

Microbial control of *O.sulcatus* by fungal

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Introduction

The black vine weevil, *Otiorhynchus sulcatus*, is a severe pest of ornamental and small fruit crops throughout the world. Both the adult and larval stages are damaging to seedlings. The adult weevils live above ground feeding on cotyledons and on the bark of seedlings at night. Root weevil larvae are subterranean, feeding on the roots of many kinds of plants including conifer seedlings in nursery beds. Until now pesticides have been the most usual way to control pest insects like *O.sulcatus*. Since pesticides also cause damages to the environment (and are forbidden in Organic farming), different insect patogen fungials are used.

Experiment

The experiment is carried out in tissue culture plates, consisting of 24 smaller separated wells. A larvae were placed individually on the surface of moist horticultural compost in 12 of these wells. Fungal isolates were applied by pipetting 25 µl of the spore suspension onto the back of each larva in the tissue culture plate wells. All fungal isolates were tested at a dose of $1,0 \times 10^7$ spores per milliliter. Control larvae were treated with 25 µl 0.05% Tween-20 and autoclaved water. Seven different fungal isolates were tested and one control. *O. sulcatus* larvae were examined for mortality 14, 21, 28 and 35 days after inoculation. Dead larvae were placed individually in moist chambers and observed for fungal growth and sporulation.

The tissue culture plates were placed in climatic chambers at 6, 12 and 18 ± 1 °C, 70% RH and 16 h of light.

Table 1 shows the observed variables in the experiment and their values.

<i>Variables</i>	<i>Description</i>	<i>Values/codes</i>
surv	Survivial of <i>O.sulcatus</i> larvae	0=dead, 1=alive
larvae	Numeric larvae identifier	1 - 1548
fung	Different species of insect killing fungal	0=control, 1-7=different fungal isolates
temp	Temperatures	6, 12 and 18 °C
plate	Identifier of each tissue plate containing a spesific number larvaes.	1-72

Table 1: Decription of the variables in this dataset.

All combinations of the 7 fungies (and control) and the 3 temperatures are done, which gives a total number of 28 treatment combinations. Numbers of replicates (tissue plate) varies and is showed in table 2.

<i>Fungi</i>	<i>Number of tissue plates (at each temperatue)</i>
0	4
1	3
2	1
3	4
4	3
5	3
6	2
7	2

Tabel 2: Number of replicates for each combination of fungi an temperature.

The purpose of this experiment is to find the best combination of fungi and temperature and it is therefore necessary to do some statistical analysis.

The whole experiment was planned and carried out by Dr. Ingeborg Klingen at Planteforsk Plant Protection Centre. [1]

Statistical method

Since the response variable, survival, in this experiment is binary (table 1), a binomial model is chosen. Illustration 1 shows the hierarchical structure in these experiment, with larvae nested under plate. It is naturally to treat the plates as random factors, since there are variation between the plates (Illustration 1).

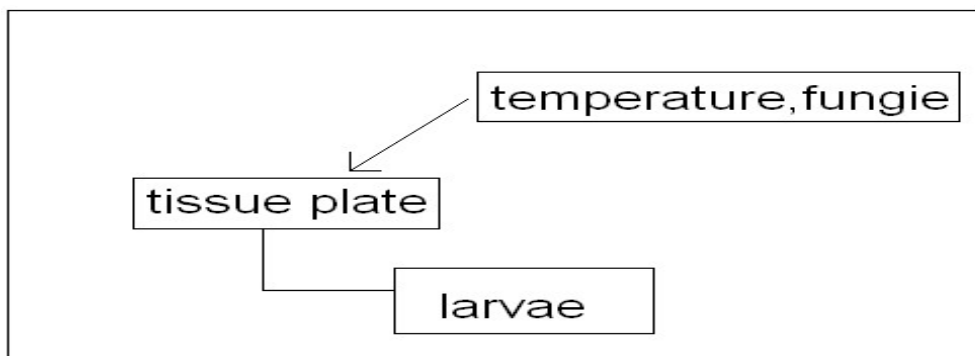


Illustration 1: The structure of the data

A 2-level logistic regression model is described by these equations.

$$y_{ij} \sim \text{binominal}(\Pi_{ij})$$

$$\text{logit}(E(\Pi_{ij})) = \mathbf{X}\boldsymbol{\beta} + \mathbf{u}_j$$

where: i is lowest level (larvae)

j is highest level (plate)

y is response

\mathbf{X} is a matrix of predictors

$\boldsymbol{\beta}$ is the coefficient vector

\mathbf{u} is random variation

This regression models will be estimated both by Generalized Mixed Models and by Markov Chain Monte Carlo method (MCMC).

