PN-ARM-488
79521

U.S.-Israel Cooperative Development Research (CDR) Program

Proposal No. C7-191

Grant No. DPE 5544-G-SS-7043-00

CHARACTERIZATION OF ORGANELLE GENOMES IN THE POTATO GERMPLASM AS AID IN BREEDING POTATOES FOR DEVELOPING COUNTRIES

PROGRESS REPORT

For the period January 1, 1988 to June 30, 1988

(Second semi-annual report)

Principal Investigator: Prof. Esra Galun
Dept. of Plant Genetics
The Weizmann Institute of Science
Rehovot 76100, Israel
Tel: +972-8-483585
Telex: 381300
Facsimile: +972-8-466966
Bitnet: LPGRUNE1 at WEIZMANN

Collaborators: Dr. P. Gregory, Dr. J. Dodds
International Potato Center (CIP)
P.O. Box 5969
Lima, Peru
Tel: 266920; 354354
Telex: 25672 PE

Recd to SS AUG 10 1989

INTRODUCTION

The establishment of the physical map of the chloroplast DNA (ctDNA) of potato (Heinhorst, Cannon, Galun, Keuschaft and Weissbach, Theor. Appl. Genet. 75, 244, 1988) on which I reported previously, furnished important tools for our project. Cloned restriction fragments of potato ctDNA are very useful as probes for analyzing ctDNA of other *Solanum* species. Moreover ctDNA fragments (obtained by endonuclease digestion) of the latter species can now be assigned specifically to potato ctDNA fragments and the alterations as well as rearrangements in ctDNA sequences of wild *Solanum* species can now be related to the standard ctDNA of potato. As shall be briefly noted below, we started to utilize cloned fragments of potato ctDNA for such purposes.

The most convenient source of organelle DNA (especially of ctDNA) are leaves from axenically cultured shoots, rather than leaves from greenhouse-maintained plants. The problem, up to recent months, was that such shoots grew sluggishly producing minute leaves. We succeeded to achieve a breakthrough in the culture of axenic shoots by suppressing ethylene generation in closed boxes: we found that the addition of a low level (2 to 8 $\mu g.ml^{-1}$) of silver thiosulfate to the culture medium causes an abrupt change: at least a threefold higher yield of plant material per box and normal rather than slim and hairy shoots were obtained (Fig. 1).

We improved our procedures for the extraction of total DNA of *Solanum* spp. and can now perform many more analyses per unit time. This is an important step which will enable the scientist form the International Potato Center (CIP), who is expected to come to us in October, to get familiar with the advanced procedures.

The latter visit is a manifestation of the collaborative work between our laboratory and the International Potato Center. As shall be detailed below the visit shall be a

prelude to a wide-range analysis of *Solanum* spp. organelles which will be undertaken in Lima, Peru.

PLANT MATERIAL FOR ORGANELLE ANALYSIS

As briefly noted above leaves constitute the plant material for the analysis of chloroplast DNA. These leaves can be obtained from greenhouse (or open-field) grown plants or from axenic shoots. The latter are a more reliable source which can be propagated at will and is free from contaminants. On the other hand, in the past a regular box yielded less than 500 mg shoots (after 3 weeks of culture) while the minimal plant material for the analysis, is 500-1000 mg. Much more plant material (i.e. 5 to 15 g) is needed to obtain ethidium-bromide stained restriction profiles from purified ctDNA (digested by several endonucleases). We found that the very high ethylene generation in boxes containing potato shoots is correlated with the suppression of shoot (especially-leaf) development and that silver thiosulfate (STS) readily negates both processes (see attached preprint of Perl, Aviv and Galun, Plant Cell Reports, in press). Consequently shoots from one box are sufficient for Southern blot analysis of *Solanum* ctDNA and 3-5 boxes suffice for stained restriction profiles of ctDNA.

We are thus propagating the plant material for ctDNA analyses as axenic shoots with STS in the culture medium. Table 1 provides information on the relevant plant material. It also contains, in an abbreviated from, the rationales of propagating the various cultivars and species. We are in continuous contact with scientists at CIP and with other investigators in order to choose appropriate *Solanum* species for organelle DNA analysis according to the guide lines detailed in our Proposal of this project.

