Research paper

Fitting Photosynthesis Irradiance Response Curves with Nonlinear Mixed-effects Models

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【Summary】

Studying photosynthesis-irradiance (P-I) relationships is fundamental to plant ecophysiology and ecological research. Important ecological questions are often inferred, based on findings from P-I studies. A P-I relationship is intrinsically nonlinear, and measurements typically contain random variations and other complicated data structures. Although rarely adopted, the best approach to analyze a P-I relationship is to use nonlinear mixed-effects (NLME) modeling. We believe that such a failing is mainly because ecophysiologists are unfamiliar with and uneasy in using the approach. Consequently, the main objective of this study is to help ecophysiologists better understand how to use NLME modeling to fit P-I and other response curves. Using data from an artificial shading experiment as an example, we outline a 'backward elimination' strategy for fitting P-I curves by NLME modeling. We also summarize our model development process that led to the final model. Compared with nonlinear models estimated by ordinary and generalized nonlinear least squares estimations, the final NLME model had the smallest estimated error variance, enabling it to detect the presence of photoinhibition where the other 2 models failed. Our study shows that an inadequate estimation approach might not only be statistically inefficient and less powerful, but may also lead to incorrect inferences.

Key words: autocorrelation, generalized nonlinear least squares, heteroscedasticity, ordinary least squares, repeated measures.

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研究報告

利用非線型混合效應模式分析光合作用−光度反應曲線

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摘 要

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分析光合作用對不同光度之反應為植物生理生態學與生態學研究之基礎。眾多重要生態學理論均由推論光合作用-光度反應曲線之關係而得。該反應曲線本質上為非線性，同時測所得之資料常包含許多複雜之資料結構與逢機變異。雖然非線性型混合效應模式為分析光合作用-光度反應曲線之最佳統計方法，但一般研究卻甚少採用該方法。究其因，主要乃因為生理生態學者對該方法之理論與使用瞭解甚少。本文之主要目的乃在利用一遮蔭試驗所得之數據為例，幫助生理生態學者瞭解與使用非線型混合效應模式分析合作用-光度反應曲線。本文除介紹如何利用非線型混合效應法配適模式外，亦利用普通最小平方法(OLSE)與廣義最小平方法(GLSE)配適相同之非線型模式，並比較三者差異。結果顯示，與其他兩種方法估算所得之結果相較，由非線型混合效應法所配適模式，因考慮逢機效應是以其所得之組內變異最小，進而得以偵測出光抑制現象之存在。本研究顯示，利用不正確之統計分析方法，不僅可能喪失統計檢測能力，更有可能導致錯誤之結論。

關鍵詞：自我相關、廣義非線型最小平方法、變異數不齊一性、普通最小平方法、重複觀測。

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INTRODUCTION

Studying photosynthesis-irradiance (P-I) relationships is fundamental to plant ecophysiology and ecological research. As a result of such studies, we can understand how plants respond and adapt to various environmental factors, such as different light regimes (e.g., Zipperlen and Press 1996), moisture regimes (e.g., Gardiner and Krauss 2001), and temperatures (e.g., Robakowski 2005). Findings from P-I studies also enable us to answer other important ecological questions, such as why plant species can coexist (e.g., Givnish 1988, Kitajima 1994).

Obtaining high-quality P-I relationship data these days is no longer a problem, but properly analyzing the data still poses a problem to many ecophysiologists for several reasons. First and foremost is the need to choose an appropriate model from existing P-I models. If photoinhibition is not present, then a typical P-I curve can be modeled by an asymptotic curve, such as a Michaelis-Menten (rectangular hyperbola) equation, a Mitscherlich-type equation, or a non-rectangular hyperbola equation. Thornley and Johnson (2000) provided a detailed discussion on candidate models when no photoinhibition is present. If photoinhibition is present, a nonlinear model, such as the one proposed by Platt et al. (1980), can be used to analyze the data. Different models reveal different aspects of P-I relationships. Some studies have compared different P-I models (e.g., Frenette et al. 1993, Pachepsky et al. 1996). However, it is still a matter of judgment to decide which model is appropriate in many instances. As important as it is, choosing an appropriate P-I model is not a statistical problem. The real statistical challenges begin after a model is chosen. The nonlinearity of the model is only a part of it.

As first pointed out by Potvin et al. (1990), many ecophysiological response studies involve repeated measurements. In the context of P-I studies, repeated measurements arise when for each of the observational units, multiple net gas exchange measurements are collected over a set of irradiance levels. In a repeated-measures experiment, responses measured on the same observational unit are usually autocorrelated. One must account for the presence of autocorrelations in the data analysis, otherwise the estimated error variance will be inflated.

Lindstrom and Bates (1990), McCulloch and Searle (2001), and Peek et al. (2002) suggested that the best way to accommodate a repeated-measures experiment in fitting nonlinear ecophysiological response curves is to use a nonlinear mixed-effects (NLME) modeling approach. When using such an approach, each plant (or leaf) is treated as a random factor and random variations are accounted for among the observational units. An NLME modeling approach can also accommodate autocorrelations and other data features by specifying a suitable error variance-covariance structure (Littell et al. 1996, Pinheiro and Bates 2000). For example, a common feature in many P-I studies is the increase in variability among the measured responses with increasing irradiance levels (e.g., Man and Lieffers 1997, Landhäusser and Lieffers 2001, Peek et al. 2002).