In order to find the best temperature, fungi or combination, we use two different tests, the Wald-test and the Likelihood-ratio test to test.

Wald-test

In order to test hypothesis between predictors in a regression model, the hypothesis is on the form:

$$H_0: \mathbf{c}'\boldsymbol{\beta} = 0$$

$$H_1: \mathbf{c}'\boldsymbol{\beta} \neq 0$$

where $\boldsymbol{\beta}$ is a coefficient vector of length $n \times 1$ and \mathbf{c} is an $n \times 1$ vector. As an example, if $\boldsymbol{\beta}$ is a 6×1 vector and we want to test the hypothesis $H_0: \beta_1 + \beta_2 - \beta_3 - \beta_4 = 0$, then $\mathbf{c} = [1 \ 1 \ -1 \ -1 \ 0 \ 0]$.

It can be showed that if H_0 is true then:

$$\frac{\mathbf{c}'\boldsymbol{\beta}}{\mathbf{c}'\mathbf{V}\mathbf{c}} \sim N(0,1)$$

where \mathbf{V} is the variance-covariance matrix of $\boldsymbol{\beta}$.

This test is not the best suited for our problem so it is decided to also use the likelihood-ratio test.

Likelihood-ratio test

Another way to test hypothesis between predictors in a logistic regression model is the likelihood-ratio test. This test is based on the deviance.

Our hypothesis is

H_0 : full model equals reduced model

$$\text{deviance} = -2 \log L_{\text{full model}} + 2 \log L_{\text{saturated model}}$$

where

$$\text{deviance} \sim \chi^2 - \text{distribution}$$

$$G^2 = -2 \log L_{full\ model} + 2 \log L_{reduced\ model}$$

$$G^2 = deviance_{full\ model} - deviance_{reduced\ model} \sim \chi^2(df)$$

where df = differences in number of parameters between the models.

L = the optimal likelihood parameters

Multiple testing with Bonferroni method

In multiple tests, the Bonferroni method tells how to reduce the significance level of each test, to achieve an overall significance of 0.05. This is done by dividing the level of significance by the number of possible combinations.

$$\frac{\text{Level of significance}}{\binom{n}{k}}$$

Multiple testing with Fisher method

Gives the single test level for pairwise comparisons and the total error rate for n multiple comparisons, much in the same way as Bonferroni but less conservative.

Results

The probability to survive in each tissue plate varies a lot. Illustration 2 shows the different probabilities at different temperatures and fungie treatments. From this, it is likely to believe that there are an effect of temperature at both 12 °C and 18 °C compared to 6 °C. There seems no effect of fungi at 6 °C but it is probably an effect of fungi at 12 °C and 18 °C for some of the species.

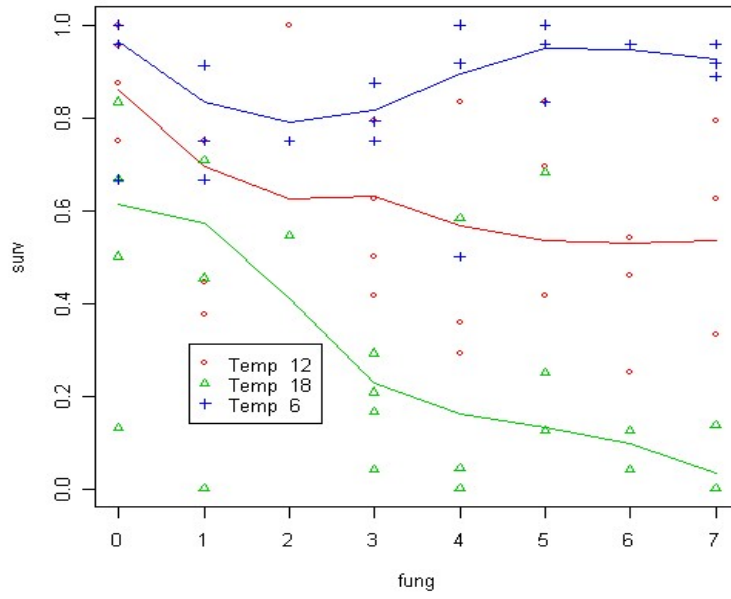


Illustration 2: The probability to survive in each tissue plate, treated with different temperatures and fungies.

