Effect of plant hormones and zinc sulphate on rooting and callus induction in in vitro propagated Coscinium fenestratum (Gaertn.) Colebr. stem and their role in estimation of secondary metabolites

Kuntal Das, Raman Dang*, Gokul Sivaraman**, Rajasekharan Punathil Ellath***, D. Roopa**** and B. Subbaya*****

Department of Pharmacognosy and Natural Product Chemistry, Krupanidhi College of Pharmacy, #12/1, Chikkabellandur, Carmelaram Post, Vathur Hobli, Bengaluru-560035, Karnataka, India
*Registrar, Delhi Pharmaceutical Sciences and Research University (DPSRU), Mehrauli-Badarpur Road, Pushp Vihar Sector 3, New Delhi-110017, India
**National Post Doctoral Fellow (N-PDF), CIMAP Research Centre, Allalasandra, GKV Post, Bengaluru-560065, Karnataka, India
***Principal Scientist, Division of Plant Genetic Resources, IIHR, Hessaraghatta Lake Post, Bengaluru-560080, Karnataka, India
****Department of P.G. Studies and Research in Wildlife and Management, Kuvempu University, Jnana Sahyadri, Shivamogga, Shankaraghatta-577451, Karnataka, India
*****Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu-641029, Tamil Nadu, India

Received March 17, 2018: Revised April 30, 2018: Accepted May 5, 2018: Published online June 30, 2018

Abstract

Coscinium fenestratum (CF) (Gaertn.) Colebr. is a hard woody climber medicinal plant belongs to the family, Menispermaceae. The plant is commonly known as tree turmeric because the stem content yellow berberine, the major active constituent. The plant is widely available in Western Ghats region but became endangered due to over exploitation and very slow germination rate. Hence, the alternate in vitro tissue culture has established for reduced germination rate and to exploit more amount of plant constituents from the stem callus. Full strength MS medium supplemented with 2, 4-D at 0.1 mg/l and kinetin at 2 mg/l gave callus growth in 46 days and the growth mechanism is observed first time through SEM study of callus. Thereafter, rooting of callus occurred in IBA and kinetin in combinations in half strength MS medium whereas direct rooting of stem occurred with IBA and zinc sulphate (IBA at 2 mg/l and ZnSO₄ at 3 mg/l) supplemented in half strength MS media along with coconut water, within 18 days. Thereafter, extracted callus is identified with TLC, followed by estimated with HPLC and HPTLC and resulted methanol extract of CF showed higher content of berberine than aqueous extract.

Key words: Berberine, callus, chromatographic method, estimation, in vitro culture, organogenesis, plant hormones, ZnSO₄

1. Introduction

Plant tissue culture is an old practice for the in vitro development of the desired plant parts from which increased number of plant secondary metabolites is isolated very easily. The main objective of plant tissue culture is to develop plantlets or callus for such plant species which are endangered or threatened due to over exploitation or the rate of seed germination is very longer time and to enhanced the active constituents by sub-cultured as per required times. This method helps to grow the plants aseptically or repeated number of multiplication of young parenchymatous cells that accumulate impurity free major plant constituents and isolation of same becomes very easy.

