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GROWTH INHIBITION OF *ASCOSPHAERA APIS* BY ROYAL JELLY AND 10-HYDROXY-2-DECENOIC ACID

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Liang-Kuang Chu, Thomas Mei-En Liu and Kai-Kuang Ho (1992) Growth inhibition of *Ascosphaera apis* by royal jelly and 10-hydroxy-2-decenoic acid. *Bull. Inst. Zool., Academia Sinica* **31**(2): 73-79. *In vitro* tests showed that Saboraud dextrose agar+yeast extract (SDA+Y) and pollen agar provided the best medium for the growth and sporulation of the fungus *Ascosphaera apis*, which could causes chalkbrood disease in the honeybee *Apis mellifera*. Honey agar alone did not inhibit the growth of *A. apis*; however, more than 5% (w/v) of royal jelly agar inhibited the vegetative growth of *A. apis*. More than 2.5% (w/v) of royal jelly in the SDA+Y medium was sufficient to delay or even inhibit the growth of *A. apis*, where as a concentration of 1% (w/v) royal jelly in the SDA+A medium promoted both hyphal growth and sporulation of *A. apis*. More than 500 μg/ml of 10-hydroxy-2-decenoic acid (10-HDA) in the SDA+Y medium was also sufficient to delay or inhibit the mycelial growth. However, 100 μg/ml of 10-HDA in the SDA+Y medium appeared to promote sporulation of *A. apis*. Our results showed that the presence of 10-HDA in honeybee larval food played an important role in inhibiting mycelial growth or promoting sporulation of *A. apis*. Nutritional factors affecting chalkbrood infection are also discussed.

Key words: Royal jelly, Chalkbrood disease of bees, *Ascosphaera apis*, Hydroxy-decenoic acid.

The composition of royal jelly has been studied for over 100 years (Planta, 1888); the antibiotic properties of royal jelly were firstly reported 50 years ago (McClesky and Melampy, 1939). Royal jelly has been found to be bacteriocidic or bacteriostatic to *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus*. It can also inhibit or delay the growth of many fungi (McClesky and Melampy, 1939; Blum *et al.*, 1959). The main antibiotic in royal jelly has been identified as 10-hydroxy-2-decenoic acid (10-HDA) (Blum *et al.*, 1959); in addition, Inoue (1982) found glucose oxidase (G.O.) in royal jelly. Thus, at least two inhibitors are included in royal jelly, one a fatty acid (10-HDA), the other an enzyme (G.O.) which produces and accumulates hydrogen peroxide.

Royal jelly is the main food source for bees during their larval stage, especially during the mass feeding stage during the first three days. Unfortunately, very few studies have focused on the relationship between royal jelly and pathogen

of honeybee larval diseases. Holst (1946) reported on the inhibition of *Bacillus larvae* by royal jelly in a cultured medium. Rose and Briggs (1969) found that larval food from resistant colonies exhibited better inhibition of *B. larvae* than larval food from susceptible colonies. Gilliam *et al.* (1978) reported that there was no inhibition of *Ascosphaera apis* by royal jelly. However, Yoshida (1985) found that the occurrence of chalkbrood disease decreased during nectar flow; when the nectar flow ended, the incidence of chalkbrood disease increased. Yoshida speculated that this variance of resistance to chalkbrood disease was due to difference in the amount of royal jelly secreted by worker bees to rear their brood. Moreover, Gochnauer and Margetts (1969) and Heath (1982) showed that *A. apis* lacks many lytic enzymes commonly found in other insect pathogens; they considered it "a relatively non-invasive parasite that kills the host by competition for primary nutrients". Milne (1983) suggested that the mechanisms of honeybee colony resistance to chalkbrood disease were complicated besides hygienic behavior.

A preliminary field survey of chalkbrood disease occurrence in Taiwan revealed that honeybee colony resistance to the disease might be related to the abundance of nectar and pollen sources (Ho *et al.*, 1990). The purpose of this study was to investigate the relationship between *A. apis* and larval food components including royal jelly, honey, pollen, and purified 10-HDA. Nutritional factors affecting the chalkbrood infection will also be discussed.

