Statistical Workshop for Repeated Measurements

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Basic Concepts

- In the biological sciences, data (measurements, observations) are routinely collected on the same experimental units at multiple times
  - Data collected over time on the same "units" are called *1)* **repeated measures** or *2)* **longitudinal data**
    - Some draw distinctions between these two labels, but we use them interchangeably (for the most part)
- Because of the unique properties of the collected data, as discussed soon, proper analysis of repeated measurements is more difficult than analysis of data collected at a single time
  - Under many circumstances, **linear mixed model analysis** is an appropriate approach for data analysis
    - This workshop teaches how to use this form of analysis

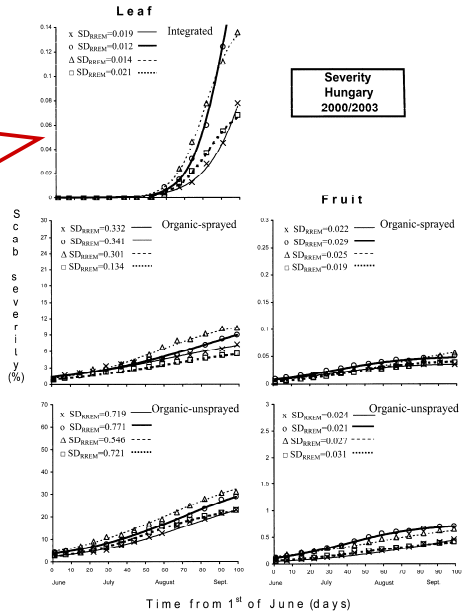
Broad Outline

- Present examples of data collected over time
  - Give reasons for collecting data over time and outline the key features of such data
  - Outline the different approaches, including the typical *incorrect* ones, which are often taken to analyze such data
- Show how one type of statistical model--*a linear mixed model*--is used (correctly) to represent data collected over time and how one chooses a particular model (i.e., how one links up a model with a particular experimental design)
- Show how to use models and statistical software (SAS) to analyze fairly typical data sets in plant pathology in which data are collected over time

Examples

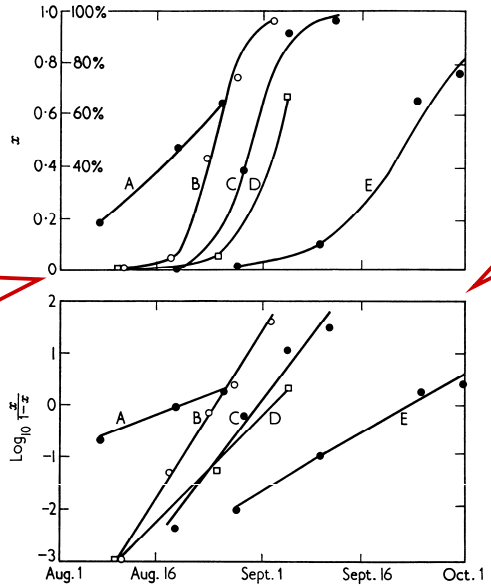
Data collected over time are common in field, greenhouse, and laboratory studies

Apple scab, with two factors: Management practice and year (within graphs)



Holb et al. (2005)

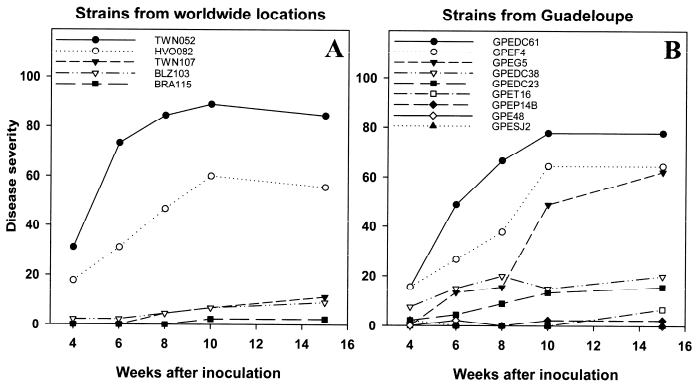
Examples



Potato late blight severity and logit transformation– Different cultivars

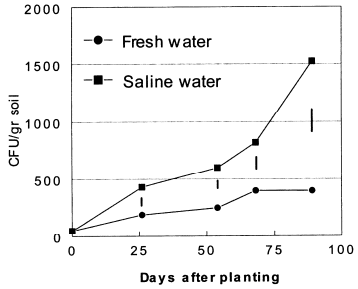
From Vanderplank (1963)

Examples

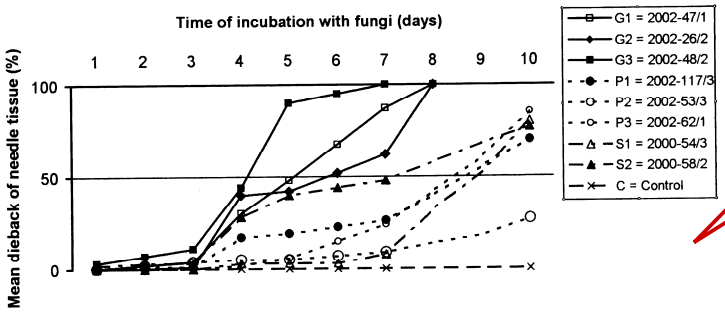


Xanthomonas albilineans on sugarcane (greenhouse study). Different isolates. Champoiseau et al. (2006)

Fusarium density on roots of tomato (field study). Triky-Dotan et al. (2005)

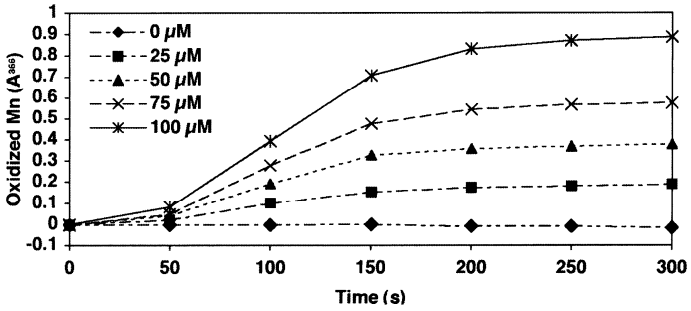


Examples

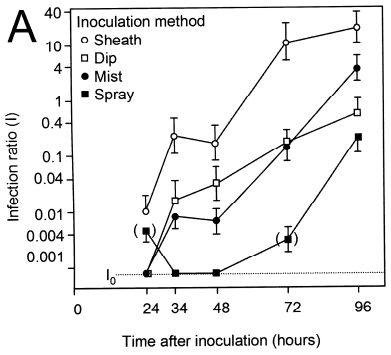


Spruce dieback (*in vitro*), with different fungal isolates.
Borja et al. (2006)

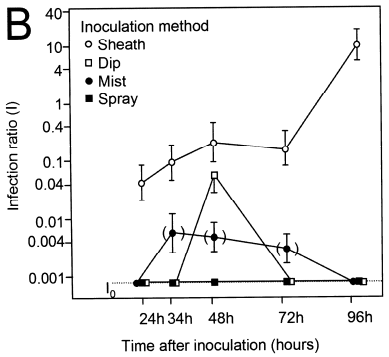
Effect of Mn concentration on rate of oxidization for *G. graminis* var. *tritici*.
Thompson et al. (2006)



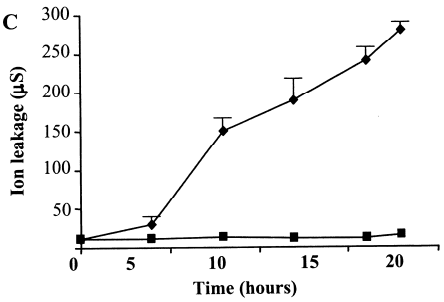
Examples



Rice blast.
Quantitative PCR results indicating # fungal cells.
Two factors:
Inoculation method and host resistance.
Berruyer et al. (2006)

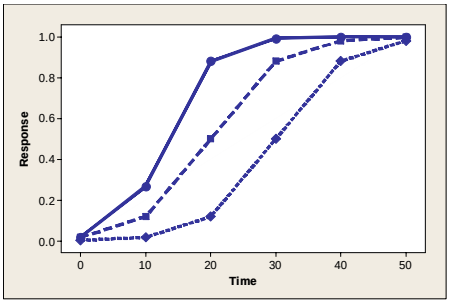


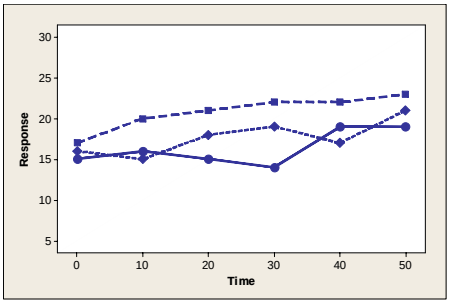
Botrytis cinerea and *Arabidopsis*
Elicitor in intercellular fluid.
Govrin et al. (2006)



Many reasons to collect data at multiple times

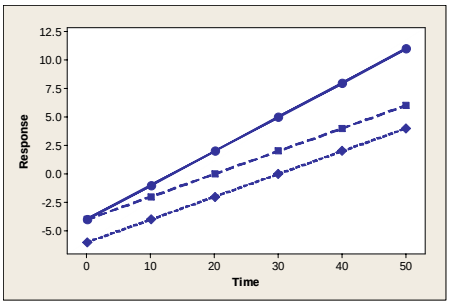
- Increased information on the effects of treatments (experimental factors) on the response variables of interest
  - At “short” or “long” times, there may be no apparent treatment effect
- Determine if the response changes with time
- Efficiency in experimental design (sometimes increased precision)





Many reasons to collect data at multiple times (continued)

- Determine if treatment affects the response (over all times)
- Determine if the effect of treatment depends on time (i.e., determine if there is an **interaction** of time & treatment)
  - Equivalently, determine if change in response over time depends on treatment
- There is often no biologically justified single time to measure disease



Key features of repeated measures

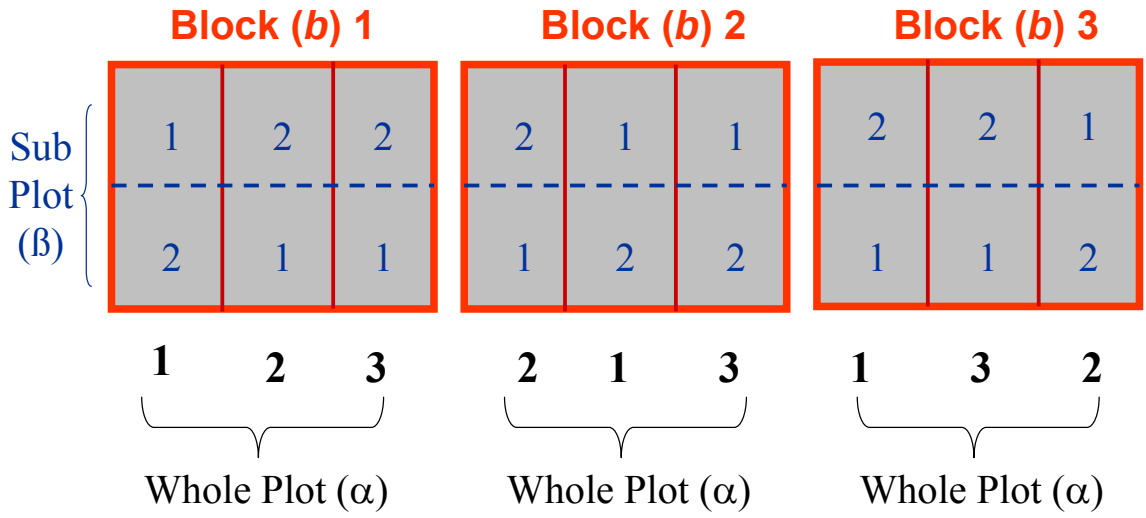
- At least **two sources of variation** (variability) in the response variable
  - **Between experimental units** (plants, plots, etc.)
    - **"Between-subject variation"**
  - **Within experimental units** (*between times* within units)
    - **"Within-subject variation"**
- **Correlation** of the response variable between times *within* experimental units
  - Because, by definition, time of measurement is not randomized (the second measurement *must* be after the first measurement and before the third), the structure or form of the correlation cannot be predicted based on the experimental design
- Often, the variability is not the same at each time, but changes over time
  - That is, there is **heterogeneity in the variances**

More on repeated measures

- Depending on experimental factors, the design could be
  - **randomized block repeated measures**
    - **Block, plus treatment, plus time**
  - **repeated measures factorial**
    - **Two or more "between-subject" factors, plus time**
  - **randomized block repeated measures factorial**
    - **Block plus two or more "between-subject" factors, plus time**
  - **Split plot repeated measures**
    - **Whole-plot factor, sub-plot factor, plus time**
- With these types of experimental designs, there is **clustering of data**, which is the cause of the correlation of data within experimental units (the **"subjects"**)
- Cluster:
  - ***collection of observations that are somehow stochastically related* (correlated)**
    - One example: splitting of experimental units

