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PRELIMINARY REPORT ON DEVELOPMENT OF SUGARCANE WOOLLY APHID (*Ceratovacuna lanigera* ZEHNTNER) CELL CULTURES

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ABSTRACT

The sugarcane woolly aphid (SWA) (*Ceratovacuna lanigera* Zehntner) successful cell cultures are reported for the first time. Primary cultures from two adult specimens were aseptically initiated with mechanical dissociation (mincing) of the adults. Adherence and growth of explants and cells were observed in Grace insect medium with 1% FBS and 1% antibiotic and antimycotic solution (10,000 U/ml penicillin, and 10 mg/ml streptomycin and amphotericin B 100 µg/ml). The interaction and effects of plant viruses, aphid viruses and endymbionts on SWA cell cultures can be studied. The SWA cell cultures can also be useful in studying the effects of chemical pesticides and to develop the biocontrol for SWA. Effects of plant extracts and of toxins can also be studied on SWA for SWA biocontrol without affecting the plants, the livestock and the humans.

INTRODUCTION

Sugarcane woolly aphid (SWA) is a foliage sucking pest. SWA earlier was known to be minor pest in India has now assumed the status of economic pest after its severe outbreak in Maharashtra during July 2002 (<http://www.icargoa.res.in/dss/sugarcane.html>) and Karnataka (<http://iisr.nic.in/download/publications/Woollyaphid-english.pdf>). SWA feeds on sugarcane (*Saccharum officinarum*) by inserting their stylets through the stomata of the plants leaves. Both nymphs and adults suck the cell sap from lower surface of leaves. They suck the sap from phloem. They excrete large amount of honey dew which falls on the leaves giving them a sticky coating on which black sooty mould (*Capnodium* sp.) develops making the leaves look all black. Due to the thick coating of sooty mould, process of photosynthesis is significantly hampered in severely infested plants thereby causing considerable reduction in cane yield (25%) and sucrose content (26.71%), whereas, during the early growth period plants may die (<http://www.icargoa.res.in/dss/sugarcane.html>) and sucrose content is reduced even up to 53.0 per cent. SWA has now spread to Tamil Nadu, Andhra Pradesh, Kerala and Gujarat in tropical India and Uttar Pradesh and Uttaranchal in sub-tropical India. In Bihar, W. Bengal and N. East States, occurrence of woolly aphid has been observed for the last 50 years. The aphid population is observed on the undersurface of leaves along the midrib or the entire under surface is covered with white flocculent, waxy secretion (<http://iisr.nic.in/download/publications/Woollyaphid-english.pdf>) appearing like wool hence called sugarcane woolly aphid.

SWA occurs on sugarcane and its wild relatives from India and Nepal, through Southeast Asia, north to Okinawa and Taiwan and south through Papua New Guinea to the Solomon Islands. (http://www.sugarresearch.com.au/icms_docs/166962_Chapter14PestManagement.pdf).

The classification of SWA

(http://animaldiversity.ummz.umich.edu/accounts/Ceratovacuna_lanigera/classification/) is:

Kingdom : Animalia
 Phylum : Arthropoda
 Subphylum : Hexapoda
 Class : Insecta
 Order : Hemiptera
 Superfamily: Aphidoidea
 Family : Aphididae
 Genus : *Ceratovacuna*
 Species : *lanigera*

There are no reports on establishment of cell culture from Sugarcane woolly aphid (SWA) (*Ceratovacuna lanigera* Zehntner) till date. There are some reports on the cultivation of primary cell cultures from different aphid species such as pea aphid *Acyrtosiphon pisum* (Tokumitsu &

