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Molecular identification and characterization of isolates of *Trichoderma* sp. indigenous Southeast Sulawesi

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This research aims to determine the certainty of species and the relationship isolate of *Trichoderma* sp. indigenous Southeast Sulawesi with another species of Trichoderma.The research was conducted in Plant Protection Laboratory, Faculty of Agriculture, Halu Oleo University and Plant Health Clinic Laboratory, Department of Pest and Plant Disease, Faculty of Agriculture, Gadjah Mada University. 11 isolates of *Trichoderma* sp. indigenous Southeast Sulawesi were used in this research namely DPA, LKP, APS, LPS, LTB, DKP, DKT, LKA, ASL, BPS and LKO. Molecular characterization used PCR technique where DNA extraction was carried out using the CTAB and primers ITS-1 and ITS-4, then Phylogenetic tree analysis using CLUSTALW. PCR results used ITS-1 and ITS-4 primers showed from 11 isolates of *Trichoderma* sp. indigenous Southeast Sulawesi have tested only six isolates namely LKP, DKP, LKA, ASL, BPS and LKO which showed the presence of DNA bands. BLAST analysis result showed that five isolates namely LKP, DKP, ASL, BPS, and LKO had the highest similarity with *Trichoderma asperellum*, while Isolate LKA had similarities with *Meyerozyma guillermondii*. Philomolecular analysis result showed that isolates of Trichoderma indigenous Southeast Sulawesi DKP had 100% similar fragments of Internal Transcribed Spacer (ITS) with *Trichoderma* sp. isolate from Riau (Indonesia), LKP isolate had 100% similar fragments of Internal Transcribed Spacer (ITS) with *Trichoderma* sp. isolate from China, and 26R/LKO isolate had 100% similar fragments of Internal Transcribed Spacer (ITS) with *Trichoderma* sp. isolate from Bogor (Indonesia).Based on molecular characterization, six isolates of *Trichoderma* sp. indigenous Southeast Sulawesi were identified as *Trichoderma asperellum* and *Meyerozyma guilliermondii*.

Keywords: Indigenous Southeast Sulawesi, molecular characterization, PCR, phylogenetic, *Trichoderma* sp.,

INTRODUCTION

Trichoderma sp. is a microorganism that will found around rhizosphere of plants, and one of the biological agents that have been widely used to control various disease-causing by pathogens in plants. They have a high antagonistic ability to fungal species, especially for pathogenic fungi. Trichoderma species (teleomorph hyprocrea) are flamentaous fungi of worldwide distribution, including agricultural habitats, which are well-known biological control agents against plant pathogenic fungi, oomycetes and even nematodes(Lorito et al., 2010; Monte 2001). The mechanisms of Trichoderma-based biocontrol rely mainly on the production of antibiotics and/or hydrolytic enzymes, as well as competition for

nutrients and the systemic activation of plant defense responses(Harman et al. 2004; Hermosa et al. 2000. Most of the Trichoderma strains used as active ingredient in commercial biocontrol products belong to selected rhizosphere-competent species such as *Trichoderma harzianum, Trichoderma atroviridae, Trichoderma asperellum* and *Trichoderma virens* (Druzhinina et al.,2011).

Trichoderma sp. produces chitinase enzymes which can degrade pathogenic fungus cell wall which consists of chitin (Gruber et al., 2011). The effectiveness of the use of *Trichoderma* sp. in controlling plant diseases depends on the source or area of origin of the antagonist agents. Erwanti reported that biological control is locally specific, where that antagonistic microorganisms found in an area will provide good results only in their origin (Erwanti 2003). Ecological fitness is an important trait of any Trichoderma strain to establish in agricultural habitats like soil, plant rhizosphere or compost materials (Weaver et al., 2005).

The survival and spread of Trichoderma in an agricultural habitat depend on its interactions with the environmental parameters as well as the biotic components of the local ecosystem (Kredics *et al.* 2003). *Trichoderma* sp. which has recently been shown to induce local and systemic defense responses in plant (Yedidia et al., 2000).

