ISSN NO 2320 - 8694

Journal of Experimental Biology and Agricultural Sciences

Volume No. 13

Issue No. 3

September- December 2025

ENRICHED PUBLICATIONS PVT. LTD

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ISSN NO 2320 - 8694

Journal of Experimental Biology and Agricultural Sciences

Managing Editor
Mr. Amit Prasad

ISSN NO 2320 - 8694

Journal of Experimental Biology and Agricultural Sciences

(Volume No. 13, Issue No. 3, Sep- Dec 2025)

Contents

MOLECULAR PROFILING OF BLAST RESISTANCE GENES IN LOCAL MALAYSIAN RICE (Oryza sativa L.)

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ABSTRACT

Paddy fields in Malaysia have been affected by rice blast disease since the early years of rice cultivation, and it continue to suffer from its devastating effects due to the rapid evolution of the causal pathogen Magnaporthe oryzae. Although Malaysia is home to various local indica rice, the potential for resistance among these resources remains limited. This study aims to investigate the distribution of blast resistance genes in Malaysian rice cultivars and to categorize them into clusters based on genetic similarities. We extracted DNA from 75 rice accessions and performed molecular genotyping for four blast resistance genes (denoted as Pi) using allele-specific functional markers: the major genes Pi33 (RM72) and Pi27(t) (Rm259), as well as the minor genes pi21 (RM1359) and Pi26 (RM413). The results of this profiling analysis were used to construct a hierarchical dendrogram for cluster analysis. The findings revealed that Pi33 had the highest distribution frequency at 60.00%, followed by Pi27(t) at 49.33%, Pi26 at 34.67%, and pi21 at 21.33%. All rice accessions were grouped into eight clusters, with four accessions, viz., Malinja, Bahagia, Serendah, and Bario, classified together in Cluster 8 for harboring 100% of the Pi genes. Meanwhile, Cluster 1 included 17 candidates with the highest similarity to the widely used resistant variety IR64. In conclusion, this study provides a framework for marker-assisted breeding to develop blast-resistant rice cultivars with multigenic resistance, contributing to sustainable rice production in Malaysia.

KEYWORDS : Blast resistance gene SSR markers Indica rice Genetic diversity Gene pyramiding

INTRODUCTION

Rice (Oryza sativa L.) is a vital staple crop for global consumption and the second-largest agricultural commodity produced in Malaysia (Department of Statistics Malaysia 2023). Despite efforts to secure a reliable rice supply to meet domestic demand, the country's self-sufficiency level hovers around 56%. In 2024, Malaysian rice granary areas, which cover approximately 280,000 to 300,000 hectares, produced only about 2.2 million tonnes of rice, 8% below the average yield (Food and Agriculture Organization of the United Nations 2024). This declining trend is heavily influenced by both biotic (pests and diseases) and abiotic (drought, flooding, and high salinity) stresses. Major biotic stresses, particularly blast disease, bacterial leaf blight (BLB), sheath blight (SB), and sheath brown rot (SBR), continue to pose significant challenges for breeders and farmers (Sabri et al. 2023). Among these diseases, rice blasts have the highest incidence in Peninsular Malaysia, potentially spreading across 5,000 hectares per year and resulting in substantial economic losses in rice production (Szulczyk 2022). Consequently, recurrent outbreaks of blast disease have prompted researchers to harness resistance ® genes to develop high-yielding rice varieties that can withstand this disease. Magnaporthe oryzae (Hebert) Barr., the fungus responsible for rice blast disease, is a filamentous and haploid ascomycetous organism that produces pyriform (pear-shaped) conidia (Pedrozo et al. 2025). M. oryzae is the most extensively studied species within the order Magnaporthales due to its high evolutionary rate, characterized by rapid mutations in pathogenicity (Mandal et al. 2023).

It is recognized as an effective pathogen due to its morphological adaptations that facilitate infection. A crucial feature of this fungus is developing a specialized structure called the appressorium. This appressorium serves as an essential infection apparatus that enables M. oryzae to penetrate various above-ground plant tissues, including leaves (leaf blast), panicles (panicle blast, also known as neck blast), nodes, culms, sheaths, and glumes throughout all developmental stages of rice via high turgor pressure (Wengler and Talbot 2025). Postinfection symptoms of leaf blast are observed as lesions or spots on the leaves that initially appear white to gray-green with darker borders. As these lesions mature, they turn whitish-gray with a necrotic red-brown border in a diamond shape. However, the severity of these lesions varies based on rice varietal resistance (Lin et al. 2018; Nurul Nahar et al. 2020; Pedrozo et al. 2025). Local rice cultivation is at risk because this pathogen thrives on airborne conidia in Malaysia's tropical climate, characterized by moderate temperatures (~24 °C), high relative humidity (~90%), high moisture levels, and at least 7 hours of leaf wetness (Pedrozo et al. 2025). Unpredictable climate change further accelerates fungal proliferation and virulence mutations, causing periodic resurgences of disease outbreaks. This evolution means some durable, resistant rice varieties with a single major R gene gradually lose their resistance and become susceptible to the disease after several planting seasons (Tian et al. 2022). Gene pyramiding through marker-assisted selection breeding (MAS) has become central to introgressing multiple major R genes into a single commercial variety to mitigate this issue. Currently, approximately 122 resistance (R) genes and alleles have been identified from the Oryza sativa Japonica cultivar (45%), the indica cultivar (51%), and other wild species (4%) (Manojkumar et al. 2020). This collection includes 66 major R genes, 17 minor R genes, and 39 cloned R genes that span all 12 rice chromosomes (Pedrozo et al. 2025). The distribution of these genes across the chromosomes is uneven, with the majority clustered on chromosomes 6, 11, and 12, which harbor 23, 29, and 25 resistance genes, respectively. In contrast, chromosomes 3 and 7 have the fewest blast resistance genes, with only one identified on each: Pi66(t) on chromosome 3 and Pi17(t) on chromosome 7 (Pedrozo et al. 2025). Major R genes typically confer broad-spectrum resistance against multiple strains of M. oryzae with varying virulence, while a small fraction of these major genes exhibit racespecific interactions that provide narrow-spectrum resistance (Qi et al. 2023). The resistance phenotype results from the action of R genes (also known as Pi-genes) that specifically recognize their corresponding avirulence (AVR) genes in the pathogen, leading to the activation of effector-triggered immunity (ETI) (Kim et al. 2020).

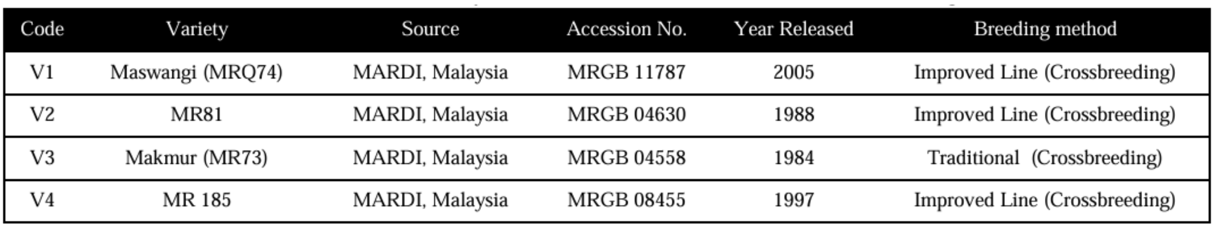
Malaysia is home to a wide variety of local indica rice, yet information regarding the potential for broad-spectrum blast resistance in Malaysian rice germplasm is still lacking. Advances in gene-specific molecular markers, such as microsatellites, can be employed to discover allelic variations in existing genetic resources, which are often overlooked due to the polymorphic effects in the gene pool. Therefore, this study aims to identify and assess resistance potential against blast using functional molecular markers that target four blast resistance genes: Pi33 and Pi27(t) (major genes), and pi21 and Pi26 (minor genes). Clustering analysis will be used to classify genotypes into distinct resistance groups for better characterization. Varieties with the most beneficial genes can be utilized as potential donor parents in marker-assisted selection (MAS) breeding.

2 Materials and Methods

2.1 Plant materials

A total of 75 local rice accessions were selected for this study, which included 63 released varieties (RVs) and 12 advanced breeding materials (ABMs) (Table 1). The seeds were collected from two institutes: the National Rice Genebank of the Malaysian Agricultural Research and Development Institute (MARDI), Seberang Perai, Pulau Pinang, Malaysia, and the Malaysia Nuclear Agency, Bangi, Selangor, Malaysia. All selected materials were germinated and cultivated in the Agrotechnology and Biosciences Division greenhouse at the Malaysia Nuclear Agency.

Table 1 Overview of Malaysian indica Rice Varieties Used for Resistance Screening



Code	Variety	Source	Accession No.	Year Released	Breeding method
V1	Maswangi (MRQ74)	MARDI, Malaysia	MRGB 11787	2005	Improved Line (Crossbreeding)
V2	MR81	MARDI, Malaysia	MRGB 04630	1988	Improved Line (Crossbreeding)
V3	Makmur (MR73)	MARDI, Malaysia	MRGB 04558	1984	Traditional (Crossbreeding)
V4	MR 185	MARDI, Malaysia	MRGB 08455	1997	Improved Line (Crossbreeding)

V5	Muda (MR71)	MARDI, Malaysia	MRGB 04557	1984	Traditional (Crossbreeding)
V6	Kadaria (MR27)	MARDI, Malaysia	MRGB 04554	1979	Traditional (Crossbreeding)
V7	Ria (IR 8)	MARDI, Malaysia	MRGB 01478	1966	Traditional (Crossbreeding)
V8	MR 103	MARDI, Malaysia	MRG 07486	1990	Improved Line (Crossbreeding)
V9	Seberang (MR77)	MARDI, Malaysia	MRGB 04559	1984	Traditional (Crossbreeding)
V10	MR84	MARDI, Malaysia	MRGB 04633	1986	Improved Line (Crossbreeding)
V11	MR 211	MARDI, Malaysia	MRGB 11629	1999	Improved Line (Crossbreeding)
V12	Pokkali	MARDI, Malaysia	MRGB 02491	1990	Traditional (Crossbreeding)
V13	Sri Malaysia I	MARDI, Malaysia	MRGB 02671	1974	Traditional (Crossbreeding)
V14	Sri Malaysia 2	MARDI, Malaysia	MRGB 02672	1974	Traditional (Crossbreeding)
V15	MR 159	MARDI, Malaysia	MRGB 08638	1995	Improved Line (Crossbreeding)
V16	Malinja	MARDI, Malaysia	MRGB 00839	1964	Traditional (Crossbreeding)
V17	Sekencang (MR7)	MARDI, Malaysia	MRGB 04552	1979	Traditional (Crossbreeding)
V18	MR 253	MARDI, Malaysia	MRGB 12095	2010	Improved Line (Crossbreeding)
V19	Sekembang	MARDI, Malaysia	MRGB 04553	1979	Traditional (Crossbreeding)
V20	Manik (MR52)	MARDI, Malaysia	MRGB 04556	1984	Traditional (Crossbreeding)
V21	MR 127	MARDI, Malaysia	MRGB 07489	1991	Improved Line (Crossbreeding)
V22	Setanjung (MR1)	MARDI, Malaysia	MRGB 04551	1979	Traditional (Crossbreeding)
V23	Bahagia	MARDI, Malaysia	MRGB 00167	1968	Traditional (Crossbreeding)
V24	Murni	MARDI, Malaysia	MRGB 01041	1972	Traditional (Crossbreeding)
V25	MR 232	MARDI, Malaysia	MRGB 12047	2006	Improved Line (Crossbreeding)
V26	MR 123	MARDI, Malaysia	MRGB 07488	1991	Improved Line (Crossbreeding)
V27	MRQ50	MARDI, Malaysia	MRGB 09345	1999	Improved Line (Crossbreeding)
V28	Pulut Malaysia I	MARDI, Malaysia	MRGB 02123	1974	Traditional (Crossbreeding)
V29	MR 220	MARDI, Malaysia	MRGB 11634	2003	Improved Line (Crossbreeding)
V30	PulutHitam 9 (PH 9)	MARDI, Malaysia	MRGB 08863	1990	Traditional (Crossbreeding)
V31	MR 220 (CL 1)	MARDI, Malaysia	MRGB 12131	2010	Improved Line (Crossbreeding)
V32	MR 220 (CL 2)	MARDI, Malaysia	MRGB 12132	2010	Improved Line (Crossbreeding)
V33	Masria	MARDI, Malaysia	MRGB 00860	1972	Traditional (Crossbreeding)
V34	MR 167	MARDI, Malaysia	MRGB 08646	1995	Improved Line (Crossbreeding)
V35	Jaya	MARDI, Malaysia	MRGB 00581	1973	Traditional (Crossbreeding)
V36	MR 106	MARDI, Malaysia	MRGB 07487	1990	Improved Line (Crossbreeding)

Code	Variety	Source	Accession No.	Year Released	Breeding method
V37	Pulut Siding	MARDI, Malaysia	MRGB 04555	1981	Traditional (Crossbreeding)
V38	Mahsuri	MARDI, Malaysia	MRGB 00826	1965	Traditional (Crossbreeding)
V39	Mahsuri Mutant	MARDI, Malaysia	MRGB 07841	1980	Mutation Breeding (γ -Ray Irradiation)
V40	Mardi Siraj 297 (MR297)	MARDI, Malaysia	MRGB 13019	2017	Traditional (Crossbreeding)
V41	Mardi Aerob 1 (MRIA 1)	MARDI, Malaysia	MRGB 12260	2014	Traditional (Crossbreeding)
V42	Kembang Sari (MRQ104)	MARDI, Malaysia	MRGB 13234	2021	Improved Line (Crossbreeding)

