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Callus Induction, Proliferation, Enhanced Secondary Metabolites Production and Antioxidants Activity of *Salvia moorcroftiana* L. as Influenced by Combinations of Auxin, Cytokinin and Melatonin

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HIGHLIGHTS

- Tissue culture technique is considered to be one of the best methods to reproduce salvia plant.
- Salvia is a medicinal plant with lots of chemical constituents.
- Salvia plays an important role in treating different fatal diseases.
- Maximum fresh and dry weights were obtained at 1.5 mg L^{-1} melatonin.
- the highest DPPH scavenging activity, total phenolics and flavonoid content were obtained at 1.5 mg L^{-1} melatonin.

Abstract: Tissue culture technique is one of the best methods to reproduce salvia plant Therefore, the aim of this research was to enhance the *in-vitro* callus proliferation and production of secondary metabolites of *S. moorcroftiana* using different combinations of auxin, cytokinin and melatonin. Initially, callus induction was optimized using indole acetic acid (IAA), 2, 4-dichlorophenoxy acetic acid (2,4-D), and naphthalene acetic acid (NAA) applied at different concentrations in combination with 1 mg L^{-1} of 6-benzylaminopurine (BAP). The results indicates that earliest days to callus induction (14.67 days) was occurred in the media fortified

with 2, 4-D+BAP (2.0+1.0 mgL-1). Whereas the highest callus initiation (100%) was induced on MS medium incorporated with 2,4-D+BAP (1+1mgL^{-1}). Furthermore, maximum fresh weight was obtained when 2,4- D + BAP at the rate of (1+ 1mg L^{-1}) was incorporated and dry weight was attained when 2,4- D + BAP at the rate of (2+1 mg L^{-1}) was added to MS media. The maximum fresh and dry weight was obtained when melatonin at rate of 1.5 mg L^{-1} was supplemented with MS media including 2,4-D + BAP (1+1mg L^{-1}), moreover the maximum DPPH scavenging activity, total phenolic and flavonoid content was noted when supplemented with melatonin at rate of 1.5 mg L^{-1}. In conclusion, among various concentrations of plant growth regulators, 2,4- D + BAP at the rate of (1+ 1mg L^{-1}) along with 1.5 g L^{-1} melatonin was the best for callus growth and production of secondary metabolites of *S. moorcroftiana*.

Keywords: Antioxidant activity; Growth regulators; Sage; Secondary Metabolites.

INTRODUCTION

Sage (*S. moorcroftiana*) belongs to the largest genus of ornamental, aromatic and medicinal plants in the family Lamiaceae distributed over tropical regions [1]. The plant is largely found in the Mediterranean Basin, Central and South America, South-East and Central Asia and is mostly cultivated for medicinal purposes. In Pakistan *S. moorcroftiana* is found in the northern areas of the Khyber Pakhtunkhwa province and Kashmir region at a height of about 5200ft [2]. *S. moorcroftiana* is a medicinal plant with lots of chemical constituents which play an important role in treating different fatal diseases. It contains chemical compounds which have anti-tumor, antibacterial, antifungal, antiviral and anti-inflammatory activity [3]. Salvia also contains several beneficial secondary metabolites like phenolic compounds, essential oils and terpenoids [4]. Sage's biological effects on human health are due to the anti-inflammatory, antibacterial, fungistatic, virostatic, astringent, eupeptic and antihydrotic properties of its components [3]. These can be used in the pharmaceutical and medical fields as a result of their hypoglycemic and antimutagenic actions and as such in the treatment of Alzheimer's Disease [5]. Sage is mixed into a combination of herbal preparations as an active ingredient and is useful for bronchitis treatment [5].

Propagation of common sage plants can be induced through cuttings and seeds, but conventional propagation methods are not practiced due to low seed germination, poor planting material and slow growth. However, the tissue culture technique is considered to be one of the best methods for reproducing salvia plants. Very limited information is available on the micro-propagation of this plant. Moreover, some other species of this genus have been tried for propagation *in-vitro* conditions such as hairy roots, callus and cell suspension [6]. Micropropagation of salvia species has been done successfully on various media such as Murashige and Skoog (MS), B5 vitamins and α- naphthalene acetic acid (NAA) for successful regeneration from explants taken from open field plants [7].

Plant growth regulators play a key role in different physiological processes during the growth and developmental stages of plants [8- 13]. Different types of cytokinin including kinetin are known to be more efficient in the proliferation of shoots such as 6-benzylaminopurine “BAP”. Auxin is also essential for *in vitro* rooting of new growth with different concentrations in salvia [14]. The micropropagation and regeneration of adventitious shoots in *S. moorcroftiana* was higher when MS media was augmented with 6-benzylaminopurine (- 0.533 mg L^{-1} BAP), naphthalene acetic acid (- 0.644 mg L^{-1} NAA) and kinetin (1.12 mg L^{-1}). BAP could also be more effective in increasing axillary buds [15].

Apart from the auxin and cytokinin groups, certain other chemicals can be used for callus induction and proliferation. Melatonin (N-acetyl-5-methoxytryptamine) is plant growth regulator and rooting agent that plays an important role in plant development and protective role in plant stress [16]. Melatonin has been reported in different angiosperms and is mostly found in tomatoes, cucumbers, higher and medicinal plants [17]. Melatonin is considered to have scavenging properties which act on reactive oxygen and hydroxyl radicals [17-18] and produce cyclic 3-hydroxymelatonin having antioxidant properties. Melatonin has similar functions like auxin, thereby promoting cell development, organogenesis, and plant growth [19]. Higher plant survival rates, greater shoot, and root development, photosynthesis, improved stomatic morphologies, high amounts of sucrose and proline, lower concentrations of ROS/RNS, lipid membrane peroxidation, and cell damage were observed in melatonin-treated plants [17]. Application of melatonin can also improve the resistance to various stresses including drought, salinity, extreme temperature, radiation, and chemical stresses. There is very limited information available on the propagation of *S. moorcroftiana* both in *in vitro* and *in vivo* conditions. Therefore, this study was aimed at evaluating the effect of plant growth regulators and melatonin on callus induction, callus growth and secondary metabolites production in *S. moorcroftiana*.

MATERIALS AND METHODS

Experimental site

Two different experiments were carried out in the Laboratory of Plant Tissue Culture, Department of Horticulture, The University of Agriculture Peshawar to optimize the plant growth regulators (auxin and cytokinin) and melatonin for callus induction and proliferation of *S. moorcroftiana*.