Exploration results have been successfully obtained 11 *Trichoderma* sp. isolates from prove from research previously(Gusnawaty & Taufik 2012). Furthermore, the 11 isolates of *Trichoderma* sp. has been tested for its potential as an antagonistic agent against *Phytophthora capsici* in vitro and against *Fusarium oxysporum* in vivo, and also against *Colletotrichum* sp. Based on morphological characterization results, there are five species of *Trichoderma* sp. namely *T. hamantum*, *T. koningii*, *T. harzianum*, *T. polysporum,* and *T. Aureoviride* (Gusnawaty et al., 2013a; Gusnawaty et al ,2013b; Gusnawaty et al. 2014a; Gusnawaty et al., 2014b). However, morphological identification has not provided certainty of species or kinship between species of *Trichoderma* sp. so that molecular methods are needed for the determination of species including *Trichoderma* sp. appropriately(Kubicek et al., 2003).

The molecular analysis was developed by Druzhininna and team using ribosomal DNA sequences (rDNA) in the area of Internal Transcribed Spacer (ITS) of *Trichoderma* sp. Sequencing of ribosomal DNA of *Trichoderma* sp. expects to be used for phylogenetic analysis(Druzhininna et al., 2005). This analysis was used to determine the relationship between species of Trichoderma isolates. The high genetic diversity of a population can occur due to mutations, gene recombination, sexual and parasexual reproduction, election factors, and gene migration from one place to another.

Morphological identification and classification have not provided species certainty, therefore it is necessary to molecularly identify of *Trichoderma* sp. and relationship (evolutionary) of each *Trichoderma* sp. isolate indigenous Southeast Sulawesi with another species.

MATERIALS AND METHODS

This research was conducted in Laboratory of Plant Protection, Faculty of Agriculture Halu Oleo University and Laboratory of Plant Health Clinic Department of Pest and Plant Disease, Faculty of Agriculture Gadjah Mada University at April to December 2015. Materials and tools were used in this research included *Trichoderma* sp. isolates indigenous Southeast Sulawesi, *Cetyltrimethyl ammonium bromide* (CTAB) 2% 700 mL, kuarsa sand, Chloroform isoamyl alcohol (CIAA), ethanol or alcohol absolute, alcohol 70%, aquabides, agarose, PCR kit (Gotaq Green), PDA (Potato Dextrose Agar) and PDB (Potato Dextrose Broth) medium, Primers ITS-1 and ITS-4, and TaKaRa PCR Thermal Cycler.

Isolation procedure

Trichoderma sp. isolates were growth on PDA medium and incubated for 7 days at 28^{o}C.

Propagation of mycelium for DNA extraction

DNA extraction begins with the preparation of *Trichoderma* sp. isolates on PDA medium then the culture was included in 50 mL of Potato Dextrose Broth (PDB) medium on an Erlenmeyer 100 mL and incubated in a rotary shaker for 4 days at 28^{o}C(Gusnawaty & Taufik 2012). Isolates that have been growth are separated from the PDB media by filtering using filter paper.

DNA Extraction

DNA extraction of *Trichoderma* sp. used CTAB method, begins with weight 0.5 gram of mycelium. Mycelium was crushed with porcelain mortar until completely smooth by adding 700 µl 2% CTAB (CTAB, Tris HCl 1M, EDTA 0.5 M, 5M NaCl, 1-B Mercato-ethanol, sterile Aquades) and a little sterile quartz sand for made more smooth. The finely scoured mycelium was transferred to

the Eppendorf tube to be heated in a 65ºC water bath for 30 minutes and shake every 10 minutes for 1 minute. After that, centrifuged for 5 minutes at a speed of 5000 rpm, the supernatant (the top clear liquid) was transferred to a fresh Eppendorf tube and added a full tube of CIAA (Chloroform: Isoamyl-Alcohol, 24:1). Then centrifuged at a speed of 12000 rpm for 10 minutes, and the supernatant contained in the tube was transferred to a fresh Eppendorf tube and added to the full tube of absolute ethanol and stored in a refrigerator at -20ºC for a night. The next day, the sample was taken and centrifuged at a speed of 12000 rpm for 10 minutes at room temperature. Then the ethanol in the top layer is poured slowly, not to let the pellets come wasted. Then, filled with 70% ethanol full tube. Shaken the tube first for 1-3 minutes and centrifuged again at a speed of 12000 rpm for 10 minutes at room temperature. After that, the top is discarded slowly not to let the pellets get wasted. Then, dried the tubes in the Laminar air flow for ±2 hours. After dried, DNA pellets were dissolved in aquabides suspension and stored in a refrigerator at - 20ºC. The extraction results are seen through electrophoresis on 0.8% gel agarose with ethidium bromide dye to ensure DNA was present in the pellet (Lestiyani 2015).

