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## Influence of Carrier Materials and Storage Temperature on Survivability of Rhizobial Inoculant

S. Kaljeet, F. Keyeo and H.G. Amir

School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

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**Abstract:** Rhizobia are bacteria that are able to nodulate leguminous plants and fix nitrogen to make it utilizable to plants thus making it beneficial as microbial biofertilizer. The aim of this study was to determine the survival of locally isolated rhizobia (*Burkholderia* sp. USM B20) from *Mucuna bracteata* in various carrier materials namely peat, rice husk and local kaolin. The carrier materials which were inoculated with the rhizobia were stored in two different temperatures; 4 and 28°C (room temperature) for eight weeks. Samples from the carrier materials were taken every week and tested for the survivability of the rhizobia in it by determining viable cell count (CFU g<sup>-1</sup>). Besides that, samples were also tested for the changes in pH and moisture content. The results showed that after eight weeks of storage, treatment of carrier peat stored at 28°C was able to sustain the highest viable cell number of rhizobia. Peat also had acceptable changes in pH value and moisture content as compared to rice husk and the mixture of rice husk and kaolin. For other treatments, the addition of kaolin to rice husk in treatment mixture of rice husk and kaolin showed that addition of kaolin increases the moisture holding stability. Thus, it was concluded that peat was the best carrier type and the addition of kaolin may increase the attributes of rice husk as potential carrier materials.

**Key words:** Rhizobia inoculant, *Burkholderia* sp., peat, rice husk, kaolin, viable cell number, *Mucuna bracteata*

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### INTRODUCTION

The close propinquity between rhizobia and their host plant allows resourceful use of the fixed nitrogen. Rhizobia are suitable to be used as potential microbial inoculants or biofertilizer (Otieno *et al.*, 2009). The microbial inoculants especially those of rhizobacteria interact with both plant root and soil and thus provide favorable effect on the plant growth and this was termed as plant growth promoting rhizobacteria (PGPR) (Ashrafuzzaman *et al.*, 2009; Mia and Shamsuddin, 2010; Verma *et al.*, 2010). The use of microbial inoculants as biofertilizers increases crop yield, environment-friendly and can be utilized as an alternative or to reduce the usage of inorganic nitrogen fertilizer (Woyessa and Assefa, 2011; Sofi and Wani, 2007; Yasmin *et al.*, 2007). However, microbial inoculants, typically those of the bacterial inoculants have a very short shelf life. The biological activity of the PGPR may decline rapidly if the handling and storage is not done in the correct manner. The usage of carrier materials for the microbial inoculants proves to be beneficial to protect the bacteria and have long been practiced (Ardakani *et al.*, 2010; Fuentes-Ramirez and Caballero-Mellado, 2005).

Among various types of carrier materials, peats are the most frequently utilized. The reason behind it was because peats were able to support high number of rhizobia and maintained its survivability due to high moisture holding capacity and large surface area. Peat can be defined as soil-like material that was formed during decomposition of carbonized plant tissues or mosses (Bashan, 1998). The usage of peat however, poses problems typically in the tropics as it is not readily available in many countries (Smith, 1992). It also harboured a lot of contaminants as the sterilization process through heat often releases toxic substances that harms the bacteria and thus reduces its viability and in time contaminants thrive in it (Bashan, 1998).

The usage of rice husk (or hull) as the microbial inoculants carrier has also been demonstrated. Rice husk is the hard covering of rice grain. During milling process, rice husk will be detached and rice grains were obtained. The rice husks were considered as agriculture waste and were usually discarded (Teixeira and Zezzi, 2004). Therefore, it started an effort to develop rice husk into something useful such as development of rice husk as biofertilizer carriers. The usage of this material as a carrier was also not preferred because of its low water retention

capability. Therefore, it became difficult for rice husk to support bacterial growth and happen to be less desirable as a carrier. However, the problem could be solved by having phyllosilicate mineral (e.g., kaolin) mixed together with the rice husk. The platy structure of kaolin is very suitable as a carrier, as it aids in the retention of water and the microbial inoculants. Moreover, kaolin particles also protect the plant as it irritates insects that were attracted to the bushes (Murray, 2002).

