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Somatic Embryogenesis in the Nickel Hyperaccumulating Shrub, *Hybanthus floribundus* (Lindl.) F. Muell.

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Abstract

This study examines the role of cytokinins and auxins in somatic embryogenesis of the West Australian nickel hyperaccumulating shrub, *Hybanthus floribundus*. Nodal segments from micropropagated shoots were inoculated on to callus induction media consisting of basic MS medium supplemented with auxins (2,4-D, dicamba or picloram at 0, 1 or 5 µM), cytokinins (zeatin or Kn at 0, 0.1, 0.5 or 1.0 µM) or combinations of both auxins and cytokinins. Four weeks after culturing calli were observed. Callus pieces were subcultured on to fresh MS medium without any plant growth regulators. Explants produced both calli and shoots in all combinations of cytokinins and auxins. Development of somatic embryos was moderate and highly dependent upon the type and concentration of plant growth regulators used. Kinetin was more effective than zeatin. Kn produced somatic embryos either with or in absence of any of the auxins. Its effective concentration was 1 µM. Anatomical investigations have confirmed somatic embryogenesis in *H. floribundus*.

Introduction

More than 150 species of the genus *Hybanthus*, family Violaceae, occur in Africa, India, Malaysia, Americas and Australia, 11 species being found in Australia. *Hybanthus floribundus* is a perennial shrub that embraces several sub-species or ecotypes (Bennett 1969, 1999) and has an insular or disjunctive distribution across the southern part of Australia (Severne 1974).

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Metal hyperaccumulation has been reported in many plant species (Brooks 1977) and *Hybanthus floribundus* is a nickel hyperaccumulator plant, taking up nickel up to 1.4% in the leaves, dry-weight basis (Cole 1973, Severne and Brooks 1972). The hyperaccumlating property makes this species suitable for exploitation in phytoremediation a process by which contaminants from soil, sediment and wastewater are removed (Raskin et al. 1997) and/or phytomining in which specific plants are grown for harvesting a crop of metals such as nickel (Nicks and Chambers 1995, Robinsone et al. 1997a,b) have listed Australian serpentine soils as potential sites for phytomining, specially by planting the Australian nickel hyperaccumulating species, *Hybanthus floribundus* and *Stackhousia tryonii*.

The success of exploiting *H. floribundus* in phytoremediation and/or phytomining however, depends on the ability to readily establish a large population of this plant species in the field. At present, propagation via seed is extremely difficult and no other means of its multiplication has been reported (Bennett 1969, 1978). The results of the present investigation indicate that this plant species can be multiplied into a large population from a small source while exploiting available genetic variation. Limited reports are available on successful *in vitro* culture of some members of the family Violaceae. Sato et al. (1995) demonstrated regeneration of plantlets from petiole derived callus of wild viola (*Viola patrinii* DC.) while Babber and Sharma (1991) studied the anatomy of vitrified structures in *Viola tricolour*. The present study focuses on the development of mature and hardened somatic embryos for encapsulation and storage as 'artificial' seeds.

Materials and Methods

Explants: Explants were obtained from micropropagated shoots. Micropropagation was achieved by initiating the shoot tip/nodal explants from mature plants on MS basal medium supplemented with 1.0 - 2.5 mg/l BA. Shoot multiplication was achieved on the medium containing 1.5 mg/l BA. Cultures were maintained on plant growth regulator-free medium for three months, to overcome carry-over effects of previously used cytokinin. Ten millimetre sections of nodal tissues from micropropagated shoots were inoculated on to callus induction medium.

Culture media: The callus induction media was MS basal medium supplemented with either auxins (2,4-D, dicamba or picloram at 0, 1 or 5 µM), cytokinins (zeatin or Kn at 0, 0.1, 0.5 or 1.0 µM) or auxin-cytokinin mixture. Media were solidified with a 0.8% agar (Biomereieux Co. Ltd.). The pH of the medium was adjusted to 5.8.

Inoculations: Nodal explants were initiated on callus induction media (one explant/tube) for four weeks before subculturing on to fresh MS medium devoid of any plant growth regulators. Each treatment and combination was replicated three times. Culture tubes were incubated in a controlled environment room that was maintained at 22 ± 2_C and 16 h photoperiod (100 $\mu Em^{-2}s^{-1}$).