Code	Variety	Source	Accession No.	Year Released	Breeding method
V43	MR 303	MARDI, Malaysia	MRGB 13001	2019	Improved Line (Crossbreeding)
V44	Jarum Mas	MARDI, Malaysia	MRGB 00573	Traditional variety	Traditional (Crossbreeding)
V45	MR 314	MARDI, Malaysia	MRGB 13012	2013	Improved Line (Crossbreeding)
V46	Rotan	MARDI, Malaysia	MRGB 03520	Traditional variety	Traditional (Crossbreeding)
V47	MR 309	MARDI, Malaysia	MRGB 13007	1996	Improved Line (Crossbreeding)
V48	MR 284	MARDI, Malaysia	MRGB 12140	2015	Mutation Breeding (γ -Ray Irradiation)
V49	MR 65	MARDI, Malaysia	MRGB 04617	1998	Improved Line (Crossbreeding)
V50	MR 319	MARDI, Malaysia	MRGB 13016	Introduced variety	Improved Line (Crossbreeding)
V51	MR 276	MARDI, Malaysia	MRGB 12127	2000	Improved Line (Crossbreeding)
V52	Serendah	MARDI, Malaysia	MRGB 01545	Traditional variety	Traditional (Crossbreeding)
V53	MR 320	MARDI, Malaysia	MRGB 13017	2013	Improved Line (Crossbreeding)
V54	MR 315 (Seri Waja)	MARDI, Malaysia	MRGB 13228	2021	Improved Line (Crossbreeding)
V55	MR 30	MARDI, Malaysia	MRGB 10519	Introduced variety	Improved Line (Crossbreeding)
V56	MR 264	MARDI, Malaysia	MRGB 12105	2009	Improved Line (Crossbreeding)
V57	MR 321	MARDI, Malaysia	MRGB 13018	Introduced variety	Improved Line (Crossbreeding)
V58	MR 272	MARDI, Malaysia	MRGB 12123	2001	Improved Line (Crossbreeding)
V59	Mardi Warna 98	MARDI, Malaysia	MRGB 13141	2018	Traditional (Crossbreeding)
V60	MR269	MARDI, Malaysia	MRGB12120	2012	Improved Line (Crossbreeding)
V61	Biris	MARDI, Malaysia	MRGB 06891	1980	Traditional (Crossbreeding)
V62	MR 307	MARDI, Malaysia	MRGB 13005	2018	Improved Line (Crossbreeding)
V63	MR 263	MARDI, Malaysia	MRGB12133	2010	Improved Line (Crossbreeding)
V64	Bario	MARDI, Malaysia	MRGB 03172	Traditional variety	Traditional (Crossbreeding)
V65	MRM 16	MARDI, Malaysia	MRGB 12130	2010	Improved Line (Crossbreeding)
V66	NMR 191	Malaysian Nuclear Agency	Not available	Advanced Variety	Mutation Breeding (Ion Beam)
V67	NMR 152 (IS21)	Malaysian Nuclear Agency	Not available	2022	Mutation Breeding (γ- Ray Irradiation)
V68	NMR 151	Malaysian Nuclear Agency	Not available	2023	Mutation Breeding (γ- Ray Irradiation)
V69	54-99	Malaysian Nuclear Agency	Not available	Promising line	Mutation Breeding (γ- Ray Irradiation)
V70	TAM-9	Malaysian Nuclear Agency	Not available	Promising line	Mutation Breeding (Ion Beam)
V71	TAM-26	Malaysian Nuclear Agency	Not available	Promising line	Mutation Breeding (Ion Beam)
V72	191-1	Malaysian Nuclear Agency	Not available	Promising line	Indirect Mutant
V73	68-1	Malaysian Nuclear Agency	Not available	Promising line	Indirect Mutant
V74	NMR 192	Malaysian Nuclear Agency	Not available	Introduced variety	Mutation Breeding (Ion Beam)
V75	57-106	Malaysian Nuclear Agency	Not available	Promising line	Mutation Breeding (γ- Ray Irradiation)

2.2 Molecular analysis using gene-specific markers

2.2.1 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from the leaves of 75 rice genotypes using young, healthy (disease-free) leaves from 14 to 21-day-old seedlings. The extraction followed the CTAB protocol described by Ab-Razak et al. (2019). Approximately 20 mg of cut leaf tissue was placed in 2 mL microtubes. 500 µl of CTAB buffer was added, and the leaf tissues were homogenized with a TissueLyser II (Qiagen, Hilden, Germany). The mixture was then incubated in a heat block ThermoMixer Comfort (Eppendorf, Hamburg, Germany) at 65°C for 20 to 30 minutes. Following this incubation, 500 µl of chloroform: isoamyl alcohol (24:1) was added, and the tubes were inverted several times to ensure proper mixing. The mixture was centrifuged at 13,200 rpm for 15 minutes. The supernatant (200-400 µl) was carefully transferred to new sterilized 1.5 mL microtubes. Chilled isopropanol was added at a 1:1 ratio to the volume of the transferred supernatant. The mixtures were then stored at -20°C for 60 minutes to allow for nucleic acid precipitation, after which they were centrifuged again at 13,200 rpm for 15 minutes. The DNA pellets were washed with 500 µl of 70% ethanol and centrifuged at 13,200 rpm for 5 minutes. The pellets were allowed to air dry. Finally, 50 µl of TE buffer was added to the dried, colorless pellets and stored at -20°C. DNA quantification and qualification were immediately assessed using a DS-11 spectrophotometer (DeNovix, Wilmington, USA).

2.2.2 Polymerase Chain Reaction (PCR) amplification for SSR Marker Analysis

Five simple sequence repeat (SSR) markers, or microsatellites, were used to study their distributions in all 75 genotypes (Table 2). These markers were selected from previously published literature identifying resistance (R) genes and their closely linked markers. Additional information, including the forward and reverse sequences, chromosome locations, annealing temperatures, and expected amplicon band sizes, was obtained from Gramene (www.gramene.org). The PCR amplification process followed the methods outlined by Aljumaili et al. (2023). The PCR reaction was conducted in a 25 µl volume, which included 5 µl of 5× PCR buffer, 3 µl of $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.2 µl of Taq DNA polymerase (Promega, Wisconsin, USA), 13.3 µl of ultrapure deionized water, 1 µl of 50 ng DNA template, and 1 µl of each forward and reverse primer. Amplification was carried out using a thermal cycler (GeneAmp System 9700 – Applied Biosystems, Foster City, CA) with a customized protocol: (I) initial denaturation at 95°C for 4 minutes, (ii) denaturation at 95°C for 15

Table 2 Selected SSR Markers for Molecular Profiling of Blast Resistance Genes in Malaysian indica Rice

SSR marker	Linked gene	Sequence (5'-3')	Chr.	Product size (bp)		Annealing temp. (°C)
				'R' allele	'S' allele	
RM 259	*Pi27(t)*	F: GGAGGTTGAGAGGAGGG R: CTTGTTGCATGGTGCCATGT	1	162	200	55
RM 1359	*pi21*	F: CTCGCGAGGAAGAAGACAAC R: CGCCGGCTGGTTAATTAATC	4	166	180	
RM 413	*Pi26*	F: GGCGATTCTTGGATGAAGAG R: TCCCCACCAATCTTGTCTTC	7	170	150	
RM 72	*Pi33*	F: CCGGCGATAAAACAATGAG R: GCATCGGTCCTAACTAAGGG	8	79	125	

*F: forward primer, R: reverse primer, Chr.: chromosome number, R allele: resistance allele, S allele: susceptible allele, Annealing temp: annealing temperature.

seconds, (iii) annealing at 55°C for 15 seconds, (iv) elongation at 72°C for 15 seconds, (v) final elongation at 72°C for 7 minutes, and (vi) cooling at 4°C post-analysis. Electrophoresis was run at 75 volts for 65 minutes in a 2.5% agarose gel. The amplified PCR products were visualized under UV light using a bioimaging system.

2.2.3 Clustering analysis

Amplified fragments of varying sizes were treated as distinct alleles. DNA bands amplified by a particular primer were recorded as present (+) or absent (-) for all samples in the study. Genetic similarities were calculated among all possible pairs using the Dice coefficient through the SIMQUAL option. This data was organized into a similarity matrix with the NTSYS-pc version 2.10e software. The similarity matrix was then utilized for cluster analysis, employing the unweighted pair-group method with arithmetic averages (UPGMA) and the sequential agglomerative hierarchical nested (SAHN) clustering algorithm to create a dendrogram (Jagadeesh and Devaki 2020).

3 Results and Discussion

3.1 Genotypic screening and distribution of four blast resistance genes in selected Malaysian indica rice varieties

This study investigated the distribution of four blast resistance genes, viz., Pi33, Pi27(t), pi21, and Pi26, within a gene pool of 75 indica rice genotypes in Malaysia. The genotypes were cultivated across various geographical regions, including coastal areas, lowlands, and highlands. Simple sequence repeats (SSRs) molecular markers were employed to identify these genes in each rice genome, targeting

DNA regions close to the Pi genes. The markers used included RM259, which is closely linked to the Pi27(t) gene; RM72, which is tightly linked to the Pi33 gene; RM1359 for the pi21 gene; and RM413 for the Pi26 gene. For validation of the molecular genotyping, IR64 and PongsuSeribu 2 (PS2) were utilized as positive control varieties, while Mr219 served as the negative control. IR64 and PS2 were positive controls because they possess multiple blast disease resistance genes, i.e., IR64 carries Pi27(t) and Pi33, while PS2 harbors pi21 and Pi26 (Table 3). Both varieties have consistently demonstrated resistance against various strains of M. oryzae, ensuring reliable positive control conditions (Pohan et al. 2024). In contrast, MR219 was selected as the negative control due to its confirmed susceptibility and lack of major resistance genes (Miah et al. 2017; Nihad et al. 2022; Mynbayeva et al. 2024).

The electrophoretic results presented in Figure 1 display the banding patterns of DNA fragments amplified by the markers. The gel shows distinct bands at specific base pairs corresponding to resistant or susceptible alleles. Results in Figures 1a-1d were limited to sixteen genotypes per marker to reduce data abundance. The overall band scoring data is summarized in Table 3, which profiles each genotype according to the presence of Pi genes. From the analysis of 75 rice accessions, four accessions viz., Malinja, Bahagia, Serendah, and Bario contained all four resistance genes (Pi33, Pi27(t), pi21, and Pi26). Additionally, sixteen genotypes carried three resistance genes (MRQ74, Sri Malaysia I, Sri Malaysia II, Manik, MR 123, Pulut Malaysia I, Mahsuri Mutant, Mardi Aerob 1, MRQ104, MR 284, MR 276, MR 30, Mr264, MRM 16, 54-99, and TAM-9). Nineteen genotypes tested positive for only two resistance genes (MR 84, Setanjung, Murni, MRQ50, MR 220, MR220 (CL 1), MR220 (CL 2), Masria, Jaya (Malaysia), Pulut Siding, MR 65, MR 319, MR 315, MR 321, MR 272, NMR191, NMR152, NMR151, and TAM-26). Furthermore, twenty-two genotypes, accounting for three-tenths of the total, exhibited at least one resistance gene (MR81, Makmur, MR 185, Muda, Kadaria, Ria, MR 103, MR 211, MR 159, Sekencang, MR 253, Sekembang, PH 9, MR 167, MR 106, Mardi Siraj 297, Jarum Mas, Rotan, MR 309, Biris, 68-1, and 57-106). The remaining fourteen genotypes did not carry any of these four resistance genes.

In Figure 2, the bar graph illustrates the percentage distribution of each Pi gene within the genome pool of the rice accessions. It was found that Pi33 was the most prevalent resistance gene, present in 60% of the resources, followed by Pi27(t) at 49.33%, Pi26 at 34.67%, and pi21 at 21.33%. These percentages indicate the presence of beneficial Pi genes distributed among our existing traditional rice cultivars and the improved cultivars released since the late 1990s by national research institutes. Resistance to M. oryzae has long been hypothesized to operate on a gene-to-gene system. This concept suggests that a specific resistance (R) gene in the host rice plant interacts with a corresponding avirulence gene (AVR) in the pathogen, triggering a defensive immune response (Kim et al. 2020). However, it has been disheartening that deploying a single major R gene has often resulted in subpar performance against more virulent strains in the fields (Wang et al. 2022). These concerns prompted this study to prioritize major and minor R genes further to explore the dynamics among genes across different spectra.

From the data in Figure 1b, the RM72 marker was used to amplify the Pi33 gene locus. Approximately 46 rice genotypes carrying the resistant alleles of this gene produced 166-bp amplicons, while susceptible lines showed 180-bp bands. This finding is particularly noteworthy, as Pi33 has been documented to provide broadspectrum, non-race-specific resistance against more than 2,000 M. oryzae isolates from 55 countries (Wu et al. 2019; Yadav et al. 2019; Yasmin et al. 2023). The gene was initially mapped to the short arm of chromosome 8 through progeny analysis from crosses between resistant (IR64) and susceptible (Azucena) cultivars. This mapping confirmed its distinct identity from neighboring resistance

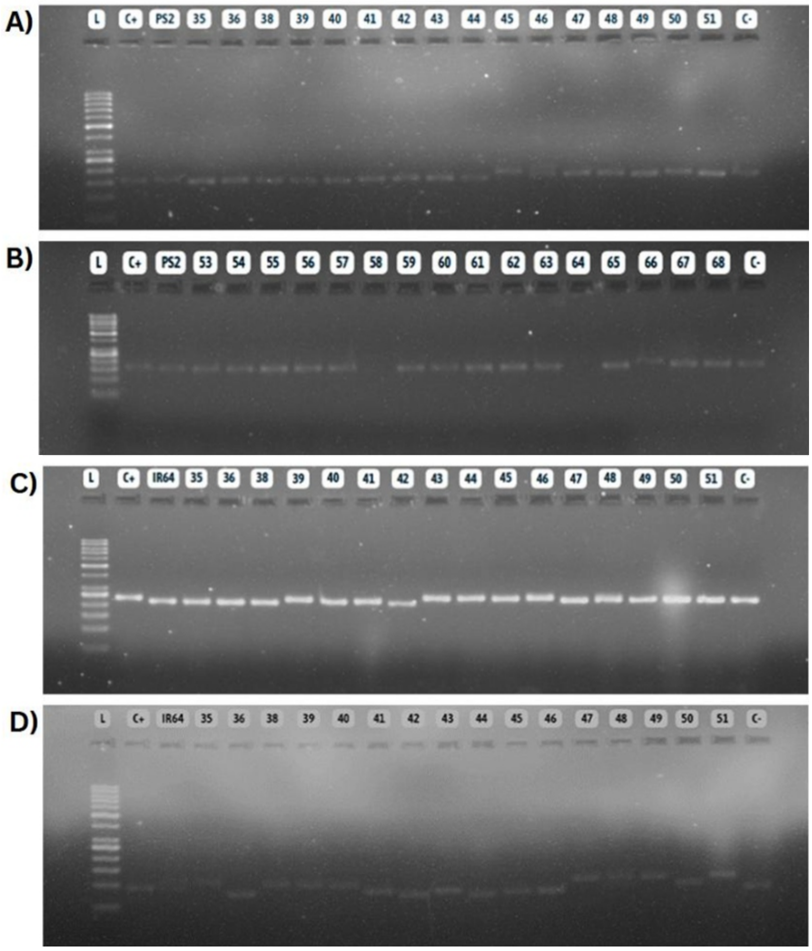


Figure 1 Agarose gel electrophoretic patterns for the presence of Pi33, Pi27(t), pi21, and Pi26 genes amplified by five SSR markers: (A) RM259, (B) RM72, (C) RM1359, and (D) RM413, L: 100 bp DNA size marker, C+: positive control that alternates between IR64 and PS2.IR64 is the positive control for (A) and (B), while PS2 is the positive control for (C) and (D), C-: MR219 as negative control, 35-68: rice accessions used in the study.

genes Pi11 and Pi-29(t) via allelism testing (Berruyer et al. 2003; Liu et al. 2005; Yang et al., 2009; Raboin et al. 2016). Therefore, Pi33 holds significant potential for developing improved rice cultivars exhibiting durable and broad-spectrum resistance. Analysis of Figure 1a indicates that the RM259 marker was employed to amplify the Pi27(t) gene locus, distinguishing resistant rice genotypes through 160-bp amplicons, in contrast to the 200-bp bands observed in susceptible lines. Pi27(t) originated from the blast-resistant donor variety IR64 (Wang et al. 2022). Located on the short arm of chromosome 1, Pi27(t) is clustered with other genes (Pit, Pish, and Pi25(t)) possessing similar protein domains, specifically the nucleotide-binding site-leucine-richrepeat (NBS-LRR) (Zhu et al. 2004). This proximity suggests these genes could interact and influence each other's expression. However, Pi27(t), as an individual gene, resists a wide range of M. oryzae isolates. Wang et al. (2014) highlighted that Pi27(t) exhibits durable resistance characteristics against M. oryzae isolates and can overcome major resistance genes commonly utilized in breeding programs in Guangdong Province. Thus, Pi27(t) is crucial in resistance breeding, complementing other major R genes to strengthen rice's defense against diverse M. oryzae populations. In Figure 1c, the RM1359 marker, designed to target pi21 on chromosome 4, differentiated resistant rice genotypes by producing 170-bp sized amplicons, while susceptible lines displayed 155-bp bands. Pi21 was successfully isolated by Fukuoka et al. (2009) and encodes for cytoplasmic proline-rich proteins, unlike most Pi genes (Zhang et al. 2016). As a quantitative resistance gene, pi21 provides only partial resistance, reducing the severity of blast disease by limiting hyphae penetration and lesion formation rather than completely preventing infection (Yasuda et al. 2015). Fukuoka et al. (2015) illustrated the value of pi21 in gene pyramiding, effectively reducing the percentage of diseased leaf area by combining pi21 with other quantitative resistance genes such as Pi34 and Pi35.

