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Abstract

Climate change is challenging plant breeders to produce better adapted plants. The EU project "RootsPlus" (www.rootsplus.eu) is working on an innovative breeding method, called root-inducing (Ri) technology, in which plants are transformed with wild-type strains of *Rhizobium rhizogenes*. This results in new interesting traits (Ri phenotype). In this work, the influence of an antimicrobial medium additive (PPM™) during pre-culture of explant donor shoots on the transformation efficiency of the apple rootstocks 'M26' and 'Selection 6' was investigated. In another experiment using 'M26' hairy roots, the effect of a culture passage in the dark on hormone-free medium (½ MS) on shoot regeneration was studied. PPM™ treatment resulted in increased transformation efficiency for 'M26', from 86.7% to 96.7%, and doubled the number of hairy roots from 5.8 to 11.3 per root-forming explant. In contrast, for 'Selection 6' the PPM™ treatment resulted in no significant differences in transformation efficiency and number of hairy roots. After 28 weeks, 19% of the 'M26' hairy roots produced shoot buds when cultured on regeneration medium with 9.1 µM Thidiazuron. The culture passage on hormone-free medium in the dark had no significant effect on this outcome. These buds subsequently developed into Ri shoots on apple propagation medium.

1. Introduction

Abiotic stresses as a result of climate change such as drought, but also biotic stresses like replant disease are problems that apple growers face and that reduce apple yields drastically (Mazzola and Manici 2012, Suran and Pravcová 2023). Plant breeding needs to produce better adapted rootstocks that also meet the many other requirements such as dwarfing and resistance to various pathogens and pests (Wang et al. 2019). "RootsPlus" is a project focusing on Ri (root inducing) technology as a breeding tool. This involves transformation with the soil bacterium *R. rhizogenes*. By integrating the bacterial T-DNA from the Ri plasmid into the plant genome and expressing the *rol* (root oncogenic locus) genes, roots are formed at the infection sites. These hairy roots can be used to regenerate Ri plants that show morphological changes such as stunted growth due to shorter internodes, small wrinkled leaves, increased branching, improved root system and altered flower morphology and flowering time (Desmet et al 2020). As wild-type strains are used, Ri plants are not considered to be genetically modified organisms (GMOs).

The success of Ri plant generation depends on efficient transformation and regeneration. Both bacterial strain and genotype impact transformation efficiency, as strains differ in inducing hairy roots (Porter and Flores 1991) and genotypes in susceptibility. Factors like genotype, bacterial strain, and plant growth regulator concentration also affect Ri plant regeneration from hairy roots (Desmet et al. 2019; Wang et al. 2019).

The objectives of this work were, firstly, to investigate the influence of pre-culturing the explant donor shoots on a medium containing the antimicrobial agent Plant Preservative Mixture (PPM^{TM}). Secondly, the regeneration of Ri shoots from hairy roots was to be improved by introducing a phase on hormone-free medium in darkness prior to the shoot regeneration phase.

2. Data, methods and procedure

2.1. Plant material

Two apple rootstocks were used for transformation with *R. rhizogenes*: 'M26' and 'Selection 6'. These genotypes were already established as in vitro shoot cultures and were propagated on apple propagation (AP) medium (MS (Murashige and Skoog 1962) salts and vitamins (Duchefa), 30 g L^{-1} sucrose, 2.2 µM benzylaminopurine (BAP), 0.5 µM indole-3-butyric acid (IBA), 7.8 g L^{-1} Plant agar (Duchefa), pH 5.8). For shoots treated with PPM^{TM} (Plant Cell Technology), 2 ml L^{-1} PPM^{TM} was added to the AP medium. Subculturing took place every four weeks for three months. All media media components and hormones (except TDZ) were autoclaved at 121 °C and 200 kPa for 20 minutes. Cultures were maintained at 24 ± 2 °C in a 16/8 h light/dark cycle with a photosynthetic photon flux density (PPFD-PAR) of 40 µmol m^{-2} s^{-1}. Plant material was used four to five weeks after the last subculturing. For transformation, young, fully expanded leaves were cut and kept in 100 ml transformation coculture medium (TCM: ½ MS salts and vitamins, 15 g L^{-1} D-glucose monohydrate, pH 5.7) for a maximum of 7 h before coculture.

