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Plant regeneration and protoplast culture of *Browollia speciosa*

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ABSTRACT

Explants from hypcotyls and cotyledons of *Browalia speciosa* were shown to regenerate plantlets.

Protoplasts were isolated from etiolated cotyledon material, and, although callus was readily obtained, plantlet regeneration was not observed using numerous hormone regimes.

ABBREVIATIONS:
M – Mannitol, 2,4-D – Dichlorophenoxyacetic acid
NAA – Naphthalene-acetic acid, BAP – Benzylaminopurine
MS medium – Murashige and Skoog (1962) medium
UM medium – Uchimiya and Murashige (1974) medium
COT – cotyledon, SH – shoot, R – root

KEY WORDS: *Browallia speciosa*, callus induction, shoot regeneration, plantlets, protoplast isolation and culture.

INTRODUCTION

Plant regeneration both from explants and protoplasts has been reported for many members of the *Solanaceae* family (Lu et al.1982). Within the genus *Browallia*, a neotropical genus containing annual herbaceous plants, plant regeneration from protoplasts has been observed from cell suspension protoplasts of *B.viscosa* (Power and Berry 1979)

Since this report, much attention was focused on other source material for protoplast isolation, with regard to high regeneration potential. Protoplasts isolated from cotyledons of green etiolated seedlings of various species have been shown to have high protoplast regeneration potential (Lu et al.1982; Ahuja et al.1983, Burger and Hackett 1982, Berry et al.1982). Cotyledon derived protoplasts, because of their special charactistics, could be used as one partner for various protoplast fusion systems.

The results of studies undertaken with seedling material of *Browallia speciosa* are described here.

MATERIAL AND METHODS

Growth of seedlings

Seeds of *Browallia speciosa* (Asmer Seeds Ltd., Leicester) were surface sterilized in 96% ethanol for 30 s followed by immersion in 10% commercial bleach solution (Domestos) and 5 washes in sterile tap water. Germination and subsequent growth of seedlings was on Murashige and Skoog (1962) medium lacking growth regulators but with 2% sucrose and 0.8% agar (pH 5.8).

Seedlings were maintained in 9 cm plastic petri dishes in the dark and 27°C for 12 days. Seedlings at the unfolded cotyledon stage were used in this study.

Callus induction from explants

Three types of explants were taken: cotyledon (COT), hypocotyl (SH) and root (R). Particular care was taken with cotyledon and hypocotyl to avoid meristematic regions.

Explants were cultured on multi-dishes with 5x5 repliplates. Each repliplate held 3.0 ml of medium.

Eleven media regimes were testet. Initial assessments were carried out using Murashige and Skoog (1962) medium supplemented with the following plant growth hormone combination:

MS + 2.0 mg/dcm³	NAA + 0.5 mg/dcm³	6-BAP	(MSP_1)
MS + 0.1 mg/dcm³	NAA + 0.5 mg/dcm³	6-BAP	(MSP_3)
MS + 2.0 mg/dcm³	IAA + 1.0 mg/dcm³	6-BAP	(MSD_3)
MS + 0.05 mg/dcm³	NAA + 0.5 mg/dcm³	6-BAP	(MSD_4)
MS + 1.0 mg/dcm³	zeatin		(MSZ)
MS + 2.0 mg/dcm³	2,4-D + 0.25 mg/dcm³	kinetin	(UM)

Cultures were maintained on the media mentioned above and in mixture with UM medium in the proportion 1:1. For each combination of media 25 explants in two independent replications were used.

Protoplast isolation and culture

Cotyledons (approx.5–7 mm long) were sliced transversely into 0.5–1.0 mm pieces and were plasmolysed for 4 hours in CPW 13M medium. The CPW salt solution consisted of: KH_2PO_4 –27.2 mg/dcm³, KNO_3 –101 mg/dcm³, $CaCl_2.2H_2O$–1480 mg/dcm³, $MgSO_4.7H_2O$ –264 mg/dcm³ , KI –0.16 mg/dcm³ , $CuSO_4.5H_2O$ –0.025 mg/dcm³ (pH–5.8)(Freason et al.1973). Slices were incubated in 2.0 ml of an enzyme mixture in plastic petri dishes (3.0 cm Nunc), in the dark for 12–14 hours on a rotory shaker (20–30 cycles per min) at 25°C The enzyme mixture consisted of 2.0% Rhozyme HP 150 (Rohm & Haas, Ltd.), 4.0% Meicelase P (Meji Seik Kaisha, Ltd.) and 0.3% Macerozyme R10 (Kinki Yakult Manuf.Co.Ltd.) in CPW 13M, pH 5.8 .

