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Methylglyoxal with glycine or succinate enhances differentiation and shoot morphogenesis in *Nicotiana tabacum* callus

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Abstract

The aim of this study was to evaluate the influence of methylglyoxal (MG) on organogenesis and regeneration of tobacco (*Nicotiana tabacum* L.) plants from callus in media containing glycine or succinate. The best improvement in shoot proliferation and shoot length was obtained in the medium supplemented with 0.1 mM MG and 0.5 mM glycine or 0.25 mM succinate. The histological studies showed vigorous development of corm like structures and shoot organogenesis from callus tissues cultured in MG supplemented media. Biochemical studies also revealed higher content of δ-aminolaevulinic acid (a precursor of chlorophyll) and of chlorophyll.

Additional key words: δ-aminolevulinic acid, benzyladenine, chlorophyll, regeneration, tobacco.

Introduction

The focus of this study is the effect of methylglyoxal (MG) on the regeneration of plants from de-differentiated callus tissue. Methylglyoxal, also called pyruvaldehyde or 2-oxopropanal (CH_3-CO-CH=O or $C_3H_4O_2$), is the aldehyde form of pyruvic acid. As a ketoaldehyde, MG exhibits strong anticancer and other diverse biological effects (Kalapos 2008, Talukdar *et al.* 2008, 2009). Similar to its effect on animal systems, MG was found to destroy crown gall tumors completely in *Nicotiana tabacum* without any adverse effect on the host plant (Ray *et al*. 2011).

Methylglyoxal was also observed to play a significant role in the formation of chlorophyll (Chl) in calluses (Roy *et al*. 2004, Ray *et al*. 2011). It has also been observed that MG has a very strong positive effect in cell to cell adhesion thereby resulting in organogenesis (Lieber 1995). Kinetin is one of the most important cytokinins; however, studies have indicated that MG may be used to replace kinetin for cell differentiation and plant regeneration (Roy *et al.* 2004). Based on these earlier reports, MG was used as a supplement with glycine and succinate to study its possible role in the process of differentiation in tobacco.

Possession of Chl is a basic difference between differentiated and undifferentiated plant cells. Therefore, the first parameter investigated was the amount of chlorophyll found in calluses after treating them with MG. Chl biosynthesis involves many intermediate steps, the formation of δ-aminolevulinic acid (ALA) being one of the most important. It has been observed that seedlings germinating in the dark turn green in the course of a few hours if they are supplied with δ-ALA (Wettstein *et al.* 1995).

Glycine and succinate are also precursors of Chl biosynthesis (Wettstein *et al*. 1995); therefore the effect of MG on Chl and ALA biosynthesis was tested independently as well as in combination with glycine or succinate. The other compounds included in this study were alanine, which induces differentiation (Zhu *et al.* 1990), and pyruvate, which is a structural analogue of MG.

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Abbreviations: ALA - δ-aminolevulinic acid; BA - N^6-benzyladenine; BAM - basal MS medium supplemented with 2.2 μM BA; Chl - chlorophyll; MG - methylglyoxal; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid.
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Materials and methods

Agar and a highly purified grade of Murashige and Skoog (1962; MS) plant salt mixture were obtained from *Hi Media Laboratories*, Mumbai, India. MG, N^6-benzyl-adenine (BA), α-naphthaleneacetic acid (NAA), and pyruvate were obtained from *Sigma*, St Louis, USA. Sodium hypochlorite, *Tween 80*, and mercuric chloride were from *SRL*, Mumbai, India, whereas glycine, succinate, alanine, and sucrose were from *Merck*, Darmstadt, Germany.

