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Running head: Detection of latent infections in potato breeding programs

Molecular detection of *Ralstonia solanacearum* to facilitate breeding for resistance to bacterial wilt in potato

Abstract

Potato bacterial wilt is caused by the devastating bacterial pathogen *Ralstonia solanacearum*. Quantitative resistance to this disease has been and is currently introgressed from a number of wild relatives into cultivated varieties through laborious breeding programmes. Here, we present two methods that we have developed to facilitate the screening for resistance to bacterial wilt in potato. The first one uses *R. solanacearum* reporter strains constitutively expressing the *luxCDABE* operon or the green fluorescent protein (*gfp*) to follow pathogen colonisation in potato germplasm. Luminescent strains are used for non-destructive live imaging, while fluorescent ones enable precise pathogen visualisation inside the plant tissues through confocal microscopy. The second method is a BIO-multiplex-PCR assay that is useful for sensitive and specific detection of viable *R. solanacearum* (IIB-1) cells in latently infected potato plants. This BIO-multiplex-PCR assay can specifically detect IIB-1 sequevar strains as well as strains belonging to all four *R. solanacearum* phylotypes and is sensitive enough to detect without DNA extraction 10 bacterial cells per ml in complex samples.

The described methods allow the detection of latent infections in roots and stems of asymptomatic plants and were shown to be efficient tools to assist potato breeding programs.

Key words: Bacterial wilt, potato brown rot, *Ralstonia solanacearum*, *Solanum tuberosum*, plant breeding, disease resistance.

1. Introduction

Bacteria can cause a range of diseases in economically important crops, leading to important losses. *Ralstonia solanacearum*, the causal agent of bacterial wilt, also referred as brown rot in potato, is one of the most devastating plant pathogens worldwide [1]. A recent taxonomic revision has led to the distinction of three separate species within the species complex [2]. In cold and temperate regions of the world, potato crops are mainly affected by *R. solanacearum* strains belonging to the phylotype IIB, sequevar 1 [3].

Potato bacterial wilt disease control is difficult due to pathogen persistence in water, soil and latently infected symptomless tubers. The use of resistant or tolerant potato varieties combined with preventive measures throughout an integrated pest management approach is highly recommended [4]. Breeding for resistance to *R. solanacearum* in Solanaceae is challenging and must combine durable resistance with desirable agronomic traits. In potato, breeding for resistance to bacterial wilt has been successfully introgressed from *S. phureja* [5], and the highly resistant wild potato *S. commersonii* Dun is currently being used [6, 7, 8].

Knowledge on pathogen distribution and multiplication in plant tissues is critical to fully exploit the potential of sources of bacterial wilt resistance through breeding programs. Asymptomatic latent infections should also be considered to avoid the selection of tolerant varieties which promote pathogen dissemination under favourable environmental conditions [9].

We present here two methodologies that we have developed to evaluate bacterial loads in inoculated potato germplasm: the use of reporter strains [10, 11] and a BIO-multiplex-PCR assay.

2. Materials

2.1 Plant varieties and plant growth materials.

1. The potato cultivar *S. tuberosum* cv. Chieftain is used as a susceptible control. Susceptible (13001.79, 13001.107) and resistant (1201.27, 09509.6) interspecific potato clones derived from different breeding lines are selected from the National Institute for Agricultural Research (INIA, Uruguay) germplasm collection.
2. Potato multiplication medium (PMM): mix 20 ml of nitrate solution (95 g/L KNO_3, 82,5 g/L NH_4SO_3. Adjust to 1 L with sterile distilled water), 20 ml of sulphate solution (18,5 g/L $MgSO_4{\cdot}7H_2O$, 1,25 mg/L $CuSO_4{\cdot}5H_2O$, 1,115 g/L $MnSO_4{\cdot}4H_2O$, 0,43 g/L $ZnSO_4{\cdot}7H_2O$. Adjust to 1 L with sterile distilled water), 20 ml of halogens solution (22 g/L $CaCl_2{\cdot}H_2O$, 41,5 mg/L

