PERTANYAAN NOMOR 4

4.Komentar untuk karya penelitian : 'Judul Artikel: Induction of systemic resistance in cucumber by Hypovirulent Binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare, Penulis: Dr. Ir. A. Muslim, M.Agr., Nama Jurnal: Tropical Life Science Research, Volume Jurnal: 30, Nomor Jurnal: 1, Tahun Terbit Jurnal: Januari 2019, Halaman: 107-120, ISSN: 1985-3718, Penerbit: Universiti Sains Malaysia': Harap lampirkan bukti-bukti korespondensinya untuk kepastian dapat digunakan sebagai pemenuhan karil syarat khusus. Tks

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ACTION	STATUS	ID	TITLE	SUBMITTED	DECISIONED
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	EO: Not Assigned • Reject (09-Jul-2017) *Archiving completed on 07-Dec-2017* view decision letter	TLSR-OA-01-2017-0002	Induction of systemic resistance in cucumber by Hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare *Files Archived*	03-Jan-2017	09-Jul-2017

2. EMAIL JAWABAN SUBMISSION PERTAMA

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Tropical Life Sciences Research - Manuscript ID TLSR-OA-01-2017-0002

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Tue, Jan 3, 2017 at 6:06 PM

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03-Jan-2017

Dear Dr. Muslim:

Your manuscript entitled "Induction of systemic resistance in cucumber by Hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare" has been successfully submitted online and is presently being given full consideration for publication in the Tropical Life Sciences Research.

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2 messages

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Reply-To: TLSR@usm.my
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09-Jul-2017

Dear Dr. Muslim:

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TLSR-OA-01-2017-0002-Induction-of-systemic-

Induction of systemic resistance in cucumber by hypovirulent binucleate *Rhizoctonia* against anthracnose caused by *Colletotrichum orbiculare*

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ABSTRACT

Treatment with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates induced systemic resistance against anthracnose infected by *Colletotrichum orbiculare* in cucumber. This is because of the different distances between HBNR and *C. orbiculare*, where the root was treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in leaves or first true leaves were treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in second true leaves. The use of barley grain inocula and culture filtrates of HBNR significantly reduced the sum of lesion diameter compared to the control ($p = 0.05$). The total lesion diameter reduction by applying barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results was also observed in treatment using culture filtrate, and the reduction of total lesion diameter by culture filtrate of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively. If cucumber root was treated with culture filtrates of HBNR, the lignin was enhanced at the pathogen penetration, which is spread along the epidermis tissue of cucumber hypocotyls. Peroxidase activity in hypocotyls in the treated cucumber plant with culture filtrates of HBNR significantly increased before and after inoculation of pathogens as compared to the control. Significant enhancement was also observed in the fast-moving anodic peroxidase isozymes in the treated plants with culture filtrates of HBNR. The results showed the elicitor(s) contained in culture filtrates in HBNR. The lignin deposition as well as the peroxidase activity is an important step to prevent systemically immunized plants from pathogen infection.

Keywords: hypovirulent binucleate *Rhizoctonia* (HBNR), induced systemic resistance, *Colletotrichum orbiculare*, cucumber

INTRODUCTION

As soon as a plant is appropriately stimulated, its resistance is intensified against a test inoculation against a pathogen. This is the phenomenon of induced resistance. It can be localized, systematic, and induced with limited infection of virulent or hypovirulent pathogens, specific non-pathogenic bacteria, cell wall fragments, plant extracts, and certain chemicals (Van Loon 2000; Walters 2010).

Concerns about impacts of agrichemicals on food safety and the environment are related to the danger of the synthetic pesticide utilization, leading plant pathologists to develop another save control for managing plant disease (Hyakumachi *et al.* 2014). Elicitors of host resistance are a potential alternative control to plant diseases (Lyon *et al.* 1995).

Several investigations have reported that cucumber anthracnose caused by *Colletotrichum orbiculare* could be effectively control by Endophytic *Streptomyce*s (Shimizu *et al.* 2009), Rhizobacteria, *Bacillus pumilus*, *Bacillus subtilis*, and *Curtobacterium flaccumfaciens* (Raupach & Kloepper 2000). Some studies show induced systemic resistance in cucumbers against antrachnose using biotic and abiotic elicitor. Meera *et al.* (1994) demontrated that sterilized fungus and *Phoma* sp. were reliable in activating the systemic resistance of cucumber against the antracnoses disease. Koike *et al.* (2001) demonstrated that fungi isolated from Zoysiagrass Rhizosphere (*Penicillium*, *Trichoderma*, *Phoma, Fusarium*, and a sterile fungus) significantly induced systemic resistance against cucumber anthracnoses. This is done through lignification enhancement and superoxide generation. Tian *et al.* (2008) reported that application of *Pieris rapae* extract onto the first true cucumber leaves effectively brought about systemic resistance against cucumber anthracnose with the enhancement of peroxidase and polyphenoloxidase. Lin *et al.* (2014) demonstrated that protein lysis buffer and a nonionic detergent agent applied to separate cell membrane complexes (Nonidet P-40) is effective to weaken cucumber anthracnose, which is influences the escalation of gene levels. This means that it is related to disease resistance (peroxidase and pathogenis associated with protein 1-1a, acidic class III chitinase, phenylalanine ammonialyase 1). Research on hypovirulent binucleate *Rhizoctonia* (HBNR) as a potential biocontrol agent against *Fusarium* diseases in tomatoes and spinach have been recently reported in our investigations with a mechanism that might be induced resistance (Muslim *et al.* 2003a, b, c). There has also been an investigation of HBNR as an agent of induced systemic resistance (ISR) on beans against *Rhizoctonia solani* or *C. lindermuthianum* (Xue *et al.* 1998); they also protected cotton against alternaria leaf spot and rhizoctonia damping-off with ISR (Jabaji-Hare & Neate 2005). However, until now, there has been no report of the use of HBNR as an agent of ISR on cucumber against anthracnose pathogen *C. orbiculare* (= *C. lagenarium*).

In general, ISR in plants is clearly defined as a set of induced defense responses, including the creation of cell wall lytic enzymes. For example, 1,3-β-glucanases and chitinases (Lowton & Lamb 1987) enhance the activities

of peroxidase and lignin deposition, callose, hydroxyproline-rich glycoprotein (Hammerschmidt & Kuc 1982; Hammerschmidt *et al.* 1982; Hammerschmidt *et al.* 1984), and phytoalexins (Ebel 1986),

This study aims to investigate HBNR capacity in inducing systemic resistance in cucumber against cucumber anthracnose. The study was designed to reveal if induced resistance in cucumber is correlated with enhanced systemic lignification and peroxidase activity.

MATERIALS AND METHODS

Isolates

Hypovirulent binucleate *Rhizoctonia* isolate of W1, W7 (AG-A), L1 (AG-Ba), and Rhv7 (unknown anastomosis group) obtained from soil samples were used as biocontrol agents. The pathogens used in this study were *Colletotrichum orbiculare* (Berk & Mont.) Arx (=*Colletotrichum lagenarium* (Pass.) Ellis & Halst.) isolate 104T, which were obtained from infected cucumber plants.

Plants

Throughout the experiment, cucumber cv. Gibai was used. Before the sowing, seeds were sterilized with 70% ethyl alcohol for one minute, and 1% of NaOCl for 20 minutes. Finally, they were rinsed in sterilized distilled water three times.

Inoculum preparation

Isolates of pathogen *C. orbiculare* were cultured on potato dextrose agar (PDA) as long as seven days without exposure to light. The temperature was maintained at 25°C. A sterilized glass bar from the cultures with added sterile water, and scraped the spore suspensions. The spore suspension was then filtered through eight layers of sterile gauze. The isolates were set as two inoculum forms: barley grain inoculum and culture filtrate.

The following procedure was used for preparation of barley grain inoculum: Each isolate was cultured in PDA for three days without light and at room temperature. Five 5 mm mycelial disks of the culture were applied to 100 grams of moist autoclaved barley grains (1:1, w/v dry barley grains/distilled water) collected in a 500 ml Erlenmeyer flask. The cultures were maintained and regularly shaken for 10 days at 25°C to produce well-colonized inoculum with HBNR. The inoculum was naturally dried for around 10 days. They were then kept refrigerated at 4°C until use.

The following procedure was used for the culture filtrate (CF): Two mycelial disks of each HBNR isolate obtained from the culture growing on PDA were put into a 20 ml flask with 50 ml of potato dextrose

broth (pH 6.5). The isolates were grown in static conditions at 23-25°C for 10 days without light. The CF separated from the mycelia. Next, the CF was filtered three times over three layers of Whatman filter paper number 2. The CF was also filtered and sterilized using millipore filtration (0.45 µm Millipore filters, Millipore Products Division, Bedford, USA).

Cucumber ISR assays

Experiments with barley grain inocula

Each sterilized plastic pot, sized ø6 cm x 7.5 cm, was filled with the colonized barley grain inocula mixture (2%, w/w) with as much as 120 grams of potting medium. The previously-sterilized (with 0.5% NaOCl) cucumber seeds were added to the mixture. Each pot was given one seed. Next, the plants were cultivated at 25ºC. This required 21 days in a growth chamber with a 14 h light (24,000 lux) per dark period. The plants that were grown in the potting medium with untreated barley grain inocula were used as a control. In this experiment, there were two replicates for each treatment. Each replicate had three plants. The experiment was repeated twice.

Experiment with culture filtrates (CF)

The plastic pots (autoclavable, ø6 cm x 7.5 cm) containing about 120 grams of potting medium were heated in autoclaves. The surface-sterilized cucumber seeds were shown in each pot. The plants were maintained in a similar manner as previously described. The first true leaves of 21-day-old cucumber plants were soaked with CF for one minute. The plants were inoculated after 24 h of incubation. One treatment comprised two replications, with three plants per replication. The experiment was repeated twice.

Challenge inoculation

The second true leaves were inoculated with 20 spore suspension drops of *C. orbiculare* (5×10^5 spores/ml). The disk of lens paper (ø 5 mm) was covered on every drop toward the run-off prevention. This was done to ensure the distribution of identical spores along the leaf surfaces. The inoculated plants were maintained for 48 h without light at 25ºC in a humid chamber (85%-90% RH). After that, for six days the inoculated plants were brought to the growth chamber. After that, all diameters and areas of *C. orbiculare* lesions on every leaf were measured.

Testing for lignin formation

The cucumber seeds were grown on damp sterilized filter paper. Next, they were incubated for a week without light at 25ºC. The roots of the seedlings were put in 5.0 ml of CF and incubated for one day. Then, with 10 µl drops of spore suspension (5×10^5 spores/ml) of *C. orbiculare*, the hypocotyls of the treated seedlings were inoculated.

Next, the inoculated seedlings were incubated for 20 h. The epidermal strips of the seedling hypocotyls were stained with toluidine blue O or phloroglucinol-HCL (Sherwood & Vance 1976). They were observed under the microscope to reveal percentage of lignification.

Protein extraction and determination

Treated cucumber root seedlings with CF of HBNR and challenge inoculated with *C. orbiculare* were prepared as described previously in *Testing for lignin formation*. Samples were collected from seedlings prior to the time of challenge inoculation and again 8-48 h after the challenge inoculation. All samples were immediately frozen at -80^{o}C until peroxidase assays were performed. Only the hypocotyls of the cucumber seedlings were used for protein extraction. These samples were homogenized in 5 ml of 0.05 M sodium phosphate buffer at pH 6.0 per 1 g sample with a cold mortar and pestle. The extract was centrifuged at 10,000 rpm for 10 minutes at 4^{o}C, and the supernatant was used to analyze the peroxidase activity. To identify the protein contents of these extracts, the Lowry method (Lowry *et al.* 1951) was used with bovine serum albumin as the standard.

Assay for peroxidase activity

Peroxidase activities were assessed following the method of Dalisay & Kuc (1995). They were determined using guaiacol, which acted as the hydrogen donor. The reaction mixture (3 ml) contained 0.25% (v/v) guaiacol in 1 mM sodium phosphate buffer at pH 6.0 with 100 mM hydrogen peroxidase. In order to catalyze the reaction, one tent ml crude enzyme extract was added and continued with colorimetrically at 470 nm min^{-1} mg^{-1} protein.

Detection of peroxidase isozymes by gel electrophoresis

Native PAGE was done with Phastsystem (Pharmacia LKB, UK). Extracts were adjusted to the same protein concentration with phosphate buffer and then loaded onto an 8-25% gradient gel. A peroxidase isoenzyme was made visible by immersing the extracts in gels of 1% 0-dianisidine solution. After 10 minutes, the gels were cleaned with distilled water. They were then placed into 0.06% H_2O_2 solution to concretely show the peroxidase isoenzyme bands.

Data analysis

The experiments in this study were designed in completely randomized designs. Total lesion numbers, anthracnose lesion diameters, and lignin formation in this study were compared using Fisher's least significant difference (LSD) test at $P = 0.05$ and $P = 0.01$.

RESULTS AND DISCUSSION

RESULTS

ISR in cucumber against anthracnose with HBNR

This study showed that the use of barley grain inoculum and CF of HBNR isolates significantly ($P = 0.05$) decreased total lesion diameter compared to the control (Table 1). However, no significant reduction was observed in total lesion number (Table 1). The reduction of total lesion diameter by barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results were also observed in the treatment with CF; the reduction of total lesion diameter by CF of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively (Table 1).

Lignin formation and peroxidase activities in cucumber hypocotyls treated with HBNR

Lignin formation was observed as the intense blue and green colors of the lignified cell walls. Cucumber hypocotyls pretreated with CF of HBNR L2, W1, W7, and Rhv7. This more significantly increased lignin deposition in places that had been infected by *C. orbiculare* compared to the control treatment (Fig. 1). Cucumber seedlings treated with CF of HBNR L2, W1, W7, and Rhv7 increased lignin deposition by 59.2%, 63.2%, 51.1%, and 64.4%, respectively.

Peroxidase activities in cucumber hypocotyls sampled at varying times before and after challenge inoculation were higher in the plant treated with HBNR compared to the control (Fig. 2). Treatment with HBNR L2, W1, W7, and Rhv7 increased peroxidase activities by 29%, 41%, 36%, and 45%, respectively, before inoculation of *C. orbiculare*, and by 28-39%, 25-43%, 30-37%, and 19-48%, respectively, after inoculation of *C. orbiculare*.

Two peroxidase isoenzymes (isoforms 1 to 2) were found in cucumber hypocotyls. The fast-moving anodic peroxidase isozymes were enhanced gradually after challenge inoculation. The peroxidase activities increased in the isoform 2 in the seedlings treated with HBNR compared to the control, at all sampling times, according to band intensity and width (Fig. 3). Interestingly, although isozyme 1 was observed after 48 h of pathogen inoculation, it was not recorded at 24 h of pathogen inoculation in the control treatment. However, it was recorded at the sampling times 24 h and 48 h after pathogen inoculation in the seedlings treated with HBNR W1.

DISCUSSION

This study reveals that treatment with HBNR isolates suppresses disease development of anthracnose in cucumber. The disease development suppression seemingly resulted from plant's ISR, as different distances between HBNR and *C. orbiculare,* where the root was employed with HBNR isolates, and *C. orbiculare* was inoculated on the leaves, or the first true leaves were treated with HBNR isolates and *C. orbiculare* was challenge inoculated on the second true leaves. Thus, HBNR and pathogen application sites were separated spatially, and no HBNR isolates could

be recovered from the second true leaves. The result of this research extends the hypothesis that the mechanism of protection from *R. solani* by HBNR is induced resistance (Cardoso & Echandi, 1987; Poromarto *et al.* 1998).

A report presented by Xue *et al.* (1998) showed that inoculation of bean hypocotyls with HBNR induced systemic resistance and protection of the roots and cotyledon to later challenges not only with the root rot pathogen *R. solani* but also with the anthracnose pathogen *C. lindemuthianum*. This study applied HBNR as barley grain inoculum, and CF induced systemic resistance in cucumber plants against *C. orbiculare*. Similar methods were used by Meera *et al.* (1994) and Koike *et al.* (2001), in which plant-growth-promoting fungi (PGPF) were applied at the root as barley grain inoculum, mycelia inoculum, or culture filtrates. This induced systemic resistance in cucumber after being challenged with *C. orbiculare* in leaves. Another study reported that germinating tomato seeds for one week in chemicals of b-aminobutyric acid (BABA) and jasmonic acid (JA) solutions promoted seed germination efficiency and induced resistance in four-week-old plants (Luna *et al.* 2016).

In this study, when HBNR CF was applied at the cucumber roots, lignin was enhanced at the attempted penetration by the pathogen in the epidermal tissues of cucumber hypocotyls. Our results also show that peroxidase activity in hypocotyls in the treated cucumber plant with HBNR significantly increased before and after inoculation of the pathogen compared to the control. Significant enhancements were also observed in the fast-moving anodic peroxidase isozymes in the plants treated with HBNR. This supports the finding by Xue *et al.* (1998) that inoculation of bean hypocotyls with HBNR induced systemic resistance, and this was positively correlated with peroxidases and 1,3-β-glucanases activity. Arora & Bajaj (1985) and Krstic *et al.* (1997) also reported that infection of mung bean and strawberry with binucleate *Rhizoctonia* resulted in an increase in peroxidase activity. Another benificial microbia using rhizobacteria also demonstrated similar results. Garcia-Cristobal *et al.* (2015) demonstrated that the plant-growth-promoting rhizobacteria (PGPR) induced resistance in young rice plants against *Xanthomonas campestris* infection through induced oxidative stress (ascorbate peroxidase), glutathione reductase, chitinase, and ß-1,3-Glucanase after pathogen inoculation.

