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AUTHOR(S):

Yano, Mariko; Inoue, Takato; Nakata, Ryu; Teraishi, Masayoshi; Yoshinaga, Naoko; Ono, Hajime; Okumoto, Yutaka; Mori, Naoki

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**Regular Article**

# Evaluation of antixenosis in soybean against *Spodoptera litura* by dual-choice assay aided by a statistical analysis model: Discovery of a novel antixenosis in Peking

Mariko Yano,[1] Takato Inoue,[1] Ryu Nakata,[2] Masayoshi Teraishi,[1] Naoko Yoshinaga,[1] Hajime Ono,[1] Yutaka Okumoto[1] and Naoki Mori[1,*]

[1] *Graduate School of Agriculture, Kyoto University, Kitashirakawa, Sakyo, Kyoto, Kyoto 606–8502, Japan*
[2] *Department of Bioscience and Biotechnology, Kyoto University of Advanced Science, 1–1 Nanjo Otani, Sogabe, Kameoka, Kyoto 621–8555, Japan*

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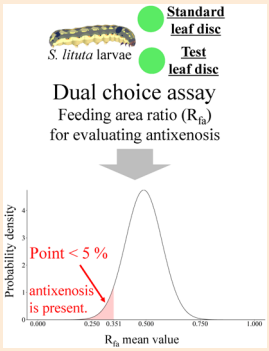
***Supplementary material***

The method for evaluating soybean (*Glycine max*) antixenosis against the common cutworm (*Spodoptera litura*) was developed based on a dual-choice assay aided by a statistical analysis model. This model was constructed from the results of a dual-choice assay in which Enrei, a soybean cultivar susceptible to *S. litura*, was used as both a standard and a test leaf disc for 2nd–5th instar larvae. The statistical criterion created by this model enabled the evaluation of the presence of antixenosis. This method was applied to four soybean varieties, including Tamahomare (susceptible), Himeshirazu (resistant), IAC100 (resistant), and Peking (unknown), as well as Enrei. Subsequently, the degrees of antixenosis were also compared by *F*-test, followed by maximum likelihood estimation (MLE). According to the results, the antixenosis of Tamahomare, Himeshirazu, and IAC100 was statistically reevaluated and Peking exhibited a novel antixenosis, which was stronger for 3rd–5th instar larvae than for 2nd instar.




***Keywords:*** Glycine max, *Spodoptera litura*, antixenosis.

## Introduction

The common cutworm (CCW), also referred to as *Spodoptera litura* (Lepidoptera: Noctuidae), is a devastating herbivorous insect pest of soybean (*Glycine max* (L.) Merr.). Application of insecticide is the most common and effective strategy for controlling *S. litura*. However, the occurrence of resistant strains is now posing a serious problem.[1,2] A sustainable strategy that combines pesticides and others measures (*e.g.*, a pheromone trap) is required for the stabilization of soybean production. The use of resistant cultivars is one of the key means. Although resistant cultivars have been bred for many years, no breeding program has succeeded sufficiently,[3] which may be due to several problems.

One major problem is the difficulty of phenotypic testing for resistance in the field. Thus, several experimental techniques in laboratory conditions have been developed.[4] These techniques are classified into two groups according to the target of the resistance mechanisms: antibiosis (adverse effects on insect development and life history) and antixenosis (hindrance effects on insect behavior).[5,6] In general, antibiosis is evaluated by the growth of insects that feed on soybean leaves (*e.g.*, larval or pupal weight), while antixenosis is evaluated by the defoliation of soybean leaves by insects (*e.g.*, consumed weight or area of leaf). However, no easy techniques with accurate of quantification and statistical correctness have been established. As a major problem, no statistical criteria are used for evaluating of resistance, given that vague criteria can cause over and under estimation of resistance.

