Effect of the Orientation of the Leaf Surface on the Growth, Development, and Reproduction of Three Populations of the Pea Aphid (Hemiptera: Aphididae)[1]

Li-Juan Wu, Jing-Wen Li, Chang Wang, Meng Zhang, Xiang-Shun Hu[2], and Tong-Xian Liu[2]

State Key Laboratory for Crop Stress Biology in Arid Areas, Key Laboratory of Crop Pest Management on the Northwest Loess Plateau, Ministry of Agriculture, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China

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Abstract The leaf disc method is widely used in laboratory studies of the pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae). We examined the effects of the orientation of the leaf discs (upright, inverted, and horizontally perpendicular to the surface) on the biological parameters of aphids from three populations (i.e., Gansu red, Gansu green, and Yunnan green) of *A. pisum*. We found that aphids feeding on inverted or horizontally perpendicular positioned leaf discs exhibited higher survival and fecundity rates than those aphids feeding on upright positioned leaf discs for all three populations. Overall, aphids in both the Gansu red and Gansu green populations performed best when feeding on the inverted leaf discs, whereas aphids of the Yunnan green population performed best in the horizontally perpendicular orientation. We postulate this may be related to body temperature adjustment by the aphids through adjusting the orientation angle between its body and the sun. Our results provide a theoretical basis for optimizing the rearing parameters of the pea aphid, reducing the systematic error of experiments, and facilitating the acquisition of more reliable data under laboratory conditions.

Key Words pea aphids, leaf disc, leaf disc orientation, feeding positions

The pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), causes economic losses to plants by sucking fluids from the host plant phloem, vectoring plant viruses, and secreting honeydew that impairs plant photosynthesis (Brault et al. 2010, Deshoux et al. 2020). *Acyrthosiphon pisum* also is a widely used biological model for studies of insect–plant interactions, symbiosis, and virus vectoring. Its complete genome sequence has been determined and reported by the International Aphid Genomics Consortium (2010).

Common rearing methods of *A. pisum* under laboratory conditions include maintaining aphids on living plants, artificial diet, or fresh in vitro leaf tissue (e.g., leaf disc) in petri dishes or other containers. Maintaining aphids on living plants is the best method in the scientific research; however, the method requires large working spaces in the laboratory or greenhouse which can be inconvenient and

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[2]Corresponding authors: X.-S. Hu (huxiangshun@163.com); T.-X. Liu (txliu@nwafu.edu.cn).

inefficient. Furthermore, observing, monitoring, collecting, and sampling aphids on whole plants is difficult. Living plants, therefore, are primarily used for aphid population preservation and colony maintenance.

Auclair and Cartier (1963) first reported the use of an artificial diet medium to rear aphids in the laboratory. Artificial diets can be prepared at any time to rear large numbers of insects that are readily available for experimentation. Use of artificial diets for rearing avoids the limitation imposed by the growth and development of the living host plants. Yet, population degradation cannot be avoided for aphids feeding on the artificial diet (Tellez et al. 2009).

The leaf disc method has many advantages for laboratory studies, including ease of operation, low cost, and ease of observation of aphids. Previous research has shown no significant difference in fecundity, longevity, body size, and developmental time between aphids reared using the leaf disc and those reared on living plants (Li and Akimoto 2018). The leaf disc method has been widely used in studies involving insect toxicology, physiology, biochemistry, ecology, molecular biology, and pesticide screening. However, the orientation of the leaf discs with respect to the surface is not clarified in most of the reported studies (Blande et al. 2004, Chen et al. 2017, Etheridge et al. 2019, Li and Akimoto 2021, Mbaluto et al. 2021, Xu et al. 2021). We observed that the orientation of the leaf disc could affect the life-history characteristics of aphids. We undertook this study to determine the effect of the orientation of leaf discs on the physiological and ecological parameters of *A. pisum* in the laboratory. Our results could provide a theoretical basis for optimizing *A. pisum* rearing parameters, reducing the systematic errors of experiments, and facilitating the acquisition of more reliable data in the laboratory.

