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BIODIVERS is a bio-science general audience magazine launched in December 2021 by the Southeast Asian Ministers of Education Organization Regional Centre for Tropical Biology (SEAMEO BIOTROP). As a scientific publication, it is designed to increase awareness and literacy on issues related to saving Tropical Biodiversity from the "Mountain to the Ocean" (MOTO). The scope of BIODIVERS includes the following (1) Ecosystem Restoration and Conservation, (2) Sustainable Use of Biodiversity, Bioenergy, Biotechnology, Food Security, and (3) Ecosystem Resilience in the face of global climate change

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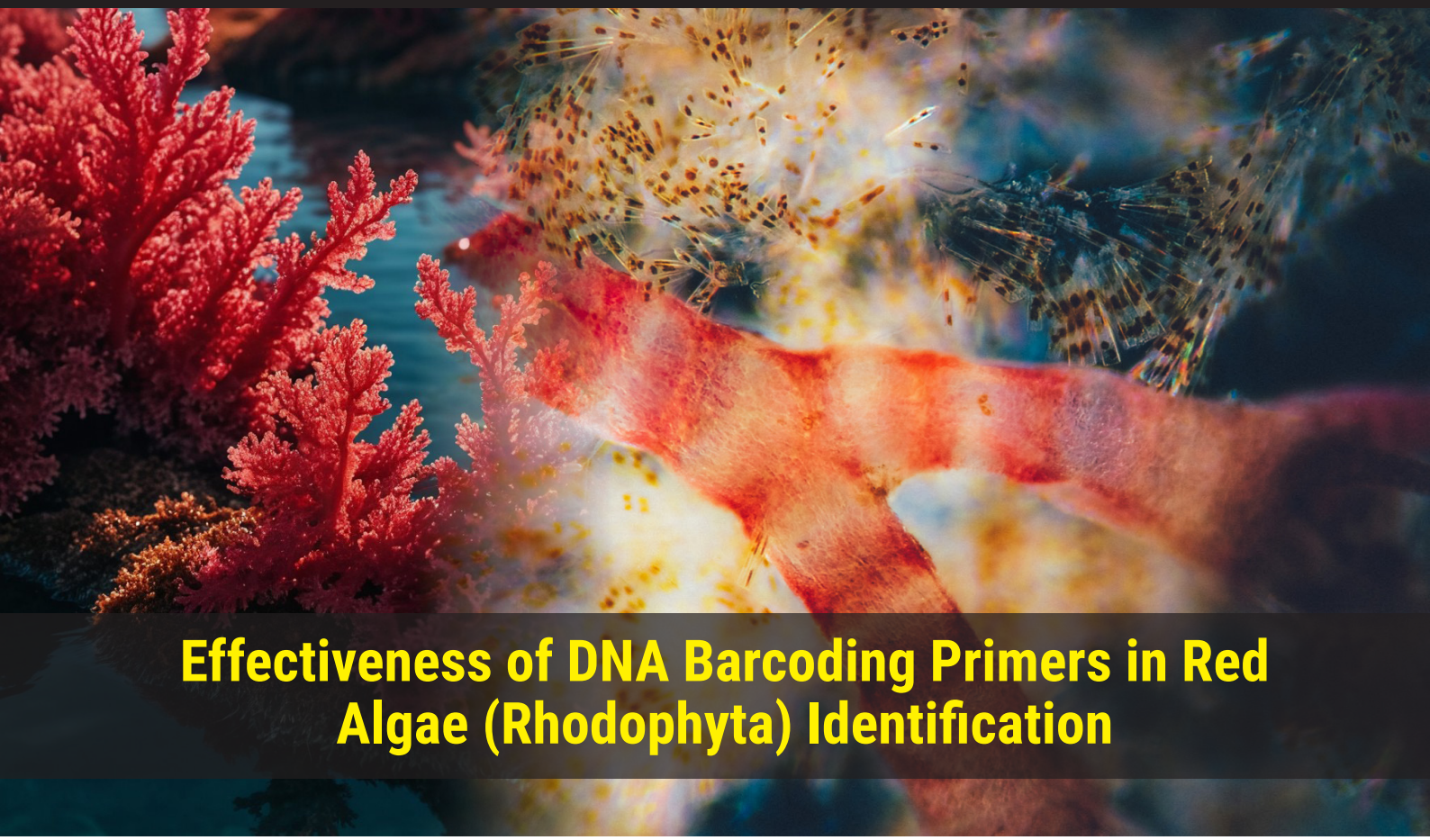

Effectiveness of DNA Barcoding Primers in Red Algae (Rhodophyta) Identification

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ABSTRACT

Red algae (Rhodophyta) are vital primary producers in marine ecosystems and are economically significant due to their wide use in food, pharmaceutical, and cosmetic industries. The significant utilization of red algae indicates that these organisms require conservation and protection from extinction, therefore, accurate identification is a must. Traditional morphological approaches face challenges due to their simplicity and plasticity; however, molecular techniques, such as DNA barcoding can overcome these limitations. This study evaluated the effectiveness of using ITS1, Cox2-3, rbcL 1, and rbcL 2 primers for barcoding seven red algae species, focusing on amplification success and sequencing quality. All of the above-mentioned primers have demonstrated a noteworthy amplification rate of success, with 100% efficacy observed for ITS1 and rbcL 2. However, only Cox2-3, rbcL 1, and rbcL 2 primers exhibited a high-quality read based on the sequencing quality score, indicating their reliability in capturing the target sequence for identification. The results strongly suggested that rbcL 2 is the optimal choice for identifying Rhodophyta due to its high amplification rate and high-quality sequencing results.

Keywords: DNA barcoding, genetic identification, primer, seaweed

INTRODUCTION

Rhodophyta, commonly known as red algae, is a division within the subkingdom Biliphyta, classified under the plant kingdom, Plantae (Ruggiero *et al.,* 2015). Red algae play a pivotal role in marine ecosystems as primary producers, significantly contributing to the maintenance of coral reefs, and providing a structural habitat for a diverse range of microorganisms through the secretion of calcium carbonate in their cells (Rajasulochana & Preethy, 2015). Certain species in this group are economically valuable because they produce carrageenan, a polysaccharide derived from algae. Carrageenan is extensively utilized across several industries, including food, pharmaceuticals, and cosmetics. The considerable utilization of red algae indicates that these organisms require conservation and protection from extinction (Samman & Achmad, 2023).

Traditional morphological identification techniques encounter challenges in diversity study. This is especially the case for red algae species that display simple, environmentally plastic or convergent characteristics, manifesting as morphological features that are difficult to define precisely (Zuccarello & Paul, 2019). In contrast to environmental and developmental factors, DNA-based molecular tools are not susceptible to such influences (Dev *et al.,* 2020). The application of a DNA-based information repository for conservation initiatives allows for the formulation and substantiation of policies through informed decision-making (Hogg *et al.,* 2022).

Over the past two decades, DNA barcoding, a novel approach utilizing DNA markers, has emerged as a reliable and rapid method for organism identification in the scientific community (Letsiou *et al.,* 2024). The DNA regions used as universal markers originate from sequences located in the nucleus, such as the Internal Transcribed Spacer 1 (ITS1), or from those within specialized organelles, such as the mitochondrial DNA (mtDNA) Cox2-3 spacer markers gene. The ITS-1 region, located between the 18S and 5.8S rDNA coding regions, is a commonly utilized marker in phylogenetic studies due to its high variability (Lee *et al.,* 2024). Similarly, the Cox2-3 spacer has been validated as a valuable marker for studying intraspecific relationships, given that this noncoding region exhibits a higher mutation rate than the surrounding genes (Zuccarello *et al.,* 2006). Moreover, the RuBisCo ribulose-1,5-bisphosphate carboxylase oxygenase-large subunit (rbcL) gene was utilized for phylogenetic studies due to its high amplification efficiency and restriction to photosynthetic organisms (Wongsawad & Peerapornpisal, 2014).

Prior research has shown that ITS1, Cox2-3, and rbcL primers are effective for delineating species boundaries within the red algae group (Achmad *et*

al., 2024; Mshiywa *et al.,* 2024; Osathanunkul *et al.,* 2018; Satriani *et al.,* 2023). Universal barcode markers must be evaluated across a more expansive spectrum, given the morphological and geographical variations and the reticulate evolution observed in plant species (Bafeel *et al.,* 2011). The success of polymerase chain reaction (PCR) is a prerequisite for barcoding, as this technique is the exclusive means of amplifying the target sequence. Consequently, maintaining high PCR success rates and its product read quality remains an important scientific objective. This study examined the effectiveness of ITS1, Cox2-3, and rbcL primers for barcoding seven species of red algae, particularly emphasizing the success rate of amplification and sequencing quality scores.

MATERIALS AND METHOD

The study was conducted at the Plant Biotechnology Laboratory, using seven red algae samples from Ambon and Lampung cultivation area and collected by a team from the Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP) Bogor, Indonesia. Sample descriptions used in the study is listed in Table 1.

Total genomic DNA was extracted according to the cetyltrimethylammoniumbromide (CTAB) method described by Doyle *et al.,* (1990), modified by adding 3% PVPP. DNA concentrations were quantified using NanoPhotometer N50-Touch (Implen, Germany), and the quality of extracted DNA was confirmed by electrophoresis on 1% agarose gels prepared with 1× TAE Buffer and stained with DNA loading dye. DNA was stored at -20 ^{o}C for later analysis.

Table 1 The codes of the seven samples utilized in the study

No.	Code	Common Name
1	LK15	Lampung 2015
2	LK18	Lampung 2018
3	BPBL	BPBL Ambon Culture
4	SCL	Sacol Lampung
5	KMT	Kotoni Maluku Tenggara
6	KW	Kotoni Wanci
7	SW	Spinosum Wanci

The specific DNA regions, ITS1, Cox2-3, rbcL 1, and rbcL 2 primers were then amplified by PCR using each primer pair. The rbcL 2 primer was designed on the basis of the complete *Kappaphycus alvarezii* and *Kappaphycus striatus* chloroplast genome available in the National Center for Biotechnology Information (NCBI) database to ensure specificity and accuracy in targeting the desired regions using the PrimeQuest website (https://www.idtdna.com/PrimerQuest). Each PCR mixture (50 µL) consisted of a 5 µL DNA template, 2 µL of each primer (10 mM), 25 µL MyTaq HS Red Mix (Bioline Reagent, Ltd., United Kingdom), and 16 µL Nuclease Free Water (NFW). The primers and PCR conditions are detailed in Table 2.

The PCR product was visualized by 1% agarose gel electrophoresis with the addition of 3 µL FloroSafe DNA Stain. Gel images were acquired with Vilber Lourmat ETX-20.M UV transilluminator (Vilber, France). The amplified PCR products were examined for the presence or absence of the band on an agarose gel to determine the percentage of primers amplification rate. The DNA amplification results were then sent to APICAL Scientific Malaysia for sequencing purposes. The Chromatogram of each sequencing product was displayed using GeneStudio software and quality scores for all sequences were determined using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Table 2 PCR primers and programs used for DNA amplification

Primer Set	Primer sequence (5'-3')	PCR condition	Reference
ITS1	F : GGTGAACCTGCGGAAGGATCATTG	95 °C, 1 min; 30 × (95 °C, 15 s; 59 °C, 15 s; 72 °C 30 s); 72 °C, 3 min; ∞ 4 °C	(Osathanunkul *et al.*, 2018)
Cox2-3	R : CCGAGATATCCATTGCCGAGAGTC	95 °C, 1 min; 30 × (95 °C, 15 s; 56 °C, 15 s; 72 °C 30 s); 72 °C, 3 min; ∞ 4 °C	(Zuccarello *et al.*, 1999)
	F : GTACCWTCTTTDRGRRKDAAATGTGATGC		
	R : GGATCTACWAGATGRAAWGGATGTC		
rbcL 1	F : AACTCTGTAGTAGAACGNACAAG	94 °C, 4 min; 35 × (94 °C, 1 min; 52 °C, 1 min; 72 °C 1 min); 72 °C, 10 min; ∞ 4 °C	(Satriani *et al.*, 2024)
	R : GCTCTTTCATACATATCTTCC		
rbcL 2	F : CATATAAAGTCGATGCTGTG	95 °C, 1 min; 30 × (95 °C, 15 s; 50 °C, 15 s; 72 °C 30 s); 72 °C, 3 min; ∞ 4 °C	(Designed in this study)
	R : CACCTGTAGCAGCAATA		

Table 3 Primer screening using ITS, Cox2-3, rbcL 1, and rbcL 2 primers in seven red algae samples

Primer set	Amplification product							Amplification rate (%)
	LK15	LK18	BPBL	SCL	KMT	KW	SW	
ITS1	+	+	+	+	+	+	+	100.0
Cox2-3	+	+	+	+	+	-	+	85.7
rbcL 1	+	+	+	+	+	-	+	85.7
rbcL 2	+	+	+	+	+	+	+	100.0

Notes: (+) = Amplified successfully; (-) = Cannot be amplified.

RESULTS

Amplification Rate Analysis

The rbcL 2 primer designed in this study was able to amplify all DNA samples and had higher amplification or success rate than rbcL 1 primer from the literature reference (Table 3). Table 3 also shows that ITS primer was able to amplify all DNA samples (Table 3; Fig. 1). On the other hand, sample "KW" could not be amplified by using Cox2-3 and rbcL 1 primers (Table3; Fig.2), which may have been caused by the incompatibility of the primers with the sample (Roux, 2009). It is worth noted from this study that PCR setup and temperature optimization enhance the amplification success during the PCR process.

Sequencing Quality Score Analysis

FastQC is a very popular tool used to provide an overview of basic quality control for next-generation sequencing data (Wingett & Andrews, 2018). The Sequence Quality Score in FastQC is a critical analysis module that assesses the quality of sequences in a FASTQ file. FastQC can visually view the quality of the segment. A warning is raised if the most frequently observed mean quality is below 27, and an error is raised if the most frequently observed mean quality is below 20 (Shi & Xu, 2016).

