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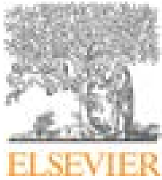

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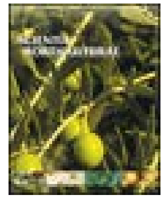

Clonal propagation of *Lomandra longifolia* by somatic embryogenesis

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ABSTRACT

A plant regeneration system based on somatic embryogenesis was developed for the efficient clonal propagation of *Lomandra longifolia*. Cultured leaf-bases, immature inflorescences and immature ovaries of *L. longifolia* formed embryogenic calli, with subsequent somatic embryo induction upon subculture to Murashige and Skoog (MS) agar medium supplemented with 2.0 mg L^{-1} 2,4-diclorophenoxyacetic acid (2,4-D), 500 mg L^{-1} casein hydrolysate, 100 mg L^{-1} myo-inositol, and 30 g L^{-1} sucrose. Of the three types of explants, immature inflorescences and immature ovaries produced only embryogenic calli, whereas leaf bases produced both embryogenic and non-embryogenic calli. Root tips cultured on 2,4-D-containing media formed a tissue that did not form somatic embryos, but instead differentiated into shoot-buds. Somatic embryo differentiation and plantlet regeneration occurred best from embryogenic calli on 2,4-D-free basal medium or MS basal medium containing 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} BAP; and the resultant in vitro-formed plantlets were successfully transferred to soil. Morphological and anatomical data describing development of calli and somatic embryos provided evidence for plant regeneration via somatic embryogenesis and, most likely, single cell origin of somatic embryos.

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1. Introduction

Lomandra is a genus of 50 species native to Australia, but two species extend to New Guinea and one of these to New Caledonia (Lee and Macfarlane, 1986). These play a vital role as understorey species, contributing to the floristic richness and biodiversity in various Australian ecosystems. Functionally, they provide food, shelter and breeding habitats for wildlife. They are excellent soil stabilizers and some species form dense stands that resist weed invasion. Our study targeted *Lomandra longifolia*, commonly named Spiny-head Mat-rush or Basket Grass, an extremely versatile and hardy plant that will grow almost anywhere. On warm summer days the flower spikes produce a pleasant perfume and the blooms are attractive in floral arrangements (Wrigley and Fagg, 2003; Stewart, 2004; New South Wales Department of Agriculture, 1966). The leaves are strap-like, forming tussocks and growing up to 1.2 m in height. A native of most Australian states, *L. longifolia* will accept waterlogged or dry soils, full sun or heavy shade, frost down to around −7 °C and tropical areas. It will also grow successfully in containers. Most *Lomandra* species are important soil stabilizing plants along river banks and streams, and are used extensively as ornamental 'grasses' in native gardens or as border plants along driveways and roadsides. *L. longifolia* grows very well in almost all areas and is often specified in revegetation planting lists. *L. longifolia* is therefore frequently required in large numbers for enhancement of degraded bushland areas and for the remediation of plant communities following disturbances caused by road construction, urban and rural development, mining and the like.

L. longifolia is usually propagated by seed. As a cross-pollinated dioecious species (Ahmad et al., 2008), it does not breed true to type and therefore, sexual propagation does not ensure that favourable genotypes (outstanding selections) are passed on to the next generation. In comparison, clonal propagation in outcrossing plants helps to preserve heterozygous genotypes that show hybrid vigour and enables retention of plants producing gametophytes of one gender only and to maintain cultivar identity. The ability to vegetatively propagate at commercially viable levels, as is possible with stoloniferous species like Bermuda grass, would alleviate the need to propagate from seed. However, our target native grass-like plant does not possess suitable structures, such as rhizomes and stolons that are amenable to vegetative propagation. Although plant numbers can be readily increased by clump division, this mode of propagation is too slow and expensive for selected genotype multiplication when required in large numbers. Methods to

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, 6-Benzelaminopurine; IAA, Indole-3-acetic acid; NAA, α-Naphthalene acetic acid; SE, Somatic embryo; TIBA, 2,3,5-Triiodobenzoic acid; 2ip, N6-(2-isopentenyl) adenine.

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improve the multiplication rate while maintaining desirable traits would therefore be of major commercial benefit to revegetation industries.

The process of somatic embryogenesis offers a viable alternative for overcoming problems of limited starting material and reducing production costs. In vitro somatic embryogenesis has been demonstrated in numerous monocotyledons (mostly Gramినaceae). Notably, somatic embryogenesis has been successfully induced in grass-like plants such as *Cyperaceae* (sedges), the north American wetland species *Scirpus robustus* (Wang et al., 2004) and recently in *Lepidosperma drummondii*, an important plant for post-mining restoration in Western Australia (Panaia et al., 2011). In the Liliaceous monocots, plant regeneration from organogenic or embryogenic callus or suspension cultures was demonstrated for several species, including *Agapanthus africanus* (Supaibulwatana and Mii, 1997), *Allium* spp. (Kim and Soh, 1996), *Asparagus officinalis* (Kunitake and Mii, 1998), *Hemerocallis* spp. (Krikorian and Kann, 1981), *Lilium* spp. (Priyadarshi and Sen, 1992; Wickremesinhe et al., 1994; Godo et al., 1998; Nakano et al., 2000) and *Tulipa gesneriana* (Famelaer et al., 1996). Somatic embryogenesis protocols have also been developed for several species of native Australian rushes (Panaia et al., 2004a,b).

