PLANT REGENERATION FROM MESOPHYLL PROTOPLASTS OF POTATO (*SOLANUM TUBEROSUM* L.) CULTIVAR DELAWARE USING SILVER THIOSULFATE (STS)

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Abstract

The use of *in vitro* potato shoot cultures for protoplast isolation is desirable for consistent protoplast qualities. Ethylene build-up in the culture vessels causes problems for leaf growth (e.g. decreasing leaf surface, dry weight and chlorophyll) in shoot culture. Fifty to one hundred µM Silver thiosulfate (STS) produced a larger leaf area as a source of plant material for protoplast isolation. STS also decreased the internode length of potato shoots but increased dry weight and chlorophyll. Regenerated plants were obtained from cultured protoplasts in a series of media based on the MS medium. Protoplasts of Delaware showed a better response in cell division and colony formation in agarose-solidified culture medium. Regenerated plants showed some degrees of aneuploid but basically were similar to the original potato plants.

Introduction

The potato (*Solanum tuberosum* L.) is the most important tuber crop grown for food worldwide. In Western Australia, the potato cultivar 'Delaware' is adapted to winter growth in the Mediterranean climate, and constitutes about 80% of the total crop [1]. Although regenerated plants from isolated protoplasts of some commercial potato cultivars, for example, cv. Desiree [18] have been reported however, regeneration system from isolated protoplasts of this cultivar has not been established. There has been a steady improvement in methods of protoplast isolation and culture for modification of plant genomes during last 15 years. This is based on the ability of protoplasts to regenerate to whole plants on culture in appropriate media. The use of protoplast culture as a breeding procedure to improve potato germplasm requires detailed information on protoplast production, manipulation, culture and shoot regeneration [35]. Once protoplast isolation, culture and plant regeneration system are established, it can be used for gene manipulation experiments for example protoplasts fusion or direct gene transformation [16,37,40]. However, the first step is to develop efficient protoplast isolation, culture and plant regeneration systems from protoplasts of the genotypes of interest [18]. Leaf mesophyll grown *in vitro* culture is a common source of plant material for potato protoplast isolation. Potato nodal cultures maintained in sealed

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vessels develop stoloniferous shoots with small leaves and aerial roots. This effect is caused by ethylene accumulation in the culture vessels [22,28]. Therefore, inhibition of ethylene action using ethylene antagonists, such as silver thiosulfate (STS), can lead to normal growth of potato tissues which consequently produce larger leaves *in vitro* and increases yield and viability of protoplasts [9,10,26,28,32,33].

In this study, we describe: 1) effects of STS on improvement of potato shoot cultures, and 2) conditions for isolation, culture and regeneration of plants from protoplasts of potato cv. 'Delaware'. The protocol described allows reproducible regeneration of plants from protoplasts of this cultivar and possibly other potato cultivars with low responsiveness to tissue culture.

Material and Methods

Establishment and Maintenance of Shoot Cultures

Virus free potato tubers cv. Delaware were kindly supplied by the Western Australian Department of Agriculture. To establish shoot cultures, tubers were washed with 5% v/v sodium hypochlorite, rinsed three times with sterile water, and plants grown in 500 ml pots containing steamed sterilised soil (Yallanbee gravelly soil)**.** Plants were fertilised with Osmocote (Grace Sierra) and commercial fertiliser (Spring, Ramprie Laboratories) in an insect-proof glasshouse. After 5-6 weeks auxillary buds with a piece of stem were removed, surface sterilised using 5% v/v sodium hypochlorite containing 1-2 drops of Tween 20, for 5-10 minutes, and then washed six times with sterile distilled water. Nodal cuttings were cultured aseptically in 250 ml glass jars containing 40-50 ml MS medium [27] supplemented with sucrose (30g/L), 6-benzylzminopurine (BAP) (0.05 mg/L) and 0.7% agar (Sigma) pH, 5.8. Tissue culture containers were sealed with Closures Suncaps, 6.0 mm clear filter (Sigma, No. C-6920). Cultures were incubated in 16 h light and 8 h dark (3000 lux, warm white fluorescent tubes) at 25°C.

