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Design and Use of Automation for Soybean Transformation (Part 2): Handling of *Agrobacterium* Infection and Plating of Explants on Media

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Abstract: At Dow AgroSciences, an efficient and robust transformation protocol has been developed for high throughput *Agrobacterium*-mediated transgenic soybean production. In this protocol, the infection of explants with *Agrobacterium* solution, and placement on cocultivation media is one of the most critical and resource intensive steps. Large scale manual process poses operational difficulties such as handling of *Agrobacterium* solution and ergonomic implications for operators. Automation of these and other steps will alleviate these risks and may potentially increase the throughput of soybean transgenic event production. To this effect, we developed an automated system that can (a) move Petri dishes using EPSON robots across different functional positions on the platform, (b) introduce up to six different types of *Agrobacterium* using a peristaltic pump system, (c) shake infecting explants to attain uniformity and consistency of infection, (d) dispense media using a Petri dish dispenser, (e) transfer individual explants onto cocultivation media using image recognition of the explant position in the infection plate, and (f) self-dispose different types of waste produced during operation. All of this was achieved using an integrated system of custom made and commercially available components coupled with robust software. The system preserves the sterility of the materials (explants) without compromising their viability and integrity while accurately executing the biological principles of the transformation method.

Key words: automation, soybean, *Agrobacterium* infection, explant transfer, robotics, imaging

1. Introduction

Soybean (*Glycine max*) is one of the most important agricultural crops in the world, with an annual crop yield of more than 200 million metric tons, and an estimated value exceeding 40 billion U.S. dollars worldwide [5] . Soybean accounts for over 97% of all oilseed production globally. Thus, reliable and efficient methods for improving the quality and yield of this valuable crop are of significant interest. Several methods have been employed to develop soybeans compatible with herbicides and resistant to insect pests by transferring genes into the soybean genome. The two major methods used are biolistic-mediated and *Agrobacterium*-mediated transformation. However, soybeans have proven to be a challenging system for transgenic engineering. The split seed *Agrobacterium*-mediated transformation protocol developed in Dow AgroSciences is a robust and highly efficient method resulting in transformation frequencies of about 15% and has been used for high throughput transgenic event production to support different projects ranging from enabling technology

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testing and new capability development to trait discovery [8]. The handling of individual shoots, plantlets, somatic embryos, or other pieces of tissue during transfer from one stage of development to another incurs a high labor input leading to high monetary cost [1, 3]. This is particularly the case for the *in vitro* stages where all tissue handling has to be carried out under sterile conditions wherein the tissue has to be cut, picked up and transferred using sterilized instruments or equipment. In recent years, there has been extensive interest in the automation of tissue culture (e.g., bioreactors, encapsulation of somatic embryos, cutting, transport, and planting through the use of robots or other devices [2, 7, 9]). Some of the most straight forward aspects of plant tissue culture that do not involve the tissue directly have already been automated:

- Nutrient media preparation.
- Environmental control of the incubators/greenhouses that contain the cultures and plantlets.
- Computer management in the laboratory and greenhouse [4].

Many of these later systems are commercially available, but some have been designed for specific commercial operations and are not widely applied. In spite of the amount of research towards automation of tissue culture, there are very few automated systems commercially available. Potential reasons for this are as follows:

- The systems are not cost effective compared with manual methods (often caused by expensive components and low production volumes).
- Failure to adequately address biological and engineering constraints together.
- Tissue cultures are not easily manipulated through automation.
- The combination of expertise necessary to develop these systems is not readily available.

The split seed *Agrobacterium*-mediated transformation protocol developed at Dow AgroSciences has several limitations that makes it a good candidate for automation. The protocol, as reported, involves preparing split seed explants from seeds that have been surface sterilized and imbibed with water for 16-20 hours. A seed is held with forceps and its embryonic axis is trimmed by about ½ to ⅔rd. The seed is split into two halves, and the seed coat is removed. These seed halves are treated with *Agrobacterium* for 30 min. Finally, the *Agrobacterium* treated half seeds are placed on solid cocultivation media in Petri plates. The drawbacks of this manual method of explant preparation are as follows:

- Reduced safety during cutting (a person has to hold the seed with forceps, then bisect the seed and cut the embryonic axis with a scalpel).
- Higher risk of ergonomic strain for researchers (manual method requires 4 hours of repetitive activity per Person depending on the number of people available and size of experiment which has significant ergonomic impact on the worker).
- Inconsistent quality and/or productivity (since many personnel are involved in preparation of the explants, uniformity of the experiment can be compromised), and high manpower requirements.

In this article, automation of the split seed *Agrobacterium*-mediated transformation protocol is described from the viewpoints of engineering design, automation construction, and the unique problems associated with the method. Tissue culture presents some unique challenges that are not usually encountered with other forms of automation developed for related fields such as micro propagation. Emphasis is placed on ensuring the biological and physiological needs of seed processing are met in the equipment design ensuring the equipment can deliver the consistency and quality expected from the process. In this report, we describe a system designed by Dow's Automation and Robotics Team to automate the procedure for placing soybean half seed explants with partial embryonic axis into *Agrobacterium* solution. The automated system strongly mimics the human

equivalent activity. Special focus was placed on the following procedures:

- Delivery of 20 ml of *Agrobacterium* solution into a Petri dish filled with approximately 30 explants using a peristaltic pumping system.
- Shaking the seeds in *Agrobacterium* solution for 30 minutes (or other time period configurable through the software). The automated system actually improves the shaking period because the seeds are continually shaken.
- Transfer of the explants from *Agrobacterium* solution onto Petri dishes with media for further culturing.

We further describe the automation of steps involving handling of *Agrobacterium* during infection of explants and placing explants onto Cocultivation Media. The automation of the steps described here will further increase efficiency towards industrialized transgenic event production and increased capacity, consistency, and reliability.

2. Materials and Methods

2.1 Manual Method for Agrobacterium Infection of Explants and Placement on Cocultivation Media

Explants were prepared manually as described by Pareddy et al. [8] or by using automation as described by McCarty et al. [6]. Briefly, surface sterilized, and overnight water imbibed soybean seed is held with forceps and ½-⅔ of the embryonic axis (found at the nodal end of the cotyledons) is trimmed using a #10 blade affixed to a scalpel. A longitudinal cut is made along the hilum to separate the cotyledons and the seed coat is removed resulting in two explants from one seed (Fig. 1a). About 30 explants are placed into a 100×25 mm Petri dish and about 30 ml of *Agrobacterium* solution added, assuring the explants are completely immersed. The explants were incubated at room temperature for 30 minutes with occasional gentle agitation (Fig. 1b). After 30 minutes, six explants were transferred onto each co-cultivation Petri dish lined with filter paper, so that the flat, adaxial side is facing the filter paper (Fig. 1c). The used *Agrobacterium* infection solution is disposed of into a biohazard waste container.

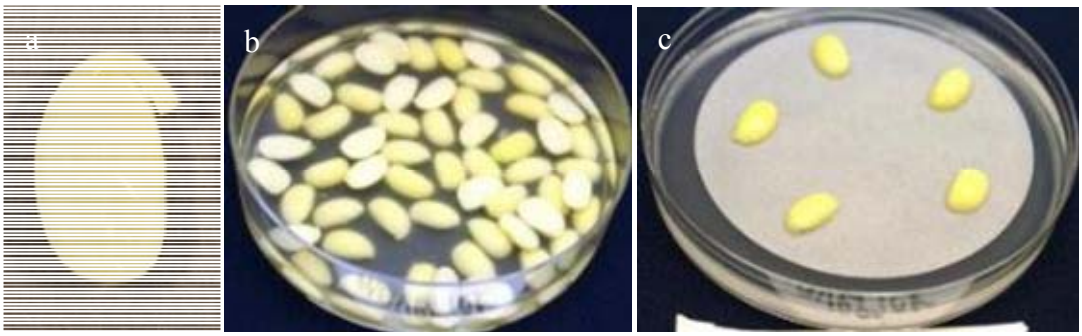

Fig. 1 *Agrobacterium* infection plates and cocultivation plates. a) Split seed explant with trimmed embryonic axis, b) Explants in *Agrobacterium* infection solution, c) Explants on co-cultivation medium.

2.2 Equipment and Tools Used for Design and Implementation of Automation for Handling of Agrobacterium Infection of Explants and Placing Explants onto Cocultivation Media

The automated system consists of the following components, depicted in Figs. 2 and 3. All the hardware components were installed on a customized table (designed by Automation By Design, ABD) and the table is located inside a large bio-safety cabinet to maintain sterility.

Photoelectric sensors to detect the container of seeds: The sensors mounted next to the delivery station are Keyence (www.Keyence.com) type LV-NH32 adjustable spot sensors. The Model LV-NH32 sensing head uses the Keyence LV-N10 amplifier electronics.

Epson Robotics: Epson robots were purchased from the vendor, and assembled onto a table designed and

built a Dow integrator, ABD (Automation By Design). All the electronics for the Epson robots, were designed and assembled by the Dow Robotics and Automation Team. To achieve the throughput required, it is necessary to use three robots. The robot chosen for this application is the Epson model C3 six axis articulated arm robot, with one main RC620+ controller and two ancillary RC620DU+ drive units. Two robots, designated as Robot A and Robot C, use three finger grippers and are installed to the left and right of the third Epson C3 designated as Robot B. Robot B is equipped with a small suction gripper. The RC620+ and RC620+DU combination provides the capability to operate the three Epson robots simultaneously, and to program them to avoid collisions with each other. The Epson brand of robot was chosen not because of its hardware capability — there are dozens of mechanically equivalent robots — but because of the

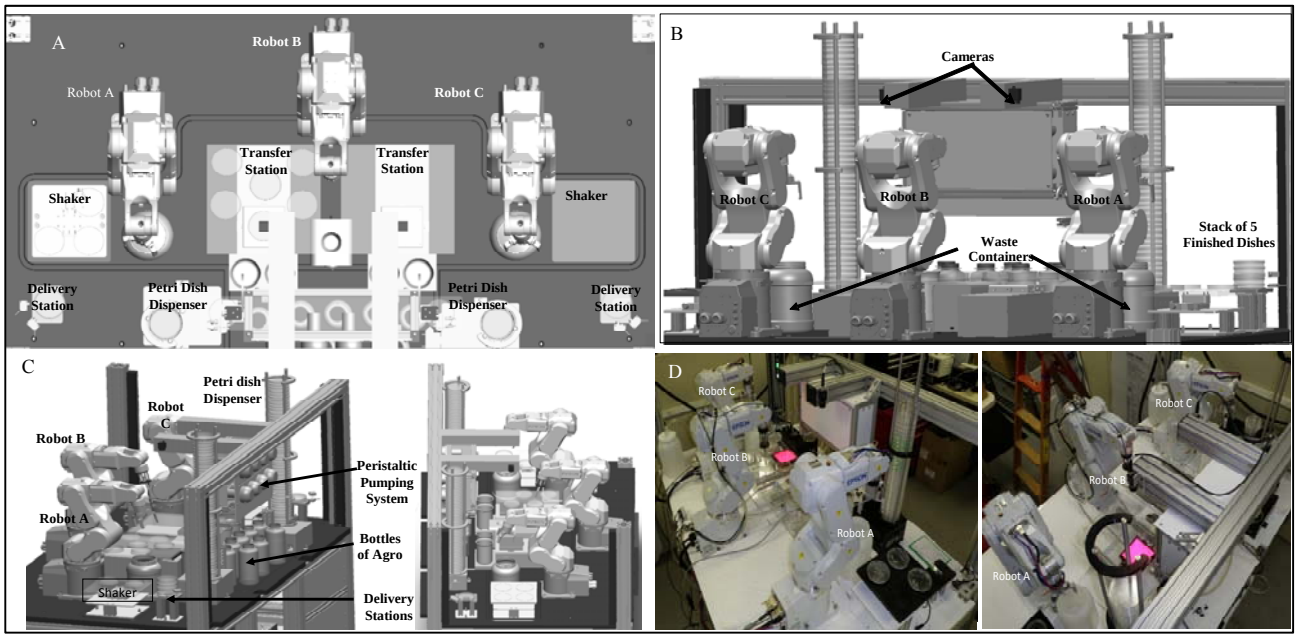

Fig. 2 CAD drawing of completed system: a) top view, b) rear view, c) iso and side views, d) photo of system.