Collection of explant material and preparation of cultured media

Moorcroftiana specie of the genus Salvia is native to the northern part of Khyber Pakhtunkhwa and Himalayan mountains of Pakistan, especially Kashmir. It grows between 5,000- and 9,000-feet elevation. This plant was introduced by botanists of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar and grown in natural environment in Medicinal Botanical Centre of PCSIR Peshawar-Pakistan.

Newly developed leaves were collected as explants from one year old plants of *S. moorcroftiana*, which are available in the Medicinal Botanical Centre of the Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar (34° 02' N, 71° 37' E), Pakistan. The plants were identified and authenticated by Dr. Hina Fazal, PCSIR under accession number 1173PCSIR. In the first experiment, three plant growth regulators (PGRs) were optimized for callus induction from leaf explants. The auxins *i.e.,* indole acetic acid (IAA), 2, 4-dichlorophenoxy acetic acid (2,4-D), and naphthalene acetic acid (NAA) were applied at the concentrations of 0.5, 1.0, 1.5 and 2.0 mg L^{-1} in combination with 1 mg L^{-1} of 6-benzylaminopurine (BAP) which were PGRs were supplemented to the media culture performed in a laminar flow unit in the culture room and then transferred to the growth room. Temperature was kept at 25±2°C with 16/8 h photoperiod. Leaf explants were thoroughly washed and sterilized with mercuric chloride ($HgCl_2$) to remove contaminants and cultured in MS-media culture (Murasheige and Skoog) having different concentrations of auxins (2,4-D, IAA, NAA) and cytokinin (BAP) (Table 1). Media without plant growth regulators were kept as control. The MS-media has 30 g L^{-1} sucrose, 7-8 g L^{-1} agar, and pH of 5.5-5.8 range was adjusted for the media.

In the second experiment, the induced callus was exposed to various concentrations of melatonin (0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) with the objective of enhancing callus growth and secondary metabolites production of *S. moorcroftiana.* Melatonin was added to the culture media before sterilization. Media were also kept saturated with an optimized level of PGRs from the previous experiment. Similarly, MS-media was having 30 g L^{-1} sucrose and 7-8 g L^{-1} agar with pH ranging from 5.5 to 5.8. The media were sterilized at 121 ℃ for 20 min. The culture was transferred to growth room at 25±2 ºC along with 16/8 hours of light and dark photoperiod respectively.

Table 1. Detail of PGRs treatments used in media culture

Treatments	Auxin (mg L^{-1})			Cytokinin (mg L^{-1})	Auxin to cytokinin ratio
	2, 4-D	NAA	IAA	BAP	
Control	-	-	-	-	-
2,4-$D_{0.5}$+BAP	0.5	-	-	1.0	0.5
2,4-D_1+BAP	1.0	-	-	1.0	1
2,4-$D_{1.5}$+BAP	1.5	-	-	1.0	1.5
2,4-D_2+BAP	2.0	-	-	1.0	2
$NAA_{0.5}$+BAP	-	0.5	-	1.0	0.5
NAA_1+BAP	-	1.0	-	1.0	1
$NAA_{1.5}$+BAP	-	1.5	-	1.0	1.5
NAA_2+BAP	-	2.0	-	1.0	2
$IAA_{0.5}$+BAP	-	-	0.5	1.0	0.5
IAA_1+BAP	-	-	1.0	1.0	1
$IAA_{1.5}$+BAP	-	-	1.5	1.0	1.5
IAA_2+BAP	-	-	2.0	1.0	2

Studied attributes

The following attributes were studied during the research experiments

Days to callus induction

Explants cultured on media were under observation on a regular basis till callus emergence. The number of days was counted from the date of culturing till callus induction.

Percent callus induction

Data on callus induction (%) was recorded with the help of the following formula:

$$\text{Percent callus induction} = \text{No of explants that produced callus/No of explants x 100}$$

Callus morphology

On full induction of callus for each treatment, callus texture and callus color were examined visually.

Callus fresh and dry weight

Fresh callus was detached from the media after 40 days and rinsed carefully, to remove the media. Callus was placed on tissue paper to drain extra water and then fresh weights were measured using a digital weighing balance. The callus was then kept in an oven at 40 $^{\circ}$C for a period of 48 h and the dry weight was calculated by using a digital balance.

Total phenolic content

The total phenolic of oven dried callus was determined according to the protocol by Singleton and Rossi [20]. The methanol-based sample was primed by taking 1.5 mg of dried callus in 5 ml methanol. Then 40 µL of the sample was further diluted by adding 3.16 mL distilled water. Folin-Ciocalteu reagent (200 µL) and 600 µL sodium carbonate (20 µL) were incorporated into the mixture and centrifuged and incubated for 25 min. Gallic acid was used as a standard for plotting a standard curve.

Total flavonoid content

The total flavonoid content (TFC, mg g^{-1} DW) was quantified as per the method used by Park and coauthors [21]. A methanol-based sample prepared for total phenolics was used. Rutin was used a as standard to calibrate the standard curve. The absorbance was recorded using a spectrophotometer.

DPPH free radical scavenging activity

DPPH-radical scavenging activity (DRSA) in treatments exposed to melatonin was quantified following the procedure explained by Ahmad and coauthors [22]. The methanol solution of each sample (1 mL) was incorporated with DPPH-solution (2 mL). Samples were protected in dark conditions for 25 min and the absorbance reading was taken through a spectrophotometer. DRSA was calculated by the equation as under.

$$\text{DRSA (\%)} = 100 \times (1 - AP/AD)$$

Where AP stands for the absorbance of shoots extract at 517 nm and AD for the DPPH solution without extract.

Statistical analysis

Data was analyzed using the statistical package (STATISTIX 8.1, Inc, Tallahassee FL, USA). The differences between means were calculated by using LSD (least significant difference) at 5% level of significance [23].

RESULTS AND DISCUSSION

Optimization of type and concentration of auxins for callus induction of S. moorcroftiana from leaf explant

During the experiment the Murashige and Skoog (MS) media was supplemented with different concentrations (0.5, 1, 1.5 and 2 mg L^{-1}) of auxins, i.e., indole acetic acid (IAA), 2, 4-dichlorophenoxy acetic acid (2,4-D), and naphthalene acetic acid (NAA) in combination with 1 mg L^{-1} of 6-benzylaminopurine (BAP). The results of this experiment are as follows.

Days to callus induction

The data regarding days to callus induction indicates a significant effect of auxin on callus induction (Table 2). The earliest callus induction (14.7 d) occurred in the medium augmented with 2 mg L^{-1} 2,4-D followed by 1 mg L^{-1} 2,4-D. While the media fortified with 2 mg L^{-1} IAA and 0.5 mg L^{-1} NAA takes the longest time of 24 d and 22 d, followed by 22 d by 1.5 mg L^{-1} BAP.

