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In-vitro cormel formation and changes of biochemical composition in calli during morphogenesis in gladiolus (*Gladiolus* × *grandiflorus*)

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ABSTRACT

The present investigation was carried out for *in-vitro* cormel formation and to study the changes of biochemical composition during shoot and root differentiation from callus in gladiolus *(Gladiolus* × *grandifforus* L.*)*. Cormel tip was used as explant for *in-vitro* regeneration on MS media supplemented with different plant growth regulators. Maximum callus formation was observed on MS medium supplemented with 4.0 mg/l 2,4-D with excellent callus growth. The inducted calli were subcultured on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA for best shooting followed by on ½ MS medium fortified with 3.0 mg/l IAA for best rooting. After two weeks of root initiation, the whole cluster of rooted plantlets was equally divided into two halves and the divided clusters were subcultured on cormel induction media. The different stages of morphogenesis, viz. non-differentiating callus, differentiating green callus, shoot differentiating callus and root differentiating callus were used for biochemical analysis. Maximum (55.0 %) and earliest (56.0 days) formation of cormels was observed on MS medium supplemented with 4.0 mg/l NAA elevated with 6% sucrose with good cormel size of up to 7.0 mm diameter. Decrease in metabolities like starch, reducing sugars and total phenols while, increase in total soluble proteins was found during shoot and root differentiation. Activities of enzymes, viz. polyphenol oxidase and peroxidase increased during shoot and root differentiation. From the present investigation, it was proved that metabolic and enzymatic activity during *in-vitro* morphogenesis help gladiolus for rapid organogenesis and multiplication through cormel formation.

Key words: Callus, Cormel tip, *Gladiolus* × *grandiflorus*, *In-vitro* cormel formation, Metabolitic variation, Regeneration

Gladiolus *(Gladiolus* × *grandifforu* L.*)* is a bulbous ornamental plant. It has a great commercial importance in cut flower industry all over the world, as well as in India due to its magnificent and colourful spikes. However, its commercial cultivation is limited by low rate of multiplication. The commercial production of corms and cormels is also greatly affected by *Fusarium* corm rot and high percentage of spoilage of corms during storage thus there is nonavailability of planting materials. Besides, one mother corm normally produces 1-2 daughter corms and about 25 cormels each season (Misra 1994). These cormels require three to four seasons to attain standard size of flowering spike and daughter corms. The production of corms and cormels doesn't fulfill the local demand of planting material. The dormancy of the corms and cormels is another problem in this regard. The other aspect, biochemical attributes are indicators of morphogenetic potential, growth and differentiation, representing differential gene action/expression or change in endogenous level of growth regulators in cell cultures (Carrillo-Castaneda and Mata 2000). Marked reductions in the number of biochemical attributes such as starch, protein, amylase, invertase, malate dehydrogenase, peroxidase and phosphorylase with a subsequent increase in soluble sugar and amino acid content precede in *in-vitro* shoot differentiation process (Verghese and Kour 1991). Biochemical changes that precede the onset of organogenesis/embryogenesis can serve as markers of differentiation processes that bring about morphological, developmental and functional specialization (Thorpe 1990). During morphogenesis certain enzymes and proteins are responsible for callus proliferation and differentiation in to shoot buds (Chawla 1991). The role of antioxidant enzymes during organogenesis and somatic embryogenesis in some species were studied in recent years (Dutta Gupta and Datta 2003 and Misra *et al.,* 2010).

So, in the sense of commercial cultivation, conventional methods of propagation are insufficient to meet the demand of planting materials. To boost up economical production, it is highly desirable to standardize *in-vitro* cormel formation and to realize metbolitic variation

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during calli morphogenesis through analysis of biochemical parameters during shoot and root differentiation form callus in gladiolus. Therefore, the present investigation was designed to explore production potential of gladiolus.

MATERIALS AND METHODS

The present investigation was conducted in the Tissue Culture Laboratory of Deparment of Floriculture Medicinal and Aromatic Plants, Faculty of Horticulture, Uttar Banga Krishi Viswavidyalaya, Pundibari, Coochbehar during the year 2011-2013.