2.2. Bacterial strains and preparation of the transformation solution

The different experiments used either the wild-type strain *R. rhizogenes* ATCC 15834, provided by Dr Frank Dunemann, Julius Kühn-Institut Quedlinburg, or the same strain with an additional C757 plasmid carrying a reporter gene (DNA Cloning Service, Hamburg) for green fluorescent protein (GFP), provided by Prof Debener, Institute of Plant Genetics, Leibniz University Hannover. The wild-type strain was used in the regeneration experiment, while the GFP-tagged strain was used in the transformation experiment. The bacterial cultures for the transformations were prepared as described by Rüter et al. (2023) by growing the bacteria in yeast extract glucose broth medium (YEG: yeast extract 1 g L^{-1}, peptone 5 g L^{-1}, glucose 10 g L^{-1}, magnesium sulfate heptahydrate 0.49 g L^{-1}, pH 7.2) to an optical density (OD_{600}) of 0.5 ± 0.1 and resuspending them in liquid TCM with 200 µM acetosyringone again to an optical density of 0.5 ± 0.01.

2.3. Transformation

The transformation was done according to Rüter et al. (2023): For each variant, 30 leaf explants were transferred abaxial side down into 100 mL of bacterial solution, wounded in an ultrasonic bath (Bandelin Sonorex Super 10 P, type DK 102 P) for 10 min at 35 kHz and 60 W L^{-1} and incubated in the bacterial solution at room temperature for 30 min. The leaves were blotted dry on sterile filter paper and transferred with the adaxial side down on TCM solidified with 7.5 g L^{-1} Plant agar and co-cultured for 3 days at 24 ± 2 °C in the dark. They were then cultured for 4 weeks under the same conditions on root induction medium (RIM) consisting of ½ MS salts and vitamins, 20 g L^{-1} sucrose, 0.5 µM IBA, 7.5 g L^{-1} Plant agar, pH 5.8, 200 mg L^{-1} cefotaxime and 100 mg L^{-1} timentin). The number of roots formed per explant was then counted. Gfp labelled roots were detected using a blue/green LED (480-530 nm) with an orange filter (NIPPON Genetics EUROPE GmbH, Germany).

Transformation efficiency was determined per vessel by dividing the number of explants with fluorescent hairy roots by the number of uncontaminated explants.

2.4. Regeneration

Roots of 'M26' resulting from transformation with *R. rhizogenes* wild-type strain ATCC 15834 were either directly transferred to regeneration medium (MS salts and vitamins, 30 g L^{-1} sucrose, 1 µM IBA and 9.1 µM TDZ and 7.8 g L^{-1} Plant agar, pH 5.8, 200 mg L^{-1} cefotaxime, 100 mg L^{-1} timentin) and cultured under a 16/8 h light/dark regime at 24 ± 2 °C or, alternatively, first transferred onto hormone-free medium (½ MS salts and vitamins, 30 g L^{-1} sucrose, 7.8 g L^{-1} Plant agar, pH 5.8, 200 mg L^{-1} cefotaxime and 100 mg L^{-1} timentin) and cultured in the dark for one month before being transferred to regeneration medium (n = 18 Petri dishes with 4 roots each, 2 repetitions). As soon as small shootbuds had formed, they were transferred to AP medium and were propagated on the same medium. The regenerated shoots were analysed by PCR for the presence of T-DNA genes and the absence of the bacterial gene virD2. The same primers and PCR conditions were used as described in Rüter et al. (2024) with the exception of the plant material used, which was 70-100 mg leaves and instead of mas1 the primer ags (F: TGAAGATGAGGAACTGCCAC, R: CAATGCTAAGGACATTACCCAC, 59°C, 452 bp provided by Philipp Rüter, Institute of Horticultural Production Systems, Leibniz University Hannover) was used. The regeneration efficiency per Petri dish was determined by dividing the number of explants that formed shoots by the number of uncontaminated explants.

2.5. Statistical analyses

All data were analyzed with R version 4.3.0 in R Studio 2023.03.1+446 (Posit Software, PBC) and plots were created with the R package "ggplot2". A linear model was used for the root data and an analysis of variance (ANOVA) was calculated. The regression model for root data considered zero inflation using the R package "pscl" 1.5.5.1, as a significant proportion of explants did not form roots. Multiple comparisons were calculated with a Tukey test using the R package "multcomp" 1.4.23 with $p < 0.05$.

