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Short Communication: Thromboplerous hyphae of the ectomycorrhizal mushroom *Rhizopogon roseolus* with and without a host tree

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Abstract. *Putra IP, Aimi, T, Shimomura N. 2022. Short Communication: Thromboplerous hyphae of the ectomycorrhizal mushroom* Rhizopogon roseolus *with and without a host tree. Nusantara Bioscience 14: 47-52.* Thromboplerous hyphae are modified hyphae found in the basidiocarp of many agarics. However, data on their development and function, as well as on their formation in pure cultures of ectomycorrhizal mushrooms, are scarce. The aim of the present study was to characterize the cytological descriptions of thromboplerous hyphae from pure cultures of *Rhizopogon roseolus* (Corda) Th.M. Fr. with or without the host pine tree *Pinus thunbergii* Parl. Thromboplerous hyphae formed on the mycelium and mycelial cords in all experimental settings. Results revealed thromboplerous hyphae were extremely melanized, smooth, cracked on the surface, and swollen at both hyphal termini. Thromboplerous hyphae produced with a host were mainly unbranched in shape in contrast to those formed without a host that possessed additional twisted, semi-twisted, and branched forms. Some thromboplerous hyphae that grew without a host had large diameters, and few showed notable septa and clamp connections. The present study also provides evidence of the initial development of thromboplerous hyphae via vegetative and tubular hyphae. This study contributes to understanding the cytology of thromboplerous hyphae grown with and without a host tree. Further investigations on the specific functions of thromboplerous hyphae are needed to deepen the current knowledge of fungal cytology.

Keywords: Cytology, host, pure cultures, *Rhizopogon roseolus*, thromboplerous hyphae

INTRODUCTION

Hyphae are the basic structure of many fungi. They constitute the mycelium of the higher fungi and form other structures (Yafetto 2018). They can be modified in many different structures to serve various functions (Kirk et al. 2008). One of the hyphae functions, which has never been studied, is the host impact on the oil production of hyphae in many fungi. In addition, there is sparse information regarding modified hyphae, such as secretory hyphae in Basidiomycota, ranging from saprophytic to ectomycorrhizal fungi. Secretory hyphae can be observed from mycelia, rhizomorphs, mycorrhiza structure, sclerotia, and basidiomes (Clémençon 2012). One of the types of secretory hyphae is the thromboplerous hyphae, which have been cytologically less observed over decades. In addition, the terminology of thromboplerous hyphae is not uniform and inconsistently used by mycologists.

Thromboplerous (Th) hyphae, previously known as oleiferous hyphae, are hyphae whose development and function remain obscure. There was a debate regarding the term between Th hyphae and oleiferous hyphae. The term "oleiferous hyphae" originates from French literature and pertains to a lipid-composed structure (Lentz 1954). However, Clémençon (2012) rejected this term since certain hyphae do not contain lipids and suggested using the term "thromboplerous hyphae" instead. Until now, Clémençon (2005) provided the only information on Th hyphae development from the saprophytic fungi. Th hyphae are easy to distinguish from undifferentiated hyphae due to their size and structure; th hyphae are melanized, refractive, contain a solid mass of homogenously dense material, and are devoid of nuclei. Furthermore, these structures' clogged, coagulated, and gelatinous consistency represents Th hyphae's "thrombo" characteristics.

Lentz (1954) reported that the oily structure of Th hyphae is found in agarics; previous studies by various authors confirmed the presence of Th hyphae in basidiocarp tissue (Smith and Zeller 1966; Vizzini and Ercole 2012; Vizzini et al. 2012; Moreau et al. 2013). However, the only comprehensive reports available on the possible mechanisms of development and function of Th hyphae are from rhizomorphs studies by Clémençon (2002; 2003; 2005). In addition, information on this particular hypha from pure cultures is scarce (Miller et al. 1983; Clémençon 2002; 2003).

