Regeneration และการส่งถ่ายจีนสู่ Quince (*Cydonia oblonga* Mill.) และ Pear (*Pyrus communis* L.)

Regeneration and Transformation of Quince (*Cydonia oblonga* Mill.) and Pear (*Pyrus commounis* L.)

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Quince (*Cydonia oblonga* Mill.) นิยมใช้เป็น rootstock ของ Pear (*Pyrus communis* L.) เนื่องจากมีขนาดเล็ก แต่มีข้อเสียคือประสิทธิภาพในการดูดธาตุเหล็กของ Quince ค่อนข้างต่ำ ดังนั้นจึงปรากฏว่าใบของ Quince จะมีสีเหลือง เนื่องจากขาดคลอโรฟิลล์ วิธีการส่งถ่ายจีนเข้าสู่ Quince และ Pear ได้ถูกนำมาใช้เพื่อปรับปรุงพันธุ์พืชทั้งสองชนิดนี้ สำหรับปัจจัยที่จำเป็นต่อการส่งถ่ายจีนที่ต้องทราบคือ การปรับปรุงวิธีการ regeneration ของ Quince และ Pear เพื่อให้มีเปอร์เซ็นต์สูงขึ้น การคัดเลือก antibiotic ที่ดีที่สุดที่จะใช้กำจัด *Agrobacterium* ในขณะที่มีผลเสียน้อยที่สุดต่อเปอร์เซ็นต์ regeneration และการคัดเลือก selectable marker ที่เหมาะสมในการคัดเลือกพืชแปลงพันธุ์ จากการทดลองพบว่า เปอร์เซ็นต์ regeneration ของ Quince สูงเกือบ 100% เมื่อเลี้ยงในอาหารที่มี thidiazuron (TDZ) ความเข้มข้น 30 μm และ α-napthalene acetic acid (NAA) ความเข้มข้น 0.3 μm และใช้ gelrite 1.6 g/l แทน agar regeneration ของ Pear rootstock RV113 สูงขึ้น เมื่อถูกกระตุ้นด้วยความเย็น antibiotic timentin ซึ่งประกอบด้วย ticarcillin และ β-lactamase inhibitor มีประสิทธิภาพสูงในการกำจัด *Agrobacterium* และมีผลเสียน้อยที่สุดต่อ regeneration เมื่อเทียบกับ cefotaxime และ carbenicillin ในการทดลองครั้งนี้เลือกใช้เวคเตอร์ซึ่งประกอบด้วย bar gene (bialaphos resistance) เนื่องจาก bialaphos ทำอันตรายต่อใบน้อยเมื่อเทียบกับ kanamycin จากการตรวจสอบโดยใช้ Gus assay (β-glucoruniadase activity) สามารถตรวจสอบได้ว่าภายใต้สภาพที่เหมาะสม เนื้อเยื่อของ Quince นั้น transformed.

Abstract

Quince (*Cydonia oblonga* Mill.) is widely used as a dwarfing rootstock for pear (*Pyrus communis* L.). The disadvantage of this rootstock is its inefficient uptake of Fe, resulting in leaf chlorosis. *Agrobacterium*-mediated gene transfer for quince and pear were devised using leaves as explants. Efforts were directed at the following: improving the regeneration system, selecting the best antibiotic to eliminate *Agrobacterium* while maintaining a high regeneration frequency, and identifying a suitable selectable marker. Regeneration of quince was improved by replacement of agar with 1.6 g/l gelrite. The regeneration frequency of control leaf disc on a medium containing 32 μm thidiazuron (TDZ) and 0.3 μm α-naphthalene acetic acid (NAA) was closed to 100%. Regeneration of the pear rootstock RV113 was enhanced by cold treatment of the shoot cultures used as explants. The antibiotic timentin, which consists of ticarcillin and a β-lactamase inhibitor, was more effective in eliminating *Agrobacterium* and less inhibitory to regeneration than cefotaxime and carbenicillin. Vectors containing the bar gene (bialaphos resistance) were chosen for transformation experiments since bialaphos caused less damage to the leaf explants than kanamycin at effective concentrations for selection of transformants. GUS (β-glucuronidase activity) assays showed that under the proper conditions, transformed tissues were obtained.

