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Utilization of the nutrients in the soluble and insoluble fractions of fungal mycelium by larvae of the stag beetle, *Dorcus rectus* (Coleoptera: Lucanidae)

MASAHIKO TANAHASHI[1] and KÔHEI KUBOTA[2]

[1]National Institute of Advanced Industrial Science and Technology (AIST), Central 6, Tsukuba, Ibaraki 305-8566, Japan; e-mail: m-tanahashi@aist.go.jp

[2]Laboratory of Forest Zoology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; e-mail: kohei@fr.a.u-tokyo.ac.jp

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Abstract. Larvae of the stag beetle, *Dorcus rectus,* feed on decaying wood, which they digest with the aid of symbiotic yeasts; however, they can be successfully reared on artificial diets containing only fungal tissue. In this study we tested whether *D. rectus* larvae can utilize fungal cell walls, which are an insoluble component of mycelium. Lyophilized *Bjerkandera adusta* mycelium cultured in potato-dextrose liquid medium consisted of a 47.6% hot-water insoluble fraction by mass, which contains 53.7% of the total nitrogen in the mycelium. *D. rectus* larvae that hatched from surface-sterilized eggs were reared for 14 days on agar-based diets containing either the soluble fraction, insoluble fraction or both, extracted from 100 mg of mycelium. The larvae increased in mass most on the mixed diet, and there was no difference in their growth on the mixed and positive control diets. Both the soluble and insoluble fractions improved larval growth compared to the negative control diet; however, the growth rates were much lower than those expected from the nitrogen dose-growth response curve obtained in a previous study. Addition of β-chitin to the soluble fraction did not positively affect larval growth. Therefore, we conclude that (1) *D. rectus* larvae need both the soluble and insoluble fractions of mycelium and (2) the larvae digest the insoluble fraction using their own enzymes.

INTRODUCTION

Solubility is one of the key factors determining food quality. Since soluble nutrients are more easily digested and quickly absorbed than insoluble ones, the amount and balance of soluble nutrients dramatically affect the growth, survival and fecundity of animals such as cows (Janicki et al., 1985), rats (Bronner, 1993), birds (Jefferies & Edwards, 2008) and insects (Awmack & Leather, 2002). Phytophagous insects feed mainly on the soluble components of plant tissues and rarely digest lignocelluloses, which are the main constituents of plant cell walls (Risebrow & Dixon, 1987; Haack & Slansky, 1987). Of plant tissues, wood is the most abundant in terms of biomass of terrestrial plants (Poorter et al., 2012). Since wood is composed of almost 90% lignocelluloses (Parkin, 1940) and contains only 2–10% water-soluble substances (Mahood & Cable, 1922; Browning, 1963; Kilic & Niemz, 2012), it is far more difficult for insects to utilize wood directly as food. Many wood-inhabiting insects have relationships with microorganisms, which help them digest wood. The best studied cases are termites and ambrosia beetles, which harbour symbiotic microorganisms in their guts or feeding tunnels that digest woody substrates (Cleveland, 1924; ; Batra, 1963; Slaytor, 1992; Breznak & Brune, 1994; Geib et al., 2009). Most other wood-feeding insects are thought to be saproxylophagous, which means they feed on decaying wood that is infested with wood-rotting fungi. Saproxylophagous insects are likely to utilize both the fungal tissues and the woody substrates that are partially decomposed and solubilized by the wood-rotting fungi.

The "lesser" stag beetle, *Dorcus rectus* (Motschulsky) (Coleoptera: Lucanidae), is the dominant saproxylic insect in lowland temperate forests in Japan (Kubota & Kubota, 2004). *D. rectus* females deposit eggs on decaying trunks and branches of hardwood trees that are usually infested with white-rot fungi (Araya, 1993; Kubota & Kubota, 2004). Hatchling larvae burrow into decaying wood and consume the surrounding wood together with fungal tissue. After the adults emerge they feed on fermented sap that exudes from tree trunks and rotten fruit. Our previous study showed that *D. rectus* larvae can be reared on an artificial diet that only contains fungal mycelium (Tanahashi et al., 2009), therefore, *D. rectus* is thought to be fungivorous even though they feed on woody material. Moreover, adult *D. rectus* females harbour xylose-fermenting yeast in their mycangium (Tanahashi et al., 2010) and inoculate this yeast along with their eggs into decaying wood (M. Tanahashi, pers. observ.). The yeast may help the larvae to digest the decaying wood and is possibly consumed by larvae as food.

