Methylgyoxal signalling in *Phaseolus vulgaris* under phosphate deficiency

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Student number: 3337863 Plagiarism declaration I declare that **Methylglyoxal signalling in common beans under phosphate deficiency stress** is my own work, that it has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete reference

01 August 2019

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I am very proud of myself to have got this far, a black girl from a small village but with all that said, this is the beginning of greater things.

List of abbreviations

M - Methylglyoxal

P - Phosphate

H_2O_2 - Hydrogen peroxide

O_2 - Superoxide

•OH - Hydroxyl radicals

MDA - Malondialdehyde

NO - Nitric oxide

SOD - Superoxide dismutase

CAT - Catalase

APX - Ascorbate peroxidase

ROS - Reactive oxygen species

NR - Nitrite reductase

EDTA - Ethylenediaminetetraacetic acid

TCA - Trichloroacetic acid

TBA - Thiobarbituric acid

NBT - 3, 3 Diindolylmethane

AM - Abuscular mycorrhizal

PSI/ II - Photosystem I/ II

G3P - Glyceraldehyde-3-posphate

DHAP - Dihydroxyacetone phosphate

TPI - Triose phosphate isomerase

Gly I/ II/ III - Glyoxalase I/ II/III

AGEs - Advanced glycation ends

NOS - Nitric oxide synthase

XOR - Xanthine oxidoreductase

Ni: NOR - Nitric dependant nitric oxide reductase

NOSLE - Nitric oxide synthase like enzymes

ETC - Electron transport chain

AtNOS1 - *Arabidopsis thaliana* nitric oxide synthases

XOH - Xanthine dehydrogenase

PTM - Post transcriptional modification

HM - Heavy metal

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Keywords

Antioxidant enzymes

Ascorbate peroxidase

Biomass

Cell death

Heavy metal

Hydrogen peroxide

Lipid peroxidation

Methylglyoxal

Nitric oxide

P. vulgaris

Phosphate deficiency

Reactive oxygen species

Superoxide dismutase

Superoxide

Abstract

In this study, we observed that phosphate (P) deficiency stunted plant growth and produced plants with poor morphological characteristics (yellow and small leaves). Furthermore, we treated plants with 0.8 mM (control) and 0.02 mM P (deficient) in addition to 6 µM methylglyoxal (MG) and we observed that the plants treated with MG had a higher germination, and better morphological characteristics (the leaves were more dark green and bigger in size) compared to the P deficient plants. However, we also observed that the P deficient plants treated with MG had low levels of both O_2^- and H_2O_2 and this could be a possible reason for the improved growth and morphological characteristics. In contrast, the P deficient plants not treated with MG had high levels of O_2^- and H_2O_2 which could be the possible reason for the observed cell death. We also performed biochemical assays including superoxide dismutase, ascorbate peroxidase, malondialdehyde content, ascorbic acid content, catalase, and most of the assays showed high levels of reactive oxygen species (ROS) and low levels of antioxidant activities in plants not treated with MG while high levels of antioxidant activities and low levels of ROS were observed in plants treated with exogenous MG. Since nitric oxide (NO) is also known to be a signalling molecule, we did a NO assay and observed that NO content increased under low exogenous doses of MG. From our findings we came to a hypothesis that MG modulates P deficiency stress in *P. vulgaris* through NO signalling or it might be that NO and MG work in tandem to modulate signalling pathways under P deficiency. Finally, we looked at the nutrient profile and the results showed that while there was a poor nutrient profile generally under P deficiency, there was an improvement in nutrient profile when MG was administered at low doses.

Chapter 1

Literature review

1.1 Introduction

About 800 years ago, the common bean *Phaseolus vulgaris* was domesticated in America and now is known to be of imperative source of food sustenance all around the world. In addition to the fact that this common bean is of incredible significance because of its caloric intake it also gives a range of supplements which incorporate folate, minerals,fibre and phytochemical. Common bean can also be stored for a period of time as it has a long shelf life and in developing countries is said to be an important food source (Castro- Guerrero *et al.* 2016).