For explants source, cormel tips with small physiological base of cv. Traderhorn were transferred into suitable cultured MS media containing 2,4-D (2.0, 4.0 and 6.0 mg/l) and NAA (2.0, 4.0 and 6.0 mg/l) alone in different concentrations for callus induction. The inducted calli were inoculated on MS media containing the three different levels of BAP (2.0, 4.0 and 6.0 mg/l) alone and in combination with NAA (0.5 mg/l) for shoot initiation. The developed micro-shoots were subcultured on rooting media containing IAA (1.0, 2.0, 3.0 and 4.0 mg/l) alone as supplement of ½ MS media.

After two weeks of root initiation (not fully developed roots), the whole cluster of rooted plantlets were taken out from cultures and equally divided into two halves in such a way that each had both shoots and roots. The divided clusters were subcultured on cormel induction media in different treatment combinations with three replicates. For *in-vitro* cormel formation NAA and IBA in different concentration (2, 4 or 6.0 mg/L) alone with elevated sucrose 6% were used. The explants were incubated in growth room for six months. Subsequent cormel development and shoot formations were achieved with few root initiation in some cultures. The effects of increased sucrose level (60 g/l) were investigated to increase cormel number and to induce early cormel formation. MS medium including all vitamins was supplemented with 6% sucrose and three levels of NAA and IBA as shown in Table 4. Throughout the study, the *in-vitro* cormel formation capacity was observed and recorded.

The calli were transferred for shoot and root differentiation on best shoot differentiation medium [MS + BAP (4.0 mg/l) + NAA (0.5 mg/l)] and on rooting medium [½ MS + IBA (3.0 mg/l)] for carrying out biochemical analysis of reducing sugar, starch, total soluble protein, total phenolic content, peroxidase (POD) and polyphenol oxidase (PPO). Reducing sugar and starch were estimated by following method of Handa *et al*. (1982) and Hassid and Neufeld (1964), respectively. The protein content was determined by the method of Bradford (1976). Total phenols were estimated as per method described by Amorim *et al*. (1977). POD and PPO activity was determined as per the procedure given by Hammerschmidt *et al*. (1982) and Mayer *et al*. (1965), respectively.

The per cent callus induction, days to callus induction, intensity and morphology of callus formation were recorded in callogenesis experiment. The per cent shoot regeneration, days to shoot regeneration, number and length of shoots, fresh and dry weight of shoots was also recorded for shoot induction experimentation. In cormel induction experiment the parameters per cent cormel induction, days to cormel induction, no. of cormels/culture, diameter of cormel (mm), perimeter of cormel (mm), fresh and dry weight of cormel (g) were recorded. The estimation of different metabolites and enzymes were done during shoot and root differentiation from callus of gladiolus.

For proper interpretation of the results, appropriate statistical test was selected depending upon the nature of the treatment and objectives of the research. All the experiments were laid out under factorial experiment in CRD (Completely Randomized Design) with three replications. The data were analyzed by Fisher's analysis of variance (ANOVA) technique and then results were interpreted.

RESULTS AND DISCUSSION

The result data, graphs and pictorial evidences of this experiment has presented in Table 1-4, Fig 1, 2, 3, 4. The callus induction was significantly affected by different concentration and combinations of 2,4-D and NAA as a supplement of MS medium (Table 1). Amongst the treatment media the maximum percentage (50.00%) of callus initiation was observed on MS medium supplemented with 4.0 mg/l 2,4-D with excellent callus growth and morphology. On the basis of overall morphology and visual growth, calli were classified and graded in different intensity classes like for excellent callus C+++, for fair callus C++, for satisfactory callus with adventitious shoot or root C+ (Table 1). The results are in agreement with the findings of Memon *et al*.

Table 1 Effect of different concentration of 2,4-D alone NAA on callus induction characters in gladiolus

Media treatment	% Callus induction (Mean±SE)	Days to callus induction (Mean±SE)	Intensity of callus formation	Callus morphology (Structure and colour)
MS + 2 mg/l 2,4-D	38.89 ± 3.25	15.0 ± 0.0	C^{+++}	Brown callus
MS + 4 mg/l 2,4-D	50.00 ± 0.00	14.3 ± 0.3	C^{+++}	Brown compact
MS + 6 mg/l 2,4-D	33.33 ± 0.00	14.0 ± 0.0	C^{++}	Brown white
MS + 2 mg/l NAA	33.33 ± 0.00	16.0 ± 0.0	C^{++}	White friable
MS + 4 mg/l NAA	44.44 ± 3.25	15.7 ± 0.3	C^{+++}	Brown white compact
MS + 6 mg/l NAA	27.78 ± 3.72	15.0 ± 0.0	C^{++}	White compact
CD	11.79	NS		
SE(m)	4.03	0.8		