The quality score of sequence reads was analyzed using fastQC software (Table 4). As we can see, sequences analyzed by using rbcL 1 & 2 primers had an average score over 36, which means the sequences had a good quality. A low-quality score (0-20) is indicative of sequences with chromatograms devoid of read peaks (Fig. 3, left section for ITS sequences). The discernible peaks between each sequence read are regarded as sequences exhibiting superior sequencing quality, with a score above 20, and may serve as an identification reference (Fig. 4).Table 4 The quality score of sequencing results in ITS, Cox2-3, rbcL 1, and rbcL 2 primers in seven red algae samples

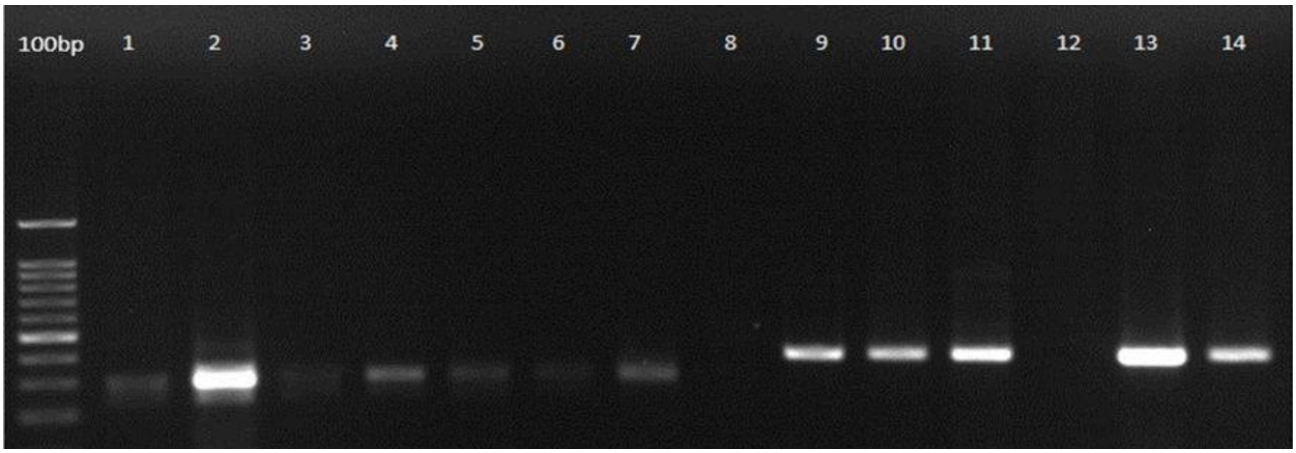

Figure 1 Amplified ITS1 (1-7) and Cox2-3 spacer (8-14) in red algae samples

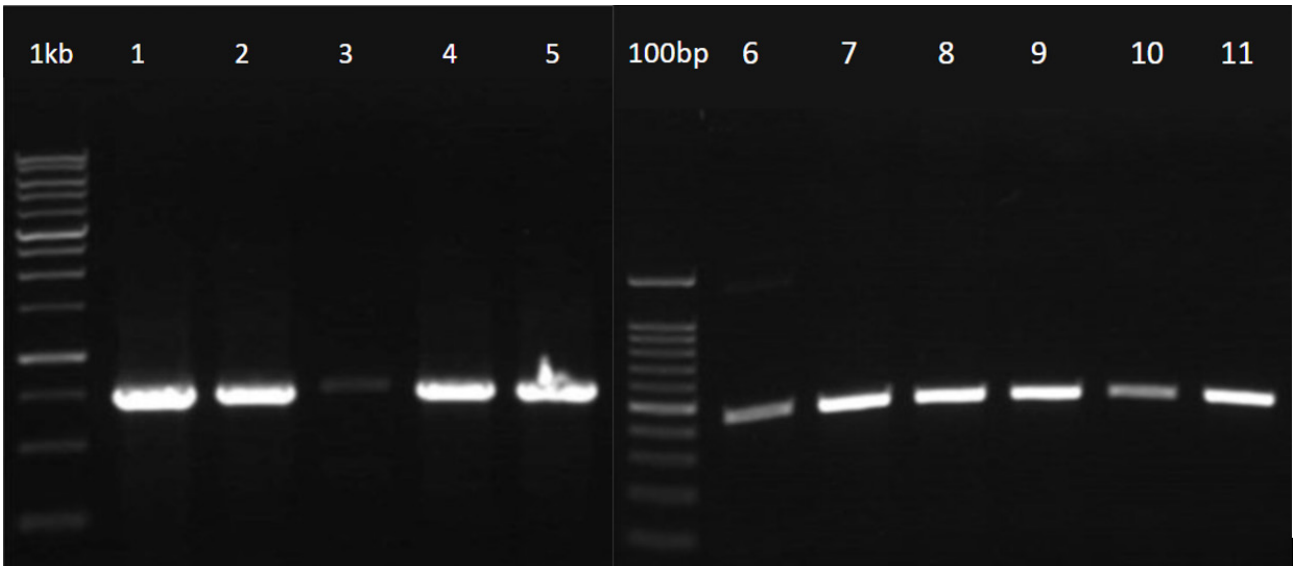

Figure 2 Amplified rbcL 1 (1-5) and rbcL 2 (6-11) in red algae samples

Table 4 The quality score of sequencing results in ITS, Cox2-3, rbcL 1, and rbcL 2 primers in seven red algae samples

Primer set	Quality score														Ave.
	LK15		LK18		BPBL		SCL		KMT		KW		SW		
	F	R	F	R	F	R	F	R	F	R	F	R	F	R	
ITS1	13.4	10.3	13.3	17.8	0.0	0.0	25.1	12.4	0.0	14.9	12.5	12.8	0.0	0.0	9.46
Cox2-3	40.8	39.9	21.2	13.0	44.6	44.1	43.9	41.7	44.9	42.0	-	-	42.9	45.1	33.15
rbcL 1	46.1	46.4	45.3	46.5	46.2	47.3	45.6	46.0	46.2	45.6	-	-	40.2	26.7	37.72
rbcL 2	41.8	39.4	41.9	45.7	42.8	42.3	44.7	44.7	47.0	47.6	45.0	45.3	43.1	42.4	43.86
Average	35.5	34.0	30.4	30.8	33.4	33.4	39.8	36.2	34.5	37.5	14.4	14.5	31.6	28.6	

Notes: <20 = low-quality QC score; 20-30 = normal quality QC score; >30 = high-quality QC score.

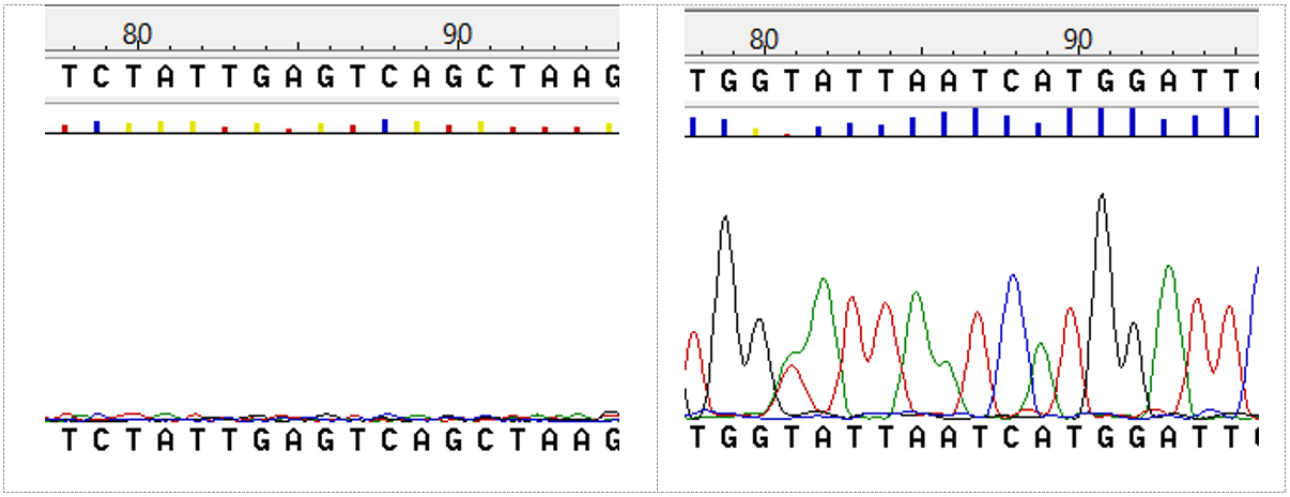

Figure 3 Sequencing chromatogram of LK18 DNA amplified by ITS1 (left) and Cox2-3 (right) primers

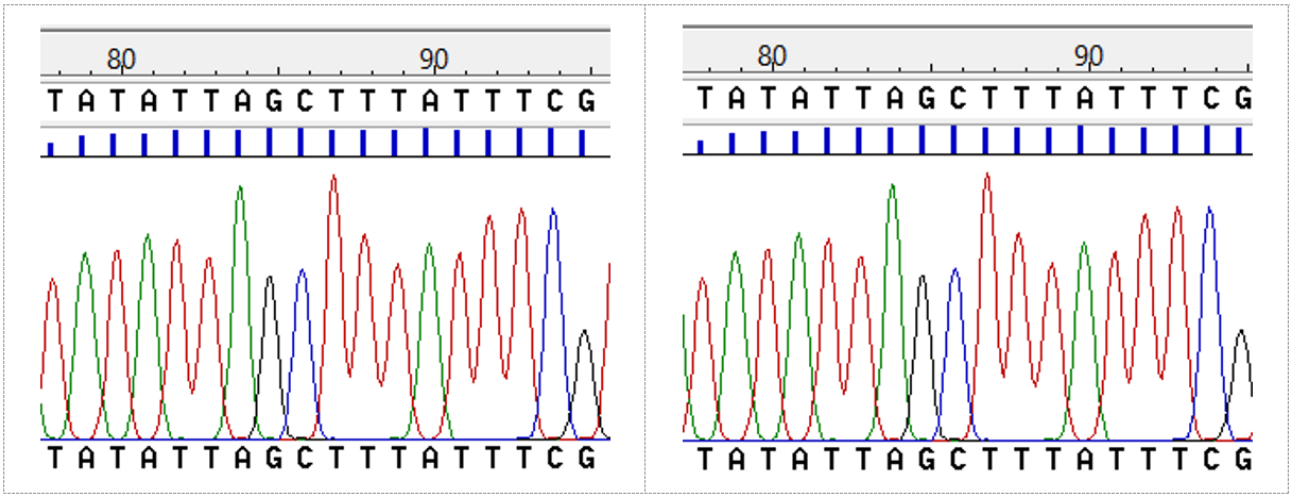

Figure 4 Sequencing chromatogram of LK18 DNA amplified by rbcL 1 (left) and rbcL 2 (right) primers

DISCUSSION

Accurate identification of many red algae to the species level using only morphological characters can be difficult. DNA barcoding developed approximately twenty years ago, is an approach that has significantly contributed to the development of the molecular biology field study (Hebert *et al.,* 2003). In its development, DNA barcoding has helped in the process of identifying plant species, both aquatic and terrestrial plants.

In this study, we successfully identified red algae using several DNA barcoding primers. As a result of this study, we found that rbcL 2 primers showed advantages over other primers. PCR results using rbcL 2 primer was able to amplify all DNA samples with an optimal sequence QC value (Table 3). This result is in accordance with the results of other studies which stated that rbcL has been effectively used in various ecological studies, including marine environments, demonstrating its adaptability and effectiveness in different biological contexts (Turk Dermastia *et al.,* 2023).

One of the important factors in the success of DNA barcoding is determined by the success of the PCR process. In PCR experiments, the primers are the key to the success of the experiment. Primers have a very important role in the process of PCR amplification (Bustin *et al.,* 2020). If the primers are too short they might hybridize with non-target sites and give undesired amplification products. In addition, the suitability of temperature is also a determining factor in the success of PCR (Roux, 2009).

Phillips *et al.,* (2019) proposed that several factors must be considered and weighed when selecting a DNA barcode, such as universal PCR amplification, range of taxonomic diversity, power of species differentiation, and bioinformatics analysis and application. Generally, for one gene marker, at least two or more pairs of primers are used, especially for ITS, Cox, and rbcL, because all of those primers are among the most commonly used universal primers (Kowalska *et al.,* 2019). As a widely used and effective tool, DNA barcoding will become more useful over time in the field of any plants.

The present barcode reference libraries are insufficient in marine macro-algal identification for Indonesian species, therefore, more efforts for DNA barcoding program of the local species is necessary to facilitate the environmental monitoring efforts, especially red algae species. Building a comprehensive local barcode reference library could contribute to resolving macro-algal taxonomy and systematics and address biogeography pertaining to the invasion of non-indigenous species. This could also result in the development and application of cost-effective and better biodiversity monitoring projects.

Some species of red algae have health, industrial, and environmental benefits (Tanaka *et al.,* 2020; Subramanian *et al.,* 2020). In species like *Kappaphycus alvarezii* barcoded in the present study and the ones occurring on the Brazilian coast (Nogueira *et al.,* 2019), various haplotypes were known to contain variable composition of antioxidants (Araujo *et al.,* 2020). Hence, the generated barcodes will be useful for taxonomic non-experts of food, pharmaceutical, and cosmetics. Further studies could be carried out to explore the possibility of linking DNA barcodes to inter and intra-species biochemical constituents of seaweeds.

CONCLUSION

Based on the result of this study, we recommend the use of rbcL 2 primer to identify red algae species because its average QC score is over 30, which is the high-quality category. Strengthening the local barcode libraries by barcoding all species could facilitate cost-effective biodiversity surveys and effective environmental barcoding programs in the near future. The generated barcode and the use of certain primers can facilitate further research (climate change, species distribution) and also for various industrial (pharmaceutical, biofuel, cosmetic, seafood, etc.) applications.

