Sucrose as a Foliar Additive to Upregulate Nitrate Reductase in *Arabidopsis* Under Elevated CO2

Grant Proposal

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Abstract (RQ)

Every year, ambient CO2 levels increase around the world. While widely acknowledged to contribute to greater plant growth, new research indicates that there is a subsequent decrease in plant protein production through the downregulation of the enzyme Nitrate Reductase. Nitrate Reductase converts nitrate into nitrite and is notably affected by increases in CO2. Sucrose is proven to increase Nitrate Reductase activity *in-vivo*. Therefore, a 5% sucrose foliar solution can be applied to samples of *a. Thaliana* to increase the enzymatic production of nitrite and encourage greater protein production. Arabidopsis lines CS76348 and CS78856 were grown under sterile conditions in normal and elevated CO2 conditions. Half of these were applied with a 5% sucrose solution weekly. After 3 weeks of growth with treatment, 20 samples of 10 plants were taken, and their Nitrate and Nitrite levels were measured spectrophotometrically using the Griess Assay. Remarkably, plant samples with the CO2 treatment exhibited XX% less NR activity compared to those grown in ambient conditions. Conversely, the sucrose treatment increased NR activity in both groups, with a greater impact under CO2 conditions of YY% compared to an increase in ZZ% under normal conditions. By using sucrose as a foliar treatment under varying environmental CO2 conditions, this research indicates that the production of nitrite via Nitrate Reductase can be increased. To confirm the use of this method in agriculture, amino acids must be subsequently measured to provide a glimpse into the true implications of increases in nitrite on plant protein production.

Keywords: protein production, nitrate reductase, elevated CO2, agriculture

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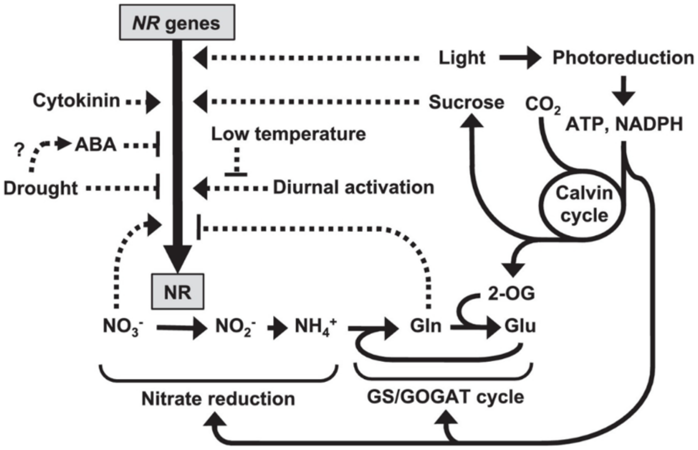
Over the last 100 years, CO2 levels in the atmosphere have increased unprecedently. An accelerating trend, these rises in fossil fuel use and growing human populations drain the planet of its resources while pumping back toxic and unwanted gases into the atmosphere throughout the process. As this occurs, the ozone layer in the upper atmosphere is depleted, exposing the earth and its organisms to greater heats and radiations that the ozone layer in its natural state would have shielded them from. Not only does this process adjust atmospheric gas distributions but greatly increases drought prevalence according to several IPCC simulations. (Radolinski et al., 2025; Sheffield, Wood, 2008). This strongly indicates a need to progress and protect modern agricultural development.

Plants supply vast amounts of nutrition to populations worldwide. For most individuals, an increase of CO2 is no concern—carbon dioxide is critical to plant growth, and many studies have proven an increase in plant activity under future climate conditions (Uddling et al., 2018). However, the effects of elevated CO2 run much deeper than photosynthesis and biomass surplus. A plant, like any organism, is made up of many components and biological machines that require balance and nutrition to stay healthy. As plant sugars and starches increase with CO2, amino acids and proteins are needed to increase concurrently to support the expanding organism. Unfortunately, under elevated CO2, this fails to occur. Unhealthy doses of carbon dioxide de-regulate critical enzymes involved in the baseline production of proteins, specifically nitrate reductase, which converts nitrate (a nitric compound from the soil) into nitrite (a nitric compound used to develop amino acids) to be synthesized further (Stitt & Krapp,1999).

