

Bioassay for Allelochemicals: Examples with **R**

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1 Introduction

Basic studies of efficacy of allelochemicals, all other things being equal, require knowledge of the relationship between plant response and dose from no effect at small doses, to complete kill at high doses. The control of weeds is determined by the size of the dose. The term ‘size of a dose’, however, is rather vague in that for some compounds, only little is needed to control weeds (e.g., many sulfonylureas herbicides) whereas for others we must apply several kg to obtain the same level of control (e.g., phenoxy acids). Whilst the size of a dose is rather well defined for herbicides this is not the case for allelochemicals, except when working with pure isolated compounds. The term toxicity is also rather vague in that it is essentially a relative term. Therefore, issuing any statement about the toxicity requires a standard compound against which a test compound can be compared.

The objective of this paper is to illustrate how the principles of assessing potency (toxic strength) of compounds in applied toxicology and pharmacology can be used in herbicide research and development and in allelopathy in that we compare potencies of compounds at some *a priori* response levels, say 50% reduction in biomass (ED50). This requires the use of dose-response relationships.

In order to facilitate the use of widely accepted dose-response relationships, without being entangled in the hassle of programming, we have developed software capable of carrying out simultaneous non-linear regression analyzes on several bioassay dose-response curves. We will demonstrate the use of the software package **drc** for analysis of series of dose-response curves. The package **drc** is an add-on package for the language and environment **R** which is open source and freely available. In order to take advantage of the **drc** package we advise the reader to familiarize him/herself with the basics of **R**, particularly how to get data in shape and be read into **R** (R Development Core Team, 2005). The functionality of **drc** is illustrated by means of examples, where the function calls in **R** and the resulting output are interwoven into the text together with the biological interpretation. The rationale behind developing this package is that a model is fitted only

once to data, and then all relevant information is extracted from this single model fit. A more comprehensive description of `drc` can be found elsewhere (Ritz & Streibig, 2005).

2 Vertical and horizontal assessment

Vertical assessment compares plant response at some preset dose levels (Fig. 1, a). This is the most common method for evaluating allelochemicals. If doses were chosen close to the upper or lower limit of the curves, differences between treatments would be less than if they were chosen in the middle part of the curve. If we are only working at dose-ranges in the middle part of the curves then, in this particular instance, differences would be almost independent of dose-levels. Consequently, the middle region is obviously the optimal part of the curve to obtain information about differences of effects.

Usually, allelochemicals are tested in two to three doses and their efficacy is compared with either the untreated control or with some standard chemicals. The nonlinear relationships in Fig. 1 show that if biologically active compounds are tested in a factorial experiment, we may get significant interactions, because the differences of effects are not constant. As this interaction is dose dependent due to the *S*-shaped curves, it may be considered trivial and of little biological significance when we appraise the action of the compounds. If we choose only dose-ranges in the middle part of the curves, then differences are constant and independent of dose-levels and the interaction would disappear, i.e. the effects are additive.

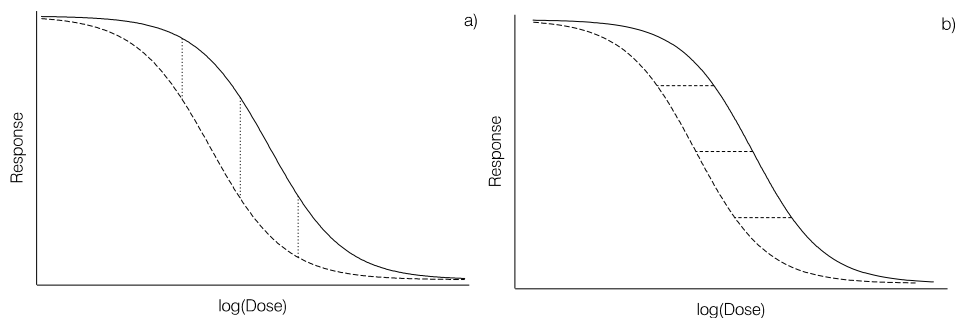


Figure 1: Vertical a) and horizontal b) comparison of dose-response curves for two biological active compounds in a plant species.

Doses of compounds giving the same response can also be compared (Fig. 1, b). This is called horizontal assessment. In this case the difference in horizontal displacement of the two curves is important. As the dose rates are on a logarithmic scale, the horizontal displacement expresses the ratio between doses yielding the same response. This ratio is also called the relative potency, R , and can be defined as

$$R = \frac{z_s}{z_t} \quad , \quad (1)$$

where z_s and z_t denote the dose of two compounds, a standard, H_s , and a test, H_t , having the same effect (equipotent doses). The relative potency tells us how much more or less test compound H_t must be used to obtain the same effect as for the dose z_s for a standard compound, H_s . If $R=1$ then the two compounds have the same potency; if $R>1$ then the test compounds, H_t , is more potent than the standard compound, H_s , and *vice versa* if $R<1$. We could consider the relative potency as being a measure of the biological exchange rate between compounds, analogous to the more common practice of exchanging currencies from say Euro to Danish Kroner (DKK). In this paper we only consider horizontal assessment.

3 The logistic dose-response curve

When plants are treated with a biologically active compound, the observed effects are mostly of two different types, graded or quantal. The response is graded or quantitative, if the results are changes in plant biomass, height, content of metabolites, photosynthetic capture of CO_2 etc. A quantal or qualitative response, also called ‘all-or-none’ response, is used if the individual plants can be classified killed/not killed, germinated/not germinated etc. With graded response each dose yields a response on a continuous scale and thus carries more information than the ‘all-or-none’ response. We only consider graded response.

