Sodium Butyrate Induces Superoxide Dismutase in Rapid-Cycling *Brassica rapa*

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Abstract: **Induction of cell differentiation, inhibition of cell proliferation and apoptosis are some of the diverse biological effects that have been shown to occur in many cell types in response to sodium butyrate — a histone deacetylase enzyme inhibitor. However, these effects have been most widely studied in animal cells. This investigation reports sodium butyrate-induced changes in the antioxidant enzyme superoxide dismutase in rapid-cycling *Brassicas*; a plant model system. Seeds of rapid-cycling *Brassica rapa* were imbibed in millimolar concentrations of sodium butyrate and grown for ten days. First true leaf tissue was utilized for enzyme assays, inhibition studies, and polyacrylamide gel electrophoresis. The results suggest that sodium butyrate induces qualitative and quantitative changes in superoxide dismutase in this plant system including induction of a novel isoenzyme and a heritable upregulation of superoxide dismutase.**

Keywords: antioxidant enzyme, novel isoenzyme, sodium butyrate, superoxide dismutase

I. INTRODUCTION

The metalloenzyme superoxide dismutase (SOD) was discovered in the late 1960's by Fridovich and McCord. It was noticed that through the univalent reduction of oxygen SOD (E.C.1.15.1.1) catalyzes the conversion of superoxide free radicals [1]. Superoxide radicals (O_2^-) are formed from leakage from electron transport chains, autoxidation, and enzymatic reaction and lead to deleterious effects to lipids, proteins, and nucleic acids [2] – [5]. The dismutation reaction, characterized by McCord and Fridovich in the late 1960s, of superoxide radicals catalyzed by SOD follows:

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

It appears that SOD is ubiquitous within all aerobic organisms while not present in anaerobic life [6]. SODs have been differentiated into four types based on the metal cofactor found at the active site: Copper-Zinc (Cu-Zn), Nickel (Ni), Manganese (Mn), and Iron (Fe), while having multiple isoforms for each type [7].

Model systems in biology such as mice, *E. coli*, *Drosophila*, and *Arabidopsis* generally lead to major advances in diverse disciplines due to the combined focus of multiple research areas. Hallmarks of

these model systems are rapid reproduction and the ability to thrive in laboratory settings. In the late 1980s Paul Williams introduced six new rapid–cycling *Brassica* model systems for plant research [8]. The brassicas are a group of nutritious vegetables which include broccoli, cabbage, collards, cauliflower, and mustard greens. *Brassica rapa* with the shortest life cycle (36 days) and the shortest time-to-flowering (14 days) of the six rapid-cycling brassicas is a highly suitable organism for investigating disease resistance, generational studies, and stage dependent enzymatic analysis.

Epigenetics is a rapidly growing field that was catalyzed by the discoveries of histone acetylation and deacetylation reactions [9] – [10]. It was later discovered that sodium butyrate, a naturally occurring four carbon fatty acid, played a role in down regulating histone deacetylation reactions in vitro and in vivo [11] – [12]. Many studies followed exposing cell cultures to numerous concentrations of sodium butyrate and noticing induction of cell differentiation, inhibition of cell proliferation and apoptosis in mammalian cells [13] – [16]. Researchers have found similarities in the effect of sodium butyrate on plant cells and mammalian cells dealing with inhibition of histone deacetylation and expression of genes and proteins [17] – [20].

In this investigation, rapid-cycling *Brassica rapa* seeds were imbibed in 5 mM sodium butyrate [a histone deacetylase enzyme (HDAC) inhibitor] for 12 hours before planting. Specific activity and molecular forms of SOD in response to sodium butyrate exposure were determined. This study also investigates lasting effects on later generations. It is our hypothesis that imbibition of rapid-cycling *Brassica rapa* seeds with sodium butyrate causes increases in superoxide dismutase levels in leaf tissue.