Maramarosch, 1966 and Peters & Black, 1971), *Hyperomyzus lactucae* L. (Peters and Black, 1970), cabbage aphid *Brevicoryne brassicae* and green peach aphid *Myzus persicae* Sulz (Hind 1971) and *Myzus persicae* (Adam and Sander 1976). These cultures were valuable in preliminary studies on their interactions with intracellular symbionts (Hinde, 1971) and with plant viruses (Peters and Black, 1970). Primary aphid cultures were used to study the uptake of viruses and viral accumulation. The cell lines have some advantage over the use of whole insects in the ease with which viral transcripts and mutant viruses can be uniformly introduced into cells (Creamer R. 1993 and Matisova, J., and Valenta, V. 1975). Since no cell lines of *Ceratovacuna lanigera* were available, we wanted to produce primary cell cultures for development of continuous cell cultures as substrate for the study of SWA biocontrol. The objective of the present study is to develop the SWA cell cultures in vitro to study the susceptibility of these cells to aphid viruses for development of the viral insecticides for SWA biocontrol. The aphid viruses sequenced to date include one DNA virus [*Myzus persicae* densovirus (van Munster et. al. 2003) and four RNA viruses. The latter include aphid lethal paralysis virus (ALPV) (van Munster et. al. 2002) and *Rhopalosiphum padi* virus (RhPV) (Moon et. al. 1998) both members of the family Dicistroviridae which infect a limited number of aphid species and the unclassified *Acyrtosiphon pisum* virus (APV) (van der Wilk et. al. 1997) together with the closely related rosy apple aphid virus (Ryabov E.V. 2007). These viruses were isolated from laboratory cultures of aphids from which significant quantities of virus could be obtained and can be used as bioinsecticides (Ryabov E.V. 2007). In addition, the viruses infecting SWA may be explored and susceptibility of SWA cell cultures to other aphid specific viruses (Ryabov E.V. 2007) can also be studied. As there is no earlier report of SWA (*Ceratovacuna lanigera*) cell cultures and SWA cell lines, this is the first report of successful cell cultures of SWA (and also the first approach to develop SWA cell line) from whole specimen mincing of winged adults with darker abdomen for studying applications of biocontrol with SWA cell cultures.

MATERIALS AND METHODS

Reagents used were Grace insect Medium (Gibco) pH 6.2, 0.25 % Trypsin (Himedia), antibiotic antimycotic solution (10,000 U/ml penicillin, and 10 mg/ml streptomycin and amphotericin B 100 µg/ml), Fetal Bovine Serum (Himedia), 0.22 µm Membrane filters (Pall Corporation), Sodium bicarbonate (Sisco Research Laboratories), parafilm (Himedia).

Preparation of Grace's insect medium (100ml) : 4.34 g of powdered medium was added to 100ml (boiled and cooled) water. The pH was 6.2 after addition of sodium bicarbonate. The medium was sterilized with a membrane filter (0.22 µm) and after addition of 1 % antibiotic and

antimycotic solution, stored at 4°C. The aliquots of freshly prepared media of 1 ml each were added in 35mm sterile petri plates sealed with parafilm and kept in a thermacool container for sterility check at room temperature.

Heat inactivation and sterilization of Fetal Bovine Serum (FBS) : The newly procured FBS was heat inactivated at 60°C for 30 minutes, cooled and sterilized (0.22 µm) and aliquots were prepared in 50 ml centrifuge tubes, sealed with parafilm and kept at 4°C.

Trypsin preparation : 0.25 gm in 100 ml of distilled water (0.25 %) Trypsin was prepared and filter sterilized (0.22 µm) and kept at 4°C.

Collection of SWA specimens: The nymphs and winged adults of SWA were collected from leaves of sugarcane (*Saccharum officinarum*) plants in the VidyaPratishthan's School of Biotechnology (VSBT) campus area. The SWA specimens were collected from December 2013 to March 2014. The infestation of SWA observed on some sugarcane plantations in December 2013 and was decreasing from beginning of March 2014. In March, the number of adult SWA specimens collected also decreased with 2 adult specimens on entire plantation area and no 'woolly' appearance on sugarcane leaves. SWA nymphs and adults were collected from the sugarcane plantation area in 2ml, 1.5 ml tubes, 50 ml tubes and in PCR tubes from the leaves as per the availability of SWA specimens. The specimen collection was done while holding the leaf with the specimen on the opened tubes, the aphids were collected inside the tubes and then quickly closing the lid of the tubes (Figure 1).

