Journal of Tropical Resources and Sustainable Science
Website : http://journal.umk.edu.my/index.php/jtrss/
eISSN : 2462-2389
Vol: 13, Issue 1, 2025, 132-137
DOI: 10.47253/jtrss.v13i1.1350

RESEARCH ARTICLE

Effects of different concentrations of *Aspergillus niger* on fermentation characteristics and nutritive value of Dwarf Napier grass silage

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ARTICLE HISTORY
Received : 27 June 2024
Accepted : 18 August 2024
Online : 30 June 2025

KEYWORDS
Aspergillus niger,
dwarf napier,
grass,
Klebsiella pneumoniae,
silage quality

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ABSTRACT

Dwarf napier grass is considered to be low-quality grass due to its low nutritional value if used at an inappropriate cutting age. This study was aimed at investigating the effect of *Aspergillus niger* on the improvement of dwarf napier grass silage quality. The 60-days old of dwarf napier grass were mixed with 7% molasses and *A. niger* at a rate of 0 (T0), 10^6 (T1) and 10^7 spores/ml (T2). Each kg of plant materials was mixed with 10.7ml fungal substrate and ensiled for 45 days. According to the results of this study, the pH tended to decrease with an increased rate of *A. niger*, while the lactic acid content tended to increase. The T1 silage showed lower ($p<0.05$) ash content than others. Non-significant ($p>0.05$) differences were observed on other proximate components. An increasing trend was observed in crude protein content with an increasing rate of *A. niger*, while no consistent trend was observed in crude fibre content. In conclusion, *A. niger* could be used as an additive to enhance the silage quality, but further research is needed to know the nature of the mode of action of *A. niger* in silage.

1. INTRODUCTION

There is increasing concern about food security as the global population is projected to reach nearly 9 billion by 2050 (United Nations, 2019). Between 2005 and 2050, the demand for meat and milk is anticipated to rise by 57% and 48%, respectively, primarily driven by higher incomes and increased consumption in developing countries (Alexandratos & Bruinsma, 2012). To fulfil the increased demand for animal products, additional feed is required for livestock production if met through food grains, which will further impact food security negatively. Hence, feed availability denotes a salient factor in animal production growth.

In Malaysia, the growth of the ruminant industry is relatively slow, owing to land topography, lack of pasture land, and high feed cost. However, Napier grass is gaining popularity among livestock farmers in the wake of its positive characteristics including high yield, easy propagation, drought resistance, and low input management (Haryani et al., 2018; Kanjak et al., 2023). Although this plant is associated with numerous beneficial characteristics, it is still considered to be a low-quality forage. Haryani et al. (2018) reported that crude protein (CP) content in napier grass varies with plant maturity with a mean value of 12%. Dwarf napier grass contains more energy and CP compared to tall varieties of napier grass. For the maintenance and production of dairy animals, a total diet usually comprised around 14-16% CP (Hasha, 2002). Therefore, it is required to enhance the CP content in dwarf napier grass and this plant can be used as a concentrate feed instead of use of expensive grains. Dwarf napier grass typically contains more than 12% CP, which is higher than the average value of 10% found in giant napier grass. However, ruminant animals require a diet containing 14-16% CP to meet their nutritional needs for optimal production. Farmers often resort to supplements to bridge this protein gap when feeding their livestock with napier grass. This study aimed to explore the possibility of increasing the CP content in dwarf napier grass through fungal fermentation. By enhancing the grass's protein content, we hope to eliminate the need for additional supplements, thereby providing a more efficient and cost-effective feeding solution for farmers. The research investigated whether treating dwarf napier grass with specific fungi can elevate its CP levels to meet or exceed the 14-16% requirement for ruminant animals.

There are some research studies in the published literature that have alluded to the use of *Aspergillus niger* and other fungus cultures in napier grass silage. Nurjana et al. (2016) reported that the addition of *Trichoderma reesei* inoculant and

the enzymes from the fungus has been able to augment the fermentation quality of the napier grass silage by increasing the CP, total digestible nutrient and reduced the neutral detergent fibre content in the silage. Moreover, the addition of *Trichoderma reesei* is not known to be associated with any negative effects on the rumen fermentability.

