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A Rapid Inoculation Method for Infection of *Ganoderma* in Oil Palm

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ABSTRACT

Basal stem rot (BSR) is a major disease of oil palm plantation caused by pathogenic fungus *Ganoderma boninense*. To overcome the disease, development of resistant or tolerant oil palms is crucial for sustainable production of palm oil. Thus a reliable and rapid method to assess resistance of oil palm to BSR is important. Here we report an inoculation technique designated as mycelium inoculation technique. This method is faster and simpler compared to the traditional technique using rubber wood block. The technique that we developed results in consistency of infection and disease can be evaluated as early as two weeks after inoculation with *G. boninense*. Furthermore, this method is also able to differentially assess resistant and susceptible palm seedlings to BSR. The results show that this new inoculation technique can be used as a routine method to infect oil palm seedlings and can be utilised for development of resistant cultivar of oil palm to *G. boninense*.

Keywords: basal stem rot, *Elaeis guineensis*, mycelium, rubber wood blocks, tolerant

INTRODUCTION

Oil palm (*Elaeis guineensis*) is a perennial crop cultivated in several tropical countries and is the most efficient producer of vegetable oil (palm oil) compared with other oil-producing plants. The expansion of oil palm industry began in 1990s and in 2005/2006, Indonesia became the largest producer of palm oil in the world after Malaysia. However, the BSR disease is becoming a major obstacle to the production and sustainability of oil palm plantations. For more than 50 years, the disease has caused serious damage to oil palm plantations in Indonesia and Malaysia. BSR disease causes a decrease in fresh fruit bunch (FFB) weight, stem weight to oil palm bunch and eventual death of the plant (Susanto 2002; Susanto *et al*. 2005).

Various attempts have been made to control *G. boninense* attacks which include mechanical, chemical and biological controls. However, no satisfactory results have been reported (Susanto 2002). One of the reasons why this pathogen is diffi-

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cult to control is because it is a soil borne pathogen that spreads and survives in soil. Moreover, the external symptoms of BSR is also difficult to detect at the early stage of infection. The common practices to limit spread of BSR usually involve methods that target sanitation such as soil mounding, isolation through trenching, and removal of diseased plant debris from plantation area. Chemical control through the use of fungicides was reported to be ineffective to control the disease (Chung 2011).

Another approach to control BSR is through breeding and the use of plants that are resistant or tolerant to *G. boninense*. However, there are constraints to the development of resistant oil palm through plant breeding. The mechanisms underpinning *G. boninense* pathogenicity and the defence response of plants are still poorly understood. This is exacerbated by the lack of simple bioassay of BSR in oil palms. For example, inoculation methods that will allow rapid and simple evaluation of BSR disease are still lacking. Naturally, infection of *G. boninense* and the progression of BSR symptoms in mature plants are slow (Panchal & Bridge 2005; Rees *et al*. 2009). This poses a challenge to screen cultivars resistant to BSR. Currently, the most commonly used artificial inoculation of *G. boninense* is using colonized rubber wood block (RWB) (Lim *et al*. 1992; Idris *et al*. 2004). This method has been utilized as a standard technique of inoculation in research of BSR disease. However, this method is laborious and time-consuming. A typical RWB inoculation requires at least six months from preparation until disease evaluation (Chong *et al*. 2012). Due to the long incubation period and difficulty in sterilizing RWB, contamination rate of other fungi is quite high. Therefore, the objective of this study is to develop a rapid, reproducible, and simple inoculation technique that can be used routinely for *in planta* assessment of BSR disease in oil palm.

MATERIALS AND METHODS

Ganoderma boninense Isolates and Oil Palm Cultivars

Ganoderma boninense used in this study was isolate B29 originated from oil palm plantations in West Sumatera (Purnamasari *et al*. 2012). Oil palm seedlings used were *tenera* (*dura x pisifera*) cultivars which were our collection (Research and Development PT Wilmar Benih Indonesia) and one cultivar (MT Gano) that was obtained from PT Socfin Indonesia (Table 1). Seedlings used were germinated seeds and acclimatized, tissue-culture-generated plantlets (ramets).