Since the influential paper of Potvin et al. (1990), many studies have adopted a repeated-measures analysis of variance approach to analyze P-I data (e.g., Kyei-Boahen et al. 2003, Heschel et al. 2004). However, in our literature review, we could only find 1 study (McElrone and Forseth 2004) that adopted an NLME modeling approach in fitting P-I curves. All other studies since 2003 that we reviewed still used a fixed-effects nonlinear modeling approach to fit P-I curves, including those that cited the work of Peek et al. (2002). Moreover, the majority of studies that used either a repeated-measures ANOVA or an NLME modeling approach to analyze P-I data did not explicitly divulge the random parameters and covariance structures used in their studies. Only a few mention the error variance-covariance structures (e.g., Heschel et al. 2004).

We believe that the reason for failing to adopt an NLME modeling approach to fit P-I curves is mainly because most ecophysiologists are unfamiliar with or uncomfortable in using this approach to conduct statistical analysis. Although an inadequate statistical model does not necessarily lead to incorrect results, the analysis will be inefficient and less powerful in a statistical sense to say the least.

The main objective of this study was to demonstrate by example how we can properly fit an NLME P-I curve using publicly available statistical software. We first briefly describe mixed-effects modeling. We also outline a strategy for fitting P-I curves by NLME modeling. Then, we analyze a real data set and summarize in a concise manner our modeling processes that led to the final NLME model. Finally, we compare the results from the final NLME model with those from 2 other modeling approaches.

Mixed-effects modeling

A brief description

In a linear fixed-effects model, the relationship among the response vector (Y), the fixed-effects design matrix (X), and an unknown parameter vector, β, can be expressed as $Y = X\beta + \varepsilon$, with ε being the random error vector that independently and identically distributed as $N(0, \sigma^2)$. Thus, $\mathrm{Var}(Y) = \sigma^2 I$, with I being an identity matrix. Here β is usually estimated based on an ordinary least-squares estimation (OLSE).

In a basic linear mixed-effects model, we have 2 additional terms, Z and γ, representing the random effects design matrix and the unknown parameter vector, respectively. The random effects represent random deviations of individual subjects from the respective population means (fixed effects). Assume that $\gamma \sim N(0, G)$, where G is the variance-covariance matrix of γ, and that the random effects are independent among subjects (e.g., individual plants). Further assume that ε and γ are independent. The relationship between the response vector, the design matrices, and parameter vectors is $Y = X\beta + Z\gamma + \varepsilon$. Var (Y) is then $ZGZ^T + \sigma^2 I$, where Z^T is the transpose of Z (Littell et al. 1996).

If the equal variance and independence assumptions on random errors are relaxed such that $\varepsilon \sim N(0, \sigma^2 R)$, then Var (Y) becomes $ZGZ^T + \sigma^2 R$, where R is the variance-covariance structure of ε (Littell et al. 1996, McCulloch and Searle 2001). The error variance-covariance structure, R, can be further decomposed into 2 components that can be used to model the correlations and to account for heteroscedasticity among the errors (Pinheiro and Bates 2000, Chaps. 5 and 7). Note that R could also be modeled using a generalized least-squares estimation (GLSE, Draper and Smith 1998, p 221-224). Thus, the main objective of using mixed-effects modeling should be to account for random effects, rather than to merely account for possible correlations and heteroscedasticity among observations.

Although the estimation processes are much more complicated in NLME modeling than in linear mixed-effects modeling, the main concept in the above description is still valid. An NLME model can be viewed as a nonlinear regression model that accounts for random effects (due to plant-to-plant variations) and other data structures (Pinheiro and Bates 2000, McCulloch and Searle 2001). That is, $X\beta$ in the linear mixed-effects models is replaced by a nonlinear function, $f(X, \beta)$.

A modeling strategy

As in linear mixed-effects modeling, in order to fit a P-I curve with NLME modeling the following need to be specified: (1) a proper G matrix for potential random parameters, (2) how fixed-effects treatments (e.g., species, temperature, irrigation, etc.) affect each parameter, and (3) a suitable R matrix to accommodate possible correlations and heteroscedasticity in the data.

Since a typical P-I curve has only 3 to 4 parameters, one can begin by considering all parameters as random and fitting the model without considering any fixed-effects treatment. The estimated G matrix is then a variance-covariance matrix with no special structure (i.e., 'unstructured', Littell et al. 1996). The estimates of the G matrix provide clues to indicate which parameters should be retained as random, and what would be a good structure to reflect the correlations among the remaining random parameters.

If there are not too many combinations of factor levels (i.e., treatments), one first assumes that all fixed-effects treatments affect all model parameters, and then the fixed-effects structure is adjusted according to the significance of each fixed-effects treatment. When deciding which parameters should be modeled by fixed-effects treatments, one may wish to be more conservative initially (i.e., by using a larger *p* value as a screening criterion).

After an initial G structure and a fixed-effects structure have been determined, the usual regression diagnostics indicate whether the equal variance and independence assumptions are met, and if not, where they are lacking.

