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TGP/8/1 Draft 1
ORIGINAL: English
DATE: June 2, 2005

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

DRAFT

Associated Document
to the
General Introduction to the Examination
of Distinctness, Uniformity and Stability and the
Development of Harmonized Descriptions of New Varieties of Plants (document TG/1/3)

DOCUMENT TGP/8

"USE OF STATISTICAL PROCEDURES IN DISTINCTNESS, UNIFORMITY AND STABILITY TESTING"

Document prepared by the Office of the Union

to be considered by the

Technical Working Party for Vegetables (TWV), at its thirty-ninth session to be held in Nitra, Slovakia, from June 6 to 10, 2005

Technical Working Party on Automation and Computer Programs (TWC), at its twenty-third session to be held in Ottawa, from June 13 to 16, 2005

Technical Working Party for Fruit Crops (TWF), at its thirty-sixth session to be held in Kofu, Japan, from September 5 to 9, 2005

Technical Working Party for Ornamental Plants and Forest Trees (TWO), at its thirty-eighth session to be held in Seoul, from September 12 to 16, 2005

Technical Working Party for Agricultural Crops (TWA), at its thirty-fourth session to be held in Christchurch, New Zealand, from October 31 to November 4, 2005

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SECTION I: INTRODUCTION

1. Aim

This introduction will briefly cover the key-elements on statistics as used in the examination of DUS. It is meant to encourage the reading of subsequent chapters that elaborate on these elements. To arrive at an objective decision when testing and comparing different items, it is important to be aware of the basic statistical notions and practices.

2. Observable characteristics and variation

The requirements are formulated and recorded in ('absolute') **observable characteristics** that are used to assess distinctness (D) between varieties, uniformity (U) within a variety and stability (S) over years for varieties. It is thus generally referred to as **DUS**-testing. The nature of the object of interest, a plant variety, makes it susceptible to all kinds of **external** influences, e.g. growing conditions like latitude, climate, soil, irrigation etc. which render variation in the expression of the characteristics observed between plants. But there are also aspects that may lead to variation (or the lack of it) in the observed characteristic of a given variety because of the type of variety e.g. the way it is propagated (e.g. cross-pollinated vs. vegetative propagation, seed/bulb/tuber size etc). We must also bear in mind that measuring a characteristic leads to yet another source of variation; the **measurement error**. All these elements of variation in our observations are to be taken into account in a model agreed by UPOV for proper **analysis**. These topics are pursued later on.

3. Why use statistical procedures?

Each observation contains a mixture of all these sources of variation and it is good practice to have some basic notion of their magnitudes (if possible). At the end of the run this will determine the **reliability** of the measurements and that is what **statistics** is all about and why we need statistical procedures for a judgement.

4. Why replicate?

The first key element of experimentation in a statistical context is **replication**. This can either be determining the number of off-types in e.g. sixty plants in only one experiment or on 20 separate plants per plot in 3-replicated blocks for 3 consecutive years. In all cases, the replication gives us a handle on hypothesis testing: is the candidate variety different from the rest by some observed characteristic that exceeds the noise that is basic to the whole experiment? This noise, present in all observations (=experimental error), acts as a yard-stick to judge differences in the experiment (i.e. distinctness). When the characteristic is less consistent, the difference for a decision needs to be accordingly greater. Moreover the observations on the replications can be used as a uniformity measure.

5. Why randomize?

The second key element is **randomization** for each experiment. This ensures that all unknown sources of variation have an equal chance of acting upon the varieties tested, and no accidental systematic effect is recorded due to e.g. germination, planting, growing, harvesting, recording etc. In those cases where specific ordering is required to observe a characteristic (e.g. comparison of colour or architecture) one should be aware that this is only (reluctantly) admissible in one replication, and one should be careful with conclusions on other

measurements. This brings our focus to a case where some grouping is required because otherwise competition would influence the observation, e.g. early varieties would hamper the development of late ones. In those cases it is crucial to randomize within and between groups of the experiment, as experiments are carried out over the years and groups should then be arranged differently to avoid systematic effects.

6. Further benefits of randomization

Proper randomization makes our conclusions valid/predictive for all similar experiments, and not only for one specific experiment of which the results might have been caused by some unknown systematic effects, e.g. ordering of varieties, some fertility trend/spot in the soil or recording time during the day. Randomization will also prevent us from working with an unrealistic small experimental error that cannot be reproduced, e.g. by close comparison of specific varieties or when all varieties are lined up in the field based on one characteristic. Randomization will also enhance objective recording of the characteristic.

7. Direct comparison and randomization

It is nevertheless common practice to plant varieties which show little difference between them side-by-side or in the same area. This allows better evaluation by the experts during the different visits and observations through direct comparison in the field for difficult cases. As already stated this does not invalidate the statistical reliability too much if it is confined to one replication in a blocked trial, or if at least different orderings are used in different blocks and in different trials. One should be aware that the yardstick used for discrimination on the other characteristics is not as reliable in this case, as the special arrangement can only be based on one or two characteristics(s). Close scrutiny in one characteristic may introduce systematic or interaction effects (bias) for others, so more care is needed in the interpretation.

8. Variety is the treatment

The third key element is the experimental (main) **treatment,** for the purposes of DUS examination these are the different **varieties**: candidate varieties and the varieties of common knowledge to be compared. Other major external sources of variation are not the object of study in this context e.g. latitude and soil. The data are observed on plants in good growing conditions. In those cases where (random) experimental conditions are thought to be relevant for the characteristic performance of the varieties (often year-by-year variation, e.g. climatic influences) this is compensated by carrying out the experiment during several years. A candidate variety can thus be compared with others using a different criterion that includes this extra source of variation (variety-by-year interaction, in or combined of year 'COY' analysis). The requirement for consistent results often implies the use of results from more than one trial for a decision. Consistency means that the general ranking between varieties should not change a lot between trials; otherwise the characteristic may not be not useful for the examination of DUS.

9. The crop makes the difference in testing

A fourth key element is the specific set of considerations that holds for a **crop**. For that reason general information is provided in this document. Depending on the crop they may or may not bare relevance on assessing distinctness in a reliable way. Two extreme examples are given. The first example is for vegetatively propagated ornamental varieties. Notes on a different colour that is consistent for all flowers during one growing season for six different

plants will often be sufficient. The second example is rapeseed, bulk samples have to be made for a reliable observation on the specific oil content of each variety and bulk samples have specific statistical implications.

For most crops the characteristics and requirements are defined in the relevant **Test Guidelines**. However, sometimes additional characteristics can be used as a complement to the Test Guidelines characteristics. A point to stress is that at all different stages, in the development of the crop, observations can be made. So it is imperative that all the facets in recording a characteristic are described properly and exhaustively to ensure that they can be compared in the long run but also understood by a novice.

10. The expert and statistical computation

During or at the end of the study, the crop expert uses all data on the same set of characteristics for all varieties in the DUS test. The use and the need of computations may vary considerably. We have seen that in some cases the notes recorded and the knowledge of the expert is sufficient. While in other cases one needs to compile a large set of replicated data from more than one growing cycle, in order to compute objective values to assess the final expert decision. However storing the data on a computer opens the possibility for easy management with routinely checks and analysis.

11. UPOV, statistical computation and harmonization

Finally, looking at the very large range of available methods and software to compute data, UPOV provides guidance on the choice and use of computational methods. Where possible software is made available. A major advantage of computations and statistics is that it is less subjective than the judgement of the crop expert. It provides data, additional controls of the data, graphical representations, and suggests decisions based on probabilities, etc., which can be understood, shared and used in a similar way by people from different countries.

12. Recapitulation with reference to subsequent chapters:

Requirements:

- Observable characteristics.

  TGP 8.3: Types of Characteristics and Their Scale Levels, gives an in-depth treatment on what a characteristic is, the different levels to look at a characteristics, the scale levels that can be distinguished among characteristics and the consequences of these levels for the statistical procedures that can be used.

Plants show variation in their characteristics:

- External – latitude, climate, soil irrigation etc.
- Internal – method of propagation; e.g.cross-pollination vs. vegetative propagation
- Intrinsic to all measures: the measurement error

Observation: an observation contains a mixture of different sources of variation, the magnitudes of which translates into the reliability of the observations; which is statistics.

Key elements to measure these sources of variation in a proper way:

- Replication:
  - Plants, plots, blocks, year -> off-types or mean character with standard deviation.

  - Hypothesis testing: experimental error (noise) yardstick for distinctness. The larger the yardstick the larger the difference needed.
  - Replication variation -> uniformity measure
- Randomisation:
  - known and unknown sources of variation
  - Valid conclusions and objective notes
  - Specific ordering gives direct comparison of few characters, can introduce systematic error in yardstick.
- Treatment:
  - Treatment is Variety
  - Plants in good growing conditions.
  - Year may influence characteristic. Need of a different yardstick (i.e. variety-by-year interaction)
  - More growing cycles: Checking the consistency of results.
- Crop:
  - Each crop has its unique requirements
  -

TGP 8.2: Experimental Design Practices covers the broad field of the above considerations that will eventually lead to a specific design on the conditions relevant to the specific crop; available designs, plot size, experimental units, field layout, complete and incomplete blocks, hypothesis testing, analysis and comparisons of units etc. Already at this stage all statistical considerations are relevant.

TGP 8.4: Validation of Data and Assumptions. With observations, the first thing to do is to check the correctness of the values. A number of examination methods are presented to spot discrepant observations. Because the recommended statistical methods for DUS testing are based on statistical theory the assumptions behind these methods are described and it is shown how these assumptions can be validated.

TGP 8.5: Statistical Methods for DUS examination, explains the way how DUS testing is done with COYD and COYU.

TGP 8.6: Examining DUS in bulk samples tells us how to do DUS examination when single observations on a plant basis are either not feasible, too expensive or time consuming.

TGP 8.7: Segregation Ratios (not yet filed), this is important for cross-pollinated species.

SECTION 2: EXPERIMENTAL DESIGN PRACTICES

2.1 Introduction

2.1.1 DUS trials are experiments for the comparison of varieties and the observation of characteristics. In this section, the emphasis will be on the comparison of varieties. Characteristics of the varieties are observed in the trial in order to assess Distinctness, Uniformity and Stability (DUS). Measurements and visually assessed data are analyzed and, using the results of the analyses, decisions are made about DUS. This section addresses a number of issues concerning the basics of statistics and experimental design. Some of the issues, e.g. variance components and sample size, assume that the characteristics are continuous, quantitative characteristics (see document TGP/8.3 'Type of characteristics and their scale levels').

2.1.2 The UPOV Test Guidelines already provide a set of recommendations for conducting the DUS test. They provide a list of characteristics and suitable test methods, but contain only a brief summary of relevant experimental designs. It is expected that the examiner conducting the tests should understand the DUS test and have good knowledge of the growing conditions for the species and the factors that can affect the expressions of the characteristics of the variety. It is important that the requirements of experimental assumptions should be well recognized (see section 4). Many environmental factors, like temperature, rainfall and sunshine, which are not under control, may influence the expression of the characteristics of the variety. Characteristics which are influenced by environmental factors in a variable way are usually relatively bad characteristics – unless the growing tests are conducted in greenhouses or other protected areas, which are less subject to such environmental factors.

2.1.3 When designing the DUS test, the examiner ('crop expert') should also take into account the features of the varieties being examined such as:

- development type (long day/short day type, winter/spring type)
- earliness (flowering, maturity)
- height of plants

Other important environmental factors include soil structure, irrigation, date of sowing (planting), fertilization and pest and disease control. These may have an influence on the variation between plots in the trial and the behaviour of the plants. The crop expert should have good knowledge of the crop and the Test Guidelines. The same procedure and protocol should be followed in all growing cycles of the test to minimize the interaction between varieties and environments. In practice, it is not usual to perform tests of stability that produce results as certain as those of the testing of distinctness and uniformity. Where appropriate or in cases of doubt, stability may be tested, either by growing a further generation, or by testing a new seed or plant stock to ensure that it exhibits the same characteristics as those shown by the previous material supplied. The test should normally be conducted in one place (location). If any important characteristics of the variety cannot be seen at that place, the variety may be tested at an additional place. In some crops, the minimum duration of the test is two independent growing cycles. That provides assurance that the observed differences between varieties are sufficiently consistent. For grasses (herbage), two separate trials, sown in successive years, are usually observed as a minimum. In some countries three separate trials are carried out for grasses. Most vegetatively propagated ornamental varieties are tested for only one growing cycle. The Test Guidelines

for the species indicate the number of plants and number of replications (usually at least two or more replications for most species). The whole plot or a representative sample of the plot is observed to assess characteristics visually. Measurements to determine distinctness and uniformity are made on a representative number of plants in accordance with the Test Guidelines. The size of the plots should be such that plants or part of plants may be removed for measuring or counting without prejudice to the observations that must be made up to the end of the growing period.

2.1.4 The varieties with which a variety under test must be compared are those varieties whose existence is a matter of common knowledge (varieties of common knowledge). Testing authorities may create a variety collection with all varieties which could be similar to candidate varieties in that country with the most similar reference varieties are selected from this collection for inclusion in the growing trial. Further guidance for the establishment and management of variety collections is provided in documents TGP/4 'Management of variety collections' and TGP/9 'Examining Distinctness'. The varieties to be grown should be divided into groups – if suitable grouping characteristics exists – to facilitate the assessment of distinctness. Characteristics, which are suitable for grouping purposes, are those in which the documented state of expression, even when recorded at different locations, can be used to select varieties of common knowledge that can be excluded from the growing trial used for examining distinctness, and/or to organize the growing trial so that similar variety trails are grouped together (see document TGP/7 'Development of Test Guidelines'). Grouping characteristics are provided in the Test Guidelines. In this way the most similar varieties are compared with each other in the trial. As far as possible and on the basis of available information the most similar varieties are placed close to each other of the same group. Relevant varieties of common knowledge and the example varieties from the variety collection should be included in the trial. Grouping of varieties according to one or more characteristics (e.g. flower colour, ploidy, heading dates in grasses) help in the arrangement of the trial and can limit the number of varieties of common knowledge which need to be included in the growing trial. Identification of varieties which need to be grown and of the most similar varieties may in some cases be based on a database containing previously established descriptions and, particularly in the case of ornamental and fruit species, by comparing the photograph of the candidate variety and photograph of existing varieties in the database.

2.1.5 The total number of varieties (i.e. varieties of common knowledge and candidate varieties) included in the trial will influence the experimental design.

2.2 Trials, experimental units and test of hypotheses

2.2.1 A plot is the experimental unit to which the varieties are allocated. A plot may contain several individual plants from the same variety. A block is a group of plots within which the varieties are randomized.

2.2.2 To decide whether a variety is uniform and whether it is distinct from other varieties statistical tests are performed. For each of these two tests we have to consider two hypotheses as specified in the following table:

	Distinctness	*Uniformity*
Null hypothesis (H0)	two varieties are not distinct	a variety is uniform
Alternative Hypothesis (H1)	two varieties are distinct	a variety is not uniform

By using a test statistic (which is a formula of the observations) a decision has to be made to accept the null hypothesis H0 and thus to reject the alternative hypothesis H1 or vice versa. The decision to reject H0 occurs if the test statistic is greater than the chosen critical value, otherwise H0 is accepted. If H0 is rejected the test is called significant.
The different types of error which can be made for distinctness are shown in the following table:

		Real situation	
		H0 true two varieties are not distinct	H1 true two varieties are distinct
Decision	H0 accept two varieties are not distinct	Correct decision	type II error (β)
	H0 reject two varieties are distinct	type I error (α)	Correct decision

The same situation for uniformity is shown in the next table:

		Real situation	
		H0 true a variety is uniform	H1 true a variety is not uniform
Decision	H0 accept a variety is uniform	Correct decision	type II error (β)
	H0 reject a variety is not uniform	type I error (α)	Correct decision

2.2.3 When doing tests there are always two types of error. They are called type I error and type II error, respectively. Let's take the test whether two varieties are different. The type I error is the error that arises when we decide that the varieties are distinct, when, in fact, they are not distinct. The type II error is the error that arises when we decide that the varieties are not distinct, when, in fact, they are distinct (valid for distinctness). The risk of type I error can be controlled easily by taking a self chosen size α of the test, whereas the risk of type II error is more difficult to control as it depends on the size of the real difference between the varieties, the random variability s, the number of replicates and the chosen α.

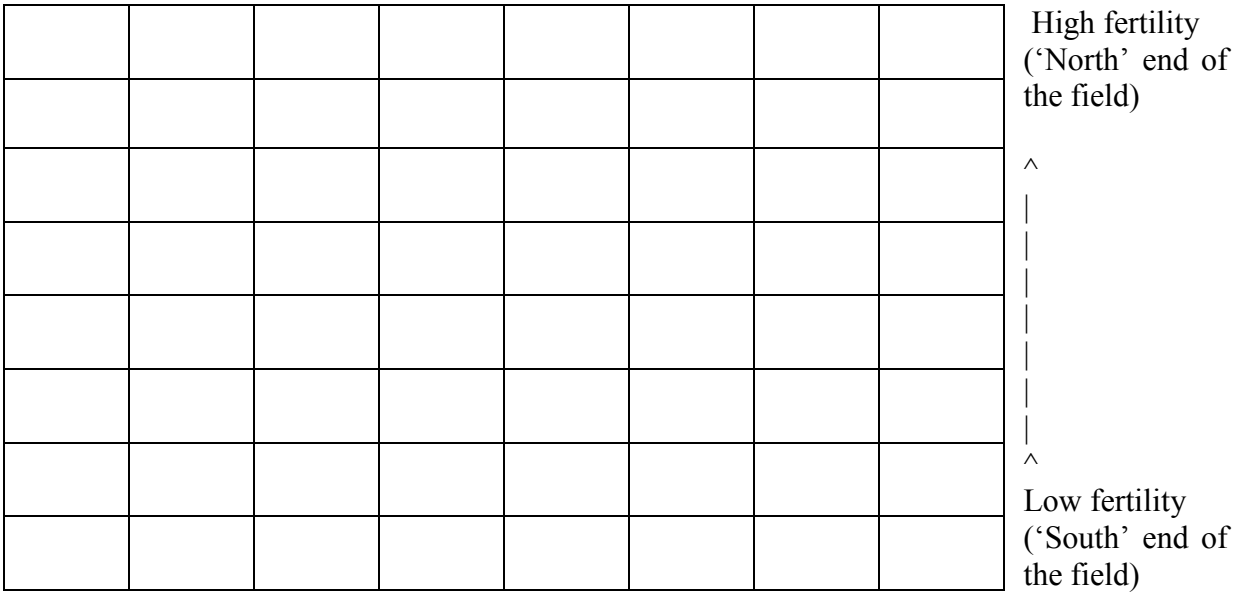
2.2.4 One of the most important requirements of experimental units is independence. That means that observations within a plot are not influenced by the circumstances in other plots. For example, if tall varieties are planted next to short ones there could be a negative influence of the tall ones to the short ones and a positive influence in the other direction. In such a case, an additional row of plants can be planted on both sides of the plot in order to avoid this dependency. Another possibility to minimize this influence is to group varieties by relevant characteristics.

2.2.5 When the same variety is assigned to a number of different plots and there is only one observation for each plot, the observations in the different plots may vary. The variation between these observations will be called the 'between-plot variability'. This variability is a mixture of different sources of variation: different plots, different plants, different times of

observation, different errors of measurement and so on. It is not possible to distinguish between these sources of variation. When there are observations of more than one, say n, plants per plot it is possible to compute two variance components: the within-plot or plant component and the plot component.

2.3 From a complete randomized design to a randomized complete block design

2.3.1 In designing an experiment it is important to choose an area of land that is as uniform as possible in order to minimize the variation between plots of the same variety. Assume that we have a field where it is known that the largest variability is in the 'north-south' direction, e.g. as in the following figure:



Let's take an example where four varieties are to be compared with each other in an experiment where each of the varieties are assigned to 4 different plots. It is important to randomize the varieties over the plots. If varieties are arranged systematically, not all varieties would necessarily be under the same conditions (see following figure).

Variety A	Variety A	Variety A	Variety A	Variety B	Variety B	Variety B	Variety B
Variety C	Variety C	Variety C	Variety C	Variety D	Variety D	Variety D	Variety D

If the fertility of the soil decreases from the north to the south of the field, the plants of variety A and B have grown on more fertile plots than the other varieties. The comparison of the varieties is influenced by a difference in fertility of the plots. Differences between varieties are said to be confounded with differences in fertility.

2.3.2 To avoid systematic errors it is always advisable to randomize varieties across the site. A complete randomization of the four varieties over the sixteen plots could have resulted in the following layout:

Variety C	Variety A	Variety A	Variety B	Variety C	Variety D	Variety B	Variety C
Variety C	Variety A	Variety D	Variety A	Variety D	Variety B	Variety D	Variety B

However, looking at the design we find that variety C occurs three times in the top row (with high fertility) and only once in the second row (with lower fertility). For variety D we have the opposite situation. Because we know that there is a fertility gradient, this is still not a good design, but it is better than the first systematic design.

2.3.3 When we know that there are certain systematic sources of variation like the fertility gradient in the paragraphs before, we may use this information by making so called blocks. The blocks should be formed so that the plots within each block are as uniform as possible. With the assumed gradients we may choose either two blocks each consisting of one row or we may choose four blocks – two blocks in each row with four plots each. In larger trials (more varieties) the latter will most often be the best, as there will also be some variation within rows even though the largest gradient is between rows. This ensures that all varieties occur an equal number of times in each block: a randomized complete block design.

Block I				Block II			
Variety A	Variety C	Variety D	Variety B	Variety A	Variety C	Variety D	Variety B
Variety B	Variety C	Variety A	Variety D	Variety C	Variety A	Variety D	Variety B
Block III				Block IV			

An alternative way of reducing the effect of any gradient between the columns is to use plots which extend over two rows, i.e. by using long and narrow plots:

Block I				Block II				Block III				Block IV			
Var A	Var C	Var D	Var B	Var A	Var C	Var D	Var B	Var B	Var C	Var A	Var D	Var C	Var A	Var D	Var B

In both designs above the 'north-south' variability will not affect the comparisons between varieties.

2.3.4 In a randomized complete block design the number of plots per block equals the number of varieties. All varieties are present once in each block and the order of the varieties within each block is randomized. The advantage of a randomized complete block design is that the standard deviation between plots (varieties) does not contain variation due to differences between blocks. The main reason for the random allocation is that it ensures that the results obtained are representative for the varieties to be compared. A side effect is that this will make the result neutral. If the plots were arranged in a systematic way (non-randomized), it might be argued that the actual order of the varieties was chosen in order to favour a certain variety in the comparison. Another feature of the randomization is that is makes the observations from individual plots 'behave' as independent observations (even though they

may not be so). There is usually no extra cost associated with blocking, so it is recommended to arrange the plots in blocks.

2.3.5 Blocking is introduced here by means of differences in fertility. Several other systematic sources of variation could have been used for blocking. Although it is not always clear how heterogeneous the field is, and therefore it is unknown how to arrange the blocks, it is usually a good idea to create blocks for many other reasons. When there are different sowing machines, different observers, different observation days, all these effects are included in the residual standard deviation if they are randomly assigned to the plots. However, these effects can be eliminated from the residual standard deviation if all the plots within each block have the same sowing machine, the same observer, the same observation day, and so on.

2.3.6 Management may affect the form of the plots. In some crops it may be easier to handle long and narrow plots than square plots. Long narrow plots are usually considered to be more susceptible to competition between varieties in adjacent plots than square plots. The size of the plots should be chosen in such a way that the necessary number of plants for sampling is available. For some crops it may be necessary also to have guard plants (areas) in order to avoid competition effects which are too large. However, overly large plots mean a waste of land and will most often increase the random variability between plots. Grouping of the varieties according to e.g. height may also reduce the competition between adjacent plots. If nothing is known about the fertility of the area, then layouts with compact blocks (i.e. almost square blocks) will most often be preferable because the larger the distance between two plots the more different they will usually be. In both designs above, the blocks can be placed as shown or they could be placed 'on top of each other'. This will usually not change the variability between plots considerably – unless one of the layouts, forces the crop expert to use more heterogeneous soil.

