

SCIENTIFIC ARTICLE

In vitro regeneration and flowering of *Portulaca grandiflora* Hook.

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Abstract

P. grandiflora is a known ornamental plant with abundant flowering. The flowers exhibit varied coloration with distinct forms and simple folded petals and/or multiple. The objective of this work was to induce regeneration via organogenesis and *in vitro* flowering of *P. grandiflora*. Nodal segments of seedlings germinated *in vitro* were used as explant source for regeneration. Kinetin (KIN) and 6-Benzylaminopurine (BA) were used for the induction of organogenesis. The treatments supplemented with 1.0 and 1.5 mg L⁻¹ BA induced the highest number of adventitious shoots with an average number of 7.0 (±1.55) e 5.4 (±0.83), respectively. The microcuttings obtained from regenerated shoots produced floral buds. The floral buds were located in the axillary and terminal regions of the microcuttings and developed in approximately 10 days of cultivation until the anthesis. The highest number of flower buds was observed in the presence of 0.75 mg L⁻¹ of gibberellic acid. This study opens new perspectives for the establishment of biotechnological tools to be applied for this important ornamental species.

Keywords: *In vitro* Flowering, Gibberellic acid, Shoot organogenesis.

Resumo

P. grandiflora é uma das plantas ornamentais com abundante floração de coloração variada com formas distintas e pétalas simples, dobradas e/ou múltiplas. O objetivo do trabalho foi induzir a regeneração pela via organogênica e o florescimento *in vitro* de *P. grandiflora*. Segmentos nodais de plântulas germinadas *in vitro* foram utilizados como fonte de explante para a regeneração. Cinetina (KIN) e 6-Benzilaminopurina (BA) foram utilizadas para a indução de organogênese *de novo*. Os tratamentos suplementados com 1.0, 1.5 mg L⁻¹ de BA induziram a formação de maior número de brotações adventícias com número médio de 7.0 (±1,55) e 5.4 (±0.83), respectivamente. As microestacas obtidas a partir de gemas regeneradas produziram botões florais. Os botões florais localizaram-se nas regiões axilar e terminal das microestacas e desenvolveram-se em aproximadamente 10 dias de cultivo até a antese. O maior número de botões florais foi observado na presença de 0.75 mg L⁻¹ de ácido giberélico. Este estudo abre novas perspectivas para o estabelecimento de ferramentas biotecnológicas a serem aplicadas para essa importante espécie ornamental.

Palavras chave: Florescimento *in vitro*, Ácido giberélico, organogênese.

Introduction

Portulaca grandiflora Hook. commonly known as “ten o’clock”, is one of the most widely cultivated annual flower plants in the tropical world by virtue of its easy cultivation, abundant flowering and flowers of varied colors such as red, yellow, pink, purple, white, orange and/or mixed. It is also found in distinct forms, with simple, folded, or multiple petals (Jain and Bashir, 2010). This ornamental plant can be grown in small spaces, gardens and pots. Additionally, *P. grandiflora* is an important medicinal plant with putative immunostimulatory and detoxifying activity in hepatitis B (Chavalittumrong et al., 2007).

Because of the ornamental potential of this plant, *in vitro* micropropagation and regeneration systems have been established for *Portulaca* species and have become relevant additional tools for their commercial production. The main regeneration pathway of *P. grandiflora* is organogenesis, which is induced mainly from nodal explants and stem apices grown in the presence of cytokinin (Srivastava and Joshi, 2009; Jain and Bashir, 2010).

In vitro flowering is probably one of the most fascinating processes in the development of plants in this culture system (Teixeira da Silva et al., 2014). The *in vitro* flowering process is a tool that can be applied for basic studies of flowering, reproduction, initiation and development of

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floral organs and senescence (Murthy et al., 2012). Several factors can influence *in vitro* flower induction, e.g., the growth medium, plant growth regulators, explant sources and photoperiod (Corbesier and Coupland, 2006; Bhat et al., 2010). Among plant growth regulators, gibberellic acid (GA_3) stands out for directly influencing *in vitro* flower bud induction and flowering (Kostenyuk et al., 1999). Studies on *in vitro* flower induction involving GA_3 have been applied to several species such as *Panax ginseng* (Lee et al., 1991), *Cymbidium goeringii* (Ho et al., 2003), *Cucumis sativus* (Kielkowska and Havey, 2015), *Rosa indica* (Bimal and Kiran, 2014) and *Solanum nigrium* (Geetha et al., 2016).

