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Polydnavirus Symbiont Detected from Calyx Tissues Wasps of Three Lepidopteran Cabbage Pests

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Parasitoid wasps are a potent biological control agent in the field. The successful of parasitism are determined by several factors, among them by the presence of polydnavirus (PDV) symbiont that could break down the immunity mechanism of its host. We explored the existence of PDV on wasps *Snellenius manilae*, *Cotesia* sp., and *Diadegma semiclausum*, a group of parasitoid on cabbage pests in Indonesia. Morphological study of PDV was done by preparing ultrasectioned calyx tissues and negative stained of extracted calyx fluid of adult parasitoids. Virogenic stroma resulted from differentiated calyx epithelial cells appeared on all three wasps. Bracovirus and ichnovirus were detected from the calyx tissues of *S. manilae* and *D. semiclausum*. The electron dense materials of PDV were distributed within nucleus and vacuolated cytoplasm of calyx cells, calyx lumen and on the surface of eggs wasps. PDVs particles were also shown in the extracted calyx fluid of *Cotesia* sp.

Key words: calyx tissues, parasitoids, polydnavirus, ultrastructure

Parasitoid merupakan salah satu musuh alami penting di lapang. Kesuksesan parasitisme ditentukan oleh beberapa faktor, antara lain polidnavirus (PDV), yaitu simbion yang dapat mematahkan sistem pertahanan inang, sehingga parasitoid dapat berkembang biak dengan sukses pada inangnya. Dalam penelitian ini, telah dieksplorasi keberadaan PDV pada tiga jenis parasitoid, *Snellenius manilae*, *Cotesia* sp., dan *Diadegma semiclausum*, yang merupakan musuh alami hama penting pada kubis. Studi morfologi PDV dilakukan dengan membuat irisan ultra preparat dari jaringan kaliks dan melakukan pewarnaan negatif pada ekstrak cairan kaliks dari parasitoid dewasa. Stroma virogen dari sel epitel yang telah terdiferensiasi tampak dari ketiga parasitoid yang diuji. Bracovirus dan ichnovirus juga terdeteksi dalam jaringan kaliks dari *S. manilae* dan *D. semiclausum*. Materi padat elektron dari PDV tampak terdistribusi dalam inti dan sitoplasma jaringan kaliks, rongga kaliks, dan permukaan telur parasitoid. Partikel PDV juga tampak dalam cairan kaliks dari parasitoid *Cotesia* sp.

Kata kunci: jaringan kaliks, parasitoid, polidnavirus, ultrastruktur

Lepidopteran larval pests are known to cause significant damages to cabbage crops. These pests harbors a complex of natural enemies, e.g. the ichneumonid wasp *Diadegma semiclausum* that are well known to control the diamondback moth *Plutella xylostella* larvae (Sastrosiswojo and Sastrodihardjo 1986; Momanyi *et al.* 2006). Several other braconid wasps such as *Snellenius manilae* and *Cotesia marginiventris* (Hymenoptera: Braconidae) have been recorded as a natural enemies of the larval pests *Spodoptera litura*, and *Trichoplusia ni*, respectively (Grasela *et al.* 2008; Ratna 2009).

One of the successes key to parasitism is the ability of the parasitic wasp to avoid the defense mechanism of its hosts (McNeil *et al.* 2010). Several studies have shown that prevention of encapsulation can be done by the symbiosis of insect with polydnavirus (PDV) (Suzuki and Tanaka 2006; Mahmoud *et al.* 2011; Provost *et al.* 2011). The case of mutualism between viruses and eukaryotic cells of ovaries tissues was reported on braconid and ichneumonid wasps (Fleming 1992; Drezen *et al.* 2003). PDV originated from braconids and ichneumonids wasps are termed as bracovirus and ichnovirus respectively (Beckage and Gelman 2004; Kroemer and Webb 2004). Studies have shown that presence of PDV symbiont inside the reproductive organ of parasitic wasps prevents the encapsulation process, through the manipulation of the larval host's physiology (Lavine and Beckage 1995; Drezen *et al.* 2003; Pruijssers *et al.* 2009). PDV is composed of a large segmented multiple circular double stranded DNA genome included in a virus particle (Drezen *et al.* 2003; Kroemer and Webb 2004). Proviral DNA or PDV segment is integrated in the wasp's genome and can pass through vertical or horizontal transmission (Lawrence 2005) and were replicated in the nucleus of calyx epithelial cells of the wasp ovaries (Drezen *et al.* 2003). The mature virions are injected into the larval host along with eggs during oviposition (Stoltz 1986; Schmidt and Schuchmann-Feddersen 1989). Bonvin *et al.* (2004) reported that viral transcript of braconid *C. inanitus* was present in the hemocytes, fat body and nervous tissue of larval host *Spodoptera littoralis*. Chen *et al.* (2007) reported that PDV genomes of *C. vestalis* (= *P. plutellae*) was expressed in the hemolymph, brain and midgut of parasitized *P. xylostella*. The viral transcript of ichneumonid *Hyposoter didymator* was also identified in the extracted tissues of parasitized larvae

