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FULL PAPER

Isolation of yeasts from palm tissues damaged by the red palm weevil and their possible effect on the weevil overwintering

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Abstract *Rhynchophorus ferrugineus* is a tropical pest of palms that has recently invaded Japan, where winter temperatures fall below 0°C. Because activities of the weevil at temperatures <13°C are extremely limited, it appears difficult for them to overwinter in Japan. However, the temperature of palm tissues damaged by this weevil has been observed to be higher than air temperature. Here, we looked for the cause of this temperature increase. First, we measured the temperature of damaged palm tissues and showed it to be between 30°C and 40°C, even in winter. Next, we isolated yeasts from the body of weevils and infested palm tissues and obtained 36 yeast strains, mostly *Candida tropicalis* and *C. ethanolica*. Then, we analyzed the soluble sugar composition in palm tissues and found that it included glucose, sucrose, and fructose. Because at least *C. tropicalis* can ferment some of these sugars, the temperature increase may be attributed to fermentation of microbes, including yeasts.

Keywords *Candida* · Fermentation heat · *Phoenix canariensis* · *Rhynchophorus ferrugineus*

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Introduction

The red palm weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Driophthoridae), one of the most serious pests of palms, was originally distributed in the south Asian tropics. However, after the 1970s, the weevil spread through Southeast, East, and west Asia including the gulf countries; North Africa, and southern Europe (Wattanapongisiri 1966; Rahalkar et al. 1985; Murphy and Briscoe 1999). In Japan, after the first record of the weevil in 1975 in the subtropical island of Okinawa, damage to palms (almost exclusively to *Phoenix canariensis* Hort) has been recorded in various places since 1998, mostly in warm, temperate western areas of the Japanese mainland, especially Kyusyu and western Honshu (Aman et al. 2000; Ito and Koizumi 2003, 2004, Ito et al. 2005; Sato and Irei 2003; Usui and Abe 2009). Palms are not indigenous to Japan but have been commonly planted on roadsides and parks in western Japan. Adult weevils are attracted to and deposit eggs in palm sheaths and stems. Larvae develop primarily in the crown region and feed on growing tissues at the shoot apex. Infestations are problematic because the weevils are usually undetectable until they have caused fatal damage to their host (Rahalkar et al. 1985).

As the Japanese mainland is in a temperate zone, this weevil has a different life cycle than that in the tropical zone. *Rhynchophorus ferrugineus* adults fly all year round in the tropical zone (Vidyasagar et al. 2000; Faleiro et al. 2002). However, their active period is limited in western Japan, where adult weevils begin to emerge from their host plant in March and flying activity lasts only from March to mid-December (Abe and Sone 2007; Abe et al. 2009). Weevil development is arrested, and they are sometimes even killed by low temperatures (Cabello 2006). The temperature 10-cm deep inside healthy *P. canariensis*

trunks has been reported to be similar to air temperature (Yoshitake et al. 2001), which becomes lower than 0°C during winter in western Japan. However, the temperature in the infested part of *P. canariensis* trunks has been observed to be higher in fall and winter, even in that area (Abe and Sone 2007; Abe et al. 2009), and thus may help weevil activity and reproduction in winter. A fermentation fragrance has been observed from such damaged tissues with high temperature, suggesting the activity of microbes with fermentation ability, including yeasts (Abe, unpublished observation). In Spain (the northern limit of *R. ferrugineus* in Europe), a similar temperature increase and plant fermentation in infested plants has been observed (Esteban-Durán et al. 1998).

