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Direct somatic embryogenesis and plant regeneration in lily

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Abstract: Aiming at *in vitro* plantlet regeneration *via* direct somatic embryogenesis in lily (*Lilium* spp.), stamens, styles, filaments and flower pedicel sections of the Oriental hybrid 'Star Gazer' and the Asiatic hybrid 'Pollyanna' were cultured on MS (Murashige and Skoog) medium supplemented with NAA (ß-naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), dicamba or picloram at different concentrations. None of 'Pollyanna' explants showed any cell proliferation and browned and died after four weeks from culture onset. Styles and filaments of 'Star Gazer' could sustain organogenesis and callus formation, according to the different auxin treatments. Flower pedicel sections of 'Star Gazer' cultured on media containing NAA, dicamba or picloram underwent direct somatic embryogenesis. When transferred to hormone-free medium, somatic embryos readily germinated forming complete plantlets.

1. Introduction

Applying biotechnology for plant breeding or mass propagation purposes requires cloning vegetative plant material by tissue cultures. Using these techniques, plantlet formation can be achieved from various types of tissues and through different regeneration pathways such as organogenesis or somatic embryogenesis. Among these, the latter offers the advantage of strongly reducing the risks of chimeral traits in the regenerated plants (Nomura and Komamine, 1985; Mariani *et al.*, 2000). This morphogenic process can occur either from unorganized tissues or directly from differentiated ones.

In breeding programs, once propagation protocols are assessed for the target crop, both embryogenic pathways can be applied depending on the research goals. During dedifferentiation and redifferentiation processes, in fact, chromosomal and genetic alterations may occur in cell lines with the consequence of the release of genetic variability (D'Amato, 1977; Evans and Sharp, 1983). This source of variation, known as somaclonal variation (Larkin and Scowcroft, 1981), represents a useful tool for *in vitro* selection programs. On the other hand, direct somatic embryogenesis is considered to be a suitable regeneration pathway to avoid somaclonal variation and produce true-to-type plants (George, 1993). This type of regeneration could be suitable for genetic transformation programs, where somaclones are undesired.

Recently, a number of protocols have been assessed to regenerate plants *via* somatic embryogenesis either from unorganized tissues or from differentiated ones in both dicots and monocots. In this regard, somatic embryogenesis in lily (*Lilium* spp.) was reported to occur from cell suspension culture (Tribulato *et al.*, 1997; Kim and Ahn, 2005) and directly from bulb scales (Haensch, 1996; Nhut *et al.* 2002).

In the present work, we report an approach for the assessment of a protocol to clone lilies *via* direct somatic embryogenesis from flower parts. Towards this goal, various types of explants, according to their demonstrated suitability in tissue culture (De Jong *et al.*, 1993; Raemakers *et al.*, 1995), were cultured under different auxin treatments in order to characterize both biological material and culture conditions suitable to regenerate plantlets directly from the explants and to highlight the putative interactions between the experimental factors.

2. Materials and Methods

The Asiatic hybrid 'Pollyanna' and the Oriental hybrid 'Star Gazer' were used as genetic material in the

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experiments. Stamens, styles, filaments and flower pedicels were collected from apparently healthy greenhouse-grown plants during late fall.

The first two flowers from the bottom of the raceme, collected when tepal colour turned from green to pink ('Star Gazer') or yellow ('Pollyanna'), were used as explant source. To avoid donor plant effect, flowers were randomly collected from at least 30 different stems. For surface sterilization, they were dipped in 80% (v/v) ethanol solution and flamed for 2-3 s. After removing the tepals, stamens (filament with anther) were excised from the receptacle and placed in Petri dishes; styles and filaments were divided into two transversal sections and placed on the medium. Pedicels were removed from the raceme and, after discarding the flower, they were surface sterilized by dipping in 70% (v/v) ethanol solution for 2 min, followed by 20 min in 2% (v/v) sodium hypochlorite solution, and then rinsed three times in sterile deionized water. Four to six transversal sections, 3-5 mm thick, per flower pedicel, representing one-third of the pedicel nearest to the receptacle, were placed upside up in 90 mm Petri dishes.

Hormone treatments consisted of ß-naphthaleneacetic acid (NAA) (5 or 15 µM), 2,4-D-dichlorophenoxyacetic acid (2,4-D) (2 or 6 µM), 3,6-dichloro-o-anisic acid (dicamba) (2 or 6 µM) or 4-amino-3,5,6-trichlorophenoxyacetic (picloram) (2 or 6 µM) added filter sterile to the cooling autoclaved basal medium. The latter was composed by basal MS (Murashige and Skoog, 1962) salt solution with vitamins, 3% (w/v) sucrose and gelled with 0.3% (w/v) gelrite. The pH of the medium was adjusted at 5.7 before autoclaving at 121°C for 20 min.

