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EFFECT OF MEDIA ON THE MYCELIAL GROWTH OF HONEY BEE FUNGAL PATHOGEN *ASCOSPHAERA APIS* MATING TYPES

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ABSTRACT

The fungal pathogen *Ascosphaera apis* significantly impacts the honey bee *Apis mellifera* by causing larval mortality and reducing colony productivity. Understanding the growth dynamics of *A. apis* under different media conditions is crucial for advancing research on its biology and pathogenicity. This study evaluates the effect of eight artificial media namely Potato Dextrose Agar (PDA), Carrot Dextrose Agar (CDA), Beetroot Dextrose Agar (BDA), Tender Coconut Water (TCW) media, Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Agar Yeast (SDAY), Sabouraud Maltose Agar Yeast (SMAY), and Malt Extract Agar (MEA) on the mycelial growth and reproductive structure formation of *A. apis*'s two mating types. Results indicated significant differences in mycelial growth across media. SDA (75.40 mm dia) and SDAY (74.83 mm dia) supported maximum growth for mating type 1 (MAT1) and, SDA (72.72 mm dia) and BDA (68.17 mm dia) yielded the maximum growth for mating type 2 (MAT2). The TCW resulted in the least growth for MAT1 (44.19 mm dia) and MAT2 (41.25 mm dia). The growth patterns showed that media supplemented with sugars generally enhanced *A. apis* growth, whereas non-sugar media did not. Furthermore, reproductive structures formed faster on PDA and CDA than on other media. The study confirms that media composition, particularly sugar content, critically influences the growth and development of *A. apis*, providing insights that could help to develop better management strategies for controlling chalkbrood disease in honey bee colonies.

Key words: *Apis mellifera*, chalkbrood, colony productivity, pathogenicity, *Ascosphaera apis*, mating types, mycelial growth, growth media, supplements, sugars, reproductive structures

Ascosphaera apis is the fungal pathogen of honey bee *Apis mellifera* causing individual larval mortality and significantly reducing the colony productivity (Spiltoir, 1955; Spiltoir and Olive, 1955; Bailey, 1967; Wood, 1998). The occurrence of chalkbrood seems to be on the rise in the recent years worldwide. The fungal infection starts from the larval ingestion of contaminated food fed by the nurse bees (Strasser, 2001; Mannino et al., 2019). Initially, the ascospores of *A. apis* are only activated under CO_2 environment that prevails in the larval gut (Nelson and Gochnauer, 1982; Koenig et al., 1987). The temperature required for the spore germination is 35°C (Bailey, 1967). The activated spore forms germ tubes followed by penetration of hyphae through the peritrophic membrane of the larva and grows out through the posterior end. When the single mating type of the fungus is present, it produces pieces of white-colored chalk-like mummies, hence the disease is named as 'chalkbrood'. Conversely, when two opposite mating types are present, spherical spore cysts are produced, comprising of spore balls with numerous ascospores that serve as the source of inoculum for further infection (Aronstein and Murray, 2010). Ascospores are protected by three layers of walls with chitin as a major component, thus favoring the prolongation of viability of ascospores for many years (Li et al., 2018; Liu et al., 1991; Bamford and Heath, 1989; Jensen et al., 2013). Since there is no approved product available for chalkbrood control, more scientific insight into the study of biology and pathogenicity is warranted. For this purpose, stable and effective cultivation of *A. apis* under *in vitro* conditions is crucial. To investigate the biology and pathogenicity of *A. apis*, it is necessary to maintain the culture by transferring it to a new growth medium. Various methods have been used to grow *A. apis* for scientific investigation. *A. apis* can grow in a wide range of artificial media in both aerobic and anaerobic environments (Bissett, 1988; Gilliam et al., 1988; Anderson et al., 1997; Johnson et al., 2005). Artificial media have a substantial impact on the germination, development, enzyme synthesis, and virulence of entomopathogenic fungi (Ibrahim, and Jenkinson, 2002; Liu et al., 2016). The nutrition of fungus grown in a controlled environment relies mostly on the carbon-to-nitrogen ratio (C/N ratio). However, the C/ N ratio was differ greatly depending on the species.

A. apis is one of the entomopathogenic fungi that exhibit heterothallism i.e., sexual reproduction occurs when compatible mating type1 (MAT1) and mating type 2 (MAT2) are joined (Aronstein and Murray, 2010). Growth of the MAT1 was faster than MAT2 of *A. apis* on different solid media and it probably depended on the specific strain (Mraz et al., 2021). Also, the speed of the reproductive structure formation is essential to harvest more ascospores to conduct in *vitro* studies. In the present study, the effect of different media on the mycelial growth of two different mating types of *A. apis* was evaluated, and the speed of reproductive structure formation on different media was also determined.

