Mitigation of glyphosate-induced plant stress in soybean using salicylic acid

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Abstract

Although currently embroiled in safety related considerations, glyphosate is a widely used and effective herbicide. It is known that glyphosate causes some decrease in the photosynthetic rate of glyphosate tolerant plants for a period of time after application, probably through some low-level of toxicity, resulting in stress to the plants. Salicylic acid (SA) is a plant hormone involved in coordinating plant responses to stress. The overall objective of this work was to determine if SA application could reverse the negative effects of glyphosate on the photosynthetic rate of glyphosate tolerant soybean plants. Research conducted for this thesis measured photosynthetic variables of soybean plant (third or fourth trifoliate stage) leaves 24 h after spray treatment with either SA and/or varying levels of glyphosate. The results indicated that the optimal (resulting in best photosynthetic rate) concentration of SA was between 6.9×10^{-5} M and 7.3×10^{-5} M which concur with other findings in the scientific literature that 10^{-5} M is an optimal rate for soybean application therefore, foliar spray of SA is recommended to alleviate the stress of glyphosate treatment on soybean plants. As for glyphosate, the goal was to identify the glyphosate concentration, when sprayed applied, resulting in the minimal reduction of soybean photosynthetic rate. The results were 16.3 to 12.1% of the recommended rate (1.67 L ha^{-1} of commercial production, at 41%, by volume, of glyphosate) indicated optimal photosynthetic rate. In addition, SA concentration was negatively correlated with intercellular CO_2 and stomatal conductance, indicating SA induced stomatal closure. Application of SA was also negatively correlated with leaf internal CO_2 concentration, a possible effect of reduced stomatal aperture, increased photosynthetic CO_2 uptake, or both. The work also showed that control soybean plants photosynthesized 13 to 31% faster than plants treated with double the recommended dose of glyphosate. The rate of photosynthesis for plants treated with half the recommended glyphosate was 13 to 29% greater than plants treated with double the recommended rate. Plants treated with the recommended rate of glyphosate photosynthesized 15 to 33% more than plants treated with double the glyphosate. Glyphosate applied at the recommended rate negatively affected the plant photosynthetic rate to a meaningful level ($p < 0.0001$), and SA application was able to partly restore this.

Keywords: Soybean, Photosynthesis, Glyphosate, Foliar, Salicylic acid

Résumé

Bien qu’actuellement impliqué dans des considérations liées à la sécurité, le glyphosate est un herbicide largement utilisé et efficace. On sait que le glyphosate provoque une certaine diminution du taux de photosynthèse des plantes tolérantes au glyphosate pendant une certaine période après l'application, probablement en raison d'un niveau de toxicité faible, entraînant un stress pour les plantes. L'acide salicylique (AS) est une hormone végétale impliquée dans la coordination des réactions des plantes au stress. L’objectif général de ce travail était de déterminer si l’application de l’acide salicylique pouvait inverser les effets négatifs du glyphosate sur le taux de photosynthèse des plants de soja tolérants au glyphosate. La recherche menée pour cette thèse a mesuré les variables photosynthétiques du soja (troisième ou quatrième stade trifoliée) des feuilles 24 h après le traitement par pulvérisation avec de l'AS et / ou des taux variables de glyphosate. Les résultats ont indiqué que la concentration optimale (résultant en le meilleur taux de photosynthèse) de SA se situait entre 6,9 × 10-5 et 7,3 × 10-5 M et le glyphosate entre 16,3 et 12,1% du taux recommandé (1,67 L ha-1). De plus, la concentration de SA était corrélée négativement avec le CO_2 intercellulaire et la conductance stomatique, indiquant une fermeture stomatique induite par l’acide salicylique. L'application de l'AS était également en corrélation négative avec la concentration de CO_2 interne dans les feuilles, un effet possible de l'ouverture stomatique réduite, une absorption accrue de CO_2 par la photosynthèse, ou les deux. Les travaux ont également montré que les plants de soja témoins présentaient une photosynthèse de 13 à 31% plus rapide que les plants traités avec le double de la dose recommandée de glyphosate. Le taux de photosynthèse des plantes traitées avec la moitié du glyphosate recommandé était supérieur de 13 à 29% à celui des plantes traitées avec le double du taux recommandé. Les plantes traitées avec le taux recommandé de glyphosate photosynthétisé de 15 à 33% de plus que les plantes traitées avec le double de glyphosate. Le glyphosate appliqué au taux recommandé a affecté négativement le taux de photosynthèse des plantes à un niveau significatif ($p < 0,0001$), et l'application de l'AS a permis de restaurer en partie ce problème.

Mots-clés: Soja, Photosynthèse, Glyphosate, Foliaire, Acide salicylique

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1.0 Introduction

Previous work at McGill's Plant Science Department determined that stomatal conductance, transpiration, leaf area and plant dry mass were all increased when soybean was treated with salicylic acid (SA) at 10^{-5} M, and photosynthetic rates increased 6 to 11% from day 2 to day 5 (Khan et al. 2002). Plants treated with SA at concentration levels of 10^{-3} M also had increased photosynthetic rates from days 3 to 5 after treatment, with a maximum increase of 7% compared to control plants (Khan et al. 2002).

Axter Agroscience has been active in the foliar fertilizer sector for more than 30 years. To measure the effectiveness of their new product, nutrients with added salicylates, on soybean, McGill University, the University of Guelph, CEROM, and the Ag-Quest research centre conducted trials since 2013, both in greenhouses and in the form of full scale farm experiments in 2015 and 2016. Axter products are liquid foliar biostimulants of different concentrations and/or combinations of nutrients that are designed to be mixed with herbicides, to alleviate the stress of herbicide treatment and, as a result, create more yield for crop producers. For soybean, at the third or fourth trifoliate stage of plant development, crop producers spray their herbicides from tanks and, for the producer's convenience, the product was designed to mix with the herbicide allowing the producer to spray only one time, making it cost efficient and less time consuming for crop producers. Trials conducted since 2013 have shown that 82% of the time, soybean treated with nutrients and salicylates yielded more than the control (herbicide only) and has shown an average increase of 316 kg ha^{-1} across crops.

Salicylic acid has been heavily studied as a plant hormone playing a key role in plant stress responses. It is the main treatment of this research. The goal of this work was to

identify, through experimentation, and using photosynthetic rate readings (LICOR 6400 portable photosynthesis meter) 24 h after foliar spray, the optimal application rate of SA, that which allowed minimal herbicide reduction, when combined with the widely used herbicide glyphosate, for soybean plants.

Keywords: Soybeans, Photosynthesis, Glyphosate, Foliar, Salicylic acid

Literature Review

2.0 Salicylic Acid

History of Salicylic acid

While SA is now widely understood in its role as a plant hormone, it was first investigated for its pharmacological properties. Medical scientists explore the therapeutic effects of various compounds, to understand their role in health and nutritional sciences. Salicylic acid was found to have numerous therapeutic effects (Raskin, 1992). Salicylic acid is a component of medicines that treat reduce fever and ease pain (Mahdi, 2010). For centuries the use of bark and leaves of willow trees was known to be an effective remedy for fever and rashes (Raskin, 1992). Thus, SA can be extracted from white willow bark and deployed as an analgesic. First Nations people in Canada also used willow preparations to relieve pain. Leaves were chewed by women during childbirth and pain extent was reduced. Observations of this persuaded researchers to explore the healing power of the willow plant. This practical action was taken by French and German scientists and the results of their research was summarized by Weissmann (Raskin, 1992). The active ingredient is salicin, a salicyle alcohol glycoside, which was named as salicylic acid (SA) by Raffaele Piria in 1838 (Raskin, 1992). In 1829, the processes for isolation, conversion, and oxidation of SA came from multiple scientists such as Raffaele Piria and Henri Leroux, which resulted in SA as we know it today, a white crystalline-like powder (Mahdi, 2010). The research conducted in the 19th century revealed that spiria and wintergreen plants contain methyl esters and glycosides that are easily convertible into salicylic acid through laboratory treatments. It was also determined that they can be prepared artificially. This synthetic preparation of SA was conducted in Germany for the first time, in 1874. Medicinal research has also shown that SA reduced levels of the inflammatory mammaliam hormone prostaglandin, because, SA has ability to block the mechanism by which archidonic acids form inflammatory prostaglandins. It has been is found that the acetyl-salicylic acid in aspirin used for heart disease, hydrolyzes into SA when consumed. In blood plasma it is converted by aryl esterase. Thus, exogenous aspirin is hydrolyzed into SA acid within mammals. Salicylic acid is a phenolic compound having an aromatic ring with a hydroxyl group (Raskin, 1992).

Although SA has been used for centuries for medicinal purposes, in 1979 it was discovered that salicylates had a protective function in plants, inducing stress responses by causing increased production of specific proteins (Raskin, 1992). SA was not evaluated in the same way as other known hormones were, during the 19th century, a time in which the concept of plant hormones was derived from observations and developmental experimentation, for example by Sachs between 1880 and 1893 (Davies, 2010). He proposed that "Morphological differences between plant organs are due to differences in their material composition"(Raskin, 1992) and hypothesized the existence of substances moving in different directions throughout the plant (Davies, 2010). Therefore, SA is relatively new in the world of plant sciences.

Salicylic acid and trials

In addition to having useful results in plants, SA has also been studied on human beings. While investigating medicinal effects of salicylic acid, its effects on skin care were also evaluated. The effects of SA on acne were studied and compared with glycolic acid peel’s effect. For this purpose 44 patients (33 women and 11 men) were recruited with facial acne and hyperpigmentation problems. The results included the guaranteed safety of both the peels without any side effect but efficacy of salicylic mandelic acid was noticed to be higher. So it is safe to use for all post and present acne conditions even with inflammatory lesions and hyperpigmentation (Garg, 2008).

Salicylic acid is a signaling molecule that plays a role in coordinating plant response mechanisms to abiotic and biotic stresses. Many experiments, as summarized below, have reported the valuable effects of SA, at low dosages, on plant growth stimulation in the absence of stress and in the presence of a range of stresses, such as salt stress (Khodary, 2004; Kaydan et al. 2006), water deficit stress (Agarwal et al. 2005), and herbicide induced stress (Ananieva et al., 2002; Ananieva et al., 2004). While we are not yet aware of the precise mechanism of the SA-regulated stress responses, a portion of this is likely due to its role in promoting reactive oxygen species (ROS) scavenging, like the increase in activity of antioxidants such as Cu, Zn-superoxide dismutase (Ananieva et al., 2002; Rao et al., 1997).

In the absence of stress, application of SA at 10^{-5} M to soybean increased stomatal conductance, transpiration, leaf area, and plant dry weight (Khan et al., 2002). Photosynthetic rates increased 6 to 11% from day 2 to day 5 after treatment (Khan et al., 2002). Plants treated with 10^{-3} M SA were also characterized by increased photosynthetic rates from days 3 to 5 after treatment, with a maximum increase of 7%, compared to control plants (Khan et al., 2002).

Fariduddin et al. (2003), found that water solutions of SA applied to foliage of 30-day-old mustard (*Brassica juncea* Czern & Coss cv. Varuna) plants were more beneficial in lower concentration (on the order of 10^{-5} M SA) than water, or higher concentrations (10^{-4} or 10^{-3} M SA). Sixty-day-old plants were characterized by an increase of 8.4% in dry mass, 9.8% in photosynthetic rate, 9.3% in carboxylation efficiency, 13% in activities of nitrate reductase and 18.5% in carbonic anhydrase, over water treatment only (Fariduddin et al. 2003). Furthermore, the number of pods and the seed yield increased by 13.7 and 8.4 %, respectively, compared to plants treated with water only.

Khan et al. (2003) and Kumar et al. (2000) concluded that stomatal conductance, transpiration rate, leaf-internal CO_2 levels, and water use efficiency all improved in soybean treated with SA. Greater SA application rates (5 and 1 mM) inhibited nodulation, thereby decreasing nodule number and dry mass of soybean, due to decreased nitrogen fixation and photosynthesis (Fariduddin, 2003).

Kuchlan et al., (2016) investigated foliar application of SA and found positive effects on seed yield, seed health, germination and seed vigour during storage (Kuchlan et al. 2016). Application of SA was effective in protecting plants from various diseases, resulting in higher quality seed, which improved the storage quality and longevity of soybean seed at 50 ppm SA, when applied at the seedling and pod filling stages (Kuchlan et al. 2016).

Salinity, as mentioned above, can diminish the growth and health of plants. Trials involving corn (*Zea maize*) showed that salt stress statistically significantly reduced Rubisco activity, photosynthetic efficiency, pigment levels, and sugar contents (Khodary, 2004). The effects of NaCl on these variables were increased with increased NaCl concentrations (Khodary, 2004). However, exogenously applied SA (10^{-2} M) reduced salt damage to *Z. maize*. SA improved salt tolerance by improving the measured plant growth aspects indicated above. SA appeared to stimulate maize salt tolerance by activating the photosynthetic process (Khodary, 2004). Kaydan et al. (2006) seed-soaked wheat in SA (10^{-2}, 10^{-4}, 10^{-6} M and control) and measured growth variables and physiological characteristics under salinity (8 ds m^{-1}) and non-salinity conditions. Reduced emergence, shoot and root dry weight, photosynthetic pigments, and decreased osmotic potential characterized NaCl-stressed wheat. Nevertheless, salinity stressed wheat that was seed-soaked in SA was improved with respect to all these attributes. These results indicated a clear positive effect of SA on wheat grown under saline conditions. The most effective concentration for effects on plant growth and physiological characteristics was 10^{-2} M SA.

Regarding water stress, Agarwal et al. (2005) applied abscisic acid (ABA) and SA as foliar spray on wheat at 25 and 40 days after sowing under water stress (−0.8 MPa) conditions and added nutrient solution. Abscisic acid and SA amplified the activity of ascorbate peroxidase, superoxide dismutase, glutathione reductase, and catalase, compared to control plants. The contents of hydrogen peroxide and thiobarbituric acid were lower in treated plants than untreated plants. The increased levels of antioxidant enzyme activity and reduction in oxidative damage was reflected in an increase of chlorophyll and carotenoid contents, relative water content, membrane stability index, leaf area, and total biomass, over unsprayed plants. Generally, lower concentrations of ABA (0.5 mM) and SA (1.0 mM) were more beneficial than higher concentrations (Agarwal et al. 2005).

For herbicide-induced stress, previous studies proposed that SA enhances tolerance to herbicides in barley plants, specifically to paraquat (Pq) (Ananieva et al. 2002). To further elaborate the role of SA in herbicide induced reactions, the study analyzed antioxidative defence mechanisms by assessing the actions of several antioxidative enzymes: superoxide dismutase, ascorbate peroxidase, glutathione, dehydroascorbate reductase (DHAR), catalase (CAT), and guaiacol peroxidase (POX). Twelve-day-old barley seedlings were supplied with 500 μmol L^{-1} SA or 10 μmol L^{-1} Pq, via the transpiration stream, and kept in the dark for 24 h. Then they were exposed to 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and measurements were taken 6 h after the onset of light exposure. Treatment of seedlings with 10 μmol L^{-1} Pq reduced the activity of APX and GR, did not affect the activity of POX and DHAR but caused a more than 40 % increase in the activity of CAT. Pre-treatment with 500 μmol L^{-1} SA for 24 h in the darkness prior to Pq application increased the activities of the studied enzymes in both the chloroplasts (SOD activity) and the other compartments of the cell (POX and CAT activities). The effect of SA pre-treatment was very clear for DHAR and POX activities. The research suggested that SA protects against Pq effects through antioxidative reactions in barley plants.

Salicylic acid stimulates plants by activating multiple defense pathways and plant growth responses (Fariduddin, 2003). Exogenously applied SA strengthens the growth and productivity of plants only when applied at relatively low concentrations. Based on the results of previous experimentation, it is hypothesized that SA, at low concentrations, is advantageous to photosynthesis and other factors that contribute to the growth of plants. Previous research indicated that SA, at relatively high concentrations, can be detrimental to plants and cause stress effects of its own (Fariduddin, 2003).

