# Establishing a protocol for sorghum (*Sorghum bicolor)* gene transformation

by

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# Abstract

Sorghum (*Sorghum bicolor)* is one of the most utilized crops in the world. Not only does it provide a staple food source for arid regions of the planet, it is also important for sustaining livestock production, as well as advancements in its use in biofuels. Despite the importance of sorghum for a large swath of the world, progress for using genetic transformation to develop more useful sorghum varieties has been far outpaced by other similar cereal crops. Thus, developing and improving a protocol to reliably transform sorghum is of great interest, in which the results will be beneficial to improving agricultural use all over the world. The objective of this study was to identify results of sorghum transformation from many sources and apply successful tissue culture and transformation techniques into one successful protocol.

Positive control was established before transformation to guarantee embryo regeneration prior to inoculation. Two different media types were established and analyzed for their production and quality of callus and shoot regeneration. A comparison was made on two medium types, referred to as “B” media [2,4-D for callus induction and 6-Benzylaminopurine (BAP)/indole-3-acetic acid (IAA) for regeneration] and “S” media [2,4-D/BAP for callus induction and 2,4-D/BAP/thidiazuron (TDZ) for regeneration], respectively. Although both these medium types yielded viable plants with roots and shoots, B media was selected for its successes in callus induction and shoot regeneration. Calluses induced embryos in B media yielded shoots that were larger, more distinct, and easier to separate compared to calluses on S media.

Once positive control was established, two bacterial strains, EHA101 and LBA4404, containing the same vector, *SbGFPTSCENH3*, were evaluated for their efficacy for targeted mutagenesis of sorghum using a CRISPR/Cas9 system. LBA4404 proved to be a more effective

bacterial strain than EHA101 in transforming sorghum embryos, with little to no bacterial overgrowth, and higher rates of green callus and shoot emergence. Of all embryos inoculated with *Agrobacterium,* five plants were fully regenerated into a viable plant on herbicide selection pressure and then acclimated in the soil. To confirm transformation, polymerase chain reaction (PCR) and gel electrophoresis analysis were performed. Our initial result from gel electrophoresis analysis of the five plants indicated that all five plants appeared to be transgenic. After sequencing the genome of the plants, their sequences were aligned and matched with the genomes sequenced from the *CENH3* construct and the extracted plasmid. Of the five plants, three of their genomes indicated a 98 to 99% match to both the *CENH3* construct and the plasmid. Upon further inspection of the chromatogram results, errors were corrected in the initial results, which showed a 100% match for these three plants. Based on these results, we can confidently say that these three plants are transgenic.

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# Introduction

Sorghum (*Sorghum bicolor)* is considered to be the 5$^{th}$ most important cereal crop in the world (Nguyen et al., 2007), with upwards of 1 billion people in semi-arid tropics depending on sorghum as the major source of dietary energy and protein (Belton and Taylor, 2004). Not only is sorghum a staple food crop for a large portion of the world's population, it has been successful for animal feed in a variety of livestock, and shows potential in the production of biofuels, ethanol, and other factory substances. Given sorghum's natural tolerance for arid climates and its' ability to retain water under drought stress, the crop will certainly be valuable for producers to utilize as global climate changes lead to varying heat and water availability around the world (Gurel et al., 2009). Since sorghum is one of the most important crops worldwide, developing improved varieties to supply to the world's population for production is of great interest. To this date, most sorghum varietal development has primarily been through conventional breeding techniques (Grootboom et al., 2010). Despite the importance of sorghum for a large swath of the world, progress for using genetic transformation to develop more useful sorghum varieties has been far outpaced by other similar cereal crops. This is due to sorghum being widely considered a recalcitrant crop when *in vitro* tissue culture and transformation techniques are attempted (Zhu et al., 1998). With climate changes becoming more likely in the future, and the fact that sorghum is one of the most drought and heat tolerant crops available, establishing a reliable method to improve the rate of developing new sorghum varieties alongside conventional breeding is becoming more crucial.

There have been two primary methods to transform cereal crops: microprojectile bombardment, and *Agrobacterium*-mediated gene transfer. The earliest success in transformation

of sorghum was reported using microprojectile bombardment (Casas et al., 1993). The first successful transformation of sorghum using *Agrobacterium*-mediated gene transfer was reported a few years later (Zhao Z et al., 2000). Both of these achievements in transformation were accomplished using sorghum genotype P898012, which is widely considered the most suitable for transformation given its high rates of regeneration and transformation (Do et al., 2016). Although transformation of sorghum to incorporate agriculturally beneficial transgenes into crop production have been reported (Girijashankar et al., 2005; Zhao ZY et al., 2003), most recent studies involving sorghum transformation have been to improve the transformation and regeneration rates using a variety of different sorghum cultivars and gene constructs (Carvalho etal., 2004; Nguyen et al., 2007; Ahmed et al.,2018; Belide et al., 2017; Liu & Godwin, 2012; Howe et al., 2006; Char et al., 2019; Si Nian et al., 2019). These most recent studies have focused on reducing the recalcitrance of sorghum growing in vitro. This has been attempted by modifying existing successful concentrations of nutrients and chemicals of in vitro growth medium to improve rates of regeneration and transformation to create a more reliable and efficient transformation system. One of the biggest hurdles for a successful sorghum transformation system is the production of phenolic compounds in vitro. These compounds, such as polyphenol oxidases and peroxidases, cause necrosis and explant death, and may also be toxic to Agrobacterium used in genetic transformation. Previous studies have shown that production of phenolic compounds can be alleviated by utilizing different methods in their transformation systems, including a cold treatment of immature seeds prior to extraction (Nguyen et al., 2007) and using antioxidants and absorbing agents (Ahmed et al., 2018). These two treatments have reduced the production of phenolic compounds and increased the transformation ratios.

A desirable outcome for transformation is the ability to reduce the timeline for creating new crop varieties. This is best achieved by producing a homozygous line due to the stability in the genes to produce generations of plants containing only that gene. Previously, this method of stabilizing a desired gene has been achieved through conventional breeding. While this method has proven successful, conventional inbreeding procedures take at least six generations to achieve nearly complete homozygosity. In order to shorten the time to reach homozygosity, techniques to implement doubled haploidy have arisen. Doubled haploidy occurs by taking a haploid pollen grain and doubling the chromosomes, which results in the plant becoming completely homozygous. Achieving double haploidy creates plants that are homozygous within one generation (Jain et al., 1996). Inducing double haploidy reduces the time, labor, and cost needed to create homozygous lines, making it an important technique to use when attempting to isolate a desirable homozygous gene in plant production systems. Double haploidy can occur within a plant spontaneously in nature or artificially. This has been artificially achieved in numerous plants including rice (*Oryza sativa*) (Tao et al., 2013), maize (*Zea mays*) (Kelliher et al., 2019), and wheat (*Triticum aestivum*) (Jing et al., 2014) by manipulating a single centromere protein, the centromere-specific histone *CENH3* (known as *CENP-A* in humans). The *CENH3* histone determines the position of the centromere, which is crucial in chromosome segregation and crossing over in meiosis (Allshire et al., 2008). Introducing a modified *CENH3* or knocking out the native protein into a genome causes chromosome segregation errors, disruption of chromatin organization, and lethality (Britt and Kuppu 2016). This disruption in segregation can result in one parent's genome not successfully integrating with the other parent's genome, resulting in half the chromosomes being inherited in the next generation, creating a haploid (Ravi and Chan, 2010). Chromosome doubling of this haploid will result in a double haploid, or

diploid, which can be referred to a true homozygous line for breeding purposes (Wang et al., 2019). To reduce the negative effects of inducing a haploid, several methods have been attempted to stabilize the transformation system. One of the most effective methods has been referred to as a "tail-swap," in which the hypervariable amino-terminal tail domain of *CENH3* is replaced with a highly conserved tail of conventional *H3* to stabilize the construct (Shamoni et al., 2015). This process reduces the rate of embryo abortions during crossing over when wildtype plants are mated to transgenic female plants, allowing the increased production of haploid plants in the next generation (Ravi and Chan, 2010). Since *CENH3* is universal in eukaryotes (Shamoni et al., 2015), this method may be extended to produce haploids in any plant species including sorghum.

# Materials and Methods

## Plant material

Sorghum genotype P898012 was chosen for evaluation of its previous successes in tissue culture and transformation. All sorghum plants were grown in the Kansas State University (Manhattan, KS) greenhouse with set-point day/night temperatures of 81/70°C (28/21°C). Seeds were planted in 9 cm boxes before being transplanted to 3-gallon pots, both containing Mycorrhizae Promix growing medium (Premier Tech, Quakertown, PA). Plants were watered daily or every other day as needed. When sorghum plants began flowering, pollen was collected onto a paper bag and then spread over same panicle from which the pollen was collected. Fertilized heads were harvested 11 to 14 days after pollen was distributed over the head. All seeds were removed and sterilized with 50% bleach and two drops of Tween 20 (Millipore Sigma, St. Louis, MO) for 10 to 15 minutes with occasional agitation. After soaking, seeds were then triple rinsed with double distilled water and dried on paper towels. Seeds were moved to cold treatment (4°C) for 2 to 3 days before immature embryos ranging from 1.0 to 1.5 mm were harvested and placed onto callus induction media for regeneration or placed onto into inoculation media for inoculation with *Agrobacterium.*

## Media types for callus induction and regeneration

Two media types from similar studies with sorghum regeneration were selected and evaluated for their efficiency and success in producing both desired sorghum callus and regeneration into full plant. Once these two media types were evaluated, a slight altered media composition was used for transformation **(Table 1)**. This media, as well as the two media types evaluated, referred to as "B" media (Do et al., 2016) **(Table 2)** and "S" media (Belide et al., 2017) **(Table 3)** and the

slightly altered media used in this study are compared in the tables below. Full plant regeneration was achieved before inoculation to serve as a positive control for both media formulations. Plant regeneration was the same process as full transformation protocol, with the exception that freshly extracted embryos were immediately placed onto callus induction media and grown into full plants. This process excluded inoculation, co-cultivation, and resting media stages.

