Calculating the Frequency of Bioluminescence Genes in *Armillaria mellea* by Screening a Sample of Mutant Isolates

By

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A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College

May 2016

ABSTRACT

Despite bioluminescence being a widespread trait in several groups of organisms and having revolutionary applications in genetic engineering, the genes that are responsible for the trait still remain largely undiscovered in most organisms. The purpose of this research was to estimate the number of genes responsible for bioluminescence in *Armillaria mellea*. This was performed by screening 154 mutagenized isolates of *Armillaria mellea* for non-bioluminescence variants that have had one gene required for proper luminescence successfully disrupted. The resulting ratio of non-bioluminescent phenotypes to normal bioluminescent phenotypes was used along with *Armillaria mellea's* total genome size and average length of each gene to estimate the number of genes that code for bioluminescence based on the probability of how many non-bioluminescent phenotypes should be observed given the sample size. From this research, we concluded with 95 percent confidence that there are between 210 and 272 genes on the *Armillaria mellea* genome that code for bioluminescence.

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Section 1: Introduction

1.1 An overview of Bioluminescence

Bioluminescence is the biochemical production and subsequent emission of light by a living organism. The phenomenon of bioluminescence has been confirmed in various widely distributed organisms belonging to diverse major groups from bacteria and protists to fish and squid, with several species in between. The trait is distributed with no discernable pattern among these genera to the point that species that share the same genus exhibit bioluminescence while other may not. The majority of these luminescent organisms are found in marine biomes, predominantly in deep-dwelling and planktonic organisms. A few examples of organisms in these marine habitats include several fish, crustaceans, shrimp, jellies, mollusks, and worms. Terrestrial bioluminescence is less frequent as it is not present in prominent high-level organisms such as flowering plants, mammals, birds, and amphibians. Nevertheless, bioluminescence can still be easily seen on land in certain fungi, click beetles, glow-worms, fireflies, and other terrestrial invertebrates. (Herring 1987, Wilson 2013).

While the specifics of bioluminescence vary from species to species, there are basic similarities shared by these organisms in terms of how they achieve bioluminescence. Bioluminescence is a form of chemiluminescence, defined as light given off as a product of a chemical reaction. The principal chemical reaction involves a luciferin substrate, oxygen, and a luciferase enzyme (Hastings 1983, Airth 1960, Ohmiya 1996). An example bioluminescent reaction that occurs in fireflies is laid out below in Figure 1.1.

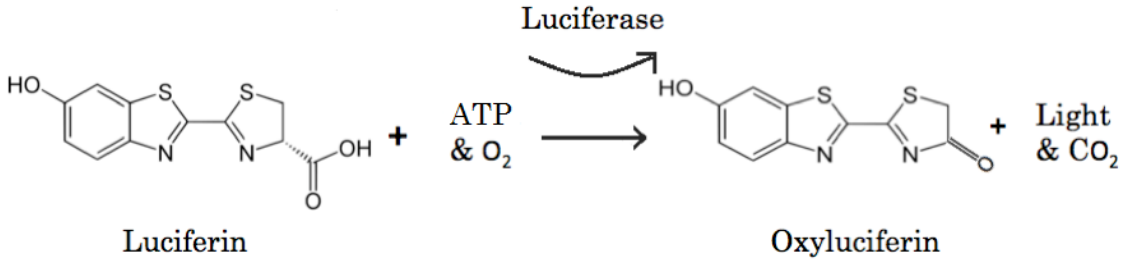

Figure 1.1: The bioluminescent reaction for fireflies in which a luciferin, oxygen, and energy in the form of ATP are converted into oxyluciferin, carbon dioxide, and light. The catalyzing enzyme, luciferase, initiates the reaction and is conserved after each reaction.

In bioluminescent reactions, a light-emitting molecule, generally referred to as a luciferin, is oxidized to produce light. Luciferase, a class of enzymes, catalyzes the oxidizing reaction of the luciferin substrate, wherein oxygen is added to the luciferin molecule (Hastings 1983). In some cases, the reaction is catalyzed not by the luciferase enzyme but by a photoprotein, a single unit that houses the reagents: luciferin, oxygen, and other varying cofactors required for light emission. The photoprotein is stable in a non-luminescent state until triggered by a certain cofactor or ion, such as Ca^{2+}. The binding if the cofactor or ion initiates a conformational change that catalyzes the bioluminescent reaction of the reagents contained in the photoprotein. This photoprotein method allows organisms to control the timing and intensity of their bioluminescence more precisely (Ohmiya 1996, Shimomura 2006).

All bioluminescent reactions require a significant amount of energy. For example, one emitted photon of green bioluminescent light requires the energy equivalent stored in eight ATP, the compound that serves as the immediate source of energy for organic processes. Additional energy is spent producing the proteins that serve as reagents and catalysts for these reactions (Wilson and Hastings 1998). In some cases, organisms spend substantial amounts of energy to produce and sustain entire photic organs that contain an

organism's bioluminescent reactions, such as a firefly's lantern. Still, despite the fact that bioluminescence is an extremely energy-expensive process, bioluminescence can be seen evolving independently in a wide range of organisms across many biomes. With the energy cost for bioluminescence so high, it reasons that the trait possess an adaptive significance, defined as some beneficial quality that increases the organism's chances of survival and subsequent reproduction.

As various organisms, many within dramatically different ecological niches, exhibit bioluminescence, it reasons the trait serves a diverse range of functions amongst these various bioluminescent species. Generally these functions fall into the three basics categories of defense, offense, and communication. In the communication category, bioluminescence is primarily for mate attraction and recognition. In the offensive category, various organisms use bioluminescence to lure potential prey, to illuminate prey, or to stun and confuse prey. In the defensive category, bioluminescence is used as a "burglar alarm" to attract secondary predators, a smoke screen or distraction, warning coloration, marine camouflage, and to trigger a startle response (Haddock et. al, 2010, Oba 2014). This being stated, the exact purpose and function of an organism's bioluminescence is difficult to experimentally verify, so often scientists are left guessing why certain organisms are bioluminescence.

1.2 Bioluminescence in research

Research of bioluminescent organisms has led to several innovations in the area of biotechnology that have revolutionized the science. For example, luciferase systems are used in genetic engineering as reporter genes, genes that are attached to a regulatory

sequence of a gene of interest to confirm the expression of that gene (Koo 2007, Xiong 2004, Yamazaki 2013). In other research, luciferase is used to determine the order of nucleotides in DNA, or DNA sequencing, by detecting the activity of DNA polymerase in a method referred to a pryosequencing (Nyrein 2001, Ronaghi 1998).

A specially blended extract of firefly lanterns is used as a method for the assay of ATP. When the firefly luciferase and lucifern comes into contact with ATP in the presence of oxygen, luminescence is triggered in proportion to the amount of ATP present. As ATP is a sign of the presence of living organisms, this assay is used to detect microbial contamination in foodstuffs and water (Selan 1992). Similarly, the bioluminescent marine bacteria *Vibrio fischeri* is being used commercially as a toxicity test as the organism demonstrates a noticeable decrease in luminescence upon coming into contact with a toxin (Parvez 2005).

1.3 Limitations of bioluminescent research

Despite the fact that the various proteins implemented by bioluminescent organisms have transformed the field of biotechnology, little is known about the genes and subsequent pathways that produce the bioluminescent substrate luciferin for any eukaryote. To date, only the genes that code for luciferase have been discovered in eukaryotes, while the genes that code for the production of luciferin in these organisms have eluded scientists. At present, the only luciferin synthesis genes successfully identified have been for bioluminescent bacteria, which are simple prokaryotic organisms.

Unlike that of eukaryotic organisms, the genes and corresponding pathways of bacterial bioluminescence are well understood. This is because prokaryotic bacteria are universally simpler than eukaryotes making bacteria a model organism for genetic analysis. Bacteria are single-celled, haploid organisms, which allows for straightforward and simple mutagenesis and clonal manipulation. Bacteria can be easily grown from a single cell cheaply and quickly with limited nutrients and attention. Consequently, researchers could easily identify any genes in prokaryotes responsible for bioluminescence by utilizing forward genetics strategy (Engebrecht and Silverman 1984, Meighen 1994).

1.4 Forward genetics and insertional mutagenesis

Forward genetics is a process that is used to identify genes or gene sets responsible for an organism's phenotype by screening for mutations of that particular phenotype and subsequently discovering its genetic cause (Engebrecht and Silverman1984, Stark 1999). For example, if researchers were investigating what genes are necessary to support the phenotype of bioluminescence in an organism, mutant organisms that exhibit the variant phenotype of non-bioluminescence would be genetically studied to find out the cause of the mutation. An organism showing a variant phenotype can either be found in the wild or created using mutagenesis. Once a set of desired mutants is produced, genetic mapping and positional cloning can be used to determine the mutational cause, the disrupted gene that participates in the inquired upon phenotype.

Inserting transferred DNA, or T-DNA for short, into an organism's genome is a common method of producing a mutation because the added DNA segment will disrupt whatever gene into which it is inserted. It is random as to which gene on the genome the T-DNA is inserted. Through many simultaneous attempts at mutagenesis, a collection of mutants can be generated such that each mutant within the collection has a different random gene disrupted. Therefore, if the collection of mutagenized isolates is large enough, there would likely exist within that collection at least one isolate for each disrupted gene in the genome. All resulting mutants exhibiting a variant phenotype are then assumed to be the result of this T-DNA being randomly inserted into a gene relating to the phenotype of interest and causing it to become abnormal or variant. Once a desired mutation is found, the known sequence of T-DNA can be readily identified in the inactivated gene using PCR in combination with DNA sequencing (Moresco 2013).

1.5 Agrobacteria used for T-DNA insertion

Agrobacteria is a class of bacteria noted for it ability to infect organisms, most commonly plants. When an *Agrobacterium* finds host cells, the bacterium transfers a portion of its genetic material into the host cells, hijacking the cells to function in the *Agrobacterium*'s favor and causing deleterious effects to the host organism. The *Agrobacterium* inserts its genetic material as T-DNA in the form of a plasmid, a circular DNA molecule separate from a cell's chromosomal DNA, which incorporates into the host genome. The host cell synthesizes this inserted T-DNA along with its own genes, resulting in the production of foreign proteins and consequential traits. Genetic engineers

have altered *Agrobacterium*'s T-DNA to infect organisms for their own purposes, adding in desired genetic material that can be used to both easily mutagenize the organism and serve as a reporter gene to confirm insertion (Krysan 1999, Zupan 1995).

1.6 Problems with insertional mutagenesis in eukaryotes

Unlike prokaryotic bacteria, eukaryotes' qualities of being multicellular and diploid make it significantly harder to study their genetics. Insertional mutagenesis, though simple and straightforward for haploid organisms such as bacteria, is not a reliable method of producing mutations in most multicellular diploid organisms that have two copies of every gene rather than one. First, the multicellular nature of these organisms would mean that the genome in every cell would have to be mutated identically to result in a variant phenotype. In order for this to be achieved the T-DNA would have to be inserted when the organism is a single celled embryo. Even if a gene is successfully mutated, the diploid nature of the organism would result in the second copy of the gene on the eukaryote's paired chromosome taking over and there will be no change in phenotype. In addition, compared to prokaryotes, eukaryotes require significantly more time, costly resources, and attention to mature properly and be viable to study.

1.7 Fungi as a candidate for forward genetics

Unlike most eukaryotes, fungi present a unique opportunity for this study. Most fungi have both a haploid and a diploid stage in their life cycle, and during this haploid

stage, the fungi is a single celled spore or mycelia. Therefore, the fungi can be manipulated and mutagenized in this haploid form similar to prokaryotic bacteria with insertional mutagenesis. *Armillaria mellea,* or *A. mellea,* is one fungus that can be studied in this way (Baumgartner et al., 2011, Baumgartner et al., 2015).

1.8 A brief overview of *Armillaria mellea*

Commonly referred to as honey fungus, *A. mellea* is one of the most widespread fungi with the organism covering temperate regions across the entire Northern Hemisphere. *A. mellea* is most known as a serious plant pathogen for causing armillaria root disease. The disease is the result of the fungi living as a parasite on its host weakening it considerably and causing growth reduction, decay, and mortality. A. mellea fungus has a wide range of hosts that include hundreds of species including shrubs, trees, and vines. Due to its diverse range of host and widespread distribution, *A. mellea* presents a serious problem to agriculture and managed ecosystems attacking timber trees, crops, and ornamentals. For this reason, it is an essential species for study.

Similarly to other fungi in its genus, *A. mellea* exhibits bioluminescence. For *A. mellea* in particular, the vegetative portion of the mushroom found in or around the soil, or mycelium, are bioluminescent as opposed to the main fruiting body, or mushroom cap, that is normally bioluminescent in other species of fungi. The specific reason for this bioluminescence in a commonly unseen portion of the fungus is still debated and heavily hypothesized (Baumgartner et al. 2011; Baumgartner et al. 2010, Baumgartner et al. 2015, Hood 1991).

1.9 Scope and Objective of Study

In this research, a forward genetic strategy was used to investigate what genes on the *A. mellea* genome result in bioluminescence. One hundred and fifty-four uniquely mutagenized isolates of *A. mellea* were screened for the variant phenotype of non-bioluminescence to determine which isolates have had one gene required for bioluminescence successfully disrupted. From the resulting data, an estimate of the number of genes responsible for bioluminescence was calculated based on the frequency that which non-bioluminescent phenotypes were produced given the total number of T-DNA insertions, the organism's genome size, and the average gene length. At the end of this project, a set of non-bioluminescent isolates and an estimate of the number of bioluminescent genes will be available for further studies into completely identifying the bioluminescence genes and subsequent protein pathways in the fungus *A. mellea.*

Section 2: Materials and Methodology

2.1 Isolate origin and transformation

Isolates of *A. mellea* were donated by Dr. Kendra Baumgartner of the University of California, Davis, from the collections held at United States Department of Agriculture - Agricultural Research Service. Agrobacterium mediated transformation was performed on the contributed isolates and confirmed using PCR by Dr. Kathyrn L. Ford of the University of Bristol, United Kingdom as per Baumgartner et al. (2015). The donated isolates were shipped and stored as mycelia inoculated agar plugs suspended in sterile deionized water until use.

2.2 Fruiting media and growth conditions

Potato dextrose agar (PDA), 1.5%, and 1.5% malt extract agar (MEA) were made and autoclaved to sterilize. Once the media had cooled to 50 °C, 30 µg/ml of hygromycin was added. The media was then poured into 5 ml, 35 mm by 10 mm, petri dishes in a sterilized cell culture hood and under ultra-violet light to avoid contamination. Once the agar solidified, the plates were inoculated with the donated mycelial agar plugs, one plug per plate. To minimize contamination, the 5 ml petri dishes were sealed in parafilm after inoculation. To simulate dark, underground conditions, these cultures were then placed in a plastic storage container completely wrapped in aluminum foil and incubated in a lab cabinet at room temperature for approximately four weeks until fully colonized.