Nitrate Reductase is an enzyme controlled by the *NR* gene. Alongside Glutamine Synthetase—another enzyme which develops plant nutrients through nitrate assimilation—, Nitrate Reductase is a key step in protein production via nitrate assimilation. Nitrate assimilation is the process which converts nitric oxides gathered from the soil through plant roots into different molecules in the order of nitrite, ammonium, and finally glutamine to be composed into amino acids (Titheradge, 1998). These amino acids are then put together in long molecular chains to form proteins, which define a plants structure and contribute greatly to its nutritional value for consumers.

Figure 1. Hormonal and photosynthetic activation of *NR* genes visual abstract. *NR* gene is activated with light and sucrose produced through photosynthesis to produce nitrite, which is reduced to glutamine and then into glutamate through products of the Calvin cycle (Yanagisawa, 2014).



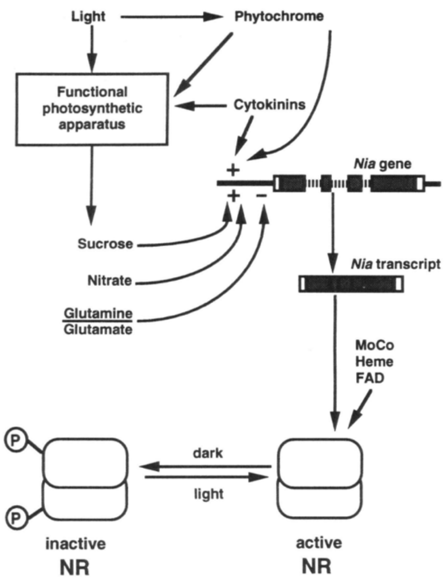


Figure 2. A schematic view of factors regulating *NR* gene activity and *Nia* transcription. Sucrose, nitrate, and cytokinin levels are key in *Nia* behavior to activate *NR* and subsequent enzymatic activity (Hoff et al., 1994)

A wide variety of factors influence this process, which consist of sucrose and glucose levels, plant hormones (cytokinin) and CO2, seen in both Figure 1 and Figure 2 which relate the relationship between Nitrate Reductase activity and enviromental factors, specifically in regard to the products and constituents of carbon metabolism (Figure 1; Figure 2; Morcuende et al., 1998). While certain hormones and sugar levels can increase the behavior of Nitrate Reductase, CO2 presence despite its relationship with sugar production does the opposite and deregulates critical nitrate reductase activity (Yanagisawa, 2014). Thus, without the up regulation of nitrate reductase, in a future climate scenario plant health, structure and crop nutritional value will reach a worrying new low.

The reduced nitrate assimilation and the subsequent loss in protein production become a problem for consumers because their crops have a significantly decreased nutritional value. Humans as omnivores especially require a balanced combination of carbohydrates, fibers, and proteins in their diets; elevated CO2 disrupts this balance on the agricultural level (Ziska, 2022). According to a recent projection, losses of 2.4%-4.3% are expected in the global availability of protein, iron, and zinc which are especially targeted in Sub-Saharan African and South-East Asian populations (Beach et al., 2019). A solution must be made to address this rising threat.

Section II: Specific Aims

This project aims to use natural sugars to increase nitrate assimilation and subsequent amino acid production under elevated CO2 conditions, which simultaneously stimulate plant growth while disrupting enzymatic and metabolic balance. An increased nitrate assimilation rate will increase the plants health and the plants nutritional value for consumers and agriculturalists.

Specific Aim 1: Develop sucrose foliar additive

The strongest application of sucrose to crops in real-world agricultural environments can be reached through a foliar additive, which is prepared in bulk beforehand with the combination of distilled water and sucrose. If sucrose was applied directly into the soil, it would disrupt microbial populations and potentially lead to a foreign growth to kill the plant. Sucrose has been observed as a surface additive in small samples of tobacco to increase *NR* activity (Morcuende et al., 1998), and previous applications of foliar additives in other fields such as the use of sucrose to reduce codling fly populations in apple trees further indicate the validity of this path (Arnault et al., 2016).

Specific Aim 2: Measure the enzymatic activity of nitrate reductase in Arabidopsis

To determine the effectiveness of a sucrose additive on nitrate assimilation in Arabidopsis plant samples, the targeted enzyme, Nitrate Reductase, must be measured through the levels of nitrate and nitrite in each plant matter. This is done through the Griess assay with additional Vanadium reduction to develop independent nitrate and nitrite measurements (Titheradge, 1998).