Among many protein sources for human consumption, the common bean *Phaseolus vulgaris* and soybean *Glyxine* max are the two most important sources as both have many benefits. However, their consumer market differs, for example, soybean is processed in China, Argentina, USA and Brazil. It is mostly used as oil and protein source in most food products. On the other hand, the common *P. vulgaris* is a staple food that does not require any modern preparing; it is the most imperative protein source food sources that provide minerals and other than the caloric admission, common bean likewise gives minerals, fibre, thiamine folate, and phytochemicals (Blair, 2013; Jha, 2015).

In addition to the benefits mentioned above, there are also economic benefits as common beans are far cheaper and affordable compared to animal proteins. Agricultural benefits include the symbiotic relationships where the plant itself interacts with N_2 fixing bacteria, thus decreasing the use of non-organic fertilizer that is bad for the soil. Given all the benefits mentioned above, it is clear that legumes are a major factor that could contribute to tackling the food security challenges currently faced (Castro-Guerrero *et al.* 2016).

Crops are helpless to biotic and abiotic pushes and relying upon the seriousness of the stress and the plant's capacity to endure them, yield could be extremely influenced. Due to drastic drought conditions in the US in 2012, there was a reduction in soybean and corn production by 10 % and 28 %; respectively. To counteract the effect pest and lack of nutrition, the use of fertilizers and pests is used. Although this solution works short term, scientists and farmers still have the task of providing food with high nutrition which is compatible with sustainable agricultural practices (Foley, 2011).

Soybean and common bean can set up advantageous relationship with nitrogen-fixing bacteria (Rhizobia) and with arbuscular mycorrhizal fungi (AM) to assist in obtaining supplements, for example, nitrogen and phosphorous (Vance, 2001; Venkateshwaran, 2013). The dramatic use of fertilizers and the release of a major greenhouse gas, N_2O is reduced, thus these symbiotic relationships are said to be economically important.

The principle impact in the production of crops is the abiotic and biotic components which incorporate high salt fixations, absence of water, temperature (certain plants are adjusted to make due in particular temperatures), absence of phosphate (P) and osmosis among numerous other more stresses (Foley, 2011). Lack of phosphate is the main plant stress of this investigation as it prompts cell harm and morphological changes in the plant. What's more, it can't be solubilised and is engaged with various metabolic processes in the plant that keep it stable. At the point when a plant is under stress (for this situation, P deficiency), reactive oxygen species, which include superoxide, hydrogen peroxide and so forth are produced which when accumulated at high amounts can cause serious harm to the cell and even kill the plant totally (Venkateshwaran, 2013). Anyway the plant may detect that it is under pressure and fortify cancer prevention agent generation to support its guard instruments, where the cell reinforcements search the ROS and changing over them into less poisonous particles (Blair, 2013; Jha, 2015). On the other hand, when the plant senses that it is under stress, the production of antioxidants is stimulated. The plant uses these antioxidants as a defence mechanism that reduces the concentrations of reactive oxygen species that destroy the plants (Foley, 2011).

Methylglyoxal (MG) is a reactive molecule found in plants which is mainly produced through tandem interaction of the enzyme glyoxalase I and glyoxalase II (Hoque et. al, 2016). If MG is over produced it becomes toxic to the plants and lead to detrimental damage however, at low concentration it is known to be a signalling molecule that improves seed germination, expands leaf size, reduce chlorosis and more (Hoque et. al, 2016). The plants defence mechanism is not enough to ensure sufficient crop production, and so it is vital that more research is done on other ways to counteract the effects of stress, thus this project is based on how we can use methylglyoxal as a signalling molecule and module the effects of P deficiency.