2.3 Bioluminescence screening

To determine if an isolate of *A. mellea* is phenotypically a non-bioluminescent mutant, the BioRad ChemiDoc MP system was used to screen the plates for any signs of bioluminescence. The BioRad ChemiDoc MP system was set for a 5-minute exposure time viewing twenty plates at a time. At the same time that the bioluminescence image was being produced, a bright-field image, similar to a traditional photograph, was taken of the isolates to indicate the amount of growth each plate had at the time of the analysis. The exposure time for this bright-field image was 0.1 second.

After each isolate was observed qualitatively, a Berthold Detection System FB 12 Luminometer was used to screen every isolate plate individually for a quantitative reading on how much light each sample emits. For each reading, the luminometer was set on a 2 second delay with 5 second reading time. In addition to the plates containing the *A. mellea* isolates, five plates containing only MEA agar were wrapped in parafilm and analyzed using the Berthold Detection System FB 12 Luminometer after being set on a lab bench under fluorescent light. These plates provided a control reading of how much stored light could be given off by the petri dish, agar, and parafilm alone. From these control readings, it was determined that if a isolate with growth was recorded to have a reading of less than 500 relative light units, then the isolate was considered a potential non-bioluminescent phenotype. The potential non-bioluminescent phenotypes were then monitored in subsequent days to ensure they remained non-bioluminescent.

2.4 Bioluminescence gene estimation

Once all the isolates were screened, a similar formula to that used by Krysan et al. (1999) was developed estimate of the number of genes *A. mellea* has on its genome that directly participate in bioluminescence. For this estimation, a gene was defined as a genomic DNA sequence, including introns and exons, from which a protein is specified. The equation takes into account an organism's estimated genome size and average gene length (Collins et al., 2013) to predict the number of required genes of bioluminescence based on the probability of how many non-bioluminescent phenotypes should be observed for a given number of integrations.

To produce such an equation, assumptions about the *A. mellea* genome and insertional mutagenesis process were made. First, the insertional mutagenesis by *Agrobacterium* was assumed to be absolutely random. Second, each insertion was assumed to effectively produce a mutant phenotype in each isolate by disrupting a protein-coding region. Thus, there are no null insertions that do not result in a variant phenotype of some kind. Third, all genes are assumed to be the same length (the average gene length) and consequently, have the same likelihood of getting disrupted. Lastly, the isolates are assumed to not have any natural variation in bioluminescent intensity.

With the above assumptions in mind, the equation outlined in Figure 2.1was used to estimate the number of bioluminescent genes in the *A. mellea* genome. From the equation a point estimate was calculated. With the point estimate calculated, a confidence interval formula was used to determine a 95% confidence interval for the estimation as outlined below in Figure 2.2 (Dowdy, 1991).

$$\frac{X}{n} \times \frac{genome\ length}{gene\ length} = \lambda$$

Figure 2.1: The equation used to estimate the number of bioluminescent gene in *A. mellea* wherein the variables are as follows:
X = the number of non-bioluminescent phenotypes in the sample size
n = the sample size, the number of unique mutated isolates screened
Genome length = total length of the *A. mellea* genome, 58,350 kilobase pairs (Collins et al., 2013)
Gene length = the average length of a gene in the *A. mellea* genome, 1.575 kilobase pairs (Collins et al., 2013)
λ = the estimated number of bioluminescent related genes on the *A. mellea* genome

$$\hat{\lambda} \pm 1.96\sqrt{\frac{\hat{\lambda}}{n}}$$

Figure 2.2: The formula used to calculate a 95% confidence interval for the point estimate wherein the variables are as follows:
$\hat{\lambda}$ = the point estimation of the number of genes or mean of all point estimates
n = the sample size, the number of trials performed to obtain λ

Section 3: Results

3.1 Confirmation of T-DNA insertion and no-grow phenotypes

The successful insertion of mutagenizing T-DNA into the genome of each *A. mellea* isolate was confirmed using PCR before the isolates were used in this research. Further confirmation that the T-DNA was successfully inserted was provided by the isolates' ability to grow on a hygromycin treated agar. Hygromycin inhibits protein synthesis in bacteria, fungus, and other eukaryotic cells. Thus, only the *A. mellea* that had the T-DNA and attached hygromycin resistant gene successfully inserted into its genome would grow, where the non-mutated *A. mellea* would be killed.

Of the 154 different isolates, a total of 6 different isolates did not exhibit satisfactory physical growth after four weeks to be viable for the experiment and were deemed no-grow phenotypes. The following no-grow isolates are as follows: Eldo17-siGFP-16, Eldo17-6-31, Eldo17-8-1, Eldo17-AmAss14, and Son202pBGgHg1222PT05 (a05) and (a15).

3.2 Use of potato dextrose agar and malt extract agar

At the start of this research, each isolate was plated on both PDA and MEA with each *A. mellea* mutants being grown on a total of four plates, two on PDA and two on PDA. This was initially done to see which agar was more practical for this research and resulted in more growth of the *A. mellea* isolates. The two agars were determined to be equally suited for growing the isolates. Therefore, only MEA was used for the remainder of the study and the majority of *A. mellea* mutants were only

plated on two MEA agar plates. Isolates on different agar were screened separately. Because of this, the agar each isolate was grown on is indicated in the text, but overall, the agar type used did not affect the screening process.

3.3 Initial screening of *A. mellea* isolates with BioRad ChemiDoc MP imaging system

Figures 3.1 to 3.20 show the images taken by the BioRad ChemiDoc MP imaging system of each mutant isolate plate in sets of 20 or fewer. In these figures, the lower image, image B, is the bioluminescence image. This bioluminescence image shows the light emission from each isolate over a five-minute period. Bioluminescence appears in this image as black on a white background, and red indicate particularly intense bioluminescence that saturates the camera at that location. In the bioluminescence image, isolates with smaller mycelia appear to be non-bioluminescent next to isolates showing more physical growth while in actuality they are just dimmer in comparison due to the light pollution from the greater bioluminescence of the neighboring isolates. In addition, no-grow isolates also appear to be non-bioluminescent phenotypes. For this reason, the top image, image A, of each figure is a bright-field image taken by the system showing the growth of the mycelium on each plate under normal LED light. This bright-field image is just like a normal photograph and only shows the physical appearance and growth of an isolate and not any bioluminescence. The bright-field and bioluminescence images were viewed together to determine which isolates were possible non-bioluminescent phenotypes and which isolates displayed normal bioluminescence.

Figure 3.1 is an example of what information was of importance in each bright-field and bioluminescence images. The names of each isolate are provided at the top of

the plate to which they correspond. These names are abbreviated in all the figures with the repeating prefix of "Eldo-17" or "Son202pBGHg" removed for simplicity and to conserve space. These removed prefixes are indicated in the following figure legend and in subsequent tables.

In Figure 3.1, the double-sided arrow is pointing to the same exact isolate in each of the images to show how each isolate corresponds to the isolate in the same position with the same name between the two images. This isolate, Son202pBGHg1222PT20 (a20), is bioluminescent. The top bright-field image shows growth in the form of the white, branching mycelium, and the bioluminescence image below shows the isolate's bioluminescence as red and black on a white background. The isolate in the bottom, left corner Son202pBGgHg1230PT03 (b03) is also bioluminescent. It is just dimmer as seen by the lack of red coloration on the bioluminescence image that indicates over saturation of the camera.

The isolates in the middle column in Figure 3.1, Son202pBGgHg1222PT05 (a05) and (a15), do not show any bioluminescence in the bioluminescence image as well. However, the above bright-field image shows no growing mycelium just the clear agar plug used to inoculate the plates. Thus, the isolates Son202pBGgHg1222PT05 (a05) and (a15) are an example of no-grow isolates. The isolate in the bottom, left corner, Son202pBGHg1222PT25 (a25) is an example of a possible non-bioluminescent isolate. The isolate shows growth in the bright-field image, but has no visible bioluminescence. This lack of bioluminescence is shown as white similar to the background on the bioluminescence image.

Figures 3.2 through 3.20, can be observed in the same way as this example, Figure 3.1. Again, all that was screened for is the presence of growth and bioluminescence, any other variations among the isolates were not noted. Intensity of bioluminescent, whether the bioluminescence appears as more red or black, can not be quantified in these bioluminescence images, only observed similar to the growth.

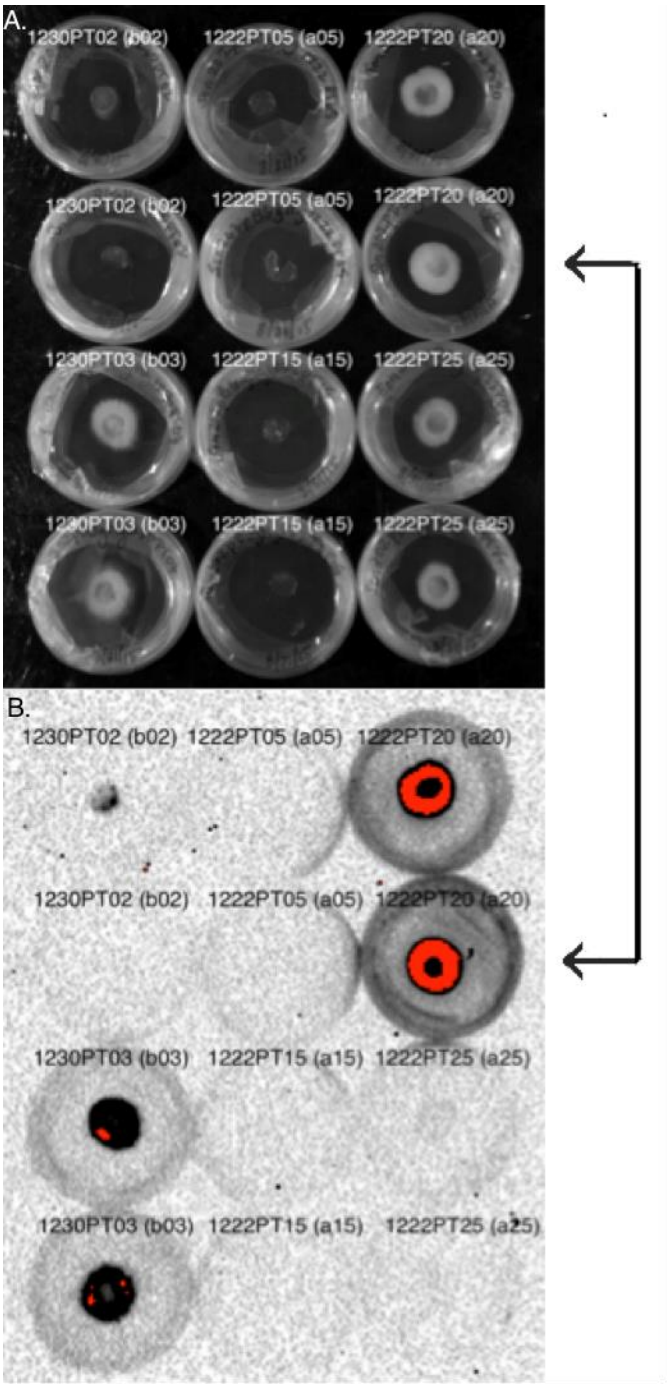

Figure 3.1: An example bright-field image (Panel A) and bioluminescence image (Panel B) taken of 12 Son202pBGHg *A. mellea* isolates with the BioRad ChemiDoc Mp imaging system. This figure is instructionally in the text to indicate what information is of importance in the images. The double-sided arrow indicates the same isolate in each image, Son202pBGHg1222PT20 (a20).

3.4 Results of secondary screening with Berthold Detection System FB 12 Luminometer

The Berthold Detection System FB 12 Luminometer was used to measure quantifiable bioluminescence intensity for each *A. mellea* isolate. The readings from the luminometer are listed in Table 3.1 through Table 3.20.

Table 3.1: A table containing the Berthold Detection System FB 12 luminometer readings for the control plates of only malt extract agar in 5 ml petri dishes wrapped in parafilm with no *A. mellea* isolate. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**
Control 1	236
Control 2	508
Control 3	432
Control 4	285
Control 5	317

Table 3.1 shows the readings from the controls, the 5 ml petri dishes containing only MEA agar wrapped in parafilm similar to the plates containing each isolate. These controls showed that a non-bioluminescent organism or object would still emit stored light that was absorbed before putting it in the luminometer. After examining the control readings, this stored light was determined to account for no greater than 500 relative light units per second (RLU/s) given the brief time that the isolates were exposed to florescent light during the screening, and thus, isolates with a reading of approximately 500 RLU/s or less were considered non-bioluminescent phenotypes. With the exception of Table 3.1, each table outlines the luminometer readings for the isolates that appear in the above figure of the same number.

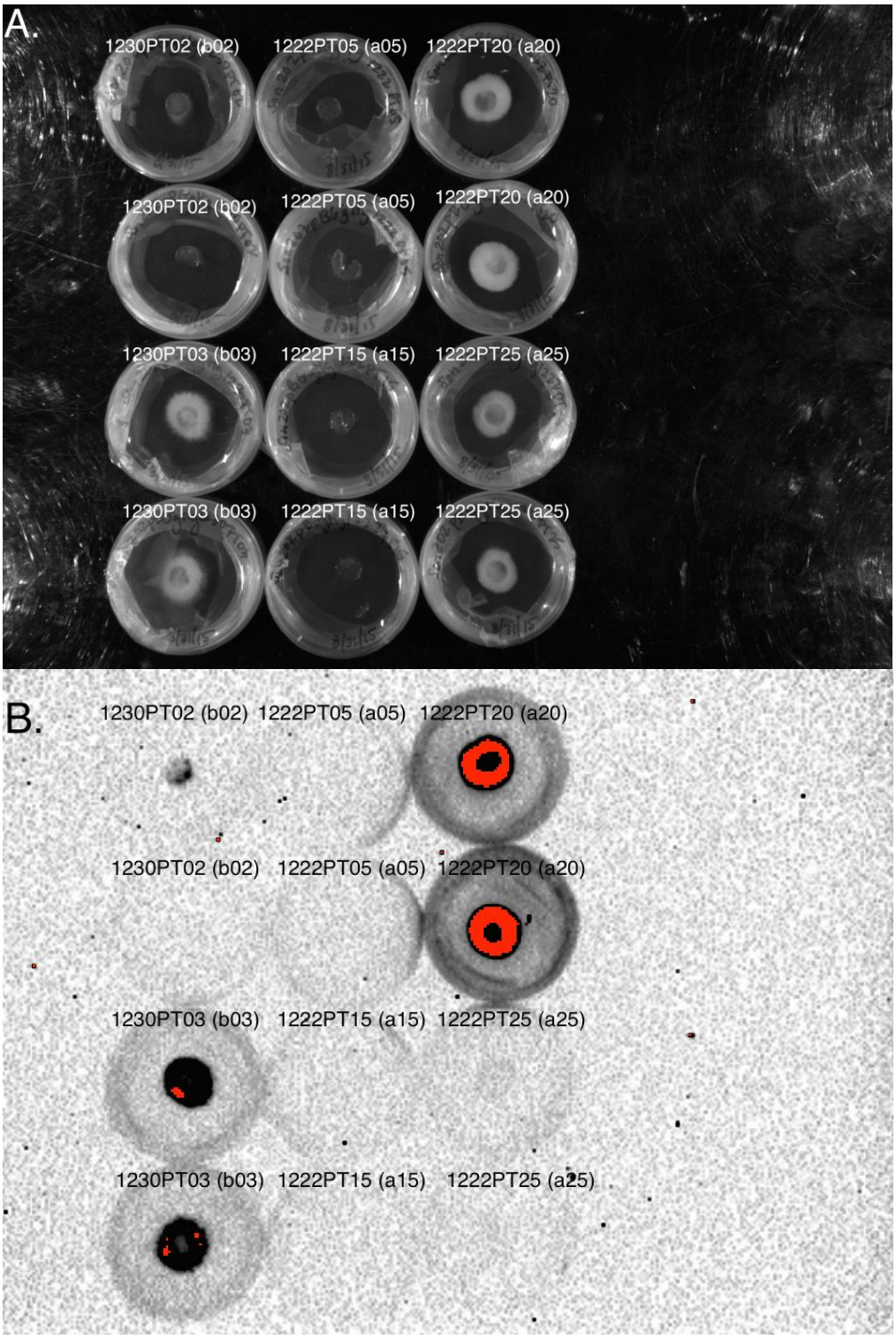

Figure 3.2: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 12 Son202pBGHg *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 12 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.2: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.2 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Son202pBGgHg1222PT05 (a05)	No-Grow	No-Grow
Son202pBGgHg1222PT15 (a15)	No-Grow	No-Grow
Son202pBGgHg1222PT20 (a20)	7030119	6805892
Son202pBGgHg1222PT25 (a25)	220	655
Son202pBGgHg1230PT02 (b02)	459704	15668
Son202pBGgHg1230PT03 (b03)	6022658	4430373

