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In vitro plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*

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ABSTRACT

Effects of plant growth regulators (PGRs) and organic elicitors (OEs) on *Coleonema pulchellum in vitro* micropropagation, secondary product production and pharmacological activities were evaluated. *In vitro*, *ex vitro* and parental plants of *C. pulchellum* were investigated for their potential to produce phenolic and pharmacological compounds. Different morphogenic characteristics of shoots were obtained with PGRs- and OEs-containing media. A higher number of normal shoots were achieved with a low concentration of thidiazuron (TDZ: 4.5 μM). Lesser numbers were found with combinations of TDZ (13.6 μM) + indole-3-acetic acid (IAA: 2.9 μM); haemoglobin (HB: 300 mg l^{-1}) or glutamine (GM: 40 μM) + benzyladenine (BA: 8.8 μM). Shoots were rooted *in vitro* and successfully acclimatized. Plant growth regulators and OEs had a significant effect on the synthesis and accumulation of phenolic compounds and flavonoids. In particular, casein hydrolysate (CH) as well as a combination of GM and BA induced high levels of total phenolics and flavonoids during *in vitro* culture. Cytokinins and OEs had a significant effect on DPPH radical scavenging and antibacterial activities of *C. pulchellum* extracts. Acclimatized *C. pulchellum* plants can be used as substitute alternative to natural populations.

1. Introduction

Coleonema pulchellum Williams (Rutaceae) is an evergreen, erect and dense shrub, which occurs from the western to the eastern Cape in South Africa. It is an ideal aromatic garden plant which remains beautiful with pink flowers throughout the year. The plant contains phenylpropenes, phenylpropanoids and terpenoids which exhibit antimicrobial properties (Brader et al., 1997).

In vitro plant cell cultures have been carried out in the recent past for biosynthesis of compounds with pharmaceutical properties (Palacio et al., 2008). Plant tissue culture techniques offer a viable tool for *in vitro* regeneration in the conservation and commercial propagation of medicinal plants (Pattnaik and Chand, 1996). The capacity for plant cell, tissue and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized since the inception of *in vitro* technology. At the present time, many valuable compounds are produced by *in vitro* plant cell cultures (Baskaran et al., 2012). However, in other cases production requires more differentiated organ cultures such as shoots and roots (Karuppusamy, 2009). No studies on *in vitro* propagation of *C. pulchellum* have been reported.

The aim of this study was to investigate the potential of *C. pulchellum in vitro* cultures to synthesize phenolic compounds and to evaluate the changes in phenolic content due to plant growth regulators and organic elicitors used in the propagation process. The antioxidant and antibacterial activities were also evaluated in *in vitro* regenerated plants in comparison with parental (wild) plants.

2. Materials and methods

2.1. Chemicals

Folin & Ciocalteu phenol reagent, gallic acid (3,4,5-trihydroxibenzoic acid), N^6-benzyladenine (BA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), thidiazuron (TDZ), casein hydrolysate (CH), glutamine (GM), mebendazole (MBZ), haemoglobin (HB), catechin hydrate, 2,2-diphenyl-1-picryl hydrazyl (DPPH), Mueller-Hinton (MH) broth, neomycin, and *p*-iodonitrotetrazolium chloride (INT) were obtained from Sigma-Aldrich Co. (Steinheim, Germany). Ferric ammonium sulphate, sodium nitrite, sodium hydroxide, aluminium chloride and sodium hydrogen carbonate (BDH Chemicals Ltd., Poole, England); agar (bacteriological) (Oxoid Ltd., Basingstoke, England); dimethyl sulfoxide (DMSO), acetone and methanol (Merck KGaA, Darmstadt, Germany) were used. All chemicals and standards used in the assays were of analytical grade.

2.2. Plant material and in vitro propagation of shoots

Young shoot-tip explants (approximately 2 cm) of mature *C. pulchellum* plants were collected from the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The explants were washed under running tap water for 10 min to remove loose dirt, disinfected with 4% (v/v) previcur for 2 min

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and then decontaminated in a 3.5% sodium hypochlorite solution for 15 min. The explants were then washed five times with sterile distilled water.

Aseptic shoot-tip explants (10 mm in length) were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar and different plant growth regulators (PGRs) and organic elicitors (OEs) alone or in combination for high-frequency rapid shoot multiplication. The concentration and combinations of PGRs are indicated in Tables 1 and 2. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and/or 0.1 N HCl before gelling with 8 g l^{-1} agar and autoclaved at 121 °C for 20 min. The cultures were maintained at 25 ± 2 °C under a 16 h photoperiod and PPF of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent light (Osram L 58W/640, Germany).

