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TECHNICAL NOTE

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AN EFFECTIVE METHOD FOR *GRAPHOSOMA LINEATUM* (L.) LONG-TERM REARING

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Binazzi F., Sabbatini Peverieri G., Simoni S., Frosinini R., Fabbricatore T., Roversi P.F. – An effective method for *Graphosoma lineatum* (L.) long-term rearing.

A simple and time-saving technique for an effective and continuous rearing of *Graphosoma lineatum* (L.) (Heteroptera Pentatomidae), an alternative host for *Trissolcus* spp. and *Ooencyrtus* spp. production, was set for entomological research and maintained for a long period. Insects were maintained in containers as rearing units; 100x35x35cm cages hosted adults; 40x30x30cm cages hosted nymphs. *Graphosoma lineatum* was fed on seeds of *Foeniculum vulgare* Mill., *Anethum graveolens* L. and *Pimpinella anisum* L. Moreover, potted young plants of *F. vulgare* were also used as additional food source. Water for insects and plants was provided by small automatic irrigation systems. When each colony cage reached the density of 100 adult couples, the number of oviposited batches was followed up for 12 weeks. Batches laid per cage were approximately one hundred per week. Therefore the overall weekly production of six adult cages was about 8400 eggs. This technique was plain and cost effective, allowing a constant egg production throughout the year.

KEY WORDS: *Graphosoma lineatum*, laboratory, rearing, Apiaceae, *Foeniculum vulgare*

INTRODUCTION

Graphosoma lineatum (L.) (Heteroptera Pentatomidae) is a widespread poliphagous species living on several plants of the Apiaceae family such as *Foeniculum vulgare* Mill., *Daucus carota* L., *Ferulago campestris* (Besser) Grecescu. In the last decades, its role as alternative host for parasitoid rearing has been investigated in the following species: *Trissolcus semistriatus* (Nees) (ASGARI *et al*., 2002; KIVAN & KILIC, 2002), *Trissolcus grandis* (Thomson)(POPOV, 1974; SUNTSOVA & SHIRINYAN, 1974; ASGHARI *et al*., 1995), *Trissolcus vassilievi* (Mayr)(POPOV 1974; ASGHARI *et al*., 1995), *Trissolcus simoni* (Mayr) (SUNTSOVA & SHIRINYAN, 1974; KIVAN & KILIC, 2004), *Ooencyrtus pityocampae* (Mercet) (BATTISTI *et al.,* 1988; TIBERI *et al.,* 1991),*Ooencyrtus masii* (Mercet) (ASKARI, 2004).

Graphosoma spp. rearing under laboratory conditions was investigated by MAKARENKO (1968). However, the method commonly adopted for *G. lineatum* rearing is based on the technique described by VOEGELE (1966) for *Graphosoma semipunctatum* (F.). This procedure was then readapted by MINEO (1970) for *Graphosoma lineatum* rearing, in order to perform *Ooencyrtus gonoceri* Viggiani mass production for the control of *Gonocerus acuteangulus* (Goeze) (Heteroptera Coreidae).

Nonetheless, when long-term rearing of *G. lineatum* was planned, some of the essential steps of the Voegele-Mineo's technique appeared often cumbersome and difficult to carry out.

In the present work, a new simple and effective method is described, which allowed *G. lineatum* rearing and egg production for multiple generations, maintaining standard biological characteristics all the year round.

MATERIALS AND METHODS

INSECT ORIGIN AND LABORATORY CONDITIONS

The first colonies were originally established at the beginning of September 2010 by field collection of wild adults and nymphs (1^{st}-5^{th} instar). Thereafter, adults were transferred into insect cages while nymphs were located into separate mesh-sided containers until adulthood. When new adults emerged in the nymph containers, they were transferred into the adult cages in order to increase the population. Although the number of insects in the rearing was consistent, every summer, wild insects were field collected and introduced into the colonies in order to minimise inbreeding within the populations .

When in each cage the *G. lineatum* colony reached the density of 200 laboratory reared adults with a stable sex ratio of 55% females, the number of laid eggs was weekly followed up for 3 months.

