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TISSUE CULTURE AND CALLUS PROTOPLAST REGENERATION OF SALPIGLOSSIS SINUATA L.

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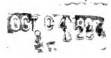

TISSUE CULTURE AND CALLUS PROTOPLAST REGENERATION OF *SALPIGLOSSIS SINUATA* L.

By

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ABSTRACT

TISSUE CULTURE AND CALLUS PROTOPLAST REGENERATION OF SALPIGLOSSIS SINUATA L.

BY

Charlene Jan Boyes

Leaf explants were aseptically cultured on Murashige and Skoog (MS) medium plus 2ip, K, BA, NAA, IAA, and 2,4-D singly or NAA and BA combinations (0.01, 0.1, 1.0, 5.0, 10.0 mg/l). Adventitious shoots arose at all MS + BA, 2ip, or K levels except at 0.01 mg/l. Callus and roots occurred on MS + NAA or IAA except at 0.01 mg/l IAA. Optimum shoot regeneration occurred from leaf callus plated on MS + 1.0 mg/l 2ip. Protoplasts were enzymatically isolated from friable callus. Continuous division to the macroscopic callus stage was obtained in a liquid MS medium modified by the deletion of ammonium ions and adding 250 mg/l glutamine, 0.1 mg/l serine, 2.0 mg/l thiamine·HCL, 0.5 mg/l IAA, 1.0 mg/l 2,4-D, 0.5 mg/l BA and 9% (w/v) mannitol. Small colonies formed callus within 2.5 months and the latter readily regenerated shoots on MS + 1.0 mg/l 2ip. High frequency rooting occurred on MS + 0.001 mg/l 2,4-D.

To Jim

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INTRODUCTION

The elicitation of morphogenetic responses through manipulation of selected explants cultured *in vitro* has been demonstrated in many plant species of economic importance (Binding, 1973; Murshige, 1974; Kartha et al., 1976). Likewise, organogenesis has been reported from a variety of explants including stem pieces (Skoog, 1944; Murashige and Skoog, 1962; Rao et al., 1973) roots (Levine, 1950; Norton and Bell, 1954) anthers (Guha and Maheshwari, 1964; Hughes et al., 1973) and embryos (Masheshwari and Baldev, 1962). Trends in somatic cell manipulation strongly suggest the potential for crop improvement through protoplast technology. A favored source of protoplasts for conducting cellular manipulation is the mesophyll tissue of leaves. Leaf protoplasts can generally be obtained with relative ease and in high yields with apparent uniformity. Regeneration, from callus derived from either explants or protoplasts, may be determined through examination of hormonal control of morphogenesis on leaf explants (Frearson et al., 1973; Kartha et al., 1976). Leaf explants have been used for morphogenetic study in *Nicotiana* (Gupta et al., 1966) and in *Lycopersicon esculentum* (Padmanabhan et al., 1974). Anthers and leaf discs of *Salpiglossis sinuata* L. have been cultured and plants regenerated from the resulting callus

(Hughes et al., 1973; Lee et al., 1977). Both reports indicated difficulty in root initiation of the regenerated shoots. However, a comprehensive study of the morphogenetic response to phytohormones in various combinations and concentrations, especially concerning rooting, has not been concluded. This inquiry was undertaken to determine 1) The morphogenetic response of Salpiglossis sinuata L. leaf explants to various phytohormones in vitro, both singly and in combination; and 2) to determine an efficient method of root initiation for regenerated shoots.

MATERIALS AND METHODS

Salpiglossis sinuata L. seeds were sown on an artificial planting mix (VSP Bay Houston Towing Co) and germinated at 22°C ± 2°C under 16 hr photoperiod at 74-84 $\mu Em^{-2}s^{-1}$ cool white tubes (General Electric). In two to three weeks, the seedlings were grown under a minimum temperature regime of 22°C night and 25°C day or higher, depending on the season. Supplemental lighting of 550 $\mu Em^{-2}s^{-1}$, cool white fluorescent tubes (General Electric) for for 16 hr coinciding with the natural daylight was used. Fertilizer solution was applied to the seedlings at a rate of 200 ppm N twice weekly using 20-20-20. Disease and insect measures were applied as needed. Basal leaves for in vitro culture were obtained from the seedlings when they were 60-90 days old.

Leaves were surface sterilized in a 5% solution of

commercial sodium hypochlorite (Clorox) for 30 min followed by six sterile distilled water rinses. Rectangular leaf pieces, 1 x ½cm, were aseptically excised from the leaves; the midrib and leaf margins were removed.

The leaf explants were cultured on Murashige and Skoog (1962) macro- and micro-elements (MS), 3% sucrose, 0.8% agar (Sigma, Grade IV). Auxins added to the basal medium included 2,4- dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA) and napthalene acetic acid (NAA) and the cytokinins 6-benzylaminopurine (6-BAP), 6(Y-Y dimethylally amino) purine (2ip), 6 furfurylamino purine (K). In some experiments, the medium of Uchimiya and Murashige (1974) (UM) was employed. The pH of all media was adjusted to 5.8 with 0.2N NaOH or 0.1N HCL. All media were autoclaved at 15 psi (1.46 Kg/cm^2) for 20 minutes. The two culture vessels employed were 60 ml glass jars with metal screw caps or 100 x15 mm Petri dishes containing 25 and 20 ml of media respectively. One explant was placed into each jar and four in each Petri dish. The culture vessels were placed under 74 to 84 $\mu Em^{-2}s^{-1}$, cool white fluorescent tubes (General Electric) with a 16h photoperiod and a temperature range of $23^oC \pm 2^o$. Each test medium was relicated eight times and each experiment was repeated at least twice. The leaf explants were routinely subcultured every four weeks. Each leaf piece was visually evaluated five weeks after initiated of the experiment for morphogenetic response(s).

