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Design and Use of Automation for Soybean Transformation (Part 1): Preparation of Soybean Explants

S. Chennareddy[1], L. McCarty[2], T. Cicak[1], G. Anthony[1], T. Parsons[2], E. Gee[3], K. Armstrong [4], P. Morabito[2], and R. Sarria[4]

1. Dow AgroSciences, West Lafayette, Indiana, USA

2. Dow Chemical, Midland, Michigan, USA

3. Dow Chemical, Collegeville, Pennsylvania, USA

4. Dow AgroSciences, Indianapolis, Indiana, USA

Abstract: This article reports on the first robotic system to prepare soybean explants for use in *Agrobacterium*-mediated transformation for industrial production of transgenic events. It is one of the most critical and labor intensive steps of the process requiring highly skilled technicians to perform repetitive work with sharp hand held tools. To deploy on a large scale, it required many such technicians that resulted in high intra- and inter-experimental variability in quality. Automation was developed for trimming the embryonic axis of imbibed seed and bisecting the seed into two halves to prepare two split seed explants. Industrial style, six-axis articulated robotic arms fitted with machine vision capability picked hydrated seeds from Petri dishes, oriented them based on the location of the hilum, and then placed the oriented seed onto a cutting device for embryo trimming and bisection. Automation of these steps resulted in reduction of man-hours required to perform the activity, added flexibility for experiment initiation, increased operator's safety by removing the need to use scalpels, and reduced the risk of ergonomic harm. The automated process improved the quality of experiments through reduction of intra-experimental variability by about 2.5 fold, and thereby improved efficiency of transgenic plant production.

Key words: automation, soybean, *Agrobacterium* infection, explant preparation, seed imaging, seed orientation, seed cutting

1. Introduction

Soybean (*Glycine max (L.) Merr.*) is a species of legume native to East Asia, widely grown for its edible bean, and classified as an oil seed crop. The soybean is economically the most important bean in the world, providing vegetable protein for millions of people and ingredients for hundreds of chemical products. Plant biotechnology involves the introduction of trait genes into plant tissues and generating stable transgenic plants through tissue culture. However, this process is expensive as it is labor intensive and requires specialized environmental controls throughout the numerous developmental stages, thus limiting larger scale deployment. Automation of all or some of the stages of tissue culture is envisaged as a way of reducing the manual handling of tissues. Economic analyses of the organogenesis and embryogenesis methods are covered in detail by Chu [2] and Cervelli and Senaratna [1]. 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. 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

Corresponding author: Lyle McCarty, Researcher, research areas/interests: robotics, automation, high throughput. E-mail: lmccarty@dow.com.

instruments or equipment. In recent years, there has been an extensive research 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 [3, 8]. 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. Reasons for this could be:

- The systems are not cost effective compared with manual methods (often caused by expensive components and slow rates of production).
- The 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 in Dow AgroSciences [9] resulted in transformation frequencies of about 15% and has been used for high throughput transgenic production to support different projects ranging from enabling technology testing and new capability development to trait discovery/development. This protocol is amenable for automation. The protocol, as reported, involves preparing split seed explants from surface-sterilized seeds 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 minutes. Finally, the *Agrobacterium* treated half seeds are placed on solid co-cultivation media in Petri plates. The drawbacks of this manual method of explant preparation are

- 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 issues for researchers. (The manual method requires 4 hours of repetitive activity per person. This may have a significant ergonomic impact on the worker if an ergonomics safety program is not in place.)
- 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 that are not usually encountered with other forms of automation developed for non-tissue culture transplants (e.g., seedlings). Emphasis is placed on ensuring the biological and physiological needs of seed processing are met by the equipment design to make sure the equipment can deliver the consistency and quality expected from the process. Special focus was placed on:

- Imaging the seed.
- Orienting the seed.
- Identifying the hilum for placement of seed on a seed nest.
- Designing a cutter for trimming the embryonic axis.
- Cutting the seed into halves.

The automation of the steps described here will further increase efficiency towards industrialized

transgenic event production with increased capacity, consistency and reliability.

2. Materials and Methods

2.1 Structure of Soybean Seed and Split Seed Explants

Soybean seeds can vary in size and be a variety of colors including yellow, green, brown, black or a mottled combination, though most commercial varieties have brown or tan seeds. The seed coat of a mature soy seed is extremely hard and water resistant so that germ that is encased within the soy seed is protected. Mature soybean seed may be spherical in shape or may be flattened and elongated to various degrees depending on variety (Fig. 1). The seed is covered by a thick, tough, brownish protective coat. Cotyledons are the food storage organs. When the seeds are imbibed in water, the seed coat softens, the cotyledons expand and elongate, and the embryonic axis and hilum are prominently visible (Fig. 1). The hilum is a funicular scar on the seed coat located on the middle of one edge that marks the point at which the seed was attached via the funiculus to the ovary tissue.

The embryonic axis is curved and covered by two cotyledons in an area called the cotyledonary node. One end of the embryonic axis called the plumule lies embedded in between the cotyledons which bear two small leaves. The other end of the embryo axis is called the radicle and it protrudes out of the two cotyledons (Fig. 2). Part of the embryo axis, located between the radicle and cotyledonary node, is called the hypocotyl. Finally, the part between the cotyledonary node and plumule is known as epicotyl (Fig. 2).

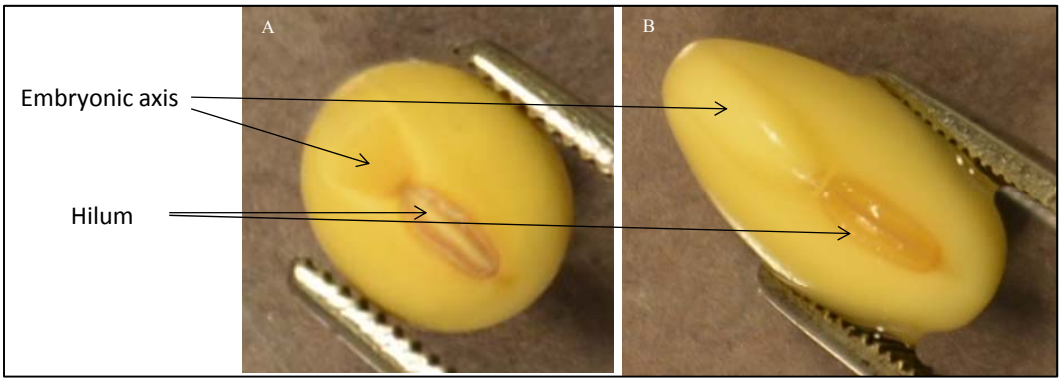


Fig. 1 Physical structure of mature dry soybean seed and 20 hour water imbibed seed, A) Mature dry soybean seed, B) 20 hr. water imbibed soybean seed.

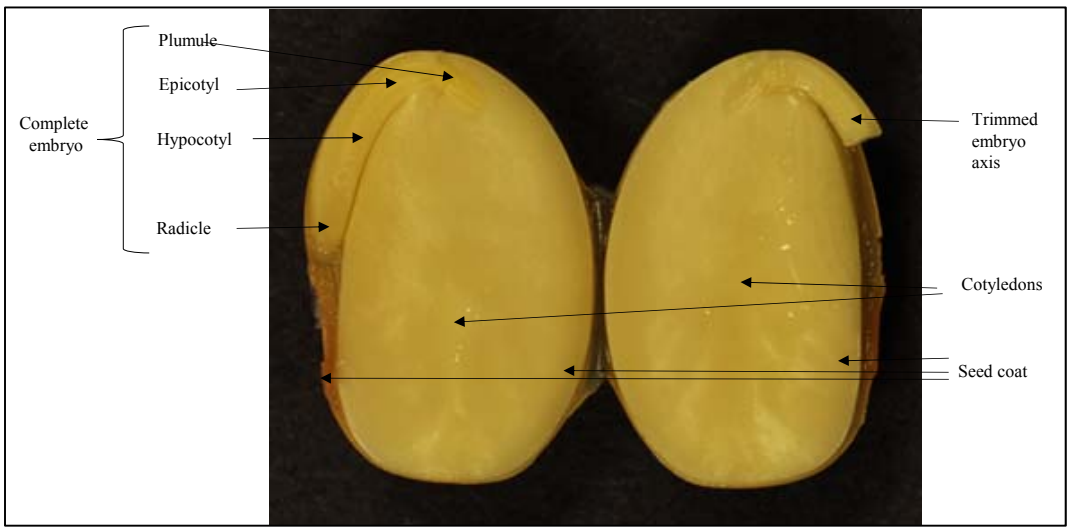


Fig. 2 Different parts of mature soybean split seed showing complete and trimmed embryonic axis. Split seed explants derived from one seed with intact embryo axis (left), and partially trimmed embryo axis (right 0).

2.2 Manual Method of Explant Preparation

Explants are prepared manually as described by Pareddy et al. [9]. Surface sterilized soybean seeds imbibed with water overnight (Fig. 3a) are held with forceps and ½-⅔ of the embryonic axis (found at the nodal end of the cotyledons (Fig. 3b) is trimmed using a #10 scalpel blade. A longitudinal cut (Fig. 3c, d) is made along the hilum to bisect the embryonic axis, separate the cotyledons, and remove the seed coat.

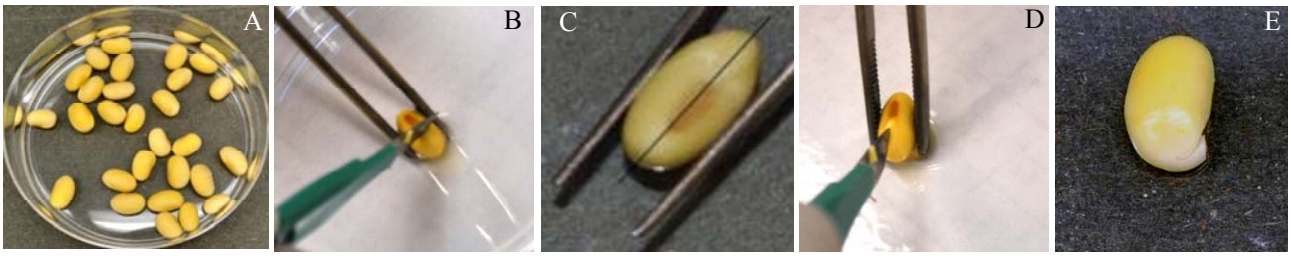


Fig. 3 Different steps of Manual Explant preparation, a) seed imbibed in water, b) trimming the embryonic axis, c) making a longitudinal cut along the embryo (the bump in the bean in the upper right corner) — the seed will be cut along the line drawn on the image, d) orienting the hilum side of the seed and bisecting the seed and e) split seed with partial embryonic axis.

