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Original Papers

ATP Assay to Determine the Viability of the Citrus Whitefly *Dialeurodes citri* (Hemiptera: Aleyrodidae) under Refrigeration

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Abstract: A bioluminescence adenosine triphosphate (ATP) assay to determine viability in sessile insect pests (e.g., whitefly nymphs) under refrigerated conditions (4 ± 1℃) was investigated to assess its applicability in plant quarantine inspections. The ATP content of living and dead *Dialeurodes citri* (Ashmead) (citrus whitefly) fourth-instar nymphs under refrigerated and unrefrigerated conditions was measured. Living *D. citri* that were not refrigerated while alive (n = 18) had 157.69 ± 30.27 pmol (mean ± SD) ATP, and refrigerated live *D. citri* exhibited higher ATP levels when they were refrigerated for longer periods. Combined data from all test groups showed that the ATP content of living *D. citri* ranged from 108.50 to 329.00 pmol, whereas the ATP content of dead *D. citri* ranged from 2.24 to 12.30 pmol 2 h after death, and from 0.11 to 12.00 pmol 24 h after death. Thus, ATP content of *D. citri* rapidly diminished after death, and differences in ATP content between alive and dead *D. citri* were evident within a short time, regardless of whether *D. citri* were unrefrigerated or refrigerated before or after death. In conclusion, the ATP assay technology can be used to determine the viability of insect pests under unrefrigerated and refrigerated conditions.

Key Words: ATP assay, *Dialeurodes citri*, plant quarantine, refrigeration, viability determination

Introduction

In import plant quarantine, the determination of quarantine pest viability have two contradictory requirements: speed and accuracy. Usually, the viability of quarantine pests is primarily determined by visual inspection based on movement and external characteristics such as color and shape. However, in the case of pest eggs or sessile insect pests (e.g., whitefly nymphs or scale insects), limited information can be gathered from visual inspections alone to determine whether the organisms are alive or dead. Therefore, in addition to visual observations, it is also desirable to develop rapid and reliable methods to determine the viability of quarantine pests. In this respect, it is interesting to note that the adenosine triphosphate (ATP) assay, with the application of bioluminescent reactions, has been used to determine viability of a few selected organisms.

Adenosine 5′-triphosphate (ATP) is a ribonucleotide produced in metabolic pathways within living cells (Lipmann, 1941), which stores chemical energy in energy-rich phosphate bonds, supplying the stored energy to sustain life and various cellular activities (Atkinson, 1965; Lipmann, 1941). However, upon cell death, ATP-degrading enzymes degrade ATP until its complete depletion (Lockshin et al., 1977; Pradet and Raymondo, 1983), leading to differences in the ATP content of living and dead cells. A wide range of studies aiming to relate this difference with cell viability has been developed in various organisms, including fungi (Yu et al., 1984), yeasts (Miller et al., 1978), and *Pantomorus cervinus* (Boheman) (Fuller's rose beetle) eggs and larvae (Forney et al., 1991; Ebina et al., 2004). Investigation on *Tetranychus urticae* Koch (two-spotted spider mite) adult females and *Panonychus ulmi* (Koch) (European red spider mite) eggs showed clear differences in the ATP content of living and dead mites in diapause and non-diapause states (Ebina and Ohto, 2007). This finding suggested that the bioluminescence ATP assay might be applicable to determine the viability of quarantine pests found in import plant inspections.

Because Ebina et al. (2004) and Ebina and Ohto (2007) only measured ATP contents under room-temperature conditions (i.e., 20 to 24℃), little is known about ATP levels and pest viability under refrigeration. However, fresh fruits, vegetables, and cut flowers are refrigerated (Matsuura, 1996; Gross et al., 2014), and transported plants are maintained at chilling temperatures, i.e., 4

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to 10℃ (Yazaki, 1997; Editorial Office of Fresh Food Systems, 2001). The present study aimed to test the suitability of bioluminescence ATP assays to determine the viability of the whitefly, an insect pest, under refrigeration.