In summary, the above strategy can be viewed as a 'backward elimination' approach. Of course, the parameter estimates might not converge in such an 'over-parameterized' approach. In that case, one might want to consider a diagonal structure for the G matrix as a start (i.e., assuming random parameters are uncorrelated) and then adjust the structure later. When the number of treatments is large, one can also use a 'forward selection' approach to determine a proper fixed-effects model structure (Pinheiro and Bates 2000).

The process leading to a satisfactory NLME model is an iterative one since components within the model do interact with one another. The choice of the structure of G affects the characteristics of R and the estimates of the fixed-effects. Modifying the fixed-effects structure also changes the estimates of G and R, which can lead to adjustments in the structures of the 2 matrices. Therefore, we iterate among fitting, examining, and modifying models. Model selection criteria help us compare the performances of different models and decide which model is better to build on.

MATERIALS AND METHODS

Materials and experimental design

The example was taken from an experiment examining the effects of artificial shading on seedling photosynthetic responses of *Castanopsis carlesii* (Fagaceae), a climax tree species in wet montane temperate forests of Taiwan.

First, seeds were germinated and grown in a controlled environment for 1 yr. On January 1998, 45 seedlings were randomly assigned to 3 light treatments (representing 24, 52, and 100% full light) with each treatment consisting of 15 seedlings. Measured with LI-190SA quantum sensors (Li-Cor, Lincoln, NE, USA), the average photosynthetic photon flux densities (PPFD, 400~700 nm μmol photons m^{-2} s^{-1}) for each of the 3 treatments during a 4-d period in February 1998 (recorded from 11:00 to 14:00 daily) were 326, 698, and 1356, respectively. The acclimation period was 18 mo, and proper irrigation and fertilization were provided to ensure good seedling growth during that period.

Net photosynthesis measurements

In August 1999, three seedlings from each treatment were randomly selected for net photosynthesis measurements. For each seedling, the first mature leaf from the third uppermost branch was selected. For each leaf, net photosynthetic rates were measured at 9 increasing PPFD levels (1, 50, 100, 300, 500, 700, 900, 1100, and 1300 μmol photons m^{-2} s^{-1}) using a portable photosynthesis system (Model 6400, Li-Cor.) The light source within the cuvette was an integrated red-blue light-emitting diode. External air was scrubbed of CO_2, mixed with pure CO_2 at a concentration of 380 μmol mol^{-1}, and controlled at a flow rate of 500 μmol s^{-1}. During measurement, the average leaf temperature was 29.3±0.1℃, and the humidity within the cuvette was controlled to approximately 50%. At each light level, an equilibration period of 15 to 20 min was allowed before any measurements were taken. All measurements were completed within 4 d, and measurements were taken between 08:00 and 16:00 on those days. Seedlings were fully irrigated 1 d prior to measurements.

P-I curve

The initial data analysis suggested that seedlings that were grown under both 24 and 100% full light exhibited photoinhibition (Fig. 1). Therefore, we adopted the model of Platt et al. (1980) to fit the data. The model had the form $P_{sn} = P_s \times (1 - e^{-\alpha \times I/Ps}) \times e^{-\beta \times I/Ps} - R_d$, where P_{sn} is the measured net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) at irradiance level I (μmol photons m^{-2} s^{-1}), P_s is the maximum rate of photosynthesis (μmol CO_2 m^{-2} s^{-1}) without photoinhibition, α is the quantum yield efficiency (μmol CO_2 μmol^{-1} photons), β is the photoinhibition coefficient (μmol CO_2 μmol^{-1} photons), and R_d is the dark respiration rate (μmol CO_2 m^{-2} s^{-1}) (Payri et al. 2001, Roberts et al. 2002).

