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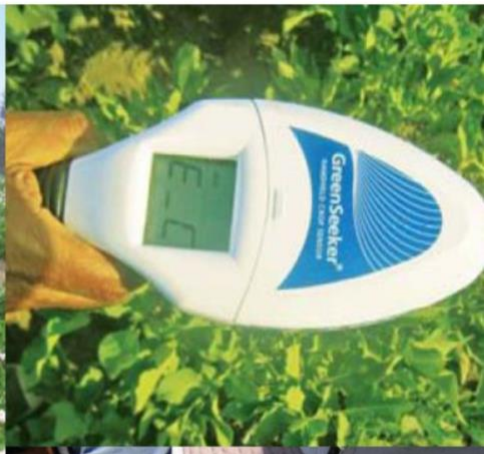

Procedures for standard evaluation and data management of advanced potato clones

Summary guide to selecting potato clones for drought tolerance under field conditions international cooperators' guide

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Roots, Tubers and Bananas
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September 2020

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Content

1. Introduction

Drought stress is a multidimensional stress and generally leads to changes in the physiological, morphological, ecological, biochemical, and molecular traits of plants (Farooq *et al*., 2009). In addition, it can negatively affect the quantity and quality of plant growth and yield (Zlatev and Lidon, 2012). Many plants have developed resistance mechanisms to tolerate drought stress, but these mechanisms are varied and depend on plant species (Hossain *et al.*, 2016). There are several options for drought tolerance mechanisms in plants, including developmental, physiological, morphological, ecological, biochemical, and molecular mechanisms. Typically, the mechanisms involved in plant tolerance to drought follow a general plan: maintaining cell water homeostasis under drought conditions (Hossain *et al.*, 2016). Impact of abiotic stresses on potato production will increase over the next decades, due to climate change and the extension of potato cultivation under drought/heat conditions (Hijmanns, 2003) since potato is extremely susceptible to drought (Monneveux *et al.*, 2013). Plant adaptation to drought involves several different morphological and physiological characteristics; however, no specific traits have been reported since drought responses change according to plant genotype and growth stage. Stem height, number of green leaves and leaf length are considered to be the parameters most sensitive to moderate drought conditions (Deblonde and Ledent, 2001); however, these traits have shown inconsistency in many cases. In the present protocol, a selection of traits is shown as a result of previous experiments and exhaustive data analysis at the International Potato Center.

2. Experimental installation

Experimental design and treatments

- **Experimental Design:** It is important to have the experiment objectives very clear while stablishing a field layout to prepare the correct number of clones needed before the experiment begins. The experimental design should be decided based on the objective of the experiment and the number of genotypes to evaluate. For a big set of clones with limited number of tuber seeds, consider an augmented block design (ABD) where un-replicated clones are planted with a group of checks repeated in each block. Checks could be advanced breeding lines, commercial varieties, or parents of the crosses that are suitable for the target region and compatible with the experimental population; however, it is recommended to have previous knowledge of its tolerance or susceptibility to drought. When the number of clones to evaluate is fewer, a strip plot design must be considered.

  In general, number of tubers for each clone could be estimated as:

$$\frac{tubers}{clone}\ needed = (5-10)\ plants * clone * replications * (2-3) treatments$$

  It is recommended to use plots of 10 plants per clone instead of 5, since some plants can be affected by diseases or not germinate.

- **Treatments:**

✓ **Normal irrigation (NI):** This is a control treatment. Here water is applied as normally required by the potato crop and determined from previous trials for normal growth at the site),

✓ **Terminal drought (TD):** This treatment can start at tuber initiation or at hilling, here irrigation is completely suspended until harvest.

✓ **Recovery (REC):** This treatment must be initiated at the same time as TD initiation. In this treatment, the crop is normally irrigated again after 25 to 30 days of drought initiation (drought effects occur approximately at 10 days after drought treatment initiation).