2.3. Rooting of shoots and acclimatization

Proliferating shoots (at least 3.0 cm in length) were obtained from shoot-tip explants on MS medium containing 4.4 μM BA, and transferred to half- and full-strength MS media supplemented with different concentrations of auxins (IAA, IBA and NAA) and 8 g l^{-1} agar. In addition, half-strength MS medium supplemented with 5.4 μM NAA, 8 g l^{-1} agar and different concentrations (10–40 g l^{-1}) of sucrose was also tested. The optimized root induction medium (half-strength MS basal medium plus 5.4 μM NAA, 30 g l^{-1} sucrose and 8 g l^{-1} agar) was used in all treated shoots for root induction. Media devoid of PGRs were used as controls. The pH of the media was adjusted to 5.8 with 0.1 N NaOH and/or 0.1 N HCl prior to addition of agar and autoclaving at 121 °C for 20 min. All cultures were maintained at 25 ± 2 °C under cool-white fluorescent light at an intensity of 40 μmol m^{-2} s^{-1} with a 16 h photoperiod.

All rooted shoots were removed gently from the rooting medium after 8 weeks, and transferred to terracotta pots (95 × 120 mm, 500 ml) containing a 1:1 (v/v) vermiculite:sand mixture and irrigated with tap water every second day. These plantlets were maintained in the greenhouse (25 ± 2 °C under natural photoperiod conditions and a midday PPF of 950 ± 50 μmol m^{-2} s^{-1}) for acclimatization. Plant survival (%) was recorded after 10 weeks.

2.4. Sample preparation

Ten-week-old *in vitro*-grown shoots, one-year-old *ex vitro*-grown plants (grown on MS + 4.5 μM TDZ) and parental plant tissues (leaves, stem and roots) of *C. pulchellum* were oven dried at 50 °C to constant weight. The dried materials were ground into fine powders using a grinder (IKA®, USA). The finely ground samples (0.1 g) were extracted with 10 ml 50% methanol in a sonication bath (Branson model 5210, Branson Ultrasonics B.V., Soest, Netherlands) for 20 min on ice. The methanolic extracts were then filtered under vacuum through Whatman No. 1 filter paper and the filtrates were immediately used for the determination of total phenolics and flavonoids.

Dried and finely ground plant extracts for antibacterial and antioxidant assays were extracted in a sonication bath on ice for 1 h (1.0 g extract in 40 ml of 70% acetone), and concentrated in a rotary vacuum evaporator (Büchi, Switzerland) at 30 °C. The concentrated extracts were then dried over a stream of cold air at room temperature, re-dissolved in 50% DMSO to known concentrations and immediately used for the determination of antibacterial and DPPH radical scavenging antioxidant activities using appropriate solvent controls.

2.5. Phytochemical analysis

The Folin & Ciocalteu assay (Singleton and Rossi, 1965) was used for the determination of total phenolic compounds. As control a blank that contained 50% aqueous methanol was used instead of sample extracts. A standard curve of gallic acid was used to quantify the total phenolic content in the extracts. Total phenolic compounds were expressed in mg gallic acid equivalents (GAE) per g dry weight (DW).

The aluminium chloride ($AlCl_3$) method was used to quantify total flavonoid content (Zhishen et al., 1999). Suitable aliquots of catechin were used to derive a standard calibration curve. Total flavonoid content was expressed in mg catechin equivalents (CE) per g DW.

2.6. DPPH (2,2-diphenyl-1-picryl hydrazyl) radical scavenging activity

The DPPH assay for the determination of free radical scavenging activity was done as outlined by Moyo et al. (2010). Methanolic DPPH (100 μM) was freshly prepared before the assay. Decrease in the purple colouration of the reaction mixtures was read at 517 nm in a Cary 50 UV–visible spectrophotometer. Ascorbic acid was used as a standard antioxidant. The assay was done using four replicates. The free radical scavenging activity (RSA) was calculated according to the formula:

$$\text{Radical scavenging activity}(\%) = 100 \times (1 - A_E/A_D)$$

where A_E is the absorbance of the reaction mixture containing the sample extract or standard antioxidant, and A_D is the absorbance of the negative control.

Table 1
Effect of plant growth regulators on adventitious shoot and root regeneration of *Coleonema pulchellum* I. Williams.