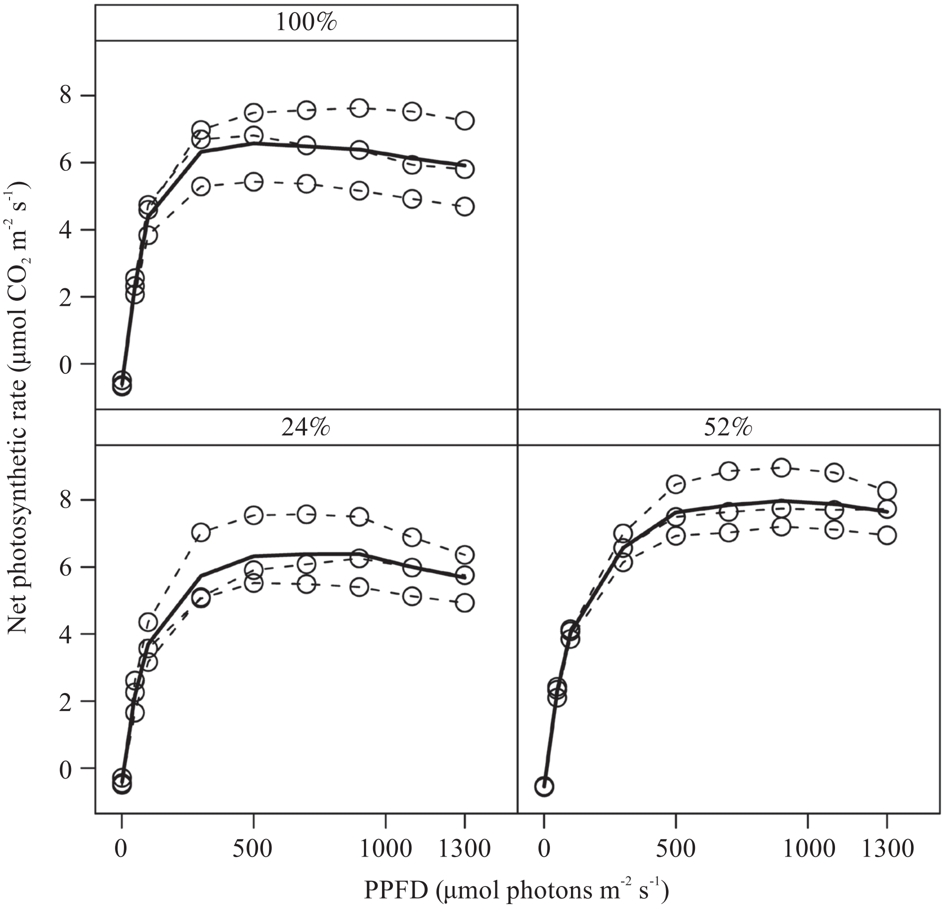

Fig. 1. Net photosynthesis measurement data for seedlings of *Castanopsis carlesii* acclimated under 24, 52, and 100% full light. Open circles represent observations from individual seedlings (dashed lines), and solid lines represent the mean trend within each treatment. PPFD, photosynthetic photon flux density.

The photoinhibition parameter (β) can be viewed as the slope of the P-I curve beyond the onset of photoinhibition (Roberts et al. 2002). With no photoinhibition ($\beta = 0$), the model reduces to a Mitscherlich-type model. In the presence of photoinhibition, β has a value of > 0. The basic form of this model (without the dark respiration rate term) has been extensively used to model P-I relationships in marine environments where photoinhibition is present (e.g., Payri et al. 2001, Suggett et al. 2001, Pringault et al. 2005). The inclusion of the dark respiration rate term not only allows us to estimate that parameter, but also to improve the numerical stability during parameter estimation.

Statistical analyses

The *NLME* package (Pinheiro et al. 2006) of R (R Development Core Team 2006) was used to analyze the data. R language is a free and comprehensive statistical package that can be obtained from the R web site (http://www.r-project.org). In *NLME*, the default estimation method (function *nlme*) estimates the parameters of an NLME model using a maximum likelihood estimation (Pinheiro and Bates 2000, Chap. 7).

We fitted a series of models based on the strategy outlined above. For model performance comparisons and selections, we used the Akaike information criterion (AIC), the Bayesian information criterion (BIC), and the Likelihood ratio testing (LRT) approach (for hierarchical models). *F*-tests were used to assess the significances of the light environments (fixed-effects treatments) on model parameters, and two-tailed *t*-tests were used to test the significance of individual fixed-effects parameters (Pinheiro and Bates 2000).

In addition to developing the 'best' NLME model, 2 additional models were fitted for comparison purposes. The first model was the 'best' nonlinear OLSE model. The second model was the 'best' fixed-effects model, but accounting for both autocorrelations and heteroscedasticity in the residuals (i.e., a model estimated by GLSE). Both models were fitted with the *gnls* function of the *NLME* package. In this study, we assumed that all random effects and errors were normally distributed, that random effects were independent among the seedlings, and that random effects and errors were independent.

RESULTS

Model fitting summary

We summarize in the Appendix the *R* codes used to develop our models. We first fitted a model by treating all parameters as random and without considering the effects of shading (model psn0 in the Appendix). We then removed parameter R_d from the list of random parameters since it had a small estimated variance. We adopted a diagonal structure for the G matrix since the estimated correlations among P_s, α, and β were small. Based on the AIC, BIC, and the *p* value of the LRT, the reduced model (psn01) performed better than the original model (Table 1).

We then introduced treatment effects into the model first by assuming that the treatments affected all of the model parameters (psn1). Then, based on *F*-tests (Table 2), we revised the model so that the light environment would affect α, β, and R_d, but not P_s (model psn11). Judging from the performance criteria, the reduced model performed as well as the full model (Table 1).

Table 1. Model performance information for the fitted nonlinear mixed-effects models

Model	df[1)]	AIC[2)]	BIC[3)]	Log-likelihood	LRT[4)] (*p* value)
psn0	15	62.84	98.75	-16.42	
psn01	8	55.45	74.60	-19.72	6.61 (0.47)
psn1	16	54.82	93.13	-11.41	
psn11	14	52.35	85.87	-12.17	1.53 (0.47)
psn2	15	40.06	75.97	-5.03	14.29 (< 0.001)
psn21	14	38.06	71.58	-5.03	≈ 0 (≈ 1)
psn3	16	28.07	66.38	1.97	13.99 (< 0.001)

1) Number of parameters in the model.
2) Akaike information criterion; for this criterion, a model with a smaller value is preferred.
3) Bayesian information criterion; as with the AIC, a model with a smaller value is preferred.
4) Likelihood ratio test. For comparing nested models only.