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Introduction

Availability of effective transformation procedures will lead to genetic improvement of pear as well as quince, a dwarfing rootstock for pear. For instance, pear may be benefit from incorporate of genes involved in disease resistance, pest resistance, and delay of ripening. Quince, which suffers from inefficient iron uptake in calcareous soils, may become a more effective rootstock by insertion of genes enhancing ion utilization. However, transformation protocols are not available yet for pear and quince. The objectives of this research are to: 1) improve regeneration of quince and pear from leaf discs; 2) establish the optional conditions for Agrobacterium-based transformation of leaf discs; and 3) generate transgenic plants.

Materials and Methods

Regeneration of quince and pear

Leaves of quince (E.M. Quince A and pear rootstock RV.113) were obtained from 3-week-old micropropagated shoots. The leaves were cut transversely, placed on modified Murashige and Skoog (MS) medium and cultured as described previously (Dolecet-Sanjuan et.al., 1991). Several medium modifications were tested for pear regeneration, including growth regulator concentrations (TDZ and NAA); replacement of 3% sucrose with 3% glucose; addition of 100 mg/1 xylose, and mannose; addition of $AgNO_3$; and decrease of the dark culture period from 21 days to 10 days, or increase to 30 days. The percentage of leaves giving shoots and the average number of shoots per regenerating leaf were determined in two repeat experiments with 10 dishes (5 leaves/ dish) per treatment.

Determination of the best antibiotic and selective agent

Preliminary experiments were performed to determine 1) the best antibiotic to eliminate Agrobacterium after co-cultivating; and 2) the efficacies of bialaphos and kanamycin as selective agents. The antibiotics tested were: cefotaxime, carbenicillin, and timentin at 0, 50, 100, 150, 200 and 250 mg/1. The bialaphos concentrations were 1, 5, 10, 15 and 20 mg/1 and kanamycin concentrations were 0, 10, 20, 30, 40, 50 and 60 mg/1.

Generation of transgenic plant

The following two vectors were transformation: pGSFR280 (Fig. 1A; De Block et.al., 1987) and pGiPTV-BAR with a 35 Spromoter (Fig. 1B; Becker et.al., 1992). Both were placed in *A. tumefaciens* strain EHA105 (obtained from E. Hood) by tri-parental mating. The transformation procedures are represented by the following flowchart:

Grow Agrabacterium in YEP medium to O.D.0.5 (550 nm)
↓
Incubate Agrobacterium in SIM medium with acetosyringone
| 5h
↓
Incubate 2-day pre-cultured leaf discs in MS with 10% SIM/Agrobacterium
| 10 min
↓
Blot dry and place on regeneration medium with acetosyringone
| 3 days in dark
↓
Transfer leaves to regeneration medium with 200 mg/1 timentin and 5 mg/1 bialaphos
| 18 days in dark
↓
Transfer leaves to fresh regeneration medium with 200 mg/1 timentin and 5 mg/1 bialaphos
| 21 days under light (16h; 40 $\mu mol.m^{-2}S^{-1}$)
↓
Transfer shoots to multiplication medium with timentin and bialaphos
| 28 days under light (16h; 40 $\mu mol.m^{-2}S^{-1}$)
↓
Transfer healthy shoots to rooting medium
| 28 days under light (16h; 40 $\mu mol.m^{-2}S^{-1}$)
↓
Perform Southern analyses on surving shoots

When pGiPTV-BAR was used as vector, β-glucuronidase (GUS) activity was determined at several points of the transformation scheme with X-Gluc as substrate.

Results and Discussion

Regeneration of qiunce and pear

Large numbers of shoots regenerated from quince leaf discs on modified MS medium containing 32 μm NAA (Fig.2). The percentage of regenerating pear (RV.113) was also highest (70%) at these concentrations of growth regulators (Fig.3), with an average of 3 shoots per leaf disc. Thus, very high concentrations of TDZ are required for regeneration of pear and quince, even though TDZ is one of the most active cytokinnins identified (Mok et.al., 1982). No significant increases were obtained in the percentage of leaves giving shoots by replacements of sucrose with glucose; addition of xylose, ribose, or mannose; addition of $AgNO_3$; or a change in the duration of the dark period. Regeneration of the pear cultivars was slightly lower than for the RV.113 rootstock (about 40-50% of leaf discs). For regeneration of all clones, the most important factor was the stage of the leaves. Young, newly expanded, leaves were the best explant source for regeneration, whereas older leaves hardly ever produced adventitious shoots.