Experience has shown that in most instances a logistic dose-response curve describes reasonably well what happens in the crop and weeds in response to dose of a herbicide or allelochemical. The logistic curve can be expressed as follows (Streibig et al. 1993):

$$y = c + \frac{d - c}{1 + \left(\frac{x}{e}\right)^b} \quad . \quad (2)$$

This curve is the four-parameter logistic curve, which is denoted **14** and is default in **drc** (an example is given in section 4).

$$y = c + \frac{d - c}{1 + \exp[b(\log(x) - \log(e))]} \quad . \quad (3)$$

sometimes the lower limit, c , can be omitted if the responses at high doses are close to zero, and then we have the three parameter logistic model, denoted **13** in **drc** (see section 4):

$$y = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \quad . \quad (4)$$

The parameters and variables in the models (2), (3) and (4) are defined in Table 1. The shape of a logistic curve is shown in Fig. 2. One of the properties of the logistic curve is that it is symmetrical curve around the parameter e (ED50).

Table 1: Interpretation of variables and parameters in the logistic dose-response curve models

Parameter/ variable	Interpretation
b	The proportional slope of the curve around the point of inflexion (e)
c	Lower limit of response (lower asymptote)
d	Upper limit of response (upper asymptote)
e	The dose reducing the response 50 % (ED50) between d and c , i.e, point of inflexion
x	Dose
y	Plant response (e.g. biomass)

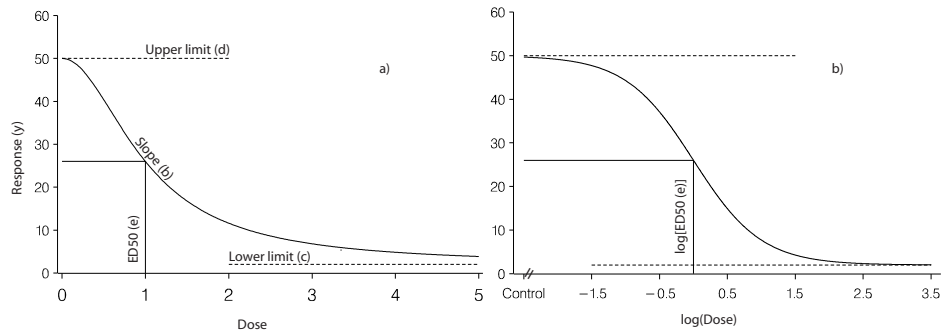


Figure 2: The logistic dose-response curve a) on non-logarithmic dose scale; b) on logarithmic dose scale. The parameters in this example are: $b=2$, $c=2$, $d=50$, $e=1$.

In contrast to linear regression, which essentially has an analytical solution, the non-linear regression only has a numerical solution. Therefore it is necessary to give some initial guesses for the non-linear regression parameters. For the less experienced researcher it may be difficult to guess the parameters and therefore the package `drc` provides self starter facilities by default. This means that the experimental data are used to obtain the initial parameter guesses. For the logistic curve this is done by using the maximum (initial d) and minimum (initial c) value of y and make a logit transformation of data as seen in equation (5) below

$$\log\left(\frac{d-y}{y-c}\right) = b(\log(x) - \log(e)) \quad . \quad (5)$$

This requires, however, that the lower limit, c , and the upper limit, d , are well described by data. If the dose-range is distributed so the response ranges from untreated control (d) to high doses, where the response is almost 0 (c), the initial guess of the slope, b , and e (ED50), are obtained by fitting a straight line to the points $(\log(x), \log((d-y)/(y-c)))$.

4 Fitting a single dose-response curve

To get started we need to load the package `drc`. This is done using the `library` function

```
> library(drc)
```

We will use the data set, `page215`, (see page 21) to illustrate our point about the assessment of potency. The data originate from Tables 3 and 4 in Gong *et al.* (2004) and the objective was to assess the inhibitory effect on plant growth. The data set `page215` contains a total of four individual response curves from the two tables, one for each of the two test species, oat and *Echinochloa crus-galli*, and the two secalonic acids denoted SAH and SAI :

```
> page215 <- read.table("page 215.txt", header = TRUE)
```

The main function in `drc` for fitting dose-response curves is the `multdrc` function which can be used to fit data from one or more bioassays. By default a four-parameter logistic model, model (3), is fitted to the data. In order to keep the example simple we first look at one dose-response curve, *viz.* that of oat and SAH,

```
> onecurve <- subset(page215, Species.Compound == "Oat-SAH")
```

which has a total of 7 observations. The `subset` function allows you to manipulate the data set `page215`. In this particular instance you select the observations being identified by the `Species.Compound` variable of Oat-SAH (see page 21). The data is displayed in the Appendix. A logistic curve fit is done:

```
> Oat.SAH.Fit <- multdrc(Root ~ Dose, data = onecurve)
```

Note that the `multdrc` does not produce any output, all the information of the logistic fit is saved in `Oat.SAH.Fit`. A summary of the fit, including estimated parameters, is obtained issuing the call

```
> summary(Oat.SAH.Fit)
```

```
A 'logistic' model was fitted.
```

```
Parameter estimates:
```

	Estimate	Std. Error	t-value	p-value
b:(Intercept)	2.6541799	0.6962051	3.8123535	0.0317
c:(Intercept)	0.0917716	0.3747035	0.2449180	0.8223
d:(Intercept)	5.5297540	0.2010299	27.5071241	0.0001
e:(Intercept)	0.0803548	0.0078816	10.1951964	0.0020

```
Estimate of residual variance: 0.08746787
```

While `Estimate` and `Std. Error` are self-explanatory, the `t-value` and `p-value` indicate whether a parameter is significantly different from zero. Furthermore the phrases also found in the output are:

- `A 'logistic' model was fit.` The logistic model has been used.
- `Estimate of residual variance: 0.0875` gives the estimated residual variance.

If you wish to have special explanation for the parameters then there is a naming facility in `multdrc`. The line

```
Oat.SAH.Fit <- multdrc(Dose, Root, fct=l4(names=c("Slope","Lower Limit",
"Upper Limit", "ED50 ")))
```

will produce the same fit, but with more informative names for the parameters. A plot of the observations and the fitted dose-response curve is displayed in Fig. 3.

