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THE ROLE OF SUPPRESSION IN THE MORPHOGENESIS AND PHYLOGENY
OF *Streptocarpus prolixus* (GESNERIACEAE)

by

RICHARD A. RAUH

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment
of the requirements for the degree of Doctor of Philosophy,

The City University of New York

2001

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ABSTRACT

THE ROLE OF SUPPRESSION IN THE MORPHOLOGY AND PHYLOGENY OF

Streptocarpus prolixus (GESNERIACEAE)

by

Richard A. Rauh

Advisor: Professor Dominick V. Basile

Genetic controls ultimately determine the suppression of proliferation that in turn determine the size and shape of any particular species. The implementation of these controls, however, depends on a complex series of interrelated pathways largely epigenetic in nature. The hypothesis that auxin, ethylene, and some hydroxyproline-containing proteins play decisive roles in this implementation has been tested in lower plants. Here we extend this testing to a flowering plant. Antagonists of the action or synthesis of auxin, ethylene, and hydroxyproline-containing proteins, are applied exogenously to the seeds and seedlings of *Streptocarpus prolixus* C.B. Clarke.

The β -glucosyl Yariv phenylglycoside (β -D-Glc)₃ is known to selectively bind to that class of cell-surface associated proteins known as arabinogalactan-proteins (AGPs). Seeds and seedlings of *S. prolixus* were treated with (β -D-Glc)₃ and α -galactosyl Yariv phenylglycoside (α -D-Gal)₃, which does not bind AGPs. With the exception of (α -D-Gal)₃, the antimetabolites all induced similar morphological

variations. They were, anisocotyly with a secondary phyllomorph, multiple phyllomorphs, anisocotyly with plumular growth, or isocotyly with plumular growth. All the variations appear related to the relative differences in time and degree of suppression of cells comprising the primary embryonic shoot meristems. The induced phenovariants copied the growth patterns of mature plants of other species in its subgenus (*Streptocarpus*). More remarkably, they also phenocopied morphotypes of species in a different subgenus (*Streptocarpella*), and the subfamily (Gesnerioideae) of the Gesneriaceae. The finding that the range and types of phenovariants induced by Hyp-protein antagonists are the same as those induced by a reagent that specifically perturbs AGPs strongly suggests that the Hyp-proteins mediating the pattern changes are hydroxyproline-containing AGPs. By extrapolation, because the induced phenovariation was due to the suppression of leaf and shoot development in *S. prolixus* and several leafy liverworts, these results support the hypothesis that certain AGPs can mediate the suppression of cell proliferation in organ primordia. The similar results produced lead to the conclusion that the same factors mediating place-dependent suppression in leafy liverwort gametophytes appear to perform the same function in flowering plant sporophytes. The results suggest that the molecular basis for the correlative inhibition of localized growth processes may be the same for all land plants.

PREFACE

Development, the generation of form in plants is a wide, complex and long studied area of investigation. The possibilities for understanding the mechanics behind this universal process are equally wide and complex. Current preoccupation would emphasize the location and discovery of the genes which regulate the process. Another approach would attempt to uncover the gene products working on each other which implement the genetic signals, by chemical/physiological processes. A more general statement of what drives the process has been stated in a hypothesis which is concerned with the balance between actively proliferating or enlarging cell populations, and the stopping, or suppression of those basic cell processes in particular locations and at particular times. This theory was developed over a period of years with experimental work done on five families of Hepatics, so that an experimental approach, rather than a molecular one had been chosen. During the course of the experimentation, a trio of factors was found to be responsible for the regulation of suppression in the liverworts. Since it was vital to the study that these factors were functioning in the living plant, an approach was undertaken to antagonize with exogenously applied substances the three factors in vivo, and to study the morphologic results. The perturbation of these naturally occurring regulators induced results that affected organ initiation, stem morphology and branching patterns in the Bryophytes. This success encouraged extension of the work in the area of flowering plants. The

criteria for the extension of the study needed to involve certain limits. It was important that the three factors were to be found working in vivo, and endogenously as they were found to be working in the liverworts. It was important that the plant chosen come from a group that displayed as much range of vegetative morphology as the leafy liverworts. It was important that the particular species studied was interpreted as being the result of regressive/reductive evolution, since the liverwort species that showed the most meaningful results were interpreted as being the results of regressive/reductive evolution. The experimental approach should be able to reveal temporarily that desuppression of primordia was present, but not expressed, and not a completely new, mutationally-derived feature.

The three factors found to be mediating the suppression of cell proliferation or enlargement in liverworts were auxin, ethylene and an arabinogalactan protein. The flowering plant chosen for the study is *Streptocarpus prolixus* C.B. Clarke a plant that appears to be the result of regressive/reductive evolution, and a member of a highly diverse genus, with an exceptional range of vegetative structure. Discussed in the following chapters is the experimental approach to test the hypothesis that the suppression of cell proliferation, in the morphogenesis and phylogeny of *S. prolixus* is controlled correlatively by the interrelated actions of auxin, ethylene and an arabinogalactan-protein. First, eight different acting antagonists of the two phytohormones and a hyp-protein produce changes in the morphogenesis of *S. prolixus*. Next, three different-acting antagonists of hyp-protein reveal possible clues into the phylogeny of *S. prolixus*. Finally, different antagonists of AGPs are tested

producing results that parallel the earlier work, strengthening the hypothesis that the hyp-proteins uncovered as components of the correlative morphoregulatory control system belong to this class of cell-surface associated proteins.

Note: The bulk of this dissertation consists of three papers covering this work. One has already been published and the other two are in review by scientific journals. Two minor papers, one reporting an unexpected result only peripherally connected to the main hypothesis and another discussing a technique developed during the course of the experimentation are in preparation and are presented as appendix chapters. The abstracts, material and methods and bibliography sections of the papers have been combined to minimize unnecessary duplication, but the introduction, result and discussion sections have for the most part been kept as they were written for submission for journal publication. Please excuse the repetition entailed.

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ABBREVIATIONS

AGP	arabinogalactan-protein
ALANAP	2-[(1-naphthalenylamino)caronyl] benzoic acid
AVG	aminoethoxyvinylglycine
Co	macrocotyledon
co	microcotyledon
3,4-dhp	3,4-dehydro-L-proline
2,2'-dp	2,2'-dipyridyl
ETOH	ethyl alcohol
G	goodness-of-fit
GA ₃	gibberellic acid
(α -D-Gal) ₃	α -galactosyl Yariv phenylglycoside or reagent
(β -D-GLC) ₃	β -glucosyl Yariv phenylglycoside or reagent
Hyp	<i>trans</i> -4-hydroxy-L-proline
<i>P</i>	probability based on a Chi-Square analysis
PCIB	<i>p</i> -chlorophenoxyisobutric acid
SEM	scanning electronic microscopy
TIBA	2,3,5-triiodobenzoic acid

CHAPTER 1
THE MORPHOGENIC EFFECTS OF VARIOUS ANTAGONISTS OF
HYDROXYPROLINE-PROTEINS, ETHYLENE
AND AUXIN IN *Streptocarpus prolixus* (GESNERIACEAE)

Introduction

"...In plants, form arises as a result of the differential effects of the driving forces of cell proliferation, directional cell partitioning, directional cell enlargement and the place-dependent suppression of growth processes..." (Basile and Basile 1993). Three roles for this place-dependent suppression in plant morphogenesis have been proposed. The first role is in establishing apical meristematic regions in the transition from diffuse to apical growth that occurs in all extant land plants manifesting apical growth. The second role of localized suppression is to stop further development of organ primordia after they are initiated. The third role is the modulation of size and shape of organs having a determinate mode of development, as indicated by the starting premise that suppression of proliferation generates form (Basile and Basile 1993).

The second role of localized suppression of organ primordia was demonstrated by extensive studies of leafy liverworts (Basile 1967, 1969, 1970, 1973a). Phylogenetic patterns were revealed by experimentally antagonizing (desuppressing) normal patterns of morphogenesis. 'Desuppression' techniques allowed for the reappearance of characters thought to be "evolutionarily lost". A reduction series from species showing an initial three leaf sequence with equal development to a derived one in which the third leaf was "lacking" could be demonstrated. The most derived member of the series could be induced to develop the most primitive form. Along with the "loss" of the third leaf, was the shift from radial symmetry to bilateral (dorsiventral) symmetry, and in the reduction in the number and kinds of branching patterns. All of

these more primitive traits were induced in species that were considered to be products of reductive/regressive evolution.

The extensive study conducted with leafy liverworts revealed some of the probable factors in the chemical control of the suppression of evolutionarily reduced organs. The insights obtained could be summarized as follows: The directional transport of auxin determines where suppression occurs. When auxin levels become superoptimal so as to trigger ethylene synthesis, suppression occurs and ethylene-mediated modification of certain hydroxyproline-proteins (Hyp-proteins) associated with the cell surface determines whether and how suppression occurs. At least three different acting antagonists of each implicated regulator produced the same effect. The results inspired confidence that auxin, ethylene and some Hyp-protein were components of the control of the "suppression system" in at least five families of leafy liverworts (Basile 1990).

Experiments conducted by Rosenblum in the Basile laboratory in the 1980s extended the range of the "Suppression Theory". A member of the genus *Streptocarpus*, in a highly derived flowering plant family, was induced to generate shoot apical meristems and nodal organization, forms that were considered "lost" in the model species (Rosenblum 1981). The study, however, was never completed at the time. Available results were sufficient to suggest that the same chemoregulatory system that functions to locally suppress cell proliferation in leaf primordia in leafy liverworts similarly functions to suppress shoot apical meristems in flowering plants. This discovery led to a reformulation of the suppression hypothesis so that it was

applicable for all land plants. It states, "...many if not most of the differences by which we distinguish the form changes that occur during the stages of ontogeny and the course of phylogeny are due to differences with respect to the time and place cell proliferation and/or enlargement is suppressed." (Basile and Basile 1993)

A model was needed to test this hypothesis in flowering plants where an organ primordia was stopped after having been initiated, to parallel the experiments conducted with liverworts. The ideal plant would have reached its present morphological state by reductive/regressive evolution.

Fortunately the flowering plant genus used earlier in the Basile laboratory by Rosenblum proved to have the necessary qualifications. The genus *Streptocarpus* (Gesneriaceae, Scrophulariales) is placed in the "most highly advanced subclass" of dicotyledons, the Asteridae, by Cronquist (1988) primarily because of its zygomorphic and fused floral parts. *Streptocarpus*, comparable to some of the liverworts studied, displays a wide range of vegetative morphologies. After exhibiting some degree of unequal development of their two cotyledons (anisocotyly) typical of its subfamily (Cyrtandroideae), species develop morphologies that can be arranged in a series that extend from the more familiar caulescent, node-internode type shoot organization to a highly derived, unifoliate form. In the latter, an extreme case of reductive evolution, the entire vegetative structure appears as a single leaf that arises from the continued development of one of the cotyledons. This leaf-like structure is termed a phyllomorph. In a manner similar to the series tracing the gradual evolution from a fully formed third leaf to a complete lack of that leaf in various liverwort genera, one

can identify variant forms between the caulescent and unifoliate phyllomorphs in the *Streptocarpus* genus, including a multiple phyllomorph rosulate-type (Jong 1970,1973) characteristic of the closely related genus *Saintpaulia* (Smith et al 1998). All the variations appear to be related to the relative differences in the time and degree to which cell proliferation in the primary, embryonic shoot apical meristems is suppressed (Rauh 1997).

Streptocarpus prolixus C.B. Clarke is the species used in this study. It is acaulescent and unifoliate throughout the early stages of its growth (approximately up to the first year), exhibiting secondary phyllomorphy about the time of flowering, and is thus classified as a plurifoliate by Hilliard and Burt (1971). Rosenblum, after working with a number of *Streptocarpus* species, settled on this species as his model plant, not only for the unifoliate habit in its early growth but for its more rapid growth and flowering and greater seed production via artificial pollination (Rosenblum 1981).

The normal post-germination growth pattern of *S. prolixus* displays steady accrescence of one of the cotyledons called the macrocotyledon. The smaller cotyledon called the microcotyledon ceases to grow, and is eventually lost. A displacement of the two cotyledons is due to the growth of the petiolode, an axis that combines the features of a stem and a petiole. At the base of the macrocotyledon a slight depression in the petiolode develops, which is named the groove meristem (Jong 1970). Any variation in this total pattern is regarded as evidence of the morphoregulatory effects of antagonizing each or any of the components of the correlative inhibitory system discovered by the Basiles.

Rosenblum's pioneering studies showed that suppression of organogenesis could be prevented if seedlings of *S. prolixus* were exposed to the phytohormone gibberellic acid (GA₃) at a critical time after seed germination. No evidence as to what causes microcotyledon or shoot apical meristems to be suppressed were confirmed by these experiments. Rosenblum's studies however, in indicating that it was possible to affect the suppressed organs, did not address the question as to whether the interrelated actions of auxin, ethylene and some hyp-protein can act to locally suppress growth and development in a flowering plant. That question is the basis for the research presented in this paper.

Results

The hypothesis that the suppression of microcotyledonary and vegetative shoot development are at least in part controlled by the interrelated actions of auxin, ethylene and some Hyp-protein in flowering plants, is confirmed by the results of this study. Phenovariation has been induced by all of the antagonists of auxin transport (Figs. 1-5), auxin action (Figs. 6-8), ethylene synthesis (Figs. 9-11) ethylene action (Figs. 12,13) and Hyp-protein synthesis (Figs. 14-22) in the early stages of morphogenesis of *Streptocarpus prolixus*. There was no visible or statistical difference between the seedling variants obtained by either the filter-paper-raft, or the solid-medium cultures. More interesting, was the similarity of types of phenovariations induced, regardless of the treatment applied or the concentrations employed (Table 1). All treatments produced a variety of morphotypes. These include anisocotyly with secondary phyllomorphs at the base of the macrocotyledon (Fig. 13) or at the juncture of the two

cotyledons (Fig. 17), *Streptocarpus* plurifoliolate-type (Type I), a precocious manifestation of the appearance of secondary phyllomorphs which in the normal growth habit of the plurifoliolate plant occurs at maturity, either along with or closely preceding the appearance of inflorescences. Less frequently observed are multiple phyllomorphs, *Saintpaulia*-type (Type II), a pattern phenocopying the rosulate habit of some species in *Streptocarpus* and the closely related genus *Saintpaulia* and other genera in the subfamily Cyrtandroideae (Fig. 22). Also observed are anisocotily with plumular growth, *Streptocarpella*-type (Type III), a condition where unequal cotyledons are a temporary early phase of caulescence typical of the *Streptocarpus* subgenus *Streptocarpella* (Fig. 16) and isocotily with plumular growth, Gesnerioideae-type (Type IV), a habit typical of most dicotyledonous plants, and of the subfamily Gesnerioideae of Gesneriaceae, (Fig. 3). The phenovariants thus displayed a range of types, that copied the phenotypes of mature plants in the same species, plants of other species in the same subgenus, and related genera, plants in a different subgenus, and plants in a different subfamily. With the exception of PCIB, where phenovariant seedlings displayed unusual growth of root hairs, the changed morphology did not display any features that could be assigned to any culture condition, antagonist, or concentration thereof (Figs. 1-28).

In the raft culture study, germination of the seeds proceeded along expected parameters. After 8-10 days, germinating seedlings could be observed in the controls and in cultures treated with four out of the six antimetabolites. The two auxin antagonists used delayed germination. Of those, the cultures treated with Alanap

began to show germination after another week, but those treated with TIBA never showed any sign of germination. The lack of germination in TIBA-treated cultures may have been caused in part by dissolving the reagent in 70% ETOH, rather than in distilled water. This may have affected the viability of the delicate, dust-like, non-endospermic seeds. In the experiments conducted with seeds inoculated onto Gellan-solidified media, the TIBA was dissolved in nutrient media, by adjusting the pH, and germination was achieved.

From 96 to 110 days after the onset of germination of the seedlings in the raft culture, the number of plants showing phenovariation was recorded. Statistical analysis of the resultant data showed that a highly significant ($P = 0.002$) amount of phenovariation was directly attributable to the use of antimetabolites (Table 1). There were 12 % of the control seedlings manifesting morphological alterations. When the effects of each antimetabolite is compared with the control, each in turn reveals a significant increase in phenovariation induced by antimetabolite over that occurring spontaneously in the control cultures. In statistical terms, for AVG $G = 7.455$, $P = 0.0063$; for Alanap $G = 9.437$, $P = 0.0021$; for 2,2'-dp $G = 11.708$, $P = 0.0006$; for 3,4-dhp $G = 5.727$, $P = 0.0167$; and for HELP $G = 22.198$, $P = <0.0001$.

The seeds sown on solid media tended to germinate poorly. The medium in this set of experiments contained (1) the auxin antagonists TIBA, Alanap and PCIB, (2) the ethylene antagonists AVG, cobalt chloride and silver nitrate and (3) the Hyp-protein antagonists 3,4-dhp, 2,2'-dp and Hyp. Silver nitrate in micromolar concentrations of 10-, 50- and 100- μm prevented germination in both experiments.

PCIB, cobalt chloride and TIBA all allowed some germination, and all produced phenovariations. Unfortunately, the total number of seedlings that developed was too small to conduct a rigorous statistical analysis. Nevertheless, all the antimetabolite-treated seedlings showed some phenovariation, while the controls showed none in the solid medium experiment. All the types of phenovariation obtained in experiments conducted with raft cultures were observed in this set of cultures. The total variation observed from all 8 antimetabolites and both culture methods included anisocotily with secondary phyllomorphs at the base of the macrocotyledon, *Streptocarpus* plurifoliate-type (Figs. 7, 8, 10, 12, 13, 15, 18, 19), anisocotily with secondary phyllomorph at the juncture of the two cotyledons, *Streptocarpus* plurifoliate-type (Figs. 4, 5, 11, 17, 20, 25, 26, 27) multiple phyllomorphs, *Saintpaulia*-type (Figs. 2, 21, 22, 28). anisocotily with some plumular growth *Streptocarpella*-type (Figs. 16, 23, 24) or isocotily with some plumular growth, Gesnerioideae-type, (Figs. 1, 3, 6, 9, 14, 26), The phenovariation induced by the antimetabolites showed no particular relationship with individual antimetabolites. All seemed to involve the desuppression of the shoot apical meristem, and in some cases, reverse the stoppage of microcotyledon growth. Generally suppressed seedling growth was observed in the plants treated with any concentration of antimetabolite. In all cases when the seedlings remained viable in continued growth, the phenovariant patterns induced proved transitory, presumably persisting only as long as the *S. prolixus* seedlings were exposed to effective (micromolar) concentrations of the antimetabolites.

