Pumpkin Treatment, Tissue Culture, and Plasmid Building

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Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

College Honors

Departmental Distinction in ~~Put Name of Department Here~~ Biology

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Thesis:

Pumpkin Treatment, Tissue Culture, and Plasmid Building

Abstract:

The idea of making a Halloween tradition better could be feasible through a proper treatment with antifungals or changing of the pumpkin genetically. This research potentially has commercial appeal. Every year jack-o-lanterns exhibit rapid unsightly decay due microbial attack as they are used as outdoor decorations. There might be a commercial market for making these pumpkins last longer and fend off the microbial growth. The purpose of this thesis is the elimination of this problem through either a spray which could prohibit such growth or genetically modifying the organism to prevent such attack. The antifungal properties of some plant extracts and essential oils are known. Several substances which inhibit fungal growth have been examined. Determining the combinations and concentrations of natural antifungal agents that are most effective in warding off fungal growth has been pursued to obtain this treatment. This will be an important step because although the end product should be prohibitive to fungal growth and as such sufficiently potent, it should also be in a concentration benign enough to not have a deleterious effect on humans. The creation of a strain of pumpkins which is more resistant to microbial attack would seem ideal. Strides have been taken towards the creation of a procedure for the tissue culture of pumpkin, using a protocol developed for squash. Additionally work has been initiated involving the construction of a plasmid which can transform first *Agrobacterium*, then pumpkin explants. The creation of the plasmid has been relatively unsuccessful, yet strides are still being taken to better understand the plasmids being used in this experiment and why they will not ligate.

Introduction:

Every year jack-o-lanterns exhibit rapid unsightly decay due to microbial attack as they are used as outdoor decorations. There might be a commercial market for making these pumpkins last longer and fend off the microbial growth. The antifungal properties of some plant extracts and essential oils are known (Hammer *et al.*, 1999). We have looked at substances which inhibit fungal growth, and have experimented with what combinations and concentrations of natural antifungal agents are most effective in warding off fungal growth. This is an important step in the process of finding the correct treatment because although the end product should be prohibitive to fungal growth and as such sufficiently potent, it should also be in a concentration benign enough to not have a deleterious effect on humans and should not be prohibitively expensive.

The initial testing for the efficacy of the artificial treatment could be done through a method which mimics that used in determining the efficacy of antibiotics. Namely, small disks coated in the agent of choice could be applied to a freshly applied fungus culture on agar. As the fungus grows, if the agent has efficacy against the microbe, there will be a zone around the disk which is comparable to the effectiveness of the agent. Through this method both the effectiveness of an agent against a microbe (denoted by any zone being present) and the effectiveness of agents relative to each other (determined by comparing the sizes of zones of inhibition of growth) can be determined (Maier *et al.*, 1974).

This need for an artificial substance to coat the pumpkin for preservation could be bypassed through the engineering of pumpkins with stronger resistance to fungal attack. Plant proteins which would aid in the plant's defense against fungal attack could be potentially incorporated

into a pumpkin genome through an *Agrobacterium* infection which would have the effect of placing desired genes into a plant's genome. One would then isolate and culture a genetically modified organism from that transformed tissue (Katavic *et al.*, 1991*).* One group of proteins of particular interest are PR proteins. PR proteins, or pathogenesis related proteins, are a group of proteins which are induced in the event of microbial attack and stimulate a systematic response which can include antimicrobial effects such as attacking the invading microbe or alerting surrounding cells to the microbial attack (van Loon, L.C., 1985). Pumpkins already have in their proteome PR-5 proteins (Cheong *et al.*, 1997). PR-5 proteins have a poorly understood mechanism of antimicrobial attack, but function in breaking (1,3)β-glucan bonds (which are found in fungal cell walls) and in cell lysis (Selitrennikoff, 2001). This PR protein is helpful in the fight against microbial attack but microbes can adjust their structure to specific PR-5 proteins (Narasimhan *et al*., 2003) (Ibeas *et al*., 2001). Therefore, since each of the different classes of PR proteins attack the fungal cell wall or cell membrane in different ways (the general sites of attack for all 5 of the PR proteins are identified in figure 1 (Selitrennikoff, 2001). The inclusion of more PR genes into the pumpkin genome would seem to be an effective way to outpace the cell wall modification carried out by fungal agents to render specific PR protein action ineffective (Selitrennikoff, 2001).

Figure 1:

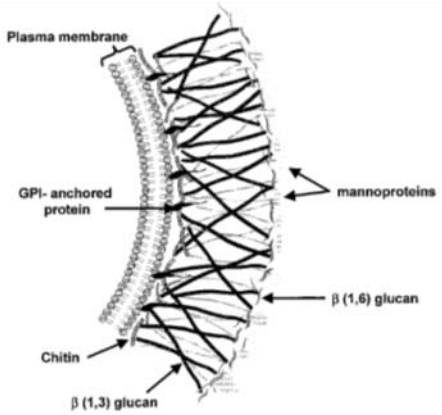


FIG. 1. Schematic of fungal cell wall. GPI, glycophosphatidylinositol.

The first steps in this process, would be the perfecting of a pumpkin tissue culture system and pumpkin cell transformation procedures. Such a transformation procedure can be found in Nanasato *et al.*'s analysis of the transformation of squash (*Cucurbita moschata*). It is the goal of this study to modify the procedure present in this paper in order to present a model of transformation for pumpkins (*Cucurbita pepo*). In this study, *Agrobacterium tumefaciens* was used as the vector for transmission of genetic material. This is possible because of the novel way in which A*grobacterium* mediates its infection of plant hosts. After a plant is injured it will secrete phenolics in order to signal the need for the healing of the wound. An *Agrobacterium* will, in response to these phenolics, export a series of virulactors along with its Ti plasmid. The protein complex will allow the plasmid entry into the nucleus by coopting cellular machinery and incorporate the Ti plasmid into the infected plant cell's genome. This new DNA segment will induce the plant cell to make amino acid – sugar conjugates called opines, which the *Agrobacterium* will use (Pitzschke *et al.*, 2010). However, if one were to replace the Ti plasmid with a plasmid of one's choosing, then the *Agrobacterium* could incorporate a gene of interest into the plant cell's genome. It was to this end that Nanasato *et al.* transformed *Agrobacterium*

with a plasmid of interest and attempted to infect C*ucurbita moschata* specimen. In order to make sure that the transformed plant could grow with the gene with relative ease the embryo of the plant, specifically the embryo's cotyledonary nodes, were isolated from C*ucurbita moschata seeds.* To induce the *Agrobacterium* to incorporate the plasmid of interest into the genome, Nanasato *et al.* used aluminum borate whiskers and agitated in order to cause a uniform injury to the explants. After this injury the transformed *Agrobacterium* were allowed to grow on the plant wound site. After sufficient growth the bacterium was killed and the transformed plant was isolated and grown using a novel tissue culture system (Nanasato *et al.*, 2011).