2.4 Randomized incomplete block designs

2.4.1 If the number of varieties becomes very large (>20-40), it may be impossible to construct blocks that will be sufficiently uniform. In that case it might be advantageous to form smaller blocks, each one containing only a fraction of the total number of varieties. Such designs are called incomplete block designs. Several types of incomplete block designs can be found in the literature. One of the most familiar types for variety trials is a lattice design. The generalized lattice designs (also called α-designs) are very flexible and can be constructed for any number of varieties and for a large range of block sizes and number of replicates. One of the features of generalized lattice designs is that some of the incomplete blocks can be (and usually are) collected to form a whole replicate. This means that such designs cannot be worse than randomized complete block designs.

2.4.2 Incomplete blocks need to be constructed in such a way that it is possible to compare all varieties in an efficient way. An example of an α-design is shown in the following figure:

Block	Sub-block	Variety			
3	5	6	5	15	19
	4	13	8	10	20
	3	2	3	4	7
	2	12	1	18	14
	1	17	11	16	9

Block	Sub-block	Variety			
2	5	4	16	6	1
	4	18	5	10	2
	3	14	7	17	8
	2	11	19	13	3
	1	15	9	20	12
1	5	4	20	5	17
	4	2	13	1	9
	3	3	6	12	8
	2	18	7	11	15
	1	16	10	14	19

In the example above, 20 varieties are to be grown in a trial with three replicates. In the design the 5 sub-blocks of each block form a complete replicate. Thus each replicate contains all varieties whereas any pair of varieties occurs either once or zero time in the same subblock.

2.4.3 The incomplete block design is most suitable for trials where grouping characteristics are not available. If grouping characteristics are available then some modification may be advantageous for trials with many varieties. At present, some work is being undertaken to examine how this can be done best.

2.5 Pairwise comparisons of some varieties

2.5.1 When pairs of varieties need to be compared very intensively it may be good to grow them in neighbouring plots. The theory used in split-plot designs may be used for setting up a design where the comparisons between certain pairs of varieties are to be optimized. When setting up the design, the pairs of varieties are treated as the whole plot factor and the comparison between varieties within each pair is the sub-plot factor. As each whole plot consists of only two sub-plots, the comparisons within pairs will be (much) more precise than if a randomized block design was used.

If, for example, four pairs of varieties (A-B, C-D, E-F and G-H) have to be compared very efficiently, then this can be done using the following design of 12 whole plots each having 2 subplots:

Pair 1 variety A Pair 1 variety B	Pair 3 variety E Pair 3 variety F	Pair 4 variety H Pair 4 variety G
Pair 3 variety F	Pair 2 variety D	Pair 1 variety A
Pair 3 variety E	Pair 2 variety C	Pair 1 variety B
Pair 4 variety G	Pair 1 variety B	Pair 2 variety C
Pair 4 variety H	Pair 1 variety A	Pair 2 variety D
Pair 2 variety D	Pair 4 variety H	Pair 3 variety E
Pair 2 variety C	Pair 4 variety G	Pair 3 variety F

In this design each column represents a replicate. Each of these is then divided into four incomplete blocks (whole plots) each consisting of two (sub)plots. The four pairs of varieties are randomized to the incomplete blocks within each replicate and the order of varieties are randomized within each incomplete block. The comparison between varieties of the same

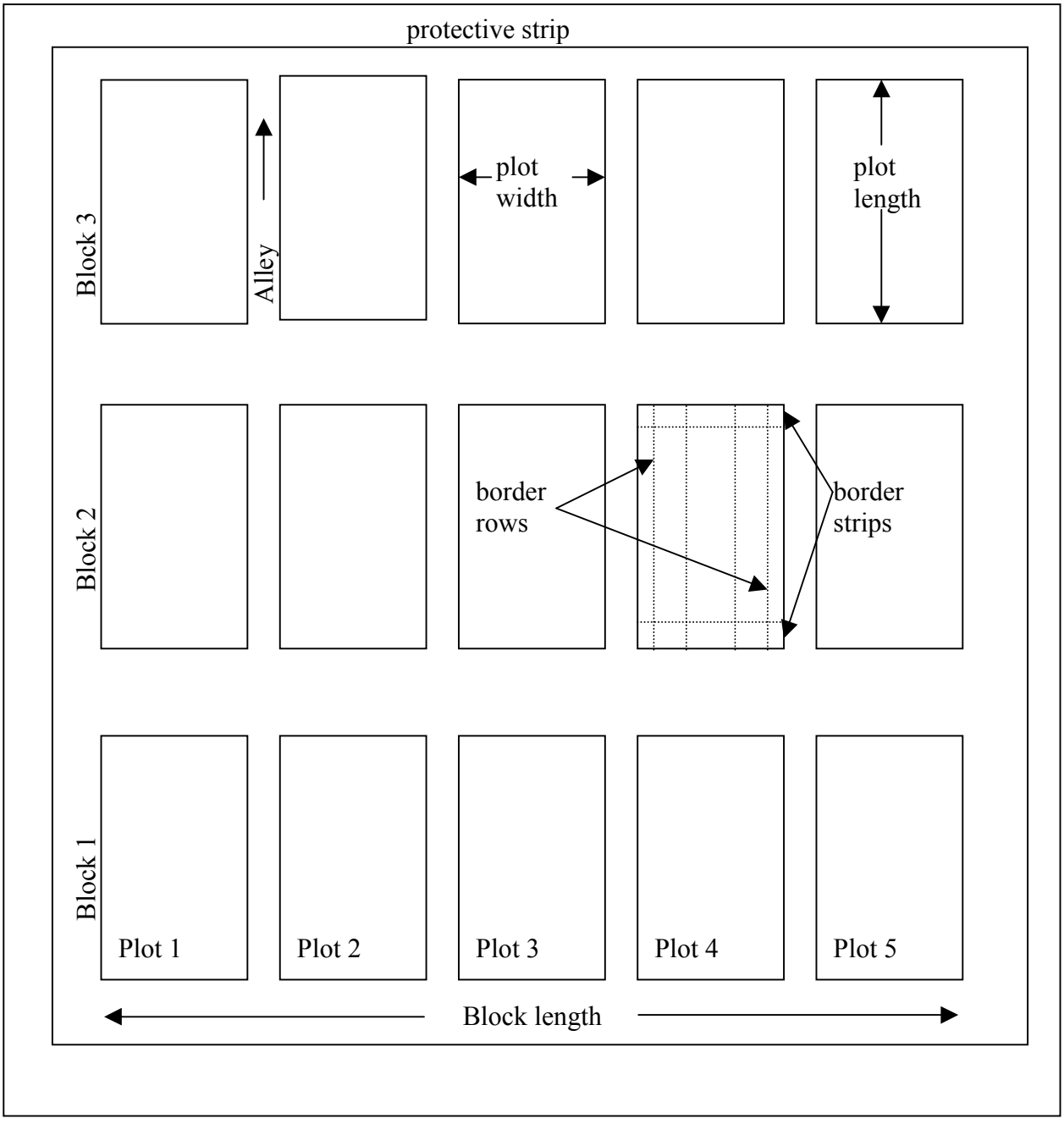
pair is made more precise at the cost of the precision of the comparison between varieties of a different pair.

2.6 Trial elements

2.6.1 An experimental unit in variety trials is a plot with one or more plants. If there is more than one plant within a plot, the observations of certain characteristics on each plant are used to estimate the mean and the variability of the characteristic. A plot is the smallest subdivision of the trial and the unit on which the varieties and the soil and plant condition should be focussed. Therefore following trial elements should be arranged accordingly:

- plot size
- shape of the plots
- alignment of the plots
- barrier rows and border strips and
- protective strips

2.6.2 The following figure may be helpful to give some explanations of the particular trial elements.



2.6.3 The four possibilities and the symbols used in the Test Guidelines to indicate the recommended method of observation for the assessment of distinctness, are as follows:

MG: single record for a group of plants or parts of plants based on measurement(s)
MS: records for a number of single, individual plants or parts of plants obtained by measurement
VG: single record for a group of plants or parts of plants based on visual observation(s)
VS: records for a number of single, individual plants or parts of plants obtained by visual observation.

2.6.4 The highest requirements on planning of the trial are based on characteristics on individual single plants (MS and VS). These characteristics determine the number of single plants and therefore the size of the plot. In some cases it is necessary to have border rows and strips to minimize the inter-plot interference and other special border effects.

2.6.5 The plot size depends upon the sample size. Furthermore, plot size and plot shape also depend on the soil conditions and on the sowing and harvesting machinery. The shape of the plot can be defined as the ratio of plot length divided by plot width. This ratio can be important for compensation of the soil variation within the block.

2.6.6 Square plots have the smallest total length of the borders (circumference). From the theoretical point of view the square shape is optimal to minimize the interference of genotypes. Grouping of the varieties can have the same effect.

2.6.7 Narrow and long plots are preferred from the technological point of view. The best length to width ratio lies between 5:1 and 15:1 and depends on the plot size and the number of varieties. The larger the number of varieties in a block the narrower the plots - but not so narrow that the inter-plot competition becomes a problem. The aim of DUS testing is to get averages of characteristics for each variety and to judge the within-variety variability by calculating the standard deviation. The averages will be used for determining the distinctness of the varieties; the standard deviations are the basis for examination of uniformity in the case of quantitative characteristics. For qualitative characteristics the number of off-types will be determined. (See document TG/1/3 “General Introduction, section 6.4.4.1.)

2.6.8 For assessment of distinctness unbiased and precise estimation of averages is necessary. The bias is difficult to calculate. Nevertheless it is common to reduce the bias by suitable precautions which are the exclusion of external influences by means of protective strips on the border of the trial. Additionally, it is often necessary to exclude border rows and strips of the plot from calculations of the average and the standard deviation. The rest of the plot without border rows and strips (effective plot size) are the basis of the unbiased and precise estimates.

2.6.9 The plants may be arranged in different ways in the trials:

- Rows of plants: This type of arrangement is used for many self-pollinated species, such as cereals. Most characteristics are assessed in an overall observation – usually using the notes stated in the Test Guidelines. In some cases it may be necessary to remove some plants from the plot in order to record some characteristics; and in that case the size of the plot should allow the removal of plants without prejudicing the observations which must be made up to the end of the growing cycle including the assessment of uniformity (see document TGP/7, ASW 6).

- Ear rows: This type of arrangement is frequently used for the assessment of uniformity in self-pollinated varieties.
- Spaced plants: This type of arrangement is used in many cross-pollinated and vegetatively propagated varieties.

2.7 Sample size

2.7.1 The Test Guidelines will usually define the sample size of one experiment. The final precision of a test based on the observations of one experiment depends for quantitative characteristics on at least three sources of variation:

- the variation between individual plants within a plot
- the variation between the plots within a block
- the variation caused by the environment, i.e. the variation in the expression of characteristics from year to year (or from location to location)

~~The recommendation to put 60 plants (3 times 20) into a variety trial is not a general one and depends on the species.~~

2.7.2 To estimate the optimal sample size when developing new Test Guidelines it is necessary to know the standard deviations, expected differences between the varieties which should be significant, the number of varieties and the number of blocks in the trial. Additionally, the crop expert has to determine the type I (α) and type II error (β). In cooperation with a statistician the crop expert can compute the optimal sample size for some characteristics and then he can determine the optimal sample size for this trial for all characteristics. Especially for the assessment of uniformity, the type II error is sometimes more important than the type I error. In some cases the type II error could be greater than 50 % and becomes unacceptable.

2.8 Analyses over years or cycles

2.8.1 The comparison between varieties is mostly based on observations from two to three years or cycles. Therefore, the number of replicates and the number of plants per plot in a single trial have an effect on the variability which is used in the COY-D and COY-U analyses (see documents TGP/9 and TGP/10). Before performing these analyses the means of the variety means and (log) standard deviations per year or cycle are calculated and then the analysis is performed on these means in the two-way variety-by-year or -cycle layout. The residual variation in these analyses is the variety-by-year or -cycle interaction.

2.8.2 The precision of the variety means in one experiment, when used for COY-D for example, is only used indirectly, because the standard deviation in that analysis is the interaction between the varieties and the years or cycles. If the differences between the varieties over the years or cycles are very large, the precision of the means per experiment are less important.

SECTION 3: TYPES OF CHARACTERISTICS AND THEIR SCALE LEVELS

3.1 Introduction

For the revision of UPOV Test Guidelines or for establishing new ones, and in order to understand the relations between the different steps of work of the crop experts during the DUS test, it is necessary to have an answer to the following questions:

1. What is a characteristic?
2. What is a process level?
3. What is a scale level of a characteristic?
4. What is the influence of the scale level on the :
   - planning of a trial,
   - recording of data,
   - determination of distinctness and uniformity and
   - description of varieties.

3.2 Different levels to look at a characteristic

Characteristics can be considered in different levels of process (Table 1). The characteristics as expressed in the trial (type of expression) are considered as process level 1. The data taken from the trial for the assessment of distinctness, uniformity and stability are defined as process level 2. These data are transformed into states of expression for the purpose of variety description. The variety description is process level 3.

Table 1: Definition of different process levels to consider characteristics

Process level	Description of the process level
1	characteristics as expressed in trial
2	data for evaluation of characteristics
3	variety description

From the statistical point of view the information level decreases from process level 1 to 3. Statistical analysis is only applied in level 2.

Sometimes for crop experts it seems that there is no need to distinguish between different process levels. The process level 1, 2 and 3 could be identical. However, in general, this is not the case.

3.2.1 Understanding the need for process levels:

3.2.1.1 The crop expert looks for characteristics to examine distinctness, uniformity and stability, and may know from Test Guidelines or his own experience that, for example, ‘Length of plant’ is a good characteristic to differentiate between varieties. There are varieties in which the plants are longer than other varieties. New varieties are expected to be uniform in this characteristic. Another characteristic could be ‘Variegation of leaf blade’.

For some varieties, variegation is present and for others not. The crop expert has now two characteristics and he knows that 'Plant length' is a quantitative characteristic and 'Variegation of leaf blade' is a qualitative one (definitions: see section 3.3 below). The expert observes the expression of the characteristics in the trial and has experience in expressions in the crop. But at this stage it has not yet been decided how the characteristics will be assessed and described. This stage of work is described as **process level 1.**

3.2.1.2 The crop expert then has to plan the trial and to decide on the type of observation for the characteristics. For characteristic 'Variegation of leaf blade', the decision is clear. There are two possible expressions: 'present' or 'absent'. The decision for characteristic 'Plant length' is not specific and depends on expected differences between the varieties and on the variation within the varieties. In many cases, the crop expert will decide to measure a number of plants (in cm) and to use special statistical procedures to examine distinctness and uniformity. But it could also be possible to assess the characteristic 'Plant length' visually by using expressions like 'short', 'medium' and 'long', if differences between varieties are large enough (for distinctness) and the variation within varieties is very small or absent in this characteristic. The continuous variation of a characteristic is assigned to appropriate states of expression which are recorded by notes. The crucial element in this stage of work is the recording of data for further evaluations. It is described as **process level 2**.

3.2.1.3 At the end of the DUS test, the crop expert has to establish a description of the varieties using notes from 1 to 9 or parts of them. This phase is described as **process level 3**. For 'Variegation of leaf blade' the crop expert can take the same states of expression (notes) he recorded in process level 2 and the three process levels appear to be the same. In cases where the crop expert decided to assess 'Plant length' visually, he can take the same states of expression (notes) he recorded in process level 2 and there is no obvious difference between process level 2 and 3. If the characteristic 'Plant length' is measured in cm, it is necessary to assign intervals of measurements to states of expressions like 'short', 'medium' and 'long' to establish a variety description. In this case, it is important to be clearly aware of the relevant level and to understand the differences between characteristics as expressed in the trial, data for evaluation of characteristics and the variety description. This is absolutely necessary for choosing the most appropriate statistical procedures in cooperation with statisticians or by the crop expert.

3.3 Types of expression of characteristics

3.3.1 Characteristics can be classified according to their types of expression or in other words according to their observed variation within the species. The consideration of the type of expression of characteristics corresponds with process level 1. The following types of expression of characteristics are defined in the General Introduction to the Examination of Distinctness, Uniformity and Stability and the Development of Harmonized Descriptions of New Varieties of Plants, (document TG/1/3, the "General Introduction", Chapter 4.4):

3.3.2 "Qualitative characteristics" are those that are expressed in discontinuous states (e.g. sex of plant: dioecious female (1), dioecious male (2), monoecious unisexual (3), monoecious hermaphrodite (4)). These states are self-explanatory and independently meaningful. All states are necessary to describe the full range of the characteristic, and every form of expression can be described by a single state. The order of states is not important. As a rule, the characteristics are not influenced by environment.

3.3.3 "Quantitative characteristics" are those where the expression covers the full range of variation from one extreme to the other. The expression can be recorded on a one-dimensional, continuous or discrete, linear scale. The range of expressions is divided into a number of states for the purpose of description (e.g. length of stem: very short (1), short (3), medium (5), long (7), very long (9)). The division seeks to provide, as far as practical, an even distribution across the scale. The Test Guidelines do not specify the difference needed for distinctness. The states of expression should, however, be meaningful for DUS assessment.

3.3.4 In the case of "pseudo-qualitative characteristics" the range of expression is at least partly continuous, but varies in more than one dimension (e.g. shape: ovate (1), elliptic (2), circular (3), obovate (4)) and cannot be adequately described by just defining two ends of a linear range. In a similar way to qualitative (discontinuous) characteristics – hence the term "pseudo-qualitative" – each individual state of expression needs to be identified to adequately describe the range of the characteristic.

3.3.5 This classification of characteristics is based on the observations made by the crop expert, on what he can see in the tests and on his general experience in the specific crop. This classification is appropriate to give general recommendations for the definition of states of expression in the Test Guidelines and to develop general rules for the assessment of distinctness, uniformity and stability.

3.4 Types of scales of data

The possibility to use specific procedures for the assessment of distinctness, uniformity and stability depends on the scale level of the data which are recorded for a characteristic. The scale level of data depends on the type of expression of the characteristic and on the way of recording this expression. The type of scale may be quantitative or qualitative.

3.4.1 Quantitatively scaled data

Quantitative data are all data which are recorded by measuring or counting. Weighing is a special form of measuring. Quantitative data can have a continuous or a discrete distribution. Continuous data result from measurements. They can take every value out of the defined range. Discrete quantitative data result from counting.

Examples:

Quantitative data	Example	Example number
- continuous	Plant length in cm.	1
- discrete	Number of stamens	2

For description of the states of expression, see Table 6.

The continuous quantitative data for the characteristic "Plant length" are measured on a continuous scale with defined units of assessment. It depends only on the costs and the

necessity to get any value in cm or in mm. A change of unit of measurement e.g. from cm into mm is only a question of precision and not a change of type of scale.

The discrete quantitative data of the characteristic "Number of stamens " are assessed by counting (1, 2, 3, 4, and so on). The distances between the neighbouring units of assessment are constant and for this example equal to 1. There are no real values between two neighbouring units but it is possible to compute an average which falls between those units.

In biometrical terminology, quantitative scales are also designated as metric scales. A synonym for metric scale is cardinal scale. Quantitative scales can be subdivided into ratio scales and interval scales.

3.4.1.1 Ratio scale

A ratio scale is a quantitative scale with a defined absolute zero point. There is always a constant non-zero distance between two adjacent expressions. Ratio-scaled data may be continuous or discrete.

The absolute zero point:

The definition of an absolute zero point makes it possible to define meaningful ratios. This is a requirement for the construction of index numbers (e.g. the ratio of length to width). An index is the combination of at least two characteristics. In the General Introduction, this special case is defined as a combined characteristic.

It is also possible to calculate ratios between the expression of different varieties. For example, in the characteristic 'Plant length' assessed in cm, there is a lower limit for the expression which is '0 cm' (zero). It is possible to calculate the ratio of length of plant of variety 'A' to length of plant of variety 'B' by division:

Length of plant of variety 'A' = 80 cm
Length of plant of variety 'B' = 40 cm

Ratio = Length of plant of variety 'A' / Length of plant of variety 'B'
= 80 cm / 40 cm
= 2.

So it is possible in this example to state that plant 'A' is double the length of plant 'B'. The existence of an absolute zero point ensures an unambiguous ratio.

The ratio scale is the highest classification of the scales (Table 2). That means that ratio scaled data include the highest information about the characteristic and it is possible to use many statistical procedures (Chapter 7).

The examples 1 and 2 (Table 6) are examples for characteristics with ratio scaled data.

3.4.1.2 Interval scale

An Interval scale is a quantitative scale without a defined absolute zero point. There is always a constant non-zero distance between two adjacent expressions. Interval scaled data may be distributed continuously or discretely.

An example for a discrete interval scaled characteristic is 'Time of beginning of flowering' measured as date which is given in section 3.4.1 (see also example 6 in Table 6). This characteristic is defined as the number of days from April 1. The definition is useful but arbitrary and April 1 is not a natural limit. It would also be possible to define the characteristic as the number of days from January 1.

It is not possible to calculate a meaningful ratio between two varieties which should be illustrated with the following example:

Variety 'A' begins to flower on May 30 and variety 'B' on April 30

Case I) Number of days from April 1 of variety 'A' = 60
Number of days from April 1 of variety 'B' = 30

$$Ratio_I = \frac{\text{Number of days from April 1 of variety 'A'}}{\text{Number of days from April 1 of variety 'B}} = \frac{60 \text{ days}}{30 \text{ days}} = 2$$

Case II) Number of days from January 1 of variety 'A' = 150
Number of days from January 1 of variety 'B' = 120

$$Ratio_{II} = \frac{\text{Number of days from January 1 of variety 'A'}}{\text{Number of days from January 1 of variety 'B}} = \frac{150 \text{ days}}{120 \text{ days}} = 1.25$$

$Ratio_I = 2 > 1.25 = Ratio_{II}$

It is impossible to state that the time of flowering of variety 'A' is twice that of variety 'B'. The ratio depends on the choice of the zero point of the scale. This kind of scale is defined as an "Interval scale": a quantitative scale without a defined absolute zero point.

The interval scale is lower classified than the ratio scale (Table 2). Fewer statistical procedures can be used with interval scaled data than with ratio scaled data (see section 3.7). The interval scale is theoretically the minimum scale level to calculate arithmetic mean values.

3.4.2. Qualitatively scaled data

Qualitatively scaled data are data which can be arranged in different discrete qualitative categories. Usually they result from visual assessment. Subgroups of qualitative scales are ordinal and nominal scales.

3.4.2.1 Ordinal scale

Ordinally scaled data are qualitative data of which discrete categories can be arranged in an ascending or descending order. They result from visually assessed quantitative characteristics.

Example:

Qualitative data	Example	Example number
- ordinal	Intensity of anthocyanin	3

For description of the states of expressions, see Table 6.

An ordinal scale consists of numbers which correspond to the states of expression of the characteristic (notes). The expressions vary from one extreme to the other and thus they have a clear logical order. It is not possible to change this order, but it is not important which numbers are used to denote the categories. In some cases ordinal data may reach the level of discrete interval scaled data or of discrete ratio scaled data (Chapter 6).

The distances between the discrete categories of an ordinal scale are not exactly known and not necessarily equal. Therefore, an ordinal scale does not fulfil the condition to calculate arithmetic mean values, which is the equality of intervals throughout the scale.

The ordinal scale is lower classified than the interval scale (Table 2). Less statistical procedures can be used for ordinal scale than for each of the higher classified scale data (see section 3.7).

3.4.2.2 Nominal scale

Nominal scaled qualitative data are qualitative data without any logical order of the discrete categories.

Examples:

Qualitative data	Example	Example number
- nominal	Sex of plant	4
- nominal with two states	Leaf blade: variegation	5

For description of the states of expressions, see Table 6.