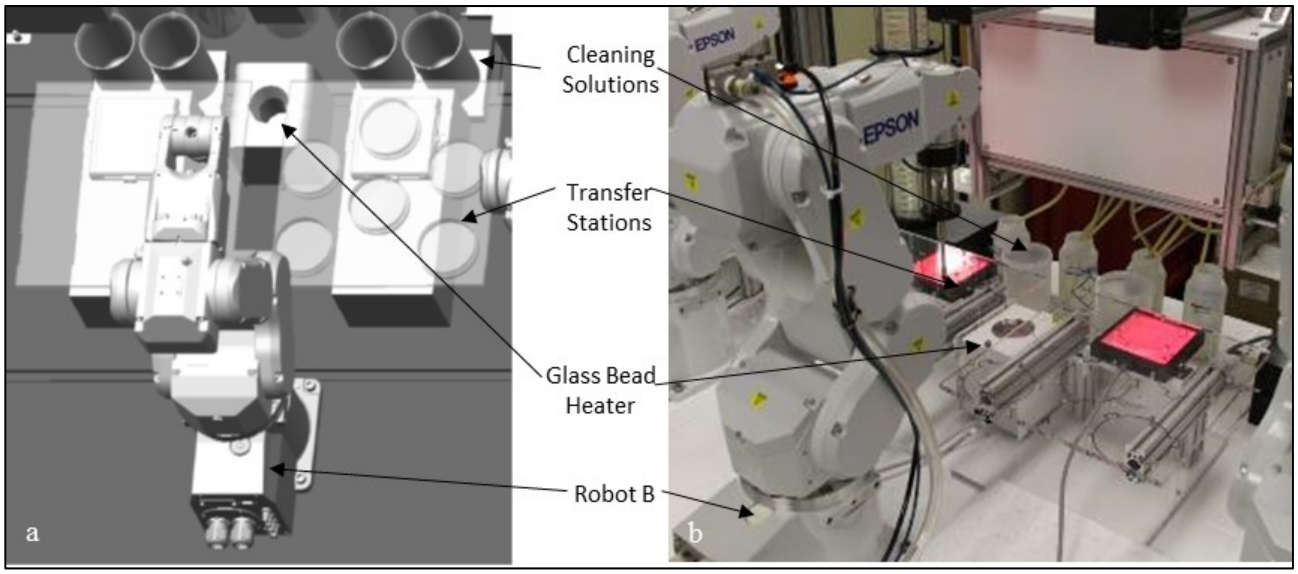

Fig. 3 Robot B hardware a) CAD drawing b) photo of system.

Epson software. Epson provides a Microsoft.NET compatible software package, allowing DOW software developers to tightly integrate the robot operation into user interfaces written using the Microsoft Visual Studio development platform.

Petri dish gripper tool: The main gripper mechanism is an SMC 3-finger; 32 mm bore gripper, part #MHSL3-32D, with type D-Y59AZ positioning grippers attached. The gripper's three fingers were designed by Dow's Mechanical Engineering Center, Midland, MI.

Peristaltic pump system: This is a combination of pump and motor (Welco brand). The peristaltic pump tubing is 3/16" Pharmed BPT tubing (Fisher Scientific, part # 14-170-04). This tubing is ideal for use in "clean-in-place and steam-in-place" cleaning and sterilization systems. It is compatible with virtually all commercial cleaners and sanitizers and can be repeatedly autoclaved (temperature range is -51°C to 135°C for up to five cycle times, without affecting overall service life.

Shakers: A Teleshake (Fisher Scientific Variomag Teleshake) magnetically coupled shaker is used to shake the explants. Magnetic coupling reduces mechanical wear, leading to longer life, plus provides the capability to design unique shaking patterns containing both rotary and side-to-side movement.

The Petri dish dispenser (media stacker): The Petri dish dispenser was built by Automation By Design, with input and testing by Dow. The dispenser, as designed, will hold up to 50 dishes of media in 0.5" high media plates. One dish is dispensed at a time using a set of four pneumatics that function to close a pair of semicircular grippers around the base of the second dish from the bottom. This gripper is mounted onto a second pneumatic that lifts the entire set of dishes off of the bottom dish. The third pneumatic extends the bottom dish of media from underneath the stack to a position for picking by the robot and the fourth pneumatic causes a small plate to rise up and contact the remaining stack of dishes. When the first gripper releases the stack of dishes, this fourth pneumatic will gently lower the stack of dishes down.

Transfer Station: There are two Transfer Stations, one between Robot A and Robot B, and the other between Robot B and Robot C. The Transfer Station is a 13" square by 3/8" thick acrylic Plexiglas plate pinned to an extruded aluminum structure. Underneath the plate is mounted a 4" square red LED (Advanced Illumination, Rochester, VT, www.advancedillumination.com, part #CB040-660-C2 Collimated Backlight). The LED provides backlighting for the explants during the Robot B pick and place process. It is controlled by the Advanced Illumination CS420 Dual Output power controller.

Cameras: A Sony XC-ES30 camera with associated JB-77 Junction Box. Computer M1614-MP 16 mm lens, F1.4, C-Mount.

Explant Gripper and gripper support: The Robot B gripper used for suctioning explants is the silicon "S-Type 2.5 Bellows, 5 mm" bellows gripper from EMI Plastics Equipment, part #3257. This gripper has a high maximum temperature rating of 250°C allowing for sterilization in heated glass beads. The bellows allows a little bit of give without breaking the vacuum. The gripper requires EMI part #3289 nipple support. The gripper is attached to a "spring loaded suspension" which allows for some movement of the gripper against the tension of the spring as the gripper contacts the explant, effectively preventing the gripper from injuring the explant.

Glass bead bath and ethanol vial: A InoTech BioScience Steri 250 dry glass bead sterilizer is used. Regular sized glass beads were replaced with 2 mm beads to prevent the beads from getting stuck inside the gripper. Also, as delivered the Steri 250 maintains its temperature at 250°C. Since the Viton gripper is rated to only 230°C, the electronics of the Steri 250 are modified to lower the glass bead temperature to 210°C. A 50 ml vial containing 70% Ethanol is placed, near the glass bead bath at a position where Robot B can access

to clean its vacuum gripper, and an operator can access from outside of the hood for replacement.

Empty jug for waste disposal: The robot disposes the used Agrobacterium dish by dropping it into a wide mouth gallon (128 oz.) high density polyethylene jugs (Fisher Scientific #50-011-66). An 8" square by 1" thick Plexiglas plate with a 6" diameter hole cut into the middle of it was pinned to the table to provide accurate placement of the waste jugs.

Bio-Safety cabinet: BioPROtect III, (The Baker Company, http://www.bakerco.com). All the hardware components were installed on a customized table (Designed by Automation by Design co,) and the table is located inside a large Baker biosafety cabinet (164 cu.ft. interior, 76"H × 102"W × 36.5"F-B, nominal) to maintain sterility. The BioPROtect® III cabinet offers clean air and containment enclosures designed for high-volume robotic and automated equipment applications, while allowing access to the interior work area through the combination of double doors or an 8" window sash opening. Its modular construction also allows disassembly for shipping, delivery and placement.

Software: The software is a combination of two programs written specifically for this instrumentation:

- An Operator Interface written in Microsoft's Visual Studio programming language Visual Basic.
- Epson SPEL Code written in Epson's SPEL+ programming language. This software controls the robots, cameras, vision imaging, shaking of explants, and transfer of explants.

Both programs run simultaneously on the Epson RC620+ controller.

3. Results and Discussion

In order to develop an automated system for the preparation of soybean explants for use in genetic transformation experiments, it was important to design and develop an integrated system that could preserve the sterility of the materials (explants) without compromising their viability and integrity. In addition the system must not alter the biological principles of the transformation system previously developed by Pareddy et al. [8]. Important concepts of this automated platform are integration of the protocol used (combining biological agents (plant and bacteria) for plant genetic transformation) with hardware, software for robotic automation, and reduced or no human intervention using cameras and image processing software to achieve the needed throughput and experimental quality. Important steps in the infection of explants and placement on cocultivation media are (a) the addition of *Agrobacterium tumefaciens* solution to the Petri dishes with explants, (b) shaking of Petri plates for 30 minutes during infection, (c) removing the used *Agrobacterium* solution, and (d) transferring the explants onto cocultivation media plates. The automation of the steps described above using custom made machine vision software to guide robotic arms in the execution of the tasks are described below. The following sections describe the results of such design and how custom equipment as well as commercially available technologies were adapted or utilized in the system.

3.1 Addition of Agrobacterium Solution to Explants and Placement on Shaker

The steps involved are as follows:

- Placement of Petri dish containing about 30 explants onto the delivery stationby the operator (Fig. 4a).
- Photoelectric sensor enabled detection of Petri dish and movement of Petri dish to the *Agrobacterium* dispensing position (Fig. 4b).
- Addition of *Agrobacterium* solution via peristaltic pumps (Fig. 4c).
- Movement of Petri dish to shaker (Fig. 4d) where it remains shaking for 30 minutes.

The dish sensors are a reflective type, where the beam from a laser inside of the sensor exits and if there is something in the path of the sensor, for example a

Petri dish, the laser light is reflected back into the sensor, detecting the dish. The Model LV-NH32 sensing head uses the Keyence LV-N10 amplifier electronics. This Keyence LV-N10 amplifier allows for the setting of detection sensing levels to most accurately detect the sensing of the dish in the beam. The amplifier's digital output is connected to a digital input in the RC620+ controller. Software written for the RC620+ controller reads that the dish has been detected, and starts additional software to process the dish of explants.

As shown in Fig. 5, there are two sensors positioned near the dish. One sensor is positioned to pick up the bottom dish in a stack, the second senses a second dish. During programming, the detection of the second dish is used to signify a stack of dishes containing two or more dishes.