Cultures were kept at 25±2°C in the dark. All explants were transferred every fourth week onto fresh medium. Sixty explants per treatment were used divided among four Petri dishes. Differences among auxin treatments were tested for significance (ANOVA) followed by Student-Newman-Keuls multiple range test to compare means (Snedecor and Cochran, 1989); all statistical analyses were performed using COSTAT software (COSTAT 2.04, 1986). Explants were observed weekly using a Leica WILD-MD 80 stereoscope.

Sixteen weeks from culture onset, somatic embryos, 2-3 mm in diameter and still attached to the explants, were transferred to hormone-free basal medium. Regenerated plantlets were grown *in vitro* under 16 hr photoperiod (35 µE m^{-2} s^{-1}; cool white fluorescent light) at 25±2°C until bulbs reached a diameter of about 10 mm. They were then potted in moist peat and transferred to a greenhouse for further growth.

One batch of embryo like structures (ELS) and mature somatic embryos, from outside the experimental plot, were collected from explants and fixed in 4% glutaraldehyde in a 20 mM phosphate buffer with 135 mM NaCl, pH 7.2, for 2 hr. Samples were then dehydrated through an ethanol series of 30, 50, 70 (overnight), 70, 90 and 100%. Subsequently they were coated with gold in a sputter coater for scanning electron microscopy for 60 s. Thereafter embryos were examined and photographed in a Zeiss DSM 940.

3. Results

Different reactions were detected between cultivars and explants. All 'Polyanna' explants browned and died within four weeks from culture onset, showing that, at least in the applied protocol, this cultivar seemed to be recalcitrant to tissue culture.

The same results were obtained with 'Star Gazer' stamens, which underwent rapid necrosis soon after culture onset. The morphogenic response differed according to the explant type as reported in Table 1. Organogenesis was detected from styles and filaments as well as friable and compact callus formation; somatic embryogenesis occurred from flower pedicel sections only (Fig. 1 a-f).

Table 1 - Morphogenic response of 'Star Gazer' explants (n=60) cultured on solidified media containing NAA, 2,4-D, dicamba or picloram

Auxins (µM)	Explants		
	Styles	Filaments	Flower pedicels
NAA 5	R, AS	R, AS CC	SE
NAA 15	R, AS, CC	R, AS, CC	SE
2,4-D 2	N	N	N
2,4-D 6	N	N	N
Dicamba 2	FC	R, AS, FC	SE, FC
Dicamba 6	AS, FC	R, AS, FC, CC	SE, FC
Picloram 2	FC	AS, FC	SE, FC
Picloram 6	AS, FC	R, FC	SE, FC

AS= adventitious shoots; CC= compact callus; FC= friable callus; N= necrosis; R= roots; SE= somatic embryos.

After 12 weeks from culture onset, somatic embryos underwent friable callus formation on media containing dicamba or picloram at both concentrations. This callus was grown on auxins containing media (data not shown) and could either represent a novel approach towards lily plantlet production or be involved in breeding programs such as *in vitro* selection.

Auxin treatments led to different morphogenic responses (Tables 1 and 2). Explants treated with 2,4-

Table 2 - Embryogenic or callogenic explants and somatic embryos differentiated from flower pedicel sections of 'Star Gazer' (n=60)

Auxins (µM)	Explants (%)		Embryos (n)	
	Embryogenic[(z)]	Callogenic	Total	Per embryogenic explant
NAA 5	1.67 a	0.0	2 a	2.00
NAA 15	3.33 a	0.0	3 a	1.50
Dicamba 2	21.67 b	8.33	52 bc	4.00
Dicamba 6	16.67 b	3.33	28 b	2.55
Picloram 2	8.33 ab	3.33	13 a	2.60
Picloram 6	36.67 c	5.0	67 c	3.05

[(z)] Values designated with the same letter within a column are not significantly different at P ≤0.05.

