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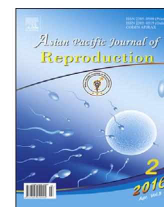

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Effect of growth regulators on rapid micropropagation and antioxidant activity of *Canscora decussata* (Roxb.) Roem. & Schult. – A threatened medicinal plant

Loganathan Kousalya*, V. Narmatha Bai

Plant Tissue Culture Laboratory, Department of Botany, Bharathiar University, Coimbatore 641046, India

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ABSTRACT

Objective: An efficient *in vitro* plant regeneration protocol for *Canscora decussata* Schult. (Gentianaceae) (*C. decussate*), a threatened medicinal herb used in Ayurvedic system of medicine was developed. Seed germination was achieved on MS growth regulator free medium.

Methods: The nodal explants were excised from the *in vitro* raised seedlings and inoculated on MS medium supplemented with various plant growth regulators such as BAP, KIN, TDZ and Zeatin individually and in combinations with or without GA_3. BAP (2 mg/L) was proved to be effective for multiple shoot induction (30.20 ± 6.53) among the cytokinin tested individually. Addition of NAA (1 mg/L) to cytokinin containing medium resulted in callus, KIN (3 mg/L) with NAA (1 mg/L) produced highest percentage of callus (82%) per explant.

Results: Among the various combination of cytokinin tested, BAP (0.5 mg/L) in combination with KIN (2 mg/L) induced highest number of multiple shoots (72.10 ± 1.05 shoot per explant). Addition of 1 mg/L of GA_3 to the above medium induced highest number of shoots (100.80 ± 3.20) with an average shoot length of 6.98 ± 0.66 cm. Rooting was optimized in half-strength MS medium supplemented with IBA at 1.0 mg/L. The plantlets were successfully transferred to hardening medium containing vermiculite with 83% survival rate. Among the antioxidant activity of methanol extract of wild-grown plants and *in vitro* regenerants tested, half-MS medium supplemented with NAA (0.5 mg/L) derived callus has promising activity for total phenolics, DPPH, ABTS, FRAP and phosphomolymbdenum assays. Total flavonoid content was found to be high in callus derived from MS medium supplemented with KIN (2 mg/L) in combination with NAA (1 mg/L).

Conclusion: Our present study suggest that *in vitro* derived callus of *C. decussata* represent a promising alternative source to meet the pharmaceutical demands for commercial formulations and the protocol could effectively be applied for the conservation of *C. decussata* Schult.

1. Introduction

Shankhpushpi is a drug of Ayurvedic 'Medhya Rasayana' category which is used to boost memory and intellect. *Canscora decussata* (*C. decussate*) Schult. (Gentianaceae) is one of the plants used as 'Shankhpushpi'. The entire plant, as well as its fresh juice is used in medicine. It is used in the popular medicine for the treatment of insanity, epilepsy and nervous debility. It has proven its therapeutic potential in acetylcholinesterase inhibition, CNS stimulation, hypertension, convulsions, tuberculosis, immunomodulation, inflammation, hepatoprotection, spermatogenesis and postmenopausal osteoporosis [1]. It is reported to contain several types of xanthones, triterpenoids, loliolide, sterols and flavonoids [2]. Studies of this plant showed hepatoprotective, antidepressant, antianxiety, antistress and antimycobacterium tuberculosis activity [3]. The presence of mangiferin in *C. decussata* can thus be correlated to the cognitive and memory enhancing activity of *C. decussata* [4].

*Corresponding author: Loganathan Kousalya, Plant Tissue Culture Laboratory, Department of Botany, Bharathiar University, Coimbatore 641046, India.
E-mail: lkousalya25@gmail.com

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Indiscriminate practice of over harvesting makes the species to become increasingly vulnerable at a point that threatens its survival [5]. Besides, these nature-harvested plants are unlikely to meet the quality standards for botanical drugs [6]. As *C. decussata* plants are short life-cycled and seasonal in nature, *ex situ* multiplication of this plant using *in vitro* techniques seems to be a viable approach for coexistence of germplasm conservation biomass utilization [7]. As the domestication of the plant using conventional techniques has not yet been successfully employed, so the present studies aim to develop a protocol for the rapid propagation of this commercially important medicinal plant.

The present research work is based on to develop an efficient and rapid propagation protocol for *C. decussata* for large-scale production of uniform raw materials for future pharmaceutical compound extraction and to analyze the antioxidant activity of the multiple shoot and callus from some selected PGR concentration and also compared with *in vivo* plants (wild-grown plants). Effective plant growth regulators in development of plants with a greater antioxidant activity were determined by 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2-Azinobis (3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), FRAP and phosphomolybdenum assays. This present study also focused on correlation of total flavonoid and phenol content for antioxidant activity of both the *in vitro* regenerants and wild plants.

2. Materials and methods

2.1. Source of plant material

The plant materials of *C. decussata* were collected from Kerala, India. An authentic sample was identified by BSI (Botanical Survey of India), Southern Circle, Coimbatore, India, and a voucher specimen has been deposited in the herbarium of BSI Coimbatore (Accession No: 1893).

2.2. Experimental procedure

Seeds collected from healthy plants were washed with running tap water followed by 5% (v/v) Teepol (detergent) treatment for 5–10 min and then treated with fungicide (1% Bavistin) for 20 min. The treated explants were washed with double distilled water. Subsequently the explants were disinfected with 0.1% mercuric chloride for 3–5 min and finally they were rinsed with sterile double distilled water under aseptic condition. MS medium [8] supplemented with 3% (w/v) sucrose was used in the experiments. The pH of the medium was adjusted to 5.7 before adding 0.8% (w/v) agar (Hi Media). Media (15 mL) were poured into (25 × 150) mm culture tubes (Borosil, Mumbai) and autoclaved at 121 °C and 1.06 kg/cm^2 pressure for 20 min. The cultures were incubated at (25 ± 2) °C under a 16 h photoperiod of 50–60 l mol/m^2/s flux density provided by cool white fluorescent tubes.

