substitute for new born calf serum in tissue culture cultivation of rabies virus.

ACKNOWLEDGEMENTS

The author is grateful to Dr. K. S. Sanjivi, Dr. V. S. Subramanian, Dr. N. S. Murali and Capt. Jayaram of the V.H.S. Medical Centre and Dr. C. S. Gangadhara Sharma, State Leprosy Officer, for all their encouragement, help and cooperation. He is also indebted to Dr. Mani Veeraraghavan and Mr. P. Gopal for their invaluable assistance throughout this study.

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Postscript

Dr. Laszlo Kato, M. D. Research Director, Catherine Booth Hospital Centre, Montreal, Canada, to whom the medium had been sent for trial, has confirmed that host grown *M. leprae* multiplied when inoculated into the medium. The maximum growth he has obtained is 8 fold in 5 days.

MORPHOGENESIS IN CULTURED LEAF DISCS OF *SALPIGLOSSIS*

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ABSTRACT

Leaf discs of *Salpiglossis* cultured in a basal medium supplemented with auxins and cytokinins either alone or in combinations displayed various morphogenetic phenomena. Leaf discs on a 2,4-dichlorophenoxyacetic acid medium produced a friable callus without any differentiation whereas on a naphthalene-acetic acid medium, they formed calluses and differentiated long roots. Combinations of kinetin and adendine induced a friable callus which contained small clusters of pink cells. On a benzyl-amino-purine or a 6 (γ,γ-dimethylallylamino) purine medium, the leaf discs expanded and differentiated numerous shoot buds. These buds on transfer to a hormone-free medium, initiated roots. Rooted plantlets when transplanted to soil in pots grew and produced flowers.

INTRODUCTION

MESOPHYLL protoplasts of several members of the Solanaceae have served as model systems in plant protoplast technology and somatic hybridization[1,2]. The basic requirement for reconstructing plants from hybrid somatic cells is the capacity of those cells to manifest morphogenesis or embryogenesis in culture. However, before proceeding with protoplast regeneration, it might be advantageous to test the regenerative potential of those individual cell lines and tissues from which they are derived. In fact, it was pointed out earlier, that "as a general approach for leaf protoplast regeneration of many systems, an accompanying study of the regeneration requirements of the appropriate leaf callus could be of value"[3]

Since *Salpiglossis* has not previously been studied for embryogenesis and also since it belongs to the Solanaceae, we thought it worthwhile to explore the regeneration requirements of the leaf tissues and the callus derived from mesophyll. This work was therefore undertaken to divise a procedure for rapid clonal multiplication of *Salpiglossis* plantlets.

MATERIALS AND METHODS

Salpiglossis sinuata (var. 'splash'), of Ball Superior Ltd. cultivated in greenhouses were used as experimental materials. The temperature and photoperiodic regimes under which plants were grown ranged between 25° 35° C (day) and 17° 22° C (night) and 16 hr light conditions. Nine mm discs along the midrib

were excised by a corkborer from surface-sterilized third and fourth leaves from the tip and cultured on a Basal medium (BM) with 0.7% agar, described previously[4]. Essentially, the BM comprised of Murashige and Skoog's minerals[5], White's vitamins[6], inositol (100 mg^{-1}) and 2% (W/v) sucrose. Auxins such as naphthalene acetic acid (NAA) and 2,4-dichlorphenoxy acetic acid (2,4-D) and cytokinins such as kinetin, benzyl amino purine (BAP), 6 (γ,γ-dimehylallylamino) purine (2-ip) and adenine were added individually or in combinations. Each treatment consisting of 20 vials was repeated at least 3 times. Cultures were incubated in a growth cabinet with 16 hr light at 26° C and 8 hr dark at 20° C.

Results

The response of leaf discs in culture varied remarkably depending on the kinds and concentration of growth regulators added to the BM. The morphogenetic responses of the discs cultured for 5 weeks on 6 different media are summarized in table 1 and discussed below.

Response to auxins

The majority of the leaf discs cultured on BM alone did not expand and eventually died within two weeks of culture (table 1, figure 1A). However, in some cultures (2 out of 20) the basal end of the midribs proliferated and produced a friable callus (figure 2). This callus was subcultured repeatedly or could be induced to differentiate shoots if transferred to media containing BAP and adenine (P_5) or BAP alone in any concentrations ranging from 10^{-7} to 10^{-5} M. Explants cultured on a medium containing NAA (P_2 medium, table 1) expanded and produced a green nodular callus. After the initiation of callus, long roots with many root hairs differentiated (figures 1B and 3). In contrast, leaf discs cultured on a 2,4-D medium (P_3) produced a friable light yellow callus which did not differentiate organs (figures 1C and 4).

