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Full Length Research Paper

In vitro regeneration, flowering, and cell culture of *Centaurea* species

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This study was conducted to establish a protocol for *in vitro* flowering of *Centaurea cyanus* and cell cultures of *Centaurea montana*. In four weeks, 50 to 60 adventitious shoots developed on leaf explants cultured in MS medium supplemented with 2.0 mg/L benzylaminopurine (BAP) and 0.1 mg/L indole-3-acetic acid (IAA). *In vitro* flowering and seed set occurred in *C. cyanus* when the shoots were incubated on MS basal medium supplemented with B5 vitamins, 100 mg/L myo-inositol and 30 g/L sucrose for 4 weeks under 16 h photoperiod. Young leaves of *C. montana* cultured on MS medium supplemented with 1.0 to 6.0 mg/L 2, 4- dichloroxypenoxyacetic acid (2, 4-D) alone or in combination with 0.5 mg/L BAP generated callus. Liquid MS medium containing 2.0 mg/L 2, 4-D produced greater fresh weight (FW), dry weight (DW) and packed cell volume (PCV) compared to MS medium with 1 mg/L 2, 4-D in a 30 day culture cycle. The results indicate that MS medium modified with appropriate phytohormones can be used to achieve efficient shoot regeneration and *in vitro* flowering of *C. cyanus* and cell cultures of *C. montana*.

Key words: *Centaurea cyanus, Centaurea montana*, cell and tissue culture, *in vitro* flowering.

INTRODUCTION

Centaurea L. is a large genus which comprised of several species (spp.), many of which are used in folk medicine (Nacer et al., 2006). The presence of 400 to 700 species in this genus makes it one of the biggest genera of the family (Dittrich, 1977; Gracia-Jacas et al., 2001; Wagenitiz and Hellwig, 1996). Several bioactive chemicals have been isolated and purified from different species of *Centaurea*. These isolates include, but are not limited to, sesquiterpene lactones (Bruno et al., 1996; Youssef, 1998), volatile constituents (Lazari et al., 2000), essential oils (Dural et al., 2003), flavanoid C-glycosides and other biologically active constituents (Ribeiro et al., 2002). Several isolates from *Centaurea* spp. have been shown to exhibit anti-inflammatory and immunological effects (Garbacki et al., 1999), cytotoxic/cytostatic effects (Koukoulista et al., 2002), and antioxidant (Kumarasamy et al., 2002) and antibacterial activities (Kumarasamy et al., 2003).

Several medicinal plants have become threatened due to climate change associated with global warming, degradation of their habitat, and their over exploitation from nature/anthropogenic pressure. Proper protection and management strategies are required for the conservation of these species. Tissue culture, a powerful tool for the rapid cloning of plants from a limited supply of explants, can assist in conservation of plant germplasm and facilitate their *in vitro* preservation (Josekutty, 2006; Tripathi, 2008).

Many plant secondary metabolites are high value, bioactive chemicals possessing structural and chemical complexities that make their artificial synthesis very difficult and expensive (Rout et al., 2000). Callus and cell suspension cultures are useful for the production of

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Abbreviations: BAP, 6-Benzylaminopurine; **Kn,** kinetin; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **2, 4-D,** 2, 4-dichlorophenoxyacetic acid;, **TDZ,** thidiazuron; **GA_3,** gibberellic acid 3.

Table 1. Frequency of callus induction on MS media containing different hormone concentrations.