In plant quarantine inspections, whitefly nymphs are intercepted on cut branches and other imported materials (Tokihiro, 2005). Because whitefly nymphs are sessile insects, it is difficult to determine their viability by visual inspection. Although *Dialeurodes citri* (Ashmead) (citrus whitefly) is a sessile pest insect, and there is little information on its viability, it was chosen as our study subject because it is larger than other whiteflies, has a translucent body, and its viability is comparatively easy to determine based on its appearance. Thus, *D. citri* fourth-instar nymphs were used in bioluminescence ATP assays to investigate pest viability under refrigerated conditions. The only unfavorable factor concerning the use of bioluminescence ATP assay is that the difference in ATP levels between living and dead whitefly individuals might decrease due to the reduction of ATP production and to the suppression of ATP-degrading enzymes under refrigeration.

Materials and Methods

Dialeurodes citri (Ashmead) fourth-instar nymphs were collected between August and September 2003 from privet trees (*Ligustrum* sp., Oleaceae) in Yokohama City (35° 26′ N, 139° 39′ E), Kanagawa Prefecture, Japan. Twenty-five branches infested with *D. citri* nymphs and measuring 20 to 30 cm were cut from trees (Figure 1).

Figure 1 Branches of the privet tree (*Ligustrum* sp.) infested with *Dialeurodes citri* fourth-instar nymphs. Tube-shaped polypropylene containers filled with tap water were attached to branches as a water supply.

The ATP content of individual *D. citri* was measured with an ATP luminometer (Model AF-70, DKK-TOA Corp., Japan), using the method reported by Ebina and Ohto (2007). "Luminescent agent" contained firefly luciferase (D-luciferin), tricine, magnesium acetate, magnesium sulfate, dithiothreitol, bovine serum albumin, sucrose, and water, and "Extractant agent" contained benzalkonium chloride, tricine, and water (DKK-TOA Corp.). The equipment used for the ATP assay was either autoclaved (120℃ for 60 min), dry-heat sterilized (120℃ for 3 h), or purchased pre-sterilized. The luminometer was calibrated against a 100-nM ATP standard solution once, each test day. A single *D. citri* nymph was placed inside each polypropylene tube containing 1 mL 5% (w/v) solution of trichloroacetic acid and crushed. The mixture was incubated at room temperature (22 to 24℃) for 10 min, with occasional swirling, to extract ATP. A 100-µL aliquot of this solution was diluted 10-fold with 40 mM Tris-acetate buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.8) to reach 1 mL volume. Next, a 100-µL aliquot of this diluted solution was pipetted into a disposable plastic measurement tube (12 mm × 55 mm), and the luminescent and extractant agents (100 µL each) were added to obtain a 300-µL solution. The disposable tube containing the sample and reagents was placed on the luminometer, and the ATP content was measured. Before measuring each test group, the luminometer was blanked three times using a solution containing only the reagents, i.e., without *D. citri*. The blank value was 0.00 (pmol/L) in all test groups; therefore, no adjustments were made during the study. The ATP content of individual *D. citri* was measured using the procedures explained above. Unrefrigerated living *D. citri* nymphs were removed from privet leaves and their ATP content was measured before lethal treatment (test group A in Table 1).

The refrigeration temperature in this experiment was set to 4 ± 1℃ to represent actual import conditions. Three groups were refrigerated for 1, 5, and 14 days while alive (test groups B, C, and D, respectively; Table 1). To ensure that the *D. citri* would remain alive under refrigeration, a tube-shaped polypropylene container with tap water was attached to the cut end of each branch comprising *D. citri* on its leaves (Figure 1) and sealed with silicone rubber to stop water inside the tube from leaking out. Branches were then sealed in a polyethylene bag, and stored in the refrigerator. Branches were taken out of the refrigerator after 1, 5, or 14 storage days and the ATP content of living *D. citri* nymphs refrigerated for these periods (test groups B to D) was measured before lethal treatment.