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Ethnomedicinal Study and Phytochemistry Analysis of Antihypertensive and Anticholesterol Plants in Sukaharja Village, Lebak Regency, Banten Province, Indonesia

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ABSTRACT

Medicinal plants are still used by people to treat many diseases, such as to lower cholesterol levels and hypertension. In Sukaharja Village, Lebak Regency, Banten Province, Indonesia, for example, the Baduy tribe still believe in practicing traditional medication. The village location is far away from the nearest health facilities. This ethnomedicinal study was conducted in the year 2023 by applying a quantitatively descriptive method. Data were collected by interviewing several respondents selected by purposive and snowball sampling methods. The list of plants was then verified by field observation, followed by species authentication, and use value (UV) calculation. Fresh samples of plants were made into simplicia and underwent phytochemical screening, including a TLC test, in a laboratory. From 50 respondents, we obtained information on 21 plant species used to lower cholesterol levels and for treating hypertension. The highest UV of plants used to lower cholesterol levels were *Peperomia pellucida* (0.56) and *Annona muricata* (0.52), while for treating hypertension were *A. muricata* (0.60), followed by *Allium sativum* (0.56). All of the plants used contained flavonoids, and selected plants examined by TLC test were revealed to be similar to quercetin. Traditional medication practices need to be preserved, which is as important as conserving medicinal plants that are precious biodiversity in Indonesia.

Keywords: Banten, cholesterol, ethnomedicine, hypertension, phytochemistry


https://www.gardeningknowhow.com/houseplants/peperomia/peperomia-pellucida-from-seed.htm

INTRODUCTION

Indonesia has one of the highest biodiversity, with many unique ecosystem and high endemicity. Ethnomedicine is a branch of ethnobotany or health anthropology that study about traditional medication which passed down for generations. This also leads to an opportunity in finding new source of medicine (Saranani *et al.,* 2021). Ethnomedicinal study is among solutions to conserve high biodiversity of medicinal plants in Indonesia.

Secondary metabolites contained in plants have pharmacological effects and, thus, have been used to treat many diseases. Hypercholesterolemia and hypertension are examples of degenerative diseases that have been treated by using medicinal plants based on traditional beliefs.

Phytochemical screening needs to be performed to qualitatively identify the metabolite that might be valued as a medicine in a plant, by using few reagents to identify the presence of flavonoids, saponins, tannins, steroids, terpenoids, and alkaloids contained in medicinal plants (Agustina *et al.,* 2016). Thin Layer Chromatography (TLC) analysis can be used to confirm the content of secondary metabolites in a plant. Maceration is among method for plant extraction that needs to be performed before running a TLC test. Maceration is carried out by using a solvent without a heating process. The principle of the TLC test is to separate the identical compound in a mixture from the extract.

Sukaharja is one of villages located in Cikulur District, Lebak Regency of Banten Province (Fig. 1), where Baduy as a traditional tribe is originated and very famous. Most of Baduy tribe work as farmers. Baduy tribe still believes in using plants to perform traditional medication, such as to treat high cholesterol levels and hypertension. Due to the village location that is far away from the nearest health facility, Baduy tribe practices traditional medication by using plants. In Sukaharja Village, we found 25 species of medicinal plants used for treating gastritis and gout arthritis (Rindita *et al.,* 2023). The latest study was carried out in Cihanjuang Village of Banten Province, in which we found 24 plant species used for curing stomach aches (Rahmadini *et al.,* 2022). This study aimed to complete the list of plants used to treat high cholesterol levels and hypertension, including the parts of plants used, the use value of the plants, and how to consume them as traditional medicine. In addition, the secondary metabolites were examined through qualitative methods, including TLC.

MATERIALS AND METHOD

Time and Location

This research was conducted in Sukaharja Village, Cikulur District, Lebak Regency, Banten Province from March until July 2023. Phytochemical screening, including TLC analysis, was performed in the Phytochemistry Laboratory in the Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. Dr. HAMKA .

Figure 1 Description of the location and situation during research in Sukaharja Village

Tools and Materials

Fieldwork: questionnaire sheets, camera for documentation, herbarium-making tools.

Phytochemical examination: analytical scale (OHAUS), test tubes and other glasswares (IWAKI, Pyrex), aluminum foil sheets, TLC plates, filter papers, hot plate (Oxone), blender (Philips), waterbath (H-WBE-8L), chamber, spray reagents for TLC, UV light (CAMAG), distilled water, Mayer reagent, Dragendorff's reagent, Bouchardat's reagent, Wagner's reagent, 70% ethanol solvent, magnesium powder, HCl 2N, quercetin, 5% FeCl3, citroborate, and toluene.

Population and Samples

The population of Sukaharja Village was 3,641 people. Sampling of population taken by using the Isaac and Michael formula (Sugiyono, 2013), obtained 50 respondents, including 9 key respondents. Sampling method for collecting respondents was conducted by using purposive and snowball sampling methods (Masturoh & Anggita, 2018). There were two types of respondents, i.e., (a) key respondent that is a person who is believed to have an ability for treating patients, and (b) common or general respondent which is the patient. The chosen respondent must be a native resident of Sukaharja Village with minimum age of 17 years old, must be healthy and communicative. There were also respondents who were previously chosen and included as respondents, but were not able to be interviewed when the interview was performed (Witjoro *et al.,* 2016).

Data Collection

The data were collected by using structured interview supported by validated questionnaire (Sugiyono, 2013). The ethical clearance was also registered to support data collection. Before the interview began, the respondents were provided with an informed consent. From the interview, the local names of plants were obtained and then confirmed with field observation and morphological identification. The field identification was documented and sent to a botanist for authentication.

Use Value Analysis

The confirmed list of plants was then being analyzed by using value calculation (Gazzaneo *et al.,* 2005). Use value is a parameter which shows the important value of each plant that has been used by local people as a traditional medicine. Use value was calculated as follows:

$$UV = \Sigma U/n$$

where: UV = use value of plant species;
U = number of citations per plant species;
n = number of respondents

Phytochemical Screening

All of the plants were sampled, dried, and processed to be made as simplicia powder (Wahyuni *et al.,* 2014). The powder was then examined by phytochemical screening process by using standard method to detect flavonoids, alkaloids, phenols, terpenoids, steroids, saponins, and tannins (Hanani, 2015; Malik *et al.,* 2016; Shaikh & Patil, 2020).

TLC Test

Only selected plants were chosen to be extracted for the TLC test. The TLC test was performed to determine one metabolite that existed in all samples. Plants chosen for the TLC test was based on UV and literature study. Maceration method was used to extract metabolites from the plants. Maceration produced crude or thick extract which was then used for TLC test (Handayani, 2016). Thick extract from plant was made by macerating 5 g of plant sample, then adding 50 mL of 70% ethanol solvent with a ratio of 1: 10, followed by soaking process for 6 hours with occasional stirring

and being left for 18 hours. The macerate was filtered and separated in another bottle. Then, the remaining residue was remacerated with 50 mL of 70% ethanol for 24 hours and stirred occasionally. The macerates were put together and then concentrated in a 50 °C waterbath to obtain a thick extract.

After obtaining a thick extract, the next step was to confirm the flavonoids compound by using TLC with quercetin as a comparison (Ladeska & Maharadingga, 2019). This step used a stationary phase in the form of silica gel GF254 plates and a mobile phase of toluene : ethyl acetate with a ratio of 3 : 7 (Maulana, 2018).

The mobile phase was introduced into the chamber with the specified comparison and was then waited until the saturation process occurs using filter paper. Condensed plant extracts and the quercetin was dissolved to obtain a concentration of 1,000 ppm. The silica gel GF254 plate was marked with an upper and lower border of 1 cm each. After the saturation process occurred in the mobile phase, the silica gel GF254 plate was stained with each sample, marked by numbers, with a comparator located on the left side. The silica gel plate was then inserted into the chamber, and the migration of eluent from the lower to the upper limit was then observed. The silica gel GF254 plate was left in place until dry and sprayed using citroborate spray reagent to qualitatively identify flavonoid group compounds. The appearance of citroborate spots is characterized by yellow, blue, or green fluorescence under UV light 366 nm and UV254 nm.

RESULTS

Total Species Found

In this study we identified 21 plant species that have been utilized by people in Sukaharja Village to treat high cholesterol levels and hypertension. These 21 plant species belonged to 17 families. Of all the species, 13 were used to lower cholesterol levels, the other 13 were used to treat hypertension, while 5 species were used for both (Table 1).

Use Value Analysis Results

A use value analysis was carried out on all species of medicinal plants used to treat high cholesterol levels and hypertension (Table 2). Leaves of soursop (*A. muricata*) and garlic bulb (*A. sativum*) showed the highest UV in treating hypertension, while the lowest UV was shown in ginger rhizome (*Zingiber officinale*). *P. pellucida* leaves and *A. muricata* leaves had the highest UV for treating high cholesterol, while *Solanum torvum* leaves had the lowest UV. Part of plants most widely used for traditional medicine was the leaves.

Table 1 Plant species, local name, family, and the utilization

No.	Species Name	Local Name	Family	Anticholesterol	Antihypertensive
1.	*Allium sativum*	Bawang putih	Amaryllidaceae	-	√
2.	*Annona muricata*	Sirsak	Annonaceae	√	√
3.	*Apium graveolens*	Seledri	Apiaceae	-	√
4.	*Centella asiatica*	Pegagan	Apiaceae	-	√
5.	*Polyscias scutellaria*	Mamangkokan	Araliaceae	√	-
6.	*Gymnanthemum amygdalinum*	Sambung nyawa	Asteraceae	√	√
7.	*Carica papaya*	Pepaya	Caricaceae	√	-
8.	*Artocarpus altilis*	Sukun	Moraceae	√	-
9.	*Moringa oleifera*	Kelor	Moringaceae	-	√
10.	*Syzygium polyanthum*	Salam	Myrtaceae	√	√
11.	*Pandanus amaryllifolius*	Pandan	Pandanaceae	√	-
12.	*Phyllanthus debilis*	Meniran	Phyllanthaceae	-	√
13.	*Peperomia pellucida*	Cacabean	Piperaceae	√	-
14.	*Piper betle*	Sirih	Piperaceae	√	-
15.	*Cymbopogon nardus*	Sereh	Poaceae	√	√
16.	*Gardenia jasminoides*	Kaca piring	Rubiaceae	-	√
17.	*Physalis angulata*	Cecenet/ciplukan	Solanaceae	√	-
18.	*Solanum torvum*	Takokak	Solanaceae	√	-
19.	*Phaleria macrocarpa*	Mahkota dewa	Thymelaeaceae	-	√
20.	*Curcuma zanthorrhiza*	Temulawak	Zingiberaceae	-	√
21.	*Zingiber officinale*	Jahe	Zingiberaceae	√	√
		Total		**13**	**13**

Table 2 Part of plants and use value of antihypertensive plants and anticholesterol plants

No.	Antihypertensive plants	Part of plants	UV	Anticholesterol plants	Part of plants	UV
1.	*Annona muricata*	Leaves	0.60	*Peperomia pellucida*	Leaves	0.56
2.	*Allium sativum*	Bulb	0.55	*Annona muricata*	Leaves	0.52
3.	*Syzygium polyanthum*	Leaves	0.51	*Cymbopogon nardus*	Stem	0.50
4.	*Apium graveolens*	Leaves	0.44	*Zingiber officinale*	Rhizome	0.48
5.	*Moringa oleifera*	Leaves	0.37	*Syzygium polyanthum*	Leaves	0.42
6.	*Cymbopogon nardus*	Stem	0.31	*Gymnanthemum amygdalinum*	Leaves	0.36
7.	*Gardenia jasminoides*	Leaves	0.26	*Artocarpus altilis*	Leaves	0.34
8.	*Curcuma zanthorrhiza*	Rhizome	0.24	*Physalis angulata*	Leaves	0.30
9.	*Phaleria macrocarpa*	Leaves	0.20	*Piper betle*	Leaves	0.24
10.	*Centella asiatica*	Leaves	0.17	*Polyscias scutellaria*	Leaves	0.18
11.	*Phyllanthus debilis*	Leaves	0.11	*Pandanus amaryllifolius*	Leaves	0.14
12.	*Gymnanthemum amygdalinum*	Leaves	0.06	*Carica papaya*	Leaves	0.08
13.	*Zingiber officinale*	Rhizome	0.04	*Solanum torvum*	Leaves	0.06

Phytochemical Screening Results

Table 3 shows phytochemical screening results from the 21 plant species used. Flavonoids were the positive compounds in all samples, while other compounds showed varying results.

Table 3 Phytochemical screening of medicinal plants in Sukaharja Village

No.	Species Name	Alkaloids	Terpenoids	Saponins	Phenols	Flavonoids	Tannins	Steroids
1.	*Allium sativum*	-	+	+	-	+	-	-
2.	*Annona muricata*	+	-	-	+	+	+	+
3.	*Apium graveolens*	-	+	-	-	+	+	+
4.	*Centella asiatica*	-	+	+	-	+	+	-
5.	*Polyscias scutellaria*	-	+	+	+	+	-	+
6.	*Gymnanthemum amygdalinum*	-	+	-	+	+	+	-
7.	*Carica papaya*	+	-	-	+	+	-	+
8.	*Artocarpus altilis*	-	+	-	-	+	-	-
9.	*Moringa oleifera*	-	-	-	+	+	+	+
10.	*Syzygium polyanthum*	-	+	+	+	+	+	-
11.	*Pandanus amaryllifolius*	-	+	-	-	+	-	+
12.	*Phyllanthus debilis*	-	+	-	-	+	-	+
13.	*Peperomia pellucida*	+	-	-	+	+	-	+
14.	*Piper betle*	-	+	-	+	+	-	-
15.	*Cymbopogon nardus*	-	+	-	+	+	-	-
16.	*Gardenia jasminoides*	-	+	-	+	+	+	+
17.	*Physalis angulata*	+	+	+	-	+	-	+
18.	*Solanum torvum*	+	+	+	+	+	-	+
19.	*Phaleria macrocarpa*	-	+	-	+	+	+	+
20.	*Curcuma zanthorrhiza*	+	-	-	-	+	-	-
21.	*Zingiber officinale*	+	+	-	-	+	-	-

Notes: + = contains metabolites; - = does not contain metabolites.

