IN VITRO REGENERATION FOR GENETIC TRANSFORMATION OF LETTUCE AND AVOCADO

By

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Abstract

Genetic engineering and gene editing are high-value technologies that can be used to improve productivity, quality, and resistance to biotic and abiotic stresses of avocado (*Persea americana*) and lettuce (*Lactuca sativa*). These technologies can have a positive impact on growers, consumers, and the environment, for instance by minimizing the use of pesticides and or enhancing the water use efficiency of crops. The main goal of this honors research study was to establish efficient *in vitro* regeneration systems for the application of genetic engineering/editing in avocado and lettuce. For avocado, immature embryos were isolated from fruits about 1.5–2.0 cm in length and cultured on Murashige and Skoog (MS) medium supplemented with sucrose, myo-inositol, thiamine-HCl, agar, and picloram. After four months, globular embryos were regenerated and transferred into a hormone-free MS medium with MS vitamins, ascorbic acid, and citric acid for embryo maturation. For lettuce transformation, cotyledons were cut from 7-day-old seedlings, and inoculated with the *Agrobacterium* strain GV3101, transformed with the pBI121 binary vector containing the *neomycin phosphotransferase* (*nptII*) gene for kanamycin selection and the *β-glucuronidase* (*uidA*) reporter gene. Forty-four kanamycin-resistant plants were regenerated and 50% were reconfirmed to carry over the *nptII* transgene by end point PCR. In conclusion, in this work, a reproducible *in vitro* regeneration system was developed for avocado, and an efficient transformation protocol was established for lettuce.

Acknowledgements

I would like to first thank the Honors University for giving me the opportunity to conduct my own research project, and making my final two years here at UCR the absolute greatest. The support they have given me does not go unnoticed. I am forever grateful for my mentors, Dr. Robert Jinkerson, Dr. Martha L. Orozco, and Dr. Patricia Manosalva for their help, support, and willingness to go the extra mile for me. Their patience, guidance, and encouragement set the ground for me to be able to bloom. I would like to thank Isua Ramirez Medina and Dr. Damaris Godinez-Vidal for their time and efforts to illuminate all that the lab requires, and Isua Martinez for her technical advice and support. If it weren't for you all, I would definitely not be where I am today. Thank you so much from the bottom of my heart.

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Introduction

There are about 7.5 billion people inhabiting the world as of April 2020 (U.S. and World Population Clock) and about 10.7% of this population are suffering from chronic undernourishment (World Hunger, Poverty Facts, Statistics 2018). Moreover, pests whether it be bacteria, fungus, viruses, weeds, insects, or animals are constantly affecting crop yields. Avocados in California have been affected by a fungus named mango anthracnose (*Glomerella cingulata*), and lettuce have been affected by a fungus named *Rhizoctonia solani*; along with various insects such as thrips (*Thysanoptera*). Even though pesticides are being used for pest control, its application is very toxic towards the environment, farmers, and consumers. The side effects of exposure to various pesticides in humans include forms of cancer and reproductive/developmental toxicity. In addition, there are about 3 million people admitted to hospitals because of pesticide usage with 220,000 deaths each year according to the World Health Organization (Klaassen, et al. 2013). However, amongst the sea of fear that these statistics bring, the process of genetic engineering and gene editing could be a way to ultimately alleviate the issue of pest control and increase yield of crops otherwise under stressful climate conditions. Through the improvement of the plants' survival rate in particular environments, a massive gain in economic strength and a reduction in famine in various countries is bound to occur. This research provides methodologies in ways to improve *in vivo* regeneration systems to possibly enhance genetic engineering efficiency in lettuce (*Lactuca sativa*) and avocados (*Persea americana*).