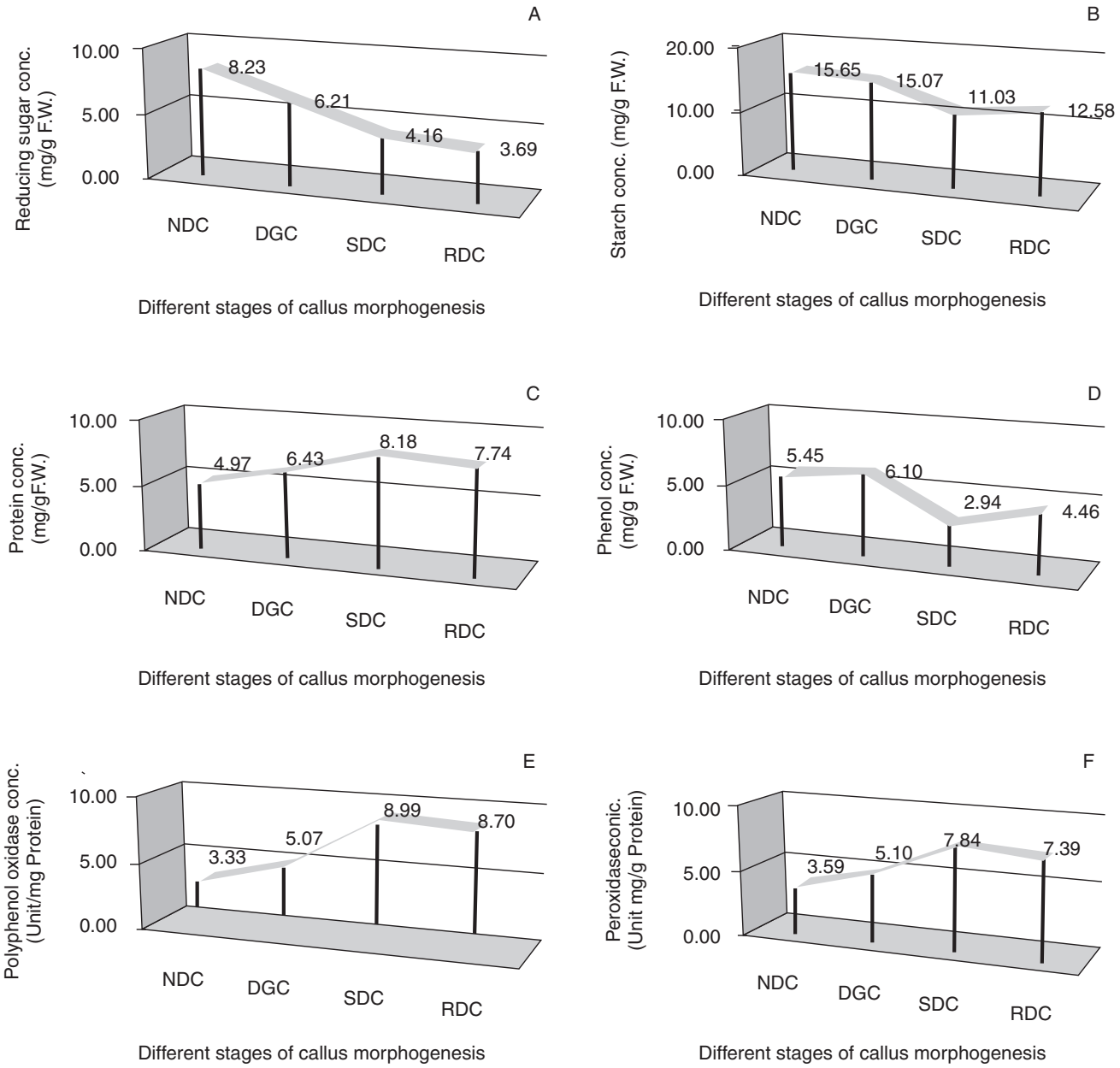

Fig 1 Changes in the activity of A. reducing sugar B. Starch C. Protein D. Phenol E. Polyphenol oxidase and F. Peroxidase in different stages of callus morphogenesis viz. Nondifferentiating callus (NDC), Differentiating green callus (DGC), Shoot differentiating callus (SDC) and Root differentiating callus (RDC) (Bars represent the mean of three replicates).