Histological studies: Somatic embryos were fixed for 48 h in FAA (formalin 10%, acetic acid 5%, ethyl alcohol 85%, v/v) and processed using the standard procedure. Serial paraffin sections were made at 10 µM thickness de-parafinised with xylene followed by absolute ethyl alcohol. Selected sections were stanined with toluidine blue (0.05% in absolute alcohol) for 5 minutes.

Results and Discussion

After four weeks of inoculation, explants turned into green, friable, cellular parenchymatous mass. This response was uniform in all treatments, indicating the presence of high endogenous content of auxins in explants. However, external application of plant growth regulators was required to trigger callus induction, as explants kept as control failed to callus (Table 1). Induced calli were organogenic and produced shoots in the auxin-free medium.

Table 1. Effect of different concentrations and combinations of plant growth regulators on callus induction and organogenesis in *Hybanthus floribundus.*

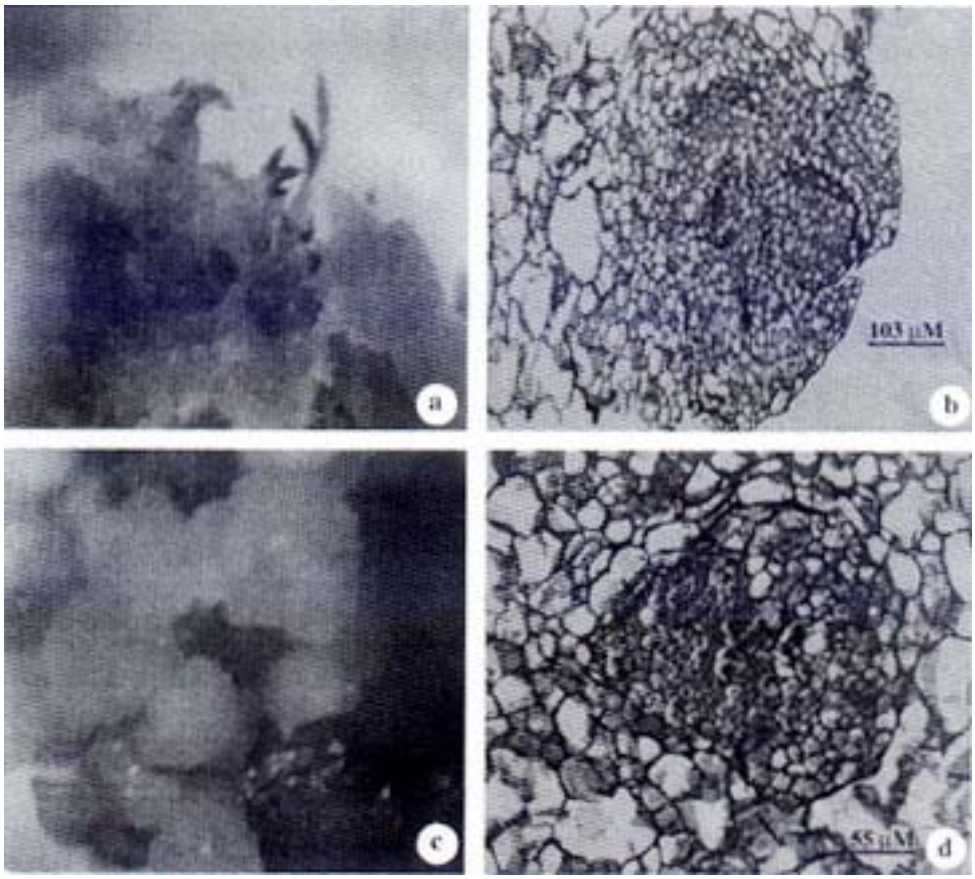
Conc. of hormone (µM)	Explants developing callus (%)	Explants producing shoot (%)	Explants producing root (%)	Explants producing somatic embryos
Control	0	0	0	0
Zeatin				
0.1	100	100	0	0
0.5	100	100	0	0
1.0	100	100	0	0
Kn				
0.1	100	100	0	0
0.5	100	100	0	0
1.0	100	100	0	100
2,4-D				
1.0	100	66	0	0
5.0	100	66	0	0
Dicamba				
1.0	100	100	0	0
5.0	100	100	0	0

(Contd.)

Table 1. (Contd.)