KI, 1,25 mg/L $CoCl_2 \cdot 6H_2O$. Adjust to 1 L with sterile distilled water), 1 ml of vitamin solution (0,05 % folic acid, 0,1% biotin, 0,1% choline chloride, 0,1% pantothenic acid, 0,1% thiamine, 0,2 % nicotinamide, 0,2% pyridoxine, 0,2% aminobenzoic acid), 0,17 g/L KH_2PO_4, 6,2 mg/L H_3BO_3, 0,25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 37,9 mg/L $Na_2EDTA.2H2O$, 27,8 mg/L $FeSO_4.7H2O$, 30 g/L sucrose, 0,2 g/L myo-inositol and 8 g/L agar. Adjust to pH 5,8 with KOH and to 1 L with sterile distilled water.

3. Glass containers with autoclavable plastic lid.
4. Sterile glass petri dishes.
5. Metal clamps and scalpels.
6. Metal sterilizer.
7. Laminar flow cabinet.
8. In vitro growth chambers for healthy plants at 22°C with cycles of 16 h light/8 h darkness.
9. Soil mix (Tref Substrate).
10. 5 cm^3 Multicell-trays.
11. 170-cm^3 individual pots.
12. Greenhouse with natural light and room temperature.
13. Growth chambers with controlled temperature, humidity and light.

2.2 Bacterial strains and bacterial culture.

1. *R. solanacearum* UY031 strain [12], *R. solanacearum* reporter strains UY031 Pps-GFP and UY031 Pps-lux [10, 11].
2. Rich B medium: 10 g/L Bacteriological peptone, 1 g/L Yeast extract and 1 g/L Casamino Acids. Add 1.5% Agar for solid media before autoclaving. Before plating, add 0.5% Glucose and 0.005% Triphenyltetrazolium chloride (TTC). Adjust to pH 7,0. Supplement with gentamicin at 5 and 75 μg/ml in liquid and solid cultures respectively for selection of reporter strains.
3. mSMSA medium [13]: 10 g/L Bacteriological peptone, 5 ml of glycerol and 1 g/L Casamino

Acids. Add 1.5% Agar for solid media before autoclaving. Before plating, add 10 ml of polymyxin B sulfate (1%), 10 ml of cyclohexide (1%), 2,5 ml of bacitracin A (1%), 500 µl of penicillin (0,1%), 500 µl of cloranfenicol (1%), 500 µl of crystal violet (1%) and 0.005% TTC. Adjust to pH 7,0.

4. Sterile Petri dishes.
5. Sterile tubes.
6. Sterile saline solution: add 9 g/L NaCl in distilled water before autoclaving.
7. Spectrophotometer.

2.3 Evaluation of bacterial colonisation using reporter strains.

1. Luminometer (LAS4000 light imager system, FujiFilm).
2. Epifluorescence microscope.
3. Confocal fluorescence microscope.
4. Glass slides.
5. Coverslips.
6. Solid vaseline.
7. 20 ml syringe.
8. 200 µl pipette tips.
9. Razor blade.
10. Agarose 1 %.

2.4 BIO-multiplex-PCR.

1. Ethanol 70%.
2. Sterile water.
3. Sterile absorbent paper.
4. Sterile plastic bags.
5. 50mM phosphate buffer: 4,26 g/L Na_2HPO_4 and 2,72 g/L. Adjust to pH 7,0 and to 1 L with

sterile distilled water.

6. Laboratory blender.
7. 1000 and 200 µl pipettes with sterile tips.
8. Sterile glass beads for liquid dissemination on Petri dishes.
9. 28°C Incubator.
10. Sterile loops.
11. Sterile tubes.
12. Centrifuge.
13. PCR tubes.
14. Thermocycler.
15. 5 units/µl Taq DNA polimerase and its concentrated buffer.
16. 25 mM $MgCl_2$
17. 5 mM Deoxyribonucleotide triphosphates (dNTPs).
18. 10 µM of IIB-1 specific primers (00876F: 5’-GGATTCAAGGTATCGCCAGA-3’; 00876R: 5’-CATAGCCGCTTCTTCTTTGG-3’) and general primers [14] (759: 5´-GTCGCCGTCAACTCACTTTCC-3´; 760: 5´-GTCGCCGTCAGCAATGCGGAATCG-3´).
19. MiliQ water.

