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# Optimized Growth and Plant Regeneration for Callus of *Lilium longiflorum* 'Nellie White'

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## ABSTRACT

Rates of growth and regeneration were compared for compact callus, friable callus, and suspension cells of *Lilium longiflorum* 'Nellie White' to determine the optimal culture conditions. The highest frequencies of embryogenic callus induction (60-90%) occurred from compact callus cultured on either picloram (0.5, 1, or 2 mg/L) or dicamba (2 mg/L). Fresh weight (FW) was higher for compact callus induced from bulb scales cultured on MS medium supplemented with picloram (0.5, 1, or 2 mg/L) compared to scales cultured on MS medium supplemented with dicamba (2, 4, or 8 mg/L). Compact callus cultured on picloram (0.5, 1, or 2 mg/L) or dicamba (2, 4, or 8 mg/L) grew slowly with a 1.2X increase in FW/month compared with suspension cells grown in 0.5 mg/L picloram that increased 1.7X in FW/month. Regeneration rates were similar (23-35 plantlets/g FW callus) for compact callus cultured on either dicamba (2 or 4 mg/L) or picloram (0.5 or 1 mg/L), but 3% of the plantlets regenerated from dicamba were phenotypically abnormal while none were abnormal with picloram. Suspension cells showed a lower regeneration rate than compact callus with a maximum of only 12 plantlets regenerated from one g fresh weight suspensions cells grown in 0.5 mg/L picloram. A fast-growing, friable callus was induced and selected from compact callus cultured on MS medium with 2 mg/L dicamba and 9% sucrose but not from 3, 6, or 12% sucrose. Friable callus grew 5X faster than compact callus and formed numerous somatic embryo-like structures when cultured on MS medium with 1% activated charcoal, but only a few embryo-like structures germinated to form plants with roots.

**Keywords:** Easter lily, regeneration, suspension cells
**Abbreviations: BA**, 6-benzyladenine; **dicamba**, 3,6-dichloro-*O*-anisic; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **FW**, fresh weight; **NAA**, α-napthaleneacetic acid; **picloram**, 4-amino-3,5,6-trichloropicolinic acid; **MS** medium (Murashige and Skoog's medium); **TDZ**, thidiazuron

## INTRODUCTION

Transformation of lilies has been reported and all reports, except those by Mercuri *et al*. (2003), Krens *et al*. (2009), and Azadi *et al*. (2010, 2011) have used the *Gus* reporter gene in an effort to establish an efficient transformation system (Watad *et al.* 1998; Irifune *et al.* 2003; Ahn *et al.* 2004; Cohen *et al.* 2004; Hoshi *et al*. 2004; Kamo and Han 2008; Li *et al*. 2008; Ogaki *et al.* 2008; Azadi *et al*. 2009; Liu *et al*. 2011; Núñez de Cáceres *et al*. 2011; Shi *et al*. 2012). The difficulty with transforming lilies using callus is that the transformation frequency is typically very low, except when using *L. longiflorum* 'Snow Queen' and *Lilium*×*formolongi* (Cohen *et al*. 2004; Ogaki *et al.* 2008; Azadi *et al.* 2009, 2010). Both 'Snow Queen' and *L.*×*formolongi* readily form callus that can be used to establish suspension cells amenable to transformation.

Callus growth rate and regeneration frequency contribute to the transformation frequency. Regeneration occurs readily from lily callus, but the callus typically consists of large, compact pieces that are slow-growing, making it difficult to accumulate enough callus for transformation experiments and to quickly select for putatively transformed callus and plants following delivery of foreign DNA.

Compact callus of various *Lilium* species has been induced from a variety of organs including filaments of 26 cultivars (Mori *et al*. 2005; Krens *et al*. 2009), filaments with anthers attached of *L. longiflorum* 'Wase Teppo Yuri' (Arzate-Fernández *et al*. 1997), leaf explants and stem nodes of *L. longiflorum* 'Georgia', Oriental hybrids 'Tiber, 'Casa Blanca' and 'Reizah,' and Lanzhou lily (Bacchetta *et al*. 2003; Kim *et al*. 2005; Sangthong *et al*. 2005; Mi and Liu 2008; Xu *et al*. 2009), bulb scales of 21 lily species (Priyadarshi and Sen 1992; Godo *et al*. 2001; Kim and Ahn 2005; Krens *et al*. 2009; Kanchanapoom *et al*. 2011), seeds of *Lilium x formolongi* (Mii *et al*. 1994), styles and flower pedicels of *L. longiflorum* 'Snow Queen' and Oriental hybrid 'Star Gazer' (Tribulato *et al*. 1997; Krens *et al.* 2009), and *in vitro*-grown bulb slices of *L. michiganense* (Ault and Siqueira 2008). Callus induction occurred in 30 of the 33 *Lilium* genotypes tested (Mori *et al*. 2005).