Discussion

To test the hypothesis that the interrelated actions of auxin, ethylene, and some hyp-protein can act to locally suppress growth and development in a flowering plant, the experimental protocol established in the work with leafy liverworts was employed (Basile and Basile 1990). The interference with the normal functioning of each of the three factors was carried out with the use of three different acting antimetabolites in the case of auxin and hyp-proteins, and two, in the case of ethylene. TIBA and Alanap are both known to be antagonists of auxin transport (Neidergang-Kamien and Leopold 1957) and PCIB of auxin action (Nakamura and Takahashi 1968). AVG is a powerful antagonist of ethylene synthesis (Yu and Yung 1979) and cobalt chloride an antagonist of ethylene action (Grover and Purves 1976). The antagonist 3,4-dhp is a specific inhibitor of prolyl hydroxylase, the enzyme that converts proline (Pro) to hydroxyproline (Hyp) (Cooper and Varner 1983, Nolan et al 1978). The antagonist 2,2'-dp is a chelator of ferrous iron that is an essential cofactor for the enzymatic conversion of Pro to Hyp (De Kock and Vaughn 1975). The antagonist trans-4-hydroxy-L-proline, is a structural analog of Pro that is able to be inserted into the position normally occupied by Pro, thereby disrupting the usual sequence of amino acids (Basile et al 1987). The result of the application of each of these eight antimetabolites on germinating seeds of *Streptocarpus prolixus* was the induction of phenovariations of remarkable similarity (Figs. 1-28). This appears to be an extension

of the findings of Basile and Basile (1990) in liverworts and Mignone and Basile (2000) in mosses. Bryophyte gametophytes and Anthophyte sporophytes seem to be reacting in a similar way, to the same plant regulators, using the same experimental approach. For example, *Plagiochila arctica* is a leafy liverwort that appears to be the product of reductive evolution. Organ primordia once thought to be "lost" develop into a third ventral leaf, when the same set of antimetabolites were applied (Basile and Basile 1984) . This is an example of the reversal of the role of localized suppression to stop organ primordia after they are initiated, which is a corollary of the Suppression Theory. In *S. prolixus*, a flowering plant that again appears to be the product of reductive evolution, suppressed shoot apical meristems are induced to develop plumules or secondary phyllomorphs. These two morphogenetic patterns phenocopy those of different genera and subgenera of the Gesneriaceae, some thought to be more primitive. It would appear to be a confirmation by interference of the second, and possibly third roles of localized suppression of cell populations, both to stop organ primordia, and by modulating the size and shape of determinate organs to thus generate form.

Continued observation of plants that showed phenovariation in early stages of morphogenesis, showed a reversion to the normal unifoliate growth habit after a period of 4 months or so, even though the phyllomorph remaining may not have been the original macrocotyledon. This is an indication that the phenovariation induced was transitory, and that the changes observed were most likely due to the perturbation of some normally carried out physiological process. The specific mechanics of how these

three factors interrelate to suppress the shoot apical meristem in these plants remains unknown, and the genetic sequences responsible for initiating the expressed pattern of growth and/or suppression in this genus have not been identified. Tsukaya (2000) in studies with *Monophyllaea*, a genus in the same subfamily Cyrtandroideae as *Streptocarpus*, uses *Arabidopsis* as a model for the mechanisms that regulate shoot apical meristem activity. He proposes that plants in *Monophyllaea* lack the gene(s) required for expression of shoot apical meristem genes at appropriate positions at the vegetative stage. With the work accomplished with examples of reductive/regressive evolution in the liverworts and with *S. prolixus* we might venture to suggest that the "lacking genes" may be unexpressed. Instead, Tsukaya suggests that there are expressed, at the base of the cotyledon, genes whose products function in the peripheral zones of authentic shoot apical meristems. Closer to the model plant studied in this paper, (Imaichi et al 2000) studied the unifoliate species *Streptocarpus grandis*. He suggests that meristems of the phyllomorph might be the result of embryonic meristematic activity that remains in the shoot apical meristem region and the cotyledons. Whether this suppressed shoot apical stem activity in *Monophyllaea* and *S. grandis* would respond to the same antimetabolite treatment as in *S. prolixus* provides a challenge for the future. The results obtained by working with *S. prolixus*, a facultative unifoliate species, have however produced positive examples. The usual early morphology of the species is altered when any of three growth regulators, auxin, ethylene, or hyp-proteins is interfered with by any of two or three different acting antimetabolites of each of these growth regulators. The "desuppression" achieved by

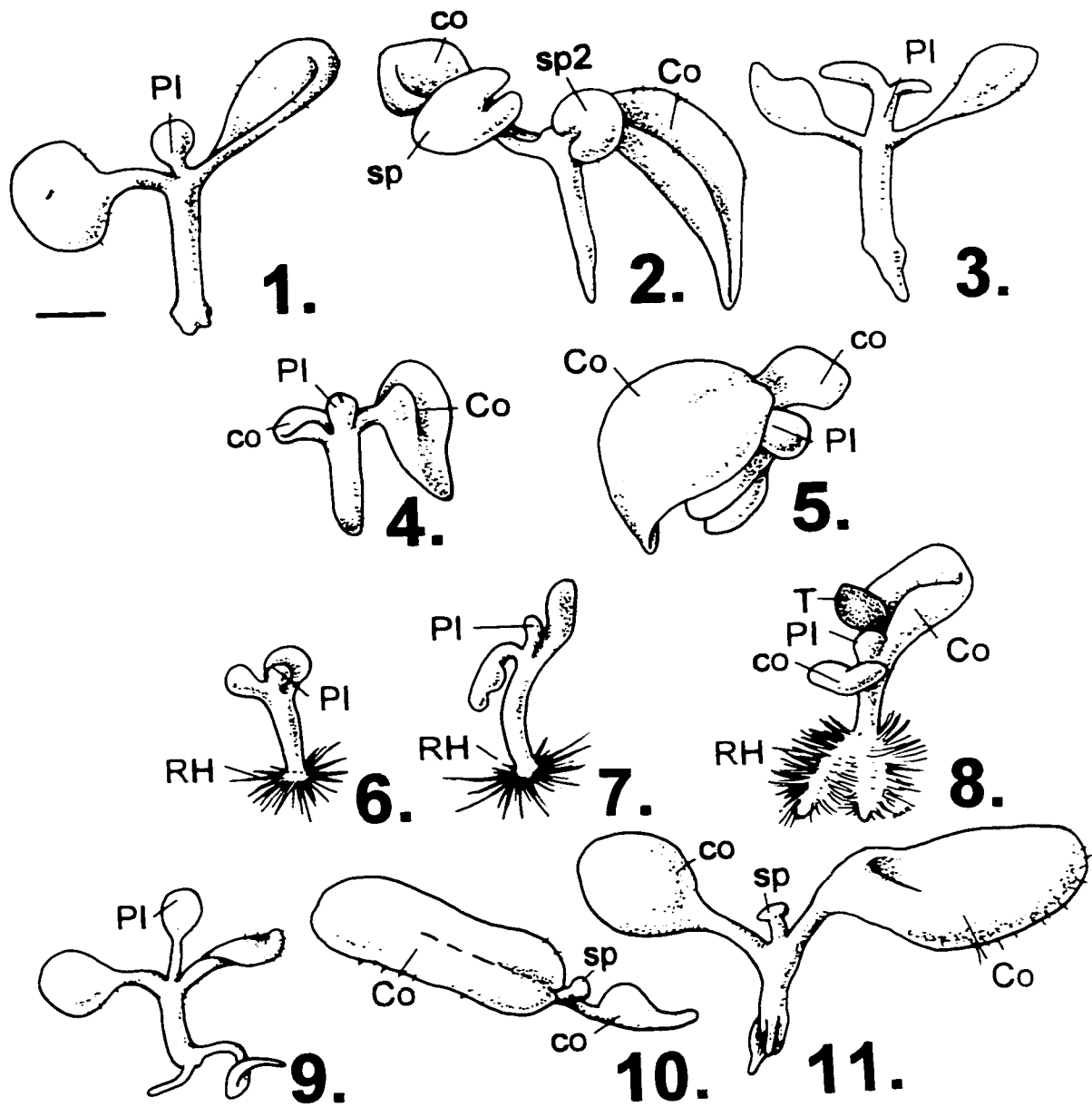
the highly derived, reduced plants in both the liverwort gametophytes and the Gesneriad sporophytes, representing the extremes of the spectrum of plant diversity suggests that the roles and means of control stipulated in the "Suppression Theory" may be generally applicable for land plants.

Table 1. A comparison of the phenovariant types that developed in response to antimetabolites expressed in percentages of the total sample number. N = sample number, I = Anisocotyly with secondary phyllomorph, *Streptocarpus* plurifoliolate-type, II = Multiple phyllomorphs, *Saintpaulia*-type, III = Anisocotyly with plumular growth, *Streptocarpella*-type, IV = Isocotyly with plumular growth, Gesnerioideae-type. %PV = percent of seedlings of all types showing phenovariation, P = probability based on a Chi Square analysis.

* The control percent tested in the special case of frequencies divided into $a = 2$ classes, shows that there is insufficient evidence to reject the null hypothesis that 10% phenovariation occurs naturally in this species (Rosenblum 1981).

Antimetabolite	N	I	II	III	IV	%PV	<i>P</i>
2,2'-dp	58	17	2	14	2	35	0.0006
3,4-dhp	79	9	0	15	1	25	0.0167
Hyp	153	7	5	18	8	37	<0.0001
AVG	54	13	0	15	2	30	0.0063
Alanap	97	6	1	12	9	29	0.0021
Control*	103	10	0	0	2	12	

Figures 1- 11. Antagonist-induced phenovariation in *Streptocarpus prolixus*. Figs. 1-8. Phenovariation induced by 50 μ M concentrations of two antagonists of auxin transport, and one antagonist of auxin action. Figs. 1-3. Phenovariations induced by Alanap, an inhibitor of auxin transport. Fig. 2. Note the multiple phyllomorphs, Fig. 3. Note the plumule with opposite leaves. Figs. 4-5. Phenovariations induced by TIBA, an inhibitor of auxin transport. Although they are accrescent there appears to be a plumule forming at the junction of the cotyledons. Figs. 6-8. Phenovariation induced by PCIB, an inhibitor of auxin action. Note the unusually long and abundant root hairs. Figs. 9-11. Phenovariations induced by AVG an antagonist of ethylene synthesis. Figs. 9,10. Phenovariations induced by a concentration of 1 μ M AVG. Fig. 11. phenovariation induced by a concentration of 10 μ M AVG. When the cotyledons are equal in length (isocotyly) they are not labeled. Co = macrocotyledon, co= microcotyledon, Pl= plumule, RH = root hairs, sp = secondary phyllomorph, sp2 = additional phyllomorph, T= testa. Bar = 1mm.



Figures 12-22. Antagonist-induced phenovariation in *Streptocarpus prolixus* (cont.).

Figs. 12-13. Phenovariation induced by cobalt chloride, an inhibitor of ethylene action.

Figs. 14-22. Phenovariations induced by different-acting antagonists of Hyp- protein

synthesis. Fig. 14-16. Phenovariation induced by 3,4-dehydroproline, an inhibitor of an enzyme essential for the hydroxylation of proline. Figs. 17-19. Phenovariation induced

by 2,2'-dipyridyl, a chelator of ferrous iron, one of the necessary cofactors for the

synthesis of Hyp-proteins. Figs. 20-22. Phenovariation induced by 4-hydroxy-L-

proline. When used exogenously, it acts as a competitive inhibitor of proline. The

changes from the normal growth pattern of *Streptocarpus prolixus*, which in the time

frame illustrated would display anisocotily alone, take a number of forms.

Abbreviations as in Figures 1-11.

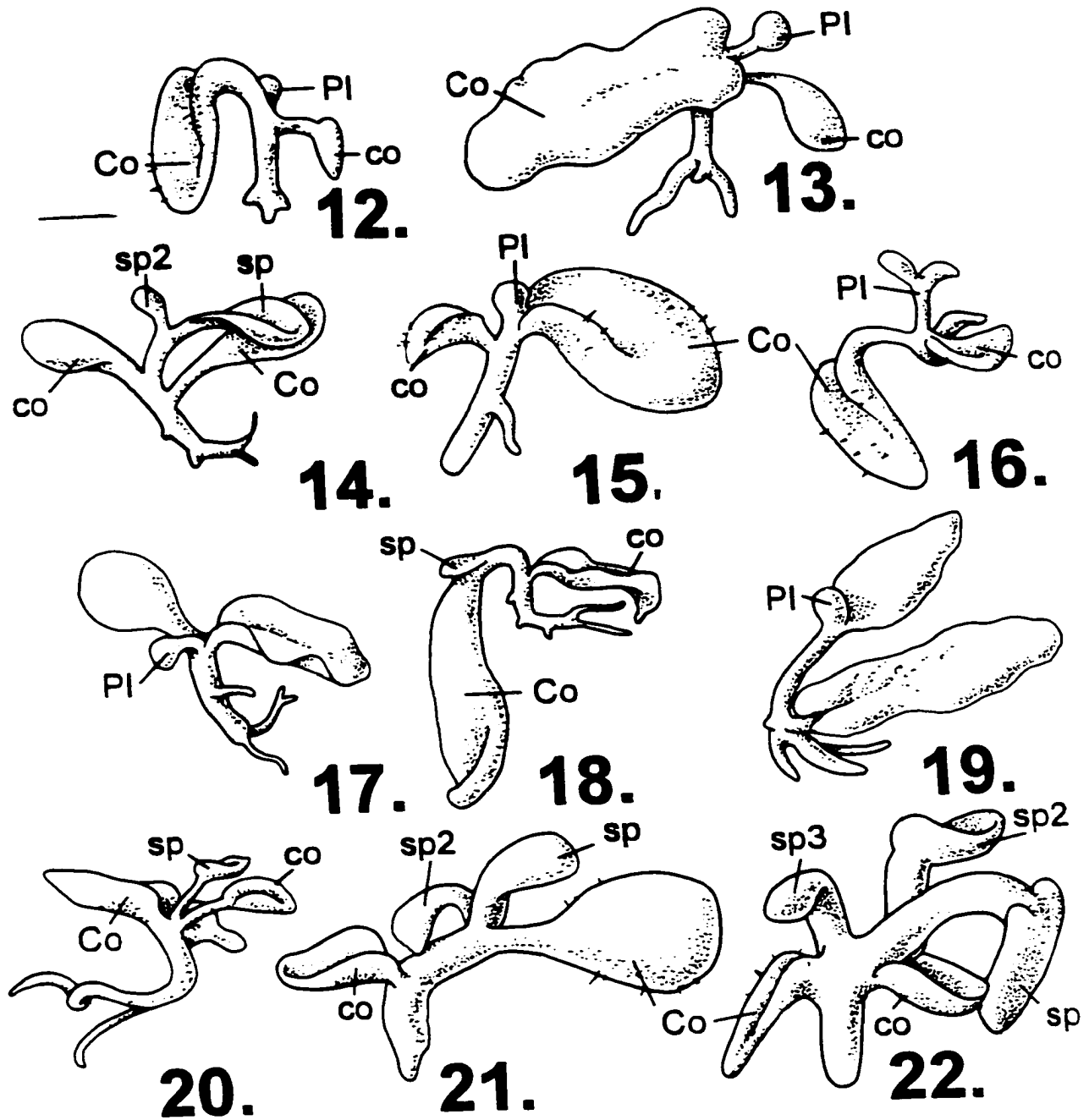
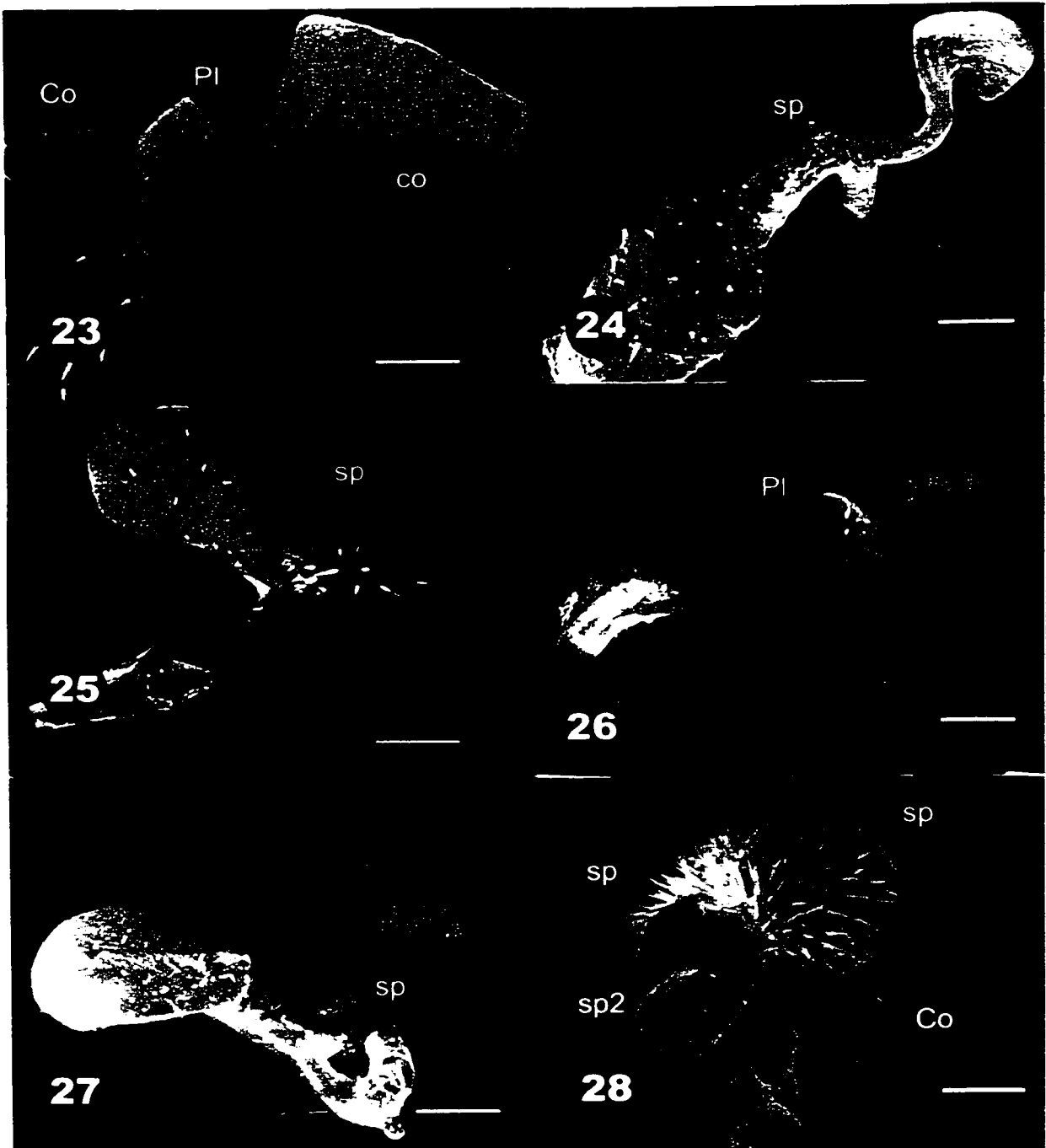


Figure 23-28. Scanning electron micrographs of phenovariants induced in *S. prolixus*. Figs. 23-24. Phenovariation induced by AVG, an ethylene synthesis inhibitor. Fig. 23. *Streptocarpella*-type III. Fig. 24. *Streptocarpus* plurifoliate-type I. Figs. 25-26. Phenovariation induced by Alanap, an auxin transport inhibitor. Fig. 25. *Streptocarpus* plurifoliate-type I. Fig. 26. Gesnerioideae-type IV. Figs. 27-28. Phenovariation induced by antagonists of Hyp-protein synthesis. Fig. 27. Phenovariation showing isocotyly and a secondary phyllomorph induced by 3,4-dehydroproline, a specific inhibitor of an essential enzyme needed for the hydroxylation of proline. Fig. 28. *Saintpaulia*-type, induced by exogenous application of Hyp, a structural analog of the normal hydroxylation of proline. The phenovariations copy phenotypes of plants in the subgenus *Streptocarpus*. More remarkably, they also phenocopy morphotypes of species in a different subgenus *Streptocarpella*, genus *Saintpaulia* and subfamily Gesnerioideae of the Gesneriaceae. Bar = 0.5mm.