3. Methods

3.1 Plant growth

1. Start *in vitro* cultures from *in vitro* plants containing 3-4 internodes grown *in vitro* in tubes or glass bottles with PMM medium.
2. Cut single-node pieces of plants and transfer them into glass bottles with fresh PMM previously autoclaved (*see* **Note 1**). Put around 10-12 single-node pieces per glass bottle (Fig. 1a).
3. Leave plantlets growing for 15-20 days in the in vitro growth chamber at 22°C with cycles of

16 h light/8 h darkness.

4. Before transfer to soil, incubate closed glass bottles with plants in a greenhouse under natural light and room temperature during 3-5 days for acclimatisation.
5. Transfer plants to soil either in plastic trays with 5-cm³ wells for bacterial wilt resistance screening (Fig. 1b), or to individual pots with 170-cm³ for evaluation of bacterial colonisation by inoculation with reporter strains (Fig. 1c) (*see* **Note 2**).
6. Incubate for 15-20 days in the greenhouse under natural light and room temperature, until plants reach a height of 10 cm.
7. Water plants frequently checking that soil mix is wet but avoiding flooding.
8. Before inoculation, incubate plants 2-3 days for acclimation in the growth chamber at 28 °C with cycles of 16 h light/8 h darkness and 65 % of humidity.

3.2 Bacterial culture and inoculum preparation

1. Streak bacterial strains from glycerol stocks kept at -80 ºC on B medium. Grow for 2 days at 28 ºC. Supplement the medium with gentamicin when reporter strains are used.
2. Pick a single colony to inoculate 20 ml of liquid rich B medium and incubate overnight at 28 ºC with orbital shaking (200 rpm) (*see* **Note 3**).
3. Centrifuge the bacterial culture, discard the supernatant and carefully re-suspend the pellet with 20 ml of sterile saline solution or sterile water.
4. Using a spectrophotometer, measure the optical density at 600 nm (OD_{600}) of the cell suspension.
5. Add the required volume of cell suspension for adjusting the desired volume of inoculum to a final concentration of 10^7 cfu/ml (consider that an OD_{600} of 0.1 corresponds to 10^8 cfu/ml).
6. Confirm the final inoculum concentration by preparing 10-fold dilutions and colony counting in rich B medium plates.

3.3 Evaluation of bacterial colonisation using reporter strains

Two types of *R. solanacearum* reporter strains are used: a bioluminescent strain for non-disruptive, macroscopic assessment of bacterial colonisation, and a fluorescent strain for microscopic evaluation of colonisation at the tissue level.

3.3.1 Evaluation of colonisation using a luminescent strain.

1. For evaluation of bacterial colonisation, use 5-10 replicate plants for each clone, grown in individual pots and arranged using a completely randomized design (Fig. 1c).
2. Prior to inoculation, damage roots slightly by making three holes of 2 cm deep in the soil of each pot with a disposable 1000 μl pipette tip.
3. Inoculate potato clones by drenching, using 40 ml of bacterial suspension of *R. solanacearum* UY031 Pps-lux strain to reach a final density of 10^6 cfu/g of soil. Inoculate plants with saline solution as negative control treatment.
4. After inoculation, record daily, for 6-10 days, bacterial colonisation of plant tissues using the Fuji Film LAS4000 light imager system with the chemiluminescence settings of incremental exposure time each 2 min and sensitivity/resolution set to high binning (Fig. 2).

3.3.2 Evaluation of colonisation using a fluorescent strain.

1. Use the same plant inoculation procedure described for the luminescent strain using instead *R. solanacearum* UY031 Pps-GFP strain.
2. Collect root and stem samples 2- and 7-days post inoculation (dpi) to follow pathogen colonisation.
3. Remove plants from pots and wash roots with tap water to remove adherent soil.
4. Surface sterilize stem and roots of each plant with ethanol 70% for 1 min, rinse with sterile water for 1 min and dry with sterile paper towels in a laminar flow cabinet.
5. Select colonized roots to be observed by confocal microscopy and put them on a glass slide with agarose 1% to maintain sample hydrated during manipulations (*see* **Note 4**).