Friable calluses were induced from leaf explants (6 to 8 mm diameter sections) excised from 6-week-old seedlings grown *in vivo* in a greenhouse. These were further treated with 10 % (v/v) aqueous sodium hypochlorite solution for 12 min, washed thoroughly with sterile distilled water, and disinfected with 0.04 % (m/v) mercuric chloride solution and 0.1 % (v/v) *Tween 80* for 7 min and then repeatedly washed with sterile distilled water. After surface disinfection, the explants were transferred and placed abaxially into *Kilner* jars (*Saffron Supplier,* Mumbai, India) containing 50 cm^3 of basal MS medium supplemented with 2.2 μM BA and 2.6 μM NAA. The pH of the medium was pre-adjusted to 5.8 before sterilization (121 ºC for 15 min). The cultures were incubated at temperature of 25 ± 1 ºC, 16-h photo-period, and irradiance inside the vessel of 42 μmol m^{-2} s^{-1} (cool fluorescent tubes, *Philips*, Kolkata, India). Shoot proliferation took place when these calluses were cultured on BAM (the basal medium supplemented with 2.2 μM BA). This concentration of BA was maintained throughout the experiment.

The effect of glycine, succinate, and alanine at various concentrations (0.1 - 0.75 mM) was studied on shoot multiplication. Four pieces of callus tissues were inoculated in each culture vessel. The number of protuberances induced, shoots per explant, and average shoot length were recorded after four weeks of subculture. Shoots less than 2 mm were considered as shoot buds. The effect of MG was studied with BAM alone and thereafter with BAM and different doses of glycine, succinate, and alanine. A stock solution of 100 mM MG in water was prepared and sterilized by passing through 0.22 μm *Millipore* (Mumbai, India) bacterial filters. Aliquots from these solutions were then aseptically added to the sterile nutrient media. The effect of pyruvate, which is a structural analogue of MG, was also studied in the same concentrations as MG.

Structural changes during the regeneration were studied using a stereomicroscope (*WILD M3Z*, Heerbrugg, Switzerland). The callus tissues were fixed in 10 % glutaraldehyde for 24 h, washed four times in phosphate buffer saline (PBS; 40 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), dehydrated in graded ethanol (10, 30, 50, 70, and 90 %) for 15 min each and then kept overnight in absolute alcohol. The material was sectioned into 5.0 μm slices with a rotary microtome and stained with haematoxylin and saffranin. The microscopic sections were then photographed with a bright field of *Olympus BX1* digital camera with *Image Pro* software (Popielarska *et al.* 2006).

The amount of ALA was measured by the method of Burnham (1970). One gram of tissue was crushed in a pre-chilled mortar and pestle with 50 mM Tris-HCl buffer, pH-7.5, to obtain a volume of 1 cm^3. The mix was centrifuged at 3 000 *g* for 10 min and 0.072 cm^3 of 80 % TCA was added. This solution was again centrifuged at 5 000 *g* for 15 min and the supernatant taken. Sodium acetate (0.167 cm^3) and 0.012 cm^3 of acetylacetone were added to 0.125 cm^3 of this supernatant and the mixture was boiled in a water bath for 15 min. After cooling, 0.437 cm^3 of Ehrlich reagent was added to the mix and after 20 min the absorbance was noted at 556 nm. Modified Ehrlich reagent was prepared fresh daily by adding 1 g of *p*-dimethylaminobenzaldehyde to 30 cm^3 of glacial acetic acid followed by 8 cm^3 of 70 % perchloric acid. The volume of the mixture was increased to 50 cm^3 with glacial acetic acid.

Chl content was estimated using the method of Arnon (1949) in acetone extract and absorbance was measured at 652 nm using spectrophotometer *U 2000*, *Hitachi*, Tokyo, Japan.

The data are presented as means ± standard deviation from six replicates for each treatment. Each experiment was repeated six times and the results were reproducible. The data are analyzed for variance (2 way *ANOVA*; Sokal and Rohlf 1987) using the software *S-Plus 2000* to detect significant differences between the means at $P \leq 0.01$.

