Demo yeast mutant analysis

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February 20, 2018 - (Update November 2020)

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1 Analysis of yeast growth data

Based on webinar by Dr. Jeremy Chacon Beginners Introduction to R Statistical Software

The mock yeast experiment table used in the webinar (yeast_example.txt) can be obtained from this short link: https://go.wisc.edu/mc5d52

1.1 Set working directory

It is always good practice to keep projects within a separate directory.

Change directory to the one on the desktop with setwd() and verify with getwd(). This command assumes that the directory exists already. Create it on your computer first if necessary, and download the yeast_example.txt (see above) within it.

setwd("~/Desktop/R_intro_2018/Yeast_demo")
getwd()

https://bitesizebio.com/webinar/beginners-introduction-to-r-statistical-software/
1.2 List all files in directory

```r
list.files()
```

```
[1] "Demo_yeast_files"    "Demo_yeast.docx"
[3] "Demo_yeast.html"     "Demo_yeast.md"
[7] "Demo_yeast.Rmd"      "mystyles.docx"
[9] "RStudio_yeast_demo.Rproj" "yeast_example.md"
[11] "yeast_example.txt"   "yeast_example.xlsx"
```

*Note:* the command `dir()` would give the same result.

1.3 List “txt” files and read data

List *.txt* files within the directory with either `list.files()` or `dir()` specifying the pattern searched:

```r
dir(pattern = ".*\txt")
```

```
[1] "yeast_example.txt"
```

Read data, specifying that the first line is a header, into variable named `yeast_eg`

```r
# yeast_eg = read.table('yeast_example.txt', header=T)
# Update due to change in R 4.0.x
yeast_eg = read.table('yeast_example.txt', header = T, stringsAsFactors = T)
```

2 Examine data

The first 6 lines of the data look like this:

```r
head(yeast_eg)
```

```
genotype   drug     treatment       OD_change
1           WT none      WT_no_drug      3.2
2           WT none      WT_no_drug      2.8
3           WT none      WT_no_drug      3.1
4           WT none      WT_no_drug      3.3
5           WT none      WT_no_drug      2.6
6           WT nocodazole WT_nocodazole 1.2
```

During an interactive session the following command will open a spreadsheet-like tab or window showing all the data in tabular format.

```r
View(yeast_eg)
```

The structure and summary of the data look like this:

```r
str(yeast_eg)
```

```
'data.frame': 20 obs. of 4 variables:
$ genotype   : Factor w/ 2 levels "mad2_del","WT": 2 2 2 2 2 2 2 2 2 2 ...
$ drug        : Factor w/ 2 levels "nocodazole","none": 2 2 2 2 1 1 1 1 1 1 ...
```
Optionally we can also create a nice looking table with some added command (that may require loading additional \texttt{R} packages, so it does not work now that’s OK.) Here is the complete dataset within the table:

\begin{verbatim}
library(knitr)
kable(yeast_eg)
\end{verbatim}

\begin{verbatim}
<table>
<thead>
<tr>
<th>genotype</th>
<th>drug</th>
<th>treatment</th>
<th>OD_change</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>none</td>
<td>WT_no_drug</td>
<td>3.2</td>
</tr>
<tr>
<td>WT</td>
<td>none</td>
<td>WT_no_drug</td>
<td>2.8</td>
</tr>
<tr>
<td>WT</td>
<td>none</td>
<td>WT_no_drug</td>
<td>3.1</td>
</tr>
<tr>
<td>WT</td>
<td>none</td>
<td>WT_no_drug</td>
<td>3.3</td>
</tr>
<tr>
<td>WT</td>
<td>none</td>
<td>WT_no_drug</td>
<td>2.6</td>
</tr>
<tr>
<td>WT</td>
<td>nocodazole</td>
<td>WT_nocodazole</td>
<td>1.2</td>
</tr>
<tr>
<td>WT</td>
<td>nocodazole</td>
<td>WT_nocodazole</td>
<td>1.5</td>
</tr>
<tr>
<td>WT</td>
<td>nocodazole</td>
<td>WT_nocodazole</td>
<td>1.3</td>
</tr>
<tr>
<td>WT</td>
<td>nocodazole</td>
<td>WT_nocodazole</td>
<td>1.9</td>
</tr>
<tr>
<td>WT</td>
<td>nocodazole</td>
<td>WT_nocodazole</td>
<td>0.7</td>
</tr>
<tr>
<td>mad2_del</td>
<td>none</td>
<td>mad2_del_no_drug</td>
<td>2.7</td>
</tr>
<tr>
<td>mad2_del</td>
<td>none</td>
<td>mad2_del_no_drug</td>
<td>2.9</td>
</tr>
<tr>
<td>mad2_del</td>
<td>none</td>
<td>mad2_del_no_drug</td>
<td>3.0</td>
</tr>
<tr>
<td>mad2_del</td>
<td>none</td>
<td>mad2_del_no_drug</td>
<td>2.5</td>
</tr>
<tr>
<td>mad2_del</td>
<td>none</td>
<td>mad2_del_no_drug</td>
<td>3.1</td>
</tr>
<tr>
<td>mad2_del</td>
<td>nocodazole</td>
<td>mad2_del_nocodazole</td>
<td>2.2</td>
</tr>
<tr>
<td>mad2_del</td>
<td>nocodazole</td>
<td>mad2_del_nocodazole</td>
<td>2.4</td>
</tr>
<tr>
<td>mad2_del</td>
<td>nocodazole</td>
<td>mad2_del_nocodazole</td>
<td>2.9</td>
</tr>
<tr>
<td>mad2_del</td>
<td>nocodazole</td>
<td>mad2_del_nocodazole</td>
<td>2.5</td>
</tr>
<tr>
<td>mad2_del</td>
<td>nocodazole</td>
<td>mad2_del_nocodazole</td>
<td>2.7</td>
</tr>
</tbody>
</table>
\end{verbatim}

