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An Optimal Standardized *in vitro* Bioassay to Evaluate Susceptibility of Green Peach Aphid, *Myzus persicae* (Sulzer)(Insecta: Hemoptera: Aphididae), to Aphicides

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복숭아혹진딧물, *Myzus persicae* (Sulzer)(Insecta: Hemoptera: Aphididae), 살진딧물 최적 *in vitro* 살충력 검정 방법 확립

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ABSTRACT: Leaf-spray *in vitro* bioassays appraise new aphicidal formulations for managing deleterious plant-feeding aphids. The formulation may utilize alternative and integrated strategies. However, leaf spraying even under controlled conditions may affect aphid reproduction and mortality. This study examines leaf spray applications for optimum and reproducible aphicidal results using tobacco leaves overlaid on cotton fabric or water agar surfaces. Infestation of the undersides of tobacco leaves with nymphs of green peach aphids was used in the assays. Spray distance and volume were optimized using water-sensitive paper to ascertain the best surface coverage. Overlays of the leaves on water agar caused less mortality and greater reproduction than the use of cotton fabric. The relative humidity of the insect-rearing chambers changed with the watering regime for the insect - rearing chambers with cotton fabric; 60% relative humidity was optimal. Relative humidity was not affected by the concentration of agar in the water agar chambers. Applications of the chemical aphicidal standard, Sulfoxaflor, under the optimized conditions exhibited similar times for lethality although the rate was faster with leaves on the cotton fabric than on water agar. These studies establish reproducible and sensitive techniques for assessing the lethality and effects on reproduction of potential aphicidal products.

Key words: Aphid, aphicides, Leaf spraying assay, Cotton fabric, Water agar, Mortality, Reproduction

초 록: 진딧물 방제제 개발을 위해 *In vitro* 경엽살포 검정방법이 널리 사용되고 있다. 이러한 신소재 진딧물 방제 제형은 종합방제와 화학농약의 대안으로 많은 연구가 진행되고 있다. 하지만, 경엽살포 검정방법은 환경이 조절되는 실내에서도 진딧물의 증식과 살충에 영향을 받는다. 본 연구에서는 담배를 기주로 하여 솜과 한천방법을 이용하여 진딧물 방제제 검정을 위한 최적 경엽살포 확립하고자 하였다. 진딧물 검정 챔버에 솜과 한천을 넣은 후 담배 잎과 진딧물 3-4령 약충을 접종하였다. Water-sensitive paper를 이용하여 경엽살포 시에 가장 표면 피복이 높은 최적 경엽살포 거리와 살포량을 확립하였다. 대조구로 물을 처리한 구에서 한천 방법이 솜 방법에 비해 살충율이 낮고, 증식율이 높았다. 솜 검정 방법에는 곤충 검정 챔버의 상대습도를 60% 이상 유지시켰을 때 가장 최적 조건이었지만, 한천 검정 방법에서는 한천의 농도에 상대습도 차이가 없었다. 최적화된 조건하에서 대조화학 농약, Sulfoxaflor, 경엽살포 시 솜 방법에서 살충율이 한천방법보다 빨랐지만, 최종 살충율은 통계적으로 유의하지 않았다. 본 연구는 살진딧물 물질을 검정 시 재현성과 활용성이 가능한 최적화된 증식율과 살충율 검정 조건을 제시하였다.

검색어: 진딧물, 살진딧물제, 경엽살포법, 솜, 한천, 살충율, 증식율

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Myzus persicae (Sulzer; Insecta: Hemoptera: Aphididae), or the green peach aphid, is a notorious pest detrimental to crops worldwide (Capinera, 2001), transmitting viral diseases (Ng and Perry, 2004) and allowing the onset of sooty mold disease on plant hosts through their honeydew secretions (Capinera, 2001). Unfortunately, current chemical aphicides for controlling green peach aphids are thwarted by progressing insecticide resistance (Bass et al., 2014; Silva et al., 2012a, 2012b). Consequently, botanical extracts are being explored as a promising alternative and integrated approaches (El-Wakeil, 2013). However, despite the plethora of potential botanical insecticide publications, insight concerning commercial-level use is limited (Isman and Grieneisen, 2014).

