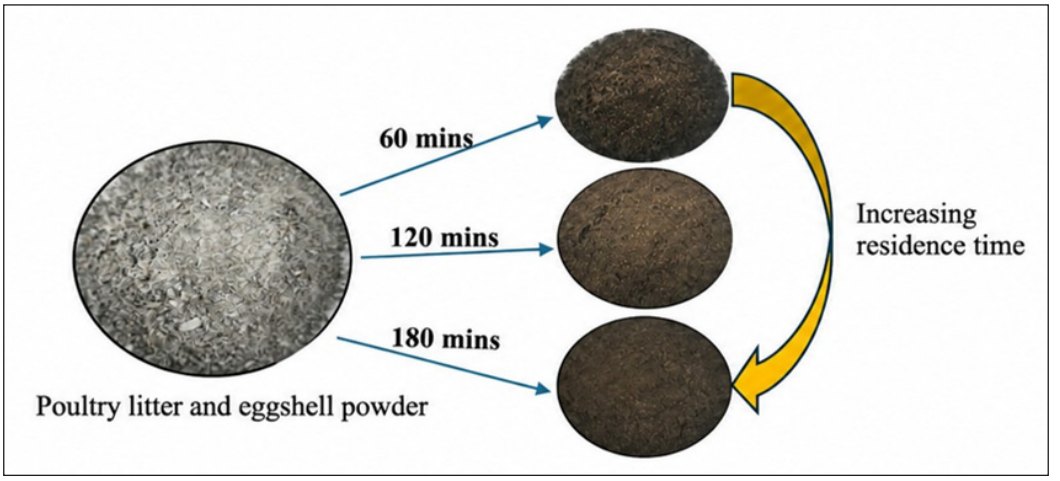

Figure 4. Hydrochars macroscopic morphologies under different residence times

Scanning Electron Microscopy (SEM)

The Scanning electron microscopy (SEM) of PL and ES powder hydrochar produced at 240 °C carbonisation temperature under 60, 120, 180 mins residence time are presented in Figure 5 and reveal a significant difference in their structure and composition, which are highly important for their use. A clear morphological transformation was observed, and it had a distinct morphology, characterised by mineral-rich, granular, and less porous surfaces under different residence times. At a residence time of 60 min, the hydrochar exhibited relatively few pore spaces, suggesting limited structural degradation and greater retention of the feedstock's original mineral constituents, including calcium carbonate derived from the eggshells (Clarke & Olea, 2024; Yang et al., 2023). Hydrothermal carbonisation of PL mixed with ES resulted in the introduction of numerous sphere-like particles in the hydrochar. These sphere-like particles presented a morphology like that of hydrochar porous carbon derived from sucrose and glucose (Ischia et al., 2023; Sevilla et al., 2021). This could be due to the breakdown of cellulose and hemicellulose into glucose during

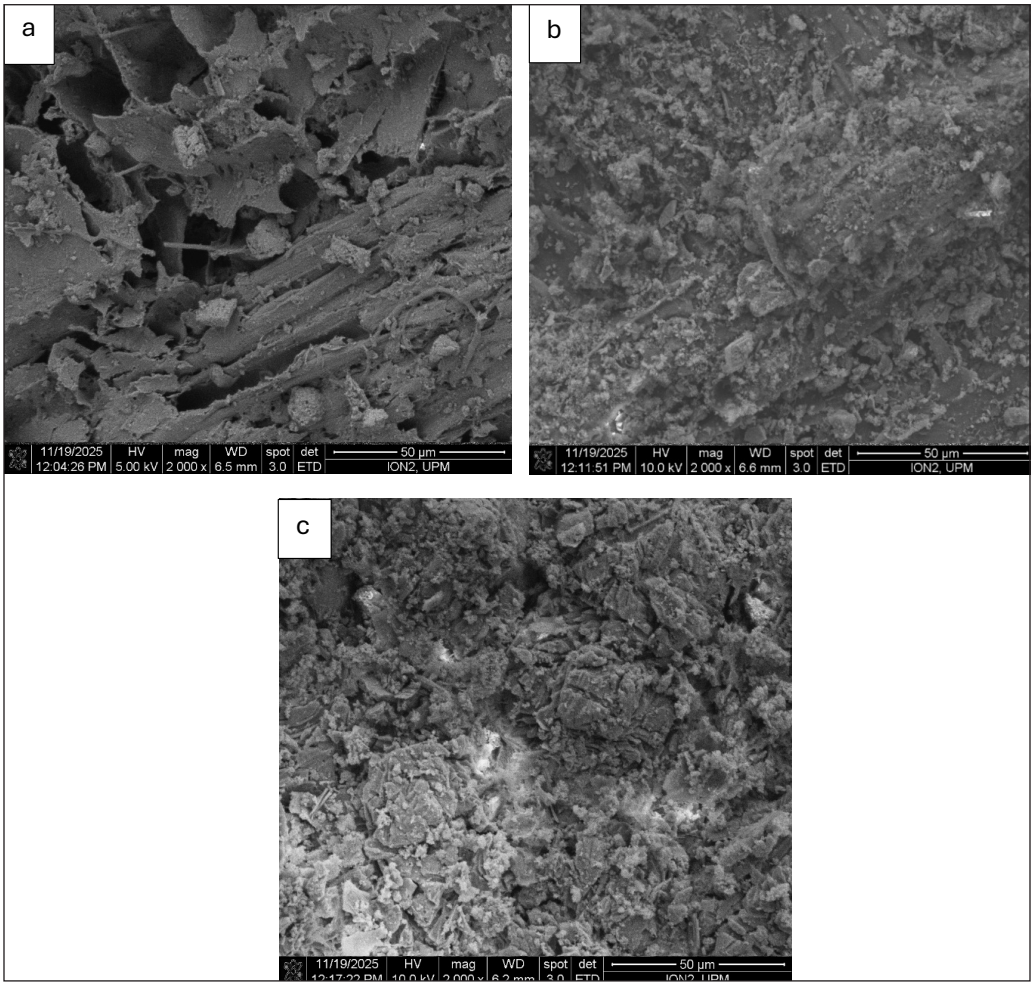

Figure 5. SEM images of a, b, and c poultry litter and eggshell powder hydrochar at 240 °C for 60, 120, and 180 mins, respectively

hydrothermal carbonisation, which enhances the formation of such spherical structures (Cheng et al., 2025).

The carbon in the hydrochar looks like irregular, dense clusters with minimal visible pore space. This is because it is usually processed at a low temperature of 240 °C, which doesn't get rid of enough volatile components that are needed for higher porosity. Clarke and Olea (2024) reported analogous dense hydrochar formations were identified in poultry manure and several nutrient-dense feedstocks treated at temperatures below 260 °C. Several brilliant, angular particles were seen in the hydrochar matrix. These particles are found to be minerals comes from ES, like calcium carbonate and other calcium compounds. Low-temperature hydrothermal processing keeps these minerals intact and evenly spread out

(Shrestha et al., 2024). Their presence offers hydrochar a significant ability to neutralise pH, helping reduce exchangeable aluminium by displacing cations (Lima et al., 2024), and lets the substance work as both an organic carbon source and a mineral liming agent. This is in line with research that shows that hydrochars that have been treated with ES work better than those that haven't (Liu et al., 2022; Shrestha, et al., 2024). Studies show that mineral exposure, not porosity, is what makes low-temperature hydrochars so good at correcting acidic soils (Liu et al., 2025; Shrestha et al., 2024).

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra Figure 6 of the poultry litter and eggshell powder hydrochar showed a broad and strong band at ~3300 cm^{-1}, corresponding to O-H stretching, which gradually decreased in intensity as HTC residence time increased, indicating gradual dehydration (Islam et al., 2021; Khan et al., 2022) The high peaks at ~1650 and 1540 cm^{-1} (Amide I and II) from poultry litter proteins decreased considerably over 2 hours; hence, confirming the thermal decay of labile nitrogenous substances (Sevilla & Fuertes, 2009). It is worth noting that the typical doublet of calcite in eggshells at 1450 and 875 cm^{-1} did not show in all treatment times, which proves the integration of stable calcium carbonate into the hydrochar structure (Chen et al., 2019). The relative proportionate rise in the salience of these mineral peaks at 180 mins would imply a concentration effect through the loss of organic matter, hence creating a mineral-enhancing carbon resource.

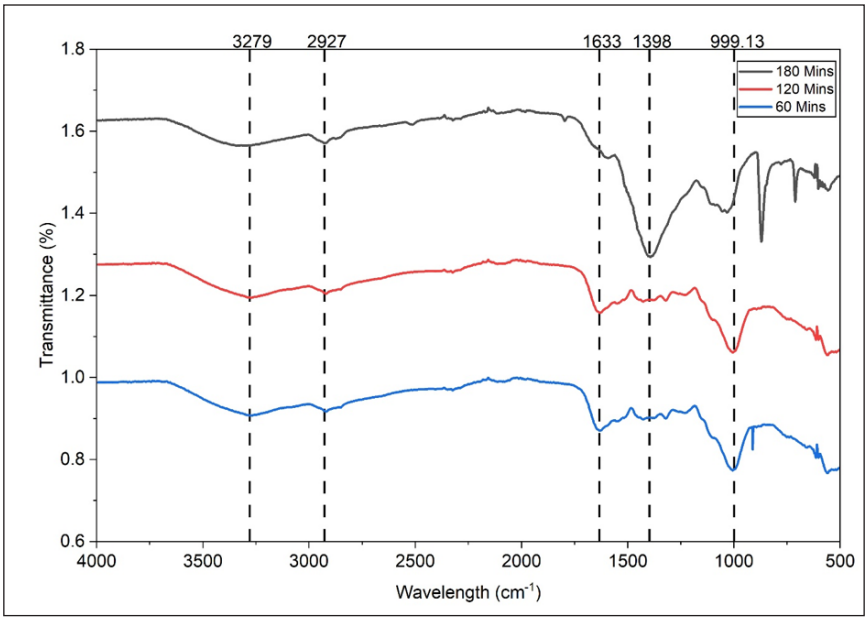

Figure 6. FT-Infrared spectra of poultry litter and hydrochar produced using hydrothermal carbonisation at 240 °C for 60, 120, and 180 mins, respectively

CONCLUSION

The study confirmed that production of hydrochar using poultry litter (PL) and eggshell (ES) waste through hydrothermal carbonisation at 240 °C acquires auspicious quality for use as a soil amendment. Porous structures, increased carbon content, reduced H/C and O/C ratios, and the presence of hydroxyl and carboxylic functional groups, showing improved carbonisation and good surface properties, were observed from the hydrochars. The embodiment of eggshell-derived calcite gave the alkaline nature of the hydrochar, making it suitable for improving soil acidity. Among the various conditions determined, 180 mins residence time gave hydrochar with the most suitable physicochemical properties. Generally, the results highlighted the potential of PL and ES-produced hydrochar as a sustainable and environmentally friendly soil amendment for improving soil fertility, increasing nutrient retention, and correcting soil acidity while encouraging the beneficial reuse of agricultural waste. It is recommended that future studies be carried out to evaluate crop performance from the produced hydrochar through controlled or field trials to determine its effectiveness in improving soil physicochemical properties, nutrient availability, and crop growth and yield, especially on acidic soil.

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Review Article

Synergistic Biological Control of Bacterial Leaf Blight of Rice by *Trichoderma, Pseudomonas* and *Bacillus*

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ABSTRACT

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae,* is one of the most destructive diseases affecting rice production worldwide. With the growing demand for environmentally friendly and sustainable agricultural practices, microbial antagonist-based biological control has emerged as a promising alternative to synthetic chemical pesticides. This review comprehensively evaluates the efficacy and mechanisms of three widely studied biocontrol agents, i.e. *Trichoderma*, *Pseudomonas* and *Bacillus,* in the management of BLB disease of rice. These biocontrol agents suppress the pathogen through multiple mechanisms, including the production of antimicrobial compounds, competition for nutrients and ecological niches, promotion of plant growth and activation of host plant defence responses. Numerous studies have demonstrated that the

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application of these beneficial microorganisms significantly reduces pathogen proliferation, disease lesion length, disease incidence and disease severity, while improving grain yield and overall crop performance. Furthermore, the combined application of these biocontrol agents has shown greater effectiveness in disease suppression and yield enhancement than the use of individual agents alone, indicating synergistic interactions among them. Overall, this review highlights the considerable potential application of *Trichoderma*, *Pseudomonas* and *Bacillus* species as sustainable and effective biological control agents for the long-term management of BLB disease. Their integration into rice production systems offers a promising strategy for integrated disease management, improved crop productivity and reduced reliance on synthetic chemical pesticides.

Keywords: Bacillus spp., bacterial leaf blight, biological control, disease severity, rice, *Pseudomonas* spp. *Trichoderma* spp., yield

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's most important staple food crops, providing the primary source of nutrition for more than half of the global population. It is the second most widely produced cereal crop, with an annual global production of approximately 645 million tonnes. Rice is cultivated in at least 114 countries, with nearly 90% of global production originating from Asian countries (Sharif et al., 2014). Beyond its role in food production, rice is deeply embedded in the economies, cultures, and livelihoods of many regions, making it indispensable to global agriculture and food security (Asma et al., 2023).

Despite its global importance, rice production is increasingly threatened by numerous biotic and abiotic stresses. Among the biotic constraints, Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most destructive diseases affecting rice worldwide (Adedibu et al., 2024) and causes severe damage to millions of hectares of rice fields annually and can result in yield losses of up to 75% under severe disease outbreaks (Adedibu et al., 2024). Consequently, effective disease management strategies are essential to minimise yield losses and ensure sustainable rice production.

Various approaches have been employed to manage BLB, including chemical control, resistant cultivars, and biological control. Chemical pesticides and antibiotics have traditionally been used to suppress the disease. However, their effectiveness under field conditions has often been inconsistent due to differences in the sensitivity of *Xoo* pathogenic races to chemical treatments (Yasmin et al., 2017). Moreover, the continuous and excessive application of pesticides and antibiotics has accelerated the emergence of resistant pathogen populations. This reduces the long-term effectiveness of these chemicals while raising environmental and public health concerns.

The cultivation of resistant rice varieties has been regarded as one of the most effective and environmentally friendly approaches for BLB management (Teja et al., 2025).

Nevertheless, this strategy also has several limitations under field application. Improving resistance against one pathogen may inadvertently increase the plant's susceptibility to other diseases (Teja et al., 2025). Furthermore, breeding disease-resistant cultivars requires balancing resistance with desirable agronomic traits, as resistance genes may negatively influence yield, grain quality, or other important characteristics (Oliva et al., 2019; Teja et al., 2025). Additional challenges, including linkage drag and limited resistance durability due to the emergence of new pathogenic races, will further complicate the development of durable resistant cultivars (Teja et al., 2025).

In response to these challenges, biological control using beneficial microorganisms has emerged as a promising and environmentally sustainable alternative for BLB management. Biocontrol agents (BCAs) and plant growth-promoting microorganisms (PGPMs) suppress plant pathogens through multiple mechanisms while simultaneously enhancing plant growth and health (Ashwini et al., 2023). These microorganisms produce a wide range of bioactive secondary metabolites, including siderophores, rhamnolipids, antibiotics, lytic enzymes, and volatile organic compounds (VOCs), which could inhibit pathogen growth through various mechanisms. They also enhance host resistance by activating plant defence responses, thereby reducing disease development (Teja et al., 2025). In addition to disease suppression, many beneficial microorganisms stimulate root and shoot development, improve nutrient uptake and promote overall plant growth, contributing to higher crop productivity (Do et al., 2023). For example, volatile organic compounds (VOCs) produced by *Bacillus* species exhibit strong antibacterial activity by inducing reactive oxygen species (ROS) accumulation and oxidative stress in plant pathogens (Ali et al., 2023). Beneficial microorganisms such as *Trichoderma* spp., *Pseudomonas* spp., and *Bacillus* spp. have demonstrated broad-spectrum activity against numerous foliar and soil-borne pathogens, including *Phytophthora* spp., *Rhizoctonia solani*, *Xanthomonas* spp. and *Pythium* spp. (Teja et al., 2025). Among these beneficial microorganisms, *Trichoderma* spp. are widely recognised for their ability to suppress plant diseases through the production of antimicrobial metabolites and the activation of plant defence mechanisms. Studies have demonstrated that *Trichoderma* significantly reduces BLB severity by inducing the rice immune system while simultaneously producing antimicrobial compounds that directly inhibit *Xoo* (Islam et al., 2023; Teja et al., 2025).

Similarly, *Pseudomonas* species, particularly *P. aeruginosa*, have shown considerable potential as biological control agents against BLB. These bacteria produce a diverse array of antimicrobial compounds, including hydrogen cyanide, phenazines, and siderophores, which effectively inhibit the growth of *Xoo*. In addition, *Pseudomonas* spp. enhance nutrient availability and induce systemic resistance in rice plants, thereby improving plant growth and increasing resistance to disease (Thammasittirong et al., 2025). *Bacillus* species also play an important role in promoting plant health and suppressing plant diseases.

Several species, including *B. amyloliquefaciens*, produce bioactive lipopeptides with strong antibacterial activity against *Xoo*. Besides directly suppressing the pathogen, *Bacillus* spp. enhance nutrient uptake, stimulate plant growth, and improve the overall vigour and productivity of rice plants (Banluehan & Tunchai, 2025).

This review aims to comprehensively evaluate the synergistic effects and biocontrol efficacy of *Trichoderma* spp., *Pseudomonas* spp., and *Bacillus* spp. in the biological management of bacterial leaf blight (BLB) of rice. These three genera were selected because they are well represented in both the commercial biopesticide industry and the existing BLB-specific research literature, accounting for a substantial proportion of currently marketed microbial biocontrol products (De Pessemier et al., 2026). Moreover, they represent distinct and complementary groups of biological control agents (BCAs) with diverse modes of action, including antibiosis, antibiotic and siderophore production, and lipopeptide-mediated antagonism. Collectively, these mechanisms provide broad functional diversity and offer considerable potential for effective BLB management. Other promising genera, such as *Streptomyces* and *Paenibacillus*, have also demonstrated biocontrol potential; however, the available BLB-specific evidence for these microorganisms remains comparatively limited. Therefore, they are beyond the scope of the present review. Overall, this manuscript provides a comprehensive assessment of the effectiveness of fungal and bacterial BCAs against BLB, with particular emphasis on their disease-suppression mechanisms, plant growth-promoting activities, and potential to enhance rice yield. The review further discusses their role in reducing reliance on synthetic pesticides and promoting sustainable rice production.

Biocontrol Potential of *Trichoderma* spp. Against BLB Disease

Disease Control Efficacy

Trichoderma spp. have shown considerable promise as BCA against a wide range of soil-borne plant diseases (Bastakoti et al., 2017; Kumar et al., 2019). Numerous studies have demonstrated their effectiveness in controlling various plant diseases, including collar rot, root rot, stem rot and sheath blight, offering an environmentally friendly alternative to synthetic chemical pesticides (Kumar et al., 2020). In addition, *Trichoderma* spp. have been reported to effectively suppress important soil-borne pathogens such as *Fusarium solani*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Bastakoti et al., 2017). Beyond disease suppression, *Trichoderma* is widely recognised as a versatile BCA because of its dual role in controlling phytopathogens and promoting plant growth, making it an important component of sustainable agricultural systems (Kumar et al., 2020). Meanwhile, Sutthisa et al. (2024) reported that several *Trichoderma* isolates enhanced the germination rate and biomass accumulation of rice seedlings, demonstrating their plant growth-promoting potential.

For management of BLB, foliar spray and soil application are the most commonly used delivery methods and have been shown to effectively reduce disease severity while improving plant health (Kalboush et al., 2017). Kamaruzzaman et al. (2021) reported that different *Trichoderma* strains exhibited in-vitro inhibition of *Xoo*, with inhibition percentages ranging from 70 to 81%. Similarly, Gangwar and Sinha (2010) screened 52 *Trichoderma* isolates and found that 23 isolates, including *T. virens*, *T. hamatum* and *T. harzianum,* achieved complete (100%) inhibition of *Xoo* growth. Among these, *T. harzianum* exhibited the strongest antagonistic activity through the production of both volatile and non-volatile antimicrobial compounds. These findings highlight the significant potential of *Trichoderma* spp. as sustainable biological control agents for the management of BLB in rice. Their ability to suppress pathogens while simultaneously promoting plant growth makes them promising candidates for incorporation into integrated disease management strategies.

Several isolates have been reported to minimise lesion length significantly. In rice leaves treated with 27 µgmL^{-1} *Trichokonins* A (TKA), lesion length decreased by 82.2% compared with untreated controls (Meehae et al., 2023; Zhang et al., 2022). Efficacy tests showed that rice treated with TKA yielded disease lesion lengths of about 3.8 cm compared to 21.2 cm in untreated rice (Zhang et al., 2022). Furthermore, Islam et al. (2023) reported that *T. asperellum* strain BDISOF08 decreased disease lesions by up to 87.5%. Details of the findings were reported in Table 1.

Table 1
Effect of Trichoderma *spp. on reduction of lesion length of bacterial leaf blight disease of rice*

***Trichoderma* spp.**	**Reduction of Lesion Length (%)**	**References**
T. asperellum,	87.5	Islam et al., 2023
T. paraviridescens,	75	
T. erinaceum	60.4	
T. longibrachiatum	82.2	Zhang et al., 2022; Teja et al., 2025; Meehae et al., 2023
Trichoderma consortia	70	Teja et al., 2025

Trichoderma treatment using the seed priming method resulted in a 75% reduction in lesion length (Islam et al., 2023). Although the percentage of disease reduction was not explicitly indicated in the report, this finding is sufficient to indicate the potential of *Trichoderma* as a BCA. On the other hand, application of *Trichoderma* as a consortium resulted in a 70% lesion length reduction when it was compared to the control treatment (Teja et al., 2025). This further demonstrates *Trichoderma's* effectiveness and synergistic response in controlling BLB of rice.

Trichoderma have shown significantly decrease in disease severity under practical applications (Zhang et al., 2022). Similarly, a field study by Gangwar (2013) revealed that *T. harzianum* managed to reduce disease severity by 59% in comparison to the untreated controls. In the study, among other isolates, *Trichoderma* isolate 40 and *T. virens* showed decreases in disease from 48-58% (Jena et al., 2022). Under field settings, the combination of foliar sprays and root dips containing *T. viride* decreased disease severity by 40% (Mandal et al., 2017). Details of the disease control efficacy report against BLB were summarised in Table 2. The application rate is a significant challenge; T. *harzianum* with high application rates (4-8 g/L) has been proven to be more successful in managing disease than lower rates, as demonstrated by Khan and Sinha (2007).

Table 2
Disease control efficacy of Trichoderma *spp. in controlling bacterial leaf blight disease of rice*

***Trichoderma* spp.**	**Disease Control Efficacy (%)**	**References**
Trichoderma harzianum	48-59	Jena et al., 2022
Trichoderma spp.	48.3	
Trichoderma virens	58	
Trichoderma viride (foliar + root dip)	40	Mandal et al., 2017
Trichoderma atroviride	81	Prismantoro et al., 2024

Trichoderma also impacts plant growth and yield. For example, *T. harzianum* has led to significantly less disease incidence while increasing grain yield. *T. harzianum* was reported to enhance grain yield from 29.67 g/plant (control) to 48.74 g/plant (Jena et al., 2022). The efficacy of *Trichoderma* may vary between isolates or strains, but all the strains or isolates tested have shown high yields. Jena et al. (2022) reported that *T. harzianum* IRRI-5 was the most efficient isolate and yielded the tallest plants with the highest number of filled grains and the highest yield per rice plant. Under controlled settings, *T. harzianum* has been shown to enhance yield properties such as the number of tillers and panicles. Number of grains per panicle and total grain yield were also improved significantly (Teja et al., 2025).

Mode of Action of Trichoderma spp.

Trichoderma spp. suppress phytopathogens through several complementary mechanisms. Their effectiveness against BLB disease infection results from a combination of direct antagonism, plant defence activation and plant growth promotion, as illustrated in Figure 1.

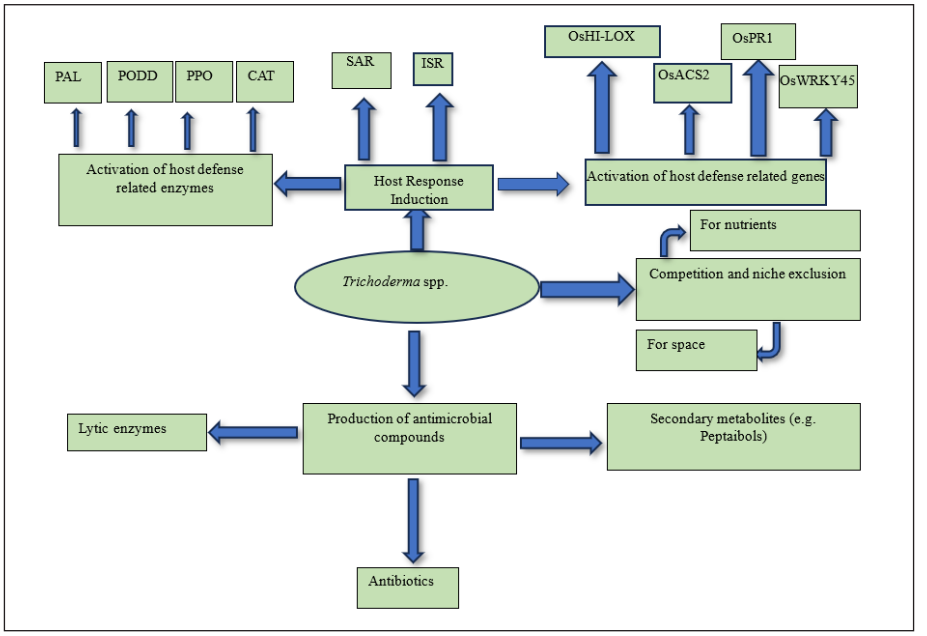

Figure 1. Illustration of the mechanisms of action of *Trichoderma* spp. for controlling bacterial leaf blight disease of rice

Note. PAL = Phenylalanine Ammonia-Lyase; POD = Peroxidase; PPO = Polyphenol Oxidase; CAT = Catalase; SAR = Systemic Acquired Resistance; ISR = Induced Systemic Resistance; OsHI-LOX = *Oryza sativa* Herbivore-Induced Lipoxygenase; OsPR1 = *Oryza sativa* Pathogenesis-Related Protein 1; OsACS2 = *Oryza sativa* 1-Aminocyclopropane-1-Carboxylate Synthase 2; OsWRKY45 = *Oryza sativa* WRKY Transcription Factor 45

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the terms used to describe how *Trichoderma* spp. boost the immune system of the rice plant (Table 3). Phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), catalase (CAT), and peroxidase (POD) are important defence-related enzymes that are activated to suppress disease infection. By reinforcing cell walls with lignin and phenolic compounds, detoxifying reactive oxygen species and synthesising antimicrobial chemicals, these enzymes help plants become less vulnerable to bacterial invasion (Islam et al., 2023). Concurrently, *Trichoderma* induces enhancement of genes related to salicylic acid (SA) and jasmonic acid (JA) signalling pathways necessary for the host defence system. Key genes are OsHI-LOX (lipoxygenase for the JA pathway), OsACS2 (ethylene biosynthesis), OsPR1 and OsPR10 (pathogenesis-related proteins) and OsWRKY45, OsWRKY62 and OsWRKY71 (transcription factors for defence). When these genes are upregulated, the rice plants enter a "primed" state that allows them to fight against the pathogen more quickly and effectively (Islam et al., 2023).

Table 3
Application of different effective strains/isolates of Trichoderma *against Bacterial Leaf Blight of rice*

Strains/Isolates	Mechanisms of Action	Efficacy of Disease Control	Evidence Level	Application Conditions/ Method	References
T. longibrachiatum SMF2 (Trichokonins A, TKA)	Direct antibacterial compound disrupts Xoo cell integrity, causing protein/ nucleic acid leakage	Lesion length reduced by 82.2% at 27 µg/mL TKA; MIC = 54 µg/mL	Laboratory (purified compound applied via detached-leaf/in-planta assay, not whole-plant field or greenhouse trial)	Purified antimicrobial compound (TKA); pathogenicity assays on rice leaves	Zhang et al. (2022)
Native *Trichoderma* isolates (BDISOF67, BDISOF91, BDISOF08, BDISOF09)	ISR: enhanced PAL, PPO, CAT, POD; upregulated SA/ JA defence genes	Enzyme activity increases (PAL 4.27×, PPO 1.77×, CAT 3.53×, POD 1.57×); lesion reduction % not reported	Greenhouse/pot trial	Rhizosphere-isolated strains; whole-plant inoculation to assess induced resistance	Islam et al. (2023)
T. harzianum (phylloplane isolate)	Field-formulated antagonism; possible volatile/non-volatile metabolite involvement	Field disease severity reduced by 48.26% and 59.22%	Field trial	Fungal bioagent formulation applied in field across seasons	Gangwar (2013)
T. harzianum, T. hamatum, T. virens, and other isolates	In-vitro antagonism via volatile/non-volatile metabolites	Up to 100% inhibition of Xoo radial growth (dual culture); up to 55.5% inhibition (diffusion, non-volatile extract, 6.25%)	Laboratory (in vitro)	Dual-culture and metabolite diffusion assays	Gangwar & Sinha (2010)
T. asperellum TAIK1 (as part of consortium)	Combined antagonism and growth promotion/induced defence (not fully resolved)	61.13% disease decrease with consortium (individual isolate contribution not separated)	Greenhouse	Native isolates applied in vivo; growth/disease assessment	Kannan et al. (2021)
T. harzianum IRRI-2 to IRRI-6 (IRRI-5 best)	In-vitro antibiosis; growth stimulation via seed treatment	IRRI-5 and IRRI-2 most effective; disease reduction % not reported	Greenhouse/pot trial	Seed treatment, then *Xoo* inoculation, then disease/ yield assessment	(Jena et al., 2022)

Note. Xoo = *Xanthomonas oryzae* pv. *oryzae*; TKA = Trichokonins A; MIC = Minimum Inhibitory Concentration; PAL = Phenylalanine Ammonia-Lyase; PPO = Polyphenol Oxidase; CAT = Catalase; POD = Peroxidase; SA = Salicylic Acid; JA = Jasmonic Acid; OsPR1 = *Oryza sativa* Pathogenesis-Related Protein 1; OsPR10 = *Oryza sativa* Pathogenesis-Related Protein 10; OsWRKYs = *Oryza sativa* WRKY Transcription Factors; OsHI-LOX = *Oryza sativa* Herbivore-Induced Lipoxygenase; OsACS2 = *Oryza sativa* 1-Aminocyclopropane-1-Carboxylate Synthase 2; IRRI = International Rice Research Institute

Certain strains of *Trichoderma* can directly inhibit the growth of *Xoo* by producing antibiotics, lytic enzymes, and secondary metabolites (including peptaibols) (Islam et al., 2023; Zhang et al., 2022). In the rhizosphere and phyllosphere, *Trichoderma* outcompete pathogens for nutrients and space (Figure 1), hence lowering the establishment and colonisation of pathogens (Islam et al., 2023; Prismantoro et al., 2024).