Picloram				
1.0	66	66	0	0
5.0	66	33	0	0
Zeatin + 2,4-D				
0.1 + 1.0	100	100	66	0
0.1 + 5.0	100	100	0	0
0.5 + 1.0	100	66	66	0
0.5 + 5.0	100	66	0	0
1.0 + 1.0	100	66	0	0
1.0 + 5.0	100	66	0	0
Kn + 2,4-D				
0.1 + 1.0	100	66	0	0
0.1 + 5.0	100	66	0	0
0.5 + 1.0	100	100	0	0
0.5 + 5.0	100	0	0	0
1.0 + 1.0	100	33	0	66
1.0 + 5.0	100	66	0	0
Zeatin + Dicamba				
0.1 + 1.0	100	100	0	0
0.1 + 5.0	100	100	0	0
0.5 + 1.0		100	0	0
0.5 + 5.0	100	0	0	0
1.0 + 1.0	100	33	0	0
1.0 + 5.0	100	66	0	0
Kn + Dicamba				
0.1 + 1.0	100	100	0	0
0.1 + 5.0	100	100	0	0
0.5 + 1.0	100	0	0	0
0.5 + 5.0	100	33	0	0
1.0 + 1.0	100	100	0	100
1.0 + 5.0	100	100	0	100
Zeatin + Picloram				
0.1 + 1.0	100	100	0	0
0.1 + 5.0	100	33	0	66
0.5 + 1.0	100	66	0	0
0.5 + 5.0	100	100	0	0
1.0 + 1.0	100	100	0	0
1.0 + 5.0	100	66	0	0
Kn + Picloram				
0.1 + 1.0	100	0	0	0
0.1 + 5.0	100	33	0	0
0.5 + 1.0	100	33	0	0
0.5 + 5.0	100	33	0	0
1.0 + 1.0	100	0	0	0
1.0 + 5.0	100	0	0	0

Histological studies revealed that shoot primordia developed in calli, but further development occurred only after their transfer to hormone-free medium (Figs. 1a, b). Anatomical studies further suggested that the origin of shoots was adventitious. Some of the parenchymatous cells of the calli differentiated into shoot primordia. Development of a dome shaped shoot meristem and primary leaves were observed (Fig. 1b).



Figs. 1a - d: Showing the development of somatic embryo. a. Callus and shoot development on MS fortified with 2,4-D 1.0 µM and zeatin 0.1 µM; b. Development of a dome shaped shoot meristem and primary leaves; c. Developing embryos on the callus surface on MS fortified with 2,4-D 1.0 µM and Kn 1.0 µM; d. Development of bipolar structures with no vascular connection to the parental vascular bundles on MS fortified with 2,4-D 1.0 µM and Kn 1.0 µM.

The progress of somatic embryo development appears to be sensitive to the type and the concentration of cytokinins used. For example, it is mostly Kn that produced somatic embryos (either alone or in combintion with auxins). Further, somatic embryo development occurred mostly at 1 µM concentration (Table 1),

although, zeatin (0.1 μM promoted formation of somatic embryos when used with picloram. These globular and cup shaped somatic embryos with tube like protuberances were first observed on the surface of calli under a dissecting microscope. Microscopic examination showed various stages of embryo development including globular, heart- and torpedo shaped structures. Embryos developed only on the callus surface in contact with media (Fig. 1c).

Anatomical studies showed zones of embryogenic cell masses. They were composed of small, isodiametric cells organised into clear embryonic structures (Fig. 1d). The latter were bipolar structures with no vascular connections with the parental vascular bundles (Fig. 1d). Cytokinin was earlier found to induce somatic embryogenesis in broad leaf plants viz. Gill et al. (1995) reported that the induction of embryogenesis in tomato requires higher hormone concentrations (15 μM) than those used in the present study. Similarly, Sagare et al. (2000) induced somatic embryos in *Corydalis yanhusuo* using 0.5 - 4.0 mg/l BA, Kn or zeatin. Also, Patel et al. (1994) achieved somatic embryogenesis in pigeon pea using only cytokinins. However, somatic embryogenesis was dependent on concentration of specific cytokinins and mineral nutrient formulation. They further reported that the induction of embryogenesis occurred only at a high cytokinin medium containing benzyladenine (22.2 μM), Kn (2.3 μM) and adenine sulphate (271 μM).

Fully developed somatic embryos germinated to produce shoots and roots in the same medium. This study has demonstrated that both organogenesis and embryogenesis are feasible in *H. floribundus* and the protocol described here can be exploited for mass propagation of this species. Further studies are required to optimise and speed up the process of somatic embryogenesis.

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