Let us return to the parameter estimates in the above summary. The parameter estimate of the lower limit, c , is not significantly different from zero (p -value = 0.82). Fig. 3 illustrates that the responses are describing the upper and middle of the curve, whilst the lower limit is less well described by experimental data (only one observation). This illustrates one of the very problems with the distribution of responses, they should be evenly distributed so both the upper and lower limits are defined as well at the middle of the curve.

```
> plot(Oat.SAH.Fit, xlab = "Concentration (mM)", ylab = "Root length(mm)")
```

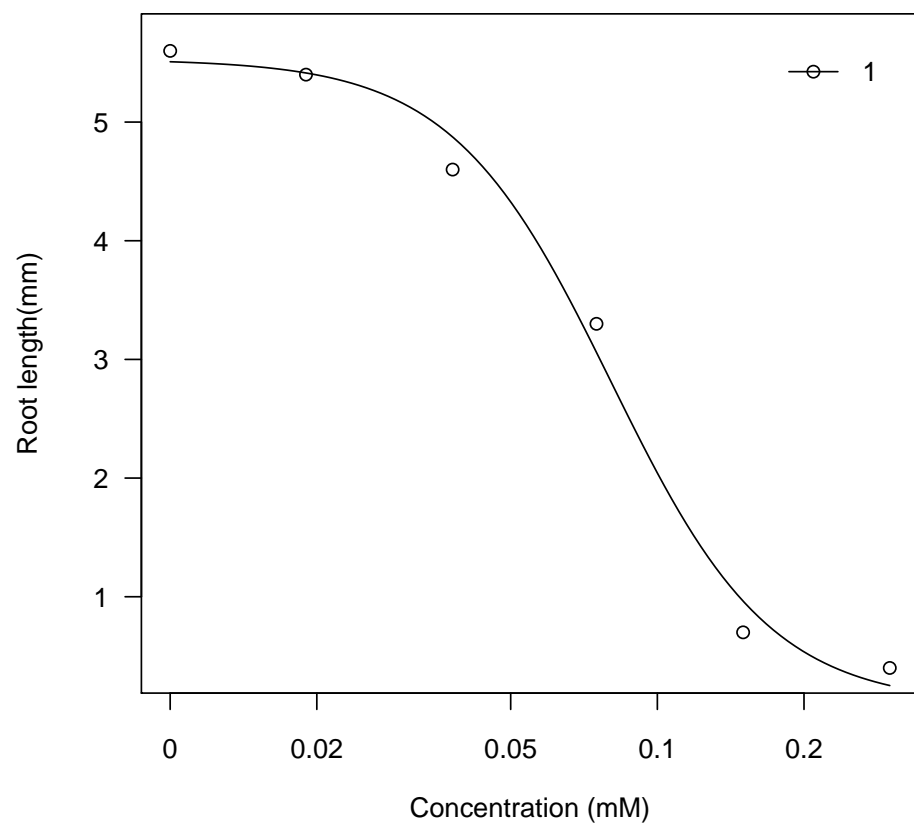


Figure 3: Non-linear regression fit for one dose-response curve for the Oat-SAH treatment (Gong *et al.* (2004))

Since the lower limit is not significantly different from zero we could use model (4), that is the three-parameter logistic model:

```
> Oat.SAH.Fit.13 <- multdrc(Root ~ Dose, fct = 13(), data = onecurve)
> summary(Oat.SAH.Fit.13)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:(Intercept)	2.5454687	0.4257559	5.9787052	0.0039
d:(Intercept)	5.5440543	0.1692710	32.7525264	5.182e-06
e:(Intercept)	0.0814925	0.0057292	14.2241282	0.0001

Estimate of residual variance: 0.06684133

Omitting the lower limit did not seriously affect the parameter estimates. Perhaps the ED50 value is not interesting because the compound should be used to control weeds, we would be more interested in say ED80 or ED90. As the EDy is a function of the slope, b , and e we can easily get the desired ED-levels with its associated standard error

```
> ED(Oat.SAH.Fit.13, c(80, 90))
```

	Estimate	Std. Error
1:80	0.14049	0.0133
1:90	0.19320	0.0267

Whether or not a four- or three-parameter logistic model is appropriate for this data set, cannot be assessed by means of a lack-of-fit test, because we only have mean response values for each dose and not the original response values.

5 Fitting several dose-response curves

As mentioned earlier one dose-response curve confers little information as to toxicity. Consequently, we usually run more than one dose-response curve as did Gong *et al.* (2004). Therefore we now wish to simultaneously fit all the response curves in the dataset [page215](#). This is done by defining which data belong to which curve. The variable to identify the curves is `Species.Compound` and this is the second argument in the specification below.

```
> All.Fit <- multdrc(Root ~ Dose, Species.Compound, data = page215)
> summary(All.Fit)
```


A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:Echin-SAI	2.9170340	0.8346208	3.4950411	0.0044
b:Oat-SAI	2.1005321	0.8655565	2.4267995	0.0319
b:Echin-SAH	4.6226801	1.8448293	2.5057495	0.0276
b:Oat-SAH	2.6541745	0.6630468	4.0029971	0.0018
c:Echin-SAI	-0.0010076	0.2251025	-0.0044760	0.9965
c:Oat-SAI	0.0564096	0.4967026	0.1135682	0.9115
c:Echin-SAH	-0.0009999	0.2231904	-0.0044801	0.9965
c:Oat-SAH	0.0917725	0.3568584	0.2571678	0.8014
d:Echin-SAI	4.0206187	0.2120884	18.9572804	2.604e-10
d:Oat-SAI	5.0098909	0.2829490	17.7059847	5.755e-10
d:Echin-SAH	4.0730072	0.1669232	24.4004862	1.353e-11
d:Oat-SAH	5.5297590	0.1914563	28.8826104	1.846e-12
e:Echin-SAI	0.0423292	0.0046071	9.1877956	8.873e-07
e:Oat-SAI	0.0633375	0.0082370	7.6893854	5.626e-06
e:Echin-SAH	0.0667753	0.0052008	12.8394723	2.269e-08
e:Oat-SAH	0.0803547	0.0075063	10.7050086	1.707e-07

Estimate of residual variance: 0.07933503

