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HORMONAL CONTROL OF ORGANOGENESIS ON LEAF EXPLANTS OF *BROWALLIA*

By

Kent James Welsh

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture

1979

ABSTRACT

HORMONAL CONTROL OF ORGANOGENESIS ON LEAF EXPLANTS OF *BROWALLIA*

By

Kent James Welsh

Hormonal control of callus, adventitious shoot and root induction on leaf explants of *Browallia viscosa* and *B. speciosa* was determined. Leaf explants were aseptically cultured on M/S salts and vitamins to which the auxins IAA, IBA, NAA and 2,4-D and the cytokinins 2 ip, K, BA and Zeatin were added singly or in various combinations. *Browallia viscosa* responded readily *in vitro* and extensive callus growth occurred on several media at 1500 lux and 25°C ± 2. Shoots arose when callus was cultured on M/S plus 2 ip, K and BA, but only consistently on 2 ip. The shoots rooted very easily in four to seven days in an artificial planting medium. *Browallia speciosa* responded slower in culture, but callus was initiated and maintained on M/S plus 0.5 or 2.5 mg/l BA and 5.0 mg/l 2,4-D or on Uchimiya and Murashige basal medium, U/M. The regenerated plants of *B. viscosa* appeared to maintain the morphological and cytological traits of the parent.

To Laura Lee, Jennifer and Melanie

ACKNOWLEDGMENTS

I would like to thank Dr. Kenneth C. Sink for his assistance in this research program and in editing this thesis. I would also thank Dr. Robert C. Herner and Dr. Harry H. Murakishi for their help in the preparation of this thesis. Thanks and appreciation also go to my parents for the upbringing and encouragement they have given to me. My wife also deserves thanks for her support, inspiration and love during time spent writing this thesis.

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INTRODUCTION

The *Solanaceae* or Nightshade family is composed of several important economic genera as well as many which are less important. Included in this family are the genera *Nicotiana* (tobacco), *Petunia*, *Lycopersicon* (tomato), *Solanum* and others. An important ornamental genus of this family is *Browallia*. There are four species within this genus, two of which, *B. speciosa* Hook and *B. viscosa* HBK, were chosen for study since they are ornamental types used as bedding plants and for hanging baskets. They are grown for their profuse masses of white or blue flowers which continue blooming throughout the summer.

The *Solanaceae* are also important since many species of this family can be manipulated *in vitro* for experimental purposes. *Browallia*, however, has received little or no attention and for this reason was selected for study. This research was conducted to gain information on the morphogenetic responses of leaf sections of *B. speciosa* and *B. viscosa* cultured *in vitro* on basal medium containing auxins or cytokinins and in combination. The main emphasis of the research was to determine: 1) the environmental and *in vitro* culture procedures for callus, root and shoot initiation on leaf explants, 2) the medium

composition for shoot regeneration from callus cultures and 3) the chromosome stability of regenerated shoots and intact plants.

LITERATURE REVIEW

Tissue culture provides an experimental system for the study of morphological responses of plant parts under rigidly defined conditions. Some of the earliest work was done by White (1934) working with tomato roots. By adding yeast to his medium, he was able to grow tomato root tips for unlimited lengths of time. The wild carrot, Daucus carota L., also provided an excellent source of material for in vitro research. Other early workers (Steward et. al., 1958; Reinert, 1959; Halperin and Wetherell, 1964; Halperin, 1966; and Linser and Neumann, 1968) conducted experiments using roots, petioles and umbellate peduncles of this species. They were able to regenerate shoots and embryos from these explants. Considerable in vitro studies have been done on the Solanaceae. Several genera, Nicotiana (Skoog and Tsui, 1948; Skoog and Miller, 1957; Gupta et. al., 1966; Walkey and Woofitt, 1968; and Uchimiya and Murashige, 1974) and Petunia (Handro et. al., 1972; Rao et. al., 1973 a,b; and Swamy and Chacko, 1973) have been studied in depth, whereas, others, Datura (Guha et. al., 1964; and Engvild, 1973), Physalis (Zenkteler, 1973), Lycopersicon (White, 1934; Norton and Boll, 1954; Padmanabhan et. al., 1974; and Ohki et. al., 1978),

Salpiglossis (Hughes et. al., 1973; and Lee et. al., 1977), Solanum (Rao and Narayanaswami, 1968 and Zenkteler, 1972) and Browallia (Power and Berry, 1979) have received less attention.

The genus Nicotiana, especially N. tabacum L. has been used extensively to gain a great deal of information on the control of morphogenesis and differentiation in vitro. Skoog and Tsui (1948) found that adenine would induce bud formation in callus and on stem internode segments of N. tabacum. They also found that NAA stimulated callus growth and root formation. Adenine plus NAA was shown to cause cell proliferation but did not cause bud formation. In 1966, Gupta et. al. were able to induce the differentiation of adventitious buds on leaves of this species.

Other species within this genus have also been examined. Walkey and Woolfitt (1968) reported that callus and adventitious shoots developed from shoot meristems of N. rustica.

Petunia has also been studied extensively. The in vitro response of P. hybrida Hort. and P. inflata R. E. Fries leaf and stem segments was determined by Handro et. al. (1972) and by Rao et. al. (1973 a,b). They observed callus, embryo, shoot, and root formation when different auxins and cytokinins were added to M/S medium.

Swamy and Chacko (1973) also found that anthers of P. axillaris (Lam) B.S.P. would develop callus and plantlets depending on the growth conditions.

Besides Nicotiana and Petunia, a number of other genera in this family have been studied. Callus, adventitious shoots and embryos have been reported from anthers (Guha et. al., 1964) and stem segments (Engvild, 1973) of Datura innoxia Mill. Norton and Boll (1954) observed shoot and callus formation from roots of a clone of Lycopersicon peruvianum (L.) Mill. Plantlets have also been induced on callus generated on explants of L. esculentum (Padmanabhan et. al., 1974). Other researchers have observed callus and adventitious shoot formation from anthers (Huhges et. al., 1973) and leaf sections (Lee et. al., 1977) of Salpiglossis sinuata L. Zenkteler (1972) reported the formation of buds on leaves of Solanum dulcamara L., S. nigrum L. and Physalis peruviana. These buds were capable of developing into shoots and plants. Power and Berry (1979) observed shoot formation from callus grown from leaves of B. viscosa HBK.