- **Plant material:** It is recommended to separate genotypes according to earliness, since It has been seen to have a great impact in data analysis. Potato genotypes with early and late growing period have different physiological behaviors and responses to drought; therefore, an analysis without taking this in consideration can lead to a false identification of drought tolerance.

Fig. 1 Hobo U30 complete weather station.

Monitoring soil and weather parameters

Weather stations should be installed and launched in the field to monitor and record relative humidity, temperature, water quantity, water pressure deficit, wind velocity, wind direction, precipitation, solar irradiation, soil temperature, and photosynthetically active radiation (PAR) every 15-30 minutes/day (Fig.1).

For soil monitoring, 3 Soil samples (100g each) must be taken at three different stages: 1) before the experiment; in order to calculate the appropriate amount of fertilizers for crop management; 2) at hilling, to have an idea of how the experiment will start; 3) at the end of the harvest; to observe nutrient consumption.

Crop management

- **Fertilization:** Soil analysis is needed for an appropriated formulation of the field fertilization dose. Fertilizers with high nitrogen content must be applied at two different stages, half before planting and half after hilling. Animal manure, di-ammonium phosphate, potassium sulfate and ammonium nitrate are types of fertilizers that could be used. It is important to apply fertilizers next to tubers and not over them, to avoid damaging sprouts. (Fig. 2)

Fig. 2 Tuber layout before fertilization.

- **Pest management:** Pests should be monitored in every plot and managed according to their incidence. Different pesticides should be applied according to the identified pest. Some recurrent pests among potato crops are: *Agrotis* spp., *Phthorimaea operculella*, *Tuta absoluta*, *Liriomyza* spp., aphids, *Leptinotarsa decemlineata*, *Russelliana solanicola*, and fungal diseases such as *Alternaria solani* and *Phytophthora infestans*.

  Not only pests can cause damages to potato crops. Weeds exponential growth can consume potato crop nutrients, adding externals factors to the experiment such as competitiveness and allelopathy. Therefore, weeding should be done as often as possible and mainly before hilling (Fig. 3). Weeding should be done manually or by using tractors.

Fig. 3 Hilling or plant coverage to allow tuber formation and bulking afterwards. (Performed at 35 to 45 days after planting.)

3. Drought tolerance traits

This summarized protocol shows a brief list of traits, selected among several agronomical and physiological traits after exhaustive data analysis. These traits can be directly used in drought breeding trials to choose drought tolerant potato genotypes. During evaluations, it is recommended to assign only one person per treatment to register values in the field book to avoid systematic errors. (Fig. 4)

Fig. 4 Example of the recommended job distribution during evaluations

Pre and post-stress traits

The next evaluations must be taken at least 3 times during the experiment. During evaluations, all collected data must be registered in the digital field book as soon as possible.

- **Stem number per plant (SNPP):** This trait should be counted once before hilling. Any further evaluation before harvest may lead to mistakes due to below ground stems ramification. It is not necessary to have more measurements since its value tends maintain. This trait has shown in many cases a high correlation to tuber yield per plant.

- **Stem diameter (SD, cm):** This trait should be measured with a vernier caliper at the bottom of the stem, just above the first node of leaves. This evaluation should be done at least once immediately after hilling and two times after drought initiation. (Fig. 5)

Fig.5 Correct stem diameter measurement example.

- **Plant height (PLAHE, cm):** Plant height should be registered from the tip of the plant to the ground level using a measuring tape placed in parallel to the main stem. This evaluation should be done once immediately after hilling, and two times after drought initiation. (Fig. 6)

Fig. 6 Proper potato PLAHE measurement.