Insects were reared in a climatized room (4.7x4.0x2.6 m). Temperature was maintained at 27±2°C; relative humidity at 45±5%. A 16:8 (L:D) photoperiod without twilights was controlled by a time switching device. Light was emitted by 14 fluorescent tubes (58W/tube) placed at 25 cm intervals on the room wall. When *G.lineatum* rearing was set up, light intensity was measured inside all insect cages by a light meter (Lutron LM-81LX). The range was between 1800 and 2200 lux, depending on the cage position and distance from the light source.

REARING CONTAINERS

Several types of containers for *Graphosoma* spp. rearing have been described (Table 1). VOEGELE (1966) noted that in *G. semipunctatum*, egg batches hatched more successfully when they were detached and positioned one

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next to the other. Moreover, HARRIS & TODD (1981) suggested to avoid solid-sided tanks such as plastic containers or glass bottles for *N. viridula* rearing, since lack of transpiration often resulted in high mortality rates. Therefore when our *G. lineatum* colonies were established, we decided either to employ mesh-sided cages or to remove batches from the adult cages, placing them onto seeds held in petri dishes. Nymph/egg rearing was thus separated from adult rearing but, unlike VOEGELE (1966), we opted for a combined rearing of all nymph instars that were thus hosted in the same container. This led to a marked reduction of labour of personnel. Two different rearing containers were thus set up: adult containers and nymph/egg containers (Fig. I, 1, 2).

STRUCTURE AND MANAGEMENT OF ADULT CONTAINERS

The cage was a light wood-frame (100x35x35cm) (Fig. I, 1); bottom and lateral short sides were made of plywood panels (5mm width) reinforcing the structure, whereas the top and long lateral sides were 1mm-mesh metal nets attached to the frame. One of the long lateral sides functioned as a door fixed to the frame by metal hinges. A polystyrene layer set at the bottom of the cage served as a robust and easy-cleaning surface. The polystyrene layer was then lined with paper sheets. On the short sides of the cage, stripes of soft absorbent paper (20x10 cm) were attached in order to form an oviposition site for *G. lineatum* females. Below the paper stripes, thick and hard cardboard tubes (10 cm length and 8 cm diameter) were placed on each side of the cage (Fig. II, 5). The inner surface of each tube was paved by pressed cotton disks (8 cm diameter). Those located at the tube ends protruded 3 cm out of both edges (Fig. II, 3). Thereafter a 20x10 cm stripe of soft absorbent paper was loosely folded into each tube in order to fill the inner volume. Dark crevices within the tubes attracted *G. lineatum* adults facilitating female oviposition on either paper or cotton disks (Fig. II, 3). In addition, two longer tubes (25x8 cm) were similarly arranged and placed along the lateral sides of the cage (Fig. II, 5).

Twice a week, cages were opened and all paper stripes and cotton disks inspected in order to collect egg batches. The presence of crevices within tubes strongly reduced the tendency of insects to oviposit on undesired substrates such as cage frame (wood, nets) or equipment (pots, seeds and plants). This behaviour is likely due to female preference for "protected spaces" during oviposition. Oviposition on cotton and/or paper was particularly practical since laid batches could be easily cut out by fine scissors and stored, reared or directly used for the experiments. On the contrary, batches laid on wood, metal parts, pots, or fennel plants had to be detached by a fine moistened brush and scalpel, with relevant waste of time and frequent egg damage.

When paper stripes and cotton disks were found to be dirty or unsuitable for oviposition, they were eliminated and replaced with new ones. Replacement was usually carried out every 10-15 days depending on population size and faecal contamination. The bottom of the cage was cleaned every two weeks. Moreover, once a week, dead bodies and exuviae were manually removed. Fungal growth or other diseases were seldom observed during rearing. Every month, cages were emptied and washed by scrubbing the wooden parts with a brush. A hot water solution of sodium hypochlorite was used in order to thoroughly clean all surfaces. The cleaned cage was then washed with water and left to air dry.