2.3 Equipment and Special Tools Used for Design Implementation

The following is a list of equipment utilized for building the automated system. Supplier (vendor) and model numbers are provided for most items. However, for all items alternative suppliers would exist.

- Two 6-axis industrial robotic arms pick, orient, and place seeds on a cutting platform (seed nest). The robotic arm used is an Epson C3-A601S 6-axis articulated arm with RC620 controller (Fig. 4).
- An orifice style vacuum pump, PIAB Model M10L, is used to generate vacuum.
- A replaceable suction gripper (Viton black with white dot three bellows gripper, EMI Plastics Equipment, part #5195) with an aluminum support (Fig. 5A) is attached to the end of each arm.
- Seed delivery platform consisting of a polished stainless steel plate at the front end of the table (Fig. 6).
- Three 4" square red Advanced Illumination LED lights powered by CS420 controllers (http://www.advancedillumination.com, Model CB0404) (Fig. 7a).
- Lighted dome to evenly illuminate an object from all sides (Fig. 8).
- Cutting device for trimming and bisecting the seed consisting of (Fig. 10):
  - An "Injector Type" blade (Blade Type 27-151) from Razor Blade Company (http://www.razorbladeco.com), made from coated stainless steel, 0.010" thick, 1.5" long and 5/16" wide. Contains two oblong holes along the edge, providing a position for gripping.
  - Blade gripper fingers, detailed in Fig. 7. The blade gripper fingers were designed and built by Dow especially for the chosen injector blade.
  - The blade gripper fingers are mounted onto an SMC Model MHZ2-20C1-M9PZ gripper.
  - The SMC gripper is mounted onto a Gimatic RT25 90 degree rotary stage for rotating the gripper and attached blade.
- Two Aerotech (www.aerotech.com) mechanical linear motor stages:
  - A model ANT95-50-L linear stage with 50 mm of travel (designated the "X-Stage" because its line of travel is in the same direction as the X direction of the 6-axis

robot's designated axes) was used to move the cutting blade into and away from the seed nest.

- A model ANT95-3-V with 3 mm of travel (designated the "Z-Stage") positions the blade vertically to properly bisect the embryo.

- Cameras: A total of six cameras are required for this dual robot system.
- Bead sterilizers: To maintain the sterility of the gripper attached to the robotic arms, two InoTech BioScience Steri 250 dry glass bead sterilizer are used, one for each robot. 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 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.
- Bio-Safety Cabinet (BioPROtect III, The Baker Company, http://www.bakerco.com). All the hardware components are installed on a customized table (designed by Automation By Design co.) and the table is located inside a large Baker biosafety cabinet (164 ft^3 interior, 76"H x 102"W × 36.5"F-B, nominal) to maintain sterility. The BioPROtect III offers clean air and containment enclosures designed for high-volume robotic and automated equipment applications, while allowing access to the interior work area through combination double doors or an 8" sash opening. Its modular construction also allows disassembly for shipping, delivery and placement.

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 biological properties of the materials (explants) without compromising their viability and integrity. The system must also maintain the biological principles of the transformation method previously developed by Pareddy et al. [9]. The hardware components listed above are represented in the CAD drawings shown in Fig. 4. The subsequent sections detail the design and function of all of these components and how custom equipment as well as commercially available technologies were adapted to build the system described here [5-7].

3.1 Automated Method of Explant Preparation

The automated system design consists of the following hardware components, depicted in Fig. 4. All the hardware components are installed on a single customized table and housed inside a large bio-safety cabinet to maintain sterility.

- Two 6-axis robotic arms for picking up, orienting, and placing seeds on a cutting platform (seed nest);
- Seed delivery platform;
- Controlled lighting dome light with cameras for seed visualization;
- Cutter for trimming and bisecting the seed;
- Glass bead bath for sterilization.

Electronic controllers located underneath the table top.

3.1.1 Robotic Arms and Vacuum Gripper

The automated system utilizes two robotic arms. However, each arm performs the same function, so other similar systems could be built using only a single arm, or even three or more arms depending upon the required throughput. Two robotic arms provide the throughput required for the current application.

The primary function of the robotic arm (Fig. 5A), is to move an uncut seed to different functional positions (pickup, imaging, cutting, and releasing seed). The robotic arms pick and release seeds using vacuum and a replaceable suction gripper. The gripper is attached to a spring loaded suspension. The spring resistance is low enough that the gripper is able pick a seed without crushing it. The purpose of the circular black pad is to reduce the effect of room lights and shadowing during various stages of the imaging process. The dimensions of the circular black pad are ¼" thick by 3" diameter.

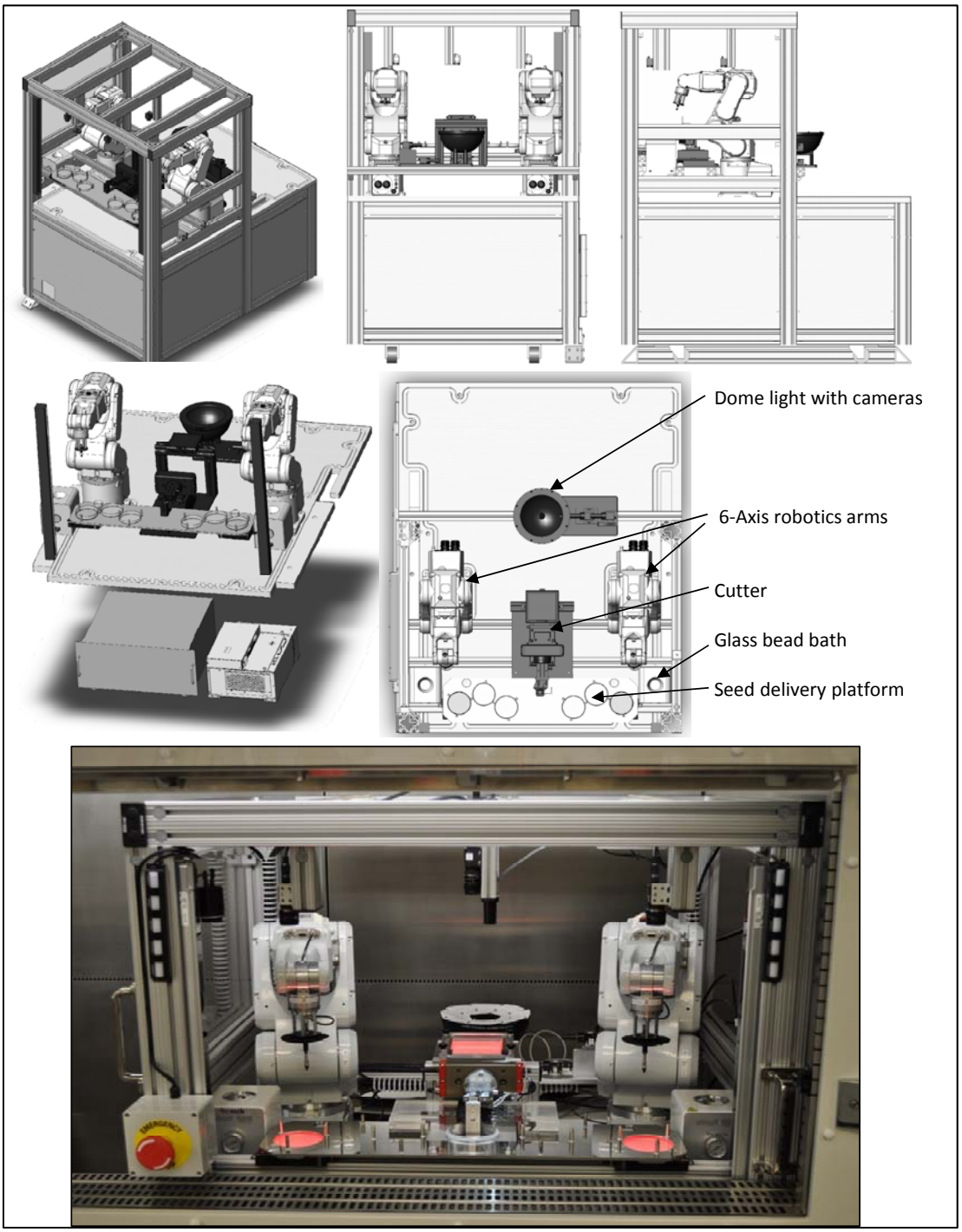


Fig. 4 CAD drawings and actual image of the automated soybean seed cutter system.

A key property of the Viton suction gripper (Fig. 5B) is a temperature rating of 446°F (230°C) allowing for heat sterilization in a bead bath. Occasionally the folds of the bellows gripper do become stuck together during sterilization. There is an automated routine designed to separate the bellows by vacuuming the gripper to a flat, sterilized surface and pulling away from the surface in 1 mm increments. A second key property of the Viton gripper (Fig. 5B), is that it is black, providing good contrast to a white seed as the seed is imaged during the process. Another important property is that it has a 65° durometer (low hardness), which combined with the

triple bellows design allows the gripper to positively contour and vacuum to the seed without damaging it.

3.1.2 Seed Delivery Platform

The seed delivery platform (Fig. 6) is a polished stainless steel plate at the front end of the table containing the following positions (for each robot):

- **Seed Delivery** position where a Petri dish of uncut seeds is placed. This position utilizes background **4" red LED under plate lighting**, described in the next section.
- **Seed drop after cutting** position where the robot drops cut seeds.
- **Dirty cutting blade drop** position where an empty Petri dish is placed to receive dirty razor blades.
- **Conical flask for ethanol** is a sterilizing position where the robot dips the gripper into a tube containing 70% Ethanol.

