

Plant regeneration from seed-derived callus of *Arctotis arctotoides*, a medicinal herb of the family Asteraceae

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WE HAVE DEvised A PROCEDURE FOR *in vitro* plant regeneration, from seed-derived callus, of *Arctotis arctotoides*, a plant noted for its medicinal uses among the rural people of the Eastern Cape province of South Africa. Callus formation was induced in basal MS salt supplemented with 3% sucrose and 1% agar at different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). After two weeks, morphogenic responses were evident in cultures and deeply stained, light-yellow calluses were produced. The highest percentage (68%) of seed explants that induced callus formation was obtained on MS medium supplemented with 2.0 mg l⁻¹ 2,4-D in the dark. When callus material was repeatedly subcultured in ½MS basal medium with a 16-h photoperiod without any hormonal supplement, adventitious shoots were produced eight weeks after the start of culture. The shoots rooted two weeks later. This is the first record of the *in vitro* regeneration of *A. arctotoides* and could serve as a foundation for further research on tissue culture, micropropagation and germplasm conservation of this noteworthy medicinal plant.

Introduction

The genus *Arctotis*, which belongs to the family Asteraceae (African daisy), occurs in southern Africa and Angola, where about 50 different species have been described. *Arctotis arctotoides* is a soft, herbaceous groundcover plant with light-green foliage and butter-yellow flowers. The

species grows in the summer rainfall areas of South Africa and Lesotho, usually in disturbed areas like road verges. The rural people of the Eastern Cape province of South Africa use the plant for the treatment of various ailments ranging from epilepsy and tuberculosis to indigestion.¹ The leaf juice, or paste, is also applied topically to treat wounds. Recent studies by Afolayan² revealed that extracts of the leaf have anti-fungal and anti-bacterial properties. The exploitation of this medicinally important plant from the wild and natural habitat, without any effort for its cultivation, has created growing pressure on its natural populations and threatens the survival of the species. *Arctotis arctotoides* is propagated by natural re-seeding. Germination is irregular and sometimes takes up to six weeks to achieve. To save this resource from progressive depletion, an alternative method for its propagation and conservation is desirable, of which one is described here.

Among the several applications of tissue culture techniques is the micropropagation and conservation of plant genetic resources of rare and endangered species. *In vitro* propagation methods have been reported to offer powerful tools for germplasm conservation and the mass propagation of many threatened plant species.³⁻⁵ With the rise in the demand for herbal medicines, tonics, cosmetics and plants with insecticidal and pesticidal properties, the potential of this technique is being exploited for use in the production of active compounds as well as the con-

servation and propagation of medicinal plants. To our knowledge, there are no published reports on the *in vitro* regeneration of *A. arctotoides* and related species. The ultimate aim of the work reported here is the development of an efficient plant regeneration system for rapid *in vitro* multiplication and conservation of this valuable germplasm. We report here the first *in vitro* procedure for plantlet regeneration from seed-derived callus of *A. arctotoides*.

Materials and methods

The experiments were carried out in the Department of Botany, University of Fort Hare, Alice. Mature achene fruits of *A. arctotoides* growing naturally on the university farm were harvested in February 2004 and used as the source of primary explants.

The collected fruits were washed in running tap water and freed of all visible particles and then rinsed in double-distilled water. They were sterilized for 20 minutes in 2.5% sodium hypochlorite solution with 2 drops of Tween 20 as wetting agent, followed by three minutes in 70% ethanol and several rinses in double-distilled water. Seed explants were carefully excised from the one-seeded achene fruit, with the aid of a stereomicroscope, under aseptic conditions. The seed testae were also removed and the kernels were placed in contact with the solid culture medium without completely immersing them. The basal medium consisted of the salt mixtures described by Murashige and Skoog⁶ with the macro elements in full and supplemented with Na₂EDTA (7.4 g l⁻¹), myo-inositol (20 g l⁻¹), thiamine-HCl (0.1 g l⁻¹), sucrose (30 g l⁻¹), and Difco-bacto nutrient agar (10 g l⁻¹). The pH was adjusted to 5.8 by adding 1 N NaOH solution dropwise before autoclaving. About 20 ml of the medium was dispensed into sterile disposable Petri plates after autoclaving at 121°C for 20 min.