Among the interspecific interactions involving insects, mutualism between insects and yeasts or yeast-like fungi is important. In well-studied cases of such mutualism, the benefits yeasts provide to insects (reviewed in Vega and Dowd 2005) are all nutritional, as follows: (1) improvement of nutritional quality—for example, two mycangial fungi of *Dendroctonus frontalis* have a positive impact on brood development and survival that likely occur through larvae and adult beetles feeding on fungi (Barras 1973; Bridges 1983; Goldhammer et al. 1990; Ayres et al. 2000); (2) supplementation of enzymes or nutrient chemicals—for example, yeast-like symbionts of *Lasioderma serricorne* and *Stegobium paniceum* provide sterols and vitamins for their hosts (Pant and Fraenkel 1954), and symbiotic yeasts provide essential nutrients, vitamins, and sterols for *Drosophila* (Sang 1978); and (3) detoxification—for example, *Candida ingens* metabolizes toxic fatty acids in cactus tissues and has a positive impact on *Drosophila mojavensis* (Starmer et al. 1982), and *Candida sonorensis* and *Cryptococcus ceranus* have been shown to metabolize 2-propanol, which is toxic to *Drosophila* larvae and adults at moderate to high concentrations in decaying cactus tissue, resulting in positive effects on three *Drosophila* species (Starmer et al. 1986). In turn, yeasts are transported to different habitats or provided with adequate moisture conditions in such mutualism. In the case presented here, if heat is produced by some yeast species and helps winter survival of the insect, it may be considered another mechanism of mutualism between insect and yeast, which is not nutritional. However, before the intensive study of such insect–yeast relationships, it is necessary to examine whether temperature increase is sufficiently high to affect weevils, whether yeasts are actually present, and whether yeasts, if present, can actually conduct fermentation.

In this study, we measured the exact temperature in infested tissues of *P. canariensis* to determine whether the temperature in these parts actually increased and to find out where and when such temperature increase occurred. We also tried to isolate yeasts from infested parts of *P. canariensis* and from weevil bodies and identify the yeasts isolated, and analyze sugar composition in palm tissues to assess whether isolated yeasts could carry out fermentation.

Materials and methods

Temperature measurements and observations inside the trunk of infested palms

Palm trees studied were those attacked by weevils and cut down by the owner or public services, except those used for sugar analysis, which limited the number and methods of sampling. To evaluate the thermal condition of weevil-infested palm tissues, nine dying *P. canariensis,* cut down from 2004 to 2008 in Kagoshima and Nichinan City were used (Table 1). Before measurement, we observed and categorized tissues into the following three phases in relation to weevil activity. (1) Phase 1: tissues still uneaten by weevils located 5–20 cm down the tunnels. (2) Phase 2: tissues just being eaten. (3) Phase 3: tissues already eaten, with the form of tissue altered and the color changed to brown. We measured the temperature of tissues in these three phases with an alcohol thermometer and compared it with air temperature. We made a slit with a hatchet and inserted the thermometer in phase 1tissues. We placed the thermometer in the end of larvae's hole in phase 2 tissues. We inserted the thermometer within phase 3 tissue, which had been broken down and become soft. Measurements were repeated when multiple samplings were possible at the time of felling.

Isolation and identification of yeasts

We used 2% malt extract agar medium plates supplemented with 0.005% chloramphenicol to isolate yeasts. Five larvae, a pupa, and two adults were used for yeast isolation from weevil bodies (Table 2). Three larvae collected from tree no.2 were dipped in 70% ethanol for 1 min to wet the surface, surface-sterilized for 1 min with 15% hydrogen peroxide (H_2O_2) solution, dipped again for 1 min in 70% ethanol, and rinsed in sterilized water. First, the mandibles of one of the larvae were cut and placed on a plate. Afterward, we cut and opened the abdomen of all the three larvae and attempted to isolate yeasts from their body fluid using a sterilized needle. The mandibles of two mature larvae collected from tree no.4 were also cut and placed on plates without sterilization. A naturally dead pupa obtained from tree no.5 was cut, its abdomen opened, and an attempt to isolate yeasts from its body fluid was made using a sterilized needle. An adult female was captured in a building in Kagoshima University. Its rostrum

Table 1 Location of felled *Phoenix canariensis* trees

Year	Day	Location		Palm tree no.
2004	10 November	Kagoshima[a]	31°534′20″N, 130°32′45″E	1
2005	6 October	Kagoshima[a]	31°34′8″N, 130°32′32″E	2
	9 October	Kagoshima[a]	31°34′11″N, 130°32′33″E	3
	20 October	Kagoshima[a]	31°34′9″N, 130°32′32″E	4
			31°34′10″N, 130°32′32″E	5
2007	5 January	Kagoshima[a]	31°34′9″N, 130°32′41″E	6
	10 April	Nichinan	31°33′32″N, 131°23′51″E	7
			31°33′32″N, 131°23′50″E	8
2008	27 May	Kagoshima[b]	31°34′3″N, 130°33′28E	9

