Plant regeneration and protoplast culture of *Browallia speciosa*

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**ABSTRACT**

Explants from hypcotyls and cotyledons of *Browallia speciosa* were shown to regenerate plantlets. Protoplasts were isolated from etiolated cotyledon material, and, although callus was readily obtained, plantlet regeneration was not observed in any of the 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 characteristics, 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 replicates. Each replica held 3.0 ml of medium. Eleven media regimes were tested. 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 (MS-P)
- MS + 0.1 mg/dcm³ NAA + 0.5 mg/dcm³ 6-BAP (MS-P)
- MS + 2.0 mg/dcm³ IAA + 1.0 mg/dcm³ 6-BAP (MS-D)
- MS + 0.05 mg/dcm³ NAA + 0.5 mg/dcm³ 6-BAP (MS-D)
- 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 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 CFW 13M medium. The CFW salt solution consisted of: KH₂PO₄ - 27.2 mg/dcm³, KNO₃ - 101 mg/dcm³, CaCl₂.2H₂O - 1480 mg/dcm³, MgSO₄.7H₂O - 264 mg/dcm³, KI - 0.16 mg/dcm³, CuSO₄.5H₂O - 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 rotary shaker (20–30 cycles per min) at 25°C. The enzyme mixture consisted of 2.0% Rhozyme HP 150 (Rhône-Poulenc, France), 4.0% Meicelase P (Meiji Seik Kaisha, Ltd.) and 0.3% Macerozyme R10 (Kinki Yakult Manuf.Colo. Ltd.) in CFW 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 CFW 21S medium (CFW salts plus 21% sucrose). Protoplasts were finally washed 3 times in CFW 13M medium and resuspended at a density 5 x 10⁴/ml of protoplast media: KP8 medium (Kao 1977), KP8M medium (Kao and Michayluk 1975), MS-P medium (MS 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 MSD4 and MSZ and hypocotyl on MSD3, MSD4 and MSZ.

Fig. 4. Morphogenic response of cotyledons on different combinations of growth substances in MS medium. supplemented with 9% manitol), MSP3, MS medium (MSP4 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 MSP1, MSP3, MSD3, MSD4, 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 (COT), 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).