2.2.1. Multiple shoot induction

Experiments were carried out on shoot induction and proliferation of *C. decussata*. The pretreated seeds were inoculated on MS medium without any growth regulator. The *in vitro* seedling derived nodal segments were cultured on various cytokinins, such as BAP, Kin, Zeatin and Thidiazuron (TDZ) (0.5–3.0 mg/L). The total number and length of shoots were calculated after 5 weeks of culture. In order to increase shoot multiplication and shoot elongation in *in vitro* derived nodal explants, different concentrations of Gibberellic acid (GA_3) in combination with BAP, KIN, Zeatin and TDZ (0.5–3.0 mg/L) was tested. For callus induction and multiple shoot proliferation, NAA (1.0 mg/L) was combined with cytokinin at different concentrations. The maximum number of shoots and shoot length were calculated after 5 weeks of culture.

2.2.2. Root induction

For root induction, excised shoots were transferred to half strength MS medium supplemented with three auxins including Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), a-Naphthaleneacetic acid (NAA) at different concentrations (0.5–3.0 mg/L). Root number and length of roots were recorded after 3 weeks of culture. Healthy plantlets with well-developed roots were potted on paper cups containing vermiculite (100%). Subsequently the plantlets were transferred into greenhouse condition. Explants inoculated onto growth regulator free MS medium were served as controls for all the above mentioned experiments.

2.3. Antioxidant studies

2.3.1. Extraction method

The air-dried powdered of *in vitro* regenerants plants and callus derived from the various treatments of PGR and wild plants was used for extracted by maceration method with methanol (48 h) and the extracts were filtered. The extracts were concentrated by rotary vacuum evaporator and then air-dried. The extracts obtained were used directly for the estimation of total phenolic content and also for the assessment of antioxidant potential through various biochemical assays.

2.3.2. Determination of total phenol & flavonoid content

The total phenol content was determined according to the method described by [9] & total flavonoid contents estimated as per described by [10].

2.3.3. In vitro antioxidant activity

The radical scavenging activity of the *C. decussata* methanol extract of wild-grown plants and *in-vitro* propagated plants and callus was evaluated using DPPH$^{\cdot}$ [11], ABTS$^{\cdot+}$ cation radical [12], ferric reducing antioxidant power (FRAP) activity [13] and phosphomolybdenum method [14].

2.4. Statistical analysis

All the experiments were conducted with a minimum of 5 replicates per treatment. The experiments were repeated three times. The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at $P < 0.05$ (SPSS 20.0 version). The results are expressed as a means ± SD of three experiments. All experiments of antioxidant studies were repeated at least three times. Results were reported as mean ± SD. The antioxidant activities of all the extracts were tested by one-way analysis of variance (ANOVA). Correlation analysis was performed using Pearson correlation (two-tailed) test.

Figure 1. *C. decussata* habit & its herbarium.

Figure 2. Effect of cytokinin and auxin on multiple shoot induction and callus induction of *Canscora decussata* from *in vitro* nodal explants.
A&B: *In vitro* seed germination and plantlet regeneration of *C. decussate* (bar-1 cm); C: Initial stage of *in vitro* derived nodal explants (bar-1 cm); D: Multiple shoot induction from MS medium + 2.0 mg/L (bar-1 cm); E: Multiple shoot induction from MS medium + BAP (2 mg/L)+KIN(0.5 mg/L) (bar-1 cm); F&G: Multiple shoot induction and elongation of single shoot from MS medium + BAP (2 mg/L)+KIN(0.5 mg/L)+GA3 (2 mg/L) (bar-1.5 cm); H: callus induction from MS medium+0.5 mg/L of NAA (bar-1 cm); I: Callus induction with roots from MS medium + KIN (3 mg/L)+NAA (1 mg/L) (bar-1 cm); J: Root induction from MS medium + 1 mg/L of IBA; (bar = 1 cm); K: *In vitro* flowering stage (bar-1.5 cm); L: Hardening stage (bar-1 cm).

3. Results

3.1. In vitro seed germination

The plant specimen with flowering stage was showed in Figure 1. A protocol for the axillary multiplication of *C. decussata* was established in present study. There are no reports on studies relating to germination of seed and micropropagation of *C. decussata*. Full-strength MS medium was used for seed germination to arise aseptic seedlings of *C. decussata* (Figure 2A & B). The pretreated seeds showed 36.2% of seed germination in the present study. The nodal explants derived from aseptically raised seedlings were used for culture initiation (Figure 2C). The germination of seeds were recorded and percentage of seed germination was calculated by the formula,

$$\text{Percentage of germination} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seed inoculated}} \times 100$$

3.1.1. Effect of cytokinin on multiple shoot induction

Preliminary experiments were conducted to study the effect of various concentrations of cytokinins such as BAP, 6-Furfurylaminopurine (KIN), TDZ, and Zeatin (0.5–3.0 mg/L) with MS basal as control medium on shoot bud induction from *in vitro* nodal explants. Each of BAP, KIN, TDZ and Zeatin separately showed a significant variation in terms of number of shoot bud induced per explant (Table 1). A maximum number of 30.20 ± 6.53 shoots per explants were induced on MS medium containing BAP (2 mg/L) (Figure 2D). In comparison to the response of nodal explants on media supplemented with cytokinins, no shoot buds were formed on MS basal media.

3.1.2. Effect of combination of cytokinin on multiple shoot induction

In order to increase the shoot multiplication, BAP (0.5–3.0 mg/L) in combination with KIN, TDZ and Zeatin (0.5–3.0 mg/L) were tested. An increase in the number of the shoots (72.10 ± 1.05) was observed when the *in vitro* nodal explants were cultured on MS medium supplemented with BAP (0.5 mg/L) and KIN (2.0 mg/L) (Table 2) (Figure 2E). GA_3 was combined with well resulted combinations of cytokinins in order to increase the multiple shoot induction as well as shoot elongation. Among these concentrations combined with GA_3 (0.5–3.0 mg/L), combination of BAP (0.5 mg/L) and KIN (2.0 mg/L) along with GA_3 (1 mg/L) induced maximum number of (100.80 ± 3.20) shoots per explants with an average shoot length of (6.98 ± 0.66) cm per shoots (Table 3) (Figure 2F & G).

Table 1

Multiple shoots induction from *in vitro* nodal explants of *C. decussata*.