Friable callus has been isolated from *L. longiflorum* 'Snow Queen' and was used to establish cell suspensions capable of plant regeneration by somatic embryogenesis (Tribulato *et al*. 1997; Cohen *et al.* 2004). Mii *et al.* (1994) established a "nodular callus" of *L.* ×*formolongi* cv. 'Azusa' that formed meristematic nodules when grown as a suspension, and plants were regenerated from protoplasts made from the suspension cells. Friable callus of *L. longiflorum* grown as a static liquid culture was reported to have a significantly higher rate of regeneration than callus (Nhut *et al*. 2006).

Regeneration from lily callus occurs by both organogenesis and somatic embryogenesis (Pelkonen and Kauppi 1999; Tribulato *et al*. 1997; Nhut *et al*. 2002, 2006). Plants regenerated from callus, suspensions, static liquid cultures, and callus grown in an airlift type bioreactor are usually phenotypically normal although abnormal flowers and changes in ploidy have been noted (Supaibulwatana and Mii 1998; Kim and Ahn 2005; Sangthong *et al*. 2005).

In the US the predominant *L. longiflorum* 'Nellie White', commonly known as the Easter Lily, has been

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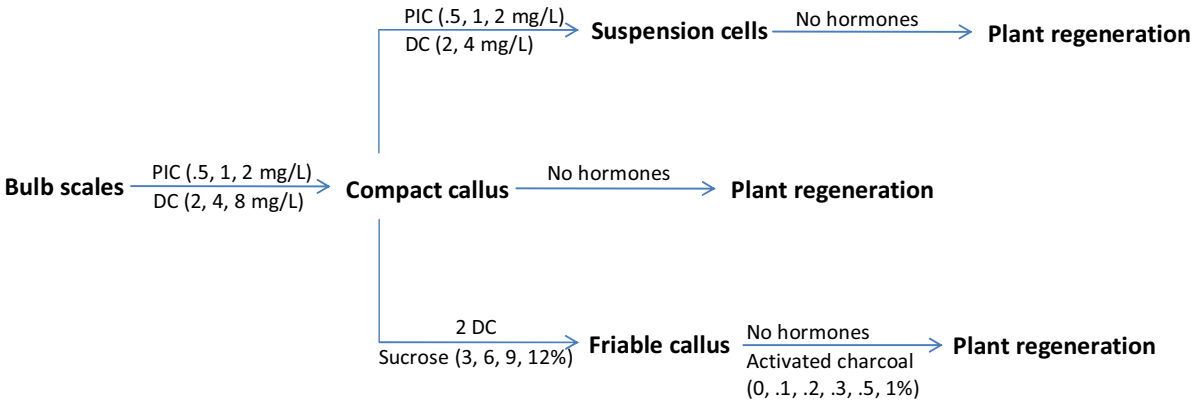


**Fig. 1 Diagram showing the experiments done using compact and friable callus and suspension cells cultured on MS medium with either 0.5, 1, or 2 mg/L picloram (PIC) or 2, 4, or 8 mg/L dicamba (DC).**

grown for >60 years (Lee Riddle, Easter Lily Foundation, pers. comm.). There is one report that callus was induced and plants regenerated from 'Nellie White' using MS medium supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L 6-benzyladenine (BA), but no information was provided on friable callus or suspension cells (Wickremesinhe *et al*. 1994). This study compares 1) callus induction and growth of both callus and suspension cells using the phytohormones 3,6-dichloro-*o*-anisic (picloram) and 4-amino-3,5,6-trichloropicolinic acid (dicamba) and 2) regeneration of plants from compact callus, friable callus, and suspension cells of 'Nellie White' to determine the optimal *in vitro* conditions for use in future genetic engineering experiments.