## *Agrobacterium* strains and vectors

Two common strains of *Agrobacterium tumefaciens,* LBA4404 and EHA101, were used in transformation of sorghum embryos. Success rates of transformation were evaluated and compared, as well as other factors relating to the quality of callus and potential overgrowth in growth mediums containing sorghum embryos/callus. Both bacterial strains contained the same construct, *SbGFPTSCENH3* (**Figure 1)**. This construct can be split in the following regions: *Sb* (*Sorghum bicolor), GFP* (green fluorescent protein)*, TS* (tail-swap)*, and CENH3* (*CENH3* protein). *CENH3* is the gene of interest used to induce haploidy in the next generation during crossing over between wildtype parent and transgenic parent. Upstream this construct is the *BAR* gene, which induces herbicide resistance in the plant when the whole construct is incorporated into the genome. This *BAR* gene is used as a selection marker, meaning that plants which do not assimilate the construct will die in the growth media where selection pressure Glufosinate (phosphinothricin) (Phytotech Labs, Lenexa, KS) is introduced (2.5 mg/L). Plants that survive the selection pressure were considered to be potentially transformed and were confirmed using PCR amplification, gel electrophoresis analysis, and DNA sequencing.

## Transformation

All *Agrobacterium* strains used for inoculation were stored in glycerol stock (-80°C). Colonies were streaked using a wire loop from the glycerol stock onto a plate containing YEP media (10 g/l bacto-yeast extract, 10 g/l tryptone, 5 g/l NaCl, 15 g/l bacto-agar) with appropriate antibiotics and then stores in an incubation chamber at 26.5°C for 3 days. Once colonies developed on the plate, a wire loop was used to take a single colony and grow in suspension culture containing liquid YEP media and the same antibiotics for 2 to 3 days. Bacterial colonies in liquid YEP were placed in a rack leaning roughly 45° on one side and spun at 225 rpm in an incubation shaker set at 27°C. Immature sorghum seeds placed in cold treatment were removed and embryos were isolated and placed into inoculation media (IM) containing 3 µl of 20mg/L acetosyringone. Acetosyringone, which is known to induce expression of *virulence A* genes and chemotaxis in *A. tumefaciens* strains, was added to induce *Agrobacterium* infection of immature embryos. Once all embryos were harvested and placed into 2 ml tubes containing inoculation media, they were placed in a hot bath at 46°C for 5 minutes while tubes containing *Agrobacterium* were centrifuged at 14000 rpm for 4 minutes. YEP media was discarded from tubes and conglomerated *Agrobacterium* at the bottom of the tube was resuspended with 1 ml inoculation media and then inserted into 2 ml tubes containing sorghum embryos for 10 to 15 minutes. Once *Agrobacterium* was introduced to the embryos, they were agitated by Vortex for 5 to 10 seconds to evenly mix the contents. After inoculation, embryos were moved onto blotting paper and dried of excess *Agrobacterium* for 20 to 30 minutes. Once the embryos were dry, they were set onto plates containing co-cultivation media (CM) for 3 days with the scutellum face up. These plates were set in a dark incubation chamber at 26°C with 25 embryos set onto each plate. After 3 days, embryos were moved onto resting media (RM) for 10 days to prevent additional bacterial growth

from outcompeting the growth of the embryos, and then were moved onto callus induction media (CIM) for 2 weeks. Embryos on RM and CIM were also cultured in dark incubation chambers at 26.5°C. Well-developed calluses were then moved onto shoot regeneration media (SIM), while embryos that did not develop well were sub cultured onto fresh CIM after 2 weeks. Embryos that did not develop into viable calluses were discarded after 4 weeks. Calluses moved onto SM were kept there for 4 to 8 weeks until viable shoots developed, with sub cultures every 2 weeks. Calluses on SIM were exposed to a 12-hour light/dark photoperiod at 24°C. Once shoots elongated and had at least two leaves, they were separated from callus and individually implanted onto root induction media (RIM). RIM was housed in magenta boxes. Once roots developed, the whole plant was moved out of the growth media, acclimated in the soil, and subjected to a 12-hour light/dark photoperiod at 24°C before being moved into the greenhouse after 3 weeks. Plants with five to six grown leaves reaching at least 5 cm were considered acclimated and suitable for DNA extraction and analysis.

## DNA extraction

Leaf samples were collected from the greenhouse from 5 cm sections of leaf tips and placed inside 2 ml tubes. The leaf segments were then flash frozen in liquid nitrogen and then ground up in the tubes. Leaf segments were treated to cycles of freezing and grinding until they formed a fine powder. This leaf powder was then mixed with 400 µl of 2% CTAB (cetrimonium bromide) buffer and 4 µl of DNase free RNase I (Thermo Fisher Scientific, Waltham, MA). Samples were then incubated in water at 55°C for 25 minutes, then mixed with 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and then centrifuged at 13000 rpm for 10 minutes. Supernatant was removed and placed into a new tube, then mixed with 400 µl isopropanol and 80

μl of 3 M sodium acetate and then incubated at 4°C for 25 minutes. After incubation, the samples were centrifuged again at 13000 rpm for 10 minutes. Supernatant was discarded and then DNA pellets were washed three times with 70% ethanol. Ethanol was removed from the tube and then DNA was resuspended in sterilized water.

DNA quality and concentration were measured with a DNA nanodrop to view 260/230 and 260/280 ratios. Based on these ratios, the best quality and concentration of DNA was chosen for PCR analysis. Three primer pairs were selected to test for the presence of the gene. These pairs were forward-GFP (5'-TGGCCAACACTCGTCACTAC -3') and reverse-GFP (5'-GCACGGGTCCATCTCCTATG -3'), forward-CENH3 (5'-AAGCAAACTGCTCGCAAGTC -3') and reverse-CENH3 (5'-GCACACAGATTCGCCACTTC -3'), and forward-BAR (5'-ACGACGCCCGGCCGACATCC -3') and reverse-BAR (5'-TGACGGGCAGGACCGGACGG -3'). To initiate PCR analysis, these primers were tested with three sets of plasmids extracted from *Agrobacterium* containing the construct *SbGFPTSCENH3* and wild type DNA. Once the proper temperature procedures were set for the primers, and wild type bands no longer appeared on the gel, DNA was extracted from possibly transgenic plants and subjected to the same modified CTAB procedure listed above. The PCR program ran to amplify the DNA fragments follows as so: 94°C for 5 minutes followed by 40 cycles of 94°C for 1 minute, 60°C for 45 seconds, 72°C for 1 minute, and 72°C for 10 minutes. Positive control tests were conducted by extracting plasmids from *Agrobacterium* used to inoculate embryos and analyzed on the gel alongside the possibly transgenic DNA. To test for negative control, wildtype DNA was extracted and ran alongside the gel to confirm that the gene present in the *Agrobacterium* is not present in the wildtype DNA. The extracted DNA was then inserted into wells in the gel, which was created with 1xTBE buffer and 1.5% TopVision agarose gel (Thermo Fisher Scientific,

Waltham, MA). The DNA was then subjected to electrophoresis at 61 volts. This combination of 1.5% agarose gel and 61 volts allowed for the DNA fragments to separate over two hours on the gel. The agent used to make DNA bands fluorescent under UV light was Biotium GelRed Nucleic Acid Stain (10,000x) at 1 μl per 25 ml of 1xTBE buffer. GeneRuler 1kb (Thermo Fisher Scientific, United States) was used to compare the lengths of DNA fragments after the bands separated to infer the size of DNA fragments showing up. Bands that resulted in desired outcomes were sent to Molecular Cloning Laboratories (MCLab, San Francisco, CA) to be sequenced to confirm if the construct was incorporated into the plant genome.