Molecular Characterization with PCR technique

DNA extraction results were amplified by PCR technique using ITS primers 5'-TCCGTAGGTGAACCTGCGG-3 '(ITS1) from 18S rDNA and 5'-TCCTCCGCTTAATTGATATGC-3' (ITS4) from 28S rDNA sequences(Gusnawaty et al. 2013a). PCR was carried out at 25 µl of reaction mixture consisting of 10 vM from both primers, 12.5 µl of PCR (Gotaq green) kit and 1 µl of template DNA. The PCR cycle used was initial denaturation for 5 minutes at 94ºC, denaturation for 1 minute at 94ºC, annealing for 2 minutes at 55ºC, and extension for 3 minutes at 72ºC and final extension for 5 minutes at 72ºC, followed by 35 cycles. The amplicon was electrophoresis on 1.2% agarose gel and visualized with Ethidium Bromide (EtBr) staining (Lestiyani 2015).

For the sequenced procedure, the PCR product was purified and then added 20 µl of product PCR with 0.66 µl 3M CH3COONa and 0.2 µl Ethachinmate (Nippon gene), added 40 µl of absolute ethanol then centrifuged at 12000 rpm for 5 minutes at 4ºC. After that, the supernatant is discarded slowly and added 100 µl of 80% ethanol then centrifuged again at a speed of 12000 rpm for 5 minutes at 4ºC. The supernatant is discarded and the pellet obtained is air dried. After drying, add 15 µl dH2O and added BigDye Terminator Cycle v3.1 sequencing (Applied Biosystems) which consisted of 1 µl buffer sequence and 0.4 µl RR24 on products with a concentration of 20 ng. Then added 1 µl of specific primer (ITS1 and ITS4) and added dH2O to a volume of 10 µl, followed by a cycle at 96ºC for 1 minute, and 30 cycles at 96ºC for 30 seconds, 55ºC for 15 seconds and 60ºC for 4 minutes. The results of sequencing product 10 µl were purified with 20 µl of absolute ethanol and 1 µl of 3M CH3COONa then incubated for 30 minutes at -80ºC and centrifuged at 13000 rpm for 10 minutes at 4ºC. The supernatant was discarded slowly and added 100 µl of ethanol 80%. Then centrifugation again at a speed of 13000 rpm for 5 minutes at 4ºC, and dried it. Samples were resuspension with deionized formamide, and heated for 2 minutes. Mounted tray on the plate and placed in the Abi Prism 3100 molecular analyzer (Applied Biosystems) for reading the samples (Klug and Cumming 2003). The sequences results are read with Finch TV version 1.4.0 and the search for a similar sequence is performed by using BLAST (http://blast.ncbi.nlm.nih.gov) (Hall 2004).

Phylogenetic Analysis

The analysis used CLUSTALW both forward and reverse nucleotides are sequenced and paired, after completion, the nucleotide bases are analyzed with CLUSTALW (http://www.genome.jp/tools/clustalw/).

Sequences that have been aligned are analyzed together with the outgroup sequence of *Trichoderm*a sp. The philomolecular analysis is carried out used distance method (Lestiyani 2015).

RESULTS

Eleven isolateshave been isolated from some District in Kendari, Southeast Sulawesi (Table 1), and have done for morphological identified as *Trichoderma* sp., and then isolates were PCR with primers ITS-1 and ITS-4. The results of the amplification showed that there were six positive isolates included in the Trichoderma species which appeared at 500 bp namely LKP, DKP, ASL, BPS, LKO and LKA (Fig. 1).The six isolates were then sequenced, and the results of the blast analysis showed that LKP, DKP, ASL, BPS and LKO isolates have the highest homology with *Trichoderma asperellum* and LKA has the highest homology with *Meyerozyma guillermondii* (Table

2). Based on GenBank data of BLAST analysis (http://www.ncbi.new.nih.gov/BLAST), the phylogenetic tree result showed isolates of *Trichoderma* sp. indigenous form Southeast Sulawesi isolate DKP has proximity to Riau isolates (Indonesia), LKP isolates have proximity to Chinese isolates and LKO have proximity to isolates from Bogor (Indonesia) (Fig. 2).