Biocontrol Potential of *Pseudomonas* spp. Against BLB Disease

Disease Control Efficacy

Exemplary potential has been shown by *Pseudomonas* to serve as biocontrol agents in sustainable agriculture. *P. oryziphila* demonstrates effective antagonistic action against *Xoo* and successfully inhibits its dispersal and growth in rice tissue (Yang et al., 2021). Research revealed that specific strains of *Pseudomonas*, such as *Pseudomonas sp. Rh323* demonstrates significant antagonistic effects against *Xoo*. In-vitro studies showed that this strain could efficiently inhibit the pathogen's growth, resulting in better plant health and higher yields in rice crops (Rangarajan et al., 2003; Yasmin et al., 2016). Certain strains of *Pseudomonas* have been shown in studies to considerably shorten the length of lesions on rice plants. Based on the findings of Rangarajan et al., (2003) treatment with *Pseudomonas* isolates, the lesion length decreased by 15-74% in normal soil and 82% in saline soil. *P. fluorescens* P-7 reduced lesion length in treated plants from 20.37 cm in the control treatment to 18.5 cm (Rangarajan et al., 2003). The severity of BLB is another parameter that is lessened considerably by *Pseudomonas* spp. treatment. Numerous studies have shown that *Pseudomonas* strains are successful in both lab and field settings. In field trials, foliar application of *P. aeruginosa BRp3* decreased the severity of BLB from 83% in the infected control to 43%. This efficacy was equivalent to the streptocyclin treatment (39%) (Yasmin et al., 2017). Inoculation with another strain, *Pseudomonas* sp. *Rh323* resulted in 80% disease reduction under field and greenhouse trials. *Pseudomonas* sp. Rh323-treated plants additionally showed increased plant dry weight, shoot and root length, and increased activity of defence-related enzymes (e.g., catalase, phenylalanine ammonia-lyase, and peroxidase), which are linked to induced systemic resistance (Yasmin et al., 2016).

Pseudomonas have demonstrated encouraging outcomes in boosting yield. *Pseudomonas* have been shown in numerous trials to be effective in lowering the severity of BLB and increasing grain yield (Gokil, 2013; Jeyalakshmi et al., 2010). *Pseudomonas* has been used in practical applications to significantly increase grain and straw yields. One study concluded that the use of *Pseudomonas* consortia in the field trials increased grain yield significantly by 31% and straw output by 10% compared to plots that were not infected (Yasmin et al., 2016). *Pseudomonas aeruginosa BRp3* inoculation increased

grain yield by 51% and straw yield by 55% in certain trials compared to non-inoculated controls (Yasmin et al., 2017). Even under pathogen pressure, *Pseudomonas* sp. application in BLB-infected rice fields has been shown to increase grain yield by up to 31% and straw yield by 10% compared to non-inoculated controls (Yasmin et al., 2016). Yasmin et al. (2016) also found that yield improvements were achieved using 20% less chemical fertiliser, indicating the effectiveness and sustainability of the approach.

Mode of Actions of Pseudomonas spp.

Rice bacterial leaf blight can be effectively controlled by *Pseudomonas* species by suppressing the pathogen and promoting plant health through a variety of ways (Figure 2 and Table 4):

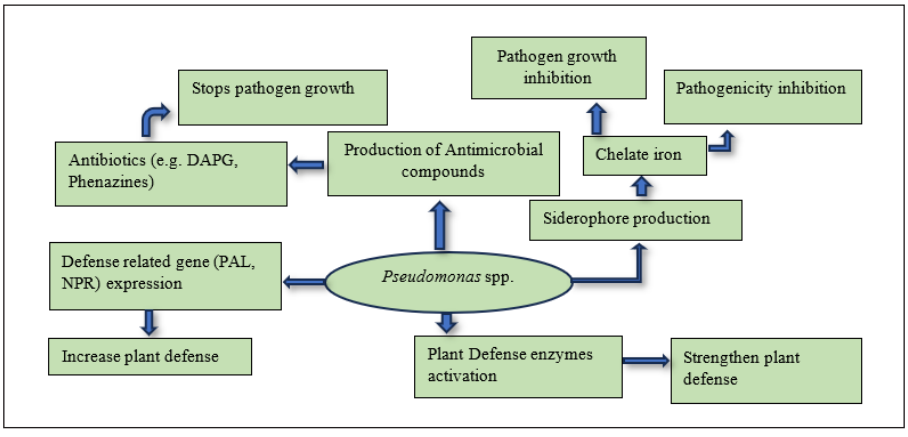

Figure 2. Illustration of the mechanisms of action of *Pseudomonas* for controlling bacterial leaf blight of rice
Note. DAPG = 2,4-Diacetylphloroglucinol; PAL = Phenylalanine Ammonia-Lyase; NPR = Nonexpressor of Pathogenesis-Related Genes 1

Antibiotics including 2,4-diacetylphloroglucinol (DAPG), phenazines, pyocyanin and pyochellin are produced by specific strains of *Pseudomonas* (Figure 2). These substances actually stop the BLB pathogen from growing (Velusamy et al., 2006; Yasmin et al., 2016, 2017). *Pseudomonas* spp. secretes siderophores that chelate iron. These inhibit the pathogen's growth and pathogenicity by depriving it of vital iron (Yasmin et al., 2016; 2017). Moreover, when rice was inoculated with *Pseudomonas* strains, defence-related enzymes such as catalase, phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase become more active. This strengthens the plant's defences against infection. Certain strains increase rice's systemic resistance by inducing the expression of defence-related genes (PAL, NPR1, etc.) (Elsharkawy et al., 2022; Yasmin et al., 2016).

Table 4
Application of different effective strains/isolates of Pseudomonas *against Bacterial Leaf Blight of rice*

Strains/Isolates	Mechanisms of Action	Efficacy of Disease Control	Evidence Level	Application Conditions/ Method	References
Pseudomonas spp. Rh323 (as part of a 4-strain consortium: E227, E233, Rh269, Rh323)	Siderophore production, phosphate solubilization; induction of plant defence enzymes (confirmed for Rh323 individually: +86% peroxidase, +53% catalase, plus PAL/PPO activity)	Co-inoculation of the consortium reduced diseased leaf area by ~80%, improved shoot length by 31%, root length by 41%, and plant dry weight by 60% versus the infected control; rhizosphere colonisation persisted up to 21 days	Laboratory (in vitro screening) + Greenhouse (net-house pot trial) + Field (consortium yield trial)	Seed inoculation and co-inoculation were investigated in growth-room, greenhouse (net house), and field consortium applications.	Yasmin et al., 2016
P. aeruginosa BRp3	Multiple secondary metabolites (siderophores, 1-hydroxy-phenazine, pyocyanin, pyochelin, rhamnolipids, HAQs); induction of plant defence enzymes	Consistent pathogen reduction in rice; grain yield +51%, straw yield +55% vs. non-inoculated control	Greenhouse (net-house pots) + Field	Used as an inoculant in net-house pot trials and field experiments (28 m^2 plots); CLSM verified rhizosphere colonisation under both sterilised and field conditions	Yasmin et al., 2017
Pseudomonas spp. E227, E233	Plant growth-promoting characteristics and siderophore production (in vitro)	In-vitro inhibition zones vs. *Xoo* (1-19 mm across strains); antagonists that enhanced plant health under stress	Laboratory (in vitro screening) + Field (as part of the same consortium trial as Rh323)	Extracted from rhizosphere, assessed in vitro, and used in seed/ co-inoculation consortia in field and greenhouse experiments	Yasmin et al., 2016
P. fluorescens BCA3; *Geobacillus thermoparaffinivorans* BCA12 (endophytic isolates)	Fluorescent pseudomonad/ endophytic bacterial antagonism (in vitro)	Strong in-vitro inhibitory zone vs. *Xoo*, ~35 mm (nutrient agar assay)	Laboratory (in vitro screening only)	Endophytic bacteria isolated from paddy roots/leaves; screened via nutrient-agar plate antagonism assay; molecular ID by 16S rRNA	Halim et al., 2020

Table 4 (*continued*)

Strains/Isolates	Mechanisms of Action	Efficacy of Disease Control	Evidence Level	Application Conditions/ Method	References
P. aeruginosa MML2212	Production of phenazine-1-carboxamide antibiotic	In-vitro antimicrobial activity vs. *Xoo*, comparable to/slightly exceeding the antibiotic rifamycin	Laboratory only (purified-compound assay)	Purified compound tested via laboratory antibacterial assays; no field disease percentage reported	Shanmugaiah et al., 2010
P. oryziphila strain 1257	LgrD-dependent nonribosomal peptides and other antimicrobial metabolites; motility/ colonization traits	Strong antagonism against *Xoo* pathovars; suppressed pathogen migration/ proliferation in rice tissue; field trial confirmed efficient biocontrol of bacterial leaf streak (BLS)	Laboratory (mutagenesis/ in vitro) + Greenhouse + Field; note: primarily tested against X. *oryzae* pv. *oryzicola* (leaf streak), not pv. *oryzae* (blight) specifically	Biosynthetic determinants identified via genome sequencing and mutagenesis; tested in vitro, in planta, greenhouse, and field trials	Yang et al., 2021

Note. Xoo = *Xanthomonas oryzae* pv. *oryzae*; CLSM = Confocal Laser Scanning Microscopy; HAQs = 4-Hydroxy-2-Alkylquinolines; BCA = Biological control agent; BLB = Bacterial leaf blight

**P. aeruginosa* is a recognised opportunistic human pathogen associated with hospital-acquired infections and is listed as a priority antimicrobial-resistance-threat organism. It is also naturally prevalent in agricultural soils, where surveyed strains frequently carry virulence and antibiotic-resistance determinants (Licea-Herrera et al., 2024). Accordingly, the efficacy data reported for BRp3 above should be read alongside this biosafety context: agricultural deployment of *P. aeruginosa*-based inoculants warrants strain-level risk screening and is subject to greater regulatory scrutiny than non-pathogenic *Pseudomonas* species (e.g., *P. fluorescens*) discussed elsewhere in this review

Biocontrol Potential of *Bacillus* spp. Against BLB Disease

Disease Control Efficacy

In sustainable agriculture, the application of *Bacillus*-based biocontrol agents provides a cost-effective and environmentally safe substitute for chemical pesticides (Shafi et al., 2017). *Bacillus* strains have been successful in suppressing *Xoo* and enhancing rice plant growth in a variety of experiments. *Bacillus* showed 50-58% inhibition against *Xoo* under greenhouse experiments and produced substantially increased plant biomass (El-Shakh et al., 2015). According to Putra and Giyanto (2014), seed treatment with *Bacillus* and *Actinomycetes* boosted seedling emergence and reduced the *Xoo* population up to 89%. Certain strains of *Bacillus* were found to secrete peptolytic enzymes that inhibited 70.9% of the growth of *Xoo* (Sousheel et al., 2021). Another study identified a strain of *B. subtilis* D13 that released volatile chemicals that markedly reduced *Xoo* colony diameter and motility (Xie et al., 2018). Additionally, *B. amyloliquefaciens* has shown antibacterial activity to *Xoo*, with the reported inhibition values of approximately 61% (Elshakh et al., 2016). Antibody potentials for *B. amyloliquefaciens* strain C2-1 against *Xoo* also showed good activity with zones of inhibition of approximately 7.4-8.9 mm over 7 days; with 14 days' action inhibition, it reduced infection by almost 60% in greenhouse testing (Meehae et al., 2023). Banluehan and Tunchai (2025) demonstrated that a number of *Bacillus* strains had high antibacterial activity, generating zones of inhibition of *Xoo* with diameters up to about 30 mm, in a dual-culture test. Reduction in disease symptoms or pathogen growth depends on strain and circumstance, with some strains showing in-vitro inhibition zones up to 30 mm and more than 60% of disease suppression in planta (Ooi et al., 2022). However, *Bacillus* strains decreased lesions between 54.7-85.3% under controlled conditions.

Disease severity has also been reduced by *Bacillus* species. It has demonstrated a significant decrease in disease severity in field trials (40-52%). For example, *Bacillus* KFP-5, KFP-7, and KFP-17 strains have been successful in decreasing BLB severity levels and stimulating rice growth (Rais et al., 2018). Endophytic *Bacillus* strains have shown the ability to reduce the severity of BLB disease (Ooi et al., 2022). The use of *B. subtilis* var. *amyloliquefaciens* (FZB 24) through different methods reduced BLB severity by 40% under glasshouse tests (Nagendran et al., 2013).

Besides these, there are promising results from Bacillus species in reducing BLB and enhancing crop productivity. Study reveals that *B. subtilis* bioformulations lower BLB incidence by 21% and possibly raise yield by as much as 50% (Wartono et al., 2015). Fresh weight and dry weight of the plant increased significantly due to the application of *B. subtilis* and *B. amyloliquefaciens*; fresh weight increased from 50.03% to 73.11%, and

dry weight increased from 64.11% to 86.65% (Ku Asmah & Sapak, 2020). Higher plant height, more tillers, longer roots, and higher chlorophyll content were all observed in treated rice plants (Ku Asmah & Sapak, 2020). Although certain yield increases were not always statistically significant, field application of antagonistic bacteria, such as *Bacillus* sp., increased grain production by up to 31% and straw yield by 10% above non-inoculated plots (Yasmin et al., 2016).

Mode of Actions of Bacillus spp.

BLB of rice is predominantly controlled by *Bacillus* species through a variety of mechanisms, such as host plant defense responses being triggered, competition for resources and habitats, generation of antimicrobial chemicals, and antagonism against *Xoo* (Figure 3).

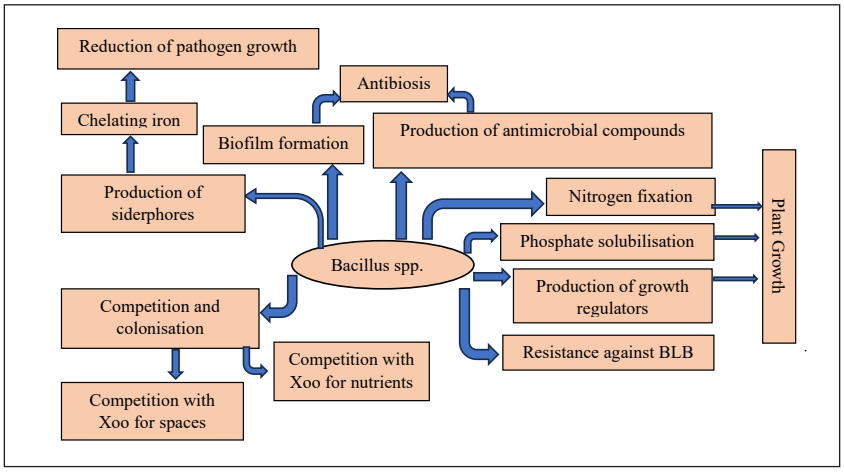

Figure 3. Illustration of mechanisms of action of *Bacillus* spp. for controlling bacterial leaf blight disease of rice

Key antimicrobial compounds produced by *Bacillus* spp., including toxins, siderophores, proteases, and antibiotics, contribute to preventing *Xoo* from growing and causing disease. For instance, the antimicrobial peptides bacitracin A and polymyxin B, which inhibit BLB pathogens, are produced by *B. subtilis* UiTMB1 (Ku Asmah & Sapak, 2020) (Table 5). It has been discovered that *Brevibacillus laterosporus* SN19-1 suppresses *Xoo* biofilm formation and enzymatic activity, leading to elevated reactive oxygen species and pathogen cellular damage (Su et al., 2024). *Bacillus* colonises in rice tissues, which then take up ecological niches and compete with *Xoo* for nutrients and space. As a result, the pathogen's capacity to cause infection is diminished. Research revealed that BLB was suppressed by high population densities of *Bacillus* sp. in rice leaf tissues (Ku Asmah & Sapak, 2020).

Table 5
Application of different effective strains/isolates of Bacillus *against bacterial leaf blight of rice*

Strains/ Isolates	Mechanisms of Action	Efficacy of Disease Control	Evidence Level	Application Conditions/ Method	References
Bacillus amyloliquefaciens A1, A2, A3 (endophytic isolates)	Carries genes for bacillomycin, fengycin, iturin, surfactin; culture filtrates inhibit growth/ biofilm and trigger plant defense/Induced Systemic Resistance	50.29-57.86% inhibition rate against *Xoo* in greenhouse; fresh weight increased 50.03-73.11%, dry weight increased 64.11-86.65%	Laboratory (in vitro antagonism/ fungal tests) Greenhouse trial	Selected endophytic strains applied in greenhouse/pot experiments; culture filtrates and live-cell applications	El-shakh et al., 2015
Bacillus velezensis Bv-303	Cell-free supernatant (CFS) antibiosis; metabolites stable to heat, acid, alkali, and ultraviolet (UV)	In-vitro growth inhibition 85.7-88.0%; in-planta leaf spray of cell-culture broth (CCB) caused 63% disease suppression.	Laboratory (in vitro)	Oxford-cup diffusion assay in vitro; *in vivo* leaf spray of cell-culture broth (CCB), cell-free supernatant (CFS), or cell suspension water (CSW); CCB is most effective	Liu et al., 2023
Bacillus subtilis UiTMB1	Antagonistic metabolites and plant growth promotion; induced systemic resistance	Glasshouse disease severity index 3.43 (treated) vs. 8.40 (untreated)	Laboratory (detached-leaf assay) Greenhouse trial	Laboratory bacterial assays and detached-leaf technique; glasshouse seed/foliar treatments prior to pathogen inoculation	Ku Asmah and Sapak, 2020
Bacillus BTH-22	Antibiosis (bacteriostatic)	Inhibition of *Xoo* growth: 3.9 cm on Nutrient Agar	Laboratory (in vitro only)	In-vitro nutrient agar antagonism assays (CRD, 6 replications)	Sayekti et al., 2025
Bacillus BTH-31a	Antibiosis (bacteriostatic)	Inhibition of *Xoo* growth: 9.3 cm on Nutrient Agar	Laboratory (in vitro)	In-vitro nutrient agar antagonism assays (CRD, 6 replications)	Sayekti et al., 2025
Bacillus Rh219 (rhizosphere isolate)	"Multiple plant growth-promoting and biocontrol traits assessed jointly, including siderophore production, phosphate solubilisation, IAA production, and antagonistic activity".	In-vitro inhibition zones 1-19 mm; the co-inoculation consortium reduced diseased leaf area by 80% vs. the infected control	Laboratory (in vitro screening), Field (consortium application tested in field trial),	Rhizosphere isolation, seed inoculation and co-inoculation in greenhouse/field trials; dual-culture and in-planta co-inoculation	Yasmin et al., 2016

By reducing the pathogen's access to iron, the iron-chelating substances like *Bacillus* inhibit pathogen growth. The formation of siderophores by a number of *Bacillus* strains contributes to their hostile actions. (Banluehan & Tunchai, 2025; Ooi et al., 2022). Nitrogen fixation, phosphate solubilisation, and the synthesis of growth regulators like indole-3-acetic acid (IAA) are among the characteristics of many *Bacillus* strains that promote plant growth and increase plant resilience against BLB (Ooi et al., 2022; Su et al., 2024).

Bacillus species induce plant innate immunity of rice plants, particularly induced systemic resistance (ISR) through jasmonic acid and ethylene signaling pathways, making them more resistant to BLB infection. To illustrate these processes, it has been shown in studies that *Bacillus* treatments enhance resistance and reduce disease severity before pathogen challenge (Shrestha et al., 2016). The antagonism against *Xoo* is mainly due to non-ribosomally produced metabolites, specifically difficidin and bacilysin. Gene knockout studies in *B. amyloliquefaciens* FZB42 showed that these two compounds are the most active agents suppressing *Xoo*. They damage the cell wall and membrane structure of the pathogen and downregulate genes involved in virulence, cell division and cell-wall synthesis (Wu et al., 2015).

INTEGRATED DISEASE MANAGEMENT PROGRAM

The successful deployment of *Trichoderma* spp., *Pseudomonas* spp. and *Bacillus* spp. for managing BLB of rice depends on strategic integration into the existing crop management system. Rather than relying on a single application method, an integrated approach should be adopted to maximise disease suppression and plant health benefits. The following methods should be followed for successful integration:

Seed and Seedling Treatment

Seed treatment is the most effective and widely used method for the integration of BCAs. Soaking seeds in BCA suspensions before sowing, for example, *T. harzianum* at 1×10^7 spores/mL and *P. fluorescens* at 1×10^8 CFU/mL, was recommended by Sai Teja et al. (2026). However, these concentrations should not be considered universal recommendations, as they may vary depending on the strain, formulation, application method, and environmental conditions. Research on basmati rice demonstrated that seed treatment with a microbial consortium (*T. harzianum* + *T. asperellum* + *P. fluorescens*) achieved maximum inhibition of BLB incidence (48.56%), disease intensity (52.42%), and lesion length (45.41%) compared to untreated controls (Sai Teja et al., 2026).

Another method is soaking 30-day-old seedlings for 20 minutes in BCA suspensions before transplanting (Sai Teja et al., 2026). The recommended concentrations are *Trichoderma* spp. at 1×10^7 spores/mL; *Pseudomonas* at 1×10^8 CFU/mL; *Bacillus* spp. at

comparable densities (Sai Teja et al., 2026). In case of foliar application, it is recommended to spray BCA suspensions at 0.1% concentration (1×10^7-10^8 CFU/mL or spores/mL) using knapsack sprayers (Sai Teja et al., 2026). In one field trial, foliar application of a BCA consortium formulation showed disease control efficacy comparable to the chemical standard (streptocycline + copper oxychloride) (Sai Teja et al., 2026). This result is based on a single study and should therefore be considered preliminary evidence rather than a generalised conclusion. Broader validation across field sites, seasons, and rice-growing regions is needed before foliar application of BCA consortia can be considered a reliable and comparable alternative to chemical control.

BCA Consortium Formulations

Single-strain inoculants often show inconsistent performance due to environmental variability, largely because an introduced strain must establish itself within a competitive, heterogeneous soil and phyllosphere microbiome, and can be readily outcompeted, suppressed, or inactivated by abiotic stress once alone (Negi et al., 2024). Consortium approaches address this limitation not simply by "adding more agents," but through specific ecological mechanisms that single strains cannot achieve on their own:

- **Niche Complementarity and Functional Redundancy:** Different BCA species or genera occupy different ecological niches (root surface versus internal tissue versus phyllosphere) and are dependent on different resources, and therefore group members can share more of the plant-associated habitat jointly and not compete with each other (Maciag et al., 2023). Functional redundancy (that is, multiple members of the consortium are capable of performing a particular biocontrol task such as antibiosis or ISR induction) provides some buffer; if one strain does not perform well in a given environment and another sub-group can take care of it, the overall anti-pathogen effect is maintained in a variety of field conditions (Maciag et al., 2023; Negi et al., 2024).
- **Metabolic Cooperation:** Consortium members can perform cross-feeding and complementary metabolic activity, where the metabolic byproducts or niche-modifying activity of one strain creates more favorable conditions for another, leading to increased defense-related outputs than either organism can produce independently. This is consistent with studies showing that miniature microbial consortia enhance plant defense signaling cascades and activate genes that encode multiple metabolic pathways (the phenylpropanoid pathway, phenylalanine ammonia-lyase pathway, total phenolics, proline accumulation, and pathogenesis-related proteins) more effectively than single-strain inoculation (Negi et al., 2024).
- **Improved Rhizosphere and Phyllosphere Colonisation:** Because consortium members can help establish each other (e.g., one strain will change local pH, exudate content, or microbiota competition before a second strain arrives) multi-strain products

generally result in much more consistent and extensive root or leaf colonisation than single strains and thus should be the basis for long-term biocontrol activities as opposed to one-off antagonistic events (Maciag et al., 2023).

- **Enhanced Resilience to Environmental Fluctuation:** Consortium members frequently differ in their optimal temperature, moisture, and pH ranges, a multi-strain formulation is more likely to retain at least partial activity across the range of conditions encountered in the field than a single strain optimised for one narrow set of conditions. This approach directly addresses the environmental-variability problem that motivates consortium use in the first place (Negi et al., 2024). Furthermore, combined mechanisms rather than single efficacy effects could explain why consortium approaches are reported to provide synergistic growth promotion effects more than single-strain treatments (Hasan Naqvi et al., 2025; Sai Teja et al., 2026).

LIMITATIONS AND PRACTICAL CHALLENGES OF BIOLOGICAL CONTROL

Although *Trichoderma, Pseudomonas* and *Bacillus* spp. have shown great potential in BLB management as discussed in detail in this review, their application as biological control agents is always limited. Their application limitations and challenges on ecological, technical and regulatory in nature, are discussed below:

Inconsistent Field Performance

One of the most fundamental limitations of biological control agents (BCAs) is the substantial disparity between their performance under laboratory or greenhouse conditions and their effectiveness in the field. Although in-vitro studies frequently demonstrate strong antagonistic activity, for example, *P. fluorescens* has been reported to inhibit *Xoo* by 59.84%, while microbial consortia achieved 68.56% inhibition under laboratory conditions (Sharma & Bora, 2022). Such efficacy does not always translate into consistent disease suppression under field conditions. For instance, *P. fluorescens* strain PDY7 reduced BLB severity by 58.83% in greenhouse experiments but achieved only 51.88% suppression under field conditions (Velusamy et al., 2013). This difference highlights the complexity of field environments, where BCA's performance is influenced by numerous interacting biotic and abiotic factors, including temperature, humidity, rainfall, soil properties, microbial competition, host physiology, and pathogen population dynamics. Inconsistent field performance has been consistently identified as a major challenge in the application of beneficial microorganisms, including *Trichoderma*, *Bacillus*, and *Pseudomonas* spp. (Gusly et al., 2026; Qadeer et al., 2025; Raju & Punamalai, 2025). The challenge is further complicated by the unpredictable nature of interactions among different BCA strains. For example, combinations of *T. harzianum* and *P. fluorescens* have been reported to exhibit both

antagonistic and synergistic interactions against BLB, indicating that combining multiple BCAs does not necessarily result in improved disease control (Jambhulkar et al., 2018). Therefore, the effectiveness of BCA-based disease management depends not simply on the presence of highly antagonistic microorganisms, but on their ability to survive, establish, multiply, and maintain effective interactions with the host plant and pathogen under dynamic field conditions (Senapati et al., 2022). Improving field consistency will consequently require greater emphasis on strain selection, compatibility testing, formulation stability, application timing, environmental adaptation, and optimisation of delivery systems.

Formulation Stability and Shelf Life

Biocontrol efficacy depends heavily on formulation type and storage conditions. *Trichoderma*-based commercial formulations, for instance, often have shelf lives of only about 3-4 months under standard storage, with propagule viability declining due to poor carrier selection, moisture exposure, or UV degradation (Otithi et al., 2026). Comparable viability and stability challenges apply to *Bacillus* and *Pseudomonas* formulations, where maintaining cell viability during storage and after field application remains a recognised obstacle for commercialisation (Schisler et al., 2004).

Environmental Dependency

BCA performance is highly dependent on local environmental and edaphic conditions rather than a fixed strain property. *Pseudomonas*-mediated lesion-length reduction, for example, has been reported to range from 15-74% in normal soil but to 82% in saline soil (Rangarajan et al., 2003), suggesting that the same strain can work very differently depending on soil chemistry. More generally, temperature, moisture, pH and soil type have a direct impact on BCA establishment and activity (Iqbal, 2026), meaning that a strain validated in one set of conditions may not perform as well elsewhere without local validation.

Interactions with Indigenous Microbiota

Introduced BCAs are not on their own and can be actively inhibited by the native soil and plant microbial community. Colonisation and persistence are known bottlenecks, with many inoculants failing to keep up with native microbiota, being preyed on by protists, or the timing of the initial application (Iqbal, 2026). These interactions can go beyond competition: in one instance, the local Stenotrophomonas maltophilia bacterium has been found to destroy *Bacillus*-produced lipopeptide antibiotics (iturin, fengycin, surfactin), which can negatively impact the biocontrol activity of the introduced BCA after colonisation (Iqbal, 2026). This shows that colonisation alone is not always sufficient to sustain the presence of the BCA, since the resident microbes can react with the active compounds of the BCA after colonisation has been successful.

Strain Compatibility in Consortium Formulations

As discussed earlier in this review on consortium approaches, compatibility between BCA species is not guaranteed and is usually medium and condition-dependent. *Bacillus velezensis* has been shown to completely inhibit *Trichoderma virens* growth in nutrient-rich synthetic media, and the same two strains are fully compatible under conditions similar to the rhizosphere (Izquierdo-García et al., 2020). Consortium design cannot safely assume that combining multiple effective single-strain BCAs will produce an effective combined formulation without empirical compatibility screening

Regulatory Barriers, Biosafety and Commercialisation

Registration of microbial biopesticides is slower and more uncertain than chemical pesticides because live organisms require more safety data. This is especially true for *Pseudomonas aeruginosa* (e.g., strain BRp3, an opportunistic human pathogen found in agricultural soils, which requires strain-level biosafety testing before being used in the field (Licea-Herrera et al., 2024), and the regulatory scrutiny and field performance remain an issue for *Pseudomonas*-based products (Giovanardi et al., 2025). By contrast, *Trichoderma* is the most established and commercially known species (registered since the late 1980s and now the most widely commercialised fungal BCA in the world (Otithi et al., 2026), and *Bacillus* and *Pseudomonas* are both still lacking in BLB-specific applications. No such product is the most developed for BLB control by a commercial product registered here so far, and the regulatory and biosafety pathways are still underdeveloped in comparison to the research base.

