RESEARCH PAPER

Mass production of *Diglyphus isaea* (Hymenoptera: Eulophidae), a biological control agent of a Korean population of potato leaf miner *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae)

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Abstract
The potato leaf miner, *Liriomyza huidobrensis* (Blanchard), is an important pest of potato throughout the world, including Korea. A method was developed for mass rearing the parasitoid *Diglyphus isaea* (Walker) using faba bean, *Vicia faba* L. (Fabaceae), as the host-plant and *L. huidobrensis* as the insect host. Faba bean plants were planted in pots and maintained in a greenhouse for about 15 d. Pots were then exposed to adult leaf miners in oviposition cages for 4 h. Plants containing late second to early third instar larvae were exposed to adult *D. isaea* in parasitoid cages for 24 h. The leaf area per pot after 15 d was 597.9 cm$^2$, which produced 103.33 larvae per pot. The number of adult parasitoids emerging per pot was 72.5; about 41% of these were female. The daily cost of parasitoid production was USD20.95 per 1000 individual parasitoids. The methodology developed for *D. isaea* could be used to rear other ectoparasitoids such as *Hemiptarsenus* spp. and *Pnigalio* sp. with different insect hosts like *L. trifolii*. This is why this mass-rearing information is important for securing test insect materials for ecological and biological study of *Liriomyza* species, and also for developing a biological control for *Liriomyza* species other than *L. huidobrensis* by mass production of associated natural enemies. We are facing rapid agro-ecosystem changes including pest systems. Continuous monitoring of *Liriomyza* in solanaceous crops is needed.

Key words: biological control, *Diglyphus*, *Liriomyza*, mass rearing, relationships.

Introduction

The genus *Liriomyza* (Diptera: Agromyzidae) includes 23 species of economic importance because they damage a wide range of field and greenhouse crops and ornamental plants (Spencer 1973; Parrella 1987; Kang 1996; Cerny et al. 2001). Invasion is increasing in new areas worldwide (Minkenberg & Van Lenteren 1986; Weintraub & Horowitz 1995; Steck 1996). Nine species of *Liriomyza* are reported in Korea (Ahn et al. 1994; Hong et al. 1996; Suh & Kwon 1998; Maharjan et al. 2014). *Liriomyza huidobrensis* has been recently noticed and considered as an emerging new pest in potato in Korea (Maharjan et al. 2014). Larvae of the leaf miner are polyphagous and cause both direct and indirect damage to plants (Musgrave et al. 1975; Minkenberg & Van Lenteren 1986). Direct damage includes leaf puncturing, feeding and laying eggs in the leaf mesophyll (Weintraub & Horowitz 1995). Larval feeding characteristics vary according to species, which can reduce photosynthesis by up to 62% (Johnson et al. 1983; Parrella et al. 1984; Heinz & Chaney 1995), accompanied by yield loss (Chandler & Gilstrap 1987). Indirect damage includes vectoring for plant diseases (*Alternaria alternate* (Fr.) Keissl) (Zitter & Tsai 1977; Matteoni & Broadbent 1988; Deadman et al. 2002).
Currently, chemical methods are the first options for controlling this invasive pest (Cox et al. 1995). However, high reliance on insecticides had been followed by the development of insecticide resistance and the subsequent reduction in natural enemy populations, which is exacerbated by improper management (Johnson et al. 1980a; Lange et al. 1980; Trumble 1981; Parrella et al. 1984; Chavez & Raman 1987; Mason & Johnson 1988; Sanderson et al. 1989; MacDonald 1991; Ferguson 2004). Thus, biological control by releasing a mass-produced biocontrol agent could be a sustainable and environmentally friendly option for this secondary pest in both protected cultivation (Parrella 1982) and open fields where additional control could be expected from natural enemies (Johnson & Hara 1987; Johnson 1993; Liu et al. 2009).