## MATERIALS AND METHODS

### Plants *in vitro*

Plants of *Lilium longiflorum* 'Nellie White' were initially established *in vitro* by surface sterilizing bulbs received from Dahlstrom and Watt Bulbs (Smith River, CA). Bulb scales were surface sterilized for 20 min in 20% (v/v) commercial bleach with 20 drops Tween-20/L followed by 3 rinses, 5 min each rinse in distilled water. Bulb scales were then cultured on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, and the following in mg/L: 6.0 BA, 0.1 NAA, 1.0 glycine, 100.0 *myo*-inositol, 1.0 thiamine, 0.5 nicotinic acid, 1.0 pyridoxine and solidified with 0.2% Phytagel (Sigma Aldrich Chemical Co., St. Louis, MO). Plants that regenerated from the bulb scales were cultured on MS medium lacking hormones and grown at 25°C under a 16 h photoperiod with cool white fluorescent lights (40-60 µmol $m^{-2}$ $s^{-1}$). Plants were transferred monthly to fresh medium and used as the source of bulb scales for all experiments.

### Compact callus induction, growth, and regeneration

Compact callus was initiated from bulb scales cultured on MS medium supplemented with either 2, 4, or 8 mg/L (9, 18, 36 µM) dicamba or 0.5, 1, or 2 mg/L (2, 4, 8 µM) picloram (**Fig. 1**). Callus cultures were maintained in the dark at 25°C and transferred monthly to fresh medium. The amount of callus induced was measured one year after bulb scales were placed on each hormone concentration. There were four plates of bulb scales, 10 bulb scales/plate, cultured for each hormone concentration, and the experiment was repeated at two different times so there were 80 bulb scales in total for each hormone concentration.

One-year-old callus that had been separated from a bulb scale explant was used for determining callus growth and regeneration. The callus in each of four plates was weighed and then cultured on MS medium with either dicamba or picloram. Initial callus fresh weight (FW) was approximately 1 gram for both callus growth and regeneration experiments. After one month the callus was weighed to determine the increase in FW ratio. The increase in FW was calculated by subtracting the initial FW from the final FW and then dividing by the initial FW; the experiment was repeated twice.

The amount of regeneration was determined by culturing four plates of callus on MS medium lacking hormones. Callus was transferred monthly to fresh medium and cultured in the dark until shoots began regenerating at which time they were transferred to the temperature and light conditions used for growing plants *in vitro*. A number of plantlets, 0.5-1 cm long, were counted during a 6 month period. Each experiment was repeated twice for callus grown one year on MS medium with either 2, 4, 8 mg/L dicamba or 0.5, 1, or 2 mg/L picloram resulting in 8 plates in total for each hormone concentration.

### Friable callus induction, growth, and regeneration

Compact callus was induced from bulb scales cultured on MS medium with 2 mg/L dicamba. A few lines of friable callus were obtained after the second and third subcultures from 3 to 7-month-old compact callus that had been chopped up into small pieces with a scalpel and then transferred to MS medium supplemented with 2 mg/L dicamba and 9% sucrose. Friable callus was induced and maintained in the dark. It was transferred monthly to MS medium with 2 mg/L dicamba and 9% sucrose. Growth of friable callus was measured as described for compact callus. Regeneration was determined by placing weighed callus (~1 g FW) in each plate containing MS medium and either 0, 0.1, 0.2, 0.3, 0.5, or 1% (w/v) activated charcoal. Callus was placed in the dark and later in the light when plantlets started to form. Plantlets were transferred to MS medium without charcoal for further development. Four plates of callus were grown for each activated charcoal concentration and the experiment was repeated twice resulting in 8 plates in total for each hormone concentration.