To date, there is no report on the micropropagation of *L. longifolia*. The aim of this study was to develop efficient micropropagation techniques for this important grass-like plant using various starting materials, such as leaf bases, immature ovaries and immature inflorescences.

2. Materials and methods

2.1. Plant material and tissue culture

Plants of *L. longifolia* grown by Leppington Speedy® Seedlings & Supplies Pty. Ltd. situated at Leppington, New South Wales, from seeds collected in the Southern Highlands region of New South Wales, were supplied in nursery trays when ≈5 cm high. These plants were grown to maturity and maintained in the experimental field of the Plant Breeding Institute, University of Sydney, Camden, NSW (Latitude: −34.016467, Longitude: 150.670687, Sea level: 87 m). Leaf bases, immature inflorescences, and immature ovaries from the tillers of a field selected elite genotype (voucher specimen of the elite genotype has been deposited in the Australian National Herbarium CANB 763118). Tiller bases (≈5.0 cm long), immature inflorescences (harvested when the inflorescence is approximately 7.0 cm in length with about 2.0 cm being visible above the leaf bases), and immature ovaries (collected about one week before flower anthesis, equivalent to stage number 13 as reported by Ahmad et al. (2008)) were excised and surface sterilized by immersion in 50% bleach (4.5% sodium hypochlorite) with 0.1% Tween-20 for 15 min, followed by 3x rinsing with sterile distilled water in a laminar flow hood. The tiller bases were then longitudinally sectioned and individual leaf bases 2–3 cm in length were cultured, whereas young inflorescences were cut into segments 0.5–1.0 cm long. The callus induction medium was MS medium (Murashige and Skoog, 1962) containing 4.43 g L^{-1} MS basal medium powder with vitamins (PhytoTechnology Laboratories), 500 mg L^{-1} casein enzymatic hydrolysate (Sigma), 100 mg L^{-1} myo-inositol (Sigma), 30 g L^{-1} sucrose, and 0.6% agar (PhytoTechnology Laboratories), supplemented with several concentrations and combinations of growth regulators. The pH of the medium was adjusted to 5.8 with 1 N KOH or HCl prior to autoclaving for 15 min at 121 °C. Seeds of *L. longifolia* (obtained from Leppington Speedy Seedlings Pty. Ltd.) were aseptically cultured on growth regulator-free basal medium for seedling development after being immersed for 30 s in 70% (v/v) ethanol, surface-sterilized for 20 min in 50% bleach (4.5% sodium hypochlorite) with 0.1% Tween-20, and rinsed four times with sterile distilled water. In the first experiment, leaf bases of the selected female plant CANB 763118 were used and the effects of different combinations of 2,4-D and BAP concentrations on callus induction and embryogenic callus formation were examined (Table 1). In a second experiment, root-tip explants obtained from in vitro-formed seedlings (four-leaf-stage) were treated with the same combinations of 2,4-D and BAP to study their effect on callus induction and embryogenic callus formation. Explants (leaf bases, immature inflorescence segments, immature ovaries and root tips) were incubated in Petri dishes (18 explants per dish) after dispensing 25 mL of semi-solid MS basal medium into each 9.0 cm diameter dish. The Petri dishes were incubated under dark conditions at 26 ± 2 °C. Three replicates (each Petri dish considered as one replicate) were performed for each treatment in a completely randomized design, and the experiments were twice repeated. Due to the high incidence of contamination experienced with the use of immature inflorescences and immature ovaries as explant sources, a very limited quantity of clean, uncontaminated callus was yielded. Subsequent experimentation was therefore limited to MS media supplemented with only the optimum concentration of 2.0 mg L^{-1} 2,4-D that had been established in preliminary work. Callus cultures were maintained by continuous subculture (every 4 weeks) of embryogenic callus fragments on MS basal medium supplemented with 2,4-D (2.0 mg L^{-1}). The callus surface was monitored for structures resembling young somatic embryos.

2.2. Somatic embryo differentiation and plant regeneration

Eight weeks after the explants were cultured on MS medium with 2.0 mg L^{-1} 2,4-D in darkness, embryogenic calli derived from leaf bases, immature inflorescences and immature ovaries were transferred to Petri dishes containing semisolid MS regeneration medium (25 mL medium per 9.0 cm diameter dish). This medium was similar to the callus induction medium but amended with combinations of NAA (0.0, 0.5, 1.0, 2.0 mg L^{-1}) and BAP (0.0, 0.1, 1.0, 2.0 mg L^{-1}) instead of 2,4-D and BAP. The Petri dishes were sealed with parafilm and incubated at 26 ± 2 °C under a 13 h light/11 h darkness photoperiod of white fluorescent light (50 μmol m^{-2} s^{-1}). Each treatment contained 10 calli and was replicated three times in a completely randomized experiment. For later stages of plant regeneration, 250 mL capacity autoclavable plastic jars (Magenta boxes) containing 50 mL basal MS regeneration medium were employed. In all cases control treatments had a growth regulator-free basal medium. Plantlets (4–5 cm in length) were potted into 5 cm pots containing vermiculite and acclimatized in a greenhouse under controlled conditions with moderate temperature (≈25 °C) and high humidity (≈98%) for further development. They were watered once each day.