*Liriomyza* leaf miner flies are known to have natural enemies (Waterhouse & Norris 1987; Murphy & LaSalle 1999). Parasitoids are the major group of natural enemies of leaf miner flies (Parrella 1987; Kang 1996). Noyes (2004) listed more than 80 species of parasitoid wasps that attack various stages of *Liriomyza* species. Genus *Diglyphus* in the family Eulopidae constitutes one of the most important parasitoid groups of *Liriomyza* spp. (Johnson & Hara 1987; Waterhouse & Norris 1987; Minkenberg 1990; Johnson 1993; Liu et al. 2009; Cisneros & Mujica 1998; Mujica & Kroschel 2011). Successful control of leaf miner species through migration of *Diglyphus* spp. has been reported in Europe (Nedstam 1987, Nuñifora & Vacante 1987). In California celery, *Diglyphus* spp. provided adequate control of *L. trifolii* (Burgess), as did pesticides (Trumble 1985). Inoculative–inundative release programs of *Diglyphus* spp. have been reported in many countries especially to control *L. trifolii* in greenhouses (Gaviria et al. 1982; Lyon 1985; Wardlow 1985; Woets 1985). Until now no information about mass-rearing of ectoparasitoids on a Korean population of *L. huidobrensis* using faba bean has been documented in Korea.

*Diglyphus isaea* is a primary parasitoid of Agromyzidae leaf miners (Sher et al. 1996; Zhu et al. 2000) and is considered as the most effective biocontrol agent of leaf miner flies including *L. huidobrensis*. *Diglyphus isaea* is a facultative gregarious parasitoid and the adult females lay one to five eggs adjacent to late leaf miner larval stages. The parasitoid larvae feed on the host for about 5 d then pupate in the mine. The adult parasitoids emerge after about 5.5 d and live for about 10 d (Minkenberg & Fredrix 1989). Morphological features such as cubital vein strongly curved at base; speculum very narrow or absent; scutellum shiny and violet; and all coxae, trochanter, basal 3/4 of femora, and tibiae dark except for base or apical 1/5–1/6 distinguish *D. isaea* from all other species of *Diglyphus* (Gonzalez et al. 1979; Zhu et al. 2000). Currently, *D. isaea* is considered as a single species with a worldwide distribution (Noyes 2002). It is common in Gangwon, north and south Chungcheon, north and south Gyeongsang and north and south Jeolla provinces in mainland Korea (R Maharjan & C Jung, 2012). This paper describes the mass production system of *D. isaea* using faba bean and potato leaf miner fly as a host-plant and insect host, respectively. Additionally, cost analysis of the rearing protocol is provided.

**Materials and methods**

**Plant production**

Faba bean, *Vicia fabea* (Fabaceae), was planted weekly in pots (18 cm diameter, 13 cm height) using commercial soil media (Plant World, Gyeonggi, Korea); three to five plants were positioned in each pot. Plants were stored on greenhouse benches and watered manually as needed. The plants were kept for 10–15 d in greenhouse conditions (28 ± 2°C, 50–70% relative humidity under conditions of 16 h light : 8 h dark (LD 16:8)). Environmental conditions were recorded by using data loggers (Easy Log-WIN-USB, Garage, Essex, United Kingdom, Lascar Electronics UK-onset computer, Garage, Essex, United Kingdom). At this time plants were about 30–40 cm tall, with sufficient leaves.

Leaf area index would allow comparison between faba bean and other potential host-plants (i.e., *Phaseolus* spp.). Areas of 175 individual faba bean leaves (dependent variable) were measured with a leaf area meter (CI-202; CID Inc., Camas, Washington, USA) and these were regressed on leaf length (independent variable) (SAS Institute 2009). This relationship was used to calculate total leaf area of each pot used in the rearing study.

