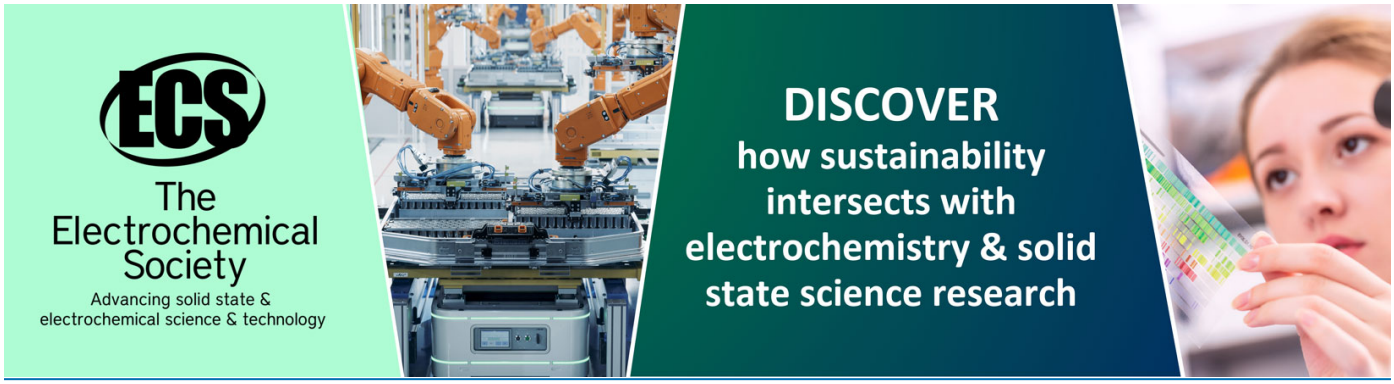
Fungi identification and test on red calliandra (*Calliandra calothyrsus*) seed as potentially antagonist agent



Fungi identification and test on red calliandra (*Calliandra calothyrsus)* seed as potentially antagonist agent

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Abstract. Red calliandra (*Calliandra calothyrsus)* is an essential economic plant and used as a renewable energy source. Seed-borne fungi can be beneficial or detrimental. One of the beneficial roles of fungi is as an antagonistic agent. Red calliandra seed is commonly infected by pathogenic fungi such as *Fusarium* sp., *Aspergillus* sp., and *Rhizopus* sp. The purpose of this research was to identify the fungi in red calliandra seeds, which are potential as an antagonistic agent, and to study their ability to inhibit the growth of pathogenic *Fusarium* sp., A*spergillus* sp. and *Rhizopus* sp. The seeds obtained are from Sukabumi, West Java, Indonesia. Fungi were isolated by soaking the seeds with 1% NaOCl for three minutes then washed with sterile aquadest. It was repeated three times. Furthermore, the seeds were sown on moist paper media and incubated until a fungus colony emerged, which is expected to be a potential antagonistic agent. The result showed that there was one isolate of fungi that was potential as an antagonistic agent; it was *Talaromyces* sp. It indicates that the antagonistic agent can be isolated from seeds. The inhibition percentages of *Talaromyces* sp. against *Aspergillus* sp., *Fusarium* sp., and *Rhizopus* sp. at seven days old were 67.92%, 59.4%, 8.89%, respectively. *Talaromyces* sp. can inhibit the growth of *Fusarium* sp. *Rhizopus* sp. and *Aspergillus* sp. in overgrowth at 14 days old. The antagonistic mechanism of *Talaromyces* sp. to *Aspergillus* sp., *Fusarium* sp. and *Rhizopus* sp. is microparasitism and competition. *Talaromyces sp.* isolated from red calliandra seeds were potential as antagonistic agents.