There are large amounts of insoluble polymers in the cell walls of fungi. However, unlike plants, chitin and/or β-1,3-glucan are the major components of cell walls of most species of fungi and yeast (Bowman & Free, 2006; Kurtzman et al., 2011). Since fungal cell walls accumulate in decaying wood over time and are usually more digestible and nutritious than lignocelluloses, they are

likely to be an important source of nutrients for saproxylophagous insects. However, few studies have focused on the utilization of fungal cell walls by saproxylophagous insects. We have already developed an agar-based artificial diet for microbe-free rearing of stag beetle larvae (Tanahashi et al., 2009). In this study, we show that (1) the agar-based artificial diet can be used to determine the nutrient requirements of stag beetle larvae, (2) they require both soluble and insoluble fractions of fungal mycelium and (3) they can digest the insoluble fraction without the aid of gut microbes.

MATERIAL AND METHODS

Insects

D. rectus females were collected in Hachioji City, Tokyo, Japan in 2005 and 2006. They were supplied with wood blocks for ovipositing on and the eggs were collected from the wood blocks every day (Tanahashi et al., 2009). The eggs were surface sterilized by treating them for 20 s with deionised water and then 99.5% ethanol, 70% ethanol twice and sterile deionized water in that order, and then incubated on moistened filter paper in Petri dishes at 25°C in the dark. Newly hatched larvae were weighed within one day of hatching and transferred to the artificial diets mentioned below.

Artificial diets

The white-rot fungus *Bjerkandera adusta* was cultured in potato dextrose liquid medium at 25°C for 10 days and the mycelium was collected by filtration as described by Tanahashi et al. (2009). The mycelium was freeze-dried and ground using a mortar and pestle to a fineness that passed through 0.5 mm mesh. For the hot water extraction, 1.2 g of lyophilized mycelium powder and 120 ml of distilled water were added to a 500-ml pear shaped flask attached to a reflux condenser and the flask placed in boiling water for 3 h. The suspension was filtered through a glass-fiber filter, the weight of which was known. Residue on the filter was rinsed with 30 ml of boiling distilled water and the water was then combined with the first filtrate. After that, the residue on the filter was rinsed with an additional 120 ml of boiling distilled water and freeze-dried for 1 day. Net dry mass of the residue was determined by weighing it to the nearest 0.01 mg on an electronic balance. The residue, which was included in the HWR diet as described below, was carefully scraped off the filter so as not to include glass fibers and then ground using a mortar and pestle to a fineness that would pass through a 0.5 mm mesh. Residual rate was calculated by dividing the net residual mass by initial mass. This procedure was repeated three times and the mean residual rate R calculated.

For the diet containing the hot-water extract (HWE diet), preservatives were added to the extract, which was then diluted with distilled water (Table 1). Fifteen ml of the solution containing the extract and the preservative (equivalent to 100 mg of mycelium) were added to 450 mg of agar powder in a 50-ml glass test tube (27.3 mm inner diameter) with a flat bottom. These test tubes were each capped with a polypropylene cap and autoclaved at 121°C for 15 min. When they had cooled to 50°C, the test tubes were removed from the autoclave, stirred several times and allowed to cool down on a clean bench. For the diet containing the residue after hot-water extraction of the mycelium (HWR diet), 100 × R mg of the residue, 450 mg agar and 15 ml preservative solution were added to each test tube and autoclaved in the same way. For the mixed diet, 15 ml of the solution containing the extract and preservative, 100 × R mg of

