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SHORT COMMUNICATION

Establishment of a primary cell culture of *Thrips palmi* (Thysanoptera: Thripidae)

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Key words. Melon thrips, in vitro, virus-vector relationship, fibroblast-like cells, orthotospovirus

Abstract. *Thrips palmi* (Thysanoptera: Thripidae) is an important pest of vegetables, ornamental plants and fruit crops. In addition to the direct damage caused by feeding, it transmits several tospoviruses. The absence of an in vitro assay system is a major bottleneck in investigating thrips-tospovirus interactions. The present study reports the establishment of a primary cell culture of *T. palmi*, which was initiated using embryonic tissue as an explant in modified Kimura's medium. Fibroblast-like cells began to be produced 3 h after tissue implantation and were the dominant cell type. They grew in size and number and covered most of the surface. This primary cell culture survived for 37 days providing sufficient time for analytical molecular studies on the replication of tospovirus and interactions with the vector components.

INTRODUCTION

Thrips palmi Karny (Thysanoptera: Thripidae) is a major pest of agricultural and horticultural crops worldwide (Guyot, 1988; Cho et al., 2000; Seal, 2004, 2013; Cannon et al., 2007; Priti et al., 2021; Jangra et al., 2022). The damage is caused by direct feeding on leaves, flowers, fruits and tender shoots. It also causes indirect damage by transmitting several tospoviruses. Tospoviruses propagate within *T. palmi*, which also serves as a host (Ghosh et al., 2021a, b; Mou et al., 2021). To date, seven tospoviruses viz. groundnut bud necrosis orthotospovirus (GBNV), melon yellow spot orthotospovirus (MYSV), calla lily chlorotic spot orthotospovirus (CCSV), watermelon silver mottle orthotospovirus (WSMV), watermelon bud necrosis orthotospovirus (WBNV), tomato necrotic ringspot orthotospovirus (TNRV) and capsicum chlorosis orthotospovirus (CaCV) are reported to be transmitted by *T. palmi* (Rotenberg et al., 2015; Ghosh et al., 2017, 2019; Chakraborty et al., 2018; Jagdale & Ghosh, 2019). Annual crop losses due to tospoviruses are estimated to be around US$ 1 billion (Pappu et al., 2009). *T. palmi*-transmitted GBNV alone accounts for over US$ 89 million losses per annum in Asia (Reddy et al., 1995). Application of insecticides and host plant resistance are ineffective in suppressing thrips on host crops and restricting the spread of tospoviruses (Mahanta et al., 2022). The use of transgenic plants is not feasible considering the wide host range and ethical issues related to genetically modified crops (Rajesh et al., 2023). Understanding the relationship between thrips and tospoviruses and interrupting the relationship is a novel option for managing the spread of tospoviruses. The present understanding of thrips-tospovirus relationships is largely based on tomato spotted wilt virus (TSWV) and Western flower thrips, *Frankliniella occidentalis*. Little is known about the biological relationship of *T. palmi* with other predominant tospoviruses. The unavailability of a thrips cell line as an experimental system greatly hampers a detailed analysis of the intra-cellular molecular interaction of the virus within host cells.

The first insect cell culture was established by Grace in 1962 and since then over 1270 insect cell lines have been established (Zhou et al., 2020). Recently, an online database of crop pest cell lines was published (Arya et al., 2022). Insect cell cultures have been a handy and imperative tool in studies on virus-vector interactions and understanding the mechanism of virus replication (Grace, 1962; Lynn, 1999; Smagghe et al., 2009; Ghosh et al., 2020; Chen et al., 2022). Maramorosch (1956) demonstrated for the first time the utility of insect cell culture in this context. He successfully multiplied the aster yellows virus in its vector's tissue, the aster leafhopper. Vector cell cultures and cell lines are widely used to study virus-vector relationships (Creamer, 1993; Leland & Ginocchio, 2007; Ghosh et al., 2020). Cell lines of several plant hoppers were used in studies on the replication and spread of rice stripe virus, southern rice black-streaked dwarf virus and rice ragged stunt virus (Ma et al., 2013; Mao et al., 2013; Lan et al., 2016; Chen et al., 2014, 2022). Cell monolayer of the leafhopper *Nephotettix cincticeps* was used to track the cellular localization of nucleorhabdovirus proteins in vitro (Zhang et al., 2018). In the case of thysanopteran vectors, the establishment of the primary cell culture of *F. occidentalis* helped in understanding how TSWV proteins function in its vector (Hunter & Hsu, 1995; Nagata et al., 1997). This paper reports the establishment of a primary cell culture of another important thysanopteran vector, *T. palmi*, which can be used for analytical molecular studies on the replication of tospoviruses and interactions with the