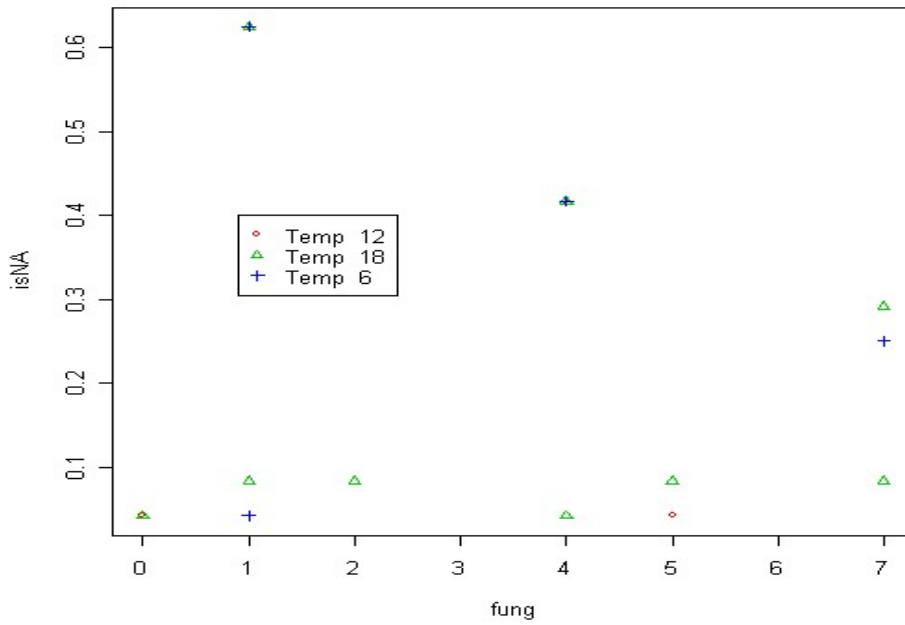


Illustration 3: Amount of missing value at different treatment combinations.

Illustration 3 shows the distribution of missing values. It is spread, but not totally randomly.

A full model with all the interactions were fitted with glmmML (R) ,IGLS (MLwiN) and MCMC (MLwiN). The predictors were treated as categorical variables.

$$\begin{aligned} \text{logit}(E(\text{surv}_{ij})) = & \beta_0 + \beta_1 \text{fung1}_j + \beta_2 \text{fung2}_j + \beta_3 \text{fung3}_j + \beta_4 \text{fung4}_j + \beta_5 \text{fung6}_j + \beta_7 \text{fung7}_j + \beta_8 \text{temp12}_j \\ & \beta_9 \text{temp18}_j + \beta_{10} \text{fung1.temp12}_j + \beta_{11} \text{fung2.temp12}_j + \beta_{13} \text{fung3.temp12}_j + \beta_{14} \text{fung4.temp12}_j + \\ & \beta_{15} \text{fung5.temp12}_j + \beta_{16} \text{fung6.temp12}_j + \beta_{17} \text{fung7.temp12}_j + \beta_{18} \text{fung1.temp18}_j + \\ & \beta_{19} \text{fung3.temp18}_j + \beta_{20} \text{fung4.temp18}_j + \beta_{21} \text{fung5.temp18}_j + \beta_{22} \text{fung6.temp18}_j + \\ & \beta_{23} \text{fung7.temp12}_j + u_j \end{aligned}$$

