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(54) **MERISTEM TRANSFORMATION METHOD USING A LIQUID SELECTION MEDIUM**

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C12N 15/82 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/8205*** (2013.01)

(57) **ABSTRACT**

The present invention provides efficient methods for transforming plant explants with an exogenous nucleic acid by culturing the explant with the exogenous nucleic acid on a liquid selection medium to select for a transformed explant. Transformed *Cannabis* or other plant explants are also provided. Plants and seeds produced by the methods are also provided and includes production of T1 plants.

FIG. 1

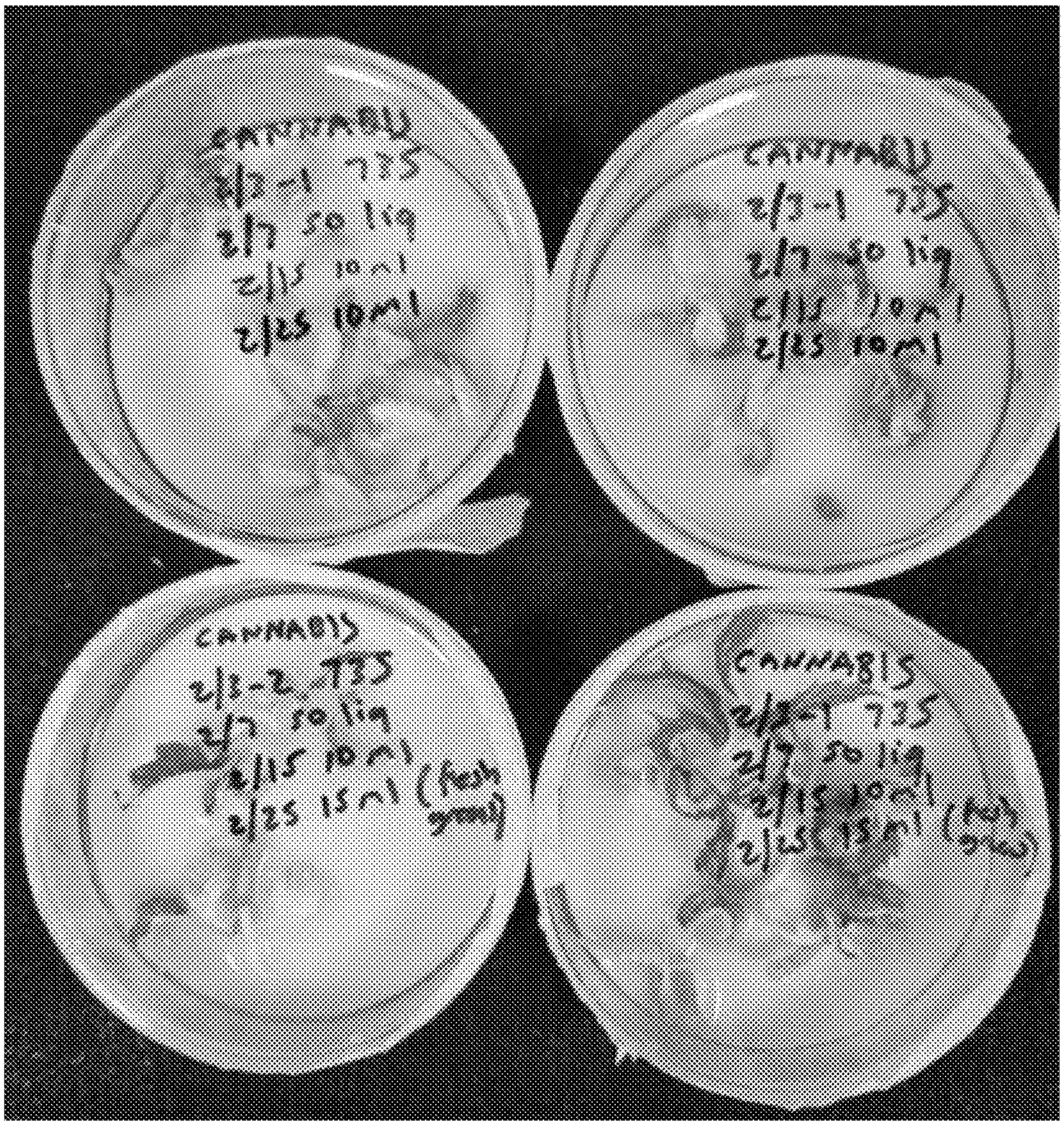


FIG. 2A

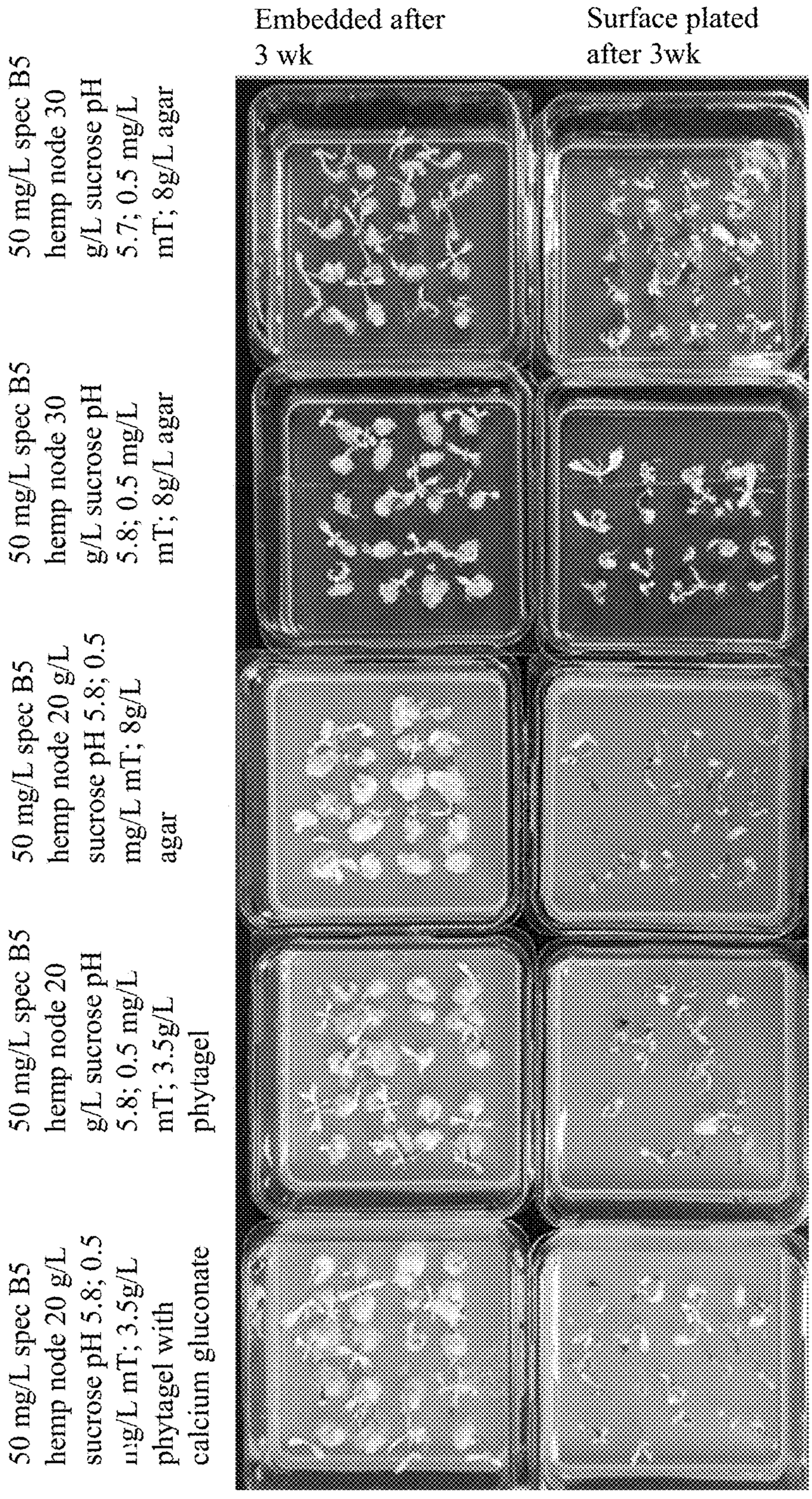

Embedded after 3 wk
Surface plated after 3wk
50 mg/L spec B5 hemp node 30 g/L sucrose pH 5.7; 0.5 mg/L mT; 8g/L agar
50 mg/L spec B5 hemp node 20 g/L sucrose pH 5.8; 0.5 mg/L mT; 8g/L agar
50 mg/L spec B5 hemp node 20 g/L sucrose pH 5.8; 0.5 mg/L mT; 3.5g/L phytagel
50 mg/L spec B5 hemp node 20 g/L sucrose pH 5.8; 0.5 mg/L mT; 3.5g/L phytagel with calcium gluconate

FIG. 2B

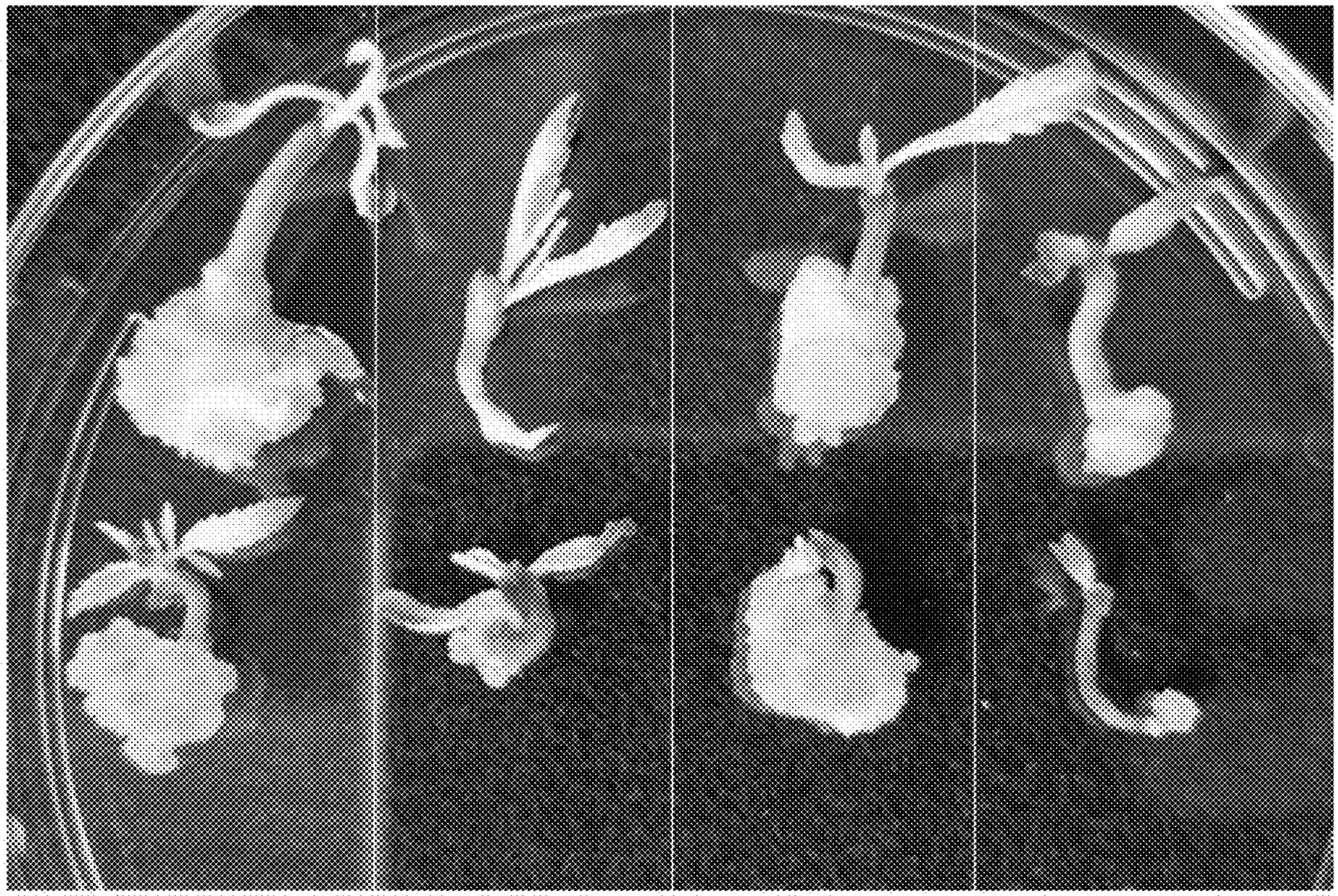

Embedded on spec hemp node media for 3.5 weeks
Surface plated on hemp node media for 3.5 weeks
Embedded hemp node media for 2.5 weeks
Surface plated on hemp node media for 2.5 weeks

FIG. 3A

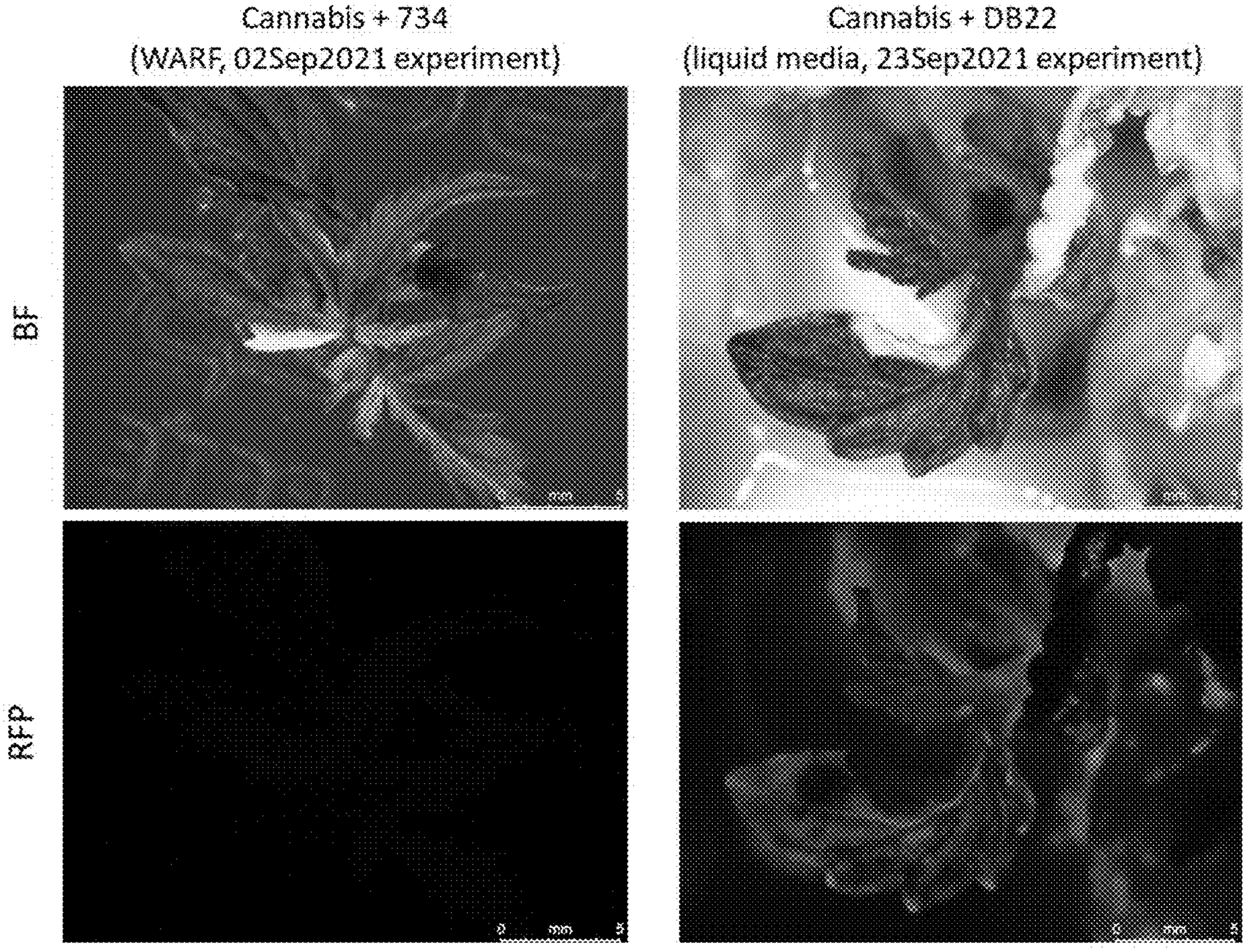

Cannabis + 734
(WARF, 02Sep2021 experiment)
Cannabis + DB22
(liquid media, 23Sep2021 experiment)
BF
RFP
500 μm

FIG. 3B

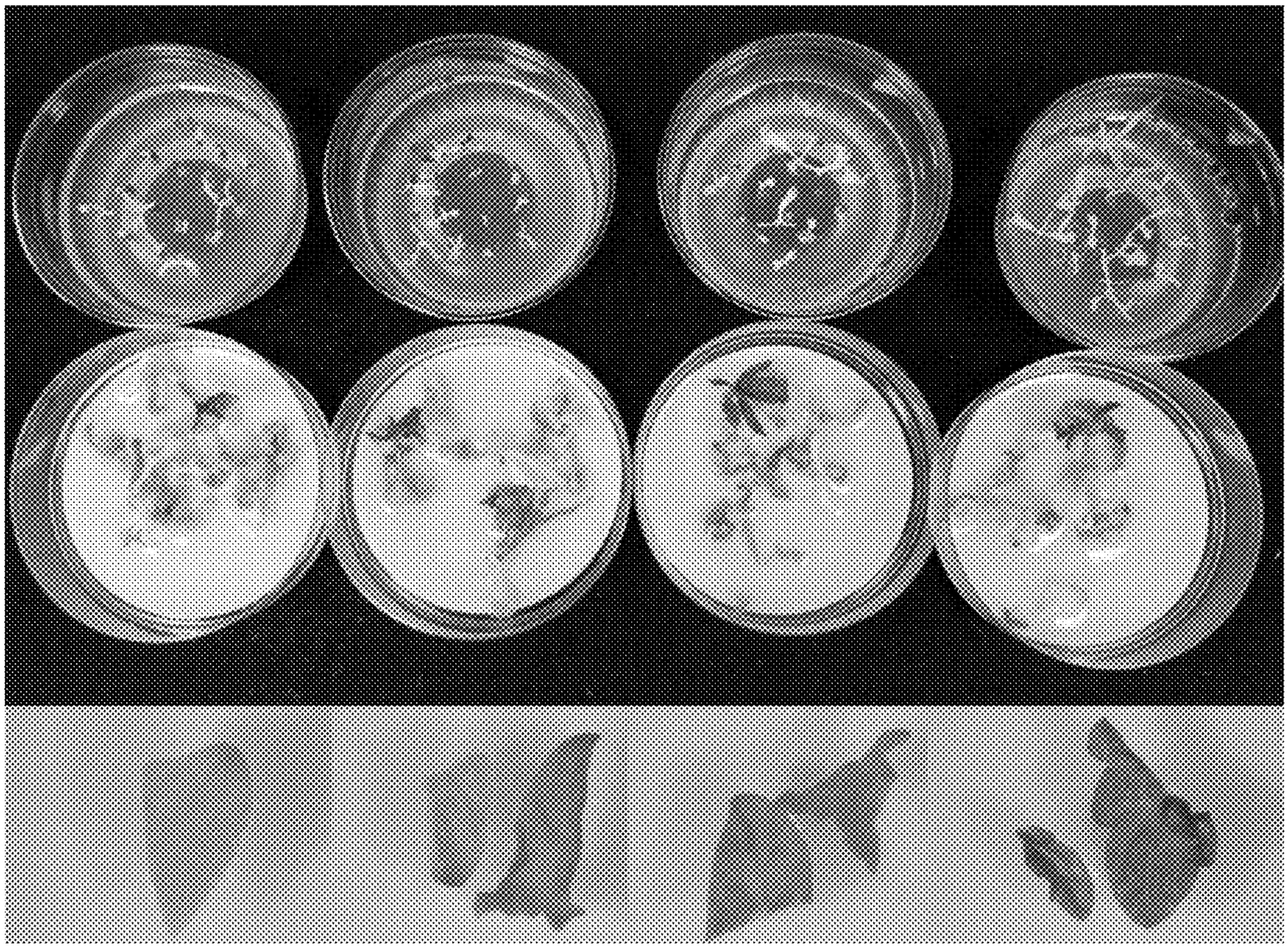


FIG. 4

FIG. 5

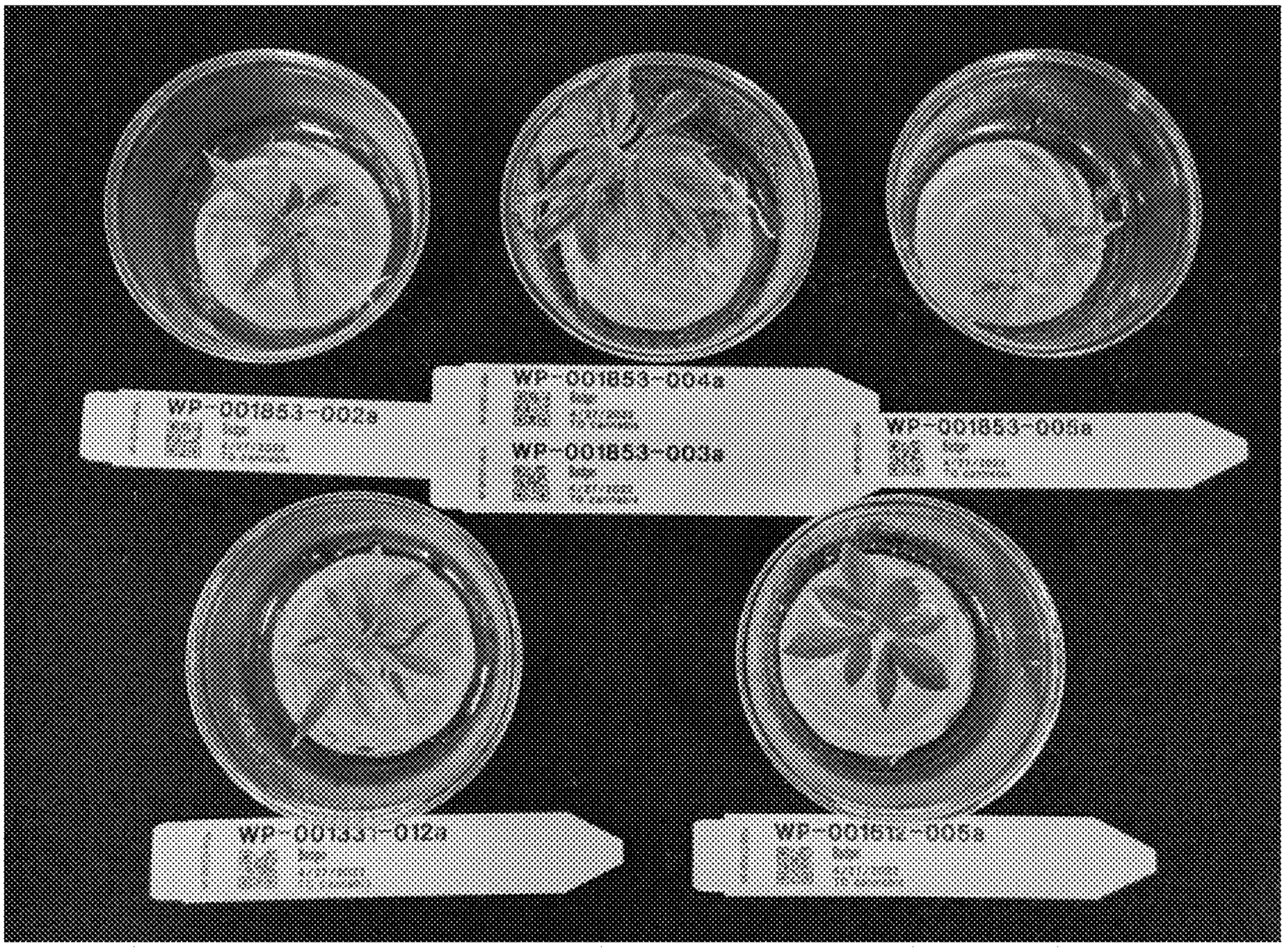
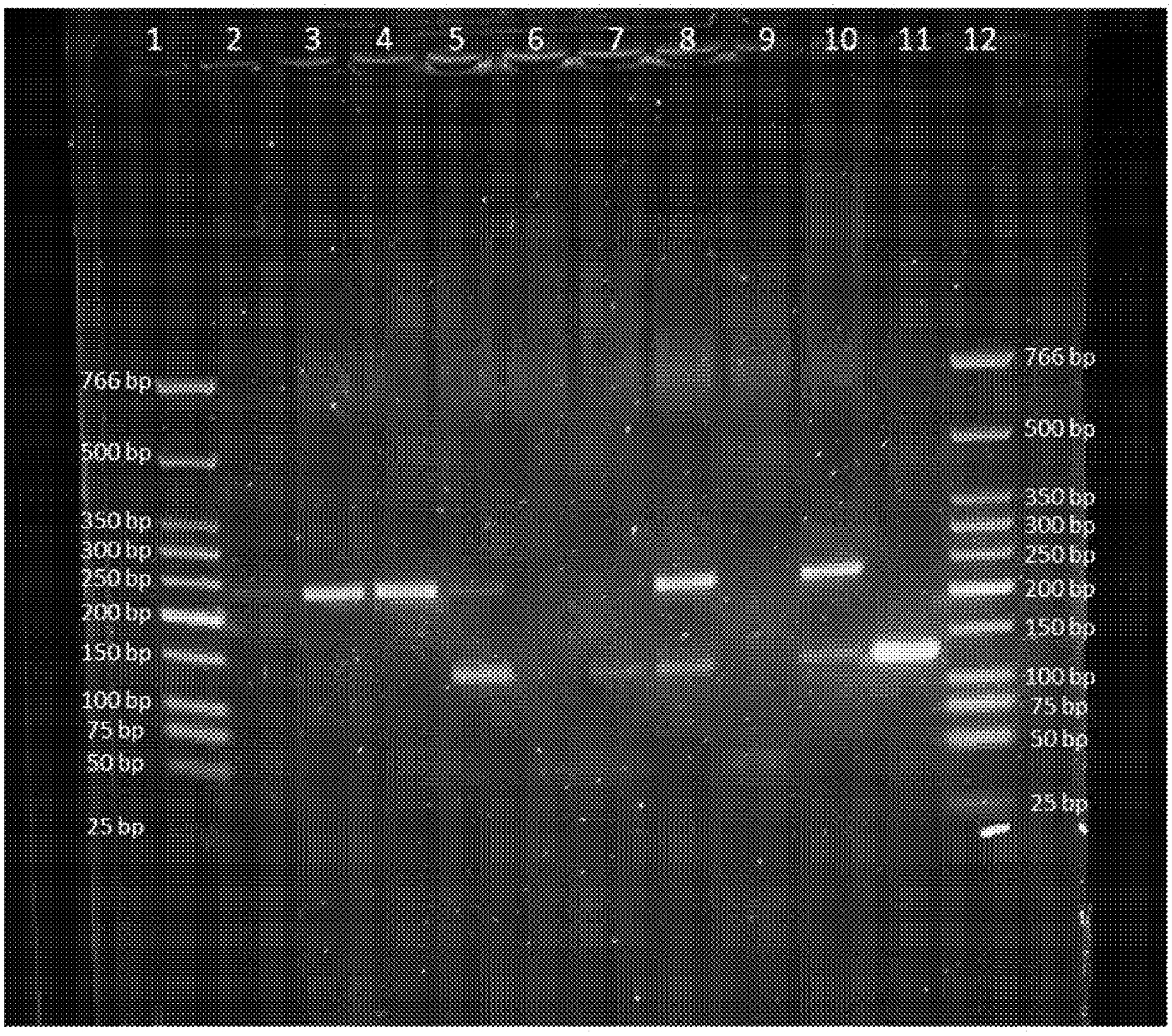