MATERIALS AND METHODS

Sporulating strains of *Ascosphaera apis* were reared on Saboraud dextrose agar + 0.2% yeast extract (SDA+Y) (20 ml/90 mm culture plate) in a 30°C incubator; 3 mm mycelium disks of *Ascosphaera apis* were then cut from the growing margin of the four-day-old colony and inoculated with the following media:

1. Saboraud dextrose agar (SDA)
   - Peptone 10 g
   - Dextrose 40 g
   - Agar 15 g
   - Distilled water 1 l
2. Saboraud dextrose agar + 0.2% Yeast extract (SDA+Y)
   - SDA 1 l
   - Bacto yeast extract (Difco) 2 g
3. Potato dextrose agar (PDA)
   - Potato 200 g
   - Dextrose 15 g
   - Agar 20 g
   - Distilled water 1 l
4. Potato dextrose agar + 0.4% Yeast extract (PDA+Y)
   - PDA 1 l
   - Bacto yeast extract (Difco) 4 g
5. 2% Malt agar (2% MA)
   - Malt extract 20 g
   - Agar 15 g
   - Distilled water 1 l
6. 0.5% Malt agar (0.5% MA)
   - Malt extract 5 g
   - Agar 15 g
   - Distilled water 1 l
7. Pollen agar (PA)
   - Fresh pollen (ripen seed of *Brassica campestris* harvested near Taichung) 125 g
   - Agar 15 g
   - Distilled water 1 l
8. Honey agar (HA)
   - Honey (longan from *Euphoria longana* harvested near Taichung) 60 g
   - Agar 15 g
   - Distilled water 1 l
9. 50% Royal jelly agar (50% RJA)
   - Fresh royal jelly 500 g
   - Agar 15 g

Distilled water 1 l

10. 10% Royal jelly agar (10% RJA)
    Fresh royal jelly 100 g
    Agar 15 g
    Distilled water 1 l
11. 5% Royal jelly agar (5% RJA)
    Fresh royal jelly 50 g
    Agar 15 g
    Distilled water 1 l
12. 1% Royal jelly agar (1% RJA)
    Fresh royal jelly 10 g
    Agar 15 g
    Distilled water 1 l
13. Czapek-Dox agar (Cz-DA)
    $NaNO_3$ 2 g
    Sucrose 30 g
    K_2HPO_4 1 g
    $MgSO_4 \cdot 7H_2O$ 0.5 g
    $FeSO_4$ 0.01 g
    KCl 0.5 g
    Agar 20 g
    Distilled water 1 l
14. Pasteur's agar (Past A)
    KH_2PO_4 1 g
    $CaHPO_4$ 0.1 g
    $MgSO_4 \cdot 7H_2O$ 0.1 g
    $CH(OH)COO(NO_4)_2$ 5 g
    Glucose 75 g
    Agar 20 g
    Distilled water 1 l

All media were autoclaved at 121.6°C for 15 min. Each petri dish (90 mm diam.) contained 20 ml of medium. The diameters of the colony and sporulating areas were recorded daily until the mycelium covered the entire plate.

Royal jelly was added to SDA+Y and diluted to 10, 7.5, 5, 2.5, and 1% (w/v). All media were then autoclaved as described above. Inoculum was prepared as previously described and its growth rate measured.

Royal jelly acid (10-HDA) was purified by the Kinoshida and Shuel method (Kinoshida and Shuel, 1975), except that the starting material was fresh royal jelly instead of lyophilized jelly. Royal jelly crude fat was extracted with ether. Saponification isolated fatty acids.

White crystalline was established by recrystallization with petroleum ether. 10-HDA purity was determined by gas chromatography (Hitachi 163, Model 833A).

Purified 10-HDA was added to the SDA+Y and diluted 5,000, 1,000, 500, 100, 50, 10 and 5 μg/ml. All media were then autoclaved as mentioned above. Four ml of medium was poured into petri dishes (52 mm diam.) and inoculated as described earlier. There were three replications for each treatment; data were subjected to oneway analysis of variance and Duncan's multiple range test.

RESULTS

In the preliminary test, both fresh royal jelly and honey were tested in an SDA+Y medium considered best for the growth of *Ascosphaera apis*. Our results showed that only fresh royal jelly could inhibit growth of *Ascosphaera apis*. The growth of *Ascosphaera apis* varied in different media (Table 1) as did sporulation.

Our comparison of four-day-old colonies of *Ascosphaera apis* with respect to individual media was a follows: SDA+Y, PA>SDA, Past A, >PDA+Y>HA>2% MA>0.5% MA>1% RJA>5%, 10%, 50% RJA. No growth was observed in RJA (>5%). Amounts of aerial hyphal growth in tested media was in the following order: PA > SDA + Y > PDA + Y. The fungus grew and sporulated better in media supplemented with yeast extract.