TLC Analysis Results

The 5 selected plants produced green and after being sprayed with citroborate spray reagent, produced a blue color (Fig. 2) with the Rf value of quercetin of 0.66 (Table 4).

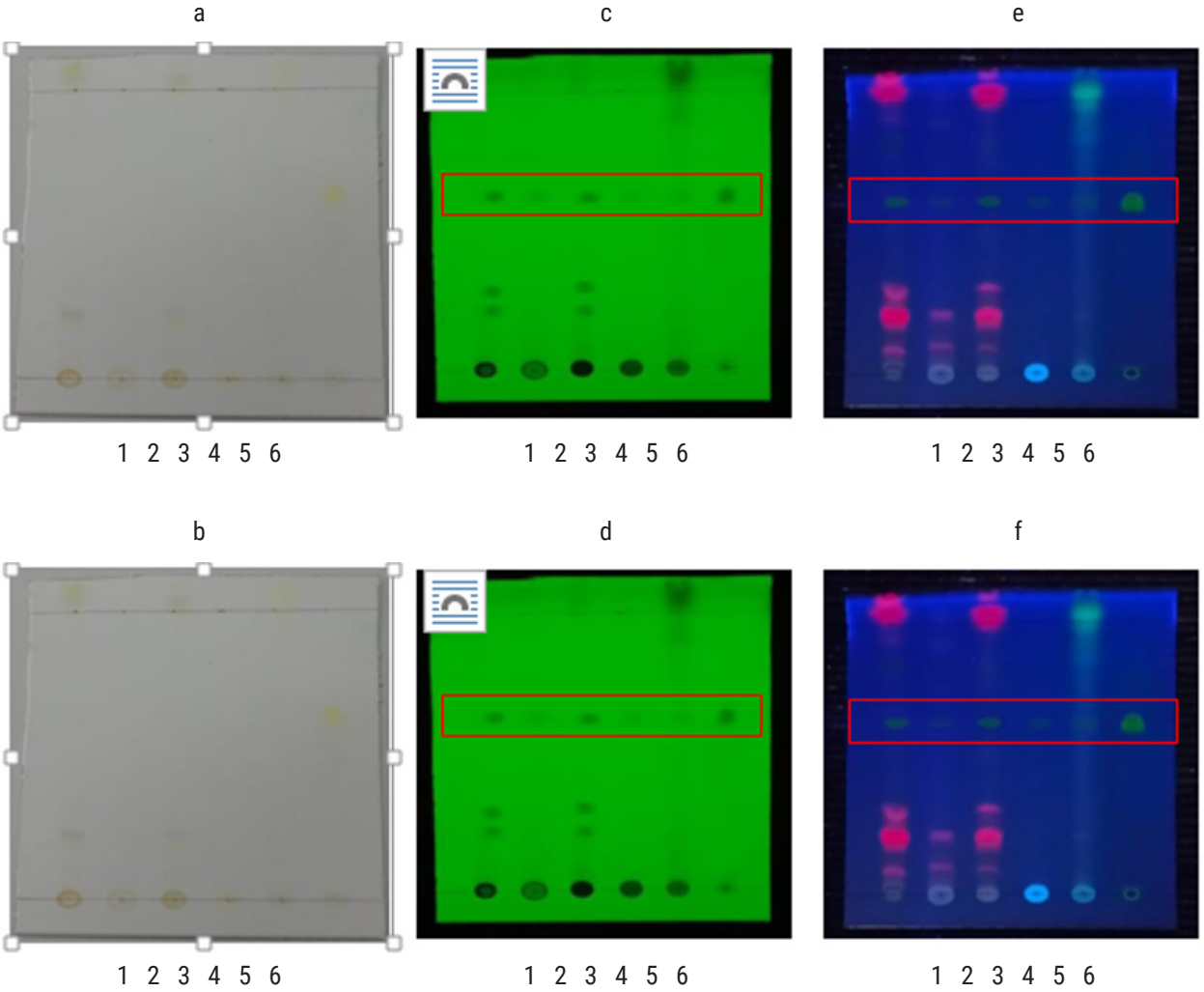

Figure 2 Results of TLC analysis

Notes: (a) Visible light before being sprayed with citroborate; (b) Visible light after being sprayed with citroborate; (c) UV254 nm before spraying with citroborate; (d) UV254 nm after spraying with citroborate; (e) UV366 nm before spraying with citroborate; (f) UV366 after nm sprayed with citroborate; Spot 1: Soursop leaves; Spot 2: Garlic bulb; Spot 3: Gardenia leaves; Spot 4: Sambung nyawa leaves; Spot 5: Ginger rhizome

Table 4 The Rf value of TLC analysis result

No	Sample	Rf value	Comparator	Nilai Rf
1.	Soursop leaves (*Annona muricata*)	0.65		
2.	Garlic bulb (*Allium sativum*)	0.64		
3.	Gardenia leaves (*Gardenia jasminoides*)	0.65	Quercetin (standard)	0.66
4.	Sambung nyawa leaves (*Gymnanthemum amygdalinum*)	0.65		
5.	Ginger rhizome (*Zingiber officinale*)	0.64		

DISCUSSION

The interview process with 50 respondents revealed that there were 21 medicinal plant species in Sukaharja Village used to treat hypertension and high cholesterol (Table 1). Cacabean (*P. pellucida*) and soursop (*A. muricata*) were the plants most often used by people in Sukaharja Village for cholesterol-lowering medicine (Table 2). Plant part mostly used was the leaves. In general, the medicinal concoction was made by boiling 5 to 7 leaves, then filtered, let cool, and drunk as medicine. Lemongrass stems (*Cymbopogon nardus*) also showed high UV. All of these three plants have been studied as having anticholesterol properties (Mazroatul *et al.,* 2016; Iswadi, 2019; Siagian *et al.,* 2022). As an antihypertensive, soursop leaves (*A. muricata*) had the highest UV, followed by garlic bulbs (*A. sativum*) and bay leaves (*Syzygium polyanthum*). The soursop leaves were used by residents, both as anticholesterol and antihypertensive. According to Suhandi *et al.,* (2022), soursop has the capability to lower blood pressure in people with hypertension. Based on previous research, those three plant species are also known to have antihypertensive properties (Suhandi *et al.,* 2022; Nwokocha *et al.,* 2011; Ismail *et al.,* 2018). To treat high cholesterol, people rarely use papaya (*Carica papaya*) and takokak leaves (*S. torvum*). However, research has been conducted on the anticholesterol properties of papaya leaves (Ademuyiwa *et al.,* 2023), while for takokak, only the fruit part is known to have the capability of lowering cholesterol (Harahap *et al.,* 2022). The plant species that are least used by residents to treat hypertension are *Gymnanthemum amygdalinum* leaves and ginger rhizomes (*Z. officinale*). However, research on these two species as antihypertensive medicinal plants has been carried out (Setiani *et al.,* 2022; Aulena *et al.,* 2021).

Use Value (UV) is an index to determine plants that have the highest use value in a community. Plants with high UV indicate a high level of confidence in the properties of these plants (Yusro *et al.,* 2020). The higher the number of people use the plant, the higher the UV has, and vice versa.

The results of phytochemical screening showed that all plant samples contained flavonoids. Testing for flavonoids using concentrated Mg powder and HCl produced yellow, blue, orange, or red colors which indicated they were positive for flavonoids (Hanani, 2015). Terpenoid compounds were also detected in most of plant samples. The test for terpenoids using chloroform and concentrated H_2SO_4 produced golden yellow, purple brown and brown rings.

For the confirmation test via TLC, this flavonoid compound was chosen because it is known to function as a cholesterol-lowering agent. According to Rachmawati *et al.,* (2019), flavonoids are known to lower cholesterol, triglyceride, and LDL levels and increase HDL levels by inhibiting 3-Hydroxy-3-methylglutary coenzyme A (HMG-CoA) reductase which functions as a catalyst in the formation of cholesterol. The comparator used is quercetin because this compound is a group of flavonoids that are often found in plants and are known to have many biological activities, such as antioxidants (Illing *et al.,* 2023).

The extraction method used in this research was the maceration method. Maceration is a method of separating compounds by immersing them in an organic solvent at room temperature. From research by Maryam *et al.,* (2023), the content of flavonoid compounds in the maceration method is higher than the other three extraction methods, namely reflux, soxhletation, and percolation. The solvent used is ethanol, because it can attract more active compounds compared to other types of organic solvents. Research carried out by Riwanti *et al.,* (2020) showed that the highest total flavonoid levels were found in 70% ethanol extract. This is thought to be influenced by the polarity of the solvent associated with research which states that the highest flavonoid content is in solvents with medium polarity. The 70% ethanol is a solvent that is more polar than 96% ethanol and more non-polar than 50% ethanol, so flavonoid compounds that are polar in nature will tend to dissolve more in 70% ethanol.

The principle of the TLC test is separation based on the distribution of two phases, namely the mobile phase and stationary phase, that follow the mobile phase polarity. The stationary phase used was silica gel GF254 plates which was able to fluorescence well in UV light with a wavelength of 254 nm (Fitriandini & Jayadi, 2021). The mobile phase is a mixture of ethyl acetate and toluene in a ratio of 3 : 7 (v/v). According to Maulana, (2018), toluene : ethyl acetate as an eluent in flavonoid compounds produces the most stable compounds. The samples were spotted on the plate, then eluted with an eluent. Stains were detected using 254 nm and 366 nm UV lamps and also using citroborate reagent. Citroborate showed a color of bright yellow at UV 366 nm for flavonoids detection. Citroborate spray reagent is a specific reagent with high sensitivity for detecting flavonoids and is specific for the ortho-dihydroxy group (Murwanto & Santosa, 2012).

CONCLUSIONS

The 50 informants used 21 plant species belonging to 17 families to treat high blood pressure and high cholesterol. The most widely used plant part was the leaves. The highest UV values for anticholesterol plants were shown by *P. pellucida* leaves and *A. muricata* (soursop) leaves, while antihypertensive plants were soursop leaves and garlic bulbs (*A. sativum*). Flavonoids was present in 5 plants having the potential to treat high blood pressure.

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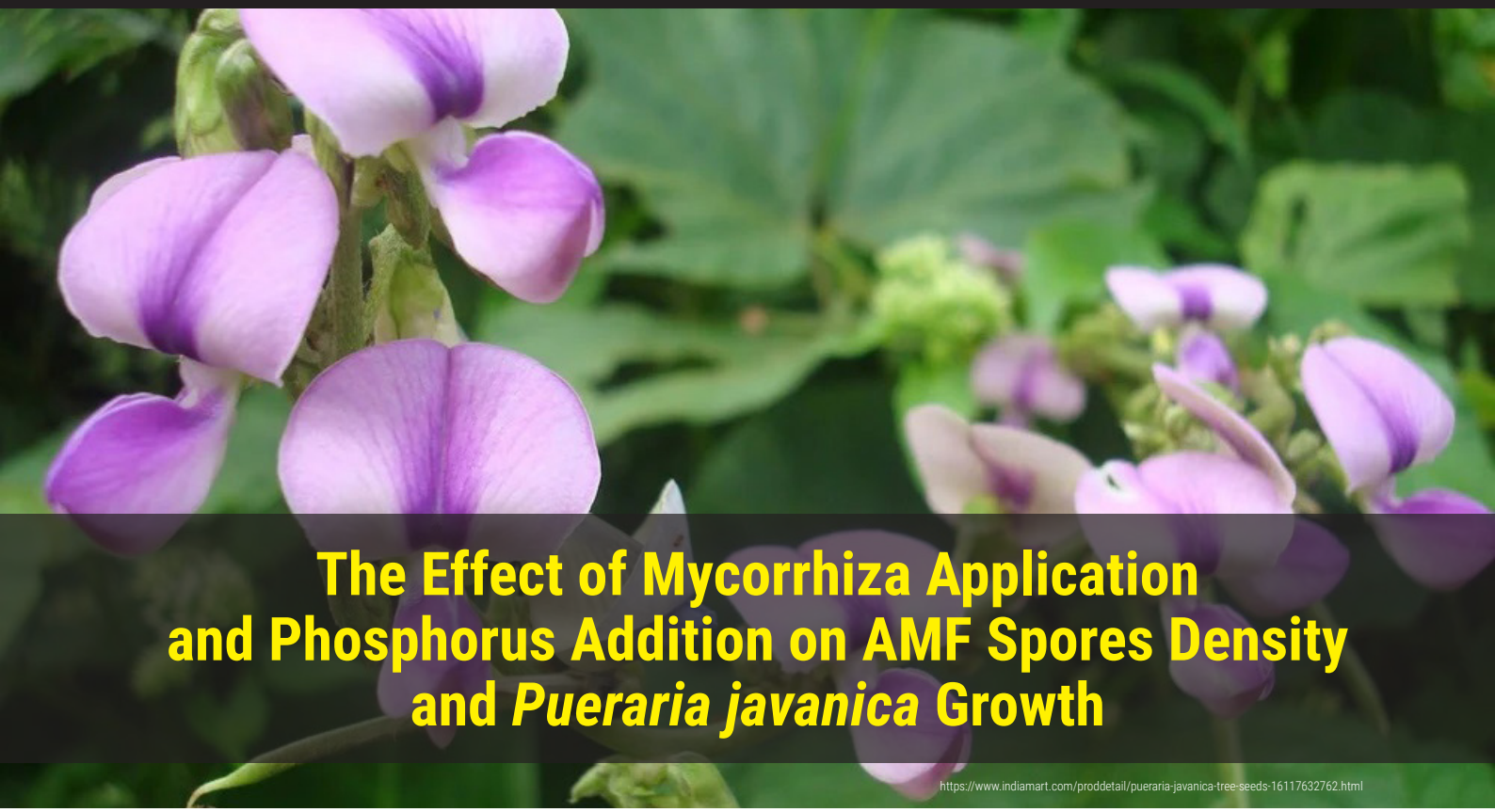

The Effect of Mycorrhiza Application and Phosphorus Addition on AMF Spores Density and *Pueraria javanica* Growth

https://www.indiamart.com/proddetail/pueraria-javanica-tree-seeds-16117632762.html

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ABSTRACT

One of the suitable host plants for AMF (Arbuscular Mycorrhizal Fungi) is *Pueraria javanica*. Several factors that can affect the growth of *P. javanica* are the availability of nutrients and the activity of potential soil microbes, such as AMF. Applying potential soil microbes (AMF) to plants can increase plant growth, chlorophyll levels, and enzyme activity, and even improve soil quality. The ability of AMF to increase plant nutrient absorption causes plants with AMF tend to have optimum growth. The study aimed to understand the effect of AMF application and phosphorus (P) nutrients addition on AMF spore density and *P. javanica* growth. The experiment used a completely randomized design with one factor (formulation). Results of the study proved that phosphorus (P) addition was able to reduce AMF spore densities (*Glomus etunicatum* and *Glomus mosseae*) 3 weeks after application (WAA). The availability of P in a fairly high amount around the root area caused plants to reduce their dependence on AMF, which resulted in a decrease in AMF colonization and AMF spore densities. On the other hand, adding P nutrients proved to increase growth parameters (plant height and number of leaves) of *P. javanica* because AMF helped the absorption of P and received carbon from plants in return. Treatment P6 (*G. etunicatum* 10 g + without phosphorus) had the highest spore density value (400.33/10 g planting media), and treatment P9 (*G. mosseae* 10 g + phosphorus 10 ppm) showed the highest increase in plant height of 13.333 ($P < 0.05$) 3 weeks after application. Meanwhile, the maximum increase in the number of leaves occurred in plants that received 10 g of *G. mosseae* and 10 ppm of P every two days for three weeks. Studying AMF spore density can significantly improve plant growth, agronomic efficiency, and agricultural sustainability.