The response of a plant tissue in vitro is mainly controlled by the medium composition and environmental conditions. And, it is clear from the work of Skoog and Tsui (1948), Steward (1958), Skoog and Miller (1957) and others that plant hormones, notably auxins and cytokinins, at least partially, are responsible for the type of growth which occurs. Of course not all plant species respond in the same way to identical hormone treatments but there are certain similarities in the types of response which occur. Auxins generally promote callus growth and/or

rooting while cytokinins seem to be required at least in some species for shoot initiation. Combinations of cytokinins and auxins produce a variety of responses from callus and root induction to shoot initiation depending on the hormones employed, their concentrations and the plant species under study.

MATERIALS AND METHODS

Plant material:

Two species of Browallia, B. speciosa Hook cv. 'Major' Blue Bells Regular and B. viscosa HBK., were used in this study. Browallia speciosa is native to Columbia. Plants of this species may attain 152.4 cm in height in natural habitats and have large flowers 3.8-5.1 cm across. The flower colors are blue, violet and white in selected varieties. Browallia viscosa is common in Peru and other South American countries. The flowers of this species reach a diameter of 1.9 cm and are usually blue with a yellow-white throat; although white flowered cultivars also exist. Browallia viscosa generally grows to a height of 30.5 cm. Extensive, detailed descriptions of these two species can be found in Hortus Third (1976).

Plants for in vitro studies were obtained from seed sown on a weekly basis. Seed was sown on a moist artificial planting medium; the seed trays were covered with clear plastic and placed under artificial continuous fluorescent light at 2600 lux at ambient room temperatures (25 ± 2°C).

After two to three weeks, the seedlings were trans-

planted to plastic trays in the greenhouse using an artificial planting medium. The seedlings were grown under natural light conditions supplemented with extended lighting, 100 lux during the night from 10 P.M. to 2 A.M. The night temperature was held at a minimum of 22°C. Fertilizer was applied to the plants twice per week using 20-20-20 at the rate of 200 ppm N. The plants were watered as needed and standard cultural practices were used to control various insects.

Leaves for *in vitro* culture were obtained from vegetative seedlings of *B. viscosa* and *B. speciosa* when they were 40-60 and 60-80 days old respectively.

Sterilization procedure:

A laminar flow hood was used for all sterile procedures. Leaves were surface disinfected by immersion in four percent commercial sodium hypochlorite solution plus 0.02 percent Tween 20. After 30 minutes, the diluted bleach solution was removed and the leaves were washed with five separate sterile water rinses.

Disinfected leaves were prepared for culture by slicing them into sections, 1/2-1 cm^2. During this procedure, the leaf margins and midrib were also removed. All the explants were subcultured to fresh medium every four weeks. The cultures were held at 25°C under continuous cool white fluorescent light at 1500 lux.

Media:

Murashige and Skoog (1962) salts and vitamins (M/S) was generally used as the basal medium. Uchimiya and Murashige (1974), (U/M) medium was also used to a limited extent. Various auxins and cytokinins as described in Table 1. were added singly or in combination to the basal medium. The growth regulators were added to the defined medium prior to autoclaving, except for IAA which was filter sterilized through a 0.22 Millipore filter and aseptically added to cooled medium (40-45°C) after autoclaving. Sucrose at 3% and 0.8% agar were routinely added to the M/S basal medium. The pH of all media was adjusted to 5.8 prior to autoclaving at 15 psi, for 20 minutes.

Table 1. Auxins and cytokinins and their abbreviations.

Auxins	Cytokinins
Indole-acetic acid, IAA Indole-butyric acid, IBA Napthalene acetic acid, NAA 2,4-dichlorophenoxyacetic acid, 2,4-D	6-furfurylaminopurine, K 6-benzylaminopurine, BA 6-(trans-4-hydroxy-3-methyl-but-2-enylamino) purine, Z 6(ν-νdimethyallylamino)-purine, 2 ip

Cytological studies:

Shoot cuttings taken from seedlings of B. speciosa, B. viscosa and from regenerated plants were dipped in 0.3% IBA and placed in perlite. The cuttings were placed on

an intermittent mist bench in the greenhouse where B. speciosa and B. viscosa rooted in ten and seven days respectively. Roots 5-10 mm in length were removed, prefixed in saturated ABN (α-bromonaphthalene) for two hours in the dark at room temperature and fixed in 3:1, absolute alcohol:glacial acetic acid (Sink and Power, 1977) overnight. Fixed roots were stored in 70% ethanol at 4°C.

Roots were subsequently hydrolyzed in 1 N HCl at 60°C for ten to fourteen minutes, placed on a slide and smeared with 1% acetocarmine stain (Darlington and La Cour, 1975). A coverslip was placed over the cell smear and the slide was gently heated. Following heating, the slide was tapped lightly with the blunt end of a wooden dissecting needle to flatten the cells and the excess stain was removed. Chromosome number was determined by counting a minimum of eight good root tip cell preparations.

Pollen viability:

The pollen fertility of the Browallia species and the regenerated plants was determined by placing a dehisced anther in a drop of 1% aniline blue (Johansen, 1940) on a microscope slide. The anthers and debris were removed and a coverslip added. Excess stain was removed from the slides and they were examined after standing for 20-25 minutes at room temperature for viable pollen.

RESULTS

Morphogenetic response of leaf explants to auxins:

The response of B. speciosa and B. viscosa leaf sections on Murashige and Skoog (1962) (M/S) basal medium with auxins incorporated is shown in Table 2. Leaf sections of B. viscosa developed extensive roots when placed on M/S+IAA, IBA or NAA.

