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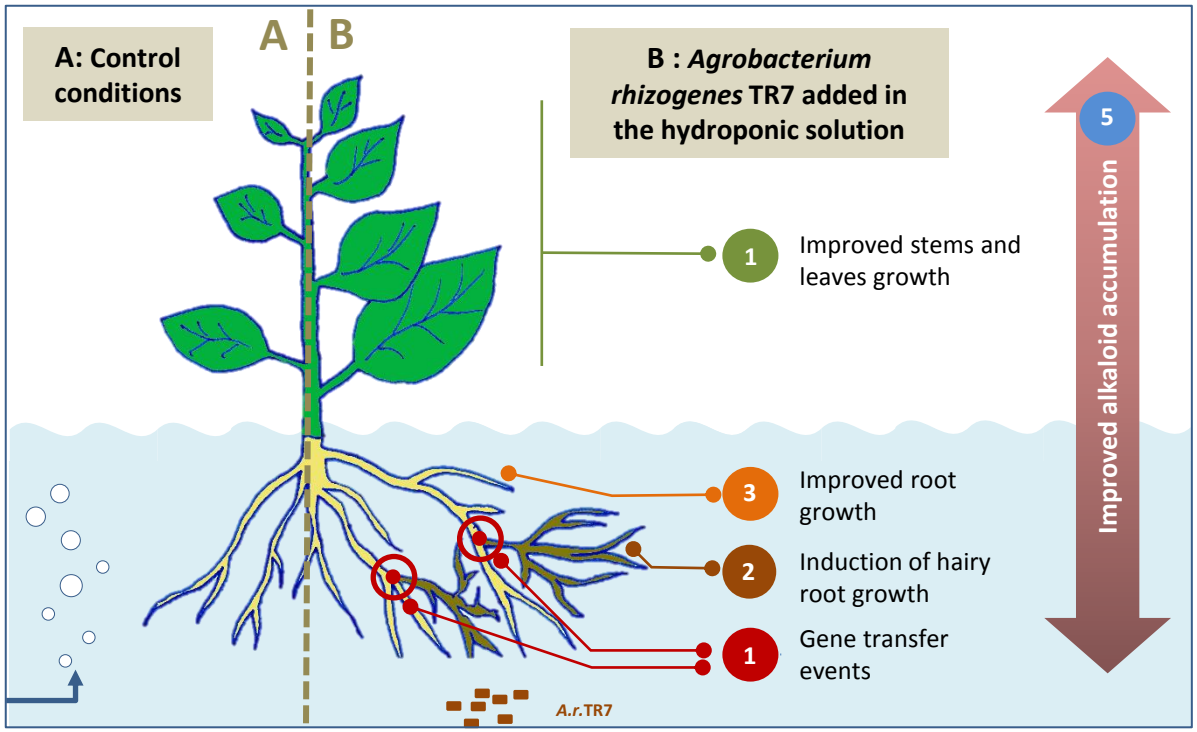
A: Control conditions
A
B
B : *Agrobacterium rhizogenes* TR7 added in the hydroponic solution
1
Improved stems and leaves growth
3
Improved root growth
2
Induction of hairy root growth
1
Gene transfer events
A.r.TR7
5
Improved alkaloid accumulation

Datura innoxia plants hydroponically-inoculated with *Agrobacterium rhizogenes* display an enhanced growth and alkaloid metabolism

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Abstract

1) Background: The production of secondary metabolites through the culture of entire plants is of great interest. Soilless culture, such as hydroponics, enables the control of plant growth and metabolism. Specific environmental conditions must be developed to maximize the productivity of medicinal plants used as efficient natural bioreactors. 2) Methods: The nutrient solution of newly established hydroponic cultures of *Datura innoxia* Mill. were inoculated with *Agrobacterium rhizogenes* (*A.r.*) wild strains (TR7, TR107, 11325 or 15834). Growth and the alkaloid contents of roots and aerial parts were analyzed. Axenic cultures were also performed with modified TR7 strains containing the *egfp* or *gus* reporter gene. *In vitro* isolated root cultures enabled the phenological and molecular demonstration of gene transfer 3) Results: *A.r.*TR 7 led to a greater improvement in plant secondary metabolism and growth. Positive expression of the reporter genes occurred. Isolation and subculture of some of the roots of these plants showed a hairy root phenotype; molecular tests proved the transfer of bacterial genes into the roots isolated from the plants. 4) Conclusions: Hyoscyamine and scopolamine productivity is enhanced after *A.r.* inoculation in the nutrient solution of hydroponic plants. Transformation events occur in the original roots of the plants. This leads to chimeric plants with a part of their roots harboring a hairy root phenotype. Such semi-composite plants could be used for successful specialized metabolite bioproduction in greenhouses.

Keywords: *Agrobacterium rhizogenes*; *Datura innoxia*; hydroponics; tropane alkaloids; chimeric plant; semi-composite plant.

1. Introduction

Higher plants can synthesize a wide range of biologically active specialized metabolites used as pharmaceuticals of generally high value (e.g. morphine, codeine, atropine, scopolamine, quinine). These structurally complex plant products are currently isolated from wild or cultivated plants, or from specific plant organs since their chemical synthesis is not economically profitable [1]. Many medicinal plants require a special climate for growth [2]. For naturally grown plants, there is a risk of over-collecting endangered species [1,2,3]. Alternative methods for producing these plant-derived drugs are therefore desirable. Biotechnological production of specialized metabolites in plant cell cultures provides obvious advantages: biomass production is generally quicker than for the whole plant; plant growth conditions and plant nutrient requirements can be easily controlled; over-collection of endangered species is avoided; geopolitical and import price fluctuations can be eliminated [4].

Callus and suspension cultures of undifferentiated cells are one of the possible biotechnological methods used to produce pharmaceutically active compounds. Despite significant effort over nearly 30 years, many limitations remain and only a few compounds produced in this way have been commercialized [5,6]. The major constraint with cell cultures is that they are metabolically unstable: cultured cells tend to produce low yields of specialized metabolites, which generally vary and decrease over months or years of culture. An alternative method of producing biologically active compounds involves the use of differentiated cultures of shoots [7] or roots [8,9]. The behavior of plant organ cultures is more predictable, and the concentrations of such products are often found to be comparable to, or higher than, those of whole plants [10,11]. Many studies have shown that transformed root cultures offer better possibilities of producing specialized metabolites [12,13]. Hairy roots, transformed with the natural vector *Agrobacterium rhizogenes* (*A.r.*; also renamed *Rhizobium rhizogenes* [14]), the causative agent of hairy root disease in plants, have been found to be suitable to produce specialized metabolites because of their stable and high productivity in hormone-free culture conditions [15]. The possibility of genetic manipulations to increase biosynthetic capacity has initiated considerable interest in them as both a fundamental research tool and a way for producing valuable products [16].

A. r. has been widely described as leading to large modifications in growth and metabolism in the case of hairy root cultures as well as for other uses [8,17]. As such, it has been considered as a biological tool for improving plant rooting [18], especially for recalcitrant [19,20] woody plants [21-25]. However, no very large-scale application in agriculture or forestry has emerged.

Since the late 1990s, we study the potential of entire plants for producing specialized metabolites in soilless conditions, such as hydroponics or aeroponics, conducted in classic “non-axenic” greenhouses [26,27]. We made evidence that the root tropane alkaloid profile was different between hydroponic cultures of *Datura innoxia* conducted in sterile and non-sterile conditions [28]. We also showed that hyoscyamine and scopolamine contents could be up to seven times higher in the hydroponic whole plant when it is co-cultivated with *A.r.*. The interaction of plant roots and *A.r.* has been studied and deciphered through a metabolomic approach including correlation network analysis [29]. Regulation points in the tropane alkaloid pathway have been identified in control roots and in plant roots after co-culture with *A.r.*. We showed that interaction between roots of whole plants and *A.r.* led to a significant growth improvement and metabolic changes in *Datura* roots and shoots. However, the mechanisms involved in the plant response were not clearly established. We assumed that a DNA horizontal transfer occurred from the wild type bacterial plasmid to the genome of the plant cell roots, but this remained to be proved.