Plant growth regulators in mg/L				Number of shoots	Shoot length (cm)
BAP	KIN	TDZ	ZEATIN		
–	–	–	–	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}
0.5	–	–	–	15.40 ± 4.87^{cd}	1.82 ± 0.35^{a}
1.0	–	–	–	22.20 ± 8.34^{b}	1.08 ± 0.73^{cd}
2.0	–	–	–	30.20 ± 6.53^{a}	1.40 ± 0.10^{abc}
3.0		–	–	19.80 ± 6.45^{bc}	1.04 ± 0.67^{cd}
	0.5	–	–	21.40 ± 3.36^{b}	1.34 ± 0.15^{bc}
	1.0	–	–	29.40 ± 5.68^{a}	1.54 ± 0.70^{ab}
	2.0	–	–	29.80 ± 4.65^{a}	1.34 ± 0.18^{bc}
	3.0	–	–	21.00 ± 2.54^{b}	1.24 ± 0.16^{bcd}
	–	0.5	–	5.80 ± 0.83^{fg}	0.42 ± 0.10^{efg}
		1.0	–	15.80 ± 0.83^{cd}	0.44 ± 0.05^{efg}
		2.0	–	11.00 ± 0.70^{def}	0.44 ± 0.05^{efg}
		3.0	–	6.20 ± 1.30^{fg}	0.52 ± 0.16^{ef}
		–	0.5	11.20 ± 0.83^{de}	0.44 ± 0.05^{defg}
		–	1.0	12.80 ± 0.83^{de}	0.84 ± 0.05^{de}
		–	2.0	9.20 ± 0.83^{ef}	0.46 ± 0.05^{efg}
		–	3.0	6.24 ± 3.37^{fg}	0.44 ± 0.05^{efg}

Data shown is the mean of five replicates ± SD. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 2

Effect of combination of Cytokinin on multiple shoot induction on *Canscora deccusata*.

Plant growth regulators (mg/L)				Number of shoots	Shoot length (cm)
BAP	KIN	TDZ	ZEATIN		
0.5	0.5	–	–	20.60 ± 2.30^{fg}	2.18 ± 0.19^{c}
0.5	1.0	–	–	37.40 ± 13.4^{d}	1.70 ± 0.12^{defg}
0.5	2.0	–	–	72.10 ± 1.05^{a}	2.68 ± 0.08^{ab}
0.5	3.0	–	–	45.40 ± 1.67^{c}	0.90 ± 0.12l^{mn}
1.0	0.5	–	–	16.00 ± 1.00^{hi}	2.18 ± 0.19^{cde}
1.0	1.0	–	–	14.40 ± 2.07^{hij}	1.70 ± 0.12^{fghi}
1.0	2.0	–	–	9.20 ± 0.83^{klmn}	1.00 ± 0.14^{klm}
1.0	3.0	–	–	20.60 ± 2.30^{fg}	0.90 ± 0.12^{klm}
2.0	0.5	–	–	15.40 ± 2.70^{hij}	1.00 ± 0.14^{klm}
2.0	1.0	–	–	18.80 ± 0.83^{gh}	2.34 ± 0.05^{bcde}
2.0	2.0	–	–	51.40 ± 5.41^{b}	2.42 ± 0.25^{bcd}
2.0	3.0	–	–	43.00 ± 6.87^{e}	2.66 ± 0.32^{ab}
3.0	0.5	–	–	21.40 ± 5.12^{f}	1.32 ± 0.13^{fghijkl}
3.0	1.0	–	–	14.00 ± 0.70^{hij}	2.40 ± 0.20^{bcd}
3.0	2.0	–	–	6.20 ± 1.30^{nopqrs}	3.38 ± 0.33^{a}
3.0	3.0	–	–	5.40 ± 0.54^{opqrst}	1.90 ± 0.15^{bcdefg}
0.5	–	0.5	–	11.40 ± 0.89^{jklm}	1.58 ± 0.13^{cdefghi}
0.5	–	1.0	–	17.80 ± 0.83^{gh}	1.82 ± 0.08^{bcdefgh}
0.5	–	2.0	–	17.40 ± 0.89^{gh}	1.52 ± 0.16^{cdefghi}
0.5	–	3.0	–	13.20 ± 1.30^{ijkl}	1.34 ± 0.20^{fghijk}
1.0	–	0.5	–	9.60 ± 0.54^{klmno}	1.46 ± 0.16^{defghij}
1.0	–	1.0	–	6.80 ± 0.44^{opqrs}	1.46 ± 0.05^{defghij}
1.0	–	2.0	–	5.20 ± 1.09^{pqrst}	1.28 ± 0.04^{fghijklmn}
1.0	–	3.0	–	3.80 ± 0.44^{qrst}	1.26 ± 0.05^{ijklmno}
2.0	–	0.5	–	16.00 ± 0.70^{hi}	1.82 ± 0.35^{bcdefgh}
2.0	–	1.0	–	14.40 ± 0.89^{hij}	1.76 ± 0.08^{bcdefghi}
2.0	–	2.0	–	13.20 ± 0.83^{ijkl}	1.30 ± 0.24^{fghijklm}
2.0	–	3.0	–	11.60 ± 0.89^{jklm}	1.26 ± 0.08^{fghijkmno}
3.0	–	0.5	–	13.40 ± 0.89^{ijk}	1.18 ± 0.16^{jklm}
3.0	–	1.0	–	16.20 ± 0.83^{hi}	1.24 ± 0.08^{ijklm}
3.0	–	2.0	–	13.20 ± 0.83^{ijkl}	1.04 ± 0.25^{klm}
3.0	–	3.0	–	12.20 ± 0.83^{ijklm}	1.34 ± 0.11^{ijkl}
0.5	–	–	0.5	9.20 ± 1.64l^{mno}	1.12 ± 0.25^{jklm}
0.5	–	–	1.0	11.80 ± 0.83^{jklm}	1.40 ± 0.45^{ghijk}
0.5	–	–	2.0	5.80 ± 1.30^{nopqrs}	1.76 ± 0.51^{efgh}
0.5	–	–	3.0	5.00 ± 0.70^{pqrst}	1.58 ± 0.25^{fghij}
1.0	–	–	0.5	7.20 ± 0.83^{nopqrs}	1.46 ± 0.18^{fghijk}
1.0	–	–	1.0	4.60 ± 0.54^{qrst}	1.32 ± 0.13^{hijkl}
1.0	–	–	2.0	4.00 ± 0.701^{qrst}	1.32 ± 0.08^{hijkl}
1.0	–	–	3.0	3.60 ± 0.894^{qrst}	1.40 ± 0.12^{ghijk}
2.0	–	–	0.5	4.60 ± 0.54^{qrst}	1.30 ± 0.07^{ijkl}
2.0	–	–	1.0	2.80 ± 0.83^{rst}	1.32 ± 0.08^{ijkl}
2.0	–	–	2.0	2.60 ± 0.893st	0.82 ± 0.083^{mno}
2.0	–	–	3.0	2.40 ± 0.54st	0.4 ± 0.32^{op}
3.0	–	–	0.5	2.80 ± 0.83^{rst}	0.4 ± 0.34^{op}
3.0	–	–	1.0	2.20 ± 0.447st	0.5 ± 0.44^{nop}
3.0	–	–	2.0	1.60 ± 0.54^{t}	0.5 ± 0.52^{nop}
3.0	–	–	3.0	1.60 ± 0.54^{t}	0.4 ± 0.36^{op}

