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TITLE PAGE

Short communication

Title: Evaluation of an aphid-rearing method using excised leaves and agar medium

Running head: Method for culturing aphids

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Abstract

The present study evaluated the effectiveness of an aphid-rearing method devised by Milner in 1981 using *Acyrthosiphon pisum* and its host plant *Vicia faba*. In the "agar-leaf method", excised leaves of *V. faba* were attached to the surface of 1% agar gel containing nutrient solution, and test aphids were transferred onto the leaves. Excised leaves grew in size and weight on the agar medium. Fecundity, longevity, body size, and developmental time to adulthood were compared between aphids reared using the agar-leaf method versus those reared on *V. faba* seedlings under the same conditions. No significant difference was detected between the two treatments for any of the four parameters, suggesting that the aphids grew and reproduced on excised leaves as successfully as on *V. faba* seedlings. This method was also useful for inducing males and oviparous females at lower temperature and in short days. Therefore, the present study confirms the effectiveness of using excised leaves on agar and suggests that this method could be applied to the rearing of other aphids, phytophagous mites, leaf miners, and leaf-gall formers.

Key words: *Acyrthosiphon pisum*, developmental time, fecundity, longevity, rearing, sexual generation, *Vicia faba*.

Several methods for culturing aphids have been developed, including the use of living plants and artificial diets (Mittler & Dadd 1962, 1964; Adams & van Emden 1972; Blackman 1988). Artificial diet systems have been used in physiological studies of aphids; however, they are reportedly not suitable for achieving a high performance and long-term maintenance of aphid colonies (Mittler & Dadd 1962; Dadd & Mittler 1966; Akey & Beek 1972; Mittler 1988). Aphids fed on artificial diets usually have smaller body size and lower growth and reproductive rates than do those that are reared on natural host plants (Akey & Beck 1972). In addition, the costs of preparing materials and difficulty in sterilizing the diets have hindered the wide use of the artificial diet method (Vanderzant 1974; Gao *et al*. 2012). Host seedlings have been commonly used to rear aphids (Adams & van Emden 1972). However, because leaves and stems hide a part of aphids, it is difficult to census aphid colonies accurately and to evaluate the population growth rate. Some researchers maintained excised leaves for rearing aphids by wrapping the petioles in wet cotton or by placing them in nutrient solution (Blackman 1988; Xiang *et al.* 2012). However, it is reported that changes in the quality of excised leaves could have detrimental or beneficial effects on aphid colonies (MacKinnon 1961; Adams & van Emden 1972; van Emden & Bashford 1976; Blackman 1988; Montllor *et al.* 1990; Num & Hardie 2012). For example, although *Schizaphis graminum* (Rondani) grew poorly on intact leaves of a resistant variety of sorghum, its growth and feeding behavior increased when the colony was reared on excised leaves of the variety (Montllor *et al.* 1990).

Milner (1981) reared aphids using leaf discs attached to the surface of 1% agar gel containing nutrient solution and obtained positive results for maintaining aphid colonies. This method has been used by several studies to maintain aphids for a short term (Lamb & MacKay 1987; Chen *et al.* 2005; Bos *et al.* 2010; Li *et al.* 2016; Insecticide

Resistance Action Committee 2016). Brodeur and Cloutier (1992) indicated that this method improves the survival of the thrips *Frankliniella occidentalis* Pergande by prolonging leaf disc quality compared to culturing of leaf discs on cotton wool and on water. However, the extent to which this method is useful for rearing aphids has not been evaluated experimentally. In the present study, using the pea aphid, *Acyrthosiphon pisum* Harris as a model species, we first determine whether aphids can reproduce stably using the "agar-leaf method", and secondly examine whether aphid growth and reproduction observed using the agar-leaf method are comparable to those achieved when aphids were reared on seedlings of *Vicia faba* Linné. We also test if this method can be used for inducing the sexual generation. Through this study, we will suggest that excised leaves "grow" on agar medium, thereby having a favorable effect on aphid population growth.