ANALYSES OF ORGANELLE DNA

The main goal of this project is to characterize the organelle compositions of *Solanum* species. The characterization of chloroplasts and mitochondria is performed by the analysis of the respective ctDNA and mtDNA.

1. Analysis of ctDNA

Most of our efforts, during the current 6 months, were devoted to the analysis of ctDNA. This analysis can be approached by two procedures, each appropriate for a different purpose. In order to establish phytogenetic relationships between species detailed data are required. Hence restriction profiles of ctDNA digested with several endonucleases should be made available. Another approach is to furnish a test which will clearly indicate that a given ctDNA differs from certain other ctDNAs. For this approach Southern blot hybridizations with appropriate probes can be utilized. The latter approach requires much less ctDNA and can even be performed with total DNA, as indicated above.

We are performing both kinds of analyses but during the past months focussed on the latter procedure. We found that the *Bam*HI no. 3 fragment of most potato cultivars (i.e. those which are known to have *S. tuberosum* cytoplasm) is missing from all the tested ctDNAs of wild *Solanum* species. We thus radiolabelled this *Bam*HI No. 3 clone and used it as probe in Southern blot hybridizations. The respective autoradiogram provided a clear distinction between potato ctDNA and the ctDNAs of other species. Other clones may be used to differentiated between two wild *Solanum* species.

We also continued to perform the full analysis of ctDNA (i.e. stained restriction profiles) of some *Solanum* species. An update of ctDNA analysis in potato cultivars and *Solanum* species is provided in Table 2.

The CIP scientist (see below - Collaboration) will perform in our lab both kinds of ctDNA analyses. But most of his time will be devoted to obtain detailed restriction profiles of ctDNA in a selected number of *Solanum* species.

2. Analysis of mtDNA

The chondriomes of potato and other *Solanum* species are much more complicated than their plastomes - which is a common feature in angiosperms. Consequently the restriction patterns of endonuclease digested mtDNA has a high number of fragments. Using a number of endonucleases to digest mtDNAs of potato and several wild *Solanum* species we choose *SalI* as the one providing the best differentiation between species. Table 2 updates the potato cultivars and *Solanum* species which were analyzed. We developed a root-culture system which shall be used in the future to give a better source of mtDNA than shoots. The mtDNA analysis should be the subject of study during the second year of our project. We hope that the CIP scientist who shall visit us (see: Collaboration) will also be trained in mtDNA analysis.

COLLABORATION

As noted in the Introduction and in accordance with our Proposal, a scientist from CIP, Felix Serquem, will come to the Weizmann Institute in October 1988. He will get acquainted with the procedures of ctDNA and mtDNA analyses. After a period of training in the methodologies he will start to perform several analyses independently so that he will master the required techniques before returning to CIP.

We maintain correspondence (by letter and telex communication) with our CIP collaborators and received plant material either from CIP or from the USDA in Beltsville. Recently, our Dr. Dvora Aviv 9who is an advisor on this project) participated in the AID

sponsored PSTC Biotechnology Conference (Washington June 6-9, 1988) where she discussed the progress of this project with Dr. N. Pallais of CIP. The principal investigator of this project (E.G.) plans to visit CIP in the spring of 1989 where a detailed plan on the utilization of the results of this project for the potato improvement at CIP, will be discussed in detail.

FUTURE WORK AND CONCLUSION

Our future work will be according to the research plan outlined in our Proposal, although in some subjects we advanced more than originally anticipated. The analyses of the plastomes of *Solanum* species are now in a very satisfactory state but should be expanded to additional species and more information, based on fragmentation of the ctDNAs with several endonucleases is required to provide the data for building the respective phylogeny. The mtDNA are at an early phase but we see no major problems in achieving the goals set in the Proposal. We may add (possibly outside the time-frame of this project) the phylogeny based on nuclear-coded genes (i.e. isozymes or RFLP analysis) to compare plastome phylogeny with nuclear-genome phylogeny in *Solanum*.

Table 1. Establishment of axenic shoot-cultures of potato *(S. tuberosum)* cultivars and of other species of *Solanum* to furnish plant material for the analysis of organelle DNA.