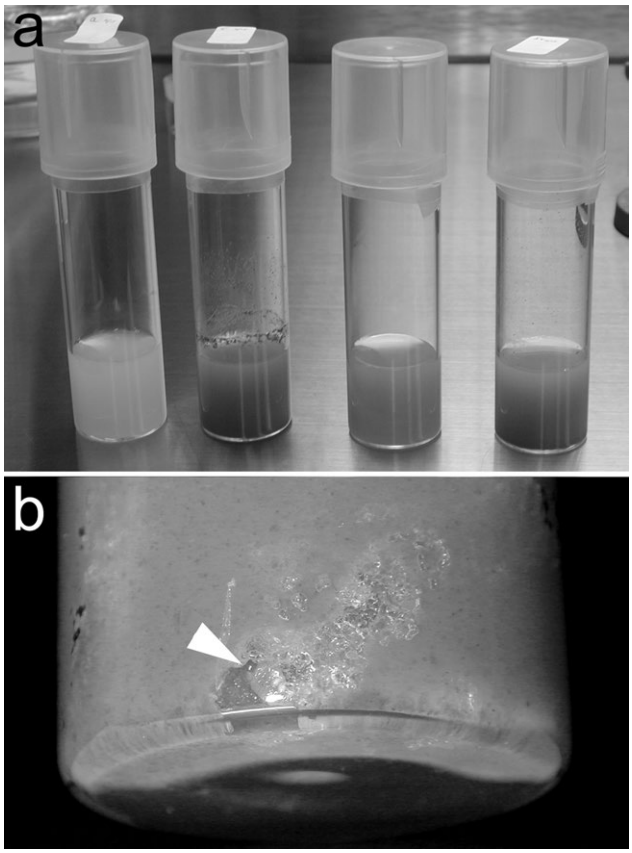

Fig. 1. Tubes containing the artificial diets used in this study. (a) Diet containing no mycelium (negative control), non-extracted freeze-dried *Bjerkandera adusta* mycelium (positive control), hot-water extract of the mycelium (HWE) and residue after hot-water extraction of the mycelium (HWR), in that order from left to right. (b) *Dorcus rectus* larva feeding on the positive control diet. The arrow indicates the head of the larva.

TABLE 1. Composition of the artificial diets for *Dorcus rectus* larvae using lyophilized *Bjerkandera adusta* mycelium.

	Final concentration	For a single diet
B.adusta mycelium (non-extracted), hot-water extract or residue	6.6 mg/l mycelium (or equivalent)	100 mg mycelium (or equivalent)
Ascorbic acid	1.0 mg/ml	15.0 mg
Sorbic acid	0.83 mg/ml	12.5 mg
Sodium hydrocarbonate	1.1 mg/ml	16.5 mg
Agar	30 mg/ml	450 mg
Distilled water		(up to 15 ml)

the residue and 450 mg agar were mixed and autoclaved. For the positive control diet (agar and preservative with 100 mg of mycelium that had not been extracted; n = 16) and the negative control diet (agar and preservative without mycelium; n = 15), we cite the data published in Tanahashi et al. (2009) because both experiments were carried out in parallel. Tubes containing these artificial diets are shown in Fig. 1.

Carbon and nitrogen content

A part (ca. 30 mg) of the freeze-dried mycelium and the residue of hot-water extraction were subjected to carbon and nitrogen analysis using an elementary analyzer (CN Corder MT-700, Yanaco, Japan). Carbon and nitrogen content of the hot water extract were estimated by subtracting each value for the residue from those for non-extracted mycelium. The carbon and nitrogen contents of each fraction are presented in two forms; (1) percentage values for the mass of each fraction and (2) percentage values for the initial mass of the non-extracted mycelium.

Rearing procedure

Immediately after weighing the hatchling larvae of *D. rectus* were placed individually on artificial diets in test tubes and reared at 25°C in the dark. After 14 days, the larvae were recovered from the test tubes and their body mass was determined again. Numbers of the larvae reared on the HWE, HWR and mixed diets were 15, 12 and 10, respectively.

In addition, three larvae were reared on HWE diets containing 30 mg of β-crystalline chitin from squid cartilage (HWE^+) in order to determine whether *D. rectus* larvae need insoluble polysaccharides as a feeding stimulant.

Detection of microbes

After the rearing period, each larva and a piece of the remaining diet were homogenised separately in sterile 1.5 ml tubes and each homogenate was spread onto potato dextrose agar (PDA: Difco) and plate count agar (PCA: Eiken Chemical) plates. The plates were incubated at 25°C for 5 days.

Data analyses

Assuming an exponential growth during the initial 14 days of rearing (Tanahashi et al., 2009), the growth rate was calculated as $\log_e$ (final body mass / initial body mass) / 14. The mean growth rate on each diet was compared using one-way ANOVA followed by Bonferroni's multiple comparisons of paired means at a significance level of 0.05.

RESULTS

Dry mass of the lyophilized *B. adusta* mycelium was nearly halved following the hot water extraction (mean residual rate $R = 0.476$). The carbon content of the extract was higher than that of the residue, whereas the nitrogen content was higher in the residue (Table 2). As a result, the C/N ratio of the residue was much lower than of the extract. The initial masses of the larvae were 6.8 ± 0.2 mg, 7.0 ± 1.3 mg and 6.7 ± 1.4 mg (mean ± s.d.) and the numbers of the larvae that survived/supplied were 15/15, 11/12 and 8/10 for the HWE, HWR and mixed diets, respectively. Larval growth recorded on the mixed diet (50.2 ± 3.4 mg) was greater than that recorded on the positive control diet (46.7 ± 7.9 mg) but they are not significantly different. The growth of the larvae that were reared on the HWE and HWR diets was significantly greater (8.9 ± 2.4 mg and 7.6 ± 2.7 mg, respectively) than that recorded on the negative control diet (4.6 ± 1.6 mg) (Fig. 2). The growth was not as good on the HWR as on the HWE diet, but not significantly different. However, the growth recorded in the HWE and HWR treatments were much lower than those estimated from the dose-growth response curves for total mycelium mass (Fig. 3a) or nitrogen concentration (Fig. 3b). Addition of β-chitin to HWE diets did not positively affect larval growth (8.9 mg for HWE^+ diet, number of survived larvae = 1), although the feeding behaviour of the larvae changed: the larva made a wider tunnel in this diet and tended to stay longer at each site. Larvae in the early stages of their development seemed to have difficulty tunneling into the agar and it is likely that this was the primary cause of death of larvae in all the treatments.