[a] Kagoshima University, Korimoto campus

[b] Kagoshima University, Shimoarata campus

Table 2 Yeasts isolation samples obtained from damaged palm trees and weevil bodies

Year	Date	Samples	No. of samples	Palm tree no. (see Table 1)
2005	6 October	Larvae body fluid	3	2
		Larvae mandible (surface-sterilized)	1	2
	20 October	Surface of the larvae mandible (nonsterilized)	2	4
		Body fluid of the dead pupa	1	5
2006	16 November	Rostrum of flying female	1	–[a]
2007	10 April	White colony between pygidium and upper wing of the adult weevil	1	7
2005	6 October	Fresh frass (from phase 2 palm tissue)	2	2
		Inner surface of the end of larvae tunnel (in phase 2 palm tissue)	2	2
		Phase 3 palm tissue	20	2
2007	5 January	Phase 1 palm tissue	11	6

[a] Weevil was caught when flying

was touched on a plate. A cocoon was obtained from tree no.7 and kept in a 500-ml sterilized bottle at 25°C. A white yeast colony was observed between the pygidium and the upper wing margin of a newly emerged adult and was isolated using a sterilized needle.

Palm tissues of the different phases were used for isolation (Table 2). Frass (approximately 1 mm × 1 mm × 2 mm) that had not yet discolored to brown (thus phase 2) were collected from the end of the tunnels and two (or three when small) pieces were placed on each plate. The inner surfaces of the end of the larval tunnels (located in phase 2 tissue) were scraped with the tip of a sterilized needle, and the needle tip was spotted on plates. Twenty pieces (1 mm × 1 mm × 2 mm) of phase 3 and 11 pieces (2 mm × 1 mm × 5 mm) of phase 1 tissues were placed on plates.

Yeasts that formed colonies on isolation plates were subcultured and their morphology observed under a differential interference contrast microscope (BX-33-FLD-2, Olympus). Most isolates showed dimorphic growth (i.e., yeast and hyphal form) and hyaline; one-celled conidia emerged directly and blastically from hyaline hyphae. These characteristics suggest that they belong to the genus *Candida* (Larone 1995). We attempted to identify these isolates down to the species level using the following three techniques.

Yeast isolates were first subcultured onto CHROMagar™ Candida (Becton Dickinson/BBL) and presumptively identified on the basis of colony morphology and distinctive color patterns. This medium allows selective isolation of yeasts and differentiation of more than ten species, including *C. albicans*, *C. tropicalis*, and *C. krusei*. Colony identification is based on the differential release of chromogenic breakdown products from various substrates following exoenzyme activity. Confirmation of their identification was performed using the API ID32C kit (bioMérieux) according to the manufacturer's instructions. Species identification from test scores was made using the manufacturer's databases. Isolates that could not be differentiated at the species level by these conventional methods were identified using molecular assays. The internally transcribed spacer (ITS) region of ribosomal DNA (rDNA) containing the ITS-1, 5.8S rDNA and ITS-2 was amplified using a primer set (ITS1 and ITS4: White et al. 1990), sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (ABI) following the manufacturer's manual, and compared with the DNA sequences deposited

in the GenBank database using the computer program BLAST. Some identified strains were deposited in the culture collection of the Medical Mycology Research Center of Chiba University and given IFM as deposit numbers, and DNA sequences were deposited in the DNA Data Bank of Japan (DDBJ) (Table 4).