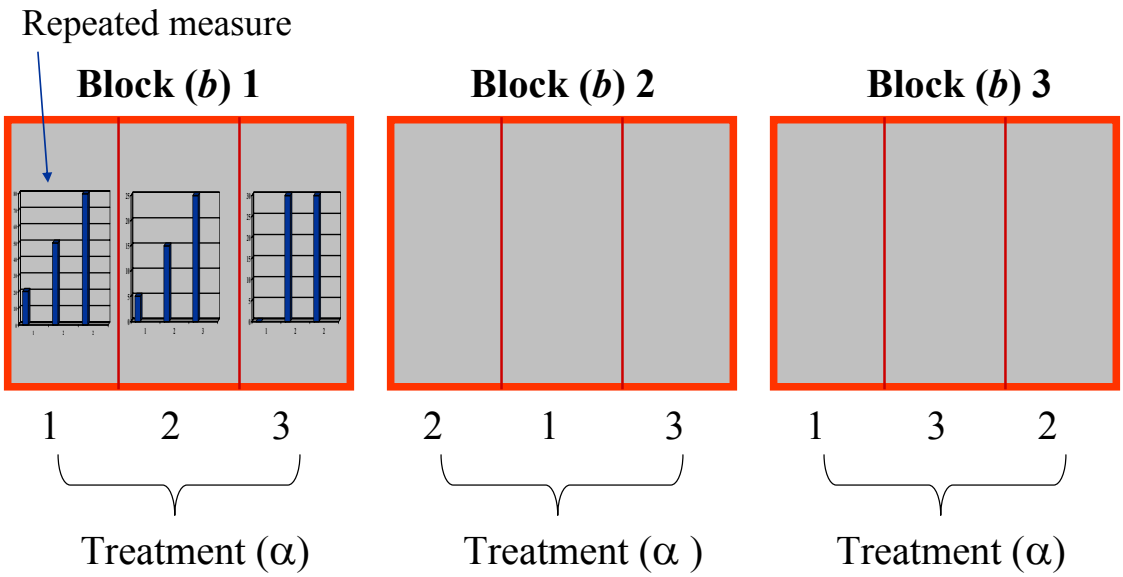
Clustering example: Splitting of experimental units:
Split Plot (two factors of interest)

α: whole-plot (e.g., irrigation method), randomly assigned within each block (large units)
β: sub-plot (e.g., cultivar), randomly assigned *within* each whole-plot (small units)



Each whole-plot constitutes a cluster, and the sub-plot observations are the within-cluster units--*variation between and within whole-plots*

More clustering: Same experimental units are measured repeatedly -- **Repeated Measures**



Cluster (the plot; experimental unit) is formed by the collection of repeated measurements. Variation between and within units.

Note on "Clusters" and "Subjects"

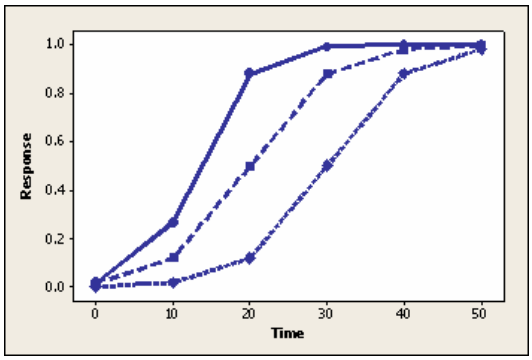
- "Subjects" are often analogous to "experimental units" in experiments
- There can be a hierarchy of "Subjects":
  - Block, whole-plots within blocks, sub-plots within whole-plots
  - Block, treatment plots within blocks, times within treatment plots
  - Block, whole-plots within blocks, sub-plots within whole-plots, times
- In some cases, there are clear ***size*** differences in the subjects

Difference between split-plots and repeated measures

- With split-plots, the levels of the sub-plot factor (the "small" experimental unit; e.g., cultivar) are randomly assigned within the whole-plot factor (the "large" experimental unit; e.g., irrigation method)
  - **Thus, the correlation of observations within a whole-plot is fixed (i.e., same correlation for each pair of cultivars)**
    - This is a consequence of the experimental design
- With repeated measures, the levels of the "sub-plot" factor (=time) are not randomly assigned within the experimental units (by definition)
  - **Thus, there is not necessarily a fixed correlation**
    - The experimental design cannot give more guidance on the nature of the correlation (one must assess the correlation structure)

Approaches to analysis of repeated measures

- There are several correct ways of analyzing data from repeated-measures experiments
  - The correct approaches all have different strengths and weaknesses
- There are also several incorrect ways, or potentially incorrect ways, of analyzing such data that have been used (unfortunately)
- We consider the continuum of approaches here (in outline), and restrict attention to one general appraoch



Approaches to analysis of repeated measures

- **The (totally) incorrect method--**
  - Ignore the unique or special aspects of *time* as a factor in ANOVA, and treat time as a 'regular' experimental factor with randomized assignment of levels to units (plots, etc.)—**ignore clustering**
  - All tests for significance and standard errors will be wrong under most circumstances (*P*-values and *SE*s will be too low)
    - Most likely outcome: high Type I error rate for tests of experimental factors of interest (higher than the nominal α):
      - Too easy to declare significance when, in fact, the effect of the factor (treatment) is not significant.
      - Too easy to find two means different when they are not, in fact, different
- **The other generally incorrect method--**
  - Analyze data from each time separately
    - Ignores the correlation of observations over time within experimental units
      - Will bias the results in treatment comparisons (affect Type I error rate and *SE*s)
    - If done for only one time (e.g., last assessment time), then OK

Approaches to analysis of repeated measures

- **The *probably* incorrect method--**
  - **Consider the experimental design a true (standard) split plot**
    - Although this approach does address the multiple types of variability (between and within experimental units), it does not allow for realistic and complicated correlation structures within experimental units
      - 'Forces' the correlation to be *fixed* (single value for all pairs of times)
    - Does not easily allow for unequal variances
    - Will give biased results for *P*-values and *SE*s under many circumstances
- **The acceptable, but inadequate, method--**
  - **Consider the experimental design a split plot, but make post-model-fitting adjustments to correct for correlation structure**
    - Greenhouse-Geisser and Huynh-Feldt corrections
    - Before the adoption of modern mixed-model methods, this was a very common approach
    - Method does not provide adequate flexibility in data analysis (to deal with a wide range of designs) and does not have the statistical *power* of other approaches

Approaches to analysis of repeated measures

- **A correct method--**
  - Replace the profiles of *Y* versus *t* for each experimental unit (e.g., plot, plant) with a single composite value (a summary response variable), such as:
    - ***Area under the disease progress curve*** **(AUDPC)**
    - **Linear *contrast* of *Y* values (e.g., last minus the first *Y* value)**
      - Or: linear, quadratic, cubic orthogonal polynomial contrasts
        - » (These are independent, but might be difficult to interpret)
  - Then, perform ANOVA on **AUDPC** (or the contrast), to determine the effects of experimental factors (e.g., treatment) on the summary response variable
    - A popular and valuable approach
    - The within-unit variability and correlation is *removed* with this approach
    - Very useful (and convenient) with *many* different times
    - *May* not be easily interpreted, especially if one is trying to determine how treatments affect the rate of disease development

Approaches to analysis of repeated measures

- **A correct but limited method (& not covered here)--**
  - **Perform a multivariate analysis of variance (MANOVA) on the data, where *Y* at each time is considered a separate variable**
    - There is a vector of responses (one for each time)
    - Use one of the multivariate tests (Wilk's Lambda, etc.) to determine treatment effects
  - Disadvantages:
    - May not be very powerful with typical sample sizes (low probability of finding a significant effect of treatment when it really has an effect)
    - Sometimes difficult to interpret results when a significant effect is found
      - How does *Y* change with time, for each treatment?
    - Difficult to handle complicated experimental designs
    - Missing values can cause major difficulties

Approaches to analysis of repeated measures

- **A correct and extremely useful contemporary approach--**
  - Use a **linear mixed model** to analyze the data
    - A very *flexible* approach and *general* method that can accommodate
      - many possible correlation structures,
      - unequal variances,
      - missing values,
      - multiple sources of variation (i.e., multiple random effects),
      - complex experimental designs (including restrictions of randomization),
      - covariance analysis
      - and many other features…
  - Use a **nonlinear mixed model** to analyze the data
    - A very powerful approach for specialized problems
    - Not covered here
- A brief introduction to models, linear models, and linear mixed models is covered

Approaches to analysis of repeated measures

- We take a **parametric frequentist** approach to data analysis in this workshop
  - **Parametric:**
    - Data can be represented with a statistical-distribution model, with parameters to be estimated
      - Assume normality here (although many other distributions could be used)
  - **Frequentist:**
    - Inference is based on the collected data *in* the experiment or survey (and probabilities related to hypothetical repetitions of the experimental), and does not use prior believes
- One can take a **nonparametric** approach to analysis (covered in an APS Workshop 2 years ago) – rank-based
  - Especially useful for ordinal data (0: no disease; 1: slight; 3: dead)
- One can also take a **Bayesian** approach to analysis (covered in an APS Workshop last year)
  - Utilizes prior beliefs in addition to collected data in statistical inference

Model

- *Abstraction of a real phenomenon or process that emphasizes those aspects relevant to the objectives of the user*
  - **Used to describe, understand, predict, compare, and make inferences about the phenomenon**
  - Models consist of terms that are:
    - **deterministic** (**systematic, structural**), for the portion of the model that does not involve uncertainty; and/or
    - **stochastic** (random)
  - Often, stochastic terms can lead to a parsimonious abstraction of the phenomenon
- **Statistical model:**
  - ***Model with stochastic components (and maybe other components [deterministic]), containing unknown constants (i.e., parameters) to be estimated***
    - ANOVA and regression models are statistical models

Statistical Model:

Response = (systematic part) + (random part)

Response = structure + error

Outcome of interest, being measured, counted, or classified (***Y***); **a random variable**

Mean (or *expected* value) of response

Function of variables and parameters (often of primary interest)

Difference between observed responses (i.e., the observations) and mean responses based on parameters; a **random variable**; estimate known as **residual** (*e*).

Characterize effects of treatments, other factors, and continuous variables on *Y*

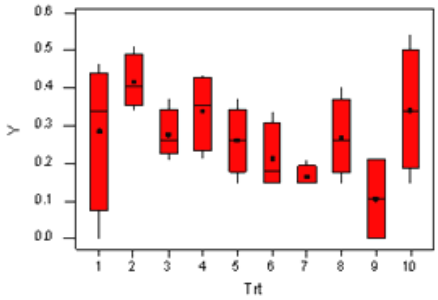
$f(X_1, X_2, \ldots; \mu, \beta_1, \beta_2, \tau, \ldots)$

Response = structure + error

$$Y = f(X_1, X_2, \ldots; \mu, \beta_1, \beta_2, \tau, \alpha, \ldots) + e$$

- Y is the response (random) variable
  - Binary, discrete, or continuous
    - We focus on continuous response variable (assume normality)
- $X_1, X_2, \ldots$ are variables that may affect the mean response variable
  - May be continuous (regression models) – e.g., temperature
  - May be "*dummy*" variables (ANOVA models) - "**factors**"
    - "**Class**" or "**category**" variables – e.g., treatment
      - e.g., $X_1 = 1$ if treatment 1, $X_1 = 0$ if not treatment 1
- Greek letters: parameters
    - **(Combine class [category, factor] variables and parameters into other parameters [e.g., τ])**
- e is the error (random variable), **normally distributed** (here)

Example data set with 10 groups (treatments)



Does treatment effect *Y*?

Which means are different from each other?

Answer questions by fitting a linear model to data.

A classical one-way ANOVA model, in statistical format:

$$Y_{ij} = \mu + \tau_i + e_{ij}, \qquad e_{ij} \sim N(0, \sigma^2)$$

Use subscripts to refer to specific treatment (i) and replication (sample; j)

- Y_{ij}: response (dependent variable) for the j-th observation in group (treatment) i (e.g., i = 1, 2, …, 10)
- μ: constant ("intercept") – could be zero
- τ_i **Group or treatment effect** (effect of group i on response) - constants (***F* test if these differ from zero**)
- e_{ij}: Error associated with group (treatment) i and observation j. (random variable with Normal distribution) Estimated σ^2: residual variance.

Without showing how (for now), the mean or expected value of Y for group 1 is $\mu + \tau_1$, The expected value of Y for group 2 is $\mu + \tau_2$, etc.