**Leaf miner production**

For oviposition, adult leaf miners were held in acrylic cages (40 cm height × 60 cm width × 80 cm length) with openings in the sides covered with meshed screens for ventilation. Conditions in greenhouses were maintained under a photoperiod of LD 16:8 and at temperatures between 21 and 28°C. Relative humidity fluctuated between 40% and 70%. Uniform numbers of leaf miners were maintained in these cages by releasing a constant number (about 200, sex ratio of male : female of 1:1) of adults daily into the cage. This action kept the population in each cage between 1000 and 1500. Preliminary experiments revealed that the variance associated with the mean number of parasitoids produced per pot stabilized with a sample size of at least six pots. Six potted faba beans were exposed in each cage for 4 h, usually between 07.00 and 11.00 h. This number of pots was chosen to assure a stable variance and for convenience because six pots fit perfectly into a standard greenhouse flat
(approximately 2000 cm²). For the purposes of documenting leaf miner and parasitoid production per pot, three replicates of six pots were monitored through the rearing process on separate days.

A short exposure time to adult leaf miners assured that all eggs were deposited in the plants at nearly the same time and that larval development would be synchronous. Pots were replaced on greenhouse benches after exposure; after 3 d, all newly hatched larvae were counted. When most larvae reached the late second or early third instar (after approximately 3–5 d from egg hatching), they were ready for exposure to the parasitoids. Plants to be used only for leaf miner production were cut at the soil line and placed on screens over sand-filled trays to collect pupae. The sand was passed through a fine screen to separate pupae. Counting the pupae was very tedious, so the relationship between the weight of pupae (independent variable) and number of pupae (dependent variable) of 100 samples encompassing 37 to 160 pupae was determined by a linear regression model (LRM) (SAS Institute 2009).

### Parasitoid production

Adult *D. isaea* were held in acrylic cages (43 cm height × 80.5 cm weight × 60.2 cm length) with openings in the sides covered with meshed screens for ventilation. These cages were located in greenhouse rooms under conditions described previously for the leaf miner. Uniform numbers of parasitoids were maintained in these cages by adjusting the number released daily into each cage (approximately 50, sex ratio of male : female 1:1). These releases kept the population in each cage at about 200–300.

Six potted faba beans, each containing second or third instars, were exposed in each cage for 24 h. After exposure, the pots were held horizontally for 2 d on tip-racks with sand-filled trays. Leaf miner larvae that survived exposure to the parasitoid emerged and were collected in these trays. Plants were then moved to another greenhouse room for parasitoid development. After about 10 d (just before parasitoid emergence from leaves), plants were cut at the soil line and placed in glass-topped sleeve cages. As they emerged, parasites were collected in small vials with hand-held aspirators. Adult parasitoids were returned to cages for parasitoid production.

### Analysis of the rearing protocol

To document the rearing procedure, pots were held individually to determine the number of leaf miners that survived parasitoid exposure per pot and the number and sex of parasitoids per pot. As discussed previously, six pots were followed through the rearing procedure on three separate days, so in total 18 pots were used to determine production statistics. On a per-pot basis, leaf area, number of newly hatched larvae, number of larvae surviving exposure to the parasitoids and number of the parasitoids were calculated. The rearing procedure was divided into the following components: potting and maintaining plants, exposing pots to leaf miner, exposing pots to parasitoids, sand-filled trays, plant and leaf miner colony maintenance while parasitoids developed, cutting plants and placing in sleeve cages, and aspirating parasitoids from cages. Each of these categories was timed during parasitoid production over a 3–4-d period, and the time required to produce 3000–4000 *D. isaea* per day was calculated.

The cost of production was divided into nonrecurring costs (e.g., cages and pots) and recurring costs (e.g., plants, soil and fertilizer) using prices from 2015. Cost of space (greenhouse rooms) and utilities was not included in the overall cost.

### Statistical analysis

Relationship between leaf area and leaf length, and pupa number and pupa weight, were calculated by LRM (SAS Institute 2009).

### Results

A significant relationship between leaf area and leaf length was found (Reg. *F* = 513.81, df = 1173, *P* < 0.0001; *R*² = 0.76) (Fig. 1). This relationship allowed rapid estimation of leaf area per pot by the use of a nondestructive method (measuring leaf length). After 15 d of plant growth, leaf area per pot was 597.9 ± 0.5 cm². Exposing six pots for 4 h in cages with 1000 to 1500 adult flies resulted in 103.33 ± 1.4 newly hatched larvae per pot. Fewer than five larvae per leaf
were usually present. So, intraspecific competition was not found to be a problem.

