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Original article

Effects of 4-CPPU on in vitro multiplication and sustainable generation of *Hibiscus rosa-sinensis* L. 'White Butterfly'

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ABSTRACT

There are more than nine thousand cultivars of *Hibiscus rosa-sinensis* L., with a series of flowers with shapes, colors and new cultivars continues as generated through both traditional and modern breeding techniques. In this study, advanced biotech methods of in vitro culture have been used to identify a technique for the efficient mass multiplication of *H. rosa-sinensis* 'White Butterfly', using phenyl urea, *N*-(2-Chloro-4-pyridyl)-*N'*-phenylurea (4-CPPU). For the first time, the effects of 4-CPPU for stimulating axillary shoot proliferation and multiple shoot regenerations from nodal explants were evaluated, and the optimal nutrient media deduced. From the diverse concentrations as 0.1, 0.5, 2.5, 5.0 & 10.0 μ M of 4-CPPU, the highest frequency of shoots was recorded at 2.5 μ M supplied in Murashige and Skoog (MS, pH-5.8) medium. After eight-weeks of culture, on an average of 6.7 shoot were obtained on this media with shoot heights of 4.2 cm from each explant. With the involvement of 0.5 μ M-IBA (indole-3-butyric acid) in MS medium the regenerated shoots were rooted and followed by successful acclimation to ex vitro conditions. The ploidy consistency among the micro-plants was analyzed using flow cytometry and compared with ex vitro grown plants. No differences in the ploidy levels were observed among the 4-CPPU induced plants, when compared with the donor plants.

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1. Introduction

There are more than 250 species in the *Hibiscus* genus, ranging from annual and perennial herbs, to small woody shrubs trees, is cogitated as one of the important group for the family in mallows in which *Hibiscus rosa-sinensis* L., is well-known to be 'Chinese hibiscus' or 'China rose' available throughout the world and offers the widest range of flower colors and shapes, of all the other species (Braglia et al., 2010). More than nine thousand cultivars of *H. rosa-sinensis* L., exists with the number is continuously increasing, due to progress using both traditional and modern breeding techniques. It's currently cultivated throughout the world and considered as an attractive potted ornamental plant in the Middle East and Europe, amongst others (Akpan, 2006). Besides its ornamental importance, Chinese hibiscus was used widely in traditional

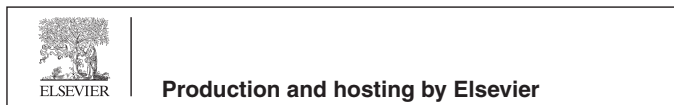
medicine to treat skin furuncles and ulcerations (Shen et al., 2017). Recently, several studies examined the medicinal assets for *H. rosa-sinensis* and found in leaves and flowers to be extracted for promoting in hair growth and healing ulcers (Adhirajan et al., 2003). It has also been shown to have significant effects when treating vein inflammation, breast pain, and indorsing for healing the wound (Afune et al., 2017; Bhaskar and Nithya, 2012).

Developing a standardized micropropagation protocol for the multiplication of *H. rosa-sinensis* would help to increase the productivity and utilization of this important plant. There are several factors affecting the successful in progress and improvement of in vitro cultures in cells and tissues in plants, including media composition (macro and micronutrients), sugar concentration, vitamin mixture, growth conditions, and alignment of growth hormones (Christensen et al., 2008). Media culturing is not optimized, adversely affects the cultured tissues and might lead to several physiological problems, and finally to the death of the tissues (Barghchi and Alderson, 1996; Van der Salm et al., 1994). Earlier studies were documented with in vitro cultivation of *H. rosa-sinensis* L., in different conditions. Rebecca and Todd (Rebecca and Todd, 2006) studied confirmed the role of various media composition in axillary shoot initiation at *H. rosa-sinensis* and found that the initiation was higher with high nutrient salt formulations

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and low thidiazuron (TDZ) concentrations. The high nutrient salt as modified Murashige and Skoog medium (Murashige and Skoog, 1962) were used for successful multiplying of *H. rosa-sinensis* nodal cuttings (Christensen et al., 2008).