Temperature and relative humidity are vital for *in vitro* aphid rearing (Mittler and Dadd, 1962; Mohammed and Hatcher, 2016; Norman and Sutton, 1967; Özder and Saglam, 2013; Venkanna and Suroshe, 2023). For example, nymphs and wingless adults are abundant at 24°C, whereas 27°C yields winged adults (Venkanna and Suroshe, 2023). Approximate optimal conditions are reportedly 25°C and 52-80% relative humidity (Mittler and Dadd, 1962; Mohammed and Hatcher, 2016; Norman and Sutton, 1967; Özder and Saglam, 2013; Venkanna and Suroshe, 2023). Specifically, Davis et al. (2006) cite 26.7°C as optimal for green peach aphid proliferation. The life cycle for this aphid is achieved in ten days at 15°C, with faster reproduction within 5-8 days at higher temperatures, 20-30°C (Kim et al., 2012).

Bioassays for aphicidal formulations frequently use spray or dip aphid-infested leaves (Chandrasena et al., 2011; Dreyer et al., 1981; Erdos et al., 2020; Hesketh et al., 2008; Liu and Stansly, 1995; Paramasivam and Selvi, 2017; Sharma et al., 2005; Vandenberg, 1996; Wattier et al., 2019). However, numerous studies report extensive aphid mortality, even by applications of water as a control (Kim et al., 2009). The studies also overlook the impact on aphid reproduction. Moreover, leaf spray method descriptions often lack details (Hesketh et al., 2008). Our previous works identified dibutyl succinate from entomopathogenic *Isaria javanica* fungi and hydrogen cyanide from probiotic, root-colonizing *Pseudomonas chlororaphis* O6 bacteria (Kang et al., 2019; Lee et al., 2019) fulfilling aphicidal activities. However, during these studies we noted inconsistencies in treatments and control sprays regarding aphid mortality and reproduction.

This paper elucidates optimized and reproducible aphicidal bioassay methodologies. We examined the efficacy of spray applications through examining effects of distance and volume. Water-sensitive paper (WSP) coated with a bromophenol blue indicator was used to determine optimal coverage (Cunha et al., 2012); Because the dye in the paper changed color when wetted, the extent of coverage was quantified for its area as discussed (Cerruto et al., 2019; Zhu et al., 2011).

Aphids possess both sexual and asexual life cycles (Ogawa and Miura, 2014; Simon et al., 2002). Our studies involved working at 21 ± 2°C, a temperature too low for winged adults to emerge, and with bioassay conditions that only allowed asexual reproduction (Venkanna and Suroshe, 2023). We evaluated impacts of relative humidity in insect-rearing chambers with nymph-infested tobacco leaf tissue or agar surfaces by varying the water added to the cotton fabric or the percent agar in the agar method.

The standard aphicide Sulfoxaflor's efficacy was used in two methods to explore its aphicidal potential by studies of the extent and timing of lethality. Sulfoxaflor is an agonist to insect nicotinic acetylcholine receptors (nAChRs) with high control efficacy against a broad spectrum of sap-feeding insects, including green peach aphids (Li et al., 2021). Sulfoxaflor has proven lethal for aphids displaying resistance to neonicotinoids and other insecticides (Sparks et al., 2013) although resistance to this chemical is observed. The standard chemical insecticide Strait^{TM}, containing 7% Sulfoxaflor, is recommended at a 2,000-fold dilution in water as the optimal 35 ppm dose. In field analyses, Sulfoxaflor displays a wide range, 2.53 to 113.93 ppm LC50 for effective dose (Li et al., 2021). Our results confirm that Sulfoxaflor had aphicidal activity in both of the methods for insect rearing although there were differences in the rate at which lethality occurred.