Chandrasekaran & Chun (2016) demonstrated that treating tomato plants with *Bacillus subtilis* CBR05 significantly enhanced the number of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and polyphenol oxidase) activities. Yanti (2015) reported that rhizobacteria enhanced peroxidase enzyme activity. The isolate PK2Rp3 (*Serratia marcescens* strain N2.4) showed the highest activity of both roots and leaves of 0.058 μg · mL^{-1} and 0.053 μg · mL^{-1}. In another experiment, Peng *et al.* (2004) found that pretreated cucumber seedlings with pectinase extract derived from *Penicillium oxalicum* BZH-2002 fermentation products resulted in induced resistance toward cucumber scab *Cladosporium cucumerinum* through the increased defense-related enzymes, polyphenol oxidasen, and peroxidase.

According to Dean & Kuc (1987) and Hammerschmidt *et al.* (1984), lignin deposition was considered a crucial phase of pathogen suppression in systemically immunized plants. Vance *et al.* (1980) reported that rapid lignin deposition might lead to the production of chemical or physical barriers to pathogen infection. Furthermore, peroxidases accelerate the ending polymerization step of lignin synthesis, resulting in the enhanced capability of protected tissue (Gross, 1979). In the other studies, (Hammerschmidt & Kuc, 1982; Ride, 1975) reported that the enhanced peroxidase activities are often related to resistance phenomenon such as the production of lignin. Peng and Kuc (1992) discovered the implications of peroxidase toward oxidative defense mechanisms in treated plants with infections. The peroxidase-generated hydrogen peroxidase directly functions as an antimicrobial agent. Further research is needed to identify other PR-proteins that may be involved in the mechanism of cucumber ISR from HBNR.

The bio-control abilities of HBNR isolates against *Fusarium* diseases in tomatoes and spinach (Muslim *et al.*, 2003a, b, c), as well as their ability to induce systemic resistance in cucumber against anthracnose, shows significant potential for their function as a bio-control agent to manage *Fusarium,* which has a very wide host, *Colletotrichum orbiculare,* and other diseases.

ACKNOWLEDGMENT

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Table 1: Effect of hypovirulent binucleate *Rhizoctonia* (HBNR) isolates on the total lesion number

and lesion diameter on leaves of cucumber plants that have been challenge inoculated with *Colletotrichum orbiculare*.

Treatments	Total lesion number[a]		Total lesion diameter (mm)[a]	
	BGI[b]	CF[c]	BGI	CF
Pathogen	19.5 a	16.8 a	123.5 b	89.3 b
HBNR L2	16.8 a	12.5 a	89.5 a	48.7 a
HBNR W1	16.0 a	13.1 a	68.7 a	48.1 a
HBNR W7	17.2 a	12.9 a	75.5 a	51.8 a
HBNR Rhv7	16.8 a	11.7 a	74.5 a	46.5 a

[a] Mean of two trials each with two replicates, with three plants per replicate. Values followed by the same letter do not differ significantly (P = 0.05) according to Fisher's least significant difference test.

[b] Plants were grown in potting medium amended with barley grain inoculum (BGI) of HBNR isolates (1%, w/w) for 21 days and challenge inoculated with 10 µl drops of 5 x 10^5 spores/ml of *C. orbiculare* at 20 locations on the second true leaves.

[c] The first true leaves of 21-day-old cucumber plants grown in potting medium were treated with culture filtrates (CF) of HBNR and challenge inoculated with *C. orbiculare* on the second true leaves as described above.

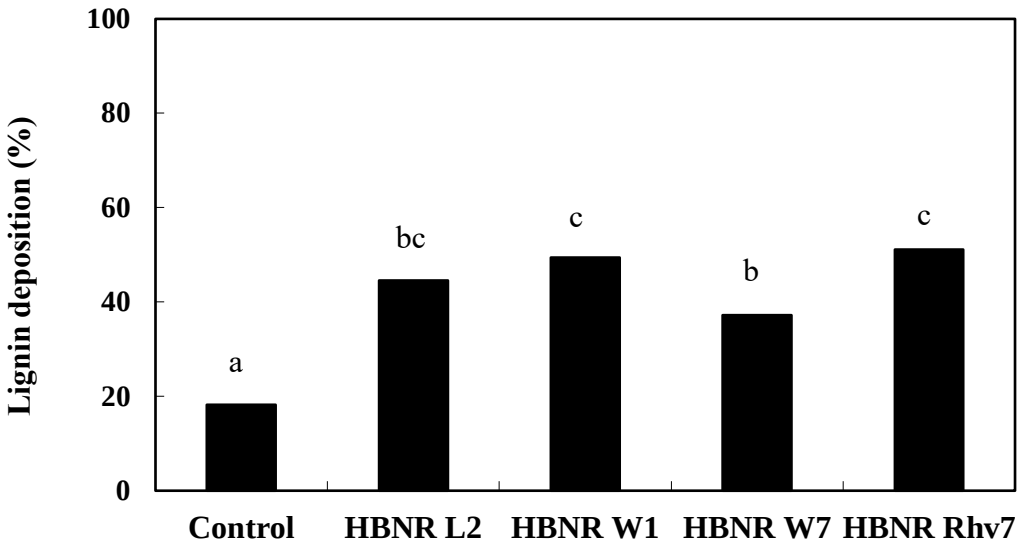

Fig. 1: Lignification of hypocotyls of cucumber seedlings induced by culture filtrates of hypovirulent binucleate *Rhizoctonia* (HBNR), following challenge inoculation with *Colletotrichum orbiculare*. The hypocotyls of treated plants were challenged with 5 µl drops of 10^5 spores/ml of *C. orbiculare* at 10 locations. Bars labeled with the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.01$).

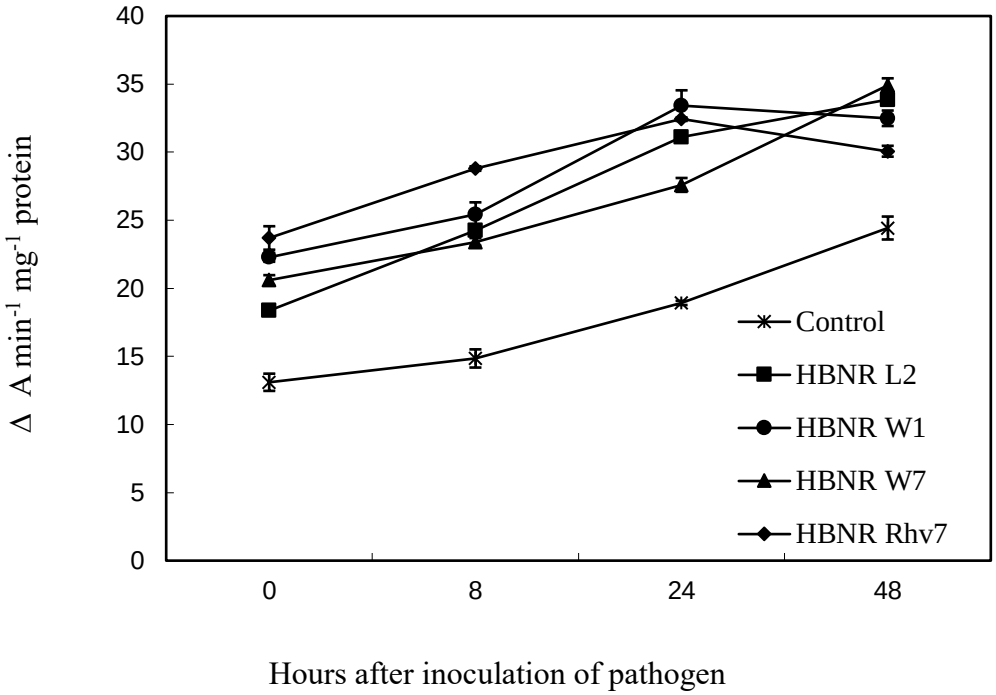

Fig. 2: Time course of peroxidase activity in hypocotyls of cucumber after treating the root with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates and challenge inoculating with *C. orbiculare*. Peroxidase activity is expressed as changes in absorbance min^{-1} mg^{-1} protein. Data are the mean of three replications with five seedlings (cucumber) per replication. Bars represent standard error of the mean. 0 h indicates time before pathogen inoculation.

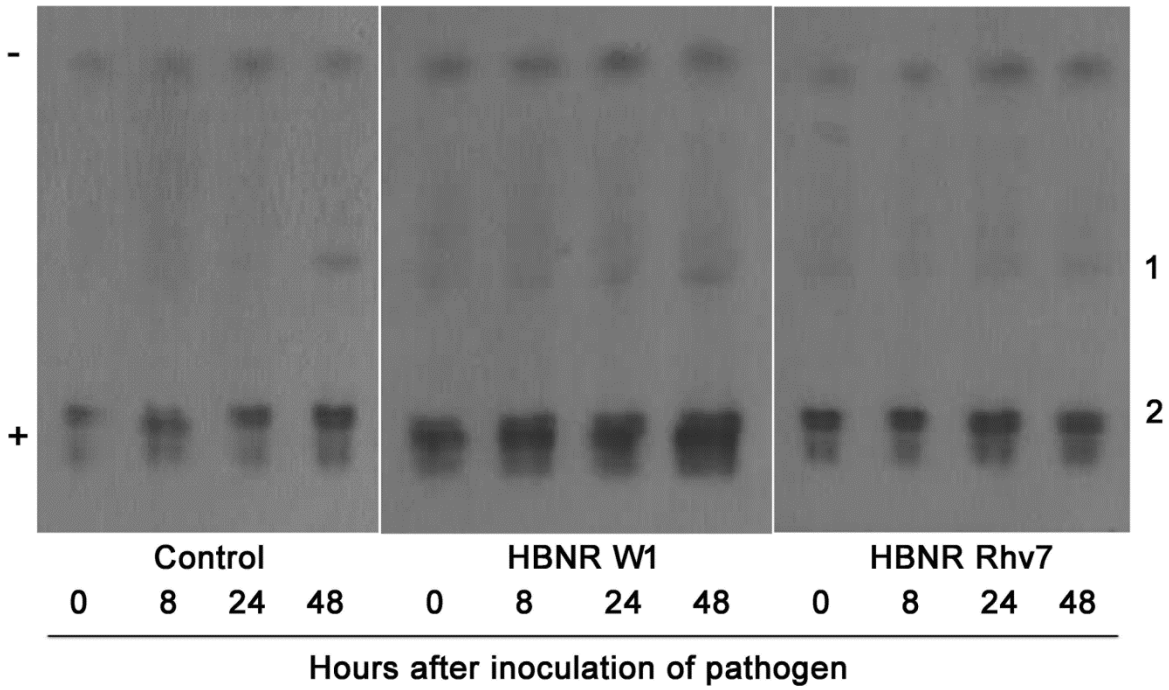

Fig. 3: Electrophoresis patterns of peroxidase isozyme cucumber seedlings treated with hypovirulent binucleate Rhizoctonia (HBNR) (Protein concentration was 0.1 mg/ml).

5. SURAT JAWABAN RE-SUBMISSION

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Tropical Life Sciences Research - Manuscript ID TLSR-OA-10-2017-0120

2 messages

Tropical Life Sciences Research
<onbehalfof+tlsr.usm+gmail.com@manuscriptcentral.com>

Sat, Oct 21, 2017 at 11:40 AM

Reply-To: tlsr.usm@gmail.com
To: a_muslim@unsri.ac.id, limpal2003@yahoo.com

21-Oct-2017

Dear Dr. Muslim:

Your manuscript entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare" has been successfully submitted online and is presently being given full consideration for publication in the Tropical Life Sciences Research.

Your manuscript ID is TLSR-OA-10-2017-0120.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at https://mc.manuscriptcentral.com/tlsr and edit your user information as appropriate.

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Thank you for submitting your manuscript to the Tropical Life Sciences Research.

Sincerely,
Tropical Life Sciences Research Editorial Office

---

a. muslim unsri <a_muslim@unsri.ac.id>
Tue, Oct 31, 2017 at 7:02 PM
To: tlsr.usm@gmail.com

Dear Tropical Life Science Risearch Editorial Office

Thank you very much for your reply our resubmit our paper/manuscript entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare" has been successfully submitted online and is presently being given full consideration for publication in the Tropical Life Sciences Research.

Sincerely

A. Muslim

[Quoted text hidden]

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Tropical Life Sciences Research - Decision on Manuscript ID TLSR-OA-10-2017-0120

4 messages

Tropical Life Sciences Research Sat, Mar 3, 2018
<onbehalfof@manuscriptcentral.com> at 10:52 AM
Reply-To: TLSR@usm.my
To: a_muslim@unsri.ac.id, limpal2003@yahoo.com

02-Mar-2018

Dear Dr. Muslim:

Manuscript ID TLSR-OA-10-2017-0120 entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare" which you submitted to the Tropical Life Sciences Research, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended MAJOR revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into https://mc.manuscriptcentral.com/tlsr and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text. Please prepare a separate MS Word document, listing all reviewer comments with author(s) rebuttal/explanation for each comment (kindly provide the page and paragraph numbers of any changes made corresponding to the reviewer comment).

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Because we are trying to facilitate timely publication of manuscripts submitted to the Tropical Life Sciences Research, your revised manuscript should be uploaded as soon as possible. If it is not possible for you to submit your revision in a reasonable amount of time, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Tropical Life Sciences Research and I look forward to receiving your revision. Final decision of your manuscript will depend on the quality and depth of
your revision.

Sincerely,
Prof. Alexander Chong
Editor-in-Chief, Tropical Life Sciences Research
TLSR@usm.my

Reviewer(s)' Comments to Author:
Reviewer: 1

Comments to the Corresponding Author

- My main concern of this manuscript is the study to determine/ to induce ISR by the host was based only on two experiments which were lignin deposition and peroxidase activity. Based on only two experiments, it is not sufficient to study induction of ISR by the host. Other

enzymes that play a role in defense mechanism against pathogens should be inlcuded in the study. For example, polyphenol oxidase, superoxide dismutase and phenyl alanine lyase.

- For lignin deposition using culture filtrate, it would be good to do chemiluminescence assay to determine the elicitor activity.

- In the challenge inoculation test, the disease assessment is not complete. How was the disease severity calculated? Is there any disease index used?

- Introduction : improve the introduction on induced systemic resistance as in the present introduction is very brief . The authors should explain the interaction between the plant and the pathogen that induce the resistance.

- The discussion need major revision as the authors did not really discuss the results but it is more like a review of ISR. They also mentioned about bacteria and othe enzymes involved as inducer of ISR.

- English language used needs improvement as there are many sentences that are not well-written.

- Other comments are indicated in the manuscript.

Reviewer: 2

Comments to the Corresponding Author
Page 1, Line 25: Using "if" in this sentence (If cucumber root was....) is not correct. Should use "when" instead.
Page 2, Line 20: Decapitalize letter E in "Endophytic".
Page 2, Line 28: Decapitalize letter R in "Rhizosphre"
Page 3, Line 14-20: What do you use for your control in this experiment?
Page 4, Line 29: Spelling error. The word "shown" should be "sown"?.
Page 4, Line 48: Repetitively using "after that". Improve the language in this paragraph.
Page 5, Line 4: How do you measure the percentage of lignification? No details on this matter. This is important for others to replicate the method.
Page 5, Line 4: Missing reference - (Sherwood & Vance 1976).
Page 5, Line 33: What is one tent ml?
Page 7, Line 44: "microbia" is an incorrect use of word. Should be microbes.
Page 8, Line 15: Fix the sentence, citation should be at the end of the sentence.
Page 8, Line 26 - 31: For your conclusion, which isolates of HBNR is the most effective? The conclusion should relate to your objectives.
Page 14, Figure 1, 2, 3: what is the control used for comparison?
Page 16, Figure 3: Explain the label: What is +, -, 1, 2 ? There are no discussion on this findings. In the result, you mention that the isozyme 1 is interesting because it was not observed after 24h. The author should discuss this findings in the discussion.

Missing references in the text:
3. Cardoso JE, Echandi E (1987).....
27. Poromarto SH, Nelson BD, Freeman TP (1998).....

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a. muslim unsri <a_muslim@unsri.ac.id>
To: TLSR@usm.my

Wed, Mar 7, 2018 at 12:32 PM

Dear Prof. Alexander Chong
Editor-in-Chief, Tropical Life Sciences Research

Thank u very much for your kindness to respond my paper. I have read the revision of our manuscript entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare.

I am doing the revision. When the revision is okay, I send it as soon as possible.

I hope our paper could be published in in your journal TLSR.

Thank u very much

Sincerely,
Ahmad Muslim, Ph.D

[Quoted text hidden]

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Wed, Apr 4, 2018 at 4:47 PM
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Boos ini forward surat dari chiief editornyo

Salam
A. Muslim

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7. ATTACHMENT PERBAIKAN PAPER DARI FEER REVIEWER

Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by *Colletotrichum orbiculare*

Journal:	*Tropical Life Sciences Research*
Manuscript ID	TLSR-OA-10-2017-0120
Manuscript Type:	Original Article
Keywords:	hypovirulent binucleate Rhizoctonia (HBNR), induced systemic resistance, Colletotrichum orbiculare, cucumber

Induction of systemic resistance in cucumber by hypovirulent binucleate *Rhizoctonia* against anthracnose caused by *Colletotrichum orbiculare*

ABSTRACT

Treatment with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates induced systemic resistance against anthracnose infected by *Colletotrichum orbiculare* in cucumber. This is because of the different distances between HBNR and *C. orbiculare*, where the root was treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in leaves or first true leaves were treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in second true leaves. The use of barley grain inocula and culture filtrates of HBNR significantly reduced the sum of lesion diameter compared to the control (p = 0.05). The total lesion diameter reduction by applying barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results was also observed in treatment using culture filtrate, and the reduction of total lesion diameter by culture filtrate of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively. If cucumber root was treated with culture filtrates of HBNR, the lignin was enhanced at the pathogen penetration, which is spread along the epidermis tissue of cucumber hypocotyls. Peroxidase activity in hypocotyls in the treated cucumber plant with culture filtrates of HBNR significantly increased before and after inoculation of pathogens as compared to the control. Significant enhancement was also observed in the fast-moving anodic peroxidase isozymes in the treated plants with culture filtrates of HBNR. The results showed the elicitor(s) contained in culture filtrates in HBNR. The lignin deposition as well as the peroxidase activity is an important step to prevent systemically immunized plants from pathogen infection.