Estimation of the number of leaf miner pupae was made easier by the strong relationship with pupal weight ($R^2 = 0.74$) ($\text{Reg. } F = 254.70, \text{df} = 1.98, P < 0.0001; R^2 = 0.74$) (Fig. 2). This estimation method is often used for leaf miner production. After exposure of pots to the parasitoid cage, $54.9 \pm 1.3$ leaf miner larvae per pot survived and pupated in the sand-filled trays. These emerged and were returned to the leaf miner colony cages as needed. Parasitoid production was $72.5 \pm 1.2$ per pot; $41\%$ of these were female. Production was equivalent to $0.12$ parasitoids per square cm of faba bean leaf.

Approximately $398.4$ min. was required to produce $4000$ adult parasitoids (Table 1). More than $50\%$ of this time was devoted to the tedious task of aspirating parasites into vials. Although other methods could be developed for parasitoid removal (e.g., anesthetization or use of the phototactic response), these methods must include some mechanism for quantifying parasitoid production as well as for calculating the sex ratio. The latter two aspects take considerable time.

The total cost of producing $4000$ $D. isaea$ was estimated to be USD$4528.40$ (Table 2). For this estimation, more time was given labor ($8$ h) than was required ($6.67$ h, Table 2) to allow some flexibility in the timing for each procedure. When production was underway, the only recurring costs that needed to be considered were labor, soil and plants. Therefore, the recurring cost of parasitoid production was USD$83.80$ per $4000$ parasitoids. This figure could underestimate the real costs because the costs and depreciation of greenhouse space, machinery and utilities were not taken into consideration.

### Discussion

Faba bean is relatively fast growing compared with other hosts such as pumpkin and cucumber; approximately $15$ d (in optimum condition) is required when a plant is suitable for parasitoid production (Table 1, Fig. 3). It is important to choose the right plant and insect host for rearing any insect and parasitoids. Salvo and Valladares (2002) reported that the size of *Liriomyza* leaf miner and its suitability for parasitoid varies with the plant host in both natural habitats and cultivated areas. The cost of faba bean and use of this plant in the rearing process would reduce the possible conditioning of the parasitoids to another host-plant. The use of the Korean population of *L. huidobrensis* as the host leaf miner, and rearing the parasitoids under general greenhouse conditions, mean that they will quickly acclimatization to the field environment, which should increase their potential parasitism. The size of parasitoids can be a useful character, although this was not quantified. Charnov *et al.* (1981) showed the solitary parasitoid adult size is closely correlated with host size.

![Figure 2](image_url)

**Figure 2** Regression analysis of leaf miner pupa number versus pupa weight ($n = 100$ samples) ($\text{Reg. } F = 254.70, \text{df} = 1.98, P < 0.0001$).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Days</th>
<th>Per pot (s)(mean ± SE)</th>
<th>Per 4000 parasitoids (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potting and maintaining plants</td>
<td>1–20</td>
<td>$53.8 \pm 1.6$</td>
<td>44.5</td>
</tr>
<tr>
<td>Exposing plants to leaf miner</td>
<td>20</td>
<td>$34.7 \pm 1.6$</td>
<td>28.4</td>
</tr>
<tr>
<td>Exposing plants to parasitoids</td>
<td>25</td>
<td>$56.2 \pm 0.7$</td>
<td>46.3</td>
</tr>
<tr>
<td>Plants placed on tip racks</td>
<td>26</td>
<td>$20.7 \pm 1.0$</td>
<td>172</td>
</tr>
<tr>
<td>Plants and leaf miner colony maintenance while parasitoids develop</td>
<td>28</td>
<td>$4.3 \pm 0.4$</td>
<td>3.5</td>
</tr>
<tr>
<td>Cutting plants and place in cages</td>
<td>40</td>
<td>$21.7 \pm 2.3$</td>
<td>18.1</td>
</tr>
<tr>
<td>Aspirating parasitoids from cage</td>
<td>43</td>
<td>–</td>
<td>240.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>398.4</td>
</tr>
</tbody>
</table>