Table 1:Eleven Indigenous isolates that have antagosnism abilty to some pathogenic fungi, and was isolated from some vegetation, in some District in Kendari, Southeast Sulawesi.

No.	Isolates Name	Origin Place	Vegetation
1	DPA	Duriasi, Konawe	Bitter melon
2	LKP	Tirawuta, Kolaka	Cowpea
3	APS	Ameroro, Konawe	Paddy
4	LPS	Tirawuta, Kolaka	Paddy
5	LTB	Lamooso, South Konawe	Sugarcane
6	DKP	Duriasi, Konawe	Cowpea
7	DKT	Duriasi, Konawe	Cucumber
8	LKA	Ngapa, North Kolaka	Cocoa
9	ASL	Besulutu, Konawe	Black pepper
10	BPS	Baruga, South Konawe	Paddy
11	LKO	Ngapa, North Kolaka	Cocoa

Source :Gusnawaty&Taufik (2012); Gusnawaty&Taufik (2013).

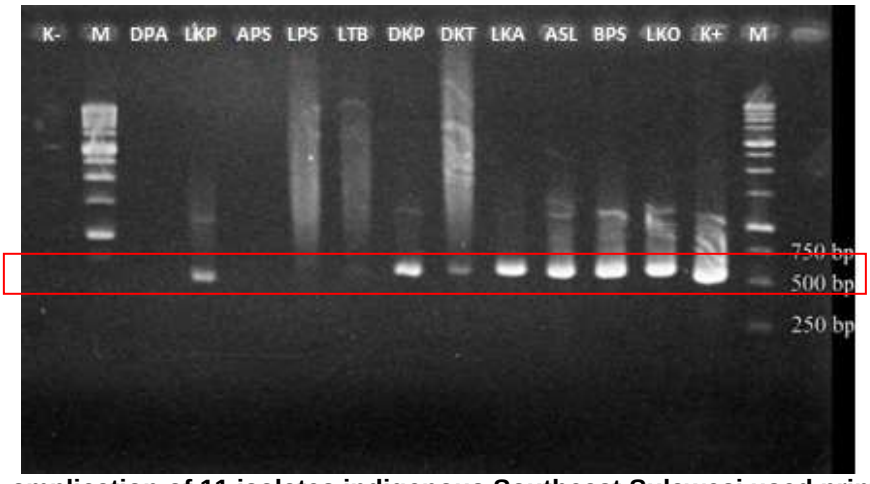

Figure 1;DNA amplication of 11 isolates indigenous Southeast Sulawesi used primers ITS-1 and ITS-4, with explanation M = Marker, K- = negative control, K+ = positive control. From 11 isolates, six isolates LKP, DKP, LKA, ASL, BPS and LKO have positive amplification with DNA band appear on 500 bp.

Table 2: BLAST-n analysis of sequencing result of six isolates

No	Isolate name	BLAST Result (*highest score*)	Identity (%)	Accession number
1	LKP	*Trichoderma asperellum*, ITS1	100	KC859432.1
2	DKP	*Trichoderma asperellum*, ITS1	100	KC859432.1
3	LKA	*Meyerozyma guillermondii*, ITS1	97	KM014576.1
4	ASL	*Trichoderma asperellum*, ITS1	100	KC859432.1
5	BPS	*Trichoderma asperellum*, ITS1	100	KC859432.1
6	LKO	*Trichoderma asperellum*, ITS1	99	KP784423.1

