Technical Report

Bilateral Project: ACIAR-*Establishing sustainable solutions to cassava diseases in mainland South-East Asia.*

Plant Health Initiative WP2: *Validation of Artificial Intelligence tools for disease recognition at large scale, available via a digital platform supporting surveillance and breeding efforts for resistance evaluations*

A protocol for measuring Cassava Bacterial Blight disease progression using machine-learning image analysis

Juan M. Pardo; Rafael Rodriguez; Jenyfer Jimenez and Wilmer J. Cuellar*

[1]*Cassava Crop Protection Team, Cassava Program, Alliance of Bioversity International and CIAT, Colombia*

*Corresponding author: w.cuellar@cgiar.org

Abstract

Cassava (*Manihot esculenta* Crantz), a starchy root crop, is the fourth most important source of dietary carbohydrate in developing countries and important source of industrial raw material in tropical regions especially in southeast Asia (SEA). *Xanthomonas phaseoli* pv. *manihotis* (Xpm) formerly known as *Xanthomonas axonopodis* pv. *manihotis* causes cassava bacterial blight (CBB) disease, a major bacterial disease of cassava worldwide. To improve CBB diagnostics and support breeding for resistance programs, we standardized a screen house protocol for the reliable, simple, space- and time-efficient and high throughput tool based on machine learning image analysis using the PhenotyperCV software. In addition, we sequenced several bacterial isolates using Nanopore technology and their pathogenicity profile was evaluated by inoculation on 3-month old plants, under greenhouse conditions. Disease symptoms were measured to calculate the area under the disease progress curve (AUDPC) as described below. This protocol allows us to identify distinct pathogenic Xpm strains and evaluate CBB at different DPI by performing symptoms measurements in an automated way. AUDPC curves are used for pathogenic characterization of additional isolates and subsequent varietal resistance screenings.

Protocol v1.0:

1. Inoculation: Through infiltration, 3 technical replicates per strain onto the leaf lobe.

2. Take photos (0,4,6,9,12 days post inoculation) image device at the same conditions. use X – Rite Passport color card to gray color correction.


3. Choose clear symptoms to create a collage.

   - Select affected area manually (GIMP or IMAGE J)
   - Create a mask: Convert picture to black and white. Only affected area white background black.

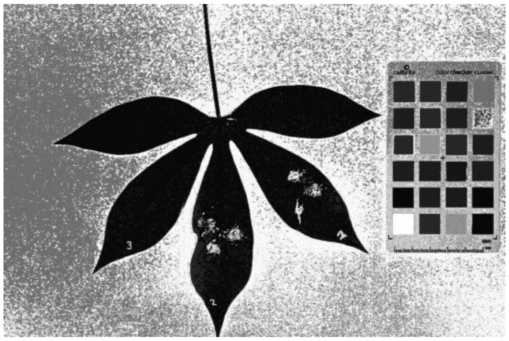
4. Machine training:

   - Use software Phenotyper (Berry et al., 2018).
   - Train naive Bayes or SVM using ML_CREATE specifying classifier type:

```
PhenotyperCV -m=ML_CREATE -method=bayes -i=collage.png -b=mask.png -class=output_bayes_classifier.yaml
```

Check the classifier prediction using either ML_PRED specifying classifier type

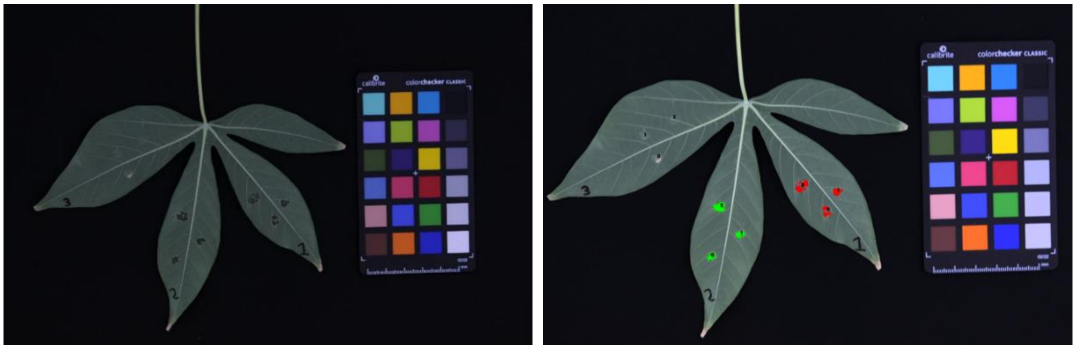
Prediction image: Affected area showed in white:



5. Routine measuring process with Phenotyper:

1. Click only the gray scale color chips from black to white
2. Feature prediction of input image
3. Threshold the prediction to isolate features

Click on the features to measure. If there are different types of features that you'd like to consider separate, currently only three different features are supported and you can specify them by which type of mouse click you use. Left = red, right = green, middle = blue