Protoplasts were released by gently squeezing and passing the tissue through a nylon sieve (45 µm pore size) and freed of cell debris by resuspension and centrifugation (100 g/10 min) in CPW 21S medium (CPW salts plus 21% sucrose). Protoplasts were finally washed 3 times in CPW 13M medium and resuspended at a density 5 x 10^4/ml of protoplast media: KP8 medium (Kao 1977), KMP8 medium (Kao and Michayluk 1975), MSP_1 9M medium (MSP_1 medium used for explant culture

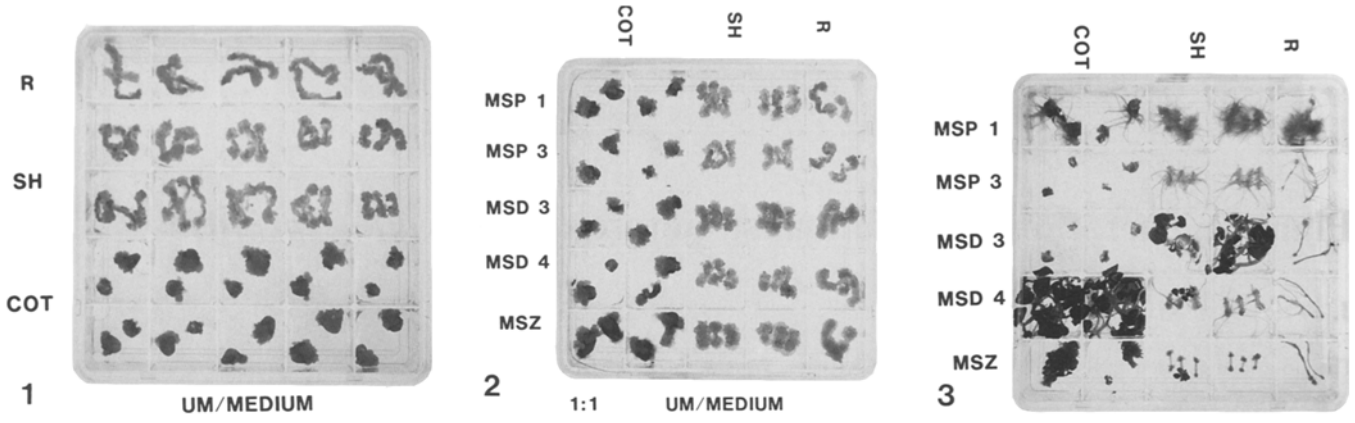

Fig.1. Callus regeneration by 12 day old seedling explant of *Browallia speciosa*; root (R), hypocotyl (SH) and cotyledon (COT) on UM medium after 4 weeks in culture.

Fig.2. Callus regeneration by seedling explants on regeneration media mixed 1:1 with callus inducing UM medium.

Fig.3. Shoot regeneration from seedling explants; cotyledon on MSD_4 and MSZ and hypocotyl on MSD_3, MSD4 and MSZ.

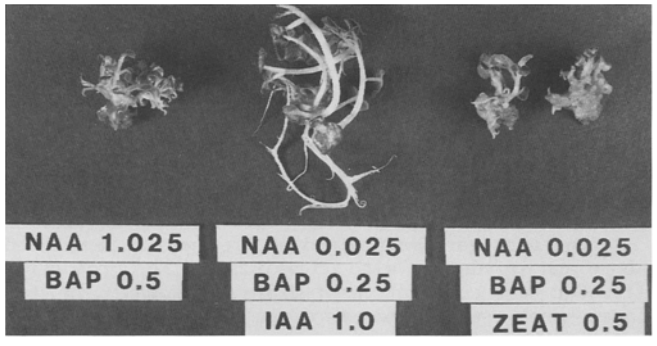

Fig.4. Morphogenic response of cotyledons on different combinations of growth substances in MS medium.

Fig.5. Regenerated plantlet in flowering stage on MSZ medium.

supplemented with 9% manitol), $MSP_3$9M medium (MSP_3 medium supplemented with 9% manitol).
Four ml of protoplast culture were maintained in a 5 cm plastic petri dish sealed with Nescofilm. The set of dishes was kept in a plastic container to maintain a high level of moisture for all cultures. Cultures were maintained for two weeks in the dark and subsequently were transferred to continuous light.
After two weeks of culture the osmotic pressure of the protoplast culture media was progressively lowered in one week steps of about 10%. The colonies were replated using the liquid-on-agar medium method (Power et al.1980).