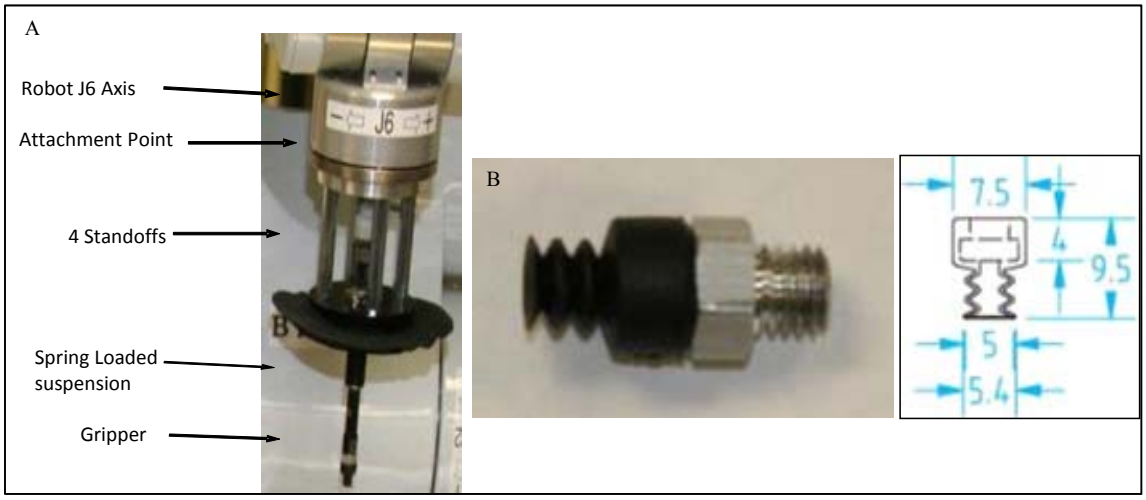


Fig. 5 A) Image of gripper assembly mounted to the robot. B) Viton black with white dot gripper.

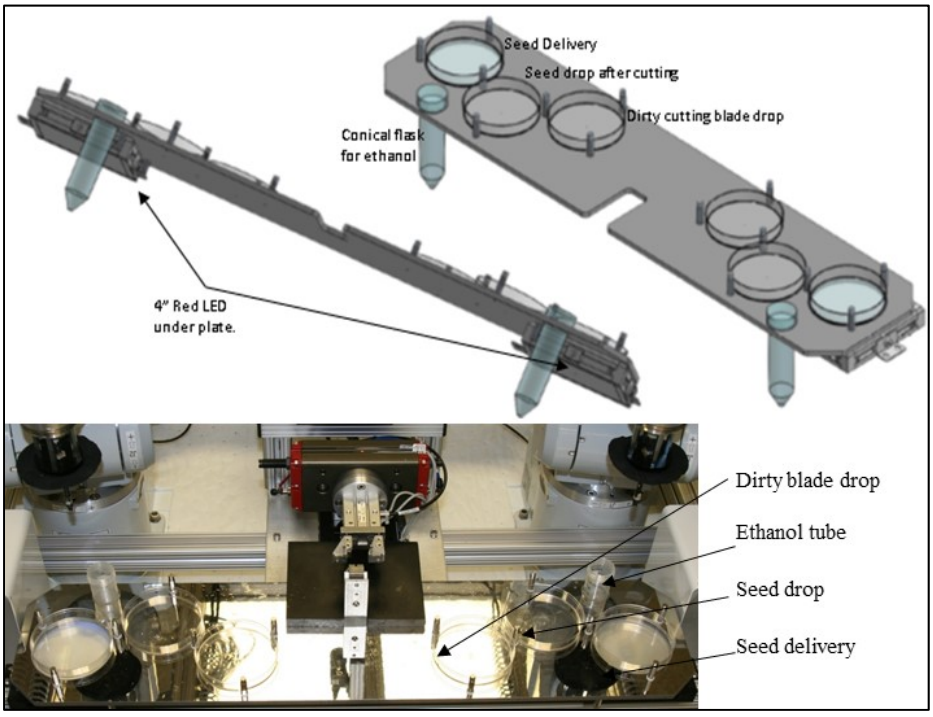


Fig. 6 Image of the polished stainless steel delivery plate.

3.1.3 LED Lighted Platforms

The system utilizes three 4" square red LED lights (Fig. 7A). Red light provides for better contrast against the white seed than a white light LED. Two of the three LED provide the same functionality — back illuminating the dish of uncut, imbibed seeds for pickup by the vacuum gripper at the positions designated for "Seed Delivery" in Fig. 6. The square LED surface is protected by a round piece of glass matching the diameter of the Petri dish delivering the seeds. Fig. 7A shows the gripper picking a seed (without the round protecting glass in place).

The third square LED (Fig. 7B) is mounted between the two robotic arms and behind the cutting device. The purpose of this LED is to back illuminate sterilized razor blades for picking by the robot vacuum gripper. These razor blades are used to bisect the seeds. The LED is covered by a structure constructed of three ½" Plexiglas sheets. The bottom sheet is 7" square on the outside with a 5" square cut out in the center, large enough to slip over the red LED. The middle sheet is 7" square and solid. Its purpose is to cover the LED and prevent the razor blades from scratching the LED surface. The top sheet, 6" square on the outside with a 4" square cutout contains the blades within the 4" lighted area. The three sheets are held together using thumb screws in each corner (Fig. 7B). The thumb screws allow for easy disassembly of the construction for cleaning.

3.1.4 Lighted Dome

An Advanced Illumination 8" diameter white LED dome light (Fig. 8B) illuminates the seed during a set of machine vision routines designed to provide to the robot the coordinates required to properly align the seed into the seed cutter. The purpose of a dome light is to evenly illuminate an object from all sides. However, in this application the white seed would be difficult to identify against a white background. Therefore a black, anodized aluminum block is mounted inside the dome light in a position where the camera would view the seed against the black background (Fig. 8A).

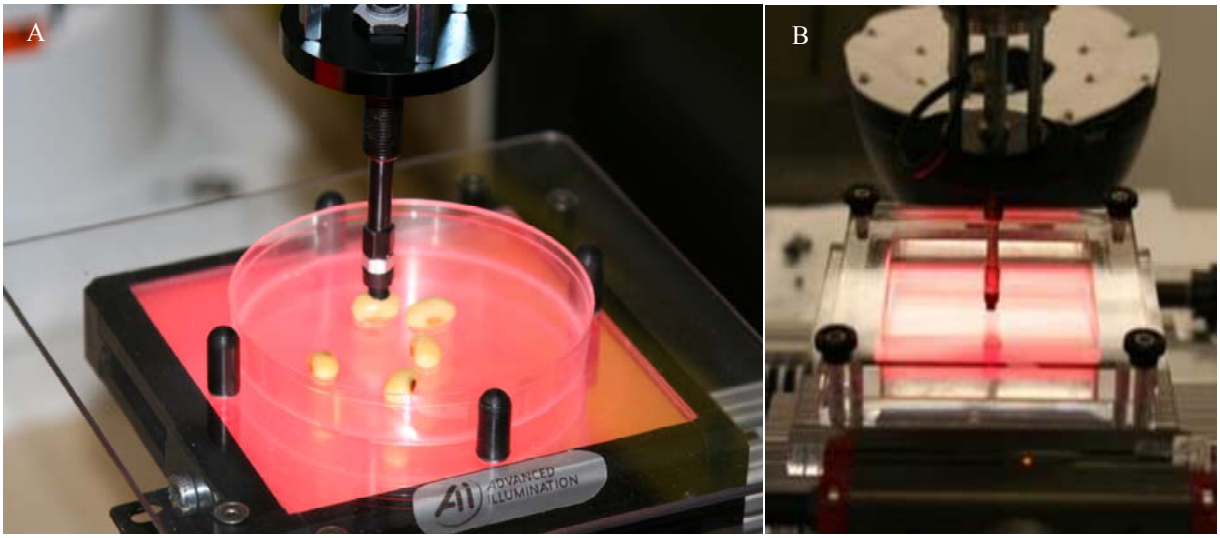


Fig. 7 A) Hydrated seeds in a Petri dish under a 4" square red LED flat light surface and suction gripper picking up located seed. B) Red LED lighting with Plexiglas covering installed for placing sterilized blades.

To reduce the possibility of room lights interfering with the vision analysis the top of the dome light is covered with a 6.5" diameter sheet of black flexible corrugated plastic sheet. The purpose of the corrugation was to reduce reflected light inside the dome from directly reflecting back down onto the top of the seed. This corrugated material was connected to a sheet of white Teflon wide enough to mount to the top surface of the dome light (Fig. 8B). A 1.25" hole was cut into the middle of the black corrugated material

and the Teflon for the robot to insert the seed into the dome without touching the silicon.

This particular dome light consists of 20 bright LED lights mounted around the top, inside edge of the dome. One unwanted property of this type of dome light is that reflections from individual lights can be seen on objects inserted into the dome. To eliminate these reflections a piece of 8.5" × 11" of ordinary white, unlined printer paper was cut into a circle and mounted between the lights and the inside of the dome to act as a diffuser.

The two robotic arms share a single dome light imaging system. The robot places the seed into lighted dome where it is evenly lit from all directions (Fig. 8A). As the seeds are illuminated, they tend to appear mostly white in images depending upon the dome light intensity. Cameras mounted outside of the dome light will snap images of the seed, and vision analysis software determines the side to side and front to back tilt angles of the seed. The system developed allows identification of the face and bottom of the seed. The bottom of the seed is imaged by using a 45 degree mirror placed below the dome light. In this system the camera is placed at the bottom of the dome light and pointed directly at the mirror to image the bottom of the seed. Holes cut into the bottom and the side of the dome light allow the cameras to view the seed from multiple positions (Fig. 9).