A classical one-way ANOVA model, in statistical format:

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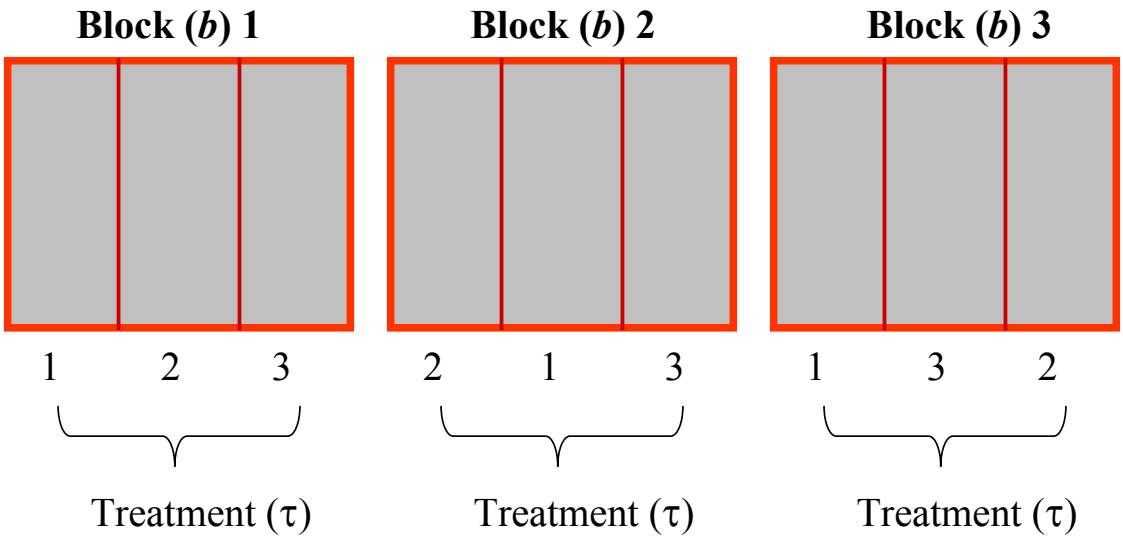
- τ_i Group or treatment effect (effect of group i on response) - parameters
- e_{ij}: Error associated with group (treatment) i and observation j. (random variable, normal distribution)
  Estimated σ^2: residual variance.

Linear: sum of ***Variables x constants***, or just constants

This is considered a linear **fixed effects** model.

Definition: *a linear model with only parameters (constants) (e.g., effects of treatment) and one random variable (the error [residual], in this case).*

Randomized Complete Block
(general schematic)



Treatments randomized *within* each block -- block can affect *Y*

Randomized Complete Block

$$Y_{ij} = \mu + \tau_i + b_j + e_{ij}\ , \quad b_j \sim N(0,\sigma_b^2),\ e_{ij} \sim N(0,\ \sigma_e^2)$$

Y_{ij}: response (dependent variable) in treatment i and block j

Note: there are now *two* variances

μ: constant ("intercept")

τ_i: Effect of the i-th level of treatment on Y

b_j: Effect of the j-th level of block on Y --consider this a ***random effect***

e_{ij}: Error associated with experimental unit in block j that received treatment i [residual]

This is considered a **linear mixed effects model** ("Mixed Model" for short).

Definition: *Linear model with at least <u>two</u> random variables (including the residual error, e), plus fixed-effects parameters, and possibly an intercept constant.*

Note: with random effects, at least some observations are <u>correlated</u>!

Fixed- *versus* random-effects factors

- **<u>Fixed effects variable (or factor)</u>**
  - Levels in the study (i.e., the particular groups) represent all possible levels of the factor, or all levels of interest by the investigator
    - e.g., fungicide treatment, biocontrol treatment, inoculum dose, cultivar, etc. **(often of primary interest)**
- **<u>Random effects variable (or factor)</u>**
  - Levels in the study represent only a *random sample* of a larger set of potential levels, or one is not interested in the specific result for each level in the study, or the effects on *Y* are stochastic in nature
    - **e.g., block, location, host or pathogen genotype (sometimes), etc.**

Whether or not a variable (factor) produces random effects is not always clear, and depends on objectives and assumptions. We only consider here random effects that are a consequence of the experimental design (i.e., from clustering of data, such as splitting and repeated measures).

Linear Mixed Effects Model ("Mixed Model")—*notation*

$$Y = (constant + fixed) + random + error$$

Response	**Fixed effects**, including constant; Greek letters (**τ, α, β**)	**Random effects**; Roman letters (***b, d, …***) lower case	Error (**residual, *e***)

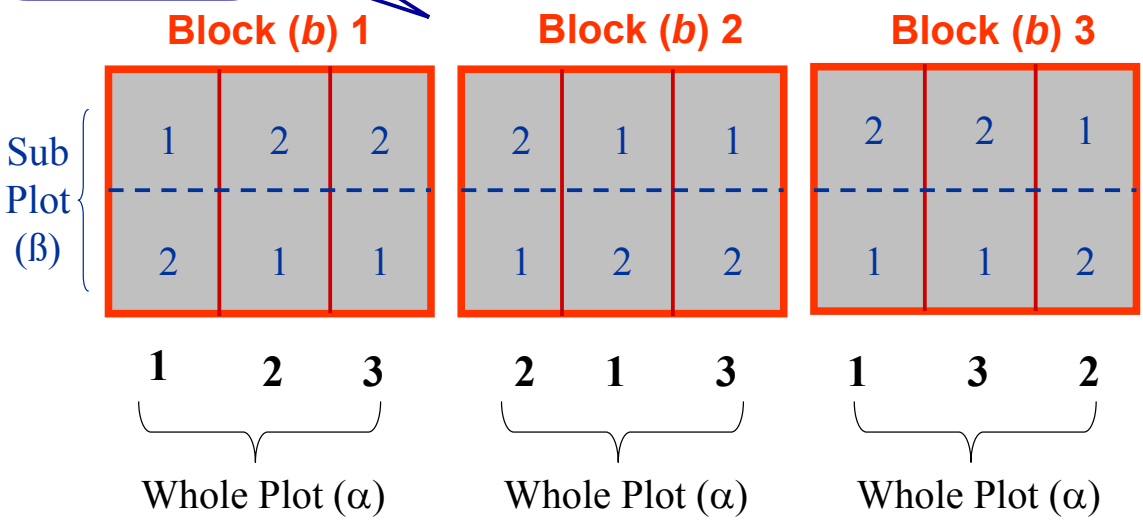
Investigators are typically interested in estimating fixed-effect parameters (e.g., "effect of treatment 1 on *Y*").

However, the estimated fixed-effect parameters and their standard errors (or the estimated means and their SEs) depend, in general, on the random-effect terms

(e.g., any variance terms affect the estimate of τ [effect of treatment] and its SE)

Splitting of experimental units:
Split Plot (two fixed-effect factors)

Each block constitutes a cluster (with variation in *Y* between clusters)



Each whole plot (within a block) also constitutes a cluster (with associated variation in *Y* between clusters)

Split Plot Design (with blocking)

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + e_{ijk},$$
$$b_k \sim N(0, \sigma_b^2),\ d_{ik} \sim N(0, \sigma_d^2),\ e_{ijk} \sim N(0, \sigma_e^2)$$

Note: there are now three variances

Y_{ijk}: response (dependent variable) for the *i-th* level of whole-plot factor, *j-th* level of sub-plot factor, and *k-th* block

μ: constant ("intercept")

α_i: Effect of the *i*-th level of whole-plot factor on *Y*

β_j: Effect of the *j*-th level of sub-plot factor on *Y*

F tests for sig. of fixed effects

$(\alpha\beta)_{ij}$ Interaction effect (effect of *i*-th whole plot and *j*-th subplot on *Y*)

b_k: Effect of the *k*-th level of block on *Y*

d_{ik}: Effect of *ik*-th experimental unit (combination of block *k* and whole-plot *i* on *Y* **[could be written as *interaction effect*, $(\alpha b)_{ik}$]**--the **"whole-plot error term"** or **between-subject variability term**

e_{ijk}: Sub-plot error associated with experimental unit in block *k* that received whole-plot *i* and sub-plot *j* [**residual--the within-subject variability term**]

Split Plot Design (no blocks, or no effect of block)

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \cancel{b_k} + d_{ik} + e_{ijk},$$
$$\cancel{b_k \sim N(0, \sigma_b^2)},\ d_{ik} \sim N(0, \sigma_d^2),\ e_{ijk} \sim N(0, \sigma_e^2)$$

There is still a random effect of *ik*-th experimental unit (the whole plot) on *Y*

Correlation:

It can be shown that the correlation of observations within the whole-plot experimental unit is given by:

$$\rho = \sigma_d^2/(\sigma_d^2 + \sigma_e^2)$$

A constant. Not realistic with repeated measures

σ_d^2 , the **between-subject variance** for the whole-plots, is the covariance of *Y* within whole-plots (same for all pairs of sub-plot levels)

All the sub-plot *Y* values have the same correlation within a whole-plot unit

Label the denominator as: $\sigma^2 = \sigma_d^2 + \sigma_e^2$

The greater the variation in *Y* among experimental units, the greater the similarity of *Y* within experimental units

Split Plot Design [no blocks, ALTERNATIVE but identical, representation]

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + e_{ijk},$$

$$b_k \sim N(0, \sigma_b^2),\ d_{ik} \sim N(0, \sigma_d^2),\ e_{ijk} \sim N(0, \mathbf{R})$$

R: Variance-covariance **matrix** of variability (incorporates both the whole-plot variance (σ_d^2) and the residual variance (σ_e^2)

Equals 0 if Y values come from different whole-plot levels (i.e., observations are independent), and equals matrix Σ if observations are from same whole plot (=**subject**).

Simple example of *three* sub-plot levels (j = 1, 2, 3):

Known as **Compound Symmetry (CS).**

$$\Sigma = \begin{pmatrix} \sigma^2 & \sigma^2\rho & \sigma^2\rho \\ \sigma^2\rho & \sigma^2 & \sigma^2\rho \\ \sigma^2\rho & \sigma^2\rho & \sigma^2 \end{pmatrix} = \sigma^2 \begin{pmatrix} 1 & \rho & \rho \\ \rho & 1 & \rho \\ \rho & \rho & 1 \end{pmatrix}$$

$\sigma^2 = \sigma_d^2 + \sigma_e^2$

Assume three treatments (i =1,2,3) and three times (j =1,2,3)

R =

i =		1	1	1	2	2	2	3	3	3
	j =	1	2	3	1	2	3	1	2	3
1	1	σ^2	$\sigma_{jj'}$	$\sigma_{jj'}$	0	0	0	0	0	0
1	2		σ^2	$\sigma_{jj'}$	0	0	0	0	0	0
1	3			σ^2	0	0	0	0	0	0
2	1				σ^2	$\sigma_{jj'}$	$\sigma_{jj'}$	0	0	0
2	2					σ^2	$\sigma_{jj'}$	0	0	0
2	3						σ^2	0	0	0
3	1							σ^2	$\sigma_{jj'}$	$\sigma_{jj'}$
3	2								σ^2	$\sigma_{jj'}$
3	3									σ^2

Each yellow block is Σ

$\sigma_{jj'} = \sigma^2 \cdot \rho$: *covariance*

Summary: For mixed models, in general, there may be *multiple equivalent* ways of representing the same experimental design and data collection protocols

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + d_{ik} + e_{ijk},$$

$$d_{ik} \sim N(0, \sigma_d^2),\ e_{ijk} \sim N(0, \boldsymbol{\sigma_e^2})$$

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk},$$

$$e_{ijk} \sim N(0, \mathbf{R})$$

Example: Split plot design with no blocks

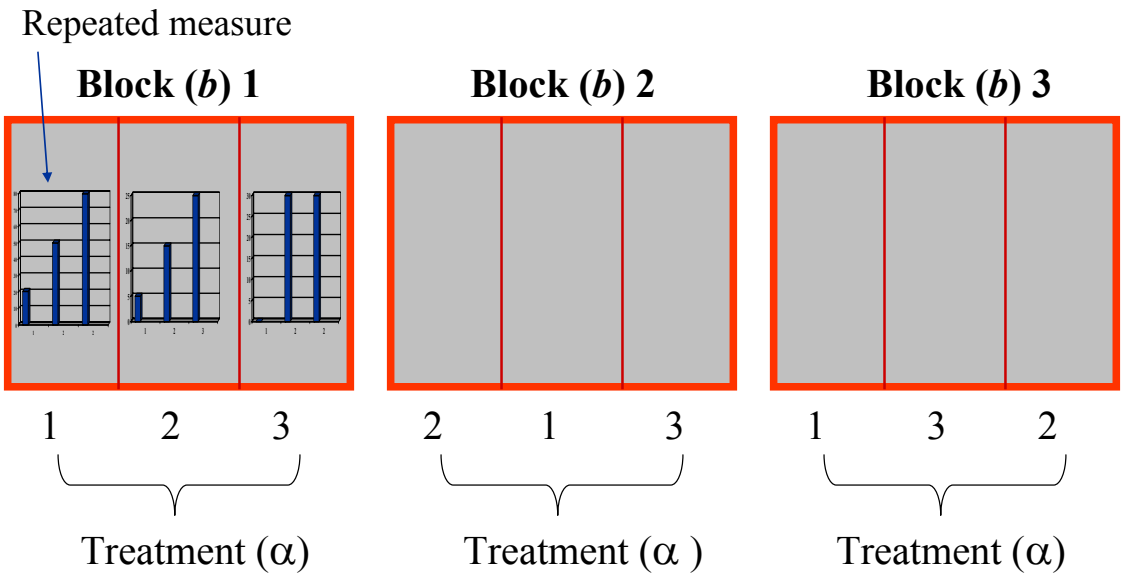
True equivalence holds when σ_d^2 is positive