Protoplast-derived callus culture

Callus, obtained from cotyledon protoplasts, was transferred to various media in an attempt to induce shoot and root differentiation. Small callus colonies were transferred to MSP_1, MSP_3, MSD_3, MSD_4 and MSZ media. In addition, and specifically for shoot regeneration from protoplast derived callus, 48 growth hormone combinations were employed using 1.0 mg/dcm³ IAA and kinetin, zeatin and 6-BAP at the concentrations 0.05, 0.1, 0.5, 1.0, 2.0, 3.0 mg/dcm³ with or without 15% coconut milk.

RESULTS AND DISCUSSION

Seedling explant culture

UM medium was used to induce friable callus from cotyledons (COL), hypocotyls (SH) and roots (R) of 12 day old seedlings (Fig.1). In the case of the root and hypocotyl, callus induction occurred over the entire explant surface but cotyledon callus regenerated only on the cut surface. On the media combinations of UM medium with other media (see material and methods) explants regenerated less friable callus than that obtained on UM medium alone. No shoot differentiation was observed (Fig.2).

Investigations carried out using different seedling explants from species of diverse families have indicated that cotyledon explants are capable of callus formation, rhizogenesis and in certain cases embryoid and bud formation (Blaydes 1966, Jelaska 1972, Rybczynski 1975).

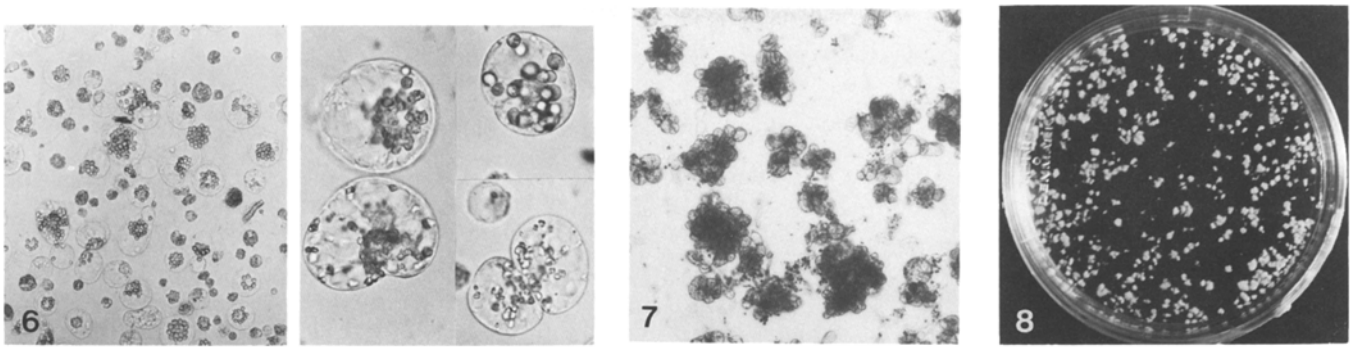

Fig.6. Cotyledon protoplasts after isolation and in KMP 8 culture medium.

Fig.7. Cell colonies derived from etiolated cotyledon protoplasts after 5 weeks of culture in KP8 liquid medium.

Fig.8. 10 week old colonies plated in liquid-on-agar KM 8 medium.

For *B.speciosa*, plantlet regeneration from cotyledons was observed after 8 weeks of culture. Shoots were induced from cotyledons over the entire explant surface. In this respect MSD_4 medium was superior to the others (Fig.3), however, MSZ stimulated multishoot formation but individual shoots were observed on MSD_4 and MSZ medium. Regenerated shoots (Fig.4) cultured on MS hormone free medium formed roots and on MSZ medium rooted plantlets flowered in the culture jars after 9 weeks (Fig.5).

Protoplast culture

Since cotyledon explants had a high regeneration capacity, protoplast isolation and culture was attempted from this material. Successful protoplast isolation from cotyledons in *Solanaceae: Datura inoxia, Hyoscyamus niger, Nicotiana tabacum, Physalis edulis, P.franchetti* has already been described (Lu et al.1982, Rybczynski and Power 1983).