Sources of Variation in Disease Control Efficacy

The wide range of disease reduction percentages reported in studies in this review (e.g. *Bacillus* lesion reduction covering 54.7-85.3%, *Pseudomonas* lesion reduction covering 15-82%) is not noise, and these are specific sources of variation which the manuscript should talk about, rather than presenting the numbers as a simple list:

1. **Pathogen Strain/Race Variability-** *Xoo* itself is genetically diverse across regions, and isolates differ in virulence and in resistance genes they can overcome, meaning a BCA's apparent success is partly a function of the *Xoo* population it was tested against, not the BCA itself (Adedibu et al., 2024)
2. **Rice Species/Varieties/Genotypes-** Host genetic background affects both baseline susceptibility and how strongly ISR-related defense pathways respond to microbial priming; consortium efficacy has been shown to differ between rice varieties tested under otherwise identical conditions (e.g., different outcomes between the Inpari 33 and Ciherang varieties reported by Teja et al., 2025).

3. **Environmental and Edaphic Conditions-** Soil salinity, pH, and moisture significantly change BCA performance, as evidenced by *Pseudomonas* lesion length loss from 15-74% in normal soil to 82% in saline soil (Rangarajan et al., 2003). The organism is more robust and performs better under one specific stress condition, rather than uniformly across all soils.
4. **Application Method and Timing-** Seed priming, seedling dip, soil application, and foliar spray are not interchangeable, and the same *Trichoderma* isolate may show a significantly different lesion-length reduction depending on the delivery method e.g., 75% via seed priming versus other values from other methods (Islam et al., 2023) and the effects are generally stronger when applied prior to infection rather than after infection is established.
5. **Dose and Formulation-** As mentioned in the field-efficacy discussion, higher application rates (e.g., T. *harzianum* at 4-8 g/L) dominate over lower rates (Khan & Sinha, 2007), and formulation quality influences the number of viable propagules actually delivered to the target tissue.
6. **Strain-Level Metabolic Variability-** Even within a species, individual strains vary in which antimicrobial compounds, siderophores, or enzymes they produce and in what quantity, which is why single-species efficacy ranges are often as wide as or wider than the ranges between genera (Ngalimat et al., 2021).

FUTURE PROSPECTIVES

The future of BLB disease management is moving beyond single-strain applications and to more sophisticated integrated approaches that incorporate the advances in nanotechnology, plant pathology, breeding, biotechnology, microbial ecology and precision agriculture. *Trichoderma* spp*., Bacillus* spp*.,* and *Pseudomonas* spp. have demonstrated measurable efficacy for BLB disease control under field conditions, but previous research indicates the need for new directions and technology to further progress in field application that would revolutionise their use in the future (Hasan Naqvi et al., 2025; Teja et al., 2025). A big way forward is from single-strain inoculants to synthetic microbial consortia (consortia of microbial communities of mutually active biocontrol agents). This approach remains largely experimental, but has shown early promise in addressing some of the field-performance limitations associated with existing single-strain BCAs (Kayla et al., 2026).

Currently, researchers are working to create "core microbiota" (the minimum set of microbes essential for plant health) and engineer them into stable synthetic communities for commercial use (Hasan Naqvi et al., 2025) . This is a transition from biological control to microbiome-based crop protection. The viability and shelf-life of BCAs in field conditions are still key issues. Two innovative approaches are being developed to address this: i) nanoencapsulation of microbial probiotics is a way to enhance resilience

to environmental conditions and preserve long-term viability (Hasan Naqvi et al., 2025). This prevents BCAs from desiccation and UV radiation, and competitive displacement by native soil microbes. However, several challenges continue to limit the practical application of nanoencapsulated BCAs, including high production costs, difficulties in scale-up, variable release kinetics, complex regulatory approval pathways, and uncertainties regarding the environmental persistence, biosafety, and potential non-target effects of nanomaterials. These limitations suggest that nanoencapsulated BCAs should still be regarded as an emerging technology rather than a field-ready approach for large-scale agricultural application (Narmatha et al., 2026). ii) genetic engineering approaches, these strategies are being explored at two levels: individual-strain engineering and whole-microbiome engineering, with the former currently being more established. At the individual-strain level, genetic modification of selected *Trichoderma*, *Bacillus* and *Pseudomonas* strains aims to enhance the production of antimicrobial compounds, improve root colonisation, and increase tolerance to environmental stresses (Ke et al., 2021). In contrast, engineering entire core microbial communities rather than individual strains remains largely experimental and conceptual, with substantial technical and ecological challenges yet to be resolved (Ke et al., 2021).

Recent studies have demonstrated the potential of engineered microorganisms for disease management. For example, the transgenic rice line UvScd1 was reported to upregulate defense-related genes and induce lesion-mimic phenotypes without compromising yield. In field experiments, *B. subtilis* engineered to produce the fungal effector UvScd1 also demonstrated control efficacy against several major rice diseases, including BLB, following foliar application (Wang et al., 2026). Nevertheless, these findings should not be interpreted as evidence that UvScd1-producing *B. subtilis* is immediately suitable for large-scale field deployment. The application of genetically modified biocontrol agents (GM-BCAs) in agriculture remains subject to significant ecological, biosafety, and regulatory concerns. The environmental release of self-replicating GM-BCAs could potentially facilitate horizontal gene transfer to indigenous microorganisms and alter the composition and functioning of soil and phyllosphere microbial communities, with potentially unpredictable long-term consequences beyond controlled experimental conditions (Lerner et al., 2026). Moreover, containing engineered, self-replicating organisms is inherently more challenging than controlling non-living chemical inputs. Although various biocontainment strategies are being investigated, including approaches designed to restrict the survival or persistence of engineered microorganisms outside their intended application sites, these technologies remain under development and have yet to be established as universally reliable and field-proven solutions (Lerner et al., 2026). Therefore, further research is required to establish the efficacy, environmental safety, genetic stability, containment, and regulatory feasibility of GM-BCAs before their widespread agricultural application can be considered.

Moreover, 16S rRNA amplicon sequencing and metagenomic analysis are being used to identify microbial communities associated with healthy rice plants and disease-suppressive soils (Hasan Naqvi et al., 2025). This reveals which native strains are most effective in specific agroecological contexts. Healthy rice rhizospheres are richer in microbial diversity and enriched in antibiotic production, siderophore biosynthesis and nutrient cycling genes (Hasan Naqvi et al., 2025). The number of microbial species present does not necessarily determine the effectiveness of disease suppression. Rather, the functional traits and activities of the microbial community may be more important. A study comparing disease-suppressive and disease-conducive soils demonstrated that suppressive soils did not necessarily contain the same bacterial species; instead, they shared similar functional genes, particularly those associated with siderophore production and other pathogen-suppressive activities. This finding suggests that disease suppression depends less on microbial diversity per se and more on the presence of microorganisms with specific beneficial functions. Thus, a rice field containing microorganisms capable of producing antibiotics, chelating iron, and cycling essential nutrients may provide greater protection against pathogens, not simply because it harbours a greater number of microbial species, but because the microbial community possesses the appropriate functional capabilities (Tracanna et al., 2021). Furthermore, native BCAs have been reported to exhibit greater effectiveness than non-native strains in terms of environmental adaptation, host colonisation, and competition for nutrients and ecological niches (Hasan Naqvi et al., 2025). This advantage may be attributed to their better adaptation to local environmental conditions and their ability to compete effectively for available resources. However, contrasting evidence indicates that introduced or non-native strains can perform equally well, or even better, when formulation and application strategies are properly optimised. For example, field trials in maize demonstrated that several *Bacillus* strains originating from outside the target location provided strong and consistent disease suppression. Their success appeared to depend more on formulation quality, application method, compatibility with the host plant, and adaptation to the prevailing environmental conditions than on their geographical origin (Arteaga-Ojeda et al., 2026). Similarly, research on *Trichoderma* has shown that the performance of the same strain can vary substantially depending on formulation type, with fresh formulations outperforming dry formulations in some cases. These findings highlight that formulation quality and delivery technology may be more critical determinants of BCA efficacy than whether a strain is native or non-native (Riaz et al., 2026). Combining genomics, transcriptomics, proteomics, and metabolomics will allow for the selection of strains with proven functional traits and decrease the trial-and-error approach in BCA development (Kayla et al., 2026).

Genetic resistance and biocontrol combination is a major future direction. CRISPR/Cas9 technology has already been used to develop BLB-resistant rice by editing susceptibility genes at all levels. At the individual level, editing the promoter regions of SWEET14 and

Xa13 (a SWEET11 allele) has been shown to confer resistance by preventing pathogen effectors from activating these genes (Yu et al., 2021). More broadly, Oliva et al. (2019) edited the promoters of all three clade-III susceptibility genes SWEET11, SWEET13, and SWEET14 simultaneously based on TALE-binding site analysis of 63 *Xoo* strains, and produced broad-spectrum resistant rice lines (Kitaake, IR64, and Ciherang-Sub1) without the yield degradation associated with resistance breeding. A similar multiplexed-editing approach was shown to successfully develop resistant rice lines against 133 Xoo strains (Xu et al., 2019). In the context of the SWEET gene family, recessive resistance gene Xa5 is a different animal (i.e., it is involved with a basal transcription factor subunit OsTFIIAγ5) that Xoo's TALEs need to activate susceptibility genes, not the single susceptibility gene in question. TALEN-based editing of the corresponding dominant TFIIAγ5 allele significantly reduced Xoo susceptibility in many mutant lines, making this gene a better target for gene editing than single-SWEET gene editing (Han et al., 2020). More advanced breeding is in process to produce transgene-free and edited rice lines through multiplexed selection, making it easier to get regulatory approval and public acceptance (Yu et al., 2021). Genetically resistant varieties can serve as the foundation for integrated disease management (IDM). BCAs can be used as complementary protection against pathogens that are able to overcome certain resistant genes. This integrated approach reduces selection pressure for resistance to either strategy alone.

CONCLUSION

BLB is a major global threat to rice production, as severe yield losses can undermine food security, particularly in major rice-producing countries and regions. The emergence of pathogen strains resistant to chemical pesticides, the adverse environmental impacts associated with excessive pesticide use, and the genetic limitations encountered in developing durable disease-resistant rice varieties have highlighted the urgent need for sustainable and effective alternatives. Based on empirical evidence, biological control can increase rice yield by up to 50%, reduce disease severity by more than 80%, shorten lesion length up to 87.5%, and suppress the growth of *Xoo* up to 80%. The efficacy of these BCAs can be further enhanced when they are applied in combination, resulting in synergistic effects and greater disease suppression compared with their individual application. The integration of *Trichoderma*, *Pseudomonas* and *Bacillus* spp. into integrated disease management programmes represents an environmentally friendly, sustainable, and promising approach for BLB management. Their adaptability, multifunctionality, and diverse modes of action can contribute to overall crop health and productivity while reducing reliance on chemical pesticides. To facilitate their wider adoption by farmers and integration into national rice disease management programmes, further research is needed to optimise formulation technologies, delivery systems and field-scale application strategies.

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CONSENT FOR PUBLICATION

The article contains no such material that may be unlawful, defamatory, or that would, if published, in any way whatsoever, violate the terms and conditions as laid down in the agreement.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest related to this article.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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Scaling Up Moringa Leaf Beverage Filtration: A Comparative Evaluation of Traditional, Laboratory-Scale and Pilot-Scale Wire Mesh Prototype System for Clarity and Mineral Retention

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ABSTRACT

Moringa oleifera, commonly called the "miracle tree," is valued in the functional food sector for its high mineral content, which includes calcium, iron, potassium, magnesium and sulphur, but the way a Moringa beverage is processed, in particular its filtration process, is a strong influence on both nutrient retention and visual clarity, a factor that is directly relevant to consumer acceptance. This study therefore compared three filtration pathways of traditional to pilot-scale production, cloth filtration, laboratory-scale filtration with Whatman No. 1 filter paper and a pilot-scale wire mesh system in terms of clarity and mineral retention. Mineral content was quantified using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) at an accredited laboratory. Clarity was visually evaluated as a relevant indicator for consumers. The three products were very different in colour, from dark brown (traditional method) to light yellow (laboratory-scale method), and the pilot scale sample had the highest single measurement values for all five minerals, which were significantly higher than the traditional and laboratory-scale samples by 71 to 200% and 100 to 278% respectively across the mineral panel. Since these values are obtained from a single measurement per sample, without replication or statistical testing, they are preliminary findings. However, the pilot-scale wire-mesh system's combination of greater

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clarity and mineral retention suggests it is a promising, scalable option for SME-level Moringa beverage production.

Keywords: Beverage production, filtration methods, functional food, mineral contents, nutrition support, Moringa leaf, visual clarity

INTRODUCTION

Background

Moringa oleifera is a fast-growing, drought-resistant tree native to the Indian subcontinent and has been widely cultivated in tropical and subtropical areas (Pareek et al., 2023; Su & Chen, 2020). It is often referred to as the 'miracle tree' for its rich phytochemical and nutritional structure. Moringa leaves have high protein, vitamins, antioxidants and minerals such as calcium, potassium, magnesium, sulphur and iron (Hodas et al., 2021; Moyo et al., 2011; Masitlha et al., 2024; Pareek et al., 2023), and a variety of phytochemicals, flavonoids, phenolic compounds, glucosinolates, saponins and carotenoids that contribute to a high antioxidant, anti-inflammatory and other health benefits (Al-Ghanayem et al., 2022; Singh et al., 2020). Moringa leaves are a valuable functional food ingredient, as they are rich in nutrients and are used in health drinks and are generally used as their nutritional value is very high (Islam et al., 2021; Peñalver et al., 2022; Wickramasinghe et al., 2020; Rahmatu et al., 2025). Figure 1 presents a visual representation of *Moringa oleifera* leaves.



Figure 1. Moringa oleifera leaves (Anzano et al., 2021)

In many communities, Moringa leaf extracts are added to beverages and teas to deliver these nutrients: fresh leaves are pulled from stems, mixed with water and strained to get a green infusion (Odey et al., 2012). However, the resulting crude beverage is darkly coloured and viscous due to fine leaf particulates, and this heavy colour and texture can reduce consumer acceptance (Abd El-Rahim et al., 2017). Some dissolved minerals are also associated with these suspended solids, and the filtration step is crucial: it has to remove unwanted particles while preserving the soluble nutrients (Daneluz et al., 2023).

Literature Review

Filtration is a critical procedure in beverage production. It removes fine particulates and suspended solids to provide a clearer, coherent product while preserving soluble nutrients

(Cescon & Jiang, 2020; Sarbatly et al., 2023). Traditional techniques like straining or coarse cloth filtration are conventional for small-scale or household Moringa beverage production, but they generally produce dark, viscous, turbid products that are not shelf-stable. This gap between traditional techniques and product quality that consumers and processors demand has resulted in filtration becoming a leading research and development area in the field of product quality management (Aziz et al., 2024).

Figure 2 shows a typical membrane filtration system in which a feed stream of suspended and dissolved material moves towards the membrane, and the filter is the primary selective barrier controlling which parts pass through it. Particles larger than the membrane pores are kept at the surface and accumulate, forming a layer of accumulated solids that can affect filtration efficiency in the long run, while dissolved substances and the liquid component of the feed pass through as permeate (or filtrate) and a support layer below the membrane provides structural strength to support the feed flow without affecting flow (Daneluz et al., 2023; Loff, 2001).

The same principle of size-selective particle retention is present in all three filtration media that we have studied in this study, but with very different levels of process control.

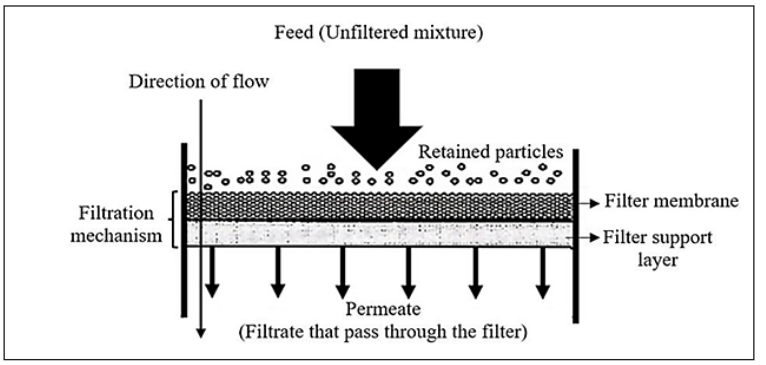

Figure 2. Schematic diagram for filtration
*Source: Nathan et al. (2008)

In *Moringa oleifera* beverage production, there are two important quality parameters to consider for clarity: clarity as measured on visual observation and mineral retention, which is important to use for calcium, potassium, magnesium, sulphur and iron, which are some of the most abundant minerals in Moringa leaves (Pareek et al., 2023). Clarity is the product of suspended fine particles and colloidal matter that are released during the extraction process and is a key factor in terms of visual appearance, mouthfeel and the acceptability of Moringa leaf beverages; poor clarity constrains commercial viability, so effective filtration must reduce turbidity while minimising the loss of soluble bioactive constituents (Columbia, 2020). Yet filtration is a trade-off for this same purpose, as minerals

are either retained in the medium, either through the presence of the suspended particles or adsorption into the filter matrix, and it is important to balance the effective removal of the particles through the filter with the mineral retention in the process (Yerena-Prieto et al., 2022).

In addition to the standard straining media, membrane-based clarification technologies such as microfiltration and ultrafiltration have been widely reported in juice and plant extract clarification, which results in fine, controlled pore sizes and high clarity (Aziz et al., 2024; Sarbatly et al., 2023; Tiwari & Saha, 2022). Tiwari and Saha (2022) applied hollow fibre microfiltration to *Moringa oleifera* leaf juice under optimal transmembrane pressure and pore size and observed significant clarity and turbidity reduction, while Daneluz et al. (2023) also demonstrated the use of microfiltration in kombucha clarification.

Despite the progress in other beverage systems, the transformation of *Moringa oleifera* leaf extracts into commercially viable beverages is significantly hampered by conventional processing methods (Hernández-Fuentes et al., 2025). Traditional preparation, which involves manual straining or coarse cloth filtration analogous to domestic tea infusion, is still prevalent in many communities and small-scale production (Mensah et al., 2025), owing to the largely unstandardised nature of such processes and lack of established operational parameters (Marobhe, 2021). These manual techniques are labour-intensive and time-consuming, and they often require repeated manual agitation, pressing, and multiple stages of cloth filtration to produce even a minimum of clarity.

Though advanced filtration technologies have shown promising capabilities on other beverage systems, systematic multi-scale comparative studies are limited, and few studies have compared traditional manual methods with optimal laboratory-scale and pilot-scale (prototype) filtration systems for Moringa leaf beverages. Recent studies on *Moringa oleifera* leaf-based functional beverages have mostly focused on extraction techniques, sensory acceptability, and basic clarification methods rather than systematic comparisons of filtration methods. Hernández-Fuentes et al. (2025) prepared Moringa leaf infusions using traditional hot water extraction followed by simple gauze filtration and concentration and demonstrated their potential therapeutic benefits for metabolic disorders.

Mensah et al. (2025) optimised a soymilk-Moringa leaf extract beverage through sensory-driven formulation and blending and percale cloth filtration to achieve acceptable sensory properties at specific blending ratios. Tiwari and Saha's (2022) hollow-fibre microfiltration method is still the most methodologically sophisticated filtration method used for Moringa leaf juice.

Combined, these studies reflect growing interest in Moringa-based beverages, but each of them focuses on small-scale extraction and basic straining or on single lab-scale clarification; none provides the systematic, multi-scale comparison across traditional,

laboratory and pilot-scale methods that this study undertakes. This is a crucial gap for efficient, scalable and reproducible production of clear and nutrient-rich Moringa beverages that are in line with consumer expectations of visual appeal, nutritional consistency and functional effectiveness today.

MATERIALS AND METHODS

Materials

All the experiments used dried Moringa leaves as the primary plant substrate and drinking water as the solvent to provide the same process in all filtration settings. To better reflect the production practices of the papers mentioned, we used three experimental setups: traditional, laboratory-scale, and pilot-scale. In the traditional setup, we used basic household utensils such as a boiling vessel and a cotton cloth filter and simulated small and medium enterprise (SME) production. In the laboratory-scale setup, we used controlled scientific equipment (3-neck round-bottom flask and heating mantle, mercury thermometer, Allihn condenser, vacuum pump and Whatman No. 1 filter paper of 11 μm pore size). The pilot-scale method used a custom-designed filtration tank with a wire mesh filter made of food-grade stainless steel to maintain sanitary processing and mechanical stability during large-volume filtration.

These three methods differ in filtration medium, processing scale, and process control, by design. This is because the traditional, laboratory, and pilot-scale Moringa beverage production is done in practice; it is not a controlled, single-variable filtration comparison. The different methods should therefore be considered as a combination of extraction and filtration at each scale, and not as a filtration medium only at each scale. A controlled single-variable follow-up study where the filtration medium is varied independently of the extraction should be considered and is discussed in the following section.

Methods

Optimum Operation for Conducting Experiment Procedures

The optimisation of extraction and filtration parameters was guided by previous studies (Fombang & Saa, 2016; Kamarudin et al., 2020; Pollini et al., 2020). The most important process parameters were temperature, leaf mass (g), water volume (mL), extraction time (min) and solid-to-liquid ratio, all of which affect bioactive compounds, nutrient solubility and clarity of the final filtration. Table 1 summarises the range of optimal operational parameters in this literature.

Table 1
Optimum operating conditions in processing Moringa leaves

Authors	Kamarudin et al. (2020)	Fombang and Saa (2016)	Pollini et al. (2020)
Filter Paper	Whatman No. 1	-	-
Temperature (°C)	82	97	60
Weight of Dried Leaves (g)	1	1	1
Volume of Water (mL)	30	20	60
Extraction Time (min)	48	35	60
Solid-liquid Ratio	1:30	1:20	1:60

Kamarudin et al. (2020) applied Response Surface Methodology (RSM) to optimise heat-assisted extraction of phenolic compounds from *Moringa oleifera* leaves, which was achieved at a temperature of 82°C, 48 minutes of extraction time and a solid-liquid ratio of 1:30 (1 g of dried sample to 30 mL of water), followed by filtration with Whatman No. 1 paper. These parameters reflect a thermally driven extraction process, where high temperatures increase solvent diffusivity and phenolic solubility, while a controlled extraction time reduces degradation of heat-sensitive compounds.

Under this parameter space, Kamarudin et al. (2020) reported that total phenolic content (TPC) and antioxidant capacity were maximised, indicating good liberation of polyphenolics such as flavonoids and phenolic acids. In addition to that, the feedback analysis of the nutrients retained by the plant showed that although some heat-sensitive micronutrients, such as ascorbic acid, may be degraded in part, antioxidant activity remains high and is even strengthened in the presence of phenolic compounds. Fombang and Saa (2016) have optimised phenolic extraction of *Moringa oleifera* leaf powder for functional tea production using RSM and found that brewing at around 97 °C, extracting for 35 minutes, using a solid-to-liquid ratio of 1:20 (1 g sample in 20 mL water), and filtering through Whatman No. 1 paper. At near-boiling temperatures, they can exploit the heat energy to rapidly disrupt and remove phenolics. But this is at the expense of heat-sensitive nutrients in the plant, as vitamin C and other glycosylated flavonoids are hydrolysed and oxidised at temperatures near 100 °C in oxygenated aqueous environments. The authors found that antioxidant activity is high, and some of these compounds are degraded to less stable aglycone forms or into secondary antioxidant species.

In contrast, Pollini et al. (2020) examined ultrasound-assisted extraction (UAE) of antioxidant compounds from *Moringa oleifera* leaves at 60 °C, with a 60-minute extraction time and a high solid-to-liquid ratio of 1:60 (1 g sample in 60 mL water), and then filtered through Whatman No. 1 filter paper. The bulk temperature of the UAE process is low enough to reduce thermal degradation by about half in comparison to conventional heat-assisted extraction, and yield of phenolics, and the high extraction yield at high temperatures

of the oxidant compounds. The method developed by Kamarudin et al. (2020) was chosen for our work as it is able to hold a good balance between high nutrient extraction and nutrient quality preservation: the temperature of 82 °C and the extraction time of 48 minutes should have enough time to release the nutrients without causing significant degradation. Whatman No. 1 filter paper (11 μm) was used for laboratory-scale filtration due to its good retention efficiency and filtration rate.

Experimental Study

The extraction parameters developed by Kamarudin et al. (2020) were tested in the laboratory and on a pilot scale to ensure their ability to be applied to larger production. At laboratory scale, 3 g of dried *Moringa oleifera* leaves were extracted with 90 mL of water and at pilot scale, 1000 g of leaves were extracted with 30,000 mL of water, keeping the same 1:30 solid-liquid ratio. Temperature (82°C) and extraction time (48 minutes) were also kept constant across all scales to ensure a good comparison of filtration efficiency and to see whether the method could be applied on a larger scale without loss of efficiency or quality. The deviation from the traditional method, and the confound it generates, is discussed in detail in the following subsection. Three samples were collected in total, representing the traditional, laboratory-scale and pilot-scale production methods, with subsequent analysis focused on the nutrient content of the resulting beverages. Each sample represents a single production run per method ($n = 1$), with no biological or analytical replicates; therefore, the reported mineral values reflect single certified laboratory measurements rather than replicated trials. This is a stated limitation of the present pilot study: no standard deviation, error bars, or inferential statistical tests (e.g., ANOVA) could be calculated, and the reported percentage differences should be read as descriptive, preliminary observations rather than statistically validated findings.

Traditional Method

Given its widespread household-level practice, owing to its simplicity and minimal operational requirements, this method was replicated in the present study to represent standard traditional practice. The process typically involves boiling herb material in approximately 230 mL of water at a temperature of around 100 °C. This boiling temperature differs from the 82 °C employed for the laboratory- and pilot-scale methods, constituting a further confounding variable, in addition to the differing extraction ratio, that may independently influence extraction yield and filtrate viscosity; this is acknowledged as a limitation in interpreting the comparison between the traditional method and the other scales.

This method closely resembles the common home preparation techniques of tea infusion (e.g., boiling water is poured over the sample and then let to steep before basic filtration, using simple tools such as sieves). This method certainly mimics the real-world consumption process, and it is very practical, but it is not able to control the temperature and extraction time, so for a scientific or industrial scale, it is not so realistic. When applied to large-scale or industrial production, this method is also labour-intensive, and in the case of filtration work, we have to do the entire process manually (often several steps). With much more labour needed, production is at least fast. Thus, the traditional method is suitable for small-scale production and not for mass production. Figure 3 illustrates the traditional method applied to filter Moringa leaves under SME conditions.

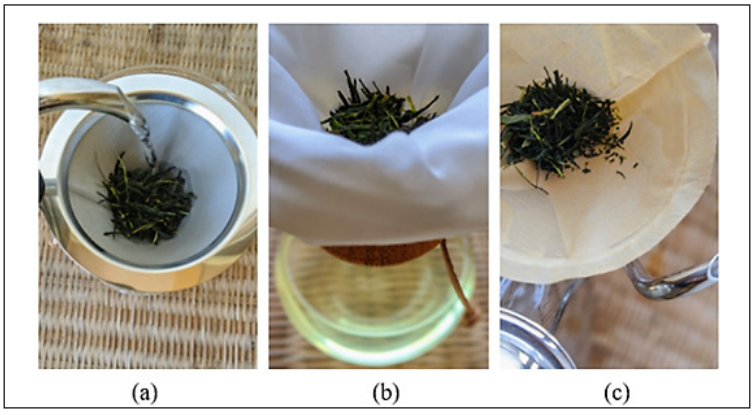

Figure 3. Traditional filtration method: (a) Pouring hot water through a cloth filter; (b) Dried leaves filtered with a cloth filter, and (c) Leaves remain in a cloth filter

Laboratory-Scale Method

The laboratory-scale method was used to test the effectiveness of Kamarudin et al.'s (2020) procedure in a controlled laboratory setting. A systematic scale-up was used to standardise the filtration process: quantities were tripled from Kamarudin et al.'s (2020) original reference value of 3 g *Moringa oleifera* leaves in 90 mL of water, and the original 1:30 solid-liquid ratio was maintained at a temperature of 82 °C for 48 minutes. Table 2 summarises the parameters of the laboratory-scale experiment.

Table 2
Parameters for laboratory-scale experiment

Parameter	Unit	Value
Dried Moringa leaves	g	3
Volume of Water	mL	90
Time	min	48
Temperature	℃	82

Figure 4 shows the laboratory-scale procedure. As shown in (a), 3 g of dried Moringa leaves were placed in 90 mL of water and heated at 82 °C for 48 minutes under carefully controlled, uniform thermal exposure (b). The extract was then filtered by Whatman Filter Paper No. 1 (c), separating the liquid extract from the residue, and the final Moringa extract product is shown in (d).

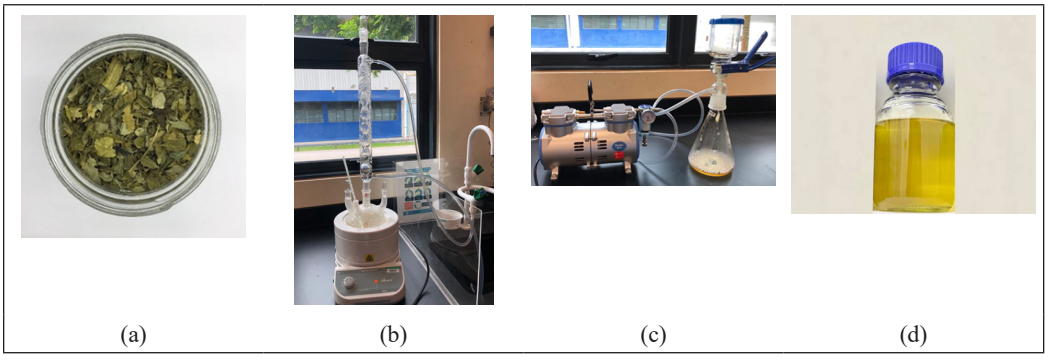

Figure 4. Laboratory-scale method: (a) Dried Moringa leaves; (b) Heating of Moringa leaves; (c) Filtration process, and (d) Moringa extract product (beverage)

Pilot-Scale Method

The pilot-scale filtration system was used under controlled conditions to evaluate the filtration tank with food-grade stainless-steel wire mesh for fluid separation, and the experimental setup was meant to mimic the food and beverage industry. The process was performed about 1000 times the base reference, using 1 kg of dried leaves and 30 L of water at 82 °C for 48 minutes; the parameters are shown in Table 3.