2.3. Light microscopy

Cultures were examined under a binocular microscope (ZEISS Stemi 2000-c stereomicroscope) and photographed with a digital camera. For histological observations, embryogenic calli at various stages of development were fixed in formalin acetic alcohol (FAA; 5 parts formalin: 5 parts glacial acetic acid: 90 parts 50% ethanol (v/v/v)) and stored in 70% ethanol. They were dehydrated through the ethanol series and then embedded in paraffin with melting point 58–60 °C for microtoming. Serial sections cut with a rotary microtome (Spencer 820: American Optical Co, Buffalo, NY, USA) at 8 μm in thickness were stained with safranin-*O* and Fast Green FCF (Sass, 1958), dehydrated through an alcohol series to absolute ethanol and mounted in DPX (BDH, Poole, UK).

The samples were examined using normal bright-field optics on a Nikon Eclipse E800 light microscope (Nikon Optical Co, Tokyo,

Table 1
Effect of 2,4-D and BAP on callus induction efficiency from *L. longifolia* leaf-bases (total number of explants tested = 1944).

Growth regulator concentration (mg L^{-1})		Percent efficiency of callus induction*	Embryogenic callus induction rate (%)**
2,4-D	BAP		
0.0	0.0	0.00h	0.00d
0.0	0.2	0.00h	0.00d
0.0	1.0	0.00h	0.00d
0.5	0.0	26.83cd	0.00d
0.5	0.2	19.47ef	0.00d
0.5	1.0	0.00h	0.00d
1.0	0.0	30.53bc	44.45ab
1.0	0.2	21.28def	24.17c
1.0	1.0	36.10b	0.00d
2.0	0.0	43.50a	55.76a
2.0	0.2	25.00cdef	40.28b
2.0	1.0	19.45ef	25.84c
3.0	0.0	25.92cde	49.17ab
3.0	0.2	18.52f	12.50cd
3.0	1.0	0.00h	0.00d
5.0	0.0	23.15def	50.56ab
5.0	0.2	5.58h	0.00d
5.0	1.0	0.00h	0.00d

* [(Number of explants producing callus/total explants sown) × 100].
** [(Number of embryogenic calli/total number of calli) × 100].
The same letter within each column shows no significant difference at $P = 0.05$ by LSD test. The results are after two subcultures.

Japan) and photographed with a Nikon Photo Head V-TP Sensicam camera (PCO CCD imaging: PCO Imaging, Kelheim, Germany) mounted on the same microscope.

2.4. Scanning electron microscopy

Various tissue samples were fixed in 2.5% glutaraldehyde (in 0.1 M potassium phosphate buffer, pH 7.1) at 20 °C for 2 h. After rinsing 3× in the same buffer (5 min each time), they were dehydrated in a graded ethanol series (50, 70, 95, and 100%). Samples were then critical point dried in CO_2 with a BAL-TEC 030 critical point dryer (Bal-Tec, Balzers, Leichtenstein), and mounted on sticky tape affixed to aluminum stubs and coated with 20 nm gold–palladium in a sputter coater (Edwards E306 A: Edwards Vacuum Systems, Crawley, UK). The mounted specimens were examined with a Phillips 505 scanning electron microscope (Philips, Eindhoven, Netherlands) operating at an accelerating voltage of 15 kV and images were captured digitally with an installed digital camera.

2.5. Data collection and analysis

The data from the two experiments were pooled and analysed statistically as one experiment. Treatments within each experiment were arranged in a completely randomized design with three replications. Each Petri dish was considered a single replicate. Data were statistically analysed and treatment means were separated according to the least significant difference (LSD) at a $P = 0.05$ using GenStat 6th edition (Payne et al., 2002). The total numbers of plants regenerated were evaluated after 60 days.

3. Results and discussion

3.1. Callus induction and surface features

3.1.1. Leaf-base culture

Callus was induced from leaf bases in darkness within 4 weeks after inoculation. A maximum of about 57% of cultures showed callusing in all combinations and concentrations of growth regulators (Table 1). Most calli produced from the original explants were yellow and non-friable with watery texture (viscous type) as shown in Fig. 1A and B. Three to four weeks after subculture to the same medium and two to three months after the initiation of the culture, the following morphogenetic responses were observed:

- Mucilaginous callus scored as organogenic (Fig. 1C).
- Translucent, root-like callus scored as organogenic (Fig. 1D).
- Friable and nodular callus, cream in colour with a high density of elongated, finger-like projections covering the callus surface and scored as embryogenic (Fig. 1E).
- Compact and white callus scored as embryogenic (Fig. 1F).
- Smooth compact callus with translucent and opaque bodies scored as somatic embryos (Fig. 1G and H).
- Friable, highly embryogenic cream coloured callus with induced embryoids at very early stages of development (Fig. 1I).
- Friable embryogenic bodies scored as differentiating somatic embryos (Fig. 1J–N).
- Smooth, compact and white embryogenic bodies scored as mature somatic embryos (Fig. 1O; labelled with stars).
- Direct somatic embryogenesis on leaf surface (Fig. 1P).

The above morphotypes differed in morphology, texture and growth rate. Embryogenic calli in all cases had slower growth rates compared with the organogenic callus morphotypes, and the friable embryogenic calli had higher growth and proliferation rates than the compact embryogenic calli.