II. Materials and Methods

A. Seeds and Plant Growth Conditions

Rapid-cycling *Brassica rapa* (RcBr) seeds were obtained from the Rapid-Cycling Brassica Collection at the University of Wisconsin at Madison. Seeds were divided into two groups for each independent experiment. The control groups were imbibed for 12 hours in 10 ml dH_2O and the experimental group in 10 ml of 5 mM sodium butyrate for the same time period. Sunshine Readi-Earth® professional

grow mix (potting soil) and the Wisconsin Fast Plant kit® (Carolina Biological Supply, Burlington, NC) were used for all plant growth experiments. Plants were grown in a Plant Light House® (Carolina Biological Supply, Burlington, NC) under 24 hour continuous lighting (230 µmol per second per square meter of irradiance). A distance of 5 – 7 cm was maintained between the light source and the plantlets. For leaf samples, plants were grown for 10 days then first true leaves were excised and stored at -80°C until used for further experimentation. Cotyledons were excised at 3 days and at 10 days post-germination then stored at -80°C until used for further experimentation.

B. Homogenization of Plant Material

All chemical reagents were obtained from Sigma-Aldrich Chemical Company, St. Louis, MO unless otherwise indicated. The homogenization solution was prepared with 50 mM potassium phosphate, pH 7.0, polyvinylpolypyrrolidone [1%], and 1X protease inhibitor cocktail (2 mM AEBSF, 0.3 µM aprotinin, 116 µM bestatin, 14 µM E-64, 1 µM leupeptin and 1 mM EDTA). Using chilled mortar and pestles, plant materials were homogenized in 1.5 ml of homogenization solution per sample at 4°C. Homogenates were centrifuged for 30 minutes at 14,000 x g (4ºC). The supernatants were stored at -80ºC until used for further experimentation.

C. Determination of Protein Concentration

Homogenates were concentrated in 30K microcentrifuge filters (Amicon® Ultra- 0.5 ml Ultracel® - 30K, NMWL 30,000; Merck Millipore Ltd; Cork, IRL) to a final volume of ~100 µl. Protein concentrations were determined using the Bicinchoninic Acid Assay Kit for Protein Determination (Sigma Chemical Co., St. Louis, MO) using bovine serum albumin as the standards. A standard curve was prepared by plotting the average net absorbance at 562 nm for each BSA standard versus the concentration in µg/ml.

D. Non-Denaturing Polyacrylamide Gel Electrophoresis

Non- Denaturing Gel Electrophoresis: All reagents and equipment used in PAGE were obtained from Invitrogen™, Carlsbad, CA. Non-denaturing Novex® 10% Tris-Glycine Mini Gels were used for all samples requiring determination of active superoxide dismutase. The samples were prepared by mixing 50 µg of protein with Novex® Tris-Glycine Native Sample Buffer (2X). The 1X Tris-Glycine Native Running Buffer was prepared by mixing 100 ml of Novex® Tris-Glycine Native Running Buffer (10X) with 900 ml of dH_2O.

Before the samples were loaded, the gels are placed inside of the XCellSureLock™ Mini-Cell Electrophoresis System then 200 mL of 1X Tris-Glycine Native Running Buffer was added to the upper chamber. The samples were loaded and 600 mL of 1X Tris-Glycine Native Running Buffer was added to the lower chamber. The XCellSureLock™ Mini-Cell Electrophoresis System was placed at 4ºC and plugged into a power source running at 15 milliamps for 10 min, 10 milliamps for 2 hours, and 5 milliamps for 2 hours.

E. SDS Polyacrylamide Gel Electrophoresis

Partially purified samples of the novel SOD isozyme were acquired from bands that were cut from non-denaturing PAGE gels that were loaded with the 5 mM sodium butyrate homogenate and stained for SOD. The bands were pulverized in 50 mM potassium phosphate buffer and centrifuged for 30 min at 14,000 x g (4ºC). Supernatants were removed, concentrated in 10K microcentrifuge filters (Amicon® Ultra- 0.5 ml Ultracel® - 10K, NMWL 10,000; Merck Millipore Ltd; Cork, IRL) and the retentates collected.