CHAPTER 2
INDUCTION OF PHYLETIC PHENOCOPIES
IN STREPTOCARPUS (GESNERIACEAE) BY THREE ANTAGONISTS OF
HYDROXYPROLINE- PROTEIN SYNTHESIS

Introduction

Plants in the family Gesneriaceae display a wide range of vegetative morphologies. The family separates into two subfamilies, based on the relative size of the cotyledons of their respective plants (Burt, 1963). In the subfamily Gesnerioideae, cotyledons remain equal, a phenomenon that is a basal synapomorphy of dicotyledons. In the subfamily Cyrtandroideae, cotyledons develop unequally, a condition known as anisocotily. Burt (1970) suggests that the evolutionary advantage of the untypical and unusual seedling morphology is tied to environmental factors. He couples anisocotily with numerous, tiny seeds lacking endosperm. Microspermy, which in other forest plants is ameliorated by symbiosis or saphrophytism, is in this case helped by the immediate production of photosynthetic tissue because of the precocious enlargement of one of the cotyledons. In some species of the genus *Streptocarpus* as well as other genera scattered throughout the Cyrtandroideae, the single cotyledon continues development to become the entire vegetative structure of the mature plant. This reaches an extreme in the genus *Monophyllaea* which completely lacks a shoot apical meristem (Cronk and Moeller 1997). The "fuzzy morphology" (Rutishauser 1995) of this self-contained organism (half leaf-like, half stem-like) has been termed a phyllomorph, and the axis, a combination stem/petiole is known as a petiolode (Jong 1970, 1973).

It is possible to arrange the morphotypes of the genus *Streptocarpus* into a series. At one extreme is this highly derived unifoliate form where the accrescent, larger cotyledon continues to grow. The subgenus *Streptocarpus* defines those species

that display this morphology and those with closely related acaulescent organization. At the other extreme is the more familiar caulescent, node-internode type shoot organization that occurs after anisocotylly, in the subgenus *Streptocarpella*. In a study by Moeller and Cronk (1997) designed to clarify the phylogenetic relationships of the genus and the related genus *Saintpaulia*, evidence was obtained that *Streptocarpus* is not monophyletic. This was supported by a further analysis by Smith et al (1998). Their results indicate a monophyletic *Saintpaulia* nested within a paraphyletic *Streptocarpus*. The subgenera, *Streptocarpus* and *Streptocarpella* are revealed as separate clades, with *Streptocarpella* being further divided geographically (Fig. 54)

The morphological development of the genus *Streptocarpus* thus appears extremely flexible, as expressed in the variety of vegetative forms exhibited by the various species (Hilliard and Burtt, 1971). Occasionally it will manifest itself in a single species. *S. nobilis* C. B. Clarke, usually a caulescent variety belonging to the subgenus *Streptocarpella*, under adverse conditions will act as a facultative unifoliolate.

In between the extremes of caulescent and monocarpic unifoliolate patterns in this series are a number of intermediate forms. Certain species in the subgenus *Streptocarpus* form rosette-like groupings of phyllomorphs, and are called “rosulates”. Jong (1978) breaks these down into two further classifications. One is “centric rosulate” in which the additional phyllomorphs are grouped about a vertical central axis; the other is “excentric rosulates”, in which the prostrate petiolode is not unlike a rhizome. The additional phyllomorphs cluster adventitiously along the dorsal side. A

third form "plurifoliate", act as unifoliate for most of their life span, but initiate additional phyllomorphs at the time of flowering.

The species used in these experiments, *S. prolixus* C. B. Clarke, is classed as a plurifoliate. It was the primary species used in experiments by Rosenblum (1981) and Rosenblum and Basile (1984) for ease and speed of growing, as well as for its facultative unifoliate habit. As may be seen from Figs. 29-32, its normal growth begins with isocotly, it soon becomes anisocotyledonous, with one cotyledon growing larger 3 or 4 days after germination. The smaller microcotyledon ceases enlargement, is gradually displaced by growth of the petiolode between the two cotyledons, and eventually falls off (see also Fig. 1 Rosenblum and Basile 1984 page 32). Rosenblum (1981) used this plant to study the morphogenetic effects of various hormones, but it also served as a model for induction of "phyletic phenocopies". The term "phyletic phenocopies" was proposed by Stebbins and Basile (1986) to define changes in form that mimic the typical form of a related phenotype, particularly one characteristic of a different taxon. The authors suggest that by inducing plants to produce these phenocopies, investigators can obtain clues to the developmental basis of evolutionary change.

Basile (1967, 1969, 1970, 1973a, 1979; Basile and Basile 1980a) in a series of experiments, induced phyletic phenocopies in species belonging to five distinct families of leafy liverworts. In each case exogenously supplied antagonists of Hyp-protein synthesis produced phenotypic changes that phenocopied characteristics of more primitive species of different genera and families of leafy liverworts (see Basile 1990,

for review). The most consistent phenovariation in Basile's series of experiments was the reappearance of a suppressed ventral leaf, and the shift in organization of the plant axis from a dorsi-ventral to a radial symmetry. In other examples, changes in leaf size and shape and more primitive branching patterns were induced (Basile and Basile 1980b, 1982).

Rosenblum (1981; Rosenblum and Basile 1984) working with *S. prolixus*, succeeded in inducing phenovariants by primarily using applications of gibberellic acid (GA₃). Although experiments with GA₃ showed that the capacity to express a node-internode type plant body was not "lost or lacking" in *S. prolixus*, the authors did not test what Basile and Basile (1984, 1993) hypothesized to be the more pivotal role of Hyp-proteins, presumably arabinogalactan-proteins (AGP's), in place-dependent suppression.

To test this hypothesis, three distinctly different-acting antagonists of the normal synthesis of Hyp-proteins were employed here, as they had previously been in the leafy liverwort experiments. The antagonist 3,4-dehydroproline (3,4-dhp) is a specific inhibitor of the enzyme, prolyl hydroxylase, the enzyme that converts proline to hydroxyproline (Cooper and Varner 1983, Nolan et al. 1978). The antagonist 2,2'-dipyridyl (2,2'-dp) is a chelator of ferrous iron that is an essential cofactor for the enzymatic conversion of proline to hydroxyproline (De Kock and Vaughan, 1975). The antagonist trans-4-hydroxy-L-proline (Hyp) is a structural analog of proline that can be inserted in the position normally occupied by proline, thereby disrupting the usual sequence of amino acids (Basile et al. 1987). The objective of the experiments

reported here was to determine whether the exogenous application of micromolar concentrations of the three Hyp-protein-antimetabolites would induce phyletic phenocopies in *S. prolixus* analogous to those induced in leafy liverworts (Basile and Basile 1984).

Results and Discussion

In all cases, the exogenous application of the antagonists of Hyp-protein synthesis "desuppressed" the normally suppressed shoot apical meristem of *S. prolixus*. This desuppression resulted in a variety of morphotypes that did not appear to be correlated with the type of inhibitor. The desuppression of the shoot apical meristem, however, apparently triggered suppression of the growth rate of other parts of the seedlings. The size of the phenovariants remained in the 1-3-mm range, whereas the control seedlings reached lengths of 13-20 mm during the same time. The pattern of development without antimetabolite inhibition was typical for *S. prolixus*. (Figs. 29-32 and #1 in Table1).

In experiments conducted with the pot-cultured plants, micromolar concentrations of the antimetabolites induced no discernible variation from the controls, whereas millimolar concentrations of Hyp induced pronounced phenovariation. The pot-cultured phenovariants displayed a variety of forms. Some phenovariants phenocopied features characteristic of species of subgenus *Streptocarpella* (Figs. 33-36), whereas other phenovariants phenocopied features characteristic of centric (Fig.37) and excentric (Fig. 38) rosulates of the subgenus *Streptocarpus*. The plant shown in Fig 38, after a time of displaying typical

cotyledonous accrescence, suddenly produced a number of small phyllomorphs simultaneously aligned along the prostrate rhizome-like petiolode. This development very closely approximates the growth pattern of the mature morphotype of *S. primulifolius* Gand., an excentric rosulate (Hilliard and Burt, 1971). However, the rhizome-like habit in this latter species is a condition that results from many seasons of continuous development. The small, additional phyllomorphs of the induced variant only lasted about 2 weeks, withering and disappearing as suddenly as they appeared, even though the antimetabolite Hyp was regularly applied at 10mM over an extended period. Another pot-grown phenovariant (Fig. 37) strongly resembled *S. gardenii* Hook., a typical centric rosulate species (Jong, 1978). The seedlings were small (total measurement across the entire plant was 17-18 mm) and very young (54 days after the onset of germination), yet they temporarily mimicked the structure of plants of several season's growth.

The results obtained when seeds and seedlings were continuously exposed to antimetabolites via filter paper platforms, however, were decidedly different from when they were periodically drenched in soil mix. In experiments conducted with the platform cultures, micromolar concentrations of all three of the antagonists were sufficient to induce phenovariation. Although some phenovariation occurs naturally (10 to 15% according to Rosenblum 1981), the antimetabolites induced noticeably higher percentages (Table 1). A 2x4 contingency table analysis of Table 1, showed a highly significant relationship between the presence of phenovariation and the type of experimental treatment. A total of 12% of controls produced phenovariants as

compared with 35% of 2,2'-dp-treated seedlings, 25% of 3,4-dhp-treated seedlings, and 37% of Hyp-treated seedlings. ($G=23.8$, 3df, $P < 0.0001$).

The observed morphotypes exhibited five different patterns:

No PV.- Normal (for *S. prolixus*) anisocotyledonous growth coupled with suppression of the shoot apical meristem and continuous growth of the macrocotyledon (Fig. 32).

I.- Anisocotyledonous, with a second phyllomorph displaced to the base of the macrocotyledon. This morphotype is a very early and untypical manifestation of the plurifoliate habit of *S. prolixus* (Figs. 40, 46, 47). A continued growth of second and third phyllomorphs arising from the base of the preceding phyllomorph mimicked the pattern of mature plants in the species *S. fanniniae* Harvey ex C. B. Clarke and *S. davyii* S. Moore (Figs. 41, 48)

II.- Multiple phyllomorphy, characteristic of the rosulate species of the subgenus *Streptocarpus* and the closely related genus *Saintpaulia* (Figs. 45, 53).

III.- Some accrescence, with plumular growth appearing in the axis of both cotyledons which phenocopies the pattern of species in the subgenus *Streptocarpella* (Figs. 42,49).

IV.- Isocotyly with plumular growth, which reproduces the growth pattern of members of the subfamily Gesnerioideae (Figs. 43, 44, 50-52).

Discussion

As stated earlier, the objective of this study was to determine whether exogenously supplied antagonists of Hyp-protein synthesis would induce phenovariation in a flowering plant family as had been previously observed in leafy liverworts. The results obtained show that they can. The Hyp-protein(s) responsible for the observed effects is not known. Evidence from studies conducted with bryophytes (Basile and Basile 1990, 1993) suggests that one or more AGPs are the likely candidates. The AGPs are Hyp-containing glycoproteins and proteoglycans associated with the plasma membranes and cell walls of all classes of land plants (Basile and Basile 1987, 1993, Clarke et al., 1978). Prior to this study, both experimental and inferential evidence had been advanced to indicate that various AGP's may function in cell recognition, cell-cell communication and morphoregulation (Basile and Basile 1990, 1993, Clarke and Knox, 1978; Clarke et al., 1978; Fincher et al., 1983; van Holst and Clarke, 1986) have found changes in the physical properties of some AGP's correlated with Hyp-protein antagonist-induced phenovariation in liverworts. Serpe and Nothnagel (1994), using an agent to perturb the functions of AGPs in living cells, suppressed cell proliferation of cell-suspension cultures of rose. Cell proliferation resumed (was "desuppressed") when the AGP-perturbing agent was removed. Thompson and Knox (1998), using the same reagent, suppressed the development of shoots in embryonic carrot cultures. Investigations to determine the nature of the Hyp-protein(s) altered by the antagonists that induced phenovariation in

S. prolixus, however, constitute the basis for a separate study. In this study, emphasis is on the phenotypic consequences of perturbing Hyp-protein synthesis.

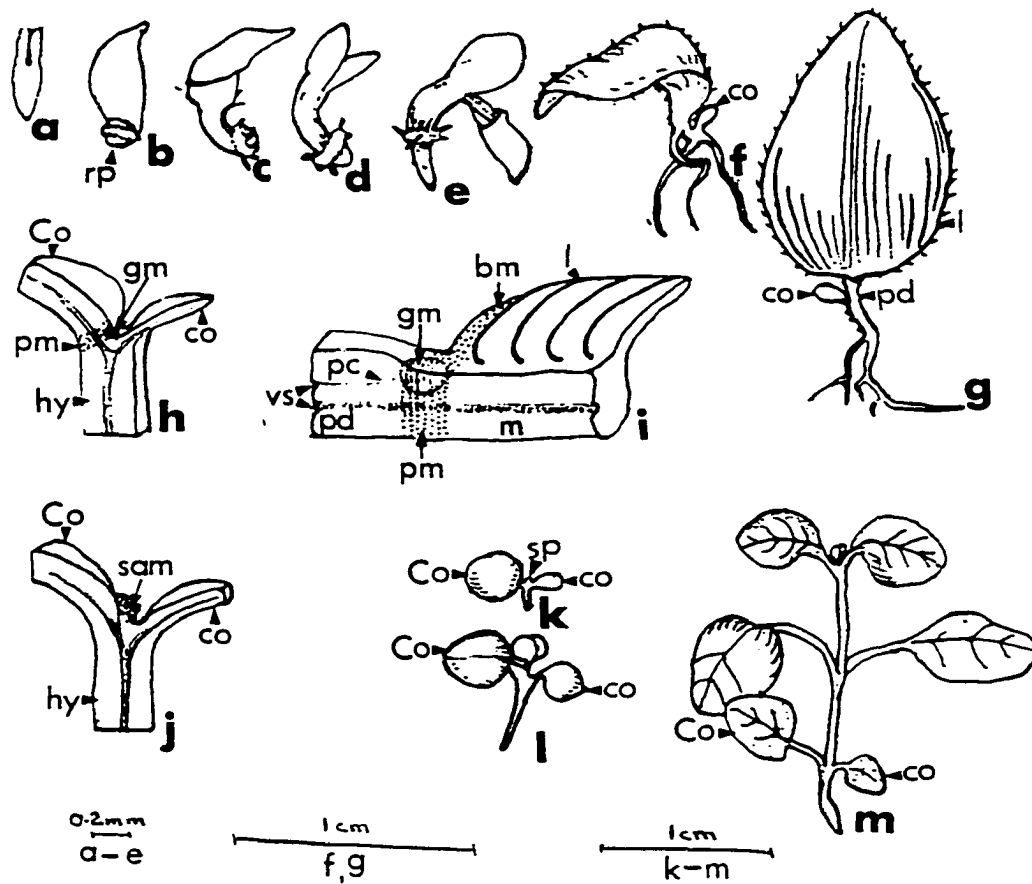
By the exogenous application of three Hyp-protein antagonists, a single species of *Streptocarpus* was induced to express morphological features extending not only through both subgenera of *Streptocarpus*, but also through two subfamilies of the Gesneriaceae. The phenovariants induced in *S. prolixus* can be divided into four basic types: anisocotyly (Cyrtandroideae) with the premature introduction of a second phyllomorph at the base of the macrocotyledon (*Streptocarpus*, plurifoliate-type), anisocotyly with multiple phyllomorphs (*Saintpaulia*-type), anisocotyly with plumular growth (*Streptocarpella*-type), and isocotyly with plumular growth (Gesnerioideae-type). All the morphotypes induced, except for number IV, are represented in the various clades shown in the molecular analysis of *Streptocarpus* and *Saintpaulia* by Moeller and Cronk (1997). The two rosulate types are not clearly defined in this tree, but appear randomly in the rosulate clade (Fig. 54). Isocotyledonous phenovariants (number IV in the table) display a character trait that appears even earlier on the evolutionary time line than is shown in Moeller and Cronk's (1997) analysis.

It is probable that the varied morphologies of the genus *Streptocarpus*, as in the liverworts previously chosen for experimentation, occur because of "differences with respect to the time and place cell proliferation and/or enlargement is suppressed" (Basile and Basile, 1993). (The unifoliate species of *Streptocarpus* and the several species of liverworts selected for experimentation were considered to be evolutionarily derived via reduction in the number and/or the kind of organs they could develop.) In

the studies using liverworts, antagonism of Hyp-protein synthesis resulted in desuppressed leaf and branch development. In the present study, antagonists of Hyp-protein synthesis lead to desuppression of the primary shoot apical meristem and microcotyledon of *S. prolixus*. It appears then, that certain Hyp-proteins have essentially the same role in mediating place-dependent suppression in plants as diverse as liverwort gametophytes and flowering plant sporophytes. The wide evolutionary divergence of these two groups suggests a molecular homology that may be the same for all land plants, and provides additional support for the relevance of the "suppression hypothesis" (Basile and Basile, 1993) in general, and the role of Hyp-proteins in place-dependent suppression in particular.