The present study aimed to evaluate the performance of nodal explants of *P. grandiflora* and to establish an *in vitro* regeneration system via organogenesis as well as induce flowering *in vitro* from a culture of microcuttings from previously regenerated plants.

Material and Methods

In vitro germination

Under aseptic conditions 100 seeds of *P. grandiflora* were surface sterilized in a laminar flow hood by immersion in 70% ethanol (v/v) for 2 minutes, followed by 15 minutes of immersion in a solution of commercial sodium hypochlorite 2.5% (v/v) added with two drops of Tween-20 dispersant 0.1% (v/v) per 100 mL of solution and rinsed four times in autoclaved distilled water. The seeds were inoculated in 250 mL flasks containing half-strength Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), MS vitamins, 100 mg L⁻¹ myo-inositol, 3.0% sucrose (w/v), and 0.8% agar (w/v) (Acumedia®). The pH of the culture media was adjusted to 5.7 ± 0.1 and then autoclaved at 121 °C and 1.5 atm for 20 min. The seeds were kept in a growth room at 25 °C ± 2 with a 16 h photoperiod for 30 days.

Shoot proliferation

For the *in vitro* induction of multiple shoot formation, nodal segments of approximately 10 mm long from 30-day-old seedlings were excised and inoculated in test tubes (200 mm × 25 mm) containing MS medium, as described above, supplemented with benzyladenine (BA) or kinetin (KIN) at the concentrations of 1.0, 2.0 and 2.5 mg L⁻¹ and in a control treatment without plant growth regulators. Thirty tubes were prepared for each treatment and one nodal segment

was inoculated per tube. After inoculation, the explants were maintained in a growth room at 25 °C ± 2 with a 16 h photoperiod for 60 days. A subculture was performed in the same medium of each treatment after 30 days of culture.

After 60 days of culture, obtained shoots were excised and transferred to MS medium without plant growth regulators for elongation and rooting. The shoots were kept in the same growth conditions described previously for 30 days.

In vitro flowering induction

Microcuttings of approximately 3 cm long from 90-day-old *in vitro* regenerated plants obtained previously were inoculated in MS medium supplemented with gibberellic acid (GA_3) at different concentrations (0.50, 0.75, 1.0, 1.5 and 2.0 mg L⁻¹) and in a control treatment without GA_3 (MS0). The medium was poured in 10 mL aliquots into sterile test tubes (200 mm × 25 mm). The pH of the culture media was adjusted to 5.7 ± 0.1 and then autoclaved at 121°C and 1.5 atm for 20 min; GA_3 was added to the culture media after autoclaving. The test tubes were sealed with stretchable polyvinyl chloride (PVC) film (9-10-µm pore). Ten replicates were performed per treatment, using one plant per test tube. Each tube was considered an experimental unit. The number of shoots roots and flowers (opening flowers and flower buds) per explant was evaluated at 60 days of culture.

Statistical analysis

The experiment was set up as a completely randomized design. Data were subjected to analysis of variance (ANOVA) and the difference between the treatment means were compared with Tukey's test at the 5% probability level, using Sisvar® software version 5.6 (Ferreira, 2011).

Results

The concentration and type of cytokinin influenced the shoot proliferation of *P. grandiflora*. In the absence of cytokinins, the nodal explants showed little bud formation. Regardless of the concentration, BA-treatments presented a higher number of shoots compared to treatments supplemented with KIN (Figure 1). The highest number of shoots per explant was observed in the treatments supplemented with 1 and 1.5 mg L⁻¹ BA, which produced 7.0 (±1.55) e 5.4 (±0.83) shoots per explant, respectively. No significant difference was observed between these treatments (Figure 1).