A nominal scale consists of numbers which correspond to the states of expression of the characteristic, which are referred to in the Test Guidelines as notes. Although numbers are used for designation there is no inevitable order for the expressions and so it is possible to arrange them in any order.

Characteristics with only two categories (dichotomous characteristic) are a special form of nominal scales.

The nominal scale is the lowest classification of the scales (Table 2). Few statistical procedures are applicable for evaluations (Chapter 7).

The different types of scales are summarised in the following table.

Table 2: Types of scales and scale levels

Type of scale		Description	Distribution	Data recording	Scale Level
quantitative (metric)	ratio	constant distances with absolute zero point	Continuous	Absolute Measurements	High
			Discrete	Counting	
	interval	constant distances without absolute zero point	Continuous	Relative measurements	↑
			Discrete	Date	
qualitative with underlying quantitative variable	ordinal	Ordered expressions with varying distances	Discrete	Visually assessed notes	↑
qualitative	nominal	No order, no distances	Discrete	Visually assessed notes	Low

From the statistical point of view a characteristic is only considered at the level of data which has been recorded, whether for analysis or for describing the expression of the characteristic. Therefore, characteristics with quantitative data are denoted as quantitative characteristics and characteristics with ordinal and nominal scaled data as qualitative characteristics.

3.5 Scale levels for variety description

The description of varieties is based on the states of expression (notes) which are given in the Test Guidelines for the specific crop. In the case of visual assessment, the notes from the Test Guidelines are usually used for recording the characteristic as well as for the assessment of DUS. As outlined in chapter 4, the notes are distributed on a nominal or ordinal scale. For measured or counted characteristics, DUS assessment is based on the recorded values and the recorded values are transformed into states of expression only for the purpose of variety description.

3.6 Relation between types of expression of characteristics and scale levels of data

3.6.1 Records taken for the assessment of qualitative characteristics are distributed on a nominal scale, for example “Sex of plant”, “Leaf blade: variegation” (Table 6, examples 4 and 5).

3.6.2 For quantitative characteristics the scale level of data depends on the method of assessment. They can be recorded on a quantitative or ordinal scale. For example, “Length of

plant" can be recorded by measurements resulting in ratio scaled continuous quantitative data. However, visual assessment on a 1 to 9 scale may also be appropriate. In this case, the recorded data are qualitatively scaled (ordinal scale) because the size of intervals between the midpoints of categories is not exactly the same.

Remark: In some cases visually assessed data on quantitative characteristics may be handled as measurements. The possibility to apply statistical methods for quantitative data depends on the precision of the assessment and the robustness of the statistical procedures. In the case of very precise visually assessed quantitative characteristics the usually ordinal data may reach the level of discrete interval scaled data or of discrete ratio scaled data.

3.6.3 A pseudo-qualitative type of characteristic is one in which the expression varies in more than one dimension. The different dimensions are combined in one scale. At least one dimension is quantitatively expressed. The other dimensions may be qualitatively expressed or quantitatively expressed. The scale as a whole has to be considered as a nominal scale (e.g. "Shape", "Flower color"; Table 6, examples 7 and 8).

3.6.4 In the case of using the off-type procedure for the assessment of uniformity the recorded data are nominally scaled. The records fall into two qualitative classes: plants belonging to the variety (true-types) and plants not belonging to the variety (off-types). The type of scale is the same for qualitative, quantitative and pseudo-qualitative characteristics.

3.6.5 The relation between the type of characteristics (process level 1) and the type of scale of data recorded for the assessment of distinctness and uniformity is described in Table 3. A qualitative characteristic is recorded on a nominal scale for distinctness (state of expression) and for uniformity (true-types vs. off-types). Pseudo-qualitative characteristics are recorded on a combined scale for distinctness (state of expression) and on a nominal scale for uniformity (true-types vs. off-types). Quantitative characteristics are recorded on an ordinal, interval or ratio scale for the assessment of distinctness depending on the characteristic and the method of assessment. If the records are taken from single plants the same data may be used for the assessment of distinctness and uniformity. If distinctness is assessed on the basis of a single record of a group of plants, uniformity has to be judged with the off-type procedure (nominal scale).

Table 3: Relation between type of characteristic and type of scale of assessed data

Procedure	Type of scale (level 2)	Distribution	Type of characteristic (level 1)		
			Quantitative	Pseudo-qualitative	Qualitative
Distinctness	ratio	Continuous	▪		
		Discrete	▪		
	interval	Continuous	▪		
		Discrete	▪		
	ordinal	Discrete	▪		
	combined	Discrete		▪	
	nominal	Discrete			▪
Uniformity	ratio	Continuous	▪		
		Discrete	▪		
	interval	Continuous	▪		
		Discrete	▪		
	ordinal	Discrete	▪		
	combined	Discrete	▪		
	nominal	Discrete	▪	▪	▪

3.7 Relation between method of observation of characteristics, scale levels of data and recommended statistical procedures

3.7.1 The scale level of data and the way of observation of characteristics are most important conditions for the application of different statistical procedures. There are four possible ways to observe characteristics (see document TGP/7 "Development of Test Guidelines"):

MG: single record for a group of plants or parts of plants based on measurement(s)
MS: records for a number of single, individual plants or parts of plants obtained by measurement
VG: single record for a group of plants or parts of plants based on visual observation(s)
VS: records for a number of single, individual plants or parts of plants obtained by visual observation.

3.7.2 The observation method depends primarily on the variation within and between varieties and affects the choice of the statistical method. All of the four observation methods may be relevant for the assessment of distinctness. For the assessment of uniformity observations must be done on individual plants. Consequently only MS or VS are appropriate. The indication of the method of observation of characteristics in the Test Guidelines refers only to the assessment of distinctness.

3.7.3 Established statistical procedures can be used for the assessment of distinctness and uniformity considering the scale level and some further conditions such as the degree of freedom or unimodality (Tables 4 and 5).

3.7.4 The relation between the expression of characteristics and the scale levels of data for the assessment of distinctness and uniformity is summarized in Table 6.

Table 4: Statistical procedures for the assessment of distinctness

Type of scale	Distribu-tion	Observa-tion method	Procedure[1] and further Conditions	Refe-rence document
ratio	continuous	MS MG (VS) [2]	R: COY-D Normal distribution, df >=20 R: long term LSD Normal distribution, df<20 NR-P: 2 out of 3 method (LSD 1%) Normal distribution, df>=20	TGP/9
	discrete			
interval	continuous			
	discrete			
ordinal	discrete	VG	R: minimum distance>=1	TGP/9
		VS	NR-D: threshold model	TWC/ 14/12
Combina-tion of ordinal or ordinal and nominal scales	discrete	VG (VS) [3]	R: state-by-state-comparison	TGP/9
nominal	discrete	VG (VS) [3]	R: each state-is clearly different from the other	TGP/9

1) R recommended
NR-P not recommended (previous method)
NR-D not recommended (method under development)
2) see remark in Chapter 6
3) normally VG but VS would be possible

Table 5: Statistical procedures for the assessment of uniformity

Type of scale	Distribu-tion	observa-tion method	Procedure[1)] and Further Conditions	Refe-rence document
ratio	continuous	MS	R: COY-U Normal distribution	TGP/ 10.3.1
	discrete	MS	NR-P: 2 out of 3 method (s^2_c<=1.6s^2_s)) Normal distribution	
interval	continuous	VS	NR-D: LSD for untransformed percentage of off-types	
	discrete			
ordinal	discrete	VS	NR-D: threshold model	TWC/ 14/12
Combina-tion of ordinal or ordinal and nominal scales	discrete		There is no case where uniformity is assessed on combined scaled data	
nominal	discrete	VS	R: off-type procedure for dichotomous (binary) data	TGP/ 10.3.2

1) R recommended
NR-P not recommended (previous method)
NR-D not recommended (method under development)

Table 6: Relation between expression of characteristics and scale levels of data for the assessment of distinctness and uniformity

Example	Name of characteristic	Distinctness			Uniformity		
		Unit of assess-ment	Description (states of expression)	Type of scale	Unit of assess-ment	Description (states of expression)	Type of scale
1	Length of plant	cm	assessment in cm without digits after decimal point	ratio scaled continuous quantitative data	cm	assessment in cm without digits after decimal point	ratio scaled continuous quantitative data
					True-type	Number of plants belonging to the variety	nominally scaled qualitative data
					Off-type	Number of off-types	
2	Number of stamens	counts	1, 2, 3, ... , 40,41, ...	ratio scaled discrete quantitative data	counts	1, 2, 3, ... , 40,41, ...	ratio scaled discrete quantitative data
3	Intensity of anthocyanin	1 2 3 4 5 6 7 8 9	very low very low to low low low to medium medium medium to high high high to very high very high	ordinally scaled qualitative data (with an underlying quantitative variable)	True-type	Number of plants belonging to the variety	nominally scaled qualitative data
					Off-type	Number of off-types	
4	Sex of plant	1 2 3 4	dioecious female dioecious male monoecious unisexual monoecious hermaphrodite	nominally scaled qualitative data	True-type	Number of plants belonging to the variety	nominally scaled qualitative data
					Off-type	Number of off-types	

Example	Name of characteristic	Distinctness			Uniformity		
		Unit of assess-ment	Description (states of expression)	Type of scale	Unit of assess-ment	Description (states of expression)	Type of scale
5	Leaf blade: variegation	1 9	absent present	nominally scaled qualitative data	True-type Off-type	Number of plants belonging to the variety Number of off-types	nominally scaled qualitative data
6	Time of beginning of flowering	date	e.g. May 21, 51st day from April 1	interval scaled discrete quantitative data	date	e.g. May 21, 51st day from April 1	interval scaled discrete quantitative data
					True-type Off-type	Number of plants belonging to the variety Number of off-types	nominally scaled qualitative data
7	Shape	1 2 3 4 5 6 7	deltate ovate elliptic obovate obdeltate circular oblate	combination of ordinal and nominal scaled discrete qualitative data	True-type Off-type	Number of plants belonging to the variety Number of off-types	nominally scaled qualitative data
8	Flower color	1 2 3 4 5 6 7 8 9 10	dark red medium red light red white light blue medium blue dark blue red violet violet blue violet	combination of ordinal and nominal scaled discrete qualitative data	True-type Off-type	Number of plants belonging to the variety Number of off-types	nominally scaled qualitative data

SECTION 4: VALIDATION OF DATA AND ASSUMPTIONS

4.1 Introduction

Statistical analyses are carried out in order to assist the crop expert when assessing candidate varieties for distinctness, uniformity and stability. In section 2, "Experimental Design Practices", aspects of designing the experiments in which the data are recorded are discussed. In section 3, "Types of Characteristics and Their Scale Levels", it is shown that the choice of which statistical methods to use depends on the type of characteristic, its scale level, and whether distinctness or uniformity is considered. In section 5, "Statistical Methods for DUS Examination", the statistical methods are described. The statistical methods are based on some theory and in order to ensure that the results can be trusted the assumptions behind the theory have to be met - at least approximately. The purpose of this section is to describe the assumptions behind the most common statistical methods used in DUS testing and to show how these assumptions may be validated. It is important to note that the COYD and COYU methods for used quantitative characteristics are based on variety means per year for COYD, and variety means of the (logarithm of the) between-plants standard deviation per year for COYU. Some methods for checking the data are described in section 4.2 "Check on Data Quality" below. In section 4.3, "Assumptions", the assumptions underlying the analysis of variance methods are given and in section 4.4, "Validation", some methods for evaluating these assumptions are given. The assumptions and methods of validation are described here for the analyses of single experiments (randomized blocks). However, the principles are the same when analyzing data from several experiments over years. Instead of plot means, the analyses are then carried out on variety means per year (and blocks then become equivalent to years). The methods described here are intended for quantitative characteristics, but some of the methods may also be used for checking qualitative characteristics on the ordinal scale. The different types of characteristics are defined in document TGP/8.3. Throughout this section data of 'Leaf: Length' (in mm) are used of an experiment laid out in 3 blocks of 26 plots with 20 plants per plot. Within each block, 26 different oil seed rape varieties were randomly assigned to each plot.

4.2 Check on data quality (before doing analyses)

4.2.1 In order to avoid mistakes in the interpretation of the results the data should always be inspected so that the data are logically consistent and not in conflict with prior information about the ranges likely to arise for the various characteristics. This inspection can be done manually (usually visually) or automatically.

4.2.2 Table 1 shows an extract of some recordings for 10 plants from a plot of field peas. For 'Seed: shape' the notes are visually scored on a scale with values 1, 2, 3, 4, 5 or 6. For 'Stem: length' the measurements are in cm and from past experience it is known that the length in most cases will be between 40 and 80 cm. The 'Stipule: length' is measured in mm and will in most cases be between 50 and 90 mm. The table shows 3 types of mistakes which occasionally occur when making manual recordings: for plant 4, 'Seed: shape' the recorded value, 7, is not among the allowed notes and must, therefore, be due to a mistake. It might be caused by a misreading a hand-written "1". The 'Stem: length' of plant 6 is outside the expected range and could be caused by changing the order of the figures, so 96 has been keyed instead of 69. The 'Stipule: length' of 668 mm is clearly wrong. It might be caused by

accidentally repeating the figure 6 twice. In all cases a careful examination has to be carried out in order to find out what the correct values should be.

Table 1 Extract of recording sheet for field peas

Plant no	Seed: shape (UPOV 1)	Stem: length (UPOV 12)	Stipule: length (UPOV 31)
1	1	43	80
2	2	53	79
3	1	50	72
4	7	43	668
5	2	69	72
6	1	96	72
7	1	51	70
8	2	64	63
9	1	44	62
10	2	49	62

4.2.3 Examination of frequency distributions of the characteristics to look for small groups of discrepant observations.

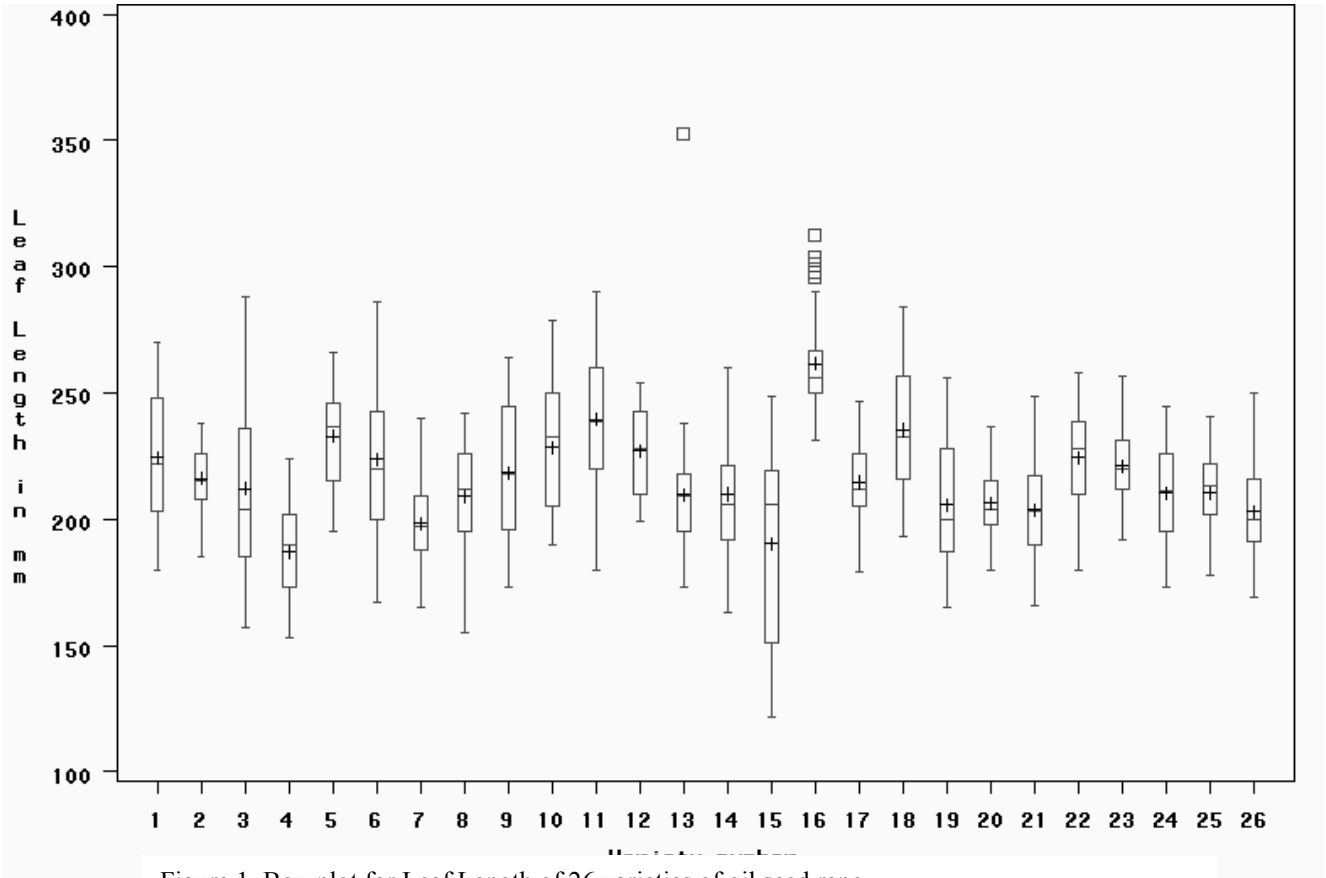


Figure 1. Box-plot for Leaf Length of 26 varieties of oil seed rape

4.2.4 Examination of scatter plots of pairs of characteristics likely to be highly related. This may often detect discrepant observations very efficiently.

4.2.5 Other types of plot may also be used to validate the quality of the data. A so-called Box- plot is an efficient way to get an overview of the data. In a Box-plot a box is drawn for each group (plot or variety). In Figure 1, all 60 Leaf Lengths of each of the 26 varieties are taken together. (If there are large block differences a better Box-plot can be produced by taking the differences with respect to the plot mean). The box shows the range for the largest part of the individual observations (usually 75%). A horizontal line through the box and a symbol indicates the median and mean, respectively. At each end of the box, vertical lines are drawn to indicate the range of possible observations outside the box, but within a reasonable distance (usually 1.5 times the height of the box). Finally, observations more extreme than that are shown individually. In Figure 1, it is seen that one observation of variety 13 is clearly much larger than the remaining observations of that variety. Also it is seen that variety 16 has large leaf lengths and that about 4 observations are relatively far from the mean. Among other things that can be seen from the figure are the variability and the symmetry of the distribution. So it can be seen that the variability of variety 15 is relatively large and that the distribution is slightly skewed for this variety (as the mean and median are relatively far apart).

4.2.6 When discrepant observations are found, the next important step will be to find out why the observations are deviating. In some cases it may be possible to go back to the field and to check if the plant or plot is damaged by external factors (e.g. rabbits) or a measurement mistake has occurred. In the last case a correction is possible. In other cases, it may be necessary to look in previous notes (or on other measurements from the same plant/plot) in order to find the reason for the discrepant observation. Generally observations should only be removed when there are good reasons. When in doubt it is recommended to discuss the problem with a statistician.

4.3 Assumptions

4.3.1 First of all it is very important to design experiments in a proper way. The most important assumptions of analysis of variance methods are:

- independent observations
- variance homogeneity
- additivity of block and variety effects for a randomized block design and additivity of year and variety effects for COYD.
- normally distributed observations (residuals)

4.3.2 In addition, one could state that there should be no mistakes in the data. However, most mistakes (at least the largest) will usually also mean that the observations are not normally distributed and that they have different variances.

4.3.3 The assumptions mentioned here are most important when the statistical methods are used to test hypotheses. When statistical methods are used only to estimate effects (means), the assumptions are less important and the assumption of normal distributed observations is not necessary.

Independent observations

4.3.4 This is a very important assumption. It means that no records may depend on other records in the same analysis (dependence between observations may be built into the model, but this is not so in the COYD and COYU or other UPOV recommended methods). Dependency may be caused e.g. by competitions between neighbouring plots, by lack of randomisation or by improper randomisation. More details on ensuring independence of observations may be found in document section 2 "Experimental Design Practices."

Variance homogeneity

4.3.5 Variance homogeneity means that the variance of all observations should be identical apart from random variation. Typical deviations from the assumption of variance homogeneity fall most often into one of the following two groups:

- The variance depends on the mean, e.g. the larger the mean value the larger the standard deviation is. In this case the data may often be transformed such that the variances on the transformed scale may be approximately homogeneous. Some typical transformations of characteristics are: the logarithmic transformation (where the standard deviation is approximately proportional to the mean), the square-root transformation (where the variance is approximately proportional to the mean, e.g. counts), and the angular transformation (where the variance is low at both ends of the scale and higher in between, typical for percentages).

- The variance depends on e.g. variety, year or block. If the variances depend on such variables in a way that is not connected to the mean value, it is not possible to obtain variance homogeneity by transformation. In such cases it might be necessary either to use more complicated statistical methods that can take unequal variances into account or to exclude the group of observations with deviant variances (if only a few observations have deviant variances). To illustrate the seriousness of variance heterogeneity: imagine a small trial with 10 varieties where varieties A, B, C, D, E, F, G and H each have a variance of 5, whereas varieties I and J each have a variance of 10. The real probability of detecting differences between these varieties when, in fact, they have the same mean is shown in Table 2. In Table 2, the variety comparisons are based on the pooled variance as is normal in traditional ANOVA. If they are compared using the 1% level of significance, the probability that the two varieties with a variance of 10 become significantly different from each other is almost 5 times larger (4.6%) than it should be. On the other hand, the probability of significant differences between two varieties with a variance of 5 decreases to 0.5%, when it should be 1%. This means that it becomes too difficult to detect differences between two varieties with small variances and too easy to detect differences between varieties with large variances.

Table 2. Real probability of significant difference between two identical varieties in the case where variance homogeniety is assumed but not fulfilled (varieties A to H have a variance of 5 and varieties I and J have a variance of 10.)

Comparisons, variety names	Formal test of significance level	
	1%	5%
A and B	0.5%	3.2%
A and I	2.1%	8.0%
I and J	4.6%	12.9%

Normal distributed observations

4.3.6 The residuals (see section 5 "Statistical Methods for DUS Examination") should be approximately normally distributed. The ideal normal distribution means that the distribution of the data is symmetric around the mean value and with the characteristic bell-shaped form (see Figure 2). If the residuals are not approximately normally distributed, the actual level of significance may deviate from the nominal level. The deviation may be in both directions depending on the way the actual distribution of the residuals deviates from the normal distribution. However, deviation from normality is usually not as serious as deviations from the previous two assumptions.

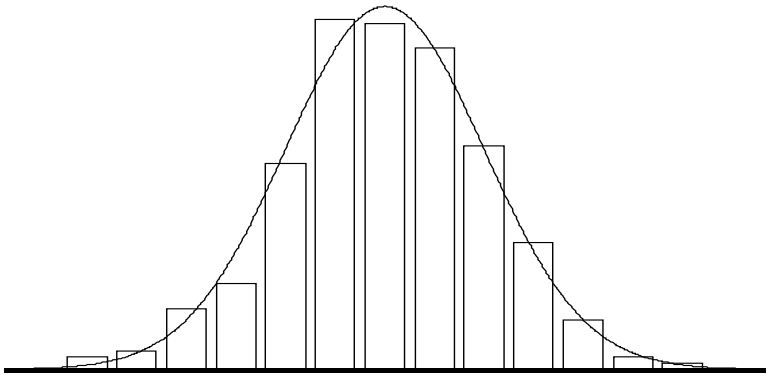


Figure 2. Histogram for normal distributed data with the ideal normal distribution shown as a curve

Additivity of block and variety effects

4.3.7 The effects of blocks and varieties are assumed to be additive because the error term is the sum of random variation and the interaction between block and variety. (For a formal description of the model see document section 5 Two-way ANOVA paragraph 5.1.7). This means that the effect of a given variety is the same in all blocks. This is demonstrated in Table 3 where plot means of artificial data (of Leaf Length in mm) are given for two small experiments with three blocks and four varieties. In experiment I, the effects of blocks and varieties are additive because the differences between any two varieties are the same in all blocks, e.g. the differences between variety A and B are 4 mm in all three blocks. In experiment II, the effects are not additive, e.g. the differences between variety A and B are 2, 2 and 8 mm in the three blocks.