Another problem is that there is no major factor conferring strong resistance on soybeans. Two qualitative trait loci (QTL)

* To whom correspondence should be addressed.
E-mail: mori.naoki.8a@kyoto-u.ac.jp
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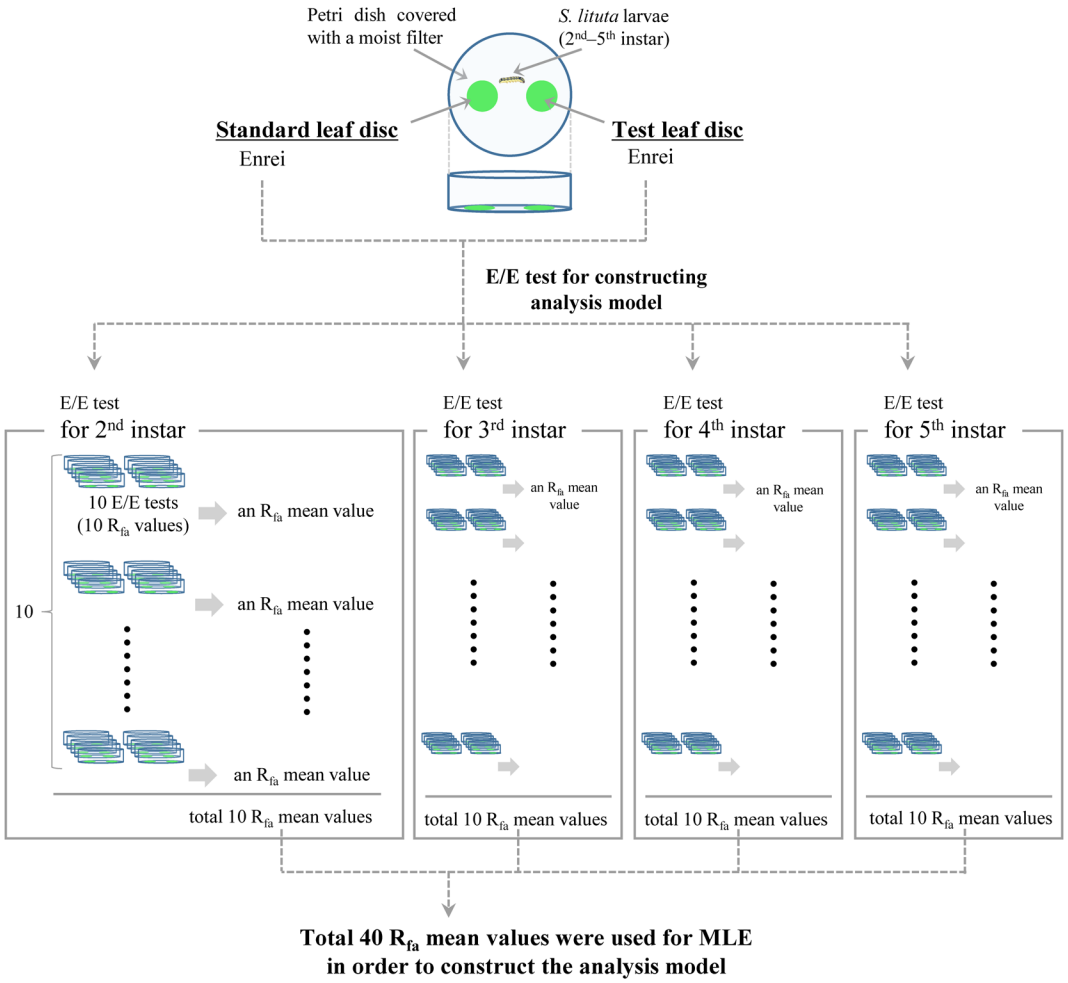


**Fig. 1.** Procedures for constructing the analysis model.

of antibiosis—*CCW-1* and *CCW-2*—were identified from the strongly resistant cultivar Himeshirazu.[7] However, the resistance of three near-isogenic lines (NILs)—*CCW-1*-NIL, *CCW-2*-NIL, and *CCW-1&2*-NIL—was not as strong as that of Himeshirazu.[3] The QTL of antixenosis was also identified from Himeshirazu.[8] Thus, gene pyramiding would be required to breed strongly resistant cultivars. Based on this idea, other resistant QTLs also were identified from wild soybeans (*Glycine soja*).[9,10] Continuous efforts to identify of other resistant genes from many varieties are required.