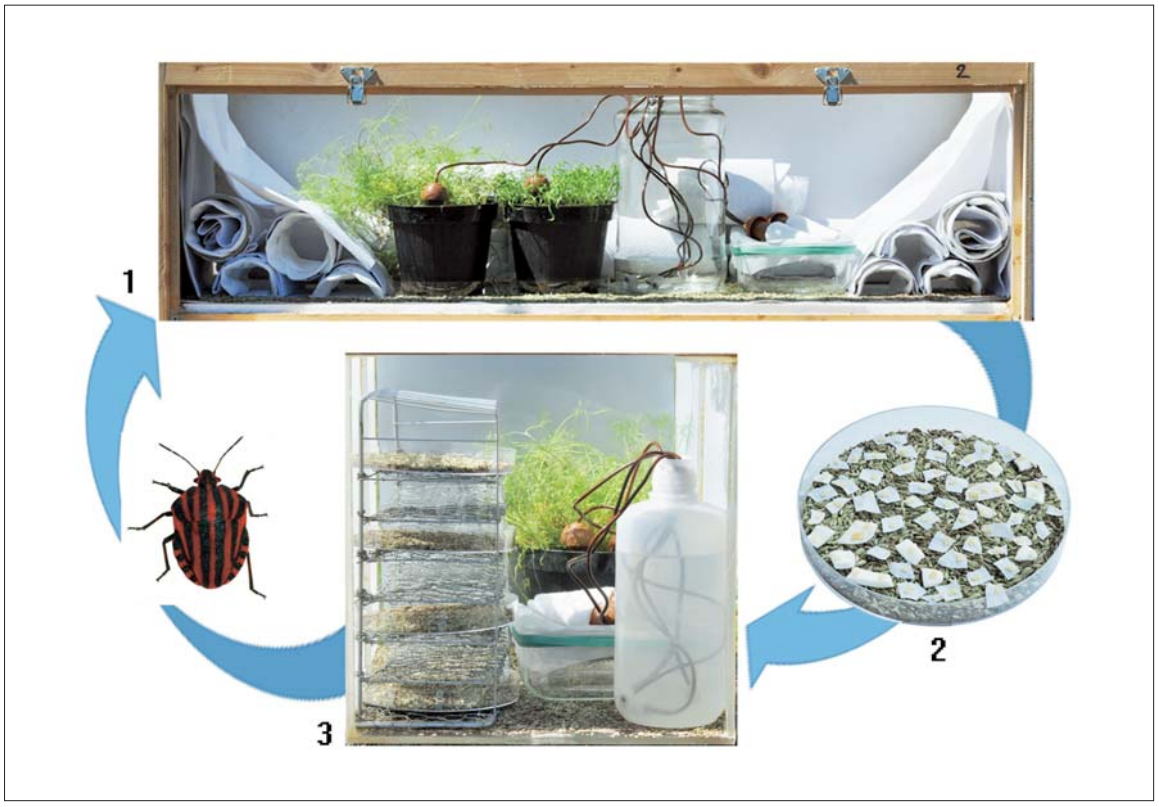

Fig. I – Rearing cycle of *Graphosoma lineatum* (L.): 1. Adult cage (petri dishes with seeds were removed). 2. Petri dish with *G. lineatum* egg batches ready to be transferred into the metal rack in the nymph cage. 3. Nymph cage (1st -5th instar).

Fig. II – 1. Metal rack with petri dishes filled with Apiaceae seeds. 2. Seepage control sprinklers with porcelain head parts enveloped in wet cotton disks. 3. Edge of a cardboard tube with protruding cotton disks and soft absorbent paper. 4. *Graphosoma lineatum* (L.) egg batches placed on Petri dishes filled with Apiaceae seeds. 5. Cardboard tubes of different sizes. 6. Seepage control sprinklers set up for providing insects and plants with water.

Structure and management of Nymph / egg containers

Containers used to rear nymphs were 40x30x30 cm cages (Fig. I, 3). All the sides were made of Plexiglas transparent panels except for the long lateral sides that were nylon-mesh material attached to the frame. The bottom of the cage was first lined with paper sheets. Afterwards, a metal rack (35x25x10 cm) was inserted into the cage and located along one of the lateral sides (Fig. II, 1). This handmade structure had been simply and inexpensively constructed by attaching metal nets and iron wire to a metal frame.

