J. Amer. Soc. Hort. Sci. 106(6):799–803. 1981.

Plant Regeneration from Leaf Mesophyll Protoplasts of Selected Ornamental Nicotiana Species[1]

J. E. Passiatore and K. C. Sink

Department of Horticulture, Michigan State University, East Lansing, MI 48824

Additional index words. Nicotiana alata, N. forgetiana, N. sanderae, regeneration, tissue culture

Abstract. **Shoot tips of *Nicotiana alata* Link & Otto, *N. forgetiana* Sander and *N. sanderae* W. Wats seedlings were established on modified Murashige and Skoog (MS) salts and vitamins medium to provide leaf material as the cell source for protoplasts. Viable protoplasts (8.0×10^5 to 1.5×10^6/ml per g fresh weight) were enzymatically isolated from *N. alata* in 2.5% Driselase plus 4.0–4.7% mannitol or sorbitol in cell protoplast wash solution (CPW). An enzyme mixture of 1.0% Driselase, 1.0% Macerozyme R-10, 1.0% Cellulase R-10, 0.5% potassium dextran sulfate and 4.0% mannitol in CPW released 4.0×10^5 to 1.0×10^6 and 7.6×10^5 to 8.5×10^5/ml protoplasts per g leaf tissue respectively from *N. forgetiana* and *N. sanderae*. Only 4 of 13 tested culture media, also exhibiting species selectivity, promoted sustained cell division of protoplasts to the macroscopic callus stage in 28–35 days. Optimum plating efficiency ranged from 15 to 37% when the protoplasts were cultured at 5.0×10^4/ml in Cool White light. Plating efficiency did not increase when cultures were placed under Gro-Lux light. Macroscopic callus was readily regenerated to shoots in 2 months on MS medium + 1.0 mg/liter zeatin (Z) or MS + 2.0 mg/liter idoleacetic acid (IAA) + 1.0 mg/liter benzylamino purine (BA). Rhizogenesis occurred in hormone-free MS medium. Regenerated plants flowered in the greenhouse and exhibited minor variation in flower form and pigmentation.**

To date, *Solanaceae* species have been found most amenable to protoplast isolation, culture, and plant regeneration (23). In devising the initial regeneration protocol for a plant species, especially dicots, greenhouse-grown seedlings are generally preferred for the isolation of protoplasts. However, as pointed out by Vasil and Vasil (23), such plant materials do not always yield consistent quantities of quality protoplasts. Conversely, sterile shoot cultures have the unique advantages of both eliminating the exposure to toxic agents to achieve sterile conditions (2), and providing uniform plant materials. Such a technique was successfully employed in this research on three *Nicotiana* species. The protoplast regeneration protocols for three ornamental bedding plant *Nicotiana* species, *N. alata, N. forgetiana* and *N. sanderae* was devised since they are closely related taxonomically to the genus *Petunia*, but are sexually incompatible (19) and therefore have potential for somatic hybridization. Regeneration is already known for *N. alata* (3, J. B. Power, personal communication), but since it was a parent of *N. sanderae*, we also included it in this study.

Materials and Methods

Source of protoplasts. Seeds of *N. alata* cv. Sensation Mixed (Joseph Harris Co., Rochester, N. Y.), *N. sanderae* and *N. forgetiana* (L. G. Burk, USDA-SEA Tobacco Research Laboratory, Oxford, N. C.) were surface sterilized by soaking in 5% diluted Clorox for 25 min, followed by 3 sterile distilled water rinses. Seeds were sown on MS (13) basal medium supplemented with (mg/liter): folic acid 0.001; kinetin (6-furfurylaminopurine) (K) 0.03; IAA 0.00875; sucrose 3.0%; agar 0.8% pH 5.8 (before autoclaving) in 60×15 mm Petri dishes and wrapped with Parafilm. The dishes were kept at 25±2°C under 28–34 $\mu Em^{-2}s^{-1}$ (400–700 nm) light (Sylvania F96T12.GRO) on a 16 hr photoperiod where germination occurred in 10–12 days. In 1 month, 2 seedlings of *N. alata* were placed on MS + (mg/liter): IAA (2.0), BA (1.0), 3% sucrose, and 0.8% agar in 60×15 mm Petri dishes and kept under 15 $\mu Em^{-2}s^{-1}$ at 25±2°C for 16 hr daily. Three seedlings of *N. sanderae* or *N. forgetiana* were placed in each 100×80 mm Petri dish containing modified MS (1/2 NO_3), sucrose 2.5%, agar 1.0%, pH 5.8 and kept under 28–34 $\mu Em^{-2}s^{-1}$ (400–700 nm). Seedling leaves were of sufficient size (2.5–3.0 × 1.0–2.0 cm) for protoplast isolation about 45 days after transfer. The stripped shoot tips were recultured and again produced leaves for protoplast isolation after 30 days. Three such shoot recultures were possible in the same Petri dish.

