Measurement of Relative Fitness of Dicarboximide-Resistant *Botrytis cinerea* in Strawberry Leaves.

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Abstract

Leaves of healthy strawberry plants were inoculated with either vinclozolin-sensitive or vinclozolin-resistant isolates of *Botrytis cinerea* at one of three concentrations: $5x10^4$, $1x10^5$, and $5x10^5$ conidia/ml. One week following inoculation, representative leaves were harvested and cut into $9mm^2$ leaf pieces. Some pieces were immediately plated onto selective *Botrytis* medium while the others were stored for 7 or 21 days in an empty petri dishes to which silica gel had been added as a desiccant. Vinclozolin-resistant *B. cinerea* isolates established fewer infections in young, healthy strawberry leaves compared to vinclozolin-sensitive strains of the fungus. However, no observable differences in relative ability to survive over a month of desiccation were noted among resistant and sensitive strains.

Introduction

Botrytis cinerea Pers.:Fr., the gray mold pathogen, causes considerable damage to Willamette Valley strawberries (9,11,12). On strawberry, *B. cinerea* can develop microscopic infections in epidermal cells of young leaves (5,20). After infection, the fungus becomes quiescent in these cells and does not begin to grow again until the leaf begins to senesce. These leaf infections carry the fungus over the winter and in spring serve

as a source of primary inoculum for fruit infections (5,10). Conidia produced on senescent leaves can infect the fruit directly, or can infect and colonize flowers before growing into the fruit.

Satisfactory control of *B. cinerea* fruit rot in strawberry depends heavily on protective fungicides (9,10,11,16,17,20) and supplemental cultural practices (9,16,19). In recent years, however, *B. cinerea* has developed resistance to several fungicides (2,3,4,5,6,10,14,11,18,21). In the Willamette Valley, resistance to benzimidazole fungicides is widespread and resistance to the dicarboximides fungicides, vinclozolin and iprodione, is becoming a problem (11). Loss of this latter fungicide class would leave small fruit growers with fewer effective options for gray mold control (11).

The acquisition of resistance to dicarboximide fungicides by *B. cinerea* is accompanied by a metabolic cost that reduces the ability of the fungus to grow and reproduce (2,3). Several reports in the literature (2,11,14) indicate that the frequency of dicarboximide-resistant strains of *B. cinerea* within a field declines over the summer, fall and winter if vinclozolin is not routinely applied to the crop. While the reason for this decline is not completely understood, it is apparent that the "fitness" of resistant strains is reduced relative to the fungicide-sensitive phenotype (3,4).

In this study, we conducted experiments on strains of *B. cinerea* that were either sensitive or resistant to the fungicide

vinclozolin to determine if they show differential abilities to infect and survive in inoculated strawberry leaves. Understanding how the these two components of fitness influence the rate of decline of dicarboximide-resistance frequency in a strawberry planting could potentially lead to improved managerial practices by growers.

Materials and Methods

Inoculation. Strawberry plants, *Frageria ananassa* Duch., cultivar Totem were grown in the greenhouse at 19°C under natural light conditions. Plants were watered, fertilized and trimmed as needed.

Isolates of *B. cinerea* were collected in 1993 from strawberry and Himalayan blackberry (*Rubus discolor*) fruit mummies in Oregon's Willamette Valley and then grown and maintained on Difco malt agar (MAS) amended with streptomycin (100 g/L). The isolates were screened for dicarboximide resistance on MAS amended with vinclozolin (Ronilan 50%, 20 mg a.i./L).