RESULTS AND DISCUSSION

The effect of cytokinins on morphogenesis - the morphogenetic response of leaf explants plated on MS + BA, K or 2ip is presented in Table 1. The results on MS + K and 2ip were essentially the same although they occurred at different concentrations. Shoots were observed at 1.0 mg/l and 5.0 mg/l K and at 0.1 mg/l and 1.0 mg/l 2ip. The biological activity (stimulation of cell division) of 2ip has been reported as being ten times greater than K (Helgeson, 1968). Concentrations above or below these levels resulted in either poor shoot development, spindly leaves or only callus. Kartha et al. (1976) found similar results on Lycopersicon esculentum cv Starfire, though no callus growth occurred at the lowest cytokinin concentrations. Rao et al. (1973) obtained shoot development with BA, K or zeatin on leaf explants of Petunia inflata. At the lowest levels of 2ip, K and BA, 0.01 mg/l, callus growth was induced, but it was slow in developing; pale green or brown in color, indicating suboptimal conditions. A purple pigment, probably anthocyanin, developed in the 0.01 mg/l K callus. Isolated incidents of pink callus were also observed, but profuse development of pink, purple callus indicated a possible stress situation. The stress may have been due to a suboptimum cytokinin level; one barely sufficient to induce and support cell division. On MS + BA and MS + 2ip (0.01 mg/l) occasionally small roots developed. This response may indicate a ratio of exogenous cytokinin to

Table 1. Effect of cytokinins on morphogenesis of *Salpiglossis* leaf explants.

Cytokinin	(mg/l)	Response	
BA	0.01	pale green callus	occasional root
	0.1	pale green callus	occasional root spindly leaves
	0.1	callus	shoots with spindly leaves development slow
	5.0	callus	few shoots
	10.0	callus	shoot primorida only
K	0.10	callus, purple	no shoots or roots
	0.1	callus, green	few shoots; development slow
	1.0	callus	multiple shoots
	5.0	callus	multiple shoots
	10.0	callus	few shoots, development suppressed
2ip	0.01	callus, pale green	very few roots
	0.1	callus	multiple shoots, development slow
	1.0	callus	multiple shoots
	5.0	callus compact	shoots
	10.0	callus compact	very few shoots, development suppressed

endogenous auxin favorable to root initiation. High levels of endogenous auxins have been reported in other Solanaceous plants (Kartha et al., 1976); in the case of Salpiglossis auxins may be low since a high level of cytokinin was not necessary to initiate roots. MS + BA as compared to MS + K and MS + 2ip was much less effective in eliciting morphogenetic responses. Shoot differentiation occurred, but elongation and normal development was inconsistent at the 1.0 mg/l level. BA is known to be less active than other structurally related cytokinins in promoting cell division (Haberlach et al., 1978), an important factor in callus growth and morphogenesis.

Effect of auxins on morphogenesis - all auxins tested induced callus growth except for IAA at 0.01 mg/l indicating it did not induce division in the leaf cells at as low a concentration as 2,4-D and NAA (Table 2). Callus induced at this level 0.01 mg/l NAA and 2,4-D, grew slowly; NAA induced callus was also quite compact. Callus induced on 0.01 mg/l 2,4-D was also compact in comparison to callus induced at higher 2,4-D levels. However, 2,4-D induced callus was not nearly as compact as that induced on NAA. Induction of callus involves the initiation of cell division and proliferation concomitant with dedifferentiation (Gresshoff, 1978). Apparently IAA at 0.01 mg/l cannot activate leaf callus to begin this process. Both NAA and 2,4-D are considered much more potent auxins and are also longer lasting than IAA (Murashige and Skoog, 1962). Thus, Salpiglossis shows

Table 2. Effect of auxins on morphogenesis of *Salpiglossis sinuata* L. leaf explants

Auxin	(mg/l)	Response
IAA	0.01	No growth
	0.1	callus, green very few roots
	1.0	callus, green/pink very few roots
	10.0	callus, green few roots
2,4-D	0.01	callus pale green
	0.1	callus pale green
	1.0	callus yellow soft
	10.0	callus pale yellow
NAA	0.01	callus green very few roots
	0.1	callus few roots
	1.0	callus multiple roots
	5.0	callus few roots one shoot
	10.0	callus pale yellow few roots

specificity for auxin induced callus growth.

NAA at all levels and IAA at 10.0 mg/l resulted in roots. Root initiation from leaf explants on MS basal medium supplemented with IAA or NAA was also observed on Petunia inflata and P. hybrida (Rao et al., 1973). Root initiation and elongation is generally promoted by high auxin levels (Gresshoff, 1978). Rhizogenesis at lower concentrations of NAA may be due to its higher level of activity as compared to IAA. Vasil and Vasil (1974) reported root formation at low NAA concentrations for Petunia callus.

All 2,4-D concentrations produced only callus. 2,4-D is widely accepted to be a suppresser of organ differentiation, especially at high concentrations (Sunderland, 1973; Gresshoff, 1978). Strong morphogenetic suppression would also result in the very slow callus growth that was evident on the leaf explants. In Petunia (Rao et al., 1973), soft friable callus formed from leaf explants and only occasional development of roots occurred on MS + 1.0 mg/l 2,4-D; later embryogenesis occurred but at a suppressed rate compared to IAA or NAA.

Effect of Benzyladenine + Naphthaleneacetic acid on Morphogenesis - Some callus formation occurred on all combinations of MS + BA + NAA (Table 3). Shoot primordia developed on 10.0 mg/l BA + 1.0 mg/l NAA or 0.1 mg/l NAA; on 1.0 mg/l BA + 0.1 mg/l NAA resulted in multiple shoot development. Salpiglossis leaf segments cultured at similar concentrations but substituting K for BA gave comparable

Table 3. Morphogenetic response of Salpiglossis sinuata leaf explants cultured on BA and NAA combinations.

Phytohormones (mg/l)		Response	
BA	+ NAA		
10	10	nodulated callus,	no shoots or roots
10	1.0	callus	shoot primordia
10	0.1	callus	shoot primordia
1.0	10	callus	no shoots or roots
1.0	1.0	callus	multiple shoots
1.0	0.1	callus	multiple shoots
0.1	10	nodulated callus	few roots
0.1	1.0	callus	few shoot primordia
0.1	0.1	callus	no shoots or root

results (Lee et al., 1977). The development of shoot primordia and shoots at these phytohormone levels may be attributed to the relatively high cytokinin to auxin ratio.