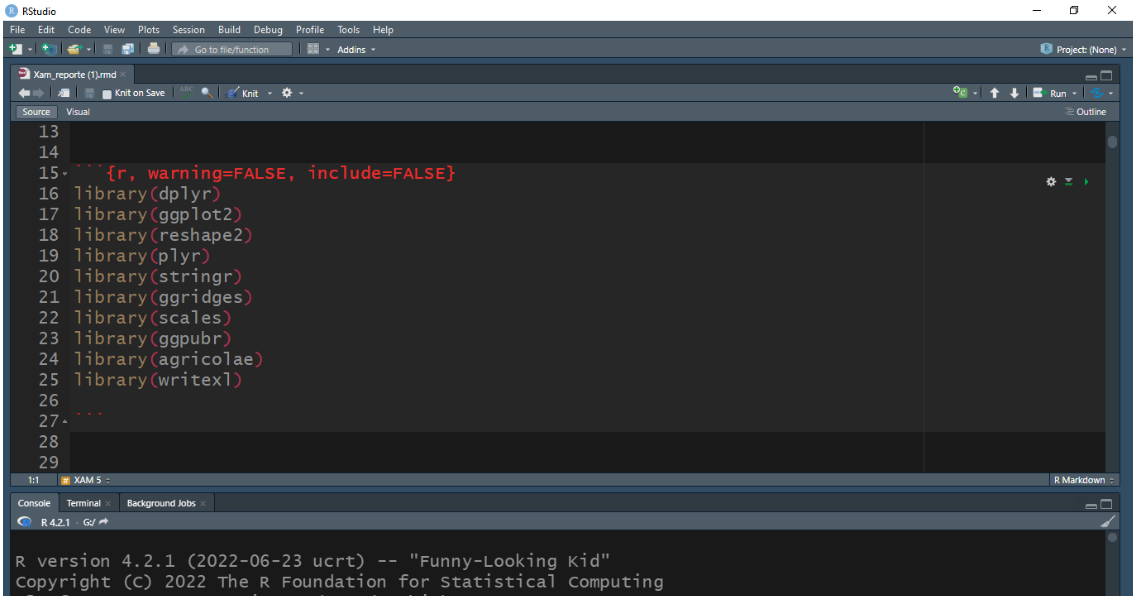


7. Data analysis: The software Phenotyper generate a .txt file:

```
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg blue 0 81 10 11.5 0.869565 12.9515 5 6 673.8 1007.6 6 674 1007.6 5.74247 3.42862 30.5698 0.897703 0.749148 0.597065 1.67486 1.33333 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg blue 1 81 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg blue 2 81 12 6.5 1.84615 7.82843 5 3 719.583 820.917 5 719.178 820.94 5.23667 2.27914 86.017 1.0628 2.46061 0.435228 2.29765 1.15314 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg green 0 81 504 553 0.911392 121.811 29 25 965.754 1065.72 14 965.668 1065.31 27.9984 22.4883 79.9993 0.627378 0.426841 0.803199 1.24502 1.29689 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg green 1 81 825 862.5 0.956522 146.811 33 36 902.778 1001.72 15 902.864 1001.95 33.3967 29.8357 42.6572 0.461794 0.481001 0.893373 1.11935 1.68188 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg green 2 81 771 798 0.966165 134.983 32 34 961.097 954.099 20 961.353 954.2 32.0342 29.1854 11.9139 0.421741 0.53175 0.911067 1.09761 1.66797 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg red 0 81 54 78 0.692308 52.0746 5 24 1151.11 960.389 6 1150.44 959.211 22.6918 5.09641 178.82 1.24532 0.250237 0.224592 4.45251 1.15098 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg red 1 81 78 339.5 0.22975 93.3266 24 25 1167.03 867.679 12 1166.57 871.373 24.1731 20.5387 170.644 0.548361 0.112537 0.84965 1.17696 1.23398 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0167.jpg red 2 81 115 373.5 0.307898 149.535 22 25 1074.9 855.522 13 1074.83 857.033 24.4824 18.6853 14.9891 0.688164 0.0646278 0.763215 1.31025 0.950073 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg blue 0 40 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg blue 1 40 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg blue 2 40 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg green 0 40 179 495 0.361616 181.279 29 26 796.246 863.57 10 793.52 859.761 32.5581 18.4978 141.698 0.929358 0.068449 0.568147 1.76011 1.16478 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg green 1 40 9 4.5 2 6.24264 4 3 720.667 842.889 6 720.604 842.898 3.29837 2.20648 68.6435 0.813682 2.90212 0.668961 1.49486 1.05664 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg green 2 40 8 3 2.66667 5 4 2 783.5 759.5 4 -1 -1 -1 -1 -1 -1 4.02124 -1 -1 1.16992 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg red 0 40 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg red 1 40 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0168.jpg red 2 40 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg blue 0 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg blue 1 41 8 3.5 2.28571 6.41421 3 3 549.125 829.125 5 -1 -1 -1 -1 -1 -1 2.4435 -1 -1 1 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg blue 2 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg green 0 41 350 657.5 0.532319 199.973 31 30 729.466 1066.9 13 729.03 1068.75 28.1828 25.8499 23.1751 0.40688 0.109985 0.917224 1.09025 1.27756 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg green 1 41 388 636.5 0.609584 207.338 29 33 807.722 991.972 11 807.684 992.157 28.4677 21.5138 30.3644 0.69896 0.113418 0.755727 1.32323 1.11349 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg green 2 41 28 22 1.27273 17.6569 6 7 711.393 959 7 711.426 959.096 7.53372 3.94958 146.046 0.975445 1.1286 0.524254 1.90747 1.08048 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg red 0 41 7 2.5 2.8 5.41421 4 2 1080.71 877.571 4 -1 -1 -1 -1 -1 -1 3.0008 -1 -1 1.05664 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg red 1 41 67 178.5 0.37535 72.9331 25 14 1129.49 819.433 8 1120.33 820.772 31.209 12.8562 93.0304 1.08449 0.158283 0.411937 2.42755 0.950073 0