Lentz (1954) argued that Th hyphae might play an essential but unknown physiological role in fungi. Currently, the only available cytological evidence on the possible functions of Th hyphae is from a study on saprophytic fungi by Clémençon (2005); therefore, there is

a need to gather data from other species and living styles of higher fungi, especially from laboratory cultures. As thromboplera are produced on saprobic and ectomycorrhiza (ECM) forming fungi, *Rhizopogon roseolus* (Corda) Th.M. Fr., an ECM fungus that is easy to isolate into pure cultures, was used in this study. Unfortunately, only Miller et al. (1983) have recently described Th hyphae from *R. roseolus* cultures, albeit with minimal information. In addition, to date, most cytological research on ECM fungi has focused only on the plant with less attention to the fungal structure (Leyva-Morales et al. 2019). Therefore, the aim of this study was to provide cytological information on Th hyphae from pure cultures of *R. roseolus* with and without the presence of the ectomycorrhizal host.

MATERIALS AND METHODS

Fungal material

Rhizopogon roseolus (= *R. rubescens* Tul. & C. Tul.) with strain number TUFC10010 was used in this study. The fungus was collected from the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan. The fungus was prepared on 2% malt extract agar with a pH of 5 and incubated for 21 days at 25°C before being used.

Plant material

Seeds of *Pinus thunbergii* Parl. were rinsed overnight in water and then surface-sterilized using 30% hydrogen peroxide. After being washed with sterile distilled water, the seeds germinated at 25°C for 1-2 weeks in water agar. One fine seedling with well-developed lateral roots was then transferred to half size of a five-fold dilution of modified Melin and Norkrans (1/5 MMN) medium in (90×20) mm Petri dish. The vertical plate contained 30 mL of solid medium with half-removed agar to provide space for pine shoots. The Petri dish was sealed with 3M™ transpore surgical tape and incubated in a controlled laboratory chamber with the following conditions: 25°C temperature, 50% relative humidity, and a photoperiod of 16-hour day at 5000 Lx.

Th hyphae in the presence or absence of ECM host

A one-month-old pine seedling was inoculated with a 7-mm round mycelial plug of *R. roseolus*. Four plugs were placed near the lateral roots. One mycelial plug was inoculated on a host-free 1/5 MMN medium. Petri dishes were then incubated for 12 weeks in identical conditions.

Morphological characterization of Th hyphae

A total of 50 Petri dishes of *R. roseolus* with the ectomycorrhizal host and ten plates without the host were examined. The cytology of Th hyphae was evaluated from mycelia and mycelial cords using stereo and optical microscopes. The samples were first observed using a Leica EZ4 stereo microscope before being fixed in a 3:1 solution of 99.5% ethanol and acetic acid and subsequently removed from the air. The samples were then mounted with distilled water and lactophenol cotton blue and observed using an Eclipse 80i light microscope (Nikon, Tokyo, Japan). Pictures were obtained using a DS-L2 digital camera (Nikon, Tokyo, Japan). The variables analyzed in the morphological assessment include Th hyphae position, surface ornaments, shape, length, diameter, septum, and clamp connection. The diameters of Th hyphae with and without hosts were subjected to a one-way analysis of variance. Mean values were ranked using the Student-Newman-Keuls test at $P < 0.05$.

RESULTS AND DISCUSSION

The present study showed Th hyphae's cytological characteristics and the host's impact on this structure. Previously, most studies on Th hyphae were only on the occurrence or absence of this structure (Wartchow and Cortez 2016; Assis et al. 2018; Gelardi et al. 2019), without information on its cytological aspects. The Th hyphae were found on fungal mycelia (Figure 1A-B) and the surface of mycelial cords at the center and periphery of the fungal colonies (Figure 1C-D), both with and without a host. These findings contrast with those of Clémençon (2003), who reported Th hyphae's presence in the agar medium's depths. In this study, we did not find Th hyphae inserted in the agar medium in any of the experimental sets, which differs from Clémençon (2003), who investigated Th hyphae of saprophytic fungi. In the current study, a non-obligate ECM fungus was inoculated with and without a host, yet the Th hyphae were consistently observed on the surface of the media.