A Novex®Tris-Glycine (10%) gel protocol was used in this study. SeeBlue® Plus2 Prestained Standard (5 µl) was used as the protein molecular weight markers for comparison. Before the samples were loaded, the gels were placed inside of the XCellSureLock™ Mini-Cell Electrophoresis System. The sample-loaded gel was electrophoresed at 125 V for 70 min. The gels were rinsed using dH_2O for 10 min and then fixed using 100 ml of 50% methanol and 7% acetic acid for 15 min with gentle agitation and then rinsed with dH_2O for 5 min. The fixing solution was washed away with dH_2O for 5 minutes. The gels were agitated in Gel Code™ (Thermo Fisher Scientific Inc., Rockford, IL) stain and washed by agitating in dH_2O. Gels were destained by agitating in 100 ml of 7% acetic acid. Gels were evaluated using the Bio-Rad Chemidoc™ + XRS system.

F. Staining for active superoxide dismutase (SOD)

The staining solution consisted of 50 mL of 0.2M Tris pH 8.0, 10 mL of 2.4 mg/mL phenazinemethosulfate (stored at -20ºC), 2 mL of 10 mg/mL nitroblue tetrazolium (stored at 4ºC), and 38 mL of deionized water [21]. The stain is light sensitive so it needs to be stored in the dark. The stain is poured onto the gel and placed into a dark place for 20-25 min, while agitating every couple of minutes. The gel is then placed under and above fluorescent lights to finish the staining process. Once the staining process was completed, digital photos of the gels were taken using the Bio-Rad Chemidoc™ + XRS system.

G. Enzyme Assays

Superoxide Dismutase Assay Kits (Trevigen®, Gaithersburg, MD) containing positive and negative controls were used to determine the specific activity of SOD in all samples.

Absorbance at 550 nm was determined using a Genesys 10™ UV-visible spectrophotometer. The total reaction volume was 1.5 ml. The volume of dH_2O used per reaction was 1367.5 µl. The reaction mixture included 60 µl of 25X reaction buffer, 7.5 µl of xanthine solution, 30 µl of NBT solution, and 25 µl of homogenate was pipetted, mixed and used to blank the spectrophotometer. Then 10 µl of XOD (xanthine oxidase) solution was pipetted into the reaction and mixed. Absorbance readings (A550 nm) were recorded every 30 seconds for 5 minutes. The first time point was recorded at 30 seconds and the final time point at 5 min 30 seconds. The rate of increase in absorbance (A) per minute was calculated using the equation:

$$\frac{A_{550}@\ 5\ \text{min. } 30\ \text{sec} - A_{550}@\ 30\ \text{sec}}{5\ \text{min}} = \Delta\ A_{550}/\text{minute}$$

The negative control included all the reagent components replacing the homogenate with 25 µl of dH_2O. The absence of an NBT competitor for superoxide radicals results in absorbance proceeding maximally. The percent inhibition was determined by using the following equation:

$$\frac{[(\Delta\ A550/\text{minute})_{\text{negative control}} - (\Delta\ A550/\text{minute})_{\text{test}}]}{(\Delta\ A550/\text{minute})_{\text{negative control}}} \times 100 = \%\ \text{Inhibition}$$

The positive control included all the reagent components excluding the homogenate which was replaced by varying concentrations of SOD: 0.5 U/ml, 1 U/ml, 5 U/ml, and 10 U/ml. The SOD concentration was plotted against percent inhibition of the rate of increase of absorbance at 550 nm due to the reduction of NBT to NBT-diformazan by the superoxide radical (O_2^-) in order to formulate a standard curve for SOD inhibition. The SOD activity (U/ml) was calculated by using the equation of the line from the generated SOD inhibition curve. The specific activity of SOD (U/mg) was calculated by using the protein concentrations (mg/ml) of each test sample with the following equation:

$$\text{Specific Activity of SOD (U/mg)} = \text{SOD Activity (U/ml)} / \text{Protein Concentration (mg/ml)}.$$