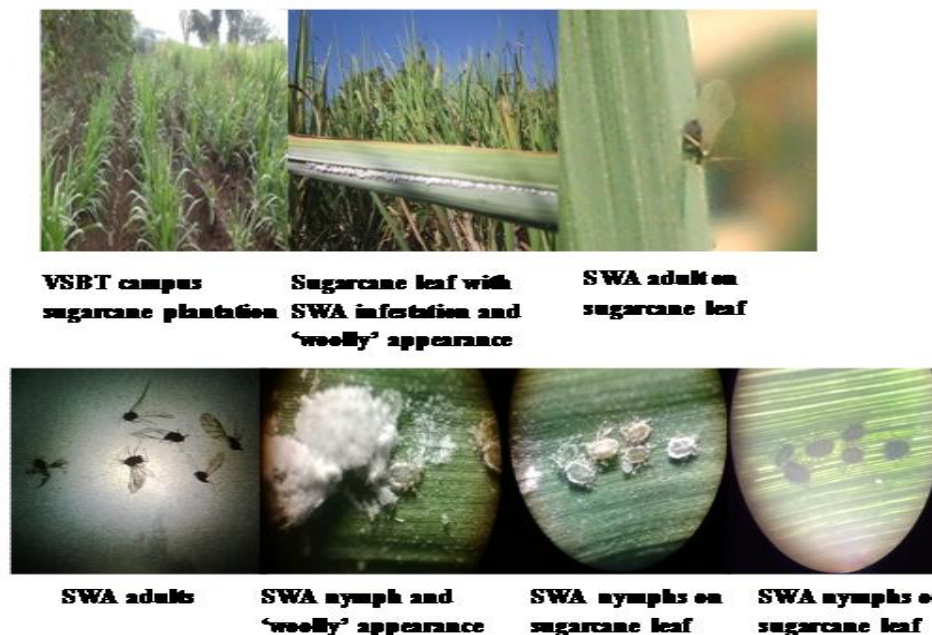


Fig. 1 : SWA on sugarcane plantation, SWA infestation, SWA nymphs and adults

Identification of SWA specimens : SWA nymphs were identified with the presence of 1 pair of cornicles on the posterior side of the abdomen projecting outwards parallel to the abdomen or away from the abdomen and adults with wings twice larger than the body apparently (Figure 2).

