



Improvement of potato (*Solanum tuberosum* L.) transformation efficiency by *Agrobacterium* in the presence of silver thiosulfate

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Abstract. Transformation of potato (*Solanum tuberosum*) through the mediation of *Agrobacterium* in the presence of the ethylene action inhibitor, silver thiosulfate (STS) was studied. STS significantly enhanced the shoot growth of *in vitro* cultured potato. The total fresh weight of plants treated with STS for 21 days was 6 times greater than the control whereas treatment of microtubers with STS did not yield significant benefits. Explants of the STS-treated potato tissues were wounded and cocultured with *Agrobacterium tumefaciens* carrying the NOS and NPT II chimeric genes. In STS-treated plants, the transformation frequency ranged from 27 to 31% for different starting materials. The cointegration of the chimeric genes into the transgenic plants was confirmed by way of the selection medium, opine assay and the detection of NPT II enzyme activity.

Key words: *Agrobacterium tumefaciens*; Ethylene action inhibitor; Neomycin phosphotransferase type II (NPT II); Opine; Potato (*Solanum tuberosum* L.); Silver thiosulfate (STS); Transformation efficiency.

Introduction

Potato (*Solanum tuberosum*) is one of the major crops in agricultural production. The improvement of quality and quantity is becoming an important affair nowadays in potato production. Except for the traditional breeding methods applied on the above mentioned improvement, a simple and widely used technique, *Agrobacterium*-mediated transformation, has been applied in most of the dicotyledonous plants. Several transformation works has been performed on potato plants (An *et al.*, 1986; Ooms *et al.*, 1987; Sheerman and Bevan, 1988; Stiekema *et al.*, 1988). While how to raise the efficiency of transformation and regeneration is becoming a limiting factor for establishing the transformation system. Recently, the cocultivation of organ explants with *Agrobacterium* was found to be able to increase the transformation frequency in potato

microtubers (Wenzler *et al.*, 1989).

In addition, some natural phenolic compounds such as acetosyringone (AS) were found that it played an important role upon improving the infection efficiency of *Agrobacterium*. Sheikholeslam and Weeks (1987) indicated that the use of synthetic AS in *Arabidopsis thaliana* increased the transformation frequency of *Agrobacterium tumefaciens*. Recent observation also indicated that AS and other natural exudates from wounded plant cells played an important role in activate of virulent (*vir*) genes of Ti plasmid (Stachel *et al.*, 1985). Such activation of *vir* genes has been proposed as a key element in the transfer of T-DNA from Ti plasmid to plant genome (Stachel *et al.*, 1986).

The materials for plant biotechnology mostly come from cultured tissues. However, it had been shown that the growth of potato was retarded in tight-closed flasks (Ammirato, 1983). Since ethylene was considered to be the major growth retardation material to *in vitro* cultured plant (Ammirato, 1983), the usage of ethylene inhibitor attempting to enhance plant

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growth was carried out in this study. Recent reports suggested that silver ion blocked ethylene action by binding to the ethylene receptors (Aharoni *et al.*, 1979; Beyer *et al.*, 1976). Cameron and Reid (1981) showed that silver ion in the form of $\text{Ag}_2\text{S}_2\text{O}_3$ was less toxic than Ag^+ in the form of AgNO_3 . Actually, silver thiosulfate (STS), an inhibitor of ethylene action was helpful for the growth to cultured potato plants (Perl *et al.*, 1988). They found thereafter that the reduction of ethylene action was helpful for potato plant to reverse to the normal growth. This study was conducted to understand the effect of STS on the growth of *in vitro* cultured potato, and to compare the responses of transformation from different organs. Various detections from the tissues of transgenic plants were also performed.

Materials and Methods

Plant Materials

In vitro cultured potato (*Solanum tuberosum* L. var. ADH69) plants were used in this experiment. The plants were routinely cultured in 0.8% agar-solidified MS medium (Murashige and Skoog, 1962) containing 2 μM silver thiosulfate (STS) every 6 weeks. These cultures were maintained in the condition of 3000 lux illumination with 16 hr daylength at 25°C. The leaves and stems of 6-week-old plant were excised and used for transformation. The microtubers obtained from 6-week-old plant cultured in MT medium (MS salts, 10 mg/l 6-BAP, 8% sucrose modified from Wang and Hu, 1982) were also used as materials.