Potassium cyanide (KCN) is an inhibitor of the Cu/Zn form of SOD. The percentage of Cu/Zn SOD specific activity was determined by exposing the leaf homogenate to 5 mM potassium cyanide (KCN) for a minimum of 10 minutes before assaying and subtracting the residual activity from the SOD total activity.

H. Statistical Analysis

Statistical analysis was performed using Student's t-test to determine the statistical significance of any differences in mean SOD specific activities of leaf homogenates (or cotyledons) under experimental conditions versus controls. Significance was determined as $P<0.05$. Results are presented as the mean (±SEM) of triplicate determinations from three independent experiments.

III. Results and Discussion

The present study was carried out to determine if sodium butyrate, a histone deacetylase enzyme [HDAC] inhibitor, would change the levels of superoxide dismutase in the leaves of rapid-cycling *Brassica rapa* following imbibition of the plant seeds. In Fig. 1, the data indicate that pre-treatment with 5 mM sodium butyrate triggered an increase in SOD activity that more than doubled the levels found in control seeds pre-treated in deionized water (0.14 ± 0.056 vs. 0.06 ± 0.035) -- a statistically significant difference.

The application of KCN, a Cu/Zn SOD inhibitor, strongly suggests that neither MnSOD nor FeSOD were affected by the butyrate treatment and that the upregulation of SOD activity was observed only in Cu/ZnSOD (Fig. 1).

A novel SOD isoenzyme (Fig. 2, blue arrow) was detected in the 5 mM sodium butyrate-treated samples that was not detected in the water-treated samples. The novel butyrate-induced SOD was the Cu/Zn form of the enzyme based upon the disappearance of the isoenzyme when stained in the presence of the inhibitor potassium cyanide (KCN). Using the standard curve (Fig. 3) generated from SDS-polyacrylamide gel electrophoresis and predetermined molecular weight markers, the molecular weight of the butyrate-induced Cu/ZnSOD was estimated to be 29.4 kilodaltons. This result is consistent with previous values of 25–35 kilodaltons for Cu/ZnSODs found in *Arabidopsis*—a well studied plant group related to the *Brassicas* [22].