vector factors and managing virus diseases. This is the first report of the establishment of a *T. palmi* cell culture.

MATERIAL AND METHODS

Rearing of *T. palmi* and collection of eggs

A population of *T. palmi* established from a single adult female was used in this study. The population was maintained on aubergine plants (var. Navkiran, Mahyco, India) under controlled conditions of 28 ± 1°C, 60 ± 10% relative humidity and 16L : 8D. The identity of the population was confirmed based on standard morphometric keys (Bhatti, 1980; Cluever & Smith, 2017) and the nucleotide sequence of *mitochondrial cytochrome oxidase I* (*COI*) (Jangra et al., 2020b).

Based on a previous report on establishing a thrips cell culture (Hunter & Hsu, 1995), embryonic tissue was chosen for initiating a culture of *T. palmi* cells. Adult females of *T. palmi* insert their eggs into leaf tissue. As the eggs are very soft, fragile and difficult to collect, they were harvested in sterile conditions using an artificial oviposition setup as reported in a previous study (Jangra et al., 2020a). In brief, 10–15 adult females of *T. palmi* were released in the bottom half of a Petri dish (35 mm d, 10 mm h, Axiva Sichem Biotech, India) and covered with a thin layer of Nescofilm (Karlan Research Product Corporation, USA). Around 500 µl of sterile water was placed on the Nescoflim, which was then covered with stretched Parafilm M (Bemis Company, Inc, USA). Pine pollen (Lost Empire Herbs, China) and 10% honey solution (Dabur, India) were provided in the Petri dish. The Petri dishes with the adult females were kept at 28°C, 60 ± 10% relative humidity and a photophase of 16L : 8D. The eggs laid in the water were collected using a '00' size Camel hairbrush after piercing the Parafilm M membrane with a sharp needle.

Identification of the best age of embryonic tissue for culturing

Based on a previous study on the embryogenesis of *T. palmi* (Jangra et al., 2020a), eggs at 15 h, 30 h, 50 h and 70 h (red eye stage) were evaluated in terms of their suitability for initiating a cell culture. Eggs at these stages in their development were collected, processed and placed in the medium as described below. Cell dissociation, survival and regeneration in the medium were recorded by observing under an inverted phase contrast microscope (EA-prime, Leedz Microimaging Ltd, England).

Preparation of medium

A modified Kimura's medium (Kimura, 1984) was used in this study to initiate a primary cell culture from embryonic tissue. This medium inludes 10 ml of 1× Schneider's Drosophila medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10 ml of 1× Medium 199 (Gibco), 0.5 ml of 1× CMRL Medium 1066 (Gibco), 77.5 mg of 0.05M L-Histidine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 70 µl of 200 mM L-glutamine (Gibco), 200 µl of penicillin-streptomycin 10,000 U/mL (Gibco), 0.8 mg of gentamycin sulphate (Himedia, Mumbai, India) and 80 µl of amphotericin B 250 mg/µl (Gibco). In subsequent changes of the medium, antibiotics were added only if needed. After mixing these components, the medium was filter-sterilized by passing through a 0.20 µM, sterile, cellulose acetate syringe filter (Minigen, Genetix Biotech Asia Pvt. Ltd, New Delhi, India). Finally, 20% by volume of heat-inactivated fetal bovine serum (Gibco) was added to the medium.