Data shown is the mean of five replicates ± SD. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 3

Effect of GA_3 in combination with cytokinins on multiple shoot induction of *C. deccusata*.

Plant growth regulators (mg/L)					Number of shoot per explant	Shoot length in cm
BA	KIN	TDZ	ZEATIN	GA_3		
0.5	2.0	–	–	0.5	46.80 ± 6.22^{c}	2.90 ± 0.15^{cd}
0.5	2.0	–	–	1.0	100.80 ± 3.2^{a}	6.98 ± 0.66^{a}
0.5	2.0	–	–	2.0	70.80 ± 6.61^{b}	4.38 ± 0.27^{b}
0.5	2.0	–	–	3.0	39.80 ± 8.87^{cd}	4.84 ± 0.58^{b}
0.5	–	1.0	–	0.5	16.20 ± 1.92^{fgh}	0.90 ± 0.12^{h}
0.5	–	1.0	–	1.0	14.40 ± 4.97^{gh}	1.10 ± 0.15^{gh}
0.5	–	1.0	–	2.0	11.60 ± 2.19^{h}	1.60 ± 0.12^{fgh}
0.5	–	1.0	–	3.0	32.80 ± 3.12^{de}	1.26 ± 0.19^{gh}
1.0	–	–	0.5	0.5	47.00 ± 4.37^{c}	2.68 ± 0.50^{cde}
1.0	–	–	0.5	1.0	64.00 ± 1.65^{b}	2.30 ± 0.66^{def}
1.0	–	–	0.5	2.0	25.00 ± 4.18^{ef}	2.04 ± 0.05^{ef}
1.0	–	–	0.5	3.0	27.20 ± 5.06^{ef}	1.66 ± 0.11^{fg}
2.0	2.0	–	–	0.5	18.75 ± 1.50^{fgh}	2.27 ± 0.41^{def}
2.0	2.0	–	–	1.0	10.16 ± 0.98^{h}	2.18 ± 0.34^{def}
2.0	2.0	–	–	2.0	12.80 ± 1.09^{gh}	3.18 ± 0.14^{c}
2.0	2.0	–	–	3.0	17.20 ± 9.85^{fgh}	4.74 ± 1.59^{b}

Data shown is the mean of five replicates ± SD. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

3.1.3. Effect of combination of cytokinin & NAA on multiple shoot induction

The effect of NAA in combination with cytokinin on multiple shoot induction and callus induction was studied. In the present study, combination of cytokinin with NAA produced lower number of shoots due to callus formation and proliferation at the base of shoot clumps. Of various combinations of NAA tested, KIN (3.0 mg/L) + NAA (1.0 mg/L) which produced 82% showed good callusing followed by TDZ (3.0 mg/L) + NAA (1.0 mg/L) (71%) (Table 4). The callus observed in NAA supplemented with cytokinin was fragile turned into compact, green and regenerative in nature with few adventitious shoot buds in the same medium. But the regenerative potential was found to be very low (Figure 2I).

Table 5

Effect of auxins on root induction of *C. deccusata*.

IBA (mg/L)	IAA (mg/L)	NAA (mg/L)	Number of roots per explant	% Of callus induction	Root length (cm)
0.5	–	–	10.40 ± 2.50^{b}	–	3.92 ± 0.57^{c}
1.0	–	–	15.80 ± 0.83^{a}	–	6.86 ± 1.31^{a}
2.0	–	–	10.00 ± 1.00^{bc}	–	3.34 ± 0.67^{c}
3.0	–	–	7.20 ± 0.83^{ef}	–	1.72 ± 0.22^{d}
–	0.5	–	5.80 ± 1.30^{g}	–	1.28 ± 0.10^{d}
–	1.0	–	9.00 ± 0.70^{cd}	–	1.52 ± 0.57^{de}
–	2.0	–	5.60 ± 1.14^{g}	–	1.32 ± 0.13^{e}
–	3.0	–	3.40 ± 0.54^{h}	–	0.54 ± 0.08^{f}
–	–	0.5	4.80 ± 0.83^{g}	72	3.76 ± 0.50^{c}
–	–	1.0	6.20 ± 0.83^{g}	34	3.80 ± 0.83^{c}
–	–	2.0	8.40 ± 0.54^{de}	20	5.60 ± 0.54^{b}
–	–	3.0	5.00 ± 1.22^{g}	13	2.20 ± 0.44^{d}
–	–	MS	0.00 ± 0.00^{i}	–	0.00 ± 0.00^{f}
–	–	½MS	0.00 ± 0.00^{i}	–	0.00 ± 0.00^{f}

Data shown is the mean of five replicates ± SD. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

3.1.4. Effect of auxin on root induction

Among the various auxins tested, IBA proved to be the most effective for root induction (15.80 ± 0.83 root per explant) (Figure 2J). Although NAA and IAA also responded for root induction but number of rooting is poor and roots were thin and delicate (Table 5). However, half strength MS medium containing NAA at 0.5 mg/L showed the highest callus induction (Figure 2H). The healthy plantlets developed on MS + IBA (1.0 mg/L) were removed from the culture tubes and washed thoroughly in sterile distilled water. Then plantlets were treated with Bavistin (1%) for 5 min and it was washed thoroughly with sterile distilled water and transferred to vermiculite (Figure 2L). The plantlets survived 83% without any phenotype changes.