At other levels of both hormones, either roots or callus alone were produced, resulting in different responses from low to high concentrations. Callus induced on the combinations of MS + BA + NAA was not superior in growth rate or quality to that induced on MS + BA or MS + NAA alone. Engvild (1973) found similar results in Datura. Synergism between auxin and cytokinin has been observed in tobacco tissue cultures (Murashige and Skoog, 1962). In this study, interaction between auxin and cytokinin was not an absolute requirement for shoot initiation; shoots occurred on the NAA + BA combinations and also on BA alone (Table 1). Roots and shoots did not develop on the same concentration of BA + NAA. The greatest obstacle in obtaining regenerated plants from Salpiglossis was in the rooting of shoots. To achieve efficient rooting ten MS based media were tested (Table 4). In addition, temperature fluctuations from 20°C to 25°C, rooting directly in an artificial planting mix and varying light levels from 11 to 80 $\mu Em^{-2}S^{-1}$ were utilized.

In Datura the spontaneous tendency for shoot formation is very pronounced; root formation is difficult (Engvild, 1973). Salpiglossis behaved in a similar manner. Kartha et al. (1976) using Lycopersicon esculentum shoots found rooting to occur on MS basal medium without phytohormones. With Salpiglossis however, rooting did not exceed 40% on this

Table 4. Root induction of regenerated shoots of Salpiglossis sinuata in MS basal medium with different addenda.

Medium (mg/l)	Light $\mu Em^{-2}s^{-1}$	% Rooting
NAA (1.0) Agar (0.35%)	11	20
NAA (0.1) Agar ().35%)	20	30
NAA (0.1) Agar (0.35%)	80	30
No hormones 20°C 3 days to 24°C	20	0
No hormones	20	20
No hormones	80	40
No hormones 1.2% Sucrose	20	33
No hormones 0.5% Sucrose	20	33
No hormones 0.4% Agar	74	33
No hormones 0.4% Agar 20°C, 3 days to 24°C	20	40
2,4-D (5.0) BA (2.5)	15	0
2,4-D (5.0) BA (2.5)	74	0
Charcoal (1%) NAA (1.0)	20	15
Charcoal (2%) NAA (1.0)	20	0
Charcoal (1%)	20	0
2,4-D (0.001)	15	25
2,4-D (0.001)	20	40
2,4-D (0.001)	80	75
Soil	80	25

The mean percentage rooting values are from 8 plates; all experiments repeated at least twice.

medium (Table 4). With Solanum xanthocarpum, roots developed on levels of 0.2 mg/l and 0.1 mg/l 2,4-D (Rao and Narayanaswami, 1968). Low levels of 2,4-D (0.001 mg/l) gave the best rooting (75%) of any test treatment of the Salpiglossis shoots.

Leopold (1964) suggested root initiation results when auxin levels increase at potential active meristematic sites on stems. The inability to readily form roots may be related in part to some type of abnormal auxin metabolism or transport. Ahuja and Hagen (1966) cultured Nicotiana tumor tissue and found it unable to root, although growth was not impaired. They suggested an inability to mobilize the auxin properly for the initiation of roots as the probable cause. Inability to perceive the exogenous auxin at the auxin receptor site could also be a cause for lack of rooting.

Generally speaking, the more vigorous Salpiglossis shoots rooted with more ease than less vigorous ones. There is much heterogeneity in Salpiglossis as is evidenced in the variable flower color and pollination habit (Lee et al., 1978); this situation may have confounded the rooting response(s).

SUMMARY

Callus and shoots were readily initiated on leaf explants of Salpiglossis sinuata cultured in vitro. Of the cytokinins tested, 2ip at 1.0 mg/l was most effective in initiating shoot regeneration on leaf sections and on callus. Culturing leaf explants on MS with combinations of BA and NAA did not enhance shoot initiation or development above that obtained with BA, 2ip or K alone. Rooting occurred with difficulty which may be attributed to:

1) an inability to mobilize endogenous auxins to the site of root initiation, or
2) lack of recognition at the receptor site for auxin.

Regenerated plants were grown to flowering in the greenhouse and were phenotypically identical to the parental line.

INTRODUCTION

The potential for crop improvement through protoplast technology has been widely discussed (Evans and Cocking, 1975; Vasil, 1976; Thomas et al., 1979) and significant advances have been made in the last decade (Takebe et al., 1971; Carlson et al., 1973; Hess et al., 1976; Vasil and Vasil, 1979). These advances in _in vitro_ methodology and their eventual application are intended to make significant contributions in plant breeding. An essential requirement for the use of protoplast technology is uniform, large populations of isolated protoplasts which can be cultured to callus and subsequently regenerated into plants. Leaves (Durand et al., 1973; Kartha et al., 1976; Shepard and Totten, 1977; Sink and Power, 1977) and callus (Power and Berry, 1979; Simmonds et al., 1979; Vasil and Vasil, 1979) and cell suspension cultures (Grambow et al., 1972; Keller et al., 1973; Kartha et al., 1974) have all proven to be good cell sources for protoplasts and have also yielded regenerated plants.

To date the vast majority of plant species that have been successfully regenerated from isolated protoplasts are food, drug and ornamental crops in the _Solanaceae_ family. In _Petunia_, five species have been regenerated from protoplasts (Frearson et al., 1973; Binding, 1974; Hayward and

Power, 1975; Sink and Power, 1977). Regenerating systems have also been developed for Atropa (Gosch et al., 1975; Krumbeigel and Schieder, 1979) Browallia, (Power and Berry, 1979) Datura, (Krumbeigel and Schieder, 1979) Lycopersicon, (Zapata et al., 1976) and Solanum (Binding and Nehls, 1977; Shepard and Totten, 1977).

A review of the methodologies used in achieving regeneration with these species clearly indicates that several prime factors regulate the in vitro responses.

One of the most important factors in obtaining uniform, large populations of isolated protoplasts is the physiological condition of the source tissue or plant. The susceptibility to digestion of the cell wall and the stability of the protoplasts are highly affected by the physiological condition prior to isolation (Uchimiya and Murashige, 1974; Shepard and Totten, 1975). Therefore, temperature and illumination as well as age and nutrition are parameters that must be determined experimentally for the species under study. Enzymatic methods of isolation, while eliminating the disadvantages inherent in mechanical methods, however, leave the protoplasts exposed for long periods of time to a variety of exogenous and endogenous enzymes. Osmotic potential can have a great deal of influence over the recovery of viable or nonviable protoplasts (Schenk and Hildebrandt, 1969). Thus, an assessment of the plasmolysis as well as pH, presence of Ca^{2+}, temperature and duration of the enzyme treatment is essential (Vasil, 1976).