To rear aphids using the agar-leaf method, we used round plastic containers (10cm diameter and 5cm height) with a lid, on which 25 holes were bored for aeration. Agar (Kanto Chemical, Tokyo) 1 g was added to 100ml nutrient solution (0.1%) for plants (Hyponex, Hyponex Japan; N:P:K = 6:10:5). The mixture was heated to the boiling point in a microwave oven and then cooled to 45^{o}C by stirring. The mixture was poured into the plastic containers to a height of 2 cm and left undisturbed until it solidified. For the agar treatment without nutrition, agar was added to 100ml distilled water. A whole leaf was excised with a pair of disinfected scissors from a *V. faba* seedling (12 – 15 days old after sprouting), and its upper surface was firmly attached to the agar medium surface immediately after excision. Leaves on the top positions were used. Test aphids were transferred on the underside of leaves. If leaves were too large or not flat, parts of them were used. While rearing aphids, we placed the containers upside down to keep the leaf surface clean from honeydew and molted skins (Fig. 1). Every four days a fresh

leaf was added to the medium, and withered leaves were removed every eight days.

In this experiment, we used an *A. pisum* clone (Nohkoh), which was collected from *V. angustifolia* on the campus of Tokyo University of Agriculture and Technology in 2011 and have been maintained clonally in the laboratory.

We have maintained the stock cultures of Nohkoh clone by transferring five newborn larvae onto a new seedling (3 – 5 days old after sprouting) of *V. faba*. Each seedling for rearing aphids was caged in a cylindrical container (30 mm diameter and 100 mm height). When they started larviposition, five newborn larvae were transferred onto a new seedling, and this procedure was repeated from generation to generation. To evade the effect of crowding, we used newborn larvae from a seedling infested by less than 30 aphids for experiments. Therefore, test aphids always developed into apterous adults.

The developmental time, fecundity and longevity of aphids (Nohkoh clone) that were reared using the agar-leaf method were compared with those of aphids that were reared on *V. faba* seedlings. During the experiments, containers for the agar-leaf method and *V. faba* seedlings, 10 – 15 days old, were placed in the same climatic chamber (MIR-254, Sanyo Corporation) that was set to 20 ± 1°C, 50-60% RH, and a 16L8D photoperiod at 5.8-7.3 W/m^2. To induce the sexual generation using the agar-leaf method, one clone of *A. pisum* (08AP2), which collected from alfalfa in Sapporo, Hokkaido, was reared at 15°C and in short days.

To examine the effect of agar medium on *V. faba* leaves, 36 leaves were collected from the top position of 11 *V. faba* seedlings (12 – 15 days old). The leaves were excised to form a rectangle (2.0cm × 3.0cm) and were randomly divided into three groups (12 rectangles for each group), each of which included leaves from the 11 seedlings. One group was used as the control, in which all leaves were oven-dried and

94 weighed using an analytical balance (H110, Sartorius Corporation). In one group (the
95 agar with nutrition treatment), six leaves were placed on agar medium containing
96 nutrient solution, and two replicates were prepared. In the remaining group (the agar
97 without nutrition treatment), six leaves were placed on agar medium containing only
98 distilled water, and two replicates were prepared. Seven days after the start of the
99 experiment, leaf area was measured using ImageJ ver.1.50i (Abràmoff *et al.* 2004) after
100 leaf images were captured into a computer, and fresh and dry leaf weight was measured
101 for both treatments. Comparisons were made between all pairs of experiments on day 0
102 and day 7 for the two agar treatments.

103 When *V. faba* leaves were placed on the surface of agar medium without aphids,
104 they remained green for seven days and had not withered 15 days later (Fig. 1). When
105 adult aphids were transferred onto the leaves on day 15, they produced first instars,
106 which successfully molted on the leaves. Seven days after the start of the experiment,
107 the dry weight of leaves that were placed on agar medium with and without nutrient
108 solution was, on average, 0.0232 g and 0.0224 g, respectively (Fig. 2A). Tukey-Kramer
109 post hoc tests showed no significant difference between the two treatments; however,
110 the leaves of both treatments were significantly heavier than those from the control
111 (0.0147 g) on day 0 (Fig. 2A). This result suggests that the leaves continued to grow on
112 agar medium (with a 58% increase in weight in the agar with nutrition treatment). On
113 day 0, there was no significant difference in fresh leaf weight between the two agar
114 treatments (Fig. 2B). However, seven days later, fresh leaf weight in the agar treatments
115 with and without nutrient solution was, on average, 0.163 g and 0.143 g, respectively. In
116 statistical tests based on the *lmer* and *lmerTest* functions in the R packages, replication
117 (containers) in each treatment was treated as a random effect. A generalized linear
118 mixed-effects model for fresh leaf weight detected a significant effect between the two