Table 3 Profiling of blast resistance genes in selected Malaysian indica rice varieties.

Code	Variety	Gene[a]				Total number of genes
		Pi33 (*RM72*)	*Pi27(t)* (*RM259*)	*pi21* (*RM1359*)	*Pi26* (*RM413*)	
Positive control	IR64	+	+	-	-	2
Positive control	PongsuSeribu 2	-	-	+	+	2
Negative control	MR219	-	-	-	-	0
V1	Maswangi (MRQ74)	+	+	+	-	3
V2	MR81	+	-	-	-	1
V3	Makmur	-	-	-	+	1
V4	MR 185	+	-	-	-	1
V5	Muda	-	-	-	+	1
V6	Kadaria	+	-	-	-	1
V7	Ria	+	-	-	-	1
V8	MR 103	-	-	-	+	1

Code	Variety	Gene[a]				Total number of genes
		Pi33 (RM72)	*Pi27(t)* (RM259)	*pi21* (RM1359)	*Pi26* (RM413)	
V9	Seberang	-	-	-	-	0
V10	MR84	+	+	-	-	2
V11	MR 211	+	-	-	-	1
V12	Pokkali	-	-	-	-	0
V13	Sri Malaysia I	+	-	+	+	3
V14	Sri Malaysia II	+	+	-	+	3
V15	MR 159	+	-	-	-	1
V16	Malinja	+	+	+	+	4
V17	Sekencang	-	-	+	-	1
V18	MR 253	-	+	-	-	1
V19	Sekembang	-	-	+	-	1
V20	Manik	+	+	-	+	3
V21	MR 127	-	-	-	-	0
V22	Setanjung	+	+	-	-	2
V23	Bahagia	+	+	+	+	4
V24	Murni	+	-	-	+	2
V25	MR 232	-	-	-	-	0
V26	MR 123	+	+	-	+	3
V27	MRQ50	+	+	-	-	2
V28	Pulut Malaysia I	+	+	-	+	3
V29	MR 220	+	+	-	-	2
V30	PulutHitam 9 (PH 9)	-	+	-	-	1
V31	MR 220 (CL 1)	+	+	-	-	2
V32	MR 220 (CL 2)	+	+	-	-	2
V33	Masria	+	+	-	-	2
V34	MR 167	-	+	-	-	1
V35	Jaya (Malaysia)	-	+	+	-	2
V36	MR 106	-	+	-	-	1
V37	Pulut Siding	-	+	-	+	2
V38	Mahsuri	-	-	-	-	0
V39	Mahsuri Mutant	+	-	+	+	3
V40	Mardi Siraj 297	-	-	+	-	1
V41	Mardi Aerob 1	+	-	+	+	3
V42	MRQ104 (Kembang Sari)	+	-	+	+	3
V43	MR 303	-	-	-	-	0
V44	Jarum Mas	-	-	+	-	1
V45	MR 314	-	-	-	-	0

Code	Variety	Gene[a]				Total number of genes
		Pi33 (*RM72*)	*Pi27(t)* (*RM259*)	*pi21* (*RM1359*)	*Pi26* (*RM413*)	
V46	Rotan	+	-	-	-	1
V47	MR 309	+	-	-	-	1
V48	MR 284	+	+	-	+	3
V49	MR 65	+	-	-	+	2
V50	MR 319	+	+	-	-	2
V51	MR 276	+	+	-	+	3
V52	Serendah	+	+	+	+	4
V53	MR 320	-	-	-	-	0
V54	MR 315 (Seri Waja)	+	+	-	-	2
V55	MR 30	+	+	+	-	3
V56	MR 264	+	+	-	+	3
V57	MR 321	+	+	-	-	2
V58	MR 272	+	+	-	-	2
V59	Mardi Warna 98	-	-	-	-	0
V60	MR 269	-	-	-	-	0
V61	Biris	-	-	-	+	1
V62	MR 307	-	-	-	-	0
V63	MR 263	-	-	-	-	0
V64	Bario	+	+	+	+	4
V65	MRM 16	+	+	-	+	3
V66	NMR191	+	-	+	-	2
V67	NMR152	+	+	-	-	2
V68	NMR151	+	+	-	-	2
V69	54-99	+	+	-	+	3
V70	TAM-9	+	+	-	+	3
V71	TAM-26	+	-	-	+	2
V72	191-1	-	-	-	-	0
V73	68-1	-	+	-	-	1
V74	NMR192	-	-	-	-	0
V75	57-106	-	+	-	-	1
	Frequency (%)	60.00	49.33	21.33	34.67	

[a] + denoted as present and - denoted as absent of gene.

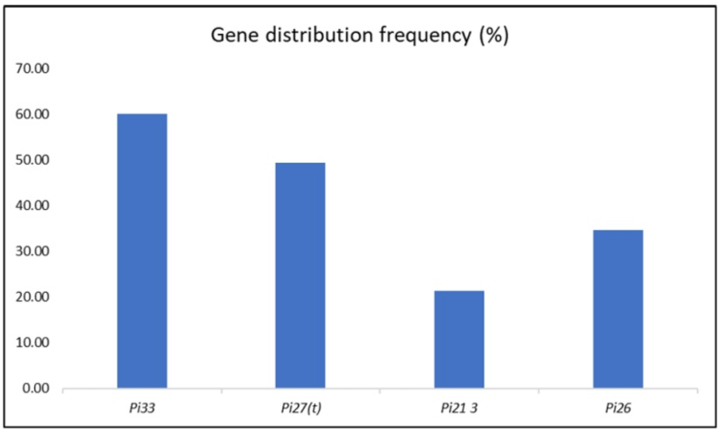


Figure 2 Distribution of blast resistance genes among backbone varieties of Malaysian indica rice.

The results obtained in Figure 1d indicate that the RM413 marker, which targets Pi26 on chromosome 7, distinguishes resistant and susceptible rice genotypes by producing a 79-bp amplicon in resistant lines and a 125-bp band in susceptible ones. Although Pi26 is classified as a minor resistance gene, it is part of an R gene cluster near the centromere on the short arm of chromosome 7, a region enriched with nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes that contribute to the evolution of rice disease resistance (Kanyange et al. 2022). While its resistance is partial, Pi26 plays a role in defense against M. oryzae isolates, particularly those prevalent in rice-growing regions of Southeast Asia (Kanyange et al. 2022; Qi et al. 2023). Both Pi26 and pi21 are significant contributors to breeding programs to enhance blast resistance in rice. Their strategic use with other resistance genes can improve durability against this devastating disease.

3.2 Molecular Clustering and Genetic Diversity Assessment in Malaysian indica Rice

Applying UPGMA with Dice's coefficient in cluster analysis provided a clear understanding of the genetic relationships among the studied genotypes, allowing for precise classification based on their genetic similarities (Ali et al. 2023). Clustering was conducted based on genetic distance, grouping accessions with similar genetic profiles while separating those with greater genetic divergence (Caguiat and Yabes 2016; Dev et al. 2022). This classification offers valuable insights into the genetic relatedness among different varieties, facilitating the identification of potential candidates for breeding programs to enhance desirable traits, such as blast resistance (Gebrie and Abebe 2022; Singh et al. 2024). The approach was applied to 75 varieties of Malaysian indica rice, providing a robust framework for understanding the genetic architecture underlying blast resistance. The SSR-based dendrogram revealed a diverse genetic structure, dividing the 75 rice accessions into eight clusters labeled Cluster 1 to Cluster 8. According to Figure 3, Cluster 1 included 15 varieties (20.0%), grouped alongside IR64, a positive control for the blast resistance genes Pi33 and Pi27(t). Cluster 2 contained 7 accessions (9.3%), while Cluster 3 comprised 13 (17.3%), forming moderately diverse sets. Cluster 4, which included 4 varieties (5.3%), was associated with Pongsu Seribu 2, a positive control that carries the resistance genes pi21 and Pi26. Cluster 5 emerged as the largest, consisting of 22 accessions (29.3%) along with MR 219, a negative control known for its susceptibility to blast disease. The presence of MR 219 in this group suggests a shared genetic background related to susceptibility. Clusters 6 and 7 each contained 5 varieties (6.7%), while Cluster 8, the smallest, included 4 varieties (5.3%). The overall distribution of accessions across these groups highlights the genetic variability within Malaysian indica rice, providing essential insights for breeding programs to enhance blast resistance.

The similarity indices among the rice varieties revealed significant differences, indicating varying levels of genetic similarity (Rezk et al. 2024). Varieties within the same cluster generally showed higher similarity indices, suggesting closer genetic relationships (Surapaneni et al. 2016). In contrast, varieties from different clusters exhibited lower similarity indices, showcasing greater genetic divergence (Anh et al. 2018). These variations in similarity indices are crucial for identifying potential parental lines for breeding programs. Crossing varieties with lower similarity indices can enhance genetic diversity and increase the likelihood of obtaining desirable traits, such as blast resistance (Surapaneni et al. 2016). Notably, the highest similarity index of 1.00 was observed between IR64, which serves as a positive control for Pi33 and Pi27(t), and several accessions within Cluster 1, including MR84, MR1, MRQ50, MR 220, MR 220 (Cl1), MR 220 (CL2), Masria, MR 319, MR 315, MR 321, MR 272, NMR 152, and NMR 151.

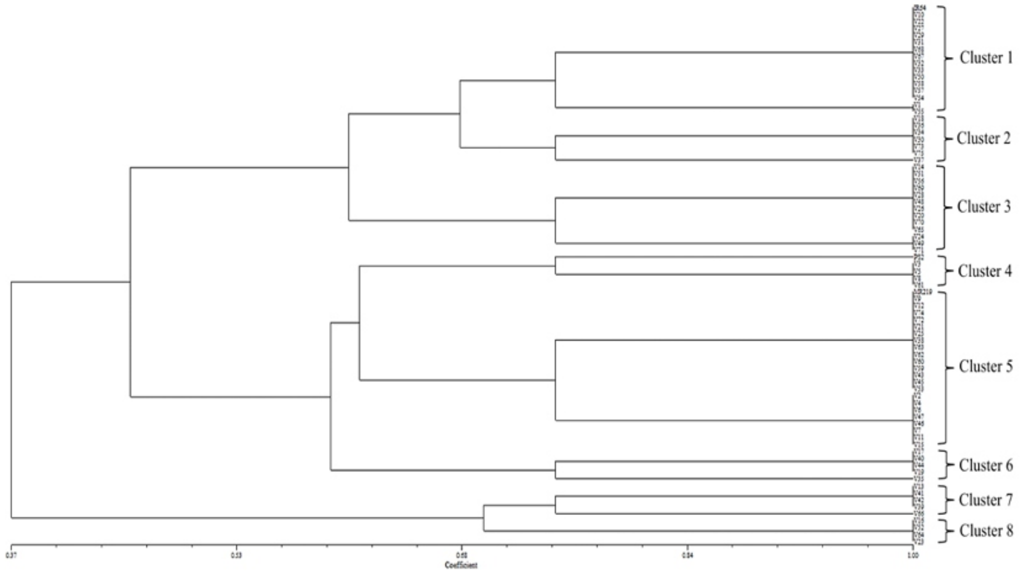


Figure 3 UPGMA-based dendrogram of 75 rice varieties constructed using SSR marker data for blast resistance traits.

This indicates a high degree of genetic similarity among these varieties. Within Cluster 1, the maximum intracluster similarity index was 0.67, observed between MR84 and MR30, indicating a moderate level of genetic similarity. In contrast, the minimum intercluster similarity index of 0.25 between MR84 and MRIA 1 belongs to different clusters, highlighting significant genetic divergence.

The genetic differences among rice varieties are reflected in their similarity indices and their phenotypic traits. For example, Mr220 and MR219 are sister lines derived from the same parent plants, MR151 and MR137. They share similar maturity periods but differ in other characteristics. MR219 is recognized for its high yield potential, exceeding 10 metric tons per hectare. It has a short maturation period of 105 to 111 days, robust culms, and resistance to bacterial leaf blight (Zuraida et al. 2011). However, its susceptibility to blast disease has led to the introduction of blastresistance genes through marker-assisted selection (Fatah 2015). In contrast, MR220CL2, a derivative of MR220, displays distinct traits, including herbicide resistance and a shorter maturity period. It also shows higher yields under various nitrogen conditions, making it a promising candidate for improving rice productivity (Misman and Zakaria 2019; Hashim et al. 2022). Similarly, MR315, developed by MARDI through backcrossing between Y1512 (MR 256) and MR 253, is an early-maturing rice variety with a maturation period of 105 to 109 days. It has a compact height of 103.3 to 106.3 cm and a high grain filling rate of 88.3 to 88.6% (Sunian et al. 2021). Initially bred in the 2006/2007 season, it underwent pedigree selection and yield evaluation at Mardi Seberang Perai. MR315 exhibits strong neck blast resistance and moderate leaf blast and sheath blight resistance, reducing dependence on fungicides. With a yield potential of 7.16 to 8.3 tonnes per hectare, reaching up to 8.3 tonnes in regions like Sungai Besar and Simpang Lima, MR315 exemplifies the advancements in breeding for higher productivity and disease resistance (Sunian et al. 2021). These variations in rice varieties reflect ongoing progress in rice breeding, driven by the need to develop superior strains with enhanced yield potential, robust disease resistance, and adaptability to diverse environmental conditions. By leveraging advanced breeding techniques, researchers and breeders aim to optimize key agronomic traits, ensuring sustainable and resilient rice production in the face of evolving climatic challenges and pest pressures.

Conclusion

This study thoroughly evaluates leaf blast resistance in 75 Malaysian indica rice varieties. It reveals that Malinja, Bahagia, Serendah, and Bario uniquely combine four resistance (R) genes: Pi33, Pi27(t), pi21, and Pi26. These varieties exhibited a 100% resistance contribution rate, indicating their strong genetic defense against Magnaporthe oryzae, the pathogen responsible for rice blast disease. The presence of both major resistance genes (Pi33 and Pi27(t)) and minor resistance genes (pi21 and Pi26) suggests a complementary interaction that enhances the durability and stability of blast resistance. Major genes provide race-specific resistance, offering immediate protection, while minor genes contribute to quantitative resistance, which ensures long-term effectiveness across various environmental conditions and pathogen populations. This combination of genes reduces the likelihood of resistance breakdown, making these varieties highly valuable for sustainable rice production. Molecular clustering analysis further supported these findings, showing a maximum similarity index of 1.00 between IR64, a positive control for Pi33 and Pi27(t), and 15 accessions in Cluster 1. Significantly, the four highly resistant varieties (Malinja, Bahagia, Serendah, and Bario) were distinctly grouped in Cluster 8, reinforcing their genetic similarity and unique resistance profile. Given their strong resistance profiles, these four varieties can be ideal genetic donors for breeding programs to develop broad-spectrum and durable blast-resistant indica rice in Malaysia. Integrating their resistance traits into elite rice lines through gene pyramiding and markerassisted selection can greatly enhance disease resilience, reduce reliance on chemical fungicides, and improve overall yield stability. This study underscores the critical role of molecular breeding in optimizing resistance management strategies, highlighting the need for continued research and innovation to ensure long-term food security in regions vulnerable to blast disease.