R: 0 if from different whole-plots. For same whole-plot, matrix **Σ:**

$$\boldsymbol{\Sigma} = \begin{pmatrix} \sigma^2 & \sigma^2\rho & \sigma^2\rho \\ \sigma^2\rho & \sigma^2 & \sigma^2\rho \\ \sigma^2\rho & \sigma^2\rho & \sigma^2 \end{pmatrix} = \sigma^2 \begin{pmatrix} 1 & \rho & \rho \\ \rho & 1 & \rho \\ \rho & \rho & 1 \end{pmatrix}$$

$\sigma^2 = \sigma_d^2 + \sigma_e^2$

Repeated Measures: Same experimental units are measured repeatedly



Primarily consider one experimental factor (α; treatment) and data collected over time (β)

Repeated Measures (with blocks)

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + e_{ijk},$$
$$b_k \sim N(0,\sigma_b^2),\ d_{ik} \sim N(0,\sigma_d^2),\ e_{ijk} \sim N(0,\mathbf{R}) \text{ or } \sim N(0,\sigma_e^2)$$

Y_{ijk}: response (dependent variable) for the *i-th* level of treatment (planting date [PD] here), *j-th* time, and *k-th* block

μ: constant ("intercept")

α_i: Effect of the *i*-th level of treatment on *Y*

β_j: Effect of the *j*-th time on *Y*

$(\alpha\beta)_{ij}$ Interaction effect (treatment x time)

b_k: Effect of the *k*-th block on *Y*

d_{ik}: Effect of *ik*-th experimental unit (each plot) on *Y* **(between-subject variability term)**

F tests:
Does treatment effect the response?
Does the response change with time, overall?
Does the effect of treatment depend on time?

e_{ijk}: Error associated with experimental unit in block *k* that received treatment *i* at time *j* [residual] – **within-subject variability term**

R: A **matrix** of variances and covariances of *Y* for each *ik* experimental unit (subject), even a constant σ_e^2 – **but probably more complex than CS—many possible choices (see next)**

Repeated Measures

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + e_{ijk},$$
$$b_k \sim N(0,\sigma_b^2),\ d_{ik} \sim N(0,\sigma_d^2),\ e_{ijk} \sim N(0,\mathbf{R})$$

With no blocks, remove the *b* term

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + d_{ik} + e_{ijk},\quad d_{ik} \sim N(0,\sigma_d^2),\ e_{ijk} \sim N(0,\mathbf{R})$$

The d_{ik} term generally (with a major exception) is redundant and should not be explicitly in the model (i.e., the between-subject variability is incorporated directly into **R** matrix for e_{ijk}, as it is for Compound Symmetry). That is, both between- and within-subject variability may be modeled directly with the residual e_{ijk} term (although some possible **R**s only deal with within-subject variability). One must be careful here, and know the syntax of software! This is tricky (*will show with examples*).

With blocks, remove the *d* term

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + e_{ijk},\ b_k \sim N(0,\sigma_b^2),\ e_{ijk} \sim N(0,\mathbf{R})$$

Without blocks, remove the *b* and *d* terms

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk},\ e_{ijk} \sim N(0,\mathbf{R})$$

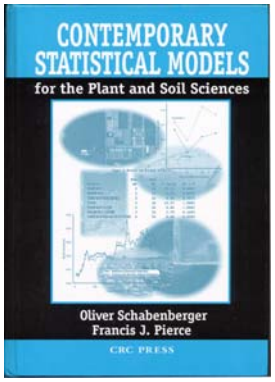
Contemporary linear mixed model analysis

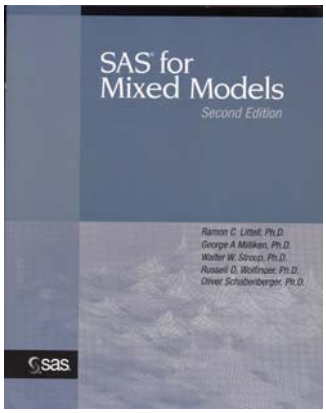
- Fit model with combination of
  - Restricted (residual) Maximum Likelihood ("REML") for random-effects terms, and
  - Estimated Generalized Least Squares ("EGLS") for fixed effects terms
  - (Combined methodology loosely called ***restricted maximum likelihood***)
- *Iterative approach*, involving estimating random- and fixed-effect terms many times. This requires:
  - sophisticated computer algorithms,
  - fast computer processing speed,
  - ample computer memory
- Warning:
  - **Until the last decade or so, most "mixed-model analyses" were not true mixed-model analyses!**

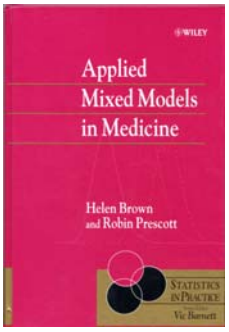
Contemporary (*correct*) linear mixed model analysis

- Hypothesis testing and inference regarding **fixed effects** are based primarily on:
  - F tests of scaled Wald statistic **("Type 3 Tests")**
    - **Degrees of freedom (*df*) may need to be estimated based on the data (different from classical *df* values).**
      - **Satterthwaite or Kenward-Roger (KR) *df* method is recommended**
    - **Tests involve contrasts. There are no Mean Squares or sums of squares in the typical analysis!**
      - **An ANOVA table is obtained for the fixed effects only.**
- Inference regarding **random effects** is based on either:
  - ***Standard normal*** statistics (only as a rough approximation when sample sizes are small or moderate)
  - ***Likelihood ratio*** tests – we will not deal with this too much
- We fit mixed models with procedures in SAS

Some good references







Some useful references

- Littell, Henry, & Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. *J. Animal Sci.* 76: 1216-1231.
- Littell, Pendergast, & Natarajan. 2000. Modelling covariance structure in the analysis of repeated measures data. *Stat. Med.* 19: 1793-1814.

Also, Chapter 4 in:

Madden, Hughes, & van den Bosch. 2007. *The Study of Plant Disease Epidemics.* APS Press, St. Paul, MN.

deals at length on the subject (strictly for analyzing epidemics)

SAS for Mixed Models

- PROC **MIXED** (main choice for *linear* mixed models & normal distribution)
- PROC **GLIMMIX** (main choice for *generalized linear* mixed models) -- new
  - For many types of non-normal distributions (Poisson, binomial, etc.)
  - Also can be used for normal data
    - Has many new options for visualizing data and efficiently presenting results
- PROC **NLMIXED** (main choice for nonlinear mixed models)

Warning (again): many other easy-to-use programs indicate they fit mixed models, but, in reality, do not really fit mixed models in the contemporary sense used here (many statistics obtained may be incorrect!). There are several other specialized programs for true mixed model analysis, but these are more for professional statisticians, and typically have far fewer options than available in SAS.

Linear Mixed Effects Model with **SAS (Mixed)**

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + e_{ijk},$$
$$b_k \sim N(0,\sigma_b^2),\ d_{ik} \sim N(0,\sigma_d^2),\ e_{ijk} \sim N(0,\mathbf{R})$$

Treat-ment, etc.,α,β,τ

Block, whole-plot effect, etc. *b, d*

Y

e

PROC MIXED *options*;
CLASS *variables*;
MODEL *response = fixed effects terms / options*;
RANDOM *random-effect terms / options*;
REPEATED *within-cluster (exp. unit) terms / options*;
LSMEANS *fixed-effects*;

Identify factor variables (compared with continuous variables)

Model-based estimates of means

REPEATED statement is for indicating the structure within-subject (residual) variability: R
Not needed if there is just a single residual variance, σ_e^2

Linear Mixed Model (Split Plot) with SAS (Mixed)

Split plot with blocks (or repeated measures [β for time], and compound symmetry [CS]):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + e_{ijk},$$

$$b_k \sim N(0, \sigma_b^2),\ d_{ik} \sim N(0, \sigma_d^2),\ e_{ijk} \sim N(0, \sigma_e^2)$$

--or--

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + e_{ijk},$$

$$b_k \sim N(0, \sigma_b^2),\ e_{ijk} \sim N(0, \mathbf{R})$$

R: matrix of variances and covariances (*Y* values sharing the same whole-plot level, or *Y* values collected over time in same plot [combination of block and α] are equally correlated]). If same whole-plot, **Σ**:

$$\mathbf{\Sigma} = \begin{pmatrix} \sigma^2 & \sigma^2\rho & \sigma^2\rho \\ \sigma^2\rho & \sigma^2 & \sigma^2\rho \\ \sigma^2\rho & \sigma^2\rho & \sigma^2 \end{pmatrix} = \sigma^2 \begin{pmatrix} 1 & \rho & \rho \\ \rho & 1 & \rho \\ \rho & \rho & 1 \end{pmatrix}$$

$\sigma^2 = \sigma_d^2 + \sigma_e^2$

Minimal code; generic syntax

```
PROC MIXED;
CLASS A B BLOCK;
MODEL Y = A B A*B;
RANDOM BLOCK A*BLOCK;
LSMEANS A B A*B;
RUN;
```

```
PROC MIXED;
CLASS A B BLOCK;
MODEL Y = A B A*B;
RANDOM BLOCK;
REPEATED /
        SUBJECT=A*BLOCK
        TYPE=CS;
LSMEANS A B A*B;
RUN;
```

Linear Mixed Effects Analysis (especially for Repeated Measures)

1. Using all the relevant fixed-effect terms in the model (including all interactions), find the most appropriate error structure (i.e., the form of **R**)
   - Base this on **-2*likelihood**, and **AIC** and **BIC** statistics
     - Smaller the better (AIC and BIC correct for number of parameters)
2. With the choice for **R**, test all the fixed effects (including interactions) for significance (*F* tests, and so on)
   - In general, one should use a denominator *df* based on the data (and not just the design): **Kenward-Roger (KR)** may be best

     ```
     MODEL Y = A B A*B / DDFM = KR;
     ```
   - *Depending on the study, one can simplify the model by removing nonsignificant fixed-effect terms, or considering time as continuous*
3. With the choice for **R** and fixed effects in the model, estimate the expected values (i.e., means) of *Y*, and SEs, for the levels of the fixed effects (treatments, time, interactions, …)
   - As relevant, use contrasts to evaluate other effects (e.g., pair-wise differences of means [multiple comparisons])

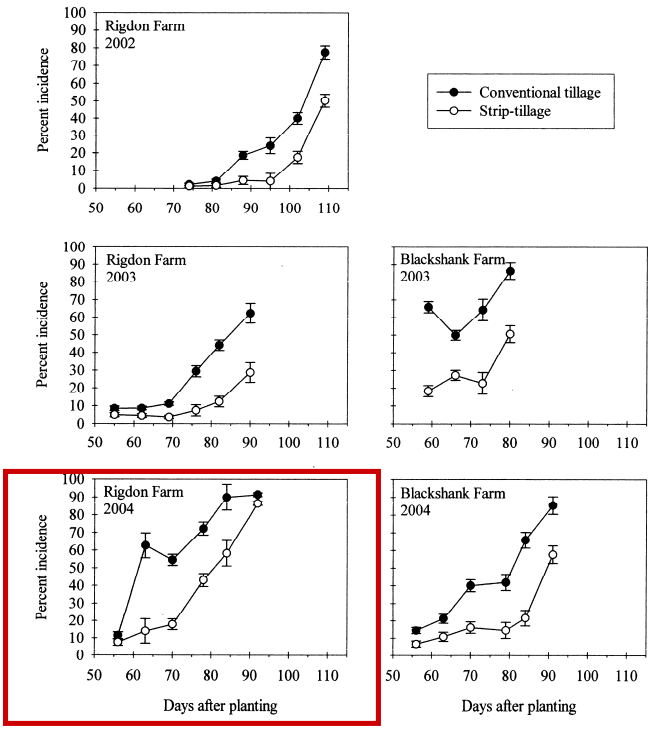
Example:

Early leaf spot of peanut (different treatments, locations, and years).
Data courtesy of Cantonwine, Culbreath, and Stevenson (*Phytopathology* 97: 187-194)

Consider only 1 location/year

Response: incidence (% leaves with symptoms)
Treatment: Tillage types (2)
Times: 6 assessment times
Replicates: 4