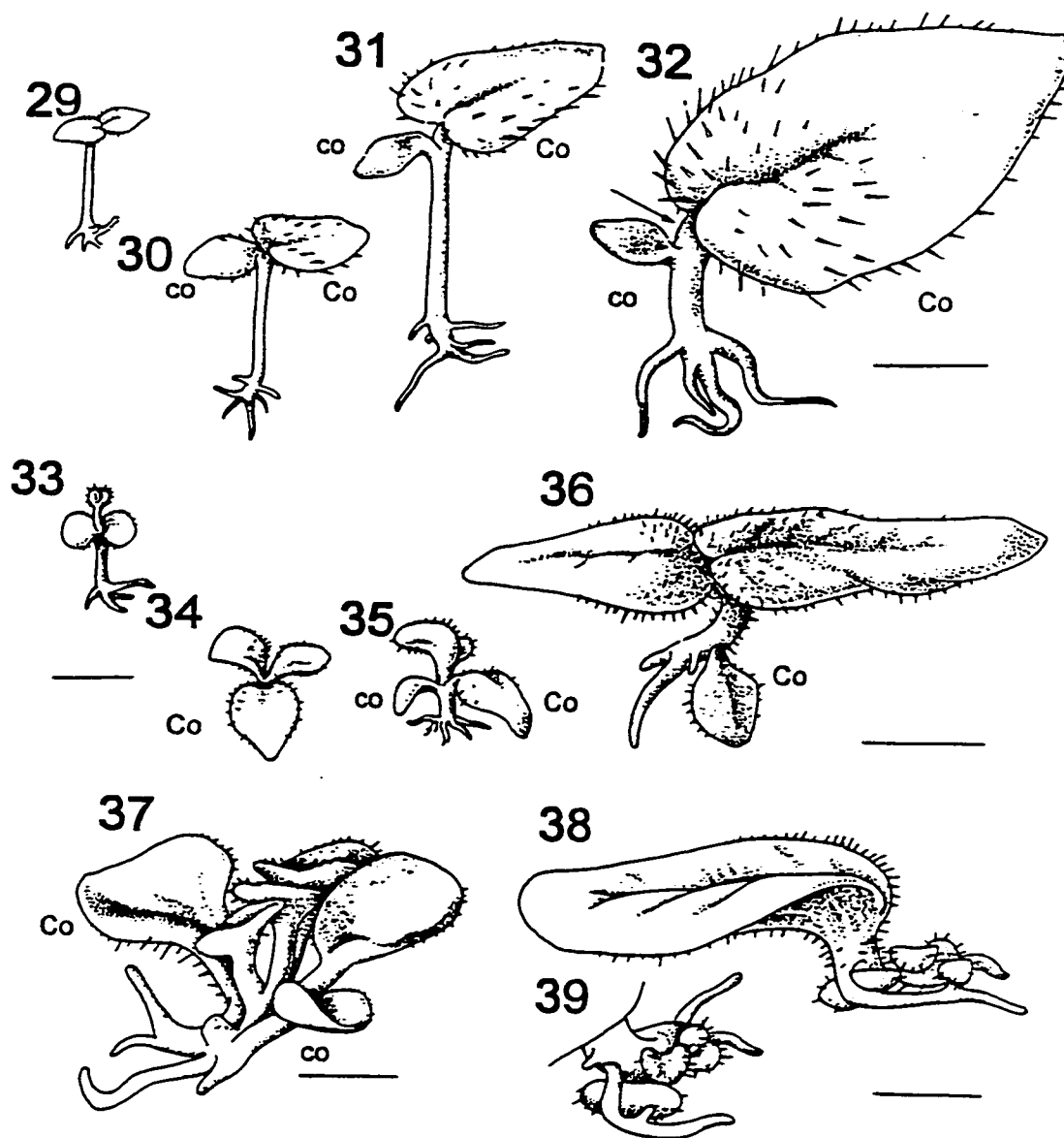
Rosenblum and Basile 1984 Figure 1. Drawing showing pertinent stages of ontogeny and histogenesis of *Streptocarpus* seedlings, redrawn from Jong 1970 (a-e, h-j) and Hilliard and Burtt, 1971 (f-g, k-m) with permission. a-j. Representative of acaulescent species, subgenus *Streptocarpus*. a,b. *S. grandis* embryo and at day 1, respectively. c,d,e. *S. gardenii*, days 3,4,6, respectively. Note that an organized primary root is lacking at germination and develops slowly subsequently. f,g. Early stages of phyllomorph development of *S. grandis*. f. Lamina development at 4 wk. g. Petiolode development at 2 months. h-i. Diagrams to show the relationship of the intercalary meristems for phyllomorph development from sections of *S. fanniniae*, subgenus *Streptocarpus*. h. A zone of meristematic cells, the petiolode meristem, extends from original embryonic shoot meristem across the hypocotyl at the base of one of the two cotyledons. Cell maturation occurs both proximal and distal to this zone. From the former, the development of the petiolode and the displacement of the embryonic meristem which becomes the groove meristem result; from the latter, the elongation of the midvein. i. A zone of meristematic cells, the basal meristem, extends laterally from the groove meristem and is contiguous with the petiolode meristem over the midrib. All tissues of the lamina, exclusive of the midrib, derive from this intercalary meristem. j-m. Representative of the caulescent species, subgenus *Streptocarpella*. j. Diagram of median l.s. of *S. nobilis* 6 wk to show initiation of a shoot apical meristem from the displaced embryonic shoot meristem. k. Seedling of *S. hilsenbergii* at same stage as j. l-m. Later stages in development of *S. hilsenbergii* showing limited macrocotyledonary accrescence and delayed shoot development characteristic of the

subgenus. **bm**, basal meristem of the lamina; **col**, collar; **gm**, groove meristem; **hy**, hypocotyl; **l**, lamina; **m**, midvein; **pc**, procambium; **pd**, petiolode; **pm** petiolode meristem; **pr**, primary root; **rp**, root primordium; **sam**, shoot apical meristem; **sp**, shoot primordium; **vs**, vascular strand.



Figs. 29-39. Normal and phenovariant seedlings of plurifoliate *Streptocarpus prolixus*.

29-32. Stages in the normal development of seedlings; note suppression of the plumule. Bar = 2mm. 29. At 6 days after germination (DAG), note equal cotyledons. 30. At 20 DAG, the upper cotyledon has begun accrescence. 31. At 30 DAG, a distinct size difference is visible between the two cotyledons. 32. At 60 DAG, note expansion of petiolode, separating the two cotyledons (arrow). 33-39. *S. prolixus* seedlings from pot cultures treated with antimetabolites. 33-36. Phenovariation #3, the appearance of a plumule with anisocotyly. 33. At 23 DAG showing plumule arising from apparently equal cotyledons and two leaf primordia. 34. Frontal view 37 DAG showing developing leaves and accrescent macrocotyledon. 35. Side view of same stage of growth clearly showing anisocotyly. Bar = 2mm. 36. Later stage (71 DAG). The two leaves continue to enlarge, but there is no indication of further shoot development. In the next month the leaf withered and eventually fell off, and the plant resumed its unifoliate pattern. The visible cotyledon is the macrocotyledon; the microcotyledon had fallen off by this stage. Bar = 5mm. 37. Side view of multiple phyllomorphs (also shown in Figs 17 and 25) phenocopying a centric rosulate habit. Bar = 2mm. 38-39. Seedling showing similarity to the excentric rosulate pattern. Bar = 2mm. 38. Side view of phyllomorph showing horizontal rhizome-like petiolode, with a number of roots and four secondary phyllomorphs. 39. Detail view, looking down the petiolode. Within the next two weeks, all of the secondary phyllomorphs withered, and the plant resumed its traditional plurifoliate habit.



Figs. 40-45. Phenovariations in *Streptocarpus* seedling morphology. 40-44. (SEMs).

S. prolixus from platform culture, treated with antimetabolites. Bar = 0.5mm. 40.

Anisocotyly, with a secondary phyllomorph appearing at the base of the

macrocotyledon (#I in Table 1). 41. Further development of this variation; the

secondary phyllomorph shows an additional phyllomorph at the base of its lamina,

phenocopying the habits of *S. fanniniae* and *S. davyii*. 42. Some accrescence, with

secondary phyllomorph in axis of the two cotyledons (#I in Table 1). 43. Isocotyly

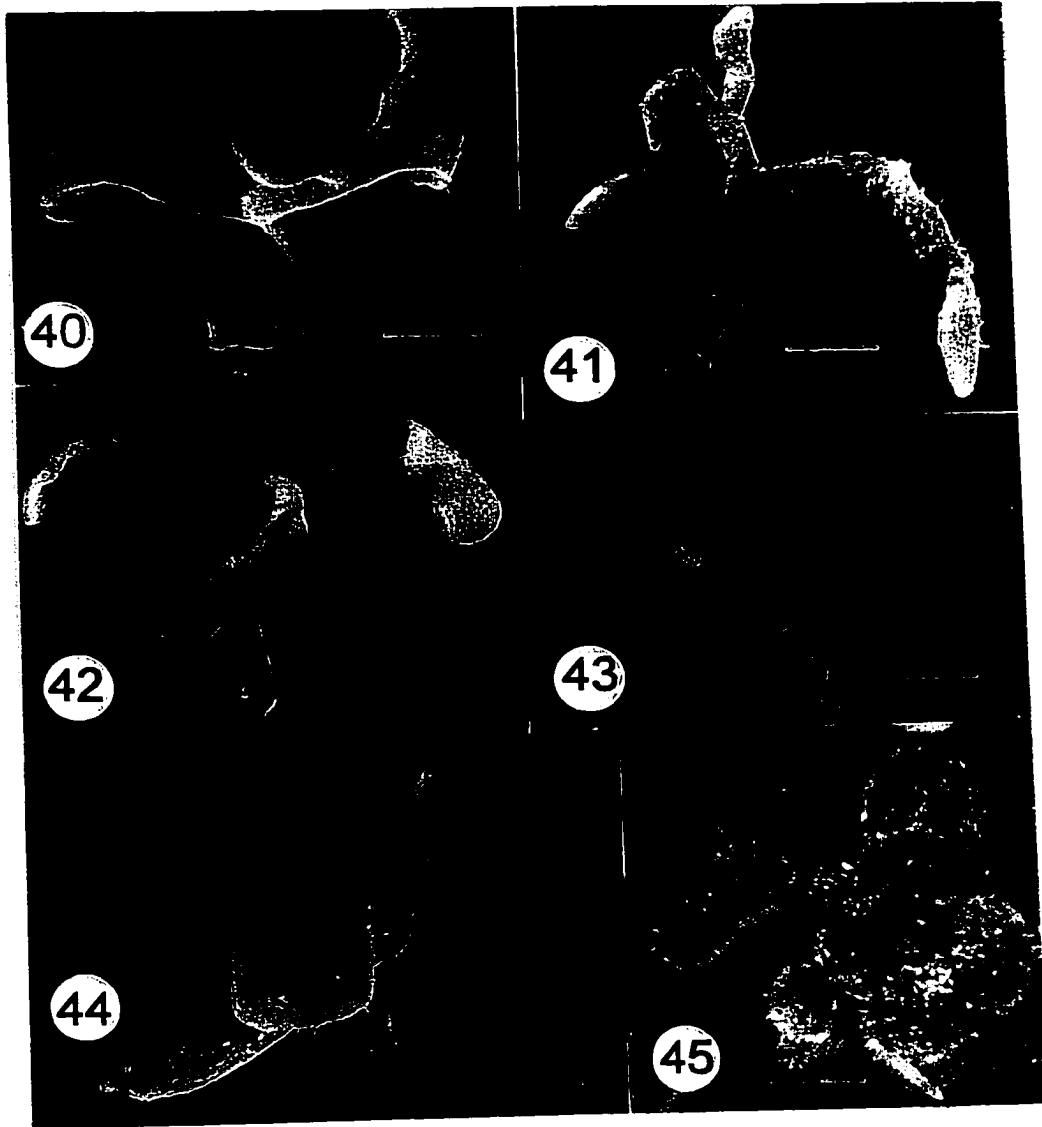
with plumule (#IV in Table 1);

note leaf primordia. 44. Further development of isocotyly with plumular growth (#IV

in Table 1), leaves show opposite decussate arrangement, typical of the subfamily

Gesnerioideae. 45. (light micrograph). Pot-grown seedling showing multiple

phyllomorphs (#II in Table 1).



Figs. 46-53. Diagrammatic interpretations of Figs. 40-45. Bar = 0.5mm. 46. Top view of Fig. 40. Note the position of the secondary phyllomorph. 47. A constructed side view of the same seedling, assisted by reference to micrographs of this angle of other seedlings showing similar morphology. 48. Phenovariation of Fig. 41, with phyllomorphs arising from the base of preceding phyllomorphs. 49. Rendering of Fig. 42. The appearance of size difference between the two cotyledons is slight because of a somewhat distorted view of the right cotyledon, which was actually 0.5mm longer than the left cotyledon. 50. Diagram of Fig. 43, ignoring damage to the specimen during mounting. Note the two leaf primordia. 51-52. Drawings of Fig. 44 from slightly different-angled SEM micrographs, making the opposite decussate leaf arrangement more apparent. 53. Top view of Fig. 45. Five secondary phyllomorphs are shown. The macrocotyledon is the larger leaf form, on the lower right. The microcotyledon is the small dark shape immediately above. Side view of the seedling is shown in Fig. 37. Abbreviations as in Figs. 29-39. Co, macrocotyledon; co, microcotyledon; sp, secondary phyllomorph; sp2, additional phyllomorph; pl, plumule; lp, leaf primordium.

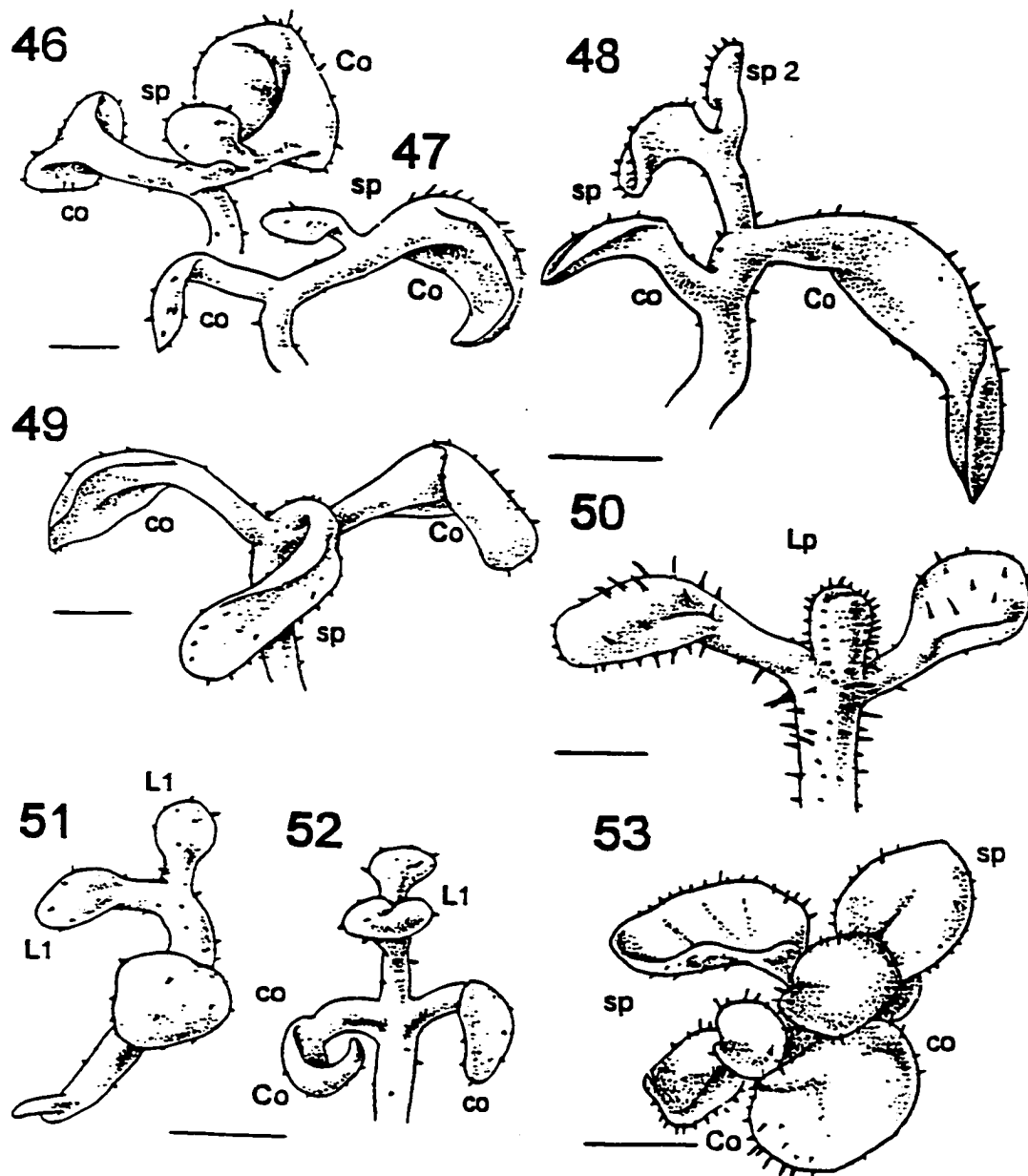
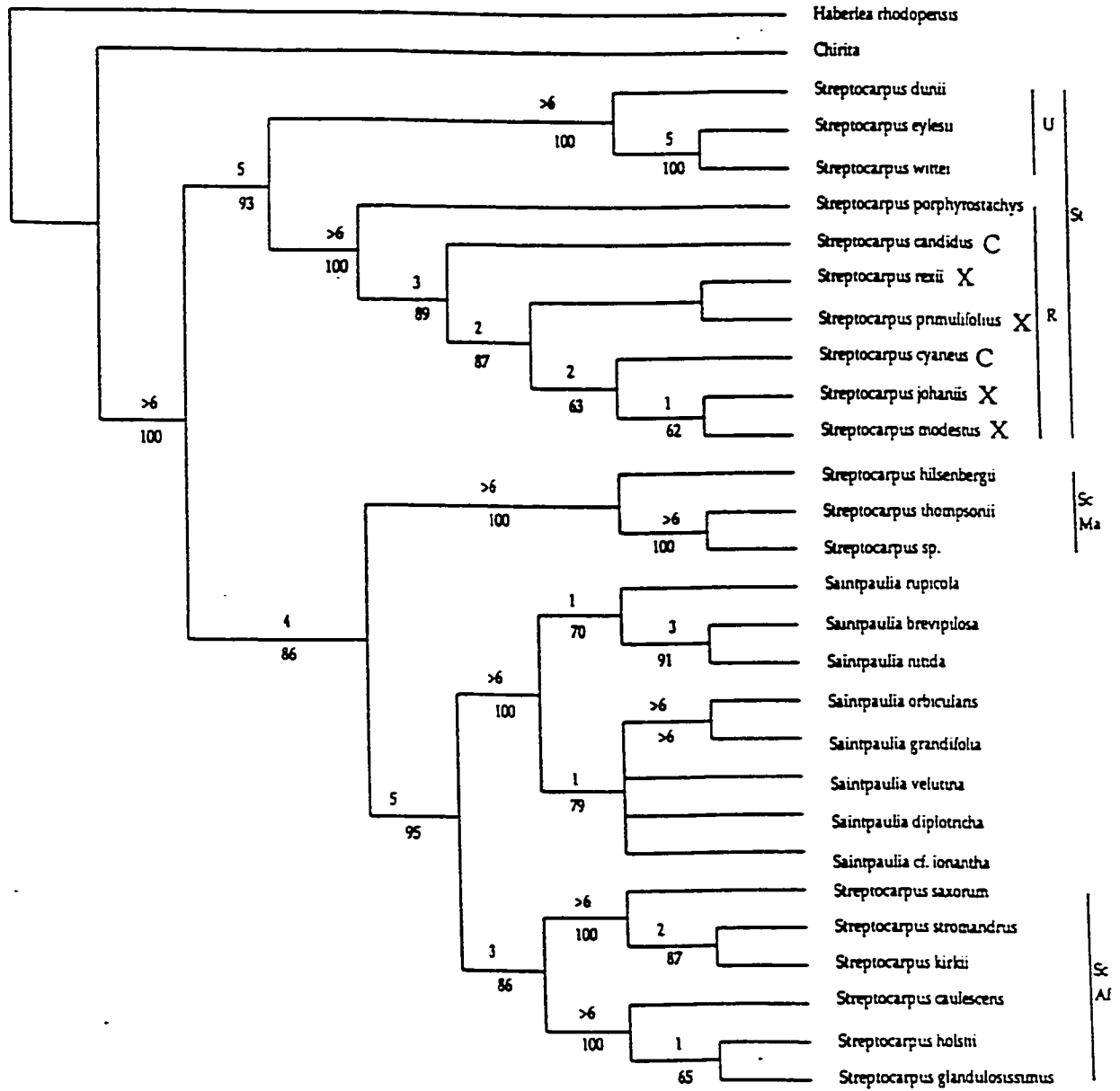