The success of microbial inoculation to promote growth of plant is vastly influenced by the number of viable cells available in the carrier materials which is introduced into the soil (Duquenne *et al.*, 1999). Thus, it is important to determine the duration of the bacterial survivability in the respective carrier materials to ensure the desired level of bacterial population remains viable for the inoculants to be effective. Besides that, the carrier material should also have the properties such as cost effective, dissolve well in water so that the bacteria can be released and able to tolerate harsh environmental conditions (FAO, 1993). Thus, the objectives of the experiment was to study the suitability of peat, rice husk and the mixture of rice husk and local kaolin, as carriers for microbial inoculants of locally isolated rhizobia stored at different temperature (4 and 28°C).

## MATERIALS AND METHODS

**Treatments and experimental conditions:** This study was conducted from August 2010 to May 2011 at School of Biological Sciences, Universiti Sains Malaysia. In this study three different carrier materials (peat, rice husk and rice husk + kaolin (3:1)) were inoculated with locally isolated rhizobia (*Burkholderia* sp. USM B20). Growth and survival of rhizobia in the carrier materials were tested at two different storage temperatures, 4 and 28°C. The carrier materials were packed into small polyethylene bags. The polyethylene bags are sturdy and permits high gas exchange, allowing for CO<sub>2</sub> losses and O<sub>2</sub> uptake as well as permitting acceptable moisture content (Thompson, 1984). Ca (OH)<sub>2</sub> was added into the bags to neutralize the carrier materials. It was mixed well in the bag and immediately sealed to maintain the moisture content. The packaged carrier materials used in this experiment were subjected to double dose of Gamma-irradiation for sterilization at 59.6 and 65.9 kGy (ISOTRON (M) Sdn. Bhd. in Kuala Ketil, Kedah). Each package of sterilized carrier materials was then inoculated with the rhizobia (>10<sup>8</sup> cells mL<sup>-1</sup>). The volume of inoculants added was 50% of the water holding capacity of the respective carrier materials. The inoculants were injected using sterile syringes into the bags that contain carrier materials aseptically. The punctured area was wiped with 70%

alcohol. The punctured hole which was resulted by the syringe, was then covered with cellophane tape (Okereke and Okeh, 2007). The bags were stored separately based on the incubation temperature (4 and 28°C). The treatments involved in the experiments were as follows: T1: Peat + rhizobia inoculum, T2: Rice husk + rhizobia inoculum, T3: Rice husk + kaolin (ratio of 3:1) + rhizobia inoculum and T4: Peat-rhizobia inoculum (control). For each treatment, four replicates were prepared in small polyethylene bags. Each bag contained 150 g of the carrier materials. The carrier materials were sampled weekly for 8 weeks for viable cell count (CFU g<sup>-1</sup>), pH determination and moisture content analysis.

**Viable cell count of inoculants in carrier materials:** A total of 1 g sample from each bag was placed into test tubes containing 9 mL of sterile distilled water. It was then mixed thoroughly to ensure complete separation of the microorganism from the carrier. Next, 10-fold serial dilution (until 10<sup>-10</sup>) was performed. Three drops of 20 µL from each dilution was plated onto YEMA (Yeast Extract Mannitol Agar) with three replicates for each dilution (Hoben and Somasegaran, 1982). All the plates were incubated at 30°C for 24 h before the colony formed was counted and the CFU g<sup>-1</sup> was determined. From this value, the log<sub>10</sub> CFU g<sup>-1</sup> was calculated. Only the number of colonies that grew in the range of 3 to 30 colonies was counted.

**pH determination of carrier materials:** The pH of the carrier materials were measured in the ratio of 1:2.5 (carrier material: water). The mixture was then shaken on the orbital shaker at 160 rpm for 30 min before it was allowed to stand for another 30 min prior to pH reading (Okereke and Okeh, 2007).

**Moisture content of carriers:** The moisture loss of the carrier materials were also determined on a weekly basis by measuring the weight loss of sample that was placed onto aluminum foil cup of known weight. It was then placed in a 60°C oven for 16 h (Feng *et al.*, 2002). The moisture content was determined based on the weight loss of sample before and after drying.