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S. frugiperda (Clavijo *et al*. 2011). These viruses disrupt the host immunosystem, thus avoiding the juvenile parasitoid from being encapsulated (Blumberg 1997).

The presence of this PDV were shown by ultrastructures studies in several braconid wasps e.g.: *Apanteles melanoscelus*, *A. paleacritae*, *Cardiochiles nigriceps*, *C. inanitus*, *C. texanus* and *Microplitis croceipes* (Stoltz *et al.* 1976; Stoltz and Vinson 1977, 1979; Hamm *et al.* 1992; Marti *et al*. 2003), *Glyptapanteles indiensis* (Chen and Gundersen-Rindal 2003), *C. plutellae* (Bae and Kim 2004), *Microplitis rufiventris* (Hegazi *et al.* 2005), and ichneumonid wasp e.g.: *Campoletis sonorensis* (Stoltz and Vinson 1979) and *H. exiguae* (Krell and Stoltz 1980). In this study, we determined the existence of bracovirus and ichnovirus on the wasp's *S. manilae*, *Cotesia* sp., and *D. semiclausum*.

MATERIALS AND METHODS

Source of Insects. Wasp *S. manilae* obtained from parasitized larvae *S. litura* was collected from taro plantation at Bogor area, while *Cotesia* sp., and *D. semiclausum* obtained from parasitized larvae and pupae *T. ni* and *P. xylostella*, respectively were collected from cabbage crops at Cianjur area. All larval hosts were fed on cabbage leaves and maintained in the laboratory under room temperature 25-27 °C and 87-100% humidity.

The developed pupal parasitoids of each group were isolated into a cage made of wooden (20 cm^3) covered with a general white-net cloth (mesh: 50 holes/inch). New emerging adult female parasitoids was paired with two day old male to allow mating. The pair was put into a plastic cylinder cage covered by a white-net cloth (diam. 10 cm, height 25 cm). Into each cage, 30 second instar larval host were introduced. Larval hosts were exposed to a pair of wasp for a period of two days. Wasps were fed with 40% honey solution that was absorbed on cotton pad. One day prior to dissection, the wasps were designed to severe from host exposure to allow accumulation of large number of PDVs in their calyx's fluids (Beckage *et al.* 1994). Morphological study of PDV was carried out by preparing ultrasectioned calyx tissues and negative stained of extracted calyx fluid isolated from the wasps (Hamm *et al.* 1992; Hegazi *et al.* 2005).