Materials and Methods

Leaf coverage efficacy from a vertical spray

Water-sensitive paper (TeeJet, 52 × 76 mm, Spraying Systems Co., Switzerland) was cut into 3.5 × 3.5 cm squares and placed onto two cotton fabric or agar layers in insect breeding

dishes. Sterile water samples were sprayed as 0.5 ml or 1 mL mists onto the paper surface for 30 seconds from 20 or 30 cm, using mini 2 mL glass bottle atomizers (5.35 × 3.62 × 3.19 inches; Brand Csdtylh 0167, Amazon, USA). After paper imaging, the Snap Card app (Department of Agriculture and Food, Western Australia) measured the extent of the color change to provide quantification of spray coverage (%). The experiment was repeated three times, with three replications per experiment.

Green peach aphid culture

Green peach aphids, provided by Dr. Duck Soo Choi from the Jeonnam Agricultural Research and Extension Services (Naju, South Korea), were maintained on tobacco (*Nicotiana tabacum* L. 'Xanthi') seedlings grown over four weeks under 40 W fluorescent lights (2000 lux, 80 µmol photons/m^{-2} s^{-1}) with a 16-hour light to 8-hour dark photoperiod. Plants were grown at 25 ± 3°C with a relative 50-60% humidity. Twenty adult apterous aphids were transferred to each four-week-old young tobacco leaves to obtain nymphs. Infested tobacco plants were placed into cages for five days to generate third- or fourth-stage nymphs (lengths > 1 mm) used in the bioassays. A stereoscopic microscope (Leica DFC 295, Leica Biosystems Nussloch GmbH 2022, Germany) using LAS version 4.13 software was used to measure insect sizes during the aphid's developmental stages on the tobacco leaves.

Cotton fabric method

One circle (9 × 9 cm) of quilting cotton fabric (2 oz thick, Happy Sewing Co., Seoul, Korea) was placed at the bottom of the insect breeding dish (10 × 4.5 cm, SPL Life Science Co., Pocheon, Korea) and wetted with sterile distilled water. Tobacco leaves from four-week-old plants were cut into 4.5 × 4.5 cm squares and centered with the underside up on the intact cotton circle. A second 9 × 9 cm circle had a 3 × 3 cm rectangular holes cut from its center before being placed over the leaf to standardize the sprayed tobacco leaf area (Fig. 1). Subsequently, sterile water was added to the cotton cloth at 2, 4, or 6 mL, daily to induce different relative humidity in the dishes. A Smart Wi-Fi Temperature Humidity Sensor (Model GKW-TH251, Hei Home, Seoul, Korea) was used to measure the dishes' relative humidity. Breeding dishes were incubated for five days under 40 W fluorescent lights (70 µmol photons/m^{-2} s^{-1}) with a 14-h light to 10-h dark cycle at 21 ± 2°C.

Agar method

The effect of different concentrations of agar, 0.5%, 0.8%, or 1.5% (w/v) agar, (Junsei, Japan) was examined in an attempt to generate water agar dishes with different relative humidities. The agar was sterilized through autoclaving at 121°C for 15 minutes before transfer of 10 mL aliquots to each well of the insect breeding dish (5.0 × 1.5 cm, SPL, Korea). Circles (5 cm diameter) were cut from leaves of four-week tobacco plants and these were transferred underside up to the agar for complete agar surface coverage (Fig. 1). Smart Wi-Fi Temperature Humidity Sensors measured the relative humidity. Breeding dishes were incubated for five days under 40 W fluorescent lights (70 µmol photons/m^{-2} s^{-1}) with a 14-h light to 10-h dark cycle at 21 ± 2°C.