Fig.54. Most parsimonious tree of 419 steps (CI = 0.79, RI = 0.90) based on analysis of nuclear ribosomal DNA internal transcribed spacer sequences, including aligned gaps.. Subgenera of *Streptocarpus* are abbreviated as follows: St. subg, *Streptocarpus*; Sc subg, *Streptocarpella*; Ma, Madagascan species; Af, African species; Sections within St subg are indicated and abbreviated as U = unifoliate, R = rosulate, C = centric, X = excentric. Adapted from Smith et al., (1998). All species in this tree, including the outgroups are anisocotyledonous. Of the rosulates according to Jong (1978) *S. candidus* and *S. cyaneus* are centric. *S. rexii*, *S. primifolius*, *S. johannis* and *S. modestus* are excentric.



CHAPTER 3
PHENOVARIATION INDUCED IN *Streptocarpus prolixus* (GESNERIACEAE)
BY β -GLUCOSYL YARIV REAGENT

Introduction

In an earlier part of the study, experiments were performed to find out whether exogenously supplied antagonists of hydroxyproline-protein synthesis would induce the same types of phenovariation in a flowering plant as had been previously induced in several species of leafy liverworts (Rauh and Basile, 2000). It was found that the exogenous application of three Hyp-protein antagonists could induce a single species of *Streptocarpus* to phenocopy features characteristic of species placed in both subgenera of *Streptocarpus*, as well as features characteristic of species placed in both subfamilies of the Gesneriaceae. The phenovariation induced in *S. prolixus* could be categorized according to four morphotypes: (I) anisocotyly with premature formation of secondary phyllomorphs at the base of the macrocotyledon (*Streptocarpus*, plurifoliolate-type), (II) anisocotyly with multiple phyllomorphs (*Saintpaulia*-type), (III) anisocotyly with plumular development (*Streptocarpella*-type), (IV) isocotyly with plumular development (Gesnerioideae-type). The first three have features characteristic of different species placed in the Cyrtandroideae. The fourth type is characteristic of species placed in a different subfamily of the Gesneriaceae, the Gesnerioideae.

Since the antagonists used were capable of interfering with the normal synthesis of any hydroxyproline-containing protein, it was not possible to identify the specific hyp-protein in which the altered synthesis resulted in the wide range of phenovariation observed. Evidence from studies conducted with bryophytes (Basile

and Basile, 1990; 1993; Basile et al. 2000), however, suggested that one or more arabinogalactan-proteins (AGPs) are likely candidates.

AGPs constitute a large family of glycoproteins and proteoglycans associated with the plasma membranes and cell walls of all plants investigated for their presence (Basile and Basile, 1987; Clarke et al., 1978; inter alia). Over the past thirty years, both experimental and inferential evidence has been accumulating to indicate that AGPs have evolved to serve a variety of functions in plants including cell, tissue, and organ identity, cell-cell communication, and morphoregulation (please see Clarke et al., 1979; Fincher et al., 1983; Keuger and van Holst, 1996; Majewska-Swaka and Nothnagel, 2000; Nothnagel, 1997; Pennell 1992; Serpe and Nothnagel, 1999; Showalter and Varner, 1989; Sommer-Knudsen et al., 1998 for reviews). Of the papers reviewed, most germane to the present study was evidence advanced to indicate that experimentally-induced changes in the size, shape, and/or distribution of organs (i.e. leaves and branches) could be correlated with experimentally induced changes in AGPs (Basile and Basile, 1990; 1993; Basile et al., 2000). Also relevant is the evidence that some AGPs may mediate changes in organogenesis by virtue of locally suppressing cell enlargement and/or cell proliferation (Ding and Zhu, 1997; Jauh and Lord, 1996; Serpe and Nothnagel, 1994; Thompson and Knox, 1998 and Willats and Knox, 1996). As mentioned above, experiments conducted in the Basile lab (loc.cit.) involved the use of antagonists of hydroxyproline-protein synthesis. They showed that Hyp antagonist-induced desuppression of cell proliferation in otherwise suppressed leaf and branch primordia could be correlated with changed electrophoretic

patterns of AGP extracts and the release of otherwise bound high buoyant density AGPs into the nutrient medium in which the plants were being cultured. The most relevant studies conducted in other labs involved the use of Yariv phenylglycosides. One of these, β -D-Glucosyl phenylglycoside (β -D-Glc)₃, is generally considered to be diagnostic for AGPs. It reversibly binds to and precipitates AGPs in the presence of low concentrations of salts. Other forms such as (β -D-Man)₃ and (α -D-Gal)₃ do not bind AGPs and can be used as controls in binding experiments. The first to find out that (β -D-Glc)₃, when added to the medium of actively growing cell suspension cultures, could suppress cell proliferation were Serpe and Nothnagel (1994). The (β -D-Man)₃ and (α -D-Gal)₃ phenylglycosides had no discernible effects. When the (β -D-Glc)₃ was washed out of the cells, proliferation resumed. Subsequently, others used this approach to perturb the function of AGPs and thereby different aspects of seedling development (Ding and Zhu, 1997; Thompson and Knox, 1998; Willats and Knox, 1996) and pollen tube growth (Jauh and Lord 1996). In each case, exposing the plants to (β -D-Glc)₃ resulted in modified patterns of growth and development. It was concluded that the modified patterns resulted because of the capacity of (β -D-Glc)₃ to perturb AGPs.

In the study reported here, seedlings of *S. prolixus* were treated with Yariv phenylglycosides in order to determine whether AGPs could be similarly implicated in altered patterns of development, particularly altered patterns of organogenesis.

Results

In the first experiment of this series, concentrations of 5 μ M and 30 μ M of the β -glucosyl Yariv reagent, and 50 μ M 3,4-dehydroproline, as well as the nutrient alone, were used. Germination was observed taking place in all dishes from a period of 6 to 15 days after seeding, with the majority of germination occurring on day 8. Measurements of the lengths of the cotyledons were taken at 18, 26, and 34 days after germination, using the 8th day of germination as the base, even though germination had started before and continued after that date. At the 18th day, most of the cotyledons were equal in length (isocotyly), and the differences in the mean length of the cotyledons between the seedlings grown in the control media and the media infused with antimetabolites showed no significant difference. The mean of the seedlings grown in the 30 μ M (β -D-Glc)₃ was actually higher than the mean of the control-grown seedlings at this point. (1.240mm for the (β -D-Glc)₃, and 1.0125mm for the control). By the 26th day after the onset of germination (DAG) the number of plants showing unequal growth of the cotyledons (anisocotyly) had increased substantially, and the measurements of the macrocotyledon indicated that the mean of the control-grown seedlings was significantly longer than any of the seedlings grown in the antagonists. The measurements taken at 34 DAG dramatically bears this out, as does the measurements taken at the time when type of phenovariation was recorded at 85 DAG. Phenovariation was apparent in the last observation period, not only due to cotyledon length but also the number of plants with distinctive changes in morphology. The results of a statistical analysis of these data and data from a later test are given in

Table 2. In order to check the significance of these data, comparisons were made individually between each treatment and the control. As a result of comparing phenovariations between the control and each treatment, 3,4dhp showed a $G = 8.459$, and a $P = 0.0036$, the $5\mu\text{M}(\beta\text{-D-Glc})_3$ showed a $G = 13.031$ and a $P = 0.0003$, and the $30\mu\text{M}(\beta\text{-D-Glc})_3$ showed a $G = 6.349$ and a $P = 0.0117$. In a later test the range of concentration of the $(\beta\text{-D-Glc})_3$ was changed, and media was prepared to include concentrations of 0, 2.5, 5, 10 and $20\mu\text{M}(\beta\text{-D-Glc})_3$. Observation of the plants 63-69DAG indicated phenovariation has taken place (Figs. 55-58), although the total number of viable seedlings had been considerably reduced, due in part to the elimination of all dishes that showed any contamination, and also due to the fact that some seedlings submerged in the Gellan medium lost the ability to photosynthesize and eventually died. A Power Curve test was run on the samples, and a least significant number at an alpha of 0.05 was determined to be 17. The sample number of three of the treatments exceeded this number. Of the 85 seedlings that were viable 85 DAG, 42 were phenovariants. A statistical analysis produced the data included in table 4 For $2.5\mu\text{M}(\beta\text{-D-Glc})_3$, the $G = 23.386$ with a $P = <.0001$, the $5\mu\text{M}(\beta\text{-D-Glc})_3$ showed a $G = 17.647$ for a $P = <.0001$ and the $20\mu\text{M}(\beta\text{-D-Glc})_3$ (not as spectacularly) showed a $G = 5.491$ with a $P = 0.0191$. The $10\mu\text{M}(\beta\text{-D-Glc})_3$ would have also showed a very high G and a probability far beyond the range of chance, but the fact that there were only 4 samples viable at the time of the measurement, placed them in a doubtful area. A third test was run to determine whether the plants would respond to $(\alpha\text{-D-Gal})_3$ as well as the $(\beta\text{-D-Glc})_3$. Measurements and observations were made at 20, 40, 65, and

90 DAG of the results on the seedlings of concentrations of 5 and 30 μ M of the two Yariv phenylglycosides. Phenovariation observed at the 90DAG was recorded in the JMP statistical analysis program (Table 3). No significant phenovariation was induced by (α -D-Gal)₃. Phenovariation induced by (β -D-Glc)₃, on the other hand, copied the changed morphotypes induced in the earlier experiments with antagonists of Hyp-proteins, auxin and ethylene. To reiterate, (β -D-Glc)₃ like the previously tested Hyp-antagonists induced *S. prolixus* to develop plumules with or without equal cotyledon growth, secondary phyllomorphs without equal cotyledon growth, or multiple phyllomorphs (Figs. 55-62).

Discussion

As stated earlier, the objective of this study was to determine whether by using Yariv phenylglycosides to perturb the function of AGPs, phenovariation comparable to that previously induced by Hyp-protein antagonists could be induced in *S. prolixus*. A qualitative appraisal of three sets of experiments indicated that one phenylglycoside that binds to AGPs could. All of the contingency analyses of data obtained from these experiments were highly supportive of the null hypothesis that the (β -D-Glc)₃ Yariv phenylglycoside affected morphological changes in the early growth patterns of *S. prolixus* in a manner similar to the changes induced by exogenous application of Hyp-protein antagonists. When a comparison test was run treating developing seedlings with both the (α -D-Gal)₃ and the (β -D-Glc)₃ a 2x4 contingency table analysis of the number of seedlings exhibiting some phenovariation was found to be highly significant. The seedlings grown in the media containing the 5 and 30 μ M (β -D-Glc)₃ showed

percentages of phenovariant plants significantly higher than the plants grown in nutrient medium alone or in media containing the 5 or 30 μ M (α -D-Gal)₃. The morphological changes (i.e. the appearance of a plumule, the equal growth of the cotyledons, the appearance of secondary phyllomorphs or multiple phyllomorphs) not only exactly phenocopied the results of tests run with different acting antagonists of Hyp-proteins, auxin and ethylene, but were also examples of changed morphology that resulted from the desuppression of normally suppressed local cell proliferation. Because there was no discernible relationships between the phenovariants produced and the concentration of Yariv phenylglycoside supplied, it is hypothesized that the time or stage of development rather than a specific concentration of (β -D-Glc)₃ was the most significant factor in inducing phenovariation. It also appeared that the previously observed phenomenon, that the desuppression of normally suppressed cell proliferation in one population of cells is accompanied by a general suppression of overall cell growth is borne out. With exogenous applications of the (β -D-Glc)₃ in different concentrations, all within biological limits, and using the same methodology which has been used with antimetabolites of Hyp-proteins, the same results are observed. In the timespan of 60 to 90 days after germination, phenovariation, in the form of the appearance of a plumule or secondary phyllomorphs in statistically significant numbers was observed. In this study, the appearance of organs normally suppressed, is the result of perturbing AGPs by a reagent well known to bind to them. Since most AGPs characterized thus far contain a high percentage of Hyp (Clarke et al, 1979, Fincher et al 1983, Showalter and Varner 1989,) it seems reasonable to

assume that the Hyp-protein hypothesized to be mediating suppression-desuppression as a result of earlier experiments belongs to the class of proteins known as AGPs. This has been found to be true in experiments with leafy liverworts, and mosses. This is the first time that the proteins affecting this type of morphoregulation in a flowering plant sporophyte can be confidently identified as belonging to the class of cell-surface-associated proteins known as AGPs.

Table 2. A comparison of the numbers and percents of phenovariation that developed in untreated and 3,4-dehydroproline and Yariv phenylglycoside-treated cultures of *S. prolixus* seedlings.

Antimetabolite	(μ Mol)	N	%	<i>P</i>
Control	0	30	3	
3,4-dhp	50	35	29	0.0036
$(\beta$ -D-Glc) ₃	2.5	19	79	<0.0001
	5	31	61	<0.0001
	10	5	75	<0.0001
	20	19	26	0.0191
	30	50	24	0.0117

(μ Mol) = concentration of antimetabolite in micromolars, N = sample number, % = percent of samples showing phenovariation, *P* = probability based on a Chi Square analysis, 3,4-dhp = 3,4-dehydroproline, (β -D-Glc)₃ = β - glucosyl Yariv phenylglycoside.