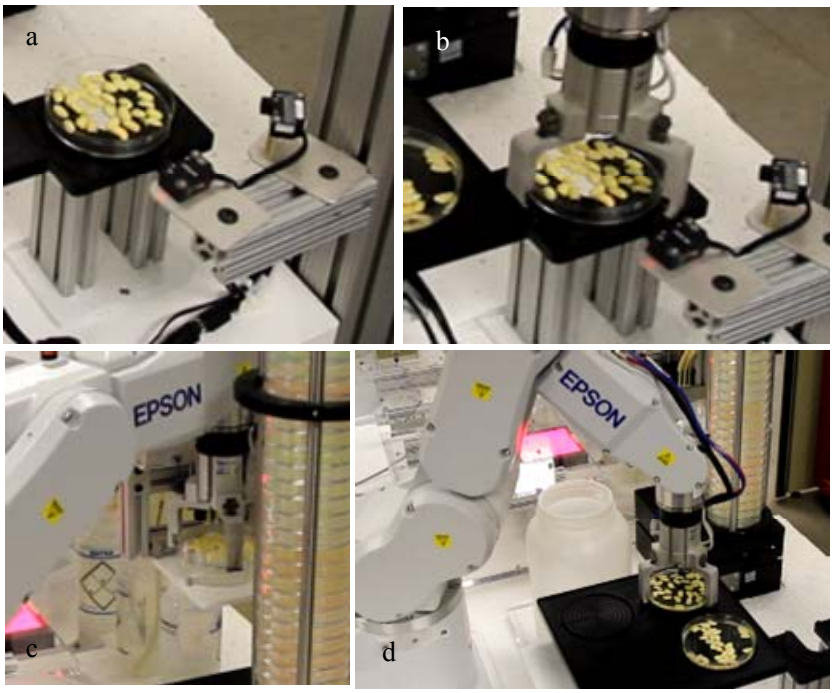

Fig. 4 a) Petri dish containing explants placed on delivery station, b) Robot A or C picking explants from delivery station c) Robot A or C places dish of explants under *Agrobacterium* dispensing tube, d) Robot A or C placing dish of explants onto the shaker.

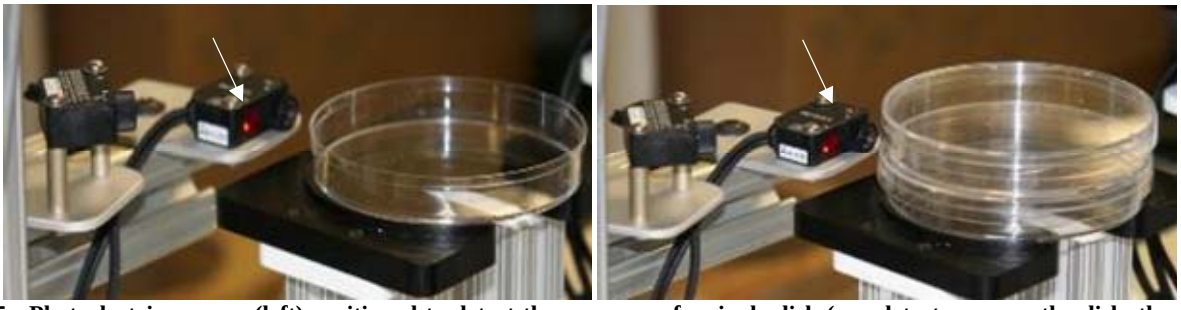

Fig. 5 Photoelectric sensors (left) positioned to detect the presence of a single dish (one detector senses the dish, the other doesn't) b) Photoelectric sensors positioned to detect the presence of up to two dishes.

3.2 Delivering a Pre-Defined Amount of an Agrobacterium Solution into the Dish of Seeds

An *Agrobacterium* delivery system is based on a system of peristaltic pumps. The peristaltic pump panel (Fig. 6) consists of the following components:

- 8 pumps and their corresponding tubes.
- Toggle switches for their corresponding pumps.
- Two Auto/Manual switches.
- Two tube holders on the side of the panel.
- Two drip pan plates under each tube holder.

Six peristaltic pumps (clear covering in Fig. 6) are designated to dispense *Agrobacterium* solutions. The remaining two peristaltic pumps (with blue covers) are mechanically the same as the other six pumps but are operated in reverse to suction used *Agrobacterium* solution after infection. A **Manual/Auto Switch** is located on the top right and top left corners of the pump front panel. When the switch is placed in the Manual position, the pumps may be operated using the toggle switches on the front of the pump panel. When in the Auto position, Epson software operates the pump, and the toggle switches are inactive. A Dow designed **tube holder** (Fig. 7) is used to hold the tubing. This holder holds up to three tubes into position where the robot can access them for delivering the *Agrobacterium* into the dish. The two tube holders, one for Robot A and one for Robot C, are pinned to the panel for easy removal and cleaning. The **drip pan plate** (Fig. 7) is a mounted flat plate approximately 4" below the tube holders. An empty catch dish is placed on it to capture drops of *Agrobacterium* that may drip from the end of the tube.

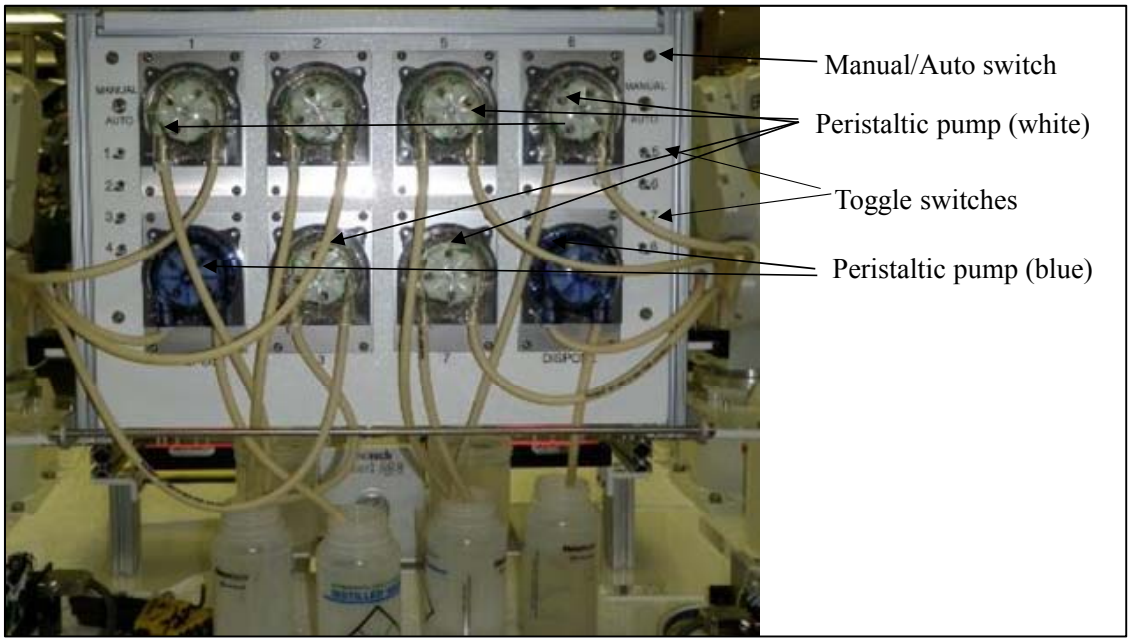

Fig. 6 Peristaltic pumping systems to dispense *Agrobacterium* solution to containers of seeds.

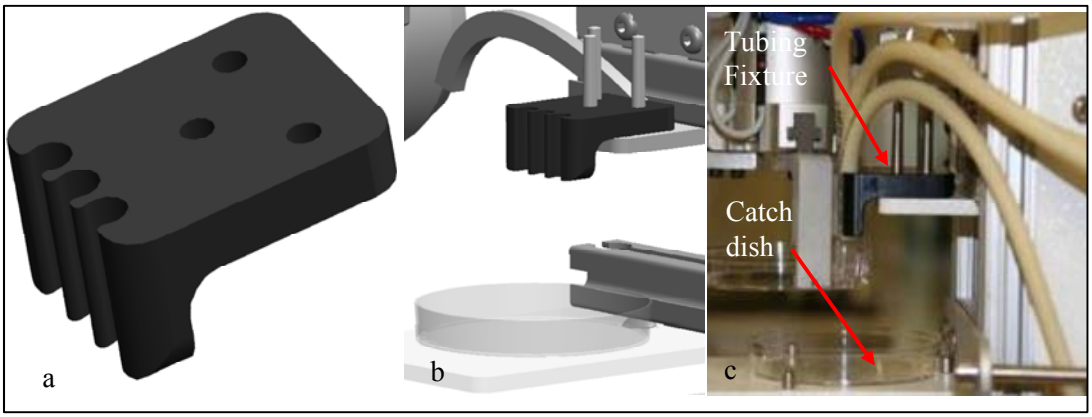

Fig. 7 a, b, and c. Detailing the holder that holds the peristaltic pump tubing into place during dispensing of *Agrobacterium*.

3.3 Shaking the Petri Dish with Explants in Agrobacterium Solution

After the *Agrobacterium* solution is dispensed, the EPSON arm (A or C) moves shaking platform designed to shake four dishes simultaneously. The first version of the shaker was a ¼" thick plate mounted onto a linear slide. That version lasted less than a year before the slide bearing wore out. It was replaced with a shaker based on the magnetically coupled Fisher Scientific Variomag Teleshake. Being magnetically coupled, the mechanical mechanisms are less likely to wear out. Fig. 8 shows the four position shaker designed for the Teleshake. It is simply a 1/16" thick sheet of Plexiglas mounted onto the Teleshake with a high temperature silicone baking sheet (purchased from Bed, Bath, and Beyond) laid on top. The silicone sheet is sticky and prevents the seeds from moving while shaking. When operated at speeds under 100 revolutions per minute the *Agrobacterium* solution does not spill, nor do the dishes move.

During the 30 minute infection time, continuous shaking of Petri dish is recommended to prevent the bacterial cells from settling to the bottom. In the manual process, explants are shaken for a few seconds every 3-5 minutes. Automating this step by placing infecting Petri dishes on shakers enabled continuous and uniform shaking for the entire infection period (30 minutes). Additionally, the shaking time is consistently maintained for 30 minutes across all Petri dishes in a run compared to the manual process where the infection time was variable based on the operator's availability.

3.4 Dispensing of Cocultivation Media Plates

A stack of pre-labeled cocultivation media dishes are manually loaded into the two media dispensers. A latch door is used to secure the plate in the stacker (Fig. 9). The media plates are labeled corresponding to the type of *Agrobacterium* strain from respective pumps on either Robot A or Robot C side. The Petri dish dispenser (Fig. 9) is capable of dispensing up to 50 dishes without needing to reload the dispenser. It is accessible within the first 12-18" of the front of the table for easy reach and is also accessible by the robot grippers.

A mechanism in the stacker lifts the stack of dishes off of the bottom dish, and a slider slides the dish out from underneath of the stack and exposes the dish for the robot to pick. There are four pneumatic mechanisms present (Figs. 10a & 1b), a pneumatic that extends and retracts the "Dish Holding Plate" for the robot to pick the dish, a pneumatic that lifts/lowers the grippers, a pneumatic that lifts/lowers the stack of dishes, and a pneumatic that opens/closes the grippers.