Acknowledgment

The authors thank the Malaysian Agricultural Research and Development Institute (MARDI) and the Malaysian Nuclear Agency for supporting the resources. The research was funded by Science and Technology Research Grant from Malaysia Toray Science Foundation (MTSF-TORAY) -100-TNCPI/PRI 16/6/2 (068/2023).

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Clearance

No ethical approval was required since this study did not involve human participation or animals.

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ISSN NO 2320 - 8694

Optimizing the Mg, Mn, and Mo co-inoculation impacts in soil using a response surface technique approach to ameliorate the N fixation efficiency of Enterobacter sp. Gg1

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ABSTRACT

Nitrogen fertilizers play a crucial role in enhancing crop productivity. However, the extensive use of synthetic fertilizers in recent years can disrupt the balance of soil nutrients, leading to deficiencies or toxicities in other essential nutrients such as magnesium (Mg), manganese (Mn), and molybdenum (Mo). This imbalance can interfere with nitrogen cycling processes, particularly affecting the activity of nitrogenase enzymes involved in ammonium production. To improve soil fertility sustainably, it is essential to adopt eco-friendly agricultural practices that maximize the activity of nitrogen-fixing bacteria. This study used an in-vitro response surface methodology to enhance ammonium production efficiency in the nitrogen-fixing bacterium Enterobacter sp. GG1. The research aimed to optimize the co-inoculation rates of Mg, Mn, and Mo to increase nitrogen fixation efficiency. The findings revealed that the three independent variables (Mg, Mn, and Mo) significantly interacted with ammonium production. The optimal conditions resulted in a maximum ammonium production of 18.48 ppm per day per gram of biomass.

KEYWORDS :N-fixation Nitrogenase enzyme Ammonium Response surface methodology (RSM) Central composite design (CCD)

INTRODUCTION

Nitrogen (N) availability significantly impacts plant growth, yield, and overall health. However, it is often a limiting nutrient in many ecosystems, meaning that insufficient nitrogen can hinder the growth of plants and other organisms (Govindasamy et al. 2023). This element is essential for forming amino acids and is fundamental in protein synthesis. Proteins are crucial for maintaining structural integrity, performing enzymatic functions, and facilitating communication in plant and bacterial cells (Zayed et al. 2023; Huang et al. 2023). Despite its importance, nitrogen availability can be constrained because the nitrogen sources present in soil, specifically nitrate (NO3-) and ammonium (Nh4 +), account for only about 2% of the total nitrogen content (Chalk and Smith 2021; Wei et al. 2023). Synthetic fertilizers have been proposed as a potential solution to the challenges faced in sustainable agricultural practices. Synthetic fertilizers typically provide only the primary macronutrients (nitrogen, phosphorus, and potassium) and often lack essential micronutrients, such as magnesium, manganese, and molybdenum. Relying heavily on synthetic fertilizers, without adequate supplementation of these micronutrients, can lead to deficiencies in the soil (Montgomery and Biklé 2021; Assunção et al. 2022). Imbalances in vital nutrients, such as magnesium, manganese, and molybdenum, can significantly affect soil fertility and the microbial processes in the rhizosphere, particularly those involved in nitrogen cycling (Li et al. 2023). Additionally, various conditions may limit effective ammonium yields by impacting nitrogenase activity. When formulating an appropriate growth medium, critical parameters include the concentrations of manganese (MnSO4·7H2O), magnesium (MgSO4·7H2O), and molybdenum (Na2MoO4·2H2O) (Markam 2022; Ghorai and Ghosh 2023). A high concentration of Mg^{2+} ions and ATP is required to enhance nitrogenase activity, combined with a low rate of the stimulating factor, Mn^{2+} ions (Bhateshwar 2022). Among the ions with strong catalytic interactions with Mg^{2+} and Mn^{2+}, oxygen (O_2) is the most dependent on proteins. Nitrogenase-activated processes involve the combination of Mg^{2+} and ATP to produce $MgATP^{2-}$. A deficiency in Mg^{2+} ions can lead to only 20% activation of the Fe protein (Ghorai and Ghosh 2022; Rajamuthuramalingam et al. 2024). Moreover, molybdenum is a cofactor in nitrogenase enzymes and plays an essential role in nitrogen metabolism. A molybdenum deficiency can impede nitrogen-fixing bacteria from converting atmospheric nitrogen, disrupting the soil's nitrogen cycle (Glass et al. 2012; Sun et al. 2021).

Managing soil nutrient imbalances through adequate fertilization, soil amendments, and crop rotation can help mitigate negative effects while promoting healthy soil microbial communities and nutrient cycling processes. Additionally, enhancing biodiversity and implementing soil conservation practices can improve soil resilience and sustainability in agricultural and natural ecosystems. This study utilized a Box-Behnken Design (BBD) to analyze the response surface thoroughly. The Box-Behnken Design is a widely used experimental response surface methodology (RSM) research framework. This approach allows for estimating quadratic effects and interactions between components while requiring fewer experimental trials than full factorial designs (Ghosh et al. 2012). Using this method, we can effectively analyze the response surface, construct a predictive model, validate its accuracy, and ultimately determine the optimal conditions for enhancing nitrogen fixation efficiency in the N-fixing bacteria Enterobacter sp. Gg1 by optimizing the co-inoculation rates of magnesium (Mg), manganese (Mn), and molybdenum (Mo).

2 Materials and Methods

2.1 Conditions for cultivation and analytical methodology

The ammonium-producing bacterium Enterobacter sp. GG1 was used in optimization trials to evaluate multiple process parameters. Strains of Enterobacter sp. GG1 were collected from the MEAB laboratory at JIS University in Kolkata (Ghorai and Ghosh 2023). The inocula were cultured at 37°C and 120 rpm in Nfb broth, which does not contain bromothymol blue, until they reached the exponential growth phase. For the experiments, 15 different trials were conducted by inoculating the Nfb broth medium with various concentrations of manganese (MnSO4·7H2O), magnesium (MgSO4·7H2O), and molybdenum (Na2MoO4·2H2O). The cultures were incubated for five days at 37°C with constant agitation at 120 rpm. After this incubation period, the bacterial cell culture was centrifuged for 10 minutes at 10,000 rpm. A volume of 1 mL of the supernatant was then collected, and the ammonium concentration was measured using the phenate method (Ghosh et al. 2012; Ghorai and Ghosh 2023).

2.2 Optimization of culture conditions for Enterobacter sp. GG1 using response surface methodology

An experimental design utilizing the 3K Box-Behnken model optimized critical process parameters for enhanced ammonium production. The Box-Behnken design offers several advantages due to three essential characteristics (Mg, Mn, and Mo). It requires fewer trial runs, and the deviation in the expected response at each point x is determined solely by the distance from x to the design center point (Box and Behnken 1960). The 3K factorial design allows for accurate estimations of second-degree quadratic polynomials and helps identify the optimal combination of values within the threedimensional observational space (Annadurai et al. 1999). To articulate the regression equation, we will describe the relationship between the coded values and the actual values as follows:

$$x_i = X_i X_i^* / \Delta X_i \qquad \text{............(Equation 1)}$$

where xi is the coded value of the ith independent variable, Xi is the uncoded value of the ith independent variable, Xi* is the uncoded value of the ith independent variable at the center point, and ΔXi is the step change value.

The Box-Behnken model has established a foundational design that includes various points, featuring several central locations and high and low values. This method determined the optimal values for three critical process variables that enhance ammonium production. In conjunction with the experimental design, Table 1 outlines the levels of these variables. The ammonium output was correlated with the concentrations of manganese (0.10, 1.05, and 2.00 mM), magnesium (0.50, 10.25, and 20.00 mM), and molybdenum (50, 1,025, and 2,000 nM) in the culture media. Fifteen trials were conducted using the 3K factorial Box-Behnken design to evaluate experimental error, with the center point replicated three times. To illustrate the relationships between the factors and the response, specifically, the yield of ammonium in parts per million (ppm), a quadratic polynomial equation was used, as detailed by the following equation:

$$N = \propto_0 + \sum_{i=1} \propto_i X_i + \sum_{i=1} \propto_{ii} X_i^2 + \sum_{i=1} \sum_{i<j=2} \propto_{ij} X_i X_j \quad \text{(Equation 2)}$$

The input variables Xi affect the response variable N, an offset term α0, a linear coefficient αi, and an interaction coefficient αij. The input values of A, B, and C that yield the maximum value of N were identified by setting the partial derivatives of the functions to zero (Box and Behnken 1960; Ghosh et al. 2012).

2.3 Statistical Analysis

Response Surface Methodology (RSM) optimizes processes by examining the relationship between input factors and output responses through statistical experiments, including Box-Behnken designs (BBD) and Central Composite Designs (CCD).

3 Results and Discussion

The ideal concentrations of essential components and their interaction effects on ammonium production were evaluated using Enterobacter sp. GG1 and response surface methodology, specifically through applying a Box-Behnken design. Table 1 outlines the statistical analysis of the test variables and the observed response values representing ammonium production. ANOVA is crucial for assessing the significance and suitability of the experimental data to the model. Table 2 presents the findings

Table 1 The Box–Behnken experimental design utilizes three independent variables to examine ammonium generation by Enterobacter sp. Gg1.

Trial	Factor1 $MnSO_4.7H_2O$ (mM)		Factor 2 $MgSO_4.7H_2O$ (mM)		Factor 3 $Na_2MoO_4.2H_2O$ (nM)		Response Ammonium productivity (ppm/day/g biomass)
	A	Code	B	Code	C	Code	
1	0.10	-1	0.50	-1	1025.00	0	9.73
2	2.00	1	0.50	-1	1025.00	0	8.28
3	0.10	-1	20.00	1	1025.00	0	8.24
4	2.00	1	20.00	1	1025.00	0	6.95
5	0.10	-1	10.25	0	50.00	-1	10.73
6	2.00	1	10.25	0	50.00	-1	9.35
7	0.10	-1	10.25	0	2000.00	1	9.72
8	2.00	1	10.25	0	2000.00	1	8.33

9	1.05	0	0.50	-1	50.00	-1	9.18
10	1.05	0	20.00	1	50.00	-1	6.67
11	1.05	0	0.50	-1	2000.00	1	10.06
12	1.05	0	20.00	1	2000.00	1	8.28
13	1.05	0	10.25	0	1025.00	0	18.6
14	1.05	0	10.25	0	1025.00	0	17.74
15	1.05	0	10.25	0	1025.00	0	19.09

NCODED = 18.48 - 0.6886A - 0.8900B + 0.0576C + 0.0370AB - 0.0026AC + 0.1838BC - 4.60A2 - 5.58B2 - 4.35C2; NACTUAL = 3.76509 + 9.93473A + 1.08802B + 0.009243C + 0.003990AB – (2.81049E-06)AC + 0.000019BC - 5.09408A2 - 0.058698B2 – (4.57538E-06)C2

Table 2 ANOVA for ammonium production by Enterobacter sp. Gg1.

Factors	Sum of Squares	df	Mean Square	F value	P value
Model	238.79	9	26.53	35.47	0.0005
A	3.79	1	3.79	5.07	0.0741
B	6.34	1	6.34	8.47	0.0334
C	0.0265	1	0.0265	0.0354	0.8581
AB	0.0055	1	0.0055	0.0073	0.9352
AC	0.0000	1	0.0000	0.0000	0.9954
BC	0.1351	1	0.1351	0.1807	0.6885
A^2	78.04	1	78.04	104.33	0.0002
B^2	114.97	1	114.97	153.69	< 0.0001
C^2	69.85	1	69.85	93.38	0.0002
Residual	3.74	5	0.7480		
Lack of Fit	2.81	3	0.9381	2.03	0.3472
Pure Error	0.9257	2	0.4628		
Cor Total	242.53	14			

from the ANOVA related to fitting the quadratic model for ammonium production. The ANOVA elements, including model F-value, coefficient of variation, and squared regression, indicate that the experimental results aligned appropriately with the model.

The coefficient of determination (R^2) has been calculated to be 0.9846, indicating a very high level of explanatory power for the model. The Model F score is 35.47, demonstrating that the model is statistically significant. The likelihood of obtaining such a high F value due to random chance is only 0.05%. P-values below 0.05 further suggest that the terms in the model are statistically significant. Key variables in the model include B, A^2, B^2, and C^2. The F value of 2.03 indicates that the lack of fit is not statistically significant when compared to pure error. The coefficient of determination (R^2) reflects the model's goodness of fit. The results are notably significant at the 98% confidence level, which suggests that the model accounts for only 2% of the total variance. Adequate precision establishes the relationship between the signal and noise in the data, with a ratio exceeding four considered more beneficial. With a ratio of 16.595, this model demonstrates a strong signal.

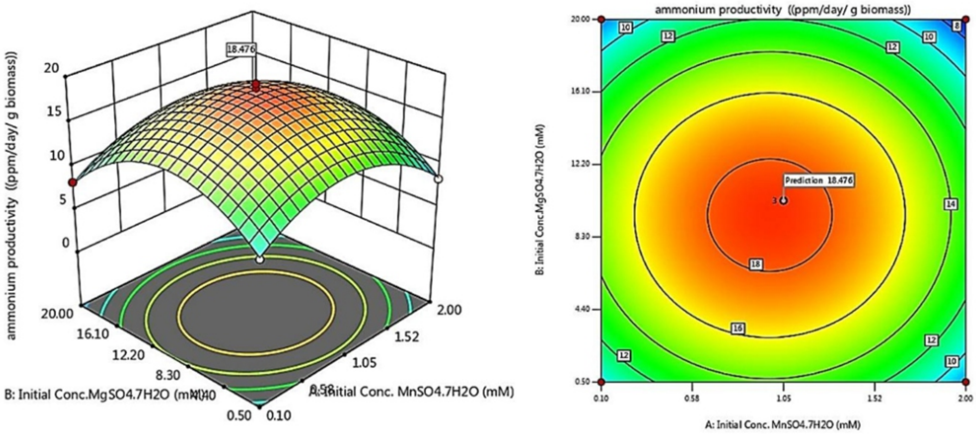

Figure 1 The maximum production of ammonium is illustrated using two-dimensional and three-dimensional contour plots. RSM plots were generated utilizing the data presented in Table 1. This study involved 15 trials conducted under the Box-Behnken design framework, focusing on ammonium production (ppm/day/g biomass) as influenced by varying concentrations of manganese and magnesium.

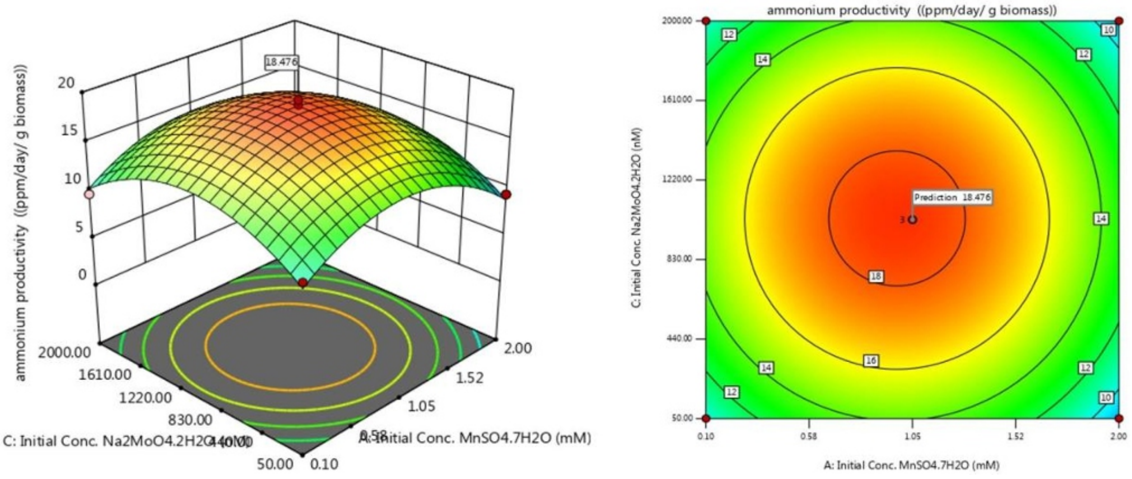

Figure 2 Maximum ammonium production is represented through two-dimensional and three-dimensional contour plots. The RSM plots were constructed utilizing the data delineated in Table 1. Fifteen experiments were conducted using the Box-Behnken design parameters, evaluating ammonium production (ppm/day/g biomass) with the concentrations of manganese and molybdenum.

