International Journal of Research

International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 07, July 2015
Available at http://internationaljournalofresearch.org

Studies on Biochemical Changes in Calli of Immature Embryos during Culturing of *Triticum aestivum* L. (var. WH711)

Sonali Malik[1]; Anita R. Sehrawat* & Krishan D. Sehrawat[2]

1. Department of Botany M.D. University, Rohtak, Haryana, India
(Email: 09sonalimalik@gmail.com)
* *Corresponding author*, Department of Botany M.D.U., Rohtak, Haryana, India
(Email: anitarsehrawat@gmail.com)
2. Department of Genetics and Plant Breeding CCS HAU Hisar, Haryana, India
(Email: krishanssehrawat@gmail.com)

Abstract

This experiment was performed to evaluate the effect of different concentrations of 2, 4-D, types of carbon sources and gelling agents on callus induction and biochemical changes in total sugar and total protein during culturing of immature embryos of wheat var. WH711. MS medium having 30.0 g/l sucrose + 9.0 g/l agar +3.0 mg/l 2, 4-D was the best (100%) for callus induction. The least response of callus formation was observed when MS medium was supplemented with Isabgol as the gelling agent in all the tested combinations. Biomass profile based on the fresh and dry weight was maximum on MS medium supplemented with 30.0 g/l glucose + 3.0 mg/l 2, 4-D + 9.0 g/l agar. But, when 2, 4-D concentration was 5.0 mg/l, the best callus growth in terms of fresh and dry weight was observed on medium having 30.0 g/l sucrose instead of glucose. Biochemical analysis of callus on MS medium containing 30.0 g/l glucose, 9.0 g/l agar and 3.0 mg/l 2, 4-D accumulated maximum amount of reducing sugar and protein, whereas the least accumulation was observed in the medium having Isabgol as the gelling agents in all the media. Maximum growth, protein and reducing sugar were accumulated on medium having glucose as the carbon source.

Keywords: *Tritcum aestivum*; 2; 4 –D; Sucrose; Isabgol; Immature embryo

Introduction

Wheat (*Triticum aestivum* L.), the most important cereal crop, ranks second as the world and Indian staple food only next to the rice. As a rabi crop it is cultivated almost in all parts of the world except for the Polar Regions (FAOSTAT, 2012). Although the total wheat producing area has decreased from approximately 221 million hectares to 216 million hectares, there is considerable increase in the production of about 100 million metric tonnes from 1993 to 2012 (USDA, 2013). India, in year 2011-12, recorded production of about 93 million tonnes (Directorate of Economics and Statistics, India 2013). An important attribute of wheat is its adaptability to varied climatic conditions which has focused the attention of researchers on improving its yield and making it tolerant to stress conditions and resistant to various diseases to solve the problem of growing food crisis in India and the world also. Cells undergo a series of morphological and biochemical changes in tissue culture which results in the formation of callus. Efficient callus formation and plant regeneration depend on various factors like genotype, explant source, growth conditions of donor plant and culture medium. Wheat immature embryos and scutella have proved to be the

most efficient sources for regenerating whole plants not only in bread wheat (Barro et al. 1999; Bahman et al. 2012). Plant cell and tissues in *in vitro* do not have autotrophic ability and so require exogenous carbon source for their energy needs. Sucrose has been the main source of carbon for such *in vitro* culture of the plants (Ahmad et al. 2007). The optimal concentration of sucrose in the medium should be enough to satisfy the needs of cell division and differentiation without having any negative osmotic effect on shoot formation (Javed and Ikram 2008). Gelling agent is the base of the medium and decides its efficiency. Agar is the most common and most preferred gelling agent for use (Afrasiab and Jafar 2011); other options include agarose and Isabgol (Aggrawal et al. 2008; Das and Gupta 2009) with good results. Exogenous supply of growth regulators is frequently necessary for calogenesis (Nurazah et al. 2009). A variety of growth regulators have been used like IAA, (Alizadeh et al. 2004); 2, 4-D (Pallegrineschi et al. 2004); 2, 4, 5-T, dicamb (Fillipov et al. 2006) etc. Synthetic growth regulators are preferred for tissue culture purposes because of instability of natural hormones in external environment like oxidation, degradation, etc. Biochemical attributes are indicator of morphogenetic potential, growth and differentiation representing differential gene action or expression (Sharma et al. 2009). Recently it has been used to study stress effects, diseases resistance and in selection of stress resistant varieties via tissue culture (Diouri and Bouiamrine 2012). In tissue culture variations obtained in the biochemical analysis can define the effect of certain factors being studied on the growth and metabolic process of the callus. Thus, the present study was undertaken to describe an optimal concentration of 2,4-D, carbon source and gelling agent on immature embryo culture and biochemical changes in total reducing sugar and total protein content during culturing.

