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A procedure for indirect shoot organogenesis of *Polianthes tuberosa* L. and analysis of genetic stability using ISSR markers in regenerated plants

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ABSTRACT

Efficient *in vitro* regeneration system was well-established in *Polianthes tuberosa* L., an attractive ornamental and aromatic plant of horticultural importance. Various explants (Crown explants, leaves, petals, flowering stems and bulb scales) and different plant growth regulators (PGRs) were evaluated for both callus induction and shoot regeneration. Results showed that the maximum callus formation was obtained in crown explants using Murashige and Skoog (MS) medium supplemented by 1.95 μM 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T). The highest rate of shoot regeneration frequency was obtained on MS medium enriched with 2.26 μM thidiazuron (TDZ) + 250 mg l^{-1} proline from crown callus. The regenerated shoots were elongated on MS medium supplemented with 2.22 μM 6-benzylaminopurine (BAP). All shoots were successfully rooted in MS medium containing 9.89 μM indole-3-butyric acid (IBA) within a 15-day under a 16/8 h photoperiod. Plantlets were successfully hardened and adapted in the greenhouse with a survival rate of 100% and demonstrated normal growth. Flow cytometry and Inter Simple Sequence Repeats (ISSR) markers analysis indicated that there was no variation between mother and regenerated plants. The established regeneration protocol would be very useful for genetic engineering of *P. tuberosa* L. leading to the production of new flower colour and shape, pest and disease resistance and fragrance modification.

1. Introduction

The genus *Polianthes* is a geophyte that belongs to the Asparagaceae family and is endemic to Mexico (González-Gutiérrez and Rodríguez-Garay, 2016). *Polianthes tuberosa* L. (Tuberose) is the most commercially popular among 15 species of genus *Polianthes* (Solano and Feria, 2007). Tuberose is an important bulbous ornamental and aromatic crop which has been cultivated especially in tropical and subtropical areas at commercial scale in flower industry (as cut flower), perfumery, cosmetic and aromatherapy industries (Jayanthi et al., 2015). Tuberose flower are used as a major biotic origin for indoor aroma because of their strong flower scent, high longevity and having a decent variety of fragrant and medicinal uses (Maiti and Mitra, 2017). Moreover, there is a high demand for different cosmetics and perfumes products of *P. tuberosa* (Maiti et al., 2014).

In commercial production of ornamental plants there is constant demand for novel cultivars with surprising characteristics, therefore genetic diversity plays an important role in flower industry (Azadi et al., 2016). However, tuberose is commercially propagated by asexual methods which this causes reducing of genetic variability (Shillo, 1992). Therefore, traditional breeding methods face some constraints and complications like lacking of genetic variability available in *P. tuberosa* L. (Datta, 2017), which could only be solved by applying in *vitro* mutation breeding or genetic engineering technologies. Therefore, the application of *in vitro* culture techniques accompanied by traditional breeding programs can be effective in increasing genetic variability and accelerating up the *P. tuberosa* L. breeding (Datta, 2017).

Indirect and direct organogenesis has the great potential to reach

Abbreviations: BAP, 6-benzylaminopurine; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; ISSR, Inter Simple Sequence Repeats; MS, Murashige and Skoog; NAA, Naphthalene acetic acid; PGRs, Plant growth regulators; TDZ, Thidiazuron; 2,4,5-T, 2,4,5-Trichlorophenoxyacetic acid; 2iP, 6-γ γ-Dimethylallylaminopurine

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Table 1
The nucleotide sequences of primers used for ISSR analysis.

Number	Primer code	Primer sequence (5' to 3')	Number of scorable bands	Reference
ISSR-1	1	AGAGAGAGAGAGAGAGC	10	Hussain et al. (2015)
ISSR-2	4	CTCTCTCTCTCTTG	4	Tamayo Contreras (2010)
ISSR-3	5	GAGAGAGAGAGAGAGATG	6	Lou et al. (2012)
ISSR-4	12	CACACACACACAAC	7	Chandrika and Rai (2010)
ISSR-5	21	GAGAGAGAGAGAGAGAC	6	Lata et al. (2016)
ISSR-6	22	ACACACACACACACACC	4	Esmaeili et al. (2014)
ISSR-7	25	ACACACACACACACACYG	4	Wang et al. (2009)
ISSR-8	26	CACACACACACACACART	6	Rahimmalek et al. (2009)
ISSR-9	29	TCCTCCTCCTCCTCCRY	5	Ibarra-Torres et al. (2015)
ISSR-10	30	GAGAGAGAGAGAGAGACG	4	Abdel-Ghani and Migdadi (2012)

the advantage of breeding programs in short time to improve the quality of plants. Previous studies have been shown that *in vitro* indirect and direct organogenesis depends on the application of different plant growth regulators (PGRs) and type of explant (Ntui et al., 2010; Scotton et al., 2013). PGRs requirement for callus induction, growth and plant regeneration varies in different explant types (Kothari et al., 2004). It has been reported that 2,4,5-T as an auxin is helpful on callus induction of some recalcitrant species including *Tylophora indica* and *Ruta graveolens* (Faisal and Anis, 2003; Ahmad et al., 2010). Crucial role of TDZ in shoot regeneration has been approved in many plant species such as *Pisum sativum, Jatropha curcas* and *Cyrtanthus mackenii* (Zhihui et al., 2009; Aishwariya et al. 2015; Kumar et al., 2018). It was reported that amino acids such as proline as an organic nitrogen source has potential to increase plant regeneration (Pawar et al., 2015).