3.2. Antioxidant activity

Plant cell and tissue cultures hold great promise for controlled production of numerous useful secondary

Table 4

Effect of NAA in combination with cytokinins in callus and multiple shoot induction of *C. deccusata*.

PGR	Callus	% of callus	Number of shoots per explant	Shoot length in cm	Number of root per explant	Root length in cm
0.5BAP+1NAA	–	–	11.20 ± 3.34^{a}	1.30 ± 0.40^{abc}	15.80 ± 3.63^{bcd}	0.88 ± 0.43^{bcd}
1BAP+1NAA	G	45	3.40 ± 2.40^{e}	0.66 ± 0.18^{ef}	18.40 ± 5.94^{bc}	0.78 ± 0.08^{cde}
2BAP+1NAA	G	56	4.40 ± 1.34^{de}	1.20 ± 0.36^{abcde}	14.60 ± 1.51^{bcd}	0.70 ± 0.15^{cde}
3BAP+1NAA	G	44	3.60 ± 0.54^{e}	0.82 ± 0.08^{cdef}	13.80 ± 5.21^{cd}	0.80 ± 0.18^{cde}
0.5KIN+1NAA	–	–	7.60 ± 2.88^{bcd}	1.56 ± 0.38^{a}	31.00 ± 9.35^{a}	1.50 ± 0.35^{a}
1KIN+1NAA	G	56	8.00 ± 3.31^{abc}	1.40 ± 0.79^{ab}	14.60 ± 5.31^{bcd}	0.88 ± 0.39^{bcd}
2KIN++1NAA	G	65	5.20 ± 2.77^{cde}	0.70 ± 0.29^{ef}	6.40 ± 2.50^{e}	0.60 ± 0.23^{de}
3KIN++1NAA	G	82	6.20 ± 1.78^{bcde}	0.76 ± 0.45^{cdef}	4.20 ± 2.58^{e}	0.70 ± 0.21^{cde}
0.5TDZ+1NAA	–	–	6.00 ± 1.00^{bcde}	1.16 ± 0.30^{abcde}	8.60 ± 1.14^{d}	0.56 ± 0.24^{de}
1TDZ+1NAA	GY	25	3.20 ± 0.83^{e}	1.30 ± 0.15^{abc}	8.60 ± 1.51^{d}	1.18 ± 0.22^{b}
2TDZ+1NAA	GY	76	3.60 ± 0.89^{e}	0.54 ± 0.15^{f}	2.40 ± 1.14^{e}	0.50 ± 0.25^{e}
3TDZ+1NAA	GY	71	3.40 ± 0.89^{e}	0.94 ± 0.72^{bcdef}	3.40 ± 0.54^{e}	0.46 ± 0.15^{e}
0.52iP+1NAA	–	–	5.60 ± 1.94^{cde}	0.90 ± 0.25^{bcdef}	3.40 ± 1.14^{e}	0.48 ± 0.20^{e}
1 2iP+1NAA	G	56	6.60 ± 2.19^{bcde}	0.68 ± 0.28^{ef}	35.00 ± 12.60^{a}	1.02 ± 0.13^{bc}
2 2iP+1NAA	G	65	9.20 ± 2.28^{ab}	0.72 ± 0.08^{def}	21.60 ± 7.66^{b}	0.44 ± 0.08^{e}
3 2iP+1NAA	G	54	8.20 ± 4.26^{abc}	1.26 ± 0.23^{abcd}	14.60 ± 2.07^{bcd}	0.78 ± 0.32^{cde}

G – Green callus, GY – Green Yellow callus

Data shown is the mean of five replicates ± SD. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

metabolites. *In vitro* cultured cells, organs and regenerated plants synthesize, accumulate and sometimes show many classes of secondary metabolites have been studied in various plant species. The literature further reveals that the regenerating callus have wide use in both basic research and industrial applications. To study the antioxidant activity, we selected those *in vitro* plants and callus which produced the best yield when treated with various plant growth regulators and compared with *in vivo* plant (nature grown). The selected concentration of tissue culturally grown plants was listed in Table 6 used to analyzed for total phenolics content and antioxidant activity by DPPH, ABTS, FRAP and phosphomolybdenum assays.

3.2.1. Determination of total phenol content

The results obtained from the assay were expressed as means standard deviation of triplicate analyses and are presented in Table 6. Highest phenol content (577.77 ± 15.18 mg GAE/g DW) was observed in the methanol extract of callus obtained from half MS containing 0.5 mg/L NAA of *C. decussata* which is also higher than that of methanol extract of *in vivo* plants. A good correlation of total phenol content with total flavonoid content (r^2 = 0.761), ABTS (r^2 = 0.922), Phosphomolybdenum (r^2 = 0.934) and FRAP assay (r^2 = 0.812) was achieved for tested samples. A negative correlation was achieved between total phenol and DPPH scavenging assay which clearly implies that increase in the phenol content which lowers the DPPH radicals (r^2 = −0.866) ($P < 0.05$) (Table 7).

3.2.2. Determination of total flavonoid content

Highest flavonoid contents (179.16 ± 10.92 mg Rutin equivalents/g DW) was observed in the methanol extracts of *in vitro* derived callus obtained from MS containing KIN (2.0 mg/L) + NAA (1.0 mg/L) which is comparatively higher than *in vivo* plant (wild plant). There is a correlation between total flavonoid content with total phenol content (r^2 = 0.761), ABTS (r^2 = 0.777), Phosphomolybdenum (r^2 = 0.802) and FRAP assay (r^2 = 0.543) for tested samples (r^2 = 0.761) (Table 7). The contribution of total flavonoid with DPPH assay was confirmed by their negative correlation because the flavonoid which tends to inhibits the DPPH radicals (r^2 = −0.695).