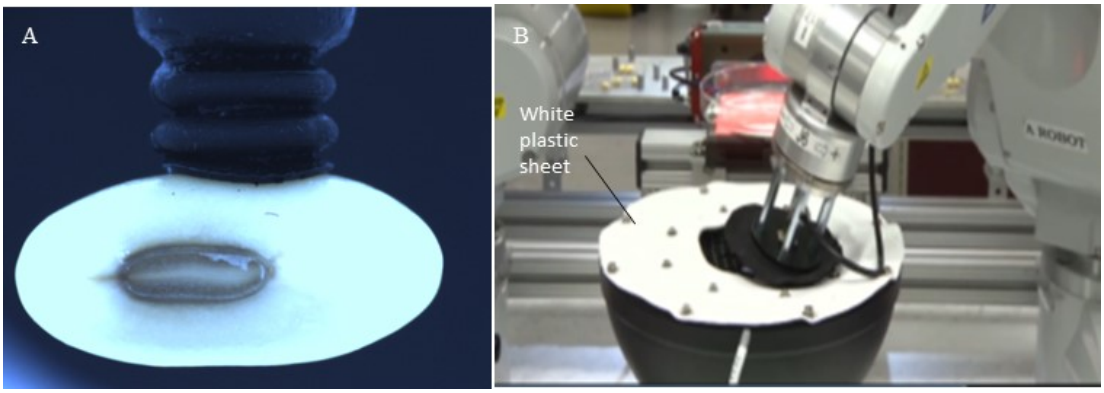


Fig. 8 A) Robot placing the suctioned seed, still held onto the gripper, into a light dome. B) Image of robot positioning seeds inside of light dome.

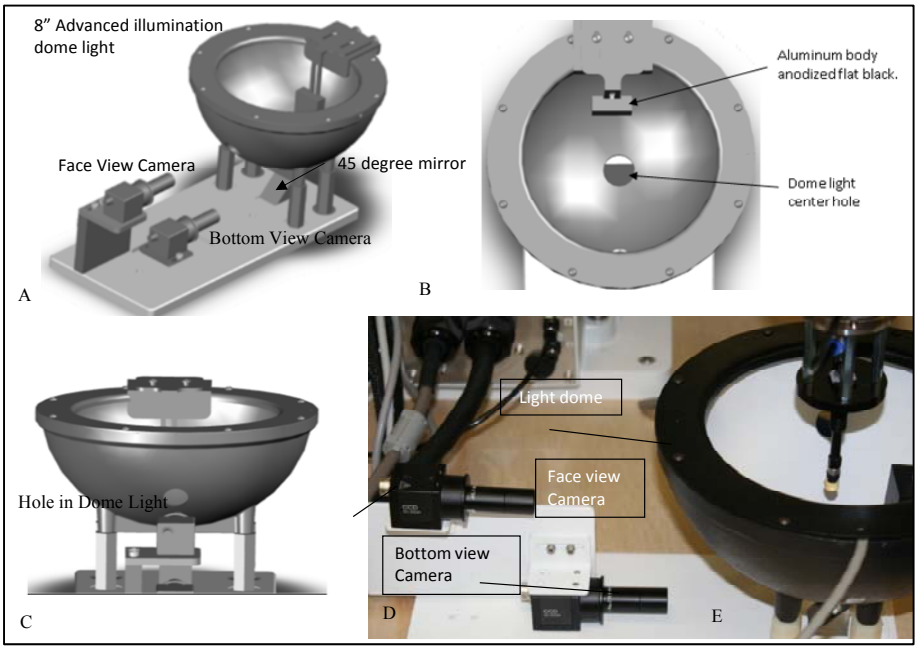


Fig. 9 Images (A-E) detailing the position of the two cameras mounted to image a seed positioned into the 8" dome light.

3.1.5 Cameras

A total of six cameras are required for this dual robot system. Two cameras are mounted above the two Seed Delivery positions (Fig. 4). One camera is mounted to image the blades (Fig. 4). Another camera is mounted above the cutter to visually inspect the cutting blade and assure it is properly seated into the gripper fingers. A high intensity pen light style LED is mounted next to that camera to illuminate the cutting area.

Two additional cameras are mounted to image the seed inside of the dome light (Fig. 9). The first camera, the "face view camera" (Fig. 9C, and E), views the seed perpendicular to the gripper axis through a hole drilled into the dome's side (Fig. 9B). The second camera, the "bottom view camera" (Figs. 9A, C and E), images the side of the seed opposite to the gripper using a 45 degree mirror (Fig. 9A) mounted below the dome light center hole (Fig. 9D).

3.1.6 Cutting Device for Trimming and Bisecting the Seed

The two robotic arms share a single cutter device (Fig. 10). This cutter is designed to both trim the embryonic node (blade vertical cut) and bisect the embryo (blade horizontal cut). After sterilization, 1 to 10 blades are placed onto the 4" red LED (Fig. 7B). A camera mounted above the LED images the blades and identifies one for the robot to pick using the Viton vacuum gripper. The robot places the blade into a set of gripper fingers (Fig. 11A) designed to hold the blade by the two oblong holes. With the gripper closed, the blade slips over the gripper fingers. The gripper opens and the fingers catch the blade and hold it rigid in position (Fig. 11B).

The machine vision software uses the Epson Geometric object to locate blades (Fig. 12A). The Geometric object compares a real time image with a stored image of the desired object and if the stored image is located in the real time image then the software passes the coordinates of the object in the image to the robot for picking the object. For the first pass through the software, the object's acceptance property is set low to 400 (out of 1000). If there are any blades in the image, the Geometric object will

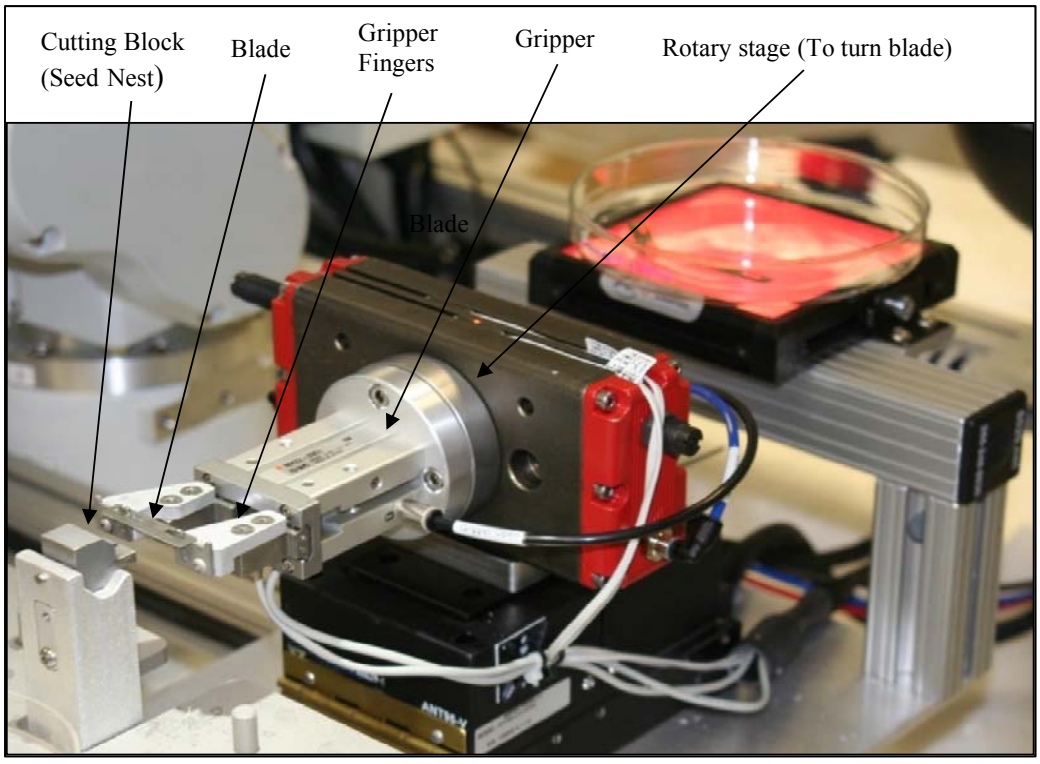


Fig. 10 Image detailing the components of the soybean seed cutter.

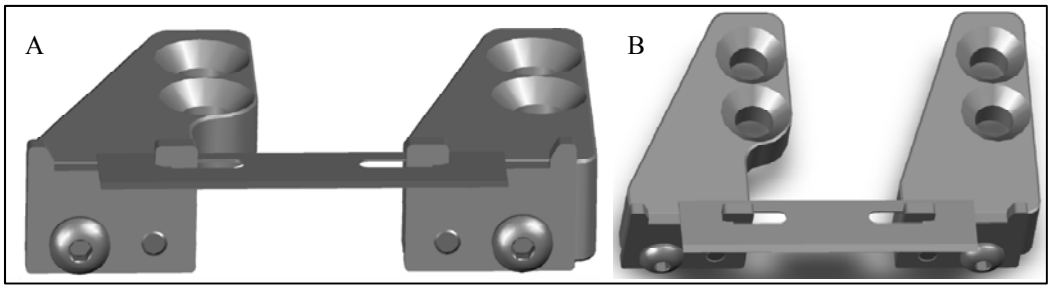


Fig. 11 Set of gripper fingers. A) Front view of gripper fingers securing a cutting blade. B) Top view of gripper fingers securing a cutting blade.

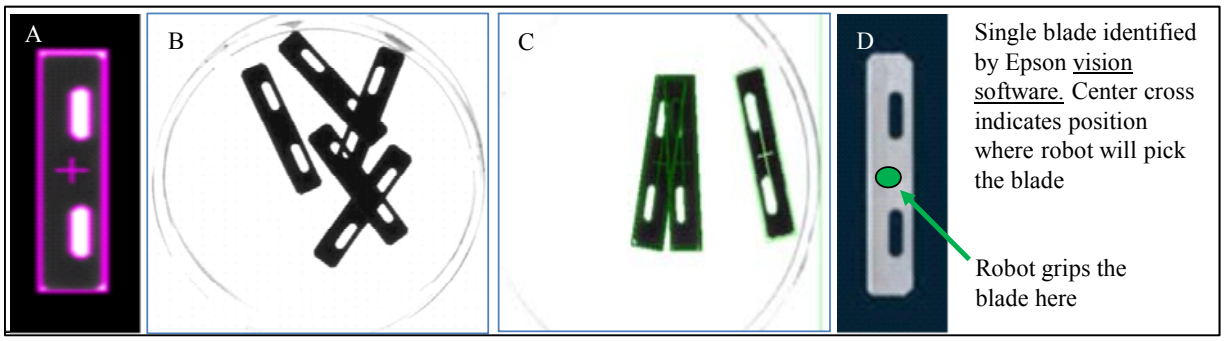


Fig. 12 A) Image of blade used to teach the geometric blade object. B) Showing overlapping blades on the red LED used by the robot to pick up a blade for insertion into the cutter. C) Showing where the robot will grip a cutting blade.

locate at least one blade. However, because the acceptance level is low it is possible that the object will identify a set of overlapping blades. Next, the Geometric object's acceptance property is set high to 950 (out of 1000), assuring that only single blades will be identified. If two blades overlap, even by a single pixel, the Geometric object will not return the location of either blade. If the Geometric object does not locate a single blade, the software resets the Geometric acceptance back to 400, and locates what it now knows is a set of overlapping blades (Fig. 12B), not a single blade. The software instructs the robot to vacuum the group of blades at the location identified as the geometric center of the overlapping blades. The robot raises the blade(s) up 1" above the surface and moves them about 1" away from where the blades were picked. The robot releases vacuum and drops the blades back onto the red LED, scattering them (Fig. 12C). The goal is to separate at least one blade from the others. The software will run through the routine of locating blades and dropping them until either a single blade is located (acceptance = 950), or 12 unsuccessful attempts were made to locate a single blade (Fig. 12D). Once a single blade is identified, the robot will pick up the blade from the red LED in the correct orientation to insert the blade correctly into the cutter's gripper fingers (Fig. 10). After cutting about 120 seed, the used blade will be swapped out for new blade.