Conidial suspensions at three concentrations ($5x10^4$, $1x10^5$, and $5x10^5$ conidia/ml) were made from both vinclozolin-resistant and a vinclozolin-sensitive isolates of *B. cinerea*. Spores were harvested from MAS plates by rubbing them off into a 1/10 strength Difco potato dextrose broth (PDB). Conidial concentrations were measured with a hemacytometer and adjusted to attain the desired concentration. In the greenhouse, the conidial suspension of each isolate was sprayed onto four

strawberry plants until the entire upper and lower surfaces of each leaf were saturated. Three of these four plants were sealed inside plastic bags containing moistened paper towels. The fourth plant was left unbagged to serve as a control of the procedure used to disinfest the leaf surfaces. Two additional plants were sprayed with 1/10 strength PDB only. After 48 h, the plants were removed from the plastic bags and placed onto the greenhouse bench for an additional 5 days. After this time, it was expected that *B. cinerea* had sufficient time to develop microscopic quiescent infections in epidermal cells (5). Seven days after inoculation, two or three representative leaves were harvested from each plant to assay for infection.

The inoculations were replicated six times by repeating the above procedure with new strawberry plants on each of six dates. A different vinclozolin-resistant *B. cinerea* isolate was used on each date; while four different vinclozolin-sensitive isolates were used (Table 1). Therefore, the experimental design included six replications of the use of two strains (vinclozolin-sensitive and vinclozolin-resistant) of *B. cinerea* at three conidial concentrations ($5x10^4$, $1x10^5$, and $5x10^5$ conidia/ml).

Leaf disc assay. An *in vitro* leaf disc assay, which was developed by Dr. John Sutton and colleagues of the University of Guelph, Ontario Canada (16,18,19,20), was used to quantify infection of leaves by *B. cinerea*. In the laminar flow hood, the selected leaves were rinsed in distilled water for 1 min, surface disinfested for 1 min in a 5% chlorox solution, and rinsed for

an additional minute in sterile distilled water. Each leaf was then cut with a razor into equal sized pieces of about 9 mm^2. Thirty leaf pieces from each plant were immediately plated on to a modified selective *Botrytis* medium (SBM)(12,13, Appendix I). The other pieces were dried in empty petri plates to which fresh packets of a silica gel desiccant (8g in cheesecloth bag) were added. Plates containing leaf pieces were sealed with parafilm for 1 wk, sampled, and then resealed for 3 wk. At 1 wk and at 1 mo post harvest, 30 dried pieces from each plant were plated onto SBM. Presence of *B. cinerea* microinfections in the leaf pieces was visually assessed by observing for pigment production on the medium after 4 or 5 days incubation.

Data analysis. For each strawberry plant, the percentage of recovered *B. cinerea* microinfections was calculated by dividing the number of leaf pieces showing the characteristic brown pigment formed by *B. cinerea* on SBM by the total number of pieces plated. For each date, *B. cinerea* incidence data were arcsine square root-transformed and analyzed with a two-way ANOVA procedure (Appendix II) in SAS (Statistical Analysis Systems, Cary NC) to evaluate the effects of conidial concentration, and vinclozolin resistance. Nontransformed recovery percentages were averaged by treatment, and plotted as a function of time in graphical arrays. An error bar representing one standard error was drawn through each point.

Results

Infection efficiency. Recovery of *Botrytis cinerea* from

strawberry leaf pieces increased significantly with conidial concentration. In addition, recovery of vinclozolin-sensitive isolates was significantly higher ($P<0.05$) than recovery of vinclozolin-resistant isolates on the initial sampling date and on leaf pieces dried for 1 mo. The background *B. cinerea* levels were approximately 2.7% as calculated from recovery in the non-bag controls.

B. cinerea was initially recovered from 6.0% $\pm$ 3.0, 12.7% $\pm$ 4.1, 16.7% $\pm$ 1.3 of strawberry leaf discs inoculated with vinclozolin-sensitive *B. cinerea* isolates at $5x10^4$, $1x10^5$, and $5x10^5$ conidia/ml, respectively (Fig. 1). In contrast, recovery from leaf pieces inoculated with vinclozolin-resistant isolates averaged 3.2% $\pm$ 1.3, 7.0% $\pm$ 3.7, and 12.3% $\pm$ 6.5, respectively over the same inoculum concentrations. This corresponded to a 27 to 47% reduction in infection efficiency by resistant *B. cinerea* strains, relative to sensitive strains.