Plant systemic acquired resistance (SAR) is a defense mechanism that is activated, typically, when a pathogen is introduced into the plant (Bektas & Eulgem, 2014). SAR is triggered in one of two ways, when pattern recognition receptors encounter microbe associated molecular patterns, activating pattern triggered immunity (PTI) (Bektas & Eulgem, 2015). Receptors that trigger PTI can be blocked by effector molecules that are secreted into plant cells by microbial pathogens (Bektas & Eulgem, 2015). Effector-triggered immunity is induced by the presence or activity of effectors blocking PTI, and it is characterized by a strong immune response that can cause death of plant cells in the area of the infection (Bektas & Eulgem, 2015). Numerous studies have shown that ETI and PTI utilize a mutual set of signaling mechanisms, together with various regulatory proteins, reactive oxygen intermediates (ROIs), SA, ethylene (ET) and jasmonic acid (JA). Levels of ROI, SA, ET, or JA often rise in plant tissues after infections (Bektas & Eulgem, 2015). The infected tissue discharges signals that are carried to the rest of the plant, where they induce SAR, which protects the plant against other possible infections from a broad spectrum of pathogens (Zewail, 2014). Effective and strong signaling establishes SAR in plant organs that are relatively far apart (Zewail, 2014). With exogenously applied salicylates, enhanced capacity to activate immune responses is established in plants, in the absence of a pathogen. Triggering of plant defenses is called ‘priming’; it can also be activated by treatment with necrotizing pathogens, inoculating with microorganisms, or treatment with natural or synthetic compounds, plant elicitors, that can activate plant defense mechanisms (Bektas & Eulgem, 2015).

SA regulates the activation of SAR and signaling networks in plants under abiotic and biotic stress conditions and stimulates various physiological processes related to plant growth and development (Pacheco et al. 2013). When SAR is activated by the accumulation of SA, the derivative SA glucoside (SAG) and SA-responsive pathogenesis-related 1 (PR1) genes are triggered in non-pathogen infected sites of the plant, and even more so when SAR-activated parts of the plant encounter a pathogen (Shah & Zeier, 2013).

SA is synthesized through two separate pathways that require different precursors: theisochorismate pathway in the chloroplast and the phenylpropanoid pathway, which is induced by phenylalanine, in the cytoplasm (Rivas-San Vicente et al. 2011). Much of the SA produced in plants is glucosylated, which in turn creates SA 2-O-b-D-glucoside (SAG) (Rivas-San Vicente et al. 2011). In *Arabidopsis* plants, the isochorismate pathway is the major source of SA during SAR (Durrant, 2004). In response to exogenous application of SA long-lasting SAR is induced, even in the absence of pathogens, leading to stimulation of plant defense mechanisms that can, under field conditions, boost crop yield.

2.1 Glyphosate

Glyphosate [*N*-(phosphonomethyl)glycine] was first synthesized in 1974, and it is currently the world's most widely used herbicide (Duke & Powles, 2008). The relative affordability, due to the homogeneous industrial production of glyphosate, commercially known as Roundup®, contributes to the popularity of this herbicide. It is currently the only non-specific and broad-spectrum herbicide that targets 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) (Duke & Powles, 2008). The foliage of plants sprayed with glyphosate take up and translocate the herbicide in the form of polyoxyethylene amine (POEA) (Van Bruggen et al. 2018). The success of glyphosate as a herbicide is also due to the creation of glyphosate-resistant soybean genotypes, with the first one appearing in 1996, followed by development of glyphosate resistant forms of other major crops. At this time about 90% of all genetically modified crops planted in the world are glyphosate resistant (GR) with 700,000 t of the herbicide used year^{-1} (Duke & Powles, 2008; Van Bruggen et al. 2018).

At first, herbicide tolerant (HT) crops, mainly soybean, regularly led to decreases in glyphosate use due to the non-existence of glyphosate resistance weeds (Bonny, 2016). However, glyphosate-only applications were consistently sprayed on the same fields, without sufficient alteration of herbicide and crop diversity, with increased end-of-season spraying to induce senescence and facilitate harvesting (Bonny, 2016). Therefore, total area and application patterns resulted in strong selection pressure for glyphosate-resistance in weed populations (Bonny, 2016; Van Bruggen, 2018). Since 1998, glyphosate resistance has been recognized in 24 plant species (Sammons & Gaines, 2014). Weeds have evolved nine mechanisms of resistance to glyphosate and 18 glyphosate resistant (GR) species are described as resistant to multiple other herbicides (Green, 2016; Sammons & Gaines, 2014). Some causes of resistance relate to replacement of the amino acid proline with either serine or threonine, within key proteins, resulting in an enolpyruvylshikimate-3-phosphate (EPSPS) resistant to glyphosate (Powles & Preston, 2006). Resistance can also be attributed to vacuolar glyphosate sequestration, gene duplication, and rapid mature leaf death, which slows or halts glyphosate translocation (Sammons & Gaines, 2014). For example, glyphosate eventually failed to control a population of the grass weed rigid ryegrass in Australia after 15 years of successful use. The weed demonstrated resistance to glyphosate in pot dose-response experiments, exhibiting 7- to 11-fold resistance when compared to a vulnerable genotype. The presence of glyphosate resistance in a major weed species indicates that an alternative mode of application or formulation in glyphosate should be considered

(Powles et al. 1998). In a more recent study, *Kochia scoparia*, in North America, has developed glyphosate tolerance as well as cross-resistances to other herbicides but can be controlled by other means such as tillage (Schwinghamer & Van Acker, 2008).

As a broad-spectrum herbicide, crop producers may, on a needs basis, diversify their weed management practices and utilize current selective herbicides such as photosystem II (PSII) inhibitors like triazine and urea herbicides, lipid synthesis inhibitors such as S-metolachlor, and inhibitors of phytoene desaturase (PDS), which also provide soil residual activity against GR weeds (Green, 2016). Due to the presence of GR weeds, many growers now use larger volumes of partially effective glyphosate or other herbicides (Green, 2016). Growers have lost the advantage of being able to use less product that was more effective than the alternatives because of GR weeds. For example, in the United States, from 1998 to 2011, soybean farmers used 28% or 0.30 kg ha^{-1} more herbicides (Perry et al., 2016). Glyphosate-tolerant (GT) soybean producers used progressively more herbicide than non-adopters of GT soybean, and the trend to larger quantities of herbicide over time was associated with the development of GR weeds (Perry et al., 2016).

Glyphosate is used worldwide; both weeds and crops are treated, however, little is known regarding glyphosate induced stress in crops that are resistant, but not completely immune, to the herbicide. Crops undergo stress due to the inability to rapidly detoxify the herbicide. From 1974 to 2014, glyphosate was sprayed and glyphosate use has risen 15-fold since 1996 (Benbrook, 2016), anecdotal evidence suggests that crop producers used 1.5-, two-, and three-times the recommended rate of glyphosate, where weeds are glyphosate-resistant. Several lines of experimentation, like the ones described in this thesis, have indicated that crops, especially soybean, endure metabolic stress after being treated with glyphosate. Several lines of experimentation, as described below, indicated that soybean cultivars experience stress when treated with glyphosate.

Although glyphosate-resistant plants are used for soybean cultivation in most countries and there is no unique method or mode of action of glyphosate use for such cultivars. Glyphosate will most likely continue to be the most applied pesticide in the world for years to come, and attention will grow in terms of measuring associated ecological and human health issues (Benbrook, 2016).

2.2 Glyphosate and Cancer

The IARC (International Agency for Research on Cancer) has suggested that there is some evidence for a connection between exposure to glyphosate and cancer in humans. Several studies have shown that people who work with glyphosate seem to be at increased risk of non-Hodgkin lymphoma cancer. A putative example of this cancer is groundskeeper Dewayne ‘Lee’ Johnson who was diagnosed in 2014, at age 42, with this particular type of cancer. A California jury ordered Monsanto to pay Johnson $289 million US in damages (https://www.cbc.ca/news/technology/monsanto-roundup-health-canada-1.4896311).

The IARC report includes a US Agricultural Health Study, which surveyed thousands of farmers and looked at whether they had increased incidences of cancer, but no link to non-Hodgkin Lymphoma was found. However, other evidence, including animal studies, has indicated links to tumours in mice and rats and this led the IARC to its ‘probably carcinogenic' classification, which would put the herbicide in group 2A, the second most toxic group, because the evidence in experimental animals was 'sufficient and the evidence in humans was limited' as explained by Kathryn Guyton, a senior toxicologist in the monographs program of the IARC, and one of the authors of the study (Cressey, 2015).

Health Canada says in light of "troubling allegations", its scientists are reviewing hundreds of studies used during the approval process for glyphosate. The decision comes after a coalition of environmental groups such as Equiterre, Ecojustice and Canadian Physicians for the Environment claimed Health Canada relied on studies that were influenced by the makers of glyphosate, Monsanto (https://www.cbc.ca/news/technology/monsanto-roundup-health-canada-1.4896311).

Health Canada's Pest Management Regulatory Agency received academic papers investigating the possibility that glyphosate causes cancer. They were initially deemed independent, but further scrutiny has suggested that Monsanto had influenced the writing these papers. At the time these papers were written and approved, Health Canada accepted the risks of glyphosate, indicating that if used correctly, according to product labels, there was little risk. Health Canada is now reviewing hundreds of case studies to assess whether or not the information validates a change to the original decision. Equiterre wants to suspend use of glyphosate until further investigation is conducted, since approval of the product was based, in part, on these papers. As discussed above, glyphosate is now commonly used in the production of crop plants such as soybean, corn, wheat, oat and other beans and grains on a regular basis. (https://www.cbc.ca/news/technology/monsanto-roundup-health-canada-1.4896311). The

Coalition says those papers prove that those specific scientific studies were either co-written, edited or otherwise influenced by Monsanto, without accurately revealing the company's role in the matter. These research papers allowed glyphosate to be sold in Canada (CBC News, https://www.cbc.ca/news/technology/monsanto-roundup-health-canada-1.4896311).

2.3 Glyphosate & Nitrogen metabolism

Glyphosate effects on nitrogen metabolism have mostly been researched in soybean in, which symbiotic nitrogen fixation supplies 40 to 70% of the plant’s total nitrogen requirement (Gomes, 2015). It is very crucial for farmers to maintain this significant nitrogen contribution, to have profitable yields and to maintain long-term soil quality, particularly in soils with less nitrogen availability and in soils where rotations are conducted regularly, with higher nitrogen consuming crops such as corn (Gomes, 2015). Glyphosate can impact plant nitrogen content through the rhizobial symbiont, or by affecting the physiology of the plant (Gomes, 2015). Organisms besides plants, possess the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzymes and are therefore affected by glyphosate (Gomes, 2015). For example, the nitrogen fixing symbiont *Bradyrhizobium japonicum*, possesses a glyphosate-sensitive EPSPS and accumulates shikimate and hydroxybenzoic acids, such as protocatechuic and/or gallic acids, upon exposure to the herbicide (Gomes, 2015). This leads to growth inhibition and cell death at high glyphosate levels (Gomes, 2015). The build-up of protocatechuic acid in soybean nodules of plants treated with glyphosate suggests a possible translocation of glyphosate to the nodules. This possibility was reinforced by the reduced nitrogenase activity of *B. japonicum* bacteroids in glyphosate treated plants (Gomes, 2015). This could be due to the accumulation of toxic substances in the shikimic acid pathway and negative effects on the synthesis of aromatic amino acids leading to increased chemical energy (ATP and PEP) spent in the shikimate pathway (Gomes, 2015).

2.4 Glyphosate & Nutrients

The nutrient status of plants treated with glyphosate has not yet been widely studied and the results available concerning glyphosate-resistant crops are inconsistent (Gomes, 2015). Some reports did not find any effect on plant nutrition (Bailey et al. 2002; Rosolem et al. 2010), while others have shown that glyphosate diminished the nutrient status of plants (Cakmak et al. 2009; Zobiole et al. 2015). In one study, conducted by Duke et al. (2012), glyphosate application at the recommended rate of 0.86 kg ha^{-1}) did not affect the mineral content of leaves or seeds of GR soybean plants grown under greenhouse and field conditions; Ca, Mg, Mn, Zn, Fe, Cu, Sr, Ba, Al, Cd, Cr, Co and Ni were all measured (Gomes, 2015). Rosolem et al. (2010) did not find any effect of glyphosate on soybean Mn nutrition, and determined that the effect of glyphosate and negative soybean nutrition status depends on several factors, such as the cultivar, stage of the plant development, and growth conditions (Gomes, 2015). Nevertheless, the herbicide molecule was first patented as a metal chelator and so it is possible that its application could affect plant nutrition and possibly alter plant growth, even in plants resistant to glyphosate (Zobiole et al.201l; Bromilow et al. 1993).

Research conducted by Cakmak et al. (2009) showed that glyphosate may induce nutritional distress by reducing the levels of certain nutrients within plants or inhibiting with their uptake, thus making the plants more vulnerable to disease (Gomes. 2015). Glyphosate, due to its chelating property, when applied to plant leaves, decreases the free activity of cationic mineral nutrients, leading to nutrient deficiency in cells (Cakmak et al., 2009; Gomes. 2015). The herbicide binds readily to divalent cations, through its carboxyl and phosphonate group, forming insoluble or very stable complexes, leading to immobilization of several divalent cations in plant leaves (Gomes. 2015). In addition, Zobiole et al. (2012) observed decreases in macro- and micronutrient contents of GR soybean plants treated with glyphosate. Glyphosate treated plants are generally less efficient in nutrient translocation and uptake, likely due to chelating effects of glyphosate leading to the formation of metal complexes within plant tissue (Gomes 2015).

Also, reduced nutrient uptake may be related to glyphosate limitation of root growth (Zobiole et al., 2012). Zobiole et al. (2011) found that glyphosate affects macronutrient accumulation in the following order: Ca > Mg > N > S > K > P whether glyphosate was applied as a single application or in sequential applications (Zobiole et al. 2011). Micronutrient accumulation is affected in the following order: Fe > Mn > Co> Zn > Cu > B > Mo and Fe > Co > Zn > Mn > Cu > Mo > B, for single and sequential treatment, respectively (Zobiole et al. 2011). Glyphosate effects on plant mineral nutrition depend on plant growth stage (Zobiole et al. 2011). For example, younger plants were more sensitive than plants treated at a later growth stage (Zobiole et al. 2011).

2.5 Glyphosate & Photosynthesis

Some herbicides were found to interfere directly with the photosynthetic electron transport. For example, 3-(3, 4 dichlorophenyl)-1, 1-dimethyl urea (DCMU) is known to block the electron flow between QA and Qa, in the photosynthetic apparatus, by competing for Qa binding sites (Gomes, 2015). Herbicides, such as glyphosate, affect photosynthesis indirectly by inhibiting the biosynthesis of carotenoids, chlorophylls, amino acids, and fatty acids. Glyphosate blocks the shikimate pathway by inhibiting the biosynthesis of secondary metabolites in plants, including compounds associated with photosynthesis, such as quinones (Gomes, 2015). It is not clear how glyphosate leads to plant death (Gomes, 2015); it has been indicated that depletion of protein stocks and depletion of reduced carbon from important pathways are possibilities, and numerous field and greenhouse studies have indicated a decreased photosynthetic rate in plants following glyphosate exposure (Gomes, 2015)

2.6 Experimentation with glyphosate

Cakmak et al. (2009) conducted greenhouse experiments to study the effects of glyphosate on plant growth and concentrations of mineral nutrients in leaves and seeds of non-GR soybean plants (Cakmak et al. 2009). The results of this study suggest that seed concentrations of Ca, Mg, Fe and Mn were greatly reduced by glyphosate and that glyphosate may inhibit uptake and transfer of these nutrients throughout the soybean plant by immobilizing them (Cakmak et al. 2009). Decreases in seed concentration of Fe, Mn, Ca and Mg due to glyphosate treatment may affect overall health of the plant and, ultimately, seed quality (Cakmak et al. 2009).