Table 6

Antioxidant activity of *in vivo* plant and *in vitro* derived plants of *C. deccussata*.

Plant extract	**Total phenol** (mg Gallic acid Equivalents/1 g)	**Total flavonoid** (mg Rutin Equivalents/1 g)	**DPPH** (IC_{50} = μg/ml)	**ABTS** (μM Trolox Equivalents/g)	**Phosphomolybdenum** (mg AAE/1 g)	**FRAP** (mM Fe (II)/1 mg)
In vivo plant	471.99 ± 0.72	165.50 ± 4.94	59.225	10590.24 ± 5.94	964.40 ± 11.08	4791.32 ± 11.40
0.5BAP+1TDZ+1GA_3	210.05 ± 2.29	93.04 ± 7.96	355.663	2111.85 ± 3.48	447.30 ± 20.06	4101.54 ± 60.00
2BAP+2KIN+0.5GA_3	176.23 ± 3.02	129.27 ± 7.39	272.450	2800.34 ± 1.64	392.40 ± 23.76	4554.23 ± 0.71
1BAP+0.5ZEATIN+3GA_3	221.32 ± 12.22	80.00 ± 0.00	261.660	1957.14 ± 5.20	293.60 ± 7.71	4384.02 ± 0.66
0.5BAP+2KIN+3GA_3	169.45 ± 8.38	67.53 ± 4.30	432.890	1191.30 ± 2.08	352.90 ± 13.90	3541.32 ± 0.44
2BAP+2KIN+3GA_3	323.63 ± 42.47	75.65 ± 6.56	90.156	3303.16 ± 5.13	591.80 ± 11.08	4785.12 ± 0.41
0.5BAP+2KIN	161.69 ± 5.10	84.34 ± 4.34	180.932	1902.99 ± 2.53	360.50 ± 11.68	4388.31 ± 0.79
1BAP+0.5ZEATIN+2GA_3	221.32 ± 16.36	100.57 ± 3.92	372.881	1639.97 ± 1.18	385.60 ± 10.20	4052.34 ± 0.95
3BAP+0.5GA_3	234.66 ± 7.64	65.50 ± 6.16	289.077	2119.59 ± 1.23	487.70 ± 20.37	4350.03 ± 0.57
0.5BAP+2KIN+2GA_3	259.87 ± 15.6	106.37 ± 6.58	268.018	1717.33 ± 2.60	445.50 ± 28.15	4493.34 ± 1.76
3BAP	212.12 ± 23.50	124.63 ± 5.65	269.575	1485.65 ± 2.29	46.79 ± 26.38	3432.23 ± 2.30
0.5BAP+2KIN+1GA_3	173.57 ± 5.55	59.71 ± 1.32	329.045	4796.43 ± 6.69	362.60 ± 3.53	2474.67 ± 0.72
1BAP+0.5ZEATIN+1GA_3	227.87 ± 3.58	65.21 ± 3.98	333.582	1910.07 ± 5.04	334.60 ± 47.13	4241.35 ± 1.76
2BAP+1NAA	388.88 ± 8.53	123.33 ± 1.44	41.790	10764.78 ± 4.53	1029.20 ± 11.45	6614.45 ± 1.92
12iP+1NAA	440.17 ± 16.23	118.05 ± 4.58	93.170	10766.65 ± 7.65	913.40 ± 137.31	8999.35 ± 1.39
2KIN+1NAA	415.81 ± 15.29	179.16 ± 10.92	139.250	10593.54 ± 5.68	1114.60 ± 5.35	7314.24 ± 40.23
3BAP+1NAA	432.90 ± 4.12	152.5 ± 4.40	28.110	10674.33 ± 6.58	120.40 ± 53.28	4023.51 ± 1.35
1BAP+1NAA	393.16 ± 17.31	135.83 ± 0.83	56.610	11334.32 ± 2.42	1254.90 ± 4.41	9226.24 ± 2.67
32iP+1NAA	407.69 ± 5.58	152.5 ± 6.29	170.040	9099.32 ± 1.27	936.84 ± 48.23	8051.24 ± 3.13
22ip+1NAA	341.02 ± 2.22	116.94 ± 4.11	105.558	9000.33 ± 2.43	102.63 ± 9.28	5272.24 ± 3.26
1KIN+1NAA	452.99 ± 9.09	156.94 ± 2.54	23.290	12004.12 ± 4.36	1157.8 ± 72.05	10479.43 ± 2.19
2TDZ+1NAA	391.02 ± 5.87	155.55 ± 5.09	104.900	9043.07 ± 3.25	981.8 ± 37.72	6684.53 ± 0.99
½MS+0.5NAA	577.77 ± 15.18	137.22 ± 0.48	20.880	12234.13 ± 4.20	1315.7 ± 25.84	13687.51 ± 1.95
3TDZ + 1NAA	481.62 ± 26.34	159.16 ± 24.59	40.160	10894.43 ± 2.42	1266.6 ± 53.53	11901.23 ± 0.84

Data shown is the mean of three replicates ± SD. Values are mean of triplicate determination (n = 3)±SD; Statistically significant at $P < 0.05$.

Table 7

Correlation between phenolics, flavonoids, and different antioxidant parameters of *in vitro* regenerated plants and wild-grown plants' methanol extract of *C. decussate*.

Parameter	TPC	TFC	DPPH	ABTS	Phosphomolybdenum assay	FRAP
TPC	1					
TFC	0.761[a]	1				
DPPH	−0.866[a]	−0.695[a]	1			
ABTS	0.922[a]	0.777[a]	−0.870[a]	1		
Phosphomolybdenum assay	0.934[a]	0.802[a]	−0.880[a]	0.961[a]	1	
FRAP	0.812[a]	0.543[a]	−0.730[a]	0.746[a]	0.796[a]	1

[a] Correlation is significant at the 0.01 level (2-tailed). TPC- Total phenol content; TFC- Total Flavonoid content; FRAP- ferric reducing antioxidant power assay (Pearson correlation).