Sugar analysis

A 5-year-old healthy *P. canariensis* tree (maximum diameter 30 cm; trunk height 40 cm), which was planted at the Experimental Nursery of Kagoshima University Forest, was cut down 15 cm from the ground, leaves were removed, and the trunk was brought back to the laboratory on 5 October 2008. Several palm-tissue cubes (approximately 5 mm × 5 mm × 5 mm) were obtained from regions near the growing point of the trunk. The tissue cubes were centrifuged together at 20,000 *g* for 10 min. The effluent was collected and recentrifuged at 5,000 *g* for 10 min, and the supernatant was passed through both strong acid cation (Dowex 50W-X8) and strong base anion (Dowex 1-X2) exchange resins. After membrane filtration [polytetrafluoroethylene (PTFE) 0.45 μm], the sample was analyzed with a high-performance liquid chromatography (HPLC) system (LC-6A system, Shimadzu, Japan) equipped with a guard column (Shim-pack SCR-C, 50 mm × 4.0 mm I.D., Shimadzu) connected to an analytical column (Shim-pack SCR-101C, 300 mm × 7.9 mm I.D., Shimadzu) and a reflective index detector (RID-6A, Shimadzu). The columns were heated to 80°C, and water (H_2O) as the mobile phase was allowed to flow at a rate of 0.8 ml min^{-1}. Each sugar was identified by its retention time.

Results

Measurement of temperature inside the tissues of infested palms

Regardless of the year and season, fermentation fragrance and high temperatures were observed in all nine sampled trees when they were cut down. Temperatures in phase 2 tissues were between 30°C and 40°C, that is, 4–26°C higher than the corresponding air temperature (Table 3a). Air, phase 2 tissue, and phase 1 tissue temperatures were 26°C and 28°C, 34–35°C and 33–35°C, and 27°C and 28–29°C, respectively (Table 3b). Temperatures in phase 1 tissues were 4–8°C lower than those in phase 2 tissues and similar (0–1°C higher) to air temperature. Air, phase 2 tissue, and phase 3 tissue temperatures were 17°C, 37°C, and 18°C, respectively (Table 3c). The temperature in phase 3 tissue was 19°C lower than that in phase 2 tissue, and similar (1°C higher) to air temperature.

Yeasts isolation and identification

Eighteen yeast strains were obtained from larvae, pupa, and adult weevils (Table 4). Ten of the 18 strains were identified as *C. tropicalis,*, three as *C. utilis*, and two as

Table 3 Air and infested tissue temperatures

Year	Date	Locality	Air temperature (°C)	Temperature in phase 1 tissues (°C)	Temperature in phase 2 tissues (°C)	Temperature in phase 3 tissues (°C)
(a) Air, and phase 2 tissue temperatures						
2004	10 November[a]	Kagoshima	17		37	
2005	6 October	Kagoshima	26		34, 35	
	9 October[a]	Kagoshima	28		33, 33, 34, 34, 35, 35	
	20 October[a]	Kagoshima	14		30, 33, 34, 37, 40	
	20 October[a]	Kagoshima	14		32	
2007	5 January[a]	Kagoshima	13		32	
	10 April	Nichinan	24		32, 34	
	10 April	Nichinan	24		34, 38	
2008	27 May	Kagoshima	28		32, 34	
(b) Air, and phases 1and 2 tissue temperatures						
2005	6 October	Kagoshima	26	27	34, 35	
	9 October	Kagoshima[a]	28	28, 28, 28, 28, 29	33, 33, 34, 34, 35, 35	
(c) Air, and phases 2 and 3 tissue temperatures						
2004	10 November	Kagoshima[a]	17		37	18

[a] Data based on Abe et al. (2009)

Table 4 Identification of yeasts using API ID32C kit, CHROMagar™ Candida and sequencing