WP-001853-002a
WP-001853-004a
WP-001853-003a
WP-001853-006a
WP-00133-012a
WP-00161-005a

FIG. 6



P140, P141 Cannabis ubiquitin endogenous primers: 233 bp expected amplicon
D80, D81 aadA primers: 129 bp amplicon
Ln 1 = Low molecular weight ladder

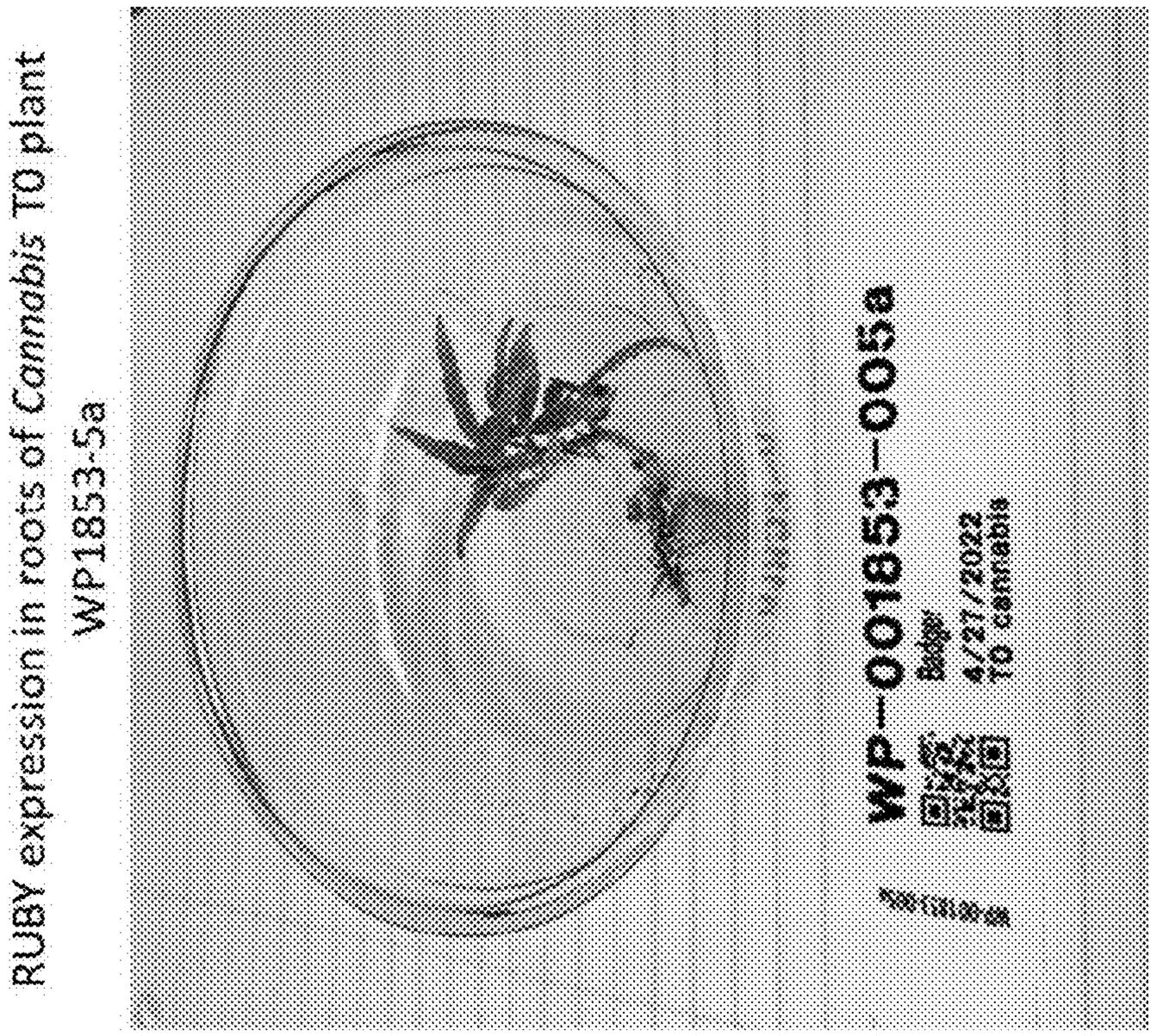
Ln 2 = 50:50 reagent negative control; P140, P141 endogenous + D80,D81 aadA primers
Ln 3= Cannabis root negative control; P140, P141 endogenous + D80,D81 aadA primers

Ln 4 = T0 WP1507-6a roots: P140, P141 endogenous + D80,D81 aadA primers
Ln 5 = T0 WP1612-5a roots: P140, P141 endogenous + D80,D81 aadA primers
Ln 6 = T0 WP1331-12a roots: P140, P141 endogenous + D80,D81 aadA primers
Ln 7 = T0 WP1853-2a roots: P140, P141 endogenous + D80,D81 aadA primers
Ln 8 = T0 WP1853-3a roots: P140, P141 endogenous + D80,D81 aadA primers
Ln 9 = T0 WP1853-5a roots: P140, P141 endogenous + D80,D81 aadA primers

Ln 10 = T1 WP421-1 roots (pos control): P140, P141 endogenous + D80,D81 aadA primers
Ln 11= 2 ng plasmid DNA; P140, P141 endogenous + D80,D81 aadA primers

Ln 12 = Low molecular weight ladder

FIG. 7


RUBY expression in roots of Cannabis T0 plant
WP1853-5a
WP-001853-005a
Badge
4/27/2022
T0 cannabis


GUS expression in roots of Cannabis T0 plant
WP1331-12a

FIG. 8

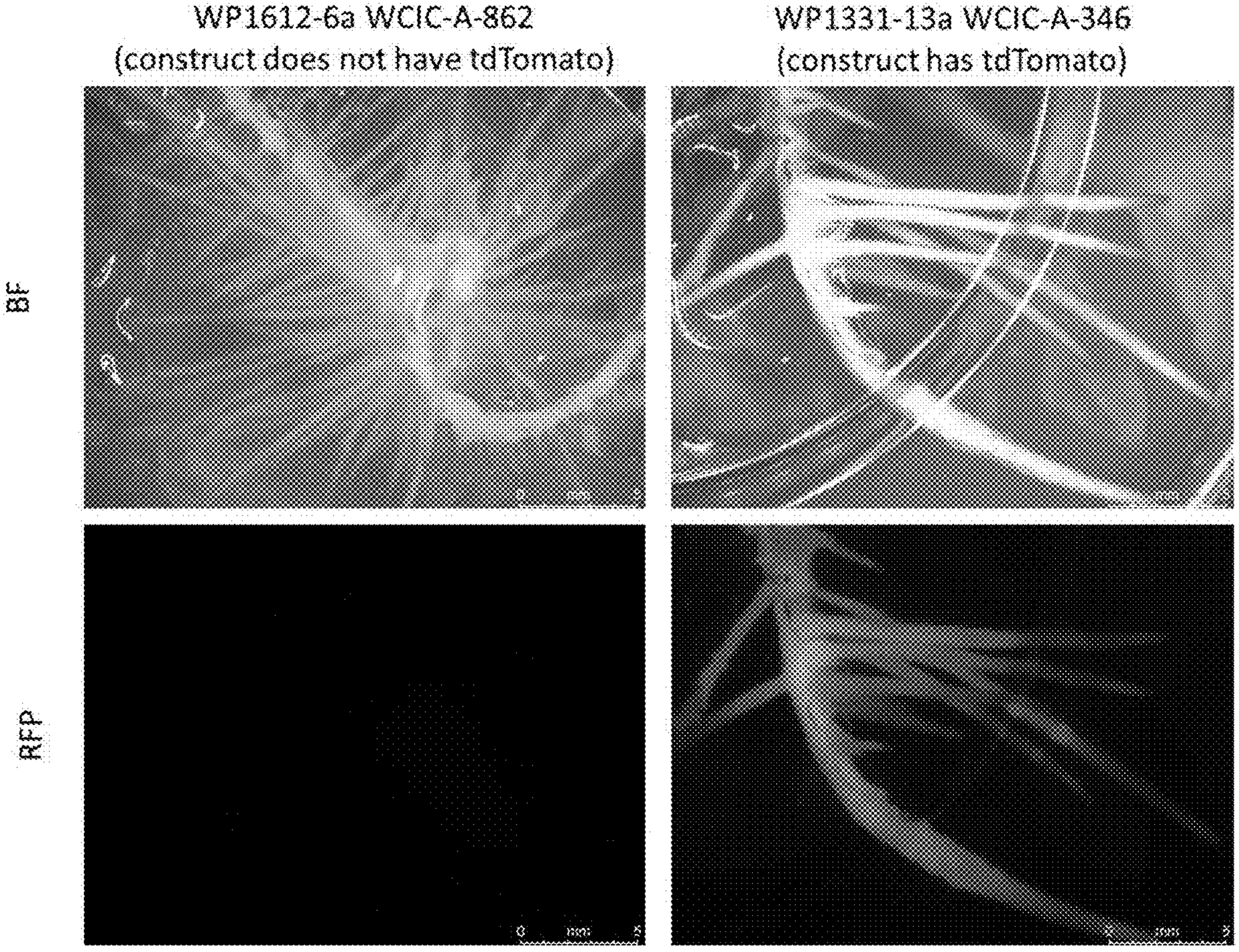

WP1612-6a WCIC-A-862
(construct does not have tdTomato)
WP1331-13a WCIC-A-346
(construct has tdTomato)
BF
RFP
0
1000
2000

FIG. 9

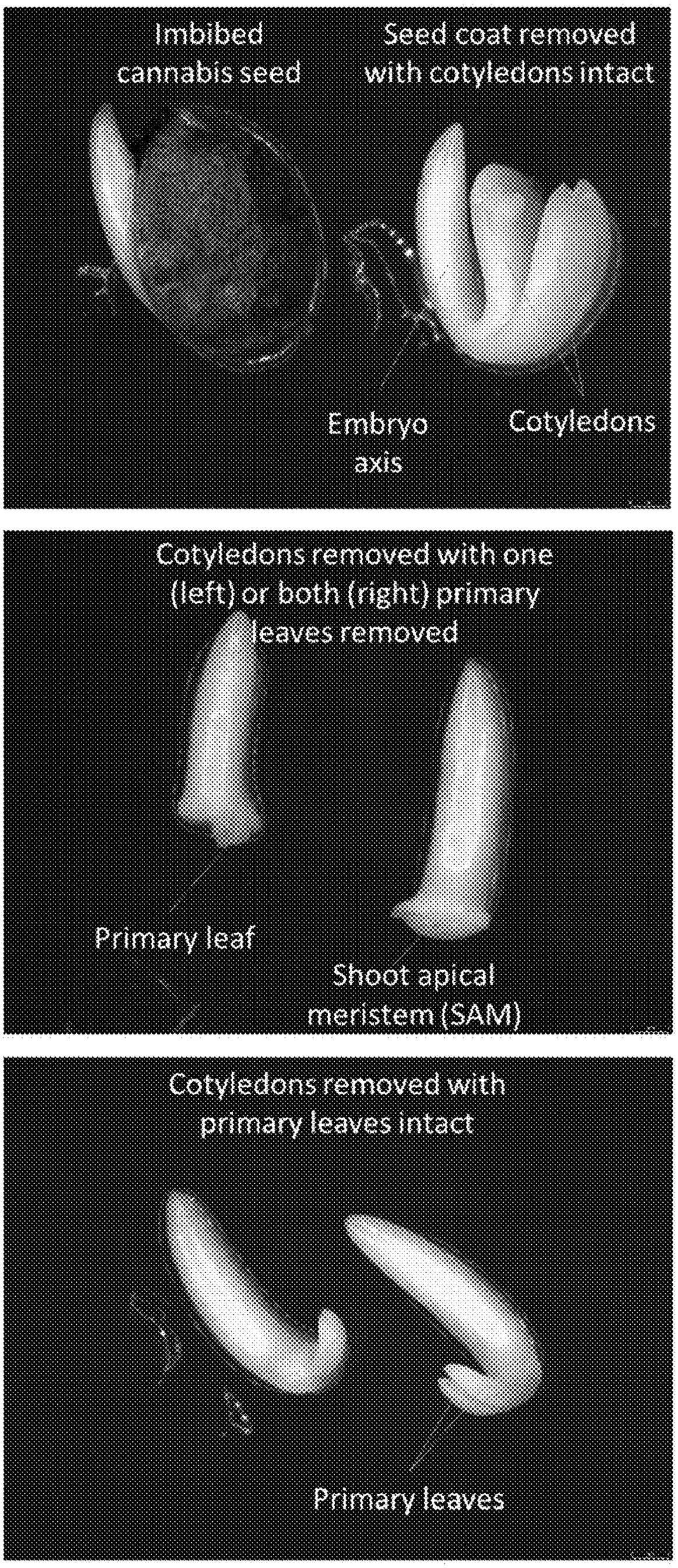

Imbibed cannabis seed
Seed coat removed with cotyledons intact
Embryo axis
Cotyledons
Cotyledons removed with one (left) or both (right) primary leaves removed
Primary leaf
Shoot apical meristem (SAM)
Cotyledons removed with primary leaves intact
Primary leaves

FIG. 10

FIG. 11

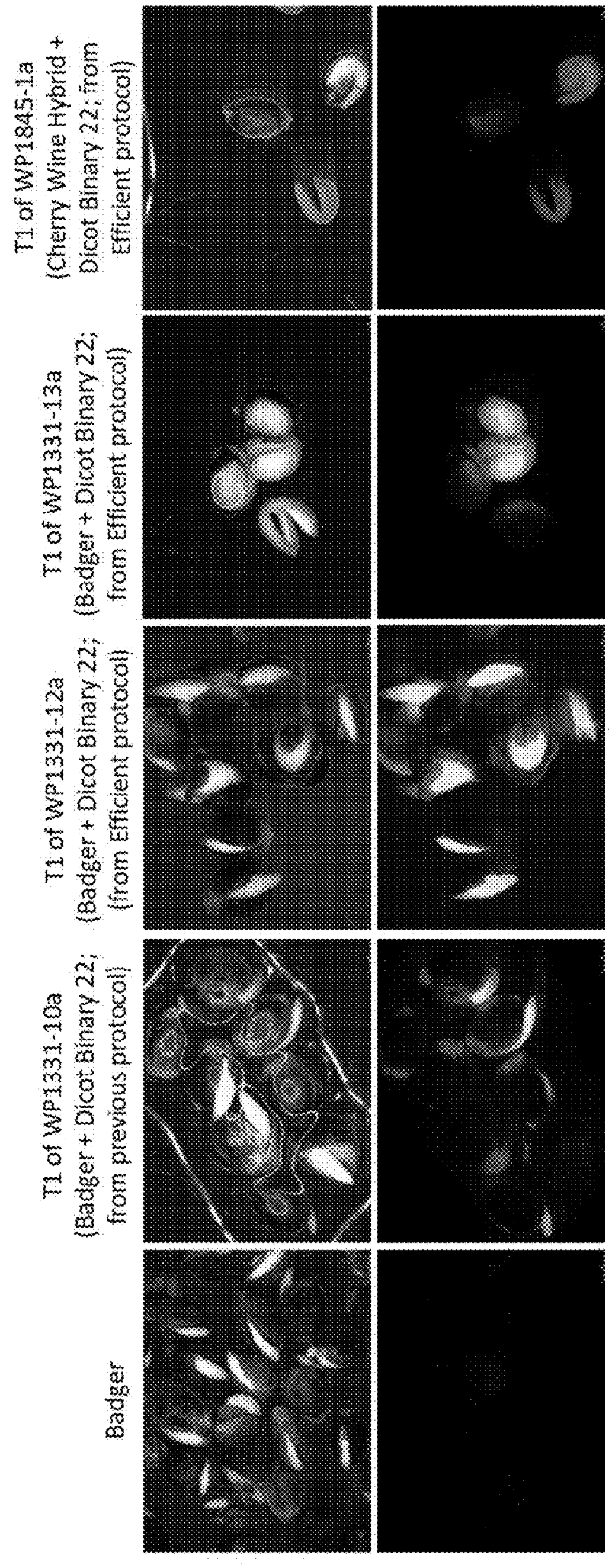

T1 of WP1845-1a
(Cherry Wine Hybrid + Dicot Binary 22; from Efficient protocol)
T1 of WP1331-13a
(Badger + Dicot Binary 22; from Efficient protocol)
T1 of WP1331-12a
(Badger + Dicot Binary 22; from Efficient protocol)
T1 of WP1331-10a
(Badger + Dicot Binary 22; from previous protocol)
Badger
BF
tdTOM

FIG. 12

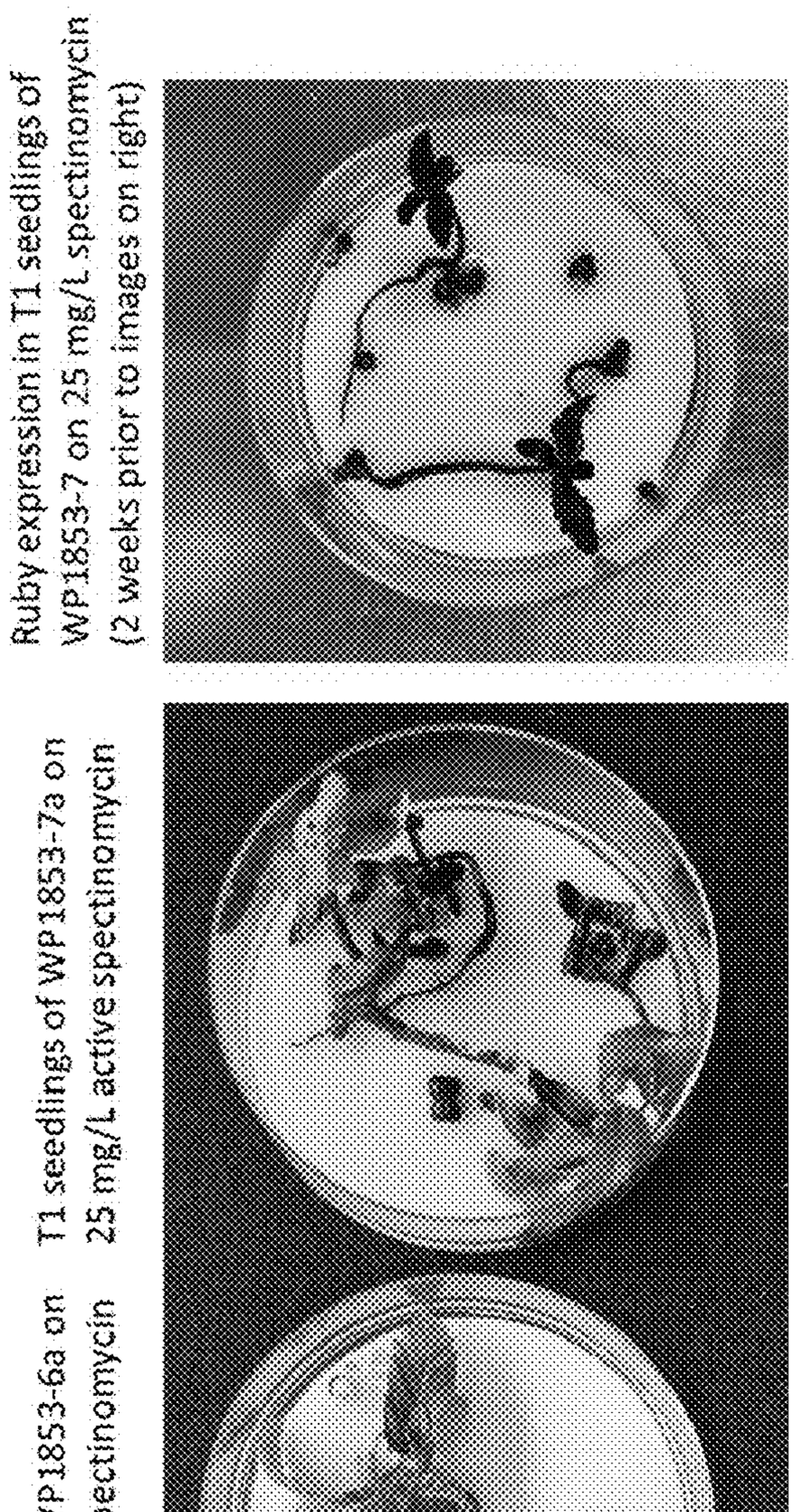

Ruby expression in T1 seedlings of WP1853-7 on 25 mg/L spectinomycin (2 weeks prior to images on right)
Badger seedlings on 25 mg/L active spectinomycin
T1 seedlings of WP1853-6a on 25 mg/L active spectinomycin
T1 seedlings of WP1853-7a on 25 mg/L active spectinomycin

FIG. 13

Species	Genotype/Line	Co-culture	Experiment	Strain / Construct	Terminator aadA	# embryos to Selection	# Greening phenotypes on 50 mg/L spec	Shoots harvested to 10 mg/L spec	T0 plants to GH	TF
Cannabis sativa	Badger 5B	2.25 ml INO + 1 ppm TDZ + nys/TBZ; 23C 16/8 photoperiod	Cannabis 10/27-1	Ar18r12v / DB22	35s	96	3	3	3	3.1%
Cannabis sativa	Badger 5B	2.25 ml INO + 1 ppm TDZ + nys/TBZ; 23C 16/8 photoperiod	Cannabis 10/27-2	Ar18r12v / DB69	EUt	114	3	1	1	0.9%

FIG. 14


WP-002283-001a
Beige
9/17/2023
To Cannabis Quality
WP-002283-002a
Beige
9/17/2023
To Cannabis Quality