agar treatments (df = 1,22.0, F = 23.4, P < 0.0001), suggesting a positive effect of nutrient solution on the growth of *V. faba* leaves. Furthermore, in both treatments, leaves on day 7 were significantly heavier than those on day 0 (Fig. 2B). On day 0, no significant difference was found in leaf area between the two agar treatments with and without nutrient solution. Seven days after cultivation, leaf area was, on average, 6.193cm^2 and 6.124cm^2 for the treatments with and without nutrient solution, respectively (Fig. 2C). There was no significant difference between the two treatments, but in both treatments leaf area on day 7 was significantly greater than that on day 0 (an increase of 10% in the agar with nutrition treatment).

To test the effect of agar medium on aphid growth and size, 12 newborn larvae, less than 24h old, were transferred from a *V. faba* seedling onto a fresh leaf on agar medium with nutrition using a fine writing brush. These aphids were referred to as the first generation. Of the newborn larvae produced by the first generation, 12 individuals (0 – 24h old) were randomly selected and transferred onto a leaf in a new container as the second generation. The third generation was reared using the same method. For each generation, developmental time to adulthood (days) was recorded. After larviposition, aphids were fixed in 80% ethanol, and their hind legs were mounted under cover glass. After the images were captured on a computer, the length of the hind leg (femur + tibia) was measured using ImageJ. For comparison, 12 newborn larvae, less than 24h old, were transferred from the same *V. faba* seedling onto another *V. faba* seedling (3 – 5 days old) and were cultivated in the same chamber. The second and third generations were established on a new seedling based on 12 newborn larvae. Developmental time to adulthood was recorded, and the length of the hind leg was measured. Two-factorial ANOVA indicated that neither of the rearing methods (agar-leaf/seedling) and generations had significant effects on developmental time to adulthood (for methods, df

144 = 1,66, F = 0.72, P = 0.40; for generations, df = 2,66, F = 0.72, P = 0.49, for the
145 interaction, df = 2,66, F = 0.24, P = 0.79). Similarly, there was no significant difference
146 in hind-leg length between the two methods or among generations (two-factorial
147 ANOVA; for methods, df = 1,66, F = 0.58, P = 0.45; for generations, df = 2,66, F =
148 1.02, P = 0.37, for the interaction, df = 2,66, F = 1.21, P = 0.30).

149 To test the effect of agar medium on aphid fecundity and longevity, one newborn
150 larva at less than 24h old was transferred from a *V. faba* seedling onto either a *V. faba*
151 leaf on agar medium containing nutrition or a new *V. faba* seedling. In both methods,
152 after larviposition, newborn larvae were counted and removed every day, and the adult
153 was observed until death. In the seedling method, seedlings were replaced every eight
154 days. Each method had 12 replicates. The result of *t*-test showed no significant
155 differences in fecundity between the agar-leaf method and the seedling method (the
156 mean fecundity is 74.58 ± 8.9 (SD) and 77.83 ± 10.7 individuals for the agar-leaf and
157 seedling methods, respectively, df = 22, t = 0.81, P = 0.42). Survival analysis indicated
158 no significant difference in longevity (from birth to death) between the two groups (the
159 mean longevity is 27.41 ± 1.9 and 26.75 ± 2.5 days for the agar-leaf and seedling
160 methods, respectively; Kaplan-Meier method, df = 1, χ^2 = 0.62, P = 0.43). These results
161 show that the agar-leaf method is as suitable as the seedling method for rearing *A.*
162 *pisum*.