Looking at the above objectives, the present study was selected a plant species which is endangered but economically important, i.e., Maramanjal. Scientifically, the plant is known as Coscinium fenestratum (CF) (Gaertn.) Colebr. (Family: Menispermaceae) is a large dioecious woody climber tree. The plant is indigenous to the Indo-Malayan region, distributed in Sri Lanka, India, Malaysia, Vietnam, Myanmar, Singapore and Thailand (Tushar et al., 2008). In India, the plant is widely spread across the Western Ghats regions of Tamil Nadu (Kanniakumari, Tirunelveli and Nilgiri districts), Kerala (Thiruvananthapuram, Wynad, Thrissur, Idukki and Palakkad districts) and Karnataka (Kodagu, Udupi, Dakshina and Uttara Kannada districts) (Sumy et al., 2000; Mohanan and Sivadasan, 2002). This tree is also known as False Calumba or Tree Turmeric due to presence of yellow colored alkaloid berberine (an isoquinoline), a medicinally active compound with numerous bioactivities (Jayaweera, 2006; Warakagoda and Subasinghe, 2014). Other constituents like protoberberine, jatrorrhizine, magnoflorine, berberrubine, thalifendine, palmitine, sitosterol, palmitic acid, oleic acid and oxyberberine are present in stem and roots of this plant (Siwon et al., 1980; Pinho et al., 1992; Agusta, 2003; Anonymous, 2005). Due to presence of various constituents, the plants have various therapeutic activities like treating digestive disorders, chronic fevers, wounds, ulcers,
jaundice, burns, skin diseases, abdominal disorders, diabetes, fever and general debility (Warrier et al., 1994; Agusta, 2003). Apart from that, it is also used in cosmetic industry and other ayurvedic products (soap, bath gels, face wash and bath oil), and have anti-diabetic (Shirwaikar et al., 2005), anti-inflammatory (Caius, 1992), antioxidant and anthelmintic activities (Das et al., 2018). These activities are possible when the plants are grown abundantly and sufficient raw materials are procured and dried woody stem has high demand in the crude drug market. Generally, propagation occurs through sexual method, i.e., done naturally by seeds but the plant takes around 13-15 years to mature. Seed germination takes longer time (6-8 months) due to hard seed coat (Harinarayanan et al., 1994; Tushar et al., 2008) and, hence the seed germination was found around 30%. Research article revealed that around 12% of the fresh fruits contain non-viable seeds (Senerath, 1991) and also vegetative propagation through stem cuttings are unsuccessful (Gunatillake et al., 2002). These all together resulted relatively slow growth rate, degradation of natural habitats, habitat specificity, no proper domestication through cultivation, illegal over exploitation and destructive collection natural populations. These difficulties are only overcome through in vitro cultivation. There are few reports on cell and suspension culture of this plant by which shoots and roots are developed (Parthasarathy, 2007; Staden et al., 2008) and also reported increased amount of berberine content, estimated by HPLC method (Talat et al., 2009; Senarath, 2010). In another study, it is revealed that petiole and leaf explants of CF formed callus on vermi-compost extract media along with coelomic fluid (Kashyap et al., 2016), but no such literature is available on development of stem callus on MS medium, containing coconut water in combination with plant growth hormones, followed by impact of zinc sulphate for early rooting of the stem as well as estimation of the berberine content through HPLC and HPTLC methods. Further, very scanty reports or no such reports on SEM study (Scanning Electron Microscopy) on proliferation of parenchymatous cells for callus growth of explants. In view of that, the present study has undertaken to establish the stem callus in MS medium, supplemented with various concentrations of plant hormones, followed by SEM study for the mechanism of callus growth and estimation of berberine content through various chromatographic methods such as TLC, HPLC and HPTLC.

2. Materials and Methods

2.1 Plant material, surface sterilization and explants selection

Seeds of C. fenestratum are collected from Dr. P.E. Rajasekharan, Principal Scientist, Plant Biotechnology Department, Indian Institute of Horticultural Research, Hessaraghatta, Bangalore. Prior to use, seeds are ex vitro germinated in plastic cup container filled with sand: coir dust (1:1) medium, placed inside laboratory and drained using 0.5 g/l topsin fungicide solution to avoid fungal infection of seeds (Figure 1). After 24 h, surface sterilized and pretreated seeds by various solvents like 3% potassium nitrate, 2000 mg/l gibberelic acid (GA), 2250 mg/l GA, and kept for germination (Figure 2). Germinated plants are used as explants for the present study.

Figure 1: A: Hard Coscinium seed, B. Fruits containing seeds.

2.2 Culture media

Murashigae and Skoog medium (MS) is procured from Hi-media, India. Full strength and half strength medium prepared for initiation of callus growth and organogenesis of the plants. Growth regulators are procured from Himedia, India and used in different concentrations for this present study, viz., 2,4-Di chlorophenoxy acetic acid (2,4-D), kinetin, indole acetic acid (IAA). Indole butyric acid (IBA), gibberellic acid (GA), 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA), zinc sulphate (ZnSO) and coconut water.

2.3 Preparation of culture medium

Various combinations of MS media are prepared with double distilled water, using sugar concentration of 3g/l along with different concentrations of required growth hormones. 0.7% agar is used as gelling agent. The media is then dispensed into culture tubes, 100 ml EM flask, 200 ml bottles with 15, 40 and 60 ml media and coconut water of 2.5, 5 and 7.5 ml, respectively on plugs or Laxbros plastic caps and made air tight. They are then sterilized in an autoclave at 121°C temperature and 103.4 kPa pressure for 20 min.