Survival. Recovery of both vinclozolin-resistant and sensitive *B. cinerea* generally declined over the month of desiccation (Fig. 2) but the rates of decline for both types of isolates were similar. After 1 wk of drying, average recovery from dried leaf pieces inoculated with vinclozolin-resistant *B. cinerea* strains was similar to the initial recoveries. At 1 mo of drying, recovery of *B. cinerea* declined to approximately 52% of the initial recovery.

Recovery of vinclozolin-sensitive *B. cinerea* strains showed a similar pattern. After 1 wk, vinclozolin-sensitive *B. cinerea*

was recovered at percentages similar to the initial recoveries. After 1 mo, recovery of *B. cinerea* declined to approximately 27% of the original recovery.

Discussion

Rates of establishment of microinfections of vinclozolin-resistant *B. cinerea* isolates in healthy strawberry leaves were 27 to 47% lower than those measured for fungicide-sensitive strains. This finding is supported by an observation (3) that dicarboximide-resistant isolates of *B. cinerea* were slower than sensitive isolates to colonize mature grape berries. As leaf infections are an inoculum source for spring fruit infections (5,20), lower establishment rates should lead to less fruit rot. This also suggests that vinclozolin-resistant spores are less likely to become established than sensitive spores during periods of low selective pressure and may be responsible for the observation that vinclozolin-resistant isolates tend to decline over periods when the fungicide is not used (3,4,10).

Studies in New Zealand vineyards (2,3) and Israeli greenhouses (21) have shown that fungal establishment within dead host tissue and debris acts as a survival niche for *B. cinerea* populations. Relative ability to survive in host tissues is an important component to investigate as dicarboximide-resistant isolates of *B. cinerea* (3,4,8) have been shown to display abnormal osmotic stress sensitivity. The hypothesis has been put forth that the ability of resistant strains to respond to periods of moisture stress may be responsible for declines in the

frequency of vinclozolin-resistant populations over the winter. In this study, however, no information was obtained to substantiate that vinclozolin-resistant *B. cinerea* phenotypes have a reduced rate of survival under dry conditions when compared to vinclozolin-sensitive strains. Our data, as well as data from Israel (21), indicates that both vinclozolin-resistant and vinclozolin-sensitive strains have the capacity to survive in dead leaves and may contribute to fruit rot the next spring.

Previous reports (19,20,21) have shown that elimination of the inoculum sources of *B. cinerea* can significantly reduce strawberry fruit rot. Results of this study indicate that removal of old leaves potentially may have a greater effect on vinclozolin-resistant *B. cinerea* strains because they must reestablish in new leaves produced in late summer and early fall. Research should be done to answer the question of how increased sanitation, such as propane flaming crop debris, or mowing and vacuuming up leaf litter, can be developed to reduce leaf infections and overwintering strains of *B. cinerea*. By reducing the amount of initial inoculum through destruction of old leaves and litter, it is believed that dicarboximide resistant populations could be kept at low enough frequencies to allow for some fungicide use without risk of control failure.

