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# Metabolic profiling to determine the cause of the increased triphenyltetrazolium chloride reduction in mannitol-treated maize callus

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ABSTRACT

When *Zea mays* callus cultures of two different genotypes were treated with the osmoticum mannitol (0.53 M) for 24 h their ability to reduce the tetrazolium derivative 2,3,5-triphenyltetrazolium chloride (TTC) to form the insoluble red compound formazan is stimulated. The formazan can be extracted with 95% ethanol for quantitation and this reaction has been used as a measure of viability since only live cells can carry out this reduction. In order to determine the cause of the increased TTC reduction caused by mannitol we carried out metabolic profiling analysis using GC–MS to identify 80 compounds. There were increases in sugar alcohols, hexoses except fructose and in total sugars. The total organic acid pools did not change and nitrogen containing compounds decreased slightly. Principle component analysis showed a large treatment effect due to changes in carbohydrate and nitrogen metabolism. These results indicate that the increased carbohydrate available for the citric acid cycle may be the cause of the increased TTC reduction observed after the mannitol treatment.



## Introduction

Tetrazolium derivatives that can be reduced by cellular redox systems to form insoluble colored products have been used to measure viability of many plant systems including seeds (Lakon, 1949), frozen tissues (Purcell and Young, 1963), heated leaves (Schaff and Clayberg, 1987) and pollen (Khatum and Flowers, 1995). The viability of cultured cells and protoplasts has also been assessed similarly including that of suspension cultures subjected to freezing and water stress (Towill and Mazur, 1975), a herbicide (Zilkah and Gressel, 1978) or callus subjected to a toxic fungal pathogen culture filtrate (Gray et al., 1986).

The insoluble red colored product formazan, in the case of 2,3,5-triphenyltetrazolium chloride (TTC), can be visualized by eye or microscopically or can be extracted with 95% ethanol to be quantitated spectrophotometrically (Duncan and Widholm, 1990).

The tetrazolium dye reduction apparently is carried out by reductants of the mitochondrial electron transport chain. Zapata et al. (1991) showed that in grapevine tissue cultured cells most of the TTC reduction was carried out by the mitochondrial alternative respiratory pathway and was sensitive to salicylhydroxamic acid.

Plant tissue cultures have been used for many studies since they have many desirable characteristics. The cells can grow rapidly as uniform tissues in axenic conditions including in liquid media so compounds can be added or removed from the medium easily. Resistant mutants can be selected using inhibitory compounds. Individual cells can be visualized microscopically and the tissues are easily extracted. Cells can be grown heterotrophically, mixotrophically or photoautotrophically depending upon the added sugar level in the medium and lighting. In most cases whole plants can be regenerated allowing micropropagation and production of transformants following particle bombardment or *Agrobacterium tumefaciens* co-cultivation and selection.

Duncan and Widholm (2004) demonstrated that type I callus and root tips of the *Zea mays* (maize) cv. Pa91 showed an increased capacity to reduce TTC to formazan after osmotic stress caused by treatment with 0.53 M mannitol leading to misleading viability estimates. Thus we carried out the studies reported here to measure the metabolites that changed during this treatment that might be involved in the increased reductant capacity to attempt to determine the cause of the TTC reduction increase.

## Materials and methods

### Tissue cultures

Callus was initiated from immature embryos of two different *Zea mays* maize inbred lines H99 and Pa91 that readily form type I regenerable callus as described by Duncan et al. (1985). Type I

*Abbreviations:* 2PGA, 2-phosphoglyceric acid; 3PGA, 3-phosphoglyceric acid; GABA, gamma-aminobutyric acid; NR, non-regenerable; PCA, Principal Component Analysis; R, regenerable; TTC, 2,3,5-triphenyltetrazolium chloride.

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cultures were maintained on D medium in the dark at 28 °C with transfer of regenerable (R) tissue every 21 d to fresh medium. The major components of D medium are N6E basal salts (Chu et al., 1975), 12 mM proline, 0.5 g $L^{-1}$ casamino acids, 28 mM $KNO_3$, 60 mM sucrose, 56 mM glucose and 8.0 g $L^{-1}$ agar (Duncan et al., 1985). The R tissue was identified visually as described by Duncan et al. (1985). The cultures of H99 and Pa91 were used 26 months and 33 months after initiation, respectively.

### *TTC reduction assay*

TTC reduction assay was performed according to Duncan and Widholm (1990, 2004): 21-day-old callus tissues were grown on D medium (Duncan et al., 1985) or D medium containing 0.53 M mannitol for 24 h in the dark at 28 °C before assay for TTC reduction to formazan. After 24 h exposure about 50 mg (FW) tissues were washed twice with 50 mM phosphate buffer (pH 7.5), then 1 mL of the same buffer and TTC with a final concentration of 23.9 mM were added. Samples were incubated for 6 h in the dark at 28 °C, collected in a 1.5 mL tube, washed three times with distilled water by centrifugation at 15,000 × *g* for 2 min, extracted twice with 1 mL of 70% ethanol for a period of 30 min at room temperature and then the formazan concentration determined spectrophotometrically at 485 nm. Heat-killed cells were used as an internal control where no TTC reduction was seen.