Table 2. Fixed-effects analysis of variance table for model psn1

Parameters	num. df[1)]	F-test value	p value[2)]
P_s intercept	1	505.91	< 0.0001
Treatment[3)]	2	1.43	0.25
α intercept	1	455.46	< 0.0001
Treatment	2	2.08	0.14
β intercept	1	13.94	0.0004
Treatment	2	3.17	0.05
R_d intercept	1	63.69	< 0.0001
Treatment	2	1.95	0.15

1) Numerator degrees of freedom. Denominator degrees of freedom were 61.
2) For initial screening, the level of significance was 0.15.
3) For treatment effects; H_0: no treatment effects.

Since the plot of standardized residuals vs. fitted values from model psn11 suggested that the variances were unequal, we used an exponential variance function with the fitted values as the covariate to model that feature. For details on modeling the residual variance structure with *NLME*, see Pinheiro and Bates (2000, Chap. 5). The new model (psn2) performed significantly better (Table 1). At this stage, parameter β was removed from the list of random parameters since its estimated variance was small, and the new model (psn21) performed as well as the old one (Table 1). Finally, the autocorrelation plot suggested that the residuals were not independent, and based on the nature of the autocorrelations, we used a second-order moving average, MA(2), process, to model the autocorrelations. The new model (psn3) performed significantly better (Table 1).

For both the OLSE- and GLSE-estimated nonlinear models (model psn.ols and psn.gnls, respectively, in the Appendix), the model performance criteria suggested that the fixed-effects treatments only affected P_s. For the GLSE model, residual heteroscedasticity was accounted for by a power variance function with the fitted values as the covariate, and its residual autocorrelations were accounted for by a second-order autoregressive, AR(2), process.

Model adequacy

The residuals vs. fitted values plot (Fig. 2a), the residuals autocorrelation plot (Fig. 2b) and the normal Q-Q plot (Fig. 2c) based on model psn3 suggested no deviation from the regular assumptions, and the Shapiro-Wilk normality test for residuals was not significant (W = 0.99, p = 0.84). The same test also suggested that the estimated random effects (P_s and the intercept of α) did not contravene the normality assumption (for P_s, W = 0.96, p = 0.83; for intercept of α, W = 0.97, p = 0.86). Therefore, we concluded that model psn3 met all of the underlying assumptions. The observed vs. fitted values plots (Fig. 3) suggested that model psn3 was adequate. Therefore, we adopted model psn3 as our final NLME model.

It is clear from Table 3 that the performance of model psn3 was superior to that of the models fitted by OLSE (psn.ols) and GLSE (psn.gnls). The estimated error variance of model psn3 was the smallest among the 3 models.

Meanings of model and parameter estimates

Random effects: Under the final NLME model, both P_s and α varied from seedling to seedling, but β and R_d did not. The estimated variances for both P_s and α (Table 4) also suggested that seedling-to-seedling variations were substantial (coefficients of variation were about 10% for both parameters). The final model also suggested that there was no correlation between the 2 random effects.