Several limitations affect development processes in standardized in vitro multiplication protocol, including spontaneous epigenetic and/or genetic changes, causing sequence changes, DNA methylation, cytological abnormalities, and mutations of in vitro propagated plants (Alatar et al., 2017). Such changes lead to losses of productivity and quality of the regenerated plants. Therefore, the current study aims to enhance the effective, reliable, and standardized in vitro multiplication protocol intended for the micro-propagation in the Chinese hibiscus (*Hibiscus rosa-sinensis* L.) using 4-CPPU. Followed by validation of 4-CPPU induced plantlets by analysis of the ploidy levels using flow cytometry. As per our knowledge, uses of 4-CPPU for in vitro regeneration of *H. rosa-sinensis* have not previously been reported.

2. Material and methods

2.1. Starting materials & disinfections

The starting material is used with nodal segments collected through hibiscus plants grown in the College of Sciences, KSU, Riyadh, Saudi Arabia. In the laboratory, the collected plant materials were thoroughly cleansed with undistilled water for minimum of half-hour; later soaked in Fairy dishwasher (5%; v/v) for 5 min and cleaned with sterile water for 4–5 times. Afterwards, plant materials stood surface decontaminated with 0.1% (w/v) mercury chloride for minimum of 3 min of exposure. Plant materials were then finally, washed through mQH₂O (sterilized) for 5 times. The preparation of explants was performed aseptically through cutting with pasteurized blades/forceps for the collected materials.

2.2. Explant culture and growth situations

MS medium (Murashige and Skoog, 1962) (MS; 1962) stayed prepared with the adjusted pH between 5.8 and 5.9 consuming either NaOH/HCl; and autoclaved at 121 °C for 20 min and 100 kPa. Cultured explants have kept in growth room at 16/8-hour day night photoperiods with an intensity of light at of 2700 lx and 24/16 ± 2 °C day/night temperatures. With several combinations and absorptions in phenyl urea: *N*-(2-Chloro-4-pyridyl)-*N*-phenylurea or Forchlorfenuron (4-CPPU; 0.1, 0.5, 2.5, 5.0 & 10.0 µM) supplemented in MS media, the explants were cultured. Regenerated plant shoot clumps were sub-cultured on new media for every two weeks until the rooting process began. The percentage and number of regenerated plants and shoots/explants were recorded for 8 weeks. The medium type was also evaluated, after the determination of the most suitable hormone concentration. The different types of media were: B5 media (Gamborg et al., 1968), Driver-Kuniyuki woody plant medium (DKW) (Driver and Kuniyuki, 1984), woody plant medium (WPM) (Lloyd and McCown, 1980), Nitsch et al (Nitsch and Nitsch, 1969); Schenk and Hildebrandt medium (SH) (Schenk and Hildebrandt, 1972).

2.3. Rooting

For in vitro micro-roots, 4–5 cm in length of regenerated shoots were selected and aseptically relocated into the rooting medium; comprises half-strength of micro and macro basal salt mixture augmented by IBA (indole-3-butyric acid) using various concentrations (0.0, 0.1, 0.5, 1.0 & 2.0 µM). After four-weeks of transfer, the percentage and number of rooted shoots were recorded.

2.4. Acclimation

After being grown for 6-weeks on rooting medium, the healthy regenerated in vitro plants were root out of the nutrient medium and cleaned thoroughly with non-sterile water. Afterwards, plantlets were transferred into potting in soil containers (10-cm diameter). The plants were maintained in the growth room under 16 days and 8 nights in photoperiods with light intensities of 50 µmol m⁻² s⁻¹ and 24/16 ± 2 °C day/night temperatures. For irrigation, half MS salt solution was used for couple of weeks and then plants were irrigated with sterilized water for a further couple of weeks. However, healthy acclimatized plants were transferred after 4 weeks to the greenhouse under normal climatic conditions and irrigated with tap water directly.

2.5. Assessment of ploidy level

Ploidy levels were assessed as per Faisal et al. (2006) and from leaf tissue(s) nuclei were extracted (approx. 100 mg) by soft chopping in 1000 µL of Galbraith buffer using very sharp edge blades. Homogenates were filtered through double layers of nylon mesh and assessed with Flow-Cytometer (Version 3.0; Coulter-Epics, US) after staining with 50 µg/mL of propidium iodide (Sigma-Aldrich, US) for 10 min.