Preparation of protoplasts. Numerous enzyme mixtures were tested for optimum release of viable leaf mesophyll protoplasts. They were derived by combining cellulase "Onozuka" R-10 (Kinki Yakult Mfg. Co.), Driselase (Kyowa Hakko, Kogyo Co.), Macerase (Calbiochem), and pectinase (Sigma Chemical Co.). Potassium dextran sulfate, mannitol and sorbitol were also tested in combination with the enzymes and all mixtures were made using CPW salts (6). Enzyme solutions were adjusted to pH 5.8 with 0.2N NaOH or 0.1N HCl before filter sterilizing (0.45μm Nalgene).

Leaves were excised and placed in 100×15 mm Petri dishes containing the test enzyme (1 g per 10 ml). They were sliced perpendicular to the midrib toward the margin at about 1.0 mm increments with a No. 11 scalpel blade while holding the entire leaf in the enzyme solution. Each dish was sealed with Parafilm and statically incubated for 3–5 hr at 26–30°C. Gentle teasing of the digested leaf strips with a Pasteur pipette toward the end of incubation aided protoplast release. Protoplasts suspended in the enzyme solution were passed through a 61μm sieve to remove debris. The protoplast mixture was transferred to 16×125 mm screw capped culture tubes and gently pelleted by centrifugation (100 g; 8 min). The supernatant was removed and replaced with CPW salts + 15% sucrose solution and the tubes were centrifuged (80 g; 5 min).

The isolation procedure for *N. alata* protoplasts was slightly modified for *N. sanderae* and *N. forgetiana*. Released protoplasts of *N. sanderae* and *N. forgetiana* were pelleted (80 g; 7 min), the supernatant was removed, and the protoplasts were resuspended in CPW salts + 15% sucrose. Subsequent centrifugation (80 g; 7 min) produced a band of intact protoplasts at the solution surface.

[1]Received for publication February 25, 1981. Michigan Agricultural Experiment Station Journal Article No. 9833. Supported in part by Society of American Florists Endowment (SAFE) grant 11-77-4 and United States-Israel Agricultural Research and Development Fund (BARD) grant I-134-79.

The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

The band of protoplasts was routinely washed twice with CPW + 15% sucrose to remove final traces of the enzyme. Finally, protoplasts were collected from the surface with a Pasteur pipette and resuspended in 10 ml of test culture medium. An aliquot was removed and counted on a haemocytometer. Subsequently, the 10 ml solution was diluted to test densities of 2.5×10^4, 5.0×10^4 and 1.0×10^5 protoplasts/ml, dispensed as 3 ml per 60×15 mm plastic Petri dish and wrapped with Parafilm. The viability of protoplasts was determined by the fluorescein diacetate (FDA) staining method of Larkin (10).

Culture of protoplasts. Selected growth regulators were tested in combination with 4 basal salt and vitamin media (7, 13, 15, 21) (Table 1). All growth regulators were autoclaved in the medium (pH 5.8) at 1.46 kg/cm^2 (121°C), for 20 min. After plating, the dishes were placed under Cool White fluorescent light, 18–22 $\mu Em^{-2}s^{-1}$, to assess the 13 initial culture media for supporting protoplast division. In a subsequent study, the 4 media sustaining protoplast divisions (Table 2) were evaluated under 3 test light regimes: 1) dark, 2) Cool White fluorescent light, 18–22 $\mu Em^{-2}s^{-1}$, and 3) Gro-Lux fluorescent light, 28–34 $\mu Em^{-2}s^{-1}$. The temperature was 25±2°C for all experiments.

A Licor 185 (sensor LI-190) was used to measure $\mu Em^{-2}s^{-1}$ (400–700nm) values. Cultures were examined daily on a Nikon inverted microscope and their viability and division assessed. Reduction of osmoticum from 4.5% mannitol was done by adding 1.0 ml of test culture containing 2.25% or 0% mannitol to each Petri dish every 2 weeks in the case of *N. alata* and *N. sanderae* and every 1 to 1.5 weeks for *N. forgetiana*. For exceptionally high plating efficiencies, half of the protoplast solution was transferred to a separate Petri dish after 3 weeks. Experiments to assess culture media and light conditions were both conducted at least 2 times with a minimum of 6 Petri dishes per experiment.

