Plant Callus Derived Scaffold for Cultured Meat Applications

A thesis submitted by

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Abstract

By 2050, the global population will surpass nine billion, demanding more food production than current agriculture can support. Cellular agriculture provides an innovative method to produce protein-rich foods in vitro, reducing environmental impact and animal cruelty. Consumers still desire the experience and nutrition of complex meat cuts, necessitating effective scaffolding in cultured meat. Traditional scaffolding methods, such as decellularized plants and electrospun scaffolds, often involve harsh preparations and degrade over time. Plant tissue culture offers a sustainable alternative, producing specialized scaffolds from living plant tissue.

This thesis investigates using plant callus scaffolds from Phyllostachys edulis (bamboo) seeds and Cucurbita maxima (Big Max Pumpkin) leaves for cultured meat production. C2C12 cells were seeded onto C. maxima callus and cultured for 21 days, showing significant adherence and stability comparable to 5% gelatin films. The callus scaffold remained stable without pH changes, degradation, or contamination. These findings suggest that plant calluses could be viable scaffolds for cultured meat and tissue engineering. Further research should explore other cell lines and callus types.

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3. Introduction

3.1. Climate Change Overview

Climate change is one of the largest challenges our society faces from long-term shifts in temperatures and weather patterns that impact everyday life. Global warming is driven by the accumulation of greenhouse gases (GHGs) such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) accumulating in the atmosphere. These GHGs trap heat and prevent it from escaping the atmosphere creating the greenhouse effect that raises the earth’s temperature causing severe weather conditions.

Climate change is influenced by natural and anthropogenic factors. Human activities such as burning of fossil fuels, deforestation, industrial pollution, and animal agriculture accelerate the accumulation of GHGs thus accelerating global warming.

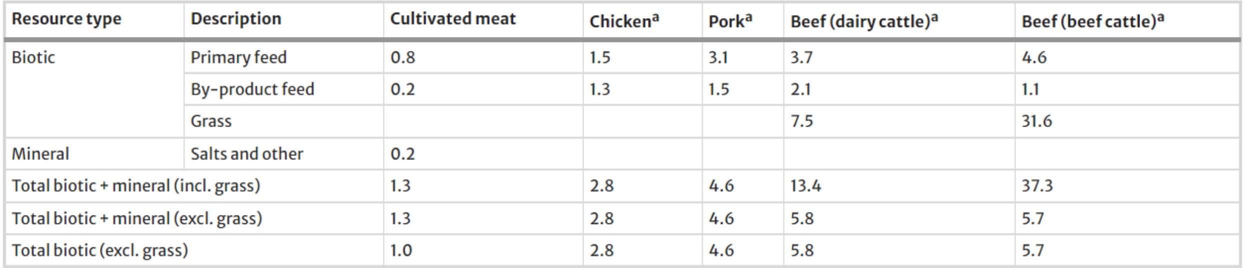
Climate change will impact the economy and society through an increase in extreme weather events such as hurricanes, droughts, and heatwaves. These events directly affect agriculture, damage infrastructure, threaten food security, and pose health risks to vulnerable populations.

3.2. Animal Agriculture Overview and Problems

Animal agriculture refers to the breeding, raising, and processing of livestock for meat, dairy, and other animal products. Commonly raised species include cattle, pigs, chickens, sheep, and goats. To sustain these animals, industrial livestock farmers deforest large areas by burning landscapes to create space for grazing and growing feed crops. This deforestation emits significant amounts of carbon dioxide and results in habitat destruction and biodiversity loss.

Livestock farming is highly water-intensive, requiring immense amounts of water for various purposes, including feed production, drinking water for the animals, waste management, and meat processing. Livestock consume feed, leading to the production of methane through digestion and manure[1]. While this is manageable on a small scale, industrial-scale livestock farming significantly impacts the global environment.

Table 1: Feed conversion ratio (FCR) of ambitious 2030 benchmarks, dry matter in:fresh meat out (kg:kg)[2]



Resource type	Description	Cultivated meat	Chicken[a]	Pork[a]	Beef (dairy cattle)[a]	Beef (beef cattle)[a]
Biotic	Primary feed	0.8	1.5	3.1	3.7	4.6
	By-product feed	0.2	1.3	1.5	2.1	1.1
	Grass				7.5	31.6
Mineral	Salts and other	0.2				
Total biotic + mineral (incl. grass)		1.3	2.8	4.6	13.4	37.3
Total biotic + mineral (excl. grass)		1.3	2.8	4.6	5.8	5.7
Total biotic (excl. grass)		1.0	2.8	4.6	5.8	5.7

Livestock farming is inefficient. Feed conversion ratio (FCR), measures the efficiency of converting feed into 1kg of meat. Table 1 shows an estimated ambitious FCR calculation for Chicken, pork, beef (dairy cattle), and beef (beef cattle) by 2030 compared to cultured meat based on a GFI study[2].

This inefficiency in feed conversion highlights that producing animal-based foods demands substantial quantities of feed, land, and water. For example, livestock farming is responsible for approximately 41% of global green and blue water use combined, with around 6% attributed to blue water use alone[2]. This significant resource requirement exacerbates environmental degradation and places additional stress on water supplies.

In addition to greenhouse gas emissions and resource inefficiency, livestock farming causes other environmental problems. Overgrazing and intensive farming practices lead to soil erosion and loss of fertility, reducing land productivity and causing sediment to build up in water bodies. Runoff from farms carries nutrients, pathogens, and chemicals into rivers, lakes, and oceans, leading to harmful algal blooms and water pollution that damage aquatic life.[3]

Animal welfare is also a major concern in livestock farming. In large-scale factory farms, animals are kept in confined spaces and prevented from engaging in natural behaviors. There is also routine use of antibiotics to prevent diseases from spreading due to poor conditions. The use of antibiotics directly contributes to the development of antibiotic-resistant bacteria, posing a significant public health risk.[4]

These challenges demonstrate the urgent need to implement sustainable and ethical alternatives to conventional animal agriculture without compromising public nutrition. Plant-based proteins and cultured meat present ways to reduce environmental impact, improve animal welfare, and enhance food security.

3.3. Plant Based Meat Solutions as a Solution

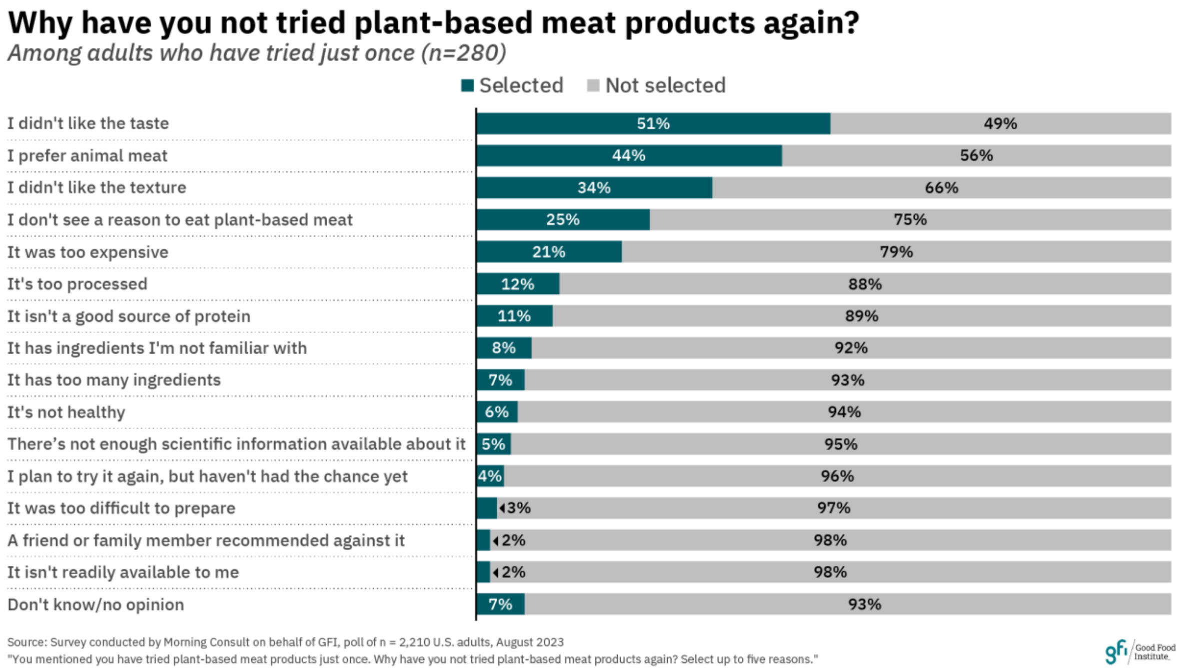
Plant based meat refers to meat alternatives derived from plant proteins. These products strive to emulate animal-based meat both structurally and nutritionally. Plant based meat is composed of protein, fat, vitamins, minerals and water not derived from animals.

Plant-based meats are designed to mimic the nutritional profile of animal meats. They strive to contain similar amounts of protein and fat, while also being lower in cholesterol and saturated fats. However, they may also have higher sodium content and sometimes lack certain micronutrients found in animal meat.

One of the main challenges in plant-based meat production is replicating the taste and texture of animal meat. Companies are investing in research and development to improve these aspects. Innovations include using advanced protein isolation techniques, developing new plant-based ingredients, and utilizing food technology to enhance flavor and texture.

The plant-based meat market has expanded significantly in the past decade, thanks to pioneers like Beyond Meat ™ and Impossible Foods ™. In 2023, the plant-based meat market was valued at $8.1 billion , and certain projections indicate it can reach $24.80 billion by 2030[6]. But consumer research shows signs of stagnant growth due to a lack of high-quality meat emulation options.

Consumer preferences are shifting, with a growing number of people seeking sustainable and ethical food options. However, many consumers still prioritize taste and texture. In December 2023, The Good Food Institute conducted a survey to consumers who purchased cultured meat in the past but did not purchase again in the following three months. Of these customers, 49% requested taste and texture being exactly like conventional meat when they try a sample. In this study, GFI concluded consumers want an identical meat substitute with minimal processing, and familiar ingredients **Figure 1.**[5]



Why have you not tried plant-based meat products again?

Among adults who have tried just once (n=280)

Source: Survey conducted by Morning Consult on behalf of GFI, poll of n = 2,210 U.S. adults, August 2023
"You mentioned you have tried plant-based meat products just once. Why have you not tried plant-based meat products again? Select up to five reasons."

Figure 1 GFI survey August 2023 among adults who have tried plant based food once, but not again (n=280)[5].

While plant-based meat presents a great bridge option to traditional animal livestock, consumers still want an identical experience to meat, and plant-based meats do not currently deliver that experience. Cellular agriculture presents a way to build a closer product to traditional livestock meat by incorporating animal meat cells such as fibroblasts, muscle cells, and adipocytes into plant-based scaffolds.

3.4. Intro to Cellular Agriculture

Cellular agriculture refers to the process of reproducing traditional meat such as chicken breast, steak, and fish from cells, not livestock. The process involves isolating cells from an animal biopsy, multiplying the cells in a media broth that contains all necessary nutrients for cell survival and growth, and the harvest of cell mass.

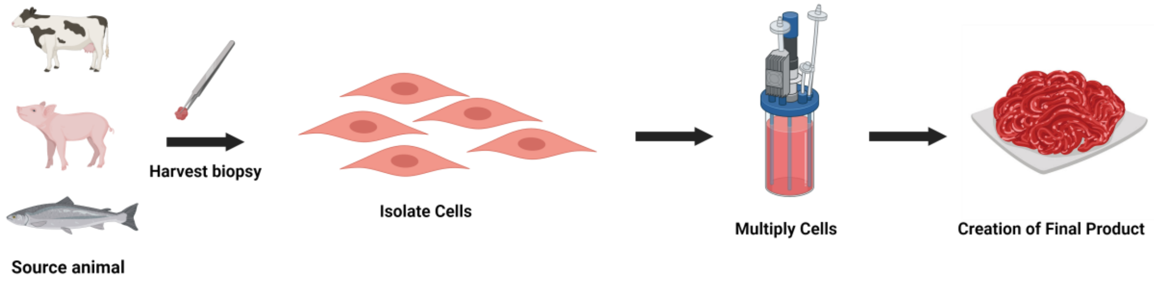


Figure 2 Overview of the Cellular Agriculture process

Figure 2 demonstrates the simplest model for cellular agriculture, cells are multiplied in a bioreactor, then harvested as a cell mass without structure or organization, yielding a ground meat-like product.

There is a wide spectrum of meat products ranging from hotdogs, sausages, and burgers to filets and steaks, with increasing structural complexity respectively. To provide the experience of complex meats, scaffolding is necessary to replicate the three-dimensional cell environment.

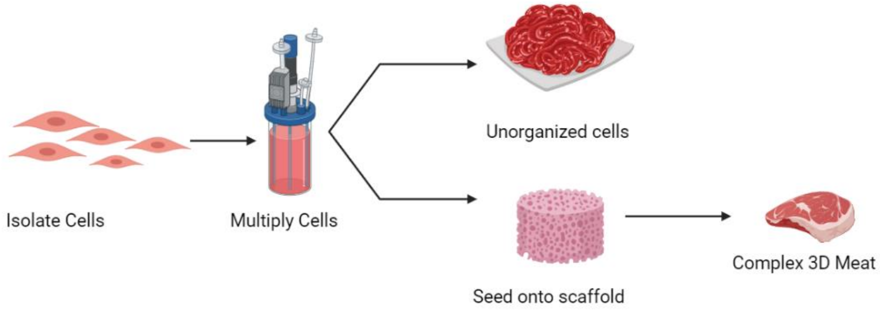


Figure 3 Comparison between cell mass cultivation versus scaffolding for a 3D structure of cultured meat.

Sensory expectations are key obstacles to obtaining high consumer acceptance for cultured meat[7]. Consumers want cultured meat products to be identical to their animal-derived counterparts[8]. The process highlighted in **Figure 2** falls short of emulating complex meat products. Scaffolding for cultured meat accelerates the path to 3D tissues shown in **Figure 3**.

3.5. Scaffolding

Scaffolding is an essential component in the production of structured 3D meats such as chicken breast, ribeye, and fish. A scaffold provides the necessary structural environment to mimic the in-vivo extracellular matrix (ECM). They provide physical and biochemical cues for muscle cells to adhere, proliferate, and differentiate into tissue that mimics the original texture and structure of conventional meat.

Scaffolding originated in the field of tissue engineering for regenerative medicine, where biocompatible structures are used to support the growth of cells into tissues and organs. In this broad field, scaffold technologies must possess biocompatibility, biodegradability, and mechanical properties like the target tissue or anatomical site for implantation. Additionally, the scaffold architecture needs to be porous to ensure the perfusion of nutrients to cells and the removal of metabolic waste within the structure. Materials in the field of tissue engineering are either synthesized from natural ECM materials which possess cell attachment group ligands Arg-Gly-Asp (RGD) binding sequences, whereas synthetic scaffolds require incorporation of these ligands[9,10].

Scaffolding for cultured meat shares many characteristics with traditional tissue engineering, including biocompatibility, biodegradability, mechanical properties, pore size, and scaffold architecture. However, cultured meat scaffolds are created with the intent of consumption, thus must be developed with food considerations in mind. The development of 3D structures for cultured meat should mimic traditional animal-derived meats by providing an environment conducive to the development of 3D muscle and fat structures. Muscle and fat are the main components of meat, so the scaffold does not need to mimic animal tissue entirely but should provide a biocompatible environment for muscle and fat cells to proliferate and differentiate, thereby improving the sensory properties of the product.

Cultured meat scaffolds should have sufficient porosity to allow for nutrient exchange without interrupting the process. To drive down costs for scaling up, cultured meat scaffolds are being designed for incorporation into the final product. Therefore, the scaffold must be edible and ideally nutritious to provide additional value to cultured meat products[11]. An ideal scaffold would be nutritious which could mean providing essential nutrients such as proteins, vitamins, and minerals that complement those found in the cultured cells. The scaffold should break down in the digestive system without causing harm or discomfort. This ensures that it integrates seamlessly into the final product, contributing to both the texture and nutritional value without requiring removal before consumption. Other scaffolds such as hollow-fiber bioreactors which are semi-permeable capillary membranes arranged in parallel arrays to mimic the in-vivo behavior of 3D culture systems have explored. However, these scaffolds are typically composed of non-digestible materials and thus additional processing steps to remove cells from the bioreactor and refresh the scaffold are required thus driving up the cost of production therefore justifying the use of incorporable scaffolds.

Researchers have made edible hydrogel and film scaffolds out of pea protein, alginate, chitosan, collagen, pectin[12], glutenin, zein, soy, cellulose, and konjac[13]. These scaffolds are created from protein dispersions, crosslinking, and gel formation. Based on research currently occurring in the Kaplan Lab, these scaffolds have been shown to degrade in media during culture over 21 days.

Electrospun scaffolds, created by applying an electric field to polymer solutions to produce ultra-thin fibers, have been explored for cultured meat applications. Researchers have electrospun various plant-based protein solutions, including zein, soy protein, chitosan, starch, and egg albumin. However, this technique's widespread adoption is hindered by the necessity of non-food-grade solvents and crosslinking agents in the electrospinning process[14]. Consequently, research on advancing electrospinning for scaffolding in cultured meat has been limited.

Researchers have explored simplifying the scaffold production process by utilizing natural scaffolds such as plant tissue through decellularization. Spinach, parsley, grass, carrots, celery, and apples have been investigated as scaffolds for mammalian cells[15, 16, 17, 18].

In the decellularization process, full plant tissues are treated with chemical solvents to remove plant cells from the tissue. This process can take up to a week and involves many toxic chemicals to produce the scaffold.

Alternatively, plant-tissue culture can be explored to create renewable, edible, living tissue scaffolds with minimal use of harsh chemicals. This method involves growing plant tissues in controlled environments, which can be used as scaffolds for cultured meat, offering a more sustainable and potentially less toxic approach.