Table 3
Parameters for pilot-scale experiment

Parameter	Unit	Value
Dried Moringa leaves	kg	1
Volume of Water	l	30
Time	min	48
Temperature	°C	82

Figure 5 illustrates the pilot-scale production process for the Moringa beverage. As shown in Figure 5a, 1 kg of dried Moringa leaves was weighed prior to the process, and 30 L of water was added to the wire mesh filter media (Fig. 5b). The mixture was heated at 82 °C for 48 minutes (Fig. 5c) to allow the bioactive compounds to migrate through and minimise thermal degradation before the beverage was collected as the Moringa extract (Fig. 5d) for downstream use.

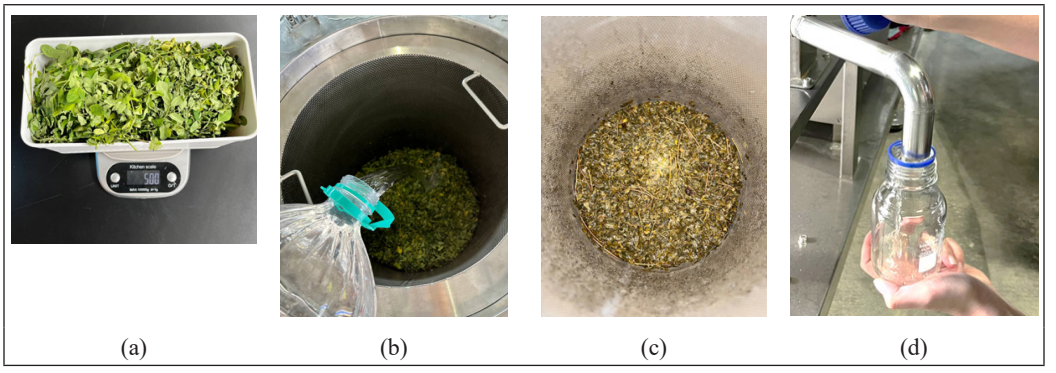

Figure 5. Pilot-scale method: (a) Dried Moringa leaves; (b) Heating process; (c) Filtration process, and (d) Moringa extract product (beverage)

The pilot-scale setup shows the technical feasibility of the wire-mesh filtration system at a larger processing volume. However, processing throughput, filtration rate, energy consumption, labour requirements, cost, and other operational feasibility aspects were not evaluated in this study. These parameters are necessary before the system's scalability for SME production can be realistically demonstrated. A second related but different topic is cleaning behaviour and fouling resistance after repeated cycles. The pilot scale was built from food-grade stainless steel to maintain a clean, corrosion-resistant surface. Cleaning validation data were not collected in this single-run study and are recommended as the next step before use.

Summary of Experimental Study

Experimental Workflow

Figure 6 illustrates the overall experimental workflow. The process commences with raw material preparation, whereby Moringa leaves are conditioned to the desired physical state prior to extraction. This is followed by the brewing stage, during which the leaves are subjected to controlled extraction under defined temperature and time parameters, and subsequently by the filtration stage, which constitutes the core separation process and varies according to methodological scale and operational setup. The resulting filtrate is then collected for further analysis, with care taken to minimise loss and contamination during transfer, before a final assessment of clarity and mineral content, the key indicators of extraction efficiency and filtrate quality. This structured workflow provides a consistent framework that enables direct comparison between the traditional laboratory-scale and pilot-scale methods.

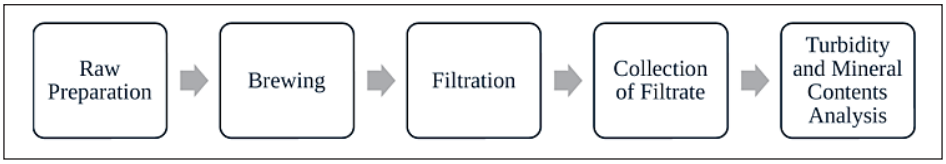

Figure 6. Experimental workflows for all methods

Table 4 compares the key experimental parameters of the three filtration methods. As mentioned above, the traditional method operates under a much less controlled environment than the standard solid-liquid ratio and 82 °C thermal control adopted by the laboratory- and pilot-scale versions.

Table 4
Summary of experimental parameters

Filtration Method	Dried Leaves (g)	Water Volume (mL)	Temperature (°C)
Traditional	1	230	100
Laboratory-scale	3	90	82
Pilot-scale	1000	30 000	82

RESULTS AND DISCUSSION

Each product was then evaluated for clarity, assessed visually based on colour (ranging from dark to light yellow to the lightest colour). Clarity was measured on a visual scale, where a lower value indicates greater transparency and a clearer product. Mineral analyses for calcium (Ca), iron (Fe), potassium (K), magnesium (Mg) and sulphur (S) were done using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) with standard sample digestion procedures.

Product Samples Collected

The experimental study produced three distinct samples, allowing direct comparison among the traditional method (which represents current standard practice, particularly in households and certain SMEs), the laboratory-scale method, and the pilot-scale method. As both the laboratory-scale and pilot-scale experiments followed the same protocol established by Kamarudin et al. (2020), this comparison also allows evaluation of the feasibility and scalability of that methodology for potential mass production. Figure 7 shows the three samples produced:

- Sample 1: Traditional Method
- Sample 2: Laboratory-scale Method
- Sample 3: Pilot-scale Method

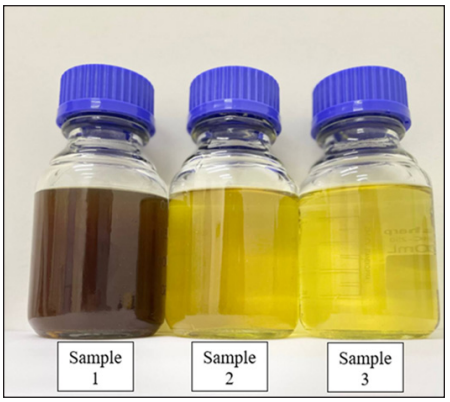

Figure 7. Product samples from three filtration methods

Product Clarity Comparison

Clarity is related to the product's visual appeal and perceived quality. In Moringa-based beverages, turbidity arises from incomplete separation of particulates in filtration; poor clarity (high turbidity) will dilute consumer acceptance and may indicate a lack of production efficiency. Sample 1 (traditional) had the lowest clarity of all three, with the dark brown colour indicating a higher visible particulate load.

That might also be detrimental to shelf stability, since a higher particulate load can accelerate sedimentation and increase the substrate available for microbial growth, though shelf life and microbial stability were not measured here. In contrast, Samples 2 and 3 became a bit lighter in colour and thus showed better particulate removal from the filtration process and a qualitative effect on the appearance of the finished product. Colour was measured only by visual observation, and instrumental turbidity measurement was not performed in this pilot study and is expected to be a focus of future research. A summary of product samples, clarity, and colour comparison is shown in Table 5.

Table 5
Summary of product samples, clarity, and colour comparison

Sample	Key Features	Clarity and Colour
1.	Basic sieves or cloth rely on gravity or minimal pressure and have no controlled parameters.	High clarity: Dark brown hue, indicating residual fine particles.
2.	Uses Whatman No. 1 filter paper, conducted under controlled laboratory conditions.	Moderate clarity: Light-yellow, suggesting effective removal of larger particles, but some fines remain.
3.	Advanced wire mesh with optimised size, conducted in a closed, controlled environment, which ensures uniform filtration.	Low clarity: Near-transparent, lightest colour, demonstrating superior removal of both large and fine particulates.

However, because Moringa extract naturally contains chlorophyll-related pigments and other colour-associated bioactive compounds, the pilot-scale sample's lighter colour cannot, on the basis of visual assessment and the current mineral panel alone, be fully distinguished from a possible partial loss of these coloured phytochemicals during filtration. Dedicated phytochemical pigment assays are recommended in future work to disambiguate desirable clarification from loss of bioactive colour compounds.

Nutrient Contents of Beverage Products

The samples were analysed at ALS Technichem (M) Sdn Bhd, Johor Bahru, Malaysia, which is a STANDARDS MALAYSIA accredited laboratory that uses the methods as well as the Association of Official Analytical Chemists (AOAC) and American Public Health Association (APHA) in terms of the metal analysis techniques. Metals are analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) to meet AOAC 999.11 and APHA 3120B standards to detect minerals and measure them in a precise manner. The Limit of Reporting (LOR) was set to 0.05 mg/100 mL for all minerals that were examined, ensuring high analytical sensitivity. The resulting nutrient content data from the certified laboratory test are presented in Figure 8.

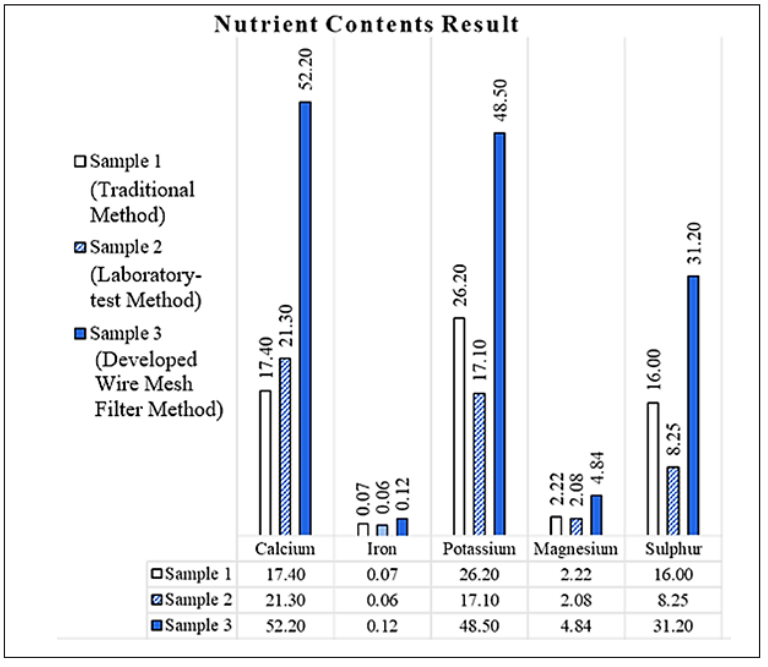

Figure 8. Nutrient contents result (mg/100 mL)

To evaluate the pilot scale method (Sample 3) against the other two methods, percentage difference calculations were performed using the same framework for all five minerals. Table 6 compares the mineral content of Moringa beverage products from the three filtration methods.

Table 6
Comparative nutrients of Moringa beverage products obtained (mg/100 mL)

Sample	Method	Ca	Fe	K	Mg	S
1	Traditional	17.40	0.07	26.20	2.22	16.00
2	Laboratory-scale	21.30	0.06	17.10	2.08	8.25
3	Pilot-scale	52.20	0.12	48.50	4.84	31.20

Sample 3 (pilot-scale) had higher single-measurement mineral values than Samples 1 and 2 for all five minerals analysed. Compared to the traditional and laboratory-scale methods, the pilot-scale sample produced 200% and 145% more calcium, 71% and 100% more iron, 85% and 184% more potassium, 118% and 133% more magnesium, and 95% and 278% more sulphur. Since these figures represent a single measurement per method rather than replicated trials, they are descriptive and not statistically valid; no inferential statistical test has been applied. The same is true for the clarity results reported above. Table 7 shows the summary of nutrient content percentage retention from the pilot-scale.

Table 7
Summary of nutrient contents percentage retention from the pilot-scale

Filtration Method	Ca	Fe	K	Mg	S
Sample 1	200	71	85	118	95
Sample 2	145	100	184	133	278

As previously noted, the traditional method's markedly lower solid-to-liquid ratio (1:230), relative to the laboratory- and pilot-scale methods (1:30), means the traditional-versus-pilot comparison reflects both extraction concentration and filtration medium simultaneously, rather than filtration alone. More notably, although the pilot-scale and laboratory-scale samples were prepared under identical extraction temperature, time and solid-to-liquid ratio, the pilot-scale sample still showed higher mineral concentrations. The same operator, heating protocol, and post-extraction handling were used across all scales to minimise procedural variability, but evaporative concentration and the exact sampling position were not independently measured or controlled, which is acknowledged as a limitation.

As a possible explanation for this laboratory vs. pilot-scale comparison, the larger surface area and lower adsorptive affinity of food-grade stainless steel wire mesh compared to cellulose-based Whatman No. 1 filter paper could reduce mineral loss through matrix adsorption during filtration. This is a plausible mechanism but not a confirmed one, since we have not directly tested it in this study; direct verification, which is a mineral analysis of the filter residue on each medium, is recommended as a specific, tractable next step.

The scope of this study was limited to the five minerals most emphasised in the nutritional literature in Moringa (calcium, iron, potassium, magnesium and sulphur) because there is a scarcity of resources and the minerals available through the accredited ICP-OES service. To separate the effect of the extraction and filtration process at these production scales rather than just taking them together would require a controlled factorial follow-up, in which the filtration method is varied independently of the extraction temperature, time and ratio, which seems to be the most logical next controlled experiment based on this pilot comparison. From a practical perspective, the pilot-scale prototype has qualitative advantages relevant to SME adoption: a reusable, food-grade stainless-steel mesh that is easier to clean than cloth, and a potential for higher single-batch throughput. However, no quantitative data on processing throughput, filtration rate, energy consumption, labour requirement and cost were collected in this study. Considered together, the confounding factors identified above, the traditional method's differing extraction ratio and the unexplained divergence between the laboratory- and pilot-scale samples under otherwise identical conditions, indicate that the clarity and mineral advantages associated with the pilot-scale system, while promising, remain mechanistically unconfirmed at this stage.

CONCLUSION

The findings of this pilot study indicate that the pilot-scale filtration system was associated with improved clarity and enhanced mineral content in Moringa beverage products, relative to both the traditional and laboratory-scale methods, consistent with the wire mesh's superior filtration efficiency and process control. These gains, however, should be interpreted in light of the confounds and open questions identified in the preceding discussion: differing extraction conditions across production scales, an unexplained divergence between laboratory- and pilot-scale results despite otherwise matched conditions, and untested operational parameters, including throughput, energy consumption, and cleaning-cycle validation. Resolving these questions, through a controlled, single-variable factorial follow-up and direct verification of the proposed adsorption-based mechanism, constitutes the necessary next step before the pilot-scale system's advantages can be confirmed and its potential scalability for SME-based functional beverage production can be more broadly generalised.

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Effect of Rock Fragment Proportion on Soil Water Availability and Maize Growth in Sandy Clay Loam Soil

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ABSTRACT

The presence of rock fragments in soil significantly impacts the soil's ability to retain water and other hydrological functions such as water storage, infiltration, evaporation, and hydraulic properties. This study evaluated soil water retention, penetration resistance, maize (*Zea mays* L.*)* growth, biomass and leaf gas exchange across five rock fragment proportions (0%, 10%, 30%, 50% and 60%; M0-M60). This experiment was conducted in a Randomised Complete Block Design (RCBD) with five treatments and three replicates, for a total of 15 experimental units. Rock fragments were 2-4 mm in diameter and were mixed with soil described as sandy clay loam. Reported plant available water capacity declined from approximately 9.00% in M0 to 3.11% in M60; M30 recorded 6.15%. In contrast, M30 had the highest mean ear weight (370.60 g plant^{-1}), root dry biomass (25.97 g plant^{-1}) and net photosynthetic rate (25.17 µmol CO_2 m^{-2} s^{-1}). Ear weight in M30 exceeded M50 but did not differ significantly from M0, M10 or M60, whereas root biomass in M30 exceeded all other treatments. In conclusion, the mixture of 30% rock fragments with sandy clay loam has the best proportions that ensure the Plant Available Water Capacity (PAWC) for maize (*Zea mays* L.*)* growth.

Keywords: Plant Available Water Capacity (PAWC), rock fragment, sandy clay loam, soil water retention, *Zea mays* L

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INTRODUCTION

A rock fragment is defined as a soil particle that is larger than 2 mm in diameter. The presence of rock fragments in soil has a substantial impact on water retention and other hydrological functions such as water storage, infiltration, evaporation, and hydraulic properties.

In many Mediterranean nations where natural environment deterioration is a major issue leading to desertification, a substantial proportion of soils contain rock pieces both at the soil surface and in the soil profile (Poesen & Lavee, 1994; Tétégan et al., 2011). Earlier studies demonstrated that rock fragments influence infiltration and crop responses to water supply (Kosmas et al., 1994). Rock fragments contents in soil may hinder the growth of plant roots and cause the soil to be unable to retain water for plant uptake. Certain proportions of rock fragments may decrease water-holding capacity. Appropriate proportions of rock fragments will provide an adequate amount of water for root uptake. The percentage of rock fragments in soil influences water availability and, by extension, plant traits. Plants cultivated in soil containing rock fragments have lower transpiration rates, particularly when well-watered. The ability of a soil to absorb water rises with its particle size. The presence of rock fragments has a major impact on hydrological processes such as water storage, infiltration and evaporation, and soil hydraulic characteristics (Ma & Shao, 2008). More recent work has shown that plants can use water stored in porous rock fragments during drought (Korboulewsky et al., 2020), and that even hard sandstone fragments can contribute to field-capacity water storage (Robertson et al., 2021). Field observations also show that fragment content modifies the spatial and seasonal distribution of soil water and temperature (Huang et al., 2023). This study addresses that question by comparing water retention, penetration resistance, maize morphology, biomass and leaf gas exchange across five proportions of 2-4 mm rock fragments.

The objectives of this study were to evaluate the effect of different rock fragments proportion on plant available water capacity (PAWC), evaporation rate, biomass, morphology growth and growth performance of maize (*Zea mays* L.*)* planted in a mixture with sandy clay loam and to evaluate the effect of rock fragment mixtures on gas exchange characteristics of maize (*Zea mays* L.). The hypothesis of this experiment is to observe the effect of different proportions of rock fragments (0%, 10%, 30%, 50% and 60%) towards the plant available water capacity (PAWC), evaporation rate, biomass, morphology, growth and growth performance and gas exchange characteristics of maize (*Zea mays* L.) planted in a mixture of sandy clay loam soil and rock fragments.

MATERIALS AND METHODS

This experiment was conducted at the University Agriculture Park, Universiti Putra Malaysia (UPM), Serdang, Malaysia. GPS coordinates: latitude 2.585660 and longitude.

101.425469. The rock fragments used in this experiment are between 2.0 mm and 4.0 mm. The sieve sizes 2 mm and 4 mm were used to ensure the range size for the rock fragments.

The soil texture used is sandy clay loam collected at UPM Serdang campus, Selangor. The soils were sieved with a 2 mm sieve size and collected for media preparation, then mixed with the rock proportion. The soil was described as sandy clay loam; its reported particle-size distribution and water-holding capacity are presented in Table 1. The sandy clay loam and rock fragments were mixed evenly and transferred into respective (16 × 16 × 16) polybags according to the treatments, with 3 replications (Table 1).

Experimental Design

The experimental design used is a Randomised Complete Block Design (RCBD) with five rock fragment proportions and three replicates, giving 15 experimental units. The treatments were M0, M10, M30, M50 and M60, corresponding to 0%, 10%, 30%, 50% and 60% rock fragments, respectively (Table 2).

Table 1
Properties of the experimental soil

Sand (%)	56.8
Silt (%)	21.6
Clay (%)	21.6
Water-holding capacity (%) VWC	66

Table 2
Treatment composition of soil and rock fragment mixtures

Label	Treatments
M0	100% sandy clay loam soil + 0% rock fragments (control)
M10	90% sandy clay loam soil + 10% rock fragments
M30	70% sandy clay loam soil + 30% rock fragments
M50	50% sandy clay loam soil + 50% rock fragments
M60	40% sandy clay loam soil + 60% rock fragments

Maize (*Zea mays* L.) was used as a test crop. One seedling was transplanted into each polybag at 7 days after sowing (DAS), and the crop duration was reported as 75 DAS (Table 3). Fertiliser application began at 14 DAS and was repeated weekly until 75 DAS at the reported rate of 5 g per polybag per application. The fertiliser type used is shown in Table 3.

Soil Properties Analysis

Soil Water Retention and Plant Available Water Capacity (PAWC)

Soil water retention was measured using the water retention pressure plate and pressure membrane method (Richards, 1941). Materials used were pressure chambers, a pressure control manifold, an air compressor and ceramic plates (100 kPa, 500 kPa and 1500 kPa plates). Determination of PAWC was done at soil water pressure of 0.1 kPa

(saturation capacity/point), 1 kPa, 10 kPa, 33 kPa (field capacity), and 1500 kPa (permanent wilting point).

Evaporation Rate

Evaporation rate is determined by the rate at which water can be conducted to the surface rather than by atmospheric conditions (Tian & Philpot, 2015). The Penman-Monteith formula is used for calculating the potential rate of evaporation, which is based on the energy balance. The resultant equation is as shown in Equation 1:

Table 3
Fertiliser recommendation for maize

Item	Value
Nitrogen (N) (kg/ha)	130
Phosphorus (P_2O_5) (kg/ha)	57
Potassium (K_2O) (kg/ha)	130
Fertiliser application rate	5 g/ polybag/ week
Plant Population Density (PPD per ha)	53 333

*Source: Department of Agriculture Malaysia (DOA) (2008)

$$ET_0 = \frac{0.408\Delta(R_n - G) + \gamma \frac{900}{T + 273} u_2 (e_s - e_a)}{\Delta + \gamma(1 + 0.34u_2)} \quad [1]$$

Where:
ET_o reference evapotranspiration [mm day^{-1}],
R_n net radiation at the crop surface [MJ m^{-2} day^{-1}],
G soil heat flux density [MJ m^{-2} day^{-1}],
T mean daily air temperature at 2 m height [°C],
u_2 wind speed at 2 m height [m s^{-1}],
e_s saturation vapour pressure [kPa],
e_a actual vapour pressure [kPa],
e_s - e_a saturation vapour pressure deficit [kPa],
D slope vapour pressure curve [kPa $°C^{-1}$],
g psychrometric constant [kPa $°C^{-1}$].

Soil Electrical Conductivity (EC)

The EC of the soil extract is obtained immediately from the digital display of a temperature-compensating conductivity meter. The electromotive force (EMF) generated by the platinum/epoxy electrode arrangement varies linearly with the EC. Plotting the measured EMF against the EC of several standard concentrations reveals this linear relationship.

Soil Compaction

Soil compaction was measured using a soil penetrometer, and readings were recorded in kilopascals (kPa). In addition, supporting soil physical properties, such as bulk density, were assessed to complement the compaction data.

Plant and Growth Performance Analysis

The maize plant's fresh weight and dry weight were taken after the cobs of maize were harvested. Maize cobs, stems, leaves, and roots were taken. To get the dry weight, the maize plant parts were put into the oven to dry. The temperature was maintained at 70 °C for 7 days. After 7 days, the plants were weighed using an electronic balance. The moisture content in the plant was also calculated based on the fresh weight and dry weight.

Plant Biomass

Biomass analysis is the dry weight of the maize plant. The maize plants were divided into 4 parts, that is, stem, leaf, ear and root. The biomass was taken to calculate the relative growth rate and absolute growth rate of the maize plant throughout the experiment.

Absolute Growth Rate (AGR)

Absolute growth rate refers to a plant's size and represents an increase in mass per unit period. AGR is also used to describe the growth of the plant, as it is based on the roots, leaves, and stem mass of the plant. The AGR was measured by the following Equation 2:

$$\text{AGR (g day}^{-1}) = \frac{(\text{fresh weight} - \text{dry weight})}{75 \text{ days planting}} \quad [2]$$

Specific Leaf Area (SLA)

(Evans and Poorter (2001) define specific leaf area (SLA) as the anticipated leaf area per unit leaf dry mass. SLA is a key characteristic in many agronomic and ecological processes, such as photosynthesis, transpiration, and field energy balance, but it can be difficult and costly to quantify. The SLA was measured using the following, Equation 3:

$$\text{SLA (cm}^2\text{g}^{-1}) = \frac{\text{Leaf area}}{\text{Leaf biomass}} \quad [3]$$

Root-shoot Ratio (RSR)

Root-shoot ratio (RSR) is important for the growth and development of plants (Andrews et al., 1999). Root-shoot ratios are thus indicative of plant response to growing conditions of maize (*Zea mays* L.). The formula is based on the dry weight of the root (g) and the shoot (g) per plant. The RSR was measured by following Equation 4:

$$\text{RSR (cm}^2\text{g}^{-1}) = \frac{\text{Root biomass}}{\text{Shoot biomass}} \quad [4]$$

Leaf Gas Exchange Analysis

Leaf gas exchange was measured one week before harvest between 08:00 and 11:00 using a LI-COR portable photosynthesis system. The measurements taken were intercellular CO_2, stomatal conductance, transpiration rate and photosynthesis. Gas exchange was measured on the same leaf on which the measuring electrode was fixed. Changes in CO_2 concentration were detected with a porometer (Li-Cor Li 6000, Lincoln, WA) that simultaneously measured the rate of transpiration during the period 8.00-11:00. Each started at a CO_2 concentration of 400 ppm and a relative humidity of 50%. The temperature in the leaf chamber was 25 °C and the light intensity 300 mmol photons m^{-2} s^{-1}. The analyser was calibrated prior to each series of measurements; the stability of the zero point was checked by means of an airline that bypassed the chamber with the leaf.

Statistical Analysis

All experimental data were statistically analysed using the analysis of variance (ANOVA) techniques in the Statistical Analysis software 9.4 (SAS Institute, Inc., Cray, NC, USA). LSD was used to compare treatment means with significant F values ($p<0.05$).

RESULTS AND DISCUSSIONS

Soil Properties Analysis

Soil Water Retention and Plant Available Water Capacity (PAWC)

Figure 1 presents the water-retention curves for the effect of rock fragments on the water content of sandy clay loam soil at different suctions. The pressure head and water content (θs) showed the water retention in the M0 (9.00 %), M10 (8.20 %), M30 (6.15 %), M50 (4.16 %) and M60 (3.11 %), which decreased based on the different rock fragment effects. There were many factors that could affect the soil hydraulic conductivity. Soil with a high percentage of clay might indicate a slow infiltration rate compared to sandy soil (Mahmood-ul-Hassan et al., 2013). High bulk density was correlated with low soil porosity and might also cause a low value in soil hydraulic conductivity. The predicted curve indicated excellent fitting for the retention curve data in the models, together with the Van Genuchten - Mualem equation (m=1-1/n), which was extracted from the retention curve model.

Figure 2 shows the plant available water capacity (PAWC) of rock fragment mixtures with soil. At 0% (8.99%) and 10% rock fragment mixtures (8.20%) showed a significant difference compared to 30% (6.15%), 50% (4.16%) and 60% rock fragment mixtures (3.11%). This indicated that the rock fragment mixture with 10% rock fragments was able to retain the highest available water compared to 30%, 50%, and 60% rock fragment mixtures. The presence of rock fragments in the soil significantly decreases Plant Available Water Capacity (PAWC) due to high rock fragments contents, which causes higher heat

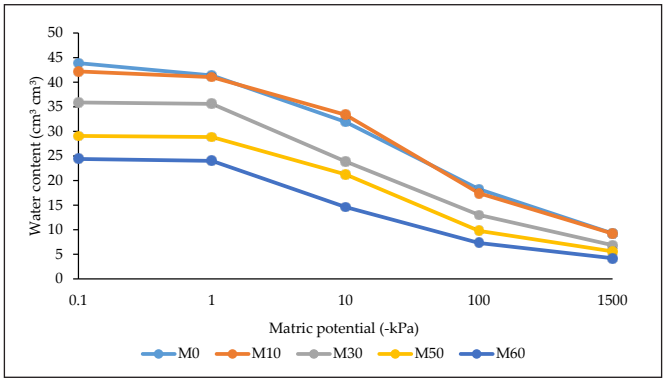

Figure 1. Effect of rock fragments on the water content of sandy clay loam soil at different suctions (kPa). Means with the same letter are not significantly different at p<0.05

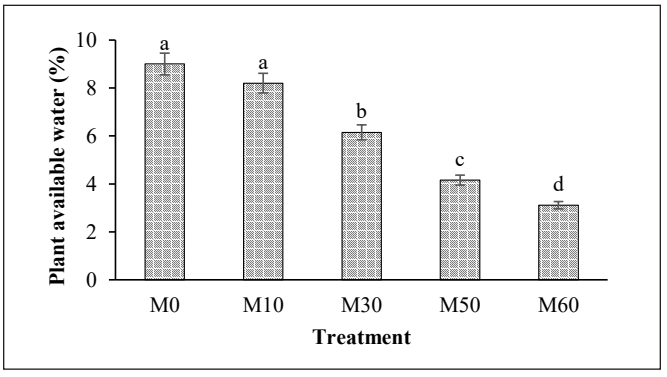

Figure 2. Effect of rock fragments on PAW of sandy clay loam soil at different suctions (kPa). Means with the same letter are not significantly different at p<0.05

conduction than stonier soil does, leading to increasing evaporation rates that result in a reduction of AWC (van Wesemael et al., 1996a; van Wesemael et al., 1996b). A thirty percent rock fragments mixture was also able to restore an adequate amount of available water content for maize establishment. Increased rock fragment proportions have been shown to (Fiès et al., 2002) that high rock fragment contents usually give a negative effect on the water-holding capacity of the soil, resulting in less Plant Available Water Capacity (PAWC).

Evaporation Rate

Rock fragment was significantly ($p<0.05$) higher in the M50 (728.50 mm day^{-1}), followed by the M60 (637.58 mm day^{-1}), M30 (531.00 mm day^{-1}), M0 (419.83 mm day^{-1}) and M10 mixture (395.08 mm day^{-1}), respectively (Figure 3). The result shows that there was no

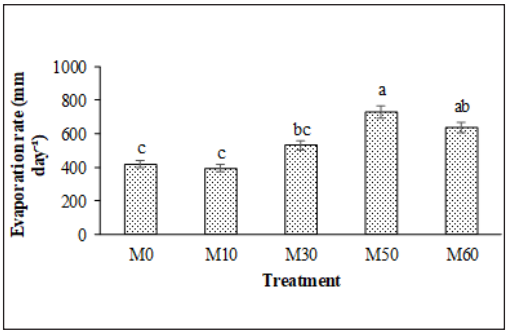

Figure 3. Effect of rock fragments on evaporation rate. Means with the same letter are not significantly different at $p<0.05$

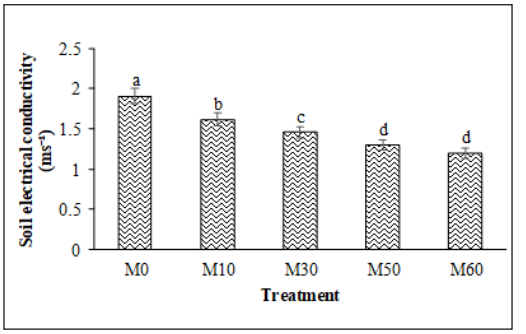

Figure 4. Effects of rock fragments on electrical conductivity. Means with the same letter are not significantly different at $p<0.05$

significant difference between the treatments of M0, M10, and M30. There was a significant difference between the M50, M60, and M10 rock fragments mixture. The mean values of M50 and M60 mixtures showed the highest evaporation rate compared to the others. According to the findings, the evaporation rate is dependent on a variety of parameters, including surface area, liquid temperature, and surrounding air. When a system's vapour pressure reaches equilibrium, the evaporation rate slows. The evaporation of soil can be influenced by rock particles. A high concentration of rock particles may increase the evaporation rate. The results of this experiment opposed the findings (Kosmas et al., 1994; van Wesemael et al, 1996b), who discovered that rock fragments induce a decrease in soil evaporation after comparing crop production in soil with and without rock.