Studies on protoplasts isolated from roots, fruits and leaves have indicated a strong and direct effect of auxins. Increased uptake of water, expansion and bursting when auxin is added indicate an effect on the membrane permeability (Gregory and Cocking, 1965; Vasil, 1976). Phytohormones, especially cytokinins and auxins, influence the ability of protoplasts to undergo division (Bui Dang Ha and MacKenzie, 1973; Evans and Cocking, 1975; Gamborg et al., 1975). Auxins are exceptional for rhizogenesis, whereas cytokinins influence initiation of shoots and have some control of cell division (Haberlach et al., 1978). Media components, organic and inorganic, also exert control over the behavior of the cell (Gamborg, 1970; Bui Dang Ha and MacKenzie, 1973; Upadhya, 1975; Shepard and Totten, 1977).

The morphogenetic potential of leaf explants of Salpiglossis sinuata has been determined. Hughes et al. (1973) derived callus from Salpiglossis anthers which differentiated into plantlets. Lee et al. (1977), utilizing Salpiglossis leaf discs and a more extensive range of hormones, reported callus, shoot and root initiation on NAA and K in various combinations. It was also reported that leaf age seemed to be a limiting factor in obtaining organogenesis. Neither of these studies reported greater than 60% rooting of the regenerated shoots.

Salpiglossis is a promising species for somatic hybridization; therefore, this investigation was undertaken to determine the potential for regeneration of protoplasts,

isolated from leaves or from callus, through manipulation of phytohormones, medium constituents and environmental factors.

MATERIALS AND METHODS

Plants for providing leaf explants as a source of callus cells for protoplast isolation were obtained from seeds sown at weekly intervals on artificial planting mix (V.S.P., Bay Houston Towing Co.) and maintained at 21^{o}C ±2^{o}C, 64 $\mu Em^{-2}s^{-1}$ cool white fluorescent tubes (General Electric) for 16 hr per day. After 2.5 weeks growth the seedlings were transplanted to artificial planting mix in plastic cell packs and placed in the greenhouse. Vegetative growth continued under natural photoperiod supplemented with a total of 550 $\mu Em^{-2}s^{-1}$ for 16 hr each day. A minimum night temperature of 22^{o}C±2^{o}C was maintained; daytime temperature fluctuated with the season. The plants were fertilized three times weekly at a rate of 200 ppm N from 20-20-20. Control of diseases and insects was according to standard cultural procedures.

Experimental plant material was also obtained from aseptically germinated seed. Shoots, 7 mm in height, from axenic cultures were excised from two-week old seedlings and cultured on Murashige and Skoog (1962) medium (MS) and Gamborg's B5 (Gamborg et al., 1968) in 60x15 mm Petri dishes. Axenic, single stem shoot cultures were maintained at 27^{o}C with a 16h photoperiod, 24 $\mu Em^{-2}s^{-1}$ from cool white

fluorescent tubes (General Electric).

Fully expanded leaves were obtained from the lower portion of seedlings 60 to 90 days after germination. They were surface sterilized with a 7% commercial sodium hypochlorite solution (5.25% NaOCl) for 30 min followed by six separate rinses with sterile distilled water. Seeds for axenic culture were surface sterilized with a 10% sodium hypochlorite solution for 30 min followed by eight separate sterile distilled water rinses. These seeds were then aseptically transferred to modified Murashige and Skoog (1962) medium in 60x15 mm Petri dishes. Germination proceeded at 27°C, 24 $\mu Em^{-2}s^{-1}$ cool white fluorescent tubes (General Electric).

Leaves were allowed to become flaccid after sterilization, and the lower epidermis was removed by peeling with the aid of jewelers' forceps. The exposed surface was placed down in a 100x15 mm Petri dish containing a cell/protoplast wash solution (Frearson et al., 1973) of 13% mannitol at a pH of 5.8. After 0.5 hr the CPW solution was removed and replaced with a solution of CPW salts and protoplast enzymes in various combinations and concentrations (Table 5). Axenic leaf tissue was removed, aseptically placed in a 60x15 mm Petri dish; sliced; then covered with an enzyme solution (Table 6).

Callus for protoplast isolation was gently pressed through a 35 μ sieve while rinsing with a CPW 8% mannitol solution. The cells were pelleted by centrifuging this

Table 5. Enzyme mixtures tested for the isolation of Salpiglossis sinuata L. leaf protoplasts.

Code	Enzyme Components
LA	2% Meicelase P,[v] 0.1% Macerozyme[w], 13% Mannitol, CPW
LC	2% Meicelase P, 0.08% Macerozyme, 13% Mannitol, CPW
LD	2% Meicelase P, 0.06% Macerozyme, 13% Mannitol, CPW
LE	2% Meicelase P, 0.05% Macerozyme, 13% Mannitol, CPW
LB	3% Meicelase P, 0.1% Macerozyme, 13% Mannitol, CPW
LBA	3% Meicelase P, 0.1% Macerozyme, 40mg Penicillian[x], 1mg Tetracycline[y], 1mg Gentamycin[z], 13% Mannitol, CPW

v Meiji Seika Kaisha
w Cal Biochem
x Sigma
y Sigma
z Sigma

Table 6. Enzyme mixtures tested for the isolation of Salpiglossis sinuata L. protoplasts from axenic shoot cultures.

Code	Enzyme Components
A1	2.5% Driselase[y], 4.5% Mannitol, CPW
A2	2.5% Driselase, o.2% Pectinase[z], 4.5% Mannitol, CPW

y Kyowa Hakko Kogyo
z Sigma

slurry at 80xg for 2 min. The supernatant was removed and the cells were resuspended in a test enzyme and CPW solution for protoplast release (Table 7).

The pH of all enzyme solutions was adjusted to 5.8 and they were sterilized by filtration through a .45 μ filter (Sybron or Satorius).