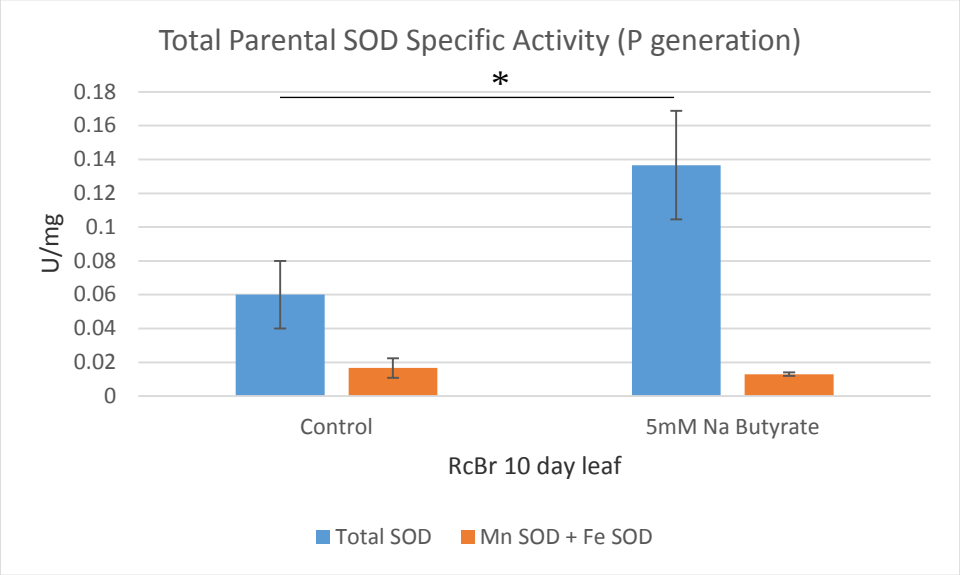

Fig. 1. The mean SOD specific activity (U/mg) for P generation control and 5 mM sodium butyrate treated RcBr in the absence and presence of KCN, an inhibitor of Cu/Zn SOD. Each bar represents the mean of triplicate experiments ± SE (*P < 0.05, Student's t-test). [Control = leaf homogenates from plants grown from seeds imbibed for 12 hr in dH_2O before planting. 5 mM Na butyrate treated = leaf homogenates from plants generated from seeds imbibed 12 hr in 5 mM Na butyrate.]

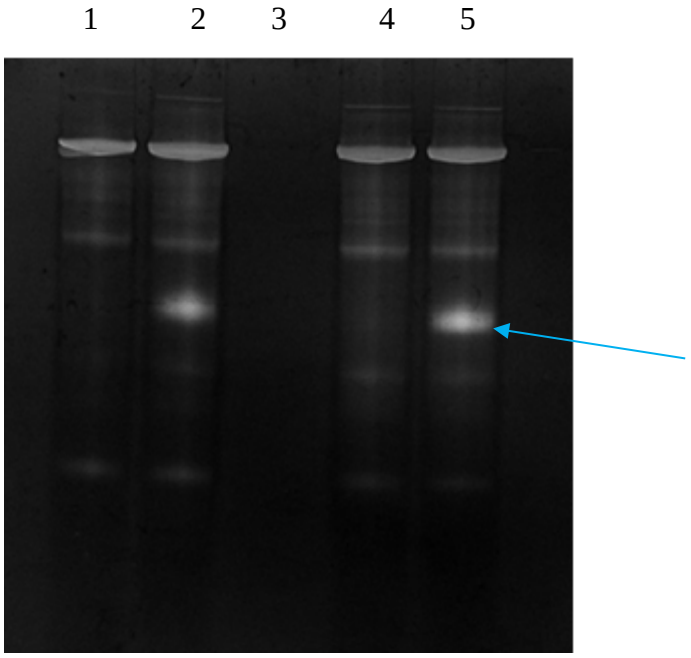

Figure 2. A non-denaturing polyacrylamide Tris-Glycine gel (10%) stained for superoxide dismutase. Samples are leaf homogenates (50 µg) loaded as follows: Lanes 1 & 4) Parental RcBr control and Lanes 2 & 5) Parental RcBr 5mM sodium butyrate-treated. Lanes 1 & 2 and 4 & 5 are results from independent duplicate experiments. Arrow indicates novel sodium butyrate-induced SOD isoenzyme. [Control = leaf homogenates from plants grown from seeds imbibed 12 hr in dH_2O before planting. 5 mM Na butyrate-treated = leaf homogenates from plants generated from seeds imbibed 12 hr in 5 mM Na butyrate.]

In order to gain some insight into the developmental stage at which sodium butyrate acts in this system, SOD isoenzyme patterns at 3 days and 10 days post-germination were established. Cotyledons are formed during embryogenesis, along with the root and shoot meristems, and therefore are present in the seed prior to germination. SOD patterns in cotyledons should indicate whether the effect of the sodium butyrate was immediate or postembryonic which is the developmental stage when first true leaves are formed from shoot apical meristem. The novel butyrate-induced SOD isoenzyme that appeared in leaf tissue was not observed in cotyledons at either time point (Fig. 4 a, b). These results suggest that the epigenetic effect of sodium butyrate is postembryonic in this plant system.