Treating napier grass with *A. niger* may enhance the protein content and digestibility of this plant. However, there is limited research on the improvement of nutrition of napier grass using *A. niger*. Therefore, the aim of this study was to investigate the effect of different concentrations of *A. niger* on fermentation characteristics and nutritive value of dwarf napier grass silage.

2. MATERIALS AND METHODS

2.1. Experimental site

The experiment was carried out at the experimental field of Agro Techno Park (ATP) and laboratory of Faculty of Agro Based Industry (FIAT), Universiti Malaysia Kelantan (UMK) during June-December 2022.

2.2. Establishment of dwarf napier grass

Rooted tiller of dwarf napier grass (*Pennisetum purpureum* Schumach) was planted in a row with spacing of 0.5 m at an experimental plot of ATP as described by (Fukagawa & Ishii, 2018). Before planting the grass, the land was cleared and ploughed using tractor to provide a good tilth. The area of experimental plot was 13m × 10m. Goat manure was applied to the experimental plot with the rate of 77g/m^2 (17 kg N/ha/year) before planting. The napier grass was cultivated under standard fertilizer management (300 kg NPK/ha/year), and irrigated twice a day, when necessary. The plants at 60 days of plant maturity were harvested, and chopped manually at 2-3 cm in length.

2.3. Fungus culture preparation

Aspergillus niger strain was purchased from local supplier, and it was derived from ATCCÒ 6275Ô in KWIK STIK form in a lyophilized pellet, a reservoir of hydrating fluid and inoculating swab. *Aspergillus niger* was cultured on Potato-Dextrose-Agar (PDA) (Güngör et al., 2018). Agar plate method used was by swabbing the fungus on to the PDA plate. Samples then were incubated at 30°C for 7 days at the laboratory of FIAT, UMK. After 7 days, fungus reached maturity by turning into dark greenish-black colour (Figure 1). After incubation, matured *A. niger* was preserved from the pure culture by cutting and preserving in glycerol at -80°C. A week before the silage was prepared, the preserved strains were sub-cultured on PDA and incubated at 30°C for another 7 days. When the fungus reached maturity, the spores were harvested for the treatments (Figure 2). The spore inoculums were prepared following the method of Kirkhouse Trust Laboratories (2003). The spores were diluted to estimate the concentration of fungus by counting the number of fungus colonies cultured. Next, the spores were then counted using a Hemacytometer based on the colony forming unit (CFU/ml) (Avin, 2019). The concentration of cultured fungus used was 10^6 spores/ml and 10^7 spores/ml. The spore inoculums were prepared. After that, the inoculums were inoculated with napier grass.

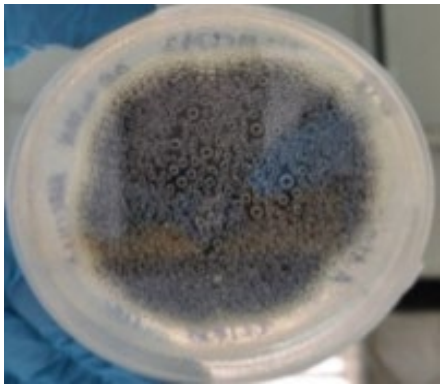

Figure 1: *Aspergillus niger* culture (7 days old).

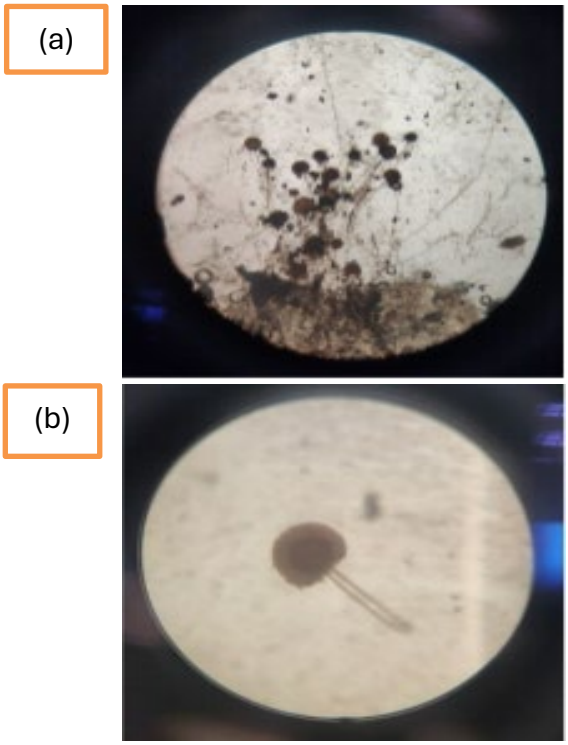

Figure 2: *Aspergillus niger* spores under microscope using (a) 10X and (b) 40X magnification.