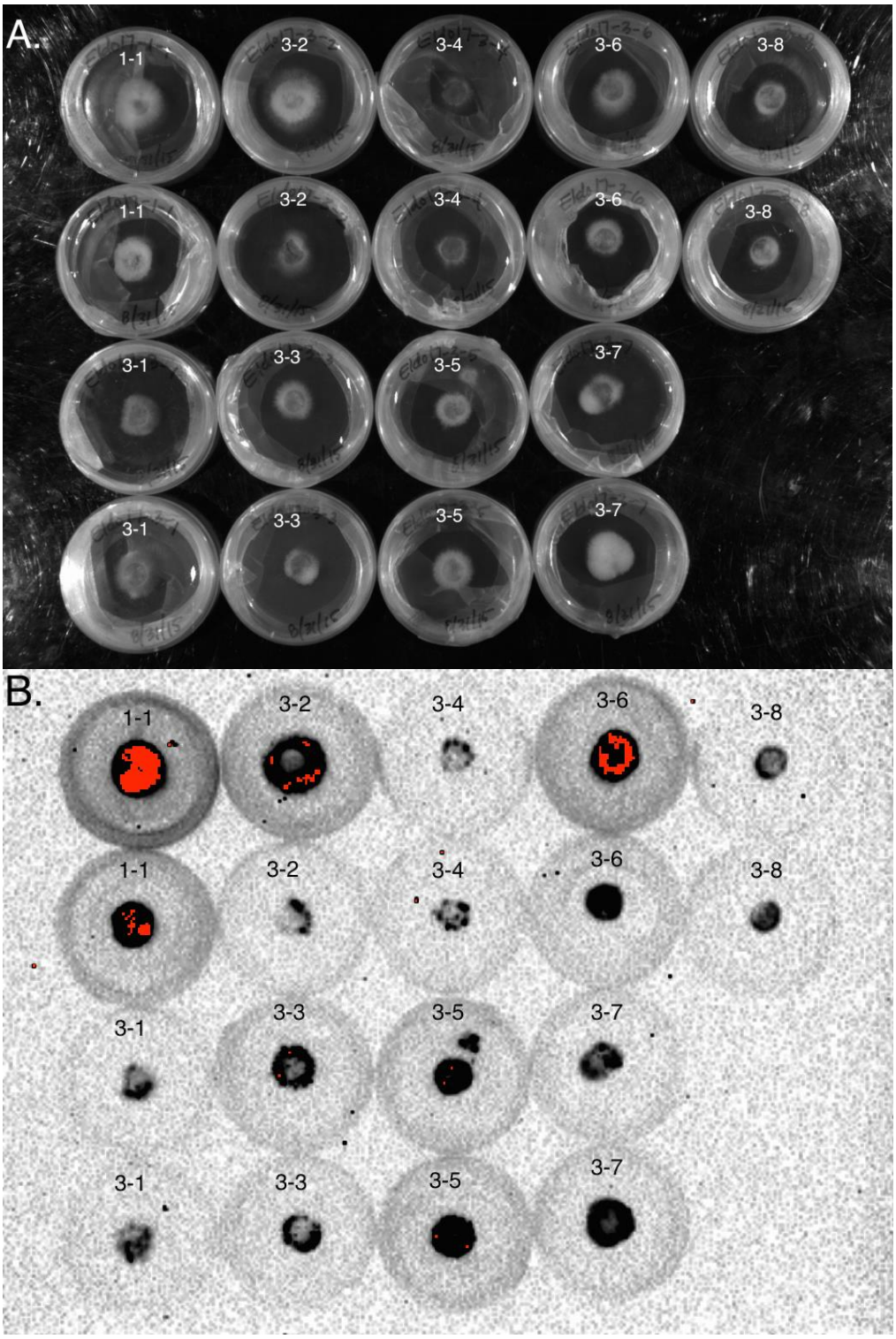

Figure 3.3: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 18 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 18 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.3: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.3 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-1-1	6773810	1541600
Eldo17-3-1	153287	177886
Eldo17-3-2	2548172	104057
Eldo17-3-3	975053	682194
Eldo17-3-4	153403	145820
Eldo17-3-5	827501	1968627
Eldo17-3-6	3390499	481440
Eldo17-3-7	437544	719901
Eldo17-3-8	194637	173223

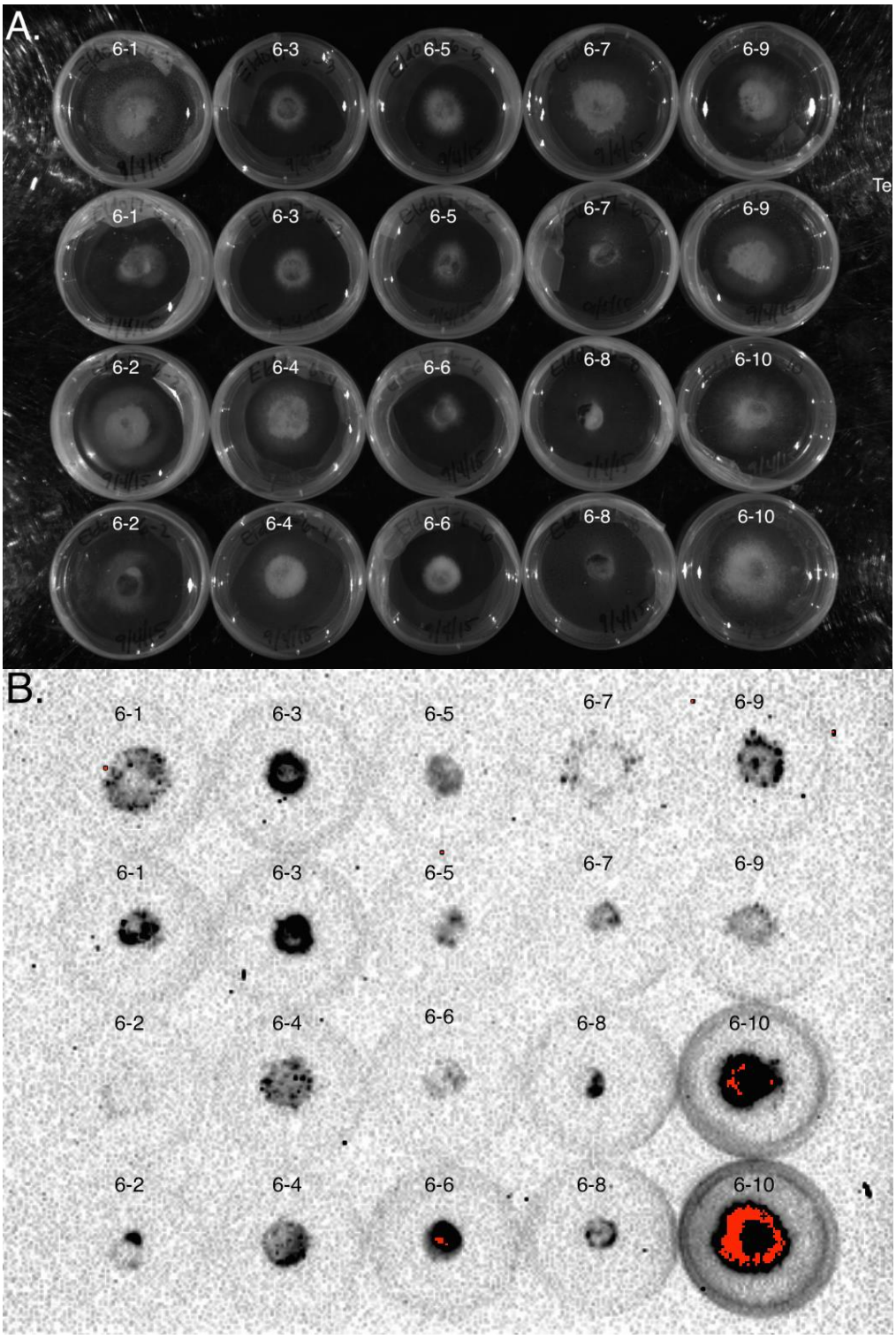

Figure 3.4: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.4: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.4 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-6-1	215129	377653
Eldo17-6-2	17520	55813
Eldo17-6-3	444541	500382
Eldo17-6-4	218194	237812
Eldo17-6-5	96449	64325
Eldo17-6-6	45720	561989
Eldo17-6-7	69656	66593
Eldo17-6-8	105546	80758
Eldo17-6-9	365452	74317
Eldo17-6-10	1832649	6258181

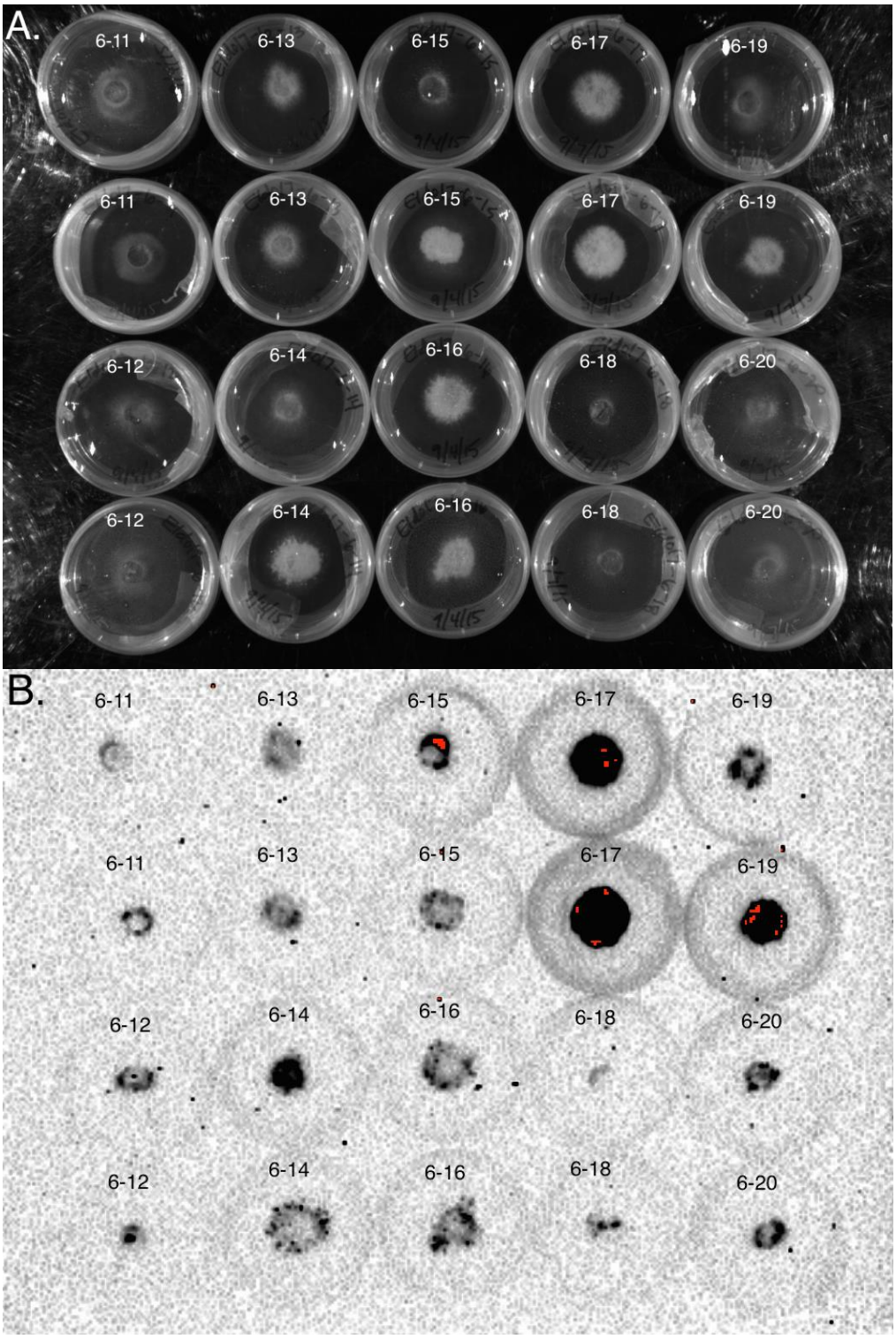

Figure 3.5: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.5: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.5 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-6-11	63296	34322
Eldo17-6-12	93651	70882
Eldo17-6-13	71173	93047
Eldo17-6-14	280398	133912
Eldo17-6-15	140837	434707
Eldo17-6-16	137648	210211
Eldo17-6-17	1443744	1752835
Eldo17-6-18	75123	14708
Eldo17-6-19	243009	1586577
Eldo17-6-20	184341	157704

