BIOLOGIA PLANTARUM 55 (2): 312-316, 2011

Methylglyoxal destroys *Agrobacterium tumefaciens* crown gall tumours in *Nicotiana tabacum* without any adverse effect on the host plant

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Abstract

Methylglyoxal (MG) is a highly reactive α-oxoaldehyde, demonstrating anticancer effect on plant neoplastic tumours. In *in vivo* studies it was observed that MG destroyed crown gall tumours in *Nicotiana tabacum* produced by *Agrobacterium tumefaciens*, without any adverse effect on the host. The efficacy of MG in comparison to other anticancer drugs *viz*. cisplatin and ellagic acid in the treatment of crown gall was investigated. A slight degeneration of galls was noted in plants treated with cisplatin and ellagic acid but the plants died subsequently. With MG however, crown galls were completely cured and the plants completed their usual life cycle by flowering and producing seeds. MG inhibited the respiration of crown gall calluses suggesting that energy depletion resulted in tumour destruction.

Additional key words: anticancer drug, cisplatin, ellagic acid, pyruvic acid, respiration.

Introduction

Agrobacterium tumefaciens is a ubiquitous soil borne pathogen responsible for crown gall disease, affecting many species of plants (Dusbábková *et al*. 1985). However, this bacterium is useful today in the genetic engineering of plants because of its ability to integrate parts of its own DNA into a plant's genome (Karami *et al*. 2009, Seong *et al*. 2009). In nature this ability results in the transformation of normal plant cells into tumour cells leading to the formation of crown galls. Galls tend to form on the stem at the soil line, on above ground stems and twigs and also on the roots. The galls are rough, hard and woody when older; young galls may be smooth and somewhat spongy. Infected plants become stunted and lack vigor because of the disrupted flow of water and nutrients from the roots (Braun 1954, Drummond 1979). The pathogen is a problem for agriculture all over the world. The proper control of crown gall disease is still a challenging task.

Methylglyoxal (MG) is a potent anticancer agent formed endogenously *via* different enzymatic and non-enzymatic reactions. Interest in the biological role of MG began almost a century ago but its enzymatic formation and breakdown in different organisms was elucidated only recently (for review see Kalapos 2008, Talukdar *et al*. 2008). In plants, of all the MG catabolyzing enzymes, the glyoxalase enzyme system has been studied to a significant extent. This enzyme system comprising of glyoxalase I and II works in tandem and converts MG to D-lactic acid. In plants there are also other routes through which MG could be catabolyzed (Smits and Johnson 1981, Paulus *et al.* 1993, Yadav *et al*. 2008). The anti-cancer effect of MG had been known for a long time and a large body of literature exists on this topic (Kalapos 2008, Talukdar *et al*. 2008). *In vitro* and *in vivo* experiments have shown that MG specifically destroys malignant cells. Subsequent studies have indicated that MG inhibits the glycolysis and mitochondrial respiration of exclusively malignant cells and renders these cells nonviable (Biswas *et al.* 1997). Although remarkable anticancer effect of MG has been reported in animal systems, very few studies have been carried out on plant systems so far (Lieber 1995). This paper describes the *in vitro* and *in vivo* effect of MG on crown gall tumours. Similar to its effect on animal system, MG has been observed to completely destroy neoplastic tumours in plant system as well. No adverse effect has been observed on the host plant.

Received 23 June 2009, *accepted* 30 April 2010.
Abbreviations: MG - methylglyoxal; MS - Murashige and Skoog; NAA - 1-naphthalene acetic acid.
Acknowledgements: The authors acknowledge the financial support of Department of Science and Technology, Government of India.
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Materials and methods

The strain *Agrobacterium tumefaciens* (ATCC 15955) for tumour induction was obtained from *Microbial Type Culture Collection*, Chandigarh, India. Cultures of *A. tumefaciens* in midlogarithmic phase were obtained by suspending a single colony in nutrient broth (beef extract 1 g dm^{-3}, yeast extract 2 g dm^{-3}; peptone 5 g dm^{-3}; and NaCl 5 g dm^{-3}) for 48 h at 28 ºC followed by subculturing in the same medium for another 24 h (Johnson *et al*. 1974).

Nicotiana tabacum. cv. Petit Havana (SR1 strain) was grown in an indoor plant room at 25 - 28 ºC with continuous ventilation and irradiance of 42 µmol m^{-2} s^{-1} for 16-h photoperiod. The tumour induction was done according to Lundeen (1974). To monitor the *in vivo* effects of different test compounds, 0.5 cm^3 of these compounds were injected directly at different sites of the tumour with a sterile needle.