In the present study, we developed a method of evaluating antixenosis in soybeans against *S. litura* larvae based on a dual-choice assay, aided by the statistical analysis model. A simple-leaf-choice assay is a general method for evaluating antixenosis, although there are no statistical criteria for the evaluation. The method we developed enabled the statistical evaluation and comparison of antixenosis. Based on this method, the antixenosis of two strongly resistant cultivars (Himeshirazu and IAC100) and a cultivar (Peking) of unknown resistance was investigated.

## Materials and methods

### 1. *Plants and insects*

Five soybean cultivars, Enrei (PI385942), Tamahomare (PI507327), Himeshirazu (PI594177), IAC100 (PI518756), and Peking (PI548402)—were used for this study. Enrei and Tamahomare are susceptible cultivars. Himeshirazu and IAC100 are strongly resistant cultivars.[11–13] Peking is a cultivar with unknown resistance. Seeds were sown in soil (Vegetable seedling soil type S; Yanmar Co., Ltd., Osaka, Japan) and vermiculite (Vermiculite GS; Nittai Co., Ltd., Osaka, Japan) in a ratio of 1 to 1 and placed in a greenhouse. *Spodoptera litura* larvae were reared on artificial diets (Insecta-LFS; Nihon Nosan Kogyo Ltd., Yokohama, Japan) under laboratory conditions of 26°C and a 16/8 hr (light/dark) cycle. The developmental stages were synchronized at each molt by collecting new larvae.

### 2. *Dual-choice assay*

In order to evaluate the antixenosis of soybean cultivars for larval instars, dual-choice assays were performed using a pair of soybean leaf disks, that had been cut out from the first trifoli-