The Th hyphae that grew with hosts were found near the pine roots, on the ECM mantle, and the extraradical mycelia. However, a larger number of Th hyphae were distributed near the plug of the fungal isolate (Figure 2A) compared to the center of the fungal colony (Figure 2B) or in any other places in both experimental settings. Previously, Clémençon (2005) proposed that Th hyphae function as energy reservoirs in fungi. While further investigation is needed to confirm this, it might explain why larger Th hyphae accumulated near the fungal isolate than in any other part of the colony in this study. Substrate colonization and symbiosis initiation require high energy from the saprophytic or mycorrhizal fungi (Cairney 1992; Smith and Read 2008).

The Th hyphae were easily distinguished from the common vegetative hyphae by their appearance (Figure 3A). The Th hyphae were thick-walled, grew up to 200 µm in length, had a pale to dark brown color in distilled water (Figure 3A-B), had rare septa, and were extremely melanized. This finding is consistent with a previous description by Moreau et al. (2013) of Th hyphae with brown content observed from the gleba of *Alpova komoviana* collected at the field. The Th hyphae are the type of secretory hyphae that differ from Heteroplera (Laticifera and Gloeoplera) by liquid and the occurrence of nuclei (Clémençon 2012) on the later types. In the secretory hyphae, a cytoplasm containing secreted substances is called deuteroplasm. The deuteroplasm is a modified cytoplasm filled with secondary metabolites seen

as droplets, heterogeneous, or homogeneous masses (Clémençon 2012).

In the Th hyphae of this study, the solid column containing homogeneous deuteroplasm was separated from the adjacent vegetative hyphae by the unclear septum, and no nuclei were evident. Therefore, th hyphae are devoid of nuclei and are considered dead hyphae (Clémençon 2012). The homogenous grain content of the hyphae protoplasm was also evident (Figure 3A, double arrow). The Th hyphae were generally smooth with some cracks on the surface (Figure 3A, arrow) and turned darker brown with age. In addition, the Th hyphae turned dark blue (Figure 3C) and sometimes green (Figure 3D) when stained with lactophenol cotton blue. Clémençon (2012) reported that higher fungi produce many types of secretory hyphae, which the morphology and reactivity can distinguish with stains and solutions.