Samples	Source of isolation	Strain no.	IFM	API ID32C kit	CHROMagar™ Candida	Sequencing	Identification	Concordance rate	DDBJ accession no.
Larvae	Fluid	1	55628	*C. tropicalis*	*C. tropicalis*		*C. tropicalis*		
	Fluid	2	55632	*C. tropicalis*	*C. tropicalis*		*C. tropicalis*		
	Mandible (surface-sterilized)	3	55627	*C. tropicalis*	*C. tropicalis*		*C. tropicalis*		
	Mandible	4	55629	*C. guilliermondii*?	*C. glabrata*?	*C. guilliermondii*	*C. guilliermondii*	100% (467/467)	AB534603
	Mandible	5		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534604
	Mandible	6	55630	*C. tropicalis*			*C. tropicalis*		
	Mandible	7	55631	*C. guilliermondii*			*C. guilliermondii*		
	Mandible	8		*C. utilis*			*C. utilis*		
Pupae	Fluid	9	55857	*C. utilis*			*C. utilis*		
	Fluid	10	55858	*C. tropicalis*			*C. tropicalis*		
	Fluid	11	55859	*C. utilis*			*C. utilis*		
	Fluid	12	55860	*C. guilliermondii*			*C. guilliermondii*		
	Fluid	13	55865	*C. tropicalis*			*C. tropicalis*		
Female (captured when flying)	Rostrum	14	55861	*C. tropicalis*			*C. tropicalis*		
	Rostrum	15	55862	*C. tropicalis*			*C. tropicalis*		
	Rostrum	16	55863	*C. tropicalis*			*C. tropicalis*		
	Rostrum	17	55864	*C. tropicalis*			*C. tropicalis*		
Female (escaped naturally from the cocoon)	White colony on the pygidium	18	57361	*C. utilis*?		*Pichia jadanii* or *C. utilis*	*C. utilis*	99.7% (361/362)	AB534605
Palm tissues (phase 2)	Fresh shaving	19	55628	?	*C. tropicalis*?	*C. tropicalis*	*C. tropicalis*	100% (267/267)	AB534606
	Fresh shaving	20		*C. tropicalis*			*C. tropicalis*		
	Fresh shaving	21		?		*Trichosporon mycotoxinivorans*	*T. mycotoxinivorans*	100% (445/445)	AB534607
	Tunnel	22	57360	*C. tropicalis*			*C. tropicalis*		
	Tunnel	23		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534608
Palm tissues (phase 1)	Tissue piece	24		?		*C. ethanolica*	*C. ethanolica*	100% (361/361)	AB534609
	Tissue piece	25		?		*C. ethanolica*	*C. ethanolica*	99.7% (362/363)	AB534610
	Tissue piece	26		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534611
	Tissue piece	27	57357	*C. tropicalis*			*C. tropicalis*		
	Tissue piece	28		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534612
	Tissue piece	29	57358	*C. tropicalis*			*C. tropicalis*		
	Tissue piece	30		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534613

Table 4 continued

Samples	Source of isolation	Strain no.	IFM	API ID32C kit	CHROMagar™ Candida	Sequencing	Identification	Concordance rate	DDBJ accession no.
	Tissue piece	31		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534614
	Tissue piece	32		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534615
	Tissue piece	33	57359	*C. tropicalis*			*C. tropicalis*		
	Tissue piece	34		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534616
	Tissue piece	35		?		*C. ethanolica*	*C. ethanolica*	100% (360/360)	AB534617
	Tissue piece	36		?		*C. ethanolica*	*C. ethanolica*	100% (362/362)	AB534618

DDBJ DNA Data Bank of Japan

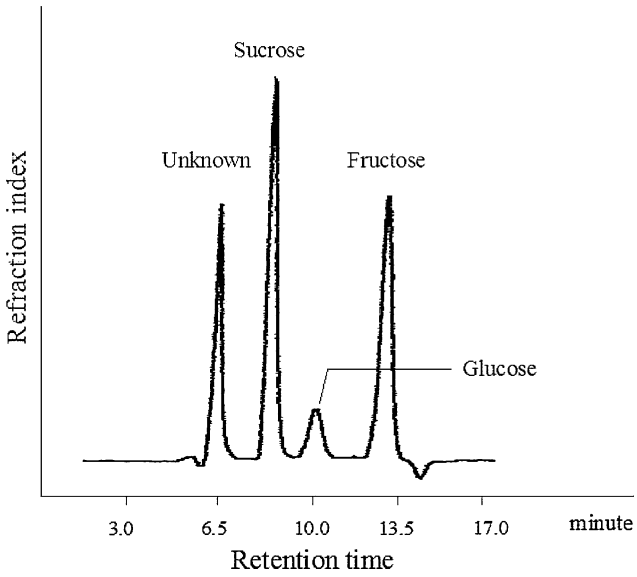

Fig. 1 Chromatograms of sugars in palm tissue analyzed with high-performance liquid chromatography (HPLC)

C. guilliermondii using the API ID32C kit and CHROMagar™ Candida. Three strains that could not be identified using the API ID32C kit or CHROMagar™ were identified by molecular sequencing as *C. guilliermondii*, *C. ethanolica*, and *C. utilis*.