Zobiole et al. (2009) investigated an decrease in macro- and micronutrients in plant-leaf tissue and in photosynthetic variables (chlorophyll content, photosynthetic rate, transpiration and stomatal conductance) with glyphosate use (single or consecutive applications). Concentrations of shoot macro- and micronutrients were found to be lower in GR-cultivars than non-glyphosate ready parental lines. Shoot and root dry biomass were affected and reduced by the herbicide, with all GR plants evaluated in two soils (Zobiole et al., 2009). Another study by Zobiole et al. (2009) demonstrated that increasing glyphosate rates caused a significant decrease in photosynthesis, nutrient uptake and macro- and micronutrient accumulation in leaf tissue samples of GR soybean plants (Zobiole et al., 2009).

Krishna et al. (2008) conducted greenhouse and growth chamber experiments to analyze glyphosate effects on growth, chlorophyll content, nodulation, and nodule leghemoglobin content of GR and non-GR soybean cultivars. In susceptible soybean genotypes, a single application of 0.28 kg ha^{-1} reduced chlorophyll content by 49% and shoot and root dry weight by 50 and 57%, respectively, at two weeks after treatment (Krishna et al, 2008). In glyphosate-resistant soybean, there were no significant effects on these variables due to a single application of 1.12 kg ha^{-1}, however, 2.24 kg ha^{-1} reduced shoot and root dry weight by 25 to 30% (Krishna et al. 2008). Larger applications also reduced root growth, and nodule number by 30% and total plant biomass by 39%, compared to untreated plants (Krishna et al, 2008). There was little or no effect on GR soybean plants at lower rates, but at higher rates, there is potential for soybean stress related injuries, with regard to chlorophyll content and dry weight of shoots and roots (Krishna et al. 2008).

Glyphosate is one of the most used herbicides in cereal-growing regions of the world. Therefore, the protein expression profile of rice leaves treated with glyphosate was analyzed to examine the effects of glyphosate on leaves of two-week old rice plants (Ahsan et al. 2008). A histo-cytochemical investigation with DAB (3,3-diaminobenzidine) attempted to localize H_2O_2 and increases in the thiobarbituric acid reactive substances (TBARS) concentration and showed that the herbicide generated reactive oxygen species (ROS), which resulted in the peroxidation and destruction of lipids in the leaves (Ahsan et al. 2008).

As indicated above, glyphosate is also toxic to the nitrogen-fixing symbiont of soybean, *Bradyrhizobium japonicum*; the EPSPS enzyme of *B. japonicum* is susceptible to this herbicide (Zablotowicz & Krishna, 2005). In a three-year field study, from 2002 to 2004, variables related to nitrogen accumulation, symbiosis and yield were observed using a hand weeded (weed free control) and four glyphosate (0.84, 1.68, 2.52+2.52, and 0.84+0.84 kg ae ha^{-1}) treatments applied at 4 and 6 weeks after planting (Zablotowicz & Krishna, 2005). Soybean plants were harvested and evaluated for nitrogenase activity (acetylene reduction assay - ARA), root respiration, nodulation, and root biomass (Zablotowicz & Krishna, 2005). Nitrogen fixation in GR soybean plants was only affected to a small degree at label use rate but was steadily reduced at rates higher than recommended (Zablotowicz & Krishna, 2005).

2.7 Glyphosate & Chlorophyll

Some studies described a reduced chlorophyll content in plants following glyphosate treatment, due to inhibition of chlorophyll biosynthesis (Zobiole et al. 20ll; Huang et al. 2012). Glyphosate may prevent chlorophyll synthesis indirectly by reducing the magnesium level in plant leaves (Cakmak et al. 2009), leading to a diminished chlorophyll content and photosynthetic rate (Zobiole et al. 2012).

Cakmak *et al.* (2009) demonstrated that foliar spray of glyphosate will decrease concentrations of cations in shoots and seeds of glyphosate-sensitive soybean plants. Similarly, by inducing iron (Fe) deficiency, glyphosate may avert the biosynthesis of aminolevulic acid (ALA), an element of the chlorophyll biosynthetic pathway (Gomes, 2015). Catalase and peroxidase, both enzymes implicated in ALA biosynthesis, are very sensitive to iron deprivation (Gomes, 2015). Glyphosate is a strong cation chelator, due to its carboxyl and phosphonate groups, binding with nutrients in plant tissues, thus making them inaccessible for biological processes, including photosynthesis (Gomes, 2015). Reddy et al. (2004) reported that inhibitory effects on chlorophyll biosynthesis due to glyphosate are primarily due to the conversion of glyphosate into aminomethyl phosphonic acid (AMPA). Gomes (2015) suggested that it is AMPA and not glyphosate that causes negative effects on chlorophyll biosynthesis, but by an as yet unidentified mechanism.

2.8 Glyphosate & Oxidative Stress

Along with the inhibition of particular target sites, glyphosate also triggers general oxidative stress in plants, which is most likely a secondary effect of the blocked shikimate pathway (Gomes, 2015). Plants have built apparatuses to manage stress provoked by reactive oxygen species (ROS), by synthesizing enzymatic and non-enzymatic antioxidants (Gomes, 2015). Among the activities of ROS-scavenging enzymes, membrane lipid peroxidation is frequently used as an indicator of oxidative stress in plants (Gunes et al. 2007). Although changes in oxidative stress indicators were reported under a wide range of stress conditions, little material is available concerning the effects of the herbicide on oxidative damage (Gomes, 2015).

Maize (*Zea mays*) leaves sprayed with glyphosate were characterized by an increased level of lipid peroxidation, gluthatione (GSH), free proline content and ion flux (Sergiev et al., 2006). In a gene expression analysis, Ahsan et al. (2008) found that

glyphosate application generates hydrogen peroxide (H_20_2), resulting in peroxidation

of lipids in rice (*Oryza sativa*) leaves (Gomes, 2015). Furthermore, these experiments also demonstrated a reduction in Rubisco activity and an increase in the accumulation of antioxidant enzymes, including ascorbate peroxidase (APX), glutathione-S-transferase (GST), thioredox h-type, nucleoside diphosphate kinase **l** (NDPK1), peroxiredoxin, and chloroplast precursor of superoxide dismutase [Cu-Zn] (SOD) in plant leaves treated with the herbicide (Gomes, 2015).

Duckweed tissues treated with glyphosate also showed higher CAT and APX stimulation than control plants, indicating that glyphosate can cause oxidative stress. Oxidative stress in pea (*Pisum sativum*), wheat and maize were also observed in plants exposed to glyphosate. An increase of malondialdehyde (MDA) (lipid peroxidation) and H_2O_2 contents, an induction of antioxidant enzymes (SOD, CAT and glutathione peroxidase - GPX), was observed (Sergiev et al. 2006; Miteva et al. 2010). Furthermore, glyphosate treatment of both roots and leaves of pea plants resulted in activation of gluthatione reductase and in the augmentation of the GST activities (Miteva *et al.*, 2010). In *Arabidopsis thaliana* treated with glyphosate, Serra et al. (2013) observed increased accumulation of inositol and ascorbate, which are oxidative-stress markers. Increase in levels of inositol and ascorbate was linked to increased oxidative stress in plants (Foyer and Noctor, 2011). Serine was also measured and shown to accumulate following glyphosate treatment; it is involved in glutathione metabolism (Foyer and Noctor, 2011), and the accumulation of serine could also be a gauge of oxidative-stress (Foyer and Noctor, 2011).

2.9 Commercial Experimentation and New Technologies

New technologies, including some that are intended to assist plants to survive stress, are becoming more widespread with new compositions and effectiveness. Some new technologies can improve crop growth and yield under conditions of stress induced by herbicide application. Since 2011, approaches to improving crop yield evolved rapidly, such as the development of synthetic elicitors (Bektas & Eulgem, 2015), use of specific amino acids (Rai, 2002. Shehata et al. 2011), seaweed based materials (Anisimov & Chaikina, 2014) and biostimulants (Smith et al. 2015), each of which is discussed below.

2.10 Synthetic Elicitors

Synthetic elicitors are external and artificial molecules that are associated with plant defenses and can enable crop plants to produce higher yields under adverse conditions, such as in the presence of pests, diseases, and abiotic stresses (Bektas & Eulgem, 2015).

Synthetic elicitor molecules can act/signal through the octadecanoid pathway, binding to localized proteins on cell membranes, and activating plant defense mechanisms through specific protein binding and recognition (Bektas & Eulgem, 2015). There are two types of synthetic molecules: *race specific* elicitors that induce defense in only the target species or specific plant cultivars (Thakur & Sohal, 2013), and *general elicitors* that induce defense in a wide range of host and non-host plants (no genotype specific genes involved) (Thakur & Sohal, 2013).

A similarity was discovered between the activation of defense in plants by general elicitors and the activation of animal innate immune systems by signaling compounds at low concentrations, unlike effects cause by toxins (Thakur & Sohal, 2013).

Several synthetic elicitor compounds have been commercialized, for example, BTH (benzo (1,2,3)-thiadiazole-7- carbothioic acid-S-methyl ester) was found to be suitable for crop defence, and has been marketed under the name of BION® (in Europe) as of 1989 and Actigard® (in the US) as of 1990 (Bektas & Eulgem, 2015). BION, the most studied resistance activator, triggers resistance in a variety of crops against a broad spectrum of diseases caused by viruses and bacteria (Oostendorp et al. 2001). In monocots, resistance is generally long-term with BION, while durability of the effect is generally shorter in dicots. The constituents of BION are translocated systemically and can replace SA in the SAR signal pathways, resulting in the same pattern of resistance and changes to the same set of molecular indicators of resistance (Oostendorp et al. 2001).

2.11 Experiments with Synthetic Elicitors

A wide range of experiments, conducted since 1998, have tested the efficacy of BTH and INA (2,6-dichloro-isonicotinic acid), another synthetic elicitor with effects similar to BTH (Bektas & Eulgem, 2015), in a range of field crops, and were evaluated in preliminary field and greenhouse experiments (Sood et al. 2013).

In 1999, field experiments were conducted with Bion® or Actigard® to evaluate the capacity of these materials to control fire blight of apples and pears in Utah (USA), Hamilton (New Zealand), and Angers (France) (Thomson et al. 1999). The active ingredient benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester (BTH) is known to induce systemic acquired resistance (SAR) against fungal diseases of plants (Thomson et al. 1999). The work showed that BTH alone provided important protection against fire blight but was not as efficient as streptomycin. Nevertheless, BTH and streptomycin combined provided up to 2 times better control of fire blight than either BTH or streptomycin alone, in the apple trials; the experiment was more successful with apples than with pears (Thomson et al. 1999).

In another research effort, increases were demonstrated in defense related enzymes and phenols activity of basmati rice, and subsequent rice yield, and rice protection against biotic and abiotic stresses were both increased, when BTH and SA were applied (Sood et al. 2013). Examination showed greatest accumulation of defense related phenols and enzymes in leaves treated with BTH and SA (Sood et al. 2013). Application of BTH and SA had positive effects through yield increases and reductions in disease levels. This research indicated that treatment of rice leaves with elicitors could be utilized to improve crop yield and protect against sheath blight under field conditions (Sood et al. 2013).

Another study was conducted under greenhouse and field conditions where soybean was sprayed with INA and BTH and evaluated for severity levels of white mold disease caused by *Sclerotinia sclerotiorum* (Dann et al. 1998). The treatments included four applications of INA to field plots from 1993 to 1995 (Dann et al. 1998). This approach reduced the severity of white mold on soybean, due to natural contamination, by 20–70%, compared with water only treatment. White mold is a serious problem in soybean; the crop is highly susceptible to the pathogen (Dann et al.1998). In the years 1998 and 1996, four applications of BTH to field plots in each year reduced white mold severity by 20–60%, with the greatest reductions detected in the more susceptible cultivars. Yields were also increased compared with controls, mainly for the susceptible cultivars and under more severe disease conditions. In greenhouse trials multiple applications of INA and BTH caused smaller lesions after leaf infection with the fungus (Dann et al. 1998). Decreases in disease severity following treatment with INA or BTH were related to enhanced induction of disease resistance (Dann et al. 1998).

A seed application (soaking) trial was conducted to determine if BTH and humic acid (HA) could induce SAR in four soybean cultivars, under greenhouse conditions, and against the pathogen *Fusarum oxysporum* (Monaim et al. 2011). Both BTH and humic acid, at two different sites in Egypt, were able to reduce and improve plant defenses against damping-off and wilt diseases when compared with water only treated soybean plants (Monaim et al. 2011). The treatments increased growth variables and seed soaking in BTH plus HA also resulted in the highest activities of the examined oxidative enzymes, followed by BTH alone, in all four of the tested soybean cultivars (Monaim et al,. 2011).

In all three of the above examples, increases in yield and growth were assessed but none of the work included evaluations in the presence of micronutrients, to determine if effects on growth and yield due to the activation of SAR are mediated by micronutrients. It is hypothesized that synthetic elicitors or SA affect plant defense mechanisms, for example, by enhancing ROS related responses, leading to enhanced stress resistance in plants and, as a result, improve crop yield.

Use of elicitors in spray tank mixes with herbicides minimized environmental damage and run-off, in an approach that was less harmful to surrounding plants, and was proposed to replace genetically modified seeds and plants as it characterized by a lower ecological risk (Thakur & Sohal, 2013).

2.12 Seaweed

NPK based biostimulants can increase the yield of crops, but NPK alone are insufficient for plants to reach their full “genetic potential.” As the global human population increases, crop yield is also increasing to meet the human demand for food (REF). Nevertheless, about 815 million people of the 7.6 billion people in the world, or 10.7% of the human population, were suffering from malnourishment in 2016 (https://www.worldhunger.org/world-hunger-and-poverty-facts-and-statistics/). The use of biostimulants contributes to the generation of higher crop yields (Koh, 2016). Seaweed extract, which has been a focus of study over the past two decades, has shown promising results and scientific evidence suggests that it acts as a highly effective biostimulant (Koh, 2016). The most active components of seaweed extract are plant hormones such as auxins, cytokinins, gibberelins, abscisic acid and ethylene (Tuhy et al. 2013). These hormones play roles in plant apical dominance, cell division, plant movements, plant development/aging, transport of nutrients, seed germination, growth regulation, bud dormancy, florescence, and fruit development (Tuhy et al. 2013).

Several studies have been conducted to test the effects of specific seaweeds. Anisimov & Chaikina (2014) used extracts of six different algal species to test the effects on growth and seedling root development of soybean: *Neorhodomela larix* and *Tichocarpus crinitus* among the red algae; *Saccharina japonica* and *Sargassum pallidum* among the brown algae; and *Ulva fenestrata* and *Codium fragile* among the green algae were investigated. *Codium fragile* resulted in the greatest effects, with an 18% increase in seedling roots, compared to the control, at a concentrations of 10^{-5} g seaweed mL^{-1} (Anisimov & Chaikina, 2014).

In India, during the 2006 rainy season, a field experiment was conducted to test growth, nutrient uptake and yield of soybean when sprayed with extracts of the red alga *Kappaphycus alvarezii* (Rathore et al. 2009). Grain yield was increased 57 and 46% after foliar application of 15 and 12,5% solutions, respectively (Rathore et al., 2009). Uptake of nutrients (N, P, K and S) was also increased following foliar applications of this seaweed (Rathore et al., 2009).

Ramarajan et al., (2012) tested the effects *Sargassum wightii* and *Ulva lactuca*, on soybean seed sprouting, growth, and biochemical aspects and concluded that these seaweeds can be applied at low concentrations and promote seed growth and seedling development of cultivated crops (Ramarajan et al. 2012).

A field experiment was conducted in 2013 in Raipur, India to study the effects of seaweed saps on crop plant nutrient and protein content, and final yield. Foliar applications of *Kappaphycussap* significantly impacted nutrient and protein contents, and yield of soybean (Lodhi et al. 2015). A 15% concentration (dilution of extract with water) of *Kappaphycussap*, along with the recommended dose of nutrients, showed the best results, causing higher N, P and K contents, as well as the highest protein content and yield (Lodhi et al,. 2015). Both seed yield and net economic return were greatest when a 15% concentration was applied (Lodhi et al. 2015).