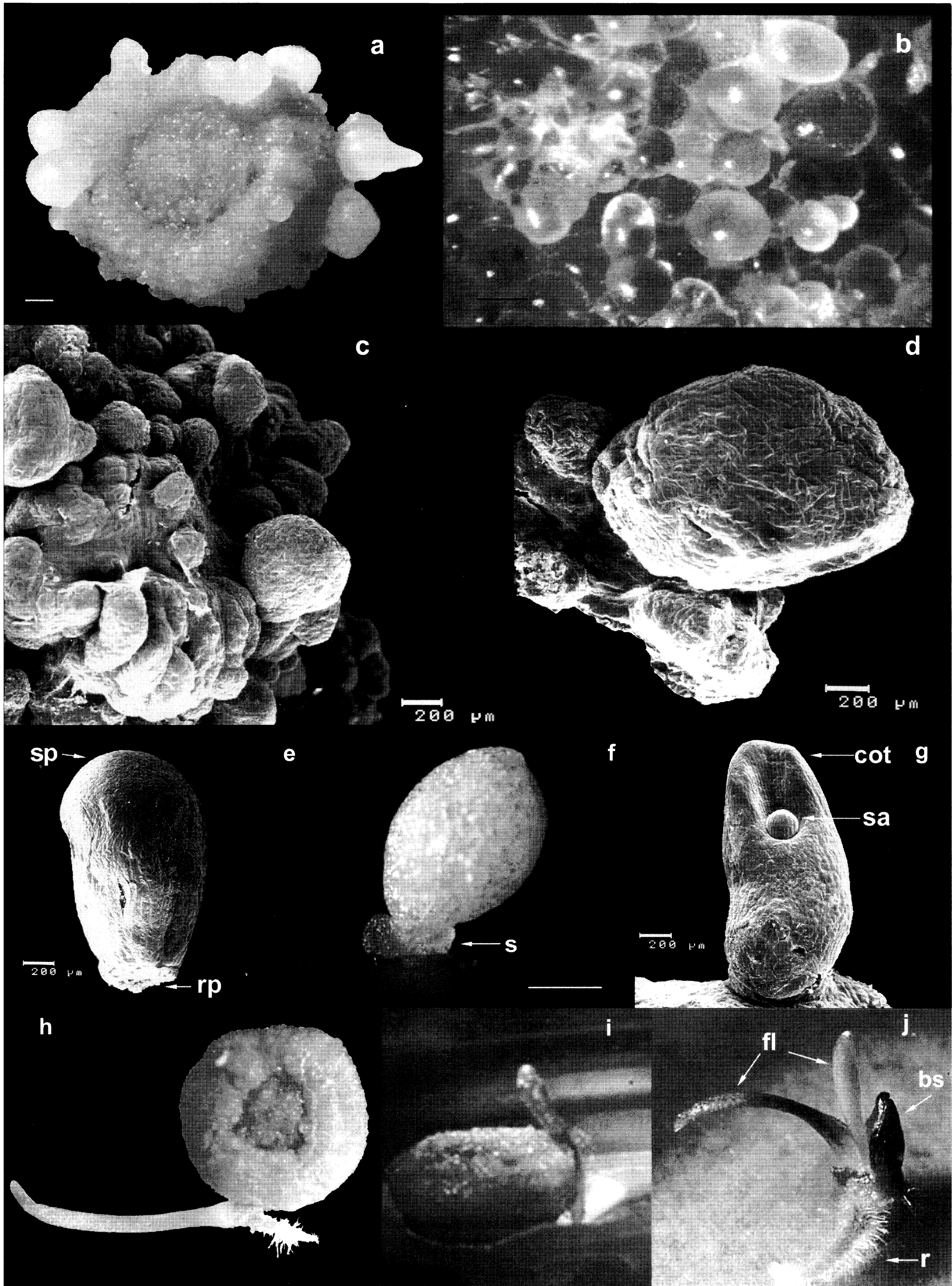

Fig. 1 - Plant regeneration *via* somatic embryogenesis in 'Star Gazer'. a) Explant after 16 weeks of induction period with attached somatic embryos. b) Round globular structures formation on the epidermis of flower pedicel sections after four weeks from culture onset. c-d) SEM of globular embryogenic structures. e) SEM of a mature somatic embryo (sp= shoot pole; rp= root pole). f) Mature somatic embryo still attached to the explant (s= suspensor). g) germination of a somatic embryo still attached to the explant (SEM - cot= cotyledon; sa= shoot apex). h-i) Plantlet formation following somatic embryogenesis on hormone free medium. j) new plantlet formation (bs= bulb scale; fl= first leaf; r= root). a, b and f bars= 1 mm.

D, demonstrating rapid necrosis, died within four weeks.

NAA induced different morphogenic responses according to the cultured explants. Somatic embryogenesis occurred from flower pedicel sections on both concentrations, although embryo yield was rather low.

Dicamba and picloram promoted organogenesis and friable callus formation from styles and filaments and direct somatic embryogenesis from flower pedicel sections. Overall, the best results were obtained with picloram 6 μM and dicamba 2 μM, with 36.6% and 21.6% respectively, of embryogenic explants obtained from cultured explants (Table 2); moreover both treatments led to the highest number of somatic embryos produced per embryogenic explant (three and four respectively).