MATERIALS AND METHODS

The mummified larvae, preferably the white mu mmies were collected from the diseased *A. mellifera* colonies at Apiary, Tamil Nadu Agricultural University, Coimbatore ($11^0$0'59''N; $76^0$55'47''E) during June 2022, for mating type separation. The fungal pathogen was morphologically and molecularly identified as *Ascosphaera apis* (Coimbatore isolate) (GenBank accession No. OQ641236). The collected mummies were subjected to mating-type separation as described by Jensen et al. (2013). Briefly, the collected mummies were surface sterilized with 10% sodium hypochlorite solution for 10 min followed by rinsing with sterile distilled water for 2 min. Sterilized mummies were cut into small pieces, placed on the PDA (Potato dextrose agar) plates, and incubated at 30-34^{0}C until mycelial growth and sporulation appeared. During incubation, the sporulation points were observed and opposite mating types were separated by continuous subculturing using a single hyphal tip isolation technique. Furthermore, the isolated opposite mating types were verified by dual test and identified by multiplex PCR assay developed by Aronstein and Colby, 2015. The separated and identified mating types were maintained in PDA media for further analysis.

A total of eight commonly used fungal growth media namely, potato dextrose agar (PDA), carrot dextrose agar (CDA), beetroot dextrose agar (BDA), tender coconut water medium (TCW), Sabouraud dextrose agar (SDA), Sabouraud dextrose agar yeast (SDAY), Sabouraud Maltose Agar Yeast (SMAY) and malt extract agar (MEA) were used for the evaluation of radial mycelial growth and speed of reproductive structure formation. All the media were autoclaved at 121^{O}C for 15 min. Six-day old cultures of both mating types were used for the experiment. For assessing radial mycelial growth of the mating types, 6 mm dia of fresh mycelium discs were taken from the edges of the plates with the help of a cork borer and placed in the middle of Petri dishes (80 mm) containing test media. All the plates were maintained in triplicates and incubated at 31-33^{O}C for seven days. The radial growth of the mycelium was measured every day with the help of a vernier caliper by drawing the two perpendicular lines at the bottom of the tested plates for up to seven days or reaching the mycelial growth to the edges of Petri plates. The speed of reproductive structure formation was evaluated by placing one disc of MAT1 and MAT2 at 6 cm distance in petri dishes (80 mm) containing different growth media. Plates were maintained in triplicate and incubated at 31-33^{0}C for 6 days. The length of each mating type was measured every day until the formation of reproductive structure at the intercepting point of two opposite mating types. Completely randomized block (CRD) with one way ANOVA was performed to statistically assess the radial mycelial growth of mating types and least significant difference (LSD) were performed to find out the significant differences among the growth media. All the statistical analyses were performed using AGRESS.

RESULTS AND DISCUSSION

The mycelial growth of *Ascosphaera apis* (Coimbatore isolate) mating types significantly differed on all tested growth media (Table 1). In case of MAT1, SDA (Fig. 1A) and SDAY showed maximum mycelial growth with a colony diameter of 75.40 mm and 74.83 mm respectively. The medium range of mycelial growth was observed in CDA (67.96 mm), BDA (67.31), and PDA (67.43). The MEA and SMAY showed mycelial growth of on-par value with colony diameters of 60 mm and 58.03 mm respectively. The least mycelial growth was observed in TCW (44.19 mm). For MAT2, the maximum mycelial growth was observed in SDA (72.72

Table 1. Effect of growth media on the mycelial growth *A. apis* mating types

Growth media	Diameter of the mycelial growth of *Ascosphaera apis* (mm)	
	MAT1	MAT2
SDA	75.40± 1.04^a	72.72± 1.78^a
CDA	67.96± 0.99^b	55.72±1.43^d
TCW	44.19± 0.47^d	41.25± 1.02^e
MEA	60.00± 1.06^c	62.86± 1.57^c
SDAY	74.83± 1.19^a	56.85± 1.63^d
SMAY	58.03± 1.58^c	58.17± 2.07^d
BDA	67.31± 0.90^b	68.17± 1.04^{ab}
PDA	67.43± 1.62^b	66.72± 1.11^{bc}
SED	0.1014	0.1372
CD (0.05)	0.2149	0.2908
CV%	1.55	2.17

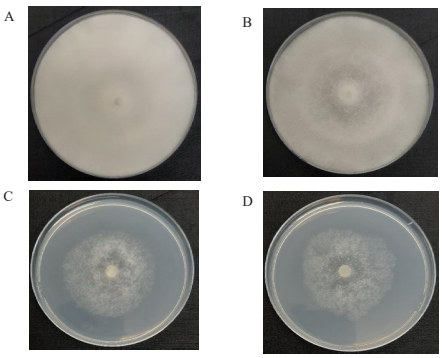

Fig. 1. Growth and reproduction of *A.apis* mating types on media. A. Fluffy mycelial growth of MAT1 on SDA; B. Fluffy mycelial growth of MAT2 on SDA; C. Thin mycelial growth of MAT1 on TCW; D. Thin mycelial growth of MAT2 on TCW