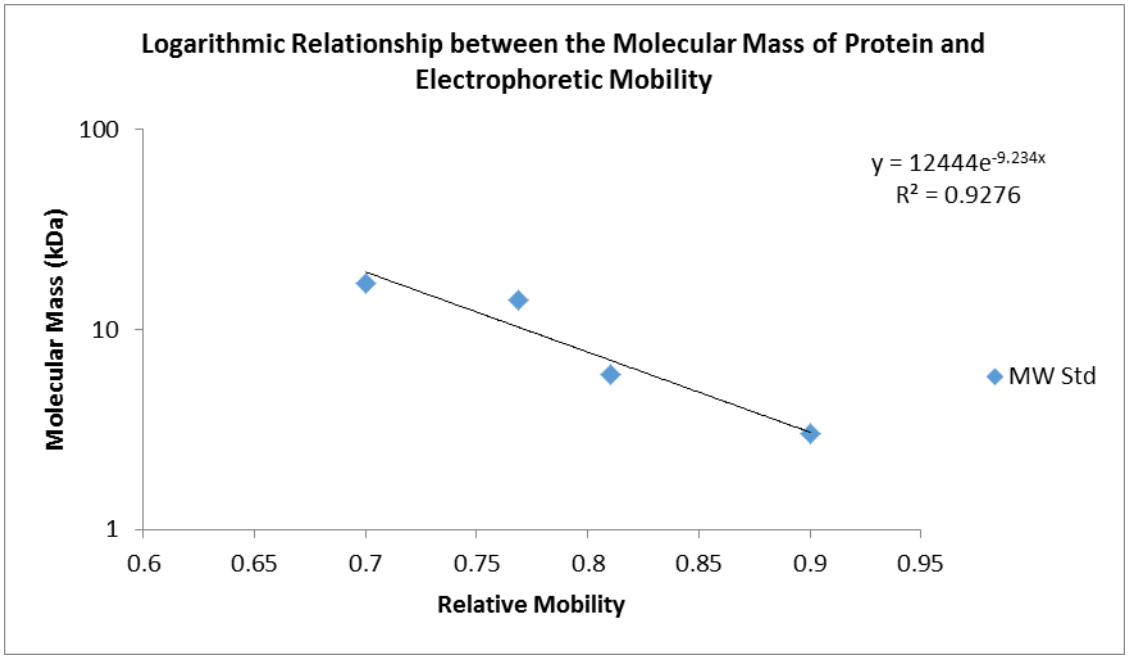

Figure 3. Relationship between the molecular mass of SOD subunits and electrophoretic mobility following SDS polyacrylamide gel electrophoresis.