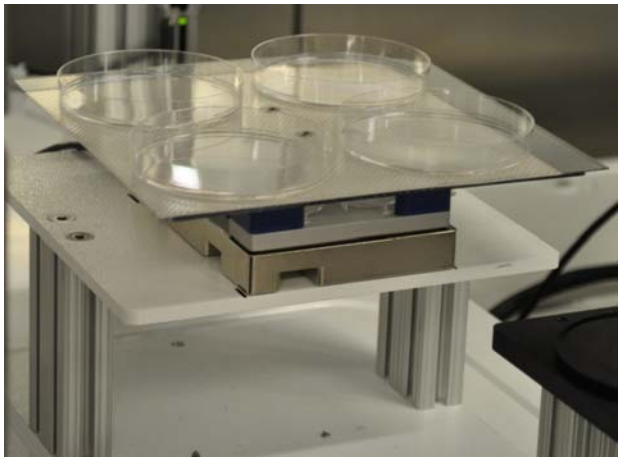

Fig. 8 Shaker used to shake four Petri dishes of seeds simultaneously.

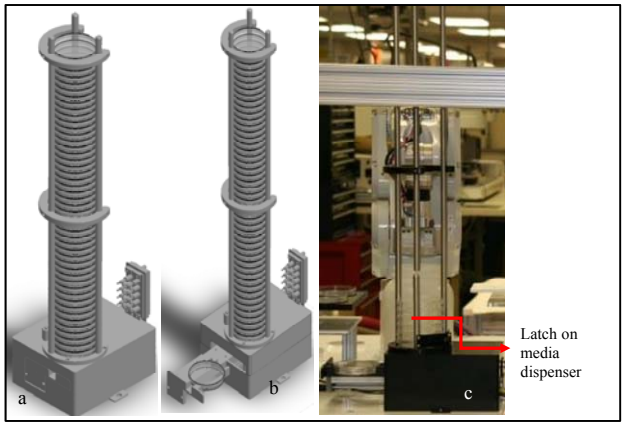

Fig. 9 a) and b) CAD images of stackers full of dishes. b) depicts the stacker presenting a dish for a robot to pick. c) photo of Petri dish dispenser.

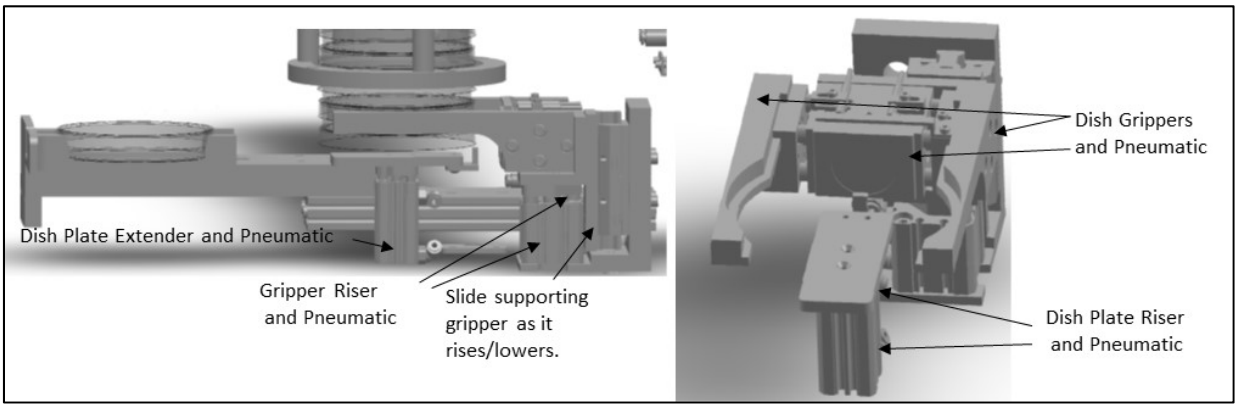

Fig. 10 Detailing interior gripper mechanisms and pneumatics.

The grippers (Fig. 10) were designed to contact along the bottom edge of the dish, where it is strongest and less likely to break. The plate lid is not contacted by the grippers, but rests on a ledge designed into the grippers. Not contacting the gripper lid is important because the lid is weaker around the bottom edge, and it would likely bow and possibly break. The ledge design also serves to help support the weight of the dish stack. Without the ledge, all of weight of the stack would rest on the contact points along the bottom edge of the dish. However, should the dish slip downwards slightly, the lid will solidly contact the ledge and the weight of the stack will then rest on the ledge. With the weight of the dishes removed from the bottom of the dish, it will stop slowly.

Dispensing of Petri plates from the stacker consists of the following actions:

1) Open Dish Grippers (Fig. 11a, b & c)
2) Close Dish Grippers and Raise Dish Grippers (Fig. 11d & e)
3) Extend Dish Holding Plate (Fig. 12a & b)
4) Retract Dish Holding Plate (Fig 12c & d)
5) Raise Stack Lowering Plate (Fig 13a)
6) Lower Stack Lowering Plate
7) Open Dish Grippers (Fig 13b)

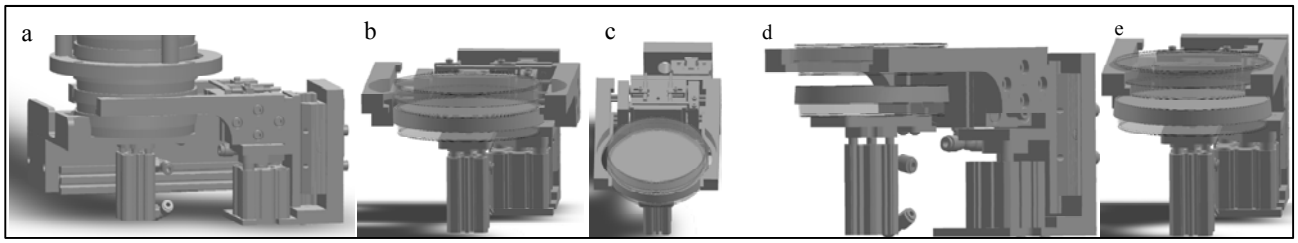

Fig. 11 a, b, and c. CAD drawings of open Dish Grippers. In these images, the Dish Grippers are ready to close onto the Petri dish that is second from the bottom dish in the stack, d and e. CAD drawings of Dish Grippers closed and raised, lifting dishes (The Dish Grippers have closed and raised, lifting the stack of dishes off of the bottom most dish.).

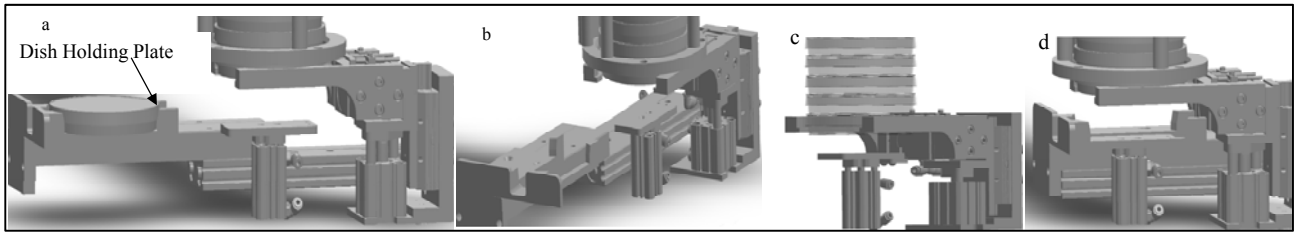

Fig. 12 CAD drawing of dish exposed for robot picking. Top) The Dish Holding Plate extends, taking the bottom most dish with it. The bottom most dish is then picked up by the robot. b) The Bottom with the bottom most dish removed, the Dish Grippers support the remaining stack of dishes, c) and d) CAD images after dish has been removed.

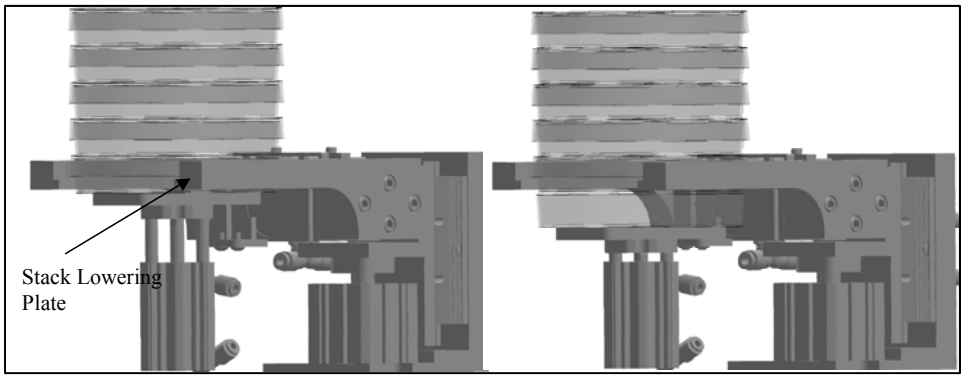

Fig. 13 a) CAD image of Stack Lowering Plate supporting stack of dishes. (The Stack Lowering Plate has risen up to support the weight of the stack.) b) The Stack Lowering Plate has lowered the dishes.

3.5 Picking and Placing Cocultivation Media on the Transfer Station

Approximately two minutes before the shaking time for a dish of explants is completed, the appropriate robot (Robot A or Robot C) removes dishes of cocultivation media from the media dispenser and places them onto the **错误！未找到引用源。**. The following steps are required:

a) The appropriate robot (Robot A or Robot C) picks the dish of co-cultivation media from the Petri dish dispenser (Fig. 14a)
b) The robot places the dish of media onto the red LED transfer position (Fig. 14b)
c) The robot opens its grippers, moves up a couple of millimeters, and then closes its gripper onto the dish lid. The lid is placed onto the transfer station at one of the five points pre-taught to the robot (Fig. 15a)
d) The robot returns to the red LED position, picks up the bottom of the dish of media, and places it onto the lid placed in Step c (Fig. 15b).

Steps **a through d** are repeated five times to place five dishes of media onto the taught positions on the transfer station (Fig. 15c).

Grippers, attached to Robot A and Robot C were designed in a way that it could both transport a Petri dish and also remove the lid (Fig. 16a and b). The Petri dishes are only contacted by three conical shaped screws. The gripper air pressure is regulated to 10 psi. This is enough pressure to hold the dish tight in the gripper, but to not crush the sides of the dish. Since the lid and bottom of the Petridish were stacked one on top of the other, it did not require an additional position on the transfer station to temporarily store the lids during explant transfer. The lids have a slight lip on top. The lip serves as a positioning mechanism for the dishes of media as the seeds are placed into them.

3.6 Place Explants on Transfer Station

When the shake time expires, the appropriate robot (Robot A or Robot C) picks the dish of explants (in *Agrobacterium*) and places them onto the **错误！未找到引用源。** red LED position (Fig. 17).

3.7 Analysis of Seed Image by Software and Transfer of Explants to Cocultivation Media

Robot B uses a pair of fixed downward cameras to

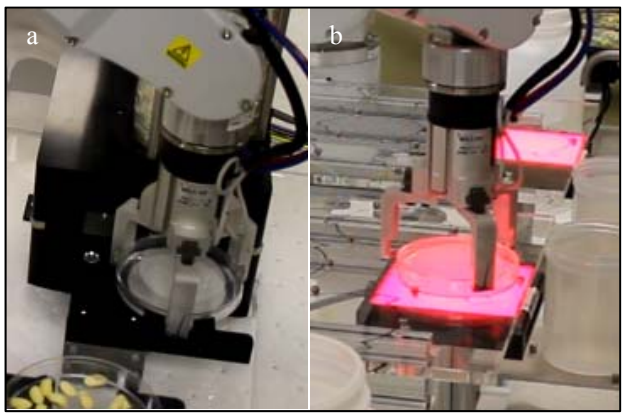

Fig. 14 Different steps of Transfer of media plates from Petri Dish Dispenser onto the Transfer Plate. a) Robot picks dish of co-cultivation media. b) Robot places dish of media onto red LED transfer position.