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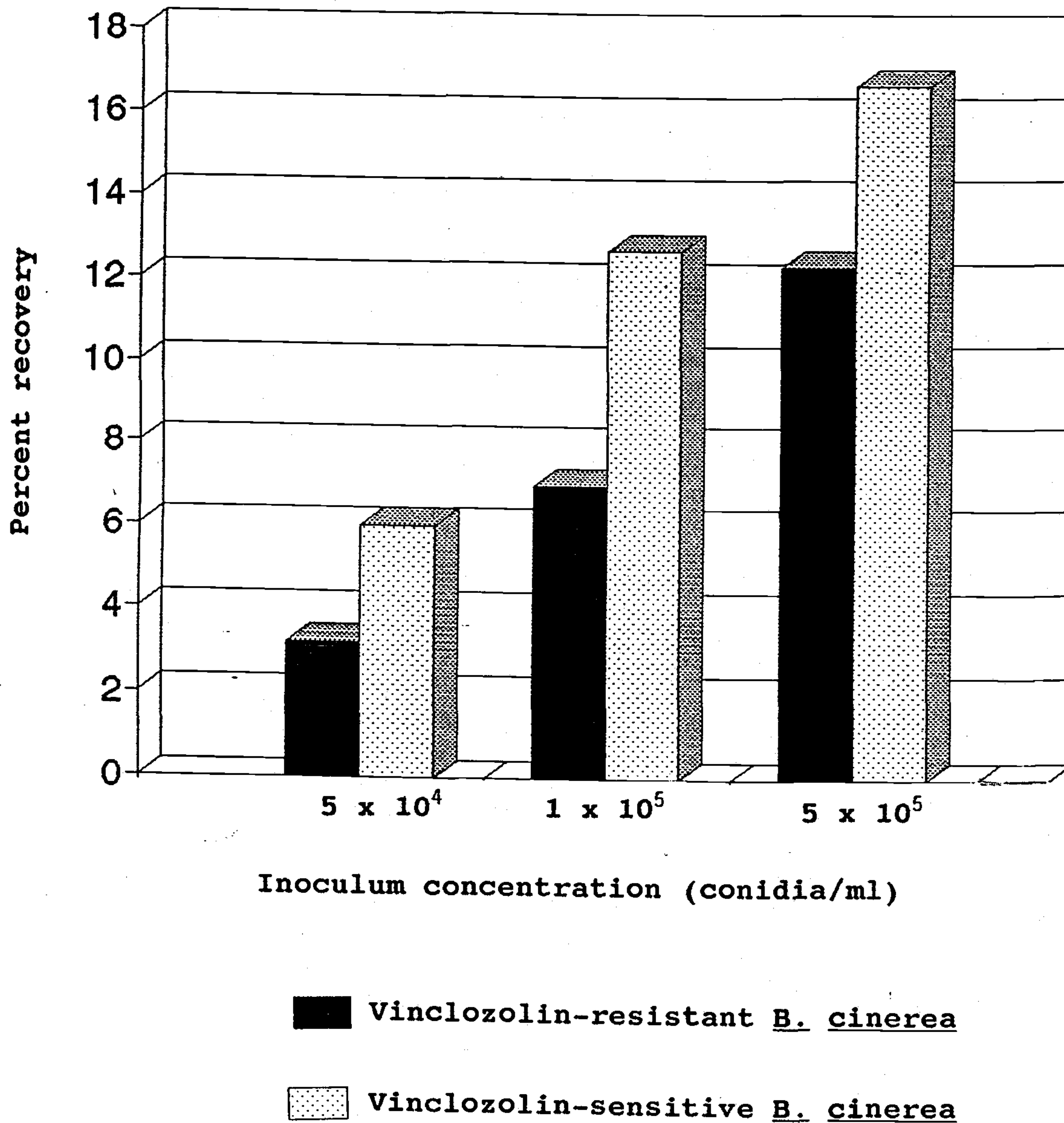
Table 1.0 *Botrytis cinerea* isolates used in this study.

Replication	Vinclozolin resistant	Vinclozolin sensitive
1	230	236
2	240	248
3	238	236
4	239	245
5	246	250
6	237	236

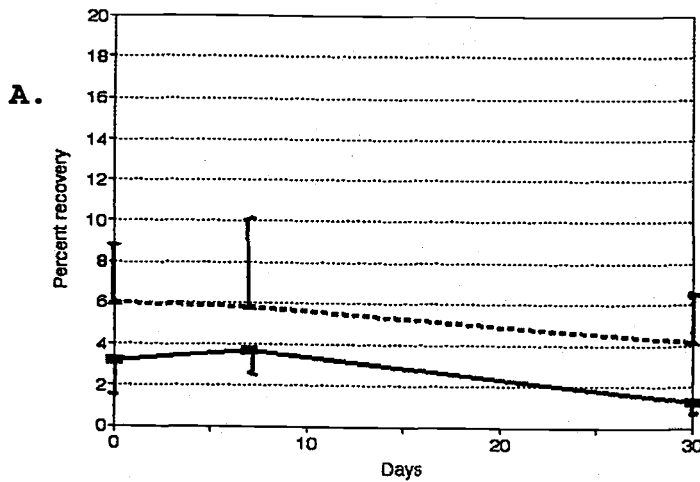
Figure 1. Percent recovery of vinclozolin-resistant and vinclozolin-sensitive strains of *Botrytis cinerea* from inoculated strawberry leaf pieces plated immediately after cutting.