The physiological activity of auxin within the plant tissue makes the difference in callus formation and morphology [24]. Furthermore, mutual action of auxins and cytokinin control the process of cell division. Auxins play a vital role in the stimulation of proteins in the cell cycle related to the cdc2/cdk2 class of cyclin-dependent kinases. Furthermore, the applications of auxins with cytokinin synergistically influence an increase in the activity of cdc2/cdk2 like kinase [25] producing more callus. Auxins to cytokinin optimum ratio are essential for maximum callus induction [26]. The present results are in close conformity with those of Hesami and Daneshvar [27] who observed compact callus of *Ficus religiosa* in NAA and IBA treatment as compared to friable callus obtained in MS media supplemented with 2, 4-D. Huang and Staden [28] also investigated days to callus induction in *S. chamelaeagnea*, and reported minimum days to callus induction on 2, 4-D and BAP medium.

Table 2. Effect of plant growth regulators for percent and days to callus induction of *S. moorcroftiana*

Treatments	Days to callus induction	% Callus induction
Control	-	
2,4-$D_{0.5}$+BAP	21.13±0.6^{bcd}	77.4^{f}
2,4-D_{1}+BAP	18.81±0.5^{f}	100.0^{a}
2,4-$D_{1.5}$+BAP	16.58±0.5^{g}	99.8^{a}
2,4-D_{2}+BAP	14.67±0.4^{h}	82.5^{c}
$NAA_{0.5}$+BAP	21.69±0.7^{bc}	77.3^{f}
NAA_{1}+BAP	18.01±0.3^{f}	66.8^{i}
$NAA_{1.5}$+BAP	20.24±0.5^{de}	71.8^{g}
NAA_{2}+BAP	23.33±0.3^{a}	96.4^{b}
$IAA_{0.5}$+BAP	20.91±0.5^{cd}	70.8^{h}
IAA_{1}+BAP	19.11±0.4^{ef}	79.4^{e}
$IAA_{1.5}$+BAP	22.14±0.4^{b}	80.7^{d}
IAA_{2}+BAP	24.24±0.3^{a}	61.0^{j}
ANOVA	***	***

Means followed by similar letters are not significantly different at 0.05 probability using LSD test

Percent callus induction

Statistical analysis revealed a significant mean difference between auxins and their concentrations. Overall, 2,4-D showed higher callus induction than NAA and IAA. The highest percentage (100%) of callus induction was observed in media fortified with 1 mg L^{-1} 2,4-D + BAP. Statistically similar results (99.8%) were observed with 1.5 mg L^{-1} 2, 4-D, followed by 2 mgL^{-1} NAA (96%). The lowest callus induction was at 2 mg L^{-1} IAA (61%) and 1 mg L^{-1} NAA (67%) (Table 2).

The de-differentiation process from active proliferated and meristematic cells tends to produce callus that leads to thicker, stiffer, swollen and visible tissues of the explant. Unorganized masses of cells generally termed callus are produced by actively divided cells on the surface of an explant. In the present study 1.5 mg L^{-1} 2,4-D produced the highest callus which encouraged most parts of the explant which shows the efficiency of 2,4-D to produce more callus as compared to IAA and NAA. As a common auxin, 2,4-D produces active proliferation from the dedifferentiation of explant cells [29]. Furthermore, growth regulator concentrations especially auxin and cytokinin in the culture medium are critical to controlling growth and morphogenesis. Generally, high concentrations of auxins and low cytokinin's in the medium promote abundant cell proliferation with the formation of callus [30]. The provision of exogenous auxin and cytokinin influence callus in various plant species. Generally, an equal ratio of auxin and cytokinin stimulates callus induction, while a high ratio of cytokinin-to-auxin or auxin-to-cytokinin induces shoot and root regeneration, respectively [26]. An excellent callus induction was observed at 3.5 and 3.0 mg L^{-1} 2, 4-D after a culture period of 4-5 weeks [31]. The present results are in close conformity with Mastuti and coauthors [32] who reported more callus induction and growth in *Physalis angulata* when growth medium was fortified with 2,4-D and Kinetin as compared to IAA and NAA. Further, Kintzios and Skoula [33] also reported almost similar results, who investigated that a higher rate of callus induction was obtained when an intermediate auxin and cytokinin concentration were used for *S. officinalis*.

Callus fresh and dry weight

The fresh and dry weight of the callus was significantly influenced by the auxins. The media supplemented with 2,4-D produced higher fresh and dry weight of callus as compared to NAA and IAA. The highest callus fresh weight (1.89 g L^{-1}) was observed at 1 mg L^{-1} 2,4-D and dry weight (1.13 g L^{-1}) at 2 mg L^{-1} 2,4-D. Media supplemented with 0.5 and 1 mg L^{-1} of IAA developed callus with the lowest fresh weight of 0.72 and 0.7 g which is statistically at par with 0.5 mg L^{-1} NAA (0.78 g L^{-1}). The minimum dry weight was observed at 0.5 mg L^{-1} IAA, followed by 2 mg L^{-1} NAA. This indicated that 2,4-D was more suitable regarding the callus weight (Table 3 and Figure 1).

Table 3. Effect of plant growth regulators for callus fresh weight, dry weight, callus color and texture of *S. moorcroftiana*

Treatments	Callus fresh weight (g L^{-1})	Callus dry weight (g L^{-1})	Callus color	Texture
Control			-	-
2,4-$D_{0.5}$+BAP	1.02±0.01^{d}	0.45±0.01^{e}	whitish-brown	compact
2,4-D_{1}+BAP	1.89±0.02^{a}	0.86±0.01^{c}	green	compact
2,4-$D_{1.5}$+BAP	1.68±0.02^{b}	0.94±0.02^{b}	whitish	friable
2,4-D_{2}+BAP	1.62±0.01^{b}	1.13±0.01^{a}	whitish	friable
$NAA_{0.5}$+BAP	0.78±0.01^{e}	0.45±0.01^{e}	whitish-brown	friable
NAA_{1}+BAP	0.98±0.01^{d}	0.85±0.02^{c}	whitish-green	Friable
$NAA_{1.5}$+BAP	1.26±0.01^{c}	0.34±0.01^{f}	green	Compact
NAA_{2}+BAP	1.53±0.02^{b}	0.24±0.01^{g}	green	Compact
$IAA_{0.5}$+BAP	0.72±0.02^{e}	0.15±0.01^{h}	green	Compact
IAA_{1}+BAP	0.70±0.03^{e}	0.30±0.01^{fg}	green	Compact
$IAA_{1.5}$+BAP	1.13±0.03^{cd}	0.60±0.01^{d}	green	Compact
IAA_{2}+BAP	1.24±0.04^{c}	0.93±0.03^{b}	green	Compact
ANOVA	***	***	***	***

Means followed by similar letters are not significantly different at 0.05% level of significance using LSD test.

