A timeline for *Eustoma grandiflorum* seedling production based on an *in vitro* germination protocol

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Abstract

Eustoma (*Eustoma grandiflorum*) is a high-rank ornamental cut flower, and propagation of plant material by seed is an important approach for high-volume production of *Eustoma*. Successful propagation using different parts of the *Eustoma* plant has been reported in numerous studies; however, to date there have been no studies comparing the timeline differences between *in vitro* and conventional germination growth methods. The objective of this study was to develop a convenient and straightforward *in vitro* protocol that decreases the lag time to germination and the seedling development periods. Seed germination and seedling growth was studied in *Eustoma* (Voyage type 2 pink) seeds using two different techniques, *in vitro* germination and conventional sowing in soil. Seeds sown under *in vitro* conditions exhibited faster (6 days) germination compared with seeds sown directly in soil (11 days). Half-strength Murashige and Skoog medium *in vitro*-grown seedlings (7 weeks) were more vigorous and produced true leaves earlier compared with conventionally-grown seedlings (15 weeks). In addition, when *Eustoma* seeds were sown *in vitro*, germination could be more easily tracked and visually compared with seeds sown in soil media. Therefore, the *in vitro* seed germination protocol developed in this study is straightforward, reproducible, and will amenable to facilitate efficient seedling production of *Eustoma* for commercial applications.

Keywords: *Eustoma grandiflorum*; *in vitro* technique; seed germination and growth rate; timeline.
Abbreviations: CCFL_Cold cathode fluorescent lamps, MES-Na_2-(N-Morpholino) ethanesulfonic sodium salt, NaOCl_Sodium hypochlorite, RH_Relative humidity, UM_Unified mayonnaise

Introduction

Eustoma [*Eustoma grandiflorum* (Raf.) Shinn] is a long day plant belonging to the family Gentianaceae. It originated as a wildflower native to a region corresponding to the area between North and South America. *Eustoma*, also known by its common name Lisianthus, ranks high among ornamental cut flowers, and is popular for its bright, colorful petals, long post-harvest life, and the ease at which it can be shipped worldwide.

In general, *Eustoma* seed is tiny and germination under conventional soil-sown conditions is sensitive to a number of environmental factors including optimal light, temperature, and moisture level (Harbaugh, 1995; 2006). A photoperiod of 16 h light is required for *Eustoma* seed germination, at least until seedling emergence (Harbaugh, 1995). An appropriate photoperiod is important for regulating germination and seedling growth because of its effect on cotyledon expansion, leaf and hypocotyl development, and photosynthesis (Williams et al., 1998). In addition to specific light requirements, seed germination requires a constant temperature of 20–25°C (Roh et al., 1989). Emergence is delayed if temperature is not maintained carefully during the germination period (Kepczynska et al., 2006; Norsworthy and Oliveira, 2006). Moreover, high temperatures inhibit seedling production at the primary stage (Ohkawa et al., 1991). Large fluctuations between day and night temperatures also affect seedling growth and development,

causing *Eustoma* seedlings grown in soil media to form rosettes (Ohkawa et al., 1993). Plants that form rosettes exhibit delayed flowering. In tropical regions, temperatures are higher and fluctuate more drastically compared with temperate regions, which are conditions that inhibit seed germination. Therefore, the environmental conditions in these areas would not support reliable seed germination for large-scale production using conventional soil media methods without facilities to control these critical parameters.

Production and propagation of plant material by seed is an important approach for high-volume production, but it is also an important as well as being the only means for enabling the evaluation of progeny from genetic crosses for selection and development of new cultivars. Conventional, in-soil germination of *Eustoma* seeds for producing seedlings has been attempted, but this technique has not proved satisfactory for commercial producers and growers, due in part to the lengthy production time (Harbaugh, 1995) and including the rosette phenotype often accompanying this propagation method (Ohkawa et al., 1993; Fukuda et al., 1994).