FIG. 15

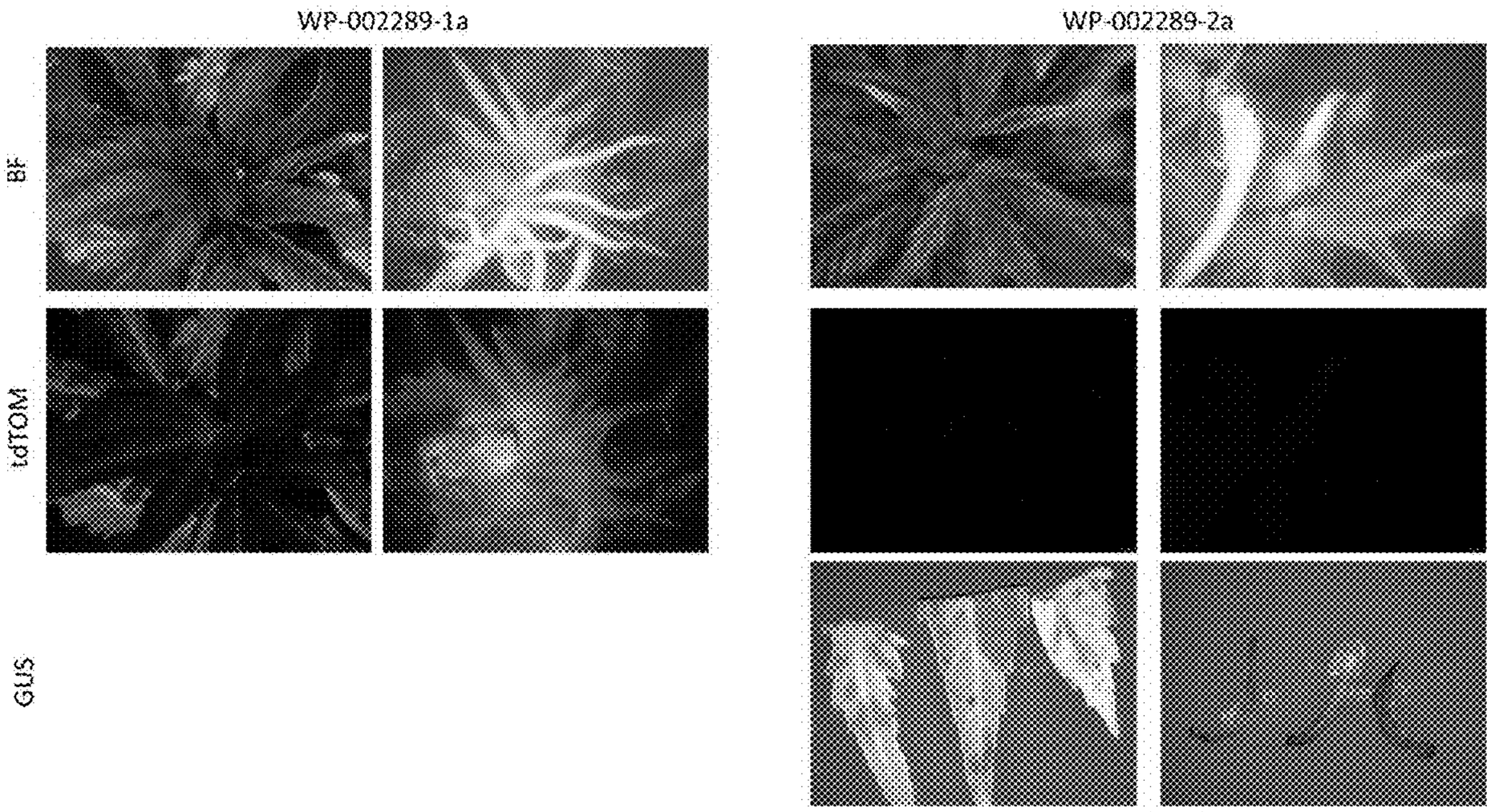


FIG. 16

Species	Genotype/Line	Experiment ID	Strain / Construct	# embryos to Selection	# Greening phenotypes on 50 mg/L spec	Shoots harvested to 10 mg/L spec	T0 plants to GH	TF
Cannabis sativa	Badger 5A	Cannabis 3/17-1	WCIC-A-346 (Ar18r12v / Dicot Binary 22)	105	10	5	4	3.8%
Cannabis sativa	Badger 5A	Cannabis 3/17-2	WCIC-A-1383 (ArPORT1 / Gaantry version of Dicot Binary 22)	208	14	4	3	1.4%
Cannabis sativa	Badger 5A	Cannabis 6/8-2	WCIC-A-1383 (ArPORT1 / Gaantry version of Dicot Binary 22)	136	14	7	4	2.9%

FIG. 17

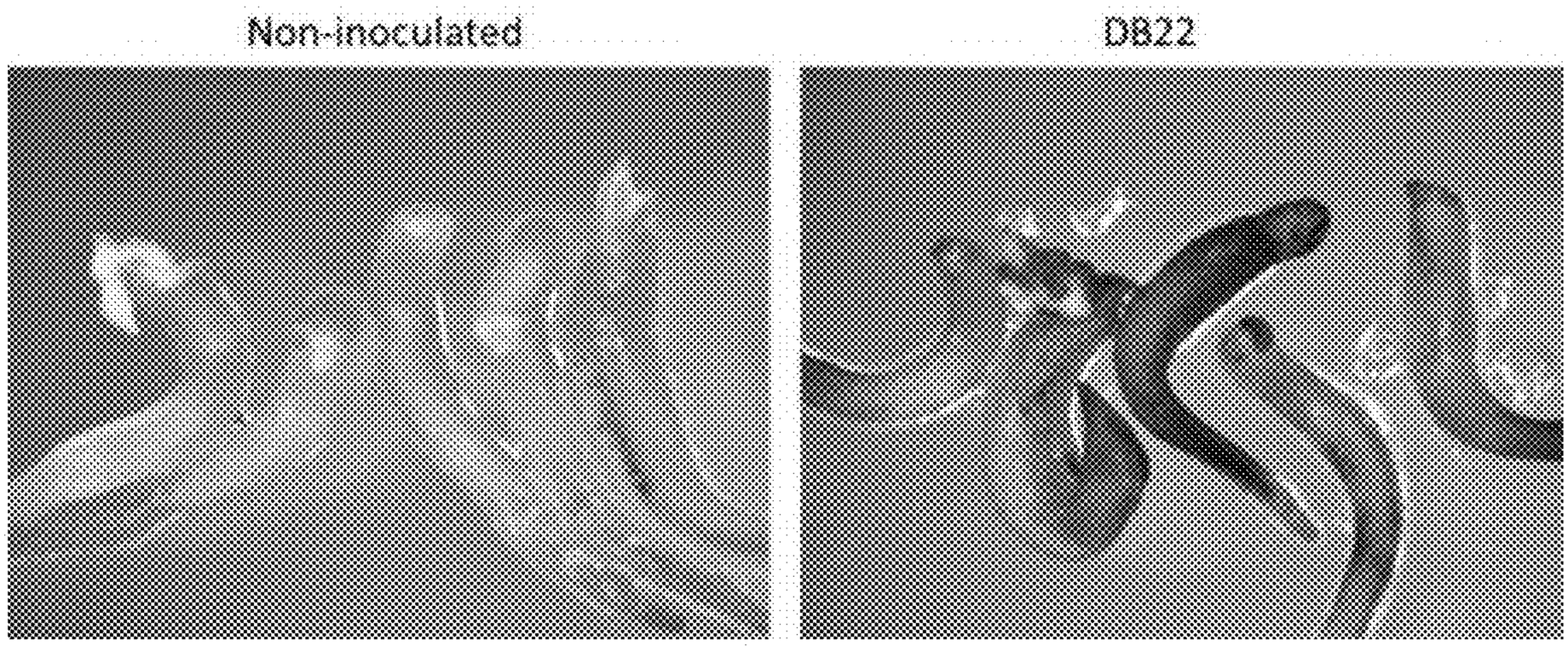


FIG. 18

Okra on non-selective solid B5 media (imaged 3 weeks post-inoculation)

Okra on 50 mg/L spec liquid Hemp node media (imaged 3 weeks post-inoculation)

Okra on non-selective liquid Hemp node media (imaged 3 weeks post-inoculation)

Okra on 25 mg/L spec liquid Hemp node media (imaged 3 weeks post-inoculation)

FIG. 19

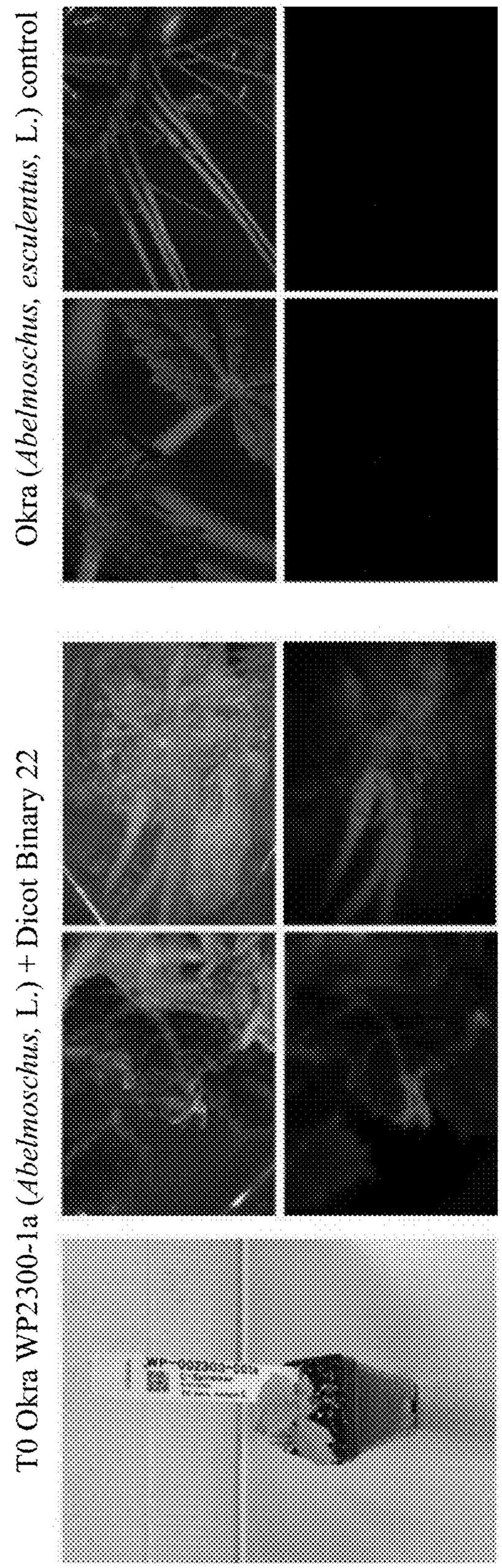

Okra (Abelmoschus, esculentus, L.) control
Okra (Abelmoschus, esculentus, L.) + Dicot Binary 22
T0 Okra WP2300-1a (Abelmoschus, L.)

FIG. 20

FIG. 21

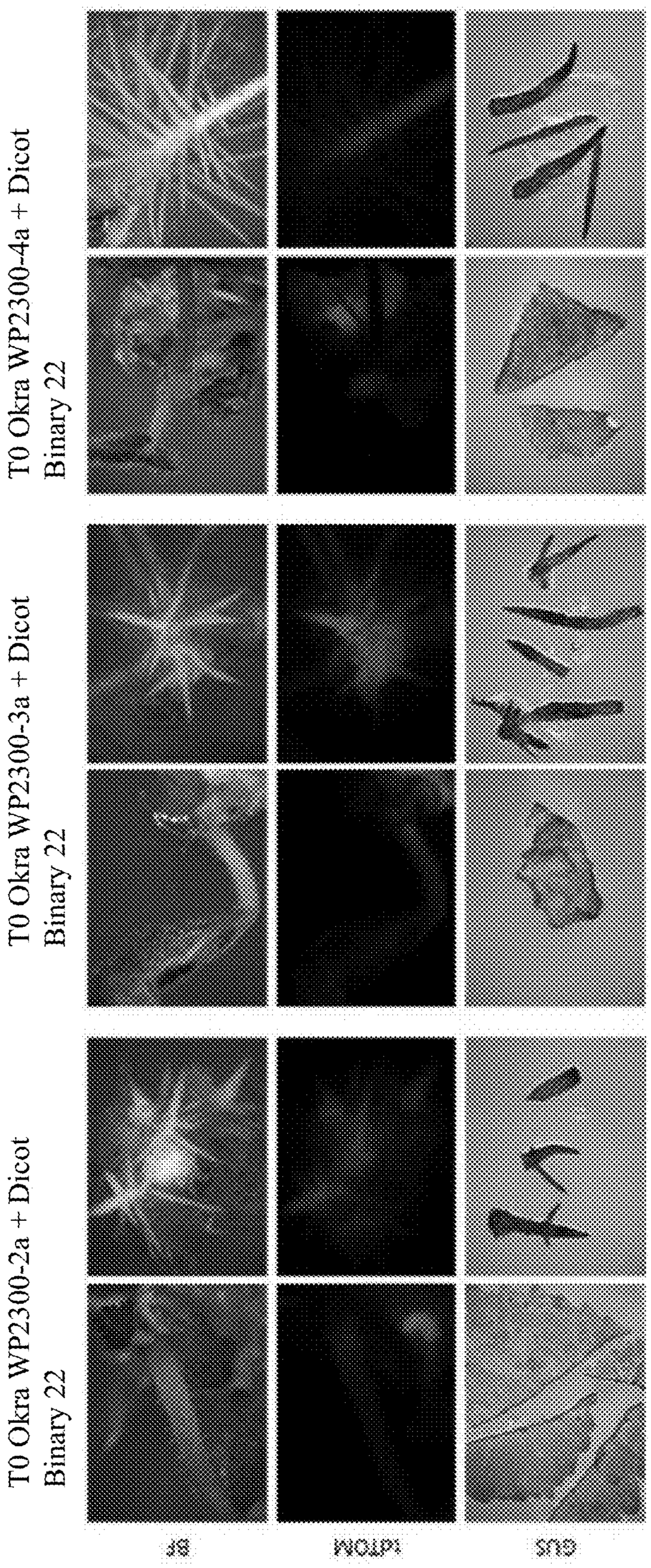

T0 Okra WP2300-4a + Dicot Binary 22
T0 Okra WP2300-3a + Dicot Binary 22
T0 Okra WP2300-2a + Dicot Binary 22
BF
uidA/GUS
GUS

FIG. 22

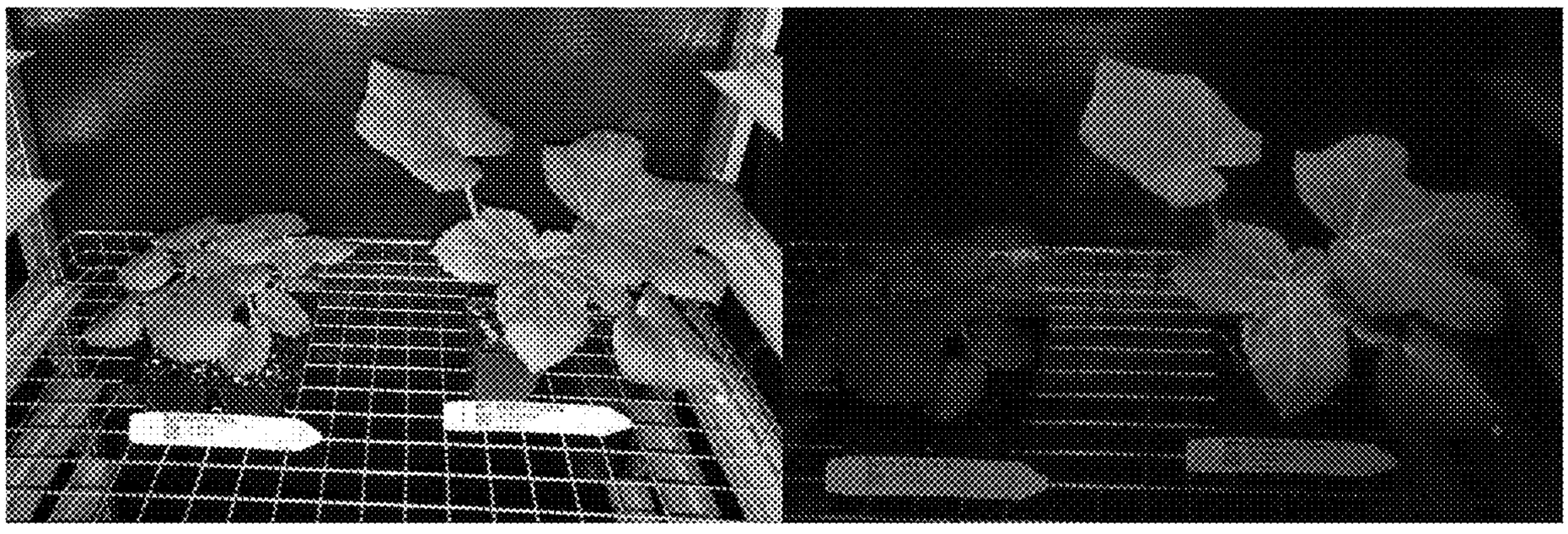


FIG. 23

FIG. 24

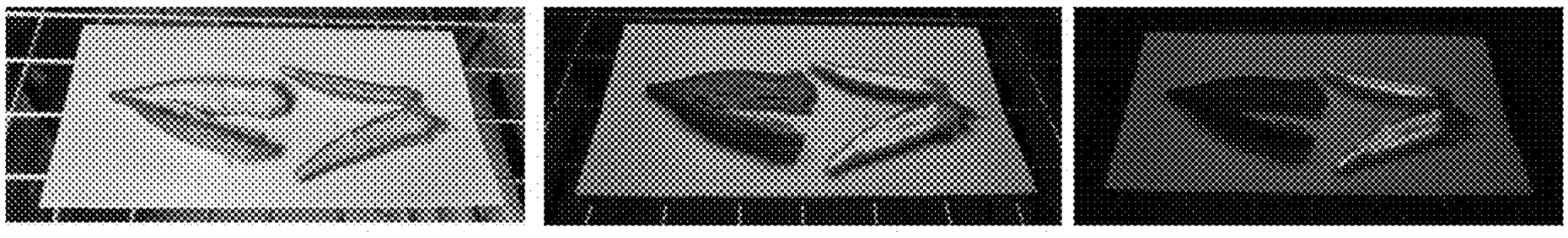


FIG. 25

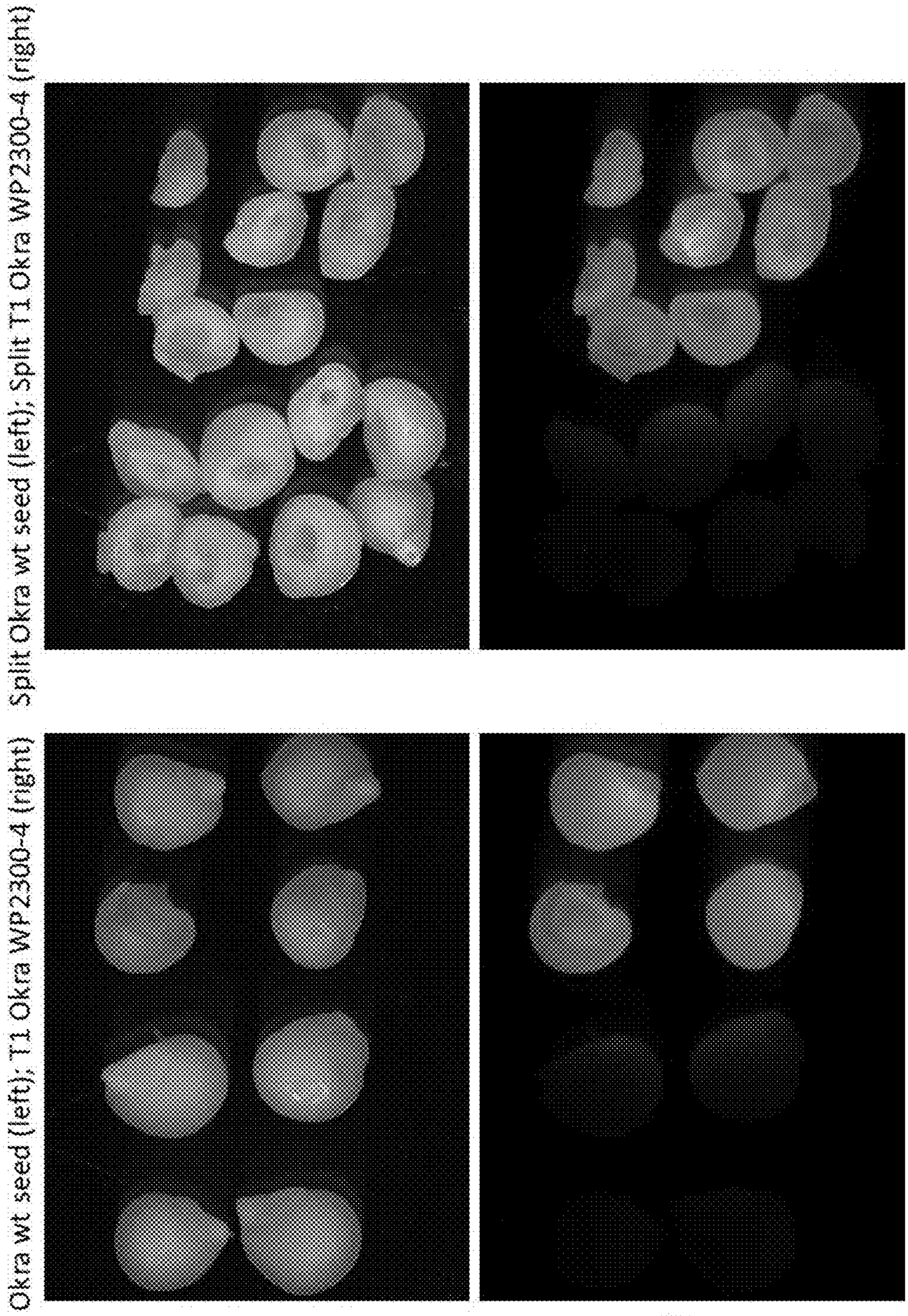

Okra wt seed (left); T1 Okra WP2300-4 (right)
Split Okra wt seed (left); Split T1 Okra WP2300-4 (right)
BF
tdTOM