Keywords: AMF, colonization, *Glomus etunicatum*, *Glomus mosseae,* nutrient absorption


https://www.eworldtrade.com/pd/ptduajayagrupindonesia/pueraria-javanica-legume-cover/1015300/

INTRODUCTION

Arbuscular Mycorrhizal Fungi (AMF) plays an important role in increasing plants' resistance to various stresses, such as flooding, salinity, temperature, heavy metals, and diseases, due to AMF ability in increasing water and minerals absorption that are important in responding to stresses and in increasing antioxidant activity, thereby preventing apoptosis (Begum *et al.,* 2019). In addition, research by Klinsukon *et al.,* (2021) found that the addition of AMF to plants can increase plant growth, chlorophyll levels, enzyme activity, and even improve soil quality (Rosita, 2021). The ability of AMF to increase nutrient absorption by plants causes plants with AMF tend to have optimum growth. The formation of arbuscular mycorrhiza occurs when fungal spores attach to the roots of the host plant and form a flat structure called the hypopodium. The presence of the hypopodium encourages the formation of hyphae that can penetrate the outer root cell tissue. The hyphae then form branches and penetrate the inner cortex cells, where the hyphae will form the arbuscular structure. The arbuscule is where the exchange of nutrients between fungi and plants occurs. The arbuscular and cytoplasm of plant cells are separated by the apoplast and perifungal membranes that facilitate the exchange of nutrients (Genre *et al.,* 2020). In addition to arbuscules, AMF also has bubble-shaped structures called vesicles that function to store nutrients. AMF reproduction occurs due to the ability of AMF to produce spores that are resistant to extreme environments (Holland & Roth, 2023).

AMF can produce spores that reproduce when in suboptimal environmental conditions. Spores can enter a dormancy stage where metabolism is drastically reduced until environmental conditions improve and germination can occur. The resistance of spores in suboptimal environments causes spores to be produced in more significant quantities in stressed environments, for example, when soil moisture is low. In addition, other factors affect spore production by AMF, such as pH and nutrients contained in the soil. Previous research by Susanti *et al.,* (2021) found that the addition of NPK (Nitrogen-Phosphorus-Potassium) at a concentration of 10 ppm can increase spore density in corn host plants. In addition to NPK, another nutrient that affects AMF is phosphorus (P). Previous research found that the higher the phosphorus content in the soil, the more limited the diversity and production of spores by AMF (Arias *et al.,* 2012). Spores produced by various AMF species have different morphologies and structures. AMF spores can sporulate or germinate when environmental conditions improve so that they can colonize the roots of host plants.

Several factors that influence this process are temperature, pH, humidity of the growing medium, nutrients in the medium, type of host plant, and even microorganisms in the environment. The sporulation time of each species varies depending on the AMF species and its environment, but generally, it takes several days to several months for the spores to germinate (Giovannetti *et al.,* 2010). One important step in identifying AMF spores is to extract and isolate AMF spores (Deveautour *et al.,* 2020). Glomus can be found in various habitats, even in polluted environments, so they show resistance to various abiotic factors (Rodrigues & Rodrigues, 2020). Some Glomus species often found are *G. etunicatum* and *G. mosseae*. Both species can increase nutrient uptake by plants so that they can be used as bio-fertilizers (Vani *et al.,* 2018). In addition, these Glomus species play a role in increasing plant resistance to salinity and drought stress (Begum *et al.,* 2019). The characteristics of *G. etunicatum* are that it has one or two layers of spore walls and is brownish-yellow, while *G. mosseae* has three layers of walls and is brownish-yellow in color (Lee *et al.,* 2006).

AMF spores in an optimal environment can germinate and colonize the host plant. One of the suitable host plants for AMF is *P. javanica*. *P. javanica* is a legume plant that can be found in Indonesia. The characteristics of this plant are that it is tolerant to various light intensities and is able to

act as a soil protector from rain. One of the properties that makes *P. javanica* often used in research is its rapid growth, which is around two months (Nursanti & Supriyanto, 2022). In addition, *P. javanica* plants have been found to be host plants for AMF colonization. *P. javanica* plants are suitable hosts for AMF because their roots are fine and strong, and their lignin content is relatively low, making the AMF colonization process easier (Lapanjang *et al.,* 2023). Factors that can affect the growth of *P. javanica* plants are the availability of water, the presence of nutrients, light intensity, and temperature. In addition, other factors that affect plant growth are internal factors of the plant, for example, hormones and genetics. One of the elements needed by plants is P, which plays an important role in plant growth and plant response to abiotic stress (Li *et al.,* 2015). Phosphorus (P) is needed in the synthesis of genetic material, the process of producing energy, and root formation. In addition, the addition of phosphorus to plants reduces damage due to abiotic stress, for example, drought and salinity (Bechtaoui *et al.,* 2021). This study aimed to study the effect of mycorrhizal application and the addition of P concentration 10 ppm on the number of spore density and growth response of *P. javanica* plants.

MATERIALS AND METHOD

Tools and Materials

The tools used in the study were sieving tools, analytical balances, scales, trays, pots, Petri dishes, pipettes, spoons, measuring cups, Erlenmeyer flasks, rulers, slides, object glasses, spore tweezers, hand counters, and stereo microscopes. The materials used in the study were zeolite media, 1000 ppm phosphate standard solution, NPK fertilizer, distilled water, Polyvinyl Lactoglycerol (PVLG) solution, aluminium foil, and filter paper. The samples in this study were *G. mosseae*, *G. etunicatum*, and *P. javanica* plants. The research procedure for the effect of adding phosphorus nutrients on the density and abundance of arbuscular mycorrhizal fungi spores was divided into four stages including *P. javanica* germination, AMF inoculation, AMF extraction and identification, and data analysis. The samples studied were two types of AMF that had been isolated and would be inoculated into *P. javanica* as the host plant. The study was conducted with a factorial randomized design and the data obtained were recorded and statistically analyzed and presented in the form of graphs and tables.

P. javanica Nursery and Seedlings

P. javanica seeds were soaked in warm water for 24 hours to facilitate the germination process. Afterwards, the seeds planted in zeolite media placed in a tray for twenty days and then transferred to sterile zeolite media as much as 300 g in a pot and left for a week (Sowmen *et al.,* 2018). During the nursery, the plants were watered with 20 ppm NPK solution every two days.

AMF Application and Phosphorus (P) Addition

The previously isolated AMF were each inoculated into the rhizosphere of the planting medium. There were ten treatments carried out, i.e., P1 (negative control) = without AMF inoculation + without phosphorus; P2 (positive control) = without AMF inoculation + 10 ppm phosphorus; P3 = *Glomus etunicatum* 5 g + without phosphorus; P4 = *Glomus mosseae* 5 g + without phosphorus; P5 = *Glomus etunicatum* 5 g + phosphorus 10 ppm; P6 = *Glomus mosseae* 5 g + phosphorus 10 ppm; P7 = *Glomus etunicatum* 10 g + without phosphorus; P8 = *Glomus mosseae* 10 g + without phosphorus; P9 = *Glomus etunicatum* 10 g + phosphorus 10 ppm; P10 = *Glomus mosseae* 10 g + phosphorus 10 ppm. Four replications were carried out for each treatment with a completely randomized design of 1 factor (formulation) so that the total treatments carried out were 40 treatments. The seedlings were maintained for three weeks and watered regularly with water or 10 ppm phosphorus fertilizer every two days based on the treatment (Nursanti & Supriyanto, 2022). Plant height, number of leaves, and leaf color were also measured and recorded before and after treatment.

Extraction and Identification of AMF

After three weeks, 10 g of zeolite media from the rhizosphere layer of each treatment were taken. The zeolite media was dissolved in water and poured into a spore filter. The sediment obtained on the 212 µm, 106 µm, and 63 µm sieves was taken and poured through Whatman filter paper. The filter paper was observed under a stereo microscope and the number and morphology of spores were counted and recorded. AMF spores were taken and mixed with a Polyvinyl Lactoglycerol (PVLG) solution on a glass slide. Spores were observed using a binocular microscope and identified based on morphology (Susanti *et al.,* 2023) and their abundance was calculated.

Data Analysis

The experiment of measuring parameters spore density and plant growth (plant height and number of leaves) was carried out for 9 weeks of planting (MST). Data were analyzed using SAS 9.0. If the results of the ANOVA test showed significance, further testing was carried out with the DMRT (Duncan Multiple Range Test) at an alpha (α) level of 5%.

RESULTS AND DISCUSSION

Analysis of variance showed that a single factor (formulation) had a significant effect on the parameters of AMF spore density and the increase in the height of *P. javanica* plants (Table 1). The density of AMF spores inoculated into plants after three weeks was calculated using the wet sieving technique.

The results of the analysis of variance showed that there was an interaction between the factors having a significant effect on AMF spore density ($P < 0.01$), increased plant height and number of leaves ($P < 0.05$). Furthermore, to determine the differences between the levels of interaction, a DMRT test was carried out. The results of the DMRT test are presented in Table 2.

AMF spore density decreased in samples treated with 10 ppm phosphorus (P) for three weeks (Table 2). This indicated that AMF samples without P had a significantly higher spore density compared to AMF which routinely received phosphorus. The data also showed that *G. etunicatum* had the potential to produce large numbers of spores in conditions without the addition of phosphorus (400.33 per 10 g of planting media). The results of this study are supported by previous research by Arial *et al.,* (2012) which showed that spore density tends to decrease with increasing nutrients in the planting media, because when nutrients are abundant, AMF tends to germinate and colonize roots. AMF colonization supports nutrient absorption by plants and encourage plant growth. Meanwhile, in plants that were not given P, more AMF spores were found. The reason is that in an environment with minimal nutrients, AMF produce more spores because AMF spores have higher stress resistance (Hopkins & Bennett, 2023). In addition, spores are a means of reproduction for AMF so that when environmental conditions improve, spores can germinate and colonize. However, the effect of phosphorus on spore density is also influenced by the dose of P where at certain concentrations P can encourage the production of large amounts of spores in certain host plants. Conversely, too high a concentration of P can inhibit spore germination so that further research on the effect of P doses on Glomus in *P. javanica* plants can be carried out (Giovannetti *et al.,* 2010; Dejana *et al.,* 2022)

Based on Table 2, compared to the control (without AMF), the administration of *G. etunicatum* and *G. mosseae* can increase the ability of plant nutrient absorption. The average increase in plant height for plants received AMF and phosphorus also tends to

Table 1 Results of analysis of variance on AMF spore density and *P. javanica* growth

No.	Parameter	Formulation
1	Increase in plant height	*
2	Increase in number of leaves	*
3	Spore density/10 g of planting media	**

Table 2 DMRT test results of the effect of formulation on AMF spore density and growth parameters of *Pueraria javanica*

Treatment	Description	AMF spore density/10 g planting media	Plant height (cm)	Number of leaves
P0	Without AMF inoculation + without phosphorus	6.67 ^{g}	7.468 ^{b}	3 ^{bc}
P1	Without AMF inoculation + 10 ppm phosphorus	6.67 ^{g}	7.633 ^{b}	6 ^{ab}
P2	*G. etunicatum* 5 g + without phosphorus	258.67 ^{b}	5.268 ^{bc}	4 ^{bc}
P3	*G. mosseae* 5 g + without phosphorus	108.33 ^{de}	1.768 ^{c}	2 ^{c}
P4	*G. etunicatum* 5 g + 10 ppm phosphorus	185.67 ^{c}	8.768 ^{ab}	5 ^{ab}
P5	*G. mosseae* 5 g + 10 ppm phosphorus	65.67 ^{f}	9.400 ^{ab}	7 ^{a}
P6	*G. etunicatum* 10 g + without phosphorus	400.33 ^{a}	8.033 ^{b}	4 ^{bc}
P7	*G. mosseae* 10 g + without phosphorus	163.00 ^{c}	9.300 ^{ab}	6 ^{ab}
P8	*G. etunicatum* 10 g + 10 ppm phosphorus	131.33 ^{d}	6.233 ^{bc}	6 ^{ab}
P9	*G. mosseae* 10 g + 10 ppm phosphorus	81.33 ^{ef}	13.333 ^{a}	10 ^{a}

be higher than the control with P (Table 2). Factors affecting plant height are the nutrients available in the planting medium. AMF addition can also increase nutrient absorption by plants. Therefore, the addition of nutrients and AMF is very important for increasing plant height (Higo *et al.,* 2020), because AMF can help increase the provision of nutrients for plants by colonizing the roots. When associated with plant roots, AMF increases plant mineral nutrient absorption (P) (Rosita *et al.,* 2020). The concentration and absorption of macro and micronutrients are higher in plants inoculated with AMF compared to plants without AMF inoculation (Rosita, 2021). Meanwhile, based on Table 2, the maximum increase in plant growth (number of leaves) compared to the two controls were the plant given AMF spores and 10 ppm P (Table 2). The maximum increase in the number of leaves occurred in plants given 10 g of *G. mosseae* and given 10 ppm P every two days for three weeks. These data support the spore density data in Table 2 because plants that tend to have a lower spore density, namely plants given 10 ppm P, experienced more maximum growth than plants given AMF and not given P. This is because P obtained can be absorbed by plants more optimally with the help of AMF which colonizes the plant roots.