6. Put solid vaseline in the border of a coverslip with syringe, making a 2-3 mm wide retaining wall (*see* **Note 5**).
7. Place the selected colonized roots (see **step 5**) on the coverslip surrounded by solid vaseline.
8. Cut 2-cm stem segments using a previously disinfected scalpel, cut from 1 cm above root. Make six to 10 cross-sections by hand with razor blade on the end of each stem segment and place them on the coverslip with root segments of each plant.
9. Add melted agarose 1% as immersion medium surrounding stem and root segments. Vaseline wall should retain melted agarose (*see* **Note 6**).
10. Immediately place a glass slide to seal the chamber (Fig. 3a).
11. Observe stem cross-sections and roots using a confocal (*see* **Note 7**) microscope (Fig. 3b).

3.4 Bacterial wilt resistance evaluation.

1. Use two replicate trays, each containing eight plants per clone. Arrange the trays in the growth chamber using a completely randomized design.
2. Prior to inoculation, damage roots slightly by making one hole of 2 cm deep in the soil of each well with a disposable 1000 μl pipette tip.
3. Inoculate each cell by adding 1 ml of bacterial suspension adjusted to 10^7 cfu/ml. Inoculate plants with saline solution as negative control treatment.
4. Record wilting symptoms until almost all the susceptible control plants (cv. Chieftain) are totally wilted (approximately 28 dpi). Disease scoring is performed using a semi-quantitative scale that ranges from 0 to 4, in which 0= no wilting, 1 = 25% of the leaves wilted, 2= 50% of the leaves wilted, 3= 75% of the leaves wilted, 4= 100% of the leaves wilted, dead plant (*see* **Note 8**).
5. Estimate the resistance level by calculating the area under disease progression curve (AUDPC) based on the average wilt scoring for each clone.

3.5 Evaluation of latent infections using BIO-multiplex-PCR.

1. The occurrence of latent infections is determined in genotypes with 0-30% of wilted plants after 28 dpi (evaluated using **Method 3.4**).
2. Collect only asymptomatic plants and wash with tap water to remove the adherent soil. Surface sterilise the plants with ethanol 70% for 1 min, rinse with sterile water for 1 min and dry with sterile absorbent paper towels.
3. Using a sterile scalpel, cut 2 cm stem segments from the basal part of each plant, just above the soil level.
4. Pool stem segments from each replicate tray of each genotype and place them into a sterile bag. Weigh stem pools, add phosphate buffer (10 ml per gram of tissue) and mix in a laboratory blender at high speed for 10 min.
5. Spread aliquots of 100 µl of stem extract onto each of two plates of mSMSA and incubate at 28 °C.
6. After 48 h wash one of the mSMSA plates with 2 mL of sterile water (*see* **Note 9**). The remaining plate is maintained at 28 °C for visual recovery and enumeration of *R. solanacearum* colonies.
7. Centrifuge washed suspensions at 8000 rpm for 5 min and re-suspend pellets in 100 µl of sterile water in Eppendorf tubes. Boil for 20 min and store on ice until used as template for amplification by multiplex-PCR.
8. Multiplex PCR: mix 5 µl of lysate; 1,5 mM MgCl2; 0,2 mM each of four dNTPs; 10 pmol of each IIB1-specific primer (00876F/00876R); 10 pmol of primers 759/760; 1,5 U Taq DNA polymerase and the buffer supplied with the enzyme in 25 µl of reaction volume.
9. Amplification program: 5 min at 96 °C, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, with a final extension step for 10 min at 72 °C.
10. See results by gel electrophoresis using agarose 1.5% for detection of both amplification products. IIB-1 specific primers 00876F/00876R and 759/760 amplify products of 342 bp and 280 bp, respectively (Fig. 4).

11. Record the number of *R. solanacearum* colonies grown in the remaining mSMSA plate after 7-10 days of incubation at 28 °C. Latently infected plants usually have a pathogen concentration of 10^3 to 10^6 cfu/g of stem tissue.