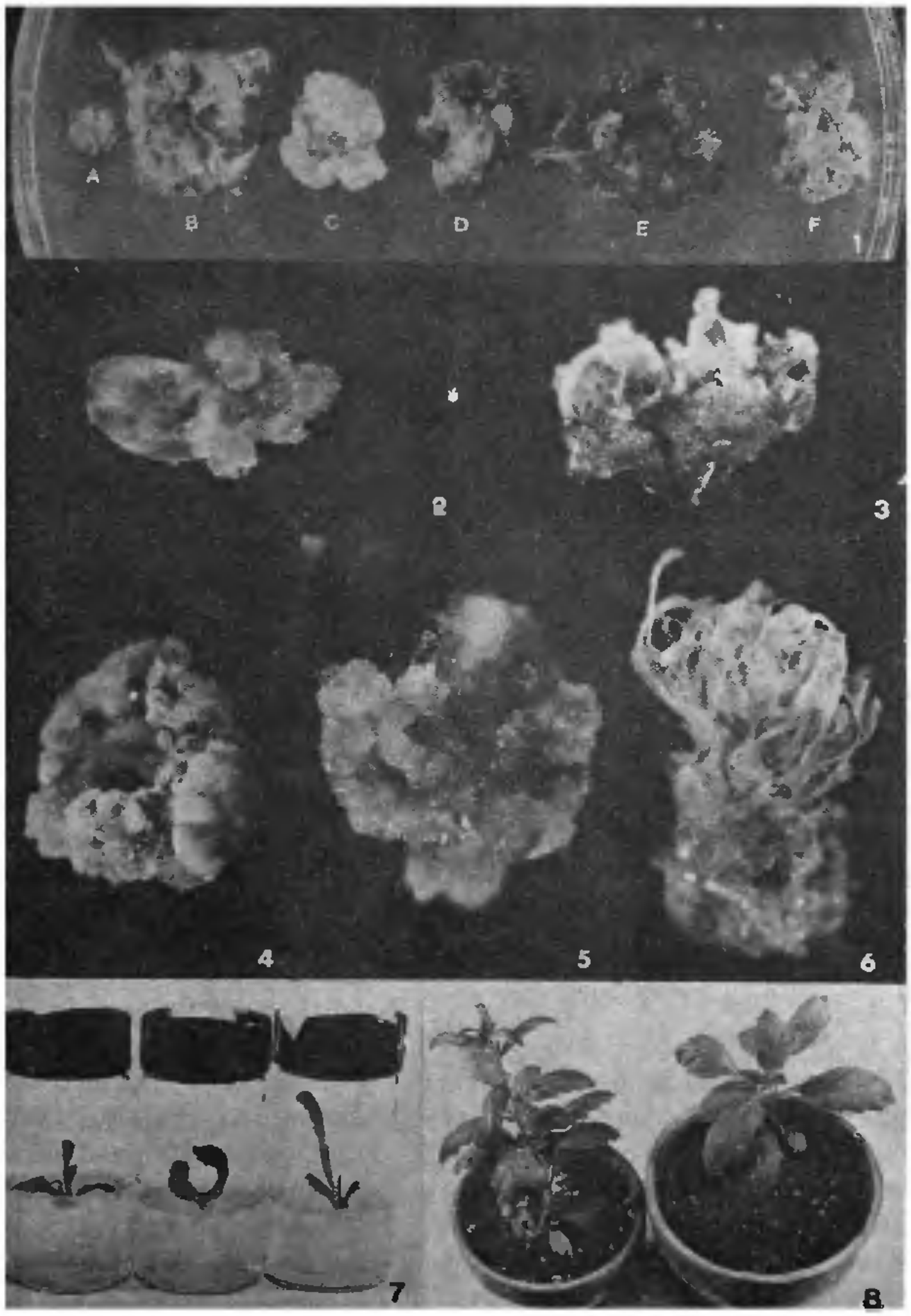
Response to cytokinins

In the presence of kinetin and adenine (P_4) the leaf discs expanded and callused (figures 1D and 5). Callus initiation started from both ends of the midribs and vein endings and eventually engulfed the entire disc surface. The friable callus contained small clusters of pink cells. These clusters formed only in cultures raised on media with adenine, as they were not present when kinetin alone was supplied. The intensity of the colour increased as the concentration of adenine was increased in the medium. However, when BAP was incorporated in the medium (P_5) the leaf discs expanded rapidly and produced a green nodular callus (figure 1E). Within 5 weeks many shoot buds differentiated and these completely filled the entire culture vial (figure 6). These buds, when isolated and transplanted to a hormone-free medium (P_1) initiated roots (figure 7). Rooted plantlets when transferred to soil in pots grew and produced flowers (figure 8.) The bud-inducing capacity of BAP was completely nullified when leaf discs were cultured on a medium (P_6) containing both BAP and 2,4-D. On such a medium,

Table 1

Morphogenetic responses of leaf discs of Salpiglossis cultured on various media

Media Code	Composition	Response observed after 5 weeks of culture
P_1	BM	90% discs died. In the remaining discs, basal end of the midrib callusing
P_2	BM + NAA (1 mg^{-1})	Green nodular callus and root differentiation of all discs
P_3	BM + 2, 4-D (0.5 mg^{-1})	Light yellow callus
P_4	BM + kinetin (1 mg^{-1}) + adenine (40 mg^{-1})	Normal callus interspersed with pinkish cell clusters
P_5	BM + BAP (1 mg^{-1}) + adenine (5 mg^{-1})	Green callus and shoot buds
P_6	(BM + BAP (0.5 mg^{-1}) + 2,4-D (-mg^{-1})	Compact white callus



Figures 1-8 *Differentiation in cultured leaf discs of Salpiglossis* **1.** Morphogenetic responses of leaf discs cultured on 6 different media for 2 weeks. (A) Control on P_1 medium, note no expansion, Disc browned. (B) Leaf disc cultured on P_2 medium initiating callus and roots. (C) Only callus proliferation on P_3. (D) Callus with pink cell clusters on discs cultured on P_4. (E) Abundant callus with shoot buds on P_5. (F) Compact callus on P_6. **2.** One of the few discs responded by callusing from the basal end of the midrib on P_1 medium (5 weeks old). **3.** A 5 week old callus and roots on discs cultured on P_2. **4.** A leaf disc cultured on P_3 showing callus all around the explant (5 weeks old). **5.** A disc cultured on P_4 for 5 weeks. Note very friable callus with pink cell clusters. **6.** A 5 week old leaf disc on P_5 medium. Note the abundance of well grown shoot buds. **7.** Isolated shoot buds from P_5 on a rooting medium. **8.** Plantlets transferred from rooted buds. Note the appearance of floral buds.