FIG. 26

FIG. 27

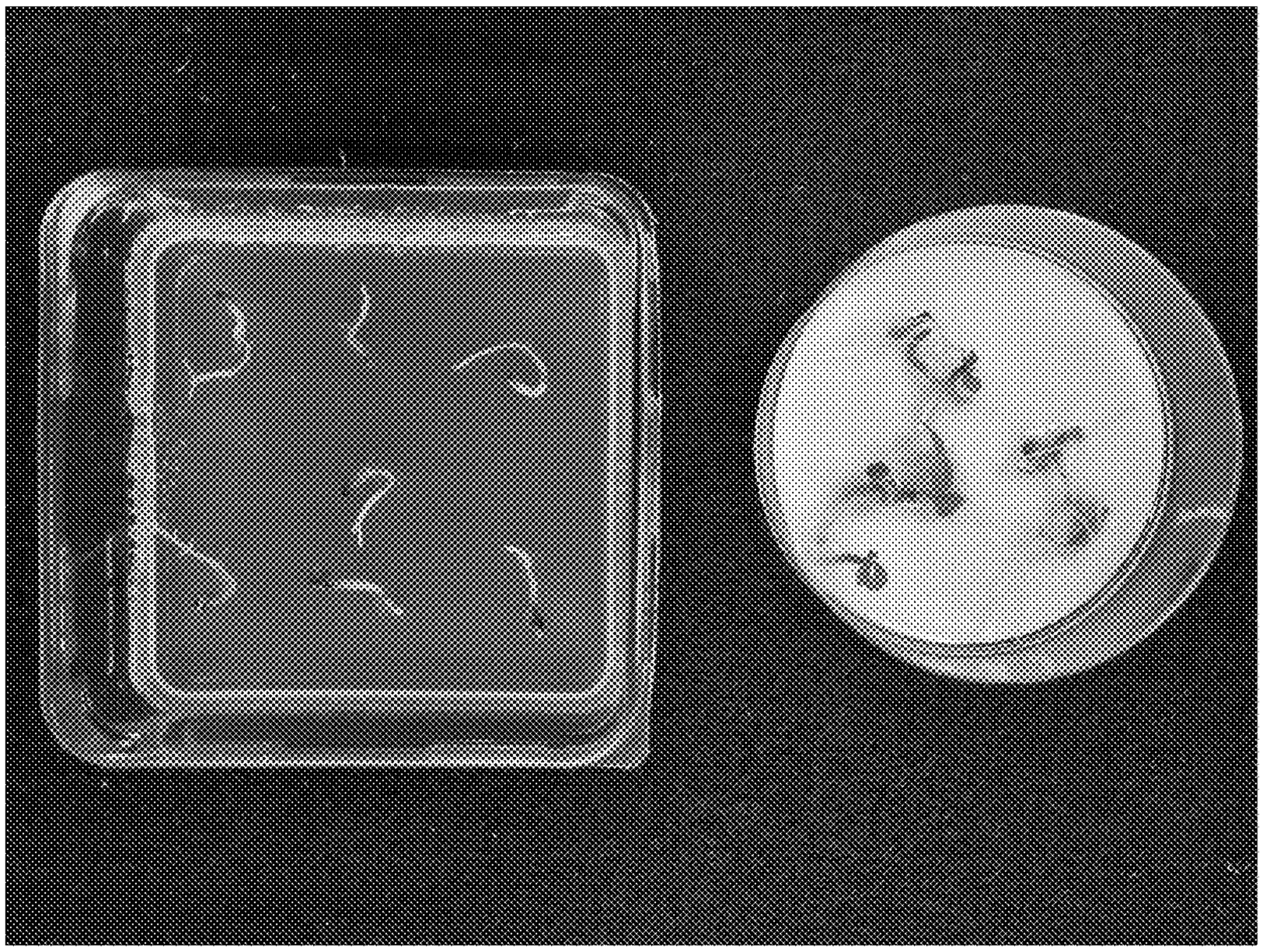


FIG. 28

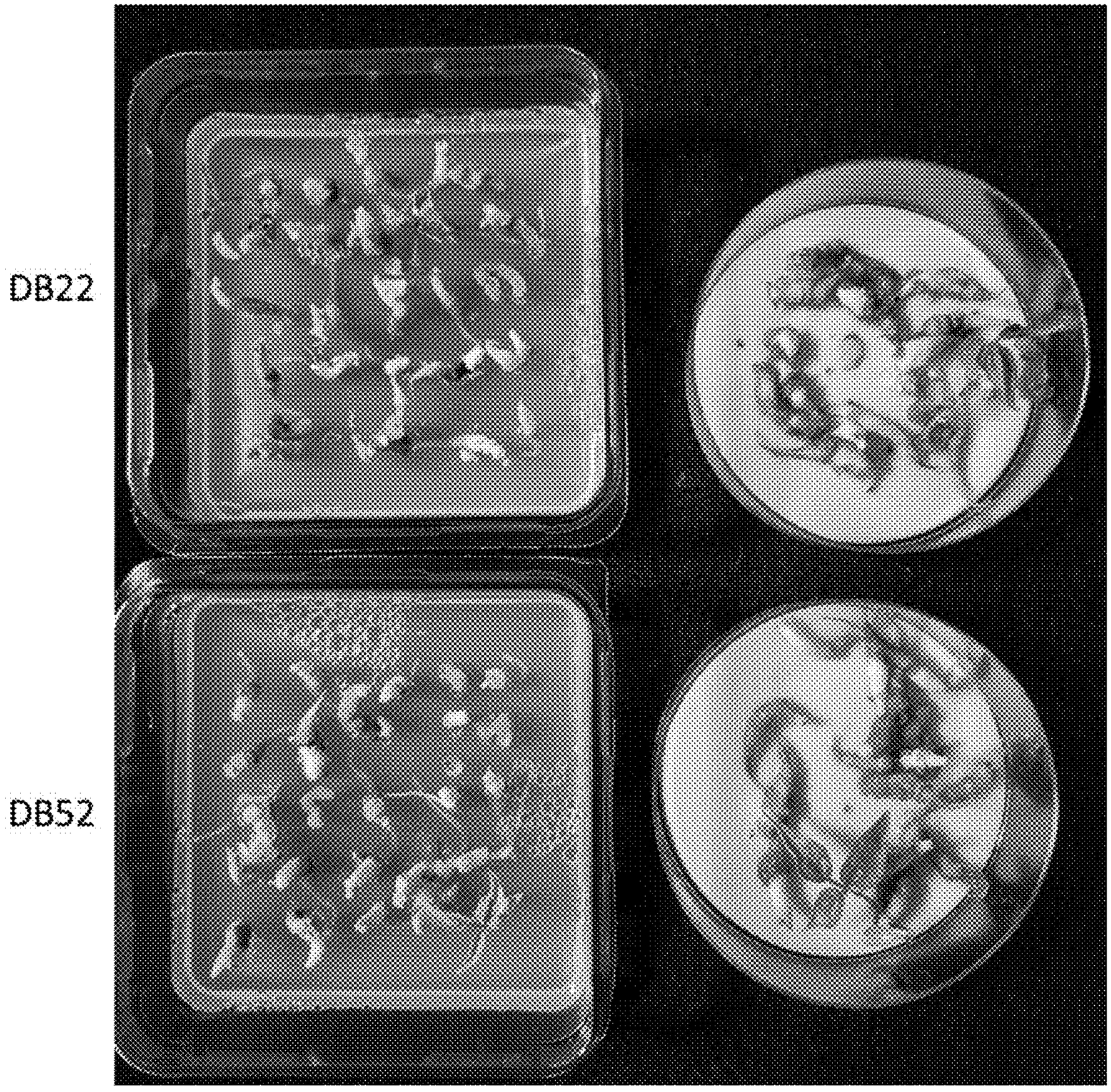


FIG. 29


Trt 1: 2 d co-culture;
25 ppm spec solid SIM;
decap 6d post SIM transfer
Trt 2: 2 d co-culture;
3d delay on liquid SIM
5 ppm spec liquid SIM;
decap 3d post SIM transfer
Trt 3: 2 d co-culture;
3d delay on liquid SIM
10 ppm spec liquid SIM;
decap 3d post SIM transfer
Trt 4: 2 d co-culture;
3d delay on liquid SIM
25 ppm spec liquid SIM;
decap 3d post SIM transfer

FIG. 30

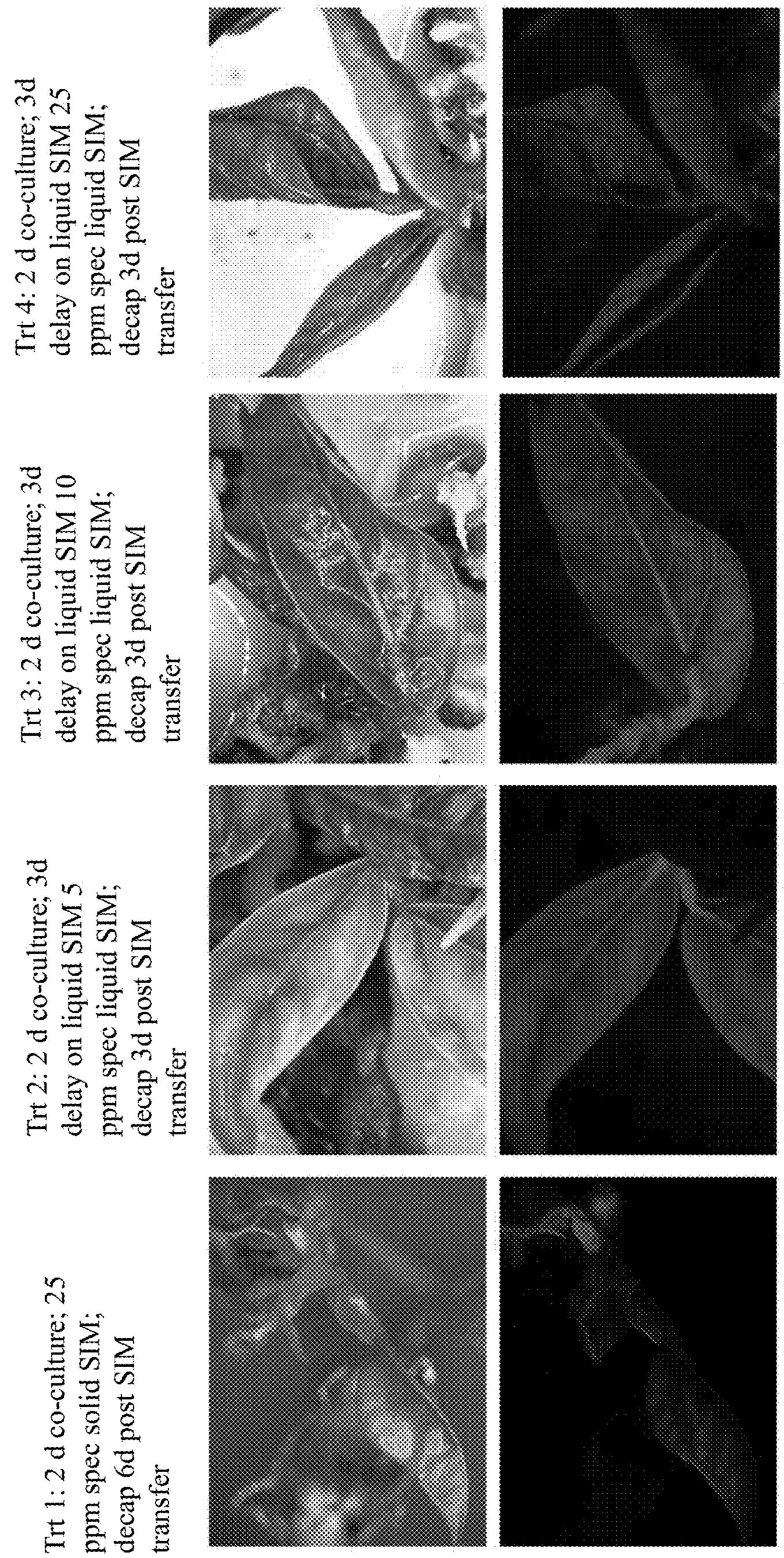

Trt 4: 2 d co-culture; 3d delay on liquid SIM 25 ppm spec liquid SIM; decap 3d post SIM transfer
Trt 3: 2 d co-culture; 3d delay on liquid SIM 10 ppm spec liquid SIM; decap 3d post SIM transfer
Trt 2: 2 d co-culture; 3d delay on liquid SIM 5 ppm spec liquid SIM; decap 3d post SIM transfer
Trt 1: 2 d co-culture; 25 ppm spec solid SIM; decap 6d post SIM transfer

FIG. 31

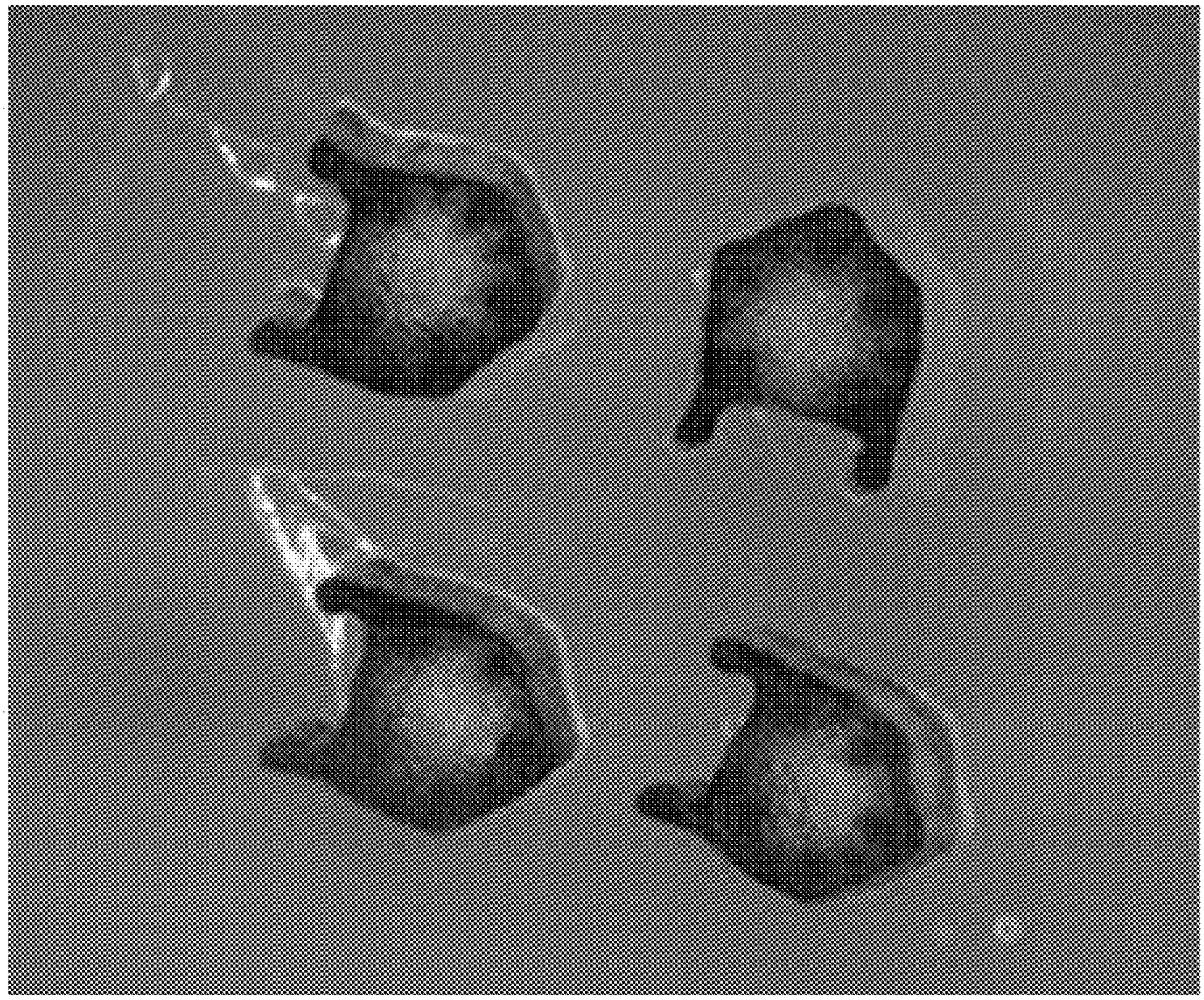


FIG. 32

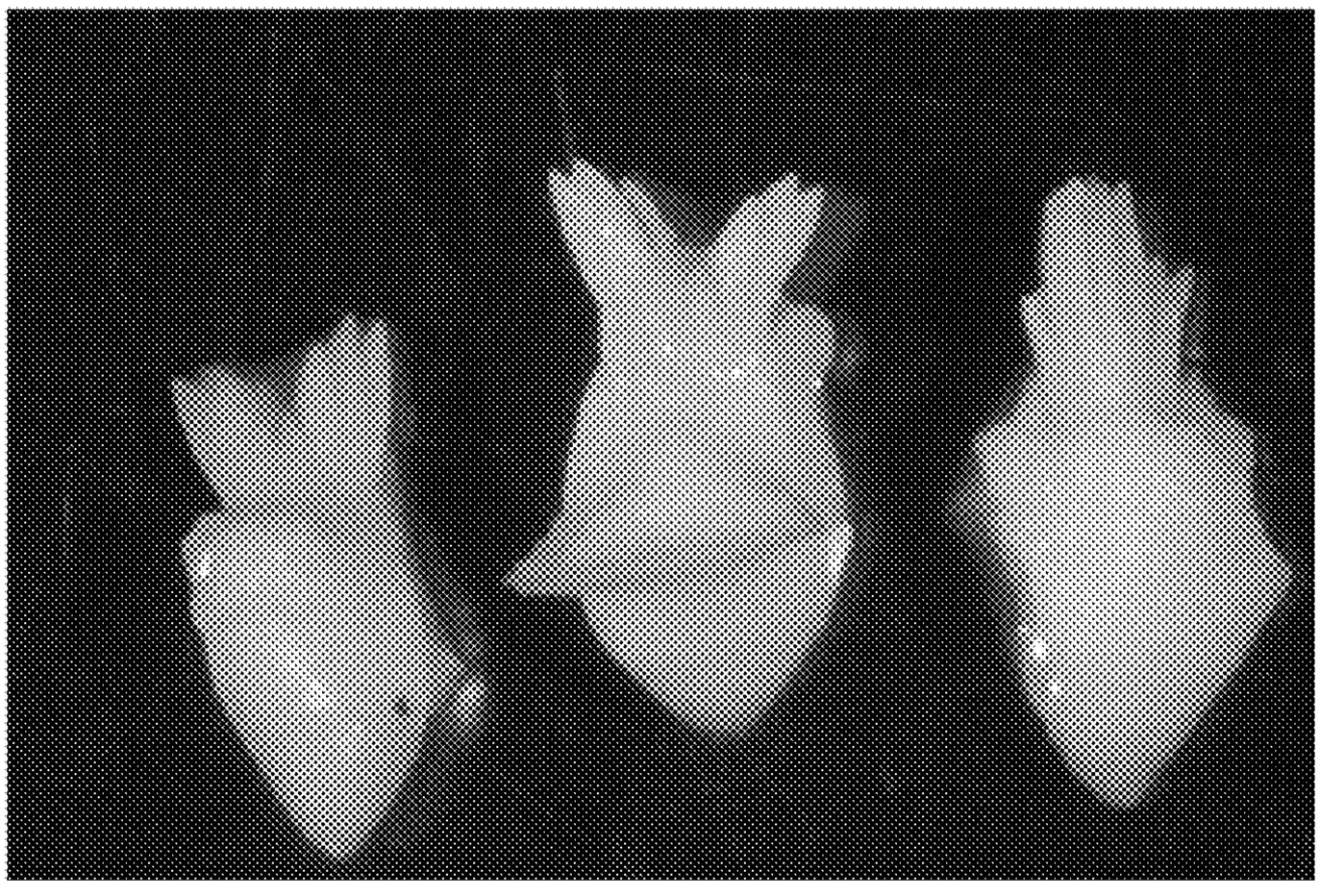
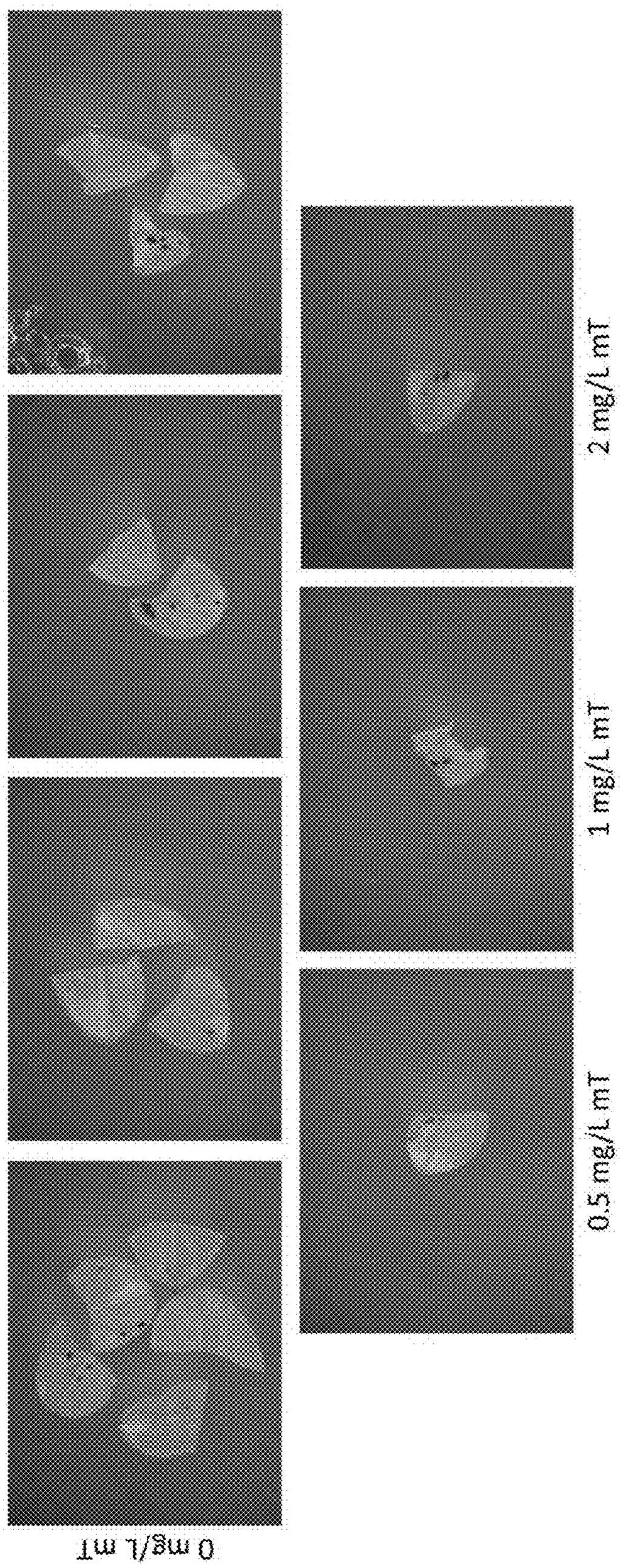


FIG. 33


0 mg/L spectinomycin
25 mg/L spectinomycin
50 mg/L spectinomycin
Semisolid Cannabis node media
Liquid Cannabis node media

FIG. 34



MERISTEM TRANSFORMATION METHOD USING A LIQUID SELECTION MEDIUM

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Application No. 63/501,510 filed on May 11, 2023, the contents of which is incorporated by reference in its entirety.

BACKGROUND

[0002] *Cannabis* has been used for millennia for medical and recreational purposes and to create paper, clothing, biofuel, and food. In recent years, the *Cannabis* industry has grown dramatically in response to expanding legalization and a flood of investor capital. Effective methods for genetically manipulating *Cannabis* are in high demand, as they would allow desirable traits (e.g., improved disease resistance, increased/decreased production of specific cannabinoids) to be introduced into these plants. However, the use of such methods in *Cannabis* has been restricted by low rates of transgenic plant regeneration. Thus, more efficient methods for introducing genes into *Cannabis* are needed in the art.

SUMMARY

[0003] In a first aspect, the present invention provides methods of transforming an explant selected from the group consisting of *Cannabis* including *Cannabis* indica, *Cannabis sativa, Abelmoschus* including *Abelmoschus esculentus*, L., *Gossypium* including *Gossypium hirsutum*, L., *Vigna* including *Vigna unguiculata*, L., and *Arachis* including *Arachis hypogaea*, L. Common names for the plants include but are not limited to hemp, marijuana, okra, cotton, cowpea and peanut.

[0004] The methods comprise (a) excising the explant from a seed by removing the seed coat and optionally cotyledons, (b) introducing the exogenous nucleic acid into the explant, and (c) culturing the explant on a liquid selection medium to select for a transformed explant.

[0005] In a second aspect, the present invention provides transformed *Cannabis* explants produced by the methods described herein.

[0006] In a third aspect, the present invention provides *Cannabis* plants grown from the explants produced by the methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0008] FIG. **1** shows *Cannabis* explants in which both primary leaves were retained during excision. The top panel shows explants that are likely transgene negative (based on their bleaching phenotype) and the bottom panel shows explants that are likely transgene positive (based on their greening phenotype). All explants were grown on 50 ppm liquid spectinomycin.

[0009] FIG. **2**A-**2**B show *Cannabis* explants cultured on a solid hemp node medium. FIG. **2**A shows callusing with both embedding (top row) and surface plating (bottom row) in a variant hemp node medium in which the MS salts are replaced with 3.21 g/L Gamborg B5 salts. The gelling agent utilized (i.e., either agar or phytagel) and meta-topolin concentration utilized are indicated above each panel. FIG. **2**B shows callusing with both embedding and surface plating (indicated below each panel) in MS-based hemp node media comprising agar (i.e., the medium described in Table 7 plus 8 g/L agar) at the indicated time points. These figures demonstrate that *Cannabis* explants callus more rapidly when they are embedded in solid hemp node medium as compared to when they are surface plated on it, which suggests that the explants have some sensitivity to this medium.

[0010] FIG. **3**A-**3**B show *Cannabis* explants cultured on liquid medium. FIG. **3**A shows the greening phenotype (top photographs) and expression of the fluorescent protein tandem Tomato (tdTomato; tdTOM) (bottom photographs) in an explant cultured on liquid medium for 2 weeks (right side) as compared to an explant grown on solid medium (left side). FIG. **3**B shows selection on solid medium (top row) or liquid medium (middle row) after 3 weeks and GUS expression in leaves produced using liquid medium (bottom row).

[0011] FIG. **4** shows chimeric phenotypes in some TO *Cannabis* plants (WP1508-4a,5a) produced using a preliminary version of the *Cannabis* transformation protocol described herein that utilizes sub-optimal selection.

[0012] FIG. **5** shows transformed TO *Cannabis* plants produced using the *Cannabis* transformation protocol described herein.

[0013] FIG. **6** shows PCR results confirming that the roots of several TO *Cannabis* plants (i.e., WP1612-5a, WP1331-12a, WP1853-2a, WP1853-3a, and WP1853-5a) produced using the *Cannabis* transformation protocol described here are transgene positive. WP1507-6a (734) rooted off selection; roots aadA negative by PCR; putative epidermal; WP1612-5a (862) rooted on selection; roots aadA positive by PCR; putative germline; WP1331-12a (DB22) rooted on selection; DNA did not amplify but roots GUS+; putative germline; WP1853-2a,3a (RUBYv1) rooted on selection; roots aadA positive by PCR; both putative germline; WP1853-5a (RUBYv1) rooted on selection; DNA did not amplify but roots RUBYv1 positive; putative germline.

[0014] FIG. **7** shows GUS expression in the roots of the *Cannabis* T0 plant WP1331-12a and RUBYv1 expression in the roots of the *Cannabis* T0 plant WP1853-5a.

[0015] FIG. **8** shows tdTOM expression in roots of the *Cannabis* T0 plant WP1331-13a. The *Cannabis* T0 plant WP1612-6a, which comprises a construct that does not include tdTOM, was used to blank the LEICA instrument.

