BIOASSAY97: A new Excel VBA macro to perform statistical analyses on herbicide dose-response data

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BIOASSAY97: A NEW EXCEL® VBA MACRO TO PERFORM STATISTICAL ANALYSES ON HERBICIDE DOSE-RESPONSE DATA

BIOASSAY97: EXCEL® ADD-IN PER L'ELABORAZIONE STATISTICA DEL DOSAGGIO BIOLOGICO CON ERBICIDI

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Summary

This paper presents BIOASSAY97, a new EXCEL® macro add-in to perform non-linear regression analysis on bioassay data. This macro has been specifically developed to comply with all the peculiarities of herbicide bioassays, even though it can be used with any kind of bioassays, especially by users with limited knowledge in statistics and computer programming. Starting from experimental data, BIOASSAY97 estimates all the most important ED-levels, such as the ED10, ED50, ED90 and any other user specified ED-levels, which are very important as decision tools in defining rational Integrated Weed Management Systems. In particular, those indicators can be used as a basis to adjust herbicide doses according to pedological, floristic and meteorological conditions. This latter aspect is particularly important as the climatic component is frequently neglected when selecting herbicide doses. Simultaneous fitting of several dose-response curves to the same dataset is also possible, in order to estimate the relative efficiency of either several herbicides or the same herbicide in different formulations or environmental conditions or weed flora situations. Three basic response models are built-in BIOASSAY97: a log-logistic symmetric model, a Gompertz model and a peaked logistic model; constraints on parameters can be introduced in several ways, according to user specified needs, to increase the flexibility of BIOASSAY97 and be able to analyse data from any type of bioassay experiments. The Box-Cox-transform-both-sides approach was built in, for the cases where the assumption of variance homogeneity is violated. Estimates are always provided with standard errors and confidence intervals; graphical analysis of residuals and F test for lack of fit are also possible to evaluate the goodness of regression. BIOASSAY97 has been extensively tested and validated, it is freeware and can be easily downloaded from the author web-site.

Key-words: Dose-response curves, pesticides, non-linear regression, simultaneous fitting

Riassunto

Viene presentata BIOASSAY97, una macro add-in di EXCEL® utilizzabile per l'adattamento di curve dose-risposta ai dati di prove di diserbo chimico. La macro è specificatamente pensata per le peculiarità del dosaggio biologico con erbicidi, ma è utilizzabile in ogni tipo di dosaggio biologico; essa consente anche ad operatori con limitate conoscenze statistiche ed informatiche di ottenere tutti gli indicatori di tossicità più importanti, quali l'ED10, l'ED50, l'ED90 e ogni altro livello di Dose Efficace, che sono estremamente utili come supporto decisionale per un più razionale diserbo chimico nell'ambito di programmi di gestione integrata della flora infestante. In particolare, le Dosi Efficaci possono essere utilizzate per adeguare la dose di diserbante alle reali condizioni floristiche, pedologiche e climatiche al momento del trattamento. Quest'ultimo aspetto è di particolare importanza, in quanto la componente meteorologica è spesso sottovalutata nella scelta delle dosi degli erbicidi. Oltre all'adattamento ai dati sperimentali di singole curve dose-risposta, BIOASSAY97 permette anche l'adattamento simultaneo di più curve di risposta allo stesso set di dati, in modo da poter stimare l'efficienza relativa di diversi erbicidi o dello stesso erbicida in diverse formulazioni o diverse condizioni floristiche e/o pedo-climatiche. In BIOASSAY97 sono disponibili tre modelli di risposta: un modello log-logistico simmetrico, il modello sigmoidale di Gompertz ed un modello sigmoidale con picco iniziale; per ciascun modello, possono essere messi vincoli sui valori dei parametri, in modo da avere la massima flessibilità d'impiego nelle diverse tipologie di dosaggio biologico. Nei casi in cui viene violata l'assunzione di omoschedasticità della risposta è disponibile la famiglia di trasformazioni di Box-Cox, secondo l'approccio TBS (transform-both-sides). Assieme alle stime vengono forniti errori standard ed intervalli di confidenza; sono implementate anche le procedure grafiche (analisi dei residui) e numeriche (eventuale test F per la mancanza di adattamento) per valutare la bontà della regressione. BIOASSAY97 è stata testata e validata, è completamente gratuita e può essere liberamente scaricata dal sito web dell'autore.

