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PATHOGENESIS-RELATED PROTEIN INDUCTION IN *Streptomyces* GanoSA1-TREATED OIL PALM SEEDLINGS DURING EARLY INFECTION BY *Ganoderma boninense*

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ABSTRACT

Basal stem rot (BSR) disease in oil palm is caused by a fungal pathogen identified as Ganoderma boninense. *In our previous study,* Streptomyces nigrogriseolus *GanoSA1 showed strong antagonistic activity against* G. boninense *PER71 in laboratory, nurseries, and field studies. This study aimed to identify the role of* Streptomyces *GanoSA1 in inducing pathogenesis-related proteins in oil palm seedlings during the early infection by the pathogen. Three-month-old commercial oil palm seedlings (D×P) were inoculated with* Streptomyces *GanoSA1 and artificially infected with* G. boninense *PER71 through the rubberwood block sitting technique. The seedlings were harvested at 0, 3, 7, 14, 28 and 60 days after inoculation (DAI) with* G. boninense *PER71. The seedlings were tested for the production of β-glucanase, peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine lyase (PAL). The* Streptomyces *GanoSA1 significantly induced the production of β-glucanase (3.04-fold), POX (1.68-fold), PPO (1.88-fold), and PAL (1.127-fold) in oil palm roots compared to control seedlings. The biological control activity by* Streptomyces *GanoSA1 might be contributed by the induction of pathogenesis-related proteins.*

Keywords: actinomycetes, artificial inoculation, defence-enzyme, *Ganoderma*.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an important crop in Malaysia, with nearly 5.84 million hectares of cultivation areas throughout the country (Parveez *et al.*, 2021). The crop, however, is susceptible to some pests and diseases. One of the severe threats is the basal stem rot (BSR) disease caused by *Ganoderma* spp. (Idris, 2012), with a potential yield loss of 68.73% (Assis *et al.*, 2020). According to Idris (2019), the incidence of BSR in 2016-2017 was 7.40%, affecting approximately 221 000 ha of oil palm cultivated areas. Cultural practices, application of chemical fungicides, and biological control were among the strategies recommended to control the disease and sustain the infected oil palm (Idris *et al.*, 2016; Naidu *et al.*, 2015; Siddiqui *et al.*, 2021; Surendran *et al.*, 2018).

Biological control is an appealing environmentally friendly option for controlling fungal plant pathogens. The introduction of antagonistic actinomycetes into the rhizosphere has the potential to alter the soil's native microorganism ecosystems. This exposure leads to the suppression of soilborne pathogens and interference of survivability or disease-producing activities by the phytopathogen. Several studies have shown that many beneficial microorganisms, such as actinomycetes, bacteria and fungi, can effectively lower plant disease incidence (Amaresan *et al.*, 2018; Gowdar *et al.*, 2018; Meena *et al.*, 2017). Many species of actinomycetes, especially those belonging to the *Streptomyces* group, are well known to produce antibiotics

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and cell-wall degrading enzymes (Amaresan *et al*., 2018; Tamreihao *et al*., 2016) and remain as major contributors to secondary metabolites (Matsumoto and Takahashi, 2017). They have been proven to potentially inhibit or affect the growth of phytopathogenic fungi (Al-Dhabi *et al*., 2019; Bettache *et al*., 2018; Dias *et al*., 2017), including *Ganoderma* sp. (Lim *et al*., 2018; Nur Azura *et al*., 2016; Pithakkit *et al*., 2015; Queendy and Roza, 2019; Shariffah-Muzaimah *et al*., 2015; Sujarit *et al*., 2020; Tan *et al*., 2002; Ting *et al*., 2014). Apart from that, a reduction in disease incidence was also observed in an *in Plantae* experiment (Dias *et al*., 2017; Idris and Shariffah-Muzaimah, 2021; Ramamoorthy *et al*., 2002; Salla *et al*., 2016; Zhang *et al*., 2016), indicating their potential to be utilised as a biocontrol agent. The biocontrol of phytopathogen may be related to mechanisms such as the production of antibiotics (Lim *et al*., 2017; Wonglom *et al*., 2019), secretion of hydrolytic enzymes (Jog *et al*., 2012; Mun *et al*., 2020; Wonglom *et al*., 2019), production of siderophores or competition for nutrient (Zeng *et al*., 2018) and induced systemic resistance in plants (Dias *et al*., 2017; Kurth *et al*., 2014; Zhang *et al*., 2016; 2020; Zhao *et al*., 2012).