Determination of the best antibiotic and selective agent

Preliminary experiments were performed to choose the best antibiotic for eliminate of *Agrobacterium* after the co-cultivation period while maintaining high regeneration capacity. A comparison of antibiotics (timentin, carbenicillin, and cefotaxime) at 0 to 250 mg/1 showed that all three antibiotics reduced the regeneration percentage (Fig.4) and the number of shoots per regenerating leaf disc. Timentin was judged the best antibiotic since 200 mg/1 was sufficient to suppress growth of *Agrobacterium* strain EHA 105, while higher concentrations of the other two antibiotics were required.

To determine whether the *nptII* (kanamycin resistance) or the bar (bialaphos resistance)

gene would be a better selectable marker for quince, leaves were grown at several concentrations of kanamycin and bialaphos at 5 mg/1 (Fig.5). Since the leaves appeared healthier in the presence of bialaphos than kanamycin, vectors with the bar gene were selected (Fig.1).

Generation of transgenic shoots

Initially, construct EHA105/pGSFR280 was used for transformation. Using the procedures outlined in the flowchart (Materials and Methods), shoots appeared at the edges of the leaves and cut end of the petioles (Fig.6). About 10 shoots were formed per 100 leaf discs. Upon transfer to multiplication or rooting medium with 5 mg/1 bialaphos, most of the shoots showed typical symptoms of bialaphos toxicity, indicating that these were escapes. A few shoots remained green and healthy, suggesting that transformation may have taken place. Southern analyses revealed clear bands hybridizing with that bar gene. These experiments indicate the transformation can occur, but at low frequencies, about one shoot per 200 leaves. This frequency is in agreement with that found for apple (about 1 in 100 leaf discs; James and Dandekar, 1991).

Further experiments with EHA/pGiPTV-BAR was used for the experiment. This vector contains the *uidA* gene, conferring GUS activity, which allows monitoring of transformation events. GUS activity tests 5 days after incubation with *Agrobacterium* demonstrated that transformation of leaf edges had taken place (Fig.6,7), but the frequency of transformed tissues was low. Experiments are in progress to further refine the protocols to increase the number of transformation sites and enhance regeneration of transformed shoots. Similar experiments are being performed with *P. communis* cultivars.

