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EFFECT OF CARBON SOURCE AND INCUBATION TEMPERATURE ON TOTAL CONTENT OF SECONDARY METABOLITES OF CALLUS CULTURE OF *SOLANUM NIGRUM*

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ABSTRACT

Solanum nigrum (Solanaceae) is known as black nightshade. The plant is extensively used to treat severe diseases such as pain, inflammation and fever. The plant is also used as an antitumorigenic, antioxidant, anti-inflammatory, hepatoprotective, diuretic, and antipyretic agent. The present study was designed to observe the effects of carbon source and incubation temperature on callus growth, and secondary metabolite content in *S. nigrum*. Murashige and Skoog (MS) basal medium supplemented with different concentrations of plant growth regulators and various concentrations of glucose, sucrose, and fructose was used for the study. The incubation temperatures such as 20, 25, 30 and 35°C were used on callus culture of *S. nigrum* for assessment of total phenolic, flavonoids and alkaloid content. The weight of biomass produced and content of secondary metabolites were determined. The results revealed that sucrose was superior to glucose and fructose for callus growth and secondary metabolites formation, and the optimum concentration of sucrose was 3%.The optimum incubation temperature was 30°C for the production of biomass and secondary metabolites. It can be concluded that sucrose as carbon source was superior to glucose and fructose for callus growth and secondary metabolite accumulation.

KEYWORDS: *Solanum nigrum* L, Total phenolics, Flavonoids, Alkaloids. Secondary metabolites.

INTRODUCTION

Solanum nigrum Linn. (Solanaceae) commonly known as Black Berried Nightshade is a short-lived perennial shrub or common herb, found in many wooded areas. *S. nigrum*, distributed throughout India, Ceylon and all temperate and tropical regions of the world (Kirtkar and Basu, 1999). The leaves are known to be used to treat diseases of nose and headache (Kirtkar and Basu, 1999), ringworm (Rana and Datt, 1997), liver and heart ailments, burns and wounds (Dafni A and Yaniv, 1994), toothache (Bocek,1984). The ethnomedical information indicated that the juice of dried leaves of *S. nigrum* is used for lowering blood sugar level (Villasenor and Lamadrid, 2006). Further, the aqueous extract of dried leaves is used as antiviral (Roychoudhury and Basu, 1983), antipyretic, anticonvulsant, sedative, antimalarial, antispasmodic & diaphoretic (Adesina, 1982), molluscicidal (Adesina et al., 1987), anti-bronchitis & anti-gastralgia (Darias et al., 1986) agent. The hot aqueous extract of the fruits and leaves as a folk medicine is used for the treatment of *diabetes mellitus* by the *Kondh* tribes of Orissa in India. Several phytoconstituents such as Quercitrin, Hyperoside (Nawwar et al.,1989), Sitosterol, Solamargine, Stigmastrol, Campesterol, Cholesterol (Bhatt and Bhatt, 1984), Solasodine (Verbist et al., 1977) are reported in the leaves.

The production of useful secondary metabolites via plant tissue and organ culture has been reported by many researchers. Many efforts have been made to improve the productivity of the plant tissue cultures, such as studies on hormone-dependency, media composition, temperature and light exposure. (Tuteja and sopory, 2008; Karuppusamy, 2009). Many researchers have tried to enhance anthocyanin accumulation through the manipulation of phytohormones in cell suspensions of strawberry *Fragaria ananassa* (Nakamura et al., 1999), *Daucus carota,*(Narayan et al., 2005) *Ipomoea batatas* and *Oxalis reclinata* (Nozue et al., 1995).

In recent years several reports demonstrated that various carbon sources influence in vitro callus growth, morphology and production of secondary metabolites in different plant species (Biahoua & Bonneau, 1999; Petersen et al., 1999; Fuentes et al., 2000).

The external conditions such as light, temperature, humidity affect growth and composition of secondary metabolites of callus (Morison and Lawlor 1999). Temperature is one of the climatic factors which act as major abiotic environmental stressor that limit plant growth and development. With respect to this phenomenon, the culture temperature also plays vital role in cell culture techniques for cell growth, morphology and secondary metabolites production. In present investigation the effect of plant growth regulator on callus growth and morphology was studied. The effect of different carbon sources and temperature on biomass and secondary metabolites production was also investigated. No such type of work on this plant has been reported so far.