Determination of plating efficiency. In order to compare the efficacy of the test culture media and light conditions on cell division and growth to callus, plating efficiencies (PEs) were calculated. PE was defined as the percent of viable protoplasts which formed cell colonies 21 days after plating. Three days after initial plating, the number of viable cells was counted. Small cell colonies were counted after 21 days; thereby, the percent of viable cells forming colonies was determined:

$$PE = \frac{\text{number of cell colonies}}{\text{number of colonies + number of live cells}} \times 100$$

Shoot regeneration, rooting and transfer to the greenhouse. Macroscopic callus, about 2.0 mm in diameter, was transferred to solid MS medium containing test growth regulators. All cultures were maintained at 25±2°C and 15–20 $\mu Em^{-2}s^{-1}$ (400–700 nm; G.E. F96T12.CW) on a 16 hr photoperiod. Regenerated shoots 1 cm or taller were excised from the callus and transferred to MS + 3.0% sucrose, 0.8% agar, pH 5.8 or 3.0% sucrose, 0.1 mg/liter naphthaleneacetic acid (NAA) and 0.35% agar to induce root formation. Rooted plants were transferred to VSP (Bay Houston Towing Co.) soilless planting medium in 6/8 Cell Paks (Geo. J. Ball, Co.) and covered with clear polyethylene to maintain high humidity. Plants were kept under 74–84 $\mu Em^{-2}s^{-1}$ (400–700 nm) on a 16 hr photoperiod and gradually exposed to ambient relative humidity in 10–20 days. Subsequently, they were transferred to the greenhouse where, under standard cultural and fertilization practices, flowering occurred.

Results and Discussion

Driselase (2.5%) plus 4.0 to 4.7% mannitol or sorbitol in CPW routinely released 8.0×10^5 to 1.5×10^6 protoplasts per fresh weight of leaf tissue from *N. alata*. A mixture of 1.0% Driselase, 1.0% Macerozyme R-10, 1.0% cellulase "Onozuka" R-10, 0.5% potassium dextran sulfate and 4.0% mannitol in CPW was found most effective, releasing 7.6×10^5 to 8.5×10^5 and 4.0×10^5 to 1.0×10^6 protoplasts per g of leaf tissue for *N. sanderae* and *N. forgetiana*, respectively. Since shoot tips were grown under sterile culture conditions and a routine isolation procedure was followed, the range of protoplast yields for each species may be attributed to the physiological variability that existed in the leaf tissues (12). The majority of liberated protoplasts were derived from palisade and mesophyll cells; other protoplasts were of epidermal origin (Fig. 1-A).

The majority of protoplasts were spherical and their chloroplasts were evenly distributed at the periphery of the plasmalemma. FDA staining confirmed the integrity of the plasmalemma and thus, protoplast viability. Damaged protoplasts appeared broken, collapsed and had irregular or severely polarized chloroplast configurations, distended plasmalemma, and did not fluoresce with FDA but rather stained red.

A 3–5 hr leaf incubation in Driselase 2.5%, 4.0–4.7% mannitol or sorbitol in CPW solution resulted in less damage to protoplasts, as determined by visual assessment, than did tissue incubated for longer durations using enzyme mixtures of lower concentrations (0.05 to 2.0% Driselase). Thus, a short incubation period was preferred, decreasing the toxic effects often resultant after long periods of enzyme exposure (4). Potassium dextran sulfate was found beneficial for *N. forgetiana* and *N. sanderae* probably due

Table 1. Culture media tested for the initiation and sustained cell division of *Nicotiana* leaf mesophyll protoplasts.

