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LETTER TO THE EDITOR

A microbiological assay for biotin determination in insects

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Dear Editor,

As the coenzyme of multiple carboxylases, biotin plays key roles in intermediary metabolism and animal nutrition (Listed, 1990; Douglas, 2017). Therefore, the analytical determination of biotin levels in diverse materials is a critical aspect of B-vitamin research. Microbiological assays are classic methods of measuring B-vitamin levels. Briefly, the growth of B-vitamin-dependent microorganisms is proportional to the amount of the specific B-vitamin in the medium, and the growth can be measured by assessing the change in the medium's turbidity. This microbiological method is highly sensitive and can detect bioactive B-vitamins in samples (Tanaka *et al*., 1987). Consequently, it has been widely used to quantify various B-vitamins (Tsuda *et al*., 2011). The importance of B-vitamins in insects has been recognized (Douglas, 2017), and they impact various aspects of insect biology (Michalkova *et al*., 2014; Nikoh *et al*., 2014; Snyder & Rio, 2015; Rio *et al*., 2016; Ju *et al*., 2019; Manzano-Marín *et al*., 2019). However, a microbiological assay for analyzing biotin in insects is lacking. Here, we investigated how experimental conditions influence the results of a biotin assay, and then we developed an efficient microbiological assay to measure biotin in insects using the whitefly *Bemisia tabaci* MEAM1, whitefly *Trialeurodes vaporariorum*, and brown planthopper *Nilaparvata lugens*, which are economically important agricultural pests worldwide.

First, we examined the effects of bacterial cells on biotin assays by measuring them at various optical density (OD) values, various incubation times and using different incubation methods (for detailed methods, please see the Supplemental information). Good linear correlations were obtained between bacterial cell growth at OD_{630}, OD_{550}, and OD_{610} values and biotin concentrations (Fig. 1A, Table 1). Some of the cultures showed growth initially when exposed to 0 ng/mL biotin but there was no bacterial cell growth at this concentration after 15 h (Fig. 1B). Moreover, the OD_{630} value at 0 ng/mL biotin was much lower than at other concentrations, indicating that this did not influence our results. Compared with after 15 and 17 h, more significant linear correlations between absorbance and biotin concentration were observed after 19 and 22 h of incubation, resulting in correlation coefficients of 0.99 and 0.97, respectively (Fig. 1C, Table 1). *Lactobacillus plantarum* is a facultative anaerobic lactic acid bacteria; therefore, incubation methods with or without oxygen may influence its growth. Thus, the effects of various bacterial cell-incubation methods on the biotin assay were investigated. Compared with the incubation of a 2 mL bacterial cell culture in a 5 mL tube (Fig. 1D), more significant linear correlations between absorbance and biotin concentration were observed after the incubation of a 0.3 mL bacterial cell culture in the well of a 96-well plate and of a 2 mL bacteria cell culture in a 2 mL tube, which resulted in correlation coefficients of 0.96 and 0.99, respectively (Fig. 1D, Table 1).

Then, to determine whether the microbiological assay developed here is suitable for the determination of biotin levels in insects, the whitefly *B. tabaci*, whitefly *T. vaporariorum* and planthopper *N. lugens* were chosen as subjects (for detailed methods, please see the Supplemental information). Good linear correlations between absorbance levels and biotin concentrations for the three insect species were observed, resulting in correlation coefficients greater than 0.95 (Table 1). Our analysis showed that the biotin titer was higher in the whitefly *B. tabaci* and planthopper *N. lugens* than in the whitefly *T. vaporariorum* (Fig. 1E; $F_{2,6} = 165.31$, $P = 0.00001$).