1.2 Phosphate deficiency in plants

Phosphorous is one of the main nutrient sources for plants due to the fact that it plays a role in a number of plant pathways such as genetic transfer, phospholipid cell membrane, nutrient transportation and many more (Lopez-Aredondo *et al.* 2014). The pathways which phosphate (P) is involved in are of great importance for the normal function of the plant, to give an example, energy produced in photophosphorylation because of the chemical processes phosphate is involved in. P is a significant molecular component in generic production, with that in mind, under stressful cell division is inhibited thus resulting in poor and slow plant maturity (Lia, 2001). Phosphate deficiency

also has morphological effects on the plant such as reduced leaf growth among other effect which result in a carbohydrate storage imbalance (Ane *et al.* 2013). This imbalance leads to carbohydrate imbalance which is often observed when leaves change colour from producing a dark purplish colour (Plaxton and Tran, 2011).

1.2.1 Morphological responses to phosphate deficiency

When plants are exposed to P stress it makes use of phosphate patches in the soil to make up for the unavailable P, and this results in an alteration in the root morphology (Lopez-Aredondo *et al.* 2014). Studies done show that under phosphate stress conditions in *Arabidopsis thaliana* result in the number of lateral roots and hairs being increased and shorter essential roots are formed (Lopez-Aredondo *et al.* 2014). Additionally, *A. thaliana* produced shoots and leaves that were reduced in size, and this resulted in an increase shoot to root weight ratios. Although morphological alteration has been observed in several diverse plants species, a small number of cultivars have a unique response to P stress. To give an example, although a reduction has been observed in the number of essential roots in common beans, various bean cultivars in the other hand show a significant increase in the number of lateral roots and hairs (Lia *et al.* 2001; Yan *et al.* 2014). The stimulation in production of shallower basal root has also been observed in many studies as a structural response when common bean were exposed to P deficiency (Lia *et al.* 2001; Yan *et al.* 2014).

1.3 Reactive oxygen species and cell damage alleviated in plants under stress

1.3.1 Reactive oxygen species

Oxygen constitutes approximately 21 % of the earth's atmospheric gases, making it available for use by plants animals and other organisms. As explained by Gill and Tuteja (2010) oxygen molecules have the potential to damage the organisms that rely on it to survive; the destructive properties of oxygen are attributed to the molecules having two unpaired electrons that have the same spin quantum number. As a result of this spin restriction, oxygen is able to accept a single electron at a time, which causes the formation of reactive oxygen species (ROS) (del Río, 2006). There are several reactive oxygen species under varying conditions which affect plants in different ways. These reactive oxygen species include superoxide radicals (O_2^-), singlet oxygen (O_2) , Hydrogen peroxide

(H_2O_2) and hydroxyl radicals (OH). These ROS are continuously produced as by-products of regular plant metabolism and are localized to different cellular components including the chloroplast, mitochondria and the peroxisome (Navrot, 2007).

One of the major sites for ROS synthesis in plants is the thylakoid membrane-bound primary electron acceptor in the chloroplast; here superoxide radicals are continuously produced during photosynthesis as a result of the partial reduction or energy transfer to oxygen (Gill, 2010). Superoxide radicals are usually the first ROS produced and may trigger the generation of other ROS including, •OH and O_2 , which results in peroxidative damage to membrane lipids and a general weakening of cellular structure. The reaction of superoxide radicals with iron (Fe_3^+) produces reduced iron (Fe_2^+) which can reduce H_2O_2 to O_2^- and OH (Gill, 2010). According to Krieger-Liszkay (2005), singlet oxygen is another ROS that is generated during photosynthesis but its formation is not a result of electron transfer to oxygen but rather insufficient energy dissipation during photosynthesis, which leads to the formation of the chlorophyll triplet state. The chlorophyll triplet state is a lower energy state which has a longer half-life and can react with $3O_2$ to generate $1O_2$, which has been proven to severely damage both photosystem I (PSI) and photosystem II (PSII) and the photosynthetic apparatus in general. Singlet oxygen is considerably damaging to plants because of its ability to oxidize a wide range of biological molecules and react with proteins, nucleic acids and lipids. Singlet oxygen is also thought to be the ROS that is responsible for light-mediated loss of PSI activity which contributes to the triggering of cell death (Krieger-Liszkay, 2008). Generated as a result of the univalent reduction of oxygen, H_2O_2 is a moderately reactive oxygen species with a longer half-life (1 ms) relative to other ROS (2-4 µs). Unlike $1O_2$ which targets membrane lipids, H_2O_2 causes oxidative damage, when present at high concentrations, by inactivating enzymes via the oxidation of the thiol groups as explained by Tewari *et a*l (2006). Aside from its destructive capabilities, H_2O_2 also acts as a signalling molecule when it is present at homeostatically favoured concentrations and is a regulator of various physiological processes including senescence, photorespiration and photosynthesis. The use of H_2O_2 as a signalling and regulatory molecule can be attributed to its relatively long half-life and high permeability across membranes (Gill, 2010; Quan, 2008). The most reactive ROS are hydroxyl radicals (OH) which can be generated from O_2^- and H_2O_2 by the Fenton reaction in the presence of iron (Gill, 2012). Hydroxyl radicals are the most destructive ROS as they can react with biological molecules and there is no characterized enzymatic