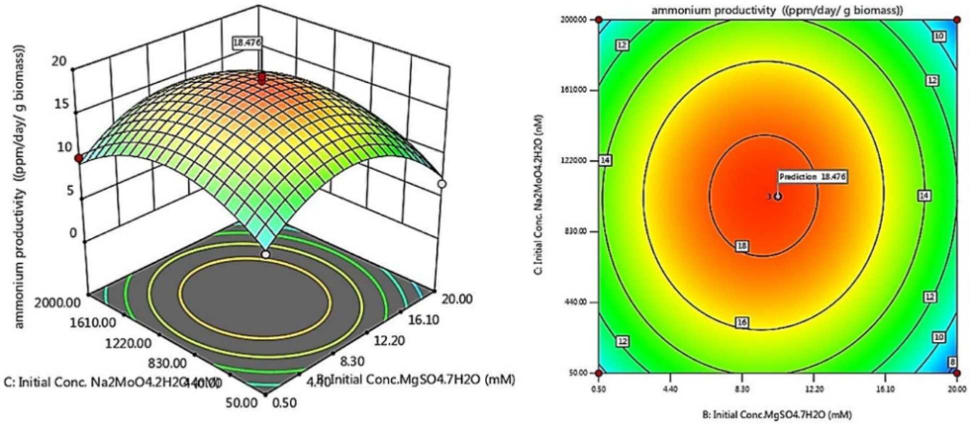

Figure 3 Maximum ammonium production is illustrated using two-dimensional and three-dimensional contour plots. RSM plots were created using the data provided in Table 1. The study involved 15 trials conducted under the Box-Behnken design framework, assessing ammonium production (ppm/day/g biomass) with varying concentrations of magnesium and molybdenum.

The experimental data revealed a low coefficient of variation (C.V. = 8.06%), indicating a significant level of precision and reliability. The contour plots of both two-dimensional and three-dimensional illustrations show the variation in ammonium production relative to the concentrations of manganese and magnesium (Figure 1), manganese and molybdenum (Figure 2), and magnesium and molybdenum (Figure 3) displayed elliptical shapes with distinctly elongated diagonals. This suggests that the three independent variables significantly interact to affect ammonium production (N). The response surface plots in Figures 1, 2, and 3 each show a distinct peak within the design boundaries, and the corresponding contour plots reveal a clear maximum. This indicates that optimal ammonium production can be achieved within the established design constraints (SI 1).

At the central concentrations of 1.05 mM manganese, 10.25 mM magnesium, and 1025 nM molybdenum, the projected ammonium output is 18.476 ppm/day/g biomass, surpassing the previously documented yield of 18.06 ppm/day/g biomass reported (Ghorai and Ghosh 2023). Validation studies were conducted in triplicate using Enterobacter sp. GG1 to confirm the projected optimal strain. The mean ammonium production rate was measured at 18.48 ppm/day/g biomass, closely aligning with the anticipated 18.476 ppm/day/g biomass value. These findings indicate that response surface tuning can effectively optimize ammonium yields and enhance nitrogen fixation.

The study thoroughly reported the optimal levels for each factor investigated that resulted in maximum ammonium production by B. subtilis. Specifically, the findings indicated that particular concentrations of glucose and yeast extract and a specific initial pH were the most conducive conditions for enhancing ammonium production (Mahat et al. 2024). This study could also elaborate on how soil nutrients such as magnesium (Mg), manganese (Mn), and molybdenum (Mo) specifically influence nitrogen fixation. The authors concluded that Response Surface Methodology (RSM) is a highly effective tool for optimizing ammonium production by Enterobacter sp. GG1. The optimal conditions identified in the soil led to a significant increase in production compared to experiments conducted under nonoptimized conditions.

Conclusion

Response Surface Methodology (RSM), utilizing a Box-Behnken design, was effectively employed to identify optimal conditions for enhancing ammonium yields. The study examined three independent variables: manganese, magnesium, and molybdenum concentrations, demonstrating significant interactive effects on nitrogen fixation. The optimal levels identified were 1.05 mM manganese, 10.25 mM magnesium, and 1025 nM molybdenum, producing the highest ammonium production of 18.48 ppm per day per gram of biomass. These RSM studies have established conditions that maximize the conversion of atmospheric nitrogen into plant-accessible ammonium through the action of Enterobacter sp. GG1. Operating under these ideal conditions improved ammonium yields by 2.72% per gram of Enterobacter sp. GG1. The findings suggest that the optimal concentrations of manganese, magnesium, and molybdenum in soil, when supplemented with Enterobacter sp. GG1 are crucial for promoting plant growth. Future research should focus on genetic modifications that enhance the production of nitrogenase enzymes, which further facilitate ammonium accumulation and optimize the potential of organic agriculture.

Acknowledgment

We sincerely thank JIS University Kolkata and the JIS Group Educational Initiatives.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Statement

Not Applicable.

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ISSN NO 2320 - 8694

Nutritional Values of Raw and Ripe Fruits of Two Commercially Viable Banana Cultivars „Grand Nain‟ and „Sabri‟: Exploring for Value Addition

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ABSTRACT

Bananas are a significant agricultural commodity valued for their delicacy and nutritional benefits. However, approximately 30% of bananas are wasted, underscoring the need for strategies to add value to this fruit. Additionally, bananas have the potential to help address a global issue: micronutrient deficiency. This study aimed to evaluate the nutritional and nutraceutical values of different parts (peel and pulp) of green and ripe bananas from two cultivars, Grand Nain and Sabri. We analyzed various nutritional and nutraceutical parameters, including ash content, crude fat, fiber, carbohydrates, protein, polyphenols, antioxidant activity, and Vitamin A content. The findings revealed that unripe banana pulps contained the highest carbohydrate content, while ripe banana peels demonstrated greater overall nutrient richness in terms of fiber, protein, minerals, and antioxidants, all of which are essential for the human body. This study highlights the potential for utilizing different parts of the banana, particularly the peels, to develop value-added products.

KEYWORDS : Grand Nain Sabri cultivar Green peel and pulp Ripe peel and pulp Nutritional values Vitamin A content

INTRODUCTION

Bananas are herbaceous perennial plants that produce delicious, nutrient-packed fruits. They are among the most widely produced fruits in the world, ranked as the fourth most valuable agricultural commodity after rice, wheat, and maize, with an estimated annual production volume of 125 million tons and a trade value of approximately 13.6 billion USD (Kumari et al. 2022). According to statistical data from the FAO (2019), India is the leading banana producer, contributing about 25% of global banana production. Bananas hold significant importance in India, serving as a major fruit crop that provides livelihood stability for thousands of people and contributes roughly 37% of the nation‟s total fruit production (Kookal and Thimmaiah 2018). Despite this, the FAO reported (2014–2016) that around 190.7 million people in India experience nutritional deficiencies. This issue primarily arises from a lack of balanced meals and poor dietary choices (Nutritional Problems in India: Types & Symptoms of Nutritional Deficiencies, acko.com).

Bananas are often referred to as “Poor Men‟s Food” due to their widespread availability and affordability, making them an economical option for addressing malnutrition. Recognized for their high nutritional value and potential to treat celiac disease, bananas are also termed “Super Food,” a classification supported by the American Medical Association, which recommends them as a healthy food for children (United Fruit Company 2018). Reports highlight bananas as a nutritious choice, rich in essential nutrients such as carbohydrates, proteins, fiber, minerals, vitamins, and bioactive compounds (including carotenoids, polyphenols, and antioxidants) that may help prevent diseases like diabetes and cancer (Borges et al. 2020). However, despite bananas being one of the most widely produced crops, over 30% are lost as waste or by-products. This waste primarily results from their shorter shelf life, mishandling, and suboptimal practices in the postharvest process. Major by-products, such as overripe bananas and nonedible parts like peels and pseudostems, contribute significantly to organic waste generation (Vu et al. 2018). This situation underscores the urgent need to develop and incorporate novel approaches to manage these wastes and convert them into valueadded products effectively.

Traditionally, bananas are consumed for their nutritional benefits and flavor, and they are commonly enjoyed fresh or used in desserts, yogurts, ice creams, puddings, and dried chips. Additionally, various by-products of bananas have practical applications; for instance, the pseudostems can be processed into fibers used for thatching, fabric, paper, natural absorbents, and cordage (Kumari et al. 2022). However, the inedible banana peel, often discarded due to a lack of scientific awareness, is rich in health-boosting phytochemicals. According to traditional medicine, banana peels are believed to treat diabetes, anemia, inflammation, snakebites, coughs, and other ailments, or they can be used as a health supplement (Vu et al. 2018). Through proper analysis, banana peels could be valuable raw material for developing nutraceutical products. Furthermore, unripe bananas contain higher amounts of phytochemicals and less sugar than ripe fruits. They also contain polyphenols, antioxidants, and resistant starch, all supporting gut health and overall well-being (Borges et al. 2020). Therefore, alternative methods for utilizing the nutritional potential of green bananas include using the unripe pulp as a vegetable or as flour in baking pancakes, bread, and other items (Subagyo and Chafidz 2018). Additionally, both raw and ripe bananas (including the pulp and peel) can be harnessed by the food, cosmetic, and health industries to produce value-added products such as pickles, healthy fat-free chips, banana cakes, cookies, nutraceuticals, supplements, medicines, creams, soaps, shampoos, and beauty powders. This uses the entire fruit and transforms waste into commercially viable products (Kumari et al. 2022).

The Grand Nain (G9) banana is a Cavendish group cultivar known globally for its excellent nutritional value, productivity, and desirable fruit quality (Manokari et al. 2022). The Sabri banana, part of the silk subgroup, is particularly popular in Bangladesh and certain regions of Northeast India. In Tripura, it is extensively cultivated as a key source of revenue and nutritional security due to its taste and dietary value (Mitra et al. 2022). While Grand Nain (G9) and Sabri are well-established commercial cultivars, most reports focus on the primary edible part of the ripe pulp. There is a notable lack of data comparing the nutritional values of the edible and non-edible parts, such as the peel and pulp, in both green and ripe stages. This area remains largely unexplored. Therefore, the present study aims to evaluate the food and nutraceutical potential of different parts (peel and pulp) of green and ripe bananas from these two Musa cultivars, Grand Nain and Sabri.

2 Materials and Methods

2.1 Banana fruit sample collection and preparation

The green (vegetable) stage and ripe bananas of selected Musa cultivars, Grand Nain and Sabri, were collected from the Nagaland University campus in Nagaland, India, and Agartala, Tripura, India. The fresh green and ripe bananas were thoroughly washed with pure water and air-dried. The peels and pulps were then separated and thinly sliced (3-5 mm) using a sterile knife before being oven-dried at 55°C for 12 hours. Once dried, the samples of peels and pulps from both stages were powdered using an electric grinder and sieved to obtain fine particles. The powdered samples were categorized and labeled as follows: a) Musa cv. Grand Nain (G9) – unripe pulp (UGP), unripe peel (UGPL), ripe pulp (RGP), and ripe peel (RGPL); b) Musa cv. Sabri – unripe pulp (USP), unripe peel (USPL), ripe pulp (RSP), and ripe peel (RSPL). These samples were then stored in airtight, sealed plastic bags at room temperature (25±3°C) for further analysis.

2.2 Proximate analyses of the samples

2.2.1 Moisture content

A sample of ten grams of freshly sliced bananas was taken as the initial weight and placed in an oven at 60°C for 10 to 12 hours until a consistent weight was achieved. The final weight was then measured. The moisture content was determined using the AOAC method (2000) and calculated using the appropriate formula:

$$\text{MC (\%)} \left(\frac{\text{Initial wt} - \text{final wt}}{\text{initial wt}}\right) * 100$$

2.2.2 Dry matter content

Dry matter content was determined by weighing the final weight of banana samples (both ripe and unripe pulp and peels) that were oven-dried at 60±2°C for 10 to 12 hours, and the content was calculated using the appropriate formula:

$$\text{DMC}\ (\%) = \left(\frac{\text{Final weight}}{\text{Initial weight}}\right) * 100$$

2.2.3 Total carbohydrate

Carbohydrate content was estimated using the Anthrone method (Sadasivam 1996), with glucose as the standard. A 100 mg sample of dried powdered material (from both ripe and unripe pulp and peel of each cultivar) was hydrolyzed with 5 ml of 2.5 N HCl in a boiling water bath for 3 hours. The mixture was then cooled to room temperature and neutralized with solid sodium carbonate until the bubbling ceased. After neutralization, 100 ml of pure water was added, and the solution was centrifuged for 10 minutes at 10000 rpm. From the supernatant, 100 µl of the sample extract was taken, and the volume was adjusted to 1 ml with ultra-pure water. Subsequently, 4 ml of Anthrone reagent was added, and the mixture was incubated for 8 minutes in boiling water. Following incubation, absorbance was measured at 630 nm, and the carbohydrate concentration was calculated using the standard graph, expressed in grams per 100 grams of dry weight equivalent to glucose.

2.2.4 Reducing sugar

The reducing sugar was estimated using the dinitro salicylic Acid (DNS) method (Sadasivam, 1996), with glucose serving as the standard. One gram of dried powdered banana samples was mixed with preheated 80% ethanol and then centrifuged at 10000 rpm for 10 minutes. A 200 µl aliquot of the sample extract was combined with 2 ml of ultra-pure water and 4 ml of DNS reagent, and the mixture was incubated in boiling water for 5 minutes. After boiling, 500 µl of 40% Rochelle salt solution was thoroughly mixed. The absorbance was measured at 510 nm using a UV-Vis spectrophotometer. The concentration was expressed as grams per 100 grams of dry weight, equivalent to the glucose standard.

2.2.5 Protein content

Nitrogen content was estimated using the micro Kjeldahl method (Goyal et al., 2022). In this procedure, three grams of dried powdered banana samples were digested in acid, then distilled and titrated until a color change from green to pink was observed. The amount of nitrogen was calculated using a specific formula:

$$\text{Nitrogen estimation (\%)} = 1.4 \times V \times N \times W$$

Where V= volume of acid used in titration (ml), N= Normality of the standard acid, and W= sample weight (grams).

Then, Protein content was calculated by multiplying the nitrogen content by 6.25 (Sadasivam 1996).