In previous studies, *Streptomyces nigrogriseolus* GanoSA1 have shown a significant suppressive effect on *Ganoderma* disease (Idris and Shariffah-Muzaimah, 2021; Shariffah-Muzaimah *et al*., 2020). The present report describes the biochemical responses with defence enzymes, such as polyphenol oxidase (PPO), peroxidase (POX), and phenylalanine ammonia-lyase (PAL), that are involved in phytoalexins and phenolic synthesis in *Streptomyces* sp. GanoSA1 treated oil palm (*Elaeis guineensis* Jacq.) seedlings infected with *G. boninense* PER71.

MATERIALS AND METHODS

Planting Materials

A total of 100 three-month-old *Dura* x *Pisifera* (D×P) oil palm seedlings used in this study were obtained from the Malaysian Palm Oil Board (MPOB). The seedlings were grown in polypropylene bags (15 × 23 cm) containing approximately 15 kg of soil mixture of commercial topsoil and sand (3:1) and maintained in a nursery (temperature variation of 30°C to 35°C) one month before treatment.

Streptomyces GanoSA1, *Ganoderma boninense* PER71 and Culture Condition

Streptomyces nigrogriseolus GanoSA1 (MT150593) was previously isolated from the rhizosphere of a healthy oil palm located in an area with severe BSR incidence in Peninsular Malaysia (1°57′10.7″N 103°22′16.6″E). This strain showed strong antagonistic activity against *G. boninense* PER71 in both *in vitro* and *in vivo* experiments (Idris and Shariffah-Muzaimah, 2021; Shariffah-Muzaimah *et al*., 2020). *Streptomyces* GanoSA1 and a virulent stock culture of *G. boninense* PER71 were obtained from the Plant Pathology and Biosecurity Unit Laboratory, Biology and Sustainability Research Division, MPOB, Malaysia. The working culture of *Streptomyces* GanoSA1 was prepared by streaking out one loopful of the glycerol stock on yeast malt extract (YME) agar. The *G. boninense* PER71 working culture was prepared by sub-culturing a stock culture onto potato dextrose agar (PDA). Plates were sealed individually and incubated at 28°C for seven days.

Powder formulation of *Streptomyces* GanoSA1 for the nursery experiment was prepared as described by Idris and Shariffah-Muzaimah (2021). The strain was cultivated in YME broth and incubated with agitation at 180 rpm for seven days. Commercial vermiculite (2-4 mm) and bio-charcoal (2-4 mm) were mixed at the ratio of 1:1 and autoclaved twice on two consecutive days for sterilisation. A total of 50 g of the vermiculite-bio charcoal powder was then inoculated with 20% of the *Streptomyces* GanoSA1 culture to achieve the concentration of 10^8 colony forming units (CFU) per gram. The inoculum of *G. boninense* PER71 for artificial inoculation in oil palm seedlings was prepared using 6 × 6 × 6 cm rubber woodblock (RWB) according to the method described by Sundram *et al.* (2015).

Experimental Design, Inoculation of *Streptomyces* GanoSA1 and Artificial Inoculation of *Ganoderma boninense* PER71 to Oil Palm Seedlings