Lettuce

Experiment 1

Methods and Materials:

Lettuce (*Lactuca sativa*) varieties Tom Thumb, Blackseed Simpson, and Buttercrunch were used in this study. The lettuce seeds were sterilized with 10% commercial bleach (5.25% sodium hypochlorite) solution including 2-3 drops of Tween-20 for 20 minutes and rinsed five times with sterilized deionized water. After sterilization, seeds were germinated on a MS inorganic salt medium (Murashige and Skoog, 1962) which incorporated 30 g/l sucrose and 8 g/l agar at a pH level of 5.8 for seven days. At this time, the cotyledons were cut; half of them wounded by running the scalpel lightly across the cotyledon in lines and half not. Once that was done, they were inoculated with the *Agrobacterium* strain, GV3101, which is transformed with the pBI121 binary vector. Initially, this was done for 10 minutes with a concentration of 10^9 *Agrobacterium* cells/ml. After every two weeks the cotyledon explants were subcultured on a 30 ml MS medium/petri dish containing the hormones and antibiotics: 0.5 mg/l 6-benzyl-aminopurine (BAP), 0.04 mg/l naphthaleneacetic acid (NAA), 100 mg/l kanamycin, 250 mg/l carbenicillin, and 125 mg/l cefotaxime. That was performed until shoots were seen (I.S. Curtis, et al.). The shoot initiation media included 0.11% of 2-(N-morpholino)ethanesulfonic acid (MES). Lastly, rooting media was 4.3 g/l MS salts, MS vitamins, 30g/l sucrose, and 100mg/l myo-inositol, and 8g/l agar.

Results:

Of the cotyledons inoculated with the bacteria, none of them grew shoots under such selective conditions, therefore, leaving no transformation yield. Ultimately, these results are only being used as a source for control data since that was the only type that grew shoots. However, the lettuce type, Buttercrunch, showed one hopeful transformation growing on kanamycin-selective medium, which died before growing completely. In Figure 1, the three different lettuce type controls, in a medium without antibiotics, are shown. Here it is noted that there is a lot of vitrification or overgrowth of the crystal like calli. In Tom Thumb it can be seen that there are shoots but the calli is very yellow. In Blackseed Simpson it can be seen that there were no shoots but a continuation of weak calli growth. Lastly, in the Buttercrunch image, it is found that although there wasn't much shoot growth, the calli is very green and capable of more induction. These observations are also supported by Figure 2. Buttercrunch has the highest percentages across all control groups, then Tom Thumb, and lastly Blackseed. Ultimately, it was determined for the next round to use Buttercrunch in a more detailed process.

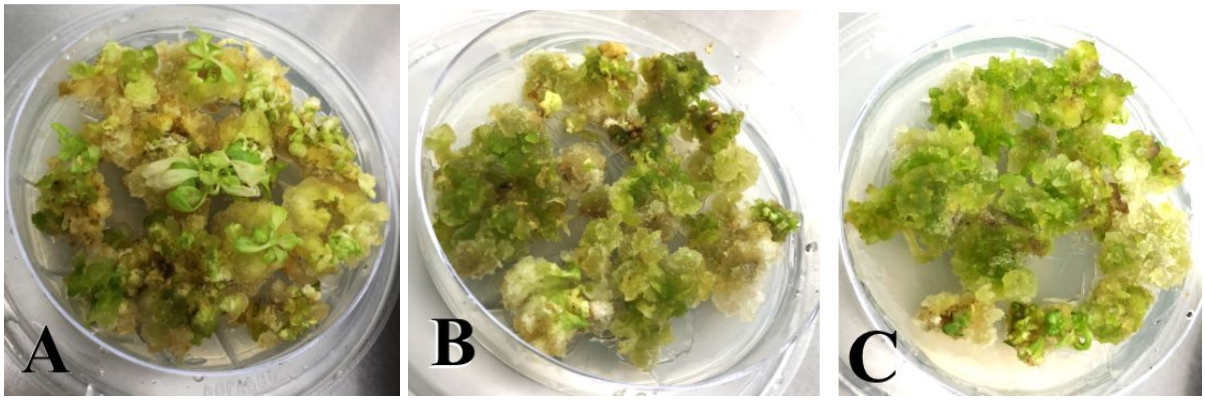


Figure 1: Resulting Control Shoot/Calli Growth of First Experiment
All are controls in rooting media. The first image on the left (A) is the variety Tom Thumb, middle image (B) is Blackseed Simpson, and the image on the right (C) is Buttercrunch.