In order to properly test this transformation procedure we are inserting a reporter gene. A reporter gene is a gene that would be easily determined to be present in a transformed organism due to the action of its gene product. In this experiment it was thought to be wise to use the insertion of the enhanced Green Fluorescent Protein (GFP) gene as the reporter gene (Chalfie, 1995). The reasons for the use of this specific protein were twofold. First it was known that the presence of this protein in the transformed pumpkin could be readily seen upon exposure to the correct wavelength of light (Chalfie, 1995). Second, it was thought that such a transformed organism could have intrinsic value of its own. As the organisms we are trying to make are to be used for the purpose of decoration increasing the organism's decorative value was seen as a potentially positive outcome.

After the *Agrobacterium* infection would take place a system for the culture of the transformed tissue to a full plant would be necessary if a fully transformed adult plant is to be achieved. Systems of tissue culture of such plants have been accomplished to great effect (Katavic *et al.*, 1991). It is hoped that a system for the transformed excised cotyledons of pumpkins can be used as a way to generate adult transformed organisms.

Materials and Methods:

Antifungal disk determination of agent efficacy

The microbial representation of the kind of microbial attack found in rotting pumpkins was obtained from a swab of a carved pumpkin allowed to decay in standard outdoor conditions. This swab was allowed for grow on a tryptic soy agar plate (TSA plate). From this plate isolates were picked and grown on TSA. Procedures for the determination of the capacity of antibacterial agents to inhibit bacterial growth were then adapted to determine the efficacy of these antifungal agents (Maier *et al.*, 1974*)*. In the anti-fungal version of this technique, fungal isolates found on decaying pumpkin matter can be swabbed onto an agar plate and then paper disks coated with the specific anti-fungal agent of interest can be placed on the agar plate. Then, just as in antibiotic testing, one will search for zones of inhibition (or a zone around the disk of inhibited growth). The size of the zone is indicative of the extent to which the agent was prohibitive of fungal growth.

Determination of treatment efficacy on carved quartered pumpkin sections

In the second phase of the testing of the antifungal agents, a spray was developed and sprayed unto a carved pumpkin medium. In this phase 6 pumpkins were quartered, scraped out, numbered with a sharpie on the outside and then placed outdoors. Quarters 1-12 were placed without a bleach pre-treatment or and quarters 13-24 were treated with bleach (Clorox bleach, 2 tsp per gallon, 8 hour treatment). This experiment was carried out from Saturday 10/19/2013, ended 11/11. The treatment used at that time was a solution of 0.01 M benzethonium chloride and 1 ml eugenol for every 10 ml of solution (corresponded to the 1:4 dilution of solution A

which was a 40% eugenol and 0.05 M benzethonium chloride solution). The state of the sections was then photographically recorded every day for 23 days.

Another trial using carved pumpkin sections was conducted in the greenhouse from November 18-29, 2013. In this trial a 1% solution A in ethanol solution was sprayed on quartered carved pumpkin sections. These sections were placed in the green house adjacent to pumpkin sections already covered in microbial contamination. A fan was used to periodically blow spores from the contaminated sections towards the trial pumpkin sections in order to expose them to microbial attack. Of the three treated sections each was sprayed to a different extent on the initial spray, the first being completely covered in spray, the second being initially not sprayed at both ends, and the third not being initially sprayed on its top end. This was done in order to understand if the treatment has efficacy in ridding the sections of already present microbes.

A determination of the carcinogenic / mutagenic qualities of the treatment was then to be determined. A miniprep on *E. coli* containing a pUC18 plasmid was performed in order to obtain DNA for the experiment. The following 5 parameters were followed (as seen in table 1).

Table 1:

Number	1	2	3	4	5
Amount of DNA added (µl)	12	12	12	12	12
Substance Added	UV light*	Ethanol	1:4 solution A	1% solution A	1% solution A
Amount of Substance	N/A	0.5	0.1**	0.1	0.5

Added (µl)					
Time of Incubation	30 minutes	12 hours	12 hours	12 hours	12 hours

*for this condition a slide was sterilized with 70% ethanol and then a bead of 12 µl of DNA was placed on the slide and the slide was placed approximately 3-4 inches from a UV source on ice and allowed to incubate for 30 minutes
**The amount added to each solution was low because of the low solubility of the components of solution A in H_2O

A study on whether this treatment would induce apoptosis in mammalian cells (mouse B6 cells) was then done. In this study a confluent culture of B6 mouse cells in DMEM + FBS in a 6 well plate was first obtained. Then in the media was changed and in each well a different condition was followed (as seen in table 2).

Table 2:

Well1	Well 2	Well 3	Well 4	Well 5	Well 6
No addition control	25 µl 95% ethanol	10 µl 1:4 solution A	25 µl 1:4 solution A	10 µl 1% solution A	25 µl 1% solution A

After these additions, the wells were allowed to incubate for 24 hours. After this incubation the media from each well was extracted, the wells were washed with PBS 1X, the cells were trypsinized (with the solutions from each of these steps being added to a tube from analysis by flow cytometry). The cells in these tubes were spun down at 1280 rpm for 5 minutes. After the supernatant was discarded, the tubes were twice filled with FACs buffer and spun at the same parameters. After this second spin and second removal of FACs buffer the pellet was resuspended in 100 µl of Annexin V Binding Buffer and 5 µl of Annexin V conjugate was added. After this 10 µl anti-PI protein was added to the solution. After a 15 minute incubation

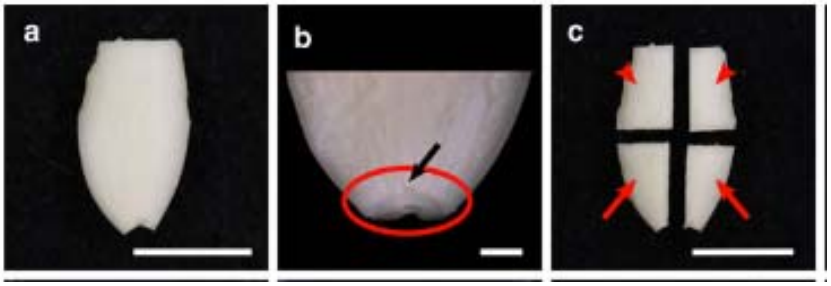
with deprivation from light 100 µl of Annexin V Binding Buffer was added to the tubes and the cells were filtered through a micron filter into FACs analysis tubes and flow cytometry was conducted.early apoptotic cells should exclude PI while late apoptotic cells should have display both PI and Annexin V.