2.4 Preparation of explants for inoculation and incubation

The stems of germinated explants is washed thoroughly with running tap water for 15 min then treated with bavistin (0.1%) for
15 min. The treated plant materials are subjected to sterilization using 0.1% mercuric chloride for 5 min. and repeated washing with sterile double distilled water under aseptic environment. A known weight of stem part is dissected and inoculated to the medium. The inoculated tubes, flasks and bottles are then incubated in the culture room at 25 ± 2°C under fluorescent light with an intensity of 60 µE/m²/sec. A photoperiod of 16 h of light and 8 h of darkness is maintained through an automatic timer and a constant relative humidity of 65-75% using an air-cooling system is also maintained.

2.5 Growth measurement

In each replication of different treatments for an individual experiment, uniform explant tissues are inoculated and incubated at 25 ± 2°C. A fixed number of replications are taken for the record in each time. Weight of the callus and its maintenance are also observed 60 days after inoculation and after every 16 days of sub-culturing, respectively.

(a) Effect of different combinations of auxin and kinetin on callus induction

Different strengths of MS basal medium along with auxins (IAA, IBA, NAA, 2, 4-D) and cytokinin (kinetin) at various concentrations are used in combinations. Stem at 1.5 to 2 cm length are sterilized and inoculated in culture tubes containing 15 ml media. Observations are recorded after 45 days of inoculation.

(b) Effect of different combinations of auxin, cytokinins and ZnSO₄ for the rooting of callus

MS half strength medium prepared and supplemented with IBA at 0.1 mg/l and cytokinins at 1 and 2 mg/l are used in combinations. Further, IBA at various concentrations and ZnSO₄ at 1, 2 and 3 mg/l are used for organogenesis of callus, using coconut water in the media. Observation upto 60 days, the rooting of callus under different treatments as well as direct rooting of the stem parts is recorded.

2.6 Mechanism of proliferation of callus

Growth of callus is occurred due to the multiplication of parenchymatous cells. Hence, callus is known as an abnormal, uncontrolled growth of parenchyma tissues. This proliferation was clearly visible under SEM study.

2.7 Preparation of extract for chemical analysis

Obtained calli (5 g dried) is refluxed 3-4 h with methanol and solvent water and analyzed for various phytochemicals present in the calli as per the standard method (Harborne, 1973; Evans, 2002).

2.8 TLC identification

Presence of berberine was identified in methanol and aqueous callus extracts by using mobile phase n-butanol, acetic acid and water (8:1:1) and silica gel G as stationary phase. Standard berberine hydrochloride is used for comparison. The plates are then derivatised in iodine chamber (Figure 5).

2.9 Estimation of berberine from extracts

HPLC and HPTLC were carried out for estimation of berberine content in both the extracted callus samples. HPTLC (CAMAG) is used for separation and estimation of berberine content in both the extracts. n-butanol, acetic acid and water (8:1:1) are used as mobile phase and silica gel GF₂₅₄ as stationary phase. The results are compared against standard berberine hydrochloride at wavelength 450 nm. The linearity of the concentration observed from 12-80 nanogram (r² = 0.9988). Sample and standard prepared 1 mg/ml concentration.

Further, efficient HPLC (Shimadzu, India) system is applied for estimation of berberine content. The condition for HPLC as column: SS WakoSil II C-18 (250 X 4.6 mm), mobile phase: methanol and water (90: 10), flow rate 1.0 ml per min and compound detected at 220 nm. Sample and standard prepared 1 mg/ml concentration using methanol as solvent. Sample further diluted within the limit of standard area for the calculation.

2.10 Statistical analysis

Callus growth was analyzed by one-way ANOVA study, followed by Tukey’s multiple comparison post test and p< 0.05 is considered as significant.

3. Results and Discussion

3.1 Seed germination

Seed of CF is very hard and germination rate is also very slow due to high content of alkaloids in the seed. Hence, various techniques are adopted earlier for breaking the dormancy (Anil Kumar et al., 2010; Ramasubbu et al., 2012; Warakagoda and Subasinghe, 2015). The same method is tried in this experiment and is resulted GA, at dose of 2500 ppm showed better result than others. Germination of seed occurred faster within 4 months but same in concentration 3000 ppm showed lower germination which is similar to the earlier reports.