Material and Method

Explant preparation and callus induction

The present work was carried out in the Department of Botany in M.D. University, Rohtak, (Haryana) India. The immature seeds of wheat var. WH711were disinfected with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min. Finally, they were rinsed in sterile distilled water 4-5 times for 3-4 min. The pH of the media was adjusted to 5.8± 0.1 and it was autoclaved at 121^{0}C at 15 psi for 20 min. Immature embryos were squeezed out of the seeds and were inoculated on MS medium (Murashige and Skoog's, 1962) with different concentrations of 2, 4-D, gelling agents and carbon sources for callus induction and biochemical changes at the time of culturing (Table 1). The cultures were incubated at 25±2˚C under photoperiod of 16/8 hours of light/dark. The data were recorded for percent callus induction, callus growth in terms of fresh weight (g) and dry weight (g) and total sugar and total protein in callus.

e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 07, July 2015
Available at http://internationaljournalofresearch.org

Table 1 Composition of culture media used

Sr. No.	Medium code	Medium Composition
1.	M1	MS + Agar (9.0 g/L) + 2,4-D (3.0 mg/L) + Sucrose (30.0g/L)
2.	M2	MS + Agar (9.0 g/L) + 2,4-D (5.0 mg/L) + Sucrose (30.0 g/L)
3.	M3	MS + Agar (9.0 g/L) + 2,4-D (3.0 mg/L) + Sugar (30.0g/L)
4.	M4	MS + Agar (9.0 g/L) + 2,4-D (5.0 mg/L) + Sugar (30.0 g/L)
5.	M5	MS + Agar (9.0 g/L) + 2,4-D (3.0 mg/L) + Glucose (30.0g/L)
6.	M6	MS + Agar (9.0 g/L) + 2,4-D (5.0 mg/L) + Glucose (30.0 g/L)
7.	G1	MS + Sucrose(30.0 g/L) + 2,4-D (3.0 mg/L) + Agar (9.0 g/L)
8.	G2	MS + Sucrose(30.0 g/L) + 2,4-D (5.0 mg/L) + Agar (9.0 g/L)
9.	G3	MS + Sucrose(30.0 g/L) + 2,4-D (3.0 mg/L) + Isabgol (24.0 g/L)
10.	G4	MS + Sucrose(30.0 g/L) + 2,4-D (5.0 mg/L) + Isabgol (24.0g/L)
11.	G5	MS + Sucrose(30.0 g/L) + 2,4-D (3.0 mg/L) + Agarose (10.0g/L)
12.	G6	MS + Sucrose(30.0 g/L) + 2,4-D (5.0 mg/L) + Agarose (10.0 g/L)

Percent callus induction was calculated as:-

$$\%\ \text{Callus induction} = \frac{\text{Number of immature embryos responding}}{\text{Total number of immature embryos cultured}} \times 100$$

Callus growth was determined by measuring fresh and dry weights (g) of calli.

Biochemical analysis

The total protein and total sugar was estimated by Lowry's method (1951) and Anthrone reagent method (Yemm and Willis 1954), respectively.