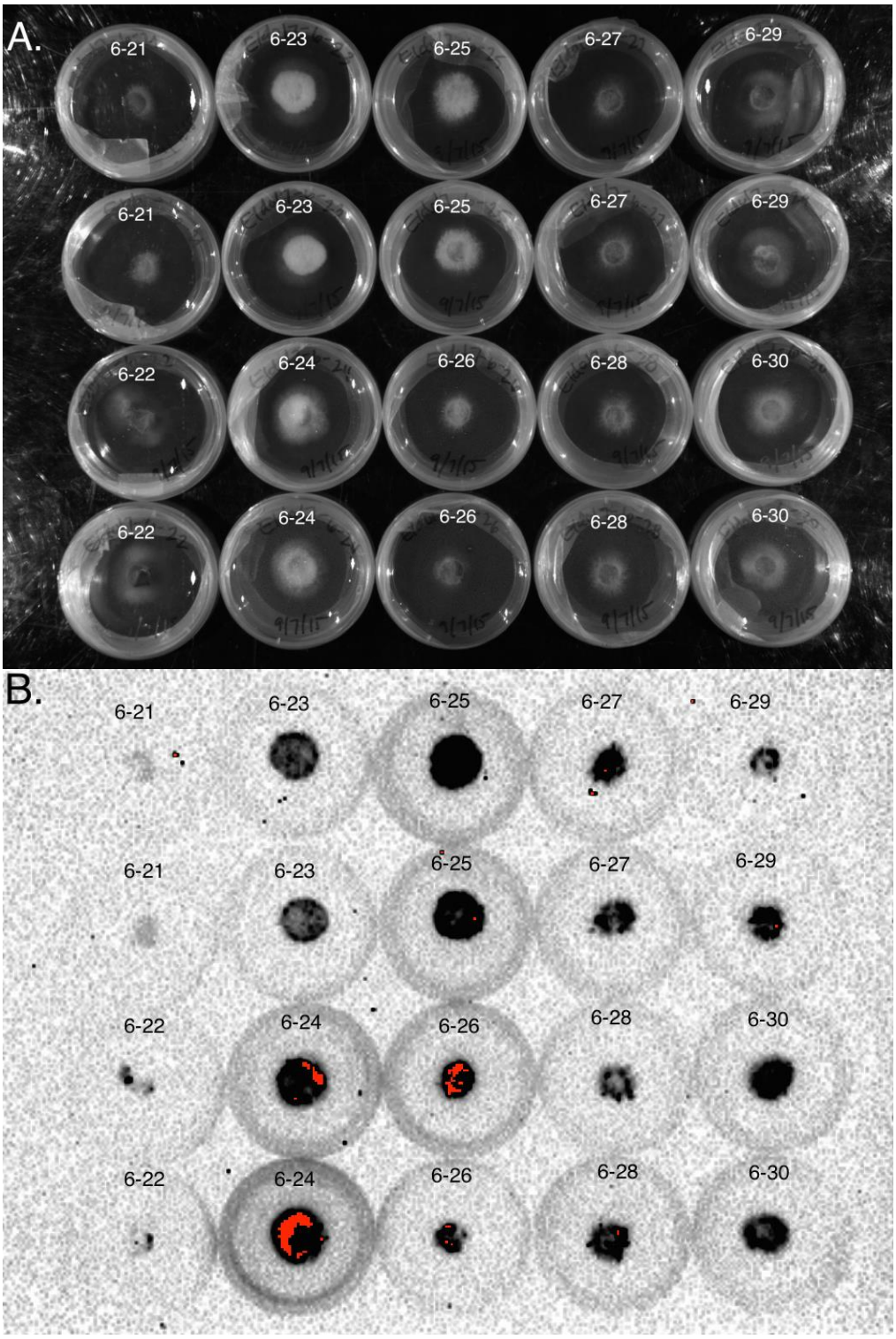

Figure 3.6: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.6: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.6 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-6-21	20347	18234
Eldo17-6-22	55341	23642
Eldo17-6-23	297287	320921
Eldo17-6-24	1552048	3530165
Eldo17-6-25	1405221	1838614
Eldo17-6-26	1356262	385552
Eldo17-6-27	440811	588536
Eldo17-6-28	247565	542018
Eldo17-6-29	185597	652754
Eldo17-6-30	609014	4536328

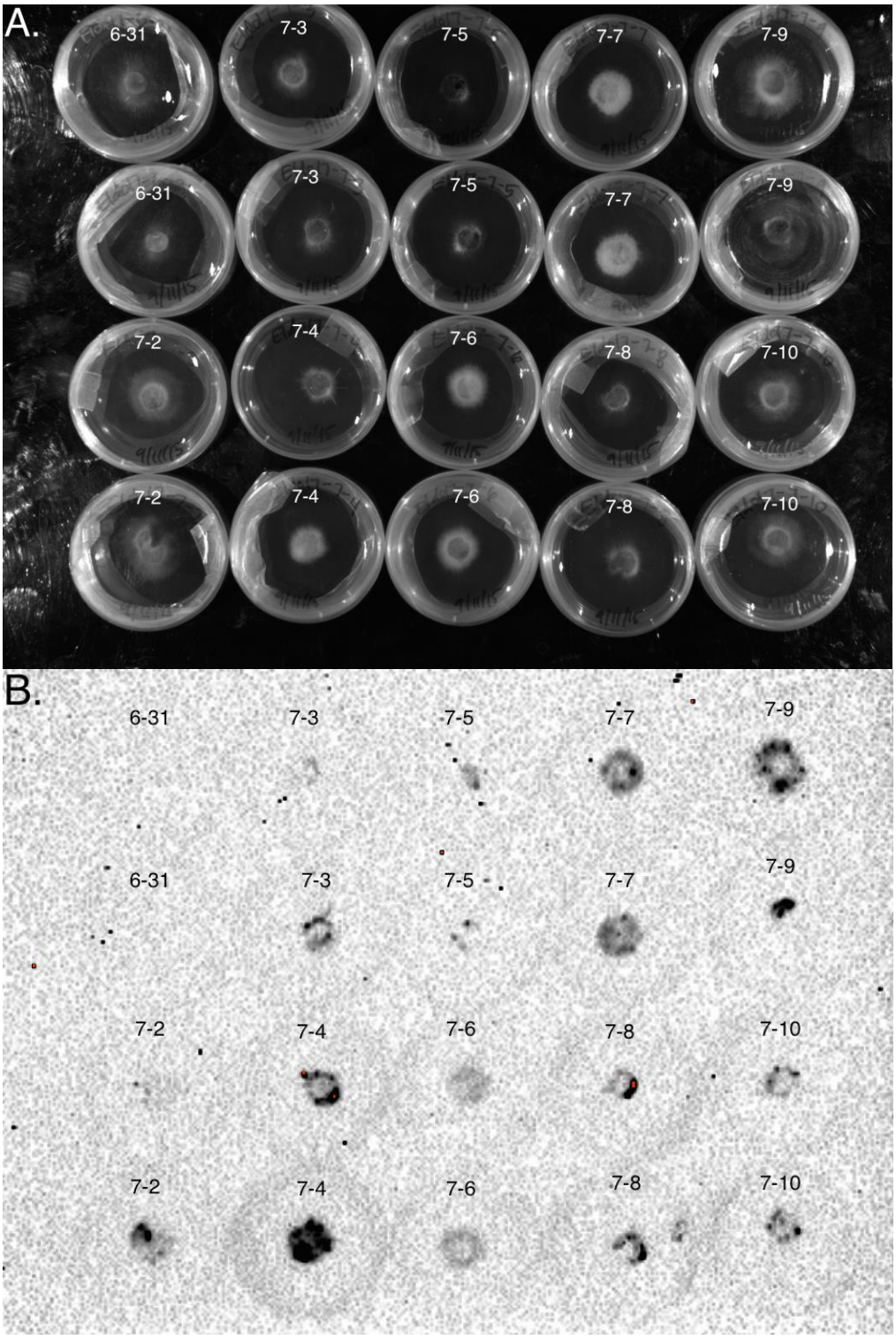

Figure 3.7: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.7: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.7 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-6-31	No-Grow	No-Grow
Eldo17-7-2	13741	93869
Eldo17-7-3	21122	76043
Eldo17-7-4	186151	516321
Eldo17-7-5	20294	13395
Eldo17-7-6	35044	48012
Eldo17-7-7	123812	95750
Eldo17-7-8	118971	116347
Eldo17-7-9	312281	128760
Eldo17-7-10	166509	99020

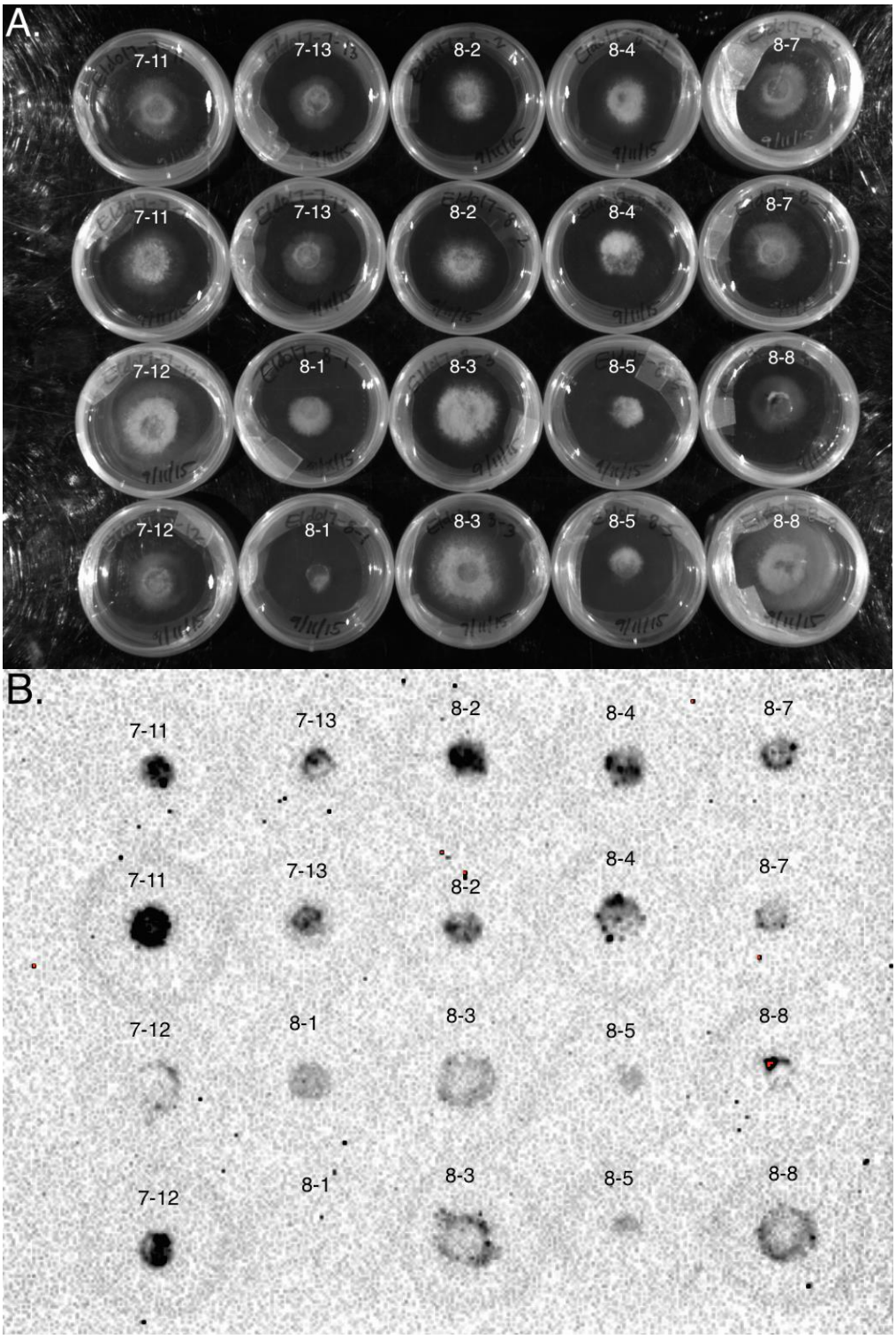

Figure 3.8: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.8: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.8 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-7-11	308029	73924
Eldo17-7-12	140451	19513
Eldo17-7-13	90157	63248
Eldo17-8-1	85530	No-Grow
Eldo17-8-2	264792	85586
Eldo17-8-3	65507	156225
Eldo17-8-4	210877	152077
Eldo17-8-5	17138	22044
Eldo17-8-7	94815	49674
Eldo17-8-8	108754	163922

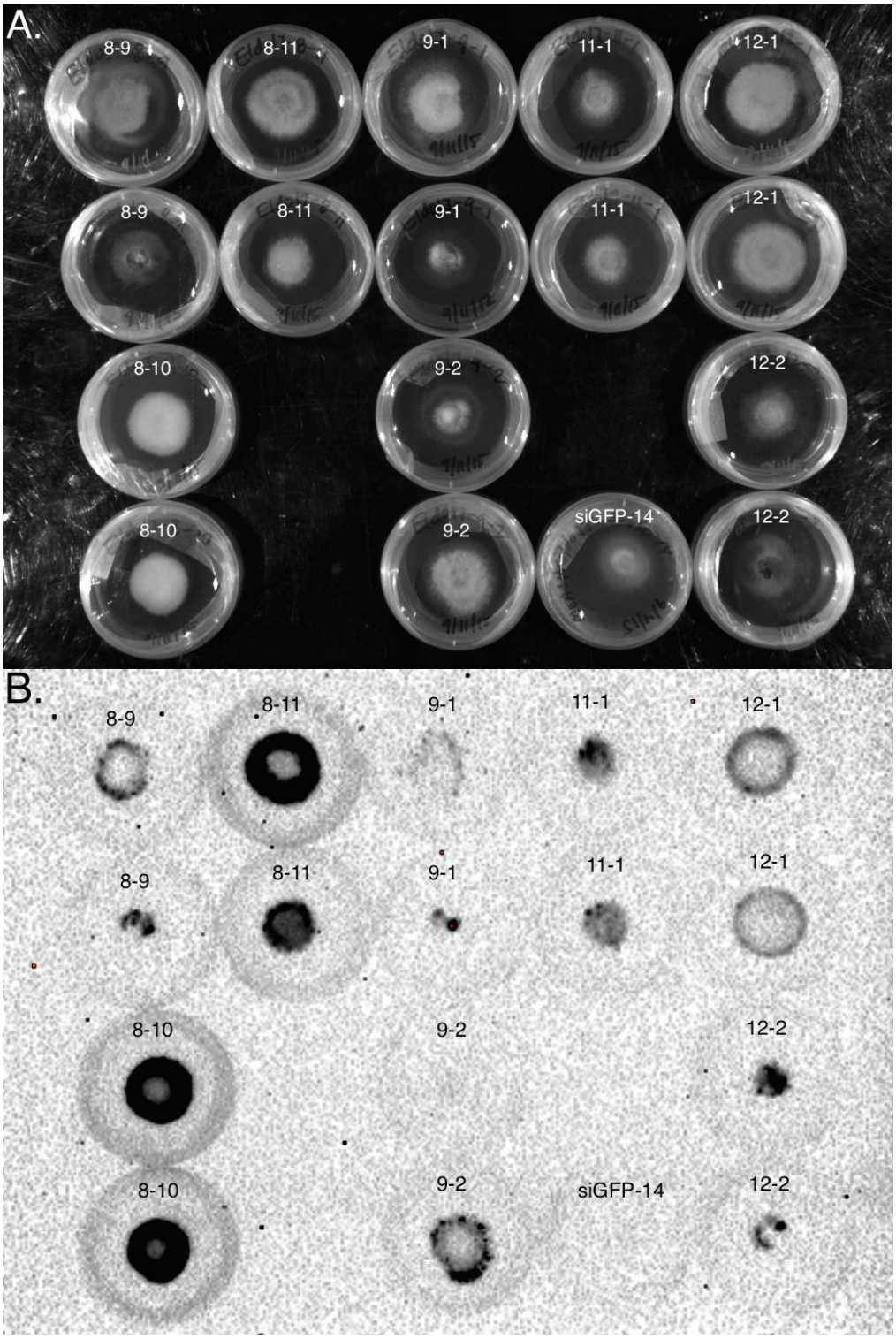

Figure 3.9: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 17 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 17 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.9: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.9 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-8-9	177597	124880
Eldo17-8-10	1844209	1483800
Eldo17-8-11	1372177	401876
Eldo17-9-1	50821	116257
Eldo17-9-2	387513	6841
Eldo17-11-1	177780	220243
Eldo17-12-1	153216	105823
Eldo17-12-2	224359	88309