When transferred to MS hormone-free medium, somatic embryos still attached to the explants readily germinated within two weeks, forming complete plantlets (Fig. 1 g-j) while callus clumps arising from the embryos did not proliferate, indicating that for further growth auxin-added media was necessary. From explants treated with dicamba 2 μM a higher number of regenerated plantlets than the previously recorded embryos was obtained (Table 3), showing that, even after transferring the explants on MS hormone-lacking medium, the auxin treatment had been an efficient stimulus in order to change cell division plans and lead to further embryo development. Abnormal structures were also formed (Table 3): a possible indication that a 16-week induction period was far too long and could negatively affect plantlet formation. This result should be kept in mind either for additional detailed studies on timing of explant exposure to auxin treatments, aiming at somatic embryogenesis, or as a possible tool for secondary embryogenesis achievement.

Table 3 - Response of somatic embryos of 'Star Gazer' when transferred to MS hormone-free medium

Auxins (μM)	Transferred embryos (n)		Pl (n)	AS (n)	DE (n)
NAA 5	2		0	0	2
NAA 15	3		2	0	1
Dicamba 2	52	→ MS	65	10	0
Dicamba 6	28		22	0	6
Picloram 2	13		7	1	5
Picloram 6	67		35	14	18

Pl= plantlets; AS= abnormal structures; DE= dead embryos.

4. Discussion and Conclusions

During the first eight weeks, tissue proliferation activity on explants was observed to occur differently. In flower pedicel sections it involved both the epidermis and the upper cutting surface (Fig. 1 b), while in styles and filaments it was only observed along the cutting surface. Round globular structures arose from the epidermis of the flower pedicel sections, while tissue proliferation observed along the cutting surface did not develop any further. This result shows how the auxin stimulus on explants was efficient to induce somatic embryogenesis from the epidermal cells, which can be considered as pre-embryogenic determined cells (Sharp *et al.*, 1982).

Flower pedicel sections were previously shown to be suitable explants for *in vitro* bulblet regeneration of *Lilium longiflorum* (Liu and Burger, 1986) on media containing NAA or IAA in combination with kinetin or N^6-benzyladenine (BA). In tissue culture the use of cytokinins, alone or together with auxins, is usually useful in promoting adventitious shoot formation but, *vice versa*, it reduces or inhibits somatic embryogenesis (Loiseau *et al.*, 1995); in our experiments auxins not combined with other plant growth regulators induced somatic embryogenesis and no organogenesis occurred.

2,4-D was shown to be suitable for induction of direct somatic embryogenesis in various monocot flower bulbs including Oriental and Asiatic lilies (Haensch, 1996; Kim and Ahn, 2005), as well as for callus induction from zygotic embryos in different lily species and hybrids (Famelaer *et al.*, 1996). In the present experiment it appeared to be lethal for the studied explants: with regard to the application of this auxin in lily tissue culture, interactions with biological experimental factors are quite relevant and should be studied in detail.

In literature, NAA has been described as a suitable growth regulator for induction of somatic embryogenesis *via* callus in *L. regale* (Pelkonen and Kauppi, 1999). So far, the use of NAA to induce direct somatic embryogenesis in lily has not been reported.

The use of dicamba or picloram in tissue culture was reported to be a suitable tool for the induction of direct somatic embryogenesis in lily hybrids (Haensch, 1996; Kim and Ahn, 2005) and indirect somatic embryogenesis in *L. longiflorum* (Tribulato *et al.*, 1997) and *L. formosanum* (Nakano *et al.*, 2000).

Embryo germination was achieved after removing the auxins from culture media. The choice to transfer the explants onto growth regulator-lacking medium was supported by the consideration that long term exposure to hormones induces either callus formation (Wernicke and Brettell, 1980) or development of abnormal structures from somatic embryos (Schiavone and Cooke, 1987), while reducing the auxin concentration or totally removing them from culture media was reported to be stimulating for further plantlet formation (Bornman, 1993; Raemakers *et al.*, 1995).

From the present experiments it is possible to highlight how the morphogenic response in lily tissue culture, besides the starting material, is genotype dependent. For a very complex genus like *Lilium* (Van Creij *et al.*, 1993), genotype choice plays a relevant role when assessing a protocol for plantlet regeneration through somatic embryogenesis.

In conclusion, according to our protocol, somatic embryos can be obtained from flower pedicel sections of the Oriental lily 'Star Gazer' on auxin-added MS media. Among the auxin treatments NAA, dicamba and picloram could promote somatic embryogenesis, and plantlet formation can be performed after transferring the explants onto hormone-free medium. As abnormal structure formation occurred, an induction period shorter than 16 weeks is preferable in order to avoid this problem. In the near future, phenotypic behavior of the regenerated plantlets will be studied along with molecular investigations with the aim of comparing our plants with commercially grown ones and to determine their putative genetic stability following the regeneration protocol.

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