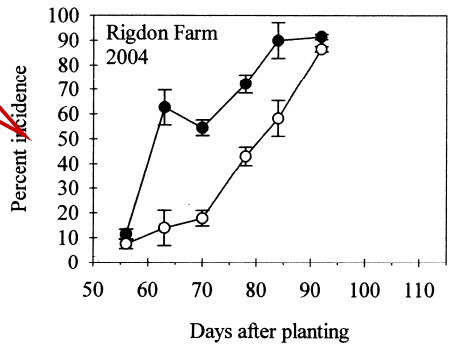
- **Does disease change over time?**
- **Does treatment affect incidence?**
- **Does treatment effect change over time (do treatment differences depend on time)?**



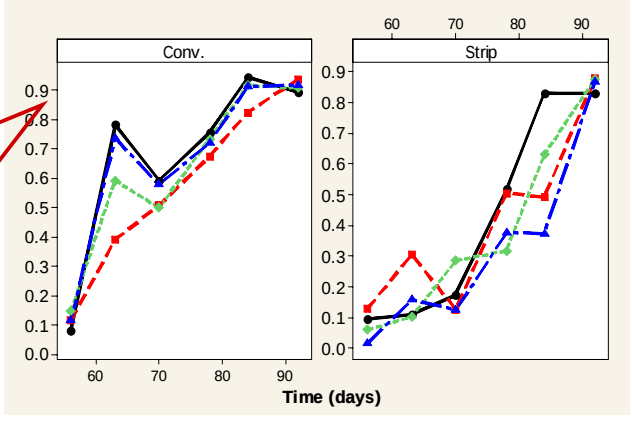
days	Mean incidence		
	treat		All
	1	2	
56	0.11	0.08	0.10
63	0.63	0.17	0.40
70	0.55	0.18	0.36
78	0.72	0.43	0.58
84	0.90	0.58	0.74
92	0.91	0.87	0.89
All	0.64	0.38	0.51

Early leaf spot example

"classical" graph



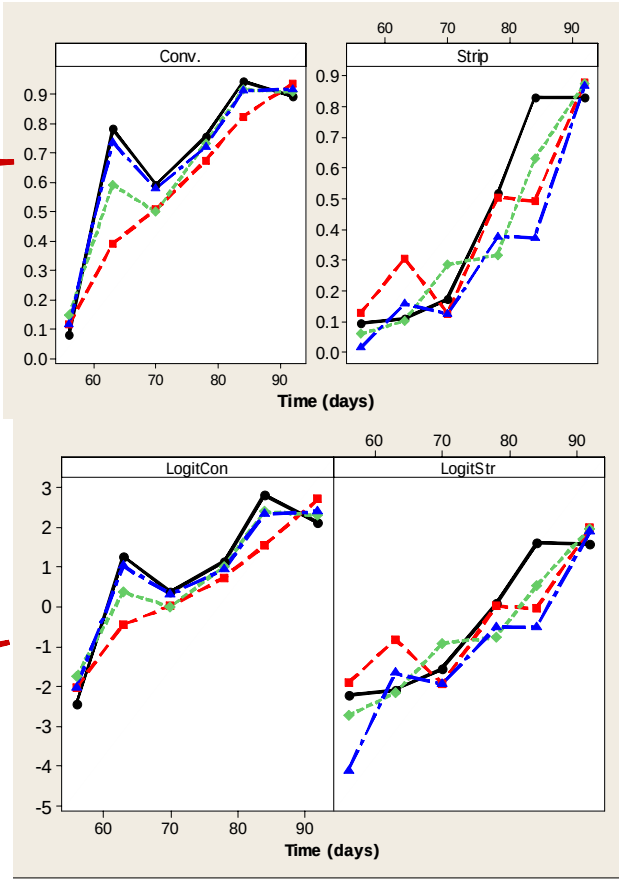
A "contemporary" graph, which emphasizes the features of the experimental design and mixed-model analysis.....
Each line corresponds to a replicate (i.e., a plot) of each treatment. The lines are for separate "subjects"– there is variability within and between subjects



Original incidence data

Often, data are transformed to be approximately normality, constant variance, and/or to obtain a linear scale
(we use *Y* for the transformed response, if a transformation is used)

Transformed to logits
For all analysis,
***Y:* logit**



Early leaf spot example (assume here that design is completely randomized + time)

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}, \qquad e_{ijk} \sim N(0, \mathbf{R})$$

Y_{ijk}: response (logit of incidence) for the *i-th* level of treatment (tillage here), *j-th* time, and *k-th* replicate

μ: constant ("intercept")

α_i: Effect of the *i*-th level of treatment on *Y* (*i*=1,2)

β_j: Effect of the *j*-th time on *Y* (*j*=1,…6)

$(\alpha\beta)_{ij}$ Interaction effect (treatment x time)

e_{ijk}: Error associated with experimental unit in replicate *k* that received treatment *i* at time *j* [residual] – **within-subject variability term**

R: A **matrix** of variances and covariances of *Y* for each *ik* experimental unit (subject) – **but probably more complex than CS**

Fit model with PROC MIXED–go to SAS file repeatEx1.sas

Early leaf spot example

***Incorrect*: single residual variance**

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk},$$
$$e_{ijk} \sim N(0, \sigma_e^2)$$

```
proc mixed data=b;
class  treat rep days;
model logit = treat|days ;
lsmeans treat|days;
run;
```

Correct: Many possible covariance structures (e.g., Compound Symmetry [CS]—see previous slide)

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk},$$
$$e_{ijk} \sim N(0, \mathbf{R})$$

```
proc mixed data=b;
class  treat rep days;
model logit = treat|days /
        ddfm=KR ;
repeated / subject=rep*treat
        type=cs;
lsmeans treat|days;
run;
```

Correct: *alternative for CS only*

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + d_{ik} + e_{ijk},$$
$$d_{ik} \sim N(0, \sigma_d^2),\ e_{ijk} \sim N(0, \sigma_e^2)$$

```
proc mixed data=b;
class  treat rep days;
model logit = treat|days /
        ddfm=KR ;
random rep*treat;
lsmeans treat|days;
run;
```

Using whole-plot random effect, d_{ik}, with single σ_e^2

Covariance Parameter Estimates				
Cov Parm	Estimate	Standard Error	Z Value	Pr Z
treat*rep	0.03022	0.04566	0.66	0.2541
Residual	0.2768	0.07148	3.87	<.0001

σ_d^2 σ_e^2

Fit Statistics	
-2 Res Log Likelihood	75.6
AIC (smaller is better)	79.6
AICC (smaller is better)	79.9
BIC (smaller is better)	79.7

Corr. = 0.030/(0.030+0.2768) = 0.098

Using explicit CS

Covariance Parameter Estimates					
Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr Z
CS	treat*rep	0.03022	0.04566	0.66	0.5082
Residual		0.2768	0.07148	3.87	<.0001

σ_d^2 σ_e^2

Fit Statistics	
-2 Res Log Likelihood	75.6
AIC (smaller is better)	79.6
AICC (smaller is better)	79.9
BIC (smaller is better)	79.7

Other residual correlation structures:
First-order autoregressive, AR(1)

Observations close in time are more similar than observations farther away in time

$$e_j = \rho e_{j-1} + e^*_j,$$

$e^*_j \sim N(0, \sigma^{*2})$ [new error], ρ: autocorrelation coefficient ($|\rho|<1$)

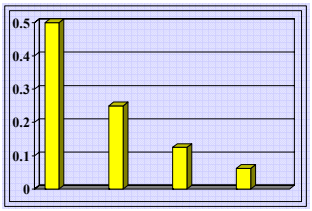
It can be shown that correlation in Y between pairs of times is:

$$\text{Corr}[Y_j, Y_{j-1}] = \rho^1 = \rho$$

For Y two time periods apart:

$$\text{Corr}[Y_j, Y_{j-2}] = \rho^2$$

In general, correlation m time periods apart is: $\text{Corr}[Y_j, Y_{j-m}] = \rho^m$



Covariance is $\rho^m\sigma^2$

Repeated Measures –
Observations become more (less) variable over time

May need a separate error variance for each time:

$\sigma^2 \rightarrow \sigma_1^2, \sigma_2^2, \sigma_3^2$

Subscript indicates the time

Frequently found that variability increases with time, although the situation could be more complex.

Can combine separate-variances for each time with different correlation structures, such as CS and AR(1)

Repeated Measures:

Covariance in Y between times within each experimental unit—some choices:

$$\sigma^2\begin{pmatrix}1 & \rho & \rho\\ & 1 & \rho\\ & & 1\end{pmatrix}$$

Compound symmetry, **CS** (like a *split plot*)

$$\sigma^2\begin{pmatrix}1 & \rho^1 & \rho^2\\ & 1 & \rho^1\\ & & 1\end{pmatrix}$$

1st order autoregressive, **AR(1)**

With ρ < 1, correlation *declines* with increasing time separation

$$\begin{pmatrix}\sigma_1^2 & \sigma_1\sigma_2\rho & \sigma_1\sigma_3\rho\\ & \sigma_2^2 & \sigma_2\sigma_3\rho\\ & & \sigma_3^2\end{pmatrix}$$

Heterogeneous CS, **CSH** Fixed correlation but changing variance

Separate variances for each time

$$\begin{pmatrix}\sigma_1^2 & \sigma_1\sigma_2\rho^1 & \sigma_1\sigma_3\rho^2\\ & \sigma_2^2 & \sigma_2\sigma_3\rho^1\\ & & \sigma_3^2\end{pmatrix}$$

Heterogeneous 1st order autoregressive, **ARH(1)**

Repeated Measures:

The most general structure for **Σ** (variance-covariance matrix for one plot, a component of **R**) allows for separate variances and covariances

$$\begin{pmatrix}\sigma_1^2 & \sigma_{12} & \sigma_{13}\\ & \sigma_2^2 & \sigma_{23}\\ & & \sigma_3^2\end{pmatrix}$$

Unstructured, **UN**

Separate variance for each time and separate covariance (e.g., σ_{23}) between all possible pairs of times

Similar to a multivariate repeated measures

In a sense, no model for R; rather any structure is allowed. However, this requires estimation of potentially many variances and covariances (i.e., estimation of many parameters)–this can be a real penalty.

Assume three treatments (i =1,2,3) and three times (j =1,2,3)

R =

i =		1	1	1	2	2	2	3	3	3
	j =	1	2	3	1	2	3	1	2	3
1	1	σ_1^2	σ_{12}	σ_{13}	0	0	0	0	0	0
1	2		σ_2^2	σ_{23}	0	0	0	0	0	0
1	3			σ_3^2	0	0	0	0	0	0
2	1				σ_1^2	σ_{12}	σ_{13}	0	0	0
2	2					σ_2^2	σ_{23}	0	0	0
2	3						σ_3^2	0	0	0
3	1							σ_1^2	σ_{12}	σ_{13}
3	2								σ_2^2	σ_{23}
3	3									σ_3^2

Each yellow block is Σ

More on correlation structures: from SAS/STAT Manual

PROC MIXED has numerous variance-covariance (correlation) structures for Σ that can be specified (a table is given in the Supplemental material)

The typical first choices may be: AR(1), CS, ARH(1), CSH, UN

> If the times are not close to being equally spaced, it is better to use SP(POW)(t) instead of AR(1), where ***t*** is continuous

Goal: *use the simplest structure that is consistent with the data* (based on AIC or BIC statistics)

> **Too simple (e.g., CS) leads to excessively high Type I errors**
>
> **Too complex leads to low power (high Type II errors)**

The advantages of choosing the best structure for Σ increase with increasing number of times

SAS/MIXED generic syntax (already shown)

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + e_{ijk},$$

$$b_k \sim N(0,\sigma_b^2), \quad e_{ijk} \sim N(0,\mathbf{R})$$

```
TYPE=   CS
        AR(1)
        CSH
        ARH(1)
        UN
        TOEP
        TOEPH
        ARMA(1,1)
        ANTE(1)
        FA(1)
        HF
         …
```

```
PROC MIXED;
CLASS A B BLOCK;
MODEL Y = A B A*B /
       ddfm=KR;
RANDOM BLOCK;
REPEATED /
       SUBJECT=A*BLOCK
       TYPE=UN;
LSMEANS A B A*B;
RUN;
```

The **A*REP** term identifies the unique plots (the experimental units), analogous to whole-plots

If there are no blocks (just replicates), then take out the RANDOM statement and use:

```
REPEATED /
SUBJECT=A*REP TYPE=AR(1);
```

Example: Finding the 'best' model (covariance structure)

Var-Cov. type	# Random Param.	-2log-likel. (-2*ll*)	AIC
(Simple)	1	76.3	78.3
CS	2	75.6	79.6
AR(1)	2	76.2	80.2
UN	***21***	***13.2***	***55.2***
CSH	*7*	*62.8*	*76.8*
ARH(1)	*7*	*61.9*	*75.9*
TOEP	6	73.8	85.8
TOEPH	*11*	*58.8*	*81.8*
ANTE(1)	*11*	*53.4*	*75.4*
FA(1)	*12*	*53.8*	*75.8*
H-F	*7*	*No conver.*	--

Sometimes, a model cannot be fit (no convergence)

At this stage, one is looking for a good fit in terms of the random effects (covariances [correlations] and variances)

Smaller "likelihood" statistics indicate a better fit.

