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Preliminary Studies on the Development of Monoclonal Antibodies Against Mycelia of *Ganoderma boninense,* the Causal Pathogen of Basal Stem Rot of Oil Palm

Shamala, S.[1*], Chris, D.[2], Sioban, O.[2] and Idris, A. S.[1]

[1]Malaysian Palm Oil Board, 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor.
[2]Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK.
Email: shamala@mpob.gov.my

ABSTRACT

This study aimed to raise specific MAbs against *G. boninense*, the causal pathogen of basal stem rot (BSR) of oil palm. Crude mycelium extract of *G. boninense* was used as immunogen to generate MAbs. Mycelium was harvested from liquid culture and freeze-dried followed by re-suspension in phosphate buffer saline (PBS). Two 10-week old BALB-C mice were immunized with the mycelial extract. The mice were boosted once before harvesting their spleens for fusion. The MAbs were fused with myeloma cells from BALB-C mice. Initial screening was carried out using plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) with mycelial immunogen of *G. boninense*. The MAbs with positive signals were verified via secondary screening and cloned for cross-reactivity test. Cross-reactivity testing was carried out with 2 other fungi namely; *Trichoderma* and *Botrytis* along with 2 different species of *Ganoderma* commonly found in oil palm plantations namely; *G. zonatum*, and *G. miniatocinctum*. This study found that the MAbs raised against *G. boninense* were not specific as the MAbs gave positive signals through the cross-reactivity test with all fungi tested in the cross-reactivity. Future work would be using these MAbs in a co-immunization program whereby the generated *Ganoderma* sp generic monoclonal antibody will be pre-mixed with the *G. boninense* mycelium immunogen to allow reduction in the potential cross-reactivity of newly generated antibodies with *Ganoderma sp.* Our efforts are also currently directed at optimizing the immunogen preparation for the production of MAbs specific to *G. boninense*.

Keywords: basal stem rot, *Ganoderma boninense*, monoclonal antibodies, mycelium and ELISA

INTRODUCTION

The oil palm, *Elaeis guineensis*, is the highest yielding among the oil-producing crops (Ariffin *et al.*, 2000). Like any other crop, the oil palm also faces a lot of pest and disease (P&D) tribulations. From seed germination right up to field planting, the crop is exposed to several P&D problems, some of which is caused by fungi. Some of the P&D problems faced by oil palm industry are the basal stem rot, brown germ, upper stem rot, *Rhinoceros* beetles and bagworm (Turner, 1981). Among these, the present most serious disease is Basal Stem Rot (BSR) caused by *Ganoderma boninense*. There are at least 4 types of *Ganoderma* species reported in oil palm plantation namely *G. boninense, G. zonatum, G. miniatocinctum* and *G. tornatum.* For the past 50 years or more, BSR had been causing serious damage to the oil palm plantation in Malaysia. BSR causes losses through reduced yield of diseased palms and through direct loss of stand due to palm death. Singh (1991) reports that yield losses can reach as high as 46% in the 15 years old oil palm of similar soils with BSR incidences of 10.9%. In coastal estates where the disease has reached epidemic proportions, the pathogen can kill up to 85% of the original stands by the time palms are 25 years old (Singh, 1991).

The disease has no indication of early infection whereby it progresses through the palm from the base without any symptoms. The symptoms begin to manifest once the infection progressed 60 – 70% in the palm. BSR is characterized by an internal dry rotting of the trunk tissues, particularly at the junction of the bowl and trunk portion of the palm. *Ganoderma* infection causes progressive destruction of the basal tissues of the oil palm trunk. Affected leaves will show external symptoms such as wilting and malnutrition after 50-60% infection by *G. boninense*. Hartley (1988) explained that foliar symptoms would be observed when the fungus has killed half of the basal stem, thus restricting the water supply and nutrients to aerial parts which eventually leads to further destructions. The first foliar symptoms are the presence of a large number of expanded spear leaves. Older fronds, on the other hand dropped down at the point of attachment to the trunk to form a skirt of dead leaves around the palm.

To date there is no effective control or methods of detecting the disease earlier. Basically good cultural practice is advisable for the management of the plantation in ways that will not allow rapid disease spread. The correct technique of land preparation at the time of oil palm replanting is regarded as an important practice for control. This method is known as clean clearing which clears out all the old planted palm debris. The control measure is done under the assumption that infection occurs by mycelial spread from one palm's roots to the other palm's roots (Ariffin *et al.*, 2000). Other cultural practices are excision of diseased tissues and digging trenches to avoid contact from palm to palm. Chemical

***Corresponding author**

treatments are considered as the immediate short-term control measures. The use of systemic fungicides, together with a correct technique of application helps reduce the progress of the disease on the palm. Attempts to control BSR in the lab and field by the use of systemic fungicide, e.g. triadimefon, carboxin, carbendazim, methfuroxam, have been made by various workers (e.g Jollands, 1983; Khairudin, 1990; PORIM 1997) and the results from these studies were all inconclusive, although some systemic fungicides and soil fumigants seem to be promising. The most recent study on chemical is the use of hexaconazole using a pressure injector, gave lower mortality rate and a hope of controlling the disease provided the disease is detected earlier (Idris, 2002).