Ultra Structural Preparation of PDV from Wasp's Calyx Tissues. Female reproductive tracts taken from 3 days old wasps were dissected and were put into fixative solution prior to ultrastructure preparation according to Hegazi *et al.* (2005). These ovaries were fixed in a primary fixative solution consisting of 8% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) (1:1, v/v) for 3 h at 0-5 °C. After fixation, they were washed out by immersing in buffer solution for 10 min. These samples were post-fixed in 2% osmium tetroxide in 0.2 M cacodylate buffer solution (1:1, v/v) for another 1 h at 0-5 °C. They were rapidly dehydrated using a grades series of ethanol solutions (concentration ranging from 30, 50, 70, and 95%, 15 min each) and were subsequently washed in ethanol absolute (three changes) for 10 min at 0-5 °C. The samples were then transferred to propylene oxide solutions for 15 min (three changes), and infiltrated with mixed solution of propylene oxide and Spurr's low-viscosity embedding resin (1:1; v/v) for overnight followed by 100% Spurr's resin for 2 h. Infiltration process was carried out by placing it on the agitator and given high speed agitation at room temperature. Each specimen was transferred to embedding block filled with fresh Spurr's resin and polymerized in the oven at 60 °C for overnight. Polymerized blocks were trimmed and sectioned on Reichert-Jung Ultra Cut type 701704 Ultramicrotome using 45 degree angle glass knives prior to provide the ultra thick (1 μm) and ultra thin (125 nm) slices of the calyx tissues. Section thickness was determined by interference colours produced by reflected light (grey for thick sections and gold for thin sections). The ultra thick sections were mounted on glass slides and stained with 1% toluidine blue in 1% of borax within 3-5 min and viewed under light microscope. The ultra thin sections were mounted on 200-mesh copper grid and stained with 2% uranyl acetate in 70% ethanol solution for 20 min. They were then washed with distilled water and stained in 4% lead citrate solution ready used for another 20 min. These grids were further floated over a drop of 0.05 M NaOH solution to reduced lead carbonate contamination and washed thoroughly with distilled water and leave it to dry. The PDV were observed from this sectioned under HITACHI transmission electron microscope.

Negative Staining of Calyx Fluid. PDV's were isolated from parasitoid by extraction of its calyx fluid (Hamm *et al.* 1992). Seventy five ovaries were each isolated from 3-4 days old wasps *S. manilae*, *Cotesia* sp., and *D. semiclausum*, in cold Ringer solution. These ovaries were washed in 2% NaHClO solution and put in 1.5 mL an eppendorf tube containing 15 μL Ringer solutions on crushed ices. Calyx fluid was obtained by maceration of reproductive tract tissues using a pestle pipette glass followed by centrifugation at 2500 rpm within 8 min. The supernatant contained PDV was transferred into another tube and centrifuged at 12000 rpm within 1 h. The second supernatant was removed and the pellet was resuspended by distilled water prior to negative staining preparation.

One drop of suspension was mixed with one drop of 1.5% fosfotungstic acid solution which was placed on a 400 mesh grid layered with 1% formvar film. Grid was viewed under the same electron microscope as above.

RESULTS

Structure of Wasps Ovaries. PDV's were assumed to be replicated in a part of ovaries of both ichneumonid and braconid wasps. Our study revealed the enlargement at the base of calyx tissues of three days old female wasps (Fig 1 and Fig 2). Oogenesis was carried out within 2 sets of paired ovarioles or egg tubes. The penultimate oocytes were located on ovarial reservoirs, then move down gradually to a pear-shaped calyx and tubular lateral oviduct. Both tubes fused to form the short common oviduct as a place of mature eggs and ended with the ovipositor.

Our result showed the presence of ultra thick sectioned calyx tissue that consisted of the outer part of

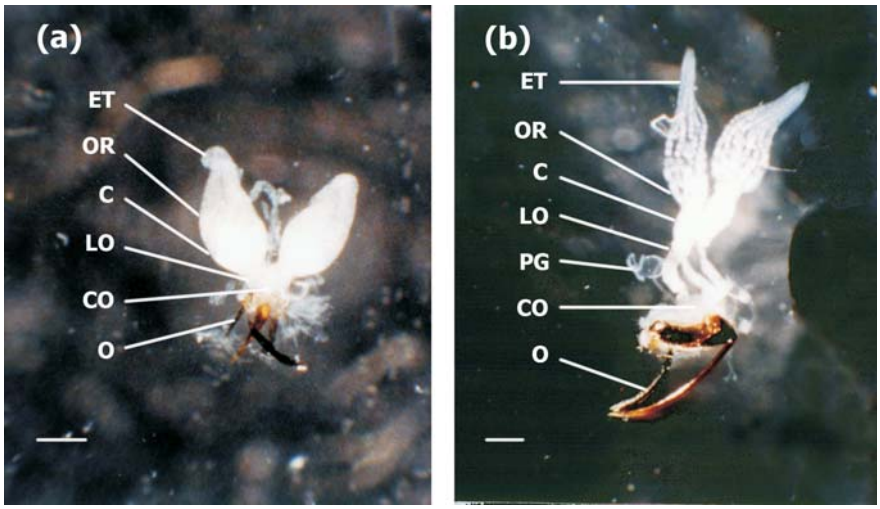

Fig 1 The reproductive tracts of adult female (a) *S. manilae* and (b) *D. semiclausum*. ET: egg tube; OR: ovarial reservoir; C: calyx; LO: lateral oviduct; CO: common oviduct: O: ovipositor: PG: poison gland. Bars = 0.5 mm.