An important approach to callus induction and growth is to determine the optimum amount of growth regulators especially auxin and cytokinin in the culture medium. The proper amount of auxin and cytokinin concentration should be optimized for more biomass and secondary metabolites production [34]. Further, callus growth generally depends on the explant nature and also the auxin and cytokinin amalgamation [35] which may be the fact that exogenously applied PGR's increase the synthesis of endogenous cytokinin and auxin which results in increment in callus growth and mass especially fresh and dry weight as for callus induction and growth, auxins and cytokinins are widely used [36]. The increase in callus biomass (fresh and dry) might also be due to the increase in cell division, elongation, differentiation in vascular tissues, root formation and rhizo-genesis, auxiliary shoot growth inhibition and embryogenesis by auxins and cytokinin [37]. Furthermore, reprogrammed differentiated cells due to methylated DNA than usual by auxin also start cell division and elongation thereby increasing the fresh and dry biomass [29]. The present results are in close conformity with Rehman coauthors [38] who reported that callus biomass was significantly increased with the application of 2,4-D furthermore the addition of BAP enhanced the role of 2,4-D which resulted in increased biomass of *Caralluma tuberculata* callus. The results are supported by Blinstrubiene and coauthors [39] who observed that media when augmented with BAP and 2,4-D significantly increased the callus fresh weight of *Stevia rebaudiana*. These results are in agreement with Aghaei and coauthors [40] on *Pistacia atlantica*, Wang and Bao [41] on *Viola wittrockiana* and Manisha and Rajesh [42] on *Tecomella undulata*. Moreover, these results are also in accordance with Andre and coauthors [43] who reported that a significant increase in callus weight was observed when treated with 2,4-D and BAP. Similar results were also observed by Rahayu and coauthors [44] on *Centella asiatica* who found that the addition of auxin to the media significantly increased callus biomass. These results are also in conformity with Fatima and coauthors [35] on *Digitalis lanata* who reported that an increase in 2, 4-D hormone was related to an increase in dry weight.

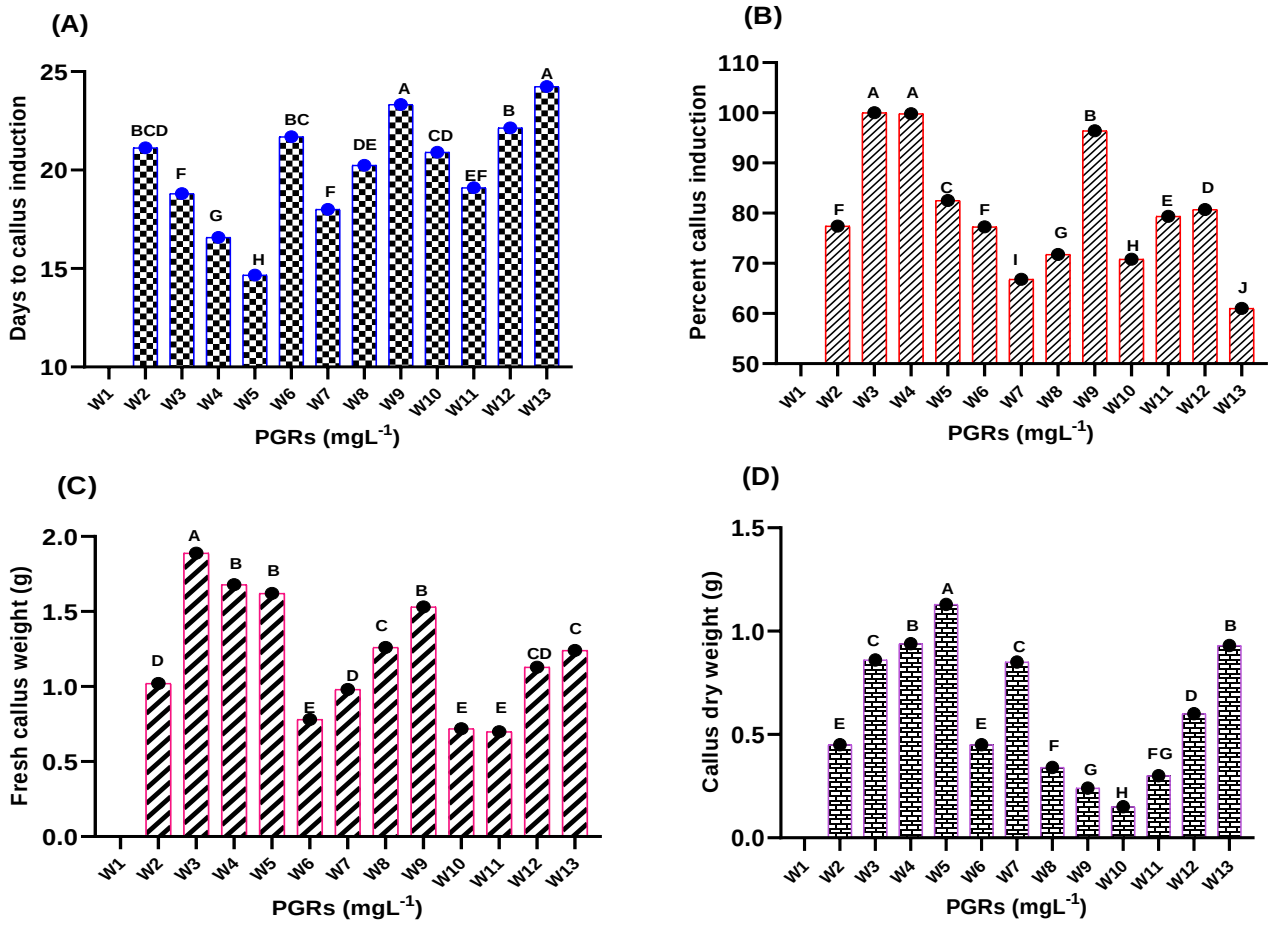

Figure 1. Effect of plant growth regulators for callus fresh weight, dry weight, callus color and texture of *S. moorcroftiana.* W1: Control, W2: 2,4-$D_{0.5}$+BAP, W3: 2,4-D_1+BAP, W4: 2,4-$D_{1.5}$+BAP, W5: 2,4-D_2+BAP, W6: $NAA_{0.5}$+BAP, W7: NAA_1+BAP, W8: $NAA_{1.5}$+BAP, W9: NAA_2+BAP, W10: $IAA_{0.5}$+BAP, W11: IAA_1+BAP, W12: $IAA_{1.5}$+BAP, W13: IAA_2+BAP.