### *Metabolic profiling by GC–MS*

The Pa91 and H99 callus tissues were harvested and the H99 callus divided into regenerable and non-regenerable parts (Duncan et al., 1985), washed twice with distilled water, lyophilized, crushed in a Qbiogene mill (Carlsbad, CA, USA) containing 3 mm glass beads and extracted with 1.5 mL of 90% methanol for 15 min at 70 °C followed by another extraction with 1.4 mL distilled water at ambient temperature. Both extracts were then combined and 1 mL of the extract was dried under vacuum. Extracted metabolites were converted into their methoximated and TMS derivatives at 30 °C for 90 min (in 80 μL of 20 mg $mL^{-1}$ methoxyamine hydrochloride in pyridine) followed by a 30-min treatment at 37 °C (with 80 μL of N-methyl-N-[trimethylsilyl]trifluoroacetamide) (Roessner et al., 2000). Trimethylsilyl adducts were analyzed as follows: 1 μL of sample was injected with a split ratio of 5:1 on the Agilent GC/MS system consisted of a 5973 MSD, a 7673A autosampler and a 5890 gas chromatograph equipped with an Supelco 30 m SPB-50 column of 0.25 mm I.D. and 0.25 μm film thickness (Supelco, Belfonte, CA, USA). Injection temperature was 230 °C, and the interface was set to 250 °C. The ion source and MS quadrupole were adjusted to 230 °C and 150 °C, respectively. The helium carrier gas was set at a constant flow rate of 1 mL $min^{-1}$. The temperature program was: 5 min isothermal heating at 70 °C, followed by an oven temperature increase of 5 °C $min^{-1}$ to 310 °C and a final 10 min at 310 °C. Acquired GC/MS spectra were recorded in the *m*/*z* 50–600 scanning range and processed using AMDIS (NIST, Gaithersburg, MD, USA) and MSD ChemStation [version D.01.00] (Agilent, Palo Alto, CA, USA) software and compared with a spectral match quality >90% with standard mass spectrum libraries NIST02 (NIST, Gaithersburg, MD, USA), WILEY7n (Palisade Corporation, NY, USA), and a custom made library built using authentic standards.

*Metabolite recovery*. The recovery of the metabolites was evaluated by the addition of authentic metabolite standards, drawn as representatives of several compound classes, to the callus samples after the extraction followed by comparison with the pure standard mixture at the same concentration (data not shown).

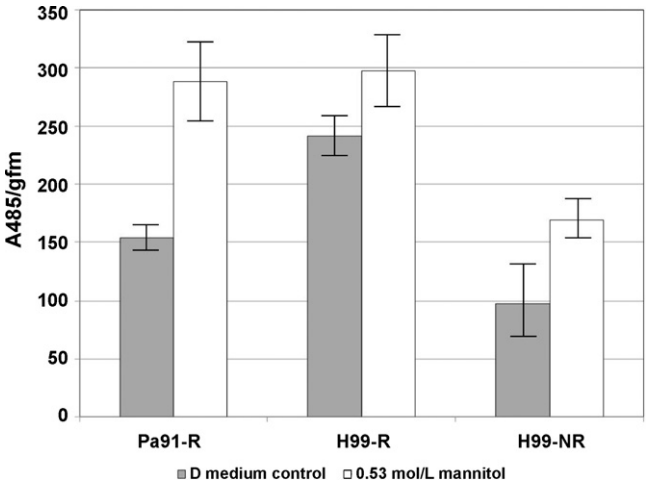


**Fig. 1.** Reduction of TTC by H99 and Pa91 regenerable (R) non-regenerable (NR) morphotype callus. The data were obtained based on three independent measurements ($n = 3$).

### *Statistical analysis*

Data sets containing 5 independent biological replicates per sample were statistically analyzed by one-way ANOVA and *t*-test using the algorithm incorporated into Microsoft Excel 2003 (Microsoft Corporation, Seattle, WA, USA). Differences were determined to be statistically significant at $P < 0.05$. To assess the metabolic changes or differences between samples and to identify metabolites involved in group discrimination multivariate analyses (PCA, PLS-DA) were performed on log-transformed (to avoid the highest intensity peaks dominating and make the values closer to a Gaussian distribution), mean-centered and Paretto-scaled data using SIMCA-P+ (12.0.0.0) program (Umetrics AB, Tvistevdgen 48 Umea 907 19, Sweden).

## Results and discussion

### *The effect of mannitol treatment on TTC reduction*

The TTC reduction capacity of Pa91 type I callus was increased after 24 h treatment with 0.53 M mannitol (Fig. 1). This was true also with H99 callus that was either visually selected as regenerable (R) or as non-regenerable (NR) in appearance according to the methods described by Duncan et al. (1985) (Fig. 1). The H99 R callus showed a higher rate of TTC reduction than the NR callus. The overall increases noted here after mannitol treatment were less than those reported in most cases by Duncan and Widholm (2004) with Pa91 callus, but were reproducible in several experiments.

### *Metabolic profiling of maize callus*

To determine how the metabolism is altered, we profiled the metabolites produced by the control and mannitol treated calli. GC/MS analysis of polar metabolites detected a total of 157 compounds in Pa91 callus and 178 metabolites in H99 R and NR calli. Of these, 75 and 80 compounds were positively identified in Pa91 and H99 calluses, respectively (Table 1) and 53 metabolites were quantified since authentic standards were available (Table 1). A total of 51 metabolites identified were altered to a statistically significant degree ($P < 0.05$) in the presence of 0.53 M mannitol in H99 calli and a total of 48 in Pa91 (Tables 1 and 2).

More metabolites (42% of total identified) were decreased in Pa91 callus (Tables 1 and 2), compared to 24% of the compounds that were increased by mannitol treatment. The H99 R calluses