Table 1 shows the effects of combinations of 2,4-D and BAP on leaf base culture of the selected female plant CANB 763118. At any concentration of BAP, 1.0 and 2.0 mg L^{-1} 2,4-D levels were the most effective in initiating callus formation. As 2,4-D concentration increased above 2.0 mg L^{-1} the percent efficiency of callus induction decreased. BAP generally had a minimizing effect on percent efficiency of callus induction except when a combination of 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} 2,4-D was used; at these concentrations 36.10% of total explants gave rise to callus, a proportion significantly higher than using 1.0 mg L^{-1} 2,4-D alone, but significantly lower than 43.50% with the optimum concentration of 2,4-D (2.0 mg L^{-1}) when used alone giving the highest efficiency of callus induction. This enhanced response may be related to interaction of the two growth regulators being present at equal concentrations and its possible effect on the endogenous auxin/cytokinin balance.

Embryogenic calli appeared after two or more subcultures in different concentrations and combinations of 2,4-D and BAP; 2.0 mg L^{-1} 2,4-D was the most effective concentration inducing

Fig. 1. Callus formation, morphotypic differentiation and maturation of somatic embryogenesis in *L. longifolia*. (A) 4-Week-old culture of leaf-bases, showing the viscous type of callus (mucilaginous). (B) A close-up view of a leaf base with a viscous type callus. (C) Proliferation of the mucilaginous callus after the first subculture. (D) Translucent, root-like callus. (E) Friable and nodular embryogenic callus with finger-like projections obtained after the second subculture. (F) Sectors of smooth, white, compact and knobby embryogenic tissue on the surface of wet, amorphous and non-embryogenic callus. (G) and (H) Smooth compact callus with differentiated somatic embryos (arrow). (I) Proliferated friable embryogenic calli. (J) A magnified view of the friable embryogenic calli in 1i. (K) Differentiating somatic embryos at the globular stage of development (arrow). (L)–(N) Differentiating somatic embryos at different stages dispersed all over the surface of the friable embryogenic callus (arrows). (O) Differentiation and maturation of somatic embryos (the arrow points to small globular somatic embryo and stars label mature embryoids). (P) Direct somatic embryos formation on leaf explant. (Q) Immature inflorescence derived callus. (R) Embryogenic white compact callus appearing on the surface of floral buds. (S)–(U) Initiation of embryogenic calli on immature ovaries and development of compact (in Fig. 1T) and friable (in Fig. 1U) calli. Scale bars = 10 mm.

compact calli. Although the embryogenic callus induction rate appeared to be about 50% and insignificantly different at 1.0, 2.0, 3.0 and 5.0 mg L^{-1} 2,4-D, the highest number of embryogenic calli induced was achieved with 2.0 mg L^{-1} 2,4-D (Table 1). Lower concentrations of 2,4-D or the addition of cytokinin (BAP) to the media had a negative effect on embryogenic callus formation. Equal concentrations of auxin and cytokinin (1 mg L^{-1}) had an inhibiting effect on embryogenic callus induction and all improvement in total percentage efficiency of callus induction was due to improved efficiency of organogenic callus morphotype. This indicated that embryogenic callus induction has more specific requirements or special signaling molecules than other callus types to allow the termination of a current gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression programme. Friable embryogenic callus appeared sometimes after prolonged subculturing of compact embryogenic callus.

In some cases, portions of the original callus induced with 2,4-D turned semi-compact, and with the appearance of bright yellow and nodular structures, somatic embryos differentiated on these semi-compact calli.

In conclusion, 2,4-D is needed to initiate high frequency callus formation from meristematic regions of cultured leaf bases. However, embryogenic callus tissue was formed (both compact and friable) after non-embryogenic calli were subcultured on media of the same composition as for the primary culture (Murashige and Skoog's basal medium) but supplemented with only 2,4-D (optimum 2.0 mg L^{-1}). Finally, globular somatic embryoids appeared on the surface of the embryogenic calli.

3.1.2. Root-tip culture

Table 2 shows the effects of 2,4-D and BAP and their combinations on callus induction from root-tips of *L. longifolia*. Like leaf bases, root-tips did not produce callus in the absence of auxin. 1.0 mg L^{-1} 2,4-D/1.0 mg L^{-1} BAP and 2.0 mg L^{-1} 2,4-D/0.2 mg L^{-1} BAP combinations produced significantly higher efficiencies of callus induction than any other combination of these two growth regulators. In both cases 100% of inoculated explants initiated yellow non-embryogenic callus.

Low (0.5 mg L^{-1}) and high (>3.0 mg L^{-1}) concentrations of 2,4-D significantly decreased the effectiveness of callus induction at all levels of BAP (Table 2). Although 13 media were effective in inducing calli, none of the calli were embryogenic or convertible into embryogenic calli even after three rounds of consecutive subculturing (Table 2). Although a few explants produced white nodes on the original callus, none of the growth was embryogenic and none had differentiated into embryogenic tissues.

Table 2
Effect of 2,4-D and BAP treatments on callus induction efficiency in *Lomandra longifolia* root-tips; embryogenic calli were not induced (total number of explants tested = 1944).

Growth regulator concentration (mg L^{-1})		Percent efficiency of callus induction*
2,4-D	BAP	
0.0	0.0	0.00h
0.0	0.2	0.00h
0.0	1.0	0.00h
0.5	0.0	12.98g
0.5	0.2	22.22f
0.5	1.0	0.00h
1.0	0.0	79.58bc
1.0	0.2	17.60fg
1.0	1.0	100.00a
2.0	0.0	87.95b
2.0	0.2	100.00a
2.0	1.0	73.15c
3.0	0.0	79.62bc
3.0	0.2	82.40b
3.0	1.0	0.00h
5.0	0.0	2.80h
5.0	0.2	58.55e
5.0	1.0	69.45d

* [(Number of explants producing callus/total explants sown) × 100].
The same letter within the column indicates no significance difference at $P = 0.05$ by LSD test.