The experiment was laid down in a complete randomised design (CRD) with four treatments, and each treatment consisted of 24 seedlings with four replicates per harvest. For each treatment, four seedlings were sampled from each replication separately on days 0, 3, 7, 14, 28 and 60 post-inoculation with *G. boninense* PER71. The four treatments were seedlings without *Streptomyces* GanoSA1 or *G. boninense* PER71, as control (T1); seedlings without *Streptomyces* GanoSA1 and with *G. boninense* PER71 (T2); seedlings with *Streptomyces* sp. GanoSA1 without *G. boninense* PER71 (T3); and seedlings with *Streptomyces* sp. GanoSA1 and with *G. boninense* PER71 (T4) (*Table 1*). Seedlings in treatments T3 and T4 were pre-treated with 50 g of *Streptomyces* GanoSA1 vermiculite bio-charcoal powder by soil mixing. Fourteen days after the pre-treatment activity, each seedling in treatments T2 and T4 were artificially inoculated with *G. boninense* PER71 using the RWB sitting technique (Idris and

Shariffah-Muzaimah, 2021; Shariffah-Muzaimah *et al.*, 2020). All of the seedlings used in this study were maintained in a nursery with standard practices as described by Esnan *et al.* (2001).

The confirmation of *Ganoderma* infection was done by referring to Rees *et al.* (2009) with modification where surface-sterilised root tissue was placed on *Ganoderma* Selective Media (GSM). The sterilisation of the root was carried out using 1% v/v sodium hypochlorite (commercial household, Clorox®).

TABLE 1. DESCRIPTION OF TREATMENT USED IN THE EXPERIMENT

Treatment	Description	
	Streptomyces GanoSA1 treatment	*Ganoderma* artificial inoculation
T1	-	-
T2	-	+
T3	+	-
T4	+	+

Determination of Pathogenesis-related Protein Activity

Pathogenesis-related proteins were determined at days 0, 3, 7, 14, 28 and 60 after the seedlings were infected with *G. boninense* PER71. For each treatment, four seedlings were sampled from each replication separately. Root samples were prepared according to Salla *et al.* (2016) and Xue *et al.* (2016). The roots were washed in running tap water to remove the soil particles, dried, and ground in liquid nitrogen using a mixer mill (MM400, Retsch, Germany) and homogenised with 1 mL (1:2, w/v) of 100 mM pH 6.5 sodium phosphate buffer. The homogenate was then centrifuged at 16 000 xg for 20 min at 4°C. The supernatant obtained represents the crude enzyme used in all enzymatic activity tested in this study except for PAL. Assay for the estimation of β-1,3-glucanase, chitinase, POX, and PAL was done by referring to Ramamoorthy *et al.* (2002), while estimation of PPO was done as described in Tuncay and Yagar (2011). The spectrophotometric readings were done by using a UV–VIS Spectrophotometer (Genesys 10S UV-VIS, USA).

β-1,3-Glucanase activity. The laminarin-dinitrosalicylic acid method was used to estimate the activity of β-1,3-glucanase in this study. The reaction mixture consisted of a 1:1 ratio of 4% laminarin and crude enzyme with a total volume of 125 µL. After 10 min of incubation at 40°C, the reaction was stopped by adding 375 µL of dinitrosalicylic acid, followed by a 5 min heating on boiling water. The reaction solution was then vortexed before transferring into a plastic cuvette. The absorbance was measured at 500 nm and enzyme activity was expressed as µg glucose min^{-1} mg^{-1} of fresh weight.

Polyphenol oxidase (PPO). The PPO activity was estimated by referring to Tuncay and Yagar (2011). A total of 1.15 mL of phosphate buffer (50 mM, pH 7) was mixed with 0.30 mL of 10 mM pyrogallol and maintained in a water bath at 25°C. The reaction was initiated by adding 0.05 mL of the crude enzyme. Enzyme activity was measured by observing changes in absorbance at 420 nm for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg^{-1} of fresh weight.

Peroxidase (POX). The method for POX activity includes the addition of 0.25 mL of the crude enzyme into a substrate solution containing 0.75 mL of 0.05 M pyrogallol and 0.25 mL of 1% hydrogen peroxide (H_2O_2) and incubated at room temperature for 2 min. The absorbance was determined at 470 nm for three min at a 30 s interval and POX activity was expressed as changes in the absorbance min^{-1} mg^{-1} of fresh weight.