Goal: **find the best fit with the fewest number of random-effect parameters**

Use AIC (or similar) statistics

In example, unequal variances (for different times) provided better fits (italics), and separate correlations for different time lags (see UN)

Ideally, one would like a simpler structure than UN (because of the many parameters)

Example: Finding the 'best' model (covariance structure)

Var-Cov. type	# Random Param.	-2log-likel. (-2*ll*)	AIC
(Simple)	1	76.3	78.3
CS	2	75.6	79.6
AR(1)	2	76.2	80.2
UN	***21***	***13.2***	***55.2***
CSH	*7*	*62.8*	*76.8*
ARH(1)	*7*	*61.9*	*75.9*
TOEP	6	73.8	85.8
TOEPH	*11*	*58.8*	*81.8*
ANTE(1)	*11*	*53.4*	*75.4*
FA(1)	*12*	*53.8*	*75.8*
H-F	*7*	*No conver.*	--

Sometimes, a model cannot be fit (no convergence)

One can formally test whether one covariance structure fits better than another, in some cases.

That is, some structures are special cases of others, such as CS being a special case of CSH, etc.

One uses a likelihood ratio test, with a chi-square distribution.

However, many potential structures are not special cases of each other, such as CS and AR(1).

Here, there is no formal test (just use AIC)

Mixed model analysis, repeated measures

- **With the chosen covariance structure, perform inference on the fixed effects**
  - Treatment, time, interaction: generally F tests
  - As already mentioned, with repeated measures, it is generally recommended to use a degrees-of-freedom calculation that is a function of the data (not just of the design). There are different options:
    - **Satterthwaite**

```
model Y = A|B / ddfm=satterth;
```

    - **Kenward-Roger** (generally, first choice, especially with missing data and correlations over time)

```
model Y = A|B / ddfm=kr;
```

- Calculate means and SEs (which depend on the covariance structure), and other interesting comparisons

Mixed model analysis, repeated measures (type=UN)

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treat	1	6	50.63	0.0004
days	5	1.82	11197.6	0.0002
treat*days	5	1.89	67.48	0.0178

The difference of two least squares means (logit scale)

Obs	Effect	treat	days	_treat	_days	Estimate	StdErr	DF	tValue	Probt
1	treat	1	_	2	_	1.3903	0.1954	6	7.12	0.0004
2	treat*days	1	56	2	56	0.6790	0.5085	6	1.34	0.2302
3	treat*days	1	63	2	63	2.2454	0.4938	6	4.55	0.0039
4	treat*days	1	70	2	70	1.7648	0.2599	6	6.79	0.0005
5	treat*days	1	78	2	78	1.2506	0.2216	6	5.64	0.0013
6	treat*days	1	84	2	84	1.8809	0.5273	6	3.57	0.0118
7	treat*days	1	92	2	92	0.5208	0.1547	6	3.37	0.0151

Standard error of the difference (SED). The **LSD is** approximately **2*SED**. However, with unequal variances, there is no single LSD!

Example: Effect of covariance on inference and SEs

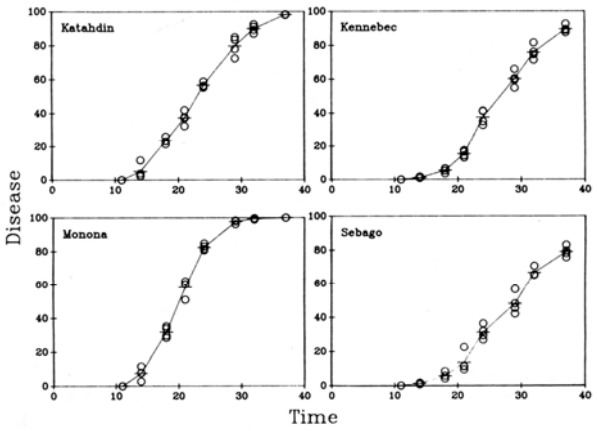
Var-Cov. type	df_N, df_D	F statistic	Prob.	Mean Trt=1,t=56 (SE)	Mean Trt=1,t=92 (SE)
(Simple)	5, 36	3.12	.0194	-2.07 (.2771)	2.388 (.2771)
CS	5, 30	3.46	.0138	-2.07 (.2771)	2.388 (.2771)
AR(1)	5, 2.67	2.93	.0309	-2.07 (.2770)	2.388 (.2770)
UN	***5, 1.89***	***67.48***	***.0178***	***-2.07 (.3596)***	***2.388 (.1094)***
CSH	*5, 11.6*	*4.74*	*.0135*	*-2.07 (.3575)*	*2.388 (.1107)*
ARH(1)	*5, 10.9*	*5.28*	*.0104*	*-2.07 (.3671)*	*2.388 (.1032)*
TOEP	5, 10.6	2.84	.0711	-2.07 (.2789)	2.388 (.2789)
TOEPH	*5, 5.06*	*4.61*	*.0584*	*-2.07 (.3683)*	*2.388 (.1207)*
ANTE(1)	*5, 6.13*	*4.57*	*.0444*	*-2.07 (.3596)*	*2.388 (.1094)*
FA(1)	*5, 6.27*	*5.11*	*.0332*	*-2.07 (.3324)*	*2.388 (.1015)*
H-F	-	-	-	-	-

Note: means are for logits; with *unbalanced* data, means are also affected by covariance

Example

- Results showed that there was unequal variability at different times (but no systematic trend), and that correlations (covariances) varied a lot, but not in a simple way with time lag
- Treatment, time, and the interaction were significant, the latter indicating that the effect of treatment depended on time, or the change in *Y* with time depended on treatment
- More detailed analysis of the data can be performed, based on above results
  - **Determine if the rate of change in *Y* with time (slope) depends on treatment (to be covered later)**
- One can also perform various types of **residual analysis** to determine if the model assumptions are reasonable
  - **Normality, outliers, proper transformation, etc.**

Example 2: potato late blight

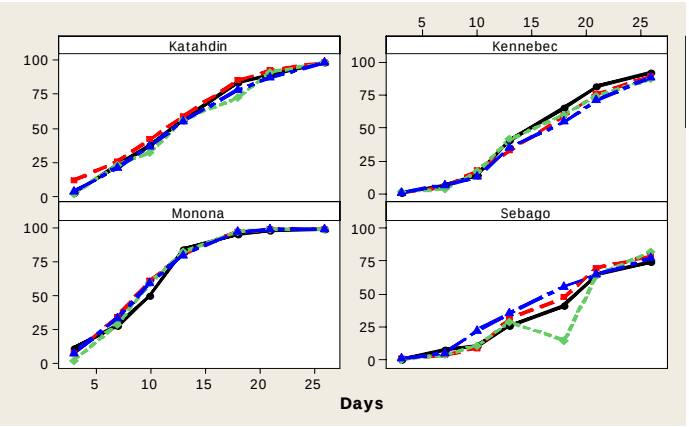


Response: **logit** of disease severity

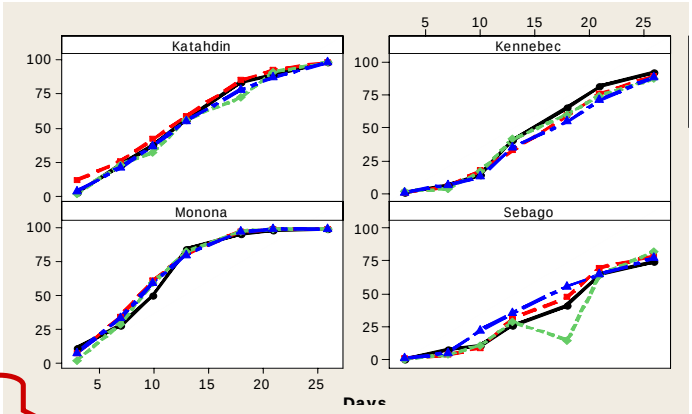
Factors:

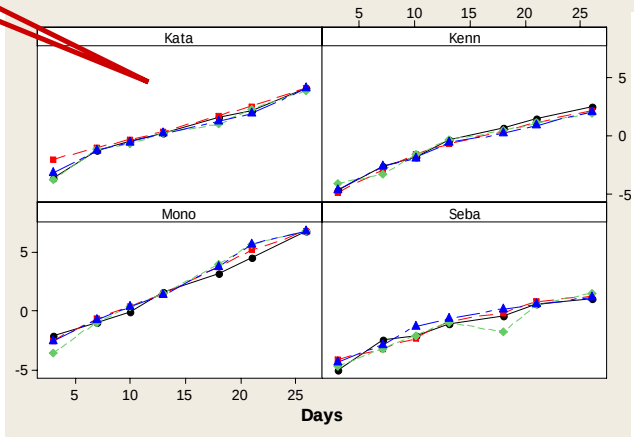
Cultivar (4),
Time (7),
Block (4)

Data from W. Fry, and given in Campbell & Madden (1990)



Example 2: potato late blight





Assignment:
Analyze these data. Find an appropriate covariance matrix, test for cultivar, time, and interaction, get means (and SEs). Don't forget there are blocks in this data set.

Data in: repeatEx2u.sas

Late Blight Example

- What is the most appropriate variance-covariance structure?
- What factors affect the response? How do you interpret these *F* tests?
- What are the least squares means for the cultivars? How do they differ?

Linear Mixed Effects Analysis (especially for Repeated Measures)

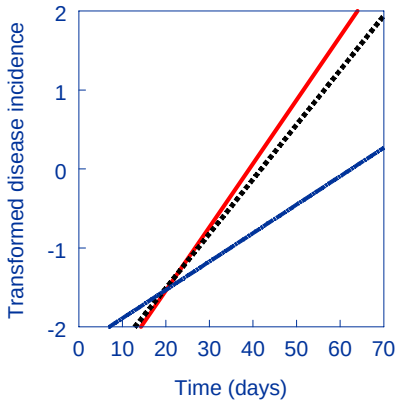
1. Using all the relevant fixed-effect terms in the model (including all interactions), find the most appropriate error structure (i.e., the form of **R**)
   - Base this on -2*likelihood, AIC and BIC statistics
2. With the choice for **R**, test all the fixed effects (including interactions) for significance (*F* tests, and so on)
   - In general, one should use a denominator df based on the data (and not just the design): Kenward-Roger (KR) may be best
   - **Depending on the type of study, one can simplify the model by:**
     - **removing nonsignificant fixed-effect terms, or**
     - **considering time as continuous**
3. With the choice for **R** and fixed effects in the model, estimate the expected values (i.e., means) of *Y*, and SEs, for the levels of the fixed effects (treatments, time, interactions, …)
   - As relevant, use contrasts to evaluate other effects (e.g., pair-wise differences of means [multiple comparisons])

Repeated Measures, continued

For some situations, it is very informative to determine the rate of disease increase over time for each level of the class (factor) variable(s).

In other words, one performs a linear mixed-model analysis of COVARIANCE .

One can determine, among other things: *are the slopes and/or intercepts (curve heights) different among treatments?*



Covariance analysis: one or more fixed-effects variables in the model are factors (class, category variables), and one or more are continuous

Do not confuse Covariance Analysis with the covariance matrix that exists for all the mixed models

Why not just perform a "regular" regression analysis to get intercepts and slopes?

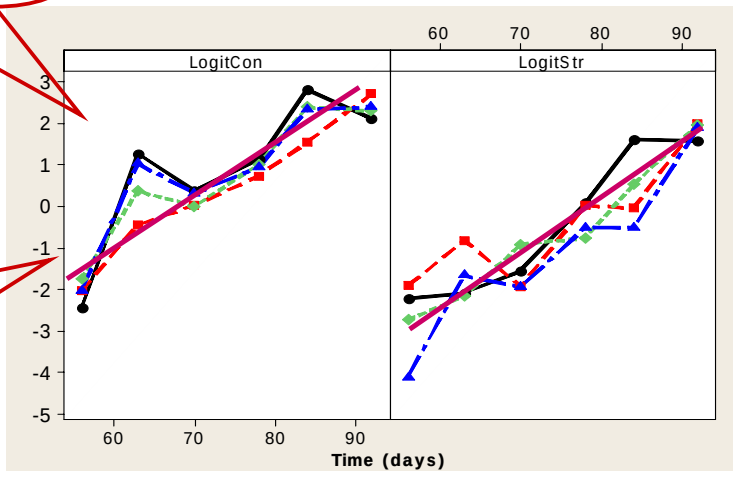
1. For a *single* profile of *Y* vs *t*, this would fine, *if* one tested for temporal autocorrelation of residuals (i.e., test for independence), and made adjustments when correlation was not zero.
   - There are other specialized programs for this (PROC AUTOREG), but these assume only a 1st order autoregressive process [AR(1)]
2. With *several* subjects (e.g., plots), there are profiles of *Y* vs *t* for each, with variation *between* subjects and *within* subjects, with associated correlation of residuals within subjects.
   - This is the fundamental basis for a mixed-model analysis
3. One *could* obtain intercepts and slopes for each of the subjects (as well as for all treatments, if relevant), and then perform mixed-model analysis on the estimated intercepts and slopes as *dependent* variables
   - These estimates are correlated however, causing problems
4. However, the mixed-model covariance analysis works in *one* step, deals with all relevant statistical issues, and is more efficient in utilizing all data at once
   - There is actually a different mixed-model covariance-analysis **("Empirical Bayes")** model, different from the main approach of this workshop, which mimics closely in one step the multiple steps of #3 – accounts for correlations, different sources of variability, etc.