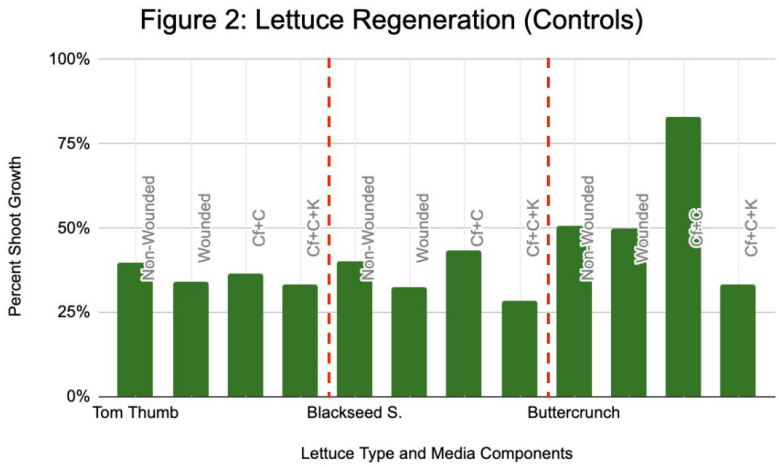


Figure 2: Chart of Control Results for Experiment One
Percentage results of the controls in the first round of the lettuce transformation. Buttercrunch leading in all media types whether wounded or not.

Experiment 2

Methods and Materials:

Buttercrunch seeds were sterilized and germinated as in the experiment 1. After cutting the cotyledons, however, they were placed in a pre-culture media consisting of MS inorganic salts, 30 g/l sucrose, 100 mg/l inositol, MS vitamins, 0.1 mg/l Indole-3-acetic acid (IAA), and 1 mg/l N-6(2-isopentenyl)-adenine (2ip) for one day. After one day, the cotyledons were inoculated with the same strain and concentration, but for 30 (treatment #1) and 60 minutes (treatment #3). Half were again wounded, 30 minute wounded is treatment #2 and 60 minute wounded is treatment #4, after inoculation by tapping with a scalpel for a total of 4 treatments. Once inoculated, the cotyledons were placed into the same pre-culture media with filter paper to co-culture for three days (72 hours). After the cotyledons were placed onto a new selective media that had MS inorganic salts, 30 g/l sucrose, 100 mg/l inositol, MS vitamins, 0.4 mg/l BAP, 0.05 mg/l NAA, 100 mg/l kanamycin, 250 mg/l carbenicillin, and 125 mg/l cefotaxime. They were subcultured every two weeks until shoots began to form (Lim, et al.).

There was a problem of vitrification and therefore the calli were cultured into four different shoot inducing selection media. The ingredients for number one is the same selective media previously stated. Number two changed the growth hormones to be 1 mg/l Zeatin and 0.1 mg/l IAA. Number three again used the same selective media but with the growth hormones 1 mg/l BAP and 0.1 mg/l IAA. Number four used a new media incorporating Schenk and Hildebrandt basal salt, 30 g/l sucrose, MS vitamins, 8 g/l agar, 0.5 mg/l kinetin, and 0.01mg/l IAA. The antibiotics were also changed to be only 100 mg/l kanamycin and 125 mg/l cefotaxime.

Rooting media were different depending on which inorganic salts were used. If using MS salts, the rooting was MS inorganic salts, 30 g/l sucrose, 100 mg/l inositol, MS vitamins, and 8 g/l agar. If using SH salts, the rooting was SH inorganic salts, 30 g/l sucrose, MS vitamins, and 8 g/l agar. Both included 100 mg/l kanamycin and 125 mg/l cefotaxime. In addition, to aid in avoiding vitrification, there were vents created in the containers by cutting a small square then covering it with surgical tape. After roots were seen the plants were transferred into soil.

Leaves from 44 shoots regenerated from all treatments were sampled for DNA extraction and polymerase chain reaction (PCR) analysis. This was done by grounding the leaf tissue and adding 300 µl of cetyltrimethylammonium bromide (CTAB) buffer. The buffer includes 10% of 1M TrisHCl at pH 8, 0.5M Ethylenediamine tetraacetic acid (EDTA) at pH 8, 5M NaCl, 3% of 99% CTAB, and 0.2% β-mercaptoethanol. They were then incubated at 65℃ for 60 minutes. One volume of chloroform was added and they were vortexed at 14,000 rpm for 8 minutes.