[0016] FIG. **9** shows *Cannabis* explant excision. The top panel shows the removal of the seed coat. The middle panel shows removal of the cotyledons and one or both primary leaves (i.e., the old excision methods). The bottom panel shows removal of only the cotyledons (i.e., the new excision methods).

[0017] FIG. **10** shows T1 Germline greenhouse-grown *Cannabis* seedlings expressing the WCIC-A-862 construct, as observed by greening/bleaching after spraying with 1000 mg/L spectinomycin.

[0018] FIG. **11** shows T1 Germline *Cannabis* seedlings expressing the DICOTBINARY22 (DB22) control construct, as observed by tdTOM presence in the T1 embryo.

[0019] FIG. **12** shows T1 Germline *Cannabis* seedlings expressing the WCIC-A-989 RUBYv1 control construct, as

determined by observing betanin presence in the developing plant, or by looking for spectinomycin resistance in developing seedlings.

[0020] FIG. **13** shows the results of alternate media schedules, including treatments of feeding *Cannabis* explants a lower volume of liquid media at greater frequency than our standard treatment.

[0021] FIG. **14** shows T0 plants recovered from this GAANTRY (Gene Assembly in *Agrobacterium* by Nucleic acid sTacking using Recombinase technologY) system, where experiments in *Cannabis* meristems employed T-DNA launched from the disarmed virulence/Ri plasmid rather than T-DNA launched from a binary plasmid.

[0022] FIG. **15** shows the first *Cannabis* T0 plants from GAANTRY rooted in the presence of spectinomycin, with one plant expressing tdTOM and the other expressing GUS.

[0023] FIG. **16** shows *Cannabis* transformation metrics from conventional binary strategy compared to GAANTRY.

[0024] FIG. **17** shows transient GUS expression in TO okra meristem explants post co-culture (right panel), compared to a non-inoculated control (left panel) using the Efficient *Cannabis* Transformation process.

[0025] FIG. **18** shows Okra explant phenotypes on non-selective MS liquid media (far left image of left panel) and on a solid B5 media (right panel). Variable spectinomycin concentrations (0, 25 or 50 mg/L) are indicated above the photographs.

[0026] FIG. **19** shows stable tdTOM expression in TO Okra roots of the first plant 7.5 weeks post-inoculation. Explants followed the "Efficient *Cannabis* meristem tfn protocol".

[0027] FIG. **20** shows TO Okra phenotypes from plants generated with the "Efficient *Cannabis* transformation" process.

[0028] FIG. **21** shows Stable tdTOM (roots) and GUS expression (roots, leaves) in TO Okra plants derived from "Efficient *Cannabis* transformation" process. Images captured 8.5 weeks post-inoculation. Explants followed "Efficient *Cannabis* meristem tfn protocol" with 25 mg/L active spectinomycin during regeneration/selection.

[0029] FIG. **22** shows stable tdTOM expression in TO Okra event WP2300-3a (right plant in both panels) ~1 month after handoff; the control plant is the left plant in both panels.

[0030] FIG. **23** shows examples of T0 Okra plant phenotypes in the greenhouse.

[0031] FIG. **24** shows Okra conventional pod/seed (left panel) vs. tdTOM expression in T1 Okra pod/seed of WP2300-4a (middle and right panels).

[0032] FIG. **25** shows Okra conventional seeds (top left panel) and conventional split seeds (top right panel) vs. tdTOM expression in T1 Okra seeds (bottom left panel) and tdTOM expression in T1 Okra split seeds (bottom right panel) of WP2300-4a.

[0033] FIG. **26** shows okra meristem explants on 25 ppm spec (left); 50 ppm spec (right) on solid (top) or liquid (bottom) Hemp node media (~3 weeks post inoculation).

[0034] FIG. **27** shows phenotypes of Cotton meristem explants on non-selective solid B5 (right), and on liquid B5 (left) after ~2 weeks.

[0035] FIG. **28** shows TO Cowpea seedlings expressing DICOTBINARY22 (DB22) and DICOTBINARY52 (DB52) using hydroponic/liquid selection media regime analogous to Efficient *Cannabis* meristem method (right) compared to standard semisolid selection media regime (left).

[0036] FIG. **29** Cowpea variety "Crowder Pea" events with a brief liquid delay phase followed by liquid selection with spectinomycin. Treatments include transferring explants to solid selection media after co-culture (std), and explants transferred to liquid media without selection for 3 days (delay), followed by transfer to liquid media with 5-25 mg/L spectinomycin selection.

[0037] FIG. **30** shows stable tdTom expression in leaves of Cowpea (Crowder pea) events generated on solid selection media (std) and from liquid media using a delay phase (3d delay followed by 5-25 mg/L spectinomycin selection).

[0038] FIG. **31** shows presence/absence of GUS expression in the vascular bundles of cross-sectioned cowpea petioles to predict germline status.

[0039] FIG. **32** shows peanut meristem explants.

[0040] FIG. **33** shows peanut meristem explants on solid (top) and liquid (middle and bottom) MS-based *Cannabis* node selection medias post co-culture with 0 mg/L (left), 25 mg/L (middle) and 50 mg/L (right) of spectinomycin approximately 2.5 weeks after inoculation.

[0041] FIG. **34** shows stable GUS expression in highly chimeric peanut shoots sonicated for 10 minutes (45 kHz) in the presence of *Agrobacterium rhizogenes* strain 18r12v (Ar18r12v)/DB22 inoculum and vacuum infiltrated. 4 day co-culture in 2.5 mL INO+lipoic acid+nystatin/TBZ+1 mg/L TDZ; 23° C. 16/8 photoperiod. Selection/regeneration on liquid Hemp Node media with 50 mg/L spectinomycin varying amounts of meta-topolin (0, 0.5, 1, or 2 mg/L meta-topolin). SAM removed after 1 week on selection; transferred to WPM after 1 month of liquid selection.

DETAILED DESCRIPTION

[0042] The present invention provides efficient methods for transforming an explant selected from the group consisting of *Cannabis sativa*, (hemp), *Abelmoschus esculentus*, L. (okra), *Gossypium hirsutum*, L. (cotton), *Vigna unguiculata*, L. (cowpea), and *Arachis hypogaea*, L. (peanut). While the examples provided herein demonstrate the methods described in *Cannabis*, okra, cotton, cowpea and peanut, those of skill in the art will appreciate that the methods provided herein may be used with other plants from similar plant species or plants from the following genera: *Cannabis, Abelmoschus, Gossypium, Vigna*, and *Arachis*. Transformed explants and plants produced by the methods are also provided.

[0043] In a previous patent application, which was granted as U.S. Pat. No. 11,512,320, the present inventors describe a method for transforming *Cannabis* meristem explants. This method produces confirmed germline events, but it requires prolonged tissue culture and laborious explant transfers, and it generally produces transformation frequencies of less than 1%. These low transformations frequencies can be at least partially attributed to poor rooting and the fact that many explants would develop a necrotic growing tip.

[0044] In the present application, the inventors describe an improved method for transforming *Cannabis* and the application of this new method to other plants. The new method offers several key benefits as compared to the old method: (1) it produces greenhouse-ready plants in significantly less time, (2) it results in a 5- to 10-fold higher transformation frequency, and (3) it requires far less manual manipulation

of explants (i.e., during both explant excision and culturing). As a result, the new method is more amenable to automation and requires fewer highly skilled personnel hours per transformed plant. In some embodiments, the method may be used to produce greenhouse-ready plants in less than 5 months, less than 4 months, less than 120 days, less than 110 days, less than 100 days. A detailed comparison of the old and new transformation methods is provided in the Examples.

[0045] The inventors also applied the method for transforming *Cannabis* to additional plant species, including an explant selected from the group consisting of *Cannabis sativa, Abelmoschus esculentus*, L., *Gossypium hirsutum*, L., *Vigna unguiculata*, L., and *Arachis hypogaea*, L. Modifications to the methods to optimize transformation efficiencies for individual species are provided herein.

Methods:

[0046] In a first aspect, the present invention provides methods of transforming an explant. As used herein, the term "transformation" refers to the genetic alteration of a cell via the direct uptake and incorporation of an exogenous nucleic acid. The methods of the present invention comprise (a) excising the explant from a seed by removing the seed coat and optionally cotyledons, (b) introducing the exogenous nucleic acid into the explant, and (c) culturing the explant on a liquid selection medium to select for a transformed explant.

[0047] *Cannabis*, which is also known as hemp, is a genus of flowering plants in the family Cannabaceae. The methods of the present invention utilize a *Cannabis* seed. A "seed" is an embryonic plant enclosed in a protective outer covering. The seed used in the present methods may be from any *Cannabis* cultivar of interest. For example, the seed may be from *Cannabis* sativa, *Cannabis* indica, or a variety developed by crossbreeding *Cannabis sativa* and *Cannabis* indica. The seed used in the present methods may also be from any cultivar of *Abelmoschus esculentus*, L. (okra), *Gossypium hirsutum*, L. (cotton), *Vigna unguiculata*, L. (cowpea), or *Arachis hypogaea*, L. (peanut).

[0048] In some embodiments, the methods may further comprise sanitizing the seed prior to step (a). Any sanitization method known in the art may be used. As used herein, "sanitization" refers to a process that removes, kills, or deactivates microorganisms. Sanitization can be achieved through various means, including heat, radiation, ultraviolet (UV) light, oxidizing gasses, plasma, high pressure, and disinfection agents. Suitable disinfection agents include, but are not limited to, chlorine, sodium hypochlorite, alcohol, and hydrogen peroxide. In the Examples, the inventors sanitized seed by incubating it in 20% Clorox™ bleach for 5 minutes. Thus, in some embodiments, the seed is sanitized using bleach (i.e., sodium hypochlorite). However, the inventors have also successfully sanitized seeds by heating them in a 50° C. water bath for 20 minutes. Thus, in some embodiments, the seed is sanitized using heat. Additional embodiments include sanitizing the seed with sulfuric acid or 15d at 4 degrees Celsius, or a combination of sulfuric acid and cold treatment, described by Liberatore et al. (2018). Thus, in some embodiments, the seed is sanitized using sulfuric acid and/or cold treatment.

[0049] In some embodiments, the methods may further comprise hydrating the seed in a hydration medium prior to step (a). The term "hydration" refers to a process in which a dry seed takes up (i.e., imbibes) water. As a seed imbibes water, enzymes within the seed are activated, increasing the metabolic activity of the seed, and preparing the seed for germination. In some embodiments, the seed is hydrated for a time sufficient for the seed to reach a moisture content of between 30% and 70%. In some embodiments, the seed is hydrated for at least 12 hours. In some embodiments, the seed is hydrated between 2 and 24 hours. The hydration step may be completed after the sanitization step.

[0050] The "hydration medium" used to hydrate the seed may be any sterile medium that supports survival of the meristematic tissue in the seed. For example, the hydration medium may comprise sterile water and/or a sterile tissue culture medium. In the Examples, the inventors utilized a hydration medium comprising sterile water, cefotaxime (antibacterial agent), Captan® (antifungal agent), and Bravo® (antifungal agent). Thus, in some embodiments, the hydration media comprises antibacterial agents (i.e., agents that kill bacteria or inhibit bacterial growth and/or reproduction) and/or antifungal agents (i.e., agents that kill fungi or inhibit fungal growth and/or reproduction).

[0051] In some embodiments, the hydration medium comprises one or more growth regulators. A "growth regulator" is a chemical that can be used to modify plant growth. For instance, growth regulators can be used to increase branching, increase rooting, suppress shoot growth, increase yields, and the like. Examples of growth regulators that can be used in the methods of the present invention include, but are not limited to, thidiazuron (TDZ), 6-benzylaminopurine (BAP), polyethylene glycol (PEG), 2,4-dichlorophenoxyacetic acid (2,4-D), PACZOL®, gibberellic acid (GA3), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), forchlorfenuron (CPPU), glyphosate, glufosinate, bialophos, hygromycin, amikacin, tobramycin, imazapyr, dicamba, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP), salicylic acid, proline, betaine, ethylene, brassinosteroids, nitrates, meta-topolin (mT), and gibberellins.

[0052] In the Examples, the inventors sanitized seeds and then hydrated them in a hydration medium before excising explants from them. Thus, in some embodiments, the method comprises sanitizing the seed and then hydrating the seed in a hydration medium prior to step (a). The inventors contemplate using a physical means to remove the seed coat would also be suitable instead of the hydration step. In either case the sanitization step is optional and can be completed in various ways as described above.

[0053] In step (a) of the present methods, an explant is excised from the seed. As used herein, the term "explant" refers to a cell or tissue that is removed from a seed and used to initiate a culture in vitro. Explants comprise meristematic tissue, which consists of undifferentiated cells that can give rise to all adult plant tissues. Plant tissues that can be used as explants include, without limitation, embryos, cotyledons, hypocotyls, leaf bases, mesocotyls, plumules, protoplasts, and embryonic axes. Explant excision may be accomplished, for example, via manual processing (e.g., using knives and forceps), wet milling using a series of rollers and spray nozzles, adjustable grinding plates, pressure, injected gasses, vacuum, or turbulence.

[0054] In preferred embodiments with respect to *Cannabis*, the explant comprises both primary leaves. In their previous *Cannabis* explant excision protocol, the inventors manually removed the seed coat, cotyledons, and one or

both primary leaves from a seed to form an explant (see FIG. **9**, middle panel). However, as is described in the Examples, the inventors discovered that they could streamline this protocol (i.e., reduce the amount of manual labor required) by retaining the primary leaves. Thus, the inventors' new explant excision protocol comprises removing only the seed coat and cotyledons from the seed (see FIG. **9**, bottom panel). Modifications of the *Cannabis* explant excision protocol were tested for explants of the additional plant species provided in the Examples. Variations included, in some cases, removal of none, only one or both primary leaves. In some embodiments leaving the cotyledon intact was beneficial. The results of these experiments are presented in the Examples.

[0055] In step (b) of the methods, an exogenous nucleic acid is introduced into the explant. As used herein, "introducing" describes a process by which exogenous nucleic acids are introduced into a recipient cell. Suitable introduction methods include, without limitation, bacteria-mediated transformation, transposition-based plant transformation, the floral dip method, viral infection (e.g., using tomato yellow leaf curl virus, tobacco yellow dwarf virus, tomato golden mosaic virus, or bean pod mottle virus), electroporation, heat shock, lipofection, microinjection, high velocity microprojection, vacuum-infiltration, direct DNA uptake, and particle bombardment. Bacteria that can be used for bacterial-mediated transformation include several species of Rhizobiaceae such as *Agrobacterium* spp., *Sinorhizobium* spp., *Mesorhizobium* spp., *Rhizobium* spp., *Ochrobacterium* spp., and *Bradyrhizobium* spp. In the Examples, the inventors transformed *Cannabis* explants using *Agrobacterium rhizogenes* strain 18r12v (Ar18r12v). Thus, in some embodiments, the exogenous nucleic acid is introduced via *Agrobacterium*-mediated transformation.

[0056] In *Agrobacterium*-mediated transformation the Transfer DNA (T-DNA), an exogenous nucleic acid is delivered into plant cells as part of a binary *Agrobacterium* vector in which it is flanked by two imperfect border repeat sequences (the Right and Left Borders; RB and LB, respectively). Prior to transformation into plant cells, this binary vector is co-transformed into *Agrobacterium* with a second vector, which must have an origin of replication which is from a different incompatibility group than that used for replication of the binary plasmid, referred to as a vir helper plasmid. The vir helper plasmid encodes proteins that mediate integration of the nucleic acid flanked by the T-DNA repeats into the genome of the plant cell. Thus, to introduce an exogenous nucleic acid into an explant via *Agrobacterium*-mediated transformation, the explant is co-cultured in a co-culture medium with an *Agrobacterium* comprising a vector comprising the exogenous nucleic acid for about 1 to 6 days. In some embodiments, the explant is co-cultured with the *Agrobacterium* for about 4 days.

[0057] In the Examples, *Cannabis* explants were transformed with an exogenous nucleic acid comprising the aadA gene. In some embodiments, an alternate terminator was used for the aadA cassette. Based on work by Diamos and Mason, (Diamos and Mason, 2018) we also examined using an alternate terminator on the aadA cassette (the EUt terminator against the standard 35s terminator on DICOTBINARY22). Although we did not see an advantage with the EUt terminator, we did obtain a T0 plant from its use and it offers an alternate embodiment to our selection cassette (FIG. **13**).

[0058] In some embodiments, the GAANTRY (Gene Assembly in *Agrobacterium* by Nucleic acid sTacking using Recombinase technologY) system may also be used for transformation of explants. We ran proof of concept experiments in *Cannabis* meristems using T-DNA launched from the disarmed virulence/Ri plasmid (Collier 2018) rather than T-DNA launched from a binary plasmid and were able to recover T0 plants from this GAANTRY (Gene Assembly in *Agrobacterium* by Nucleic acid sTacking using Recombinase technologY) system (FIG. **14**). The first TO plants from GAANTRY rooted in the presence of spectinomycin, with one plant expressing tdTOM and the other expressing GUS, are shown in FIG. **15**.

[0059] The "co-culture medium" used for *Agrobacterium*-mediated transformation may be any medium that supports the growth and survival of the explant. In some embodiments, the co-culture medium comprises one or more growth regulators (see examples of growth regulators above). In the *Cannabis* Examples, the inventors utilized the co-culture medium described in Table 6, which comprises dicot INO medium, nystatin (antifungal agent), thiabendazole (antifungal agent), and thidiazuron (growth regulator). Thus, in some embodiments, the co-culture medium comprises the growth regulator thidiazuron. The co-culture medium may be modified, or alternative co-culture mediums may be used for different tissues or species. As described in the Examples below, we retained the 1 mg/L TDZ in INO-based co-culture, but other cytokinins (ex. BAP) could be used in co-culture and at different concentrations. In addition, solidified co-culture media could be utilized by adding a solidifying agent, such as agar, agarose, phytagel or others to INO media.

[0060] In some embodiments, the methods further comprise force treating the explant prior to or following step (b) to aid in the uptake of the exogenous nucleic acid. Examples of suitable force treatment methods include, without limitation, sonication, vortexing, centrifugation, heat-shock, increased pressure, vacuum infiltration, desiccation, and addition of chemicals (e.g., TDZ, glyphosate, metolachlor). In the Examples, the inventors force treated explants via sonication at 45-55 kHz for 20 seconds. Thus, in some embodiments, the explants are sonicated.

[0061] In step (c) of the methods, the explant is cultured on a liquid selection medium to select for transformed explants. A "selection medium" is a medium that comprises a selection agent. A "selection agent" is an agent that changes the phenotype, kills, or prevents the growth of cells that do not comprise a selectable marker (i.e., a gene that protects cells from an otherwise toxic compound). Thus, ideally, only explants that are transformed with a selectable marker can grow on the selection medium. Examples of suitable selection agents include antibiotics (e.g., spectinomycin, streptomycin) and herbicides (e.g., imazapyr). In the Examples, the explants were transformed with an exogenous nucleic acid comprising the aadA gene, which confers resistance to spectinomycin, and spectinomycin was used in the selection medium. Thus, in preferred embodiments, the selection medium comprises spectinomycin. While 50 mg/L of spectinomycin was used in the liquid selection medium in the Examples, the inventors have also achieved bleaching of non-transformed cells with as little as 10-15 mg/L spectinomycin and have used up to 150 mg/L spectinomycin in other dicot meristem systems. Thus, in some embodiments, the selection medium comprises 10-150 mg/L spectinomycin. In

other embodiments, the selection medium comprises 20-100 mg/L, 30-80 mg/L, or 40-60 mg/L spectinomycin.

[0062] Any liquid medium that supports the growth and survival of transformed explants may be used as the selection medium. Suitable base media for use in the selection medium include, without limitation, B5 medium, DKW, WPM-based medium, MS salts-based medium, and ½×MS salts-based medium. Different plants and tissues may require different base media selected from the group consisting of B5 medium, DKW medium, WPM-based medium, MS salts-based medium, and ½×MS salts-based medium, and possibly further modifications necessary, as described below and in the Examples. In addition to the base medium, the selection medium should comprise at least one selection agent and may additionally comprise additives such as antibacterial agents, antifungal agents, growth regulators, and micronutrients. In the Examples, the inventors used the selection medium described in Table 7, which includes MS salts, sucrose, Cleary's 3336 (antifungal agent), meta-topolin (growth regulator), carbenicillin (antibacterial agent), cefotaxime (antibacterial agent), timentin (antibacterial agent), and spectinomycin (selection agent). Additional embodiments of the selection medium may contain ammonium nitrate and potassium nitrate, or both. In some embodiments, the liquid selection medium is hemp node media (MS-based) and comprises 1600-3000 mg/L ammonium nitrate. In a preferred embodiment, the hemp node medium comprises 2500 mg/L ammonium nitrate. In some embodiments, the liquid selection medium is DKW and comprises 0-1500 mg/L potassium nitrate. In a preferred embodiment, the DKW medium comprises 950 mg/L potassium nitrate.

[0063] The selection medium used with the present invention is a liquid selection medium, meaning that it does not solidify at room temperature. Thus, the selection medium used with the present invention may not comprise agar or other gelling agents. *Cannabis* explants may form callus when cultured on the agar-based hemp node medium that was used as the selection medium in the inventors' previous *Cannabis* transformation method (i.e., the method described in U.S. Pat. No. 11,512,320). This previous method was labor intensive, as it required that the that the explants were transferred one-by-one to fresh solid media every 2-3 weeks. In addition, it also required callus to be manually removed with a scalpel in some cases also greatly increasing the workload. As is described in Example 1, the inventors discovered that using a liquid formulation of hemp node medium as the selection medium minimized the time required to provide fresh media and also reduces callusing to the extent that callus removal is unnecessary. With liquid selection medium, explants can be passaged (i.e., transferred to fresh media) by simply adding fresh media to the culture dish rather than moving each fragile explant to a new culture dish by hand. Spent media may be removed from the culture dish prior to adding fresh media. Thus, the use of liquid selection medium dramatically decreases the amount of manual labor required in this step of the method because the explant are not moved from one culture dish to a fresh culture dish. In some embodiments, the explants are not transferred to a new culture dish during the selection process. This also decreases the cost of supplies for use in the methods as compared to methods in which the explants must be transferred to new culture dishes every 2-3 weeks. In some embodiments, a delay between steps (b) (introducing the nucleic acid) and step (c) (culturing in the liquid selection medium) of the method may be employed. The delay may be 1, 2, 3, 4, or 5 days or longer. In a preferred embodiment, a three-day delay is employed.

[0064] In subsequent experiments, the inventors tested alternate media schedules, modified media, and additional plant species. In addition, transformation frequencies for T1 *Cannabis* plants are provided in Example 3. As described in Example 4, alternate media schedules involving feeding explants a lower volume of liquid media at greater frequency than the standard treatment did not appear advantageous save for offering greater flexibility to the feeding schedule (Table 11). Also described in Example 4, the inventors examined alternate medias during the selection/regeneration phase (Table 12). The first set of these experiments examined varying levels of ammonium nitrate and potassium nitrate in the media. However, lowering the ammonium nitrate concentration did not appear advantageous over the standard (although in this set the standard treatment did not produce TO plants). The inventors did obtain a T0 plant by increasing the ammonium nitrate concentration from the std MS level (1650 mg/L) to 2500 mg/L. Additionally, plants were regenerated using DKW media, which has a comparable level of ammonium nitrate but a lower amount of potassium nitrate than MS media. The inventors also examined the impact of Phytoax cytokinin replacing meta-topolin in the regenerative media. However, Phytoax did not appear advantageous over meta-topolin, but the experiment did demonstrate generation of T0 plants using DKW media as an alternative to MS media. The inventors then tested using one or more liquid selection mediums, including a liquid formulation of hemp node medium, as the selection medium for other plant species, and in most cases found superior results compared to using a solid medium. In some embodiments, modifying the liquid hemp node medium produced better results, depending on the species. In other embodiments, using an alternative liquid selection medium other than the liquid hemp node medium produced better results. Example 5 shows successful germline TO Okra transgenic plant production through the Efficient *Cannabis* Transformation process, illustrating an advantage from using liquid selection medium. Example 6 shows greater regeneration of Cotton explants when grown on a liquid medium. Example 7 illustrates successful TO cowpea transgenic plant production using a hydroponic/liquid media regime analogous to the Efficient *Cannabis* Transformation process with modifications, including a 3-day liquid delay phase prior to transferring to the liquid selection medium. Example 8 describes the results of testing Peanut meristem explants on solid and liquid MS-based *Cannabis* node selection medias post co-culture, with an advantage to using the liquid medium. Although stable TO Peanut plants were not recovered in these experiments, recovery of regenerating highly chimeric Peanut plants stably expressing GUS does suggest feasibility of this strategy to those skilled in the art. These experiments demonstrate that different timing of steps and feeding schedules, different media compositions and different growth regulators may be used and still achieve the improvements in transformation efficiency described herein by using a liquid selection medium in step (c) of the method.