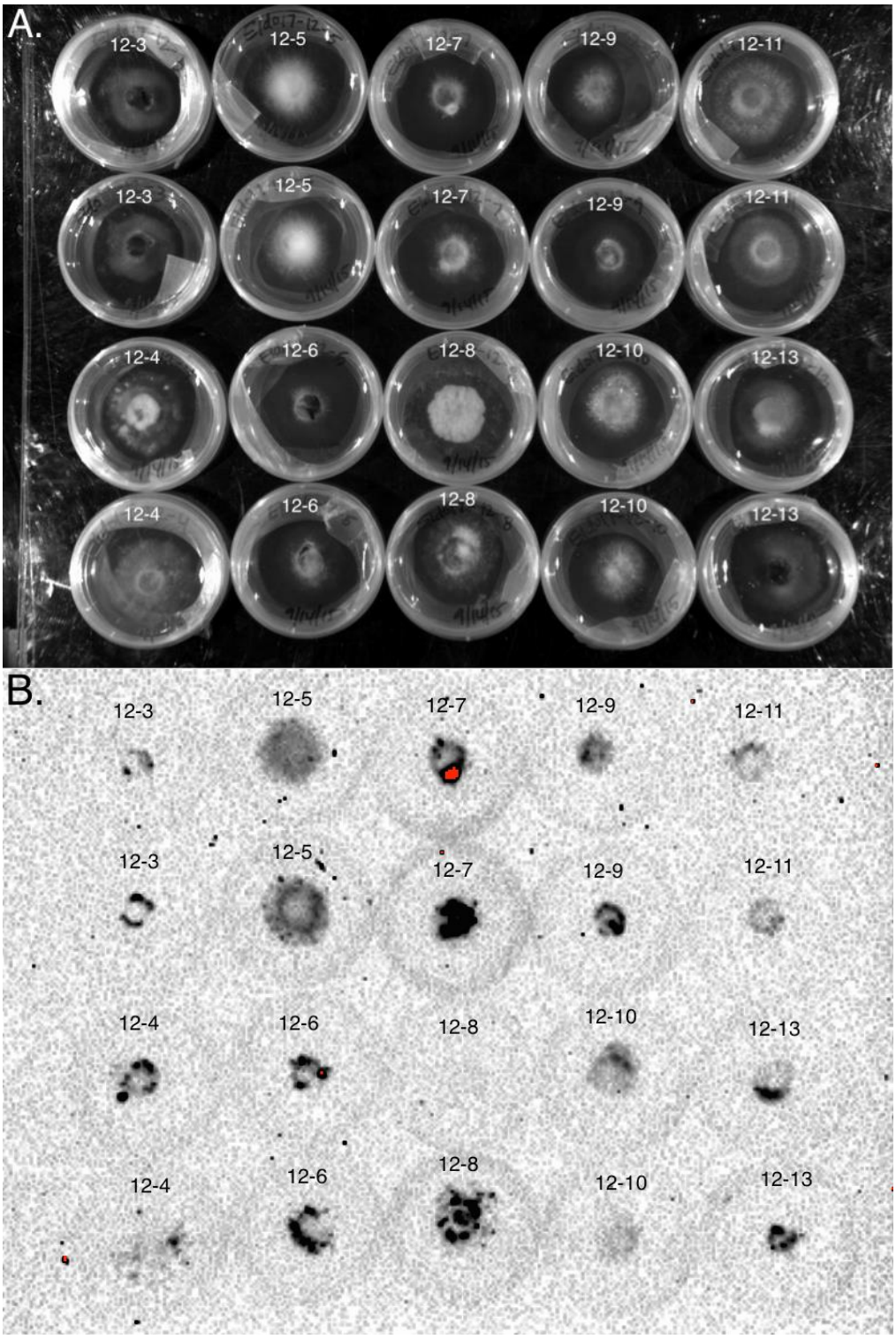

Figure 3.10: A) A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.10: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.10 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-12-3	42555	65786
Eldo17-12-4	190999	78791
Eldo17-12-5	241711	346427
Eldo17-12-6	182169	222373
Eldo17-12-7	652456	738830
Eldo17-12-8	7050	394344
Eldo17-12-9	91087	165614
Eldo17-12-10	115825	30553
Eldo17-12-11	65029	56694
Eldo17-12-13	83015	120479

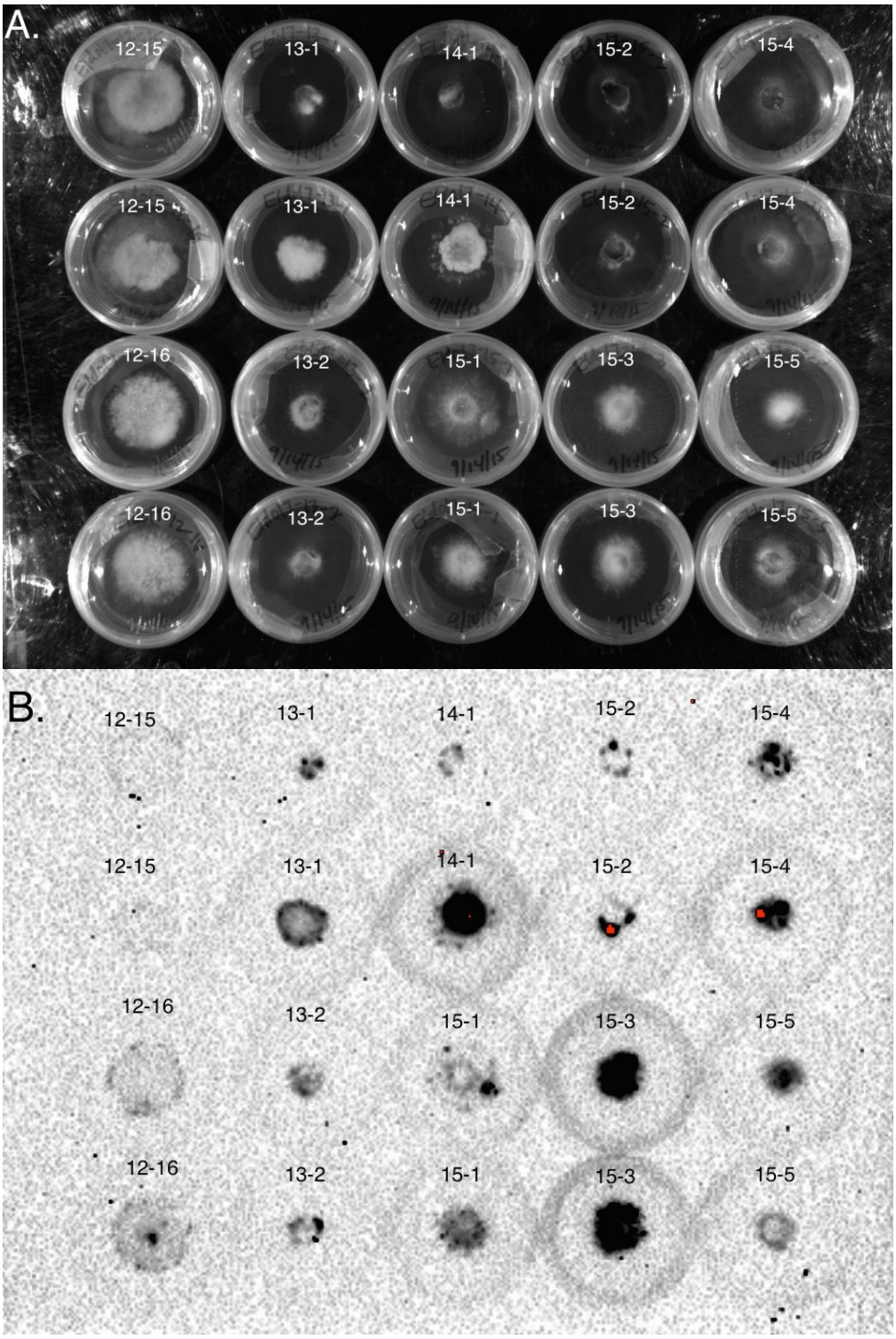

Figure 3.11: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.11: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.11 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-12-15	15298	15168
Eldo17-12-16	55924	127377
Eldo17-13-1	57910	161228
Eldo17-13-2	57425	63276
Eldo17-14-1	1009479	31580
Eldo17-15-1	142486	142359
Eldo17-15-2	42027	266493
Eldo17-15-3	863106	1172886
Eldo17-15-4	247153	758018
Eldo17-15-5	217294	55476

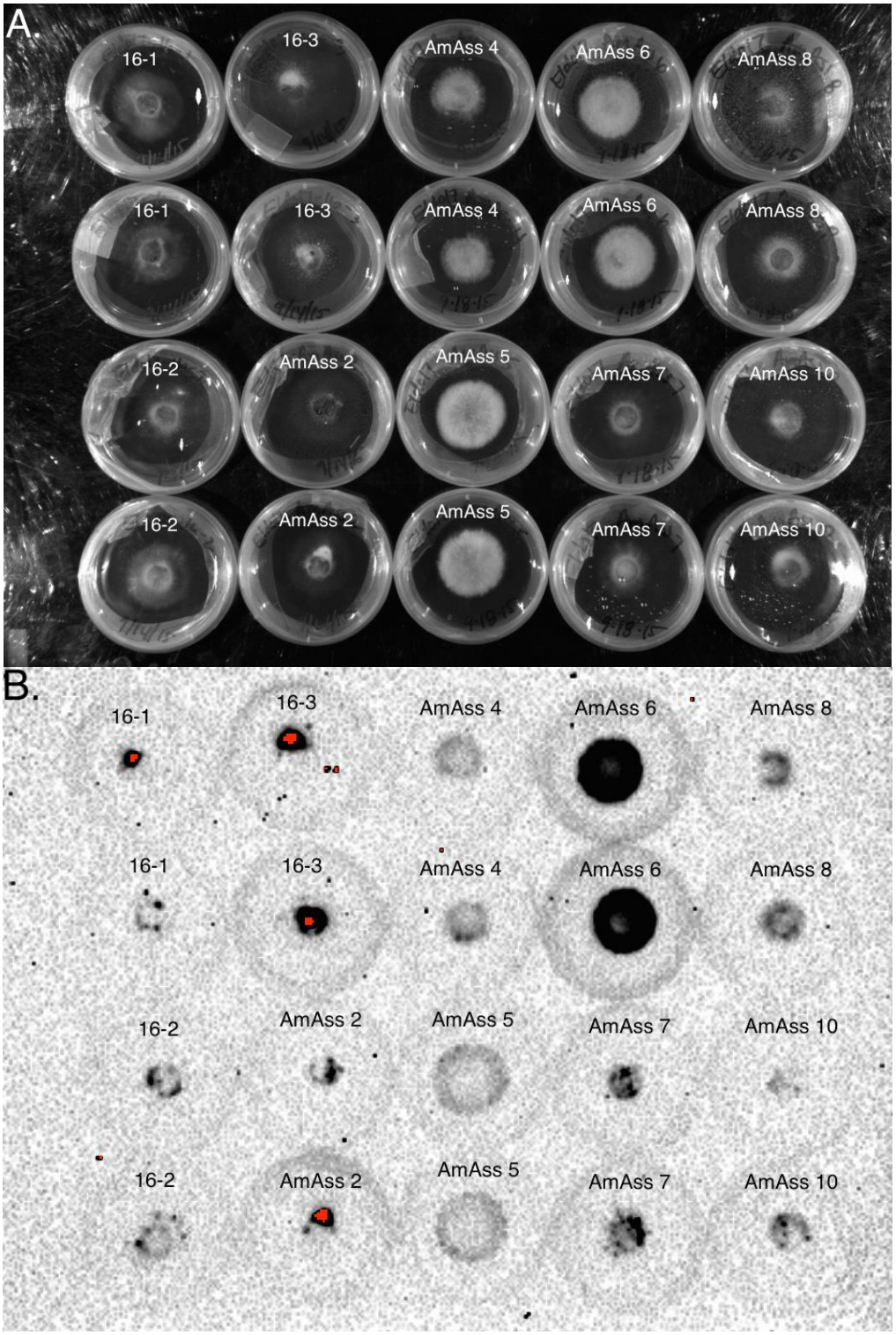

Figure 3.12: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.12: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.12 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-16-1	290906	51293
Eldo17-16-2	87691	97259
Eldo17-16-3	631167	820739
ELDO17-AmAss2	86313	574391
ELDO17-AmAss4	63296	78292
ELDO17-AmAss5	81181	77936
ELDO17-AmAss6	793625	852708
ELDO17-AmAss7	161791	372078
ELDO17-AmAss8	92499	114058
ELDO17-AmAss10	27967	98059