(2010) and Shaheenuzzaman *et al.* (2011).The time required for callus induction was recorded not to be significantly differed among the treatment media. The significantly highest mean shoot regeneration (50.00%) exhibited in MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA (Table 2). The MS medium fortified with 6.0 mg/l BAP and 0.5 mg/l NAA took minimum days (13.0) for initiation of shoots from callus. The maximum number (2.7) of shoots were observed after 4 weeks of inoculation on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA which was significantly superior over other media treatments (Table 2). Prasad and Gupta (2006) reported that without combination of BAP and NAA calli did not induce shoot regeneration. This statement is supported by the results of Goo *et al*. (2003) who recorded more shoot induction from callus of cormel shoot tip on MS medium with lower levels of NAA (0.01 mg/L) in combination with higher levels of BAP (1.0 mg/L). The maximum (83.33%) and earliest (7.6 days) root formation was obtained on medium ½ MS fortified with 3.0 mg/l IAA, whereas the ½ MS media containing 2.0 mg/l IAA recorded maximum number (5.3) of roots (Table 3). Stimulated rooting in auxin supplemented media had been reported by Beura *et al*. (2003). Similar result was found on rhizogenesis by Memon *et al.* (2010).

In this investigation higher level of auxins (IBA and NAA) had advanced the cormel induction along with higher

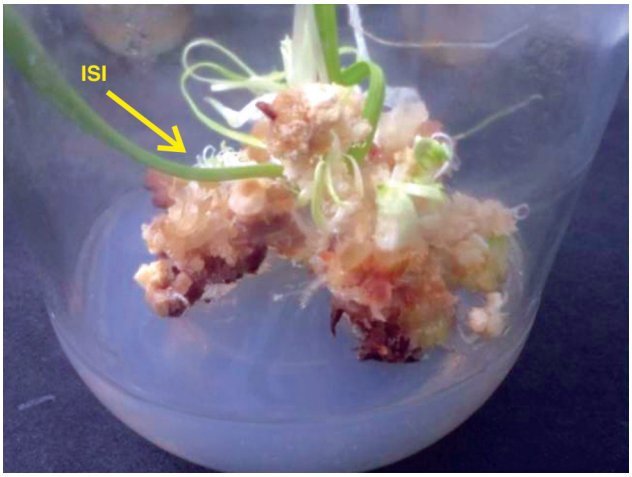

Fig 2 Depicting the response of callus for indirect shoot initiation on MS medium supplemented with BAP (2.0 mg L^{-1}) in combination with NAA (0.5 mg L^{-1}) (ISI, indirect shoot initiation).

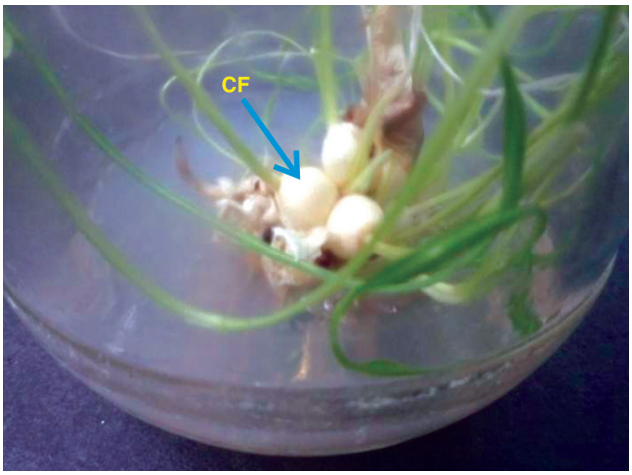

Fig 3 Depicting best *in-vitro* cormel multiplication of gladiolus on MS medium supplemented with NAA (4.0 mg L^{-1}) with elevated sucrose (6%). (CF, cormel formation).