3 Data exploration

3.1 Accessing column data

Accessing specific columns in the data table can be done in 2 ways:

- Using the \$ sign between the name of the dataset and the name of the column. For example: \texttt{yeast_eg}\$genotype
- The \texttt{with()} function allows a more elegant writing. The first argument is the dataset, here \texttt{yeast_eg}. The second command will be typically be a function into which is specified the name of the column to use. For example: \texttt{with(yeast_eg,summary(genotype))}.
3.1.1 Exploratory plots

The following command will plot the genotype on the horizontal x axis and the OD change on the vertical y axis:

```
with(yeast_eg, plot(genotype, OD_change))
```

![Plot of genotype vs. OD change]

Note: Using the `$` nomenclature would create the exact same plot: `plot(yeast_eg$genotype, yeast_eg$OD_change)`.

We can observe that the OD change is higher, on average for `mad2_del` as indicated by the thick line within the box representing the median.

Thus for now it appears that the growth rate is greater in `mad2_del` even when we add the drug `nocodazole` which should stop the cells from growing.

But to confirm this hypothesis we need to look at the data a few different more ways.

We can now look at the effect of the drug on the OD change.

```
with(yeast_eg, plot(drug, OD_change))
```
Now we see on the plot that the growth rate is lower in the presence of the drug *nocodazole* compared to no drug added. This suggests that treatment worked as expected as the drug on average should stop growth rate.

The *treatment* variable, the 3rd column of data, summarizes all 4 treatments into one variable (genotype and presence of drug). This is making plotting easier to see all treatments on the same plot.

```r
with(yeast_eg, plot(treatment, OD_change, las=3))
```

*Note:* the rotating command (*las*) can alter the style of axis labels: 0=parallel, 1=all horizontal, 2=all perpendicular to axis, 3=all vertical. If labels are horizontal and not showing, simply extend the size of the display with the mouse.
The plot indicates that:

- WT cells without drug grow very fast.
- WT cells with drug grow much more slowly, as expected.
- mutant cells grow faster in absence of drug.
- mutant cells grow almost as fast in presence of drug.

Therefore for mutants cells this suggest that the drug is not causing the cell cycle to stop as we expect for the WT cells further suggesting that gene mad2 may be important for cell cycle rest.

BUT can we trust our eyes with this plot?
Are the differences we see real?
The next step is to perform some statistics.

4 Statistical test: two-way ANOVA

4.1 Calculate ANOVA

We can do a 2-way ANOVA because we have a continuous response variable (OD_change) and 2 factors (genotype and drug treatment) and we want to look at the interaction between those 2 factors.

In summary we want to know if the effect of drug differs depending on the genotype.

R was created as a statistical language and therefore it is easy to calculate an ANOVA with the aov() function. However, it is necessary to know how to tell R the model formulation for the experiment.

 response ~ predictor1 + predictor2

- ~: separates response from predictor
- +: adds more predictors

The formula with + would give answer for main effect.

To specify interaction use * to specify all possible interactions and main effects:

 response ~ predictor1 * predictor2

For our experiment we would want the main effects and also the interactions between the predictors.

In our case we would write the formula with the response as OD_change separated by tilde ~ and then genotype as the first main effect, then asterisk * and then drug which is the second main effect.

By using the asterisk * the ANOVA results will include both main effects and the interactions between the 2 factors.