Keywords: hypovirulent binucleate *Rhizoctonia* (HBNR), induced systemic resistance, *Colletotrichum orbiculare,* cucumber

INTRODUCTION

As soon as a plant is appropriately stimulated, its resistance is intensified against a test inoculation against a pathogen. This is the phenomenon of induced resistance. It can be localized, systematic, and induced with limited infection of virulent or hypovirulent pathogens, specific non-pathogenic bacteria, cell wall fragments, plant extracts, and certain chemicals (Van Loon 2000; Walters 2010).

Concerns about impacts of agrichemicals on food safety and the environment are related to the danger of the synthetic pesticide utilization, leading plant pathologists to develop another save control for managing plant disease (Hyakumachi *et al.* 2014). Elicitors of host resistance are a potential alternative control to plant diseases (Lyon *et al.* 1995).

Several investigations have reported that cucumber anthracnose caused by *Colletotrichum orbiculare* could be effectively control by Endophytic *Streptomyces* (Shimizu *et al.* 2009), Rhizobacteria, *Bacillus pumilus*, *Bacillus subtilis*, and *Curtobacterium flaccumfaciens* (Raupach & Kloepper 2000). Some studies show induced systemic resistance in cucumbers against antrachnose using biotic and abiotic elicitor. Meera *et al.* (1994) demontrated that sterilized fungus and *Phoma* sp. were reliable in activating the systemic resistance of cucumber against the antracnoses disease. Koike *et al.* (2001) demonstrated that fungi isolated from Zoysiagrass Rhizosphere (*Penicillium*, *Trichoderma*, *Phoma, Fusarium*, and a sterile fungus) significantly induced systemic resistance against cucumber anthracnoses. This is done through lignification enhancement and superoxide generation. Tian *et al.* (2008) reported that application of *Pieris rapae* extract onto the first true cucumber leaves effectively brought about systemic resistance against cucumber anthracnose with the enhancement of peroxidase and polyphenoloxidase. Lin *et al.* (2014) demonstrated that protein lysis buffer and a nonionic detergent agent applied to separate cell membrane complexes (Nonidet P-40) is effective to weaken cucumber anthracnose, which is influences the escalation of gene levels. This means that it is related to disease resistance (peroxidase and pathogenis associated with protein 1-1a, acidic class III chitinase, phenylalanine ammonialyase 1). Research on hypovirulent binucleate *Rhizoctonia* (HBNR) as a potential biocontrol agent against *Fusarium* diseases in tomatoes and spinach have been recently reported in our investigations with a mechanism that might be induced resistance (Muslim *et al.* 2003a, b, c). There has also been an investigation of HBNR as an agent of induced systemic resistance (ISR) on beans against *Rhizoctonia solani* or *C. lindermuthianum* (Xue *et al.* 1998); they also protected cotton against alternaria leaf spot and rhizoctonia damping-off with ISR (Jabaji-Hare & Neate 2005). However, until now, there has been no report of the use of HBNR as an agent of ISR on cucumber against anthracnose pathogen *C. orbiculare* (= *C. lagenarium*).

In general, ISR in plants is clearly defined as a set of induced defense responses, including the creation of cell wall lytic enzymes. For example, 1,3-β-glucanases and chitinases (Lowton & Lamb 1987) enhance the activities of peroxidase and lignin deposition, callose, hydroxyproline-rich glycoprotein (Hammerschmidt & Kuc 1982;

Hammerschmidt *et al.* 1982; Hammerschmidt *et al.* 1984), and phytoalexins (Ebel 1986),

This study aims to investigate HBNR capacity in inducing systemic resistance in cucumber against cucumber anthracnose. The study was designed to reveal if induced resistance in cucumber is correlated with enhanced systemic lignification and peroxidase activity.

MATERIALS AND METHODS

Isolates

Hypovirulent binucleate *Rhizoctonia* isolate of W1, W7 (AG-A), L1 (AG-Ba), and Rhv7 (unknown anastomosis group) obtained from soil samples were used as biocontrol agents. The pathogens used in this study were *Colletotrichum orbiculare* (Berk & Mont.) Arx (=*Colletotrichum lagenarium* (Pass.) Ellis & Halst.) isolate 104T, which were obtained from infected cucumber plants.

Plants

Throughout the experiment, cucumber cv. Gibai was used. Before the sowing, seeds were sterilized with 70% ethyl alcohol for one minute, and 1% of NaOCl for 20 minutes. Finally, they were rinsed in sterilized distilled water three times.

Inoculum preparation

Isolates of pathogen *C. orbiculare* were cultured on potato dextrose agar (PDA) as long as seven days without exposure to light. The temperature was maintained at 25°C. A sterilized glass bar from the cultures with added sterile water, and scraped the spore suspensions. The spore suspension was then filtered through eight layers of sterile gauze. The isolates were set as two inoculum forms: barley grain inoculum and culture filtrate.

The following procedure was used for preparation of barley grain inoculum: Each isolate was cultured in PDA for three days without light and at room temperature. Five 5 mm mycelial disks of the culture were applied to 100 grams of moist autoclaved barley grains (1:1, w/v dry barley grains/distilled water) collected in a 500 ml Erlenmeyer flask. The cultures were maintained and regularly shaken for 10 days at 25°C to produce well-colonized inoculum with HBNR. The inoculum was naturally dried for around 10 days. They were then kept refrigerated at 4°C until use.

The following procedure was used for the culture filtrate (CF): Two mycelial disks of each HBNR isolate obtained from the culture growing on PDA were put into a 20 ml flask with 50 ml of potato dextrose broth (pH 6.5). The isolates were grown in static conditions at 23-25°C for 10 days without light.

The CF separated from the mycelia. Next, the CF was filtered three times over three layers of Whatman filter paper number 2. The CF was also filtered and sterilized using millipore filtration (0.45 µm Millipore filters, Millipore Products Division, Bedford, USA).

Cucumber ISR assays

Experiments with barley grain inocula

Each sterilized plastic pot, sized ø6 cm x 7.5 cm, was filled with the colonized barley grain inocula mixture (2%, w/w) with as much as 120 grams of potting medium. The previously-sterilized (with 0.5% NaOCl) cucumber seeds were added to the mixture. Each pot was given one seed. Next, the plants were cultivated at 25°C. This required 21 days in a growth chamber with a 14 h light (24,000 lux) per dark period. The plants that were grown in the potting medium with untreated barley grain inocula were used as a control. In this experiment, there were two replicates for each treatment. Each replicate had three plants. The experiment was repeated twice.

Experiment with culture filtrates (CF)

The plastic pots (autoclavable, ø6 cm x 7.5 cm) containing about 120 grams of potting medium were heated in autoclaves. The surface-sterilized cucumber seeds were shown in each pot. The plants were maintained in a similar manner as previously described. The first true leaves of 21-day-old cucumber plants were soaked with CF for one minute. The plants were inoculated after 24 h of incubation. One treatment comprised two replications, with three plants per replication. The experiment was repeated twice.

Challenge inoculation

The second true leaves were inoculated with 20 spore suspension drops of *C. orbiculare* (5 x 10^5 spores/ml). The disk of lens paper (ø 5 mm) was covered on every drop toward the run-off prevention. This was done to ensure the distribution of identical spores along the leaf surfaces. The inoculated plants were maintained for 48 h without light at 25°C in a humid chamber (85%-90% RH). After that, for six days the inoculated plants were brought to the growth chamber. ~~After that,~~ all diameters and areas of *C. orbiculare* lesions on every leaf were measured.

Testing for lignin formation

The cucumber seeds were grown on damp sterilized filter paper. Next, they were incubated for a week without light at 25°C. The roots of the seedlings were put in 5.0 ml of CF and incubated for one day. Then, with 10 µl drops of spore suspension (5 x 10^5 spores/ml) of *C. orbiculare,* the hypocotyls of the treated seedlings were

inoculated. Next, the inoculated seedlings were incubated for 20 h. The epidermal strips of the seedling hypocotyls were stained with toluidine blue O or phloroglucinol-HCL (Sherwood & Vance 1976). They were observed under the microscope to reveal percentage of lignification.

Protein extraction and determination

Treated cucumber root seedlings with CF of HBNR and challenge inoculated with *C. orbiculare* were prepared as described previously in *Testing for lignin formation*. Samples were collected from seedlings prior to the time of challenge inoculation and again 8-48 h after the challenge inoculation. All samples were immediately frozen at -80^{o}C until peroxidase assays were performed. Only the hypocotyls of the cucumber seedlings were used for protein extraction. These samples were homogenized in 5 ml of 0.05 M sodium phosphate buffer at pH 6.0 per 1 g sample with a cold mortar and pestle. The extract was centrifuged at 10,000 rpm for 10 minutes at 4^{o}C, and the supernatant was used to analyze the peroxidase activity. To identify the protein contents of these extracts, the Lowry method (Lowry *et al.* 1951) was used with bovine serum albumin as the standard.

Assay for peroxidase activity

Peroxidase activities were assessed following the method of Dalisay & Kuc (1995). They were determined using guaiacol, which acted as the hydrogen donor. The reaction mixture (3 ml) contained 0.25% (v/v) guaiacol in 1 mM sodium phosphate buffer at pH 6.0 with 100 mM hydrogen peroxidase. In order to catalyze the reaction, one tent ml crude enzyme extract was added and continued with colorimetrically at 470 nm min^{-1} mg^{-1} protein.

Detection of peroxidase isozymes by gel electrophoresis

Native PAGE was done with Phastsystem (Pharmacia LKB, UK). Extracts were adjusted to the same protein concentration with phosphate buffer and then loaded onto an 8-25% gradient gel. A peroxidase isoenzyme was made visible by immersing the extracts in gels of 1% 0-dianisidine solution. After 10 minutes, the gels were cleaned with distilled water. They were then placed into 0.06% H_2O_2 solution to concretely show the peroxidase isoenzyme bands.

Data analysis

The experiments in this study were designed in completely randomized designs. Total lesion numbers, anthracnose lesion diameters, and lignin formation in this study were compared using Fisher's least significant difference (LSD) test at $P = 0.05$ and $P = 0.01$.

RESULTS AND DISCUSSION

RESULTS

ISR in cucumber against anthracnose with HBNR

This study showed that the use of barley grain inoculum and CF of HBNR isolates significantly ($P = 0.05$) decreased total lesion diameter compared to the control (Table 1). However, no significant reduction was observed in total lesion number (Table 1). The reduction of total lesion diameter by barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results were also observed in the treatment with CF; the reduction of total lesion diameter by CF of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively (Table 1).

Lignin formation and peroxidase activities in cucumber hypocotyls treated with HBNR

Lignin formation was observed as the intense blue and green colors of the lignified cell walls. Cucumber hypocotyls pretreated with CF of HBNR L2, W1, W7, and Rhv7. This more significantly increased lignin deposition in places that had been infected by *C. orbiculare* compared to the control treatment (Fig. 1). Cucumber seedlings treated with CF of HBNR L2, W1, W7, and Rhv7 increased lignin deposition by 59.2%, 63.2%, 51.1%, and 64.4%, respectively.

Peroxidase activities in cucumber hypocotyls sampled at varying times before and after challenge inoculation were higher in the plant treated with HBNR compared to the control (Fig. 2). Treatment with HBNR L2, W1, W7, and Rhv7 increased peroxidase activities by 29%, 41%, 36%, and 45%, respectively, before inoculation of *C. orbiculare*, and by 28-39%, 25-43%, 30-37%, and 19-48%, respectively, after inoculation of *C. orbiculare*.

Two peroxidase isoenzymes (isoforms 1 to 2) were found in cucumber hypocotyls. The fast-moving anodic peroxidase isozymes were enhanced gradually after challenge inoculation. The peroxidase activities increased in the isoform 2 in the seedlings treated with HBNR compared to the control, at all sampling times, according to band intensity and width (Fig. 3). Interestingly, although isozyme 1 was observed after 48 h of pathogen inoculation, it was not recorded at 24 h of pathogen inoculation in the control treatment. However, it was recorded at the sampling times 24 h and 48 h after pathogen inoculation in the seedlings treated with HBNR W1.

DISCUSSION

This study reveals that treatment with HBNR isolates suppresses disease development of anthracnose in cucumber. The disease development suppression seemingly resulted from plant's ISR, as different distances

between HBNR and *C. orbiculare*, where the root was employed with HBNR isolates, and *C. orbiculare* was inoculated on the leaves, or the first true leaves were treated with HBNR isolates and *C. orbiculare* was challenge inoculated on the second true leaves. Thus, HBNR and pathogen application sites were separated spatially, and no HBNR isolates could be recovered from the second true leaves. The result of this research extends the hypothesis that the mechanism of protection from *R. solani* by HBNR is induced resistance (Cardoso & Echandi, 1987; Poromarto *et al.* 1998).

A report presented by Xue *et al.* (1998) showed that inoculation of bean hypocotyls with HBNR induced systemic resistance and protection of the roots and cotyledon to later challenges not only with the root rot pathogen *R. solani* but also with the anthracnose pathogen *C. lindemuthianum*. This study applied HBNR as barley grain inoculum, and CF induced systemic resistance in cucumber plants against *C. orbiculare*. Similar methods were used by Meera *et al.* (1994) and Koike *et al.* (2001), in which plant-growth-promoting fungi (PGPF) were applied at the root as barley grain inoculum, mycelia inoculum, or culture filtrates. This induced systemic resistance in cucumber after being challenged with *C. orbiculare* in leaves. Another study reported that germinating tomato seeds for one week in chemicals of b-aminobutyric acid (BABA) and jasmonic acid (JA) solutions promoted seed germination efficiency and induced resistance in four-week-old plants (Luna *et al.* 2016).

In this study, when HBNR CF was applied at the cucumber roots, lignin was enhanced at the attempted penetration by the pathogen in the epidermal tissues of cucumber hypocotyls. Our results also show that peroxidase activity in hypocotyls in the treated cucumber plant with HBNR significantly increased before and after inoculation of the pathogen compared to the control. Significant enhancements were also observed in the fast-moving anodic peroxidase isozymes in the plants treated with HBNR. This supports the finding by Xue *et al.* (1998) that inoculation of bean hypocotyls with HBNR induced systemic resistance, and this was positively correlated with peroxidases and 1,3-β-glucanases activity. Arora & Bajaj (1985) and Krstic *et al.* (1997) also reported that infection of mung bean and strawberry with binucleate *Rhizoctonia* resulted in an increase in peroxidase activity. Another benificial microbia using rhizobacteria also demonstrated similar results. Garcia-Cristobal *et al.* (2015) demonstrated that the plant-growth-promoting rhizobacteria (PGPR) induced resistance in young rice plants against *Xanthomonas campestris* infection through induced oxidative stress (ascorbate peroxidase), glutathione reductase, chitinase, and ß-1,3-Glucanase after pathogen inoculation.

Chandrasekaran & Chun (2016) demonstrated that treating tomato plants with *Bacillus subtilis* CBR05 significantly enhanced the number of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and polyphenol oxidase) activities. Yanti (2015) reported that rhizobacteria enhanced peroxidase enzyme activity. The isolate PK2Rp3 (*Serratia marcescens* strain N2.4) showed the highest activity of both roots and leaves of 0.058 μg · mL^{-1} and 0.053 μg · mL^{-1}. In another experiment, Peng *et al.* (2004) found that pretreated cucumber seedlings with

pectinase extract derived from *Penicillium oxalicum* BZH-2002 fermentation products resulted in induced resistance toward cucumber scab *Cladosporium cucumerinum* through the increased defense-related enzymes, polyphenol oxidasen, and peroxidase.

According to Dean & Kuc (1987) and Hammerschmidt *et al.* (1984), lignin deposition was considered a crucial phase of pathogen suppression in systemically immunized plants. Vance *et al.* (1980) reported that rapid lignin deposition might lead to the production of chemical or physical barriers to pathogen infection. Furthermore, peroxidases accelerate the ending polymerization step of lignin synthesis, resulting in the enhanced capability of protected tissue (Gross, 1979). In the other studies, (Hammerschmidt & Kuc, 1982; Ride, 1975) reported that the enhanced peroxidase activities are often related to resistance phenomenon such as the production of lignin. Peng and Kuc (1992) discovered the implications of peroxidase toward oxidative defense mechanisms in treated plants with infections. The peroxidase-generated hydrogen peroxidase directly functions as an antimicrobial agent. Further research is needed to identify other PR-proteins that may be involved in the mechanism of cucumber ISR from HBNR.

The bio-control abilities of HBNR isolates against *Fusarium* diseases in tomatoes and spinach (Muslim *et al.*, 2003a, b, c), as well as their ability to induce systemic resistance in cucumber against anthracnose, shows significant potential for their function as a bio-control agent to manage *Fusarium,* which has a very wide host, *Colletotrichum orbiculare,* and other diseases.

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Table 1: Effect of hypovirulent binucleate *Rhizoctonia* (HBNR) isolates on the total lesion number and lesion diameter on leaves of cucumber plants that have been challenge inoculated with *Colletotrichum orbiculare*.

Treatments	Total lesion number[a]		Total lesion diameter (mm)[a]	
	BGI[b]	CF[c]	BGI	CF
Pathogen	19.5 a	16.8 a	123.5 b	89.3 b
HBNR L2	16.8 a	12.5 a	89.5 a	48.7 a
HBNR W1	16.0 a	13.1 a	68.7 a	48.1 a
HBNR W7	17.2 a	12.9 a	75.5 a	51.8 a
HBNR Rhv7	16.8 a	11.7 a	74.5 a	46.5 a

[a] Mean of two trials each with two replicates, with three plants per replicate. Values followed by the same letter do not differ significantly (P = 0.05) according to Fisher's least significant difference test.