3.2 Growth measurement

i. Effect of different combinations of auxin and kinetin on callus induction

The effect of different combinations of auxins and kinetin on callus induction of *C. fenestratum* in different strengths of MS medium is given in Table 1. The results suggest that the growth of callus initiated in full strength of MS media, supplemented with 2, 4-D at 0.1 mg/l and kinetin at 2 mg/l. The highest growth of callus is observed in full strength MS media (5.48 cm) than half strength MS media (2.10 cm) (Negative results are not reflected in Table 1). It was revealed that a combination of 2,4-D, BAP and kinetin (2.0, 2.0, 1.0 µM, respectively) in MS medium enhances the callus production in *C. fenestratum* (Khan et al., 2008) in 46 days. Our present study is also followed the same trend for the callus development (Figure 3).

Table 1: Effect of plant growth regulators for callus initiation

| Treatments (mg/l) | Callus growth | Diameter of callus (cm) |
|------------------|---------------|------------------------|
|                  | ½ strength    | Full strength          | ½ strength   | Full strength |
| 2,4 D 1+ Kinetin 0.1 | No            | No                     | ---          | ---          |
| 2,4 D 1+ Kinetin 0.2 | Yes           | Yes                    | 0.80 ± 0.11  | 1.12 ± 0.22  |
| 2,4 D 1+ Kinetin 0.3 | Yes           | Yes                    | 0.90 ± 0.12  | 1.00 ± 0.31  |
| 2,4 D 0.1+ Kinetin 1 | Yes           | Yes                    | 1.00 ± 0.20  | 2.20 ± 0.11† |
| 2,4 D 0.1+ Kinetin 2 | Yes           | Yes                    | 2.10 ± 0.10† | 5.48 ± 0.20**|
| 2,4 D 0.1+ Kinetin 3 | Yes           | Yes                    | 1.43 ± 0.10  | 3.42 ± 0.31**|

n = 6; readings ± SEM; significant at p<0.05**
ii. Effect of different combinations of auxin, cytokinins and ZnSO₄ for the rooting of callus

MS half strength medium prepared and supplemented with IBA at 0.1, 0.2, 0.3, 1, 2 and 3 mg/l and kinetin at 1 mg/l are used in combinations. Further, IBA at concentration 2 mg/ml and ZnSO₄ at various concentrations 1, 2 and 3 mg/l are used for organogenesis of callus, using coconut water in the media. It is seen that IBA is effective against rooting of callus whereas added application of ZnSO₄ at concentration 3 mg/ml along with IBA 2 mg/ml in combinations showed direct rooting in stem within 18 days in half strength MS medium mixed with coconut water (Table 2, Figure 4). Literature survey revealed that root initiation occurred after the 3rd week of transferring the shoots to the rooting medium and the rooting percentage greatly depends on strength of MS medium (Senarath, 2010). Our results showed rooting of stem occurred within 18 days which is lesser time than that of reported earlier. This may be due to added zinc sulphate which acts synergistic activity along with IBA. It was reported that 50 µm zinc sulphate concentration significantly caused boosting of leaf area and both fresh and dry weight of shoot and root development (Nejad et al., 2014) which is also positive in our present study.

Table 2: Effect of plant growth regulators on rooting of Coscinium stem

| Treatments (mg/l) | Root growth | Length of Root (cm) |
|------------------|-------------|---------------------|
|                  | ½ strength | Full strength       | ½ strength | Full strength |
| IBA 1+ Kinetin 1 | No          | No                  | ---        | ---          |
| IBA 2+ Kinetin 1 | Yes         | Yes                 | 2.01       | 0.67         |
| IBA 3+ Kinetin 1 | No          | No                  | No         | No           |
| IBA 0.1+ Kinetin 1| No          | No                  | No         | No           |
| IBA 0.2+ Kinetin 1| No          | No                  | No         | No           |
| IBA 0.3+ Kinetin 1| No          | No                  | No         | No           |
| IBA 1+ ZnSO₄ 1  | No          | No                  | No         | No           |
| IBA 2+ ZnSO₄ 1  | No          | No                  | No         | No           |
| IBA 3+ ZnSO₄ 1  | No          | No                  | No         | No           |
| IBA 1+ ZnSO₄ 2  | No          | No                  | No         | No           |
| IBA 2+ ZnSO₄ 2  | Yes         | No                  | 1.02 ± 0.10| No           |
| IBA 3+ ZnSO₄ 2  | Yes         | No                  | 0.92 ± 0.20| No           |
| IBA 1+ ZnSO₄ 3  | No          | No                  | No         | No           |
| IBA 2+ ZnSO₄ 3  | Yes         | Yes                 | 2.6 ± 0.11 **| 1.10 ± 0.10 |
| IBA 3+ ZnSO₄ 3  | Yes         | Yes                 | No         | No           |

n =6; readings ± SEM; significant at p<0.05**

3.3 Mechanism of proliferation of callus

Scanning Electron Microscopy is carried out first time to observe the proper proliferation of parenchyma cells for growth of callus. It is seen that uncontrolled abnormal growth (Figure 5). There are various scale used for observation of growth mechanism of callus.