Callus morphology

Callus of whitish brown color and compact texture were observed in media fortified with a combination of 2,4 D (0.5 mg L^{-1}). On the other hand, callus with the same whitish brown color but having afriable texture were observed in media supplemented with NAA (0.5 mg L^{-1}). The calluses of green color and compact texture were found in MS media augmented with 2,4 D (1 mg L^{-1}), NAA (1.5 mg L^{-1}), NAA (2 mg L^{-1}), IAA (0.5 mg L^{-1}), IAA (1 mg L^{-1}), IAA (1.5 mg L^{-1}) and IAA (2 mg L^{-1}). A callus of whitish green color with a friable texture was observed in media containing growth hormones 2,4-D (1.5 mg L^{-1}) and 2,4-D (2 mg L^{-1}). One of the media amongst all supplemented with NAA (1 mg L^{-1}) developed callus of whitish green color with the friable texture (Table 3, Figure 2 and 3).

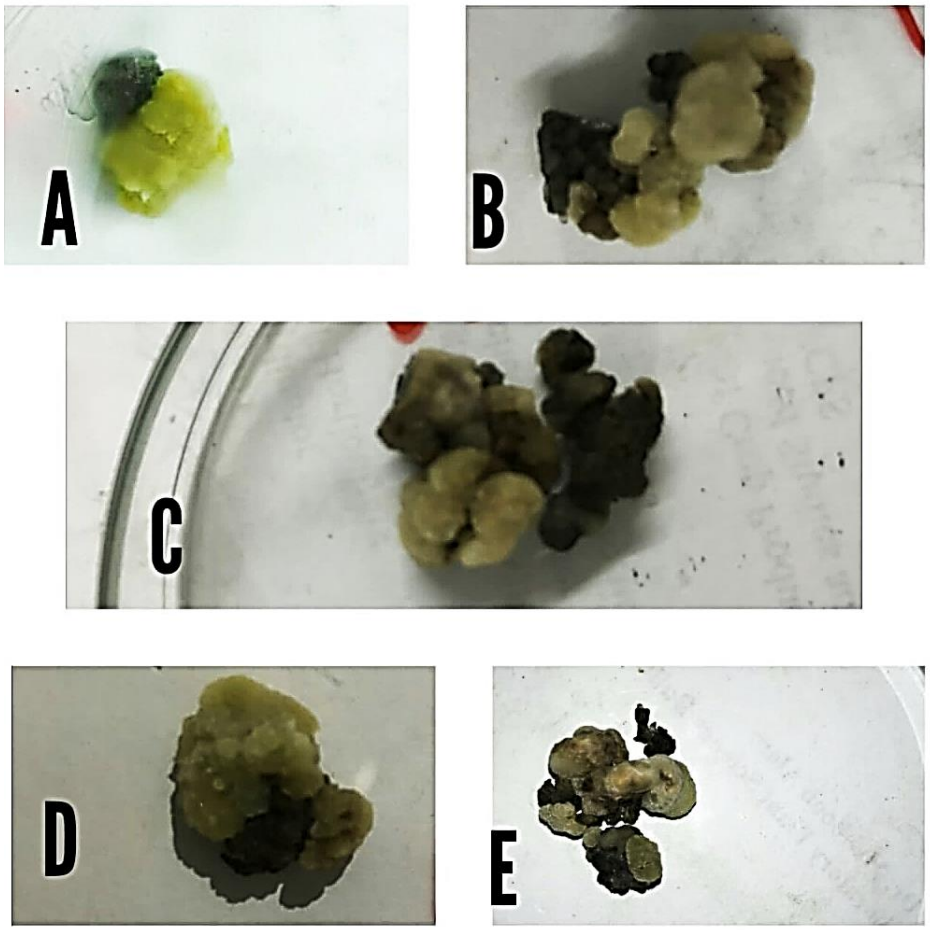

Figure 2. Callus proliferation under various levels of Melatonin. A) Control, B) 0.5, C) 1, D) 1.5, and E) 2 mg L^{-1} of Melatonin keeping 2,4-D + BA

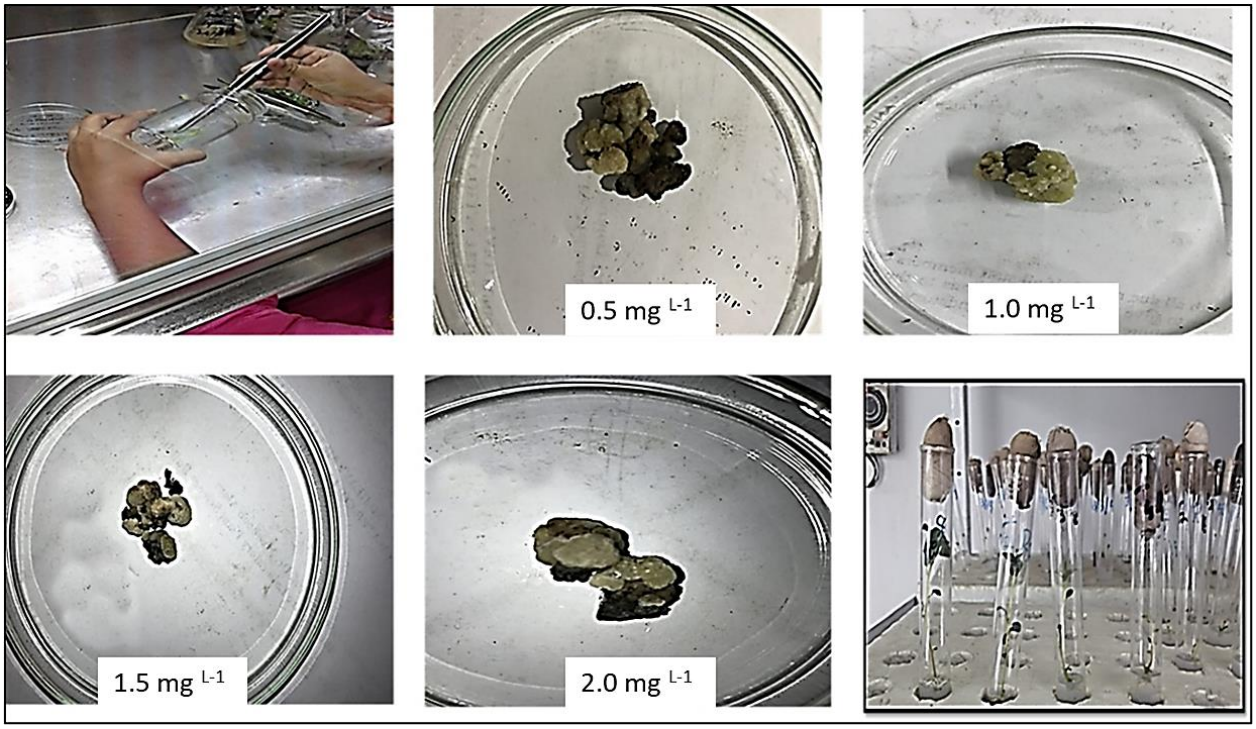

Figure 3. Sampling and culturing of nodal cuttings for multiplication under laminar flow unit, callus formation and plants formation after successful multiplication through *in-vitro* propagation.