On the M/S+IAA media there was a gradual increase in the number, length and degree of branching of roots as the level of IAA increased from 0.01 mg/l to 5.0 mg/l and at 10.0 mg/l they decreased. At 0.01 mg/l IAA, one or two small, branched roots were observed per explant. Numerous, well developed and highly branched roots were observed at 5.0 mg/l IAA. Less root development occurred at 10.0 mg/l IAA. The roots which developed at all IAA levels were white and branched; callus growth was not observed.

After 6 weeks of culture, differences in root initiation between the IBA levels were not distinguishable. Numerous, fine white, branched roots were observed in each culture.

Leaf sections placed on M/S+NAA at 0.01-1.0 mg/l all responded similarly. They were dark green, with numerous,

Table 2. The morphogenetic response of leaf explants of two Browallia species cultured in vitro for 6 weeks on M/S basal medium containing auxins.

Auxin	Conc. mg/l	B. speciosa	B. viscosa
MS	-	-	-
MS+IAA	0.01	N/T	R
	0.1	N/T	R
	1.0	N/T	R
	5.0	N/T	R
	10.0	N/T	R
MS+IBA	0.01	-	R
	0.1	-	R
	1.0	-	R
	5.0	-	R
	10.0	-	R
MS+NAA	0.01	-	R
	0.1	-	R
	1.0	-	R
	5.0	-	-
	10.0	-	-
MS+2,4-D	0.01	-	-
	0.1	-	C
	1.0	-	C
	5.0	-	C
	10.0	-	-

-, no response; C, callus; R, roots; N/T, not tested.

highly branched, white roots. All leaf explants were dead after 6 weeks when cultured at the two highest NAA concentrations. Very minute quantities of light brown callus were observed at the cut edges of leaf pieces cultured on IBA and NAA.

2,4-dichlorophenoxyacetic acid (2,4-D) evoked a different morphogenetic response than the other three auxins. Leaf sections of B. viscosa had little or no callus formation at 0.01 mg/l 2,4-D, increased to large amounts at 0.1 mg/l 2,4-D and decreased in quantity at the higher concentrations. At 10.0 mg/l 2,4-D no callus production occurred. The callus initiated on 2,4-D was yellow-white, soft and friable in texture.

Leaf sections cultured on M/S basal medium without auxins gave no response or very infrequently produced roots.

Browallia speciosa did not respond to auxin supplements. After 4 weeks in culture all of the leaf sections were dead regardless of auxin type or concentration, except for IAA which was not tested.

Morphogenetic response of leaf explants to cytokinins:

Callus or callus plus shoots were initiated on leaf sections of B. viscosa when cultured on M/S basal medium containing the 3 cytokinins: K, BA or 2 ip (Table 3). Most leaf explants plated on K or BA died within 6 weeks.

Table 3. The morphogenetic response of *Browallia viscosa* leaf explants and callus cultured on basal medium containing cytokinins. Observations taken 6 and 12 weeks after plating the leaf explants and callus respectively.

Cytokinin	Conc. mg/l	Leaf Sections	Callus initiated on 2,4-D 0.1 mg/l	1.0 mg/l
MS	-	-	C	C
MS+K	0.01	-	C	C,S
	0.1	-	C,S	C,S
	1.0	C	C	C,S
	5.0	C,S	C,S	C
	10.0	C	C	C
MS+BA	0.01	-	C,S	C,S
	0.1	-	C	C,S
	1.0	-	C	C
	5.0	C	C	C
	10.0	C	C,S	C
MS+2 ip	0.01	-	C,S	C
	0.1	C	C,S	C
	1.0	C,S	C,S	C,S
	5.0	C,S	C,S	C,S
	10.0	C,S	C,S	C,S

-, no response; C, callus; S, shoots and/or leaf primordia.

Table 3. The morphogenetic response of Browallia viscosa leaf explants and callus cultured on basal medium containing cytokinins. Observations taken 6 and 12 weeks after plating the leaf explants and callus respectively.

Cytokinin	Conc. mg/l	Leaf Sections	Callus initiated on 2,4-D 0.1 mg/l	1.0 mg/l
MS	-	-	C	C
MS+K	0.01	-	C	C,S
	0.1	-	C,S	C,S
	1.0	C	C	C,S
	5.0	C,S	C,S	C
	10.0	C	C	C
MS+BA	0.01	-	C,S	C,S
	0.1	-	C	C,S
	1.0	-	C	C
	5.0	C	C	C
	10.0	C	C,S	C
MS+2 ip	0.01	-	C,S	C
	0.1	C	C,S	C
	1.0	C,S	C,S	C,S
	5.0	C,S	C,S	C,S
	10.0	C,S	C,S	C,S

-, no response; C, callus; S, shoots and/or leaf primordia.

Occasionally, very small amounts of green callus were observed at the cut edges of explants cultured on media containing the three highest levels of K. Also, one small shoot was observed at 5.0 mg/l K. Small to medium quantities of compact green callus were also observed on basal medium containing BA at 5.0 and 10.0 mg/l. No shoot initiation was observed on M/S medium to which BA was added. Shoot initiation and growth was consistent only when 2 ip was employed as the cytokinin. The first shoots were observed after 4 weeks at 1.0 mg/l 2 ip. Within 6 weeks after leaf sections were placed in culture, leafy growth and shoots were observed on more than 85% of the explants on 1.0, 5.0 and 10.0 mg/l 2 ip (Figure 1). A few green shoots, 2 cm in height and leaf-like structures were present at 1.0 mg/l 2 ip. A small number of roots were observed on the callus and on several of the shoots at this 2 ip concentration. Larger shoots, up to 5 cm in height, some with flowers and well developed, branched roots were observed at 5.0 mg/l 2 ip. At 10.0 mg/l 2 ip, shoots and leafy growth 2 cm tall were observed with very few roots also present on the callus.