Shooting media [MS + PGRs (μM)]				Rooting media [1/2 MS + 5.4 μM NAA]		
	Shoots [explant^{-1}] [#]	Shoot length [cm]	Morphogenic characteristics	Roots [shoot^{-1}] [#]	Root length [cm]	Morphogenic characteristics
MS (control)	0	0	0	0	0	0
4.4 BA	6.8 ± 1.12 g	3.76 ± 0.31bc	Normal shoots	8.0 ± 1.14ab	3.80 ± 0.37ab	WR and WFC
13.3 BA	12.6 ± 1.08ef	3.40 ± 0.50bc	Normal shoots	7.0 ± 1.26ab	3.24 ± 0.58ab	GR and WFC
22.2 BA	9.4 ± 0.51 g	3.20 ± 0.37bc	Normal shoots	6.2 ± 0.86ab	2.60 ± 0.51bc	GR and WFC
4.2 *m*T	7.0 ± 0.70 g	4.20 ± 0.58b	Normal shoots	9.0 ± 1.30a	4.58 ± 0.64a	WR and WFC
12.4 *m*T	15.0 ± 1.00de	6.40 ± 0.93a	Normal shoots	7.6 ± 1.12ab	4.00 ± 0.54ab	WR and WFC
20.7 *m*T	16.8 ± 1.02de	3.66 ± 0.18bc	Normal shoots	6.6 ± 0.51ab	3.60 ± 0.51ab	GR and WFC
4.5 TDZ	25.0 ± 1.84b	3.10 ± 0.37bc	Normal shoots	7.2 ± 1.01ab	2.60 ± 0.24bc	WR and WFC
13.6 TDZ	37.0 ± 2.09a	2.60 ± 0.19 cd	Shoot-tip necrosis	4.8 ± 0.58 cd	2.64 ± 0.46bc	GR and GFC
22.7 TDZ	21.4 ± 1.28bc	1.96 ± 0.27ef	Shoot-tip necrosis + GFBC	3.8 ± 0.34e	2.26 ± 0.37c	GR and GFC
13.6 TDZ + 2.2 BA	14.0 ± 1.41ef	1.72 ± 0.24f	Shoot-tip necrosis + GFBC	4.2 ± 0.37de	3.20 ± 0.34ab	GR and GFC
13.6 TDZ + 2.1 *m*T	13.2 ± 1.35ef	1.82 ± 0.30f	Shoot-tip necrosis + GFBC	6.6 ± 0.68ab	4.28 ± 0.58ab	GR and GFC
13.6 TDZ + 2.7 NAA	18.4 ± 1.20 cd	2.16 ± 0.39de	Shoot-tip necrosis + GCBC	7.6 ± 0.50ab	4.40 ± 0.56a	GR and GFC
13.6 TDZ + 2.9 IAA	23.2 ± 1.90bc	2.04 ± 0.34de	Normal shoots + GCBC	6.0 ± 0.28bc	3.52 ± 0.42ab	GR and GCC

GFBC = Green friable basal callus, GCBC = Green compact basal callus, WR = White root, GR = Green root, WFC = White friable callus, GFC = Green friable callus and GCC = Green compact callus.
Values are mean ± standard error (SE). Means followed by same letters in each column are not significantly different ($p = 0.05$) using Duncan's multiple range test.

Table 2
Effect of organic elicitors and plant growth regulators on adventitious shoot and root regeneration of *Coleonema pulchellum* I. Williams.

Shooting media OEs [mg l^{-1}] + PGRs [μM]				Rooting media [1/2 MS + 5.4 μM NAA]		
	Shoots [explant^{-1}] [#]	Shoot length [cm]	Morphogenic characteristics	Roots [shoot^{-1}] [#]	Root length [cm]	Morphogenic characteristics
MS (control)	0	0	0	0	0	0
300 CH	8.2 ± 1.35e	2.66 ± 0.28b	Shoot-tip necrosis + RG + GFBC	10.2 ± 1.20ab	9.80 ± 0.66b	GR and WFC
300 MBZ	8.6 ± 1.02e	2.72 ± 0.08b	Shoot-tip necrosis	8.2 ± 0.37bc	5.60 ± 0.51c	GR and WFC
40 GM	14.0 ± 1.41bc	4.40 ± 0.67a	Shoot-tip necrosis	12.0 ± 0.71a	13.24 ± 0.89a	GR and WFC
300 HB	12.6 ± 1.28 cd	3.26 ± 0.21b	Normal shoots + GCBC	9.4 ± 0.42bc	8.46 ± 0.54b	GR and WFC
300 CH + 4.5 TDZ	16.8 ± 1.15bc	2.68 ± 0.19b	Hyperhydric shoots + GCBC	5.8 ± 0.56ef	3.16 ± 0.70d	GR and GCC
300 MBZ + 4.5 TDZ	15.6 ± 1.20bc	2.92 ± 0.15b	Hyperhydric shoots + GCBC	4.6 ± 0.50f	2.80 ± 0.37d	GR and GCC
40 GM + 4.5 TDZ	19.4 ± 1.04a	3.06 ± 0.18b	Shoot-tip necrosis	7.6 ± 0.54 cd	3.62 ± 0.58d	GR and GCC
300 HB + 4.5 TDZ	16.2 ± 1.02bc	2.98 ± 0.26b	Hyperhydric shoots + GCBC	5.4 ± 0.40ef	3.00 ± 0.45d	GR and GCC
300 HB + 8.8 BA	12.2 ± 1.17 cd	4.40 ± 0.51a	Normal shoots	7.0 ± 0.71 cd	3.82 ± 0.58 cd	GR and GCC
40 GM + 8.8 BA	14.0 ± 1.30bc	5.46 ± 0.92a	Normal Shoots	6.4 ± 1.36de	4.60 ± 0.93 cd	GR and GCC

RG = Root growth, GFBC = Green friable basal callus, GCBC = Green compact basal callus, WR = White root, GR = Green root, WFC = White friable callus, GFC = Green friable callus and GCC = Green compact callus.
Values are mean ± standard error (SE). Means followed by same letters in each column are not significantly different ($p = 0.05$) using Duncan's multiple range test.