Phenylalanine lyase (PAL) activity. A mixture of ice-cold 0.1 M sodium borate buffer, pH 7, 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone was added to 1.0 g of the root sample and homogenised evenly by using a mixer mill (MM400, Retsch, Germany). The homogenate was filtered through a cheesecloth, and the filtrate obtained was centrifuged at 16 000 xg for 15 min. A 0.4 mL of the supernatant was then mixed with 0.5 mL of 0.1 M borate buffer, pH 8.8 and 0.5 mL of 12 mM of L-phenylalanine and incubated for 30 min at 30°C. The absorbance of the solution was measured at 290 nm. The amount of trans-cinnamic acid formed from the conversion of L-phenylalanine was calculated using the Beer-Lambert formula (molar absorptivity of 9630/M/cm). The enzyme activity was expressed as µmol of trans-cinnamic acid min^{-1} mg^{-1} of fresh weight.

Statistical Analysis

All experiments were carried out in triplicate. The results are presented as mean ± standard deviation (SD). The data were analysed by analysis of variance (ANOVA) using SAS® software version 15 for Windows. Analysis of the differences was conducted using the least significant difference (LSD) test, and a $p<0.05$ was considered to demonstrate a significant difference.

RESULTS AND DISCUSSION

Confirmation of *Ganoderma boninense* PER71 on Roots Sample

The ability of *Streptomyces* spp. to promote plant growth had previously been reported in several plants such as chilli pepper, chickpea, banana, rice, cucumber, tomato and sunflower (El-Tarabily, 2008; El-Tarabily *et al.*, 2009; Gopalakrishnan *et al.*, 2011; Goudjal *et al.*, 2014; Patil *et al.*, 2011; Pornsuriya and Sunpapao, 2014; Thakker *et al.*, 2013). The results on the effect of *Streptomyces* GanoSA1 on oil palm growth have been reported and elaborated by Idris and Shariffah-Muzaimah (2021) and Shariffah-Muzaimah *et al.* (2020). Based on our observation during sampling, lesions on the root tissues can be seen on the roots of seedlings inoculated with *G. boninense* (*Figure 1a*), especially in the area near the inoculum source. The presence of *Ganoderma* was confirmed on GSM (*Figure 1b*). A white fluffy colony with a brown pigmented halo was observed on GSM, indicating the appearance of the basidiomycete. The same observable colony was also reported by Naidu *et al*. (2018) from the cross-sections of the infected tissues indicating the possible appearance of the pathogenic *G. boninense*. Based on the observations during harvest, seedling height, and root number on T3 treatment were higher compared to other treatments (*Figure 1c*).

Effect of *Streptomyces* GanoSA1 on Pathogenesis-related Proteins of Oil Palm with or without *Ganoderma boninense* PER71

In our previous study, *Streptomyces* GanoSA1 demonstrated its potential to lower the disease incidence caused by *G. boninense* in oil palms under greenhouse and field conditions (Idris and Shariffah-Muzaimah, 2021; Shariffah-Muzaimah *et al.*, 2020). Previous works on *Streptomyces* GanoSA1 have focused more on the strain's effectiveness under laboratory, nursery, and field studies with limited work focusing on the mechanism. Therefore, this current study explored the role of the strain in triggering induced systemic resistance in oil palm. To assess the induction of resistance in oil palm seedlings, we examined the production of plant defence-related enzymes in seedlings treated with different treatments at different time intervals. The results demonstrated that *Streptomyces* sp. GanoSA1 enhanced the production of these enzyme activities in oil palm roots with or without *G. boninense* PER71.