Parole chiave: *Curve dose-risposta, agrofarmaci, regressione non lineare, adattamento simultaneo*

Introduction

It has been clearly recognised that the use of chemical means within an Integrated Weed Management System (IWMS; Shaw, 1982) has to meet some basic requirements (Berti *et al.*, 2001):

1 - herbicides are to be sprayed only when this practise is economically convenient;
2 - active ingredients are to be chosen according to their eco-toxicological profile, in order to minimise environmental side-effects and negative interferences on weed population dynamics;
3 - herbicide dose should be as low as possible according to pedo-climatic and floristic conditions (factor-adjusted dose; Kudsk, 1989).

In order to comply with this latter aspect, farmers should evaluate the real field conditions and select the lowest dose to obtain a satisfactory weed control; such a dose has been defined as the Minimum Lethal Herbicide Dose (MLHD; Haage *et al*., 2002). It has been recognised that in several conditions MLHD may be 1/4th or 1/8th with respect to the dose reported on herbicide labels, depending on weed species (Covarelli *et al*., 2000), weed size (Onofri *et al*., 1995), adjuvants (Covarelli and Onofri 1995). Within this frame, the meteorological component should be as well regarded as a key-factor: it has been pointed out that climatic conditions at the time of application (temperature, humidity and rainfall…) greatly influence herbicide performances, by affecting their retention, penetration and translocation (Price, 1983). The effect of meteorological variables on herbicide efficacy should be studied by using the same statistical approach that is suggested for agronomic variables, i.e. it is necessary to quantitatively measure the effect that the climate may have on MLHDs (Kudsk *et al.*, 1990). This is of a particular importance to bring the climatic component within the decisional process that leads to the selection of herbicide doses, which is a frequently neglected aspect.

Indeed, there is great evidence that the use of MLHD in field may help to achieve a reduction in the use of herbicides, which is not only advisable, but has also become compulsory in several North-European countries (Haage *et al*., 2002).

Apart from MLHD, other indexes may be extremely important for rational herbicide usage, such as the No Observable Effect Level, i.e. the lowest herbicide load that does inflict a measurable effect on a living organism, such as a higher plant species (NOEL; Pestemer and Günther, 1993). NOELs permit the calculation of safe recropping intervals, to avoid possible negative side effects of herbicides within the farming system.

The above considerations imply that the determination of MLHDs, NOELs and other quantitative indexes of herbicide phytotoxicity should be regarded as an important aspect of IWMS. Even though models may play a future role in the assessment of such indexes, so far the most successful strategy has been to empirically measure MLHDs and NOELs in appropriately planned field or greenhouse experiments and build up databanks, eventually organised within expert systems such as Pl@nteInfo (Jensen *et al*., 2000), that include a specific weed-module (Rydhal and Pinnschmidt, 2004).

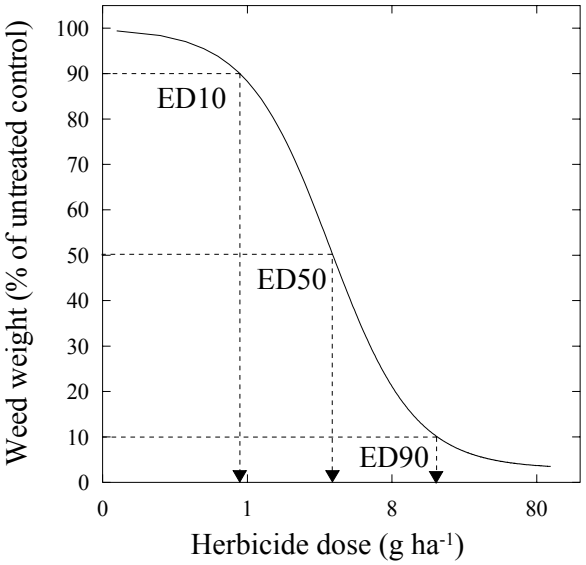

Fig. 1 - Example of a dose-response curve. The most important ED-levels are outlined (ED10, ED50 and ED90)

Fig. 1 - *Esempio di curva log-logistica dose-risposta. Sono evidenziate le Dosi Efficaci (ED) più utilizzate in pratica (ED10, ED50 ed ED90)*