Tobacco callus was obtained by culturing leaf explants in Murashige and Skoog (1962, MS) medium with 0.5 mg dm^{-3} 6-benzylaminopurine (BAP), 0.5 mg dm^{-3} 1 - naphthalene acetic acid (NAA), solidified with 0.8 % agar (normal callus - NC). Axenic cultures of crown gall tumour tissues (CG) were grown on standard hormone free MS medium with minor modifications in the composition of vitamins (Bourgin *et al*. 1979). Calluses were maintained by subculture at 15 - 20 d interval.

A stock solution of 100 mM MG in water and ellagic acid (100 mM) in Tris-HCl buffer (pH 9.0) was prepared and sterilized by passing through *Millipore* bacterial filters (0.22 µm). Required aliquots from these solutions were then aseptically added to the sterile nutrient media.

Growth index (GI) was calculated according to Godoy-Hernandez and Vazquez-Flota (2006) as the ratio of the final (M_F, after 30 d) and the initial biomass (M_0) [$GI = (M_F - M_0)/M_0$]. Chlorophyll was extracted from calluses by 80 % acetone and estimated by measuring absorbance at 652 nm (Roy *et al*. 2004). Crown gall cell suspensions were monitored for cell death by estimating Evans blue spectrophotometrically at 600 nm (Baker and Mock 1994). Oxygen consumption by tissue slices of one month old normal and crown gall calluses was determined using a Clark type electrode connected to a *Hansatech* (Norfolk, UK) oxygraph using a standard 2 cm^3 reaction medium containing, 40 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (PBS, pH 7.3) with 3 mM glucose at 30 ºC. For histological studies crown gall and normal calluses were grown in presence and absence of MG under *in vitro* condition. The calluses were fixed in 10 % glutar-aldehyde for 24 h, washed four times with PBS (pH 7.3) followed by dehydration in graded ethanol series (10, 30, 50, 70 and 90 %) for 15 min each; and then kept overnight in absolute alcohol. The material was sectioned 5 µm thick with a rotary microtome and stained with haematoxylin and saffranin and studied under light microscope. The microscopic sections were photographed under bright field of *Olympus BX1* (Tokyo, Japan) digital camera with *Image Pro* (Tokyo, Japan) software (Popielarska *et al.* 2006).

Beef extract, yeast extract, peptone and sodium chloride were procured from *Merck*, Darmstadt, Germany. Agar and Murashige and Skoog (MS) plant salt mixture, Evans blue were obtained from *HiMedia*, Mumbai, India. Methylglyoxal, 6-benzylaminopurine and 1-naphthalene acetic acid (NAA), were obtained from *Sigma*, St. Louis, USA. Cisplatin was obtained from *Biochem Pharmaceutical Industries*, Mumbai, India.

Each experiment was repeated at least six times and the results were found to be similar. A comparison between the experimental groups was done using Student's two tailed *t*-test. Values of $P \leq 0.05$ were considered significant.

Results

Plants treated with water died after 15 d of the gall infection (Fig. 1*A*) whereas plants injected with 5 mM MG were cured within 12 - 15 d (Fig. 1*C*) On increasing the dose of MG to 10 mM, destruction of tumour was noted within 5 - 6 d. The plants remained healthy in both the cases and completed their usual life cycle by flowering and producing seeds within 40 d after the start of the treatment. Further, MG showed no adverse effect on the uninfected tobacco plant. To investigate whether the destruction of crown gall is MG specific we also injected cisplatin (1 µM), ellagic acid (5 mM) and pyruvic acid (5 mM) separately into crown gall in a similar procedure. Injection of cisplatin, a widely used anticancer drug (Cassidy *et al.* 2002) degenerated the tumours but the plant died after 20 d of the start of treatment (Fig. 1*E*). When cisplatin was injected into an uninfected healthy tobacco plant, it died within 2 - 4 d. No significant degeneration of tumour was noted when the plants were treated with ellagic acid, a phytochemical having anticancer properties (Aggarwal and Shishodia 2006, Edderkaoui *et al.* 2008). The plant however, did not survive (Fig. 1*F*). Pyruvic acid which is structurally similar and metabolically related to MG also showed no effect on tumour growth and plant died within 20 d due to continuous growth of the tumour. (Fig. 1*D*). These results clearly indicate the specificity of MG in the treatment of crown galls with respect to other anticancer drugs.