Callus growth was also initiated when leaf sections of B. viscosa were plated on M/S+2 ip. Callus growth increased progressively from zero at 0.01 mg/l 2 ip to large quantities at 5.0 mg/l 2 ip. Only medium amounts were present at 10.0 mg/l 2 ip. The callus at all 2 ip levels was compact, yellow-green and nodular.

Figure 1. Adventitious shoot initiation on leaf sections of Browallia viscosa cultured on M/S basal medium plus 2 ip after 6 weeks in culture. Concentrations of 2 ip from left to right: 0.0, 0.01, 0.1, 1.0, 5.0 and 10.0 mg/l.

Figure 1.

The intensity of shoot formation in relation to the different cytokinins is presented in Table 4. Shoot initiation and growth occurred on leaf sections at: M/S+5.0 mg/l K and 1.0-10.0 mg/l 2 ip. The number of shoots initiated at 1.0 and 5.0 mg/l 2 ip was very similar with less shoot formation at 10.0 mg/l. Shoot development did occur at 5.0 mg/l K, but was not consistent.

Leaf sections of B. speciosa were also plated on M/S+ K, BA and 2 ip, however, after 4 weeks in culture all of the explants had died.

Morphogenetic response of callus to cytokinins:

Induction of shoots was also observed when callus derived from leaf explants of B. viscosa was cultured on K, BA or 2 ip (Tables 3 and 4). Callus initiated on 2,4-D at 0,1 and 1.0 mg/l was used to conduct these cytokinin experiments.

Most of the callus which originated on 0.1 mg/l 2,4-D died following plating on M/S+K. Small to medium amounts of yellow-brown callus were observed at each K level, which subsequently turned brown and died. Shoot formation did occur however at 2 K concentrations. At 0.1 mg/l K, one explant of 4 was greenish-white with numerous, small, dark, green leaves. These leafy growths were 2-3 mm high. Shoots, 2-3 mm tall with well developed leaves and flower buds were observed at 5.0 mg/l K on one piece of callus.

Table 4. Frequency of regenerated shoots on Browallia viscosa leaf explants and callus cultured on basal medium with cytokinins for 6 and 12 weeks respectively.

Cytokinin	Conc. mg/l	Leaf Sections	Callus initiated on 2,4-D 0.1 mg/l	1.0 mg/l
MS	-	-	-	-
MS+K	0.01	-	-	++
	0.1	-	++++	+
	1.0	-	-	+++
	5.0	+	++++	-
	10.0	-	-	-
MS+BA	0.01	-	+	++
	0.1	-	-	+
	1.0	-	-	-
	5.0	-	-	-
	10.0	-	++++	-
MS+2 ip	0.01	-	+++	-
	0.1	-	++	-
	1.0	++	++	++
	5.0	++	+	++++
	10.0	+	+	+

-, no shoots; +, less thn 5; ++ 5-20; +++, 20-50; ++++, >50.

The callus ranged in color from light brown to greenish-white at 0.01-1.0 mg/l K. Shoots and/or leaves were also observed at these K levels. Shoots, leaves and small amounts of callus were initiated at 0.01 mg/l K. The shoots were green and up to 2 cm in height. Dark green, leafy appendages and moderate amounts of callus were observed at 0.1 mg/l K. At 1.0 mg/l K, large amounts of compact callus, small leaves and 1.5 cm long shoots developed. Only one explant initiated shoots at each K level. Moderate amounts of greenish-white callus growth were observed at 5.0 and 10.0 mg/l K, but no shoot initiation occurred.

Callus cultured on M/S+BA responded very similar to that placed on K. All of the callus derived from 2,4-D at 0.1 mg/l was dead or dying after 12 weeks. At each BA level, small to medium amounts of yellow-brown callus developed before death occurred. Shoots were initiated at 0.01 and 10.0 mg/L BA. Only one explant per treatment developed shoots. Three green shoots, 4 mm tall were observed at 0.01 mg/l BA, while numerous leaves and small, 5 mm shoots originated on BA at 10.0 mg/l.

Callus induced on 2,4-D at 1.0 mg/l and transferred to M/S+BA continued growth. Large amounts of semicompact to very compact, brown to greenish-white callus developed on the explants at each BA level. Leaves and shoots occurred at 0.01 mg/l BA with only leaves present at 0.1 mg/l BA. In each instance, only one of four cultures

developed shoots.

Callus from 0.1 and 1.0 mg/l 2,4-D responded similary when subcultured on 2 ip (Tables 3 and 4). Medium to large quantities of yellowish-green, nodular callus developed at each 2 ip concentration. Semicompact callus was observed at 0.01 mg/l 2 ip and became progressively more compact as the level of 2 ip increased to 1.0 mg/l. The callus on 1.0-10.0 mg/l 2 ip was very hard and compact. As with leaf sections, shoot proliferation was most consistent on M/S basal medium containing 2 ip. However, there were differences in the number and type of shoot growth depending on the callus source and concentration of 2 ip.

Shoots and/or leaves arose on 0.01-10.0 mg/l 2 ip with callus derived from 0.1 mg/l 2,4-D. The highest number and size of shoots occurred at 0.01 mg/l 2 ip and decreased as the 2 ip level increased. Approximately 25 shoots, 2 cm in height and leaves were initiated on 2 ip at 0.01 mg/l. Less than 10 shoots and leaves occurred at 0.1 mg/l 2 ip. Only leaves were initiated at 1.0-10.0 mg/l 2 ip. Shoot initiation occurred on one of four explants at 0.01, 0.1, 5.0 and 10.0 mg/l 2 ip. At 1.0 mg/l 2 ip, 50% of the explants developed shoots.