Callus extract preparation

Dried sample was grinded with distilled water and filtered and washed. The residue was again grinded in more distilled water and filtered. The filtrate was centrifuged at 10,000 rpm for 15 minutes. Supernatant was collected and final volume of sample extract was made 20 ml by adding more distilled water. This extract was used for analysis.

Preparation of reagents for protein estimation

Complex-forming reagent was prepared immediately before use by mixing the following stock solutions: SS1 (Stock Sol. 1) was prepared by mixing 1% Na_2CO_3 and 0.1N NaOH solution. Making the final volume to 50 ml. SS2 (Stock Sol. 2) was prepared by dissolving 250 mg $CuSO_4$.5H2O and 0.5 g sodium citrate in 50 ml distilled water. Reagent A was prepared by mixing 1ml of SS2 in 50 ml of SS1. Reagent B (Folin's reagent) was prepared by mixing Folin and distilled water in equal amounts (1:1). Sock solution of standard protein (e.g., bovine serum albumin) containing 2 mg/ml protein was prepared in distilled water.

Standards were prepared by diluting the stock solution with distilled water.

Preparation of reagents for reducing sugar estimation

Fresh anthrone reagent was prepared by dissolving 0.4 g anthrone in 200 ml of ice-cold 95% H_2SO_4.Standard glucose stock was prepared by dissolving 100 mg glucose in 100 ml distilled water. For working standard -10 ml stock was mixed with 90 ml distilled water to prepare working solution.

Estimation of protein in callus

One ml callus extract was taken in a test tube. To this 10 ml Reagent A was added. After 10 min. 1.0 ml Reagent B was added. After waiting for 1hour the absorbance at 750 nm was recorded. The amount of color produced in the callus extract was dependent on amount of the proteins. The amount of total proteins in the samples was estimated via reading absorbance at 750 nm of the resulting solution against standard curve. Three replicates of each sample were used for estimation.

Estimation of reducing sugar in callus

Two ml distilled water was taken in a test tube. To this 1ml extract and 4 ml anthrone reagent was added. The amount of total carbohydrates in the samples was estimated via reading absorbance at 620 nm of the resulting solution against standard curve. Three replicas of each sample were used for analysis.

Result and Discussion

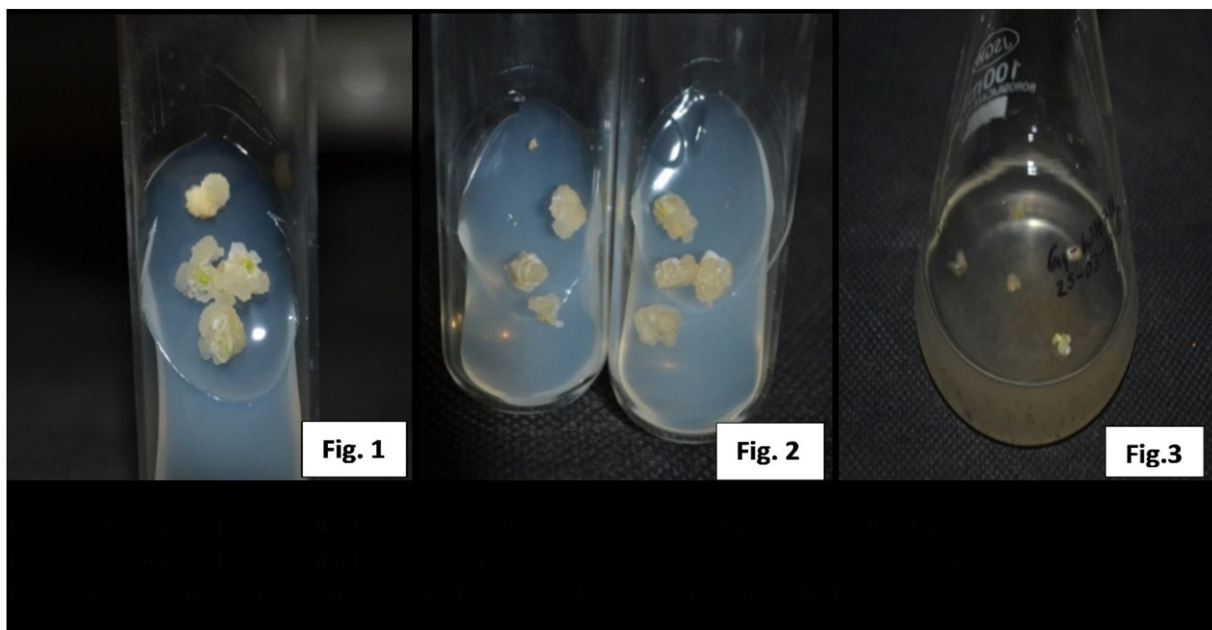
Effect of different concentration of 2, 4-D, carbon sources and gelling agents on callus induction and callus growth