A. Available cultivars and species

S. tuberosum cultivars	
Desiree	Standard source of reference
Atzimba	Able to yield tubes in warm areas
Atlantic	Parent for hybrid TPS
Mirka	Widely used European cultivar
Kufri-Jyoti	Cultivar developed in India
Serrana	Tuberous wild type
LT-1	Parent for hybrid TPS (late tubering)
DTO-28	Parent for hybrid TPS
Y-245.7 (CMS)	CMS, probably *S. stoloniferum* cytoplasm
Ticause	ssp.*andigena*
S. brevidens	Representative wild *Solanum*
S. chacoense	Representative wild *Solanum*
S. berthaultii	Distantly related wild *Solanum*
S. eutuberosum	Very distantly related wild *Solanum*
S. commersonii	Representative wild *Solanum*
S. venturii	Representative wild *Solanum*
S. nigrum	Very distant species from *S. tuberosum*

B. Additional Species*

S. demissum
S. chomatophilum
S. tarijense
S. raphanifolium
S. piurae
*S. microdontum***
*S. phureja***
*S. polydemium***
*S. sparsipillum***
*S. stoloniferum***
*S. bukasovii***

* These species were reported to show resistances to pathogens and thermal stresses; the latter resistances could be organelle-controlled.

** These species are being sent by the USDA, ARS, North-Central Region, Potato Introduction station, Sturgeon Bay, WI, via the USDA Plant germplasm Quarantine Center, Beltsville MD.

Table 2. Update of ctDNA and mtDNA analyses in potato cultivars and *Solanum* species (up to June 30, 1988).

Cultivar or species	Chloroplast DNA	mitochondrial DNA
Solanum tuberosum		
cv. Desiree	+	+
cv. Atzimba	+	+
cv. Atlantic	+	+
cv. Mirka	+	+
cv. Kufri-Jioti	+	+
Serrana (wild)	+	+
cv. LT-1	-	+
cv. DTO-28	-	+
cv. Y-245.7 (CMS)	+	+
cv. Ticausi (andigena)	-	+
Solanum brevidens	-	+
S. berthaultii	+	+
S. eutuberosum	+	+
S. commersonii	-	+
S. venturii	-	+
S. nigrum	+	+

Figure 1. Potato shoots cultured in boxes containing media without (right) or with (left) 2 $\mu g.ml^{-1}$ silver thiosulfate

Ethylene and *in vitro* culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene

Plant Cell Reports (In press)

Avihai Perl, Dvora Aviv and Esra Galun

Department of Plant Genetics, Weizmann Institute of Science Rehovot 76100, Israel

ABSTRACT

Ethylene release by potato shoots cultured in closed boxes was suppressed by the addition of silver thiosulfate to the culture medium. Shoots cultured in the presence of silver thiosulfate produced appreciably more tissue and the yield of protoplasts per unit tissue mass was vastly increased, resulting in an 8 fold increase of protoplast yield per shoot. Exposure of pricked leaves to macerating enzymes facilitated ethylene generation. Leaves of shoots which were previously cultured in silver thiosulfate containing medium generated much less ethylene than leaves from control shoots and this generation could be further reduced by the addition of acetylsalicylic acid during maceration. The capability of polyethylene glycol treated potato protoplasts to produce microcalli was vastly increased by the addition of silver thiosulfate during exposure of protoplasts to $Ca(NO_3)_2$ following the polyethylene glycol treatment. Similarly, when a plasmid (pCAP212) containing an expressible gene for chloramphenicol acetyltransferase was introduced into potato protoplasts through a polyethylene glycol treatment, the transient expression of acetyltransferase was very much increased by the addition of a short incubation of the protoplasts with silver thiosulfate.

Abbreviations: AOA, (aminooxy)acetic acid, ASA, acetylsalicylic acid ; AVG, aminoethoxyvinyl-glycine; CAT, chloramphenicol acetyltransferase; MV, methyl viologen; PEG, polyethylene glycol; STS, silver thiosulfate.

INTRODUCTION

It is a common observation that tightly closing culture-vessels containing potato shoots results in visible growth retardation (e.g. Hussey and Stacey 1981, 1984). Ethylene was implicated with various phenomena of *in vitro* cultured plant tissue (see Ammirato 1983). Evidence for a correlation between elevated ethylene production and reduced viability of *Nicotiana* protoplasts was reported by Cassells et al. (1980). The role of macerating-enzyme preparations was investigated by Facciotti and Pilet (1981). The latter authors reported on ethylene release during the isolation of *N. sylvestris* protoplasts. They observed higher release by haploid than by diploid protoplast suspensions and related the lower division capability of the former protoplasts to the higher ethylene levels. A rapid burst of ethylene production during exposure of *N. tabacum* leaf-discs to Cellulysin was related to a specific factor in this enzyme preparation, apparently inducing ACC synthase (Anderson et al. 1982, 1985). Similarly Mussell et al. (1986) reported on elevated ethylene release during exposure of ort leaves to specific macerating-enzyme preparations.