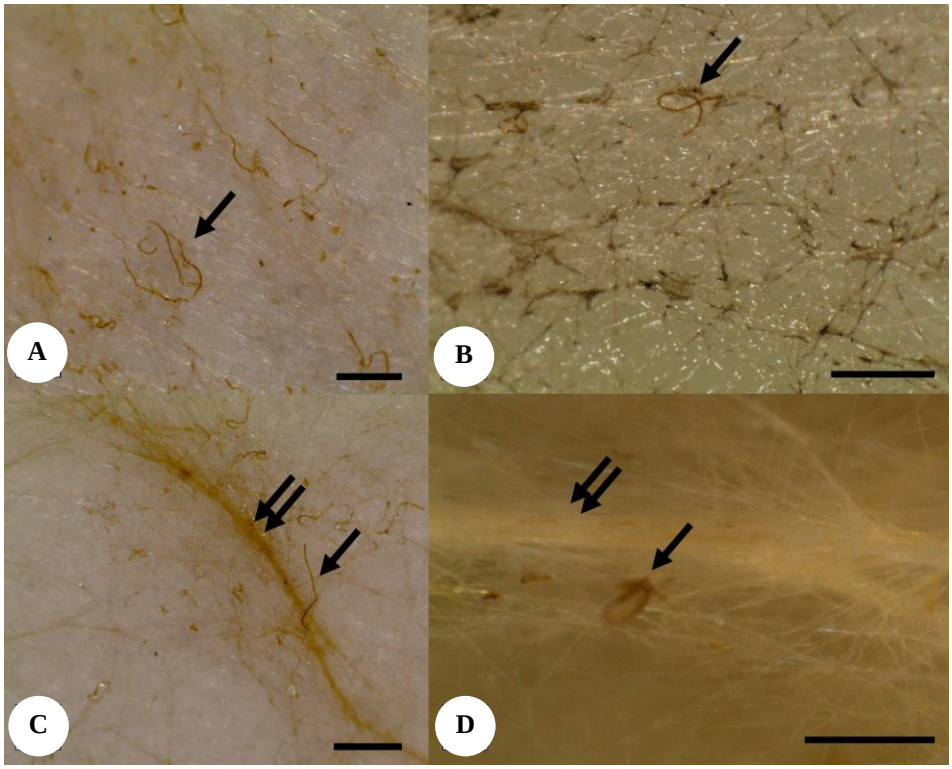

Figure 1. A-B. Thromboplerous hyphae (arrow) on the mycelia of *Rhizopogon roseolus* with and without a host, C-D. Thromboplerous hyphae (arrow) on the mycelial cords (double arrow) of *Rhizopogon roseolus* with and without a host. Bars = 200 µm

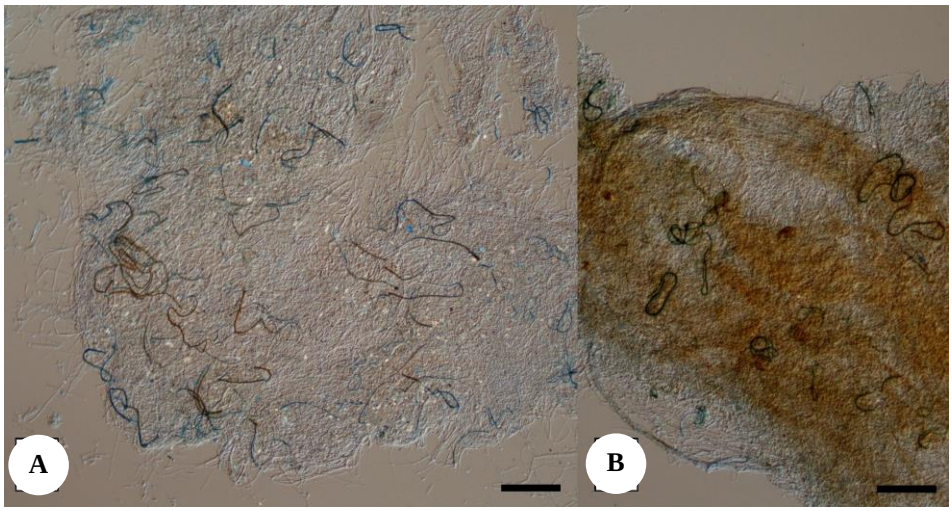

Figure 2. A larger number of *Rhizopogon roseolus* thromboplerous hyphae were found near the fungal inoculum (A) compared to the center of the fungal colony (B) with and without a host. Samples were stained with lactophenol cotton blue. Bars = 100 µm