<i>Parameters</i>	<i>GlmmML(R)</i>	<i>IGLS(MLwiN)</i>	<i>MCMC(MLwiN)</i>
Intercept	2.67899(0.5894)	2.147(0.480)	2.554(0.707)
β for fung1	-1.206990.8424	-0.787(0.736)	-1.088(1.099)
β for fung2	-1.51744(1.1832)	-0.238(0.869)	-0.136(1.269)
β for fung3	-1.18535(0.7597)	-0.724(0.655)	-1.036(0.990)
β for fung4	-0.97943(0.8445)	-0.697(0.725)	-0.651(1.026)
β for fung5	0.16124(0.8900)	0.449(0.778)	0.501(1.119)
β for fung6	0.62356(0.9541)	0.989(0.859)	0.929(1.162)
β for fung7	-0.05363(0.8789)	0.338(0.778)	0.183(1.104)
β for temp12	-0.42959(0.7965)	0.112(0.654)	0.196(0.925)
β for temp 18	-2.53382(0.7451)	-2.004(0.633)	-2.438(0.932)
β for fung1temp12	--0.97058(1.1402)	-1.416(0.999)	-1.559(1.451)
β for fung2temp12	13.85723(367.8814)	10	10
β for fung3temp12	-0.65881(1.0329)	-1.160(0.894)	-1.304(1.326)
β for fung4temp12	-1.35668(1.1387)	-1.659(0.984)	-2.135(1.414)
β for fung5temp12	-1.72773(1.1613)	-2.095(1.012)	-2.533(1.460)
β for fung6temp12	-3.23725(1.2068)	-3.584(1.073)	-4.054(1.472)
β for fung7temp12	-1.81957(1.1491)	-2.260(1.009)	-2.545(1.419)
β for fung1temp18	0.68788(1.1124)	0.359(0.988)	0.412(1.452)
β for fung2temp18	1.56475(1.6048)	0.278(1.330)	0.214(1.948)
β for fung3temp18	-0.62786(1.0128)	-0.950(0.897)	-0.823(1.342)
β for fung4temp18	-0.95677(1.1444)	-0.913(0.998)	-1.496(1.435)
β for fung5temp18	-1.03219(1.1273)	-1.212(0.999)	-1.375(1.465)
β for fung6temp18	-3.52868(1.2442)	-3.727(1.132)	-3.926(1.535)
β for fung6temp18	-3.38372(1.2590)	-3.487(1.136)	-4.017(1.610)
Random error (u _j)	0.761(0.116)	0.478(0.138)	1.357(0.433)

Tabel 3: Estimation of the parameters by glmmML(R), IGLS(MLwiN) and MCMC(MLwiN)

In table 3 the result of estimation with glmmML(R), IGLS(MLwiN) and MCMC(MLwiN) is shown.

There are relative big differences between the estimates (they are all on a logit scale). The result from the MCMC and the IGLS seems to differ less than the glmmML. When using a simple z-test, many of the estimates turns out to be not significant at all. But since the purpose of this project is to find the best combination of fungi and temperature and not the best model, we will not attend to reduce the model. During the further analysis, the glmmML function in R is used.

A large estimate is made for the interaction fungi2.temp12 ($\exp(13.86)=7.75 \cdot 10^5$). The reason for this, is simply that there are only one tissue plate treated at this combination of temperature and fungi, and that all the larvaes survived. There will therefore be an increase in survival, by a factor of $7.75 \cdot 10^5$, when this combination is true. Theoretically, it should be infinitely high, and it is therefore been used an offset of 10 in the calculations done in MLwiN.

From the IGLS estimation in MLwiN, residual plots are made. Illustration 4 shows a normal probability plot at logit scale. Here we expect normal distribution, and it seems to fit very well. Illustration 5 shows the residuals plotted against the predicted value. Also this one looks very good.

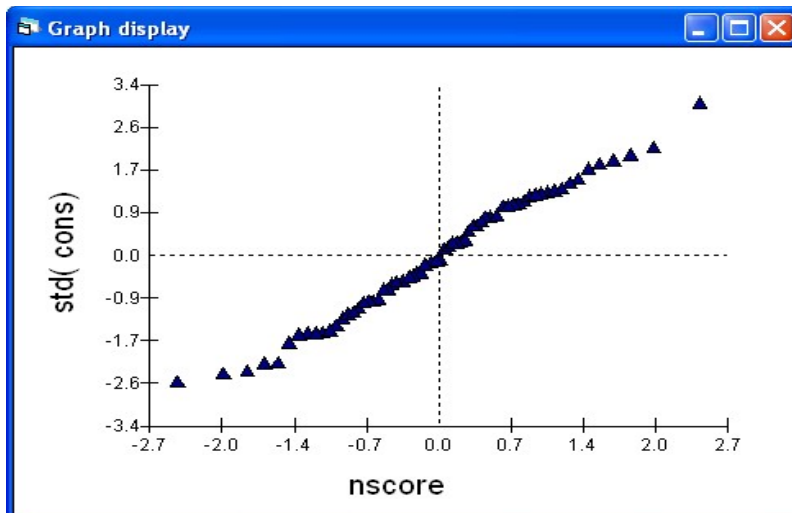


Illustration 4: Plot of standardised residual against normal score.

