Improved rooting and advanced micropropagation.

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It is well known that the rooting ability of woody species is difficult and that the rooting ability decline with maturity.

Objectives

• To improve rooting ability of the apple rootstock M26 and M)/29 and the pear rootstock BP10030 using gene technology.

• To evaluate the influence of the transgenic rootstocks on several grafted cultivars
Apple rootstocks M26 and M9/29

• dwarfing rootstock
• commonly used in the world
  • difficult to root
  • bad anchorage

Pear rootstock BP10030

• no *Pyrus* rootstocks available
• Quince (*Cydonia oblonga*)- main rootstock
  • not winter hardy
  • incompatible with some cultivars
  • BP10030-*Pyrus* type
• dwarf and winter hardy, but difficult to root
The rolB gene used to improve rooting originates from the soil bacteria *Agrobacterium rhizogenes*.
The gene construct used in Agrobacterium mediated transformation: pCMB-B:GUS
Transformation procedure

In vitro grown-shoots

Young leaves wounded with scalpel or forceps

Leaves co-cultured with agrobacterium for 3 days in the dark on callus induction medium

Callus induction medium + cef. and kan. in the dark for 2-6 weeks

Shoot induction medium + cef. and kan. in the dark until shoots appear
Apple leaf explants grown on the selection medium
Shoots regenerated from leaf explants on the selection medium
Verification of putative transformed clones of M9/29 by PCR and Southern hybridisation of the rolB gene
In vitro rooting of apple M9/29 on the IBA-free rooting medium
## Rooting in vitro without auxin

<table>
<thead>
<tr>
<th>Plants</th>
<th>% rooting</th>
<th>No of roots per rooted shoot</th>
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<tbody>
<tr>
<td>M26 control</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Transgenic clone 1</td>
<td>100</td>
<td>5.5 ± 2.1</td>
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<tr>
<td>Transgenic clone 2</td>
<td>94</td>
<td>5.5 ± 3.5</td>
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<td>M9/29 control</td>
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<td>1</td>
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<tr>
<td>Transgenic clones 1-4</td>
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<td>7.4 ± 2.8</td>
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<tr>
<td>Transgenic clones 5-9</td>
<td>83</td>
<td>5.6 ± 1.5</td>
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<tr>
<td>Pear control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Transgenic clone 1</td>
<td>100</td>
<td>3.1 ± 1.0</td>
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Apple M9/29 and pear cuttings
Can transformed rootstocks affect growth and development of the cultivar grafted onto them?
Field trial with transgenic apple rootstocks

Transformed and untransformed M26 and M9/29 were planted in the field 2001.

Twenty trees of each type of rootstock were budded 2002 with five cultivars.

The rootstocks were cut back 2003.
Cultivars used in the field trial

- Jonagold
- Elstar
- Aroma
- Elise
- Discovery
Field trial on apple rootstocks with the rolB gene
Budding in the field
Field trial with transgenic rootstocks 2011
Plant height (cm) of five apple cultivars grafted onto transgenic and non-transgenic apple rootstocks. Data collected from 10 trees for each combination and given as average of 5 years (2003-2007).

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<td>Aroma</td>
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<tr>
<td>Jonagold</td>
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<td>119</td>
<td>138</td>
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<td>109</td>
</tr>
</tbody>
</table>
Fruit colour, form and quality

Results from fruit weight, diameter, firmness and colour showed no consistent changes when comparing transgenic rootstocks with their control counterparts. Regarding vitamin C, phenolics and titratable acidity no significant differences were observed whereas total soluble solids increased for Jonagold and Elstar grafted on transgenic rootstocks.

Field trial with rolB transgenic pear rootstocks

2006: In vitro produced non transgene pear rootstock BP10030 and two rolB transgenic clones and BP10030-rolB1 and BP10030-rolB2 were planted in the field.

2007: These rootstocks were budded with the 3 cultivars Clara Frijs, Alexander Lukas and Conference and 20 trees of each rootstock /cultivar combination were used.

2009: 12 trees of each combination were selected planted in 4 rows.
Stability of transgene expression under field conditions

Rooting of cuttings from field grown materials

RT-PCR of the rolB expression in non grafted rolB transgenic rootstocks

1-5= Jonagold
6-10 Elise

RT-PCR of the rolB mRNA in scion cultivars grafted on transgenic rootstocks
Advanced micropropagation
Traditional micropropagation, a costful procedure
Several attempts have been made to reduce labour costs by scaling up the procedure

- The first attempt to reduce the labour costs was mechanical blending.
- Based on homogenisation of in vitro established shoots followed by regeneration from the homogenate.
- Clumps of multiple shoots were aseptically transferred to a sterile blender containing sterile culture medium.
- This method has been used for species with high regeneration capacity like ferns, *Saintpaulia*, *Spathiphyllum* and hybrid *Begonia*.
Micropropagation by mechanical blending