the discs developed a white compact callus without any signs of differentiation (figure 1F). In order to study the effect of BAP on budding frequency, BAP alone was incorporated in the medium in the following concentrations, 0, 10^{-7}, 10^{-6}, 5×10^{-6}, 10^{-5} and 10^{-4} M. As evident from figure 9, 10^{-5} M was the optimum concentration for bud production. Essentially similar responses were observed when the discs were cultured in a medium containing the above conentrations of 2-ip.

Figure 9. Effect of various concentrations of BAP on the production of shoot buds from in vitro cultured leaf discs of *Salpiglossis*. Each vial from right to left is representative of 5 replicates (except the first one where this disc was the only one still surviving) had media fortified with 0, 10^{-7}, 10^{-6}, 5×10^{-6}, 10^{-5} and 10^{-4} M BAP. Photographed after 6 weeks of culture.

Discussion

A number of general and specific points concerning the nutritional and hormonal requirements for growth and morphogenesis of *in vitro* grown *Salpiglossis* leaf tissues are apparent in these observations. Generally, as reported by many earlier workers auxins tended to promote root initiation or callus formation and cytokinins shoot buds in cultured leaf discs[7,8]. One of the interesting observations which is reminiscent of the classical experiments on tobacco tissue cultures of Skoog and Miller[9] was that auxins, cytokinins, auxin-cytokinin and cytokinin-cytokinin interactions elicited well-defined and clear cut morphogenetic responses in these cultured leaf discs. As briefly reported earlier[10] the responses of the discs to the individual auxins tested in culture were variable. For example, NAA induced callus and roots. However, when shoot buds differentiated from discs cultured on P_5 medium, were transfered to a NAA medium they failed to initiate any roots. This indicated a differential physiological competency probably in terms of auxins/cytokinins balance of leaf discs and shoot buds differentiated from these discs. This observation is in agreement with that of Lee *et al*[11], who observed similar responses in their experiments which were reported when this work was completed. If 2,4-D was employed in the place of NAA, the leaf discs initiated a light yellow callus instead of the green nodular callus. Further, there was no root initiation and even at very low concentrations 2,4-D was inhibitory for root differentiation. A similar response was reported in cultured leaf discs of *Petunia inflata* and *P. hybrida* by Rao *et al*[12], even though in *Salpiglossis* leaf discs no root differentiation occurred in response to 2,4-D. The potency of 2,4-D in stimulating callus proliferation in *in vitro* cultured explants is widely known and our results confirm those observations.