# Results

## Media types

Two media types were chosen to be evaluated in their efficiency of regeneration as well as their effects on callus growth. These two media concentrations compared were referred as “B” media (2,4-D for callus induction and BA/IAA for regeneration) and “S” media (2,4-D/BA for callus induction and 2,4-D/BA/TDZ for regeneration), respectively. Both these medium types yielded viable plants with roots and shoots induced by their environments, but each medium had a different impact on the development of these calluses. As the calluses developed, the differences between the embryonic response to “B” media and “S” media was noticeable. “B” callus induction media created calluses that were crumbly in appearance, and easier to separate into individual fragments. “S” callus induction media produced calluses that were more globular in appearance, and harder to separate individual parts. Both sets of media produced a viable callus for regeneration within about 3 to 4 weeks. The shoots grown in “B” media were wider, more distinct, and easier to isolate from the callus **(Figure 2).** The shoots grown in “S” media were smaller, more clumped together, and difficult to isolate from other apical meristems **(Figure 3)**. Although “S” media produced shoots that were harder to isolate and use, the three different media stages used for shoot growth (shoot induction, shoot regeneration, and shoot outgrowth) created calluses that grew indefinitely in petri dishes. After selecting “B” media for further culturing, the ratios of callus induction, green spot regeneration, and shoot induction were evaluated. 1500 embryos were placed on “B” media stages to examine the ratios (**Table 4)** of these tested embryos, two weeks on CIM resulted in 1140 embryos producing callus (76.1%). These calluses were then moved onto regeneration media (SM) for two weeks, resulting in 748

calluses producing green spots (49.8%) with 258 of these green spots being viable shoots (17.8%).

Using Glufosinate (phosphinothricin) as a selection pressure proved to work nearly 100% of the time. Any embryo that did not incorporate the construct was not able to withstand the selection pressure, inducing a darkening of callus and rapid cell death within 2 to 3 weeks of initial introduction of the herbicide **(Figure 4).** This allowed simple observations of callus to determine early possibility of achieving a transgenic plant, and ease of separating developing green spots/shoots on callus from dead cells that had died off. Occasionally some "escapes," (non-transgenic green spots on callus), emerged on the selection pressure, but these were subjected to additional sub cultures on growth medium with selection pressure present. Additional sub cultures of green callus either eliminated the green spot, confirming the callus was not transgenic, or caused it to develop into a shoot that is potentially transgenic.

The introduction of polyvinylpolypyrrolidone (PVPP) into both "B" and "S" media solutions, as well as implementing a cold treatment for immature seeds at 4°C for 1 to 3 days, reduced both the frequency and concentration of phenolic compounds emerging around growing callus. On average, growing callus would produce rings of phenolic compounds between 2 to 10 mm around the center of the callus. The size and concentration of the rings would be larger and darker with more dying/dead cells on the growing callus. The concentration of PVPP in all stages of growth medium reduced the concentration of any phenolic compounds being produced around callus by acting as an absorbing/antioxidant agent to prevent the black rings of phenolic compounds from spreading farther compared to calluses grown without PVPP **(Figure 5).** Both the addition of PVPP and usage of 1 to 3 days of cold treatment also increased the number of surviving embryos. In tandem, these techniques reduced the concentration of phenolic

compounds produced around callus, as well as reduced the size of the ring of phenolic compounds by 2 to 4 mm, if not preventing the formation of the phenolic compounds entirely. Removal of black/dying tissues from callus also helped to reduce the production of phenolic compounds. It appeared that phenolic compound production was significantly increased around these dead/dying tissues than healthier cells on the callus.

## *Agrobacterium*

The rates of success in transformation were evaluated and compared between two common strains of *Agrobacterium tumefaciens,* LBA4404 and EHA101 using "B" media as the growth medium, based on our initial research. During the early attempts at transformation, the problem of bacterial overgrowth emerged among embryos inoculated with EHA101. Original media concentrations contained 250 mg Clavacillin, which limited bacterial overgrowth in embryos inoculated with LBA4404. However, this proved to be unsuccessful in limiting overgrowth with embryos inoculated with EHA101. A significant portion of embryos inoculated with EHA101 in media containing 250 mg Clavacillin were outcompeted by the overgrowth of bacteria in vitro, while embryos inoculated with LBA4404 rarely experienced any bacterial overgrowth. Increasing the concentration of Clavacillin from 250 mg to 500 mg in resting media (RM) significantly reduced the amount of overgrowth for embryos inoculated with EHA101 but was still present in the system. Embryos inoculated with LBA4404 saw almost no bacterial overgrowth once the concentration of antibiotics was increased. This change in the concentration of Clavacillin had no impact on development of callus outside of controlling bacterial overgrowth **(Figure 6).**

Between a range of 3429 and 4039 embryos were inoculated with LBA4404, and between 3575 and 4185 inoculated with EHA101. Of these attempted transformations, 38 green spotted calluses emerged for embryos treated with LBA4404, versus only 16 green spots emerging for those treated with EHA101. Additionally, 13 of the calluses showing green spots for LBA4404 treated embryos induced distinct shoots, 5 of which grew into plants with three or more leaves and soon induced roots as well. Green spots on callus that were inoculated with EHA101 did not grow into any viable shoots. **(Table 5; Figure 7)**.

## PCR, gel electrophoresis, and DNA sequencing analysis

A total of five plantlets were obtained from embryos inoculated with LBA4404 and were grown into full plants and then acclimated in the soil. Once plants reached five to six leaves at least 5 cm in length, their DNA was extracted and analyzed on gel electrophoresis. After isolating DNA and amplifying through PCR, the DNA was ran on gel electrophoresis with the three sets of primers. Results were considered acceptable if the DNA from positive control (extracted plasmid) appeared on the gel and the DNA from negative control (wildtype plant) did not appear on the gel in accordance with DNA extracted from the potential transgenic plants. DNA amplified with GFP primers produced one result showing that one of the five experimental plants aligned with the extracted plasmid **(Figure 8)**. DNA amplified with CenH3 produced three results, two of which showed all five plants were transgenic, and one result where four of the five plants were transgenic **(Figure 9).** DNA amplified with BAR primers produced four results, all of which showing all five plants were potentially **(Figure 10).** To confirm assimilation of gene construct, the DNA of the five plants was sequenced and aligned with the DNA sequences of the CENH3 construct and the extracted plasmid. Initial results of the compared sequences

indicated a 98 to 99% match to both the CENH3 construct and the plasmid for the sequences of three of the five potentially transgenic plants **(Figure 11)**. Upon further inspection of the chromatogram results, errors were corrected in the initial results. With these errors replaced from the initial results, they showed a 100% match for the three plants (**Figure 12)**.

# Discussion

## Media types

Both media formulations “B” media (Do et al., 2016) and “S” media (Belide et al., 2017) evaluated in this study developed viable shoots and fully regenerated plants **(Figure 2; Figure 3)**. The decision of which one to use was dependent more on the desired outcome for the type of callus produced than it did with the efficiency in producing callus and regenerated shoots. Callus and regenerated shoots were considered more desirable from “B” media in our studies for two reasons. First, for the ability to easily separate individual apical meristems and second, the ability to develop larger regenerated shoots emerging from the callus. These two major differences between the two media types made “B” media more desirable. However, “S” media does has an advantage, as it produces callus that can grow indefinitely *in vitro*. This is an important quality, as it allows researchers to have callus tissue readily available that can be used indefinitely for new apical meristems generations, as opposed to using a callus once and the need to continually grow new plants for additional immature embryos to inoculate. While there were differences in callus formation, there was little difference in the ability of these media to regenerate callus and plant tissues. This suggests that both media types are equally sufficient in callus production and full plant regeneration, and that the primary difference between both media is the form of callus induced and regenerative tissues they produce.

Similar studies have shown that phenolic compound production is one of greatest hinderances to transformation efficiency when attempting to transform sorghum (Do et al., 2016; Ahmed et al., 2018). Results obtained from this research were comparable to the findings of referenced studies when cold treatment was used and an antioxidant compound

(polyvinylpolypyrrolidone, PVPP) was added to the growth medium. Similar studies have used PVPP as well as other antioxidant/absorbing agents including polyvinylpyrrolidone (Liu and Godwin, 2012), activated charcoal (Nguyen et al., 2007), and lipoic acid (Belide et al., 2017). While this study did not compare these additives and their efficiency in reducing phenolic compounds, similar reductions in the concentration and spread of phenolic compounds were observed. This suggests the addition of any of these antioxidant/absorbing agents will reduce the production of phenolic compounds. While these studies used a variety of different additives to reduce the production of phenolic compounds, some also suggest a time frame of 1 to 3 days of cold treatment at 4°C for immature seed prior to inoculation (Nguyen et al.,2007). The findings of this research confirm the claims made by studies referenced above. It appears that a cold treatment of harvested seeds prior to inoculation with *Agrobacterium* is essential in reducing the production of phenolic compounds, and therefore essential to producing quality callus. A combination of both cold treatment applications and antioxidant/absorbing agent use in the transformation system appear to create more desirable callus regardless of the media and concentrations (**Figure 5)**.