```
> plot(All.Fit, ylim = c(0, 7))
```

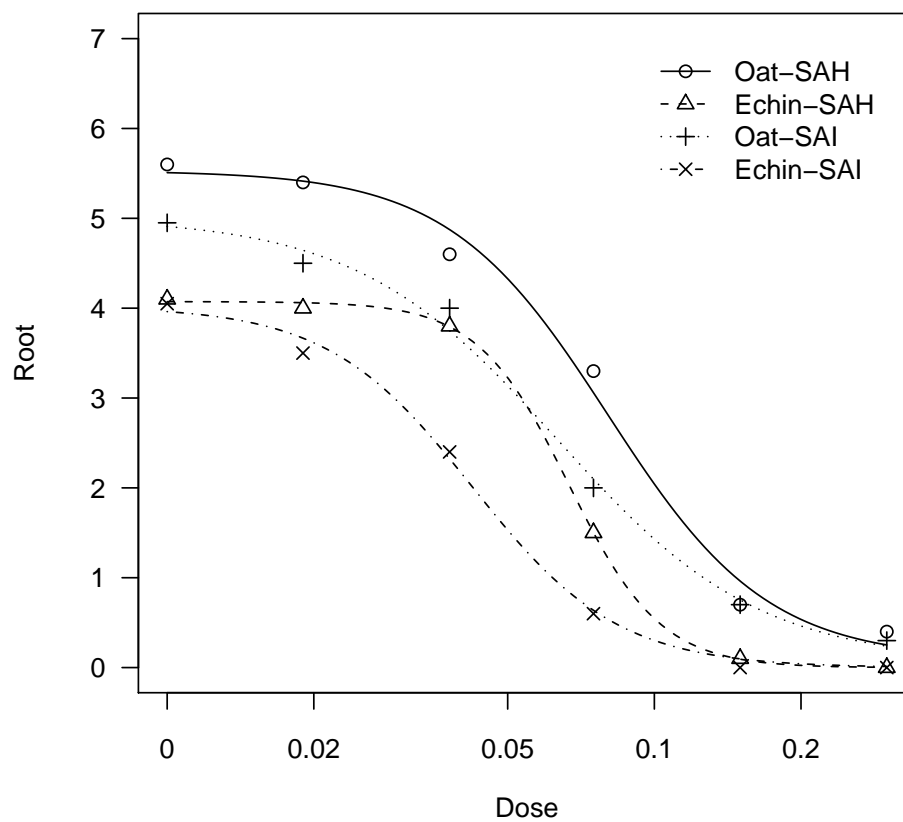


Figure 4: Non-linear regression fit of all four dose-response curves from Gong *et al.* (2004). Note that the argument `ylim=c(0,7)` is defining the response axis so the legends are separated from the curves.

The regression model seemed to describe the response curves within reasonable limits, judged by the fit in Fig. 4. In section 6 we will demonstrate that when working on original data we have some strong tools to statistically assess how well a dose-response model describes the data.

It is obvious from the parameters and the Fig. 4 that oat generally had higher upper limits than did *Echinochloa crus-galli*. The parameters for the lower limits, however, are not significantly different from zero and even `c:Echin-SAI` has an illogically negative lower limit.

Apparently the data can be described by a model with zero as lower limit, model (4):

```
> All.Fit.13 <- multdrc(Root ~ Dose, Species.Compound, fct = 13(),
+   data = page215)
> summary(All.Fit.13)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:Echin-SAI	2.9188467	0.6295943	4.6360756	3e-04
b:Oat-SAI	2.0202678	0.3807812	5.3055873	1e-04
b:Echin-SAH	4.6257211	1.4955810	3.0929258	7e-03
b:Oat-SAH	2.5454721	0.4029956	6.3163777	1.026e-05
d:Echin-SAI	4.0204546	0.1813394	22.1708781	1.943e-13
d:Oat-SAI	5.0292574	0.2004656	25.0878807	2.838e-14
d:Echin-SAH	4.0729025	0.1438160	28.3202289	4.231e-15
d:Oat-SAH	5.5440520	0.1602216	34.6024065	2.013e-16
e:Echin-SAI	0.0423206	0.0036547	11.5797752	3.442e-09
e:Oat-SAI	0.0639121	0.0058326	10.9577190	7.584e-09
e:Echin-SAH	0.0667688	0.0043446	15.3683145	5.308e-11
e:Oat-SAH	0.0814925	0.0054229	15.0275289	7.434e-11

Estimate of residual variance: 0.05988557

Fixing the lower limits at zero did not change the parameter estimates much.

The question is now how to compare the potencies of the two compounds in the two species. We could, for example compare the relative potencies, as defined by equation (1), among compounds and species at the ED50 response level. The comparisons are given by means of the selectivity index (SI)

```
> SI(All.Fit.13, c(50, 50))
```

	Estimate	Std. Error	t-value	p-value
Oat-SAH/Echin-SAH:50/50	1.22052	0.11359	1.94127	0.0701

Oat-SAH/Oat-SAI:50/50	1.27507	0.14401	1.91005	0.0742
Oat-SAH/Echin-SAI:50/50	1.92560	0.20993	4.40903	0.0004
Echin-SAH/Oat-SAI:50/50	1.04470	0.11709	0.38173	0.7077
Echin-SAH/Echin-SAI:50/50	1.57769	0.17059	3.38639	0.0038
Oat-SAI/Echin-SAI:50/50	1.51019	0.18974	2.68883	0.0161

In contrast to the output from `summary`, where the p -value indicates if a parameter is different from zero, the p -value in the output from `SI` indicates whether the relative potency is different from 1. In other words it tests whether two compounds have the same potency at the ED50 level.