3. Results and discussion

3.1. Improvement of R. rhizogenes transformation in apple with PPM™

This experiment was carried out to investigate whether PPM™ treatment influences the transformation with *R. rhizogenes* through comparing the transformation efficiency between untreated and PPM™ treated pre-cultures of two apple rootstocks. Leaf explants of 'M26' treated with PPM™ produced an average of 11.3 roots per explant, significantly higher than the control average of 5.8 roots (Fig. 1). In contrast, explants of 'Selection 6' produced on average 1.3 roots per explant in both treatments, showing no significant differences in the number of roots per explant between the two treatments. The PPM™ treatment increased the transformation efficiency of 'M26' by 10%, while it remained almost the same for 'Selection 6'. Both genotypes showed a high transformation efficiency: 'Selection 6' reached 73.3% in the control and 70% after PPM™ pret-reatment. For 'M26', 86.7% was determined in the control and 96.7% after PPM™ pre-treatment.

According to Romadanova et al. (2022), the PPM™ treatment reduces the abundance of the endophyte *Bacillus megaterium* or even eliminates it. It is therefore plausible, that *R. rhizogenes* has less competition in the leaf, resulting in an increase transformation

efficiency. Additionally, Romadanova et al. (2022) observed varying responses across different apple genotypes, a pattern also seen between 'M26' and 'Selection 6' in this study. These differences may be attributed to genotypic variability in responsiveness to the treatment and differences in endophytic bacterial communities. The transformation efficiency was high compared to previous work, which reached 78% in 'Jork9' (Pawlicki-Jullian et al. 2002), 2% in 'Ringo' (Yamashita et al. 2004) and 37% in 'Baccata' (Wu et al. 2012). These results illustrate how strongly transformation efficiency can vary depending on the specific *R. rhizogenes* strain used and the genotype of the apple rootstock.