Because hydroponic or aeroponic culture of medicinal plants can be performed at industrial scale to produce specialized metabolites, and because the co-culture of such plants with *A.r.* (added in the nutrient solution) can enhance growth and specialized metabolite biosynthesis, it may be possible to recommend the co-culture of plants with *A.r.* on an industrial scale. A prerequisite is to clarify the way in which *A.r.* impacts the plant. It is thus necessary to choose a biological system as a model. Our choice was *Datura* belonging to the Solanaceae family. It is composed of many species including *innoxia*, *metel* and *stramonium*. Such plants produce tropane alkaloids, and particularly scopolamine and hyoscyamine that are used as anticholinergic drugs [30]. For decades, *D. innoxia* has been used as a model for medicinal plants, especially for studies devoted to biotechnological research on hairy root cultures. Many studies were thus carried out to establish and optimize plant growth conditions and phytochemical production [31-33]. The effects of feeding precursors [9], elicitors [31] and heavy metals [34] were also tested. The effect of the biotic environment on tropane alkaloids was less studied, particularly in whole plants.

In the present work, our first objective was to assess the effects of four different *A.r.* strains on the development, growth, and hyoscyamine and scopolamine production of hydroponic *D. innoxia* plants. The second objective was to study the best bacterial strain and to determine if gene transfer really occurred during the early co-culture. Finally, the aim was to build a model of plant root-bacteria interaction that could be applied as a generic tool for specialized metabolite production in whole plants cultivated in greenhouses (*i.e.* in semi-controlled conditions).

2. Materials and Methods

2.1. Biological material

Seeds of *Datura innoxia* Mill., initially obtained from Genbank (Gatersleben, Germany) were germinated under greenhouse conditions. *Agrobacterium rhizogenes* TR7, TR107, 11325 and 15834 wild-type strains (BCCM™/LMG Bacteria Collection, University of Gent) and the derived *A.r.* TR7-*egfp* and TR7-*gus* were grown at 28°C in liquid YMB medium [35] for 24 h. Cells were harvested by centrifugation (3 min, 1100 g), resuspended in distilled water and adjusted to the desired concentration before being used as inoculum. For the construction of the two targeted strains, TR7-*gus* and TR7-*egfp*, plasmid pTDE4 (kindly provided by the group of Prof. Van Montagu, Gent) bearing the GUS reporter gene, and plasmid pPE113 (kindly provided by Prof. F. Guérineau, University of Picardie Jules Verne) coding for a modified Green Fluorescent Protein (EGFP), were introduced, respectively, by electroporation into *A.r.* TR7. The pTDE4 T-DNA contained i) the GUS gene under the control of *CaMV 35S* promoter and followed by the *nos* polyadenylation signal, and, ii) the *nptII* gene between the *nos* (nopaline synthase) promoter and the *ocs* (octopine synthase) polyadenylation signal. The pPE113 T-DNA contained i) the *2X35S* promoter, the *egfp* gene and the *CaMV polyA* sequence and, ii) a *nos-nptII-nos* fusion.

2.2. Non-axenic hydroponic cultures

Seeds of *Datura innoxia* Mill. were sown in the greenhouse in a 1:1 (v/v) mixture of sand/humus and pots were watered daily. After four to five weeks, plants were transferred into hydroponic culture under greenhouse conditions at a day/night temperature of 27/23°C with a photoperiod of 16 h. An amount of 160 µmol of photons.m^{-2}.s^{-1} was provided to the plants by sodium lamps (Philips Son-T Agro 400). The hydroponic system consisted of an extruded polystyrene plate, drilled, floating on a 40-L container (L 56 x W 36 x H 23 cm) with ~30 L of mineral MS nutrient solution [36] as previously described [27]. Young *Datura innoxia* plants were inserted into the holes of plates and roots were kept in the dark. The nutrient solution was inoculated at 3.10^5 CFU.ml^{-1} with an *A.r.* wild-type solution and was continuously aerated by a capillary placed at the bottom of the tray [27]. Plant density was 90 plants.m^{-2}, *i.e.* each tray contained 18 plants. To study growth and development parameters, *Datura innoxia* plants were cultivated in eight trays (2 per modality), each containing a different *A.r.* wild-type strain (TR7, TR107, 11325 and 15834) for five weeks. Plants cultivated in the same conditions without bacteria were used as a control (2 more trays). The total weight of the raft was measured over time on one of the trays. At day 21, 3 plants were used for morphological analysis on the replicate 1 of each tray (root morphology analysis –using WinRHIZO- was not possible later because of root growth was too extensive). For the replicate 2 of each tray, at days 2, 9, 17, 23 and 35, 3 plants were sacrificed for each experimental measurement (fresh and dry weight, alkaloid levels).

2.3. Axenic hydroponic cultures of *Datura innoxia* plants

Seeds of *Datura innoxia* Mill. were surface-sterilized and germinated in glass jars (10 x 5 cm diameter) containing MS/2 medium (22°C, 60% relative humidity, 16 h photoperiod, irradiance of 160 µmol m^{-2} s^{-1}). After eight weeks, plantlets with three to four leaves were transferred, under sterile conditions, to hydroponic bottles containing 1 L MS solution with one plant per bottle.

Each sterile (autoclaved) culture system was composed of a bottle, a tubing with a 0.2 µm filter for 0.1 VVM air bubbling into the nutrient solution and a 44 X 20.5 cm polypropylene cover bag (Sunbag, Sigma, France).

Plants were grown in the growth chamber (60-75% relative humidity, 16 h photoperiod, 25°C day vs. 20°C night, irradiance of 180 µmol m^{-2} s^{-1}). In order to check that the culture had been conducted in sterile conditions, a sample of the nutrient solution was used for microbial testing. The sterile hydroponic solution

was then inoculated with one of the different *A. rhizogenes* strains -the wild-type TR7, the tagged TR7-*egfp* or the TR7-*gus* - with ~0.7 10^5 CFU.ml^{-1}. In these conditions, no microorganism other than the added *A.r.* can lead to a physiological response of the plant.

2.4. *In vitro* isolation of root lines from the roots of axenic plants inoculated by *A.r.*

After 25 days of culture, roots of the different inoculated *D. innoxia* plants were excised, carefully rinsed to eliminate a maximum of bacteria and cultured individually on solid half-strength MS medium (MS/2) supplemented with 30 g.L^{-1} sucrose and 300 mg.L^{-1} cefotaxime. These explants were subcultured 6 more times in the same conditions. As a bacteriostatic, cefotaxime was used in order to eliminate the bacteria gradually throughout the subcultures. The roots were then transferred into liquid B5 medium [37] supplemented with 30 g.L^{-1} sucrose and kept in a rotary shaker (200 rpm) at 25°C in the dark. These cultures were performed without cefotaxime in order to check that no viable *A.r.* remained.

2.5. Measurement of plant growth and root architecture

Three plants per treatment were harvested in order to measure plant growth. Measurements were typically made once a week, for five weeks. Shoots and roots were separated, weighed (fresh biomass), oven dried at 80°C for 72 h, weighed again (dried biomass) and ground for further analysis.