### Suspension cell growth and regeneration

Suspension cells were initiated by placing 3 bulb scales covered with compact callus in 25 ml liquid MS medium supplemented with the same hormone concentration as used for its callus induction i.e. either picloram (0.5, 1, or 2 mg/L) or dicamba (2 or 4 mg/L). After one month, callus that had fallen off the bulb scale was transferred to fresh medium. Suspension cells were cultured for 6 months in MS medium with either 0.5 or 1 mg/L picloram or 2 mg/L dicamba and used for regeneration experiments. Suspension cells could not be maintained 6 months in MS medium supplemented with either 2 mg/L picloram or 4 mg/L dicamba so they were unavailable for the regeneration experiments. Cells were grown in the dark at 25°C on a gyratory shaker at 120 rpm. Each month the medium was completely removed and approximately half of the cells placed in 25 ml fresh medium.

Growth was determined by weighing the suspension cells (FW) to be subcultured into 25 ml MS medium supplemented with either 0.5 mg/L picloram or 2 mg/L dicamba. Suspension cells subcultured in MS with 1 mg/L picloram were not included in the growth experiment because they died after approximately 9 months on this concentration of picloram. The final FW was mea-

sured four weeks later. Four flasks of cells were used for each phytohormone concentration and the experiment was repeated twice. Regeneration was determined by weighing suspension cells (1-2 g FW) and culturing them on solid MS medium in the dark and later in the light when they began regenerating plantlets. The number of 0.5-1 cm long plantlets regenerated was counted during a six month period. Four plates were regenerated for each phytohormone concentration and the experiment was repeated twice. A total of 8 flasks of cells were used to determine the growth and regeneration for each hormone concentration.

### Statistics

Data was analyzed using SigmaStat (SPSS, Inc. Chicago, IL). One-way Analysis of Variance was performed and means shown by different letters are significantly different at $P < 0.05$ according to the Dunn's multiple comparison test (**Figs. 1-5, 7**).

## RESULTS AND DISCUSSION

### Compact callus induction

The amount of compact callus induced from bulb scales cultured on MS medium with picloram was significantly higher than on dicamba (**Fig. 2**). Picloram, 0.5 mg/L, was optimal for callus induction from bulb scales although the amount was not statistically significant compared to bulb scales cultured on either 1 or 2 mg/L picloram. A high percentage of the bulb scales (80-90%) cultured on MS medium with either 0.5, 1, or 2 mg/L picloram had embryogenic callus (**Fig. 3**). Dicamba, 4 and 8 mg/L, were less effective for the induction of embryogenic callus, although 58% of the bulb scales induced embryogenic callus on 2 mg/L dicamba which was not statistically different than picloram. Other researchers have cultured bulb scales of *L. longiflorum* on MS medium supplemented with either 1 mg/L picloram, 5.4 µM (1 mg/L) α-napthaleneacetic acid (NAA) and 1.1 µM (0.24 mg/L) thidiazuron (TDZ), 1 mg/L NAA and 1 mg/L BA, 4.5 µM (1 mg/L) 2,4-D and 1.1 µM (0.24 mg/L) BA to induce callus capable of regeneration (Priyadarshi and Sen 1992; Supaibulwatana and Mii 1998; Watad *et al.* 1998; Nhut *et al*. 2002; Mori *et al*. 2005). It is not known which of these hormones resulted in the most callus induced as the amount of callus was not determined. Ault and Siqueira (2008) used *L. michiganense* and found a high frequency of callus induction, 91-98%, from transversely sectioned bulbs cultured on MS medium with either 1-8 mg/L dicamba, 1-8 mg/L picloram, or 1-8 mg/L kinetin combined with NAA.

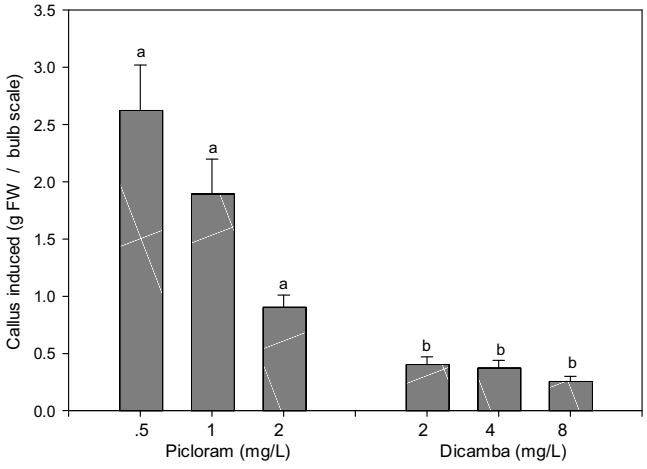


**Fig. 2 Compact callus induced from *L. longiflorum* 'Nellie White' bulb scales cultured on MS medium with either picloram (0.5, 1, or 2 mg/L) or dicamba (2, 4, or 8 mg/L), and the amount of callus (gFW) was measured after one year.** Four plates of bulb scales (10 bulb scales/plate) were cultured on each hormone concentration and the experiment was repeated twice. Bars indicate mean ± SE.