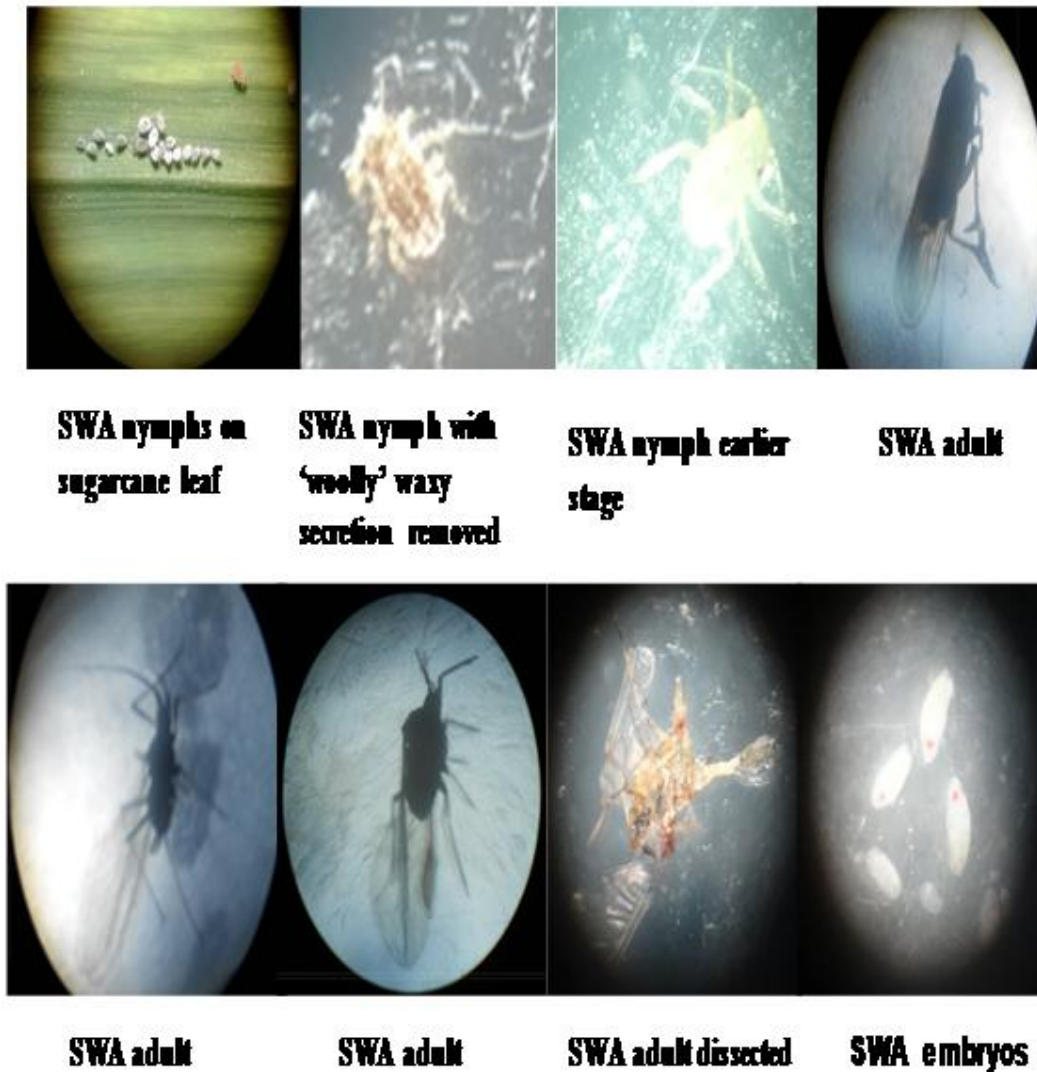


Fig 2 : SWA nymphs, adults and embryos

Surface sterilization and seeding of cells and explants : The adults of SWA were surface sterilized with immersing them in absolute alcohol for 1 minute. Initial approach was to culture cells of the SWA embryos as the source of rapidly dividing cells. Then, the nymphs underneath the sugarcane leaves of earlier and subsequent instar stages were collected for cell cultures. The

approach was to mince the whole adults after surface sterilization. The adults were kept immersed in antibiotic and antimycotic solution to prevent microbial contamination of the specimens for more than 5 minutes till mincing aseptically (mechanical dissociation) in Laminar AirFlow (LAF) cabinet. Also, the specimens were kept immersed in 0.25% trypsin (enzymatic dissociation, cold trypsinization) for more than 1 hour at 4⁰C and minced aseptically in LAF cabinet and the activity of trypsin was inhibited with the addition of sterile FBS.

Two winged adult SWA specimens (darker abdomen) were collected to initiate the cell cultures. They were immersed in absolute alcohol for more than 1 minute. First, 1ml sterile medium was added in two new sterile 35 mm petri plates. 10 µl of FBS was added. The two specimens were dried in LAF after immersing in absolute alcohol. Each specimen was minced and torn in abdomen with two sterile dissecting needles aseptically in LAF for about 2 minutes. Both the SWA cultures were sealed with parafilm and labeled and kept in the thermacool container swabbed with absolute alcohol at room temperature.

Microscopical analysis of cell morphology :

Daily observations of cultured SWA cells were done in Zeiss phase contrast inverted microscope at 5X, 10X and 40X magnification and were photographed at 5X, 10X and 40X magnification and also with digital zoom in phase contrast with digital camera.