## RESULTS

**Viable cell count of rhizobia in carrier materials:** In Fig. 1a, for storage temperature of 4°C, carrier material peat was the best amongst all 3 types of treatment. Treatment of rice husk only and mixture of rice husk and kaolin (3:1) did not performed as well as peat especially after week 4 where the difference became apparent. Only treatment of

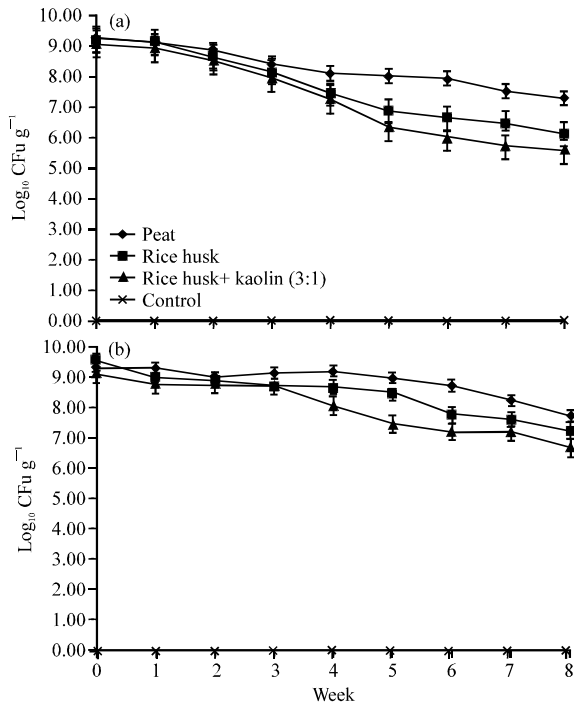


Fig. 1(a-b): Changes in viable cell count for rhizobia (*Burkholderia* sp. USM B20) in different carrier materials at (a) 4°C and (b) 28°C

peat manages to maintain optimum viable cell count which was higher than  $10^7$  CFU  $g^{-1}$ . In Fig. 1b, for storage temperature of 28°C, carrier material peat still maintained as the best treatment as it recorded the highest viable cell count amongst all 3 treatments. However, in this particular temperature 28°C, the other 2 treatment of rice husk only and mixture of rice husk and kaolin (3:1) manage to maintain its viable cell count above  $10^7$  CFU  $g^{-1}$ . For control treatment of both temperature, as can be seen in Fig. 1a and b, no growth of bacteria were detected. This was relevant since control treatments were without inoculation. As a whole, the overall viable cell count ( $\log_{10}$  CFU  $g^{-1}$ ) of rhizobia of both storage temperatures, decreased over time for all carrier materials tested. However, peat was considered to have a better advantage over the rest of the carrier materials as it maintained optimum viable cell count ( $>10^7$  CFU  $g^{-1}$ ) of inoculants for eight weeks of storage for both temperatures tested. The reduction in number of bacteria over time was lesser for peat compared to other carrier materials tested. It was also interesting to point out that in terms of temperature (4 and 28°C), all treatments performed better under the temperature of 28°C than carrier stored in 4°C.

**pH changes of rhizobia in carrier materials:** In Fig. 2a, for pH changes under storage temperature of 4°C, pH of

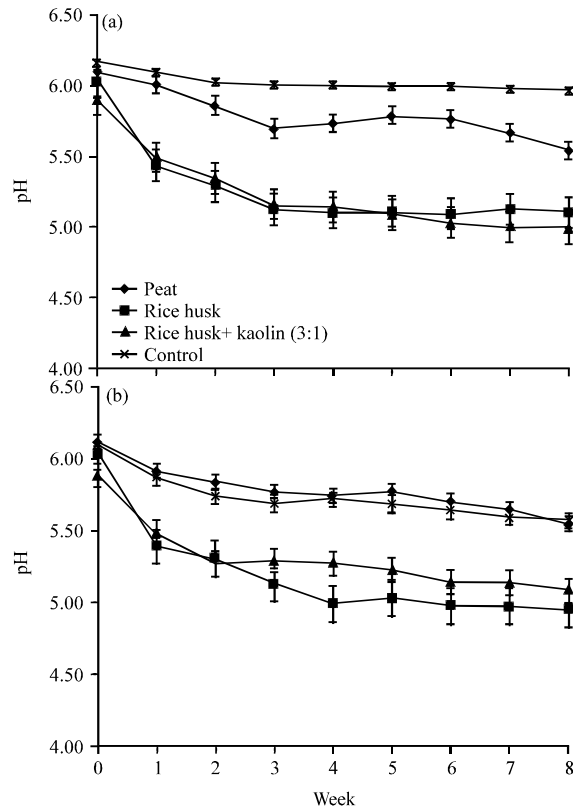


Fig. 2(a-b): Changes in pH for rhizobia (*Burkholderia* sp. USM B20) in different carrier materials at (a) 4°C and (b) 28°C

treatment rice husk only and mixture of rice husk and kaolin (3:1) recorded the largest drop. This was followed by peat with smaller pH changes. Control treatment as expected, did not have significant changes. In Fig. 2b, for pH changes under storage temperature of 28°C the trend of pH changes was the same as in Fig. 2a. However, a notable difference was pH of peat whereby it was more stable in temperature of 28°C. It has shown to mimic the trend of control treatment that only has minor pH changes.