3.1.3. Immature inflorescence and immature ovary culture

Immature inflorescences and immature ovaries can be prolific sources of explants to multiply desirable genotypes (Ahmad et al., 2008). However, in our experiments there was a high incidence of contamination when we attempted to use these plant parts, especially with samples taken from field-grown plants.

Callus tissue was initiated from juvenile cultured inflorescences (Fig. 1Q) or from floral buds of older immature inflorescences (Fig. 1R) in MS basal media supplemented with 2.0 mg L^{-1} 2,4-D and was ready for transfer after an incubation period of 4 weeks. Callus tissue was also initiated from immature ovaries (Fig. 1S–U) in the same media and under the same conditions. The subcultured callus, light yellow or cream in colour and friable in texture, proliferated vigorously with globular somatic embryo formation (Fig. 1I), but did not develop beyond the globular stage as long as 2,4-D was present; omission of 2,4-D by transferring to MS media lacking 2,4-D resulted in further growth and development of the embryoids until reaching the maturity stage as shown in Fig. 1J–N. However, white compact embryogenic calli (Fig. 1F and G) had differentiated into somatic embryos (Fig. 1O) after transfer to MS media without 2,4-D.

Table 3 summarizes the effects of MS basal media supplemented with 2.0 mg L^{-1} 2,4-D on callus induction and embryogenesis when immature inflorescences and immature ovaries were used as explant sources. About 87% of inoculated segments of immature inflorescences produced callus on most floral buds, and 100% of the induced calli has differentiated into either friable or compact embryogenic calli in approximately equal proportions. Both types of embryogenic calli differentiated into somatic embryos.

About 52% of inoculated immature ovaries initiated callus, and 100% of these were embryogenic and differentiated into mature somatic embryos once transferred to MS media without 2,4-D.

Friable calli could be maintained long-term when subcultured every four weeks to a media supplemented with 2.0 mg L^{-1} 2,4-D. This frequent subculturing was necessary, not only to refresh the culture medium and prevent necrosis, but also because of the vigorous callus growth and the development of embryoids to maturity

Table 3
Effect of 2,4-D on callus induction efficiency from immature inflorescences and immature ovaries from *L. longifolia* (total number of explants tested = 108).

Treatment	Immature inflorescences		Immature ovaries	
	Percent* efficiency of callus induction	Embryogenic** callus induction rate (%)	Percent* efficiency of callus induction	Embryogenic** callus induction rate (%)
2,4-D (2.0 mg L^{-1})	86.67	100	51.67	100

* [(Number of explants producing callus/total explants sown) × 100].
** [(Number of embryogenic calli/total number of calli) × 100].