After three weeks of culture, leaves were removed from three other plants and scanned at 200 dpi. Leaf area was then determined by the software WinFOLIA (version 2002a). Root morphology of the same three plants was analyzed by the software WinRHIZO (versions 2002b and 2002c, Regent, Canada). Roots were spread in a tray containing water, scanned and analyzed using the parameters recommended by Bouma *et al*. [38]. Number of root apices, total root length and root diameters were measured by WinRHIZO. Root area and volume were then calculated. It was not technically possible to analyze root architecture at dates later than 3 weeks (impossible to spread the roots in the tray because of their too extensive growth).

2.6. Sample extraction and HPLC analysis

After five weeks of culture, three plants per treatment were harvested. Shoots and roots were separated and immediately frozen at -18°C. Ground freeze-dried tissues (30 ± 5 mg) were extracted with 1.5 mL of an 80/20 (v/v) 0.1 M HCl–95% EtOH mixture. After 30 min, with 3 × 1 min stirring at ambient temperature, the samples were centrifuged at 12000 *g* for 10 min. The supernatant was directly injected into the HPLC apparatus after filtration through a 0.2 µm filter (Minisart RC15, Sartorius, Aubagne, France).

As previously described [39,40], hyoscyamine and scopolamine levels were measured with an HPLC system consisting of: a TSP P100 pump coupled to a TSP AS100 automatic injector (ThermoElectron, France); a UV WAT 484 detector (Waters, France) used at 204 nm; an AZUR® V3.0 software (Datalys, France); and a 4.6 mm i.d. × 250 mm, 5 µm, 120Å YMC ODS-AQ reverse phase column (AIT, France). The mobile phase (flow: 0.8 mL.min^{-1}) was composed of 0.05% formic acid in an 86/14 (v/v) water/acetonitrile mixture.

2.7. Evaluation of the transgenic state of plant roots

The histochemical GUS assay was carried out by incubating root fragments in GUS assay buffer at 37°C overnight and decolorizing with 95% ethanol prior to observation [41].

Imaging of EGFP fluorescence was performed using a fluorescence microscope (Nikon Eclipse E-800) with an excitation filter (450-490 nm). Fluorescent images were recorded using a digital camera.

Total DNA was isolated from fresh roots (500 mg) using the "DNeasy Plant Mini Kit" (Qiagen). For the PCR analysis, the following PCR mixture was used: 200 ng of total DNA, 12.5 µM of each primer, 200 µM d NTPs, 1.5 u *Taq* DNA polymerase (Q-Biogene), and buffer supplied by the enzyme manufacturer in a total volume of 25 µL. The *rolA* specific primers used (*rolA* DIR cagaatggaattagccggacta / *rolA* REV cgtattaatcccgtaggtttgttt; Sevon et al., 1997) generated a 310 bp PCR product; the *virA* primers (*VirAAr*85D ccatcttgtgagcggcaacga / *VirAAr*230R tactcagcgcacgagtgaagctg; [42]) generated a 145 bp PCR product; the *egfp* primers (*egfp*222D ctaccccgaccacatgaagcag / *egfp*395R ttgaagtcgatgcccttcagctc) generated a 173 bp product and the *gus* primers (35SDIR100 cactatccttcgcaagacccttcctctatataaggaa / *gus*284R ccgtaatgagtgaccgcatcgaa) generated a 384 bp fragment.

PCR amplifications were performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C (for the *rolA* gene) or 55°C (for the *gus* gene) or 62°C (for the *virA* and *egfp* primers) for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

The PCR reaction mixtures (25 µL) were loaded directly onto a 1.5% agarose gel for electrophoretic analysis. A DNA ladder (Eurogentec) was used as a molecular weight marker for the PCR-amplified double-stranded DNA fragment.

2.8. Statistical analysis

All data were analyzed using the Statbox Pro variance procedure. Means were classified according to the Newman-Keuls test. Data followed by different letters are significantly different ($p<0.05$).

3. Results

3.1 Inoculation of the nutrient solution of hydroponic *Datura innoxia* plants by different strains of *Agrobacterium rhizogenes*

3.1.1 Growth and development of *D. innoxia* plants

Shoot and root dry matter of *D. innoxia* plants grown in the greenhouse under non-axenic hydroponic conditions was determined on three plants per condition at days 2, 9, 17, 23 and 35 (Fig. 1). These plants were grown with or without one of the four *A.r.* strains. Leaf area per plant, root length, root area, root volume and root apex number were already higher after three weeks in plants with *A.r.* than in controls, regardless of the strain (Fig. 2). Because the root growth was too extensive, it was not possible to spread, scan and analyze the root morphology with WinRHIZO at later dates.

After 35 days, the presence of *A.r.* had increased dry matter accumulation in shoots and roots compared to control plants (Fig. 1). TR7 strain led to the highest dry matter accumulation in whole plants (17.8 gDW for shoots and 4.7 gDW for roots). The mean plant biomass per plant was doubled in TR7 compared to control plants (22.5/11.5 gDW per plant). Based on the culture density of 90 plants per square meter, this corresponds to a biomass production of 2.025 kg DW.m^{-2} for *D. innoxia* hydroponic culture inoculated with TR7, and only 1.035 kg DW.m^{-2} for the control plants after 35 days of culture.

3.1.2 Tropane alkaloid levels in *D. innoxia* plants

Hyoscyamine and scopolamine contents were also measured in shoots and roots after 35 days of culture (Fig. 3). *A.r.* TR7 strain significantly increased the hyoscyamine content in shoots compared to controls (Fig. 3). TR7 and 11325 had the same positive effect for hyoscyamine in the roots of inoculated plants. The

scopolamine concentration was enhanced in the shoots when TR7, 11325 and 15834 strains were added to the nutrient solution. It was significantly enhanced in the roots only with the 11325 strain. The inoculation with TR7 bacterial strain enhanced the hyoscyamine and scopolamine levels from 2 to 8 in roots and shoots. This led to an estimated total accumulation of 2.5 g of alkaloids per square meter after a culture of 35 days. The total productivity could thus be determined as 71 mg of hyoscyamine and scopolamine produced per day and per square meter of hydroponic culture.

To validate these results at a larger scale, the same kind of experiment was carried out at a pilot scale (Fig. S1.1 to S1.9). Two parallel hydroponic cultures of 252 plants were conducted in two 3 m^2 [L: 5 m x W: 0.6 m x H: 0.1 m; 200 L of nutrient solution] containers under the same greenhouse conditions. One was inoculated by *A.r.* TR7, the other was the control. This assay confirmed the previous ones and demonstrated the value of inoculating *D. innoxia* with *A.r.* TR7 strain: increased plant growth (Fig. S1.1 to S1.7**)** and higher hyoscyamine and scopolamine contents in shoots and roots (Fig. S1.8). Compared to the control, the initial addition of *A.r.* TR7 into the nutrient solution of the pilot-scale hydroponic system did not lead to evident modification of the total microflora that grew spontaneously in the nutrient solution (Fig. S1.9).

3.2. Evaluation of the transgenic state of roots

3.2.1 Culture of *Datura* in axenic hydroponic conditions and inoculation of the nutrient solution with modified *A.r.* TR7 containing the *egfp* or *gus* reporter gene

Because the inoculation of the nutrient solution by dead *A.r.* TR7 (boiled twice in a microwave oven) did not lead to growth (Fig. S2.1) or alkaloid level (Fig. S2.2 and S2.3) improvement, we hypothesized that the positive effect of *A.r.* TR7 could be related to gene transfer events. To check this hypothesis, two genetically modified *A.r.* strains called TR7-*gus* and TR7-*egfp* were constructed by transfection with the two plasmids pTDE4 and pPE113, respectively. These two strains have two available plasmids: pRi and either pTDE4 or pPE113. The vir proteins can transfer one or both T-DNAs into the plant genome and can therefore generate transformed tissues with the rol genes and/or the reporter genes.