- **Canopy reflectance (CR_NDVI):** Normalized Difference Vegetation Index (NDVI) is calculated as a value between 0 to 1 resulting from the relation of visible and near-infrared light reflected by vegetation, and it is used to estimate biomass and changes in leaf water content. Relative vegetation index (reflectance at 800 nm/reflectance at 650 nm) and normalized difference vegetation index (NDVI) ([reflectance at 800 nm−reflectance at 650 nm]/[reflectance at 800 nm + reflectance at 650 nm]) strongly correlates with leaf area index and biomass in potato. Greenseeker® is one of the most practical hand-held sensors for NDVI measurements to evaluate complete plots on foot. To take measurements, the Greenseeker® must be placed perpendicular to the canopy being careful of not creating shadow below the sensor. This evaluation should be done once before drought initiation (right after hilling) and at least 2 times after drought initiation. For better results, it is recommended to evaluate this trait using high resolution NDVI cameras attached to flying drones.

Fig. 6 Greenseeker® equipment. a) Correct usage during field evaluation; b) perpendicular view of the equipment.

- **Canopy temperature Depression (CTD):** The change of canopy temperature in relation to environmental air temperature is known as canopy temperature depression (CTD). This trait is an indication of how capable is transpiration to reduce leaf temperature under a demanding environmental load (since a major role of transpiration is leaf cooling); moreover, CTD is an easily measured manifestation of crop metabolic and physiologic response to the environment and has been recognized as an indicator of overall plant water status and used in such practical applications such as evaluation of plant response to environmental stress, irrigation scheduling, cultivar comparison for water use and tolerance to heat and drought. In order to measure the temperature deviation of plant canopies in comparison to ambient temperature, it is necessary to ensure integration of most of the leaves and coverage of different regions of the plot. It is recommended to have an elevated place to stand above the field level to take several overlapping images that cover the whole experiment (Fig. 7a). The images should be taken between 9:30 hrs to 15:30 hrs on sunny days without wind, in high air temperature and low relative humidity; the emissivity value should be set at 0.97. The images should be analyzed using FLIR Reporter Professional Software.

$$CTD = \frac{air\ temperature\ [Ta]}{canopy\ temperature\ [Tc]}$$

CTD is positive when the canopy is cooler than the air.

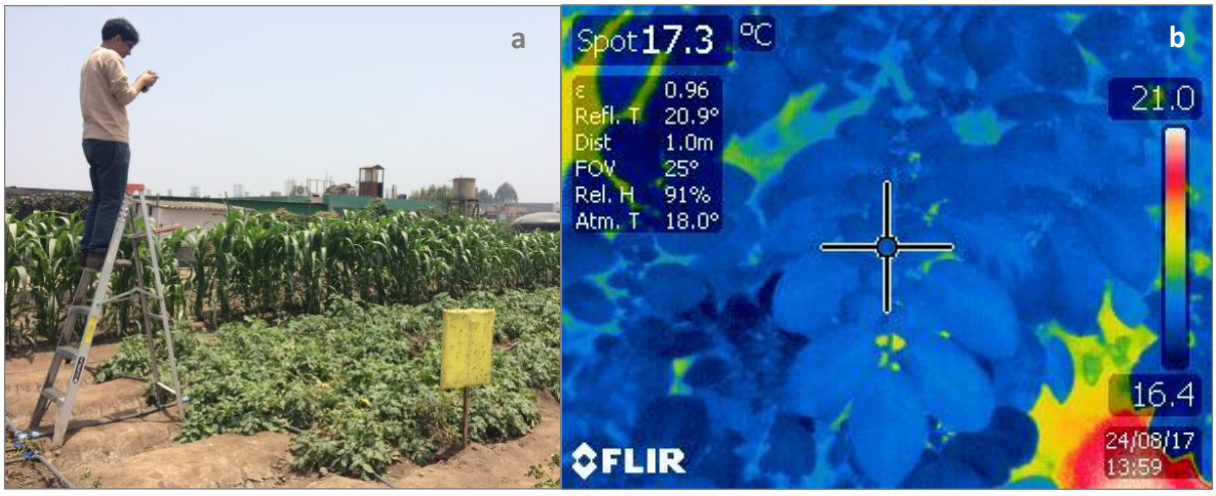

Fig. 7 Thermal imaging. a) Recommended distance for thermal imaging. b) Thermal picture example, note that the information in the superior left corner must be manually updated in the camera every 30 to 60 minutes.