Materials and Methods

Pea aphids. Three populations of *A. pisum* were initially collected from field-grown plants. Two populations were collected from Lanzhou, Gansu, China (N 36°6′36″, E 103°42′0″), and were named “Gansu red” and “Gansu green” because of the characteristic coloration of the aphids. Aphids of the third population were characterized by small-sized green bodies and were collected from Yuxi, Yunnan, China (N 24°21′0″, E 102°32′24″), and aptly named “Yunnan green.” The three populations were maintained separately on seedlings of *Vicia faba* (L.) (cultivar ‘Jinnong’) for more than 10 generations in an environmentally controlled chamber (GZP-250A, Nanjing Hengyu Instrument Manufacturing Limited Co., Suzhou, Jiangsu, China) housed in the Key Laboratory of Applied Entomology, Northwest A&F University, at 18 ± 0.5°C and 70 ± 5% relative humidity under a 14:10 h L/D photoperiod regime.

Experimental design. Our assays were conducted in 24-well plates (well diameter 15.6 mm). An 8-mm-diameter hole was punched through the cover of each well for air exchange, and the hole was covered with a gauze screen to prevent aphid escape. To maintain freshness of leaf discs, 600 μl of 1.0% agar was added to each well in the plates and allowed to cool and harden into a gel. Leaf discs (diameter 15 mm) were cut from leaves excised from *V. faba* seedlings using a manual punch, and then placed individually on the agar surface with the upper leaf

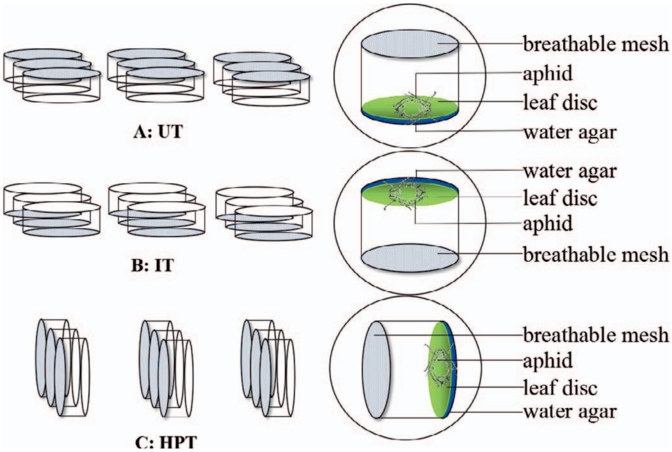

Fig. 1. Orientation of the abaxial surface of leaf discs: upright orientation treatment (UT) (A); inverted orientation treatment (IT) (B); and horizontally perpendicular orientation treatment (HPT) (C).

surface in contact with the agar in each well. Each leaf disc was pressed gently using a brush to ensure contact with the agar surface.

The experiment was a two-factor design. One factor was the orientation of the leaf surface (upright, inverted, and horizontally perpendicular). The other factor was the three pea aphid populations (Gansu red population, Gansu green population, and Yunnan green population). A total of nine treatments were composed. Each treatment used two 24-well plates, each well with one leaf disc, and only one aphid admitted to feeding on the leaf disc in one well (total of 48 aphids per treatment). The life-history traits of each aphid were recorded every day during the course of observations.

In the upright orientation treatment, the openings of the well plates were facing upward so that the leaf abaxial surface was also facing up; therefore, with the aphids feeding on the abaxial surface, the aphids were feeding with the ventral surface facing down and the dorsum facing up (Fig. 1A). In the inverted orientation treatment, the openings of the well plates were inverted so that the leaf abaxial surface was facing downward, and the ventral surface of the aphids was facing upward as the aphids fed on the leaf disc abaxial surface (Fig. 1B). In the horizontally perpendicular orientation treatment, the openings of the well plates were oriented horizontally perpendicular to the lab surface so that the leaf abaxial surface was also horizontally perpendicular to the surface. The ventral surface of the aphid, therefore, was also facing horizontally perpendicular to the surface when feeding on the leaf abaxial surface (Fig. 1C).