However, the relative differences are not dramatically large. From a weed management point of view, the ED50 might not be the best response level to consider when looking for allelopathic potential. ED90 might be a more appropriate response level to consider. The `SI` function can calculate selectivity indices for any EDy levels, for instance at ED90 levels:

```
> SI(All.Fit.l3, c(90, 90))
```

	Estimate	Std. Error	t-value	p-value
Oat-SAH/Echin-SAH:90/90	1.79942	0.33605	2.37885	0.0302
Oat-SAH/Oat-SAI:90/90	1.01877	0.22915	0.08193	0.9357
Oat-SAH/Echin-SAI:90/90	2.15040	0.44136	2.60651	0.0191
Echin-SAH/Oat-SAI:90/90	0.56617	0.12825	-3.38264	0.0038
Echin-SAH/Echin-SAI:90/90	1.19506	0.24737	0.78852	0.4419
Oat-SAI/Echin-SAI:90/90	2.11077	0.51080	2.17459	0.0450

At the ED90 response level, there are now four relative potencies that are significantly different from 1. The difference in the results between ED50 and ED90 levels is caused by the shape of the response curves. At high effect levels the intrinsic differences among curves may be change in comparison to lower effect levels (see also Fig. 4).

6 Fitting bioassays and statistics

Until now we have only had the mean values taken from Gong *et al.* (2004). As pointed out in section 4, we could not statistically test whether the logistic model was a appropriate model to use.

From Inderjit *et al.* (2002) we have original data and we can statistically assess whether the response fit is appropriate and the data follow the logistic curve as illustrated by Streibig *et al.* (1993). The selected data are the effect of p -hydroxybenzoic acid (HBA) or ferulic acid (FA) on perennial ryegrass root and shoot length. The objective of the bioassays was to study the joint action of various phenolic acid mixtures on plant growth. Only the pure compounds are used here. We consider root length as response.

```
> Inderjit <- read.table("inderjit.txt", header = TRUE, , dec = ",")
```

The structure of the data is seen in the Appendix. The bioassay was a completely randomized design with five doses of each of the two compounds. There was three replications for each compound and dose and six replications for the untreated control, which was common for both compounds. LR denotes root length and LS shoot length. The fit of the four parameter logistic curves with common upper limit is obtained as follows:

```
> Inderjit.ls <- multdrc(LR ~ Dose, TRT, collapse = data.frame(TRT,
+      TRT, 1, TRT), data = Inderjit)
```

Control measurements detected for level: control

```
> summary(Inderjit.ls)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:FA	2.88210	0.52355	5.50488	3.472e-06
b:HBA	1.08500	0.19671	5.51580	3.359e-06
c:FA	0.38636	0.22538	1.71430	0.0953
c:HBA	-0.93911	1.03596	-0.90651	0.3709
d:(Intercept)	6.93485	0.16627	41.70853	9.813e-32
e:FA	3.81129	0.24574	15.50963	1.484e-17
e:HBA	7.59854	2.30085	3.30248	0.0022

Estimate of residual variance: 0.2319723

In order to let the response curves share a common upper limit, the

```
collapse=data.frame(TRT,TRT,1,TRT)
```

argument instructs `multdrc` to let the d -parameter be a common parameter for both curves. The `data.frame` reflects which parameters are being shared among response curves and it takes the parameters in alphabetic order (b, c, d, e). From the summary it is obvious that the two lower limits are not significantly different from zero, and the parameter estimate for `c:HBA` is even negative. A test for lack of fit below (`anova(Inderjit.ls)`), is not significant. The test for lack of fit supports the idea that the logistic model is describing the data appropriately. This is also supported by the distribution of data and regression lines in Fig. 5.

```
> anova(Inderjit.ls)
```

Lack-of-fit test

	ModelDf	RSS	Df	F value	p value
Two-way ANOVA	29	7.3429			
DRC model	35	8.1190	6	0.5108	0.7951

```
> plot(Inderjit.ls, ylim = c(0, 8))
```

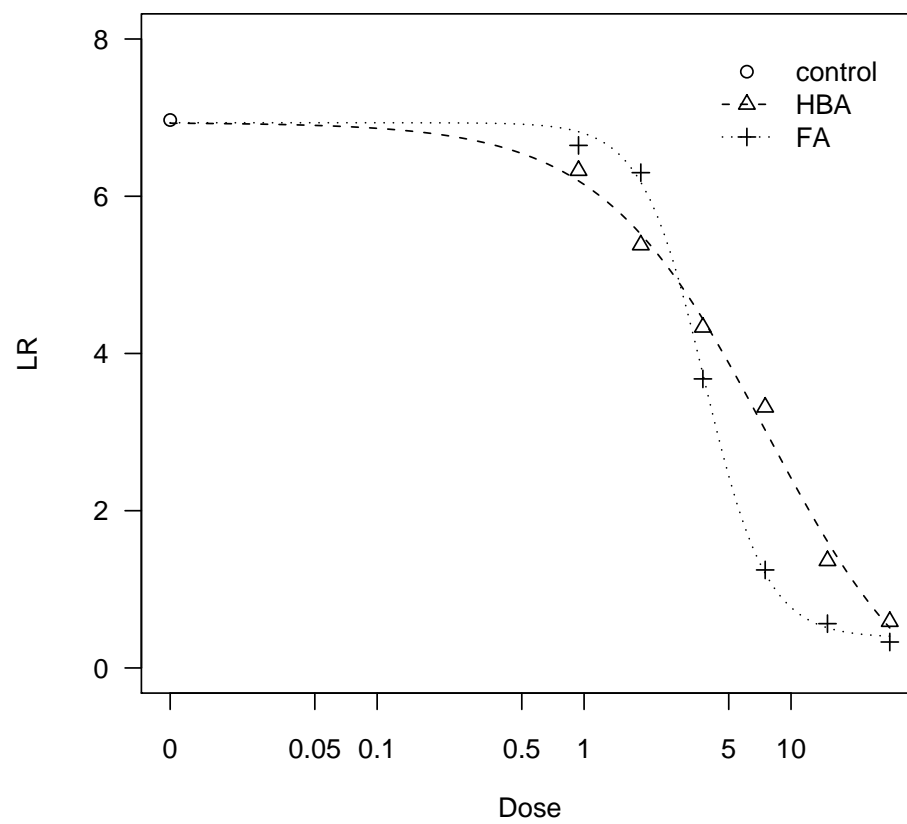


Figure 5: Non-linear regression fit of root length on *p*-hydroxybenzoic acid (HBA) or ferulic acid (FA) (Inderjit *et al.*, 2002).

But a closer look at the variation in data shows that the variation is much more pronounced at low doses than at high doses; that is the variance of responses is not homogeneous. Briefly, it means that the assumption of constant variance cannot be entertained. It might have an impact on the parameter estimates and the estimated standard errors in particular and also affect various statistical tests. We can in **drc** make a graphical visualization of the variation of the residuals. A plot of the predicted versus the residuals illustrated in Fig. 6 shows heterogenic variance.