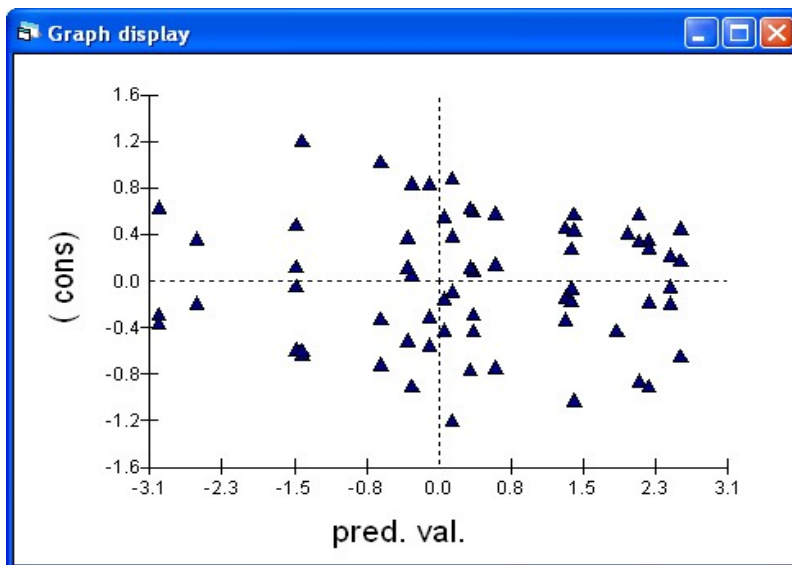


Illustration 5: Plot of standardised residual against fixed part prediction.

In the MCMC estimation, after ~100 000 iterations, the trace of the different β s looks very autocorrelated (each value looks quite correlated to the preceding value), see illustration 6. This is also expected, since the full model, with all possible interactions is used. Illustration 7 shows this large autocorrelation at β_0 . The kernel density estimate of the posterior distribution looks fine.