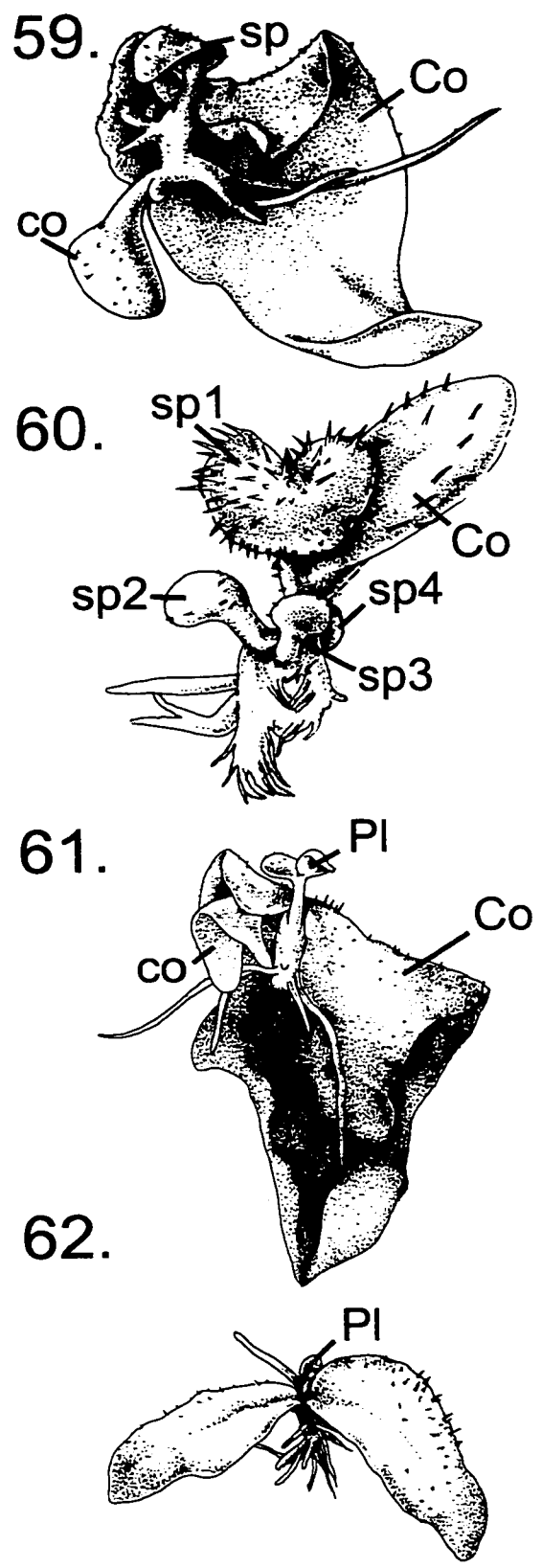
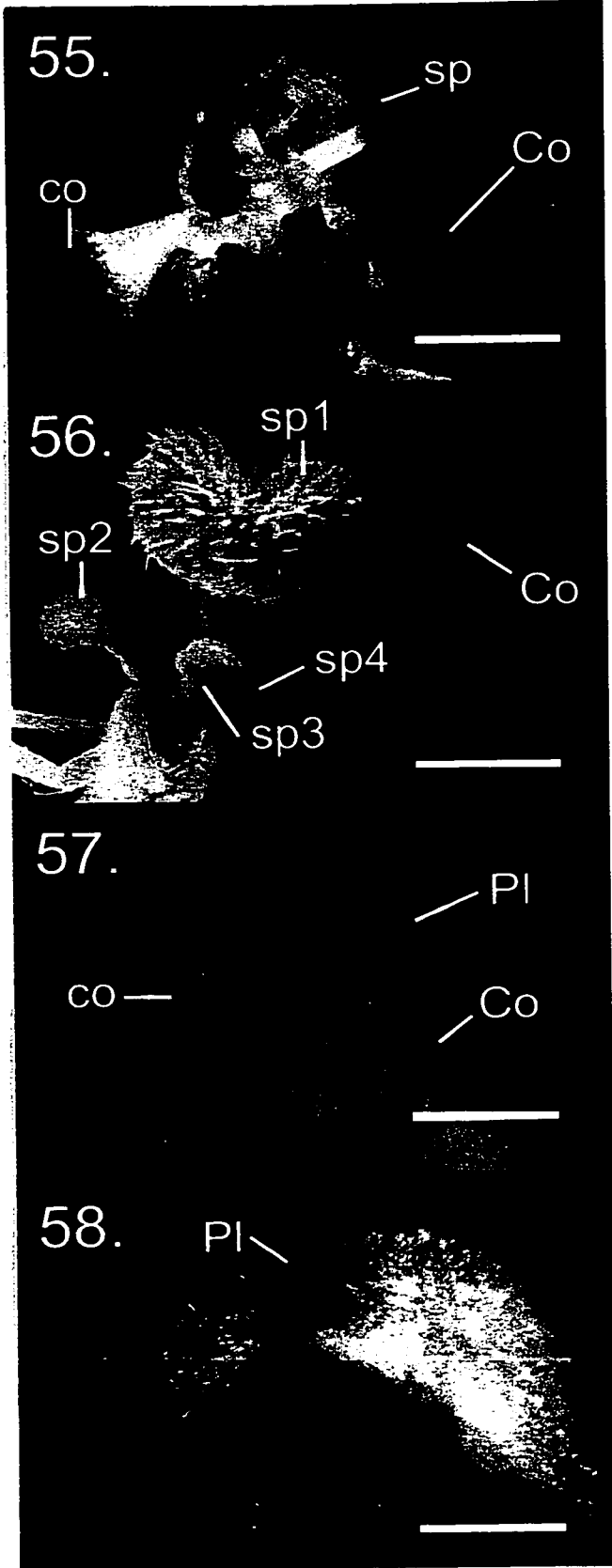
Table 3. A comparison of the numbers and percents of phenovariants that developed in cultures of *S. prolixus* with α -D-galactosyl and β -D-glucosyl Yariv phenylglycosides.

Note that the different strengths of Yariv induced results that had no significant difference, but that the difference between the reagents that bind AGPs (β -D-Glc)₃ and those that do not (α -D-Gal)₃, is highly significant with a $P = 0.0005$. (α -D-Gal)₃ = α -D-galactosyl Yariv phenylglycoside, (β -D-Glc)₃ = β -D-glucosyl Yariv phenylglycoside, N = sample number (viable seedlings at 90DAG), % = percent of samples showing phenovariation.

Antimetabolite	(μ Mol)	N	%
Control	0	30	3
(α -D-Gal) ₃	5	64	9
	30	48	8
(β -D-Glc) ₃	5	55	31
	30	58	31

Figures 55-58. Micrographs of phenovariants of *S. prolixus* induced by β -lucosylYariv phenylglycoside showing the same four types induced in earlier experiments by antagonists of Hyp-proteins. They are, 55. anisocotyly with a secondary phyllomorph (*Streptocarpus*, plurifoliate-type)(Type I), 56. multiple phyllomorphs (*Saintpaulia*-type)(Type II), 57. anisocotyly with a plumule (*Streptocarpella*-type)(Type III), and 58. isocotyly with a plumule (Gesnerioideae-type)(Type IV). co = microcotyledon, Co = macrocotyledon, sp = secondary phyllomorph, sp 1-4 = additional phyllomorphs, Pl plumule, Bar = 500 μ m.

Figures 59-62. Drawings of the whole plants shown in figures 55-58. in an attempt to clarify the phenovariation shown in the micrographs. The drawing have been oriented so that the primary axis (the petiolode) is vertical. 59 = 55, 60 = 56, 61 = 57, 62 = 58. Bar = 0.05mm.



MATERIALS AND METHODS

Plant materials -- Seeds of *Streptocarpus prolixus* C. B. Clarke used in this study were from the Seed Fund of the American Gloxinia-Gesneriad Society, and from plants grown to maturity from these seeds and hand-pollinated by the author. Seeds were stored at 5-6° C until needed.

Preparation of Inocula -- Seeds were surface sterilized in a "Swinney" syringe adapter filter housing between two disks of Miracloth (Chicopee Mills, Inc NYC) using a B-D Cornwall pipetting syringe to pump the sterilizing solution past the seeds, according to the "washing machine" protocol developed by Basile (1973b). Various percentages of Clorox (5.25% sodium hypochlorite) were tested from 30 seconds to 7 minutes to determine which condition produced minimal contamination without impairing seed viability. Surface sterilization was then achieved by either pumping 2% Clorox past seeds for five minutes, or 3% Clorox for four minutes.

Culture Medium -- For the axenic culture of seeds and seedlings, a basal medium of Knop's macronutrients (0.125g/L KNO_3 , 0.5g/L $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.125g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125g/L KH_2PO_4) supplemented with Hutner's "metals 49" adjusted to a pH of 5.8 was used. (Basile 1978).. Three protocols for growing seeds were used. In one a commercial soil-less mix (Grace Sierra Metromix 360TM, E. C. Geiger, Inc. Harleysville, PA) in 5.5 x 4.7-cm. plastic pots supported the seeds. In the second

protocol, 110mm discs of Whatman #4 filter paper were folded to form platforms (rafts) approximately 4cm square and 4cm high and placed within culture ("baby food") jars covered with Magenta Caps™ (Sigma, St Louis, MO). Aliquots of 27mL of the basal medium were pipetted into each of the jars, which were then autoclaved for 20 minutes at 250°F at 15 psi. This amount of aqueous medium, plus the 3mL added containing the concentrated the antimetabolites allows the filter paper to wick the solution to the seeds without submerging them. In a third protocol a basal medium of Knops was solidified with Gellan (Research Organics Cleveland, OH), at 4.5g/L in 60mm Petri dishes. Seeds were sown in a proscribed pattern in each Petri dish and sealed with Parafilm.

Application of Antagonists -- Antagonists were added as filter-sterilized solutions to the culture jars or to partially cooled heat sterilized Gellan nutrient solution prior to pouring into sterile Petri dishes in prescribed amounts. The antimetabolites used were made up from reagents obtained from Sigma (St Louis MO), except as noted. They were aminoethoxyvinylglycine (AVG), cobalt chloride, 2,3,5-triiodobenzoic acid (TIBA), 2[(1-Naphthlenylamino) carbonyl] benzoic acid sodium salt (Alanap) (a gift from the Uniroyal Co. Inc. Naugatuck, CT), p-chlorophenoxyisobutric acid (PCIB), 2,2'-dipyridyl (2,2'-dp), 4-hydroxy-L-proline (HLP) and 3,4-dehydroproline (3,4-dhp). α -galactosyl and β -glucosyl Yariv phenylglycosides had been prepared by Dr. D.V. Basile and Dr I Ganjian (Ganjian and Basile 1997). The antagonists were all dissolved in nutrient medium and cold-filter sterilized using 2 cc B-D Plastic Disposable

Syringes, Pro-X Filter units, 0.22µm Hydrophilic PVDF membrane and B-D 18g 1 1/2 hypodermic needles. before adding to the media. Final (working) concentrations of the antimetabolites were 10, 50 and 100mM, except in the case of AVG where the concentrations were 1, 5, and 10mM. The Yariv reagents were added to the medium to produce working concentrations of 2.5, 5, 10, 20 and 30µM. Solutions of Hyp were supplied at higher, millimolar concentrations in a continuance of the pot culture experiments.

Uniform inoculation procedures -- The method of inoculating cultures differed when seeds were inoculated onto plastic pots, paper rafts, or Gellan-solidified media. Twenty to thirty seeds were placed in each pot and then drenched with water or solutions of antimetabolites at seeding time, and for every 7 days thereafter for 2 months. When solidified media was used, 2x180mm wooden applicators sharpened to a fine point were flame sterilized at each use to transfer surface-sterilized seeds one at a time from the "washing machine" housings. They were planted on Gellan-solidified media in the Petri dishes in five rows of five seeds with a single seed in the center position of a sixth row to maintain orientation. This process was carried out in sequence, one row at a time, for each control culture and each one of replicate cultures containing antimetabolites to achieve maximal random choice of seeds. Once a set was completely seeded, the dishes were numbered, dated, identified as to treatment, and sealed with Parafilm. This process was repeated until five replicates of the control and each experimental culture was inoculated.

In the case of the raft culture, two slight variations from the original method (Basile1973a) were used. One entailed transferring all of 50-60 surface sterilized seeds together with the Miracloth discs from the filter housings directly on to the filter paper rafts. In the other, seeds clinging to the Teflon O-rings were also spread on the raft platform, using a sterile forceps. Each jar thus contained somewhat more than 50 to 60 seeds. The jars were covered with Magenta Caps™ (Sigma) and sealed with Parafilm. Again five replicates were made of the control and each concentration of antimetabolite. All of these operations were performed in a Laminar Flow Hood.

Incubation of cultures -- The pot cultures were incubated in an aquarium tank on water-saturated capillary matting, with the tank covered with clear acrylic. All cultures were maintained in Percival (Boone, IA) model WE-106 Plant Growth Chambers. The pot cultures were maintained at 20°C, 80% humidity. Light with a photoperiod of 14 hrs was provided from a mixture of incandescent and cool white fluorescent lamps at 4520 lux for pot culture. The raft and Petri dish cultures were maintained at 18°C under cool white fluorescent lamps at 3875 lux for a 14 hr photoperiod.

Measurements -- Measurements were made under a Bausch and Lomb Binocular Microscope, along the midrib of each cotyledon. Observations of the presence of phenovariation were made at the same time. Phenovariation was noted with the appearance of a third or more leaflike forms, and/or the appearance of a plumule.

Documentation -- Depending on size and relative complexity of the plants that developed under the different experimental conditions, different means of illustrating salient morphological features were employed. These include photography through either a dissecting or scanning electron microscope, or carefully drawing the plants under the dissecting scope, using a background of millimeter square graph paper to insure accurate measurement. Photomicrographs were taken on Kodak Polapan film with either an Olympus OM4 35mm camera mounted on a Zeiss Operational Dissecting Microscope, or on Polaroid 339 film on a Polaroid MicroCam attachment mounted on a Bausch and Lomb Stereo Zoom 7 microscope. A number of the seedlings measuring 1-3mm were fixed, dehydrated, critical point dried in a Tousimis Samdri-790 (Rockville, MD), and sputter coated with gold with use of a Technics Hummer II (Technics Corp., Alexandria, VA). The seedlings were then photographed at low magnification (25kV) on a Hitachi S-2700 scanning electron microscope with a Polaroid camera attachment. Photographs of the pot-grown plants were taken with an Olympus OM-2 mounted on a Zeiss dissecting microscope using Kodak TP-2415 black and white panchromatic and Kodak Kodacolor 100 color print films. Micrographs were traced using Micron Technical Pens on clear Denril vellum acetate sheets, to clarify the photographs.

Statistics -- Data was entered into the JMP 3.2.2 Statistical software program of SAS Institute Inc using the "Fit X by Y" subprogram. Tables were converted graphically

using Deneba Canvas 6. Polaroid images were scanned into Adobe Photoshop 5.5, to remove unnecessary artifacts from the background and to add scale bars and figures.

APPENDIX 1

A METHOD FOR DETERMINING THE SURFACE AREA OF IRREGULAR SHAPES

Abstract

The superficial, epidermal cells of plant leaves although tabular in nature, and consequently of near uniform thickness (Eames and McDaniels 1947, Esau 1967) are highly irregular shapes, not unlike the pieces of a jig-saw puzzle. To make a determination of the square millimeter area of these complex forms enclosed by the cell walls, line tracings of the cells were made. Area was arrived at by weighing a cut-out representation of a cell, and comparing it to a measured and weighed full sheet of the same paper. The dimensions of the full sheet, divided by its weight, multiplied by the weight of the individual cut-out shape converts into an accurate area delineation of the shape without a need to resort to counting squares, or applying the trapezoidal rule, or requiring the use of digitizing tablets and a Fourier transform computer program.

Introduction

The subfamily Cytandrioidae of the family Gesneriaceae is characterized by the uneven growth of the cotyledons. It is a condition known as anisocotyly -- the continued growth of one of the cotyledons, identified as the macrocotyledon (Co), while the other slows, and eventually stops and is known as the microcotyledon (co). The area of the Co illustrated in figure 1 for example, is 6.8 times larger than the co

from the same plant. (Fig. 1). Is this enlargement of the one cotyledon due to cell elongation, or to cell proliferation? To test this, a comparison was made of the surface dimensions of epidermal cells, whose uniform depth may be considered a constant, and therefore not a factor in this type of size analysis. The dramatic increase in the size of the one cotyledon was manifest in the length and width of the lamina, and not as much in the thickness, which is another reason that volume measurement of the cells was not undertaken. A suggestion from Dr. Dwight Kincaid, that the measurement of the area of the irregularly shaped epidermal cells might be most easily achieved through a process of comparative weight encouraged the development of this protocol, which can easily be applied to two dimensional irregular shapes of all kinds.

Materials and Methods

Anisocotyledonous seedlings of *Streptocarpus prolixus* C.B. Clarke, grown in Knops nutrient media were picked out of their dishes at different stages of growth, during the period extending from 30 to 60 DAG. Seedlings at sequential stages of the development of the Co relative to the co were killed and cleared in 80% ethyl alcohol at 80°C for 24 hours. The cleared cotyledons, after removing the thicker petiolode and roots, were mounted on slides in 30% glycerol. They were then micrographed using a Polaroid camera mounted on a Leitz Orthoplan Light Microscope, at a magnification of 562.5 times (45x10x1.25times) according to the indicators on the microscope. The micrographs were made at high magnification in order to minimize the depth of field of the image, and enhance the outlines of the cell walls of the superficial cells (Fig. 2). Images were taken from the apex, base, and middle areas of

both the macrocotyledons and microcotyledons. The cells from the three regions were extremely varied in form. The cells had interlocking lobed margins and differences in overall appearance from squat to round to elongated, so that any attempt to compare the cells by measuring the longest axis were meaningless. The cell walls visible on these micrographs were traced on clear acetate sheets (Fig. 3). In addition to the cotyledons a prepared micrometer slide etched to 0.01mm was photographed at the same enlargement (Fig. 4). One-to-one Xerox copies were made of all the tracings at the same time and on the same photocopier, at the same settings, along with extra copies, and blank sheets of copy paper, run through the copier. The full sheets of the paper were measured, with great care taken not to touch the paper with anything but sterile forceps or gloved hands, during the measuring, folding and weighing process. Ten sheets, some with Xerox images and some without were weighed on an analytical balance that reads to five decimal places, and an average weight determined. The measurements of the sheets were consistent for all ten papers. Using a fresh bladed #11 Exacto Knife, individual cells from the Xerox copies of the plant surfaces were cut from the images, against a cleaned Alvin Cutting Mat, and then placed into marked envelopes using a blunt ended forceps. Only those cells that had complete circumferences within the micrograph image were used (Fig. 5). Tracings of the measured scale was also made (Fig.4) and a square area, drawn using ten increments of the scale was made. The square tracing was likewise photocopied, the square cut out and weighed following the same procedure as the cells. Each individual paper cut-out cell was weighed, using the same balance on which the folded full sheets were

weighed. Similar Polaroid micrographs were taken of the Co and the co from the same plant at an enlargement of 17.5 times using a dissecting scope (Fig. 1). This was to determine the area difference between the macrocotyledon and the microcotyledon from which some of the cells were micrographed, and again a protocol of tracing, photocopying, cutting and weighing was carried out.

Results and Discussion

In order to determine whether the differences in the size of macrocotyledons compared to microcotyledons was due to differences in cell number or cell size, a simple procedure for comparing the size/area of their surface cells was developed.