Seaweed extracts play more than just a role in affecting nutrients and plant hormones, they also act on defense mechanisms against several pathogens. The macroalga *Ulva armoricana* has been shown to induce plant defence responses in a study conducted by Jaulneau et al. (2011). Three crop species were tested (common bean, cucumber and grapevine) along with three different mildew pathogens: *Erysiphe polygoni*, *E. necator* and *Sphareotheca fuliginea*. Disease symptoms were decreased by up to 90% by seaweed treatment, with the greatest decreases in disease symptoms observed for the highest applied dosage (concentration - 1:9 dilution, 6 g L^{-1} dry matter) for all three cultivars (Jaulneau et al. 2011).

A broad taxonomic range of algae can affect crop plants and increase nutrient uptake, growth, and yield. They can trigger defense responses against specific pathogens, or general pathogen resistance (Khan, 2009). Further research is necessary to determine whether or not combinations of specific algae with SA and nutrients can increase plant growth and induce SAR, or enhance crop yield.

2.13 Amino Acids

Amino acids are the “building blocks” of proteins, essential components of metabolism (enzymes) and structure in plants (Rai, 2002); proteins are polymers of amino acids. Plants produce amino acids from the essential elements carbon and oxygen from the air, hydrogen from soil water and nitrogen from soil or air, in the case of symbiotic nitrogen fixation (Rai, 2002). Plants use the required biochemistry to combine nitrogen and elements of carbohydrates, leading to the synthesis of L-amino acids (Rai, 2002). Free amino acids fulfill a wide range of functions in plants, beyond being constituents of proteins, such as: osmolytes, regulating ion transport, controlling stomatal opening, and detoxification of heavy metals (Rai, 2002). Amino acids also play a role in synthesis of enzymes, gene expression, and redox-homeostasis and are well known biostimulants, which have positive effects on plant growth and yield, and minimize injuries caused by a range of abiotic factors (Rai, 2002; Shehata et al. 2011).

In addition, due to increasing global food demand, there is increased pressure on the availability of irrigation water and the use of salt contaminated land for crop production, which have pivotal roles in the development of crops. Salt stress is one of the most difficult abiotic stresses to overcome and so has significant effects on crop yield over some very large areas of crop production (Sadak et al. 2014).

Diluted seawater is now beginning to be a viable option for growth of certain crops, under specific conditions in which amino acid applications reduce salt and heavy metal stresses and promote crop growth. For example, when faba beans were exposed to salt concentrations of 3.13 or 6.25 dS m^{-1} in diluted seawater, this led to reductions of mono and disaccharides, polysaccharides, fresh and dry weight of shoots, number of leaves per plant, seed yield, shoot length and concentrations of photosynthetic pigments (Sadak et al. 2014). Plants grown in seawater had higher contents of Na^+ and Cl^- and decreased levels of K^+, K^+:Na^+, Ca^{2+}, Mg^{2+} and P^{3+} (Sadak et al. 2014). Seawater treatment also decreased total protein contents in seeds (Sadak et al. 2014). Concentrations of 500, 1000 or 1500 mg L^{-1} of amino acid as a foliar spray greatly increased the measured variables that had been negatively affected by seawater salinity (Sadak et al. 2014). The highest dose of amino acid (1500 mg L^{-1}) resulted in the greatest effect, reducing the damaging outcome of seawater salinity stress (Sadak et al, 2014).

Faba bean (*Vicia faba* L. cv. Calvor 103) plants were tested with with NaCl and $CaCl_2$ at concentrations inducing soil osmotic potentials (ψ_{soil}) from 0 to -1.2 MPa. Plants were then sprayed with glycinebetaine (8.5 µM) and proline (8.7 µM) solutions (Gadallah, 1999). Bean plants respond to soil salinity by reducing leaf water content and osmotic potential (Gadallah, 1999). Salinity decreased the contents of chlorophyll, dry mass, soluble proteins, total free amino acids, and Na^+, Ca^{2+}, and Cl^- contents. Proline and glycinebetaine applications resulted in diminished membrane injury, improved growth and K^+ uptake and enhanced over all chlorophyll contents (Gadallah, 1999).

Other experiments, conducted in Egypt, studied the mitigating effects of the commercial products Sway and Sea Weed, combinations of both amino acids and seaweed. Seaweed extracts were applied at 1, 2 and 4 mL L^{-1} and free amino acids at 2, 4 and 8 mL L^{-1} (Zewail, 2014). The two products were used as foliar spray with applications to bean plants three times, at 10 day intervals, during the growing season, starting at 25 days after seeding (Zewail, 2014). Seaweed, at 2 mL, and amino acid at 4 mL, showed the best results in terms of plant height, number of branches, stem diameter, total leaf area, dry weight and specific leaf weight, and resulted in improved levels of these variables when compared to the control treatment (Zewail, 2014).

During the winter seasons of 2008 and 2009, amino acids and seaweed extract, as biostimulants, were studied in Beheira, Egypt. Celeriac plants were tested with regard to effects on their chemical composition, yield, quality and growth when amino acids at 500 and 750 ppm and seaweed extract at 1000 and 2000 ppm were applied to the crops. Results were compared with a check treatment (Sheheta et al. 2011). Spraying with seaweed and amino acids at higher concentrations resulted in increased plant height, and fresh and dry weight of leaves, when compared to the control (Sheheta et al. 2011). Spraying seaweed extract alone, at the higher rate, resulted in the highest values for photosynthetic pigments, and P and K content of leaves (Sheheta et al. 2011). Amino acids alone, at both rates, increased N content compared to the control; plants treated with either amino acids or seaweed extract at higher rates had increased green tissue (leaves) production (Sheheta et al., 2011).

2.14 Biostimulants

In the last 10 years, much research has been focused on the evolution of crop plants, their complex relationship with microbes and the molecular signaling mechanisms used to communicate between plants and their associated phytomicrobiome (Smith et al. 2015). Plants also detect negative byproducts produced by potential pathogens and activate pathogen-response systems (Smith et al. 2015). This intercommunication enhances facets of plant development and productivity. Research findings, such as those of Prudent et al. (2014), suggest that manipulating signals can increase the characteristics of the phytomicrobiome community to trigger or affect plant responses to foliar, soil, and climatic conditions. Prudent et al (2014) hypothesized that the application of thuricin 17, a molecule produced by the PGPR *Bacillus thuringiensis* NEB17, could enhance soybean tolerance to water stress (Prudent et al. 2014). Results indicated that thuricin 17 application under water stress increased plant biomass by 17 %, inhibiting the (negative) effect on biomass due to water stress. This effect was attributed to changes to root structures, which resulted in a 37 % increase in root length and 55 % increase in nodule biomass, and small increases in leaf area and photosynthetic rate (Prudent et al. 2014).

Microbes produce elicitor molecules that initiate symbiotic relationships with plants, e.g. lipochitooligosaccharide (LCO), and modify plant development, e.g. thuricin 17. LCO and thuricin 17 enhanced plant growth of legumes and non-legumes, e.g., *Arabidopsis thaliana* responded positively to treatment with the bacterial signal compounds in the presence of salt stress (up to 250 mM NaCl). Shotgun proteomics of unstressed and 250 mM NaCl stressed *A. thaliana* rosettes (7 days post onset of stress), in combination with the LCO or Th17 revealed enhanced production of many known, unknown, and putative proteins. As a result, PEP carboxylase, Rubisco-oxygenase large subunit, pyruvate kinase, and proteins of photosystems I and II were some of the notable proteins whose concentrations were increased by the signal compounds. These results suggest that the proteome of *A. thaliana* rosettes is substantially altered by the bacterial signals tested, and more so under salt stress, thereby affecting plant development under high salt stress conditions (Subramanian et al. 2016).

Subramanian et al. (2016) conducted research on soybean germination under unstressed and salt stressed conditions, in the presence of LCO and Th17. Germination was examined on seeds treated using two concentrations of Th17 (10^{-9} and 10^{-11} M) or LCO (10^{-6} and 10^{-8} M), combined with 100, 125, 150, 175, and 200 Mm NaCl. Salt stress strongly delayed seed germination, and this effect was greater at greater levels of salinity stress. LCO at 10^{-6} M and Th17 at 10^{-9} M enhance soybean seed germination in the presence of salinity stress and did this most effectively at 100 mM NaCl, when measured at 48 h after the onset of germination. In the absence of stress addition of LCO and Th17 did not result in statistically significant increases in overall soybean seed germination rate (Subramanian, 2016).

Biostimulants of plant growth, such as bacteriocins like Bac IH7, promote growth of plants such as tomato and musk melon (Subramanian & Smith, 2015). Thuricin 17 is the only bacteriocin studied extensively for plant growth promotion, including at the molecular level. Th17 works as a bacteria-to-plant signal mechanism, enhancing plant growth in legumes and non-legumes. In *Arabidopsis thaliana* and soybean, Th17 increased concentrations of the phytohormones IAA and SA at 24 h post treatment. At the proteome level Th17 treatment of 21 day old *A. thaliana* rosettes led to > 2-fold increases in activation of the carbon and energy metabolism pathway proteins, 24 h post treatment. Th17 treated plants, at 250 mM NaCl, retained important levels of the light harvesting complex, photosystems I and II proteins and energy and antioxidant pathways were activated, so that rosettes could grow and develop better in the presence of salt stress. For soybean, Th17 promoted seed germination in the presence of NaCl stress and was most effective at 100 mM NaCl. The 48 h post germination proteome suggested efficient and speedier partitioning of storage proteins, and activation of carbon, nitrogen and energy metabolisms in Th17 treated seeds both under optimal and salt stress (100 mM NaCl) conditions (Takishita et al. 2018).

Synthetic elicitors can trigger systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants; this is also an important attribute of plant growth promoting rhizobacteria (PGPR). Certain PGPRs can trigger ISR in plants and make them less susceptible to ensuing attack by pathogens, thereby contributing to disease mitigation and protection (Takishita et al. 2018).

Factors responsible for ISR induction by PGPR include residues or sub-structures produced by the PGPR. Induction of these responses occurs when these PGPR-produced factors are detected by pattern recognition receptors (PRRs) in plant roots, often in the form of microbe-associated molecular patterns (MAMPs), such as bacterial flagellin or fungal chitins. How plants differentiate useful microbes and detrimental pathogens is not fully understood, but MAMPS such as flagellin, lipopolysaccharides, and peptidoglycan fragments are known to be ISR elicitors. Likewise, compounds that are secreted by PGPR, such as iron-regulated metabolites, SA and antibiotics can also act as elicitors of ISR (Takishita et al. 2018).

Plant hormones play a crucial part in the aftermath of molecular recognition of PGPR; multiple signaling actions take place within the plants. The difference between ISR and SAR is that, typically, ISR is thought to be dependent on the plant hormones jasmonic acid (JA) and ethylene (ET) and is different from pathogen-induced-SAR, in which signaling is largely comprised of SA. Although the signaling pathways are different, both SAR and ISR are characterized by a supervisory protein non-expressor of PR genes1 (NPR1), but its role must be different since SAR activation controls activation of pathogensis-related (PR) genes, SA related responsive genes, while ISR stimulation does not. More and more evidence indicates that a multitude of signaling pathways can be present, depending on the type of PGPR, pathogens and host plants. Moreover, ISR-inducing PGPR that use the SA-pathway, and not the JA/ET pathway, have been reported (Maurhofer et al. 1994; De Meyer and Höfte, 1997; Maurhofer et al. 1998; Audenaert et al. 2002; Barriuso et al. 2008; van de Mortel et al. 2012). In addition, gibberellins (Navarro et al. 2008), auxins (Kazan and Manners 2009), cytokinins (Giron et al. 2013), and brassinosteroids (Nakashita et al. 2003) have also been established to function as modulators of the plant immune signaling systems; hormone crosstalk is believed to exist, providing plants with capacity to maximize responses related to their growth, protection and overall productivity (Takishita et al. 2018).

3.0 Objectives

The goal of this research is to determine whether or not SA inhibits the negative effects of glyphosate on the photosynthetic rate of GR soybean. Four experiments were motivated by the hypotheses. First, 10^{-2} M to 10^{-8} M salicylic acid dosages were tested, to determine the dosage of SA that optimized plant photosynthesis. Previous studies indicated a relationship between photosynthetic rate and yield when product nutrient solution that included SA was applied in field experiments (Axter Agroscience). A transient increase in photosynthetic rate followed herbicide application, which was followed by an increase in yield under field conditions (Axter Agroscience). The goal of the second experiment was to model the photosynthetic rate response to glyphosate (foliar spray application).

In the third experiment, three dosages of SA (10^{-2}, 10^{-5}, and 10^{-8} M) were spray-applied, along with the recommended dose of glyphosate (equivalent to 1.67 L ha^{-1}). The goal of the third experiment was to determine the photosynthetic response of soybean to SA under glyphosate treatment conditions. The three experimental dosages of SA were determined based on the results of the first experiment, i.e., 10^{-2} M resulted in the lowest photosynthetic rate and 10^{-5} M the highest.

The goal of the final experiment was to identify the optimal concentration of SA, resulting in the greatest photosynthetic rates in the presence of glyphosate induced stress.

The statistics of all four experiments were assisted by Dr. Timothy Schwinghamer to evaluate the efficacy, distribution, and evaluation of each function in the experiments. In Experiment 1: Salicylic acid dosage experiment, generalized linear mixed methods were used to model the nonnormal distributions of the observed variables (McCulloch & Neuhaus, 2005). In the second, third, and fourth experiment, since multiple factors measured, i.e., photosynthetically active radiation, greenhouse temperature, and concentration in Experiment 2; SA concentration, intercellular CO_2, and conductance to water being tested in experiment 3; and SA, intercellular CO_2, leaf temperature, and air temperature in Experiment 4. The multivariate data was modeled using redundancy analyses (RDAs) that extracted and summarized the variation in the response variables that were explained by the experimental variables (Buttigieg & Ramette, 2014). An RDA is a direct gradient analysis procedure that condenses linear relationships between components of response variables that are "redundant"or “explained by” a set of explanatory variables. To do this, RDA extends multiple linear regression by allowing regression of multiple response variables on multiple explanatory variables (Buttigieg & Ramette, 2014).

4.0 Methods

Four experiments were carried out in 2017, to test the mitigating effects of SA on the photosynthetic rates of soybean plants, at the Cérom Grain Research Center (Beloeil, Québec, Canada). The tests were conducted under environmentally controlled conditions in a research greenhouse. Temperatures of the greenhouse are listed below for all experiments. Measurements of soybean photosynthesis for Experiments 2 and 3 were taken during March, 2017.

Experiment 1: LICOR Reading (6400XT portable photosynthesis system, LI-COR Inc., Lincoln Nebraska, USA): February 17, 2017:

(min 23.1 ^{o}C - max 32.1 ^{o}C) average 26.5 ^{o}C

Experiment 2 and Experiment 3: LICOR Reading: March 28, 2017:

(min 24.2 ^{o}C - max 30.9 ^{o}C) average 26.5 ^{o}C

Experiment 4: LICOR Reading: August 3, 2017:

(min 20.9 ^{o}C - max 35.5 ^{o}C) average 26.6 ^{o}C

Plants were sprayed on a ‘spray-bench’ with Weathermax glyphosate (the Synagri Company, Saint-Hyacinthe, Québec, Canada) (www.synagri.ca) and SA: 2-(HO) $C_6H_4CO_2H$ (Sigma- Aldrich, Oakville, Ontario, Canada) (www.sigmaaldrich.com) and maintained by the technicians at the Cérom facility. At the third trifoliate leaf stage of soybean plant growth, the experimental treatments were applied: SA treatments, dilute

In water or glyphosate, were applied to the foliage of the soybean plants. Photosynthetic measurements were taken with the LI-COR 6400XT portable photosynthesis system (Li-Cor, Lincoln, Nebraska, USA), 24 h after treatment with glyphosate, SA or both, while on spray bench developed to simulate field application of sprayed materials. All photosynthetic measurements are taken in Micro mole of C02 per meter square leaf area per second. The spray bench was developed by the Industrial Research Center of Quebec (CRIQ) in 1977, and was then used for the *Ministère de l'Agriculture des Pêcheries et de l'Alimentation du Québec* (MAPAQ). In Experiments 1, 2, and 3, five outcome variables were measured: photosynthetic rate (Photo), conductance to water (Cond), intracellular CO_2 levels (Ci), transpiration (Trmmol), and leaf temperature (Tleaf). Three exogenous variables were also measured: SA, photosynthetically active radiation (PARo), and air temperature (Tair). Multivariate statistical analytic methods and redundancy analysis (RDA) were performed using SAS and R. The multivariate method of RDA assessed the hypothesis that a linear combination of the explanatory variables explained a statistically significant portion of the total variance of the dependent variables. We used RDA because there were multiple response variables and multiple treatment variables. An RDA approach allowed us to partition the variance of the response variables that were explained by the treatment variables. The multivariate approach as it fit best with the design of the experiments.