## *Agrobacterium*

Previous studies have heavily related the efficiency of transformation to the *Agrobacterium* strain used (Do et al., 2016; Ahmed et al., 2018). Our study found that LBA4404 was more affective at producing potentially transgenic plants (green spots) compared to EHA101. In the time period this study was conducted, LBA4404 also managed to produce more viable shoots, and also produced fully regenerated plants with roots and shoots, whereas EHA101 did not produce any shoots **(Table 5)**. This is similar to previous research, in which Ahmed et al., (2018)

observed a higher transformation rate with LBA4404 compared to EHA101; however, other research has shown the opposite (Do et al., 2016) in which EHA101 was designated the more efficient *Agrobacterium* for transformation. One possible reason LBA4404 appeared to be the superior bacterium strain in this study is due to the much higher rates of overgrowth for EHA101 compared to LBA4404. EHA101 is considered to be a more aggressive and virulent strain of *Agrobacterium* (Hood et al., 1986), which would explain its higher rates of survival on selection pressure, and higher rates of callus death in vitro. As a more virulent strain, this would explain why EHA101 overgrowth continued to be an issue, even as the concentration of Clavacillin was increased in the growth medium **(Figure 6)**. Since embryos inoculated with LBA4404 showed little to no signs of bacterial overgrowth, produces a much higher rate of green callus and shoots, and was the only one to create potentially transgenic plant **(Figure 12)**, LBA4404 should certainly be considered one of the most efficient *Agrobacterium* strains to use in a transformation system.

## PCR, gel electrophoresis, and DNA sequencing analysis

With the purported transgenic lines aligning with the positive control, and no negative control bands appearing on the same system, all five lines appeared to be transgenic. While *GFP* primer amplified DNA only produced one acceptable result **(Figure 8)**, *CENH3* (**Figure 9)** and *BAR* (**Figure 10)** produced three and four acceptable results respectively. This is likely due to *CENH3* and *BAR* primers optimally working at the same annealing temperature used (60°C) while *GFP* did not optimally perform at the same temperature. To fully confirm assimilation of our gene construct into our five lines, their DNA were sequenced and analyzed in comparison to the *CENH3* construct and extracted plasmid. With three of these five plants having a 100% match to

the construct and plasmid **(Figure 12)** after corrections, we can confidently say that these three plants are transgenic. This will need to be further confirmed using qPCR and examining *GFP* gene expression.

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# Figures


**Figure 1. Design of the construct used for sorghum transformation**

Map of T-DNA region of the binary vector *SbGFPTSCENH3* used for transformation. RB, right border; LB, left border; *Tvsp*, 3' terminator from soybean vegetative storage protein gene; *BAR*, phosphinothricin acetyl transferase used to induce herbicide resistance for negative control *in vitro*; *TEV*, Tobacco Etch Virus translational enhancer; *p2x35s*, tandem repeat of the cauliflower mosaic virus (*CaMV*) *35S* promoter; *p4x35s*, tandem repeat of the cauliflower mosaic virus (*CaMV*) *35S* promoter; Sb (*Sorghum bicolor*) refers to the species type; *GFP* (Green Fluorescent Protein) is the reporter gene used to analyze protein synthesis in transgenic plants; *TS* (Tail-swap) refers to the technique used to stabilize this construct; *CENH3* (*CENH3* protein) is the gene of interest used to induce haploidy in the next generation during crossing over between wildtype parent and transgenic parent; *tocs*, octopine synthase terminator; *tnos*, nopaline synthase terminator.

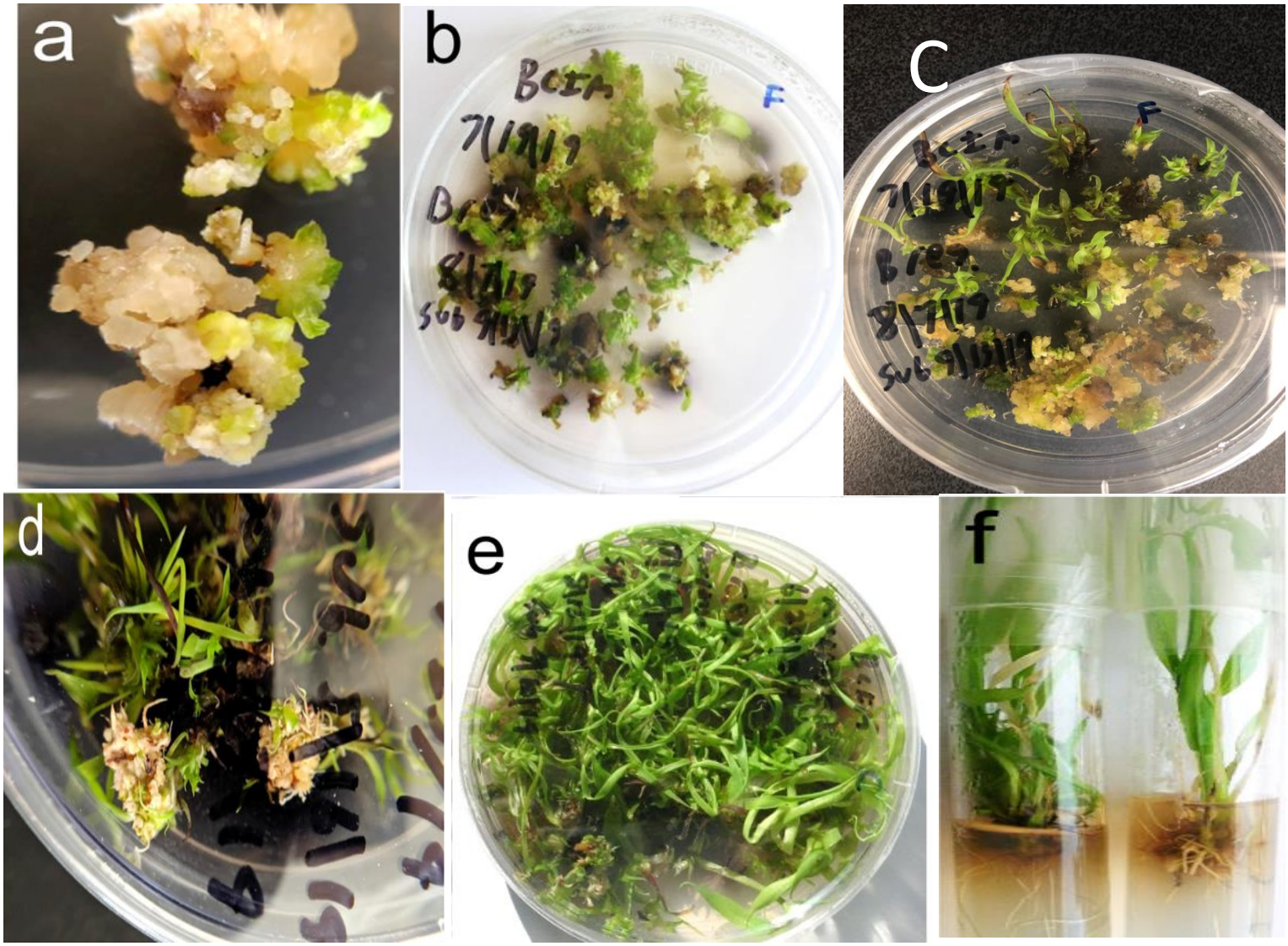


**Figure 2. Callus induction and regeneration on "B" media**

Callus initiation and induction from sorghum immature embryo on "B" media (a). Early active shoot growth on calluses grown in B media (b). Calluses induced on "B" media grew crumbly in shape and with more distinct apical meristem growth (c and d) with multiple distinct shoots emerging from induced callus (e). Root induction of regenerated non-transgenic plant (f) occurred when shoot was viable and large enough to move to rooting media.