The limiting factor of controlling the BSR disease is the lack of a reliable diagnostic method, which directly allows the early detection of the disease. The use of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) for detecting pathogenic fungi in infected plants has been applied widely. However immunoassay techniques offer greater simplicity and less equipment than those of DNA probe analysis such as PCR (Darmono, 2000). ELISA offers advantages in term of speed, ease of use and costs compared to PCR based assay (Utomo and Niepold, 2000). Successful detection of root-infecting fungi in infected plants by ELISA has been reported previously. One of the closest pathogens causing root diseases from the same class of *Ganoderma* is *Heterobasidion annosum*. It is one of the common basidiomycete organisms responsible for the decay of conifers. Successful detection by ELISA was reported for this fungus by polyclonal antibodies (Avramenko, 1989). However limitations of such sera can be useful in western blotting (Vigrow *et al.,* 1991) but their application to simple assay systems such as ELISA is limited if cross-reactivity is extensive especially in the case of fungi where higher cross-reaction can be expected (Galbraith and Palfreyman, 1994). Eventually other workers (Galbraith and Palfreyman, 1994) developed monoclonal antibodies against *H. annosum* that has specificity in detection of the pathogenic strain. Similar understanding also applies to *Ganoderma,* as there are at least 4 different *Ganoderma* species in the oil palm plantations with *G. boninense* being pathogenic against oil palm (Idris, 1999). Another successful detection using ELISA is the detection of *Armillaria*, a root disease pathogen for a wide range of woody plants, has been undertaken successfully with monoclonal antibodies (Fox and Hahne, 1989; Priestley *et al*., 1994). To date, immunological methods have not been widely applied on the detection of *G. boninense* though the development of polyclonal antibodies against the organism was reported (Darmono *et al*., 1993; Utomo and Niepold 2000). Monoclonal antibodies will be the best diagnostic tool due to its specificity and uniformity of detection. Thus this study was carried out to determine the possibility of raising a specific MAb for the detection of *G. boninense* in the infected palm.

MATERIALS AND METHODS

Preparation of Immunogen

G. boninense was grown in liquid culture of potato dextrose broth (PDB) for 2 weeks under ambient temperature and light conditions. The mycelium extract was removed from the liquid culture by filtering through a muslin cloth leaving behind the mycelium extract. It was filtered again with vacuum pump to remove the attached liquid media. Sterile water was added followed by the same amount of phosphate buffer saline (PBS) to facilitate the filtration and decant as much of the liquid media. The filtered mycelium was then inserted into bijou bottles and freeze dried overnight.

After freeze drying, the material was snap frozen with sufficient amount of liquid nitrogen in pestle depending on the amount of material. When enough liquid nitrogen covered the pestle, the mycelia were grinded with little movement to break the mycelia. Mycelia was removed and weighed immediately to avoid being moistured. It was then suspended in PBS (kept in 4ºC) at 1.5ml/0.2g and mixed thoroughly for an even distribution. If the resuspended material is very viscous, more PBS was added. The mixture was whirl mixed with vortex for 30 seconds followed by centrifuging mixture at 4500rpm for 10 minutes at 4ºC. Supernatant was removed and referred as soluble immunogen.

Protein Concentration

Protein concentration was carried out based on a commercial Bicinchoninic Acid (BCA) protein assay reagent kit (PIERCE). Protein concentration was carried out to determine the minimum level of coating concentration of protein on wells with sufficient amount of antigen for the immunization and ELISA protocol. This was to allow the detection specificity by MAbs that were raised on microtitre plates when screening was carried out.

Immunization

Two BALB-C mice of 10-week old were immunized intraperitoneal with soluble antigen of *G. boninense* (Campbell, 1986). Three injections were carried out at 2 weeks interval with tail bleed carried out on the 6th week to determine that both mice were secreting antibodies detecting *G. boninense* (Figure 1). Once identified, final boosting carried out 4 days prior to the fusion. Both mice were killed on the 7th week for the fusion experiment and referred as Fusions 240 and 243.

Fusion

Mice were killed and the spleens were harvested. For a continuous supply of antibodies, the harvested MAbs were fused with myeloma cells for hybridoma cells in assisting further screening of the MAbs. Fusion procedure for

hybridoma cells was carried according to Campbell (1986).