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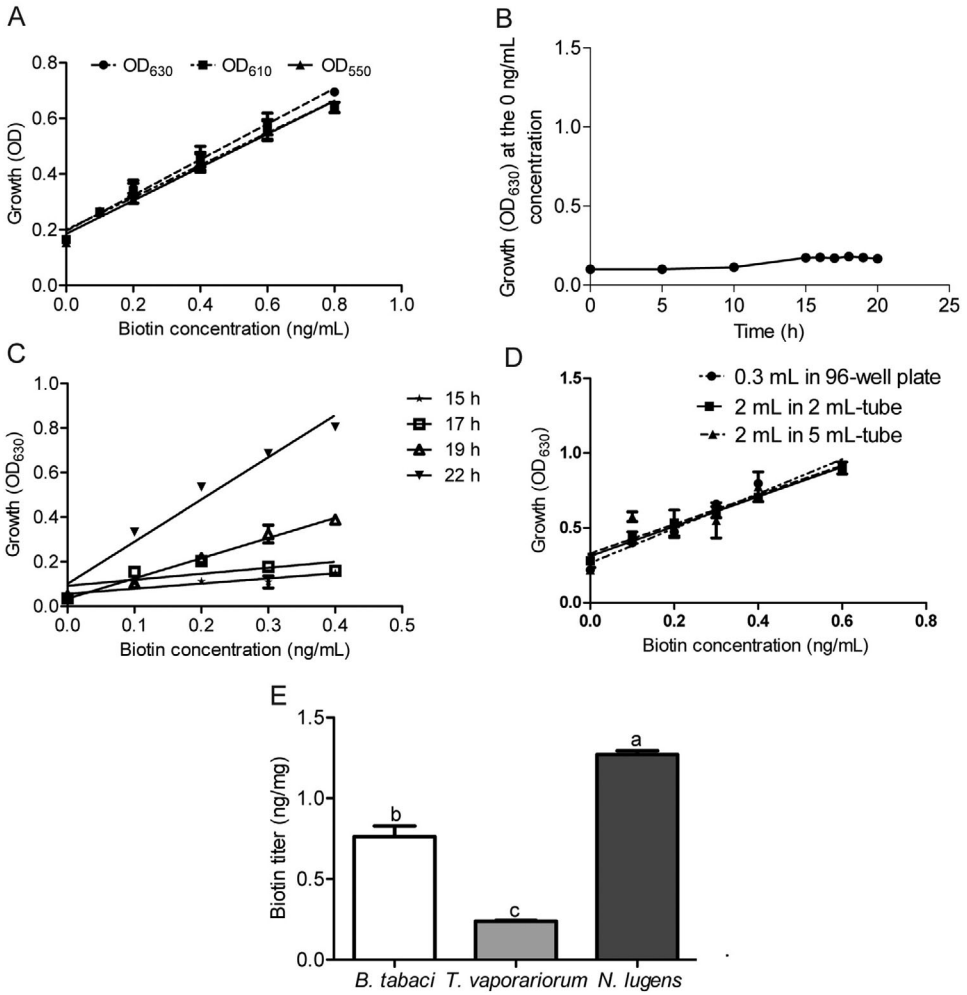

Fig. 1 Standard curves for biotin concentration using *Lactobacillus plantarum* cells at various measure conditions and the biotin titer in the whitefly and planthopper. (A) Standard curves for biotin concentration using *L. plantarum* cells with optical densities (ODs) measured under various OD values. $N = 3$. The measurements were taken at 20 h. (B) The growth dynamics of bacterial cells at the 0 ng/mL concentration of biotin. $N = 3$. (C) Standard curves for biotin concentration using *L. plantarum* cells at F3 for various incubation times. $N = 3$. (D) Standard curves for biotin concentration using *L. plantarum* cells at F3 under various incubation methods. $N = 3$. The measurements were taken at 20 h. (E) The biotin titer in the whitefly *Bemisia tabaci* and *Trialeurodes vaporariorum* and planthopper *Nilaparvata lugens* measured using the microbiological assay. $N = 3$. Error bars represent one standard error. Different letters above the bars indicate significant statistical differences at $P < 0.05$.