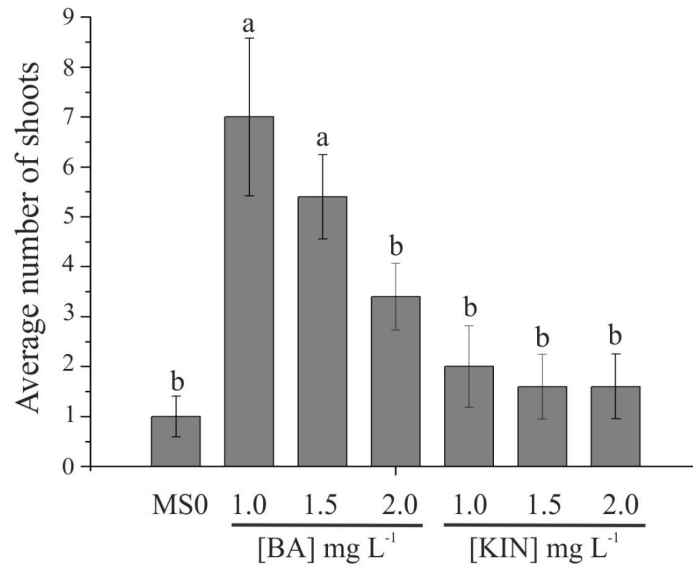


Figure 1: Number of adventitious shoots obtained from *P. grandiflora* nodal segments. Error bars denote the standard error. Means followed by the same letters in each of the graphs do not differ from each other by the Tukey test at the 5% probability level.

The regeneration of shoots from nodal explants of *P. grandiflora* initiated at the cut surfaces of the explants but, mainly, in regions located far from the cut surface of the explant (Figure 2A-B). The regeneration process occurred directly without callus formation. After 10 days of culture, organogenic structures were observed on the surface of

the explants. These structures later differentiated into new adventitious shoots (Figure 2A-B). During shoot development, no morphological differences were observed among the BA- and KIN-Treatments. Clusters of multiple shoots were observed after 60 d in BA-supplemented medium and 30 d in MS medium without plant growth regulators (Figure 2C).

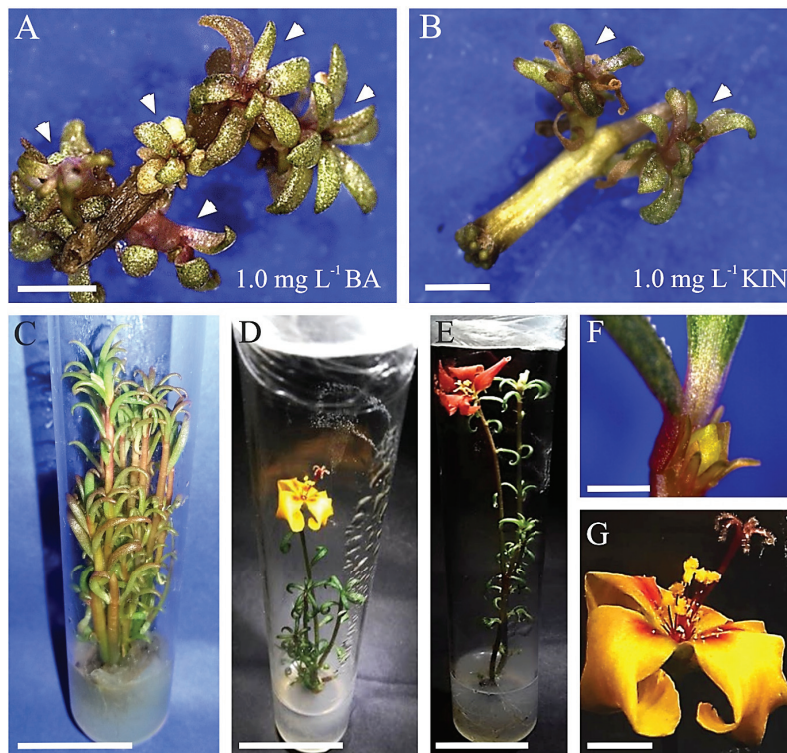


Figure 2: *In vitro* shoot regeneration and flowering of *P. grandiflora*. (A-B) Nodal segments cultured in media supplemented by 1.0 mgL⁻¹ BA (A) and 1.0 mgL⁻¹ KIN (B). Note the number of adventitious shoots produced in both treatments (white arrowheads). (C) Clusters of multiple shoots; (D-G) *In vitro* flowering. Note the flower bud in the axillary region of the plant (F) and the axial flower in anthesis evidencing male and female organs (G). Bars. A-B = 0.5 mm; C-E = 20 mm; F-G = 4 mm.