2.4. Experimental design

The chopped grasses were mixed with 7% molasses and *A. niger* at a rate of 0 spores/ml (T0, control), 10^6 spores/ml (10.7ml of the fungal substrate/kg of mixtures) (T1) and 10^7 spores/ml (10.7ml of the fungal substrate/kg of mixtures) (T2).

The mixtures (500 g/bag) were compacted and stored in plastic bag under anaerobic fermentation at room temperature for 45 days. There were 3 treatments, and each treatment had 3 replications (1 bag/replication). After fermentation, the samples were analysed for fermentation characteristics and proximate analysis.

2.5. Agronomic parameters

After 60 days of plant growth, growth parameters such as plant height, leaf length, leaf width, stem circumference and leaf stem ratio (LSR) were determined on 5 randomly selected plants. For estimation of LSR, leaf and stem fractions were separated, chopped and dried in an oven for 48 hours at 70 °C for dry weight determination.

2.6. Physical characteristics of silage

The organoleptic test was conducted by including colour and odour by filling out a questionnaire assessing the characteristics of colour and odour by panellists by using the senses of sight and odour as described by Umami et al. (2023). The number of panellists was determined to be 5 people so each silage sample received 5 replicates.

2.7. Chemical analysis

Titratable acidity test was used to determine the lactic acid content in the silage. The sample of 5 g of silage was weighed and placed in the beaker. Then, 50 ml of distilled water were added into the beaker. The sample was cooked at the boiling point on a hot plate, till carbon dioxide was removed, and left it to cooled. Using a filter paper, the required sample were filtered, and the filtrate was collected. Next, five drops of 1% phenolphthalein were added into the filtrate. The filtrate was titrated with 0.1N NaOH until its colour changes to dark cherry colour (Amin et al., 2004). The volume of 0.1 N NaOH titrated was recorded. Total acidity is expressed as (%) of lactic acid. The percentage of lactic acid was calculated by using the following formula:

$$Lactic\ acid\ (\%) = \frac{V \times N \times 9}{W} \times 10$$

Where;

V = volume of titrated 0.1N NaOH; N = normality of NaOH; 9 = molecular weight of lactic acid; W = weight of fresh sample; 10 = Dilution factor

The pH value of the silage was determined by using a pH meter. Approximately 15 g of silage sample was weighed and put into a beaker, 60 ml of distilled water was added, mixed and let it rest at room temperature for 30 min. Then, the pH value of each treatment of silage was determined by placing the pH meter in the silage sample as recommended by Bernardes et al. (2019).

The silage samples were dried in an oven at 70°C for 48 hours, ground using blender, and sieved to size of 1-mm with sieve shaker. The dried and ground samples were analysed for dry matter (DM), CP, ether extract (EE), crude fibre (CF) and ash contents following the method of AOAC (2005). Organic matter (OM) was determined by subtracting percent ash content from one hundred. The nitrogen free extract (NFE) was calculated using the following formula:

% NFE = 100 % – (% EE + % CP + % Ash + % CF).

2.8. Statistical analysis

All the data were analysed using one-way ANOVA by using SPSS software (ver. 27). All differences among the treatments were evaluated using Duncan's Multiple Range Test at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Agronomic characteristics

Table 1 shows the agronomic characteristics of dwarf napier grass, which was harvested at 60 days of plant maturity. Agronomic characteristics of plants has substantial effect towards forage productivity and nutrient composition. As shown in Table 1, the LSR of dwarf napier grass was 2.6:1, which indicated that the leaf amount is 2.6 times higher than the stem. Zailan et al. (2016) also reported that the leaf fraction of dwarf napier grass was four times higher than the stem fraction. As the harvesting age increases, the LSR of napier grass decreases. The LSR has a significant impact on the nutritional content of the grass since leaves contain more nutrients and less fibre than stems (Kebede et al., 2016).