Depending on how clear the supernatant was, chloroform was added and was vortexed again for 8 minutes at 14,000 rpm. Once clean, 1 volume of isopropanol was added into the recovered supernatant. These were then placed in -20℃ for 30 minutes. The isopropanol was removed after vortexing at 14,000 rpm for 10 minutes. The DNA was then rinsed with 20% ethanol and the ethanol was removed after 5 minutes vortex at 14,000 rpm. It was allowed to dry before adding 30 µl of sterile water; DNA was quantified by Nanodrop. The PCR used specific primers for neomycin phosphotransferase (*nptII*) gene, which is the kanamycin resistant gene in the pBI121 binary vector. The primer sequences for *nptII* are KanR: 5' ACT GAA GCG GGA AGG GAC T 3' and KanF: 5' AAG GCG ATA GAA GGC GAT G 3'.

Results:

All the 30 minute wounded cotyledons had bacterial overgrowth and weren't able to continue growing, eventually turning brown and dying. All the other treatments: 30 minutes no wounding, 60 minutes no wounding, and 60 wounding (60-W) had shoot regeneration/growth. The representation of overall progression of growth before switching to four different selective media to aid in the later vitrification, is shown below in Figure 3.

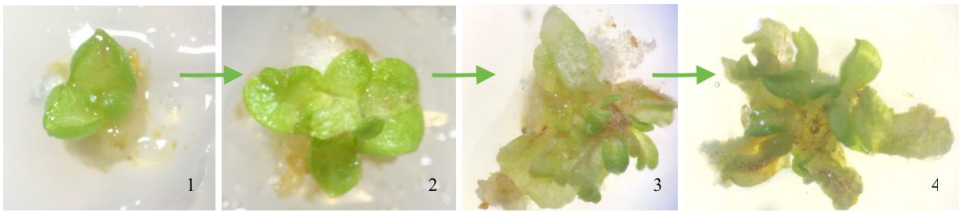


Figure 3: Overall Progression of Shoot Development
Showing shoot growth of the same Buttercrunch that was inoculated for 60 minutes with wounding. First and second image starting from the left was under the microscope with a 20x objective lens and the third and fourth image was obtained with a 10x objective lens. The third/fourth image shows the beginning of the eventual vitrification.

The growth after placing them into four different selective mediums are shown in Figure 4, and the percentages of actual growth, shoot and calli, is shown in Figures 5 and 6. Overall, it was found that media number 4 was the best to induce shoot formation without the continuation of calli formation; 97.5% of the calli regenerated shoots. It was also found that inoculating for 30 minutes without wounding is the better methodology shown by having higher averages of shoot formation and overall better quality of the plant as shown in Figure 4. In addition, 30 minutes without wounding didn't have the same amount of browning as the others do.

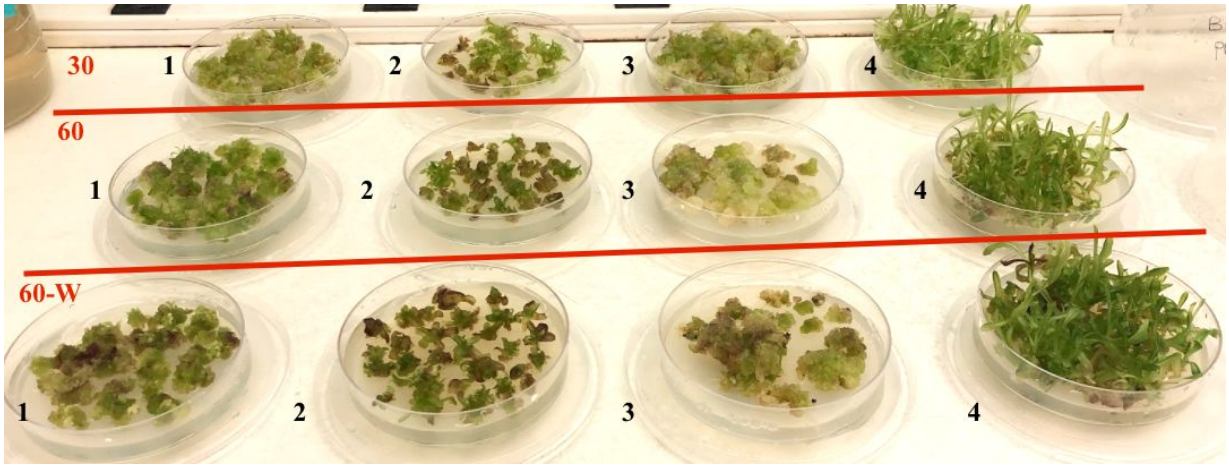


Figure 4: Shoot/Calli Development in all Various Mediums
In this image all groups are shown. The top row is 30 minute without wounding, the middle row is 60 minute without wounding, and the bottom row is 60 minute with wounding. The columns are separated by which selective media is being used, the numbers are corresponsive with the number of the selective media.