AMF is able to produce several organic acid compounds and even phosphatase enzymes that help the absorption of phosphate, especially inorganic phosphate, by plants (Shi *et al.,* 2023). AMF form a symbiotic relationship with plants, where AMF helps the absorption of nutrients P and receives carbon from plants in return. If plants receive sufficient nutrients from the environment, their need for mycorrhizae decreases, which can decrease mycorrhizal activity and spore production.

CONCLUSION

The addition of nutrients and P can reduce the density of mycorrhizal spores (*G. etunicatum* and *G. mosseae*) after 3 weeks after application (WAA). The decrease in AMF colonization and spore densities occur when nutrients, especially phosphorus, is sufficiently available around the root area. The addition of phosphorus nutrients can increase growth parameters (plant height). AMF helps in nutrients P absorption and receive carbon from plants in return. Treatment P6 (*G. etunicatum* 10 g + without phosphorus) had the highest spore density value (400.33/10 g of planting media) and treatment P9 (*G. mosseae* 10 g + phosphorus 10 ppm) resulted in the highest plant height increase of 13.333 ($P < 0.05$) 3 weeks after application. The maximum increase in the number of leaves occurred in plants received 10 g of *G. mosseae* and 10 ppm of P every two days for three weeks. Studying the density of mycorrhizal spores provides significant benefits in improving plant growth, agronomic efficiency, and agricultural sustainability.

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Risk Management of Genetically Modified Organism Product: Experience from Indonesia, Malaysia, and Thailand

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ABSTRACT

Genetically modified organisms (GMOs) have become a subject of significant debate and monitoring due to their potential implications for human health, environmental impact, and socioeconomic considerations. As a result, risk management strategies and regulatory frameworks have been developed to assess and mitigate the potential risks associated with GMOs. This review focuses on examining the current landscape of risk management approaches and regulations pertaining to GMOs. The review analyzes the key components of risk management, including risk assessment, risk communication and bioethics. It explores the role of regulatory authorities in establishing guidelines for the evaluation and approval of GMOs, ensuring their safety for human consumption and minimizing potential environmental risks. The study also investigates the involvement of international organizations in harmonizing regulations and facilitating global trade of GMO products.

Keywords: bioethics, biotechnology, Genetically Modified Organisms, regulation, risk management

INTRODUCTION

Agriculture is one of main sectors that has a big impact on public welfare in a country, because large food production is a key to reach food security. Nevertheless, there are several issues that prevent food production from remaining stable and one of them is pest infestation. Based on previous study, global economic losses caused by pest infestations can reach up to 220 billion dollars every year (PPID IPB, 2022). This enormous amount could be increased if there is no solution in the near future.

The field of biotechnology has generated a solution to inhibit an increase in economic losses caused by pest infestation. By genetic engineering, crops can build up their own defense against pests (Talakayala *et al.,* 2020). It can also inhibit the production of some compounds such as ethylene (Schaller, 2017). Ethylene is a naturally occurring plant hormone that regulates various physiological processes in plants, including fruit ripening, leaf aging, and abscission (the shedding of leaves, flowers, or fruits) (Liu *et al.,* 2015). Ethylene produced in gaseous form can accelerate the ripening of fruits or vegetables. Therefore, by inhibiting its production, it can extend product shelf life after being harvested (Schaller, 2017).

Products circulating in the United States are generally found in two types, namely *Bt* crops and HT crops. *Bt* crops are crops that have been genetically modified by adding genes through the bacterium *Bacillus thuringiensis* (*Bt*) which produces insecticidal proteins. This protein is toxic to some insects (specifically), such as corn borers, cotton bollworms, and tobacco budworms (Abbas, 2018). *Bt* crops can help farmers reduce the use of chemical insecticides which can harm the environment and human health. Meanwhile, HT crops are crops that are genetically engineered to be tolerant to certain herbicides. This makes it easy for farmers to kill all kinds of weeds without having to worry about damaging the crop (USDA, 2022).

With various advantages that was mentioned above, land development for planting GMO crops has increased to 194.4 million ha since 1996 which was only 1.7 million ha (ISAAA, 2019). In addition, in recent years, various developing countries have planted more GMO crops than industrialized countries with a total planting area of 56% of the global total with the commodities planted as follows (Fig. 1) (ISAAA, 2019).

To further demonstrate the growing significance of GMOs, the adoption of GMOs in the United States shows an increase since 1996 (Fig. 2) (USDA, 2022).

The increase in adoption is closely related to the superiority of GMO products compared to conventional products. Although genetic engineering seems to be a bright solution for agriculture problem, in its application, this method reaps controversy from the public. GMO, as the product of biotechnology, has generally been unwelcome (Arcieri, 2016). This is because people still doubt the safety of these products for human health and the environment.

Therefore, assessments and regulations are required for GMO products before they are released to the market. The assessment and regulation that applied must be able to assess product safety both in terms of health and the environment. This can prevent any adverse effects that come after being consumed and prevent negative impacts on the ecosystem. It can also be an effective way of gaining the trust of the public.

Genetically Modified Organism

Genetically Modified Organism (GMO) is an organism whose genetic properties have been altered for various purposes. In short, GMO is a genetic engineering product. GMO can be derived from microorganisms, animals or plants. One of the method to making them is by using recombinant DNA technology to enable specific functions, such as enhanced productivity or disease & pest resistance.

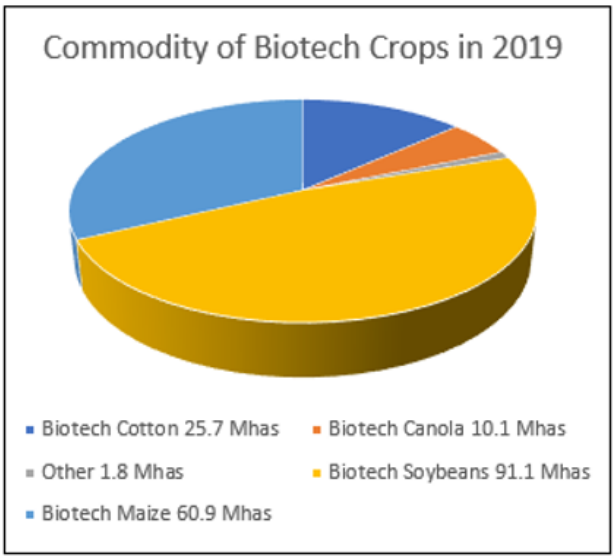

Figure 1 Commodity of Biotech Crops in 2019
Source: ISAAA, (2019)

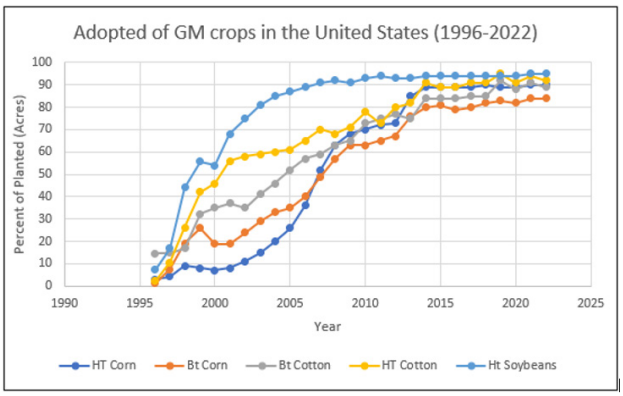

Figure 2 Adoption of GM crops HT (herbicide-tolerant) and *Bt* (*Bacillus thuringiensis*) in the United States
Source: USDA, (2022)

GMO transgenic is produced from a combination of a host, vector and genetic material. The process itself involves insertion of DNA Recombinant to the host. Recombinant DNA usually consists of genes of interest, terminator, promoter and marker genes. All these parts are introduced into plants usually by two methods: biolistic transformation and agrobacterium tumefaciens-mediated transformation (Chaurasia *et al.,* 2020).

Biolistic transformation allows for direct introduction of DNA or RNA into cells. In a brief explanation, DNA or RNA construct is coated onto gold or tungsten particles. The gene gun then releases the particles with high-pressure helium gas and directly penetrate the cell wall (Batles *et al.,* 2017). The second method, Agrobacterium tumefaciens-mediated transformation involves the help of bacteria to deliver genes of interest into a host plant. After entering the plant nucleus, rDNA is capable of integrating into the genome system and inherit desired traits in the next breeding (Hwang *et al.,* 2017). Besides that, there are also various other methods of making GMOs.

In addition, GMOs can also be made using genome editing techniques. In its definition, genome editing is a method or technology that allows scientists to change the structure of a DNA. This technology allows adding, removing, or changing a genome in a specific location. One of the well-known technologies for genome editing is CRISPR-Cas9 technology. CRISPR-Cas9 itself is a bacterial defensive mechanism against various viral infections. This protective mechanism generally consists of the Cas9 protein as a DNA cutter and Guide RNA as a guide for the location of the cut. CRISPR-Cas9 works by cutting off a part of a sequence from a genome (in this case the sequence derived from a virus) and inactivating it so that viral particles cannot be produced. CRISPR-Cas9 was then developed into a genome editing method by changing the guide RNA from Cas9 so that it attaches to the desired sequence. The CRISPR-Cas9 method has a good level of accuracy, effective and relatively affordable. By doing this genome editing, the GMO produced can have various desired traits such as lowering ethylene production (in fruits) to inhibit fruit ripening, eliminating hereditary defects or diseases in plants, animals or humans, increasing product resistance from pest and disease, and many more (Medline Plus, 2022).

Potential Risk of GMO

When GMO insulin was first introduced as the first GMO product in the medical field in 1982, people saw genetic engineering as an accelerator in the advancement of medical technology (FDA, 2023). It is because genetic engineering method is seen

as a new solution that can solve various diseases that have been a common cause of patient death, such as cancer, tumors, etc. (Teferra, 2021). However, when GM foods were first introduced in 1990s, people began to debate about the safety and ethics of the manufacture and consumption of these products. The use of GMOs in daily products is still a hot debate to this day (FDA, 2023).

In the manufacture of GMO products themselves, there are various risks that can cause harm. These risks are generally classified into 4 categories, which is human & animal health, socioeconomic and environment. In human and animal health, the GMOs that are produced have the potential to be toxic and can cause allergies. Some plants deliberately inserted a gene that can produce toxic compounds. It is intended so GMO plants can produce their own defense system against pests. Therefore, it is necessary to determine how much toxic compound is produced by a GMO so that it does not cause any adverse effect on humans or animals that will consume it. In addition, the insertion of a new gene in an individual can create a new protein which is one of the causes of allergic effects. Some allergic effects can cause severe symptoms or even end in death. Therefore, there is a need for testing that can capture the allergic effects of GMOs in a potentially allergic subset of the human population (EFSA, 2011).

In the environment, poisons that are deliberately formed through genetic engineering in crops can also cause death to useful non-target organisms, such as butterflies or bees. This can disrupt the ecosystem balance of the natural surroundings and reduce natural biodiversity. In addition, there is also the possibility of unintentional breeding between GM crops and domestic crops in the nature. This breeding can transfer transgenes that exist in GM crops to wild plant, resulting genetic contamination in nature which can cause various disasters such as the growth of super weed plants (herbicide tolerant weed). In addition, plants that are tolerant to pests will trigger the creation of super pests, or pests that are tolerant to pesticides (ISAAA, 2018).

From a socioeconomic perspective, the sale of GMO production in the market will lead to dependence of farmers on companies that create GMO seeds while controlling prices and seed supply. This happens because GMOs are not something that is easily made in general by various groups. The price gap between conventional seeds and GMO seeds can create inequality among farmers as poorer smallholders will be left behind by their competitors. In addition, there is a possibility that the introduction of GMOs will result in economic losses. This possibility arises from the consideration of consumer perceptions as a determining factor for the success of GMO products in the market. If consumer perceptions of GMO products are poor, there will not be many consumers who want to buy GMO products. As a result, the income earned is not enough to cover the production costs of the product and results in economic losses (LaHorgue, 2019).

Benefits of GMO

Apart from the various risks that exist, GMO products also come with various advantages and opportunities. By carrying out proper risk management, GMOs that pass the assessment can become goods that are superior to conventional products. In United States, several GMO products have been on the market for a long time, such as maize, soybean, cotton, potatoes, etc. (FDA, 2022). All of these products have passed various assessments in accordance with the established standards. This makes GMO products have the same quality, nutrition and safety as conventional products (Bawa & Anilakumar, 2013). Some GMOs are even made to increase the nutritional value of the product (FDA, 2022). Although there are no studies yet that say clearly that GMOs can have a negative effect on body health. Recent studies have shown that consuming GMO products can increase the number of tumors in mice. Even so, the study was later retracted due to unreliable data (Hefferon, 2015).