/home/lab/Desktop/Assay6/dpi4/CM681-2/Img0169.jpg red 2 41 67 54.5 1.22936 23.4853 18 5 1044.33 746.582 8 1044.42 746.218 16.5675 6.30232 95.4086 1.11319 1.52649 0.380403 2.62879 1.05708 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg blue 0 25 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg blue 1 25 7 6 1.16667 13.042 5 7 409 535 4 413 540 11.9045 8.14675 8.0445 0.794551 0.517152 0.684344 1.46125 0.792481 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg blue 2 25 16 11 1.45455 13.6569 4 5 545.562 505 6 545.705 505 4.51642 3.59538 0 0.638644 1.07802 0.796067 1.25618 1.23348 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg green 0 25 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg green 1 25 3 6 0.5 25.1868 22 13 776.667 907 3 -1 -1 -1 -1 -1 -1 0.0594273 -1 -1 0.773976 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg green 2 25 253 583.5 0.43359 186.972 31 32 673.162 794.996 13 676.739 794.164 28.3617 24.2753 61.7842 0.53681 0.0909447 0.855918 1.16834 1.21269 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg red 0 25 26 72.5 0.358621 47.2471 16 11 1072.62 689.154 7 1078.81 698.797 27.6534 21.4494 120.493 0.669851 0.146364 0.77565 1.28924 0.864858 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg red 1 25 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0170.jpg red 2 25 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg blue 0 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg blue 1 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg blue 2 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg red 0 41 67 543 0.123389 99.7613 36 23 758.478 965.91 9 747.588 966.396 46.1698 21.2308 83.387 1.03938 0.0845981 0.459842 2.17466 0.96356 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg red 1 41 287 805.5 0.3563 277.259 39 30 817.899 938.317 13 817.395 936.317 33.1018 25.1271 105.938 0.694138 0.0469161 0.759086 1.31737 1.27991 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg red 2 41 513 908.5 0.564667 276.234 40 31 829.187 823.24 18 828.41 822.847 32.757 28.4387 73.1275 0.513478 0.0844834 0.86817 1.15185 1.63197 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg red 3 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg red 4 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0171.jpg red 5 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0172.jpg blue 0 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0172.jpg blue 1 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0172.jpg blue 2 41 0 0 -nan 0 0 0 -nan -nan 0 -1 -1 -1 -1 -1 -1 -nan -1 -1 -nan 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0172.jpg green 0 41 489 888.5 0.550366 230.807 37 35 824.446 981.041 12 825.487 980.225 34.72 28.2242 116.286 0.611703 0.115351 0.81291 1.23015 1.34702 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0172.jpg green 1 41 562 686 0.819242 166.826 37 28 891.609 902.546 10 891.391 902.861 31.7388 23.1851 80.8765 0.734173 0.253758 0.730495 1.36893 1.34255 0
/home/lab/Desktop/Assay6/dpi4/CM4919-1/Img0172.jpg green 2 41 175 514.5 0.340136 179.249 33 29 824.937 867.943 11 821.361 865.07 31.5238 22.9316 54.5708 0.738325 0.0684436 0.727438 1.37469 1.40668 0
```

The measuring value in the .txt file is in term of pixels.

8. The automated statistical analysis is performed using R Studio Software and the report is generated with the R Markdown tool.