A new era for commercialization of *Eustoma* has opened up through development of rapid and high-volume production techniques based on *in vitro* multiplication of plant material from various plant parts including the leaf and apical shoot tip (Semeniuk and Griesbach, 1987; Paek and Hahn, 2000; Esizad et al., 2012; Kaviani, 2014), flower bud or anther (Rezaee et al., 2012), internode (Semeniuk and Griesbach,

1987; Rezaee et al., 2012), and hypocotyl. Consequently, different media recipes with varying concentrations of various components such as carbohydrates (Akbari et al., 2014), vitamin and plant growth regulators (Kaviani, 2014; Winarto et al., 2015), hormones (Paek and Hahn, 2000), and solidifying agents (Semeniuk and Griesbach, 1987; Rezaee et al., 2012) as well as different concentrations of the same media mixture have been developed to propagate *Eustoma* from different plant parts. However, these previous studies have not addressed the timeframe for production or resulting plant quality, aspects that are important for commercial applications. Therefore, adoption of *in vitro* protocols would enable manipulation of these parameters to improve germination and seedling growth rates for commercial *Eustoma* production in tropical regions. Seed germination protocols have already been reported and established for other plants including *Aloe ferox* Mill. (Bairu et al., 2009), *Brasiliorchis picta* (Dos Santos et al., 2016), *Cymbidium aloifolium* (L.) (Pradhan et al., 2014) and *Annona crassiflora* Mart. (Ribeiro et al., 2009) with various aims including the preservation of endangered plant species. Development of methods for the efficient germination of *Eustoma* seeds and growth of seedlings *in vitro* should similarly be achievable and applicable as an alternative method for *Eustoma* production. Previously, Damiano et al. (1989) reported the micropropagation and tissue culture germination of *Eustoma* seed, but did not present a clear protocol or a timeline relevant for commercial applications. In this paper, we present streamlined methods to enable research and production laboratories and or seed companies to rapidly adopt the technique of germinating *Eustoma* seeds *in vitro*. In summary, we (i) describe and compare the morphological characteristics of the *Eustoma* seedlings resulting from *in vitro* and conventional culture (ii) compare the production timelines obtained using *in vitro* and conventional culture and (iii) describe the detailed procedures to produce seedlings using an *in vitro* seed germination technique. This study represents the first report on a method of germination and propagation of *Eustoma* by seed *in vitro* together with a comparison of the production timelines and seedling phenotypes obtained from seeds grown *in vitro* versus conventional soil culture.

Results and discussion

In vitro technique

Rapid seed germination under regulated light and optimal temperature was visually apparent for seeds sown *in vitro*. The seedlings emerged *in vitro* six days after seeding (Fig. 1 and 3) and exhibited rapid growth and development to the fourth true leaf pair by seven weeks. The germination percentage, and shoot and root length were significantly greater for seeds sown *in vitro* compared with those sown in conventional technique; however, there was no significant difference in total fresh biomass between the two techniques (Table 1). The seedlings germinated *in vitro* appeared to be healthy and vigorous (Fig. 1). The rapid development of the seedlings *in vitro* not only reduced the duration of production but also the days to leaf pair is shorter for commercial production (Fig. 3). The *in vitro* technique utilized in this study employed an artificial growth medium consisting of macro- and micro-salts that provided essential elements and sucrose for controlling growth and various metabolic activities (Gibson, 1984), a combination of nutrients known

to support photomixotrophic metabolism and optimal development (Coupe et al., 2006; Fila et al., 2006). Sucrose influences the maintenance of osmotic function in plant cells, directly participating in glycolytic and pentose phosphate pathways that speed up the growth and development of the seedling (Zha et al., 2007; Gago et al., 2014). Indeed, early *in vitro* studies reported that sucrose acts on and significantly affects seed germination, shoot emergence, leaf and root development, and seedling development (Wang and Ruan, 2014; Huh et al., 2016). Establishing protocols for seed germination and seedling growth is critical for improved seedling quality and large-scale production of crops (Subedi and Ma, 2005).