OBSERVATIONS

After 24 h, in one SWA culture, increased adherence of explants and cells was observed compared to another SWA culture done simultaneously. The morphology of adhered cells was spindle shaped, bipolar, multipolar and cluster of cells. Round shaped cells were also observed. Primary cultures of SWA were successfully obtained. It was observed that the cells and tissue explants adhered within twenty four hours (Cells seeded in afternoon and observed next day morning) to the substratum. Cells adhered individually and also from the adhered explants and clusters with cytoplasmic processes. Initially, the cell morphologies were round, spindle shaped, branched like string of beads and rectangular like. There was no microbial contamination. There were tissue explants and some cells were with bipolar and long cytoplasmic processes like fork and some circular cells were observed upto five days. Fresh change of complete medium was given after two weeks. The cells and explants were detached from the bottom (Figure 3, 4, 5).



**SWA cell cultures
incubation at room
temperature**

**SWA cell cultures
incubation at room
temperature**

40X
**Adherence of explant
with cytoplasmic
processes**

40X
**Adherence of explant
with cytoplasmic
processes**



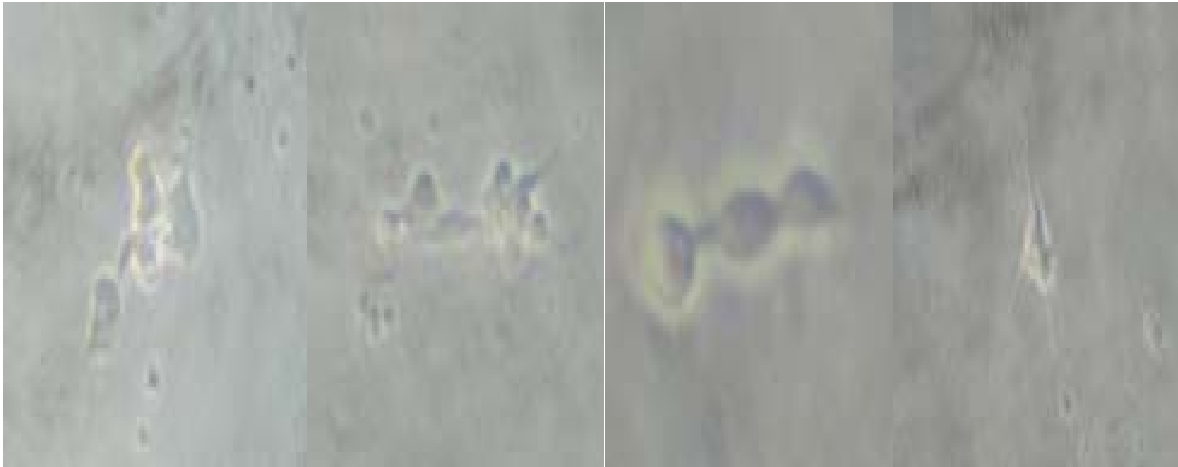
40X
**SWA cells appearing to
adhere**

40X
**SWA cell with tripolar
cytoplasmic processes**

40X
**SWA spindle shaped
cell with two round
cells**

40X
Round cells

Fig 3 : SWA cell cultures incubation at room temperature, explants and cell morphologies



40X

40X

40X

40X

Three to four very small tissue fragments. Spindle shaped cells seems to develop from three fragments

A small fragment of which all cells are attached and seem to grow

The chain consists of the original cell and two new ones attached to each other

Tissue fragment with the fork at the tip of one of the cytoplasmic processes



40X

40X

40X

40X

A growing tissue fragment of three cells

A larger fragment with developing spindle cells. Some cells in the fragment expand

A fragment of which all cells are attached and seem to grow

One spindle shaped cell

Fig 4 :SWA explants and cell morphologies



40X

40X

40X

40X

These cells are attached and seem to grow with cytoplasmic processes

A tissue fragment with one developing cell and one or more may start to develop

Three cells shaped and the central one may start

Adherence of explant with cytoplasmic processes



40X

40X

40X

40X

Adherence of explants with cytoplasmic Processes and cells

Appearance of adherence of cells

Adhered spindle shaped cells

Adhered cells with cytoplasmic processes

Fig 5 : SWA explants and cell morphologies

RESULTS

SWA cells were adhered in Grace insect media and the cell cultures of SWA were obtained by using Grace insect media with FBS. Cells were viable as observed upto 5 days in culture.