Inoculation of *Ganoderma boninense*

Artificial inoculation of *G. boninense* was performed using mycelium inoculation technique (Chong *et al*. 2012) with some modifications. Inoculum of *G. boninense* B29 was prepared by cutting mycelia of 6-day-old culture on potato dextrose agar (PDA) into 1x1 cm^2 plugs using a sterile inoculated into 100 mL potato dextrose scalpel. The mycelial plugs

Table 1 Oil palm cultivars used in this study

Cultivar	Type	Source
TS1	Seedlings	Wilmar
TC17	Tissue culture	Wilmar
TC20	Tissue culture	Wilmar
TC68	Tissue culture	Wilmar
TC39	Tissue culture	Wilmar
TC57	Tissue culture	Wilmar
TC24	Tissue culture	Wilmar
TC27	Tissue culture	Wilmar
TC18	Tissue culture	Wilmar
MT Gano	Seedlings	Socfindo

were then broth (PDB) and incubated for 4 days at 28 °C with agitation. After 4 days, the mycelium cultures were blended using a sterile hand held blender (Philips® HR1603) for 3 minutes with the addition of 0.002% Tween® 20. The concentration of mycelia was adjusted to 10^5 fragments mL^{-1} using a haemocytometer.

For oil palm seedlings, roots of 4-month-old oil palm plants were removed carefully from soil and soaked in the mycelium suspension for 30 minutes before replanting. As a control, plant roots were immersed in sterile water for 30 minutes. The inoculated plants were then replanted in sterile soil in 70-mm pots and grown in glasshouse. Soil was sterilized using an autoclave with liquid cycle (121 °C, 20 minutes, 100 kPa) then put at room temperature for 2 days. After 2 days, the soil was sterilized again with the same conditions prior to potting. Plants in pots were placed in a greenhouse with ambient relative humidity (ranges between 70 and 90%), kept at 30 °C, watered twice daily and fertilized once a week [8 g 0.625 g L^{-1} of N:P:K:Mg (15:15:6:4) fertilizer for 100 seedlings]. The progression of disease symptoms was observed every week. For each line of seedling, ten plants were used for this test and each plant was planted in one pot. For ramets, the plantlets were acclimatized for 2.5 months prior to inoculation with *G. boninense*. After acclimatization, the ramets were inoculated using the same methods as for the germinated seedlings.

Evaluation and Assessment of Basal Stem Rot Disease

After two weeks of incubation, disease was evaluated and scored. Disease scoring was measured on a scale of 0-4 according to the observed symptoms of the disease, where 0 = healthy plants; 1 = slightly chlorotic spots on the leaves; 2 = chlorosis in 1-2 leaves; 3 = chlorosis on many leaves (> 2 leaves); 4 = dead plants (Abdullah *et al*. 2003).

The disease ranking is an ordered categorical (ordinal) variable that has non-Gaussian distribution, thus the statistical model applied was non-parametric. Statistical test was conducted using a general linear model to compare significance of scores between cultivars and probabilities were adjusted further by using a Tukey *post-hoc* test ($P < 0.05$). Statistical analysis was conducted using the R statistics program (R Core Team 2016).

Verification of *Ganoderma boninense* Infection

To ensure that the observed symptoms of BSR was caused by *G. boninense* B29, the pathogen was re-isolated from the infected roots and molecular tests were performed to identify the isolated fungus. Roots of healthy (control) and diseased oil palms were cut, washed with water and surface-disinfected using 1% (v/v) sodium hypochlorite solution for 5 minutes then rinsed using sterile distilled water. Each root piece was then grown on *Ganoderma* selective medium (Chong 2010) and incubated at 30 °C for one week.

After one week of incubation, observation was made on the fungi that grew around the infected roots. These fungal colonies were re-isolated and grown on PDA for six days. Mycelia that grew on the media were collected and genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen). Fungal identification was performed by analyzing DNA sequences in the internal transcribed spacer region I (ITSI), region II (ITSII), and the 5.8S ribosomal DNA (rDNA). DNA sequences within these regions (ITSI, ITSII, and

5.8S rDNA) were amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplified DNA fragments were then sequenced (Applied Biosystems® 3130 Genetic Analyzer, Thermo Scientific) and verified.