Figure 5: Media Type vs Calli Growth

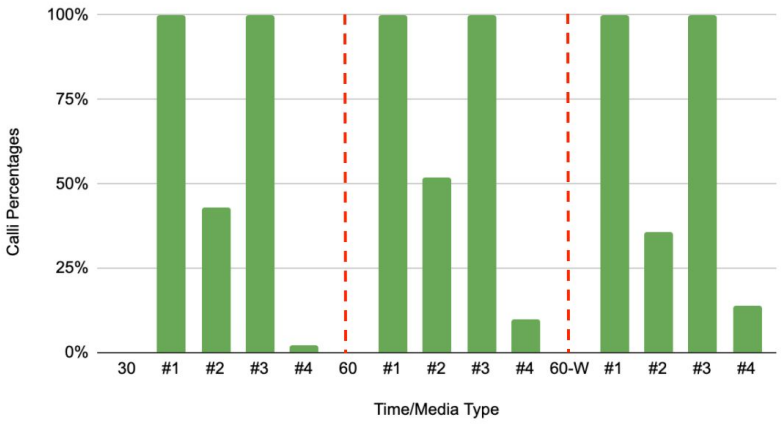
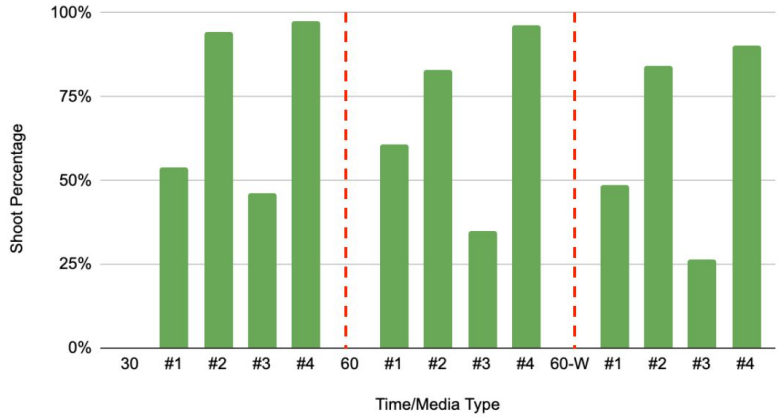


Figure 6: Media Type vs Shoot Growth



Figures 5 and 6: Percentage Development of Calli and Shoot in Various Mediums 1-4

These tables are representing the calli (Figure 5) and shoot (Figure 6) percentage growth throughout all subcultures. They show media number 4 having the best outcome of higher shoot formation and lower calli formation.

The PCR results showed that 50% of the 44 plant samples amplified a band of 750 bp; corresponding to *nptII* gene. This is shown in Figure 7. Moreover, of the 22 samples showing the expected PCR amplicons, about 60% were from the 30 minute non-wounded group being 13 of the 22. The 60 minute non-wounded had 2 of the 22 and the 60 minute wounded had the remaining 7 bands to itself.