3.2.3. DPPH scavenging assay

Among all the extract of *in vitro* derived plants and callus obtained from various PGR containing media and wild-grown plants (Table 6), highest DPPH radical scavenging activity, *i.e.* lowest IC_{50} value, was observed in methanol extract of callus derived from half MS medium supplemented with 0.5 mg/L of NAA (IC_{50} = 20.88 µg/mL). This was followed by the methanol extracts of callus from 1.0 mg/L KIN+1 mg/L NAA (IC_{50} = 23.29 µg/mL). The IC_{50} values of callus extract from 0.5 mg/L NAA was lower than all the extracts of *in vitro* and *in vivo* plant (wild-grown plant) extracts.

3.2.4. ABTS assay

The ABTS radical scavenging activity of methanol callus extract obtained from *in vitro* from MS medium+0.5 mg/L NAA has highest ABTS radical scavenging activity was observed (12 234.13 ± 43.20 µM TEAC/g DW) followed by methanol extract of *in vitro* callus from MS medium containing KIN at 1.0 mg/L + NAA at 1.0 mg/L (12 004.12 ± 2.81 µM TEAC/g DW) (Table 6). The ABTS assay of the *Canscora deccusata* (*C. deccusata*) extracts calculated as Trolox equivalents/g extracts (TEAC/g).

3.2.5. Phosphomolybdenum assay

Highest reducing power showed in methanol extract of *in vitro* callus from MS medium containing 0.5 mg/L NAA (1 315.78 mg AAE/g) which was followed by MS medium containing TDZ (3.0 mg/L) +NAA (1.0 mg/L) (1 266.66 mg AAE/g) (Table 6). We observed that the total phenolic content have more ability to reduce Mo^{+} ion than the total flavonoid content of *C. decussata*. The phosphomolybdenum assay of the *C. deccusata* extracts calculated as ascorbic acid equivalents/g extracts (AAE/g).

3.2.6. FRAP assay

The ferric ion-reducing activities of *C. decussata* extracts is calculated as µmol Fe(II)/g extract. Among the various samples tested, methanol extract of callus derived from MS medium containing 0.5 mg/L NAA of *C. decussata* showed stronger Ferric reducing power (13 687.51 ± 1.95 µmol Fe(II)/g extract) which was consistent with the results obtained from the DPPH and ABTS assays (Table 6). The FRAP assay of the *C. deccusata* extracts calculated as Fe ion (II) equivalents/g extracts.

3.2.7. Correlation analysis

A good negative correlation was observed for all other assays such as ABTS (r^2 = −0.870), phosphomolybdenum assay (r^2 = −0.880) and FRAP assay (r^2 = −0.730) (Table 7). The inhibition of DPPH radicals tends to have the negative correlation with all the assays tested. Correlations among the ABTS, FRAP, and phosphomolybdenum assays were positively high and ranged between 0.74 and 0.96: the highest correlation was between ABTS and phosphomolybdenum (0.97) and the lowest correlation was between ABTS and FRAP (0.746) ($P < 0.05$) (Table 7). From the correlation analysis, it is evident that the phenolics and flavonoids in the methanolic extract of callus derived from MS medium containing 0.5 mg/L NAA were responsible for highest antioxidant activity in all assays tested (Table 7). On the basis of the current findings, we conclude that MS medium supplemented with 0.5 mg/L NAA yields high total phenol content as well as higher antioxidant activity.

4. Discussion

Recently, Gaikwad *et al.* [15] and Sethiya *et al.* [16] were studied *in vitro* propagation and pharmacological activities of *C. decussata* Schult., respectively. But, the critical examination on their figures which clearly shows it is only the species of *Canscora diffusa* (*C. diffusa)* resembles in vegetative forms as *C. decussata* and it might have been misidentified as *C. decussata* (C.f. Figure 1). The growth habit of the plant species of *C. decussata* showed in Figure 1. From this result, we can conclude that our study is the first report on micropropagation and antioxidant activity of *C. decussata*. The objective of this study is to develop an effective *in vitro* regeneration protocol and to evaluate the antioxidant of both wild-grown and *in vitro* regenerated plants of *C. decussata*.

MS medium supplemented with BAP (2 mg/L) was effective for shoot multiplication in nodal segments of *C. decussata*. The effect of BAP on multiple shoot formation has also been studied in various medicinal plant species such as *Ceropegia noorjahaniae* (*C. noorjahaniae*) [17] *Gymnema sylvestre* (*G. sylvestre*) [18] and *Stevia rebaudiana* (*S. rebaudiana*) [19]. Any further increase in concentration more than optimum level of all cytokinins tested did not improve any parameters of shoot multiplication. In this research, application of cytokinin such as BAP in combination with KIN resulted in high-frequency shoot regeneration in *C. decussata*. The synergistic effect of BAP and KIN in promoting shoot multiplication has been reported earlier in *Swertia chirata* (*S. chirata*) [20,21], *Stevia rebaudiana* (*S. rebaudiana*) [22] and *Achryrantes aspera* (*A. aspera*) [23]. *In vitro* flowering was also observed when the culture was stored for longer period on the same medium (Figure 2K). Addition of GA_3, not only increases shoot elongation but also increases the shoot multiplication. Similarly results were obtained in *Gentiana triflora* (G. *triflora*) [24], *S. chirata* [21], *Enicostema axillare* (E. *axillare*) [25] & *G. sylvestre* [26].

NAA play an important role in callus induction. NAA induced callus when combined with all cytokinin containing medium. Similar, synergistic effect of auxins with cytokinins in callus induction was reported by in *Salvia officinalis* (*S. officinalis*) [27], *Salvadora oleoides* (*S. oleoides*) [28] and *Eustoma grandiflorium* (*E. grandiflorium*) [29]. Exogenous application of cytokinin and auxin in a specific ratio may help to maintain the required ratio which favoured callus production. The replication and proliferation of callus was due to the essence of NAA, because this hormone belongs to auxins groups and these groups of hormones usually cause the cell elongation, tissue swelling, cellular division (callus formation), adventitious roots formation, prevention from adventitious and adverse branches and often embryogenesis in suspension cultures [30]. Superiority of NAA for callus induction has also been reported in different plant species, viz., in *Erigeron breviscapus* (*E. breviscapus)* [31] *Rosmarinus officinalis* (*R. officinalis*) [32] and *E. grandiflorium* [29].