We are engaged in protoplast-manipulations of potato for the study of nuclear-genome/plastome interactions (e.g. Perl et al. 1988; Galun et al. 1988). Consequently, we were interested in following ethylene release during various phases of the operations which result in cybrid and transgenic potato plants. Moreover, we searched for treatments which will reduce ethylene release and concomitantly improve growth and cell-division capabilities of *in vitro* cultured potato tissue and cells.

MATERIALS AND METHODS

Plant material. *In vitro* cultured shoots of *Solanum tuberosum* cv. Bintje were originally obtained from Dr. G.S. Bokelmann (ITAL, Wageningen). Shoot segments with at least one node were routinely planted in Magenta GA7 boxes containing 50 ml of agar-solidified (1%) MS medium (Murashige and Skoog 1962) with only 1% sucrose. Ten shoots per box were cultured for about three weeks at 25°C under continuous cool-white fluorescent illumination providing 60-70 $\mu E\ m^{-2}\ s^{-1}$. The shoots were either segmented and replanted or used for protoplast isolation basically as described by Binding et al. (1978). The boxes were closed with their regular covers; the covers were either sealed with "Parafilm" bands ("sealed" boxes) or not sealed ("unsealed" boxes).

Protoplast isolation. One gram of "shoots" (i.e. shoots including leaves) was pricked by a multineedle stainless-steel pricker and transferred to 10 ml of maceration fluid. This fluid contained NM (Nagy and Maliga 1976) medium supplemented with 0.35% Cellulase Y-C, 0.025% Pectolyase Y23 (both from Seishin Pharmaceutical Co., Tokyo, Japan) and 0.075% Macerozyme R-10 (Kinki-Yakult, Nichimomiya, Japan). Maceration was for 16 h in the dark at 25°C. Protoplasts were filtered though a Nitex screen (100 μm diameter pores) and washed twice by centrifugation (75 g for 5 min) in CPW medium (Frearson et al. 1973).

Protoplast fusion and culture. Protoplast fusion was performed as described (Aviv and Galun 1985) but was mediated by 40% (w/v) PEG 3350 MW (Sigma). Homofusion was performed; hence only Bintje protoplasts served as fusion partners. The fused

protoplasts were diluted to a density of $2.10^4 \cdot ml^{-1}$ with culture medium which consisted of macroelements of Adams and Townsend (1983), microelements and vitamins of Kao and Michayluk (1975), with the addition of 1 $\mu g \cdot ml^{-1}$ naphthaleneacetic acid, 0.5 $\mu g \cdot ml^{-1}$ zeatin and 0.1 $\mu g \cdot ml^{-1}$ 2,4-D. Three ml of this suspension were plated in each 5 cm petri dish. The plates were maintained at 24°C, in the dark, for 3 days and then exposed to low light intensity (10 $\mu E \cdot m^{-2} \cdot s^{-1}$). The osmolarity was reduced and the medium composition was changed by periodical additions of 0.3 ml MS containing 1 $\mu g \cdot ml^{-1}$ zeatin and 0.5 $\mu g \cdot ml^{-1}$ naphthalenacetic acid.

Evaluation of shoot and root yields. For each treatment, 20 shoots were harvested three weeks after plating and their fresh weight was measured. The roots of those shoots were gently released from the agar, washed with running water, dried by filter paper and weighed.

Ethylene measurements. To enable gas sampling from cultured shoots in sealed boxes, holes were made in the covers and rubber valves were inserted. The valves were tightly fitted into the holes. To avoid ethylene release from the rubber, the valves were autoclaved and ventilated before insertion into the holes. Likewise, to sample gas during protoplast release, Erlenmeyer flasks (25 ml) containing 1 g shoot-tissue and 10 ml maceration fluid were sealed with rubber valves. Samples of gas (1 ml) were taken by a syringe, through these valves and injected into a gas chromatograph (Carlo-Erba 6C-6000 with an aluminum oxide column and a flame ionization detector).