Basal medium	Ref No.	Growth regulator (mg/liter) NAA	2,4-D	BA
MS	13			
P-I		2.0	–	0.5
PD		1.5	0.5	1.0
35		5.0	–	0.5
MS-G[w]	13			
I		3.0	–	1.0
II		2.0	–	0.5
III[x]		–	–	1.0
IV[y]		–	1.0	0.5
V		0.5	1.0	0.5
F_5	6	2.0	–	1.0
NT	14	3.0	–	1.0
UM[z]	21	0.6	–	–
B-5	7			
P-I		2.0	–	0.5
R-5		–	1.0	0.5

[w]MS salts and vitamins + (mg per liter): 1000 meso-inositol, 2.0 thiamine HCl, 250.0 L-glutamine, 0.1 L-serine, 30,000 sucrose.
[x]+ IAA 2.0 mg/liter
[y]+ IAA 0.5 mg/liter
[z]+ Kinetin 0.1 mg/liter

Fig. 1. (facing page) Regeneration of leaf mesophyll protoplasts of *Nicotiana alata*. A. Freshly isolated protoplasts. 390×. B. First cell division, 3 days after plating. 1500×. C. Second division, approx. 10 days. 1500×. D. Small cell colony stage after 21 days. 352×. E. Callus obtained after 4–5 weeks. 320×. F. Shoot regeneration from callus obtained after 2 months, actual size. G. Rooted plantlet, actual size. H. Small plants ready for transfer to greenhouse conditions after approx. 4 months, actual size. Similar stages were observed for *N. forgetiana* and *N. sanderae*.