There was a clear difference in rooting response of PGR-treated and untreated regenerated shoots of *C. decussata*. In the presence of auxin, regenerated shoots rooted earlier and had a much higher rooting rate than untreated shoots. IBA is a common auxin used for inducing rooting in several Gentianaceae plant species in *S. chirata* [20] and *Gentianella austriaca* [33]. Likewise, IBA has been shown to be very effective in root induction as in various cases including *Garcinia indica*

(*C. indica*) [34], *Ceropegia noorjahaniae* (*C. noorjahaniae*) [17] and *Terminalia arjuna* [35]. Similar results were achieved in *Swertia corymbosa* (*S. corymbosa*) [36] & *Eustoma grandiflorum* [37]. The effectiveness of IBA in root formation may be due to its easier uptake/transport, constancy greater than other auxins, and successive gene activation.

Phenols are compounds that have the ability to destroy radicals because they contain hydroxyl groups [38]. These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and form stable phenoxyl radicals; hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of phenolic compounds is very important in order to determine the antioxidant capacity of plant extracts [39–41]. Our results are in agreement with a previous report where a positive correlation between high Total phenol content and Total flavonoid content and antioxidant activities in *Artemisia absinthium* L. (*A. absinthium)* [42,43]. The antioxidant potential in various medicinal plants has been shown to be mainly due to phenolic compounds [44–47]. The results imply that both phenol and flavonoid content contributed in all the antioxidant assays tested.

The DPPH method is a preferred method because it is fast, easy and reliable and does not require a special reaction and device. DPPH is a stable, synthetic radical that does not disintegrate in water, methanol, or ethanol. The free radical scavenging activities of extracts depend on the ability of antioxidant compounds to lose hydrogen and the structural conformation of these components [48,49]. The IC_{50} values of callus extract from 0.5 mg/L NAA was lower than all the extracts of *in vitro* and *in vivo* plant (wild-grown plant) extracts. This shows that NAA played an important role for the antioxidant activity of *C. decussata* in *in vitro* cultures.

The ABTS radical cation decolourization assay is another method commonly used to assess antioxidant activity. ABTS free radical on incubation with sodium persulfate forms ABTS cation, which is deep blue in colour and is highly reactive towards antioxidants. When mixed with an antioxidant, an electron is donated to the ABTS radicals which is converted to a non-radical form. Decrease in colour intensity indicates the reduction of the ABTS radical. ABTS assay is consistent with the results of DPPH where callus derived from MS medium + NAA at 0.5 mg/L shows highest activity (Table 7).

The phosphomolybdenum assay is successfully used to quantify vitamin E in seed, and being simple and independent of other antioxidant assays commonly employed, it was decided to extend its application to plant extract [14]. We compare and evaluated for the capacity to reduce Mo (VI) to Mo (V), a green phosphate by the antioxidant compound present in the samples. This reduction ability was expressed in ascorbic acid equivalents (AAE). The FRAP assay mainly depends on the reducing capacity of Fe^{3+}–Fe^{2+} conversion and serves as a significant indicator of its potential antioxidant activity. The antioxidant activities have been attributed to various reactions, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continuous proton abstraction and radical scavenging activity [50]. The FRAP is often used as an indicator of phenolic antioxidant activity. The antioxidant potential of sample was estimated by their abilities to reduce Fe (III)-TPTZ to Fe (II)-TPTZ [13]. From Pearson correlation coefficient test, we can confirm the total phenol and flavonoid content were responsible for the antioxidant activity of all the assays. There are studies in the literature that report a positive correlation between antioxidant activity and the quantity of phenolic compounds [49,50].

Callus culture is very useful to obtain commercially important secondary metabolites. The potential of *in vitro* plant culture systems for the production of an enormous variety of antioxidant compounds has been recognized. Addition of NAA had stimulatory effect on the level of flavonoids and total phenolics in the majority of the treatments. This may be due to the induction of callus in NAA added medium. Earlier studies have been undertaken on the investigations of total phenolic content in callus culture of various medicinal plants. Similarly, phenolics associated enhanced antioxidant activities over wild plants have been reported for the callus culture of *Habenaria edgeworthii* (*H. edgeworthii)* [51] and cell suspension and *in vitro* shoot cultures of *Ruta graveolens* (*R. graveolens*) [52].

In *in vitro* cultures, especially after the addition of NAA (0.5 mg/L) to the medium, those total phenolics and antioxidant activity was significantly high compared to field-grown plants. This may be due to the presence of NAA which induces high stress level which tends to accumulated more phenolics by producing callus in *in vitro* condition. Stress conditions during *in vitro* cultivation may have stimulated polyphenol production, and plant growth regulator cytokins and auxins might have been responsible. NAA regenerated callus proved to be better for the accumulation of secondary metabolite. Therefore, the protocol developed in the present study can be efficiently used for the large-scale production of secondary metabolites in pharmaceutical industries.

Although the previous study on *C. decussata* of Gaikawad *et al*. 2015 has made a misidentification of *C. diffusa* as *C. decussata*. By this, the present study is the first report on efficient rapid regeneration protocol for *C. decussata*. Due to over exploitation of natural populations and difficulty in the cultivation of *C. decussata*, it become threatened and going to be extinct in few years. The protocol standardized in the present study enabled high rate of mass multiplication and could be applied for pharmaceutical industries for isolation of selective bioactive compounds. The *in vitro* callus derived from half strength MS medium supplemented with NAA (0.5 mg/L) has a stronger antioxidant activity compared to field-grown plants and could be used for the extraction of bioactive compounds for large-scale production in the field of pharmacy and medicine without disturbing the natural habitat of this threatened plant sps. The *in vitro* produced bioactive compounds by callus culture are of medically huge interest is a viable alternative in comparison to traditional methods, being able to exceed the productivity of *in situ* plant. Further investigation on the phytochemistry to these calli is of great curiosity in order to elucidate which molecules are responsible for the higher antioxidant activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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