Incubation and isolation proceeded in three ways:

1) Leaf pieces from greenhouse grown material were held in the enzyme for 16 hr at 27°C in the dark. The enzyme was removed and replaced with a 21% (w/v) sucrose in CPW solution, placed in a 125x16 mm screw capped tube and centrifuged at 100xg for 10 min. The band of protoplasts that floated to the surface during centrifugation were resuspended in test medium.
2) The leaf pieces from axenic shoot cultures were incubated for 5 hr at 27°C in the dark. The released protoplasts were gently pelleted at 80xg for 2 min, the supernatant removed and the pellet resuspended in a 15% (w/v) sucrose CPW solution. The protoplasts were separated from undigested cellular material by centrifuging at 100 xg for 10 min. The resultant band at the solution surface was removed and placed in test culture medium.
3) Friable callus was incubated for 5 hr at 27°C on a rotary shaker at 50 rpm. The enzyme mixture plus released protoplasts was centrifuged at 80 xg for 2 min, the supernatant removed and replaced with 15%

Table 7. Enzymes tested for the isolation of *Salpiglossis sinuata* L. callus protoplasts.

Code	Enzyme Components
C	2% Cellulase R-10[z], 1% Macerozyme, 1% Driselase, 8% Mannitol, CPW
CM	2% Cellulase R-10, 1.4% Macerozyme, 1% Driselase, 8% Mannitol, CPW
4	1% Driselase, 0.5% Pectinase, 8% Mannitol, CPW
5	1% Driselase, 0.75% Pectinase, 8% Mannitol, CPW
CP	2% Cellulase R-10, 0.75% Pectinase, 1% Driselase, 8% Mannitol, CPW

[z]Kinki Yakult

(w/v) sucrose in CPW. Centrifugation proceeded at 100 xg for 10 min; the meniscus of protoplasts was collected and placed in test culture medium. All freshly isolated protoplasts were subsequently counted and diluted to a final density of $2.5x10^4$ to $2.0x10^5$/ml. The protoplasts were plated as 4 ml in 60x15 mm Petri dishes at 27°C, 28 $\mu Em^{-2}s^{-1}$ cool white tubes (General Electric) under a 16 hr photoperiod.

Media tested for protoplast division included Murashige and Skoog (1962), Uchimiya and Murashige (1974), a modified Durand's (1973), Nagata and Takebe (1971), Kao and Michayluk (1975), and Gamborg's B5 (Gamborg et al., 1968). Various combinations and concentrations of auxins and cytokinins were added to these media. Mannitol at 9% (w/v) was added to all liquid medium as the osmotic stabilizer. All water was double distilled in a Pyrex glass still and all media were autoclaved at 15 psi for 20 min. Four replicates of each test media were conducted.

Shoot tips from regenerated protoplasts were dipped in 1000 ppm indole-butyric acid (IBA) and placed in perlite. Under intermittent mist in the greenhouse, the shoot tips rooted in 12 to 14 days with 75% efficiency. Roots 1 to 2 cm long were removed and pre-fixed in a 0.1% solution of colchicine for 2.5 hr at 23°C in the dark. Fixing occurred overnight in a 3:1 absolute alcohol:glacial acetic acid solution, the tips were then stored in 70% ethanol at 4°C. Hydrolysis was in 1N HCl at 60°C for 11 min. The root tip

was smeared and stained with a 1% aceto-carmine solution (Darlington and LaCour, 1975). The number of root tip cells counted to establish the chromosome number was 8 to 10.

RESULTS AND DISCUSSION

Of the enzymes tested (Table 5) for protoplast release from leaf material, LA gave the lowest yield and concomitant low quality protoplasts as evidenced by their shrunken condition and emerging vacuoles. Variations of this enzyme mixture, LC, LD, LE gave better yields yet digestion was not always complete and the protoplasts were often damaged. The protoplasts were released but their vacuoles emerged through the plasma membrane and bursting occurred frequently.

Utilizing the same level of pre plasmolysis, LB and LBA gave infinitely better results. LB efficiently converted cells to protoplasts that were spherical with the chloroplasts evenly distributed about the cell periphery. However, bacterial contamination necessitated the use of antibiotics; enzyme LBA. Protoplasts obtained from this enzyme treatment were also spherical with even distribution of the chloroplasts. Within three days chloroplasts had moved to the center of the cell, cytoplasmic strands were evident. The antibiotics employed were not sufficient to overcome the apparent endogenous contamination of greenhouse grown leaf cultures. The bacterial contamination was persistent and could not be controlled by a standard

antibiotic regime.

Axenic shoot and callus cultures circumvented the contamination problem, but the former gave very poor protoplast yields. Enzyme A1 released a fair amount of spherical protoplasts. The yield was much greater though with enzyme A2; however, the vacuoles consistently emerged after 24 hr culture, apparently due to pectinase damage of the protoplasts. Pectinase is known to contain toxic basic proteins that could cause this problem (Evans and Cocking, 1975).

Callus was subsequently employed as the protoplast source. Enzyme C (Table 7) yielded spherical protoplasts with active cytoplasmic streaming; systrophy occurred within 14 hr. However, the released number of protoplasts from callus was very low. CM improved the yields only slightly. Enzymes 4 and 5 gave increased yields, but many were dead (brown in color) and shrunken. The protoplasts that survived displayed cytoplasmic strands and were spherical. A combined form of other test enzymes, CP converted callus to protoplasts at a rate of 6.0×10^5 from 1 g of callus tissue.

Testing of enzymes resulted in a combination (CP) that would allow the release of spherical intact protoplasts.

The effectiveness of an enzyme mixture may be attributed to three causes:

1) The callus may superficially appear to be uniform though it is not necessarily so. Timing of callus

subculture has an influence on the effectiveness of the enzyme (Uchimiya and Murashige, 1974). There are generally higher yields from rapidly growing cells (Constabel, 1975). Cell walls of many cultured lines may be resistant to crude cellulase preparations, though the use of Driselase and Macerozyme often eliminates this problem (Evans and Cocking, 1975).

2) The shape of the cell itself and its physical arrangement may also be a factor in the ability of the enzyme(s) to convert cells to protoplasts. In maize and wheat it has been suggested that the cells have an interlocking structure (Evans and Cocking, 1975). It is conceivable that cells would be specific for types of enzymes as if it were a key to these cells and others would not be quite specific enough to work (Raj and Herr, 1970).

3) The enzyme must be highly specific; without specificity isolations may be carried out, but at the expense of the cell. Pectinase is effective in Lycopersicon fruit (Gregory and Cocking, 1965) and Solanum nigrum (Raj and Herr, 1970). This study indicated the effectiveness of pectinase on Salpiglossis. Specifically, it carries polygalaturonase which attacks the middle lamella and facilitates the separation of the cells. However, it may also weaken the plasmalemma destroying the integrity of the cell.