Dialeurodes citri were subjected to the following lethal treatment: nymphs were removed, one by one, from the leaves and individually placed in a sterilized 1.5-mL polypropylene tubes, which were stored at -35℃ for 24 h to kill the nymphs.

Table 1 ATP content in *Dialeurodes citri* fourth-instar nymphs under various conditions: unrefrigerated or refrigerated, and dead or alive.

Temperature condition before lethal treatment			ATP content before lethal treatment		Temperature condition after lethal treatment		ATP content after lethal treatment					
							after 2 h		after 4 h		after 24 h	
			ATP (pmol)	(n)			ATP (pmol)	(n)	ATP (pmol)	(n)	ATP (pmol)	(n)
unrefrigerated		A[*1]	157.69 ± 30.27[*2] (108.50-221.00)[*3]	(18)	unrefrigerated	E	4.16 ± 0.85 (2.27-5.64)	(18)	3.67 ± 0.98 (2.28-5.40)	(18)	1.24 ± 0.94 (0.11-3.21)	(18)
					refrigerated (4 ± 1℃)	F	8.53 ± 2.10 (5.55-12.30)	(18)	7.57 ± 1.93 (4.76-13.20)	(18)	3.35 ± 0.90 (1.82-5.36)	(18)
refrigerated (4 ± 1℃)	1 day	B	187.30 ± 36.23 (142.50-259.50)	(17)	unrefrigerated	G	4.77 ± 1.24 (2.24-6.23)	(12)			1.30 ± 0.81 (0.13-2.65)	(12)
					refrigerated	H	7.00 ± 1.59 (5.24-10.30)	(12)			3.59 ± 1.22 (2.32-6.51)	(12)
	5 days	C	194.50 ± 33.30 (131.00-248.00)	(18)	unrefrigerated	I	3.98 ± 0.88 (2.61-5.65)	(12)			0.87 ± 0.64 (0.19-2.36)	(12)
					refrigerated	J	5.90 ± 1.79 (2.52-9.27)	(12)			2.36 ± 1.15 (0.29-3.95)	(12)
	14 days	D	295.80 ± 33.55 (259.00-329.00)	(5)	unrefrigerated	K	3.84 ± 0.82 (3.04-4.95)	(5)			2.62 ± 5.25 (0.15-12.00)	(5)
					refrigerated	L	6.39 ± 1.46 (4.50-8.10)	(5)			2.92 ± 0.95 (1.34-3.78)	(5)

*1. Test groups were assigned letters from A to L. *2. Values (mean ± SD) represent mean ATP content (± SD) (pmol). *3. Values (minimum value – maximum value) represent ATP content ranges (in pmol).

Unrefrigerated living *D. citri* nymphs whose ATP contents were measured after lethal treatment (test groups E and F), were removed from the leaves and killed, whereas refrigerated living *D. citri* nymphs whose ATP contents were measured after lethal treatment (test groups G to L), were stored in the refrigerator for 1, 5, or 14 days and then removed from the leaves and killed. After the lethal treatment, test groups were further divided into two groups: one was kept in the laboratory at room temperature (unrefrigerated), whereas the other was refrigerated. Following the lethal treatment, tubes containing dead *D. citri* were removed from the freezer and kept in the appropriate environment (laboratory or refrigerator) for a maximum of 24 h. The ATP content of each dead *D. citri* was measured at 2 and 24 h after lethal treatment. In the case of *D. citri* unrefrigerated conditions while alive (test groups E and F), ATP content was measured at 2, 4 and 24 h after lethal treatment (Table 1). In a preliminary experiment using *P. cervinus* and spider mites, we confirmed that ATP content does not decrease during lethal freezing treatment (Ebina et al., 2004; Ebina and Ohto, 2007). Thus, the time point at which *D. citri* were removed from the freezer was set as the experimental time of death.

Overall, the ATP content of living *D. citri* was measured in four groups (A to D) and that of dead *D. citri* was measured in eight groups (E to L). Twenty-two measurements were taken for all groups, including nymphs' ATP content while alive and after 2, 4, and 24 h after lethal treatment (Table 1). Five to 18 *D. citri* individuals were measured at each measurement point (Table 1).