For *in vitro* flower induction, microcuttings obtained from adventitious shoots formed in BA-supplemented media (Figure 2C) were subcultured in the presence and absence of GA₃. After 60 days of culture, no significant differences were observed in the number of shoots and roots among the treatments (Figure 3A-B). However, the number of flowers of *P. grandiflora* was clearly affected by the GA₃ treatments,

exhibiting typical dose-dependent performance (Figure 3C). In the GA₃ absence and in media supplemented with low GA₃ concentrations, the number of flowers increased with increasing GA₃ concentrations. The highest number of flowers was observed in plants cultured under 0.75 mg L⁻¹ GA₃ (Figure 3C). At higher GA₃ concentrations (1 and 2 mg L⁻¹), flowering plants were not observed (Figure 3C).

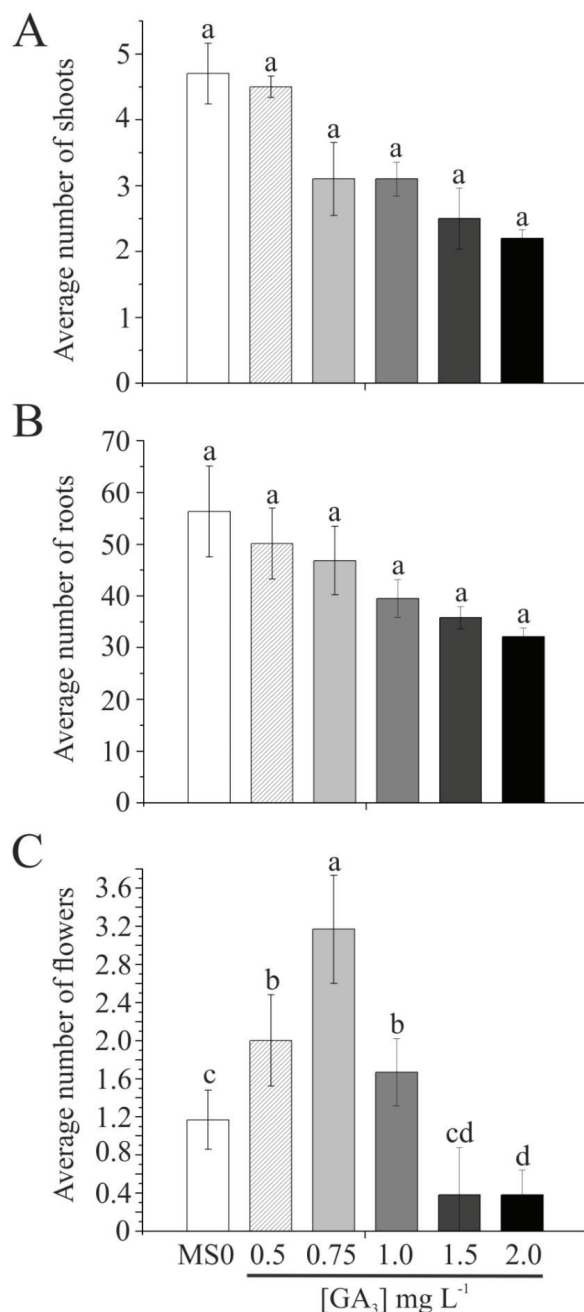


Figure 3. *In vitro* flowering of *P. grandiflora* 60 days of culture. Average number of shoots (A), roots (B) and flowers (C). Error bars denote the standard error. Means followed by the same letters in each of the graphs do not differ from each other by the Tukey test at the 5% probability level.

The flower buds were located in the axillary and terminal regions of the microcuttings and developed in approximately 10 days of culture, until entering anthesis (Figure 2D-F). The flowers remained in anthesis for approximately 24 h. Hermaphrodite *P. grandiflora* flowers and male and female reproductive structures were visible (Figure 2G).

Discussion

In the present study, we have reported *in vitro* induction of organogenesis and flowering in microcuttings of *P. grandiflora*. The success in this regeneration pathway depends on the type of explant and the plant growth regulators, which stand out as the main key factors for *in vitro* morphogenesis (Duclercq et al., 2011; Xu and Huang, 2014). The use of the nodal segment in the present study was based on the recurrent use of this explant source to induce multiple shoot formation in *Portulaca* species (Srivastava and Joshi, 2009; Jain and Bashir, 2010). It is believed that micropropagation from meristematic explants such as nodal segments can provide greater genetic stability to the genotype, since meristematic activation is less prone to somaclonal variation events commonly observed in neoformation processes of organs (Torres et al., 1998).