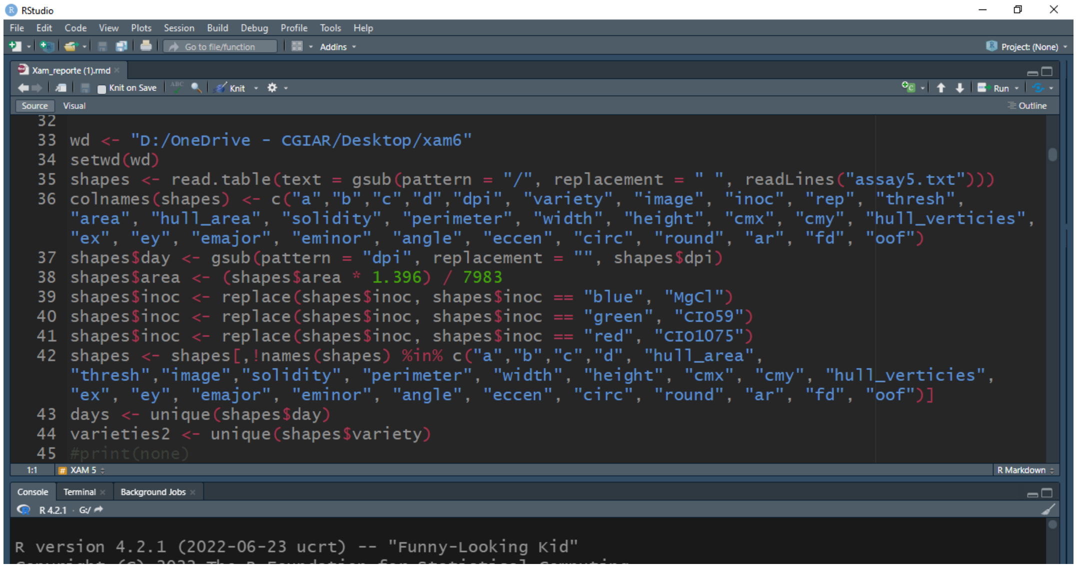


~~~
```{r, warning=FALSE, include=FALSE}
library(dplyr)
library(ggplot2)
library(reshape2)
library(plyr)
library(stringr)
library(ggridges)
library(scales)
library(ggpubr)
library(agricolae)
library(writexl)

~~~

```
R version 4.2.1 (2022-06-23 ucrt) -- "Funny-Looking Kid"
Copyright (C) 2022 The R Foundation for Statistical Computing
```

9. The .txt file generated by Phenotyper should be uploaded in the R Markdown tools. The data could be replaced from pixels to square centimeters; the bacterial strain ID should be assigned using a color code.



```
wd <- "D:/OneDrive - CGIAR/Desktop/xam6"
setwd(wd)
shapes <- read.table(text = gsub(pattern = "/", replacement = " ", readLines("assay5.txt")))
colnames(shapes) <- c("a","b","c","d","dpi", "variety", "image", "inoc", "rep", "thresh",
"area", "hull_area", "solidity", "perimeter", "width", "height", "cmx", "cmy", "hull_verticies",
"ex", "ey", "emajor", "eminor", "angle", "eccen", "circ", "round", "ar", "fd", "oof")
shapes$day <- gsub(pattern = "dpi", replacement = "", shapes$dpi)
shapes$area <- (shapes$area * 1.396) / 7983
shapes$inoc <- replace(shapes$inoc, shapes$inoc == "blue", "MgCl")
shapes$inoc <- replace(shapes$inoc, shapes$inoc == "green", "CIO59")
shapes$inoc <- replace(shapes$inoc, shapes$inoc == "red", "CIO1075")
shapes <- shapes[,!names(shapes) %in% c("a","b","c","d", "hull_area",
"thresh","image","solidity", "perimeter", "width", "height", "cmx", "cmy", "hull_verticies",
"ex", "ey", "emajor", "eminor", "angle", "eccen", "circ", "round", "ar", "fd", "oof")]
days <- unique(shapes$day)
varieties2 <- unique(shapes$variety)
#print(none)
```