Growth index of calluses was calculated. Normal calluses grown in presence of MG upto a concentration of 0.25 mM showed small increase in growth as compared to control. However, the growth of calluses was inhibited by approximately 10 % when MG concentration was

increased to 0.5 mM. In contrast, 0.1 mM MG inhibits the growth of crown gall calluses significantly, and MG at a concentration of 0.25 and 0.5 mM completely destroys the crown gall callus (Table 1).

Chlorophyll content of normal callus were found to increase with increasing concentration of MG in MS medium and optimum concentration was found to be 0.25 mM. MG at a concentration of 0.1 mM also induces chlorophyll formation of crown gall calluses but to a lesser extent in comparison to normal calluses. Further increase in concentration of MG leads to degeneration of crown gall tissues (Table 1).

Death of cells in crown gall callus increase with an increase in concentration of MG and all cells died when MG was used at 0.5 mM concentration. In contrast cell death of normal callus was not significant even at a concentration of 0.5 mM MG.

Fusion and enlargement into irregular shape and size of the crown gall callus was noted after incubation for 30 d in MS medium (Fig. 1*H*). Whereas under similar conditions addition of 0.1 mM MG to the medium regenerates the cells to normal both in shape and size (Fig. 1*I*). Normal callus treated with 0.5 mM MG also induces cell enlargement and chlorophyll biosynthesis as evidenced from histological staining (Fig. 1*G*) and chlorophyll estimation (Table 1). In case of CG, the

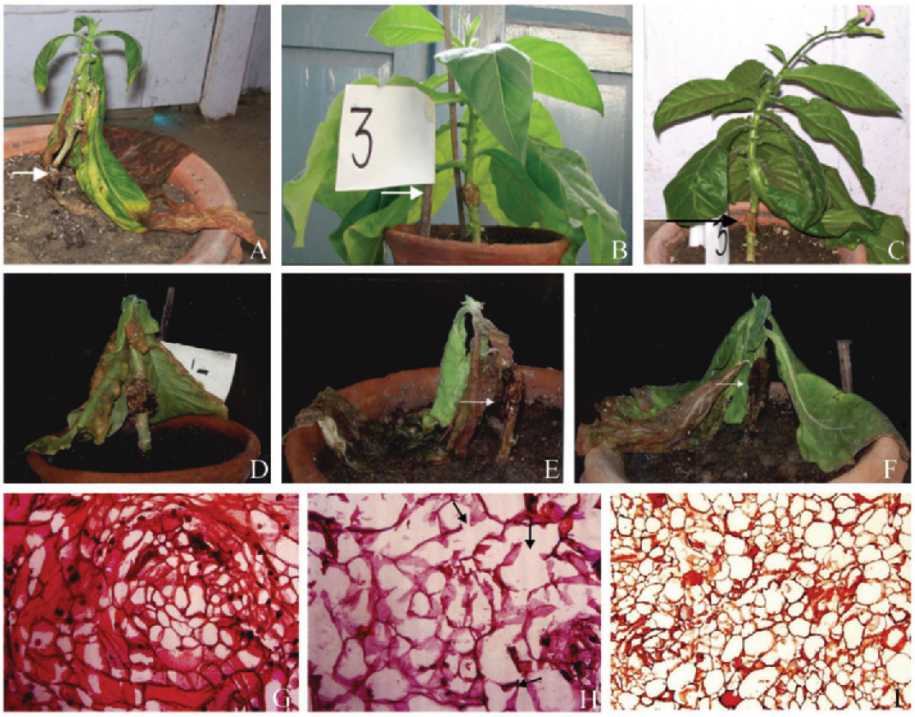

Fig. 1. Treatment of *Nicotiana tabacum* harboring crown gall by different test compounds and histogical examination. *A* - Control plants injected with water. *B* - Crown gall infected plants before start of the treatment. *C,D,E,F* - Plants treated with 5 mM MG, 5 mM pyruvic acid, 1 μM cisplatin and 5 mM ellagic acid. Histological sections of *in vitro* calluses: *G,H* - normal (NC) and crown gall (CG) callus before treatment, *I* - crown gall after 0.1 mM MG treatment.

Table 1. Growth index and chlorophyll (Chl) content of normal callus (NC) and crown gall (CG) grown *in vitro* with different concentration of MG. Means ± SD, $n = 6$; n.d - not detectable (calluses were totally destroyed).