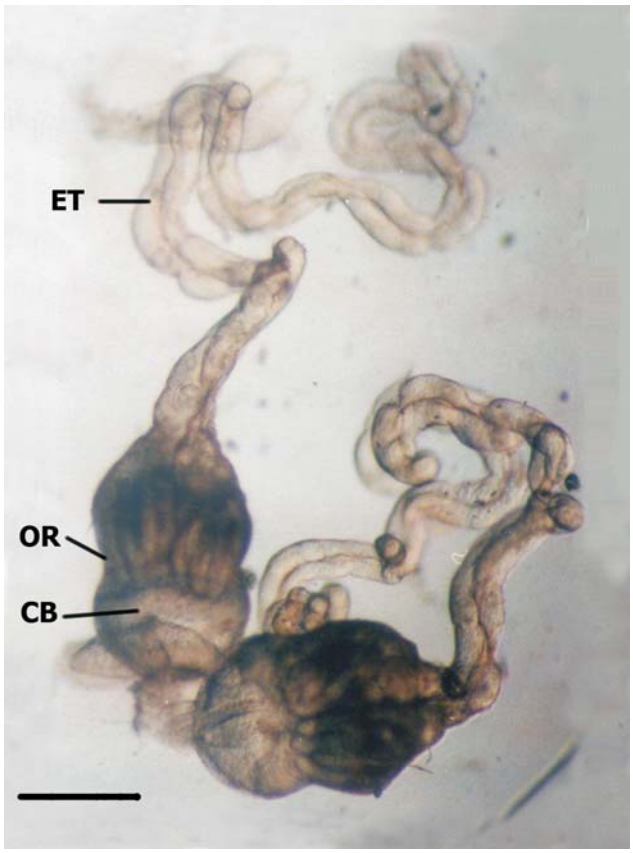

Fig 2 Enlargement picture part of ovaries *S. manilae*. ET: egg tube; OR: ovarial reservoir; CB: calyx based; Bar = 0.5 mm.

tissues, layered by basal lamina, followed with epithelial cells (Fig 3). In the center region, a lumen calyx contained eggs are surrounded by calyx fluids. Virogenic stroma from the epithelial cells of *S. manilae* appears to have a bud stalk-like shaped which supported a group of cells developed into calyx lumen and almost half of lumen filled with eggs (Fig 3a). These cells mostly consisted of relatively big nucleus that is thought as a place of replicated PDV. A different appearance was seen in the wasp *Cotesia* sp., i.e. the layers of vacuolated calyx epithelial cells that are more clearly surrounding the calyx lumen which are filled with less egg (Fig 3b). Bud cells was not shown in this sectioned. As in *S. manilae* here we saw virogenic stroma with obviously protruded nucleus, which fulfills almost three-part of the calyx lumen. In contrast, in ichneumonid wasps *D. semiclausum*, the virogenic stroma was not spread out circularly around the lumen calyx, but instead laid on spot in a certain area (Fig 3c). The vacuolated cytoplasm of calyx epithelial cell with small nucleus deeply inserted between eggs.

PDV in Calyx Tissues and Calyx Fluid. Result of low magnification of the electron microscope convinced that active calyx epithelial cells are vacuolated. The nuclei have irregular shapes. The electron dense materials of PDV appeared within calyx