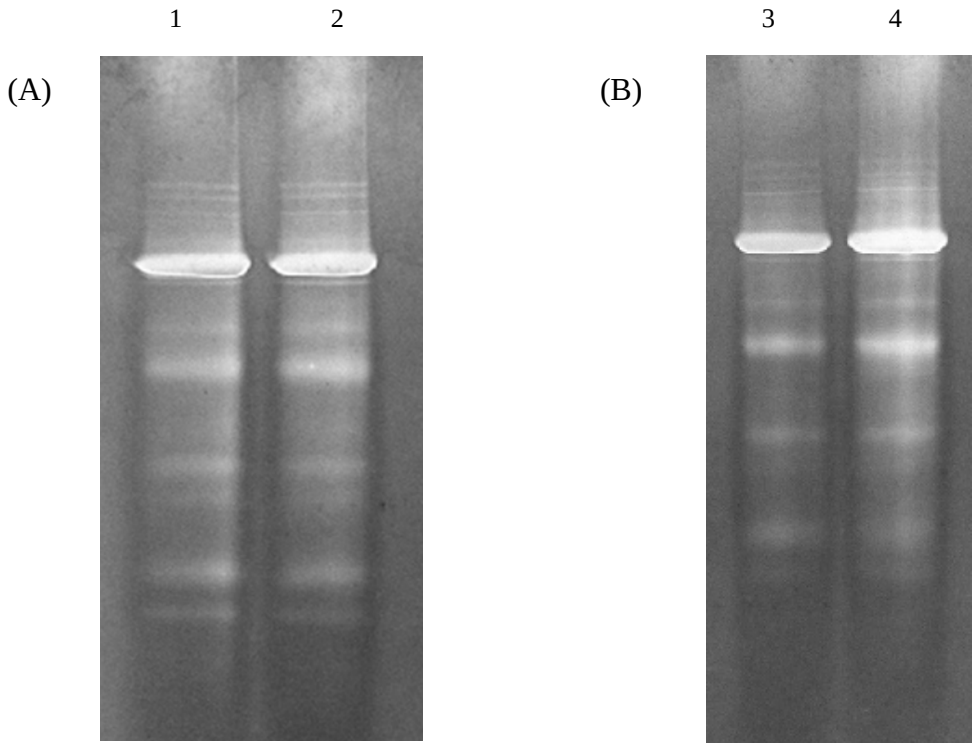

Figure 4. A non-denaturing polyacrylamide Tris-Glycine (10%) gel stained for superoxide dismutase. (A) Three day cotyledon homogenates (50 µg): Lane 1) *Brassica rapa* control and Lane 2) *Brassica rapa* 5 mM sodium butyrate treated. [Control = cotyledon homogenates from plants grown from seeds imbibed 12 hr in dH_2O before planting. 5 mM Na butyrate treated = cotyledon homogenates from plants generated from seeds imbibed overnight in 5 mM Na butyrate.] (B) Non-denaturing polyacrylamide Tris-Glycine (10%) gel stained for superoxide dismutase with ten day cotyledon homogenates (50 µg) from (lane 3) 12 hr water-treated and (lane 4) 12 hr sodium butyrate -treated seeds.

The novel Cu/ZnSOD isoenzyme induced in the leaves of the parental butyrate-treated plants was not observed in the leaves of the F_1 offspring of the parental generation nor the F_2 offspring (Fig. 5). However, compared to leaves from control plants, total SOD specific activity was significantly higher in leaves of plants from the two subsequent generations after the initial butyrate treatment of RcBr seeds (Fig. 6). These data suggest that the histone deacetylase (HDAC) inhibitor sodium butyrate can play a significant role in increasing levels of the antioxidant enzyme superoxide dismutase and contribute to heightened resistance to oxidative stress.

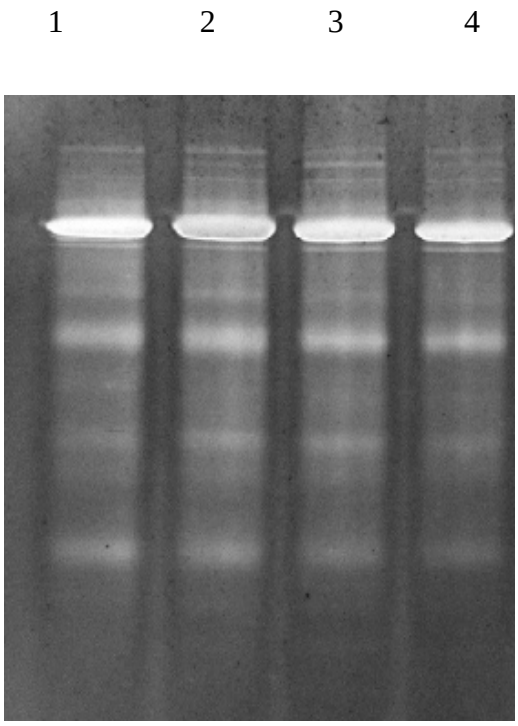

Figure 5. A non-denaturing polyacrylamide Tris-Glycine (10%) gel stained for superoxide dismutase. Samples are leaf homogenates (50 µg): Lanes 1) F_1 RcBr control. Lane 2) F_1 RcBr 5mM sodium butyrate-treated. Lanes 3) F_2 RcBr control. Lanes 4) F_2 RcBr 5 mM sodium butyrate-treated. [Control = leaf homogenates from plants grown from seeds imbibed 12 hr in dH_2O before planting. 5 mM Na butyrate treated = leaf homogenates from plants generated from seeds imbibed 12 hr in 5 mM Na butyrate]