Axenic cultures of hydroponic *Datura* were performed (Fig. S3.1 and S3.2) and used for the co-culture with the different TR7 strains. After 25 days, the different root systems of *D. innoxia* inoculated with *A.r.* TR7 wild-type, TR7-*egfp* and TR7-*gus* were more developed than that of the control. To determine the presence of EGFP protein, the roots were observed with UV light under a microscope. Control roots (Fig. 4a) never showed any kind of yellow-green fluorescence. In contrast, some of the TR7-*egfp* roots showed the typical yellow-green fluorescence due to the presence of EGFP protein. The observation of pieces of TR7-*egfp* roots clearly indicated that EGFP accumulated only in some parts of the root system; for example, in the tips (Fig. 4b) and the proximal parts of some roots (Fig. 4c). Likewise, histochemical analyses of the roots of TR7-*gus*-inoculated hydroponic plants showed some gus activity after 25 days (Fig. 5a) in contrast to controls (Fig. 5b). However, this staining was not homogeneous (Fig. 5a and 5c) and showed a chimeric distribution in the roots (Fig. 5d). Only some parts of the root system thus appeared to be *gus*-positive.

3.2.2 Isolation of roots from *A.r.* TR7 inoculated *Datura* and *in vitro* characterization of their hairy root-like phenotype after eradication of the bacteria

To determine whether gene transfer occurred during the plant-root interaction, some root apices from the different hydroponic plants were isolated and transferred to Petri dishes on MS/2 medium without hormone

but with cefotaxime at 300 mg/L (Fig. 6a to 6d). After 18 days of culture (Fig. 6e to 6h), differences were noted in the root growth and phenotypes. The roots from control plants did not grow, even after a long time (Fig. 6e). In comparison, some of the roots from the *A.r.* co-cultured plants presented a fast and significant growth with numerous ramifications like hairy roots (examples in Fig. 6f to 6h). Some others were thick with a positive geotropism. In some cases, some of the root apices did not develop, like control roots (not visible on these Petri dishes).

The isolated roots were then subcultured six more times on solid agar medium without phytohormone but with cefotaxime 300 mg/L in order to remove any remaining agrobacteria. Some explants were transferred onto solid medium without cefotaxime to check that no living bacteria could grow again. In addition, small explants (1.5 – 2 cm) of the rapidly well-developed roots were isolated and cultured in liquid B5 medium without phytohormones and without cefotaxime. No bacterial contaminant occurred in the cultures. The roots quickly developed and presented all the attributes of hairy roots (Fig. S4.1) with a two hundred-fold increase in the fresh biomass (from 0.02gFW to values of 4 to 4.3g for TR7/TR7-egfp/TR7-gus; Fig. S4.2). Compared to the control roots, the alkaloid contents were six to seven times larger (Fig. 7).

3.2.3 Molecular proof of gene transfer occurrence

To establish that transformation events had definitely occurred, we checked the integration of the different foreign genes in the *in vitro* isolated root lines that grew without additional phytohormone in liquid medium. These cultures contained no viable *Agrobacterium* cells: no bacteria grew during the microbiological tests performed on the root media and tissues. Using PCR, no *virA* gene could be detected in all these root cultures. Furthermore, the positive controls (*A.r.* TR7) were all positive, confirming that the molecular test was efficient. Thus, it could be concluded that no more -even dead- *Agrobacterium* remained in the flasks (Fig. 8a to 8c; see *A.r.* TR7).

The occurrence of (i) the *rol A* gene from the plasmid pRi TR7 in the wild-type TR7 (Fig. 8a to 8c), (ii) the *egfp* gene from the plasmid pPE113 in TR7-*egfp* (Fig. 8b) and (iii) the *gus* gene from the plasmid pTDE4 in TR7-*gus* (Fig. 8c) was confirmed by PCR analysis in root lines from each inoculation experiment. The systematic presence of the *rolA* gene thus proved that a gene transfer had systematically occurred; *i.e.* a genetic transformation by *A.r.*. A difference in the intensity of amplified genes could also be observed. This may be explained by the still chimeric state of the root biomass in the Erlenmeyer flasks (some roots may have integrated more than one DNA copy). The *egfp* gene was recovered in 4 of the 6 "TR7-*egfp*" tested root lines (Fig. 8b). Of the seven tested root lines, two were positive for the *gus* gene after amplification (Fig. 8c). The combined presence of these genes (*rolA* + [*egfp* or *gus*]) proved that a double transformation event had occurred. The *rol*A gene was systematically present and lead to the hairy root phenotype whereas the *egfp* or the *gus* gene are present in some independent transformation.

4. Discussion

Hairy root (HR) cultures have been widely studied as a model of choice for the characterization of Specialized Metabolic (SM) pathways and for the development of the biotechnological production of plant metabolites [2,43-49]. Typically, HRs are cultured *in vitro*, in Erlenmeyer flasks and in different types of higher scale bioreactors. The initiation of such HRs generally starts by the inoculation of plant material (seedlings, plant part) with *Agrobacterium rhizogenes* (*A.r.*). It is well known that such a bacterium can transfer some plasmidic DNA (ri-tDNA) into the genome of a plant cell. The transformed cell display a modified

phytohormone balance and sensitivity [50] and tend to divide and differentiate into root tissues that can be multiplied *in vitro* [51-53]. Such *in vitro* plant material is of very great interest because it generally has a relatively high growth rate and the levels of Specialized Metabolites are comparable to or higher than those of the roots in entire plants. These characteristics are stable throughout the subcultures. Nevertheless, with the aim of the industrial production of plant specialized metabolites, it is necessary to overcome the difficulty of both i) scaling up and ii) maintaining the axenic characteristic (*i.e.* sterility) of the culture.

In our strategic perspective, we chose here to use entire plants cultivated in open air conditions (*i.e.* non-sterile) and with the possibility of an easy scale-up of the cultures carried out in greenhouses. Soilless culture, like hydroponics (or aeroponics), opens up such perspectives. It also enables a better control of plant growth parameters (nutrient solution, temperature, humidity, light, etc.). Plant biomass containing large amounts of Specialized Metabolites of interest can be produced by optimizing the process. We demonstrate here that inoculation of the nutrient solution of hydroponic *D. innoxia* with chosen *A.r.* strains led to improved growth and alkaloid production. This work also reveals the mechanisms by which the plant-bacteria interaction acts on the productivity of the system. Due to *A.r.*, the novel plants now harbor a chimeric root system composed of “normal roots” and “transformed roots”. The latter, like *in vitro* HRs, probably grow faster and synthesize more alkaloids. Furthermore, they most probably induce an increase in growth and secondary metabolism in neighboring “normal roots”. These modifications would influence the aerial parts, which may grow more and accumulate more alkaloids.

In addition, in our experiments the aerial parts of the plants were normal. They did not have the phenotype observed in *Datura* plants regenerated from hairy roots. In an unpublished experiment, we obtained these kinds of plants regenerated from a more than ten-year-old *D. innoxia* hairy root strain cultivated *in vitro*. The regenerated plants were smaller than controls (dwarf phenotype as described by Casanova *et al.* [54]). Their internodes were shorter, and their leaves were wrinkled. Flowering occurred but no fruit production was observed. In the case of the *Datura* plants described in this article, the shoots were absolutely comparable to those of control plants. No modification of architecture or fertility was observed.

Cultivating medicinal plants in hydroponics (or aeroponics), optimizing culture condition parameters and inoculating the nutrient solution with chosen *A.r.* to produce semi-composite plants could be a strategy of generic interest to produce plant secondary metabolites. Bacterization of the nutrient solutions with a synthetic inoculum composed of different *A. rhizogenes* strains could be suggested in greenhouse-controlled conditions [4, 55] or for the seedlings before the greenhouse culture.