Additionally, the biotin titer obtained using the microbiological assay developed in this study for the planthopper *N. lugens* was comparable to that obtained using liquid chromatography–mass spectrometry (LC–MS) in a previous study (Ju *et al*., 2019), indicating that our method is reliable. A further recovery test demonstrated that a high recovery rate with a low relative standard deviation was obtained (Table 2), which confirmed that our approach is reliable for assaying biotin levels in insects.

Multiple experimental conditions may influence the results of the B-vitamin assay. Our results revealed that incubating bacterial cell cultures for at least 19 h and in 96-well plates or 2 mL tubes was suitable. Additionally, incubation time influences the microbial cell density, thereby affecting cell growth during the biotin assay. We demonstrated that extending the incubation time to more than 19 h was essential for obtaining good linear correlations between absorbance and biotin concentration. Additionally, *L. plantarum* cells can live with and without oxygen. Various incubation methods result in different oxygen levels. However, no effects of the incubation chamber on biotin assays had been previously examined.

Table 1 Standard curves for biotin concentration and coefficients of correlation between cell growth and biotin concentration using *Lactobacillus plantarum* cells with optical densities (ODs) measured under various OD values, under various incubation times and methods, and for three species of insects.

Factors	Levels	Standard curve	Coefficients of correlation
OD value	OD_{550nm}	$y = 0.567x + 0.1856$	0.9875
	OD_{610nm}	$y = 0.5791x + 0.2$	0.9826
	OD_{630nm}	$y = 0.6397x + 0.196$	0.9907
Incubation time (h)	15 h	$y = 0.2302x + 0.0559$	0.8017
	17 h	$y = 0.2698x + 0.0918$	0.4398
	19 h	$y = 0.9067x + 0.0344$	0.9881
	22 h	$y = 1.8931x + 0.1006$	0.9669
Incubation methods	0.3 mL per well of 96-well plate	$y = 1.1552x + 0.2658$	0.9568
	2 mL in 2 mL tube	$y = 0.9922x + 0.3119$	0.9905
	2 mL in 5 mL tube	$y = 0.98x + 0.3294$	0.8384
Insects	*Bemisia tabaci*	$y = 1.3431x + 0.3775$	0.9548
	Trialeurodes vaporariorum	$y = 0.2357x + 0.452$	0.9661
	Nilaparvata lugens	$y = 0.1829x + 0.4403$	0.971

Table 2 Recovery for biotin in the whitefly *Trialeurodes vaporariorum* added with standard solutions ($n = 6$).

Added biotin standard (ng)	Found biotin in samples (ng)	Recovery rate (%)	Relative standard deviation
0.20	0.19 ± 0.003	94.86 ± 0.52	1.27
1.00	1.00 ± 0.04	100.27 ± 1.74	4.25

We found that incubating 2 mL bacterial cell cultures in 5 mL tubes was not good for bacterial growth, resulting in poor linear correlations between absorbance and biotin concentration. This may be caused by the amount of oxygen present in the remaining 3 mL space during the assay, which influenced bacterial growth. Our study optimized the biotin assay conditions and will promote the use of microbiological assays to measure other B-vitamin levels.

In summary, to our knowledge, except for vitamin B9, no other B-vitamins have been measured using a microbiological assay in insects (Snyder & Rio, 2015). Therefore, this is the first study to use a microbiological assay to measure biotin in insects. Because the method we developed is highly sensitive, rapid, and accurate, we believe that this approach will be useful for assaying biotin levels in other insects. Moreover, the microbiological assay developed in this study may also advance research on other B-vitamins in entomology.

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Author contributions

J.B.L., F.R.R. and Y.Z.H. designed the experiments. F.R.R., B.B. and J.S.H. performed the experiments. F.R.R., Y.Z.H. and J.B.L. analyzed the data. J.B.L. and Y.Z.H. wrote the paper. All authors edited and approved the paper.

Disclosure

The authors declare no competing interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Materials and methods