Bacterial Strain

Agrobacterium tumefaciens C58 containing the cointegrated plasmid pfdA2 (kindly provided by Dr. Klaus Geider) (Baltes *et al.*, 1987) was used for all the infections. The plasmid described above carries neomycin phosphotransferase, which confers kanamycin resistance. The bacteria were grown in 10 ml YEB medium (pH 7.2, each liter contains beef extract 5 g, yeast extract 1 g, peptone 5 g, sucrose 5 g, and MgSO_4 0.5 g) containing 50 $\mu\text{g}/\text{ml}$ kanamycin for 24 hr. The bacteria concentration was measured the absorbance at 600 nm by spectrophotometer. The infection bacteria number used for inoculation was about 6.9×10^7 per ml.

Transformation, Regeneration and Selection

Leaves and stems were cut into 1–2 cm fragments and placed into 9-cm petri dishes containing 10 ml MS liquid medium. The 25 μl *Agrobacterium* solution was added to each petri dish and incubated for 2 days under low light intensity (about 300 lux). Afterwards, the tissues were taken from the inoculated solution and blotted dry on sterilized filter papers. The infected leaf and stem fragments were transferred to the selection medium PDM1 (each liter contains MS salts, 1 g casein hydrolysate, 40 mg adenine sulfate, 0.4 mg GA3, 300 mg 2,4-D, 0.3 mg kinetin, 100 mg myo-inositol, 0.5 mg thiamine-HCl, 0.5 mg folic acid, 15 mg L-glutamine, 0.25 mg 6-BAP, 20 g mannitol, 0.5 g MES, 500 mg cefotaxime, and 100 mg kanamycin, pH 5.8) under 300 lux illumination. The tissues were then subcultured on fresh PDM1 medium weekly. After several weeks, the callus formed visualizable by naked eyes among these inoculated tissues. After this stage, the tissues with developing callus were transferred to PDM2 medium (modified PDM1 medium, 200 mg cefotaxime but lacking 2,4-D) for shoot development. The regenerated shoots were excised and cultured on the root medium PDM3 (MS salts, R3 vitamins, 30 g sucrose, 100 mg kanamycin, 200 mg cefotaxime, pH 5.8) under 3000 lux illumination for rooting. Plants with regenerated shoots and roots were transferred to soil for further growth. In the comparison of different inoculation method in transformation, we dipped the explants in *Agrobacterium* culture in a test tube for 1–2 min. Explants were then transferred to PDM1 medium. The continuing procedure was the same as that described above.

For microtubers transformation, microtubers were cut into discs with 2 mm thickness. The manipulation of microtubers with *Agrobacterium* was the same as that of leaves and stems described above. But the composition of selection medium was modified from 3C5ZR medium (Sheerman and Bevan, 1988) (each liter contains MS salts, R3 vitamins, 3 μM NAA, 5 μM zeatin, 3% sucrose, 500 mg/l cefotaxime, 100 mg/l kanamycin, pH 5.8).

Nopaline Assay

The transgenic calli and transgenic plants were assayed for the presence of nopaline. The assay was performed by paper electrophoresis (Otten and Schilperoot, 1978) with at least four replicates. Fifty to

one hundred mg samples were grounded with 50–100 μ l liquid LS medium (mg/ μ l) (Linsmaier and Skoog, 1965) which contained 0.1 M arginine and 2-keto-glutaraldehyde, and incubated overnight at 28°C. Spot the sample on the Whatman 3MM chromatography paper and electrophoresis was run at 200 V for 50 min. The reaction mixtures were separated on Whatman 3MM chromatography paper, and the nopaline spots were examined under UV-irradiation after staining with phenanthrenequinone.

NPT II Enzyme Assay

Leaves of transgenic plants were assayed for the expression of NPT II gene by means of dot-blot assay according to Radke *et al.* (1988) with some modifications. For the NPT II assay, tissues transformed from different organs were assayed at least for four replicates. Leaf tissue (50–100 mg) was grounded in eppendorf tube containing equivalent amounts (50–100 μ l) of extraction buffer (2.5 mM Tris, pH 6.8, 0.143 mM β -mercaptoethanol, 0.27 mM leupeptin), then centrifuged for 15 min at 4°C. Twenty μ l of the supernatant was added to 10 μ l of reaction buffer A (67 mM Tris-Maleate, 42 mM MgCl₂, 400 mM NH₄Cl, 1.7 mM dithiothreitol, 0.4 mg/ml kanamycin sulfate) or reaction buffer B (identical to reaction buffer A but no kanamycin). Five μ l of ATP solution (1.0 μ Ci [γ -³²P] ATP per 5 μ l, 0.75 mM ATP dissolved in reaction buffer B) was added to each reaction mixture. The samples were incubated in 30°C water bath for 30 min. Using a "Hybri-Dot" (BRL) blotting apparatus, reaction samples were blotted onto three layers of Whatman P81 ion exchange papers placed on top of one Whatman 3 mm paper. All the papers were washed twice with distilled water for 4 min. After that, the blots were incubated for 60 min at 65°C in 10 ml proteinase K solution (1.0 mg/ml proteinase K in 1% SDS), and washed subsequently once with distilled water for 4 min and then washed three times with 85°C distilled water for 3 min. After dried, blots were exposed to X-ray film with an intensifying screen.