Processing of egg tissues and placing in medium

The *T. palmi* eggs were incubated in sterile distilled water at 28°C. Eggs of a particular age were collected in a microcentrifuge tube and sterilized by washing them 4–5 times with sterile distilled water and 70% ethanol. The residual ethanol was allowed to evaporate inside a biosafety cabinet (Air Science, USA). The eggs were washed with nystatin (100 U, Himedia) and penicillin-streptomycin (20 µl in 1 ml) and centrifuged at 4000 rpm for 1 min. The antibiotics were removed by gentle pipetting. Thereafter, 2 ml of Tyrode's salt (Himedia) was added and the eggs were centrifuged at 4000 rpm for 1 min. The eggs were teased apart in a pre-sterilized micropestle and then incubated with trypsin-EDTA 10× (4 µl in 100 µl, Gibco) for 5 min. After incubation, 1.5 ml of freshly prepared medium was added to the micro-centrifuge tube and centrifuged at 4000 rpm for 3 min. This step was repeated 3–4 times to inactivate trypsin-EDTA. The medium with the cells was placed on different cell culture substrates and adequate fresh medium was added to re-suspend the cells. The cells were then kept in a cell culture incubator at 28°C. A medium supplementation of 50% was done at weekly intervals to provide fresh nutrients for the growth and development of the cells. The cells were regularly monitored under an inverted phase contrast microscope (EA-prime, Leedz Microimaging Ltd) at different magnifications and phases.

Evaluation of substrate

Three substrates: 40 ml cell culture flasks vented (SPL Life Sciences Co. Ltd. Gyeonggi-do, Korea) and non-vented (Nunc Thermo Fisher Scientific), coverslip (22 × 22 mm, Himedia) and 8-well cell culture slide (SPL Life Sciences Co. Ltd.), were evaluated for their suitability for initiating a cell culture. The embryonic tissue was processed and placed in the medium as described above. In the case of the coverslip culture, the eggs were transferred directly to a coverslip in a sterile Petri dish (35 mm d, 10 mm h, Axiva Sichem Biotech, New Delhi, India) with the help of a sterile Camel hair brush. Then they were washed by adding a drop of Tyrode's salt solution. The eggs were teased apart using a pre-sterilized micropestle and incubated with trypsin-EDTA (4 µl in 100 µl, Gibco) for 5 min. The cells were washed three times with 150 µl of freshly prepared medium. Then fresh medium (200–300 µl) was added to re-suspend the cells.

Cell viability assay by dual staining

The primary cell culture of *T. palmi* was stained one week after implantation with hematoxylin and eosin (H&E). This procedure is described by Fisher et al. (2008). Briefly, the cells in the primary cell culture were washed three times with sterile distilled water, incubated with Mayer alum hematoxylin solution for 2 min and washed. The cells were then incubated with eosin Y solution for 10 min, washed and dehydrated using a sequence of ethanol concentrations of 60%, 90% and 100%. The stained cell culture was observed under an inverted phase contrast microscope.

PCR confirmation of *T. palmi* cells

The *T. palmi* cells from the primary culture were transferred to a 1.5 ml micro-centrifuge tube. Cell lysis was undertaken in 20 µl PBS buffer. The PCR was carried out in 25 µl reaction volume containing 1 µl cell lysate, 2.5 µl 10× PCR buffer (Thermo Fisher Scientific), 0.4 µM of each *T. palmi*-specific forward and reverse primers (AG35F–AG36R) targeting inter transcribed spacer 2 (ITS2) region (Jangra et al., 2020b), 260 µM dNTPs (Thermo Fisher Scientific) and 2 U Dream*Taq* polymerase (Thermo Fisher Scientific). The following reaction conditions were followed, initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 60.4°C for 45 s and extension at 72°C for 30 s followed by a final extension at 72°C for 10 min. The PCR products were identified using a 1% agarose gel stained with GoodView (BR Biochem, New Delhi, India) and visualized using a gel documentation system (MaestroGen Inc, Hsinchu City, Taiwan).