Treatment of Potato Tissue Cultures with the Ethylene Inhibitor (Silver Thiosulfate, STS)

A stock silver thiosulfate solution was prepared by mixing 400 µM silver nitrate and 1600 µM sodium thiosulfate in a 1:1 ratio, and used at concentrations of 50, 100 and 200 µM in culture media. MS medium without phytohormones was autoclaved then STS solutions were filter sterilised and added to the medium. 30 ml of MS medium containing STS was poured into 130 ml sterile poly carbonate tissue culture containers. Four potato auxillary buds from cv. Delaware were placed in each container and for each treatment there were 4 replicates. After 4 weeks all explants were collected and leaf areas and internode lengths were measured using graphic paper (excluding the bottom leaf). For statistical analysis of the data Duncan multiple range tests was performed [13].

Protoplast Isolation, Culture and Plant Regeneration

Four to five weeks after growth of potato node cultures cv. Delaware on MS + STS media (50-100 µM) approximately 0.5-1.0 gram of the large leaves were removed, cut into thin (0.5-1.0 mm) strips with a sterile scalpel, and soaked overnight at 4°C in preconditioning medium as described by Fish *et al.* [15]. Leaves were preplasmolysed in wash solution [14] containing 7.5 % mannitol pH 5.6, for 0.5-1 h. Protoplasts were released by gently swirling the leaf slices for 4-5 h at 40 rpm at 25-28°C in an enzyme solution containing the major salts of Fish and Karp [14], macerozyme R-10 (0.15%) "Yakult, Tokyo, Japan", cellulase R-10 (0.7%) "Yakult, Tokyo, Japan", and mannitol (7.5 %), pH 5.6., then centrifuged at 700 rpm for 5 min. Protoplasts were resuspended and washed twice in wash solution. The density of protoplasts was determined by counting in a haemocytometer, and the viability of protoplasts was also examined by fluorescein diacetate "FDA" (5 mg/L) as described by Tempelaar and Jones [37]. Then isolated protoplasts were washed twice with liquid culture medium A (Table 1) and cultured in medium A solidified with 0.45% agarose type VII low gelling temperature (Sigma, catalogue No. A-6560) at final density of $3\text{-}4\times10^4$/ml. To plat the protoplasts in agarose, 1.0 ml of double strength culture medium A with 1.8% agarose (after melting) were mixed at 45°C, then added to 1.0 ml of protoplast suspension in 5 cm sterile petri dishes. The protoplasts and agarose were mixed gently by swirling or pipetting the culture medium. The dishes were sealed with Nescofilm and incubated at 25°C in the dark. Protoplast division after 8-10 days and plating efficiencies (proportion of initial protoplasts which undergo sustained division to colonies) were calculated 3-4 weeks following culture. To overcome the browning problem, agarose-cultured protoplasts were cut into small pieces as described by Shillito *et al.* [32] and were transferred to liquid medium C (Table 1) and maintained on a shaker at 40 rpm. After 5-6 weeks small calli (1-2 mm) were transferred to medium S (Table 1). After 6-8 weeks small shoots were transferred to medium R (Table 1) for elongation and rooting. Shoots were subcultured every 3-4 weeks on MS or medium R. Root-tip squash preparations for chromosome analysis of regenerated plants was carried out as described by Karp (1991) [23] with some modifications.

Table 1. Media composition for protoplast culture (A), callus formation (C), shoot induction (S) and root initiation (R)

Component	Medium A*	Medium C	Medium S	Medium R
Macronutrients	MS*	MS	MS	MS
Micronutrients	MS*	MS	MS	MS
Vitamins				
Glycine	2.0 (mg/L)	2.0 (mg/L)	2.0 (mg/L)	2.0 (mg/L)
Myo-Inositol	100 (mg/L)	100 (mg/L)	100 (mg/L)	100 (mg/L)
Nicotinic Acid	0.50 (mg/L)	0.50 (mg/L)	0.50 (mg/L)	0.50 (mg/L)
Pyridoxine-Hydrochloride	0.50 (mg/L)	0.50 (mg/L)	0.50 (mg/L)	0.50 (mg/L)
Folic acid	0.5 (mg/L)	0.5 (mg/L)	0.5 (mg/L)	0.5 (mg/L)
Biotin	0.05 (mg/L)	0.05 (mg/L)	0.05 (mg/L)	0.05 (mg/L)
Casein-Hydrolysate	500 (mg/L)	400 (mg/L)	—	—
Adenine sulfate	40 (mg/L)	40 (mg/L)	80 (mg/L)	80 (mg/L)
Glutamine	—	100 (mg/L)	146 (mg/L)	—
Coconut milk	20 (ml/L)	—	—	—
Hormones				
NAA*	1.0 (mg/L)	0.1 (mg/L)	—	—
6-BAP	0.5 (mg/L)	0.5 (mg/L)	—	—
IAA**	—	—	0.1 (mg/L)	—
Zeatin*	—	—	1.0 (mg/L)	—
Others				
MES	976 (mg/L)	976 (mg/L)	976 (mg/L)	—
Mannitol	—	4%	3%	—
Glucose	7.5%	—	—	—
Agarose type VII (Sigma)	0.45%	—	—	—
Sucrose	2.5 (g/L)	2.5 (g/L)	2.5 (g/L)	30 (g/L)
Agar	—	7.0 (g/L)	7.0 (g/L)	7.0 (g/L)
pH	5.6	5.6	5.6	5.8

