TISSUE CULTURE SYSTEM FOR THE ARTIFICIAL PATHOGENICITY OF *Ganoderma boninense*

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Ramets development from tissue of selected *Dura* x *Pisifera* (DxP) palms is seen as the most promising technique towards more uniformity and higher efficiency in oil palm plantations. Cloning is a process where identical or true-to-type 'photocopies' of selected palm which is known as ortet, are reproduced by developing plantlets from leaf tissue of DxP oil palms with desirable characteristics (Mutert and Fairhust, 1999). Therefore, tissue culture system would be the ideal condition to investigate plant-pathogen interaction whether at molecular or at cellular level. The possibility of establishing uniform characteristics among oil palm ramets allows an opportunity to develop the tissue culture system for artificial pathogenicity study using any pathogen. In this case, *Ganoderma boninense* is the main focus for the use of the established tissue culture. Artificial infection technique has been developed to understand the specific plant-pathogen interaction without any noise created by external factors such as soil and environment. Pathogenicity trials were mainly conducted in the nursery scale, either using seed germinating technique (Idris *et al*., 2006) or sitting technique (Sundram 2013), using *Ganoderma* colonised rubber wood block as inoculum. However, the entire process is very time-consuming as preparation and trials will take up to eight months in order to obtain the desired results. Rubber wood sawdust acts as a suitable substrate for inoculum. Instead of eight weeks of incubation, preparation of *Ganoderma* colonised rubber wood sawdust only requires 7 to 10 days. In addition, nursery trials also have to encounter external factors such as changes in temperature, humidity, pest attack or soil microbiota changes. These factors may cause and capable of creating variables and eventually interrupting the entire experiment. This could be a challenge especially for studies with molecular and physiological approaches that requires uniformity in the physiological process. Therefore, the objective for this study is to develop a tissue culture system that allows pathogenicity trials to be conducted in a short period of time under controlled environment with uniformed infection.

THE TECHNOLOGY

To begin with, molecular approaches require uniformity in the physiological process within its biological samples to establish a good expression of genomic change for analysis. However, this becomes a challenge when uniformity is not established especially in the conventional system using soil whereby infection is below ground, which preludes the assumption that infection is indeed taking place. Therefore, the *in vitro* approach provides the baseline for infectivity process that requires uniformity. The interference noise in molecular analysis in turn will be reduced and confidence in genomic expression work will be increased. This includes molecular analysis such as metagenomics, transcriptomics, metabolomics and proteomics to further elucidate the genomic data of *Ganoderma* pathogenicity in oil palm. Other than this, the development of biomarkers will benefit from this technique. This technique will also be applicable for screening of biological control agents. The severity in disease infection could be assessed and potential biocontrol agent could be identified for the next action plan. The technique uses lesser space and resources compared to the *in planta* system. Therefore, the establishment of infection method on tissue culture material will be able to help researchers to reduce the number of experimental trials, especially to understand the behaviour of pathogens or biological control before conducting a large scale of screening in nursery trials.

NOVELTY OF TECHNOLOGY

The developed *in vitro* tissue culture pathogenicity technique is the first to establish the natural

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manifestation of *Ganoderma* infection without any wounding or any form of pressure to recreate *Ganoderma* infection that were demonstrated earlier by two publications (Govender and Wong, 2017; Goh *et al*., 2016). The former technique developed by Universiti Putra Malaysia (UPM), Serdang, Selangor used wounding, while the latter developed by Nottingham University, Semenyih, Selangor used pressure to recreate infection in the tissue culture system. However, this system uses none of the technique in the two publications instead naturally infects the axenic samples. Conventionally, the infection of oil palm ramets without infection is conducted by inoculating mycelia agar to the basal area of the plant. However, the success rate of infection was less than 60% as some of the mycelia failed to grow during the incubation period. The established tissue culture system was found to be uniform with manifestation of disease (repeated at three different time frames) observed within two weeks of inoculation process. In addition, the sequence of infection process resembled to that of in *planta* system.