Fast-growing plants, such as grasses, are often grown for their ability to quickly generate large amounts of biomass with little energy requirement (sunlight-driven processes). This biomass, rich in lignocellulosic material, is typically processed into biofuels, paper, and other industrial products. Lignocellulosic biomass consists of cellulose, hemicellulose, and lignin, making it a versatile and sustainable raw material. Fast growing plants present ideal systems to explore for scaffolding for cultured meat to drive value to achieve significantly reduced energy use and costs. By utilizing their rapid growth characteristics and robust tissue structures, we can develop renewable, edible scaffolds that support the growth of cultured meat, potentially offering a more efficient and sustainable alternative to traditional scaffolding materials. Ideally lignocellulosic plants typically considered waste streams (bamboo, corn husk, wheat straw, hemp, etc…) would be ideal scaffolding materials for cultured meat.

Due to the one-year nature of this study, pumpkin, which is a fast-growing plant but not considered lignocellulosic or waste, was explored as an alternative to bamboo because of the difficulties associated with the plant tissue culture of bamboo.

3.6. Plant Tissue Culture

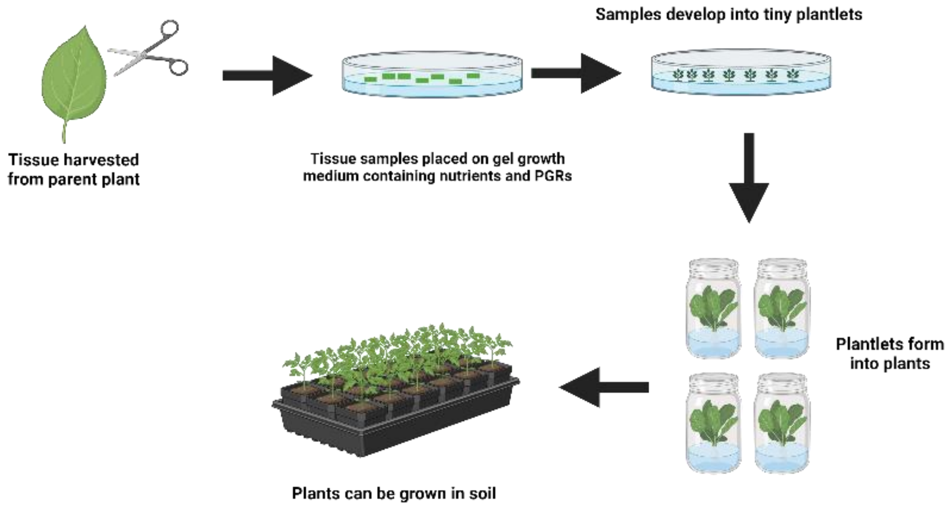


Figure 4 Plant Tissue Culture overview

Plant tissue culture refers to the techniques used to grow plant cells under sterile conditions in a nutrient rich medium that mimics the in-soil conditions. It is often used for micropropagation where a cutting from a plant is placed in a nutrient solution containing auxins and cytokinins, hormones that signal plant growth pathways. Auxins stimulate cell elongation and cell division, cytokinins stimulate cell division in meristematic tissues. By combining auxins and cytokinins in specific ratios, the morphology of a plant can be controlled[19].

Media for plant tissue culture can be either liquid or solid depending on the inclusion of a gelling agent such as agar, or gellan gum. It is based on Murashige and Skoog Basal Medium (MS) which is a widely used plant tissue culture medium created by Toshio Murashige and Folke Skoog (1962)[20]. Murashige and Skoog identified the essential macro and micronutrients required for developing plant tissues in vitro across a wide range of species.

MS medium contains the following compounds: ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3), potassium dihydrogen phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), ferrous sulfate ($FeSO_4$), disodium EDTA, manganese sulfate ($MnSO_4$), zinc sulfate ($ZnSO_4$), copper sulfate ($CuSO_4$), boric acid (H_3BO_3), sodium molybdate (Na_2MoO_4), thiamine hydrochloride, pyridoxine hydrochloride, nicotinic acid, sucrose, and optional, gelling agents such as agar or gellan gum[20].

In addition to the MS basal medium and gelling agents, hormones, also referred to as plant growth regulators (PGRs) regulate growth, development, and differentiation of plant tissues. Auxins such as Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), and Naphthaleneacetic Acid (NAA) promote root initiation and development. Cytokinin such as 6-Benzylaminopurine (BAP or BA), Kinetin, and Thidiazuron (TDZ) promote shoot formation and development[21].

Specific combinations of these PGRs result in plants developing tissue in highly customized environments. The reason for this is plants respond to external stimuli and sense changes in their environment. To shed leaves, develop flowers, or initiate defense mechanisms, plants synthesize these hormones for cell communication[19].

Figure 5 Photo of *C. maxima* callus creating shoots, roots, and callus from initial leaf explant due to hormone signaling imbalance in an experiment.

These PGRs are incorporated into media at low doses, do not have nutritious values, and are not phytotoxic. PGRs can be used to obtain specific advantages such as resilience towards abiotic and biotic stress, improved structure, and added secondary metabolites[19].

By combining approximately equal ratios of cytokinin: auxin, plants develop a callus, which is a mass of unorganized, undifferentiated plant cells. Since plants are totipotent, the callus can regenerate into a full plant if cultured on the appropriate medium as shown in **Figure 5**.

PGRs are often dedicated for herbicide and pesticide use cases. One example of this is the synthetic auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D). 2,4-D was the first synthetic herbicide to be commercially developed. It is an herbicide that kills dicot pants, without affecting monocot plants – such as weeds (dicot) versus grasses (monocot) on a lawn. It is a synthetic compound that mimics natural auxins at the molecular level. At high concentrations, 2,4-D leads to abnormal cell growth, and plant death in dicots[22]. 2,4-D is an essential compound in callus formation.

3.7. Callus culture

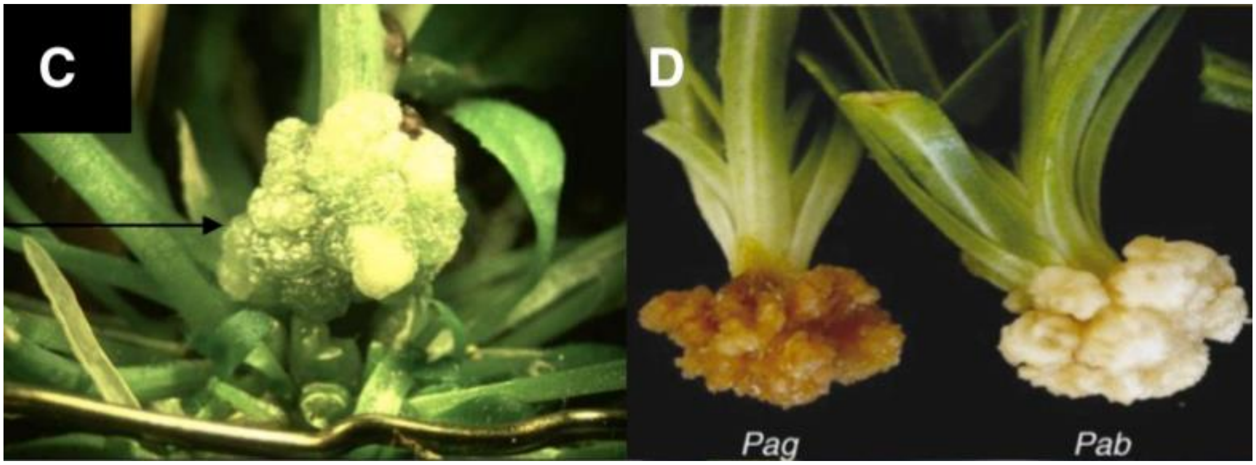


Figure 6 Photo of callus formation naturally occurring in nature on Aradopsis[23].

Plants develop callus in a natural response to a wound acting as a protective barrier, similar to a callus on human skin[24].Callus are composed of totipotent plant cells which enable the total regeneration of plant tissue. Calli can be induced to differentiate into complete plantlets, which can then be developed into mature plants. This is a valuable tool for propagating rare or commercially important plants or rescuing diseased plants. Calli also can remain in dormant stages in storage for extended periods of time allowing preservation of endangered species.

Plant callus can be created through tissue culture techniques for micropropagation or secondary metabolite farming. Plants can be genetically modified using callus as a substrate for genome transformation since the cells are undifferentiated and can be induced to form full plants with desired traits, such as resistance to pests, diseases, or herbicides.

Plants have evolved to adapt to their environment by producing molecules that react to environmental stressors, also known as secondary metabolites. These secondary metabolites occasionally have medicinal and commercialization value for humans. Alkaloids, flavonoids, terpenoids, and ginsenosides are examples of medicinal secondary metabolites found in plants and can also be extracted from plant callus. Traditionally, plants were overharvested to acquire these valuable secondary metabolites for medical purposes but callus culture enables a scalable and sustainable way to harvest these metabolites[25].

Callus can be cultured in suspension or in solid state. Suspension cultures involve isolating individual callus cells and placing them on shakers in media in the dark in a MS based medium with species-specific PGR combinations. The suspension culture is simple, effective, and scalable for producing valuable secondary metabolites[26].

Alternatively, callus can be cultured on solid gel mediums containing necessary nutrients and PGRs to induce callus growth (solid culture). Callus form compact masses of undifferentiated plant cells when grown on solid media. As the callus continues to grow, and the cells are further from media contact, friable (fragile) callus will form which can easily be broken apart and put into suspension culture to proliferate. Compact calluses are frequently used for plant regeneration whereas the friable callus is used for secondary metabolite harvesting.

3.8. Callus as a Living Scaffold

Researchers have explored decellularized, dead, plant tissue as scaffolds for tissue engineering purposes. However, these methods often involve harsh chemical washes that can leave residual chemicals in the scaffold, potentially compromising its safety, biocompatibility, digestibility, and degradability. Callus cultures offer an alternative approach to creating plant-based scaffolds with minimal processing steps, reduced maintenance, and fewer harsh chemicals.

This specific study explores the efficacy of *Cucurbita maxima* (*C. maxima*) “Big Max Pumpkin” as a callus scaffold for cultured meat applications. By leveraging the natural growth and regenerative properties of callus tissues, this study aims to develop a sustainable, edible, and biocompatible scaffold that can support the growth of cultured meat cells, providing a viable alternative to traditional scaffolding methods.

4. Specific Aims:

4.1. Specific Aim 1: Isolate and Proliferate a Fast-Growing Plant Callus Scaffold

For a scaffold to be suitable for cultured meat, it should be biocompatible, biodegradable, edible (digestible), supportive of cell adhesion and growth, and nutritious. Additionally, plant-tissue culture derived scaffolds should be nutritious, sustainably sourced, non-competitive with the food supply, and fast-growing.

This study aims to assess the potential of *Phyllostachys edulis* (Moso bamboo) and *Cucurbita maxima* (Big Max pumpkin) as sources for plant tissue culture-derived scaffolds. These species were selected for their rapid growth rates in nature, with the hypothesis that this characteristic will translate to accelerated callus formation in vitro.

Aim 1 involves selecting and sterilizing C. maxima seeds to ensure aseptic conditions, germinating the seeds, and inducing callus formation from leaf explants. It also includes optimizing the culture conditions, such as media composition and PGR combinations, to promote rapid and robust callus growth, as well as evaluating the growth rate, morphology, and regenerative capacity of the callus.

4.2. Specific Aim 2: Analyze Adhesion, and Growth of Mammalian Cells on Callus Scaffold

According to a study by Kong et al., pumpkin seed protein isolate rivals gelatin as a coating for edible microcarriers for cultured meat applications[27]. Kong et al. isolated pumpkin seed protein from pumpkin seeds then coated edible alginate microcarriers and seeded them with C2C12s and chicken muscle satellite cells. This study will explore if pumpkin callus can function as a scaffold similarly to the pumpkin-coated microbeads. It is expected that C2C12s will adhere to pumpkin callus with mildly high seeding{Citation} efficiency and will proliferate and differentiate on the scaffold due to the natural RGD ligand content in pumpkin.

Specific aim 2 includes preparing the pumpkin callus scaffold and ensuring it maintains its structure and properties, seeding the scaffold with C2C12 cells, assessing cell adhesion, proliferation, and viability on the pumpkin callus scaffold, and comparing the performance of the pumpkin callus scaffold to traditional scaffolding materials.

5. Design Goals

Regenerative Plant Tissue Culture Scaffolds

The primary objective is to create a regenerative, sustainable plant tissue culture callus-based scaffold that supports cell adhesion for biomedical and biotechnological applications. The design goals include ensuring biocompatibility by promoting cell viability and proliferation, achieving edibility by making the scaffold safe for incorporation into the final cultured meat product, and focusing on sustainability by utilizing eco-friendly, renewable materials to achieve a sustainable production process.

6. Methods

6.1. Seed selection from *Phyllostachys edulis* (Moso Bamboo)

Phyllostachys edulis (Moso bamboo) is one of the edible bamboo species. It was ranked as 5/5 taste on guaduabamboo.com, and it is known to be one of the most commonly eaten bamboo species[28]. *P. edulis* seeds (Amazon B0CGCZBB2S) were ordered from Amazon for the experiment. Seeds were germinated in petri dishes and paper towels to test validity.

6.2. Rhizome selection from USDA *P. edulis*

Rhizome for *P. edulis* (PI 647946, Grif 13948, Phyllostachys edulis (Carrière) J. Houz.) was ordered from the USDA National Plant Germplasm System. Rhizome was planted in the Tufts University Greenhouse located on the third floor of Barnum Hall (163 Packard Avenue in Medford, MA). Samples were cut using gardening scissors.

6.3. Seed selection of C. maxima (Big Max Pumpkin)

One of the fastest growing accessible species from a seed is pumpkin. *C. maxima* is a fast-growing vine plant that produces large pumpkins that are often sold as novelty at fairs and contests, thus the species was selected for exploration of a fast-growing, sustainable, and regenerative plant scaffold. *C. maxima* were chosen over traditional pumpkin (*C. pepo*) because *C. maxima* have been selectively bred to be larger and faster growing to create giant pumpkins. *C. maxima* seeds (Amazon B08FCVY248) were ordered from Amazon were germinated in paper towels over a week to explore germination rates.

6.4. Germination Media Preparation

Germination media was prepared using standard ½ strength formulation. 1.13 g/L MS Basal Medium (Plant Cell Technology, USA) and 8 g/L Agar (Plant Cell Technology, USA) were measured in powder form and added to 500 mL of ultrapure distilled water (Invitrogen, USA) under medium mixing at room temperature. The pH was calibrated to ~5.70 using HCl and NaOH measured with a pH probe. The resulting solution was autoclaved on the Liquid 20 cycle and the molted solution was aliquoted in 20 mL parts into 100 mm x 20mm petri dishes (Corning, USA). The petri dishes are then cooled at room temperature in the biosafety cabinet to form a solid, semi-transparent gel, wrapped with parafilm, and stored at 4°C.

6.5. C. maxima Callus Media Preparation

Callus media for leaf explants was prepared using the formulation from Yuan et al[29]. 4.54 g/L MS Basal Medium (Plant Cell Technology, Washington DC), 8 g/L Agar (Plant Cell Technology, Washington DC), 30 g/L Sucrose (Millipore Sigma, USA) 4 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) (Research Products International, USA) 1 mg/L BA (Thermo Fisher, USA) AND 0.1 mg/L Zeatin (Millipore Sigma, USA) were added to 500 mL of ultrapure distilled water (Invitrogen, USA). Certain experiments explored the removal of BA and Zeatin, as well as different concentrations of 2,4-D (2-8 mg/L). The pH was calibrated to ~5.70 using HCl and NaOH measured with a pH probe. The components were combined with stirring, then autoclaved on the liquid-20 cycle in the small autoclave. The resulting solution was aliquoted into 90 mm, and 30 mm petri dishes at 20 mL, and 10 mL respectively and let cool at room temperature to form a solid, semi-transparent gel, then wrapped with parafilm, and stored at 4C.

Callus media for root explants was prepared using the formulation from Balen et al. for the formation of embryogenic callus in hairy roots of pumpkin (*Cucurbita pepo* L.*)* 2 mg/L IAA (Millipore Sigma, USA), 0.5 mg/L BA (Millipore Sigma, USA), 10 mg/L Thiamine HCl (Millipore Sigma, USA), 30 g/L sucrose (Millipore Sigma, USA), 8 g/L agar (Plant Cell Technology, USA), and 4.2 g/L MS Basal medium (Plant Cell Technology, USA) were combined in 500 mL ultrapure distilled (Invitrogen, USA) water under light room temperature stirring then autoclaved on the liquid-20 cycle in the small autoclave. The resulting solution was aliquoted into 100 mm x 20 mm petri dishes (Corning, USA), let cool at room temperature, wrapped with parafilm, and stored at 4C.

6.6. Sterilization of C. maxima Seeds

C. maxima pumpkin seeds (Amazon B08FCVY248) were fully submerged in ~200 mL of ultrapure distilled water (Corning, USA) overnight to help with germination. After submersion in water, *C. maxima* pumpkin seeds were fully submerged in a 1% sodium hypochlorite solution created from 12.12 mL Clorox Germicidal Bleach (Clorox, USA) and 87.88 mL ultrapure distilled water under light stirring for 30 minutes. The seeds were removed from the sodium hypochlorite solution and rinsed five times with ultrapure distilled water. Then, the beaker containing the seeds was transferred to the biosafety hood for germination.

6.7. Aseptic Germination of C. maxima Seeds

Inside the biosafety cabinet, the sterile *C. maxima* seeds were placed on a Kimwipe to air dry. One to four *C. maxima* seeds were placed on germination petri dishes with sufficient space as described in **6.4** using sterile forceps. These dishes were then re-wrapped with parafilm and placed in a 27C incubator with a 16-hour LED light cycle for five to ten days, until leaves were formed.

6.8. C. maxima Leaf Isolation and Processing for Callus Induction

After leaves were formed in the germination step as described in **6.7**, the germination petri dish containing *C. maxima* seeds was brought into the biosafety cabinet. Using sterile forceps, and a one-time use sterile scalpel, leaves were isolated from the shoot of the pumpkin plant and placed into a sterile petri dish. Leaves were cut into 1cm x 1cm squares exposing tissue on all four sides. The squares were placed into the callus media dish as described in **6.5** using sterile forceps and cultured in the dark at 27°C in an incubator covered in tinfoil for seven to fourteen days to form callus.

6.9. C. maxima Callus Subculture and Isolation

Orange and pale callus formed on leaves that were incubated on solid callus medium described in **6.5**. These calluses were isolated from their leaves using sterile forceps and one-time use scalpels after two weeks of growth and sub-cultured on solid callus medium from **6.5**. Each petri dish contained three to five calluses.