Treatments with ethylene inhibitors. The STS stock solution (6 mM, or 1.5 mg ml^{-1}) was prepared by combining (at an 1:1 ratio) 12 mM $AgNO_3$ and 96 mM $Na_2S_2O_3 \cdot 5H_2O$. ASA was obtained from Sigma and dissolved in ethanol (as 0.5M stock solution). STS and ASA solutions were filter-sterilized and diluted as required.

Transient expression. A plasmid vector (pCAP 212, see Velten and Schell 1985) containing genes coding for kanamycin resistance and CAT activity was kindly provided by Prof. J. Schell (MPI, Cologne). Direct transformation of isolated protoplasts, using this vector was performed according to Kruger-Lebus and Potrykus (1987) with the addition of a $Ca(NO_3)_2$ step as in our above mentioned protoplast fusion procedure. The enzymatic assay for CAT activity was as described by Herrera-Estrella et al. (1983).

RESULTS

Ethylene release by in vitro cultured shoots. When potato shoots are cultured in sealed boxes their leaf-blades hardly expanded and their stems were very slim and hairy. Culture in unsealed boxes, allowing gas exchange resulted in increased leaf blades, thicker stems and less hairs. Ethylene may be involved in all these morphological characteristics. We thus performed a preliminary survey in which we added $AgNO_3$ (0.1-100 $\mu g \cdot ml^{-1}$), AVG (0.1-100 $\mu g \cdot ml^{-1}$) or AOA (0.1-25 $\mu g \cdot ml^{-1}$) to the shoot culture medium in unsealed boxes. The rationale of using $AgNO_3$ was based on the reported suppression of ethylene effects by silver ions (e.g. Beyer 1976, 1979; Imaseki 1986) and on the report of Purnhauser et al. (1987) who showed the shoot regeneration from scutellar calli of wheat and from *Nicotiana plumbaginifolia* calli was substantially improved by adding $AgNO_3$ as an ethylene inhibiting agent to the culture medium. This silver salt did not provide the expected improvement in potato-shoot growth; we therefore tried STS (0.1-50 $\mu g \cdot ml^{-1}$). Some

Figure 1. Potato shoots cultured in unsealed boxes in media with (left) or without (right) STS.

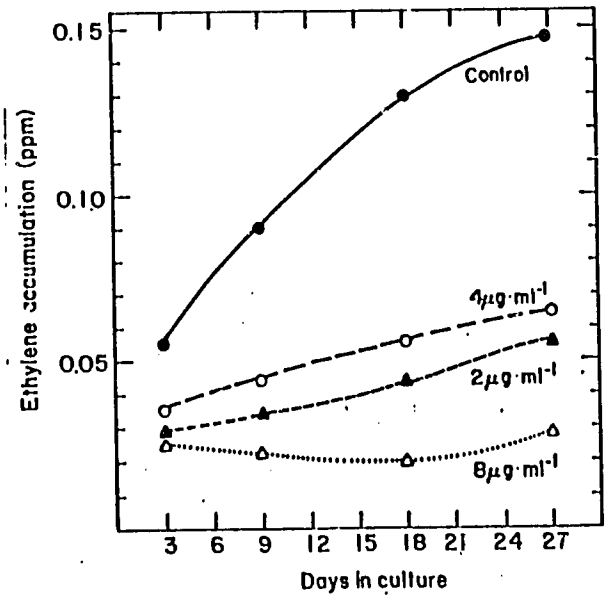

Figure 2. Ethylene accumulation during *in vitro* growth of potato shoots.

concentrations (2-8 $\mu g \cdot ml^{-1}$) vastly increasing shoot and root development (Table 1) and leaf blade size (Figure 1) as compared to growth in the control, unsealed, boxes. STS was therefore utilized in further tests on the role of silver ions in ethylene generation. To test whether or not this drastic change in morphology is correlated with ethylene generation we added 2, 4 or 8 $\mu g \cdot ml^{-1}$ STS to the culture medium in which the potato shoots were planted. The gas inside the control boxes and in boxes containing STS was then sampled periodically, up to 3 weeks (Figure 2). The ethylene analyses substantiated our assumption: ethylene accumulated in the sealed boxes and this accumulation was suppressed by STS.