Table 3. Artificial plot means of Leaf Length in mm from two experiments showing additive block and variety effects (left) and non-additive block and variety effects (right)

Experiment I			
Variety	Block		
	1	2	3
A	240	242	239
B	244	246	243
C	245	247	244
D	241	243	240

Experiment II			
Variety	Block		
	1	2	3
A	240	242	239
B	242	244	247
C	246	244	243
D	241	242	241

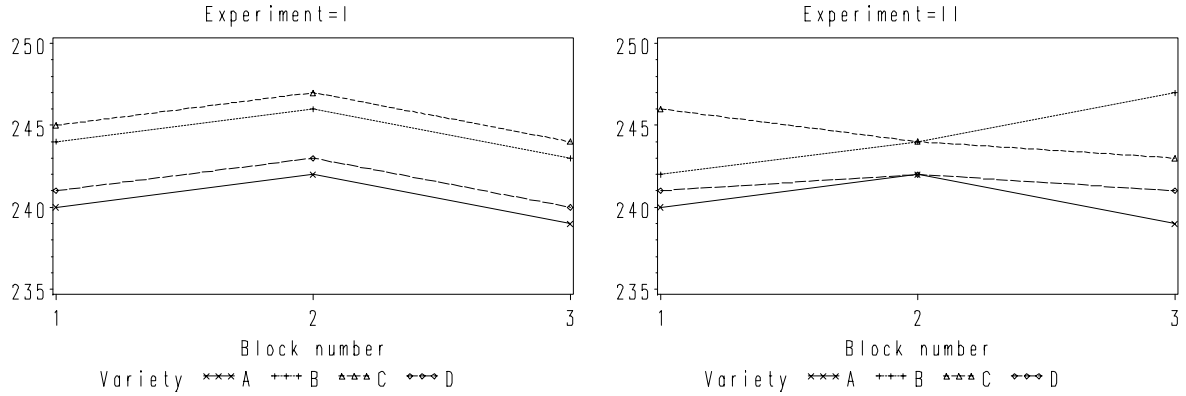


Figure 3. Artificial plot means from two experiments showing additive block and variety effects (left) and non-additive block and variety effects (right) using same data as in table 2

4.3.8 In Figure 3 the same data are presented graphically. Plotting the means versus block numbers and joining the observations from the same varieties by straight lines produces the graphs. Plotting the means versus variety names and joining the observations from the same blocks could also have been used (and may be preferred especially if many varieties are to be shown in the same figure). The assumption on additivity is fulfilled if the lines for the varieties are parallel (apart from random variation). As there is just a single data value for each variety in each block, it is not possible to separate interaction effects and random variation. So in practice the situation is not as nice and clear as here because the effects may be masked by random variation.

4.4 Validation

4.4.1 The purpose of validation is partly to check that the data are without mistakes and that the assumptions underlying the statistical analyses are fulfilled.

4.4.2 There are different methods to use when validating the data. Some of these are:

- look through the data
- produce plots to verify the assumptions
- make formal statistical tests for the different types of assumptions. In the literature several methods to test for outliers, variance homogeneity, additivity and normality may be found. Such methods will not be mentioned here partly because many of these depend on assumptions that do not affect the validity of COYD and COYU seriously and partly because the power of such methods depends heavily on the

sample size (this means that serious lack of assumptions may remain undetected in small datasets, whereas small and unimportant deviations may become statistically significant in large datasets)

Looking through the data

4.4.3 In practice this method is only applicable when a few observations have to be checked. For large datasets this method takes too much time, is tedious and the risk of overlooking suspicious data increases as one goes through the data. In addition, it is very difficult to judge the distribution of the data and to judge the degree of variance homogeneity when using this method.

Using Figures

4.4.4 Different kinds of figures can be prepared which are useful for the different aspects to be validated. Many of these consist of plotting the residuals in different ways. (The residuals are the differences between the observed values and the values predicted by the statistical model).

4.4.5 The plot of the residuals versus the predicted values may be used to judge the dependence of the variance on the mean. If there is no dependence, then the observations should fall approximately (without systematic deviation) in a horizontal band symmetric around zero (Figure 4). In cases where the variance increases with the mean, the observations will fall approximately in a funnel with the narrow end pointing to the left. Outlying observations, which may be mistakes, will be shown in such a figure as observations that

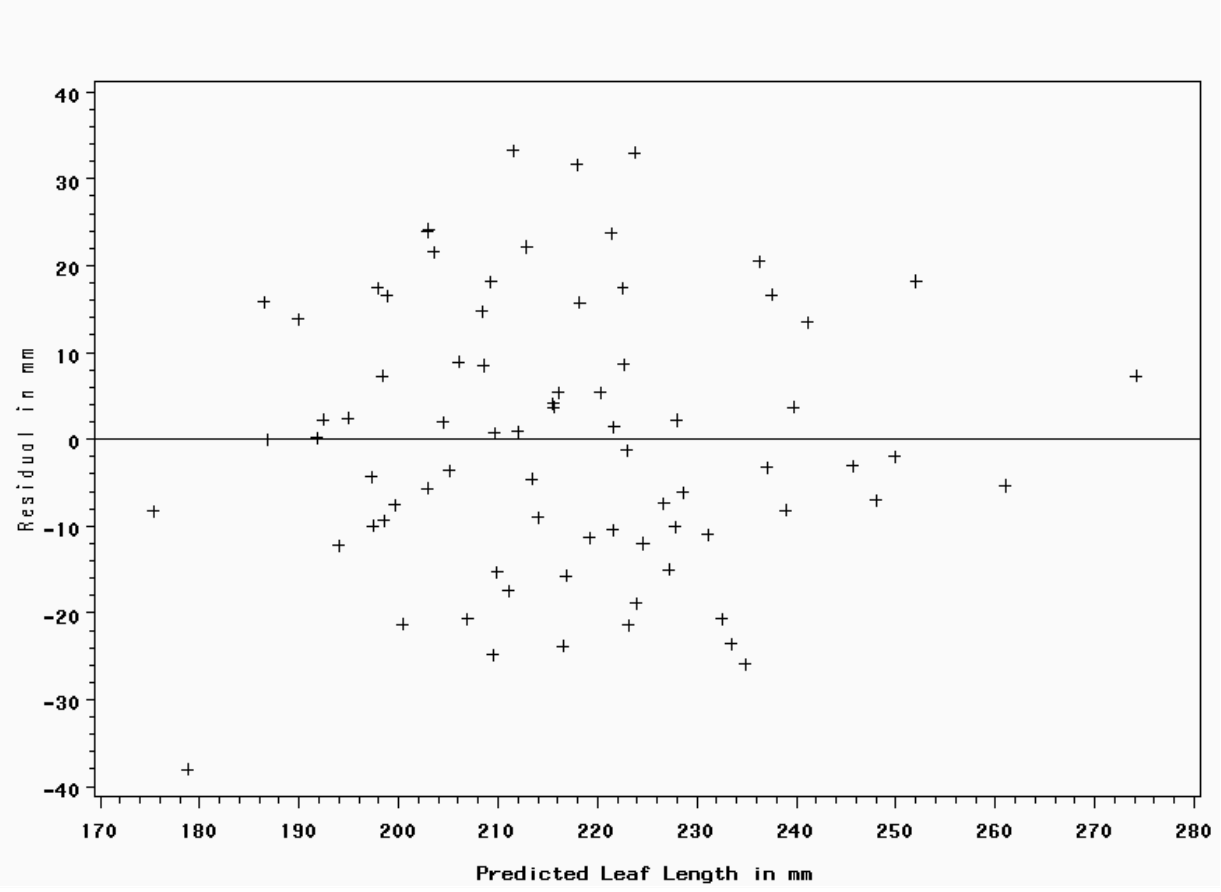


Figure 4. Plot of residuals versus plot predicted values for Leaf Length in 26 oil seed rape varieties in 3 blocks

clearly have escaped from the horizontal band formed by most other observations. In the example used in figure 4, no observations seem to be outliers (the value at the one bottom left corner where the residual is about -40 mm may at first glance look so, but several observations have positive values of the same numerical size). Here it is important to note that an outlier is not necessarily a mistake and also that a mistake will not necessarily show up as an outlier.

4.4.6 The residuals can also be used to form a histogram, like Figure 2, from which the assumption about the distribution can be judged.

4.4.7 The range (maximum value minus minimum value) or standard deviation for each plot may be plotted versus some other variables such as the plot means, variety number or plot number. Such figures (Figure 5) may be useful to find varieties with an extremely large variation (all plots of the variety with a large value) or plots where the variation is extremely large (maybe caused by a single plant). It is clearly seen that the range for one of variety 13's plots is much higher than in the other two plots. Also the range in one of variety 3's plots seems to be relatively large.

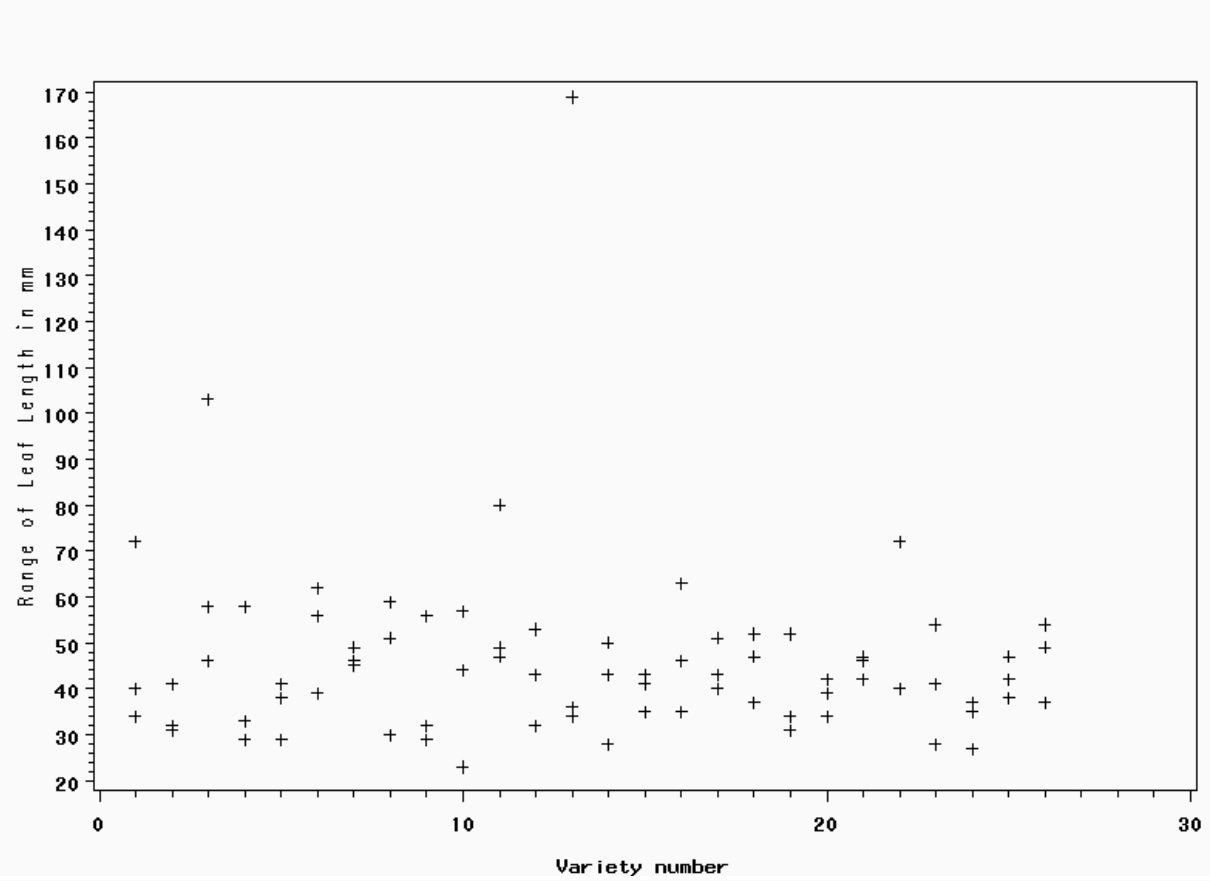


Figure 5. Differences between minimum and maximum of 20 leaf lengths for 3 plots versus oil seed rape variety number

4.4.8 A figure with the plot means (or variety adjusted means) versus the plot number can be used to find out whether the characteristic depends on the location in the field (Figure 6). This, of course, requires that the plots are numbered such that the numbers indicate the relative location. In the example shown in Figure 6, there is a clear trend showing that the leaf length decreases slightly with plot number. However most of the trend over the area used

for the trial will - in this case - be explained by differences between blocks (plot 1-26 is block 1, plot 27-52 is block 2 and plot 53-78 is block 3).

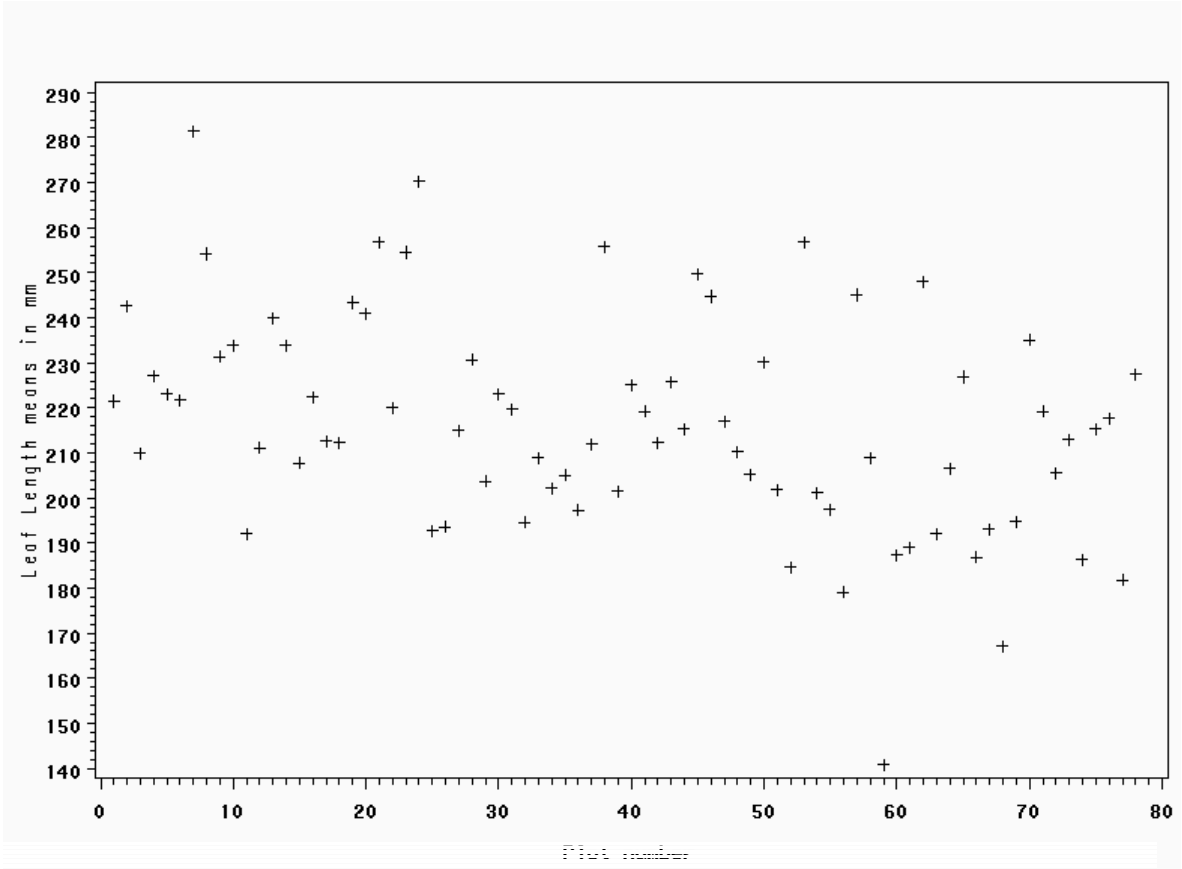


Figure 6. Plot means of 20 Leaf Lengths versus plot numbers

4.4.9 The plot means can also be used to form a figure where the additivity of block and variety effects can be visually checked at (see Figure 3).

4.4.10 Normal Probability Plots (Figure 7). This type of graph is used to evaluate to what extent the distribution of the variable follows the normal distribution. The selected variable will be plotted in a scatter plot against the values "expected from the normal distribution." The standard normal probability plot is constructed as follows. First, the residuals (deviations from the predictions) are rank ordered. From these ranks the program computes the expected values from the normal distribution, hereafter called z-values. These z-values are plotted on the X-axis in the plot. If the observed residuals (plotted on the Y-axis) are normally distributed, then all values should fall onto a straight line. If the residuals are not normally distributed, then they will deviate from the line. Outliers may also become evident in this plot. If there is a general lack of fit, and the data seem to form a clear pattern (e.g. an S shape) around the line, then the variable may have to be transformed in some way.

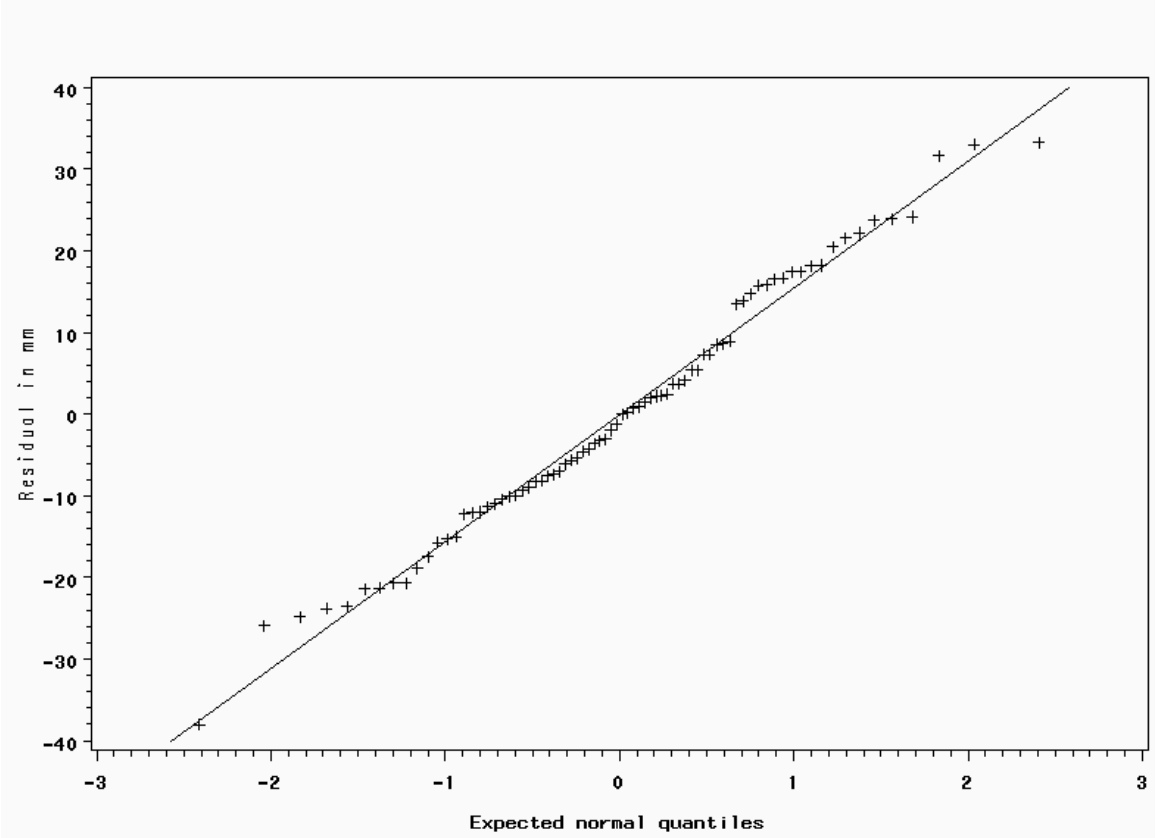


Figure 7. Normal probability plot for the residuals of Leaf Length in 26 oil seed rape varieties in 3 blocks

SECTION 5: STATISTICAL METHODS FOR DUS EXAMINATION

5.1 Analysis of variance

5.1.1 Introduction

5.1.1.1 The analysis of variance (ANOVA) of data from a designed experiment has two purposes. Firstly it subdivides the data's total variation into separate components with each component representing a different source of variation, so that the relative importance of the different sources can be assessed. Secondly it provides an estimate of the random variation in the data. This may be used as an estimate of precision when comparing means calculated from the data.

5.1.1.2 ANOVA can take many forms. Just two forms will be considered in detail here. These are the two forms which arise as part of the statistical techniques used in COY. At their simplest, both operate on an $n \times m$ table of data. They are the:-

– Two-way ANOVA, e.g. as used in the analysis of variety-by-year means for a characteristic for v varieties grown in each of y years in the Combined Over Years Distinctness (COYD) (see appendix A4) criterion. The paired t-test is a special case of two-way ANOVA.

– One-way ANOVA, e.g. as used in the analysis of variety-by-year adjusted log(SD+1)'s (a measure of uniformity) for a characteristic for v reference varieties grown in each of y years in the Combined Over Years Uniformity (COYU) criterion.

5.1.1.3 The particular form an ANOVA takes depends on the origins of the data. This determines the model for the data, i.e. what factors are likely to cause the data to vary, which in turn determines what components the total variation is divided into, and hence the form of the ANOVA.

5.1.2 Two-way ANOVA

5.1.2.1 The data model

5.1.2.1.1 In two-way ANOVA, the $n \times m$ table of data corresponds to nm data values classified by two factors: Factor 1 with m levels and Factor 2 with n levels. Usually, only one of the factors will be of interest while the other will be present simply because it explains variability in the data. For example (Example A), each data value might be the mean over all plants in a plot for a characteristic from a trial with vb plots laid out in b blocks (Factor 1) of v varieties (Factor 2), the factor of interest.

Example A: data from a trial with *v* varieties and *b* blocks

	Block 1	Block 2	Block 3	…	Block *b*
Variety 1	-	-	-	…	-
Variety 2	-	-	-	…	-
Variety 3	-	-	-	…	-
…	…	…	…	…	…
Variety *v*	-	-	-	…	-

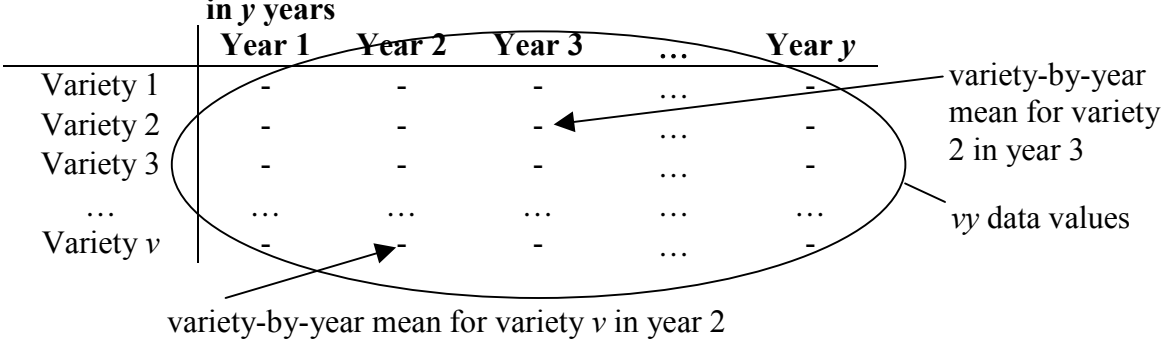
mean of all plants in the plot in block *b* with variety 2

vb data values

mean of all plants in the plot in block 2 with variety 3

5.1.2.1.2 Alternatively, for the above COYD example (Example B), the data values might consist of the *vy* variety-by-year means for a characteristic for *v* varieties (Factor 2, the factor of interest) grown in each of *y* years (Factor 1).