RESULTS AND DISCUSSION

Mycelium Inoculation Technique Provides a Rapid and Consistent Pathogenicity Test

The mycelium inoculation technique developed in this study has enabled a rapid and simple infection of *G. boninense* into oil palm seedlings. Symptoms of BSR disease began to appear a week after inoculation. After two weeks, BSR symptoms in the crowns and leaves have progressed significantly as shown by necrotic crowns and leaves, severe wilting, and rotten stems, especially at the base (Figure 1). Longitudinal sections of the stem of inoculated plants revealed browning and rotten crowns, whereas healthy plants did not show these symptoms.

In addition, root observations showed that plants with necrotic and rotten stems also exhibited root rot (Figure 2). Mycelial colonization was also observed in infected plants especially at the root area. In contrast, uninoculated plants were healthy and did not show symptoms of BSR disease. Based on the observed BSR symptoms, there was no difference between the plants originating from tissue culture (ramet) and seedlings.

The technique was also capable of differentiating the levels of tolerance or susceptibility of several cultivars of oil palms including cultivars in which the tolerance levels are already known (TS1 and MT Gano). This technique was developed based on a simple hypothesis that *G. boninense*, like some other plant pathogens, will be able to infect plants even though it is grown on defined media and inoculated artificially. Using this inoculation technique, the whole process only took 24 days, 10 days to grow *G. boninense* culture and 14 days for bioassay of BSR disease. The technique was much faster than the standard method that uses RWB that typically takes about six months until the disease can be assessed. The differences between these two methods are summarized in Table 2.

Mycelium inoculation employed in our study was more effective because there was direct contact between pathogen and plant roots. In the rubber wood block technique, direct contact between plant roots and the pathogen is crucial in successful infection compared to the size of the wood block used (Rees *et al*. 2007). In our method, because roots were dipped into the mycelial suspension, it ensured direct contact between roots and the pathogen. In addition, the method also gave faster results than a method reported in a previous study conducted by Chong *et al*. (2012) where detection of ergosterol concentration in inoculated plants takes 3-6 weeks after inoculation.

Table 2 Comparison of mycelium inoculation methods and rubber wood blocks

Parameters	Mycelium inoculation technique (this study)	Rubber wood block
Time to prepare inoculum	10 days	1 month
Incubation time	2 weeks	5 months
Inoculum concentration	Quantified	Not quantified
Risk of contamination	Low	High
Levels of complexity in preparation	Simple	Complicated