**Moisture content of rhizobia in carrier materials:** In Fig. 3a, for moisture content of carrier under storage temperature 4°C, treatment of rice husk only, recorded the highest moisture content (75% during initial experiment until 42% during final week) compared to other treatments which were peat and mixture of rice husk and kaolin (3:1). The other treatments have approximately the same moisture content percentage towards the end of week 8 (39% for peat and 35% for mixture of rice husk and kaolin). The same trend was detected in moisture content of carrier under storage temperature of 28°C (Fig. 3b). Rice husk still recorded the highest moisture content

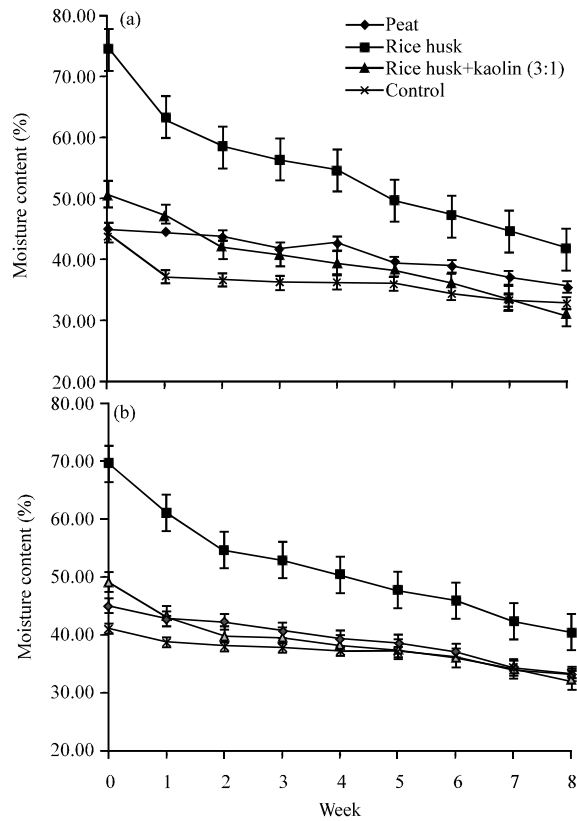


Fig. 3(a-b): Changes in moisture content (%) for rhizobia (*Burkholderia* sp. USM B20) in different carrier materials at (a) 4°C and (b) 28°C

(70% moisture) compared to other carrier materials during the initial assessment and sampling. Nonetheless it was also apparent that rice husk treatment suffered the most moisture loss for both temperatures. The moisture content of rice husk decline rapidly towards the latter week, this in turn made rice husk moisture content seem to be unstable. For other treatments, although it initially was not recorded to have a high moisture content, it had shown stable moisture content throughout the 8 weeks of storage.

### DISCUSSION

The effects of carrier material and storage temperature on the viable cell number, pH and moisture content of the biofertilizer are important because the overall functioning and reliability of the biofertilizer to increase crop yield may be affected by it. Thus, it is important to emphasis on proper method of storage and temperature in which will prolong the shelf-life of the biofertilizer. Selection of the proper type of carrier materials is also very important. It should be able to sustain a high amount of bacterial inoculants for as long as possible. A high number of

bacteria present and the capability to sustain for a long period of time ensures that the biofertilizer is in good condition and is readily applied to the soil.

Over the storage time of eight weeks, the viable cell count of all carrier materials tested showed a decline for both temperatures. The material peat showed the best result where peat recorded the highest viable cell count for both temperatures of 4 and 28°C. Meanwhile the other 2 treatments of rice husk only and mixture of rice husk and kaolin (3:1) showed acceptable viable cell count only in temperature of 28°C. This showed the advantage of peat as being stable in adapting and surviving in both temperatures. To compare between the storage temperature of 4 and 28°C, the viable cell counts of rhizobia in peat incubated at 4°C was rather low compared to similar sample incubated at 28°C. Similar results were also seen for viable cell count of rhizobia in other carrier material treatment where better growth and survival of rhizobia in the rice husk was recorded for samples incubated at 28°C. This showed that these particular rhizobia were able to persist in peat at the highest possible number at 28°C. The results also showed that lower storage temperature had reduced growth and survival of the inoculants tested. This showed that temperature plays a critical role in growth and survival of any bacteria as supported by Roughley (1970). He stated that continuous storage of rhizobia in sterilized peat at 4°C has in fact restricted the initial multiplication and the maximum numbers were not achieved until after 26 weeks of storage. Besides that, if the inoculants stored at 4°C were to be used, it was important to transfer them into incubation at 26°C for at least one week before being used. This is because higher temperature promotes bacterial growth while lower temperature may slow down the bacteria multiplication. The temperature of 28°C in this experiment may be the most suitable temperature storage for these particular rhizobia inoculants and temperature of 4°C may slow down the rhizobia growth.