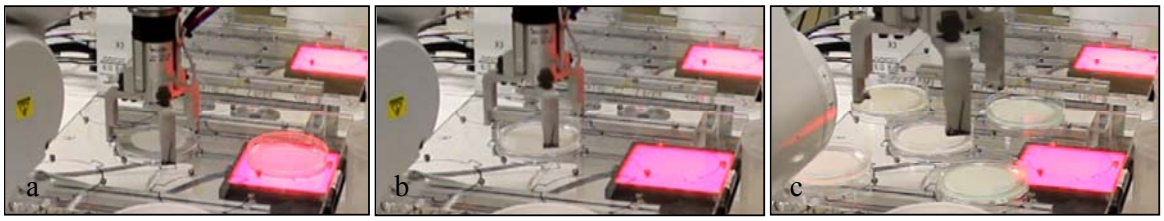

Fig. 15 a) Robot placing lid onto transfer station. b) Robot placing dish of media onto lid. c) Image showing five dishes of media placed onto the transfer station.

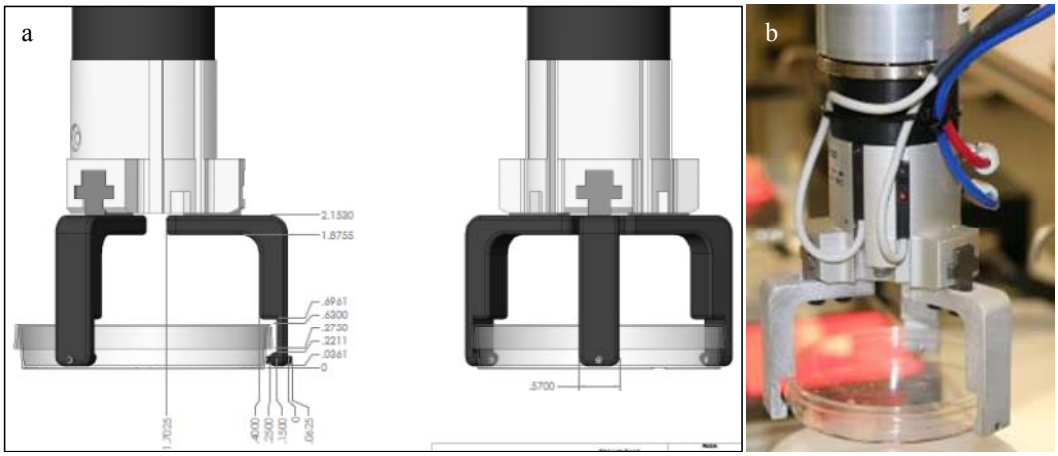

Fig. 16 a) Drawing and b) photo of Dow designed gripper showing gripper with dish and lid for Petri dish manipulation.

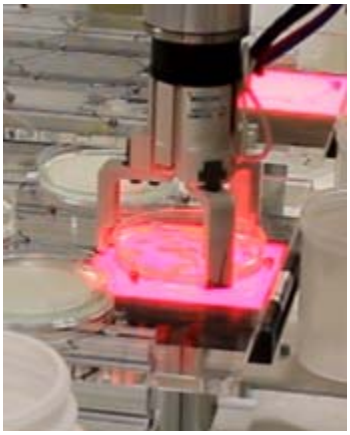

Fig. 17 Robot placing dish of explants onto red LED position.

vision explants in *Agrobacterium* solution placed onto the Plexiglas 错误！未找到引用源。 position above the two CB040 red LED. The visioning components are:

- A 4" square, red LED panel, (Advanced Illumination, Rochester, VT, http://www.advancedillumination.com, part #CB040-660-C2 Collimated Backlight). The LED panel provides backlighting for the explants during the Robot B pick and place process. Controlled by Advanced Illumination CS420 Dual Output power controller.
- A Sony XC-ES30 camera with associated JB-77 Junction Box.
- Computer M1614-MP 16 mm lens, F1.4, C-Mount.

Fig. 18 shows the visioning system detail. Fig. 18b is a photo from behind Robot B, and the two cameras are mounted above the red LED. In Fig. 18, Robot A is to the right and Robot C is to the left.

3.7.1 Image Capture and Analysis

The Epson six axis articulated arm (Robot B) can calibrate the robot's position in space with respect to the image of explants. A unique capability of this application is the use of the vision software to locate and pick explants that have irregular shapes. A black and white camera was used, thereby the red LED light appears as a bright white image through the camera and the seeds appear as black blobs (Fig. 19). By setting the camera aperture correctly, it is possible to practically eliminate all traces of the Petri dish and its shadow to

eliminate chances of the imaging software identifying the Petri dish wall or its shadow as a seed. A detailed description of the imaging software algorithms designed to transfer explants from the *Agrobacterium* solution onto the co-cultivation media follows.

Because the depth of *Agrobacterium* solution in the dish is greater than the height of an explant, it is possible for explants to overlap each other. However, it is a requirement that only individual explants are picked and placed onto the cocultivation media. A technique for identifying overlapping explants and separating them was built into the software.

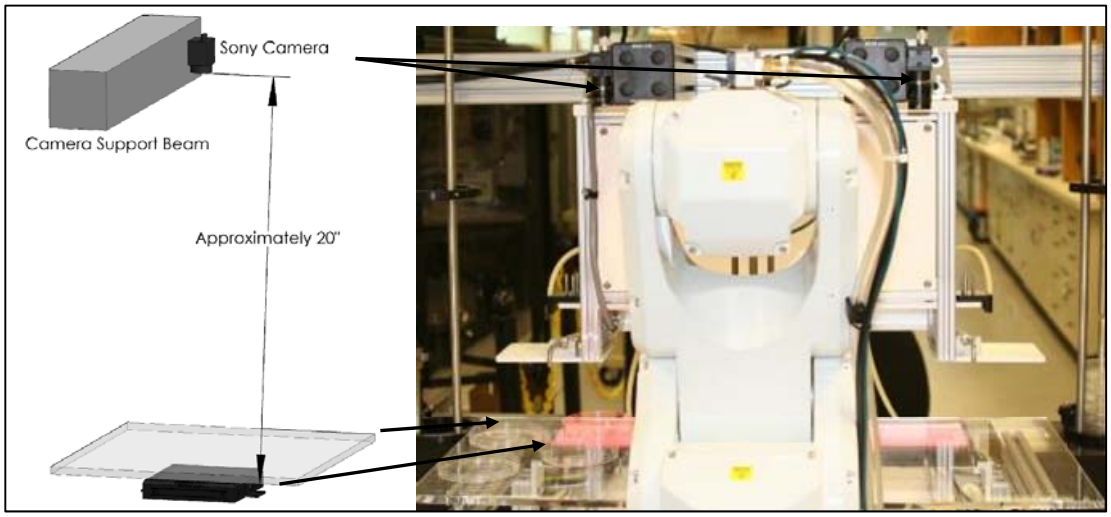

Fig. 18 Vision system for picking explants from *Agrobacterium*. The photo shows the two cameras to the left and right of Robot B imaging the red LED below.

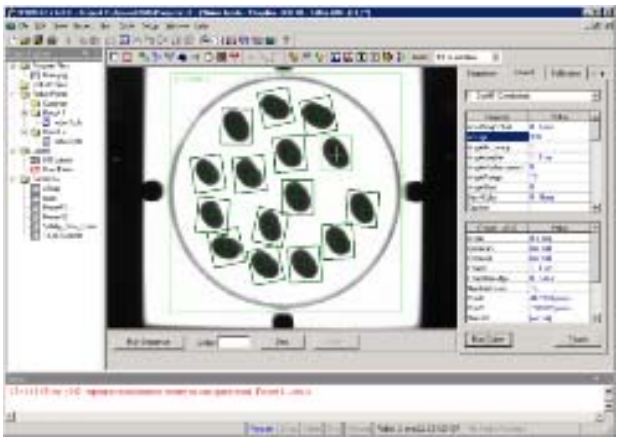

Fig. 19 Explants on the Petri dish appear black when a black and white camera is used to image against the red LED.

The right images of Figs. 20 and 21 demonstrate the results of seven image operations performed on the original image on the left:

(1) Use an "ImageOp" vision object to binarize the image. Shadows disappear and the explants appear very sharp and black.

(2) Use a "Blob" vision object to locate the dish positioning pin in the upper left of the image.

(3) Center an "ImageOp" object on the pin blob, and binarize the pin to make it disappear from the image (If not removed from the image, the software may mistake the pin for an explant).

(4) Use a "Blob" vision object to locate the dish positioning pin in the upper right of the image.

(5) Center an "ImageOp" object on the pin blob, and binarize the pin to make it disappear from the image.

Use a "Blob" vision object to locate overlapping explants. The area of a single explant is approximately 1000 pixels. Since the purpose of the blob is to detect overlapping explants, the MinArea property is set to 1200 (Fig. 20)

(6) Use a "Geometric" vision object to locate single explants. The important properties for the geometric are as follows:

- ScaleEnable – True

- ScaleFactorMax – 1.5
- ScaleFactorMin – 0.8
- NumberToFind – 1

Fig. 22 shows the explant model used to train Epson geometric object.

The camera images and software sequences are mapped into the robot's coordinate system, and many of the imaging operations, in this case the geometric object, return the robot's XY coordinates for picking the explant. The Fig. 28 flow chart depicts the software logic used by Robot B to transfer explants from the *Agrobacterium* solution onto the cocultivation media (Fig. 23).

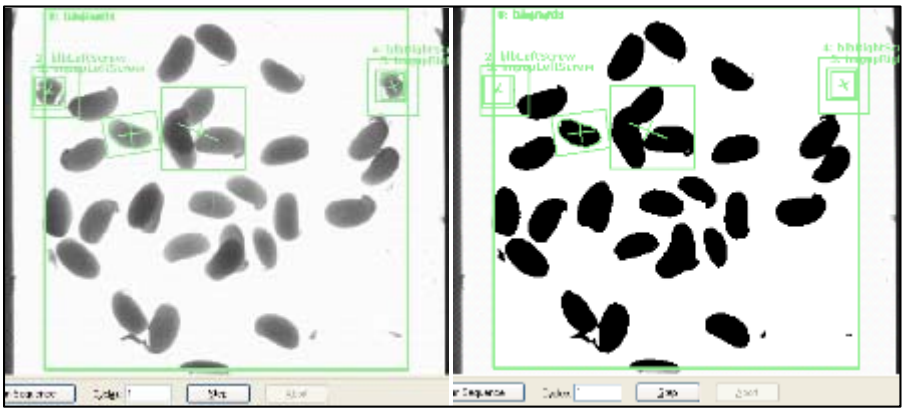

Fig. 20 Screen shot of Epson software showing how the binarization and blob vision objects are able to remove the positioning pins from the image.