After isolation, the protoplasts were spherical with peripherally situated chloroplasts. They were cultured in

various media (Table 8) that resulted in changes in their shape, size and organelle distribution. With leaf protoplasts, a spherical shape was often retained for several days. In one medium, P1, the cells remained alive and spherical for seven days. However, in the majority of the test media the chloroplasts became polarized, the vacuoles emerged and burst. Occasional budding did occur; when observed, the protoplasts did not divide.

Axenic derived leaf protoplasts lived no longer than four days in any of the test media (Table 9). Within 24 hr after plating, the chloroplasts became polarized. Shortly thereafter the cells became brown and shrunken.

Callus protoplasts were tested in 24 media (Table 10). The majority of these media maintained the protoplasts in a spherical shape, with even distribution of the chloroplasts. Within three or four days after plating budding often occurred and the protoplasts rapidly declined. In PM, low in ammonium and containing organic nitrates, the protoplasts clustered together and became reniform. However, in less than two weeks they had become brown. Only occasionally did cytoplasmic streaming and systrophy take place. One test medium, MSG4, did invoke profuse systrophy and cytoplasmic streaming (Table 11).

In MSG4 the protoplasts aggregated and became reniform in two to three days. During this time the cells also expanded. The extent of this enlargement appeared to depend upon physiological condition of the callus prior to isolation

Table 8. Test media for leaf protoplasts of *Salpiglossis sinuata*

Code	Hormones
MS basal	
P1	2.0 mg/l NAA, 0.5mg/l BA
PD	1.5 mg/l NAA, 0.5 mg/l 2,4-D, 1.0 mg/l BA
35	5.0 mg/l 2,4-D, 2.0 mg/l BA
A	3.0 mg/l NAA, 0.1 mg/l BA
Gamborg basal	
B/5	1.0 mg/l NAA, 1.0 mg/l 2,4-D, 2 mg/l BA
Modified Durand	
F/5	2.0 mg/l NAA, 1.0 mg/l BA

Table 9. Test media for protoplasts from axenic shoot cultures of *Salpiglossis sinuata*

Code	Hormones
Modified MS basal medium[z]	
GI	3.0 mg/l NAA, 1.0 mg/l BA
GII	2.0 mg/l NAA, 0.5 mg/l BA
GIII	2.0 mg/l IAA, 1.0 mg/l BA

[z]Minus NH_4NO_3 plus 250 mg L glutamine, 0.1 mg/l serine, 2.0 mg/l thiamine.

and the plasmolyticum used (Bui Dang Ha and MacKenzie, 1973). The change in shape also indicated the presence of a new cell wall (Grambow et al., 1972).

The changes in organelle distribution and protoplast size and shape have been documented in Pisum sativum (Constabel et al., 1973) and many other species (Nagata and Takebe, 1970; Vasil and Vasil, 1979). Budding and cell wall regeneration are also well documented (Evans and Cocking, 1975; Vasil, 1976).

Several different combinations of growth hormones were tested to determine the optimal medium constituents for sustained division of Salpiglossis protoplasts. In no case did first division occur although the cells remained viable for up to seven days. With the addition of L-glutamine (250 mg/l) and L-serine (0.1 mg/l) and the deletion of ammonium nitrate, division did occur and was sustained. The reduction or deletion of NH_4NO_3 has proven beneficial in the culture of potato protoplasts (Upadhya, 1975; Shepard and Totten, 1977). In asparagus, the addition of L-glutamine was advantageous to division of protoplasts (Bui Dang Ha and MacKenzie, 1973). Bayley et al. (1972) demonstrated that the addition of L-glutamine eliminated the need for reduced nitrogen in soybean callus. Joshi and Ball (1968) determined that glutamine and casein hydrolysate in the medium resulted in substantial increases in growth. However, both of these studies (Bayley et al., 1972; Joshi and Ball, 1968) showed that ammonium ions would satisfy any

requirement for reduced nitrogen the soybean or peanut cell required. This need for ammonium ions and the failure to use nitrate successfully was attributed to a lack of an efficient nitrate reductase or an interchangeable factor with glutamine (Bui Dang Ha and MacKenzie, 1973; Bayley et al., 1972; Joshi and Ball, 1963).

Interchangeability may be questionable. Solanum tuberosum protoplasts did not divide until the ammonium ions were toally deleted. This was found to be the case herein with Salpiglossis. Utilization of the division medium (Table 11) with the addition of NH_4NO_3 did not result in division. Further, deletion of glutamine and serine once NH_4NO_3 was added did not result in division of the protoplasts. Halperin and Witherell (1965) reported ammonium ions and glutamine not to be interchangeable in embryo formation.

Ammonium salts may only be inhibitory in the respect that an organic compound is needed for division to commence. Gamborg (1970) suggested a reduced organic nitrogen source such as glutamine can be much more desirable than ammonium salts. Glutamine may in fact provide a necessary carbon skeleton in addition to supplying nitrogen (Gamborg, 1970).

The elimination of ammonium ions and addition of glutamine to the test medium for the Salpiglossis protoplasts may have enabled the cell to prepare for division by rapidly synthesizing new proteins and nucleic acids.

The actual stimulation of division is probably

facilitated by the phytohormones. In studies on bindweed (Calystegia sepium) a definite requirement was found for both auxins and cytokinins for division to begin. However, work on Nicotiana suggested that only the presence of a cytokinin is necessary for cell division. Added auxin did increase the rate of division (Evans and Cocking, 1975).

The division medium for Salpiglossis (Table 11) contains both an auxin source and a cytokinin. This medium, duplicated exactly, but substituting NAA for IAA (Table 10) did not result in division. NAA is generally considered a much stronger and longer lasting auxin than IAA (Murashige and Skoog, 1962). NAA in combination with 2,4-D may act synergestically to completely inactivate the process of cell division. This may be especially so if there is a high level of endogenous auxin. High levels of endogenous auxins have been reported in Solanaceous plants (Kartha et al., 1976). The possibility of a specific auxin receptor with a higher affinity for IAA than NAA or 2,4-D also exists. Vreugdenhil et al. (1979) demonstrated the occurrence of a particle-bound auxin receptor in tobacco pith. They suggested that a similar receptor exists on the outer side of the plasma membrane of tobacco leaf protoplasts. Auxin receptors show affinity for different growth regulators (Vreugenhil et al., 1979). Tobacco, however, has a higher affinity for IAA than 2,4-D. It is entirely possible that Salpiglossis callus protoplasts have a higher affinity for IAA than NAA; thus, stimulating division where NAA did not.