Nevertheless, the choice of the carrier materials used for the production of biofertilizer is also important as the chemical and physical characteristics of the materials differed from one another. The type of carrier materials used may affect the viability of the rhizobia inoculated in it. It should suit and be able to sustain high number of bacteria and also as many types of strains as possible (Bashan, 1998). It is to be noted that the carrier materials that were chosen for this purpose were easily sterilized apart from being non-toxic towards the rhizobia. Mahdi *et al.* (2010) stated the suitability of various carrier materials for the development of biofertilizer were in the order of peat>lignite>charcoal>soil>rice husk. Peat that was used as carrier in these studies is the most commonly

used material in the rhizobia inoculant production industry. In this form, the bacteria were metabolically active and it is known that bacterial multiplication continues during the storage period and it is governed to the nutrients available in the peat component, moisture and temperature in which it is stored (Bashan, 1998). As can be observed from the result, peat showed better results as compared to other materials used as carrier materials. It was able to sustain the number of bacteria of more than  $10^7$  cells  $g^{-1}$  even after eight weeks of storage. In a study reported by Feng *et al.* (2002), the viability of bacteria in peat as carrier material existed and maintained in high number at  $10^8$  cells  $g^{-1}$  even after 85 days. The results also showed that rice husk alone might not be able to compete in terms of desirability of the carrier materials as compared to peat. However, rice husk was still considered as the alternative to peat in carrier selection. The fact that rice husk was easily available as agriculture waste and cheap, making it extremely sought-after to be used as carrier materials for biofertilizer. One of the strategies to increase the quality of rice husk was by the addition of clay material such as kaolin. Hence, the addition of treatment mixture of rice husk and kaolin (3:1). However, the usage of peat as carrier material was still far more superior as compared to rice husk.

As for the control treatment, no growth of bacteria or contamination was recorded. This proved the double dose of Gamma-irradiation that was subjected to the sample between the minimum and maximum level of 59.6 and 65.9 kGy, respectively was a great success in sterilizing the carrier materials. Biofertilizer carrier under gamma radiation treatments was proven to show positive result on enhancing plant growth after inoculation (Karem *et al.*, 2000). However, proper inoculation together with the best quality carrier and bacteria itself do not guarantee an increase to the crop especially in terms of nodulation or yield. Another factor to be considered was the presence of indigenous rhizobia or contaminants that can cause lack of response to the biofertilizer inoculant that was applied. This was supported by the study on leguminous crop by Chemining'wa *et al.* (2007) whereby the lack of nodulation was attributed to the presence of highly competitive indigenous bacteria that restricted the occupancy of nodules by strain. Good aseptic techniques applied during the sampling process also proved to be the factor that prevented contamination to the carrier materials. Hafeez *et al.* (1989) stated that growth and survival of rhizobia during storage was much better in the sterilized carriers than in non-sterilized carrier material. The advantage that sterilized carrier materials has over the non-sterilized carrier was that it exclude antagonistic effect of harmful microorganisms besides ensuring a more

precise determination of the total number of viable rhizobia. Rhizobia in sterilized peat were much more tolerant at a higher level of moisture and were optimal in the range of 40-60%. With this it proves the point that sterilization process is very much important in the development of biofertilizer.