Plant hormones (mg/L)	Responsive explants (%)	Callus
0	0	0
2,4-D 1.0	75	2
2,4-D 2.0	45	1
2,4-D 3.0	20	1
2,4-D 4.0	12.5	1
2,4-D 5.0	15	1
2,4-D 6.0	12	1
2,4-D 3.0 BAP 0.5	70	3
2,4-D 4.0 BAP 0.5	38.34	2
2,4-D 5.0 BAP 0.5	30	1
2,4-D 6.0 BAP 0.5	28	1

*Callus: 0= no callus, 1= very little callus, 2=proliferating callus, 3=fast growing callus.

bioactive chemicals *in vitro,* for cell line selection to enhance alkaloid production (Josekutty, 1998), for genetic engineering using gene targeting, and for cryopreservation of genetic resources (Kim et al., 2003). Plant callus and cell culture can be used to biosynthesize and produce secondary metabolites *in vitro* (Josekutty, 1998; Zhou and Wu, 2006). Flower-heads of *Centaurea cyanus* are used in European traditional medicine, for example, in the treatment of minor ocular inflammation (Bruneton, 1995). Anti-inflammatory and immunological effects of *C. cyanus* flower extract have been experimentally verified by Garbacki et al. (1999). Gonnet (1996) demonstrated that *Centaurea montana* is a source of major flavonoid glycosides that could be used in different pharmaco-medicinal assays. Montamine, a unique dimeric indole alkaloid extracted from the seeds of *C. montana* showed cytotoxic activity against CaCo-2 colon cancer cells (Shoeb et al., 2006).

Therefore, the present study was aimed towards developing an efficient protocol for *in vitro* shoot regeneration and flowering in *C. cyanus* to ensure year-round availability of its medicinal flowers. This study also aimed to optimize media and culture conditions for callus and cell cultures of *C. montana* to produce bioactive chemicals *in vitro.*

MATERIALS AND METHODS

Chemicals and supplies

All chemicals and supplies (tissue culture grade) used in this study were obtained from Phytotechnology Laboratories, USA.

Plant material and decontamination of explants

Seeds of *C. montana* and *C. cyanus* were obtained from VIS seeds company, California, USA. They were germinated at 20°C in the greenhouse. Young, fully opened leaves from 4.0 week old seedlings were cleaned with soap and running tap water. The clean leaves were surface sterilized with dilute Clorox® (0.1% sodium hypochlorite solution) for eight minutes with continuous agitation in a laminar flow hood. Surface sterilized leaves were rinsed four times with sterile MilliQ water, blot dried on sterile filter papers, and cultured in 100 × 15 mm Petri plates.

Medium constituents and culture conditions

MS medium (Murashige and Skoog, 1962) containing different concentrations of plant growth regulators were used for shoot induction and further multiplications. A combination of 20 g/L sucrose and 7.0 g/L agar was common to all of the semisolid media except for the *in vitro* flowering medium. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. Ten explants per plate (with explants being 0.5 to 1.0 cm leaf segments) were plated with the abaxial side in contact with the medium. All cultures were incubated at 25 ± 2°C under 16 h photoperiod and irradiance of 30 µmol $m^{-2} s^{-1}$ provided by fluorescent lights unless stated other-wise.

Direct shoot regeneration and *in vitro* flowering of *C. cyanus*

Direct shoot regeneration from leaf explants of *C. cyanus* was carried out on MS medium supplemented with 2.0 mg/L BAP and 0.1 mg/L IAA following the protocol developed for regeneration of *C. montana* (Alaiwi et al., 2011). The leaf explants were subcultured biweekly on to fresh medium for sustained development and adventitious shoot regeneration. These *in vitro* shoots were transferred to a hormone free MS medium containing 30 g/L sucrose, B5 vitamins (Gamborg et al., 1968) and 100 mg/L myo-inositol and were incubated under 16/8 h light/dark cycle for two weeks to induce *in vitro* flowering (bolting). The bolted shoots were maintained on this medium for a further 4 weeks to effect blooming and seed set.