Mechanical blending of in vitro established *Begonia cheimántha*. A) dentist drill to cut the explants, B) homogenized tissue and C) cultures for transfer to soil or new blending.
Introduction of the temporary immersion principle

D. RITA system from Alvard et.al, 1993

E. Twin flasks system from Escalona et al., 1999
Five treatments were used for shoot multiplication (2 weeks) and elongation (3 weeks) of the apple rootstock M26 in the temporary immersion system. The basal medium was MS. The containers were filled with 150 ml liquid medium. For 1-3 the frequency of medium immersion was 8 times per day and duration of 5 min and 16 times per day with duration of 2 min for 4-5.
Plants of the apple rootstock M26 rooted in a RITA container.

The shoots were rooted in the rooting medium consisting of Lepoivre macro-and micro nutrients, Walkey vitamins and 1.2 μmol IBA for 4 days in the dark and then on IBA free rooting medium in light for 3 weeks.
Semi-automated micropropagation using Temporary Immersion System (TIS)

Five-litre twin glass vessels were used. A) vessels with nutrient solution B) vessels with plant material. The liquid medium is drained from the medium vessel to the plant vessel and vice versa by means of compressed air (C) being passed through a three-way solenoid valve controlled by a programmable timer (D).
Plants propagated in the twin vessels

Rhubarbs
Plants propagated in the twin vessels

Blueberry
Plants propagated in the twin vessels

Strawberry
Plants propagated in the twin vessels

Apple

Birch

Raspberry

Raspberry

Syringa
Problems encountered with existing equipment

1. The glass vessels are rather heavy especially since both the media and the plant vessel have to be transferred to the laminar flow together.

2. The culture vessels should have a larger bottom area and less height in order to keep more plants with possibility to expand.

3. Certain plant species are very sensitive to hyperhydricity. For this reason the plants should be placed in such a way to avoid contact with the liquid medium that can remains after the plant vessels have been drained off.
We have now developed a commercially attractive bioreactor for large scale in vitro plant production.

1. It is easy to handle
2. It is transparent,
3. It is autoclavable,
4. The immersion time of nutrients and gas exchange can be controlled using air pumps and a timers.
5. The bioreactors can be placed above each other saving place in the climate chamber.
The figures below show the construction and details of the bioreactor.

1. Outer container with 3 inlets/outlets for gas exchange, 2E shows the middle filter connected to a plastic tube on the inner chamber, 3 filters, plastic tubes, clamps, nuts and silicon rings to be connected to the 3 inlets/outlets on the outer container, 4A inner chamber with 3 grooves on the long side and connection to the middle filter, 5B basket with 3 rows of small holes, 6C frame with 4 legs, 7 lid with 4 flaps and an inner silicon ring.
Description of the bioreactor

1. The bioreactor 180x150x150 mm is made of polycarbonate
2. The inner chamber, basket and legs are made of polypropylene
3. The outer container is filled with 500 ml of nutrients
4. The sterile plant material (shoot tips or nodal segments) are placed on the basket.
5. The legs are placed on the basket to press down the basket when pressure is applied
6. The filters on two of the outlets are connected to a dual timer and air pumps
7. The immersion time and frequency is set by the timer
Assemble of the bioreactors
Advantages of the new bioreactor

The use of liquid nutrient solution will eliminate costs for agar, the gelling agent, which is used in conventional in vitro propagation.

The size of the bioreactor will allow a larger amount of plants in each unit and explants can be placed randomly which will reduce labour costs for transferring to new containers.

The rooting will take place in liquid medium which facilitate planting in soil afterwards.

The gas phase in plant tissue culture is important and due to three outlets in this system enrichment of air can be regulated separately.
Large scale production
Large scale production
Different plant species tested in the bioreactors

Birch

Rhubarbs

Echinacea

Raspberry

Blueberry
Different plant species tested in the bioreactors

Chestnut

Banana

Cordyline

Blackberry
Potato production in Plantform bioreactor

Pictures from In Vitrosur
Sevilla Spain
Plants coming out form the bioreactor is easy to acclimatize
Acclimatization after 4 weeks in the greenhouse

Echinacea

Rubus
Conclusions

The newly developed Plantform bioreactor functions well for both large scale micropropagation and basic research.

It is convenient to work with: one unit with low weight.

It is easy to fill medium, filters/tubes quickly applied,

It is easy to change medium.

No shoot positioning needed.

Easy to connect to pumps and timers.

More plants on less space.

Environmentally friendly.

www.plantform.se