Carrier materials stored under different temperatures also showed declining pattern of pH. The reduction in pH for rice husk and mixture of rice husk and kaolin (3:1) was greater as compared to peat regardless of its temperature (Fig. 2a and b). The reduction in pH reading for all carrier materials could have been due to bacteria that regulate their activities to ensure their survival in the carrier materials. It may also be due to production of acidic substances by the bacteria during its metabolism that causes reduction of the pH values of the carrier for both incubation temperature tested. Report by Bazilah *et al.* (2011) noted that higher temperature of 30°C compared to the lower temperature (10 and 20°C) may cause increased number of bacteria and this will in turn promote the production of waste by bacteria and therefore changes the pH drastically. Contrary to that result, in terms of temperature effect on the pH value of different type of carriers, there were not much difference between 4 and 28°C. Both temperatures showed no effects on the values of pH. For both storage temperatures, the level of pH for each carrier materials gradually decreased regardless of its temperature. The dropped values of pH may be caused by the decreasing number of viable rhizobia in the carrier itself. As clearly seen in Fig. 1a and b, the viability of rhizobia in the carrier dropped steadily until week 8 and similar pattern can be observed in the lowering of pH values in Fig. 2a and b. Since the viable number of rhizobia in carrier material rice husk and mixture of rice husk and kaolin (3:1) have more significant drop of viable cell, the pH decrease was also very apparent. The death of bacteria may have released acidic substances when the bacteria lysed in its surrounding. This naturally will lower down the pH values of the carrier it was inoculated to. With lower pH, it might probably be responsible to further lower the number of viable rhizobia. This in turn became a cycle reaction of viability and level of pH whereby the death of rhizobia in the carriers will decrease the level of pH. Lower pH of the carrier materials will create unsuitable growth condition for rhizobia, making the viable cell further decrease.

In standard quality assurance for biofertilizer, another parameters used for quality assessment of the biofertilizer other than viable cell count and pH was moisture content at the time of its manufacture. The minimum moisture content of the carrier material should be in between 35-40% (Mishra, 2002). From the result in Fig. 3a and b of

moisture content (%), rice husk recorded the highest moisture content compared to other carrier materials during the initial assessment and sampling for both temperatures. In the final week, it was clear that the moisture content for rice husk dropped drastically for both of the temperatures. For other types of carrier tested, a steady drop of moisture content was recorded but not as severe as the moisture loss of rice husk carrier. The moisture loss treatment between mixture of rice husk and kaolin (3:1), peat and control did not differ greatly from one another. This showed that moisture loss in rice husk can be prevented with the addition of clay material such as kaolin. A small loss of moisture was to be expected especially during storage of the carrier. But the percentage of loss should not be too great that it could decrease the viability of inoculants. In a study by Feng *et al.* (2002), it was reported that after 85 days of storage at 30°C, the moisture content of carrier tested only decreased from 52 to 49% on a wet basis (only 3% moisture loss overall). Thus, he reasoned that the rhizobia were unlikely to be subjected to desiccation stress because the low loss of water from the peat used as carrier material. In addition, detail analysis of the peat particle using electron microscope showed that the structure of the peat itself which was highly irregular and has many cervices may have acted to protect the rhizobia itself. The usage of rice husk as a carrier material has also been previously studied extensively. Rice husk proves to be more economic as it was a by-product of the rice milling. Studies done by Hafeez *et al.* (1989) showed that rice husk has the ability in terms of having higher water holding capacity as compared to peat. However, this was not enough to make it a better substitute for peat as a carrier material for biofertilizer. The peat was still superior as compared to the suggested rice husk. Therefore, to increase the desirability of rice husk as carriers, the addition of kaolin with a proper and correct amount may increase the quality of carrier itself. In terms of the treatment of mixture for rice husk and kaolin (3:1), it has better attributes in term of moisture content (the ability to retain moisture) but did not manage to have pH stability as good as peat.

### CONCLUSION

The objective of the study which was to find the best carriers for microbial inoculants of locally isolated rhizobia was accomplished successfully with peat still being a more superior carrier material as compared to rice husk and also the mixture of rice husk and kaolin (3:1). However, it was also shown that the addition of kaolin to rice husk (as depicted in the treatment of rice husk and

kaolin mixture (3:1)) showed increased desirability of rice husk as carriers. But further studies need to be establish to find the best ratio of rice husk and kaolin in order for it to become a superior carrier. Various factors come in to play in the development of biofertilizer. Besides the factor of sterility of the carrier materials, the particle size of the ground and sieved materials were also crucial to determine the biofertilizer to work at its best. Temperature also plays an important role in the survival of the rhizobia that was inoculated into the carrier materials. The storage of the inoculated carrier materials at 4°C reduces its viability and growth; however, it still remains good to a certain period of time. The storage temperature of 28°C was the best suited for this particular rhizobia. Peat as carrier material remains the best to be used in biofertilizer. As for the rice husk and also the mixture of rice husk and kaolin (3:1), the performance matches those of peat at 28°C, yet markedly lower.

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