level of sugar (6%) (Table 4, Fig 1B and 1C), this might be due to the sugar facilitates early cell division and cell elongation and supply nutrition for the cormel production to the plantlets. The maximum per cent (55.00%) and number (5.7) of cormel formation is noticed in MS medium fortified with 4 mg/l NAA and 6% elevated sucrose which was significantly superior over other media treatment combinations. The minimum percentage (27.78%) of cormel induction was noticed in the medium MS fortified medium with 2 mg/l IAA and 6% sucrose. Sinha and Roy (2002) obtained maximum cormels from rooted shoots on medium having sucrose 6% and IBA 2.0 mg/L in *Gladiolus primulinus*. Although, Memon *et al.* (2010) reported successful cormel formation on medium containing IBA (1 mg/L) with high levels of sucrose (7%) in variety Traderhorn and *Peter Pears*. The higher level of NAA produced more number of cormels than IBA used because IBA facilitates contractile root formation in the culture. Fresh weight of the cormel might trend to increase in relation to the growth

Fig 4 A closer view of *in-vitro* produced mature cormels from regenerated shoot with contractile root system.

regulators used. The weight of cormel was increased with the concentration of auxins IBA and NAA upto the level of 6.0 mg/L and this might be due to the higher concentration of auxins exogenously forced to increased

Table 2 Effect of different concentration of BAP alone or in combination with NAA on shoot regeneration characters in gladiolus

Media treatment	% shoot regeneration from callus (Mean±SE)	Days to shoot regeneration from callus (Mean±SE)	No. of shoots/ explants (after 8 weeks of inoculation) (Mean±SE)
MS + 2 mg/l BAP	26.67 ± 4.22	16.0 ± 0.0	4.0 ± 0.6
MS + 4 mg/l 2,4-D	33.33 ± 4.22	15.7 ± 0.3	5.7 ± 0.9
MS + 6 mg/l 2,4-D	20.00 ± 0.00	14.7 ± 0.3	3.7 ± 0.3
MS + 2 mg/l BAP + 0.5 mg/l NAA	41.67 ± 4.10	14.0 ± 0.0	6.3 ± 0.3
MS + 4 mg/l BAP + 0.5 mg/l NAA	50.00 ± 8.66	13.7 ± 0.3	8.0 ± 1.2
MS + 6 mg/l BAP + 0.5 mg/l NAA	33.33 ± 4.10	13.0 ± 0.6	4.3 ± 0.3
CD	9.89	0.96	
SE(m)	3.22	0.30	

Table 3 Effect of different concentrations of IBA on root formation in gladiolus

Media treatments	% Root induction (Mean±SE)	Days to root initiation (Mean±SE)	No. of roots/ Shoot (Mean±SE)
½ MS + 1 mg/l IAA	61.11 ± 3.25	10.0 ± 0.0	4.7 ± 0.3
½ MS + 2 mg/l IAA	77.78 ± 11.77	8.3 ± 0.3	5.3 ± 0.3
½ MS + 3 mg/l IAA	83.33 ± 10.41	6.7 ± 0.3	4.0 ± 0.0
½ MS + 4 mg/l IAA	64.12 ± 4.26	9.3 ± 0.3	4.2 ± 0.3
CD	13.01	1.0	0.9
SE(m)	4.75	0.3	0.3

Table 4 Effect of different concentration of NAA and IBA with sucrose (6%) on cormel characters in relation to *in-vitro* cormel formation in gladiolus (after 2-3 months of inoculation)