2.2.6 Crude fat

Crude fat was determined using the Soxhlet apparatus, following the AOAC method (2000) and utilizing petroleum ether as the solvent. A sample of 5 g of dried, powdered banana was pre-dried in an oven and then cooled. This sample was placed in a cellulose extraction thimble, covered with cotton, and inserted back into the Soxhlet extractor. Petroleum ether was added to the extraction flask, and the mixture was extracted for 6 hours at a temperature of 60-80°C. After extraction, the flask with its contents was ovendried and subsequently cooled in a desiccator. The crude fat content was calculated as the difference between the initial and final weights of the extraction flask. The formula used for calculating crude fat is as follows:

$$\text{Crude fat (\%)} = \frac{[(\text{Weight of flask} + \text{Extracted fat}) - (\text{weight of empty flask})]}{\text{Wight of sample}} * 100$$

2.2.7 Ash content

The ash content was determined by incinerating pre-weighed, ground, and dried samples in a muffle furnace at 550°C for 5 hours until white or grey ash was achieved (AOAC 2000). The ash content was then calculated using a specified formula:

$$\text{AC (\%)} = \frac{\text{weight of ash}}{\text{Total weight of sample}} * 100$$

2.2.8 Crude fibre

The crude fiber content was determined following the method described by Madhu et al. (2017). A 3 g sample of defatted dried banana powder was boiled in 200 ml of 1.25% sulfuric acid (H_2SO_4) for 30 minutes. After boiling, the mixture was filtered using a muslin cloth, and any remaining acid was washed off with boiling water. The residue was then boiled again with 200 ml of 1.25% sodium hydroxide (NaOH) for another 30 minutes. This mixture was also filtered and washed with boiling water and alcohol. The residue was transferred to a pre-weighed crucible (W1) and dried at 100 ± 3°C for 4 hours. Once dried, the crucible was cooled in a desiccator and weighed (W2). The dried samples were ignited at 600°C for 1 hour, cooled in a desiccator, and weighed again (W3). The crude fiber content (%) was calculated using the appropriate formula:

$$\%\ \text{Crude fibre} = \frac{(\text{W2} - \text{W1}) - (\text{W3} - \text{W1})}{\text{W1}} * 100$$

2.3 Antioxidant activity and nutraceutical compounds analyses

2.3.1 Antioxidant activity /2, 2- diphenyl-1-picrylhydrazyl free radical antioxidant activity

The antioxidant activity was assessed using the DPPH free radical scavenging assay (Baliyan et al. 2022), with Trolox serving as the standard. The powdered dried banana samples were extracted overnight using an 80% methanolic solvent in a cool, dark environment. After extraction, the mixture was centrifuged at 10000 rpm for 15 minutes to obtain the sample extracts. Ten concentrations of the sample extracts, ranging from 0.5 mg/ml to 5 mg/ml, were prepared by diluting the extracts with 80% methanol to a final volume of 1 ml and then adding 3 ml of a 0.1 mM DPPH solution. The mixtures were thoroughly mixed and incubated for 30 minutes at room temperature in the dark. Absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm. IC50 values were calculated using the appropriate formula:

$$\%\ \text{DPPH radical scavenging} = \frac{(\text{absorbance of control} - \text{absorbance of specimen})}{\text{absorbance of control}} * 100$$

IC50 value is the concentration of sample required to scavenge 50% of the DPPH free radical and is calculated by plotting % DPPH activity against sample concentration.

2.3.2 Total phenol content

The total phenol content was estimated using the Folin-Ciocalteau Reagent colorimetric method (Fu et al. 2011), with gallic acid as the standard. For the analysis, 10 g of powdered dried banana samples (both unripe and ripe pulp, as well as peels) were incubated overnight in 80% methanol. This mixture was placed in a shaker and kept in a dark environment. After incubation, the supernatant was collected following centrifugation at 10000 rpm for 15 minutes and subsequent filtration. To prepare the sample for analysis, 20 µl of the extract was mixed with pure water to reach a final volume of 3 ml, and the mixture was agitated vigorously. Next, 500 µl of Folin-Ciocalteau phenol reagent was added, followed by 2 ml of 7% sodium carbonate. The mixture was then incubated in the dark for 90 minutes. The absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 765 nm, and the total phenol content (TPC) values were calculated using the appropriate formula:

$$TPC = \frac{C * V * DF}{Wt} * 100$$

TPC values were expressed as gallic acid equivalent (GAE) g/100 g dry weight.

2.3.3 Total flavonoid content

The total flavonoid content was estimated using the aluminium chloride colorimetric method (Sahreen et al. 2010), with quercetin as the standard. One gram of powdered dried banana samples (from both unripe and ripe pulp and peels) was incubated overnight with 80% methanol in a shaker. The mixture was then centrifuged at 10000 rpm to extract the sample. Next, 10 µl of the sample extract was combined with 30% methanol to achieve a final volume of 4 ml. 150 µl each of AlCl3·6H2O (0.3 M) and sodium nitrite (0.5 M) were added. The mixture was vigorously mixed and incubated for 5 minutes, after which 1 ml of 1 M NaOH was introduced. The absorbance of the reaction mixture was measured at 510 nm, and the values were expressed as quercetin equivalents in grams per 100 grams of dry weight.

2.3.4 ß-Carotenoid content

The concentration of β-carotenoid was determined using the method of Aremu and Nweze (2017). One gram of dried powdered banana samples was incubated overnight in 10 mL of methanol in a shaker and kept in the dark. To separate the β-carotene layer, 10 mL of hexane was added using a separation funnel, and sodium sulfonate was included to remove moisture. The absorbance was measured at 436 nm using a UV-Vis spectrophotometer, with hexane used as the blank. The concentration of β-carotenoids was calculated using the appropriate formula:

$$\beta - \text{Carotenoid} \left(\frac{U}{mL}\right) = \frac{(Abs * V * DF * 100)}{wt} * Y$$

V = Total extract volume, DF = Dilution factor; Wt=Sample weight; Y = Dry matter content of the sample.

Vitamin A content was estimated by multiplying it with the conversion factor 0.167µg RE (1µg β-carotene= 0.167µg Retinol Equivalent).

2.4 Statistical Analysis

All data results are presented as means ± standard deviations (n=3). The figures were created using Origin software 2021. Significant differences were analyzed using One Way ANOVA in MS Excel 2019, and Pearson‟s correlation test was employed to determine correlation using GraphPad Prism 10.

3 Results

3.1 Proximate composition

The nutritional compositions of both the pulp and peel of raw and ripe fruits from the two cultivars, Grand Nain and Sabri, are presented in Table 1. The differences in the studied parameters, including moisture content, dry matter, total carbohydrates, protein, ash, and crude fiber were found to be non-significant both within and between the cultivars. However, significant differences were observed in reducing sugar (both within and between cultivars) and fat content (within cultivars), but no significant differences were found between the cultivars.

3.1.1 Moisture content

The moisture content of the studied cultivars, as shown in Table 1, ranged from 70.2 to 87.85% for G9 and from 69.67% to 85.58% for Sabri. The highest moisture content was found in the ripe peel of G9 (87.85%) and Sabri (85.58%). In contrast, the lowest moisture content was observed in the unripe pulp of G9 (70.21%) and Sabri (69.67%). The peel had a higher moisture content in both cultivars than the pulp. Furthermore, a significant increase in moisture content was noted as the fruit ripened in both cultivars.

3.1.2 Dry matter content

The dry matter content in the banana samples varied significantly between the two cultivars. For G9, it ranged from 12.16 to 29.79%, while for Sabri, it ranged from 14.64 to 30.33%. The highest dry matter was found in the raw pulp of both cultivars, with 29.79% in G9 and 30.33% in Sabri. Conversely, the lowest dry matter content was observed in the ripe peel of G9 at 16.56% and the raw peel of Sabri at 14.64% (Table 1). As the fruits ripened, there was a gradual decline in dry matter content in both the peel and pulp of G9. Specifically, the dry matter in the pulp decreased from 29.79% when unripe to 24.67% when ripe. In the peel of G9, it reduced from 16.56% to 12.16%. In Sabri, the trend was slightly different; the dry matter content in the pulp decreased from 30.33% (unripe) to 27.76% (ripe). However, unlike G9, the peel of Sabri exhibited a slight increase in dry matter content, rising from 14.64% (unripe) to 16.42% (ripe).

3.1.3 Carbohydrate content

The carbohydrate content ranged from 12.29 to 80.40 g per 100 g in the G9 variety and from 20.46 to 79.65 g per 100 g in the Sabri variety (Table 1). The highest carbohydrate content was found in the unripe pulp of G9 (80.40 g) and Sabri (79.65 g), while the lowest was in the ripe peel of G9 (12.29 g). The carbohydrate content in the pulp was similar for both cultivars; however, Sabri had a higher carbohydrate content in the peel than G9. Additionally, a gradual decline in carbohydrate content was observed in both the pulp and peel as the fruit ripened.

3.1.4 Reducing sugar content

The reducing sugar content ranged from 2.34 to 7.87 g/100g in the G9 cultivar and from 1.73 to 10.37 g/100g in the Sabri cultivar. The highest amount of reducing sugar was observed in the ripe pulp of Sabri (10.37g), while the lowest was found in the raw peel of Sabri (1.73 g) (Table 1). It was noted that the reducing sugar content in both cultivars significantly increased as the bananas ripened. Additionally, the levels of reducing sugar in the ripe pulp and peel of G9 were found to be nearly identical.

Table 1 Comparative proximate composition of unripe and ripe pulps and peels of Grand Nain and Sabri banana cultivars

Banana Cultivars	Parameters	Moisture content (%)	Dry matter content (% in DW)	Total carbohydrate (g/100g in DW)	Reducing sugars (g/100g in DW)	Protein (g/100g in DW)	Fat content (% in DW)	Ash content (% in DW)	Crude fibre content (% in DW)
Cv. Grand Nain	Unripe Pulp	70.21±2.23	29.79±2.23	80.40±6.26	2.34±0.79	3.33±0.30	1.71±0.14	5.71±0.19	0.87±0.13
	Unripe Peel	83.44±2.16	16.56±2.16	33.55±4.21	3.44±1.11	6.93±0.23	8.07±1.32	10.08±0.44	9.59±0.64
	Ripe Pulp	75.33±0.85	24.67±0.85	39.58±2.79	7.71±1.67	3.97±0.33	0.17±0.11	4.58±0.21	0.88±0.09
	Ripe Peel	87.85±3.21	12.16±3.21	12.29±1.64	7.87±1.25	6.11±0.08	6.80±0.35	11.26±0.36	9.72±0.43
Cv. Sabri	Unripe Pulp	69.67±1.12	30.33±1.21	79.65±4.36	2.56±1.19	3.37±0.25	0.25±0.02	2.22±0.91	0.76±0.07
	Unripe Peel	85.36±1.46	14.64±1.46	53.28±8.28	1.73±1.17	4.38±0.15	7.52±3.44	6.61±0.82	10.67±2.40
	Ripe Pulp	72.23±2.32	27.76±2.32	42.16±6.12	10.37±1.45	3.67±0.19	0.53±0.25	2.64±0.11	1.32±0.15
	Ripe Peel	85.58±2.42	16.42±2.42	20.46±1.68	6.41±0.34	6.87±0.53	3.31±0.72	8.74±0.15	6.57±1.12

Data represents the mean of three replicates ±standard deviation.

3.1.5 Protein content

Although not very high, both cultivars were found to have a good protein content ranging from 3 to 7%. As shown in Table 1, the protein content in G9 ranged from 3.33 to 6.93 g per 100 g, while in Sabri, it was reported to be between 3.37 and 6.87 g per 100 g. It revealed that the protein levels were similar in both cultivars. The highest protein content was observed in the peels of unripe G9 (6.93 g) and ripe Sabri (6.11 g). In contrast, the lowest protein content was found in the unripe pulp of Sabri (3.38 g) and G9 (3.33 g). In both cultivars, there was a slight increase in protein content in the ripe pulp compared to the raw stage. However, in cultivar G9, the protein content in the ripe peel decreased compared to the unripe peel. In contrast, there was a significant increase in protein content in the ripe peel of cultivar Sabri, which was 6.87% compared to 4.38% in the unripe peel.

3.1.6 Fat content

As shown in Table 1, the fat content ranged from 0.17 to 8.07% in the G9 cultivar and from 0.25% to 7.52% in the Sabri cultivar. Both cultivars exhibited high fat content in their peels at both the green and ripe stages. For G9, the fat content was recorded 8.07% in the green stage and 6.8% in the ripe stage. In contrast, the Sabri cultivar showed fat content levels of 7.52% in the green stage and 3.31% in the ripe stage.

3.1.7 Ash content

According to Table 1, the ash content in the G9 cultivar ranged from 4.58 to 11.26%, while in the Sabri cultivar, it ranged from 2.22 to 8.74%. This study indicates that the G9 peel has a higher ash content, ranging between 10 and 11%, compared to the Sabri peel, which has an ash content of 6.6 to 8.74%. Additionally, both cultivars" peels exhibit a higher ash content than their respective pulps.

3.2 Antioxidant activity and nutraceutical compounds

The results of the bioactive compounds in G9 and Sabri are presented in Table 2 and Figure 1. We observed no significant differences ($p > 0.05$) in the total phenols, flavonoids, and antioxidant activity values. In contrast, for Vitamin A, there was a significant difference ($p < 0.05$) in the values between both intra- and inter-cultivars.

3.2.1 Antioxidant activity

The IC50 values indicating radical scavenging antioxidant activity ranged from 1.43 to 4.77 µg/ml for G9 and from 0.79 to 5.56 µg/ml for Sabri (Table 2. The highest radical scavenging activity was observed in the peels of unripe G9 (1.43 µg/ml) and ripe Sabri (0.79 µg/ml), both of which exceeded the antioxidant levels found in the edible portions of the bananas.

3.2.2 Total phenolic content

The total phenolic content (TPC) ranged from 67.28 to 359 mg/100g in the G9 cultivar (Figure 1a) and from 52.86 to 284.4 mg/100g in the Sabri cultivar (Figure 1b). The highest levels were found in the ripe peel of G9 (359.8 mg) and Sabri (284.4 mg). Notably, the total phenolic content in the peels was significantly higher than that in the pulp. Additionally, a steady increase in TPC values was observed in the ripened fruits of both cultivars.

3.2.3 Total flavonoid content

The total flavonoid content (TFC), as shown in Figure 1, ranged from 29 to 88 mg/100g in the G9 variety and from 18 to 103 mg/100g in the Sabri variety. The highest TFC was found in the ripe peel of the G9 variety, measuring 88 mg/100g (Figure 1a), while the Sabri variety had a peak TFC of 103 mg/100g (Figure 1b). There were significant differences in flavonoid content

Table 2 Comparative values of antioxidant activity, β-carotenoid and Vitamin A contents in unripe and ripe banana pulps and peels of banana cultivars Grand Nain and Sabri

Cultivars	Parameters	Antioxidant activity (IC_{50} µg/ml)	β-Carotenoid content (µg/100g)	Vitamin Am content (µg/100g)*
Cv. Grand Nain	Unripe G9 Pulp	1.95±0.12	53.7±5.03	8.62±0.98
	Unripe G9 Peel	1.43±0.29	465±18.61	84.81±3.11
	Ripe G9 Pulp	4.77±1.82	60.29±3.04	9.82±0.51
	Ripe G9 Peel	1.81±0.39	275.4±29.6	48.95±7.1
Cv. Sabri	Unripe Sabri Pulp	4.98±3.82	68.41±0.09	10.90±1.66
	Unripe Sabri Peel	5.56±0.33	550.7±35.1	91.97±8.35
	Ripe Sabri Pulp	2.13±0.19	65.29±3.07	10.88±0.51
	Ripe Sabri Peel	0.79±0.1	255.2±5.2	42.46±0.87

Data represents mean of three replicates ±standard deviation.