Nymphs' viability was visually assessed. A separate group of fourth-instar nymphs of *D. citri* was placed under a stereoscopic microscope (Model Stemi 2000C, Carl Zeiss Microscopy GmbH, Jena, Germany) and a biological microscope (Model BX50, Olympus Co., Ltd., Tokyo, Japan) to observe body color, shape, and the presence or absence of pulsation in the dorsal vessel. Through this last observation, we verified that refrigerated living *D. citri* were indeed alive, and that the *D. citri* subjected to lethal treatment were dead.

Results

The ATP content of *D. citri* under unrefrigerated conditions while alive, which was measured immediately after insect collection, was 157.69 ± 30.27 (108.50–221.00) pmol (mean ± SD [minimum value – maximum value]). When unrefrigerated living *D. citri* were not refrigerated after lethal treatment, their ATP content after 2, 4, and 24 h was 4.16 ± 0.85 (2.27–5.64) pmol, 3.67 ± 0.98 (2.28–5.40) pmol, and 1.24 ± 0.94 (0.11–3.21) pmol, respectively. The ATP content rapidly decreased after death (Table 1 and Figure 2). When unrefrigerated living *D. citri* were refrigerated after lethal treatment, their ATP content after 2, 4, and 24 h was 8.53 ± 2.10 (5.55–12.30) pmol, 7.57 ± 1.93 (4.76–