Consequently, other points must be clarified. The first one is would such semi-composite (chimeric) plants obtained with a wild strain of *A.r.* be considered genetically modified organisms? The answer is no, based on legal requirements in European countries. Also, is there a risk of using a pathogenic bacterial (*A.r.*) species in a greenhouse? Based on the recent review of Bosman *et al.* [56]), the answer is yes. Some biovars from *Agrobacterium* species are causative agents of pathology, the hairy root disease, particularly in the hydroponic cultivation of tomato, cucumber and eggplant. However, some other *Agrobacteria* are not pathogenic. Lastly, in the case of problems within the greenhouse, some methods are available to eradicate or at least minimize the presence and/or the effects of the pathogenic bacterium [21].

In this work, *A.r.* was used to improve the production of plant roots (and subsequently aerial parts) which then produce more metabolites of interest. In *Datura*, roots are known to be the main biosynthetic site of tropane alkaloid synthesis prior to further translocation into shoots and preferentially the youngest leaves [57]. The increase in the root biomass and number of root apices could have directly led to a higher

biosynthetic potential of the root system. Therefore, the whole plant contained more tropane alkaloids after partitioning and remobilization from roots to leaves.

As a conclusion, we suggest here producing plant secondary metabolites from medicinal plants through the soilless culture of entire plants and with an optimized inoculation of the nutrient solution containing a chosen strain of *A.r.*. The final plant biomass should be greater and may contain higher levels of phytochemicals than non-inoculated control plants.

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Appendix A: Supplementary data 1; Effects of *Agrobacterium rhizogenes* TR7 on growth and tropane alkaloid content of *Datura innoxia* cultivated in a large-scale hydroponic system; including Fig. S1.1-9.

Appendix B: Supplementary data 2; Effects of microwaved boiled *Agrobacterium rhizogenes* TR7 initial inoculation on the growth and tropane alkaloid levels of hydroponic *Datura innoxia* plants; including Fig. S2.1-3.

Appendix C: Supplementary data 3; Inoculation of modified *Agrobacterium rhizogenes* TR7 strains in the nutrient solution of axenic-hydroponic cultures of *Datura innoxia*; including Fig. S3.1-2.

Appendix D: Supplementary data 4; Characterization of growth and tropane alkaloid levels in the axenic and stabilized liquid cultures of roots isolated from axenic hydroponic *Datura innoxia* plants in which the nutrient solution had been inoculated with *Agrobacterium rhizogenes* TR7, TR7-egfp or TR7-gus strain; including Fig. S4.1-3.

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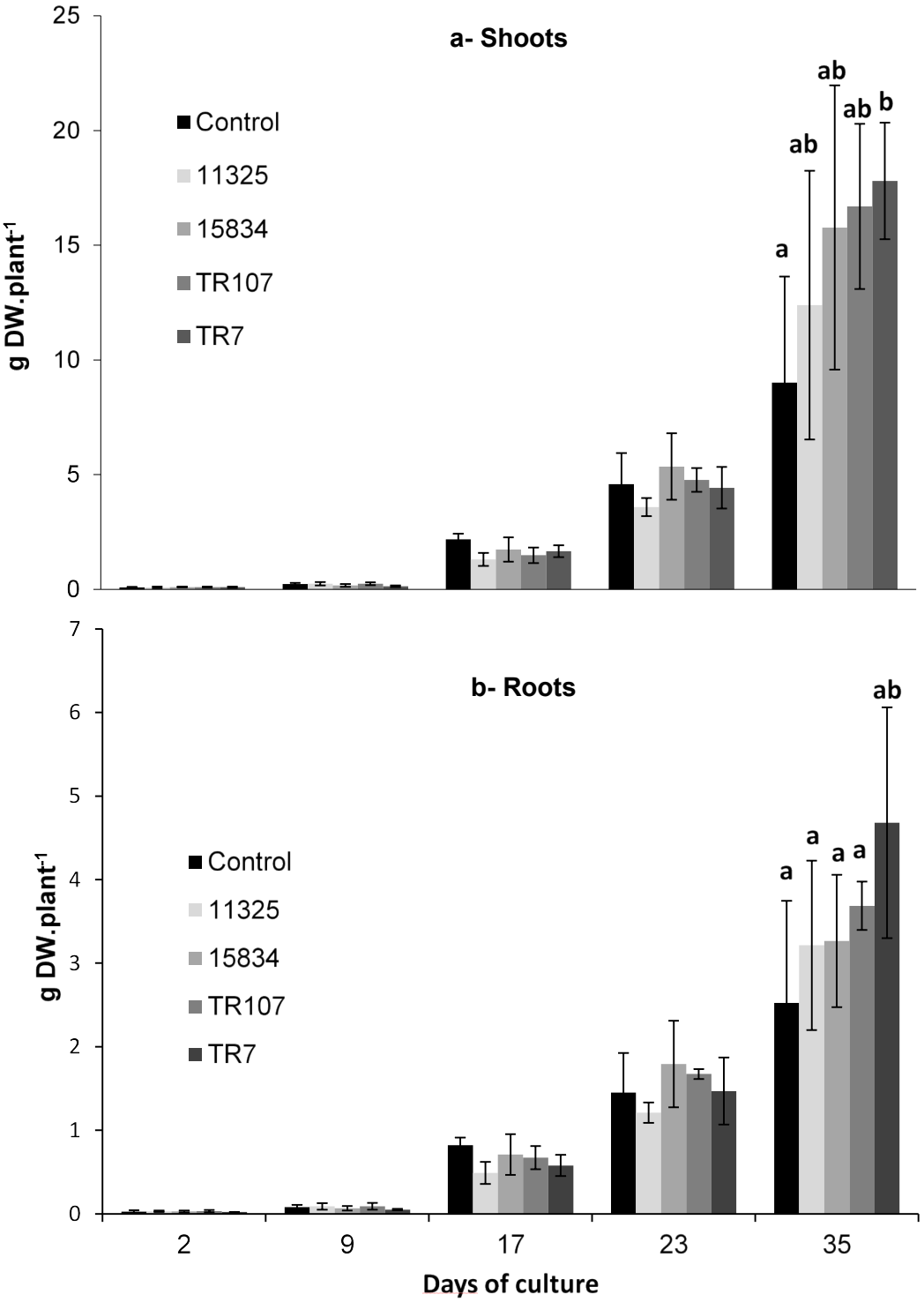

Figure 1: Dry matter accumulation per *D. innoxia* plant in shoots (a) and roots (b) in the presence or not of four different *Agrobacterium rhizogenes* strains. The values are means of 3 plants ±standard error; for day 35, significant ANOVA at $p<0.05$ was followed by a Student Newman Keuls test at $p<0.05$ (different letters for significant differences).