Once as seed has been imaged in the light dome and the orientation of its axes is known to the robot, it is moved to the cutting block (seed nest). The robotic arm with attached Viton suction cup stabilizes the seed while the dual axis stage trims and bisects the seed (Fig. 13 A and B). The seed nest is manufactured from steel and mounted to a magnetized pedestal. The pedestal and seed nest are contoured in a manner that helps to secure the seed nest to the pedestal. Since the pedestal is only held in position with a magnet, the pedestal can be easily removed for sterilization. A heat sterilized seed nest is used during every run.

3.1.7 Bead Sterilizers

To sterilize the gripper, the robot moves the gripper into the ethanol and runs a routine where the gripper moves up, down and side-to-side quickly for 5 seconds,

then moves from ethanol into the glass bead sterilizer, and runs a routine to move the gripper up and down quickly inside of the sterilizer (Fig. 14).

3.1.8 Software

The software is a combination of two programs written specifically for this instrumentation. Both programs run simultaneously on the Epson RC620+ controller.

- An operator interface written in Microsoft's Visual Studio Visual Basic programming language.
- Epson robot machine code, written in Epson's SPEL+ programming language. This software controls the robots, cameras, lights, and cutter.

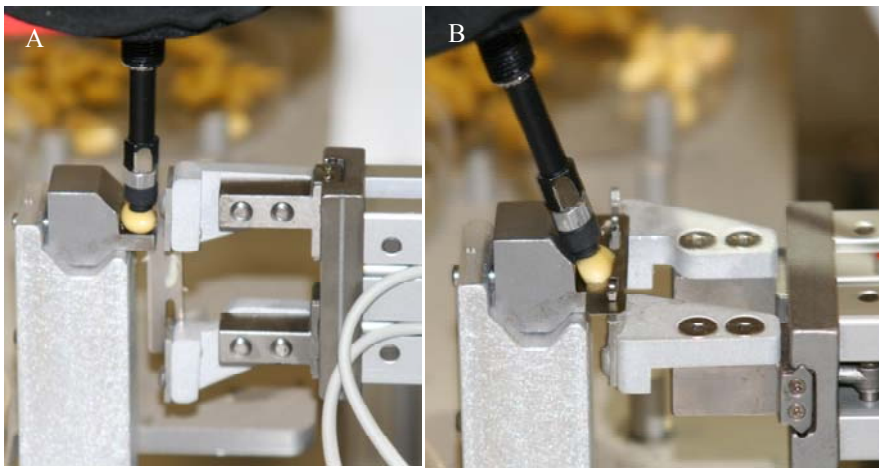


Fig. 13 A) Image of cutter trimming the embryonic node of soybean seed. B) Image of cutter in horizontal position for bisecting seed.

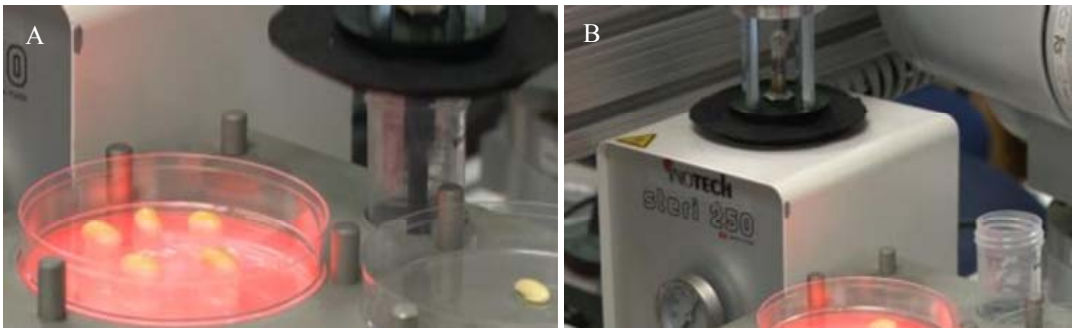


Fig. 14 A) Robot placing gripper into flask of ethanol. B) Robot gripper inside of Inotech Steri 250 glass bead sterilizer.

3.1.9 Sequence of Imaging for Seed Picking

A monolayer of seeds (Fig. 15A) is presented to the robot by the operator onto a platform lighted from underneath with red LED (Fig. 15B). A camera mounted above the red LED images the seeds. The camera is calibrated to return the seed coordinates with respect to the robots vacuum gripper frame of reference to provide the coordinates of a single seed to the robot.

To accurately bisect a seed, the robot must be able to position the seed into the cutter with the hilum directed towards the cutting blade. Therefore, it is crucial for the robot to vacuum the seed in a manner that the hilum is not underneath the vacuum cup. A property of a soybean seed that enables this to work is that a hydrated a soybean seed naturally develops a flat side, and when laid on its flat side the seed hilum is oriented parallel to its container, in this case a Petri dish (Fig. 16A). Thus when the robot gripper attaches to the flat side the hilum is not underneath the gripper, but is at a right angle to the gripper (Fig. 16B).

Another property of the soybean seed that proves useful is that for most (not all) seeds the side of the seed

containing the hilum is slightly straighter than the other side (Fig. 16C). The underlying machine vision software selecting the seed is based on an Epson Geometric vision object. As described earlier, the Geometric vision object uses pattern matching to locate the object. The pattern the machine vision software uses to compare against for picking the seed from the Petri dish has a distinct flat side (Fig. 16B). Therefore, when the machine vision software computes the angle for picking the seed from the dish, often it will be picked so that the hilum is already correctly oriented with respect to the gripper (Fig. 16D).

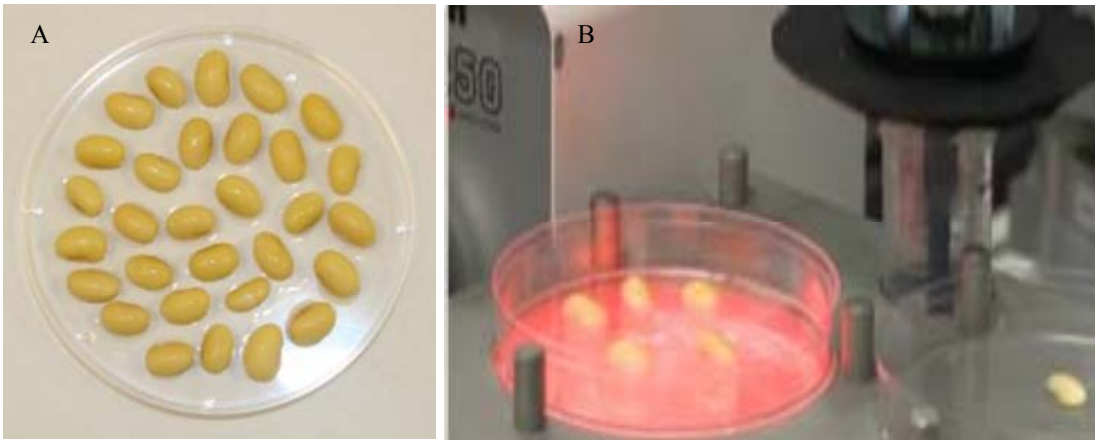


Fig. 15 A) A monolayer of seeds placed correctly in a Petri dish for cutting. B) Side image of seeds in Petri dish under LED lighting.

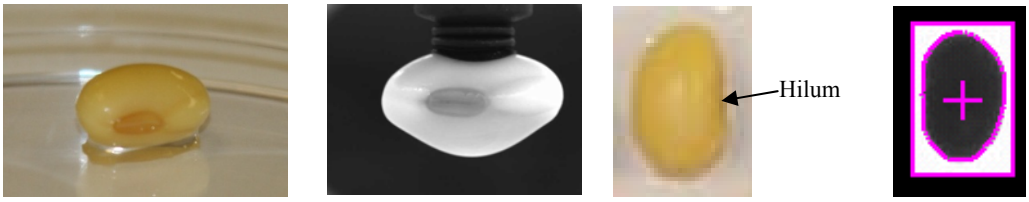


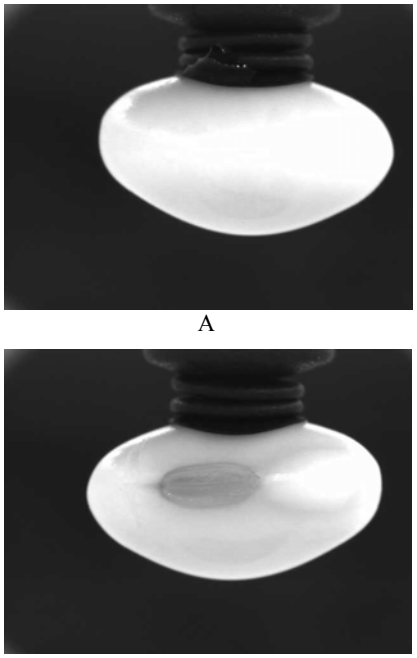
Fig. 16 A) Seed on Petri dish, hilum is parallel to dish. B) Seed attached to vacuum gripper, hilum perpendicular to gripper axis. C) Hilum is located on flatter side of seed. D) Seed model used to train Epson Geometric object.