```
> plot(fitted(Inderjit.ls), residuals(Inderjit.ls))
> abline(h = 0)
```

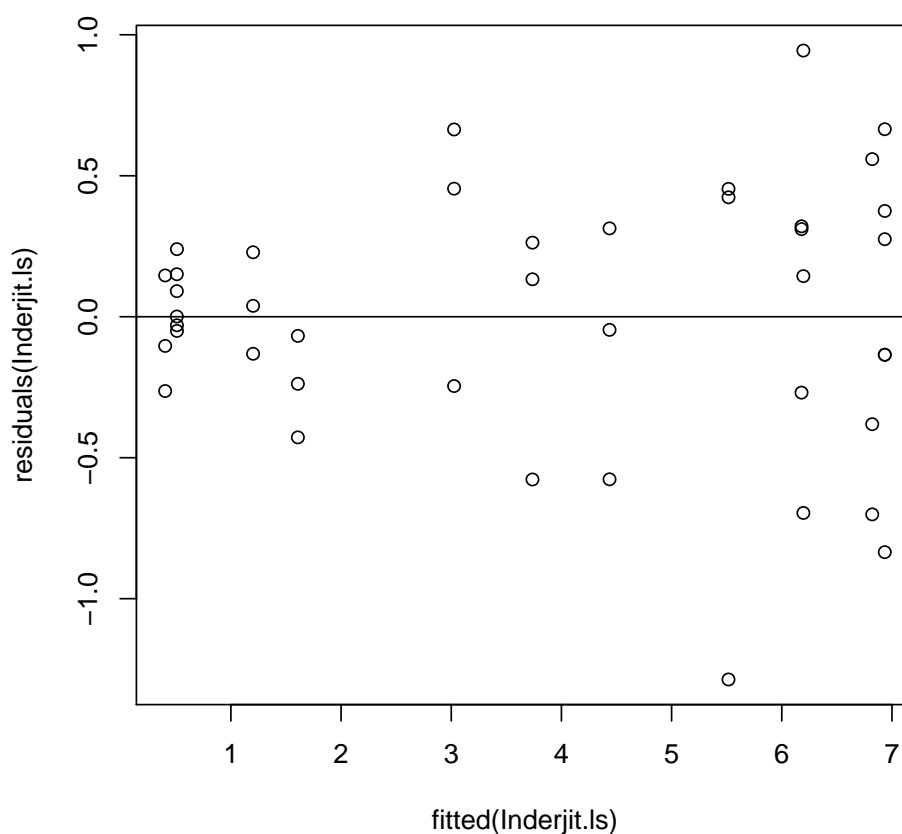


Figure 6: Plot of residuals versus predicted (fitted) values. It is obvious that the variance at low predicted values (high doses) are lower than the high predicted values (low doses). This is often the case when we have large differences between the maximum and minimum responses.

The problem of heterogeneous variances is common in biology but it can be dealt with by using the Box-Cox transformation in a so-called transform both sides technique (Streibig *et al.*, 1993). Consequently, we could re-fit the model with a Box-Cox transformation before further analysis, notice the argument `boxcox=T`:

```
> Inderjit.boxcox <- multdrc(LR ~ Dose, TRT, boxcox = T, collapse = data.frame(TRT,
+   TRT, 1, TRT), data = Inderjit)
```

Control measurements detected for level: control

```
> summary(Inderjit.boxcox)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:FA	2.668026	0.340567	7.834069	3.314e-09
b:HBA	1.164271	0.200003	5.821256	1.328e-06
c:FA	0.317887	0.085675	3.710380	0.0007
c:HBA	-0.558914	0.561421	-0.995534	0.3263
d:(Intercept)	6.935612	0.216204	32.078967	7.619e-28
e:FA	3.837436	0.231875	16.549620	1.995e-18
e:HBA	6.723966	1.137844	5.909391	1.016e-06

Estimate of residual variance: 0.06039547

Heterogeneity adjustment: Box-Cox transformation

Estimated lambda: 0.5

Confidence interval for lambda: [0.299,0.674]

```
> anova(Inderjit.boxcox)
```

Lack-of-fit test

	ModelDf	RSS	Df	F value	p value
Two-way ANOVA	29	1.8015			
DRC model	35	2.1138	6	0.8380	0.5509

The Box-Cox transformation was justified in that the optimal λ was 0.5 and the confidence interval for λ was between 0.299 and 0.674. It means that a square root transformation is required. Furthermore, the lower limits for the two compounds are now different; c:HBA is taking a rather large negative value (-0.56) but it is not different from zero. As

mentioned previously a negative lower limit is in this case illogical. The lower limit of FA, `c:FA` is positive and definitely different from zero. In order to get rid of the negative lower limit we assume that both curves have a common lower limit

```
> Inderjit.bboxcox.common <- multdrc(LR ~ Dose, TRT, bboxcox = T,
+   collapse = data.frame(TRT, 1, 1, TRT), data = Inderjit)
```

Control measurements detected for level: control

```
> summary(Inderjit.bboxcox.common)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:FA	2.610883	0.350012	7.459408	8.273e-09
b:HBA	1.541319	0.163085	9.451014	2.747e-11
c:(Intercept)	0.273100	0.084313	3.239139	0.0026
d:(Intercept)	6.827802	0.219320	31.131758	6.313e-28
e:FA	3.927215	0.245507	15.996379	3.065e-18
e:HBA	5.538348	0.553478	10.006445	6.106e-12

Estimate of residual variance: 0.06681839

Heterogeneity adjustment: Box-Cox transformation

Estimated lambda: 0.5

Confidence interval for lambda: [0.299,0.674]

Also it is notable that the Std. Errors have changed for some of the parameters and perhaps more importantly the estimate `e:HBA` dropped from 7.60 to 6.72 and a bit further to 5.54. The drop in `e:HBA` is caused by the relative large change in the lower limit of -0.56 to 0.27. `e:FA` did not change dramatically. Finally, the test for lack of fit is not significant so we can tentatively entertain the idea that, after transformation, the four parameter logistic model with common upper and lower limits describes the dose-response data within acceptable limits.

The only thing left to do is to find out if there is any difference in potency between the two compounds. As was the case in section 4 we wish to look at the ED50 effect level but also the ED90 as the objective of the study was to find some allelochemicals that can help control weeds:

```
> SI(Inderjit.bboxcox.common, c(50, 50))
```

	Estimate	Std. Error	t-value	p-value
HBA/FA:50/50	1.41025	0.23811	1.72293	0.0935

```
> SI(Inderjit.bboxcox.common, c(90, 90))
```

	Estimate	Std. Error	t-value	p-value
HBA/FA:90/90	2.52881	0.69592	2.19681	0.0346

Only at the ED90 response level the relative potency between the compounds is slightly, significantly different from 1.

An analysis for shoot length (LS) follows the same lines as for root length. There was, however, no reason to do a Box-Cox transformation (analysis not shown) on the basis of the distribution of the responses.

```
> Inderjit.shoot <- multdrc(LS ~ Dose, TRT, collapse = data.frame(TRT,
+   TRT, 1, TRT), data = Inderjit)
```

Control measurements detected for level: control

```
> summary(Inderjit.shoot)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:FA	1.461031	0.499500	2.924984	0.0060
b:HBA	0.884817	0.499899	1.769991	0.0854
c:FA	0.434604	1.520977	0.285740	0.7768
c:HBA	0.475426	6.541076	0.072683	0.9425
d:(Intercept)	5.777982	0.169843	34.019457	1.038e-28
e:FA	13.037960	6.154547	2.118427	0.0413
e:HBA	31.184933	82.065223	0.380002	0.7062

Estimate of residual variance: 0.2471985

```
> anova(Inderjit.shoot)
```

Lack-of-fit test

	ModelDf	RSS	Df	F value	p value
Two-way ANOVA	29	7.9551			
DRC model	35	8.6519	6	0.4234	0.8573

The test for lack of fit is not significant. The ED50 for shoot length is much higher than for root length. Furthermore, the Std. Error for e:HBA, ED50, is so large that it is not significantly from zero. The same applies to the lower limits for both compounds. The reason for the large variation is illustrated in Fig. 7; the dose-range was not wide enough to describe the whole curve. It is conveniently reflected in the very high Std. Error for the e:HBA and e:FA, that is for the ED50 values.

```
> plot(Inderjit.shoot, conName = "Control", xlim = c(0, 100),
+      ylim = c(0, 8))
```

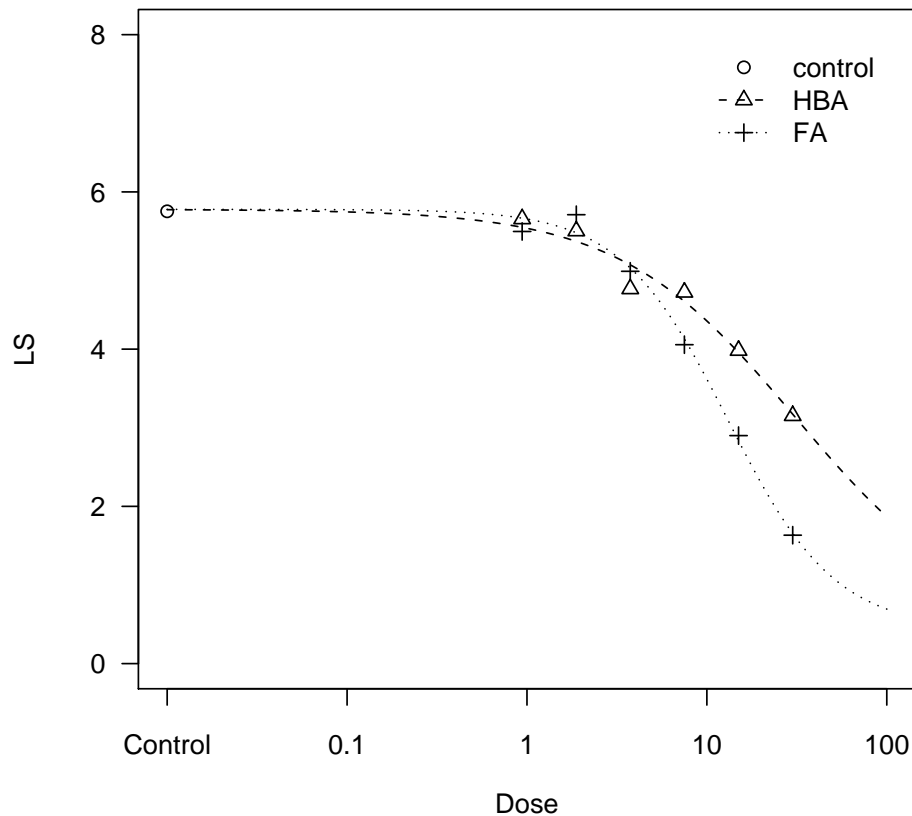


Figure 7: Non-linear regression fit of shoot length on *p*-hydroxybenzoic acid (HBA) or ferulic acid (FA) Inderjit et al. (2002).

For the same reason the lower limit is also rather indeterminate, we have no observations to support any of the lower limits. Therefore we could as well fit the model without lower limit, model (4),

```
> Inderjit.shoot.no.c <- multdrc(LS ~ Dose, TRT, fct = 13(),
+   collapse = data.frame(TRT, 1, TRT), data = Inderjit)
```

Control measurements detected for level: control

```
> summary(Inderjit.shoot.no.c)
```

A 'logistic' model was fitted.