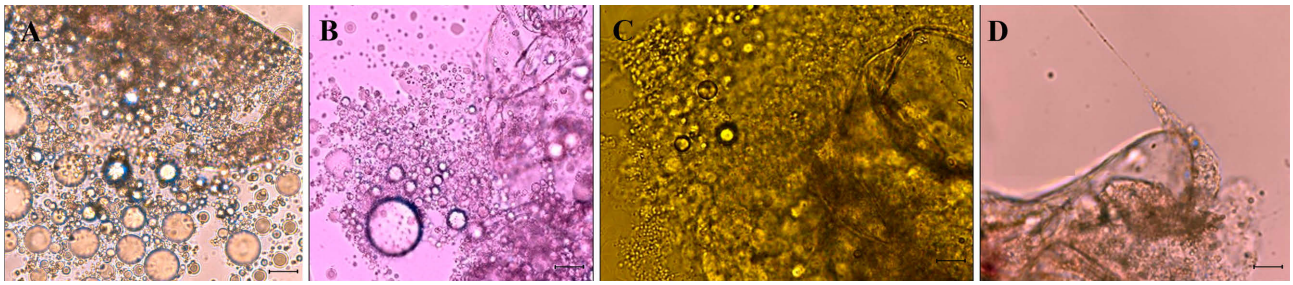

Fig. 1. Identification of the best age of embryonic tissue of *T. palmi* for the establishment of a primary cell culture. Embryonic tissues that were 15 h (A), 30 h (B), 50 h (C) and 70 h-old (D) were tested. A few fibroblast-like cells were produced by embryonic tissue at the red-eye stage (70 h). Scale bar = 50 µm.

RESULTS

Thrips palmi population

The adults in the stock culture had the characteristic morphological features of *T. palmi*, a pale yellowish quadrangular head and seven segmented antennae. Three brick-red ocelli were visible in a triangular formation. Outside of the ocellar triangle, there is a pair of inter-ocellar setae. The presence of a pointed ovipositor at the abdomen's apex distinguished females from males. The females were larger than the males. In addition, the nucleotide sequence of 676 bp *COI* showed 100% homology with *T. palmi* (NCBI accession no. OP622868), which confirmed the identity.

Determination of the best age of embryonic tissue

The suitability of eggs of different ages (15, 30, 50 and 70 h) was tested using coverslip culture. In the case of 15 h-old embryonic tissue, numerous small (~2–10 µm) and medium cells (~10–40 µm) and a few big cells (~40–50 µm) were recorded (Fig. 1A). The cells were round in shape and firmly attached to the surface of the coverslip and although no growth or cell division was recorded, however, the presence of multinucleate cells indicated nuclear division. The 30 h-old embryonic tissues produced round small and medium cells as described above (Fig. 1B), along with a few clumps of tissue consisting of single cells, but no growth or cell division was recorded. Similarly, the 50 h-old embryonic tissue (Fig. 1C) produced clumps of tissue and single cells and most of them were attached to the substrate, but like 15 h and 30 h-old embryonic tissues, no growth was recorded. The eggs at the red-eye stage 70 h post-oviposition produced a large number of tissue clumps along with a few round small and medium cells (Fig. 1D). A few fibroblast-like cells were observed in this culture 3 h after tissue implantation. The level of contamination was higher at this age of tissue than at the earlier-aged tissues. In brief, the cellular dissociation was good in early-aged embryonic tissues as a larger number of single cells were observed, however, no growth was observed. Fibroblast-type cells were developed only from the embryonic tissue at the red-eye stage (70 h). Based on the above observations, red-eye stage embryonic tissue was used for generating the primary cell culture.