2.7. Antibacterial microdilution assay

The minimum inhibition concentration (MIC) of the extracts was evaluated using the serial micro-dilution assay (Eloff, 1998). Four bacterial strains Gram-positive (*Enterococcus faecalis* ATCC 19433 and *Staphylococcus aureus* ATCC 12600) and Gram-negative (*Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 10145) were used. Overnight bacterial cultures were diluted with sterile Mueller-Hinton (MH) broth to a final inoculum concentration of 10^6 colony forming units (CFU)/ml. Plant extracts were re-suspended in 50% DMSO to a concentration of 25 mg/ml. A positive control, neomycin (100 μg/ml) was used against each bacterial strain. Sterile distilled water, 50% DMSO, bacteria-free MH broth, and each bacterial strain, with no extract added completed the set of controls. The assay was done in triplicate. INT (0.2 mg/ml) dissolved in distilled water was used as the indicator of bacterial growth after incubation at 37 °C for 1 h.

2.8. Statistical analysis

Data were collected after 10 weeks of culture for shoot multiplication and 8 weeks for rooting experiments. All PTC experiments were repeated three times with 50 explants per treatment. The data were subjected to one-way analysis of variance (ANOVA) using SPSS version 18.0 for Windows (Chicago, USA). Significantly different means were separated using Duncan's multiple range test ($p = 0.05$).

3. Results and discussion

3.1. Influence of PGRs on shoot regeneration

Shoot-tip explants of *C. pulchellum* were cultured on MS medium with different cytokinins [benzyladenine (BA), *meta*-topolin (*m*T) and thidiazuron (TDZ)] at various concentrations (4.2–22.7 μM) to evaluate their potency on shoot multiplication. Different morphogenic characteristics were noticed in these *in vitro* cultures (Table 1). The shoot regeneration rate varied significantly between the control and PGR-containing treatments after 10 weeks of culture (Table 1). TDZ (13.6 μM) produced significantly higher shoot numbers (37.0 shoots per explant) after 10 weeks of culture (Table 1; Fig. 1A). However, these shoots exhibited shoot-tip necrosis symptoms (Table 1). On the other hand, BA, *m*T and lower concentrations of TDZ (4.5 μM) produced normal shoots (Table 1; Fig. 1B). The present study indicates that shoot-tip necrosis, a physiological disorder commonly observed in *in vitro* cultures affecting a wide range of plants, depends on the type and concentration of cytokinin in the culture medium. It is a phenomenon whereby the apical shoot initially becomes brown and later dies (Lakshmi and Raghava, 1993; Bairu et al., 2009). Benzyladenine was successfully used to control shoot-tip necrosis in chestnut cultures (Piagnani et al., 1996). Excessive yellowing of leaves and necrosis was observed at higher concentrations of 2-isopentenyladenine (2iP), kinetin or TDZ in Mexican redbud plants (Mackay et al., 1995). In the present study, various combinations and concentrations of PGRs were used to develop high-frequency rapid normal shoot multiplication from shoot-tip explants. A combination of TDZ (13.6 μM) and IAA (2.9 μM) significantly increased normal shoot production compared to other treatments (Table 1; Fig. 1C), indicating cytokinin–auxin synergistic effects in shoot proliferation. These cultures induced different types of basal callus after 10 weeks of culture (Table 1). A low auxin concentration in combination with high cytokinin concentration promoted shoot proliferation in other species of Rutaceae (Bohidar et al., 2008; Vennel Raj and Basavaraju, 2012).