The RDA assumes that the variance of the (dependent) response variables can be explained by the linear additive combination of the independent variables.

4.1 Experiment 1: Salicylic acid dosage experiment

Salicylic acid (SA) was diluted in water then applied to the foliage of plants at seven levels: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M (in February of 2017). Plants at the third trifoliate stage were sprayed on Cérom's spray bench. Each treatment group consisted of six plants. Therefore, there were 48 observations in experiment 1. Only 44 observations were retained due to small or broken plants in 4 instances. The SA dosage treatments were randomized in the greenhouse.

For the multivariate analysis, there were five outcome variables and three exogenous variables in Experiment 1. The photosynthetic variables measured by the LICOR portable photosynthesis meter were affected by the experimental levels of SA concentration, photosynthetically active radiation, and air temperature:

(Observed photosynthesis variables) = (Exogenous variables)

(Photo, Cond, Ci, Trmmol, Tleaf) = (SA, PARo, Tair)

4.2 Experiment 2: Glyphosate dosage experiment

The second experiment was also conducted at the Cérom Grain Research Center (March, 2017). Five levels of glyphosate were used: 0.5, 1.0, 1.5, 2.0, and 3.0-times the recommended dosage (1.67 L ha^{-1}). The experiment was structured following a completely randomized block design (RCBD) and photosynthetic readings were taken 24 h after spray treatment. Out of 30 photosynthesis readings taken in Experiment 2, 29 were used; one experimental unit was omitted because of damage to the plant. The RDA model was: (Photo, Cond, Ci, Trmmol, Tleaf) = (Glyphosate, PARo, Tair)

4.3 Experiment 3: SA × glyphosate interaction

In the third experiment, photosynthesis readings were taken in March of 2017. The experiment was organized following a RCBD with six blocks, five replications, and five treatments. The five spray treatments for this experiment were: control (water only), glyphosate (1.67 L ha^{-1}), 10^{-2} M SA + glyphosate, 10^{-5} M SA + glyphosate and 10^{-8} M SA + glyphosate. The recommended rate of glyphosate (1.67 L ha^{-1}) was used in all treatments except for the control. Therefore, the model for the Redundancy analysis (RDA) of this experiment was Observations = SA + Glyphosate + PARo + Tair. The values of the variance inflation factor (VIF) were 1.068827 for salicylic acid, 1.162170 for glyphosate, 1.602571 for PARo and 1.609207 for Tair. The VIF values were less than 10, and therefore the explanatory factors were retained in the model.

4.4 Experiment 4: Optimal levels of SA x Glyphosate

In the final experiment of this study, combinations of glyphosate and SA were applied as 16 different treatments. The concentrations of the treatments were selected based on the results of the previous experiments (Table 1). This experiment was performed twice. In the first replication of the experiment, there were 58 observations. In the second replication, there were 44 observations. The experiment was structured following an RCBD design in the Cérom Grain Research Center greenhouse. The values of the variance inflation factor were 1.089764 for salicylic acid, 1.004000 for glyphosate, 1.019746 for PARo and 1.091744 for Tair, and therefore the explanatory variables were retained in the RDA. The values of the two experiments were tested for homogeneity of variance and pooled for final statistical analyses.

Table 1. Treatment application levels of glyphosate and concentration of SA solutions for Experiment 4, for both replications of the experiment.

Glyphosate Rate*	SA Rate
0	0
0	10^{-4} M SA
0	10^{-5} M SA
0	10^{-6} M SA
0.835 L ha^{-1}	0
0.835 L ha^{-1}	10^{-4} M SA
0.835 L ha^{-1}	10^{-5} M SA
0.835 L ha^{-1}	10^{-6} M SA
1.67 L ha^{-1}	0
1.67 L ha^{-1}	10^{-4} M SA
1.67 L ha^{-1}	10^{-5} M SA
1.67 L ha^{-1}	10^{-6} M SA
3.34 L ha^{-1}	0
3.34 L ha^{-1}	10^{-4} M SA
3.34 L ha^{-1}	10^{-5} M SA
3.34 L ha^{-1}	10^{-6} M SA

*Indicates application of commercial product which is 41% glyphosate, by volume.

4.5 Overview of experiments

This work was composed of a progression of four experiments. The general purpose of each experiment was as follows:

1. Experiment 1 – To determine if effective SA levels match corresponding levels found in literature and to warrant further experimentation based on those findings.
2. Experiment 2 – To determine negative effects of glyphosate on the photosynthetic rates of glyphosate-tolerant soybean plants across a range of application rates.
3. Experiment 3 – Based on Experiment 1, three selected levels of SA (10^{-2}, 10^{-5} and 10^{-8} M) were evaluated to determine if the optimum rate of SA application is constant across a range of glyphosate levels.
4. Experiment 4 – Evaluated the three most advantageous levels of glyphosate (10^{-4}, 10^{-5} and 10^{-6} M), as determined in experiment 1 and combined it with 4 different levels glyphosate, for a total of 16 treatments, to find the optimal combination of SA and glyphosate, with regard to photosynthetic rate, for glyphosate tolerant soybean plants.

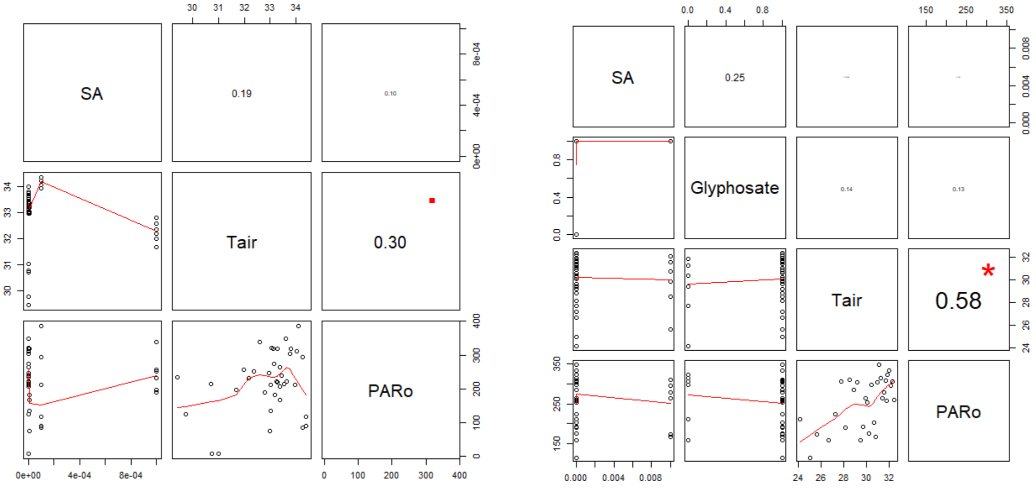
5.0 Results

In experiments 1 and 2, an initial analysis of the correlations of the experimental variables indicated that air temperature was correlated with photosynthetically activate radiation, but the value of the correlation coefficient was less than 0.7 and therefore the explanatory variables were retained for the respective RDAs. Based on the combined data, the air temperature was weakly but nonetheless significantly correlated with SA in contents of the plants, which was due to the designs of the experiments in the greenhouse. Figure 1 shows panel plots with the Pearson product moment correlations shown in the upper part above the diagonal, and scatterplots overlaid with a nonparametric regression below the diagonal. The hollow circles in the plots represent observed values. The red lines represent the nonparametric regressions. Red asterisks indicate statistically significant correlations. It is important that the correlations are less than 0.75 between the independent variables for redundancy analysis. Redundancy analysis was used as a multivariate approach because there were multiple independent and dependent variables.

Figure 1. Panel plots of nonparametric regressions and correlations between experimental variables, for all four experiments.

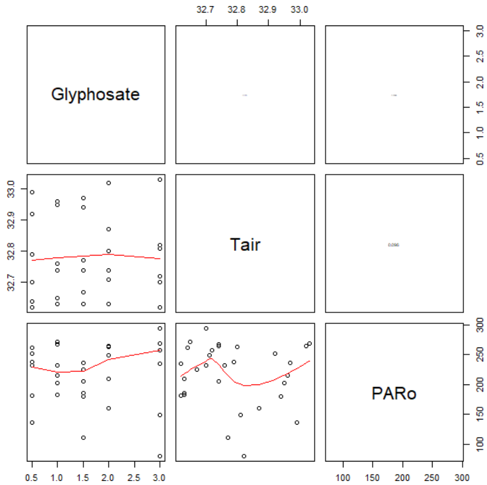
(a) Experiment 1

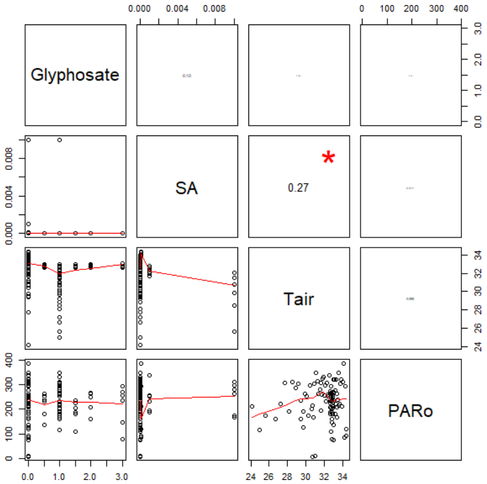
(c) Experiment 3



(b) Experiment 2

(d) Combined Experimental Data



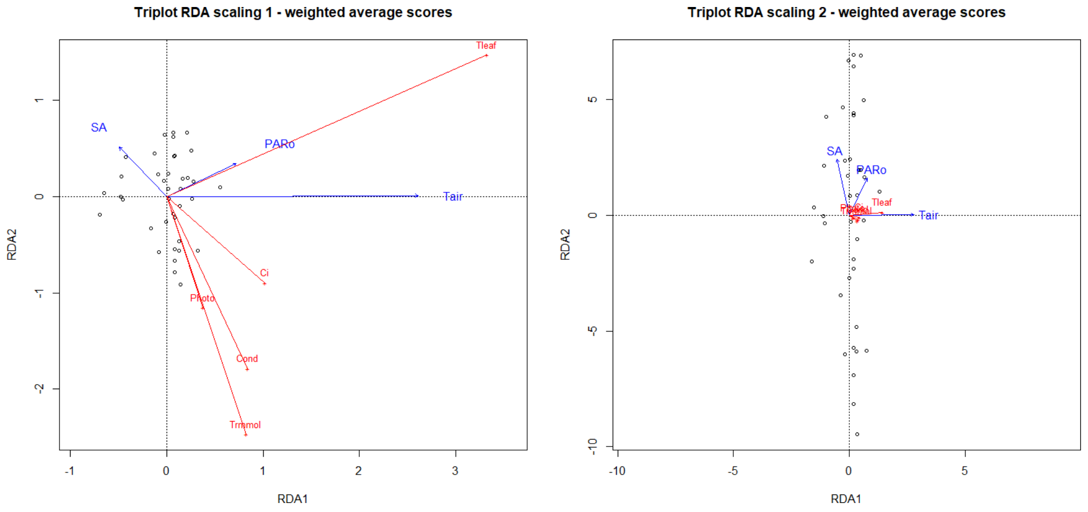


5.1 Experiment 1: SA Dosage Experiment

The blocks in this experiment were randomized in the greenhouse but not complete with respect to the placement of treatments. The photosynthetic rate of plants was correlated to the location in the greenhouse ($r = 0.37040$, $p = 0.0133$). Therefore, the position in the greenhouse was included as a variable in a generalized linear mixed model (SAS PROC GLIMMIX) and the position was controlled statistically. The values of photosynthetic rate, SA level and location were standardized before modelling, using SAS PROC STANDARD with MEAN = 0 and STD = 1. The Gamma distribution was specified using DIST = GAMMA in the MODEL statement. The Gamma distribution was selected based on the model fit statistics (Bayesian information criterion). The SAS PROC GLIMMIX output for experiment 1 indicated evidence for a moderate negative effect on soybean photosynthetic rate due to SA, ($F_{1,41} = 4.68$, $p = 0.0364$). These results motivated further research regarding the effects of SA on photosynthetic rate using a completely randomized block design.

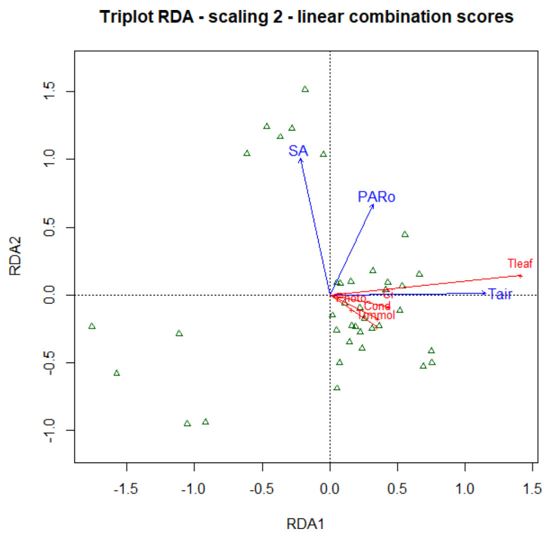
The multivariate analysis was a redundancy discriminant analysis (RDA) that included the photosynthetic variables measured by the LICOR portable photosynthesis meter, SA concentration, photosynthetically active radiation and air temperature (Figure 2).

Figure 2. (a) Distance triplot (scaling 1) with weighted average scores where the matrix of canonical eigenvectors **U** and the matrix of centered response data **Y** were used to compute **YU** to obtain an ordination in the space of the original variables, (b) distance triplot with linear combination scores where the matrix of fitted values $\hat{\mathbf{Y}}$ was used to calculate $\hat{\mathbf{Y}}\mathbf{U}$ and the ordination in the space of variables **X**, (c) correlation triplot (scaling 2) with weighted average scores, (d) correlation triplot with linear combination scores (Palmer 1993). Angles between vectors indicate strength of correlation between factors. The points represent experimental soybean plants. Blue arrows indicate vectors of quantitative explanatory variables. Red lines indicate response variable vectors.


(a)

(c)


Triplot RDA - scaling 1 - linear combination scores


Triplot RDA - scaling 2 - linear combination scores

The partitioning of the inertia (variance) is shown (Table 1). Therefore, the canonical coefficients of the RDA explain 19.67% of the total variation (in correlation) and 19.67% of the variability of the plant photosynthesis variables can be explained by the exogenous variables, and 80.33% of the variability in the photosynthesis variables was not explained by the explanatory variables, for experiment 1.

Table 2. Partitioning of variance based on RDA results from greenhouse experiment with soybean showing the effect of SA and photosynthetically active radiation, experiment 1.