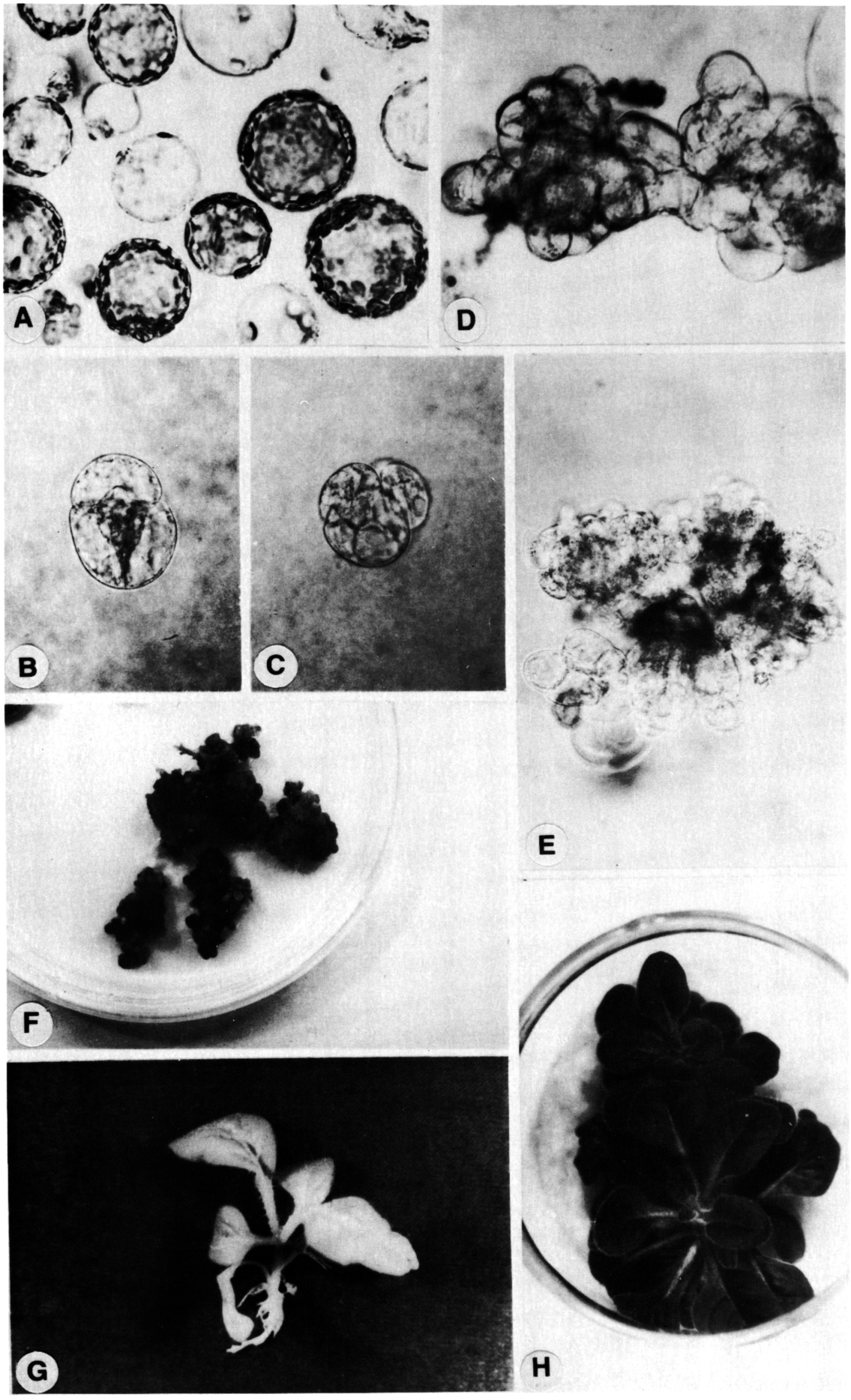

Table 2. Plating efficiencies for 3 species of *Nicotiana* at 3 densities and cultured under 28-34 $\mu Em^{-2}s^{-1}$ Gro-Lux light.

Species	Medium	Plating efficiency[z] (%) Density/ml 2.5×10^4	5.0×10^4	1.0×10^5
N. alata	MSP-I	0	32	31
	NT	0	37	34
	MS G-1	–	15	–
	MS G-II	–	0	–
N. sanderae	MS P-I	0	32	36
	NT	0	30	32
	MS G-I	–	0	–
	MS G-II	-	32	–
N. forgetiana	MS P-I	0	13	17
	NT	0	33	34
	MS G-I	–	0	–
	MS G-II	–	0	–

[z]PE = [no. of cell colonies/(no. of colonies + no. live cells)] x 100

to a protective effect on the plasmalemma which further aided in increasing the number of intact protoplasts of higher quality as revealed by FDA (16). Takebe and coworkers (20) suggested that macerozyme contains protein(s) toxic to tobacco cells and that dextran sulfate protects protoplasts from damage by blocking their action. Such polyanions are known to electrostatically bind protein and thus inhibit the enzymatic activity of various basic proteins (1).

Preplasmolyzing leaf tissue in CPW + mannitol prior to enzyme treatment did not influence the quality or quantity of protoplasts released. Tobacco and soybean mesophyll cells isolated from greenhouse grown leaf tissue were obtained without preplasmolysis but 0.6 or 0.3 M sorbitol was included in the maceration solution (5). High photosynthetic rates were found and thus, exposure to a plasmolysing agent appears not to be a general requirement.

A developmental sequence was observed in the protoplasts that was common to the 3 *Nicotiana* species following isolation (Fig. 1). Similar sequences have been reported in other species (3, 6, 15, 17). At 24 hr after plating, protoplasts became slightly enlarged and chloroplasts were polarized toward one end or rearranged unevenly throughout the cytoplasm. Subsequently, cells became ovoid and chloroplasts appeared to decrease in number. Changes in shape were indicative of cell wall synthesis (17) and evidence of cytoplasmic streaming and chloroplast degradation suggested the onset of cellular dedifferentiation. At 72 hr after plating, systrophy, rosetting of chloroplasts around the nucleus occurred, cells became reniform and first division began (Fig. 1-B) followed in about 8–9 days by second division (Fig. 1-C).

Precise determination of PEs was impeded by the strong aggregation of non-dissociable protoplast clumps in the culture medium; thus, PEs could only be approximated (17, 18). Clumping was possibly related to the quantity of calcium ions present from $CaCl_2 \cdot 2H_2O$ which could neutralize the net negative surface charge on protoplasts. Thus, aggregation occurred through attractive and constant van der Waals forces (14). The least aggregation, evaluated visually, was observed in B5 medium (150 mg/liter $CaCl_2 \cdot 2H_2O$) and the greatest amount was observed in MS, MS-G, UM (440 mg/liter $CaCl_2 \cdot 2H_2O$) and F5 (850 mg/liter $CaCl_2 \cdot 2H_2O$) media. PE did not appear to be related to degree of protoplast aggregation.