Soil Electrical Conductivity

Figure 4 shows the effect of rock fragment mixtures on electrical conductivity (mS cm^{-1}). The results showed that there was no significant difference between M50 (1.30 mS cm^{-1}) and M60 rock fragment mixtures (1.20 mS cm^{-1}), but there was a significant difference between M0 (1.91 mS cm^{-1}), M10 (1.52 mS cm^{-1}) and M30 (1.46 mS cm^{-1}) treatments. This possibly happened because the available water content was high in M0 to M30 mixtures. An EC meter was used to measure the potential electrical conductivity transported through the soil mixtures. The result showed that the increased amount of rock fragment content could possibly affect the EC value or the salinity of the soil. Apparently, EC was highly dependent on soil water content content (Mi et al., 2016).

Soil Compaction

Soil compaction tended to decrease as the proportion of rock fragments in the soil increased. The results of comparisons of mean soil compaction for each rock-fragment content

showed that rock-fragment M0 was higher than rock-fragment contents of M10, M30, M50 and M60 (Figure 5). A significant difference was observed in the M0 rock-fragment mixing (539.72 kPa) and M60 rock-fragment mixing (333.02 kPa). However, there was no significant difference in M10 (380.10 kPa), M30 (376.91 kPa) and M50 (355.99 kPa). The decrease in compaction was based on the percentages of mixing rock fragments and soil that based on the pores and structure size. In addition, soil compaction is mostly related to soil bulk density (Panayiotopoulos et al., 2003) and a similar result was observed in this experiment. According to (Horn et al., 1995; Lipiec & Hatano, 2003; Panayiotopoulos et al., 2003), soil compaction can cause the degradation of inter-aggregate pores, as well as a reduction in soil hydraulic conductivity and air permeability. Furthermore, Lipiec and Hatano (2003) stated that in order to obtain an accurate measurement of the effects of soil compaction in all types of soil, it should not only consider soil bulk density but also soil strength, aeration, and moisture.

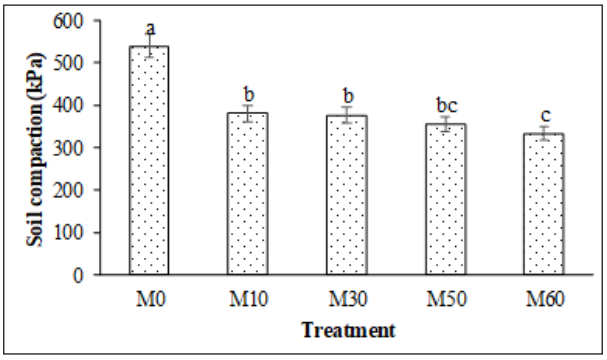

Figure 5. Effects of rock fragments on soil compaction. Means with the same letter are not significantly different at $p<0.05$

Plant and Growth Performance Analysis

Maize Growth Morphology

Table 4 shows the ear weight (g plant^{-1}) of M30 (370.6 g plant^{-1}), which was identified as the highest compared to M50 rock fragment mixtures (265.9 g plant^{-1}). From the results, there was no significant difference between treatments of M0, M10 and M60 rock fragment mixtures, but there was a significant difference between treatments of M30 and M50 rock fragment mixtures. The ear weight differs because the maize plants have more than one ear. Ear weight could be different, correlated to the ability of rock fragment mixtures to hold water. Based on Figure 2, M0 rock fragments were able to retain more water content compared to the other treatments. The ability to retain adequate water content might help plant photosynthesis and increase ear size, and thus increase the ear weight per plant (Sekucia et al., 2020).

Table 4
*Effect of rock fragment on growth morphology of maize (*Zea mays L.*). Means with the same letter are not significantly different at p<0.05*

Parameter	Percentage of Rock Fragment in Sandy Clay Loam				
	0%	10%	30%	50%	60%
Ear Weight (g plant^{-1})	319.13ab	295.20ab	370.60a	265.88b	288.66ab
Stem Diameter (cm plant^{-1})	3.45ab	3.90a	2.94ab	2.52b	2.83b
Plant Height (cm plant^{-1})	113.50a	115.89a	117.44a	89.17b	97.95ab
Leaf Area (cm^2 plant^{-1})	4186.40ab	3896.00ab	4846.70a	2426.30c	3209.50bc

Stem diameter in Table 4 shows that there was no significant difference between treatments of M0 and M30 rock fragment mixtures, and there was also no significant difference between M50 and M60 rock fragment mixtures. But the M10 rock fragment mixture showed a significant difference compared to M50 and M60 rock fragment mixtures. This was possibly because 0% to 30% rock fragment mixtures provide adequate water content for maize growth. In contrast, daily stem contraction demonstrated complex and loose interactions with soil and plant water status, as well as stomatal conductance; changes in stem diameter are widely used to measure plant water status (Torres-Ruiz et al., 2013).

Plant height in Table 4 indicated that the plant height (cm plant^{-1}) of M30 (117.4 cm plant^{-1}) was the highest compared to M50 rock fragments mixture (89.2 cm plant^{-1}). From the result, there was no significant difference between the treatments of M0, M10, M30 and M60 rock fragment mixtures. Treatments of M50 and M60 rock fragments mixture were also shown no significant difference. The plant height differs probably because of 0% to 30% rock fragment mixtures had good porosity within the mixtures and better aeration for root growth. The plant height was also related to water holding capacity based on Figure 5. A limited water supply reduced the plant height (Sari-Gorla et al., 1999).

Table 4 shows that the leaf area (cm^2 plant^{-1}) of M30 (4846.7 cm^2 plant^{-1}) was the highest compared to M50 rock fragments mixtures (2426.3 cm^2 plant^{-1}). The M30 rock fragments mixture showed a high amount of leaves present, and the value of leaf area might also increase. These might explain why the leaf area differs between the treatments. Crop plant growth and development are essential for establishing a normal plant structure capable of carrying out all physiological and metabolic activities and providing potential yield. Different ratios of rock fragment mixes resulted in water stress, which may impede maize growth and development. Growth and development include several component parameters that were estimated using various qualities such as plant height, leaf area, root structural and functional attributes, plant biomass, plant fresh weight, plant dry weight, and stem diameter. Drought stress reduces plant height, stem diameter, biomass, and leaf area (Anjum et al., 2017).

Biomass and Growth Performance of Maize

Table 5 shows the leaf biomass (g plant^{-1}) of M30 (36.13 g plant^{-1}) compared to the M50 rock fragments mixture (23.2 g plant^{-1}). The leaf biomass influenced the number of leaves per plant, and the M30 rock fragments mixture of maize plants showed the highest number of leaves and increased their leaf biomass value. It was established that increasing inputs, primarily water content at the farm level, will improve biomass production in addition to boosting the harvest index. (Pandey et al., 2000).

Table 5 shows the ear biomass of M30 (84.7 g plant^{-1}) compared to the M60 rock fragments mixture (61.1 g plant^{-1}). The ear biomass in this data was influenced by the number of ears per plant. The maize plant from the M30 rock fragments mixture had more than one ear per plant compared to the other treatments. The 10% to 30% rock fragment mixtures of maize plants also received enough nutrients because the presence of rock fragments provided adequate porosity for plant growth. The rock fragment mixtures of 50% and 60% gave unfavourable conditions for maize to grow because water was easily leached due to the high macro-pores present in the maize planting media (Fortin, 1994)

There was the highest stem biomass (g plant^{-1}) at the M30 rock fragment mixture (35. 5 g plant^{-1}) compared to M50 (22.9 g plant^{-1}) (Table 5). The root biomass (g plant $^{-1}$) of M30 (26.0 g plant^{-1}) was the highest compared to the M60 rock fragment mixture. The 30% rock fragments mixture gave more aeration and more micropores for maize growth in the mixtures and encouraged the widening of root establishment in the polybag (Chow et al., 2007). Apparently, the 30% rock fragments mixture showed the highest maize root biomass and the biggest root size compared to the other treatments. The presence of rock fragments in soil layers may have an impact on their hydraulic properties. Previous research has demonstrated that rock pieces on the soil surface can have a variety of effects on soil moisture, runoff generation, overland flow hydraulics, and interrill and rill erosion (Poesen & Lavee, 1994; Wang et al., 2012). For example, in cultivated soils, rock fragments affect both moisture content and plant growth. Kosmas et al. (1994) and Tétégan et al. (2011) demonstrated that soils produced from sandstone and conglomerates with rock fragments are less affected by dry periods than soils on marls without rock fragments, resulting in improved maize biomass output. It seems that 0% to 30% rock fragment mixtures in this study might not hinder root and maize growth performance.

Table 5 shows that the M30 rock fragments mixture (221.9%) had the highest moisture content compared to M50 (143.5%). The results, it showed that the 30% rock fragments mixture stored more water content compared to the other treatments. The results of this experiment were comparable to those of Ma and Shao (2008), who discovered that the favourable effect of rock fragments varied according to the range of rock fragment concentration, which might affect biomass between 10% and 50% rock fragment mixtures.

Table 5
*The effect of rock fragments on biomass and growth performance of maize (*Zea mays L. *Means with the same letter are not significantly different at p<0.05*

Parameter	Percentage of Rock Fragment				
	0%	10%	30%	50%	60%
Leaves Biomass (g plant^{-1})	30.69ab	26.01bc	36.13a	23.17c	24.99bc
Ear Biomass (g plant^{-1})	75.50ab	69.79ab	84.72a	77.42ab	61.14b
Stem Biomass (g plant^{-1})	27.63b	33.27a	35.45a	22.92c	23.2bc
Root Biomass (g plant^{-1})	17.03b	16.09b	25.97a	17.82b	15.57b
Total plant moisture content (% plant^{-1})	175.01bc	190.02ab	221.91a	143.55c	160.48bc

Specific Leaf Area (SLA)

Figure 6 shows the Specific Leaf Area (SLA), and there was a significant difference in SLA due to different percentages of rock fragment mixtures. There was a significant difference between M30 and M60 rock fragment mixtures. SLA of the M30 rock fragment mixture was the highest recorded in this experiment. SLA was formed by two components, which are leaf area and leaf biomass

Absolute Growth Rate (AGR)

Figure 7 shows the total absolute growth rate (g day-1) of M30 (8.7 g day-1) compared to the M50 (5.5 g day^{-1}) rock fragments mixture, which was the lowest among the other percentages in this experiment. AGR is also used to explain plant growth because it is based on the root, leaf, stem, and leaf mass of the plant. AGR is the most basic metric for measuring plant growth rate. However, limits will arise because it is only used to compare the development of the same plant at the same age or original size. Growth is defined as an increase in plant size, which is directly proportional to an increase in the number and size of cells. According to Romano (2002), the absolute growth rate varied according to the quantity of rock fragments, as did the dry mass of roots, leaves, stems, and fruits.

Root-Shoot Ratio (RSR)

For the M30, M50 and M60 rock fragment mixtures (means of 0.33, 0.43 and 0.39 g^{1} g^{-1}, respectively) was significantly different from M0 and M10 (0.34 g^{1} g^{-1}) (Figure 8). In general, the effect of RSR reflects dry matter partitioning between the shoot and root of higher plants, and it has been concluded that changes in RSR occur in response to a wide range of environmental factors, including rock fragments (Andrews et al., 1999; Ding et al., 2025). Furthermore, particular percentages of rock fragments may boost root growth while limiting aboveground biomass production (Du et al., 2017).

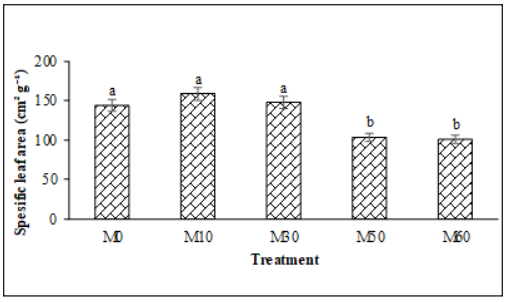

Figure 6. Effects of rock fragments on Specific Leaf Area of maize (*Zea mays* L.). Means with the same letter are not significantly different at p<0.05

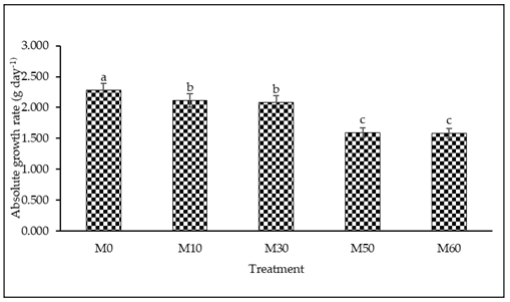

Figure 7. Effects of rock fragments on the Absolute Growth Rate of maize (*Zea mays* L.). Means with the same letter are not significantly different at p<0.05

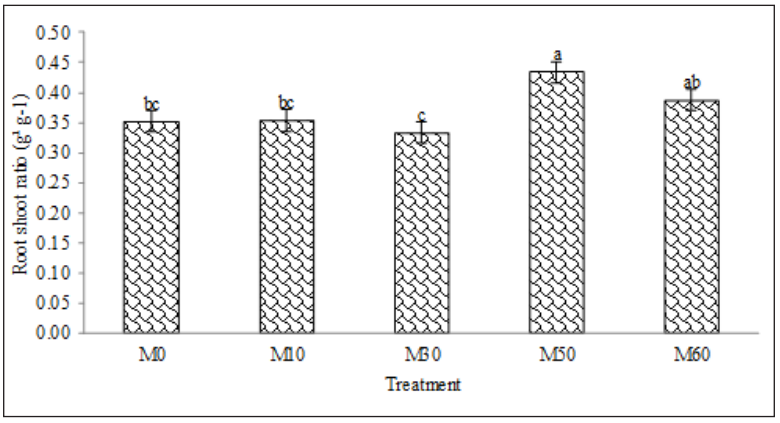

Figure 8. Effects of rock fragments on the root-shoot ratio of maize (*Zea mays* L.). Means with the same letter are not significantly different at p<0.05

Rock fragments can influence plant growth by changing soil physicochemical qualities and controlling plant root systems (Carrick et al., 2013; Estrada-Medina et al., 2013), although there is no agreement on the effects of rock fragments on plant growth. For example, Poesen and Lavee (1994) found that rock pieces promote plant growth, particularly in arid conditions. Soils containing rock fragments have been shown to produce high-quality grapes (Seguin, 1971) and to boost blueberry seedling development (Jackson et al., 1972). Munn et al. (1987) discovered a negative effect of rock pieces on grass productivity in irrigated reclaimed areas in Colorado (USA), while Kongsrud and Nes (1999) found a similar effect on strawberry yield. In contrast, Du et al. (2017) found that a rock fragment concentration of 30% had a beneficial effect on korshinsk peashrub growth and biomass, whereas a rock fragment level of 50% had a detrimental effect on plant growth. This disparity is most likely due to variances in plant species, soil conditions, and rock fragment composition. For example, Du et al. (2017) hypothesised that rock shards

would have a negative impact on root development in sandy soil but would be beneficial for air and water circulation in clayey soil, increasing the root growth environment. Similarly, Babalola and Lal (1977) found that rock fragments had a stronger inhibitory effect on root development of maize seedlings in sandy soil than in sandy loam or clay soil.

Leaf Gas Exchange Analysis

Table 6 shows the photosynthesis rate (A_{net}) ($\mu molm^{-2}s^{-1}$) of the 30% (25.17 $\mu molm^{-2}$ s^{-1}) rock fragments mixture (highest) compared to the M60 rock fragments mixture (14.70 $\mu molm^{-2}$s-1) (lowest) among the other treatments. The adaptation of photosynthesis to water stress had previously attracted significant interest, and there had been a long-standing debate about which was the primary limitation for photosynthesis. Stomatal closure or metabolic impairment (Galmés et al., 2007).

Stomatal Conductance (G_s) in Table 6 presented the G_s ($molm^{-2}$ s^{-1}) of the M30 (0.375 $molm^{-2}$ s^{-1}) (highest) rock fragments mixture compared to the M60 (0.154 $molm^{-2}$ s^{-1}) (lowest) rock fragments mixture. Stomata are plants' major regulatory mechanisms for reducing water loss, and they may do it swiftly. Stomata are responsive to environmental stimuli, which cause them to open or close. The primary function of stomata is to allow carbon dioxide in to stimulate photosynthesis while also allowing water to exit when it evaporates, cooling the leaves. Light, water, temperature, and CO_2 level within the leaf all have an impact on stomatal opening and shutting. Stomata will open in the daylight and close in the darkness. However, stomata can close in the middle of the day if water is limited, CO_2 builds up in the leaf, or the temperature is too high. (Driesen et al., 2020).

CO_2 intercellular concentration (C_i ($\mu molCO_2$ m^{-2} s^{-1}) of the M30 (223.67 μmolCO2 m^{-2} s^{-1}) rock fragments mixture was the highest, and the M60 rock fragments mixture was the lowest. A plant's stomata were open during transpiration, allowing gas exchange between the atmosphere and the leaf. Open stomata allowed water vapour to leave the leaf while also allowing carbon dioxide (CO_2) to enter. Carbon dioxide was required for photosynthesis to occur. Transpiration exceeds CO_2 uptake because water molecules diffuse more rapidly, are driven by a steeper concentration gradient from the hydrated leaf to the drier atmosphere, and traverse a shorter diffusion pathway compared to CO_2, which has a lower atmospheric concentration and must travel a longer internal distance to reach the chloroplast (Hwang et al., 2012). The greater the stomatal aperture, the easier it is for carbon dioxide to enter the leaf and fuel photosynthesis (Evans & von Caemmerer, 1996). However, this enormous aperture would allow the leaf to lose a significant amount of water, increasing the danger of dehydration or water-deficit stress. Plants with slightly open stomata lose less water per CO_2 molecule taken in, resulting in improved water use efficiency (the ratio of water lost to CO_2 acquired) (Lawson & Blatt, 2014). Plants with higher water use efficiency could endure periods of low soil moisture (Bassiouni et al., 2023).

Table 6
*Effects of rock fragments on gas exchange of maize (*Zea mays *L.) Means with the same letter are not significantly different at p<0.05*

Parameter	Percentage of Rock Fragment				
	0%	10%	30%	50%	60%
Photosynthesis Rate (A_{net}) ($\mu molm^{-2}s^{-1}$)	21.43b	22.57b	25.17a	18.10c	14.70d
Stomatal Conductance (G_s) ($molm^{-2}s^{-1}$)	0.17b	0.28 ab	0.38a	0.16b	0.15b
CO_2 Intercellular (C_i) ($\mu molCO_2\ m^{-2}s^{-1}$)	133.33b	204.33a	223.67a	193.33ab	140.33b
Transpiration Rate (E_L) ($\mu molH_2O\ m^{-2}s^{-1}$)	1.53b	2.48ab	3.18a	1.55ab	1.47b

Transpiration Rate (E_L) is the evaporation of water from a plant. Roots absorb water from the soil and transfer it as a liquid to the leaves via xylem in the leaf; tiny pores allow water to escape as a vapour (Hwang et al., 2012). From the analysis of variance, there was a significant difference in E_L due to different percentages of rock fragment mixtures. Table 6 shows that the EL ($\mu molH_2O\ m^{-2}s^{-1}$) of M30 (3.18 $\mu molH_2O\ m^{-2}\ s^{-1}$) was the highest, and the M60 (1.47 $\mu molH_2O\ m^{-2}s^{-1}$) rock fragment mixture was the lowest compared to the other treatments. Less than 5% of the water received by plants is retained for growth. (Korboulewsky et al., 2020) studied and explained that the presence of rock fragments can make Plants waste much water, the path water travels through plants, how plants can compensate for excessive water loss to prevent stress circumstances, and how the environment influences plant water loss. As the proportion of rock fragments increases, soil temperature increases, water-holding capacity decreases, transpiration increases, and plant moisture content decreases due to high transpiration (Huang et al., 2023). High vapour pressure deficit is caused by limiting hydraulic conductance in the plant, which restricts the passage of water from roots to transpiration sites on the leaf surface (Schönbeck et al., 2022). A substantial portion of the hydraulic resistance can be located in the leaf, especially the extravascular component (Cochard et al., 2004).

Relationship between Transpiration and Photosynthesis

Figure 9 shows the positive correlation between transpiration rate (E_L) and photosynthesis rate (A_{net}). The correlation was 0.53. The relationship was influenced by the total amount of moisture content in maize plants cultivated in different proportions of rock fragment mixtures. Water evaporated from the surface of air-exposed leaf cells in actively growing plants. This water was replaced by extra water absorbed from the earth. Liquid water stretched through the plant from the soil water to the leaf cell surfaces, where it was changed from a liquid into a gas by the process of evaporation and transpiration (Meinzer, 2002). Thus, when photosynthesis increased, the transpiration rate increased, which might also have influenced the increase in total moisture content of the maize plant.

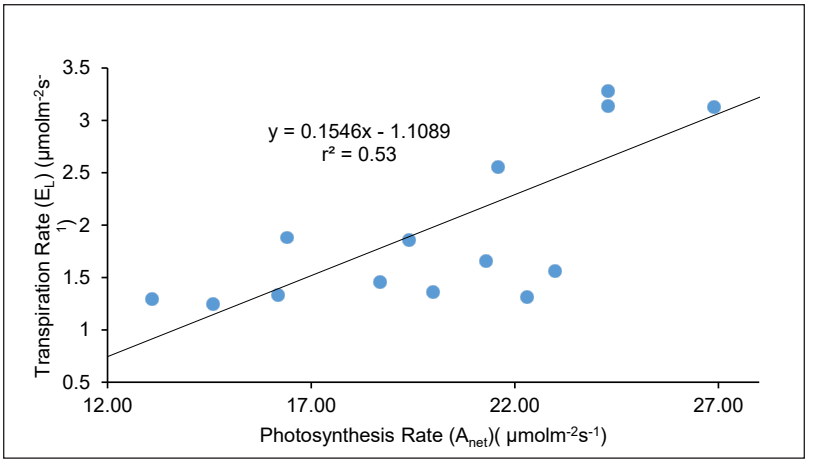

Figure 9. Relationship between transpiration rate (E_L) and photosynthesis rate (A_{net})

Water consumption was influenced by both the water content and the rock particles. Transpiration first remained consistent, but eventually reduced as the water content fell. Increased rock fragment content in the soil considerably reduced transpiration, particularly under well-watered circumstances (>60% field capacity). Hydrogen bonding between adjacent water molecules maintains a continuous water column within the xylem, enabling transpiration at the leaf surface to generate the tension that pulls water upwards through the plant. This mechanism is described by the cohesion-tension theory of sap ascent (Mott, 2007). Under water stress, increased stomatal resistance limits both water loss via transpiration and CO_2 uptake for photosynthesis. Water stress can also affect the biochemical processes of photosynthesis by altering protein hydration and enzyme activity (Zargar et al., 2017).

CONCLUSION

In general, this study has shown significant differences between the percentage of rock fragment mixtures in soil on water-holding capacity, biomass, growth morphology and the growth performance of maize. The optimal proportion of rock fragments was 30% due to the good performance shown in soil water-holding capacity, maize biomass, growth morphology and growth performance. Soils amended with 30% rock fragments also showed favourable characteristics that enhance the ability to retain available water and good macro- and micropores for aeration. In conclusion, the mixture of 30% rock fragments with sandy clay loam soil was the best proportion that could provide Plant Available Water Capacity (PAWC) for the growth of maize.

LIMITATION AND RECOMMENDATION FOR FUTURE RESEARCH

This study was conducted under controlled conditions using sandy clay loam soil and maize (*Zea mays* L.) as the test crop, which may limit the direct applicability of the findings to other soil types, crops, and field environments. The experiment also focused on short-term plant growth responses and did not evaluate the long-term effects of rock fragments on soil structure, nutrient dynamics, root development, and crop productivity under varying climatic conditions. Furthermore, the rock fragments used were within a specific size range (2-4 mm), and different fragment sizes, shapes, and mineral compositions may produce different effects on soil physical properties and plant performance. Therefore, future research should investigate the long-term impacts of rock fragments under field conditions across diverse soil types and crop species, while also evaluating different rock fragment characteristics and their interactions with soil moisture, nutrient availability, and plant physiological responses to better understand their role in sustainable soil and water management.

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Efficacy and Synergistic Potential of Aqueous Plant Extracts Against Whiteflies, *Bemisia tabaci* Genn. in Chilli Plants

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ABSTRACT

The whitefly, *Bemisia tabaci,* causes serious yield losses by transmitting plant viruses and through aggressive feeding on the plant sap. Decades of overreliance on chemical insecticides for whitefly control have driven pest resistance and a detrimental impact on the environment, necessitating the development of sustainable alternatives. This study evaluates the potential of sustainable aqueous extracts against *B. tabaci* on chilli. Bioassays were performed using extracts produced from dried plant samples (10g) extracted using 100ml of distilled water at a 1:10 (w/v) ratio at room temperature. Adult mortality was recorded at 24 hours post-treatment and expressed as mean percentage ± SD. Statistical significance was determined by ANOVA and the Tukey HSD test. Lethal concentration (LC) values were derived via probit analysis. Among the extracts tested, the lowest LC50 value was shown by *A. indica* (63677.9ppm), *C. citratus* (65385.6ppm) and *P. acidus* (80449.5ppm). Synergistic combinations of these extracts were evaluated using LC25 and LC50 in a ratio of 1:1. Synergistic effects were observed in the combination of *A. indica* (LC25) with *P. acidus* (LC50) as well as *P. acidus* (LC25) with *C. citratus* (LC50). These findings highlight the potential of botanical extracts in mitigating whitefly infestation on chilli.

Keywords: *Bemisia tabaci*, efficacy, lethal concentration, plant extracts, synergism

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INTRODUCTION

The increasing popularity of international cuisines and spicy foods has driven the growing demand for chillies, from fresh to processed. Cultivating chilli (*Capsicum annuum* L.) crops can be a highly rewarding endeavour for farmers and home gardeners alike. Despite being known for their spiciness, chilli plants are also susceptible to pests. The whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is one of the primary pests of chilli. This pest's aggressive feeding behaviour and role as a vector for plant viruses disrupt plant physiology and productivity (Yadav et al., 2022). The ability of the whiteflies to rapidly populate within the plant intensifies the damage and contributes to significant yield losses due to the production of chilli fruits with low market viability (Kammara et al., 2023). The common approach to managing pests and diseases in chilli plant cultivation is the use of chemical pesticides. The underregulated and indiscriminate application of pesticides during the preharvest or postharvest stages often results in pesticide residues that pose risks to human health and the environment and hinder international trade (Tambo et al., 2024; Yami et al., 2025). These risks are exacerbated by the flooding of the market with illegal pesticides, often leading to the misuse of chemicals beyond recommended levels (Rahman, 2021). In the single year of 2020, agricultural activities in Malaysia consumed around 36 000 metric tons of pesticides, mostly organophosphates, pyrethroids, glyphosate, and 2,4-D-dimethylammonium (Malhotra & Aman, 2024). In addition, the level of understanding among Malaysian farmers regarding the environmental and human health risks of pesticides remains moderate, as reported in a study on pesticide usage awareness among farmers in Malaysia (Sabran & Abas, 2021). Many farmers apply these pesticides without proper protective measures, increasing their risk of exposure, and illegally use some organophosphates banned by the government, which eventually results in health problems (Hossain et al., 2010). Without safe management, awareness and sensibility on the pesticide usage by farmers, it can cause a detrimental impact not just on the environment but also on humans and other organisms, including the pest's natural enemy. Despite the growing emphasis in the academic literature on incorporating biopesticides into crop protection strategies (Chandler et al., 2011; Jalloh et al., 2026), chilli cultivation in Malaysia still relies heavily on synthetic insecticides. However, the emergence of resistance in whitefly biotypes has rendered many of the conventional approaches ineffective (Horowitz et al., 2020). These challenges highlight a need for plant-derived alternatives that demonstrate efficacy against whiteflies without causing adverse effects to the environment and end users. Furthermore, research into the potential synergy of these extracts may offer a way to leverage multiple bioactive compounds to overcome resistance in whiteflies. Therefore, the present study aims to evaluate the efficacy and synergistic effects of aqueous plant extracts against whiteflies in chilli plants.

METHODOLOGY

Culturing *B. tabaci*

Colonies of the whiteflies, *B. tabaci,* utilised in this study, were from a field collection in an infested commercial greenhouse located in Kampung Ampang Baru, Ipoh, Perak (4° 35' 18.55282"-101° 8' 15.63179"). Chemical management consisting of weekly applications of the neonicotinoid insecticide Imidacloprid was utilised in the greenhouse. Collection of the pest sample was carried out in the morning during the inactive period of whiteflies (Iqbal et al., 2022). Leaves containing adults and nymphs were picked carefully and confined in an insect-proof cage. The whiteflies colonies were then maintained in insect-proof cage on untreated chilli plant of 4 months old, prior to the bioassay to establish a culture. This acclimation period eliminates any acute residual effects from prior field exposure. The insect cage was constructed from a clear plastic container measuring 62cm × 33cm × 36cm (length × width × height) with a grid-like lid. The opening of the container was secured with fine mesh fabric, preventing the escape of the whiteflies while ensuring ventilation. The lid has a rectangular opening, 13cm × 11cm (length × width), allowing access for monitoring and collection of the insect without fully removing the lid. If the existing chilli plant deteriorates or shows signs of imminent death, a new chilli plant is introduced in the cage to maintain the infestation. Illumination was provided through an LED light with a diameter of 5.2cm, operating voltage ≤36V. The LED light has an adjustable clip that allows it to be affixed to the top of the container. The colonies in the rearing cage were maintained throughout the experiment period at 27-29 °C and 78 ± 10% relative humidity.