A concentration of more than 2.5% (w/v) royal jelly in the SDA+Y medium was sufficient to delay or even inhibit the growth of *Ascosphara apis*. On the contrary, 1% (w/v) royal jelly in the

Table 1
Growth and sporulation of *Ascosphaera apis* in different media

Medium	Colony (mm diam.) days			Sporulating area (mm diam.) days	
	2	3	4	3	4
SDA	26^{c}	57^{b}	72^{bc}	10^{f}	33^{c}
SDA+Y	30^{a}	65^{a}	79^{a}	49^{a}	66^{a}
PDA	22^{d}	44^{d}	54^{e}	28^{d}	35^{c}
PDA+Y	24^{d}	52^{c}	66^{d}	38^{c}	51^{b}
2% MA	13^{f}	24^{f}	34^{g}	18^{e}	20^{d}
0.5% MA	4^{gh}	11^{g}	18^{h}	4^{gh}	4^{e}
PA	28^{b}	59^{b}	74^{ab}	40^{b}	55^{b}
HA	15^{e}	37^{e}	45^{f}	12^{f}	25^{d}
50% RJA	3^{h}	3^{h}	3^{j}	0^{h}	0^{e}
10% RJA	3^{h}	3^{h}	3^{j}	0^{h}	0^{e}
5% RJA	3^{h}	3^{h}	3^{j}	0^{h}	0^{e}
1% RJA	6^{g}	8^{g}	9^{i}	0^{h}	4^{e}
Cz-DA	6^{g}	10^{g}	14^{h}	0^{h}	4^{e}
Past A	20^{d}	55^{bc}	69^{cd}	25^{d}	38^{c}

SDA: Saboraud dextrose agar; SDA+Y: Saboraud dextrose agar+0.2% yeast extract; PDA: Potato dextrose agar; PDA+Y: Potato dextrose agar+0.4% yeast extract; MA: Malt agar; PA: Pollen agar; HA: Honey agar; RJA: Royal jelly agar; Cz-DA: Czapek-Dox agar; Past A: Pasteur's agar. Means in each column not sharing the same letter(s) were significantly different at 5% level according to Duncan's multiple range test.

SDA+Y medium promoted both hyphal growth and sporulation of *Ascosphaera apis* ($p<0.05$) (Table 2).

Ten-hydroxydecenoic acid ($>500\ \mu g$/ml) in the SDA+Y medium was sufficient to delay or inhibit mycelial growth ($p<0.01$). However, 100 μ/ml of 10 μg/ml of 10-HAD in the SDA+Y medium appeared to promote sporulation of *Ascosphaera apis* ($p<0.01$) (Table 3).

Table 2
Inhibition of *Ascosphaera apis* by royal jelly added to SDA+Y

Royal jelly (w/v)	Colony (mm diam.) days			Sporulating area (mm diam.) days		
	3	5	13	3	5	13
1%	43^{a}	90^{a}	90^{a}	21^{a}	70^{a}	90^{a}
2.5%	19^{b}	57^{c}	90^{a}	0^{c}	46^{b}	90^{a}
5%	5^{d}	9^{d}	41^{b}	0^{c}	0^{d}	38^{b}
7.5%	3^{d}	3^{e}	3^{d}	0^{c}	0^{d}	0^{c}
10%	3^{d}	3^{e}	3^{d}	0^{c}	0^{d}	0^{c}
Water agar	11^{c}	13^{d}	16^{c}	0^{c}	0^{d}	0^{c}
SDA+Y	41^{a}	75^{b}	90^{a}	11^{b}	27^{c}	44^{b}

Means in each column not sharing the same letter were significantly different at 5% level according to Duncan's multiple range test.

Table 3
Inhibition of *Ascosphaera apis* by 10-Hydroxy-2-decenoic acid (10-HDA) added to SDA+Y

10-HDA	Colony (mm diam.) days			Sporulating area (mm diam.) days		
(μg/ml)	2	3	4	2	3	4
5,000	3.0[a]	3.0[a]	3.0[a]	0[a]	0[a]	0[a]
1,000	6.7[b]	12.0[b]	19.7[b]	0[a]	6.0[b]	10.3[b]
500	12.7[c]	23.3[c]	42.0[c]	0[a]	11.0[c]	24.7[e]
100	20.7[d]	35.7[d]	52.0[d]	0[a]	21.0[d]	42.0[e]
50	18.7[d]	35.0[d]	51.3[d]	0[a]	18.0[d]	38.7[e]
10	20.7[d]	36.0[d]	52.0[d]	0[a]	21.6[d]	38.0[e]
5	20.7[d]	36.0[d]	51.3[d]	0[a]	20.0[d]	37.3[e]
CK	20.7[d]	36.0[d]	52.0[d]	0[a]	18.3[d]	32.0[d]

Means in each column not sharing the same letter were significantly different at 1% level according to Duncan's multiple range test.

DISCUSSION

Both fresh and autoclaved royal jelly inhibited the growth of *Ascosphaera apis*, but honey did not. Apparently, glucose oxidase was not the main inhibitor; 10-hydroxydecenoic acid may be solely responsible for the inhibition of *Ascosphaera apis*. Thus, our results may strongly challenge the viewpoint of Gilliam *et al.* (1978).

The condition of additives in media may differ considerably from that in the intestines of honeybee larvae. It was found that—in inoculated honeybee larvae reared with royal jelly in a plastic queen cup—the more royal jelly larvae consumed, the more resistant they were against the fungal infection; larvae subjected to starvation showed the highest rate of infection (Chu, 1988). It seems that nutritional factors (larval food sources) determine larval defense against *Ascosphaera apis* attack.