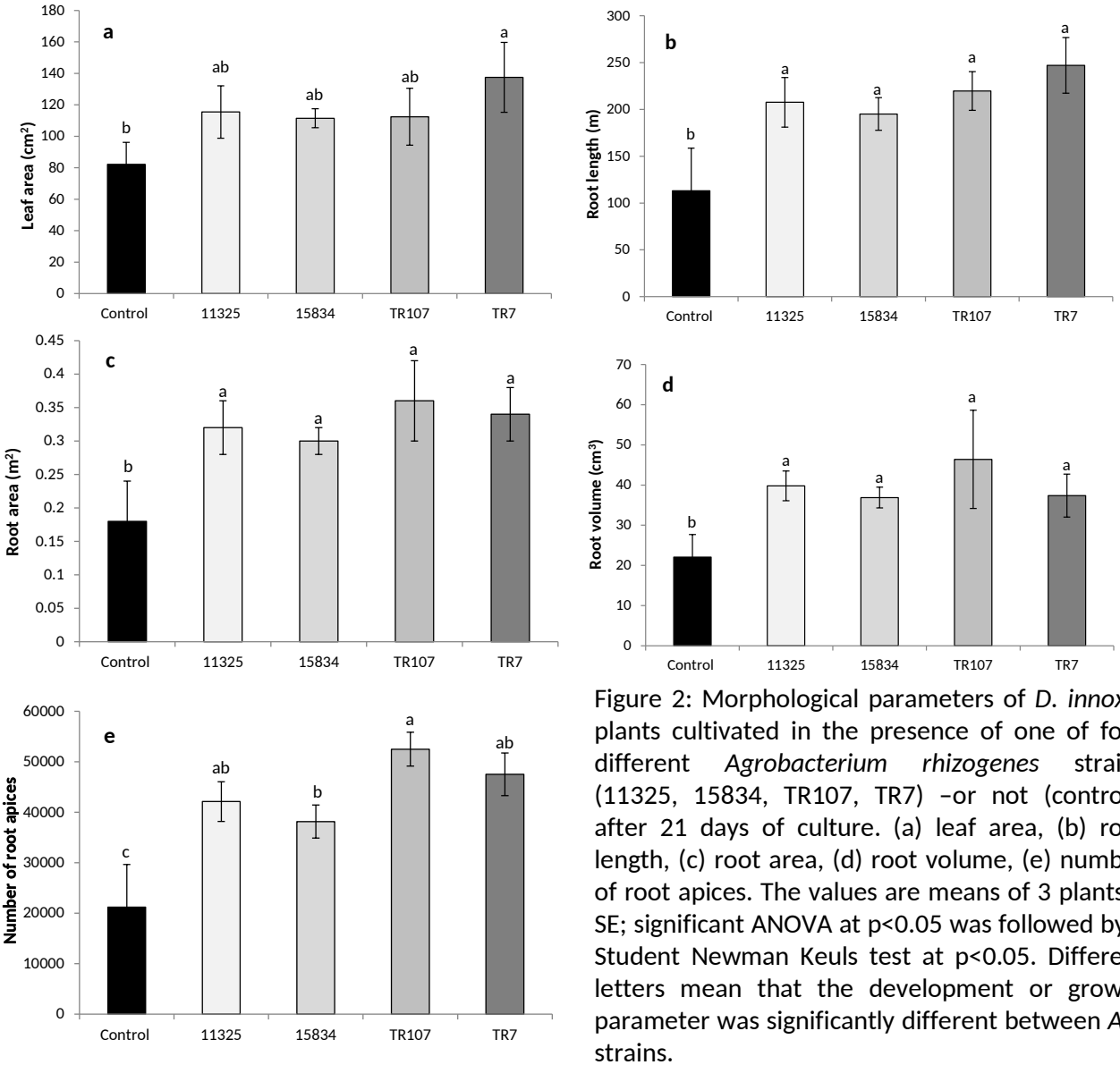

Figure 2: Morphological parameters of *D. innoxia* plants cultivated in the presence of one of four different *Agrobacterium rhizogenes* strains (11325, 15834, TR107, TR7) –or not (control)- after 21 days of culture. (a) leaf area, (b) root length, (c) root area, (d) root volume, (e) number of root apices. The values are means of 3 plants ± SE; significant ANOVA at $p<0.05$ was followed by a Student Newman Keuls test at $p<0.05$. Different letters mean that the development or growth parameter was significantly different between *A.r.* strains.

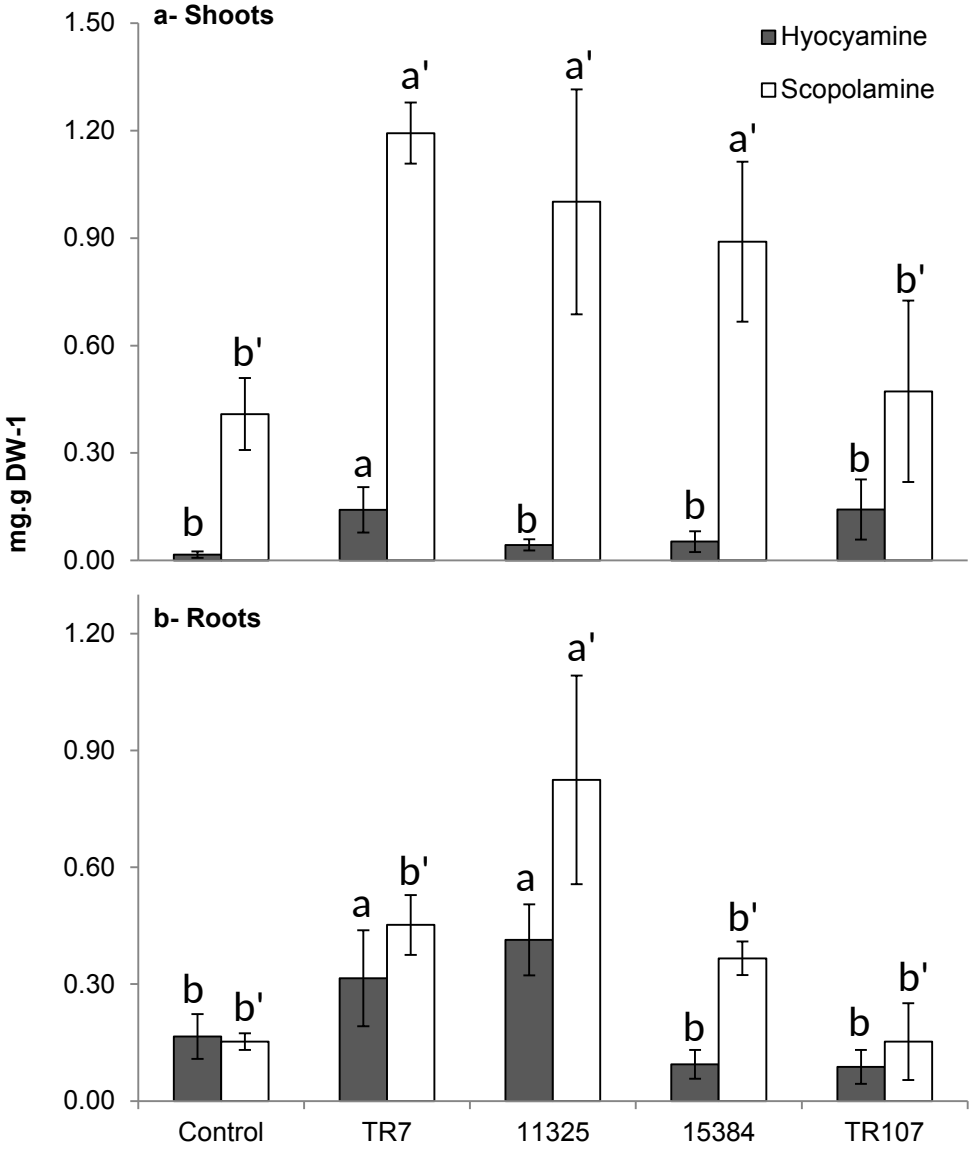

Figure 3: Hyoscyamine and scopolamine contents in 35-day-old shoots (a) and roots (b) of non-axenic *D. innoxia* hydroponic cultures in which the nutrient solution was initially inoculated -or not (control)- with four different *Agrobacterium rhizogenes* strains (TR7, 11325, 15834, TR107). The values are means of 3 plants ± standard error. Significant ANOVA at $p<0.05$ was followed by a Student Newman Keuls test at $p<0.05$. Different letters mean that the tropane alkaloid accumulation was significantly different from the corresponding control.