```
R version 4.2.1 (2022-06-23 ucrt) -- "Funny-Looking Kid"
```

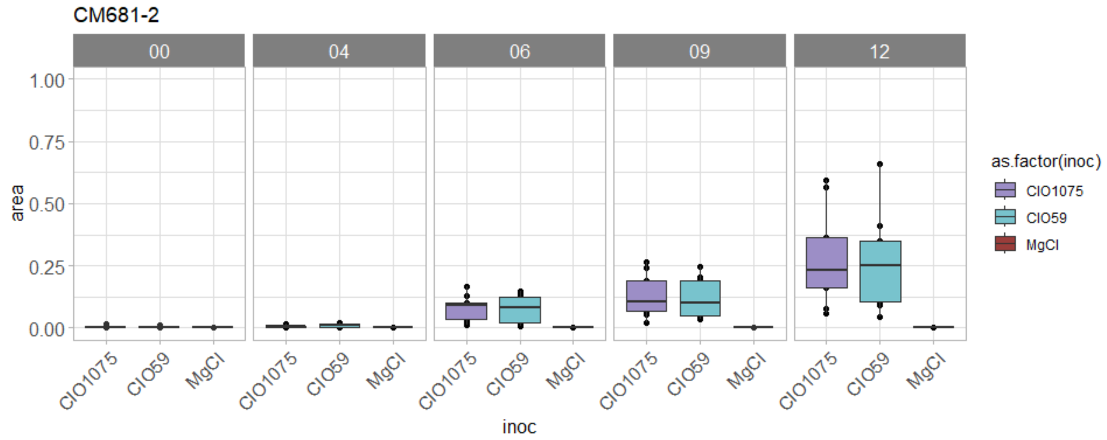
10. The report is generated as a word document as follows:

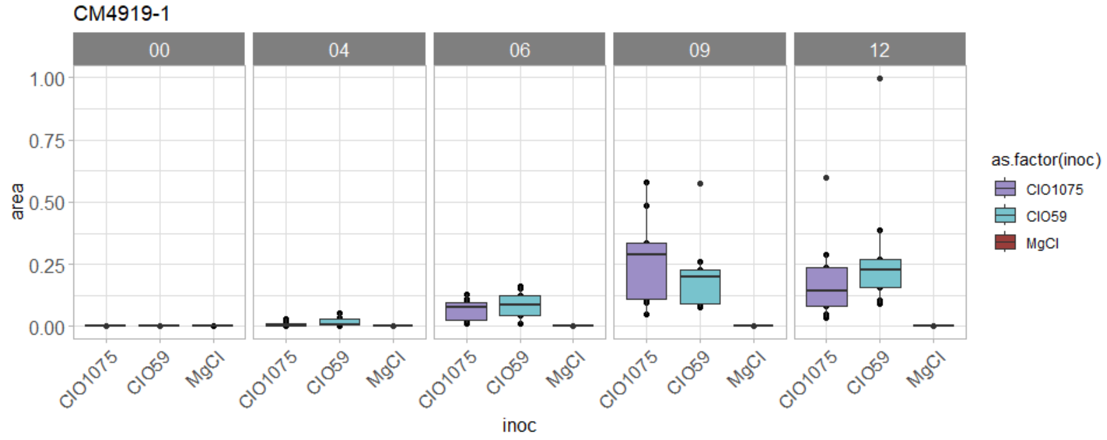
XPM 5

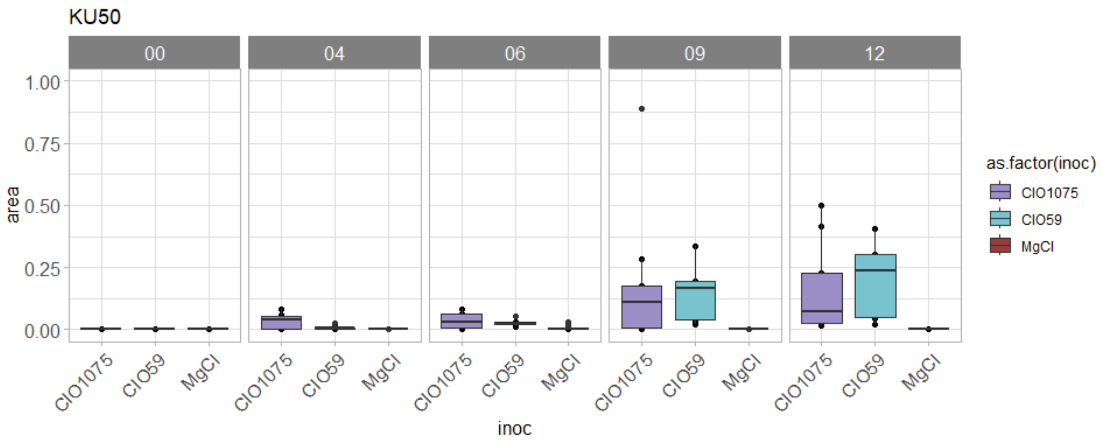
Author: JMP

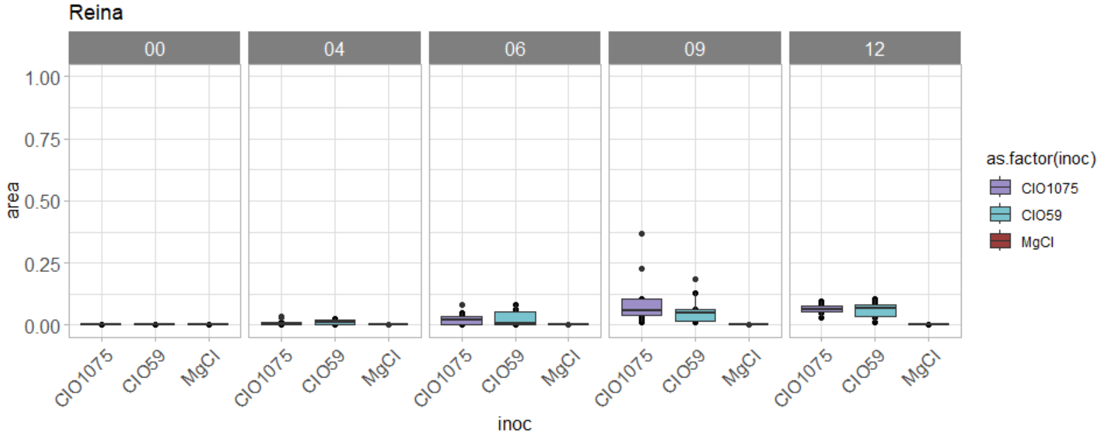
Date: 2023-01-12

Boxplot Areas cm^2 (raw data) – Variety

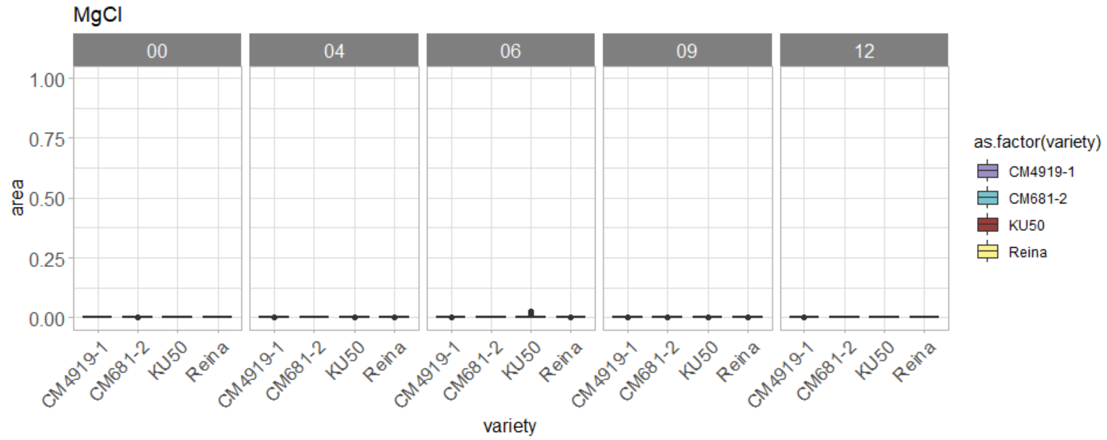


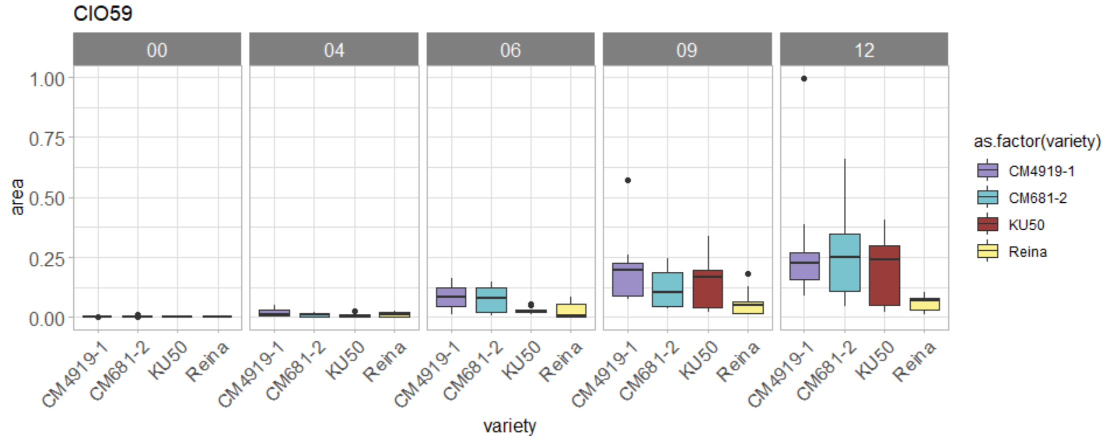


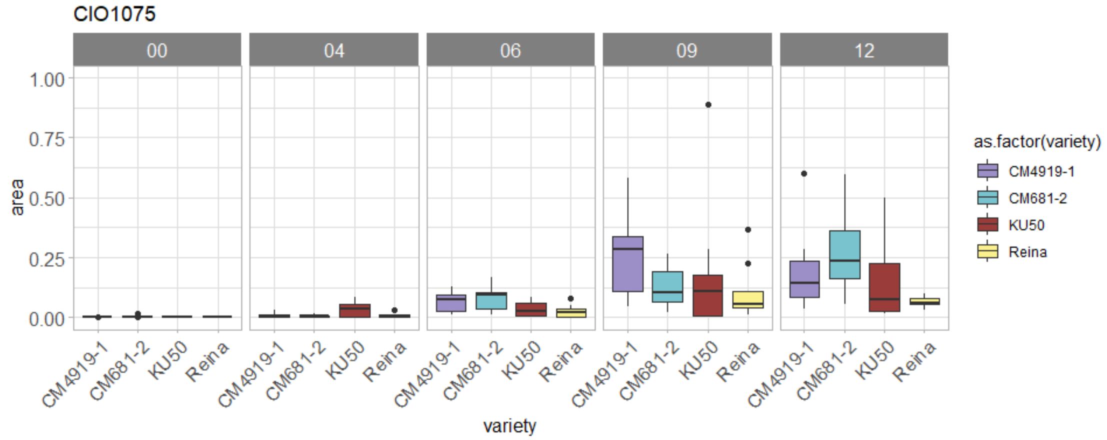

KU50
00
04
06
09
12
area
1.00
0.75