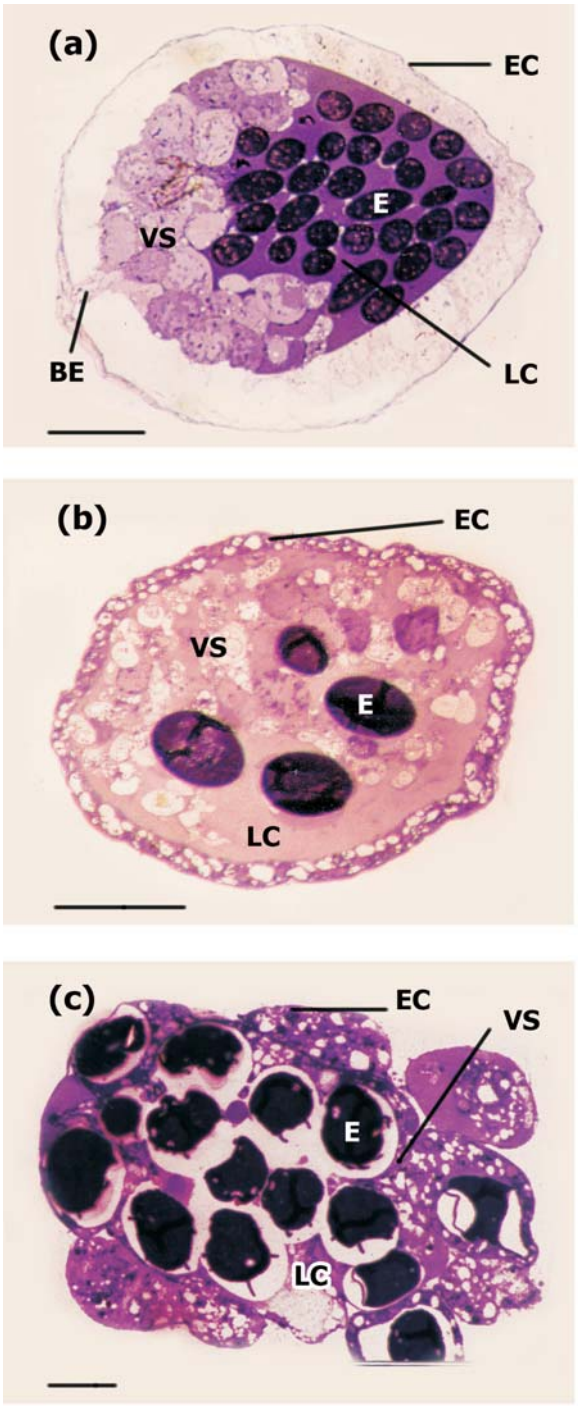

Fig 3 Transversal sections of calyx tissues of wasps (a) *S. manilae*, (b) *Cotesia* sp., and (c) *D. semiclausum*. EC: epithelium calyx cells; BE: budding epithelium; VS: virogenic stroma; E: eggs, LC: lumen calyx; Bars: 1 mm.

nuclei and cytoplasm. It also seems to spread in the surface of the eggs of both parasitoids *S. manilae* and *D. semiclausum* (Fig 4a and 4b). Fig 4c also showed that the PDV materials had been lysed from the cells spreading within the vacuoles of calyx lumen.

Under high magnification it is shown that cytoplasm of the calyx epithelial cells contained different morphological structure of PDV particles that differs between the two group species of wasps (Fig 5). PDVs from braconids wasp *S. manilae* are typically bracovirus which is recognized as a group of

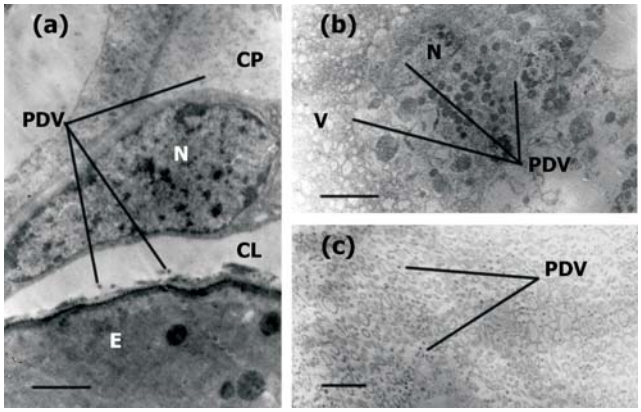

Fig 4 Calyx tissues (a) *S. manilae*, (b) *D. semiclausum*, and (c) calyx lumen *D. semiclausum*). CL: calyx lumen; CP: cytoplasm; E: eggs; N: nucleus; PDV: particles dense virion; V: vacuolated cytoplasm; Bars = 1 μm.