To this aim, herbicide trials have to be appropriately planned as biologic assays, applying the same principles that are common to ecotoxicological studies with other xenobiotic compounds. In other words, it is necessary to plan experiments aimed at defining for each combination 'herbicide/weed-species/formulation/climatic condition' the relationship between herbicide dose and weed control efficacy (fig. 1). From these empirically determined dose-response relationships it is possible to extrapolate some important Effective Dose levels (ED), such as the ED10, ED50 and ED90, which respectively denote the dose causing 10%, 50% and 90% weed control. ED10 may be assumed as No Observable Effect Level (NOEL; Pestemer and Günther, 1993), while ED90 can be assumed as MLHD. The ratio between ED50 for two herbicide preparations or "herbicide/weed species/formulation/climatic condition" combinations (relative potency or efficacy) may be also determined and used as an indication on how much the dose can be reduced or adjusted according to the actual pedo-climatic and floristic situation at the moment of herbicide spraying (Onofri *et al*., 1997).

In order to determine ED-levels and relative potencies it is necessary to carry out simultaneous non-linear regression analysis on several bioassay runs. Rational techniques for analysing herbicide bioassays have been thoroughly revised and developed by Streibig *et al*. (1993 a and b); they require the use of some statistical software

on a personal computer. Generic statistical programmes or specific bioassay programmes may be used, even though they may be either expensive or difficult to use. Furthermore, herbicide bioassays have some peculiarities such as: (1) responses are normally quantitative (i.e. weight of weeds surviving the treatment) rather than quantal; (2) lower asymptote (response at very high doses) is generally different from zero; (3) in some cases response curves are not symmetric; (4) growth stimulation at low doses is often observed with some herbicides families (Fig. 2). Facing such peculiarities with generic statistical programmes may either be time consuming or require a certain programming effort.

Therefore, it is of interest to develop specific computer programmes to analyse herbicide bioassays; such programmes should be as easy to use as possible, to enable scientists, technicians and students to perform bioassay analysis, regardless of their statistic and computer skills. Some good tools for herbicide bioassay have already been developed within statistic environments such as SAS (SAS Institute, 1985; Seefeldt *et al*., 1995) or R (R Development Core Team, 2003; Ritz and Streibig, 2004); both require some advanced statistic and computer skills to be used.

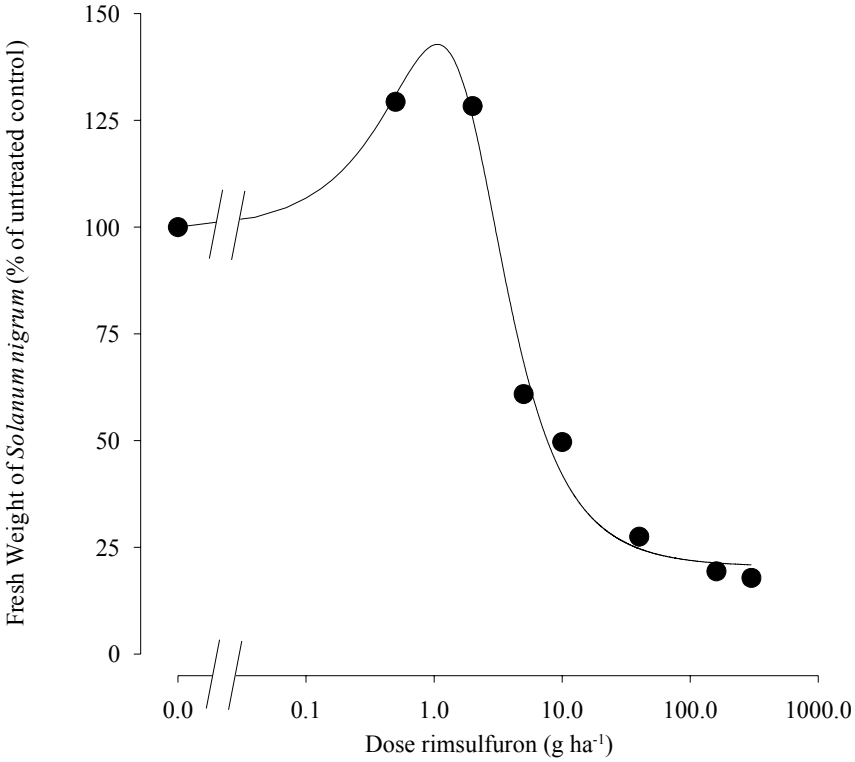

Fig. 2 - Effect of rimsulfuron on fresh weight of *Solanum nigrum* three weeks after treatment. The symbols are observed values, while the solid line is the fitted response, showing an apparent stimulation at low herbicide doses (Onofri, unpublished data).