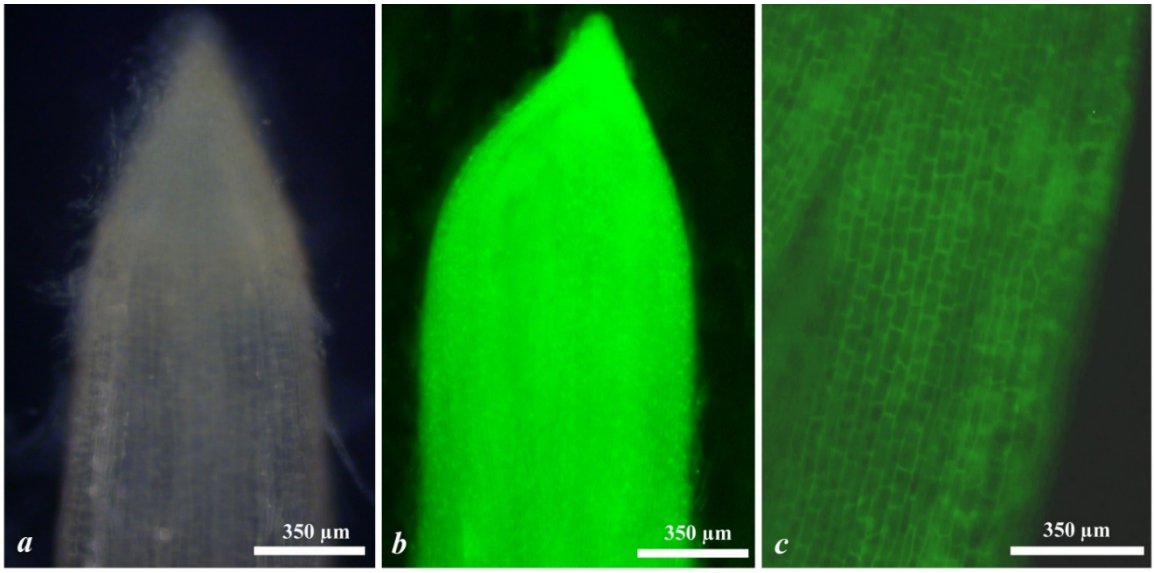

Figure 4: Fluorescence in a root of a hydroponic plant cultivated with *A. rhizogenes* TR7-*egfp*. Root apex from a control plant (a; no fluorescence), and fluorescent root apex from a co-cultured plant (b). Distribution of fluorescent signal in the root, above the apex (c).

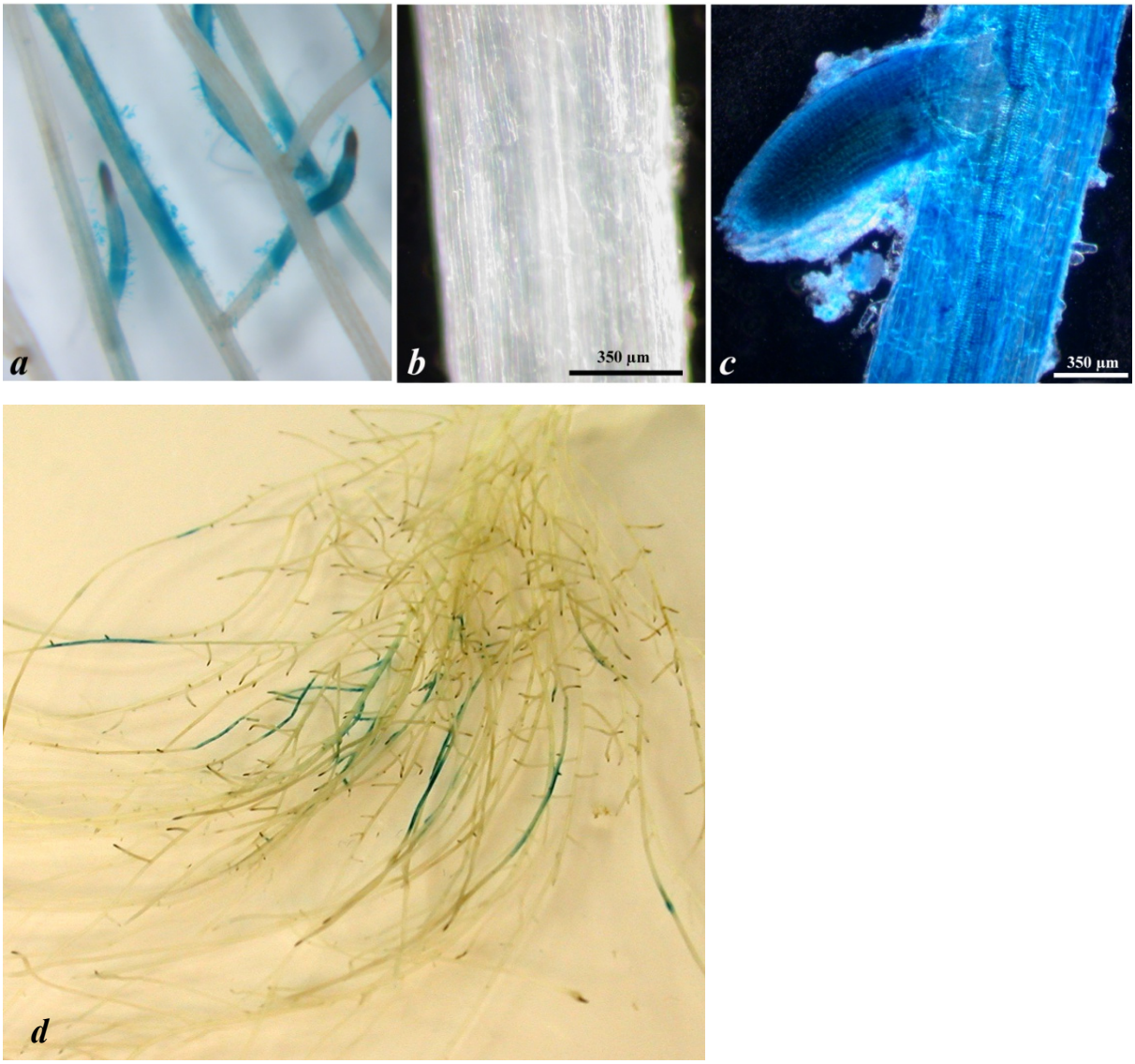

Figure 5: Blue color from the metabolization of X-gluc substrate in roots of axenic hydroponic plants co-cultured with *A. rhizogenes* TR7-*gus*: (a) non-uniform blue color in roots, (b) root of a control plant, (c) emergence of a lateral blue root from a blue-colored root. (d) Root system of a 21-day-old *A.r. TR7-gus* plant showing a chimeric distribution of blue color that reveals x-gluc activity and thus the distribution of potential sites of transformation with the *gus* gene. Control plants never showed any coloration.