A (in millimeters) = area of full sheet of paper

W (in grams) = weight of full sheet of paper

w (in grams) = weight of paper cutout

$A/W \times w$ = area of paper cutout

The full sheets of paper measured 216mm by 355.5 mm for an area measurement of 76788mm². The individual weights of ten sheets of paper ranged from 5.863g to 6.033 with a mean of 5.9632g. Dividing 76788mm² by 5.963g gives 12,877 mm²/g. For example, a single cell cutout weighing 0.03922g multiplied by 12877mm²/g is 505.04mm². The enlarged square, made from ten 0.01mm increments measured 57mm by 57mm for a total of 3249mm². The measurement of the ten 0.01mm increments (or 0.1mm) thus indicated an actual enlargement through the camera of 570 times rather than the 562.5 times determined by the readings on the microscope. The square cutout weighed 0.24297g -- multiplied by 12877mm²/g equals

3128.7mm². Thus the actual area determined by weight compared to the area determined by use of the micrometer scale has an error of only 0.0369. These measurements however are the dimensions of the surface area of the enlarged images of the Xerox copy. Although the determination of the relative cell sizes (areas) of the Co compared to the co is the main objective for developing the technique described here, it is deemed useful to find out if it provides a reasonable method for determining actual cell size. To find the actual cell dimensions it is necessary to divide by a factor of 570x570 or 324900. The size of the epidermal cell in question would therefore be 0.0015544mm². To check the validity of this result, the area of the photocopied square, 3249mm² divided by the actual area it represented (0.1mm x 0.1mm or 0.01mm²) confirms the magnification factor of 324900 times. However, for the purpose of this analysis it was the relative rather than the actual areas of the cells that was important. Therefore as long as all measurements were treated equally, weighed under the same conditions, and multiplied by a constant number, the determination of the actual cell size was secondary. The information required in this case is whether there was a significant size difference in the cells of the macrocotyledon and microcotyledon. By roughly measuring the Xeroxed cell images, (taking the longest dimensions in perpendicular axes and multiplying), a maximum estimate of the rectangular area occupied by the cell is provided. For the cell in question these were 27mm by 24mm, giving an area measurement of 648mm². The determined dimension, (505.04mm²) taking into account the incursions made into the rectangle by the irregular shape of the cell, is therefore well within the range of possibility. The

comparisons between the macrocotyledon and the microcotyledon from a single seedling indicated a size difference of 6.8 times between the smaller and the larger cotyledon. This factor varied widely depending on the age of the seedling, but always increased as the age of the seedling increased since the macrocotyledon gets larger while the microcotyledon growth slows and eventually stops. The results of a procedure developed to compare the relative surface areas of epidermal cells of the macro and microcotyledons of *S. prolixus* are summarized in Table 1. The data shows that the mean area of cells of the two types of cotyledons are very similar. More importantly, when subjected to statistical analysis, the data indicates that there is no significant differences between the cell areas from macro or microcotyledons. It is concluded therefore that the increase in size of the macrocotyledon relative to the microcotyledon is due to an increase in the degree of cell proliferation rather than cell enlargement. The hypothesis that the increase in size was a result of proliferation, rather than elongation or enlargement was thus validated. The protocol of area measurement thus provides an accurate and facilitating tool for investigations of this kind.

Figure 1. A micrograph of the two cotyledons (at 17.5x magnification) of a seedling of *Streptocarpus prolixus* at 30DAG. The microcotyledon on the left shows the lamina and a flattened portion of the attached petiolode.

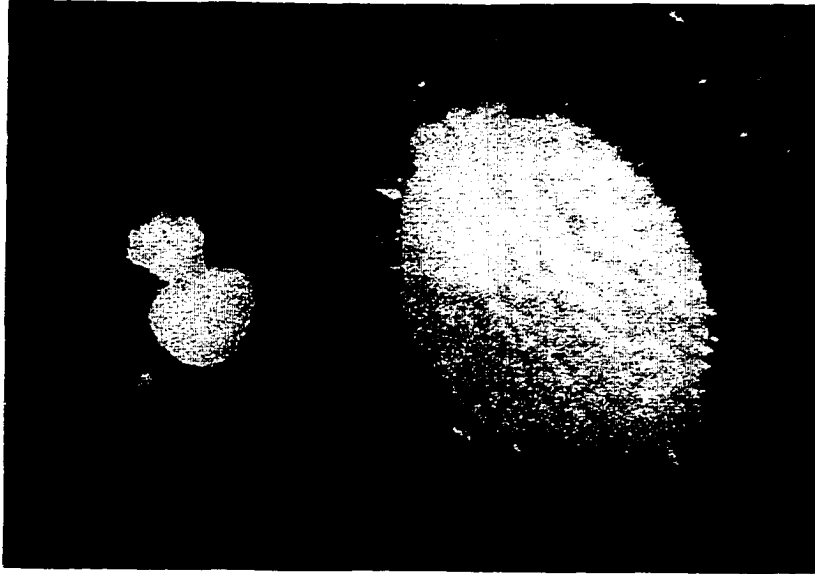


Figure 2. Micrograph of epidermal cells of the microcotyledon shown in Fig. 1 (570x)

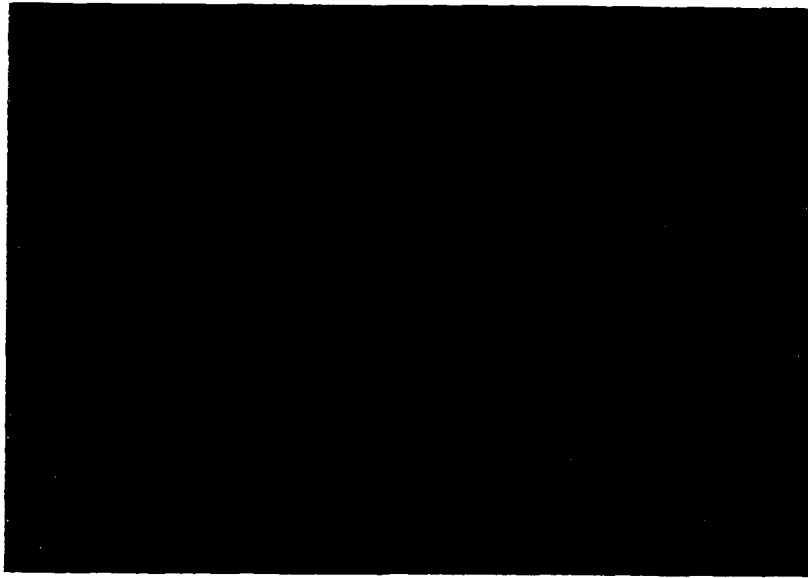


Figure 3. A micrograph (570 times) taken under the same conditions as figure 2 of the epidermal cells of the macrocotyledon shown in figure 1 with an overlay of the cell walls traced on acetate. A piece of white paper is inserted under the acetate part way to enhance the tracings.

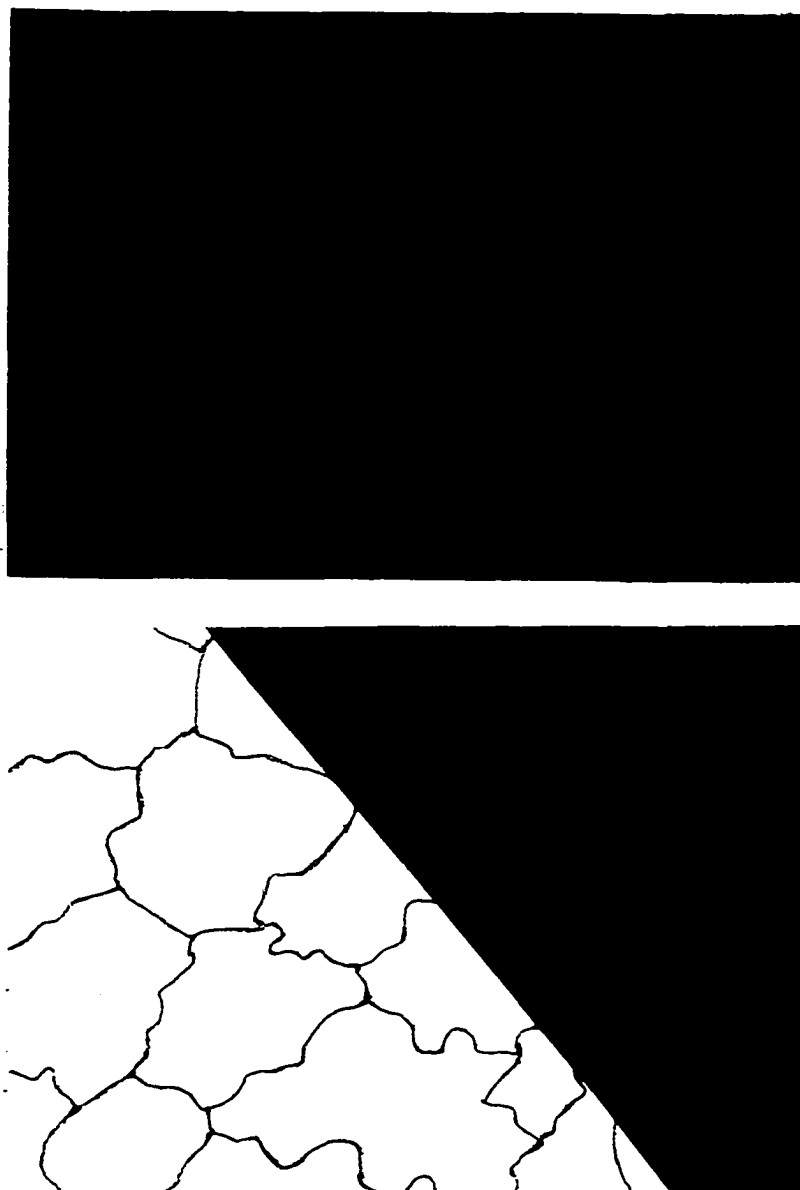


Figure 4. Micrograph of a portion of a stage micrometer. The increments are 0.01mm at the same enlargement (570 times) as the epidermal cell images. The square was made by tracing 10 increments.

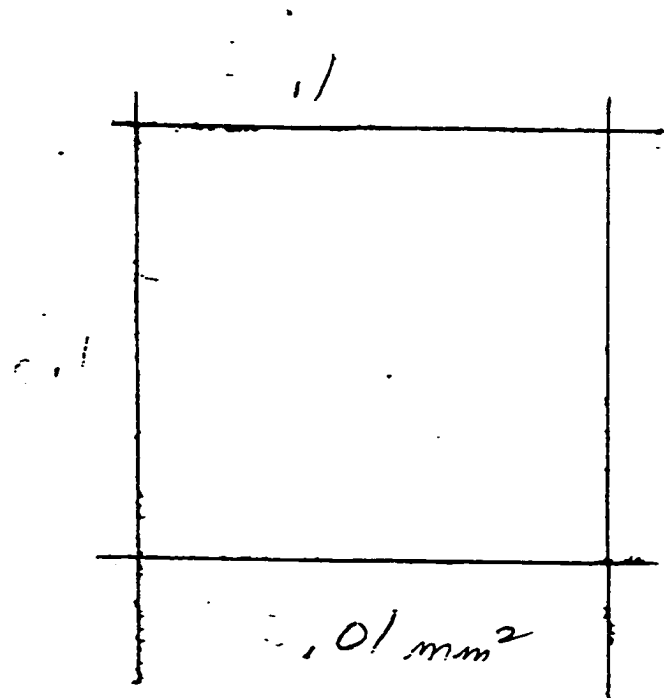
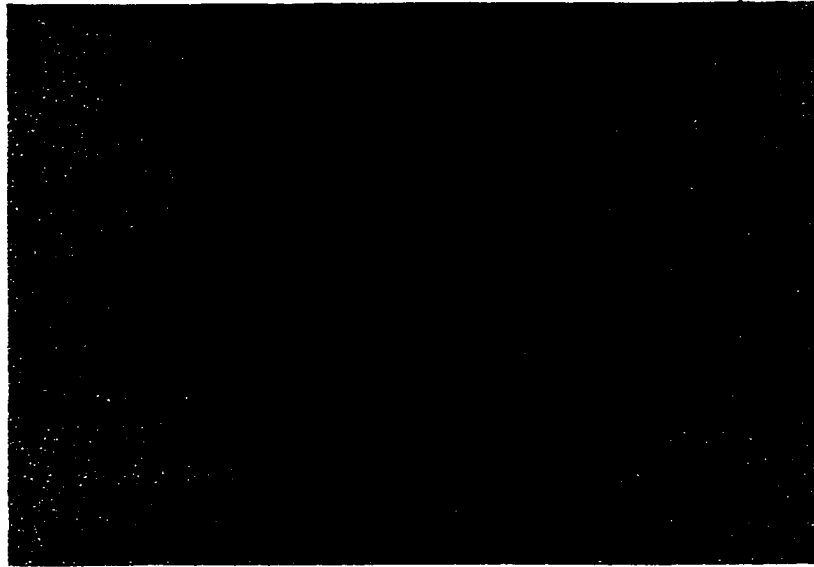


Figure 5. A photocopy of the tracing of the enlarged epidermal cell walls of the macrocotyledon shown in figure 3. The photocopy of the center-left cell (*) was cut out and weighed 0.03267g. This calculated to an area measurement of 420.69mm². Measuring the same image by counting squares gave a number of 418+mm².

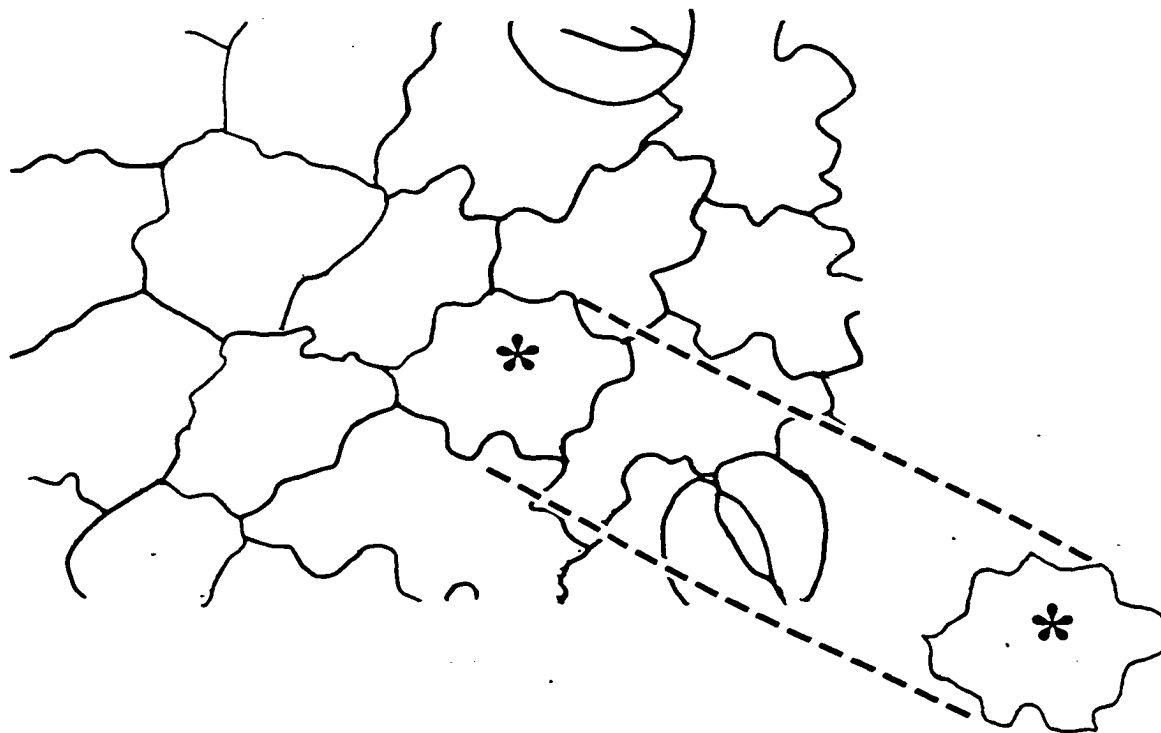


Table 1. The results of an Anova comparing the surface areas of >50 epidermal cells taken from the microcotyledons and macrocotyledons of *S. prolixus* seedlings at 30DAG to 60DAG. The bottom graph shows the results of a test to determine sample size taken from the same data. The extremely large size is another indication of the lack of significant difference between the two cell sizes.

Epidermal cell area	Mean	<i>P</i>
Co	512.939	0.8868
co	517.445	

Significance Level	Least Significant Number
0.05	23046
0.01	39803

APPENDIX 2
A VERY LOW CONCENTRATION OF β -GLUCOSYL YARIV REAGENT
STIMULATES CELL PROLIFERATION IN THE EARLY STAGES OF
MORPHOGENESIS

Abstract

In treating seeds of *Streptocarpus prolixus* C.B. Clarke with a range of concentrations of the arabinogalactan-binding β -glucosyl Yariv phenylglycoside (β -D-Glc)₃, an unsuspected pattern of growth became apparent with seedlings grown in 2.5 μ M concentrations of (β -D-Glc)₃. The seedlings so treated were larger and more vigorous than the control plants and plants grown in higher concentrations of (β -D-Glc)₃ for twenty to forty days after germination (DAG). Eventually this stimulation was diminished, and the plants grown without antimetabolites reached or surpassed the size of all the plants that had been treated with any concentration of (β -D-Glc)₃. This is another instance attesting to the critical relationship between the place and time effective concentrations of morphoregulatory molecules are present in the spatial and temporal regulation of plant morphogenesis.

Introduction

Yariv reagents were developed in an attempt to generate artificial antigens to monosaccharides, (Yariv et al 1962) but the discovery that certain of these reagents selectively bind, stain and precipitate arabinogalactan-proteins (AGPs) has made them important molecular probes for this class of proteoglycans and glycoproteins (Clarke

et al 1979, Fincher et al 1983, Nothnagel 1997). Recently, the ability for those Yariv phenylglycosides that selectively bind to AGPs has been used on living plants to induce morphological changes. These changes were concentration dependent in certain cases (Serpe and Nothnagel 1994), and apparently not in others (Ding and Zhu 1997, Rauh and Basile unpublished, Thompson and Knox 1998). In these instances a Yariv reagent, by perturbing the normal functioning of AGPs and producing morphological changes, was acting as an antimetabolite.

In a number of experiments where antimetabolites of auxin, ethylene, hydroxyproline-proteins and AGPs produced phenovariations in liverworts, mosses and *S. prolixus* (Basile 1990, Mignone and Basile 2000, Rauh and Basile 2000, Rauh and Basile, unpublished, Rosenblum 1981), it was demonstrated that the effectiveness of the antimetabolite fell within a critical range of concentration, usually between 1 to 100 micromolars. In experiments conducted with suspension-cultured *Rosa* cells, cell proliferation was reduced in the presence of $10\mu\text{M}(\beta\text{-D- Glc})_3$ and completely inhibited at $50\mu\text{M}$ (Serpe and Nothnagel 1994). Subsequently, Thompson and Knox (1998) found that suspension-cultured embryogenic carrot cells treated with concentrations of $3.5\mu\text{M}(\beta\text{-D- Glc})_3$ or less responded in the same way as controls, as opposed to cultures treated with $3.5\mu\text{M}$ or greater which produced “plants” with normal appearing roots but lacking shoots. After first using a range of concentrations from 2 to $50\mu\text{M}$ which established the effect of $(\beta\text{-D- Glc})_3$ they carried out further experiments solely with $5\mu\text{M}$ and $30\mu\text{M}(\beta\text{-D- Glc})_3$ which produced the same results,

noting that the number of roots produced was not concentration dependent (Thompson and Knox 1998).