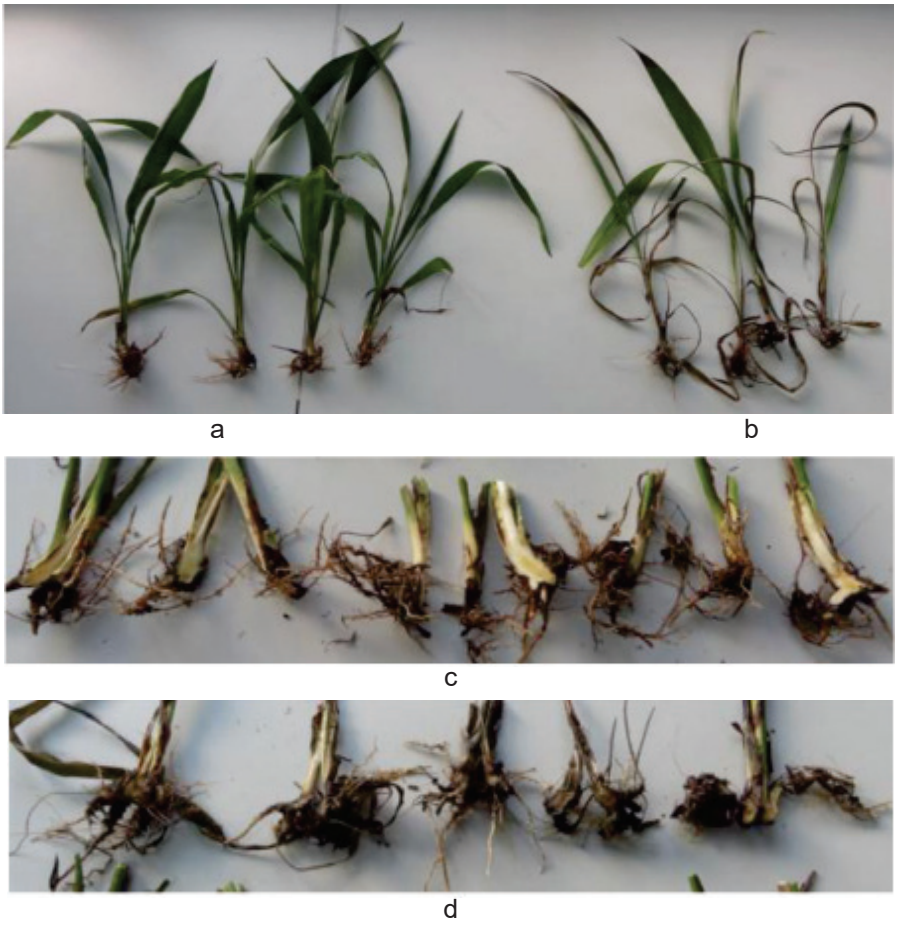


Figure 1 Symptoms of basal stem rot disease in tolerant (a and c) and susceptible plants (b and d) to *Ganoderma boninense*. Inoculation of *G. boninense* was performed using mycelium inoculation technique. a Leaves and stems of tolerant plants did not show symptoms of basal stem rot (BSR) disease, b while necrotic tissues were seen in susceptible plants c longitudinal slice of healthy and d infected stems.

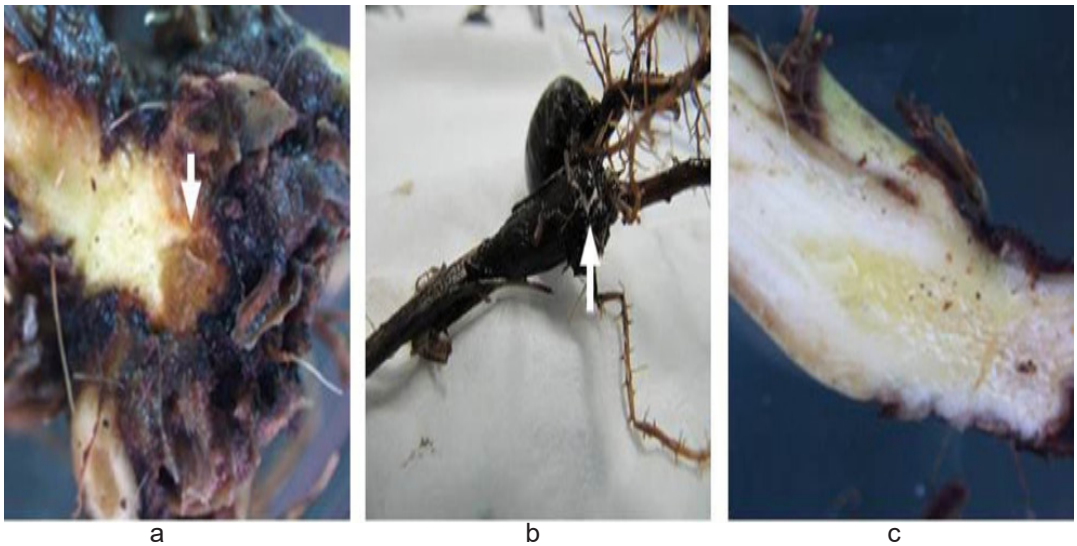


Figure 2 Necrotic and rotten crown of an infected plant a as indicated by the arrow. b Colonization of mycelia at the base of the infected stem (arrow). c Longitudinal section of crown of a healthy plant.

This is because the mycelium inoculation method used immersion technique that allowed longer contacts between mycelia and plant roots than the spray method used by Chong *et al*. (2012).

Another important factor is that in this study, in contrast to the rubber wood block technique, concentration of inoculum was determined and quantified. Concentration of 10^5 mycelia mL^{-1} of *G. boninense* was sufficient to infect plant roots and provided consistent pathogenicity levels. Considering that *G. boninense* is a weak competitor in the soil (Idris *et al*. 2004), the determination of quantifiable concentration of inoculum becomes crucial to the effectiveness of infection.