No shoots developed on M/S+2 ip at 0.01 or 0.1 mg/l when callus from 1.0 mg/l 2,4-D was used. Shoots and leaves did occur, however at 1.0-10.0 mg/l 2 ip. At 1.0 mg/l 2 ip, 10-20 well developed shoots, 1.5 cm in height

and leafy growth occurred. These shoots were dark green and appeared morphologically normal. Numerous, small leaf-like structures and shoots were present at 5.0 mg/l 2 ip. Over 50% of the cultures at 1.0 and 5.0 mg/l 2 ip developed shoots. The number of shoots decreased at 10.0 mg/l 2 ip to less than five. Only one of 4 cultures at 10.0 mg/l 2 ip developed shoots.

Callus from 0.5 mg/l BA plus 5.0 mg/l 2,4-D was subcultured to M/S+Z at 1.0 mg/l to determine shoot regeneration potential. No shoots were observed at this Z concentration after 12 weeks of culture. The callus was greenish-white, compact and slow growing.

Callus of B. speciosa initiated on 0.5 or 2.5 mg/l BA plus 5.0 mg/l 2,4-D was subcultured to M/S basal medium with K, BA or 2 ip. Twevle weeks after subcultures were initiated, the callus on all media was either dead or turning brown and dying. The exception to this response was that callus obtained from 2.5 mg/l BA plus 5.0 mg/l 2,4-D placed on 1.0 mg/l Z remained greenish-white, semicompact and increased slightly in quantity. A few single roots, less than 5 per culture, with numerous root hairs were also present.

The only difference in response between the two callus sources was in the quantity of callus growth before death occurred. Only small amounts of callus developed on explants derived from 0.5 mg/l BA plus 5.0 mg/l 2,4-D plated on K, BA or 2 ip regardless of concentration. Explants from

2.5 mg/l BA plus 5.0 mg/l 2,4-D initiated medium-large quantities of callus on all levels of the three cytokinins.

Morphogenetic response of leaf explants to auxin/cytokinin combinations:

Leaf sections of B. viscosa and B. speciosa were plated on BA plus NAA media (Figures 2 and 3). The media combinations included each growth regulator at five levels: 0.01, 0.1, 1.0, 5.0 and 10.0 mg/l. With both species, callus growth increased from the lowest level of each hormone to the highest level; however, their rate of growth was not the same.

Callus was initiated on leaf explants of B. viscosa cultured on all but the lowest BA+NAA combinations (Figure 2). Yellow-green, compact callus was observed frequently and the largest quantity of callus growth was produced on leaf pieces placed on 10.0 mg/l BA plus 10.0 mg/l NAA. Roots did not develop on BA plus 0.01 or 0.1 mg/l NAA combinations. However, many white, branched roots were observed on 0.01, 0.1, 1.0 mg/l BA on 1.0, 5.0 and 10.0 mg/l NAA. These cultures were nearly covered with roots. Root number decreased gradually at the same NAA levels when combined with 5.0 or 10.0 mg/l BA. Only a few, poorly developed roots were observed on the 10.0 mg/l BA plus 1.0, 5.0 or 10.0 mg/l NAA treatments.

Browallia speciosa grew very slowly on all BA+NAA

Figure 2. Morphogenetic response of leaf sections of *Browallia viscosa* to BA+NAA combinations after 6 weeks in culture. Concentrations of BA from top to bottom and NAA from left to right: 10.0, 5.0, 1.0, 0.1 and 0.01 mg/l.

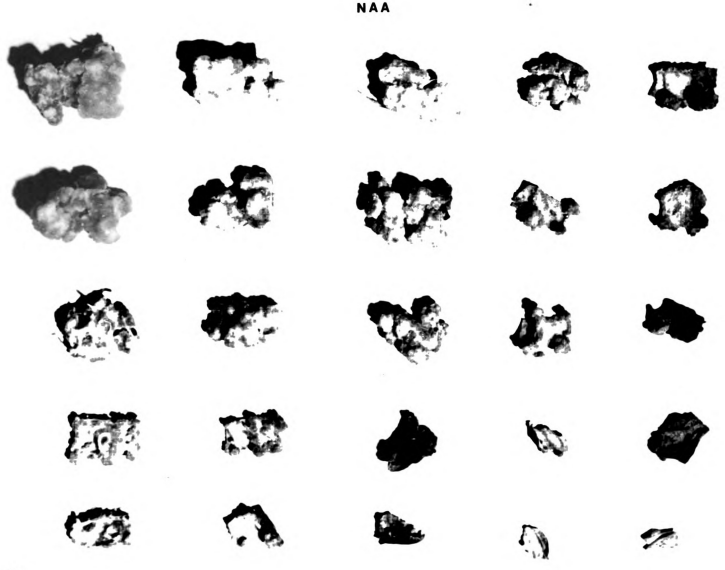

Figure 2.

Figure 3. Morphogenetic response of leaf sections of *Browallia speciosa* to BA+NAA combinations after 6 weeks in culture. Concentrations of BA from top to bottom and NAA from left to right: 10.0, 5.0, 1.0, 0.1 and 0.01 mg/l.

Figure 3.

combinations (Figure 3). After 6 weeks, only small to medium amounts of yellow-brown callus growth occurred even at the highest BA+NAA concentrations. The largest amount of callus was observed at 5.0 mg/l BA plus 10.0 mg/l NAA.

Shoots were not initiated on any of the BA+NAA combinations on either Browallia species.

Investigations were also carried out with leaf sections of B. speciosa and B. viscosa on M/S with 2.5 or 0.5 BA plus 5.0 mg/l 2,4-D and on U/M (0.25 mg/l K plus 2.0 mg/l 2,4-D) medium. Callus was initiated and grew slowly on leaf explants of B. speciosa on each of these media. However, callus removed from the original explants and subcultured on the same medium increased in growth rate. The callus produced on each of these media was whitish-yellow and friable. Medium quantities of callus growth occurred on 2.5 mg/l BA plus 5.0 mg/l 2,4-D, with large amounts on 0.5 mg/l BA plus 5.0 mg/l 2,4-D and on U/M.