*?-Naphthalene acetic acid **Indole-3-acetid acid; MS-Murashige and Skoog [27]

Results

Effect of Silver Thiosulfate (STS) on Leaf Area and Internode Length

The presence of silver thiosulfate in the culture media significantly increased leaf area and internode length of nodule shoot cultures of cv. Delaware. In comparison to control (MS medium, no STS) concentrations of 50 µM, 100 µM and 200 µM STS increased leaf area size significantly and the leaf was almost two fold more than that of the control (Fig. 1) Statistical analysis of leaf size showed that the differences between control and treatments with 50 µM and 100 µM STS was significant ($p < 0.0001$). However, the difference between control and application of 200 µM STS was not significant. The results of measuring the internode section of potato shoots treated with 3 different concentrations of STS showed that STS treatments decreased the internode length of potato shoots cv. Delaware. The internide data also showed significant differences ($p < 0.0001$) between control and all treatments with STS, but no significant differences among three levels of STS were observed.

Protoplast Isolation, Culture and Plant Regeneration

The yield of protoplasts from digested leaves of potato cv. Delaware using the techniques described in the material and methods, was 2-2.5×10^6 protoplasts/g fresh weight of leaf, and the viability was 80-95%. On

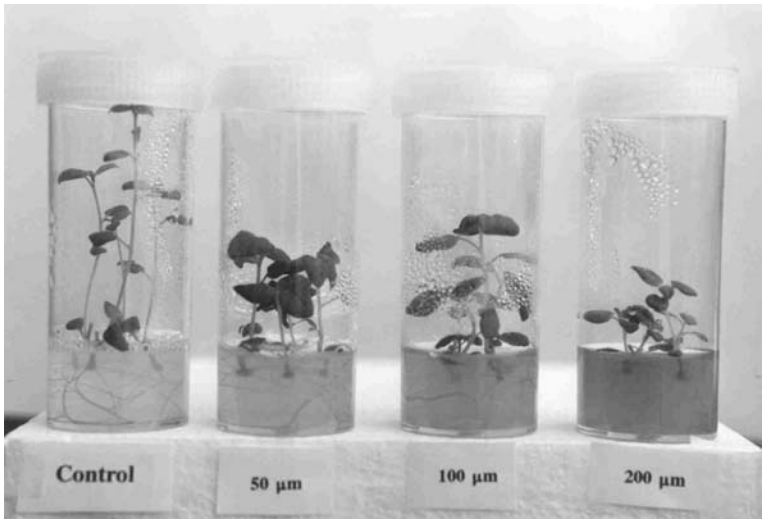

Figure 1. Effects of Different concentration of STS on potato explant**.**

culture, the first protoplast divisions/were observed 6-10 days after incubation. The results of a series of experiments for optimising of culture medium are summerised as following:

The effects of two culture media, VKM medium and medium A (Table 1), were examined on protoplast division and colony formation. VKM medium resulted in a division frequency of 13% whereas in medium A it was 28%. The protoplasts division was higher when protoplasts were cultured in medium A solidified with agarose (20%) rather than in liquid medium A (6%). Experiments on drop culture showed that this technique did not improve protoplast division. During culture of protoplasts cv. Delaware browning decreased the frequency of division and therefore most of the protoplasts showed no more than 3-4 divisions. This was possibly because of phenolic compounds produced by metabolic activity or by dead or damaged protoplasts. Also polarised browning was observed around the cell wall. To overcome the browning problem, activated charcoal was used (1%w/v) as recommended by Carlberg *et al.* [12] and Fish [17], however, it did not improve the division frequency and plating efficiency of cultured protoplasts. However, when segments of agarised cultured protoplasts were incubated in a liquid culture medium C on gyratory shaker as recommended by Shillito *et al.* [32] colonies derived from protoplasts started to develop and became white. The use of liquid culture medium C reduced browning to a far greater extent than the other techniques. Application of this technique resulted in a range of 5-20 calli per petri dish which continued their growth.