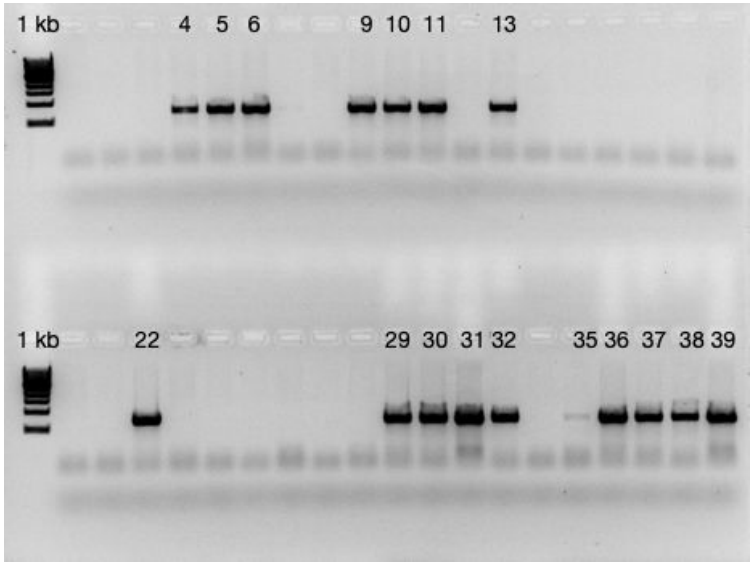


Figure 7: PCR Analysis of Kanamycin-Resistant Plants Regenerated from Different Treatments This is the image of the PCR that shows the bands around 750 bp. The numbers correspond with the plants' assigned numbers.

Avocado

Methods and Materials:

After collecting about 745 Duke 9 immature avocado fruits from the UCR field, they were all measured in centimeters and classified in 5 different groups according with their size (diameter): 1) mini, less than 1.5 cm; 2) small, 1.5 to 2 cm; 3) medium-small, 2 to 2.5 cm; 4) medium, 2.5 to 3 cm; and 5) large, larger than 3 cm. After the separation, the avocados were sterilized with 20% commercial bleach and 2-3 drops of Tween 20 per 100 ml sterilant for twenty minutes. They were then washed three times with sterile deionized water (Cruz-Hernández, et al. (1998) and Sánchez, et al. (2005)).

From each size, 30 fruits were placed into one of four culture mediums: A, B, C and D. Therefore, 120 fruits in total were used from each size. The four media include two with hormones and two without. A is 140 g/l sucrose, 8.6 g/l MS salts, 1 g/l malt extract, citrus vitamins, and 7 g/l agar. B is the same as A with the addition of 5 μM kanamycin. C is 4.3 g/l MS salts, 30 g/l sucrose, 100mg/l myo-inositol, 0.4mg/l thiamine-HCl, and 8g agar. Lastly, D is the same as C but with the addition of 0.1mg/l picloram. With sterile scalpels and tweezers that have also been dipped into 70% ethanol and then put into a flame for a few seconds, each fruit was cut longitudinally and the embryo was taken out and placed into the media. There were about 5 fruits per each plate and roughly 6 plates. The plates were then labeled and sealed with parafilm and then put into the dark at 25°C for about 3 weeks (Palomo-Ríos, et al., 2016). After 2 subcultures, they developed some globular embryos and the formation of small yellow callus appeared which is shown in Figure 8.

After consistent subculture every 4-5 weeks, it was noted that media C and D had actual pro-embryonic masses as seen in Figure 10 image number one. Therefore, those types of media were used for all the next subcultures. Once globular somatic embryos turned white and opaque they were moved into somatic embryo enhancing media (SEEM), shown in Figure 10 image 5, including 4.3 g/l MS salts, 30 g/l sucrose, 100 mg/l myo-inositol, MS vitamins, 150 mg/l ascorbic acid, 150 mg/l citric acid, and 8 g agar. After the somatic embryos got larger, they were moved into the same media but with an increase of sucrose to 45g/l, an increase of agar to 10 g/l, and 20% sterilized coconut water.

Using particle bombardment methods with pK7WG2D.0 plasmid DNA (Sanford, et al., 1993), one plate of size small avocado's pro-embryonic masses were separated into two. One was bombarded once and the other was bombarded twice. They were then placed into their same media but now containing 100 mg/l kanamycin.

Results:

The beginning media A and B did not produce any pro-embryonic masses and therefore deemed unuseful. Moreover, most of the avocado embryos from the sizes large, medium, and medium-small did not grow into the somatic embryos capable of germination. Size small had the highest amount ending with 222 somatic embryos. Figure 9 shows how small is leading with its highest percentage of 37%, and that media C is better at inducing the embryos than media D. Overall, the process of growth is shown in Figure 10.