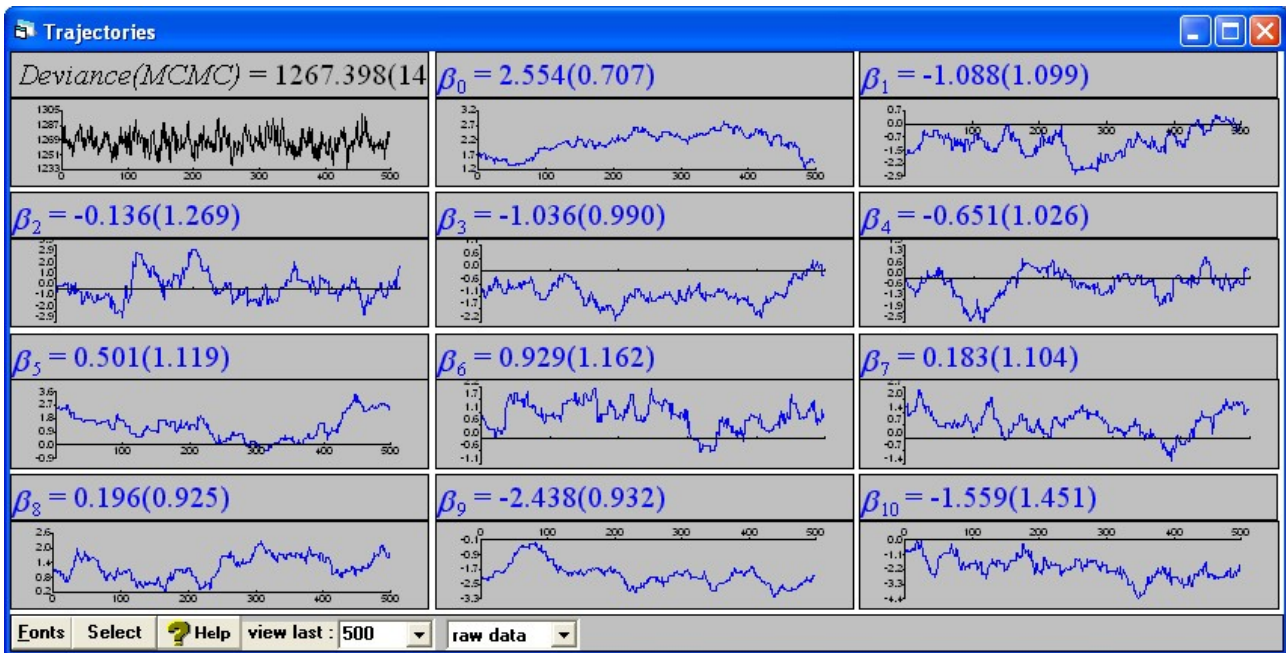


Illustration 6: Trace for the beta values

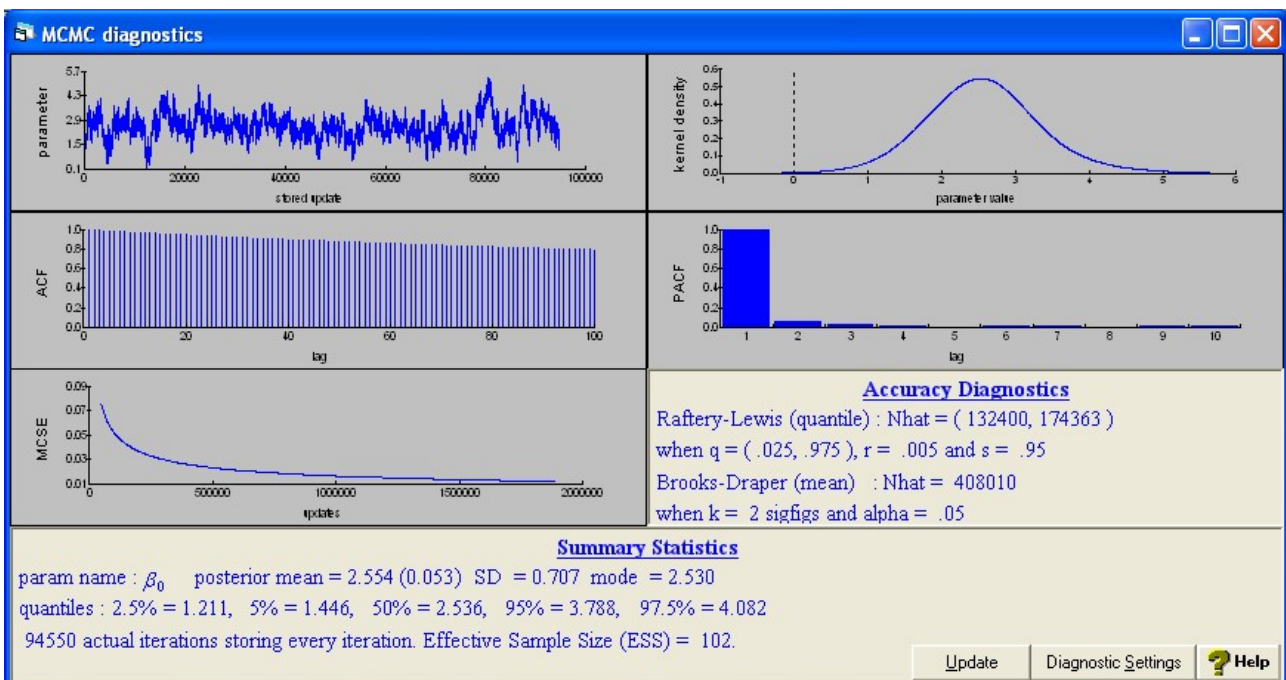


Illustration 7: Diagnostic picture of beta 0

<i>Hypothesis H_0</i>	<i>Likelihood-ratio test p-value</i>
No effect of temperature at 6 °C	0.19
No effect of temperature at 12 °C	$7.53 \cdot 10^{-5}$
No effect of temperature at 18 °C	$2.38 \cdot 10^{-7}$
No effect of fungi at 6 °C	0.23
No effect of fungi at 12 °C	0.0037
No effect of fungi at 18 °C	0.00056
Random effects are not significant	0

Table 4: Results of different multiple hypothesis

Table 4 shows that it is a clear effect of 12 °C and 18 °C, as expected from illustration 2. There are also a clear effect of fungi at 12 °C and 18 °C. We can not prove any effect of fungi at 6 °C at all. It is also a significant random effect. All tested using a significant level of 0.05.