The results showed a significant difference in the activities of β-1,3-glucanase between all the treatments used in this study (*Figure* 2). On days 3, 7 and 14 days after treatment, glucanase activities were higher in seedlings treated with *Streptomyces* (T3 and T4) compared to the untreated (T1). The independent presence of *Ganoderma* enhanced the production of glucanase at the highest point up to 1.94-3.20 folds higher than the other treatments at seven days after inoculation (DAI). However, the activity was observed as a short-lasting response as the production tended to decline at 14, 28 and 60 DAI and reflected a downward trend toward the end of the experiment. Meanwhile, the presence of *G. boninense* PER71 in *Streptomyces*-treated seedlings (T4) had the highest activity of glucanase. In the treatment, the optimum activity reached 3.800 ± 0.01 μmol min^{-1} mg^{-1} fresh weight at 60 DAI, which was significantly higher compared to *Streptomyces* GanoSA1, *G. boninense* PER71 and control treatment with 2.758 ± 0.96, 0.5648 ± 1.15 and 1.252 ± 0.46 μmol min^{-1} mg^{-1} fresh weight, respectively. An increased amount of glucanase was reported as one of the main responses by the plant due to infection and pathogenesis by pathogens, which frequently occur before the visible symptoms are observable (Sahebi *et al.*, 2018). Treatment with biological control agents not only induced higher glucanase production in plants but also contributes to the production of the enzymes itself as one of the

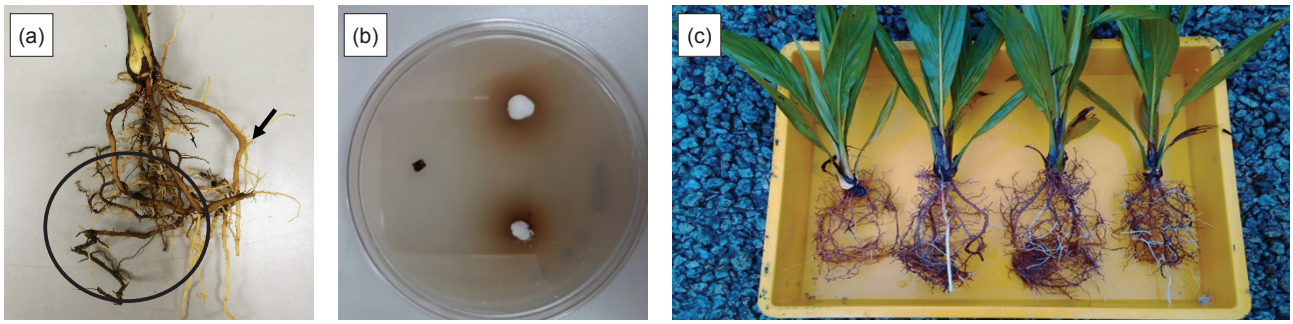

Figure 1. Effect of Streptomyces nigrogriseolus *GanoSA1 treatment and* Ganoderma boninense *PER71 inoculation on oil palm seedlings, (a) Oil palm seedlings root showing lesion (circle) and healthy (arrow) symptoms, and (b)* Ganoderma *Selective Medium (GSM) added with root pieces from T2 and T4 treatment, and (c) seedlings observed during harvesting at 14 DAI (From left to right, T1, T2, T3 and T4 where, T1 = untreated and uninoculated seedling (control), T2 = seedling inoculated with* G. boninense *PER71, T3 = seedling treated with* S. nigrogriseolus *GanoSA1 and T4 =seedling inoculated with* G. boninense *PER71 and treated with* S. nigrogriseolus *GanoSA1).*

