ATP assay for determining the viability of the two-spotted spider mite (*Tetranychus urticae* Koch) and the European red mite (*Panonychus ulmi* (Koch)) (Acari: Tetranychidae) during diapause

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Abstract

A bioluminescent adenosine triphosphate (ATP) assay for determining viability in adult females of the two-spotted spider mite, *Tetranychus urticae*, and eggs of the European red mite, *Panonychus ulmi*, (both diapause and non-diapause state), was investigated. ATP contents in living and dead individual mites (adult females or eggs) were measured. The mites of the lethal treatment group were killed by freezing at -35°C . Mean ($\pm\text{SD}$) ATP contents for non-diapause and diapause *T. urticae* female adults were 34.39 ± 5.14 pmol and 26.40 ± 2.67 pmol, respectively. Corresponding values for non-diapause and diapause *P. ulmi* eggs were 1.12 ± 0.19 pmol and 0.87 ± 0.16 pmol, respectively. ATP contents in living mites in diapause were therefore approximately 77% of those in non-diapause mites. A wide variance in the ATP contents (0.00–0.94 pmol) was found in individual *P. ulmi* diapause eggs at 2 and 4 h after treatment; therefore, their mean ATP contents did not show a rapid decrease. We speculated that some *P. ulmi* diapause eggs survived for several hours after treatment by cold hardiness, maintaining metabolic activities until death. However, with the exception of *P. ulmi* diapause eggs, ATP contents in both mite species rapidly decreased at 2 h after treatment. At 24 h after treatment, ATP contents in both mite species had decreased significantly to 0.4–2.7% of the pre-treatment values, regardless of whether they were in a non-diapause or diapause state. Thus, based on our findings with *T. urticae* adult females and *P. ulmi* eggs, the ATP bioluminescence assay could be applicable to the determination of pest viability, regardless of their diapause state.

Key words: ATP assay; determining viability; *Tetranychus urticae*; *Panonychus ulmi*; plant quarantine

INTRODUCTION

Adenosine 5'-triphosphate (ATP) is a ribonucleotide produced in metabolic pathways within cells; it stores and provides the energy required to sustain life and various other activities by means of energy-rich phosphate bonds (Lipmann, 1941; Atkinson, 1965). When the cell dies, ATP-degrading enzymes break down ATP until it is completely depleted (Lockshin et al., 1977; Pradet and Raymond, 1983; Forney et al., 1991). Accordingly, since ATP contents differ between living and dead organisms, the reductions in ATP contents after death have been studied in some organisms, for example, yeast (Miller et al., 1978) and fungal spores (Yu et al., 1984), as a means of determining their viability. With respect to insect pests, the eggs and

larvae of the Fuller rose beetle, *Pantomorus cervinus* (Boheman), have been investigated, and they showed clear reductions in ATP contents after lethal treatment (Forney et al., 1991; Ebina et al., 2004). These reports suggest the potential for ATP bioluminescence assays to determine the viability of quarantine pests intercepted at plant quarantine inspections.

Import plant quarantine is conducted at seaports and airports in Japan to prevent the invasion of plant pests or diseases endemic to exporting countries. Viability determination for quarantine pests is required in the shortest possible time so as not to impede the efficient flow of imported plants and plant products. Accordingly, the viability of quarantine pests is primarily determined by visual inspection based on external characteristics, such as

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color and shape; however, for some 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 such inspections, pests in a diapause state are occasionally found, such as diapausing spider mites. Diapause is a physiological state of arrested development, which enables an organism to more easily survive a period of unfavorable conditions (Dickson, 1949; Jeppson et al., 1975). It is therefore anticipated that diapausing pests will show decreased ATP contents due to reduced metabolic activity. It is further speculated that when ATP contents in the diapause state of pests are very low, or when the decrease in ATP contents following lethal treatment is very small, the use of an ATP bioluminescence assay to determine viability might not be appropriate due to minimal differences in ATP contents between living and dead organisms. Therefore, we investigated the ATP bioluminescence assay method to determine the viability of adult females of the two-spotted spider mite, *Tetranychus urticae* Koch, and of the eggs of the European red mite, *Panonychus ulmi* (Koch) in both diapause and non-diapause states. The choice of these organisms was based on their availability for experimentation.