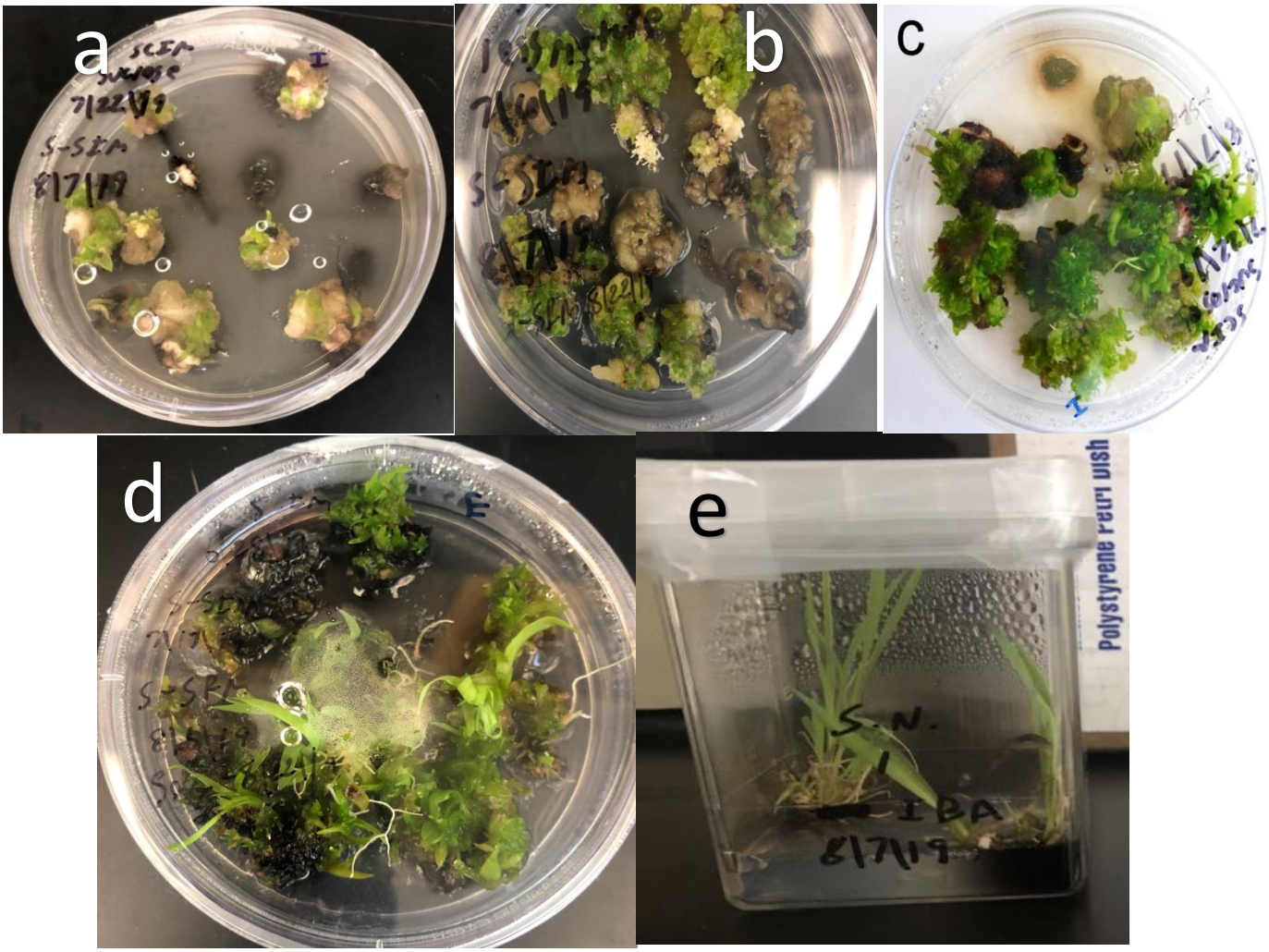


**Figure 3. Callus induction and regeneration on "S" media**

Callus initiation and induction from sorghum immature embryos on "S" media (a). Calluses induced on "S" media grew more globular and grew less distinct apical meristems (b and c) than "B" media, but grew a larger number of apical meristems out of each callus indefinitely (d). Root induction of regenerated non-transgenic plant occurred when shoot was viable and large enough to move to rooting media, but was harder to isolate individual meristems resulting in more shoots per callus than intended in rooting media (e).

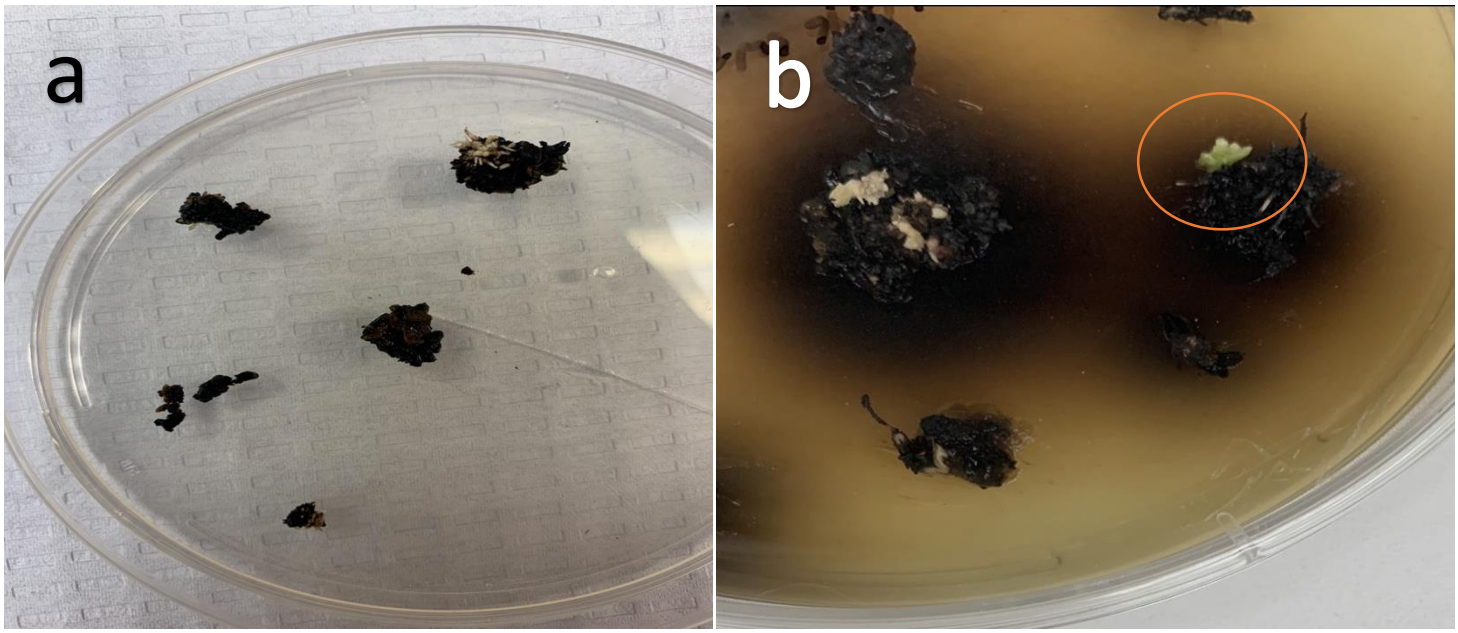


**Figure 4. Use of selection pressure**

Developed calluses set on glufosinate (phosphinothricin) selection pressure for 3 weeks. Callus cell death induced by selection pressure (a). Green spot on callus (circled) separate from dead tissue (b). Green spots on callus can be isolated from dead tissue and sub cultured onto fresh media to continue regenerative growth. All dying/dead calluses were considered non-transgenic and discarded. Green spots were assumed to be possibly transgenic and were sub cultured until they died or grew into apical meristems.

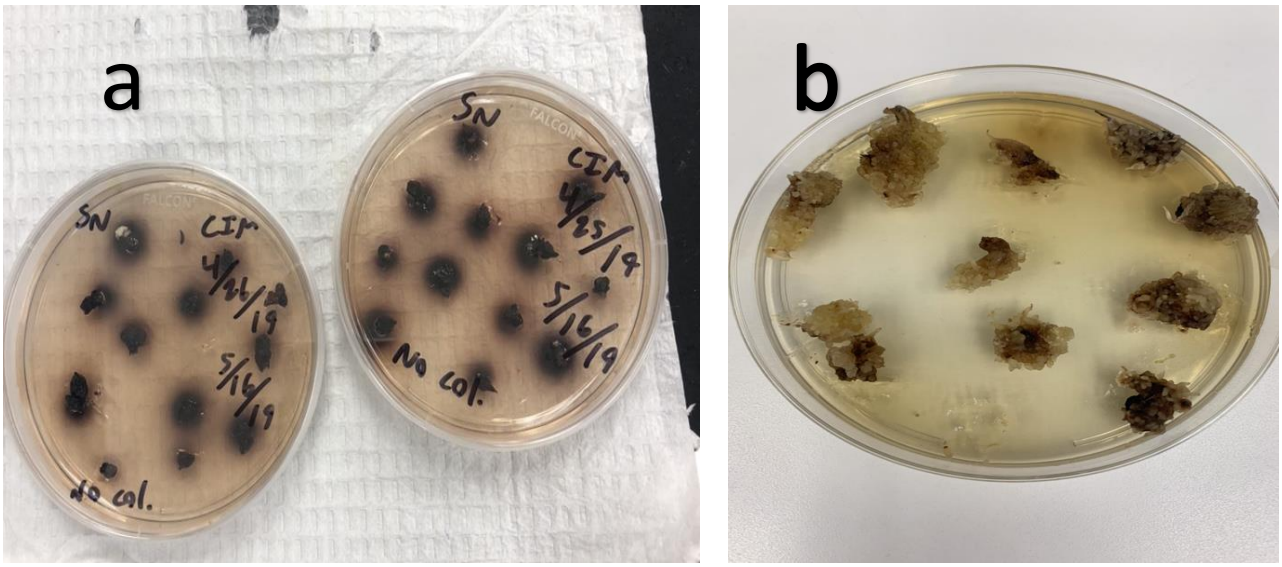


**Figure 5. Use of antioxidant to reduce production of phenolic compounds**

Embryos grown on callus indication media (CIM). Calluses grown without cold treatments or antioxidant polyvinylpolypyrrolidone (PVPP) produced larger rings of phenolic compounds that were higher in concentration surrounding the callus (a). These calluses also had higher rates of cell death. Calluses grown after cold treatment on CIM with PVPP produced healthier calluses that were able to regenerate at higher rates (b) due to the reduction of phenolic compounds being produced.