MATERIALS AND METHODS

Plant Material

The leaves of *S. nigrum* were collected from herbal garden of Hamdard University New Delhi. India and authenticated by Taxonomist. The leaves sections were used as explants for *in vitro* study. The plant herbarium was deposited in Department of Pharmacognosy and Phytochemistry, faculty of Pharmacy, Hamdard University for further reference. Vide voucher number DPP/FP/JH/SN-050.

Surface sterilization of explants

The leaf explants were washed thoroughly with 2 % Tween-20 followed by running tap water for 10 min and then washed thrice with distilled water. The explants were then dipped in 70% ethanol for 1 min, followed by surface sterilization with 0.1% mercuric chloride for 10 min and finally rinsed thrice with sterile double distilled water. The explants were surface-dried on sterile filter paper and transferred on Murashige and Skoog (MS) basal medium supplemented with different concentrations and combinations of plant growth hormones (Murashige and Skoog, 1962).

Culture media and culture conditions for callusing

The surface sterilized explants were transferred on Murashige and Skoog (MS) medium containing 3% sucrose and 0.8% agar. The pH of all cultures were adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The explants were cultured on Murashige and Skoog (MS) medium fortified with different plant growth regulators alone or in combination for callus induction (Table1). All the cultures were incubated at 25 ± 2 ^{o}C for 16 hours photoperiod in a BOD incubator (YORCO Pvt Ltd India). The observations and results were recorded and presented in (Table1).

Effect of carbon source

The callus cultures were subcultured on MS medium fortified with 2, 4-D 0.5 mg/L and NAA 2 mg/L. In addition to sucrose, two other carbohydrates (fructose and glucose) were used at 3, 4 and 6 % in MS medium to observe the effects of these carbon sources on biomass and secondary metabolites production. The effect of various carbon sources on biomass and secondary metabolites production was observed and recorded.

Effect of Incubation temperatures

The callus cultures were subcultured on MS medium fortified with 2, 4-D 0.5 mg/L and NAA 2 mg/L and they were kept under various temperatures such as 20 ^{0}C, 25 ^{0}C, 30 ^{0}C and 35^{0}C. The effect of various incubation temperatures on biomass and secondary metabolites production was observed and recorded.

Determination of Total Phenolics Content

The quantity of phenols was determined according to the Folin and Ciocalteau's procedure. In this procedure, gallic acid was used as a standard where concentrations of 0.01, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mg/ml of gallic acid were prepared in methanol. Concentrations of 1 mg/ml of callus extract were prepared in methanol. 0.125 ml of each sample was mixed with 0.625 ml of a ten-fold diluted Folin-Ciocalteau's reagent and 0.5 ml of 7.5% sodium carbonate ($Na2CO_3$). The mixture was allowed to stand for 30 minutes at room temperature before the absorbance was read at 760 nm (UV Spectrophotometer, Shimadzu, Japan). All determinations were performed in triplicates. The total phenolic content was expressed as percentage in mg gallic acid equivalent (GAE) per gram of plant material on dried basis (Makkar et al., 1997).

Determination of Flavonoids content

Total flavanoid content was determined by Aluminium Chloride ($AlCl_3$) colorimetric method. Quercetin was used as standard in concentrations of 0.01, 0.02, 0.04, 0.06, 0.08, 0.10 mg/ml quercetin in methanol. Concentrations of 1 mg/ml of callus extract were prepared in methanol. 0.5 ml of each sample was added with 0.5 ml of 2% $AlCl_3$ in methanol. The mixture was allowed to stand for 1 hour at room temperature before the absorbance was read at 420 nm. All determinations were performed in triplicates. The total flavonoids content was expressed as percentage in mg quercetin equivalent per gram of plant material on dried basis (Chang et al., 2002)

Determination of Alkaloids

3.5 gram of the callus was prepared in a beaker and 200 ml of 10 % acetic acid in ethyl alcohol was added to the extract sample. The mixture was covered and allowed to stand for 4 h. The mixture then filtered and the extract was allowed to become concentrated in a water bath until it reached to one quarter of the original volume. Concentrated ammonium hydroxide was added until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed (Mustapha and Harun, 2014).

Experimental Design and Data Analysis

The study was conducted according to Completely Randomized Design (CRD). All the experiments were done in triplicate and the data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test (DMRT), using the SPSS software version 17. Values expressed are mean of three replicate determinations ± standard error.