163 To examine the effect of agar medium on aphid population growth, a newly molted
164 adult was transferred onto a leaf on agar medium containing nutrition and was allowed
165 to reproduce for 15 days. The larvae produced were not removed. This experiment was
166 repeated 12 times. Nine days after the first larviposition (on day 10), the
167 second-generation aphids started to produce the third generation. Colonies starting from
168 a single *A. pisum* increased in size to 244.2 ± 18.5 (SD) individuals on average, by day

15. The maximum colony size ranged from 209 to 272.

To examine whether this method is useful for inducing males and oviparous females, we transferred a fourth instar larva (G0) of 08AP2 clone on a leaf on agar medium that was kept in a 8L16D photoperiod at 15°C and allowed to produce larvae (G1). G1 larvae were individually transferred on a new leaf on agar medium and reared under the same conditions, and nine replicates were prepared. G1 females produced a total of 32.8 (range: 24 – 45) offspring, on average, with 22.7 (19 – 28) oviparous females, 8.0 (0 – 12) apterous males, and 2.1 (0 – 6) viviparous females. Thus, all the G1 females produced oviparous females and, except one, males.

This study showed that the agar-leaf method has several benefits for rearing aphids. First, host leaves attached to the agar medium grew in size and weight, suggesting that leaf conditions remained suitable for test aphids for approximately two weeks. The addition of nutrient solution to agar medium led to an additional increase in the fresh weight of leaves. As a result, the growth, reproduction and longevity of aphids reared using the agar-leaf method were comparable to those obtained using the seedling methods. It is reported that excised leaves of *Lolium temulentum* maintain the capacity of photosynthesis, containing markedly increased amounts of soluble carbohydrate a few days after excision (Housely & Pollock 1985). In addition, the amounts of amino acids, especially asparagine, increase in the non-protein fraction a few days after excision (Thompson *et al.* 1966; Montllor *et al.* 1990). However, it remains to be clarified why excised leaves of *V. faba* on agar can provide aphid colonies with sufficient nutrition for long time and how leaf growth after excision is related to aphid nutrition.

The result from the agar-leaf method implies that the flow of phloem sap is not essential for aphid growth and reproduction. Because of excision of leaves, the supply

of phloem sap would have been stopped, whereas inducible plant defenses against aphid feeding (Louis & Shah 2015) might have been moderated (Klinger *et al.* 2005; Num & Hardie 2012). Montllor *et al.* (1990) indicated that the green bug, *Schizaphis graminum* exhibits higher growth and more frequent feeding behavior when the colony was placed on excised leaves of a resistant variety of sorghum than on intact leaves.

Second, the agar-leaf method uses materials that are easily available, e.g. sealed transparent containers and excised host leaves. During the experiments, host leaves were kept clean, and this condition contributed to aphids settling on the leaves. Colony size was readily determined by taking photos from above. This characteristic is suitable for precise evaluation of aphid colony size and reproductive rates.

Third, the induction of the sexual generation is facilitated using the agar-leaf method. Experiments for inducing aphid sexual generations have been conducted by cultivating host plants at low temperatures and in short days (Simon *et al.* 1991). In the case of *A. pisum*, these conditions lead to spindly growth in *V. faba*, so that test aphids have to be transferred to new plants in succession to evaluate the sex ratio (Li & Akimoto, personal observation 2017). However, the agar-leaf method uses one container only for evaluating the sex ratio of a single test aphid.

The agar-leaf method has been used for the rearing of other aphids (*Aphis gossypii* Glover, Chen *et al.* 2005; *Myzus persicae* (Sulzer), Bos *et al.* 2010; *Megoura crassicauda* Mordvilko, this study) and for the predaceous mite – thrips – plant system (Brodeur & Cloutier 1992). Further experiments are required to determine whether or not the agar-leaf method is effective in rearing phytophagous mites, leaf miners, and leaf-gall formers.