As mention before, the purpose of this experiment is to find the best combination of fungi and temperature. There are a significant effect of both fungi and temperature at 18 °C (table 7). Illustration 8 shows that the smallest estimated probabilities of survival can be found at 18 °C. The analysis is therefore continued at this level.

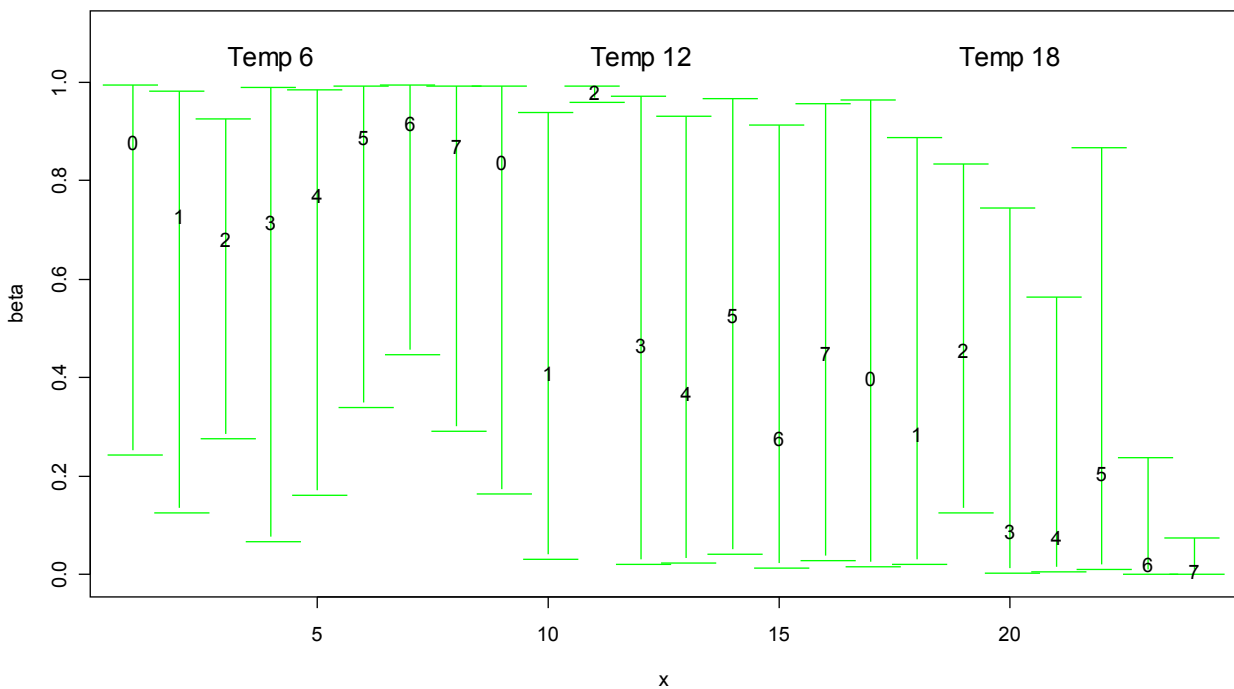


Illustration 8: The estimated combine effect of fungi and temperature for all combinations.

<i>Hypothesis</i>	<i>Wald test p-value</i>	<i>Likelihood-ratio test p-value</i>
Fungi 0 at temp18 = Fungi1 at temp18	0.31	0.47
Fung 0 at temp 18 = Fungi 6 at temp18	0.0014***	0.00032***
Fungi 1 at temp18 = Fungi 3 at temp18	0.042*	0.092*
Fungi1 at temp18 = Fungi6 at temp18	0.003**	0.007*
Fungi1 at temp18 = Fungi7 at temp18	0.0012***	0.00198**
Fungi2 at temp18 = Fungi4 at temp18	0.044*	0.088
Fungi2 at temp18 = Fungi6 at temp18	0.039*	0.013*
Fungi2 at temp18 = Fungi7 at temp18	0.0028**	0.005*
Fungi3 at temp18 = Fungi4 at temp18	0.44	0.87
Fungi3 at temp18 = Fungi7 at temp18	0.04*	0.07
Fungi4 at temp18 = Fungi5 at temp18	0.09	0.19
Fungi4 at temp 18 = Fungi6 at temp 18	0.14	0.2
Fungi5 at temp18 = Fungi7 at temp18	0.003**	0.004*
Fungi6 at temp18 =Fungi 5 at temp18	0.007*	0.015*
Fungi6 at temp18 = Fungi7 at temp18	0,31	0.31
Fungi6 at temp12 = Fungi7 at temp12	0.85	0.30
Fungi7 at temp12 = Fungi1 at temp 18	0.72	0.89