Aphid reproduction and mortality when sprayed with water as a control

Tobacco leaves resting atop cotton fabric or agar were infested with 20 third/fourth stage nymphs using a soft brush by their transfer from previously infested leaves. Surfaces were sprayed with 1 mL of sterile water from a 20 cm vertical position for 30 seconds. Breeding dishes were incubated for five days under 40 W fluorescent lights (70 µmol photons/m^{-2} s^{-1}) with a 14-h light to 10-h dark cycle at 21 ± 2°C. This duration allowed the nymphs to mature to wingless adults and the birth of new nymphs on the leaf surface. The following formulation calculated how treatment affected reproduction:

Reproduction (%) = (Insect quantity on specific day/initial third or fourth stage nymphs deposited (i.e., 20) for each leaf) × 100

Mortality was calculated from (B-A/B) × 100, where A is the total aphid number before treatment and B is number of the live aphids after treatment. Nymphs/adult viability was mea-

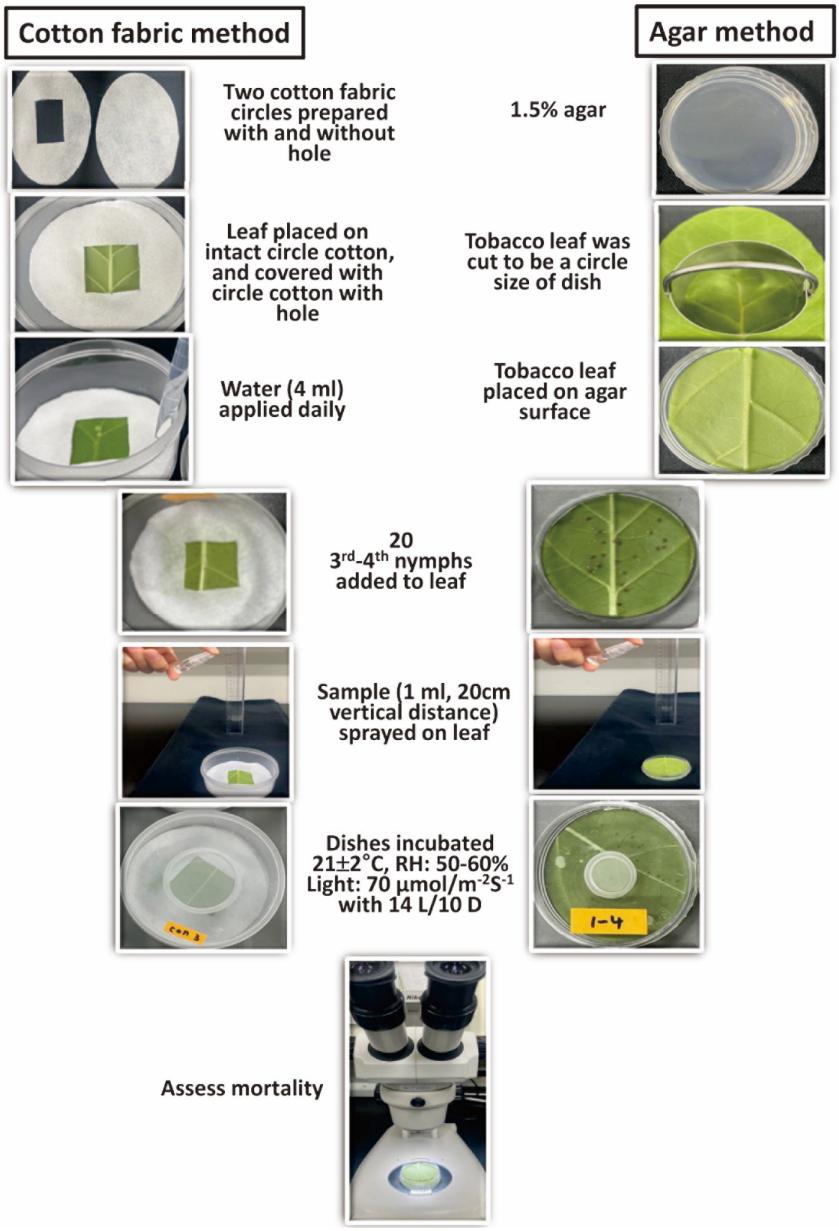

Fig. 1. Optimal *in vitro* bioassay procedures for assessing green peach aphicidal efficacy. An optimal 21 ± 2°C and a 50%-60% relative humidity was maintained for five days. Insect viability was measured under stereoscopic microscopy; gentle prodding with a fine brush at defined times classified insects without movement as dead.

sured under a stereoscopic microscope (Model C-LEDS, Nikon Imaging Japan Inc., Japan) with a fine brush for prodding at defined times and classifying insects without response as dead.