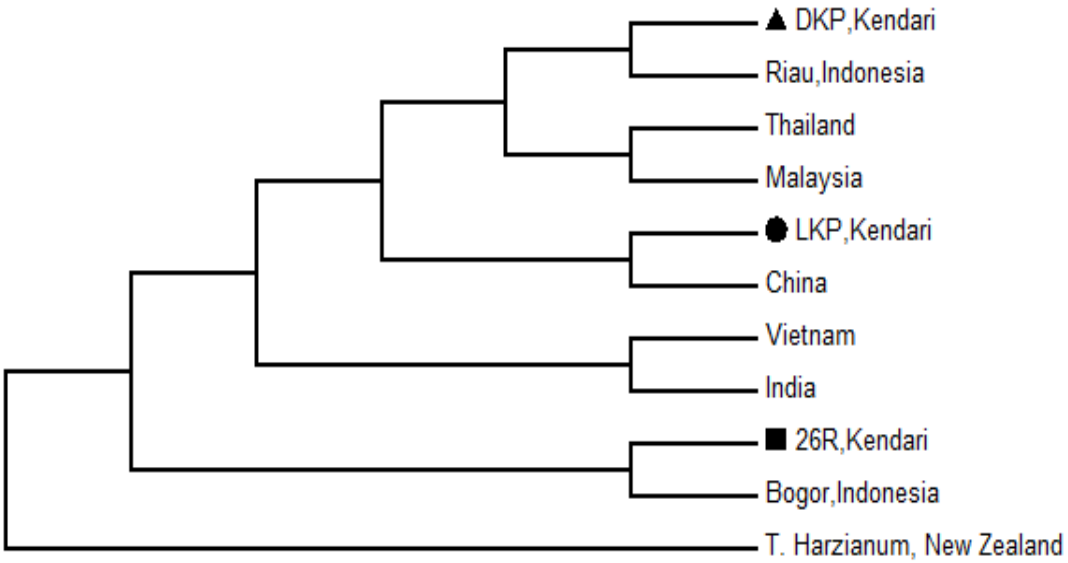

Figure 2;Phylogenetic tree analysis of *T. asperellum* indigenous from Southeast Sulawesi with *Trichoderma* sp. from another region. With explanation ▲= DKP isolate, ● = LKP isolate and = 26R(LKO) isolate. ■

DISCUSSION

Trichoderma commonly available in the soil and root ecosystems has gained immense importance as biological control ability against several plant pathogens(Kubicek & Harman 1998). The DNA prosses characteristics that are suitable for the identification of fungal isolates at the species level. The molecular techniques have been used in Trichoderma research, in order to species identification dan characterization. This rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome(Hibbett 1992). They also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies (Bruns et al. 1991; CW Magill 1992).

Identification of a species in precession is an important first step, including the species of Trichoderma sp. Morphological characterization carried out often mistakenly identified of the species. Along with the development of molecular technology is very helpful in ensuring a species such as Trichoderma sp. The next step was BLAST (Basic Local Alignment Search Tool) used for surely that primers used can cut the right region or ITS fragment on Trichoderma sp. and also for identification and compare interspecies of Trichoderma sp. from some country which was saved on NCBI database and has a genetic relationship with Trichoderma sp.

The results of this study showed that of the eleven isolates were tested used primers ITS-1 and ITS-4 successfully identified six isolates were amplified based on DNA bandsappera in 500 bp. Five isolates successfully identified as T. asperellum and one isolate namely M. guillermondii. The six isolates have been tested based on the morphological identification and in vitro test that has been done previously, they also have antagonistic abilities againts several pathogenic fungi such asPhytophthoracapsici, FusariumoxysporumandColletotrichum sp. (Gusnawaty et al., 2013a; Gusnawaty et al., 2013b; Gusnawaty et al., 2014a; Gusnawaty et al., 2014b).

Trichoderma asperellum can multiply rapidly in the rhizosphere, and a parasitic fungus that can attack and take nutrients from other fungi. The use of T. asperellum based products has grown exponentially in the las few years since this species was included in the list of biocontrol microorganisms for registration proposes in the European Union, as well as in Australia, Canada, China, India, New Zealand, South Africa, and the

USA. T. asperellum was split from Trichoderma viridae species complex using DNA sequencing(Lieckfeldt et al. 1999). More recently, multilocus genealogies of four genomic regions showed that T. asperellum sensu lato consists of two cryptic species,T. asperellum, and Trichoderma asperelloides(Samuels et al., 2010). Strains identified as T. asperellum have been shown a wide spectrum for plant disease control (Mbarga et al., 2012; Narasimha-Murthy et al., 2013; Trillas et al., 2006).