Tabel 5: Hypothesis testing, using both Wald test and Likelihood-ratio test. Rejection at * : p -level=0,05(46.4) , ** : p -level=0,003 (0.0511) (Fisher) and *** : p -level=0,0018(0.0325) (Bonferroni). The numbers in the parentheses are the total probability of reject a true H_0 when doing 28 comparisons. All the hypothesis is total effects compared with the intercept.

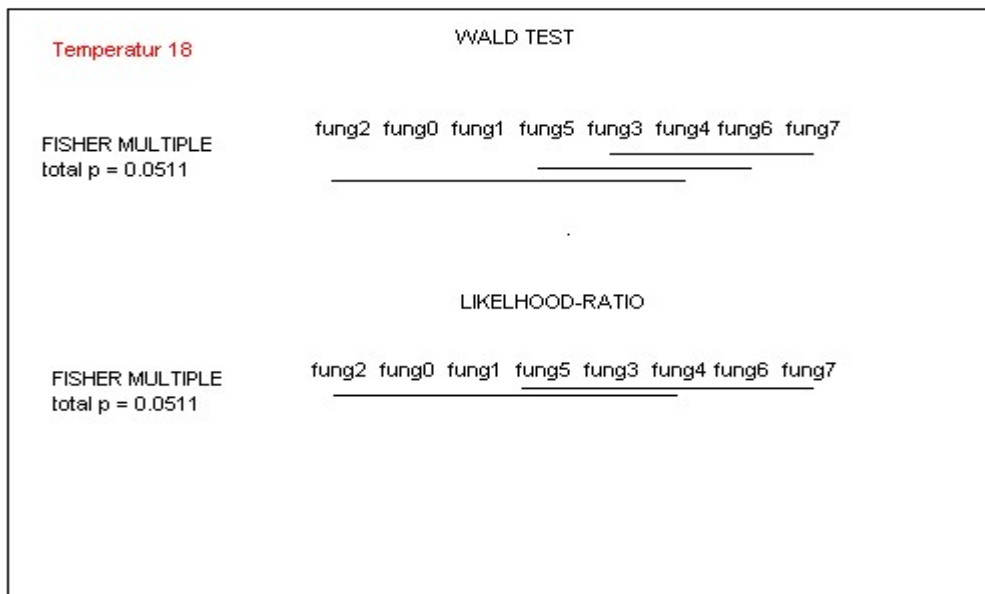


Illustration 9: The equality/inequality between the different fungies at temperature 18. Fungies that are connected is regarded as equal. The effects of the fungi is ordered after size with fung2 as the smallest.

Table 5 shows the result of different comparisons between fungies at temperature 18 °C. The results from this table is showed in illustration 9. From the Wald test it can be concluded that fungi 7 is a better choice than fungi 6, since fungi 7 only lies in one group, while fungi 6 lies in two (can also equal fungi 5).

In the likelihood-ratio test, both fungi 6 and fungi 7 could be the best choice.

Conclusion

Based on the statistical analysis it is concluded that the best combinations of fungi and temperature is 18 °C with fungi 6 and 7. The Wald test supposed that fungi 7 is the best, while the likelihood-ratio test gave both fungi 6 and fungi 7. Since the likelihood-ratio test is assumed to be a better test we hold on the conclusion given from this.

There is an large difference in the estimates from the three different estimations method for these model. The IGLS(MLwiN) and the MCMS(MLwiN) seems to be more similar than the glmmML(R). Therefore it is a large uncertainty of these result and it propose to try different software.

References

[1] Klingen, L. *Norwegian Crop Research Institute, Plant Protection Centre.*