3.2. Influence of organic elicitors and PGRs on shoot regeneration

The importance of organic elicitors (OEs: casein hydrolysate, haemoglobin, glutamine and mebendazole) alone and in combination with cytokinin (TDZ and BA) was examined for rapid shoot proliferation (Table 2). Organic elicitors showed significant rapid shoot proliferation in *C. pulchellum*. The promotional effects of elicitors on shoot regeneration have been reported in other plant species (Baskaran and Jayabalan, 2005; Baskaran and Van Staden, 2011). In this study, GM and HB treatments increased the number of shoots after 10 weeks of culture (Table 2; Fig. 1D). The HB treatment produced normal shoots as well as green compact basal callus (GCBC) after 10 weeks of culture. Shoots produced in the CH treatment exhibited shoot-tip necrosis symptoms, root growth and green friable basal callus (Table 2; Fig. 1E). A combination of TDZ and GM significantly increased shoot number (Table 2), but the shoots were also necrotic after 10 weeks of culture (Table 2; Fig. 1F). Combinations of CH, MBZ or HB with TDZ produced hyperhydric shoots as well as green compact basal callus (Table 2; Fig. 1G). Hyperhydricity is characterized by a glassy or swollen appearance to the tissue, usually resulting in reduced multiplication rates, poor quality shoots and tissue necrosis (Ziv, 1991). Their organs are translucent, in some cases less green and easily breakable (Ziv, 1991). In this study, combinations of HB or GM and BA produced normal shoots with a significantly higher shoot length (Table 2; Fig. 1H). These organic elicitors can be considered to mimic PGRs in the culture medium. Hyperhydricity has been reported to be influenced by cytokinin type and concentration (Kadota and Niimi, 2003), which is in agreement with our present findings. The current investigation showed that OE and PGR combinations in the MS medium enhanced adventitious shoot multiplication.

3.3. Effect of medium strength, sucrose concentration and auxins on in vitro rooting and acclimatization

Excised shoots from MS medium containing 4.4 μM BA were rooted using both half- and full-strength MS media with different types of

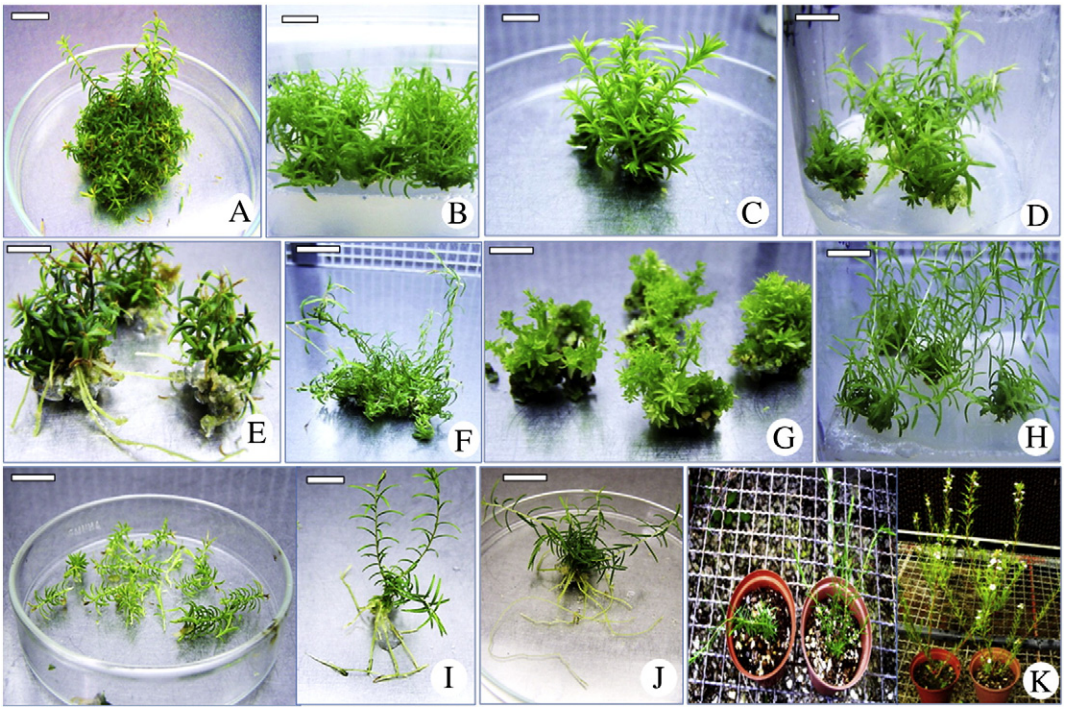

Fig. 1. *In vitro* propagation from shoot-tip explants of *C. pulchellum*. (A) Multiple shoots developing with shoot-tip necrosis from shoot-tip explants on MS medium with 13.6 μM TDZ after 10 week of culture (*Bar*, 10 mm), (B) Shoot multiplication on MS medium with 4.5 μM TDZ (*Bar*, 10 mm), (C) Shoot multiplication with green compact basal callus on MS medium with 13.6 μM TDZ and 2.9 μM IAA (*Bar*, 10 mm), (D) Adventitious shoot regeneration with green compact basal callus on MS medium containing 300 mg l^{-1} HB (*Bar*, 10 mm), (E) Shoot multiplication with shoot-tip necrosis, root growth and green friable basal callus on MS medium containing 300 mg l^{-1} CH (*Bar*, 10 mm), (F) Shoot multiplication plus shoot-tip necrosis on MS medium containing a combination of 40 μM GM and 4.5 μM TDZ (*Bar*, 10 mm), (G) Shoot multiplication with hyperhydric shoots and green compact basal callus on MS medium with 300 mg l^{-1} CH and 4.5 μM TDZ (*Bar*, 10 mm), (H) Production of adventitious shoots on MS medium with 300 mg l^{-1} HB and 8.8 μM BA (*Bar*, 10 mm), (I) Excised shoots from shooting medium and rooting of shoots (*Bar*, 10 mm), (J) Rooting of shoots derived from GM treatment (*Bar*, 10 mm), (K) Acclimatized plants with *ex vitro* flowering after 10 months.