3.4 TLC identification of CF extracts

Two different extracts, viz., methanolic and aqueous extracts were prepared and resulted higher percentage yield in methanol extract (24.8% w/w) than aqueous one (16.3% w/w), but both contain saponins, alkaloids, flavonoids, resins, etc. Thereafter, TLC study has carried out with the various solvent systems and revealed the n-butanol, acetic acid and water is the mobile phase that identified and separated chemical constituents when compared with the
standard berberin hydrochloride. The Rf is calculated and resulted 0.63 (Figure 6) after derivatised in iodine chamber.

Figure 6: TLC plate of extracted CF stem callus.

Earlier scientific literatures evident that berberine identification was carried out using various TLC solvent systems (Rojsanga et al., 2006; Krishna et al., 2011), looking at that we are also used different solvent system with standardized ratio for the separation and identification of berberine in the CF extracts.

Figure 7: HPTLC of callus extract of CF.

3.5 Estimation of berberine in the extract

HPTLC method is applied for the separation and estimation of the berberine content in both the MECF and AECF using same solvent system as TLC used. Results showed that Rf values are coincided at 0.63 when scanned at 450 nm. The finger printing as well as the tracks are showed in Figures 7 A, B, C and D. Many literatures also revealed the estimation of berberin through HPTLC method using various solvent systems (Rojsanga et al., 2006; Krishna et al., 2011; Jayaprakasam and Ravi, 2014).

3.6 HPLC method

Further, effective separation and estimation of CF extracts are carried out using HPLC method using methanol and water solvent system at 220 nm. Finally the berberine content is estimated by calculated Rt of standard and sample (Rt = 3.074 min.) (Figures 8 a, b and c).
Figure 8(a): HPLC of standard berberine hydrochloride.

Results of standard graph

| Peak No. | Peak ID | Ret. time | Height  | Area    | Conc.   |
|----------|---------|-----------|---------|---------|---------|
| 1        |         | 3.074     | 65154.414 | 565060.625 | 95.7911 |
| 2        |         | 3.565     | 1352.586 | 24827.801 | 4.2089  |
| **Total**|         |           | 66507.000 | 589888.426 | 100.0000|

System evaluation

| Peak No. | Peak ID | Ret. time | Half peak width | Theoretical levels | Resolution | Tail factor | Asymmetry |
|----------|---------|-----------|-----------------|--------------------|------------|-------------|-----------|
| 1        |         | 3.074     | 0.100           | 5147.950           | 0.000      | 0.647       | 0.500     |
| 2        |         | 3.565     | 0.297           | 800.002            | 1.303      | 0.964       | 0.928     |

Figure 8(b): HPLC graph of AEIF.
### Results of AECF graph

| Peak No. | Peak ID | Ret. time | Height | Area   | Conc.  |
|----------|---------|-----------|--------|--------|--------|
| 1        |         | 2.035     | 73666.961 | 144483.125 | 57.4882 |
| 2        |         | 2.270     | 52032.164  | 364193.375  | 14.4908 |
| 3        |         | 2.572     | 30384.494  | 306517.625  | 12.1960 |
| 4        |         | 3.070     | 19667.855  | 224781.406  | 8.9438  |
| 5        |         | 3.375     | 6232.493   | 124087.844  | 4.9373  |
| 6        |         | 3.997     | 1832.170   | 28167.869   | 1.9439  |
| **Total**|         |           | 183816.137 | 2492581.244 | 3.997   |

### System evaluation

| Peak No. | Peak ID | Ret. time | Half peak width | Theoretical levels | Resolution | Tail factor | Asymmetry |
|----------|---------|-----------|-----------------|-------------------|------------|-------------|-----------|
| 1        |         | 2.035     | 0.337           | 203.408           | 0.000      | 0.726       | 0.498     |
| 2        |         | 2.270     | 0.115           | 2164.899          | 0.517      | 1.382       | 1.763     |
| 3        |         | 2.572     | 0.158           | 1463.371          | 1.098      | 0.877       | 0.754     |
| 4        |         | 3.070     | 0.300           | 700.459           | 0.575      | 4.853       | 8.706     |
| 5        |         | 3.375     | 0.300           | 700.459           | 0.575      | 4.853       | 8.706     |
| 6        |         | 3.997     | 0.257           | 1344.392          | 1.123      | 2.434       | 3.868     |