- **Chlorophyll content index (ChlSPAD, SPAD units):** Chlorophyll content index is measured using SPAD 502 Plus Chlorophyll meter (Konica Minolta). It measures the leaf absorbance in the red and near-infrared spectrums. Using these two absorbance values, the equipment calculates a numerical value denominated SPAD (Soil Plant Analyzer Development), this value is proportional to the amount of chlorophyll content in 6 cm^2 of the plant leaf. SPAD reading should be taken from 3 leaflets per plant at the third fully develop mature leaf. This evaluation should be done once before drought initiation and at least 2 times after drought initiation.

Fig. 8 SPAD equipment. Showing the correct position of usage.

- **Leaf Area (LFA, cm2):** To measure this trait 3 leaflets are placed in a fabricated picture table made of a white or black clipboard with a squared plastic 2cm^2 red sticker centered at 2 to 4cm of the bottom (Fig. 9). Pictures must be taken with a cellphone or a digital camera perpendicularly to the picture table at a distance in which only the clipboard background, the leaflets and the red square are displayed. After taking pictures, the complete set of pictures can be processed using the EasyLeafArea program (Easlon & Bloom, 2014) (https://github.com/heaslon/Easy-Leaf-Area/archive/master.zip). It is recommended to use the program before taking pictures in the field to test the correct form for taking pictures.

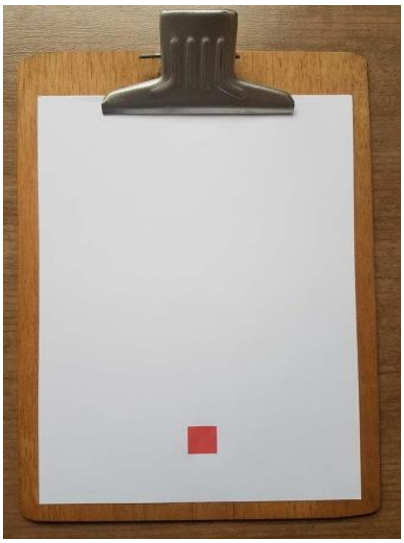

Fig. 9 Example of a fabricated picture table for determination of Leaf Area.

Harvest traits

- **Number of plants harvested (NPH):** The number of plants taken in consideration for harvest should be counted and recorded.

- **Total number of tubers/plant (TNTPL):** The number of tubers for each plant should be counted in all treatments. Only tubers that have twice the diameter of the stolon where they are inserted at the stem are qualified to be considered.

- **Total tuber fresh weight (TTFW, g):** Tubers must be stored in plastic nets and weighted with a hand-held digital balance. Digital balances with multiple unit choices must be established in gram and should be monitored constantly, since this may lead to multiple mistakes.

- **Fresh and dry weight of non-tuber organs:** The total weight (g) of each non-tuber plant organ must be recorded using an analytical balance. For better management purposes, it is recommended to weight and store the total plant biomass in 2 groups: 1) leaves and stems must be stored in a single labeled Kraft bag, which must be weighted for total aerial part fresh weight/plant (APFW, g); the resulting weight must be written in the same bag with a blue pen; 2) roots and stolons should be processed in the same way as leaves and stems. It is highly important to use the same size of Kraft bags for all samples in order to tare the bag at the end of the procedure deducting an average Kraft bag weight from all values; It is strongly recommended not to tare bag when weighing fresh and dry, otherwise this might cause a systematic error.

  After Harvest, all bags must be dried in an oven for 2 days at 80°C or 4 days at 65°C; for further Aerial part dry weight/plant (APDW, g) calculation, all dried materials must be weighted as soon as possible to avoid humidification, and the result must be written in the same bag with a red pen. All information must be typed in the digital field book as soon as possible.