The ANOVA result is stored in variable m1 specifying interactions between genotype and drug written in the format using the with() function:

\[
\text{m1} \leftarrow \text{with(yeast_eg, aov(OD_change ~ genotype * drug))}
\]

We can look at the ANOVA results:

m1

Call:

 aov(formula = OD_change ~ genotype * drug)

Terms:

   genotype  drug  genotype:drug Residuals
Sum of Squares 1.4045 4.9005 2.3805 1.6320
Deg. of Freedom 1 1 1 16
Residual standard error: 0.3193744
Estimated effects may be unbalanced

However the ANOVA table itself has more information. It is obtained with the `summary()` function:

Show ANOVA summary:

```
summary(m1)
```

<table>
<thead>
<tr>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>genotype</td>
<td>1</td>
<td>1.405</td>
<td>1.405</td>
<td>13.77</td>
</tr>
<tr>
<td>drug</td>
<td>1</td>
<td>4.901</td>
<td>4.901</td>
<td>48.04</td>
</tr>
<tr>
<td>genotype:drug</td>
<td>1</td>
<td>2.380</td>
<td>2.380</td>
<td>23.34</td>
</tr>
<tr>
<td>Residuals</td>
<td>16</td>
<td>1.632</td>
<td>0.102</td>
<td></td>
</tr>
</tbody>
</table>

---

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The table shows the degrees of freedom (Df.), the sum of squares (Sum Sq.), the mean square (Mean Sq.), the F-statistic (F value,) and the p-value (Pr>(F)). At the bottom the Signif. codes provides a level of statistical significance for the results highlighted in the table next to the p-value.

The p-value indicates that the genotype has a significant effect on growth rate, as expected, since we observed that on average the WT grows slower than the mutant because it got arrested in its growth with drug.

We also see a significant of the drug, as indeed the drug on average decreases the growth rate of the cells.

Importantly, we see a significant interaction between the genotype and the drug. While WT cells stopped growing when we added the drug, the mutant mad2_del cells did not stop growing with the drug added.

### 4.2 Plot ANOVA

It is important to check the model assumptions. This can be done visually with the plot command on the ANOVA model.

```r
par(mfrow=c(2,2))
plot(m1)
```
par(mfrow=c(1,1))

The parameter function `par()` instructs how to split the plot area in rows and columns, and how many of which. Here we’ll split in 4 quadrants: 2 rows and 2 columns. After the plot the area is reset to a single spot.

5 Publication quality plots

5.1 Installation of ggplot

The package `ggplot` is a modern package that makes beautiful plots. `gg` stands for “grammar of graphics.”

It may be necessary to install the package on your computer. The following command will do that, or it can also be done within RStudio with a graphical interface.

```
install.packages('ggplot2')
```

Note: installation other, dependent packages is likely to occur as well.

5.2 Plot with ggplot

The `library()` command will load the requested package and others dependencies.

```
library(ggplot2) # load the package for this session.
```

On the original video, the plot command was shorter for a less fancy plot:

```
ggplot(yeast_eg, aes(x = drug, y = OD_change, color = genotype))+ geom_boxplot()+ theme_bw()+ scale_x_discrete('')+ scale_y_continuous(expression(paste('Growth Rate (', Delta, ' OD600)')))
```
However the `opts` option is no longer supported and an error could occur if run. The only difference is that the legend is outside the plot rather than within the plotting area.

The following code provides a colored fill within the box and separates the boxes so that they do not touch. Some of the background grids are also removed.

```r
ggplot(yeast_eg, aes(x = drug, y = OD_change, fill = genotype)) +
  geom_boxplot(position=position_dodge(1)) +
  theme_bw() +
  scale_x_discrete('') +
  scale_y_continuous(expression(paste('Growth Rate (', Delta, ' OD600)'))) +
  theme(legend.position=c(0.8, 0.35), panel.border = element_blank(), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), axis.line = element_line(colour = "black"))
```

Note: in RStudio the plot will appear within the bottom right quadrant by default. To make the plot appear in a separate window, the following commands can be used depending on the type of computer used. In addition, specific dimensions can be specified in inches.

<table>
<thead>
<tr>
<th>Computer type /OS</th>
<th>Command</th>
</tr>
</thead>
<tbody>
<tr>
<td>Windows</td>
<td>windows</td>
</tr>
<tr>
<td>Macintosh</td>
<td>quartz()</td>
</tr>
<tr>
<td>Linux</td>
<td>x11</td>
</tr>
</tbody>
</table>

In the video the command is `windows(height=3, width=3*1.3)`

6 Saving a plot in PDF

While there are different ways to saving to PDF, artifacts of errors can occur. The Cairo package is a specific package for creating PDF reproducibly.
6.1 Installation of Cairo

If you need to install this package you can run the following command or use the RStudio graphical interface:

```r
install.packages('Cairo')
```

Load the package with:

```r
library(Cairo)
```

Initialize the PDF file. Specified sizes are in inches by default.

```r
CairoPDF(file='growth_rate.pdf', width=3*1.3, height=3)
```

Now CairoPDF is waiting for the plot to be given.

Issue the `ggplot` above command again.

Note that no plot will be visible at that time.

It is then necessary to “close” the PDF file called a “device” hence the name of the function used.

```r
dev.off()
```

7 Conclusions

- This was a complete data analysis in very few lines of code
- The R code is re-usable
- The R code is reproducible
- Essential points to remember:
  - write code in a script
  - run lines by highlighting and the use `control+enter` to run it
  - Get help on functions with `?` or `??`
  - Web search engines can provide many solutions to questions