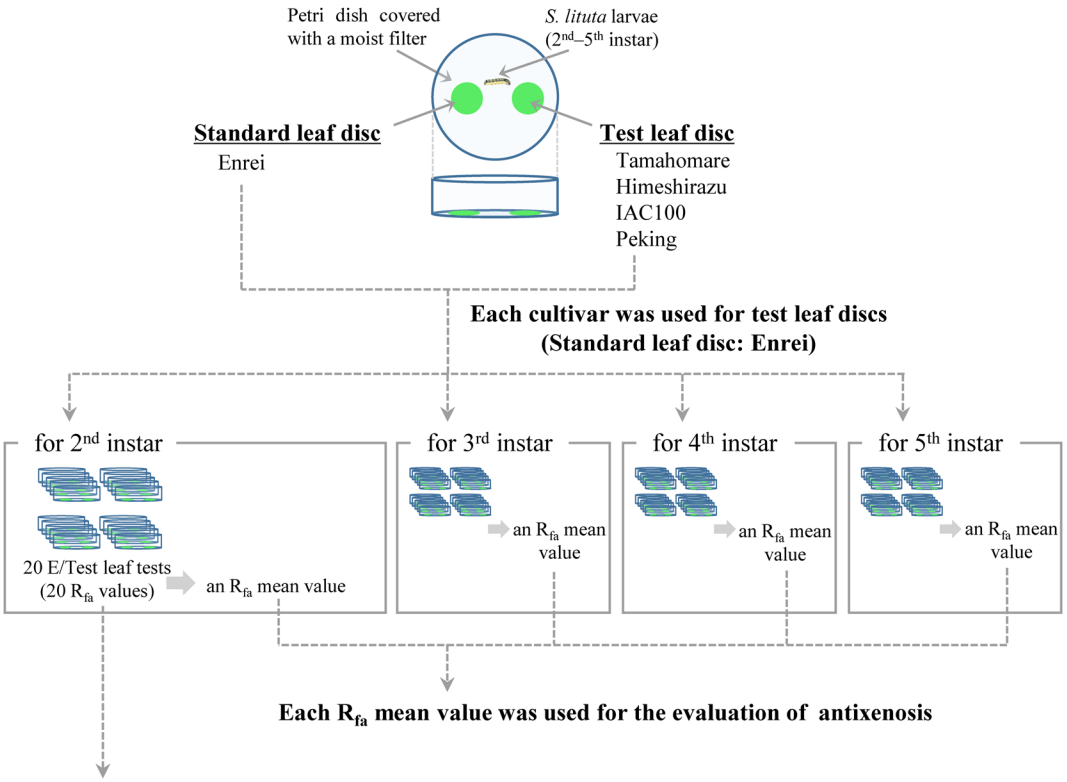


**Fig. 2.** Procedures for evaluating and comparing the antixenosis.

ate leaves of soybeans at the V4–5 stage[14] with punches. Among soybean leaf disk pairs, one was a standard leaf disk of Enrei, and the other was a test leaf disk of one of the cultivars described in the *Plants and insects* section. They were symmetrically laid on the bottom of Petri dishes covered with a moist filter paper, with the adaxial side up. One *S. litura* larva (4–6 hr after molting) was placed in each Petri dish. After sealing the lid with paraffin tape, larvae were kept in an insect-rearing room at 26°C for 16 hr (2nd–4th instar larvae) or 12 hr (5th instar larvae). The sizes of leaf disks and Petri dishes were changed according to the larval instar: $\phi$ 10 mm disk and $\phi$ 55 mm dish for 2nd instar, $\phi$ 18 mm disk and $\phi$ 90 mm dish for 3rd instar, $\phi$ 25 mm disk and $\phi$ 90 mm dish for 4th instar, and $\phi$ 35 mm disk and $\phi$ 125 mm dish for 5th instar.

Then, leaf disks were put on white paper with double-stick tape and scanned with a multifunction printer at 600 dpi. The obtained images were saved as PDF files and processed by ImageJ with Fiji plug-ins (https://fiji.sc) in order to measure the feeding area. The row image was exchanged with the split channel command (blue) and binarized with a minimum threshold value of 121; afterward, the retained leaf area was measured. Subsequently, the feeding area was calculated by subtracting the retained leaf area from the original leaf area, which was averaged out of 20 non-defoliated leaves. The antixenotic index, the feeding area ratio ($R_{fa}$), was calculated by following equation:

$$R_{fa} = \frac{A_{test}}{A_{test} + A_{standard}}$$

where $A_{test}$ refers to the feeding area of the test leaf disk; $A_{standard}$ refers to the feeding area of the standard leaf disk (Enrei).

### 3. *Data collection and model construction*

To construct the analysis model, we conducted dual-choice assays, in which Enrei was used for both the standard and test leaf disk (denoted by E/E test below) (Fig. 1). In the E/E test, the $R_{fa}$ values were obtained for 2nd–5th instar larvae of *S. litura*. In each instar, 10 $R_{fa}$ values were averaged to give one mean value; and then, repeating this procedure 10-times gave 10 $R_{fa}$ mean values. Namely, 40 $R_{fa}$ mean values were obtained (*i.e.*, 10 $R_{fa}$ mean values for four instars). The normality of the $R_{fa}$ mean values in each instar was verified by a normal probability plot and Shapiro-Wilk (SW) test. If the plots are aligned in a straight line, the observed values are considered to follow a normal distribution. The parameters in the SW test are described with test statistic (W), degree of freedom (df), and *p*-value (*p*). Here, the test statistic W is a value that indicates how well the plots are aligned in a straight line in a normal probability plot. Hartley's test and one-way analysis of variance (ANOVA) were also performed to confirm the homoscedasticity of the $R_{fa}$ mean values among 2nd–5th instars. The parameters in Hartley's test are de-