Callogenesis was induced using six

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different concentrations (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg l⁻¹) of 2,4-dichlorophenoxyacetic acid (2,4-D) and two photoperiod regimes (in the dark or continuous light). The Petri dishes were sealed with parafilm and the cultures were maintained at 25 ± 3°C in a growth chamber. Light was provided by cool white fluorescent tubes with an intensity of 4000 lux. All operations and inoculations were carried out under sterile conditions in a laminar airflow cabinet. There were at least 30 explants per culture condition. Since culture for 48 hours in the 2,4-D medium is sufficient to initiate callus formation and development,⁷ the explants were transferred to a hormone-free medium after the first three days in the induction medium. They were then transferred to fresh medium every fortnight and observed for six weeks, during which time the number of explants forming callus per treatment was recorded and a visual evaluation of colouration and browning degree was made. Initiation was assumed to be unsuccessful in those explants that showed no callus formation after six weeks in culture.

Light-yellow, friable and organogenic calluses obtained from the explants were transferred to hormone-free ½MS medium with sucrose (15 g l⁻¹) and agar (6 g l⁻¹) levels reduced to half strength for callus proliferation and shoot differentiation. The pH of the medium was adjusted to 5.8. The plant material was incubated at 25 ± 3°C with a 16-h photoperiod in the growth chamber, and subcultured in fresh medium every two weeks. We investigated six treatments and four replications with three callus fragments in each treatment. The size (diameter), morphology, habituation (capacity of the callus to continue growing) and further morphogenic responses of the calluses were observed for six weeks.

Results and discussion

Calluses began forming within two weeks of incubation on MS medium with or without growth regulators.

Several factors have been reported to influence the efficiency of *in vitro* culture. Among these are the concentration of PGR in the induction medium,⁸ chilling at 4°C,⁹ and stress such as desiccation. Optimum concentration of growth regulators is, however, the principal factor in callus induction and proliferation. Different types of plant growth regulators have been reported to induce callus and plant regeneration from seed explants of many plant species.^{10–12} Among these regulators, 2,4-D is the principal synthetic auxin used

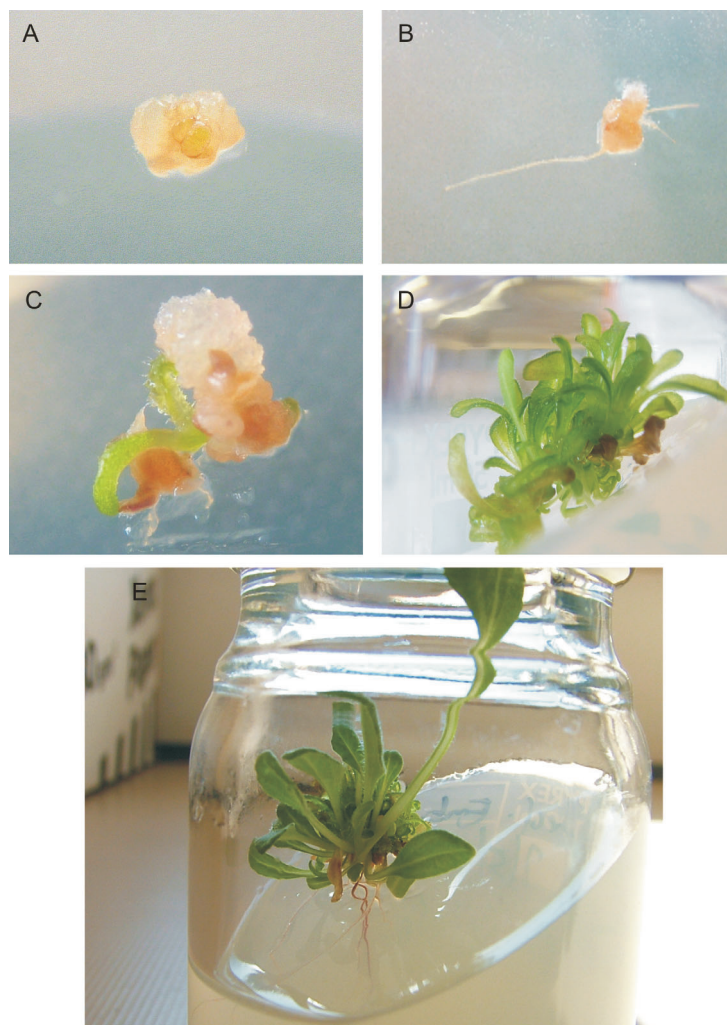


Fig. 1. Plant regeneration from seed-derived callus of *Arctotis arctotoides*. **A**, Callus formation after two weeks in hormone-free medium (explant initiated in MS medium supplemented with 2.0 mg l⁻¹ 2,4-D for three days). **B**, Shoot differentiation and profuse callusing at eight weeks in culture. **C**, Well-developed shoots at 12 weeks in culture. **D**, Callus forming roots 12 weeks in culture (culture medium was MS supplemented with 4.0 mg l⁻¹ 2,4-D). **E**, *In vitro* rooted *Arctotis arctotoides* in hormone-free MS medium.