Table 1: Agronomic characteristics of dwarf napier grass.

Characteristics	Dwarf napier grass (60-days old, first cut)
Plant height (cm)	89.30
Leaf length (cm)	99.53
Plant circumference (cm)	7.17
Leaf width (cm)	3.03
Leaf to stem ratio	2.6:1

3.2 Physical characteristics of silages

The colour of silage can be varied based on the types of crops ensiled, although normal ranges for grass silage are olive green, greenish yellow or golden (Jianxin & Jun, 2002). High-quality silage typically retains the green or yellow colour of the original plant material, indicating that it has been well-preserved (Jianxin & Jun, 2002). From the results of this study, it can be considered that the silages were well preserved as it achieved the normal range of discolouration (Table 2). The odour of silages shown in this study ranged from mild acidic smell (T0) to sweet sour fruity smell (T1 and T2). The odour in T0 silage might be caused due to the low lactic acid production and high

in acetic acid (Opinya, 2020). Properly fermented silage should also have a pleasant, slightly sweet or acidic smell and be free from mould. This indicates that the silage has undergone good fermentation, maintaining its nutritional value and palatability for livestock (Jianxin & Jun, 2002). Good silage usually preserves well the original colour of the standing plant. When green raw material produces silage with green or yellow colour, it can be considered of good quality. While colour can give some clues about the fermentation process and the potential quality of the product, it should not be used as the sole indicator of nutritive value or palatability. Other factors like smell, texture, and an analysis of the nutrient content are also crucial in determining the overall suitability of the feed for animals.

Table 2: Physical characteristics of dwarf napier grass silages treated with different concentrations of *A. niger*.

Parameter	Treatment		
	T0	T1	T2
Colour	Olive green	Olive green	Olive green
Odour	mild acidic	sweet sour fruity	sweet sour fruity

T0 = Grass treated without *A. niger*; T1 = Grass treated with *A. niger* (10.7 ml fungal substrate/kg of silage; 10^6 spores/ml); T2 = Grass treated with *A. niger* (10.7 ml fungal substrate/kg of silage; 10^7 spores/ml).

3.3 pH value and lactic acid content

There were no significant ($p>0.05$) differences on pH values among the treatments (Table 3). The average pH values of all silages were ranged from 4.53 to 6.00. The T1 and T2 silages can be considered as moderate quality as the normal range of pH value for grass silage is between 4.3-4.7 (Kung & Shaver, 2001). Heuzé et al. (2020) suggested that if the napier grass contains high moisture content and when the nutritional value is at the peak, it could be an impediment to utilising it as silage because it causes undesired fermentation with significant nutrient loss. In this study, the moisture content was consistent among treatments, so other factors likely contributed to the increased pH in the T0 silage. The natural microbial population in T0 silage might not have been optimal for efficient fermentation, leading to insufficient lactic acid production.

Like pH values, there were no significant ($p>0.05$) differences on lactic acid contents among the treatments (Table 3), and it was ranged from 3.72 to 6.41%. Thus, all the silages in this study can be considered as good silage, because the desirable lactic acid content for well fermented silages generally ranges between 4-7% (% DM) or its content should be at least 65-70% of the total lactic acid as reported by Ward (2009). The addition of *A. niger* might prompt the increase of lactic acid content among the treatments, because there was a trend to increase the lactic acid content in silages with increased rate of *A. niger*. However, there was insufficient information in published literatures about the utilisation of *A. niger* that could increase the lactic acid content. One hypothesis could be that *A. niger* breaks down more complex carbohydrates like cellulose or hemicellulose, which are not easily fermentable by lactic acid bacteria (LAB), into simpler sugars like glucose. These simpler sugars could then be utilized more effectively by LAB to produce lactic acid. Another possibility is that the breakdown of proteins into peptides and amino acids by *A. niger* could provide additional nitrogen sources, supporting the growth and activity of LAB, thereby increasing lactic acid production. Experiments designed to explore the relationship between *A. niger* addition and lactic acid production in silage could provide more concrete evidence and help clarify the mechanisms involved.

Table 3: pH value and lactic acid content of dwarf napier grass silages treated with different concentrations of *A. niger*.