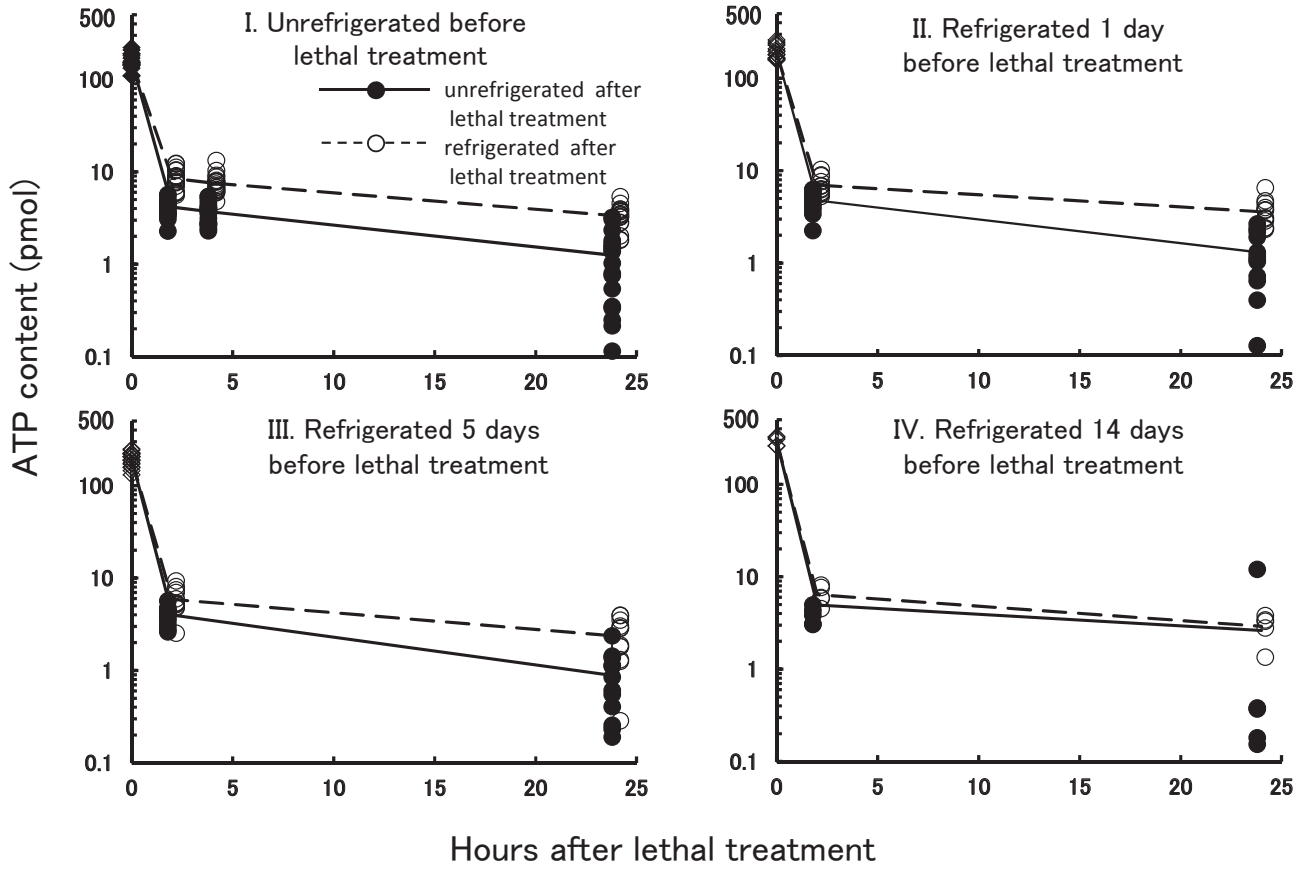

Figure 2 Reduction in ATP content after lethal treatment in *Dialeurodes citri* fourth-instar nymphs. Each line represents the change in mean ATP content, and each point represents the ATP content for individual nymphs; solid lines and closed circles (●) represent unrefrigerated nymphs after lethal treatment; dashed lines and open circles (○) represent refrigerated nymphs after lethal treatment. Values at "zero (0) hours" on the x-axis indicate the ATP content of living nymphs; closed diamonds (◆) indicate unrefrigerated nymphs (graph I); open diamonds (◇) indicate refrigerated nymphs (graphs II to IV).

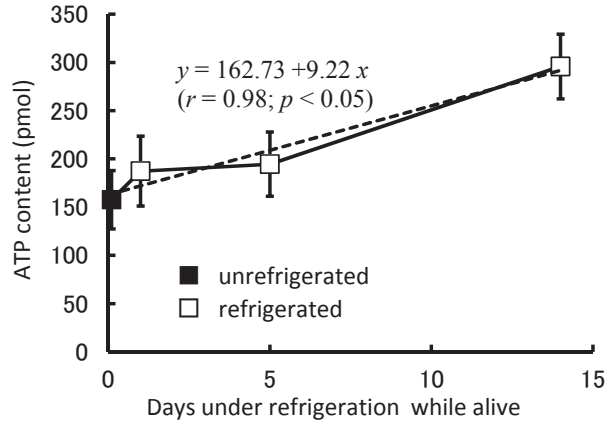

Figure 3 Increase in ATP levels in refrigerated living *Dialeurodes citri* fourth-instar nymphs. Each point represents mean (± SD) ATP content. The dashed line indicates the regression (y = 162.73 + 9.22x; r = 0.98; p < 0.05). Values at "zero (0) days" on the x-axis indicate the mean ATP content (± SD) of the unrefrigerated group (■).

13.20) pmol, and 3.35 ± 0.90 (1.82–5.36) pmol, respectively. Similarly, their ATP content decreased rapidly after death (Table 1 and Figure 2). Regardless of whether the measurement was taken 2, 4, or 24 h after lethal treatment, the ATP content of *D. citri* refrigerated after lethal treatment was, on average, higher than that of nymphs unrefrigerated after lethal treatment (Table 1 and Figure 2).

The ATP content of living *D. citri* kept refrigerated while alive was 187.30 ± 36.23 pmol (refrigerated for one day), 194.50 ± 33.30 pmol (refrigerated for five days), and 295.80 ± 33.55 pmol (refrigerated for 14 days); the ATP content of living *D. citri* increased with increasing refrigeration time (Figure 3). The ATP content of living *D. citri* refrigerated for 14 days was 1.88 times higher than that of living *D. citri* kept unrefrigerated (i.e., when ATP was measured immediately after collection). Based on the regression equation defining the relationship between the number of days under refrigeration while alive (day x) and mean ATP content (pmol, y), y = 162.73 + 9.22x (r = 0.98; p < 0.05) (Figure 3), the ATP content of living refrigerated *D. citri* increased about 9 pmol/day.