It is known that number of farmers in India who have committed suicide are around 15,000 with a peak in 2004, the year *Bt* cotton was first commercialized in India. In 2007, there was a significant reduction in farmer suicides by up to 25%. This data proves that the introduction of *Bt* cotton to India has solved many problems in agriculture while promoting the mental health of farmers (Smyth, 2020).

Genetically modified organisms (GMOs) have been instrumental in addressing specific issues, such as reducing pest and disease infestations on plants. GMO crops have commonly been developed with three prevalent traits: resistance to pest infestations, tolerance to herbicides, and resistance to harmful microorganisms (FDA, 2023). These traits significantly affect the development of a farmer's farm, starting from reducing costs for using pesticides and using herbicides which are much easier because there is no need to worry about damaging crops. The use of herbicides is also not necessary after carrying out soil tilling which can maintain the health of the soil and the worker's energy.

Some GMOs are also specifically designed to increase profits for consumers. For instances, production of GMO soybeans that can improve the health of oil and apples which do not experience a browning reaction when cut. The development of GMOs can also reduce the possibility of food loss can also increase people's access to food and make prices affordable.

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GMO Classification

In general, according to Health and Safety Department University of Edinburg (2022), GMO can be classified as Class 1 to Class 4 based on the risk the GMO poses to human health and environment (Table 1).

This classification plays a crucial role in determining the strategies for containing the product in question, taking into account several key criteria. Firstly, the evaluation considers the product's ability to cause harm or damage. This includes assessing the potential risks associated with its usage or exposure. Secondly, the severity of the harm or damage that could result from the product is taken into consideration. This helps in understanding the magnitude of the potential consequences. Additionally, the assessment includes an examination of the risk of spreading harm or damage to the population, gauging the likelihood of transmission or adverse effects on individuals. Furthermore, the potential harm to the environment or the possibility of economic loss is evaluated, considering the broader impacts beyond human health. Lastly, the availability of vaccines and effective treatments is factored in, as this can influence containment strategies and mitigation efforts. By considering these diverse criteria, an appropriate containment approach can be developed based on the specific characteristics and risks associated with the product.

In determining the risk level of a GMO product, several assessment considerations are needed which include risks to human health and their impact on the environment. In class determination, it is generally carried out to evaluate the high potential for a disaster from GMOs and the magnitude of the consequences. This relationship can be represented in the following formula.

$$Risk = Likelihood\ X\ Consequences$$

The results of the calculations can produce risks that are effectively zero, low, medium/low, medium, or high (Health and Safety Department University of Edinburgh, 2020). The results of the calculations are then adjusted to the level of risk in the following matrix (Table 2).

Table 1 GMO Classification

Class	Containment	Description
1	Level 1	Unlikely to give adverse effects to human or environment.
2	Level 2	May cause human disease or danger to employees, but it is not possible to spread to the community. This class is also not possible to cause significant environmental damage.
3	Level 3	May cause severe human disease and has serious threat to employees. This class is also possible to spread to the community but there is usually effective prophylaxis or treatment available. This class is possible to cause significant environmental damage or economic loss.
4	Level 4	May cause severe human disease and has serious threat to employees. This class can also spread to the community with no effective prophylaxis or treatment available. This class is possible to cause significant environmental damage or economic loss

Table 2 Risk Assessment Matrix

Consequences of Hazard	Likelihood of Hazard			
	High	Medium	Low	Negligible
Severe	High	High	Medium	Effectively Zero
Modest	High	Mediun	Medium/Low	Effectively Zero
Minor	Medium/Low	Low	Low	Effectively Zero
Negligible	Effectively Zero	Effectively Zero	Effectively Zero	Effectively Zero

GMO Risk Assessment

GMO Risk assessment is an effort to assess the risks posed by GMO products. Conducting a risk assessment is required for any commercial activity involving the use of GMOs. Risk assessment is commonly used in assessing potential risks to humans, animals, plants, or other aspects related to the environment. The assessment must be carried out by a person who is competent in his field. The work itself is categorized into several sections such as assessments on hosts, vectors, genetic materials, GMOs, types of activities, containment levels, classes, and others. The purpose of conducting this assessment is none other than to minimize adverse effects that may arise due to GMO products. This assessment also aims to select a suitable, sufficient and proportionate control method (Health and Safety Department of the University of Edinburgh, 2022).

In its method, the GMO assessment must be able to cover various important points such as composition, nutrition and comparison with conventional products, toxicity, allergenicity, molecular characteristics (such as stability of the inserted gene), potential of harming important microorganism, effect on non-target organisms, unintended effect on target organisms (such as resistance development), effects on biogeochemical processes (such as in the nitrogen cycle) (EFSA, 2019).

Points that have been mentioned above are useful in assessing the quality of the risk assessment itself. In carrying out a risk assessment, the initial step that is usually taken is to understand the overall molecular characteristics of the GM plant. Followed by an analytical comparison of the differences between the GM plant and the original. In more detail, understanding these molecular characteristics involves a comparative analysis of composition, phenotypic, and agronymic. This comparison is made to ensure that the molecular characteristics of the GM plant do not fall far beyond the range of natural variation. The results of this comparative analysis then build a risk assessment procedure for a GMO product (EFSA, 2011).

Some GMOs were created to produce toxins independently to create mechanisms of protection from pests. However, these toxic compound can potentially be produced in excessive amounts. Therefore, a toxicological study is needed to ensure that the toxic content produced is correct (Giraldo *et al.*, 2019). Toxicological studies of GMO should be performed with method described by Organisation for Economic Co-operation and Development (OECD) and in accordance with the quality assurance principles laid

by Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances. Some guidelines that can be used in testing the toxicity of chemical compounds in GMO risk assessment are as follows (Table 3) (EFSA, 2011).

Any adaptation of the protocol or use of any method different from the main protocol must be clearly explained and justified. In addition, GMO product toxicity testing must be carried out in a facility that can perform well. It's required to ensure that the test results that appear are high quality data. Toxicity potential is not only tested on GMO products, but also on the expression of new genes in them. The outcome of the toxicity test should indicate the availability of information regarding the adverse effects of expression of new proteins and other novel constituents created by genetic modification in particular along with specific dose levels.

Allergenicity assessment also must be conducted to see if there is any adverse reaction from consuming GMO food. This is because food allergy is a public health problem that is common and very important. Unlike, toxic reactions, allergies are deviations from the body's immune response to a compound which causes the individual to experience serious symptoms or even death. One type of allergy that has severe reactions and can create life-threatening conditions is IgE-mediated food allergy. Therefore, it is necessary to conduct specific studies that focus on the emergence of this allergic reaction due to consuming GMO products. Usually, the types of chemical components that often cause food allergies are proteins. Compounds resulting from protein breakdown can create allergic reactions, including new protein breakdown products (Herman *et al.,* 2022; EFSA, 2011).

Allergenicity is a symptom commonly experienced by a portion of the human population. The causes of allergies can vary from genetic, geographic or environmental factors. Therefore, in testing, it is necessary to interact between food and several individuals who have an allergic background. In addition, it is also necessary to ensure that the source of the transgene given to GMO products is not an allergen. If the new gene in a GMO plant is proven allergenic, testers should test for potential changes in allergenicity in all foods derived from that GM crop. This is recommended because there is a possibility that genetic modifications may induce unintended effects.

Nutritional assessment needs to be done in producing GMO products. This test is useful for demonstrating that there are no nutritional disadvantages in GMO products compared to conventional products. These tests include the effect of the presence of new protein expression on changes in nutritional value, changes in the levels of endogenous constituents in GM plants and their product derivatives, and potential changes in the total diet for consumers. If testing of the nutritional content of GMO products is not in accordance with conventional products, further assessment is required (EFSA, 2011).

Table 3 OECD guidelines for testing of chemicals

OECD Number	Title
402	Acute Dermal Toxicity
406	Skin Sensitisation
407	Repeated Dose 28-day Oral Toxicity Study in Rodents
408	Repeated Dose 90-Day Oral Toxicity Study in Rodents
410	Repeated Dose Dermal Toxicity: 21/28-Day
415	One-Generation Reproduction Toxicity
416	Two-Generation Reproduction Toxicity Study
417	Toxicokinetics
421	Reproduction/Developmental Toxicity Screening Test
471	Bacterial reverse mutation test
473	In-vitro mammalian chromosome aberration test
474	Mammalian erythrocyte micronucleus test
475	Mammalian bone marrow chromosome aberration test
476	In-vitro mammalian cell gene mutation test
479	In-vitro sister chromatid exchange (SCE) assay in mammalian cells
482	DNA damage and repair, unscheduled DNA synthesis in mammalian cells in vitro
487	Draft guideline on: In-vitro mammalian cell micronucleus test


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Risk Assessment Procedure

Risk assessments need to be conducted with adequate procedure. The procedure itself consists of hazard identification, dose-response assessment, exposure characterization, and risk conclusion. In the first step, it is necessary to identify the various types of risks or hazards that may occur due to GMO products. Afterwards, dose-response study needs to be conducted to determine the critical level above which the risk that has been found becomes a threat. Based on previous studies, the next step is to identify the different routes through which the hazard can pose a threat. The last step is to understand perceived risk and recommend necessary action (Carzoli *et al.,* 2018).

As an example, *Bt* maize is one of the GMO products that has been declared safe. This product has gone through all the assessments that have been mentioned above. One of the major concerns about the release of *Bt* maize is how it will affect consumer's health, whether it is human or animal. A series of assessments carried out to check if Cry protein (toxic compounds that produce in *Bt* maize) can give an adverse effect to the consumer (Carzoli *et al.,* 2018).

Step 1: Hazard identification

Hazard identification begins with looking for evidence or signs of poisoning that occurred after the product was consumed. This identification is usually done using animals as models that represent the human body. Based on this test, no signs of poisoning were found after consuming *Bt* Maize.

Step 2: Dose-response assessment

The resulting data of this assessment is quantitative and is usually assessed before negative effects due to poison occur. This value is commonly called the No Observed Adverse Effect Level (NOAEL) which is expressed in units of milligrams of the compound per kilogram of body weight per day (mg/kg/day). The formula commonly used in determining NOAEL is as follows:

$$Exposure\ Dose\ (ED) = \sum \frac{Residu\ Concentration\ x\ Food\ Comsumtion}{Body\ Weight}$$

To estimate the risk to humans, toxicological results to animals must be multiplied 10-fold into the NOAEL value as a form of avoiding uncertainty from potential differences due to species and sensitivities of some sub-populations. Based on NOAEL values obtained from toxicological animal models, the potential risk can be calculated in the human population consuming products containing *Bt* Cry protein.

Step 3: Exposure characterization

Exposure characterization. In animal studies conducted on various models, no toxicity was observed from any class of Cry proteins, even at the highest dose used in acute oral feeding experiments. The US Environmental Protection Agency acknowledges oral toxicity studies involving animals, which included doses exceeding 5000 mg of Cry protein per kilogram of body weight. By considering 5000 mg of Cry protein per kilogram of body weight as a hypothetical No-Observed-Adverse-Effect Level (NOAEL) and applying a 1000-fold uncertainty factor to account for variations among species and sensitive populations, the reference dose for humans is calculated to be 5 mg of Cry protein per kilogram of body weight. Assuming a Cry protein concentration of one part per million in maize grains and an average human body weight of 70 kg, it would require consuming 350 kg of maize per day to reach the dosage of 5 mg of Cry protein per kilogram of body weight. Considering the unlikelihood of consuming such a large quantity of *Bt* maize, the hazard identification studies suggest that the exposure to Cry proteins from the consumption of *Bt* maize by humans or animals does not pose a risk. Furthermore, research on other known toxic proteins indicates that they are usually harmful at low doses. Therefore, increasing the amount of potential Cry protein consumed is unlikely to result in any adverse effects not previously observed in the described studies.

Step 4: Risk conclusion

Risk conclusion. Based on the previous studies, it was found that the consumption of Cry proteins didn't have any adverse effects, leading to the conclusion that chronic studies were unnecessary. The proteins are digested in the stomach, which makes acute exposure the primary concern. Furthermore, *Bacillus thuringiensis* (*Bt*) has been used as an insecticide for many years without any reported negative effects on human health. This collective evidence supports the notion that there is no significant risk associated with the consumption of Cry proteins from *Bt* crops for humans or animals.

Risk Communication of GMO

According to (WHO 2017), risk communication is a real-time exchange of information, advice or opinions between an expert and an individual or community who is faced with danger whether in health, economy or social. The purpose of implementing risk communication is to enable effective distribution of information to someone who has the potential to have a risk or hazard in order to make the right decisions to reduce the effects of the threat. Risk communication has proven to be an important tool that can be used in an emergency situation. In the field of biotechnology, risk communication is the main key in controlling risk assessment and management of the development, importation, and use of GM crops. In the assessment process, risk communication plays a role in ensuring the scope and limits of GMO risk are clearly defined.

Risk communication also includes explaining to stakeholders how the regulatory system works, how regulatory decisions are made and the meaning of each decision. In distributing information, there is a risk of perception that can disrupt the flow of information. For example, many people will be confused when it is explained that one of the risks of using a GM crop is that it will turn into a new invasive species. This kind of misunderstanding can lead to the decisions taken by the recipients of the information will be less effective or even wrong. Therefore, various ways are needed to increase the effectiveness of risk communication.

Based on the article that was published by (ICGEB 2018), the first step to make an adequate risk management is developing a risk communication strategy. Risk communication can begin by explaining how the regulatory program works, what obligations the applicant has for the government, how data will be collected and how decisions can be made. Regulators must publish all regulations, policy statements, and guidance documents to support the administration of regulatory programs. Furthermore, regulators can plan risk communication campaigns based on the most likely regulatory scenario so that risk communication can be implemented efficiently.

The next step is to identify the stakeholder. Stakeholder can be divided into various sub-populations and have different interests. Some stakeholders sometimes have a narrow focus. For example, some stakeholder only care about the impact of implementing GM crops regulations on trade. There are also some stakeholders who have a broad interest but don't understand the technology behind the development of biotechnology. Therefore, regulators must be able to devise a risk communication plan that can cover various classes of audiences and develop appropriate messages for each stakeholder group.