Parameter	Treatments (mean ± standard deviation)			*p*-value
	T0	T1	T2	
pH	6.00 ± 2.21	4.71 ± 0.37	4.53 ± 0.12	0.378
Lactic acid (%)	3.72 ± 2.46	4.20 ± 1.02	6.41 ± 1.73	0.236

T0 = Grass treated without *A. niger*; T1 = Grass treated with *A. niger* (10.7 ml fungal substrate/kg of silage; 10^6 spores/ml); T2 = Grass treated with *A. niger* (10.7 ml fungal substrate/kg of silage; 10^7 spores/ml).

3.4 Proximate components

There were no significant ($p>0.613$) differences on DM content among the treatments (Table 4). The average DM content of silages ranged from 12.38% - 13.43%. The optimum DM content for grass silage is about 15% - 40% when mature (Nash, 2020), but the DM content of napier grass silage in this study did not achieve the optimum level. The grass should be wilted to get optimum moisture prior to silage making at 65-75%. Carvalho et al. (2015) reported that using *A. niger* and *Rhizopus sp.* for solid-state fermentation of cactus pear has decreased the DM content. During the fermentation of glucose in aerobic media, about half of the carbon is oxidised to CO_2 to provide the energy required by the microbes, while the other half is transformed into cellular material. In fact, similar findings were reported by Nurjana et al. (2016). The conversion of carbohydrates and sugars to lactic acid or volatile fatty acids reduced the DM content of napier grass silage due to lactic acid bacteria used to produce lactic acid and volatile fermentation products. Moreover, there might also few factors to be considered such as harvesting period, plant maturity, soil types and irrigation which might be the cause for high moisture in napier grass (Islam et al., 2023).

The average CP content of napier grass silages in this study were ranged from 11.51% to 14.09%. Crude protein content in forages is critical for different species of livestock that consume them. The rumen microbes need protein to maintain the rumen fermentation and digestion. More than 7% CP are needed in the diet of ruminant animals to support their rumen activity. The addition of different concentrations of *A. niger* in this study tended to improve the CP content of dwarf napier grass

silages, although the differences were not significant (p>0.05) among the treatments (Table 4). As reported by Mangisah et al. (2010), the CP content of water hyacinth leaf fermented with *A. niger* increased due to the increase in sporulating resulted from an increase in *A. niger* biomass, thereby slightly increased the CP content.

Table 4: Proximate components of dwarf napier grass silages treated with different concentrations of *A. niger*.

Components	Treatments (mean ± standard deviation)			*p*-value
	T0	T1	T2	
DM (%)	12.82 ± 0.92	13.43 ± 0.72	12.38 ± 1.85	0.613
CP (%)	11.51 ± 0.68	12.15 ± 0.27	14.09 ± 2.41	0.158
EE (%)	4.25 ± 1.07	3.08 ± 0.62	2.54 ± 0.68	0.102
CF (%)	32.45 ± 2.34	30.76 ± 2.11	31.10 ± 2.04	0.623
Ash (%)	11.77 ± 0.38^{b}	10.12 ± 0.21^{a}	12.59 ± 1.10^{b}	0.012
OM (%)	88.23 ± 0.38^{a}	89.88 ± 0.21^{b}	87.41 ± 1.10^{a}	0.012
NFE (%)	40.02 ± 2.91	43.90 ± 2.68	39.68 ± 5.26	0.380

^{ab}Means with different superscripts in a row differ significantly (p<0.05). T0 = Grass treated without *A. niger*; T1 = Grass treated with *A. niger* (10.7 ml fungal substrate/kg of silage; 10^{6} spores/ml); T2 = Grass treated with *A. niger* (10.7 ml fungal substrate/kg of silage; 10^{7} spores/ml). DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fibre; OM, organic matter; NFE, nitrogen free extract.

Like DM and CP contents, there were no significant (p>0.05) differences on EE content among the treatments. The EE contents of silages tended to decrease from 4.25% to 2.54% with the increased rate of *A. niger*. Muhammad Ikhwan et al. (2016) reported that soy husk fermented in higher content of *A. niger* has resulted in fat degradation. It occurred because of the fungus's exponential development. Not only that, Ng et al. (2002) also reported that the reduction in fat in fermented palm kernel oil cake flour was caused by the conversion of fat into a single protein biomass. Hence, the results of this study are in line with the findings of Muhammad Ikhwan et al. (2016) and Ng et al. (2002) as there were reduction to the EE contents when higher concentration of *A. niger* were added into silages.