RESULTS AND DISCUSSION

Effects of plant growth regulators on biomass production and morphology of callus

The auxins 2,4-D, IAA and NAA are commonly used to induce callus in plant tissue culture studies. Auxin, mainly 2,4-D have been reported to be as most effective plant growth regulator for callus induction and growth of callus (Sun et al., 2006) and (Burbulis et al., 2007). In present study, different type and concentration of auxins were tested to induce the formation and subsequent growth of callus from leaf explants. The auxins 2,4-D; NAA and IAA were added to MS basal medium. In all combinations tested, the callus initiation did not occur without plant growth regulators (PGRs control) after eight weeks of culture. The addition of auxins in culture medium allowed the induction and proliferation of various types of callus from leaf explant. Callus induction began at the periphery before expanding gradually to completely cover the explants. Variability was observed in the colour and texture of callus depending on the type of auxins. With 2,4-D, callus was friable and white. In the presence of NAA greenish white callus was produced. On the medium containing the IAA, induction of compact and brown callus was recorded. The induction of different morphological types of callus in the presence of various concentrations of auxins has been already reported in *Curcuma mangga*. (Sundram et al., 2012). The explants inoculated on MS medium supplemented with plant growth hormone showed callus induction. MS medium

amended with 2,4-D 0.5 mg/L with NAA 1.0 mg/L produced greenish white friable callus with maximum fresh weight (3.5 ± 0.08) and dry weight (0.40 ± 0.04) (Table-1). MS medium fortified with 2,4-D 0.5 mg/L with IAA 1.0 mg/L produced white coloured friable callus with maximum fresh weight (2.9 ± 0.06) and dry weight (0.32 ± 0.09). No callus induction was noted from explants placed on the control medium. This result shows that the presence of auxins in MS basal medium is essential to inducing callus in *S.nigrum*. A plant growth regulator is a key factor responsible for callus initiation and development in plant cell cultures. Similar observations were reported by Haouala et al. (2010) for *Gerbera jamesonii*, and Ahmed et al. (2011) for *L. nodiflora*. When the medium is fortified with low concentrations of NAA leaf explants did not induce callus. The effectiveness of high concentrations of NAA has been demonstrated in callus induction in *L. junelliana* (Hector et al., 1999) and *Quercus suber*. (El Kbiach et al., 2002). Gupta et al., (2001) reported in *L. alba* that high concentrations of NAA inducing callus formation from explant. Similar results have also been observed in *Cucumis sativus* (Saeid et al., 2013) and *Gmelina arbore*. (Sukartiningsih et al., 1999). The low concentrations of NAA are insufficient to induce callus formation while high concentrations of 2, 4-D exert a depressive effect. Evans et al., (2003) reported that synthetic auxins like 2,4-D may possess herbicidal property at high concentrations which might inhibit callus formation.

Effects of carbon source on biomass production

Bishnoi et al., (2000) reported that carbohydrates have two roles in culture medium, both as source and as osmotic pressure regulator, which are both important for callus induction. Nowak et al. (2004) stated that sugars do not only act as an external energy source but also contributes to the osmotic potential of the medium which would allow the absorption of mineral nutrients in medium, essential to the cell growth and thereby the optimal osmotic pressure necessary for optimal proliferation. Then, it is necessary to investigate the best carbon source for callus induction (Mendoza and Kaeppler, 2002). On media supplemented with different types of carbohydrates, callus was induced from leaf explants. Statistical analysis revealed that type of sugar affected callus induction and cell proliferation (Table-2, figure 2). The significant effect of carbon source on the frequency of callus formation and callus growth was also reported in many plants such as olive (Gracia et al. 2002) and rice (Shahnewaj and Bari, 2004). The rate of callus formation in medium supplemented with sucrose is significantly higher than those obtained on media containing fructose. In terms of cell proliferation, sucrose followed by glucose exhibited the highest callus biomass. The