System of pumpkin tissue culture

Figure 2:

Cucurbita pepo seeds taken and, after the removal of their coat, were then, rehydrated, moved to solution of 99 ml DI water, 1 ml Clorox, and 100 µl Tween-20 for 10 minutes, washed 3 more times in water, and finally placed into shoot-induction medium (which has 1 mg/L BAP) abaxial side down, after which each plate was parafilmed. These plates were then allowed to incubate at 28 degrees Celsius for 24 hours while being deprived of light (figure 2).

Figure 3:



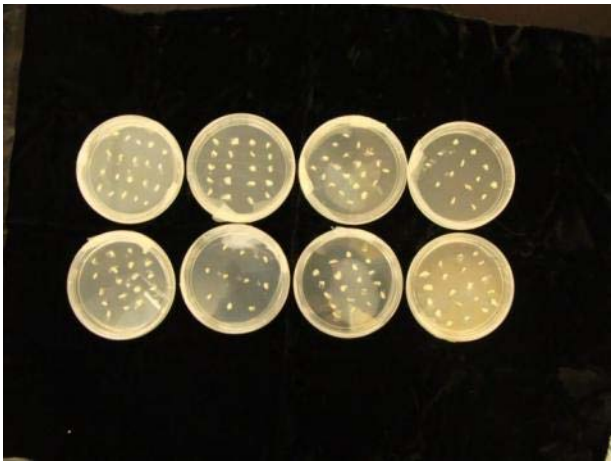
(Nanasato *et al.*, 2011) After 24 hours in the dark the seeds were cut in halves and then in fourths while taking care not to remove or injure the cotyledonary nodes (figure 3). The bottom two fourths were placed cotyledon down into fresh SI medium and left in the dark for 3 days at 28 degrees Celsius (figure 3). SI medium is shoot induction medium and its formulation can be seen in table 3.

Table 3:

30 g	Sucrose
100 mg	i-inositol
20 ml	Solution 1
20 ml	Solution 2
5 ml	Solution 3
5 ml	Solution 4
5 ml	Solution 5
10 ml	Solution 6
5 ml	Thiamine
5 ml	Nicotinic Acid
5 ml	Pyridoxine
8 g	Bactoagar

Amount Added / L	Stock Solution/ Substance	Constituents	Conc. In Stock (g/L)	Final Conc. (mg/L)
20 ml	Solution 1	NH_4NO_3	82.5	1650
20 ml	Solution 2	KNO_3	95	1900
5 ml	Solution 3	H_3BO_3, KH_2PO_4, KI, $Na_2MoO_4.2H_2O$, $CoCL_2.6H_2O$	1.24, 34.0, 0.166, 0.05, 0.005	6.2, 170, 0.83, 0.25, 0.025
5 ml	Solution 4	$CaCl_2.2H_2O$	88	440
5 ml	Solution 5	$MgSO_4.7H_2O$, $MnSO_4.4H_2O$, $ZnSO_4$, $CuSO_4.5H_2O$	74, 3.38, 1.72, 0.005	370, 16.9, 8.6, 0.025
10 ml	Solution 6	$Na_2.EDTA$, $FeSO_4.7H_2O$	3.72, 2.78	37.2, 27.8

Figure 4:



It is as this point where we would injure the seeds with aluminum borate whiskers, then place *Agrobacterium tumefaciens* onto those wounds. The wound exudates secreted by the plant in response to the wounding would cause the *Agrobacterium* to incorporate its genetic material (which we would replace with the gene of interest) into the plant genome. From these transformed cells we would continue on with the procedure. At this point the explants are transferred into the shoot inducing (SI) medium and allowed to continue until shoots develop. Once shoots have developed the shoot should be extracted from the explant and placed into shoot elongation medium (SEM). SEM is half strength SIM with 0.1 mg/L BAP. This medium should induce formation of roots. After roots form one can transfer the plantlet into soil and slowly wean the plantlet off of 100% humidity to ambient humidity and allow it to grow into a full plant. Due to high rates of loss in culture the antibiotics penicillin and streptomycin as well as the antifungal fungizone were all placed in the SIM and SEM to reduce the rates of contamination. This change was met with success in that the attrition was greatly reduced. We also found that the Jack-o-lantern cultivar was less susceptible to contamination than Connecticut Field variety. See figure 5 for example of shoot and root induction of pumpkin embryo explants.

Figure 5:

Transformation of bacteria:

Strides have been taken in creating the plasmid with which the pumpkin will be transformed. An enhanced GFP gene was amplified by PCR and then cut and ligated into the PEV plasmid (a binary plasmid that can propagate in both *E. coli* and *Agrobacterium*). This will be used to transform DH5α *E. coli* bacteria which will be used to create and infinite supply of this plasmid. The plasmid will then be extracted from the DH5α bacteria and used to transform *Agrobacterium*. This *Agrobacterium* will be used to transform the pumpkin explants. We have thus far completed PCR of the GFP gene from a plasmid containing an enhanced GFP sequence (kind gift from Dr. Adam Hersperger) and isolation of PEV plasmid through a miniprep of bacteria containing the PEV plasmid. The PEV plasmid is a low copy number plasmid, meaning it is present very few times per bacterium. This fact made the miniprep method insufficient as a method to effectively isolate enough plasmid for a ligation procedure. A midiprep, which uses a

higher volume of bacteria to gain the same volume of isolate DNA, was then carried out in order to isolate enough plasmid. With enough plasmid isolated the pPEV plasmid was cut using EcoR1 and BamH1. Upon closer examination of the plasmid map a problem was found here because it was discovered that this cut resulted in the cutting off of the poly-A tail from the potential mRNA to be produced. The idea of a double cut site was then given up and a single BamH1 cut was then pursued to prevent this problem.

In order for this to be achieved a BamH1 cut site had to be engineered into the enhanced GFP gene. Therefore PCR was performed on the enhanced GFP plasmid obtained from Dr. Hersperger.

The 5'primer:

5'GCGGATCCATGGTGAGCAAGGGCGAGGAGCTG 3'

1 2 3

3' primer:

5' GCGGATCCTTACTTGTACAGCTCGTCCATGCC 3'

1 2 3

1= GC clamp (make sure it extends 2 bases on the 3' primer)

2 = BamH1 cut site

3 = matches the template

With these primers a BamH1 cut site can be made a part of the amplified GFP. This is possible because the because if one borders such a small sequence within the larger plasmid with a GC

clamp and a longer sequence which matches the plasmid to be amplified then the primer should anneal to the plasmid while creating the desired cut sites within the new sequence. This PCR was performed because of the desire for these cut sites but also because of the desire for only the GFP gene. The plasmid we were using was approximately similar in size to the pPEV plasmid (see figure 9 lane 2). By the polymerase chain reaction this sequence within the larger plasmid could be targeted and amplified in order to gain a solution of the GFP plasmid bordered by the two cut sites shown.