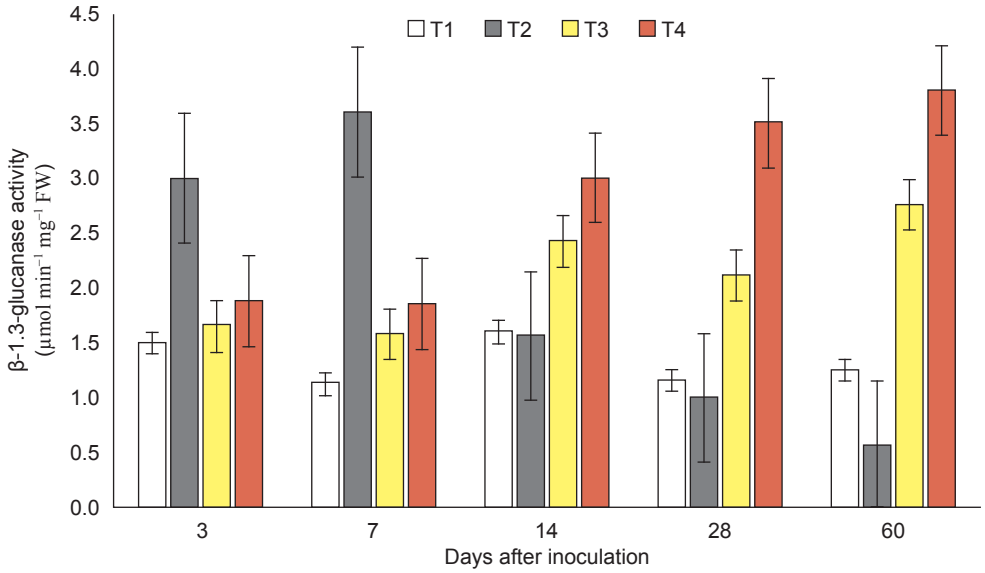

Figure 2. Induction of β-1,3-glucanase activity by Streptomyces nigrogriseolus *GanoSA1 in oil palm roots challenged with or without the* G. boninense *PER71. [T1 = untreated and uninoculated seedling (control), T2 = seedling inoculated with* G. boninense *PER71, T3 = seedling treated with* S. nigrogriseolus *GanoSA1 and T4 = seedling inoculated with* G. boninense *PER71 and treated with* S. nigrogriseolus *GanoSA1]. Vertical bars indicate standard deviations of four replications.*

important mechanisms during the pathogen control activities (Meena *et al.*, 2017). The POX activity in seedlings inoculated with *G. boninense* PER71 either treated with or without *Streptomyces* GanoSA1 was higher compared to seedlings with *Streptomyces* GanoSA1 only and control treatment. Significance differences in POX activity in all treatments were observed from day 14 onwards (*Figure 3a*). Based on the observation, the activities were enhanced when the pathogen was present. The POX activity reached a maximum level of 1.955 ± 2.15 and 2.143 ± 1.56 μmol min^{-1} mg^{-1} of fresh weight, in T3 and T4 treatments, respectively at 60 DAI, which was 3.6-4.0 folds higher compared to those acquired in the seedlings untreated with *Streptomyces* GanoSA1 and seedlings uninoculated with *G. boninense* PER71.

Based on the present study, PPO activity in all seedlings showed higher activity compared to PPO activity at control seedlings showing the lowest activity of all tested time points ranging from 0.02-0.08 μmol min^{-1} mg^{-1} of fresh weight (*Figure 3b*). At 3, 7 and 14 DAI, seedlings inoculated with *G. boninense* PER71 with or without *Streptomyces* GanoSA1 exhibited increased PPO activity in comparison to other treatments. PPO activity in seedlings treated with *Streptomyces* GanoSA1 with the inoculation of the pathogen continues to be expressed higher in 7, 14, 28 and 60 DAI than in the other three treatments (*Figure 3b*). Dias *et al.* (2017) reported a similar response on tomato plants inoculated with *Streptomyces* sp. The increase of both enzymes at early and later points in time, respectively in the evaluation period is related to the defence response. The PPO response in a plant may be associated with the production of toxic phenol molecules; meanwhile, the POX activity might be responsible for the oxidation of phenols for strengthening the cell walls to act as a barrier in constraining the spread of the pathogen (Dias *et al.*, 2017). Remarkably, *Streptomyces* GanoSA1 induced stronger POX and PPO production, especially with the presence of the *G. boninense* PER71. PPO activity in our study demonstrated little variation in the *Streptomyces* GanoSA1-treated seedlings, especially in the early DAI. This phenomenon may be related to the recognition of non-harmful plant-microbe interaction by oil palm seedlings which leads to low expression, as observed in our results. *Ganoderma boninense* PER71 inoculation caused an obvious rise in PAL activity 7 DAI compared with the other treatments; however, the activity was reduced thereafter (*Figure 3c*). Pre-treatment with *Streptomyces* GanoSA1 modulated the response of PAL. Seedlings treated with *Streptomyces* GanoSA1 alone showed higher PAL activity compared to treatments of control and *G. boninense* alone at 14 DAI onwards, but activity was not as much as in *G. boninense* PER71-inoculated seedlings. The amount of PAL in seedlings with the presence of the pathogen was 3.09-fold and 1.60-fold higher than those obtained in the control group, *G. boninense* PER71 only, and *Streptomyces* GanoSA1 only, respectively (*Figure 3c*).