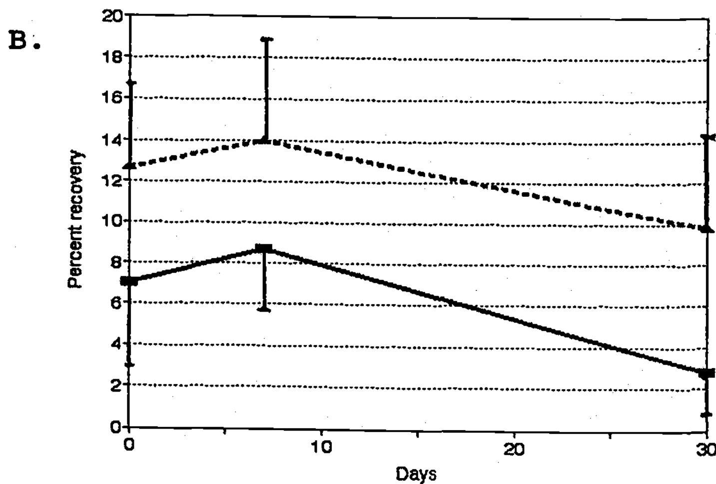


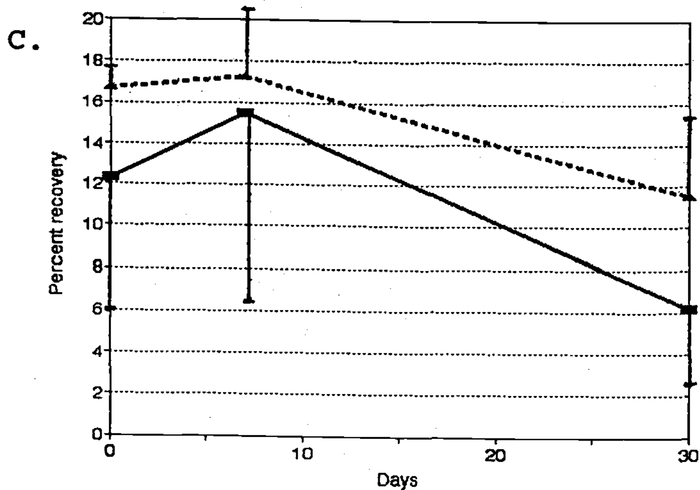
Vinclozolin-resistant B. cinerea

Vinclozolin-sensitive B. cinerea

Figure 2. Survival of vinclozolin-resistant (—— solid line) and vinclozolin-sensitive (- - dashed line) strains of B. cinerea in dried strawberry leaf pieces treated with three inoculum concentrations A) 5 x 10^4 conidia/ml, B) 1 x 10^5 conidia/ml, C) 5 x 10^5 conidia/ml. Lines through points represent one standard error.







Appendix I. Discussion on Selective *Botrytis* Media (SBM)

The medium used in this project was Selective *Botrytis* Medium (SBM) developed by Kritzman and Netzer (13). Alterations to this medium were based on Johnson (12) and personal experience. The media is easy to use and allows for the rapid identification of the fungus (13). Eighteen grams nutrient agar. 20 g dextrose, and 5 g potato dextrose agar per liter distilled water were autoclaved for twenty minutes. Five grams of tannic acid (a tannic acid/sterile water slurry gave the best mixing results) were added immediately to the hot agar. Prior to pouring, these ingredients were added: $4x10^{-4}$ g Maneb (manganese ethylene bisdithiocarbamate), $7x10^{-3}$ g Terraclor (pentachloronitrobenzene, PCNB), $2.5x10^{-2}$ g chloramphenicol, and 100 mg of streptomycin sulfate. The pH of the medium was then adjusted to 6.0 using NaOH (about 300 micrograms of 10 M NaOH). This resulting media was more of a differential media than a true selective one as many different species of fungus grew on it. However, only *Botrytis spp.* produced a warm honey-brown pigment on the media. The fungus presence was further distinguished by its rapid spread across the media.