Results

STS Treatments

The effect of silver thiosulfate (STS) on the growth of potato plant *in vitro* cultured was shown in Table 1. Results showed that STS treatment enhanced

Table 1. Effect of silver thiosulfate (STS) on the shoot growth, mass tuberization in potato plant

STS (2 μ M)	Total weight (g \pm SE)	No. of tuber per flask*
+	1.81 \pm 0.21	45 \pm 13.2
–	0.3 \pm 0.05	46 \pm 12.8

*Average of ten 500 ml-Erlenmeyer flasks with 150 ml solidified MT medium.

the growth of potato plant. After grown on MS medium for three weeks, the fresh weight of plantlet was 6 times greater than that without STS. However, we didn't find any improvement in tuberization of *in vitro* cultured potato. No great difference was found on the potato tuberization when they were grown in the MT medium either with or without STS treatment.

Transformation and Regeneration

The leaves or stems of STS treated plants were used for the *Agrobacterium*-transformation. Our observations showed that the formation of transformed callus occurred after inoculation for 3 weeks. The viability of transformed callus could be selected through the culture on the medium containing kanamycin, whereas the controls couldn't survive and form callus on the selection medium. Transformation frequency was measured as the percentage of explants based on numbers of treated tissues which regenerated into shoots on the medium containing kanamycin. These plantlets were also found to be positive in the detection of opine compounds and NPT II enzyme activity (Figs. 1, 2). Table 2 showed that transformation frequencies were not much variable, 30% from leaf inoculation, 31% from stem inoculation, and 27% from microtuber inoculation. In this study, 232 calli transformed from 430 leaf segments within 3–4 weeks growth on the selection medium and 129 transformed plants were subsequently regenerated from these transformed calli within 3 months (one of multiple shoots in each transgenic callus was counted as one transformed plant). In stem transformation, we got 139 calli transformed from 278 stem segments and 87 plants regenerated from transgenic calli. The evaluation of STS effect on the transformation frequency was extended until the callus stage. In stem transformation, the formation frequency of transgenic callus from STS-treated plants was 5 times higher than from controlled plant (Table 3). The