**Table 1.** $R_{fa}$ mean values and SW test in E/E test for each instar

| Instar | $R_{fa}$ mean values | | | | | | | | | | SW test | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | W | df | *p*-value |
| 2nd | 0.604 | 0.394 | 0.505 | 0.461 | 0.696 | 0.601 | 0.509 | 0.603 | 0.510 | 0.584 | 0.955 | 10 | 0.731 |
| 3rd | 0.424 | 0.520 | 0.550 | 0.399 | 0.600 | 0.379 | 0.659 | 0.454 | 0.400 | 0.438 | 0.901 | 10 | 0.227 |
| 4th | 0.579 | 0.363 | 0.479 | 0.482 | 0.473 | 0.519 | 0.446 | 0.538 | 0.405 | 0.402 | 0.978 | 10 | 0.955 |
| 5th | 0.489 | 0.447 | 0.514 | 0.366 | 0.442 | 0.541 | 0.371 | 0.508 | 0.520 | 0.370 | 0.873 | 10 | 0.109 |

scribed with the test statistic ($F_{max}$), degree of freedom (df), the number of level (k), and *p*-value (*p*). The test statistic $F_{max}$ is the value obtained by dividing the largest variance of the levels by the smallest one. After confirming the normality of the total 40 $R_{fa}$ mean values, they were used to construct the analysis model by maximum likelihood estimation (MLE) (Supplemental Information S1).

*4. Evaluation of antixenosis*

To evaluate the presence of antixenosis in Tamahomare, Himeshirazu, IAC100, and Peking, these cultivars were used for the test leaf disk in a dual-choice test (Fig. 2). In all cultivars, 20 $R_{fa}$ values were obtained for each instar of the larvae. Subsequently, the 20 $R_{fa}$ values were averaged out to give one $R_{fa}$ mean value in each. These $R_{fa}$ mean values were used to evaluate the presence of antixenosis using the analysis model. The original 20 $R_{fa}$ values were also used to compare the degrees of antixenosis, as described in the Results section.

*5. Statistical analysis*

We used BellCurve for Excel ver. 3.20 (Social Survey Research Information Co., Ltd.) for the statistical analysis and plotted data using RStudio (RStudio, Boston, MA, USA).

## Results

*1. Construction of an analysis model*

The results of dual-choice assay of the E/E test were used to construct the analysis model that can statistically evaluate antixenosis. The $R_{fa}$ mean values are shown in Table 1. The SW test (Table 1) and normal probability plot (Supplemental Fig. S1) indicate that the $R_{fa}$ mean values in each instar were normal. The homoscedasticity of the $R_{fa}$ mean values among 2nd–5th instars was assumed (Hartley's test, $F_{max}$=2.046, *df*=9, *k*=4, *p*=0.725) and no significant difference among 2nd–5th instars was observed (ANOVA, $F_{3, 36}$=1.956, *p*=0.138). Thus, the distribution of $R_{fa}$ mean values was not different among 2nd–5th instars; that is, all 40 $R_{fa}$ mean values in Table 1 were obtained from the same approximate normal distribution regardless of which instar (2nd–5th) instar. Thus, after confirmation of the normality of total 40 $R_{fa}$ mean values by normal probability plot (Fig. 3) and SW test (*W*=0.963, *df*=40, *p*=0.211), the approximate normal distribution was obtained by MLE, which gave *N* (0.489, 0.084). This normal distribution was used for the analysis model of antixenosis as follows.

*2. Evaluation of antixenosis using an analysis model*

Using the approximate normal distribution *N* (0.489, 0.084), the presence of antixenosis in the four cultivars—Tamahomare, Himeshirazu, IAC100, and Peking—was evaluated for 2nd–5th instar larvae. When the $R_{fa}$ mean value of a tested cultivar is less than 0.351 [*i.e.*, the lower 5 percentage point of *N* (0.489, 0.084)], antixenosis is said to be present. Here, an $R_{fa}$ mean value lower than 0.351 can be observed rarely in E/E testing (Supplemental Fig. S2). The obtained results are summarized in Table 2. Himeshirazu and IAC100 (resistant) showed antixenosis against 2nd–5th instar larvae, while Tamahomare (susceptible) did not. However, a resistance-unknown variety, known as Peking, also showed antixenosis against 3rd–5th instar larvae.