mm) (Fig. 1B) and BDA (68.17 mm) followed by PDA (66.72) and MEA (62.86). SMAY and SDAY showed a medium range of mycelial growth with an average colony diameter of 58.17 mm and 56.85 mm. TCW was the least-performing growth media with a colony diameter of 41.25 mm for MAT2. SDA and SDAY generally supported higher mycelial growth compared to other media for both MAT1 and MAT2. TCW consistently showed the lowest mycelial growth for both MAT1 and MAT2 (Fig. 1C, D). *Ascosphaera apis* exhibited enhanced growth on medium supplemented with sugar such as PDA, CDA, BDA, SDA, SDAY, SMAY, and MEA. However, its development was slower in non-sugar supplemented media like TCW. This aligns with Heath's (1982) observation, noting that *A. apis* thrives on sugar-rich substrates due to its efficient utilization of various sugars like arabinose, dextrose, mannose, galactose, sucrose, maltose, lactose, trehalose, glucose, fructose, as well as dextrin and starch. The growth of mycelium for both mating types increased gradually until 6th days (Fig. 2, 3). However, the mycelial growth of MAT1 was faster on SDA and CDA and reached the edges of the Petri plates on 5th day, while MAT2 grew faster only on SDA and reached the edges of the Petri plates on 5th day. In addition, both mating types produced fluffy mycelial growth on the SDA than other growth media. Overall, SDA, CDA, TCW, and SDAY yielded faster mycelial growth for MAT1 than MAT2. These results were consistent with Claussen (1921) and Mauriziov (1935), who asserted that the MAT1 exhibits a higher growth rate than the MAT2. Whereas, MEA, BDA, and PDA supported their growth nearly equal to both mating types when grown separately. These results are in line with Evison et al. (2015), Bissett (1988), and Anderson and Gibson (1998), they did not observe any variations based on the development of different mating types. These disparities

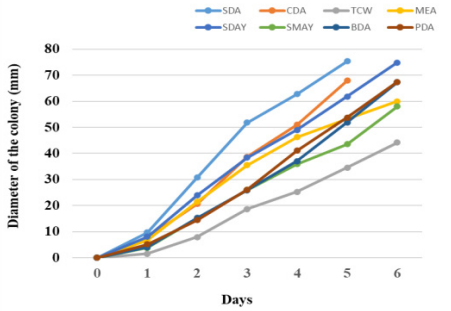

Fig. 2. Growth of *A. apis* MAT1 on media

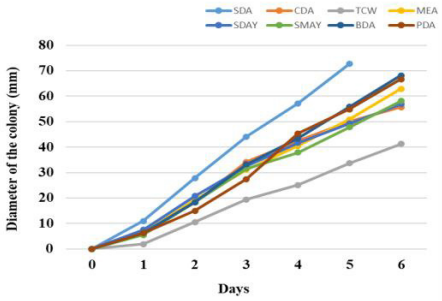

Fig. 3. Growth of *A. apis* MAT2 on media

between the growth of the mating types probably depend on a particular strain of *A. apis*. Furthermore, the reproductive structure formation test revealed that PDA and CDA formed the reproductive structures on 5th day (Fig. 4) and MEA and BDA were formed on 6th day. In all these four media MAT1 grew faster than MAT2. The development speed of *Ascosphaera apis* mating types, which also reflects their degree of virulence, is essential because of the superinfection model of evolution, in which strains compete rather than cooperate (Evison et al., 2015). A less aggressive and less prevalent mating types may possess an evolutionary competitive edge (Brown et al., 2002) in utilizing poor nutrient source more effectively. The evolutionary benefit of slower-growing mating types lies in their improved competitiveness in certain situations; for instance, their slow growth allows them to utilize poor nutrient sources more effectively. Conversely, a more virulent mating type requires more nutrients to sustain its rapid growth. Another benefit is the limited reproduction rate, since a mating type that grows quicker is likely to

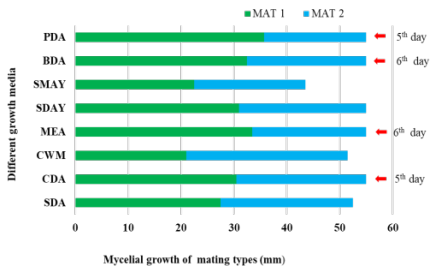

Fig. 4. Growth competition of mating types on media-arrow indicates the reproductive structure formation

be more prevalent. The presence of white mummies in the brood indicates presence of only one mating type while presence of black mummies indicates presence of both mating types. In an experiment, Gilliam (1986) found 35% of the mummies were white. This might help reduce ascospore loads in infected beehives, promoting a balanced evolution between parasite and host.

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CONFLICT OF INTEREST

No conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

VK: Sample collection, isolation and evaluation of growth media; drafting the manuscript. MRS: Advisor for the research work, conceptualization and for drafting the manuscript and reviewing the manuscript. VRS, SK and VB corrected the drafted manuscript.

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