Ten-HDA content in natural royal jelly is about 1.8-2.0% (Howe *et al.*, 1985), while worker jelly and modified worker jelly only contain 1.1% and 0.75%, respectively—enough to inhibit the growth of *Ascosphaera apis* (Okada, 1980). It is reasonable to speculate that when 10-HDA content in larval intestines is high enough (>500 μg/ml), it can delay or inhibit the mycelial growth of *Ascosphaera apis*. Field observations showed that larvae exhibited resistance to *Ascosphaera apis* (i.e., the occurrence of chalkbrood disease decreased or ceased naturally without artificial control) when nectar flow began (Yoshida, 1985; Chu, 1988). In addition apiaries located in areas rich in nectar and pollen sources reduce the incidence of chalkbrood disease (Ho *et al.*, 1991).

When 10-HDA content in larval intestines became favorable to growth and sporulation of *Ascosphaera apis*, food competition between larvae and pathogen occurred. This might be due to penetration of intestines by outgrowths of *Ascosphaera apis*—resulting in larval death. According to field surveys, incidences of chalkbrood increased when nectar flow ended (Yoshida, 1985; Gilliam, 1986; Chu, 1988). Nutritional stress may be the predisposing factor for chalkbrood occurrence.

Bailey (1966) postulated a cool-temperature hypothesis for chalkbrood infection however, it is hard to explain

why heavy infections have been seen in honeybee colonies during hot seasone (Mehr *et al.*, 1976), including when the average monthly temperature was 29°C (Gilliam, 1978). Based on our observations, we postulate a nutritional hypothesis for chalkbrood infection. Honeybee larvae may ingest either spores or mycelium of *Ascosphaera apis* when they are 3-4 days old; the spores then germinate in larval guts which are nearly anaerobic and conducive to spore germination (Bailey, 1966). If the larval intestines are filled with food maintaining a high content 10-HDA (>500 μg/ml), this will directly inhibit or delay mycelial growth of *Ascosphaera apis*. Moreover, the nearly anaerobic conditions will not allow vegetative growth of *Ascosphaera apis*. The mycelium of *Ascosphaera apis* will be excreted during pupation. On the other hand, if the larvae are starved for several days, resulting in 10-HDA content reduction in larval intestines, as well as promoting vegetative growth of *Ascosphaera apis* due to the high air content that therefore diffuses into the gut, the fungus will then penetrate the intestine and invade all larval tissues except those of the tracheal system (Carrera *et al.*, 1987; Chu *et al.*, 1989).

Our hypothesis may explain why weak colonies (De Jong, 1976), an excess of brood (Seal, 1957), colonies suffering from other diseases and pests (Deans, 1940; Heath, 1982), and a dearth of nectar and pollen (Yoshida, 1985; Gilliam, 1986; Chu, 1988) can all enhance chalkbrood infection. We may conclude that our nutritional hypothesis may be more concerned with chalkbrood development, and therefore still remain compatible with a cool-temperature hypothesis (Bailey, 1966). Chalkbrood is a stress-related disease; nutrition and temperature may be the two most important factors affecting the resistance of honeybee larvae to *Ascosphaera apis*.

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蜂王漿及癸烯酸對蜂囊菌 (*Ascosphaera apis*) 生長之抑制作用

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以蜂王漿，蜂蜜，花粉等蜜蜂幼蟲食物配製成六種培養基及其他八種培養基進行蜜蜂白堊病病原眞菌蜂囊菌 (*Ascosphaera apis*) 之生長測試試驗，結果顯示 Saboraud dextrose agar+0.2% yeast extract (SDA+Y) 及花粉瓊脂 (Pollen agar) 爲蜂囊菌最佳生長及產胞培養基。蜂蜜瓊脂 (Honey agar) 對蜂囊菌不具抑制作用，然而含5%以上之蜂王漿者 (Royal jelly agar) 却可以抑制蜂囊菌之生長。

2.5% (w/v) 以上蜂王漿添加於 (SDA+Y) 中，具延緩甚至抑制蜂囊菌菌絲之生長，但是添加 1% 者反有促進生長及產胞之功效。

蜂王漿中重要抗生物質癸烯酸 (10-Hydroxy-2-decenoic acid) 經純化後添加於 SDA+Y 中，500 μg/ml 以上卽可延緩甚至抑制蜂囊菌菌絲之生長，然而相反地，100 μg/ml 却可促進蜂囊菌產胞，顯示蜜蜂幼蟲腸內食物所含之癸烯酸在控制腸內蜂囊菌生長及產胞方面扮演一個重要角色。文中並討論食物因子對蜜蜂白堊病發病之影響。