In vitro INFECTION OF OIL PALM RAMETS WITH *Ganoderma boninense* PER71

One-month old tissue culture oil palm ramets were used for the development of *in vitro* infection studies. *Ganoderma boninense* PER71 was used as the fungal inoculum and was maintained on potato dextrose agar (PDA). The fungal inoculum was prepared by inoculating 7 days old *G. boninense* PER71 to sawdust formulation and allowed to colonise at 28°C for 7-10 days (*Figure 1*). A 7 days old *G. boninense* PER71 on PDA was used as a comparison method. Oil palm ramets were infected with different types of methods: i) (T1) non-inoculated; ii) (T2) *G. boninense* PER71 agar disc (10 mm diameter); iii) sawdust colonised with *G. boninense* PER71. Each treatment was replicated for three times and observed for the next 15 days. All ramets were incubated in the dark at 28°C.

Figure 1. Rubber wood sawdust mixture used as Ganoderma boninense *inoculum for infection of tissue culture oil palm ramets.*

PHYSIOLOGICAL ASSESSMENT ON OIL PALM RAMETS

Morphology and symptoms on the infected ramets from two different techniques were observed and evaluated. The successful rate of infection was recorded by assessing the *Ganoderma* mycelia success in colonising the oil palm ramets. The severity of internal tissue and extend of decay at the infection site were observed by cutting the bole area into longitudinal slices using sterilised scalpel. Lactophenol blue was used stain *G. boninense* fungal cell walls by adding one drop to the sliced area (Goh *et al*., 2016). The physiological assessment of oil palm ramets was conducted and they were categorised into different severity classes of infection. The finding of this study showed the effectiveness and consistency of using *G. boninense* PER71 colonised sawdust in the infection of ramets compared to mycelia disc. *Figure 2* shows the difference between using mycelia disc and *G. boninense* PER71 colonised sawdust as fungal inoculum. The disadvantage of using mycelia disc was the inconsistency in infection (*Figures 2a* and *2b*). The successful rate of infection by inoculating mycelia disc as fungal inoculum was less than 60%, where some mycelia disc failed to grow or were contaminated incubation process. However, *G. boninense* PER71 colonised sawdust as fungal inoculum, gave successful rate of infection at 100% within one month with manifestation of infection observed in ramets (*Figure 2c*).

Physiological and morphological of the ramets between the treatments were compared (*Figure 3*). The colonisation of mycelia on the bole area of ramets can be seen on T3. However, the mycelia disc (T2) took a longer time to colonise especially when the mycelia disc failed to attach on the bole area. The leaves for T3 started to turn brown on the area where the colonisation occurred. This proved that *G. boninense* has initiated infection in the plant system by colonising plant tissues. The internal tissue of the ramets was further investigated.

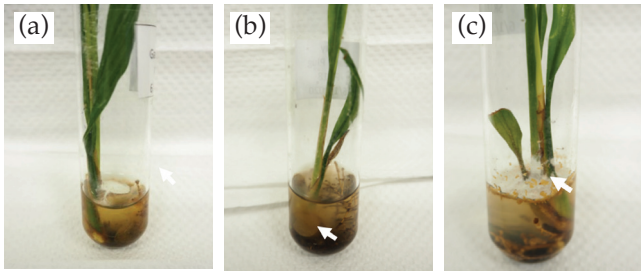

Figure 2. Consistency of Ganoderma boninense *growth, comparing between two different inoculation techniques: (a) Mycelia disc infecting ramets (b) Mycelia disc that failed to grow, and (c)* G. boninense *colonised sawdust as fungal inoculum.*

Figure 4 shows the effectiveness of using *G. boninense* colonised sawdust infecting ramet. The ramets in T1 were healthy without any inoculation of fungus, therefore no sign of decaying was observed. A slight decay of tissue was observed in T2 ramets, indicating *G. boninense* at early stage of infecting tissue of the plant. The decay in bole tissue of T3 ramets was severe and advanced compared to the T2 ramets. This might be due to nutrients provided by sawdust as substrate which allows *Ganoderma* to sustain and aggressively infect during the whole process. Therefore, this showed that *G. boninense* colonised sawdust represented as effective fungal inoculum for the infection study using oil palm ramets to ensure consistency in infection towards the plant. The microscopic observation further observes the extend of decay caused by *G. boninense* in just 15 days of incubation (*Figure 5*). The staining intensity of lactophenol blue indicates the colonisation of mycelia present in the internal stem tissues of ramets. T3 ramets were intensively stained as compared to T1 and T2 ramets.