Error variance-covariance structure: The parameter estimates for the MA(2) pro-

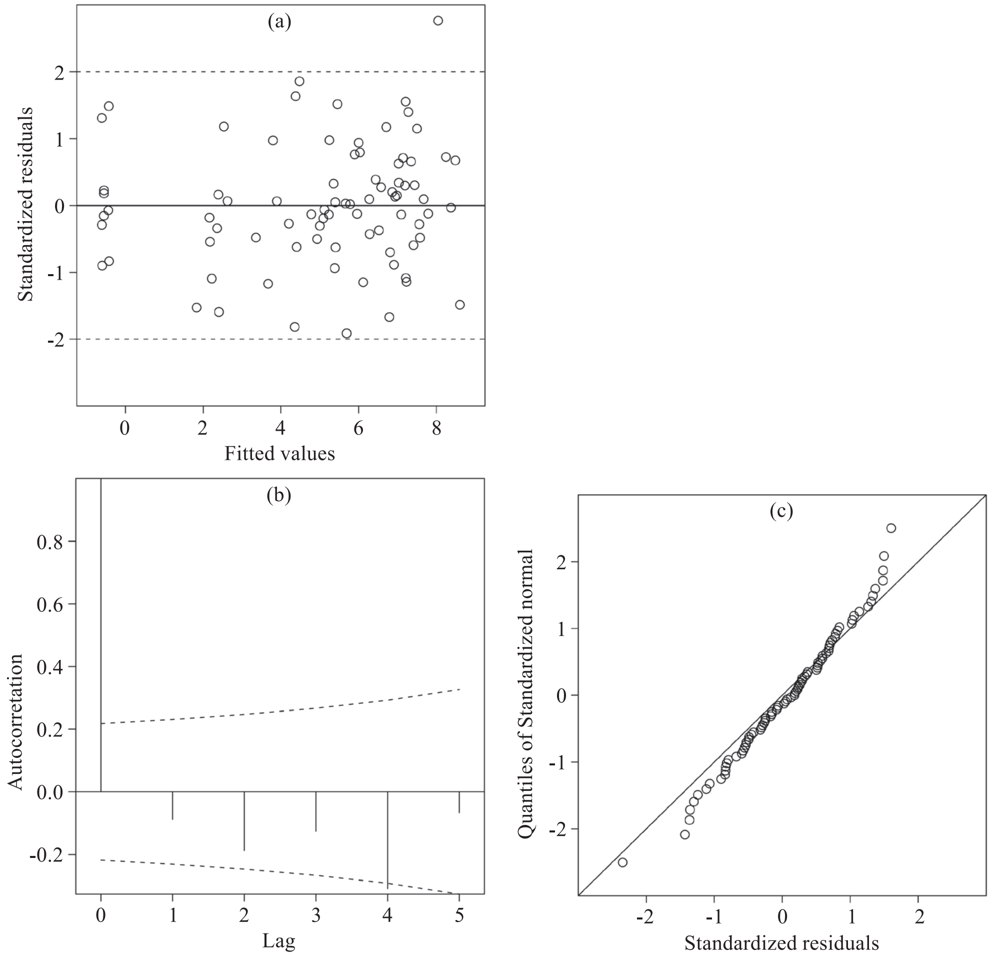

Fig. 2. Diagnostic plots of residuals of the final nonlinear mixed-effects model (model psn3). (a) Residuals vs. fitted values plot, (b) autocorrelation plot with 95% confidence interval bounds for no autocorrelation, and (c) residual normality Q-Q plot.

cess and the variance function suggested that our measurements indeed had a complicated error structure (Table 4). Net photosynthesis measurements were autocorrelated up to lag 2, and the measurement variability rose exponentially with increasing irradiance levels. Judging from the model performance criteria (Table 1), the residuals diagnostic plots (Fig. 2), and the error structure parameters estimates (Table 4), we successfully modeled that structure.

Treatment effects: From Table 5, we could infer how different light treatments affected photosynthesis characteristics of the seedlings. The estimated mean P_s value for the seedlings was about 7.53 µmol CO_2 m^{-2} s^{-1}. The 3 light treatments did not affect the mean P_s level of seedlings grown under different light environments. Shading did affect the mean responses of the quantum yield efficiency, dark respiration rate, or photoinhibition. Seedlings grown under full light had a higher mean quantum yield efficiency and a higher dark respiration rate than those

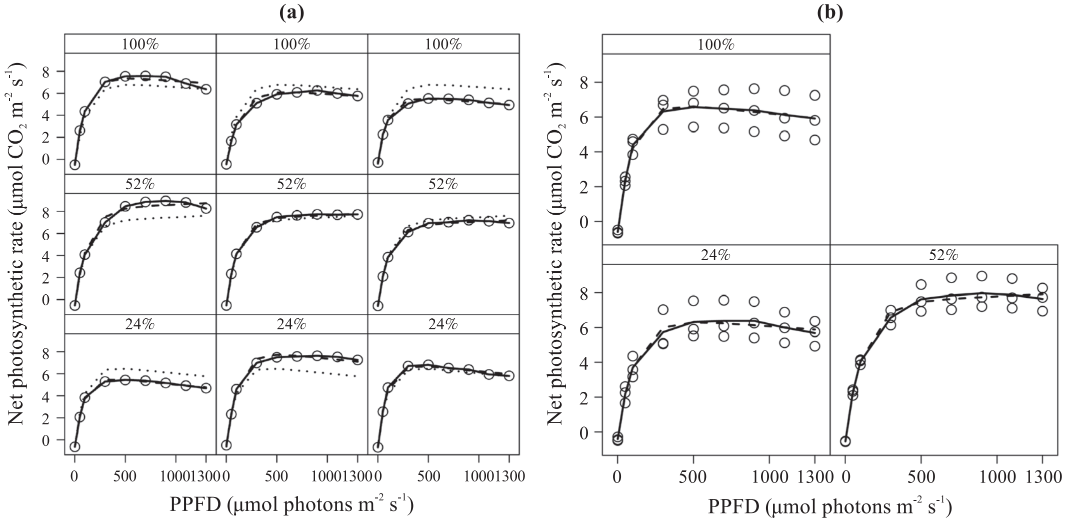

Fig. 3. Observed and fitted response plots based on the final mixed-effect nonlinear model. (a) Individual seedlings, and (b) mean trends for each treatment. Solid lines represent observed trends, dotted lines represent fitted values based on the fixed-effects alone, and dashed lines represent fitted values based on the mixed-effects. PPFD, photosynthetic photon flux density.

Table 3. Model performances and the estimated error variance ($\hat{\sigma}_\varepsilon^2$) of the chosen P-I curve fitted by the ordinary least-squares estimation (psn.ols), generalized least-squares estimation (psn.gnls), and nonlinear mixed-effects estimation based on the maximum likelihood (psn3). Refer to the text and Appendix for the features of each model

Model	df[1)]	AIC[2)]	BIC[2)]	Log-likelihood	$\hat{\sigma}_\varepsilon^2$
psn.ols	7	188.66	205.42	-87.33	0.546
psn.gnls	10	77.44	101.38	-28.72	0.165
psn3	16	28.07	66.38	1.97	0.010

[1)] Number of parameters in the model.