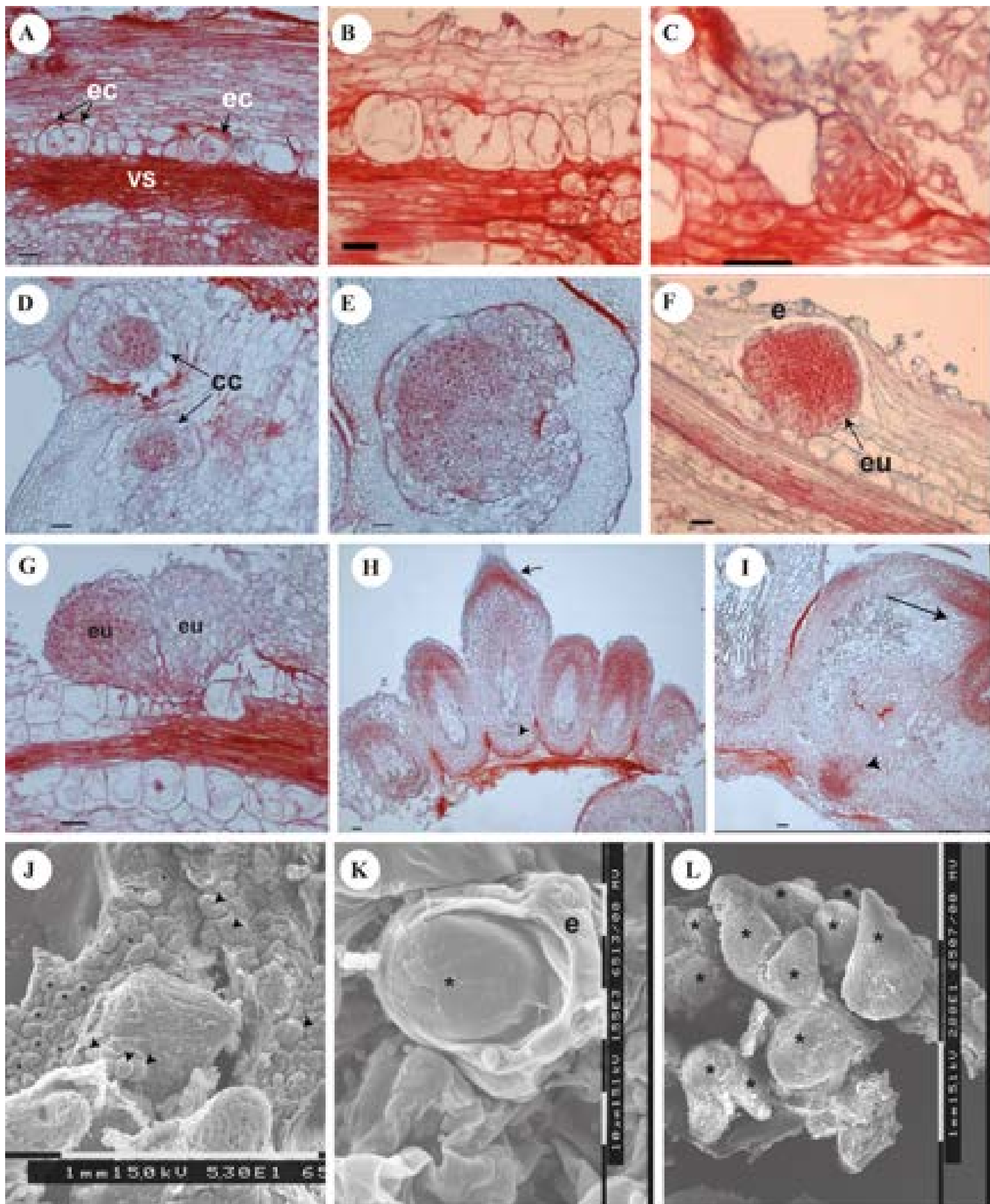

Fig. 2. Ontogeny of somatic embryos of *L. longifolia* in longitudinal sections and scanning electron micrographs. (A) Embryogenic callus originated from leaf bases showing single enlarged cells with dense cytoplasm and thick walls alongside the vascular system. (B) A close-up view of the embryogenic cells. (C) A cluster of small and cytoplasmic dense cells after few mitotic divisions. (D) Clusters of very small cells with a dense high cytoplasmic content separated from each other by groups of loose and vacuolated parenchyma cells. (E) Embryogenic unit or globular aggregate formation with cytoplasm-rich cells. (F) A growing embryogenic unit pushing through the epidermis. (G) Enlarged embryogenic units protruding through the epidermis. (H) Development of transition units with an apical (arrow) and a basal (arrow head) region. (I) Establishment of polarity by organization of a root meristem at one end of the proembryo (arrow head) and a shoot meristem at the other end (arrow). (J)–(L) SEM micrographs showing in (J). Early embryogenic culture of the granular surface (stars) with embryogenic units (arrow heads); in (K). Bipolar transition unit (star) and in (L). Late embryogenic cultures with clusters of somatic embryos (stars indicate individual somatic embryos). Abbreviations: cc, cell cluster; e, epidermis; ec, embryogenic cell; eu, embryogenic unit; vs, vascular system. Scale bar in (A)–(I) = 50 μm.

followed by germination, probably as a consequence of depletion of 2,4-D.

3.1.4. Histological analysis

Histological analyses were aimed at elucidating the origin and development of somatic embryo formation in tissue-cultured *L. longifolia*. After several mitotic divisions in some enlarged cells, alongside the vascular system and far away from the leaf epidermis (Fig. 2A and B), many small cytoplasm-rich cells with small nuclei (relative to cells and nuclei of surrounding tissue) were seen. These were most likely the embryogenic cells of the callus tissue, responsible for differentiation of meristematic zones (cell clusters) (Fig. 2C