Slow callus growth occurred when B. viscosa was cultured on the same medium. The callus which developed was compact, whitish-yellow and no shoot or root organization was observed.

Shoot growth:

Shoots that arose on callus derived from both 0.1 and 1.0 mg/l 2,4-D and subcultured on 1.0 or 5.0 mg/l 2 ip were easily rooted on M/S medium without growth regulators, on

M/S+IAA at 0.01, 0.1, 1.0, 5.0 and 10.0 mg/l, or in an artificial planting medium. Some adventitious roots also occurred on shoots after their elongation on shoot regeneration media. Terminal shoot cuttings placed in a moist, artificial planting medium of peat moss and vermiculite at a 1:1 ratio and held at 100% relative humidity rooted in 4-7 days. Roots also developed within 7 days on M/S basal medium and on M/S+IAA (Figure 4). The number and length of roots decreased as the IAA concentration increased. Well developed, slightly branched roots were induced on shoots cultured on 0.01 mg/l IAA. At this IAA level, 3-5 roots, 5-38 mm in length were produced on each explant. Shoots placed on M/S medium developed roots very similar to those occurring at 0.01 mg/l IAA.

Shoots rooted in the artificial planting medium were readily acclimated to greenhouse conditions. The plants grew vigorously and within two weeks were transferred to 10 cm pots using a sterilized 1:1:1, soil:sand:peat moss planting medium.

Cytology and morphology of regenerated plants:

Flowering Browallia viscosa plants originating from callus cultured on 2 ip were evaluated visually for morphological traits, cytologically and by pollen viability studies. All regenerated plants appeared normal when first placed in the greenhouse, but later, abnormal vegetative

Figure 4. Root development on regenerated shoots of *Browallia viscosa* cultured on M/S basal medium plus IAA after 1 week in culture. Concentrations of IAA from left to right: 0.01, 0.1, 1.0, 5.0 and 10.0 mg/l.

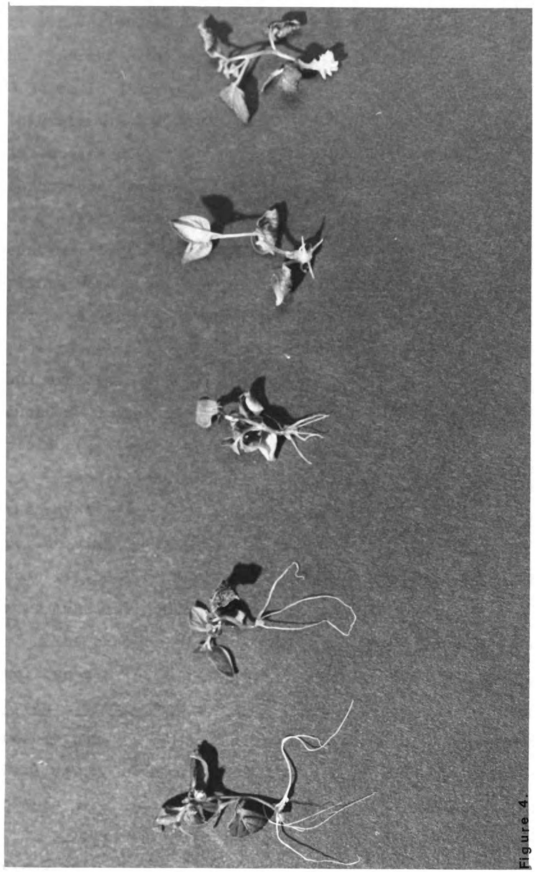

Figure 4.

growth was observed on some of them. One plant was one half normal size and covered with crinkled blue flowers, 0.94 cm in diameter. Only 50% of pollen collected from this plant was viable. This plant, subsequently did not produce seed upon self-pollination. Unfortunately, the chromosome number of this plant was not determined.

Several other plants also varied morphologically in contrast to the parent B. viscosa. The leaves of these plants were twice as large as those found on parent plants, had more hairs on the leaf surface, and had heavier stems. Flowers which developed on these plants were of normal size and exhibited 90% pollen viability, but seed set was reduced more than 50%. These plants were found to be tetraploids (2n=4x=44) with 44 chromosomes.

Most regenerated plants upon visual evaluation matched the parent for major morphological traits. Eighty percent pollen viability was observed for these plants. Seed from some of them was germinated and the seedling populations were likewise normal.

The chromosome number of each Browallia species as well as some of the regenerated plants was determined. Browallia speciosa and B. viscosa are diploids with chromosome numbers of 44 and 22 respectively as reported by Darlington and Wylie (1955). Most of the regenerated plants were diploids but several tetraploids were found.

Pollen viability studies were conducted on B. speciosa, B. viscosa and on the regenerated plants. Pollen

from flowers of the former species was found to be 80% viable while 94% viability was observed with B. viscosa. Pollen viability of regenerated plants ranged from 50-90%.

DISCUSSION

Although B. speciosa and B. viscosa belong to the same genus only slight similarities existed in their response in vitro. Leaf sections and callus of B. viscosa cultured on M/S plus auxins, cytokinins or their combinations resulted in definitive morphogenetic responses while explants of B. speciosa cultured under identical conditions died or in only a few instances initiated callus growth. These differenced in in vitro morphogenetic response between B. speciosa and B. viscosa were not entirely unexpected since there are obvious differences in growth rate, growth habit and flower size between the two species. There are at least three possible explanations for the observed in vitro differences: 1) a cultivated vs a wild species, 2) taxonomic differences and 3) cytological differences.

Cultivated vs wild species:

Browallia speciosa is a species that has been adapted by breeding efforts for ornamental cultivation while B. viscosa is a wild type. It is quite probable that selection for flower color and size, profuse blooming and growth

habit has indirectly modified the ability of B. speciosa to respond in vitro.