After the GFP PCR both the PPEV and GFP plasmids were cut with the BamH1 restriction endonuclease. The PPEV plasmid then was treated with the alkaline phosphatase enzyme. This treatment prevented the religating back together of these BamH1 cut sticky ends within this linearized PPEV plasmid. These two sequences were then ligated back together using the T4 ligase. The ligated plasmid should then be used to transform competent DH5α *E. coli* bacteria. These bacteria allow the production of a virtually unlimited supply of this plasmid. This plasmid can then be extracted from the DH5α bacteria by midiprep and used to transform A*grobacterium tumefaciens*. This transformed *Agrobacterium* could then be used to transform the previously decribed pumpkin explants.

Results:

In the pumpkin antifungal treatment experiment the results of the antifungal disks experiment (Table 4) showed during its duration that, out of the agents examined, clove oil and grapefruit seed extract were the most effective agents. It was then that an examination of what component of each substance caused the prohibitive effect was carried out. In the case of the grapefruit seed extract it was found out through the paper by Bekiroglu *et al.* that this product was being spiked with a chemical called benzethonium chloride (Bekiroglu *et al.*, 2008). This is a dangerous and potentially toxic chemical (this is being placed without warning into oils to be consumed). In the case of clove oil it was found in the paper by Alma *et al.* that the primary components of it were eugenol and eugenol acetate (Alma *et al.*, 2007). These two components were what was first explored out of the many components of clove bud oil. As can be seen in table 4, the eugenol had a pronounced effect, while eugenol acetate had no effect. Then the idea of what combinations of effective agents would work well when combined was pursued. Table 4 shows that the only combination which showed an increase in efficacy is the combination of eugenol and benzethonium chloride. Knowing that this was the combination which would be effective and their both being soluble in ethanol, solution A was made. Solution A was 40% eugenol and 0.05 M benzethonium chloride in 95% ethanol because these substances are not water soluble. Table 5 shows the results of the dilution of solution A using the antifungal disk method of testing. From this it can be seen that the 1:4 dilution was the most effective in retaining the effect over a long period of time.

Table 4:

Name of agent	Radius from rim (cm)	Diameter of zone (cm)
Juglone	0.2	1.3
Clove oil full concentration	0.36*	1.72*

Clove oil 1:1	0.3*	1.6*
Clove oil 1:2	0.25*	1.5*
Grapefruit seed extract (GSE) + clove oil 1:1	0.70*	2.27*
Olive leaf oil	0	1
Clove	0*	1*
Cinnamon	0	1
Oregano	0	1
Coconut oil	0	1
Cayenne	0	1
Fungus fighter	0.6	1.12
GSE full concentration	0.30*	1.62*
GSE 1:1	0.1*	1.2*
GSE 1:2	0.04*	1.08*
Benzethonium chloride 1:2	0.1*	1.20*
Benzethonium chloride 1:4	0.12*	1.24*
Benzethonium chloride 1:20	0.05*	1.10*
Benzethonium chloride + clove oil + fungus fighter	0.32*	1.63*
Benzethonium chloride + clove oil + fungus fighter	0.35*	1.70*
Benzethonium chloride + clove oil	0.42*	1.85*
Fungus fighter + juglone	0.025	1.05
Eugenol, 80%	0.35	1.7
Eugenol, 40%	0.3	1.6
80% Eugenol + 16% Eugenol Acetate	0.3	1.6
Eugenol Acetate, 16%	0	1
Eugenol Acetate, 8%	0	1
40% Eugenol + 8% Eugenol Acetate	0.20	1.53

(all were done with controls in which radius from rim = 0 cm, diameter of zone = 1 cm (length of disk))

*= possessed an unmeasured zone of semi-inhibition

Table 5:

		Full concentration	1:2 dilution	1:4 dilution
24 hours	Diameter of zone	1.6	1.6	1.4
	Radius from disk	0.3	0.3	0.2
48 hours	Diameter of zone	1.6	1.6	1.4

	Radius from disk	0.3	0.3	1.4
72 hours	Diameter of zone	1.6	1.6	1.4
	Radius from disk	0.3	0.3	1.4
96 hours	Diameter of zone	1.6	1.6	1.4
	Radius from disk	0.3	0.3	1.4
1 week	Diameter of zone	1.6	1.6	1.4
	Radius from disk	0.3	0.3	1.4
2 weeks	Diameter of zone	1.6*	1.6*	1.4
	Radius from disk	0.3*	0.3*	1.4
1 month	Diameter of zone	1.6*	1.6*	1.4
	Radius from disk	0.3*	0.3*	1.4
2 months	Diameter of zone	1.6*	1.6*	1.4
	Radius from disk	0.3*	0.3*	1.4

*= some fungus grew on disk and into the agar, the zone is a zone of semi-inhibition and is therefore invalid

Through these results it could be seen that the 1:4 dilution of the solution A was the most effective so this solution was continued on to the trials involving carved pumpkins. These trials involving involved 6 pumpkins which were carved and quartered as discussed in the materials and methods section as can be seen in figures 6 and 7.

Figure 6:

Clorox 8 hour soak
Top row #1-3, #4-6; Bottom row # 7-9, #10-12
Control (#1-3), 1:4 Solution A, one time (#4-6), Control (#7-9), 1:4 solution A weekly (#10-12)
Figure 7:

No soak
Top row #13-15, #16-18; Bottom row #19-21; #22-24
Control (#13-15), 1:4 Solution A (#16-18), Control (#19-21), 1:4 Solution A weekly (#22-24)

Figure 8:

Progression of experiment

a)

Day 12:

b)

Day 13:

c)

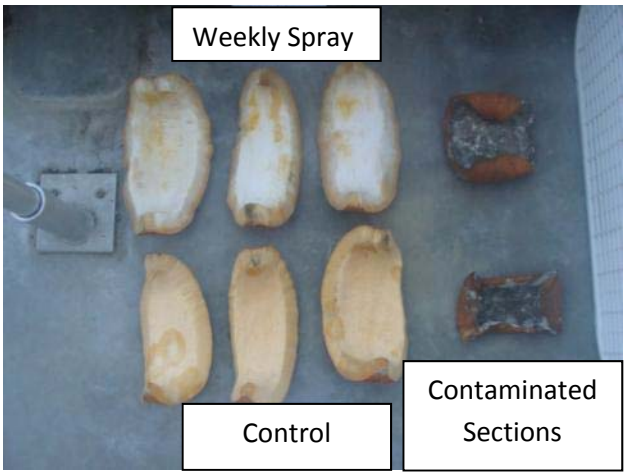
Day 23:

Here, in figure 8, what can be seen is the length of time it takes for microbial growth to occur on these media. It can be seen in figure 6a that it took about 12 days for all controls to be completely covered in growth; while at 13 days (figure 7b) growth could begin to be seen on the single treatment pumpkins. However, at day 23 (the day of the termination of the experiment) there was no growth on the pumpkin quarters which were treated once per week in both the Clorox soak and no soak models. It is at this stage that the experiment was terminated due to severe shriveling and water loss of the pumpkin sections. This loss was most likely due to the much larger amount of surface area in these sections than in a conventional carved pumpkin.

Figure 9:

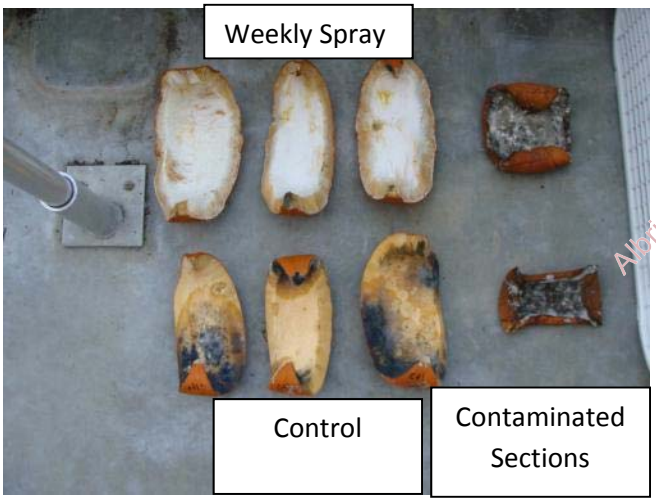
In figure 9 a)-e) a study of the efficacy of a 1% solution A treatment in preventing microbial attack and in the riding a pumpkin of an already present contamination can be seen.

a) Day 0



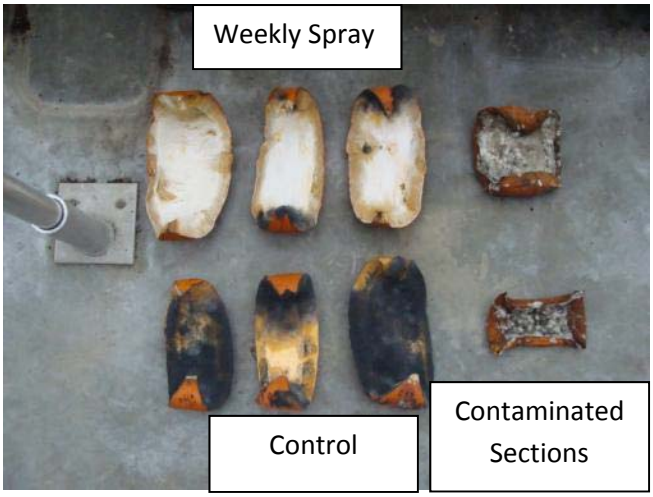
Top row: (left to right) #1 fully sprayed, #2 not sprayed at bottom or top tips initially, #3 not sprayed at top tip initially.
Bottom Row: all unsprayed for duration of experiment

b) Day 2



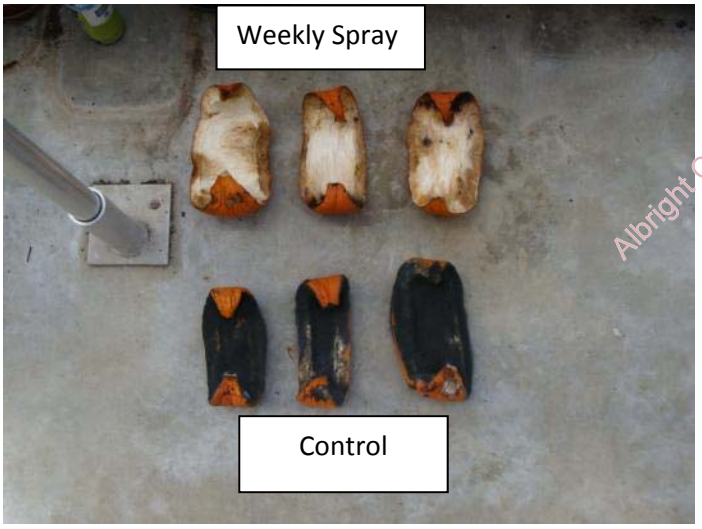
By day 2 it can be seen that, after two days of the periodic fanning to distribute spores on pumpkins, the controls are beginning to have pronounced contamination while the weekly spray group are beginning to show some spots of contamination at unsprayed tips.

c) Day 4



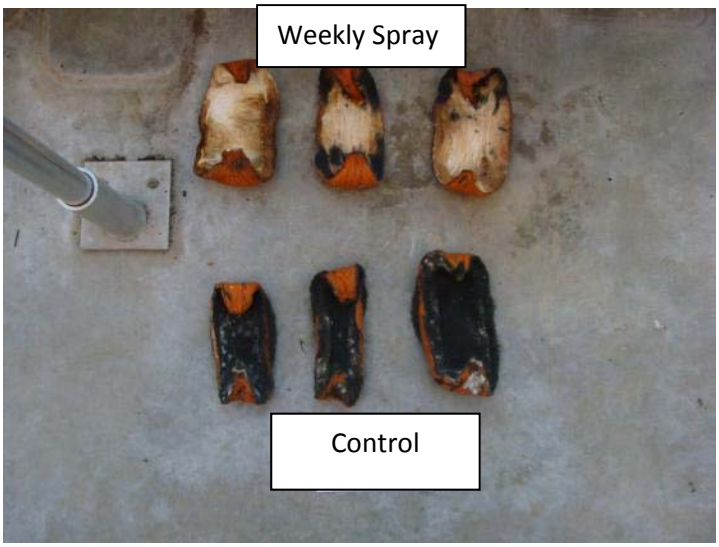
By day 4 it can be seen that the controls are almost completely overrun with contamination. The weekly spray group is also displaying some contamination at point of non-spray. However, the points of contamination appear to be spreading less quickly than the control group.