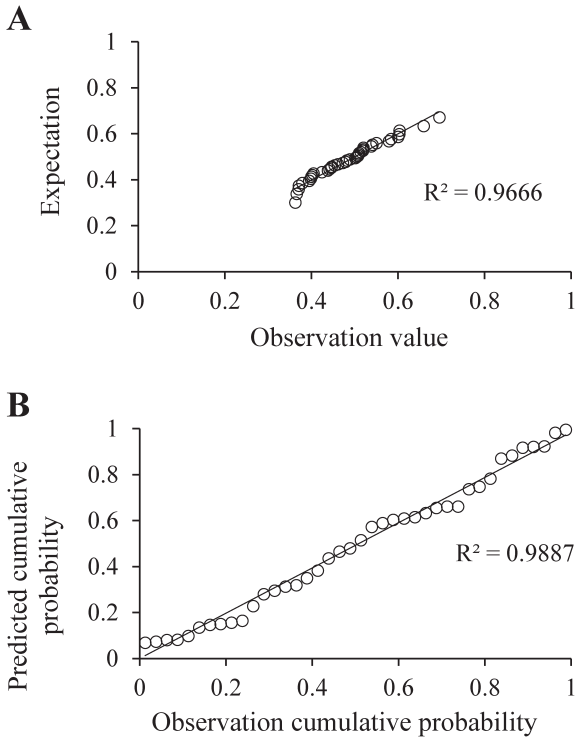


**Fig. 3.** Normal probability plots of 40 $R_{fa}$ mean values obtained from E/E test for 2nd–5th instars. The linear regression line and coefficient determination ($R^2$) are described in (A) normal Q–Q plot and (B) normal P–P plot. In (A), the *y*-axis represents the expected value if the observation value follows a normal distribution, and the *x*-axis represents the observation value itself. In (B), the *y*-axis represents the expected value of the cumulative probability if the observation values follow a normal distribution, and the *x*-axis represents the cumulative probability based on the rank of the observation values.

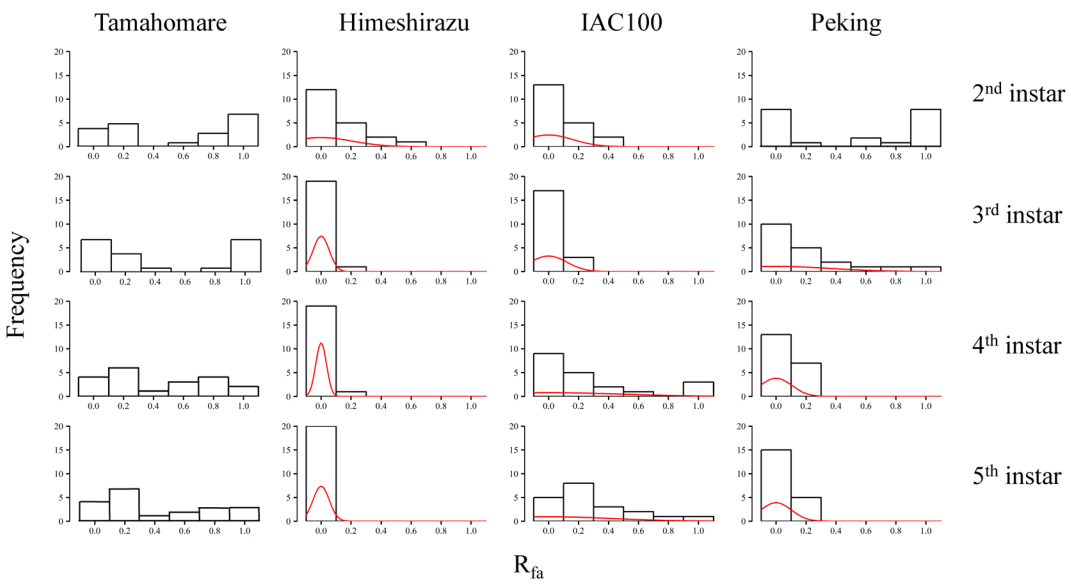


**Fig. 4.** Histograms of $R_{fa}$ values and probability density curves of approximately truncated normal distributions (red colored) in each variety and instar where antixenosis is found.