Egg batches collected from the adult cages were placed onto a 5 m-thick layer of seeds held in 140 mm Petri dishes (Fig. II, 4). Petri dishes were then transferred into the nymph cages and inserted into the metal racks. When

egg batches hatched, nymphs started their development on the seeds. Afterwards, the more active immature stages (from 3rd to 5th instar) tended to move away from the Petri dishes to reach a young potted plant where they continued their development until the adult stage. Nymphs were often observed to climb on the metal rack and on the inner surface of the cage in order to reach the plant. When petri dishes were replaced, care was taken not to discard young nymphs hidden within the seeds.

On the other hand, as soon as new adults emerged they were transferred into the adult cages. To this end, plastic containers with screw-on lids were used for temporary holding and/or transfer of adults.

Nymph cages were emptied and completely cleaned every two weeks. A hot water solution of sodium hypochlorite was often used to clean Plexiglas panels and nylon mesh. The cleaned cage was then washed with water and dried before reuse.

FOOD

Graphosoma spp. food preferences have been thoroughly investigated in order to design effective rearing conditions (Table 1). Moreover its biological parameters after feeding on various food sources were evaluated by several authors such as ASGHARI *et al.,* (1995), KARSAVURAN (1996), YUCE ORS & KARSAVURAN (2004), KOCAK & BARIS (2008) and KOCAK *et al.,* (2009).

In the rearing technique developed by VOEGELE (1966) and readapted by MINEO (1970), seeds and stored plant material of the giant fennel *F. communis* represent an essential food source. Unfortunately, details concerning its laboratory cultivation and handling are not provided, making its management complex and laborious. In these methods either seeds or plant material need to be harvested and stored when long-term rearing is planned. At our rearing conditions, *G. lineatum* never accepted dried and/or stored plants of any of the commercially available or naturally occurring Apiaceae such as *F. vulgare, A. graveolens, D. carota*, *F. campestris*. Likewise, other plants such as *C. maculatum* and *H. platytaenium*, though reported to be suitable as a food source, are often commercially unavailable all the year round and difficult to handle within cages.

To our knowledge, when short-time experiments are planned, supplying *G. lineatum* with umbels, tops, leaves and seeds of its host plant is generally sufficient for its adequate feeding. However, when long-term rearing or continuous experiments are planned, a different technique has to be designed in order to maintain the populations.

Therefore, in our method, a feeding protocol was selected including plant species that matched the following criteria: i) reported to be natural hosts of G. *lineatum*; ii) inexpensive and commercially available throughout the year; iii) easy to cultivate and with seeds suitable for long-term storage; iv) easy to handle within cages.

To this end, four 140-mm Petri dishes filled with equal

Table 1 – Parameters selected for *Graphosoma* spp. long-term laboratory rearing.

	VOEGELE (1966)	MINEO (1970)	KIVAN & KILIC (2002)	Present study
Host Species	*Graphosoma semipunctatum*	*Graphosoma semipunctatum* / *Graphosoma lineatum*	*Graphosoma lineatum*	*Graphosoma lineatum*
Temperature (°C)	30	30	26±1	27±2
Relative Humidity (%)	40-70	40-70	not reported	45±5
Container (for immature stages)	Box (4x9.5x6.5 cm) (1st - 3rd stage nymphs)	Box (12.5x12.5x3.5 cm) (Egg - 1st stage nymphs)	Cage (size not reported)	Cage (40x30x30 cm) (Eggs and all Nymph instars)
	Cage (22x45x60 cm) (3rd - 5th stage nymphs)	Cage (29x54x67 cm) (2nd - 5th stage nymphs)		
Container (for adults)	Cage (22x45x60 cm)	Cage (29x54x67 cm)		Cage (100x35x35 cm)
Oviposition substrate	Cloth stripes	*Ferula communis* seeds and cloth stripes	Paper stripes (2x20 cm)	Paper stripes and cotton disks
Food source (for nymphs)	*Ferula communis* seeds held in boxes (1st - 3rd stage nymphs)	*Ferula communis* seeds held in boxes (Egg - 1st stage nymphs)	Dried umbelliferous seedlings from *Daucus sativus, Pimpinella anisum* and *Foeniculum vulgare*.	Seeds of *Foeniculm vulgare, Anethum graveolens* and *Pimpinella anisum*
	Ferula communis umbels and wheat sprouts held in trays (3rd - 5th stage nymphs)	*Ferula communis* seeds and wheat sprouts held in trays (2nd - 5th stage nymphs)		
Food source (for adults)	*Ferula communis* seeds attached to paper stripes	*Ferula communis* seeds and wheat sprouts held in trays	Dried umbelliferous seedlings from *Daucus sativus, Pimpinella anisum* and *Foeniculum vulgare*.	Seeds and young plants of *Foeniculm vulgare, Anethum graveolens* and *Pimpinella anisum*
Water supply/ availability	Cotton layer, moistened twice a day, located on top of the nymph rearing boxes	Cotton layer, moistened twice a day, located on top of the nymph rearing boxes	Water daily added	Automatic watering systems