0.50
0.25
0.00
CIO1075
CIO59
MgCl
inoc
as.factor(inoc)
CIO1075
CIO59
MgCl


Reina
00
04
06
09
12
area
1.00
0.75
0.50
0.25
0.00
CIO1075
CIO59
MgCl
inoc
as.factor(inoc)
CIO1075
CIO59
MgCl

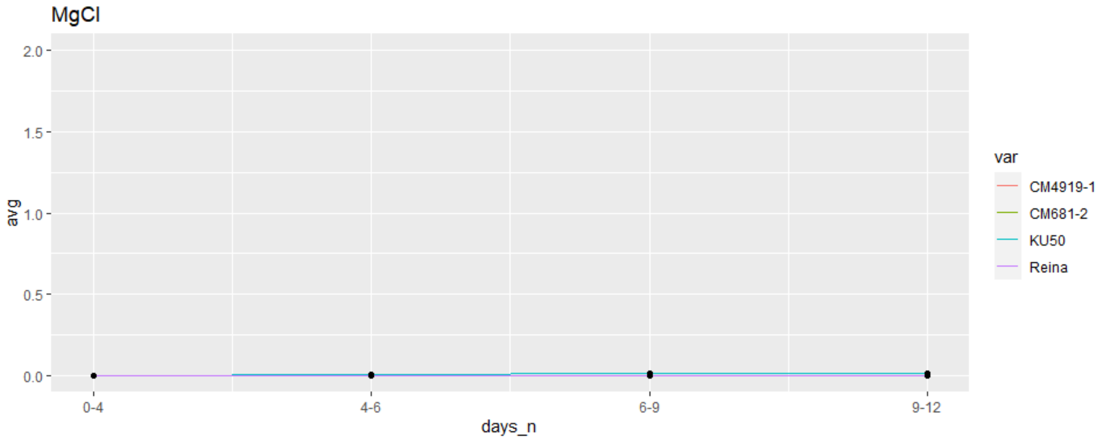
Boxplot Areas cm^2 (raw data) – Strain

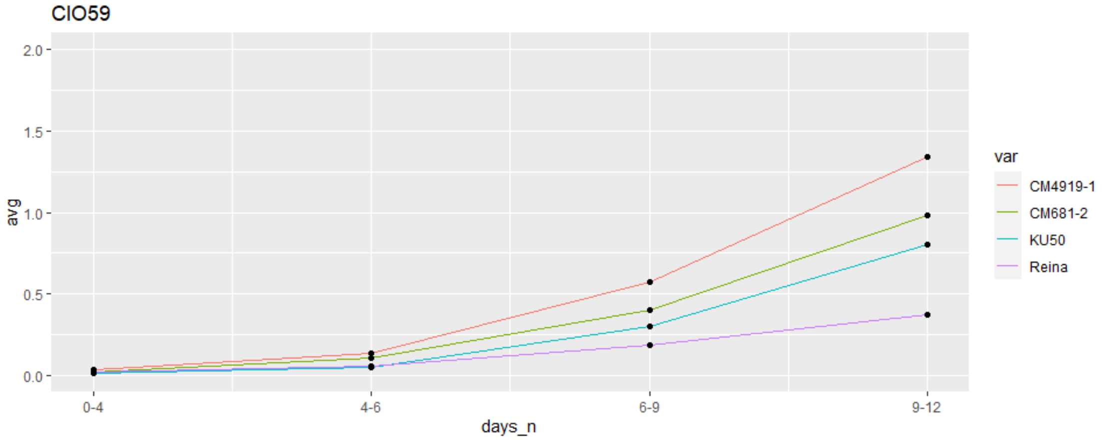


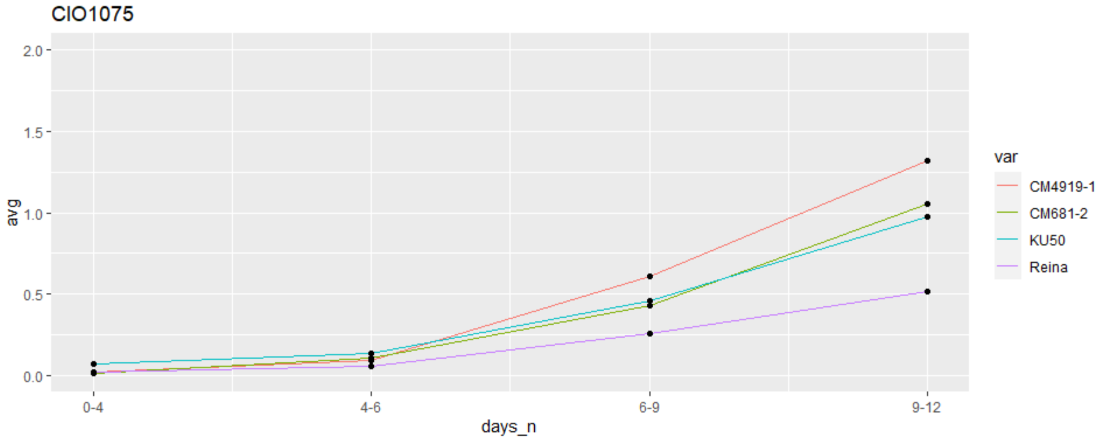




Lines graphics for AUDPC by Strain


MgCl


ClO59


ClO1075

Variance analysis Test

	Df	Sum Sq	Mean Sq
rep	2	0.53798	0.26899

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