auxins (Fig. 1I). Both half- and full-strength MS media containing 5.4 μM NAA induced higher numbers of roots after 8 weeks of culture (Table 3). Root length was significantly higher in full-strength MS medium containing 5.4 μM NAA after 8 weeks of culture (Table 3). These cultures produced normal shoots and roots as well as white friable basal callus (Table 3). In this study, concentrations of sucrose (10–40 g l^{-1}) were also tested in half-strength MS medium with 5.4 μM NAA. Different rates of root growth were observed after 8 weeks of culture (Table 3). The medium containing 30 g l^{-1} sucrose was more effective in induction of root growth compared to other sucrose concentrations (Table 3). The present study revealed the importance of sucrose concentration on root induction in *C. pulchellum*. A similar trend was also observed in Persian walnut (Vahdati et al., 2004). Half-strength MS medium supplemented with 5.4 μM NAA was selected as the standard growth medium for root induction in subsequent experiments.

All the PGR and OE-treated shoots produced roots with different morphogenic characteristics (Tables 1 and 2). Root induction rate varied significantly between the shooting media after 8 weeks of culture (Tables 1 and 2). GM-produced shoots were the most amenable to root induction, and produced significantly higher root numbers (12.0 roots per shoot) and root lengths (13.24 cm) after 8 weeks of culture (Table 2; Fig. 1J). These cultures induced green roots and white friable basal callus

Table 3
Effect of medium strength, auxins and sucrose concentration on root induction from *in vitro* shoots of *Coleonema pulchellum* I. Williams incubated in MS medium with 4.4 μM BA.

Medium strength + Auxins (μM)	Sucrose concentration (g l^{-1})	Morphogenesis of root and shoot growth			
		Roots [shoot^{-1}] [#]	Root length [cm]	Shoot length [cm]	Morphogenetic characteristics
½ MS (control)		0	0	2.80 ± 0.37de	Single shoot
½ MS + 5.7 IAA		5.6 ± 0.24 cd	3.40 ± 0.24c	6.60 ± 1.07ab	Normal shoot + GR + GFC
½ MS + 4.9 IBA		2.2 ± 0.37e	4.24 ± 0.37bc	4.72 ± 0.58bc	Normal shoot + WR
½ MS + 5.4 NAA		8.6 ± 1.29a	3.82 ± 0.42bc	4.48 ± 0.47bc	Normal shoot + WR + WFC
MS (control)		0	0	3.46 ± 0.51d	Single shoot
MS + 5.7 IAA		6.4 ± 0.51bc	3.20 ± 0.37c	6.82 ± 1.24a	Normal shoot + GR + GFC
MS + 4.9 IBA		1.6 ± 0.32ef	2.00 ± 0.32 cd	3.80 ± 0.37bc	Normal shoot + WR + GFC
MS + 5.4 NAA		8.2 ± 0.80a	7.42 ± 1.24a	5.86 ± 0.58bc	Normal shoot + WR + WFC
½ MS + 5.4 NAA	10	4.0 ± 0.32d	2.16 ± 0.45c	3.64 ± 0.42c	Normal shoot + WR
½ MS + 5.4 NAA	20	6.6 ± 0.40bc	3.60 ± 0.81c	4.40 ± 0.51bc	Normal shoot + WR + WFC
½ MS + 5.4 NAA	30	8.4 ± 0.71a	7.20 ± 1.28a	4.56 ± 0.84bc	Normal shoot + WR + WFC
½ MS + 5.4 NAA	40	5.2 ± 0.58 cd	5.18 ± 1.16b	3.72 ± 0.73c	Normal shoot + WR + WFC

WR = White root, GR = Green root, WFC = White friable callus and GFC = Green friable callus.
Values are mean ± standard error (SE). Means followed by same letters in each column are not significantly different ($p = 0.05$) using Duncan's multiple range test.

Table 4
Effect of plant growth regulators and organic elicitors on secondary metabolite production and radical scavenging activity of *in vitro* shoots, acclimatized and natural-grown plants of *Coleonema pulchellum* I. Williams.