1. Introduction

Red calliandra (*Calliandra calothyrsus*) as one of the legume plants, has much utility, such as for fodder, apiculture, fuel, fiber, erosion control, shade, nitrogen-fixing, soil improver, ornamental, boundary, and also suitable for intercropping [1]. This species is an essential economic plant and used as a renewable energy source. For these reasons, this plant has been developed in the community forest. In planting programs, the limiting factor is disease in seeds, seedlings, plants and post-harvest products

These diseases can harm the economy and need to be controlled. Control with chemical pesticides has adverse effects, such as pathogen resistance, costly, and hazardous to the environment. The use of antagonistic agents can be a solution as an environmentally friendly pest control. Seed-borne fungi can be beneficial or detrimental. One of the beneficial roles of fungi is as an antagonistic agent. Some types of fungi often found in forest plant seeds include *Fusarium* sp., which is a fungus often found in

the storage, and *Aspergillus* sp. as well as *Rhizopus* sp., which is the storage fungi [2]. Seed-borne fungi of red calliandra, among others, are *Aspergillus* sp. [3], *Fusarium* sp. [4] and *Rhizopus* sp.

Seed-borne pathogenic fungi can affect seed quality and the production of seedlings [5]. One of them, *Fusarium* sp. carried in red calliandra seeds, can cause damping off. Seed-borne fungi, both saprophytic and also pathogenic fungi, inhibited seed germination, and seedling emergence [6]. Antagonistic agents that are often used to control disease in plants can originate from fungi and bacteria. Some fungi genera serve as an antagonistic agent, namely *Gliocladium* sp., *Penicillium* sp. [7], *Trichoderma* sp., *Talaromyces* sp. [8].

Antagonistic agents are found in the soil, such as *Trichoderm*a sp., *Gliocladium* sp. [9], *Penicillium* sp. *Talaromyces* sp. [10], but not much information on antagonistic agents from seed. The purpose of this research was to identify the fungi in red calliandra seeds, which are potential as antagonistic agents, and to study their ability to inhibit the growth of pathogenic fungi *Fusarium* sp., *Aspergillus* sp. and *Rhizopus* sp.

2. Materials and methods

2.1. Study site

The seeds obtained are from Sukabumi, West Java, Indonesia. The research was conducted at the pest and disease laboratory, BP2TPH, from January to May 2019.

2.2. Fungi identification at red calliandra seed as potentially antagonist agent

Fungi were isolated by soaking the seeds with 1% NaOCl for three minutes then washed with sterile aquadest. It was repeated up to three times. Furthermore, the seeds were sown on moist paper media and incubated until a fungus colony emerged, which is expected to be a potential antagonistic agent. Fungi identification was made using the identification key book [11] and other literature [12]. The potential fungi as an antagonist agent were isolated into pure cultured.

2.3. Antagonistic tests

The dual culture method was used to test between the antagonistic fungus and saprophytic/pathogenic fungi of seven-day-old culture. The saprophytic/pathogenic fungi of red calliandra seed included *Fusarium* sp., *Aspergillus* sp., and *Rhizopus* sp. The lower part of the Petri dish base was marked by two pieces, each with a distance of 3 cm from the edges of the petri dish. Furthermore, the mycelial plug (5 mm diameter) of the fungi colony was placed at one point, while the other was placed with the mycelial plugs of the saprophytic/pathogenic fungi colony. Then, the plates were incubated for 14 days in the ambient room.

2.4 Observations

Macroscopic observation consists of antagonist fungi colony observation in PDA medium, inhibition percentage measure, and observation of fungi colony interaction types in agar medium. While the microscopic observations are morphology observation of antagonist fungi (hyphae, spores) and observation hyphae interaction using the microscope. The inhibitory percentage observation was done by measuring the radius of *Fusarium* sp, *Aspergillus* sp., and *Rhizopus* sp. colony toward and against the fungi of antagonistic agents at the age of seven days.

$$Inhibitory\ percentage = \frac{r_1 - r_2}{r_1} x\ 100\% \quad (1)$$

Remarks

r_1 = Colony radius of *Fusarium* sp., *Aspergillus* sp. and *Rhizopu*s sp. were opposed to antagonistic agents

r^2 = Colony radius of *Fusarium* sp., *Aspergillus* sp. and *Rhizopus* sp. which are toward antagonistic agents

Interactions between fungal colonies on agar medium are based on Dickinson & Boardman (1971) in [13]:

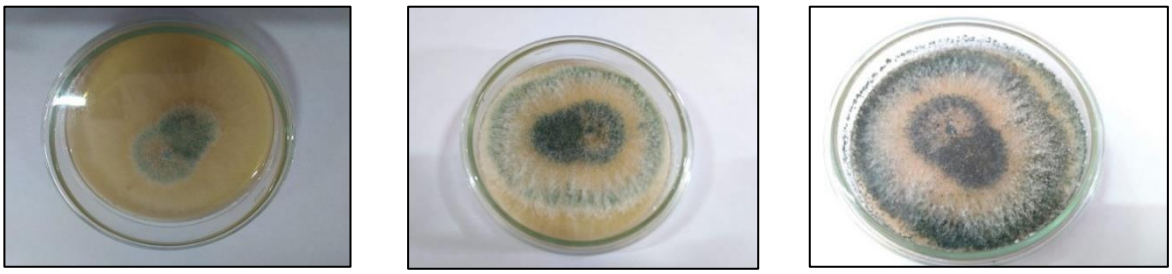
A. Both fungi can grow mingle with each other without any microscopic signs of interaction.
B. Mixing up the growth where the fungus observed is growing into an opposing fungus either above or below the colony or the fungus observed has stopped growing and is overgrown by other colonies.
   The two colonies approach each other until they almost touch and then form a mild barrier line 0.1 – 2 mm
C. Inhibit each other at a distance of > 2 mm.

3. Results and discussions

3.1. Fungi identification at red calliandra seed as potentially antagonist agent

Observation results showed that fungus colonies appeared green in the incubation period of two weeks (Figure 1). Usually, saprophytic/pathogenic fungi appear before seven days. The macroscopic observation result showed that the fungus' characteristics on day three were light green with white edges (Figure 1a), colony diameter of 4.3-6.7 cm, (an average of 5.2 cm). However, some isolates were still colored white or greenish-white. On the 5th day, the colony diameter was 6-9 cm (7.75 cm on average). The edge of the colony was generally dark green, but some were light green or greenish-white (Figure 1b). On the 7th day, almost all isolates covered the cup (8-9 cm in diameter), with the edges were still white. The growths of fungus colonies on PDA media are listed in Table 1.

On the 3rd day, the colony was greenish-white; on the 4th day, several isolates covered the entire cup, and on the 7th day, all isolates covered the entire cup surface and were green in color. In line with research conducted by Achmad and Nurhayati [14], the fungus colonies are dark green, grow fast, spread and cover the entire surface of the cup. The observations showed that the fungus mycelium was white while the conidia was green.



(a) (b) (c)

Figure 1. (a) Fungal colony on PDA at the 3rd day (left), (b) the 5th day (center), (c) the 7th day (right).

Table 1. *Talaromyces* sp. growth in PDA medium for seven days.

Days to-	Diameter (cm)		Color
	Range	means	
3	4.3-6.7	5.2	white, green
4	5- 9	7.25	green
5	6- 9	7.75	green
6	7-9	8	green
7	8-9	8.5	green

The identification result of this fungus based on [15] is *Talaromyces* sp., which is a sexual/teleomorphic stage of *Penicillium s*p. (Figure 2) [16] transferred all species of *Penicillium* subgenus *Biverticillium* to *Talaromyces*. As seen in Figure 1a, the characteristics of these fungi are green, penicilli biverticillate-symmetrical (phialides lanceolate, ascospores subglobose, or ellipsoidal,

ranging within the size 3-4.5 µm as in Figure 1b). *Talaromyces sp.* has white mycelium and green conidia [17,18]. Thus Talaromyces sp. can be isolated from the seeds of forest plants such as calliandra seeds.