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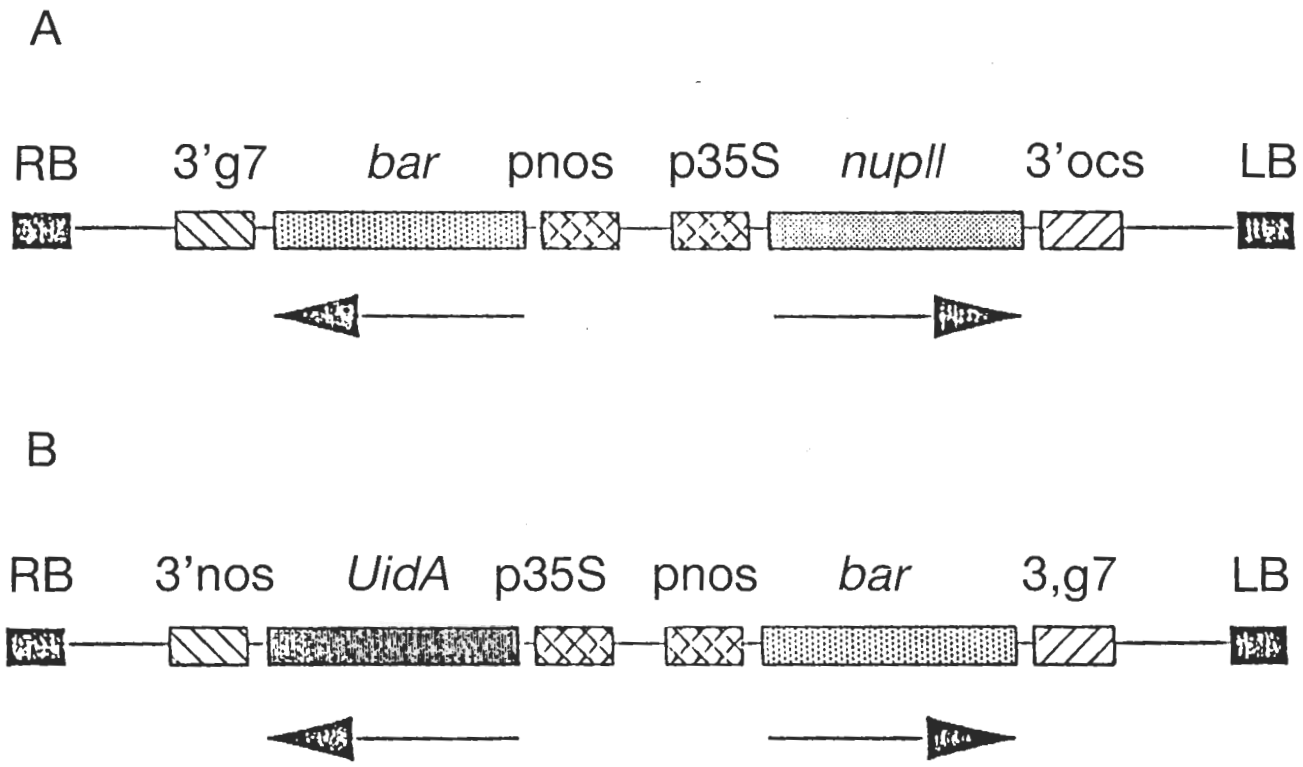

Fig. 1 Vectors used for transformation: A. pGSFR280 (De Block *et al.*, 1987); B. pGiPTV-BAR (Becker *et al.*, 1992). bar: bialaphos resistance; nptII: kanamycin resistance; uidA: B-glucuronidase (GUS)

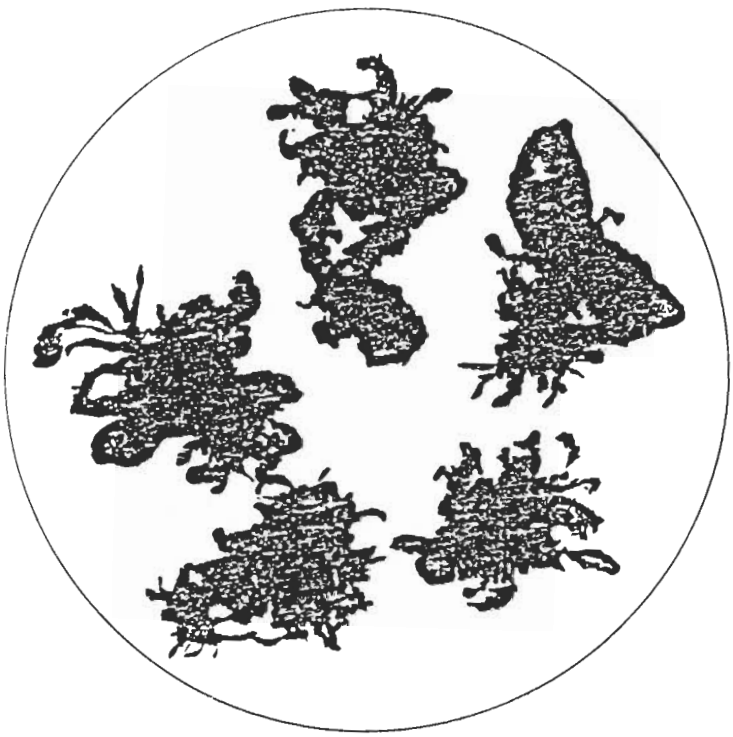

Fig. 2 Regenerating shoots on quince leaves cultured on medium containing gelrite for 8 weeks.