Manipulation of plant growth regulators is essential to optimize callus induction. The results presented in this study showed that 2, 4-D (3.0 mg/l) responded better for callus induction as compared to 5.0 mg/l 2, 4-D in almost all the tested combinations (Table 2, Fig. 4). Hundred percent callusing (Fig. 1) was achieved on MS medium having 2, 4-D (3.0 mg/l) + sucrose (30.0 g/l) + agar (9.0 mg/l) followed by MS medium having glucose (97.93%, Fig.2) as the carbon source.

Table 2 Callus induction from immature embryos on MS media having 2, 4-D (3mg/L) and 2, 4 –D (5mg/L) with different types of carbon sources and gelling agents

2,4-D used							
3mg/l				5mg/l			
Medium Code	% callusing	Days to callus initiation	Nature of Callus	Medium Code	% callusing	Days to callus initiation	Nature of Callus
M1	100%	3-4	Creamish ,fragile	M2	97.87%	3-5	Creamish ,fragile
M3	93.75%	3-5	Creamish, compact	M4	93.75%	3-5	Creamish, compact
M5	97.93%	3-4	Yellowish green	M6	95.81%	3-5	Creamish yellow

G1	100%	3-4	Creamish ,fragile	G2	97.87%	3-5	Creamish ,fragile
G3	91.62%	3-5	Creamish	G4	85.37%	3-7	Creamish
G5	95.81%	3-4	Creamish ,compact	G6	91.66%	3-6	Yellowish compact



Our results are consistent with others, 2,4-D at low concentration is the best for callus production and regeneration (Chauhan et al. 2007). While the higher concentration proved determinant for regeneration (Khurana et al. 2002). Callus initiation was observed after 3-4 days of culture with the swelling of embryos. Callus induced varied with regard to color on different combinations (Table 2). Parmar et al. (2012) reported that different types and concentrations of auxins for mature embryos culture responded differently for callus induction as well as regeneration. In the present study beside growth regulators, the type of carbohydrates and gelling agents also played important role in callus induction, callus growth and accumulation of total proteins and total reducing sugar. The poor response in term of callus induction was observed on the MS medium with market sugar as the carbon source (93.75%, Table 2). Sul and Karbon (1998) reported that the type of carbon source effect *in vitro* growth and the part that is used as explant. The type of sugar and their concentration in the medium affect the growth of the plants *in vitro*. It is believed that addition of sugar to the culture medium promotes the growth *in vitro* and compensate for the low or negative net photosynthesis rate as a result of poor photosynthetic ability, this increasing the survival rates of tissue section cultured *in vitro* (Demo et al. 2008). Sucrose not only acts as an external energy source but also contribute to the osmotic potential of the medium (Nowark et al. 2004). This would permit the absorption of mineral nutrients present in the medium, essential to the cell growth. The difference in the suitability of the

various carbon sources as reported by various researchers is indicative of the fact that one carbon source may not be suitable for the *in vitro* culture of all plants but rather the type of plant and the culture condition may play a role. By changing the composition of media such as plant growth regulators, carbon sources and gelling agents it is possible to improve the dissociation (Akaneme and Eneobong 2008). In our result it was observed that using agar with sucrose was superior (100% callusing) followed by agarose (95.81%) and the Isabgol (91.62%) as the gelling agents in the media with 3.0 mg/l 2, 4-D. Same trend was observed when the concentration of 2, 4-D was 5.0 mg/l with all the three gelling agents (Table 2, Fig.4).