Specific Aim 3: Observe a change in nitrate reductase activity with foliar additive

Across all treatments groups, an increase in nitrate reductase activity which correlates to an increased rate of nitrate assimilation would be observed in treatments groups containing the sucrose additive (Stitt & Krapp, 1999). *NR* activity will be similarly measured to across different levels of CO2 to expand on existing understanding of the enviromental impact CO2 exerts purely on *NR* activity (Ziska, 2022).

The expected outcome of this work is an agricultural application of sucrose to crop leaves to increase nutritional output in a future climate scenario.

Section III: Project Goals and Methodology

Relevance/Significance

As CO2 levels increase in most agricultural settings, efforts need to be made to retain crop nutritional value and quality as select enzymatic activities weaken. Much of the world faces hunger crises, which will grow not only with overpopulation but increases in drought as predicted with heightened CO2 levels and a loss of crop nutritional content, which is proven and observed under growing levels of CO2 (Beach et al., 2019). Moreover, disruptions in nitrogen metabolism in general ecology can weaken endangered plants and allow for invasive species to thrive, like how invasive species benefit greater from nitrogen deposition compared to native species (Xiang et al., 2024).[1]

Innovation

Methodology

Arabidopsis due to its feasibility and the extent of its pertaining research in botanical fields has been selected as an experimental sample to model the sucrose foliar additive with. The project begins with the growth of multiple Arabidopsis varieties in agar. After germination and sufficient growth, seedlings are periodically treated with sucrose foliar additive, which was prepared once beforehand. These seedlings are also ordered into four treatments groups defined by their CO2 level and treatment status, resulting in four groups: a control group (normal CO2 level, no sucrose treatment), a group for sucrose in normal conditions (normal CO2 level, sucrose treatment), a predicted future control group (elevated CO2 level, no sucrose treatment) and a treated future plant group (elevated CO2 level, sucrose treatment).

Plants samples are taken from Arabidopsis after set exposure to foliar treatment, and ground with liquid nitrogen through mortar and pestle into a homogenous powder. The powder is mixed with a liquid base and combined in a centrifuge for several minutes, before being applied with the Griess reagents (sulfanilamide and N-(1-naphthyl) ethylenediamine hydrochloride). Vanadium III and hydrochloric acid solution is treated to the remaining sample to reduce the nitrate to nitrite, which is measured once more with the Griess regents. Measurements are compared to a standard curve, which will be analyzed to yield the total enzymatic action of

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[1] Xiang et al. (2024) found that increased nitrogen deposition in western North America landscapes increased the competitive advantage of invasive species due to increased nitrogen uptake parameters and suitability to the changing climate.

nitrate reductase in the samples. ANOVA testing for variance will be subsequently utilized to interpret the data between control and treatment groups to draw a justified conclusion.

Specific Aim #1: Developing a sucrose foliar additive

The objective is to develop a sucrose-water foliar spray to apply to our Arabidopsis plants. This will encourage enzymatic activity and activation in plant samples while engaging minimally with the surrounding environment through direct application to plant leaves and tissue.

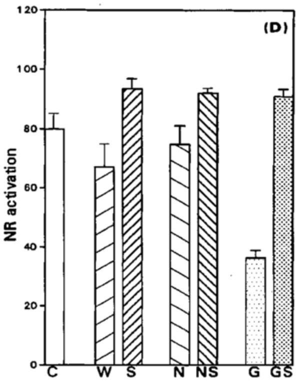

Figure 3. *NR* activation (amount of enzyme which converted one mM of nitrate to nitrite per minute) across 6 treatment groups: water, W; sucrose, S; nitrate, N; nitrate sucrose, NS; glutamine, G; glutamine sucrose, GS; and one control, C. Only sucrose containing treatments exhibited increased enzymatic activation (Morcuende et al.., 1998).

Justification and Feasibility. Nitrate reductase, controlled by the *NR* gene, can be activated and upregulated by a variety of enviromental conditions. Carbon levels stored in the plant through sugars, primarily sucrose and glucose, have a profound enviromental impact. Not only is carbon required to power enzymatic activity, but enhanced sucrose supply increases signaling to *NR* and leads to heightened enzymatic activity and activation, a previous study finding that sucrose containing treatments exhibiting the greatest impact on the *NR* activation (Figure 3). Therefor through the development of sucrose as a pure and direct additive to plant tissue, the Nitrate Reductase enzyme can be further activated and aid in the vital production of plant proteins and nutrition under adverse conditions.