6.10. Bio resin 3D Print Design for confined growth

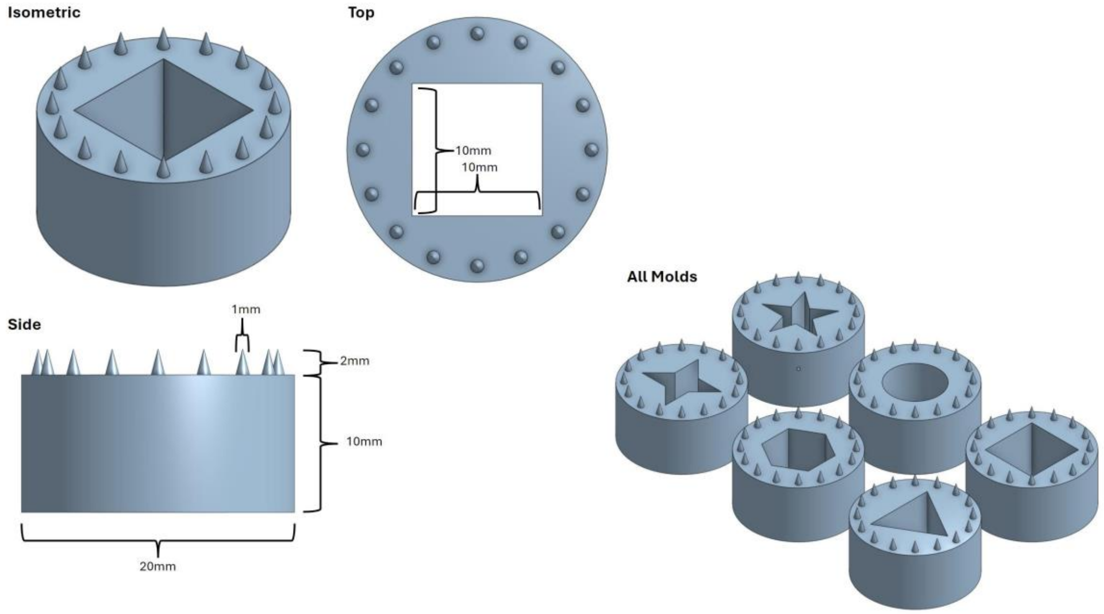


Figure 7 OnShape CAD Mockup of 3D molds for confined callus growth

Callus molds were first modeled in a CAD software, OnShape. An STL file for each mold was created and imported into the slicing software, PreForm 3.37.3. Molds were sliced with a layer height of 50um. Molds were then printed on the Formlabs Form 3B using Biomed Clear V2 Resin (Formlabs, USA). After printing, each mold was washed three times in an IPA bath to remove residual resin. Molds were then autoclaved for sterilization.

6.11. C. maxima Scaffold Formation

Compact, pale orange callus without signs of contamination (slimy, fuzzy, abnormal formations) were chosen for scaffolds. These calluses were isolated from their original culture dish and placed into a sterile petri dish in the biosafety cabinet. The callus was squished with even pressure using a 10 cm x 10 cm 3D printed bioresin square as described in **6.10** until slight juices came out of the callus ~ 1mm in height. 6mm x 1mm samples were created with a biopsy punch and placed into a sterile petri dish then transferred to their respective wells in the 24-well ultralow attachment (ULA) well plate (Corning, USA). Sterile forceps were used for every step.

6.12. Confined Growth Plating

Leaves were isolated and cut according to **6.8** and placed on callus induction media until signs of callus germination were present. Autoclaved 3D printed bioresin molds described in 6.10 were placed into petri dishes containing callus induction media. Slightly germinated callus leaves were placed into the 3D confinements on callus media so that there was either callus or exposed leaf tissue touching the gel. These petri dishes were parafilmed and cultured in the dark at 27C.

6.13. TPA Analysis of the Callus Scaffold

Three biological replicates from three separate callus plates were isolated for texture profile analysis (TPA) testing using a TA.XTPlus 100 Connect (Stable Micro Systems, USA) to analyze mechanical properties. 6mm x 3mm biopsy samples from these callus plates were isolated and compared with three 6mm x 3mm 12.5% gelatin scaffolds as described in **6.18**. The control was created at 12.5% to more closely emulate the hardness of the callus. TPA was used to acquire hardness, resilience, cohesion, springiness, gumminess, and chewiness data from the gelatin controls and callus. The results suggested that the 12.5% gelatin composition was not as close of a control as anticipated thus the data was removed but can be found in the appendix **Figure 28.**

Hardness, indicating material stiffness, is measured by the peak force during the first compression cycle (F1). Chewiness, representing ease of biting, is calculated as the product of hardness, cohesiveness, and springiness. Cohesiveness reflects material consistency and is derived from the ratio of force-time curve areas between the second and first compression cycles. Springiness measures material recovery after deformation, calculated as the ratio of times to reach maximum load in the second cycle compared to the first. Resilience quantifies material deformation after compression cycles, determined by the ratio of upstroke (A3) to downstroke (A4) areas in the first compression cycle.[30].

6.14. C2C12 Media Preparation

C2C12 media was prepared using standard formulation. 10% Fetal Bovine Serum (FBS) (Thermo Fisher, USA), 1% Antibiotic/Antimycotic (anti/anti) (Thermo Fisher, USA), and 2.5 µg/mL Puromycin (Thermo Fisher, USA) was added to DMEM + Glutamax (Thermo Fisher, USA) Media was stored at 4°C.

6.15. Maintenance of C2C12 + GFP

C2C12 + GFP was received at passage 33, passaged at 90% confluency in T-75 flasks (Thermo Fisher, USA) and fed every three days, and incubated at 37°C and 5% CO_2 (Thermo Scientific, HERAcell vios 250i LK, USA).

6.16. Gelatin Film Preparation

2D 5% Gelatin films were prepared using the protocol from another master's student performing concurrent research. 5% bovine skin gelatin (Millipore Sigma, USA) was dissolved in ultrapure water with medium stirring for 1 hr @ 37°C. The resulting solution was cooled to room temperature under medium stirring then 1% wt microbial transglutaminase (MTG) (Sigma-Aldrich, USA) was added. The resulting solution was deposited onto 6mm PDMS substrates and let air dry overnight. Gelatin films were placed into their respective wells on a 24 ULA well plate (Corning, USA) and soaked in 70% ethanol for 30 minutes.

6.17. 3mm x 3mm 5% Gelatin Scaffold Preparation

3mm x 3mm 5% gelatin cylinder scaffolds were prepared in a PDMS mold. The gel solution was created as stated in **6.16** but instead of depositing onto a substrate, it was deposited into a

3mm x 3mm PDMS mold, placed in a sealed 15 mL falcon tube, and solidified at 37C overnight to maintain moisture. The next day, the scaffolds were harvested from the PDMS mold by carefully scooping them out with a scapula and placing them into an Eppendorf tube. The scaffolds were transferred to their respective wells in the ULA and soaked in 70% ethanol for 30 minutes for sterilization. After the ethanol sterilization, appropriate media was added to the wells and incubated overnight at 37°C and 5% CO_2 (Thermo Scientific, HERAcell vios 250i LK, USA).

6.18. 6mm x 3mm 12.5% Gelatin Scaffold Preparation

6mm x 3mm 12.5% gelatin cylinder scaffolds were prepared in a PDMS mold. The gel solution was created as stated in **6.16** but instead of depositing onto a substrate, it was deposited into a 6mm x 6mm PDMS mold with a concentration of 12.5% gelatin, placed in a sealed container falcon tube, and solidified at 37C overnight. The following day, 6mm x 6mm scaffolds were harvested by carefully scooping them with a spatula then cut into 6mm x 3mm using 3D printed 6mm x 3mm guides and a razorblade. The scaffolds were stored in falcon tubes until further use.

6.19. C2C12 Preliminary Experiment Design

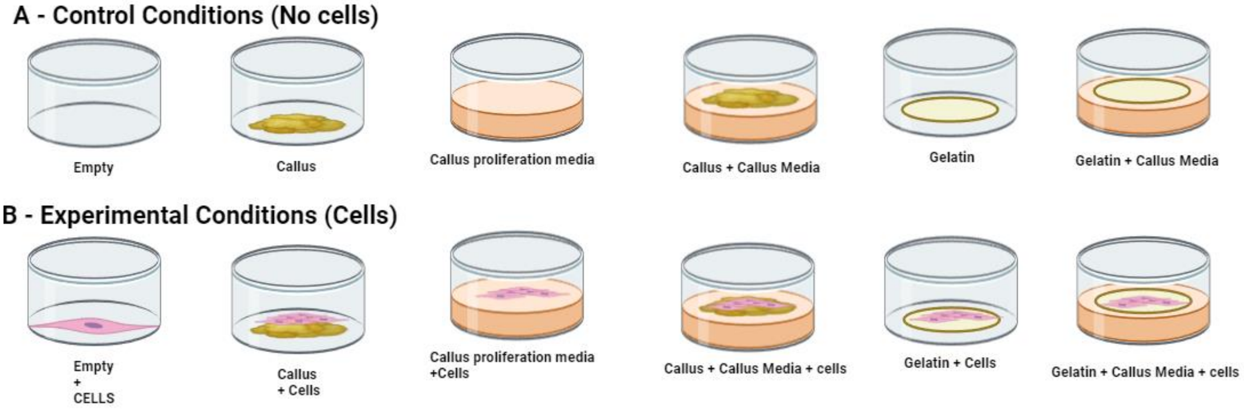


Figure 8 C2C12 Preliminary Scaffold Testing Experiment. A) Control conditions – no cells. These wells will be used as the no-cell signal for metabolic assays. B) Experimental conditions - cells. These wells will be used as the cell signal for metabolic assays. Gelatin is used as a direct callus comparison because cells are known to adhere, proliferate, and differentiate on the material. All wells received C2C12 liquid media.

To rapidly optimize the culture condition, three different combinations were explored. Empty well, scaffold, and scaffold with solid callus proliferation media. All experiments were performed in a 24-well ultralow attachment (ULA) well plate (Corning, USA). In the first experiment, the callus was crushed into a pseudo-2-D film with a height of ~1mm. If the callus was crushed further, it would lose structure and cohesion and become pulpy. 12mm biopsy punches of callus proliferation media were obtained using a sterile biopsy punch and placed into respective wells in the 24-well ULA plate. 12mm biopsies were used to ensure there was enough surface area for 6mm callus and gelatin scaffolds to be seated on them. Gelatin 2D films were created as described in **6.16**. Each condition was compared with a gelatin control in place of the callus. C2C12s were seeded at 69,000 cells per well. Biological replicates refer to the different callus samples which were taken from separate leaves. Empty well groups were used to confirm ultralow attachment signal. Fluorescent signal from PrestoBlue™ metabolic assay from the control groups (no cells) were subtracted from the experimental groups (cells) to determine the cell signal in isolation. The experiment was cultured for 21 days at 37°C and 5% CO_2. The growth media was replaced 24 hours after a PrestoBlue™ analysis, and 48 hours between days. PrestoBlue ™ metabolic analysis was performed on days 1, 3, 7, 10, and 14.

6.20. PrestoBlue™ Assessment of Cell Viability

After scaffold seeding, the cells were cultured in growth media for 21 days. The growth medium was replaced 24 hours after a PrestoBlue™ analysis, and 48 hours otherwise. Metabolic activity was observed at 1, 3, 7, 10, 14, and 21 days using a 10% PrestoBlue ™ Cell Viability Reagent (Invitrogen A13261), a fluorescence-based assay. PrestoBlue ™ uses resazurin, a non-fluorescent blue dye, which is reduced to resofurin, a fluorescent red dye by metabolically active cells. The amount of fluorescence is proportional to the number of living cells and can be measured using a fluorescence-based plate reader that reads at an excitation wavelength of 560 nm, and emission of 590 nm[31]. Scaffolds without cells were used as a control group and compared to cells cultured on *C. maxima* callus and gelatin scaffolds. The signal from cells alone was determined by subtracting the non-cell condition from the cell condition.

6.21. Statistical analysis

All statistical analysis was done using GraphPad Prism 10.2.3 (GraphPad, San Diego, CA, USA). Unless specifically stated, all data is expressed as mean ± standard deviation. Comparisons for multiple groups used a one-way ANOVA. For comparisons between two groups, a Welch's *t*-test was used. A p value of <0.05 was used as the threshold of statistical significance.

6.22. Photos of Callus

Microscope photos of the callus were taken on a HAYEAR 4K HDMI Microscope Camera Kit (HAYEAR HY-6110). Images were uploaded to a PC and scale bars were added using FIJI-Image J 1.54j (National Institutes of Health (NIH), Rockville, MD, USA) downloaded from https://imagej.net/ij/. Other photos of the callus were taken on an iPhone 14 Pro (Apple, USA).

7. Results

7.1. Troubles with Callus Germination from *P. edulis* seeds

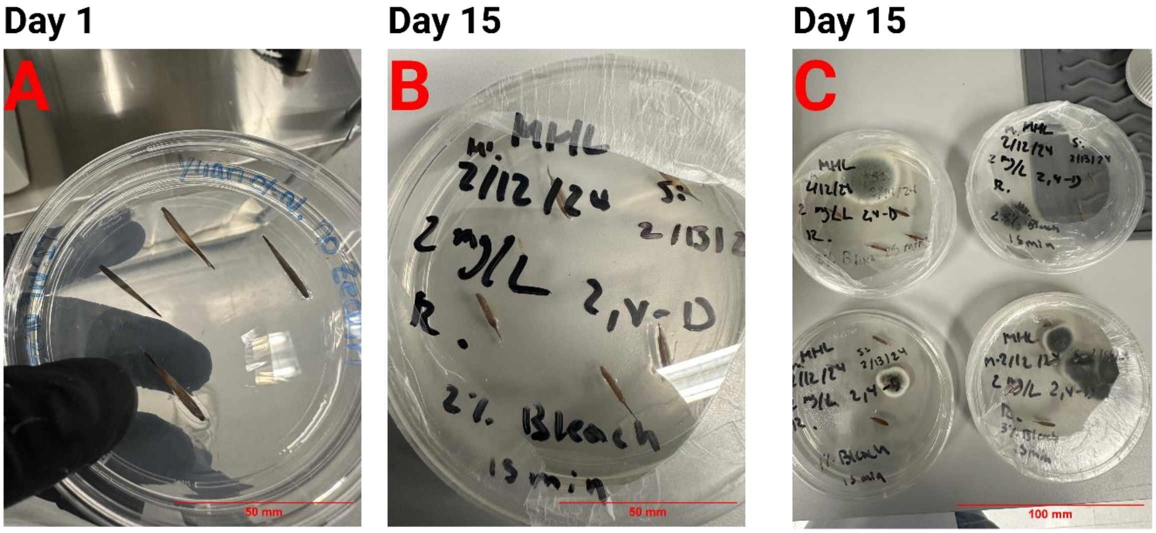


Figure 9 *P. edulis* seeds not germinating. A) Day 1 of plating fresh seeds. B) Day 15 with no contamination, no results. C) Day 15 with contamination.

Figure 10 *P. edulis* seeds germinating in paper towel. Scale bar 40mm.

Figure 9 shows that *P. edulis* seeds did not show any signs of callus formation while following the protocol from Yuan et al for direct callus induction from seeds. There were signs of contamination following 2% bleach sterilization cycles indicating some of the samples were not sterile upon inoculation. Samples that did not show contamination also did not germinate callus. There were suspicions that the seeds from this supplier were duds which was confirmed by attempting to germinate the seeds in a paper towel as shown in **Figure 10**.

7.2. Troubles with Callus Germination from *P. edulis Rhizome*

A rhizome is an underground stem that grows horizontally and produces new roots and shoots thus allows the bamboo to spread. Unlike bamboo seeds, rhizomes are available yearround for harvest and experimentation.

Figure 11 Bamboo rhizome callus initiation issues. A) Photo of *P. edulis* germplasm rhizome from the USDA. B) Photo of bleach sterilization of rhizome samples. C) Rhizome callus induction. D) Contamination after 5 days in culture.

Figure 11 shows the challenges encountered from working with the bamboo rhizome. Since the rhizome originates from the soil, it requires extensive cleaning and sterilization steps. In this experiment, the rhizome was rinsed with water and sterilized in a 10% bleach solution as shown in **Figure 11b**. Despite these efforts, after only 5 days in culture, there were intense signs of contamination as depicted in **Figure 11d.**

7.3. Aseptic Germination of *C. maxima* Functionality

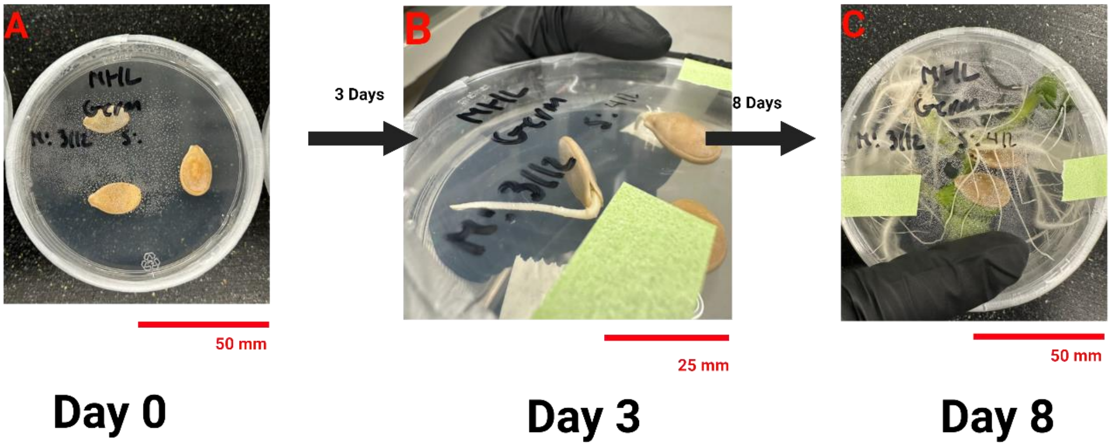


Figure 12 Aseptic germination of *C. maxima* seeds in sterile environment over 8 days. A) 3 seeds after sterilization and plating. B) Seed showing signs of germination after 3 days. C) full stem, leaf, and root growth after 8 days in the incubator.