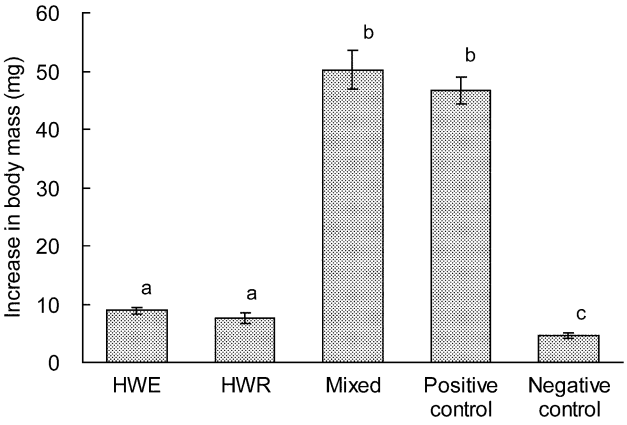

Fig. 2. Growth response of newly hatched *Dorcus rectus* larvae reared on each of the artificial diets. Values are the mean increase in mass of the larvae reared for 14 days on the hot-water extract of *Bjerkandera adusta* mycelium (HWE), hot-water residue (HWR), both extract and residue (mixed), positive control and negative control diets. Bars show ± SE. Different letters above each column indicate a significant difference at the 5% level using one-way ANOVA followed by Bonferroni's multiple comparisons of paired means.

TABLE 2. Carbon and nitrogen contents (mean ± s.d.) of the hot-water extract and residue of lyophilized *Bjerkandera adusta* mycelium.

Materials	n	Total mass (%)	Carbon (%)	Nitrogen (%)	C/N
B. adusta mycelium (total)	5	100.00	43.79 ± 0.38	4.23 ± 0.18	10.37 ± 0.41
Hot-water extract (soluble fraction)	3	52.37 ± 1.07	51.15 ± 0.30 (26.79 ± 0.16)*	3.74 ± 0.04 (1.96 ± 0.02)*	13.69 ± 0.05
Hot-water residue (insoluble fraction)	3	47.63 ± 1.07	35.70 ± 0.33 (17.00 ± 0.16)*	4.77 ± 0.04 (2.27 ± 0.02)*	7.48 ± 0.01

* percentage values based on total mass of the non-extracted mycelium.