When $5\mu\text{M}$ and $30\mu\text{M}$ ($\beta\text{-D-Glc}$)₃ were applied to germinating *S. prolixus* seedlings, significant phenovariation was induced. (Rauh and Basile unpublished). The normal growth pattern of development for *S. prolixus* is for the two cotyledons to develop unequally. The accrescent cotyledon (the macrocotyledon) continues enlarging and eventually becomes the entire vegetative body of the mature plant, while the other cotyledon (the microcotyledon) stops growing, and eventually falls off. The addition of ($\beta\text{-D-Glc}$)₃ to the nutrient media induces a number of morphological effects. Most notably, the otherwise suppressed shoot apical meristem as well as the otherwise suppressed microcotyledon are desuppressed. The addition of ($\beta\text{-D-Glc}$)₃, in concentrations above $2.5\mu\text{M}$, however, consistently reduced the overall growth of the seedlings, whether or not desuppressed organogenesis was evident (fig 1). Reduced overall growth coupled with desuppressed microcotyledonary and plumular development is the only previously observed response of *S. prolixus* when seedlings were treated with known antagonists of hyp-proteins (Rauh and Basile 2000). We report here evidence that a concentration of $2.5\mu\text{M}$ ($\beta\text{-D-Glc}$)₃ not only desuppresses the microcotyledons and plumules, but stimulates the overall growth of the seedlings for a period after germination.

Results

The first indication that the growth of seedlings cultured on a medium containing $2.5\mu\text{M}$ ($\beta\text{-D-Glc}$)₃, was outpacing those on the unsupplemented (control)

medium as well as those exposed to higher concentrations was confirmed at a measurement taken at 30 DAG of the first set of experiments (Fig. 2). It appeared that the lowest of four concentrations of the reagent supplied tended to “stimulate” growth of the seedling for 20 to 40 DAG. Because the reagent has a distinct red color, the relative concentrations of the reagent in newly prepared dishes can be correlated easily with their relative pink hues, which are darker with increased concentrations. During the course of the experiment this coloration gradually lightens and eventually disappears. Whether the change in color is due to absorption by the plants, or fading from exposure to the light source (or both) was not determined. Petri dishes which were left uncultured and stacked in a partly illuminated area were not observed to change color, but since conditions for this observation were not identical with the cultured dishes no definite conclusion could be reached. When the phenomenon of the increased cotyledon growth was measured (at intervals over incubation periods) there was no visible variation in the relative color of the dishes by 30 DAG. To insure that the concentrations of $(\beta\text{-D-Glc})_3$ had actually been prepared as designed, the experiment was repeated. It was noted that the newly prepared dishes showed color gradation corresponding to the increased concentrations of the $(\beta\text{-D-Glc})_3$. Again the same apparent “stimulation” of growth was noted in those dishes containing the $2.5\mu\text{M } (\beta\text{-D-Glc})_3$, and a similar fading of the coloration over time. Measurements of the macrocotyledons were taken at 5, 30, 40 and 80 DAG (fig. 3), and these corresponded to the measurements taken of the first set of seedlings subjected to the same concentrations (fig. 2). The seedlings treated with $2.5\mu\text{M } (\beta\text{-D-Glc})_3$ exhibited significantly greater lengths up to 40DAG in both experiments. By 80DAG,

however, the length of the macrocotyledon in the control cultures surpassed that of the Yariv reagent-treated cultures.

Although there was a significant difference in the growth rates of the cotyledons early in the incubation period, there was no significant difference in the kind and degree of phenovariation induced in the plants exposed to 2.5 μ M (β -D- Glc)₃ and those exposed to higher concentrations by 80 DAG. (Table 1)

Discussion

Apparently, the outwardly observable morphogenic effect of using (β -D- Glc)₃, to perturb plant cell-surface AGPs depends on a number of factors. Two factors for which there is some experimental evidence are differences in species employed and stage of development at which the perturbation is attempted.

When 10 to 50 μ M (β -D- Glc)₃ was added to culture medium in which *Arabidopsis thaliana* seedlings were grown, shoot development was normal but root growth was inhibited and epidermal cell bulging was observed (Ding and Zhu 1997). Different results were obtained when (β -D- Glc)₃ was applied to embryogenic cell-suspension cultures of *Daucus carota*, depending on the stage of somatic embryogenesis perturbed. Roots without shoots were produced at one stage while at another stage entire plants developed, but the roots were greatly elongated and shoot apices radially expanded. At still other stages, (β -D- Glc)₃ inhibited overall growth (Thompson and Knox 1998).

In a series of experiments conducted with non-embryogenic cell suspensions of 'Paul's Scarlet' rose (*Rosa sp*) perturbation with (β -D- Glc)₃ caused inhibition of cell proliferation (Serpe and Nothnagel 1994). The results were concentration-dependent,

within limits of 10 μ M to 50 μ M, the increase in concentration correlated with a decrease in proliferation.

The few studies cited above should suffice to support the proposition that different plant species and or different cell populations manifest different morphogenic effects when (β -D- Glc)₃ is used to perturb their cell-surface AGPs. Although the manifest effects differed with the different experimental systems, the basis for the differences can reasonably be attributed to the capacity for given concentrations of (β -D- Glc)₃ to suppress or desuppress cell proliferation and or cell enlargement in different populations of cells comprising the developing plant body when the AGPs were perturbed. Obviously, with cell suspension cultures, the only manifest effect would be whether or not cell proliferation was suppressed. In somatic embryogenesis, the developing plant body progresses through a series of stages, each of which may contain different populations of cells with respect to their reaction to the perturbing effects of (β -D- Glc)₃. The observed differences in the morphogenetic effect of perturbing AGPs may be due, in part, to differences in the stage of development achieved by the embryo before dormancy set in, and in part, due to differences in the normal pattern of development of the species or cultivar. *Daucus carota* and *Arabidopsis thaliana* are two plants that follow very different patterns of development and may respond differently to perturbations by similar concentrations of (β -D- Glc)₃.

The pattern of development of *S. prolixus* is distinctly different from both *D. carota* and *A. thaliana*. Embryogenesis in seeds of *S. prolixus* does not progress beyond what may be considered a torpedo stage before the onset of dormancy.

Relative to those species in which seed embryos progress to the stage where plumules and radicles are established prior to dormancy, cell proliferation and differentiation can be considered “precociously suppressed”. If the assumption that all the cells of *S. prolixus* seed embryos were precociously suppressed when the seeds were inoculated onto the culture media is valid, then it follows that the apparent stimulation of growth produced by $2.5\mu\text{M}$ ($\beta\text{-D-Glc}$)₃ was the result of desuppressing cell proliferation in the germinating seeds and developing seedlings more effectively than the endogenously generated promotive processes normally do.

The morphogenetic consequences of perturbing AGPs in *S. prolixus* was in one sense concentration dependent. When concentrations above $2.5\mu\text{M}$ were used, only certain organ primordia were desuppressed. When $2.5\mu\text{M}$ was used, growth and development over the entire plant body, rather than only the otherwise suppressed microcotyledons, shoots and phylloforms, were desuppressed. It appears then that whether total rather than partial desuppression is achieved by perturbing AGPs in *S. prolixus* depends on the concentration used. Why this is so is not obvious from the results of this study or information gleaned from the relevant literature. What does appear to be supported by this study and those cited herein, is the hypothesis that AGPs play a critical role in regulating where and when, in developmental time and space, cell proliferation and or cell enlargement are suppressed or desuppressed. Hopefully, the finding that an initial supply of ($\beta\text{-D-Glc}$)₃ was sufficient to stimulate *S. prolixus* growth and development over an extended period, due at least in part to desuppressed cell proliferation, will eventually contribute to the understanding of how AGPs help to locally mediate growth suppression.

Figure 1. Petri dish culture of *S. prolixus* seedlings at 30DAG. From top left to right. the first dish shows seedlings grown in nutrient alone. The second with 2.5 μ M (β -D-Glc)₃ added to the medium, the third with 5 μ M, on the lower row the left dish with 10 μ M, and the right with 20 μ M. It is apparent that the very low concentration of the Yariv phenylglycoside has stimulated the growth of the seedlings more than any of the other treatments.



Figure 2. These graphs indicate the mean lengths of macrocotyledon lengths at 5, 20, 30 and 40 DAG of seedlings of *S. prolixus* from the first set of experiments using 2.5, 5, 10 and 20 mM concentrations of (β -D- Glc)₃. The seedlings grown in media treated with 2.5 μ M (β -D- Glc)₃ begin to show significantly greater lengths beginning with 30 DAG, and increase this difference by 40 DAG. This stage marked the maximum difference of length of the plants grown in the very low concentration of the Yariv reagent. As the time increased stimulation was diminished, and the expected pattern of significantly larger plants grown without antimetabolites resumed. All Y axes in this figure are 10 mm. The bars along the X axes are treatments of *S. prolixus* seedlings in μ M concentrations of (β -D- Glc)₃.

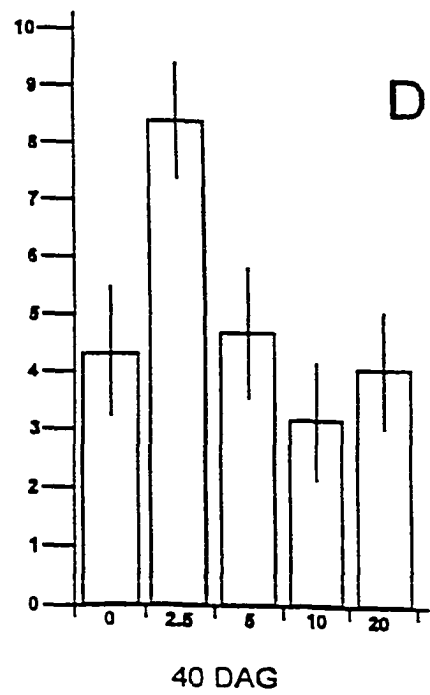
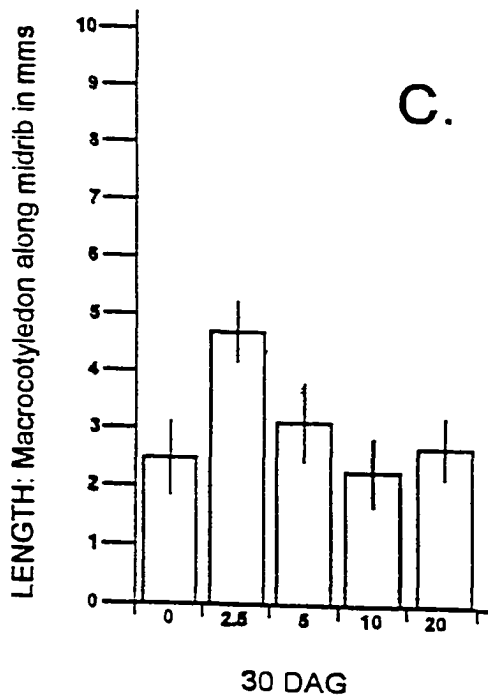
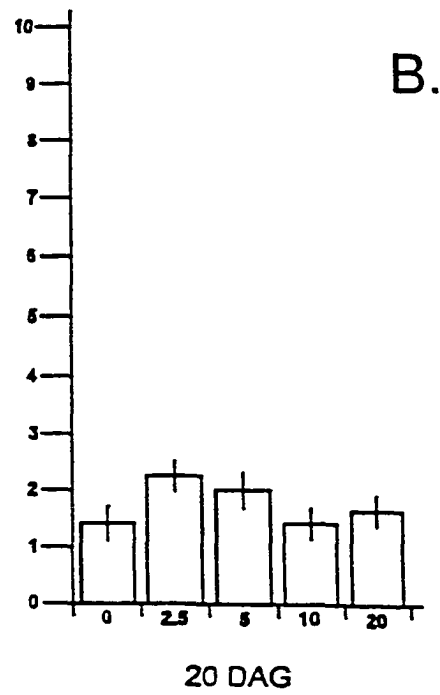
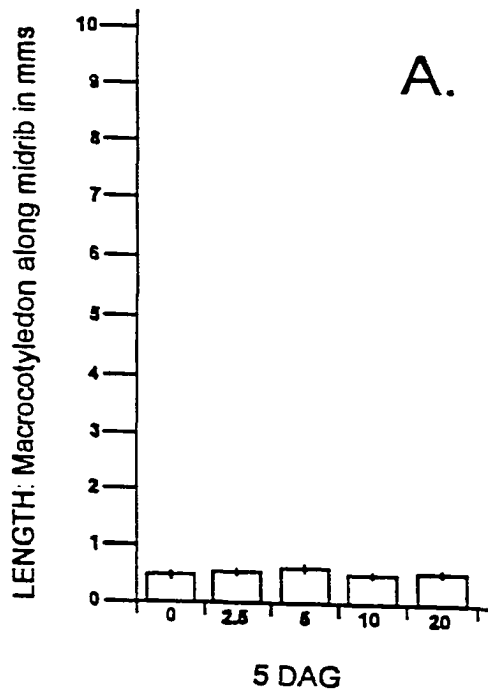
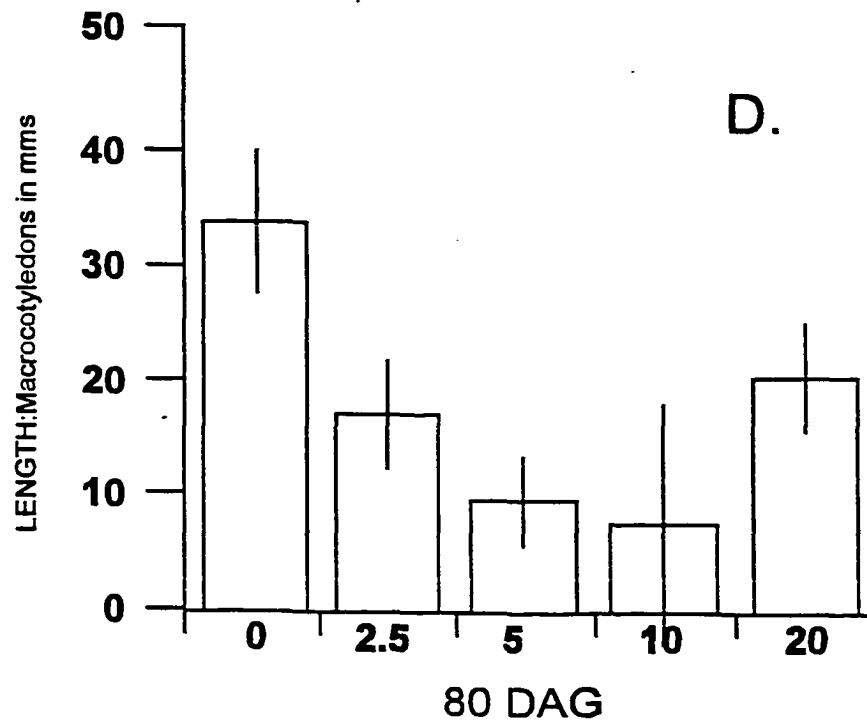
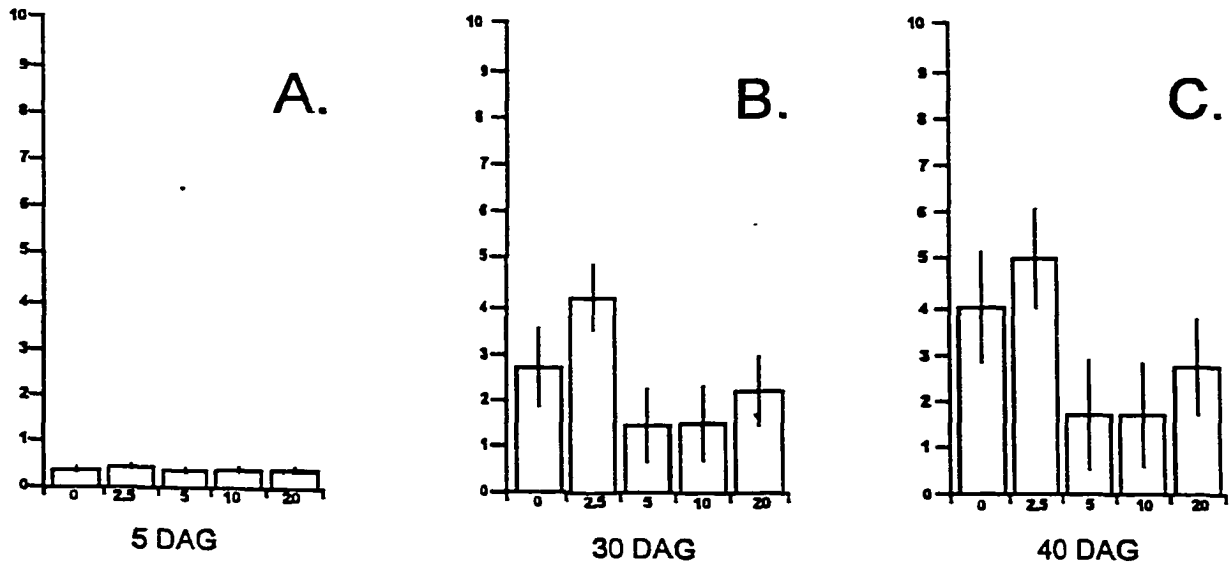


Figure 3. These graphs indicate the mean lengths of macrocotyledon lengths at 5, 30, 40 and 80 DAG of seedlings of *S. prolixus* from the first set of experiments using 2.5, 5, 10 and 20 mM concentrations of $(\beta\text{-D- Glc})_3$. The seedlings at 30 DAG show a stimulation of growth significantly greater than the control-grown plants and plants grown in higher concentrations of $(\beta\text{-D- Glc})_3$. By 40 DAG the control-grown plants have moved to eliminate the significance of the difference, even though mean of the plants grown in the $2.5\mu\text{M } (\beta\text{-D- Glc})_3$ remains higher. By 80DAG the control-grown seedlings are significantly larger than any of the seedlings grown with the addition of the Yarov reagent. At this stage the seedlings grown in the $2.5\mu\text{M } (\beta\text{-D- Glc})_3$ show no significant difference from seedlings grown in the higher concentrations of the Yarov reagent. The Y axes of the graphs show mean length of the macrocotyledon (measured along the midrib) in millimeters. A = 5 DAG, B = 30 DAG, C = 40 DAG and D = 80 DAG. The scale of A,B and C is 10mm. The scale of D is 50mm. The bars along the X axes are treatments of *S. prolixus* seedlings in μM concentrations of $(\beta\text{-D- Glc})_3$.



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