proportions of *F. vulgare, A. graveolens* and *P. anisum* seeds (20g/plant species/Petri dish) were randomly placed into the adult cages. Alternatively, combinations of *F. vulgare* and *A. graveolens* or *F. vulgare* and *P. anisum* could be employed for the same purpose. The cage floor was fully covered by fennel seeds that formed a 5mm layer. In addition, one or two young potted plants of *F. vulgare* were introduced to improve *G. lineatum* rearing providing the insects with their natural host plant. Plants were changed when dieback occurred, whereas seeds were added every week and fully substituted every 15 days.

In the nymph cages, insects could equally rely on either mixed seeds of *F. vulgare, A. graveolens* and *P. anisum* held in the petri dishes (Fig. II, 1) or fennel seeds laid to cover the cage floor. Moreover a potted fennel plant was placed on the opposite side of the rack. Plants were changed when required, while seeds were weekly added and completely replaced every two weeks.

Plants were cultivated in a climatized room at standard conditions of 25±1°C temperature, 50±10% relative humidity (RH) and a photoperiod of 16:8 (L:D). The role of these plants was essential representing an extra food source and/or a natural resting site where adults or nymphs could hide.

On the other hand, all seeds were stored in large baskets in a climatized and ventilated room at 10±1°C, 30±10% (RH)

WATER

Pentatomid colonies are generally provided with water. To this end, moistened cotton (Table 1) or other devices such as plastic "diet" cups with absorbent cotton wicks are often used (HARRIS & TODD, 1981).

These systems soon appeared unsuitable for long-term rearing since wicks or cotton layers needed constant replacement. Moreover a direct water supply for potted fennel plants was also required to allow insect survival and adequate growth. When *G. lineatum* rearing was planned, a new method for providing either plants or insects with water was developed: small automatic irrigation systems were set up close to the pots (Fig. II, 6).

In the adult cages, this system included a 2-liter tank and two watering devices made of porous porcelain head parts connected to plastic pipes. The porcelain heads were completely inserted into the soil of potted plants whereas plastic pipe ends were fully immersed into the water container. Water flowed through the plastic pipes reaching the porcelain head parts by capillarity.

The same automatic irrigation device was employed to provide the insect with constant fresh water. The heads of other two sprinklers were first cleaned and then completely enveloped with wet cotton disks (Fig. II, 2). This system allowed the cotton to remain constantly moistened, ensuring a permanent water source for the pentatomids. The sprinklers were then placed on a 1mm-mesh plastic net covering a glass container (15x15x5cm) that served to collect the extra water, preventing liquid spillage on the cage floor. When required, the extra water was eliminated from the glass container whereas the big 2-liter tank was weekly cleaned and refilled with fresh new water. Likewise, a similar system for water supply was arranged in the nymph cage. This time a smaller 1-liter tank was used for reasons of space.

Since *G. lineatum* is capable to walk on the pipes reaching the containers, care was taken in capping them. The 2-liter container had a pierced screw-on lid, which allowed the passage of pipes but prevented that of adults (Fig. II, 6). On the contrary, in the nymph cage, cotton was used for capping the 1-liter tank in order to freely insert the pipes preventing the entrance of nymphs. When plant dieback occurred, insects were observed to move onto the moistened cotton seeking for water. Cotton might thus act as a second-choice option for insects, representing an extra water source when fennel cannot provide the necessary amount of liquids. Since water can represent a source of transfer for pathogens (HARRIS & TODD, 1981), cotton enveloping the porcelain head parts was weekly replaced.