Optimized *in vitro* bioassays with Sulfoxaflor, a chemical standard aphicide

Sprays (1 mL) of 2,000 × diluted commercial Strait^{TM} with 7% Sulfoxaflor (35 ppm when applied; Dongband Agro Co. Seoul, Korea) or 1 mL of sterile water as the control were applied to the leaves in the dishes to compare how the assay method affected aphicide efficacy. The water agar dishes contained 1.5% agar and the cotton fabric dishes were amended with 4 ml water daily. The sprays were applied for 20 ± 2 times within 30 seconds from a 20 cm vertical height.

M. persici mortality was measured at defined times (3, 6, 9,

12, 24, 27, 30, 33, 36, 48, 51, 54, 57, 60, and 72 h) after spray treatments and calculated aphid mortality. Mortality correction, i.e., mortality caused by the insecticide, was determined by Abbott's formula (T-C/100-C) × 100, where T was mortality from the insecticide, and C was mortality within the control (Abbott, 1925). Sulfoxaflor's median lethal time (LT) for 50% or 90% losses (LT50 and LT90) were calculated using a complementary log-log model (Finney and Stevens, 1948). Survival analysis was completed with the Kaplan-Meier Survival Analysis Log-Rank (Goel et al., 2010; Kaplan and Meier, 1958). Each experiment was repeated three times with three replicates.

Data analyses

All experiments were independently performed three times each with three replicates per treatment. Data were analyzed through ANOVA ($P < 0.05$) using SPSS (version 23, SPSS Inc., Chicago, IL, USA). Duncan's multiple range test ($P < 0.05$) further elucidated variations between measurements if the F test revealed significant differences. Similarly, Tukey's post-test compared LT50 and LT90 values between methods when the variance analysis results were significant at a 95% confidence level using SPSS. The LT50 and LT90 were also assessed through Probit analysis with SPSS. Survival was evaluated with the Kaplan-Meier Survival Analysis Log-Rank, and ANOVA compared median and maximum survival rates using SPSS.

Results and Discussion

Spray optimization

Water-sensitive paper coverage was examined by applying volumes (1 or 2 mL samples) from defined vertical distances, 20 or 30 cm. The variation in color change of the papers is shown in Fig. 1. Table 1 displays the effect of height and volume on surface coverage. A 1 mL sample sprayed from 20 cm exhibited the highest coverage. Less coverage occurred with the 30 cm and the lower application volume 0.5 mL (Table 1 and Supplemental Fig. 1).

Table 1. Spray distance and volume effects on surface coverage

Distance (cm), amount (mL)	Coverage (%)*	
	Cotton fabric	Agar
30 cm, 1.0 mL	52.3 ± 14.6 b	76.6 ± 6.7 b
30 cm, 0.5 mL	19.3 ± 10.8 c	24.5 ± 2.3 d
20 cm, 1.5 mL	86.5 ± 2.1 a	87.9 ± 1.9 a
20 cm, 1.0 mL	84.6 ± 1.2 a	84.2 ± 0.9 a
20 cm, 0.5 mL	45.7 ± 3.2 b	44.6 ± 2.4 c

*Spray coverage was determined using water-sensitive paper. Data represent the means and standard error from three independent experiments each with three replicates. One-way analysis of variance (ANOVA; $P < 0.01$ was considered significant) analyzed data with IBM SPSS software version 23 (IBM Corp., Armonk, NY, USA); Duncan's multiple range test ($P < 0.01$) further compared differences if the F test was significant. Letters indicate statistical differences between spray distance and amount combinations in each method.