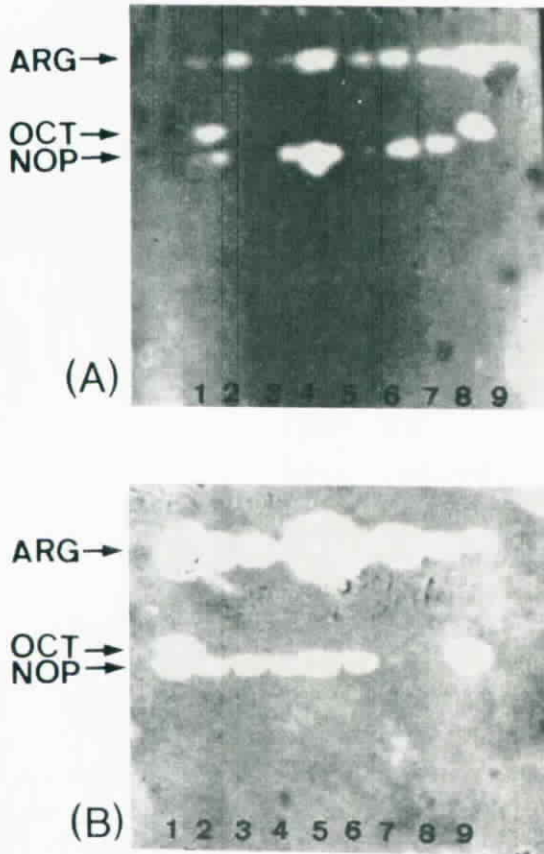


Fig. 1. (A) Opine assay from transformed plant tissue regenerated from leaf inoculation. Lane 1 standard, lane 2 and 9 controls, lane 3 transformed callus, lane 4 stem of transformed plant. Lane 5, 6, 7 leaf of transformed plant. Lane 8 the octopine type callus from transformed tobacco inoculated by *A. tumefaciens* Ach5. (B) Opine assay of plant or tissue transformed from microtuber or stem. Lane 1 standard. Lane 7, 8 stem fragment and tuber disc from non-infected plant, respectively. Lane 2, 3, 4 calli from stem transformation, stem and leaf of transformed plant regenerated from calli of stem transformation. Lane 5, 6 stem and leaf of transformed plant regenerated from microtuber transformation. Lane 9 Tuber disc after inoculation for one week.

results of leaf transformation were similar to stem transformation (data not shown). Following the opine assays, the results showed that nopaline was really present in these kanamycin-resistant calli, but not in the non-transgenic calli.

The discs of *in vitro* cultured potato microtubers (less than 6 months old) were also inoculated with *Agrobacterium*. It was found that 98 shoots were emerged from 360 microtuber discs following incubation on the selection medium for 2 weeks. The shoots were grown continuously until it was about 5 cm in length for the subsequent 3-4 weeks (Fig. 3), and then were excised and planted onto rooting medium containing 100 $\mu\text{g/ml}$ kanamycin and 200 $\mu\text{g/ml}$ cefotaxime for root formation. The observations showed that lots of the root formed from the nodes, while few of them rooted from the cutting surface. This phenomenon could also be observed from other experiments of both stem and leaf transformations. When the roots formed following grown on rooting medium for 2 weeks, the transformed plants were transferred to the pot in green-

Table 3. Effect of STS (2 μM) treatment and different infection medium on the transformation frequency. Stem tissues were inoculated in this experiment

Plant*	Inoculation**	Transgenic callus frequency
materials	method	
+	I	139/278 (50.0%)
	II	17/102 (16.7%)
-	I	5/50 (10.0%)
	II	3/50 (6.0%)

*Plant materials cultured on MS medium with or without STS.

**Inoculation method I and II were described as Methods and Materials.

Table 2. Transformation frequency of potato plant mediated by *Agrobacterium tumefaciens* C58 (*pfDA2*)

Infection region	Total NO.	Transformed callus* or Transgenic plant	Transformation frequency (%)	
			Callus	Plants
LEAF	430	232 (129)	53.9	30.0
STEM	278	139 (87)	50.0	31.3
MICROTUBER	360	(98)	-	27.2

*The number in parenthesis means transgenic plant regenerated from callus after 7 weeks culture.

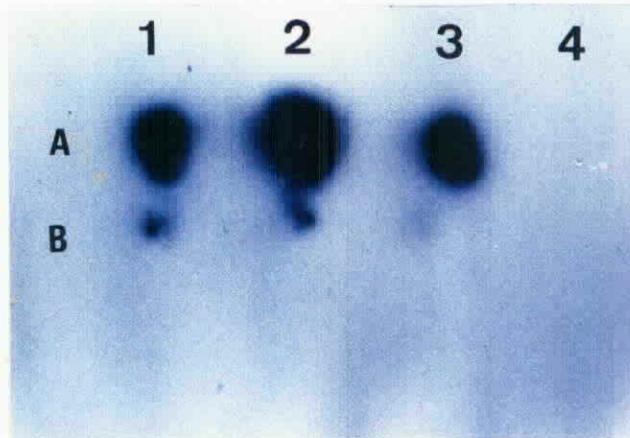


Fig. 2. The dot blot assay of neomycin phosphotransferase II enzyme from the leaf tissue extracts of transgenic potato plants. Row A represents reactions that contained kanamycin while row B represents reactions that did not contain kanamycin. Lane 1, 2, and 3 is NPT II enzyme activity in leaf extracts of transgenic plant transformed from leaf, stem, and microtuber, respectively. Lane 4 is control.