Cotyledons of *B.speciosa* gave cytoplasmic dense protoplasts (2.3 x 10^3 per cotyledon pair) with a size range of 12-45um. After 2-3 days of culture the first asymmetric and regular protoplast divisions were observed. Cell division was generally more rapid in those protoplasts having larger storage bodies (Fig.6). This accentuation of division was particularly evident in KP8 medium. Plating efficiency (number of dividing protoplasts expressed as the percentage of the total protoplast population) of protoplast culture was estimated after two weeks of the culture with KP8, KMP8 and $MSP_1 9M$ media giving 4.32, 12.75 and 0.26% respectively for five independent isolations. The plating efficiency was almost zero for $MSP_3 9M$ medium. By contrast, plating efficiency for cell suspension protoplasts of *B.viscosa* was 30% (Power and Berry 1979).

The cell division ability of cotyledon protoplasts, presented by plating efficiency described for *D.inoxia, H.niger* and *N.tabacum* (Lu et al.1982) and for mesophyll protoplast of few species of the genus *Physalis* (Bapat and Schieder 1981, Gupta 1986) and *Lycopersicon* (Zapata et al.1981) suggested that this character is species and/or media dependent.

Cell colonies (Fig.7) were maintained by a regular decrease in the osmotic pressure but the rate of the dilution could not exceed 10% of the previous osmotic pressure. After 10 weeks of culture yellowish colonies, 3-4 mm in size were obtained and subcultured in K8 and KM8 medium using the liquid-on-agar method (Fig.8).

Protoplast-derived calli of *B.speciosa* were placed on MS medium supplemented with various hormone combinations (see material and methods) in an attempt to induce shoot and plant regeneration. All these cultures were unsuccessful. Here it is necessary to say that members of *Solanaceae* family are very often used as the "model plants" in somatic cell genetics , but still there are examples of species which require a great deal to improve their morphogenic potential. In the case of *Physalis minima* morphogenic response of leaf and stem explants in various growth hormone regimes has been described with extensives shoot formation (Bapat and Rao 1977). The mesophyll protoplast derived callus tissue of this species did not show shoot regeneration due to the lack of totipotency or to the incompatibility of growth hormone combinations used (Bapat and Schieder 1981). Only by using different culture conditions and "shooter" strain of *A. tumefaciens* could shoot regeneration of mesophyll protoplast cultures of *P.minima* be achieved and this improved somatic cell genetic manipulation of this species (Steffen et al.1986, Patel et al.1987, Gupta 1986).

Protoplasts of *B.speciosa* cotyledons could be induced to divide to give callus but unlike the original explant, the callus was not able to undergo organogenesis. This is in marked contrast to the results obtained for suspension protoplast cultures of *B.viscosa*.

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REFERENCES

Ahuja PS, Hadiuzzman S, Davey MR, Cocking EC (1983) Plant Cell Reports 2: 101-104

Bapat VA, Rao PS (1977) Z.Pflanzenphysiol. 85: 403-416

Bapat VA, Schieder O (1981) Plant Cell Reports 1: 69-70

Berry SF, Lu DY, Pental D, Cocking EC (1982) Z.Pflanzenphysiol. 108: 31-38

Blaydes DF (1966) Physiol. Plant. 19: 748–753

Burger DW, Hackett WP (1982) Physiol. Plant. 56: 324–328

Freason EM, Power JB, Cocking EC (1973) Dev. Biol. 33: 130–137

Gupta PP (1986) Plant Sci. 34: 151–154

Jelaska S (1972) Planta 102: 278–280

Kao KN (1977) Mol.Gen.Genet. 150: 225–230

Kao KN, Michayluk MR (1975) Planta 126:105–110

Lu DY, Pental D, Cocking EC (1982) Z. Pflanzenphysiol. 107: 59–63

Murashige T, Skoog F (1962) Physiol. Plant.15: 473–479

Patel GK, Bapat VA, Rao PS (1987) Proc.Indian Acad. Sci.(Plant Sci.) 97: 333–335

Power JB, Berry SF (1979) Z. Pflanzenphysiol.94: 59–63

Power JB, Berry SF, Chapman JV, Cocking EC (1980) Theor. Appl. Genet. 57: 1–4

Rybczynski JJ (1975) Gen. Pol. 16: 161–166

Rybczynski JJ, Power JB (1983) Protoplast 1983, Poster Proceedings of 6. International Protoplasts Symposium, Basel, Switzerland 1983: 32–33

Steffen A, Eriksson T, Schieder O (1986) Theor. Appl. Genet. 72: 135–140

Uchimiya H, Murashige T (1974) Plant Physiol. 54: 936–940

Zapata FJ, Sink KC, Cocking EC (1981) Plant Science Letters 23: 41–46