A foliar additive will be approached to do this because of its feasibility on the large, agricultural scale. Foliar additives containing sucrose have been explored in other environments, including as a natural deterrent to codling flies in apple trees, with strong success (Arnault et al., 2016). While nutrients can be applied directly to growth media and soil, this encourages the uncontrolled growth of microbes and weeds which disturbs ecological balance and may develop confounding errors in data. Many different studies explore ranges of nutrient-concentrations between 5-0.01% sucrose, making a 5:95 sucrose to distilled water solution a safe and versatile treatment option.

Summary of Preliminary Data. Preliminary sucrose solution was prepared with the combination of 10ml distilled water and 500mg sucrose, settling into a little lower than 5% sucrose. This was sterilized with a syringe filter, which filtered about 2ml of solution before structural collapse. This solution has been applied to two samples (about 7 plants).

Figure 4: something related to sucrose foliar additive

Expected Outcomes. The expected outcome of completing this objective is a resolute solution that can be applied with a spray. This includes a chosen spray system and tool that must be acquired. If I were to measure sucrose levels in various samples of the liquid, the change in sucrose should not be statistically significant at a 0.05 significance level.

Potential Pitfalls and Alternative Strategies. We expect that this solution will not remain constant overtime due to the accumulation of waste and evaporation. This can be avoided through storage in clean containers in low temperature. Water can be added as evaporation requires, and a new solution should be made regularly.

Specific Aim #2: Measure the enzymatic activity of nitrate reductase in Arabidopsis

The objective is to successfully grow a preliminary sample of Arabidopsis and measure a portion of its nitrate and nitrite content. This allows for complete experimentation of a sucrose foliar additive on samples of Arabidopsis.

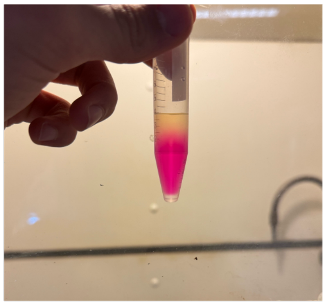

Figure 5: Pink Azo Dye Formation

Justification and Feasibility. Arabidopsis is a staple laboratory plant, being easy to propagate and having many genetically altered varieties (source). For the purposes of the experiment, wildtype Arabidopsis belonging to the different climates of Russia, Iran, and New Zealand will be grown to increase sample biodiversity and replicate real work scenarios.

Arabidopsis has been chosen not for its genetic maneuverability, but for its presence as a species that has braved many diverse environments across many continents. Its response to changes in CO2 and sucrose signaling will thus also represent that of many other native species, which provides additional insight into the feasibility and use of sucrose as an enviromental treatment to threatened ecologies with reduced biodiversity because of changes in CO2.

Summary of Preliminary Data. Sodium Nitrite was obtained from Meat Curing Salt #2, in which approximately <0.5grams was mixed with 10ml of each Griess Reagent. These were combined into a liquid solution, where pink azo dye immediately formed (Figure 2). The dye lasted for over one hour, and with a standard curve and be used to indicate the quantity of nitrite in the sample.

Expected Outcomes. The expected outcome of completing this objective is a range of known nitrite and nitrate quantities corresponding to each plant sample, which indicates their Nitrate Reductase activity.

Potential Pitfalls and Alternative Strategies. We can expect that vanadium reduction of nitrate complicates the standard curve and magnifies errors. This can be avoided by vanadium preparation immediately before use and using existing algorithms to predict NR activity solely from nitrite measurement.

Specific Aim #3: Observe a change in Nitrate Reductase Activity with Foliar Additive

The objective is to cause and observe a change in plant nitrogen metabolism when exposed to the sucrose foliar additive. This will be used to validate the effectivity of sucrose additives in increasing crop protein production.