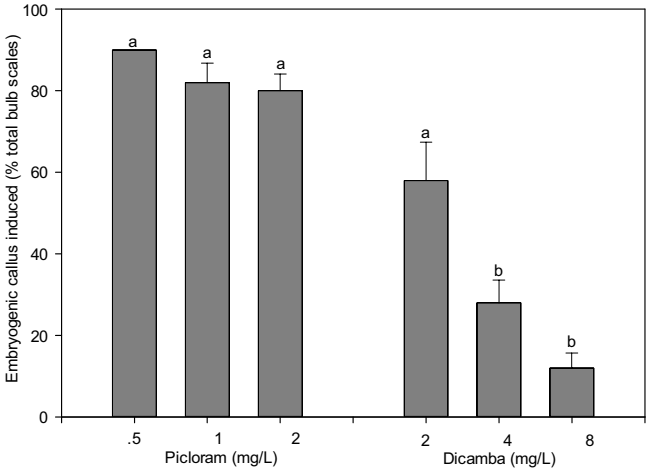


**Fig. 3 Embryogenic callus of *L. longiflorum* 'Nellie White' induced from compact callus cultured one year on MS medium supplemented with either picloram (0.5, 1, or 2 mg/L) or dicamba (2, 4, or 8 mg/L).** The presence of embryogenic callus was observed after six months of culture. Four plates of bulb scales (10 bulb scales/plate) were cultured for each hormone concentration and the experiment was repeated twice. Bars indicate mean ± SE.

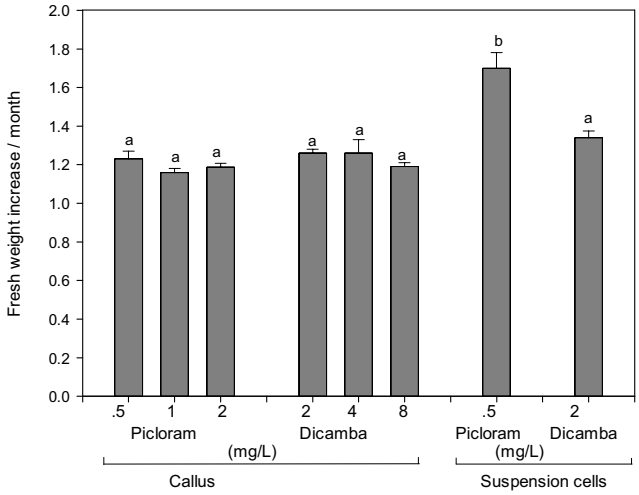


**Fig. 4 Compact *L. longiflorum 'Nellie White'* callus and suspension cells grown on MS medium with picloram (0.5, 1, or 2 mg/L) or dicamba (2, 4, or 8 mg/L), and the amount of growth measured after one month.** Four plates of callus or four flasks of suspension cells were cultured and measured for FW for each hormone concentration, and the experiment was repeated twice. Bars indicate mean ± SE.

### Compact callus and suspension cell growth

Suspension cells of 'Nellie White' were established because generally suspension cells grow faster than callus. Optimal growth of 'Nellie White' occurred with suspension cells cultured in MS medium supplemented with 0.5 mg/L picloram and this was significantly higher than growth on 2 mg/L dicamba (**Fig. 4**). Suspension cells grown in either 1 or 2 mg/L picloram or 4 or 8 mg/L dicamba could not be maintained for over 6 months. Suspension cells increased 1.7-fold in FW as compared to compact callus that increased 1.2-fold in FW. Growth was similar for compact callus grown on either 0.5, 1, or 2 mg/L picloram and either 2, 4, or 8 mg/L dicamba indicating its growth was not dependent on the auxin concentrations tested. Both 'Nellie White' suspension cells and callus do not grow nearly as fast as *L. longiflorum* 'Snow Queen' and *L.* ×*formolongi*. Tribulato *et al.* (1997) and Cohen *et al.* (2004) reported the establishment of a fast-growing suspension from *L. longiflorum* 'Snow Queen' that was subcultured every 2 weeks whereas in our study four weeks were needed. A suspension