Fig. 2 - *Effetto del rimsulfuron sul peso fresco di* Solanum nigrum*, rilevato tre settimane dopo il trattamento. I simboli mostrano i dati osservati, la linea mostra il modello di regressione, da cui risulta un'evidente stimolazione a basse dosi (Onofri , dati non pubblicati)*

The aim of this work was to develop BIOASSAY97, a free EXCEL® VBA macro, specifically designed to perform non-linear regression analysis of bioassay data, which would enable users with a limited background in statistics to work directly within the most common spreadsheet. An example will be given by using some real bioassay data.

General features

The macro was written in the VBA language of EXCEL® 97 and runs as an add-in to EXCEL 97 or higher. The add-in macro SOLVER.XLA is also needed to use BIOASSAY97 and must be installed from the original EXCEL installation package before running BIOASSAY97.

BIOASSAY97 performs the following job:

1 - read bioassay data from the spreadsheet;
2 - performs the necessary transformations, as requested from the user;
3 - performs non-linear regression analysis on bioassay data referring either to single dose-response experiments or to multiple dose-response experiments (simultaneous fitting);
4 - returns ED-levels, relative efficiencies and all the graphical and numerical tools to assess the goodness of fit.

Input data must meet the following criteria:

1 - they are to be organised in a database, with observations in rows, and variables in columns. Basically, at least one dose column and one response column are needed; one further column variable (with herbicide/preparation code) is required in the case of simultaneous fitting of several response curves. The first row is reserved for variable names;
2 - response variable must be numeric and measured on a continuous scale (fresh weight, dry weight, herbicide efficacy on a percentage scale, etc..). Quantal responses should be appropriately transformed to meet the basic assumptions for regression analysis (Streibig *et al*., 1993 a and b).

Specifications

Types of analyses

All the rationale and theories behind BIOASSAY97 have been taken by Streibig *et al.*, 1993 (a and b). Basically, BIOASSAY97 manages two types of analysis:

1 - single dose-response curve;
2 - multiple dose-response curves.

The user will use the first type when analysing one single dose-response curve, aiming at measuring ED levels, while he will use the second type of analysis when his interest is in comparing several dose-response curves and estimating relative potencies of herbicide preparations.

Dose-response models

Three types of dose-response models can be fitted into bioassay data:

1 - log - logistic model (sigmoidal symmetric responses on log-dose);
2 - Gompertz model (logistic asymmetric responses);
3 - peaked model (Brain and Cousens, 1989; logistic responses with stimulation at low doses).

A log-logistic model would fit in most of the cases, while a Gompertz model should be selected in case of clearly asymmetric responses (Streibig *et al.*, 1993a and b). The peaked model is suitable in case of response stimulation at low doses, but its usage should be carefully evaluated, as its mathematical properties are not as good as those of the other two sigmoidal models. In the case of multiple curve analysis only the first two models can be selected in BIOASSAY97.

Each model can handle both decreasing curves (for example based on growth of test-species, that is expected to decrease, as dose increases; fig. 1) or increasing curves (for example based on growth inhibition, that is expected to increase, as dose increases). The macro itself will decide whether an increasing or a decreasing curve is required and will provide itself for the necessary adjustments on the model.

Constraints can be put on the lower and/or upper asymptotes. This might be necessary to reach convergence or to improve the estimates of the other parameters. In particular, constraints might be needed:

1 - for biological reasons;
2 - whenever the lower asymptote is not significantly different from 0 or negative;
3 - dealing with curves based on percentage weed control, which are often supposed to range from 0 to 100%;
4 - when asymptotes are measured or known without any experimental error.

In the case of multiple curve analyses, curves may be forced to assume the same lower and/or higher asymptotes and/or the same slope (parallel curve analysis).

Estimation of parameters

The estimation of model parameters is reached by the optimisation procedure provided by the SOLVER.XLA add-in (Microsoft Corporation, 1992); the user has to provide reasonable starting values, which is rather easy with herbicide bioassays. Basically, the highest and the lowest observed response values can be used as the starting points for the higher and the lower asymptotes respectively. A starting value for the inflection point can be relatively easily estimated from observed data (just choose a dose value which gave a response approximately half way between the higher and the lower asymptote), while a value of 1 for the slope should be considered appropriate in most cases. Likewise, a value of 1 should be appropriate for the parameter describing stimulation, if needed.