lowest cell proliferation was observed with media containing fructose. These results indicate that callus induction from leaf explants in *S. nigrum* depends strongly on the nature of the carbon source. The favorable effect observed with sucrose in this study could be explained by the fact that this sugar is more easily assimilated by cells of *S. nigrum.* In contrary to results recorded in present study, better response to callus formation was obtained on glucose supplemented media by many reserchers (Kouakou et al., 2008 and Burbulis et al., 2007). Thompson and Thorpe (1987) reported that sucrose is the carbon source most frequently used for callus induction and plant regeneration. The results obtained in present investigation showed that sucrose is always the best carbon source for callus. Out of the four types of carbohydrates used, sucrose has promoted the maximum callus formation. Different concentrations of this sugar have been investigated. A highly significant effect of sucrose concentration was revealed by statistical analysis. Callus growth increased regularly to reach highest values at a concentration of 3%. Beyond this concentration, a significant reduction was observed in callus induction growth and cells proliferation (Figure 2). Callus formation from leaf explants depends on the concentration of sucrose in the medium. A significant effect of the concentration of the carbon source in the culture medium on the frequency of callus formation was also noted in many plants such as rice (Shahnewaj and Bari, 2004) and olive (Gracia et al., 2002). Similarly, Al-Khateeb (2008) found a significant difference in callus weight with *Phoenix dactylifera* when explants were placed on media fortified with different concentrations of carbon source. Gerdakaneh et al., (2009) has also been reported in *Fragaria xananassa* variability in response to callus formation in the presence of different concentrations of sugar. In this study, 3% was the optimum concentration of sucrose to maintain maximum callogenesis response from leaf explants of *S. nigrum*. The increase in callus weight was observed from 2% to 3% of sucrose might be due to a gradual increase in the osmotic stress induced by the medium increasingly concentrated with sugar (Juhasz et al., 1997). Addition of sugar in culture medium acts as an osmotic agent that may induce osmotic stress. This increasing osmotic stress probably causes greater accumulation of secondary metabolites. However, above certain concentration of sugar 3%, osmotic stress led to a decrease in cell growth (Kim and Kim, 2002).

Effect of carbon source on secondary metabolites production

To study the effect of carbon source on secondary metabolite production (total phenolics, flavonoides and alkaloids) the callus cultures were inoculated on MS medium containing different type of sugars in different concentrations. The content of secondary metabolites was

highest in the case of callus cultured at 3% sucrose followed by glucose and fructose. The rate of consumption was attributed to manipulation of sucrose, while autoclaving the medium, is converted into glucose and fructose thus glucose being utilized first supports the growth of cells and tissues while fructose is less efficient. Similar results were reported earlier. Kadkade, (1982) reported that sucrose was the best carbon source for the production of podophyllotoxin in callus culture and glucose, fructose and galactose were less effective. Bondarev et al (2003) registered optimum accumulation of steviol glycoside in callus cultured on medium containing 3 % sucrose. Addition of carbon sources increased the production of paclitaxel in callus cultures (Ketchum et al., 2003). Vineesh et al., (2007) observed that MS medium fortified with 3% sucrose was the best for the production of camptothecin.

Effect of various incubation temperatures on biomass and morphology of callus

Temperature is one of the important climatic factors which act as major abiotic environmental stressor that limit plant growth and development. With respect to this phenomenon, the culture temperature also plays vital role in cell culture techniques for cell growth, morphology and secondary metabolites production. The callus culture of *S. nigrum*. maintained on MS medium containing 2,4-D (0.5 mg/L) + NAA (1mg/L) under the exposure of various incubation temperatures such as 20, 25, 30 and 35°C for 4-12 weeks to study the morphogenetic response, total phenolic, flavonoids content and alkaloids content of the callus. The callus grown under various temperatures showed difference in callus biomass and slight variation in callus morphology. The influences of incubation temperatures 25 ± 2°C and 30 ± 2 °C on growth of rice callus were studied earlier. Summart et al., (2008) reported significant difference in biomass growth and callus morphology was reported when they were cultured at above mentioned temperature. Similar results were found in our investigation. The callus grown under 20 and 30°C were produced 2.8 ± 0.05 and 4.8 ± 0.05 gram respectively (Figure 3). Moderate amount of callus was proliferated (3.9 ± 0.08 gram) under the temperature 25°C. Under all the temperature stress the texture of the callus was compact but there was insignificant variation in the colour. The temperature 20 and 25°C influenced brown colour callus formation where as 30 and 35°C alter the colour in to blackish brown (Figure1).