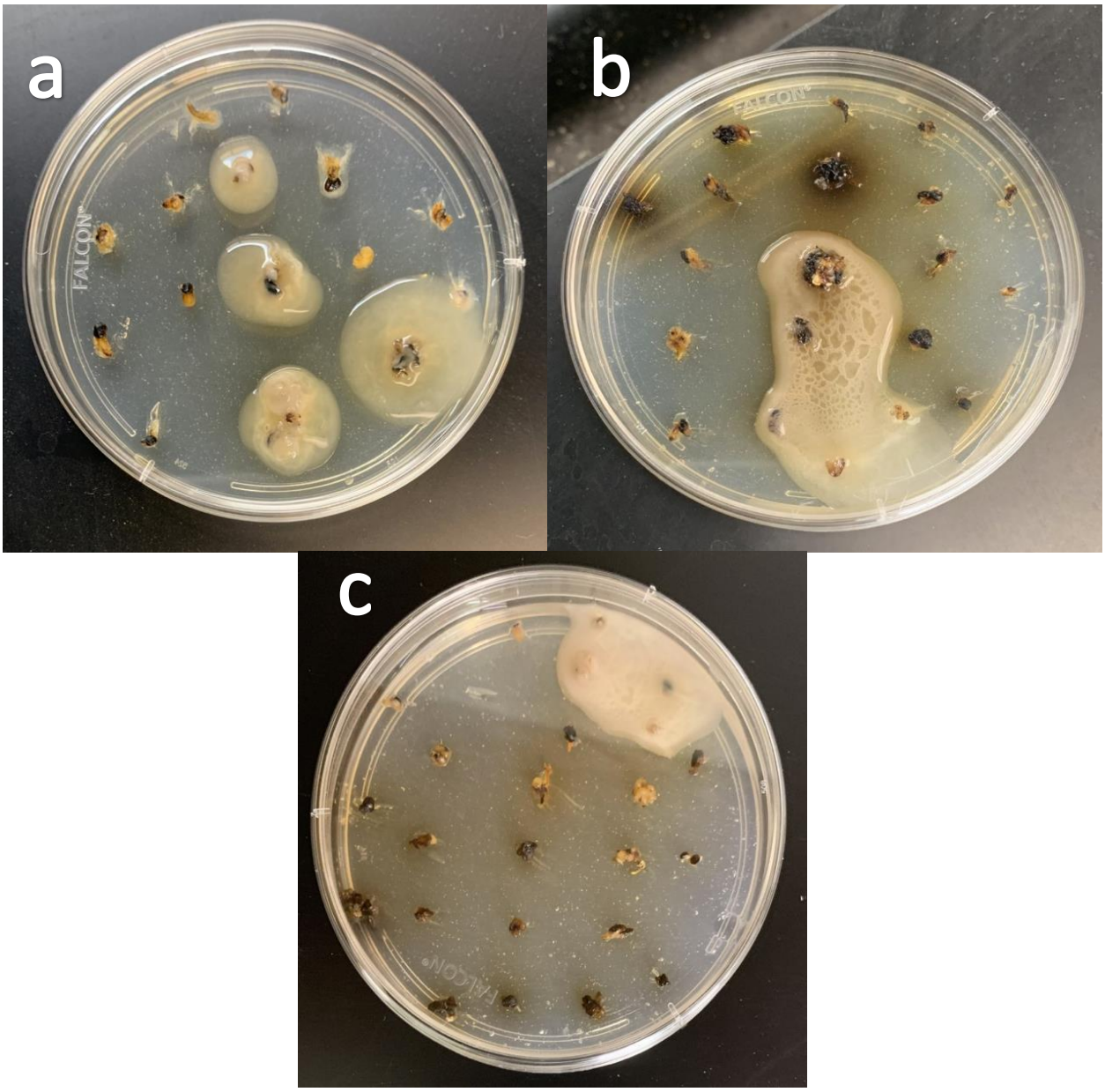


**Figure 6. Bacterial overgrowth in embryos inoculated with EHA101**

Overgrowth can start to emerge on individual calluses (a) or by starting from one callus and spreading to others present in the growth media (b). Increasing the concentration of antibiotics appeared to limit bacterial overgrowth of EHA101, but did not completely eliminate it (c).

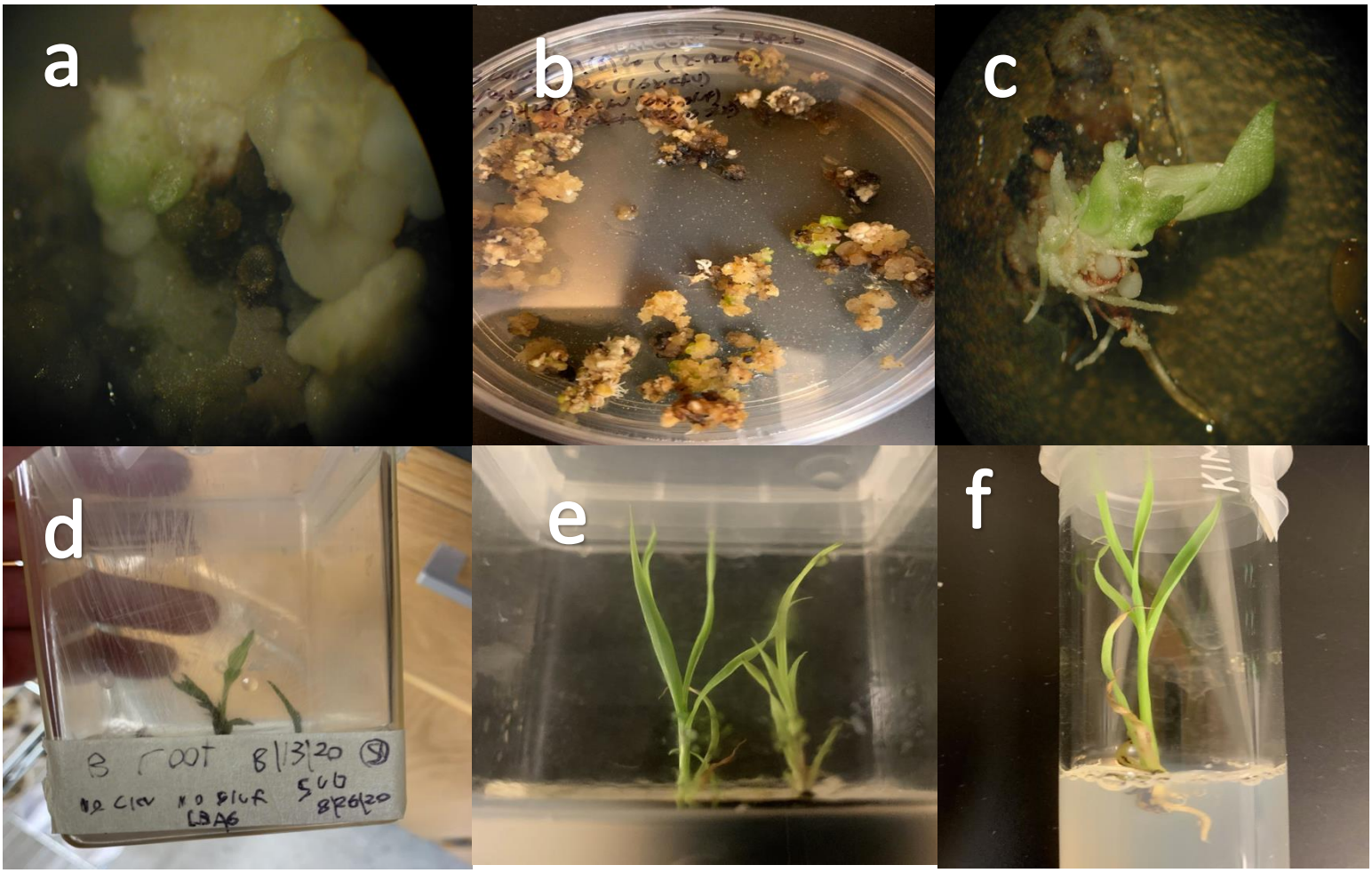


**Figure 7. Green spots on inoculated callus regenerated into full viable plant**

Green spots on callus can be seen under microscope (a) that grow larger (b) until they were able to produce a viable shoot (c). once viable shoots develop one to two leaves, they were moved into magenta boxes (d) with root induction media (RM). Once shoots developed three or more leaves (e), they were isolated in tubes with root induction media (f) to allow for more space to grow, as well as eliminate any chance that plants would compete in vitro. Only embryos treated with LBA4404 managed to create viable shoots.

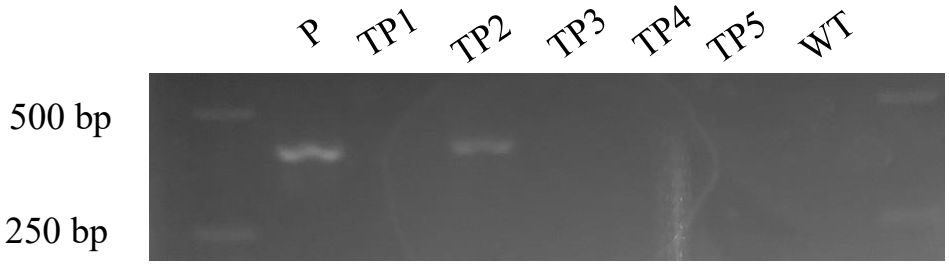


**Figure 8. Gel electrophoresis analysis of *GFP* primer amplified DNA**

The length of the GFP DNA fragment is 412 base pairs. DNA amplified with *GFP* primers gave one adequate result. Transgenic line 2 (TP2) was the only line to be confirmed with *GFP* DNA fragment in the extracted plasmid (p).