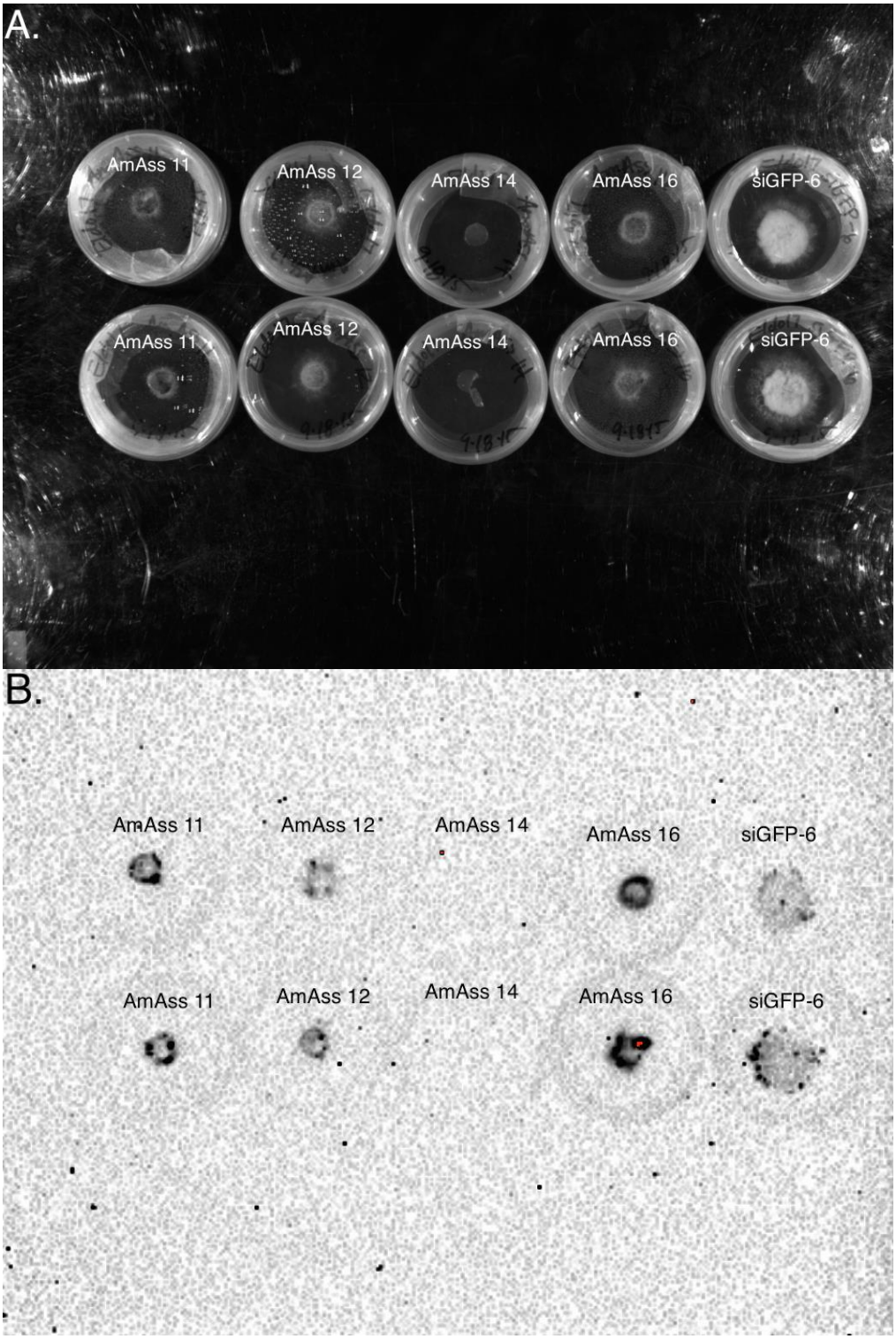

Figure 3.13: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 10 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 10 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.13: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.13 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
ELDO17-AmAss11	125872	141179
ELDO17-AmAss12	54402	74817
ELDO17-AmAss14	No-Grow	No-Grow
ELDO17-AmAss16	149695	268595
Eldo17-siGFP-6	87121	171186

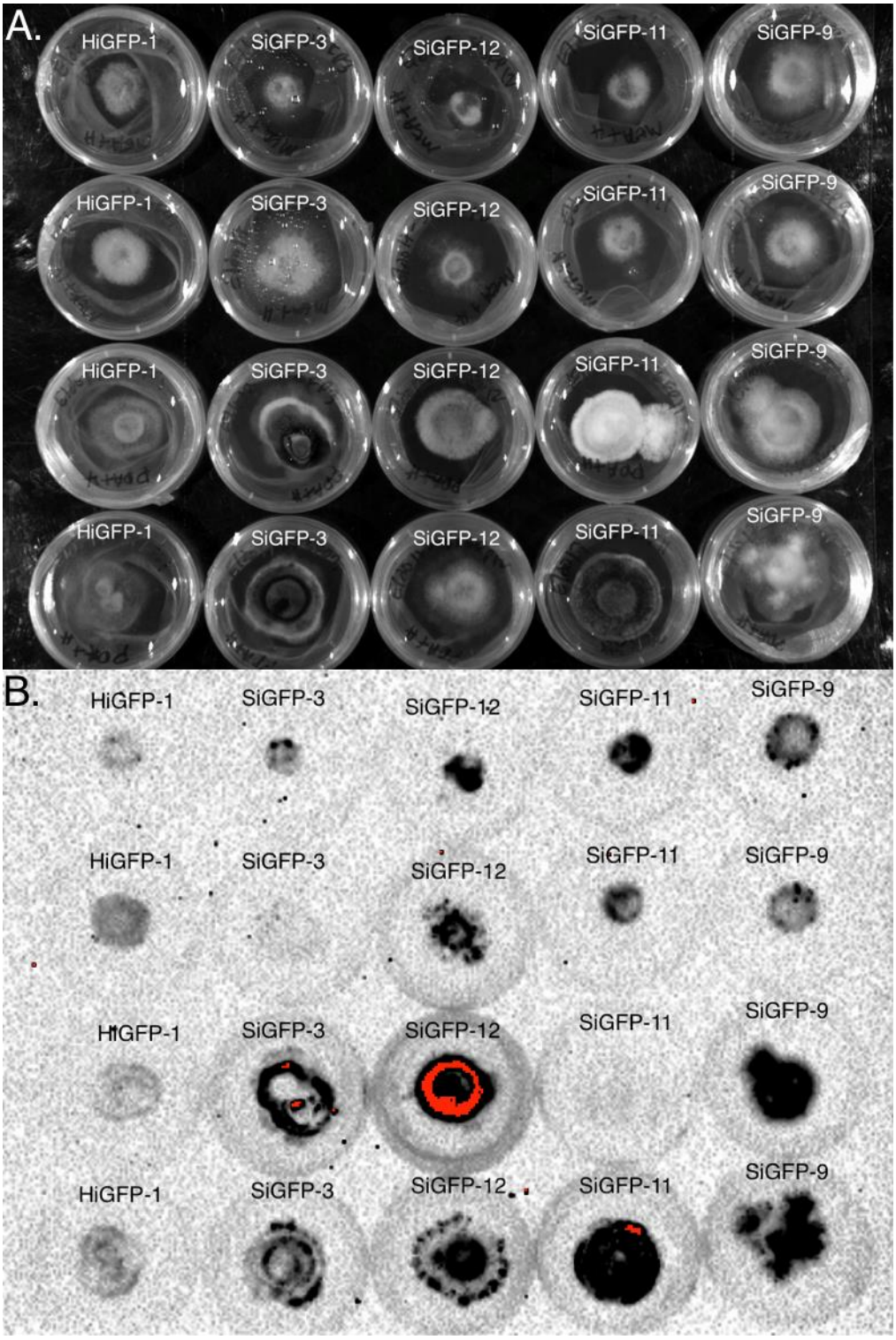

Figure 3.14: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system with bioluminescence appearing as black and red on a white background. In the images, the plates in the top two show *A. mellea* isolates on MEA agar, and the bottom two rows show *A. mellea* isolates on PDA.

Table 3.14: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.14 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	Luminometer Reading (RLU\s)		Agar
Eldo17-hiGFP-1	117013	36728	MEA
Eldo17-hiGFP-1	239034	195706	PDA
Eldo17-siGFP-3	26020	7509	MEA
Eldo17-siGFP-3	13482	50449	PDA
Eldo17-siGFP-9	1571754	1161898	MEA
Eldo17-siGFP-9	2559566	4313681	PDA
Eldo17-siGFP-11	100989	138958	MEA
Eldo17-siGFP-11	27267	986541	PDA
Eldo17-siGFP-12	74012	386756	MEA
Eldo17-siGFP-12	3428452	1632210	PDA

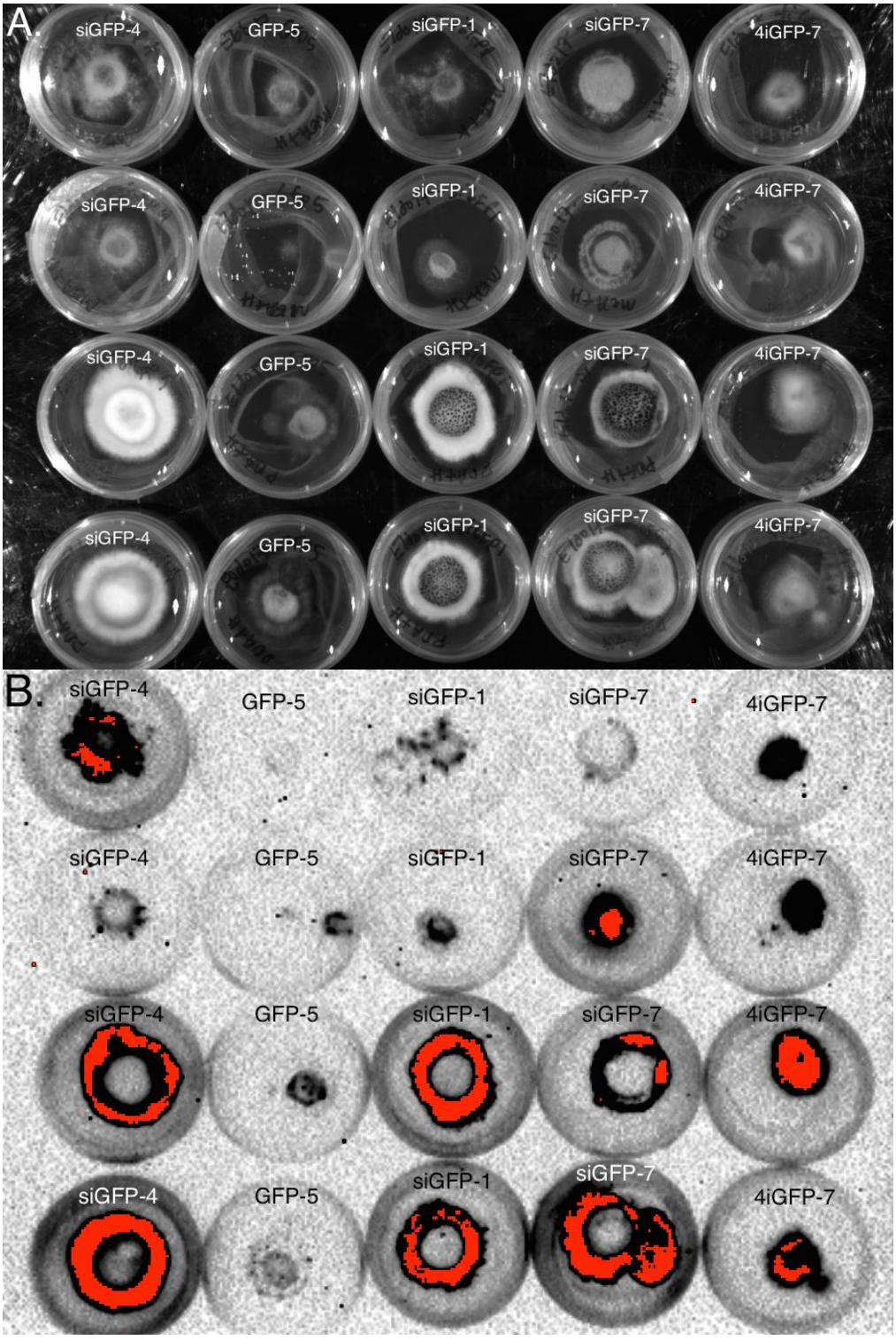

Figure 3.15: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system with bioluminescence appearing as black and red on a white background. In the images, the plates in the top two show *A. mellea* isolates on MEA agar, and the bottom two rows show *A. mellea* isolates on PDA.

Table 3.15: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.15 on malt extract agar and potato dextrose agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	Luminometer Reading (RLU\s)		Agar
Eldo17-GFP-5	36903	31657	MEA
Eldo17-GFP-5	763111	314585	PDA
Eldo17-4iGFP-7	1045620	1641344	MEA
Eldo17-4iGFP-7	17168112	838544	PDA
Eldo17-siGFP-1	50995	222778	MEA
Eldo17-siGFP-1	40853	165297	PDA
Eldo17-siGFP-4	32066	227884	MEA
Eldo17-siGFP-4	93571	741568	PDA
Eldo17-siGFP-7	30437	4803	MEA
Eldo17-siGFP-7	26891	328690	PDA

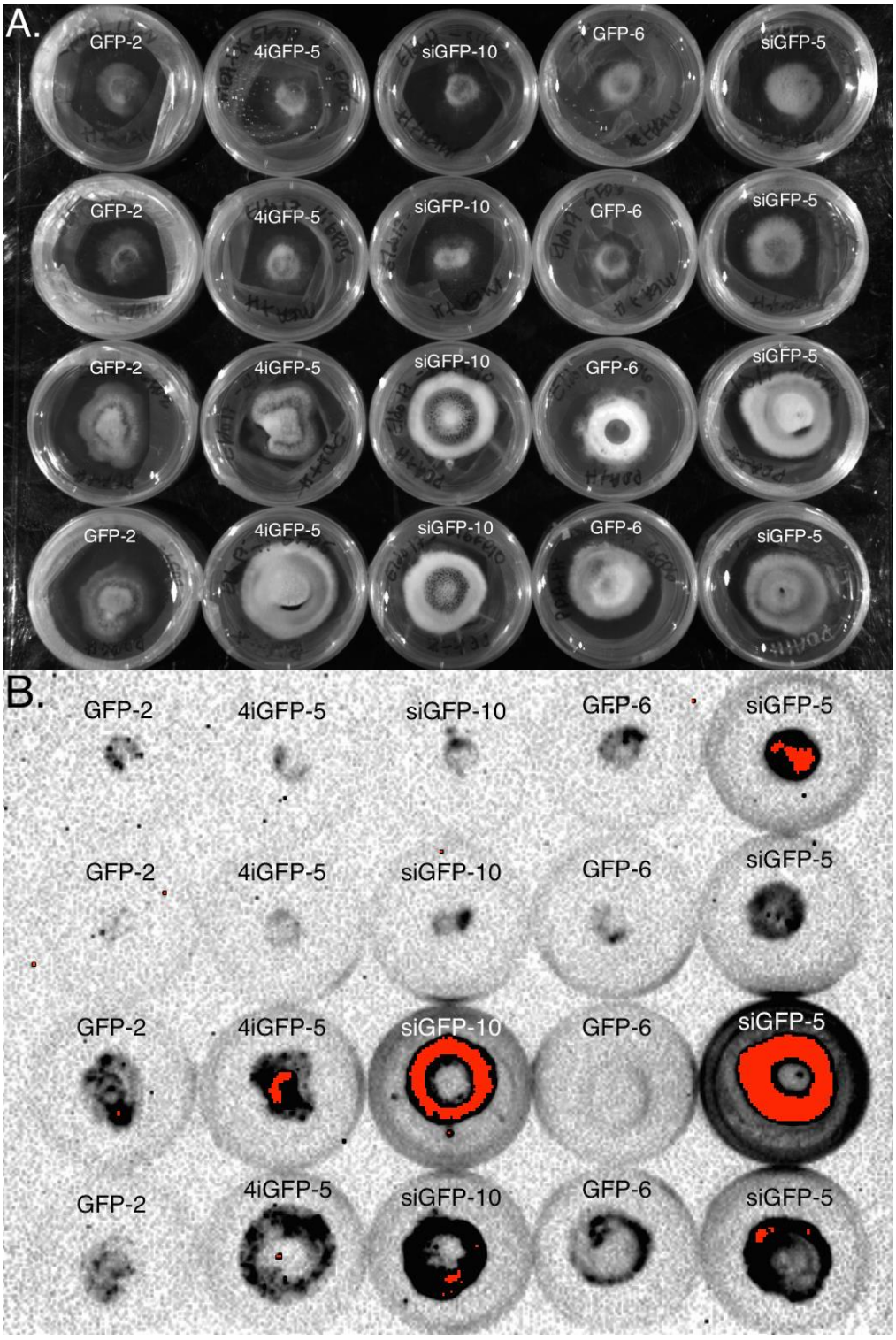

Figure 3.16: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system with bioluminescence appearing as black and red on a white background. In the images, the plates in the top two show *A. mellea* isolates on MEA agar, and the bottom two rows show *A. mellea* isolates on PDA.