Effect of various incubation temperatures on secondary metabolites accumulation

The total phenolics, flavonoids and alkaloids content were considerably altered in the callus tissue under various incubation temperatures. The maximum content of total phenolic, flavonoids and alkaloid 8.6 ± 0.19, 4.2 ± 0.02 and 7.3 ± 0.09 respectively were estimated in the callus tissues grown under the temperature of 30°C (Table 3). The lowest temperature at 20°C was highly reduced the above mentioned secondary metabolites of callus tissue. Moderate amount of secondary metabolites was produced under the temperature 25°C and 35°C (Table 3). Thanonkeo et al., (2006) reported that temperature played an important role in the growth and isoflavone production from callus cultures of *Pueraria candollei*. Over two fold of growth and three fold of isoflavone production were observed at 32 ± 2ºC. Influence of incubation temperatures on total phenolic, flavonoids content and free radical scavenging activity of callus from *Heliotropium indicum* was reported by Kumar et al., 2012.

Temperature strongly influences metabolic activity and plant ontology, and high temperatures can induce premature leaf senescence (Morison and Lawlor 1999). Carotenoids in Brassicaceae, including β-carotene, were found to be slightly decreased after thermal treatments(Morison and Lawlor 1999). Elevated temperatures increase leaf senescence and root secondary metabolite concentrations in the herb *Panax quinquefolius* (Juchoum et al., 2007). Elevated temperatures by 5°C would reduce photosynthesis and biomass production of *P. quinquefolius*, on the contrary storage ginsenoside is reported to be enhanced (Gera et al., 2007). Several studies have examined the effects of increased temperatures on secondary metabolite production of plants (Morison and Lawlor 1999). Lower soil temperatures caused an increase in levels of steroidal furostanol and spirostanol saponins (Szakiet et al., 2010) Temperature variations has multiple effects on the metabolic regulation, permeability, rate of intracellular reactions in plant cell cultures(Morison and Lawlor 1999). Changing the culture temperature may change the physiology and metabolism of cultured cells and subsequently affect growth and secondary metabolite production (Morison and Lawlor, 1999). Temperature range of 17–25°C is normally used for the induction of callus tissues and growth of cultured cells. Yu et al., (2005) reported the influence of temperature and light quality on production of ginsenoside in hairy root culture of *Panax ginseng*. Chan et al., (2010) reported that *Melastoma malabathricum* cell cultures incubated at a lower temperature range (20 ± 2°C) grew better and had higher anthocyanin production than those grown at 26±2°C and 29±2°C. Optimum temperature (25°C) maximizes the anthocyanin yield as demonstrated in cell cultures of *Perilla frutescens* (Zhong and Yoshida, 1993) and strawberry (Zhang et al.,

1997) Lower temperature favors anthocyanin accumulation, but reduces cell growth. For strawberry cell culture, maximum anthocyanin content was obtained at 15°C and it was about 13-fold higher than that obtained at 35°C (Zhang et al., 1997). For suspension cultures of *Perilla frutescens*, anthocyanin production was remarkably reduced at the relatively high temperature of 28°C, whereas 25°C was optimal for the productivity of the pigment (Zhong and Yoshida, 1993). Similar observations on optimal productivity of anthocyanin in cell suspension cultures of *Daucus carota* was reported (Narayan et al., 2005) Pigment release from hairy root cultures of *Beta vulgaris* under the influence of different temperatures was reported (Thimmaraju et al., 2005).

Table 1: ***Effect of plant growth hormone on biomass and morphology of callus induced from leaf segment of S. nigrum.***

Plant growth hormone (mg/L) MS + IAA (1) + 2,4-D	**Fresh wt (gram)**	**Dry wt (gram)**	**Morphology of callus**	
0.00	0.00^{a}	0.00^{a}	**Colour**	**Texture**
0.1	1.2 ± 0.08^{b}	0.10 ± 0.06^{c}	Reddish brown	Compact
0.5	2.9 ± 0.06^{e}	0.32 ± 0.09^{d}	White	Friable
1.0	1.6 ± 0.06^{d}	0.14 ± 0.05^{c}	Brown	Compact
1.5	1.4 ± 0.05^{c}	0.09 ± 0.06^{b}	Blackish brown	Compact
2.0	1.1 ± 0.07^{b}	0.06 ± 0.06^{b}	Dark brown	Nodular
MS + NAA (1) + 2,4-D				
0.00	0.00^{a}	0.00^{a}	--	--
0.1	1.4 ± 0.04^{c}	0.13 ± 0.05^{c}	Brown	Compact
0.5	3.5 ± 0.08^{e}	0.40 ± 0.04^{d}	Greenish white	Friable
1.0	1.7 ± 0.03^{d}	0.16 ± 0.05^{c}	Reddish brown	Nodular
1.5	1.2 ± 0.01^{b}	0.10 ± 0.06^{b}	Dark brown	Compact
2.0	1.0 ± 0.02^{b}	0.08 ± 0.06^{b}	Light brown	Nodular

In a same column, values followed by the same letters are not significantly ($P < 0.05$) different.