Treatment	Total phenolic content (mg GAE/g DW)	Flavonoid content (mg CE/g DW)	DPPH radical scavenging activity (%)		
			31.25 μg/ml	62.5 μg/ml	125 μg/ml
13.3 BA	12.52 ± 0.30de	4.13 ± 0.11d	45.8 ± 2.16	77.3 ± 3.20	91.4 ± 0.09b
12.4 *m*T	9.85 ± 0.26hij	3.24 ± 0.06gh	23.7 ± 1.44	46.0 ± 2.91	65.4 ± 2.30g
13.6 TDZ	10.24 ± 0.24hi	3.54 ± 0.04fg	52.0 ± 4.83	80.9 ± 2.14	91.6 ± 0.09b
13.6 TDZ + 2.9 IAA	10.93 ± 0.44fgh	3.83 ± 0.04def	72.9 ± 2.21	84.4 ± 1.10	85.9 ± 2.49cd
300 CH	14.63 ± 0.06c	4.76 ± 0.09c	60.4 ± 1.39	86.3 ± 0.77	87.5 ± 0.57bc
300 MBZ	9.14 ± 0.14ij	2.99 ± 0.07hi	28.8 ± 1.86	57.9 ± 1.64	81.6 ± 1.18de
40 GM	9.73 ± 0.21hij	2.91 ± 0.05hij	13.0 ± 2.21	30.8 ± 2.44	59.0 ± 3.91h
300 HB	8.65 ± 0.39j	2.69 ± 0.02ij	24.5 ± 4.10	52.2 ± 1.72	78.5 ± 1.45e
300 CH + 4.5 TDZ	9.15 ± 0.34ij	2.70 ± 0.08ij	42.3 ± 1.62	61.1 ± 2.27	84.8 ± 0.98cd
300 MBZ + 4.5 TDZ	11.48 ± 0.07efg	3.09 ± 0.09h	42.0 ± 2.93	67.5 ± 6.10	88.6 ± 1.87bc
40 GM + 4.5 TDZ	11.84 ± 0.30ef	3.59 ± 0.12efg	54.3 ± 2.15	79.6 ± 3.33	87.1 ± 0.77bc
300 HB + 4.5 TDZ	10.59 ± 0.17gh	3.24 ± 0.06gh	26.3 ± 0.57	47.4 ± 0.82	78.4 ± 1.04e
300 HB + 8.8 BA	13.11 ± 0.32d	3.93 ± 0.08de	75.1 ± 1.37	86.3 ± 0.28	88.8 ± 0.79bc
40 GM + 8.8 BA	9.27 ± 0.34ij	2.61 ± 0.10j	16.4 ± 6.17	40.2 ± 0.89	70.7 ± 3.93f
Acclimatized plants (R)	6.60 ± 0.14k	3.11 ± 0.14h	0.8 ± 0.83	12.6 ± 2.32	24.9 ± 0.90i
Acclimatized plants (S + L)	19.66 ± 0.28b	7.57 ± 0.30b	79.5 ± 0.78	83.2 ± 0.49	87.5 ± 0.59bc
Natural plants (R)	6.76 ± 0.56k	2.07 ± 0.05k	29.8 ± 3.21	52.6 ± 3.68	79.1 ± 0.71e
Natural plants (S + L)	36.86 ± 1.05a	12.89 ± 0.22a	91.8 ± 0.14	93.7 ± 0.08	93.8 ± 0.07b
Antioxidant control (Ascorbic acid)			96.4 ± 0.05	96.5 ± 0.03	96.9 ± 0.22a

S + L — stem and leaves; R — roots.
Values are mean ± standard error (SE). Means followed by same letters in each column are not significantly different ($p = 0.05$) using Duncan's multiple range test.

(Table 2). The present study indicates that beneficial root growth depends on type as well as concentration of PGRs and OEs in the media (Tables 1 and 2).

The *in vitro*-rooted shoots were removed from the culture media after 8 weeks and successfully acclimatized (100%) in a vermiculite:soil mixture (1:1 v/v) in the greenhouse (25 ± 2 °C) under natural photoperiod conditions with a midday PPF of 950 ± 50 μmol m^{-2} s^{-1} (Fig. 1K).

3.4. Phytochemical and antioxidant properties of C. pulchellum extracts

The results on the effect of cytokinins and OEs are presented in Table 4. Total phenolic and flavonoid contents were higher in the leaves and stems compared to the roots for both acclimatized and natural plants. Shoot cultures provide a viable option for the production of secondary metabolites, especially where the compounds are synthesized and/or stored in the aerial plant parts (Amoo et al., 2012). Cytokinin type and OEs had a significant effect on total phenolic and flavonoid contents. In particular, casein hydrolysate significantly upregulated the levels of total phenolic compounds and flavonoids (Table 4). Total phenolic and flavonoid contents were also upregulated in *in vitro C. pulchellum* cultures treated with BA. Different types and concentrations of PGRs have been reported to regulate developmental processes and modify the concentration of secondary metabolites in plants differently (Palacio et al., 2008; Baskaran et al., 2012). A combination of GM and BA significantly increased the *in vitro* accumulation of phenolics and flavonoids, compared to when the compounds were used singly. The results show that the concentration of secondary metabolites in *in vitro* cultures is influenced by the composition of the medium (Abbasi et al., 2010; Baskaran et al., 2012). Natural phenolic compounds and flavonoids are widely known for a range of biological activities such as antimicrobial, anti-cancer and cardiovascular regulatory effects (Brader et al., 1997; Palacio et al., 2008; Moyo et al., 2010).