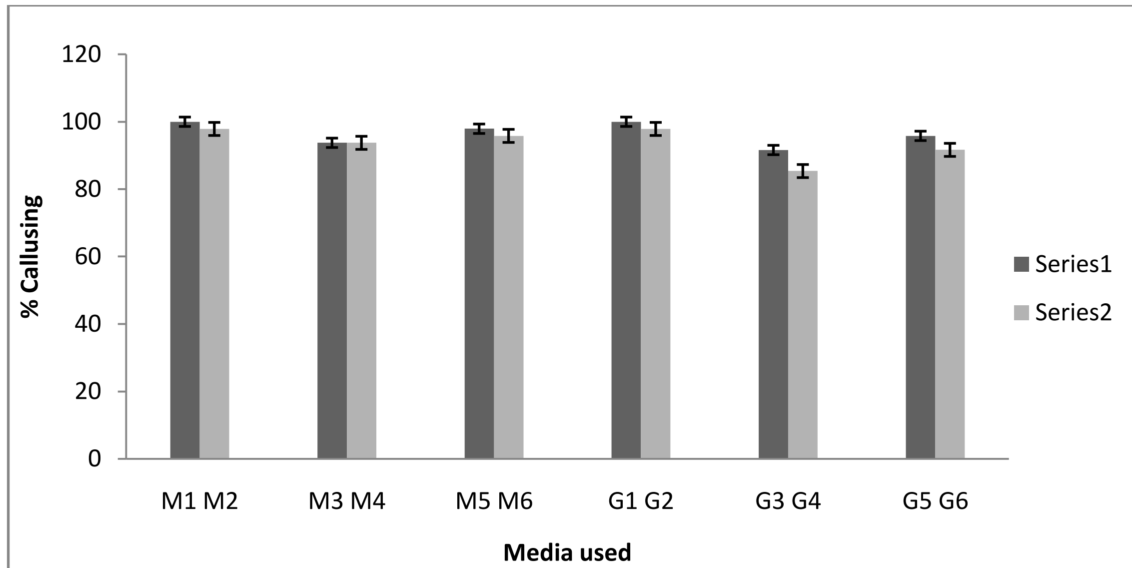


Fig.4 Callus induction on different MS media (Series1: 2, 4 -D 3.0 mg/l & Series2: 2, 4 -D 5.0 mg/l)

In general, above results recommended that agar with sucrose and 3.0 mg/l 2, 4-D gave the highest callus induction as compared with agarose and Isabgol. Meanwhile, agar with sucrose and 5.0mg/l of 2, 4-D also responded better when used with agarose and Isabgol as the gelling agent. Isabgol was the poor gelling agent for all the tested media combinations (Fig. 3). Arregui et al. (2003) also found the tuberization was higher when phytgel was used rather than DifcoBacto agar for potato cultivars. Replacement of commercial agar with phytagel or gellan gum promoted the high frequency of hyperhydricity. This gelling agent was reported to produce hyperhydricity in apple (Singha 1982) and pear (Kadota and Niimi 2003).The study on biomass profile based on fresh and dry weight of calli showed that the accumulation of biomass was maximum on MS medium having glucose as the carbon source with 2, 4-D (3.0 mg/l and 5.0 mg/l) and agar as the gelling agent (Fig.5). The dry matter accumulation was maximum on the MS medium with glucose (30 g/l) + agar (9.0g/l) + 2, 4-D (3.0 mg/l,), Fig. 6.