*3. Comparative analysis of antixenosis among varieties and instars*

Histograms of 20 $R_{fa}$ values in each cultivar for 2nd–5th instar larvae are shown in Fig. 4. It was predicted that the distributions of $R_{fa}$ values at the level where antixenosis is found are truncated normal distribution (lower limit 0, upper limit 1). The mean values and standard deviations of the normal distributions before truncation were estimated by MLE (Supplemental Table S1). These results show that the mean values were commonly 0 if antixenosis was present. The variances in each cultivar for 2nd–5th instar larvae are summarized in Table 3. The differences in variances can be attributed to the difference in degree of antixenosis. A large variance implies larvae freely selected diets due to weak antixenosis of the tested cultivar, while a small variance means that larvae mainly selected one preferred diet due to strong antixenosis of the tested cultivar. Therefore, we used an *F*-test to analyze the variances of the normal distributions before evaluating the degree of antixenosis between cultivars and/or instars. For instance, the degree of antixenosis between Himeshirazu and IAC100 for 3rd instar larvae was compared as follows. The variance ratio of these cultivars (with the larger value in the numerator) was 5.17 ($1.48\times10^{-2}/0.29\times10^{-2}$, Table 3), which was more than 2.17 (*i.e.*, the upper five percentage points of $F(19, 19)$) (Supplemental Information S2). Therefore, the antixenosis of Himeshirazu was stronger than that of IAC100 for 3rd instar larvae. According to this procedure, the relationship between the degree of antixenosis and larval instar was revealed (Table 3). In Himeshirazu, the degree of antixenosis was stronger for 3rd–5th instar larvae than that for 2nd instar larvae. Conversely, in IAC100, the degree of antixenosis was weaker for older larvae (4th–5th instars). In Peking, antixenosis strongly worked for 4th and 5th instar larvae even though it was ineffective against 2nd instar larvae (see Table 2).

## Discussion

The purpose of this study was to evaluate the antixenosis of four soybean cultivars to *S. litura* larvae through statistical analysis. We constructed an analysis model for evaluating the presence of antixenosis using the normal distribution. So far, antixenosis have not been evaluated based on any statistical criteria. In other words, antixenosis has been evaluated using vague criteria. Based on this model, we conducted statistical and comparative analyses of antixenosis. In previous studies, antixenosis in Himeshirazu to *S. litura* has been well studied.[3,7,8] Oki *et al.*[8] showed that a QTL, *qRslx-1*, is associated with antixenosis locat-

**Table 2.** $R_{fa}$ mean value in each variety and instar

| Instar | $R_{fa}$ mean value | | | |
|---|---|---|---|---|
| | Tamahomare | Himeshirazu | IAC100 | Peking |
| 2nd | 0.565 | 0.146† | 0.109† | 0.501† |
| 3rd | 0.453 | 0.032† | 0.087† | 0.209† |
| 4th | 0.414 | 0.061† | 0.279† | 0.075† |
| 5th | 0.421 | 0.045† | 0.291† | 0.077† |

Note. The value smaller than 0.351 was indicated with the letter †, which means the presence of antixenosis.

**Table 3.** The variance of the normal distributions before truncation in each variety and instar

| Instar | Variance | | | |
|---|---|---|---|---|
| | Tamahomare | Himeshirazu | IAC100 | Peking |
| 2nd | — | $4.33\times10^{-2}$ a | $2.64\times10^{-2}$ a | — |
| 3rd | — | $0.29\times10^{-2}$ b | $1.48\times10^{-2}$ a | $13.26\times10^{-2}$ a |
| 4th | — | $0.13\times10^{-2}$ b | $25.13\times10^{-2}$ b | $1.10\times10^{-2}$ b |
| 5th | — | $0.29\times10^{-2}$ b | $17.91\times10^{-2}$ b | $1.04\times10^{-2}$ b |

Note. The different letters indicate significant differences between instars in each variety ($F$ test with Bonferroni correction, $p<0.05$, $k=6$, $a=0.0083$). —: Values are not abtained because the values do NOT follow truncated normal distribution.