The Mycelium Inoculation Technique was Able to Differentiate Tolerance of Oil Palms to Basal Stem Rot Disease

Evaluation of tolerance or susceptibility levels of oil palm lines was observed two weeks after inoculation. Among ten different cultivars tested, it was shown that TS1, TC27, and TC18 had the highest number of diseased plants showing high were susceptible to *G. boninense* (Figure 3). Cultivars TC24 and TC18 were also susceptible to G. boninense even though the proportion of the diseased plants was not as high as the three cultivars mentioned above. On the other hand, TC20, TC68, and MT Gano cultivars showed the lowest number of diseased plants, which means that these cultivars were tolerant to *G. boninense*. Meanwhile, TC17 and TC39 also showed tolerance to *G. boninense* although it was not as strong as TC20, TC68, and MT Gano.

Based on the differences in the disease severity, statistical test showed that the plants could be considered into three groups: susceptible (TS1, TC27, TC18), moderate tolerant (TC57, TC24), and tolerant (TC17, TC20, TC39, and MT Gano) (Figure 4). Amongst the tolerant group, TC20, TC68, and MT Gano were the most tolerant cultivars to *G. boninense*. The division of tolerance and susceptibility was solely based on our statistical interpretation of the data since there is no comprehensive reference on the classification of tolerance in oil palms in the current literature.

The effectiveness of mycelium inoculation technique was tested using a number of oil palm cultivars originated from both seedlings and tissue culture clonals. The consistency of *G. boninense* pathogenic-

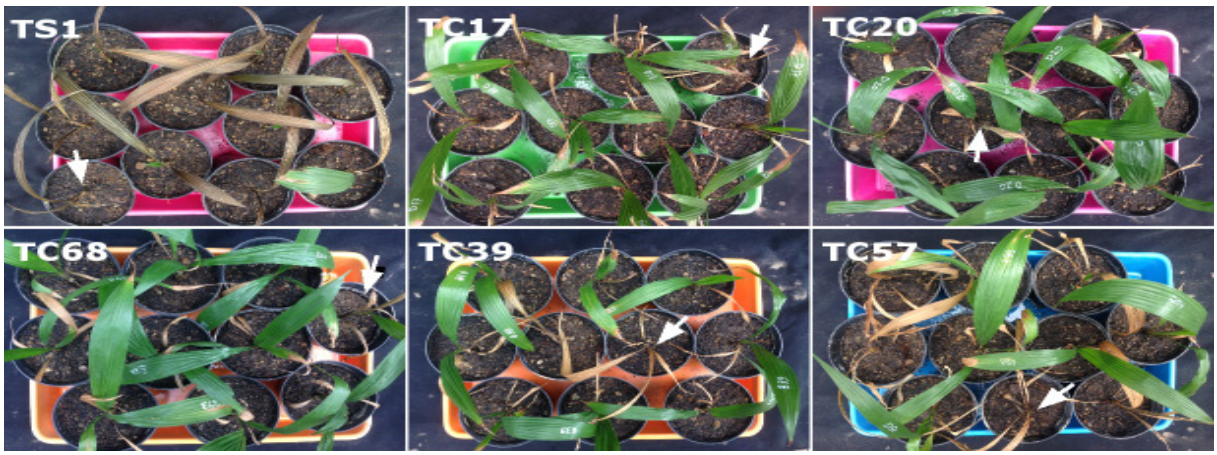


Figure 3 Pathogenicity test of *Ganoderma boninense* B29 on several oil palm cultivars for selection of tolerant cultivars using mycelium inoculation technique developed in this study. Examples of infected and necrotic plants are shown by white arrows.

ity in both types of oil palm materials indicated that this method can be used for plants of various origins. In addition to consistency, the bioassay also produced the expected levels of tolerance or susceptibility in some of the cultivars. For example, TS1 is known to be susceptible to *G. boninense* and the results confirmed the susceptibility of TS1. In contrast, MT Gano was tolerant to *G. boninense*, consistent with the report released by PT Socfin Indonesia, the company that produces the cultivar.