*Based on three replicates of the entire process. For 4000 parasites, 50 pots are required with 80 parasites per pot. –, not calculated.*
Although host sizes of *L. huidobrensis* larvae were not measured in this study, studies by Spencer (1973) and Musundire et al. (2012) showed that under uniform conditions, *L. huidobrensis* larvae are larger than those of *L. sativae* and *L. trifolii*, suggesting that this species should receive a higher allocation of female progeny compared with larvae of *L. sativae* and *L. trifolii*. Thus the female proportion of the parasitoid *D. isaea* population would be higher when produced from larger host insect species. The female proportion in any *D. isaea* population in augmentative and mass rearing is important, because females are more important as they are directly responsible for pest population regulation (Ode & Heinz 2002). In this rearing procedure, the female proportion was 41%, which was significantly lower than the maximum values of 70–75% of Ode and Heinz (2002) and Musundire et al. (2012). Production of *D. isaea* as a biological control agent is expensive, with a large and complex production system. For this reason, improvement of the female : male ratio in the population is an important quality management option. Ode and Heinz (2002) advocated the host–size-dependent sex ratio theory with high-quality, larger size host insect production using agromyzid leaf miners. Further, Musundire et al. (2012) described that *Liriomyza* species and *D. isaea* can be used for further larger scale field studies aimed at optimizing field releases and adapting mass rearing of *D. isaea* on different plant species. Field-collected populations are being added regularly for colony vigor. The cost of USD20.95 per 1000 parasitoids (recurring costs only) is much lower than current prices charged for leaf miner parasitoids in the Korean market. Some of the sources of *D. isaea* in Korea (e.g., Dongbu Ceres Co., Nonsan, Korea) currently charges USD44.00 for 250 parasitoids. By using our method or a modification if it, mass production of *D. isaea* in the greenhouse environment or even in the field could be possible, which could contribute to the biological control of leaf miner flies. More vigorous testing of the system could improve the productivity and quality leading to ecologically efficient and economically achievable management of invasive *Liriomyza* pests.

![Figure 3 Schematic of overall rearing procedures for *D. isaea* using faba bean as a host-plant and *L. huidobrensis* as the insect host. (Modification based on the training from International Potato Center (CIP), Lima, Peru).](image)

**Table 2** Cost estimates for the production of 4000 *Diglyphus isaea* per day using faba bean as the host-plant and *Liriomyza huidobrensis* as the insect host

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Unit cost (USD)</th>
<th>Total cost (USD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labor Technician (1 full time)</td>
<td>8 h</td>
<td>5.9</td>
</tr>
<tr>
<td>Materials Nonrecurring costs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pots</td>
<td>50</td>
<td>0.3</td>
</tr>
<tr>
<td>Aspirator</td>
<td>1</td>
<td>12.0</td>
</tr>
<tr>
<td>Test tubes</td>
<td>7</td>
<td>0.8</td>
</tr>
<tr>
<td>Vials</td>
<td>20</td>
<td>0.6</td>
</tr>
<tr>
<td>Vial holders</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>Analytical balance</td>
<td>1</td>
<td>1250.0</td>
</tr>
<tr>
<td>Trays</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>Cages</td>
<td>12</td>
<td>900</td>
</tr>
<tr>
<td>Tipping racks</td>
<td>4</td>
<td>350.0</td>
</tr>
<tr>
<td>Holding racks</td>
<td>4</td>
<td>150.0</td>
</tr>
<tr>
<td>Recurring costs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil mix</td>
<td>70 kg</td>
<td>1.3</td>
</tr>
<tr>
<td>Plants (seed)</td>
<td>150</td>
<td>0.10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Acknowledgements

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