ed on Chr 7. In the present study, we confirmed the antixenosis of Himeshirazu to 2nd–5th instar larvae of *S. litura*. Antixenosis in IAC100[13] was also evaluated in detail. Furthermore, Peking has a novel antixenosis, which has clearly stronger effects on older larvae. It is well known that older *S. litura* larvae have high resistance to several pesticides—such as cyhalothrin and fenpropathrin in pyrethroid, carbaryl in carbamate, tebufenozide in IGRs, and abamectin in macrolide antibiotics[15] —and plant allelochemicals due to the highly developed detoxification ability of larval enzymes (*e.g.*, cytochrome P450, carboxylesterase, and glutathione-*S*-transferase).[15–18] Antixenosis in Peking is not overcome by such abilities; moreover, it works stronger on older larvae. The combined utilization of this resistance and pesticides would provide an effective protection program against *S. litura* larvae of all ages.

Although the underlying mechanisms of this novel antixenosis in Peking remain unknown, they are possibly associated with some unique traits. Peking is known to have some resistance against various pests. The molecular basis of this resistance has been revealed recently. Resistance to soybean cyst nematodes (*Heterodera glycines* L.; SCN) is conferred by some genes in two major loci: *rhg1* on Chr 18 and *Rhg4* on Chr 8.[19] "Peking-type" SCN resistance exhibits a broad spectrum of races due to the "Peking-type" haplotype (*rhg1-a* and *Rhg4-a*) and epistatic interactions between genes.[20–25] It is suggested that such a genetic variant is rare.[22,24] Peking is also known to have the *Rsv4* gene on Chr 2 for resistance to soybean mosaic virus (SMV).[26] Ishibashi *et al.*[27] revealed that the *Rsv4* gene encodes the RNase H family protein with dsRNA-degrading activity. The specific gene structure in *Rsv4* was 3.6 kbp indel, which was present in *rsv4* of susceptible cultivars, Enrei, and Williams 82. This resistance-related gene structure was rare in cultivated soybeans, but frequently found in Chinese and Korean soybean landraces and wild soybeans (*Glycine soja*).[27] Based on this information, Peking may have many rare genes conferring exclusive resistance against pests. The unique antixenosis against *S. litura* found in this paper is one example, and it could be helpful in developing novel target sites and insecticides.

The presence of antixenosis was assessed by the analysis model with a statistically significant level created from the normal distribution of the $R_{fa}$ mean values of the E/E test. This analysis model was constructed with 40 $R_{fa}$ mean values obtained by the E/E test for four instars (2nd–5th instars). In other words, we repeated the E/E test 400 times. However, we suppose that the number of E/E tests could be reduced to 50 times while still ensuring statistical correctness because the reliable normal distribution can be constructed only with 5 $R_{fa}$ mean values for at least any one of 2nd–5th instar larvae. Here, we reconstructed four analysis models for each instar and assessed their reliability as follows. In each instar, we randomly extracted five $R_{fa}$ mean values from the 10 $R_{fa}$ mean values described in Table 1. Afterward, analysis models for each instar were constructed by MLE using those extracted values. Subsequently, the lower 5 percentage points for each analysis model were calculated: 0.413, 0.392, 0.352, and 0.378 for 2nd–5th instars, respectively (Supplemental Table S2). Any of these values gave the same results in the evaluation of antixenosis (see also Table 2).

This is the first report revealing the presence of a novel antixenosis resistance against *S. litura* larvae in Peking. The antixenosis in Peking became stronger against older larvae; however, the detailed mechanisms remain unknown. Further investigation would help to find new target sites for insecticides against *S. litura* and other insects. As shown in this study, the mathematical analysis of antixenosis using analysis models, followed by image processing enables the statistical evaluation and comparison of antixenosis. Our analysis model could be applied to evaluating the antixenosis of various plants and antixenotic activities of pesticides.

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## Electronic supplementary materials

The online version of this article contains supplementary material, which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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