3.1.10 Sequence of Imaging for Seed Alignment into Cutter

After picking the seed from the Petri dish delivery plate, the robot places the seed into the lighted dome and uses the two dome imaging cameras to calculate the correct angles for the robot to hold the seed in the cutter.

First, the seed hilum is identified (Fig. 17). If the Geometric pattern matching was successful for picking the seed (Section 3.1.9) the hilum will be found in the first image. If unsuccessful and the hilum is not identified in the first image, the robot will automatically rotate a seed 180 degrees for a second image. Most often the hilum is found in one of these two images. If it is not then the seed is discarded.

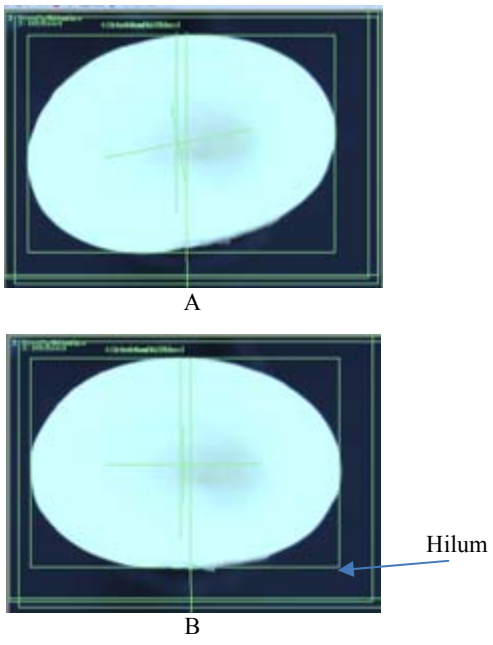
After locating the hilum, the machine vision software acquires an image of the seed opposite to the side vacuumed onto the gripper through the camera positioned to view the seed using the 45° mirror. After locating the seed, the software calculates the principal axis, determines the angle that the seed must rotate to orient the hilum (based on the flat edge of the seed) squarely towards the camera, and commands the robot to perform that rotation. Fig. 18 shows an image of the seed before and after rotation. In Fig. 18B, the principal axis of the seed is now rotated so as to be parallel with the bottom edge of the image and the hilum is also located at the bottom. This is the edge that will eventually be presented to the cutting blade.



A

B

Fig. 17 A) An image of a seed that was not correctly identified by the Geometric pattern matching, B) The same seed rotated 180o to reveal the hilum.



A

B

Fig. 18 A) Image of a seed before rotation, B) Image of seed after rotation, hilum aligned to bottom edge of image.

Next, the images collected by the dome camera are used to locate the edges of the seed (Fig. 19).

Now a series of machine vision objects called a "blob" are dropped along each end of the seed. The purpose of the blob is to locate the center of mass of the seed on each end. In Fig. 20 the red boxes outline the region of the seed ends that is being imaged and the blue dots represent the center of mass identified by the blob. A line is drawn between the center of mass at each end of the seed and the angle required to center the seed with respect to the bottom of the image is calculated. That angle is used by the robot to tilt the seed parallel to the bottom of the image. This is the angle required to align the embryo with the cutting blade so that the blade will correctly bisect the embryo. Fig. 20 shows a series of images collected during this process.

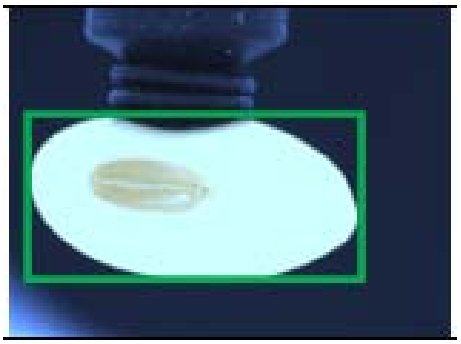


Fig. 19 Edges of seed are located.

In the next step, depicted in Fig. 21, the angle required to rotate the hilum's center of mass (green dots) to coincide with the center of mass of the two ends of the seed is calculated. The edges of the seed are located. On one edge a blob object is used to locate the center of mass of the edge of the seed (blue dots). Another blob object locates the center of mass of the hilum. Now the angle needed to rotate the seed either towards (to bring the hilum down from the top of the image) or away from (to bring the hilum up from the bottom of the image) the camera is calculated. The robot is instructed to rotate the hilum. In the images shown in Fig. 21, the robot gripper tilts toward the camera to rotate the hilum downward.

Note how in the previous image sets (Figs. 18-20) the seed is bright white against the black background. The embryo cannot be discerned. To locate the embryo the intensity of the dome light is reduced as can be seen in Fig. 21.

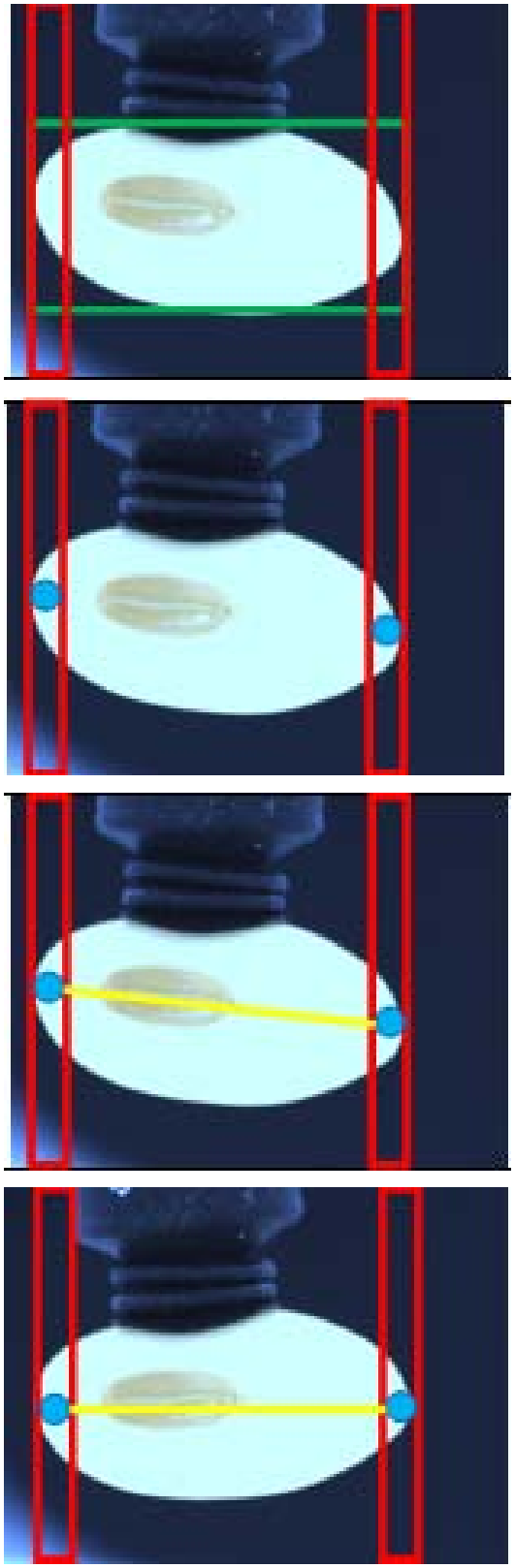


Fig. 20 A series of images depicting how the machine vision software identifies the edges of a seed and uses the center of mass at each edge to calculate the angle that the seed is tilted in the gripper.

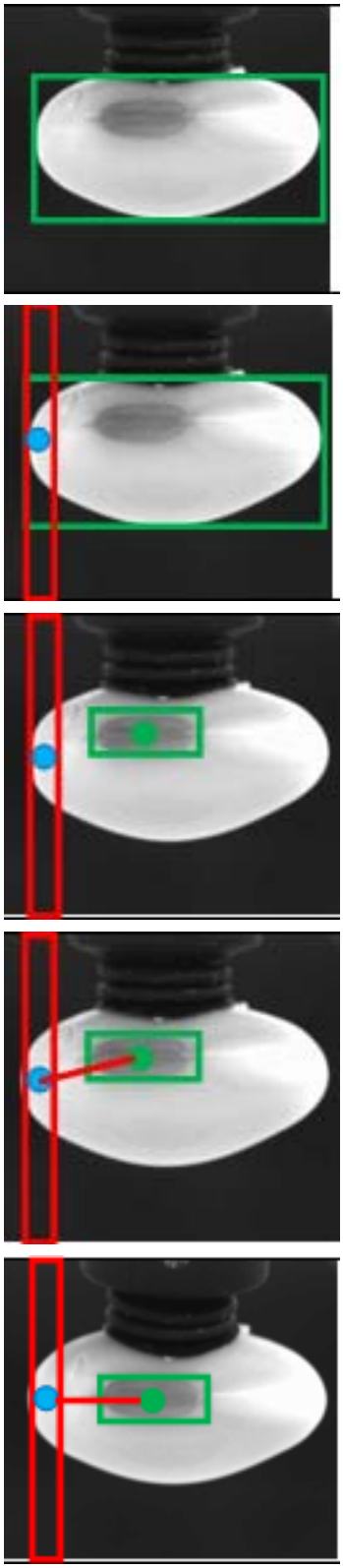


Fig. 21 A series of images depicting how the machine vision software identifies the edge and hilum of a seed and uses the center of mass at each edge to calculate the angle that the seed is tilted in the gripper.

Next, the embryo is located. Because the hilum tissue appears darker than the rest of the seed, in Fig. 21, it is clear to the human eye that the embryo is to the right of the hilum (viewing the image). This darker hilum tissue is used as a unique marker from which the location of the embryo can be inferred using the Epson machine vision Geometric object. The model taught for object matching is shown in Fig. 22. Note that the edge of the hilum is included in the model, on the right side of the image. The embryo is to the left of the hilum.

Including the hilum in the model is key. When the Geometric object compares an unknown seed image with the model (Fig. 22) and determines that the hilum is on the right, then the Geometric object will return a rotational value of 0, meaning that the image does not need to be rotated to match the model. However, if the Geometric object were to detect the hilum on the left side, then a rotational value of 180 is returned, indicating that the embryo is to the right of the hilum (in that image).