Callus and cell culture of *C. montana*

Variants of MS medium containing 1.0 to 6.0 mg/L 2, 4-D alone or in combination with BAP (0.5 mg/L) were used to induce callus from cultured leaf explants of *C. montana* (Table 1). The leaf explants were incubated either in light or dark to induce callus. The percentage of explants producing callus was calculated by dividing the number of explants producing callus by the fifth week of incubation with the total number of cultured explants and multiplying by 100. The cultures were subcultured onto fresh medium every two weeks to sustain growth. Quality of the generated callus was ranked based on a visual assessment. Friable calli were transferred to liquid MS

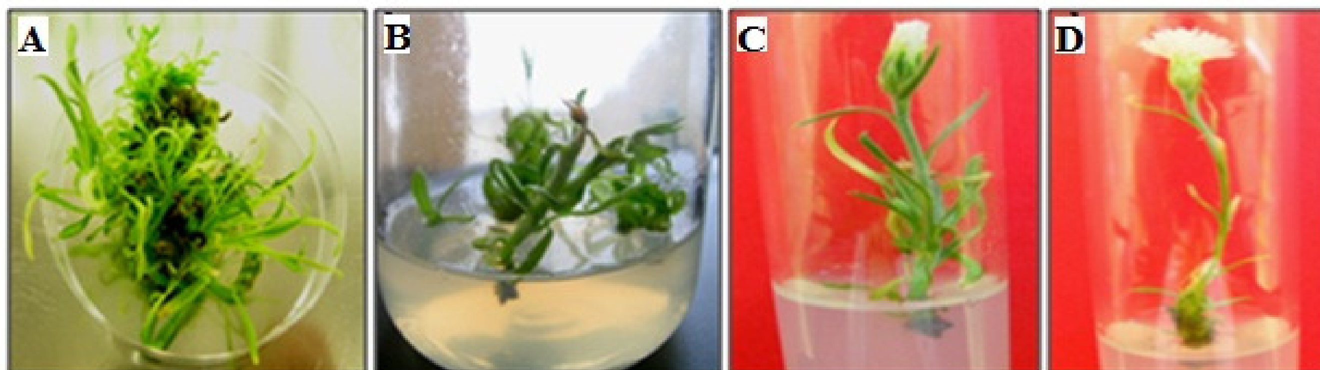

Figure 1. (A) *In vitro* flowering of *C. Cyanus,*(B) shoot separation and flower bud induction, (C) and (D) blooming at maturation.

medium supplemented with 1.0 and 2.0 mg/L 2, 4-D to initiate and maintain cell culture. Effects of 1.0 and 2.0 mg/L 2, 4-D on the growth kinetics of the cell culture were studied and growth parameters like packed cell volume (PCV), fresh weight (FW) and dry weight (DW) through the lag phase, log phase and stationary phase were recorded.

Statistical analysis

Each treatment consisted of at least 30 explants for shoot and callus induction experiments. The data was analyzed with Microsoft Excel / XL STAT by comparing variance and means for statistically significant differences using Duncan's multiple-range test at 95% confidence level ($p < 0.05$).

RESULTS AND DISCUSSION

Direct shoot regeneration of *C. cyanus*

Young leaf explants of *C. cyanus* cultured on MS medium containing 2.0 mg/L BAP and 0.1 mg/LIAA for four weeks regenerated 50 to 60 shoots per explant. Most of these shoots developed into proper, elongated shoots (≥ 10 cm) in four weeks following a subculture at the end of the second week into fresh medium of the same composition (Figure 1A).

MS medium has been the favored medium for tissue culture of *Centaurea* spp(Alaiwi et al., 2011; Cuenca et al., 1999; Cuenca and Amo-Marco, 2000; Kurt and Erdag, 2009; Mallón et al., 2010). Cuenca et al. (1999) reported micropropagation of *Centaurea paui* and *Centaurea spachii* respectively from inflorescence stem explants. They recorded the induction of 4.8 ± 0.5 shoots per explant using MS media supplemented with BAP after four weeks in culture. In addition, micropropagation of *Centaurea zeybekii* (Kurt and Erdag, 2009) and *Centaurea ultreiae* (Mallón et al., 2010) was demonstrated using MS medium supplemented with BAP. These reports suggest that BAP is the preferred cytokinin to generate multiple shoots in *Centaurea* cultures although at much lower frequency as compared to our results with *C. montana* (Alaiwi et al., 2011) and *C. cyanus*. Higher rates of shoot regeneration obtained in our experiments may be due to the synergistic effects of using a combination of BAP (2.0 mg/L) and IAA (0.1 mg/L).