4. Notes.

1. Carry out micro-propagation procedures inside the laminar flow cabinet. Cut single-node pieces with metal clamps and scalpels inside glass petri dishes previously autoclaved. Sterilize metal clamps into the metal sterilizer every 3-4 plants. Before autoclaving, melt PMM and transfer 30-50 ml inside the glass bottles. Single-node stem pieces should be in contact with PMM for plant growth.
2. Before transfer plants to soil, remove agar from roots carefully avoiding root damage. Rinse plants in tap water 2-3 times until no agar remains adhere to the roots.
3. Pick a colony with the typical morphology of *R. solanacearum*: large, fluidal, and either entirely white or white with a red centre.
4. Put whole root system on a glass slide with agarose 1% to observe roots on epi-fluorescence microscope and to maintain samples hydrated. Then, when colonized roots are selected transfer root pieces to a new glass slide with agarose 1%.
5. Put a 200 µl pipette tip in the syringe tip to make a finest solid vaseline retaining wall.
6. Add agarose immediately after placing stem cross-sections. Tissue segments could dry out if agarose addition is delayed.
7. Fluorescence can also be detected using an epifluorescent microscope, although, interference with the chlorophyll autofluorescence often makes interpretation difficult. The intensity of the GFP signal and the microscope fluorescence filters are key variables that have to be evaluated in each case. The use of a confocal microscope highly improves signal specificity and resolution and is always effective.

8. Scoring of disease symptoms has some degree of subjectivity. It is strongly recommended that the same person carries out the whole symptom recording to avoid experimental bias.
9. The mSMSA plate should be washed only when no evident growth or pin-point colonies are observed after 48 hs of incubation. If confluent growth is observed the washing should be replaced by collecting the cells with a sterile toothpick and diluting in 100 μl of sterile water, before following with the lysis and amplification procedure by multiplex PCR.

5. References

1. Mansfield J, Genin S, Magori S et al. (2012) Top 10 plant pathogenic bacteria in molecular plant pathology. Mol Plant Pathol 13:614-629.
2. Safni I, Cleenwerck I, De Vos P et al. (2014) Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. Int J Syst Evol Microbiol 64:3087–3103.
3. Siri MI, Sanabria A, Pianzzola MJ (2011) Genetic diversity and aggressiveness of *Ralstonia solanacearum* strains causing bacterial wilt of potato in Uruguay. Plant Dis 95:1292–1301.
4. Huet G (2014) Breeding for resistances to *Ralstonia solanacearum*. Front Plant Sci 715:1-5.
5. Fock I, Collonnier C, Purwito A et al. (2000) Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja*. Plant Sci. 160:165-176.
6. Carputo D, Aversano R, Barone A et al. (2009) Resistance to *Ralstonia solanacearum* of sexual hybrids between *Solanum commersonii* and *S. tuberosum.* Am J Potato Res. 86:196-202.

7. Siri MI, Galván G, Quirici L et al. (2009) Molecular marker diversity and bacterial wilt resistance in wild *Solanum commersonii* accessions from Uruguay. Euphytica. 165:371-382.
8. Gonzalez M, Galvan G, Siri MI et al. (2013) Resistencia a la marchitez bacteriana de la papa en *Solanum commersonii* Dun. Agrociencia. 17:45-54.
9. Gutarra L, Kreuze J, Lindqvist-Kreuze H, De Mendiburu F (2014) Variation of resistance to different strains of *Ralstonia solanacearum* in highland tropics adapted potato genotypes. Am J Potato Res 92:258-265.
10. Cruz APZ, Ferreira V, Pianzzola MJ et al. (2014) A novel, sensitive method to evaluate potato germplasm for bacterial wilt resistance using a luminescent *Ralstonia solanacearum* reporter strain. Mol Plant Microbe Interact 27:277–285.
11. Ferreira V, Pianzzola MJ, Vilaró F et al. (2017) Interspecific potato breeding lines display differential colonisation patterns and induced defense responses after *Ralstonia solanacearum* infection. Front Plant Sci 8:1424-1437.
12. Guarischi-Sousa R, Puigvert M, Coll NS et al. (2016) Complete genome sequence of the potato pathogen *Ralstonia solanacearum* UY031. Stand Genomic Sci 11:7–14.
13. Elphinstone JG, Hennessy J, Wilson JK, Stead DE (1996) Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. EPPO Bull. 26:663–678.
14. Opina N, Tavner F, Hollway G et al. (1997) A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia Pac J Mol Biol Biotechnol 5:19-30.