Similar contrasting in vitro morphological responses have been observed with other Solanaceous species. Observations taken from a study of inbred Petunia species as well as Petunia hybrida cv Comanche (Power et. al., 1976) indicated that all of the inbred species regenerated much easier than the cultivar Comanche. Tal et. al. (1977) compared morphogenetic potentials between wild and cultivated species of tomato. They found that the wild species exhibited a higher morphogenetic potential than the cultivated ones. In addition, it was noted that the morphogenetic responses were dependent on the auxin or cytokinin employed. This dependency on a specific hormone has been shown to be due to genetic control (Izhar and Pover, 1976) and has a high degree of heritability. In studies with several inbred Petunia species they observed that certain lines required 2,4-D and others NAA for growth in culture. Hybrids between these lines were shown to be capable of growth on a wider range of hormones than the parents. Frearson et. al. (1973) also concluded that the inability of certain Petunia hybrida varieties to regenerate when plated on identical medium was due to genetic differences between these varieties. In a comparison between two lines of Lycopersicon esculentum (Ohki et. al., 1978) it was observed that one line 'Porphyre' had a higher morphogenetic capacity than the other line 'Apedice'. Reciprocal

crosses were made between these two parents and the hybrids were also screened for morhpogenetic potential. They concluded that the *in vitro* response of these hybrids was genetically controlled.

These findings suggest that the differential *in vitro* response between *B. viscosa* and *B. speciosa* may be due to genetic variation possibly as a result of breeding efforts.

Taxonomic differences:

It has been suggested (Sink and Power, 1977) that variations in response between several *Petunia* species were a reflection of taxonomic differences among these species. And, upon visual comparison of *B. speciosa* and *B. viscosa* it became quickly apparent that taxonomic differences occur between these two species. *Browallia speciosa* has a dense growth habit with large blue or white flowers while *B. viscosa* has an open growth habit and small blue flowers with a yellow eye. These differences in turn reflect variable environmental conditions under which the plants have adapted in their native habitat. And, since in these studies both *Browallia* species were grown and cultured under identical conditions the observed differences between *B. speciosa* and *B. viscosa* must be due to their genotypes. From this study the conclusion may be drawn that the differences in *in vitro* morphogenetic responses between *B. speciosa* and *B. viscosa* are due to

genetic variation. Taking these arguments a step further, it can be suggested that the observed hormonal specificity of these two species must also be genetically controlled.

In 1976, Power et. al. hypothesized that the ability of certain Petunia species to regenerate was correlated with their taxanomic relationships (differences). If this hypothesis is accepted then the large differences in morphogenetic responses between B. speciosa and B. viscosa would indicate that they are not closely related taxonomically. This would explain why B. viscosa has the ability to regenerate under the culture conditions used herein and why B. speciosa did not.

Cytological differences:

Although both Browallia species have a basic chromosome number of x = 11 and are diploid, B. speciosa has been shown to have twice as many chromosomes (44) as B. viscosa (22) (Darlington and Wylie, 1955). This difference in the number of chromosomes also could be responsible for the different response of B. speciosa and B. viscosa in culture. It is obvious that difference in response between closely related species with a different basic chromosome number such as between Petunia parviflora (x = 9) and P. axillaris, P. violaceae and P. hybrida (all x = 7) (Sink and Power, 1977) is at least partially due to the different base chromosome number, but this could also be true between

species where the basic chromosome number is the same but the diploid chromosome number is different. This seems to be a good explanation for the differences in *in vitro* response between *B. speciosa* and *B. viscosa*.

While these three explanations have been presented as being somewhat distinct from each other this is not neccessarily the case. The three suggestions are interrelated with the central concept being that the differences in morphogenetic response between *B. speciosa* and *B. viscosa* are due to genetic variation. The cause of the genetic variation is of primary concern. From these suggestions also comes the conclusion that the hormone requirements for growth of *B. speciosa* and *B. viscosa in vitro* are probably genetically controlled.

With these conclusions in mind, it becomes evident that a discussion of the hormonal specificity for the growth or non-growth of *B. viscosa* and *B. speciosa* in culture is relevant.

Auxin specificity:

There were few similarities in the response of leaf sections of *B. viscosa* and *B. speciosa* when plated on M/S medium containing auxins. Leaf sections of *B. viscosa* cultured on M/S plus IAA, IBA or NAA developed extensive root systems. In each case, numerous fine white roots were observed at each concentration of auxin except with

NAA at 5.0 and 10.0 mg/l. And, in this situation it is possible that these levels may have been too high for root initiation. Other researchers have observed similar effects with different Solanaceous species. Gupta et. al. (1966) reported that IAA stimulated root development directly on leaves of Nicotiana tabacum. Root initiation has also been observed on leaf explants of Petunia inflata and P. hybrida when supplemented with IAA or NAA (Rao et. al., 1973 a, b).

Contrasting results were obtained when 2,4-D was used as the auxin. Loose, friable callus developed on leaf pieces cultured on 2,4-D at 0.1 or 1.0 mg/l. Likewise, 2,4-D has been shown to cause prolific cell growth with Petunia (Rao et. al., 1973 a, b), Datura (Engvild, 1973) and Solanum (Rao and Narayaswami, 1968).

Very different results were obtained when leaf sections of B. speciosa were cultured on M/S plus IAA, IBA, NAA or 2,4-D. Within four weeks after being placed into culture all of the explants had died.

These very contrasting responses between B. viscosa and B. speciosa are a reflection of the genetic differences between them. Browallia viscosa appears to have simple genetic requirements for root initiation since the results are very similar with IAA, IBA or NAA. However the requirements for callus induction are more specific since 2,4-D is required for the initiation and growth of loose friable callus. Browallia speciosa represents a more

complex system of genetic control than B. viscosa since it did not respond to the incorporation of individual auxins in vitro.