The frequency of calli formation (calli derived from protoplasts) in liquid culture medium C was 0.1-0.5%. When small calli were 1-2 mm in size, they were transferred to solidified medium C under light and the calli became light green. They were then transferred to S medium and dark green and granular calli were obtained. After 6-8 weeks on S medium shoot initials developed on the surface of the granular green calli. The percentage of calli producing shoots was 20-30%, and the number of shoots or shoot buds per callus on medium S was 1 to 4. When small shoots were transferred to medium R they elongated and formed roots. The sequences of plant development from mesophyll protoplasts of potato cv. Delaware are illustrated in Figure 2a-h.

To obtain a higher frequency of shoots, we used different plant growth regulators, (e.g. in medium S the concentration of Zeatin was increased from 1.0 (mg/L) to 2.0 and 4.0 (mg/L), or Zeatin was replaced with 1.0 mg/L Thiadiazuron (TDZ), then 20 callus derived from protoplasts were treated with these modified S medium, only green callus grew vigorously on medium S supplemented with 2.0 (mg/L) or 4.0 (mg/L) of Zeatin. When medium S containing TDZ was used, most of the calli became brown and died. It was observed that when calli were transferred from liquid medium C to solid medium C dark treatment for a 1-2 weeks helps calli to grow better. The results of chromosome counting showed a range of chromosome numbers between 40 to 48. This variation may be a result of squashing technique used leading to errors in chromosome counting, or to the presence of aneuploid plants.

Discussion

STS Treatment

Effects on leaf area. The development of aerial roots, branching and scale leaves on cultured potatoes shoots