Plants have a wide range of defence responses that can be activated in response to attacks by a pathogen (Agrios, 2005). The usage of beneficial microbes has been reported as one of the efficient biotechnological tools to stimulate plant metabolism and reduce the risk of phytopathogen infection

(Bonanomi *et al.*, 2018; Cappellari *et al.*, 2017; Meena *et al.*, 2017; Russo *et al.*, 2012; Siddiqui *et al.*, 2015). It is known from the literature that the bacterial genera of *Pseudomonas*, *Bacillus*, *Azospirillum* and *Streptomyces* are some of the common genera which have been reported with promising PGPR activity. Additionally, a countless number of actinomycetes from the genus of *Streptomyces* have been reported to contribute to plant resistance against various phytopathogens (Alekhya and Gopalakrishnan, 2014; Gopalakrishnan *et al.*, 2015; Hata *et al.*, 2015; Jacob *et al.*, 2016; Kamal and Sharma, 2014; Lyu *et al.*, 2017; Manhas and Talwinder, 2016; Sangdee *et al.*, 2016; Srividya *et al.*, 2012), which possibly occur through a plant growth promotion activity (Lehr *et al.*, 2008; Schrey and Tarkka, 2008; Viaene *et al.*, 2016) and/or induction of defence-related enzymes (Dias *et al.*, 2017; Salla *et al.*, 2014; 2016; Shao *et al.*, 2018; Singh and Gaur, 2017; Xue *et al.*, 2016; Zhang *et al.*, 2016). This gram-positive filamentous genus is well-known for its potential in producing various secondary metabolites with diversified biological activities and functions (Olanrewaju and Babalola, 2019; Pacios-Michelena *et al.*, 2021).