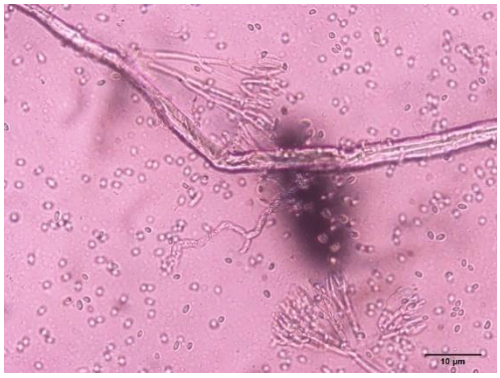


Figure 2. Morphology *Talaromyces* sp.

Talaromyces sp is known as a type of fungi that is potential as an antagonistic agent [19]. Some types of fungi that their growth can be inhibited are *Phytophthora palmivora*, *P. parasitica*, *Peronophythora litchii*, *Colletotrichum capsici*, *C. gloeosporioides*, *Pestalotiopsis guepinii*, *Phyllosticta* sp. [20].

3.2. Antagonist test for Talaromyces sp. and Fusarium sp., Aspergillus sp., Rhizopus sp.
Talaromyces sp. can inhibit the growth of *Fusarium* sp. and *Aspergillus* sp. until seven days old with more than 50% of inhibition (Table 2). From Table 2, it can be seen that the percentage of inhibition of *Fusarium* sp. by *Talaromyces* sp. is relatively fixed while *Aspergilus* is increasing. It is because *Fusarium* growth tends to be slow. Growth of *Aspergillus* sp. is faster, but the size of the *Talaromyces* sp. approaching radius is shorter than those that are away from *Talaromyces* sp. The small inhibition percentage of *Rhizopus* sp by *Talaromyces* sp. is caused by the rapid growth of *Rhizopus* so that when the *Talaromyces* sp. approaches and is away from radius are almost the same until seven days old.

Table 2. Percentage of inhibition *of Fusarium* sp., *Aspergillus* sp., *Rhizopus* sp. by *Talaromyces* sp.

Fungi species	Inhibition percentage (%)				
	The 3rd day	The 4th day	The 5th day	The 6th day	The 7th day
Fusarium sp.	57.81	63.37	60.43	61.71	59.4
Aspergillus sp.	18.31	44	57.05	67.78	67.92
Rhizopus sp.	23.63	16.67	12.5	10.83	8.89

Antagonism mechanism consisted of mycoparasitism, antibiosis, and competition. It can be seen in Figure 3 that after seven days old, *Talaromyces* can inhibit the growth of *Fusarium* sp. Even if *Talaromyces* is already dominant, *Aspergillus* sp. is still growing with almost the same growth rate. However, the two types of fungi still do not dominate each other.

Interactions among fungal isolates were categorized as antagonism and mutual antagonism [20]. Based on Dickinson & Boardman (1971) in [13], the interaction of *Talaromyces* s.p with *Fusarium* sp. and *Rhizopus* sp. is intermingling growth (overgrowth by the antagonist) as depicted in Figure 4. In contrast, the growing interaction *of Talaromyces* sp. with *Aspergillus* sp. is mutual intermingling, which means that there is no hyphal interaction with each other. According to [21], the dual culture of some *Penicillium* species with *Aspergillus* produced mutually intermingled growth without any zone of inhibition, showing that the production of the antibiotic by both fungi was failed.

From Figures 3 and 4, it appears that the highest inhibition is against *Fusarium* sp. This result is supported by [22] research, stating that the growth of *Talaromyces*sp. is fast and can inhibit the growth of pathogenic fungi within ten days.