Table 3.16: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.16 on malt extract agar and potato dextrose agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	Luminometer Reading (RLU\s)		Agar
Eldo17-GFP-2	97407	17377	MEA
Eldo17-GFP-2	2643408	86001	PDA
Eldo17-GFP-6	30324	61398	MEA
Eldo17-GFP-6	1245143	20532	PDA
Eldo17-4iGFP-5	21421	22885	MEA
Eldo17-4iGFP-5	119231	218341	PDA
Eldo17-siGFP-5	1119997	444217	MEA
Eldo17-siGFP-5	4210935	9838950	PDA
Eldo17-siGFP-10	51395	70057	MEA
Eldo17-siGFP-10	83990	230604	PDA

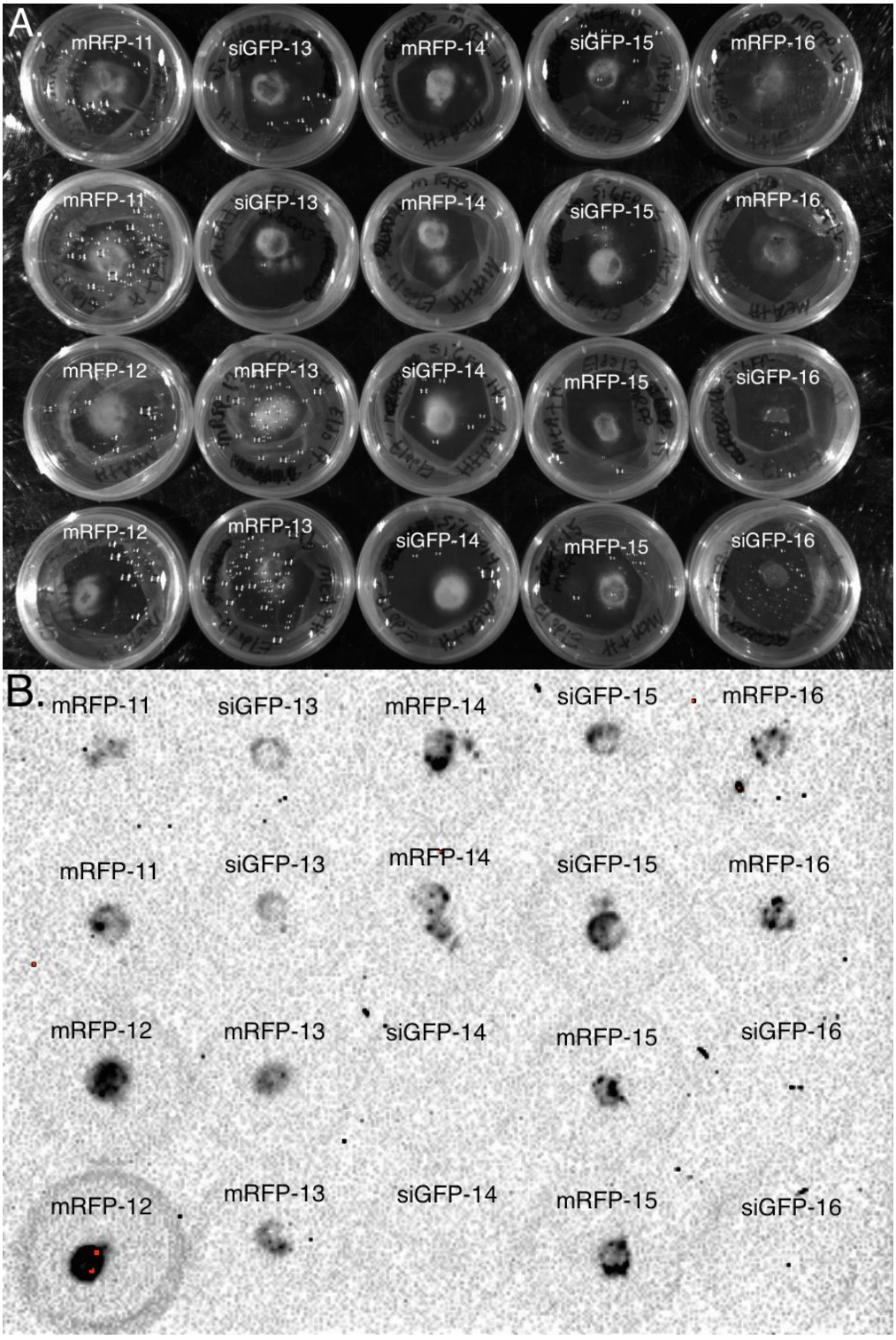

Figure 3.17: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.17: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.1 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-mRFP-11	62302	51319
Eldo17-mRFP-12	60023	128965
Eldo17-mRFP-13	152747	163671
Eldo17-mRFP-14	71661	38830
Eldo17-mRFP-15	52467	77314
Eldo17-mRFP-16	47951	63571
Eldo17-siGFP-13	180636	66188
Eldo17-siGFP-14	216	4547
Eldo17-siGFP-15	173206	59374
Eldo17-siGFP-16	No-Grow	No-Grow

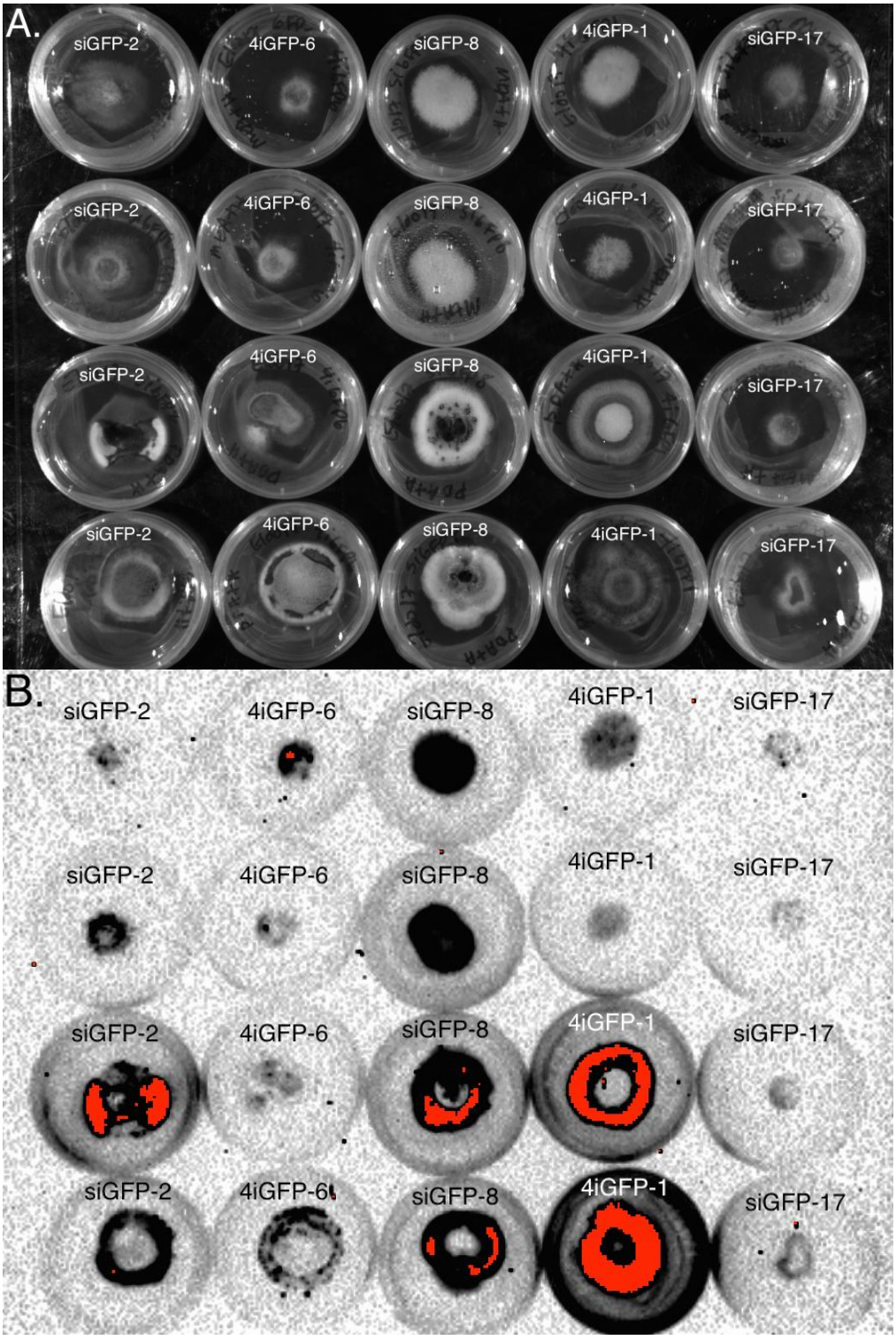

Figure 3.18: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system with bioluminescence appearing as black and red on a white background. In the images, the plates in the top two show *A. mellea* isolates on MEA agar, and the bottom two rows show *A. mellea* isolates on PDA.

Table 3.18: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.18 on malt extract agar and potato dextrose agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	Luminometer Reading (RLU\s)		Agar
Eldo17-4iGFP-1	146802	330563	MEA
Eldo17-4iGFP-1	6307676	3170083	PDA
Eldo17-4iGFP-6	21865	63107	MEA
Eldo17-4iGFP-6	38526	5848071	PDA
Eldo17-siGFP-2	92048	290521	MEA
Eldo17-siGFP-2	109537	628497	PDA
Eldo17-siGFP-8	1069888	1234259	MEA
Eldo17-siGFP-8	233759	109801	PDA
Eldo17-siGFP-17	102519	20738	MEA
Eldo17-siGFP-17	45233	47543	PDA

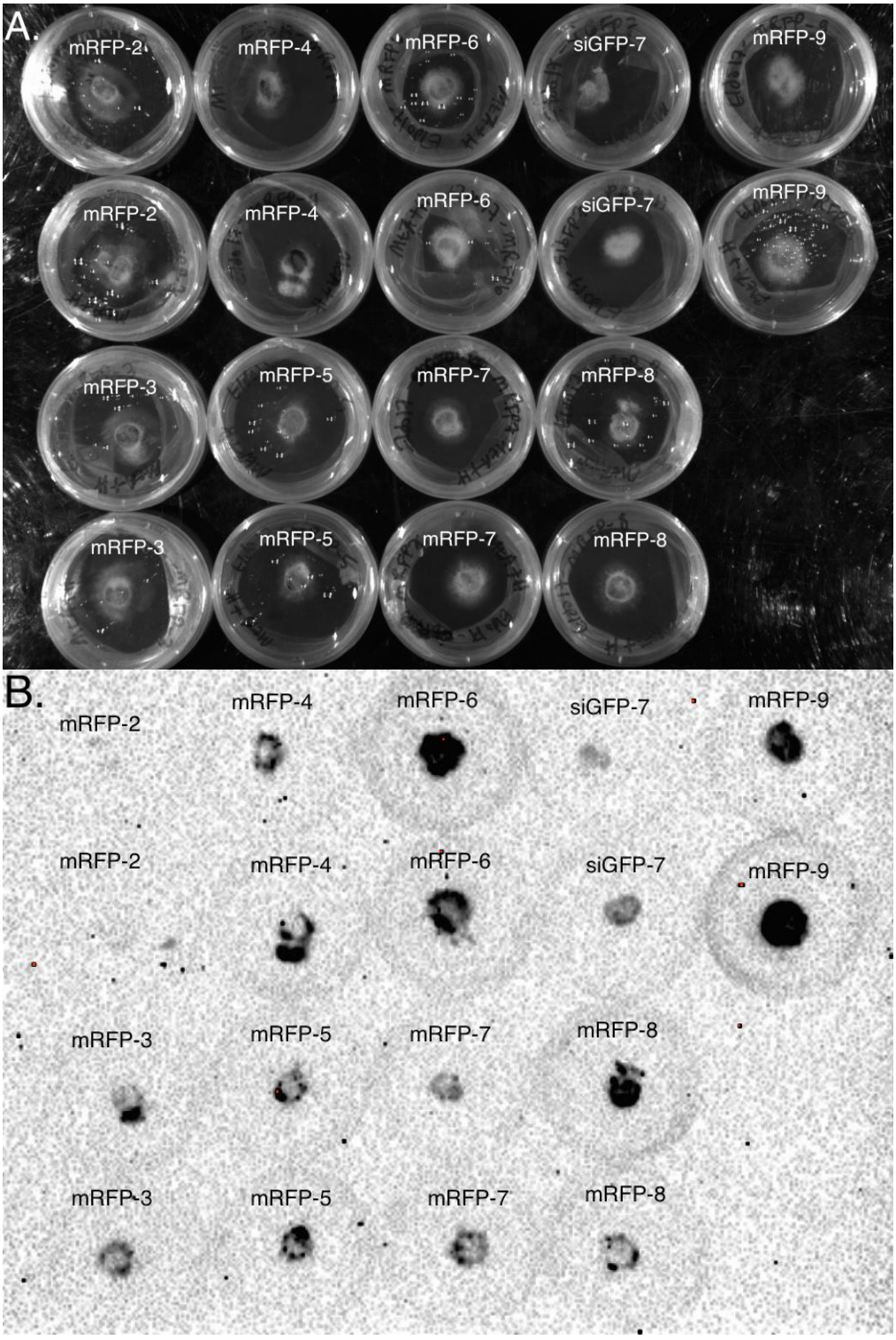

Figure 3.19: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 20 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 20 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.19: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.19 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-mRFP-2	7392	4676
Eldo17-mRFP-3	11790	203772
Eldo17-mRFP-4	90538	89971
Eldo17-mRFP-5	54918	241437
Eldo17-mRFP-6	261981	632455
Eldo17-mRFP-7	10961	63247
Eldo17-mRFP-8	277638	113008
Eldo17-mRFP-9	92418	791020
Eldo17-siGFP-7	30437	4803