After the message for each audience is well made, the regulator must be able to determine an effective way to spread the message. Dissemination of the message must be done in a creative way to increase its effectiveness. Several ways can be done with the help of information technology such as the internet which includes the dissemination of messages through social media, websites, etc. By carrying out these steps, it is hoped that the message created can be conveyed effectively.

GMO Analytic Standardization Based on ISO

International Organization for Standardization (ISO) has standardized several analytical methods for detecting GMOs and their derivative products. The standard is regulated in standard number ISO 6498:2012, ISO 21569 concerning Qualitative nucleic acid-based methods, ISO 21570 concerning Quantitative Nucleic acid-based methods, ISO 21571 regarding Nucleic acid extraction, and ISO 21572 concerning protein based methods. Specifically, information regarding protein detection methods can be seen in standardization ISO number 21572.

As an example, there is standardization in screening for GMO in cotton and textiles which is regulated in standard number ISO/IWA 32:2019. In general, this standard was created to provide laboratory guidance worldwide in assessing cotton, cotton fiber, genetically modified cotton plants, etc. in a standardized manner. This document is intended for non-GM and textiles production lines but can be applied to any production line that wants to check the presence of GM cotton.

ASEAN Guidelines Regarding GMO

Based on the results of the 44th AMAF meeting conducted by (ASEAN, 2022), regulated guidelines were obtained for conducting proficiency testing (PT), analysis, validation, & verification on genetically modified organisms (GMO). As one of procedure that has been regulated by ASEAN, PT is used as a tool or method in demonstrating the competence and capability of a laboratory in carrying out specific test analysis. PT is also commonly used in validating and demonstrating laboratory measurement processes by comparing test results from one laboratory to another. In other words, PT is an essential element in knowing laboratory quality. The guidelines and regulations provided by ASEAN are based on the criteria stated in ISO/IEC 17043:2010.

In the ASEAN guidelines, proficiency testing for GMOs requires several technical requirements which include personnel, facilities, environment and equipment, methods of analysis, reporting of results and documentation. At the personnel stage, there are the following requirements:

1. Laboratories shall appoint one of their permanent employees.
2. The designated personnel must be qualified and competent in carrying out the process of proficiency test samples.
3. Laboratory management must include a plan in handling PT scheme and communicate it to personnel about their responsibilities and roles in PT sample.
4. Personnel must have good knowledge and skills related to the PT sample.
5. Laboratory management can delegate authority to appointed personnel to choose a good PT scheme.
6. The appointed personnel must be able to plan and carry out a good sample test using the right test methods and instruments.
7. Appointed personnel must have the capability to express opinions and interpret results.

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8. The designated personnel must be able to evaluate the results obtained and be able to perform statistical analysis on the data that has been obtained.

In terms of facilities, environment, and equipment are as follows:

1. Participants must be able to ensure that the laboratory used is adequate in handling and analyzing GMO PT samples.
2. Participants can ensure that the laboratory environment such as temperature, humidity, and cleanliness of the work area does not adversely affect the PT sample analysis process.
3. Participants can ensure segregation of the work area to minimize cross contamination.
4. PT samples must be stored separately from various materials and reagents to prevent cross contamination.
5. Analysis of the sample pt should be carried out in the correct order and workflow in order to ensure the accuracy of the test.
6. Access and use of the work area must be monitored and strictly regulated regarding staff allowed access to minimize sources of contamination in the PT sample during analysis
7. PT samples must be able to be analyzed separately from other samples to prevent cross-contamination.
8. Participants must be able to ensure that the reagents used are not expired, the equipment used has been properly maintained, and calibration is carried out on a scheduled basis.

In the analysis of methods and procedures, there are the following requirements:

1. The participant must use a chosen test method, calibration or measurement procedure, which must be consistent with the routine procedure.
2. Participants must be able to ensure that the results obtained after the PT sample analysis are valid and reliable.

In reporting the results, the results from the PT must be able to be submitted to the PT provider within the allotted time based on the format or instructions that have been given. Results can be a copy of the number or weight-for-weight (% w/w) percentage or as a statement e.g. “Detected” or “Not Detected” depending on the format provided by the PT provider. After submitting the results, the PT provider will evaluate the final results of the report to all participants. The performance of the participants in the PT must be able to be evaluated as “Satisfactory” or “Not Satisfactory” depending on the evaluation results of the PT. Participants should be able to review the final evaluation report and ask for suggestions for better results.

After that, documentation was carried out on the basis of the results of the PT sample analysis which had been well recorded. This includes associated worksheets, operators, instrument print out of results, final evaluation reports from pt providers and various related documents such as test methods, work instructions, and laboratory standard operating procedures (SOPs).

GMO Regulations in Indonesia

In Indonesia, the supervision and control of genetically engineered agricultural crop varieties is regulated by the Indonesia Ministry of Agriculture in regulation No. 50 Year 2020. This regulation stands on the basis of considering that genetically engineered plant products

apart from having advantages, also have risks to human health, animals, and environment. To minimize this risk, it is necessary to have supervision and control. Under supervision, GMO plants are carried out through routine monitoring and reporting of cases by permit owners. This monitoring was carried out in the third year since agricultural GMO crops have been circulating in the territory of the Republic of Indonesia. Monitoring is carried out for 3 consecutive years to determine the impact on livestock health and the environment.

Routine monitoring is carried out through farmer questionnaire surveys, analysis of scientific papers and analysis of agricultural environmental data. The survey was carried out by an independent survey institution or university using a questionnaire according to Format-1 as listed in the attachment which is an integral part of Ministerial Regulation No. 50 Year 2020. The survey was carried out using the multi-stage cluster sampling method with a sample taken of at least:

1. 3 regencies/cities if agricultural GMO crops are grown in one province.
2. 3 regencies/cities in 2 provinces if agricultural GMO crops are grown in two provinces.
3. 3 provinces if agricultural GMO crops are grown in 3 or more provinces.

Meanwhile, the scientific work that has previously been alluded to is explaining the impact of agricultural GMO crops on the health of livestock and the environment. Impacts can be in the form of negative impacts and/or positive impacts on the circulation of agricultural GMO plants on the health of livestock and the environment.

Analysis of agricultural environmental data in the planting area is carried out by an independent survey agency funded by the permit owner. Environmental data were obtained from supervisors from various supervisory networks including plant pest and disease inspectors, veterinary medicine, seed inspectors, pesticide supervisors, livestock feed supervisors, and irrigation water quality supervisors.

The implementation of routine monitoring is submitted to the Minister in writing through the head of the agency in the form of a routine monitoring report. The report is conducted once in 12 months by attaching an analysis of farmer questionnaires, scientific papers on the impact of agricultural GMO crops on the health of livestock and the environment, and analysis of data in the area of planting agricultural GMO crops.

Monitoring reports are submitted in Format-2 as listed in the Ministry of Agriculture Regulation No 50 Year 2020. Report inspection is carried out within a maximum period of 14 days from receipt by the Head of the Agency. If the inspection results are declared incomplete, the routine monitoring report is returned to the permit owner by the head of the agency.


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The assessment is carried out by the head of the agency who is assisted by the agricultural GMO plant supervisory team before being reported to the Minister with the consideration that the agricultural GMO plants in circulation do not have a detrimental impact on the health of livestock and the environment or vice versa.

Case reports are submitted within a maximum period of 10 days after the negative impact is known. Case reports are prepared in Format-3 as stated in the Ministry of Agriculture Regulation No 50 Year 2020. The report review will be carried out by the Head of the Agency and the Agricultural GMO Plant Supervision Team.

Apart from that, there are various other rules related to GMO requirements before being released to the market. These requirements are regulated in the Regulation of the Government of the Republic of Indonesia Number 21 Year 2005 concerning the biosafety of genetic products. The requirements listed include, description and purpose of use, detected genetic changes and phenotypes, clear identity regarding GMO taxonomy, physiology and reproduction, organisms used as gene sources, engineering methods used and procedures,

molecular characteristics of GMOs, gene expression transformed into GMO, method of extermination in case of irregularities.

GMO Regulations in Thailand

The Minister of Public Health of Thailand regulates the circulation of GMO products through notification (No. 431) B.E. 2565 (2022) regarding the regulation of foods containing genetically modified organisms (GMOs). The notification stipulates that control measures are necessary to protect the health of consumers. This supervision includes the requirement that GMOs that are produced, imported or sold must pass a biological food safety assessment which will then be reviewed by the Food and Drug Administration of Thailand.

The notification prohibits all manufacture, import, or trading of GMO products except under 2 specific conditions. The two conditions are that GMOs have been approved by the Thai FDA or have passed the required assessment. GMOs that have been legalized will be included in Annex 1 (positive list). Annex 6 (temporary approval list) lists GMO products that have not been approved, but can still be produced, imported and sold while the assessment is still being carried out. This temporary permit is valid for 5 years but can also be revoked at any time if the product fails to be assessed. Developers must submit documents or evidence specified in Annex II for GM Plants, Annex III for GM Microorganisms or Annex IV for GM animals to the National Center for Genetic Engineering and Biotechnology (USDA, 2023).

In its assessment, GMO products must meet the following criteria:

1. Does not pose a health risk compared to conventional methods.
2. The GMO product has the same nutritional value and required properties as conventional product.
3. Meet the food quality and standards set by the Ministry of Public Health Thailand
4. Meet various applicable qualities and other standards required in the assessment results and some supporting documents or evidence.

Packaged products containing GM ingredients greater than or equal to 5% by weight with detectable GMOs and recombinant protein resulting from biotechnology must have a label indicating that the product contains GMOs. The same applies to products that contain less than 5% GMOs of plant or animal origin. This labeling is regulated by the Minister of Public Health Thailand through notification No. 432 Re: Labeling of GM Foods. If the importer is unable to provide specific information regarding the raw materials in the product (USDA, 2022).

GMO Regulations in Malaysia

Regulations in the distribution and assessment of GMO products in Malaysia are regulated by the Ministry of Natural Resource and Environment of Malaysia under the Biosafety Act 2007. Legalization of GMO products in Malaysia requires one of the following conditions:

1. Accepted by the National Biosafety Board
2. Notified by the National Biosafety Board

Approval means that all import activities or processes involving GMO products require a permit from the National Biosafety Board. Licensing can take the form of a certificate of acceptance. Any process involving GMOs may be commercialized only after the certificate has been issued.

Notification means that all participants who export or import goods involving GMOs require a notification from the National Biosafety Board. The NBB will then provide a notification statement letter to the participant who submitted the notification. Any process involving GMOs may be commercialized only after the declaration has been issued.

Trafficking of illegal GMOs will result in violation of the law with a maximum fine of RM 250,000 and/or imprisonment for a maximum of 5 years (individual). Meanwhile, companies will be fined a maximum of RM 500,000.

In the distribution of GMO products in Malaysia, regulations were made based on food regulations regulated in the Malaysian Food Act 1983 which forced every GMO product to have a label. This regulation is useful for informing consumers about GM foods and the substances contained in them. GMO content itself should be at less than 3% of the food composition. Labeling GMO products makes it easier to trace GM products at every stage of marketing, making it easier to control (Sanmugam *et al.,* 2021).

Bioethics Regarding GMO

The rapid development of biotechnology generate various new innovations. As time goes by, more and more "unnatural" entities have appeared in biotechnology. The topic of genetic engineering always triggers an interesting topic of conversation, but it is also a deep concern (Arcieri, 2016). The problem of genetic products is not always related to the safety of its consumption, but it's not uncommon to be involved in religious debates which judge that changing an organism's genetic system is an immoral act that should not be done by humans (CABI, 2001). With all of these controversy, traditional bioethics cannot cover all these areas. Therefore, bioengineering ethics was established to regulate ethics in engineering biological resources.

The regulations previously mentioned were created because of bioethical reforms. This aims to prevent indiscriminate planting of genes that can lead to unwanted results. The inclusion of GMOs into bioethics is nothing but to keep the process of genetic modification of living things in a humane and responsible way.

Existing GMO regulations are useful for ensuring that there are no adverse effects on the health or safety of humans, animals and the environment. On environmental factors, modified genes present in GMOs may be released into the wild causing various ecosystem problems such as species invasion, decreased biodiversity, or the growth of wild plants that have too strong pest resistance. While in human and animal health factors, allergenicity and toxicity produced by modified genes, especially in antibiotic resistance can lead to unintended catasthrophy. Based on these various considerations, GMO products usually take 10 years and cost millions of dollars just to reach consumers (Uzochukwu *et al.,* 2022).

Bioethics does not only cover GMOs, but also one of the methods for making them, like genome editing. Genome editing technology itself has been the subject of debate for more than 50 years. The debate includes the topic of bioethical boundaries and regulatory practices of genome editing. Even though it has been running for a very long time, the results of the debate still have not found a satisfactory result (Mandrioli, 2022).

CONCLUSION

While genetic engineering is developing rapidly in this era, there are various consequences and risks that come with it. GMO products are like a double-edged sword, providing new breakthroughs in solving problems from various sectors such as agriculture, food or medical. However, without proper risk management, regulations and bioethics, these innovations can become a threat to humans, animals and even the environment. Therefore, various international organizations have established standardization and strict assessment procedures for all activities related to GMOs. This standardization is then adopted in every country in the world with various modifications to suit the social and cultural environment in each country.

The risk management that is carried out needs to emphasize the safety of GMO products for the environment, nature, and human and animal health. Various lengthy tests need to be carried out to ensure safety, starting from the level of toxicity produced, allergenicity, to the effects of new traits developed on living creatures in natural ecosystems such as bees or butterflies. It should also be noted that decisions in implementing regulations can also be influenced by various subjective factors such as political interests, the state, miscommunication etc. So we need the right way of communication through the design of risk communication.

It should also be underlined that the development of genetic engineering must be based on humanity principles and global needs which include humans and all parts of nature and not running on the subjective goals of certain groups. Therefore, all GMO-related activities must not go beyond the limits set by bioethics. With these things, the risks and threats from developing GMOs can be minimized and kept under control.

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