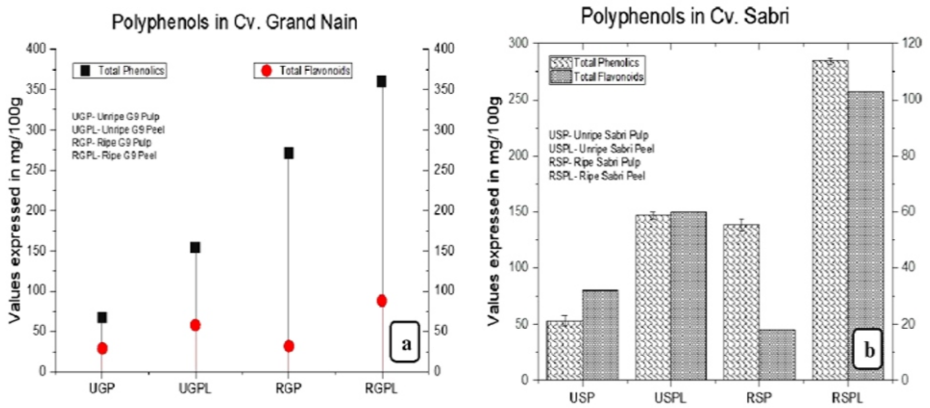

Figure 1 Total phenolic and flavonoid contents in different parts and stages of fruits; a. Cv. Grand Nain or G9, b. Cv. Sabri.

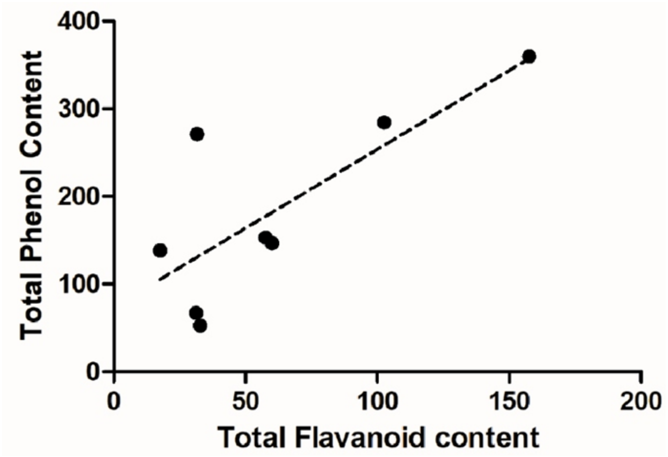

Figure 2 Correlation between total phenol and flavonoid contents. Correlation coefficient, r = 0.7738, 95% confidence interval = 0.1518 to 0.9568, Coefficient of determination, r2 =0.5988.

between the pulp and the peels, with the peels containing a higher concentration of flavonoids. Additionally, it was observed that flavonoid content increased gradually as the fruits ripened.

3.2.4 ß-carotenoid and vitamin A

Beta carotenoid levels (Table 2) ranged from 53.7 to 465 µg/100g (equating to Vitamin A levels of 8.62 to 84.81 µg/100g) in the G9 cultivar and from 65.29 to 550.7 µg/100g (Vitamin A levels of 10.88 to 91.97 µg/100g) in the Sabri cultivar. The highest concentration of Vitamin A was observed in the unripe peel of G9 (84.81 µg/100g) and Sabri (91.97 µg/100g). Both cultivars‟ peels demonstrated higher values than their pulps, with a slight decrease in these values noted during the ripened stage.

4 Discussion

4.1 Proximate composition

4.1.1 Moisture content

A notable increase in moisture content was observed in the ripe fruits of both banana cultivars, as moisture levels elevated during ripening. This increase is primarily due to the breakdown of carbohydrates. The peels of bananas contain a higher moisture content than the pulp, which occurs because of osmotic water transfer from the peel to the pulp, resulting in elevated moisture and sugar levels (Kookal and Thimmaiah 2018). The findings of this study align with reports by Hapsari and Lestari (2016) on ripe dessert (AAA) and cooking bananas (ABB), as well as Dotto et al. (2019) regarding unripe cooking bananas. Additionally, Khawas and Deka (2016) reported that the moisture content in culinary bananas (ABB) ranges from approximately 57 to 65%, which is lower than that of the G9 and Sabri cultivars. Moisture content is influenced by various factors, including genetic traits, farming techniques, and environmental conditions (Ansari et al. 2023).

4.1.2 Dry matter content

The current study observed a gradual decline in the dry matter content of the fruits as they ripened. Similar findings were reported by Chauhan and Jethva (2016), who suggested that the decrease in dry matter during ripening may be due to the transfer and conversion of moisture and starch.

4.1.3 Carbohydrate content

Different forms of carbohydrates are essential components of a healthy diet because they provide the body with energy. Upon digestion, complex carbohydrates convert to glucose, which is the brain‟s primary fuel. The optimal daily intake of carbohydrates is around 200-300 grams per day. The results obtained in the pulp of G9 in the present study align with the findings of Khoza et al. (2021). Additionally, a study by Sardá et al. (2016) on Cavendish and other banana cultivars reported that Sao Paulo cultivars had similar carbohydrate values, viz., 76.24g and 81.43g, which are comparable to those found in the current research. In this study, there was a noticeable decline in the total carbohydrate content of both the peel and pulp of raw and ripe bananas across both cultivars; however, the content of reducing sugars increased. Unripe bananas contain about 70-80% carbohydrates, predominantly in the form of starch and polysaccharides (Philips et al. 2021).

4.1.4 Reducing sugars content

In both cultivars studied, there was a gradual increase in the total sugar content of ripened fruits. This observation aligns with the findings of Kookal and Thimmaiah (2018). The increase in reducing sugars during ripening primarily results from the degradation or hydrolysis of starch into soluble sugars by various enzymes. This process converts starch reserves into soluble sugars, making the pulp of the ripe banana sweet and easily digestible (Khawas and Deka 2016; Kookal and Thimmaiah 2018; Philips et al. 2021).

4.1.5 Protein content

Protein is essential for overall health, supporting immunity, maintaining pH balance, regulating fluid balance, facilitating tissue repair, promoting cell regeneration, and encouraging body growth and structure. The current study‟s findings align with those of Kumar et al. (2019) and Khoza et al. (2021) regarding the raw G9 pulp. Additionally, earlier research by Baiyeri et al. (2011), Bezerra et al. (2013), and Sardá et al. (2016) has also reported similar results, reinforcing the present study‟s conclusions. However, a study by Thatayaone et al. (2022) observed lower protein content in G9 compared to the current research findings. The present study also notes increased protein content as the fruit ripens, which is supported by previous studies (Akaninwor and Sodje 2005; Adegunwa et al. 2017; Kookal and Thimmaiah 2018). This increase in protein content during ripening is attributed to enhanced enzyme activity and protein synthesis (Onwuka and Onwuka 2005).

4.1.6 Fat content

In general, bananas are not a good source of fat. Fats, which are part of the larger class of lipids known as triglycerides, are present in small amounts in our diet to support various bodily functions. These functions include aiding in vitamin absorption, producing hormones such as estrogens, testosterone, and cortisol, and storing energy in the form of fat (Ahmed et al., 2023). The fat content in the edible portion of bananas is very low, often negligible. However, banana peels contain a higher fat content, which can be extracted for commercial purposes. Past studies by Sardá et al. (2016) and Thatayaone et al. (2022) reported similar findings regarding the pulp of Cavendish bananas and G9 varieties. The results of the current study on the Sabri variety are consistent with those of earlier reports. Additionally, a study by Khoza et al. (2021) on the green flour of the G9 variety found a comparatively lower fat content.

4.1.7 Ash content

Ash refers to the inorganic residue left after samples have been subjected to high ignition, which eliminates organic matter. This ash residue revealed the composition of various minerals, primarily potassium, sodium, calcium, magnesium, copper, manganese, and zinc (Preethi et al. 2025). In the current study, the peels exhibited higher ash content, suggesting their potential use as a food supplement (Table 1). These findings align with some reports regarding the pulp of the G9 variety (Khoza et al. 2021; Thatayaone et al. 2022). Research on Cavendish bananas conducted by Sardá et al. (2016) showed similar results for the cv. Sabri variety, although the ash content was significantly lower than that of the G9. Additionally, studies by Kookal and Thimmaiah (2018) and Dotto et al. (2019) on dessert and cooking bananas indicated comparatively lower ash contents compared to the results of the present study.

4.2 Antioxidant activity and nutraceutical compounds

4.2.1 Antioxidant activity

Antioxidants neutralize, reduce, or prevent oxidative damage to target molecules, commonly known as free radicals or reactive oxygen species (ROS). Free radicals or ROS are atoms or molecules with unpaired, highly reactive electrons, capable of instantly attacking cells, DNA, and other molecules. This can lead to harmful effects and damage to the human body, contributing to various chronic illnesses (Ao and Deb 2019; Baliyan et al. 2022). A study conducted by Sulaiman et al. (2011) on eight Malaysian cultivars (Mas, Kapas, Berangan, Rastali, Raja, Nangka, Awak, and Nipah) found antioxidant values similar to those observed in the present study. Conversely, other studies have reported lower antioxidant values than our findings (Khawas and Deka 2016; Siji and Nandini 2017). In our study, the peels of the fruits exhibited a higher concentration of antioxidants than the pulp. This indicates that the peels can serve as a valuable source of antioxidants, which can be extracted and processed into health-beneficial products. These results align with the findings of Someya et al. (2002).

4.2.2 Total phenolic content

Phenolic compounds are a diverse group of bioactive secondary metabolites that exhibit a range of biological activities. They possess anti-inflammatory, antibacterial, and antioxidant properties associated with numerous health benefits. Scientific research has demonstrated their ability to help prevent various diseases, including diabetes, as well as degenerative disorders such as cancer, cardiovascular disease, Alzheimer‟s, and Parkinson‟s disease (Sulaiman et al. 2011; Singh et al. 2016; Elbagoury et al. 2022). Research shows that the total phenolic content in fruit peels is significantly higher than in the pulp. This finding is supported by studies conducted by Someya et al. (2002) and Sulaiman et al. (2011). Additionally, a study by Elbagoury et al. (2022) on the G9 cultivar reported results consistent with those of the present study. Similarly, Bashmil et al. (2021), in their research on six banana cultivars, found that the phenolic content ranged from 54 to 132 mg/100g in peels and from 38 to 128 mg/100g in pulp. Furthermore, our study observed a gradual increase in polyphenol content as the fruits of both cultivars ripened. In a study by Khoza et al. (2021) on G9 green flour, a higher total phenolic content (TPC) of 524.87 mg/100g was reported. In contrast, Bashmil et al. (2021) found slightly lower TPC values in their research on Australian-grown bananas. The discrepancies in TPC values may be attributed to factors such as variations in handling and maintenance during postharvest, prolonged storage, oxidative reactions during storage, temperature conditions, or the selection and drying methods of samples (Kookal and Thimmaiah 2018; Elbagoury et al. 2022).

4.2.3 Total flavonoid content

Flavonoids are the most prominent class of phenolic compounds found in nearly all plants and are well-known for contributing colors to fruits and vegetables. They act as scavengers that protect against reactive oxygen species (ROS) and free radicals, which can lead to various illnesses and the aging process (Singh et al. 2016; Bashmil et al. 2021). In a study by Ali et al. (2019) on the „Grand Nain‟ cultivar, the flavonoid content increased gradually as the fruit ripened. The flavonoid values for the ripe pulp of this cultivar were similar to those of the ripe pulp of the „Sabri‟ cultivar, but there was a notable difference when compared to the ripe pulp of the „G9‟ cultivar in the current study. Additionally, the peels of both cultivars contained significantly higher amounts of flavonoids than the pulp, which correlated with the total phenolic content (TPC) and antioxidant values (Someya et al. 2002; Sulaiman et al. 2011). In some studies, the total flavonoid content (TFC) reported values as high as 407.08 mg/100g in raw „G9‟ flour (Khoza et al. 2021) and 110 to 131 mg/100g in three different cultivars of ripe bananas (Kookal and Thimmaiah 2018). The variations in TFC across different studies can be attributed to factors such as sample type, extraction methods, solvents used, duration, and temperature (Bashmil et al. 2021).

4.2.4 ß-carotenoid and vitamin A

Carotenoids are precursors of vitamin A that are naturally abundant in many foods and are often referred to as provitamin A. These fat-soluble pigments give fruits and vegetables their characteristic yellow, orange, or red colors, enhancing flavor and aroma and attracting pollinators. Carotenoids are well-known for their anti-aging properties and numerous health benefits, including preventing leukemia, retinitis pigmentosa, lung cancer, breast cancer, and various skin conditions (Maoka 2020; Molina et al. 2023). Studies from various researchers have reported the betacarotenoid content in Cavendish and selected banana cultivars, which ranged from 5 to 117.2 µg/100g in the pulp and from 49 to 241 µg/100g in the peels. These findings are consistent with the results of the present study (Arora et al. 2008). In research conducted by Siji and Nandini (2017), beta-carotenoid levels were significantly lower, and reported 2.19 µg/100g in the pulp and 21.19 µg/100g in the peels. The notable variations in carotenoid content can be attributed to the degradation of carotenoids during storage and the effects of repeated thawing and transferring of samples, which can lead to the destruction of carotenoids during postharvest activities.

Conclusions

This study focused on two Musa cultivars, „Grand Nain‟ and „Sabri‟, examining both their pulp and peel‟s unripe and ripe stages. The findings emphasize the significance of nutrient distribution and provide a comprehensive dataset for both cultivars at these two stages, which can facilitate further research. Understanding how to utilize the entire banana and determining the best stage for specific purposes is essential. Both cultivars exhibited nutrients that can help address nutrient deficiencies many countries and regions face. While the nutrient compositions of the two cultivars were similar, „Sabri‟ showed a higher carbohydrate and reduced sugar content. This difference explains the contrasting texture and sweetness between the two types. The study underscored the importance of unripe bananas and their inedible peels, highlighting ways to maximize banana usage. Unripe pulp is particularly rich in carbohydrates, indicating a significant presence of resistant starch, known for its beneficial effects on the digestive system. The peels are nutrient-rich, containing high levels of fiber, protein, minerals, antioxidants, and bioactive compounds essential for health. As a result, pharmaceutical companies can utilize these peels to extract and formulate health supplements, medications, and food additives. The unripe pulp and peels of bananas can also contribute to developing value-added products and enhancing existing offerings. The unripe pulp can be used as a vegetable, while the unripe pulp and peels can improve product quality, making them superior, nutrient-rich, gluten-free, and fiber-rich options. By utilizing the entire banana fruit, it is possible to create more valuable products and support a healthier environment.

Acknowledgement

The authors sincerely thank the UGC NON-NET, GBPNIHESDsponsored NMHS R&D Project, DBT-sponsored Biotech Hub, UGC-SAP (DRS-III), DST-FIST (Level 1), and the Department of Botany, Nagaland University for their support and facilities.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Ethical Approval

No ethical issues were associated with the present study as it did not include human participants, animals, or any sensitive data.

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ISSN NO 2320 - 8694

Biological Performance of Laying Quail Supplemented with Alphitobius diaperinus Larval Meal

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ABSTRACT

The lesser mealworm (Alphitobius diaperinus) is an insect pest that can potentially serve as an important protein source in poultry diets. The high protein content of A. diaperinus larvae makes them a viable alternative for inclusion in quail feed during rearing. This study analyzed quail performance when their diet is supplemented with A. diaperinus larval meal in the rearing phase. This experiment utilized a completely randomized design with four treatments and five replications. The treatments involved replacing fish meal in the diet with A. diaperinus larval meal at 0%, 5%, 10%, and 15%. The results revealed that substituting A. diaperinus larval meal did not significantly affect ($p>0.05$) daily feed consumption, egg weight, or shell thickness. However, it significantly influenced body weight gain ($p<0.05$). While the weight gains at 10% and 15% larval meal inclusion were not substantially different, both were greater than those of the control group. Overall, substituting up to 15% of A. diaperinus larval meal in quail diets had no adverse effects on feed consumption, growth, egg weight, or shell thickness. Therefore, A. diaperinus larval meal may be a suitable alternative to fish meal in quail diets, offering economic benefits and opportunities for sustainable A. diaperinus larval culture.