HANDLING THE INSECTS AND THE EGGS

G. lineatum adults were easily collected and temporarily stored (6-8h) for transportation in plastic containers with snap-on lids. Boxes were often filled with seeds and host plant material in order to form an adequate environment.

From a more general point of view, the pentatomid could be easily handled in either laboratory or field. On field capture, collected specimens often emitted a typical defensive odour but, after caging, this behaviour tended to decrease. Nonetheless unnecessary handling should be avoided. Mating individuals are, in fact, particularly sensitive and tend to flee when they are stressed. When necessary, young instars were transferred by a fine moistened brush from petri dishes to nymph cages or vice versa. However needless transfer of nymphs should be always avoided, since brush hairs, though very soft, can easily trap or damage the delicate *G. lineatum* 1^{st} instars.

CONCLUSION

In the last 4 years, this method enabled us to rear *G. lineatum* with a maximum labour of about 6h per week when the rearing room was at its maximum capacity of six cages for both adults and nymphs (1200 adults and 6000 nymphs). At the conditions set, in our climatized room, no *G. lineatum*, ever entered diapause whereas egg production and insect development were constant throughout the year.

VOEGELE (1966) noted that *G. semipunctatum* nymphs could successfully thrive even at very high densities such as 600 individuals/237cm^3 (1^{st}-3^{rd} instar) or 2,500 individuals/59,400 cm^3 (4^{th}-5^{th} instar). Likewise, in our colonies, *G. lineatum* instars (1^{st}-3^{rd}) were often observed to form dense masses at the corners of cages, effectively withstanding overcrowding. Similarly, the 3^{rd} and 5^{th} instars were equally gregarious when feeding on potted fennel plants. However, in order to reduce labour and save time, we decided to rear nymphs and adults at densities that never exceeded respectively the 1000 and 200 units per cage.

Some pentatomids such as *N. viridula,* often decline in viability and vigour with successive generations due to inbreeding. Moreover *N. viridula* is often subject to frequent mortality in the 4^{th} and 5^{th} instars and imaginal molt. Cannibalism is also reported in 4^{th} and 5^{th} instars preying on molting late instars and adults (HARRIS & TODD, 1981).

On the contrary, cannibalism, molting abnormalities or decrease in insect size were never noted throughout the period of our *G. lineatum* rearing.

VOEGELE, (1966) reported an average of 20.000 eggs laid by 300 couples of *G. semipunctatum* in three weeks of rearing at 30°C.

In *G. lineatum*, preliminary experiments showed that, such a level of production could be only achieved when large quantities of insects were field collected, caged and provided with fresh plant material of their natural host plant. However this feeding regime was only sustainable during summer periods when fresh umbels, tops, and stems of Apiaceae are suitable for harvesting. Unfortunately, insects in our colonies, never accepted stored and/or dried plant material except for seeds. Therefore, we had always to rely on stored seeds and cultivated plants. Moreover, speeding *G. lineatum* cycle up to 30°C as in *G. semipunctatum*, though improving egg production, usually resulted in an increased food consumption and faecal contamination. The management of such a cycle was thus time consuming leading to excessive labour of personnel.

As a consequence, all environmental parameters were always maintained at the same standard conditions described above (27±2°C, 45±5% RH and 16:8 L:D) throughout the rearing period.

In the 12 weeks of our follow up, the number of oviposited batches for each colony cage was approximately 100 per week. Since every batch is about 14 eggs, the production of each cage was about 1400 eggs per week. Therefore, when the room was at its maximum capacity of six nymph and adult cages the overall production was approximately 8400 eggs per week.

When compared with previous *Graphosoma spp.* rearing protocols, our technique simplified either rearing steps or feeding regimes. However, we emphasize that, though our *G. lineatum* rearing conditions are adequate either for behavioural studies at this latitude or for continuous egg supply, these parameters are not necessarily those, which will ensure a maximum rate of population increase. Nonetheless, overall results showed that *G. lineatum* is an ideal candidate for long term rearing. In fact, at the conditions set in our rearing, diapause was never induced and egg production was continuous. Moreover, handling of insects was reduced and food sources were inexpensively bought and easily managed. Finally, either high density of individuals or inbreeding never led to increased mortality rates or insect malformations.

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