The next step in the image analysis required to properly place the seed into the cutter for embryo trimming is to determine where to trim the embryo with the vertical cut. The edge of the hilum is located. The edge of the seed where the embryo is located is determined. Then it is a simple matter to measure the number of pixels between the two edges and divide the result in half. The resulting number of pixels is equated to millimeters using a calibration table that determines the number of mm/pixel in the image. Fig. 23 shows an example of where the embryo will be vertically trimmed.

Seeds are naturally different sizes. It is important not to cut too far into the seed while performing the vertical embryonic node trim. Therefore the distance the cutting blade must travel to contact the embryo to trim it must be calculated. To perform this calculation the bottom of the seed is imaged and the leading edge of the seed at the position where the embryo will be trimmed is determined. Fig. 24A indicates the trim position. Fig. 24B is an image of the same seed on the side opposite to the gripper. The red line indicates the embryo trim location. At that position the software uses a vision "Edge" object to first detect the edge of the seed closest to the bottom of the image (Fig. 24C), then to detect the edge of the seed closest to the top of the image. The green line (Fig. 24D) is the width of the seed at the position where the seed will be trimmed.

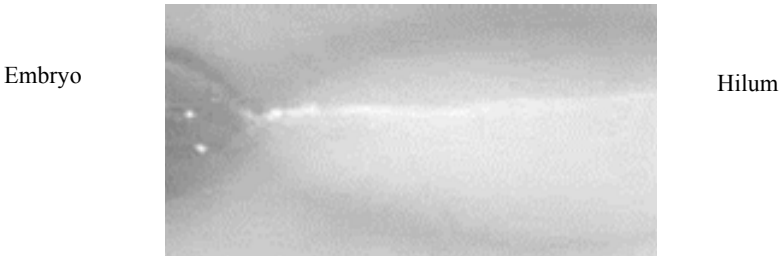


Fig. 22 An example of an image used to teach the Epson Geometric object about the shape of the embryo and its relationship to the hilum.

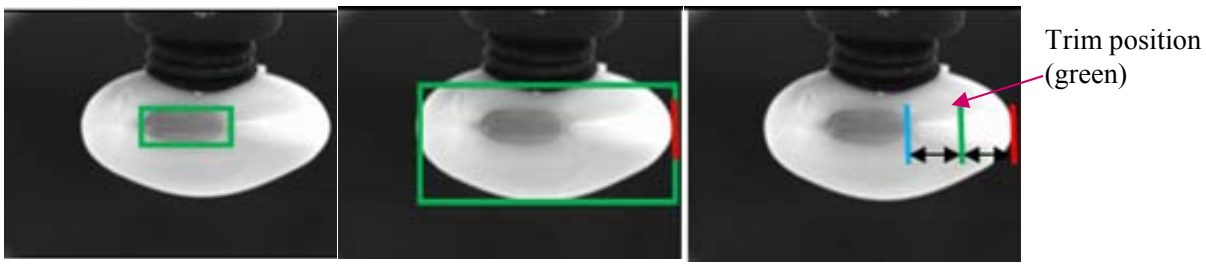


Fig 23 A series of images depicting where the embryo will be trimmed.

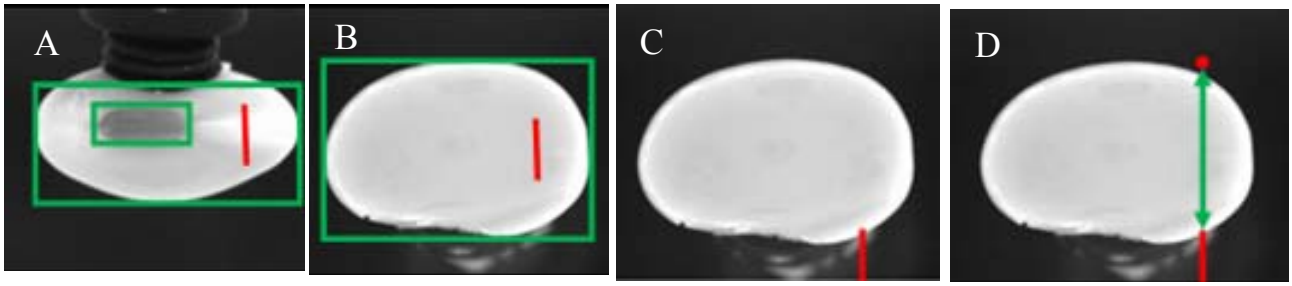


Fig. 24 The steps used by the Epson machine vision software to calculate the width of the seed at the position of the seed where the embryo will be trimmed.

The distance from the back wall of the seed nest to the leading edge of the blade is known (14.55 mm as seen in Fig. 25). Subtracting the width of the seed at the embryonic node trimming position (9.0 mm in Fig. 25) from the distance between the back of the seed nest and the leading edge of the blade calculates the distance the blade must travel to trim the embryonic node (5 mm). The blade will travel an additional 1.5 mm to trim the node.

At this point the software has all of the angles and distances needed to successfully position the seed into the cutter for trimming the embryonic axis vertically.

The final required calculation determines the distance (Z travel) the Aerotech Z-stage should position the blade to bisect the embryo along its embryonic axis. Then the seed is imaged, the bottom edge is located (Fig. 26A), and the center of mass of the embryo is located (Fig. 26B). The difference between those two measurements is the height from the bottom of the seed to the position of the blade to bisect the embryo.

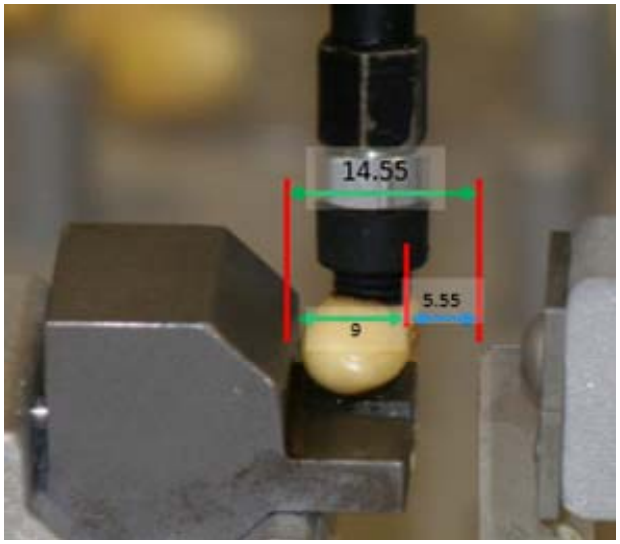


Fig. 25 Showing the distance the cutting blade will travel (5.55 mm) before contacting the seed.

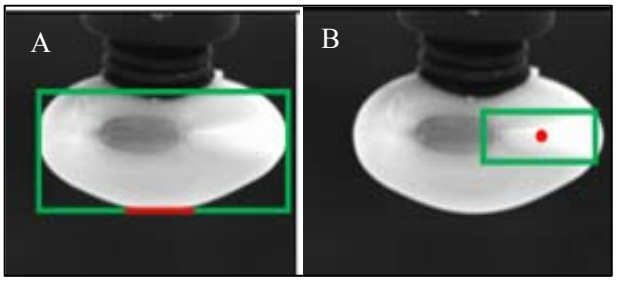


Fig. 26 Showing the distance to set the cutting blade relative to the bottom of the seed for bisecting the embryo along its axis.

3.1.11 Sequence for Cutting of Seeds

After the software has learned the location of the embryo and how to level the seed for placement into the cutter, the next step is to cut the seed. Different stages of cutting are shown in Fig. 27A-E.

1) The seed is positioned onto the seed nest. Note the slight angle of the gripper assembly towards the left top of the image. This is the tilt described by Fig. 21. It is a little hard to discern in the image, but the top of gripper is also tilting towards the reader. This is the tilt described by Fig. 20.
2) The node is trimmed by moving the blade to the position described by Figs. 24 and 25. Note that the blade is turned vertically at this time.
3) The blade is returned to its starting position and rotated horizontally.
4) The blade is moved to a position to trim the embryonic axis.

Note that the blade does not cut completely through to contact the seed nest. This keeps the soybean seed coat intact on the side against the seed nest thus holding the two seed halves and allowing both seed halves to be removed from the cutter as a single unit.

Fig. 28 shows examples of two successfully trimmed soybean seeds. In addition to the horizontal cut bisecting the seed, note the small vertical cut to the right of the hilum that trims the embryo. It should also be noted that despite the seed bisection, it can still be handled as a single unit, because the seed coat on the opposite side of the hilum is not completely cut on purpose to hold together the two halves. After the seed is cut, the seed is considered fully processed and the robotic arm holding it in place will move it to a designated plate where all the processed seed is stored. From there, an operator will manually complete the removal of the seed coat, releasing the seed halves.

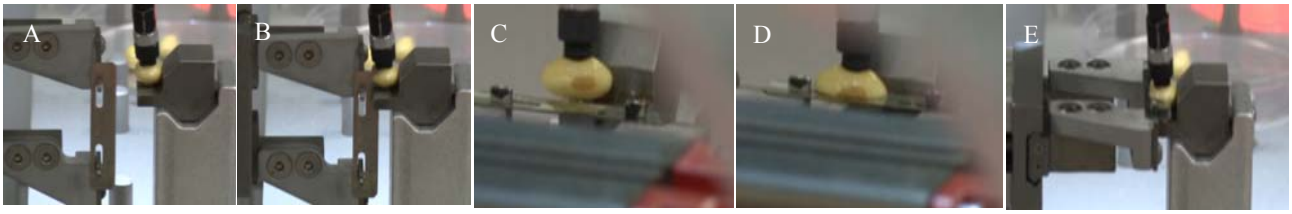


Fig. 27 A-E shows different steps of seed cutting. A) seed being held at the seed nest while the cutter is positioned for the trim of the embryonic axis, B) the embryonic axis being trimmed, C) seed being held at the seed nest while the cutter is positioned for the bisect (view of seed hilum), D) seed being bisected, and E) seed being held at the seed nest while the cutter is cutting the seed (side view).