Figure 12 demonstrates that *C. maxima* seeds with overnight submersion in ultrapure water will survive in 1% bleach for 30 minutes to sterilize and will germinate in a sterile environment containing ½ strength MS medium to produce shoots and leaves without contamination within 8 days of initiation. Roots, leaves, and stems form before 8 days; thus, the callus isolation could be started sooner than 8 days. Biological variation and inactive seeds slightly vary germination speed.

7.4. Callus induction of *C. maxima* from Aseptic Leaves

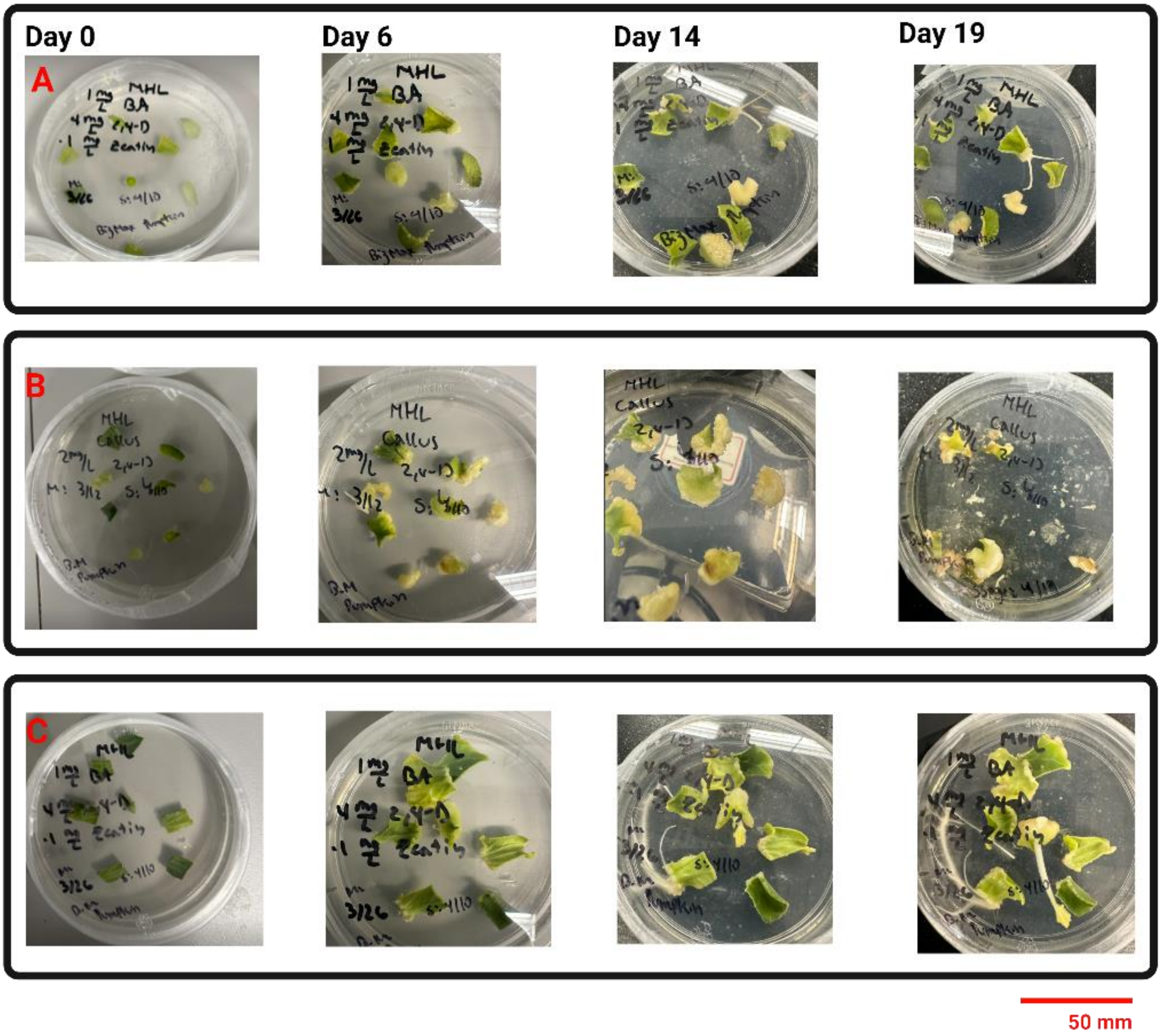


Figure 13 Callus formation of *C. maxima* over 19 days initiated on April 10th. A) 1 mg/L Ba, 4 mg/L 2,4-D, 0.1 mg/L Zeatin. B) 2mg/L 2,4-D. C) 1 mg/L BA, 4 mg/L 2,4-D, 0.1 mg/L Zeatin. Scale bar = 50 mm.

Induction of callus occurred when the leaves from *C. maxima* were isolated and cut into 1cm x 1cm squares with 4 sides of exposed tissue. Callus formation varied between biological samples. All samples prepared with proper sterile technique remained sterile. Pale orange callus began forming within 6 days of induction with culture in the dark at 27C.

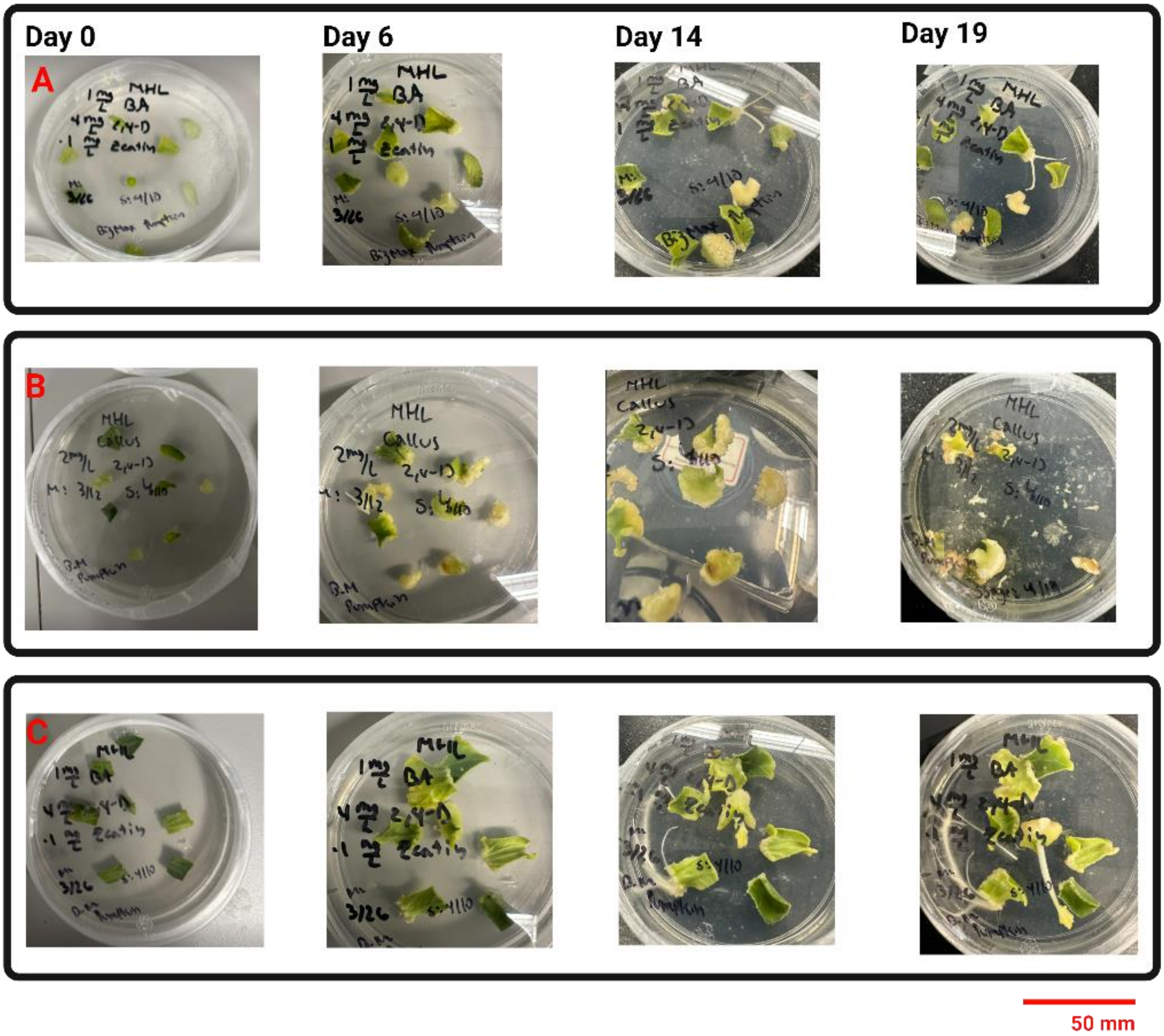
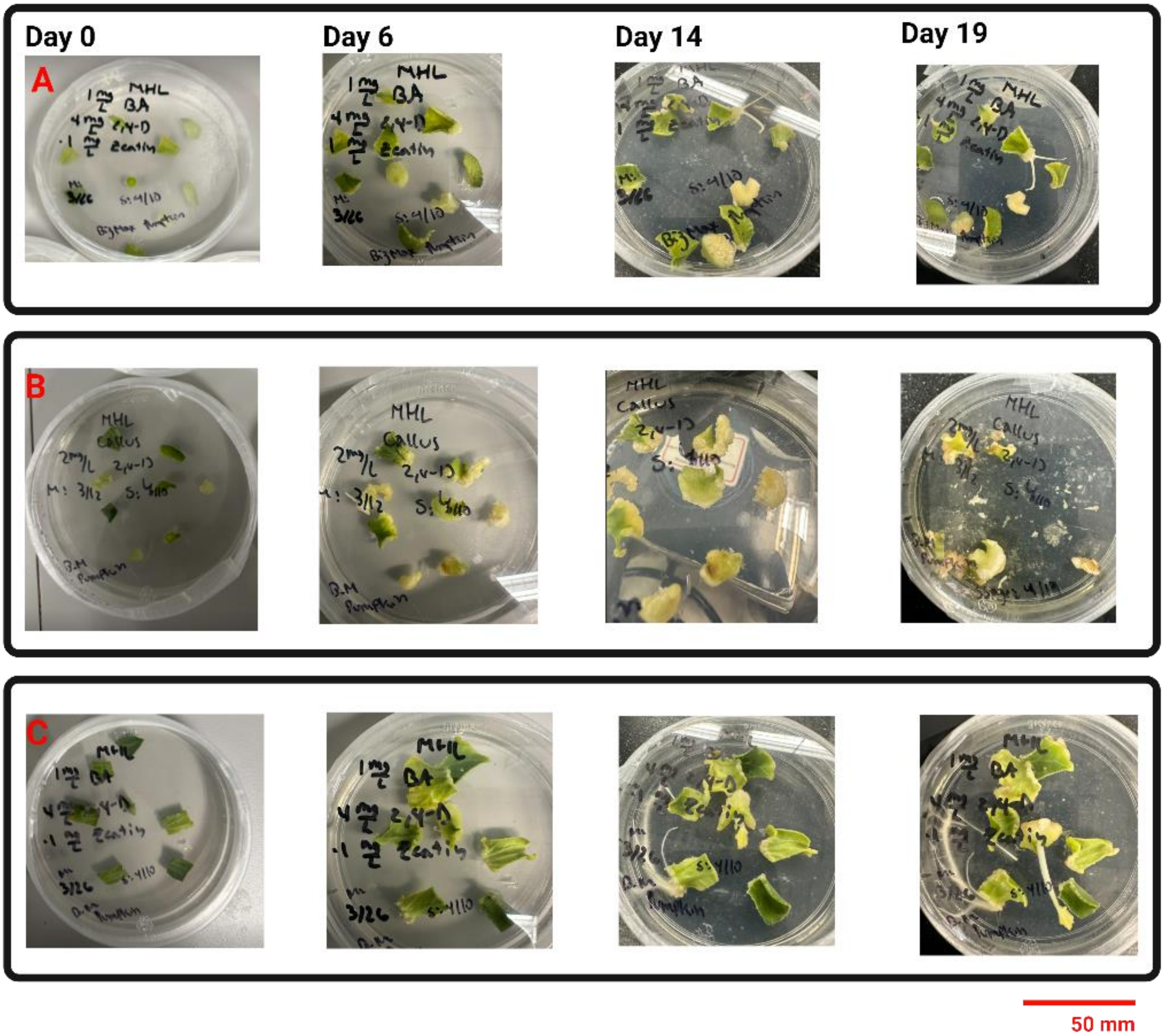


Figure 13 displays the callus formation process. Leaves slightly curl as undifferentiated, randomized callus cells develop from cut and exposed tissue. Callus forms after 5-6 days and continues to develop over time. When media is supplemented with solely 2,4-D, only callus forms. Media supplemented with 1 mg/L BA, and 0.1 mg/L Zeatin (**Figure** 13**a, c)** resulted in minor shoot and root formation from initial callus.



7.5. Callus induction of C. maxima from Aseptic Hairy Roots

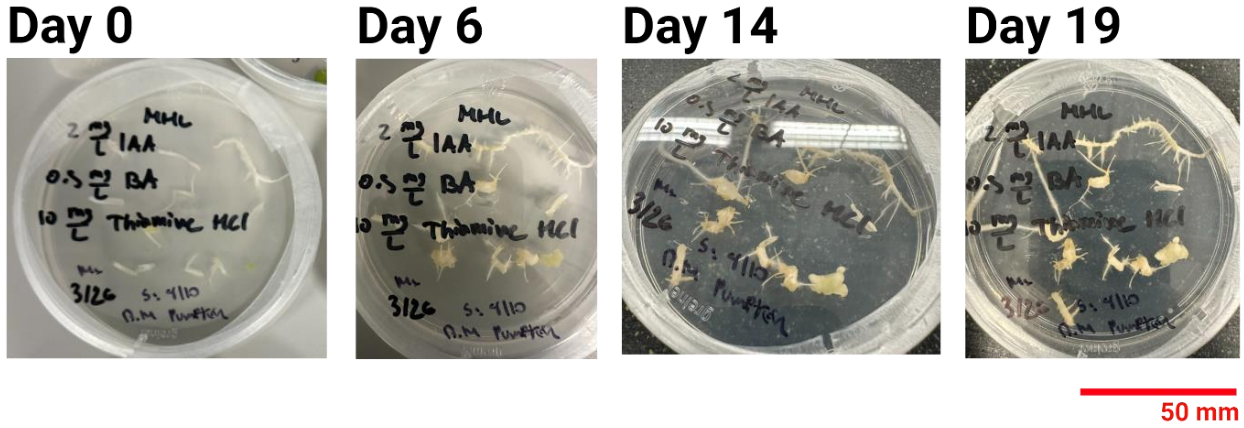


Figure 14 Callus formation from hairy root of aseptically germinated *C. maxima*. Scale bar = 50mm.

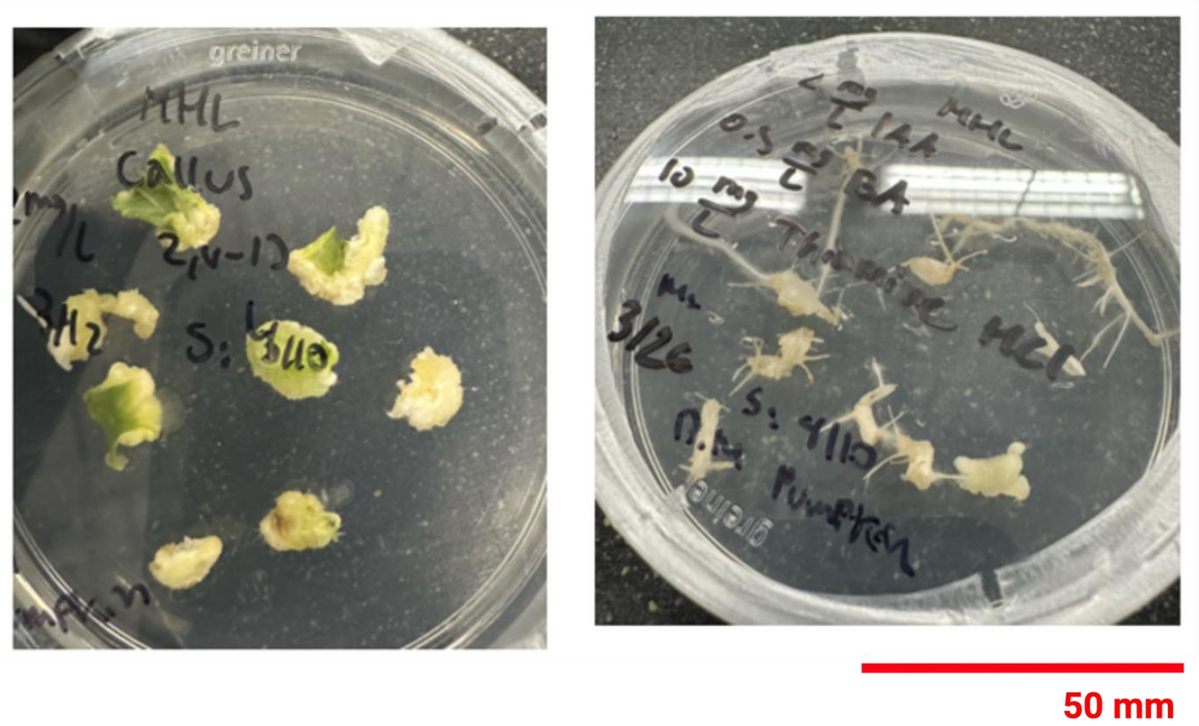


Figure 15 Two weeks of growth comparing hairy root callus versus leaf callus

Hairy roots were isolated from aseptically germinated *C. maxima* seeds and placed onto media composed of 2 mg/L IAA, 0.5 mg/L BA, and 10 mg/L Thiamine HCl. **Figure 14** shows roots began forming callus after 6 days, like the leaf explants. **Figure 15** shows that the root callus did not proliferate as much as the leaf callus. Due to the time constraint of the experimental timeline, and the design goal for rapidly germinating calluses, root callus was not further explored, and leaf callus was selected for scaffolding. Further research should be conducted into comparing root callus versus leaf callus for scaffolding.