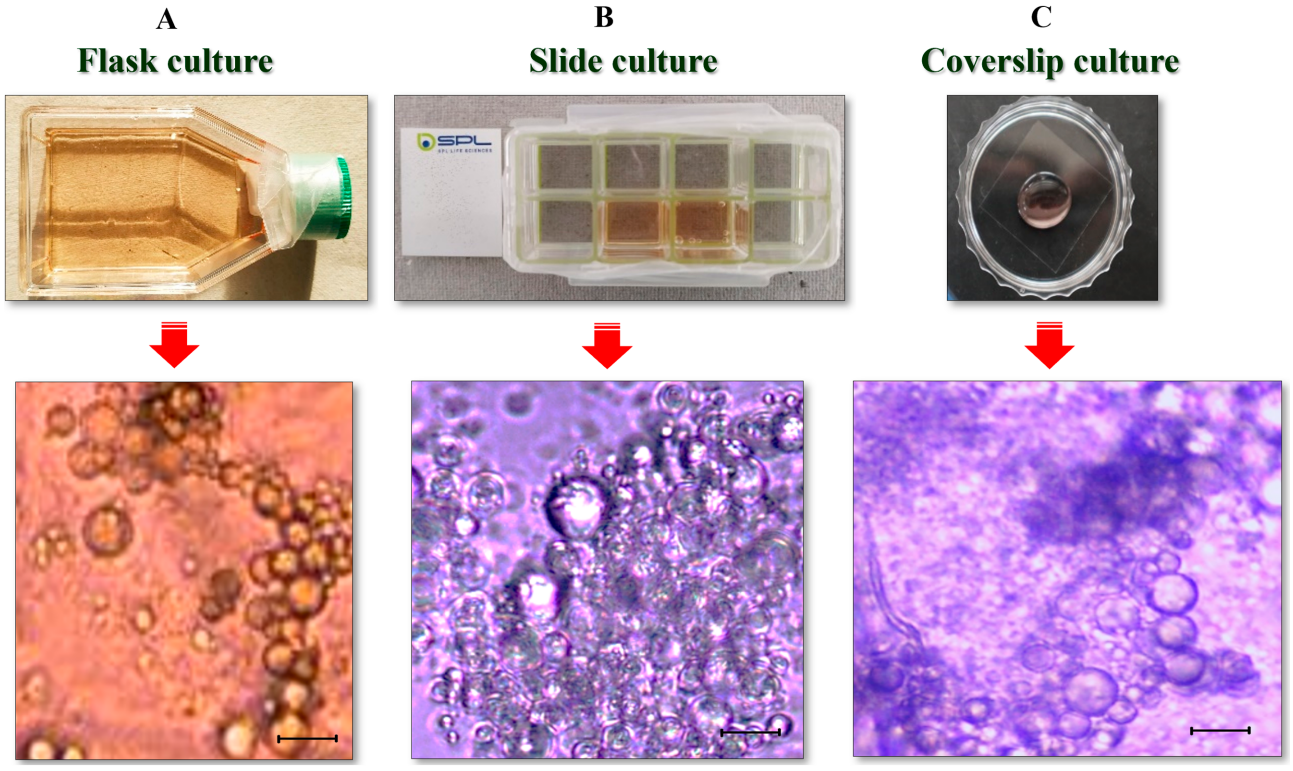

Fig. 2. Evaluation of substrates for the establishment of a primary culture. The different substrates evaluated were: Cell culture flask (A), 8-well cell culture slide (B) and coverslip (C), of which the non-vented culture flask was the most suitable for culturing cells of *T. palmi* in terms of cell attachment, multiplication and survival. Scale bar = 50 µm.