Appendix II. ANOVA Results

ANOVA Program

```
options ps=60;
Data area;
infile "b:\curarea.prn";
input day resist conc area;
proc anova;
class day resist conc;
model area = day resist conc resist*conc;
means day resist conc resist*conc/lsd;
run;
```

ANOVA Data

Replication	Resistance	Concentration	Percent Recovery: Initial	One week	One month
1	0	1	17.35	6.05	0.00
1	0	2	21.42	12.17	19.47
1	0	3	23.23	23.23	22.34
1	1	1	6.05	12.17	12.17
1	1	2	17.35	21.42	13.63
1	1	3	19.47	22.34	18.43
2	0	1	8.57	10.52	0.00
2	0	2	16.19	14.96	0.00
2	0	3	26.57	23.23	6.05
2	1	1	6.05	6.05	8.57
2	1	2	8.57	13.63	0.00
2	1	3	22.34	20.46	8.57
3	0	1	0.00	10.52	0.00
3	0	2	6.05	0.00	6.05
3	0	3	21.42	20.46	19.47
3	1	1	6.05	12.17	6.05
3	1	2	0.00	6.05	8.57
3	1	3	6.05	6.05	0.00
4	0	1	12.17	10.52	10.52
4	0	2	22.34	25.76	19.47
4	0	3	25.76	12.17	18.43
4	1	1	8.57	6.05	0.00
4	1	2	16.19	18.43	6.05
4	1	3	13.63	10.52	12.17
5	0	1	10.52	6.05	17.35
5	0	2	19.47	33.21	30.37
5	0	3	24.94	30.37	31.09
5	1	1	14.96	12.17	6.05
5	1	2	6.05	10.52	14.96
5	1	3	8.57	12.17	0.00
6	0	1	24.94	29.63	21.42
6	0	2	32.51	29.63	18.43
6	0	3	22.34	33.21	13.63
6	1	1	14.96	16.19	0.00
6	1	2	28.13	25.76	6.05
6	1	3	39.23	50.12	27.35

Replications 1-6

Resistance

- **0 = vinclozolin-sensitive _B._ _cinerea_**
- **1 = vinclozolin-resistant _B._ _cinerea_**

Concentration

- **1 = 5 x 10^4 conidia/ml**
- **2 = 1 x 10^5 conidia/ml**
- **3 = 5 x 10^5 conidia/ml**

```
Means with the same letter are not significantly different.
          T Grouping              Mean       N  DAY
                   A            27.018       6  6
                   B            17.478       6  1
                   B
                   B            16.443       6  4
                   B
                   B            14.715       6  2
                   B
                   B            14.085       6  5
                   C             6.595       6  3
              T tests (LSD) for variable: WEEK
             Alpha= 0.05  df= 25  MSE= 58.5441
                 Critical Value of T= 2.06
            Least Significant Difference= 9.0981
          T Grouping              Mean       N  DAY
                   A            30.757       6  6
                   B            17.415       6  5
                   B
                   B            16.230       6  1
                   B
                   B            14.808       6  2
                   B
                   B            13.908       6  4
                   B
                   B             9.208       6  3
             T tests (LSD) for variable: MONTH
             Alpha= 0.05  df= 25  MSE= 60.07223
                 Critical Value of T= 2.06
            Least Significant Difference= 9.2161
          T Grouping              Mean       N  DAY
                   A            16.637       6  5
                   A
           B       A            14.480       6  6
           B       A
           B       A            14.340       6  1
           B       A
           B       A    C       11.107       6  4
           B            C
           B            C        6.690       6  3
                        C
                        C        3.865       6  2
              T tests (LSD) for variable: INIT
             Alpha= 0.05  df= 25  MSE= 30.18361
                 Critical Value of T= 2.06
            Least Significant Difference= 3.7717
         T Grouping              Mean       N  RESIST
                  A            18.655      18  0
                  B            13.457      18  1
              T tests (LSD) for variable: WEEK
             Alpha= 0.05  df= 25  MSE= 58.5441
                 Critical Value of T= 2.06
            Least Significant Difference= 5.2528
```