Molecular Analysis Confirmed that Infection in the Plants was Caused by *Ganoderma boninense*

Isolation of the fungi from the infected root showed that the fungal isolates were morphologically the same as *G. boninense* B29 (Figure 5). Sequencing of the ITS regions of these isolates showed sim-

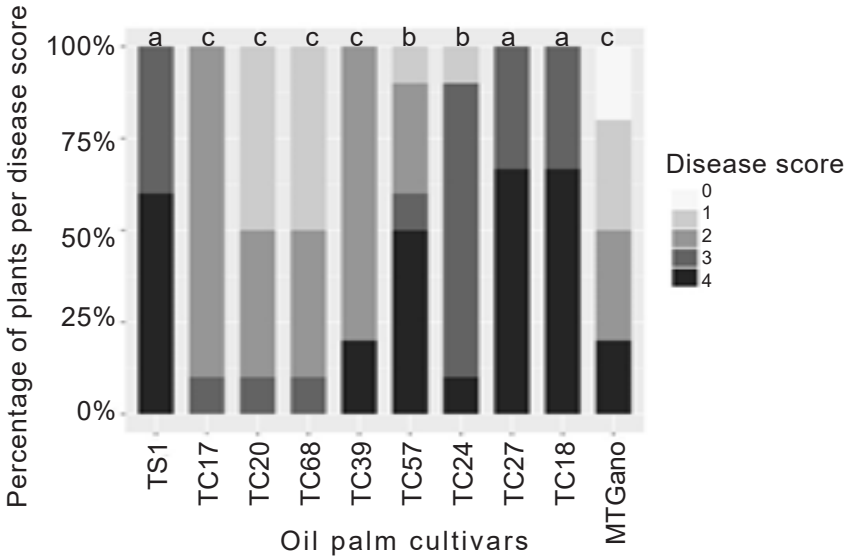
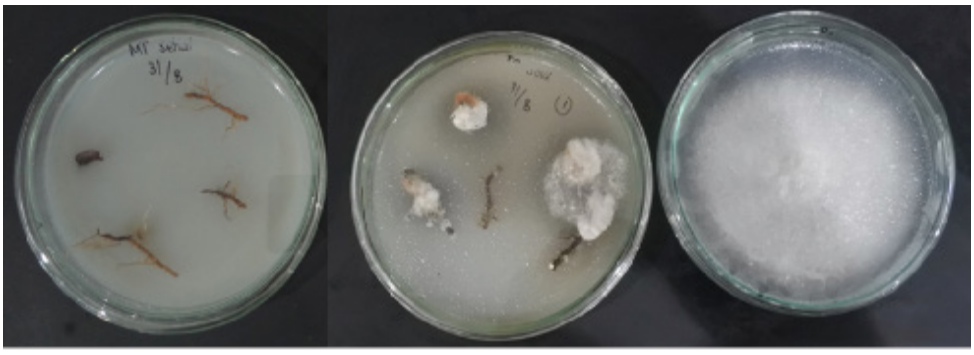


Figure 4 Basal stem rot (BSR) disease assessment in several oil palm cultivars inoculated with *Ganoderma boninense* B29 using mycelium inoculation technique. Disease scoring of BSR wilt according to Abdullah *et al*. (2003) at two weeks after inoculation. The area for each colour code indicates the percentage of plants having the corresponding disease scores. At least ten plants with each plant in individual pot were used in this assay. Control is non-inoculated plants treated only with water. Bar charts labelled with different letters indicate significant differences ($P < 0.05$).



a b c

Figure 5 Re-isolation of *Ganoderma boninense* B29 from roots of infected plants. a Plant roots inoculated onto *Ganoderma* selective medium (GSM). b Mycelia of *G. boninense* from infected roots began to grow on the medium. c Pure culture of *G. boninense* isolated from the infected roots.

ilarities with the published sequences of *G. boninense* (95.4% similarity) (data not shown).

Together, results presented here suggest that the new inoculation method can assist plant breeding process by selecting resistant or tolerant cultivars of oil palms to *G. boninense*. It also can assist other bioassays such as application of synthetic or organic fungicides to control BSR thus allowing assessment of efficacy of the fungicides. Further, the rapid and reproducible *in planta* assay of disease tolerance would be useful for identifying and functional analyses of genes that could play role in tolerance of oil palm to *G. boninense*.

CONCLUSION

In conclusion, a new inoculation technique was developed to facilitate rapid and reliable infection of *G. boninense* B29 into oil palm. *In planta* disease assays showed that the BSR disease produced by this method was reproducible and it required a shorter incubation time with simpler preparation compared with the existing method of inoculation such as RWB. In addition, the consistency of this technique was further confirmed in the assessment of BSR in several cultivars of oil palm with different levels of tolerance to *G. boninense*.

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