Results and discussion

The procedures described above as well as studies carried out in our laboratory earlier indicate a significant role of MG in the process of differentiation (Roy *et al.* 2004, Ray *et al.* 2011). These studies have shown that the Chl content of calluses increases with the concentration of MG from 0.1 to 0.25 mM MG and then begins to drop at 0.5 mM MG. The growth index of the calluses, however, is the highest at 0.1 mM MG and steadily drops at higher concentrations (Fig. 1). For the callus growth and differentiation, 0.1 mM MG is therefore considered to be the optimal concentration and is used in all subsequent studies. As a baseline, diffe-rentiation was first studied with different concentrations of BA alone. Maximum protuberance induction was observed with 2.2 μM BAP but this concentration did not promote shoot morphogenesis significantly. Addition of MG, however, resulted in extensive ramification of shoots. Subsequent tests were carried out on the effect of other compounds

such as glycine, succinate, pyruvate, and alanine in combination with MG. Glycine in combination with MG showed remarkable shoot organogenesis (Table 1). Succinate also showed shoot organogenesis but to a lesser extent than with glycine (Table 2). Pyruvate has a structural similarity with MG and is formed from it by several dehydrogenases (Van der Jagt and Davison 1977, Ray and Ray 1982). Alanine had been reported to induce differentiation in barley (Zhu *et al*. 1990). However, both alanine and pyruvate had no effect on the process of differentiation in tobacco callus.

Two sets of experiments were carried out, with and without MG, supplemented with either glycine or succinate at various concentrations. After 10 - 12 d of culture, callus tissue showed surface expansion and the formation of small globular-shaped structures (Tables 1 and 2). It was observed that maximum number of protuberances was produced when only BA was present in the medium. In the presence of 0.5 mM glycine and 0.1 mM MG, the number of protuberances and shoot buds

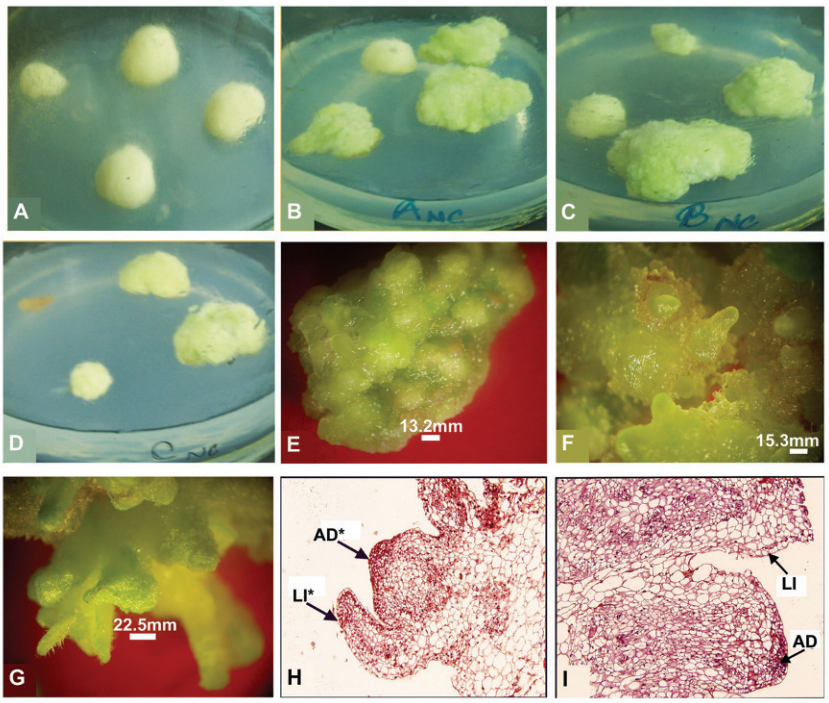

Fig. 1. Effect of MG on calluses: *A* - control, *B* - 0.1 mM MG, *C* - 0.25 mM MG, and *D* - 0.5 mM MG. Stereomicroscopic view of calluses: *E* - appearance of protuberance induction with BA alone, *F* - shoot bud induction with BA + MG, and *G* - shoot formation with BA + MG + glycine (0.5 mM). Histological view of regeneration in calluses: *H* - 0.1 mM MG + 0.5 mM glycine and *I* - 0.1 mM MG + 0.25 mM succinate (AD*- prominent apical dome, AD - apical dome, LI* - prominent leaf initial, LI- leaf initial).

Table 1. Effect of MG and glycine (GL) on organogenic protuberance induction and shoot regeneration from 1 g callus derived from leaf explants. The concentrations used for MG and BA were 0.1 mM and 2.2 μM, respectively. All the values $P \leq 0.01$ were considered significant (*F* - Fisher values).