2.6. Data collection and analysis

In the present investigation, 20 explants of apiece concentration was used; each experimentation was repeated thrice. Data on the shoot regeneration and root inductions were documented after 8 and 6 weeks respectively, and analyzed using SPSS software (SPSS Inc, Chicago, US). Significant alterations between the values of different experiments were assessed with Tukey's HSD-tests ($p \leq 0.05$).

3. Results and discussion

The results indicate the in vitro morphogenic responses of explants and the developmental process are mainly stimulated through incorporation of different plant growth hormones in the nutrient media. In the maximum experiments, cytokinins are frequently used growth regulators for the initiation and multiplication of shoots in vitro and BA has been recognized as most effective followed through kinetin. While in the present investigation, for the first time, we have evaluated the effects of 4-CPPU on the in vitro morphogenic responses of the apical meristem containing an axillary bud harvested from a identified field grown plants of *H. rosa-sinensis* 'White Butterfly'. Like TDZ, 4-CPPU is also a phenylurea, having cytokinin activity and stimulating growth and shoot organogenesis in vitro, principally because of its relative tolerance to the endogenous cytokinin oxidases, the key enzyme of cytokinin degradation, along with the ability to produce endogenous cytokinins (Kamínek, 1992; Arinaitwe et al., 2000). The different concentrations of 4-CPPU used had significant effects ($P \leq 0.05$) on the in vitro shoot induction and multiplication (Table 1; Fig. 1A). While the treatments without any growth adjuvant, served as controls and exhibited no shoot regeneration ($P \leq 0.05$). On an average of 1.3 ± 0.35 shoots/explants were observed in 41.0 ± 2.1% of the cultured explants, at low concentrations (0.1 µM) of 4-CPPU. It was observed that the regeneration potential of explants pruned with growing concentrations of CPPU. A similar trend was reported in kiwifruit, where the higher concentrations of CPPU suppressed shoot organogenesis (Caboni et al., 2009).

Table 1
Effect of 4-CPPU on efficiency of axillary shoot regeneration in *H. rosa-sinensis*.

4-CPPU (μM)	% Regeneration	Mean shoot numbers	Mean shoot length (cm)
0.0	–	–	–
0.1	41.0 \pm 2.1 ^c	1.3 \pm 0.35 ^d	3.0 \pm 0.12 ^a
0.5	58.3 \pm 1.7 ^c	3.2 \pm 0.25 ^c	3.7 \pm 0.15 ^a
2.5	90.0 \pm 2.9 ^a	6.7 \pm 0.47 ^a	4.2 \pm 0.17 ^a
5.0	76.6 \pm 1.6 ^b	4.3 \pm 0.61 ^b	3.7 \pm 0.14 ^a
10	68.4 \pm 1.7 ^b	2.1 \pm 0.34 ^d	2.3 \pm 0.10 ^d

Means followed by the same superscript alphabet within columns are not significantly different by Tukey's HSD test.

Various concentrations of 4-CPPU were tested and the highest percent of shoot regeneration were documented on the media containing 2.5 μM 4-CPPU. On this media, 6.7 \pm 0.47 shoots were achieved from an explant; of an average of shoot length is 4.2 \pm 0.17 cm (Fig. 1B). In this current study, it has observed that the higher concentrations, greater than 2.5 μM of 4-CPPU, had inhibitory effects and resulted in basal callusing from the explants and stunted shoot growth. These results are consistent with earlier reports on *Ixora coccinea* (Lakshmanan et al., 1997); *Cassia-Siamea* (Parveen et al., 2010); the media with high concentrations of growth regulators stimulated the callus growth and resulted in decreased regeneration potentials of the explants. The basal calluses were removed immediately, and the regenerated shoot buds were transferred to fresh nutrient media.

To optimize the method for the efficient and substantial regeneration of this, and other important garden plants, different medias were evaluated for their optimal concentrations. In most of the studies, the MS medium was the first choice as it contained a balanced nutrient composition and further, different nutrient media were evaluated with the most significant formulation of hormonal combinations to achieve a maximum number of plant propagules. In addition to the MS various nutrient media viz., the B5 medium (Gamborg et al., 1968), DKW medium (Driver and Kuniyuki, 1984), WPM (Lloyd and McCown, 1980), Nitsch et al (Nitsch and Nitsch, 1969), and SH media (Schenk and Hildebrandt, 1972) were used with the optimal 4-CPPU concentrations (Fig. 2). Among the different media used, the nodal explants of the China rose exhibited the most significant responses in the MS medium with 2.5 μM 4-CPPU.