[b] Plants were grown in potting medium amended with barley grain inoculum (BGI) of HBNR isolates (1%, w/w) for 21 days and challenge inoculated with 10 µl drops of 5 x 10^5 spores/ml of *C. orbiculare* at 20 locations on the second true leaves.

[c] The first true leaves of 21-day-old cucumber plants grown in potting medium were treated with culture filtrates (CF) of HBNR and challenge inoculated with *C. orbiculare* on the second true leaves as described above.

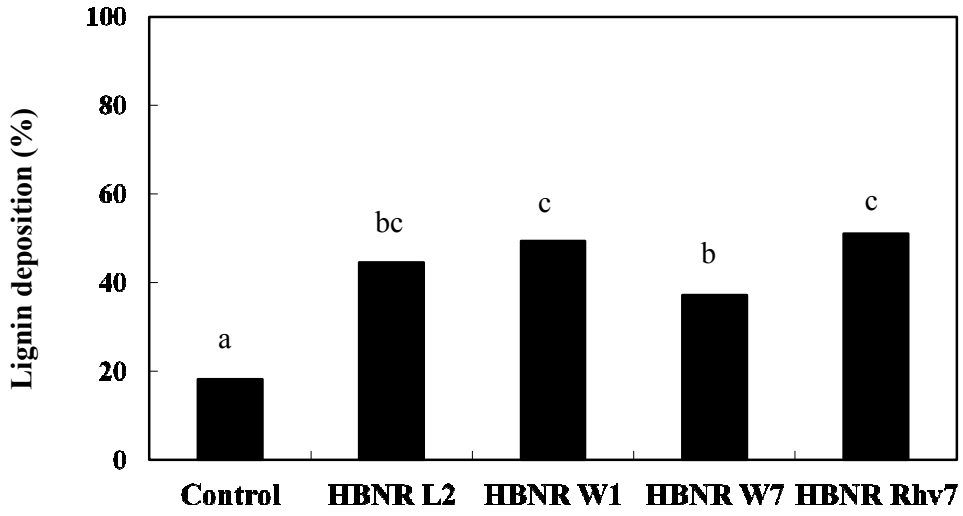

Figure 1: Lignification of hypocotyls of cucumber seedlings induced by culture filtrates of hypovirulent binucleate *Rhizoctonia* (HBNR), following challenge inoculation with *Colletotrichum orbiculare*. The hypocotyls of treated plants were challenged with 5 µl drops of 10^5 spores/ml of *C. orbiculare* at 10 locations. Bars labeled with the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.01$).

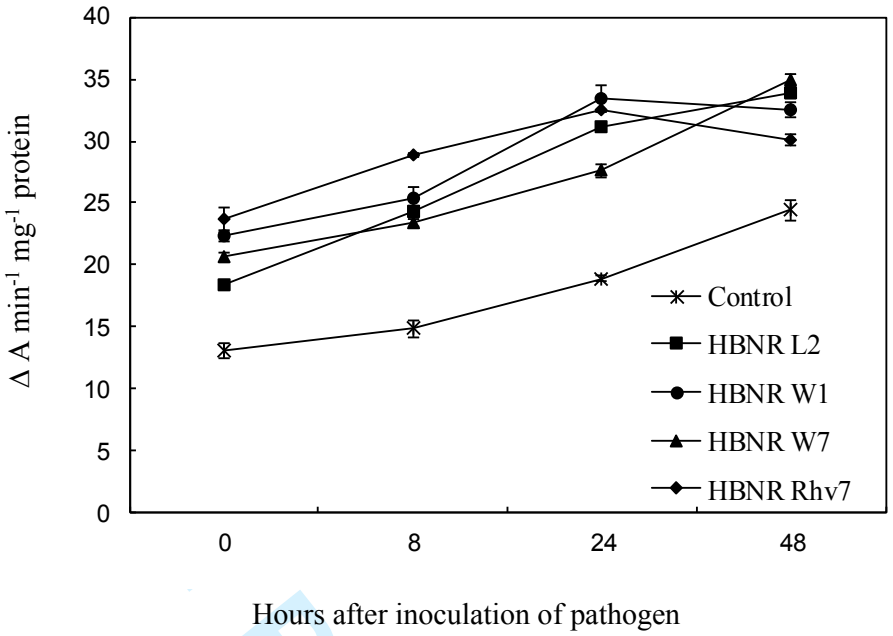

Figure 2: Time course of peroxidase activity in hypocotyls of cucumber after treating the root with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates and challenge inoculating with *C. orbiculare*. Peroxidase activity is expressed as changes in absorbance min^{-1} mg^{-1} protein. Data are the mean of three replications with five seedlings (cucumber) per replication. Bars represent standard error of the mean. 0 h indicates time before pathogen inoculation.

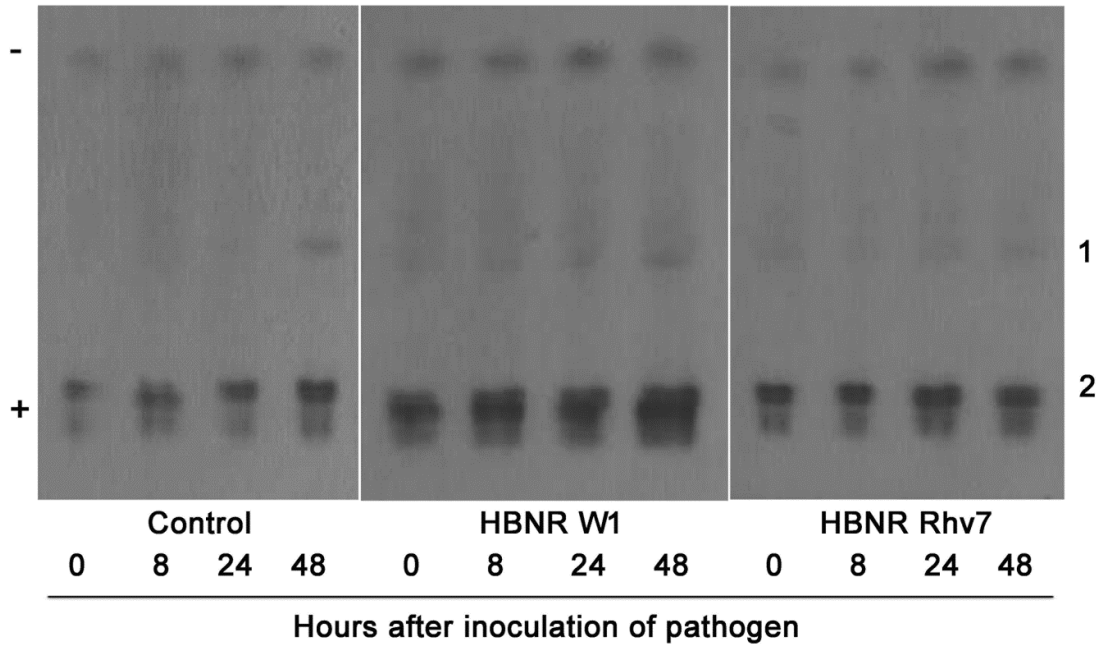

Figure 3: Electrophoresis patterns of peroxidase isozyme cucumber seedlings treated with hypovirulent binucleate Rhizoctonia (HBNR) (Protein concentration was 0.1 mg/ml).

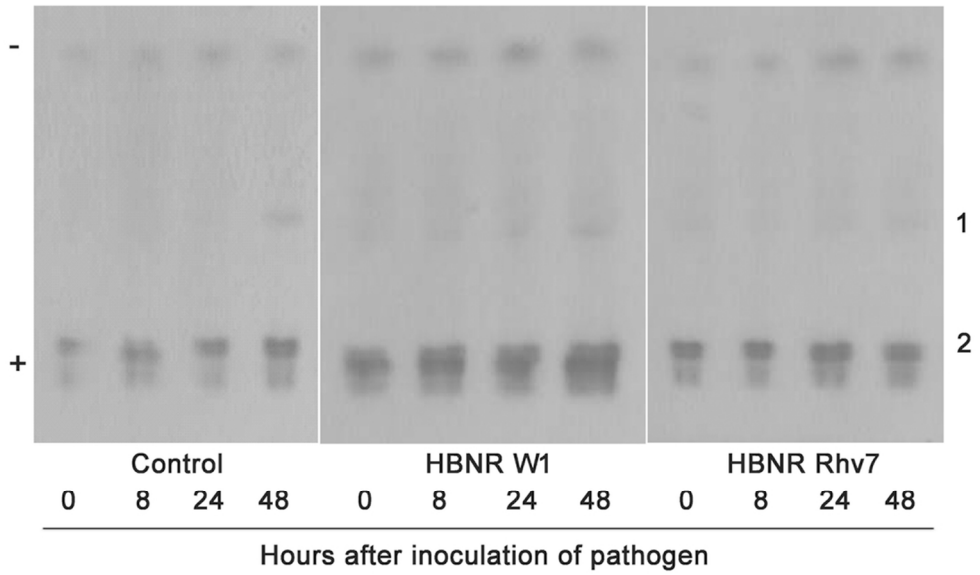

Figure 3

96x58mm (300 x 300 DPI)

8. ATTACHMENT REPORT KESIMPULAN PERBAIKAN PAPER DARI FEER REVIEWER

TLSR-OA-10-2017-0120
Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare

- My main concern of this manuscript is the study to determine/ to induce ISR by the host was based only on two experiments which were lignin deposition and peroxidase activity. Based on only two experiments, it is not sufficient to study induction of ISR by the host. Other enzymes that play a role in defense mechanism against pathogens should be inlcuded in the study. For example, polyphenol oxidase, superoxide dismutase and phenyl alanine lyase.

- For lignin deposition using culture filtrate, it would be good to do chemiluminescence assay to determine the elicitor activity.

- In the challenge inoculation test, the disease assessment is not complete. How was the disease severity calculated? Is there any disease index used?

- Introduction : improve the introduction on induced systemic resistance as in the present introduction is very brief . The authors should explain the interaction between the plant and the pathogen that induce the resistance.

- The discussion need major revision as the authors did not really discuss the results but it is more like a review of ISR. They also mentioned about bacteria and othe enzymes involved as inducer of ISR.

- English language used needs improvement as there are many sentences that are not well-written.

- Other comments are indicated in the manuscript.

a. muslim unsri
<a_muslim@unsri.ac.id>

Reminder: Tropical Life Sciences Research

4 messages

Tropical Life Sciences Research <onbehalfof@manuscriptcentral.com>
Reply-To: tlsr.usm@gmail.com
To: a_muslim@unsri.ac.id, limpal2003@yahoo.com

Sat, Mar 17, 2018 at 11:47 AM

17-Mar-2018

Dear Dr. Muslim:

Recently, you received a decision on Manuscript ID TLSR-OA-10-2017-0120, entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare." The manuscript and decision letter are located in your Author Center at https://mc.manuscriptcentral.com/tlsr.

This e-mail is simply a reminder that your revision is due in one week. If it is not possible for you to submit your revision within one week, kindly contact the Editorial Office, or we will consider your paper as a new submission.

Sincerely,
Suhana Sobi
Tropical Life Sciences Research Editorial Office
tlsr.usm@gmail.com

---

a. muslim unsri <a_muslim@unsri.ac.id> Fri, Mar 30, 2018 at 2:32 PM
To: tlsr.usm@gmail.com

Dear Dr. Suhana Sobi

I am so Sorry I am now still try to revise our manuscript. I try to submit our revision in few day later, Please Give me few days to submit our revision.
Thank u very much for your kindness

Sincerely Regard
A. Muslim
[Quoted text hidden]

TLSR USM <tlsr.usm@gmail.com> Fri, Mar 30, 2018 at 4:43 PM
To: "a. muslim unsri" <a_muslim@unsri.ac.id>

Dear Dr. Muslim,

I have extended the submission date to 6 April 2018.
Thank you.

Regards,
Miss Suhana Sobi
Tropical Life Sciences Research Editorial Office
[Quoted text hidden]
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Tropical Life Sciences Research
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We are indexed in

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a. muslim unsri <a_muslim@unsri.ac.id> Fri, Mar 30, 2018 at 9:04 PM
To: TLSR USM <tlsr.usm@gmail.com>

Dear Miss Suhana Sobi

Thank you so much for your kindness to extend date of submission of our revision.

thank you

Best Regard
Dr. A. Muslim
[Quoted text hidden]

a. muslim unsri
<a_muslim@unsri.ac.id>

Reminder: Tropical Life Sciences Research

1 message

Tropical Life Sciences Research <onbehalfof@manuscriptcentral.com>
Reply-To: tlsr.usm@gmail.com
To: a_muslim@unsri.ac.id, limpal2003@yahoo.com

Sat, Mar 31, 2018 at 11:59 AM

31-Mar-2018

Dear Dr. Muslim:

Recently, you received a decision on Manuscript ID TLSR-OA-10-2017-0120, entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare." The manuscript and decision letter are located in your Author Center at https://mc.manuscriptcentral.com/tlsr.

This e-mail is simply a reminder that your revision is due in one week. If it is not possible for you to submit your revision within one week, kindly contact the Editorial Office, or we will consider your paper as a new submission.

Sincerely,
Suhana Sobi
Tropical Life Sciences Research Editorial Office
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Tropical Life Sciences Research - Manuscript ID TLSR-OA-10-2017-0120.R1

2 messages

Tropical Life Sciences Research Fri, Apr 6, 2018
<onbehalfof@manuscriptcentral.com> at 5:59 PM
Reply-To: tlsr.usm@gmail.com
To: a_muslim@unsri.ac.id, limpal2003@yahoo.com

06-Apr-2018

Dear Dr. Muslim:

Your manuscript entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare" has been successfully submitted online and is presently being given full consideration for publication in the Tropical Life Sciences Research.

Your manuscript ID is TLSR-OA-10-2017-0120.R1.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail

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Thank you for submitting your manuscript to the Tropical Life Sciences Research.

Sincerely,
Tropical Life Sciences Research Editorial Office

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a. muslim unsri <a_muslim@unsri.ac.id> Fri, Apr 6, 2018 at 7:58 PM
Draft

[Quoted text hidden]

Dear Prof. Alexander Chong
Editor-in-Chief, Tropical life Science Research

Thank you very much for your generous suggestions. We have revised the manuscript according to your suggestion and the two reviewer.

Reviewer 1

1. My main concern of this manuscript is the study to determine/ to induce ISR by the host was based only on two experiments which were lignin deposition and peroxidase activity. Based on only two experiments, it is not sufficient to study induction of ISR by the host. Other enzymes that play a role in defense mechanism against pathogens should be inlcuded in the study. For example, polyphenol oxidase, superoxide dismutase and phenyl alanine lyase.

   Answer :

   We agree that Other enzymes that play a role in defense mechanism against pathogens should be inlcuded in the study. For example, polyphenol oxidase, superoxide dismutase and phenyl alanine lyase. However, We do not agree that in our Experimnent is not sufficient to study induction of ISR, becuase Induced Resistance involved multimechanisms. acoording to Van Loon (2000) typical defence responses of plants to pathogen are synthesis of Phytoalexins, accumulation of pathogenesis-related proteins (PRs) and reinforcement of cell wall. Peroxidase activity was systemiically increased in induced cucumber leaves, and peroxidase activity increased sooner in induced than in non-induced plants after challenge with *C. cucumerinum* or *C. lafenarium* (Hammerschmidt and kuc, 1982; Hammerschmidt et al 1982), resulting in lignification of the fungus bound to wall components of the host. Lignification plays central role in the plant resistance aginst fungal infection (Xue et al. 211).

2. For lignin deposition using culture filtrate, it would be good to do chemiluminescence assay to determine the elicitor activity.

   Answer :

   Since we would like to evaluate hypovirulent binucleate Rhizoctonia (HBNR) as elicitor after challenged with pahtogen in cucumber plant (in situ). We believe that our experimets (in situ) with record lignin depositoin in hypocotyl is enough to determine HBNR as a eliciter to lignin deposition

3. In the challenge inoculation test, the disease assessment is not complete. How was the disease severity calculated? Is there any disease index used?

   Answer:

In our experiment, pathogen was inoculated by placing 20 drop in separated point on single leaf (each drop was 10 µl) of spore suspension of *C. orbiculare* (5 x 10 spores/ml). So evaluation of diseases severity was based on lesion area of single inoculation. In this case, measurement based on lesion area is more accurated than diseases index. Diseases index was develop when inoculation was done on whole surface of leaf area (by spraying).

4. Introduction : improve the introduction on induced systemic resistance as in the present introduction is very brief . The authors should explain the interaction between the plant and the pathogen that induce the resistance.

Answer

We agree and are grateful for this suggestion. To improve the introduction on the interaction between the plant and the pathogen that induce the resistance. We inserted additional sentence:
Various agents both abiotic and biotic induscer (e.g., virulent or avirulent pathogens, nonpathogen microorganisms, cell wall fragments, plant extracts, and synthetic chemicals) have been documented to induce resistance after challenging with pathogen attack, both locally and systemically (Walters et al. 2005). Plants possess various inducible defense mechanisms to protect themselves against pathogens. These defense mechanisms include preexisting physical and chemical barriers, as well as inducible defense responses. The pre-existing biochemical defense mechanisms include phenolics, phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, 5-alkylated resorcinols and dienes (Osbourn 1996). The inducible defenses include the production of reactive oxygen species (ROS), hypersensitive response, reinforcement of cell wall, phytoalexins production and pathogenesis-related (PR) proteins (Mellersh and Heath 2004).

5. The discussion need major revision as the authors did not really discuss the results but it is more like a review of ISR. They also mentioned about bacteria and othe enzymes involved as inducer of ISR.

Answer

We agree and are grateful for this suggestion. To improve the discussion, We inserted several sentence sentence in our manuscript:

Enhanced lignin deposition was positively correlated with significant reduce of lesion development. Lignin may improved plant resistance against fungal infection through enhanced physical barrier and chemical direct toxicity through their toxic derivated such as phenolic compound (Xue, 2017).