MATERIALS AND METHODS

In the experiment, *T. urticae* adult females and *P. ulmi* eggs were used. The difference in the diapause stages of these mites (Lees, 1953; Veerman, 1985) accounts for the different stages used in this study.

T. urticae were provided by the Faculty of Horticulture, Chiba University (Chiba Prefecture, Japan), and were maintained at the Research Division, Yokohama Plant Protection Station (YPPS), Ministry of Agriculture, Forestry and Fisheries of Japan (Kanagawa Prefecture, Japan). These mites were reared on bean (*Phaseolus vulgaris*) leaves placed on paper towels soaked in water in a plastic Petri dish (90 mm diameter) at 20°C and 60–70% RH under a light-dark photoperiod of 16L:8D. Diapause adult females were selected from those reared from eggs under a short photoperiod

(8L:16D) at 18°C. Based on the reports by Lees (1953) and Gotoh and Shinkaji (1981), diapause females were identified by a vivid orange pigmentation and the absence of black spots.

P. ulmi were collected from an agricultural test field at the Aomori Prefectural Agriculture and Forestry Research Center in Kuroishi-shi (40°38'N; 140°37'E), and were maintained at the Research Division, YPPS. The mites were reared on the leaves of potted apple (*Malus domestica*) seedlings (40 cm height, one plant per pot) at 20°C and 60–70% RH under a photoperiod of 16L:8D. Non-diapause eggs were collected from those deposited on apple leaves under a photoperiod of 16L:8D. Diapause eggs were obtained from those deposited on apple twigs (30 cm length) infested by adult females, which had been kept under a short photoperiod (8L:16D) at 15°C. Based on the report by Lees (1953), eggs deposited on twigs and pigmented dark red were identified as being in diapause. After allowing the laying of diapause eggs for 14 days, we cut twigs on which eggs had been deposited into pieces of appropriate length and transferred the pieces to glass tubes (25 mm × 200 mm), which were then sealed with cotton wool. Following the reports by Lees (1953) and Tsugawa et al. (1966), the diapause eggs were maintained at 10°C (under a short photoperiod) for 30 days after collection, and then at 5°C (under complete darkness) for 40–60 days. These low temperature treatments resulted in high hatching ratios of diapause eggs. The hatching ratios of the diapause and non-diapause *P. ulmi* eggs were both approximately 90%.

ATP contents were measured using a Model AF-70 ATP Tester Luminometer (DKK-TOA Corp., Japan). Reagents used for measuring ATP contents were “Luminescent Agent[®]” (containing firefly luciferase, D-luciferin, tricine, magnesium acetate, magnesium sulfate, dithiothreitol, bovine serum albumin, sucrose and water) and “Extractant Agent[®]” (ibid. containing benzalkonium chloride, tricine and water) (DKK-TOA Corp.). Usually, the above mentioned luminometer and reagents are used for microbial control by counting bacteria attached to a surface. The laboratory ware (forceps, glassware, etc.) used for the ATP assay was either autoclaved (120°C, 60 min) or dry heat sterilized (120°C, 3 h), unless it had already been sterilized when purchased and was being used for the first

time.

ATP content measurements were conducted in accordance with the methods described by Ebina et al. (2004). Luminometer calibration was carried out daily prior to taking measurements using a 100 nM ATP standard solution. Individual mites (one adult female or one egg) were crushed in 1 ml of a 5% (w/v) solution of trichloroacetic acid in 1.5 ml polypropylene tubes. The mixture was left for 10 min with occasional swirling to extract ATP. An aliquot of the mixture (100 μ l) was diluted with 40 mM Tris-acetate buffer containing 1 mM ethylenediaminetetraacetic acid (pH 7.8) to a final volume of 1 ml. An aliquot of this diluted solution (100 μ l) was pipetted into a disposable plastic measurement tube (12 mm $\times$ 55 mm) to which was added 100 μ l of Luminescent Agent and 100 μ l of Extractant Agent giving a total 300 μ l of solution. Measurements of ATP contents, which required approximately 10 s, were made by placing each disposable tube containing the sample material and reagents in the luminometer. For each test group, blank values (background luminescence) were obtained using a solution of reagents only (without the mite sample). Blank values were subsequently subtracted from mite sample results to give measurements of mite ATP contents corrected for the influence of reagents.