In vitro flowering of *C. cyanus*

Adventitious shoots regenerated on MS medium supplemented with 2.0 mg/L BAP and 0.1 mg/LIAA were used in the *in vitro* flowering study. Bolting occurred when isolated, large shoots (≥10 cm) were incubated on hormone free MS medium supplemented with B5 vitamins, 30 g/L sucrose and 100 mg/L myo-inositol under 16 h photoperiod for two weeks. Blooming and seed set occurred when the bolted shoots were further incubated in the same medium for 4 to 6 weeks (Figure 1B to D). Although, *in vitro* flowering of plants is not a new phenomenon (John and Nadgauda, 1999), it has not been reported, to our knowledge, in any species of *Centaurea*. Natural flowering is a complex process that is induced by light and temperature cues from nature (Ausín et al., 2005). These natural cues cause changes in the hormonal regime of the plants that lead to their flowering (Bernier and Périlleux, 2005; Heggie and Halliday, 2005). Normally, *Centaurea* spp. will only flower during the long, warm days of summer. *In vitro* flowering in *C. cyanus* is significant because the medicinally important part of this species is the flower.

In vitro flowering has been reported in many crops and a process for inducing flowers and/or seeds under *in vitro* conditions has been reported previously by two of the authors on this paper in several crop species such as Festuca-Loilum, maize and soybean (Goldman et al., 2010; Rudrabhatla and Goldman, 2009).

Culture medium (especially sucrose concentration), hormones (Kn, BAP, Paclobutrazol, GA_3, TDZ), and the photoperiod are key variables reported to influence flowering *in vitro*. Singh et al. (2006), reported 2 to 3 mg/L Kn and photoperiod of 12 to 16 h as important factors conducive for *in vitro* flowering in Kinnow mandarin.