### Figure 8(c): HPLC graph of MECF
Results of MECF graph

| Peak No. | Peak ID | Ret. time | Height      | Area      | Conc.  |
|---------|---------|-----------|-------------|-----------|--------|
| 1       |         | 1.940     | 16974.535   | 266202.688| 21.0077|
| 2       |         | 2.107     | 10625.940   | 84754.539 | 6.6885 |
| 3       |         | 2.382     | 4371.208    | 39732.684 | 3.1356 |
| 4       |         | 3.073     | 46628.488   | 437294.688| 66.0761|
| 5       |         | 3.565     | 667.818     | 3631.600  | 0.2866 |
| 6       |         | 4.057     | 1595.774    | 12497.084 | 1.7487 |
| 7       |         | 4.373     | 960.333     | 13391.933 | 1.0568 |
| Total   |         |           | 81824.096   | 857505.216| 100.0000|

System evaluation

| Peak No. | Peak ID | Ret. time | Half peak width | Theoretical levels | Resolut ion | Tail factor | Asymmetry |
|---------|---------|-----------|-----------------|--------------------|-------------|-------------|-----------|
| 1       |         | 1.940     | 0.232           | 388.496            | 0.000       | 0.649       | 0.321     |
| 2       |         | 2.107     | 0.133           | 1383.005           | 0.457       | 3.409       | 5.818     |
| 3       |         | 2.382     | 0.137           | 1682.466           | 1.019       | 2.153       | 3.306     |
| 4       |         | 3.073     | 0.210           | 1186.562           | 1.995       | 0.987       | 0.993     |
| 5       |         | 3.565     | 0.093           | 8082.366           | 1.621       | 1.067       | 1.093     |
| 6       |         | 4.057     | 0.125           | 5834.657           | 1.318       | 0.823       | 0.644     |
| 7       |         | 4.373     | 0.272           | 1435.689           | 0.798       | 0.949       | 0.926     |

We observed that both the HPTLC and HPLC graphs, the amount of berberine content is estimated for both the extracts and the results are tabulated in Table 3.

Table 3: Estimation of berberine content in various extracts

| Methods | Extract | Berberine present (mg) | %RSD |
|---------|---------|------------------------|------|
| HPTLC   | MECF    | 1.23                   | 1.12 |
|         | AECF    | 0.97                   |      |
| HPLC    | MECF    | 1.34                   | 1.54 |
|         | AECF    | 0.99                   |      |

From these two methods, it is observed that methanol extract of CF showed higher amount of berberine content than aqueous extract. HPLC showed better amount of berberine (1.34 mg) content than HPTLC (1.23 mg) for MECF. Earlier literature also revealed the same results where methanolic extract showed higher percentage of berberine content than other extract (Jayaprasakasam and Ravi, 2014). Further, it is also revealed that percentage yield of extract also higher in methanol extract than others (Arawwwala and Wickramaarachchi, 2012; Akowuah et al., 2014) and the same trend followed in the present study where methanol extract of callus showed higher percentage of yield than aqueous extract. It is also proved that content of active constituents are also depends on the percentage of yield (Dent et al., 2013; Das et al., 2016; Das et al., 2017). Our present study is also reported the similar results that influence direct correlation with the amount content of the active constituents.

4. Conclusion

The present study established the in vitro culture of C. fenestratum stem in full strength MS medium, supplemented with various plant growth regulators (combinations of 2, 4-D at 0.1 mg/l and kinetin at 2 mg/l) in various concentrations within 46 days. First time, SEM study is reported the mechanism for callus growth via abnormal proliferation of parenchyma cells. Thereafter, organogenesis of stem cellus developed in combinations of IBA and kinetin combinations in half strength MS medium. Further, direct rooting of stem in half strength MS medium is observed within 18 days when IBA combined with zinc sulphate is used along with coconut water in the medium (IBA 2 mg/l and ZnSO4 3 mg/l combination). Grown callus further extracted with methanol and aqueous solvents and estimated for berberine content by HPLC and HPTLC where methanol extract showed higher content of berberine than aqueous extract. Further, study required hardening of plant in huge quantities for conversion of endangered species to cultivated plant and to discover new biomolecules for various therapeutic activities.