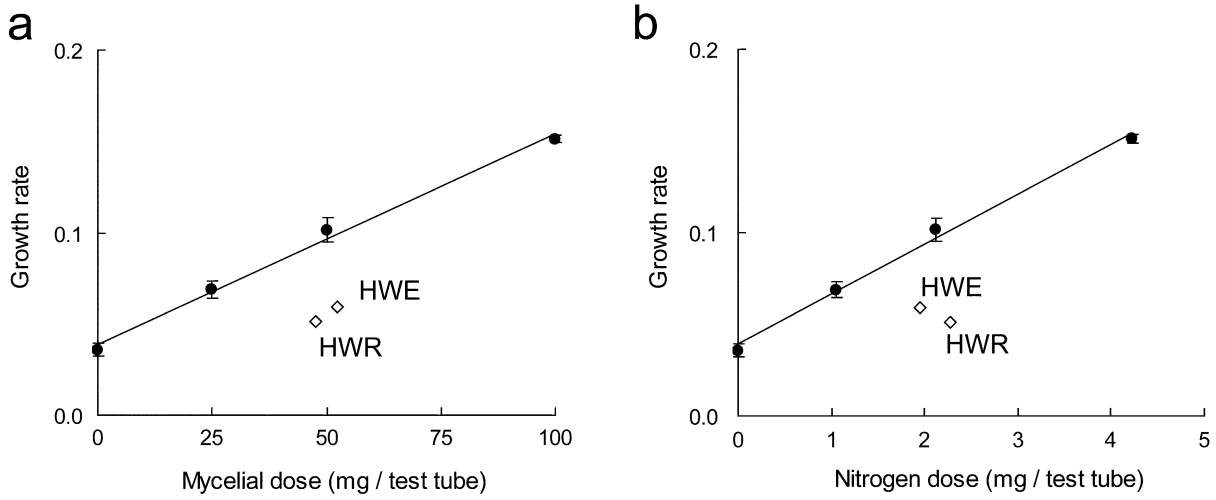

Fig. 3. Growth rate of newly hatched *Dorcus rectus* larvae reared on diets containing (a) different doses of mycelium and (b) nitrogen (after Tanahashi et al., 2009). Bars show ± SE. Open diamonds indicate the mean growth rate when reared on diets containing the hot-water extract (HWE) and hot-water residue (HWR) of *Bjerkandera adusta* mycelium. There is only one point for each diet as the average mass was used to compare the growth on the different diets in this study.

No fungal or bacterial colonies developed on the PDA or PCA plates on which the homogenates of the larvae or remains of the diets were spread.

DISCUSSION

Newly hatched larvae of *D. rectus* significantly increased in mass when fed on the agar-based artificial diet that contained lyophilized mycelium of *B. adusta*. The larvae hardly increased in mass when fed on the artificial diets that contained only the hot-water extract or the residue of the lyophilized mycelium, both of which represent almost half the mass of the mycelium. Larval growth was fully restored when the two fractions were mixed, suggesting that the extraction process did not affect the quality or quantity of nutrients. No fungi, yeasts or culturable bacteria were detected in the larvae or in the diets on which larvae were reared. As a result, we conclude that the larvae of *D. rectus* need both the soluble and insoluble components of mycelium for normal growth and their own enzymes can digest the insoluble components.

The hot-water soluble fraction, which corresponds to the HWE diet, contained 52.4% of the mass of the lyophilized mycelium of *B. adusta* and 46.3% of the nitrogen in the mycelium. The HWE diet would also have contained soluble sugars, amino acids and minerals, which are essential or non-essential nutrients. However, the mean growth of the larvae of *D. rectus* on HWE diets was much lower than that expected from the nitrogen dose-growth response curve published by Tanahashi et al. (2009). Therefore we hypothesized that the HWR diet also includes some essential nutrients and/or feeding stimulants.

The hot-water insoluble fraction, which corresponds to the HWR diet, contained 47.6% of the mass of the lyophilized *B. adusta* mycelium and had a C/N ratio that was much lower than that of the soluble fraction; therefore, the insoluble fraction is likely to be a potential nitrogen source. Fungal cell walls, which account for a large part of the water insoluble fraction, consist mainly of polysaccharides such as chitin (co-polymer of *N*-acetyl-glucosamine and *N*-glucosamine) and β-1,3-glucan (Selitrennikoff, 2001). Chitin is known to be an important nitrogen source for mycorrhizal fungi (Leake & Read, 1990) and bacteria (Streichsbier, 1983; Killiny et al., 2010). However, it is still uncertain whether insects can utilize chitin as a nutrient; for example, predatory insects are unlikely to digest the chitinous exoskeleton of their insect prey (Wilder et al., 2010). Glucosamine content of lyophilized *B. adusta* mycelium cultured on birch wood determined using acid hydrolysis analysis (Jones & Worrall, 1995) was 1.71% and the nitrogen content of purified chitin of the edible mushroom *Pleurotus ostreatus* is 5.9% (Tshinyangu & Hennebert, 1996). Applying those conversion factors to our results, chitinous nitrogen represents only 2.4% of the total nitrogen in *B. adusta* mycelium and 5.1% of that in the water insoluble fraction. This indicates that chitin was not the major source of nitrogen for the larvae and other nitrogen-rich substrates such as superficial mannoproteins and other membrane-associated proteins, which are combined with the chitin-glucan matrix layer (Selitrennikoff, 2001), might account for the high nitrogen content of the insoluble fraction.

Insoluble polysaccharides often aid swallowing in phytophagous insects. Silkworm needs cellulose in order to swallow mulberry leaves (Hamamura et al., 1962). Cellulose is commonly added to the artificial diets of lepidopteran insects and some wood-feeding insects as substitute for wood (Dubois et al., 2002; Gindin et al., 2009). In our experiment, agar might partially compensate for the lack of cellulose as *D. rectus* larvae fed normally on the artificial diets. However, larvae changed their feeding behaviour when β-chitin was added to the HWE diet, suggesting that they use insoluble polysaccharides as a feeding signal. *D. rectus* larvae may not be able to digest

chitin since the addition of β-chitin to the HWE diet did not positively affect larval growth. However, chitin is possibly digested by gut microorganisms in nature and the nutritional importance of fungal chitin should be addressed in future studies.

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