mechanism that can be used to keep hydroxyl radical concentrations in a steady state equilibrium, as a result of increased concentrations of hydroxyl radicals always result in cell death (Gill, 2010).

1.3.2 Superoxide (O_2^-)

Superoxide (O_2^-) is a ROS molecule which is mostly formed in the mitochondria and the chloroplast by and enzyme called NASPH oxidase which is found in the plasma membrane and low levels in the peroxisome (Hossain *et al.* 2011; Mourato *et al.* 2012). A study done by Mourato and colleagues in 2012 characterised O_2^- as being medium reactive due to the fact that it does not diffuse across the cell membrane and so the only way to reduce its levels is through a mechanism where it is scavenged by superoxide dismutase (SOD) which basically utilises O_2^- present converting it to H_2O_2 (Held, 2012).

1.3.3 Hydrogen peroxide (H_2O_2)

Like superoxide, H_2O_2 is primarily produced in the mitochondria and peroxisomes as a by-product when SOD is detoxifying O_2^-. H_2O_2 can penetrate through the membrane unlike O_2^- and it has a half-life of 1 ms thus making it the least ROS molecule (Hossain *et al.*, 2012; Moutato *et. al*, 2012). With H_2O_2 being known for its harmful effects to the cell, the oxidative damage caused when overproduced stimulates the activity of antioxidants that the plants use as a defence mechanism which include CAT and APX among others (Ahsan *et al.* 2003). Although H_2O_2 can really be toxic to the plant and cause damage, it also serves as a signalling molecule. In an intermediate concentration, H_2O_2 can improve the plants defence to biotic stresses through regulating gene expression and signalling pathways (Singll-Pareek *et al* . 2006; Hossain *et al* . 2012).

1.3.4 Hydroxyl ions (•OH)

The most reactive ROS molecule that results damage in the plant when not metabolised is the hydroxyl radical (•OH) (Cartea *et al.* 2011; Held, 2012). It is formed through Fenton and Fenton like reactions and from H_2O_2 and O_2^-. Additionally no enzyme is known to degrade it thus it causes great damage to DNA, lipids, proteins and photosynthetic pigments (Mourato *et al.* 2012).

1.3.5 Lipid peroxidation

The change that occurs in membranes morphology and function is known as lipid peroxidation and largely serves as an oxidative damage indicator (Yadav, 2010). As we know that an

overproduction of ROS occurs when the plant is under stress, the effect of these ROS results in the degradation of polyunsaturated fats which make up the membrane (Sinha *et al.* 2005; Verna & Dubey, 2003). When lipid peroxidation occurs, a compound known as malondialdehyde is produced which can be measured and used as an indicator of cellular damage (Wahsha *et al.* 2012). The effect that lipid peroxidation poses to the plants include permeability of the cell across the membrane, this means that vital ions which include potassium are lost through leakage (Zhang *et al.* 2007). To counteract the effect of ROS overproduction, plants use antioxidants such as ascorbate peroxide, superoxide dismutase, catalase and many other more as defence mechanisms (Sinha *et al.* 2012)