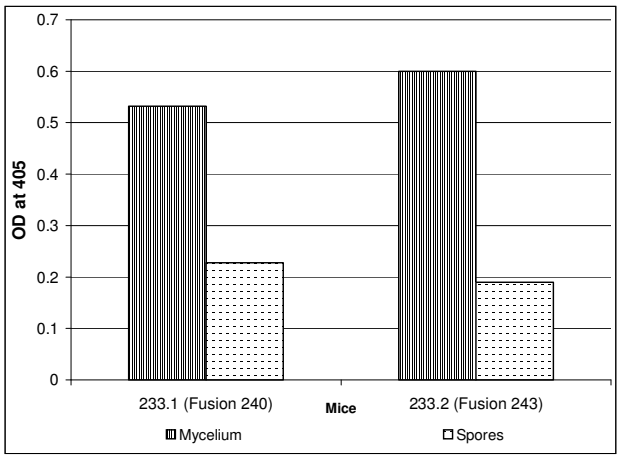

Figure 1: Graph Indicating 6 Weeks Tail Bled for Soluble Immunised Mice

ELISA

Test tissue culture supernatant (TCS) from Fusions 240 and 243 were screened for the presence of antibodies recognizing the soluble antigen of *G. boninense.* Each fusion and clone retesting involves a standard PTA-ELISA protocol. Wells were coated at 5µg/L of the soluble antigen in coating buffer and incubated overnight at 4℃ in microtitre plate wells, blocked with PBS/Tween/TCS/Anti Mouse IgG/p-NPP (p-nitrophenyl phosphate). Antibody binding was detected by microplate reader at 405nm according to optical density (OD).

Cross-reaction with Fungi Associated in Oil Palm Plantation

Specificity of the selected MAbs was determined by PTA-ELISA using the fungi commonly found in the oil palm plantation. Fungi tested were *G. miniacinctum, G. zonatum, Trichoderma* and *Botrytis*. *G. miniacinctum, G. zonatum* were isolated from fruiting body that appeared on oil palm trunk while *Trichoderma* and *Botrytis* were isolated from the soil using the soil dilution method of Rose Bengal Agar (Ilias, 2000). Spores of *G. boninense* that were obtained from the fruiting of the pathogen was also included in the cross-reactivity test.

RESULTS

From the two sets of fusions (240 and 243) carried out, 44 wells were detected with hybridoma growth. Screening was carried out to determine the specificity of the growing hybridomas. Screening was based on the OD reading obtained through the ELISA test. Fusion 240 had 12 good cell lines while Fusion 243 had only 6. However, only 2 cell lines from Fusion 240 showed good growth. Eventually 20 cell lines were selected for cloning for further testing. Cloning on cell lines from Fusion 243 was unsuccessful as measured through ELISA results thus cross-reactivity test was aborted.

The supernatant of the cloned cell lines (Fusion 240) were tested for cross-reaction with fungi associated with oil palm including *G. zonatum, G. miniatocinctum, Trichoderma* and *Botrytis*. The 20 cell lines that were cloned for cross-reactivity test were tested using PTA-ELISA and OD results were recorded. From the OD readings, it was found that all the selected cloned cell lines cross-reacted positively with all the fungi included in this test (Figure 2).

DISCUSSION

Fusion 240 (soluble immunogen) gave raise to many hybrids (22%) that were successfully cloned twice by limiting dilution techniques. Extensive cross reactivity testing of these cell lines demonstrated that antibodies have been raised towards a common fungal marker throughout the *Ganoderma* genus and other fungi as well. As for Fusion 243, it yielded 41% hybrids by day 19-post fusion. However, screening against the soluble antigen as previously conducted generated only 6 promising cell lines for Fusion 243 and were eventually cloned. Neither of these hybrids tolerated the cloning process as illustrated by poor ELISA responses when re-tested against immunogens and other closely related fungal isolates.

MAbs proved to be superior to polyclonal antisera where its specificity for one single epitope, homogeneity and availability in unlimited amounts was essential in the development of diagnostic kit against any pathogen. Any immunological technique, its entire success depends upon the completion of series sequential steps. The nature of the immunogen, immunization process, cell fusion procedure, cloning and screening assays for antibody production are among areas where difficulties can be encountered (Ouelette and Benhamou, 1987). However, immunization process, cell fusion procedure, cloning and screening assays are procedures that are now becoming standard techniques (Ouelette and Benhamou, 1987). Difficulties were encountered in the optimization of immunogen preparation whereby the preparation varies for different types of fungi. Galbraith and Palfreyman (1994) used crude extract of *H. basidion* to obtain a specific MAbs hence the preliminary effort in raising MAbs in this study used crude extract from the pathogen. Immunogen preparation may have contributed to the inability of raising specific MAbs (Ouelette and Benhamou, 1987). Efforts are being directed in optimizing the preparation of a purified immunogen by running the immunogen on a gel in order to separate the higher and lower molecular weight fractions. Further to this, western blotting from these gels would be carried out and eventually probed with the general antibody. Based on the signals obtained, the fractions could be narrowed down for immunization. Efforts are also currently being directed to a