KEYWORDS Alphitobius diaperinus Biological performance Feed consumption Quail Shell thickness Weight gain

INTRODUCTION

The lesser mealworm (Alphitobius diaperinus) is a pest insect belonging to the family Tenebrionidae. These insects are problematic in poultry farms as they feed on chicken feed and litter. Their presence poses significant economic challenges for poultry farmers worldwide (Renault and Colinet 2021). Additionally, A. diaperinus can be found in various grain storage facilities and poultry houses. Adult beetles and their larvae can damage cage structures by gnawing through the walls and ceilings of wooden buildings, where they also lay eggs and hide pupating larvae (Del Valle et al. 2016; Lyons et al. 2017; Dzik and Mituniewicz 2020). Despite their pest status, A. diaperinus larvae are recognized for their high protein content. In Indonesia, these larvae are frequently used to feed domestic birds and offer a food source for several reptiles and ornamental fish species. The poultry feed industry relies heavily on fishmeal, leading researchers to seek alternative protein sources.

Certain insect species, including the black soldier fly (Hermetia illucens), house flies (Musca domestica), house crickets (Acheta domesticus), field crickets (Grylloides assimilis), banded crickets (Gryllodes sigillatus), yellow mealworms (Tenebrio molitor), and lesser mealworms (A. diaperinus), show potential as nutritious and protein-rich sources for fish and poultry (Veldkamp and Bosch 2015; Khalifah et al. 2023). From an ecological perspective, insects like A. diaperinus can be cultivated to help address environmental issues (Mutafela and Nasilele 2015). A. diaperinus larvae are included in the European Union Commission Regulation (EU 2017/893), identifying insect species safe for animal protein sources in livestock feed (Gasco et al. 2018). These insects can be mass-produced and harvested at the third instar stage, making them a viable protein source for animal feed (Adámková et al. 2016; Janssen et al. 2017; Rumbos et al. 2019). Besides protein and fat, A. diaperinus also contains essential minerals, including zinc, manganese, magnesium, iron, calcium, and phosphorus (Roncolini et al. 2020). The protein content in A. diaperinus larval meal has been reported to range from 49 to 65% (Vrabec et al. 2015; Brandon et al. 2018; Urbanek et al. 2020), with a relatively low chitin and fat content, ranging from 13.4 to 24.3%. Insect larvae are also low in chitin and exhibit high digestibility (De Marco et al. 2015; Józefiak et al. 2016; Kurečka et al., 2021). According to Janssen et al. (2017), the ideal nutritional composition of insects for animal feed is observed during the third instar larval stage, where A. diaperinus has considerably lower chitin content than Tenebrio molitor and Hermetia illucens larvae.

Quails (Coturnix coturnix japonica) require high protein levels in their diets, particularly during their growing phase (Emadinia et al. 2020). The National Research Council recommends that the protein content of a quail's diet during the production phase should be between 20 - 24%, while the energy content should be at least 2900 Kcal/kg. This study aimed to evaluate the performance of quails during the rearing phase when their diet is supplemented with A. diaperinus larval meal. Ultimately, this research seeks to shift public perception of these insects from harmful pests to beneficial organisms while promoting sustainable cultivation practices that provide alternative protein sources for poultry feed, thereby reducing reliance on fishmeal.

2 Materials and Methods

The study was conducted at the Laboratory of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, Indonesia, for approximately 60 days in 2023. It utilized a completely randomized design with four treatments and five replicates. A total of 100 female quails, each 15 days old and in the growing phase, were used for this experiment. The weights of the quails ranged from 80 to 84 grams. Each unit consisted of five quails housed in a battery cage with dimensions of 35 cm × 20 cm

× 37 cm. The treatments involved replacing A. diaperinus larval meal in the quail diet at four levels, i.e., 0%, 5%, 10%, and 15% of the total ingredients (Table 1). According to Emadinia et al. (2020) and the NRC (1994) standard, the diet for the rearing quails was formulated to contain at least 20% protein, with a minimum caloric value of 2900 Kcal/kg. Table 1 presents the formulation of the treatment diets, while Table 2 shows the nutritional value calculations for each treatment.

Biological performance parameters included measurements of feed consumption, weight gain, egg weight, and shell thickness. Observations were conducted, and data were collected over six weeks.

2.1 Palatability measurements

Average feed consumption per bird per day was used to indicate feed palatability. In this study, each bird was given up to 30 grams of feed twice a day, i.e., once at 6:30 a.m. and again at 3:00 p.m., with water provided ad libitum. Any remaining food was removed and weighed daily in the morning before being replaced with fresh feed. To determine feed consumption, we calculated the amount of feed provided minus the amount left over, divided by the length of the maintenance period, and then multiplied by the number of quails in each experimental unit.

2.2 Weight Gain

The body weight of the quails was measured using digital scales at the start of the treatment and then once a week for four weeks. Weighing occurred in the morning before the quails were fed. The average weight gain per bird over the four weeks was calculated by subtracting the initial weight at the beginning of each week from the final weight at the end of the study period.

Table 1 Quail diets formulated to replace A. diaperinus larvae meal

Ingredient (%) Experimental diets

Ingredient (%)	Experimental diets			
	P0	P1	P2	P3
Yellow Corn	52	52	51	51
Rice Bran	10	10	11	11
Soybean Meal	13	13	13	13
Fish Meal	15	10	5	0
Mealworm Powder	0	5	10	15
Coconut Oil Cake	8	8	8	8
Vitamins and minerals (Premix)	2	2	2	2
Total (%)	100	100	100	100

P0 = Control treatment (without A. diaperinus larval meal), P1 = Diet treatment with 5% A. diaperinus larval meal, P2 = Diet treatment with 10% A. diaperinus larval meal, P3 = Diet treatment with 15% A. diaperinus larval meal

Table 2 Calculation of nutritional value in each treatment diet*

Nutritional Content	P0	P1	P2	P3
Protein (%)	20.50	20.58	20.71	21.00
Crude Fiber	7.67	7.00	6.84	6.67
Fat (%)	7.52	8.01	8.50	9.01
Ca (%)	3.73	3.44	3.17	2.88
P (%)	0.76	0.7	0.63	0.57
ME (Kcal)	3002.27	3014.48	3006.68	3108.88

*The nutritional value of each ingredient.

2.3 Egg Weight Measurement

Egg samples were collected on nesting days, which occur 5-7 days after the first egg is laid. Five egg samples were taken from each cage and weighed, and the average weight of eggs per head per cage was calculated.

2.4 Measurement of shell thickness

All egg samples were cracked open, and the eggshells were removed. Each shell was measured three times for thickness at three different locations, i.e., the blunt end, the pointed end, and the equator. The measurements were taken using a digital caliper, and the average of these measurements represented the shell thickness of each egg.

2.5 Data Analysis

SPSS 24 software was used to analyze the mean ± standard deviation data. The ANOVA test was used to identify significant treatment differences, and further tests were performed using Tukey’s test.

3 Results and Discussion

Table 3 presents the measurement results for four biological performance parameters: daily feed consumption, weight gain, egg weight, and shell thickness.

3.1 Feed Consumption

The ANOVA analysis indicated that substituting larval meal at 5% to 15% level in the diet had no significant effect ($p > 0.05$) on average feed consumption. Quails consumed between 25.17 and 25.58 grams per head per day on average during the growing phase. This average consumption during the rearing phase of the quails was consistent with the study by Li et al. (2023), which reported an average daily feed intake of 25.19 grams per head per day for laying quail. The lack of treatment effect on feed consumption in this study suggests that diets containing A. diaperinus larval meal do not affect the palatability of feed for laying quail. Physically, A. diaperinus larval meal appears drier than fish meal, light brown in color, and has no strong odor. According to Janssen et al. (2017), A. diaperinus larval meal contains a low level of chitin (0.304%), which is less than the chitin found in T. molitor larvae (0.624%) and H. illucens (0.529%) based on dry matter content. Chitin is an N-acetyl-Dglucosamine polymer and is a major component of the exoskeletons of crustaceans, insects, and some fungi. It contains polysaccharides that animals cannot digest (Tabata et al. 2017). A. diaperinus larvae are also noted to contain all essential amino acids, with an index of 71.8% (Kurečka et al. 2021). Palatability is a crucial factor influencing the level of feed consumption, affected by taste, smell, and color, which are physical and chemical characteristics of the feed (Sadarman et al. 2022). Additionally, metabolic needs and satiety can influence feed palatability (Mc Crickerd and Forde, 2016). The level of feed palatability can serve as a measure of feed consumption efficiency. An indication that the feed is palatable is when birds demonstrate eating behavior that leaves little to no residue, as observed in this study.

Table 3 Biological parameters of laying quails after treatment

Treatment	Biological Parameter			
	Feed Consumption (g/head/day)^{ns}	Overall Weight Gain (g/head)	Egg Weight (g/egg)^{ns}	Shell Thickness (mm)^{ns}
P0	25.17±0.58	146.08 ± 2.72^{a}	11.10 ± 0.97	0.18 ± 0.06
P1	25.58±0.25	151.35 ± 3.43^{a}	10.74 ± 0.82	0.16 ±0.02
P2	25.17±0.50	165.27 ± 2.66^{b}	11.37 ± 0.86	0.18 ±0.03
P3	25.52±0.16	169.97 ±3.22^{b}	11.22 ± 0.56	0.15± 0.04

ns = non-significant, Significantly different at $p<0.05$ for different superscripts within the same row, legends for P0, P1, P2, and P3 are the same as those mentioned in Table 1.

3.2 Body weight gain

The ANOVA analysis revealed that varying the flour substitution of A. diaperinus larvae in the feed significantly affected the weight gain of laying quails during the rearing phase ($p < 0.05$). The highest weight gain was observed in the P3 treatment, which reached 169.97g (Table 3). The Tukey test indicated that there was no significant difference between the P0 and P1 treatments ($p > 0.05$), but both were significantly different from the P2 and P3 treatments ($p < 0.05$). These findings suggest that substituting 10% and 15% of the feed with A. diaperinus larvae resulted in better growth than the control group. In this study, the weight gain of quails during the grower phase was calculated based on the average weekly weight gain over four weeks. The total body weight gain in the control group was 6.08 g/head/day, which was not significantly different from that of the rearing quails. This finding aligns with a study by Khan et al. (2023), which recorded a total body weight gain of 159.06 g/head over four weeks, equivalent to 5.47 g/head/day. Similarly, Reda et al. (2020) reported a gain of 5.91 g/head/day, equivalent to 165.48 g/head. Previous research using larval meal from Hermetia illucens also demonstrated similar growth trends in laying quail (Widjastuti et al. 2014). The improved growth can be attributed to diets containing A. diaperinus larval flour, enhanced feed efficiency compared to the control diet, which did not include A.

diaperinus larval meal. This improved feed efficiency may result from the high digestibility of A. diaperinus larval meal. According to Bosch et al. (2014), the digestibility of T. molitor and A. diaperinus larval proteins is approximately 91%. Additionally, the protein content of A. diaperinus larval meal reaches 62% of dry matter, and its chitin content is lower than that of T. molitor and H. illucens larvae (Janssen et al. 2017). Feed digestibility gives insight into livestock's ability to utilize feed, as high and low digestibility reflect the amount of feed that animals can effectively digest (Zhang et al. 2017).

3.3 Egg Weight

The effect of supplementing A. diaperinus larval meal in the diet on quail egg weight during the first week of laying is presented in Table 3. Statistical analysis indicated no significant effect ($p > 0.05$) on egg weight; the egg weights across all treatments were not significantly different from those in the control group. The average egg weight in the control group was reported to be 11.10 g per egg, while the average weights for the treatments with 5%, 10%, and 15% A. diaperinus larval meal were 10.74g, 11.37g, and 11.22g, respectively. Therefore, substituting A. diaperinus larval meal at a level of 15% did not affect egg weight in this trial. According to Silaban et al. (2019), quail egg weights typically range from 10 to 11.91 g per egg, accounting for about 8% of the mother's body weight, placing them within the normal range. Compared to other studies, this average is higher than the egg weights reported in commercial or standard feed treatments. For instance, Ibrahim et al. (2018) reported an average egg weight of 9.63 g per egg in their control group, Akramullah et al. (2023) noted 9.79 g per egg, and

Li et al. (2023) found an average of 10.93 g per egg. Using the simple additive weighting method described by Abadi et al. (2018), quail eggs can be classified into five criteria based on their weight: very high, high, medium, low, and very low. In this classification system, quail eggs weighing between 10 and 11 g per egg are categorized as very high quality.

3.4 Eggshell thickness

Table 3 presents the mean thickness of quail eggshells for each treatment. The statistical analysis of the eggshell thickness data indicated that the supplementation of A. diaperinus larval meal at 15% did not significantly affect egg weight ($p > 0.05$). This implies that the egg weights across all treatments were comparable to those of the control group. Specifically, the mean egg weight for the control was 11.10 g/egg, while the weights for the predefined three treatments were 10.74 g, 11.37 g, and 11.22 g. Therefore, replacing A. diaperinus larval meal at 15% did not impact egg weight in this study. Furthermore, the analysis also demonstrated that the substitution of A. diaperinus flour did not significantly affect shell thickness ($p > 0.05$). The mean shell thickness in the control group was 0.18 mm, while the treatment groups showed values ranging from 0.15 mm to 0.18 mm. This indicates that incorporating A. diaperinus flour in the diet can still support consistent eggshell thickness at the beginning of the laying period. According to Abadi et al. (2018), eggshell thickness can be categorized into several ranges, with thicknesses between 0.13 mm and 0.2 mm falling into the low category. Multiple factors influence shell quality, including genotype, age, oviposition time, and the diet's balance of minerals and calcium-phosphorus elements. The thickness of the shell is also related to the time required for formation but is primarily influenced by genotype (Ketta and Tůamová 2016). Increasing dietary calcium can enhance shell quality and thickness (De Souza et al. 2016). Gül et al. (2022) observed a decrease in shell thickness with age in laying quails, noting that at 56-60 weeks, the eggshell thickness measured 0.372 mm compared to 0.354 mm at 20-24 weeks. The study concluded that the eggshell quality of older quails is superior to that of younger ones, while egg size, feed intake, and folliclestimulating hormone levels significantly increase with age. Higher energy and protein levels in the diet can also contribute to increased thickness and strength of eggshells (Lotfi et al. 2018).

Conclusion

The results of this study indicate that A. diaperinus larval meal can potentially be an animal protein source for quail, serving as a substitute for fish meal. To promote its sustainable use, it is important to change public perceptions of A. diaperinus insects, viewing them not as pests but as a high-value protein source. Additionally, developing methods to produce larval meals on an industrial scale is essential. Feeding quails with A. diaperinus larval meal, up to 15% of their diet, does not negatively impact feed intake, egg weight, or shell thickness. However, it influences female quails' weight gain during their growing phase. Therefore, if A. diaperinus larval meal can be consistently produced on an industrial scale, it holds promise as an alternative protein source for quails.

Acknowledgment

The researcher would like to thank Universitas Negeri Semarang for supporting the DIPA LPPM UNNES Fund research for number 10.12.4/UN37/PPK.10/2023.

Ethical Approval

The Ethical Clearance for this research was obtained from the Health Research Ethics Commission (KEPK) Faculty of Sports Sciences, Universitas Negeri Semarang (No. 202/KEPK/EC/2023).

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