Nymph rearing for in vitro bioassay

Our study established that green peach aphid life cycles on tobacco leaves involved four stages with maturation to adult from the first nymph stage requiring 7 d. The nymphs increased in length at each stage (Supplemental Fig. 2). After nine days, apterous female adults birthed 20-40 first-stage nymphs, completing an asexual cycle (Supplemental Fig. 2). The third or fourth stage nymphs were produced by transfer of the female aphids after 5 d.

Relative humidity optimization in insect breeding dishes

Relative humidity (RH) within breeding dishes potentially influences green peach aphid reproduction and mortality. RHs in dishes containing cotton fabric differed by daily water addition volume. RHs ranged between 49 to 64% with a 1 ml or 2 mL daily addition, 65 to 71% from 3 or 4 mL, and 74 to 77% from 5 or 6 mL (Supplementary Table 1). The application of 5 to 6 ml submerged some of the aphids. For water agar dishes, the air space above the agar ranged between 62 to 70% RH with no significant difference due to agar content (Supplementary Table 1).

Water spray effects on aphid mortality and reproduction: comparing insect rearing conditions

The optimal *in vitro* bioassays must have minimal effects on aphid mortality and reproduction in the control treatments. To explore these consequences, we utilized the optimal spray conditions: 1 mL volume, 20 ± 2 times for 30-second spray, a 20 cm vertical height, of sterile water. Our results indicated that there was higher mortality and less reproduction in the cotton fabric method than the agar method (Fig. 2). For the

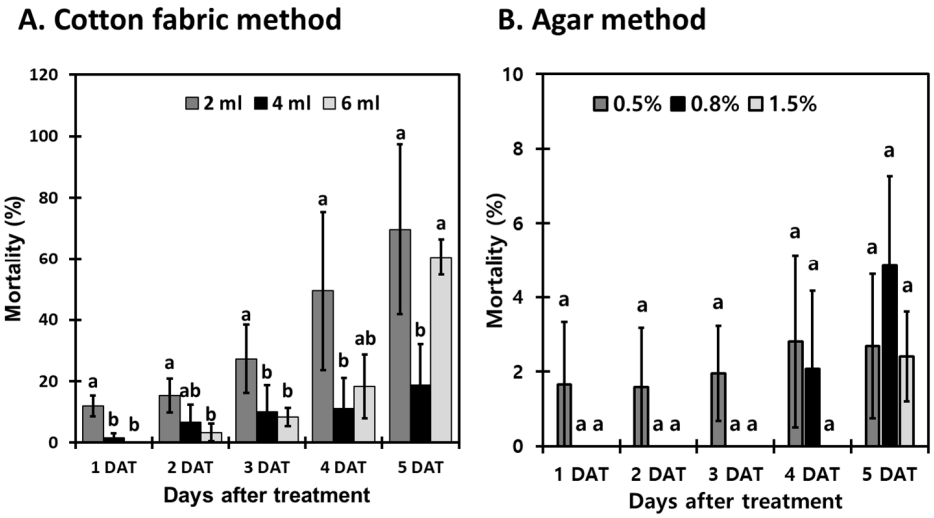

Fig. 2. Water spray effects on aphid mortality. Optimized conditions for spraying (1 mL sterile water over 30 seconds from 20 cm) onto infested leaves (20 nymphs per leaf) were used. Mortality was classified as insects without movement after a gentle prod as dead daily for 5 d after spraying. Data represent three independent experiments means and standard error with three replicates each. One-way analysis of variance (ANOVA; $P < 0.05$ was considered significant) analyzed data using IBM SPSS software version 23 (IBM Corp., Armonk, NY, USA); Duncan's multiple range test ($P < 0.05$) further compared differences if the F test was significant, and lowercase letters indicate statistical differences.