Crude fibre is a carbohydrate that is difficult to digest by ruminants. High CF content in diet influence to decline the feed intake due to its high complex carbohydrate as it declines appetite of the animal. Crude fiber is challenging for ruminants to digest due to its complex and resistant structure, primarily composed of cellulose, hemicellulose, and lignin. While ruminants have specialized microbes in their rumen that can ferment cellulose and hemicellulose, the process is slow and not entirely efficient. The presence of lignin in feed reduces the digestibility of the entire fibre complex, as it physically and chemically protects the carbohydrates within the plant cell wall from microbial attack (Jung & Allen, 1995). Iyayi and Zaid (2004) reported that fungi could degrade CF. This is because during the fermentation process microbial enzymes are secreted by the fungi which degrade the cell wall components and cause a decrease in CF content of T1 (30.76%) and T2 (31.10%) silages than in T0 (32.45%) silage. These results are also in line with the study of Mangisah et al. (2010) who reported that the rise in CF during the fermentation phase of vegetable waste was attributable to the use of the nutrients given by the fungi, and the decrease can be related to breaking the non-starch polysaccharide for fungi protein (Rajesh et al., 2010). Degradation of CF is important because as the fibre content in silage is high, the nutritive value of silage is low. Further, high fibre content is assumed as low digestibility.

Unlike the DM, CP and EE contents, the ash contents of napier grass silages were significantly (p<0.05) different among the treatments. The ash content of T1 silage was significantly (p<0.05) lower (10.12%) than the T0 (11.77%) and T2 (12.59%) silages. However, no significant (p>0.05) difference was observed between T0 (11.77%) and T2 (12.59%) silages. Ash is the total mineral content in a forage or feed of animal. The normal ash content of forages is usually near 9.0%, and forages containing more than 10% ash are likely contaminated with increasing amounts of soil (Hoffman, 2005). Sitindaon et al. (2021) reported that fermentation process enhances the biomass of the fungi which affect in other chemical composition. This result is also in line with Güngör et al. (2017) who assumed that the enzymes generated by *A. niger* causes the sour cherry to be degraded and the mineral compounds inside it to be released, resulting in an increase in total ash. The organic matter (OM) is everything that is the diet except for the ash content. Based on the report by Nurjana et al. (2016), the DM and OM contents of napier grass silage treated with *Trichoderma reesei* were decreased. This was probably because the breaking down process of fibre into fermentable carbohydrate. In addition, Mangisah et al. (2010) also reported the reduction in organic matter was caused using carbohydrate as an energy source for fungal cell biomass development and growth, as well as citric acid synthesis. Therefore, this might be the reason for the decreased of OM content in T2 (87.41%) silage compared to T0 (88.23%) and T1 (89.88%) silages. Organic matter in feed is vital for ruminants because it provides the essential nutrients that support energy production, protein synthesis, and overall health (Van Soest, 1994).

The average NFE contents of Napier grass silages ranged from 39.68% to 43.90%. Muhammad Ikhwan et al. (2016) reported that the NFE content increased during the 4th day of fermenting of water hyacinth treated with *A. niger* which might be occurred due to high content of glucose from *A. niger* to hydrolyse cellulose. Nevertheless, there were no difference (p>0.05) on NFE content among the treatments. During fermentation process, the reduction of NFE might be occurred due to utilisation of nutrients by fungi for its energy source (Rajesh et al., 2010).

4. CONCLUSION

It can be concluded that *A. niger* application did not significantly influence on the fermentation characteristics and nutritive value of dwarf napier grass silages, except the ash content. The T1 silage showed lower ash content followed by T0 and T2 silages. An increasing trend on CP content in silages was observed with the increased rate of *A. niger* application. Further research is needed to understand the mode of action of *A. niger* in dwarf napier grass silage. In addition, different species of fungi (e.g., *Saccharomyces cerevisiae* and baker's yeast) needed to be explored to enhance the quality of tropical forage.

ACKNOWLEDGEMENT

This research work was funded by Universiti Malaysia Kelantan (Grant No. R/PRO/A0700/01597A/003/2022/01135).

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