	Proportion	Cumulative Proportion
Total	5.0000	1.0000
Constrained	0.9834	0.1967
Unconstrained	4.0166	0.8033

The eigenvalues sum up to total ‘‘inertia”. That is, they each explain a certain proportion of the total variance that is shown in Table 2. The largest eigenvalue indicates that the largest amount of the total (constrained) variance is explained by RDA. Limited by the number of descriptors or explanatory variables, there were three RDA (constrained) axes and five PCA (unconstrained) axes. The second canonical coefficient was associated with the contribution of SA to the variability in photosynthesis. It explained 0.925% of the total variance (4.703% of the constrained variance) (Table 3) of soybean photosynthetic variables. Therefore, SA was secondary to the effect of air temperature in terms of its contribution to the variance of photosynthetic variables. Nevertheless, the model for experiment 1 was statistically significant as indicated by the permutations test (*p* = 0.011).

Table 3. Eigenvalues for the RDA of experiment 1.

	Importance of components		
	RDA1	RDA2	RDA3
Eigenvalue	0.9063	0.04625	0.03085
Proportion Explained	0.1813	0.00925	0.00617
Cumulative Proportion	0.1813	0.19051	0.19668

Table 4. Accumulated constrained eigenvalues of experiment 1.

	RDA1	RDA2	RDA3
Eigenvalue	0.9063	0.04625	0.03085
Proportion Explained	0.9216	0.04703	0.03137
Cumulative Proportion	0.9216	0.96863	1.00000

The variable scores (canonical coefficents of the RDA) represent the position of soybean photosynthesis variables on the RDA axes (Table 4). The scores are coordinates used to ordinate points and vectors. The response variables can be located on the RDA triplot based on the RDA scores: photosynthetic rate, conductance to water, intercellular CO_2, and transpiration rate are in the lower right quadrant; while leaf temperature is in the upper right quadrant. Each of the canonical axes is a linear combination (i.e., a multiple regression model) of all explanatory variables (Table 5).

Table 5. Variable scores, greenhouse-grown photosynthesis (observed) loading on canonical axes for experiment 1.

	RDA1	RDA2	RDA3
Photo	0.1607	-0.11115	-0.21757
Cond	0.3581	-0.17194	-0.01847
Ci	0.4333	-0.08608	0.18523
Trmmol	0.3496	-0.23777	0.02770
Tleaf	1.4147	0.14127	-0.03419

Table 6. Biplot scores for constraining variables for experiment 1.

	RDA 1	RDA 2	RDA 3
SA	-0.1897	0.87223	-0.45080
PARo	0.2761	0.57690	0.76874
Tair	0.9997	0.01149	0.02122

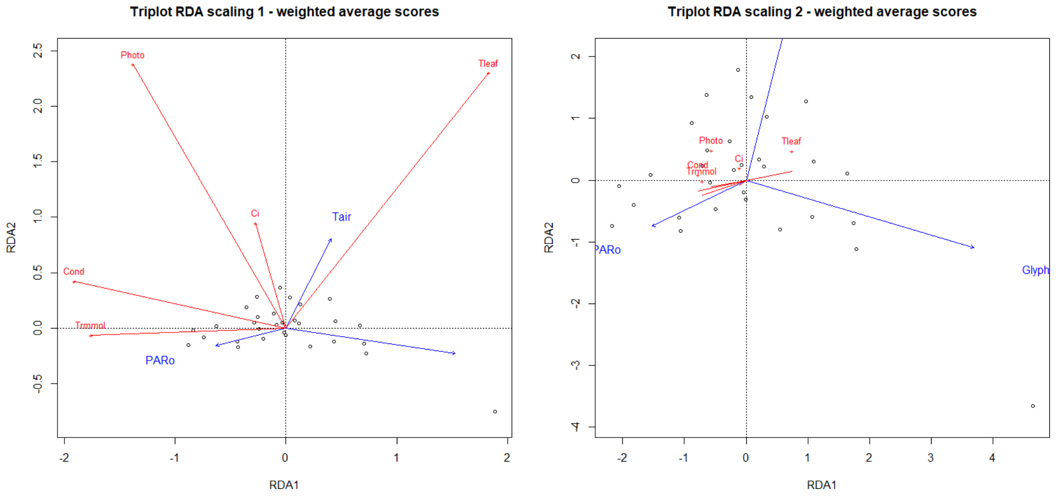
The global test of the RDA model was a permutation test (999 permutations). It indicated that the overall model was statistically significant (p = 0.011), indicating clear, and in this case negative, effects of herbicide on the measured photosynthetic variables, and that these effects were at least in part, reversed by SA application at the time of herbicide application. Tests of the canonical axes indicated that the first axis explained a statistically significant amount of the variance in the data.

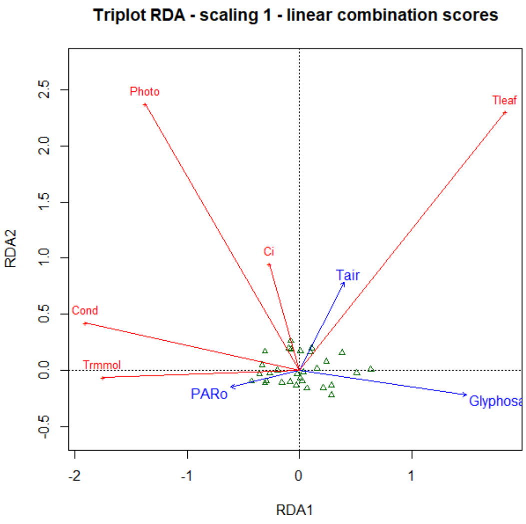
5.2 Experiment 2: Glyphosate dosage experiment

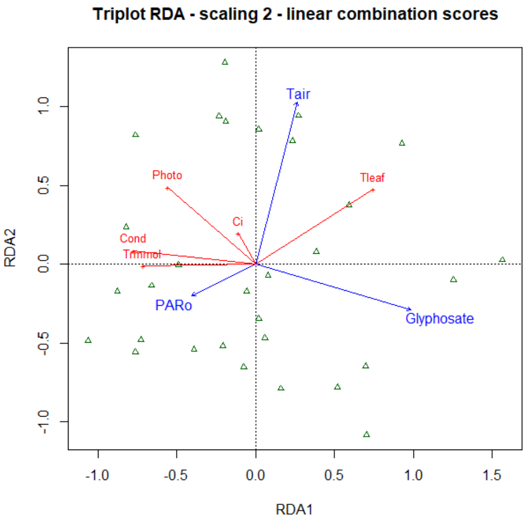
In this experiment, photosynthetically active radiation was correlated to the greenhouse temperature, and the concentration of glyphosate was negatively correlated to conductance to water (Figure 3).

Figure 3. (a) distance triplot and (b) correlation triplots based on the RDA for experiment 2. There is a positive correlation between glyphosate and leaf transpiration and a negative correlation between glyphosate and photosynthetic carbon assimilation (Photo).

(a) (b)







In experiment 2, 20.95% of the variability in the plant photosynthesis variables can be explained by the exogenous variables, and 79.05% of the variability in the photosynthesis variables cannot be explained by the explanatory variables but can be attributed to factors that were not included in the model (Table 6).

Table 7. Partitioning of correlations for Experiment 2

	Inertia Proportion	
Total	5.000	1.0000
Constrained	1.047	0.2095
Unconstrained	3.953	0.7905

The eigenvalues explain the proportions of the total variance. The largest eigenvalue was associated with RDA1 that explained the largest proportion of the total variance (Table 9).

Table 8. Eigenvalues for experiment 2.

Importance of components:			
	RDA1	RDA2	RDA3
Eigenvalue	0.8248	0.21028	0.01220
Proportion Explained	0.1650	0.04206	0.00244
Cumulative Proportion	0.1650	0.20702	0.20946

19.67% of the variability in the plant variables can be explained by the exogenous variables, and 84.55% of the variability in the plant variables cannot be accounted for by the explanatory variables (Table 10).

Table 9. Accumulated constrained eigenvalues for experiment 2.

Importance of components:			
	RDA1	RDA2	RDA3
Eigenvalue	0.8248	0.2103	0.01220
Proportion Explained	0.7876	0.2008	0.01165
Cumulative Proportion	0.7876	0.9883	1.00000

The response variables can be located on the RDA triplot based on the RDA scores: photosynthetic rate, conductance to water, and intercellular CO_2 are in the upper left quadrant; transpiration rate is in the lower left quadrant; and leaf temperature is in the upper right quadrant (Table 9).

Table 10. Variable scores for experiment 2.

	RDA1	RDA2	RDA3
Photo	-0.5588	0.48775	0.01493
Cond	-0.7745	0.08664	0.06649
Ci	-0.1100	0.19436	-0.15151
Trmmol	-0.7128	-0.01318	-0.02501
Tleaf	0.7445	0.47229	0.03404

RDA 1 shows inhibition to H_2O with glyphosate and RDA 2 shows that air temperature is correlated with photosynthetic rate (Table 12).

Table 11. Biplot scores for constraining variables for experiment 2.

	RDA1	RDA2	RDA3
Glyphosate	0.9104	-0.2697	0.3139
PARo	-0.3754	-0.1846	0.9083
Tair	0.2419	0.9519	0.1881

The global test indicates a low level of statistical significance for the explanation of photosynthesis by Experiment 2 exogenous variables (Table 12 and 13).

Table 12. Permutation test for RDA under reduced model for experiment 2.

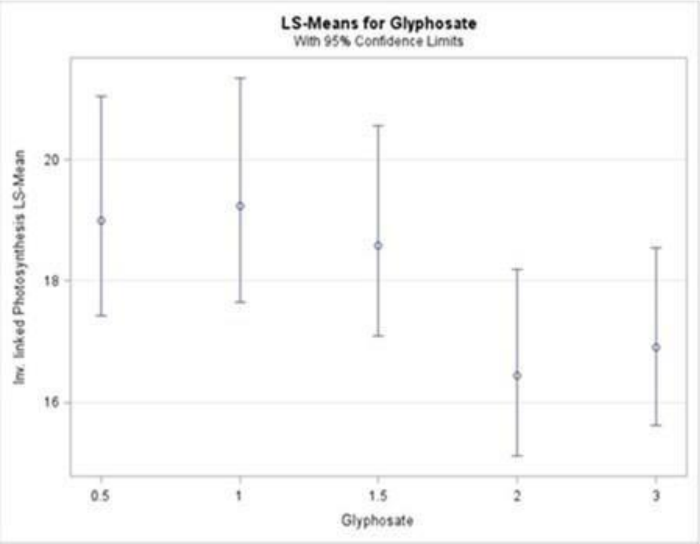
	Df	Variance	F Pr(>F)
Model	3	1.0473 2.2964	0.071 .
Residual	26	3.9527	
Signif. codes:	0		0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1‘ ’ 1

Table 13. Tests of the canonical axes for experiment 2.

	Df	Variance	F Pr(>F)
RDA1	1	0.8248	5.4257 0.107
RDA2	1	0.2103	1.3832 0.520
RDA3	1	0.0122	0.0803 0.938

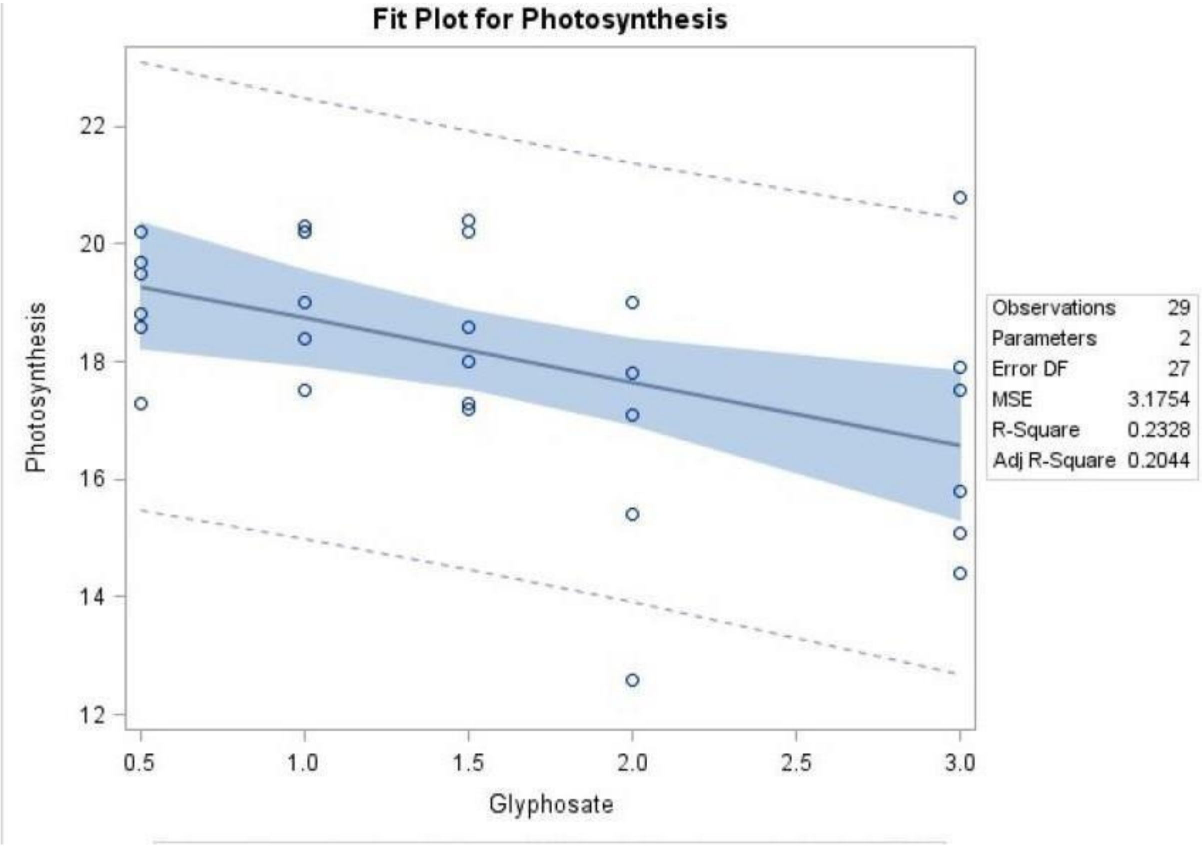
The negative correlation between glyphosate rate and photosynthetic rate that was indicated by the RDA plot was shown explicitly by a generalized mixed model (SAS PROC GLIMMIX in SAS). The type III test of fixed effects from the PROC GLIMMIX output indicated that the *F*-value for this experiment was $F_{4,19} = 3.03$, $p = 0.0434$. Therefore, there was moderate evidence that glyphosate affected the photosynthetic rate of greenhouse-grown soybean plants. Nevertheless, after adjustment for multiple comparisons, no statistically significant difference was detectable between the treatments.

Figure 4. Levels of photosynthetic measurements when compared with different levels of glyphosate. All photosynthetic measurements are taken in Micro mole of CO_2 per meter square leaf area per second, for experiment 2.



The figure below shows a simple linear regression of photosynthetic rates correlated with glyphosate rates (SAS PROC REG). The greater the application rate, the lower the photosynthetic rate of soybean plants, which were detected with the LICOR 6400 meter. The "Analysis of Variance" shows that the regression model is significantly better than using merely the mean photosynthetic rate to predict the photosynthetic rate (p = 0.0080). The R^2 of the model is 23.28%, which indicates that we expect 23.28% of the variance in photosynthesis to be explained by the regression model. The "Parameter Estimates" table gives us the regression model, which is a simple linear regression: Photosynthesis = 19.82 - 1.09 X glyphosate, where glyphosate indicates the rate (0, 0.5, etc) of glyphosate application.