Cytokinin specificity:

Contrasting morphogenetic responses were also evident between explants of B. viscosa and B. speciosa when cultured on M/S medium containing cytokinins. Browallia viscosa exhibited a very specific cytokinin requirement. Approximately 85% of leaf sections and slightly over 50% of callus cultures developed shoots when placed on M/S basal medium containing 2 ip. In comparison, less than 10% of explants cultured on K or BA initiated shoots. The largest quantities of callus were also observed on cultures containing 2 ip. Ohki et. al. (1978) reported a similar cytokinin specificity with Lycopersicon esculentum when 2 ip was compared with K and BA. With tomato however, the auxin, IAA in combination with 2 ip was required to obtain optimum shoot proliferation.

Power and Berry (1979) reported infrequent shoot initiation when callus derived from leaf sections of B. viscosa was subcultured on M/S plus a BA+NAA combination or on M/S plus Z. However, under the cultural and environmental conditions used in this research, no shoots were observed on the same media formulations.

The response of explants of B. speciosa to cytokinin

treatment was quite different from that of B. viscosa and represents a more complex hormonal requirement. Leaf and callus explants of this species were either dead or dying within 4 or 12 weeks respectively after being subcultured to M/S plus K, BA or 2 ip.

As with the auxins, it is clear from the cytokinin results that there is a great deal of genetic variation between B. viscosa and B. speciosa. This is represented by the very specific cytokinin requirement of B. viscosa and the inability of B. speciosa to respond to any cytokinin treatment.

Cytokinin/auxin specificity:

Cytokinin/auxin combinations stimulated morphogenetic responses with both Browallia species that were not otherwise induced. Also, each species exhibited preferences towards particular cytokinin/auxin combinations. Leaf sections of B. viscosa initiated varying amounts of callus and roots on BA+NAA combinations depending on the cytokinin/auxin concentrations. In comparison, the growth rate of B. speciosa was much slower than that of B. viscosa on the same BA+NAA combinations.

Browallia speciosa did however surpass B. viscosa in in vitro response with respect to the amount and quality of callus growth on M/S with BA plus 2,4-D or on U/M medium (0.25 mg/l K + 2.0 mg/l 2,4-D).

Browallia viscosa thus shows a preference for BA plus NAA while BA or K plus 2,4-D are required for the growth of B. speciosa in vitro. Similar preferential morphogenetic responses have been observed (Izhar and Power, 1977) between several inbred Petunia species. Following in vitro studies with F_1 hybrids between the Petunia species with the same and different preferences they suggested that the specific hormonal requirements were genetically controlled. Their finding again indicate the genetic variation between B. viscosa and B. speciosa.

Sustained callus growth was also observed when callus of B. speciosa from a BA+2,4-D combination was subcultured to M/S plus Z at 1.0 mg/l. Shoot regeneration, however, was not observed on leaf or callus explants of B. speciosa in these investigations.

It is interesting to note that leaf sections of B. speciosa died when exposed to individual cytokinin or auxin levels but remained alive when they were combined in the basal medium. Even under these conditions, only callus growth could be induced. These findings lend support to the hypothesis that the morphogenetic responses of B. speciosa are under a rather complex system of genetic control. This suggests the need for changes in the experimental or environmental parameters which must take place before the regeneration of this species can be realized.

Rooting:

While growing B. viscosa seedlings in the greenhouse it was noted that rooting occurred when the stems of the plants came in contact with the soil. Cuttings also rooted easily when placed in a mist propagation bench. Thus, it was not surprising that shoots which developed in culture rooted readily in an artificial planting medium or upon subculture to M/S basal without growth regulators or M/S plus IAA at 0.01 mg/l. Kartha et. al. (1977) observed similar results with shoots of Lycopersicon esculentum cv Starfire. They suggested that the ease of rooting was due to the presence of high levels of endogenous auxins in the tomato stems. If B. viscosa also has a high endogenous auxin content this may account for the ease of root formation.

Cytology-morphology of regenerated plants:

The majority of plants regenerated from callus were normal with respect to cytological and morphological traits. A few were not normal in appearance, but this can be expected in plants derived from callus cultures (Murashige and Nakano, 1966 and 1967; Ohki et. al. 1978).

Conclusions:

It has been suggested (Frearson, 1973 and Kartha et. al., 1976) that the response of leaves of a species to certain *in vitro* cultural conditions can be used as an indicator of the ease or difficulty of using that species in protoplast studies. Thus, the results gained here with *B. viscosa* and *B. speciosa* are valuable in considering them for further somatic cell studies. Both species merit further consideration although in different areas. *Browallia viscosa* has already been regenerated from protoplasts (Power and Berry, 1979) and is a candidate for further *in vitro* genetic manipulations. The fact that *B. speciosa* does not respond in culture may make it amenable as part of a selection system for protoplast fusion studies.

SUMMARY

Three hypothesis have been suggested as possible explanations for differences in *in vitro* morphogenetic responses between *B. viscosa* and *B. speciosa*: 1) cultivated vs wild species, 2) taxonomic differences and 3) cytological differences. Upon cosideration of these hypothesis, it has been concluded that the morphogenetic differences are due to genetic variation between the two species either as a result of one of these suggestions or more likely due to a combination of them.

Browallia viscosa:

Callus, shoots and roots were initiated on leaf and callus explants of *B. viscosa* cultured *in vitro*. Only the cytokinin 2 ip was effective in initiating shoot development. Shoots of *B. viscosa* rooted easily when placed on M/S medium alone or with low levels of auxin or on an artificial planting medium. Growth regulators were not required for a high percentage rooting of shoots. Plants could be regenerated from callus cultures and were successfully grown to flowering in the greenhouse. Most of the regenerated plants were observed to be identical to the

parent, B. viscosa by following visual, cytological and pollen viability evaluation.

Browallia speciosa:

Browallia speciosa did not respond in culture. Callus development occurred only when leaf sections were cultured on cytokinin/auxin combinations. Prolific callus development occurred on M/S basal medium with 0.5 or 2.5 mg/l BA plus 5.0 mg/l 2,4-D and on U/M. No shoots developed on leaf explants or callus cultures of B. speciosa in vitro.

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