NOVA Program

```
)ptions ps=60;
)ata area;
.nfile "b:\curarea.prn";
.nput day resist conc area;
)roc anova;
:lass day resist conc;
nodel area = day resist conc resist*conc;
neans day resist conc resist*conc/lsd;
:un;
```

ANOVA Data

Replication	Resistance	Concentration	Area under curve
1	0	1	0.466667
1	0	2	2.255556
1	0	3	4.238889
1	1	1	1.127778
1	1	2	2.761111
1	1	3	3.461111
2	0	1	0.544444
2	0	2	1.205556
2	0	3	2.994444
2	1	1	0.427778
2	1	2	0.855556
2	1	3	2.45
3	0	1	0.466667
3	0	2	0.155556
3	0	3	3.344444
3	1	1	0.777778
3	1	2	0.388889
3	1	3	0.194444
4	0	1	0.972222
4	0	2	4.316667
4	0	3	2.333333
4	1	1	0.233333
4	1	2	1.788889
4	1	3	1.127778
5	0	1	1.205556
5	0	2	7.272222
5	0	3	7
5	1	1	0.972222
5	1	2	1.205556
5	1	3	0.7
6	0	1	5.444444
6	0	2	5.483333
6	0	3	5.288889
6	1	1	1.322222
6	1	2	3.538889
6	1	3	11.86111

Replications 1-6

Resistance
- **0 = vinclozolin-sensitive *B. cinerea***
- **1 = vinclozolin-resistant *B. cinerea***

Concentration
- **1 = 5×10^4 conidia/ml**
- **2 = 1×10^5 conidia/ml**
- **3 = 5×10^5 conidia/ml**

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
DAY	6	1 2 3 4 5 6
RESIST	2	0 1
CONC	3	1 2 3

Number of observations in data set = 36

Dependent Variable: AREA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	134.0651427	13.4065143	3.63	0.0043
Error	25	92.4369465	3.6974779		
Corrected Total	35	226.5020892			

R-Square	C.V.	Root MSE	AREA Mean
0.591894	76.75895	1.922883	2.50509258

Source	DF	Anova SS	Mean Square	F Value	Pr > F
DAY	5	81.24962546	16.24992509	4.39	0.0052
RESIST	1	10.88389036	10.88389036	2.94	0.0986
CONC	2	40.29795761	20.14897880	5.45	0.0109
RESIST*CONC	2	1.63366928	0.81683464	0.22	0.8033

T tests (LSD) for variable: AREA

Alpha= 0.05 df= 25 MSE= 3.697478

Critical Value of T= 2.06

Least Significant Difference= 2.2865

T Grouping	Mean	N	DAY
A	5.490	6	6
B	3.059	6	5
B			
B	2.385	6	1
B			
B	1.795	6	4
B			
B	1.413	6	2
B			
B	0.888	6	3

T tests (LSD) for variable: AREA

Alpha= 0.05 df= 25 MSE= 3.697478

Critical Value of T= 2.06

Least Significant Difference= 1.3201

T Grouping	Mean	N	RESIST
A	3.055	18	0
A			
A	1.955	18	1

T tests (LSD) for variable: AREA
Alpha= 0.05 df= 25 MSE= 3.697478
Critical Value of T= 2.06
Least Significant Difference= 1.6168

T Grouping		Mean	N	CONC
	A	3.750	12	3
	A			
B	A	2.602	12	2
B				
B		1.163	12	1

Level of RESIST	Level of CONC	N	AREA Mean	AREA SD
0	1	6	1.51666667	1.94786345
0	2	6	3.44814833	2.71339278
0	3	6	4.19999983	1.71446660
1	1	6	0.81018517	0.41697644
1	2	6	1.75648167	1.19773136
1	3	6	3.29907383	4.36266163