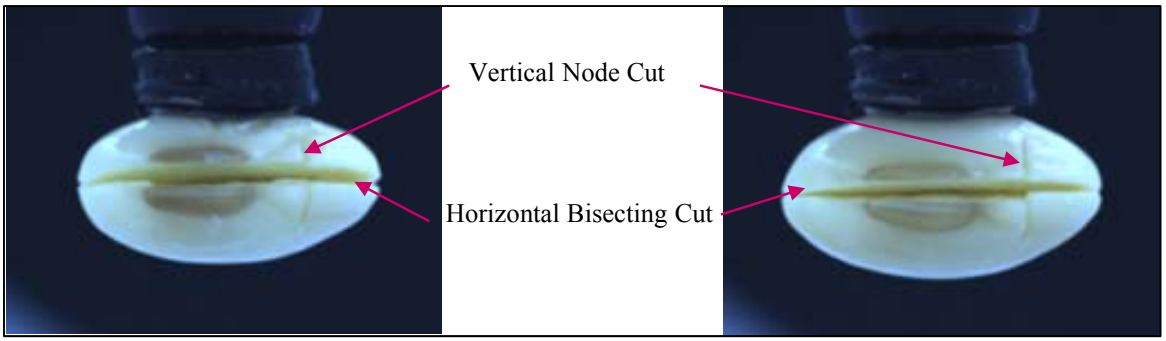


Fig. 28 Examples of two successfully cut soybean seeds.

3.2 Operator Workflow

Fig. 29 graphically depicts the manual workflow followed by an operator to process seeds using the automated system.

- An operator:
  - Manually places 3 to 4 sterile blades on red LED
  - Attaches new grippers on robot A and B
  - Places two 50 ml tubes of 70% ethanol on the table
  - Places a sterile seed nest on the cutting post
- Two Petri dishes of approximately 30 uncut seeds each are presented to the robot by the operator.
- Petri dishes containing processed (trimmed and bisected seeds) plates are removed and replaced by new dishes of seeds and automated processing resumes.
- In a manual, parallel process, the processed dishes of seeds are transferred to a laminar flow hood where the operator manually removes the seed coats, removes the loose cut end of the embryonic axis, and discards any incorrectly processed seed.

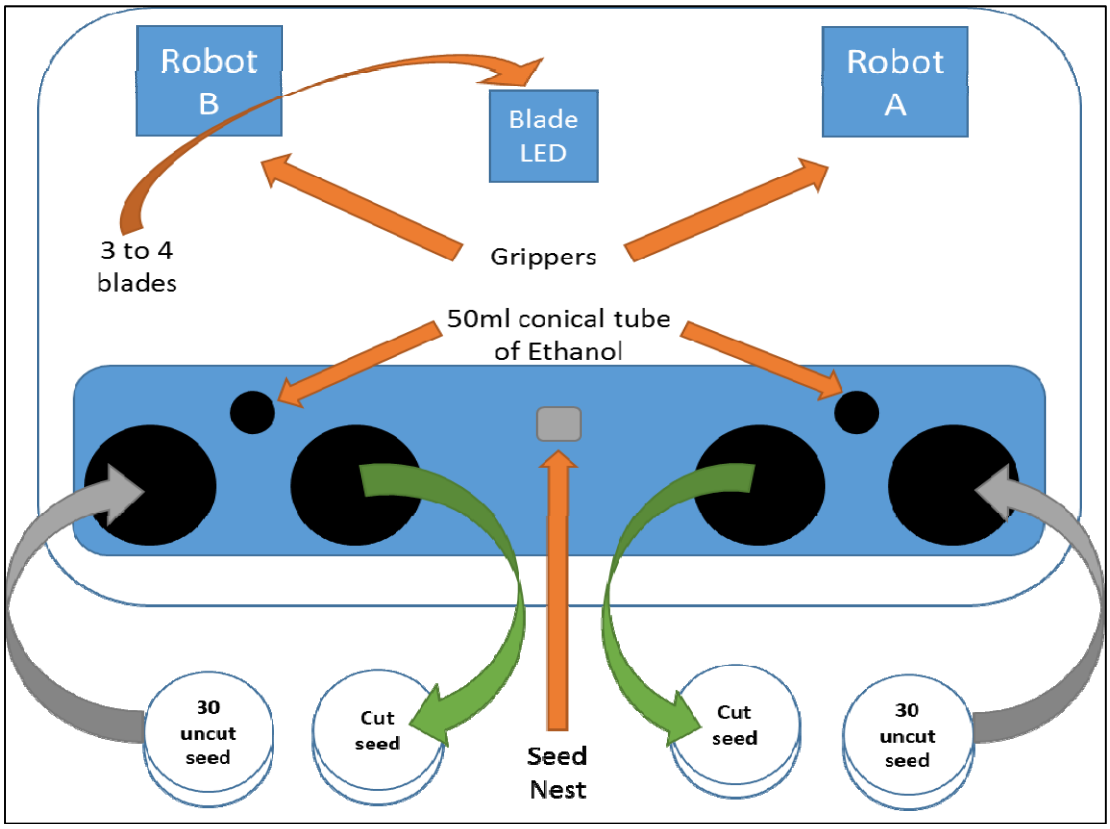


Fig. 29 Work flow diagram.

New dishes of seeds are presented to the automated system until the desired number of seeds are processed.

3.3 Processing Time and Explant Survival

Whether explants are obtained manually or with the automated system, typically about 500 to 600 seeds (20 Petri dishes containing approximately 30 seeds each) are processed in a session to acquire about 1000 to 1200 explants. Two metrics are helpful to compare the performance of the automated seed cutter with the performance of an operator:

Processing time: With automation, it takes 17 minutes for the two robots to cut 60 seeds. On the other hand, it takes about 16 minutes for an operator to cut 60 seeds manually. Hence, in terms of processing time per seed, the robot performed on par with the manual method. However, the manual method was ergonomically challenging and therefore not safe for a single human to perform the intricate act of bisecting 600 seeds continuously. Consequently, multiple operators (3-6) had to be assigned to the manual task to enable large scale experimentation. Automation involved only one operator supplying and retrieving Petri dishes of uncut and cut seeds. Automation is limited only by the number of simultaneously operating units, and does not require additional operators, since there is sufficient downtime between plates for an operator to handle simultaneously operating units. It should be noted here that although the manual seed coat removal step in the automated process is an additional step, it did not amount to additional operator time since it overlapped with the robot cutting time.

The quality of the processing and consistency of good quality cuts: We defined quality of processing by the percentage of explants that survive a 5-day culturing (co-cultivation phase that followed after the processed seeds were infected with *Agrobacterium* solution). The purpose of this metric was to determine if the robotic handling caused any undesired

mechanical damage to seeds which may result in reduced viability. Cut seeds were manually inspected for correctness (equal bisects with completely trimmed embryonic axis) during the seed coat removal step and only the correctly processed seeds were advanced to the co-cultivation step. We found that the explant survival rate was about 88% which was comparable to what we achieved when seeds were manually processed (91%) (Table 1). However, a comparison of plate-to-plate standard deviation showed a statistically significant reduction in the intra experiment variability when automation was used, that is 1.7% with automation versus 4.4% with the manual method (Tukey HSD $p = 0.015$). This is a dramatic reduction (2.5 fold) in intra experimental variation. It can be attributed to a single operator inspecting seeds processed using automation, whereas, the manual method involved multiple operators cutting and inspecting seeds.

Therefore, we concluded that by automating the seed processing step, we not only achieved performance on par with manual method but also better consistency in terms of explant survival and better utilization of scientists skills for less repetitive tasks. Most importantly, biosafety, safety and ergonomics of the process were improved considerably for the operators.

The complexity associated with custom designing robots to automate the soybean seed processing step produced a unique set of challenges. It was hard to achieve errorless output (perfectly bisected seed with partially trimmed axis) at every input (an imbibed seed). The types of errors included the inability of the system to identify the hilum or undesirable output quality such as unequal bisects and/or untrimmed embryonic axis. This error prone processing is attributed to the inability of our system in its current form to compensate for the heterogeneity of input material. In this case, the soybean seed is a biological entity that is heterogeneous for size, shape, color, length of hilum, seed coat banding pattern, etc. These variables affected the software interpretation of images. It was absolutely necessary to minimize the misinterpretations by using optimal settings for factors such as light, shadow, gripper air pressure, and camera angles. Several design improvements were incorporated from the original prototype to improve the quality and efficiency of the system.

Table 1 Seed survival rate of explants cut through automated cutter and manual cutting.

Method	% explants survived	Plate-to-plate Standard deviation
Automation	87.9%	1.7%
Manual	90.5%	4.4%

Sterility of the explants was a major concern during the development of the automation. The designed equipment was installed in a Baker biosafety cabinet (model BioPROtect III) and several features were incorporated including the use of a detachable autoclavable seed nest and 220°C bead baths for sterilizing the vacuum grippers. The accumulation of seed debris on the seed cutter blades made it necessary to wipe the blade with the vacuum gripper after each cut and replace the blade after several plates of seed were processed. Lighting conditions during imaging of the seed had to be controlled through several modifications of the dome light and robot which included the addition of matte black material both at the opening of the dome light and above the gripper.

As with any other newly automated system, learnings from early trial runs and their resulting adaptations were incorporated into the design. For example, we realized from our initial runs that the way seeds were presented to the robot played a significant role in determining the success of processing correctly. To improve the presentation, we placed the seeds in a thin layer of water to protect them from drying and sticking to the gripper; we made sure seeds were placed such that they lay on their side and the hilum was parallel to the plate surface; and we positioned them in a plate such that they were not touching each other or the wall of the plate. The number of seeds per plate was also doubled thus decreasing the frequency of human interaction.

Automating the seed processing step opened up several opportunities to improve the process as stated above and extend automation to other steps in the soybean transformation process such as infection of processed seeds with *Agrobacterium* solution and placement of infected seeds on co-cultivation medium. In the future, we envision an automated process with minimal human intervention and efficiencies that are several fold better than conventional methods.

4. Conclusions

The Dow AgroSciences soybean transformation protocol currently in use for transgenic seed production is a highly efficient system that has several steps which are amenable to automation. To that end, equipment was developed for the automation of two steps in this transformation process: trimming of the embryonic axis and bisecting of the seed. The equipment consisted of: two robotic arms, a cutting device, and a method to visualize and orient the imbibed seed. This automated system is capable of cutting and trimming explants in a manner that resulted in a significant decrease in experiment variation (2.5×reduction). It also eliminates the need for direct handling of scalpels; thereby increasing safety during seed cutting, and reduces repetitive hand motions for trimming and bisecting.

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