In our study, When plant treated with barley grain inoculum and CF of HBNR isolates, significant reduction was observed in total lesion diameter. However, no significant reduction was observed in total lesion number. This result indicated that increased lignification and peroxidase activities observed in this study did not restrict total penetration of *C. orbiculare*. The data suggest that the involvement of other defence mechanism(s) acting at the level of restricting lesion development to fungal infection.

Lignification and peroxidase activities, alone or collectively, are not sole determinants for induced systemic resistance. Van Loon (2000) indicated that induced resistance is the result of multimechanisms. Therefore it is neccessary to investigate further other mechanisms, alone or collectively, involved in systemic resistance against *C. orbiculare*.

Reviewer 2

1. Page 1, Line 25: Using "if" in this sentence (If cucumber root was....) is not correct. Should use "when" instead.

   Answer:

   We have revised the sentence as reviewer's suggestion.

2. Page 2, Line 20: Decapitalize letter E in "Endophytic".

   Answer:

   We agree and we have revised the "Endophytic" to be "endophytic"

3. Page 2, Line 28: Decapitalize letter R in "Rhizosphre"

   Answer:

   We agree and we have revised the"Rhizosphre" to be rhizosphre

4. Page 3, Line 14-20: What do you use for your control in this experiment?

   Answer:

   We inform you that we do not use other microorganism as a control. The control is untreated plant with HBNR, where plant treated with SDW or barley grain only. This information is inserted in method.

5. Page 4, Line 29: Spelling error. The word "shown" should be "sown"?.

   Answer:

   We have revised the sentence as reviewer's suggestion. . The word "shown" is changed to "sown"

6. Page 4, Line 48: Repetitively using "after that". Improve the language in this paragraph.

   Answer:

   We have revised the sentence as reviewer's suggestion.

7. Page 5, line 4: How do you measure the percentage of lignification? No details on this matter. This is important for others to replicate the method.

   Answer:

   We insert how to measure the percentage of lignification :

   Spores of *C. orbiculare* germinated 90% or more on cucumber hypocotyls. The degree of lignin deposition was evaluated by determining the percentage of germinated spores together with appressoria around which lignin depositions were induced. For each treatment 100 germinated spores were evaluated.

8. Page 5, Line 4: Missing reference - (Sherwood & Vance 1976).

   Answer:

   We agree and we have inserted the reference in our text in References

9. Page 5, Line 33: What is one tent ml?

   Answer:

   It is our mistake, we mean one tenth.

10. Page 7, Line 44: "microbia" is an incorrect use of word. Should be microbes.

    Answer:

    We have recvised "microbia" to be “microbes”

11. Page 8, Line 15: Fix the sentence, citation should be at the end of the sentence.

    Answer:

    We have revised the sentence as reviewer’s suggestion.

12. Page 8, Line 26 - 31: For your conclusion, which isolates of HBNR is the most effective? The conclusion should relate to your objectives.

    Answer:

    Since the result of Induced systemic Resistance in cucumber against antracnose with HBNR where treatment of barley grain inoculum and CF of HBNR isolates has no significant each other among them to decreased the total lesion diameter. Althogh there are sligh difference of their ability to reduce lesion diameter. So we mention that they has a great potential as the biocontrol agent to control *Colletotrichum orbiculare* and other diseases.

13. Page 14, Figure 1, 2, 3: what is the control used for comparison?

    Answer:

We use sterilized distilled water as control. We have insert the sentence in the legend of the figure.

14. Page 16, Figure 3: Explain the label: What is +, -, 1, 2 ? There are no discussion on this findings. In the result, you mention that the isozyme 1 is interesting because it was not observed after 24h. The author should discuss this findings in the discussion.

    Answer:

    We delete the the label +, - . the labels are katoda and anoda. It is not necessary to show this label in the ficture.
    The label 1, 2 are the isozyme 1 and izozyme 2 of the peroxidase.

15. Missing references in the text:

    3. Cardoso JE, Echandi E (1987).....

    27. Poromarto SH, Nelson BD, Freeman TP (1998).....

    Answer:

    The references have already involve in the text both in Discussion (page 7 line 10-11) and in the referencien no. 3 and no. 27.

13. DOKUMEN REVISI MANUSCRIPTS HASIL PERBAIKAN AUTHOR

Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by *Colletotrichum orbiculare*

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Keywords:	hypovirulent binucleate Rhizoctonia (HBNR), induced systemic resistance, Colletotrichum orbiculare, cucumber

Induction of systemic resistance in cucumber by hypovirulent binucleate *Rhizoctonia* against anthracnose caused by *Colletotrichum orbiculare*

ABSTRACT

Treatment with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates induced systemic resistance against anthracnose infected by *Colletotrichum orbiculare* in cucumber. This is because of the different distances between HBNR and *C. orbiculare*, where the root was treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in leaves or first true leaves were treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in second true leaves. The use of barley grain inocula and culture filtrates of HBNR significantly reduced the sum of lesion diameter compared to the control (p = 0.05). The total lesion diameter reduction by applying barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results was also observed in treatment using culture filtrate, and the reduction of total lesion diameter by culture filtrate of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively. ~~If~~ When cucumber root was treated with culture filtrates of HBNR, the lignin was enhanced at the pathogen penetration, which is spread along the epidermis tissue of cucumber hypocotyls. Peroxidase activity in hypocotyls in the treated cucumber plant with culture filtrates of HBNR significantly increased before and after inoculation of pathogens as compared to the control. Significant enhancement was also observed in the fast-moving anodic peroxidase isozymes in the treated plants with culture filtrates of HBNR. The results showed the elicitor(s) contained in culture filtrates in HBNR. The lignin deposition as well as the peroxidase activity is an important

step to prevent systemically immunized plants from pathogen infection.

Keywords: hypovirulent binucleate *Rhizoctonia* (HBNR), induced systemic resistance, *Colletotrichum orbiculare,* cucumber

INTRODUCTION

As soon as a plant is appropriately stimulated, its resistance is intensified against a test inoculation against a pathogen. This is the phenomenon of induced resistance. It can be localized, systematic, and induced with limited infection of virulent or hypovirulent pathogens, specific non-pathogenic bacteria, cell wall fragments, plant extracts, and certain chemicals (Van Loon 2000; Walters 2010).

Concerns about impacts of agrichemicals on food safety and the environment are related to the danger of the synthetic pesticide utilization, leading plant pathologists to develop another ~~save~~ sustainable control for managing plant disease (Hyakumachi *et al.* 2014). Elicitors of host resistance are a potential alternative control to plant diseases (Lyon *et al.* 1995).

Several investigations have reported that cucumber anthracnose caused by *Colletotrichum orbiculare* could be effectively control by ~~Endophytic~~ endophytic *Streptomyces* (Shimizu *et al.* 2009), Rhizobacteria, *Bacillus pumilus*, *Bacillus subtilis*, and *Curtobacterium flaccumfaciens* (Raupach & Kloepper 2000). Some studies show induced systemic resistance in cucumbers against antrachnose using biotic and abiotic elicitor. Meera *et al.* (1994) demonstrated that ~~sterilized fungus~~a sterile fungus and *Phoma* sp. were reliable in activating the systemic resistance of cucumber against the ~~antracnoses~~anthracnose disease. Koike *et al.* (2001) demonstrated that fungi isolated from ~~Zoysiagrass~~ zoysiagrass (*Zoysia tenuifolia*) ~~Rhizosphere~~ rhizosphere (*Penicillium*, *Trichoderma*, *Phoma, Fusarium*, and a sterile fungus) significantly induced systemic resistance against cucumber anthracnoses. This is done through lignification

enhancement and superoxide generation. Tian *et al.* (2008) reported that application of *Pieris rapae* extract onto the first true cucumber leaves effectively brought about systemic resistance against cucumber anthracnose with the enhancement of peroxidase and polyphenoloxidase. Lin *et al.* (2014) demonstrated that protein lysis buffer and a nonionic detergent agent applied to separate cell membrane complexes (Nonidet P-40) is effective to weaken cucumber anthracnose~~, which is~~ by triggering genes ~~influences the escalation of gene levels. This means that it is~~ related to disease resistance (peroxidase and pathogenis associated with protein 1-1a, acidic class III chitinase, phenylalanine ammonialyase 1). Research on hypovirulent binucleate *Rhizoctonia* (HBNR) as a potential biocontrol agent against *Fusarium* diseases in tomatoes and spinach have been recently reported in our investigations with a mechanism that might be induced resistance (Muslim *et al.* 2003a, b, c). There has also been an investigation of HBNR as an agent of induced systemic resistance (ISR) on beans against *Rhizoctonia solani* or *C. lindermuthianum* (Xue *et al.* 1998); they also protected cotton against alternaria leaf spot and rhizoctonia damping-off with ISR (Jabaji-Hare & Neate 2005). However, until now, there has been no report of the use of HBNR as an agent of ISR on cucumber against anthracnose pathogen *C. orbiculare* (= *C. lagenarium*).

In general, ISR in plants is clearly defined as a set of induced defense responses, including the creation of cell wall lytic enzymes. For example, 1,3-β-glucanases and chitinases (Lowton & Lamb 1987) enhance the activities of peroxidase and lignin deposition, callose, hydroxyproline-rich glycoprotein (Hammerschmidt & Kuc 1982; Hammerschmidt *et al.* 1982; Hammerschmidt *et al.* 1984~~), ~~); and phytoalexins (Ebel 1986~~),~~).

Various agents both abiotic and biotic inducer (e.g., virulent or avirulent pathogens, nonpathogen microorganisms, cell wall fragments, plant extracts, and

synthetic chemicals) have been documented to induce resistance after challenging with pathogen attack, both locally and systemically (Walters *et al.* 2005). Plants possess various inducible defense mechanisms to protect themselves against pathogens. These defense mechanisms include preexisting physical and chemical barriers, as well as inducible defense responses. The pre-existing biochemical defense mechanisms include phenolics, phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, 5-alkylated resorcinols and dienes (Osbourn 1996). The inducible defenses include the production of reactive oxygen species (ROS), hypersensitive response, reinforcement of cell wall, phytoalexins production and pathogenesis-related (PR) proteins (Mellersh & Heath 2004).

This study aims to investigate HBNR capacity in inducing systemic resistance in cucumber against *C. orbiculare*~~cucumber anthracnose~~. The study was designed to reveal if induced resistance in cucumber is correlated with enhanced systemic lignification and peroxidase activity.

MATERIALS AND METHODS

Isolates

Hypovirulent binucleate *Rhizoctonia* isolate of W1, W7 (AG-A), L1 (AG-Ba), and Rhv7 (unknown anastomosis group) obtained from soil samples were used as biocontrol agents. The pathogens used in this study were *Colletotrichum orbiculare* (Berk & Mont.) Arx (=*Colletotrichum lagenarium* (Pass.) Ellis & Halst.) isolate 104T, which were obtained from infected cucumber plants.

Plants

Throughout the experiment, cucumber cv. Gibai was used. Before the sowing, seeds were sterilized with 70% ethyl alcohol for one minute, and 1% of NaOCl for 20 minutes. Finally, they were rinsed in sterilized distilled water three times.

Inoculum preparation

Isolates of pathogen *C. orbiculare* were cultured on potato dextrose agar (PDA) as long as seven days without exposure to light. The temperature was maintained at 25°C. A sterilized glass bar from the cultures with added sterile water, and scraped the spore suspensions. The spore suspension was then filtered through eight layers of sterile gauze. The isolates were set as two inoculum forms: barley grain inoculum and culture filtrate.

The following procedure was used for preparation of barley grain inoculum: Each isolate was cultured in PDA for three days without light and at room temperature. Five 5 mm mycelial disks of the culture were applied to 100 grams of moist autoclaved barley grains (1:1, w/v dry barley grains/distilled water) collected in a 500 ml Erlenmeyer flask. The cultures were maintained and regularly shaken for 10 days at 25°C to produce well-colonized inoculum with HBNR. The inoculum was naturally dried for around 10 days. They were then kept refrigerated at 4°C until use.

The following procedure was used for the culture filtrate (CF): Two mycelial disks of each HBNR isolate obtained from the culture growing on PDA were put into a 20 ml flask with 50 ml of potato dextrose broth (pH 6.5). The isolates were grown in static conditions at 23-25°C for 10 days without light. The CF separated from the mycelia. Next, the CF was filtered three times over three layers of Whatman filter paper number 2. The CF was also filtered and sterilized using millipore filtration (0.45 µm Millipore filters, Millipore Products Division, Bedford,

USA).

Cucumber ISR assays

Experiments with barley grain inocula

Each sterilized plastic pot, sized ø 6 cm x 7.5 cm, was filled with the colonized barley grain inocula mixture (2%, w/w) with as much as 120 grams of potting medium. The previously-sterilized (with 0.5% NaOCl) cucumber seeds were added to the mixture. Each pot was given one seed. Next, the plants were cultivated at 25°C. This required 21 days in a growth chamber with a 14 h light (24,000 lux) per dark period. The plants that were grown in the potting medium with untreated barley grain inocula were used as a control. Each inocula of HBNR isolate was inoculated on six plants as replication and t~~In this experiment, there were two replicates for each treatment. Each replicate had three plants. T~~he experiment was repeated twice.

Experiment with culture filtrates (CF)

The plastic pots (autoclavable, ø6 cm x 7.5 cm) containing about 120 grams of potting medium were heated in autoclaves. The surface-sterilized cucumber seeds were s~~h~~own in each pot. The plants were maintained in a similar manner as previously described. The first true leaves of 21-day-old cucumber plants were soaked with CF for one minute. The plants were inoculated after 24 h of incubation. Each CF of HBNR isolate was applied on six plants as replication and ~~One treatment comprised two replications, with three plants per replication. T~~the experiment was repeated twice.

Challenge inoculation

The second true leaves were inoculated with 20 individual drops (each drop was 10 µl) of spore suspension ~~drops~~ of *C. orbiculare* (5 x 10^5 spores/ml). The disk of lens

paper (ø 5 mm) was covered on every drop toward the run-off prevention. This was done to ensure the distribution of equal numbers of spores ~~identical spores~~ along the leaf surfaces. The inoculated plants were maintained for 48 h without light at 25°C in a humid chamber (85%-90% RH). After that, for six days the inoculated plants were brought to the growth chamber. ~~After that, a~~The total number per leaf and diameter of lesion per inoculated drop~~ll diameters and areas of *C. orbiculare* lesions on every leaf~~ were measured.

Testing for lignin formation

The cucumber seeds were grown on damp sterilized filter paper. Next, they were incubated for a week without light at 25°C. The roots of the seedlings were put in 5.0 ml of CF and incubated for one day. Then, with 10 µl drops of spore suspension (5 x 10^5 spores/ml) of *C. orbiculare,* the hypocotyls of the treated seedlings were inoculated. Next, the inoculated seedlings were incubated for 20 h. The epidermal strips of the seedling hypocotyls were stained with toluidine blue O or phloroglucinol-HCL (Sherwood & Vance 1976). They were observed under the microscope to reveal percentage of lignification.

Spores of *C. orbiculare* germinated 90% or more on cucumber hypocotyls. The degree of lignin deposition was evaluated by determining the percentage of germinated spores together with appressoria around which lignin depositions were induced. For each treatment 100 germinated spores were evaluated.

Protein extraction and determination

Treated cucumber root seedlings with CF of HBNR and challenge inoculated with *C. orbiculare* were prepared as described previously in section of t~~T~~esting for lignin formation. Samples were collected from seedlings prior to the time of challenge

inoculation and again 8-48 h after the challenge inoculation. All samples were immediately frozen at -80°C until peroxidase assays were performed. Only the hypocotyls of the cucumber seedlings were used for protein extraction. These samples were homogenized in 5 ml of 0.05 M sodium phosphate buffer at pH 6.0 per 1 g sample with a cold mortar and pestle. The extract was centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was used to analyze the peroxidase activity. To ~~identify~~ determine the protein contents of these extracts, the Lowry method (Lowry *et al.* 1951) was used with bovine serum albumin as the standard.

Assay for peroxidase activity

Peroxidase activities were assessed following the method of Dalisay & Kuc (1995). They were determined using guaiacol, which acted as the hydrogen donor. The reaction mixture (3 ml) contained 0.25% (v/v) guaiacol in 1 mM sodium phosphate buffer at pH 6.0 with 100 mM hydrogen peroxidase. In order to catalyze the reaction, one-tenth ml crude enzyme extract was added and continued with colorimetrically at 470 nm min^{-1} mg^{-1} protein.

Detection of peroxidase isozymes by gel electrophoresis

Native PAGE was done with ~~Phastsystem~~ a PhastSystem (Pharmacia LKB, UK). Extracts were adjusted to the same protein concentration with phosphate buffer and then loaded onto an 8-25% gradient gel. A peroxidase isoenzyme was made visible by immersing the extracts in gels of 1% ~~0~~o-dianisidine solution. After 10 minutes, the gels were cleaned with distilled water. They were then placed into 0.06% H_2O_2 solution to concretely show the peroxidase isoenzyme bands.

Data analysis

The experiments in this study were designed in completely randomized designs. Total lesion numbers, anthracnose lesion diameters, and lignin formation in this study were compared using Fisher's least significant difference (LSD) test at $P = 0.05$ and $P = 0.01$.

RESULTS AND DISCUSSION

RESULTS

ISR in cucumber against anthracnose with HBNR

This study showed that the use of barley grain inoculum and CF of HBNR isolates significantly ($P = 0.05$) decreased total anthracnose lesion diameter compared to the control (Table 1). However, no significant reduction was observed in total lesion number (Table 1). The reduction of total lesion diameter by barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results were also observed in the treatment with CF; the reduction of total lesion diameter by CF of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively (Table 1).