Transformation of data.

Data do not always meet the basic assumptions for regression analyses. Typically, whenever the difference between the highest and the lowest observed response is higher than one order magnitude, variances will not be constant across all the treatments. Among the Box and Cox (1964) families of transformations, the following one has been chosen and implemented into BIOASSAY97:

$$W = \begin{cases} \left(Y^{\lambda} - 1\right) \Big/ \lambda \dot{Y}^{\lambda - 1} & per\ \lambda \neq 0 \\ \dot{Y} \log Y & per\ \lambda = 0 \end{cases}$$

where W is the transformed variable, Y is the untransformed variable, λ is the transformation parameter and $\dot{Y}$ is the geometric mean of the observations.

By default λ is set to 1 (no transformation); a value of 0.5 means that a square root transformation is performed, while a value of 0 means that a logarithmic transformation is performed. Often, a value of 0.25 has been found to be appropriate for dose-response analyses.

A maximum likelihood value for λ can be chosen following the procedure proposed by Box and Draper (1987).

In order to obtain parameter estimates on the original scales, a Transform Both Sides technique has been adopted on BIOASSAY97 (according to Streibig *et al.*, 1993a).

Iteration process and results.

The iterative procedure for parameter estimation is carried out by the EXCEL Solver. If convergence cannot be reached, a warning message is displayed and the analyses is stopped. Very often this depend on overparameterisation and/or wrong starting values for parameters. A more careful selection of model and/or starting parameter values should improve the analysis.

When convergence is reached, results are displayed on a new worksheet, that is added to the current workbook. By default, the program itself will calculate the most use-

ful ED levels, together with confidence limits. Indeed, in the case of decreasing curves, ED10, ED30 and ED50 are calculated, while in the case of increasing curves, ED50, ED70 and ED90 are calculated. The calculation of any other user-defined ED-level is possible.
Confidence limits are calculated by including each ED level in the model as an explicit parameter, as shown by Jensen and Streibig (1994). This reparameterisation is not possible with the peaked curve of Brain and Cousens; in this case, confidence limits for ED-levels are calculated by using the inference band for the expected response, as shown by Bates and Watts (1988) and Snedecor and Cochran (1989).

Validation of the macro

BIOASSAY97 has been extensively validated by using bioassay data of various types and purposes; results were compared to those obtained by SAS; differences were always within acceptable ranges, considering the iterative nature of the optimisation process.

One example

The following bioassay data are obtained from a research study carried out in the greenhouse at the Department of Agroenvironmental and Forest Biology, Section of Weed Science (CNR, Legnaro, PD). Plants of *Lolium multiflorum* were treated with a sulphonylurea herbicide (chlorsulfuron) at increasing doses, with or without an inhibitor of detoxification (Bravin *et al*., 2004). Such an inhibitor is obviously expected to increase herbicide phytotoxicity; the aim of the experiment was to quantitatively measure that increase.
Fresh weights of *Lolium multiflorum* plants (tab. 1) were subjected to non-linear regression analysis, by using BIOASSAY97; among the available response models it was decided to fit the log-logistic one:

$$FW = C + \frac{D - C}{1 + \exp\{b[\log(Dose) - \log(ED50_i)]\}} \quad \text{(Eq.1)}$$

where *FW* is the fresh weight (g) of test plant, *C* is the lower asymptote (weight of plant at very high herbicide doses), *D* is the higher asymptote (weight of plant in the untreated control), *b* is the slope of the curve around its inflection point, *ED50* is the Effective Dose 50, estimated half-way between the higher and lower asymptote (Streibig *et al.*, 1993 b), and *i* is the herbicide preparation (with or without inhibitor). Indeed, this model assumes two curves (one for each preparation) with only one different parameter, i.e. the ED50 (parallel curves; in total we have 5 parameters to be estimated). Of course, other types of models may

Tab. 1 - Dose-response data obtained on a bioassay with *Lolium multiflorum* treated with chlorsulfuron with or without an inhibitor of herbicide detoxification (malathion). Data from Bravin *et al*., 2004
Tab. 1 *- Dati ottenuti da un biosaggio su* Lolium multiflorum *trattato con chlorsulfuron da solo o in miscela con un inibitore della detossificazione (malathion). Dati da Bravin et al., 2004*