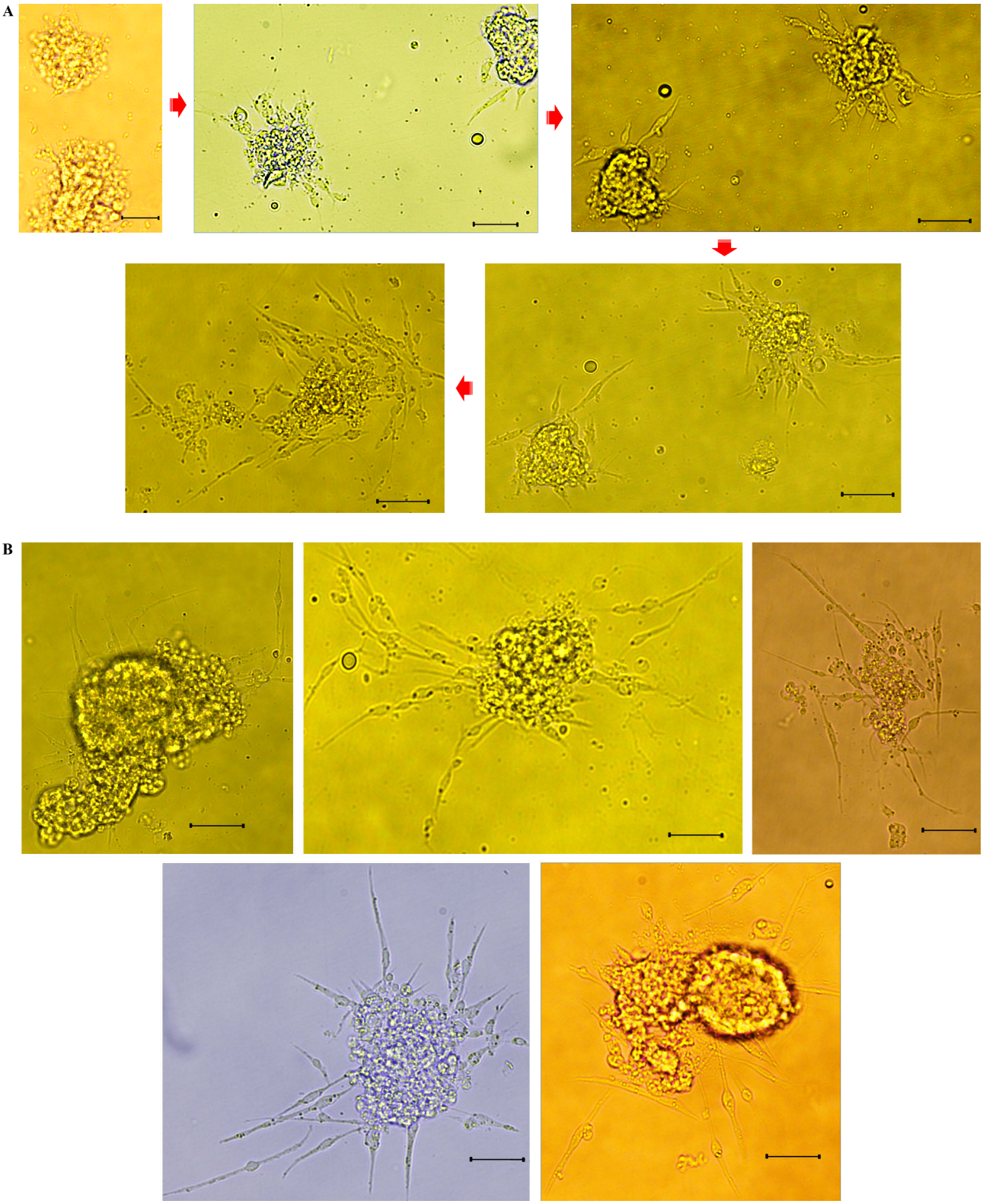

Fig. 3. Primary cell culture of *T. palmi*. A – A large number of clumps of tissue and a few round cells were recorded just after implantation. Fibroblast-like cells started developing 3 h after implantation then greatly increased in number and covered most of the surface, and formed a network 6 days after implantation. The spindle-shaped fibroblast-like cells attained a length of around 600 µm within a week. Some of the clumps of tissue were pulsating, which indicated they were still alive. B – Fibroblast-like cells that developed from large clumps of tissue in the primary cell culture of *T. palmi*. Scale bar = 50 µm.

Evaluation of substrate

Of the different substrates evaluated, non-vented flask culture was found to be suitable for culturing the cells of *T. palmi* in terms of cell attachment, multiplication and survival. A greater number of fibroblast-like cells was recorded only in the non-vented cell culture flask (Fig. 2), which was used to establish the primary cell culture of *T. palmi*.