The ATP content of *D. citri* refrigerated for one day while alive, subjected to lethal treatment, and then unrefrigerated or refrigerated was 4.77 ± 1.24 pmol or 7.00 ± 1.59 pmol after 2 h, and 1.30 ± 0.81 pmol or 3.59 ± 1.22 pmol after 24 h, respectively. The ATP content of *D. citri* refrigerated for five days while alive, subjected to lethal treatment, and then placed under

unrefrigerated or refrigerated conditions was 3.98 ± 0.88 pmol or 5.90 ± 1.79 pmol after 2 h, and 0.87 ± 0.64 pmol or 2.36 ± 1.15 pmol after 24 h, respectively. The ATP content of *D. citri* refrigerated for 14 days while alive, subjected to lethal treatment, and then placed under unrefrigerated or refrigerated conditions was 3.84 ± 0.82 pmol or 6.39 ± 1.46 pmol after 2 h, and 2.62 ± 5.25 pmol or 2.92 ± 0.95 pmol after 24 h, respectively. At every designated time after lethal treatment in all test groups, the ATP content of *D. citri* refrigerated after lethal treatment was, on average, higher than that of nymphs unrefrigerated after lethal treatment (Table 1 and Figure 2). The reduction in ATP content of dead *D. citri* refrigerated while alive (graphs II to IV in Figure 2) was similar to the reduction in ATP content of dead *D. citri* unrefrigerated while alive (graph I in Figure 2), regardless of the length of the refrigeration period. As mentioned above, the ATP content of dead *D. citri* decreased rapidly in all test groups (Table 1 and Figure 2).

Discussion

The ATP content of *D. citri* fourth-instar nymphs decreased to 2.6% (4.16 pmol) that of live nymphs, when the former were subjected to lethal treatment and unrefrigerated for 2 h; when fourth-instar nymphs were refrigerated for the same time after lethal treatment, ATP decreased to 5.4% (8.53 pmol) that of live nymphs. This rapid and clear reduction in ATP content after lethal treatment is presumed to occur because ATP production ceases after cell death and ATP is degraded by ATP-degrading enzymes. Similar findings were reported for *P. cervinus* eggs and larvae (Forney et al., 1991; Ebina et al., 2004) and *T. urticae* adult females (Ebina and Ohto, 2007).

When live *D. citri* were refrigerated, their ATP content increased with increasing refrigeration days (Figure 3). ATP is consumed during primary active transport across cell membranes; in the guinea pig red cell sodium pump, when temperature decreases, the turnover number at 5℃ decreases to only 0.4% that at 37℃ (Ellory and Willis, 1982). These authors also shown that refrigeration suppresses ATP consumption through a reduction in metabolic activity. Thus, refrigeration might have caused the reduction of metabolic activity and suppression of ATP consumption observed in live *D. citri*; however, ATP production did not decrease conspicuously.

After live *D. citri* (with or without refrigeration) were subjected to lethal treatment, their ATP content declined similarly among individuals (Figure 2). In other words, irrespective of whether or not nymphs were refrigerated while alive, and irrespective of the refrigeration period, dead *D. citri* showed similar ATP content degradation rates after death. This result suggests that refrigeration before death does not affect the biochemical reactions in which ATP-degrading enzymes participate after nymphs' death.