Cytokinins, such as BAP, kinetin and 2-ip induced bud development, with BAP and 2-ip being the most effective. However, an important observation was while BAP and 2-ip was effective for bud induction alone, kinetin had to be supplied along with an auxin (Raman, Unpublished). Similarly, a cytokinin-auxin combination was necessary for *in vitro* shoot induction in cultured leaf discs of 12 mutant clones of tomato[13] whereas BAP alone triggered shoot buds in discs of *Petunia inflata* and *P. hybrida*[12].

In a detailed study on leaf discs of tomato cultivar Starfire, Kartha *et al*[14] observed that combinations of BAP and IAA were more effective than those of BAP and NAA in inducing shoot buds *in vitro*, whereas kinetin in conjunction with either IAA or NAA failed to induce shoot differentiation. In contrast, only in the

presence of coconut milk (CM) supplemented with kinetin did tobacco leaves produce shoot buds in culture[15]. Further, they also observed that rootdifferentiation in those leaves occurred only when the media contained both CM and IAA. However, in the present study, we observed that either BAP or 2-ip was effective in inducing shoot buds in cultured leaf discs of *Salpiglossis*. These differential responses of explants may be a reflection of varying levels of endogenous plant growth regulators such as auxins and cytokinins in these plant genera.

Suchr differences in the endogenous levels of plant growth regulators were detected even in single gene mutants and its wild genotypes. For example, we observed differential sensitivity to various plant growth substances, especially gibberellins by *in vitro* grown immature "single" and "double" flower buds of *Nigella damascena*[4]. This differential sensitivity paralleled a positive correlation in both the endogenous levels and the compartmentalization profiles of extractable gibberellin-like substances in these two genotypes, albeit we were uncertain as to the cause and effect relationship[16].

Addition of 2,4-D along with BAP induced embryo formation, though fewer in number than formed in explants cultured on 2,4-D alone in *Petunia inflata*, and *P hybrida* leaf discs[12]. In contrast the presence of 2,4-D and BAP together, completely inhibited the shoot bud differentiation and produced a compact white callus in *Salpiglossis* leaf discs.

Leaf discs cultured on a medium containing both kinetin and adenine proliferated to form a callus with patches of pink cell clusters. Further, we observed that the intensity of the pink coloration increased with increasing concentrations of adenine in the medium. Earlier, Nitsch and Nitsch[17] reported that the intensity of the pink colouration of *in vitro* formed flower buds of *Plumbago* increased with increasing levels of adenine in the medium. Thus, our present observations and those of Nitsch and Nitsch[17] confirm that the influence of adenine may be similar in at least some differentiating as well as dedifferentiating plant tissues in culture.

The extensive bud regeneration capacity of the leaf discs in *Salpiglossis* provides a system by which rapid multiplication of a selected horticultural variant of this plant could be accomplished without much modifications. A similar simple *in vitro* procedure for the rapid multiplication of *Streptocarpus* and Gloxinia from cultured pedicels was reported earlier from our laboratory[18]. Recently, we conducted a feasibility study for the vegetative propagation of *Zea mays* from the dormant axillary buds[19]

Thus our present study has demonstrated that *Salpiglossis* leaf discs are morphogenetically very plastic and a subtle change in the hormone composition in media may elicit various responses. This plasticity and the ease with which various differentiation phenomena that can be induced, certainly makes this system a prime choice for any future protoplast work and eventually somatic hybridization technology in plants.

ACKNOWLEDGEMENTS

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to Prof. R. I. Greyson and the author thanks him for helpful discussions during the course of this work and for his critical comments on the manuscript.

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