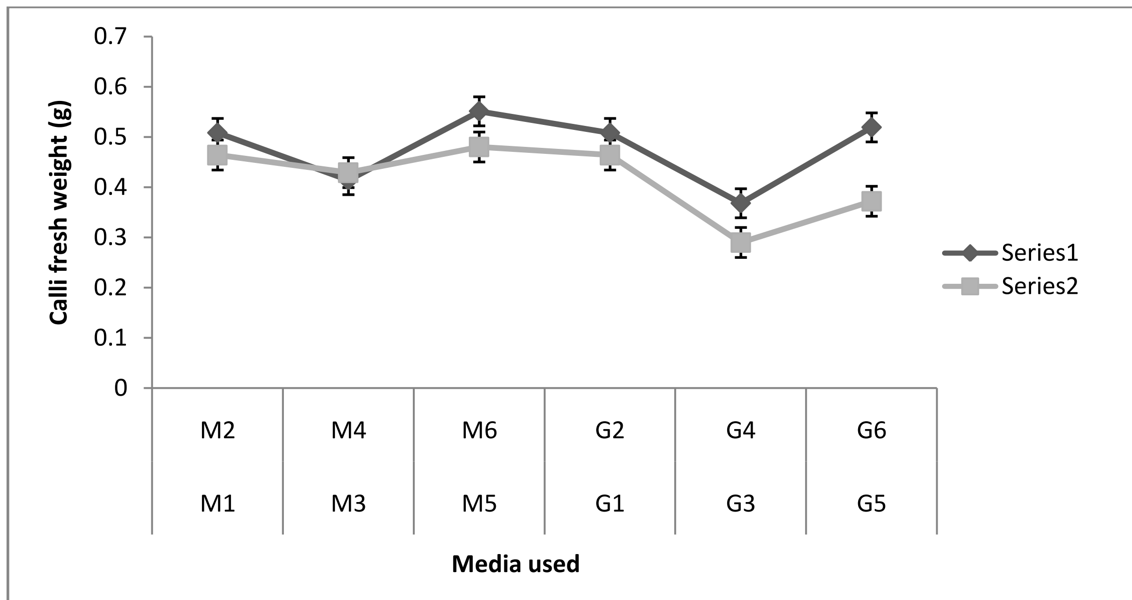


Fig. 5 Fresh-weight of calli grown on different media having 2, 4-D 3.0 mg/L (Series1: M1, M3, M5, G1, G3, G5) and 2, 4-D 5.0 mg/L (Series2: M2, M4, M6, G2, G4, G6)