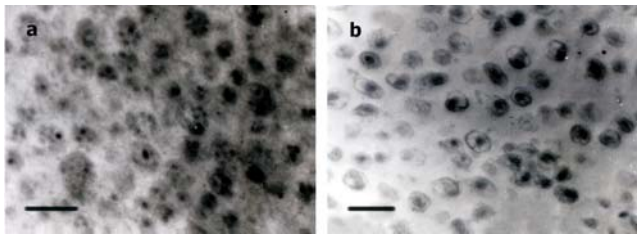

Fig 5 PDV particles of the calyx tissues (a) *S. manilae* (b) *D. semiclausum*. Bars = 0.5 μm

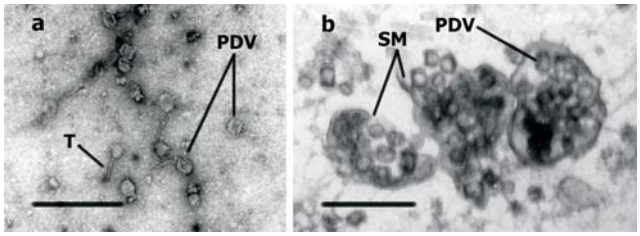

Fig 6 Polydnavirus particles in calyx fluid of *Cotesia sp.* (a) PDVs with and without tails (b) PDVs within the sack. T: tail or protrusion membrane; SM: enveloped membrane are being lysis; Bars = 0.2 μm.

cylindrical nucleocapsid particles each surrounded by a single unit membrane, compared with PDVs from ichneumonid wasp *D. semiclausum* known as ichnovirus which is a single particle of lenticular nucleocapsid surrounded by double membranes.

The result of investigation under negative staining of calyx fluid extracts revealed that the PDV shape appeared as a circular DNA particles surrounded by a membrane (Fig 6a). Two shapes of virion existed with and without membrane protrusion or tail-like appendages. Fig 6b showed that capsid of bracovirus of *Cotesia* sp. was bursting from its envelope.

DISCUSSIONS

Our result showed that the ovarial reservoirs were full of eggs, which is indicated by the enlargement of based calyx. The swollen tissues consisted of developed epithelial cells producing PDV, and the lumen calyx filled with eggs and the released PDVs

(Stoltz *et al.* 1976; Marti *et al*. 2003). This is consistent with the result of Hegazi *et al.* (2005) which revealed that PDV started to appear in the calyx cells of the mid-age pupae of the braconid wasp *M. rufiventris* and it is very abundant in the calyx fluid of pharate adult.

Here, we observed the expansion of calyx epithelial layers forming clusters of cells possessing large irregular shaped nuclei and vacuolated cytoplasm in the side region beneath the lamina basal that is considered as a virogenic stroma. This virogenic stroma was more developed in the braconids *S. manilae* and *Cotesia* sp. as indicated by the presence of PDV in almost half or two-third areas of its lumen. It is interesting to note that those tissues originated from the growth point of epithelial cells, protruding in one side, and then formed a bud-shape like pedicel. This was not seen in the ichneumonids *D. semiclausum* calyx tissues. Bracovirus seems to be replicated in these cells producing enlargement of nuclei, occupying most of the cell volume at the end of virus replication (Drezen *et al.* 2003; Marti *et al.* 2003). The virus particles are then released into and dispersed within a calyx lumen (Beckage and Gelman 2004). The budding structures were also reported in calyx epithelium cells of *C. nigriceps* and *M. croceipes* that seems to characterize ichnovirus (Stoltz *et al.* 1976; Stoltz and Vinson 1979).

Using high magnification, the electron dense materials appeared in the nucleus and vacuolated cytoplasm of the epithelium calyx cells and also in the egg's surface and calyx fluid of all three wasps. There is indication that PDVs are being replicated within the cells followed by lysis and then discharged into the calyx lumen. Hegazi *et al.* (2005) also mentioned that vacuolated cytoplasm appeared in the wasp calyx cells and PDVs were also found on the surface of egg chorion on the lumen of lateral oviduct.