Lignin formation and peroxidase activities in cucumber hypocotyls treated with HBNR

Lignin formation was observed as the intense blue and green colors of the lignified cell walls. Cucumber hypocotyls pretreated with CF of HBNR L2, W1, W7, and Rhv7~~. This more~~ significantly increased lignin deposition in places that had been infected by *C. orbiculare* compared to the control treatment (Fig. 1). Cucumber seedlings treated with CF of HBNR L2, W1, W7, and Rhv7 increased lignin deposition by 1.45-fold, 1.71-fold, 1.04-fold, and 1.81-fold~~59.2%, 63.2%, 51.1%, and 64.4%~~, respectively, relative to control.

Peroxidase activities in cucumber hypocotyls sampled at varying times before and after challenge inoculation were higher in the plant treated with HBNR compared to the control (Fig. 2). Treatment with HBNR L2, W1, W7, and Rhv7 increased peroxidase activities by 40%, 70%, 57%, and 81%, ~~29%, 41%, 36%, and 45%,~~ respectively, before inoculation of *C. orbiculare*, and by 39-64%, 33-94%, 43-58%, and 23-94%, ~~28-39%, 25-43%, 30-37%, and 19-48%,~~ respectively, relative to control after inoculation of *C. orbiculare*.

Two peroxidase iso~~en~~zymes (isoforms 1 ~~to~~ and 2) were found in cucumber hypocotyls. The fast-moving anodic peroxidase isozymes were enhanced gradually after challenge inoculation. The peroxidase activities increased in the isoform 2 in the seedlings treated with HBNR compared to the control, at all sampling times, according to band intensity and width (Fig. 3). ~~Interestingly,~~l ~~although i~~sozyme type 1 had a minor activity band and was observed after 48 h of pathogen inoculation either on inoculated or non-inoculated with pathogen~~, it was not recorded at 24 h of pathogen inoculation in the control treatment. However, it was recorded at the sampling times 24 h and 48 h after pathogen inoculation in the seedlings treated with HBNR W1.~~.

DISCUSSION

This study reveals that treatment with HBNR isolates suppresses disease development of anthracnose in cucumber. The disease development suppression seemingly resulted from plant's ISR, as ~~different~~ separated ~~distances~~ inoculation sites between HBNR and *C. orbiculare*, where the root was employed with HBNR isolates, and *C. orbiculare* was inoculated on the leaves, or the first true leaves were treated with HBNR isolates and *C. orbiculare* was challenge inoculated on the second true leaves. Thus, HBNR and pathogen application sites were separated spatially, and no HBNR isolates could be recovered from the second true leaves. The result of this research

~~extends~~ supports the ~~hypothesis~~ evidence that the mechanism of protection from *R. solani* by HBNR is induced resistance (Cardoso & Echandi, 1987; Poromarto *et al.* 1998).

A report presented by Xue *et al.* (1998) showed that inoculation of bean hypocotyls with HBNR induced systemic resistance and protection of the roots and cotyledon to later challenges not only with the root rot pathogen *R. solani* but also with the anthracnose pathogen *C. lindemuthianum*. This study applied HBNR as barley grain inoculum, and CF induced systemic resistance in cucumber plants against *C. orbiculare*. Similar methods were used by Meera *et al.* (1994) and Koike *et al.* (2001), in which plant-growth-promoting fungi (PGPF) were applied at the root as barley grain inoculum, mycelia inoculum, or culture filtrates. This induced systemic resistance in cucumber after being challenged with *C. orbiculare* in leaves. Another study reported that germinating tomato seeds for one week in chemicals of b-aminobutyric acid (BABA) and jasmonic acid (JA) solutions promoted seed germination efficiency and induced resistance in four-week-old plants (Luna *et al.* 2016).

In this study, when HBNR CF was applied at the cucumber roots, lignin was enhanced at the attempted penetration by the pathogen in the epidermal tissues of cucumber hypocotyls. Enhanced lignin deposition was positively correlated with significant reduce of lesion development. Lignin may improve plant resistance against fungal infection through enhanced physical barrier and chemical direct toxicity through their toxic derivatives such as phenolic compounds (Xue & Yi 2017). Our results also show that peroxidase activity in hypocotyls in the treated cucumber plant with HBNR significantly increased before and after inoculation of the pathogen compared to the control. Significant enhancements were also observed in the fast-moving anodic peroxidase isozymes (isoform 2) in the plants treated with HBNR. Isoform 1 may have less significant role in induce resistance since it showed a minor activity and found on

both inoculated and non-inoculated hypocotyl. This supports the finding by Xue *et al.* (1998) that inoculation of bean hypocotyls with HBNR induced systemic resistance, and this was positively correlated with peroxidases and 1,3-β-glucanases activity. Arora & Bajaj (1985) and Krstic *et al.* (1997) also reported that infection of mung bean and strawberry with binucleate *Rhizoctonia* resulted in an increase in peroxidase activity. Peng *et al.* (2004) found that pretreated cucumber seedlings with pectinase extract derived from *Penicillium oxalicum* BZH-2002 fermentation products resulted in induced resistance toward cucumber scab *Cladosporium cucumerinum* through the increased defense-related enzymes, polyphenol oxidase, and peroxidase.

Another benificial microbia using rhizobacteria also demonstrated similar results. Garcia-Cristobal *et al.* (2015) demonstrated that the plant-growth-promoting rhizobacteria (PGPR) induced resistance in young rice plants against *Xanthomonas campestris* infection through induced oxidative stress (ascorbate peroxidase), glutathione reductase, chitinase, and ß-1,3-Glucanase after pathogen inoculation.

Chandrasekaran & Chun (2016) demonstrated that treating tomato plants with *Bacillus subtilis* CBR05 significantly enhanced the number of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and polyphenol oxidase) activities. Increased peroxidase activity is also well observed in rhizobacteria-induced systemic resistance. Chandrasekaran & Chun (2016) demonstrated that treating tomato plants with *Bacillus subtilis* CBR05 significantly enhanced the activities of antioxidant enzymes including peroxidase. Yanti (2015) reported that rhizobacteria enhanced peroxidase enzyme activity. The isolate PK2Rp3 (*Serratia marcescens* strain N2.4) showed the highest activity of both roots and leaves of 0.058 μg · mL^{-1} and 0.053 μg · mL^{-1}. In another experiment, Peng *et al.* (2004) found that pretreated cucumber seedlings with pectinase extract derived from *Penicillium oxalicum* BZH-2002 fermentation products resulted in induced resistance toward cucumber scab *Cladosporium cucumerinum*

through the increased defense-related enzymes, polyphenol oxidasen, and peroxidase.

According to Dean & Kuc (1987) and Hammerschmidt *et al.* (1984), lignin deposition was considered a crucial phase of pathogen suppression in systemically immunized plants. Vance *et al.* (1980) reported that rapid lignin deposition might lead to the production of chemical or physical barriers to pathogen infection. Furthermore, peroxidases accelerate the ending polymerization step of lignin synthesis, resulting in the enhanced capability of protected tissue (Gross, 1979). In the other studies, (Hammerschmidt & Kuc, 1982; Ride, 1975) reported that the enhanced peroxidase activities are often related to resistance phenomenon such as the production of lignin (Hammerschmidt & Kuc, 1982; Ride, 1975). Peng and Kuc (1992) discovered the implications of peroxidase toward oxidative defense mechanisms in treated plants with infections. The peroxidase-generated hydrogen peroxidase directly functions as an antimicrobial agent. In our study, when plant treated with barley grain inoculum and CF of HBNR isolates, significant reduction was observed in total lesion diameter. However, no significant reduction was observed in total lesion number. This result indicated that increased lignification and peroxidase activities observed in this study did not restrict total penetration of *C. orbiculare*. The data suggest that the involvement of other defense mechanism(s) acting at the level of restricting lesion development to fungal infection. Lignification and peroxidase activities, alone or collectively, are not sole determinants for induced systemic resistance. Van Loon (2000) indicated that induced resistance is the result of multi-mechanisms. Therefore it is necessary to investigate further other mechanisms, alone or collectively, involved in systemic resistance against *C. orbiculare*. Further research is needed to identify other PR-proteins that may be involved in the mechanism of cucumber ISR from HBNR.

The bio-control abilities of HBNR isolates to induce systemic resistance in cucumber against anthracnose and to enhance lignin deposition and peroxidase activity

as well as their effectiveness ~~of HBNR isolates~~ against *Fusarium* diseases in tomato~~es~~ and spinach in our previous studies (Muslim *et al.*, 2003a, b, c)~~, as well as their ability to induce systemic resistance in cucumber against anthracnose,~~ shows significant potential ~~for~~ as ~~their function as~~ a bio-control agent to manage ~~*Fusarium,* which has a very wide host,~~ *Colletotrichum orbiculare*~~,~~ and other diseases.

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Table 1: Effect of hypovirulent binucleate *Rhizoctonia* (HBNR) isolates on the total lesion number and lesion diameter on leaves of cucumber plants that have been challenge inoculated with *Colletotrichum orbiculare*.

Treatments	Total lesion number[a]		Total lesion diameter (mm)[a]	
	BGI[b]	CF[c]	BGI	CF
Pathogen	19.5 a	16.8 a	123.5 b	89.3 b
HBNR L2	16.8 a	12.5 a	89.5 a	48.7 a
HBNR W1	16.0 a	13.1 a	68.7 a	48.1 a
HBNR W7	17.2 a	12.9 a	75.5 a	51.8 a
HBNR Rhv7	16.8 a	11.7 a	74.5 a	46.5 a

[a] Mean of two trials each with six plants per treatment. Values followed by the same letter do not differ significantly ($P = 0.05$) according to Fisher's least significant difference test.

[b] Plants were grown in potting medium amended with barley grain inoculum (BGI) of HBNR isolates (1%, w/w) for 21 days and challenge inoculated with 10 μl drops of 5 x 10^5 spores/ml of *C. orbiculare* at 20 locations on the second true leaves.

[c] The first true leaves of 21-day-old cucumber plants grown in potting medium were treated with culture filtrates (CF) of HBNR and challenge inoculated with *C. orbiculare* on the second true leaves as described above.

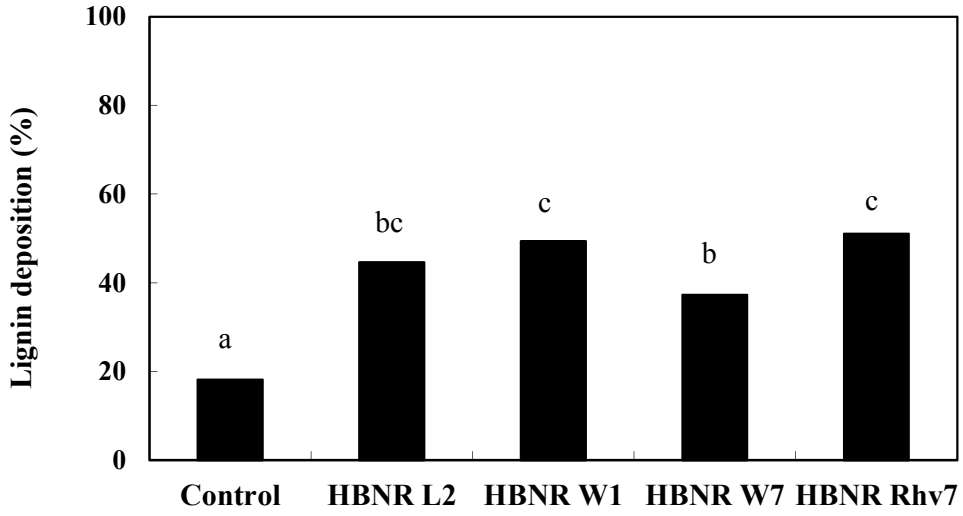

Fig. 1: Lignification of hypocotyls of cucumber seedlings induced by culture filtrates of hypovirulent binucleate *Rhizoctonia* (HBNR), following challenge inoculation with *Colletotrichum orbiculare*. Cucumber seedlings treated with sterilized distilled water were used as control. The hypocotyls of treated plants were challenged with 5 μl drops of 10^5 spores/ml of *C. orbiculare* at 10 locations. Bars labeled with the same letter are not significantly different according to Fisher's least significant difference test (P = 0.01).

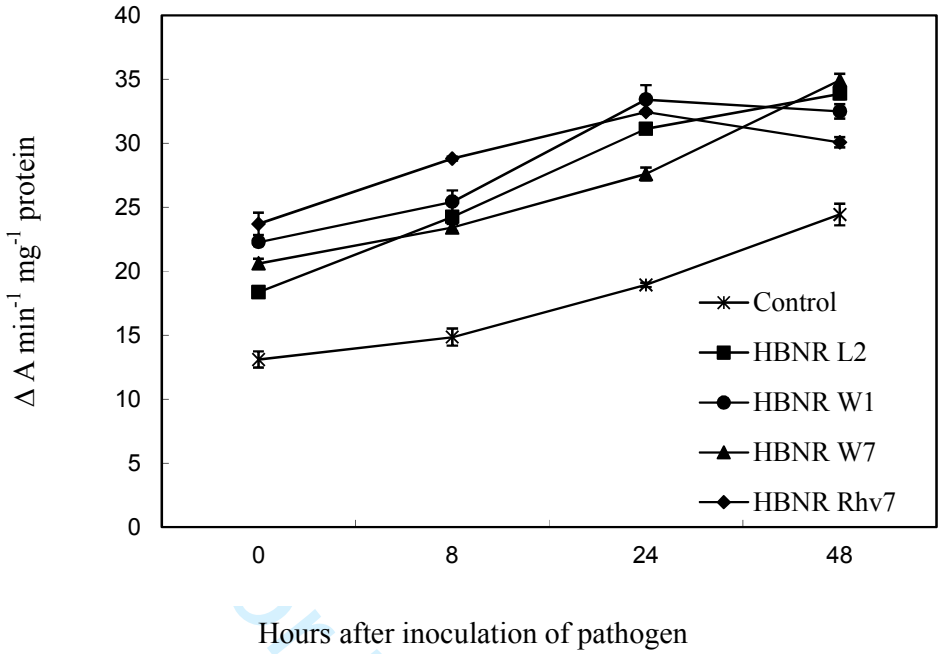

Fig. 2: Time course of peroxidase activity in hypocotyls of cucumber after treating the root with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates and challenge inoculating with *C. orbiculare*. Peroxidase activity is expressed as changes in absorbance min^{-1} mg^{-1} protein. Cucumber seedlings treated with sterilized distilled water were used as control. Data are the mean of three replications with five seedlings (cucumber) per replication. Bars represent standard error of the mean. 0 h indicates time before pathogen inoculation.

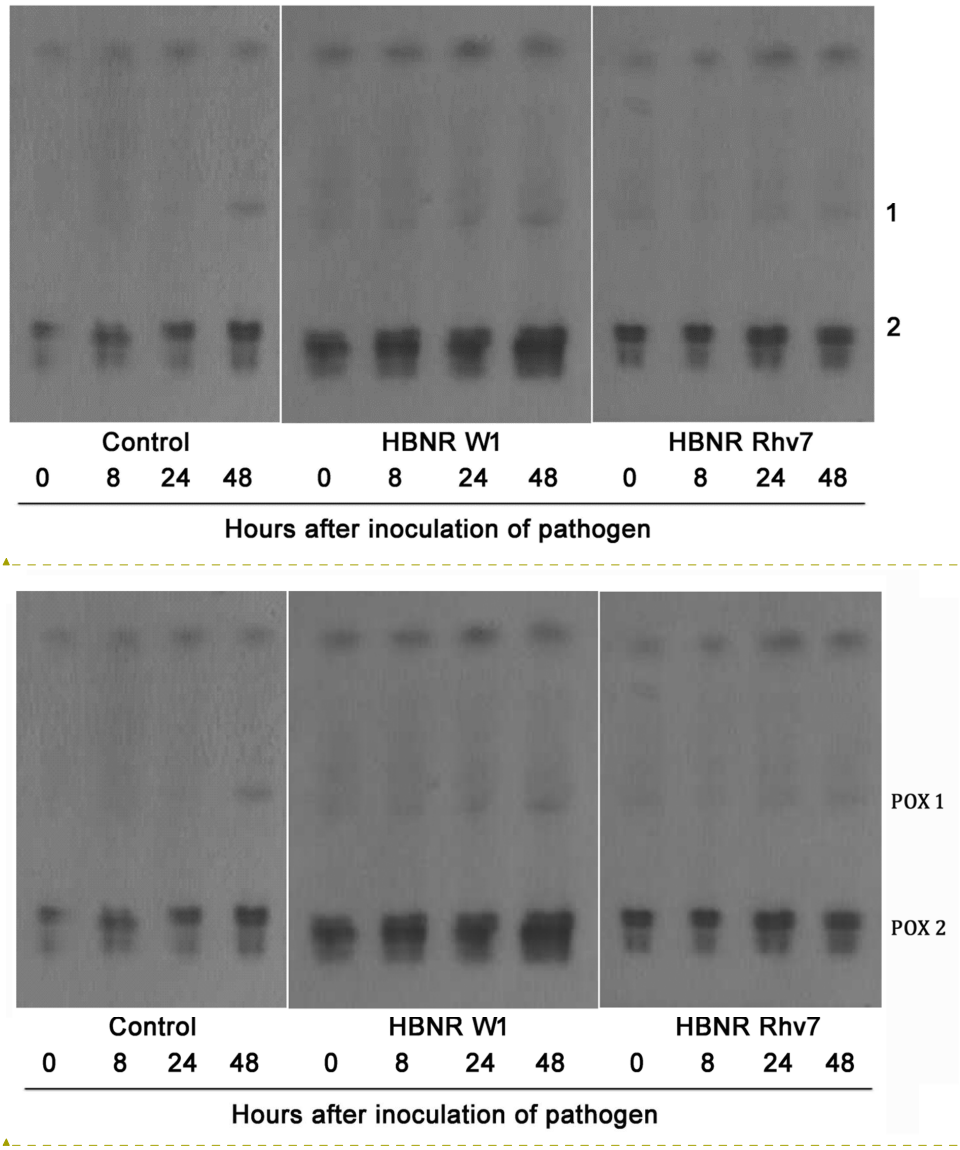

Fig. 3: Electrophoresis patterns of peroxidase isozyme (POX 1 and POX 2) cucumber seedlings treated with hypovirulent binucleate *Rhizoctonia* (HBNR) (Protein concentration was 0.1 mg/ml). Cucumber seedlings treated with sterilized distilled water were used as control.