Five yeast strains were obtained from larvae tunnels and frass (Table 4). Two of the five strains were identified as *C. tropicalis* using the API ID32C kit and CHROMagar™. The three other strains were identified by sequencing as *C. tropicalis*, *C. ethanolica* and *Trichosporon mycotoxinivorans*.

Thirteen yeast strains were obtained from phase 1 tissues (Table 4). Of these strains, three were identified as *C. tropicalis* using the API ID32C kit, and the remaining ten were all identified as *C. ethanolica* by sequencing. Yeasts were not isolated in phase 3 tissues.

Sugar analysis

Palm tissue contained sucrose (1.23%), fructose (1.11%), and glucose (0.28%) as soluble sugar (Fig. 1).

Discussion

Most yeasts isolated in this study belonged to the genus *Candida*, an almost completely cosmopolitan genus (Fujita and Yamagata 1981; Kurtzman and Fell 1998; Pfaller et al. 2000). Among the members of the genus, *C. tropicalis* and *C. ethanolica* were dominant, and a few strains of *C. utilis* and *C. gilliermondii* were also isolated. All isolated *Candida* species except for *C. ethanolica* have constant fermentation abilities (Table 5, Kurtzman and Fell 1998).

Table 5 Fermentation ability of the four *Candida* species isolated (data from Kurtzman and Fell 1998)

	Glucose	Sucrose	Maltose	Galactose	Lactose	Raffinose	Trehalose
C. tropicalis	+	v	+	+	−	−	+
C. ethanolica	v	−	−	−	−	−	−
C. utilis	+	+	−	−	−	w	−
C. guilliermondii	+	+	−	v	−	+	+

+ Positive, − negative, *w* weak, *v* variable (±, w/−)

Candida tropicalis can ferment glucose, maltose, galactose, and in some cases sucrose; *C. utilis* can ferment glucose, sucrose, and weakly raffinose; and *C. guilliermondii* can ferment glucose, sucrose, raffinose, trehalose, and in some cases galactose. *Candida ethanolica* has shown variable reactions to glucose but has no reaction to other sugars. In this study, tissues of *P. canariensis* crowns were shown to contain sucrose, glucose, and fructose. Therefore, if the *Candida* species described here invade such tissues, at least three of them are able to ferment some of the sugars present (i.e., glucose and sucrose). Fermentation by ordinary yeasts, including *Candida* species, produces heat. If glucose contained in 1 kg of undiluted solution of palm tissues analyzed here (0.28% = 2.8 g) are used for alcohol fermentation, 3.69 kJ heat will be produced. Therefore, it is suggested that the high temperature of damaged tissues observed in the study may be caused by fermentation produced by these yeast species, which is supported by observation of fermentation fragrance from damaged tissues.

Rhynchophorus ferrugineus undergoes complete metamorphosis. Cabello (2006) reported lethal lower temperatures of 10°C and 5°C for eggs and larvae and a minimum developmental threshold of 15°C and 13°C for larvae and pupae, respectively. In addition, activity of adult weevils (i.e., moving) drastically decreases <20°C; they can hardly move, and they fail to mate and oviposit <15°C (Abe, unpublished observations). Thus, weevils require temperatures >13°C to develop and >15°C to propagate. Yoshitake et al. (2001) reported that the temperature 10-cm deep inside the trunk of healthy palm trees is similar to air temperature, which is within 0–1°C. In Kagoshima City, monthly mean temperatures from December to March 2007 were 8.5–13.3°C according to Japan Meteorological Agency. Therefore, the activity of weevils should be difficult during the winter season. However, damaged palm tissues maintained a temperature of 30–40°C, which is sufficiently high for the development and propagation of the weevils, regardless of air temperature. Thus, it is suggested that heat observed in damaged tissues may promote weevil development during the winter season.