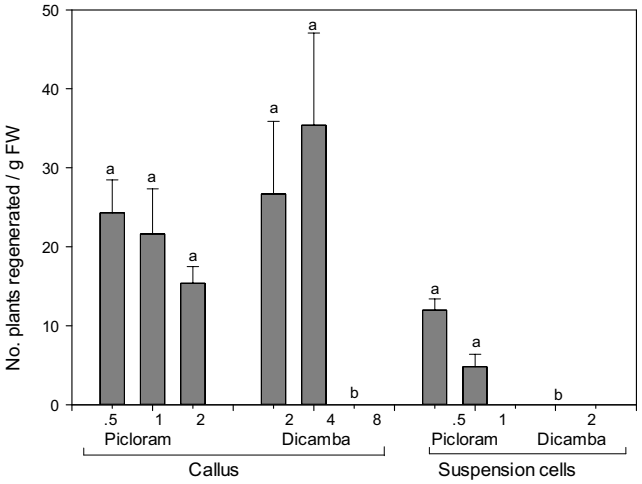


**Fig. 5 *L. longiflorum* plantlets regenerated from compact callus and suspension cells cultured one year on either picloram (0.5, 1, or 2 mg/L) or dicamba (2, 4, or 8 mg/L).** Four plates of callus or suspension cells placed on regeneration medium in plates were cultured for cells that had been grown on each hormone concentration. Bars indicate mean ± SE.

of *L.*×*formolongi* multiplied rapidly and 1 g FW cells was used to inoculate 40 ml liquid medium every 3 weeks (Mii *et al.* 1994) whereas in our study 2-3 g FW cells were subcultured into 30 ml of liquid medium every four weeks.

## Callus and suspension regeneration

A high regeneration frequency is critical for obtaining transformed plants. Optimal regeneration (35 plantlets/g FW callus) occurred from compact callus cultured 1 year on MS medium with 4 mg/L dicamba (**Fig. 5**) although 3% of the plantlets were phenotypically abnormal. In comparison, plantlets regenerated from callus cultured 1 year on MS medium with either 0.5, 1, or 2 mg/L picloram were all phenotypically normal. The number of plantlets regenerated (15-24 plantlets/g FW callus) was higher for callus that had been induced and grown on either 0.5, 1, or 2 mg/L picloram than suspension cells cultured in either 0.5 or 1 mg/L picloram although the difference between these numbers was not statistically significant (**Fig. 5**). There were no plants regenerated from suspension cells grown in 2 mg/L dicamba and callus grown on 8 mg/L dicamba.

## Friable callus induction, growth, and regeneration

Friable callus was induced and selected because it frequently grows faster than compact callus (Kamo and Hodges 1986). The concentration of sucrose affected induction of friable callus. A few lines of friable callus were obtained by finely chopping up 3-7-month-old compact callus with a scalpel and then transferring the callus to MS medium supplemented with 2 mg/L dicamba and 9% sucrose. Friable callus was not induced from compact callus cultured on either 3, 6, or 12% sucrose. Seven lines of friable callus were induced following the second or third subculture onto 9% sucrose. No new lines of friable callus were found one year after the third subculture. Friable callus of 'Nellie White' was a lighter yellow color than compact callus (**Fig. 6A**). Tribulato *et al.* (1997) induced friable callus from styles and pedicels of the Easter lily 'Snow Queen' when the explants were cultured on MS medium with either 2 µM (0.44 mg/L) dicamba or picloram. Very little callus of 'Nellie White' was induced from bulb scales cultured on only 2 µM dicamba, indicating the sensitivity of 'Snow Queen' to auxin.