Table 10. Test media for callus protoplasts of *Salpiglossis sinuata*.

Basal medium	Hormones
MS basal	
P1	2.0 mg/l NAA, 0.5 mg/l BA
PM1	3.0 mg/l NAA, 1.0 mg/l BA
PM3	2.0 mg/l NAA, 1.0 mg/l BA
PM3	3.0 mg/l NAA, 0.5 mg/l BA
35	5.0 mg/l 2,4-D, 0.5 mg/l BA
PD	1.5 mg/l NAA, 0.5 mg/l 2,4-D, 1.0 mg/l BA
CG4	0.5 mg/l IAA, 1.0 mg/l 2,4-D, 0.5 mg/l BA
N26	0.5 mg/l NAA, 1.0 mg/l 2,4-D, 0.5 mg/l BA
Modified MS	
GI	3.0 mg/l NAA, 1.0 mg/l BA
GII	2.0 mg/l NAA, 0.5 mg/l BA
GIII	2.0 mg/l IAA, 1.0 mg/l BA
GIV	0.5 mg/l IAA, 1.0 mg/l 2,4-D, 0.5 mg/l BA
GV	0.5 mg/l NAA, 1.0 mg/l 2,4-D, 0.5 mg/l BA
PM	3.0 mg/l NAA, 1.0 mg/l BA
Uchimiya and Murashige	
UM	2.0 mg/l 2,4-D, 0.25 mg/l K
UMP	0.6 mg/l NAA, 0.1 mg/l K
Gamborg	
B5	2.0 mg/l NAA, 2.0 mg/l 2,4-D, 2.0 mg/l BA
Modified Durand	
F5	2.0 mg/l NAA, 1.0 mg/l BA
Kao and Michayluk	
KM	1.0 mg/l NAA, 0.2 mg/l 2,4-D, 0.5 mg/l z
Nagata and Takebe	
NT	3.0 mg/l NAA, 1.0 mg/l BA

Table 11. Test medium supporting the division of protoplasts from Salpiglossis callus, (mg/l).

Basal medium

a) salts;
KNO_3 (1700), $MgSO_4 \cdot 7H_2O$ (370), $MnSO_4 \cdot 4H_2O$ (17), $CuSO_4 \cdot 5H_2O$ (0.025), $ZnSO_4 \cdot 7H_2O$ (8.6), $CaCl_2 \cdot 2H_2O$ (440), KI (0.83), $CoCl_2$ (0.025), KH_2PO_4 (170), H_3BO_3 (6.2), $Na_2MoO_4 \cdot 2H_2O$ (0.25)

b) Iron in a solution of 3.724 g/l of the disodium salt of ethylene diaminetetraacetic acid, and 2.784 g/l of $FeSO_4 \cdot 7H_2O$ (10 ml/l)

c) thiamine HCl (2.0)

d) myoinositol (1.0g)

e) organic nitrate sources
L-glutamine (250) L-serine (0.1)

f) Hormones
IAA (0.5), 2,4-D (1.0), BA (0.5)

g) carbon sources
sucrose (30 g/l)

h) Osmoticum
Mannitol (90 g/l)

During the initial period (48 hr) of culture, approximately 25% of the isolated protoplasts died. The remaining protoplasts tended to aggregate and become reniform in shape and within five days entered first division. The initial period of incubation prior to division appeared to vary depending upon the physiological condition of the callus. In some cases first division was not initiated until the fourteenth day of culture. Density was also important in the initiation of division (Evans and Cocking, 1975). Protoplasts plated at 5×10^4 /ml divided much earlier than those at $2/5 \times 10^4$ /ml. At a density of 2.5×10^4, protoplasts showed cytoplasmic streaming and systrophy; yet only occasionally divided after ten to twelve days. At densities of 1.0×10^5 /ml and 2.0×10^5 /ml the protoplasts remained spherical with even distribution of the chloroplasts, finally becoming brown and dying. The plating efficiency of 5.0×10^4 /ml was determined to be 28% whereas 2.5×10^4 /ml was 12%.

Once first division had commenced, second division was observed in about six days (Figure 1). The cells became light green in color indicating chlorophyll synthesis and specialization of the plastids by the twenty-sixth day of the culture. At this time micro-colonies had formed (Figure 1).

After 2.5 months growth, colonies of 2 to 3 mm in diameter were aseptically transferred to shoot regeneration medium (Table 12) where they continued to grow. Colonies

Figure 1. Regeneration of isolated callus protoplasts of Salpiglossis sinuata. a. Freshly isolated protoplasts from 7 day-old callus subcultures. x 200. b. First division of isolated protoplast, 6 days after plating. x 125. c. Second division 14 days after plating. x 125. d. Two-month old cell colony. x 200. e. Shoots produced from callus on MS + 1.0 mg/l 2ip. actual size. f. Adventitious root initiation on shoots cultured on MS + 0.001 mg/l, 2,4-D. actual size.

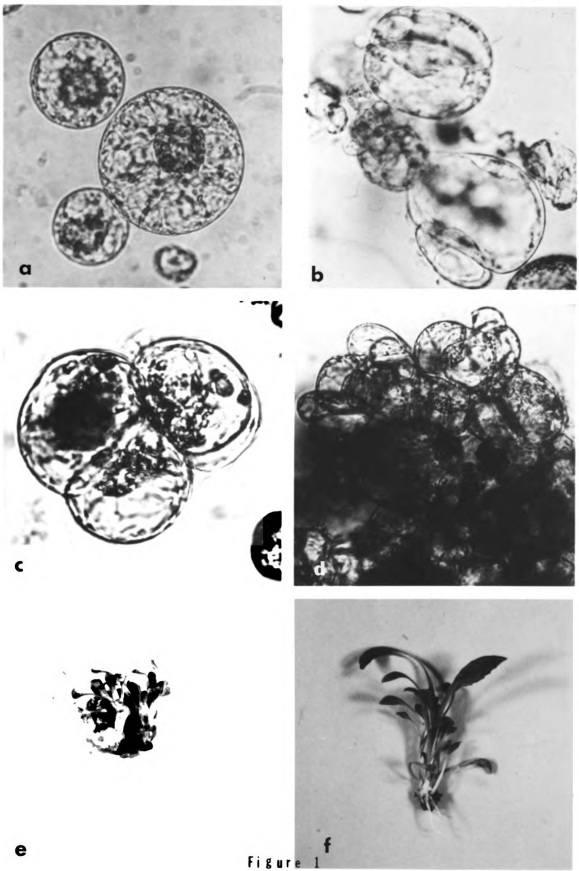

Figure 1

Table 12. Media for the regeneration of shoots and roots from callus derived from protoplasts of Salpiglossis sinuata (mg/l).