Two different ultrastructures of ichnovirus and bracovirus particles found in the lumen of oviduct or calyx of the epithelium cells was elaborated by several authors. Ichnovirus particles found in the calyx fluid of *H. exigua* consist of a nucleocapsid seen as electron dense materials surrounded by inner and outer membranes (Krell and Stoltz 1980). On the other hand, bracovirus particles is a package containing a single large virus particles (Drezen *et al.* 2003). The spherical shape of mature virion *M. rufiventris* (Braconidae) were covered individually by nucleocapsids that consist of a single unit membrane (Hegazi *et al.* 2005). This bracovirus was also discovered in the calyx fluids *C. texanus* (Stoltz *et al.* 1976). Our result showed that several nucleocapsid particles of *Cotesia* sp. were located within the sack and the individual capsid covered with a single membrane. Some of them had a protrusion of tail-like shaped. This is similar to what was found by Stoltz and Vinson (1977), where the electron-dense nucleocapsids particles per envelope appeared in the nucleus of calyx epithelial cells of braconids wasps, *A. paleacritae* (Stoltz and Vinson 1977) and *C. congregata* (Drezen *et al.* 2003). In general, PDVs could be integrated in the calyx cells, but the nature ancestral virus in braconids and ichneumonids was probably specific between families (Drezen *et al.* 2003; Federici and Bigot 2003; Kroemer and Webb 2004). The morphology of nucleocapsid of both virions reflected the differences of original ancestral virions. Kroemer and Webb (2004) explained that the multiprotein double membrane of bracovirus was recognizably different compared to lipid membrane found in ichnovirus. A characterized protrusion membrane were shown in bracovirus *C. congregata*, *C. melanoscela* and *M. croceipes* and ichnovirus *C. sonorensis* wasps (Stoltz *et al.* 1976; Stoltz and Vinson 1977; Beckage *et al.* 1994). However, this protrusion membrane was not shown in bracovirus *C. marginiventris* (Hamm *et al.* 1992).

The evidence of bursting PDV from the sack was shown in our study. According to Stoltz and Vinson (1977), nucleocapsid particles of calyx fluid *A. melanoscelus* were visible within envelopes and the negative stained non-protrusion membrane baculovirus-like particles were released from disrupted envelopes. The envelope structures contained one or several PDVs have also been reported in *C. marginiventris* and it seems a characteristic of bracovirus where the virion is released through a lysis process (Hamm *et al.* 1992). The different process of released PDV has been explained by several authors that cell lyses from envelopes was found in the case of PDV that was released in bracovirus and that budding process happens without damaging the cells in the case of PDV released in ichnovirus (Stoltz and Vinson 1979; Fleming 1992; Drezen *et al.* 2003; Wyler and Lanzrein 2003; Bonvin *et al.* 2004).

Observation of the negative staining from the calyx fluid extracts showed that PDV is only found in the wasp *Cotesia* sp. It might be possible that the critical time for a calyx tissues extraction has influenced the production of a mature released PDVs. Hegazi *et al.* (2005) explained that the ovarial reservoir of *M. rufiventris* can be clearly distinguished at 3 days old pupae and PDV is visible in the calyx lumen of pharate adult or one day prior to adult emergence. PDV began to replicate during the late stages of pupal development that coincides with melanization of the pharate adult cuticle (Kroemer and Webb 2004) or in a newly emergence wasp (Lavine and Beckage 1995). In the ovary of *C. plutellae*, PDV was reported to be present on 5-day old wasp (Bae and Kim 2004). Beckage *et al.*

(1994) elaborated that newly emerging wasp aged less than 24 h had significantly less virus compared to 3-4 days old mature females. Based on our result, the epithelial calyx tissues of 3 days old *D. semiclausum* appeared to be intact and has no vacuolated cytoplasm. This result suggested that the virus has not fully grown in the wasp, or that the mature virion had not been released into calyx lumen due to the fact that its epithelial cell differentiation is still in process. However, the evidence showed in the wasp *M. rufiventris*, showed that the virogenic stroma calyx fluid started to be visible prior to adult eclosion. At this time, the lumen contained one or at least a single egg and this stroma is continuously producing during the whole life span of the adult females (Drezen *et al.* 2003).

This study reveals that bracovirus and ichnovirus wasp symbionts of the lepidopteran cabbage pests are very important in preventing encapsulation. These findings can be used to increase the success of biological control agent in the field.

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