In vitro shoot growth and protoplast yield as affected by STS. To further quest the correlation between shoot growth in unsealed boxes and STS mediated ethylene suppression, we planted shoots in boxes without or with 2 μg ml^{-1} STS. After three weeks the weight of the shoots and their roots was determined. The shoots were then exposed to macerating enzymes and the protoplast yields were determined (Table 1). The yield of shoots grown in the presence of STS was about 2.5 fold higher than without STS. Moreover the yield of protoplasts per 1 g tissue from STS containing medium was also over trifold higher than from shoots cultured in the absence of STS. The addition of STS

to the shoot culture medium thus caused an 8 fold higher protoplast yield, per shoot. Many repetitions of such tests provided the same results. Higher levels of STS did not improve protoplast yields. It should be noted that the division capability of purified protoplasts from STS treated shoots was not higher than the division capability of protoplasts from control shoots: the plating efficiency of protoplasts from either source (i.e. percent of dividing protoplasts) ranged from 8 to 15.

Table 1. Improvment of shoot growth and protoplast yield by the addition of STS to the shoot-culture medium*

Shoot culture medium	Shoot and root yields (mg ± SE)		Protoplast yield (per g tissue)
	shoots	roots	
Without STS	88 ± 3	36 ± 2	$3.8\ 10^5$
With STS	226 ± 7	56 ± 3	$1.2\ 10^6$

* Shoot sections (20, in two boxes) were planted in unsealed boxes containing 50 ml shoot-culture medium, without or with STS (2 $\mu g \cdot ml^{-1}$). Yield of shoots and roots was evaluated after 21 d of culture.

Ethylene formation during protoplast release. Ethylene generation was reported (e.g. Anderson et al. 1982, 1985) to be stimulated during enzymatic release of protoplasts. We wanted to lower this ethylene generation. One candidate to lower ethylene generation was STS; the other candidate was ASA (see: Rabinowitch and Fridovich, 1983). Furthermore, in studies reviewed by Rabinowitch and Fridovich *(loc.cit.)* the superoxide radical (O_2^-) was implicated, in plants, with the conversion of ACC to ethylene and this radical was reported to increase following MV application. We therefore asked further if MV added during tissue-maceration will increase ethylene release and if it does will ASA and/or STS suppress such an increase. The results of an experiment, summarized in Table 2, provided very clear answers to these questions. Corroborating results of other authors *(loc. cit.)* exposure of potato tissue to macerating enzymes elevated ethylene generation (over five-fold). This elevated generation of ethylene was partially or completely suppressed by STS or ASA, respectively As expected, MV increased the macerating-enzymes' mediated generation of ethylene (about two fold) ; this increase was almost completely suppressed by ASA. Addition of STS to the maceration fluid of shoots grown in STS-did not suppress ethylene generation. This unexpected observation could result from a carryover effect; also, STS does not directly suppress ethylene generation but inhibits it's effect.

Colony formation capability and transient expression of an alien gene following exposure to PEG in the presence and absence of STS. Both protoplast-fusion and direct gene-transformation require a step of exposure to PEG followed by an abrupt dilution of the PEG, causing severe stress to the protoplast as well as a series of structural changes. We suspected that the induction of ethylene release by the PEG treatment is involved in the reduction of protoplast vitality. We therefore performed a mock fusion experiment in which Bintje protoplasts were first treated with a PEG solution. The PEG-containing protoplast-suspension was diluted with $Ca(NO_3)_2$, in the absence or presence of STS. We found that a concentration of 100 $\mu g \cdot ml^{-1}$ STS substantially increased the number of calli which developed after the PEG-$Ca(NO_3)_2$ treatment of protoplasts. In a typical test 17 and 212 colonies were formed without and with STS, respectively.

Table 2. Ethylene generation during leaf-incubation and protoplast isolation*

Experimental conditions and treatments	Ethylene generation (pmoles·g^{-1} tissue)
Without enzymes in maceration fluid	
Shoots cultured without STS	2460[f]**
Shoots cultured with STS	1060[h]
With enzymes in maceration fluid	
Shoots cultured without STS	
No MV added during maceration	
- Without STS and ASA	13000[b]
- With STS added to maceration fluid	8000[c]
- With ASA added to maceration fluid	2020[g]
MV added during maceration	
- Without ASA	26600[a]
- With ASA added to maceration fluid	2800[e]
Shoots cultured with STS	
- Without STS & ASA during maceration	5300[d]
- With STS added to maceration fluid	8100[c]
- With ASA added to maceration fluid	2320[f]

Levels of STS, ASA and MV were 10$\mu g \cdot ml^{-1}$, 50μM and 1mM, respectively.