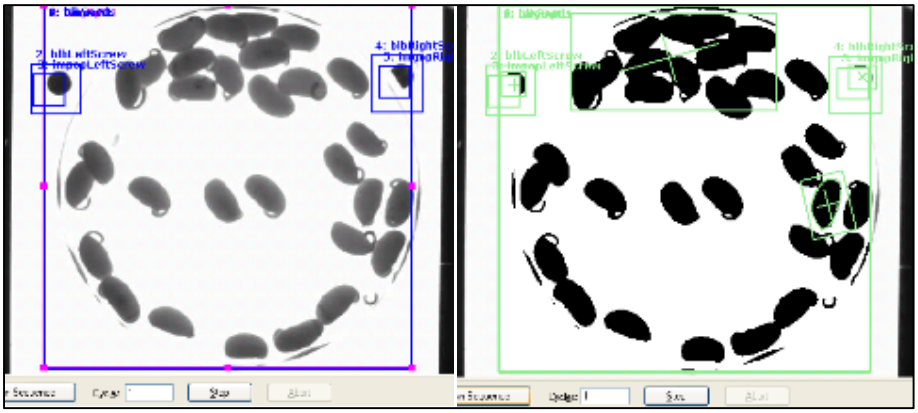

Fig. 21 Showing a group of overlapping explants identified by a blob vision object. (The original image of the explants is shown on the left.)

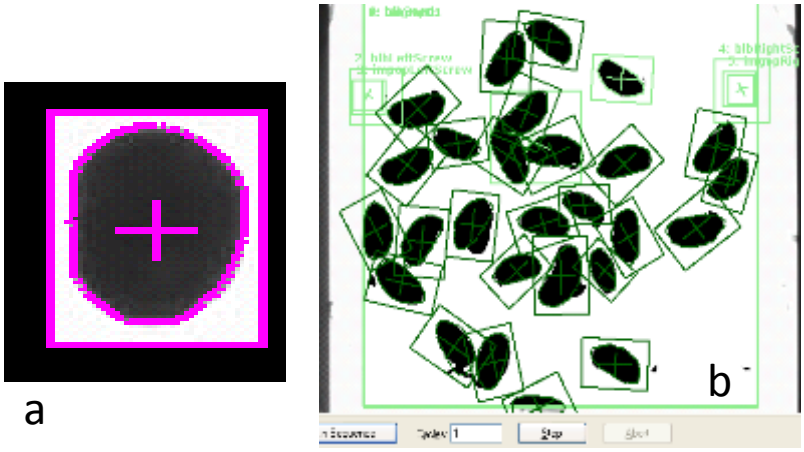

Fig. 22 a) Explant model used to train Epson geometric object. b) Screen shot of Epson software identifying multiple explants using the Geometric vision object.

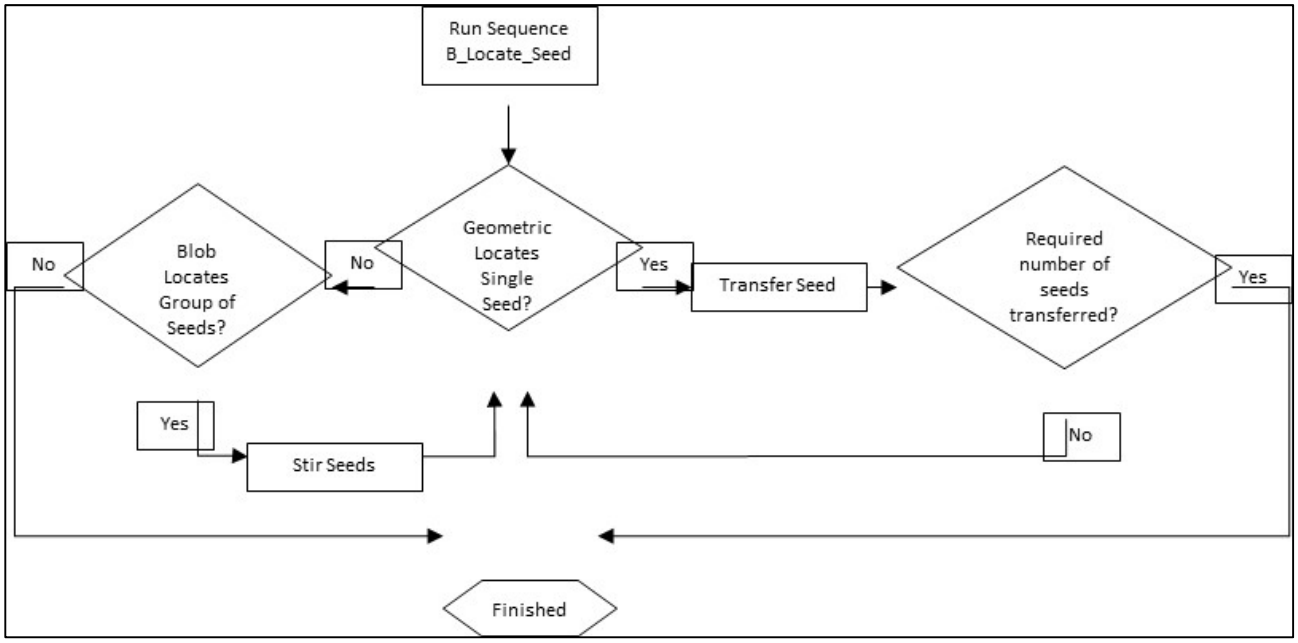

Fig. 23 Flow chart depicting software logic used by Robot B to transfer explants.

Note that the explants are floating in *Agrobacterium* solution during the transfer process. The purpose of keeping them in solution is to provide a means to separate them easily. The vision blob object locates the center of mass of a group of overlapping explants. Robot B places its gripper approximately 10 mm above the center of mass and turns on a stream of air to blow onto the explants. Because the explants are still in the *Agrobacterium* solution, they will separate. The robot backs up and the imaging sequence will run again to determine if the explants have separated, and a single explant is visible.

3.7.2 Picking up Explants by Robot B Gripper

A gripper and spring loaded suspension was attached to the robot B arm (Fig. 24a and b). Using this type of suspension allows the robot to always move to the same position above the explants in the Petri dish to pick a seed from the dish without fear of crushing the seed into the dish when picking the seed. A unique aspect of this equipment is the ability to pick the half seed explants using vacuum while they remain in the liquid *Agrobacterium* solution, without suctioning the *Agrobacterium* solution. A slight positive flow of air pressurizes the suction gripper as it is placed onto an explant. The gripper pushes the explant to the bottom of the container, with the effect that no liquid remains on the interior of the gripper.

3.7.3 Placing Explants on Cocultivation Media Dishes

Robot B places six explants in a circle at equal distances (60 degrees) around the cocultivation media dish. The software calculates the positions to place the explants onto each co-cultivation media dish — the positions are not hard-coded into the software. By changing a single variable, Number_Explants_Per_Dish, the operator can change the number of explants required for a dish of media, and the software will assure the explants will be placed equal distances apart. However, the five cocultivation media dish positions on the transfer station, shown in Fig. 25, are hard coded into the software (Fig. 25a and b). Increasing the number of co-cultivation media positions would be relatively straight-forward in the software. The limitation of five is due to the physical reach of each robot, with respect to the area shared by either Robot A and Robot B, or by Robot C and Robot B. The area

shared is not much larger than the size of the existing transfer station, which holds six dishes (five media dishes and the red LED position). It would be difficult to add another dish onto that plate. Increasing the number of media positions would require a change in the layout, and possibly a change in the size of the robot.

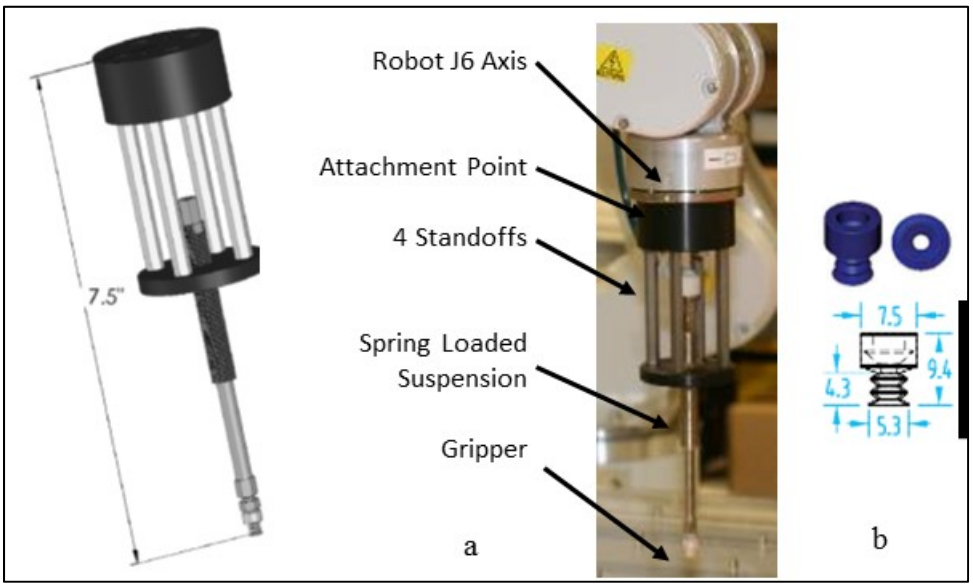

Fig. 24 a) Image of gripper assembly mounted to the robot. b) "S-Type 2.5 Bellows, 5 mm" gripper.

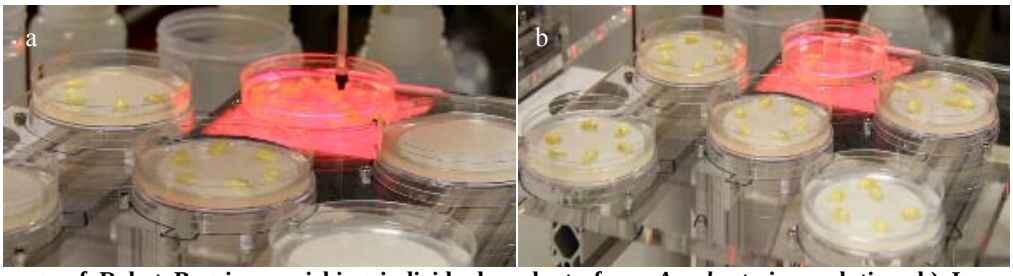

Fig. 25 a) Image of Robot B gripper picking individual explants from *Agrobacterium* solution. b) Image of explants distributed onto 5 dishes of co-cultivation media.

The five co-cultivation media positions and the red LED light position are taught positions for both Robot A and Robot C. The mid-point of each transfer position is a taught position referenced to the robot placing the media onto the transfer station (Fig. 26). The points are taught at the center of the dish. The "1 Transfer" and "3 Transfer" positions are also taught for Robot B. Using stored taught point coordinates for Robots A and C, the software code can calculate the positions of the 10 co-cultivation media dishes with respect to Robot B. It is not necessary to teach the mid-point of each of the five media positions with respect to Robot B. The software has access to the taught points for all robots. For Robot B, only the mid-point of the red LED positions are taught. Since those positions are also taught with respect to Robot A and Robot C, the software can calculate where the media plates are located with respect to Robot B.