Conventional technique

In contrast to the results observed for seeds sown using the *in vitro* technique, germination of seeds sown in the soil-based medium (conventional seed sowing in soil) took eleven days (Fig. 3); even under the uniform, optimal conditions provided, seed germination was $84.3 \pm 0.58\%$ (Table 1). However, the survival rate was higher for the plants grown using conventional seed sowing in soil compared with the *in vitro* technique (Table 1). Due to the seed germination to seedlings development accomplishes in soil-based medium, transfer condition that faces the minimal environmental stresses for the survival of seedlings in conventional technique compared with *in vitro*, however; other attributes of *Eustoma* seedlings were greater *in vitro* (Table 1). Growth and development to the fourth leaf pair for the seedlings sown in soil required at least fifteen weeks (Fig. 2 and 3). These results are in line with previous data indicating a period of 10–20 weeks for production of transplant-ready (4–5 true leaf pair stage) *Eustoma* seedlings from seed using conventional, soil sowing techniques (Halevy and Kofranek, 1984; Matsuo and Shirasaki, 1990; Harbaugh, 1995). According to previous reports, *Eustoma* seed germination in soil is finicky, requiring great skill, daily maintenance, and appropriate facilities to provide the optimal environmental conditions. Slow seed germination and lengthy production timeline have a great bearing on the overall production cost and resulting potential economic damage to seedling producer.

Materials and Methods

Plant material

The *Eustoma* (Voyage type 2 pink) seeds (*Eustoma grandiflorum*) used in this experiment were obtained from Sakata Seed Corporation, Japan. Eustoma seeds were left in a sunny place to dry and then kept in a seed storage chamber at 4°C for one week. Seed from any commercial seed company, nursery, or garden center can be used to demonstrate the applicability and efficacy of this technique.

Media preparation for the in vitro technique

For *in vitro Eustoma* seed germination, half-strength macro-micro salt (Murashige and Skoog, 1962) supplemented with $30.0\ \mathrm{g\ L^{-1}}$ (3.0%) sucrose (Sigma Aldrich, USA) was used as the growing medium (Fig. 4). Phytagel ($3.0\ \mathrm{g\ L^{-1}}$; Sigma Aldrich, USA) was added and dissolved by heating the medium in a microwave oven set on high for 4 min. The medium was mixed in an Erlenmeyer flask using a magnetic

Table 1. Comparison of *in vitro* and conventional technique on growth and development of *Eustoma.*

Culture technique	Germination percentage (%)	Shoot length (mm)	Root length (mm)	Total fresh biomass (g)	Survival rate (%)
in vitro	96.09 ± 0.40	56.6 ± 0.82	72.2 ± 0.62	1.1 ± 28.2	82.3 ± 0.93
Conventional	84.3 ± 0.58	48.7 ± 0.94	63.4 ± 0.80	0.8 ± 22.7	92.3 ± 0.61

Traits are mean ± SE ($n = 10$) and significantly different ($p < 0.05$) using Tukey's HSD test.
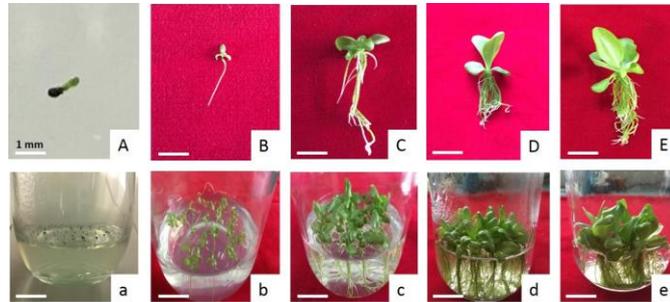
Fig 1. Development of *Eustoma* (*Eustoma grandiflorum*) seedlings from germination to seedling stage *in vitro*. A and a, seed emergence and germination (6 days); B and b, first true leaf pair stage (1 week); C and c, second true leaf pair stage (3 weeks); D and d, third true leaf pair stage (5 weeks); E and e, fourth true leaf pair stage (7 weeks). Scale bar = 2 cm.
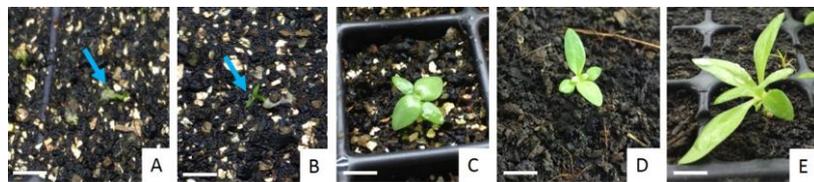