All the treatments exhibited a dose-dependent increase in DPPH radical scavenging activity (Table 4). *In vitro* treatment using cytokinins and organic elicitors had a significant effect on DPPH radical scavenging activity, in particular BA, TDZ and CH as well as their respective combinations. Natural antioxidants play a crucial role in ameliorating the detrimental effects of oxidative stress caused by reactive oxygen species as well as being food additives that inhibit the oxidation of nutrients in foodstuffs. There is a strong correlation between disease pathogenesis and oxidative stress levels, hence the use of natural antioxidant compounds is considered an effective therapeutic approach against several diseases (Moyo et al., 2010). The potential use of *in vitro* technology for the synthesis of antioxidant compounds from medicinal plants has been widely reported (Matkowski, 2008; Abbasi et al., 2010; Amoo et al., 2012; Khateeb et al., 2012).

3.5. Antibacterial activity of C. pulchellum extracts

The antibacterial activity of *C. pulchellum* extracts is presented in Table 5. The tested extracts had better activity against Gram-positive *Enterococcus faecalis*. *In vitro* extracts derived from *m*T and CH-treated plants exhibited high antibacterial activity against *E. faecalis* with MIC values of 0.781 and 1.041 mg/ml, respectively. Furthermore, CH-treated *C. pulchellum* plants had a low MIC (1.56 mg/ml) against the Gram-negative bacteria *E. coli*. Isolated phenylpropenes from *C. pulchellum* had good antimicrobial activity (Brader et al., 1997). The authors attributed the observed antimicrobial activity to the reactivity of the phenolic hydroxyl group (Brader et al., 1997). In a similar study, Khateeb et al.

Table 5
Effect of plant growth regulators and organic elicitors on antibacterial activity of *in vitro* shoots, acclimatized and natural-grown plants of *Coleonema pulchellum* I. Williams.

Treatment	Minimum inhibitory concentration (mg/ml)			
	S. a	*E. f*	*P. a*	*E. c*
13.3 BA	2.604	1.302	6.25	6.25
12.4 *m*T	3.125	0.781	6.25	6.25
13.6 TDZ	3.125	1.562	6.25	6.25
13.6 TDZ + 2.9 IAA	6.25	1.302	>6.25	6.25
300 CH	3.125	1.041	6.25	1.562
300 MBZ	3.125	1.562	>6.25	6.25
40 GM	3.125	3.125	>6.25	6.25
300 HB	3.125	3.125	6.25	6.25
300 CH + 4.5 TDZ	3.125	1.562	>6.25	6.25
300 MBZ + 4.5 TDZ	6.25	2.604	6.25	6.25
40 GM + 4.5 TDZ	6.25	2.083	>6.25	6.25
300 HB + 4.5 TDZ	6.25	1.562	6.25	6.25
300 HB + 8.8 BA	3.125	1.562	6.25	6.25
40 GM + 8.8 BA	6.25	1.562	6.25	6.25
Acclimatized plants (R)	3.125	6.25	>6.25	6.25
Acclimatized plants (S + L)	2.083	1.562	6.25	3.125
Natural plants (R)	3.125	1.562	>6.25	6.25
Natural plants (S + L)	3.125	0.781	6.25	3.125
Neomycin (μg/ml)	0.0061	>100	0.0732	0.122

(2012) reported the antimicrobial activity of *in vitro*-derived secondary metabolites of *Cichorium pumilum*. The present study shows that *C. pulchellum* contains biologically potent therapeutic phytochemicals with high antibacterial and antioxidant activities.

4. Conclusions

The present study demonstrated that *C. pulchellum* shoot-tip explants are a good starting material for *in vitro* culture establishment. A combination of TDZ and IAA produced a significant number of normal shoots while lower TDZ concentrations favourably affected the induction of normal shoots. The best rooting was achieved with half-strength MS medium containing 30 g l^{-1} sucrose and NAA. *In vitro* and *ex vitro*-grown *C. pulchellum* treated with combinations of OEs and PGRs exhibited high antioxidant and antibacterial activities. This study showed that *in vitro* regenerated *C. pulchellum* plant tissues and organs contain the active phytochemical constituents, which have medicinal properties. The present micropropagation system can be recommended for the mass propagation of *C. pulchellum* plants. The acclimatized *in vitro*-derived plants can be used as an alternative to natural populations of *C. pulchellum*.

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