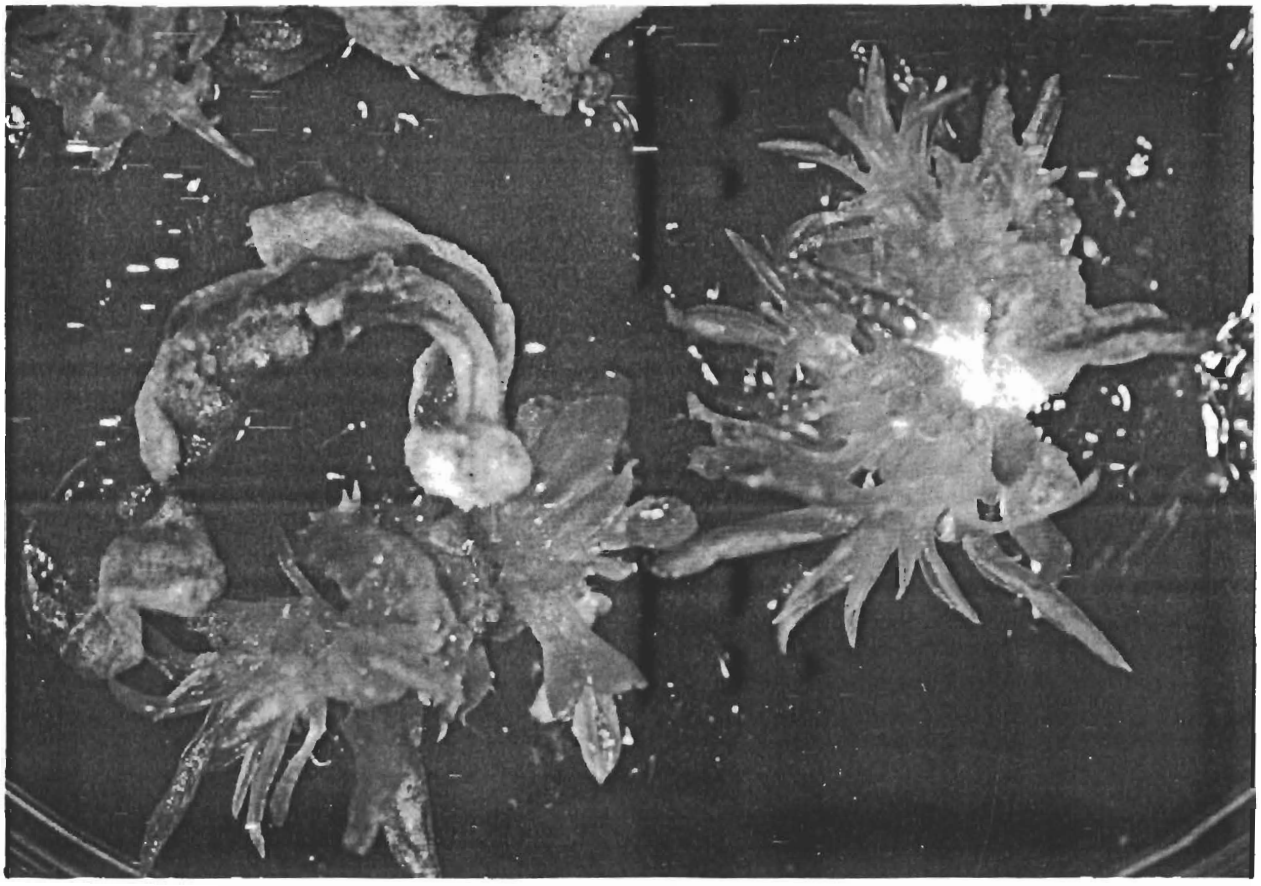

Fig. 3 Regenerating shoots on pear (RV.113) leaves cultured for 8 weeks.