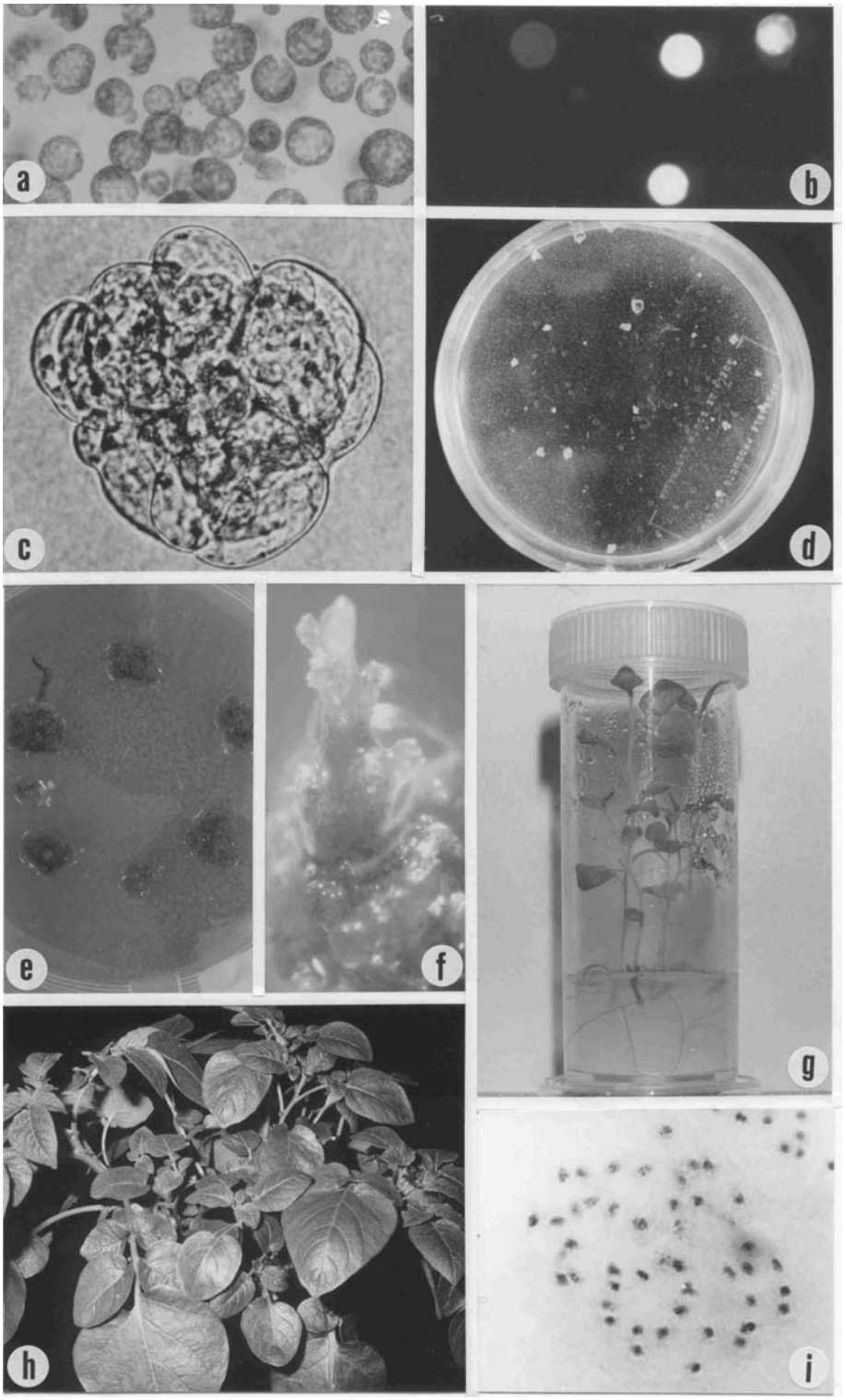

Figure 2. Steps in plant regeneration from protoplasts of potato cv. Delaware. a) isolated protoplasts, b) isolated protoplasts stained with FDA (yellow-green protoplasts are viable), c) divided protoplasts after 10 days in medium A, d) early stage of callus formation in medium C, e) early stage of shoot formation in medium S, f) shoot formation from callus, g) regenerated plant from protoplast in vitro, h) regenerated plant grown in the glasshouse, i) chromosome preparation from a regenerated plant.

usually occurs if cultured in tightly closed-vessels. This phenomenon has also been reported for other plants, for example *Brassica campestris* L. [26]. This is caused by accumulation of ethylene and depletion on oxygen in the atmosphere of the tissue culture container. Ethylene reduces the frequency of adventitious shoot regeneration from explants and retards potato growth [3,9,11], and also inhibits callus growth of rice [2]. Untreated potato nodal shoot cultures of cv. Delaware were not a suitable material for protoplast isolation. The effects of ethylene in potato tissue culture may be due to the interaction between endogenous growth-promoting hormones and ethylene [10]. Perl *et al.* [28] reported that ethylene inhibits cell division and cytodifferentiation in lettuce pith explants, a similar report by Apelbaum and Burg [4] indicating that cell division frequency is reduced by 95-98% in the hook region of *Pisum sativum* treated with 50 µM ethylene. Similar to the previous report by Perl *et al.* [20], the inhibition of ethylene action by STS treatment resulted in the recovery of potato growth to a normal level [9,10,39] and produced larger leaves and supplied more material for protoplasts isolation. We found that optimum concentration of STS for normal potato tissue cultures of cv. Delaware was in a range between 50 to 100 µM. When 200 µM STS was used the leaf area was lower than when treated with 50µM or 100µM STS. This could be as a result of high concentration of STS and possibly toxicity of STS. In a similar experiment the possible toxicity of STS at high concentrations (700 µM) on callus culture of *Oryza sativa* L. and nodal culture of *Carica papaya* L. has already been reported [2].

Effects on internode length. Inhibition of ethylene action by STS or ethylene synthesis by ancymidol has been shown affects plant growth and development. For example ancymidol inhibits internode elongation [12], and STS prevents glycium enlargement. It has been reported that the presence of ethylene interferes with gibberellic acid function in plant tissues. Stewart *et al.* [34] reported an antagonism between gibberellic acid (GA) and ethylene in regulation of internode elongation of pea seedlings. Since both cell division and cell elongation are influenced by the action of gibberellins, it can be concluded that the application of STS as ethylene inhibitor action and ancymidol as ethylene synthesis inhibitor on potato nodal culture resulted in a similar pattern of inhibition of stem elongation (Aso. Pfro. Jen McComb, personal communication) These results are similar to those which Coolbaugh and Hamilton [12] observed with Alaska Peas.

Protoplast Isolation and Culture

It has been shown that the responsiveness of plants to protoplasts isolation, culture and regeneration is genotype dependent [16,18,21,22,23,29]. The optimum enzyme combination and concentration for protoplast isolation of Delaware were cellulase R-10 (0.7%) and Macerozyme R-10 (0.15%), higher concentrations of enzyme yielded more protoplasts but there was more debris and non viable protoplasts. Because protoplasts of Delaware are fragile and sensitive to digesting enzyme, this could be due to the toxicity of enzymes [19]. Tavazza and Ancora (1986) [35] found that in an enzyme solution containing 1.0% cellulase R-10 and 0.25% macerozyme R-10, potato cv. Desiree and cv. Kenebec yielded 1.56×10^6 and 1.21×10^6 protoplasts /gram fresh leaves. In our experiments cv. Delaware yielded more protoplasts with lower concentration of these enzymes.