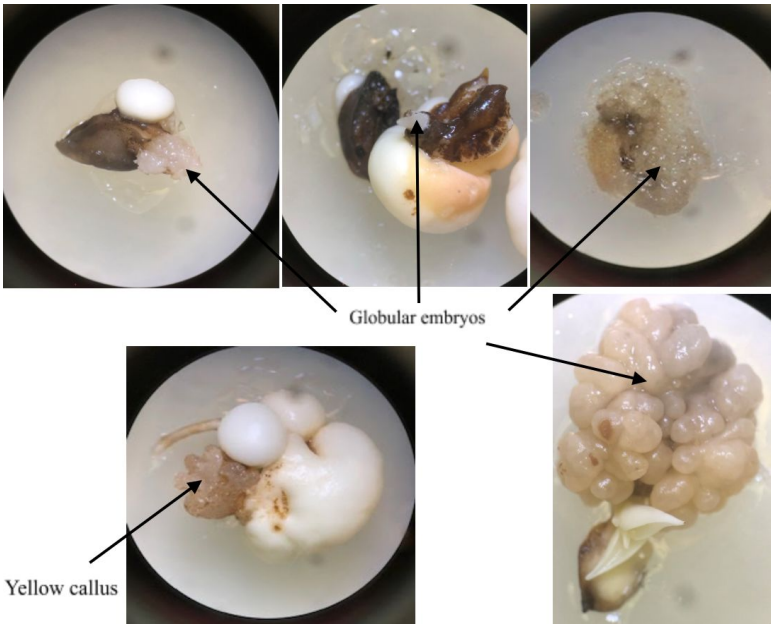


Figure 8: Development of Globular Embryos and Yellow Calli in Avocado
All these images are showing embryos from media A in size mini except the bottom right. That image is from media B (with hormone) size small. The microscope is at 10X magnification.

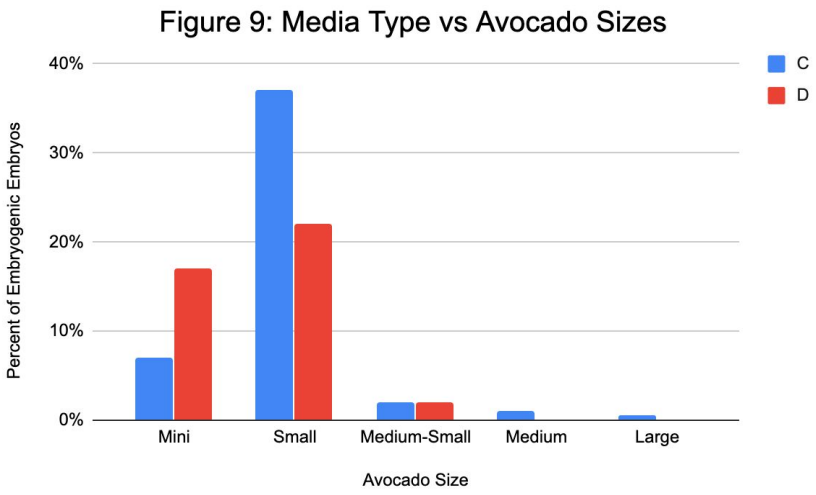


Figure 9: Percentage of Globular Somatic Embryo Development in Mediums C and D
This chart shows how media C is more efficient in inducing globular somatic embryos while the size small is the best measurement to try to induce.