Cryopreservation : 400µl of FBS, 10µl of antibiotic and antimycotic solution and 100µl of glycerol is added to subsequent SWA cell culture and kept at 4°C, in freezer, then kept at -60°C.

CONCLUSION : The concentration of FBS may be optimized for further increased growth of cells and for promotion of adherence of cells and monolayer formation and SWA cell lines development.

DISCUSSION : The cultures from adult SWA were started with one specimen and subsequently the fresh cultures were done from more than 1 adult specimen, 5 specimens, from adults with green abdomen upon the availability of adult SWA on sugarcane leaves in VSBT sugarcane plantation. The present results are reported from the 5 days SWA cultures and upto medium change subsequently. The development of cells was followed daily initially with microscopic observations and subsequently it was noticed that there is adherence of many explants and the cells started to migrate from the explants and adhere. In first five days there was no degeneration of cells. In earlier reports on aphid cell cultivation, the ovarian and embryonal tissues were used. In this preliminary report, the whole specimens were dissected aseptically for starting cell cultures. As per cation and amino acid content of the body fluid of aphids, the amount of sodium and calcium is low and that potassium and magnesium occurs in high amounts (Peters and Black 1971). The cations and amino acids composition in body fluids of SWA and the media be designed according to the nutritional requirements of tissues and cells of SWA. In our earlier SWA cultures, the FBS was not added and no cell adherence was observed. After addition of same medium to the sterile petri plate, and with the addition of 10 µl of FBS (1% FBS) the cell adherence was observed the next day indicating similar cation requirement of SWA tissues and cells as per pea aphid (Peters and Black 1971) tissues and cells requirements in cultures. When 20µl and 300µl of FBS was added, no adherence observed. Hence, cation analysis and hemolymph analysis of SWA has to be done for media optimization and new media preparation for growth of SWA cell cultures and development of SWA cultures. Presently 1% FBS addition to Grace insect media depending on the observations in SWA may be better for development of SWA cultures and SWA cell lines. Also, another approach is to add biomaterial such as fibroin from silkworm (*Bombyx mori*) cocoon as bioscaffold for cell adherence instead of serum as serum is quite costly. Although aphid cells have a considerable capacity of survival in adverse environments, they may require a medium of specific composition to permit proliferation so that

subculturing is possible. Hence, biochemical composition of hemolymph be studied to formulate such a medium (Peters and Black 1971). Similarly, in our observations from SWA culture, cells did adhere and grew, although for subculturing of cells and development of SWA cell lines, the media be optimized based on SWA hemolymph and additives like silkworm hemolymph can be added. The studies on rate of cell proliferation, survival and subculturing of the cells are essential for the optimization of the media and are thus proposed for development of cell lines with cell growth measurements and characterization of SWA cell lines.

Hemolymph collection from SWA for development of hemocyte cultures and hemocyte cell lines and to study the immune function of hemocytes like silkworm hemocytes may also be done. The SWA cells may be substrates for the study of aphid viruses, plant viruses. Cell susceptibility of SWA aphid viruses and to baculoviruses is proposed in vitro and smeared on leaves for in vivo SWA biocontrol study. The SWA specific and other aphid species specific viruses (*Myzus persicae* densovirus, ALPV, RhPV, APV, rosy apple aphid virus mentioned above) susceptibility to SWA cultures and their genes may be explored to kill and control SWA with transgenic and silencing of SWA genes approaches. Hence, establishment of cell culture from sugarcane woolly aphid SWA (*Ceratovacuna lanigera* Zehntner) is a novel work.

The interaction and effects of endosymbionts on SWA cell cultures can be studied. The SWA cell cultures can also be useful in studying the effects of chemical pesticides and to develop the biocontrol for SWA. Effects of plant extracts and of toxins can also be studied on SWA for SWA biocontrol without affecting the plants, the livestock and the humans.

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