- **Fresh and dry weight of tubers:** For each evaluated plant, around 200 g of chopped tubers are prepared as the fresh weight of a tuber sample (FWTS, g) to calculate dry matter content (DMC). The tuber sample must be stored in a labeled Kraft bag to start the drying process in an oven for 4 days at 80°C. All dried samples must be weighted to evaluate the dry weight of the tuber sample (DWTS, g) as soon as possible to avoid humidification; the resulting value must be written in the same bag with a red pen.

4. Data management and statistical analysis

Calculated variables

These traits are obtained using mathematical calculations based on previous evaluated traits.

- **Slopes:** The slopes will only be calculated in traits evaluated in different points through time. This trait could be calculated either using Microsoft Excell "=slope()" function with DAPs as time values, or using the formula below:

$$TRAIT\_SLP = \frac{\sum(DAP\_EV - DAP\_AV)(TRAIT\_EV - TRAIT\_AV)}{\sum(DAP\_EV - DAP\_AV)^2}$$

Being:

DAP_EV: number of DAP during an evaluation

DAP_AV: average DAP when evaluations were realized

TRAIT_EV: value obtained for a trait in an evaluation

TRAIT _AV: average value of evaluations of a specific trait

- **Dry Harvest index (HI_Dry):** This trait is used in agriculture to quantify the yield of a crop species versus the total amount of biomass that has been produced. Generally, this value is expressed in percentage (Cabello *et al.*, 2014).

$$dHI = \frac{TTDW}{TBDW} * 100$$

- **Drought indices:** Drought indices based on yield loss under stress conditions in comparison to normal state have been used for screening drought tolerant genotypes.

  - ✓ **Drought tolerance index (DTI):** It is a useful tool for determining high yield and stress tolerance potential of genotypes (Fernandez, 1992). Rosielle and Hamblin (1981) have demonstrated that a lower stress tolerance index is a result of a close yield between normal irrigation and drought treatments, which is interpreted as drought tolerance.

$$DTI = \left(\frac{TTWP_p}{\overline{GMTTWP_p}}\right)\left(\frac{TTWP_s}{\overline{GMTTWP_s}}\right)\left(\frac{\overline{GMTTWP_s}}{\overline{GMTTWP_p}}\right) = \frac{(TTWP_p)(TTWP_s)}{\left(\overline{GMTTWP_p}\right)^2}$$

Being:

$TTWP_p$ = TTWP of a given genotype in a non-stress environment;

$TTWP_s$ = TTWP of a given genotype in a stress environment;

$\overline{GMTTWP_p}$ = great mean of TTWP in non-stress environment; and

$\overline{GMTTWP_s}$ = great mean of TTWP in stress environment.

The higher the value of DTI for a genotype, the higher its stress tolerance and yield potential.

- ✓ **Drought susceptible index (DSI):** Fischer and Maurer (1978) suggested the DSI for measurement of yield stability in variable environments. In spring wheat cultivars, Guttieri *et al.* (2001) using DSI, suggested that a DSI values above or equal to 1 indicates susceptibility to drought stress.

$$DSI = \frac{1 - \left(\frac{TTWP_s}{TTWP_p}\right)}{SI} = \frac{1 - \left(\frac{TTWP_s}{TTWP_p}\right)}{1 - \left(\frac{\overline{GMTTWP_s}}{\overline{GMTTWP_p}}\right)}$$

The smaller the value of DSI, the greater is the stress tolerance. Selection based on DSI favors genotypes with low yield potential and high yield under stress conditions. In plants exposed to severe drought, DSI has been found to be around 0.7, which works as a good differentiating pattern between tolerant and non-tolerant plants.

Statistical analysis

All Data content in the digital field book must be examined and cleaned to obtain consistent data prior Statistical analysis. In this section, the statistical analysis methodology using R is explained. Note this section gives explicit instructions of what to do with the codes.