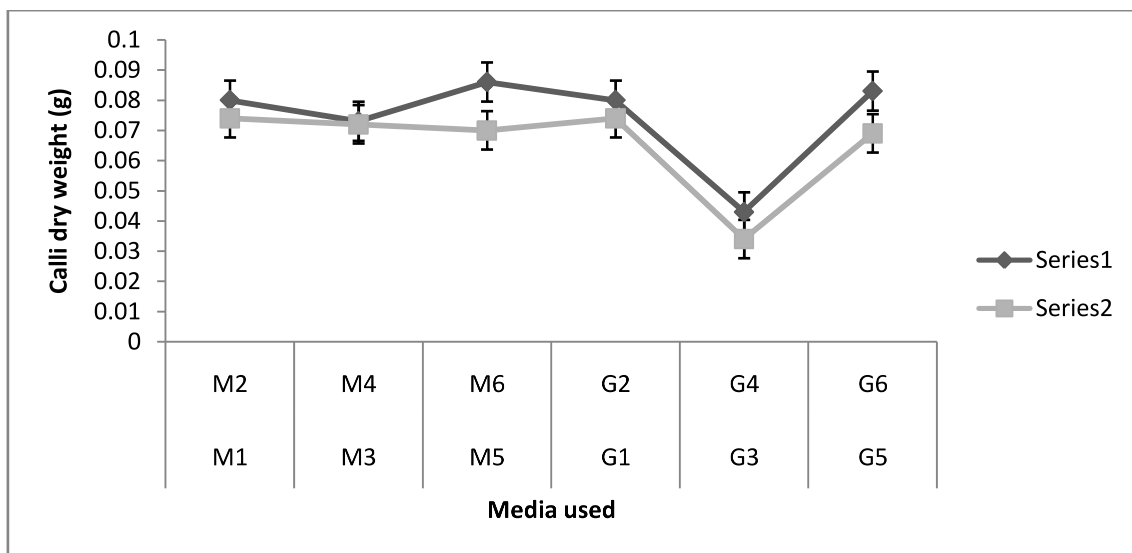


Fig. 6 Dry-weight of calli grown on different media having 2, 4-D 3.0 mg/L (Series1: M1, M3, M5, G1, G3, G5) and 2, 4-D 5.0 mg/L (Series2: M2, M4, M6, G2, G4, G6)

The use of different types and concentrations of carbohydrates on callus fresh weight has been reported for *Abies* species (Salaj et al. 2004). The water potential of the medium is very important to the growth and development of plants in *in vitro* since it determines the movement of water and minerals elements into the plant material and also maintains a better turgor for the plant cells. One of the factor that affect the water potential of the media is the type and concentration of sugar used

(Buah et al. 2000). Even though the cost analysis was not done, it is believed that the replacement of laboratory grade sucrose with table sugar, and isabgol could have a positive effect in reducing the cost of *in vitro* regeneration.

Biochemical Analysis

Biochemical changes that precede the onset of organogenesis can serve as a markers of differentiation process that bring about morphological, developmental and functional specialization (Thorpe 1980). Results in the present study on biochemical analysis showed that the total protein and reducing sugar were maximum accumulated in callus on MS medium having 30.0 g/l glucose + 9.0 g/l agar + 3.0 mg/l 2, 4-D and 5.0 mg/l 2, 4-D (Fig. 7 & 8).This may be due to the fast ready availability of carbon source in the form of glucose as compared to the sucrose and market sugar. Rathod et al. (2014) also observed the biochemical changes during *in vitro* organogenesis of *Tylophora indica*. The results of biochemical changes during *in vitro* indicated that there was periodic quantitative change in various metabolites takes place by changing the culturing conditions.