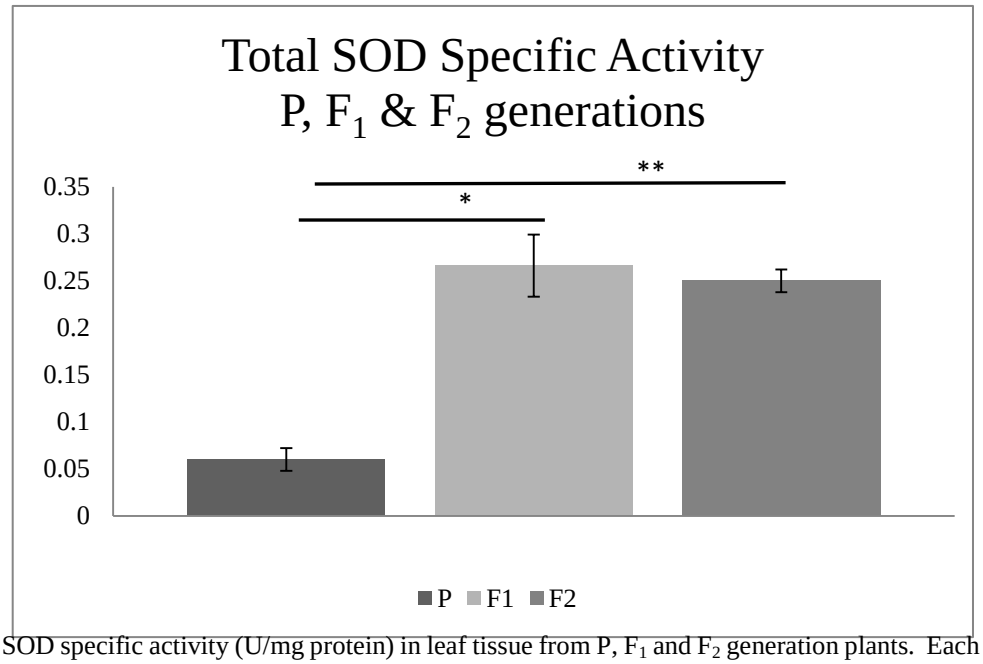

Figure 6. Total SOD specific activity (U/mg protein) in leaf tissue from P, F_1 and F_2 generation plants. Each bar represents the mean from triplicate experiments ± SE (*P < 0.05, **P<0.01, Student's t-test). [P = leaf homogenates from plants grown from seeds imbibed for 12 hr in dH_2O before planting, F_1 = leaf homogenates from plants produced by crossing P generation plants that were grown from seeds imbibed 12 hr in 5 mM Na butyrate before planting, F_2 = leaf homogenates from plants produced by crossing F_1 plants.]

IV. CONCLUSION

The experimental data support our hypothesis that imbibition of rapid-cycling *Brassica rapa* seeds with sodium butyrate before planting induces higher levels of the enzyme in the first true leaves of the resulting plantlets. In addition, butyrate treatment also caused the expression of a novel Cu/ZnSOD isoenzyme. The effects of the HDAC inhibitor caused phenotypic changes (increased SOD levels) that persist for at least two successive generations following initial butyrate treatment. The epigenetic mechanism appears to be postembryonic since the biochemical effects were not observed in cotyledons which are formed during an earlier developmental stage than leaf tissue. It remains to be determined if other plant-derived biomolecules are induced by the treatment described in this investigation.

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