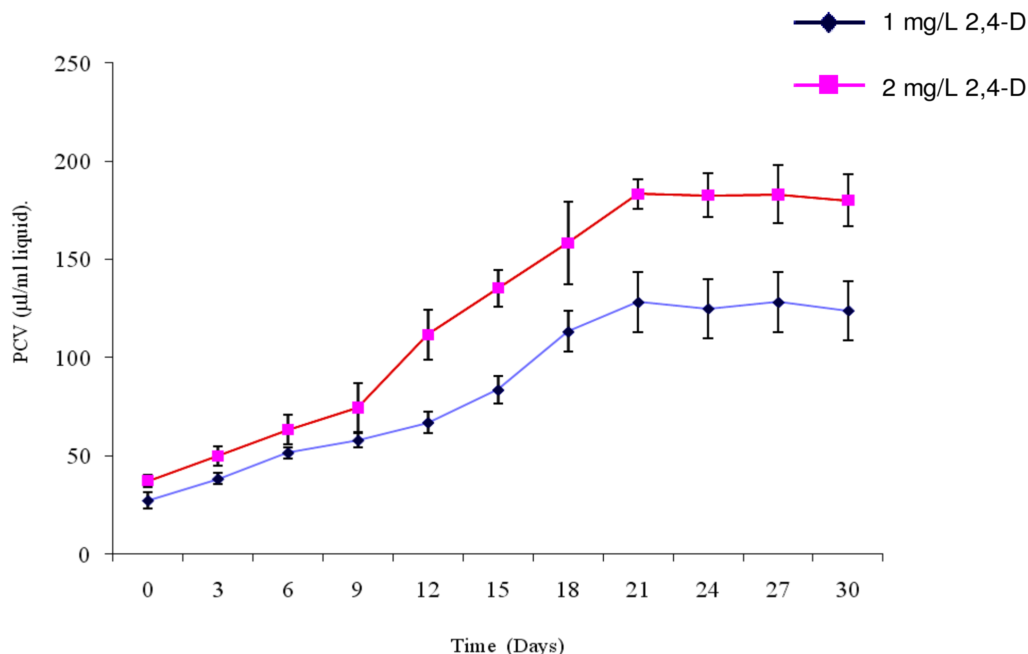

Figure 2A. Effect of 2, 4-D concentration on *C. montana* cell suspension culture growth characteristics (PCV) over a period of 30 days. Error bars indicate ±SD.

Paclobutrazol (0.25 to 0.50 mg/L) in the medium induced *in vitro* flowering in *Dendrobium* shoot cultures (Te-chato et al., 2009). Britto et al. (2003) reported that *Ceropegia bulbosa* cultures incubated on MS medium supplemented with 0.5 mg/L BAP and 1.0 mg/L NAA exhibited *in vitro* flowering but the same species produced only micro-tubers when maintained on MS medium containing Kn and IBA. High levels of sucrose (30 g/L) in the medium favored *in vitro* flowering in mandarin (Singh et al., 2006), gentian (Zhang and Leung, 2000), and bamboo (Lin et al., 2004). Jana and Shekhawat (2010) found that plant growth regulators adenine sulfate and carbohydrates influence *in vitro* flowering of *Anethum graveolens*.

Our results are different from the earlier observations because *in vitro* flowering occurred when the shoots of *C. cyanus* were transferred from a BAP containing medium to a hormone-free medium. Our observations are similar to *in vitro* flowering of cassava observed by Tang et al. (1983). Tang et al. (1983) noticed *in vitro* flowering in cassava when the shoots were maintained for 17 to 21 days on a culture medium supplemented with 0.125 mg/L BAP, 0.09 mg/L IAA, and 0.17 mg/L GA_3 and were further incubated on a hormone free medium containing 100 mg/L myo-inositol for 2 to 4 weeks. In our present study, cultures on a medium containing BAP and IAA had been maintained under a 16 h photoperiod before transferring to the hormone-free MS medium supplemented with myo-inositol and higher concentrations (30 g/L) of sucrose. We think that the flower induction may have taken place while the shoots were incubated in a medium containing BAP under long day conditions. In addition, myo-inositol and elevated levels of sucrose may also have assisted in the induction of *in vitro* flowering of *C. cyanus*. Further development did occur during the incubation on the basal medium under long day conditions.

This *in vitro* flowering system in *Centaurea* allows for generation of clean flowers intended for medicinal use in required quantities throughout the year and unaffected by the seasons. In addition, this system can also be used to study the physiological and molecular basis of the complex process of flowering.

Callus and cell culture of *C. montana*

75% of the *C. montana* leaf explants cultured on MS medium supplemented with 1.0 mg/L 2, 4-D produced callus compared to 45% of explants on a medium containing 2.0 mg/L 2, 4-D. Medium containing 3.0 mg/L 2,4-D and 0.5 mg/L BAP was more effective in producing callus from leaf explants when compared to medium containing 3.0 mg/L 2, 4-D alone (Table 1). However, the medium containing 3.0 mg/L 2, 4-D along with 0.5 mg/L BAP generated non-friable callus. Therefore, callus generated on MS medium supplemented with 1.0 mg/L 2, 4-D were serially subcultured onto MS medium containing 2.0 mg/L 2, 4-D to obtain friable callus. The friable callus obtained after three consecutive subcultures in MS media containing 2.0 mg/L 2, 4-D was used to initiate the cell culture. Cell cultures in MS medium containing 2.0 mg/L 2, 4-D yielded greater PCV, FW, and DW of cells compared to the cell cultures grown in MS medium containing 1.0 mg/L 2, 4-D (Figure 2 A to C).