The optimum osmolarity of solutions for protoplast isolation and culture differs with genotype and species of potato (e.g. 0.3 M mannitol for *Solanum chacoense* and 0.4 M mannitol recommended for *Solanum tuberosum* by Grun and Chu [19]. We found 7.5% mannitol was the optimum osmolarity for potato cv. Delaware and a higher concentration of mannitol caused protoplasts to shrink and protoplast division was reduced (data not shown).

Growth and shoot initiation of potato protoplasts depends on the composition of the culture media and genotype [36]. Protoplasts cultured from potato cv. Delaware gave relatively low division and plating frequencies. When effects of culture media on protoplast division and plating efficiency were examined, we observed that VKM medium resulted in fewer protoplast divisions than A medium. In VKM medium protoplasts divided only 3-4 times. The same pattern of cell division was observed by Binding *et al.* [7] when they cultured protoplasts of dihaploid clone H^2 140 in KM medium. In our experiments when VKM culture medium was replaced with A medium the frequency of division improved. Similar to our finding, Foulger and Jones [18] reported higher division and plating efficiency of potato cv. Desiree, King Edward and Maris piper by modifying the MS culture medium.

Protoplasts from potato cv. Delaware cultured on liquid VKM and A medium showed a higher division frequency than protoplasts cultured in agarose solidified medium. Improvement in culturing protoplasts in agarose-solidified media could be due to the physical property of the medium [32,35].

Browning was a serous problem for culture of Delaware protoplasts. Previous reports indicate that protoplasts of dihaploid clones and commercial cultivars of potato have shown a similar phenomenon [7,20.35]. The reason for polarised browning is unknown, but this could be due to local changes in pH on one side of the protoplasts, or alterations of components, particularly Fe^{2+} ions in the culture medium. However, this

phenomenon needs to be studied in more details.

Some reports have shown that when light green calli were transferred to the shoot formation medium, dark green calli were obtained [3,17,20]. A similar pattern of growth was observed when light green calli of potato cv. Delaware were transferred to S medium.

It has already been observed that potato cv. Delaware shows a low responsiveness in tissue culture and plant regeneration [1]. The frequency of shoot formation from p-calli of cv. Delaware cultured on S medium was 20-30%, which was similar to potato cv. Russet Burbank reported by Shepard and Totten [31]. However, using Zeatin (higher concentrations) or TDZ did not improved frequency of shoot formation. It could be a result of inhibitory effects of high concentration of Zeatin on shoot regeneration.

It has been reported that shoot formation from calli derived from protoplasts is genotype dependent. Thomas [38] observed p-calli of cv. Maris Piper did not produce any shoots on regeneration medium. However, in our experiments from p-calli of potato cv. Delaware after 6-8 weeks in culture medium S, 35 shoot buds and shoots were obtained. Despite the low percentage of callus formation from protoplasts of Delaware, the frequency of shoot formation was 20-30% which was similar to other *Solanum* species e.g. *Solanum torvum* [20].

There are numerous reports in the literature about changes in both chromosome number and structure in plants regenerated from potato protoplasts. One of the common changes (aneuploidy) has been reported for many varieties for example, Russet Burbank [30], Fortyfold [24], Maris Bard [15]. Similar to these reports, we observed that a majority of regenerated plants of cv. Delaware were aneuploid. Some reasons can be suggested for chromosome variation a) Choice of starting material may affect the degree of chromosome variation, b) it may happen in the callus phase, c) the condition of protoplasts culture and plant regeneration particularly, hormones and osmolarity of protoplasts culture medium [16,24,25]. Therefore, appropriate conditions have to be stabilised in order to reduce or avoid chromosome variation. It seems majority of chromosome changes occur in callus phase, and this phenomenon has been reported in considerable evidences. Despite of the chromosome variation in regenerated plants from protoplasts cv. Delaware, the morphology of normal tetraploid and aueuploid plants was similar.

Improvement of protoplast yield of potato cv. Delaware using STS provides suitable starting material for regeneration of plants from protoplasts. The plant regeneration system for this cultivar assists genetic manipulations by the techniques of protoplasts fusion or direct gene transformation (electroporation).

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