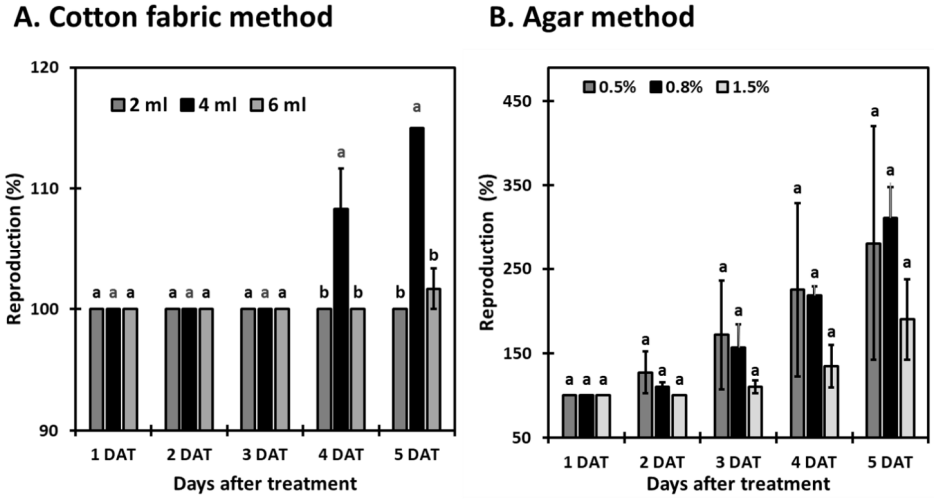

Fig. 3. Water spray effects on green peach aphid reproduction. Optimized conditions for spraying (1 mL sterile water over 30 seconds from 20 cm) onto infested leaves (20 nymphs per leaf) were used. Reproduction was assessed for five days post-treatment with the infested leaves. Reproduction (%) = (Insect quantity on specific day/initial third or fourth stage nymphs deposited (i.e., 20) for each leaf) × 100. Thus, 100% conveys no change in insect number. Data represent three independent experiments' means and standard error. One-way analysis of variance (ANOVA; $P < 0.05$ was considered significant) analyzed data using the IBM SPSS software version 23 (IBM Corp., Armonk, NY, USA); Duncan's multiple range test ($P < 0.05$) further compared differences if the F test was significant, and lowercase letters indicate statistical differences.

cotton fabric method, mortality varied with daily water volume; adding 4 mL water/dish caused least mortality, approximately 20% mortality (Fig. 2B). Mortality was independent of agar concentration in the agar method and was about 5% at 5 d after the water spray application (Fig. 2B).

We theorize that the cotton fabric method's high mortality resulted from unfavorable RH conditions due to 2 ml or 6 mL daily water supplements; with 6 mL aliquots, aphids were submerged in water. Our results substantiate that the optimum RH green peach aphid nymph development was achieved in the 60 to 70% range using 4 mL applications daily. Reproduction also differed between methods. Both adults and nymphs were counted regardless of their live or dead status with increases in insect numbers only occurring with 4 mL supplementation (Fig. 3A). High reproduction, three-fold was observed on water agar dishes independent of agar concentration (Fig. 3B).

Testing Sulfoxaflor's aphicidal potential

Sulfoxaflor's insecticidal effect on green peach aphids was assessed using the optimal spray application conditions (Supplemental Table 2) and compared between the cotton fabric and agar methods. Simultaneous studies with duplicate dishes confirmed higher and accelerated mortality in the cotton fabric than in the agar method. Sulfoxaflor exhibited a 100% nymph mortality in both assays within 72 h post-application. Although the extent of mortality was identical between methods after 48 h, mortality occurred more rapidly in the cotton fabric dishes (LT50=18.4 h) than with the agar method (LT50 29.6 h; Table 2). However, similar LT90s were obtained, approximately 53 h for the cotton fabric dishes and 54 h for the agar method (Table 2). The Kaplan-Meier survival analysis (Fig. 4) confirmed that the time of lethality significantly differed ($P < 0.001$) differed with the control water sprays, but there was no significant difference ($P = 0.270$) existed between methods for Sulfoxaflor treatments (Fig. 4). Sulfloxaflor's LT50 and LT90 were similar between methods, as evidenced by probit and Kaplan-Meier survival analyses (Table 2 and Fig. 4).