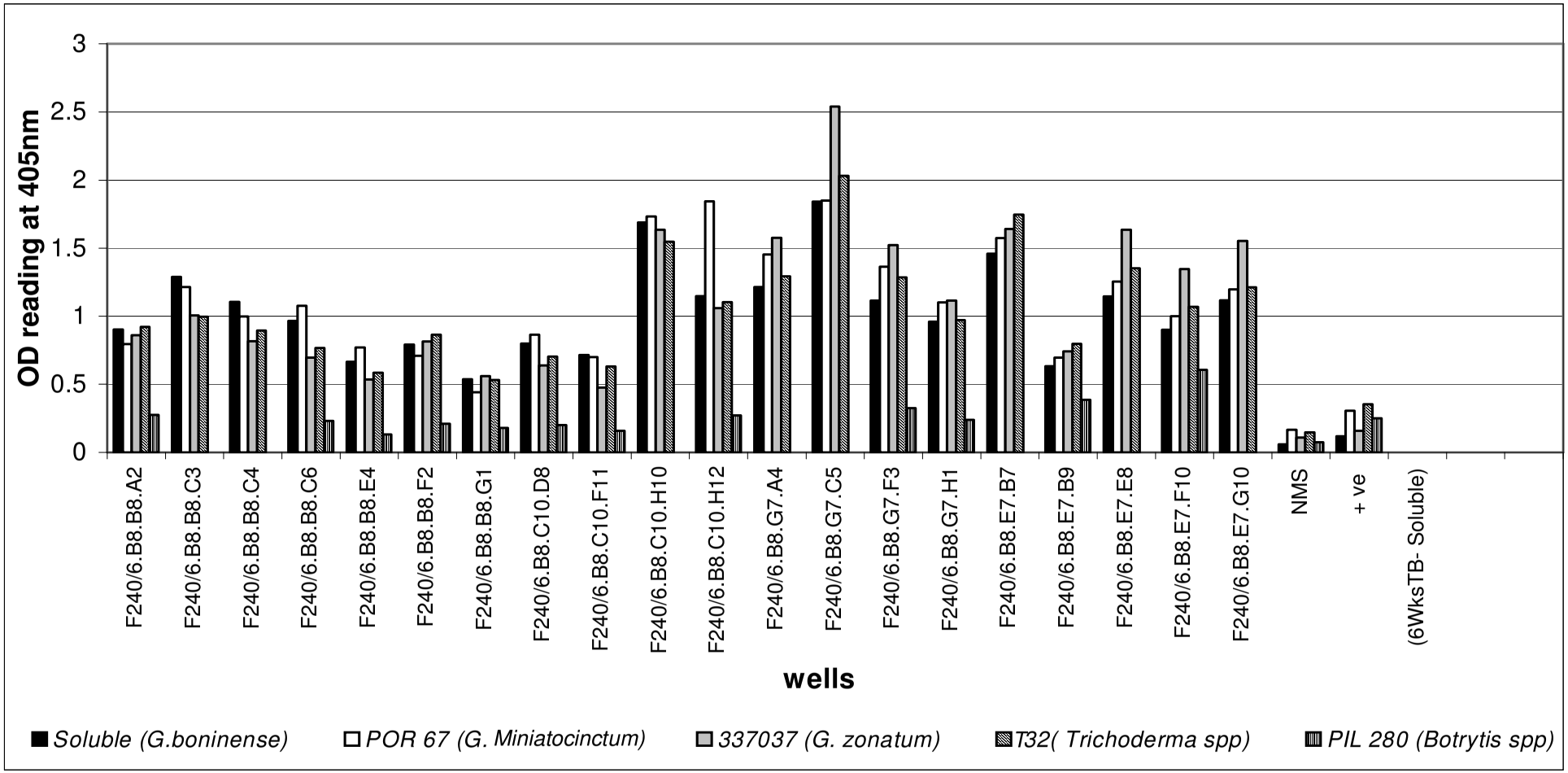

Figure 2: Graph Indicating Cross Reactivity of Fusion 240 MAb (Soluble) with Other Fungi

co-immunization program whereby the generated *Ganoderma* generic MAbs (F240) from this study will be pre-mixed with the *G. boninense* soluble immunogen. The resulting mixture will then be used as the immunogen for future immunization. This period of pre-absorption would allow a reduction in the potential cross-reactivity of newly generated antibodies with *Ganoderma.*

CONCLUSION

This study is a preliminary effort in developing specific MAbs. From this study, general MAbs that were generated provided valuable information and could be useful for future research work. MAbs can be generated from *G. boninense,* however more research needs to be carried out before a specific MAb could be generated.

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