* Potato shoots were cultured with or without STS, harvested leaves were pricked and incubated with or without maceration enzymes.

** Values followed by the same letter are not significantly different ($P \geq 0.05$) Numbers means of five replications.

Direct transformation of plant protoplasts with recombinant-DNA constructs also involves PEG-$Ca(NO_3)_2$ treatment. We asked whether or not the transient expression of a DNA sequence conferring resistance to chloramphenicol will be improved by providing STS during the $Ca(NO_3)_2$ dilution. Using Bintje protoplasts and plasmid pCAT212 we performed a transformation experiment followed by a CAT test as described in Figure 3. The protoplasts for the two treatments (i.e. with or without STS), each with three replications, were from the same protoplast-isolation batch. Acetylation of ^{14}C-chloramphenicol (expressed in the assay as the appearance of radioactive spots away from the application site) was caused by homogenates of transformed protoplasts whether or not STS was added during $Ca(NO_3)_2$ dilution. But, the addition of STS (lanes 2, 6 and 7) clearly improved the acetylation: the radioactivity near the site of homogenate application (i.e. the non-acetylated chloramphenicol) was very low or not detectable. This allows two interpretations for the STS effect: STS improved the rate of transformed protoplasts or STS affected the metabolic activity of transformed protoplasts. Further experimentation is required to clarify the role of STS in improving the CAT assay results but presently the addition of STS during transformation of potato protoplasts is recommended.

DISCUSSION

Ethylene generation in sealed containers, containing *in vitro* cultured potato shoots, was previously reported

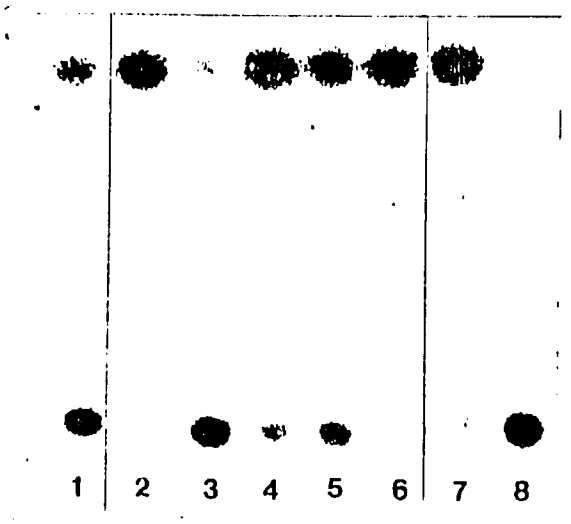

Figure 3. Chloramphenicol acetyltransferase (CAT) activity in transformed potato protoplasts. Lane designation: Lane 1 - positive control (0.1 units of enzyme, Sigma); lanes 2, 6 & 7 - protoplasts transformed in the presence of 100 $\mu g \cdot ml^{-1}$ STS; lanes 3,4 & 5 - protoplasts transformed in the absence of STS; Lane 8 - negative control (untransformed protoplasts).

and implicated with the production of stoloniferous shoots and small leaves (Hussey and Stacey 1981, 1984). Our study provided kinetic information on the ethylene build-up under such conditions. Moreover we showed that the accumulation of ethylene can be suppressed by the addition of STS. The reduced ethylene accumulation was correlated with normal development of the potato shoots. In addition to having a much higher tissue mass, the shoots which grew in the presence of STS yielded three times more protoplasts, per unit tissue mass, than control shoots. Two explanations are plausible. The ratio of leaf-blades to stem tissue is much higher in shoots cultured in the presence of STS; because the leaf-blades are the source of protoplasts, the change in ratio may cause the increase of protoplast yield. It is also possible that the tissue of shoots grown in the presence of STS is more "resistant" to the maceration stress. Several authors reported on the induction of ethylene during the exposure of leaves to maceration enzymes (e.g. Facciotti and Pilet 1981; Anderson et al. 1982; Mussell et al. 1986). We found the same phenomenon in potato but also showed that this ethylene generation is much lower when the leaves were derived from shoot cultured in STS. The vast increase in ethylene generation caused by the addition of MV during maceration is noteworthy. Recently, Ishii (1987) found that exposure of oat leaves to macerating enzymes caused an elevated generation of supperoxide. Since the latter radical was implicated in the past with ethylene synthesis (Rabinowitch and Fridovich 1983) the evidence from oats reconciles with our results. Furthermore, when we added ASA, which was reported to inhibit ethylene synthesis in cultured plant cells (Leslie and Romani 1986), the MV effect was largely suppressed: the generation of ethylene by the potato leaves, exposed to macerating enzymes *and* MV, was reduced to the level of ethylene generation in the absence of both enzymes and MV. A very recent publication (Lentini et al. 1988) reported that norbornadiene reversed ethylene damage in *Brassica* shoots grown in sealed containers.