In conclusion, we established an optimal spray application method for full tobacco leaf coverage. Our assays comprised tobacco leaf infestation with third- or fourth-stage green peach aphid nymphs, at 21-24°C to restrict winged aphid formation, and only allowed asexual reproduction. Our water spray control confirmed that a 60-70% RH supported optimal aphid health (mortality and reproduction) in both *in vitro* bioassay methods; however, the cotton fabric method exhibited heightened aphid mortality and lower reproduction compared with the agar method. Although the agar incubation dishes displayed more substantial aphid reproduction and less mortality than the wet cotton fabric, the commercial aphicide Sulfoxaflor expressed similar aphicidal effects within a two-day application window. Compared to the agar method, Sulfoxaflor treatments with the

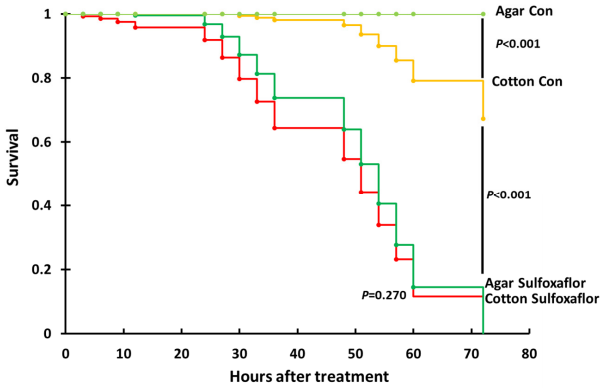

Fig. 4. Kaplan-Meier survival plots of green peach aphids (n = 90) treated with sterile water (control) versus Sulfoxaflor. Green peach aphid mortalities were monitored at defined hours post-treatment. Data points represent the average of three experiments with three replicates each. One representative data set is shown. *P* values indicate significant differences between treatments as determined by the Log-Rank test.

Table 2. Probit analysis of in vitro cotton fabric- and agar-bioassays to estimate Sulfoxaflor's LT50 and LT90 on green peach aphid nymphs

Method	LT50 (95% CL)	LT90 (95% CL)	Regression line and coefficient
Cotton fabric	18.4 h (14.0-22.7)	52.7 h (41.1-76.4)	Y=2.6776x-3.2812, R^2=0.8932
Agar	29.6 h (28.3-30.9)	54.1 h (51.2-52.7)	Y=4.7903x-6.972, R^2=0.9671

The regression line equation reflects the relationship between aphicidal activity and the 2,000-fold diluted Sulfoxaflor treatment. LT50 and LT90 convey lethality times for 50 and 90% loss, and the 95% Confidence limit (CL) demonstrates a 95% overall parameter in this range.

cotton fabric method demonstrated accelerated lethality, potentially due to this environment being more stressful. This study revealed potential processes that hinder reliable bioassay methods for aphicides and provided standardization. Our standardized optimal method, such as the agar insect-rearing dishes, will permit a more reliable assessment for developing aphicides from botanical extracts.

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Statement for Authorship Position & Contribution

Cho, K.H: Chonnam National University, Student in MS, Involved in experiment implementation, statistical analysis, and wrote the first draft.

Kim, H.J: Jeollanamdo Agricultural Research & Extension Services, Researcher; Involved in experiment conception and design, aphid preparation, statistical analysis, and wrote the first draft.

Kim, Y.C: Chonnam National University, Professor; Involved in experiment conception and design, result analysis and interpretation, manuscript preparation, and financial grant acquisition.

All authors read and approved the manuscript.

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