Example B: data for COYD example with *v* varieties grown in *y* years



5.1.2.1.3 If *x* represents one of the *nm* data values in the *n*×*m* table of data, the model explaining the variation in the data is as follows:

$$x = \text{Factor 1 effect} + \text{Factor 2 effect} + \left(\begin{matrix} \text{Factor 1} \times \text{Factor 2} \\ \text{interaction effect} \end{matrix} + \text{random variation} \right)$$

Thus each of the *nm* data values is made up of a sum of effects. The "Factor 1 effect" and "Factor 2 effect" are due to the particular levels of Factors 1 and 2 influencing the data value. An interaction between two factors is when the effects of one factor differ, i.e. are inconsistent, from level to level of the other factor. So a Factor 1×Factor 2 interaction is when Factor 1 effects differ from level to level of Factor 2. In the above model the remainder, or residual, of the data value is the amount, additional to its Factor 1 effect and its Factor 2 effect, which appears to be due to its particular combination of Factor 1 and 2 levels. This amount might be partly due to a genuine interaction or it might be just due to random variation. As there is just a single data value for each combination of Factor 1 and 2 levels it is not possible to determine which.

5.1.2.1.4 For Example A, the model explaining the variation in the data is as follows:

$$x = \text{block effect} + \text{variety effect} + \left(\begin{matrix} \text{variety} \times \text{block} \\ \text{interaction effect} \end{matrix} + \text{random variation} \right)$$

In this example each of the *vb* data values (one from each of the *vb* plots) is made up of the sum of a "block effect", due to the block the plot is in, plus a "variety effect", due to the variety sown on the plot, plus a remainder. This remainder, or residual, represents the amount that is additional to the variety and block effects. As there is just a single data value for each variety in each block, it is not possible to separate interaction effects and random variation. However, as the variety effects are not expected to differ from block to block, or in other words any variety×block interaction is expected to be negligible, the residual is likely to be due to random variation.

5.1.2.1.3 For Example B (COYD), the model explaining the variation in the data is as follows:

$$x = \text{year effect} + \text{variety effect} + \left(\begin{array}{c} \text{variety} \times \text{year} \\ \text{interaction effect} \end{array} + \text{random variation} \right)$$

Here each of the *vy* variety-by-year means is made up of a sum of effects. The "year effect" is an amount due to the year the variety-by-year mean was recorded in. The year effects might or might not be the same for all years. The "variety effect" is an amount due to the variety of the variety-by-year mean, and might or might not be the same for all varieties. The remainder, or residual of the variety-by-year mean represents the amount that is additional to its variety effect and its year effect, which appears to be due to that particular variety in that particular year. This amount may be partly due to a genuine variety×year interaction effect or it may be just due to random variation caused by the means having been calculated from different plants grown on different plots, and possibly due to measurement error. As there is just a single variety-by-year mean for each variety in each year it is not possible to distinguish between interaction effects and random variation.

5.1.2.2 The two-way analysis of variance table

5.1.2.2.1 Two-way ANOVA produces a table as follows:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Factor 1	m - 1	-	-	-
Factor 2	n - 1	-	-	-
Residual	(n - 1)(m - 1)	-	-	
Total	nm - 1	-		

[As the ANOVA computations are likely to be done by computer, details are not given here. Details can be found at the following references: Dagnelie (1998 and 1981), Kala (2002), Mead *et al.* (1993), and Sokal and Rohlf (1995).]

5.1.2.2.2 For Example A, the two-way ANOVA table is as follows:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Block	b - 1	-	-	-
Variety	v - 1	-	-	-
Residual	(b - 1)(v - 1)	-	-	
Total	vb - 1	-		

5.1.2.2.3 For Example B (COYD), the two-way ANOVA table is as follows:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Year	y - 1	-	-	-
Variety	v - 1	-	-	-
Residual	(y - 1)(v - 1)	-	-	
Total	vy - 1	-		

5.1.2.2.4 The total variation in the data is measured by the Total Sum of Squares, which is the sum of the squared deviations of all the data from their mean, i.e. $\sum(x-\bar{x})^2$. It is subdivided into "sums of squares" representing the three component sources of variation included in the data model: variation due to Factor 1, variation due to Factor 2 and residual variation. These sums of squares are divided by their degrees of freedom (df) to give "mean squares", which are directly compared in order to assess the relative magnitude of the different sources of variation. This is done in the final column where the F-ratio's are the ratios of each of the Factor 1 and Factor 2 mean squares to the residual mean square. Providing the assumptions discussed below about the data are valid, these F-ratios can be used to perform "F-tests" of the significance of the variation due to each of Factors 1 and 2, i.e. used to test whether Factors 1 and 2 have significant effects. This is done by comparing the F-ratios with critical values from F tables on the df of the numerator and the denominator mean squares: if the F-ratio is greater than or equal to the 100×p% critical value, the factor is declared to have a significant effect at the p% level. Otherwise, it is does not have a significant effect at the p% level.

5.1.2.2.5 The residual mean square is a variance. It estimates the combined variation due to any Factor 1×Factor 2 interaction and random variation. Hence, it is often referred to as the "Factor 1-by-Factor 2 mean square", e.g. the "variety-by-block mean square" in Example A or the "variety-by-year mean square" in the COYD example (Example B).

5.1.2.2.6 Statistical theory shows that in two-way ANOVA it is appropriate to use the residual mean square to estimate the variance or standard errors of means calculated from the data. This is the case whether the Factor 1×Factor 2 interaction is assumed to be negligible, such as in Example A, or not, such as in the COYD example (Example B).

5.1.2.2.7 Worked examples of two-way ANOVA are given in Appendix A1. These are of the same types as Examples A and B above.

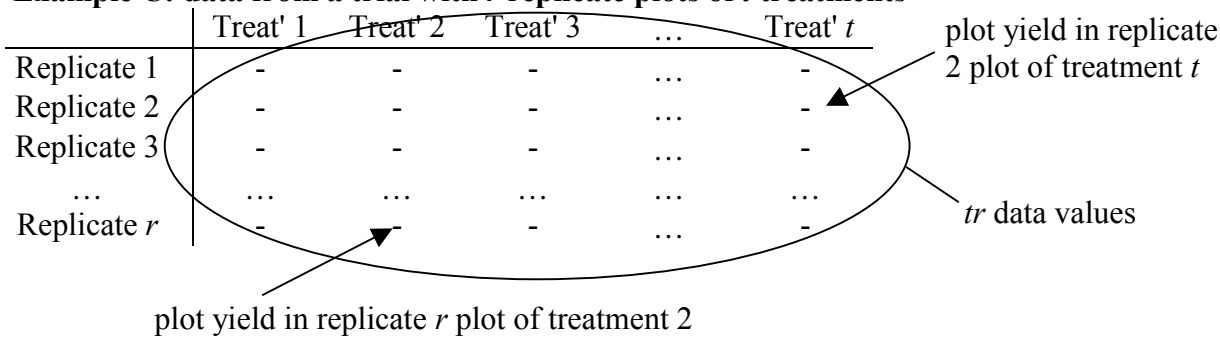
5.1.3 One-way ANOVA

5.1.3.1 The data model

5.1.3.1.1 In one-way ANOVA, the $n \times m$ table of data corresponds to data classified into m groups by a single factor of interest such that there are n independent replicates within each group. It is important to note that the replicates are different within each group, i.e. there is nothing in common between the i^{th} replicate in one group and the i^{th} replicate in another group. For example (Example C), each data value might be the plot yield from a trial with tr plots laid out with r replicate plots of each of t treatments (the factor of interest).

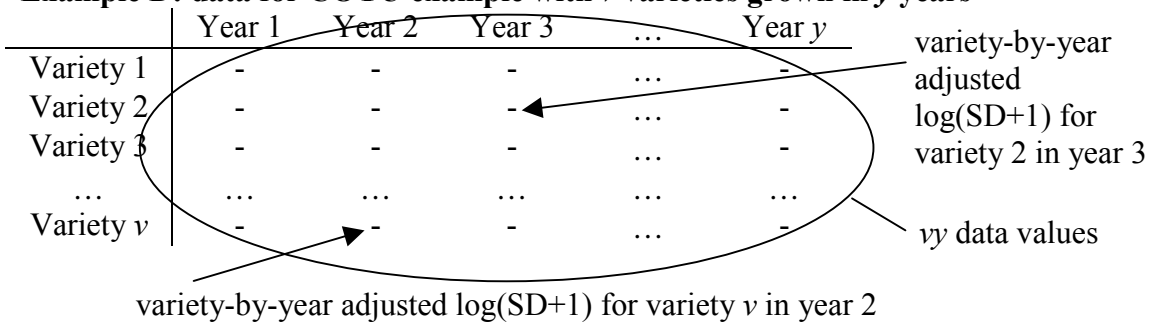
Alternatively each data value might be the mean over all plants in a plot for a characteristic for the *tr* plots.

Example C: data from a trial with *r* replicate plots of *t* treatments



5.1.3.1.2 Alternatively, as in the above COYU example (Example D), they might consist of variety-by-year adjusted log(SD+1)'s for *v* reference varieties (replicates) grown in each of *y* years (the factor of interest) for a characteristic.

Example D: data for COYU example with *v* varieties grown in *y* years



5.1.3.1.3 It might surprise the reader to see the adjusted log(SD+1)'s of the *v* varieties within a year regarded just as replicates, and not as a second factor like in two-way ANOVA. Year is included as a factor in the ANOVA because the overall levels of uniformity, as measured by the adjusted log(SD+1)'s, can be expected to vary from year to year. However, by regarding the uniformity values of the *v* varieties within a year as replicates, this allows the variation between them to be used as an estimate of the random variation in uniformity between the reference varieties, which are all considered to be uniform. It will be seen in the following that this estimate of the random variation in uniformity among the (uniform) reference varieties is used to compare the uniformity of a candidate variety with the mean uniformity of the (uniform) reference varieties. This is done in order to see whether the uniformity of the candidate variety is extreme relative to the uniformity of the reference varieties.

5.1.3.1.4 If *x* represents one of the *nm* data values in the $n \times m$ table of data, the model explaining the variation in the data is as follows:

x = Factor effect + random variation

Thus each of the *nm* data values is made up of the sum of a "Factor effect", which is due to the particular level of the factor influencing the data value, plus a residual amount which is random variation. This means that the variation between data values within a group is considered to be random variation.

5.1.3.1.5 For Example C, the model explaining the variation in the data is as follows:

x = treatment effect + random variation

Here each of the *tr* plot yields is the sum of a "treatment effect", due to the treatment the plot receives, plus a residual amount due to random variation.

5.1.3.1.6 For Example D (COYU), the model explaining the variation in the data is as follows:

x = year effect + random variation

Here each of the *vy* variety-by-year adjusted log(SD+1)'s, i.e. measures of uniformity, is the sum of a "year effect" plus a residual amount due to random variation. This is equivalent to recognising that uniformity is likely to vary from year to year, and is expected to vary at random from variety to variety within a year. Note: the absence of a variety effect in the model indicates that, within a year, apart from random variation, uniformity is expected to be the same for all reference varieties.

5.1.3.2 The one-way analysis of variance table

5.1.3.2.1 One-way ANOVA produces a table as follows:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Factor	m - 1	-	-	-
Residual	$m(n$ - 1)	-	-	
Total	nm - 1	-		

[Again, details of the ANOVA computations are not given here but may be found in DAGNELIE (1998 and 1981), Kala (2002), Mead et al (1993), and Sokal and Rohlf (1995).]

5.1.3.2.2 For Example C, the one-way ANOVA table is as follows:-

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Treatment	t - 1	-	-	-
Residual	$t(r$ - 1)	-	-	
Total	tr - 1	-		

5.1.3.2.3 For Example D (COYU), the one-way ANOVA table is as follows:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Year	y - 1	-	-	-
Residual	$y(v$ - 1)	-	-	
Total	vy - 1	-		

5.1.3.2.4 The total variation in the data is subdivided into "sums of squares" representing the two component sources of variation in the data model, i.e. variation due to the factor of interest and residual or random variation. The sums of squares are divided by their degrees of freedom (df) to give directly comparable "mean squares" used to compare the two sources of variation. This is done in the final column where the F-ratio is the ratio of the factor mean square to the residual mean square. Providing the assumptions discussed below about the data are valid, comparison of the F-ratio with critical values from F tables on m - 1 and $m(n$ - 1) df provides an "F-test" of the significance of the variation due to the factor of interest, i.e. whether that factor has a significant effect. If the F-ratio is greater than or equal to the 100×p% critical value, the factor of interest is declared to have a significant effect at the p% level. Otherwise, it is does not have a significant effect at the p% level.

5.1.3.2.5 The residual mean square is a measure pooled over groups of the variation in the data from replicate to replicate within a group. Thus it is a variance and estimates the random variation in the n×m table of data that has been analysed. Consequently, it can be used to estimate the variance or standard errors of means calculated from the data.

5.1.3.2.6 Worked examples of one-way ANOVA are given in Appendix A2. These are of the same types as Examples C and D above.

5.1.3.3 Assumptions about the data

5.1.3.3.1 Two assumptions are necessary for one-way and two-way ANOVA. They are that:

(a) the variability of the data in the n×m table of data is the same for the different levels of the classifying factors.

Thus, for two-way ANOVA it is assumed that the variability of the vb means is the same for all varieties and for all blocks in Example A, and that the variability of the vy variety-by-year means is the same for all varieties and for all years in Example B (COYD).

For one-way ANOVA it is assumed that the variation in the plot yields between the replicates within a treatment is the same for all treatments in Example C. Likewise, in the one-way ANOVA COYU example (Example D) it is assumed that the variation between the adjusted log(SD+1)'s of the different varieties in a year is the same from year to year;

(b) the model describes the data adequately in that the effects of the classifying

factors are additive. For example (Example A), it is assumed that the expected difference in the data values for two varieties is the same in one block as it is in any other block.

Failure of this assumption will lead to large residuals, as the residual is the part of a data value which is not explained by the additive factor effects. This in turn will lead to a large residual mean square, which will give large standard errors of means, and so large differences will be required between factor means in order for them to be declared significant.

An example of such a failure would be when a variety×year interaction occurs, i.e. when the variety effects are inconsistent over years in the two-way ANOVA COYD example (Example B). Here only large differences between varieties would be declared significant.

5.1.3.3.2 For F-tests of ratios of ANOVA mean squares and t-tests based on ANOVA mean squares (or the equivalent use of LSD's based on ANOVA mean squares) it is also necessary to assume that the data values are independent and that the random variation in the data has an approximately Normal distribution. In COYD the use of the LSD is not dependent on the significance of the F-test.

5.1.3.4 The precision of means and the differences between means

Let $\bar{x}_1$ and $\bar{x}_2$ be factor means of r_1 and r_2 data values from the $n \times m$ table of data that has been analysed by ANOVA (or from equivalent data). The precision of a mean $\bar{x}_i$, where $i = 1$ or 2, is measured by its standard error ($\mathrm{SE}(\bar{x}_i)$), which is estimated by

$$\mathrm{SE}(\bar{x}_i) = \sqrt{\frac{RMS}{r_i}}$$

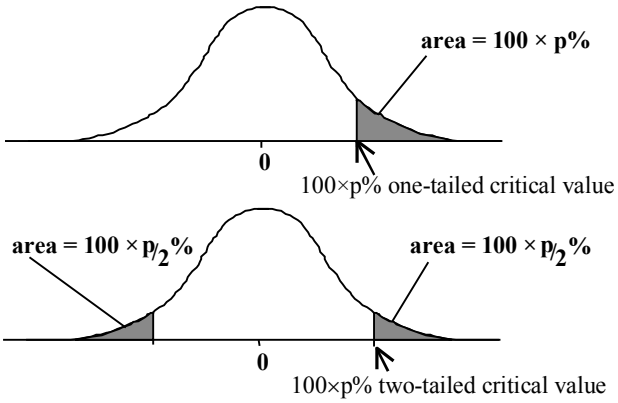
Where *RMS* is the residual mean square from the ANOVA of the $n \times m$ table of data. The precision of the difference in two means, $\bar{x}_1 - \bar{x}_2$, is measured by its standard error, ($\mathrm{SE}(\bar{x}_1 - \bar{x}_2)$), which is estimated by

$$\mathrm{SE}(\bar{x}_1 - \bar{x}_2) = \sqrt{RMS\left(\frac{1}{r_1} + \frac{1}{r_2}\right)}$$

5.1.3.5 Comparisons of means

The significance of the difference between $\bar{x}_1$ and $\bar{x}_2$ can be tested by either:

– Comparing the two sample t-statistic $\mathrm{t} = \dfrac{\bar{x}_1 - \bar{x}_2}{\mathrm{SE}(\bar{x}_1 - \bar{x}_2)}$ with critical values from Student's t-tables on the df of the *RMS*. If it is known *a priori* which mean will be larger, the critical values should be the one-tailed values, and the test is called a "one-tailed test". If it is NOT known *a priori* which mean will be larger, the critical values should be the two-tailed values, and the test is called a "two-tailed test".



If the absolute value of t is greater than or equal to the 100×p% critical value, the means are declared to be significantly different at the p% level. Otherwise, the means are declared to be not significantly different at the p% level.

– Comparing the absolute difference between the means, $|\bar{x}_1 - \bar{x}_2|$, with the 100×p% least significant difference (LSD), i.e. comparing

$$|\bar{x}_1 - \bar{x}_2| \text{ with } t \times \mathrm{SE}(\bar{x}_1 - \bar{x}_2)$$

where *t* is the 100×p% critical value from Students' t-tables on the df of the *RMS*. The critical value should be the one-tailed value if it is known *a priori* which mean will be larger, and two-tailed otherwise.

If the absolute difference between the means is greater than or equal to the 100×p% LSD, the means are declared to be significantly different at the p% level. Otherwise, the means are declared to be not significantly different at the p% level.

5.1.4 Higher-order ANOVA's

5.1.4.1 ANOVA has been introduced as the subdivision of the total variation among the data values in an *n*×*m* table of data such that it:

– allows a comparison of the different sources of variation

– provides an estimate of the random variation affecting the *nm* data values

The *n*×*m* table of data may consist of means calculated from a higher order table of data such as an *l*×*n*×*m* table of data or a *k*×*l*×*n*×*m* table of data. For example, in the above two-way ANOVA COYD example (Example B) the *v*×*y* table of data are variety-by-year means for *v* varieties in each of *y* years and these can be viewed as having been calculated from an *l*×*v*×*y* table of plot means from trials with *l* blocks and *v* varieties in each of *y* years. Alternatively they can be viewed as having been calculated from a *k*×*l*×*v*×*y* table of measurements on *k* plants in each plot of trials with *l* blocks and *v* varieties in each of *y* years.

5.1.4.2 If the *n*×*m* table of data takes the form of means calculated from a higher order table, the experimenter can analyse the data using a multi-way ANOVA that is a logical extension of the two-way ANOVA. In this the total variation is subdivided into components for each of the factors classifying the data table plus components for two-way, three-way and all higher order interactions between the factors. As with the two-way ANOVA, the

components of variation can be compared using ratios of mean squares. Also the residual mean square is a variance which estimates the random variation at the level of the data values in the table of data that has been analysed.

5.1.4.3 Given data values in a more-than-two-way table of data, the experimenter has the choice of analysing it by multi-way ANOVA or by calculating an $n \times m$ table of means and using two-way ANOVA. If the data values in the $n \times m$ table are the means of r of the original data values, then the mean squares in the multi-way ANOVA are r times the size of the equivalent mean squares in the two-way ANOVA. Thus whichever approach is used, the relative sizes of the mean squares are the same and the variances estimated by the residual mean square in the two-way ANOVA can be derived from the mean squares in the higher order ANOVA. However, it is important for the purposes of COYD that the variety means are compared using variances or standard errors based on the variety-by-year mean square as an estimate of random variation, such as is provided by the residual mean square in two-way ANOVA.

5.1.5 Unbalanced data and the method of Fitting Constants

5.1.5.1 An $n \times m$ table of data that has a data value present in each of the nm table cells is balanced. If data values are missing from one or more cells it is unbalanced or incomplete.

5.1.5.2 Although the data for one-way ANOVA was introduced for simplicity as having equal (n) replication in each of the m groups, i.e. balanced, this is not a necessary requirement for one-way ANOVA. If, instead of being balanced, the data consist of a total of w data values unequally replicated within the m groups, the computations are straight forward, and the ANOVA table looks similar to that for one-way ANOVA except that the df differ. The total df is w-1, the factor df is m-1, and the residual df is calculated by subtracting the factor df from the total df, i.e. (w-1) - (m-1).

5.1.5.3 By contrast, the data for two-way ANOVA must be balanced. If the data is unbalanced, i.e. some of the $n \times m$ table's cells have no data, two-way ANOVA cannot be used. Instead, some other method of analysing two-way data such as the method of Fitting Constants or Fitcon (Yates (1933)) or restricted maximum likelihood (REML) (Patterson and Thompson (1971)) must be used. Unbalanced data would arise in the above two-way ANOVA COYD example (Example B) if one or more of the v varieties either was not present or failed to grow in one or more of the y years. It also arises in the calculation of Long Term LSD for use in Long Term COYD. In this a table of variety-by-year means that extends over more years and varieties than are present in the test years is to be analysed. As not all varieties are present in all years, this table is unbalanced. Like two-way ANOVA, Fitcon subdivides the total variation in the data into different components for the different sources of variation, and the residual mean square provides an estimate of the random error variation. The df are as for the two-way ANOVA except that the total df is w-1 where w is the total number of data values, and the residual df is calculated by subtracting the df for each of Factor 1 and Factor 2 from the total df, i.e. (w-1) - (m-1) - (n-1).

5.1.5.4 If the data is unbalanced in either one-way ANOVA or two-way analysis such as Fitcon or REML, the standard errors needed for LSD's or t-tests for comparing factor means are more complicated because they differ depending on which factor mean is being compared with which. In one-way ANOVA this simply affects the replication of the factor means (r_1 and r_2) and the standard errors are calculated as given above. In two-way analysis the

standard errors are further complicated because they depend on the pattern of missing values for the two factor means being compared. However they are easily calculated by a computer program.

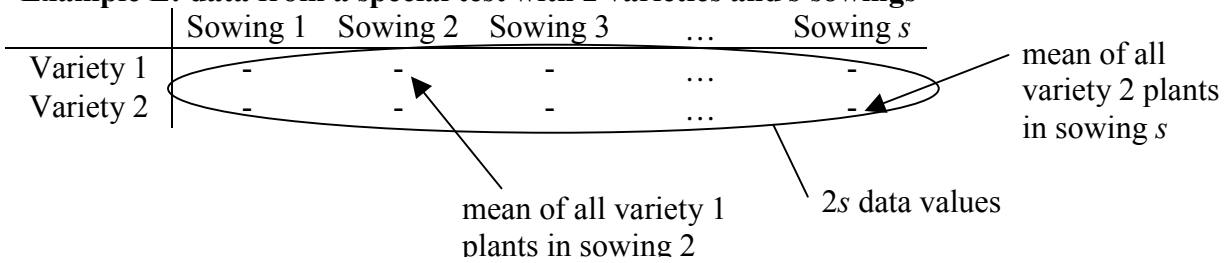
5.1.6 The paired t-test

The paired t-test is a special case of two-way ANOVA where the factor of interest has just two levels. Thus, it is used when there is an $n \times 2$ table of data corresponding to $2n$ data values classified by two factors: Factor 1 with n levels and Factor 2, the factor of interest with 2 levels. Examples of the paired t-test include:

(a) Each data value might be the plot yield from a trial with $2b$ plots laid out in b blocks (Factor 1) of 2 treatments (Factor 2);

(b) in DUS testing when a special test is set up to test distinctness between two varieties using additional characteristics. A number of plants of each variety are sown in a trial. This is repeated in time with s independent sowings. In this example (Example E) the data values are the $2s$ means for the additional characteristic, each based on a fixed number of established plants, from the s sowings (Factor 1) of the two varieties (Factor 2).