- **Pre-analysis:** Before using the codes presented in this brief methodology description, the following R packages must be loaded:

- Matrix
- Lme4
- Readxl
- st4gi
- scales
- lattice
- ggplot2
- gridExtra
- tidyr
- factoextra
- MASS
- klaR
- plyr
- reshape2
- polycor
- survival
- Formula
- Hmisc
- Caret
- Tcltk
- Tkrplot
- Sp
- SpatialEpi
- Biotools
- randomForest
- RcolorBrewer

In order to load the packages mentioned above, use the following codes to install missing packages not added by default in R:

```
install.packages(c('Matrix', 'lme4',"devtools", 'readxl', 'scales', 'ggplot2', 'lattice', 'gridExtra', 'tidyr', 'factoextra', 'MASS', 'klaR', 'plyr', 'reshape2', 'polycor', 'survival', 'Formula', 'Hmisc', 'caret', 'tcltk', 'rpanel', 'tkrplot', 'sp', 'SpatialEpi', 'biotools', 'randomForest', 'RColorBrewer'))

devtools::install_github("reyzaguirre/st4gi")
```

- **Descriptive statistics:** The following code will create a data matrix in which every trait will be displayed in the first column followed by the minimum, maximum, mean and standard deviation values in subsequent columns with their respective order.

```
# Code to Load the field book: the names in red must be changed for the address and name of the field book which will be used.

setwd('C:/Users /Dropbox/data_analysis/drought/Hidap')

temp1 <- data.frame(read_excel("PTDrought022217_ICA.xlsx", 1, na = "NA"))


# Code to Re-order the complete data matrix to 3 columns: Treatment, Trait, and Values. All evaluations, and values of each evaluation will appear right next to the original Treatment column.

temp2 <- temp1[,!names(temp1) %in% c("ORD", "BLOCK", "PLOT", 'FACT',"CIPNUMBER")] #type every column title which is not a trait or a treatment

melted <- melt(temp2, id.vars=c("TREAT"))

melted <- na.omit(melted)


# Code to calculate values for every trait by each treatment, and to generate a data matrix with the results.

temp2 <- ddply(melted, c("TREAT", "variable"), summarise,

      min = min(value),

      max = max(value),

      mean = mean(value),

      sd = sd(value))
```

- **Correlations analysis:** To obtain a better idea of how the data behaves within evaluated traits, Spearman correlation analysis is done to see if there is any unexpected behavior or to eliminate collinearity. The following codes will create a data matrix of every trait correlation by a selected treatment.

```
# Code to load the field book – the names in red must be changed for the address and name of the field book which will be used.

setwd('C:/Users /Dropbox/data_analysis/drought/Hidap')

temp1 <- data.frame(read_excel("PTDrought022217_ICA.xlsx", 1, na = "NA"))
```

```
# Code to order the data frame and to enumerate rows according to “ORD” column, leaving only the treatment column and all which correspond to traits.

temp2 <- temp1[,!names(temp1) %in% c("BLOCK", "PLOT", 'FACT',"CIPNUMBER")]

temp2 <- temp2[,-1]

rownames(temp2) <- temp1[,1] #note “ORD” column must be first in the field book


# Code to select one treatment, to erase the treatment column, and to omit missing values.

temp2 <- temp2[temp2$TREAT == "TD",] #letters in red must be changed for the desired treatment, as shown in the field book.

temp2 <- temp2[,-1]

temp2 <- na.omit(temp2)


# Code to rearrange the data matrix, to calculate each trait correlation for the chosen treatment, and to save the results in a data matrix called “correlations1”.

flattenCorrMatrix <- function(cormat, pmat) {
 ut <- upper.tri(cormat)
 data.frame(
  row = rownames(cormat)[row(cormat)[ut]],
  column = rownames(cormat)[col(cormat)[ut]],
  cor  =(cormat)[ut],
  p = pmat[ut]
 )
}

res<-rcorr(as.matrix(temp2))

correlations1 <- flattenCorrMatrix(res$r, res$P)
```