Protoplast survival 3 days after plating ranged from 47–78%, and dividing cells formed small colonies (20–30 cells) in about 21 days (Fig. 1-D). Colonies subsequently produced callus (Fig. 1-E). Occasionally, a small portion of the colonies suffered osmotic shock upon mannitol reduction, became necrotic and died. Shepard and Totten (18) suggested that an unreasonably high uptake of organic and inorganic substances resulting from the reduced osmoticum produced a toxic effect on potato colonies. Cellular ribonuclease levels are increased when cells suffer osmotic shock and thus, RNA degradation is thought to occur under conditions of lower mannitol concentrations (11); hence, injuring colonies. PEs of the 3 species, at 2 densities (Table 1), 5.0×10^4 to 1.0×10^5, for *N. sanderae* and *N. forgetiana* were comparable. However, the diffusion of beneficial substances by protoplasts and presumed necessary for division and growth, possibly became deficient at 2.5×10^4/ml density (8, 15). Alternatively, at 2.5×10^4 the concentration of salts may have been sufficiently high that toxic effects occurred (18). After 3 weeks, protoplast cultures were divided in half and fresh medium with a reduced osmoticum was added. Gleba (8) reported that preculturing protoplasts for 1–3 days at 1.0×10^4 — 1.0×10^5 protoplasts/ml significantly improved the PE of tobacco. Preculturing protoplasts in a "conditioned" medium could supplant the need for nutrient enriched medium. A density of 5.0×10^4 protoplasts/ml permitted a constant growth rate throughout the early stages of development and was used routinely in screening other media. Protoplasts of all 3 *Nicotiana* species divided to form macroscopic callus in MSP-I and NT media (Table 2). However, only MS G-I supported the growth to callus of *N. alata*; whereas only MS G-II promoted division and growth of *N. sanderae* (Table 2). MS G-I is identical to NT medium for growth regulators, has the same salts as MS P-I, but differs from the latter by containing 1.0g meso-inositol, 2.0 mg/liter thiamine·HCl, 250.0 mg/liter L-glutamine and 0.1 mg/liter L-serine, and lacks NH_4NO_3. *N. sanderae* and *N. forgetiana* remained viable for 7–10 days in MS G-I, but they did not divide. MS G-II contains the same salts, vitamins, growth regulators as MS G-I, but the concentration of the latter is lower (Table 1). Thus, it appears that the ability to tolerate lower ammonium levels and the amino acids, glutamine and serine, in MS G-I medium may have been contributed to *N. sanderae*, by its original *N. alata* parent. The capacity of *N. sanderae* to grow in MS G-II medium whereas neither of its ancestral parents did, suggests that gene complementation in the hybrid permits division and growth. Izhar and Power (9) proposed that several genes are responsible for and exert control over the progressive stages of protoplast development *in vitro*. Genetic complementation of the parental genomes in hybrids thus could activate all required genes and result in a complete growth cycle.

Protoplasts exposed to Gro-Lux (GL) irradiance were found to enter first division 1–2 days earlier than those exposed to other light sources. Beneficial responses have been reported for tobacco protoplast cultures using GL of 1000 lux (22). We found that GL was promotive only for *N. sanderae* compared to CW and the effect was even lower than that obtained by dark(D) and culture in NT medium (Table 3). Lighting conditions did not influence the PEs of *N. alata* or *N. forgetiana*. Transferring cultures from D to GL was tried only with *N. sanderae* and the resultant PE did not exceed that in GL alone as has been shown for *N. tabacum* (12).

Two media were selected for shoot regeneration based on earlier experiments (unpublished data) employing callus derived from leaf sections of the three *Nicotiana* species. Prolific shoots, 5–10 per callus, occurred on both MS + 1.0 mg/liter Z and MS + 1.0 mg/liter Z and MS + 1.0 mg/liter BA + 2.0 mg/liter IAA for all 3 species except for *N. forgetiana* on the latter. No variation in

Table 3. Plating efficiencies (%) of 3 *Nicotiana* species in division media under 3 light regimes; density 5.0×10^4 protoplasts/ml.

Species	Medium	Plating efficiency (%) Dark (D)	Light regime Gro-lux (GL) 28-34 $\mu Em^{-2}s^{-1}$ (400-700 nm)	Light regime Cool white (CW) 18-22 $\mu EM^{-2}s^{-1}$ (400-700 nm)
N. alata	NT	34	37	30
	MS P-I	22	32	28
	MS G-I	14	15	15
N. sanderae	NT	48	30	16
	MS P-I	27	32	24
	MS G-II	33	32	17
N. forgetiana	NT	32	33	35
	MS P-I	12	13	12

shoot regenerative ability was noted relative to the protoplast culture medium in which the callus was derived. Shoot nodules appeared in 3–4 weeks and following reculture were visible in another 2–3 weeks (Fig. 1-F). Rooting (100%) occurred within 6–10 days when regenerated shoots were inserted in MS + 3.0% sucrose, 0.8% agar or 3.0% sucrose, 0.1 mg/liter NAA and 0.35% agar (Fig. 1-G, H). Sufficient levels of naturally occurring auxin may have been present in the regenerated shoots as exemplified by a high rate and efficiency of rhizogenesis.