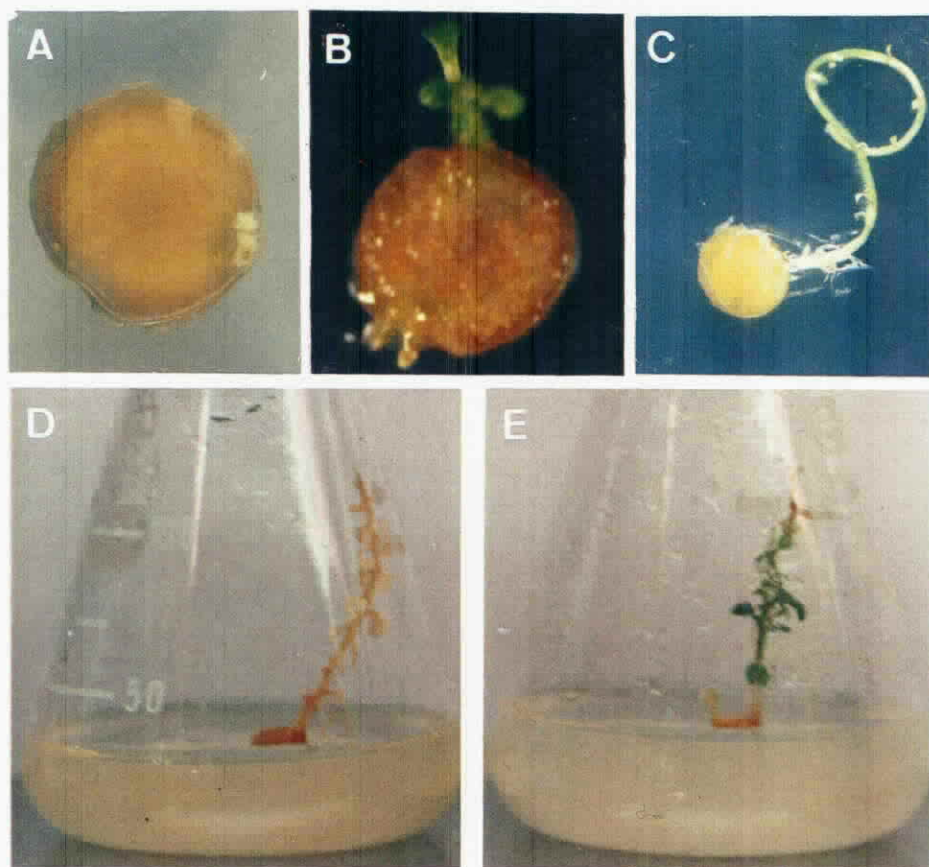


Fig. 3. Microtuber transformation. Shoot regenerated from the tuber disc after inoculated and cultured in the medium containing kanamycin for 3 weeks (A), 4 weeks (B), and 5 weeks (C), respectively. Transformed plant could be grown in the medium containing kanamycin (D), but non-infected plant died in the same medium (E).

house for further growth.

Analysis of Transformants

The plants transformed from different organs were sampled to detect the expression of nopaline. From the results of paper electrophoresis, it showed that all transformed tissues contained nopaline but not in the controls (Fig. 1).

The activity of NPT II enzyme was also usually utilized for evaluating the gene expression of transformants. Leaves of the transformed plants were extracted and detected for the activity of NPT II by dot blotting. The results were shown in Fig. 2. Lane 1, 2, and 3 represented the NPT II activity in leaf extracts of transgenic plant regenerated from leaf-, stem-, and microtuber-transformation, respectively. The results showed that all the transformed plants really expressed the enzyme activity, but not in controls (lane 4).

Discussion

The phenomenons of stoloniferous shoots and small leaves in potato plants were commonly occurred as it was cultured in tightly closed-vessels. According to our observations, the tissues of transformed stoloniferous shoots or small leaves usually died immediately after transferring to the selection medium. Therefore, those leaves and stems were not suitable for using as a good target explant for *Agrobacterium*-mediated transformation. It has been shown that accumulated ethylene in sealed containers could result in the retardation of potato growth (Ammirato, 1983). Thus, the usage of silver thiosulfate (STS), an inhibitor of ethylene action, was attempted to promote the growth condition. Similar to the previous report (Perl *et al.*, 1988), the inhibition of ethylene action by STS treatment resulted in the recovery of potato growth to a normal growth status in this experiment. The morphogenesis of STS-treated potatoes *in vitro* was similar to that in out-of-vessel cultivation. In addition, *in vitro* culture provided sterile and healthy materials. So, these healthy plants with larger and stronger leaves and stems could be beneficially used for the subsequent transformation experiments.