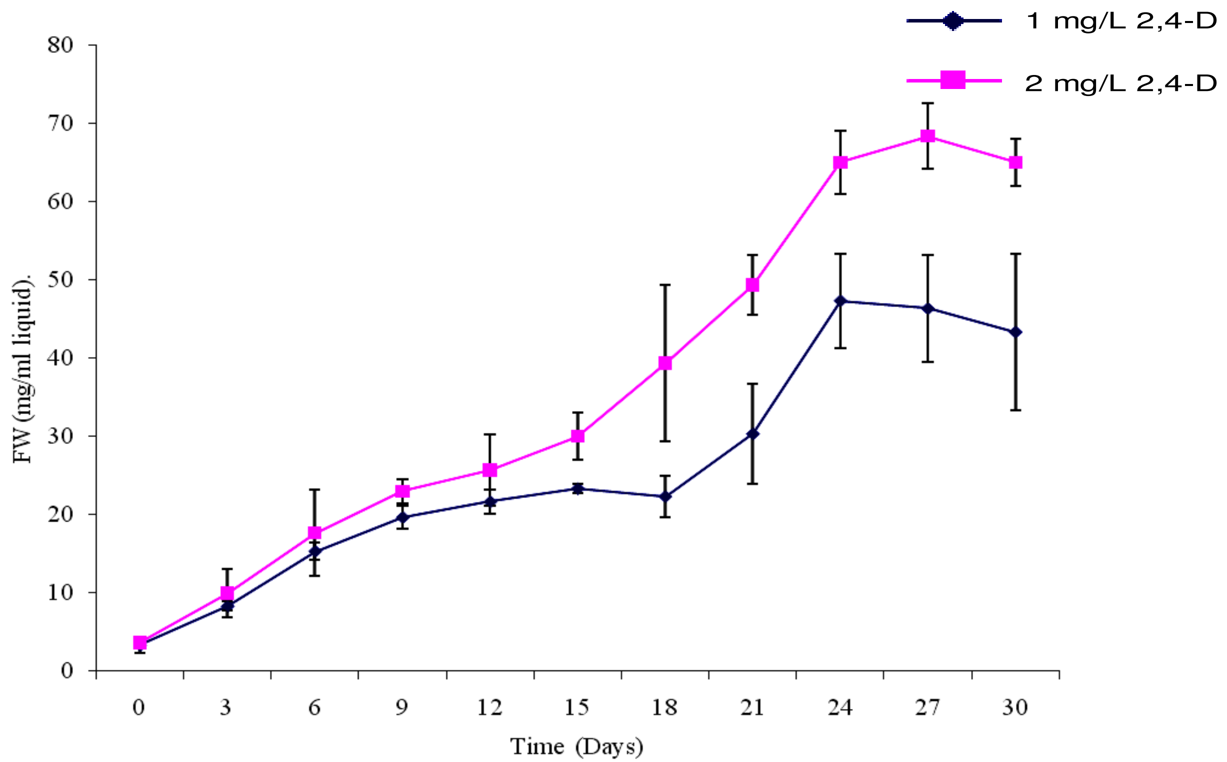

Figure 2B. Effect of 2, 4-D concentration on *C. montana* cell suspension culture growth characteristics (Fresh weight) over a period of 30 days. Error bars indicate ± SD.