On this media an average of 6.7 shoots were recorded after 8 weeks of culture, and it was considered to be the most apt media for in vitro shoot induction cum plant regeneration of 'white butterfly'. While, the NN media produced the lowest number 3.2 \pm 0.15 of shoots per explant. The results obtained in the present

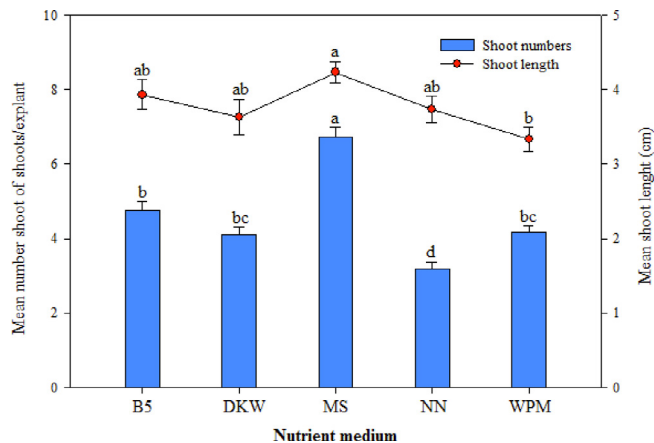


Fig. 2. Efficiency of different nutrient media supplied with the optimum concentration of 4-CPPU for the shoot regeneration potential in *H. rosa sinensis*.

study are in agreement with earlier tissue culture studies on *Mucuna pruriens* (Faisal et al., 2006); *Aquilaria malaccensis* (Saikia et al., 2013); *Cymbidium aloifolium* (Pradhan et al., 2013) and *Solanum lycopersicum* (Alatar et al., 2017), where, MS medium had significant effects in comparison to the other nutrient media.

The success of a protocol with tissue culture is mainly depends on efficient root inductions to get healthy plantlets before being transferred to ex-vitro conditions. To get complete plantlets, in vitro regenerated shoots individually excised from shoot clumps were transferred to different rooting inducing media. It is well documented that auxins have significant roles in the development of roots. IBA, most widely used auxin for initiation of roots from micro-shoots. In this study, the individual micro-shoots of *H. rosa sinensis* were re-located to MS nutrient medium having 0.1–2.5 μM IBA (Table 2). Micro-shoots were best rooted on media con-

Table 2

Effects of IBA on efficiency micro-root formation from 4-CPPU induced shoots of *H. rosa sinensis*.

4-CPPU (μM)	% Regeneration	Mean root numbers	Mean root length (cm)
0.0	–	–	–
0.1	76.6 \pm 1.6 ^b	3.6 \pm 0.21 ^{ab}	3.9 \pm 0.31 ^{ab}
0.5	97.0 \pm 2.1 ^a	5.6 \pm 0.32 ^a	4.5 \pm 0.12 ^a
1.0	77.4 \pm 1.7 ^b	4.1 \pm 0.17 ^b	4.0 \pm 0.23 ^a
1.5	60.3 \pm 1.7 ^c	3.0 \pm 0.12 ^b	3.2 \pm 0.14 ^b
2.0	41.0 \pm 2.1 ^d	1.3 \pm 0.25 ^c	2.0 \pm 0.15 ^c

Means represented by the same superscript alphabet within columns are not significantly different by Tukey's HSD test.