[2)] AIC, Akaike information criterion; BIC, Bayesian information criterion.

grown under 24% full light. Seedlings grown under 52% full light were not photoinhibited at higher irradiance levels, whereas seedlings grown under both 24 and 100% full light were photoinhibited.

In summary, P_s was a normally distributed random variable with an estimated mean of about 7.53 and an estimated variance of about 0.703 under the final NLME model. The quantum yield efficiency (α) was also a normally distributed random variable with an estimated variance of 3.9×10^{-5}. Seedlings grown under full light had the highest average quantum yield efficiency and dark respiration rate, followed by those under 52 and 24% light intensities. The patterns in the estimated mean values of α and R_d suggested that it would cost more for seedlings to have a higher quantum yield efficiency. This was also found in other studies (e.g., Aleric and Kirkman 2005). The photoinhibition parameter (β) suggested that photoinhibition did

Table 4. Parameter estimates for the components in the G and R matrices of the final nonlinear mixed-effects model

Matrix	Parameter	LCL [1)]	Estimate	UCL [1)]
G	$\sigma^2_{P_s}$	0.51	0.70	1.38
	σ^2_{α}	1.4×10^{-5}	3.9×10^{-5}	1.3×10^{-4}
R	θ_1 [2)]	0.21	0.61	1.00
	θ_2 [2)]	0.07	0.56	0.87
	δ [3)]	0.09	0.16	0.23
	σ^2_{ε}	0.004	0.010	0.025

1) Lower and upper 95% confidence limits.
2) Coefficients for the second-order moving average process.
3) Coefficient for the exponential variance function.

Table 5. Fixed-effects parameter estimates and their respective 95% confidence interval of the final nonlinear mixed-effects model (psn3)

Parameter	LCL [1)]	Estimate	UCL [1)]
P_s	6.89	7.53	8.17
α 24%	0.059	0.068a [2)]	0.076
α 52%	0.065	0.073ab	0.082
α 100%	0.072	0.080b	0.089
β 24%	0.0001	0.0007a	0.0012
β 52%	-0.0010	-0.0005b	-0.0002
β 100%	0.0003	0.0009a	0.0145
R_d 24%	0.39	0.50a	0.60
R_d 52%	0.52	0.63ab	0.73
R_d 100%	0.56	0.68b	0.80

1) Lower and upper 95% confidence limits.
2) Within a parameter, estimates followed by different letters significantly differ ($p < 0.05$) based on a conditional *t*-test.

occur at higher irradiance levels for seedlings grown under 24 and 100% full light. Seedlings grown under full light also showed a slightly higher, although not statistically significant, photoinhibition effect.

DISCUSSION

By conventional standards, the models fitted by OLSE (psn.ols) and GLSE (psn.gnls) were excellent. For example, the squared Pearson's correlation coefficient (r^2) between the fitted and observed values was above 0.92 for both models. Based on this standard, one would conclude that both models had accurately summarized the photosynthetic characteristics of the seedlings. However, the results from both models differed completely from that of the final NLME model. Both psn.ols and psn.gnls suggested that light treatments only affected P_s of the chosen P-I curve ($p < 0.001$), and seedlings grown under 52% full light had the highest average P_s (Table 6). Further, both models also suggested that there was no strong evidence of photoinhibition ($p > 0.1$, Table 6). Nevertheless, the data suggested that a certain degree of photoinhibition did occur for seedlings grown under 24 and 100% full light (Fig. 1).

Based on the 3 models, the estimated photoinhibition parameters were small (0.0005 for model psn.ols, 0.0002 for model psn.gnls, and 0.0007 for model psn3). Thus, all 3 models gave evidence that some degree of photoinhibition did occur. However, because the final NLME model did account for seedling-to-seedling variations, as well as the heterogeneity and autocorrelations in the data, it had an estimated error variance small enough to reject the null hypothesis of no photoinhibition

Table 6. Parameter estimates and their respective 95% confidence interval of the final ordinary least-squares estimation and generalized least-squares estimation models

Parameter	OLSE model (psn.ols)			GLSE model (psn.gnls)		
	LCL [1)]	Estimate	UCL [1)]	LCL	Estimate	UCL
P_s 24%	6.21	7.07a [2)]	7.93	5.59	6.36a	7.13
P_s 52%	7.81	8.71b	9.60	7.77	8.60b	9.43
P_s 100%	6.45	7.31a	8.18	6.22	7.00c	7.78
α	0.052	0.067	0.081	0.067	0.074	0.081
β	-0.0003	0.0005	0.0014	-0.0004	0.0002	0.0009
R_d	0.03	0.51	0.98	0.34	0.55	0.75

[1)] Lower and upper 95% confidence limits.

[2)] Within a parameter, estimates followed by different letters significantly differ ($p < 0.05$) based on a conditional *t*-test.

across the 3 light treatments (i.e., it was statistically more powerful). Both the OLSE- and GLSE-fitted models failed to reject the same null hypothesis because their estimated error variances contained information that was not explained by the model structure.