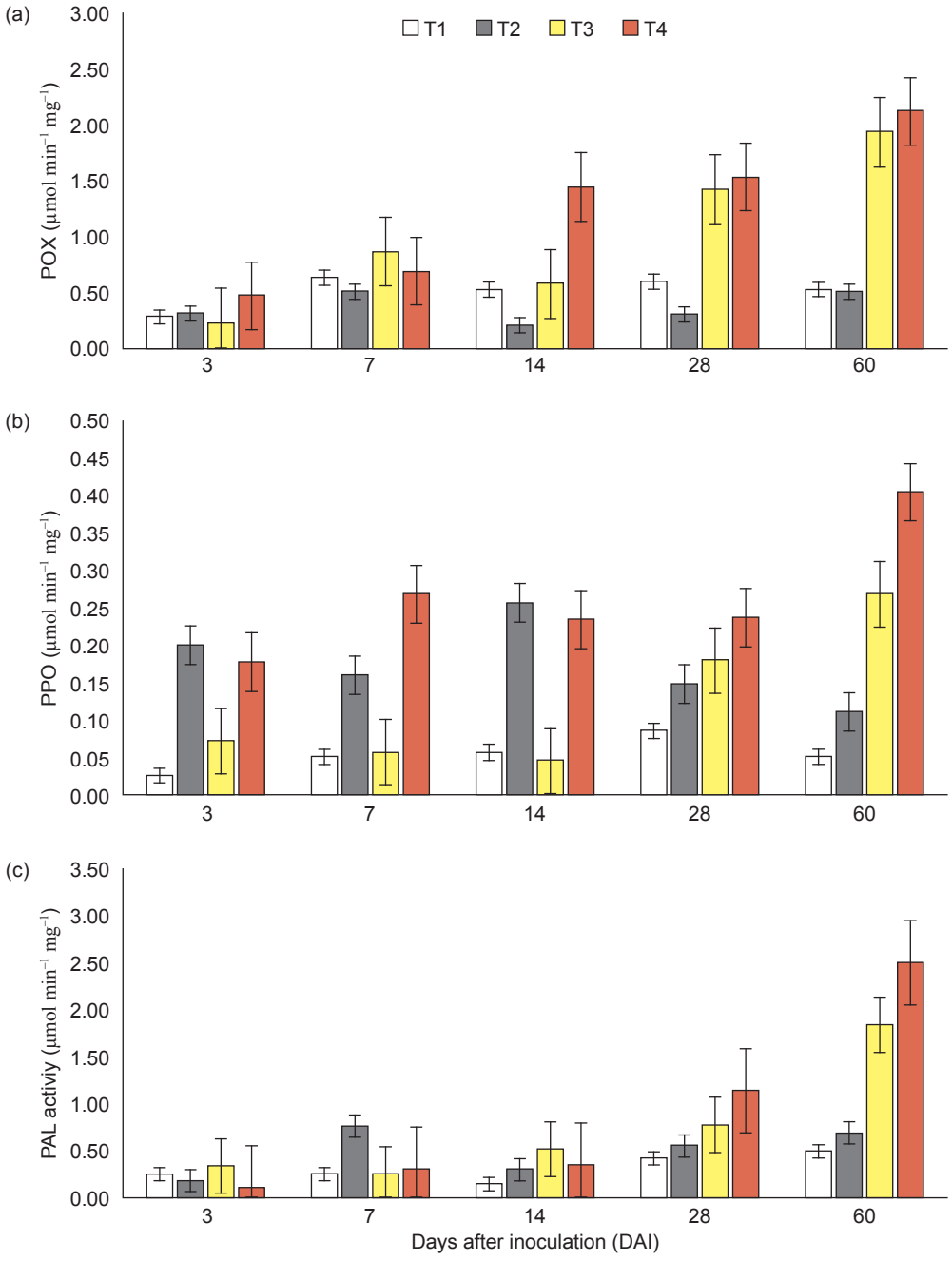

Figure 3. Changes in (a) peroxidase (POX), (b) polyphenol oxidase (PPO), and (c) phenylalanine lyase (PAL) activity induced by Streptomyces nigrogriseolus *GanoSA1 in oil palm roots challenged with or without the pathogen* G. boninense *PER71. Vertical bars indicate standard deviations of four replications.*

This report concurs with earlier works on the induced systemic resistance that usually results from the rhizosphere colonisation by PGPR, such as *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp., and *Streptomyces* spp. (Pieterse *et al.*, 2014; Zhang *et al.*, 2020). This study showcased the ability of *Streptomyces* GanoSA1 in enhancing the production of pathogenesis-related enzymes related to inducing systemic resistance in oil palm. These findings are supported by the observation of other researchers, who reported on increased activity of glucanase, POX, PPO and PAL when plants were treated with *Streptomyces* sp. (Dias *et al.*, 2017; Salla *et al.*, 2016; Singh and Gaur, 2017; Zhang *et al.*, 2016), *Streptomyces* bioactive compounds (Zhang *et al.*, 2020), *Trichoderma* strain (Musa, 2017; Paudzai *et al.*, 2019) and *Pseudomonas fluorescens* (Ramamoorthy *et al.*, 2002; Saikia *et al.*, 2004).

CONCLUSION

This study demonstrated the potential role of plant defence enzymes in developing systemic disease resistance through *Streptomyces* GanoSA1 against BSR disease in oil palm. The potential *in vivo* antagonism of the strain, along with reducing the incidence of the *Ganoderma* disease and the modulation of defence-related enzymes, PPO, POX and PAL, highlighted the biological control traits of the strain. It indicates a possible rapid induction of enzymes involved in phenylpropanoid metabolism and PR proteins in response to treatment with biocontrol agents. To the best of our knowledge, this is the first report which demonstrate the effect of *Streptomyces* spp. to enhance the production of pathogenesis-related proteins in oil palm with or without *Ganoderma* infection. Further studies on a gene related to protein expression would strengthen the finding. Field evaluation on a large scale with a longer timeframe and impact on the productivity of oil palm would provide a novice finding on the strain's effectiveness under a natural environment for sustainable oil palm planting.

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