Example E: data from a special test with 2 varieties and *s* sowings



The analysis can be done in either of the two following ways:

5.1.6.1 The paired t-test using a one-sample t-test of differences

5.1.6.1.1 The difference in the two data values (one data value for each of the two levels of Factor 2, the factor of interest) for each of the n levels of Factor 1 is obtained. This produces n differences denoted d_i, i=1,…,n. The mean difference and the variance of the differences are calculated and used to produce the one sample t-statistic as follows:

$$t = \frac{\bar{d}}{SE(\bar{d})}$$

where $\bar{d} = \frac{1}{n}\sum_{i=1}^{n} d_i$ is the mean difference, and $SE(\bar{d}) = \sqrt{\frac{s_d^2}{n}}$ is the standard error of the mean difference, and $s_d^2 = \frac{1}{n-1}\sum_{i=1}^{n}(d_i - \bar{d})^2$ is the variance of the differences, which is more easily

computed as $s_d^2 = \frac{1}{n-1}\left[\sum_{i=1}^{n} d_i^2 - \left(\sum_{i=1}^{n} d_i\right)^2 \Big/ n\right]$.

Providing the assumptions about the data discussed above are valid, this t-statistic can be compared with critical values from Student's t-tables on *n*-1 df. The critical values should be one-tailed values if the sign of the mean difference is known *a priori*, and two-tailed values otherwise. The test is of whether the mean difference is significantly different from zero, i.e. whether Factor 2 has a significant effect.

If the absolute value of t is greater than or equal to the 100×p% critical value, the mean difference is declared to be significantly different from zero at the p% level. Otherwise, the mean difference is declared to be not significantly different from zero at the p% level.

5.1.6.2 The paired t-test using two-way ANOVA

5.1.6.2.1 A standard two-way ANOVA of the *n*×2 table of data will provide an ANOVA table with F-ratio's of each of the Factor 1 and Factor 2 mean squares to the residual mean square. Providing the assumptions about the data discussed above are valid, comparison of these F-ratios with F tables on the df of the numerator and the denominator mean squares will provide "F-tests" to test whether Factors 1 and 2 have significant effects.

5.1.6.2.2 For Example E, the two-way ANOVA table is as follows:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Sowing	*s* - 1	-	-	-
Variety	1	-	-	-
Residual	*s* - 1	-	-	
Total	2*s* - 1	-		

5.1.6.2.3 The residual mean square from the two-way ANOVA can be used to estimate the variance or standard errors of means calculated from the data. These can be used to calculate a two-sample t-statistic (or the equivalent LSD), which can be used to test the significance of the difference between the two factor means for Factor 2, i.e. test whether Factor 2 has a significant effect

5.1.6.2.4 Whichever method of analysis is used, the significance of the test of the effect of Factor 2 will be the same. In other words the significance of the t-test in the former method will be the same as the significance of the F-test of the Factor 2 effects and the significance of the t-test of the two Factor 2 means in the latter method.

5.1.6.2.5 A worked example of a paired t-test of the same type as Example E is given in Appendix A3.

SECTION 6: EXAMINING DUS IN BULK SAMPLES

6.1 Introduction and abstract

In some crops samples are bulked before certain characteristics are examined. The term "bulk sampling" is used here for the process of merging some or all individual plants before recording a characteristic. There are different degrees of bulking ranging from: 1) merging pairs of plants, 2) merging 3 or 4 up to all plants within a plot up to 3) merging all plants within a variety. The degree of bulking may play an important role in the efficiency of the tests. Bulking is usually only applied where the measurement of the characteristic is very expensive or very difficult to obtain for individual plants. Some examples are seed weight in cereals and peas and beans, and erucic acid content in rapeseed. This section describes some of the consequences of bulk sampling. It is shown that the test of distinctness (using COYD, see Appendix A4 of section 5) may be expected to be relatively insensitive to the degree of bulking, but that the efficiency of the tests for uniformity (using COYU, see document TGP/10, section 2) must be expected to decrease when the data are bulked. The COYU test for uniformity cannot be carried out if all plants within a plot are bulked.

6.2 Distinctness

6.2.1 In the COYD method for examining distinctness the basic values to be used in the analyses are the annual variety means. As bulk sampling also gives at least one value for each variety per year, it will usually still be possible to use the COYD method for distinctness purposes for any degree of bulking, as long as at least one value is recorded for each variety in each year and that the bulk samples are representative for the variety. However, some problems may be foreseen: the assumption of data being normal distributed may be better fulfilled when the mean of many individual measurements are analyzed instead of the mean of fewer measurements or, in the extreme, just a single measurement.

6.2.2 The efficiency of the test of distinctness may be expected to be lower when based on bulked samples than when it is based on the mean of all individual plants in a year. The loss will be from almost zero upwards, depending on the importance of the different sources of variations. The variation which is relevant for the efficiency of variety comparisons is formulated in the following model.

$$\sigma^2_{total} = \sigma^2_{vy} + \sigma^2_{p} + \sigma^2_{i} + \sigma^2_{m}$$

where

σ^2_{total} is the total variance of a characteristic used for comparing varieties

The total variance is regarded as being composed of four sources of variations:

1: σ^2_{vy} the year in which the variety is measured

2: σ^2_{p} the plot in which the measurement was taken

3: σ^2_{i} the plant on which the measurement was taken

4: σ^2_{m} the inaccuracy in the measurement process

6.2.3 In cases where the data are not bulked the variance of the difference between two variety means, σ^2_{diff}, becomes:

$$\sigma^2_{dif} = 2\left\{\frac{\sigma^2_{vy}}{a} + \frac{\sigma^2_p}{ab} + \frac{\sigma^2_i}{abc} + \frac{\sigma^2_m}{abc}\right\}$$

where

a is the number of years used in the COYD method

b is the number of replicates in each trial

c is the number of plants in each plot

6.2.4 Assuming that each bulk sample has been composed in such a way that it represents an equal amount of material from all the individual plants which have been bulked into that sample, the variance between two varieties based on *k* bulked samples (each of *l* plants) becomes:

$$\sigma^2_{dif} = 2\left\{\frac{\sigma^2_{vy}}{a} + \frac{\sigma^2_p}{ab} + \frac{\sigma^2_i}{abkl} + \frac{\sigma^2_m}{abk}\right\}$$

where

k is the number of bulk samples

l is the number of plants in each bulk sample

6.2.5 Thus if all plants in each plot are divided in *k* groups of *l* plants each and an average measurement is taken for each of the k groups, then only the last term in the expression for σ^2_{diff} has increased (as *kl* is equal to *c*). For many characteristics it is found that the variance caused by the measurements process is small and hence the bulking of samples will only have a minor effect on the conclusions reached by the COYD method. Only if the variance caused by the measurement process is relatively large can bulking have a substantial effect on the distinctness tests using COYD.

6.2.6 To illustrate the effect, the variances for comparing varieties were estimated (by the use of estimated variance components) for different degrees of bulking. The calculations were based on the weight of 100 seeds of 145 pea varieties grown in Denmark during 1999 and 2000. In this example, the contribution to the variance caused by the measurement process was relatively very small, which means that bulking will have a low influence on the test for distinctness. In a 3 year test with 30 plants in each of 2 blocks, the variance on a difference between two varieties was estimated to be 2.133 and 2.135, for no bulking and a single bulk sample per plot, respectively. It should be noted that tests for uniformity are impossible if only 1 bulk per plot is used (see section 6.3).

6.2.7 For other variables the variance component due to the measurement process may be relatively more important. However, it is likely that in most practical cases this variance component will be relatively small.

6.2.8 In some cases each bulk sample is not drawn from a specific set of plants (say, plant 1 to 5 in bulk sample 1, plant 6 to 10 in bulk sample 2 etc.), but bulk samples are formed from mixed samples of all plants in a plot. This means that different bulk samples may contain material from the same plants. It must be expected that similar results apply here, although, in this situation, the effect of bulking may have an increased effect because there is no guarantee that all plants will be equally represented in the bulk samples.

6.3 Uniformity

Bulking within plot

6.3.1 In COYU the test is based on the standard deviation between individual plants (within plots) as a measurement of uniformity. The log of the standard deviations plus one are analyzed in an over-years analysis; i.e. the values $Z_{vy} = \log(s_{vy} + 1)$ are used in the analyses. The variance on these Z_{vy} values can be regarded as arising from two sources, a component that depends on the variety-by-year interaction and a component that depends on the number of degree of freedom used for estimating the standard deviation, s_{vy} (the fewer degrees of freedom the more variable the standard deviation will be). This can be written (note that the same symbols as used in the distinctness section will be used here with different meaning):

$Var(Z_{vy}) = \sigma_{vy}^2 + \sigma_f^2$

where this variance can is regarded as being composed of two sources of variations:

1: σ_{vy}^2 the year in which the variety is measured

2: σ_f^2 the number of degrees of freedom using in estimating s_{vy}

σ_f^2 is approximately $\frac{1}{2v}\left(\frac{\sigma}{\sigma+1}\right)^2$ when then recorded variable is normally distributed and the standard deviations do not vary too much. This last expression reduces to $0.5/\nu$ when $\sigma >> 1$. Here σ is the mean value of the s_{vy} values and ν is the number of degrees of freedom used in the estimation of s_{vy}.

6.3.2 The variance caused by the year in which the variety is measured may be assumed to be independent on whether the samples are bulked or not, whereas the variance caused by the number of degrees of freedom will be increase when bulked samples are used because a lower number of degrees of freedom is available.

6.3.3 The variance of a difference between a Z_{vy} for a candidate variety and the mean of the reference varieties' Z_{vy} values may be written:

$$\sigma_{dif}^2 = \left(\sigma_{vy}^2 + \sigma_f^2\right)\left(\frac{1}{a} + \frac{1}{ar}\right)$$

where

a is the number of year used in the test

r is the number of refference varieties

6.3.4 To illustrate the effect of bulking in the test for uniformity, an estimate was made using the same data as for the illustration in 8.6.2, paragraph 7. For a test using 50 reference varieties in 3 years with 30 plants per variety in each of 2 plots per trial the variance for comparing the Z_{vy} value for a candidate variety and the mean of the reference varieties' Z_{vy} will be 0.0004 if no bulking is done. This can be compared to 0.0041, 0.0016 and 0.0007 when 2, 4 and 10 bulk samples per plot were used. Thus, in this example, the effect of bulking has a great influence on the test for uniformity. The variance increased, approximately by a factor of 10 when changing from individual plant records to just 2 bulk

samples per plot. This means that the degree of non-uniformity must be much higher for it to be detected when 2 bulk samples are used instead of individual plant records.

Bulking across plots

6.3.5 Bulking across plots means that part of the between plot (and block) variation will be included in the estimated standard deviation between bulked samples. If this variation is relatively large it will tend to mask any differences in uniformity between varieties. In addition some noise may also be added because the ratio of material from the different plots may vary from bulk to bulk. Finally the assumptions for the present recommended method, COYU, may not be fulfilled in such cases. Therefore it is recommended to bulk only within plots.

Taking just one bulk sample per plot

6.3.6 In general, if all plants in a plot are bulked such that only a single sample is available for each plot, it becomes impossible to calculate the within plot variability and in such cases no tests for uniformity can be performed. In rare cases, where non-uniformity may be judged from values that can only be found in mixtures, non-uniformity may be detected even where a single bulk sample for each plot is used. For example, in the characteristic “erucic acid” in oil seed rape, values between 2% and 45% can only arise because of a lack of uniformity. However this only applies in certain special cases and even here the non-uniformity may only show up under certain circumstances.

SECTION 7: THE GAIA METHODOLOGY

The GAIA method has been developed by experts from France to calculate a phenotypic distance between two varieties. The principle is to compute a phenotypic distance between a pair of varieties, which is a sum of distances for individual characteristics. For each species, this system must be calibrated to determine the weighting given to the difference in each characteristic used and the threshold for the phenotypic distance used to eliminate varieties from the growing trial.

7.1. Weighting of characteristics

7.1.1 Weighting is defined as the contribution in a given characteristic to the total distance between a pair of varieties. For each species, this system must be calibrated to determine the weight which can be given to each difference and to evaluate the reliability of each characteristic in a given environment and for the genetic variability concerned. For that reason the role of the crop expert is essential.

7.1.2 Weighting depends on the size of the difference and on the individual characteristic. The weightings are defined by crop experts on the basis of their expertise in the crop and on a "try-and-check" learning process and stored in the GAIA database. Experts can give zero weighting to small differences, thus, even if two varieties have different observed values in many characteristics, the overall distance might be zero. The same weighting is attributed to any pair of varieties whose absolute differences between observed values are the same for a given characteristic.

7.1.3 The weighting should be simple and consistent. The following three rules are given:

(i) the distances for the characteristic should be integer values, i.e. 0, 1, 2, 3, etc. where 3 is considered to be about 3 times greater than 1;

(ii) if for a characteristic a given difference "expressed as an absolute value" is considered as a double distance for character *a* compared to character *b*, the distance value for this difference should be double that in character *a* than it is in character *b*;

(iii) define the values by "try-and-check" (see Diagram 1.)

7.1.4 The following simple example shows the computation of the distance between two varieties on the basis of a qualitative characteristic:

Example: taking the characteristic "Shape of ear", observed on a 1 to 3 scale, the crop experts have attributed weighting to differences which they consider significant:

Shape of ear:
1 = conical
2 = conico-cylindrical
3 = cylindrical

Comparison between difference in notes and weighting		
	Different in notes	Weighting
conical (1) vs. conical (1)	0	0
conical (1) vs. conico-cylindrical (2)	1	2
conical (1) vs. cylindrical (3)	3	6
conico-cylindrical (2) vs. conico-cylindrical (2)	0	0
conico-cylindrical (2) vs. cylindrical (3)	1	2
cylindrical (3) vs. cylindrical (3)	0	0

When the crop experts compare a variety 'i' with conical ear (note 1) to a variety 'j' with cylindrical ear (note 3), they attribute a weighting of 6 etc. The weightings are summarized in the form of a weighting matrix:

Weighting matrix 'i'				
		Variety i		
Variety 'j'		**1**	**2**	**3**
	1	0	2	6
	2		0	2
	3			0

When the crop experts compare a variety i with conical ear (note 1) to a variety j with cylindrical ear (note 3), they attribute a weighting of 6.

7.2. Determining "Distinctness Plus"

The threshold for the phenotypic distance used to eliminate varieties from the growing trial is called "Distinctness Plus" and is determined by the crop expert at a level which is higher than the difference needed to establish distinctness. This ensures that all pairs of varieties having a distance equal or greater than the threshold (Distinctness Plus) would be distinct in the growing trial. The Distinctness Plus threshold must be based on experience gained with the varieties of common knowledge and must minimize the possibility of not including in the growing trial a pair of varieties which should be further compared in the field.

7.3. Computing GAIA phenotypic distance

7.3.1 The principle is to compute a phenotypic distance between two varieties, which is the total distance between a pair of varieties as the result of the addition of the weightings of all characteristics (see section 2 of this Annex). Thus, the GAIA phenotypic distance is:

$$dist(i,j) = \sum_{k=1,nchar} W_k(i,j)$$

where:

$dist(i,j)$ is the computed distance between variety i and variety j.

k is the k^{th} characteristic, from the *nchar* characteristics selected for computation.

$W_k(i,j)$ is the weighting of characteristics k, which is a function of the difference observed between variety *i* and variety *j* for that characteristic k.

OV_{ki} is the observed value on characteristic *k* for variety *i*.

$$W_k(i,j) = f\left(\left|OV_{ki} - OV_{kj}\right|\right)$$

7.3.2 Varieties are compared in pairs in different combinations of pair-wise comparisons, e.g.:

- compare two varieties,
- compare a given variety to all varieties in the variety collection,
- compare all candidate varieties to all the other candidate varieties and the varieties in the variety collection,
- compare all possible combinations.

7.4. The GAIA software

7.4.1 The GAIA software allows the computation of the phenotypic distance using qualitative, quantitative or electrophoretic characteristics, which can be used alone or in combination. The user can decide on the type of data and the way it is used:

(i) select all the available characteristics, or different subsets of characteristics.

(ii) define different weighting values:

- experts can choose different values as the weighting/distance for a characteristic (1, 2, 5, etc.);
- some crops have more characteristics than others;
- the crop expert can use all available information, or a subset of characteristics only.

(iii) the way the Distinctness Plus threshold is used:

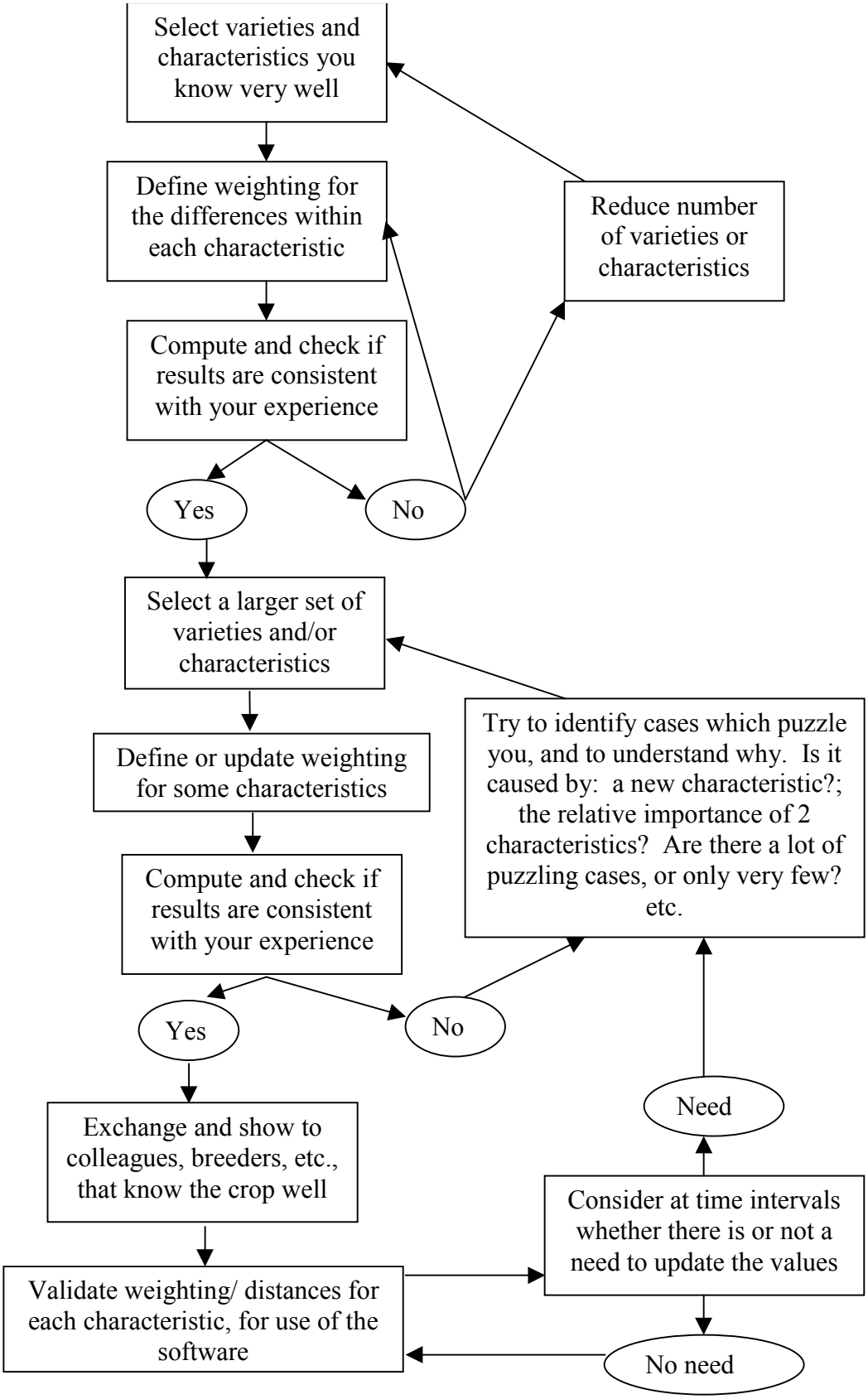
- a low Distinctness Plus threshold, which helps to find the more difficult cases (to identify similar varieties- very often used by crop experts);
- intermediate Distinctness Plus threshold (different levels according to the needs);

- a large Distinctness Plus threshold when there is a need to have a comparison which uses all the available characteristics;
- a Distinctness Plus threshold greater than the maximum distance possible on all characteristics to see all available raw data and the weightings for each characteristic

7.4.2 The software provides a comprehensive report for each pair-wise comparison. It computes an overall distance, but also provides all the individual absolute values and the distance contribution of each characteristic (see section 6 of this Annex).

7.4.3 In order to minimize computation time, as soon as the threshold is achieved for a comparison between two given varieties, the software proceeds to the next pair of varieties. Remaining characteristics and their raw values will not be shown in the summary output, and will not contribute to the distance.

Diagram 1: "Try-and-check" process to define and revise the weightings for a crop



7.4.4 Section 6 of this Annex provides a screen copy of a display tree which shows how the expert can navigate and visualise the results of computations.

7.4.5 GAIA software has been developed with WINDEV-7.5. The general information (species, characteristics, weighting, etc.), the data collected on the varieties and the results of computations are stored in an integrated database. Import and export facilities allow for other information systems to be used in connection with the GAIA software. ODBC allows access to the GAIA database and to other databases simultaneously.

7.4.6 For qualitative characteristics, 1 or 2 notes per variety can be used. In general, two notes are present when there are two trial locations. For electrophoresis data, only one description can be entered per variety. For quantitative characteristics at least 2 values (different trials, repeats, etc.) are necessary and the user selects which to use in the computation.

7.4.7 GAIA is most suitable for self-pollinated and vegetatively propagated varieties, but can also be used for other types of varieties.

7.5. Using the GAIA methodology

The GAIA methodology can be used:

(i) to eliminate from subsequent growing cycles all pairs of varieties reaching or surpassing the GAIA distance threshold;

(ii) to focus on close varieties, having a GAIA distance lower than the threshold, for the next growing cycle(s).

7.5.1 *Using phenotypic distance in the first growing cycle*

7.5.1.1 A crop that has a large variety collection and uses only quantitative characteristics on a 1 to 9 scale; the GAIA methodology allows the selection of varieties to be included in the growing trial. This can be used to plan the first growing cycle trials as well as the subsequent growing cycles.

7.5.1.2 In crops with relatively few candidates and a small variety collection, which enables the crop expert to sow all candidates (e.g. an agricultural crop), and the appropriate reference varieties, in two or three successive growing cycles. The same varieties are sown in growing cycles 1, 2 and 3, in a randomized layout. The software will help to identify the pairs with a small distance, to enable the expert to focus his attention on these particular cases when visiting the field.

7.5.2 *Using phenotypic distance after the first growing trial*

7.5.2.1 After one growing cycle (e.g. in the examination of an ornamental crop), the absolute data and distance computations are an objective way to confirm the opinion or the decision of the expert. There might be cases where pairs of varieties have a small distance, but nevertheless the expert has clear evidence of distinctness. If more growing cycles are necessary before a decision is taken, the software helps to identify on which cases the expert will need to focus.