Acknowledgements

Authors are thankful to Rajiv Gandhi University of Health Sciences, Bangalore, India for financial assistance of Rs. 1.5 Lakhs as research grant (Principal Investigator) to carry out the present investigation (Order No. RGU: Adv. Res.: Proposal-P-171: 2015-16 DATE: 06-01-2016).

Conflict of interest

We declare that we have no conflict of interest.

References

Agusta, A. (2003). Coscinium fenestratum (Gaertner) cebelr. Inlemens, RMHJ and bunyaphrathatsara, N. Plant Resour. South East Asia: Med. Poisonous Plants, 3: 139-140.

Akowuah, GA.; Okechukwu, P.N. and Chiam, N.C. (2014). Evaluation of HPLC and spectrophotometric methods for analysis of bioactive constituent berberine in stem wtraits of Coscinium fenestratum. Acta. Chromatographica., 26(2):243-254.
Anilkumar, C.; Chitra, C.R.; Bindu, S.; Prajith, V. and Mathew, P.J. (2010). Dormancy and germination of Coscinium fenestratum (Gaertn.) Colebr. seeds. Seed Science and Technology, 38:585-594.

Anonymous. (2005). Quality standards of Indian medicinal plants. Indian Council of Medical Research, New Delhi, 2:119-128.

Arawawala, L.D.A.M. and Wickramaarachchi, W.A.N. (2012). Berberine content in Coscinium fenestratum (Gaertn.) Colebr grown in Sri Lanka. Pharmacologia, 3(12):679-682.

Caius, J.F. (1992). The medicinal and poisonous plants of India. Jodhpur, India, pp:141-142.

Das, K.; Dang, R.; Sivaraman, G. and Rajasekharan, P.E. (2018). Phytochemical screening for various secondary metabolites, antioxidant and anthelmintic activity of Coscinium fenestratum fruit pulp a new biosource for the novel drug discovery. Turk. J. Pharm. Sci., 5(2): (unpublished).

Das, K.; Deb, S.; Karanth, T.; Upreti, S. and Dang, R. (2016). Effect of cultural condition on element contents in raw material vis-à-vis impact of solvent nature on estimation of phytochemicals and screening of anthelmintic activity of Melia dubia Cav. leaf. Ann. Phytomed., 5(2):58-68.

Das, K.; Reka, R.; Ibrahim, M.A.; Yahya Ahmed, S. and Dang, R. (2017). Effect of geographic location on Phlebodium decumanum (Wild.) J. Sm. for its phytoconstituents and establishment of antioxidant and novel anthelmintic activity of its aqueous and methanolic leaf extracts. Ann. Phytomed., 6(1):101-106.

Dent, M.; Uzelac, D.; Penic, M.; Brnic, M.; Bosiljik, T. and Levaj, B. (2013). The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in dalmatian wild sage (Salvia officinalis L.) extracts. Food Technol. Biotechnol., 51(1):84-91.

Evans, W.C. (2002). Trease and Evans Pharmacognosy, 15th edition. W.B Saunders Company Ltd, London. pp:137-139, 230-240.

Gunatillake, L.A.U.; Gunatillake, C.V.S.; Tennakone, K.U.; Dassanayake, M.D. and Wanigasundara, W.P.D. (2002). Development of propagation techniques for medicinal plant species Solanum album, Coscinium fenestratum, Piper longum and Hemidesmus indicus. Sri Lanka conservation and sustainable use of medicinal plant project, Ministry of Indigenous Medicine Disaster Relief Research Report MP/RP/04. Postgraduate institute of Science, University of Peradeniya, Peradeniya.

Harborne, J.B. (1973). Phytochemical methods: A guide to modern techniques of plant analysis, 13th Ed. Chapman and Hall, Ltd. London. pp:5-15.

Harinarayanan, M.K.; Mustafa Anand, P.R.; Jayanthi, A. and Reetha, A. (1994). Some preliminary observations on daraharidra-A vanishing medicinal plant. Aryavaidyan, 8:18-23.

Jayaprakashan, R. and Ravji, T.K. (2014). Development and validation of HPTLC and RP-HPLC methods for the estimation of berberine in Coscinium fenestratum extract and its formulation. American J. Pharmatech. Res., 4(3):206-218.

Jayaweera, D.M.A. (2006). Medicinal Plants (Indigenous and Exotic) used in Ceylon, National Science Foundation of Sri Lanka, Colombo, Sri Lanka, 470-71.