Early leaf spot (example 1), *revisited*

On a logit scale, it appears that a straight line provides a reasonable fit to the disease progress curves

This can all be addressed by using linear mixed models

It also appears that the heights of the lines are different, and *maybe* the slopes (rates)



Repeated Measures: covariance analysis

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \cancel{b_k + d_{ik}} + e_{ijk},$$

$$\cancel{b_k \sim N(0,\sigma_b^2),\ d_{ik} \sim N(0,\sigma_d^2)},\ e_{ijk} \sim N(0,\mathbf{R})$$

Can be fitted with PROC MIXED using almost identical syntax as before

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}, \qquad e_{ijk} \sim N(0,\mathbf{R}),$$ unstructured cov.matrix

$$Y_{ijk} = \mu + \alpha_i + \beta t_j + (\delta_i t_j) + e_{ijk}, \qquad e_{ijk} \sim N(0,\mathbf{R}),$$ unstructured cov.matrix

β is now a single parameter (constant), giving an overall slope for all data

δ_i Is the effect of the i-th treatment on the slope (an interaction term)

Time as a continuous variable (t)

Always maintain the same **R** structure chosen for the fuller model

Repeated Measures: covariance analysis *(another view)*

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}, \qquad e_{ijk} \sim N(0,\mathbf{R})$$

$$Y_{ijk} = \mu + \alpha_i + \beta t_j + (\delta_i t_j) + e_{ijk}, \qquad e_{ijk} \sim N(0,\mathbf{R})$$

***t*: time in days**

-- or --

$$Y_{ijk} = (\mu + \alpha_i) + (\beta + \delta_i) t_j + e_{ijk}, \qquad e_{ijk} \sim N(0,\mathbf{R})$$

Intercept for treatment *i*

Slope for treatment *i*

```
proc mixed data=b;
class  treat rep days;
model logit = treat|t / ddfm=kr solution ;
repeated / subject=rep*treat type=un;
run;
```

Variable **t** is not a "class" factor

Need **"solution"** option to see all parameters

Mixed Models (*an aside*):
Must consider parameterization

$$Y_{ik} = \mu + \alpha_i + e_{ik}, \quad e_{ik} \sim N(0, \sigma_e^2)$$

Example: one-way layout (with only residual variance)

A constant (parameter). If there were no treatments, then this would be the overall mean for all data

Effect of each treatment on expected *Y*.

However, with **4** treatments, for example, there are **5** fixed-effects parameters, μ, α_1, α_2, α_3, α_4. **The model is overparameterized,** meaning there is no unique set of parameters for a given data set. This overparameterization is necessary for calculating valid *F* tests, etc., for effects, and causes no problems in determining means and SEs.

There are several ways of dealing with this overparameterization. The SAS approach is to always assign **0** to the last level of the factor (factors), e.g., $\alpha_4 = 0$.
Expected (means) for four treatments are, thus:

$E(Y_1) = \mu + \alpha_1$ $\quad$ $E(Y_2) = \mu + \alpha_2$

$E(Y_{13}) = \mu + \alpha_3$ $\quad$ $E(Y_4) = \mu + \alpha_4 = \mu + 0 = \mu$

Repeated Measures: covariance analysis
Example: 2 treatments (factor levels)

$$Y_{ijk} = \mu + \alpha_i + \beta t_j + \delta_i t_j + e_{ijk}, \quad e_{ijk} \sim N(0, \mathbf{R})$$

Treatment 1: $Y_{1jk} = (\mu + \alpha_1) + (\beta + \delta_1) t_j + e_{1jk}$

Treatment 2: $Y_{2jk} = (\mu + \alpha_2) + (\beta + \delta_2) t_j + e_{2jk} = (\mu + 0) + (\beta + 0) t_j + e_{2jk}$

```
proc mixed data=b covtest ;
class  treat rep days;    *---Time (t) is not a factor;
model logit = treat|t / ddfm=KR solution ; *---Or: treat t treat*t ;
repeated / subject=rep*treat type=un;
run;
```

	Solution for Fixed Effects						
	Effect	**treat**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
μ	**Intercept**		-12.6180	0.1892	6	-66.69	<.0001
α_1	**treat**	**1**	2.9505	0.2676	6	11.03	<.0001
α_2	**treat**	**2**	0	.	.	.	.
β	**t**		0.1561	0.001908	6	81.82	<.0001
δ_1	**t*treat**	**1**	-0.02210	0.002698	6	-8.19	0.0002
δ_2	**t*treat**	**2**	0	.	.	.	.

Intercepts are:
1) -12.6180 + 2.9505 = -9.668
2) -12.6180 + 0 = -12.618

Slopes are:
1) 0.1561 – 0.02210 = 0.134
2) 0.1561 + 0 = 0.1561

Repeated Measures: covariance analysis

$$Y_{ijk} = \mu + \alpha_i + \beta t_j + \delta_i t_j + e_{ijk}, \qquad e_{ijk} \sim N(0, \mathbf{R})$$

PROC MIXED can be used directly to obtain all slopes and intercepts directly.

Remove the "intercept" and the "main effect" of time: the investigator must understand the parameterization and software syntax.

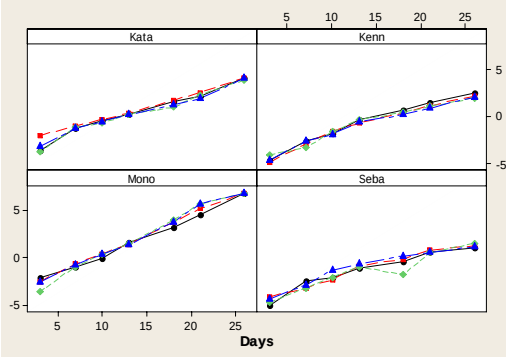
Continue analysis of the early leaf spot data (Example 1), and learn how to directly obtain intercepts and slopes, and compare them with contrast statements.

repeatEx3.sas

Continue analysis of potato late blight data (Example 2):

Obtain slopes and intercepts for each cultivar, and compare these…

repeatEx2u.sas



Covariance analysis (general points)

- PROC MIXED statements are very similar to those already used (time is no longer a factor (not in CLASS statement))
- However, unlike the situation with only class (factor) variables, the actual **solution** to the fixed-effect terms in the model (e.g., α_1, α_2, …, β, etc.) is needed, not just means (i.e., linear combinations of the terms).
- Generally, the fit will be 'poorer' if time is continuous (since one is fitting a model with a **straight-line** through the points) than when it is a factor
  - With time as a factor, much more complicated relationships between *Y* and time are allowed (quadratic, asymptotic)
  - Note: always find the covariance matrix structure with time as a factor, and then switch to time as continuous (no need to reassess the best covariance structure)
- Other continuous variables can be incorporated in the model from the start (never considered as a factor):
  - e.g., weather variables for each experimental unit

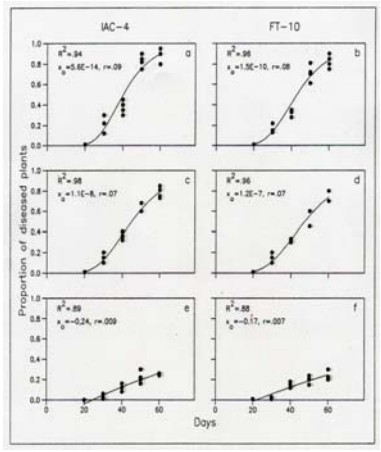
More complex experimental designs

- Factorial + time (with or without blocks)
- Split plot + time (with or without blocks)
- Time + subsampling (within plots)
- Doubly repeated measures
  - (e.g., collect samples ("harvest") of leaves or fruit at several times from same plots, then measure disease on these samples at several times (for each "harvest"))
- Spatially repeated measures, with or without temporal repeated measures
- Random-effect factors of direct interest
  - e.g., random location effect or random pathogen strain effect

Repeated measures example (factorial)

- Data set:
  - Effects of planting date and cultivar on disease progress of soybean bud blight
    - Almeida et al. (1994)
    - A subset of dataset was used in Madden et al. textbook (2007), with a different transformation
- Terms in model:
  - Block, ***b*** (random)
  - Planting date, **α** (fixed)
  - Time, **β** (fixed), use just last 4 dates (no variation on first assessment)
  - Cultivar or "var", **γ** (fixed)
  - Interactions
- Response: transformed incidence (*Y*) ["Gompertz" transformation here]

Numerous questions to ask about the main effects and interactions



repeatEx4.sas

Factorial Repeated Measures (with blocks)

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{il} + (\beta\gamma)_{jl} + (\alpha\beta\gamma)_{ijl} + b_k + e_{ijkl}$$
$$b_k \sim N(0,\sigma_b^2),\ e_{ijkl} \sim N(0,\mathbf{R})$$

Y_{ijk}: response (dependent variable) –Gompit of incidence

μ: constant ("intercept")

α_i: Effect of the *i*-th level of planting date on *Y*

β_j: Effect of the *j*-th time (days) on *Y*

γ_l: Effect of the *l*-th variety on *Y*

b_k: Effect of the *k*-th block

$(\alpha\beta)_{ij}$,etc.: Interactions

e_{ijkl}: Residual

R: A **matrix** of variances and covariances of *Y* for each *ikl* experimental unit (subject)

Try different structures

```
proc mixed data=alv covtest;
title2 'repeated measures, cs' ;
class var PD dayc block ;
model ystar = var|PD|dayc / ddfm=kr ;
random block;
repeated / sub=block*var*PD type=CS;
```

Example

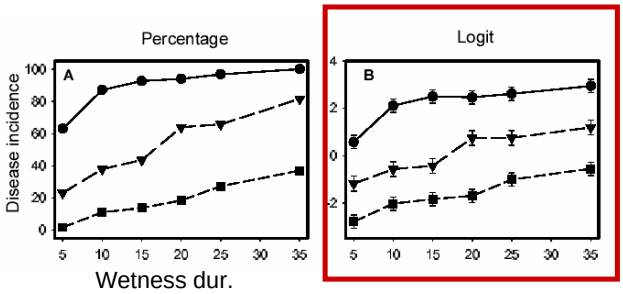
Groups of plants inoculated at single temperatures, and plants removed from chamber after different periods of time (wetness durations). Leaflets of three ages were assessed for disease.

Data set:

Effects of Wetness duration (W), Temperature (T), and leaflet age (A) on incidence (and severity) of Phomopsis leaf blight of strawberry (Nita et al., *Plant Dis*. 87: 579-584).

Terms:
R: repetition (block) – random
T: (6 levels)
W: (6 levels)
A: (3 levels)
+ Interactions, + random effects

Although disease only measured once, both W and A are repeated measures, since there is, by definition, no randomization of wetness durations or leaflet ages. Moreover, the clustering of W (and A) within T*R experimental units results in correlations. But selected plants at each W were randomly selected from the total, so W might be considered a sub-plot in a split-plot design, + time.