For each test group, we tested 18 *T. urticae* adult females and 10–18 *P. ulmi* eggs. For the lethal treatment groups, with the exception of *P. ulmi* diapause eggs, the mites were killed by freezing at -35°C for 24 h; however, the *P. ulmi* diapause eggs were killed by freezing at -35°C for 7 days since diapause eggs have been reported to possess cold hardiness (MacPhee, 1961; Tsugawa et al., 1966). It was confirmed that following lethal treatment, all *T. urticae* adult females were dead, and *P. ulmi* eggs were incapable of hatching. It was also confirmed that ATP contents did not decrease during the freezing treatment; therefore, the point at which the mite was placed at room temperature after treatment was set as the experimental time of death. ATP contents in individual mites were measured at 2, 4, and 24 h for *T. urticae* adult females and *P. ulmi* non-diapause eggs, and at 2, 4, 8, and 24 h for *P. ulmi* diapause eggs after the treatment (i.e., after the mites were placed at room temperature). Based on the study by Ebina et al. (2004), the measurement at 24 h after treatment was to determine the

ATP contents of dead mites (i.e., depleted ATP contents), while those at 2 and 4 h after treatment were to observe the rate of decline in ATP contents.

RESULTS

The results for *T. urticae* adult females are shown in Fig. 1. ATP contents (mean $\pm$ SD) in non-diapause living females and diapause living females per individual were 34.39 ± 5.14 pmol and 26.40 ± 2.67 pmol, respectively. Thus, ATP contents in diapause females were 76.8% of those in non-diapause females. For non-diapause females, ATP contents in mites subjected to lethal treatment decreased to 0.45 ± 0.22 pmol at 2 h after treatment (1.3% of that in live non-diapause females), and to 0.15 ± 0.12 pmol at 24 h after treatment (0.4% of that in live non-diapause females) (Fig. 1). Similarly, for diapause females, ATP contents decreased to 0.79 ± 0.10 pmol at 2 h after treatment (3.0% of that in live diapause females), and to 0.18 ± 0.06 pmol at 24 h after treatment (0.7% of that in live diapause females) (Fig. 1). Thus, ATP contents in *T. urticae* adult females decreased rapidly during the 2 h after treatment, and remained at a low level until 24 h after treatment, regardless of whether the female mite was in a non-diapause or diapause state.

The results for *P. ulmi* eggs are shown in Fig. 2. ATP contents (mean $\pm$ SD) in non-diapause and

Fig. 1. Reduction in ATP contents in *Tetranychus urticae* adult females after freezing treatment (lethal treatment) at -35°C for 24 h. Lines represent mean ATP content, and each point represents data for one individual ($n=18$); dotted line with open circles indicates non-diapause state; solid line with closed circles indicates diapause state. * Data at 0 h on the X-axis indicate ATP content in living mites.

Fig. 2. Reduction in ATP contents in *Panonychus ulmi* eggs after freezing treatment (lethal treatment) at -35°C for 24 h (for non-diapause eggs) or 7 days (for diapause eggs). Lines represent mean ATP content, and each point represents data for one individual ($n=10-18$); dotted line with open circles indicates non-diapause state; solid line with closed circles indicates diapause state. * Data at 0 h on the X-axis indicate ATP content in living mites.

diapause live *P. ulmi* eggs per individual were 1.12 ± 0.19 pmol and 0.87 ± 0.16 pmol, respectively. Thus, ATP contents in diapause eggs were 77.7% of those in non-diapause eggs. ATP contents in non-diapause eggs decreased to 0.10 ± 0.09 pmol at 2 h after treatment (8.9% of that in live non-diapause eggs), and to 0.03 ± 0.05 pmol (2.7% of that in live non-diapause eggs) at 24 h after treatment (Fig. 2). In contrast, diapause egg ATP contents decreased to 0.43 ± 0.24 pmol at 2 h after treatment and to 0.41 ± 0.34 pmol at 4 h, an overall less prominent decrease (Fig. 2); however, at 24 h after treatment, diapause egg ATP contents had declined to 0.02 ± 0.01 pmol (2.3% of that in live diapause eggs), which was almost as the same as that recorded in non-diapause eggs (Fig. 2).

In addition to these results, it has been proved that ATP contents (mean $\pm$ SD; $n=5$) in individual living *T. urticae* eggs were 1.36 ± 0.09 pmol (Ebina, unpublished); therefore, it was considered that the different ATP content between *T. urticae* females (34.39 ± 5.14 pmol) and *P. ulmi* eggs (1.12 ± 0.19 pmol) was due to their different body size.