Molecular Identification of an Adult Whitefly Sample

The sample of wild strain of adult whitefly, *B. tabaci*, collected was subjected to a molecular identification process. The whitefly specimen is submitted to the Centre for Insect Systematics (UKM), Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). DNA from the adult *B. tabaci* was extracted using NucleoSpin DNA Insect Kit (Macherey-Nagel, Germany). The universal primers, forward primer LCO1490 (5'GGTACAACAAATCATAAAGATATTGG-3') and reverse primer HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA-3') were utilised for Polymerase Chain Reaction (PCR) amplification (Folmer et al., 1994). PCR reactions were set up in a final reaction volume of 25 µL utilising a Techne Prime Thermocycler. The reaction mixture consisted of 12.5 uL Mastermix, 1 uL of each primer, 5 uL ddH_2O and 5 uL of DNA template. The PCR thermal cycling conditions were optimised for hemipteran targets, with initial denaturation for 30s at 95 °C, annealing for 1min at 47°C - 52 °C, extension for 30s at 72 °C, and a final extension for 10min at 72 °C. The PCR products were then electrophoresed at 100V on a 1.5% agarose gel for 20 min. Bands were visualised under

UV light using an Accuris Instruments transilluminator to confirm successful amplification of the target fragment (Syahrawati et al., 2025). Sequencing analysis was performed, and all obtained sequences were edited using Sequencher version 4.1.4, and sequence similarity was compared with those in the GenBank database using the BLASTn program of the National Centre for Biotechnology Information (NCBI).

Plant Sample Collection and Preparation of Aqueous Plant Extract

Seven locally available plants were sourced either from a local grocery store or collected from nature (Table 1). The plant species were chosen based on the following selection criteria: possessing insecticidal activities based on past literature search, ease of local availability, and accessibility. The plants collected were identified through structural characteristics and anatomical features. Further validation of the plant species was evaluated against the taxonomic standards of the Royal Botanic Garden, Kew, electronic data repository (Plants of the World Online [POWO], 2026). The plant parts used were washed thoroughly with tap water to remove dirt, insects and foreign elements before being left to air-dry at room temperature under shade. The air-dried plants were then pulverised using a Waring laboratory blender. The ground plant materials were immersed in distilled water at a 1:10 (w: v) solid to solvent ratio for 24 hours at room temperature to obtain an aqueous extract of each plant (Osmić et al., 2019). All the extracts obtained were filtered by using Whatman No.1 filter paper to obtain a homogenous extract that was used immediately for the bioassay. The efficacy of the extracts was assayed across a concentration range from 25,000 ppm to 100,000 ppm to establish the lethal concentration values (Wallace et al., 2021).

Table 1
List of plant samples screened for potential biopesticide activity against adult whitefly, B. tabaci

Scientific Name	Common Name	Family	Plant Parts Used	Source
Allium cepa	Onion	Amaryllidaceae	Bulb	Store bought[1]
Azadirachta indica	Neem	Meliaceae	Leaves	Collected[2]
Cymbopogon citratus	Lemongrass	Poacea	Leaves	Store bought[1]
Murraya koenigii	Curry leaves	Rutaceae	Leaves	Collected[3]
Ocimum sanctum	Holy basil	Lamiaceae	Leaves	Collected[2]
Phyllanthus acidus	Star gooseberry	Phyllanthaceae	Leaves	Collected[3]
Plectranthus amboinicus	Indian borage	Lamiaceae	Leaves	Collected[2]

Note. [1]- Segi Fresh Klebang, Chemor, Perak; [2]- 4° 36' 42" North, 101° 6' 48" East; [3]- 4° 41' 30" North, 101° 6' 26" East

Bioassay



Figure 1. The bioassay arena was utilised to evaluate the *B. tabaci* adult mortality

The adult mortality bioassay was conducted in accordance with the IRAC Susceptibility Test method number 008-2009 with slight modifications (Insecticide Resistance Action Committee [IRAC], 2009). Clear plastic cups of 80mm, 70mm, and 50mm (height: top diameter; base diameter) with a 250ml volume capacity, were used as the bioassay arena. The opening of the plastic cup was covered with a circular piece of fine netting material and closed with the plastic lid of a 60mm × 15mm (diameter × depth) petri dish with a circular hole of 30mm diameter. A 1-inch steel nail was used to pierce a hole in the bottom of the plastic cup. An uncontaminated young chilli leaf with a petiole approximately 25mm was then dipped in the tested extracts for 30 sec by holding the leaf by the petiole. The leaf was left to air dry for 5 minutes, and the petiole of the test leaf was passed through the hole in the bottom of the plastic cup. The petiole was then wrapped with wet cotton wool and aluminium foil to maintain turgidity of the leaves throughout the experiment (Figure 1). Ten individual whiteflies per replicate were evaluated. Distilled water represented the negative control. Commercially available organic pesticide, Baba Mr Ganick®, Aphid Buster with major active ingredient plant alkaloid, Faboideae, was used as a positive control. The percentage mortality was determined at 24 hours. A whitefly adult was considered dead if it did not respond to probing with a fine paintbrush. The 24-hour mortality data were corrected for control mortality by applying Abbott's formula (Piepho et al., 2024). To satisfy the assumptions of the One-Way Analysis of Variance (ANOVA) test, an arcsine square root transformation was performed. The assumption of normality and homogeneity of variance were confirmed using the Shapiro-Wilk test and Levene's test, respectively.

Synergistic Assay

Based on the probit analysis and findings from the bioassay, extracts with the lowest LC25 and LC50 values were selected for evaluation of their synergistic potential. Binary mixtures were prepared by using the sublethal, LC25 and lethal, LC50 concentrations of the aqueous plant extracts. The combinations of *A. indica*, *P. acidus* and *C. citratus* were made by mixing the extract in a ratio of 1:1 at the specific lethal doses. Individual extracts at LC25 and LC50 and 12 binary mixtures were assayed for lethal effects using the leaf

dip bioassay. Four replicates of each combination were used for the bioassay. Distilled water represented the negative control. The percentage mortality of adult whitefly was determined at 24 hours. Whitefly, without any detectable movement, even upon probing with a soft paintbrush, was classified as dead (Bandyopadhyay et al., 2014; Kulawardhana et al., 2018; Kumrungsee et al., 2022). All bioassays were conducted in the laboratory at 28 ± 2°C and 50 ± 10% relative humidity.

Data Analysis

All bioassays were done in quadruplicate, and the values were shown as the mean of adult whitefly mortality in percentage values along with their standard deviations (± SD). A one-way ANOVA test was performed to determine the mean difference of adult whitefly mortality in percentage between the treatment groups. Mean separation was performed using the Tukey HSD test. The adult whitefly mortality rate at different concentrations of the aqueous plant extracts was subjected to probit analysis to determine the lethal concentrations that caused 50% (LC50) and 95% (LC95) mortality. The data is log-transformed in order to satisfy the assumption of probit analysis. The analysis was performed using Statistical Package for the Social Sciences (IBM SPSS) version 27. The rate of mortality for adult whitefly in individual extracts was determined at LC25 and LC50. The percentage of mortalities due to the binary mixtures was obtained and compared to the expected mortalities based on the method proposed by Colby (1967). The expected efficacy was calculated using the formula as in Equation 1:

$$\% E = A + B - (AB/100) \quad [1]$$

Where A and B are the mortality values for the individual extracts.

The efficacies of the combinations were determined to be antagonistic, additive or synergistic by X^2 comparisons as in Equation 2:

$$X^2 = (O_m - E)^2 / E \quad [2]$$

Where O_m *is* the observed mortality due to binary mixtures, E is the expected mortality, X^2 with df= 1 and $\alpha = 0.05$ is 3.841. X^2 values > 3.841 with observed mortality greater than expected mortality represent a synergistic effect, X^2 values > 3.841 with observed mortality lower than the expected mortality constitute an antagonistic effect of the plant extracts mixtures, while X^2 values < 3.841 depict additive effects.

RESULTS AND DISCUSSION

Molecular Analysis of *B. tabaci*

Molecular characterisation in this study revealed that the whiteflies collected from the field in Kampung Ampang Baru, Ipoh, Perak have 100% sequence similarity with the *Bemisia tabaci* isolate WF-NP with Gen Bank Accession number OR646686 (Table 2). The isolation source is indicated as a leaf from a field in Coimbatore, Tamil Nadu, India, in the host plant Bhendi in the Tamil native language, or Okra (*Abelmoschus esculentus*). The insect sample in this study also shows 99% sequence similarity with the *Bemisia tabaci* isolate TNAU01 in host plant Okra, *Bemisia tabaci* isolate Coimbatore, *Bemisia tabaci* isolate Salem_Brinjal, and *Bemisia tabaci* isolate Asia 1 New Delhi Brinjal (Table 2) in host plant brinjal (*Solanum melongena*), respectively.

Table 2
Comparison of the generated sequence of B. tabaci*, isolated in this study from an infested vegetation area in Kampung Ampang Baru, Ipoh, Perak, with* B. tabaci *isolates available at NCBI*

Isolate Name	The Most Similar Sequences in Genbank	
	Gen Bank Accession Number	Sequence Similarity (%)
Bemisia tabaci isolate WF-NP	OR646686.1	100
*Bemisia tabac*i isolate TNAU01	MN911178.1	99.84
Bemisia tabaci isolate Coimbatore	MW488192.1	99.84
Bemisia tabaci isolate Salem_Brinjal	MW488189.1	99.84
Bemisia tabaci isolate Asia 1 New Delhi Brinjal	OQ706117.1	99.70

B. tabaci is a cryptic species complex with 46 cryptic species that includes Africa, Asia I, Asia II 1-12, Asia 1-India, Asia III, Asia IV, Asia V, Australia, Australia/Indonesia, China1-7, Indian Ocean, Ru, Middle East Asia Minor I-II (MEAM), Mediterranean (MED), MEAM K, New World 1-2. Japan 1-2, Uganda, Italy 1, and Sub-Saharan Africa 1-5 (Rehman et al., 2021). Although the interspecific differences may be subtle, the different cryptic species of *B. tabaci* also differ in terms of insecticide resistance, host preference, parasitoids associated with endosymbiont faunas and virus transmissions (MacLeod et al., 2022).

The findings of this study are consistent with prior research that reported the largest number of sequences presented from Malaysia were the Asia 1 species, which is also predominantly distributed in other countries of South East Asia (Mahmood et al., 2022). An earlier study also established an introductory map of *B. tabaci* species in Malaysia that shows recovery of the Asia-1 species in 9 out of 10 states sampled (Shadmany et

al., 2019). Another research has provided evidence for significant levels of resistance of Asia- 1 whiteflies against selected organophosphate, pyrethroid and neonicotinoid insecticides in cotton growing regions in India. The study strongly suggests that large-scale organophosphate insecticide usage by the Indian farmers, long-term exposure of *B. tabaci* to the insecticide, regular outbreak of crop diseases in endemic areas and mandatory seed dressing treatment resulted in increased resistance to the chemical insecticides (Naveen et al., 2017). Meanwhile, in a study conducted in Malaysia on a wild strain of adult *B. tabaci* belonging to the Asia 1 biotype, a high level of resistance against diafenthiuron was detected (Shadmany et al., 2015). The insecticide resistance often led farmers to increase the application frequency, often with higher doses, which further intensifies the resistance. Pest control failure due to insecticide resistance not only poses a severe threat to the crop's productivity but also reduces the quality of the produce.

Efficacy of Aqueous Plant Extracts Against Adult Whitefly, *B. tabaci*

All the aqueous extracts investigated in this study exhibited insecticidal activity against the adult whitefly. The percentage mortality of adult whitefly resulting from the tested extracts bioassayed against adult whitefly at different concentrations is presented in Table 3.

Table 3
Efficacy of different extracts against adult whitefly B. tabaci *24 hours post-treatment*

Treatment	Mean ± SD [95% CI] [a]
Allium cepa	73.68 ± 13.59 [52.06, 95.31]
Azadiracta indica	76.32 ± 10.08 [60.28, 92.35]
Cymbopogon citratus	89.47 ± 14.89 [65.79, 100.00]
Murraya koenigii	55.26 ± 15.79 [30.14, 80.39]
Ocimum sanctum	65.79 ± 10.08 [49.75, 81.83]
Organic pesticide	84.21 ± 13.59 [62.59, 100.00]
Phyllanthus acidus	86.84 ± 10.08 [70.81, 100.00]
Plectranthus amboinicus	57.89 ± 28.51 [12.54, 100.00]

Note. [a] One-way ANOVA was conducted on mortality data following an Abbott correction and an arcsine square root transformation. Shapiro-Wilk $W = 0.94$; $p = .094$; Levene's test $F = 0.42$; $p = .879$; ANOVA $F = 2.10$; $p = .083$

At the highest concentration, all the extracts produced more than 50% mortality of adult whitefly; nevertheless, chilli leaves treated with the aqueous extracts of *C. citratus* exhibited the highest percentage mean mortality (89.47%), followed by *P. acidus* (86.84%) and *A. indica* (76.32%) (Figure 2).

The lethal concentrations that cause 25%, 50% and 95% mortality of *B. tabaci* adults exposed to the treatments are shown in Table 4. Lowest LC25 values were given by *A. indica* (29530.11ppm), *C. citratus* (39445.68ppm), *P. amboinicus* (43299.98ppm) and *P. acidus* (52271.03ppm). Meanwhile, the lowest LC50 value was presented by the aqueous extract of *A. indica* (63678.0ppm), followed by *C. citratus* (65385.6ppm) and *P. acidus* (80449.5ppm). This indicates the extracts have high insecticidal activity against the whiteflies at lower concentrations. Lowest LC95 values were obtained from the aqueous extracts *C. citratus* (224246.2ppm), followed by the extracts *P. acidus (*230245.9ppm)*, M. koenigii* (250182.0ppm) and *O. sanctum* (261826.8ppm) (Table 4). Among the seven plant extracts, *C. citratus* and *P. acidus* were the most toxic to whiteflies after contact at high concentrations. Determination of the lethal concentrations is important for formulating an effective biopesticide as well as recommending an effective concentration to end users for targeted pest control while preventing excessive application. This ensures long-term efficacy of the biopesticides and minimises any risk of resistance development.

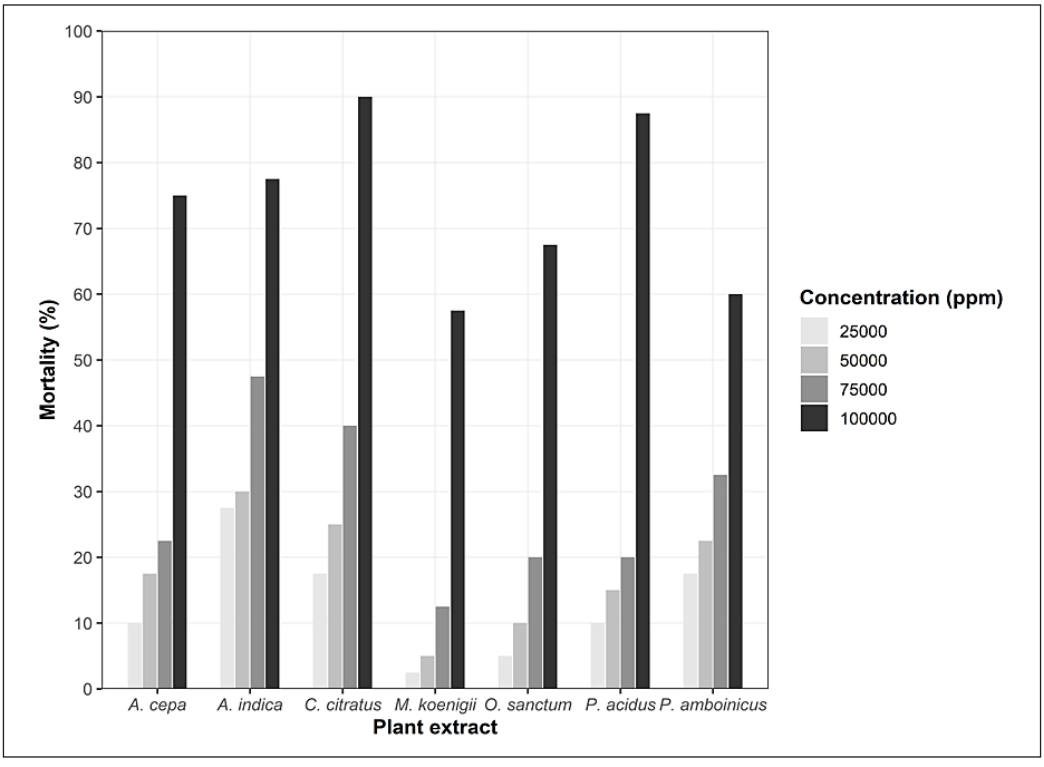

Figure 2. Percentage mortality of *B. tabaci* exposed to aqueous extracts at different concentrations(ppm) at 24 hours post-treatment

Table 4
Probit analysis of B. tabaci *exposed to selected aqueous extracts at different concentrations produced at 24 hours of extraction*

Plant Extract	n	Slope ± SE	LC25 [95% CI]	LC50 [95% CI]	LC95 [95% CI]	χ^2	df	p-value
Allium cepa	160	3.03 ± 0.60	52600.5 [43632.9, 63411.2]	87815.2 [71750.4, 107476.9]	306454.8 [164449.4, 571084.8]	28.45	14	0.012[b]
Azadiracta indica	160	2.02 ± 0.48	29530.1 [20114.0, 43354.3]	63677.9 [50350.8, 80532.5]	414791.2 [161938.2, 1062452.6]	12.32	14	0.581[a]
Cymbopogon citratus	160	3.07 ± 0.54	39445.7 [31784.6, 48953.4]	65385.6 [55676.4, 76788.1]	224247.3 [139443.7, 360624.5]	26.54	14	0.022[b]
Murraya koenigii	160	4.42 ± 1.01	74802.3 [65488.7, 85440.4]	106266.9 [87344.4, 129288.9]	250180.3 [145125.5, 431283.4]	46.75	14	< .001[b]
Ocimum sanctum	160	3.75 ± 0.76	63144.3 [54281.0, 73454.8]	95491.0 [79386.7, 114862.2]	261827.7 [153367.8, 446989.0]	22.92	14	0.062[a]
Phyllantus acidus	160	3.60 ± 0.65	52271.0 [44258.1, 61734.8]	80449.4 [68782.5, 94095.4]	230246.0 [145714.8, 363814.8]	56.31	14	< .001[b]
Plecranthus amboinicus	160	1.86 ± 0.50	43300.0 [31695.4, 59153.3]	99900.0 [68881.2, 144887.2]	767343.1 [193645.6, 3040686.0]	16.11	14	0.307[a]

Note. n = total number of whiteflies; $\chi 2$ = Pearson goodness-of-fit chi-square. Control mortality was corrected using the Abbott Formula, Slope = change in mortality probit per unit change in log10(concentration)
[a] $P > 0.05$ = the model is fit,
[b] $P < 0.05$= significant heterogeneity, Heterogeneity factor was used in the calculation of 95% CI

In order to facilitate the adoption of alternative pest control methods by farmers, it is necessary that their preparation and extraction processes be easy and viable (Nascimento et al., 2022). The plants used for the preparation of extracts in this study are commonly found and cultivated locally. This local availability ensures a consistent supply and increases their feasibility for application in small-scale farming. The selected plants also represent a diverse range of phytochemical compounds that have known insecticidal activity. The aqueous extraction carried out in this study using water as a solvent is a simple and sustainable extraction method without application of high heat or ultrasonic waves that can degrade bioactive compounds such as phenols and thermolabile compounds such as flavonoids (Mungwari et al., 2024). Water is considered the greenest extraction solvent as it is non-toxic to human health and the environment.

The recovery of phytochemicals from plant materials is influenced by many factors, including the solubility of the compounds in the extraction solvent, the choice of solvent, the degree of polymerisation of the bioactive compound, the interaction of the active compound with other plant constituents, as well as the extraction technique and duration (Yannick Stéphane et al., 2021). Water is a highly polar solvent with the ability to extract a wide range of polar substances, while also being low-cost, non-toxic and non-flammable (Abubakar & Haque, 2020). A previous study has reported that extraction using water resulted in a higher yield of extract compared to methanol (Singh et al., 2017).

Past literature has also revealed the efficacy of aqueous plant extracts against other phytophagous insects. The aqueous extracts (10% w/v, i.e., 100g of plant powder per litre of water) of ten pesticidal plant species, *A. indica, Ocimum basilicum, Nicotiana tabacum, C. citratus, Tephrosia vogelii, Aloe vera, and Lantana camara. Trichillia emetica, Vernonia amygdalina* and *Lippia javanica* were assessed for their contact toxicity and antifeedant properties against the fall armyworm, *Spodoptera frugiperda*. The study demonstrates that out of the ten screened species, the four most promising for efficacy were shown by the extracts of *L. javanica, O. basilicum, C. citratus,* and *A. indica* (Phambala et al., 2020). Another study reported that 10% aqueous extract of unpeeled neem seed sprayed on a papaya plant significantly stops the feeding of aphid, *Aphis nerii* and showed significantly higher mortality compared to the control (Hernández-Castro et al., 2005).

The bioassay in this study revealed the lowest lethal concentration value, expressed by *A. indica, C. citratus* and *P. acidus. A. indica* is widely recognised for its biopesticidal activity stemming from a range of bioactive compounds, including limonoids such as azadirachtin, salannin, meliantriol, and nimbin. However, the extraction of limonoids into aqueous solvents such as water is limited, the mortality of the adult whitefly in this study is most likely triggered by water-soluble bioactive constituents such as saponins, phenolic compounds, tannins, flavonoids and cardiac glycosides present in neem (Kumar et al.,

2018). These compounds are also known to induce strong antifeedant activity and inhibit insect growth (Agbo et al., 2019; Juma et al., 2022). A past study reported that neem-derived saponins exhibited strong insecticidal activity by inhibiting acetylcholinesterase (AChE) in the insect pests' nervous system. Inhibition of AChE is responsible for antifeedant behaviours, repellence and mortality in insect pests (Sami et al., 2018).

The primary bioactive constituent largely attributed to the pesticidal activity of *C. citratus* is the monoterpene, citral, composed of two isomers: geranial and neral, along with other compounds such as myrcene, limonene and linalool (Faria & Barbosa, 2024; Pinto et al., 2015). Although these major compounds are predominantly lipophilic with minimal aqueous solubility in water, the presence of these compounds, even at low concentrations, is known to exert significant insecticidal activity due to their high intrinsic toxicity to insects (Kobenan et al., 2021). Phytochemical analysis in previous studies has revealed the presence of water-soluble secondary metabolites: tannins, saponins, reducing sugars, glycosides, flavonoids, and other phenolic compounds in the *C. citratus* (Alzobaay & Kadhim, 2018; Unuigbe et al., 2019). Saponins are natural surfactants that can act as a natural emulsifying agent (Akbari et al., 2023; Kregiel et al., 2017), which may facilitate the dispersion of hydrophobic compounds like terpenes to form microemulsions that contribute to the insecticidal activity of the lemongrass extract. *P. acidus* is not only used for its edible fruit but also for a wide spectrum of diseases (Tan et al., 2020); however, its insecticidal activity has not been conclusively established and still remains an area of ongoing investigation. Previous studies have characterised the bioactive compounds found in *P. acidus*, including cyclohexene-1-(2-propenyl), jatrorrhizine, ethoxyquin, and isocorilagin. These bioactive substances are known to exert insecticidal activities by disrupting the midgut, interfering with the neurotransmission and enzymatic activity, ultimately causing death in insect pests (Divekar et al., 2022; Gope & Rawani, 2025).

Synergistic Interaction of Selected Individual Extracts at Sublethal and Lethal Concentrations

Individual extracts of *A. indica*, *P. acidus* and *C. citratus* at 25% and 50% sublethal and lethal concentrations were evaluated against the adult whitefly using the leaf-dip bioassay. Application of the binary extract of *P. acidus* at LC25 and *C. citratus* at LC50 and *A. indica* at LC25 and *P. acidus* at LC50 shows a synergistic effect with X^2 values > 3.841. Meanwhile, the following combination of *A. indica* and *P. acidus* at LC25:LC25, *A. indica* and *C. citratus* at LC25:LC25, *A. indica* and *C. citratus* at LC50:LC50, *A. indica* and *P. acidus* at LC50:LC25, *P. acidus* and *C. citratus* at LC50:LC25, *A. indica* and *C. citratus* at LC50:LC25 and *A. indica* and *C. citratus* at LC25:LC50 showed an additive effect with X^2 values < 3.841. A combination of the

extract *P. acidus* and *C. citratus* at LC25:LC25, *A. indica* and *P. acidus* at LC50:LC50 and *P. acidus* and *C. citratus* at LC50:LC50 reveals antagonistic effects with observed mortality values lower than the expected mortality (Table 4).

The binary extract of *A. indica* and *P. acidus* shows an additive effect at sublethal concentration, LC25; however, when the concentration of both extracts increased to LC50, the interaction becomes antagonistic. The mixtures of *A. indica* and *C. citratus* give an additive effect at both LC25 and LC50. The research findings show that a higher concentration of *A. indica* than *P. acidus* and *C. citratus* exhibits an additive effect. Increased concentration of *C. citratus* than *A. indica* also shows an additive effect. Nevertheless, when the concentration of *P. acidus* is higher than *that of A. indica, it causes a* synergistic effect. A similar concentration of *P. acidus* combined with *C. citratus* gives an antagonistic effect at both LC25 and LC50. Higher concentration of *P. acidus* than *C. citratus* gives an additive effect, but when the concentration of *C. citratus* increases, it induces a synergistic effect (Table 5).

A combination of extracts provides a broader spectrum of insecticidal activity with its multicomponent nature, delaying any potential resistance development (Selvamuthukumaran et al., 2024). The varied mechanism of action of the bioactive compounds in the extracts, which includes repellence, antifeedant, insecticidal and growth regulators, could act additively or synergistically (Dougoud et al., 2019). The synergistic or additive effects between extracts by which the lethal effects increase and the concentration of individual extracts can be reduced without affecting the efficacy can be exploited further in the development of a cost-effective biopesticide. Past research has also reported the effectiveness of combining extracts in managing pests. Earlier research found that the mixture of a common weed, *Datura stramonium* and neem, *A. indica* extracts was able to suppress the population of whiteflies, *B. tabaci,* on tomato, *Solanum lycopersicum* (Maina et al., 2025).

Prior study also reported synergistic interaction between the combined extracts of cinnamon, *Cinnamomum zeylanicum,* with *C. citratus* that exhibited high acaricidal activity compared to individual treatment against the larvae of *Rhipicephalus (Boophilus) microplus* (Singh et al., 2019). Synergistic effect between herbaceous shrub, Indian catmint, *Anisomeles malabarica* and *P. emblica* caused statistically significant larvicidal activity against the malaria vector, *Anopheles stephensi* (Jayakumar et al., 2014). In the current study, the synergistic effect of the mixture of *P. acidus* and *C. citratus* and *A. indica* and *P. acidus* at a specific lethal concentration resulted in higher mortality of adult whitefly compared to the individual treatments. This existing literature suggests that the combinations of extracts result in promising efficacy against insect pests.

Table 5
Mortality of the whitefly adults 24 h post-treatment with binary mixtures of aqueous plant extracts at their LC25 and LC50 values

Extract A	Extract B	Lethal Concentration	Final concentration of extract (ppm)	Adult mortality (%)				χ^2	Effect
				Individual extract		Binary Mixtures			
				Observed A	Observed B	Expected	Observed		
Azadirachta indica	*Phyllanthus acidus*	LC25:LC25	14785.06: 26135.52	22.5	17.5	36.1	30	1.02	Additive
Phyllanthus acidus	*Cymbopogon citratus*	LC25:LC25	26135.52: 19722.84	17.5	25	38.1	25	4.52	Antagonistic
Azadirachta indica	*Cymbopogon citratus*	LC25:LC25	14785.06: 19722.84	22.5	25	41.9	52.5	2.70	Additive
Azadirachta indica	*Phyllanthus acidus*	LC50:LC50	31838.95: 40224.70	30	25	47.5	32.5	4.74	Antagonistic
Phyllanthus acidus	*Cymbopogon citratus*	LC50:LC50	40224.70: 32692.80	25	30	47.5	17.5	18.95	Antagonistic
Azadirachta indica	*Cymbopogon citratus*	LC50:LC50	31838.95: 32692.80	30	30	51.0	60	1.59	Additive
Azadirachta indica	*Phyllanthus acidus*	LC50:LC25	31838.95: 26135.52	30	17.5	42.3	45	0.18	Additive
Phyllanthus acidus	*Cymbopogon citratus*	LC50:LC25	40224.70: 19722.84	25	25	43.8	50	0.89	Additive
Azadirachta indica	*Cymbopogon citratus*	LC50:LC25	31838.95: 19722.84	30	25	47.5	47.5	0.00	Additive
Azadirachta indica	*Phyllanthus acidus*	LC25:LC50	14785.06: 40224.70	22.5	25	41.9	55	4.11	Synergistic
Phyllanthus acidus	*Cymbopogon citratus*	LC25:LC50	26135.52: 32692.80	17.5	30	42.3	72.5	21.66	Synergistic
Azadirachta indica	*Cymbopogon citratus*	LC25:LC50	14785.06: 32692.80	22.5	30	45.8	37.5	1.49	Additive

Synergistic interaction often relies on the content, interaction between the different bioactive compounds, susceptibility of the target pest, as well as concentrations and ratios of individual components. A previous study on the synergistic interaction of thymol with *Piper ribesioides* extracts and isolated active compounds against *Spodoptera exigua* concluded that the synergism between plant extract and secondary metabolite depends on the composition of the extract and specific doses used (Kumrungsee et al., 2022). The higher potency of combined extracts at lower concentrations offers economic advantages in developing a cost-effective pest control approach as well as reducing the risk of resistance development. Smallholder farmers with limited technical resources can benefit from pest management strategies based on locally available resources.

CONCLUSION AND IMPLICATION OF THE STUDY

This study shows the potential of plant-derived insecticides, with all evaluated extracts achieving over 50% mortality against adult whiteflies. The aqueous extracts of *A. indica*, *C. citratus* and *P. acidus* exhibited promising pesticidal activity against adult whitefly with lower LC50 values. Additionally, the bioassay performed also revealed synergistic interaction between the mixtures of *P. acidus* and *C. citratus*, as well as *A. indica* and *P. acidus* under laboratory conditions.