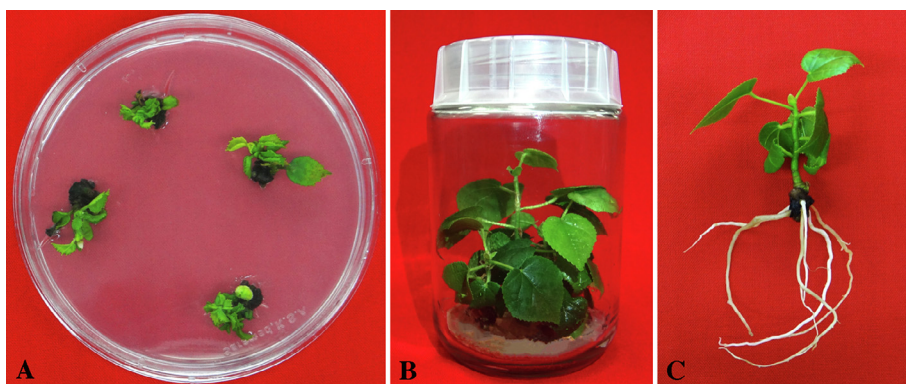


Fig. 1. 4-CPPU induced in vitro shoot induction and plant regeneration in *H. rosa-sinensis* L. A. Shoot induction on MS + 4-CPPU (2.5 μM); B. Proliferated shoots after 8 weeks of culture. IBA (0.5 μM) induced micro-roots.

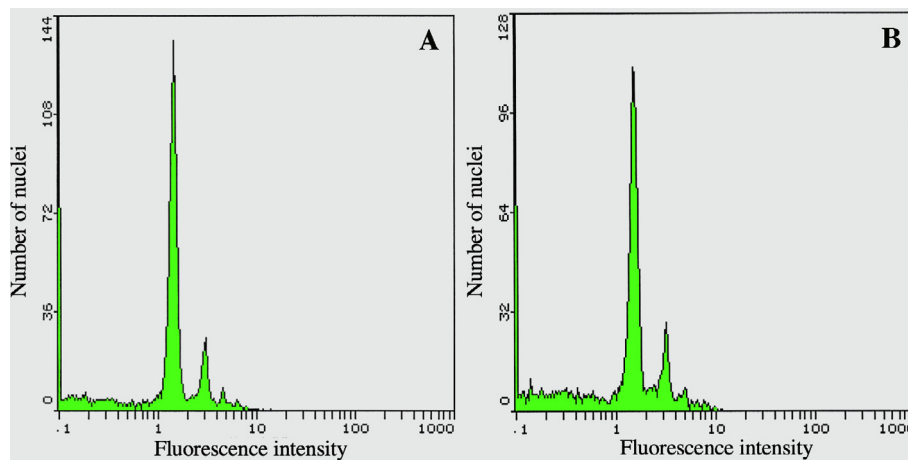


Fig. 3. Flow-cytometric histogram profiles of *H. rosa sinensis*. A. 4-CPPU induced plantlets; B. Normally grown plants.

tains 0.5 μ M IBA (Fig. 1C). After 6 weeks, on this media an individual micro-shoot induced 5.6 ± 0.32 roots with a typical length of 4.5 ± 0.12 cm. While, media containing the high concentrations of IBA suppressed the root inductions and only $41.0 \pm 2.1\%$ of the regenerated shoots were able to induce micro-roots. The stimulating effect of IBA on in vitro root development in *H. rosa sinensis* was in consistent with earlier reports on several ornamental plants (Parveen et al., 2010; Sivanesan et al., 2011; Aslam et al., 2013; Kale et al., 2018; Sarropoulou and Maloupa, 2019). The micro-plants with roots and shoots were acclimatized for ex-vitro conditions with a 90% survival rate. The micro-propagated plants transferred for ex-vitro conditions were grown well and there were no visual variances in the growth and morphology were observed among the plants. Producing clones is a most censorious factors due to somaclonal variation that arises as a direct consequences of plant cell and tissues cultured in vitro. In recent years, flow cytometry has been widely used for the estimation of DNA and ploidy levels of in vitro plants and it appears to be an efficient and reliable method for characterization in the regenerated plants (Obae and West, 2010; Vujović et al., 2012; Faisal et al., 2014; Faisal et al., 2018; Leite et al., 2019). In this study, the microprogrammed plants derived from the axillary buds were verified by flow cytometry and no differences in ploidy were recorded, as it was clearly evident in the histogram generated by flow cytometric analysis of the nuclei isolated from both the tissue culture as well as ex vitro grown plants (Fig. 3).

The present study concludes the initial report of in vitro culture of *H. rosa sinensis* using a phenyl urea (4-CPPU). An efficient and reliable system was developed for rapid and large-scale multiplication and sustainable supply of these valuable ornamental plants. Furthermore, the ploidy level of the regenerated plantlets was analyzed first to substantiate the uniformity of the tissue culture raised plants.

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