The lack of statistical power reflected on other parameter estimates as well. For example, the estimated treatment effects on the dark respiration rate were about the same for all 3 models (Tables 5, 6). However, the standard errors for the estimated treatment effects from the pns.ols, pns.gnls, and psn3 models were about 0.24, 0.10, and 0.06, respectively. Because differences among the treatment effects were small in comparison to the estimated standard errors (Table 5), both the OLSE and GLSE models were unable to detect the effects of light treatment on the dark respiration rate. In these respects, our example demonstrated the consequences of using an inadequate modeling approach to fit P-I curves.

NLME modeling can be extended to incorporate multilevel random effects as well (e.g., Zhao et al. 2005). For example, in many P-I relationship studies, more than 1 leaf from each plant is measured. Measurements then have 2 levels (i.e., an among-plant level and a within-plant but among-leaf level) of random effects. A mixed-effects model can be used to accommodate the additional levels of random effects. In that case, one might need to specify a variance-covariance structure for each level of random effects. Of course, one could also just average the measurements from different leaves within each plant and use a single-level NLME model. However, under that approach, one would assume that all random effects are due to plant-to-plant variations, which might not be true. An advantage of using a multilevel NLME modeling approach is that the real sources of variations can be identified and additional questions answered. The ability to accommodate multilevel random effects has been regarded as 1 of the advantages of the *NLME* package (Smith 2003).

Detecting the presence of photoinhibition is important in some ecophysiological studies (e.g., Senevirathna et al. 2003, Robakowski 2005). Rather than estimating the cardinal points of a chosen P-I curve first and then fitting the model or only comparing those cardinal points, the model and the estimation approach used in this study provide an integrated manner to detect both the presence and the degree of photoinhibition. The basic model form of Platt et al. (1980) can also be used to model other ecophysiological response curves as well. For example, instantaneous water-use efficiency often shows significant declines at higher irradiance levels. The Platt et al. (1980) model can be used to model the relationship between instantaneous water-use efficiency and irradiance.

Ecophysiological response curves are intrinsically nonlinear, and measurements usually have a complicated data structure. Both factors contribute to difficulties in analyzing and fitting data. With advances in statistical computing and the availability of reliable software, we should strive to use the best statistical estimation methods available to analyze ecophysiological response curves. Although using an inadequate estimation approach does not necessarily lead to incorrect results, it can occur, as our example has shown. We hope that this study will help ecophysiologists overcome their uneasiness with using an NLME modeling approach to analyze their data in the future when the need arises.

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APPENDIX

Summary of model fitting procedures and R codes (in italic) for the example

All notations follow Pinheiro and Bates (2000) and Pinheiro et al. (2006).

Step 1: Define the Platt et al. (1980) model. *ps* corresponds to P_s, *a* corresponds to α, *b* corresponds to β, and *rd* corresponds to R_d in the model. Refer to text for model details.

picurve = function (x, ps, a, b, rd) (ps(1 - exp (-a*x/ps))*(exp (-b*x/ps))) - rd*

Step 2: First model. Data of psng have a special grouped data structure grouped by individual trees. See Pinheiro and Bates (2000, Chap. 3) for details.

psn0 = nlme (psn ~ picurve (ppfd, ps, a, b, rd), fixed = (ps + a + b + rd ~ 1), random = (ps + a + b + rd ~ 1), start = c (7, 0.05, 0.001, 0.5), data = psng)

Step 3: Revise the model. The G matrix now has a diagonal structure for the remaining random parameters.

psn01 = update (psn0, random = pdDiag (ps + a + b ~ 1))

Step 4: Incorporate treatment effects into the model, by assuming that treatments affects all parameters.

psn1 = update (psn01,fixed = (ps + a + b + rd ~ treat), start = c (7,0,0,0.05,0,0,0.001,0,0,0.5,0,0))

Step 5:Treatment affects parameters *a*, *b* and *rd*, but not *ps*.

psn11 = update (psn1,fixed = list (ps ~ 1, a + b + rd ~ treat), start = c (7,0.05,0,0,0.0002,0,0,0.5,0, 0))

Step 6: Fit an exponential variance function with fitted values as the covariate to account for heteroscedasticity in the residual variance.

psn2 = update (psn11, weights = var Exp ())

Step 7:Remove parameter *b* from the list of random parameters.

psn21 = update (psn2, random = pd Diag (ps + a ~ 1))

Step 8: Fit an MA(2) process to account for autocorrelations among residuals within each tree. Final nlme model.

psn3 = update (psn21, corr = cor ARMA (q = 2))

Fit the 'best' nonlinear OLSE model. Only P_s was modeled as a function of treatments.

psn.ols = gnls (psn ~ picurve (ppfd, ps, a, b, rd), params = list (ps ~ treat, a + b + rd ~ 1), start = c (7,0,0,0.05,0,0.5), data = psng)

#Fit the 'best' nonlinear GLSE model. Variance function is a power function with fitted values as the covariate, and residual autocorrelations are modeled by a second-order autoregressive process, AR(2). Only P_s was modeled as a function of the treatments.

psn.gnls = gnls (psn ~ picurve (ppfd, ps, a, b, rd), params = list (ps ~ treat, a + b + rd ~ 1), start = c (7,0,0,0.05,0,0.5), weights = var Power(), corr = cor ARMA (p = 2), data = psng)