First-instar aphids ($<$24 h old) were randomly selected from the appropriate colony, weighed using a microbalance (Sartorious MSA 3.6P-000-DM, Gottingen, Germany), and placed individually onto a leaf disc in a well. Aphids were observed daily. Upon molting into adults, the aphids were weighed and development time recorded. The number of nymphs produced by each resulting adult aphid was recorded, and the newborn nymphs were removed immediately. Aphids were monitored until death. The leaf discs were replaced every 2 d.

Statistical analysis. Life-history data from each treatment were analyzed using the paired bootstrap test of TWOSEX-MSChar (V2018.05.04). The age-stage survival rate (S_{xj}) was calculated based on the age-stage-structure matrix. The formulae for the parameters were calculated as follows:

$$\text{Net rate of reproduction: } R_0 = \sum l_x m_x;$$

$$\text{Mean generation time: } T = \frac{\sum x l_x m_x}{\sum l_x m_x} = \frac{\sum x l_x m_x}{R_0};$$

$$\text{Intrinsic rate of increase: } r_m = \frac{\ln R_0}{T};$$

$$\text{Finite rate of increase: } \lambda = e^{r_m},$$

where x is the age of aphid in days, m_x is the mean number of nymphs produced by the aphids at an age of x days, and l_x is the survival rate of the aphids at an age of x days (Chi and Su 2006, Chi et al. 2019, Huang et al. 2018).

The variance and significance of difference in the longevity, weight gain, and fecundity of the three pea aphid populations reared under the three different orientations of the leaf discs were analyzed using one-way analysis of variance, followed by Tukey's test, at a significance level of $P < 0.05$. All statistical analyses were performed using the IBM SPSS Statistics package 19 (SPSS Inc., Chicago, IL). The line/scatter diagrams were plotted using the SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA).

Results

Aphid survivorship. Plots of percentage survival over time showed that survivorship of the three aphid populations did not differ among the three orientation treatments for the first 20 d (Fig. 2). Survivorship in all three populations decreased after the 20th day. In the Gansu red and Gansu green populations, the decline in survivorship occurred later in the inverted orientation treatment than in the upright and horizontally perpendicular treatments (Fig. 2A, B). Although percentage survival also declined after the 20th day in the Yunnan green population, leaf and aphid orientation had no effect on extending survivorship (Fig. 2C).

Aphid longevity, weight gain, and fecundity. Aphid longevity, weight gain, and fecundity differed significantly among the three populations (longevity: $F = 38.33$; df $= 2, 423$; $P < 0.001$; weight gain: $F = 8.52$; df $= 2, 423$; $P < 0.001$; fecundity: $F = 9.41$; df $= 2, 423$; $P < 0.001$) and among the three orientation treatments (longevity: $F = 49.84$; df $= 2, 423$; $P < 0.001$; weight gain: $F = 51.14$; df $= 2, 423$; $P < 0.001$; fecundity: $F = 9.41$; df $= 2, 423$; $P < 0.001$). The statistical analysis also detected significant interactions of population $\times$ orientation (longevity: $F = 4.80$; df $= 4, 423$; $P < 0.001$; weight gain: $F = 1.48$; df $= 4, 423$; $P = 0.21$; fecundity: $F = 5.46$; df $= 4, 423$; $P < 0.001$). While there were significant differences in these life-history parameters