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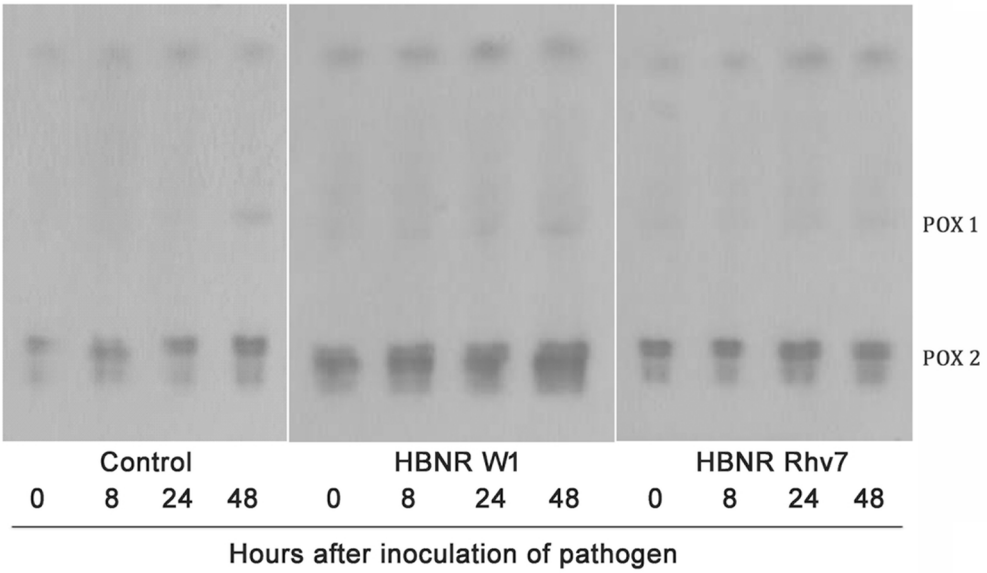

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08-May-2018

Dear Dr. Muslim:

It is a pleasure to accept your manuscript entitled "Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by Colletotrichum orbiculare" in its current form for publication in the Tropical Life Sciences Research.

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Suwandi fp <suwandi@fp.unsri.ac.id> Tue, May 8, 2018 at 4:21 PM
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Suwandi, Ph.D.
Phytopathology Laboratory, Department of Plant Protection
Faculty of Agriculture, Sriwijaya University

Jl. Palembang-Prabumulih Km.32 Indralaya 30662 Indonesia
Tel./Fax. +62-711-580663 E-mail:
suwandi@fp.unsri.ac.id

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Dear Prof. Chong,

This is with reference to our article entitled: Induction of systemic resistance in cucumber by hypovirulent binucleate Rhizoctonia against anthracnose caused by *Colletotrichum orbiculare* (manuscript ID:TLSR-OA-10-2017-0120.R1), kindly find attached a completed and signed Copyright Transfer Form.

We would like to express our sincere appreciation for the thorough revision, changes and comments. We are very grateful to your and the anonymous reviewers' time, great effort and

support on our manuscript. I have learned a lot from you and the reviewers about writing a manuscript.

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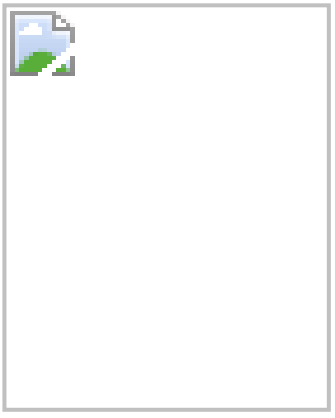

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TLSR 30(1) 2019 - Article 7

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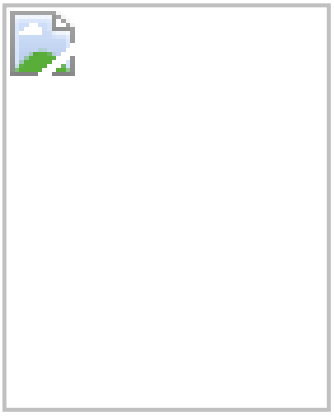

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Suwandi, Ph.D.
Phytopathology Laboratory, Department of Plant Protection
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Assalamualaikum boss, berikut ini saran kesimpulan papernya,
Cucumber anthracnose caused by Colletotrichum orbiculare could be effectively controlled using HBNR isolates through an induce systemic resistance mechanism.

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Wandi
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Dear Miss Suhana Sobi
Publication Officer TLSR USM,

Herewith we send our final revision of our article. After reading throughout our article, there are some minor revision we made in abstract and introduction. Please find the revision in the bubble comments in the article. We have inserted the conclusion.

Abstract
Revision 1:
"Treatment with hypovirulent binucleate Rhizoctonia (HBNR) isolates induced systemic resistance against anthracnose infected by Colletotrichum orbiculare in cucumber. This is because of the different distances between HBNR and C. orbiculare, where the root was treated with HBNR isolate and C. orbiculare was challenged and inoculated in leaves or first true leaves were treated with HBNR isolate and C. orbiculare was challenged and inoculated in second true leaves "

Please replaced with "**Treatment with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates induced systemic resistance against anthracnose infected by *Colletotrichum***

orbiculare **in cucumber, as there were no direct interaction between HBNR and *C. orbiculare*."**

Revision 2: Please delete "sum of"

Introduction:
"As soon as a plant is appropriately stimulated, its resistance is intensified against a test inoculation against a pathogen. This is the phenomenon of induced resistance. It can be localised, systematic, and induced with limited infection of virulent or hypovirulent pathogens, specific non-pathogenic bacteria, cell wall fragments, plant extracts, and certain chemicals (Van Loon 2000; Walters 2010)"

Please replaced with "**Induced resistance is the phenomenon in which a plant, once appropriately stimulated, exhibits an enhanced resistance upon challenge inoculation with a pathogen. It can be localized as well as systemic, and can be induced by limited pathogen infection, virulent or avirulent pathogens, certain non-pathogenic bacteria, cell wall fragments, plant extracts, and certain chemicals (Van Loon 2000; Walters 2010).**"

Thank you very much for publishing our paper in TLSR Journal.

Best Regards,
A. Muslim

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To: a. muslim unsri <a_muslim@unsri.ac.id>

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Tropical Life Sciences Research, 30(1), 107–120, 2019

Induction of Systemic Resistance in Cucumber by Hypovirulent Binucleate *Rhizoctonia* Against Anthracnose Caused by *Colletotrichum orbiculare*

[1]A. Muslim*, [2]Mitsuro Hyakumachi, [3]Koji Kageyama and [1]Suwandi Suwandi

[1]Department of Plant Protection, Faculty of Agriculture, Sriwijaya University, Jl. Raya Palembang-Prabumulih, Km. 32, Inderalaya, Ogan Ilir 30662, Indonesia
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To link to this article: https://doi.org/10.21315/tlsr2019.30.1.7

Abstract. Treatment with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates induced systemic resistance against anthracnose infected by *Colletotrichum orbiculare* in cucumber. This is because of the different distances between HBNR and *C. orbiculare*, where the root was treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in leaves or first true leaves were treated with HBNR isolate and *C. orbiculare* was challenged and inoculated in second true leaves. The use of barley grain inocula and culture filtrates of HBNR significantly reduced the sum of lesion diameter compared to the control (p = 0.05). The total lesion diameter reduction by applying barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results was also observed in treatment using culture filtrate, and the reduction of total lesion diameter by culture filtrate of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively. When cucumber root was treated with culture filtrates of HBNR, the lignin was enhanced at the pathogen penetration, which is spread along the epidermis tissue of cucumber hypocotyls. Peroxidase activity in hypocotyls in the treated cucumber plant with culture filtrates of HBNR significantly increased before and after inoculation of pathogens as compared to the control. Significant enhancement was also observed in the fast-moving anodic peroxidase isozymes in the treated plants with culture filtrates of HBNR. The results showed the elicitor(s) contained in culture filtrates in HBNR. The lignin deposition as well as the peroxidase activity is an important step to prevent systemically immunised plants from pathogen infection.

Keywords: Hypovirulent Binucleate *Rhizoctonia* (HBNR), Induced Systemic Resistance, *Colletotrichum orbiculare,* Cucumber

* Corresponding author: a_muslim@unsri.ac.id

INTRODUCTION

As soon as a plant is appropriately stimulated, its resistance is intensified against a test inoculation against a pathogen. This is the phenomenon of induced resistance. It can be localised, systematic, and induced with limited infection of virulent or hypovirulent pathogens, specific non-pathogenic bacteria, cell wall fragments, plant extracts, and certain chemicals (Van Loon 2000; Walters 2010).

Concerns about impacts of agrichemicals on food safety and the environment are related to the danger of the synthetic pesticide utilisation, leading plant pathologists to develop another sustainable control for managing plant disease (Hyakumachi *et al.* 2014). Elicitors of host resistance are a potential alternative control to plant diseases (Lyon *et al.* 1995).

Several investigations have reported that cucumber anthracnose caused by *Colletotrichum orbiculare* could be effectively control by endophytic *Streptomyce*s (Shimizu *et al.* 2009), Rhizobacteria, *Bacillus pumilus*, *Bacillus subtilis*, and *Curtobacterium flaccumfaciens* (Raupach & Kloepper 2000). Some studies show induced systemic resistance in cucumbers against antrachnose using biotic and abiotic elicitor. Meera *et al.* (1994) demonstrated that a sterile fungus and *Phoma* sp. were reliable in activating the systemic resistance of cucumber against the anthracnose disease. Koike *et al.* (2001) demonstrated that fungi isolated from zoysiagrass (*Zoysia tenuifolia*) rhizosphere (*Penicillium*, *Trichoderma*, *Phoma, Fusarium*, and a sterile fungus) significantly induced systemic resistance against cucumber anthracnoses. This is done through lignification enhancement and superoxide generation. Tian *et al.* (2008) reported that application of *Pieris rapae* extract onto the first true cucumber leaves effectively brought about systemic resistance against cucumber anthracnose with the enhancement of peroxidase and polyphenoloxidase. Lin *et al.* (2014) demonstrated that protein lysis buffer and a non-ionic detergent agent applied to separate cell membrane complexes (Nonidet P-40) is effective to weaken cucumber anthracnose by triggering genes related to disease resistance (peroxidase and pathogenis associated with protein 1-1a, acidic class III chitinase, phenylalanine ammonialyase 1). Research on hypovirulent binucleate *Rhizoctonia* (HBNR) as a potential biocontrol agent against *Fusarium* diseases in tomatoes and spinach have been recently reported in our investigations with a mechanism that might be induced resistance (Muslim *et al.* 2003a, 2003b, 2003c). There has also been an investigation of HBNR as an agent of induced systemic resistance (ISR) on beans against *Rhizoctonia solani* or *C. lindermuthianum* (Xue *et al.* 1998); they also protected cotton against alternaria leaf spot and rhizoctonia damping-off with ISR (Jabaji-Hare & Neate 2005). However, until now, there has been no report of the use of HBNR as an agent of ISR on cucumber against anthracnose pathogen *C. orbiculare* (= *C. lagenarium*).

In general, ISR in plants is clearly defined as a set of induced defense responses, including the creation of cell wall lytic enzymes. For example, 1,3-β-glucanases and chitinases (Lowton & Lamb 1987) enhance the activities of peroxidase and lignin deposition, callose, hydroxyproline-rich glycoprotein (Hammerschmidt & Kuc 1982; Hammerschmidt *et al.* 1982; Hammerschmidt *et al.* 1984); and phytoalexins (Ebel 1986).

Various agents both abiotic and biotic inducer (e.g., virulent or avirulent pathogens, nonpathogen microorganisms, cell wall fragments, plant extracts, and

synthetic chemicals) have been documented to induce resistance after challenging with pathogen attack, both locally and systemically (Walters *et al.* 2005). Plants possess various inducible defense mechanisms to protect themselves against pathogens. These defense mechanisms include preexisting physical and chemical barriers, as well as inducible defense responses. The pre-existing biochemical defense mechanisms include phenolics, phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, 5-alkylated resorcinols and dienes (Osbourn 1996). The inducible defenses include the production of reactive oxygen species (ROS), hypersensitive response, reinforcement of cell wall, phytoalexins production and pathogenesis-related (PR) proteins (Mellersh & Heath 2004).

This study aims to investigate HBNR capacity in inducing systemic resistance in cucumber against *C. orbiculare*. The study was designed to reveal if induced resistance in cucumber is correlated with enhanced systemic lignification and peroxidase activity.

MATERIALS AND METHODS

Isolates

Hypovirulent binucleate *Rhizoctonia* isolate of W1, W7 (AG-A), L1 (AG-Ba), and Rhv7 (unknown anastomosis group) obtained from soil samples were used as biocontrol agents. The pathogens used in this study were *Colletotrichum orbiculare* (Berk & Mont.) Arx (=*Colletotrichum lagenarium* (Pass.) Ellis & Halst.) isolate 104T, which were obtained from infected cucumber plants.

Plants

Throughout the experiment, cucumber cv. Gibai was used. Before the sowing, seeds were sterilised with 70% ethyl alcohol for one minute, and 1% of NaOCl for 20 min. Finally, they were rinsed in sterilised distilled water three times.

Inoculum Preparation

Isolates of pathogen *C. orbiculare* were cultured on potato dextrose agar (PDA) as long as seven days without exposure to light. The temperature was maintained at 25°C. A sterilised glass bar from the cultures with added sterile water, and scraped the spore suspensions. The spore suspension was then filtered through eight layers of sterile gauze. The isolates were set as two inoculum forms: barley grain inoculum and culture filtrate.

The following procedure was used for preparation of barley grain inoculum: Each isolate was cultured in PDA for three days without light and at room temperature. Five 5 mm mycelial disks of the culture were applied to 100 g of moist autoclaved barley grains (1:1, w/v dry barley grains/distilled water) collected in a 500 mL Erlenmeyer flask. The cultures were maintained and regularly shaken for 10 days at 25°C to produce well-colonized inoculum with HBNR. The inoculum was naturally dried for around 10 days. They were then kept refrigerated at 4°C until use.

The following procedure was used for the culture filtrate (CF): Two mycelial disks of each HBNR isolate obtained from the culture growing on PDA were put into a 20 mL flask with 50 mL of potato dextrose broth (pH 6.5). The isolates were grown in static conditions at 23-25°C for 10 days without light. The CF separated from the mycelia. Next, the CF was filtered three times over three layers of Whatman filter paper number 2. The CF was also filtered and sterilized using millipore filtration (0.45 µm Millipore filters, Millipore Products Division, Bedford, USA).

Cucumber ISR Assays

Experiments with barley grain inocula

Each sterilised plastic pot, sized ø 6 cm x 7.5 cm, was filled with the colonized barley grain inocula mixture (2%, w/w) with as much as 120 grams of potting medium. The previously-sterilised (with 0.5% NaOCl) cucumber seeds were added to the mixture. Each pot was given one seed. Next, the plants were cultivated at 25^{o}C. This required 21 days in a growth chamber with a 14 h light (24,000 lux) per dark period. The plants that were grown in the potting medium with untreated barley grain inocula were used as a control. Each inocula of HBNR isolate was inoculated on six plants as replication and the experiment was repeated twice.

Experiment with culture filtrates (CF)

The plastic pots (autoclavable, ø6 cm x 7.5 cm) containing about 120 g of potting medium were heated in autoclaves. The surface-sterilized cucumber seeds were sown in each pot. The plants were maintained in a similar manner as previously described. The first true leaves of 21-day-old cucumber plants were soaked with CF for 1 min. The plants were inoculated after 24 h of incubation. Each CF of HBNR isolate was applied on six plants as replication and the experiment was repeated twice.

Challenge inoculation

The second true leaves were inoculated with 20 individual drops (each drop was 10 µl) of spore suspension of *C. orbiculare* (5 x 10^5 spores/mL). The disk of lens paper (ø 5 mm) was covered on every drop toward the run-off prevention. This was done to ensure the distribution of equal numbers of spores along the leaf surfaces. The inoculated plants were maintained for 48 h without light at 25^{o}C in a humid chamber (85%-90% RH). After that, for six days the inoculated plants were brought to the growth chamber. The total number per leaf and diameter of lesion per inoculated drop were measured.

Testing for Lignin Formation

The cucumber seeds were grown on damp sterilized filter paper. Next, they were incubated for a week without light at 25°C. The roots of the seedlings were put in 5.0 mL of CF and incubated for one day. Then, with 10 µl drops of spore suspension (5 x 10^5 spores/mL) of *C. orbiculare,* the hypocotyls of the treated seedlings were inoculated. Next, the inoculated seedlings were incubated for 20 h. The epidermal strips of the seedling hypocotyls were stained with toluidine blue O or phloroglucinol-HCL (Sherwood & Vance 1976). They were observed under the microscope to reveal percentage of lignification.

Spores of *C. orbiculare* germinated 90% or more on cucumber hypocotyls. The degree of lignin deposition was evaluated by determining the percentage of germinated spores together with appressoria around which lignin depositions were induced. For each treatment 100 germinated spores were evaluated.

Protein Extraction and Determination

Treated cucumber root seedlings with CF of HBNR and challenge inoculated with *C. orbiculare* were prepared as described previously in section of testing for lignin formation. Samples were collected from seedlings prior to the time of challenge inoculation and again 8-48 h after the challenge inoculation. All samples were immediately frozen at -80°C until peroxidase assays were performed. Only the hypocotyls of the cucumber seedlings were used for protein extraction. These samples were homogenized in 5 ml of 0.05 M sodium phosphate buffer at pH 6.0 per 1 g sample with a cold mortar and pestle. The extract was centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was used to analyze the peroxidase activity. To determine the protein contents of these extracts, the Lowry method (Lowry *et al.* 1951) was used with bovine serum albumin as the standard.