7.5.2.2 In cases where there are many candidate and reference varieties and there is a wide variability in the species (e.g. a vegetable crop); on the one hand there are already obvious differences after only one cycle, but on the other hand some varieties are very similar. In order to be more efficient in their checks, the crop experts wish to grow "similar" varieties close to each other. The raw results and distances will help to select the "similar" varieties and decide on the layout of the trial for the next growing cycle.

7.5.2.3 In crops in which there are many similar varieties, for which it is a common practice to make side-by-side comparisons, GAIA can be used to identify the similar varieties after the first cycle, in particular, when the number of varieties in a trial increases, making it less easy to identify all the problem situations. The software can help to "not miss" the less obvious cases.

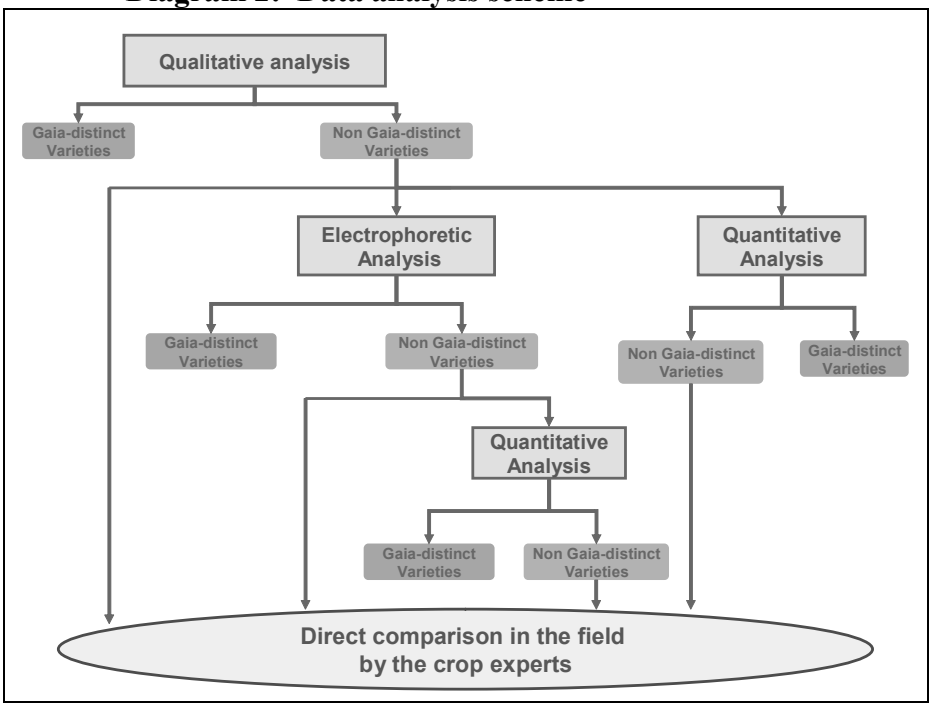
7.5.2.4 In vegetatively propagated ornamental varieties, the examination lasts for one or two growing cycles: after the first growing cycle, some reference varieties in the trial are obviously different from all candidates, and their inclusion in the second growing cycle is not necessary. When the number of varieties is large, the raw data and distance(s) can help the expert to detect reference varieties for which the second growing cycle is unnecessary.

7.6. Example with qualitative, electrophoretic and quantitative characteristics (Zea mays data)

7.*6.1 Introduction*

7.6.1.1 The software can use qualitative, quantitative and/or electrophoretic data. These types of data can be used alone or in combination, as shown in Diagram 2.

Diagram 2: Data analysis scheme



7.6.1.2 In this example, it is assumed that the crop expert has decided to use a Distinctness Plus threshold $\mathbf{S_{dist}}$ of 10 (see section 2 of this Annex).

7.*6.2 Qualitative Analysis*

7.6.2.1 For each characteristic, weightings according to differences between levels of expression are pre-defined in a matrix of distances.

7.6.2.2 "Shape of ear": observed on a 1 to 3 scale, the crop experts have attributed weightings greater than zero to differences which they consider significant:

1 = conical
2 = conico-cylindrical
3 = cylindrical

		Variety 'i'			
			1	2	3
Variety 'j'	1	0	2	6	
	2		0	2	
	3			0	

When the crop experts compare a variety 'i' with conical ear (note 1) to a variety 'j' with cylindrical ear (note 3), they attribute a weighting of 6.

7.6.2.3 "Length of husks", observed on a 1 to 9 scale, the crop experts have defined the following weighting matrix:

1 = very short
2 = very short to short
3 = short
4 = short to medium
5 = medium
6 = medium to long
7 = long
8 = long to very long
9 = very long

		Variety 'i'								
		1	2	3	4	5	6	7	8	9
Variety 'j'	1	0	0	0	2	2	2	2	2	2
	2		0	0	0	2	2	2	2	2
	3			0	0	0	2	2	2	2
	4				0	0	0	2	2	2
	5					0	0	0	2	2
	6						0	0	0	2
	7							0	0	0
	8								0	0
	9									0

The weighting between a variety 'i' with very short husks (note 1) and a variety 'j' with short husks (note 3) is 0. Experts consider a difference of 3 notes is necessary in order to recognise a non-zero distance between two varieties. Even if the difference in notes is greater than 3, the experts do not increase the distance more than 2.

7.6.2.4 The reason for using a lower weighting for some characteristics compared to others can be that they are less "reliable" or "consistent" (e.g. more subject to the effect of the environment); and/or they are considered to indicate a lower distance between varieties.

7.6.2.5 The matrix for a qualitative analysis for 5 characteristics for varieties A and B:

	Ear shape	Husk length	Type of grain	Number of rows of grain	Ear diameter	
Notes for variety A (1 to 9 scale)	1	1	4	6	5	
Notes for variety B (1 to 9 scale)	3	3	4	4	6	
Difference observed	2	2	0	2	1	
Weighting according to the crop expert	*6*	*0*	*0*	*2*	*0*	$D_{qual} = 8$

In this example $\mathbf{D_{qual} = 8 < S_{dist}}$ varieties A and B are declared "GAIA NON-distinct" and can be passed on to electrophoretic analysis.

7.6.3 Electrophoretic analysis

7.6.3.1 The electrophoretic characteristic is a homozygous allele in the UPOV Test Guidelines (see Diagram 3). The software does not allow the use of heterozygous alleles.

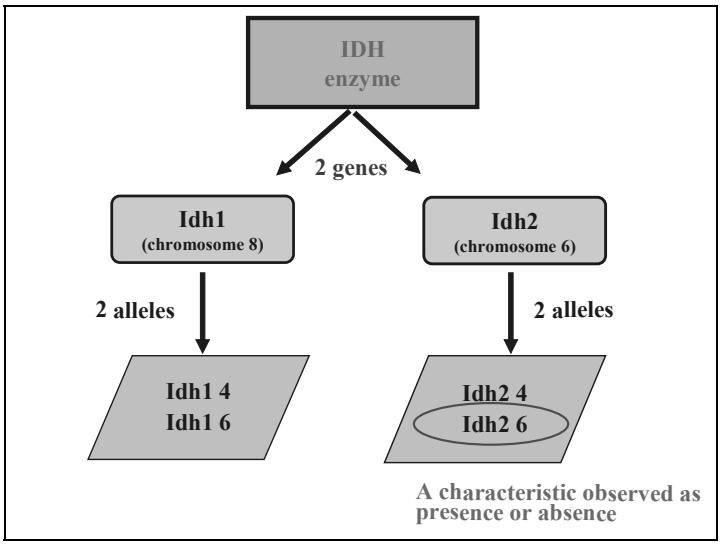


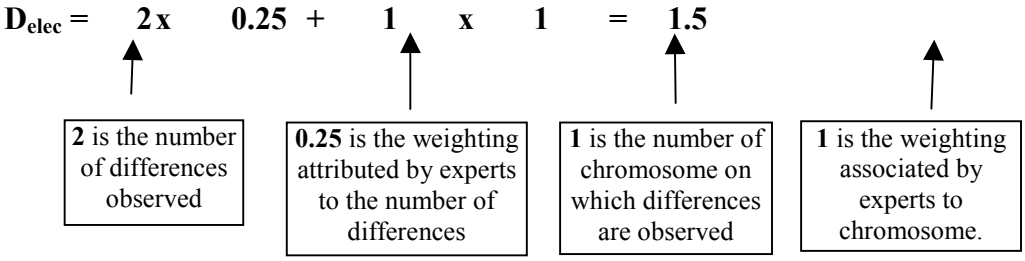
Diagram 3: The Isocitrate Deshydrogenase (IDH) enzyme has two genes (Idh1 and Idh2) located on two different chromosomes. Each of them has two alleles which are observed as 1 (presence) or 0 (absence).

7.6.3.2 Electrophoretic characteristics are noted as 0 or 1 (absence or presence). The decision rule, used to give a weighting to two varieties, is the addition of the weighting number of differences observed and the weighting number of chromosomes related to these differences (see example below):

	Chromosome 8		Chromosome 6	
	Idh1 4	Idh1 6	Idh2 4	Idh2 6
Variety A	0	1	1	0
Variety B	0	1	0	1
Difference	0	0	1	1

7.3.3 In this example, varieties A and B are described for 4 electrophoretic 6.characteristics:

Idh1 4, Idh1 6, Idh2 4 and Idh2 6. The software looks at differences and gives the phenotypic distance using the following computation:



7.6.3.4 This formula, which might be difficult to understand, was established by the crop experts in collaboration with biochemical experts. Both the *number of differences* and the *number of chromosomes on which differences are observed* are used. Thus, less importance is attached to differences when these occur on the same chromosome, than when they occur on different chromosomes.

7.6.3.5 After qualitative and electrophoretic analysis, the phenotypic distance between varieties A and B is equal to:

$$\mathbf{D = D_{qual} + D_{elec} = 8 + 1.5 = 9.5}$$

The phenotypic distance is *lower than* $\mathbf{S_{dist}}$, *therefore varieties A and B are considered "GAIA NON-distinct".*

7.6.3.6 It is not possible to establish distinctness solely on the basis of electrophoretic analysis. It is necessary to have a minimal phenotypic distance in qualitative analysis in order to take into account the electrophoresis results. This minimal phenotypic distance must also be defined by crop experts.

7.6.4 Quantitative Analysis

7.6.4.1 For each quantitative characteristic, the comparison of two varieties is made by looking for consistent differences in at least two different experimental units. Experimental units are defined by the user depending on data present in the database. It can, for example, be the data from two geographic locations of the first growing cycle, or 2 or 3 replications in the case of a single geographical location.

7.6.4.2 For a comparison to be made, the two varieties must be present in the same experimental units. The differences observed must be greater than one of the two threshold values (or minimal distances), fixed by the crop experts.

- $\mathbf{D_{min\text{-}inf}}$ is the lower value from which a weighting is attributed,

- $\mathbf{D_{min\text{-}sup}}$ is the higher minimal distance. These values could be chosen arbitrarily or calculated (15% and 20% of the mean for the trial, or LSD at 1% and 5%, etc.)

7.6.4.3 For each minimal distance a weighting is attributed:

- $\mathbf{D_{min\text{-}inf}}$ a weighting P_{min} is attributed;

- $\mathbf{D_{min\text{-}sup}}$ a weighting P_{max} is attributed;

- the observed difference is lower than $\mathbf{D_{min\text{-}inf}}$ a zero weighting is associated.

7.6.4.4 Varieties A and B have been measured for characteristics "Width of blade" and "Length of plant" in two trials.

7.6.4.5 For each trial, and each characteristic, the crop experts have decided to define ($\mathbf{D_{min\text{-}inf)}}$ and $\mathbf{D_{min\text{-}sup}}$ by calculating respectively the 15% and 20% of the mean for the trial:

	Width of blade		Length of plant	
	Trial 1	Trial 2	Trial 1	Trial 2
$\mathbf{D_{min\text{-}inf}}$ = 15% of the mean	1.2 cm	1.4 cm	28 cm	24 cm
$\mathbf{D_{min\text{-}sup}}$ = 20% of the mean	1.6 cm	1.9 cm	37 cm	32 cm

7.6.4.6 For each characteristic, the crop experts have attributed the following weighting:

A weighting $\mathbf{P_{min}} = 3$ is attributed when the difference is greater than $\mathbf{D_{min\text{-}inf}}$.

A weighting $\mathbf{P_{max}} = 6$ is attributed when the difference is greater than $\mathbf{D_{min\text{-}sup}}$.

	Width of blade		Length of plant		
	Trial 1	Trial 2	Trial 1	Trial 2	
Variety A	9.9 cm	9.8 cm	176 cm	190 cm	
Variety B	9.6 cm	8.7cm	140 cm	152 cm	
Difference	0.3 cm	1.1 cm	36 cm	38 cm	
Weighting according to the crop expert	0	0	3	6	D_{quan} =?

7.6.4.7 In this example, for the characteristic "Width of blade", the differences observed are lower than $\mathbf{D_{min\text{-}inf}}$, so no weighting is associated. On the other hand, for the characteristic "Length of plant" one difference is greater than the $\mathbf{D_{min\text{-}inf}}$ value and the other is greater than the $\mathbf{D_{min\text{-}sup}}$ value. These two differences are attributed different weightings.

7.6.4.8 The user must decide which weighting will be used for the analysis:

- minimalist option: the weighting chosen is that attributed to the lowest difference;
- maximalist option: the weighting chosen is that attributed to the highest difference;
- mean option: the weighting chosen is the mean of the others.

7.6.4.9 In this example, the crop experts have decided to choose the lowest of the two weightings, so the phenotypic distance based on quantitative characteristics is $\mathbf{D_{quan} = 3}$.

7.6.4.10 In summary, at the end of all analysis, the phenotypic distance between varieties A and B is:

$$\mathbf{D = D_{qual} + D_{elec} + D_{quan} = 8 + 1.5 + 3 = 12.5 > S_{dist}}$$

7.6.4.11 The phenotypic distance is greater than the distinction threshold $\mathbf{S_{dist}}$, fixed by the crop experts at 10, so varieties A and B are declared "GAIA-distinct".

7.6.4.12 In this example, the use of electrophoresis data "confirms" a distance between the two varieties; but on the basis of qualitative and quantitative data alone, the threshold is exceeded (8 + 3 = 11 is greater than 10).

7.6.4.13 If the threshold had been set at 6, the difference on the characteristic ear shape would have been sufficient, as variety A is conical and variety B is cylindrical, which is already a clear difference.

1 = conical
2 = conico-cylindrical
3 = cylindrical

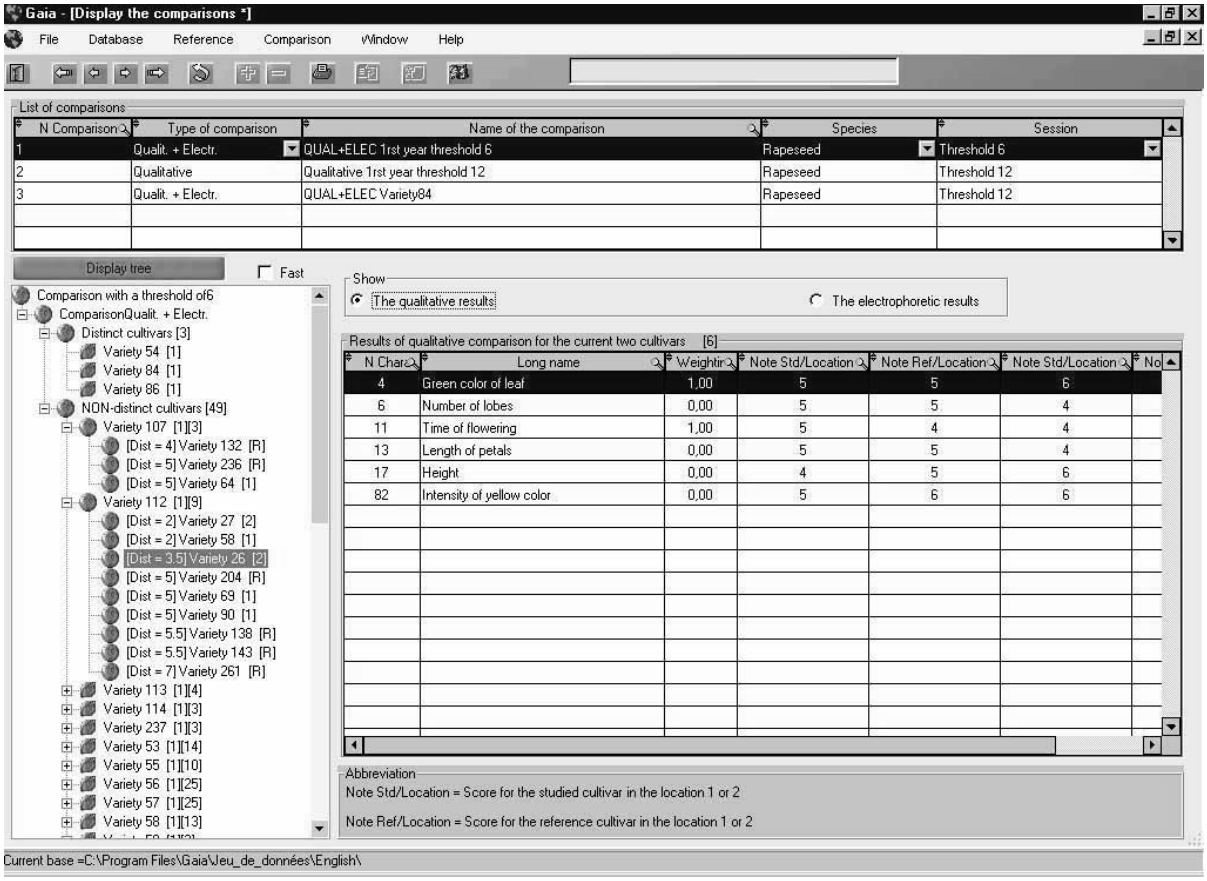
Variety i			
	1	2	3
1	0	2	6
2		0	2
3			0

7.6.5 Quantitative and qualitative analysis on the same characteristics

7.6.5.1 For some crops, it is common practice to produce notes on a 1 to 9 scale for quantitative characteristics. Sometimes the transformation process is very simple, sometimes it is a complex process where all available data are used, but with a special manipulation of example varieties to adjust the raw values to the notes on the scale.

7.6.5.2 GAIA can include both as two separate characteristics: the original quantitative scale; and the "transformed into qualitative notes" scale. They are associated in the description of the characteristics. Using the knowledge of this association, when quantitative and qualitative characteristics are both present, only one characteristic is kept, in order to avoid the information being used twice.

7.7 GAIA screen copy.



The upper part shows 3 different computations which have been kept in the database.

The display tree on the left shows results for a [qualitative + electrophoresis at threshold of 6] computation.

Distinct cultivars [3] demonstrates that 3 varieties were found distinct from all others. There was a total of 52 (49 + 3) cultivars in the computation.

The display tree is used to navigate through all possible pairs.

The user can expand or reduce the branches of the tree according to his needs.

NON-distinct cultivars [49]. Forty-nine cultivars were found “not distinct from all others” with a threshold of 6.

The first variety, *Variety 107*, has only 3 close varieties, whereas the second, *Variety 112*, has 9 close varieties, the third, *Variety 113*, 4 close varieties, etc.

The raw data for *Variety 112* and *Variety 26* are visible for the 6 qualitative characteristics observed on both varieties.

Variety 112 [1][9] indicates variety 112 is in the first year of examination *[1]*; and has 9 close varieties according to the threshold of 6 *[9]*.

[dist=3.5]Variety 26 [2] indicates variety 26 has a GAIA distance of 3.5 from variety 112, which is in second year of examination.

The third column is the weighting according to the pre-defined matrices. The notes for both varieties are displayed for the two available locations (Std stands for "studied" which are the candidate varieties).

In this screen copy the varieties have been numbered for sake of confidentiality, the crop experts can name the varieties according to their need (lot or application number, name, etc.).

7.8 Final remark

The above example was described in order to explain how GAIA uses different types of characteristics in a practical case. The efficiency of the use of GAIA depends on the species.

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APPENDICES

APPENDIX A1

Example of two-way ANOVA (same type as Example A)

1. A trial with four blocks of five varieties of Kale was sown with 15 plants per plot. The data below are the plot means over all the plants in a plot for the "petiole length in mm" characteristic.

	Block			
Variety	I	II	III	IV
J	361	375	361	399
K	388	383	376	401
L	356	386	365	382
M	383	373	385	405
N	386	385	389	413

2. Two-way ANOVA of the data produces the following table:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Block	3	2116.00	705.33	9.95
Variety	4	1316.30	329.08	4.64
Residual	12	850.50	70.88	
Total	19	4282.80		

3. From F-tables the 5%, 1% and 0.1% critical values on 4 and 12 df are 3.259, 5.412 and 9.633 respectively. Comparison of the Variety F-ratio with these shows that there is a significant variety effect at the 5% level ($P<0.05$). The residual mean square or variety-by-block mean square is an estimate of the random variability of the 20 values in the above data table. It may be used to estimate the variance or standard errors of means calculated from the data.

4. The variety means are the means of 4 data values and are as follows:

Variety	Mean
J	374.0
K	387.0
L	372.2
M	386.5
N	393.2

5. Their standard error, $\mathrm{SE}(\bar{x})$, is estimated by

$$\mathrm{SE}(\bar{x}) = \sqrt{\frac{RMS}{r}} = \sqrt{\frac{70.88}{4}} = 4.21$$

6. The standard error of the difference in two means, ($SE(\bar{x}_1 - \bar{x}_2)$), is estimated by

$$SE(\bar{x}_1 - \bar{x}_2) = \sqrt{RMS\left(\frac{1}{r_1} + \frac{1}{r_2}\right)} = \sqrt{70.88\left(\frac{1}{4} + \frac{1}{4}\right)} = 5.95$$

7. The significance of the difference between pairs of variety means can be tested by comparing the absolute difference between pairs of means with, for example, the 5% LSD, where

$$5\%\ \text{LSD} = t \times SE(\bar{x}_1 - \bar{x}_2) = 2.179 \times 5.95 = 12.97$$

and t is the 5% two-tailed critical value from Students' t-tables on 12 df. Thus varieties J and K are significantly different at the 5% level, whereas varieties J and L and varieties M and N are not significantly different at the 5% level etc.

Example of two-way ANOVA (same type as Example B)

8. This example illustrates the calculation of the COYD criterion. The data are the variety-by-year means for 11 varieties of italian ryegrass in three years for the "plant width in cm at ear emergence" characteristic.

Variety	Year 1	Year 2	Year 3
L	60.66	61.47	55.18
N	58.91	62.28	55.66
O	54.46	56.68	51.32
P	57.69	54.75	54.94
Q	56.57	57.62	51.46
R	51.33	53.40	49.18
S	58.59	59.08	51.67
T	63.47	58.94	54.97
V	66.14	65.49	60.15
W	62.63	63.90	58.84
AC	60.36	58.42	58.51

9. Two-way ANOVA of the data produces the following table:

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Year	2	148.821	74.4106	26.843
Variety	10	383.679	38.3679	13.841
Residual	20	55.443	2.7721	
Total	31	587.944		

10. From F-tables the 5%, 1% and 0.1% critical values on 10 and 20 df are 2.348, 3.368 and 5.075 respectively. Comparison of the Variety F-ratio with these shows that there is a very highly significant variety effect at the 0.1% level ($P<0.001$). The residual mean square or

variety-by-year mean square is an estimate of the random variability of the 33 values in the above data table. It may be used to estimate the variance or standard errors of means calculated from the data.