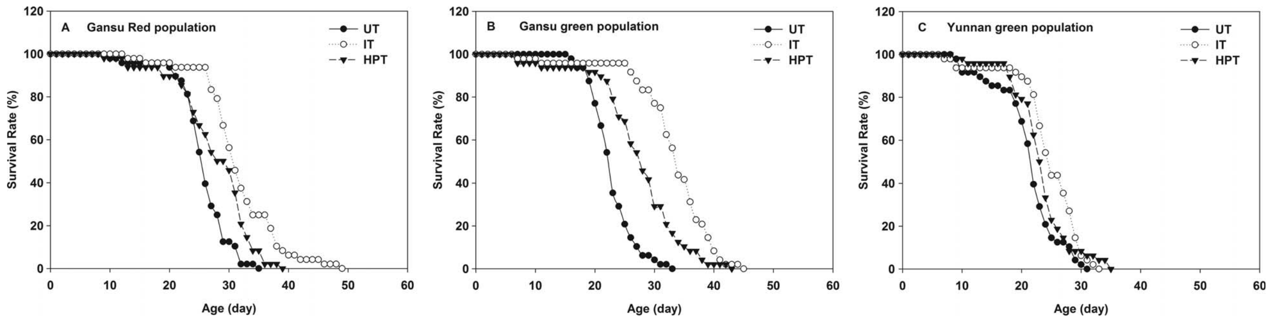

Fig. 2. Survivorship of Gansu red (A), Gansu green (B), and Yunnan green (C) populations of *A. pisum* in response to upright orientation (UT), inverted orientation (IT), and horizontally perpendicular (HPT) orientation of the abaxial surface of leaf discs.

among the three populations, our objective remained with determining impacts of leaf and aphid orientation; therefore, our focus was on response to orientation.

Longevity was greatest in aphids that fed on inverted leaf discs in each of the three populations (Table 1). Mean (± SE) longevity in the inverted treatment in the Gansu red (31.83 ± 0.92 d) and Gansu green (33.40 ± 0.98 d) populations was significantly higher than in either the upright or horizontally perpendicular treatments. Longevity in the Yunnan green population was significantly greater in the inverted treatment (24.81 ± 0.78 d) than in the upright treatment (21.38 ± 0.74 d) but did not differ significantly from the horizontally perpendicular treatment (23.42 ± 0.70 d) (Table 1).

Leaf orientation had little to no significant effect on mean weight gain within each population (Table 1). The greatest overall weight gain (1.59 ± 0.07 mg) was recorded with Gansu red aphids in the horizontally perpendicular treatment, which was not significantly different from the inverted treatment (1.56 ± 0.08 mg) but significantly different from the upright treatment (1.27 ± 0.06 mg). The least weight gain (1.16 ± 0.07 mg) was recorded in the Yunnan green population with the upright treatment, but this mean weight gain did not differ from those of the Yunnan green aphids in the inverted or horizontally perpendicular treatments. No significant differences were observed among the orientation treatments in the Gansu green population.

Fecundity differed significantly among the orientation treatments within each population (Table 1). In the Gansu red and Gansu green populations, the mean (± SE) number of offspring produced per adult was greatest in the inverted treatment (Gansu red = 53.50 ± 2.03; Gansu green = 62.17 ± 2.77) followed by the horizontally perpendicular (Gansu red = 46.31 ± 2.95; Gansu green = 47.23 ± 2.92) and then the upright (Gansu red = 31.88 ± 2.03; Gansu green = 30.06 ± 1.23) treatments. In contrast, the fecundity of aphids from the Yunnan green population was significantly greater in the horizontally perpendicular treatment (61.77 ± 2.92) than in the upright treatment (43.54 ± 3.00), but did not differ significantly from the inverted treatment (54.42 ± 3.38). The upright and inverted treatments also did not differ significantly.

Aphid life table parameters. The intrinsic rate of increase (r_m) of the Gansu red and Yunnan green aphid populations was greatest in the inverted (Gansu red = 0.273 ± 0.005 d^{-1}; Yunnan green = 0.325 ± 0.005 d^{-1}) and horizontally perpendicular (Gansu red = 0.273 ± 0.006 d^{-1}; Yunnan green = 0.329 ± 0.008 d^{-1}) treatments with values that were significantly greater than that of the upright (Gansu red = 0.256 ± 0.006 d^{-1}; Yunnan green = 0.306 ± 0.005 d^{-1}) treatment (Table 2). Similarly, the net reproduction rate (R_0) was highest in the inverted and horizontally perpendicular treatments in the Gansu red and Yunnan green populations (Table 2). The R_0 values in the Gansu green population showed statistical differences among the treatments as inverted > horizontally perpendicular > upright.