At every test time after lethal treatment (2, 4, and 24 h), the mean ATP contents of *D. citri* refrigerated after death was higher than that of nymphs unrefrigerated after death (Table 1 and Figure 2). Thus, the rate of ATP reduction in *D. citri* refrigerated after death was slower than that of nymphs unrefrigerated after death. According to Forney et al. (1991), the ATP content of insects killed by freezing decreased rapidly after death, but if they were killed by immersion in 55℃ water for 3 min, the rate of ATP content reduction after death slowed until practically stopping 4 h after death. This is likely because ATP-degrading enzymes were partially inactivated by the hot water treatment (Forney et al., 1991). Similarly, the rapid reduction in ATP content after lethal treatment observed in the present study might be due to the degradation of ATP by ATP-degrading enzymes. Taken all previous reports and the present results into consideration, the 4℃ refrigeration environment seems to have helped suppressing the activation and effectiveness of ATP-degrading enzymes.

In *D. citri* unrefrigerated after lethal treatment, the ATP content 2 h after death was 1.3% to 2.6% that of live nymphs, and in *D. citri* refrigerated after lethal treatment the ATP content was 2.2% to 5.4% that of live nymphs. Thus, the difference in ATP content between live and dead *D. citri* was noticeable 2 h after lethal treatment under both conditions. In other words, although refrigeration after death suppressed the function of the ATP-degrading enzymes, the action of ATP-degrading enzymes led to clear difference in ATP contents between live and dead *D. citri*, irrespective of nymphs refrigeration after death.

The use of ATP assays to determine insect pest viability must meet two conditions: an ascertainable difference in ATP content between live and dead insects, and a rapid reduction in ATP content shortly after death. Combined data from all test groups showed that the ATP content of living *D. citri* ranged from 108.50 to 329.00 pmol, whereas the ATP content of dead *D. citri* ranged from 2.24 to 12.30 pmol 2 h after death, and from 0.11 to 12.00 pmol 24 h after death (Table 1). Thus, ATP content of *D. citri* rapidly diminished after death, and differences in ATP content between alive and dead *D. citri* were evident within a short time, regardless of whether *D. citri* were unrefrigerated or refrigerated before or after death. As the biochemical reaction associated with ATP transfer of chemical energy within cells (ATP production and ATP consumption) is common to all eukaryote cells (Alberts et al., 2008), we suppose that ATP assays under refrigeration can be applied to other organisms. From our findings, we conclude that ATP assay technology can be used to determine viability of insect pests under unrefrigerated and refrigerated conditions.

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和文摘要

冷蔵下に置かれたミカンコナジラミ *Dialeurodes citri* (Hemiptera: Aleyrodidae) の生死を判定するための ATP 分析法（ATP アッセイ）

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植物検疫において、寄主植物に固着して動かない害虫（たとえばコナジラミ類若虫等）は目視による生死の判定が難しいため、生物発光現象によるアデノシン三リン酸（以下 ATP）分析法（ATP アッセイ）を利用した昆虫の生死判定技術の開発が検討されてきた。本調査では冷蔵条件が ATP 測定値にどのように影響するかといった従来の調査が扱わなかった点に着目し、非冷蔵条件下（室温）及び冷蔵条件下（4 ± 1℃）のそれぞれにおいて、生存時及び死亡後それぞれの *Dialeurodes citri*（ミカンコナジラミ）4 齢若虫の 1 個体毎の ATP 量を計測した。その結果、非冷蔵条件下の生存供試虫（n=18）の ATP 量は 157.69±30.27 pmol（平均値 ± 標準偏差）であり、生存時に冷蔵条件下に置かれた生存供試虫の ATP 量は冷蔵日数に従って増加した。全試験区のデータを合わせると、生存供試虫の ATP 量が 108.50-329.00 pmol の範囲であることに対し、致死処理後 2 時間経過後で 2.24-12.30 pmol の範囲、致死処理後 24 時間経過後で 0.11-12.00 pmol の範囲となった。このように、生存時及び死亡後において非冷蔵条件下または冷蔵下条件下のいずれに置かれても、供試虫の生存時と死亡後との ATP 量の差異は死亡後短時間で明瞭となることが確認された。以上のことから、冷蔵下においても ATP アッセイを利用する昆虫の生死判定は実用の可能性があるものと結論づけられた。

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