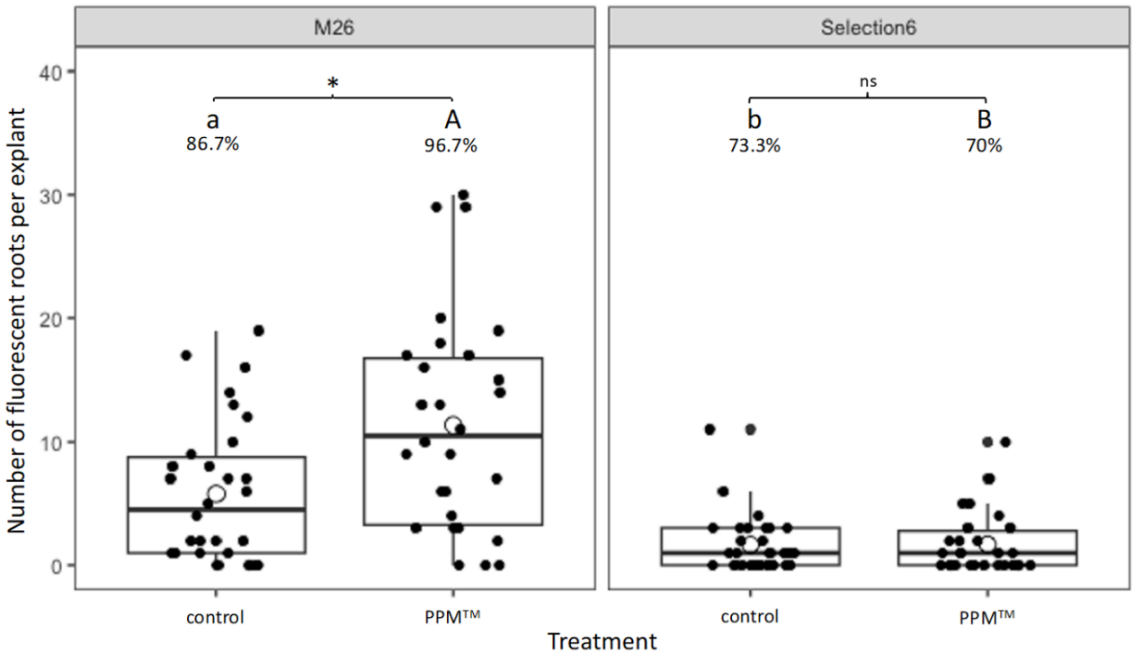

Figure 1. Hairy root formation on leaf explants of two apple genotypes after pre-culture (3 subcultures) on apple propagation medium with PPM™ (2 mL L^{-1}) and without (control), using the strain ATCC 15834, 5 weeks post transformation. Circles represent mean values; percentages indicate average transformation efficiency of all vessels. Letters denote significant differences between genotypes within each treatment (lowercase for control, uppercase for PPM™), and asterisk between treatments within each genotype, ns (not significant), based on Tukey's test (zero-inflated regression model with negative binomial distribution, $p < 0.05$). n = 3 vessels with 10 explants each, 1 repetition.

3.2. Regeneration of apple Ri plants

In this experiment, the shoot regeneration efficiency was compared between a treatment with a phase on hormone-free medium (½ MS) in the dark and a treatment in which the roots were placed directly on the regeneration medium with 16/8h light. In both treatments, the first cluster of shoot buds regenerated between the 10th and 12th week from callus that had formed on the roots after 8 weeks (Fig. 2). No significant difference was found when comparing the two treatments. This showed that a dormant period on hormone-free medium in darkness did not increase the rate of shoot regeneration from hairy roots. It is important to note that a high shoot regeneration efficiency of 19% was achieved in 'M26' roots. Lambert and Tepfer (1992) observed shoot initiation only on a single root of 'M26' and noted that regeneration is more difficult than transformation. However, similar high regeneration efficiencies were obtained by Yamashita et al. (2004), who reported 13.8% for 'Jork9,' and

by Pawlicki-Jullian et al. (2002), with 16.7% for 'Ringo'. Wu et al. (2012) even achieved a regeneration efficiency of 73%. However, in all previous studies, only regenerates on roots that were still attached to the shoots were generated. In this work, however, it could be shown that Ri plant regeneration is also possible in detached roots in medium with 9.1 µM TDZ. According to Li et al. (2014), TDZ was more suitable than BAP for shoot regeneration on leaves of 'M26'. Whole Ri shoots were obtained after transferring the cluster of shoot buds on AP medium (Fig. 2). A total of 19 bacteria-free lines were successfully generated and propagated. Molecular analysis confirmed that 95% of these lines were true Ri shoots, each carrying at least part of the bacterial T-DNA. Approximately 50% of the Ri lines contained truncated T-DNA fragments. These results align well with those of Rüter et al. (2024) who reported a similar rate of 91% for true Ri shoots.

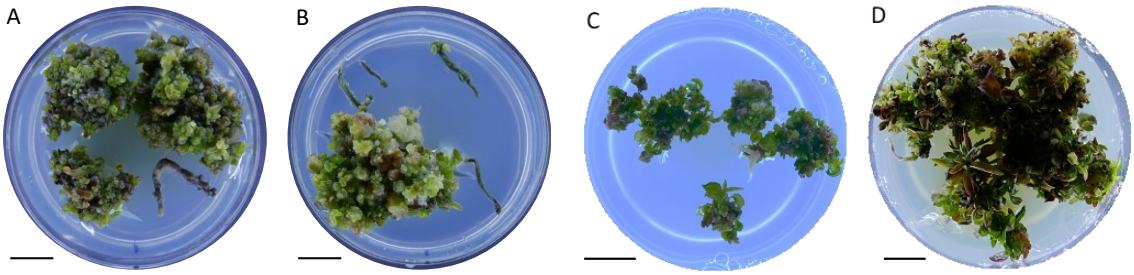

Figure 2. Examples of roots with callus and shoot buds after 28 weeks on regeneration medium: (A) without and (B) with phase on hormone-free medium in darkness. (C) Shoot buds transferred to apple propagation medium after 4 weeks, and (D) further shoot development on apple propagation medium after 8 weeks (some symptoms of hyperhydricity). bar = 1 cm.

4. Conclusions

This study shows that PPM™ pre-treatment significantly increases Rhizobium rhizogenes transformation efficiency and root formation in apple rootstock 'M26'. Genotypic differences were evident as 'Selection 6' showed no such response, highlighting the variability in transformation between apple genotypes. In addition, efficient regeneration of Ri shoots was achieved from detached hairy roots, reaching a 19% efficiency on medium containing 9.1 µM TDZ. Molecular analysis confirmed that 95% of the regenerated shoots were Ri shoots, supporting the reliability of the protocol to produce transformed plants. Future work will focus on morphological and stress physiological assessments of Ri plants.