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1. Inorganic salts:

   NH_4NO_3 (1650), KNO_3 (1900), $mgSO_4$ $7H_2O$ (370), $MNSO_4$ $4H_2O$ (22.3), $ZNSO_4$ $4H_2O$ (8.6), $CuSO_4$ $4H_2O$ (0.025), $CaC1_2$ $2H_2O$ (440), KI (0.83), $CoC1_2$ $6H_2O$ (0.023), KH_2PO_4 (170), H_3BO_3 (6.2) NA_2 N_oO_4 $2H_2O$ (0.25)

2. Iron in a solution of 3.724 g/l of EDTA NA_2 and 2.784 g/l $FeSO_4$ $7H_2O$

3. Organic components:
   Nicotinic acid (0.5) pyridoxine HC1 (0.5) thiamine HC1 (0.1) glycine (2.0) myo-inositol (100)

4. Carbon source

   Sucrose 30 g/l

5. Agar 8 g/l

6. Hormones:

   Shoots : 2ip (1.0)
   Roots: 2,4-D (0.001)

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less than 1 mm in diameter generally failed to grow on this medium when removed from liquid culture. The callus rapidly increased in size and in twenty-one days shoot primordia were observed with the aid of a stereoscope. Growth slowed abruptly at this point; the callus remained green and friable. Shoots elongated and could be excised in fourteen more days (Figure 1). Shoots of separate callus origin were transferred to the root induction medium (Table 12). This transfer was preceded by a quick dip of each shoot in 1000 ppm IBA. After placing in the root induction medium, roots were evident fourteen days in 75% of the cultures (Figure 1).

Hughes et al. (1973) and Lee et al. (1977) have shown that phytohormone manipulation in the culture medium can result in morphogenesis and plantlets in Salpiglossis callus. Regeneration of plants from protoplasts has been reported in several Solanaceous plants (Frearson et al., 1973; Binding and Nehls, 1977; Zapata et al., 1946; Shepard and Totten, 1977; Hayward and Power, 1975; Sink and Power, 1977; Binding, 1974; Nehls, 1978). Regeneration of plants from protoplasts derived from callus has also been reported (Bui Dang Ha and MacKenzie, 1973; Power and Berry, 1979; Vasil and Vasil, 1979).

Regenerated plantlets examined cytologically were found to have a somatic chromosome number of 2n=44 except in one case where the chromosome number was determined to be 88. A normal diploid count is 44 (Darlington and

Wylie, 1955; Hughes et al., 1973). Instability of callus in culture has been demonstrated (Evans and Cocking, 1975). Chromosome aberrations in in vitro cell cultures are known to occur, the frequency of which increases with the time in culture (Sunderland, 1973).

RECOMMENDATIONS

The results herein indicate that Salpiglossis sinuata could be a potential parental species in somatic hybridization attempts. The success of such experiments could be improved threefold with respect to Salpiglossis: 1) increasing the yield of protoplasts, 2) increasing the plating efficiency, and 3) minimizing genetic aberration in the protoplast callus source. The yield of protoplasts may be increased by testing further combinations of enzymes used in the isolation step. The use of new enzyme combinations may unintentionally lead to increased plating efficiency since protoplast viability is dependent to some extent on the concentration of enzymes and the length of incubation time. More extensive use of the initial callus derived from leaf sections and their early subcultures may serve to optimize the results obtained in increasing yield and plating efficiency. Furthermore, this practice should aid in maintaining the genetic stability of the callus cultures and thus the resultant protoplasts and regenerated plants.

Selection systems are readily apparent for the recovery of heterokaryons using Salpiglossis in fusion experiments based on its limited or non-division in several culture media. This in vitro response may be linked to the use of the alternate parent as a chlorophyll deficient type that divides. Thus, the ability for protoplast division may be transferred during fusion to the heterokaryon cells from the albino parent. The heterokaryon cells would also be

assumed to carry chlorophyll synthesis competency from Salpiglossis and thus be distinguished from the albino parent cells by their green pigmentation.

Several closely related Solanaceous genera merit consideration for fusion studies with Salpiglossis. These include Browallia, Nicotiana and Petunia, all of which can presently be regenerated from protoplasts. For some of them, chlorophyll deficient mutants are also available.

SUMMARY

Protoplasts were successfully isolated from leaves and callus of Salpiglossis sinuata. Callus provided a sterile source of protoplasts and isolation techniques were pursued. Enzyme containing pectinase was found to be most effective in converting callus to high quality protoplasts in large quantities. Effectiveness of the enzyme is attributed to physiological condition of the callus, specificity of the cells for certain enzymes and complete degradation of the middle lamella. Division occurred when protoplasts were cultured in a modified MS medium containing 250 mg/l glutamine, 0.1 mg/l serine, 2.0 mg/l thiamine and deleting NH_4NO_3. The enhancement of division with glutamine is well documented (Bayley et al., 1972; Bui Dang Ha and MacKenzie, 1973), as are the possible toxic effects of ammonium ions (Shepard and Totten, 1977; Upadhya, 1975). The division medium also contained IAA, 2,4-D and BA; substitution of NAA for IAA did not result in division. This suggests a possible affinity for IAA at the auxin receptor site. Callus and regenerated shoots occurred within three months after isolation. The shoots were rooted and the plants were examined cytologically. One aberrant plant was found of the ten examined. Instability of callus, from which the protoplasts were derived; genetic and epigenetic has been demonstrated (Vasil, 1976; Ogihara and Tsunewaki, 1979).

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