d) Day 7



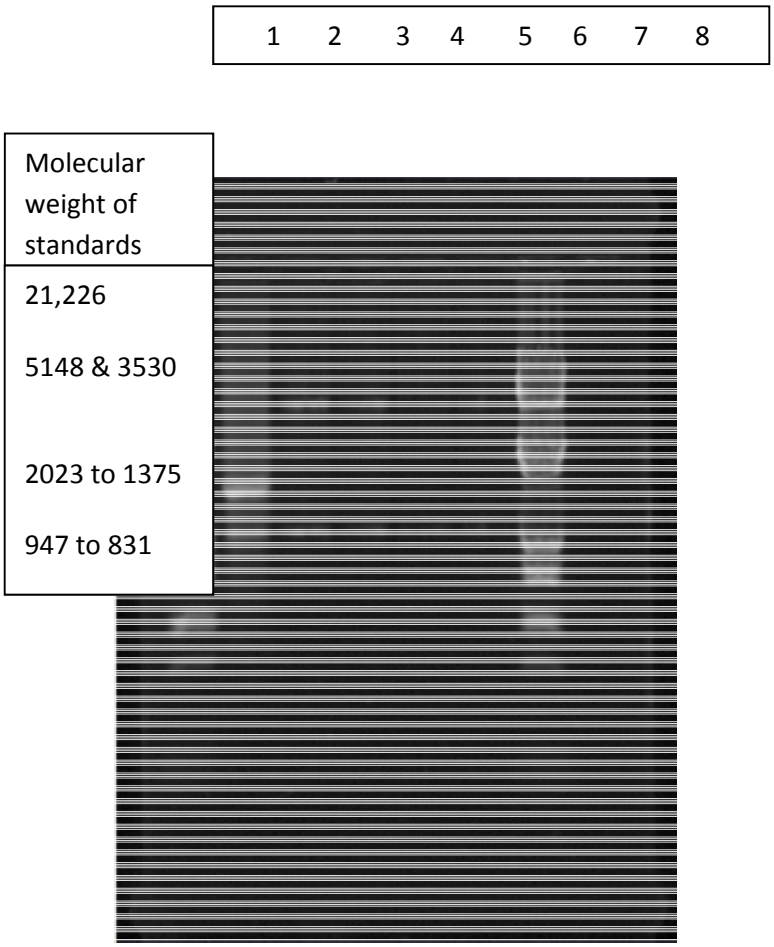
Controls are completely overtaken by contamination. No contamination of the evenly sprayed quarter. Contamination on the other weekly spray sections is limited to where not sprayed but the zone of contamination on sections 2 and 3 is slowly growing.

e) Day 11



Section 1, which was completely sprayed in the initial spray, still shows no contamination. Sections 2 and 3 have their zones of contamination still growing and it appears that that will continue despite treatments. Controls are still completely covered with microbial contamination.

Figure 10:

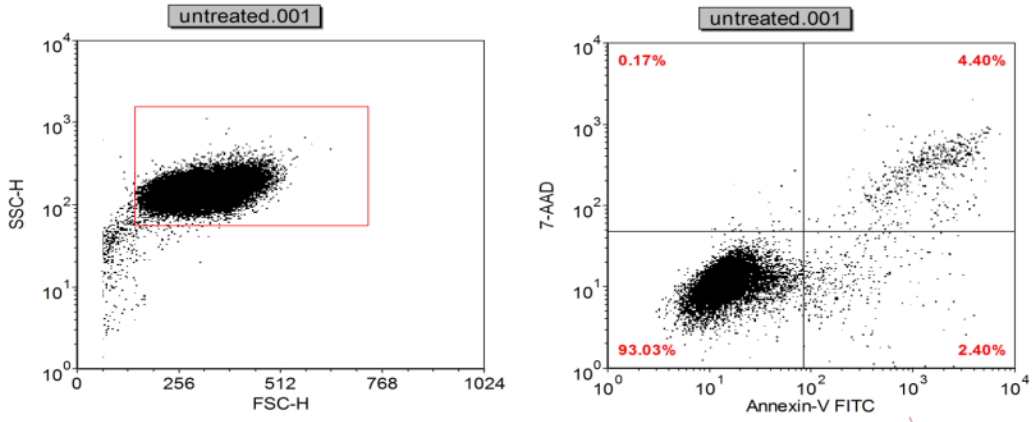


Lanes 1 and 7 are standards. Lane 2 is the UV exposed DNA. Lane 3 is the 0.5 µl ethanol treated DNA. Lane 4 is the 0.1 µl 1:4 solution treated DNA. Lane 5 is the 0.1 µl 1% solution A treated DNA. Lane 6 is the 0.5 µl 1% solution A treated DNA. Nothing was loaded in Lane 8.

Figure 11:

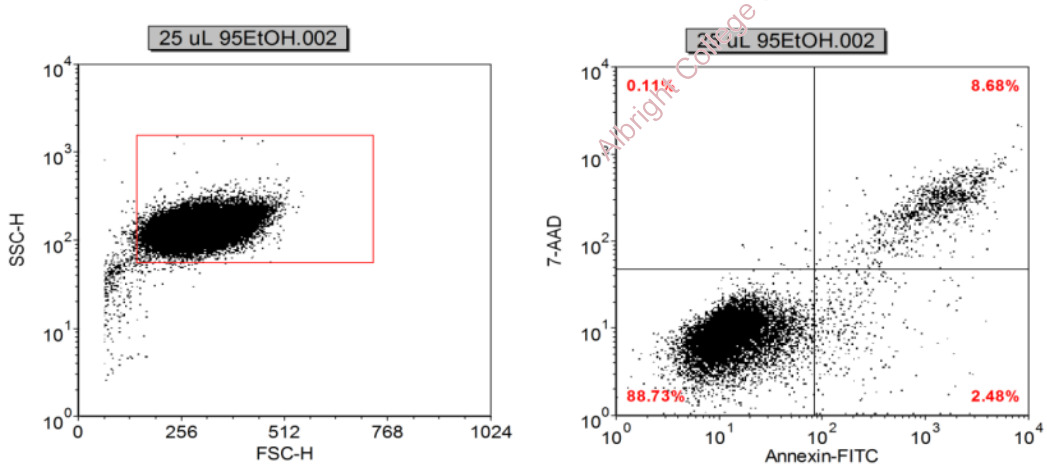
The results of the study of treatment induced apoptosis in mammalian (B6 mouse cells) cells. The bottom left quadrant represents healthy cells. The bottom right quadrant represents cells which are undergoing the early stages of apoptosis (stain for Annexin V). The top right quadrant represents cells which are undergoing late stages of apoptosis (as their cellular membranes have begun to break down, allowing the access of the 7-AAD to DNA while Annexin V is still expressed at the surface).

a)



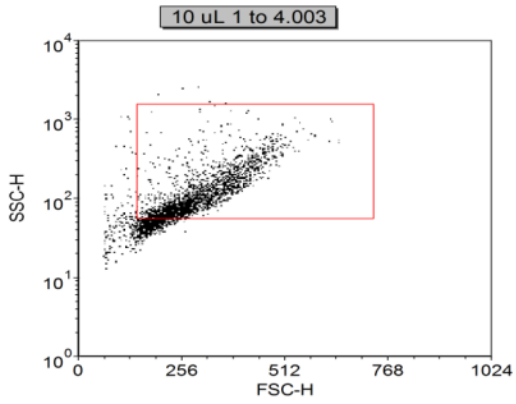
No treatment control.