Multiple levels of subjects:
blocks,
block*temperature ("Whole plot" variation),
block*temperatue*wetness ("sub-plot" variation),
block*temperature*wetness*age (residual)

repeatEx5.sas

Doubly Repeated Measures (as split-split-plot)

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{il} + (\beta\gamma)_{jl} + (\alpha\beta\gamma)_{ijl} + b_k + d_{ik} + f_{ijk} + e_{ijkl},$$

$$b_k \sim N(0,\sigma_b^2),\ d_{ik} \sim N(0,\sigma_d^2),\ f_{ijk} \sim N(0,\sigma_f^2),\ e_{ijkl} \sim N(0,\sigma_e^2)$$

Y_{ijk}: response (dependent variable) – logit of incidence

μ: constant ("intercept")

α_i: Effect of the *i*-th level of **temperature** on *Y (whole plot)*

β_j: Effect of the *j*-th **wetness duration** on *Y (sub plot)*

γ_l: Effect of the *l*-th leaflet **age** on *Y (sub-sub plot = "time"-like term)*

$(\alpha\beta)_{ij}$,etc.: Interactions

b_k: Effect of the *k*-th block on *Y*

d_{ik}: Effect of *ik*-th experimental unit on *Y ("whole plot" error)* **(run of the exp. at a given temp.)**

f_{ijk}: Effect of *ijk*-th experimental unit on *Y ("sub-plot" error)* **(run of exp. at a given temp. at a given wetness duration)**

e_{ijkl}: Residual *("sub-sub plot" error)*

Doubly Repeated Measures (as split-plot + time)

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{il} + (\beta\gamma)_{jl} + (\alpha\beta\gamma)_{ijl} + b_k + d_{ik} + e_{ijkl},$$

$$b_k \sim N(0,\sigma_b^2),\ d_{ik} \sim N(0,\sigma_d^2),\ e_{ijkl} \sim N(0,\mathbf{R})$$

Y_{ijk}: response (dependent variable) – logit of incidence

μ: constant ("intercept")

α_i: Effect of the *i*-th level of **temperature** on *Y (whole plot)*

β_j: Effect of the *j*-th **wetness duration** on *Y (sub plot)*

γ_l: Effect of the *l*-th leaflet **age** on *Y ("time"-like term)*

$(\alpha\beta)_{ij}$,etc.: Interactions

b_k: Effect of the *k*-th block on *Y*

d_{ik}: Effect of *ik*-th experimental unit *(run of exp. in chamber at a temp.)* on *Y*

e_{ijkl}: Residual

Could expand more, by considering *structure* for the sub-plot variability term

R: A **matrix** of variances and covariances of *Y* for each *ijk* experimental unit (subject) -- try different structures

Repeated Measures—doubly repeated (again)

One could argue that there is no natural hierarchy—***age within wetness*** or ***wetness within age***—but a *simultaneous* clustering within block-temp.

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{il} + (\beta\gamma)_{jl} + (\alpha\beta\gamma)_{ijl} + b_k + e_{ijkl}$$

$$b_k \sim N(0,\sigma_b^2)\ ,\ e_{ijkl} \sim N(0,\mathbf{R})$$

Y_{ijk}: response (dependent variable) – logit of incidence

μ: constant ("intercept")

α_i: Effect of the *i*-th level of **temperature** on *Y*

β_j: Effect of the *j*-th **wetness duration** on *Y*

γ_l: Effect of the *l*-th leaflet **age** on *Y*

$(\alpha\beta)_{ij}$,etc.: Interactions

b_k: Effect of the *k*-th block on *Y*

e_{ijkl}: Residual

R: A **matrix** of variances and covariances of *Y* for each ***ik*** experimental unit (subject). **Requires a MULTIVARIATE covariance structure**

… `type=un@ar(1)` or `type=un@cs` or `type=un@un;`

(See SAS Manual for more details -- this is the approach in Nita et al. -- not covered here)

Repeated Measures: Conclusions

- In general, mixed models are extremely valuable for analyzing data collected over time in multiple experimental units (subjects)
  - Although there are other useful approaches, for most investigators, mixed models should be used (especially, linear mixed models)
- With programs such as PROC MIXED of SAS (and others), investigators have extremely powerful tools available for properly analyzing data from all kinds of experimental designs (including repeated measures)
- The key to proper analysis is to choose an appropriate mixed model, which entails specifying the fixed and random effects in the model, as well as the structure of the variance-covariance matrix of the residuals (**R**)

Repeated Measures: Conclusions, *continued*

- With repeated measures, the experimental design alone cannot be used to decide on the structure for $\boldsymbol{R}$ -- one must allow the data to indicate which structure is reasonable
  - Statistical inference and interpretation (for the fixed and the random effects) follow naturally, once a reasonable model is chosen (including $\boldsymbol{R}$)
- As indicated at the beginning of the workshop, nonlinear mixed models and generalized linear mixed models are also of great value for many analyses (maybe the subject of another workshop)
- As always in data analysis, investigators should consult with a statistician whenever possible

Repeated Measures: Addendum

- Longer list of covariance matrix structures available in PROC MIXED of SAS
- Introduction to Estimation and Hypothesis Testing: the use **`ESTIMATE`** and **`CONTRAST`** statements with PROC MIXED of SAS
  - *See separate file (…supplemental) for this information*

Table 46.7. Covariance Structure Examples

Description	Structure	Example
Variance Components	VC (default)	$\begin{bmatrix} \sigma_B^2 & 0 & 0 & 0 \\ 0 & \sigma_B^2 & 0 & 0 \\ 0 & 0 & \sigma_{AB}^2 & 0 \\ 0 & 0 & 0 & \sigma_{AB}^2 \end{bmatrix}$
Compound Symmetry	CS	$\begin{bmatrix} \sigma^2+\sigma_1 & \sigma_1 & \sigma_1 & \sigma_1 \\ \sigma_1 & \sigma^2+\sigma_1 & \sigma_1 & \sigma_1 \\ \sigma_1 & \sigma_1 & \sigma^2+\sigma_1 & \sigma_1 \\ \sigma_1 & \sigma_1 & \sigma_1 & \sigma^2+\sigma_1 \end{bmatrix}$
Unstructured	UN	$\begin{bmatrix} \sigma_1^2 & \sigma_{21} & \sigma_{31} & \sigma_{41} \\ \sigma_{21} & \sigma_2^2 & \sigma_{32} & \sigma_{42} \\ \sigma_{31} & \sigma_{32} & \sigma_3^2 & \sigma_{43} \\ \sigma_{41} & \sigma_{42} & \sigma_{43} & \sigma_4^2 \end{bmatrix}$
Banded Main Diagonal	UN(1)	$\begin{bmatrix} \sigma_1^2 & 0 & 0 & 0 \\ 0 & \sigma_2^2 & 0 & 0 \\ 0 & 0 & \sigma_3^2 & 0 \\ 0 & 0 & 0 & \sigma_4^2 \end{bmatrix}$
First-Order Autoregressive	AR(1)	$\sigma^2 \begin{bmatrix} 1 & \rho & \rho^2 & \rho^3 \\ \rho & 1 & \rho & \rho^2 \\ \rho^2 & \rho & 1 & \rho \\ \rho^3 & \rho^2 & \rho & 1 \end{bmatrix}$
Toeplitz	TOEP	$\begin{bmatrix} \sigma^2 & \sigma_1 & \sigma_2 & \sigma_3 \\ \sigma_1 & \sigma^2 & \sigma_1 & \sigma_2 \\ \sigma_2 & \sigma_1 & \sigma^2 & \sigma_1 \\ \sigma_3 & \sigma_2 & \sigma_1 & \sigma^2 \end{bmatrix}$
Toeplitz with Two Bands	TOEP(2)	$\begin{bmatrix} \sigma^2 & \sigma_1 & 0 & 0 \\ \sigma_1 & \sigma^2 & \sigma_1 & 0 \\ 0 & \sigma_1 & \sigma^2 & \sigma_1 \\ 0 & 0 & \sigma_1 & \sigma^2 \end{bmatrix}$
Spatial Power	SP(POW)(*c*)	$\sigma^2 \begin{bmatrix} 1 & \rho^{d_{12}} & \rho^{d_{13}} & \rho^{d_{14}} \\ \rho^{d_{21}} & 1 & \rho^{d_{23}} & \rho^{d_{24}} \\ \rho^{d_{31}} & \rho^{d_{32}} & 1 & \rho^{d_{34}} \\ \rho^{d_{41}} & \rho^{d_{42}} & \rho^{d_{43}} & 1 \end{bmatrix}$
Heterogeneous AR(1)	ARH(1)	$\begin{bmatrix} \sigma_1^2 & \sigma_1\sigma_2\rho & \sigma_1\sigma_3\rho^2 & \sigma_1\sigma_4\rho^3 \\ \sigma_2\sigma_1\rho & \sigma_2^2 & \sigma_2\sigma_3\rho & \sigma_2\sigma_4\rho^2 \\ \sigma_3\sigma_1\rho^2 & \sigma_3\sigma_2\rho & \sigma_3^2 & \sigma_3\sigma_4\rho \\ \sigma_4\sigma_1\rho^3 & \sigma_4\sigma_2\rho & \sigma_4\sigma_3\rho & \sigma_4^2 \end{bmatrix}$
First-Order Autoregressive Moving-Average	ARMA(1,1)	$\sigma^2 \begin{bmatrix} 1 & \gamma & \gamma\rho & \gamma\rho^2 \\ \gamma & 1 & \gamma & \gamma\rho \\ \gamma\rho & \gamma & 1 & \gamma \\ \gamma\rho^2 & \gamma\rho & \gamma & 1 \end{bmatrix}$
Heterogeneous CS	CSH	$\begin{bmatrix} \sigma_1^2 & \sigma_1\sigma_2\rho & \sigma_1\sigma_3\rho & \sigma_1\sigma_4\rho \\ \sigma_2\sigma_1\rho & \sigma_2^2 & \sigma_2\sigma_3\rho & \sigma_2\sigma_4\rho \\ \sigma_3\sigma_1\rho & \sigma_3\sigma_2\rho & \sigma_3^2 & \sigma_3\sigma_4\rho \\ \sigma_4\sigma_1\rho & \sigma_4\sigma_2\rho & \sigma_4\sigma_3\rho & \sigma_4^2 \end{bmatrix}$
First-Order Factor Analytic	FA(1)	$\begin{bmatrix} \lambda_1^2+d_1 & \lambda_1\lambda_2 & \lambda_1\lambda_3 & \lambda_1\lambda_4 \\ \lambda_2\lambda_1 & \lambda_2^2+d_2 & \lambda_2\lambda_3 & \lambda_2\lambda_4 \\ \lambda_3\lambda_1 & \lambda_3\lambda_2 & \lambda_3^2+d_3 & \lambda_3\lambda_4 \\ \lambda_4\lambda_1 & \lambda_4\lambda_2 & \lambda_4\lambda_3 & \lambda_4^2+d_4 \end{bmatrix}$
Huynh-Feldt	HF	$\begin{bmatrix} \sigma_1^2 & \frac{\sigma_1^2+\sigma_2^2}{2}-\lambda & \frac{\sigma_1^2+\sigma_3^2}{2}-\lambda \\ \frac{\sigma_2^2+\sigma_1^2}{2}-\lambda & \sigma_2^2 & \frac{\sigma_2^2+\sigma_3^2}{2}-\lambda \\ \frac{\sigma_3^2+\sigma_1^2}{2}-\lambda & \frac{\sigma_3^2+\sigma_2^2}{2}-\lambda & \sigma_3^2 \end{bmatrix}$
First-Order Ante-dependence	ANTE(1)	$\begin{bmatrix} \sigma_1^2 & \sigma_1\sigma_2\rho_1 & \sigma_1\sigma_3\rho_1\rho_2 \\ \sigma_2\sigma_1\rho_1 & \sigma_2^2 & \sigma_2\sigma_3\rho_2 \\ \sigma_3\sigma_1\rho_2\rho_1 & \sigma_3\sigma_2\rho_2 & \sigma_3^2 \end{bmatrix}$
Heterogeneous Toeplitz	TOEPH	$\begin{bmatrix} \sigma_1^2 & \sigma_1\sigma_2\rho_1 & \sigma_1\sigma_3\rho_2 & \sigma_1\sigma_4\rho_3 \\ \sigma_2\sigma_1\rho_1 & \sigma_2^2 & \sigma_2\sigma_3\rho_1 & \sigma_2\sigma_4\rho_2 \\ \sigma_3\sigma_1\rho_2 & \sigma_3\sigma_2\rho_1 & \sigma_3^2 & \sigma_3\sigma_4\rho_1 \\ \sigma_4\sigma_1\rho_3 & \sigma_4\sigma_2\rho_2 & \sigma_4\sigma_3\rho_1 & \sigma_4^2 \end{bmatrix}$
Unstructured Correlations	UNR	$\begin{bmatrix} \sigma_1^2 & \sigma_1\sigma_2\rho_{21} & \sigma_1\sigma_3\rho_{31} & \sigma_1\sigma_4\rho_{41} \\ \sigma_2\sigma_1\rho_{21} & \sigma_2^2 & \sigma_2\sigma_3\rho_{32} & \sigma_2\sigma_4\rho_{42} \\ \sigma_3\sigma_1\rho_{31} & \sigma_3\sigma_2\rho_{32} & \sigma_3^2 & \sigma_3\sigma_4\rho_{43} \\ \sigma_4\sigma_1\rho_{41} & \sigma_4\sigma_2\rho_{42} & \sigma_4\sigma_3\rho_{43} & \sigma_4^2 \end{bmatrix}$
Direct Product AR(1)	UN@AR(1)	$\begin{bmatrix} \sigma_1^2 & \sigma_{21} \\ \sigma_{21} & \sigma_2^2 \end{bmatrix} \otimes \begin{bmatrix} 1 & \rho & \rho^2 \\ \rho & 1 & \rho \\ \rho^2 & \rho & 1 \end{bmatrix} =$

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