The synergistic combinations of these plant extracts highlight their potential for optimising biopesticide formulations against *B. tabaci*. Utilising natural insecticides promotes sustainable agricultural practices and is generally safer for application on edible plants, as they are less likely to persist in the environment. Ultimately, incorporating biopesticides into small- scale farming can potentially reduce reliance on synthetic pesticides and minimise their associated risks.

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Production of Antioxidant-Rich Feather Hydrolysate by *Alcaligenes faecalis* MEK1 and Its Evaluation in Mung Bean Under Salinity Stress

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ABSTRACT

Poultry feather waste is a protein-rich resource whose valorisation is constrained by the recalcitrant structure of keratin. This study evaluated feather bioconversion by *Alcaligenes faecalis* MEK1 and the antioxidant and seed-priming properties of the resulting hydrolysate under salinity stress. Temperature, initial pH, feather concentration, and incubation time were evaluated using reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency. Antioxidant capacity was determined using DPPH, ABTS, nitric oxide scavenging, and ferric-reducing assays, followed by mung bean germination and salinity-stress experiments. Based on the combined responses, 37 °C, pH 8.0, and 1.5% (w/v) feather substrate were selected as working conditions. Reduction-associated activity reached 129.30 U/mL at 72 h, followed by maximum keratinase activity of 5.74 U/mL at 96 h, whereas soluble protein concentration and feather degradation reached 1.18 g/L and 74.90%, respectively, at 168 h. The hydrolysate exhibited concentration-dependent antioxidant activity across the four assays. Seed priming with 0.5% (v/v) hydrolysate increased germination from 87.87% to 98.24%, reduced mean germination time from 2.874 to 1.758 days, and increased root length from 5.918 to 7.060 cm. Under 100 mM NaCl, hydrolysate treatment reduced hydrogen peroxide from 18.9 to 10.7 µmol/g fresh weight and malondialdehyde from 28.6 to 15.4 nmol/g fresh weight while increasing superoxide dismutase, catalase, and peroxidase activities. These findings demonstrate the potential of MEK1 feather hydrolysate as a seed-priming material for improving early mung bean responses under controlled salinity stress.

Keywords: Antioxidant, feather hydrolysate, feather waste valorisation, mung bean, salinity stress, seedling vigour

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INTRODUCTION

Feather waste is a valuable protein-rich byproduct of poultry processing, but its valorisation is limited by the recalcitrant keratin structure. Feather keratin contains high proportion of sulfur-rich proteins stabilised by disulfide bonds, hydrogen bonding, and hydrophobic interactions, making the substrate insoluble and resistant to ordinary proteolysis (Chaitanya Reddy et al., 2021; Das et al., 2024; Kumar et al., 2026). Conventionally, feather waste has been disposed of through chemical treatment, open-air burning, incineration, and landfilling, which help reduce waste accumulation but often require high energy input, generate undesirable secondary waste, and result in the loss of valuable peptides and amino acids (Chaitanya Reddy et al., 2021; Kumar et al., 2026). Therefore, recent studies suggest that microbial conversion offers a more sustainable alternative because keratinolytic microorganisms can hydrolyse the feather keratin under milder conditions through keratinases and associated biochemical reactions that enhance the protein solubilisation and preserve the resulting hydrolysate (Brandelli et al., 2010; Qiu et al., 2020). This approach is relevant for circular waste management because it helps in the conversion of feather waste from the poultry industry into valuable protein hydrolysates, enzymes and nutrient-rich products with high potential in agriculture and industrial biotechnology (Enciso-Tenorio et al., 2025; Nad et al., 2024; Park et al., 2023).

Microbial feather degradation should not be defined only as a keratinase-driven bioprocess because native feather keratin remains protected by disulfide-stabilised structural packing. Biological reduction weakens these disulfide constraints and increases the exposure of peptide regions for keratinase-mediated hydrolysis into soluble proteins, peptides and amino acids (Qiu et al., 2020; Q. Wang et al., 2025). This reductive capacity provides a biological substrate-controlled opening mechanism compared to severe thermal or chemical solubilisation, which may compromise the quality of functional properties of the resulting hydrolysate through process severity and amino acid loss (Karuppannan et al., 2021; Sharma & Kango, 2021). Combined reductase and keratinase systems have been shown to accelerate feather degradation and increase soluble and functional bioactive protein or amino acid recovery compared with keratinase-centric systems, supporting the need to evaluate the reductive and proteolytic activities together (Fan et al., 2026; Ji et al., 2024; Li et al., 2023). Therefore, *A. faecalis* MEK1 feather hydrolysate is positioned in this study as a biologically produced hydrolysate generated through coordinated reductase-associated and keratinase activities for functional antioxidant application.

The potential agricultural value of feather hydrolysate is associated with its soluble peptides and amino acids, which may support nutrient availability, osmotic adjustment, and stress-responsive metabolism during seed germination. Protein-derived hydrolysates have been reported to improve germination, root and shoot development, biomass accumulation, and crop productivity after application (Adelere & Lateef, 2023; Kaur et al., 2021;

Roy & Jílková, 2025). Under salinity, amino acid- and peptide-based biostimulants may also enhance antioxidant defense, including superoxide dismutase, catalase, and peroxidase activities, thereby limiting hydrogen peroxide accumulation, lipid peroxidation, and associated growth inhibition (Gezgincioğlu & Atici, 2023; Zuluaga et al., 2023). These reported responses provide a scientific basis for evaluating MEK1 feather hydrolysate in salt-stressed mung beans. However, osmotic adjustment and nutrient uptake were not measured directly in the present study; therefore, hydrolysate effects were interpreted using germination, seedling growth, antioxidant enzyme activities, and oxidative-damage markers. Recent studies have shown that feather hydrolysates can exhibit DPPH, ABTS, and ferric-reducing activities and may improve germination, growth, or yield in mung bean, spinach, French bean, and other crops (Cavello et al., 2021; Devi et al., 2024; Ismail et al., 2025; Kaur et al., 2021; Kshetri et al., 2020; Stanly & Umesh, 2024). Studies involving *Alcaligenes* species have mainly focused on keratinase production, feather degradation, amino acid recovery, or organic-fertiliser applications (Otuyelu et al., 2025; Yusuf et al., 2016). However, limited research has integrated reduction-associated and keratinolytic activities with hydrolysate antioxidant performance and plant responses under salinity within the same experimental design. The effective concentration of an *A. faecalis*-derived hydrolysate for mung bean seed priming and its association with salinity-related oxidative stress also remain insufficiently defined. Therefore, the novelty of this study lies in producing feather hydrolysate using *A. faecalis* MEK1, characterising its antioxidant activity, and evaluating its concentration-dependent effects on mung bean germination and oxidative-stress responses under controlled salinity.

This study hypothesised that feather hydrolysate produced by *A. faecalis* MEK1 through reduction-associated and keratinolytic activities would improve mung bean germination and seedling vigour under salinity, accompanied by enhanced antioxidant defense and reduced oxidative damage. Feather bioconversion was evaluated across temperature, pH, substrate concentration, and incubation time using reduction-associated activity, keratinolytic activity, soluble-protein release, and degradation efficiency as responses. The resulting hydrolysate was subsequently assessed for antioxidant activity, concentration-dependent seed-priming effects, seedling growth, and biochemical responses to 100 mM NaCl. Accordingly, this study aimed to determine whether *A. faecalis* MEK1 could convert recalcitrant feather waste into an antioxidant-active hydrolysate capable of improving mung bean performance under controlled salinity stress.

MATERIALS AND METHODS

Materials, Chemicals, and Reagents

All chemicals and reagents used in this study were of analytical grade unless otherwise stated. Nutrient agar and nutrient broth were obtained from Oxoid Ltd. (Basingstoke, UK).

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate heptahydrate, sodium chloride, sodium hydroxide, hydrochloric acid, trichloroacetic acid, potassium persulfate, potassium ferricyanide, ferric chloride, methanol, phosphoric acid, thiobarbituric acid, sulfosalicylic acid, ninhydrin, hydrogen peroxide and sodium hypochlorite were purchased from Merck KGaA (Darmstadt, Germany). 5,5′-Dithiobis-2-nitrobenzoic acid, L-cysteine, L-tyrosine, DPPH, ABTS, Trolox, ascorbic acid, sodium nitroprusside, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, nitroblue tetrazolium, riboflavin, methionine and guaiacol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent and bovine serum albumin were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Fresh chicken feathers were collected from a poultry slaughterhouse in Putrajaya, Malaysia, while mung bean seeds were obtained from a local agricultural supplier in Selangor, Malaysia. Distilled water was used for all reagent preparation and experimental procedures.

Microorganism Identity, Revival, and Inoculum

A. faecalis MEK1 was used as a potent bacterial strain. The strain was previously identified by full-length 16S rRNA gene sequencing using the universal primers 27F and 1492R. Sequence similarity was determined using BLASTn, and phylogenetic analysis was performed using MEGA11 based on the maximum likelihood method with 1000 bootstrap replicates. The 16S rRNA gene sequence was deposited in GenBank with accession number PX096348.1. For inoculum preparation, *A. faecalis* MEK1 was revived from glycerol stock on nutrient agar. The agar was incubated at 37 °C for 24 h. Single colony was transferred into 10 mL nutrient broth and incubated at 37 °C with agitation at 150 rpm for 18 h. The bacterial culture was adjusted to OD_{600} 0.8 ± 0.05 before use.

Preparation of Chicken Feather Keratin Substrate

White chicken feathers were collected from a poultry slaughterhouse in Putrajaya, Malaysia. The feathers were used as the keratin substrate for the study. The feathers were washed with tap water and mild alkaline detergent to remove blood, dirt, and organic matter, followed by rinsing with distilled water. The cleaned feathers were dried at 60 °C until dry and cut into 0.5 to 1.0 cm fragments. The prepared feather substrate was sterilised at 121 °C for 15 min before use in submerged feather keratin bioconversion.

Submerged Feather Keratin Bioconversion

Keratin bioconversion was done in 250 mL Erlenmeyer flasks containing 100 mL minimal basal media supplemented with chicken feather keratin substrate. The minimal basal media contained 0.4 g potassium dihydrogen phosphate, 0.3 g dipotassium hydrogen phosphate,

0.1 g magnesium sulfate heptahydrate, and 0.5 g sodium chloride in 1 L. Each flask was inoculated with 1% v/v *A. faecalis* MEK1 inoculum and incubated at 35 °C, initial pH 7.0, and 150 rpm for 7 days. The initial chicken feather keratin substrate concentration was at 1.0% w/v unless otherwise stated in the optimisation experiment.

Hydrolysate Harvesting and Clarification

After submerged feather keratin bioconversion, the culture mixture was filtered by vacuum filtration to separate the liquid feather hydrolysate from residual feather material. The recovered hydrolysate was centrifuged at 10,000 × g for 10 min, and the supernatant was collected as crude feather hydrolysate for subsequent enzymatic, soluble protein, antioxidant, and plant bioassay analyses. Residual feather material retained after filtration was used for determination of feather degradation efficiency.

Preparation of Chemical Feather Hydrolysate Comparators

Chemical feather hydrolysate comparators were prepared using NaOH-treated and NaOH plus autoclave-treated chicken feather keratin substrate. For NaOH treatment, feather substrate was mixed with 1 M NaOH at 1.5% w/v chicken feather keratin and incubated at 35 °C for 120 h. For NaOH plus autoclave treatment, the feather substrate was mixed with 1 M NaOH and autoclaved at 121 °C for 15 min. After treatment, all the hydrolysates were cooled, neutralised to pH 7, centrifuged at 10,000×g for 10 min, and filtered to obtain the soluble fraction. The soluble protein concentration of each hydrolysate was determined at approximately 0.65 g/L before antioxidant analysis to allow comparison with *A. faecalis* MEK1 feather hydrolysate.

Enzymatic Activities, Soluble Protein and Feather Degradation Assays

Keratinase activity was determined using crude feather hydrolysate as the enzyme source. The hydrolysate was centrifuged at 10,000×g for 10 min, and the supernatant was mixed with 0.05 M phosphate buffer at pH 7.4 and 1% w/v sterilised chicken feather substrate. The reaction mixture was incubated at 37 °C for 60 min and terminated with 0.4 M trichloroacetic acid. After centrifugation at 5,000×g for 10 min, absorbance was measured at 280 nm. Keratinase activity was calculated from an L-tyrosine standard curve and expressed as U/mL using Equation 1. 1 unit represents the amount of enzyme required to release 1 µmol of L-tyrosine per min under assay conditions.

$$Keratinase\ Activity\ (U/mL) = \frac{\mu mol\ of\ L-tyrosine}{volume\ (mL) \times time\ (min)} \quad [1]$$

Reductase-associated activity was determined using Ellman's assay based on sulfhydryl group release from chicken feather substrate. The crude enzyme was mixed with a Tris-EDTA buffer containing 30 mM Tris-HCl and 3 mM EDTA at pH 8.2, along with 1% w/v chicken feather substrate. The reaction mixture was incubated at 37 °C for 60 min and terminated with 0.4 M trichloroacetic acid. After centrifugation at 5,000×g for 10 min, the supernatant was reacted with 3 mM DTNB for 10 min at 25 °C, and absorbance was measured at 412 nm. Reductase-associated activity was calculated from an L-cysteine standard curve and expressed as U/mL using Equation 2. 1 unit represents the release of 1 μmol of sulfhydryl groups per min under the assay conditions.

$$Reductase-associated\ Activity\ (U/mL) = \frac{\mu mol\ of\ sulfhydryl\ groups}{volume\ (mL) \times time\ (min)} \quad [2]$$

For both keratinolytic and reduction-associated activity assays, substrate, enzyme, and reagent blanks were prepared by omitting the enzyme preparation, substrate, or both components, respectively, while maintaining the same final reaction volume. A zero-time control was prepared by adding the reaction-stopping reagent immediately after enzyme-substrate mixing without incubation. The heat-inactivated enzyme control was prepared by heating the enzyme preparation at 95 °C for 5 min, followed by immediate cooling on ice for 5 min, before analysis under the corresponding assay conditions. Complete inactivation was assumed from the severity of the heat treatment because residual activity was not independently quantified. For the TCA-precipitated control, the enzyme preparation was treated with 15% (w/v) trichloroacetic acid before substrate addition, incubated briefly to allow protein precipitation, and centrifuged at 8000 rpm for 10 min at 4°C; the resulting protein-depleted supernatant was then subjected to the respective assay, with its pH adjusted to the assay conditions where required. The same blank definitions, heat-inactivation procedure, and TCA-precipitation approach were applied consistently to both enzyme assays to correct background signals and determine whether the measured responses were attributable to active soluble proteins.

Soluble protein concentration was determined using the Bradford assay with bovine serum albumin as the standard, using Equation 3, and absorbance was measured at 595 nm. Feather degradation efficiency was determined by the dry weight loss of residual feather material after filtration and drying at 60 °C until constant weight. Degradation efficiency was calculated as the dried unhydrolysed feather keratin substrate using Equation 4.

$$Soluble\ Protein\ Concentration\ (g/L) = \frac{Absorbance\ at\ 595\ nm}{Slope\ of\ standard\ curve} \quad [3]$$

$$Feather\ Degradation\ (\%) = \frac{(Wo - Wt)}{Wo} \times 100\% \quad [4]$$

W_0 represents the initial dry feather mass before treatment, while Wt represents the remaining dry feather mass after treatment or incubation

One Factor at Time Optimisation

Keratin bioconversion was optimised using one factor at a time. The tested parameters included incubation temperatures at 25 to 50 °C, initial pH at 5.0 to 11.0, feather keratin substrate concentrations at 0.5 to 2.0% w/v, and incubation time up to 7 days. The effects of each parameter were evaluated based on reduction-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency. The optimum condition was selected based on the combined response of enzyme activities, protein solubilisation, and feather degradation.

Antioxidant Capacity of Feather Hydrolysate

The antioxidant capacity of *A. faecalis* MEK1 feather hydrolysate and chemical feather hydrolysate comparators was determined using DPPH radical scavenging, ABTS radical scavenging, nitric oxide scavenging, and ferric reducing power assays. Hydrolysate samples were tested at 0.02 to 0.65 g/L soluble protein. For DPPH analysis, the sample was mixed with 0.1 mM DPPH solution and incubated in the dark at 25°C for 30 min before absorbance was measured at 517 nm. For ABTS analysis, ABTS radical solution was prepared using ABTS and potassium persulfate, incubated in the dark for at least 12 h, diluted to an absorbance of 0.70 at 734 nm, and reacted with the sample for 6 min before measurement at 734 nm. Nitric oxide scavenging activity was determined using sodium nitroprusside and Griess reagent, with absorbance measured at 546 nm. Ferric reducing power was determined using potassium ferricyanide and ferric chloride, with absorbance measured at 700 nm. Ascorbic acid was used as the positive control for DPPH, nitric oxide scavenging, and ferric reducing power assays, while Trolox was used as the standard for ABTS analysis. Radical scavenging activity was calculated relative to the blank using Equation 5, and IC50 values for DPPH, ABTS, nitric oxide scavenging, and ferric reducing power were estimated from dose-response curves using nonlinear regression.

$$Radical\ Scavenging\ (\%) = \frac{A_c - A_s}{A_c} \times 100\% \quad [5]$$

Where A_c is the absorbance of the control or blank reaction and A_s is the absorbance of the sample.

Determination of Hydrolysate Working Concentration for Mung Bean Seed Priming

The seed-priming and germination procedure was adapted from previous studies evaluating feather-derived or animal-protein hydrolysates as plant biostimulants (W. Wang et al., 2022), with modifications to the hydrolysate concentration and priming duration. Mung bean seeds were sterilised with 1% w/v sodium hypochlorite for 15 min and rinsed with distilled water. The sterilised seeds were primed with feather hydrolysate at 0, 0.25, 0.5, 1.0, and 2.0% v/v for 6 h. After priming, 5 seeds were transferred onto sterile filter paper in each Petri dish. The seeds were incubated at 25 °C for 7 days. Germination and early seedling growth were evaluated based on germination percentage (Equation 6), mean germination time (Equation 7), germination index (Equation 8), root length, shoot length, total seedling length, fresh weight, and dry weight. The hydrolysate concentration that induces growth without inhibiting it was selected for salinity stress validation.

$$Germination\ (\%) = \frac{N_g}{N_t} \times 100\% \quad [6]$$

Where N_g is the number of germinated seeds and N_t is the total number of seeds.

$$Mean\ germination\ time = \frac{\sum n_i t_i}{\sum n_i} \quad [7]$$

Where n_i is the number of seeds germinated on day i, and t_i is the incubation time in days.

$$Germination\ index = \frac{\sum G_i}{\sum T_i} \quad [8]$$

Where G_i is the number of seeds germinated each day and T_i is the corresponding day of counting.

Salinity Stress Validation and Plant Biochemical Assays

Mung bean seedlings were evaluated for the stress-mitigating effect of MEK1 feather hydrolysate under salinity stress, with slight modifications to previously reported procedures (Shafique et al., 2019; Singh & Choudhary, 2025). Surface-sterilised seeds were primed for 6 h with either distilled water or 0.5% (v/v) feather hydrolysate and then transferred to sterile filter paper in Petri dishes. Salinity stress was imposed immediately after seed placement by moistening the filter paper with 100 mM NaCl solution. Four treatments were established: water-primed seeds supplied with distilled water as the untreated control, hydrolysate-primed seeds supplied with distilled water, water-primed seeds exposed to 100 mM NaCl, and hydrolysate-primed seeds exposed to 100 mM NaCl. The non-

saline treatments received an equal volume of distilled water, and the filter papers were maintained with the corresponding solution once throughout the experiment. All treatments were conducted in triplicate and incubated at 25 °C for 7 days. Thus, the NaCl-treated seedlings were exposed continuously to 100 mM NaCl from the beginning of germination until harvest. Fresh seedlings were collected on day 7 for the determination of hydrogen peroxide, proline content, malondialdehyde, superoxide dismutase, and catalase responses.

Hydrogen peroxide content was measured using the potassium iodide method and expressed as µmol/g fresh weight. Lipid peroxidation was determined as malondialdehyde content using the thiobarbituric acid reactive substances assay (Aguilar Diaz De Leon & Borges, 2020). Absorbance was recorded at 532 nm and corrected at 600 nm to remove nonspecific turbidity.

Proline content was measured using the acid ninhydrin method described by (Forlani & Funck, 2020). Fresh tissue was extracted in sulfosalicylic acid, reacted with acid ninhydrin, and the absorbance was measured at 520 nm. Proline concentration was expressed as µmol/g fresh weight. Crude enzyme extract was prepared from fresh seedling tissue by buffer homogenisation and centrifugation. Soluble protein concentration in the extract was measured by the Bradford assay using bovine serum albumin as the standard.

Superoxide dismutase activity was determined using the nitroblue tetrazolium inhibition method of Durak et al. (1993), where one unit represented 50% inhibition of NBT reduction. Catalase activity was measured by following hydrogen peroxide decomposition at 240 nm according to Hadwan et al. (2024). Peroxidase activity was measured from hydrogen peroxide-dependent substrate oxidation with adjustment from Nelson and Parsonage (2011). All antioxidant enzyme activities were normalised to soluble protein concentration and expressed as U/mg protein.

Statistical Analysis

All experiments were performed in triplicate, and results were expressed as average ± standard deviation. Raw data were organised using Microsoft Excel 365, while statistical analysis and graph preparation were performed using GraphPad Prism version 10. Data from one factor at a time of optimisation, antioxidant assays, and hydrolysate working concentration experiments were analysed using one-way analysis of variance followed by Tukey's multiple comparison test. For salinity stress validation, one-way analysis of variance was used to determine the effects of NaCl and feather hydrolysate. IC50 values for DPPH, ABTS, and nitric oxide scavenging activities were estimated from dose-response curves using nonlinear regression. Statistical significance was accepted at $p < 0.05$. Phylogenetic analysis of *A. faecalis* MEK1 was performed separately using MEGA11.

RESULTS AND DISCUSSION

Effect of Temperature, Initial pH and Feather Keratin Substrate Concentration

Temperature, initial pH, and feather substrate concentration affected feather bioconversion by *A. faecalis* MEK1, with different response patterns observed across reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency (Figures 1 to 3). Temperature showed the clearest optimum at 37 °C, where reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency reached 88.76 U/mL, 4.00 U/mL, 0.98 g/L, and 61.60%, respectively. These values were higher than those at 25 °C by 43.80%, 115.40%, 56.70%, and 54.70%, while all responses declined at 50 °C. For pH, reductase-associated activity was highest at pH 8.00 with 88.24 U/mL, while keratinase activity was highest at pH 7.00 with 4.19 U/mL and remained close between pH 8.00 and 9.00. However, soluble protein concentration and feather degradation efficiency increased further at pH 11.00, reaching 1.17 g/L and 77.10%, suggesting that higher alkalinity favored solubilisation and mass loss more than balanced enzymatic activity. For feather substrate concentration, reductase-associated activity peaked at 1.25% w/v with 90.23 U/mL, whereas keratinase activity, soluble protein concentration, and degradation efficiency were highest at 1.50% w/v with 4.06 U/mL, 0.99 g/L, and 63.23%, respectively. All responses declined at 2.00% w/v. Based on the combined response rather than a single maximum value, 37 °C, pH 8.00, and 1.50% w/v feather substrate were selected as the working conditions for MEK1 feather bioconversion.

The selected 37 °C, pH 8.00, and 1.50% w/v feather substrate agree with previous optimisation of feather-based keratinase production by *A. faecalis* IHB B 6507 and with other bacterial keratinolysis studies that commonly report mesophilic temperature, neutral to mildly alkaline pH, and moderate feather loading as suitable conditions for keratinase production and feather conversion (Akram et al., 2021; Otuyelu et al., 2025). However, the MEK1 result shows why reductase-associated activity should be included in process optimisation. Keratinase activity alone would favor pH 7.00, while soluble protein release and feather degradation would favor pH 11.00. In contrast, pH 8.00 retained keratinase activity and gave the highest reduction-associated activity, suggesting a more balanced condition for biological feather conversion. This agrees with studies showing that keratin degradation depends on proteolysis and weakening of the disulfide rigid structure either through sulfur metabolism, disulfide reduction, or combined reductase and protease systems (Fan et al., 2026; Ji et al., 2024; Kumari et al., 2024; Luo et al., 2026; Qiu et al., 2020).

Evidence from wool keratin studies also supports the same principle, where disulfide bond reduction improves protease accessibility, although these studies should be used only as structural support for feather keratin interpretation (Khumalo et al., 2020; Li et al., 2024; Q. Wang et al., 2025). Accordingly, pH 8.0 was selected as a balanced working condition because it maintained both reductase-associated and keratinolytic activities

without selecting the process solely based on alkaline solubilisation or feather mass loss. This supports a broader definition of keratinolytic performance in potency of keratinolytic microorganisms, reduction of disulfide bonds, keratinase activity, soluble protein release, and feather degradation are considered together.

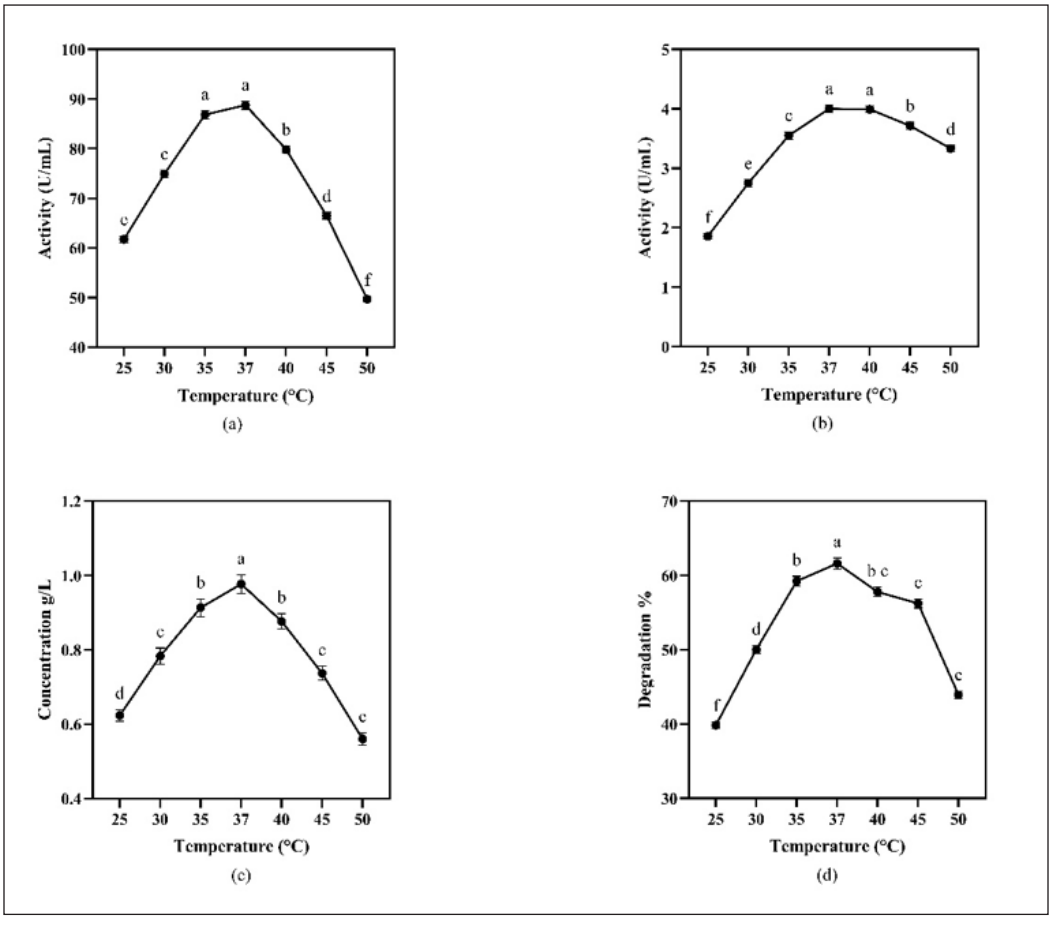

Figure 1. Effect of incubation temperature on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

Note. Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among incubation temperatures were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

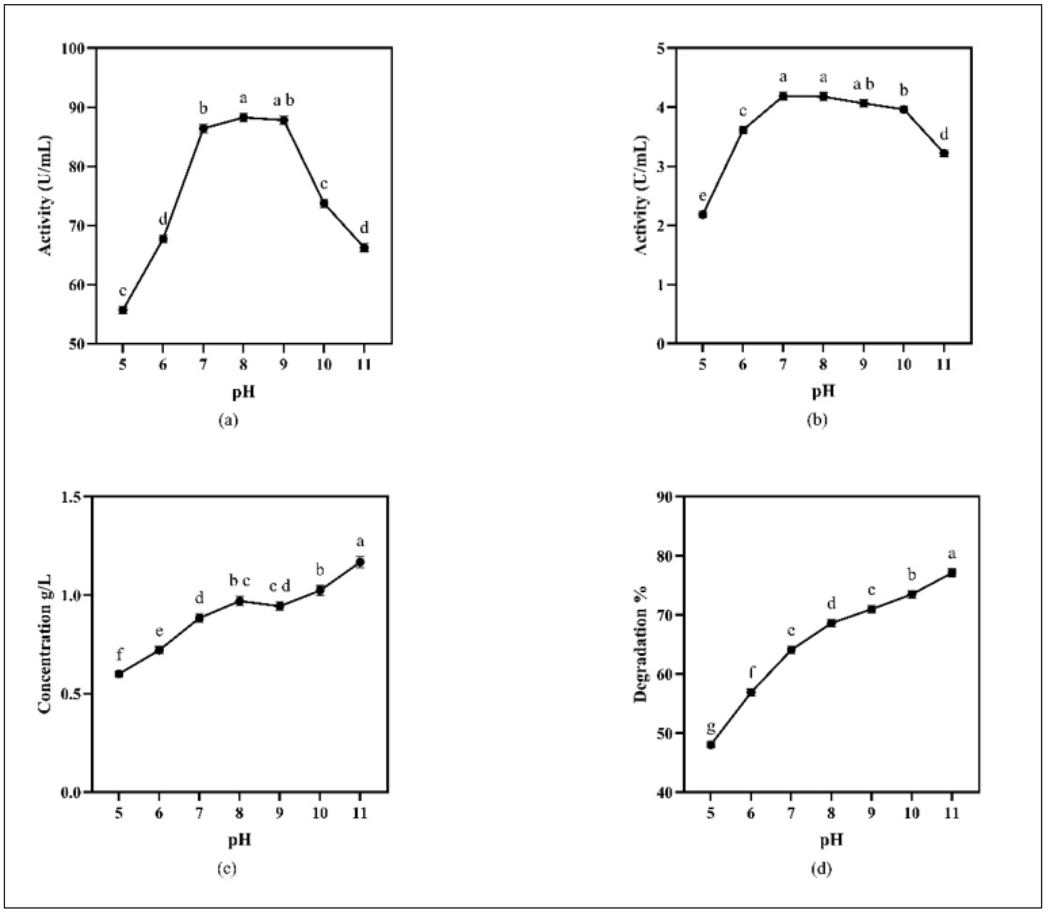

Figure 2. Effect of initial medium pH on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