7.6. C. maxima subculture Results

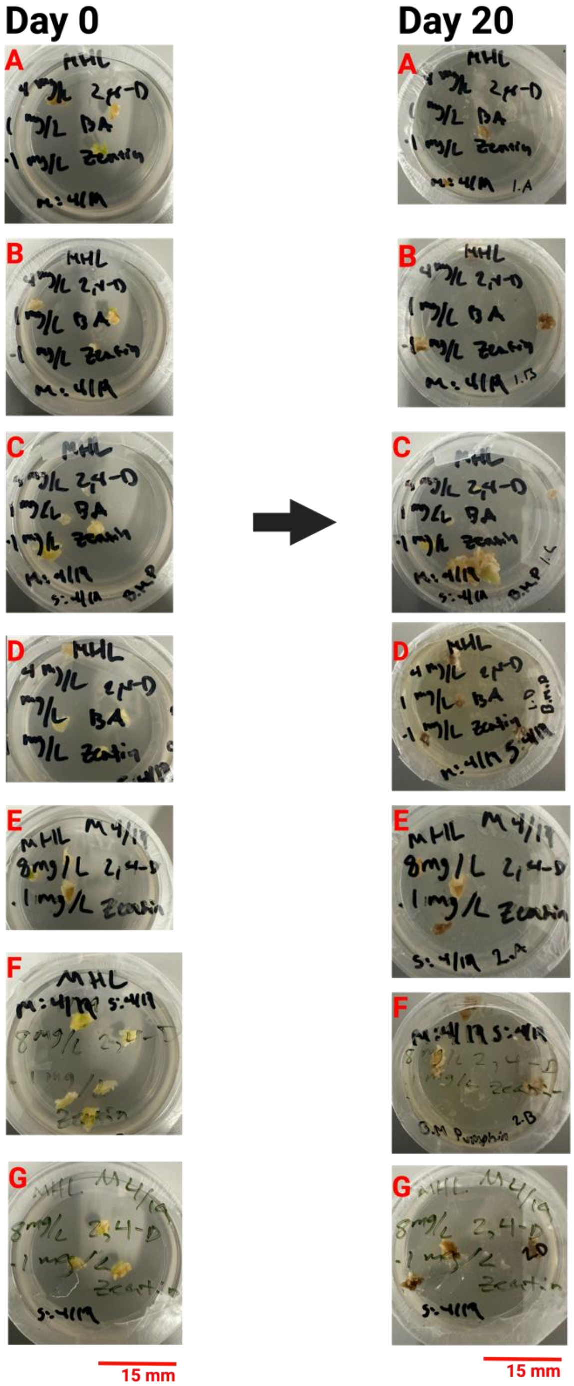


Figure 16 Callus isolated from leaf tissue and maintained over 3 weeks. A, B, C, and D display 4 mg/L 2,4-D. E, F, and G display 8 mg/L 2,4-D. Both groups have 1 mg/L BA and 0.1 mg/L Zeatin.

C. maxima callus survived and slightly proliferated when isolated from the leaf and placed into a separate petri dish. Callus maintained orange, compact, shapes for at least one month on the same medium and remained healthy and sterile when transferred between plates. If sterile practice was violated, the callus became contaminated with bacteria. If the media was not changed for over one and a half months, the callus became black, and the media turned orange (**Figure 16d)**.

Figure 16 shows various isolated callus samples over 3 weeks. Groups A, B, C, and D are compared with E, F and G to examine how increased concentration of 2,4-D impacts isolated callus health over the month. Too much of 2,4-D will kill plant cells. Sample G shows blackened spots on the callus indicating cell death. Samples E and F turned hard and dark orange. Samples A, B, C, and D turned slightly harder but also increased in size. Sample C showed the greatest growth in size over the two weeks in isolation. Sample D became slightly contaminated over the three weeks as indicated by orange media.

7.7. Microscope images of *C. maxima*

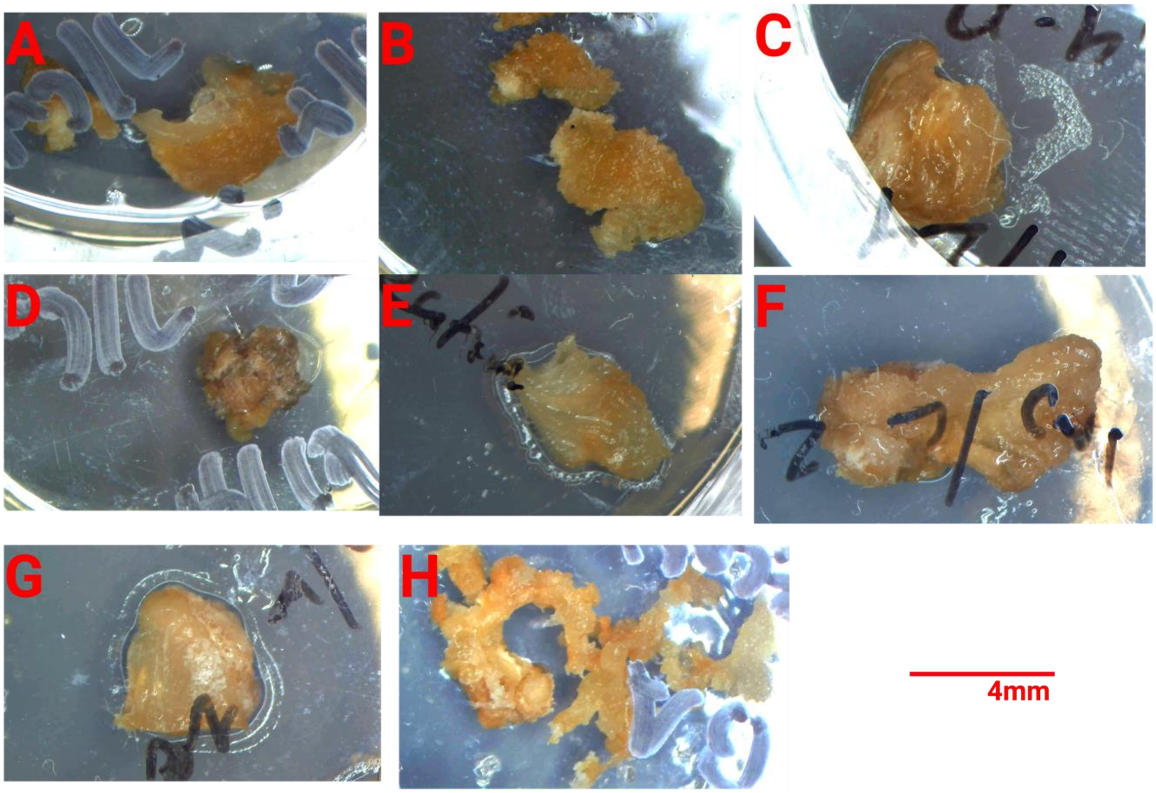


Figure 17 Microscope photos of *C. maxima* callus with scale bar 4mm.

Figure 17 depicts photos of various *C. maxima* callus were taken using the HAYEAR 4k HDMI Microscope Camera Kit to display morphology. Photos were taken from above the petri dish. Each callus photo shows a slight layer of shiny reside which may be rich with RGD peptides conducive to cell binding. Further proteomics assessment should be conducted. **D** shows a hardened and dying callus, which does not glisten like the other samples. It is also a darker orange, transitioning to grey or black indicating cell death. **H** shows a callus that has been harvested for a scaffolding experiment and has regenerated over time.

7.8. Callus confined growth

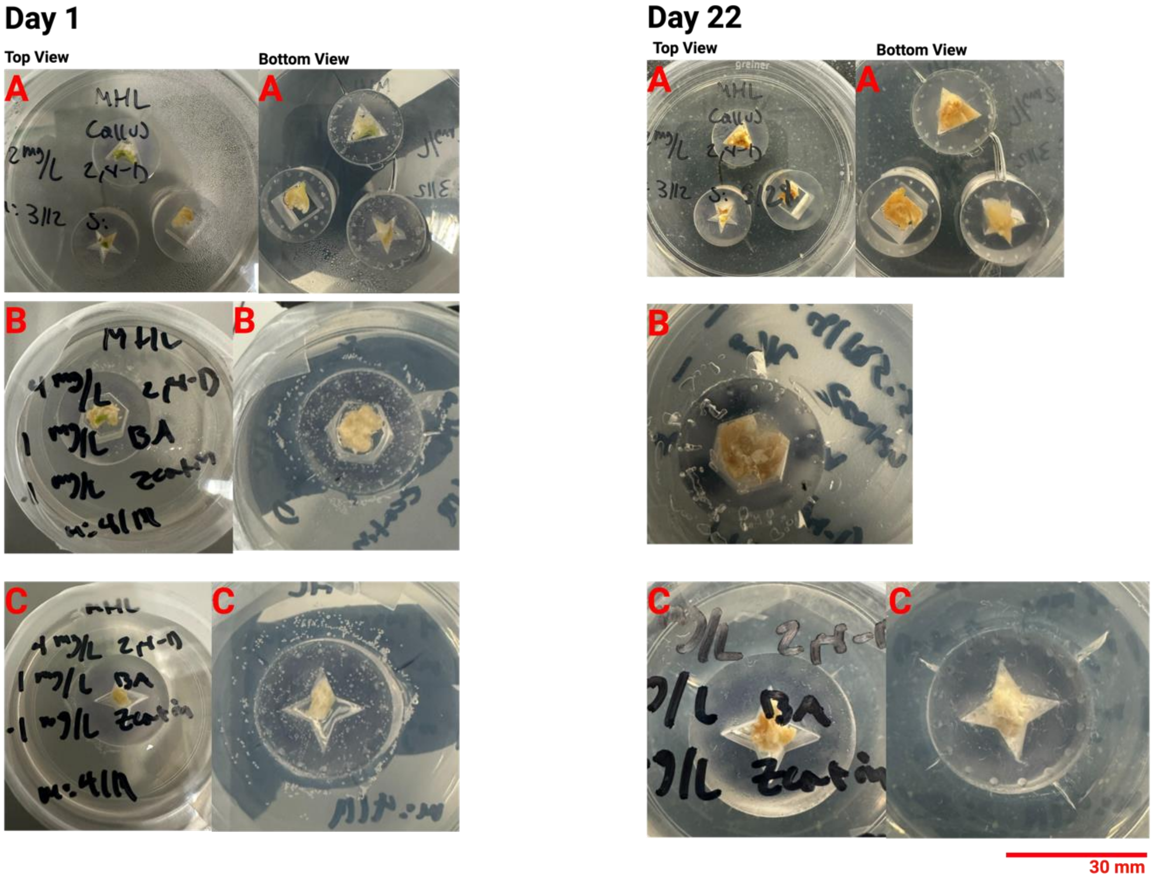


Figure 18 Photos of callus confined growth over 3 weeks. A) Triangle, star, and square molds on 2,4-D media. B) Hexagon mold on 4 mg/L 2,4-D, 1 mg/L BA, 0.1 mg/L Zeatin mold. C) Star mold on 4 mg/L 2,4-D, 1 mg/L BA, and 0.1 mg/L Zeatin mold. Top and bottom view is shown for all samples.

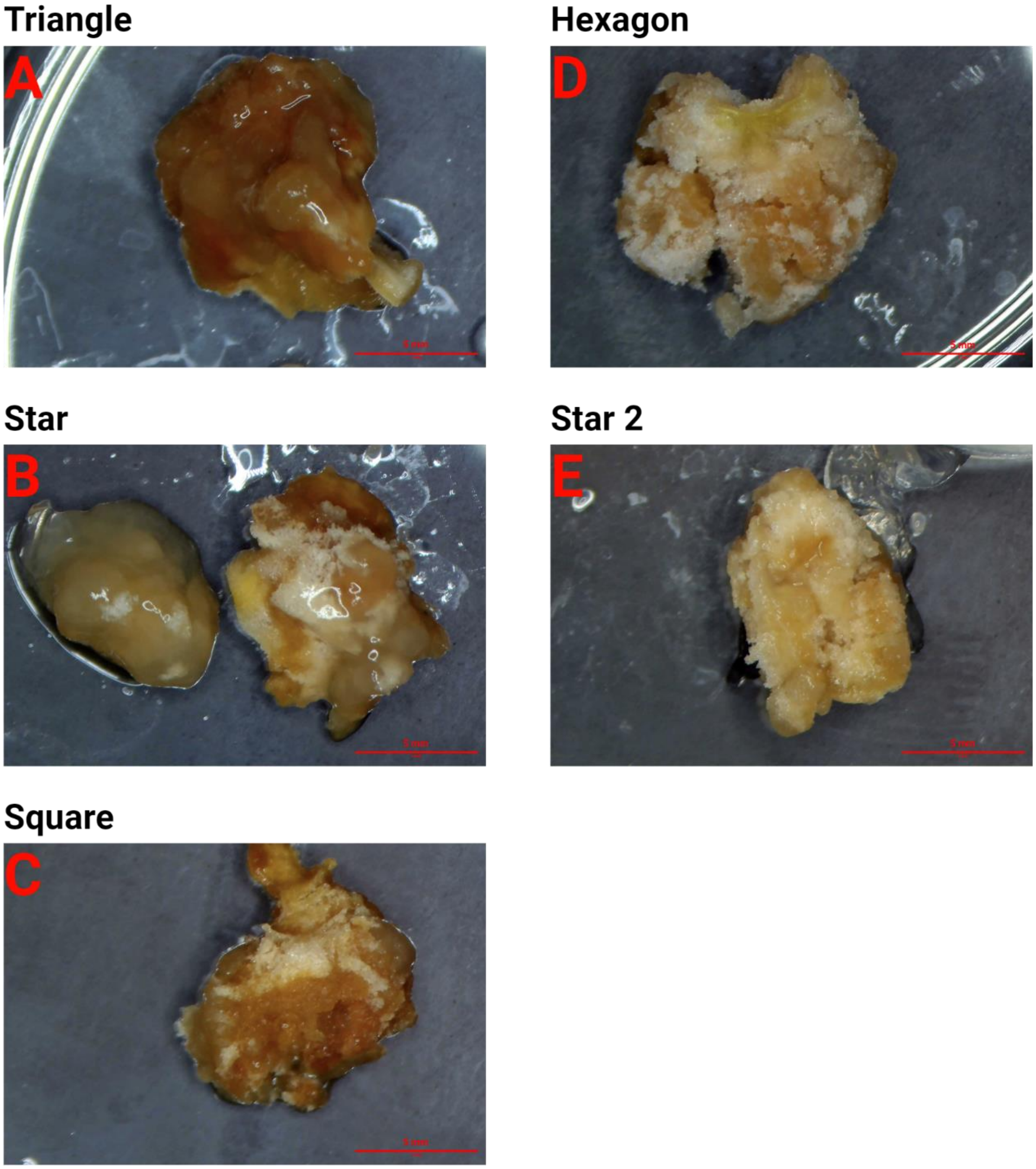
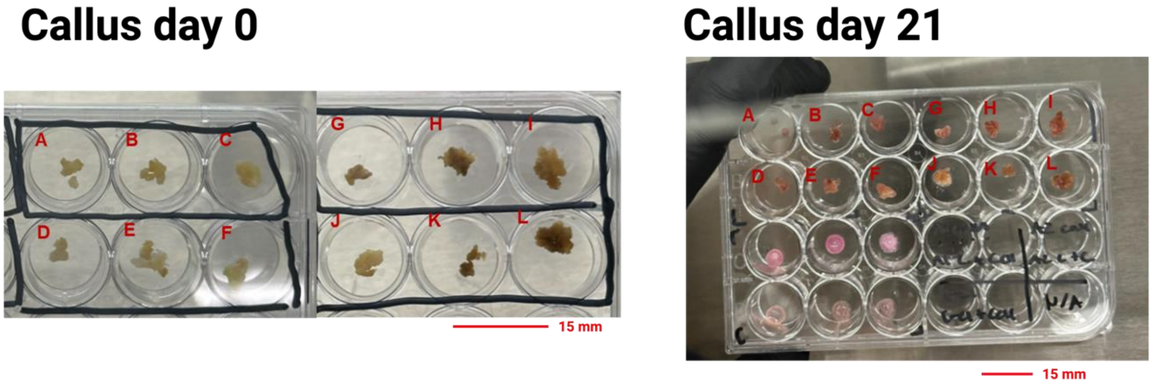


Figure 19 Microscope photos of callus after removal from their respective shape molds after five minutes removal (time to transfer from BSC to microscope).

Figure 18 shows callus growth in confined molds over three weeks. The molds had increasing complexity. Low complexity molds were the square, hexagon, and triangle. High complexity were the stars. **Figure 18a** shows the callus was able to grow into the triangle and star mold, but not the square mold. **Figure 18b** shows the callus can grow into the hexagon Molds were limited by callus growth. Some molds, such as the star mold in **Figure 18**a started growing under the mold into the media. To avoid this in the future, the molds should be more secured into the dish, and apply pressure to maintain callus shape.

7.9. Callus Degradation over 21 Days in Media


Callus day 0

Callus day 21

Figure 20 Callus degradation in media over 21 days. A) Callus on day 0. B) Callus on day 21 and gelatin films.