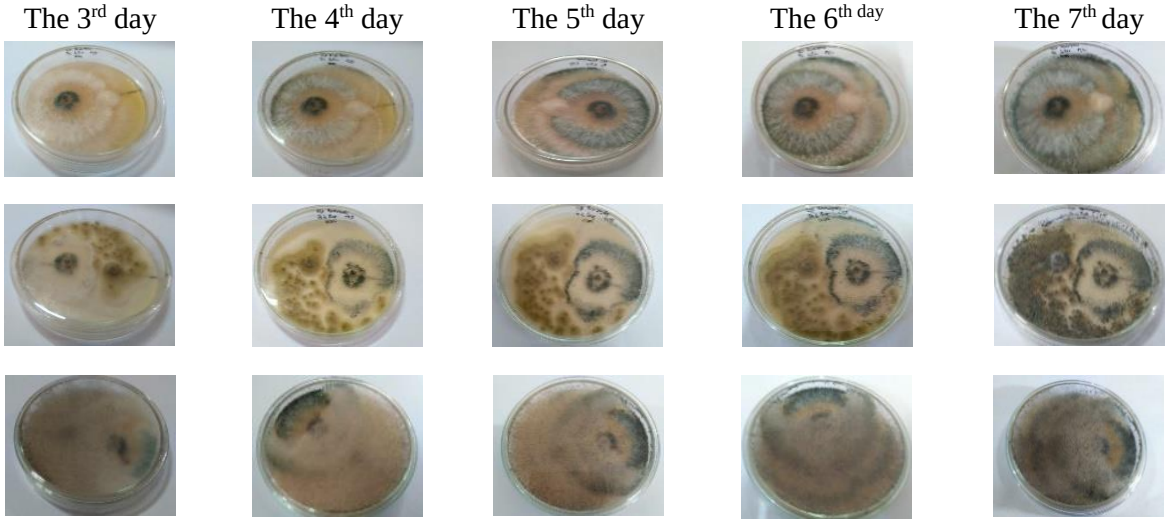
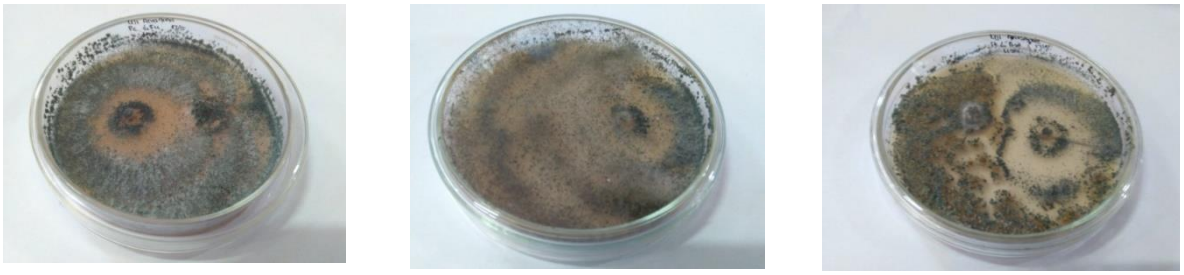


Figure 3. Antagonistic test between *Talaromyces* sp. and *Fusarium* sp., *Rhizopus* sp., *Aspergillus* sp. until seven days.

Talaromyces sp. was able to suppress the *Fusarium* sp. growth, *Aspergillus* sp., and *Rhizopu*s sp. and it was even overgrowth at 14 days old (Figure 4). At 14 days, the dominant mechanism for the three types of pathogenic fungi was mycoparasitism, space and nutrition competition. According to [23], mycoparasitism is the main mechanism to control *Sclerotium rolfsii,* which is related to chitinase activity. In contrast, the activity of glucose-oxidase and antifungal compounds (antibiotics) plays a role in inhibiting the germination, growth, and melanization of *S. rolfsii* microsclerotia.

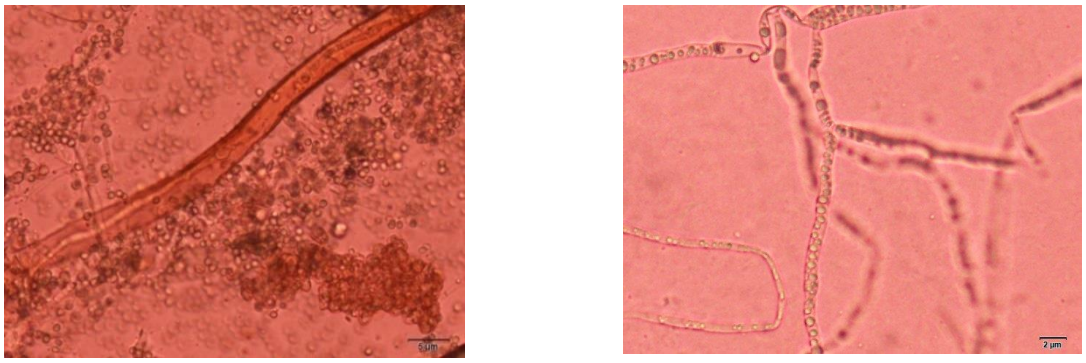


(a) (b) (c)