Note. Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among initial pH levels were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

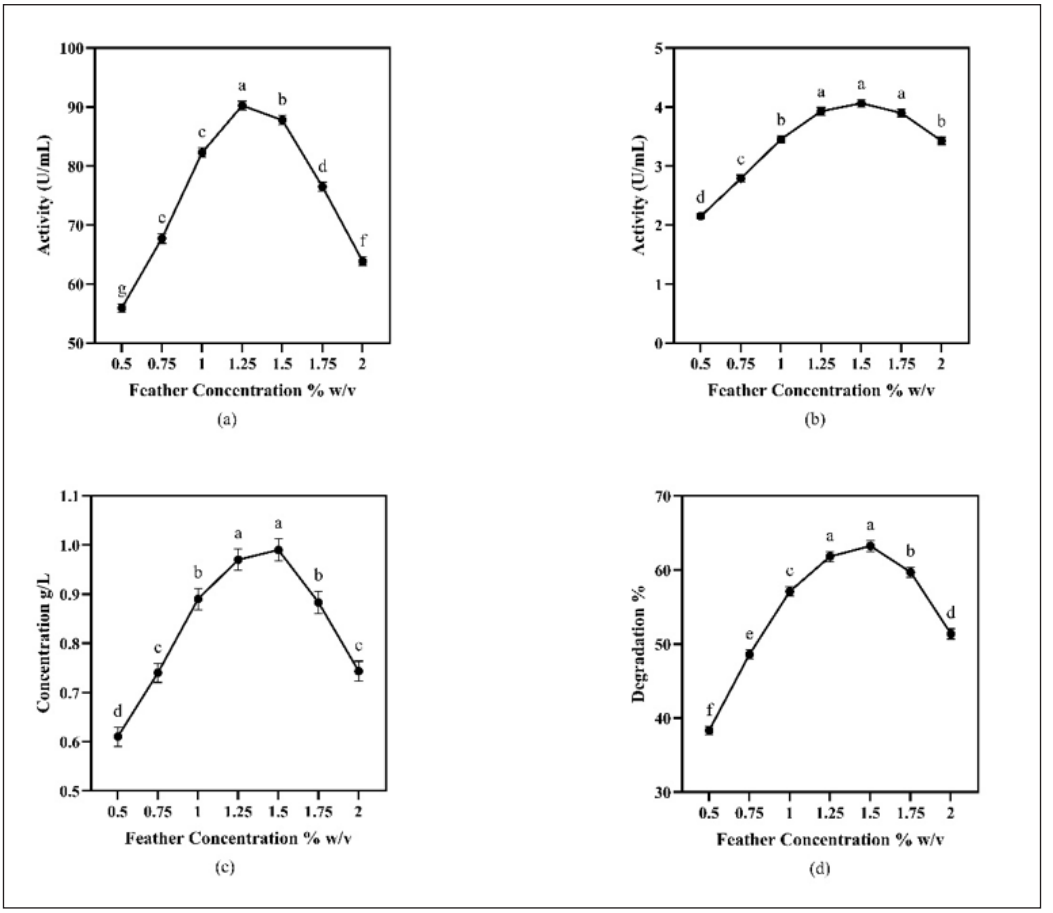

Figure 3. Effect of chicken feather keratin substrate concentration on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

Note. Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among feather substrate concentrations were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

Effect of Incubation Time on Feather Bioconversion

Incubation time affected reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency during *A. faecalis* MEK1 feather bioconversion (Figure 4). Reductase-associated activity peaked earlier at 72 h with 129.30 U/mL, then declined to 73.50 U/mL at 168 h. Keratinase activity reached its highest later at 96 h with 5.74 U/mL, while soluble protein concentration and feather degradation efficiency continued to increase until 168 h, reaching 1.18 g/L and 74.90%, respectively. Compared with 24 h, the maximum keratinase activity, soluble protein concentration, and feather

degradation efficiency increased by 373.29%, 408.62%, and 477.49%, respectively. This result suggests that sulfhydryl group release was more evident during the earlier stage of feather bioconversion, while keratinase activity, protein release, and feather degradation became more noticeable during the later stage.

Reductase-associated activity peaked at 72 h, keratinase activity peaked at 96 h, and soluble protein concentration, together with feather degradation efficiency, reached their highest values at 168 h. The temporal sequence is consistent with a reduction-assisted model of keratinolysis in which weakening of disulfide constraints may increase substrate accessibility to subsequent proteolytic attacks.

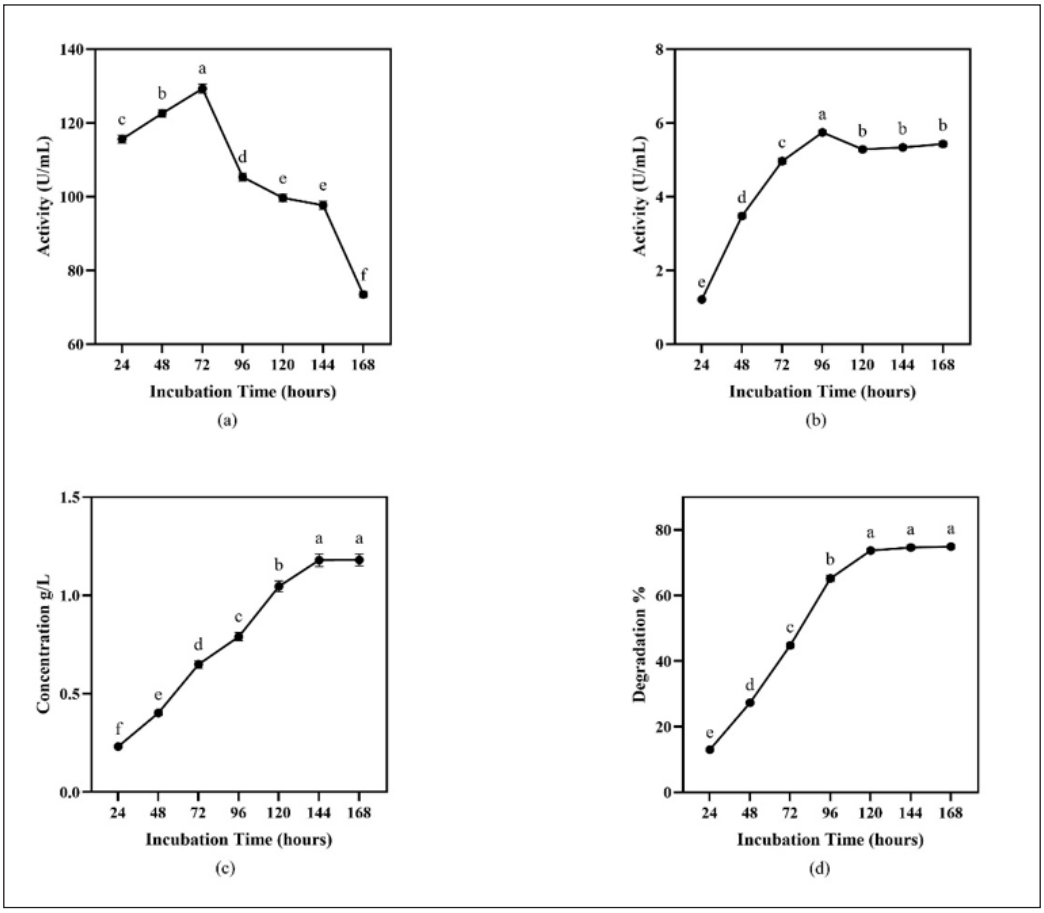

Figure 4. Effect of incubation time on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

Note. Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among incubation times were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

However, the present time-course experiment does not independently establish that the earlier reductase-associated response caused the subsequent increase in keratinase activity or feather degradation. Similar time-dependent patterns have been reported in *Bacillus* sp. P45, *Bacillus* sp. MBRL 575, *Stenotrophomonas maltophilia* R13, and *Chryseobacterium* sp. RBT, where sulfhydryl group release increased during early fermentation, followed by keratinase activity, soluble protein release, or feather degradation (Daroit et al., 2010; Gurav et al., 2020; Jeong et al., 2010; Kshetri et al., 2020).

Mechanistic-related studies also support this pattern by showing that sulfur metabolism, cysteine and cystine cycling, disulfide bond reduction, and coupled reductase and protease activity can contribute to keratin degradation, which we suggest that *A. faecalis* MEK1 may follow a similar reduction-assisted keratinolysis pattern (Lai et al., 2023; Peng et al., 2019; Qiu et al., 2020; B. Wang et al., 2024; Zhou et al., 2023). In *Alcaligenes* sp., previous studies have shown feather degradation and keratinase production by *Alcaligenes* sp. AQ05 001 and *A. faecalis* IHB B 6507, but the temporal relationship between reductase-associated activity, keratinase activity, protein release, and feather degradation has not been clearly validated (Otuyelu et al., 2025; Yusuf et al., 2016). The MEK1, therefore, adds a process-level insight by showing that in *Alcaligenes faecalis* sp., the reductase-associated activity appeared earlier than the keratinase activity, while soluble protein release and feather degradation increase over time.

Antioxidant Capacity of Feather Hydrolysates

The antioxidant capacity of *A. faecalis* MEK1 feather hydrolysate differed among assays, with clearer activity observed in DPPH radical scavenging activity, nitric oxide scavenging activity, and ferric reducing power than in ABTS radical scavenging activity (Figure 5). In the DPPH assay, MEK1 feather hydrolysate showed the lowest IC50 at 0.1933 g/L, compared with 0.2519 g/L for NaOH-treated hydrolysate and 0.4585 g/L for NaOH plus autoclave-treated hydrolysate. A similar pattern was observed for nitric oxide scavenging activity, where MEK1 feather hydrolysate recorded the lowest IC50 at 0.1674 g/L, followed by NaOH-treated hydrolysate at 0.2040 g/L and NaOH plus autoclave-treated hydrolysate at 0.2210 g/L. In ferric reducing power, MEK1 feather hydrolysate showed stronger reducing activity at lower concentration, although NaOH-treated hydrolysate showed a higher fitted maximum response. In contrast, ABTS radical scavenging activity showed that NaOH-treated hydrolysate had a slightly lower IC50 than MEK1 feather hydrolysate, while MEK1 feather hydrolysate showed the highest fitted maximum response.

The antioxidant capacity of MEK1 feather hydrolysate supports the earlier decision to avoid selecting bioconversion conditions based only on maximum solubilisation or feather mass loss. MEK1 feather hydrolysate showed lower IC50 values than the chemical hydrolysates in DPPH radical scavenging activity and nitric oxide scavenging activity, and

it also showed stronger ferric reducing power at lower concentration. The results indicate that microbial feather bioconversion produced a hydrolysate with comparatively strong antioxidant activity in several assay systems, although it did not produce the highest response in every assay. Differences in peptide size, amino-acid composition, and redox-active residues may have contributed to the assay-dependent responses. However, these compositional characteristics were not determined.

In contrast, NaOH plus autoclave-treated hydrolysate showed weaker activity in several assays, suggesting that severe chemical and thermal treatment may increase keratin solubilisation but compromise the resulting hydrolysate. This agrees with studies showing that antioxidant capacity in feather hydrolysates is influenced not only by soluble protein concentration, but also by peptide size, composition of amino acids, and redox-active residues such as tyrosine, cysteine, methionine, tryptophan, and histidine (Alahyaribeik et al., 2021; Bezus et al., 2021; Fakhfakh et al., 2011; Jana et al., 2022; Pei et al., 2023). Together, these findings show that MEK1 can convert feather keratin into an antioxidant active product under milder processing conditions. This supports its potential use as a value-added feather hydrolysate, while reducing dependence on chemical treatment during feather valorisation.

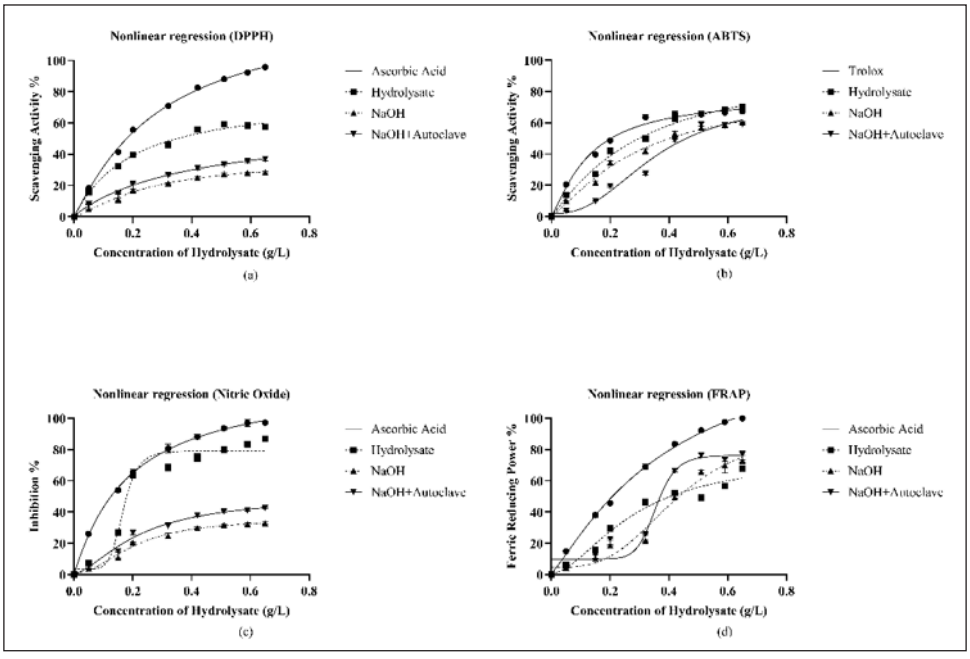

Figure 5. Antioxidant capacity of feather hydrolysates. (a) DPPH radical scavenging activity, (b) ABTS radical scavenging activity, (c) nitric oxide scavenging activity, and (d) ferric reducing power

Note. Ascorbic acid was used as the positive control for DPPH radical scavenging activity, nitric oxide scavenging activity, and ferric reducing power, while Trolox was used as the standard for ABTS radical scavenging activity.

Determination of Effective Hydrolysate Concentration for Seed Priming

Hydrolysate concentration influenced mung bean germination and early seedling growth, with 0.5% v/v giving the best overall response (Figure 6). At this concentration, root length increased from 5.918 to 7.060 cm, shoot length from 0.988 to 1.676 cm, and total seedling length from 6.906 to 8.736 cm compared with the control. Fresh weight and dry weight also increased from 0.818 to 1.188 g and from 0.188 to 0.284 g, respectively. Germination percentage improved from 87.87% to 98.24%, while mean germination time decreased from 2.874 to 1.758 days. In contrast, higher hydrolysate concentrations at 1.0% and 2.0% v/v reduced most growth responses relative to 0.5% v/v. These results suggest that MEK1 feather hydrolysate improved germination and seedling growth within an effective concentration range, rather than showing a dose-dependent increase. Therefore, 0.5% v/v was selected as the working concentration for subsequent salinity stress validation because it produced the strongest non-inhibitory response across germination, seedling growth, and biomass accumulation.

The MEK1 feather hydrolysate was effective only within a suitable dose range, rather than increasing seedling growth. This pattern agrees with previous reports showing that feather hydrolysates can improve seed germination, root growth, shoot growth, biomass,

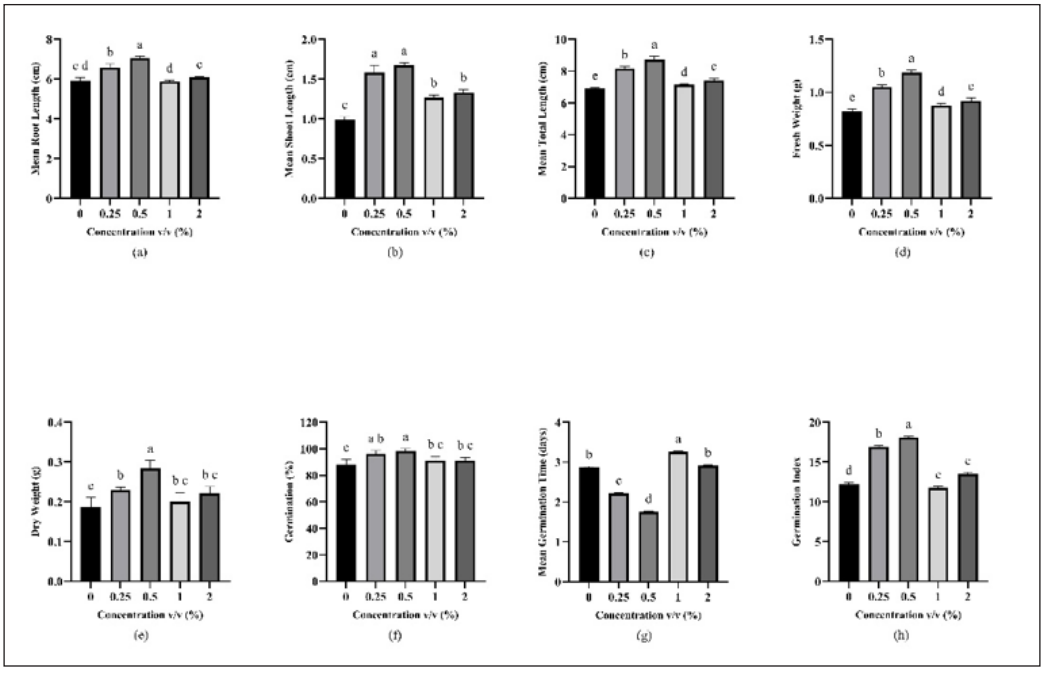

Figure 6. Effect of feather hydrolysate concentration on mung bean germination and early seedling growth. (a) mean root length, (b) mean shoot length, (c) mean total seedling length, (d) fresh weight, (e) dry weight, (f) germination percentage, (g) mean germination time, and (h) germination index

and early seedling establishment, with the best response commonly observed at low or intermediate concentrations (Adelere & Lateef, 2023; Bhange et al., 2016; Devi et al., 2024; Kaur et al., 2021; Paul et al., 2013; Stanly & Umesh, 2024). Similar findings have also shown that higher hydrolysate concentrations may reduce plant growth responses, possibly due to excess soluble peptides, amino acids, salts, or nitrogen compounds that can affect early seedling growth (Broutin et al., 2025; Jain et al., 2016; Możejko & Bohacz, 2023). In this study, 0.5% v/v MEK1 feather hydrolysate improved germination speed, seedling length, and biomass, suggesting that this concentration supported early growth without causing inhibition due to high concentration. This result provides a practical working concentration for seed priming and supports further testing of MEK1 feather hydrolysate under salinity stress, where the objective was to determine whether the hydrolysate could protect seedlings under stress rather than only improve growth under normal conditions.

Salinity Stress Validation of Hydrolysate-Treated Mung Bean Seedlings

Salinity stress increased oxidative stress markers and reduced antioxidant enzyme activity in mung bean seedlings, while 0.5% v/v MEK1 feather hydrolysate reduced these stress effects (Figure 7). Hydrogen peroxide increased from 8.4 µmol/g FW in the control to 18.9 µmol/g FW under 100 mM NaCl, while malondialdehyde increased from 11.2 to 28.6 nmol/g FW. In the salt plus hydrolysate treatment, hydrogen peroxide decreased to 10.7 µmol/g FW and malondialdehyde decreased to 15.4 nmol/g FW, corresponding to reductions of 43.4% and 46.2% compared with salt stress alone. Proline increased from 9.6 µmol/g FW in the control to 17.3 µmol/g FW under salt stress, but decreased to 11.2 µmol/g FW when hydrolysate was supplied with salt. Salt stress also reduced superoxide dismutase, catalase, and peroxidase activities to 48.7, 15.6, and 10.3 U/mg protein, respectively, while salt plus hydrolysate increased them to 61.8, 29.5, and 18.7 U/mg protein. The hydrolysate-only treatment remained close to the control, suggesting that 0.5% v/v MEK1 feather hydrolysate did not impose detectable oxidative stress under non-saline conditions.

Treatments were controlled, 0.5% v/v feather hydrolysate, 100 mM NaCl, and 100 mM NaCl plus 0.5% v/v feather hydrolysate. The stress validation result shows that MEK1 feather hydrolysate was associated with reduced oxidative damage and stronger antioxidant enzyme responses. The decrease in hydrogen peroxide and malondialdehyde suggests lower reactive oxygen accumulation and lower lipid peroxidation, while the recovery of superoxide dismutase, catalase, and peroxidase activities suggests stronger enzymatic antioxidant defense under salt exposure. The lower proline level in the salt plus hydrolysate treatment should be interpreted as a reduced stress burden rather than loss of osmotic adjustment, because it occurred together with lower oxidative damage

and higher antioxidant enzyme activity. This trend agrees with studies showing that protein hydrolysates and feather-derived hydrolysates can improve plant stress tolerance by supporting antioxidant defence and reducing oxidative injury during abiotic stress (Devi et al., 2024; Gezgincioğlu & Atici, 2023; Kaur et al., 2021; Stanly & Umesh, 2024; Zuluaga et al., 2023).

In relation to the earlier antioxidant assays, the salinity result provides biological validation that the hydrolysate was not only active in chemical antioxidant models but also associated with lower oxidative damage in seedlings. The observed response may involve combined contributions from soluble peptides, amino acids, antioxidant-active components, and modulation of endogenous antioxidant defense. However, their relative contributions were not determined.

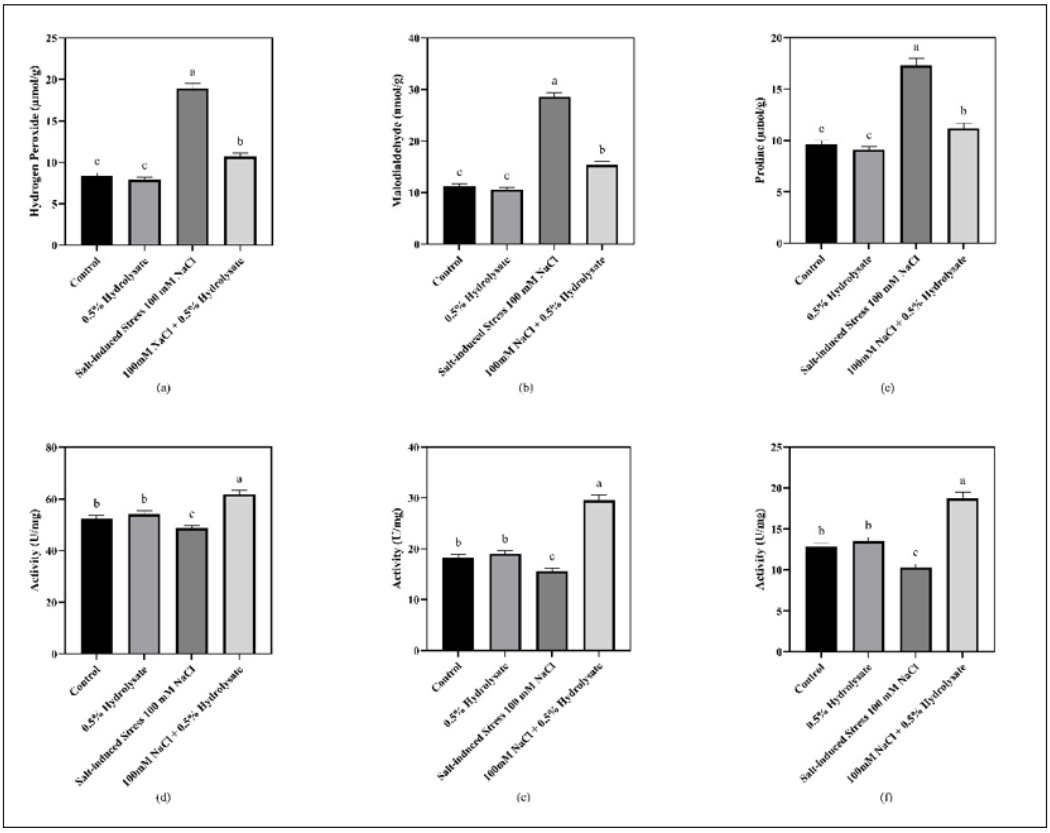

Figure 7. Effect of MEK1 feather hydrolysate on oxidative stress markers and antioxidant enzyme activity in mung bean seedlings under salinity stress. Panels show (a) hydrogen peroxide content, (b) malondialdehyde content, (c) proline content, (d) superoxide dismutase activity, (e) catalase activity, and (f) peroxidase activity

CONCLUSION

A. faecalis MEK1 converted chicken feather keratin into an antioxidant feather hydrolysate through submerged fermentation. The optimum conditions were observed at 37 °C, pH 8.00, and 1.50% w/v feather substrate supported reductase-associated activity, keratinase activity, soluble protein release, and feather degradation. The time course pattern suggests that reductase-associated activity occurred earlier than peak keratinase activity, followed by soluble protein accumulation and feather degradation. This finding suggests that MEK1 feather bioconversion is better explained by combining sulfhydryl release and proteolysis, rather than keratinase activity alone. The hydrolysate also showed consistent antioxidant activity in DPPH radical scavenging, nitric oxide scavenging, and ferric reducing power assays, while chemical treatment did not consistently improve antioxidant performance. At 0.5% v/v, MEK1 feather hydrolysate improved mung bean germination, reduced germination time, and enhanced root growth, shoot growth, and biomass. Under salinity stress, hydrolysate reduced hydrogen peroxide, malondialdehyde, and proline accumulation while enhancing superoxide dismutase, catalase, and peroxidase activities. Further work should validate its performance under soil, greenhouse, and field conditions and identify the active peptide or amino acid fractions responsible for the observed plant response.

IMPLICATION OF THE STUDY

The main implication of this study is that chicken feather waste should be viewed as a recoverable source of functional hydrolysate. *A. faecalis* MEK1 provides a mild biological route for converting feather keratin into an antioxidant-active hydrolysate with measurable effects on mung bean germination and salinity response. The study also suggests that MEK1 is a potent keratin degrader due to its ability to reduce disulfide bonds and proteolysis. This also prevents the process from being evaluated only by mass loss or chemical solubilisation, which may not reflect hydrolysate quality. The antioxidant assays and seedling results show that MEK1 hydrolysate has functional value at a practical working concentration of 0.5% v/v. At this concentration, it improved germination, seedling growth, and biomass, while under salinity stress, it reduced oxidative damage and supported antioxidant enzyme activity. These findings position MEK1 feather hydrolysate as a potential natural seed priming material for early stress protection. More broadly, the study strengthens the link between poultry waste valorisation, microbial bioconversion, and crop stress management through a product that can be evaluated by both biochemical function and plant response.

LIMITATION AND RECOMMENDATION FOR FUTURE RESEARCH

Advanced optimisation of microbial feather conversion has increasingly shifted from sequential one-factor testing toward multivariable statistical designs that can identify significant process variables, quantify factor interactions, and generate predictive operating

models. Plackett-Burman screening has been used to identify influential parameters such as feather concentration, pH, temperature, inoculum level, and nutrient composition, followed by response surface methodology using central composite or Box–Behnken designs to refine the selected factors and maximise keratinase production or feather hydrolysis (Derhab et al., 2024; Ismail et al., 2025). These approaches have produced approximately 1.5- to 13.3-fold improvements over unoptimised conditions and have generally shown close agreement between predicted and experimentally validated responses (Ismail et al., 2025; Sharma et al., 2022). Statistical optimisation has also supported scale-up in solid-state and semi-pilot fermentation systems, although performance remains sensitive to substrate heterogeneity, oxygen and moisture transfer, pH control, enzyme stability, and reactor configuration (El Salamony et al., 2024). More computational approaches, including artificial neural networks, genetic algorithms, Bayesian optimisation, kinetic modelling, and model-based bioreactor control, may offer additional advantages for highly nonlinear or multi-response systems, but their application to feather-hydrolysate production remains comparatively limited. These methods were not used in the present study and are relevant mainly as future strategies for simultaneous optimisation of enzyme activity, soluble-protein release, feather degradation, product bioactivity, and scale-up efficiency.

Although microbial feather hydrolysates and related protein hydrolysate biostimulants have produced promising responses under controlled conditions, their agronomic effectiveness remains dependent on crop species, dose, soil properties, environmental conditions, and application method. Feather hydrolysate increased barley biomass in pot experiments but did not significantly improve barley yield under field conditions, demonstrating that laboratory or greenhouse responses may not translate directly into agronomic performance. Concentration-dependent effects have also been reported, with effective application levels ranging from 0.2-0.4% (v/v) for maize seedling elongation to approximately 5% (v/v) for broader improvements in plant growth, soil fertility, and microbial activity (Jagadeesan et al., 2023). Formulation and storage conditions, therefore, require systematic evaluation because peptide composition, soluble protein concentration, antioxidant activity, and microbial quality may change during storage, although short-term stability at 4 °C has been reported for chicken-feather protein hydrolysate (Jagadeesan et al., 2023). Microbial feather conversion can reduce the accumulation of keratin-rich waste and recover amino acids and bioactive peptides, but residual feather solids, microbial biomass, process water, energy consumption, and downstream treatment requirements must be considered when evaluating its overall environmental benefit (Jagadeesan et al., 2023; Jain et al., 2016). Accordingly, claims of circularity, reduced fertiliser dependence, or commercial feasibility remain provisional in the absence of comparative life cycle and techno-economic assessments. The present study did not include field validation, formulation development, long-term storage testing, waste-stream characterisation, or

formal techno-economic analysis. These aspects should therefore be evaluated before the laboratory-observed improvements in mung bean germination, seedling vigour, and oxidative-stress responses are interpreted as evidence of field performance or commercial applicability.

ACKNOWLEDGEMENT

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