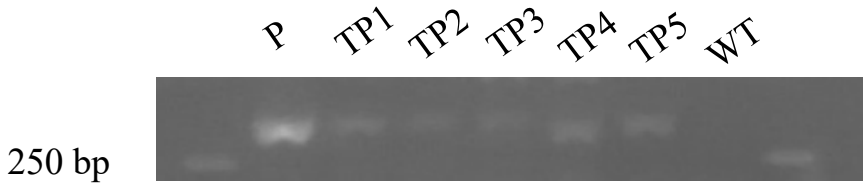


**Figure 9. Gel electrophoresis analysis of *CENH3* primer amplified DNA**

The length of the *CENH3* DNA fragment is 332 base pairs. DNA amplified with *CENH3* primers gave three adequate results. Two of these gel results showed all 5 plants (TP1-5) were transgenic, and one result where four out of the five plants were transgenic except for transgenic line 4 (TP4) when compared with plasmid DNA (p) and wildtype DNA (WT). Other gel results are not shown.

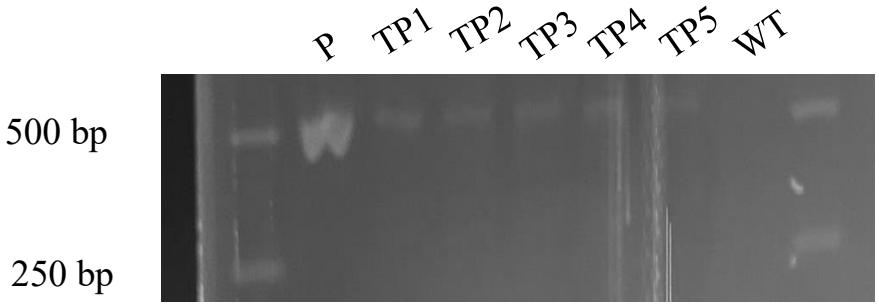


**Figure 10. Gel electrophoresis analysis of *BAR* primer amplified DNA**

The length of the *BAR* DNA fragment is 530 base pairs. DNA amplified with *BAR* primers gave four adequate results. All four of these gel results showed all five plants (TP1-5) were transgenic (a-d) when compared with plasmid DNA (p) and wildtype DNA (WT). Other gel results are not shown.

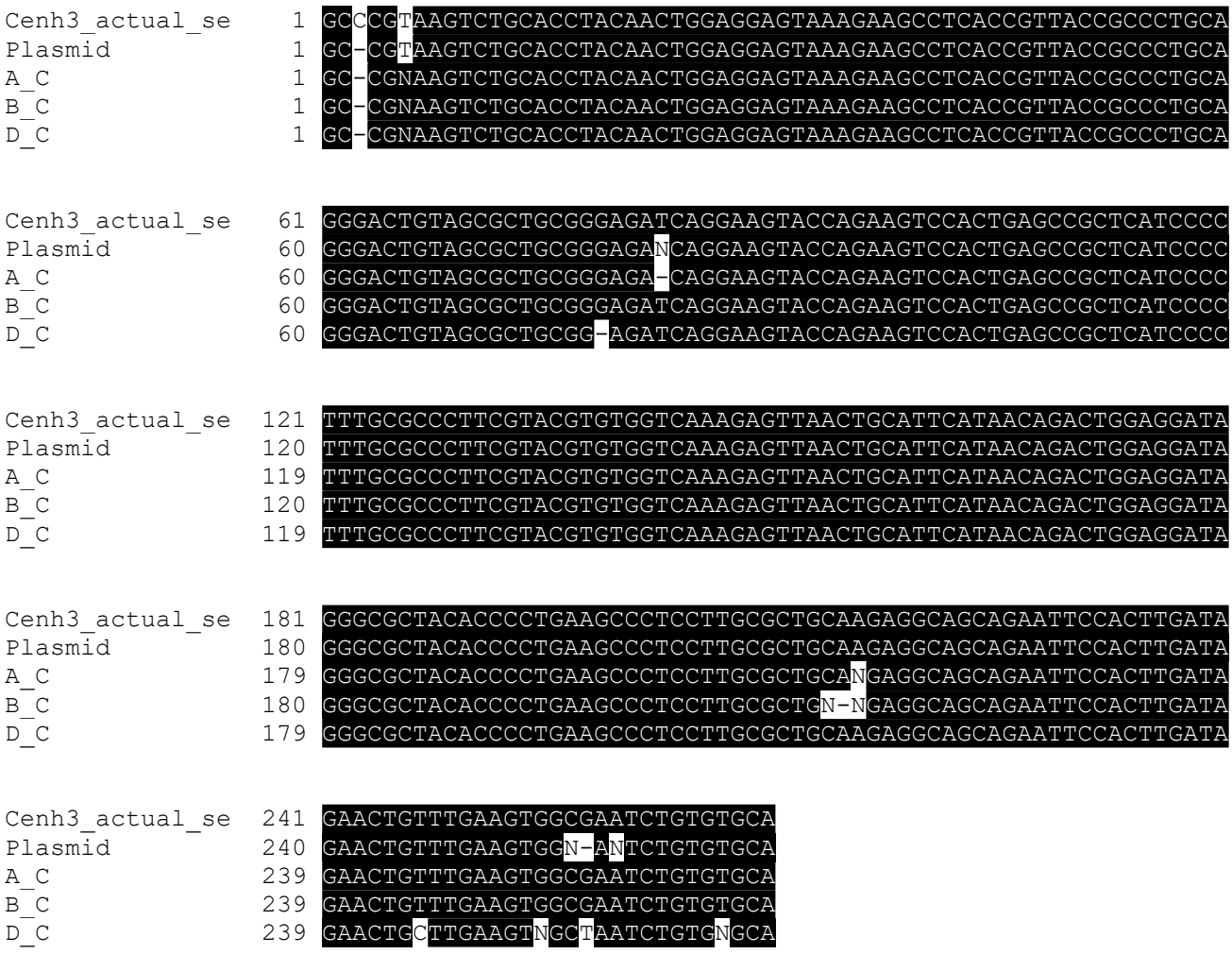


**Figure 11. Comparison of three transgenic plants to CENH3 construct and plasmid prior to corrections**

Potentially transgenic lines A, B, and D were aligned with plasmid extracted from *Agrobacterium* and *CENH3* construct sequence. Prior to errors being corrected, these three plants showed a 98 to 99% match with construct DNA. Errors were later corrected by analyzing associated chromatogram data and replacing errors with closest readings of base pairs.