Dewi states that forward and reverses primers function to limit the area of DNA to be amplified. The ITS-1 and ITS-4 primers are base markers in the core genome region of the rDNA coding(Dewi 2012). PCR results from 11 isolates of *Trichoderma* sp. showed there were only six isolates which showed good and uncontaminated quality single band (Fig. 1.). The six isolates namely LKP from Kolaka, DKP from Konawe, LKA and LKO from North Kolaka, ASL from Konawe, and BPS from Konawe, all amplification fragments showed up around 500 bp. The unsuccessful amplification of the 11 Trichoderma isolates tested may be caused by various factors such as some isolates not the genus of Trichoderma or DNA damage and contamination during the extraction process to PCR. The impure DNA could interfere with the primary attachment of the site and would inhibit the activity of polymerase enzymes which function to DNA polymerize(Runtunuwu and Hartono 2002). To validate BLAST results, sequence analysis using phylogenetic trees was carried out. Phylogenetic relationship analysis and intergeneric level using this approach have a higher level of accuracy than BLAST(Brinkman and Leipe 2002).

Phylogenetic analysis sequences of all *Trichoderma* sp. indigenous of Southeast Sulawesi was carried out by comparing them with *Trichoderma* sp. sequences from other regions those in Genebank. Comparison of the sequence of *Trichoderma* sp. can be known by aligning all the sequences to be analyzed first. Alignment is carried out with the aim to determine the homology level of the DNA sample base sequence analyzed by comparison species. Furthermore, the ITS DNA sequence alignment results were analyzed using the MEGA 6.0 program to reconstruct phylogenetic trees. Then, data were analyzed using the Neighbor-Joining method. This is because Neighbor Joining can be used on all data regardless of evolutionary distance. Saitou and May suggested that Joining Neighbor could show phylogenetic trees with the principle of minimum evolution and efficient in choosing the correct topology(Saitou and Nei 1987).

The genetic distance indicates the level of gene differences between populations or species. Paramarta reported that genetic distance can indicate whether or not the genetic relationship between nucleotide sequences which is observed(Paramanta 2014). Isolate DKP indigenous from Konawe Kendari Southeast Sulawesi have 100% similarities of ITS fragments with *T. asperellum* isolate from Riau (Indonesia), isolate LKP indigenous from North Kolaka Kendari Southeast Sulawesi have 100% similarities of ITS fragments with *T. asperellum* isolate from China, and 26R/LKO indigenous from North Kolaka Kendari Southeast Sulawesi have 100% similarities of ITS fragments with *Trichoderma* isolate from Bogor (Indonesia).

The genetic diversity of *T. asperellum* is due to differences in area and vegetation of sampling. According to Donald, high genetic diversity of a population can occur due to mutations, gene recombination, sexual and parasexual reproduction, selection factors, and gene migration from one place to another(McDonald 1997). The results of the analysis showed that all isolates clustered randomly because the pattern of distribution was not influenced by geographical distribution. This is read from the dendrogram that isolates from one region (indigenous Southeast Sulawesi) which turned out to have a close distance to other regions. Based on the phylogenetic tree, geographically isolated Southeast Sulawesi was related and one group with isolates from other regions.

CONCLUSION

Based on research it concluded that molecular detection of *Trichoderma* sp. Southeast Sulawesi indigenous can be amplified with ITS-1 and ITS-4 primers with 500 bp amplicons, and the result of the phylogenetic analysis showed five isolates LKP, DKP, ASL, BPS, and LKO were close to *Trichoderma asperellum* and LKA closed to *Meyerozyma guillermondii*. Isolate DKP indigenous Southeast Sulawesi had 100% similar fragments of Internal Transcribed Spacer (ITS) with *Trichoderma* sp. isolate from Riau (Indonesia), LKP isolate had 100% similar fragments of Internal Transcribed Spacer (ITS) with *Trichoderma* sp. isolate from China, and 26R/LKO isolate had 100% similar fragments of Internal Transcribed Spacer (ITS) with *Trichoderma* sp. isolates from Bogor (Indonesia).

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

GHS designed and performleed the experiments and also wrote the manuscript. MT, R and LOSB collected sample, molecular analysis and data analysis. NPP reviewed the manuscript. All authors read and approved the final version.

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