Sample	MG [mM]	Chl [μg g^{-1}(f.m.)] 0 d	10 d	20 d	30 d	Growth index 30 d
NC	0	2.0 ± 0.34	3.0 ± 0.40	3.6 ± 0.38	4.0 ± 0.36	10.6 ± 0.34
	0.10	2.0 ± 0.34	4.7 ± 0.37	10.0 ± 0.35	18.0 ± 0.38	11.4 ± 0.35
	0.25	2.0 ± 0.34	7.8 ± 0.41	13.0 ± 0.37	25.0 ± 0.32	10.4 ± 0.35
	0.50	2.0 ± 0.34	5.0 ± 0.38	11.0 ± 0.38	22.0 ± 0.39	8.7 ± 0.40
CG	0	0.9 ± 0.32	1.2 ± 0.34	1.6 ± 0.35	2.0 ± 0.31	40.3 ± 0.40
	0.10	0.9 ± 0.32	1.8 ± 0.35	3.1 ± 0.34	4.0 ± 0.35	23.7 ± 0.30
	0.25	0.9 ± 0.32	n.d.	n.d.	n.d.	2.0 ± 0.37
	0.50	0.9 ± 0.32	n.d.	n.d.	n.d.	n.d.

calluses were totally destroyed at 0.25 and 0.5 mM MG.

In order to understand the mechanism of destruction of crown gall by MG we have investigated the inhibitory effect of MG on the respiration of tissue slices taken from untreated CG, using an oxygraph. When MG was added to these slices it was found to inhibit respiration to the extent of 31, 45 and 76 % at concentrations of 2.5, 5.0 and 10.0 mM, respectively (Table 2). In contrast MG even at a concentration of 10 mM did not inhibit the respiration of normal calluses significantly (data not shown).

Table 2. Effect of MG on crown gall and normal callus respiration rate measured as a rate of oxygen consumption [nmol(O_2) mg^{-1}(f.m.) min^{-1}]

	Control	2.5 mM	5 mM	10 mM
Crown gall	7.0±0.17	4.8±0.20	3.8±0.17	1.8±0.40
Normal callus	5.0±0.12	-	-	4.7±0.14

Discussion

This study clearly demonstrates that MG can completely destroy the crown gall present on tobacco plant without any adverse effect on the host. All the other compounds used in this experiment were not only less effective in destroying crown galls, they were also found to adversely affect the host, eventually destroying the host plant itself. Cisplatin destroyed both the plant and tumour indicating further evidence of toxicity of this widely used antitumour drug. A recent study has shown the anticancer effect of cisplatin only on crown gall under *in vitro* condition. However whether cisplatin had any adverse effect on whole plant had not been investigated in that study (Babula *et al.* 2007). The specificity of MG was further highlighted by the fact that ellagic acid showed no significant effect against crown gall, while pyruvate was totally ineffective.

It was reported earlier from different laboratories including our own that MG is an effective agent against a wide variety of malignant animal cells. This paper for the first time demonstrates the *in vivo* anticancer effect of MG on plant systems thus demonstrating the broad spectrum effects of this compound. It is generally assumed that each type of cancer is fundamentally different; however, the anticancer effect of MG against such diverse types of malignant cells begs the question whether all types of tumour cells have common altered site(s) and MG acts on these site(s). In several previous studies carried out in our laboratory it has been observed that MG specifically inhibits mitochondrial complex I in malignant cells. In this paper we also show that crown gall respiration was inhibited by 2.5 mM MG, whereas normal calluses remained unaffected up to 10 mM MG.

It is generally assumed that cancer cells derive most of the energy from glycolysis, but numerous evidences have pointed that this might not be the case (Zu and Guppy 2004, Moreno-Sánchez *et al.* 2007). In fact, as an added evidence, it had been observed that *A. tumefaciens*-induced tumour of *Ricinus cummunis* is 'mainly oxygen-respiration dependent' (Pradel *et al.* 1999). In our preliminary *in vitro* experiments we observed no significant amount of lactic acid formation either in normal or crown gall calluses (data not presented).

Crown gall is a common disease of plant with no effective treatment so far. The complete destruction of crown gall by MG without affecting the host plant appears to be potentially important in the field of horticulture. MG seems to be a cost-effective treatment for crown gall disease. Further studies to investigate the efficacy of MG in treating and destroying crown galls in other plants and cash crops would be of great significance in the field of horticulture.

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