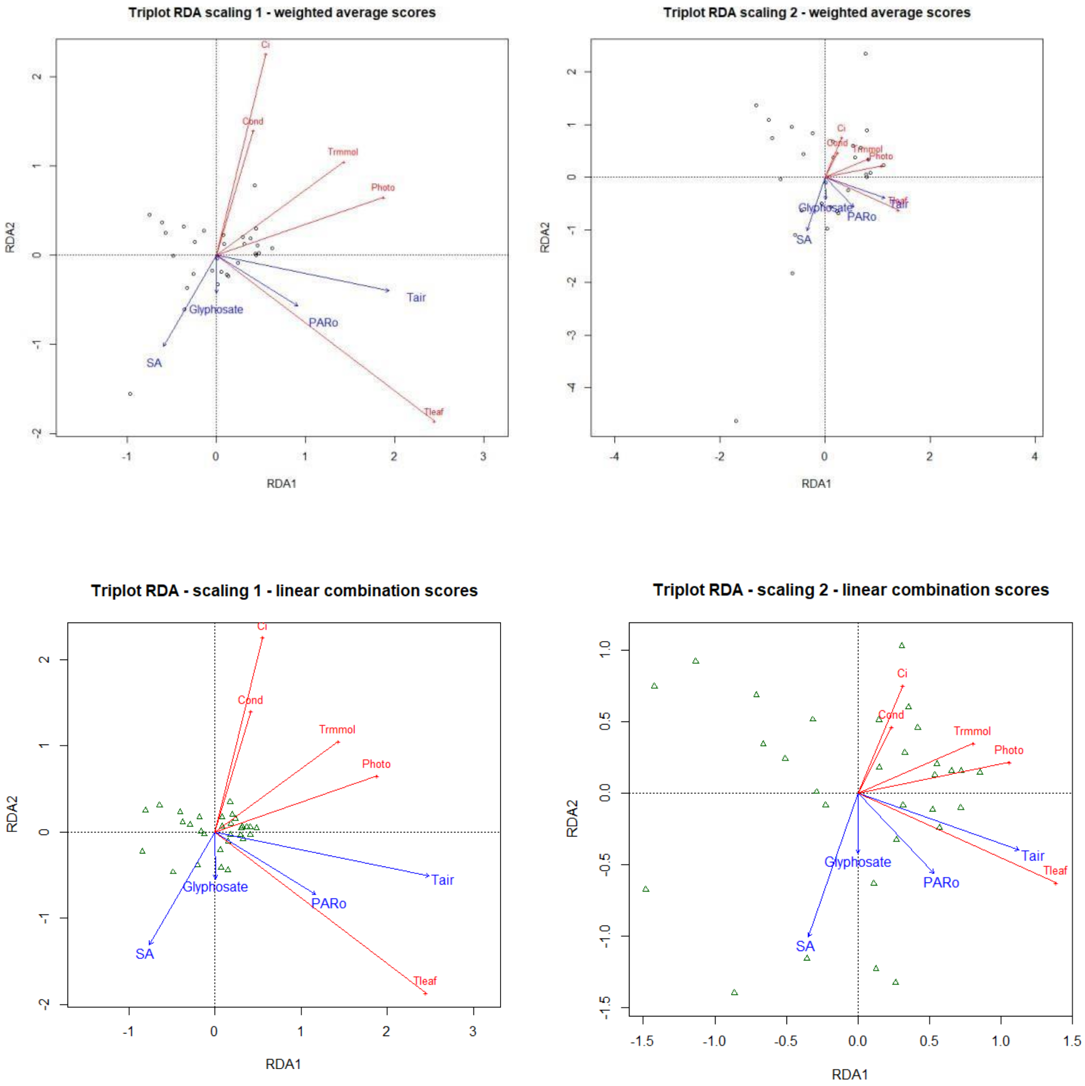
Figure 5. Fit plot for the regression of soybean photosynthetic rate on glyphosate application rate. The blue band indicates the 95% confidence limits, and the dashed lines indicate the 95% prediction limits for experiment 2.



5.3 Experiment 3: SA + Glyphosate

The RDA for experiment 3 indicated that the SA concentration was negatively correlated with intercellular CO_2 and conductance to water (Figure 6). The RDA for experiment 3 also indicated that SA was independent of air temperature and leaf temperature, expressed in the correlation triplots as right angles between the vectors. Due to the experimental design, SA was independent of leaf temperature and is negatively correlated with stomatal conductance (*Cond*) and intercellular CO_2 concentration.

Figure 6, (a) distance triplot and(b) correlation triplots based on the RDA, for experiment 3.


(a) (b)

For experiment 3, 43.97% of the variability in the plant photosynthesis variables can be explained by the exogenous variables, and 56.03% of the variability in the photosynthesis variables cannot be explained by the exogenous variables (Table 16).

Table 14: Partitioning of correlations for experiment 3

Inertia Proportion		
Total	5.000	1.0000
Constrained	2.198	0.4397
Unconstrained	2.802	0.5603

Table 15: Importance of components: Eigenvalues, and their contribution to the correlations, for experiment 3.

	RDA1	RDA2	RDA3
Eigenvalue	1.6073	0.5585	0.02696
Proportion Explained	0.3215	0.1117	0.00539
Cumulative Proportion	0.3215	0.4332	0.43855

Table 16. Importance of components: Accumulated constrained eigenvalues, for experiment 3.

	RDA1	RDA2	RDA3	RDA4
Eigenvalue	1.6073	0.5585	0.02696	0.005746
Proportion Explained	0.7311	0.2540	0.01226	0.002610
Cumulative Proportion	0.7311	0.9851	0.99739	1.000000

Table 17: The response variables can be located on the RDA triplot based on the RDA scores: photosynthetic rate, conductance to water, intercellular CO_2, and transpiration rate are in the upper right quadrant; and leaf temperature is in the upper left quadrant, for experiment 3.

	RDA1	RDA2	RDA3	RDA4
Photo	1.0634	0.2176	-0.005438	0.09295
Cond	0.2383	0.4670	0.188003	-0.03319
Ci	0.3141	0.7544	-0.160778	-0.03955
Trmmol	0.8112	0.3505	0.055757	-0.01413
Tleaf	1.3880	-0.6225	-0.024310	-0.04830

Table 18. Biplot scores for constraining variables, for experiment 3.

	RDA1	RDA2	RDA3	RDA4
SA	-0.288053	-0.8416	-0.440818	0.12017
Glyphosate	0.001622	-0.3511	-0.150578	-0.92416
PARo	0.444943	-0.4683	0.737939	0.19534
Tair	0.943696	-0.3299	0.009224	-0.02271

The global test of the RDA model was a permutation test (999 permutations). It indicated that the overall model was statistically significant ($p = 0.001$) (Table 20).

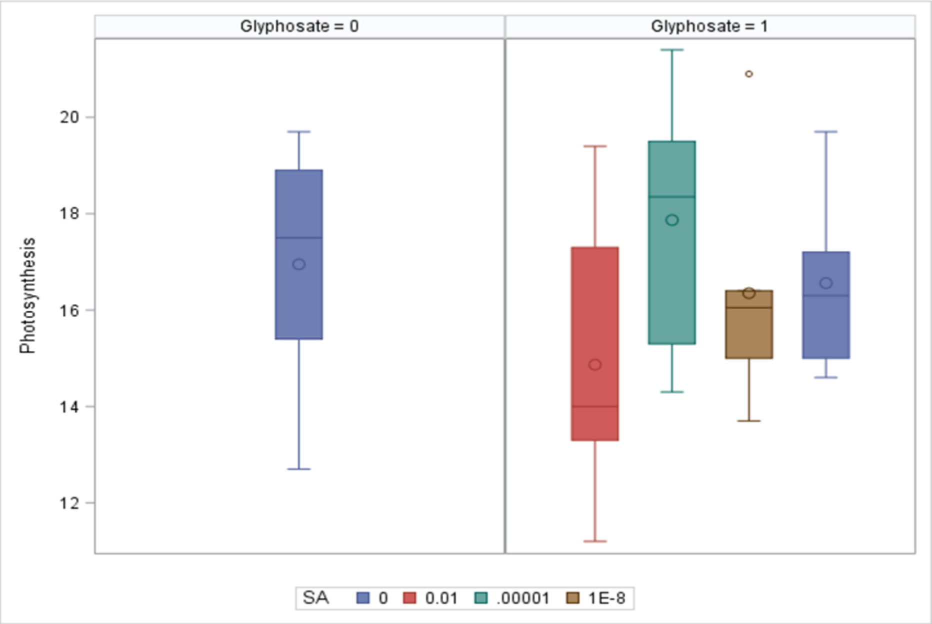
Table 19. Global test of RDA, for experiment 3.

	Df Variance	F	Pr(>F)
RDA1	1 1.60733	14.3433	0.001 ***
RDA2	1 0.55845	4.9835	0.101
RDA3	1 0.02696	0.2406	0.985
RDA4	1 0.00575	0.0513	0.981
Residual 25 2.80152			

There was no evidence to contradict the null hypothesis (that the mean photosynthetic rate was equal for different levels of the nested treatments) ($F_{4,19} = 1.59$, $p = 0.2181$).

Although results were not significant, there is consistency between results found in Experiments 1 and 3.

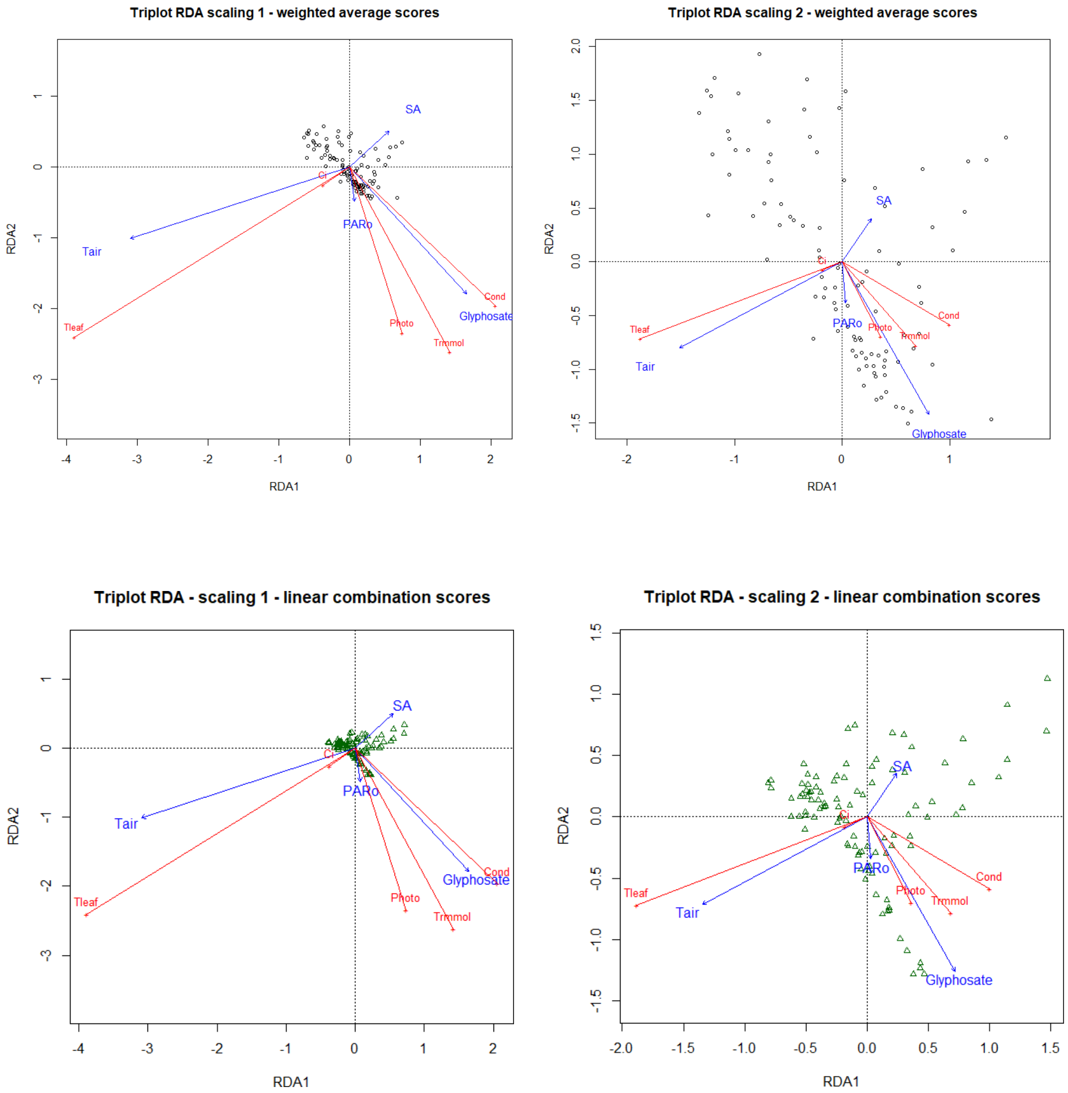
Figure 7. Photosynthetic measurement rates with and without glyphosate using three levels of SA (10^{-2} M, 10^{-5} M and 10^{-8} M), for experiment 3.



5.4 Experiment 4: combination of 16 treatments

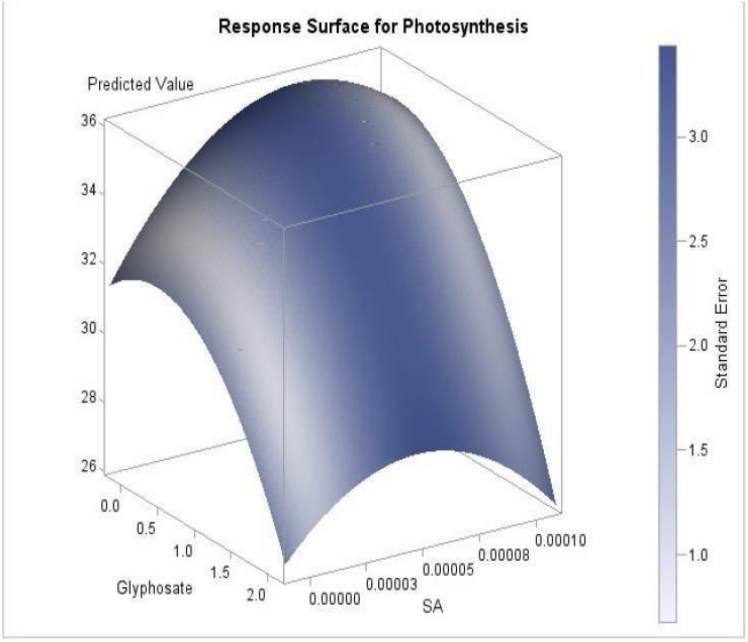
In the RDA for the final experiment, we see again the negative correlation between SA and intercellular CO_2. However, in this experiment, SA is negatively correlated with leaf temperature and air temperature because of the artificial conditions in the experimental space. SA concentration is independent to transpiration and conductance to water as well. This approach doesn't consider the quadratic relationship between SA and photosynthetically active radiation.

Figure 8. (a) distance triplot and (b) correlation triplots based on the RDA for the combined data


(a) (b)

In part 1, although the data did not contradict the null hypotheses that the mean photosynthetic rates for the respective levels of glyphosate and SA were equal, we can expect to find a maximum photosynthesis value where SA is applied at a rate of 0.000073889 (7.3 x 10^{-5} M) and glyphosate is applied at a rate of 0.121518 (12.1%), as indicated by the response surface (Figure 9).. Based on this experimental data, that combination could be expected to maximize the photosynthetic rate of soybean (Figure 9 in the presence of glyphosate. The statistical significance of the response surface (Figure 9) relates to the overall shape of the surface, and not to comparison among specific elements of the surface, so that the overall shape indicates the effects of glyphosate and SA, each across a range of concentrations, and the interaction between these two effects. In part 2 of the experiment, the optimal (stable) point is a maximum where SA is 0.000069061 (6.9 x 10^{-5}) and glyphosate is at 0.163183 (16.3% of recommendation) which are similar and consistent with findings of part 1 of Experiment 4.

Figure 9. Response surface indicating the effect of SA application, across a range of concentrations, to soybean plants treated with a range of glyphosate levels, for experiment 4.