BENEFITS OF THE TECHNOLOGY

The system described provides an excellent tool for numerous experimentations. Other key benefits of the technique include costing, time factor and space along with a few additional advantages that has been highlighted in *Table 1*. *Table 1* provides the benefits of technique when compared to the conventional technique.

PROSPECTUS CLIENTS AND ECONOMIC ANALYSIS

This technology will be valuable for a wide range of researchers, universities, research institutes and

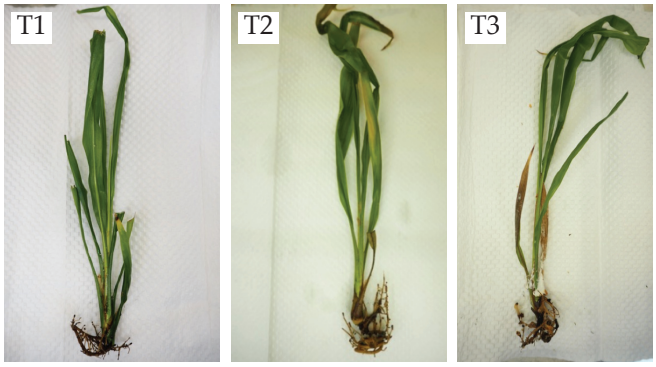

Figure 3. Physiological and morphological comparison between the treatments: (T1) non-inoculated; ii) (T2) Ganoderma boninense *PER71 mycelia disc (10 mm diameter) and (iii) rubber wood sawdust colonised with* G. boninense *PER71.*

Figure 4. Internal tissue and extent of decay at the bole area of ramet; (i) (T1) non-inoculated; (ii) (T2) Ganoderma boninense *PER71 mycelia disc (10 mm diameter); (iii) rubber wood sawdust colonised with* G. boninense *PER71.*

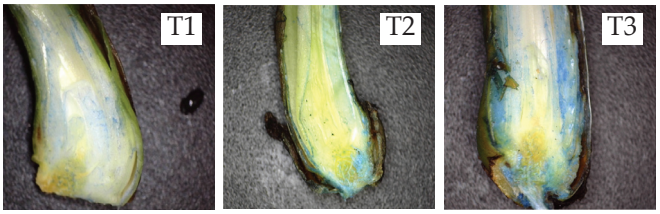

Figure 5. Microscopic observation using Dinolite on the stem region of oil palm ramets on day 15 after staining with lactophenol blue dye; (i) (T1) non-inoculated; (ii) (T2) Ganoderma boninense *PER71 mycelia disc (10 mm diameter); (iii) rubber wood sawdust colonised with* G. boninense *PER71. Scale bar: 1.0 mm.*

TABLE 1. COMPARISON BETWEEN CONVENTIONAL ARTIFICIAL PATHOGENICITY TECHNIQUE AND TISSUE CULTURE SYSTEM

Conventional	Tissue culture
Require wounding and pressure	No wounding or pressure
Time frame: 8-10 months	Time frame: 1 month
Influenced by environmental and abiotic factors	Nil
60%-70% infection	100% infection
Expensive (seedlings, rubber wood blocks, soil, polybags, labour, nursery, fertiliser)	Cost effective
Symptoms development varies	Uniformity in symptom development
Unsuitable for molecular and biochemical experimentation	Suitable for molecular and biochemical experimentation

plantation with research facilities. Fundamental studies would highly benefit from this technique while laboratories that conduct screening of biocontrol agents or germplasms would find the technique very affordable that provides infection with high precision. The invention of this tissue culture system for a uniform infection on oil palm ramets creates an opportunity to conduct the numerous physiological *in vitro* and molecular projects that may require a consistency and controlled environment. It will give a great impact especially to the research industry, where different applications can be used on a single tissue culture system. The cost of conducting the pathogenicity system in MPOB is estimated at RM3500.

CONCLUSION

Overall, the development of this tissue culture system for artificially infection using sawdust as *Ganoderma* inoculum was proven to be time and cost effective approach for studies on early disease development in oil palm ramets. This method allows natural manifestation of *Ganoderma* to the oil palm ramets without any need of wounding. Utilisation of sawdust as an inoculum was originally inspired by the nursery pathogenicity trials, where the rubber wood blocks were used for infection. This current invention provides an important methodology for many researchers that require to study on oil palm plants *in vitro* before conducting large scale nursery work.

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