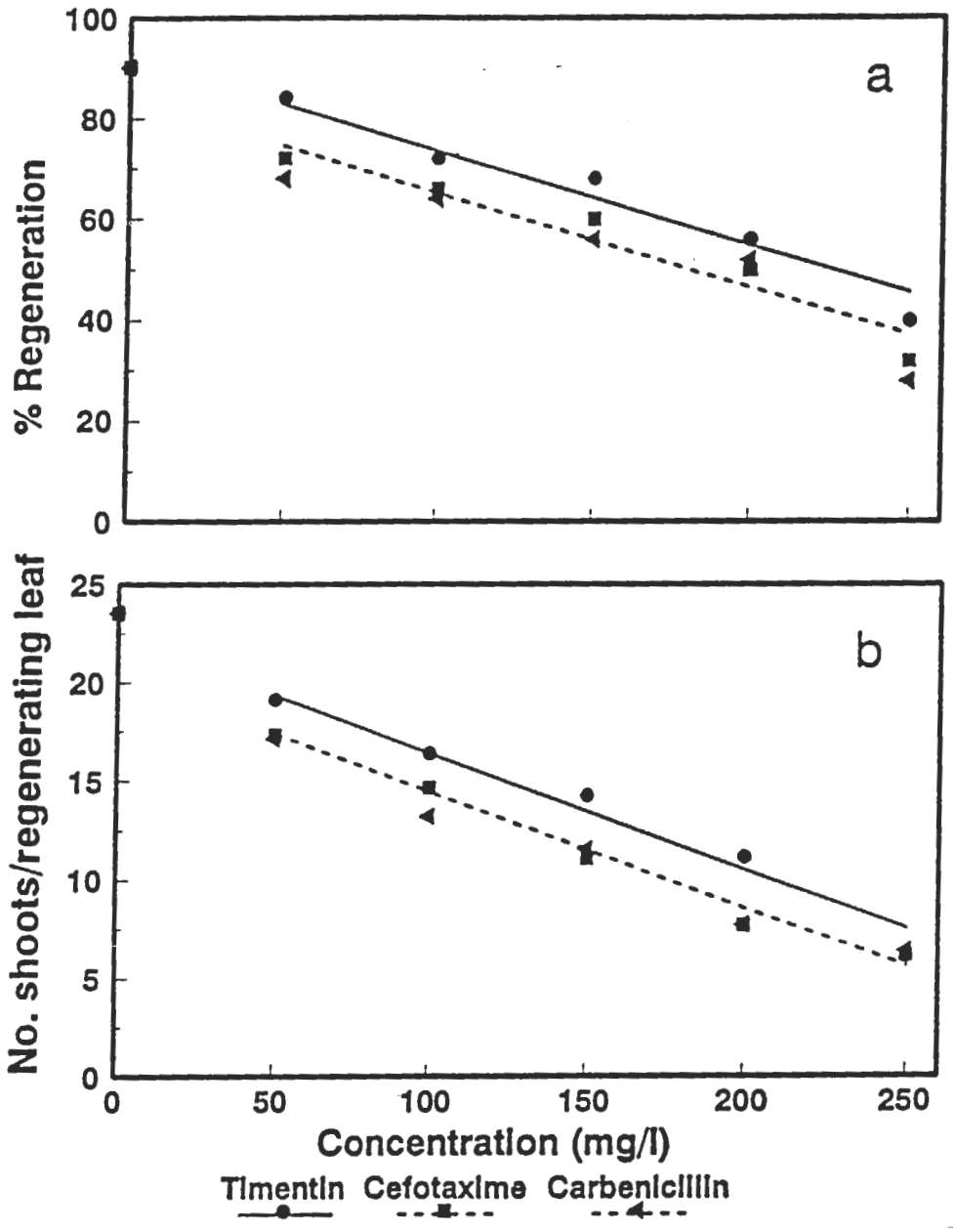

Fig. 4 Effects of timentin, cefotaxime, and carbenicillin on regeneration percentage (a) and number of shoots per regenerating leaf (b) after a culture period of 8 weeks of quince leaves on regeneration medium.