7.10. Presto Blue Fluorescence for *C. maxima* Scaffolds C2C12s

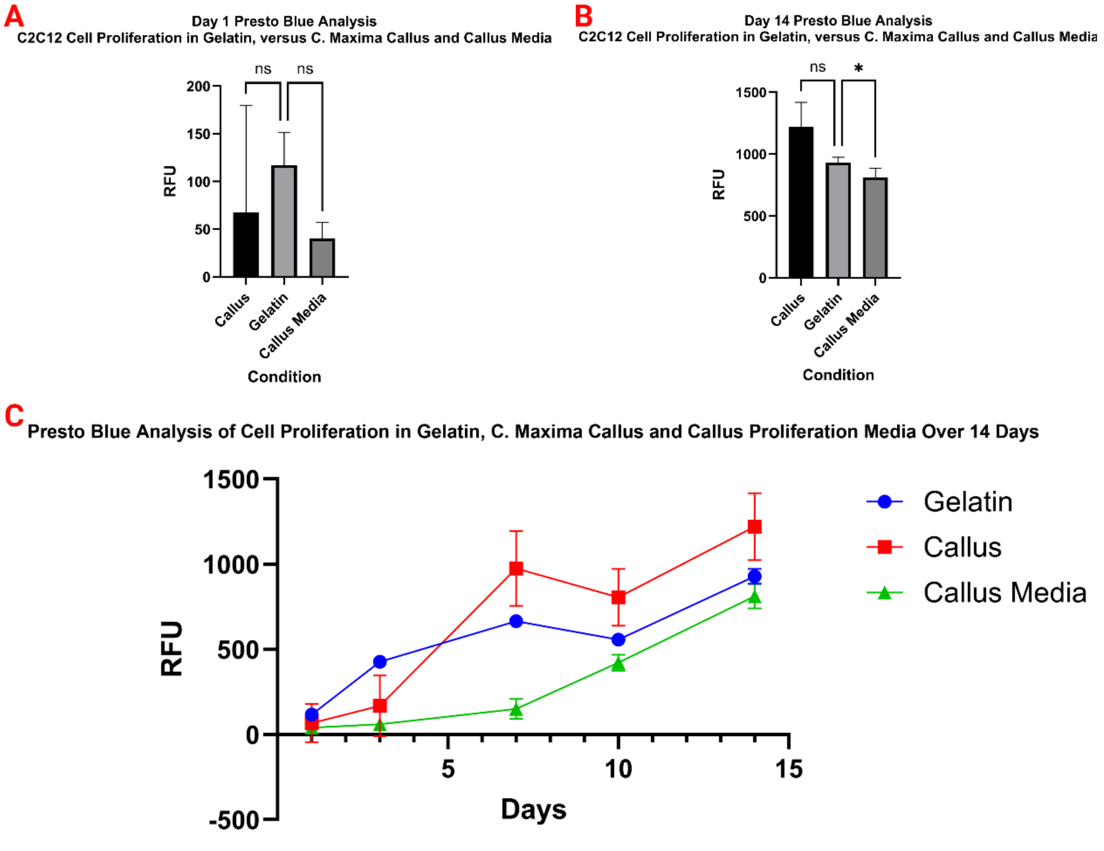


Figure 21 Presto Blue Data for C2C12 Proliferation over 14 Days. A) Day 1 data. B) Day 14 Data. C) Line graph over time (n=3). One-way ANOVA test was performed. * Indicates $p < 0.05$. ns indicates no significance.

Figure 21 shows the metabolic activity for cells seeded onto either the callus, gelatin film, or scaffold over 14 days. **Figure 21a** shows the metabolic data for cells one day after seeding 69,000 C2C12s per scaffold well. **Figure 21b** shows the metabolic data for the cells after 14 days. **Figure 21c** shows the metabolic data over 14 days.

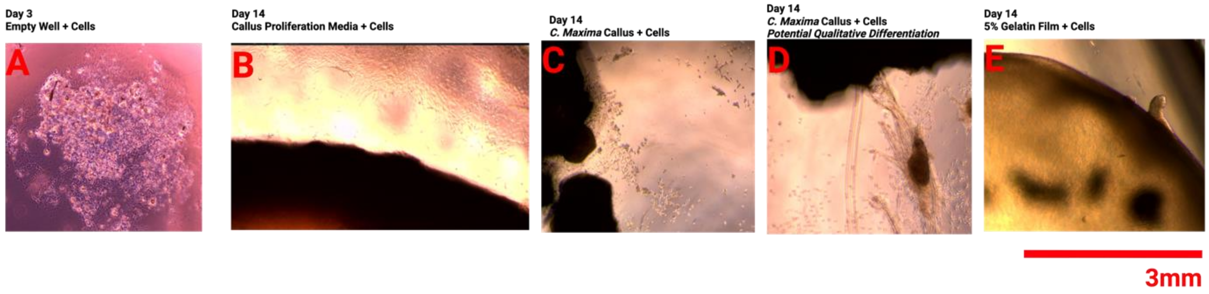


Figure 22 Photos of the bottom of ULA well plate. A) Empty well + cells showing senescence on day 3. B) Cells aggregated around edge of well in callus proliferation media well on day 14. C) Cells adhered in clusters around callus scaffold but attached to bottom of plate on day 14. D) Potential C2C12 differentiation near callus scaffold on day 14. E) Lack of cells adhered to bottom of ULA for 5% gelatin film.

Upon closer inspection, it appeared that cells were sticking to the bottom of the ULA well plates for the callus media and callus wells as shown in **Figure 22**. **Figure 22a** shows dead cells that were not able to adhere to the bottom of an empty well in the ULA well plate. However, **Figure 22b, Figure 22c,** and **Figure 22d** show C2C12 cells aggregating and attaching to the bottom of ULA well plates. There is qualitative evidence of differentiation in **Figure 22d** as indicated by the tube like strucutre.

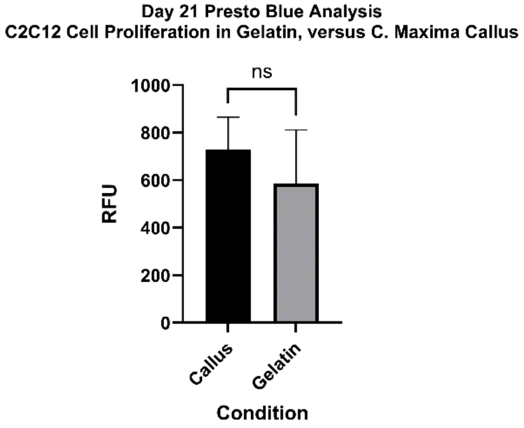


Figure 23 Day 21 PrestoBlue data for the callus scaffold versus 5% Gelatin film. Scaffolds were moved to a fresh well for analysis to prevent signal from adhered cells to bottom of well. Welch's *t*-test was used for comparison. ns indicates no significance.

Figure 23 represents the pure cell viability on the *C. maxima* scaffold versus the 5% Gelatin film. The scaffolds were moved to fresh wells thus no signal could be picked up from the cells on the bottom. The callus proliferation media scaffold was not examined because it was deemed irrelevant for the scope of this project.

7.11. TPA Analysis of *C. maxima* compared to other materials

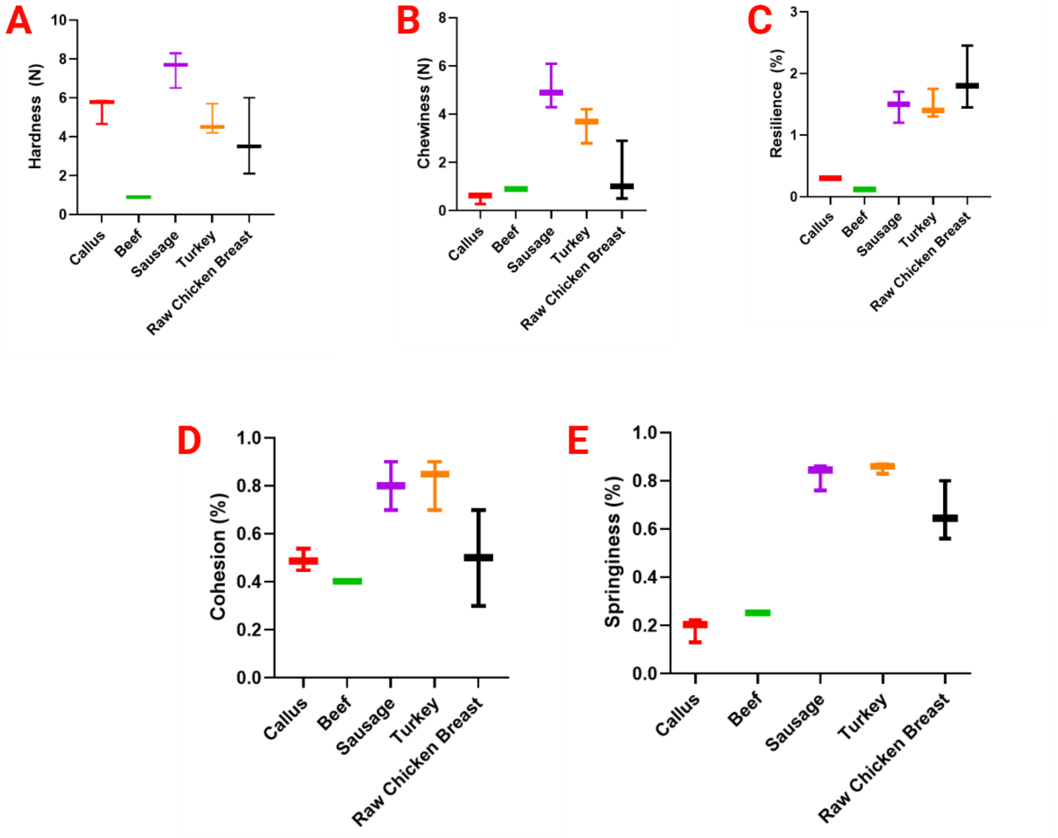


Figure 24 Box plot of the a) hardness, b) chewiness, c) resilience, d) cohesion, and e) springiness of four traditional meats (beef, sausage, turkey, raw chicken breast) compared to callus scaffolds (no cells). Data for sausage, turkey, and raw chicken breast was acquired from Parades et al.[30]

8. Discussion

Climate change, coupled with a growing population and diminishing land resources, necessitates alternative solutions for food production[32]. While the animal agriculture industry has traditionally provided protein and nutrition, new technologies such as plant-based proteins and cultivated meat offer sustainable and nutritious alternatives. Cultivated meat, produced by culturing animal cells in a controlled environment, can replicate or even surpass traditional livestock products. A global dynamic model, and life cycle assessment by Wali et al shows a global transition to cellular agriculture by 2050 could reduce greenhouse gas emissions by 52%, and use 83% less land than traditional agriculture.[33]

Unstructured meat products, like burgers and sausages, are easier to produce with cellular agriculture as they avoid the challenges of creating complex tissue structures. However, consumers demand alternatives identical to traditional meat, making the production of whole cuts, such as steaks or chicken breasts, essential. Structured cultured meat replicates the taste, texture, mouthfeel, and juiciness of traditional meat products. Scaffolding provides a three-dimensional framework for these complex cultured meat products, enabling cells to attach, proliferate, and differentiate to form structured tissues.

Specific Aim 1 for this study is to identify, isolate, and proliferate a fast-growing regenerative plant callus scaffold. Originally, *P. edulis* (Moso bamboo) was explored as a candidate for deriving callus scaffolds to satisfy Aim 1. Bamboo is known for its rapid growth rate, which is among the fastest of any plant species. This rapid growth theoretically translates into fast in-vitro callus growth making bamboo a cost-effective and scalable option for producing large quantities of scaffold material.

However, Bamboo is a difficult species for tissue culture due to inaccessible explant sources. **Figure 9** shows *P. edulis* seeds did not germinate callus as indicated by the protocol from Yuan et al for somatic embryogenesis of bamboo callus from Moso bamboo seeds[29]. **Figure 10** shows that the *P. edulis* seeds did not germinate under paper towel conditions either, thus indicating the seeds were inactive. Upon consultation with Dr. Ellmore of the Tufts University Biology department, it was determined that germinating bamboo seeds were hard to find, and it was unlikely that these *P. Edulis* seeds from Amazon were legitimate. Bamboo produces seeds when it flowers, and it flowers on intervals of 20 to 100 years, thus bamboo seeds are very hard to source[34].

The USDA National Germplasm Resources Laboratory contains living genetic resources such as seeds, tissues, or cells for various plant resources. The only germplasm the USDA had for bamboo was rhizome since seeds were too hard to acquire. Thus, *P. edulis* rhizome was explored as an explant source for callus formation.

Figure 11 highlights the difficulty experienced with bamboo rhizomes. Despite harsh sterilization protocols as shown in **Figure 11b**, there was still intense contamination of bamboo rhizome when trying to obtain a callus (**Figure 11D**). The literature provides little evidence of successful callus induction from bamboo rhizome, and the attempt to initiate a culture from bamboo rhizome was a final effort to create a callus from bamboo.

Due to the one-year timeframe of this study, and the failures of bamboo rhizome and seeds, another species was chosen for further exploration. The intense sterilization procedures required for bamboo rhizome were impractical for this research, as they would increase processing time and cost and risk chemical contamination of the scaffolds.

Pumpkin seed protein was identified as a promising edible microcarrier in a study by Kong et al[27]. Therefore, the next species chosen for exploration to satisfy Aim 1 were Cucurbita maxima *(C. maxima)* “Big Max Pumpkin” seeds.

A study by Kong et al, coated edible microbeads with pumpkin seed protein to test cytoaffinity as a gelatin replacement using C2C12s, and chicken muscle cells. In this study they found that pumpkin seed protein has relatively poor solubility compared to other plant proteins such as mung-bean, soybean, red lentil, broad bean, chickpea, pea, rapeseed, oat, and chia seed. However, pumpkin seed protein still demonstrated the ability to coat microbeads and had the best performance as an ECM material for cell culture[27].

It is well known that ligands play a crucial role in the ECM for influencing cell attachment and proliferation through interaction with cell-surface receptors. More specifically, the arginine-glycine-aspartic acid (RGD) peptide family is known as the most prominent ligand for ECM integrin receptors[35]. Historically, animal gelatins isolated from porcine, bovine, goat, and rat tissues have been used as ECM mimics for in-vitro tissue regeneration. However, these are animal derived components which do not fit within cellular agriculture, or sustainable bioengineering. The study from Kong et al concluded that pumpkin seed protein contained high levels of RGD and DGEA peptide sequences which influence cell attachment through integrin receptors.

To dissolve the pumpkin seed protein to coat the edible microbeads, Kong et al ground the seeds into a powder, followed by several centrifugation and pH-based precipitation reactions to isolate the protein powder. The precipitated protein powder was then solubilized, frozen, and lyophilized.

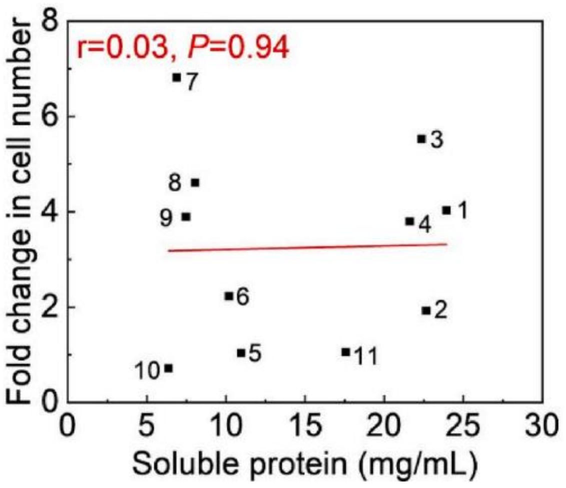


Figure 25 Protein solubility versus fold change of C2C12s during a 3-day culture (Kong et al). [27]

According to **Figure 25**, Kong et al reported a solubility of ~9 mg/mL for pumpkin seed protein, but a fold change in cell number of ~7 which indicates the low solubility resulted in high cell attachment. Kong et al concluded that the bioactive molecules in pumpkin seed protein of RGD and DGEA could be used as an ECM mimic in place of gelatin.

A major inefficiency in Kong's work is the processing required to produce the pumpkin seed proteins for coating, which had low solubility. Despite solubility issues, the study was able to coat edible alginate microcarriers with sufficient pumpkin seed protein for cell proliferation and attachment. Edible microcarriers are excellent substrates for the scale up of biomass production of cultivated meat because they increase the surface area to volume ratio for cell attachment and growth. Microcarriers have been proposed as a temporary substrate for cell attachment and proliferation, or incorporated into the final product, as shown with edible microcarriers[36]. Microcarriers lack in providing the structural and sensory experience from traditional meat in cultivated meat. Plant based proteins that are made into 3D scaffolds through extrusion, bioprinting, or gel formation present better ways to emulate the 3D structure. Pumpkin seed protein has a low solubility, thus would not be a great ingredient for these plant-based scaffolds because it would be difficult to isolate the required protein mass to make a quality scaffold. Thus, plant tissue culture presents a way to create new plant-based scaffolds that contain RGD peptides for cell adhesion without intense processing steps.

Figure 12 demonstrates that *C. maxima* seeds proved to germinate within three days of treatment, sterilization, and plating into the ½ strength MS germination dish under a 16-hour light cycle. These plants remained sterile unless the closed petri dish was opened outside the hood, or sterile practices were violated. The plants survived as long as the solid media was not completely consumed, maintaining root contact with nutrients. This technique presents a high-throughput scaffold production method with minimal processing steps, establishing an aseptic culture environment. This is advantageous for creating a sterile environment for cultured meat production and satisfaction of specific aim 1.