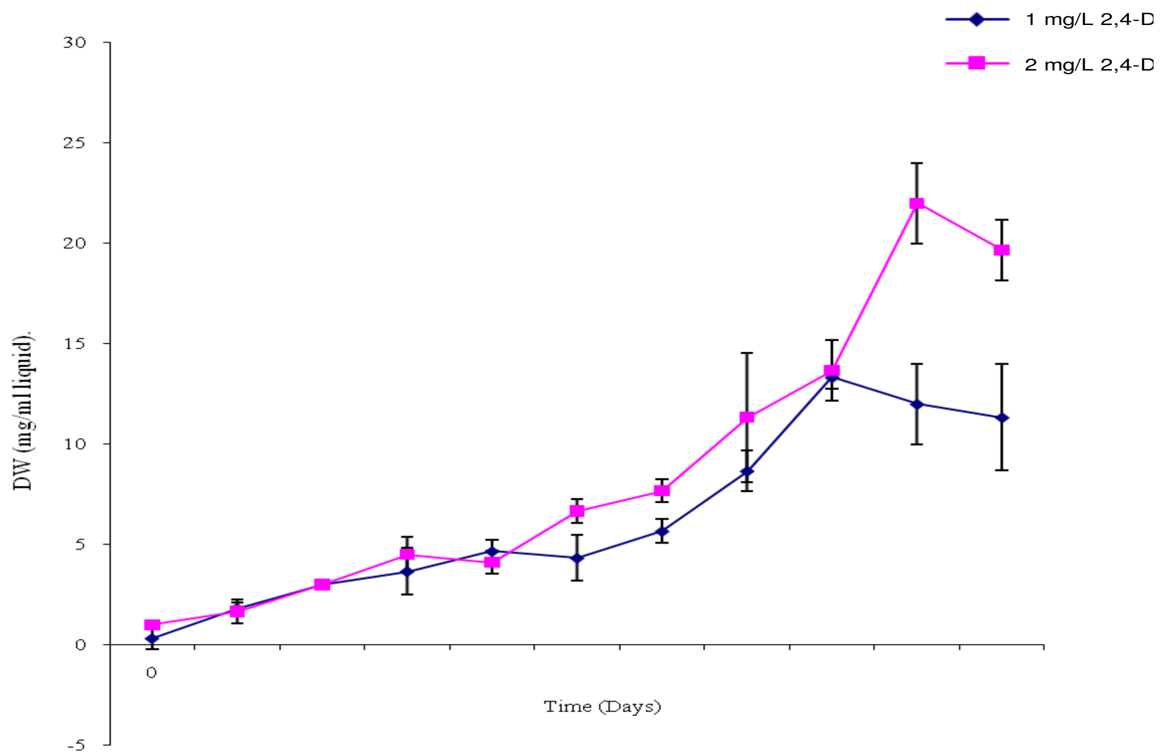

Figure 2C. Effect of 2, 4-D concentration on *C. montana* cell suspension culture growth characteristics (Dry weight) over a period of 30 days. Error bars indicate ± SD.

Vidal et al. (2004) studied callus culture of *C. solstitialis* using three modifications of MS medium, namely MS-CAL, MS-CAR and MS-T1. MS-CAL contained 0.5 mg/L Kn and 2iP along with 0.1 mg/L of NAA. MS-CAR had 2.0 mg/L 2, 4-D. MS-T1 contained 2.0 mg/L NAA and 2, 4-D along with 0.2 mg/L 2iP. Vidal et al. (2004) reported that medium MS-CAL, containing a combination of cytokinins and an auxin (Kn, 2iP or NAA), favored callus growth when compared to medium MS-CAR that contained 2.0 mg/L 2, 4-D or medium MS-T1 that included NAA, 2,4-D (2.0 mg/L) and 0.2 mg/L 2iP. Similar to Vidal et al. (2004), we observed that MS medium supplemented with a combination of auxin and cytokinin (2, 4-D and BAP) produced faster growing callus from cultured leaf discs of *C. montana* compared to slower growing callus obtained from MS medium supplemented with 2, 4-D alone. We therefore decided to generate callus cultures on MS medium containing 2.0 mg/L 2, 4-D alone since this medium generated friable callus suitable for initiating cell cultures. This cell culture system could be used to produce valuable secondary metabolites such as montamine.

Conclusions

Methods for *in vitro* flowering of *C. cyanus* as well as callus and cell culture of *C. montana* were developed. These protocols may be used to generate clean flowers of *C. cyanus* throughout the year for medicinal purposes and to produce bioactive phytochemicals from callus and cell cultures of *C. montana.*

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