DISCUSSION

It was observed that ATP contents in *T. urticae* diapause adult females and *P. ulmi* diapause eggs were approximately 77% of those in non-diapause

mites (*T. urticae* diapause adult females, 76.8%; *P. ulmi* diapause eggs, 77.7%). Although the individuals studied represent two different species and have different diapause stages (adult or egg), their ATP production and consumption in the diapause state can be regarded as similar. The physiological features associated with the diapause state are generally known to include markedly reduced metabolic activity (Danilevskii, 1961); this, in turn, is likely to account for a reduction in ATP demand and suppression of ATP production, thereby resulting in a decrease in ATP contents. ATP consumption of diapause mites was also considered to be lower than that of non-diapause mites. While McEnroe (1961) reported that oxygen consumption via external respiration in diapause adult females of *T. urticae* was 19.3% of that in non-diapause females, the reduction in ATP content during diapause was not as pronounced as that in oxygen consumption. Results from the present study showed that ATP contents in mites in diapause, which is a state of suppressed metabolic rate and other activities, were relatively similar to those in the non-diapause state.

ATP contents in *T. urticae* adult females and *P. ulmi* eggs decreased significantly in the 24 h following lethal treatment, regardless of whether they were in a non-diapause or diapause state (Figs. 1 and 2). With the exception of *P. ulmi* diapause eggs, ATP contents in mites of both species decreased rapidly at 2 h after treatment. These findings are in accordance with studies showing that ATP contents of *P. cervinus* larvae and eggs are reduced at 2 h after lethal treatment to 1% or less of the values measured when alive (Forney et al., 1991; Ebina et al., 2004). As noted above, rapid decreases in ATP contents are considered attributable to the fact that while ATPs are actively produced by metabolic processes in living cells, they are readily broken down by ATP-degrading enzymes after cell death.

Mean ATP contents in *P. ulmi* diapause eggs did not show a rapid decrease (Fig. 2); however, some *P. ulmi* diapause eggs were observed to have significantly lowered ATP contents at 2 and 4 h after treatment (Fig. 2). This indicates the possibility that *P. ulmi* diapause eggs could display a rapid postmortem decrease in ATP contents. If ATP contents decrease uniformly and slowly after treatment, this should occur in all individuals. Figure 2

shows a wide variance in ATP contents (0.00–0.94 pmol) in *P. ulmi* diapause eggs at 2 and 4 h after treatment. It is speculated that this variance is presumably attributable to the cold hardiness of *P. ulmi* diapause eggs. Organisms in diapause are known to have higher survival rates under low temperature conditions than those in non-diapause states, as shown in a previous cold hardiness study on diapause pupae of the flesh fly, *Sarcophaga crassipalpis* (Lee and Denlinger, 1985). Similarly, *P. ulmi* diapause eggs are reported to show cold hardiness and enhanced freezing tolerance when maintained at or below -30°C (MacPhee, 1961); therefore, non-diapause and diapause eggs are believed to display differential sensitivity to lethal treatment (freezing at -35°C) implemented in this study. Diapause eggs eventually died after lethal treatment, but some of the eggs survived for several hours, maintaining metabolic activities until death. In other words, it was assumed that although ATP contents in *P. ulmi* diapause eggs rapidly decreased after they died, there were individual differences in the length of time prior to death. At 8 h after treatment, no individuals (diapause eggs) kept the same level of ATP contents as in live eggs, and as a result, their mean ATP content was apparently lower than in live eggs (Fig. 2); thus, it is considered that the number of live eggs decreased over time following lethal treatment. At 24 h after treatment, ATP contents in *P. ulmi* diapause eggs exhibited a decrease similar to that observed in non-diapause eggs (Fig. 2). These findings appear to indicate the practical applicability of the present technique to *P. ulmi* diapause as well as non-diapause eggs.

With a view to applying ATP bioluminescence assays to viability determinations of quarantine pests, it is essential to confirm clearly observable differences in ATP contents before and after the death of organisms. The results of the present study indicate that the above requirements are satisfied with respect to diapause mites. Based on our findings with *T. urticae* adult females and *P. ulmi* eggs, the ATP bioluminescence assay could be applicable to the determination of pest viability, regardless of their diapause state.

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