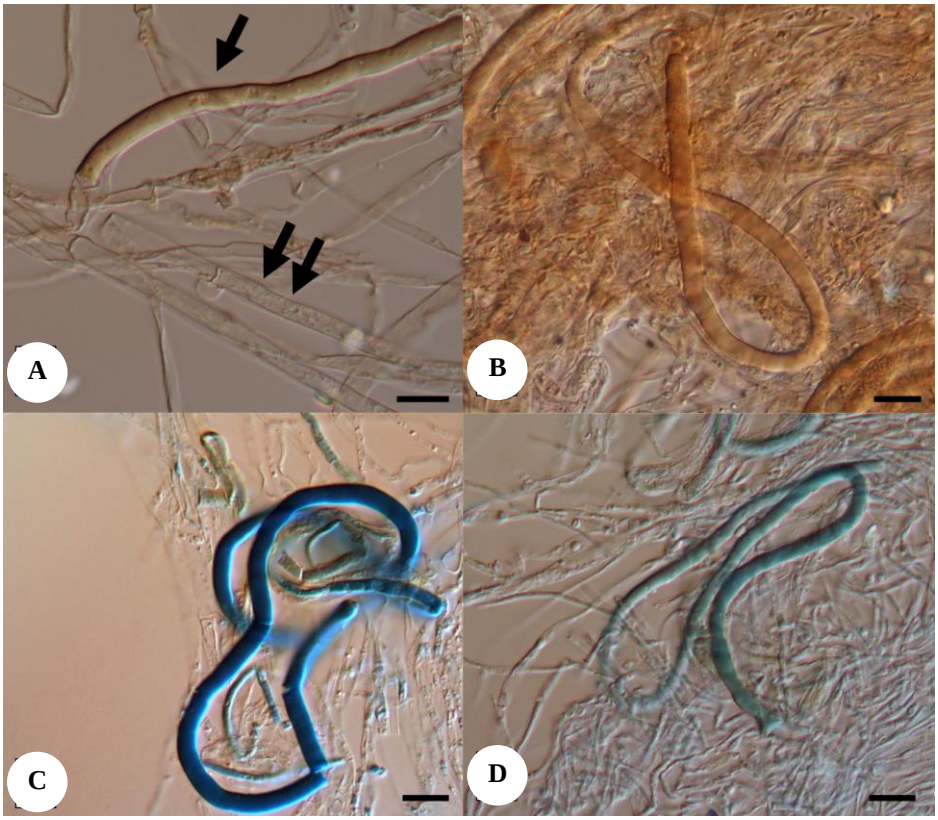

Figure 3. The common vegetative hyphae (double arrow) and melanized hyphae (arrow) with cracks on the surface (A), Thromboplerous (Th) hyphae mounted with distilled water showed brown content of Th hyphae and prominent oil drop near the hyphae (B), Th hyphae heavily stained with lactophenol cotton blue (C), Th hyphae that turned green after stained with lactophenol cotton blue (D). Bars = 10 µm

The Th hyphae were inflated at the distal or proximal ends of the hyphal terminal (Figure 4A-B). Thrombocytes of Th hyphae, usually found on old cultures (Clémençon 2002), are swollen cells at the ends of the structures. The Th hyphae of *R. roseolus* were reminiscent of oil but less refractive, a finding that contrasts with the description of Miller et al. (1983). Clémençon (2005; 2012) classified Th hyphae as secretory hyphae that never release latex. However, drops of oil were prominent near Th hyphae (Figure 3B), which changed the color of the fungal colony from white to cream. The deuteroplasm does not flow out but expands out of the damaged hyphae (Clémençon 2005; 2012).

The Th hyphae that grew with and without hosts were cytologically identical except for a few differences in shape and size. Th hyphae that grew with a host were mostly unbranched (Figure 4B), whereas those that grew without a host possessed semi-twisted to twisted (Figure 4C) and branched hyphae (Figure 4D). Clémençon (2003; 2005; 2012) reported that Th hyphae commonly grew without branches. In contrast, the present study is the first report of branched Th hyphae in fungi. Septa were evident in the middle of the Th hyphae and in the terminals where branches formed (Figure 4C-D). Th hyphae that grew without a host had slightly larger diameters than those that grew with a host (diameter 3.96 µm ± 0.7, range 3.1-5.03, n=9; diameter 4.47 µm ± 1.62, range 1.72 - 7.65, n=23, respectively). The presence of a host had no significant impact on the diameter of the Th hyphae (P> 0.05). Some of the Th hyphae that grew without a host produced exceptionally large (>9 µm) diameters (Figure 4A). Judging from the size, we suggest that large Th hyphae were derived from tubular hyphae, while vegetative hyphae initially formed the more common and smaller-sized Th hyphae.