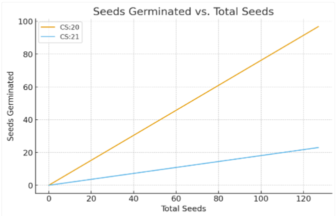

Figure 6: CS20 and CS21 Arabidopsis plant line germination rates

Justification and Feasibility. Nitrate Reductase activity is regulated in plants by a variety of factors, most significantly exposure to light, plant sugars, plant hormones levels, and CO2. Sucrose functions as a signaling molecule and a metabolic inducer for nitrate assimilation through a two step-process, significantly increasing Nitrate Reductase activity. Sucrose functions as a signaling molecule by activating sugar-sensing pathways, involving specifically Trehalose-

6-phosphate, which work to signal to the plant a sufficiency of carbon. As *NR* transcripts are upregulated through the carbon signaling, increased sucrose content becomes correlated with the carbon skeletons needed for nitrogen assimilation, providing an increase in enzyme activity with the resources needed to bolster the output of nitrite.

Beyond its implications as an internal product of photosynthesis, sucrose has been observed *in vivo* to increase nitrate assimilation through physical interaction with detached foliar surfaces of tobacco. Plants in this study were grown at consistent enviromental conditions, and after uniform growth detached leaves were left to incubate in liquid sucrose and glucose solutions for a period of 8 hours, at which point a variety of factors including *NR* gene activation and Nitrate Reductase activity were measured across treatment groups. Sucrose-containing treatments consistently exhibited heightened *NR* gene activation and enzymatic activity across all treatment groups (Morcuende et al., 1998).

Summary of Preliminary Data. Arabidopsis plant lines CS20 and CS21 were stratified at -80°C for three days before completing germination under artificial lights. The germination rate and plant health was both observed for both plant lines, where the CS20 plants line (wild type Arabidopsis) germinated at a much higher rate than the CS21 plant line (abscisic acid deficiency). 64 out of 84 CS20 seeds germinated compared to only 23 of 127 CS21 seeds. Plant growth behavior changed between the varieties as well, where wild type Arabidopsis (CS20) grew more uniformly at a slower rate.

Expected Outcomes. The expected outcome of completing this objective is measuring an observable change in Nitrate Reductase activity in Arabidopsis that is directly correlated to the level of sucrose foliar additive.

Potential Pitfalls and Alternative Strategies. We can expect that vanadium reduction of nitrate complicates the standard curve and magnifies errors. This can be avoided by vanadium preparation immediately before use and using existing algorithms to predict NR activity solely from nitrite measurement.

Section III: Resources/Equipment

Equipment and Materials

100 seeds from both Arabidopsis plant lines CS76348 and CS78856 from the ARBC were used as sample organisms. Tween-20 Detergent and Bleach were used to sterilize seeds before germination in agar plates, which included the Murashige and Scoog MES and Basal Salt solution. Granulated Sucrose was combined with autoclaved distilled water at 5% m:v for preparation of sucrose foliar solution. Placebo water solution only incorporated autoclaved distilled water, with no mineral additives.

Sample organisms were processed by freezing in liquid nitrogen before being ground in a chilled mortar and pestle. 70% ethanol, or varying quantity, was used in order to extract grounds from ceramic mortar. Eppendorf model 5417 C/R was used at 12000 rpm to extract supernatant from plant-ethanol solutions. Microcentrifuge vial warmer was used for 30hours to minimally heat and ultimately evaporate ethanol from each sample vial.

The Griess assay was prepared by pre-mixing both reagents and preparing a Vanadium III Hydrochloride solution for nitrate reduction. Sulfanilamide reagent was prepared by dissolving 1 gram of Sulfanilamide with 100ml of 5% orthophosphoric acid. The complementary solution was prepared through the solution of 0.1g N-(1-naphthyl)ethylenediamine hydrochloride with 100ml distilled water. Both solution were refrigerated and stored away from light. Vanadium solution was prepared with the solution of 39mg Vanadium III, 50ml distilled water and 50ml hydrochloric acid, which was all similarly stored, covered, and refrigerated.

Standard curve was prepared with a solution of sodium nitrite and distilled water. Sodium Nitrite was obtained through meat curing salt, which possesses 6.25% sodium Nitrite. Protocol calls for 89mg of sodium nitrite per 100ml water, so 1104mg of meat curing salt was dissolved in 100ml water to provide the necessary nitrite content. This solution was diluted 1:100 to produce 100uM sodium nitrite stock, which was further diluted into 75, 50, and 24ul stocks.

Section V: Ethical Considerations

Section VI: Timeline

Section VII: Appendix

Section VIII: References

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