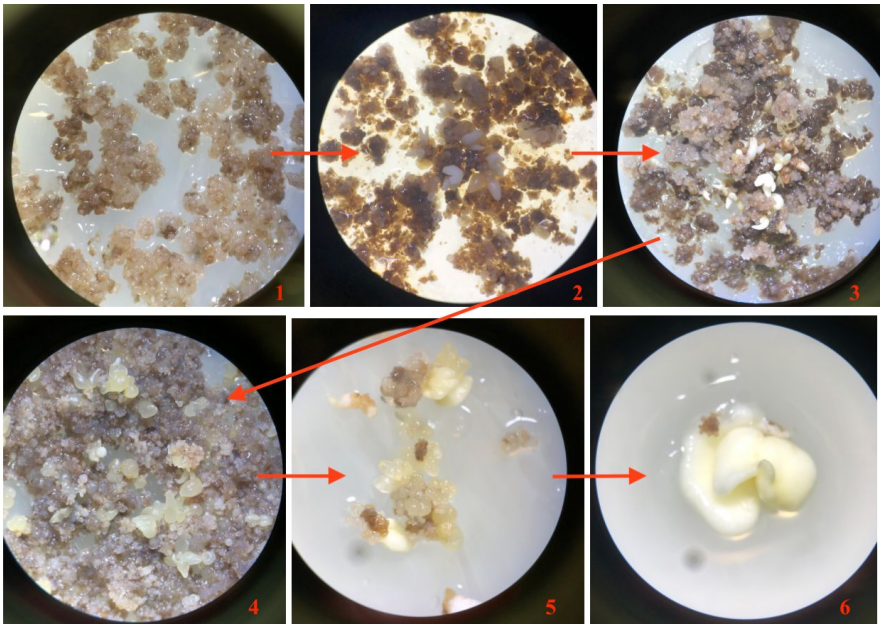


Figure 10: Overall Development of Somatic Embryos in Avocado
This image shows the process of the avocado's somatic embryo growth ending before germination (1-6). The magnification is about 13X.

Both plates showed GFP after 48 hours. Shown in Figure 11, somatic embryo growth was found in both kinds of bombarded avocado plates. There was about 40% growth of embryos in 1X bombarded and 63% growth in 2X bombarded. Unfortunately no pictures of the GFP were able to be taken at the time.

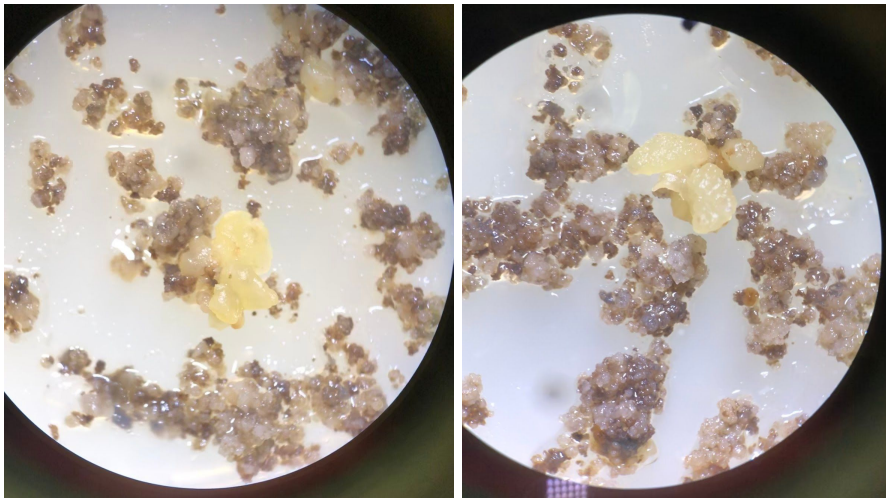


Figure 11: Somatic Embryo Growth in Selective Media After Bombardment
Both images show somatic embryo growth one month after bombardment. The first image on the left is 2X bombarded and the second image on the right is 1X bombarded.

Discussion:

In this study, an efficient *Agrobacterium*-mediated transformation protocol has been established for lettuce. This methodology involves the preculture of cotyledon explants for one day before inoculation with Agrobacterium for 30 minutes without wounding then coculturing on that same media. Next, culture on selection medium number 1 for callus induction, transfer of antibiotic-resistant calli to shoot and root induction medium number 4, and finally transfer rooted plants to soil. Using this methodology, 44 putative transgenic plants were regenerated, of which 50% were confirmed by PCR. The problem of vitrification was significantly reduced using this methodology and with the help of the containers having vents; by increasing the gas exchange. Ultimately, 97.5% of the calli regenerated shoots. This protocol is currently being used with a different lettuce variety named Conquistador. At the moment, there is a high yield shoot formation; PCR has yet to be done. Moreover, for future prospects, the lettuce plants will be allowed to grow to further develop their own seeds. There is now a need to see whether or not the introduced genetic material can pass onto the offspring.

The avocado transformation with particle bombardment is very promising. Having only done two plates, both are growing transformed embryos. There is, however, a need to do more research on this methodology in terms for transformation. This part of the project was forced to be cut short due to the closure of the campus. For the future, the methodology will be narrowed down, as done with the lettuce, to using only small fruits between 1.5 and 2.0 cm in diameter. Given the yield with that size was much higher than the others, it can be used as a model stepping stone. In the end, genetic transformation/engineering is a forthcoming field that will

eventually provide food for all without the worries of pests ruining our crops and pesticides ruining our health.

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