Within 5 months of protoplast isolation, regenerated plants at anthesis were assessed for pollen viability and seed set. Seeds were produced on *N. alata* through sib-pollinations. Regenerated plants displayed a slight decrease (75%) in viable pollen (aniline blue) when compared with seed grown plants (89%). Overall, regenerated plants appeared morphologically identical to seed grown plants. Upon careful examination, however, split corollas, flower size variations or slight flecking of the petals was apparent, indicating that genetic changes may have occurred. The successful regeneration of isolated protoplasts from these three ornamental *Nicotiana* spp. now permits their use in various somatic cell genetic manipulations.

Literature Cited

1. Bernfeld, P. 1963. Polyanionic inhibitors. p. 437–472. In: R. M. Hochester and J. H. Quastel (eds.) Metabolic Inhibitors 2. Academic Press, New York.
2. Binding, H. 1975. Reproducibly high plating efficiencies of isolated protoplasts from shoot cultures of tobacco. Physiol. Plant. 35:225–227.
3. Bourgin, J.-P., Y. Chupeau, and C. Missonier. 1979. Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. Physiol. Plant 45:288–292.
4. Cassels, A. C. and M. Barlass. 1976. Environmentally induced changes in the cell walls of tomato leaves in relation to cell and protoplast release. Physiol. Plant. 37:239–246.
5. Cataldo, D. A. and G. P. Berlyn. 1974. An evaluation of selected physical characteristics and metabolism of enzymatically separated mesophyll cells and minor veins of tobacco. Amer. J. Bot. 61:957–963.
6. Frearson, E. M., J. B. Power, and E. C. Cocking. 1973. The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol. 33:130–137.
7. Gamborg, O. L., R. A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Expt. Cell Res. 50:151–158.
8. Gleba, Y. Y. 1978. Microdroplet culture: tobacco plants from single mesophyll protoplasts. Naturwissenschaften 65:158–159.
9. Izhar, S. and J. B. Power. 1977. Genetical studies with *Petunia* leaf protoplasts: I. genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci. Lett. 8:375–383.
10. Larkin, P. J. 1976. Purification and viability determination of plant protoplasts. Planta 128:213–216.
11. Lazar, G., G. Borbely, J. Udvardy, G. Premecz, and G. L. Farkas. 1973. Osmotic shock triggers an increase in ribonuclease level in protoplasts isolated from tobacco leaves. Plant Sci. Lett. 1:53–57.
12. Meyer, Y. 1974. Isolation and culture of tobacco mesophyll protoplasts using a saline medium. Protoplasma 81:363–372.
13. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
14. Nagata, T. and G. Melchers. 1978. Surface charge of protoplasts and their significance in cell-cell interaction. Planta 142:235–238.
15. Nagata, T. and I. Takebe. 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20.
16. Otsuki, Y. and I. Takebe. 1969. Isolation of intact mesophyll cells and their protoplasts from higher plants. Plant & Cell Physiol. 10:917–921.
17. Pelcher, L. E., O. L. Gamborg, and K. N. Kao. 1974. Bean mesophyll protoplasts: production, culture and callus formation. Plant Sci. Lett. 3:107–111.
18. Shepard, J. F. and R. E. Totten. 1975. Isolation and regeneration of tobacco mesophyll cell protoplasts under low osmotic conditions. Plant Physiol. 55:689–694.
19. Sink, K. C. and J. B. Power. 1978. Incongruity of interspecific and intergeneric crosses involving *Nicotiana* and *Petunia* species that exhibit potential for somatic hybridization. Euphytica 27:725–730.
20. Takebe, I., Y. Otsuki, and S. Aoki. 1968. Isolation of tobacco mesophyll cells in intact and active state. Plant & Cell Physiol. 9:115–124.
21. Uchimiya, H. and T. Murashige. 1974. Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells. Plant Pysiol. 54:936–944.
22. Uchimiya, H. and T. Murashige. 1976. Influence of the nutrient medium on the recovery of dividing cells from tobacco protoplasts. Plant Physiol. 57:424–429.
23. Vasil, I. K. and V. Vasil. 1980. Isolation and culture of protoplasts. p. 1–19. In: I. K. Vasil (ed.) Perspectives in plant cell and tissue culture. Suppl. 11B. Intern. Rev. Cytol. Academic Press, New York.