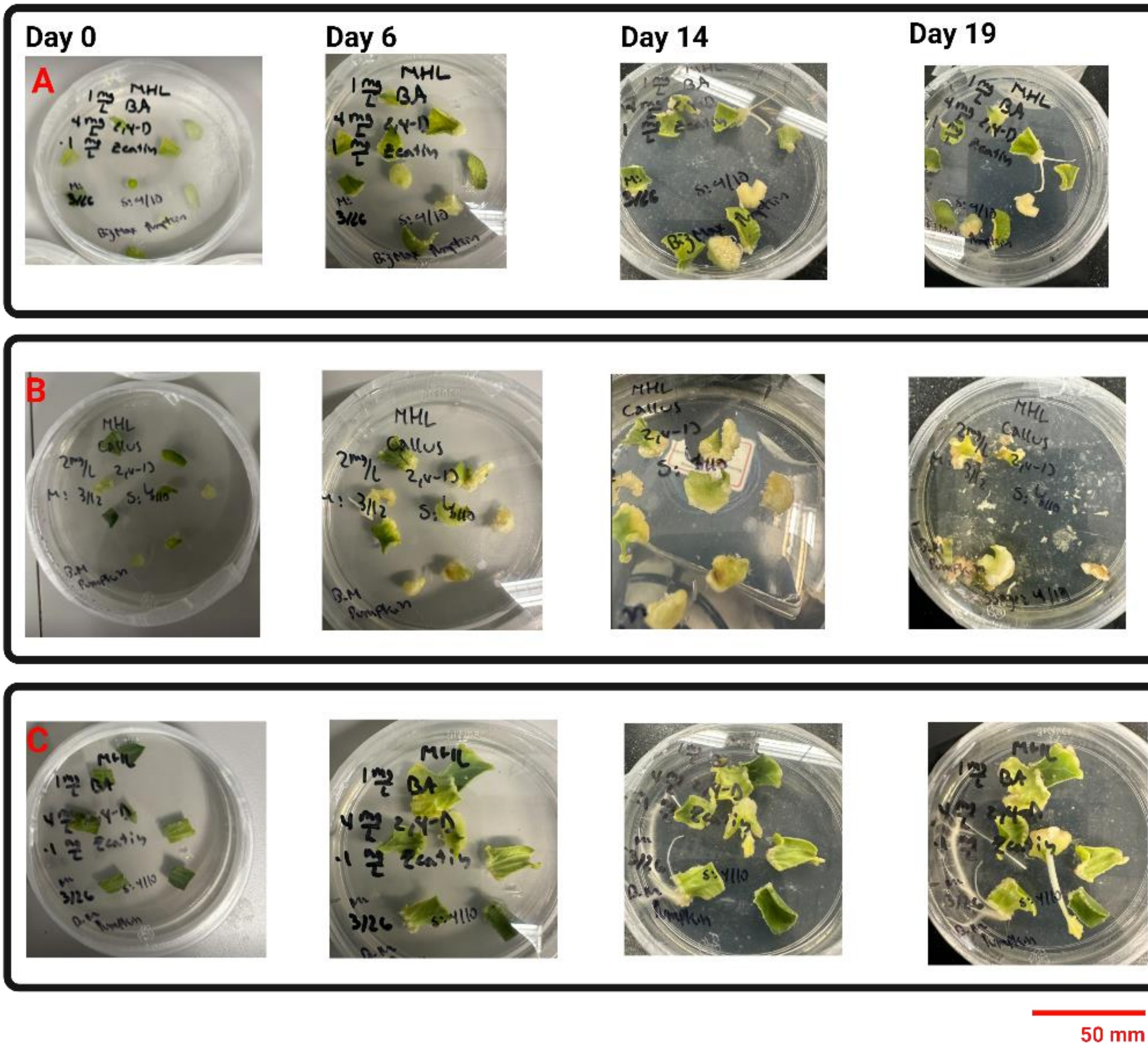


Figure 13 shows that between five to ten days after initiating the aseptic culture, the leaves were harvested, and cut into ~1 cm x 1 cm squares which were put onto a callus induction medium containing 2 or 4 mg/L 2,4-D, 0.1 mg/L Zeatin, 1 mg/L BA, or 2 mg/L 2,4-D alone. All plates contained full-strength MS, 30 g/L sucrose, and 8 g/L agar. Approximately six days after plating, leaves begin to develop pale, compact, orange callus. The callus continues to grow for up to 30 days. Fresh callus tissue is yellowish in color, but darkens to orange, then brown over time.

Figure 14 shows the induction of a callus derived from the hairy roots of pumpkin, which is a different explant source. **Figure 15** demonstrates the callus derived from leaves grows faster than the callus derived from hairy roots. Furthermore, there is less material needed to generate leaf callus than callus derived from hairy root.

Figure 12,

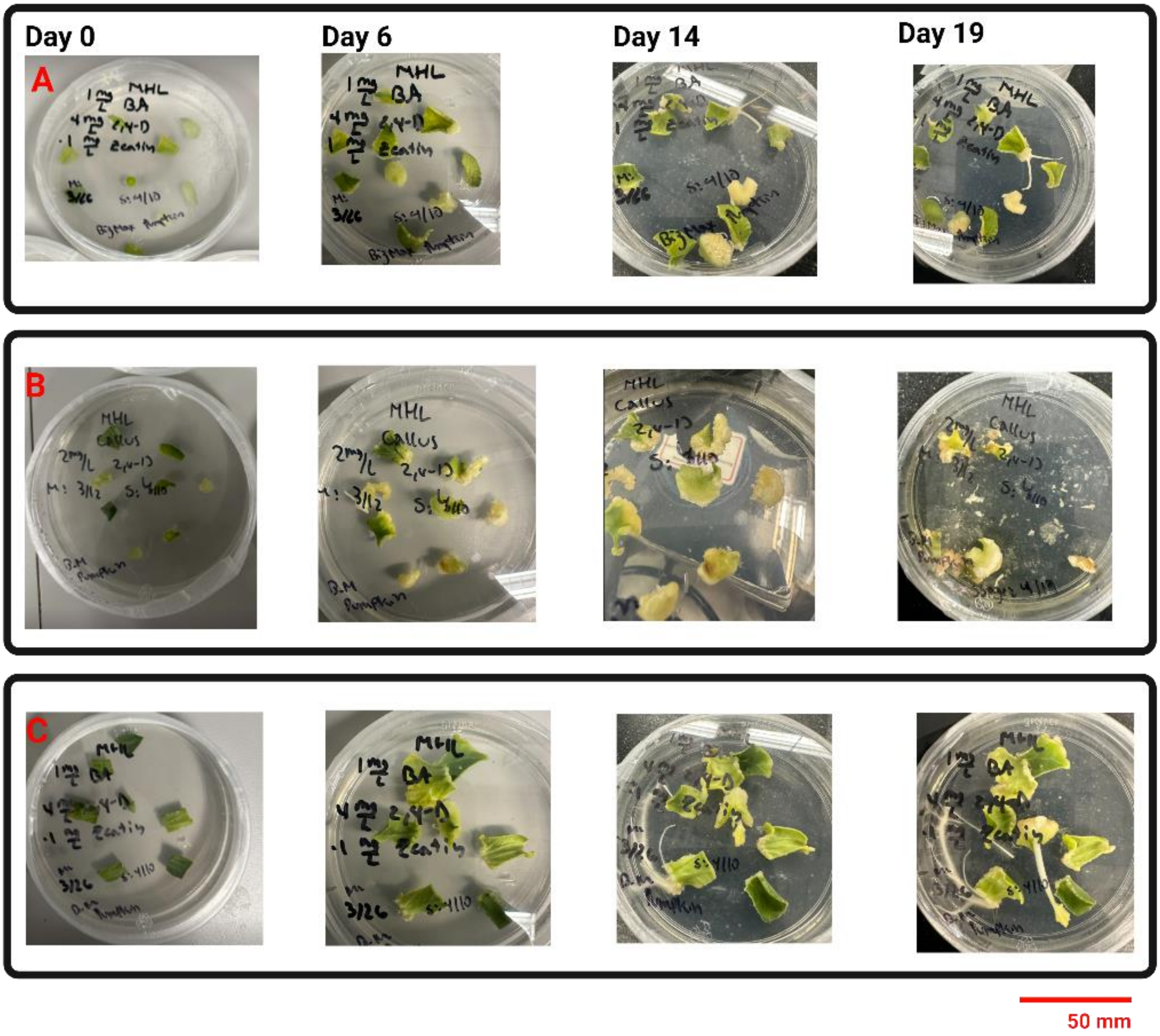


Figure 13, Figure **14** demonstrate the ability to develop a leaf-derived or root-derived callus from *C. maxima* seeds within days of seed germination presenting a rapidly growing living scaffold suitable for cultured meat applications. This is an improvement from the bamboo species, which struggled to show any signs of life in-vitro culture. In addition, *C. maxima* seeds are accessible, easy to store, sterilize, and initiate culture which are key advantages over bamboo. In addition, there are two potential callus sources for scaffolding. Due to the one-year timeframe of this study, leaf callus was selected for further exploration for Aim 2, but further research into *C. maxima* hairy root derived callus to satisfy Aim 1 should be explored.

Figure 16 shows that callus isolated from leaf tissue were able to survive for at least three weeks on various callus proliferation mediums. Some of these plates continued to survive for another month after these pictures concluded **(Figure 29)**. The remaining callus were used for various experiments and testing. **Figure 16g** shows that some subculture mediums containing 8 mg/L 2,4-D resulted in hardening and blackening of callus indicating 8 mg/L was too high of a concentration. *C. maxima* callus survived and slightly proliferated on the 4 mg/L 2,4-D subculture plates. **Figure 16c** shows one callus sample plate doubling in size over the three weeks. Other plates slightly proliferated, but not as much as **Figure 16c**. This may be due to biological variation or isolation techniques. Further experimentation should be conducted focused on the proliferation of calluses on subculture medium. This study demonstrates *C. maxima* leaf derived callus can stay alive and continue to proliferate when placed on appropriate subculture medium providing a useful maintenance media for scaffolding preparation for cultured meat and satisfying Aim 1.

Figure 17 shows high-resolution images of the calluses in isolation. **Figure 17d** shows a callus that has hardened and began to die due to high concentrations of 2,4-D. **Figure 17d** is visibly different than the remaining samples because there is no glossy texture and orange color, indicating **Figure 17d** has poor health. **Figure 17** shows the morphology of callus scaffolds before isolation for scaffolding. **Figure 17h** shows a callus sample that was flattened and harvested using 3mm x 3mm biopsy punches, then placed back onto the callus proliferation medium. The callus remained alive and regenerated over time, as well as puffed back up with hydration from the media.

Figure 18 demonstrates the ability for the callus to grow in confined spaces into specific shapes over three weeks. This experiment also highlighted the need for callus growth optimization. The calluses used for this experiment came from various leaf tissues, resulting in different growth rates, which may have prevented them from completely filling the shape molds. Despite this, **Figure 18a** shows that the callus was able to grow into the shape of a triangle, and star. **Figure 18b** shows that the callus was able to take the shape of a hexagon, and **Figure 18c** shows that the callus was able to take the shape of a star. In **Figure 18a**, the callus began growing under the mold into the media indicating that the mold system should be redesigned to contain the callus appropriately by applying pressure.

Figure 19 shows the shapes of the callus after removal from the confined shape molds. All callus quickly puffed up after removal from the mold once they were not constrained. The figure that maintained shape the most was a triangle as depicted in **Figure 19a.** Other shapes such as the stars in **Figure 19b** and **Figure 19e** showed slight characteristics of the shape mold, but those characteristics were quickly lost over the ten minutes it took to transfer from the lab the incubators were in to the microscope.

Despite slight loss of shape in the callus, it was still able to grow in the confinement. The confined growth system is important for callus as a scaffold because it further reduces the processing steps needed for scaffolding. In the future, an additional step to make sure the callus maintain shape should be added. This capability to grow the callus in the mold has substantial implications for the field of bioengineering, particularly in the production of cultured meat. By utilizing these methods, scaffolds can be designed and used to guide the growth of callus into the precise shapes of various meat cuts, such as chicken breasts, steaks, or fish fillets. This not only enhances the feasibility of producing lab-grown meat that closely mimics the texture and appearance of traditional meat products but also opens new possibilities for custom-designed meat products. The confined growth experiments show that the callus satisfies Aim 1 and can be used for additional research for scaffold molding to further decrease processing steps in tissue engineering and cultured meat.

Production of scaffolds from living plant tissue bypasses intense processing steps. Some plant proteins have poor solubility and must be dissolved in toxic solvents. Decellularization involves multiple days of treatment with harsh chemicals. Plant tissue culture presents a way to grow these scaffolds in highly customizable sterile environments to form 3D structures as shown in **Figure 18**.

Texture profile analysis (TPA) uses a double compression test to determine data about texture characteristics such as springiness, cohesiveness, chewiness, and resilience for various food products. **Figure 24** demonstrates the mechanical properties of the *C. maxima* callus compared to ground beef, sausage, turkey (cold cut), and raw chicken breast. Each material varied in their hardness, chewiness, resilience, cohesion, and springiness, and these are characteristics that define specific foods. The callus had similar hardness to turkey, and similar chewiness, resilience, springiness, and cohesion to beef. These results are without cells, but a future TPA experiment with cells should be conducted to analyze how cells change the properties of the material. The mechanical properties of the scaffold reveal that the plant-based material is already mechanically like cold cut turkey and beef, thus this information suggests bovine cell incorporation should be explored with the callus scaffold. 7.11

Specific Aim 2 demonstrates the efficacy of the *C. maxima* callus derived from Specific Aim 1 as a scaffold. To demonstrate the material efficacy as a scaffold, C2C12 cells are seeded onto the material to analyze adhesion and growth of mammalian cells on the *C. maxima* callus.

Figure 20 demonstrates that over 21 days of culture, the callus had minimal signs of degradation. The calluses that were pulpy on day one remained pulpy on day 21 (**Figure 20a, b, k**). The callus that were compact on day one stayed compact on day 21. (**Figure 20c, d, e, f, g, h, i, j, l**). There was slight browning of the callus over time, but the callus did not turn black or turn hard. The callus had a slight pinkish tint from the phenol red indicator in the media. The callus did not cause any contamination, and the media did not change from red for callus without cells on them, indicating the callus pH is close to 7.2 due to the phenol red indicator (ideal conditions for cell culture) [37].

The lack of degradation for the callus demonstrates a key requirement for scaffold material development. Other plant based protein dispersions and films degrade over time in media, but the callus maintained shape which is important for developing 3D tissues and whole cut cultured meat products.

Plants contain many bioactive secondary metabolites which are found in plant organs such as leaves, stems, roots, and flowers. These secondary metabolites provide advantages to the plant for survival in their natural environment, but are not necessary for plant growth, development and reproduction[26]. Some common secondary metabolites are caffeine, capsaicin, vanillin, menthol, and limonene [38]. Callus culture can be used to harvest these secondary metabolites for industrial use cases without growing entire plants.

In this specific study, callus from *C. maxima* is studied for cultured meat applications. Pumpkins in the Cucurbita family contain secondary metabolites such as carotenoids: beta-carotene, alpha-carotene, lutein, zeaxanthin, vitamin E, ascorbic acid, phytosterols, selenium, and linoleic acid. These secondary metabolites have antidiabetic, antioxidant, anticancer, and anti-inflammatory effects[39].

In a study by Dr. Andrew Stout et al, C2C12s and bovine satellite cells were engineered to endogenously produce antioxidant carotenoids: phytoene, lycopene, and beta-carotene which provide nutritional value, and protection against diseases associated with red and processed meat consumption. In his study, he concluded that these engineered cells produce carotenoids that are able to reduce lipid oxidation than traditional beef, thus potentially increasing shelf-life, color stability, and flavor of a cultured meat product which can further lower the cost of production while creating a nutritious product[40].

These engineered antioxidants and other secondary metabolites can be found in *C. maxima* and other plant callus thus presenting a way to increase shelf-life, color stability, and flavor of cultured meat products through a 3D scaffold.

A scaffold is irrelevant if cells do not adhere and proliferate on the material. C2C12s are an immortalized mouse myoblast cell line that is commonly used in biomedical research, including cultured meat. C2C12s are a well-established model system for studying muscle cell growth and differentiation. In addition, C2C12s are able to adapt to serum-free or serum-reduced cultures which further drives down the cost of production for cultivated meat[41].

Figure 21 shows that C2C12 cells adhere to callus, gelatin, and callus media after 1 day. **Figure 21b** shows that after 14 days, an ANOVA one-way test determines that cells on callus versus gelatin are non-statistically different wheras cells do not prefer callus media. **Figure 21c** shows the growth over 14 days of C2C12 cells on callus, gelatin, and callus media. For the first 4 days, there were less cells on callus scaffolds than in gelatin as shown by the lower fluorescence units. After 5 days, callus scaffolds consistently had a higher cell signal than gelatin. The metabolic assay performed in this specific study was not quantitative thus the exact number of cells was not determined.

Figure 21 shows that the C2C12s also adhered to the callus media, but not as well as the callus in isolation. This is important because eventually a co-culture system involving the growth of the callus simultaneously with cells incorporated would further drive down processing steps for creating 3D meats. In an ideal co-culture system, the C2C12s would only adhere and proliferate on the callus scaffold, and not on the solid callus media. In this experimental design, media filled the entire well, thus cell media is consumed by cells both on the callus scaffold and attached to the callus media gel. This is not ideal for a co-culture system because media would need to be replenished more often thus increasing production cost. In future designs for a co-culture system, mammalian cell media should only be used for the growth of mammalian cells on the callus scaffold, thus further design improvements isolating the two media compartments should be investigated.

Despite using an ULA well plate for the experiment, **Figure 22** shows C2C12 cell attachment to the surface of the well plate in wells with callus proliferation media + *C. maxima* callus + cells (**Figure 22b)** and *C. maxima* callus + cells (**Figure 22c**). **Figure 22c** even shows qualitative C2C12 differentiation by the formation of a tube structure, but immunostaining should be used to confirm this is differentiation.

One possible explanation for this is that the cells are adhering to callus debris on the bottom of the plate. Another possible explanation is the callus is coating the bottom of the well plate with juices that may contain the RGD peptides that influence cell attachment. **Figure 22a** shows that cells cultured in the same Corning 24-well ULA plate did not adhere to the surface and died.

To ensure the signal recorded in the presto blue was from the cells on the callus and the gelatin, the callus and gelatin control and experimental wells were moved to a fresh, empty ULA well and PrestoBlue^{TM} was used. **Figure 23** demonstrates the signal for cells on callus and gelatin were still significant, and this specific instance did not have any cells on the bottom of the well plate. A Welch's *t*-test confirmed the signal from cells on both the callus and the gelatin film were not statistically significantly different thus evidence suggests C2C12s adhere and proliferate substantially on *C. maxima* callus scaffolds.

6mm 2D Gelatin films were used as the control group for preliminary testing because they are a known material that cells adhere and proliferate on[13]. Originally, 2D callus film scaffolds were to be explored in this study. A 2D film scaffold is 1-dimensional and therefore has minimal thickness. The callus used for the scaffold study initially had a thickness of approximately 1 cm and were crushed down to as close to a 2D film as possible. Upon flattening, the callus lost its structure and turned into a pulp. Thus, without further processing steps, it was incredibly difficult to turn the callus into a 2D film. Instead, the callus was turned into a pseudo 2D film with a diameter of 6mm and a thickness of approximately 1mm as demonstrated in

Figure 26 and **Figure 27**. The surface area of the pseudo callus film is approximately 75.40 mm^2 whereas the 2D gelatin film would have as surface area of approximately 28.27 mm^2. Therefore, the C2C12s had approximately 2.6x more surface area for proliferation and adherence on the callus scaffolds than the 2D gelatin films, despite only a 1mm height difference.

The demonstration of C2C12s to proliferate on the callus scaffold is crucial for the efficacy of *C. maxima* callus as a material for making 3D scaffolds. If C2C12s did not survive on the material, it is likely that other cells would die as well. Since C2C12s adhered, additional cell lines such as chicken fibroblasts, bovine satellite cells, and fish cells should be explored.

In this specific thesis study, *C. maxima* callus scaffolds produced leaf derived callus are evaluated as a new scaffold material for mammalian cells C2C12 cells. There is little evidence in the literature of plant callus scaffolds for tissue engineering, thus there is limited data to compare these results to.