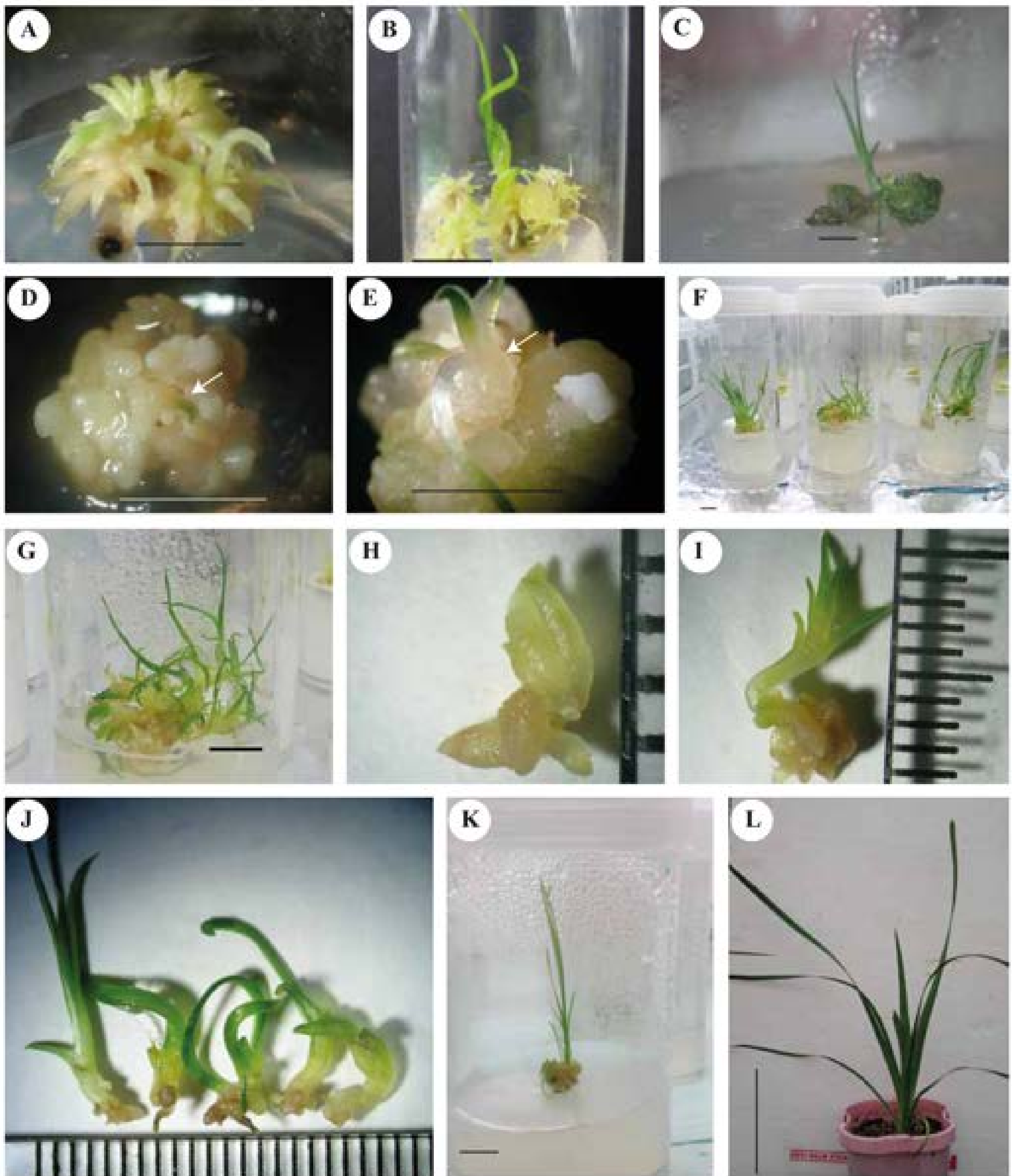

Fig. 3. Plant regeneration from different types of calli from *L. longifolia*. (A) Regeneration from root-like morphotype of organogenic callus. (B) and (C) Plantlets regeneration from compact organogenic callus showing shoot formation in (B), shoots and roots in (C). (D) Compact embryogenic callus with germinating somatic embryos. (E) Close-up view to a germinating somatic embryo on a compact embryogenic callus. (F) Regeneration of plantlets from friable embryogenic callus on transfer to growth regulator-free MS basal medium. (G) A close-up view to one of the tubes in (F). (H) Germinating somatic embryo at its earliest stage. (I) Germinating somatic embryo in more advanced stage with developed leaves. (J) Germinating somatic embryos with well-developed shoot and root structures ready for transfer to pots. (K) Somatic embryo-derived plantlet on plant growth regulator free medium. (L) Somatic embryo-derived plant established in the greenhouse. Scale bars = 10 mm in (A) to (G) and (K); ruler divisions = 1.0 mm in (H) to (J) and scale bar = 15 cm in (L).

Table 4
Effect of BAP and NAA treatments on plant regeneration efficiency from embryogenic callus in *Lomandra longifolia* (Total number of calli tested = 960).

NAA level ($mg L^{-1}$)	BAP level ($mg L^{-1}$)			
	0.0	0.1	1.0	2.0
	*Percent efficiency of plant regeneration by combination of BAP and NAA			
0.0	96.30ab	22.22g	0.0h	0.0h
0.5	90.74bc	68.52d	0.0h	0.0h
1.0	46.30e	100.00a	0.0h	0.0h
2.0	37.03f	85.19c	0.0h	0.0h

* Efficiency = percent of calluses producing roots and/or shoots [(No. of explants producing roots and/or shoots/total number of calluses evaluated) × 100].
Any two values within the table followed by the same letter are not significantly different at $P = 0.05$ by the LSD test.

and D).These cell clusters appeared as islets in the parenchymous leaf tissue and are assumed to be the proembryonal complexes (the sites of somatic embryo origin). This was followed by periclinal and anticlinal divisions leading to unorganized globular aggregates of cytoplasmically-rich cells or "embryogenic units" (Fig. 2E, F and J), that developed into bipolar "transition units", consisting of an apical protoderm-like region with small cells and larger, more vacuolated, cells in the central region (Fig. 2G, H and K). These "transition units" marked the transition from unorganized to organized growth and determined the initiation of somatic embryogenesis before maturing into somatic embryos with distinct meristematic shoot and root regions (Fig. 2I and L).

In summary, the embryogenic potential of callus tissue of *L. longifolia* was situated in large embryogenic cells. These cells divided into aggregates of small isodiametric cells containing central nuclei and dense cytoplasms.

Fransz and Schel (1991) suggested that somatic embryogenesis in immature embryo-derived maize callus progresses through three identifiable stages, viz., embryogenic units, transition units and embryoids. Histological analysis of leaf bases-derived callus in *L. longifolia* in the present study appears to support that model.

Histological and morphological aspects of somatic embryos were consistent with the general definition of somatic embryos given by Haccius (1978) and Rose et al. (2010) that somatic embryos are organized bipolar structures arising from a single cell and having no vascular connection with the maternal tissue.

3.1.5. Direct somatic embryogenesis

Globular somatic embryos developed directly from the surfaces of explants after about 4 weeks (Fig. 1P). This direct somatic embryogenesis was noticed only on leaf bases at very low frequency (seven of a few hundred using the 2.0 $mg L^{-1}$ 2,4-D treatment).