Parameter estimates:

	Estimate	Std. Error	t-value	p-value
b:FA	1.35081	0.19443	6.94757	3.330e-08
b:HBA	0.84809	0.17650	4.80494	2.573e-05
d:(Intercept)	5.79275	0.15521	37.32174	2.869e-31
e:FA	14.83638	1.54631	9.59468	1.400e-11
e:HBA	37.34556	8.42995	4.43011	1e-04

Estimate of residual variance: 0.2342373

```
> anova(Inderjit.shoot.no.c)
```

Lack-of-fit test

	ModelDf	RSS	Df	F value	p value
Two-way ANOVA	29	7.9551			
DRC model	37	8.6668	8	0.3243	0.9500

The ED50 value for HBA is now significantly different from zero and with a much lower Std. Error than when including the lower limit. The ED50 for FA did not change as dramatically as did HBA, but its Std. Error was also considerably reduced. This example clearly shows that routine fitting without taking into account the distribution of responses may lead to dubious conclusions. The relative potency:

```
> SI(Inderjit.shoot.no.c, c(50, 50))
```

	Estimate	Std. Error	t-value	p-value
HBA/FA:50/50	2.5172	1.4314	1.0599	0.2961

```
> SI(Inderjit.shoot.no.c, c(90, 90))
```

	Estimate	Std. Error	t-value	p-value
HBA/FA:90/90	6.6017	3.1667	1.7689	0.0852

is not significant at the ED50 level. At the ED90 level the relative potency is also not significant, as there is in fact no data to substantiate the HBA curve at this very response level.

7 Concluding remarks

The package `drc` contains several other facilities, for example alternative regression models that are not symmetrical. The `collapse()` argument is flexible in that we can reduce parameters and test whether common parameters can be shared among dose-response curves. This has been shown for the four-parameter and three-parameter logistic models. In the examples given here the biological lesson learnt is that fitting non-linear dose response curves is not a trivial matter and cannot be run routinely without proper statistical tests and a close look at the parameter estimates and their biological meaning (see Table 1).

The great advantage of using regression model to describe dose-response curves in bioassay with allelochemicals or any other biologically active compounds is that we can test various hypotheses about the action of the compounds; and on the basis of few statistical tests perhaps reduce parameters and thereby in a parsimonious way present experimental data in a biologically meaningful way, independent of pre-set doses. Last but not least we can tell the reader how far our experimental data can take us without compromising scientific principles. That said, the experienced experimenter knows that any response curve is just a crude and simplified way of looking at biological variation, and in several instances the regression models do not always fit. Whether the models fit or not, by choosing a response function on the basis of biologically knowledge of how a system operates is far better than denying the sound use of statistical methods.

Appendix

Dataset used in the chapter:

```
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```

	Species.Compound	Dose	Root
1	Oat-SAH	0.000	5.5
2	Oat-SAH	0.010	5.7
3	Oat-SAH	0.019	5.4
4	Oat-SAH	0.038	4.6
5	Oat-SAH	0.075	3.3
6	Oat-SAH	0.150	0.7
7	Oat-SAH	0.300	0.4
8	Echin-SAH	0.000	4.0
9	Echin-SAH	0.010	4.2
10	Echin-SAH	0.019	4.0
11	Echin-SAH	0.038	3.8
12	Echin-SAH	0.075	1.5
13	Echin-SAH	0.150	0.1
14	Echin-SAH	0.300	0.0
15	Oat-SAI	0.000	5.5

16	Oat-SAI	0.010	4.4
17	Oat-SAI	0.019	4.5
18	Oat-SAI	0.038	4.0
19	Oat-SAI	0.075	2.0
20	Oat-SAI	0.150	0.7
21	Oat-SAI	0.300	0.3
22	Echin-SAI	0.000	4.0
23	Echin-SAI	0.010	4.1
24	Echin-SAI	0.019	3.5
25	Echin-SAI	0.038	2.4
26	Echin-SAI	0.075	0.6
27	Echin-SAI	0.150	0.0
28	Echin-SAI	0.300	0.0

> *onecurve*

	Species.Compound	Dose	Root
1	Oat-SAH	0.000	5.5
2	Oat-SAH	0.010	5.7
3	Oat-SAH	0.019	5.4
4	Oat-SAH	0.038	4.6
5	Oat-SAH	0.075	3.3
6	Oat-SAH	0.150	0.7
7	Oat-SAH	0.300	0.4

> *Inderjit*

	TRT	Dose	LR	LS
1	control	0.00	7.31	5.64
2	control	0.00	7.21	5.63
3	control	0.00	7.60	7.10
4	control	0.00	6.80	4.88
5	control	0.00	6.10	5.33
6	control	0.00	6.80	5.94
7	HBA	0.94	5.50	5.34
8	HBA	0.94	6.34	5.44
9	HBA	0.94	7.14	6.19
10	HBA	1.88	5.97	5.47
11	HBA	1.88	5.94	5.45
12	HBA	1.88	4.23	5.59
13	HBA	3.75	4.75	4.71
14	HBA	3.75	3.86	4.05

15	HBA	3.75	4.39	5.54
16	HBA	7.50	2.78	4.66
17	HBA	7.50	3.48	4.94
18	HBA	7.50	3.69	4.57
19	HBA	15.00	1.37	4.21
20	HBA	15.00	1.18	3.74
21	HBA	15.00	1.54	4.00
22	HBA	30.00	0.51	3.32
23	HBA	30.00	0.66	3.11
24	HBA	30.00	0.60	3.03
25	FA	0.94	6.12	5.35
26	FA	0.94	6.44	5.86
27	FA	0.94	7.38	5.28
28	FA	1.88	5.91	5.67
29	FA	1.88	6.50	5.22
30	FA	1.88	6.49	6.24
31	FA	3.75	4.00	5.90
32	FA	3.75	3.87	4.02
33	FA	3.75	3.16	5.05
34	FA	7.50	1.43	4.13
35	FA	7.50	1.24	4.04
36	FA	7.50	1.07	4.00
37	FA	15.00	0.75	2.69
38	FA	15.00	0.46	2.88
39	FA	15.00	0.48	3.13
40	FA	30.00	0.55	1.10
41	FA	30.00	0.30	1.50
42	FA	30.00	0.14	2.30

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