Usually, a single explant comprises of callus having various strains which considerably affect the amount of consistency, color and morphogenetic competence of the callus [29]. The color and texture of the callus

can be altered by using different concentrations and types of PGR's (alone and in combination) present in MS media [45]. Application of exogenous PGR's also affects the internal concentration of enzymes and plant hormones. Endogenous plant hormones are synthesized with the help of these exogenous plant hormones [46] thereby affecting callus color, texture and nature. Auxin is dynamic for callus growth and development; it causes various distinct effects with the help of diverse levels used in the culture medium [47]. It is considered that auxin promotes physiological modification, which mainly starts the process of cell division and differentiation. The manipulation of endogenous cytokinin is responsible for the cell aggregation that leads to compact structured callus [48]. Similar results were also observed by Elaleem and coauthors [49] who investigated the medium preparation, concentration and combination of PGR's affect the callus texture, color and nature. Rajaram and coauthors [50] achieved green compact callus of *caralloma fimbriata* callus in the MS medium supplemented with 2, 4-D and BAP. Furthermore, Sreelatha and coauthors [51] attained a compact, green callus from an explant of *Caraloma stalagmifera* cultured on MS medium using 2, 4-D and Kn.

Effect of melatonin on the callus growth and accumulation of phenol, flavonoids and antioxidant activities in callus culture of S. microftiana.

Fresh and dry weight

The fresh and dry weight of the callus was significantly influenced by the different concentrations of melatonin and in combination with the contact level of PGR's auxins. A significant increase in *S. moorcroftiana* callus fresh weight was obtained under different concentrations of melatonin and in combination with constant levels of PGR's (1 mgL^{-1} 2, 4-D and 1 mgL^{-1} BAP). The maximum fresh and dry weight accumulation (2.53 and 1.92 mg L^{-1}) was recorded on MS medium fortified with 1.5 mgl^{-1} melatonin, respectively, followed by fresh (2.11 mg L^{-1}) and dry (1.28 mg L^{-1}) weight in tissue culture media fortified with melatonin at 1 mg L^{-1}. Whereas minimum fresh and dry weight biomass accumulation (1.66 and 0.75 mg L^{-1}) was observed on MS medium containing 2 mg L^{-1} melatonin. This indicated that melatonin is suitable for better fresh and dry weight of callus (Table 4 and Figure 4).

Table 4. Effect of various concentrations of melatonin for callus fresh and dry weight of *S. moorcroftiana*

Melatonin (mg L^{-1})	callus fresh weight (mg L^{-1})	callus dry weight (mg L^{-1})	DPPH activity	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
0	1.87±0.02^{c}	0.86±0.01^{d}	80.98±0.6C	0.86 ±0.01D	0.89±0.01D
0.5	1.74±0.02^{d}	1.16±0.01^{c}	80.02±0.5D	1.12 ±0.01C	1.12 ±0.01C
1	2.11±0.02^{b}	1.29±0.01^{b}	82.11±0.8B	1.29±0.01B	1.33±0.01B
1.5	2.53±0.03^{a}	1.92±0.01^{a}	87.20 ±0.7A	1.92±0.02A	2.19±0.02A
2	1.66±0.01^{e}	0.75±0.01^{e}	79.83 ±0.7D	1.05±0.01C	1.12 ±0.01C
ANOVA	***		***	***	***

Means followed by similar letters are not significantly different at 0.05% level of significance using LSD test