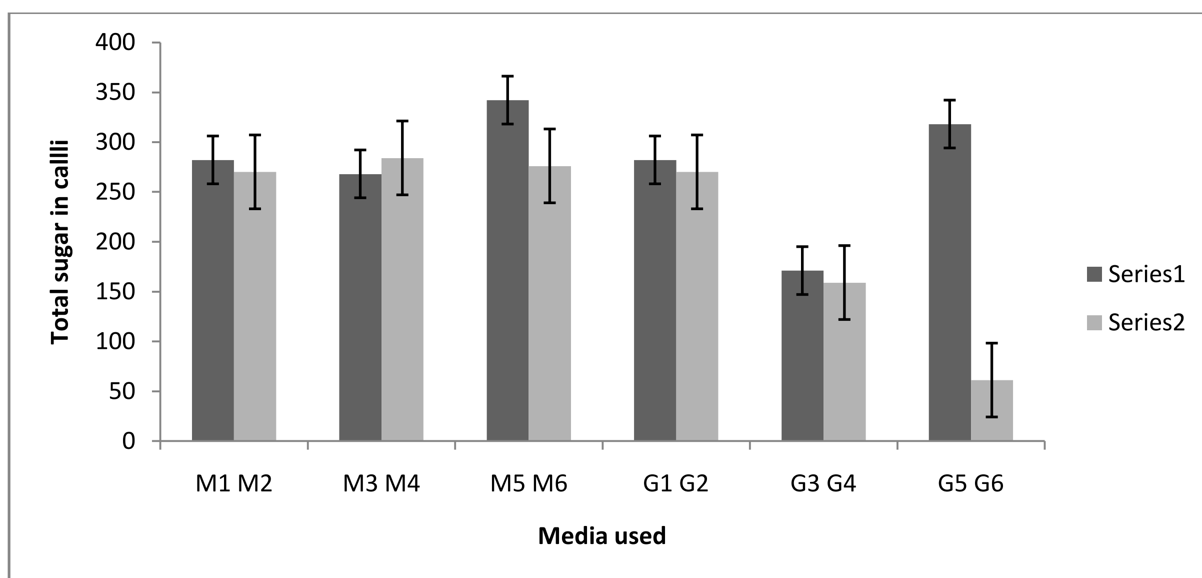


Fig. 7 Quantification of glucose in callus grown on medium having different 2, 4 –D (3.0 mg/L) and 2, 4-D 5.0 mg/L. (Series1 – 2,4-D 3.0 mg/L; Series2 – 2, 4-D 5.0 mg/L)

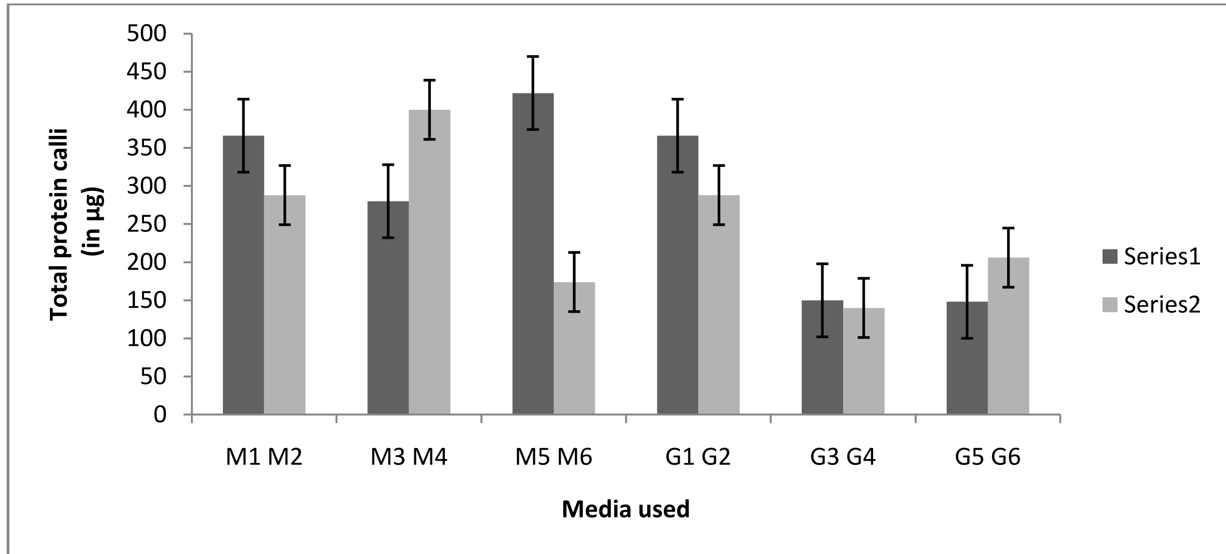


Fig. 8 Quantification of protein in callus grown on medium having different 2, 4-D (3.0 mg/L) and 2,4-D 5.0 mg/L. (Series1 – 2, 4-D 3.0 mg/L; Series2 – 2,4-D 5.0 mg/L).

Conclusion

Callus induction and biomass accumulation were observed on MS medium with different concentrations of 2, 4-D, carbon sources and gelling agents. The most effective medium for callus induction was MS + sucrose (30.0 g/l) + agar (9.0 g/l) + 2, 4-D (3.0 mg/l). Maximum callus growth in term of fresh and dry matter, total protein and reducing sugar accumulation was observed when glucose instead of sucrose was the carbon source. These finding provide the basic information on the morphology, development and biochemical changes in callus during culturing.

Acknowledgment

The authors would like to thank the Department of Botany, Maharshi Dayanand University, Rohtak (Haryana), India for the laboratory facilities.

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