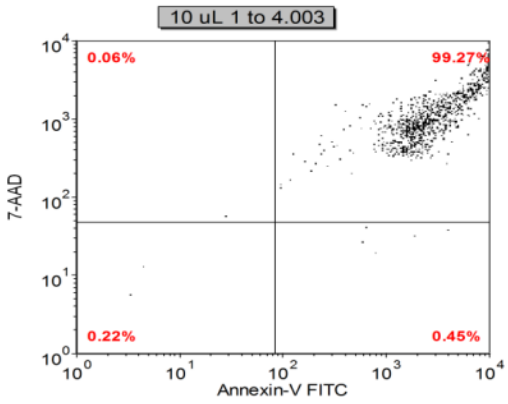
b)



25 µl ethanol

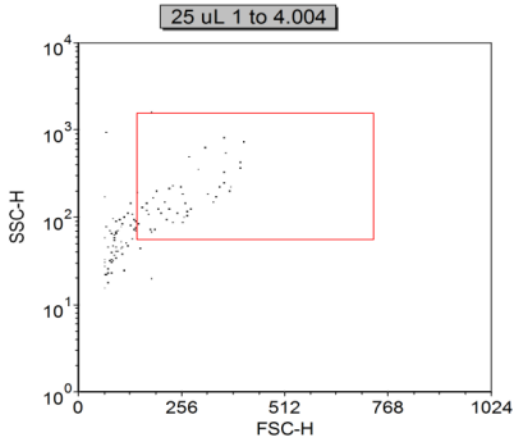
c)

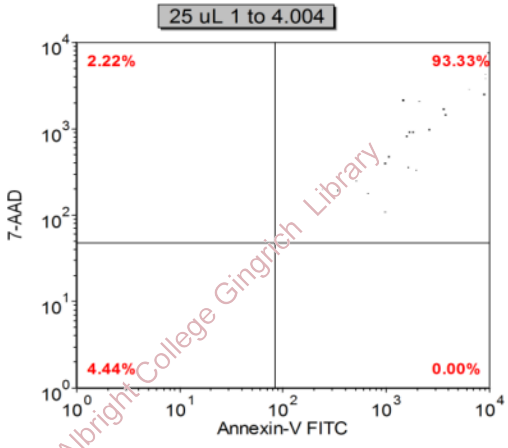




10 µl 1:4 dilution solution A

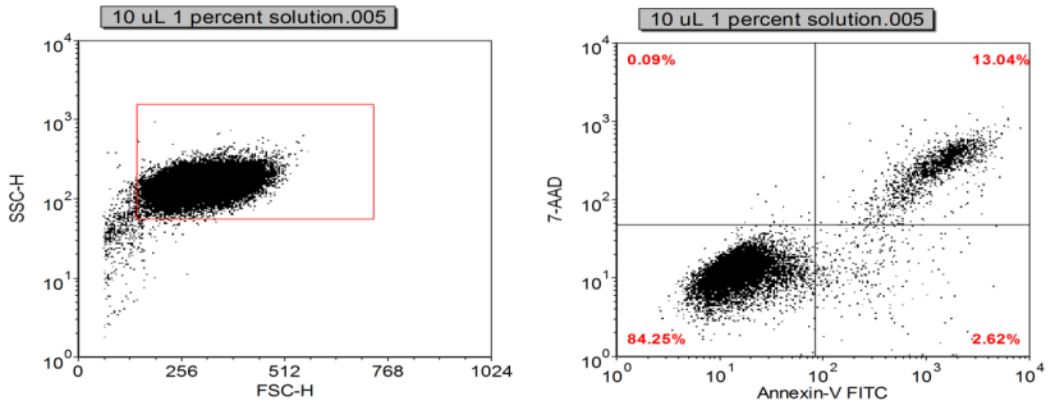
d)





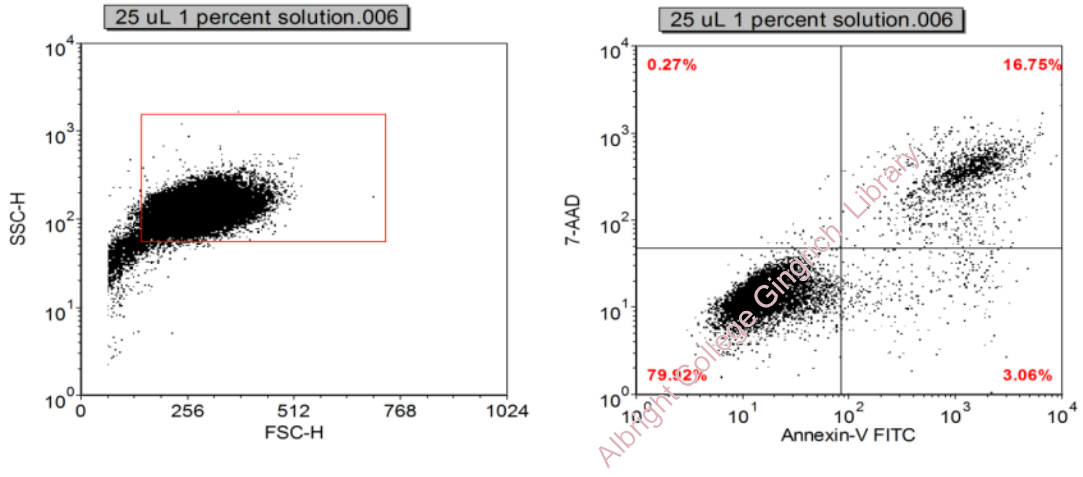
25 µl 1:4 dilution solution A

e)