3.8 Sterilization of Robot B gripper

A system requirement was to include the capability for the robot transferring the explants from *Agrobacterium* onto the co-cultivation media to sterilize the portion of the robot tool that touches the explant. After the transfer is complete for each individual dish of explants, the gripper must be cleaned

before the next dish is processed to avoid cross contamination. The process for cleaning the gripper is as follows:

- Robot B will immerse the gripper in the alcohol and turn on the vacuum for one second. With the amount of vacuum applied, one second is enough time to draw ethanol through the gripper and into the PIAB vacuum generating device.
- Robot B moves the tip of the gripper up and out of the ethanol. The vacuum is turned back on. This draws air into the gripper to begin the drying process. It also helps to prevent ethanol from dripping out of the gripper as it moves to the next step (Fig. 27a)
- Robot B moves the gripper to the Inotech glass bead heated bath and places the gripper into the heated bath for 10 seconds (Fig. 27 b & c)
- Removes gripper and returns to Robot B HOME position

3.9 Disposal of Used Agrobacterium Solution and Empty Petri Dish

3.9.1 Siphoning out of Agrobacterium Solution

The siphoning of the used bacterial solution (i.e., liquid waste) from the container after the explants have been removed is achieved by using the two blue peristaltic pumps (Fig. 9). Robot A or Robot C picks the Petri dish with used *Agrobacterium* solution from its corresponding transfer station and moves it to the waste *Agrobacterium* siphoning position (Fig. 28a). Waste *Agrobacterium* is pumped into a labeled biohazard waste flask, and properly disposed.

3.9.2 Empty Dish Waste Disposal

After the *Agrobacterium* solution is siphoned out, the empty Petri dish (solid waste) is disposed of into a plastic bin. This jug will hold up to 20 empty dishes. When full, the operator simply has to place a lid onto the container, label the container, and submit it for disposal.

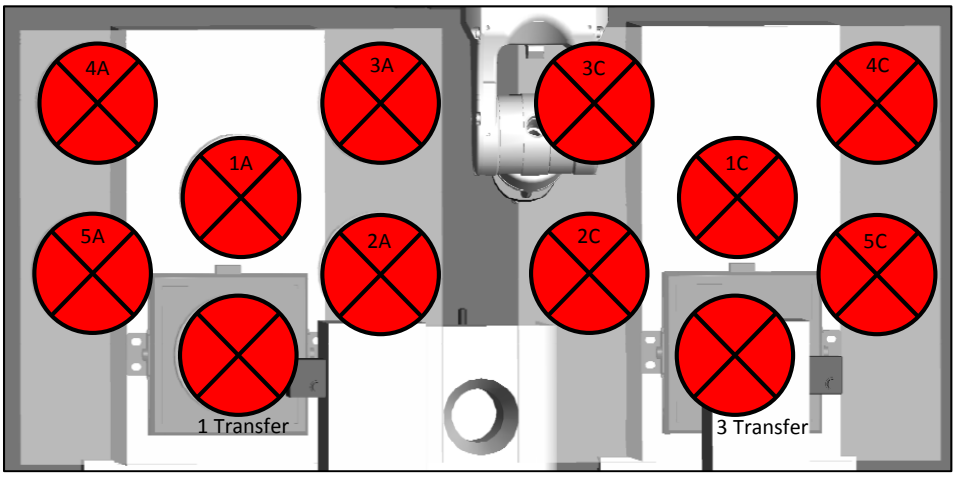

Fig. 26 Identification of the Robot A and Robot C taught positions on the transfer stations.

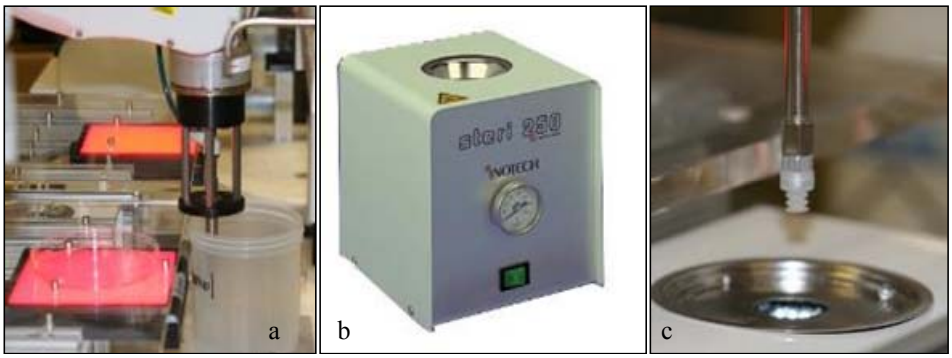

Fig. 27 a) Robot placing gripper into flask of ethanol. b) Inotech Steri 250 glass bead sterilizer. c) Gripper moving into glass bead sterilizer.

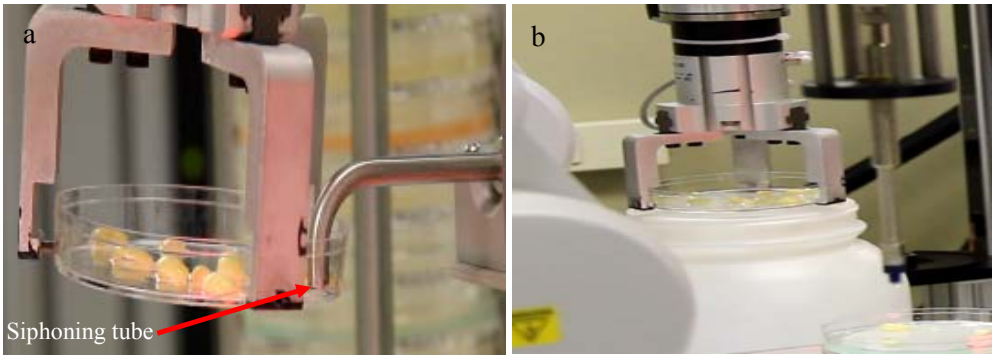

Fig. 28 a) Sipping used *Agrobacterium* and b) disposing of empty dish.

3.10 Transfer of Cocultivation Media Plates with Explants to Delivery Station

After the waste is disposed, the robot (Robot A or Robot C) is commanded to place the lids back onto the cocultivation media, which now contains the explants, and place the dishes of explants onto the delivery station for the operator to remove. The steps involved are as follows:

a) The robot grips the bottom of the dish (Fig. 29a) and transfers it to the red LED position (Fig. 29b).

b) The robot returns the same position in step a, grips the lid (Fig. 29c), returns to RED LED and places the lid on the Petri dish (Fig. 29 d).

c) Robot places covered dish onto the dish Delivery Station (Fig. 29d).

Steps **a through c** are repeated until all the five plates have been stacked on the delivery station (Fig. 30). The Delivery Station, previously identified in Figs. 7 & 8, is used as the stacking position for processed cocultivation dishes with infected explants.

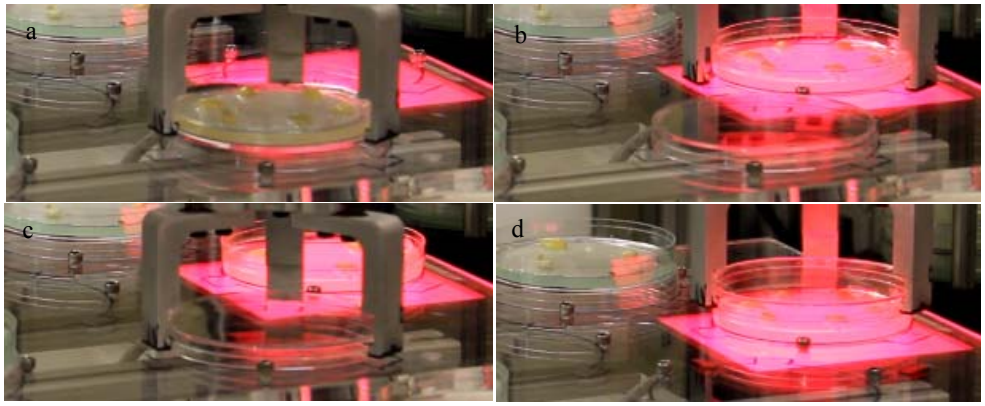

Fig. 29 a) Robot gripping dish of media containing explants. b) Robot placing dish of media containing explants onto the red LED position, c) Robot gripping lid. d) Robot placing lid onto media dish.

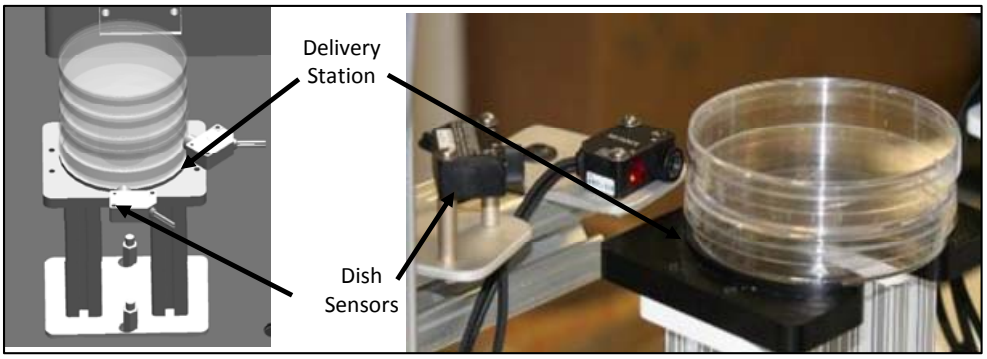

Fig. 30 Delivery Station detailing Keyence sensors detecting single and multiple dishes.

The second, upper sensor (leftmost sensor in the right photo) picks up the placement of a second dish on top of the first dish. Therefore the second sensor serves as a mechanism for the system software to know that dishes of finished explants are on the delivery station. The software uses both sensors to decide the correct action for the operator or robot:

- No dishes present for either sensor - OK for robot to place dishes of co-cultivation media with explants onto the delivery station.
- Dish present only in front of the lowest sensor — operator has placed a dish of explants onto the delivery station. Software will schedule pickup of seeds.
- Dish present in front of both sensors; dishes of finished seeds on station. The software will notify the operator via an audible alarm that the dishes are available.

3.11 Safety Features: Light Curtain and Emergency Stop

A light curtain was used for personnel safety, and the robots stop moving when the operator enters the robotic work area if the software program is running. There are two sets of light curtains:

1) A set across the front of the table since only the front side of this system was accessible to the operator. This set will pause the robot as personnel access the plate delivery station for the left side of the table (Robot A), the Petri dish dispensers, and the peristaltic pump *Agrobacterium* delivery station.
2) Set on the right side of the table. This set will pause the robot as personnel access the delivery station for the right side of the table (Robot C).

Because the entire automated system is in the BioProtect hood, there is no need to place light curtains or other operator protection devices on the left or back sides of the table.

The Epson software monitors a pair of input bits from the light curtain, and pauses the robots when the light curtain is broken. Normally, the light curtain would be wired directly into the Epson safety latching electronics. However, in this system the light curtain is wired as a digital input into the Epson electronics for two reasons:

1) It was necessary to break the light curtain to train robot positions during development and troubleshooting. It is not possible to break the light curtain and move the robot while teaching positions if the light curtain is wired directly into the safety latching circuitry.
2) The Epson controller cuts power to the robot motors when the light curtain is broken and the curtain is wired into the Epson safety latch circuitry. The process of restarting the motors and bringing the system back on-line takes 5 to 10 seconds.