Medium	Number of protuberances	Number of shoot buds	Number of shoots	Mean shoot length [mm]
Control	0	0	0	-
BA	30 ± 3.0	10 ± 3.0	0	-
BA + MG	10 ± 3.0	30 ± 2.0	8 ± 2.0	5.0 ± 0.66
BA + 0.25 mM GL	13 ± 3.0	8 ± 2.0	20 ± 3.0	7.3 ± 1.0
BA + 0.25 mM GL + MG	6 ± 2.0	10 ± 3.0	24 ± 3.0	10.0 ± 2.0
BA + 0.50 mM GL	12 ± 2.0	17 ± 3.0	28 ± 3.0	8.4 ± 0.92
BA + 0.50 mM GL +MG	6 ± 3.0	2 ± 1.0	51 ± 3.0	15.0 ± 1.8
BA + 0.75 mM GL	7 ± 2.0	21 ± 3.0	7 ± 2.0	5.5 ± 1.16
BA + 0.75 mM GL+ MG	15 ± 3.0	38 ±2.0	18 ± 2.0	6.2 ± 1.0
BA + 1.00 mM GL	6 ± 2.0	14 ± 3.0	8 ± 3.0	5.2 ± 0.9
BA + 1.00 mM GL + MG	9 ±3.0	16 ± 3.0	11 ± 2.0	5.4 ±1.0
F values	37.0	85.0	115.0	47.0

Table 2. Effect of MG and succinate (S) on organogenic protuberances induction and shoot regeneration from callus. The concentrations used for MG and BA were 0.1 mM and 2.2 μM, respectively. All the values $P \leq 0.01$ were considered significant.

MS	Number of protuberances	Number of shoot buds	Number of shoots	Mean shoot length [mm]
Control	0	0	0	-
BA	26 ± 2.0	5 ± 3.0	0	-
BA + MG	22 ± 3.0	11 ± 3.0	6 ± 2.0	2.0 ± 0.4
BA + 0.05 mM S	24 ± 2.0	14 ± 3.0	10 ± 2.0	25.0 ± 0.3
BA + 0.05 mM S + MG	18 ± 3.0	17 ± 2.0	12 ± 3.0	3.0 ± 0.5
BA + 0.10 mM S	15 ± 3.0	14 ± 3.0	16 ± 3.0	3.7 ± 0.7
BA + 0.10 mM S + MG	12 ± 3.0	21 ± 3.0	18 ± 3.0	5.0 ± 0.9
BA + 0.25 mM S	20 ± 3.0	13 ± 3.0	23 ± 3.0	8.0 ± 1.0
BA + 0.25 mM S + MG	23 ± 2.0	16 ± 2.0	31 ± 3.0	15.0 ± 2.5
BA + 0.50 mM S	24 ± 3.0	11 ± 2.0	9 ± 3.0	2.0 ± 0.2
BA + 0.50 mM S + MG	21 ± 3.0	14 ± 3.0	13 ± 2.0	23.0 ± 0.4
F values	13.0	11.0	41.0	86.0

were minimum, but the mean shoot length and the number of shoots produced were maximum (Table 1). A similar but less pronounced effect was observed when glycine was replaced by succinate in a medium supplemented with MG (Table 2). The maximum number of shoots produced was observed at a concentration of 0.5 mM of glycine with MG (Table 1). The batches treated with 0.25 mM succinate and MG yielded fewer shoots (Table 2).

The callus morphology varied with the number of days in culture as well as with the progression of differentiation. The callus tissue during initiation and in the very early days of development was soft and yellow. After 15 d in culture, calluses turned green and became protuberant with the appearance of several corm-like structures on the surface. Some of these protuberances eventually differentiated into meristemoids from which tiny shoot buds formed after 15 d. These differentiated shoot buds grew into small shoots within 30 d in culture. The leaf initials with the central shoot apices were found to differentiate from these callus-regenerated shoot buds. Stereomicroscopic analysis of the callus tissue clearly indicates that with BA alone, shoot morphogenesis was negligible (Fig. 1*E*). However, the rate of shoot morphogenesis was found to increase when the medium was supplemented with 0.1 mM MG (Fig. 1*F*) and 0.1 mM MG plus 0.5 mM glycine (Fig. 1*G*). This is the first report on the positive effect of a combination of MG, glycine, and BA on shoot morphogenesis and plant regeneration from tobacco callus.