10 µl 1% solution A

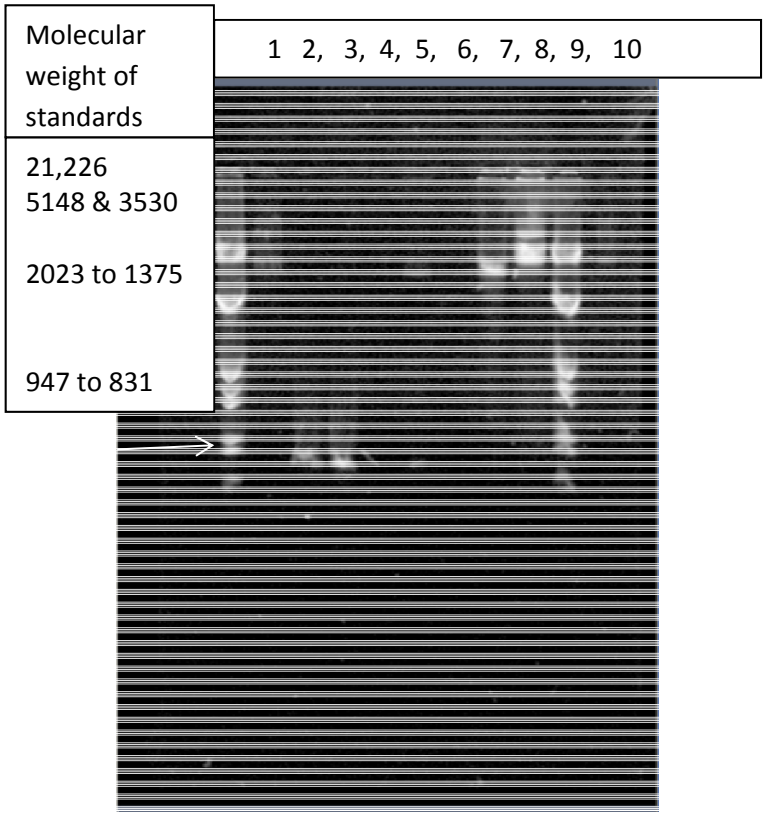
f)



25 µl 1% solution A

Bacterial Transformation and Plasmid Ligation

Figure 9:



1 and 10 are molecular markers. Lane 2 is the larger plasmid the GFP gene was amplified from. Lane 3 is a PCR reaction of GFP and 4 is that reaction post BamH1 cut. Lane 6 is the ligation. Lane 8 is the cut PPEV and lane 9 is the uncut PPEV.

Difficulties have been encountered in ligating the plasmids together, a crucial, early step in the process of creating the modified organism. It can be visualized that the cutting through the action of the restriction endonuclease is effective in that each of the cut bands electrophoresed to a greater extent than the uncut bands. This difference in the positions of the bands shows their being cut because a linearized piece of DNA should move through the gel matrix with greater

ease than a still circularized plasmid. However one can visualize the problems in lane 6, in which the "ligated" plasmid should be. Instead both plasmids can be seen together in this lane, but with no overlap. It would appear that the cutting is, in fact, effective, yet the ligation will not take place.

Discussion:

Pumpkin Antifungal Treatment

Through the mimicking of a procedure for the determination of the efficacy of antibiotics, it was found that a combination of eugenol and benzethonium chloride was ideal for making an antifungal treatment. This treatment was then taken and applied to pumpkins in a time course and it was found that if applied weekly that it could last the life of a pumpkin once cut and on display outdoors. The loss of water does not seem to be an issue because it was seen in all samples, control and treated. The idea that the treatment could work indefinitely if applied for the lifetime of the outdoor-decorative function is an exciting one with much promise. Continued trials are ongoing in the determination of the toxicity of the treatment with regard to invertebrates.

Our second goal with regard to the antifungal treatment was to determine the best concentration of the active ingredients of solution A would be in order to achieve a safe and monetarily effective treatment; as well as the determination of whether this treatment could be used proactively as well as preventatively. The treatment appears effective when applied to the whole area of the pumpkin. However, as the seen in the sections which had a small amount of contamination which continued to spread as the days passed, it would appear that the treatment

can only prevent the microbial attack of the pumpkin segments and not eliminate already present contamination.

The determination of the mutagenicity / carcinogenicity of the treatment yielded results which were indicative of a lack of either function in the treatment. The incubation of each of the DNA isolates should have yielded, if the substance being tested were mutagenic / carcinogenic, some cleavage of DNA which could be reflected in a difference in the band pattern in an agarose gel. Such an occurrence was noted in the positive control (the UV exposed DNA). In this positive control the higher DNA band traveling further down the gel when compared to the untreated negative control is indicative of the DNA being in shorter strands and therefore cleavage of DNA. The fact that this was not present in lanes 3-6 of figure 8 and of those wells being similar to the negative control is indicative of this treatment not having such an effect on DNA. This does not reflect mutagenicity from mechanisms other than cleavage of the DNA and such tests as the Ames test.

The Ames test is a procedure which uses several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis which allows for the detection of mutagens acting via many different mechanisms including whether the product of the metabolism of the suspected mutagen is mutagenic. In this procedure the bacteria are spread on an agar plate with a small amount of histidine. This small amount of histidine in the growth medium allows the bacteria to grow for an initial time and have the opportunity to mutate. When the histidine in the plate is depleted only bacteria that have mutated to gain the ability to produce its own histidine will survive; therefore, the mutagenicity of a substance would be proportional to the number of colonies observed (Ames *et al.*, 1973).

The product of the mammalian cell apoptotic study was a determination of how toxic the treatments are and in what doses. The results (figure 9) show that the 1:4 treatment is without question far too dangerous for feasible every day use, as it induced apoptosis (programmed cell death) in almost all the cells it came in contact with. However, the results of the 1% solution are grounds for optimism in that 1% solution A's effect on the cells, which was induced apoptosis in 15-20% of cells in culture when compared with ethanol's inducing apoptosis in approximately 11% of the culture. Further study into how deleterious an effect this treatment could have topically on animals is warranted.

Bacterial Transformation

The lack of ligation of the GFP and pPEV together is troubling and can be explained in a variety of ways. It would appear that the PCR is working well and the primers are effective. This can be seen in the amplification of the GFP gene, which would not have been possible without the 5' and 3' primers. One reason for the DNA segments not ligating together could be the continued presence of the DNTPs from the PCR reaction which are being ligated onto the sticky ends by the ligase. Such DNTPs would not be visualized on the gel because the way DNA is shown in these gels is through the intercalating function of ethidium bromide. There is no way to intercalate with DNTPs, as they are not in the double helical conformation required. Another explanation would be that the function of the T1 DNA ligase has ceased and that the enzyme has expired. This also seems untrue because this same result was obtained using fresh DNA ligase.

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