The finite rate of increase (λ) values ranged from 1.292 ± 0.008 d^{-1} in the upright orientation of the Gansu red aphids to 1.389 ± 0.011 d^{-1} in the horizontally perpendicular treatment of the Yunnan green population (Table 2). The statistical differences among the treatment means in each population were basically the same sequence as observed with r_m and R_0 values. Within the Gansu red and Yunnan green populations, λ values were statistically higher in the inverted and horizontally

Table 1. Mean (± SE) longevity, weight gain, and fecundity of *A. pisum* aphids from three populations feeding leaf discs in different spatial orientations.*

		Leaf Disc Orientation			Statistics	
Parameter	Aphid Population**	Upright	Inverted	Horizontally Perpendicular	*F*	*P*
Longevity (d)	GR	25.73 ± 0.64b	31.83 ± 0.92a	27.85 ± 0.91b	13.86	<0.001
	GG	23.02 ± 0.51c	33.40 ± 0.98a	27.60 ± 1.03b	35.61	<0.001
	YG	21.38 ± 0.74b	24.81 ± 0.78a	23.42 ± 0.70ab	5.46	0.005
Weight gain (mg)	GR	1.27 ± 0.06b	1.56 ± 0.08a	1.59 ± 0.07a	6.11	0.003
	GG	1.40 ± 0.05a	1.55 ± 0.06a	1.40 ± 0.08a	1.91	0.152
	YG	1.16 ± 0.07a	1.37 ± 0.07a	1.26 ± 0.05a	2.46	0.089
Fecundity†	GR	31.88 ± 2.03c	53.50 ± 2.03a	46.31 ± 2.95b	19.39	<0.001
	GG	30.06 ± 1.23c	62.17 ± 2.77a	47.23 ± 2.92b	43.69	<0.001
	YG	43.54 ± 3.00b	54.42 ± 3.38ab	61.77 ± 2.92a	7.96	0.001

* Means within rows followed by the same lowercase letter are not significantly different ($P < 0.05$; Tukey's test).
** Populations: GR, Gansu red; GG, Gansu green; YG, Yunnan green.
† Fecundity: number of offspring produced per aphid.

Table 2. Life table parameters (mean ± SE) of *A. pisum* aphids from three populations feeding on leaf discs in different spatial orientations.*

Parameter	Aphid Population**	Leaf Disc Orientation		
		Upright	Inverted	Horizontally Perpendicular
Intrinsic rate of increase (r_m)	GR	0.256 ± 0.006b	0.273 ± 0.005a	0.273 ± 0.006a
	GG	0.275 ± 0.005b	0.291 ± 0.005a	0.275 ± 0.006b
	YG	0.306 ± 0.005b	0.325 ± 0.005a	0.329 ± 0.008a
Net reproductive rate (R_0)	GR	31.87 ± 2.02b	53.50 ± 2.41a	46.31 ± 2.91a
	GG	30.06 ± 1.23c	62.17 ± 2.76a	47.23 ± 2.85b
	YG	43.54 ± 2.94b	54.42 ± 3.39a	61.77 ± 3.30a
Finite rate of increase (λ)	GR	1.292 ± 0.008b	1.314 ± 0.007a	1.314 ± 0.007a
	GG	1.317 ± 0.007b	1.338 ± 0.007a	1.317 ± 0.008b
	YG	1.357 ± 0.007b	1.384 ± 0.007a	1.389 ± 0.011a
Generation time (T)	GR	13.53 ± 0.28 b	14.59 ± 0.27 a	14.06 ± 0.31 ab
	GG	12.35 ± 0.22 b	14.18 ± 0.23 a	14.01 ± 0.24 a
	YG	11.48 ± 0.21 b	12.69 ± 0.16 a	13.07 ± 0.18 a

* Means within rows followed by the same lowercase letter are not significantly different ($P < 0.05$; paired bootstrap test).

** Populations: GR, Gansu red; GG, Gansu green; YG, Yunnan green.

perpendicular treatments than in the upright treatment, and the λ value of the inverted treatment was higher than the values of the upright and horizontally perpendicular treatments in the Gansu green population.

The mean generation time (T) ranged from 11.48 ± 0.21 d in the upright orientation of the Yunnan population to 14.59 ± 0.27 d in the inverted orientation of the Gansu red population (Table 2). The mean generation time of the Gansu red population in the inverted treatment was significantly longer than in the upright treatment (13.53 ± 0.28 d) but did not differ significantly from the horizontally perpendicular treatment (14.06 ± 0.31 d). In the Gansu green population, T was statistically higher in the inverted (14.18 ± 0.23 d) and horizontally perpendicular (14.01 ± 0.24 d) treatments than in the upright treatment (12.35 ± 0.22 d). The T values obtained with the inverted (12.69 ± 0.16 d) and horizontally perpendicular (13.07 ± 0.18 d) treatments of the Yunnan population were statistically equivalent but statistically longer than the upright orientation (11.48 ± 0.21 d).