Fig 2. Seedling development of *Eustoma* (*Eustoma grandiflorum*) plants seeded in conventional, soil medium. A, seed germination (11 days); B, first true leaf pair stage (3 weeks); C, second true leaf pair stage (5 weeks); D, third true leaf pair stage (8 weeks) and E; fourth true leaf pair stage (15 weeks). Scale bar = 2 cm.
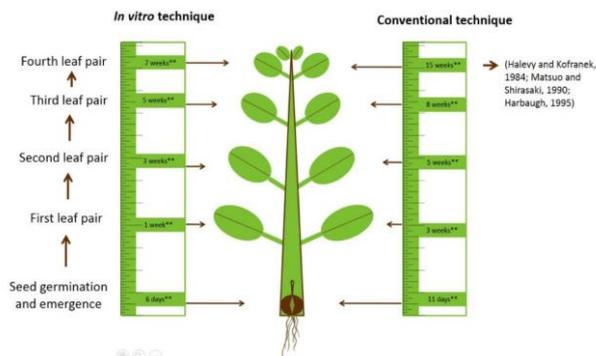

Fig 3. A timeline for *Eustoma* (*Eustoma grandiflorum*) seed germination through various stages of seedling development. A flow chart outlining the timeline (in days and weeks) for the various seedling developmental stages of plants obtained from seeds sown *in vitro* versus by plants obtained from seeds sown by the conventional technique (in soil). (Numbers in each column represent the days to germination and weeks to respective number true leaf pair. ** showing significant difference with Tukeys HSD test at $p < 0.05$. Traits are mean ± SE and $n = 10$)
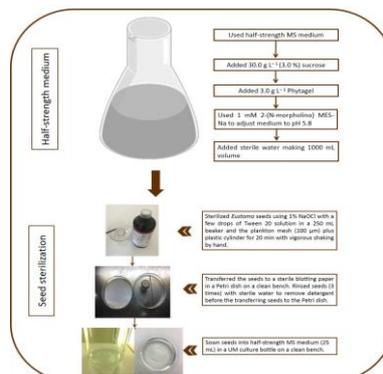

Fig 4. Scheme for the preparation of a 1000 ml of half-strength MS medium and the seed sterilization technique for *Eustoma* seed germination and growth *in vitro*.

stir bar and a stir plate (AS ONE, Japan) set at 600 rpm min^{-1} for 3 min. The medium was adjusted to pH 5.8 with 1 mM 2-(N-morpholino) ethanesulfonic sodium salt (MES-Na) (Sigma Aldrich, USA). The culture medium was sterilized by autoclaving at 121°C and 1.5 kgf cm^{-2} for 15 min (Tomy BS-305; Tomy Seiko Co., Ltd., Japan). Following autoclaving, the medium was distributed aseptically in 25 mL aliquots to sterile UM (Unified mayonnaise clear glass culture bottle with plastic cap) bottles (AS ONE, Japan) under laminar air flow (Bio Clean Bench; Sanyo Electric Co., Ltd., Japan) and then allowed to solidify for 20 min, after which it was stored until use.