Several evidences suggested that ethylene was involved in the alteration of cell wall components (Eisinger, 1983; Prasad and Cline, 1987). We thought that the reverse action of ethylene-retarded potato

growth by STS may be due to the alteration of cell wall components. Those cell wall components such as glucan were convenient for bacteria binding to plant cell. It has also been demonstrated that ethylene activated the responsive genes of pathogen defence (Ecker and Davis, 1986). The inhibition of ethylene action by STS might decrease the defence ability of plants to pathogen. Therefore, in the present study, the increase of transformation frequency in STS-treated potato tissues might be correlated with the binding of bacteria to plant cell wall.

It has been observed that some phenolic compounds such as AS, were one of the factors in improving the infection efficiency of *Agrobacterium*. These compounds were considered to play an important role in activating virulent (vir) gene which is essential for the transfer of T-DNA (Koukolikova-Nicola *et al.*, 1985; Stachel *et al.*, 1986a). In this experiment, tissues were incubated in liquid medium with *Agrobacterium* for 2 days. Stachel *et al.* (1986) indicated that when the plant tissues were wounded and soaked in liquid medium, plant cells will release AS to liquid medium. Additionally, it has been reported that plant tissues would also release wounded ethylene under stresses (Abeles, 1972). The injury caused by cutting in the process of inoculation and the subsequent submergence in liquid-infection conditions might produce considerable amount of stress-induced ethylene. Ethylene has also been found that it could stimulate the biosynthesis of phenolic compounds (Lieberman, 1979). These phenolic compounds including AS might improve the transfer of T-DNA into potato cell. This is one of the possible reasons that the frequency of transgenic callus formed from inoculated tissues in liquid-infection medium was higher than in solid infection medium (Table 3).

Except for factors mentioned above, there were several other factors such as amounts of *Agrobacterium*, low light intensity, frequency of transferring tissues to a new selection medium could also affect the transformation frequency (An *et al.*, 1986). In the present investigation, we also found that frequent transferring (every week) of the cocultured tissues to a new selection medium would effectively enhance the development of transformed tissues. In contrast, the lower frequency caused by less frequent transferring might be due to untransformed tissues which died on the selection medium containing kanamycin and released some toxic compounds.

Similar to others (Ishida *et al.*, 1989; Sheerman and Bevan, 1988; Stiekema *et al.*, 1988), we found that there was an advantage in tuber transformation, that shoots could grow directly from microtuber slice instead of the stage of transient callus formation which was needed in the processes of stem and leaf transformation (An *et al.*, 1986; Twell and Ooms, 1988; De Block, 1988; Shahin and Simpson, 1986). However, STS-treatment and liquid infection medium seemed to have better efficiency on raising the transformation frequency in both leaf and stem tissues than the microtuber tissue. In addition, we didn't find any phenotypic variation of transgenic potato plant transformed either from leaf or stem transformation.

When the explants was attempted to be used for gene transfer, it would be convenient if there was a selection scheme whereby transformed cell could be separated from untransformed cells. The NPT II gene was found feasible to be used as a selectable marker in potato plant, because control tissues could not grow in the medium containing kanamycin. Transformed calli and shoots remained green while untransformed tissues were bleached on the medium containing kanamycin.

We have demonstrated the ability to transfer and regulate the expression of bacterial gene in potato by stem-, leaf-, and tuber-transformation. During the infection and culture processes, ethylene might act as an important factor for the transformation. The relationship between the transformation and plant hormones are under investigated. It is possible to apply this cocultural system described here to other *Solanum* species.

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利用硫代硫酸銀增進馬鈴薯轉殖效率之研究

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利用乙烯作用抑制劑硫代硫酸銀 (silver thiosulfate, STS) 添加於培養基可使無菌培養之馬鈴薯植株獲得較佳之生長。經處理 21 天，其植株總重量為對照之 5 倍，葉片面積也較對照組大很多，且徒長叢生的現象亦消失，莖部變為粗短，利用經此處理後之植株當作轉殖材料，其轉殖效率可顯著提高。藉由農桿腫瘤菌 (*Agrobacterium tumefaciens*) 的感染接種，將帶有 neo 基因之 Ti 質體轉殖入馬鈴薯植株之試驗結果，不同部位經感染後，其轉殖效率為葉片 30%，莖片段 31%、塊莖 27%。經抗性篩選培養、opine 之檢測以及 NPT II 酵素活性之檢定方式皆證實外來基因已成功地嵌入轉殖植株，而確定馬鈴薯轉殖系統之建立。