Table 2: ***Effect of carbon source on secondary metabolites production*.**

Sugars	**Concentration %**	**Total Phenolics (mg/gm)**	**Total flavonoids (mg/gm)**	**Total alkaloids (mg/gm)**
Glucose	3	6.2 ± 0.16^{b}	0.81 ± 0.06^{c}	3.9 ± 0.08^{c}
	4	4.5 ± 0.31^{d}	0.54 ± 0.01^{d}	2.5 ± 0.05^{d}
	6	2.4 ± 0.09^{f}	0.28 ± 0.06^{e}	1.8 ± 0.03^{e}
Fructose	3	4.8 ± 0.11^{d}	0.63 ± 0.04^{b}	2.3 ± 0.07^{d}
	4	3.0 ± 0.12^{e}	0.40 ± 0.02^{d}	1.2 ± 0.05^{e}
	6	1.2 ± 0.09^{g}	0.11 ± 0.06^{e}	0.08 ± 0.01^{f}
Sucrose	3	7.5 ± 0.12^{a}	2.5 ± 0.08^{a}	5.5 ± 0.06^{a}
	4	5.7 ± 0.23^{c}	$1.9. \pm 0.03^{b}$	$4.1. \pm 0.03^{b}$
	6	4.2 ± 0.17^{d}	1.4 ± 0.07^{b}	2.6 ± 0.04^{d}

In a same column, values followed by the same letters are not significantly (P< 0.05) different.

Table 3: ***Effect of temperature on secondary metabolites production.***

Temperature ^{0}C	Total Phenolics (mg/gram)	Total flavonoids (mg/gram)	Total alkaloids (mg/gram)
20	5.2 ± 0.13^{d}	1.6 ± 0.03^{d}	3.8 ± 0.02^{c}
25	7.8 ± 0.15^{b}	2.8 ± 0.06^{c}	6.2 ± 0.08^{b}
30	8.6 ± 0.19^{a}	4.2 ± 0.02^{a}	7.3 ± 0.09^{a}
35	7.2 ± 0.11^{c}	3.4 ± 0.07^{b}	6.2 ± 0.04^{b}

In a same column, values followed by the same letters are not significantly (P< 0.05) different.

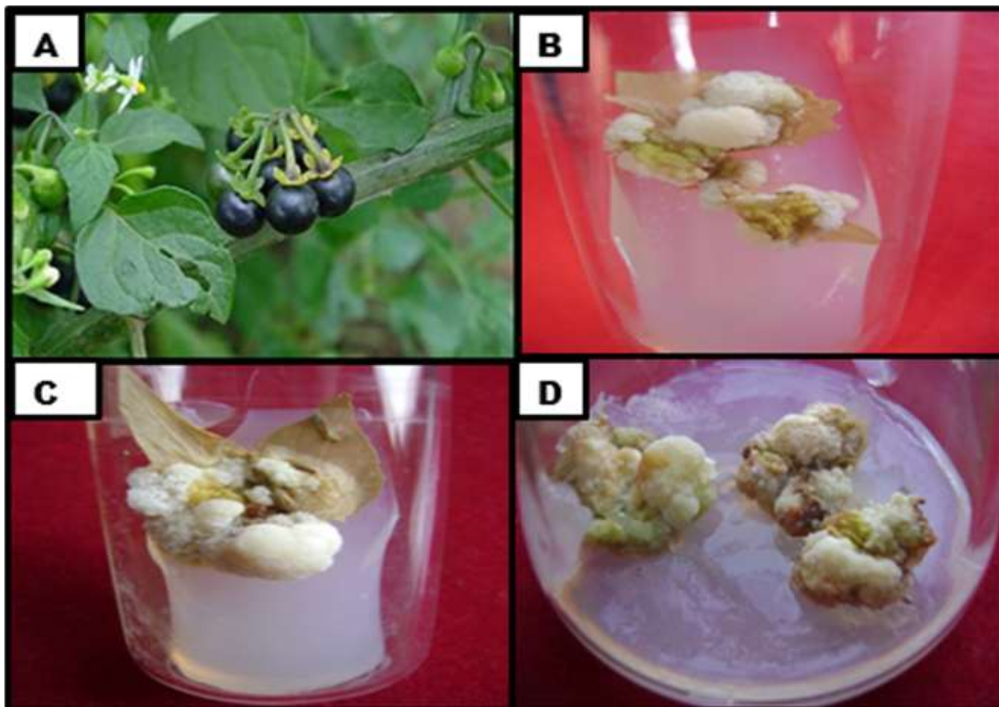

Figure 1: Effect of carbon source and temperature on biomass production of *S.nigrum*.