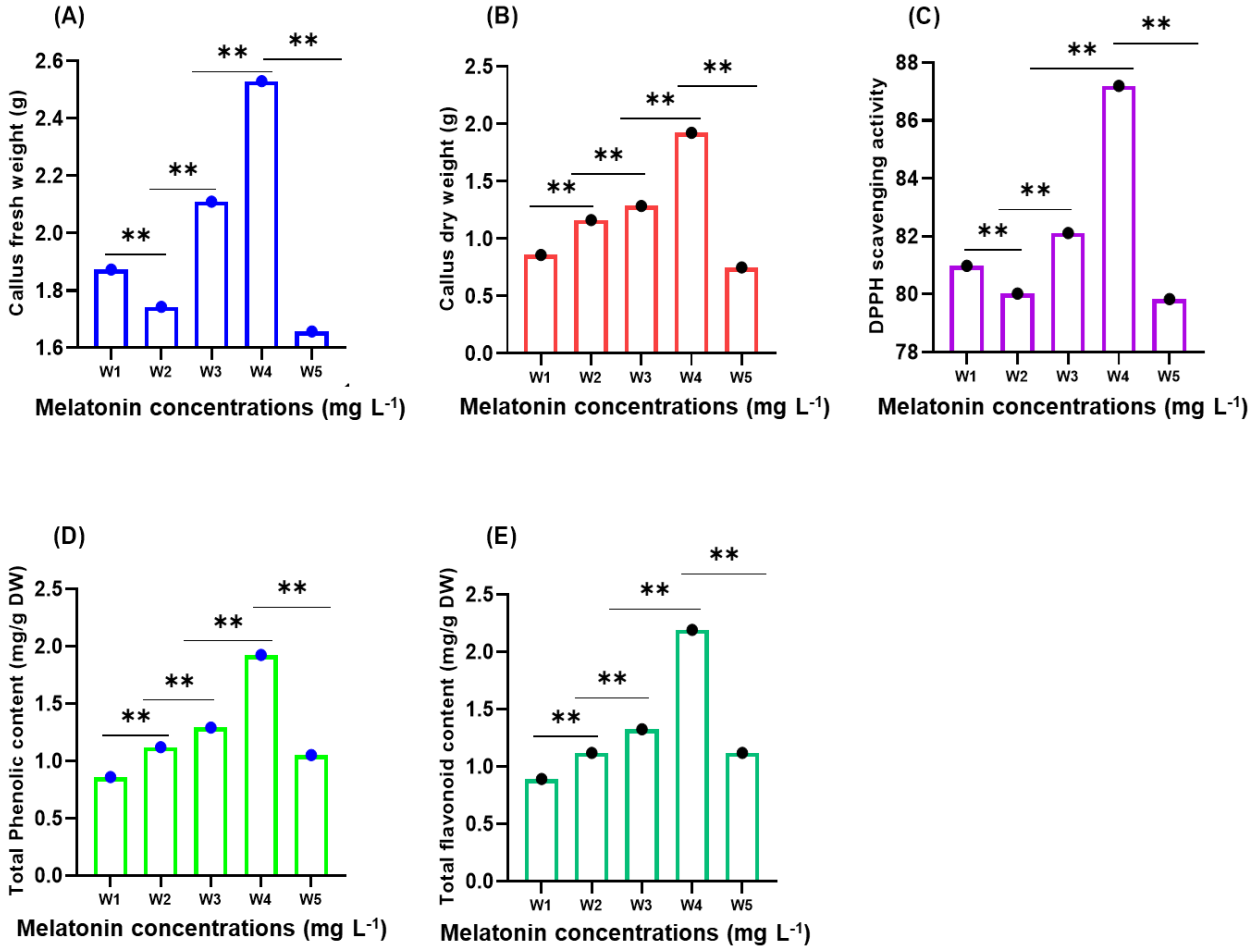

Figure 4. Callus fresh weight (A), dry weight (B), DPPH (C), total phenols (D) and total flavonoids (E) of *S. moorcroftiana* as affected by various concentrations of melatonin. W1: Control, W2: 0.5 mg L^{-1} melatonin, W3: 1.5 mg L^{-1} melatonin, W4: 1.5 mg L^{-1} melatonin, W5: 2.0 mg L^{-1} melatonin.

Melatonin is considered as a promoter of plant growth, development and adaptation [52] and is known to have a stimulatory effect on callus fresh weight and growth which may be because the growth is triggered due to the melatonin stimulated biosynthesis of IAA [53]. Melatonin in combination with auxin also induces the organogenic pattern of plants [54]. Melatonin plays an important role in regulation of photosynthesis, callus growth, formation and root regeneration of explants [55- 65]. The increase in fresh and dry biomass after treatment with melatonin is due to the role of melatonin in cell expansion which increases the cell volume thereby increasing the callus biomass [57]. Furthermore, melatonin has also a role in inhibition of ACC oxidase activity, hence showing greater similarity to auxin in some physiological responses [58], resulting in increased the fresh and dry biomass. In the present study it was also observed that increase in melatonin content from 1.5 to 2 mg L^{-1} decreased the fresh and dry biomass. This might be due to the fact that melatonin in higher concentrations generates ROS which leads to cell apoptosis thereby preventing cellular growth and proliferation [59]. The present results are in conformity with Hernández-Ruiz and Arnao [58] in Lupin cotyledons who reported that a significant increase in biomass was noted in the presence of melatonin. Similar results were also recorded in *Cucumis sativus* [55], *Punica granatum* [60] and *Oryza sativa* [61]. Moreover, a decrease in biomass accumulation of *Fagonia indica* was observed with a higher concentration of melatonin [62].

DPPH free radical scavenging activity

Different concentration of melatonin with constant level of plant growth regulators (1 mg L^{-1} 2, 4-D and 1mg L^{-1} BAP), the antioxidant activity (DPPH) in terms of free radical scavenging activity was determined in *S. moorcroftiana* callus (Table 4). The maximum antioxidant activity (87.197 %) was observed in callus fortified with 1.5 mg L^{-1} melatonin in combination with 1mgL^{-1} 2,4-D and 1mg L^{-1} BAP which was followed by 1 mgL^{-1} melatonin concentration (82%) in combination with 2,4-d and BAP (1mg L^{-1}), while that of minimum

DPPH radical scavenging activity (80%) was recorded in 2 mg L^{-1} melatonin in combination with PGR's which is statistically at par with 0.5 mgL^{-1} melatonin (80.81%).

The DPPH assay reflects the capability to scavenge free radicals, which incorporates hydrogen radicals from potential antioxidants [10]. Melatonin has been reported to enhanced phytochemical constituents and improved bio-reductive capacity [17]. Callus produced in the presence of melatonin proved to have the highest reducing power (RP) because melatonin enhanced the phytochemical constituents (flavonoids and phenolics) in callus cultures by acting as a good reductone (reductones are terminators of free radical chain reactions). Melatonin has been reported to have higher antioxidant activity in different plants than other PGRs [63]. Melatonin also activities antioxidant enzymes [64] which protect plants from oxidative damage and increases the efficiency of the mitochondrial electron transport chain [65] which results in improved antioxidant activity. Bioactive compounds like flavonoids and phenolics acted as an electron donor and scavenge free radicals by converting them into more stable products and electron donation capacity of the plant depends on the amount of bioactive compounds present in it. Similar results were also found by Arnao and Hernandez-Ruiz [16] in *Lupinus albus*. Moreover, Liang and coauthors [66] also confirmed the present results, who reported improved phenolic and antioxidant compounds in the leaves of *Actinidia* spp. (kiwifruit) by exogenous application of melatonin.

Total phenolic content

Under various concentrations of melatonin with a combination of constant levels of plant growth regulators (1 mg L^{-1} 2,4-D and 1mg L^{-1} BAP) (Table 4). The total phenolic content of *S. moorcroftiana* callus was recorded. The maximum total phenolic content was accumulated in callus proliferated on MS medium augmented with 1.5 mg L^{-1} melatonin (1.92 mg g^{-1} DW) in combination with PGR's, which was followed by 1 mg L^{-1} melatonin (1.29 mg g^{-1} DW). However, the minimum total phenolic content yield (0.86 mg g^{-1} DW) was recorded on controlled treatment. While that of maximum melatonin concentration (2 mgL^{-1}) gives the yield of (1.05 mg g^{-1} DW) which is statistically similar to 5 mg L^{-1} melatonin concentration (1.12 mg g^{-1} DW).