In conclusion, our study demonstrated that STS can cause vast improvements in several potato-protoplast manipulation procedures. It also provides a basis for further studies on ethylene generation and on ethylene damage to plant protoplasts.

ACKNOWLEDGEMENTS

We are grateful to Bat-Chen Israel for assistance during part of the experimental work. This study was supported by a German-Israeli Biotechnology grant, provided by Israel's National Council for Research and Development and the BMFT of the Federal Republic of Germany and by a grant (DPE-5544-G-SS-7043) from the Agency for International Development (Washington, USA).

REFERENCES

Adams TL, Townsend SA (1983) Plant Cell Rep 2: 165-168

Ammirato PV (1983) In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of Plant Tissue Culture Vol 1, Macmillan, New York. pp 82-123

Anderson JD, Chalutz E, Mattoo AK (1985) In: Cellular and Molecular Biology of Plant Stress, Alan R Liss Inc. New York, NY, pp 263-280

Anderson JD, Mattoo AK, Lieberman M (1982) Biochem Biophys Res Commun 107: 588-596

Aviv D, Galun E (1985) J Heredity 76: 135-136

Beyer EM Jr (1976) Plant Physiol 58: 268-271

Beyer EM Jr (1979) Plant Physiol 63: 169-173

Binding H, Nehls R, Schieder O, Sopory SK, Wenzel G (1978) Physiol Plant 43: 52-54

Cassells AC, Cocker FM, Austin S (1980) Plant Sci Lett 19: 169-173

Facciotti D, Pilet PE (1981) Z. Pflanzenphysiol 104: 401-407

Frearson EM, Power JB, Cocking EC (1973) Develop Biol 33: 130-137

Galun E, Perl A, Aviv D (1988) In: Applications of Plants Cell and Tissue Culture (Ciba Foundation Symposium 137) Wiley, pp 97-112 (in press)

Herrera-Estrella L, Depicker A, Van Montagu M, Schell J (1983) Nature 303: 209-213

Hussey G, Stacey NJ (1981) Ann Bot 48: 787-796

Hussey G, Stacey NJ (1984) Ann Bot 53: 565-578

Imaseki H (1986) In: Takahashi N. (ed) Chemistry of Plant Hormones. CRC Press, Boca Raton, FL, pp 249-264

Ishii S (1987) In Vitro Cell Develop Biol 23: 653-658

Kao KM, Michayluk MR (1975) Planta 126: 105-110

Kruger-Lebus S, Potrykus I (1987) Plant Mol Biol Rep 5: 289-294

Lentini Z, Mussell H, Mutschler MA, Earle ED (1988) Plant Sci 54: 75-81.

Leslie CA, Romani RJ (1986) Plant Cell Rep 5: 144-146

Murashige T, Skoog F (1962) Physiol Plant 15: 473-497

Mussell H, Earle E, Campbell L, Batts LA (1986) Plant Sci 47: 207-214

Nagy JF, Maliga P (1976) Z Pflanzenphysiol 78: 453-455

Perl A, Aviv D, Galun E (1988) Proc Intern Congress of Plant Tissue Culture-Tropical Crops, Bogota, September 21-25, 1987 (in press)

Purnhauser L, Medgyesy P, Czako M, Dix PJ, Marton L (1987) Plant Cell Rep 6: 1-4

Rabinowitch HD, Fridovich I (1983) Photobiol. 37: 679-690

Velten J, Schell J (1985) Nucleic Acid Res 13: 6981-6997