Established friable callus of 'Nellie White' grew quickly and increased at least 5-fold in FW after one month, and its growth rate was unaffected by the sucrose (3, 6, 9, or 12%) or dicamba (0.5, 1 or 2 mg/L) concentration in the

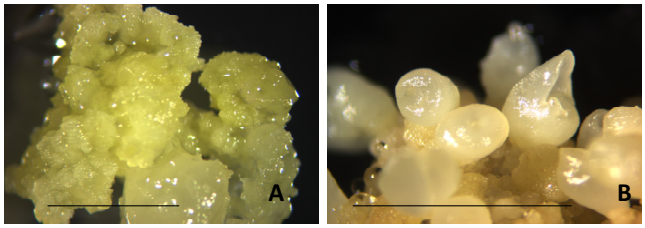


**Fig. 6** (**A**) Friable callus of *L. longiflorum* 'Nellie White' cultured on MS medium with 2 mg/L dicamba and 9% sucrose. (**B**) Embryo-like structures formed from friable callus cultured on MS medium with 1% activated charcoal and lacking hormones. Magnification bar equals 1 cm.

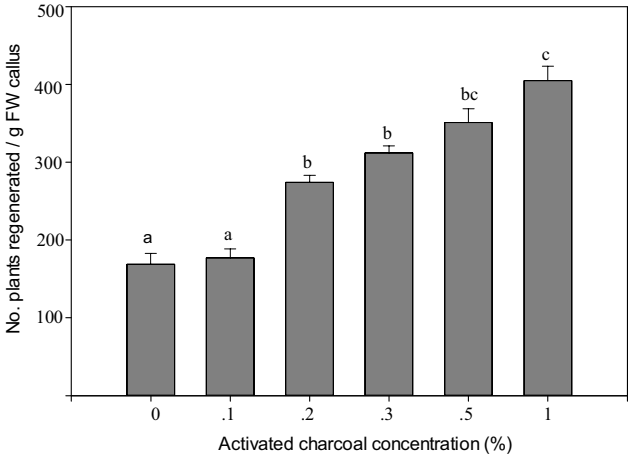


**Fig. 7 Regeneration of *L. longiflorum 'Nellie White'* plantlets from friable callus was stimulated following 7 weeks on MS medium with either 0.1, 0.2, 0.3, 0.5, or 1% activated charcoal.** Four plates of callus were cultured for each concentration of activated charcoal and the experiment was repeated twice. Bars indicate mean ± SE.

MS medium (data not shown). Several unsuccessful attempts were made to establish suspension cells from the friable callus. Tribulato *et al.* (1997) established fast-growing suspension cultures from friable callus of 'Snow Queen'.

Somatic embryo-like structures developed from friable callus cultured on hormone-free MS medium (**Fig. 6B**). The addition of activated charcoal (0.2-1.0 mg/L) stimulated the development of somatic embryo-like structures from friable callus (**Fig. 7**), but few of these embryo-like structures developed further to form intact plants. Nhut *et al.* (2001) showed that activated charcoal stimulated the regeneration of lily shoots directly from thin layer stem explants. The few plants regenerated from friable callus that developed to form roots were phenotypically normal when grown in the greenhouse. Tribulato *et al.* (1997) were able to regenerate intact plants from friable callus only when the callus had been cultured on filter paper for regeneration. Nhut *et al.* (2006) established static liquid cultures of *L. longiflorum* from friable callus, then transferred embryogenic callus that formed to solidified MS medium containing 1 mg/L NAA and 0.2 mg/L TDZ for successful regeneration of numerous plants.

## CONCLUSION

Picloram (0.5 mg/L) was optimal for compact and embryogenic callus induction from bulb scales of 'Nellie White'. Suspension cells grown on 0.5 mg/L picloram increased 1.7-fold in FW/month which was significantly faster than callus growth. Regeneration rates were higher for callus that had been cultured on dicamba than picloram, but 3% of the plantlets were phenotypically abnormal using dicamba, and no abnormalities were observed using picloram. A fast-growing friable callus capable of increasing 5-fold in FW/month was established, but the regeneration rate of intact plantlets with roots was very low for this type of

callus. It was concluded that callus and suspension cells of 'Nellie White' grown using MS medium with 0.5 mg/L picloram should be best for transformation experiments based upon their induction, growth, and regeneration rates. Results show that each lily cultivar differs in the hormone and its concentration required for optimal callus induction and growth of both callus and suspension cells.

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