Figure legends

Fig 1. Representative photographs of the different potato plant growth stages. **(a)** In vitro plants growing on PMM in glass bottles. **(b)** Plants grown in plastic multicell-trays for bacterial wilt resistance evaluation. **(c)** Plants grown in individual pots for inoculation with reporter strains.

Fig 2. Bioluminescence imaging of *Ralstonia solanacearum* UY031 Pps-lux strain coloninising different potato genotypes. (a) Susceptible potato cultivar *Solanum tuberosum* cv. Chieftain. **(b)** Resistant potato clone 11201.27. Light gray indicates background luminescence and black regions are colonized tissue by light-emitting bacteria. Images were acquired at 3 and 6 days post inoculation (dpi).

Fig 3. Evaluation of bacterial colonisation using the *Ralstonia solanacearum* fluorescent reporter strain UY031 Pps-gfp. **(a)** Confocal visualisation chamber containing stem cross-sections and root segments. **(b)** Representative confocal fluorescence micrographs of stem cross-section (Resistant potato clone 09509.6, left) and root (Susceptible potato clone 13001.79, right). Images were acquired 7 days post inoculation (dpi). Dark arrows show bacterial colonisation.

Fig 4. Gel electrophoresis showing positive results of the multiplex-PCR for detection of latent infections in asymptomatic potato plants inoculated with *Ralstonia solanacearum* (IIB-1).