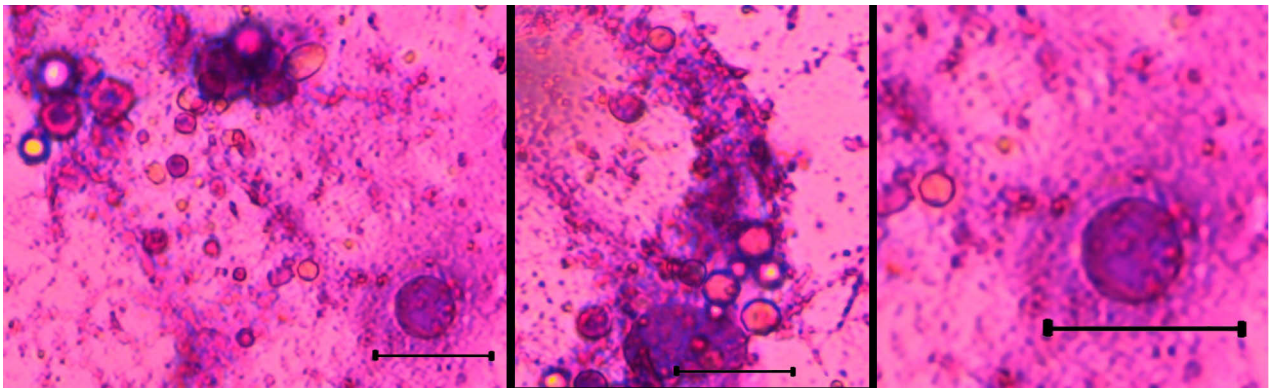

Fig. 4. Staining of the primary cell culture of *T. palmi* using H&E. Hematoxylin stained the nuclei of the cells dark blue and eosin stained the cytoplasmic and extracellular structures pink. Scale bar = 50 µm.

Establishment of primary cell culture of *T. palmi*

The embryonic tissue from the red-eye staged (70 h) eggs was used to establish the primary cell culture in non-vented flasks. A large number of clumps of tissue and a few round-shaped single cells were recorded immediately after tissue implantation. Migration of fibroblast-like cells was recorded 3 h after implantation (Fig. 3) in the large clumps of tissue from which they spread over the surface of the non-vented flask. The fibroblast-like cells were the dominant cell type. No further growth was recorded in the single isolated round-shaped cells. The fibroblast-like cells increased rapidly in number and size. This cellular multiplication was not preceded by a lag phase as they started multiplying within 3 h of tissue implantation. Initially, the cells in the large clumps of tissue were up to 50 µm in length and they increased in size with time. Around 5–10 cells were recorded on the first day and up to 20–40 cells 24 h later and then the number increased exponentially and covered most of the surface forming a network 6 days after tissue implantation. The spindle-shaped fibroblast-like cells attained a length of around 600 µm within a week. Thereafter, there was a sudden reduction in the rate of multiplication. Most of the clumps of tissue were attached to the surface and they were the only clumps that produced fibroblast-like cells. The unattached clumps were washed away during the first medium supplementation. The growth of fibroblast-like cells was rapid in the first week. Thereafter, the rate of growth gradually declined. Pulsation in some of the clumps of tissue was observed up to 30 days post-implantation indicating that the clumps were still alive. The cell culture survived for 37 days with weekly supplementation of fresh medium.

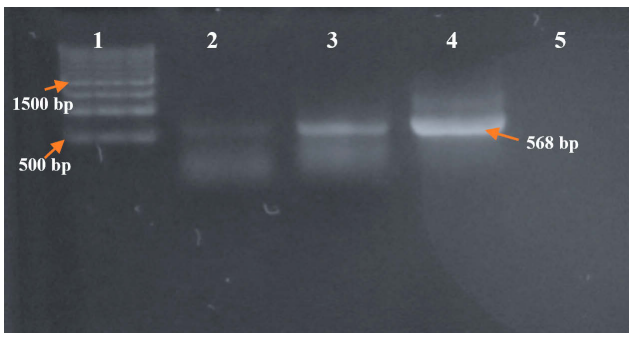

Fig. 5. PCR confirmation of the identity of the cells of *Thrips palmi* in the primary culture. Lane 1: 100 bp plus DNA ladder, lane 2–3: PCR amplicons of 568-bp specific to *T. palmi* using cell lysate as template, lane 4: positive control and lane 5: non-template water control.

Cell viability assays using dual staining

Hematoxylin stained the nuclear components of the cells (anionic components) a dark blue colour, whereas the counterstain eosin differentially stained the nuclear and cytoplasmic components of the cells (Fig. 4). The cytoplasm (cationic components) stained a pink or purple colour. Dual staining discriminated between the cytoplasmic and nuclear contents and indicated that the cells were viable. More than one nucleus was visible inside the cells.