Seed sterilization for the in vitro technique

The seeds were disinfected in a laminar flow chamber. Seeds (0.15 g) were placed in a 3 cm diameter plastic sieve cylinder fitted with plankton mesh (100 μm mesh and 5 × 5 cm square net) and placed in a 250 mL beaker. Sterilization solution was prepared by adding 50 mL of 1% NaOCl (Sigma Aldrich, USA) along with 500 mL autoclaved sterile water in a separate, 500 mL beaker. A few drops of Tween 20 (Sigma Aldrich, USA) solution were added with the sterilization solution to wash the seeds in the 250 mL beaker. The seeds held in the mesh were then surface sterilized by adding sterilization solution followed by soaking for 20 min (Sauer and Burroughs, 1986). The seeds were then washed three times with sterile water to remove the detergent. Vigorous shaking by hand was important to ensure thorough washing and removal of seeds clinging to the plastic sieve cylinder. The seeds were subsequently rinsed three times with sterile water (about 50 mL each time) in a beaker to remove the seeds clinging to the plastic sieve cylinder. After rinsing, the seeds were transferred to sterile blotting paper in a Petri dish in the flow bench. A sterile L-shaped hockey stick (130 mm L × 50 mm W (bottom) × 4 mm diameter) (Sigma Aldrich, USA) was used to distribute the seeds on the surface of the growth medium contained in the UM culture bottle. There were 20–25 seeds per UM culture bottle, evenly distributed on the surface of the germination medium (Fig. 4). Higher seed densities reduce germination and development (Rasmussen and Johansen, 1989). Following seed distribution, all UM culture bottles were sealed with two layers of parafilm. All cultures were kept in a growth chamber at 23 ± 2°C under a 16 h light photoperiod with a light intensity of 98 μmol m^{-2} s^{-1} (LA-105; NK-System, Japan) provided by cool white fluorescent lamps (Tubular fluorescent lamps; Toshiba Lighting & Technology Taiwan Co., Ltd., Japan Light Analyzer). Cultures were examined every other day and contaminated cultures discarded if found.

Seed sowing in soil for the conventional culture

To understand the efficacy and merits of seed germination *in vitro*, seed germination was also tested using conventional methods by direct sowing in soil (Tanekura No. 42; Sumirin Agricultural Industry Co., Ltd., Japan). The germination experiment was conducted in a cold cathode fluorescent lamp (CCFL) growth chamber (Bio Multi Incubator; NK-System, Japan) under a 16 h photoperiod with 60–70% relative humidity (RH). Seed was sown on the surface (not buried) of Tanekura No. 42 soil contained in cells of plastic cell trays (Cell trays with 144 to 228 cells, each cell 2.5 × 2.5 cm); 50 cells, each planted with one seed each were used for germination and seedling growth tests. The plastic, seeded cell tray was subsequently covered with a polyethylene sheet to minimize moisture loss. A water contained plastic bowl (2.0 mL) was placed under the plastic cell tray to maintain moisture. RH was found to be 70–75%. The seeds were considered as germinated when emergence (hypocotyl) was observed. The germination period was 9–11 days. The rate of germination of the seeds sown in the soil was estimated using a modified Timson's index of germination velocity = Σ G/t, where G is the percentage of seed germination at one-day intervals and t is the total germination period (Khan and Ungar, 1984). A high mean value represents a high rate of germination.

Statistical analysis

For each treatment, there were ten replications and the results were expressed as mean ± standard error (SE). Data from after the *in vitro* technique at 7 weeks and conventional sowing in soil at 15 weeks were analyzed using one-way ANOVA and significant differences were calculated after applying Tukey's HSD test at $p < 0.05$ using KaleidaGraph-4.5.0 (Synergy Software, USA).

Conclusion

This protocol paper incorporates practical, technical, and horticultural aspects relevant to the efficient germination of *Eustoma* seeds and growth of resulting seedlings *in vitro*. We present a simple and efficient *in vitro* technique for *Eustoma* seed germination, which effectively shortens the seed germination and seedling development periods. Short plant production timelines are crucial for reducing costs and commercial viability. In addition, the technique and results for seed germination and seedling growth of *Eustoma* described in detail in this study supports the importance of these parameters for improved seedling quality and large-scale production. This technique can be applied to enable eustoma seedling production in tropical regions.

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