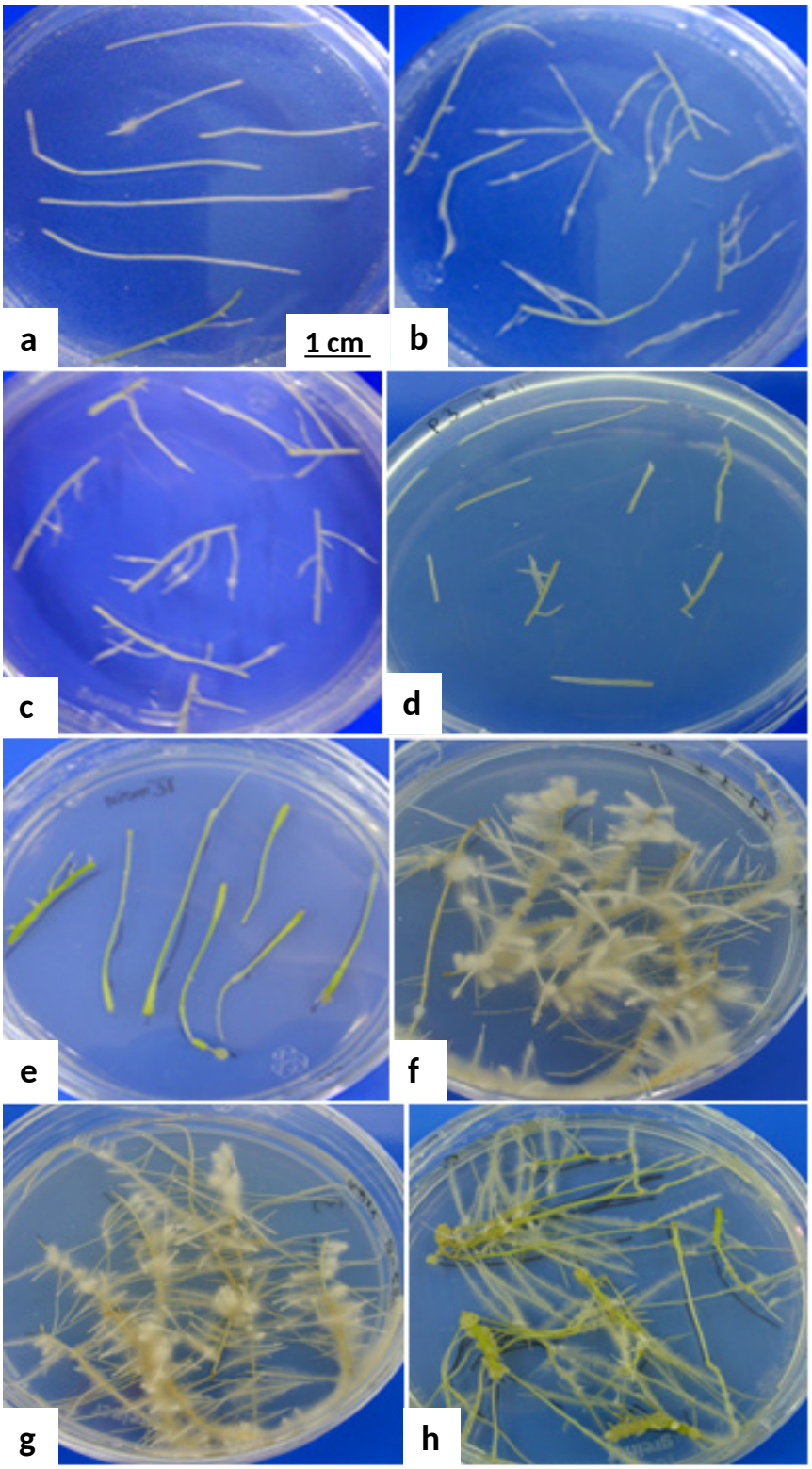

Figure 6: *In vitro* development of roots isolated from hydroponic axenic plants. The culture was performed without phytohormone but with cefotaxime 300 mg/L to avoid bacterial growth. Petri dishes (a) to (d) are initial roots from control, TR7, TR7-*egfp* and TR7-*gus* plants, respectively. (e) to (h) are the same Petri dishes after 18 days of culture.

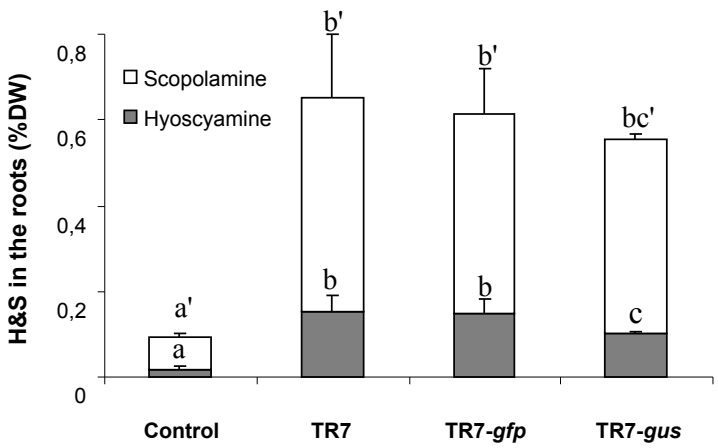

Figure 7: Hyoscyamine and scopolamine levels measured in the different *in vitro* isolated hairy root lines. Control corresponds to analyses performed on control roots from axenic hydroponic plants. Analyses of TR7/TR7-gfp/TR7-gus were performed 16 days after the 7th *in vitro* subculture in liquid medium. The values are means of 3 plants ± standard error. Significant ANOVA at $p<0.05$ was followed by a Student Newman Keuls test at $p<0.05$. Different letters mean that the tropane alkaloid accumulation was significantly different from the corresponding control.

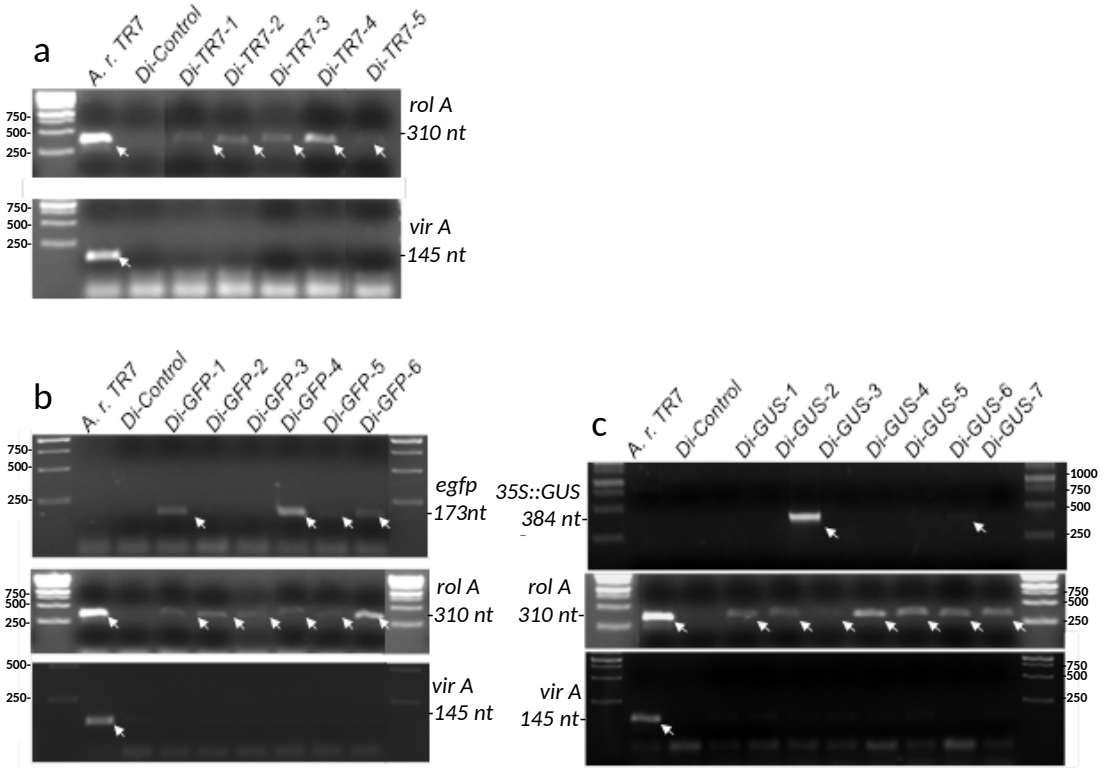

Figure 8: PCR analysis of the *vir*A, *rol*A, *egfp* and *gus* genes in the different root lines. (a) TR7, (b) TR7-*egfp*; (c) TR7-*gus*.