Assay for Peroxidase Activity

Peroxidase activities were assessed following the method of Dalisay and Kuc (1995). They were determined using guaiacol, which acted as the hydrogen donor. The reaction mixture (3 mL) contained 0.25% (v/v) guaiacol in 1 mM sodium phosphate buffer at pH 6.0 with 100 mM hydrogen peroxidase. In order to catalyze the reaction, one-tenth ml crude enzyme extract was added and continued with colorimetrically at 470 nm min^{-1} mg^{-1} protein.

Detection of Peroxidase Isozymes by Gel Electrophoresis

Native PAGE was done with a PhastSystem (Pharmacia LKB, UK). Extracts were adjusted to the same protein concentration with phosphate buffer and then loaded onto an 8-25% gradient gel. A peroxidase isoenzyme was made visible by immersing the extracts in gels of 1% *o*-dianisidine solution. After 10 min, the gels were cleaned with distilled water. They were then placed into 0.06% H_2O_2 solution to concretely show the peroxidase isoenzyme bands.

Data Analysis

The experiments in this study were designed in completely randomised designs. Total lesion numbers, anthracnose lesion diameters, and lignin formation in this study were compared using Fisher's least significant difference (LSD) test at P = 0.05 and P = 0.01.

RESULTS

ISR in Cucumber Against Anthracnose with HBNR

This study showed that the use of barley grain inoculum and CF of HBNR isolates significantly (P = 0.05) decreased total anthracnose lesion diameter compared to the control (Table 1). However, no significant reduction was observed in total lesion number (Table 1). The reduction of total lesion diameter by barley grain inoculum of HBNR L2, W1, W7, and Rhv7 was 28%, 44%, 39%, and 40% respectively. Similar results were also observed in the treatment with CF; the reduction of total lesion diameter by CF of HBNR L2, W1, W7, and Rhv7 was 45%, 46%, 42%, and 48%, respectively (Table 1).

Table 1: Effect of hypovirulent binucleate *Rhizoctonia* (HBNR) isolates on the total lesion number and lesion diameter on leaves of cucumber plants that have been challenge inoculated with *Colletotrichum orbiculare*.

Treatments	Total lesion number[a]		Total lesion diameter (mm)[a]	
	BGI[b]	CF[c]	BGI	CF
Pathogen	19.5 [a]	16.8 [a]	123.5 [b]	89.3 [b]
HBNR L2	16.8 [a]	12.5 [a]	89.5 [a]	48.7 [a]
HBNR W1	16.0 [a]	13.1 [a]	68.7 [a]	48.1 [a]
HBNR W7	17.2 [a]	12.9 [a]	75.5 [a]	51.8 [a]
HBNR Rhv7	16.8 [a]	11.7 [a]	74.5 [a]	46.5 [a]

Notes: [a] Mean of two trials each with six plants per treatment. Values followed by the same letter do not differ significantly (P = 0.05) according to Fisher's least significant difference test.
[b] Plants were grown in potting medium amended with barley grain inoculum (BGI) of HBNR isolates (1%, w/w) for 21 days and challenge inoculated with 10 μl drops of 5 x 10^5 spores/ml of *C. orbiculare* at 20 locations on the second true leaves.
[c] The first true leaves of 21-day-old cucumber plants grown in potting medium were treated with culture filtrates (CF) of HBNR and challenge inoculated with *C. orbiculare* on the second true leaves as described above.

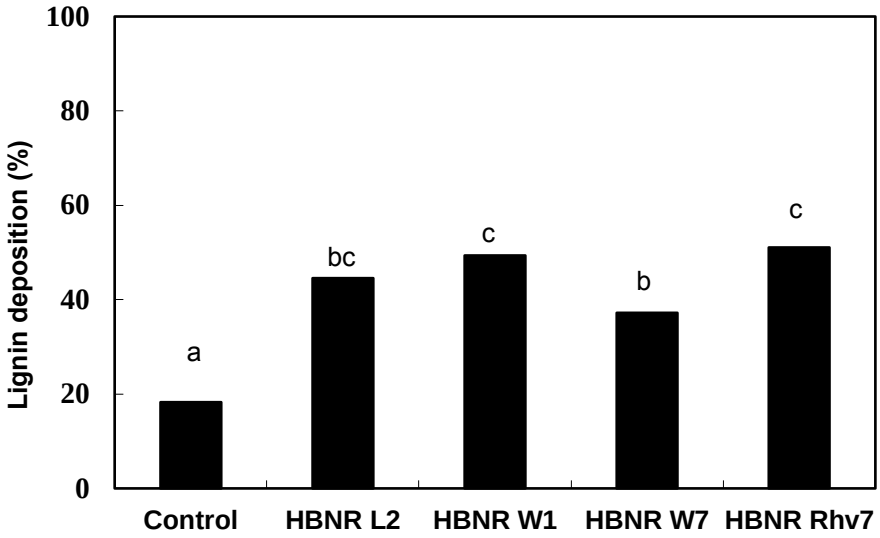

Fig. 1: Lignification of hypocotyls of cucumber seedlings induced by culture filtrates of hypovirulent binucleate *Rhizoctonia* (HBNR), following challenge inoculation with *Colletotrichum orbiculare*. Cucumber seedlings treated with sterilized distilled water were used as control. The hypocotyls of treated plants were challenged with 5 μl drops of 10^5 spores/ml of *C. orbiculare* at 10 locations. Bars labeled with the same letter are not significantly different according to Fisher's least significant difference test (P = 0.01).

Lignin Formation and Peroxidase Activities in Cucumber Hypocotyls Treated with HBNR

Lignin formation was observed as the intense blue and green colours of the lignified cell walls. Cucumber hypocotyls pretreated with CF of HBNR L2, W1, W7, and Rhv7 significantly increased lignin deposition in places that had been infected by *C. orbiculare* compared to the control treatment (Fig. 1). Cucumber seedlings treated with CF of HBNR L2, W1, W7, and Rhv7 increased lignin deposition by 1.45-fold, 1.71-fold, 1.04-fold, and 1.81-fold, respectively, relative to control.

Peroxidase activities in cucumber hypocotyls sampled at varying times before and after challenge inoculation were higher in the plant treated with HBNR compared to the control (Fig. 2). Treatment with HBNR L2, W1, W7, and Rhv7 increased peroxidase activities by 40%, 70%, 57%, and 81%, respectively, before inoculation of *C. orbiculare*, and by 39-64%, 33-94%, 43-58%, and 23-94%, respectively, relative to control after inoculation of *C. orbiculare*.

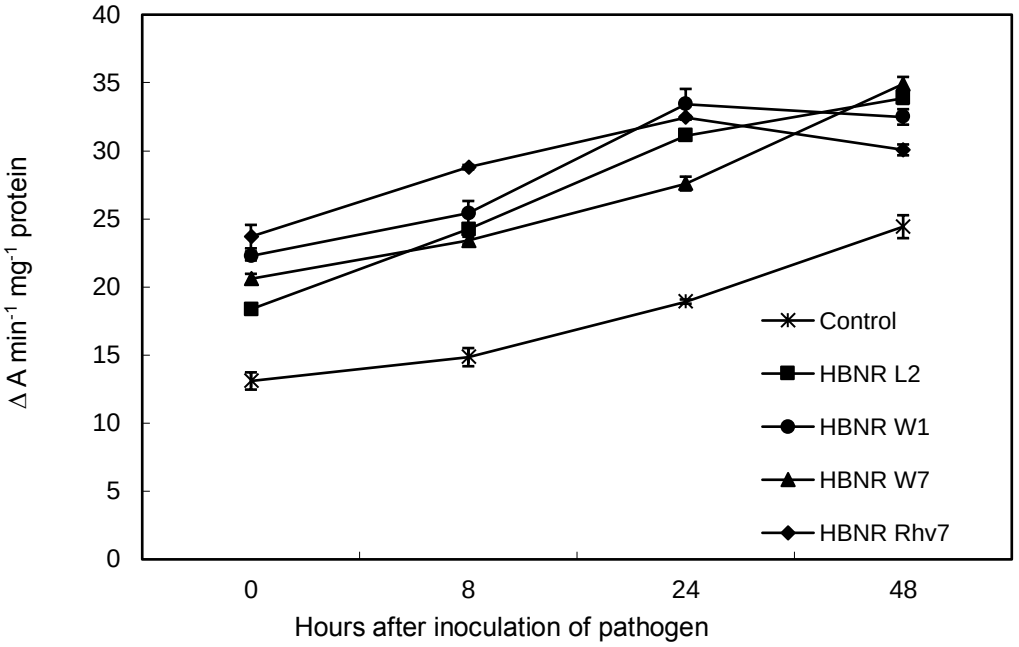

Fig. 2: Time course of peroxidase activity in hypocotyls of cucumber after treating the root with hypovirulent binucleate *Rhizoctonia* (HBNR) isolates and challenge inoculating with *C. orbiculare*. Peroxidase activity is expressed as changes in absorbance min^{-1} mg^{-1} protein. Cucumber seedlings treated with sterilized distilled water were used as control. Data are the mean of three replications with five seedlings (cucumber) per replication. Bars represent standard error of the mean. 0 h indicates time before pathogen inoculation.

Two peroxidase isozymes (isoforms 1 and 2) were found in cucumber hypocotyls. The fast-moving anodic peroxidase isozymes were enhanced gradually after challenge inoculation. The peroxidase activities increased in the isoform 2 in the seedlings treated with HBNR compared to the control, at all sampling times, according to band intensity and width (Fig. 3). Isozyme type 1 had a minor activity band and was observed after 48 h of pathogen inoculation either on inoculated or non-inoculated with pathogen.

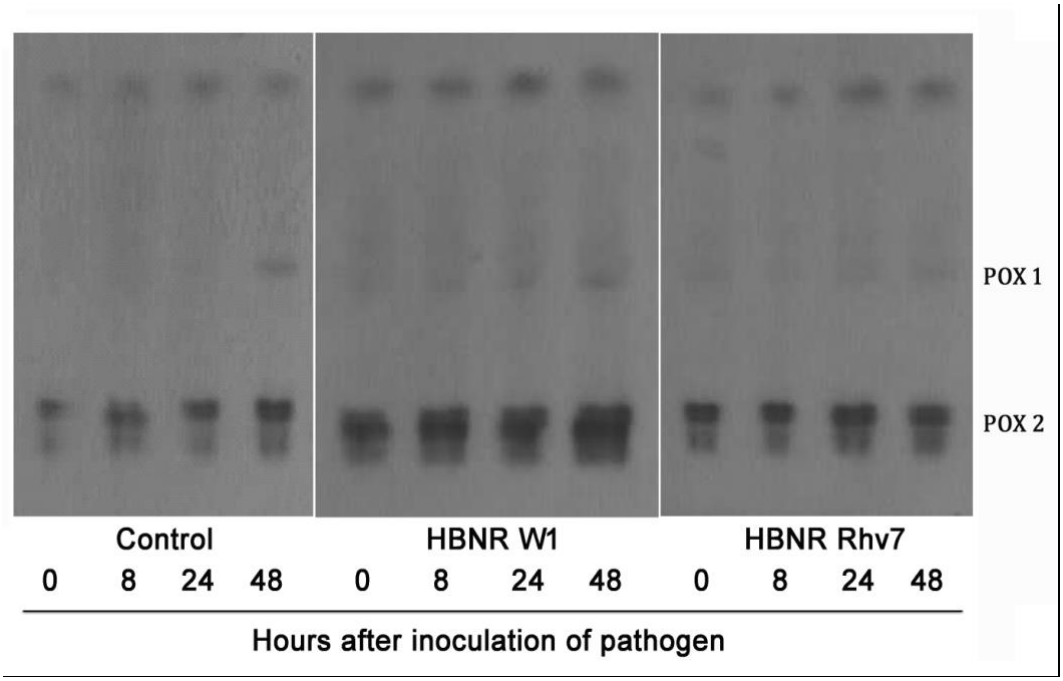

Fig. 3: Electrophoresis patterns of peroxidase isozyme (POX 1 and POX 2) cucumber seedlings treated with hypovirulent binucleate *Rhizoctonia* (HBNR) (Protein concentration was 0.1 mg/mL). Cucumber seedlings treated with sterilized distilled water were used as control.

DISCUSSION

This study reveals that treatment with HBNR isolates suppresses disease development of anthracnose in cucumber. The disease development suppression seemingly resulted from plant's ISR, as separated inoculation sites between HBNR and *C. orbiculare*, where the root was employed with HBNR isolates, and *C. orbiculare* was inoculated on the leaves, or the first true leaves were treated with HBNR isolates and *C. orbiculare* was challenge inoculated on the second true leaves. Thus, HBNR and pathogen application sites were separated spatially, and no HBNR isolates could be recovered from the second true leaves. The result of this research supports the evidence that the mechanism of protection from *R. solani* by HBNR is induced resistance (Cardoso & Echandi 1987; Poromarto *et al.* 1998).

A report presented by Xue *et al.* (1998) showed that inoculation of bean hypocotyls with HBNR induced systemic resistance and protection of the roots and cotyledon to later challenges not only with the root rot pathogen *R. solani* but also with the anthracnose pathogen *C. lindemuthianum*. This study applied HBNR as barley grain inoculum, and CF induced systemic resistance in cucumber plants against *C. orbiculare*. Similar methods were used by Meera *et al.* (1994) and Koike *et al.* (2001), in which plant-growth-promoting fungi (PGPF) were applied at the root as barley grain inoculum, mycelia inoculum, or culture filtrates. This induced systemic resistance in cucumber after being challenged

with *C. orbiculare* in leaves. Another study reported that germinating tomato seeds for one week in chemicals of b-aminobutyric acid (BABA) and jasmonic acid (JA) solutions promoted seed germination efficiency and induced resistance in four-week-old plants (Luna *et al.* 2016).

In this study, when HBNR CF was applied at the cucumber roots, lignin was enhanced at the attempted penetration by the pathogen in the epidermal tissues of cucumber hypocotyls. Enhanced lignin deposition was positively correlated with significant reduce of lesion development. Lignin may improve plant resistance against fungal infection through enhanced physical barrier and chemical direct toxicity through their toxic derivatives such as phenolic compounds (Xue & Yi 2017). Our results also show that peroxidase activity in hypocotyls in the treated cucumber plant with HBNR significantly increased before and after inoculation of the pathogen compared to the control. Significant enhancements were also observed in the fast-moving anodic peroxidase isozymes (isoform 2) in the plants treated with HBNR. Isoform 1 may have less significant role in induce resistance since it showed a minor activity and found on both inoculated and non-inoculated hypocotyl. This supports the finding by Xue *et al.* (1998) that inoculation of bean hypocotyls with HBNR induced systemic resistance, and this was positively correlated with peroxidase. Arora and Bajaj (1985) and Krstic *et al.* (1997) also reported that infection of mung bean and strawberry with binucleate *Rhizoctonia* resulted in an increase in peroxidase activity. Peng *et al.* (2004) found that pretreated cucumber seedlings with pectinase extract derived from *Penicillium oxalicum* BZH-2002 fermentation products resulted in induced resistance toward cucumber scab *Cladosporium cucumerinum* through the increased defense-related enzymes, polyphenol oxidase, and peroxidase.

Increased peroxidase activity is also well observed in rhizobacteria-induced systemic resistance. Chandrasekaran and Chun (2016) demonstrated that treating tomato plants with *Bacillus subtilis* CBR05 significantly enhanced the activities of antioxidant enzymes including peroxidase. Yanti (2015) reported that rhizobacteria enhanced peroxidase enzyme activity. The isolate PK2Rp3 (*Serratia marcescens* strain N2.4) showed the highest activity of both roots and leaves of 0.058 $\mu g \cdot mL^{-1}$ and 0.053 $\mu g \cdot mL^{-1}$.

According to Dean and Kuc (1987) and Hammerschmidt *et al.* (1984), lignin deposition was considered a crucial phase of pathogen suppression in systemically immunised plants. Vance *et al.* (1980) reported that rapid lignin deposition might lead to the production of chemical or physical barriers to pathogen infection. Furthermore, peroxidases accelerate the ending polymerization step of lignin synthesis, resulting in the enhanced capability of protected tissue (Gross 1979). In the other studies, the enhanced peroxidase activities are often related to resistance phenomenon such as the production of lignin (Hammerschmidt & Kuc 1982; Ride 1975). Peng and Kuc (1992) discovered the implications of peroxidase toward oxidative defense mechanisms in treated plants with infections. The peroxidase-generated hydrogen peroxidase directly functions as an antimicrobial agent. In our study, when plant treated with barley grain inoculum and CF of HBNR isolates, significant reduction was observed in total lesion diameter. However, no significant reduction was observed in total lesion number. This result indicated that increased lignification and peroxidase activities observed in this study did not restrict total penetration of

C. orbiculare. The data suggest that the involvement of other defense mechanism(s) acting at the level of restricting lesion development to fungal infection. Lignification and peroxidase activities, alone or collectively, are not sole determinants for induced systemic resistance. Van Loon (2000) indicated that induced resistance is the result of multi-mechanisms. Therefore, it is necessary to investigate further other mechanisms, alone or collectively, involved in systemic resistance against *C. orbiculare*. Further research is needed to identify other PR-proteins that may be involved in the mechanism of cucumber ISR from HBNR.

The abilities of HBNR isolates to induce systemic resistance in cucumber against anthracnose and to enhance lignin deposition and peroxidase activity as well as their effectiveness against *Fusarium* diseases in tomato and spinach in our previous studies (Muslim *et al.* 2003a, 2003b, 2003c), shows significant potential as a bio-control agent to manage *Colletotrichum orbiculare* and other diseases.

CONCLUSION

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