The purpose of Aim 2 was to explore if mammalian cells could adhere and proliferate on callus scaffolds, which was demonstrated with C2C12s. The signal from C2C12s seeded on the callus scaffold compared to a callus without cells was significant, indicating C2C12s successfully adhered to the callus material. However, the signal comparison between callus and the gelatin film may not be an accurate representation of scaffold efficacy. When in media, both the callus and the film floated in the well. Therefore, cells were able to attach to all facets of the scaffold. The pseudo 2D film callus scaffold had more locations for cell attachment thus allowing for more surface area for growth and therefore providing a higher Presto Blue metabolic assay signal.

Ultimately, C2C12 cells adhered to the callus scaffold, demonstrating the callus worked to support C2C12s. Exact cell numbers, immunostaining, or images were not quantified in this experiment and should be explored in the future for more detailed information on the scaffold.

One of the ideal elements of a scaffold is minimal processing for preparation. This experiment demonstrates the callus can be grown, harvested, and placed into media with no further processing steps. This is an improvement from decellularization, or traditional plant-based scaffolds made from protein dispersions or hydrogels.

Stout et al demonstrated that secondary metabolites can be engineered into C2C12s and iBSCs to improve the sensory, nutrition, and stability of cultured meat products[40]. Since plant callus already have secondary metabolites, specific species selection can provide improved benefits to cells cultured on callus scaffolds. *C. maxima* contains Carotenoids: beta-carotene, lutein, zeaxanthin, alpha-carotene, alpha- and beta-cryptoxanthin, 9-cis-beta-carotene, 13-cis-beta-carotene, luteoxanthin, violaxanthin, neoxanthin. Phenolic acids: gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, chlorogenic acid, caffeic acid, ferulic acid, sinapic acid. Flavanols: rutin, kaempferol, isoquercetin, astragalin, myricetin, quercetin. Tocopherols: alpha-tocopherol, gamma-tocopherol. Minerals: potassium, calcium, sodium, magnesium, iron, zinc, copper, manganese.[42]. Carotenoids and phenolic acids can improve antioxidant capacity, reducing the oxidative stress the final product. Tocopherols can help reduce lipid oxidation, and minerals assist with improving the nutritional value of cultured meat.

Overall, the secondary metabolites found in *C. maxima* callus may improve the nutritional, sensory, and shelf live value of cultivated products. To confirm this hypothesis, isolation of the secondary bioactive compounds from media should be performed as well as antioxidant testing. The scope of this thesis did not cover testing media or cells for secondary metabolite treatments.

It is ideal for callus scaffold candidates to already possess desirable secondary metabolites. Calli are genetically modifiable to insert, delete, or amplify genes that produce secondary metabolites. This is a concept derived from secondary-metabolite farming through genetic modification[26].

Callus genome modification is performed with agrobacterium-mediated transformations, or particle bombardment. This uses a naturally occurring soil bacterium called *Agrobacterium tumefaciens*. The agrobacterium delivers a plasmid vector containing the desired gene(s) into the callus cells, which integrates into the plant genome altering the cells[43]. Alternatively, callus can be bombarded with DNA-coated microparticles that force into the callus cells which integrate the DNA into the plant genome upon penetration[44].

The *Stevia rebaudiana* is the plant responsible for producing the zero-calorie natural sweetening compounds steviol glycosides. In a study by Sharma et al, steviol glycoside production was enhanced through the modification of the *S. rebaudiana* genome using agrobacterium transfection. This study demonstrated it was possible to insert genes to modify stevia production pathways. This same concept can be applied to *C. maxima* callus to produce increased antioxidants for cultured meat products, or accelerate callus proliferation[45].

One of the key components of media are growth factors and albumin. In a study from Lee et al, human recombinant FGF2, EGF, and recombinant human serum albumin were produced through transgenic rice suspension cells (*Oryza sativa L. cv. Dongjin*). These growth factors were used to differentiate and maintain human-induced pluripotent stem cells into neural stem cells

(NSCs) which could further differentiate into neuronal and glial cells[46]. This study indicates serum-free, or serum reduced media components can be produced from secondary metabolites of transgenic callus. There is incentive for researchers to explore transgenic calluses that are biocompatible, edible, fast growing, and contain secondary metabolites of interest to drive down the cost of cultured meat production with a highly efficient scaffold.

As humanity continues to push boundaries into space, ensuring a sustainable and nutritious food supply for astronauts on long-term space missions becomes a crucial challenge. Cultured meat and plant tissue culture offer promising solutions for space farming providing the capability to produce fresh, high-quality, and nutritious, food in a confined resource limited environment of space. Both cultured meat and plant cultures can be cultivated in bioreactors that have a small footprint and precisely controlled conditions (temperature, media composition, oxygen, shear environment, etc…) that allow for consistent batch-to-batch reproducibility, and productivity optimization.

These bioreactors can be integrated into spacecrafts and space habitats enabling continuous food production and decreasing resupply missions from Earth. The renewable system approach recycling waste streams highlighted in LCAs in bioreactors ensures minimal waste generation and recycling of nutrients[2]. Furthermore, the ability to grow a variety of enhanced crops using plant tissue culture and growing enhanced cultured meat on demand provides astronauts with a diverse and balanced diet, crucial for maintaining health in space. As we prepare for future missions to Mars and beyond, the development and refinement of these technologies will play a pivotal role in ensuring that humanity can thrive in the harsh environments of space, paving the way for permanent settlements and the continued exploration of our solar system.

Cultured meat presents a promising technology to mitigate the negative sustainability effects from traditional livestock farming. Plant tissue culture presents a way to grow plants in vitro in highly customizable environments, shapes, and compositions to use for the improvement of cultured meat. A combination of these two technologies may result in decreased production cost for cultured meat, and even an improvement to traditional meat (higher nutritional value, lower cost, controlled environment to prevent cancer). Lastly, this technology shows promise for providing nutritious food sources in isolated areas, such as space, or heavily isolated islands.

9. Conclusion

The fabrication of leaf derived callus from *C. maxima* pumpkin seeds represents a significant step forward in the field of scaffolding for cellular agriculture. At the time of this study, there is limited information about callus used as scaffolds for tissue engineering. This study accomplished Aim 1 of isolating and creating a callus from *C. maxima* seeds. Aim 2 was accomplished by demonstrating C2C12 cells were able to adhere and proliferate on the callus material for at least 21 days. This study demonstrates the ability to rapidly form a callus in sterile environment from *C. maxima* seeds which can be incorporated into cell culture media without additional processing steps. This opens a range of new possibilities for the creation of combined plant and animal cell scaffolds for cultured meat. Plants themselves have been used as sustainable, cheap, regenerable medicinal products for a very long time. Some examples include aloe vera, chamomile, garlic, ginger, ginseng, lavender, peppermint, turmeric, cannabis, milk thistle, elderberry, licorice root, sage, thyme, etc… Plant tissue culture presents a way to selectively modify the genome of plants to control secondary metabolite production, thus using plants like a bioreactor. By combining this with cultured meat, researchers can efficiently create scaffolds with positive feedback cycles that improve the nutrition, value, and structure of cultured meat, while decreasing production time, costs, and improving sustainability. This specific study demonstrated preliminary analysis of using plant callus as scaffolds for cultured meat. Further work should be done to validate, test, and expand the selection of plant species and cell lines for cultured meat research.

10. Future Work

Due to the one-year timeline for this project, the research was mainly preliminary. Thus, most next steps in this study should be repeated experiments, validation, and cell line exploration. In this study, one plant callus cell line was isolated for scaffolding – *C. maxima*. To get a better understanding of the potential for callus scaffolds, multiple callus scaffolds should be analyzed from different species, containing different secondary metabolites. Species of interest include bamboo, red lentil, rapeseed, algae, and other fast growing lignocellulosic plants.

In this study, C2C12s successfully were seeded and proliferated on the callus scaffold. To consider a callus scaffold viable for cultured meat, additional cell lines such as chicken, mackerel, bovine, tuna, and salmon should be tested. These should be compared to seeding efficacy on other scaffolds.

Furthermore, more specific quantitative data should be acquired from this study. This study was preliminary and demonstrated C2C12s proliferate according to Presto Blue ^{TM}. Quantitative analysis using Cyquant ^{TM} assays can be performed figure out how many cells attach to the scaffold after 1 day of seeding to further seeding efficiency data.

It is also important to understand cell morphology on the scaffold. To accomplish this, immunostaining and confocal image analysis should be conducted on the scaffold after at least 7 days of seeding. Staining for differentiation and cell alignment will further elucidate the morphology and interactions of the cells with the scaffold.

A major assumption is the *C. maxima* callus has RGD peptides that influence cell attachment. To confirm this, proteomics analysis using mass spectroscopy should be performed to confirm their presence.

Secondary metabolite engineering of the scaffolds should also be explored. Crispr/CAS-9 through agrobacterium mediated transfection should be attempted to insert or modify genes producing metabolites of interest to increase the value of the callus scaffold.

Lastly, this technology has space applications, thus simulation of isolated cultures in extreme environments such as space should be performed to test if the callus would be viable for cultured meat applications in space.

For all these studies, the key interests are cell proliferation, morphology, attachment ratio, growth, and mechanical properties. Further testing of the callus as a cultured meat scaffold will highlight its potential as a material of interest.

11. Appendix and Supplementary Photos

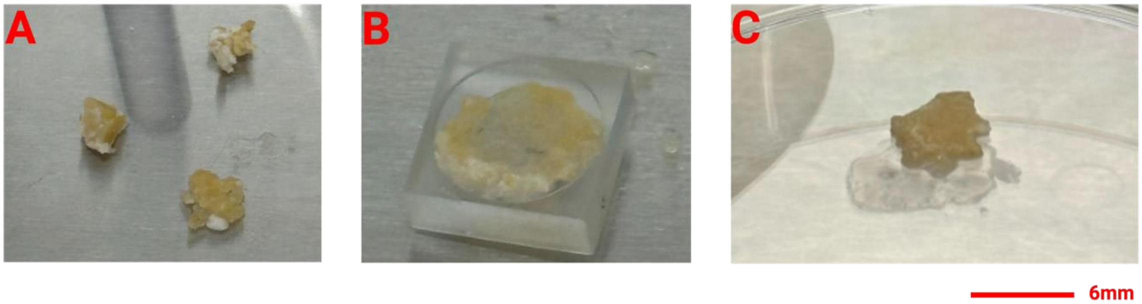


Figure 26 Callus scaffold fabrication process. A) callus pre-flattening. ~1 cm height. B) callus post "2D film." Turned into a pulp and was impossible to harvest. C) Callus as a pseudo-2-D film used for the experiment. Scale bar 6mm.

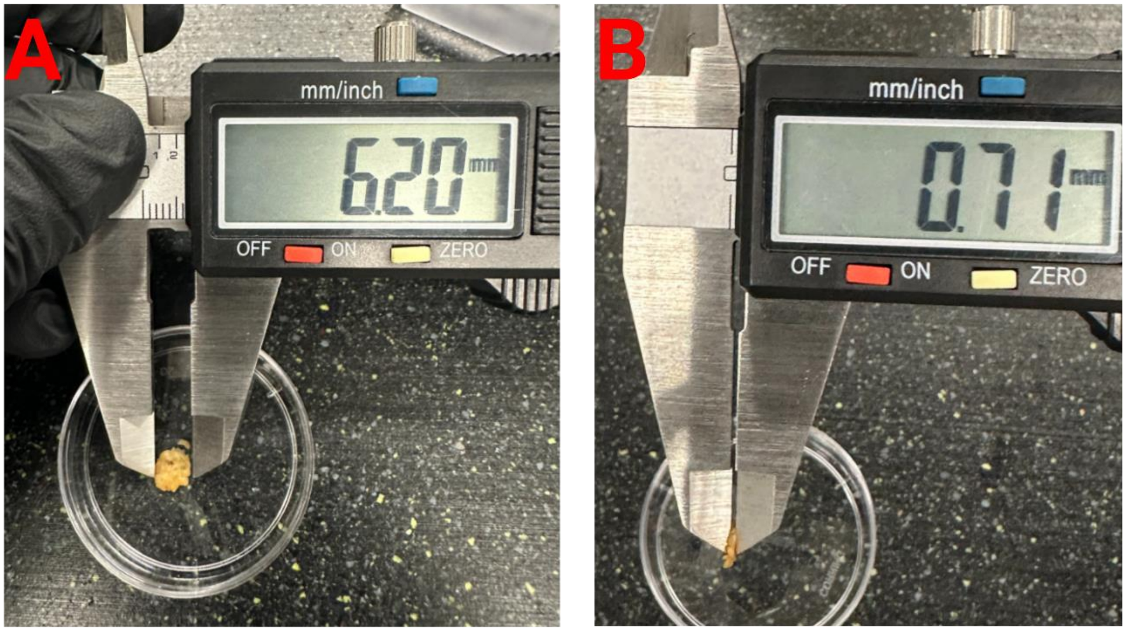


Figure 27 Photos of nonsterile callus that was flattened to get measurements for pseudo 2D-film. A) Pseudo film was approximately 6mm in diameter. B) Pseudo 2D film was approximately 0.7mm in diameter (~1mm).

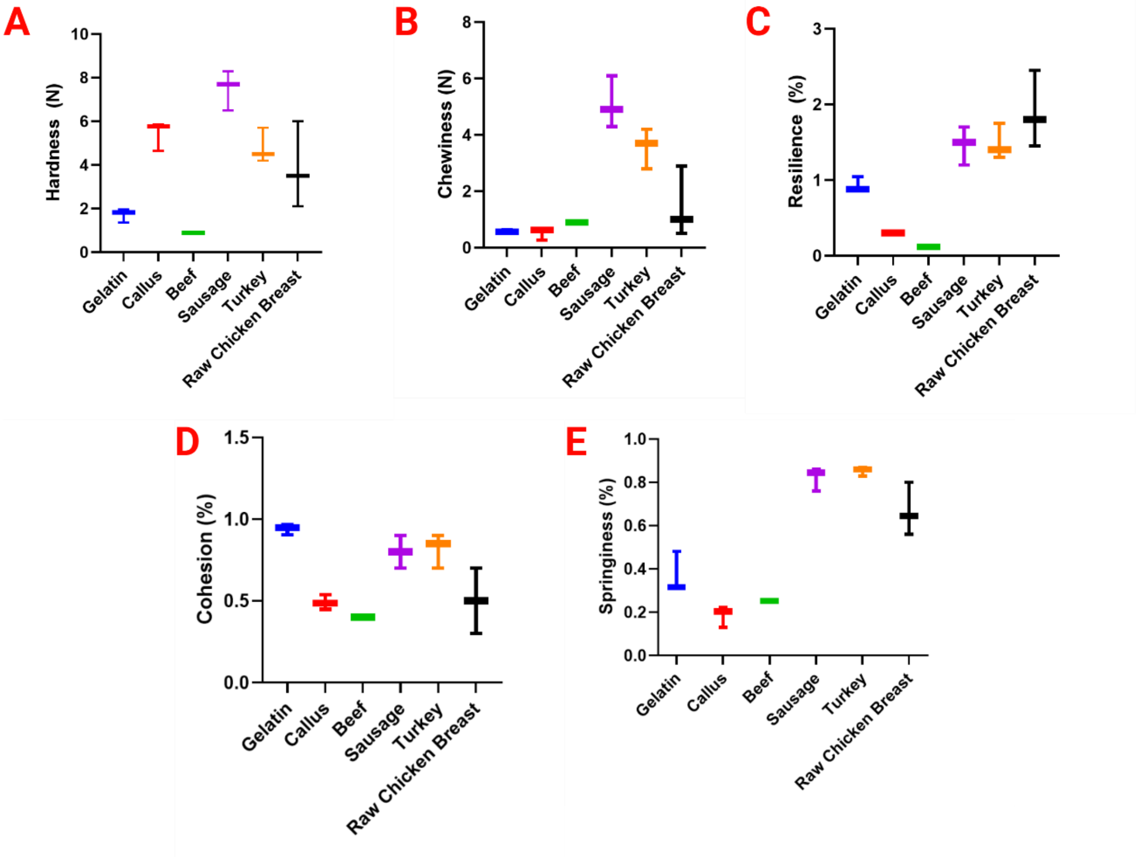


Figure 28 TPA figure including gelatin 12.5% 3x 6mm scaffolds

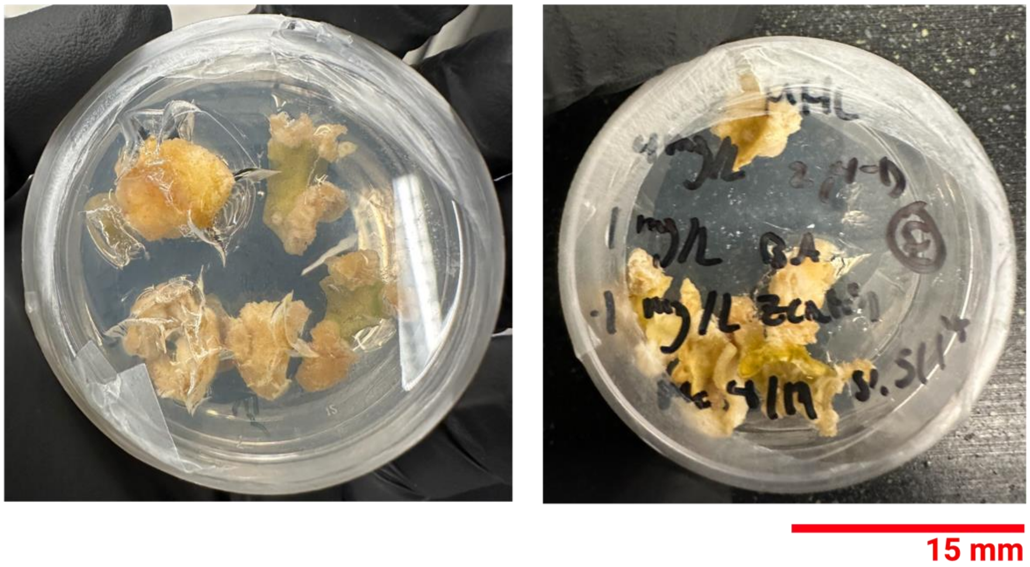


Figure 29 Extended growth of a callus plate over two and a half months. Scale bar 15 mm.

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