Chlorsulfuron dose (g a.i. ha^{-1})	**Fresh weight of *Lolium multiflorum* (g plant^{-1})**	
	with inhibitor	**without inhibitor**
0.00	1.477	1.315
1.875	0.863	-
3.75	0.521	0.878
7.50	0.225	0.520
11.25	0.181	0.430
22.50	0.116	0.189
45.00	0.098	0.151
67.50	-	0.124

Tab. 2 - Parameters of log-logistic dose-response curve (eq. 1 and Fig. 3) for data reported on Table 1. Standard errors are in brackets
Tab. 2 *- Parametri del modello log-logistico (Eq. 1 e Fig 3) adattato ai dati in tabella 1. Tra parentesi gli errori standard*

Parameter	**Estimate (standard error)**	
	with inhibitor	**without inhibitor**
Higher asymptote (g)	1.40 (0.033)	
Lower asymptote (g)	0.09 (0.031)	
Slope	1.58 (0.185)	
ED50 (g ha^{-1})	2.32 (0.189)	5.04 (0.390)

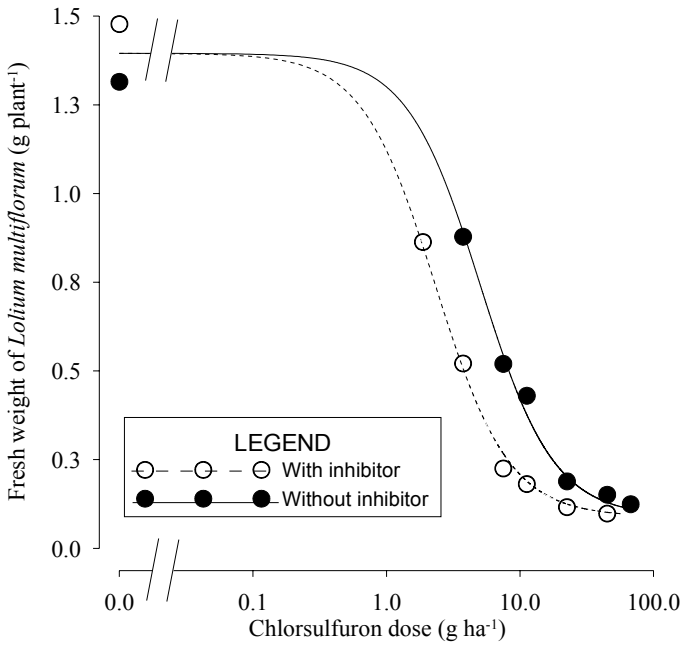

Fig. 3 - Observed data (symbols) and fitted curves (Eq. 1 and Tab. 2) for biassay results reported in tab.1
Fig. 3 - *Dati osservati (simboli) e curve dose risposta (Eq. 1 e Tab. 2) stimate a partire dai dati riportati in tab.1*

be more appropriate to other kinds of bioassays and may be chosen in BIOASSAY97.
After convergence is reached, results show that the two response curves are parallel (Fig. 3), but are significantly different (F tests are easily performed with results provided by BIOASSAY97; see Bates and Watts, 1988); the addition of the inhibitor decreases the ED50 of chlorsulfuron from 5.0 to 2.3, with a relative potency of 2.17 (Tab. 2). This model does not show any significant lack of fit to experimental data (Fig. 3; an F test for lack of fit can also be performed with BIOASSAY97).

Conclusions

The bioassay work carried out during previous years and the intensive use of BIOASSAY97 by users with any kind of statistic and computer backgrounds (students, technicians and researchers) has shown that this tool has the flexibility and the simplicity to accomplish the main needs of those working with herbicide bioassays. Thanks to its characteristics, BIOASSAY97 is also interesting in plant protection for any kind of bioassays with xenobiotic substances as well as in agrometeorology, to quantitatively measure the effect of climatic factors on pesticide performances and dosages. This aspect is particularly interesting to provide farmers with a support to include the climatic aspect within the process of selecting pesticide doses, which is often a neglected issue.
BIOASSAY97 is a freeware, virus-free macro and can be downloaded from the INTERNET (URL: http://www.agr.unipg.it/disaprov/agronomia/onofri.htm or http://www.unipg.it/~onofri), or requested to the author by e-mail. It is a part of a collection of macros (REGLIN97 and ANOVA97) that can be used to perform statistical analysis of data from agricultural experiments of various types.

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