Prior reports on the development and function of Th hyphae are scarce, and studies have been conducted exclusively on saprophytic fungi (Clémençon 2003; 2005). In this study, the cytoplasm of tubular hyphae, which had a diameter similar to that of Th hyphae, condensed into a solid homogeneous mass (Figure 5C). In addition, granules of melanizing protoplasms (Figure 5D) and empty portions of Th hyphae (Figure 5B) were also evident. Granules (Figure 5D) were always found near mature Th hyphae. The results of this study provide evidence of the initial development of Th hyphae in *R. roseolus*. Clémençon (2012) previously reported the gradual change of *Amanita citrina* protoplasm from a meromorphic (granular) to a more thrombomorphic state. In addition, Clémençon (2005) argued that Th hyphae develop from tubular hyphae based on their diameter. The present study found many smaller hyphae melanizing to Th hyphae (Figure 5B-D) than tubular hyphae. Therefore, the results suggest that Vegetative mycelia dominate th hyphae production and not the tubular hyphae.

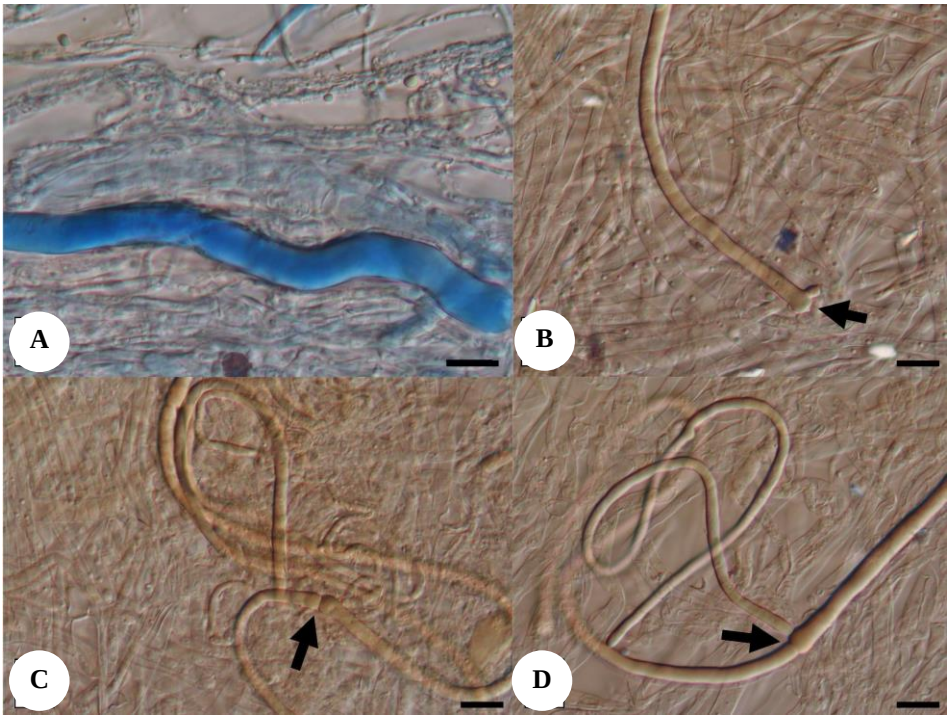

Figure 4. A. The large thromboplerous hyphae formed by specimens without a host stained with lactophenol cotton blue; B. The typical unbranched thromboplerous hyphae grown with a host, note the thrombocytes (arrow); C. The twisted shape of thromboplerous hyphae grown without a host, note the septa (arrow); D. The branched thromboplerous hyphae grown with a host, note the branch node (arrow). Bars = 10 µm