inoc	2	20.29297	10.14648	127.55	0.00024
Residuals	4	0.31819	0.07955	NA	NA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
variety	3	4.8847	1.62823	10.3769	0.00001
inoc:variety	6	2.5452	0.42421	2.7035	0.01848
Residuals	90	14.1219	0.15691	NA	NA

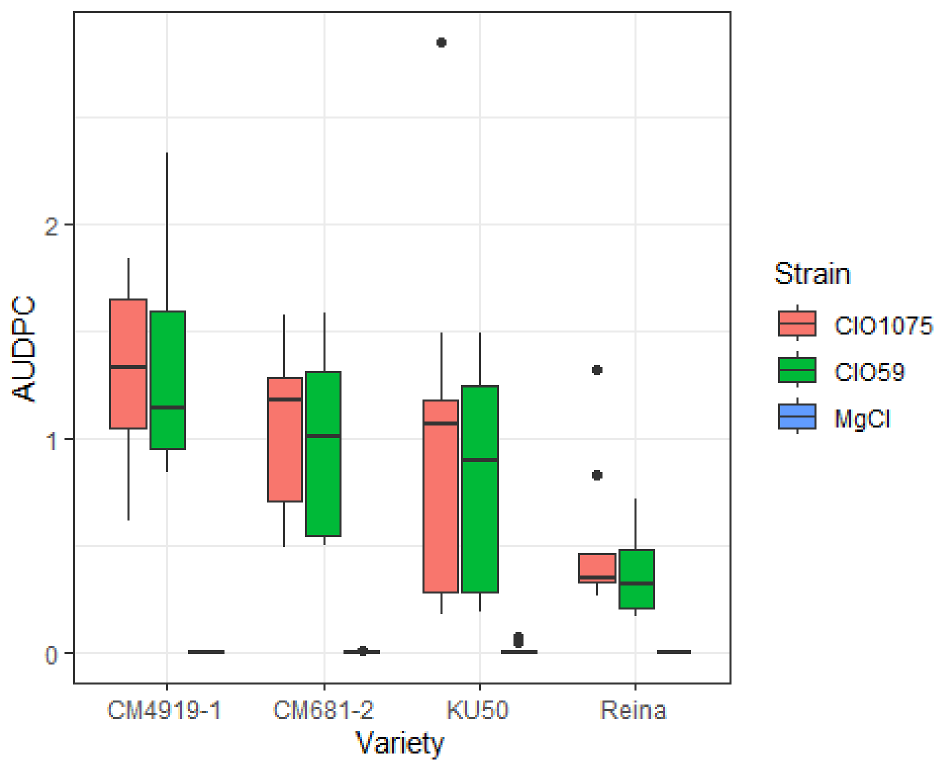
Comparing means Test

	as.numeric(acumAuc)	groups
CM4919-1	0.88773	a
CM681-2	0.68042	ab
KU50	0.59662	b
Reina	0.29575	c

	as.numeric(acumAuc)	groups
CIO59:CM4919-1	1.34408	a
CIO1075:CM4919-1	1.31732	a
CIO1075:CM681-2	1.05654	ab
CIO59:CM681-2	0.98403	ab
CIO1075:KU50	0.97317	ab
CIO59:KU50	0.80252	bc
CIO1075:Reina	0.51407	cd

	as.numeric(acumAuc)	groups
CIO59:Reina	0.37267	de
MgCl:KU50	0.01417	e
MgCl:CM4919-1	0.00178	e
MgCl:CM681-2	0.00068	e
MgCl:Reina	0.00051	e

Boxplot graphs for AUDPC (12DPI)



References

Lliott K, Berry JC, Kim H. et al. (2022). A comparison of ImageJ and machine learning based image analysis methods to measure cassava bacterial blight disease severity. *Plant Methods*, 18, 86.

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