Kashyap, S.; Kapoor, N. and Kale, R. D. (2016). Coscinium fenestratum: Callus and suspension cell culture of the endangered medicinal plant using vermicompost extract and coelomic fluid as plant tissue culture media. American Journal of Plant Sciences, 7(6):899-906.

Khan, T.; Krupadanam, D. and Anwar, S.Y. (2008). The role of phytohormone on the production of berberine in the calli cultures of an endangered medicinal plant Coscinium fenestratum. African Journal of Biotechnology, 7(18):3244-3246.

Krishna, C.; Sujatha, K.; Polisetty, H. and Reddy, C.U. (2011). Standardization of Coscinium fenestratum with reference to berberine by high performance thin layer chromatography. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2(2):226-229.

Mohanan, N. and Sivadasan, M. (2002). Flora of Agasthyamala. Bishen Singh Mahendra Pal Singh, Dehra Dun, India, pp:65.

Nejad, R.H.; Najafi, F.; Arvin, P. and Firuzeh, R. (2014). Study different levels of zinc sulphate (ZnSO4) on fresh and dry weight, leaf area, relative water content and total protein in bean (Phaseolus vulgaris L.) plant. Bulletin of environment, Pharmacology and Life Sciences, 3(6):144-151.

Parthasarathy, V.A. (2007). High tech propagation of horticultural crops: accent of recalcitrance. Recent trends in horticultural biotechnology (eds. R. Kesharachandran, P.A. Nazeem, D. Girija, P.S. John and K.V. Peter). New India Publishing Agency. New Delhi, India, pp:85-91.

Pinho, P.M.M.; Pinto, M.M.M.; Kijjoua, A.; Phuradai, K.; Díaz J.G and Herz, W. (1992). Protoberberine alkaloids from Coscinium fenestratum. Phytochemistry, 31:1403-1407.

Ramasubbu, R.; Chandra Prabha, A. and Kumuthakalavalli, R. (2012). Seed biology of Coscinium fenestratum (Gaertn.) Colebr. A critically endangered medicinal plant of Western Ghts. Journal of Medicinal Plants Research, 6(6):1094-1096.

Senarath, W.T.P.S.K. (2010). In vitro propagation of Coscinium fenestratum (Gaertn.) Colebr. (Menispermaceae): An endangered medicinal plant. J. Natn. Sci. Foundation Sri Lanka., 38(4):219-223.

Senerath, M.A.B.D. (1991). Biological studies on Coscinium fenestratum Colebr. (Menispermaceae). M.Phill Thesis. University of Peradeniya, Peradeniya.

Shirwaikar, A.; Rajendra, K. and Punitha, L.S.R. (2005). Antidiabetic activity of alcoholic stem extract of C. fenestratum in streptozotocin nicotinamide induced type-2 diabetic rats. Journal of Ethno-pharmacology, 97:369-374.

Siwon, J.; Verpoorte, R.; Van Essen, G.F.A. and Svendsen, A.B. (1980). Studies on Indonesian medicinal plants. III: The alkaloids of Coscinium fenestratum. Planta Med., 38:24-32.

Staden, J.V.; Zuzimalova, E. and George, E.F. (2008). Plant growth regulators II. Cytokinins, their analogues and antagonists. Plant propagation by tissue culture, 3rd edition, (eds. EF George, MA Hall, GD Klerk), pp:205-226.

Sumbi, O., Ved, D.K. and Krishnan, R. (2000). Tropical Indian Medicinal Plants: Propagation methods. FRLHT, Bangalore, India, pp:114-115.

Talat, K.; Krupadanam, G.L.D. and Anwar, S.Y. (2009). Isolation and characterization of enhanced berberine from the calli cultures of Coscinium fenestratum L: An endangered medicinal plants of Western Ghat, India. Biosciences, Biotechnology Research Area., 6(1):197-202.

Tushar, K.V.; George, S.; Remashree, A.B. and Babachandran, I. (2008). Coscinium fenestratum (Gaertn.) Colebr: A review on this rare, critically endangered and highly-traded medicinal species. J. Plant Sci., 3:133-145.

Warakagoda, P. S. and Subasinghe, S. (2015). Studies on seed germination of Coscinium fenestratum (Menispermaceae): A threatened medicinal plant. International Journal of Minor Fruits, Medicinal and Aromatic Plants, 1(1):37-46.

Warakagoda, P.S. and Subasinghe, S. (2014). In vitro seed germination of Coscinium fenestratum (Gaertn.) Colebr. Annual Research and Review in Biology, 4(23):3549-3565.