The generalized linear mixed model indicated that, based on Bonferroni-adjusted limits at 95% confidence, control plants would photosynthesize 13 to 31% more than plants treated with 2 × the recommended dose of glyphosate which has never been reported before. Plants treated with half the recommended dose of glyphosate would photosynthesize 13 to 29% more than plants treated with 2 × standard glyphosate levels. Plants treated with standard glyphosate would photosynthesize 15 to 33% more than plants treated with 2 × the rate of glyphosate. Glyphosate affected the plant photosynthetic rate to a highly statistically significant degree $F_{3,35} = 33.08$, $p < .0001$

The canonical coefficients of the RDA of the combined-data explain 32.56% of the total variation of the soybean photosynthesis variables (Table 22). For photosynthesis variables. Some 67.44% of the variability cannot be explained by the explanatory variables.

Table 20: Partitioning of correlations for experiment 4.

	Inertia	Proportion
Total	5.000	1.0000
Constrained	1.628	0.3256
Unconstrained	3.372	0.6744

Table 21: Eigenvalues, and their contribution to the correlations for experiment 4.

	RDA1	RDA2	RDA3	RDA4
Eigenvalue	1.1709	0.44541	0.01088	0.0006049
Proportion Explained	0.2342	0.08908	0.00218	0.0001200
Cumulative Proportion	0.2342	0.32326	0.32544	0.3255600

Together the first two axes of the RDA explain 99.29% of the constrained variance.

The first axes explains 71.93% and the second explains 27.36% of the total axes (Table 24).

Table 22: Accumulated constrained eigenvalues, for experiment 4.

	RDA1	RDA2	RDA3	RDA4
Eigenvalue	1.1709	0.4454	0.01088	0.0006049
Proportion Explained	0.7193	0.2736	0.00668	0.0003700
Cumulative Proportion	0.7193	0.9929	0.99963	1.0000000

Table 23: Variable scores for experiment 4.

	RDA1	RDA2	RDA3	RDA4
Photo	0.3555	-0.69877	-0.07177	-0.0390024
Cond	0.9950	-0.58565	0.09119	-0.0005575
Ci	-0.1838	-0.07736	0.18334	-0.0102478
Trmmol	0.6840	-0.78044	-0.02830	0.0318358
Tleaf	-1.8813	-0.71798	0.00647	0.0049123

The response variables can be located on the RDA triplot based on the RDA scores: photosynthetic rate, conductance to water, and transpiration rate are in the lower right quadrant; while intercellular CO_2 and leaf temperature is in the lower left quadrant.

If the angles (between the vectors associated with variables located in the same quadrants on the correlation triplot) were relatively small, then the positions of these variables on the triplot represents an association and correlation.

Table 24. Biplot scores for constraining variables for experiment 4.

	RDA1	RDA2	RDA3	RDA4
SA	0.15770	0.2332	-0.37700	0.88241
Glyphosate	0.47013	-0.8279	-0.29759	0.07072
PARo	0.01985	-0.2243	0.83950	0.49451
Tair	-0.88381	-0.4674	0.01664	-0.01291

When the data are combined, the RDA model explains the variance in soybean photosynthesis variables significantly ($p < 0.001$) and well according to the global permutations test (999 permutations).

6.0 Discussion

The four experiments carried out in this research showed promising results that are consistent with several other experiments conducted with SA. For example similar results were found in other published literature, Khan et al. (2002) determined that stomatal conductance, transpiration, leaf area and plant dry mass were all increased when soybean plants were treated with SA at 10^{-5} M. Fariduddin et al. (2003), found that application of water solutions of SA to foliage of 30-day old mustard plants (*Brassica juncea* Czern & Coss cv. Varuna) resulted in greater dry mass and photosynthetic rates at a lower concentration (10^{-5} M) of SA than those sprayed with water only, or with higher dosages of SA (10^{-4} or 10^{-3} M). The optimal concentration of SA, with regard to photosynthetic rates, including with glyphosate spray, from the last two experiments conducted in this work, also indicated that the optimum response in soybean plants occurred in the 6.9 x 10^{-5} to 7.3 x 10^{-5} M range. Although, SA responses at specific rates match the responses of fully grown plants, at more advanced development levels in other experiments, optimal photosynthetic rates suggest, at an early stage of plant development, alleviation of glyphosate-induced stress by SA. Also, the research reported here is restricted to greenhouse conditions with a limited number of plants. More research needs to be conducted to test whether the ranges found in SA combined with glyphosate will correspond to the same findings, with regard to maximum yield, plant dry mass and leaf area, for fully developed (all the way to seed maturity) soybean plants.

Although GR soybean technology is used in most parts of the world, there are no exact or particular fertilizer recommendations for use in conjunction with this technology; a recommendation that would limit or minimize the detrimental effects of herbicide, mainly glyphosate, and few studies have been directed at determining the influence of glyphosate on GR soybean nutrient status (Zobiole et al. 2010). There are also no quantifiable results from research conducted that would identify at what percentage damage is being done at, i.e. the degree of yield reduction. This research is the first study to quantify glyphosate damage to photosynthetic rate. Some trials including Zobiole et al. (2010), studied cultivar maturity groups on different soil types, and revealed a significant decrease in macro- and micronutrients in leaf tissues, and in photosynthetic variables (chlorophyll, photosynthetic rate, transpiration rate and stomatal conductance) as affected by glyphosate use (single or sequential application). Regardless of glyphosate applications, concentrations of shoot macro- and micronutrients were found to be lower in the near-isogenic GR-cultivars than their respective non-GR parental lines. Shoot and root dry biomass were reduced by glyphosate for all GR cultivars studied, on both soil types; there were also lower levels of nutrients in tissues of the glyphosate treated plants (Zobiole et al. 2010).

Like soybean, the respiration of quackgrass (*Elymus repens*) treated with glyphosate significantly reduced total net photosynthesis 9 days after treatment. Glyphosate reduced total photosynthesis more in quackgrass than in wheat (Sprankle et al. 1975).

Zobiole et al. (2010) found that increasing glyphosate rates revealed a significant reduction in photosynthesis, macro- and micronutrient accumulations in leaf tissues and also decreases in overall nutrient uptake. The reduced biomass in GR soybean genotypes represents additive effects of the decreased photosynthetic variables, as well as lower availability of nutrients in tissues of the glyphosate treated plants.

Soybean growers have reported that some glyphosate-resistant soybean varieties are visually damaged by glyphosate. The effect of increasing rates of glyphosate on lignin and amino acid content, photosynthetic rate and dry biomass for the early maturity group soybean cultivar BRS 242 GR was studied by Zobiole et al. (2010). Plants were grown in nutrient solution and treated with various rates of glyphosate, either as a single or in consecutive applications. All measured variables evaluated were affected by increasing glyphosate rates. The effects were more noticeable as glyphosate rates increased, and were more extreme with a single application than sequential applications at lower rates. Similar findings were found in this research as well in which higher concentration of glyphosate led to a decrease in photosynthetic rate.

Gomes (2015) addressed how glyphosate affects physiological processes such as photosynthesis. Despite its precise and well understood site of action, glyphosate can boost oxidative stress, although it is not known how the inhibition of the shikimate pathway is related to oxidative stress (Gomes. 2015). The photosynthetic apparatus is very sensitive to reactive oxygen species build up, by interacting with organic compounds, ROS can induce damage to DNA, lipids and proteins (Gomes. 2015). To alleviate oxidative stress due to ROS promotion, plants deploy enzymatic (superoxide dismutase [SOD], catalase [CAT], ascorbate peroxidase [APX], glutathione peroxidase [GPX] and glutathione reductase [GR]) and non-enzymatic mechanisms, such as ascorbate and glutathione systems (Gomes. 2015). These antioxidant systems, as well as lipid peroxidation, are oxidative stress markers, which were shown to be moderated by glyphosate (Gomes. 2015).

It is commonly assumed that glyphosate kills weeds by disturbing the EPSPS synthase enzyme and thus disrupting the synthesis of aromatic amino acids. However, this herbicide has several other secondary derivatives or auxiliary effects on plant physiology which may also explain glyphosate effects in general. The harmful effects of glyphosate could be related to its attributes on other physiological processes, such as photosynthesis, mineral nutrition, hormone production and oxidative status. The alteration of these processes could be directly linked to the harmful effects of glyphosate observed on plant growth and production (Gomes, 2015). As a metal chelator, glyphosate could deny plants important nutrients which have key roles as enzymatic co-factors, biomolecule constituents and anti-oxidative activities (Gomes 2015). Oxidative stress, such as lipid peroxidation caused by glyphosate is known to strongly injure the cell integrity and lead to cell death, and with increased ROS production, it can interfere with photosynthetic processes such as decreasing the chlorophyll content, photochemical efficacy and carbon metabolism, leading to reductions in overall plant growth (Gomes, 2015).

There are multiple research efforts and experiments conducted that show an increase of antioxidant activity when SA is applied. For instance, Razmi et al. (2015) showed that the activities of superoxide dismutase and ascorbate peroxidase, and the concentrations of hydrogen peroxide, proline and total protein were increased in response to SA applications (Razmi et al. (2015). Brendao et al. (2014) showed that SA increased the content of betacyanins, total phenols and flavonoids and the non-enzymatic antioxidant capacity of leaves of *Alternanthera tenella* 0, 12, 36 and 48 h after SA treatment. As a result the antioxidant activity of the leaves of *A. tenella* is encouraged by SA and can be attributed to the increase in betacyanin content, compounds with recognized antioxidant activity (Brendao et al. 2014).

The percentage of the recommended full rate of glyphosate that would be optimal to maximize photosynthesis was found to be 16.3 and 12.1%. This would be an ideal level for plant development according to this research, but realistically, crop producers may be skeptical of using only 16.3 and 12.1% of the recommended glyphosate dose due to heavy weed pressure in fields. Already we are seeing resistance to the full recommended dose, as mentioned above; since 1998, 24 plant species have evolved glyphosate resistance and weeds have evolved nine different mechanisms of resistance to glyphosate (Sammons & Gaines, 2014; Green, 2016). Perhaps a lower recommended dose, in increments, can be tested in small on-farm experiments to see how well the weed pressure can be managed at various application rates, to determine if the recommended dose is actually in surplus and, as such, could be reduced. As mentioned in the glyphosate section of the literature review, there are no methods or directions regarding when glyphosate should be discontinued in a field, so perhaps starting at a lower dosage of glyphosate in a relatively ‘new’ field, that hasn’t been sprayed with the herbicide in recent years, to determine if we then see weeds being a minimal problem and photosynthetic rates allowing production of maximum yield.

As resistance to glyphosate and other herbicides continues to grow (Sammons & Gaines, 2014; Green, 2016), there will be a continuous need for better, more efficient biostimulants, to help deal with the stress associated with application of higher levels of herbicide spray-applied on the crop plant, and to also help deal with competition for nutrients in the field, due to intense competition with weeds. Companies are now turning to more creative and experimental ways to increase yield, not relying only on more conventional methods, such as application of NPK. Instead, companies are combining primary and secondary nutrients with amino acids, seaweed extracts, antioxidants and other substances, to make certain their products are still competitive and present in the biostimulant market.

Due to the increasing demand for crop yield, to feed the growing world population, and the pressure from crop producers to develop crops that can be deployed for this purpose, more and more herbicides are used on crops, to control weeds and to ensure a productive crop growing season. For example, as mentioned in the sections above, in the United States, from 1998 to 2011, soybean farmers used 28% or 0.30 kg ha^{-1} more herbicide (Perry et al. 2016). Therefore, some farmers may use more than simply the recommended rate. Zobiole et al. (2010) were able to detect a decrease in photosynthetic variables using glyphosate resistant (GR) soybean types and glyphosate, but to our knowledge, no study has shown or has quantified at the level of the decrease, or when using more than the recommended dose becomes a serious problem. In the work reported here we showed that control soybean plants photosynthesized 13 to 31% more ($p < 0.05$) than plants treated with 2x the recommended dose of glyphosate. The rate of photosynthesis for plants treated with 0.5x glyphosate was 13 to 29% greater than plants treated with 2x glyphosate levels. Plants treated with standard 1x glyphosate photosynthesized 15 to 33% more than plants treated with 2x glyphosate. Glyphosate affected the plant photosynthetic rate to a meaningful level ($F(3,35) = 33.08$, $p < .0001$). Using more glyphosate can, perhaps, ensure that challenge from weeds as the crop is emerging may be beneficial, but can affect yield based on detrimental effects at early stages of plant development (3rd or 4th trifoliate) and photosynthetic measurements (Li-Cor 6400xt). Although, antioxidant activity was not measured when using multiple doses of glyphosate, photosynthetic measurements have shown, at a statistically significant level, a decrease in photosynthetic rate when using higher levels of glyphosate, which can be attributed to oxidative stress (Gomes, 2015). In this research, we quantified how glyphosate affects the photosynthetic rate of a GR soybean, providing percentage reductions, along with the levels of SA that result in maximum photosynthetic activity when glyphosate has been added. The levels of SA concur with other researcher's findings in which 10^{-4} M, 10^{-5} M and 10^{-6} M are shown to optimize photosynthetic rate (Khan et al. 2002; Fariduddin et. al 2003).

Exogenous levels of SA help promote photosynthetic rate under stress conditions in various crop species, such as mustard (Fariduddin et al. 2003; Nazar et al. 2015), cucumber (Liu et al. 2009) and wheat (Khan et al. 2013). Its mode of action depends on multiple aspects, such as species of the plant, the environmental conditions (light, temperature, etc.) and the concentration of SA (Janda et al. 2014). When SA is added exogenously, it can deliver protection against numerous sorts of stress; however, above a certain level of concentration it can also be damaging to the plant (Janda et al. 2014). Although numerous phenolic compounds have been shown to have direct consequences on the photosynthetic electron transport chain, the widely held consensus has suggested that the effect of SA on the photosynthetic apparatus is indirect, originating from its effect on stomatal conductivity. ROS also play a decisive role in both the upstream and downstream developments of SA signalling (Janda et al. 2014). Recent results strongly suggest that controlled SA levels are important in plants for ideal photosynthetic performance and for acclimation to varying environmental conditions, including stressful ones (Janda et al. 2014).

The RDA for experiment 3 indicated that the SA concentration was negatively correlated with intercellular CO_2 and conductance to water (Figure 6). In the RDA for experiment 4, we see again a negative correlation between SA and intercellular CO_2, like the findings of Khokon et al. (2011), who measured stomatal responses of arabidopsis to treatment with SA. The reduced leaf internal CO_2 levels could be due to reduced stomatal aperture, restricting CO_2 entry into the leaf, because photosynthetic CO_2 uptake rates at the photosynthetic reaction centres was increased, or possibly both. Given that SA application caused both reduction in stomatal aperture and enhanced photosynthetic rates it seems likely that the latter (both) was the situation. Because SA is involved in plant stress responses it affects many plant physiological processes including stomatal movement. In this case SA induced stomatal closure. In experiments 3 and 4, we observed similar findings along with a negative correlation with SA and intercellular CO_2. The SA effect could be part of signaling/response related to systemic acquired resistance, which primes the plant by closing up the stomata to retain water and thus retaining or limiting conductance to water and CO_2.

7.0 Conclusions

This work illustrated and confirmed the previous observation that glyphosate does inhibit the photosynthetic rates of glyphosate tolerant soybean plants and demonstrated that SA can play a role in restoring the photosynthetic rates of glyphosate treated glyphosate tolerant soybean plants. This research showed that the concentration of SA most effective at restoring photosynthetic rates was 10^{-5} M which matches with other findings in the literature. It also indicated that SA induced a stress response that both increased plant photosynthetic rate and decreased the degree of stomatal opening and also quantified, in percentage, levels of photosynthetic measurements when compared to varying treatments of glyphosate.

Future directions

Although photosynthetic rates are strong indicators regarding how the plant will grow and create yield, the results obtained in this research go only as far as the third or fourth trifoliate stages of soybean plant development and the results obtained are all related to photosynthetic rate 24 h after spray application, and do not go all the way to seed yield. Therefore, this research provides an intriguing indication of the mode of action by which salicylic acid diminishes the negative impacts of glyphosate on soybean growth and yield, more research is required to confirm that the photosynthetic effects demonstrated here carry through to final seed yield.

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