In callus grown with 2.2 μM of BA alone, a few scattered meristematic regions were observed but no shoot bud formation was seen. Addition of MG to the media led to the emergence of apical dome and leaf initials. In the presence of glycine, irrespective of concentration, the tissue organization was disrupted resulting in the reduction of the number of shoot buds. The tissue was found to be more disoriented with the loss of cell to cell adhesion. However, glycine at 0.5 mM in combination with MG produced more number of shoots (Fig. 1*H*). Succinate stimulated the production of shoot buds at various concentrations. Addition of MG did not affect the number of shoot buds produced. Moreover this combination did not promote shoot formation. It was, however, noticed that 0.5 mM succinate produced cells that were enlarged with negligible meristematic activity. Addition of MG resulted in enhanced scattered meristematic regions and compact cells which seemed to be less enlarged (Fig. 1*I*). For regeneration in combination with 0.1 mM MG, optimum concentrations of glycine and succinate were 0.5 and 0.25 mM, respectively.

As mentioned earlier, when callus was grown in a medium containing MG, considerable greening was observed from the 10th day onwards and regeneration was optimum on the 30th day of culture. Chl and ALA content were therefore measured at the end of 30-d culture period. Chl content was 25 μg g^{-1}(f.m.) in medium with 0.5 mM glycine and 0.1 mM MG. Succinate (0.25 mM) with 0.1 mM MG also increased Chl content but less than glycine. The control samples which were grown in MS had negligible amounts of Chl. During the process of regeneration, changes in ALA content were similar to changes in Chl content in all the cases mentioned above.

In several publications, the role of different organic and inorganic compounds on the morphogenesis from explants has been studied. Significant roles of nitrate, ammonium ions, and other compounds on the initiation and formation of shoots have been observed (Ramage and Williams 2002). Similarly, polyamines have been found to be involved in root regeneration from cultured explants (Shen and Galston 1985, Evans and Malmberg 1989) and have a wide range of effects on plants. Even the type of the culture vessel has an impact, ventilated vessels improve regeneration and growth of plantlets significantly (Mohamed and Alsadon 2011). The role of different sugars on somatic embryogenesis and the growth of callus had also been studied (Blanc *et al*. 2002). A significant role of serine in root development (Muñoz-Bertomeu *et al*. 2009) and of auxin in both leaf and root development (Park *et al.* 2011) of *Arabidopsis*

thaliana has been demonstrated. The molecular mechanisms by which the hormones function had also been studied by investigating gene expression (Yin *et al*. 2008). An analysis of leaf anatomy and morphogenesis during early developmental stages in a mutant of *Arabidopsis thaliana* had also been studied (Janosevic *et al*. 2011). However, with the exception of a few studies, the precise molecular mechanisms which are causative of these changes have not been ascertained.

The results presented in this study clearly indicate that MG is a potent agent to promote differentiation in plants. Along with BA, it can induce differentiation to an extent which is significantly better than with the BA alone. Further improvement in shoot proliferation and shoot length was observed when the medium was additionally supplemented with 0.5 mM glycine or 0.25 mM succinate.

Since it has been observed that formation of both ALA and Chl increased in calluses treated with MG, it is quite possible that MG induces mRNA expression of the enzymes of the Chl biosynthetic pathway. Of the many biological effects of MG, its interaction with both DNA and proteins are well-known (reviewed in Kalapos 2008, Talukdar *et al.* 2008, 2009). MG might interact with a repressor molecule and deactivate it and/or might have a direct interaction with DNA base pairs, or might even stimulate the translation process. On the other hand, MG may directly stimulate activity of the enzymes responsible for the biosynthetic pathway of Chl. This is possibility supported by the observation that the effect of MG was augmented by the precursors of Chl, glycine and succinate. Although this study was carried out with *Nicotiana tabacum* calluses, it is very likely that these MG effects will be observed in other plant systems as well and may have commercial implications.

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