to induce callogenesis because of its capacity to stimulate cell division in the tissues of several plants efficiently. A range of 2,4-D concentrations tested in our experiments indicated callus formation from the seed explants with or without 2,4-D supplement. The first morphogenic response was observed after one week in the medium. Within two weeks of culture, callus developed all over the surface of the seed explants. The calluses were deeply stained, light-yellow and friable in texture (Fig. 1A). The frequency of callus induction ranged between 4% and 68% on MS medium containing different concentrations of 2,4-D under the two photoperiod regimes (Table 1). The photoperiod conditions appeared to have no effect on callogenesis. However, differences based on 2,4-D concentrations were observed. The induction percentage of callus initially rose and then fell with increasing concentration of the hormone.

The highest rate (68%) of callus induction was obtained in MS basal medium supplemented with 2.0 mg l⁻¹ 2,4-D in continuous darkness (Table 1). The efficacy of this hormone for the induction of callus in many plants has been reported by various workers;^{13–16} concentrations in the range 1.0–3.0 mg l⁻¹ 2,4-D are generally used.

Although high (3.0–5.0 mg l⁻¹) and low (1.0 mg l⁻¹) concentrations of 2,4-D were capable of inducing explants to produce calluses, the latter grew very slowly, with the average size ranging from 1.6 mm to 5.2 mm (Table 1). Calluses obtained from initiation medium without hormonal supplement did not develop further, turned brownish and died. Those obtained from growth medium supplemented with 3.0–5.0 mg l⁻¹ 2,4-D developed only roots (Fig. 1B) and did not form shoots twelve weeks after culture. On the other hand, calluses obtained from explants cultured in MS supplemented

Table 1. Effect of various concentrations of 2,4-D and photoperiod on callus induction and organogenesis from seed explants of *Arctotis arctotoides*.

[2,4-D] (mg l ⁻¹)	Photoperiod ^a	Morphogenic response ^c	Rate of callus induction ^b (%)	Mean callus size (mm)	Mean number of roots ^c	Mean number of shoots ^c
0.0	+	Callus	6	2.1	0.0	0.0
	-	Callus	4	1.7	0.0	0.0
1.0	+	Callus	35	2.4	0.0	0.0
	-	Callus	26	3.6	0.0	0.0
2.0	+	Shoots and roots	58	8.7	4.5	8.5
	-	Shoots and roots	68	10.2	6.1	12.4
3.0	+	Roots	37	4.1	8.2	0.0
	-	Roots	21	5.2	7.3	0.0
4.0	+	Roots	33	2.1	10.2	0.0
	-	Roots	38	3.3	8.8	0.0
5.0	+	Callus	10	1.6	0.0	0.0
	-	Callus	7	2.2	0.0	0.0

^a+ indicates continuous light; - indicates continuous darkness.^bData collected six weeks after culture initiation.^cData collected 12 weeks after culture initiation.

with 2.0 mg l⁻¹ 2,4-D were fast growing, light yellow in colour and friable, reaching an average size of between 8.7 mm and 10.2 mm within six weeks. The formation of green spots that characterized shoot differentiation was observed eight weeks after the start of culture. In the absence of auxin and under a 16-h photoperiod, the greenish tissue mass showed further cell division and underwent shoot differentiation. This indicated that 2,4-D is an essential hormonal constituent of the medium and an optimum concentration (2.0 mg l⁻¹) is required for shoot formation in *A. arctotoides*. On further subculturing in hormone-free medium, the shoots proliferated and grew into green healthy plantlets (Fig. 1C). The optimum number of shoots (12 or 13) was obtained from the explants started on medium supplemented with 2.0 mg l⁻¹ 2,4-D. Further manipulation of the mix of growth regulators in the medium may still lead to enhanced shoot differentiation.¹⁷ Rooting of shoots (Fig. 1E) was achieved in the same hor-

none-free medium after twelve weeks in culture.

This procedure was relatively rapid as plantlets were obtained within three months of the start of culture. The regeneration mechanism appears to achieve a higher rate of shoot induction than regeneration via somatic embryogenesis.¹⁶ The protocol described in this report could serve as a foundation for further research on micropropagation and germplasm conservation of *Arctotis arctotoides*.

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