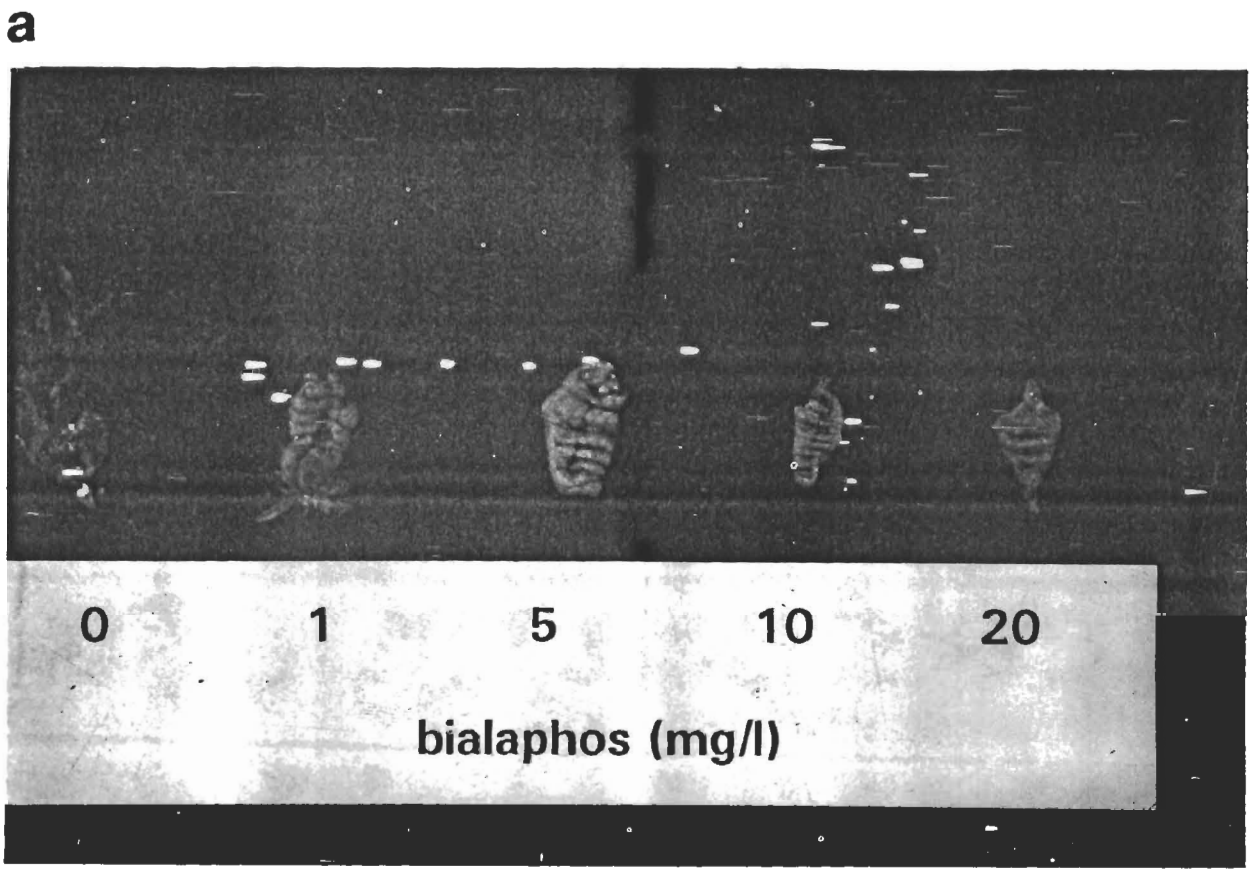

Fig. 5 Effects of bialaphos (a) and kanamycin (b) on regeneration of quince leaves.