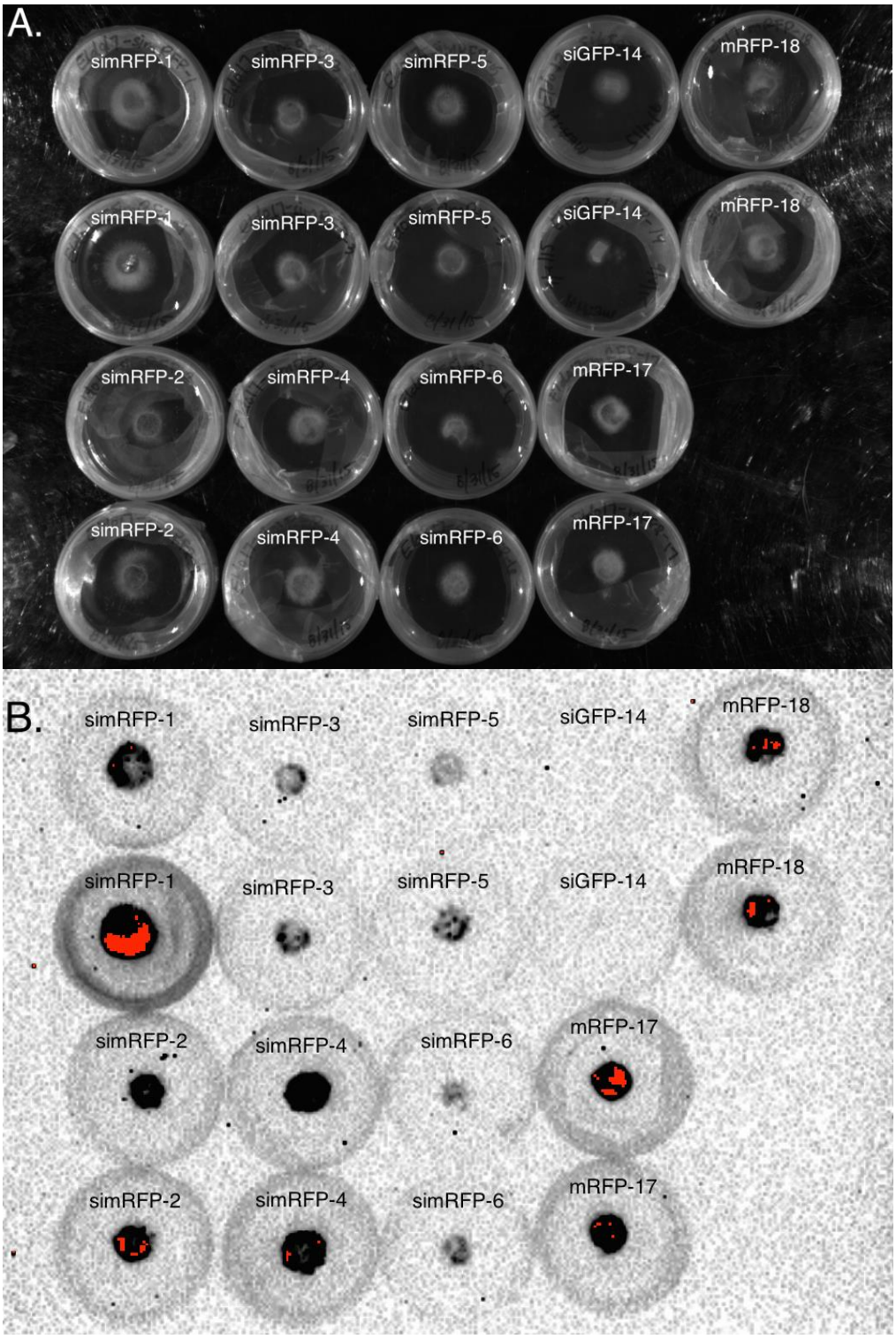

Figure 3.20: A) A bright-field image taken with the BioRad ChemiDoc Mp imaging system of 18 Eldo17 *A. mellea* isolates under LED light at exposure time of 0.1 sec. B) A bioluminescence image of the same 18 isolates at an exposure time of 5 min. taken with the BioRad ChemiDoc MP imaging system. In this image, bioluminescence appears as black on a white background, and red indicates particularly intense bioluminescence that saturates the camera at that location.

Table 3.20: A table containing the Berthold Detection System FB 12 luminometer readings for the set of isolates in Figure 3.20 on malt extract agar. Berthold Detection System set for a 2 sec. delay time with a 5 sec. reading time. Luminometer readings are in relative light units per second.

Isolate	**Luminometer Reading (RLU\s)**	
Eldo17-simRFP-1	584982	4372204
Eldo17-simRFP-2	618335	1068201
Eldo17-simRFP-3	110646	133159
Eldo17-simRFP-4	1306116	865123
Eldo17-simRFP-5	40406	205098
Eldo17-simRFP-6	47390	74192
Eldo17-siGFP-14	576	664
Eldo17-mRFP-17	1325903	906867
Eldo17-mRFP-18	893463	699292

3.5 Isolates of interest

As seen in the above Figures 3.2 through 3.20, the vast majority of the isolates showing growth were determined to be bioluminescent. In Tables 3.2 through 3.20, bioluminescence was further confirmed with the majority of luminometer readings being in the high thousands, which is in the range of bioluminescence.

Out of the 154 individual isolates, the isolate Son202pBGgHg1222PT25 (a25) in Table 3.2 and Figure 3.2 was determined to be the only non-bioluminescent phenotype. Despite having substantial growth, the luminometer readings for the isolate were consistent with that of the control group of isolates in Figure 3.1.

In Table 3.17, isolate Eldo17-siGFP-14 had conflicting readings with one isolate reading indicating a non-bioluminescent phenotype which was under the threshold of 500 RLU/s and another noticeably above 500 RLU/s indicating bioluminescence. Figure 3.17 supported the reading of non-bioluminescence as no bioluminescence was shown in the bioluminescence image while the bright-field image showed considerable growth. Follow up analysis in Table 3.20 and Figure 3.20 revealed that Eldo17-siGFP-14 still exhibited bioluminescence in both isolates despite being particularly dim.

3.6 Estimation number of *A. mellea* bioluminescence genes

With all the isolates successfully screened for the presence of a bioluminescent phenotype, one non-bioluminescent *A. mellea* mutant was observed out of the 154 total mutated isolates. Using this ratio of desired phenotype in the given sampling size a formula was used to produce an estimation of the number of genes that directly participate in bioluminescence on the *A. mellea* genome given its known total size and average length of each gene (Collins et al 2013). The statistical equation that was used is

outlined along with its variables in Figure 2.1 (Dowdy, 1991).

From this equation, a point estimate of 240 genes was calculated. However, a confidence interval was needed to account for standard deviation in the random sample of mutants. The number of non-bioluminescent mutations was assumed to follow a Poisson distribution, a frequency distribution in which the probability of a number of events occurs in a fixed space independently of one another. The confidence interval formula used for a 95% confidence interval given that the process follows a Poisson distribution is outlined in Figure 2.2 (Dowdy, 1991). From these calculations, it was estimated with 95% confidence that 210 to 272 genes are present on the *A. mellea* genome that code for bioluminescence.

Section 4: Discussion

For this research, an estimation of the number of genes *A. mellea* has on its genome that directly participate in bioluminescence was calculated. The screening of *A. mellea* isolates for bioluminescence was straightforward, and resulted in one non-bioluminescent phenotype out of 154 total screened isolates. From this, the estimation was produced given the percentage of non-bioluminescent mutations observed in a screened population of random mutants, the estimated size of the *A. mellea* genome, and average length of a gene on that genome. Thus, with a 95 percent confidence interval, it was determined that there are somewhere between 210 to 272 genes on the *A. mellea* genome that code for bioluminescence.

For clarification, the equation used to produce the estimation of the number of bioluminescence genes can be compared to throwing darts at a dartboard. In this analogy, the size of the dartboard represents the total size of the *A. mellea* genome, and randomly thrown darts are the number of mutated isolates used in the study. The bull's-eye can be thought of as the number of bioluminescent genes. Thus, a hit on the bull's-eye is the same as getting a non-bioluminescent mutant. Given the number of random throws at this dartboard, the size of the dartboard, and number of times the bull's-eye is hit, an estimation can be made on the size of the dartboard's bull's-eye. This estimation of the size of the bull's eye on the dartboard is analogous to the estimated number of bioluminescence genes on the genome.

It is impossible to conduct any statistical analysis without making some necessary assumptions about the data; this calculation is no different. Assumptions were carefully made so that an accurate estimation of the total number of genes that directly participate

in *A. mellea* bioluminescence could be predicted given current knowledge. These assumptions include:

1. Insertion by the *Agrobacterium* into the host genome was absolutely random.
2. All genes have the same probability of being disrupted
3. Each insertion successfully produced a mutant phenotype
4. Natural variation in bioluminescent intensity is null.

The estimation of bioluminescence genes is only as valid as its given assumptions about the *A. mellea* genome and insertional mutagenesis. Inaccurate or oversimplified assumptions could result in inaccurate conclusions, but these assumptions were necessary to produce estimation.

The following is one example on why assumptions are necessary and how a statistical estimation can differ depending on the validity of that assumption. The lengths of all *A. mellea* bioluminescence genes are unknown. Therefore, it had to be assumed that all bioluminescence genes in the *A. mellea* genome were of average gene length for that particular genome as determined by Collins (2013). If, in actuality, the bioluminescence coding genes were determined to be smaller than average gene length on average, the genes would have a less likely chance of getting disrupted in contrast to larger genes. Thus, it would reason, the resulting estimation of genes would be falsely higher than previously stated.

Future researchers can use this estimation on the number of genes that code for bioluminescence in determining how many T-DNA transformed isolated are realistically necessary to conclude their research into the bioluminescent genes and protein pathways of *A. mellea*. This should save the researchers valuable resources in creating and growing

only as many T-DNA insertions as are sufficient given the eventual diminishing returns when investing in creating additional mutants to screen.

This research can be continued by producing more mutated isolates to add to the sample size of the estimation and further improve the estimation by narrowing down the confidence interval on the exact number of bioluminescent genes of *A. mellea.* Additionally, the screened isolates of *A. mellea* from this study will be used for further studies into identifying which genes were disrupted during the mutation process through forward genetics. This will lead to the identification of all bioluminescent genes and subsequent protein pathway in the fungus *A. mellea*.

Appendix I: DEFINITIONS AND ABBREVIATIONS

- Adaptive Significance, *noun* – the beneficial qualities, in terms of increased survival and reproduction, that a trait conveys
- *Agrobacterium*, *noun* – a subdivision of Bacteria well known for its ability to transfer DNA between itself and other host organisms, primarily plants and fungi
- Armillaria mellea, *noun* – a fungus commonly referred to as honey fungus notable for causing Armillaria root rot and exhibiting mycelia bioluminescence
- Bioluminescence, *noun* – the emission of biochemically produced light by an organism; bioluminescent, *adj*.
- BioRad ChemiDoc MP Imaging System, *noun* – an instrument designed to detect and image chemiluminescence among other documenting functions.
- Chromosome, *noun* – the packed and organized structure within a cell containing a single molecule of DNA
- Diploid, *adj*. – the state of containing two complete sets of chromosomes
- Deoxyribonucleic acid (or DNA), *noun* – a double-stranded nucleic acid that contains the genetic information for cell function.
- Enzyme, *noun* – a protein that acts as a catalyst produced by cells to generally increase the rate of a reaction without changing the reactions products or getting used up in the reaction.
- Eukaryote, *noun* – an organism characterized by a cell or cells that contain DNA in the form of chromosomes contained in a nucleus. Eukaryotes tend to have larger cells with complex genomes. Examples of eukaryotes include animals, plants, and fungi; Eukaryotic, *adj*.

- Forward Genetics, *noun* – a process that is used to identify genes or gene sets responsible for a particular phenotype of an organism by screening for a particular phenotype and subsequently discovering its genetic cause
- Gene, *noun* – the functional unit of heredity; a segment of DNA that codes for a particular protein that is collectively responsible for an organism's phenotype
- Gene regulation, *noun* – the alterations made at any stage in gene expression, the process by which a gene is used in the production of its functioning gene product. This includes the various methods in which genes are switched on and off, to what extend the gene is expressed, and the like.
- Genetics, *adj.* – of or relating to genes or heredity
- Genome, *noun* – the complete set of genetic material contained in an organism
- Genotype, *noun* – a set of alleles that determines an organism's expression of a particular characteristic or trait
- Haploid, *adj* – the state of having a single set of unpaired chromosomes
- Hydrolysis, *noun* – a chemical reaction in which the interaction of a compound with water results in the decomposition of the compound
- Hygromycin, *noun* – an antibiotic produced by bacteria that kills bacteria, fungi, and other eukaryotic cells by inhibiting protein synthesis
- Insertional Mutagenesis, *noun* – formation or development of a mutation by the insertion of one or more bases
- Isolate, *noun* – a separate entity; used in this experiment to refer to a unique, secluded mutant of *Armillaria mellea* fungus
- Luciferase, *noun* – any of the group of enzymes in bioluminescent organisms that

catalyze the oxidation of luciferins to produced light

- Luciferin, *noun* – a general term for the light-emitting compound found in bioluminescent organisms that act as substrates to both luciferases and photoproteins
- Luminometer, *noun* – a sensitive photometer capable of measuring very low intensities of light such as that given of by a bioluminescent organism
- Malt Extract Agar (or MEA), *noun* –a culture medium
- Mutagenesis, *noun* – the process of creating a mutation by which the genetic information of an organism is changed in a stable manner
- Mutant, *noun* – an organism or gene that is undergoing or resulting from a mutation causing it to differ from the norm or typical form common in nature
- Mutation, *noun* – a change in the genetic structure of a gene that may cause a change in the phenotype of an organism
- Mycelium, *noun* – mass of branching hyphae that constitutes the vegetative part or body of a fungus
- Organism, *noun* – an individual living thing that can react to stimuli, reproduce, grow, and maintain homeostasis
- Photoprotein, *noun* – a type of enzyme in bioluminescent organisms that contains within itself all the required factors and reagents needed for light emission.
- Polymerase Chain Reaction (or PCR), *noun* – a process used to amplify, or copy, a segment of DNA
- Potato Dextrose Agar (or PDA), *noun* – a culture medium
- Phenotype, *noun* – an observable trait that is the result of a genetic contribution
- Prokaryote, *noun* – an organism characterized by the lack of a distinct nucleus and

membrane-bound organelles. They tend to be small and have simple genomes; Prokaryotic, *adj*.

- Pryosequencing, *noun* – a method of DNA sequencing
- Relative light units, *noun* – the units given to a measurement produced by the luminometer that are relative to other measurements taken by the same luminometer
- Substrate, *noun* – a substance acted upon by an enzyme
- Transfer-DNA (or T-DNA), *noun* – transferred DNA, a portion of a plasmid of some species that is inserted into the genome of a host cell

All definition derived from the Biology-Online Dictionary website as of April 2016.

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