Media treatment	% Cormel formation (Mean±SE)	Days to cormel formation (Mean±SE)	No. of cormel/ culture (Mean±SE)	Fresh weight (g) of cormel (Mean±SE)	Dry weight (g) of cormel (Mean±SE)	Relative dry weight (Mean±SE)	Diameter (mm) of cormels (Mean±SE)	Perimeter (mm) of cormels (Mean±SE)
MS + 2.0 mg/L NAA + 6% Sucrose	44.44 ± 3.25	63.7 ± 0.9	4.0 ± 0.0	0.57 ± 0.01	0.52 ± 0.01	0.90 ± 0.01	5.3 ± 0.3	16.8 ± 1.1
MS + 4.0 mg/L NAA + 6% Sucrose	55.56 ± 6.49	58.3 ± 0.8	5.7 ± 0.3	0.72 ± 0.06	0.69 ± 0.06	0.91 ± 0.03	7.0 ± 0.0	22.0 ± 0.0
MS + 6.0 mg/L NAA + 6% Sucrose	50.00 ± 5.62	56.0 ± 1.7	4.3 ± 0.3	1.08 ± 0.07	0.97 ± 0.08	0.92 ± 0.08	6.0 ± 0.6	18.9 ± 1.8
MS + 2.0 mg/L IBA + 6% Sucrose	27.78 ± 3.72	66.3 ± 1.2	4.0 ± 0.0	0.25 ± 0.02	0.23 ± 0.01	0.90 ± 0.04	3.7 ± 0.9	11.5 ± 2.8
MS + 4.0 mg/L IBA + 6% Sucrose	38.89 ± 3.25	62.7 ± 1.5	3.3 ± 0.3	0.35 ± 0.02	0.33 ± 0.01	0.92 ± 0.06	5.3 ± 0.7	16.8 ± 2.1
MS + 6.0 mg/L IBA + 6% Sucrose	33.33 ± 0.00	59.0 ± 0.6	2.7 ± 0.3	0.44 ± 0.02	0.42 ± 0.02	0.96 ± 0.00	4.0 ± 0.6	12.6 ± 1.8
CD	12.09	3.7	0.9	0.13	0.12	NS	1.8	5.7
SE(m)	4.25	1.2	0.3	0.04	0.03	0.05	0.6	1.8

the cell volume and weight in cormels. Similarly dry weight might trend to increase in relation to the fresh weight of the cormels.

The minimum time (56.0 days) was required for cormel formation in MS medium containing 6 mg/l NAA and 6% sucrose which was at par (58.3 days) with the media MS + 4 mg/l NAA+ 6% sucrose. Maximum cormel fresh weight (1.08 g) and dry weight (0.97 g) was achieved in MS + 6 mg/l NAA+ 6% sucrose medium in 6 to 8 weeks (Table 4). In the present study the experiment was also conducted on the size enhancement studies of *in-vitro* produced cormels using high sucrose concentration (Plate 4). The maximum size of the cormels in terms of both diameter (7.0 mm) and perimeter (22 mm) was noticed in MS medium fortified with 4 mg/l NAA and 6% elevated sucrose, which was decreased with the concentration of NAA and IBA (Table 4). Ahmad *et al.* (2000) obtained good size cormels (upto 10 mm diameter) on the increasing levels of sucrose from 6 to 9%. The diameter of cormels was increased with the concentration of auxins IBA and NAA upto the level of 6.0 mg/L and this might be due to the higher concentration of auxins exogenously forced to developed more the cell volume and weight in cormel. Similarly the perimeter might trend to increase in relation to diameter. Similar results on *in-vitro* cormel formation were reported by Dharmasena *et al.* (2011) and Jala (2013).

Analysis of various cellular metabolites during organ differentiation in callus provides a reasonable and promising approach towards an understanding of the biochemical basis of developmental pathway. To understand the differentiation events in callus tissues on regeneration medium, metabolites associated with organ differentiation in callus tissue were studied.

Maximum reducing sugar (8.23 mg/g F W) and starch (15.65 mg/g F W) content was recorded during non-differentiating (NDC) stage of callus (Fig 1A and 1B) whilst total soluble protein (8.18 mg/g F W) and total phenol (6.10 mg/g F W) was found to be maximum at shoot differentiating (SDC) and differentiating green callus (DGC) stages of morphogenesis (Fig 1C and 1D), respectively. The reducing sugar content was maximum at callusing as compared with those at organogenesis and root induction which might be due to the hydrolysis of reserve polysaccharides and depletion of reducing sugars to meet the energy requirement during organogenesis (Panigrahi *et al.* 2007). However, in root differentiation, the reducing sugar content declined upto root initiation and later on increased during root formation (Fig 1A). The depletion depicts the utilization of sugars during differentiation (Singh *et al.*, 2006). Similar result on protein, starch and reducing sugar activity in callus during mophogenesis was found by Jana and Shekhawat (2012). The cycle of starch accumulation/mobilization reflects a potential causative role of starch in organogenesis and suggests that starch is used both during organ initiation and later in its development. Organogenesis is a high-energy-requiring process. Starch degradation results in the formation of glycolytic intermediates that will subsequently be catabolized and yield high amounts of ATP (Mangat *et al*. 2006). In the present study, it was found that starch content in callus declined during the process of root and shoots differentiation and increased only after shoot development (Fig 1B). Similar result on starch activity in callus during morphogenesis was found by Jana and Shekhawat (2012). The protein concentration varied among different develop-mental stages (Fig 1C). With the development of shoot, formation of protein was maximum. This might be due to synthesis of certain amino acids/polypeptides required to initiate shoot bud formation. It was observed that total soluble proteins in root and shoot forming calli