Figure 4. Antagonistic test between *Talaromyces* sp. and *Fusarium* sp. (a), *Rhizopus* sp. (b), *Aspergillus* sp. (c)

Although *Rhizopus* sp. is still alive at the age of seven days, hyphae interference occurs, so this is categorized as antagonism (Figure 5a). According to [23], mycoparasitism involves cell wall-degrading enzymes, such as β1,3-, β-1,4-, and β-1,6-glucanases, cellulase, and chitinase. At the age of seven days, coagulation and granulation occurin the hypha of *Fusarium* sp., and the hyphal folding (Figure 5b). Coagulation and granulation in hypha of the pathogen showed the lysis [24]. In line with the research conducted by [21], during contact between *A. niger* hypha and *Pencillium* sp. hypha., there was no hyphae interaction. However, *Penicillium* sp. growing above *A. niger* would not completely inhibit the growth.It would be antagonistic if *Penicillium* sp. Were overgrowth. According to [25], *T. convolutes* produces talaroconvolutins, which can inhibit the growth of *A.fumigatus* and *A.niger*.

Based on the results, it is found out that *Talaromyces* sp. can inhibit overgrowth for *Fusarium* sp., at seven days old, while for *Rhizopus* sp. and *Aspergillus* sp., occur more than seven days. Thus,*Talaromyces* sp. has a high potential to suppress diseases caused by *Fusarium* sp., such as damping off.



(a) (b)

Figure 5. (a) Hyphal interference of *Rhizopus* sp. (b) Coagulation and granulation in the hyphae and hyphal folding of *Fusarium* sp.

4. Conclusion

One of the potential fungi as antagonist agent isolated from the seed of calliandra is *Talaromyces* sp. The main antagonistic mechanisms of *Talaromyces* sp. to *Aspergillus* sp., *Fusarium* sp., and *Rhizopus* sp. are mycoparasitism and competition.

Acknowledgments

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5. References

[1] Orwa C, Mutua A, Kindt R and Jamnadass R S A 2009 Agroforestree database:a tree reference and selection guide version [Online] accessed from http://www.worldagroforestry.org/publication/agroforestree-database-tree-reference-and-selection-guide-version-40

[2] Schmidt O, Grimm K and Moreth U 2002 Molecular identity of species and isolates of the Coniophora cellar fungi *Holzforschung*. **56**(6): 563–571

[3] Ardiansyah 2017 Potency of Citronella (*Cymbopogan nardus) for controlling of fungus pathogen carried by Red Calliandra Seeds* (*Calliandra calothyrsus*) (in Indonesian) [Skripsi] (Bogor: Institut Pertanian Bogor) p 28

[4] Hidayati N 2017 Identification of pathogen causes of damping off diseases on kaliandra seedlings (in Indonesian) *J Pemuliaan Tanam Hutan* **12**(2): 135–142

[5] Cram M M and Fraedrich S W 2009 Seed diseases and seedborne pathogens of North America. *Tree Plant Notes*. **53**(2): 35–44

[6] Abdulwehab S A, El-Nagerabi S A F and Elshafie A E 2015 Leguminicolous fungi associated with some seeds of Sudanese legumes *Biodiversitas* **16**(2): 269–280

[7] Jabnoun-Khiareddine H 2009 Biocontrol of tomato verticillium wilt by using indigenous *Gliocladium* spp. and *Penicillium* sp. isolates. dyn Soil *Dyn Plant*. **3**(1): 70–79

[8] Dubey S 2015 Geomicrobilogical Study of Kaliadah Palace Ujjain with special R]reference to fungi and restoraton measures (India: Vikram University) p 304