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221

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292

293 Figure legends

294

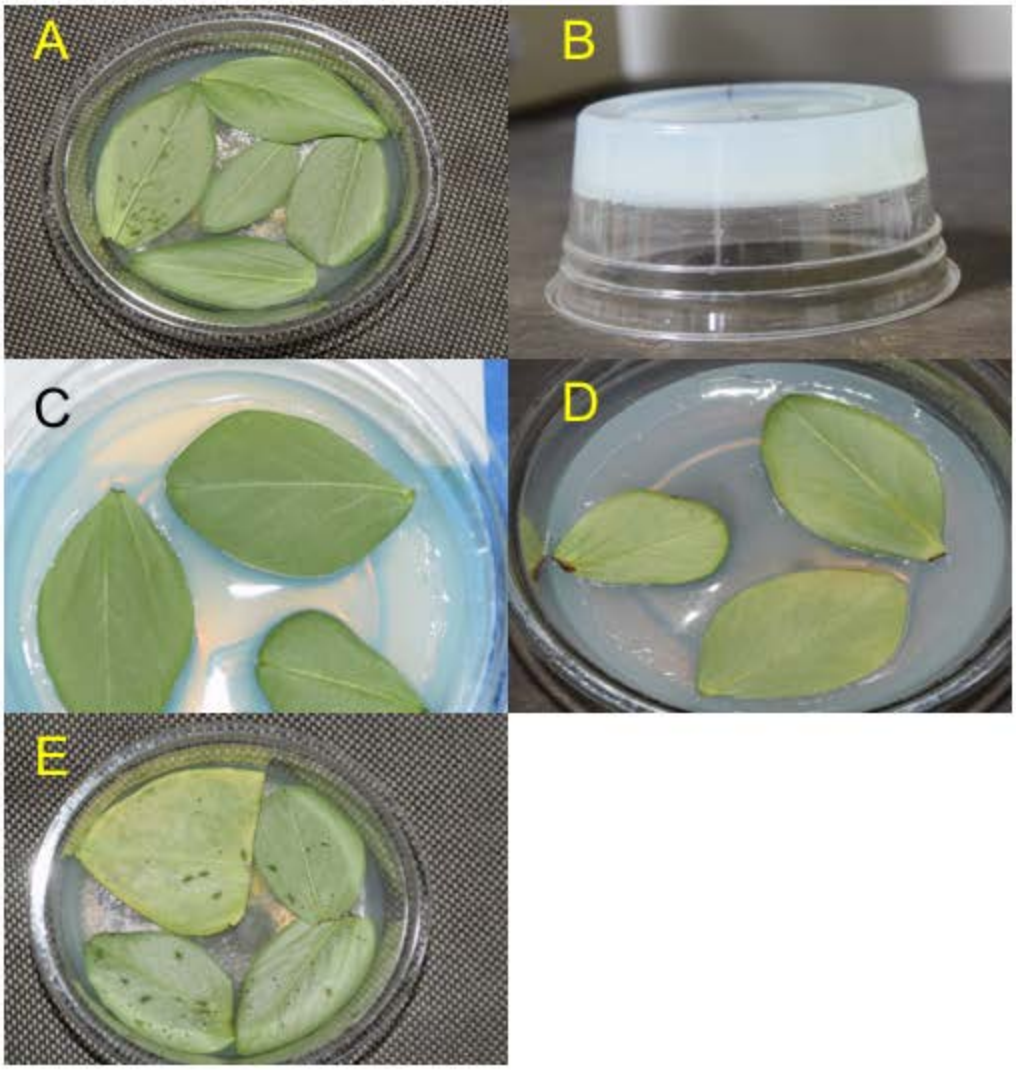
295 Figure 1 Agar-leaf method for rearing aphids. A, *Acyrthosiphon pisum* and excised *Vicia*
296 *faba* leaves on agar medium; B, lateral view; C, leaves at the start of the experiment; D,
297 the same leaves as in C 15 days after the start of the experiment; E, males, oviparous
298 females, and eggs of *Acyrthosiphon pisum* on agar medium.