There are several reports on micropropagation from mature bulbs and shoot elongation from meristems of *P. tuberosa* (Krishnamurthy et al. 2001; Sangavai and Chellapandi, 2008 and Naz et al., 2012). They described a micropropagation protocol with a high multiplication rate using meristems of bulb as an explant on the MS medium supplemented with BAP. Therefore, lack of an efficient *in vitro* regeneration protocol is the main barrier for the improvement of tuberose in term of use of biotechnology methods like gene transfer technologies. *In vitro* plant regeneration of tuberose is an unique technique for production of new cultivars with commercial traits such as novel flower color, change plant architecture and fragrance (Datta, 2017).

To the best of our knowledge, there is no published data demonstrating the successful use of crown explants for indirect shoot regeneration in tuberose. Therefore, the primary target of this study was to introduce an efficient protocol for plant regeneration *via* indirect organogenesis.

2. Materials and methods

2.1. Plant materials and sterilization

P. tuberosa L. cv. Pearl Double were used as source of explants in this study. For *in vitro* explants, bulbs roughly 2–3 cm in diameters were used. Various preliminary disinfection methods were evaluated. The highly effective sterilization technique was used in all following process. The dry tunic and old roots of the bulbs were removed and soaked under running tap water for 30 min, and then bulbs were placed in heated bath (Benmari method, 53 °C) for 1 h. Bulbs were surface-sterilized in 70% (v/v) ethanol for 30 s and followed by placing in flasks with a solution of 4% (w/v) sodium hypochlorite and agitated for 30 min. Then bulbs were washed three times for 10 min with sterilized water. After removing the damaged tissue explants, they were cultured on a MS medium (Murashige and Skoog, 1962) supplemented with 2.22 μM BA.

2.2. Culture conditions

Throughout the research full strength MS medium containing 3% (w/v) sucrose (Duchefa Biochemic B.V. Haarlem, The Netherlands) and 7 g l^{-1} agar (plant agar, Duchefa Biochemic B.V. Haarlem, The Netherlands) was used. The pH of media was adjusted to 5.8 before autoclaving at 121 °C and 1.5 kg cm^{-1} pressure for 20 min. The cultures were incubated at 24 ± 2 °C in the light intensity of 80 μ mol m^{-2} s^{-1} emitted by cool white fluorescent lamps and 60–65% relative humidity. All tissue culture chemicals were provided from Duchefa Biochemie (Haarlem, The Netherlands).

2.3. Callus induction

For callus induction, 5 types of explants (Crown explant and leaves from mature *in vitro* plants, petals, flowering stem and bulb scales from soil-grown plants) in basal MS medium supplemented with different PGRs including 2,4,5-T, 2,4-D, Naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) alone or in combination with BA and kinetin were used. Based on our results, crown explants of 2-weeks-old *in vitro* plantlets (as explant) and different concentration of 2,4,5-T (0, 1.95, 3.91, 5.87, 7.82, 9.78 μM) were selected for further callus induction experiments (Table 1). All cultures were incubated at 24 ± 2 °C in the light for 10 days. After callus formation, calli were separated from the initial explants and cultured on regeneration medium (Fig. 1). The callus formation was evaluated as follows:

$$\text{Callus formation rate (\%)} = \frac{\text{Number of calli induced}}{\text{Number of cultured explants}} \times 100$$

2.4. Shoot regeneration from callus

In this study, different cytokinins including TDZ, zeatin, kinetin, 6-γ, γ-Dimethylallylaminopurine (2ip) (Duchefa Biochemic B.V. Haarlem, Netherlands) and BAP were examined for shoot regeneration. Based on our finding, TDZ was chosen for further regeneration experiments. To increase the rate of regeneration, interaction of TDZ and proline was considered. Calli were transferred to shoot regeneration medium supplemented with 2.26 μM TDZ and various proline concentrations (0, 50, 100, 150, 200 and 250 mg l^{-1}). All explants were maintained equally for 45 days at 24 ± 2 °C in the light intensity of 80 μmol m^{-2} s^{-1} emitted by cool white fluorescent lamps and 60–65% relative humidity. The numbers of calli regenerated were evaluated in each treatment as follows:

$$\text{Shoot regeneration rate (\%)} = \frac{\text{Number of calli regenerated into shoots}}{\text{Number of cultured callus}} \times 100$$

2.5. Shoot elongation and rooting

The regenerated shoots were isolated from calli, and transferred to