[9] Celar F and Valic N 2005 Effects of *Trichoderma* spp. and *Gliocladium roseum* culture filtrates on seed germination of vegetables and maize *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz* **112**(4): 343–350

[10] Ramos S M S, Cruz R, do Nascimento B R, Machado A R, da Cossta A F de S M C M and de O N T 2018 *Penicillium* and *Talaromyces* communities of sugarcane soils (*Saccharum officinarum* L.): ecological and phylogenetic aspects *J Agric Sci.* **10**(4): 335–350
[11] Barnett H L H B 1998 *Illustrated genera of imperfect fungi* ed B B Hunter (Minnesota: The American Phytopathological Society) p 200
[12] Houbraken J and Samson R A 2011 Phylogeny of *Penicillium* and the segregation of Trichocomaceae into three families *Stud Mycol*. **70**: 1–51
[13] Fakhrunnisa, Hashmi M H and Ghaffar A 2006 *In vitro* interaction of *Fusarium* spp., with other fungi *Pakistan J Bot.* **38**(4): 1317–1322
[14] Achmad and Nurhayati P W 2004 Genus of fungi on mangrove soils polluted by heavy metals in Muara Angke DKI Jakarta (in Indonesian) *J Manaj Hutan Trop*. **10**(2):14–21
[15] Stolk A C and Samson R A 1972 *Studies on Talaromyces and related genera II studies in mycology* No. 2. ed R A Samson (Utrecht Netherlands : Westerdijk Fungal Biodiversity Institute) p 65
[16] Samson R A, Yilmaz N, Houbraken J, Spierenburg H, Seifert K A, Peterson S W, et al 2011 Phylogeny and nomenclature of the genus *Talaromyces* and taxa accommodatedin Penicillium subgenus Biverticillium *Stud Mycol*. **70**: 159–183
[17] Barbosa R N, Bezerra J D, Souza-Motta C M, Frisvad J C, Samson R A, Oliveira N T H J 2018 *New Penicillium and Talaromyces species from honey, pollen and nests of stingless bees.* ed AV Leeuwenhoek 111 pp 1883–1912
[18] Pelo S and Mavumengwana V 2020 Diversity and antimicrobial activity of culturable fungal endophytes in *Solanum mauritianum Int. J. Environ. Res. Public Health.***17**(2): 1-11
[19] Manoch L and Dethoup T 2011 A potential use of *Talaromyces* species as biological agents against plant pathogenic fungi *Thai J Agric Sc*i. **44**(2): 81–91
[20] Dethoup T, Manoch L, Visarathanonth N, Chamswarng C and Kijjoa A 2007 Morphology and distribution of *Talaromyces flavus* from soil and potential use as a biological control agent against plant pathogenic fungi *Thai J Agric Sci.* **40**(1): 37–50
[21] Khokhar I, Haider M S, Mukhtar I and Mushtaq S 2013 Biological control of *Aspergillus niger,* the cause of black-rot disease of *Allium cepa* L. (onion), by *Penicillium* species *J Agrobiol*. **29**(1): 23–28
[22] Suciatmih, Antonius S, Hidayat I and Sulistiyani T R 2014 Isolation, identification and evaluation of antagonism to *Fusarium oxysporum* f.sp. *cubense* (Foc) under *in vitro* conditions from endophytic fungi of *Musa* sp. (in Indonesian) *Ber Biol*. **13**(1): 71–83
[23] Madi L, Katan T, Katan J and Henis Y 1997 Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms *Phytopathology*. **87**(10): 1054–1060
[24] Padmaja M, Swathi J, Narendra K, Sowjanya K M, Jawahar Babu P and Satya K K 2013 *Trichoderma* sp. as a microbial antagonist against *Rhizoctonia solani*. *Int J Pharm Sci*. **5** (SUPPL.4): 322–325
[25] Suzuki S, Hosoe T, Nozawa K, Kawai K I, Yaguchi T and Udagawa S I 2000 Antifungal substances against pathogenic fungi, talaroconvolutins, from *Talaromyces convolutus*. *J Nat Prod*. **63**(6): 768–772