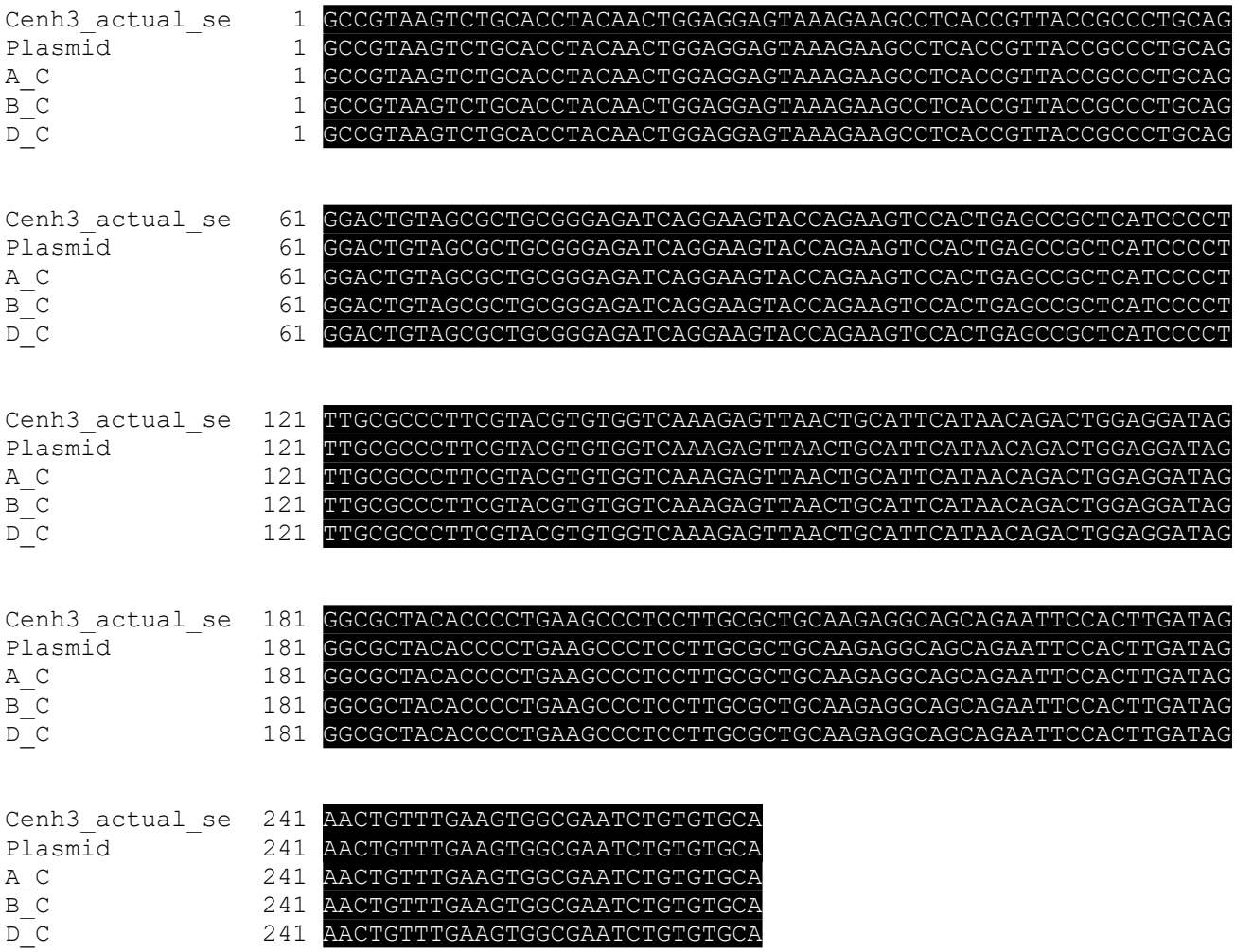


**Figure 12. Comparison of three transgenic plants to CENH3 construct and plasmid with included corrections**

Potentially transgenic lines A, B, and D were aligned with plasmid extracted from *Agrobacterium* and *CENH3* construct sequence. Using associated chromatogram data, errors were replaced with closest readings of base pairs. After corrections were included in the alignment, all three plants showed an 100% match to extracted construct DNA.

## Table 1.

## Altered "B" media compositions used for callus induction and full plant regeneration in the transformation system of sorghum (*Sorghum* bicolor)

| Medium Components | Concentration of components | | | | | | |
|---|---|---|---|---|---|---|---|
| | Units per liter | Inoculation (IM) | Co-cultivation (CM) | Resting (RM) | Callus Induction (CIM) | Shoot Induction (SM) | Root Induction (RM) |
| MS salts | g | 4.3 | 4.3 | 4.3 | 4.3 | 4.3 | 4.3 |
| MES | g | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Proline | g | - | 0.7 | 1.0 | 1.0 | - | - |
| 2,4-D | mg | 1.5 | 1.5 | 1.5 | 1.5 | - | - |
| Sucrose | g | 68.5 | 20 | 30 | 30 | 30 | 30 |
| Glucose | g | 36 | 10 | - | - | - | - |
| Vitamin B5 (100X) | ml | 10 | 10 | 10 | 10 | 10 | 10 |
| Ascorbic acid | mg | - | 10 | - | - | - | - |
| BAP | mg | - | - | - | - | 1 | - |
| IAA | mg | - | - | - | - | 1 | - |
| IBA | mg | - | - | - | - | - | 1 |
| Agar | g | - | 8 | 8 | 8 | 8 | 8 |
| PVPP | g | - | 10 | 10 | 10 | 10 | 10 |
| AS | µM | 100 | 100 | - | - | - | - |
| $CuSO_4$ | mg | - | - | 0.16 | 0.16 | 0.16 | 0.16 |
| Asparagine | g | - | - | 1 | 1 | - | - |
| $KH_2PO_4$ | g | - | - | 1 | 1 | - | - |
| Clavacillin | mg | - | - | 500 | 375 | 375 | - |
| Glufosinate | mg | - | - | - | 2.5 | 2.5 | - |
| pH | | 5.2 | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 |
| Timeframe | | 10-15 min | 3 days | 10 days | 2 weeks | 4-8 weeks | 3-4 weeks |

## Table 2.

## Original "B" media compositions

| Medium Components | Concentration of components | | | | | | |
|---|---|---|---|---|---|---|---|
| | Units per liter | Inoculation (IM) | Co-cultivation (CM) | Resting (RM) | Callus Induction (CIM) | Shoot Induction (SM) | Root Induction (RM) |
| MS salts | g | 4.3 | 4.3 | 4.3 | 4.3 | 4.3 | 4.3 |
| MES | g | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Proline | g | - | 0.7 | 1.0 | 1.0 | - | - |
| 2,4-D | mg | 1.5 | 1.5 | 1.5 | 1.5 | - | - |
| Sucrose | g | 68.5 | 20 | 30 | 30 | 30 | 30 |
| Glucose | g | 36 | 10 | - | - | - | - |
| Vitamin B5 (100X) | ml | 10 | 10 | 10 | 10 | 10 | 10 |
| Ascorbic acid | mg | - | 10 | - | - | - | - |
| BAP | mg | - | - | - | - | 1 | - |
| IAA | mg | - | - | - | - | 1 | - |
| IBA | mg | - | - | - | - | - | 1 |
| Agar | g | - | 8 | 8 | 8 | 8 | 8 |
| PVPP | g | - | 10 | 10 | 10 | 10 | 10 |
| AS | μM | 100 | 100 | - | - | - | - |
| $CuSO_4$ | mg | - | - | 0.16 | 0.16 | 0.16 | 0.16 |
| Asparagine | g | - | - | 1 | 1 | - | - |
| $KH_2PO_4$ | g | - | - | 1 | 1 | - | - |
| Cefotaxime | mg | - | - | 400 | 300 | 300 | 300 |
| Glufosinate | mg | - | - | - | 2.5 | 2.5 | - |
| pH | | 5.2 | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 |
| Timeframe | | 10-15 min | 3 days | 10 days | 10-15 days | 4-6 weeks | 2-3 weeks |

## Table 3.

## Original "S" media compositions

| Component | Osmotic medium (CIM-OS) | Pre-selection Medium (CIM-PS) | Callus induction (CIM-G25) | Shoot induction (SIM-G35) | Shoot Regeneration (SRM-G25) | Shoot out growth (SOG-G15) | Root Induction (RIM-G15) |
|---|---|---|---|---|---|---|---|
| MS (g/l) | 4.33 | 4.33 | 4.33 | 4.33 | 4.33 | 2.2 | 4.33 |
| **Growth Regulators** | | | | | | | |
| 2,4-D (mg/l) | 1.0 | 1.0 | 1.0 | 0.5 | - | - | - |
| BAP (mg/l) | 0.5 | 0.5 | 0.5 | 1.0 | 1.0 | - | - |
| TDZ (mg/l) | - | - | - | - | 0.5 | - | - |
| NAA (mg/l) | - | - | - | - | - | - | 1.0 |
| IAA (mg/l) | - | - | - | - | - | - | 1.0 |
| IBA (mg/l) | - | - | - | - | - | - | 1.0 |
| **Amino Acids** | | | | | | | |
| L-Proline (g/l) | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | - | - |
| **Antioxidants** | | | | | | | |
| L-Lipoic acid (mg/l) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| L-cysteine (mg/l) | - | 50 | - | - | - | - | - |
| Ascorbic acid (mg/l) | - | 15 | - | - | - | - | - |
| **Others** | | | | | | | |
| Peptone (g/l) | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 |
| Myo-inositol (g/l) | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| CuSO4 (mg/l) | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| PVP (g/l) | - | - | - | - | - | - | 2 |
| **Sugars** | | | | | | | |
| Maltose (g/l) | - | 30 | 30 | 30 | 30 | - | - |
| Sucrose (g/l) | - | - | - | - | - | 15 | 15 |
| Mannitol (g/l) | 36.4 | - | - | - | - | - | - |
| Sorbitol (g/l) | 36.4 | - | - | - | - | - | - |
| **Antibiotics** | | | | | | | |
| Geneticin (mg/l) | - | - | 25 | 30 | 25 | 15 | 15 |
| **Solidifying agent** | | | | | | | |
| Type A agar (g/l) | 4.25 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| pH | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 |
| Culture duration | 24 hours | 3-4 days | 4 weeks | 2 weeks | 2 weeks | 2 weeks | 2-3 weeks |

**Table 4. Callus induction and regeneration ratios of "B" media**

| Ratio of callus induction/regeneration of 1500 embryos into full plants when using "B" media | | | |
|---|---|---|---|
| **Number of Embryos Tested** | **Number of calluses developed from 1500 embryos** | **Number of green spots emerged on 1140 developed calluses** | **Number of shoots emerging from 748 green spots** |
| **1500 (100% of embryos)** | **1140 calluses induced (76% of embryos)** | **748 green spots on induced callus (49.9% of embryos)** | **258 shoots on emerged green spots (17.6% of embryos)** |

1500 immature embryos were tested to determine rates of callus induction and shoot regeneration for four weeks. Two weeks after embryos were placed on callus induction media (CIM), 1140 were induced to form callus (a). After two weeks on CIM, these calluses were moved onto regeneration media (SM). After two weeks on SM, 748 of those induced calluses grew green spots (b) and 258 of those green spots were viable shoots (c).

**Table 5.**

**Number of embryos inoculated and regeneration ratios of *Agrobacterium* strains**

| *Agrobacterium* strain | Number of embryos inoculated | Number of green callus spots emerged | Number of shoots emerged | Number of shoots that grew roots | Number of plants acclimated in soil | Number of plants confirmed transgenic |
|---|---|---|---|---|---|---|
| EHA101 | 3575-4185 | 16 | 0 | 0 | 0 | 0 |
| LBA4404 | 3429-4039 | 38 | 13 | 7 | 5 | 3 |