were higher during root and shoot differentiation than in controlled callus. Since, during differentiation the cells are quantitatively changing their activities, new proteins have to be synthesized, thus the protein concentration is high during differentiation. Similar observation was also reported by Mohapatra and Rath (2005). Similar result on protein in callus during mophogenesis was found by Hasbullah *et al*. (2012). Present investigation showed gradual decrease in phenolic content during differentiation (Fig 1D). Phenols participate in formation of cross-linking of cell wall constituents which is catalyzed by peroxidase (Mader and Fussel, 1982). Changes in the levels of metabolites and enzymes during differentiation from callus culture may be helpful in our understanding of the biochemical basis of developmental pathway of gladiolus. It seems that the increased content of phenolics during formation of callus is associated with their growth regulating properties, and decreased content during organ formation is associated with lignifications during cellular differentiation and organization into shoot primordia. Similar result on phenolic activity in callus during organogenesis was found by Sharma *et al.* (2009).

The developmental stages even differ greatly in their enzyme activities like there is a great increase in polyphenol oxidase (PPO), peroxidise (POD) activities during differentiating green callus stage (Fig 1E and 1F). Both the PPO (8.99 unit/mg Protein) and POD (7.84 unit/mg Protein) were recorded to be maximum during shoot differentiating (SDC) stage of calli morphogenesis. Different *in-vitro* stages showed a significant polyphenol oxidase activity. The maximum polyphenol oxidase activity was showed in differentiating shoots which is supposed to highest meristematic activity.

Different *in-vitro* stages showed a significant peroxidase activity (Fig 1F). The maximum peroxidase activity was showed in differentiating shoots which is supposed to highest meristematic activity. Similar result on enzymatic activity in callus during organogenesis was found by Sharma *et al.* (2009). Peroxidase activity seems to be sensitive to plant hormones and its activity may be important in controlling of auxin degradation, lignification, growth and differentiation Samantaray *et al*. (1999). The increased peroxidase activities during differentiation might be due to accumulation or synthesis of high phenolic substances (Vanhoof *et al*. 1976). This result is in agreement with *Gladiolus hybridus* where CAT and POX activity increased while SOD activity decreased during shoot organogenesis (Dutta Gupta and Datta 2003). It is also in consonance with antioxidant enzyme changes in saffron as reported by Sharifi and Ebrahimzadeh (2010). Similar result on enzymatic activity in callus during organogenesis was found by Sharma *et al.* 2009 and Rathod *et al.* 2014.

The study concludes that maximum callus induction with excellent callus growth followed by shooting and rooting as subcultured separately formation was observed on MS medium supplemented with 4.0 mg/l 2,4-D, MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA and ½ MS fortified with 3.0 mg/l IAA, respectively. Two weeks old rooted plantlets produced maximum (55.0%) and earliest (56.0 days) cormels when subcultured on MS medium supplemented with 4.0 mg/l NAA elevated with 6% sucrose with good cormel diameter. The *in-vitro* cormels were found with the regenerated plants which can increase the propagation potentiality of gladiolus. Biochemical analysis indicated decrease in metabolities like starch, reducing sugars and total phenols during calli morphogenesis from NDC to RDC while increase in total soluble proteins during shoot and root differentiation. Activities of enzymes, viz. polyphenol oxidase and peroxidase increased during shoot and root differentiation. From the present investigation, it was proved that metabolic and enzymatic activity during *in-vitro* morphogenesis help gladiolus for rapid organogenesis and multiplication through formation of cormels.

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