- **Lineal Discriminant Analysis (LDA):** Since a clone’s behavior under water-stress that has similar behavior as the same clone over Normal Irrigation treatment (NI) can be interpreted as a tolerant behavior, the predictive capacity of a group of traits to classify a new clone in different treatment outputs is an important evaluation to interpret tolerant behaviors, and it can be analyzed through LDA.

```
# Code to load the field book and to eliminate missing values– the names in red must be changed for the address and name of the field book that will be used.

setwd('C:/Users /Dropbox/data_analysis/drought/Hidap')
```

```
temp1 <- data.frame(read_excel("PTDrought022217_ICA.xlsx", 1, na = "NA"))

temp2 <- na.omit(temp2) #Omitir NAs#

# Traits which have 0 by default in some cases must be fixed in order to run this code; otherwise, a complete row of information might be erased.


# Code to generate a Train and Test data set

set.seed(1)

intrain <- sample(nrow(temp2), round(0.50*nrow(temp2)))

train <- temp2[intrain, ]

test <- temp2[-intrain, ]


# Code to calculate the predictive value of a group of selected traits.

lda1cv <- lda(as.factor(TREAT)~ SD_110DAP + TTFW + CR_90DAP, data=temp2, CV = T)

sum(diag(prop.table(table(temp2$TREAT, lda1cv$class))))

lda1train <- lda(as.factor(TREAT)~ SD_110DAP + TTFW + CR_90DAP, data=temp2, subset = intrain, CV = F)

sum(diag(prop.table(table(test$TREAT, predict(lda1train, temp2[-intrain, ])$class))))
```

Lineal Discriminant Analysis Plot: In order to visualize the result of the discrimination capacity of a group of selected traits, the LDA is plotted as follows.

```
# Code to load the field book and to omit missing values – the names in red must be changed for the address and name of the field book that will be used.

Take in consideration that omitting missing values of traits that might have 0 by default will erase a complete row of values.

setwd('C:/Users /Dropbox/data_analysis/drought/Hidap')

temp1 <- data.frame(read_excel("PTDrought022217_ICA.xlsx", 1, na = "NA"))

dallpc <- na.omit(dallpc) #Omitir NAs#


# Code to generate colors and categorical order for ordered display

# http://research.stowers.org/mcm/efg/R/Color/Chart/index.htm # see this link to choose different colors

myColors <- colors()[c(258, 144, 75)]

dallpc$TREAT <- factor(dallpc$TREAT, levels=c("NI", "REC", "TD"))

names(myColors) <- levels(dallpc$TREAT)
```

```
colScale <- scale_colour_manual(name = "Treat",values = myColors)


# Code to generate a Train and Test data set

set.seed(1)

intrain <- sample(nrow(temp2), round(0.50*nrow(temp2)))

train <- temp2[intrain, ]

test <- temp2[-intrain, ]


# Code to Plot the discrimination coefficients in a bi-dimensional graph.

 # Note that traits written in red must be replaced for the selected traits of interest.

lda2train <- lda(as.factor(TREAT)~ DSI_a + SD_Slp + ChCI_Slp + CR_Slp, data=dallpc, CV = F)

prop.lda =  lda2train$svd^2/sum(lda2train$svd^2)

plda <- predict(object = lda2train, newdata = dallpc)

dataset = data.frame(Stress = dallpc["TREAT"], lda = plda$x)

p1.1 <- ggplot(dataset) + geom_point(aes(lda.LD1, lda.LD2, colour = Stress), size = 2) + xlim(-4, 4) + ylim(-
5, 1) +

  labs(x = paste("LD1 (", percent(prop.lda[1]), ")", sep=""),

       y = paste("LD2 (", percent(prop.lda[2]), ")", sep="")) +

  ggtitle('Linear Discriminant analysis\nTraits: DSI, SD_Slp, ChCI_Slp, CR_Slp') +

  theme(plot.title = element_text(hjust = 0.5, size = 10))

grid.arrange(p1.1 + colScale)
```

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