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Literature

Desmet S, De Keyser E, van Vaerenbergh J, Baeyen S, van Huylenbroeck J, Geelen D, Dhooghe E (2019) Differential efficiency of wild type rhizogenic strains for *rol* gene transformation of plants. Appl Microbiol Biotechnol 103: 6657-6672. https://doi.org/10.1007/s00253-019-10003-0

Desmet S, Dhooghe E, De Keyser E, van Huylenbroeck J, Müller R, Geelen D, Lütken H (2020) Rhizogenic agrobacteria as an innovative tool for plant breeding: current achievements and limitations. Appl Microbiol Biotechnol 104: 2435-2451. https://doi.org/10.1007/s00253-020-10403-7

Lambert C, Tepfer D (1992) Use of *Agrobacterium rhizogenes* to create transgenic apple trees having an altered organogenic response to hormones. Theor and Appl Genet 85 (1): 105-109. https://doi.org/10.1007/BF00223851

Li BQ, Feng CH, Hu LY, Wang MR, Chen L, Wang QC (2014) Shoot regeneration and cryopreservation of shoot tips of apple (*Malus*) by encapsulation–dehydration. In Vitro Cell Dev Biol Plant. 50: 357-368. https://doi.org/10.1007/s11627-014-9616-2

Mazzola, M, Manici LM (2012) Apple Replant Disease: Role of microbial ecology in cause and control. Annu Rev of Phytopathol. 50: 45-65. https://doi.org/10.1146/annurev-phyto-081211-173005

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Pawlicki-Jullian N, Sedira M, Welander M (2002) The use of *Agrobacterium rhizogenes* transformed roots to obtain transgenic shoots of the apple rootstock Jork 9. PCTOC 70: 163-171. https://doi.org/10.1023/A:1016387004712

Porter JR, Flores H (1991) Host range and implications of plant infection by *Agrobacterium rhizogenes*. CRC Crit Rev Plant Sci 10:387-421. https://doi.org/10.1080/07352689109382318

Romadanova NV, Tolegen AB, Kushnarenko SV, Zholdybayeva EV, Bettoni JC (2022) Effect of Plant Preservative Mixture™ on endophytic bacteria eradication from in vitro-grown apple shoots. Plants 11 (19): 2624. https://doi.org/10.3390/plants11192624

Rüter P, Eeckhaut T, Dhooghe E, De Keyser E, Doan MH, Bartels J, Winkelmann T (2024) Optimization of *Rhizobium rhizogenes*-mediated transformation, regeneration and characterization of *Malus domestica* BORKH. Ri lines. PCTOC 157 (2): 32. https://doi.org/10.1007/s11240-024-02742-4

Rüter P, Wehrenberg F, Bartels J, Debener T, Winkelmann T (2023) Optimization of *Rhizobium rhizogenes* -mediated transformation for a diversity set of rose genotypes. Acta Hortic 225-234. https://doi.org/10.17660/ActaHortic.2023.1383.27

Suran P, Pravcová G (2023) Drought stress affects productivity and fruit size of new apple cultivars. Acta Hortic. 1372: 149-154. https://doi.org/10.17660/ActaHortic.2023.1372.20

Wang Y, Li W, Xu X, Qiu C, Wu T, Wei Q, Ma F, Han Z (2019) Progress of apple rootstock breeding and its use. HPJ 5: 183-191. https://doi.org/10.1016/j.hpj.2019.06.001

Wu J, Wang Y, Zhang LX, Zhang XZ, Kong J, Lu J, Han Z-H (2012) High-Efficiency Regeneration of *Agrobacterium rhizogenes*-induced hairy root in apple rootstock *Malus baccata* (L.) Borkh." PCTOC 111 (2): 18389. https://doi.org/10.1007/s11240-012-0182-1

Yamashita H, Daimon H, Akasaka-Kennedy Y, Masuda T (2004) Plant regeneration from hairy roots of apple rootstock, *Malus prunifolia* Borkh. var. Ringo Asami, strain Nagano No. 1, transformed by *Agrobacterium rhizogenes*. JJSHS 73 (6): 505-510. https://doi.org/10.2503/jjshs.73.505