A: Natural plant with flowers and fruits, B: Proliferation of leaf callus on MS medium with 3 % sucrose at 30 ^{0}C (4 weeks old), C: : Proliferation of leaf callus on MS medium with 3 % sucrose at 30 ^{0}C (8 weeks old), D: : Proliferation of leaf callus on MS medium with 3 % sucrose at 30 ^{0}C (12 weeks old).

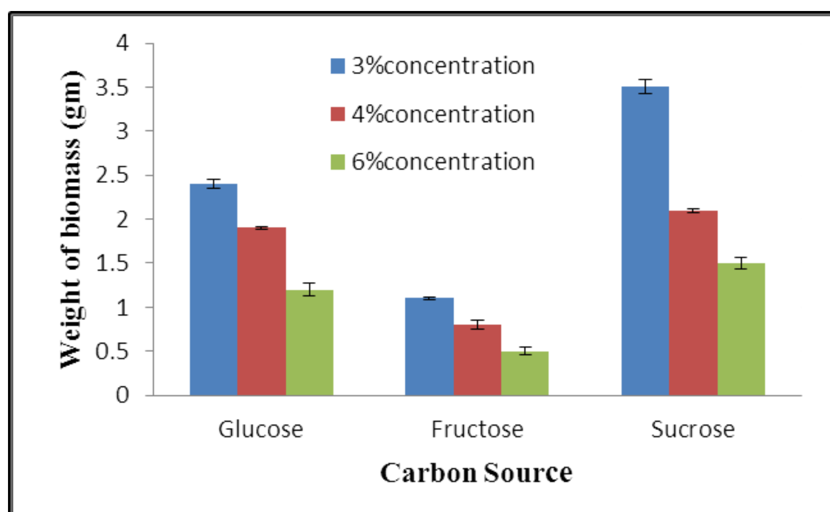

Figure 2: Effect of different carbon sources on biomass production.

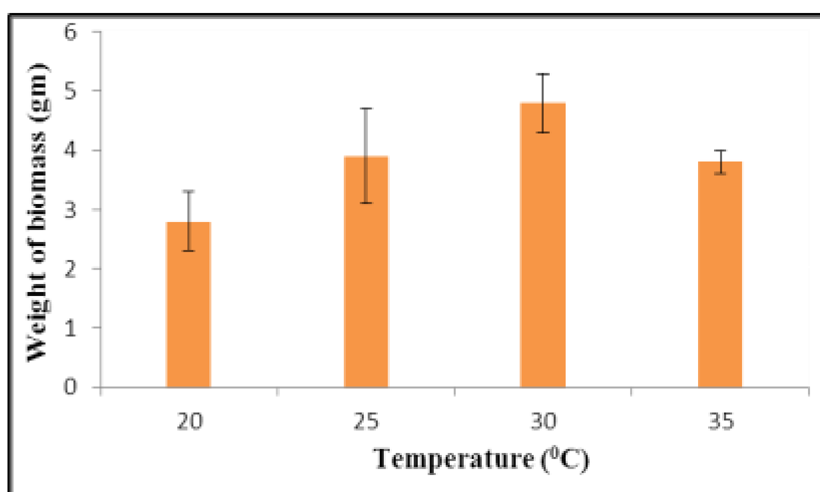

Figure: 3 Effect of different temperature on biomass production.

CONCLUSION

The callus grown under various incubation temperatures was studied for their biomass, total phenolic, flavonoids and alkaloids content. In callus morphology there was no difference in the texture but the callus colour among the treatments show slight variations. There was positive correlation between the callus growth and total secondary metabolites content among the various treatments. However the callus produced at 30°C shows maximum amount of secondary metabolites. Sucrose as carbon source was found the best for biomass and secondary metabolites production.

Conflict of interest

The authors declare no conflicts of interest.

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