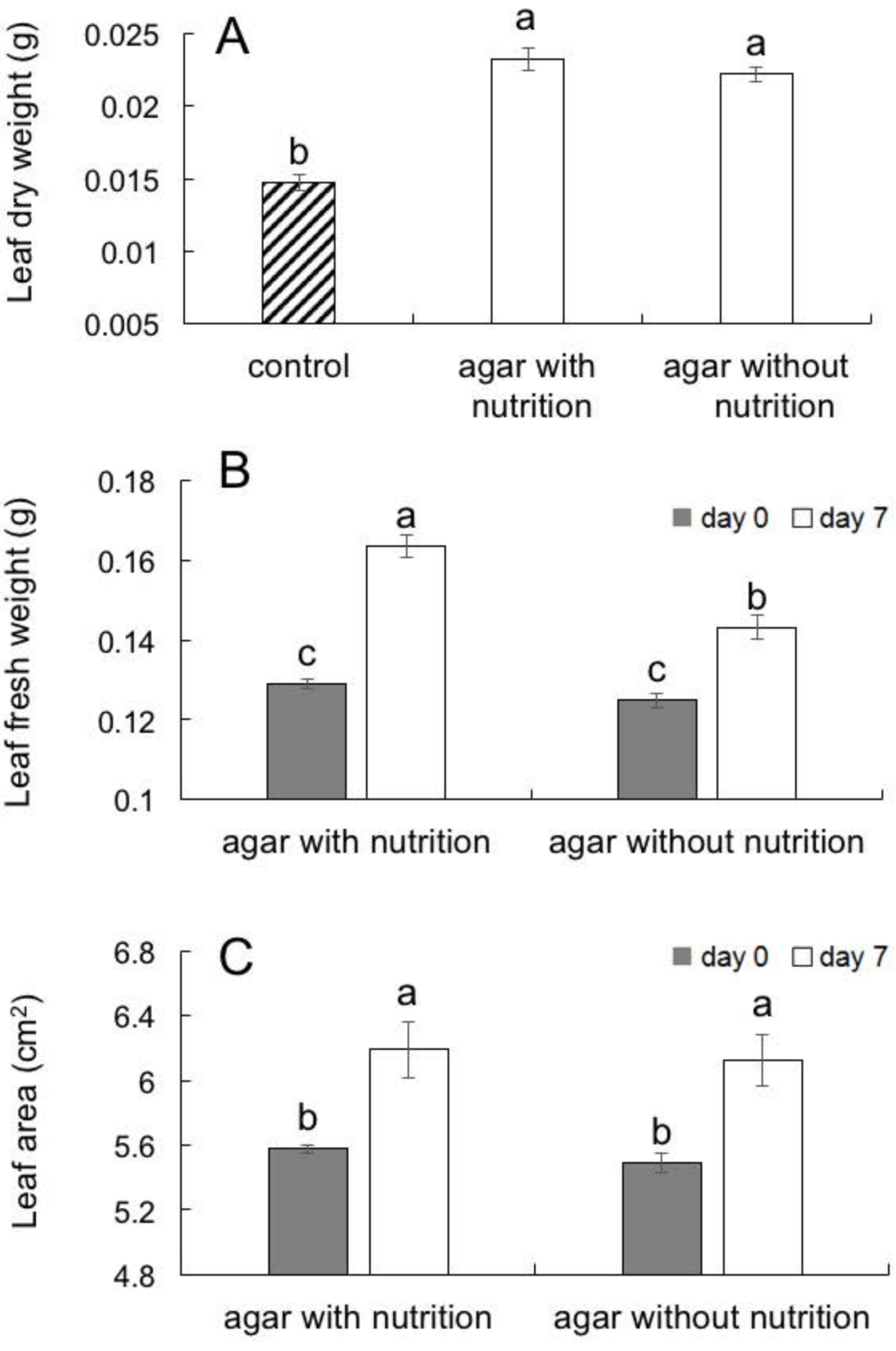
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300 Figure 2 Effects of agar medium on the weight and area of excised *Vicia faba* leaves.
301 Comparisons were made among the control and two treatments using Tukey-Kramer
302 method. Different letters indicate significant differences at a significance level of 0.05.
303 A, dry leaf weight; B, fresh leaf weight; C, leaf area.

304

305


A
B
C
D
E


A
Leaf dry weight (g)
0.025
0.02
0.015
0.01
0.005
a
a
b
control
agar with nutrition
agar without nutrition
B
Leaf fresh weight (g)
0.18
0.16
0.14
0.12
0.1
day 0
day 7
a
b
c
c
agar with nutrition
agar without nutrition
C
Leaf area (cm²)
6.8
6.4
6
5.6
5.2
4.8
day 0
day 7
a
a
b
b
agar with nutrition
agar without nutrition