[0065] In some embodiments, the methods further comprise (d) culturing the transformed explant on a rooting medium. Any medium that supports the growth and rooting of transformed explants may be used as the rooting medium. Suitable base media for use in the rooting medium include,

without limitation, woody plant medium (WPM)-based medium, ½×Murashige and Skoog (MS)-based medium, Linsmaier and Skoog (LS) medium, White's Medium, and Gamborg (B5) medium. Ideally, the rooting medium comprises rooting auxins, such as indole acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA). In the Examples, the inventors demonstrate that the use of a WPM-based rooting medium enhanced the level and rate of rooting as compared to the ½×MS-based rooting medium used in the previous method. Thus, in some embodiments, the rooting medium is WPM-based. In addition to the base medium, the rooting medium may further comprise additives such as antibacterial agents, antifungal agents, growth regulators, gelling agents, and selection agents. In the Examples, the inventors used the rooting medium described in Table 8, which includes WPM salts, sucrose, agar (gelling agent), IBA (growth regulator), cefotaxime (antibacterial agent), timentin (antibacterial agent), and spectinomycin (selection agent).

[0066] In the Examples, the inventors tested the minimal level of the selection agent spectinomycin that could be used in the rooting medium to allow for selection of successful transformants and found that 10 mg/L spectinomycin is sufficient while 5 mg/L spectinomycin allows non-transgenic shoots to root. However, the inventors have successfully produced transgenic *Cannabis* plants using rooting media containing concentrations of spectinomycin ranging from 0 to 60.2 mg/L. Thus, in some embodiments, the rooting medium comprises 5-100 mg/L, 7-60 mg/L, or 9-11 mg/mL spectinomycin.

[0067] The methods of the present invention offer several major advantages over the inventors' previous method for transforming *Cannabis* (i.e., the method described in U.S. Pat. No. 11,512,320). One such advantage is that the methods of the present invention produce greenhouse-ready plantlets in less than 100 days post-inoculation. In the Examples, the new methods produced greenhouse-ready plantlets within 60-71 days of inoculation, whereas the old methods produced greenhouse-ready plantlets within 103-255 days of inoculation (Table 2). Thus, the new method reduces the time to greenhouse by at least 30 days as compared to the old method. In some embodiments, the methods of the present invention produce greenhouse-ready plantlets in less than 90 days, less than 85 days, less than 80 days, less than 75 days, less than 70 days, less than 65 days, less than 60 days, less than 55 days, or less than 50 days. A plantlet is considered "greenhouse-ready" after it has developed roots that are at least 2 cm long and leaves.

[0068] Another advantage is that the methods of the present invention have a transformation frequency of greater than 1%. In Examples 1 and 2, the new methods produced transformation frequencies ranging from 1.5 to 3.8% whereas the old methods produced transformation frequencies ranging from 0.1 to 0.3% (Table 2). Thus, the new methods have a transformation frequency that is about 5- to 10-fold higher than that of the old methods. In some embodiments, the methods have a transformation frequency of 1-5%. Example 3 describes further work producing stable T1 *Cannabis* plants, while previously, germline rates (T1) were predicted from TO *Cannabis* shoots rooting on selection and/or presence of transgene in TO root tissue. The production of T1 *Cannabis* plants having transformation frequencies of 1-5% provides a significant improvement in *Cannabis* transformation efficiency, especially given the difficulty of transforming this incalcitrant species. Transformation efficiencies for additional species tested herein are also provided in the Examples. "Transformation frequency" is calculated by dividing the number of T0 or T1 plants produced by the number of T0 or T1 explants inoculated, respectively.

[0069] In the methods of the present invention, *Cannabis* explants are transformed with an exogenous nucleic acid. The terms "nucleic acid," "oligonucleotide," and "polynucleotide" are used interchangeably to refer a polymer of DNA or RNA. A nucleic acid may be single-stranded or double-stranded and may represent the sense or the antisense strand. A nucleic acid may be synthesized or obtained from a natural source. The nucleic acids used with the present invention are "exogenous," meaning that they originate outside of *Cannabis* or would represent inclusion of an additional copy of a *Cannabis*-derived nucleic acid from the same or a different variety of *Cannabis*.

[0070] The exogenous nucleic acid used with the present invention may include a novel nucleic acid that is not found in the *Cannabis* genome, a modified version of a nucleic acid found in the *Cannabis* genome, or an extra copy of a nucleic acid found in the *Cannabis* genome. In some embodiments, the exogenous nucleic acid is used to reduce or silence the expression of a nucleic acid found in the *Cannabis* genome, e.g., via RNA interference (RNAi). In some embodiments, the exogenous nucleic acid encodes or includes a guide RNA (gRNA) that is used to perform CRISPR/Cas-mediated gene editing (CRISPR) on the *Cannabis* genome. CRISPR can be used to edit an endogenous gene (e.g., correct a mutation or modify the product produced by the gene), disrupt expression of an endogenous gene (e.g., by inserting a stop codon, a frameshift mutation, or a nonsense mutation), modify a regulatory sequence to upregulate or downregulate expression of an endogenous gene, or insert an exogenous gene (e.g., a gene encoding a novel product). In these embodiments, the exogenous nucleic acid may further encode a Cas enzyme or a Cas enzyme may be introduced by other means.

[0071] The exogenous nucleic acid used with the present invention may confer a desirable trait or phenotype to the transformed *Cannabis* plant. In some embodiments, the exogenous nucleic acid confers a trait of agronomic interest, such as resistance to a disease, insect, or pest; tolerance to an herbicide or environmental stress; growth enhancement (e.g., increased plant size, growth rate, or nitrogen fixation), or a plant product improvement (e.g., increased yield, nutritional enhancement, improved flavor, altered fruit ripening). In some embodiments, the exogenous nucleic acid causes the plant to produce a novel product (e.g., a pharmaceutical, an industrial enzyme).

[0072] In some embodiments, the exogenous nucleic acid modulates the expression or activity of an endogenous *Cannabis* gene selected from the group consisting of tetrahydrocannabinolic acid (THCA) synthase, cannabidiolic acid (CBDA) synthase, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, O-methyltransferase (CsOMT21), lipid transfer protein 2 (LTP2), prenyltransferase 3 (CsPT3), and prenyltransferase 1 (CsPT1). For example, *Cannabis* plants that have low THC content can be generated by reducing or eliminating expression of THCA synthase and/or CBDA synthase; *Cannabis* plants with increased trichome numbers can be generated by increasing expression of LTP2; *Cannabis* plants with increased cannabigerol

(CBG) and cannabidiol (CBD) production can be generated by increasing expression of CsPT1 or CsPT3; *Cannabis* plants with increased chrysoeriol, cannflavin A, and cannflavin B production can be generated by increasing expression of CsOMT21; and glyphosate resistant *Cannabis* plants can be generated by mutating the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) gene. The sequences of these genes and the proteins that they encode as well as examples of gRNAs that can be used to target the THCA and CBDA genes are provided in U.S. Pat. No. 11,512,320, which is hereby incorporated by reference in its entirety.

[0073] In some embodiments, the exogenous nucleic acid comprises a promoter or another regulatory element. As used herein, the term "promoter" refers to a DNA sequence that defines where transcription of a nucleic acid begins. RNA polymerase and the necessary transcription factors bind to the promoter to initiate transcription. Promoters are typically located directly upstream (i.e., at the 5' end) of the transcription start site. However, a promoter may also be located at the 3' end, within a coding region, or within an intron of a gene that it regulates. Promoters may be derived in their entirety from a native or exogenous gene, may be composed of elements derived from multiple regulatory sequences found in nature, or may comprise synthetic DNA. A promoter is "operably linked" to a nucleic acid if the promoter is positioned such that it can affect transcription of the nucleic acid.

Explants and Plants:

[0074] In another aspect, the present invention provides transformed explants produced by the methods described herein.

[0075] The present invention also provides plants grown from the explants produced by the methods described herein. The term "plant" is used broadly herein to refer to a plant at any stage of development or to part of a plant, including a plant cutting, a plant cell, a plant cell culture, a plant organ, a plant tissue, a plant seed, a plantlet, or a harvestable plant part (e.g., flowers, pollen, seedlings, cuttings, tubers, leaves, stems, fruit, seeds, roots).

[0076] In preferred embodiments, the explants or plants produced by the methods are germline transformants. A "germline transformant" is a transformed explant or plant in which the exogenous nucleic acid has been transformed into cells that will give rise to pollen or an ovule, such that the exogenous nucleic acid is passed on to seed produced by the plant.

[0077] The present disclosure is not limited to the specific details of construction, arrangement of components, or method steps set forth herein. The compositions and methods disclosed herein are capable of being made, practiced, used, carried out and/or formed in various ways that will be apparent to one of skill in the art in light of the disclosure that follows. The phraseology and terminology used herein is for the purpose of description only and should not be regarded as limiting to the scope of the claims. Ordinal indicators, such as first, second, and third, as used in the description and the claims to refer to various structures or method steps, are not meant to be construed to indicate any specific structures or steps, or any particular order or configuration to such structures or steps. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to facilitate the disclosure and does not imply any limitation on the scope of the disclosure unless otherwise claimed. No language in the specification, and no structures shown in the drawings, should be construed as indicating that any non-claimed element is essential to the practice of the disclosed subject matter. The use herein of the terms "including," "comprising," or "having," and variations thereof, is meant to encompass the elements listed thereafter and equivalents thereof, as well as additional elements. Embodiments recited as "including," "comprising," or "having" certain elements are also contemplated as "consisting essentially of" and "consisting of" those certain elements.

[0078] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure. Use of the word "about" to describe a particular recited amount or range of amounts is meant to indicate that values very near to the recited amount are included in that amount, such as values that could or naturally would be accounted for due to manufacturing tolerances, instrument and human error in forming measurements, and the like. All percentages referring to amounts are by weight unless indicated otherwise.

[0079] No admission is made that any reference, including any non-patent or patent document cited in this specification, constitutes prior art. In particular, it will be understood that, unless otherwise stated, reference to any document herein does not constitute an admission that any of these documents forms part of the common general knowledge in the art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein. All references cited herein are fully incorporated by reference, unless explicitly indicated otherwise. The present disclosure shall control in the event there are any disparities between any definitions and/or description found in the cited references.

[0080] The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

Example 1

[0081] In the following example, the inventors describe an improved method for transforming *Cannabis*.

Protocol Modifications:

[0082] Simplified excision method. Retaining both primary leaves of the *Cannabis* explant during excision increases the efficiency of isolating this relatively delicate

tissue. Both greening phenotypes and T0 *Cannabis* plants have been obtained using this simplified excision method (FIG. **1**).

[0083] Liquid selection medium. *Cannabis* explants undergo extensive callusing (i.e., unorganized callusing due to hyperhydricity as opposed to embryogenic or organogenic callus) at the hypocotyl when cultured on agar-based hemp node medium post co-culture, which requires labor-intensive manual callus removal with a scalpel every 2-3 weeks for every explant. Phytagel-based hemp node medium and surface plating were tested as alternatives to agar-based hemp node medium. These modifications did not reduce callusing, but surface plating did delay callusing slightly (FIG. **2**). However, a liquid formulation of hemp node medium was found to dramatically reduce callusing (i.e., to the degree that callus no longer needed to be removed from the explants to recover transgenic plants) and to produce precocious greening and shooting relative to the solid medium, with most of these greening explants expressing the transformation reporter tdTOM and/or GUS (FIG. **3**). For example, callus did not need to be removed manually every approximately 3 weeks from each explant.

[0084] Reduced level of selection agent in rooting medium. The level of the selection agent spectinomycin included in the ½×Murashige and Skoog (MS)-based rooting medium was titrated back to determine the minimal level at which L1 epidermal events could be separated from germline events (Table 1). Levels as low as 10 mg/L spectinomycin were found to be sufficient to enrich shoots for germline transmission (as determined by either T1 progeny analysis or the presence of transgene in T0 roots). A germline event was obtained using 5 mg/L spectinomycin in the rooting medium, but it was determined that non-transgenic shoots are capable of rooting at this lower level of spectinomycin.

TABLE 1

Germline transformation of *Cannabis* meristem explants using different levels of selection during rooting

Spectinomycin	# rooting *Cannabis* plants	# germline events	% germline events
0 mg/L	22	10	45%
60.2 mg/L	7	6	86%
39 mg/L	5	5	100%
10 mg/L	17	17	100%
5 mg/L	1	1	100%

[0085] Modified rooting medium. A woody plant medium (WPM)-based rooting medium with increased indolebutyric acid (IBA) and no carbenicillin was tested as an alternative to the ½×MS-based rooting medium used in the previous method and was found to enhance the general level and rate of rooting in *Cannabis* meristem transformation. Further, the new rooting medium was found to rescue shoots obtained using the previous method that had failed to root (i.e., the shoots produced plantlets after being transferred from the old rooting medium to the new rooting medium).

Protocol Comparison:

[0086] A new protocol that includes the simplified excision method, liquid culture, and modified rooting medium discussed above was compared to the *Cannabis* meristem transformation protocol previously disclosed in U.S. Pat. No. 11,512,320. The new protocol was found to provide enhanced transformation frequency and efficiency (i.e., reduced labor per plant and time to greenhouse) as compared to the previous protocol (Table 2). Specifically, the new protocol was found to dramatically increase the number of transgenic *Cannabis* plants generated with a given number of explants and to decrease the time from shoot harvest to rooting (Table 3).

[0087] Early variants of the new *Cannabis* transformation protocol that use liquid medium were tested prior to its full development. Reduced selective pressure in the liquid medium (i.e., 15 mg/L and 25 mg/L spectinomycin) was tested but resulted in no plants, which may have been due to an insufficient advantage of aadA-transformed cells compared with untransformed cells. T0 plants that were initially selected for using 15 mg/L spectinomycin but were transferred to 50 mg/L spectinomycin were recovered, but the resulting plants were occasionally splotchy (i.e., they had bleached regions or spotting but were otherwise green, see FIG. **4**), suggesting chimerism. In these initial tests, the older ½×MS-based rooting medium was initially used, and then shoots were transferred to the WPM-based rooting medium where they subsequently rooted. Initial use of the ½×MS-based rooting medium added time to the protocol (i.e., days from inoculation to greenhouse) relative to use of WPM-based rooting medium alone. Some of the plants were rooted on non-selective WPM-based rooting medium, and some likely L1 (non-germline) epidermal events (based on null results in aadA root PCR assays) were sent to greenhouse. These plants were not counted toward total T0 plants or transformation frequency. The transformation metrics for these early iterations of the new protocol are included in the last five rows of Table 2.

TABLE 2

Comparison of transformation metrics for the *Cannabis* meristem transformation protocol described in U.S. Pat. No. 11,512,320 ("old"), the improved protocol of the present invention ("new"), and early versions of the improved protocol that utilized insufficient selection ("early"). In all cases, the data was generated using the *Agrobacterium rhizoenes* strain 18r12v (Ar18r12v) and the subcellular location of aadA1a was At_dTP aadA.

Protocol	*Cannabis* variety	Explant excision	Construct	Selection medium	Manual callus removal	Rooting medium	# Explants	# Shoots	# T0 plants	Average time to greenhouse	TF	Germline Lines (T1)	Germline TF
Old	Badger	1 primary leaf manually removed	WCIC-A-862	50 ppm spec SOLID hemp node medium	Every explant, at each subculture (every 14-21 days)	10 ppm spec ½X MS hemp rooting medium	1009	11	3	132 days	0.3%	2	0.2%

TABLE 2-continued

Comparison of transformation metrics for the *Cannabis* meristem transformation protocol described in U.S. Pat. No. 11,512,320 ("old"), the improved protocol of the present invention ("new"), and early versions of the improved protocol that utilized insufficient selection ("early"). In all cases, the data was generated using the *Agrobacterium rhizoenes* strain 18r12v (Ar18r12v) and the subcellular location of aadA1a was At_dTP aadA.

Protocol	*Cannabis* variety	Explant excision	Construct	Selection medium	Manual callus removal	Rooting medium	# Explants	# Shoots	# T0 plants	Average time to greenhouse	TF	Germline Lines (T1)	Germline TF
New	Badger	primary leaves left intact	WCIC-A-862	50 ppm spec LIQUID hemp node medium	None	10 ppm spec WPM hemp rooting medium	80	4	3	71 days	3.8%	3	3.8%
New with GA3	Badger	primary leaves left intact	WCIC-A-862	50 ppm spec LIQUID Hemp node medium + 1 ppm GA3	None	10 ppm spec WPM hemp rooting medium	80	3	3	69 days	3.8%	2	2.5%
Old	Badger	1 primary leaf manually removed	WCIC-A-346	50 ppm spec SOLID hemp node medium	Every explant, at each subculture (every 14-21 days)	non-selective ½X MS hemp rooting medium, and 5 ppm spec WPM chickpea BRM*	516	5	3	122 days	0.6%	3	0.6%
New	Badger	primary leaves left intact	WCIC-A-346	50 ppm spec LIQUID hemp node medium	None	10 ppm spec WPM hemp rooting medium	134	5	2	68 days	1.5%	2	1.5%
New	Badger	primary leaves left intact	WCIC-A-989	50 ppm spec LIQUID hemp node medium	None	10 ppm spec WPM hemp rooting medium	246	14	7	60 days	2.8%	7	2.8%
New w non-selective rooting medium	Cherry Wine Hybrid	primary leaves left intact	WCIC-A-346	50 ppm spec LIQUID hemp node medium	None	non-selective WPM hemp rooting medium**	44	1	1	54 days	2.3%	1	2.3%
New	Badger	primary leaves left intact	DB22 WCIC-A-346	50 ppm spec LIQUID Hemp node	None	10 ppm spec WPM Hemp rooting	284	8	4	91 days	1.4%	3	1.1%
Old	Badger	1 primary leaf manually removed	WCIC-A-589	50 ppm spec SOLID hemp node medium	Every explant, at each subculture (every 14-21 days)	10 ppm spec ½X MS hemp rooting medium	1303	5	1	103 days	0.1%		
Old	Badger	1 primary leaf manually removed	WCIC-A-590	50 ppm spec SOLID hemp node medium	Every explant, at each subculture (every 14-21 days)	10 ppm spec ½X MS hemp rooting medium	1560	8	3	255 days	0.2%		
Old	Badger	1 primary leaf manually removed	WCIC-A-734	50 ppm spec SOLID hemp node medium	Every explant, at each subculture (every 14-21 days)	10 ppm spec ½X MS hemp rooting medium	870	14	3	169 days	0.3%		

TABLE 2-continued

Comparison of transformation metrics for the *Cannabis* meristem transformation protocol described in U.S. Pat. No. 11,512,320 ("old"), the improved protocol of the present invention ("new"), and early versions of the improved protocol that utilized insufficient selection ("early"). In all cases, the data was generated using the *Agrobacterium rhizoenes* strain 18r12v (Ar18r12v) and the subcellular location of aadA1a was At_dTP aadA.

Protocol	*Cannabis* variety	Explant excision	Construct	Selection medium	Manual callus removal	Rooting medium	# Explants	# Shoots	# T0 plants	Average time to green-house	TF	Germ-line Lines (T1)	Germ-line TF
Early	Badger	1 primary leaf manually removed	WCIC-A-735	15 ppm spec LIQUID hemp node medium	None	10 ppm spec ½X MS hemp rooting medium	132	1	0	n/a	0.0%		
Early	Badger	1 primary leaf manually removed	WCIC-A-735	25 ppm spec LIQUID hemp node medium	None	10 ppm spec ½X MS hemp rooting medium	128	2	0	n/a	0.0%		
Early	Badger	1 primary leaf manually removed	WCIC-A-735	50 ppm spec LIQUID hemp node medium and 15 ppm spec LIQUID hemp node medium	None	10 ppm spec ½X MS hemp rooting medium, then 10 ppm spec WPM hemp rooting medium	80	1	1	118 days	1.3%		
Early	Badger	primary leaves left intact	WCIC-A-735	50 ppm spec LIQUID hemp node medium and 15 ppm spec LIQUID hemp node medium	None	10 ppm spec ½X MS hemp rooting medium, then 10 ppm spec WPM hemp rooting medium	321	11	4	87 days	1.2%		
Early	Badger	1 primary leaf manually removed	WCIC-A-346	50 ppm spec LIQUID hemp node medium and 15-25 ppm spec LIQUID hemp node medium	None	10 ppm spec ½X MS Hemp rooting, then 10 ppm spec WPM Hemp rooting	140	7	3	77 days	2.1%		

TF: transformation frequency.

Germline Lines (T1): Number of T1 germline lines.

Germline TF: transformation frequency of T1 germline lines.

*inclueds confirmed germline 1331-1a, 2a events and 1331-10a (tdTOM positive roots)

[0088] includes confirmed germline 1331-1a,2a events and 1331-10a (tdTOM positive roots)

TABLE 3

A breakdown of the average times to greenhouse for methods described in the first three rows of Table 2

Protocol	Days from inoculation to shoot harvest	Days from shoot harvest to rooting	Total days for inoculation to greenhouse
Old	46	86	132
New	39	32	71
New with GA3	36	33	69

T0 Plant Analysis:

[0089] The new *Cannabis* meristem transformation protocol has been used to generate transformed T0 *Cannabis* plants (FIG. **5**). Multiple assays (i.e., PCR for aadA expression, GUS assay, RUBYv1 expression, and fluorescent detection of tdTOM) have demonstrated that the TO plants are transgene positive in their roots, which is an early indicator of positive germline status (FIG. **6-8**, Table 4).

TABLE 4

Putative germline status of Cannabis T0 events generated using the new *Cannabis* meristem transformation protocol

Cannabis plant ID	Binary construct	Assay	Result
WP1612-5a	WCIC-A-862	PCR for aadA	aadA positive
WP1331-12a	WCIC-A-346	GUS assay	GUS positive
WP1853-2a	WCIC-A-989	PCR for aadA	aadA positive
WP1853-3a	WCIC-A-989	PCR for aadA	aadA positive
WP1853-5a,b	WCIC-A-989	RUBYv1 detection	RUBYvl positive
WP1845-1a	WCIC-A-346	tdTOM detection	tdTOM positive
WP1331-13a	WCIC-A-346	tdTOM detection	tdTOM positive
WP1853-6a	WCIC-A-989	RUBYvl detection	RUBYvl positive
WP1612-9a	WCIC-A-862	PCR for aadA	aadA positive
WP1853-7a	WCIC-A-989	RUBYv1 detection	RUBYv1 positive
WP1331-14a	WCIC-A-346	GUS assay	GUS positive

Protocol:

1) *Cannabis* Seed Sanitization and Hydration—Day Before Inoculation:

[0090] In a 50 ml centrifuge tube, measure approximately 10 ml of seed.

[0091] In a laminar flow hood (LFH), add in 25-30 ml 20% Clorox® to the centrifuge tube, place cap back on tube, place tube on its side (for better seed coverage) and incubate for 5 minutes.

[0092] Pour the contents of the tube through an autoclaved strainer, collecting seed in the strainer and allowing Clorox® to collect in a waste bucket. Rinse the seed with ~500 ml sterile distilled water.

[0093] Transfer sanitized seed to an autoclaved beaker. Remove excess liquid with a 1000 μL pipette.

[0094] Prepare hydration medium.

[0095] In LFH, add 62.5 L cefotaxime 100 mg/ml stock to 50 ml sterile distilled water (SDW) for a final concentration of 125 mg/L.

[0096] Place 500× Captan/Bravo stock solution (30 mg/ml Captan, 15 mg/ml Bravo in sterile distilled water, with stir bar, stored at 4° C.) on stir plate and allow time for resuspension of the fungicides. Then, in LFH, dilute the stock 500× by adding 100 μL 500× Captan/Bravo stock to 50 ml SDW for a final concentration of 60 mg/L Captan and 30 mg/L Bravo.

[0097] In LFH, shake the rehydration medium to resuspend contents and pour over the 5-10 ml sanitized seed. Cover and place at 37° C. in the dark overnight.

2) *Agrobacterium* Preparation-Day Before Inoculation, Early Afternoon:

[0098] In LFH, pipette 50 ml LB medium into a 250 ml glass baffle flask.

[0099] Most of the WCIC binary constructs use kanamycin as a bacterial selectable marker (stock at 50 mg/ml=50,000 ppm). In LFH, dilute kanamycin stock 1000× in LB by adding 50 μL (of 50 mg/ml stock) to 50 ml LB to for a final concentration of 50 ppm.

[0100] Thaw glycerol stock of Ar18r12v comprising the desired binary construct. In LFH, add ~50 μL of the glycerol stock to the LB+kanamycin.

[0101] Grow overnight in shaker set at ~28 C and ~200 RPM.

[0102] Note: The *Agrobacterium* cultures can be started at any time, but you will need to add more/less thawed glycerol stock depending on how long they are allowed to grow.

3) Explant Excision-Day of Inoculation:

[0103] In LFH, rinse imbibed *Cannabis* seed 3-5× with SDW. Pour into a petri plate for excision. The petri plate may optionally contain a sterile Whatman filter paper to minimize transfer of wounding exudates from the excision step to the final explants.

[0104] Remove seed coats with a #11 blade and forceps and place embryos in a fresh dish of SDW.

[0105] Rinse embryos 5× with SDW and transfer to a second petri dish with sterile filter paper.

[0106] Remove cotyledons from embryo with a #11 blade and forceps under a microscope.