Phenolics are low molecular weight anti-oxidative compounds found in various plant species, which are useful against several disorders [67-69]. Physiological responses are positively affected by exogenously applied melatonin [66]. Melatonin also has a beneficial function in signaling by inducing various metabolic pathways and stimulating the production of different secondary metabolite plant substances [70]. Biomass accumulation and phenolic production are directly proportional and strongly dependent on the activation of key enzymes (tyrosine ammonia lyase) responsible for phytochemical production [71] which is greatly activated by melatonin thereby increasing phenolic and antioxidant activity. Melatonin is considered as an antioxidant which directly detoxifies ROS and reactive nitrogen species and also stimulates many antioxidant enzymes indirectly which suppresses pro-oxidant enzyme activity thereby increasing antioxidant activity such as phenolic [72] etc. The up regulation of phenylpropanoid genes by melatonin also results in polyphenol metabolism thereby increasing the phenolic content [73]. The results are in agreement with Riaz and coauthors [74] who observed that Melatonin significantly increased TPC values. Similar results were also found by Sumaira and coauthors [75] and Sheshadri and coauthors [76] also reported that Melatonin greatly enhances the biosynthesis of phytochemicals.

Total flavonoid content (TFC)

In culture media addition of melatonin in combination with constant level of plant growth regulators (1 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP) resulted in significant variation of total flavonoids content accumulation in *S. moorcroftiana* callus culture. Maximum total flavonoid production (2.19 mg g^{-1} DW) was obtained in callus cultured on MS medium supplemented with 1.5 mg L^{-1} melatonin, which was closely followed by 1mg L^{-1} melatonin (1.33 mg g^{-1} DW). However, the maximum level of melatonin is statistically at par with 0.5 mg L^{-1} melatonin concentration (1.12 mg g^{-1} DW) and (1.12 mg g^{-1} DW) respectively. However, the minimum total flavonoid content yield is recorded on control treatment with 0 level of melatonin (0.89 mg g^{-1} DW) (Table 4). Flavonoids are plant secondary metabolites that show *in vitro* and *in vivo* antioxidant activity due to the availability of free hydroxyl (OH^-) groups, particularly 3-OH [77-78]. Flavonoids are a class of phenolic compounds having antimicrobial, antioxidant, anti-inflammatory and allergenic activities [79-83]. Callus having different TFC strongly depends upon PGR type and concentration [82]. The results are in line with those of Riaz and coauthors [74] who reported that application of Melatonin in combination with NAA significantly increased the total flavonoids content in the callus. Similarly, Sheshadri and coauthors [76] also reported that melatonin greatly enhances the biosynthesis of phytochemicals.

CONCLUSION

The current results suggest that using various PGRs and melatonin concentrations significantly enhanced the callus induction and secondary metabolites production in the callus culture of *S. moorcroftiana* (Figure 5). The application of 2,4-D+BAP at rate of 1+1 mg L^{-1} significantly increased the callus induction and callus growth. Callus culture supplemented with 1.5 mg melatonin L^{-1} significantly increased the phenols, flavonoids and DPPH scavenging activity of the callus culture.

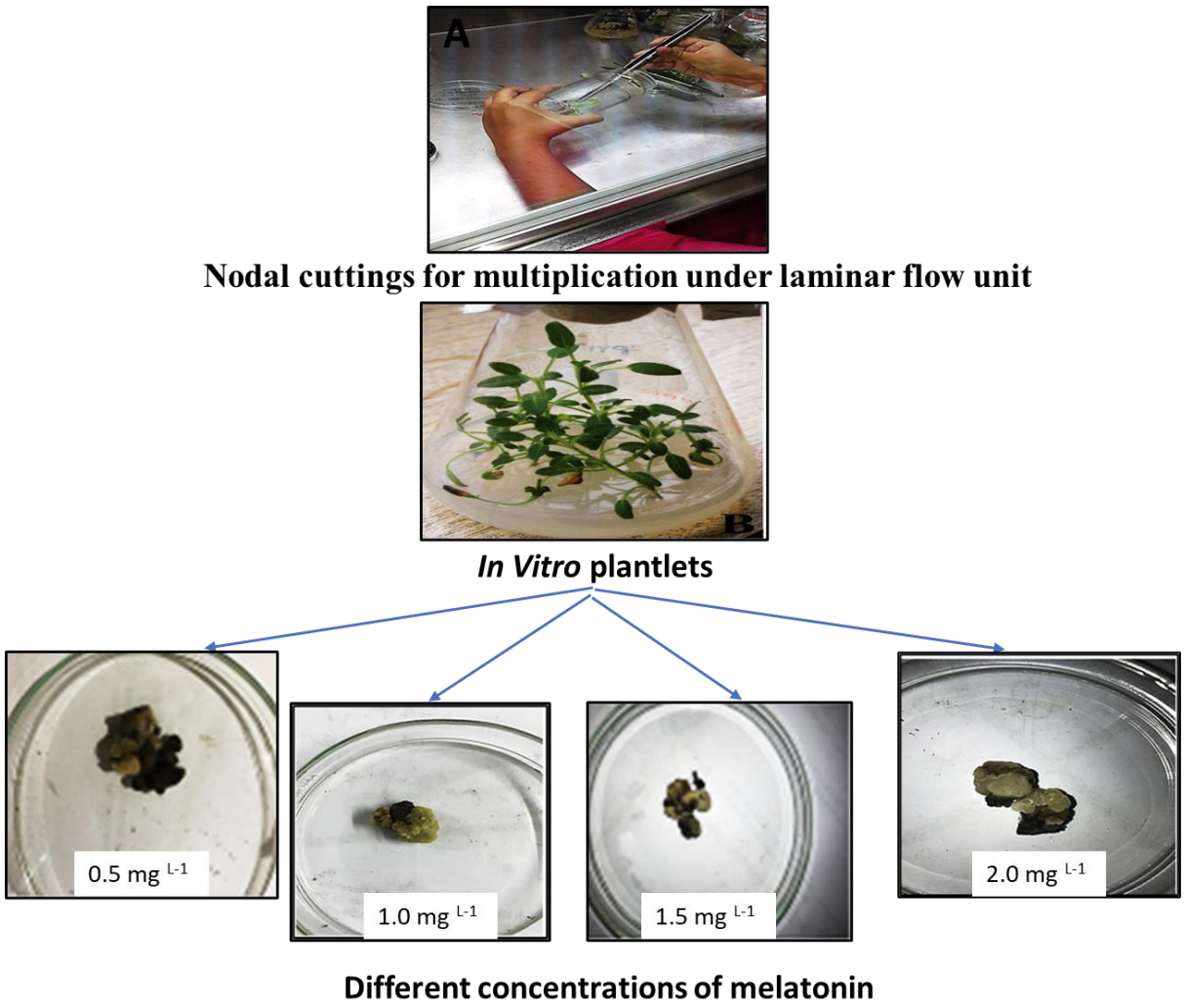

Figure 5. Graphical abstract that shows the effect of different concentration of melatonin on callus shape and color.

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