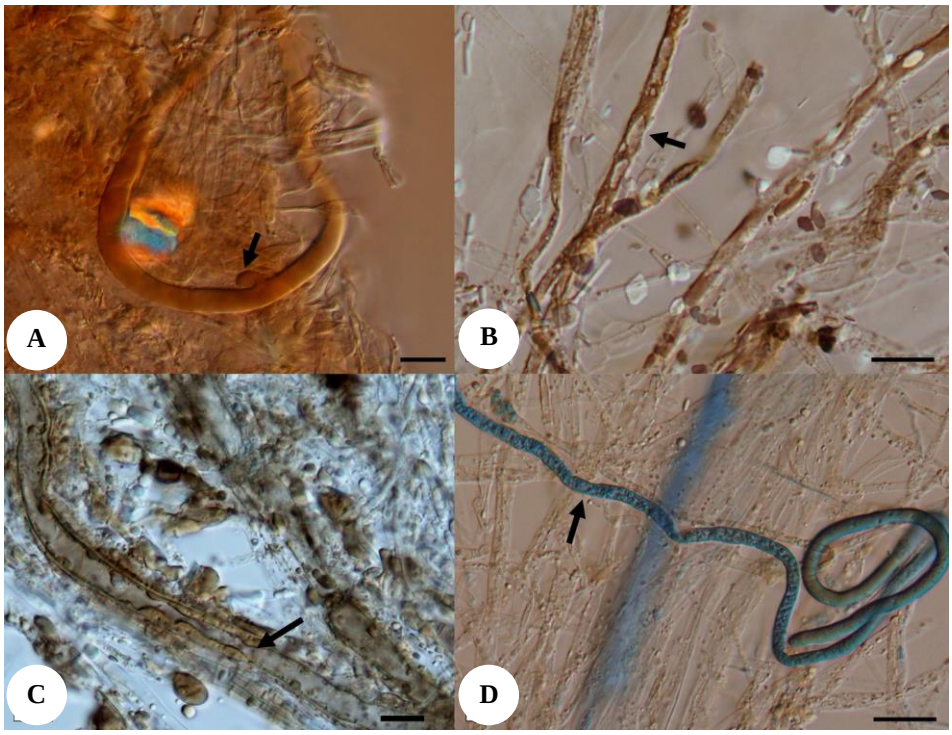

Figure 5. A. Clamp connection (arrow) of thromboplerous hyphae grown without a host, B. Melanizing hyphae showing a large vacuole (arrow), C. Tubular hyphae with condensing protoplasm (arrow), D. The homogenous deuteroplasm (arrow) of the initial formation of thromboplerous hyphae. Bars = 10 µm

Clémençon (2005) observed many clamp connections and incomplete septa in Th hyphae from the rhizomorph of *Ossicaulis lignatilis*. In this study, only a few Th hyphae that grew without a host had prominent septa (Figure 4C-D) and clamp connections (Figure 5A) compared to those that grew with a host. The clamp connection was also filled with the homogenously solid deuteroplasm. The occurrence of septa on Th hyphae in this study contrasts with the Th hyphae of *R. roseolus* described by Miller et al. (1983). Furthermore, Clémençon (2005) assumed that Th hyphae are used as reservoirs by fungi as he observed the presence of intrahyphal hyphae inside Th hyphae. While no intrahyphal hyphae were found inside Th hyphae in the present investigation. However, this study suggests that Th hyphae play an essential role in *R. roseolus* as it is produced in all stages of development of the fungal colony. However, the investigation of cytological aspects and hyphal features of *R. roseolus* was sparse (Martín and Gracia 2000; Putra et al. 2021), and more research should be done to reveal the undescribed phenomenon of the cytological characters of ECM fungi with and without a host.

The results of this study deepen our current understanding of the cytology of Th hyphae from laboratory studies. Studies on ultrastructure observations and 3D models of Th hyphae are underway, hopefully revealing more information on this aspect. In addition, further studies are needed to determine the specific roles of Th hyphae growing with or without a host in ECM fungi.

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