[0107] Transfer explants to a fresh dish of SDW. (Grab embryo by the cotyledon to avoid damaging the hypocotyl.)

[0108] About mid-way through this step, spin down and resuspend the *Agrobacterium* inoculum.

[0109] Rinse explants 3-5× with SDW. Limit their "sitting time" in SDW.

4) Inoculum Prep-Day of Inoculation:

[0110] Check that *Agrobacterium* has grown (LB should be turbid). In LFH, take a 0.8-1 ml sample and place in a cuvette. Add 0.8-1 ml of LB to separate cuvette to use as a blank. If you don't have a lot of *Agrobacterium*, you can take 100 μL of your culture and add it to 900 μL LB in your cuvette. The resulting optical density (OD) will be approximately 1/10× of the OD of your culture.

[0111] Read the OD660 of the culture in a spectrophotometer by setting wavelength to 660 nm and placing the LB blank in position 1 and the sample in position 2. The OD660 should ideally be between 0.4 and 1.2.

[0112] If the OD660 is low and you need a lot of inoculum, you can put culture back in shaker and let it grow longer.

[0113] If the OD660 is too high (>1.4), dilute your sample 1/10× to make sure the readings are accurate.

[0114] Cells should be harvested in log phase-Inoculum from stationary phase cultures generally appear clumpy and non-uniform.

[0115] In LFH, pour the contents of the culture flask into a 50 ml centrifuge tube. Centrifuge for 20 min at 3000 rpm using a H6000A rotor (2619×g) with the brake set at 5.

[0116] In LFH, decant supernatant and use 10 ml of dicot INO medium (Table 5) to resuspend the bacterial pellet by pipetting this medium up and down.

[0117] In LFH, add additional dicot INO medium to bring target OD660 between 0.3 and 0.4. (Use dicot INO medium as a blank.)

[0118] Pour culture back into baffle flask.

[0119] Optionally, add 200 mM acetosyringone stock to a final concentration of 100 μM.

[0120] Shake the culture at 23° C. at room temperature and 175 rpm for at least an hour.

5) Inoculation and Co-Culture:

[0121] Remove water from explants in petri plate. With a sterile scoop or sterile forceps, gently transfer explants to an inverted plantcon. Remove excess liquid with a 1000 ml pipette.

[0122] In LFH, add inoculum to explants to cover them (usually at least 25-30 ml per plantcon).

[0123] Expose explants to 20 seconds of sonication at 45-55 kHz, then incubate for 30 min on a shaker at ~75 rpm.

[0124] To prepare co-culture medium (Table 6), in LFH, pipette 25 ml dicot INO medium into a 50 ml centrifuge tube or similar sterile container and add:

[0125] 25 μl nystatin/thiabendazole (TBZ) stock (50 mg/ml nystatin+10 mg/ml TBZ stock diluted 1000× to 50 ppm and 10 ppm, respectively), and

[0126] 25 μL thidiazuron (TDZ) stock (1 mg/ml TDZ stock diluted 1000× to 1 ppm).

[0127] Note: TDZ inhibits native plant cytokinin oxidase and acts as a cytokinin. Both encourage growth/budding in secondary meristems and reduce germination response in SAM.

[0128] In LFH, remove excess inoculum to remove un-attached *Agrobacterium*.

[0129] Optionally, rinse the explants 1× with SDW and fully remove water.

[0130] In LFH, prepare co-culture plantcons by placing a square piece of sterile filter paper in plantcon bottoms and pipette co-culture medium onto the filter paper.

[0131] Note: Different amounts of co-culture medium can be used. For example, 1.25 ml, 1.5 ml, 1.75 ml, 2.25 ml, and 2.5 ml have all been used successfully.

[0132] In LFH, move explants onto filter papers with sterile forceps (~35 explants per plankton).

[0133] Place co-culture plantcons in a 23° C. 16 h light/8 h dark photoperiod Percival for 4 days.

6) Selection:

[0134] Optional 3 day delay prior to transferring explants to liquid selection medium.

[0135] In LFH, transfer explants to liquid selection medium.

[0136] Prepare filter beds by placing 4 sterile 8.2 cm filter papers in a deep-dish petri plate. Add 15 ml liquid selection medium, i.e., hemp node medium supplemented with 50 mg/L active spectinomycin, 0.5 mg/L meta-topolin, 250 mg/L carbenicillin, 200 mg/L cefotaxime, and 150 mg/L timetin (Table 7).

[0137] Transfer 16-20 explants to each plate.

[0138] Wrap plate with venting tape.

[0139] Place in a 27° C. 16/8 photoperiod Percival.

[0140] Every ~10 days, add 10 ml fresh liquid hemp node medium (with the same additives) to each plate. When humidity is low, you may need to do this about every 7 days.

[0141] Optionally, transfer greening phenotypes (spectinomycin will bleach the non-transformed cells) to a fresh filter bed with 15 ml liquid hemp node medium (with the same additives) as they develop.

7) Shoot Harvest and Rooting:

[0142] Harvest greening shoots that are at least 0.5 cm long. Use a microscope to check the health of the shoots and determine where to cut them (want to cut at a node to increase rooting potential).

[0143] Use a #11 blade to embed shoots into rooting medium, i.e., WPM hemp rooting medium comprising 10 mg/L active spectinomycin and 5× indole-3-butyric acid (IBA), without carbenicillin (Table 8).

[0144] Place shoots in a 27° C. 16/8 photoperiod Percival for about 4-8 weeks.

[0145] Transfer shoots to the greenhouse once they have developed decent roots (e.g., at least 2 cm long) if their leaves are still green.

Media Used in Protocol:

[0146]

TABLE 5

dicot INO medium

Ingredients and notes	Amount to add per liter
Phytotechnology Laboratories B5 salts G398	1.284 g
Glucose	30 g
MES hydrate (Alfa Aesar CAS 4432-31-9)	2.8 g
pH to 5.4 with IN KOH	
autoclave	

TABLE 6

Co-culture medium: dicot INO medium with TBZ and TDZ

Ingredients and notes	Amount
Dicot INO medium	1 L
Nystatin/thiabendazole (TBZ) (50 mg/ml nystatin and 10 mg/ml TBZ stock)	1 ml
Thidiazuron (TDZ) (1 mg/ml stock)	1 ml

TABLE 7

Selection medium: liquid hemp node regeneration medium	
Ingredients and notes	Amount to add per liter
MS Salts complete with vitamins (PhytoTech M519)	4.43 g
Sucrose	30 g
Cleary's 3336	0.06 g
pH to 5.7 with IN KOH	
autoclave	
Meta-topolin (mT) (1 mg/ml)	0.5 ml
Carbenicillin (100 mg/ml)	2.5 ml
Cefotaxime (100 mg/ml)	2 ml
Timetin (150 mg/ml)	1 ml
Selection	as needed

TABLE 8

Rooting medium: WPM hemp rooting medium with 5X IBA, without carbenicillin	
Ingredients and Notes	Amount to add per liter
WPM salts (Phytotechnology Laboratories WPM L449)	2.41 g
Sucrose	15 g
pH to 5.6 with KOH	
Agar (Sigma A7921)	8 g
Autoclave	
IBA stock (1 mg/ml)	2.55 ml
Cefotaxime (100 mg/ml stock)	2 ml
Timentin (150 mg/ml stock)	1 ml
Selection	as needed
Container	ice cream dishes
Distribution	~35 ml/dish

Example 2

[0147] In the following example, the inventors describe several methods that are hybrids of the new *Cannabis* transformation protocol described herein and the previous protocol described in U.S. Pat. No. 11,512,320. These hybrid methods were assessed as part of the development of the new protocol. Table 9 outlines the major differences between the old, hybrid, and new protocols. Table 10 details the generation of T0 *Cannabis* plants using a variety of protocols, which are as classified as "old," "hybrid," or "new" based on the following criteria:

[0148] Old:

[0149] One primary leaf of the explant was left intact

[0150] Shoots were developed on solid selection medium

[0151] T0 plants were sent to the greenhouse as rooted hypocotyls or rooted shoots on ½×MS-based medium

[0152] Hybrid:

[0153] One or both primary leaves of the explant were left intact

[0154] Shoots were developed on solid or liquid selection medium

[0155] T0 plants were sent to the greenhouse as rooted shoots on ½×MS- or WPM-based medium

[0156] New:

[0157] Both primary leaves of the explant were left intact

[0158] Shoots were developed on liquid selection medium

[0159] T0 plants were sent to the greenhouse as rooted shoots on WPM-based medium

TABLE 9

Comparison of the old, new, and hybrid *Cannabis* transformation protocols

Protocol step	Old protocol	Hybrid protocols	New protocol
Seed sanitization	5 min in 20% Clorox, or 1 min in 70% EtOH followed by 5 min in 20% Clorox	5 min in 20% Clorox, or 1 min in 70% EtOH followed by 5 min in 20% Clorox	5 min in 20% Clorox
Prime step (interval between seed sanitization and imbibition)	~2 hours	~2 hours or none	none
Imbibition medium	WCIC BGM	WCIC BGM or SDW	SDW
Imbibition time	overnight	overnight	overnight
Imbibition temperature	23° C. or 37° C.	23° C.- 37° C.	37° C.
Preculture	None (*Agrobacterium*) or EJW1 (particle bombardment)	None or EJW1 (*Agrobacterium*)	None
Selection	aadA (spectinomycin and streptomycin)	aadA (spectinomycin)	aadA (spectinomycin)
Selection targeting	CTP	CTP, At dTP	At dTP
Primary leaves	At least one primary leaf removed (*Agrobacterium*) or both primary leaves removed (particle gun)	At least one primary leaf removed (*Agrobacterium*)	Retained
Co-culture	3-5 d 23° C. 16/8 photoperiod	3-5 d 23° C. 16/8 photoperiod or dark	3-5 d 23° C. 16/8 photoperiod
Selection / regeneration medium for shoot development	Solid MS-based medium with spectinomycin	Solid or liquid MS-based medium with spectinomycin	Liquid MS-based medium with spectinomycin

TABLE 9-continued

Comparison of the old, new, and hybrid *Cannabis* transformation protocols

Protocol step	Old protocol 5 min in 20% Clorox, or 1 min in 70% EtOH followed by 5 min in 20% Clorox	Hybrid protocols 5 min in 20% Clorox, or 1 min in 70% EtOH followed by 5 min in 20% Clorox	New protocol 5 min in 20% Clorox
Seed sanitization			
Transfer regime	Explants transferred to fresh medium every 2-4 weeks with manual removal of callus from each explant hypocoty1	Explants transferred to fresh medium every 2-4 weeks with manual removal of callus from each explant hypocoty1	No callus removal; ~10 ml fresh medium added every ~10 days
T0 sent to greenhouse	Rooted hypocotyl or rooted shoot	Rooted shoot	Rooted shoot
Medium used for root development	Solid MS-based medium with either streptomycin or non-selective (hypocotyl); or solid 1/2 MS-based medium (shoot)	Solid 1/2 MS-based medium; solid WPM-based medium with spectinomycin	Solid WPM-based medium with spectinomycin
Selection level during root development	150 mg/L streptomycin or 0-62.5 mg/L spectinomycin	0-62.5 mg/L spectinomycin	10 mg/L spectinomycin

TABLE 10

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Pro-tocol	Con-struct	*Can-nabis* Geno-type	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP421-1	Old	DB19	Cherry Wine Hybrid (3WS)	5 min 20% Clorox; 23° C. imbibition in BGM	2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin Hemp Node	Whole explant	50 mg/L streptomycin Hemp node	10/10 leaves GUS+, tdTOM+, CTP+ by PCR	Germline segregating 1:1
WP421-2	Old	DB19	Cherry Wine Hybrid (3WS)	5 min 20% Clorox; 23° C. imbibition in BGM	2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	3 weeks 75 mg/L active spectinomycin Hemp Node	Whole explant	50 mg/L streptomycin Hemp node without mT; then Soy BRM (½X MS) without selection	Initial leaves chimeric GUS positive, but not detected later in GH plant	Event tossed (escape)
WP1182-1a	Old	DICOT BOMB13	Cherry Wine Hybrid	5 min 20% Clorox; 37° C. imbibition in BGM; explant precultured on EJW1 o/n at 27° C. 16/8 photoperiod	n/a PARTICLE GUN; 1.2 ng DNA/ug 0.6 um gold DLR	4 weeks 100 mg/L active spectinomycin Hemp Node	Whole explant	50 mg/L streptomycin hemp node; then Soy BRM without selection	10/10 leaves GUS+; CTP+ by PCR	Epidermal; 0 POS; 6 null
WP1182-1b	Old	DICOT BOMB13	Cherry Wine Hybrid	5 min 20% Clorox; 37° C. imbibition in BGM; explant precultured on EJW1 o/n at 27° C. 16/8 photoperiod	n/a PARTICLE GUN; 1.2 ng DNA/ug 0.6 um gold DLR	4 weeks 100 mg/L active spectinomycin Hemp Node	Whole explant	50 mg/L streptomycin hemp node; then Soy BRM without selection	10/10 leaves GUS+; CTP+ by PCR	No T1 seed set (We attempted to masculinize this clone to pollinate the -1a event to produce homozygotes)
WP1331-1a	Old	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	1.5 ml co-culture; 5 d 23 C. dark	6 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	5 mg/L active spectinomycin Chickpea BRM (WPM BRM)	Chimeric; 3/5 leaves aadA+ by PCR	Germline; 3 POS: 8 null

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1331-2a	Old	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	1.25 ml co-culture; 5 d 23 C. dark	6 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	5 mg/L active spectinomycin Chickpea BRM; then Hemp rooting medium without selection	Uniform tdTOM+ throughout plant	Germline; 9 POS; 6 null
WP1300-2a	Old	DB19	Badger	122° F. heat for 20 min; 37° C. imbibition in H2O	Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	12 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	39 mg/L active spectinomycin Hemp Rooting with 500 mg/L activated charcoal		Germline: 4 POS; 4 null
WP1319-1a	Old	DB19	Badger	5 min 20% Clorox then 37° C. imbibition in BGM, OR 122° F. for 20 min, SDW, 37° C. overnight	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	3 weeks 30 mg/L active spectinomycin ½X MS Hemp Node; 2 weeks on 60.2 mg/L active spectinomycin Hemp Node; 2.5 weeks 60.2 mg/L active spectinomycin WPM	Rooted shoot	60.2 mg/L active spectinomycin Hemp Rooting	Leaf sample tdTOM+, GUS+, and aadA+ by PCR	Germline: 3 POS; 8 null
WP1345-1a	Old	WCIC-A-591	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	6 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	60.2 mg/L active spectinomycin Hemp Rooting	4/4 leaves aadA+ by PCR	Germline: 5 POS; 3 null
WP1345-2a	Old	WCIC-A-591	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	6 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	60.2 mg/L active spectinomycin Hemp Rooting	3/5 leaves aadA+ by PCR	Germline: 1 POS; 3 null
WP1345-3a	Old	WCIC-A-591	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	12 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	60.2 mg/L active spectinomycin Hemp Rooting	3/4 leaves aadA+ by PCR	Germline: 8 POS, 9 null
WP1345-4a	Old	WCIC-A-591	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	8 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	39 mg/L active spectinomycin Hemp Rooting	2/3 leaves aadA+ by PCR	Germline: 4 POS; 6 null

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1345-5a	Old	WCIC-A-591	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	1.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node (surface plated)	Rooted shoot	50 mg/L active spectinomycin Hemp rooting medium; then 10 mg/L active spectinomycin Hemp rooting medium	2/2 leaves aadA+ by PCR (3^{rd} leaf no endogenous band)	Germline: 2 POS; 4 null
WP1345-6a	Old	WCIC-A-591	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	8 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	39 mg/L active spectinomycin Hemp Rooting with 500 mg/L activated charcoal	4/4 leaves aadA+ by PCR	Germline: 2 POS; 1 null
WP1346-1a	Old	WCIC-A-592	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	6 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	60.2 active spectinomycin Hemp Rooting	Autoflowering; endogenous bands did not amplify in PCR; cutting of this plant gave 0/4 leaves aadA+ by PCR	Epidermal (cuttling likely negative): 0 POS, 16 null
WP1346-2a	Old	WCIC-A-592	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	9 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	60.2 active spectinomycin Hemp Rooting	4/4 leaves aadA+ by PCR	Germline: 1 POS; 5 null
WP1346-3a	Old	WCIC-A-592	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	9 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	60.2 active spectinomycin Hemp Rooting	4/4 leaves aadA+ by PCR	Germline: 4 POS; 3 null
WP1346-4a	Old	WCIC-A-592	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 4 d 23 C. 16/8 photoperiod	8.5 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	39 mg/L active spectinomycin Hemp Rooting with 500 mg/L activated charcoal	4/4 leaves aadA+ by PCR	Germline: 4 POS; 1 null
WP1530-1a	Old	WCIC-A-591	Cherry Wine Hybrid	5 min 20% Clorox; 37° C. imbibition in BGM	Rinsed 1X post inoculation; Approximately 2.5 ml co-culture; 5 d 23 C. 16/8 photoperiod	7 weeks 60.2 mg/L active spectinomycin Hemp Node	Rooted shoot	39 mg/L active spectinomycin Hemp Rooting with 500 mg/L activated charcoal	4/4 leaves aadA+ by PCR	Germline: 1 POS; 2 null

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1344-1a	Old	WCIC-A-590	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp rooting medium	Died in GH nursery	
WP1344-2a	Old	WCIC-A-590	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	2 ml co-culture; 4 d 16/8 photoperiod	7 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp rooting medium	small branch has 3/3+ leaves aadA by PCR; large branch had 0/3+ aadA leaves and was pruned	
WP1345-11a	Hybrid	WCIC-A-591	Badger	5 min 20% Clorox; 37° C. imbibition in BGM; explants then precultured on EJW1 37° C. dark o/n	1.75 ml co-culture; 3 d 16/8 photoperiod	10 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	50 mg/L active spectinomycin Hemp rooting medium; then 10 mg/L active spectinomycin Hemp rooting medium	Branching plant; both branches 3/3 leaves aadA+ by PCR	
WP1345-12a	Old	WCIC-A-591	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	2.5 ml co-culture; 4 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin modified Hemp Node (premix MS salts with B5 vitamins; phytagel replacing agar)	Rooted shoot	50 mg/L active spectinomycin Hemp rooting medium; then 10 mg/L active spectinomycin Hemp rooting medium		
WP1502-1a	Old	WCIC-A-589	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2 ml co-culture; 5 d 16/8 photoperiod	6 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp rooting medium		
WP1507-1a	Old	WCIC-A-734	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2 ml co-culture; 5 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp rooting medium	Plant died in nursery	
WP1507-2a	Old	WCIC-A-734	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2 ml co-culture; 5 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp Rooting		

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1331-3a	Hybrid	DB22	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	2.25 ml co-culture; 4 d 16/8 photoperiod	3.5 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	Leaves aadA+ by PCR and GUS+; roots aadA negative by PCR and GUS negative; putative epidermal	
WP1593-1a	Old	WCIC-A-730	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 5 d 16/8 photoperiod	8 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	Leaves aadA+ by PCR and roots aadA negative by PCR; putative epidermal	
WP1331-4a	Hybrid	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 75 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp Rooting minus carb	Leaves and roots tdTOM; leaves aadA+ by PCR; putative germline	
WP1612-1a	Old	WCIC-A-862	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	8 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp Rooting	Leaves and roots aadA+ by PCR; Putative germline	
WP1331-5a	Hybrid	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	Roots tdTOM positive; Putative germline	
WP1331-5b	Hybrid	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	Roots tdTOM positive; Putative germline	
WP1331-6a	Hybrid	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	Roots tdTOM negative; Putative epidermal	
WP1507-3a	Old	WCIC-A-734	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2 ml co-culture; 4 d 16/8 photoperiod	8 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp Rooting with 5X IBA	Roots aadA negative by PCR; putative epidermal	

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1612-2a	Old	WCIC-A-862	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp Rooting minus carb with 5X IBA		
WP1331-7a	Hybrid	DB22	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 75 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp Rooting minus carb with 5X IBA	Roots not expressing tdTOM but GUS+' putative germline	
WP1331-8a	Old	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	8 weeks 50 mg/L active spectinomycin Hemp Node with Glucose replacing Sucrose	Rooted shoot	Non-selective Hemp rooting with 5X IBA minus carb	Roots not expressing tdTOM but GUS+' putative germline	
WP1331-9a	Hybrid	DB22	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture with Sucrose replacing Glucose; 4 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node with Glucose replacing Sucrose	Rooted shoot	10 mg/L active spectinomycin WPM Hemp Rooting minus carb with 5X IBA	tdTOM positive roots; putative germline; autoflowering	
WP1845-1a	New	DB22	Cherry Wine Hybrid	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	Non-selective WPM Hemp Rooting minus carb with 5X IBA	tdTOM positive roots; putative germline	
WP1344-3a	Old	WCIC-A-590	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	2.5 ml co-culture; 4 d 16/8 photoperiod	6 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin with 5X IBA minus carb		
WP1344-4a, b	Hybrid	WCIC-A-590	Badger	5 min 20% Clorox; 37° C. imbibition in BGM	1.75 ml co-culture; 3 d 16/8 photoperiod	9 weeks 50 mg/L active spectinomycin B5-based Hemp Node medium	Rooted shoot	10 mg/L active spectinomycin WPM Hemp Rooting minus carb with 5X IBA		
WP1508-1a	Hybrid	WCIC-A-735	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	10 mg/L active spectinomycin with 5X IBA minus carb	Roots aadA positive by PCR; putative germline	

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1508-2a	New	WCIC-A-735	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin LIQUID Hemp Node	Rooted shoot	Non-selective WPM Hemp Rooting minus carb with 5X IBA	Roots aadA negative by PCR; putative epidermal	
WP1851-1a, b	Hybrid	LBA4404 thy- + H/DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	2 ml co-culture supplemented with 50 mg/L thymidine; 4 d 16/8 photoperiod	1 week 50 mg/L active spectinomycin Hemp Node; then 2 weeks 50 mg/L active spectinomycin Hemp Node without antibiotics	Rooted shoot	Non-selective WPM Hemp Rooting minus carb with 5X IBA	tdTOM positive roots; putative germline; autoflowering	
WP1331-10a	Old	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	9 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	tdTOM positive roots; putative germline	
WP1507-4a, b	Old	WCIC-A-734	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2 ml co-culture; 4 d 16/8 photoperiod	6 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp Rooting minus carb with 5X IBA	Roots aadA positive by PCR; putative germline; -b plant autoflowering	
WP1612-3a	Old	WCIC-A-862	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	5 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin Hemp Rooting minus carb with 5X IBA; shoot dipped in 1000 mg/L IBA	Roots aadA positive by PCR; putative germline	
WP1344-5a, b	Hybrid	WCIC-A-590	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	2.25 ml co-culture; 4 d 16/8 photoperiod	12 weeks 50 mg/L active spectinomycin Hemp Node without mT	Rooted shoot	10 mg/L active spectinomycin WPM with 5X IBA minus carb		
WP1507-5a, b	Hybrid	WCIC-A-734	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2 ml co-culture; 4 d 16/8 photoperiod	4 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp WPM Rooting minus carb with 5X IBA	Roots aadA positive by PCR; putative germline	
WP1331-11a	New	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp WPM Rooting minus carb with 5X IBA	Roots not expressing RFP but expressing GUS; putative germline	

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1508-3a	New	WCIC-A-735	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	6 weeks LIQUID 15 and 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb		
WP1612-4a	New	WCIC-A-862	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb		
WP1853-1a	New	DICOT RUBYv1 (WCIC-A-989)	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	3.5 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb		
WP1851-2a, b	Hybrid	LBA4404 thy- + H/DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	2 ml co-culture supplemented with 50 mg/L thymidine; 4 d 16/8 photoperiod	1 week 50 mg/L active spectinomycin Hemp Node; then 3 weeks 50 mg/L active spectinomycin Hemp Node without antibiotics	Rooted shoot	Non-selective Hemp WPM Rooting minus carb with 5X IBA	Roots not expressing RFP but expressing GUS; putative germline	
WP1507-6a	Hybrid	WCIC-A-734	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	7 weeks 50 mg/L active spectinomycin Hemp Node	Rooted shoot	Non-selective Hemp WPM Rooting minus carb with 5X IBA	Roots PCR negative for aadA; putative epidermal	
WP1612-5a	New	WCIC-A-862	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4.5 weeks LIQUID 50 mg/L active spectinomycin Hemp Node with 1 mg/L GA3	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Roots aadA+ by PCR; putative germline	
WP1331-12a	New	DB22	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture;	4 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Roots GUS+; putative germline	

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
WP1853-2a	New	DICOT RUBYv1 (WCIC-A-989)	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	4 d 16/8 photoperiod Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	3.5 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Roots aadA+ by PCR; putative germline	
WP1853-3a	New	DICOT RUBYv1 (WCIC-A-989)	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	3.5 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Roots aadA+ by PCR; putative germline	
WP1853-4a	New	DICOT RUBYv1 (WCIC-A-989)	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	3.5 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb		
WP1853-5a	New	DICOT RUBYv1 (WCIC-A-989)	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	4.5 weeks LIQUID 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Roots expressing DICOT RUBYv1; putative germline	
WP1508-4a	New	WCIC-A-735	Badger	1 min 100% EtOH, then 5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	8 weeks LIQUID 15 and 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Splotchy, likely chimeric	
WP1508-5a	New	WCIC-A-735	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	8 weeks LIQUID 15 and 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Splotchy, likely chimeric	
WP1508-6a	New	WCIC-A-735	Badger	5 min 20% Clorox; 37° C. imbibition in SDW	Primary leaves INTACT; Rinsed 1X post	8 weeks LIQUID 15 and 50 mg/L active	Rooted shoot	10 mg/L active spectinomycin WPM rooting with		

TABLE 10-continued

Comparison of various protocols used to generate of T0 *Cannabis* plants

Event ID	Protocol	Construct	*Cannabis* Genotype	Seed conditions	Co-culture	Regeneration	Whole explant/ Shoot	Rooting medium	T0 GUS/ tdTOM/ PCR	T1 segregation (1:1 expected for non-chimeric single copy event crossed with wild type)
					inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	spectinomycin Hemp Node		5X IBA minus carb		
WP1612-6a	New	WCIC-A-862	Badger	5 min 20% Clorox; 37° C.	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	8.5 weeks LIQUID 15 and 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb		
WP1612-7a	New	WCIC-A-862	Badger	5 min 20% Clorox; 37° C.	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d 16/8 photoperiod	8.5 weeks LIQUID 15 and 50 mg/L active spectinomycin Hemp Node + 1 ppm GA3	Rooted shoots	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb		
WP1331-13a	New	DB22	Badger	5 min 20% Clorox; 37° C.	Primary leaves INTACT; Rinsed 1X post inoculation; 2.25 ml co-culture; 4 d dark	3.5 weeks LIQUID 15 and 50 mg/L active spectinomycin Hemp Node	Rooted shoot	10 mg/L active spectinomycin WPM rooting with 5X IBA minus carb	Roots RFP+, putative germline	

Example 3

[0160] While previously, germline rates (T1) were predicted from TO *Cannabis* shoots rooting on selection and/or presence of transgene in TO root tissue, the following example illustrates the generation of stable T1 *Cannabis* plants. Transformation frequencies and rates for T1 plants are reported in Table 2. T1 Germline status was established with the WCIC-A-862 construct by observing greening/bleaching of greenhouse-grown seedlings sprayed with 1000 mg/L spectinomycin (FIG. **10**). T1 Germline status was established with the DICOTBINARY22 control construct by observing tdTOM presence in the T1 embryo (FIG. **11**). T1 Germline status was established with the WCIC-A-989 DICOT RUBYv1 control construct by observing betanin presence in the developing plant, or by looking for spectinomycin resistance in developing seedlings (FIG. **12**).