Discussion

The survivorship curves plotted in this study were curvilinear and, of the three types of survivorship curves, most resembled the Type I curve indicating that the majority of the population can live to the mean longevity, while only a few individuals die before living to the physiological longevity (Sedaratian et al. 2011, Thirakhupt and Araya 1992, Xiao et al. 2012). From these results, we concluded that *A. pisum* nymphs had a high survival rate and that the leaf disc method was effective for bioassays and for rearing these aphids. Our results further showed that orientation of the leaf discs can impact aphid survival rates when using the leaf disc method in pea aphid rearing or bioassays. In the Gansu red and Gansu green populations, survival was extended with maintenance of aphids on inverted leaf discs (Fig. 2). Survivorship of the aphids from the Yunnan green population did not appear to be significantly affected by leaf disc orientation. One possible reason for the longer survival of the aphids on the inverted leaf discs may be related to deposition of honeydew produced by the aphids as they feed on the *V. faba* leaves (Leroy et al. 2011). In the inverted position, the honeydew would likely not accumulate on the aphid or leaf disc surfaces, thereby reducing interference with life activities or even aphid mortality owing to accidental adhesion to honeydew deposits.

Aphid fecundity also was impacted by leaf disc orientation. The highest levels of fecundity occurred with the inverted leaf discs in the Gansu red and Gansu green populations and with the horizontally perpendicular leaf surfaces in the Yunnan green population. All three populations exhibited lowest fecundity when reared on the upright-oriented leaf discs, thus indicating that the upright orientation of leaf discs in *A. pisum* rearing and bioassays may not be desirable in terms of pea aphid fecundity. Many factors, including the concentration of amino acids in plant leaves (Gao et al. 2018), may affect pea aphid fecundity, but to our knowledge this is the first study to demonstrate that feeding orientation impacts fecundity. Leaf disc orientation, however, had no effect on aphid weight gain.

These results were reflected in the life table parameters calculated from our data. Regardless of the population, the intrinsic rate of increase (r_m), net reproductive rate (R_0), finite rate of increase (λ), and mean generation time (T) were significantly lower in aphids maintained on upright-oriented leaf discs than on inverted or horizontally perpendicular discs.

Residing and feeding on the abaxial surface of the leaf or at the leaf–stem juncture of the host plant provides some protection from natural enemies and adverse effects of natural elements (e.g., rain, sunlight) and honeydew deposition for pea aphids and their progeny (An et al. 2006). We found that orienting the abaxial leaf surface in an upright and exposed position resulted in pea aphids exhibiting significantly lower performance in the life-process parameters we measured, particularly those parameters related to survival, development, and reproduction. Aphid performance was best in either the inverted or horizontally perpendicular orientation of the leaf discs. Performance, as measured in these life-processes parameters, could be inherently linked to orientation of the aphid and its leaf surface.

Photoperiod is a major environmental factor that regulates seasonal polyphenism in insects (Matsuda et al. 2020, Nijhout 2003). Furthermore, photoperiod changes with latitude which may be an explanation of the differences we observed with the Yunnan green population versus the two Gansu populations. The Yunnan population originated at a latitude (N 24°21′0″) that is farther south than that of the Gansu populations (N 36°6′36″). Perhaps performance of the Yunnan population aphids is affected by feeding on foliage oriented perpendicular to the horizon, which may be related to the body temperature adjustment of the aphids by adjusting the angle between its body direction and the sun. If our hypothesis is correct, this indicates that the pea aphid reared in the laboratory environment maintains its feeding habit of the natural environment, a habit that has been adapted to gravity and the different sunlight angles at different latitudes. Thus, the laboratory environment for rearing the pea aphid should simulate its natural environment.

Acknowledgments

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