3.1.6. Somatic embryo differentiation and plant regeneration

Embryogenic callus was selected from leaf bases, immature ovaries and immature inflorescence-derived callus and was cultured in regeneration media (MS basal medium supplemented with combinations of NAA and BAP).

The effects of NAA and BAP combinations on plant regeneration are shown in Table 4. Plant regeneration was inhibited after calli had been transferred to media containing 1 $mg L^{-1}$ or more BAP in the absence or in combination with any concentration of NAA used in this study. The lowest concentration of BAP (0.1 $mg L^{-1}$) had a negative effect on regeneration compared with calli grown on media lacking growth regulators. However, combining 0.1 $mg L^{-1}$ BAP with NAA increased the efficiency of regeneration to reach the maximum at 1.0 $mg L^{-1}$ NAA before significantly decreasing at 2.0 $mg L^{-1}$ NAA to 85.19%. The effect of NAA when used as the only source of growth regulator had an inhibiting pattern when increased although there was no significant difference between 0.5 $mg L^{-1}$ NAA and the control media which lacked growth regulators. Within four weeks (Fig. 4) from transfer to regeneration media without growth regulators, or with a combination of 1.0 $mg L^{-1}$ NAA and 0.1 $mg L^{-1}$ BAP, somatic

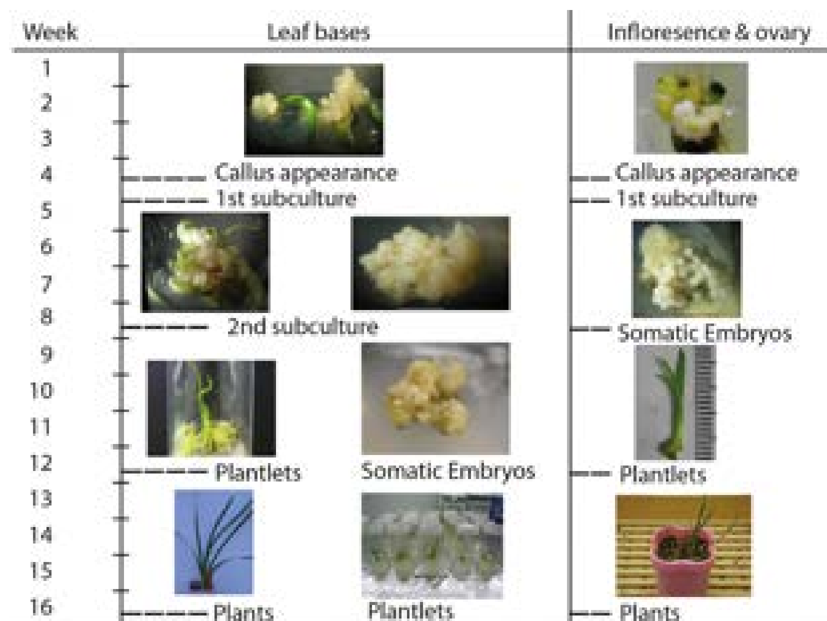

Fig. 4. Schematic diagram of the *L. longifolia* micropropagation in weeks through organogenesis and embryogenesis pathways using leaf bases and inflorescences/immature ovaries as sources of explants.

embryos continued differentiating beyond the globular stage (Fig. 1J–O) and germinating mature somatic embryos were evident within the compact embryogenic callus (Fig. 3D and E) and the white, friable callus (Fig. 3F–K). Although shoot formation due to organogenesis was observed, most shoots were formed by outgrowths of embryos. Plants regenerated from embryoids established well in the greenhouse (98%) under controlled conditions (moderate temperature and high humidity) after potting in 5 cm pots containing vermiculite (Fig. 3L).

Other growth regulators, such as TIBA (4.0 mg L^{-1}), 2ip (0.5 mg L^{-1}), IAA (0.5 or 1.0 mg L^{-1}), and picloram (10.0 mg L^{-1}), were used in the regeneration media, but none were effective (at the concentration used in this study) in inducing regeneration of plants. When organogenic callus was transferred to regeneration media supplemented with 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} BAP, initially to check its regenerability, most of the callus turned into green tissue, but shoot formation occurred in only 15% of callus pieces (Fig 3A–C). Therefore, embryogenic calli, and specifically the friable embryogenic type, were the most successful for plant regeneration in *L. longifolia*.

Although our embryogenic callus-derived regenerants were not examined cytogenetically, none exhibited visible phenotypic abnormalities. However, the formation of embryos directly from leaf cells (Fig. 3A–C) may also provide an opportunity for in vitro cloning, which importantly avoids the problem of callus culture-induced variability. Attempts are currently underway to optimize the direct embryo induction process to obtain embryogenic suspension cultures from leaf tissues. As the leaf-base and floral parts culture experiments in this study were conducted on female plants with only one genotype (CANB 763118), the effect of gender and genotype requires further study.

4. Conclusion

In summary, experiments in this study show that leaf bases or floral parts of *L. longifolia* can be used for somatic embryogenesis. The developed protocol is simple, easy to carry out and can provide large numbers of embryos and plants for mass propagation in a short period of time (Fig. 4). This capability opens prospects for using biotechnological approaches for *L. longifolia* improvement. Further research is aimed at culturing calli of *L. longifolia* in liquid media systems to simplify the procedure of inducing formation of high qualitative and quantitative somatic embryos, for developing scale-up technology employing bioreactors for mass propagation, and for comparative studies of somatic and zygotic embryogenesis.

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