Wiring the light curtain into the Epson as a digital input allows the Epson software to pause both robots when the light curtain is broken without shutting off the robot motors. If the motors do not shut off, the robots can begin moving again in less than one second after the light curtain is cleared. However, there was a safety

concern that if the Epson digital input/output circuitry became inactive (accidentally unplugged, for example) then the robots may not stop when the light curtain is broken. As a continuous check on the input circuitry, a hardwired watchdog timer was added. The output of the watchdog timer is connected into the Epson's safety latch circuitry and if the watchdog timer trips, then the robots will stop moving and their motors will shut off. The watchdog timer's reset input is connected to an Epson digital output. While the Epson software is running, the software sends a signal to reset the watchdog once per second. If the watchdog does not receive the reset (from the Epson digital output), then the watchdog timer will trip.

When the software starts, there is an initialization routine that runs to check the watchdog and light curtain circuitry. During that routine the operator is instructed to break the light curtain beam. If the software does not recognize the light curtain being broken, then the software stops and does not allow the processing of explants. If an emergency occurs with the operation of the automation all equipment can be turned off using the emergency stop.

3.12 Operator Workflow

These are the operations an operator must follow in order to prepare the system at the beginning of each day:

- Gripper on Robot B is hand screwed into position.
- A vial containing 70% ethanol is placed on the deck and the glass bead sterilizer is turned on.
- Sterile tubes are installed on the peristaltic pumps as appropriate for the type and number of *Agrobacterium* solutions in use.
- Cocultivation media plates are stacked in each Petri dish dispenser as appropriate for the type and number of *Agrobacterium* solutions in use.
- Waste containers for liquid *Agrobacterium* waste and solid waste are placed in their respective positions
- *Agrobacterium* solution(s) (up to six different types) are positioned under the peristaltic pump panel with tubes inserted into them.

The computer user interface (Fig. 31) is set-up by assigning pumps to their respective A*grobacterium* solution based on experiment plan or based on how the cocultivation Petri dishes and *Agrobacterium* solution are sequentially placed for a run.

The software is initialized and Petri dishes of 30 explants are presented to the robot by an operator when the delivery station status indicates by turning green that it is active. From the point when the Petri dish is filled with *Agrobacterium* and placed on the shaker till the point when Robot B transfers the explants on cocultivation media, the real-time position of the Petri dish with explants is shown on the operator form which is indicated as the shaker plate position and Robot B status boxes, respectively. As plates of explants are delivered, they are picked up by the operator. The sequence of steps is illustrated in Fig. 32.

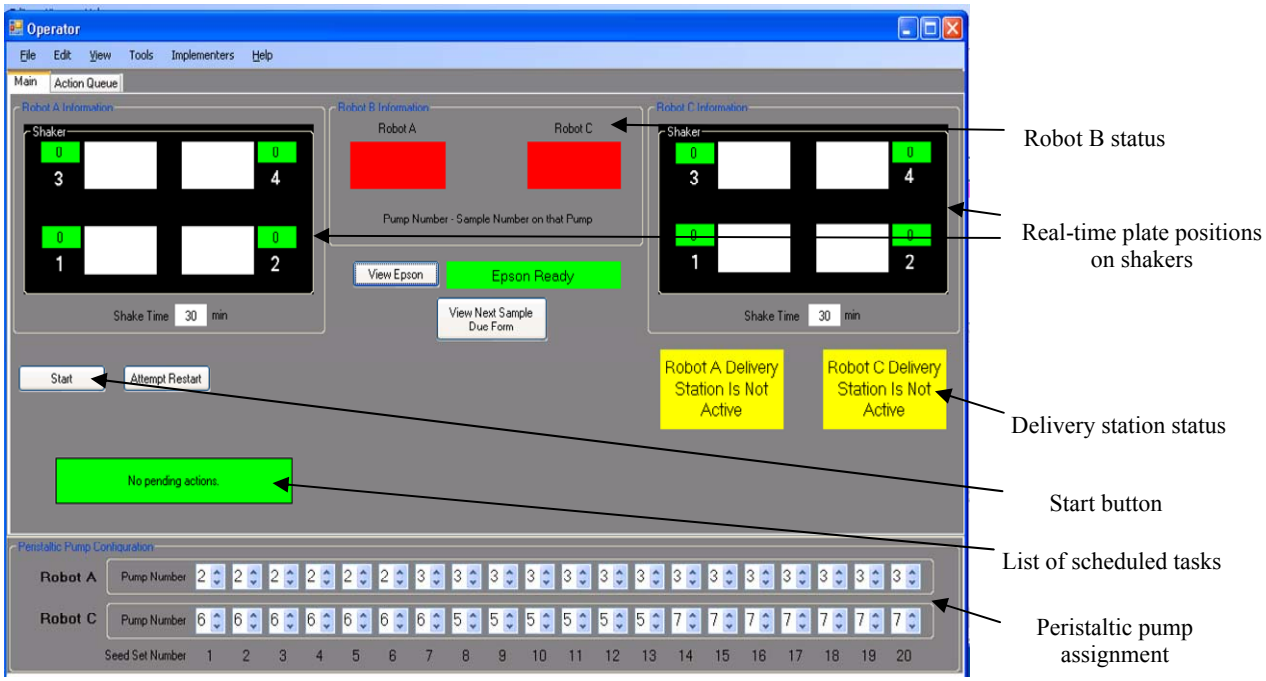

Fig. 31 Screen shot of operator form used to interface between the operator and machine.

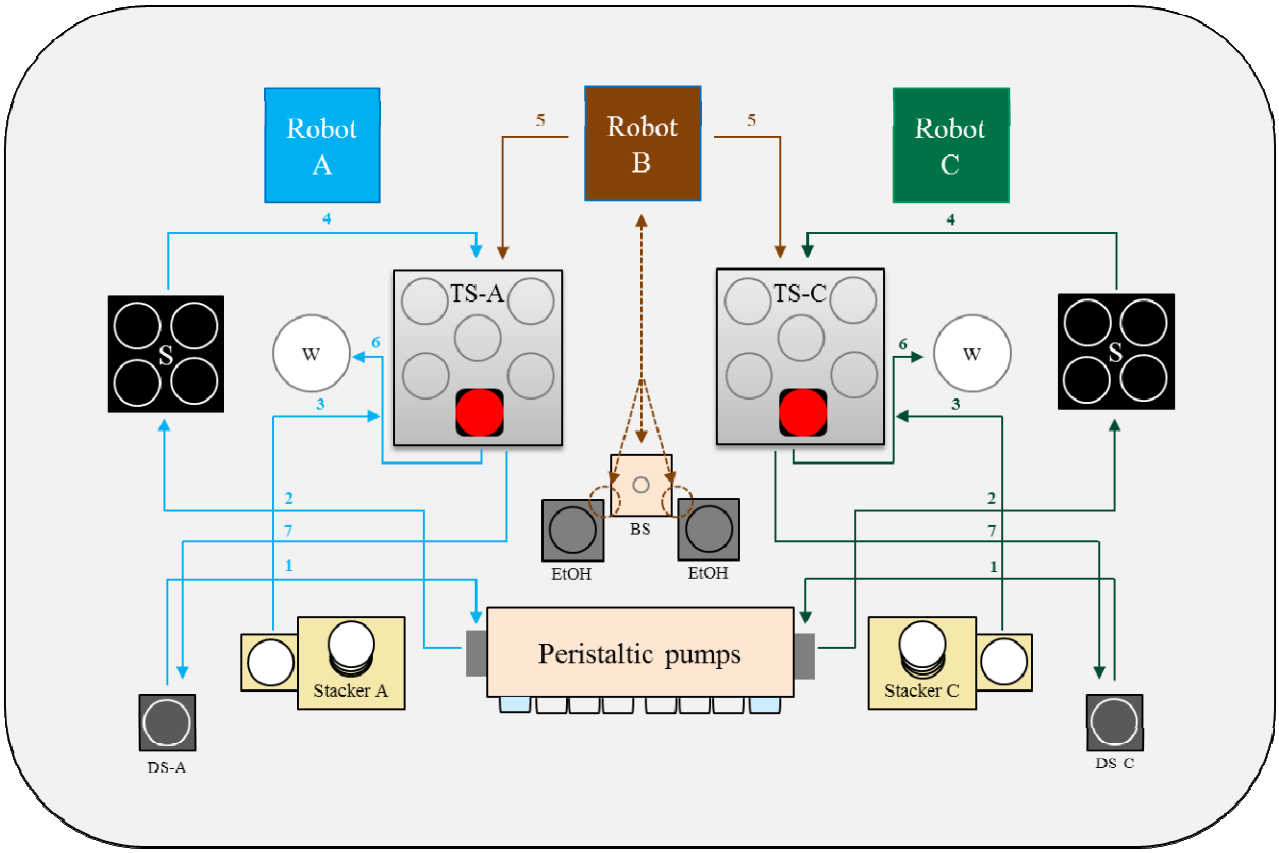

Fig. 32 Work flow diagram: 1) Transfer infection plate of 30 explants to the peristaltic pump and addition of *Agrobacterium*, 2) Transfer of filled infection plate to the shaker followed by 30min of agitation, 3) Movement of 5 cocultivation plates from the

plate stacker to the transfer station followed by removal of the lid and setting of the bottom of the plate on top of the lid, 4) Transfer the infection plate from shaker to the transfer station, 5) Transfer of explants from the infection plate to the cocultivation plates, 6) Disposal the infection plate, 7) Placement of the lids back on the cocultivation plates and transfer of the plates to the plate pickup and drop off station for the operator to collect. S – Shaker, W- Waste disposal container, TS-A & C – Transfer station A & C, BS- Bead sterilizer, EtOH – Vials for 70% Ethanol, DS-A & C – Delivery station A & C

The system was validated for speed (the amount of time it took to process one Petri dish of 30 explants) and traceability (the ability of the equipment to switch between up to six different *Agrobacterium* solutions without error). The speed at which Table B was able to complete a run averaged about 35 minutes. One of the significant challenges to be addressed during design of this equipment was that of material traceability since up to six different *Agrobacterium* treatments could be run in a single operation and a single point mix-up in one of the tasks could result in compromising the entire operation. Several changes were made to the user-interface to avoid such mix-ups and allow the operator to recover if a mistake were to happen. Some of the enhancements include a) the addition of status boxes (shaker and Robot B) showing real-time status of Petri dishes and their positions, b) a list of scheduled tasks and c) a Next Sample due form indicating the time (in seconds) left to place a new petri plate of explants on the delivery station and warning when not to place a plate. These enhancements ensured that even if a mix-up were to happen, the operator would know exactly the completed and to-be-completed tasks and would be able to recover the system without compromising the entire operation.

4. Conclusions

The soybean transformation process currently being used in the West Lafayette Trait Product Development site is a proprietary and highly efficient transformation system that has several steps amenable to the implementation of an automated solution. To that end, equipment was developed for the automation of two steps in this transformation process:

- The trimming of the embryonic axis and bisecting of the seed into two seed halves, described in a separate publication [2]
- *Agrobacterium* infection of the explants and placing infected explants into Petri dishes containing growth media.

The second system has been described in this report. It is designed with: three robotic arms (two for manipulating plates and one for transferring explants), two shakers, peristaltic pumps (for transferring *Agrobacterium*), plate stackers (for holding co-cultivation plates), a method for visually locating seeds in plates, and a method for software scheduling multiple infections. The automation reduces the need for direct handling of *Agrobacterium* cultures, and reduces man-hours needed for the infection process.

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