The supplementation with cytokinin was essential for the induction of morphogenetic responses in *P. grandiflora* nodal segments. Molecular signaling of cytokinin is necessary for the maintenance and differentiation of shoot buds. During *in vitro* organogenesis, cytokinins promote the expression of essential genes to induce the formation of new stem meristems (Su et al., 2011). However, the right concentration of plant growth regulators is essential for the specification of the morphogenetic response. In the present study, the treatments supplemented with 1.0 mg L⁻¹ and 1.5 mg L⁻¹ BA showed the highest number of adventitious shoots and no statistical differences were detected between them.

BA-treatments induced a higher number of shoots compared to treatments supplemented with KIN. The higher BA efficiency in inducing multiple shoots may be related to its lower susceptibility to enzymatic degradation (Magyar-Tabori et al., 2010). According to Hahman (2006), BA is a purine-cytokinin that persists in the culture medium. In addition, it is chemically more stable than other purine-derived cytokinins (Klem et al., 2004). It is possible that the BA conjugated amount in the medium was lower than KIN, thus presenting a higher free form quantity readily available for the nodal explants (Buah et al., 2010).

Gibberellins regulate many plant developments programs such as; germination, shoot elongation and flowering induction (George, 2008). In the present study, exogenous GA₃ supplementation may have increased the cellular GA concentration (Gupta and Chakrabarty, 2013), which played an important role in flowering induction in

P. grandiflora, since the number of flowers increased with increasing GA₃ concentrations until 0.75 mg L⁻¹, treatment that induced the highest number of flowers. Similar results were observed in some others ornamental plant species as: *Petunia × atkinsiana* D. Don (Kulpa and Nowak, 2011), *Trichodesma indicum* (Mahesh and Jeyachandran, 2013) and *Rose* sp. cv. Apricola (Demirci and Canli, 2015). On the other hand, in the present study no significant differences were observed in the number of shoots or roots of plants cultured in the presence of GA and without PGRs (MS0). This suggests that GA₃ may play a limited role in *P. grandiflora* development although, the GA₃-treatments showed a tendency to reduce the number of roots. Bryan et al. (1969) reported that GA₃ inhibited rooting of *Azukia* sp. cuttings which no longer showed stem elongation. However, in the literature the GA effects on root system architecture are controversial and, apparently, species-dependent.

Regardless of the type of system *in vivo* and/or *in vitro* flowering is achieved when intact or regenerated plants reach maturity and are able to advance from the vegetative to the reproductive phase. According to Silva et al. (2014), *in vitro* flowering system has contributed to the progress of research in the flowering mechanism. *In vitro* flowering can also be applied as a tool to accelerate breeding programs for the commercial production of plants *in vitro* or specific compounds of floral organs (Ziv and Naor, 2006; Kulpa and Nowak, 2011).

Conclusions

In summary, the present paper reports the establishment of an *in vitro* system for the regeneration and flowering of *P. grandiflora*. We showed that the best cytokinin concentrations to induce multiple shoot formation were 1 or 1.5 mg L⁻¹ BA, whereas flowering induction was promoted by 0.75 mg L⁻¹ GA₃. We believe the current results will help elucidate the *in vitro* performance of this species as well as open new prospects for the establishment of biotechnological tools to be applied for this important ornamental species.

Author Contribution

C.FC.⁰⁰⁰⁰⁻⁰⁰⁰²⁻¹⁶⁰²⁻⁸¹¹⁸: wrote the paper and design experiments; **W.F.S.**⁰⁰⁰⁰⁻⁰⁰⁰²⁻¹⁰⁴⁰⁻⁶³⁷⁶: design experiments; **C.S.S.**⁰⁰⁰⁰⁻⁰⁰⁰¹⁻⁶⁷⁰¹⁻⁹¹⁹³ and **M.D.M.**⁰⁰⁰⁰⁻⁰⁰⁰¹⁻⁵⁴¹⁹⁻⁷⁰⁰³: designed experiments; **I.F.C.**⁰⁰⁰⁰⁻⁰⁰⁰³⁻¹⁶⁵⁷⁻³⁴⁸ and **D.I.R.**⁰⁰⁰⁰⁻⁰⁰⁰¹⁻⁶⁶⁸³⁻⁰⁹⁶¹: review the paper; **M.L.S.**⁰⁰⁰⁰⁻⁰⁰⁰¹⁶⁹²⁸⁻²⁸⁵: designed the research project and wrote the paper.

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