Figure 1

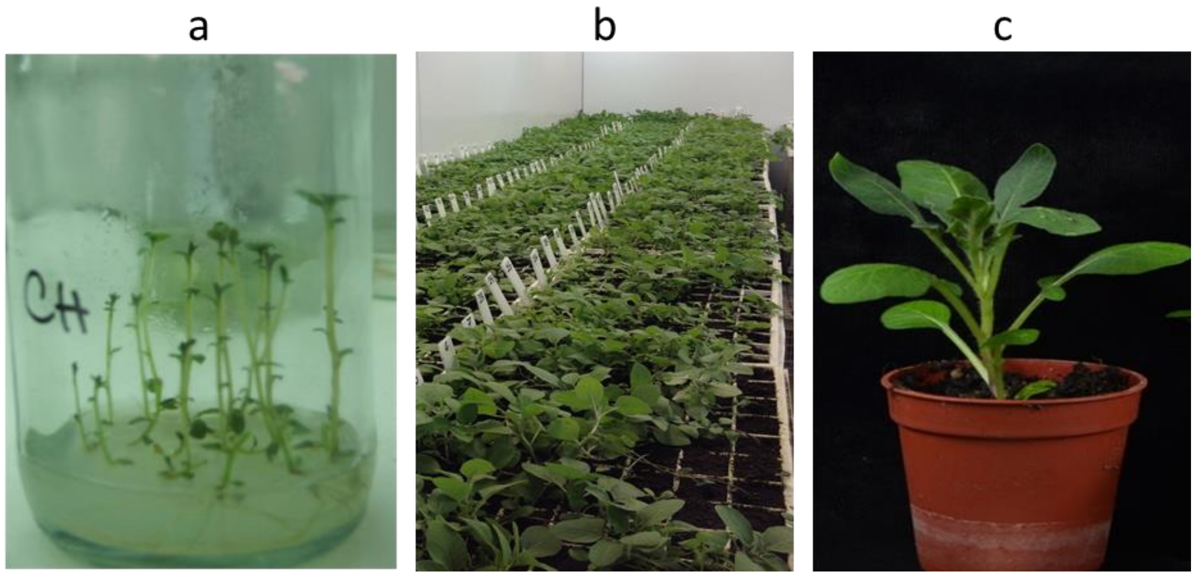

Figure 2

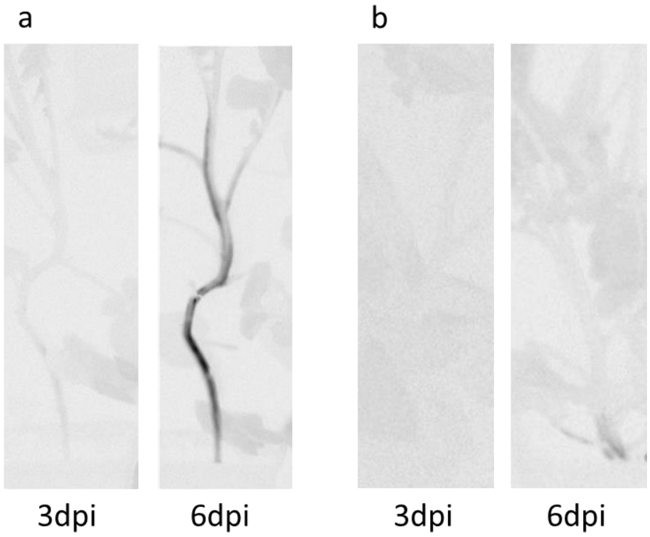

Figure 3

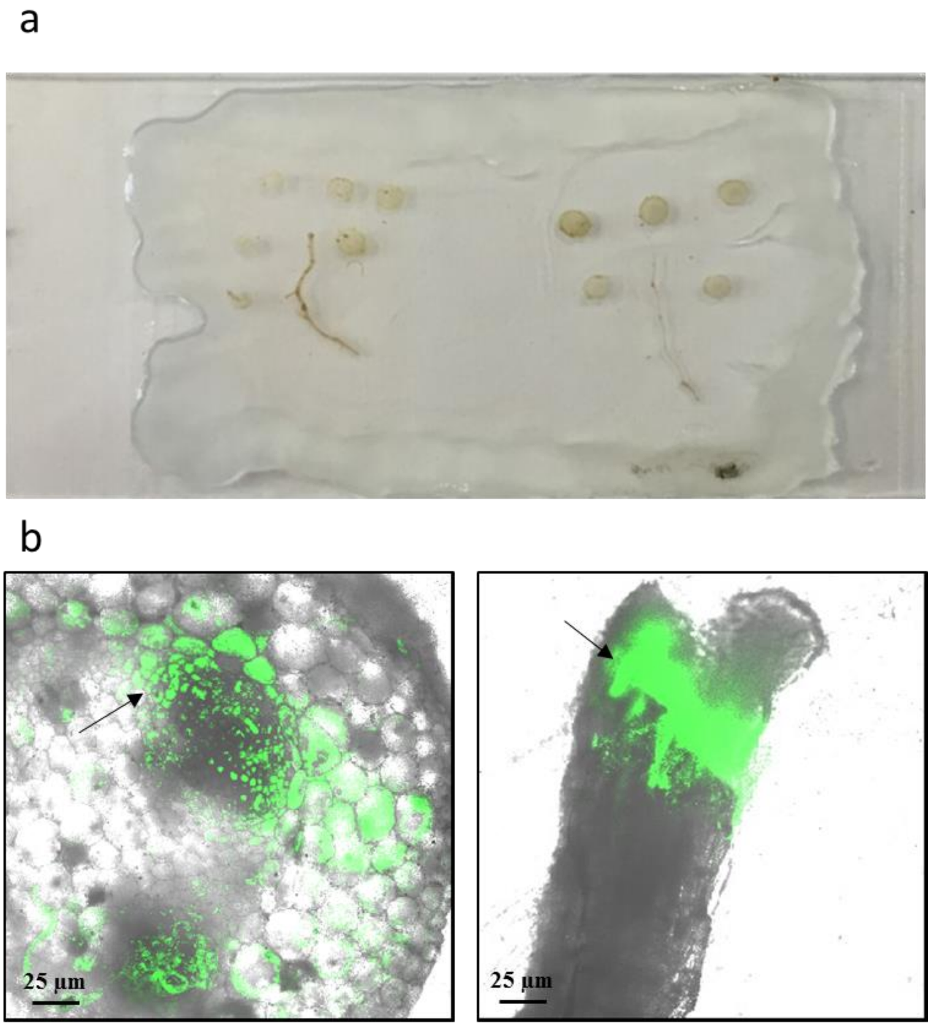

Figure 4