PCR confirmation of *T. palmi* cells

The identity of the *T. palmi* cells in the primary culture was confirmed by the presence of the sharp amplicon of 568 bp specific to *T. palmi*. No amplification was observed in the non-template water control (Fig. 5).

DISCUSSION

Insect cells have been successfully cultured in vitro for over 35 years. The medium, culturing and conditions are well-known for many insects and new cell lines are routinely being developed (Ghosh et al., 2020). The primary cell culture and permanent cell lines of insect vectors are widely used to study virus-vector relationships (Creamer, 1993; Leland & Ginocchio, 2007; Ghosh et al., 2020). Here, reported for the first time is the successful establishment of a primary cell culture of the thysanopteran insect vector, *T. palmi*.

It is likely that the study of thrips-tospovirus interactions at the cellular level will help in understanding the functions of tospovirus proteins in virus infection and dissemination by arthropod vectors. The use of cell lines to explore thrips-tospovirus interactions, however, has been hindered by difficulties in establishing continuous cell lines for thrips. As an alternative, primary cell cultures of thrips could be a promising way of studying the thrips-tospovirus cellular interactions. Although primary cell cultures of *F. occidentalis* and *Thrips tabaci* are reported (Hunter & Hsu, 1995; Nagata et al., 1997), no cell culture of *T. palmi* has been established and reported to date.

In this study, embryonic explants from eggs at different stages in their development were tested for their suitability for producing a primary cell culture. The embryonic tissue at the red-eye stage (70 h) proved best in terms of producing fibroblast-like cells. The embryonic tissue at the earlier embryonic stages produced many single cells, but no fibroblast-like cells. Nagata et al. (1997) and Hunter & Hsu (1995) also used embryonic tissue for establishing primary cell cultures for *F. occidentalis* and *T. tabaci*. Fibroblast-like cells only migrated from red-eye stage embryonic tissue in *F. occidentalis* and *T. tabaci* (Nagata et al., 1997). In the present

study, fibroblast-like cells started developing 3 h after implantation in the medium. In previous studies, however, fibroblast-like cells are recorded 24–48 h post-implantation (Hunter & Hsu, 1995; Nagata et al., 1997). The difference might be due to the species of thrips, medium composition or substrate. A modified Kimura's medium was used in the present study. A similar medium was used by Nagata et al. (1997) to establish *F. occidentalis* and *T. tabaci* cell cultures. Hunter & Hsu (1996), however, used Hunter's Thrips medium to establish a primary cell culture of *F. occidentalis*. The 96-well culture plate was previously used for establishing a primary cell culture of thrips (Nagata, 1999). In the present study, however, the non-vented culture flask was found to be better than other substrates in terms of growth and attachment of *T. palmi* cells in vitro, with the spindle-shaped cells increasing in number and size with time and covering most of the surface. A primary cell culture of *F. occidentalis* survived for up to 100 days, with a monolayer of epithelial cells developed 10–20 days after tissue implantation (Hunter & Hsu, 1995). In the present study, the primary cell culture of *T. palmi* survived for up to 37 days after implantation in the medium and the pulsation of some tissue fragments was recorded for up to 30 days, which indicated the suitability of the medium and procedure for the establishment of an in vitro primary cell culture of *T. palmi*.

The primary cell culture of *T. palmi* developed in the present study will help determine how tospoviruses infect and replicate in this species of thrips, which could help in the identification of potential ways of controlling these viruses. It is also the essential first step towards developing a permanent cell line of *T. palmi*.

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AUTHORS' CONTRIBUTIONS. AG conceived the experiments. HD and SJ performed the experiments and generated scientific data. AG and SJ analyzed the data. SJ and HD drafted the manuscript. AG edited and wrote the final manuscript. All authors read and approved the final manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE. Not applicable.

CONSENT FOR PUBLICATION. All authors agreed.

COMPETING INTERESTS. The authors declare that they have no competing interests.

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