Efficient *Cannabis* Transformation Using Gaantry System:

[0161] The GAANTRY (Gene Assembly in *Agrobacterium* by Nucleic acid sTacking using Recombinase technologY) system may also be used for transformation of explants. We ran proof of concept experiments in *Cannabis* meristems using T-DNA launched from the disarmed virulence/Ri plasmid (Collier 2018) rather than T-DNA launched from a binary plasmid and were able to recover T0 plants from this GAANTRY (Gene Assembly in *Agrobacterium* by Nucleic acid sTacking using Recombinase technologY) system (FIG. **14**). The first TO plants from GAANTRY rooted in the presence of spectinomycin, with one plant expressing tdTOM and the other expressing GUS, are shown in FIG. **15**. Transformation metrics from conventional binary strategy compared to Gaantry are given in FIG. **16**.

Example 4

[0162] Additional modifications to the protocols described in Examples 1-3 are described below.

Protocol Modifications:

[0163] Alternate media schedules. We examined feeding explants a lower volume of liquid media at greater frequency than our standard treatment, but this did not appear advantageous save for offering greater flexibility to the feeding schedule (Table 11).

TABLE 11

Alternative Media Schedule Experiments

Species	Genotype/ Line	Experiment ID	Strain/ Construct	# embryos to Selection	Transfer regime	# Greening phenotypes on 50 mg/L spec	Shoots harvested to 10 mg/L spec	T0 plants to GH	TF
Cannabis *sativa*	Badger 5A	*Cannabis* 7/14-1	WCIC-A-346 (Ar18r12v/ DB22)	70	2.3 ml co-culture volume, 15 ml initial liquid media, 10 ml subsequent feedings every ~10 days	3	1	1	1.4%
Cannabis sativa	Badger 5A	*Cannabis* 7/14-2	WCIC-A-346 (Ar18r12v/ DB22)	80	2.5 ml co-culture volume, 15 ml initial liquid media, 10 ml subsequent feedings every ~10 days	4	3	2	2.5%
Cannabis sativa	Badger 5A	*Cannabis* 7/14-3	WCIC-A-346 (Ar18r12v/ DB22)	80	2.3 ml co-culture volume, 15 ml initial liquid media, 5 ml subsequent feedings every ~7 days	1	1	1	1.3%
Cannabis sativa	Badger 5A	*Cannabis* 7/14-4	WCIC-A-346 (Ar18r12v/ DB22)	80	2.5 ml co-culture volume, 15 ml initial liquid media, 5 ml subsequent feedings every ~7 days	2	2	0	0.0%

[0164] Modified media. We also examined alternate medias during the selection/regeneration phase, as described in Table 12 below. The first set of these experiments examined varying levels of ammonium nitrate and potassium nitrate in the media. We found lowering the ammonium nitrate concentration did not appear advantageous over the standard (although in this set the standard treatment did not produce TO plants). We did obtain a T0 plant by increasing the ammonium nitrate concentration from the std MS level (1650 mg/L) to 2500 mg/L. We also obtained plants from DKW, which has a comparable level of ammonium nitrate but a lower amount of potassium nitrate than MS media. For example, we obtained plants utilizing a modified DKW, which contains 950 mg/L potassium nitrate compared to the standard 0 mg/L potassium nitrate.

TABLE 12

Modified Media Experiments

Species	Genotype/ Line	Experiment ID	Strain/ Construct	# embryos to Selection	Transfer regime (with 0.5 mg/L mT)	Ammonium nitrate	Potassium nitrate	# Greening phenotypes on 50 mg/L spec	Shoots harvested to 10 mg/L spec	T0 plants to GH	TF
Cannabis sativa	Badger 5A	*Cannabis* 9/14-1	WCIC-A-346 (Ar18r12v/ DB22)	96	Hemp node media (MS-based) without ammonium nitrate	0 mg/L	1900 mg/L	2	0	0	0.0%
Cannabis sativa	Badger 5A	*Cannabis* 9/14-2	WCIC-A-346 (Ar18r12v/ DB22)	96	Hemp node media (MS-based) with 400 mg/L ammonium nitrate	400 mg/L	1900 mg/L	6	0	0	0.0%
Cannabis sativa	Badger 5A	*Cannabis* 9/14-3	WCIC-A-346 (Ar18r12v/ DB22)	96	Hemp node media (MS-based) with 800 mg/L ammonium nitrate	800 mg/L	1900 mg/L	2	2	0	0.0%
Cannabis sativa	Badger 5A	*Cannabis* 9/14-4	WCIC-A-346 (Ar18r12v/ DB22)	96	Hemp node media (MS-based) with 1650 mg/L	1650 mg/L	1900 mg/L	1	0	0	0.0%

TABLE 12-continued

Modified Media Experiments

Species	Genotype/ Line	Experiment ID	Strain/ Construct	# embryos to Selection	Transfer regime (with 0.5 mg/L mT)	Ammonium nitrate	Potassium nitrate	# Greening phenotypes on 50 mg/L spec	Shoots harvested to 10 mg/L spec	T0 plants to GH	TF
					ammonium nitrate (STD)						
Cannabis sativa	Badger 5A	*Cannabis* 9/14-5	WCIC-A-346 (Ar18r12v/ DB22)	48	Hemp node media (MS-based) with 2500 mg/L ammonium nitrate	2500 mg/L	1900 mg/L	1	1	1	2.1%
Cannabis sativa	Badger 5A	*Cannabis* 9/29-1	WCIC-A-346 (Ar18r12v/ DB22)	96	Hemp node media (MS-based) with 1650 mg/L ammonium nitrate (STD)	1650 mg/L	1900 mg/L	1	1	0	0.0%
Cannabis sativa	Badger 5A	*Cannabis* 9/29-2	WCIC-A-346 (Ar18r12v/ DB22)	96	DKW media	1416 mg/L	0 mg/L	5	4	1	1.0%
Cannabis sativa	Badger 5A	*Cannabis* 9/29-3	WCIC-A-346 (Ar18r12v/ DB22)	96	modified DKW media	1416 mg/L	950 mg/L	12	6	3	3.1%
Cannabis sativa	Badger 5A	*Cannabis* 9/29-4	WCIC-A-346 (Ar18r12v/ DB22)	96	WPM media	400 mg/L	0 mg/L	0	0	0	0.0%

[0165] We also examined the impact of replacing meta-topolin with Phytoax cytokinin in the modified cytokinin experiments, as shown in Table 13 below. Phytoax did not appear advantageous over meta-topolin, but again obtained T0 plants using DKW media as an alternative to MS media.

TABLE 13

Meta-topolin v. Phytoax in Media

Species	Genotype/ Line	Co-culture	Experiment	# embryos to Selection	Transfer regime	# Greening phenotypes on 50 mg/L spec	Shoots harvested to 10 mg/L spec	T0 plants to GH	TF
Cannabis sativa	Badger 5D	2.25 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	*Cannabis* 10/6-1	112	Hemp node media (MS-based) with 1650 mg/L ammonium nitrate with 0.5 mg/L mT (STD)	5	3	1	0.9%
Cannabis sativa	Badger 5D	2.25 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	*Cannabis* 10/6-2	112	Hemp node media (MS-based) with 1650 mg/L ammonium nitrate with 0.5 mg/L Phytoax	8	3	1	0.9%
Cannabis sativa	Badger 5D	2.25 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	*Cannabis* 10/6-3	112	DKW media with 0.5 mg/L mT	7	2	2	1.8%
Cannabis sativa	Badger 5D	2.25 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	*Cannabis* 10/6-4	112	DKW media with 0.5 mg/L Phytoax	2	0	0	0.0%

Example 5

[0166] The following example describes efficient transformation in Okra (*Abelmoschus esculentus*, L.). We successively obtained germline TO Okra transgenic plants through

the Efficient *Cannabis* Transformation process. For Okra transformation we used a greater co-culture volume (2.5 ml/plantcon) then we generally use for *Cannabis* as Okra meristem explants physically larger than *Cannabis* meristem explants (although we have generated germline events from this volume in *Cannabis* as well). We imaged transient GUS expression in Okra meristem explants post co-culture, as shown in FIG. **17** (right panel), compared to a non-inoculated control (left panel). Okra explant phenotypes are shown in FIG. **18** on non-selective MS liquid media (far left image of left panel) and on a solid B5 media (right panel), along with inoculated explants on 25-50 mg/L spectinomycin selection (middle and right images in left panel). Stable tdTOM expression in T0 Okra roots of the first plant 7.5 weeks post-inoculation is shown in FIG. **19**. Explants followed the "Efficient *Cannabis* meristem tfn protocol". TO Okra phenotypes from plants generated with the "Efficient *Cannabis* transformation" process are shown in FIG. **20**. Stable tdTOM (roots) and GUS expression (roots, leaves) in T0 Okra plants derived from "Efficient *Cannabis* transformation" process are shown in FIG. **21**. Images captured 8.5 weeks post-inoculation. Explants followed "Efficient *Cannabis* meristem tfn protocol" with 25 mg/L active spectinomycin during regeneration/selection.

[0167] In the pilot Okra meristem transformation tests, we used hand excised meristems (primary leaves intact) from seed surface sanitized in 20% Clorox 5 min; rinsed; imbibed for ~20 h in H2O at 37 C; rinsed, inoculated and sonicated 20s 45 kHz; incubated 30 min, inoculum removed; explants co-cultured on 2.5 ml INO+1 ppm TDZ+nys/TBZ; 23 C 16/8 photoperiod. Transformation metrics in Pilot Okra meristem transformation test are shown in Table 14 below.

[0168] FIG. **22** shows stable tdTOM expression in TO Okra event WP2300-3a (right plant in both panels) ~1 month after handoff; the control plant is on the left in both panels (FIG. **22**). FIG. **23** shows examples of T0 Okra plant phenotypes in the greenhouse. FIG. **24** shows Okra conventional pod/seed vs. tdTOM expression in T1 Okra pod/seed of WP2300-4a). FIG. **25** shows Okra conventional seeds (top left panel) and conventional split seeds (top right panel) vs. tdTOM expression in T1 Okra seeds (bottom left panel) and tdTOM expression in T1 Okra split seeds (bottom right panel) of WP2300-4a.

[0169] Transformation metrics from a follow-up experiment of Okra transformation (TO plants not sent to GH) are shown in Table 15 below.

TABLE 14

Transformation metrics in Pilot Okra meristem transformation test

Species	Genotype/Line	Experiment ID	# embryos to Selection	First selection media	# Shoots	#T0 plants	Putative TF	# Germline Plants (T1 pos seed)	Germline TF
Okra	Clemson Spineless OG	Okra 4/27-1	5	Non-selective B5 (solid)	n/a	n/a	n/a	n/a	n/a
Okra	Clemson Spineless OG	Okra 4/27-2	25	25 mg/L spectinomycin B5 (solid)	1	0	0.0%	0	0.0%
Okra	Clemson Spineless OG	Okra 4/27-3	25	150 mg/L spectinomycin B5 (solid)	1	1	4.0%	1	4.0%
Okra	Clemson Spineless OG	Okra 4/27-4	7	Non-selective hemp node with 0.5 ppm mT (liquid)	n/a	n/a	n/a	n/a	n/a
Okra	Clemson Spineless OG	Okra 4/27-5	20	25 mg/L spectinomycin hemp node with 0.5 ppm mT (liquid)	4	3	15.0%	3	15.0%
Okra	Clemson Spineless OG	Okra 4/27-6	20	50 mg/L spectinomycin hemp node with 0.5 ppm mT (liquid)	5	2	10.0%	0 (1 plant tossed before seed)	n/a

TABLE 15

Transformation Metrics of Okra Using Solid v. Liquid Media and Different Rates of Spectinomycin Selection

Species	Genotype	Comments	Experiment ID	# embryos to Selection	First selection media	# Shoots	#T0 plants	Putative TF
Okra	Clemson Spineless OG	Hand excised (primary leaves intact) from seed surface sanitized in 20% Clorox 5 min; rinsed; imbibed for ~20 h in H2O at 37 C.; rinsed, inoculated and sonicated 2 min 45 kHz; incubated 30 min, inoculum removed; explants co-cultured on 2.5 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	Okra 6/1-1	50	25 mg/L spectinomycin Hemp node with 0.5 ppm mT (solid)	2	2	4.0%
Okra	Clemson Spineless OG	Hand excised (primary leaves intact) from seed surface sanitized in 20% Clorox 5 min; rinsed; imbibed for ~20 h in H2O at 37 C.; rinsed, inoculated and sonicated 2 min 45 kHz; incubated 30 min, inoculum removed; explants co-cultured on 2.5 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	Okra 6/1-2	50	50 mg/L spectinomycin Hemp node with 0.5 ppm mT (solid)	7	6	12.0%
Okra	Clemson Spineless OG	Hand excised (primary leaves intact) from seed surface sanitized in 20% Clorox 5 min; rinsed; imbibed for ~20 h in H2O at 37 C.; rinsed, inoculated and sonicated 2 min 45 kHz; incubated 30 min, inoculum removed; explants co-cultured on 2.5 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	Okra 6/1-3	48	25 mg/L spectinomycin Hemp node with 0.5 ppm mT (15 ml liquid on 4 filter papers)	20	8	16.7%
Okra	Clemson Spineless OG	Hand excised (primary leaves intact) from seed surface sanitized in 20% Clorox 5 min; rinsed; imbibed for ~20 h in H2O at 37 C.; rinsed, inoculated and sonicated 2 min 45 kHz; incubated 30 min, inoculum removed; explants co-cultured on 2.5 ml INO + 1 ppm TDZ + nys/TBZ; 23 C. 16/8 photoperiod	Okra 6/1-4	51	50 mg/L spectinomycin Hemp node with 0.5 ppm mT (15 ml liquid on 4 filter papers)	16	9	17.6%

[0170] FIG. **26** shows okra meristem explants on 25 ppm spec (left); 50 ppm spec (right) on solid (top) or liquid (bottom) Hemp node media (~3 weeks post inoculation). Okra regeneration appears enhanced on liquid media relative to solid media, as in *Cannabis*.

Example 6

[0171] The following example describes efficient transformation in Cotton (*Gossypium hirsutum*, L.). We also examined phenotypes of Cotton meristem explants on non-selective solid B5 (right), and on liquid B5 (left) after ~2 weeks (FIG. **27**) and noted a greater rate of regeneration/greening in the Cotton explants on liquid media, suggesting possible utility in this crop as well.

Example 7

[0172] The following example describes efficient transformation in Cowpea (*Vigna unguiculata*, L.). We used a variant of the Cowpea transformation process described by Che et. al. to examine the possibility of using a liquid selection media for Cowpea meristem transformation. The Liquid Cowpea shoot induction media (SIM) is analogous to the Efficient *Cannabis* meristem method, but differs from the protocol described in Table 7 by replacing meta-topolin with 0.5 mg/L BAP and 0.5 mg/L kinetin. The liquid Cowpea SIM differs from Che's SIM by removing agar, has no MES, and has pH 5.7 rather than Che's 5.6. We were able to establish proof of concept of generating TO Cowpea seedlingswith DB22 and DB52. and a hydroponic/liquid selection media regime analogous to Efficient *Cannabis* meristem method. However, the liquid selection media (right) did not appear advantageous relative to standard semisolid selection media regime (left) without additional changes, as shown in FIG. **28** and Table 16.

TABLE 16

Pilot Tests of Liquid Media in Cowpea

Species	Genotype/ Line	Strain	Binary	Experiment	Co-culture	Explants to 25 ppm spec SIM cct	First Selection Media (24 h light 26 C.)	Decap-itated	# greening explants transferred to WPM 27 C. 16/8 photoperiod	# shoots	# rooting shoots	putative TF
Cowpea	IT86D-1010	Ar18r12v	DB22	Cowpea 5/24-1	2 d cc in 23 C. 16/8 photoperiod (Cowpea INF media, 700 uL/ plate)	125	25 ppm spec SIM cct	Jun. 2, 2023	22	22	15	12%
Cowpea	IT86D-1010	Ar18r12v	DB52	Cowpea 5/24-2	2 d cc in 23 C. 16/8 photoperiod (Cowpea INF media, 700 uL/ plate)	125	25 ppm spec SIM cct	Jun. 2, 2023	9	7	5	4%
Cowpea	IT86D-1010	Ar18r12v	DB22	Cowpea 5/24-3	2 d cc in 23 C. 16/8 photoperiod (Cowpea INF media, 700 uL/ plate)	110	LIQUID 25 ppm spec cct	Jun. 2, 2023	6	5	5	5%
Cowpea	IT86D-1010	Ar18r12v	DB52	Cowpea 5/24-4	2 d cc in 23 C. 16/8 photoperiod (Cowpea INF media, 700 uL/ plate)	110	LIQUID 25 ppm spec cct	Jun. 2, 2023	2	2	0	0%

[0173] We then tested Cowpea variety "Crowder Pea" with a brief liquid delay phase. For example, a 3d delay means 3 days on liquid media without selection (after co-culture), followed by varying levels of selection on liquid media. There appeared to be some advantage in using a 3 day liquid delay phase followed by liquid selection at 25 mg/L spectinomycin (FIG. **29**, Table 17). Stable tdTomato expression in Crowder Pea events generated on solid media and on liquid media using delay phase is shown in FIG. **30**. We examined presence/absence of GUS expression in the vascular bundles of cross-sectioned cowpea petioles to predict germline status (FIG. **31**). Explants from these experiments were all inoculated with *Agrobacterium rhizogenes* strain 18r12v (Ar18r12v)/DICOTBINARY22.

TABLE 17

Transformation metrics in Cowpea with Liquid Media and Delay Phase

Species	Geno type/ Line	Explants to 25 ppm spec SIM, 26 C. 24 h light after co-culture	First Selection Media (24 h light 26 C.)	Second Selection Media (24 h light 26 C.)	# greening explants transferred to WPM 27 C. 16/8 photoperiod	Shoots harvested to 25-50 ppm spec WPM rooting	#rooting shoots	Putative TF	#TO0 plants predicted germline (petiole)	Predicted germline TF (petiole)
Cowpea	Crowder Pea	100	25 ppm spec SIM cct (embedded)	n/a	8	4	2	2.0%	1	1.0%
Cowpea	Crowder Pea	90	3 d delay: 0 ppm LIQUID spec SIM cct (5 ml)	10 ml of 5 ppm spec LIQUID SIM cct	20	16	3	3.3%	1	1.1%
Cowpea	Crowder Pea	90	3 d delay: 0 ppm LIQUID	10 ml of 10 ppm spec LIQUID SIM cct	6	6	3	3.3%	1	1.1%

TABLE 17-continued

Transformation metrics in Cowpea with Liquid Media and Delay Phase

Species	Geno type/ Line	Explants to 25 ppm spec SIM, 26 C. 24 h light after co-culture	First Selection Media (24 h light 26 C.)	Second Selection Media (24 h light 26 C.)	# greening explants transferred to WPM 27 C. 16/8 photoperiod	Shoots harvested to 25-50 ppm spec WPM rooting	#rooting shoots	Putative TF	#TO0 plants predicted germline (petiole)	Predicted germline TF (petiole)
			spec SIM cct (5 ml)							
Cowpea	Crowder Pea	90	3 d delay: 0 ppm LIQUID spec SIM cct (5 ml)	10 ml of 25 ppm spec LIQUID SIM cct	8	6	4	4.4%	4	4.4%

Example 8

[0174] The following example describes efficient transformation in Peanut (*Arachis hypogaea*, L.). We also ran small experiments of Peanut meristem explants (FIG. **32**) on solid and liquid MS-based *Cannabis* node selection medias post co-culture with three rates of spectinomycin (0 mg/L, 25 mg/L and 50 mg/L) and noted a greater rate of regeneration in the Peanut meristem explants on liquid selection media compared to explants on solid selection media approximately 2.5 weeks after inoculation (FIG. **33**). We did not recover stable TO Peanut plants from these pilot tests, but did recover regenerating highly chimeric Peanut plants stably expressing GUS (FIG. **34**), suggesting feasibility of this strategy to those skilled in the art.

What is claimed:

1. A method of transforming an explant with an exogenous nucleic acid, the method comprising:

a) excising the explant from a seed by removing the seed coat and optionally cotyledons,

b) introducing the exogenous nucleic acid into the explant, and

c) culturing the explant on a liquid selection medium to select for a transformed explant.

2. The method of claim **1**, wherein the seed is selected from the group consisting of a *Cannabis* seed, an *Abelmoschus* seed, a *Gossypium* seed, a *Vigna* seed, and an *Arachis* seed.

3. The method of claim **1**, further comprising sanitizing the seed prior to step (a).

4. The method of claim **1**, further comprising hydrating the seed in a hydration medium prior to step (a).

5. The method of claim **1**, wherein the explant comprises both primary leaves.

6. The method of claim **1**, wherein the exogenous nucleic acid is introduced by co-culturing the explant in a co-culture medium with *Agrobacterium* comprising the exogenous nucleic acid for about 1 to 6 days.

7. The method of claim **6**, wherein the co-culture medium comprises one or more growth regulators.

8. The method of claim **6**, wherein the co-culture medium is the medium described in Table 6.

9. The method of claim **1**, further comprising force treating the explant prior to or following step (b).

10. The method of claim **9**, wherein the force treatment is selected from the group consisting of sonication, vortexing, centrifugation, heat-shock, pressure, vacuum infiltration, and addition of chemicals.

11. The method of claim **1**, wherein the liquid selection medium comprises 10-150 mg/L spectinomycin.

12. The method of claim **11**, wherein the liquid selection medium is selected from the group consisting of the medium described in Table 7, B5 medium, DKW medium, WPM-based medium, MS salts-based medium, and ½×MS salts-based medium.

13. The method of claim **1**, wherein callus formation is minimized compared to callus formation using a solid selection medium.

14. The method of claim **1**, wherein the method further comprises:

d) culturing the transformed explant on a rooting medium.

15. The method of claim **14**, wherein the rooting medium comprises 5-100 mg/L spectinomycin.

16. The method of claim **1**, wherein the method produces greenhouse-ready plantlets in fewer than 100 days after introducing the exogenous nucleic acid into the explant.

17. The method of claim **1**, wherein the method results in a transformation frequency of greater than 1%.

18. The method of claim **1**, wherein the exogenous nucleic acid encodes or includes a guide RNA.

19. A transformed explant produced by the method of claim **1**.

20. A plant grown from the explant of claim **19**, wherein the plant is a T1 plant.

21. A seed produced from the plant of claim **20**.

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