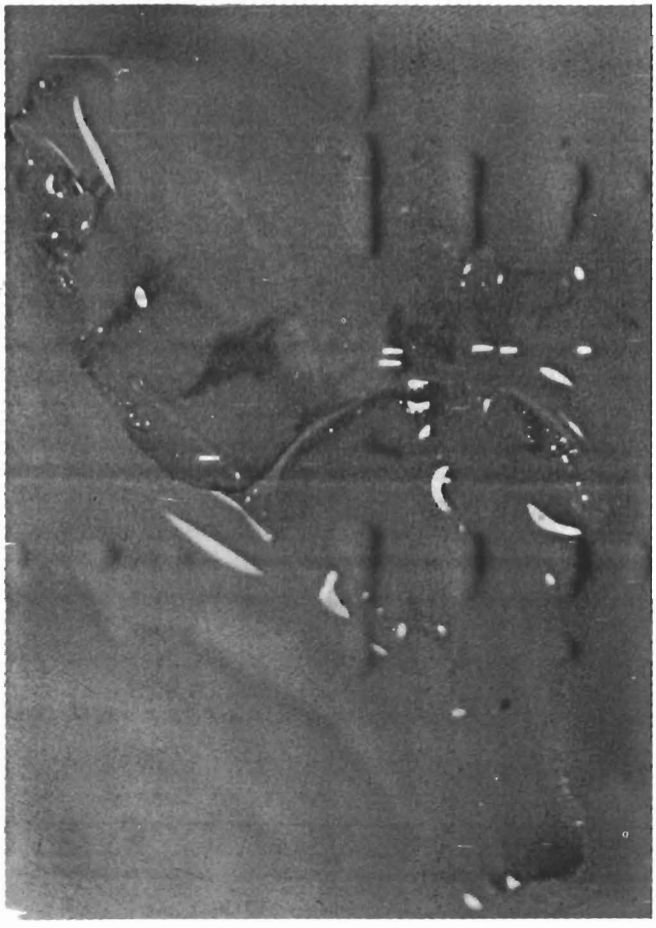

Fig. 6 Leaf of quince (experiment 2-2) showing GUS activity 6 days after incubation with *A. tumefaciens* EHA105/pGPTV-BAR

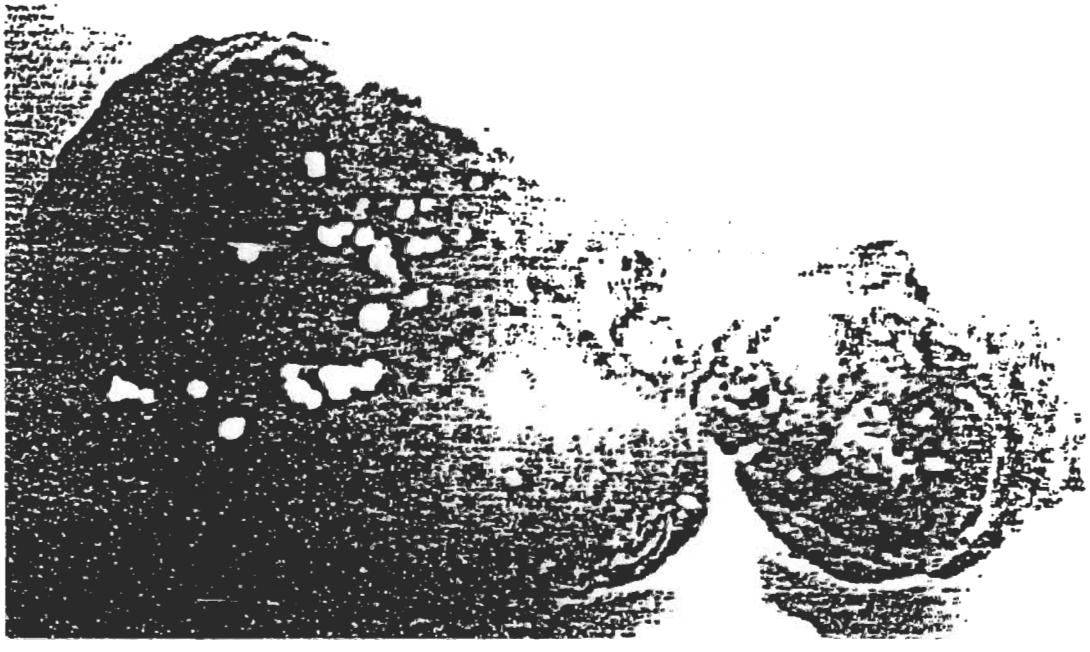

Fig. 7 Callus on quince leaves showing GUS activity after a culture period of 6 weeks following incubation with *A. tumefaciens* EHA105/pGPTV-BAR.