11. The variety means are the means of 3 data values and are as follows:

Variety	Mean
L	59.103
N	58.950
O	54.153
P	55.793
Q	55.217
R	51.303
S	56.447
T	59.127
V	63.927
W	61.790
AC	59.097

12. Their standard error, $\mathrm{SE}(\bar{x})$, is estimated by

$$\mathrm{SE}(\bar{x}) = \sqrt{\frac{RMS}{r}} = \sqrt{\frac{2.7721}{3}} = 0.9613$$

13. The standard error of the difference in two means, ($\mathrm{SE}(\bar{x}_1 - \bar{x}_2)$), is estimated by

$$\mathrm{SE}(\bar{x}_1 - \bar{x}_2) = \sqrt{RMS\left(\frac{1}{r_1} + \frac{1}{r_2}\right)} = \sqrt{2.7721\left(\frac{1}{3} + \frac{1}{3}\right)} = 1.3594$$

14. The significance of the difference between pairs of variety means can be tested by comparing the absolute difference between pairs of means with the 1% LSD where

$$1\%\ \mathrm{LSD} = t \times \mathrm{SE}(\bar{x}_1 - \bar{x}_2) = 2.845 \times 1.3594 = 3.868$$

and t is the 1% two-tailed critical value from Student's t-tables on 20 df. Thus varieties L and N are not significantly different at the 1% level, whereas varieties L and O and varieties L and Q are significantly different at the 1% level etc.

15. For more information on the COYD criterion see document TGP/9.7.

APPENDIX A2

Example of one-way ANOVA (same type as Example C)

1. The data below are the plot yields in Kg from a glasshouse experiment consisting of 18 pots (plots) of potato plants to which 3 replicates of each of six fungicide treatments were allocated at random.

Replicate	Treat' 1	Treat' 2	Treat' 3	Treat' 4	Treat' 5	Treat' 6
1	1.07	0.23	1.07	0.66	1.07	0.91
2	0.74	0.54	0.63	0.85	1.31	0.94
3	0.89	0.57	1.08	0.78	1.50	0.66

2. One-way ANOVA of the data produces the following table:-

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Treatments	5	1.1236	0.2247	6.48
Residual	12	0.4161	0.0347	
Total	17	1.5398		

3. From F-tables the 5%, 1% and 0.1% critical values on 5 and 12 df are 3.106, 5.064 and 8.892 respectively. Comparison of the Treatment F-ratio with these shows that there is a highly significant treatment effect at the 1% level ($P<0.01$) on the plot yield. The residual mean square is an estimate of the random variability of the 18 values in the above data table. It may be used to estimate the variance or standard errors of means calculated from the data.

4. The treatment means are the means of 3 data values and are as follows:-

Treatment	Mean
1	0.900
2	0.447
3	0.927
4	0.763
5	1.293
6	0.837

5. Their standard error, $\mathrm{SE}(\bar{x})$, is estimated by

$$\mathrm{SE}(\bar{x}) = \sqrt{\frac{RMS}{r}} = \sqrt{\frac{0.0347}{3}} = 0.1075$$

6. The standard error of the difference in two means, $(\mathrm{SE}(\bar{x}_1 - \bar{x}_2))$, is estimated by

$$\mathrm{SE}(\bar{x}_1 - \bar{x}_2) = \sqrt{RMS\left(\frac{1}{r_1} + \frac{1}{r_2}\right)} = \sqrt{0.0347\left(\frac{1}{3} + \frac{1}{3}\right)} = 0.1521$$

7. The significance of the difference between pairs of treatment means can be tested by comparing the absolute difference between pairs of means with, for example, the 5% LSD, where

$$5\%\ \mathrm{LSD} = t \times \mathrm{SE}(\bar{x}_1 - \bar{x}_2) = 2.179 \times 0.1521 = 0.3313$$

and t is the 5% two-tailed critical value from Student's t-tables on 12 df. Thus treatments 1 and 2 and treatments 1 and 5 are significantly different at the 5% level, whereas treatments 1 and 3 are not significantly different at the 5% level etc.

Example of one-way ANOVA (same type as Example D)

8. This example illustrates a stage in the calculation of the COYU criterion. The data are the variety-by-year adjusted log(SD+1) of the "days to ear emergence" characteristic for 11 reference varieties of perennial ryegrass in three years. The data have been adjusted for any relationships between log(SD+1) and mean values for the characteristic. The data are as follows:

Variety	Year 1	Year 2	Year 3
R1	2.36	2.13	2.30
R2	2.32	2.00	2.00
R3	2.42	2.10	1.95
R4	2.43	1.96	2.06
R5	2.52	2.14	1.96
R6	2.36	1.84	2.16
R7	2.43	2.19	1.80
R8	2.44	1.70	1.91
R9	2.52	2.16	2.24
R10	2.33	2.23	2.09
R11	2.28	1.78	1.96

9. One-way ANOVA of the data produces the following table:-

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Year	2	1.011	0.5053	25.06
Residual	30	0.605	0.0202	
Total	32	1.616		

10. From F-tables the 5%, 1% and 0.1% critical values on 2 and 30 df are 3.316, 5.390 and 8.773 respectively. Comparison of the Year F-ratio with these shows that there is a very highly significant year effect at the 0.1% level ($P<0.001$) on uniformity. However, this F-test is of minor importance in calculating the COYU criterion. Of real importance is the overall mean adjusted log(SD+1) for all the reference varieties, and the residual mean square. The residual mean square provides an estimate of the random variability of the 33 values in the

above data table, i.e. the variation between reference varieties within years. It may be used to estimate the variance or standard errors of means calculated from the data. In particular, it allows the overall mean of the reference varieties to be compared with a candidate variety's mean adjusted log(SD+1).

11. The overall mean adjusted log(SD+1) is 2.154. It is the mean of the reference varieties' 33 data values. Its standard error, $\mathrm{SE}(\bar{x})$, is estimated by

$$\mathrm{SE}(\bar{x}) = \sqrt{\frac{RMS}{r}} = \sqrt{\frac{0.0202}{33}} = 0.0247$$

12. If $\bar{x}_1$ represents the overall mean adjusted log(SD+1) and $\bar{x}_2$ a candidate variety's mean adjusted log(SD+1) which is the mean of the equivalent of 3 data values, then the standard error of the difference in the two means, ($\mathrm{SE}(\bar{x}_1 - \bar{x}_2)$), is estimated by

$$\mathrm{SE}(\bar{x}_1 - \bar{x}_2) = \sqrt{RMS\left(\frac{1}{r_1} + \frac{1}{r_2}\right)} = \sqrt{0.0202\left(\frac{1}{33} + \frac{1}{3}\right)} = 0.0857$$

13. The significance of the difference between $\bar{x}_1$, the overall mean adjusted log(SD+1) and $\bar{x}_2$, a candidate variety's mean adjusted log(SD+1) can be tested by comparing

$$\left|\bar{x}_1 - \bar{x}_2\right| = \bar{x}_2 - \bar{x}_1 \text{ with } t \times \mathrm{SE}(\bar{x}_1 - \bar{x}_2) = 3.118 \times 0.0857 = 0.2672$$

Where *t* is the 0.2% one-tailed critical value (one-tailed because the candidate's mean is only rejected if it is larger than the overall mean) from Student's t-tables on 30 df. This is equivalent to comparing

$$\bar{x}_2 \text{ with } 0.2672 + \bar{x}_1 = 2.4212$$

14. Thus if a candidate variety has a mean adjusted log(SD+1) greater than or equal to 2.42*, this mean is significantly greater than the mean of the reference varieties at the 0.2% level, and hence the candidate variety is considered to be significantly non-uniform compared to the reference varieties. If the candidate variety's mean adjusted log(SD+1) is less than 2.42*, the mean will be considered to be not significantly different from the mean of the reference varieties at the 0.2% level, and hence the candidate variety not significantly different from the reference varieties in uniformity.

* The above is based on the conventional use of the two sample t-test. However, in order to give the benefit of the doubt to the breeder, in the practical application of COYU the accepted procedure is to reject a variety as non-uniform if its mean adjusted log(SD+1) is *greater than* the uniformity criterion, and accept it as uniform if its mean adjusted log(SD+1) is *less than or equal to* the uniformity criterion.

15. For more information on the COYU criterion see document TGP/10.3.1.

APPENDIX A3

Example of a paired t-test (same type as Example E)

1. A special test was set up in order to test distinctness between two varieties of perennial ryegrass using the additional characteristic "Seedling width of vegetative leaf". Thirty plants of each variety were sown at each independent sowing in a glasshouse, and when the plants were established, the "Seedling width of vegetative leaf" was observed in mm on each plant, and the mean for each variety calculated. There were 6 independent sowings and the data below are the 12 means: one for each variety for each sowing.

	Sowing					
Variety	I	II	III	IV	V	VI
J	6.9	7.9	5.2	5.8	6.5	4.8
N	5.9	6.7	3.8	5.3	3.6	3.6

2. The data will be analysed using each of the described methods.

The paired t-test analysis using a one-sample t-test of differences

3. Subtracting the variety N data value from the variety J data value for each sowing gives the following differences:-

	Sowing					
	I	II	III	IV	V	VI
Difference (d_i)	1.0	1.2	1.4	0.5	2.9	1.2

4. The mean difference $\bar{d}$, the variance of the differences s_d^2, and the standard error of the mean difference $SE(\bar{d})$ are calculated from the differences as follows:-

$$\bar{d} = \frac{1}{n}\sum_{i=1}^{n} d_i = \frac{1}{6}\sum_{i=1}^{6} d_i = \frac{1}{6}(1.0 + 1.2 + 1.4 + ... + 1.2) = \frac{1}{6} \times 8.2 = 1.37$$

$$s_d^2 = \frac{1}{n-1}\left[\sum_{i=1}^{n} d_i^{\,2} - \left(\sum_{i=1}^{n} d_i\right)^2 \Big/ n\right] = \frac{1}{6-1}\left[\sum_{i=1}^{6} d_i^{\,2} - \left(\sum_{i=1}^{6} d_i\right)^2 \Big/ 6\right]$$

$$= \frac{1}{5}\left[\sum_{i=1}^{6} 1.0^2 + 1.2^2 + 1.4^2 + ... + 1.2^2 - (1.0 + 1.2 + 1.4 + ... + 1.2)^2 / 6\right]$$

$$= \frac{1}{5}\left[14.5 - (8.2)^2 / 6\right] = 0.6587$$

$$SE(\bar{d}) = \sqrt{\frac{s_d^2}{n}} = \sqrt{\frac{0.6587}{6}} = 0.3313$$

5. These are used to calculate the one sample t-statistic as follows:-

$$t = \frac{\bar{d}}{SE(\bar{d})} = \frac{1.37}{0.3313} = 4.12$$

6. From Student's t-tables the 5%, 1% and 0.1% two-tailed critical values on 5 df are 2.571, 4.032 and 6.869 respectively. Comparison of the t-statistic with these shows that the mean difference is highly significantly different from zero at the 1% level (P<0.01), i.e. Variety has a highly significant effect. From the sign of the mean difference it is apparent that variety J has a greater "Seedling width of vegetative leaf" than variety N.

The paired t-test analysis using two-way ANOVA

7. Two-way ANOVA of the data produces the following table:-

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio
Sowing	5	13.8900	2.7780	8.44
Variety	1	5.6033	5.6033	17.01
Residual	5	1.6467	0.3293	
Total	11	21.1400		

8. From F-tables the 5%, 1% and 0.1% critical values on 1 and 5 df are 6.608, 16.26 and 47.18 respectively. Comparison of the Variety F-ratio with these shows that there is a highly significant Variety effect at the 1% level (P<0.01). The residual mean square or variety-by-sowing mean square is an estimate of the random variability of the 12 values in the above data table. It may be used to estimate the variance or standard errors of means calculated from the data.

9. The variety means are the means of 6 data values and are as follows:-

Variety	Mean
J	6.18
N	4.82

10. Their standard error, $\mathrm{SE}(\bar{x})$, is estimated by

$$\mathrm{SE}(\bar{x}) = \sqrt{\frac{RMS}{r}} = \sqrt{\frac{0.3293}{6}} = 0.2343$$

11. The standard error of the difference in two means, ($\mathrm{SE}(\bar{x}_1 - \bar{x}_2)$), is estimated by

$$\mathrm{SE}(\bar{x}_1 - \bar{x}_2) = \sqrt{RMS\left(\frac{1}{r_1} + \frac{1}{r_2}\right)} = \sqrt{0.3293\left(\frac{1}{6} + \frac{1}{6}\right)} = 0.3313$$

12. The significance of the difference between the two variety means can be tested by calculating the two sample t-statistic:

$$\mathrm{t} = \frac{\bar{x}_1 - \bar{x}_2}{\mathrm{SE}(\bar{x}_1 - \bar{x}_2)} = \frac{6.18 - 4.82}{0.3313} = 4.12$$

From Student's t-tables on 5 df, the 5%, 1% and 0.1% two-tailed critical values are 2.571, 4.032 and 6.869 respectively. Comparison of the t-statistic with these shows that the difference in the variety means is highly significant at the 1% level ($P<0.01$), i.e. variety J has a greater 'Seedling width of vegetative leaf' than variety N.

APPENDIX A4

5.2 The Combined Over-Years Distinctness Criterion (COYD)

5.2.1 Summary

5.2.1.1 To distinguish varieties on the basis of a quantitative characteristic we need to establish a minimum distance between varieties such that, when the distance calculated between a pair of varieties is greater than this minimum distance, they may be considered as "distinct" in respect of that characteristic. There are several possible ways of establishing minimum distances from Distinctness, Uniformity and Stability (DUS) trials data. Here is described what is known as the Combined-Over-Years Distinctness (COYD) method.

5.2.1.2 The COYD method involves:

- for each characteristic, taking the variety means from the two or three years of trials for candidates and established varieties and producing over-year means for the varieties;

- calculate a least significant difference (LSD), based on variety-by-years variation, for comparing variety means.

- if the over-years mean difference between two varieties is greater than or equal to the LSD then the varieties are said to be distinct in respect of that characteristic.

5.2.1.3 The main advantages of the COYD method are:

- it combines information from several seasons into a single criterion (the "COYD criterion") in a simple and straightforward way;

- it ensures that judgements about distinctness will be reproducible in other seasons; in other words, the same genetic material should give similar results, within reasonable limits, from season-to-season;

- the risks of making a wrong judgement about distinctness are constant for all characteristics.

5.2.2 Introduction

5.2.2.1 In order to decide if two varieties are distinct in respect of a measured characteristic, a criterion is needed which will determine whether the differences found in DUS trials are clear and sufficiently consistent. The Combined-Over-Years Distinctness (COYD) method provides such a criterion.

5.2.2.2 This paper describes:

- the principles underlying the COYD method;

- UPOV recommendations on the application of COYD to individual species;

– details of ways in which the procedure can be adapted to deal with special circumstances. This includes when there are small numbers of varieties in trial;

– the computer software which is available to apply the procedure.

5.2.3 The COYD Method

5.2.3.1 The COYD method aims to establish for each characteristic a minimum difference, or distance, which, if achieved by two varieties in trials over a period of two or three years, would indicate that those varieties are distinct with a specified degree of confidence.

5.2.3.2 The method uses variation in variety expression of a characteristic from year-to-year to establish the minimum distance. Thus, characteristics which show consistency in variety ranking between years will have smaller minimum distances than those with marked changes in ranking.

5.2.3.3 Calculation of the COYD criterion involves analysing the variety-by-year table of means for each characteristic to get an estimate of the varieties-by-years variation, which is used in the next step: to calculate an LSD. Usually data for all candidate and established varieties which appeared in trials over the two or three test years are included in the table, the analysis is by analysis of variance (see TGP/8.5 for details), the varieties-by-years mean square is used as the estimate of the varieties-by-years variation, and the resulting LSD is known as the COYD LSD. However, where there are small numbers of varieties in trial, the approach is different.

5.2.3.4 Where there are small numbers of varieties in trial, the table used to calculate of the COYD criterion is expanded with means from other varieties and earlier years, a different method of analysis is used to get a varieties-by-years mean square to estimate the varieties-by-years variation, and the resulting LSD is known as the Long-Term LSD. This is discussed later.

5.2.3.5 Equation [1]

$$LSD_p = t_p \times \sqrt{2} \times SE(\bar{x})$$

where $SE(\bar{x})$ is the standard error of a variety's over-year mean calculated as:

$$SE(\bar{x}) = \sqrt{\frac{\text{varieties - by - years mean square}}{\text{number of test years}}}$$

and t_p is the value in Student's t table appropriate for a two-tailed test with probability p and with degrees of freedom associated with the variety-by-years mean square. The probability level p that is appropriate for individual species is discussed under UPOV RECOMMENDATIONS ON COYD below.

5.2.3.6 An example of the application of COYD to a small data set is given in Figure 1. Statistical details of the method are in Annex IV and in document TGP/8.5, "Statistical Methods for DUS Examination." Further information about the COYD criterion can be found in Patterson and Weatherup (1984).

5.3 UPOV Recommendations on COYD

5.3.1 COYD is recommended for use in assessing the distinctness of varieties where:

– the characteristic is quantitative;

– there are some differences between plants (or plots) of a variety.

– observations are made on a plant (or plot) basis over two or more years;

5.3.2 A pair of varieties is considered to be distinct if their over-years means differ by at least the COYD LSD in one or more characteristics.

5.3.3 The UPOV recommended probability level p for the t_p value used to calculate the COYD LSD differs depending on the crop and for some crops depends on whether the test is over two or three years. The testing schemes that usually arise in distinctness testing are described in Annex VI.

5.4 Adapting COYD to special circumstances

5.4.1 Differences between years in the range of expression of a characteristic. Occasionally, marked differences between years in the range of expression of a characteristic can occur. For example, in a late spring, the heading dates of grass varieties can converge. To take account of this effect it is possible to fit extra terms, one for each year, in the analysis of variance. Each term represents the linear regression of the observations for the year against the variety means over all years. The method is known as modified joint regression analysis (MJRA) and is recommended in situations where there is a statistically significant ($p \leq 1\%$) contribution from the regression terms in the analysis of variance. Statistical details, and a computer program to implement the procedure, are described in the appendices.

5.5 Small numbers of varieties in trials: Long-Term COYD

5.5.1 It is recommended that there should be at least 20 degrees of freedom for the varieties-by-years mean square in the COYD analysis of variance. This is in order to ensure that the varieties-by-years mean square is based on sufficient data to be a reliable estimate of the varieties-by-years variation for the LSD. Twenty degrees of freedom corresponds to 11 varieties common in three years of trials, or 21 varieties common in two years. Trials with fewer varieties in common over years are considered to have small numbers of varieties in trial.

5.5.2 In such trials the variety-by-year tables of means can be expanded to include means for earlier years, and if necessary, other established varieties. As not all varieties are present in all years, the resulting tables of variety-by-year means are not balanced. Consequently, each table is analysed by the least squares method of fitted constants (FITCON) or by REML, which produces an alternative varieties-by-years mean square as a long-term estimate of variety-by-years variation. This estimate has more degrees of freedom as it is based on more years and varieties.

$$\text{degrees of freedom} = \left(\begin{array}{c} \text{No. values in expanded} \\ \text{variety - by - year table} \end{array} \right) - (\text{No. varieties}) - (\text{No. years}) + 1$$

5.5.3 The alternative varieties-by-years mean square is used in equation [1] above to calculate an LSD. This LSD is known as a "Long-Term LSD" to distinguish it from COYD LSD based on just the test years and varieties. The Long-Term LSD is used in the same way as the COYD LSD is used to assess the distinctness of varieties by comparing their over-year (the test years) means. The act of comparing the means of varieties using a "Long-Term LSD" is known as "Long-Term COYD".

5.5.4 Long-Term COYD should only be applied to those characteristics lacking the recommended minimum degrees of freedom. However, when there is evidence that a characteristic's LSD fluctuates markedly across years, it may be necessary to base the LSD for that characteristic on the current two or three-years of data, even though it has few degrees of freedom.

5.5.5 Figure 2 gives an example of the application of Long-Term COYD to the italian ryegrass characteristic "Growth habit in spring" (UPOV Char 6). A flow diagram of the stages and DUST modules used to produce Long-Term LSD's and perform Long-Term COYD is given in Figure B2 in Annex V.

5.6 Marked year-to-year changes in an individual variety's characteristic

Occasionally, a pair of varieties may be declared distinct on the basis of a t-test which is significant solely due to a very large difference between the varieties in a single year. To monitor such situations a check statistic is calculated, called F_3, which is the variety-by years mean square for the particular variety pair expressed as a ratio of the overall variety-by-years mean square. This statistic should be compared with F-distribution tables with 1 and *g*, or 2 and *g*, degrees of freedom, for tests with two or three years of data respectively where *g* is the degrees of freedom for the variety-by-years mean square. If the calculated F_3 value exceeds the tabulated F value at the 1% level then an explanation for the unusual result should be sought before making a decision on distinctness.

5.7 Implementing COYD

The COYD method can be applied using the DUST package for the statistical analysis of DUS data, which is available from Dr. Sally Watson, Biometrics Division, Department of Agriculture for Northern Ireland (DANI), Newforge Lane, Belfast BT9 5PX, United Kingdom. Sample outputs are given in Annex V.

Figure 1: Illustrating the calculation of the COYD criterion

Characteristic: Days to ear emergence in perennial ryegrass varieties

Varieties	Years 1	2	3	Over Year Means	*Difference (Varieties compared to C2)*	
Reference		Means				
R1	38	41	35	38	*35*	*D*
R2	63	68	61	64	*9*	*D*
R3	69	71	64	68	*5*	*D*
R4	71	75	67	71	*2*	
R5	69	78	69	72	*1*	
R6	74	77	71	74	*-1*	
R7	76	79	70	75	*-2*	
R8	75	80	73	76	*-3*	
R9	78	81	75	78	*-5*	*D*
R10	79	80	75	78	*-5*	*D*
R11	76	85	79	80	*-7*	*D*
Candidate						
C1	52	56	48	52	*21*	*D*
C2	72	79	68	73	*0*	-
C3	85	88	85	86	*-13*	*D*

ANALYSIS OF VARIANCE

Source	df	Mean square
Years	2	174.93
Variety	13	452.59
Variety-by-years	26	2.54

$LSD_p = t_p * \sqrt{2} * SE(\overline{X})$

$LSD_{0.01} = 2.779 * 1.414 * \sqrt{(2.54/3)} = 3.6$

Where t_p is taken from Student's t table with $p = 0.01$ (two-tailed) and 26 degrees of freedom.

To assess the distinctness of a candidate, the difference in the means between the candidate and all other varieties is computed. In practice a column of differences is calculated for each candidate. In this case, varieties with mean differences greater than, or equal to, 3.6 are regarded as distinct (marked *D* above).

Figure 2: Illustrating the application of Long-Term COYD

Characteristic: Growth habit in spring in italian ryegrass varieties

Varieties	Years 1	2	3*	4*	5*	Mean over test years	*Difference (Varieties compared to C2)*	
Reference			Means					
R1	43	42	41	44				
R2		39	45					
R3	43	38	41	45	40	42	*6*	*D*
R4	44	40	42	48	44	44.7	*3.3*	*D*
R5	46	43	48	49	45	47.3	*0.7*	
R6	51	48	52	53	51	52	*-4*	*D*
Candidate								
C1			43	45	44	44	*4*	*D*
C2			49	50	45	48	*0*	
C3			48	53	47	49.3	*-1.3*	

* indicates a test year

The aim is to assess the distinctness of the candidate varieties C1, C2 & C3 grown in the test years 3, 4 & 5.

The trial has a small number of varieties in trial because there are just seven varieties in common over the test years 3, 4 & 5 (data marked by a black border).

FITCON analysis of the variety-by-years table of means expanded to nine varieties in five years gives: varieties-by-years mean square = 1.924, on 22 degrees of freedom

Long-term $LSD_p = t_p * \sqrt{2} * SE(\overline{X})$

Long-term $LSD_{0.01} = 2.819 * 1.414 * \sqrt{(1.924/3)} = 3.19$

Where t_p is taken from Student's t table with $p = 0.01$ (two-tailed) and 22 degrees of freedom

To assess the distinctness of a candidate, the difference in the means between the candidate and all other varieties is computed. In practice a column of differences is calculated for each candidate. In the case of variety C2, varieties with mean differences greater than, or equal to 3.19 are regarded as distinct (marked *D* above).

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