In this study, *Candida* isolates were obtained from all developmental stages of the weevils examined, including flying adult weevil, and from damaged palm tissues, suggesting that yeasts may have been transferred by adult weevils to newly infested palms. However, other possibilities can also be considered; for example, leakage of sugars from damaged tissues may facilitate reproduction of yeasts, which may be carried in the air or stay on the surface or the inner parts of palm tissues. From this study, it cannot be ascertained which of these is correct. Looking at yeast species composition found in different sources, *C. tropicalis* was mainly isolated from weevils, both *C. tropicalis* and *C. ethanolica* were obtained from tunnels or frass, and *C. ethanolica* was predominant in undamaged tissues near tunnels, but a few *C. tropicalis* strains were also isolated. This difference may indicate that different yeast species have different habitat preferences or transmission pathways. Further study is necessary to confirm their colonization processes.

The process of fermentation along with weevil activity may be speculated from the relationships between the presences of *R. ferrugineus*, isolated *Candida* species, and temperature increases for different conditions of palm tissues (Table 6). Remarkable temperature increases were only observed in phase 2 tissues. In such tissues, *R. ferrugineus* was active, and yeasts such as *C. ethanolica* and *C. tropicalis* were isolated. These tissues were generally being destroyed by *R. ferrugineus* and fluids were oozing, which should make the tissues more conducive for microbes, including yeasts to ferment sugars. The isolated yeasts were not only *C. ethanolica*, which is reported to show a variable fermentation reaction only for glucose, but also *C. tropicalis*, which can surely ferment at least glucose. Therefore, it is suggested that microbes, including yeasts, could ferment sugars in these palm tissues, which might produce heat, leading to a significant temperature increase. In phase 1tissues, a temperature increase was not observed. In these tissues, yeasts were isolated, but the majority of strains were *C. ethanolica*, which has only a variable fermentation ability for glucose. Moreover, *R. ferrugineus* was absent, and therefore the tissues were sound. These conditions may be the cause of the lack of

Table 6 Relationships between presence of *Rhynchophorus ferrugineus*, isolated *Candida* species, and temperature increase for different conditions of palm tissues

Condition of palm tissues	Phase 1	Phase 2	Phase 3
Presence or absence of weevil	−	+	+
Isolated *Candida* species	*C. ethanolica* (10/13)[a]	*C. tropicalis* (3/5)	−
	C. tropicalis (3/13)	*C. ethanolica* (1/5)	−
Increase in temperature	−	+	−

+ Present, − absent

[a] Number of isolated strains of the species/total number of isolated strains

temperature increase in such tissues despite the presence of yeasts. In phase 3 tissues, temperature increase was also not observed. In these tissues, weevils were present, but yeasts were not isolated. Moreover, the tissues had already been destroyed completely and had started decaying. It is considered that such palm tissues were unsuitable substrates for microbes, including yeasts, for both fermentation and growth.

Japan is the northern limit of distribution of *R. ferrugineus* in east Asia, where the weevil's flight is limited by low winter temperatures. However, probably because of the heat, which is suggested here to be produced by fermentation, the weevils seems to be able to develop and reproduce in palm trees even during winter. In other words, if the weevil invades other temperate areas with low winter temperatures, they may be able to establish themselves as long as their host plants can grow and permit reproduction of the microbes with fermentation ability. The temperature increase in damaged tissues reported in Spain (Esteban-Durán et al. 1998) appears to be one example of such a case.

This study suggests that the weevil can develop and reproduce inside the palm tree, regardless of air temperature, with the help of fermentation heat. If this fermentation is conducted by the yeast species isolated here, this may be a new type of benefits derived from yeast. The weevils, in turn, seem to provide the substrate for yeast reproduction through adult weevils attacking and larvae feeding on the plant tissues, even if yeasts were not transported by the weevil. Thus, this may be the first identified case of heat-mediated mutualism between insect and yeast. To prove this, however, more direct evidence that shows these yeast species to be the main cause of temperature increase, and data on population-level effects for both the weevil and yeasts by this association is necessary.

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