1.4 Antioxidant enzymes that act as a defence mechanism against ROS accumulation

1.4.1 Superoxide dismutase (SOD)

As mentioned above, SOD is a mechanism that is utilised to convert O_2^- to H_2O_2 with the use of cofactors such as Mn, Zn, Cu and Fe. This happens when superoxide radicals are produced at high concentrations (Almeselmani *et al*. 2006; Mourato *et al*. 2012). The location of SOD has been said to be in various cell components such as peroxisome and mitochondria (MnDOSs), chloroplast (FeSODs) and cytosol (Cu/ZnSODs) Beuttner, 1998; Braodbent *et al.* 1998; Mourato *et. al*, 2021). A study done by Buetner in 1998 illustrated that the only antioxidant that acts as the first line of defence on ROS is SOD as it scavenges O_2^- thus converting it to H_2O_2 due to the fact the H_2O_2 can diffuse across the membrane because it has a longer life. The presence of high H_2O_2 accumulation in turn triggers other antioxidants in the plant that also act as defence mechanisms (Beuttner, 1998; Braodbent *et al.* 1998; Mourato *et al.* 2012).

1.4.2 Ascorbate peroxidase (APX)

When H_20_2 is available at high levels in the plant, ascorbate peroxidase (APX) in tandem with ascorbate (APX cofactor) available comes into play to degrade H_2O_2 and thus keeping it at low concentrations (Almeselmani*,* 2006; Cartea*,* 2011). Ascorbate peroxidase is localised apoplast, vacuoles, chloroplast and cytosol (Mourato et al. 2012). Ascorbate peroxidase plays a vital role in H_2O_2 degradation because although it is not the only antioxidant that scavenges H_2O_2 but it has a greater scavenging ability compared to CAT (Mourato et al. 2012).

1.4.3 Catalase (CAT)

Like APX, CAT also scavenges H_2O_2 in tandem with peroxidases and converts it into H_2O. When there is excess intracellular H_2O_2 available, CAT and peroxidases are the two principal antioxidants that maintain the levels (Blokhina et al. 2013; Held , 2012). Catalase is mainly localised in the peroxisomes. In the mechanism that CAT utilises, Fe or Mn (CAT cofactors) are oxidised by singlet H_2O_2 radicle and the H_2O_2 that has bound to the cofactor is transported to a new substrate (Mhamdi *et al.* 2010). Basically APX and CAT work together to scavenge H_2O_2 and because APX has a higher scavenging ability, CAT only comes in to play to scavenge excess H_2O_2 once APX has been activated.

1.5 Methylglyoxal (MG)

Methylglyoxal is known as a reactive molecule because it reacts with and maintains RNA, DNA and proteins, however if MG accumulate in the plant cells at high level it becomes toxic to the plant and lead not only to cell, but also inhibit growth of roots, germination of roots and photosynthesis among many other effects. Although MG is toxic at high concentration, when it is at low concentrations between 30-75 µM it has been reported to be a signalling molecule which can improve regulation of diverse metabolic reactions such as homeostasis, cell proliferation and many other more (Hoque *et. al*, 2016). Methylglyoxal regulates stress by controlling the opening and closure of the stomata, ROS production and expression of stress responsive genes (Kaur *et al* . 2015).

1.5.1 MG synthesis and detoxification

Methylglyoxal is produced in mainly three pathways which include glycolysis and through intermediates of photosynthesis reaction which are triose sugar phosphates glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), and finally through metabolic pathways such as respiration (Bless, 2015). The main location of MG production in plants is mitochondria, cytosol and chloroplast. MG production rate and location vary based type of cell or tissue and the organ of the plant (for example roots or leaves) and the morphology of the plant (Kaur *et al.* 2015).

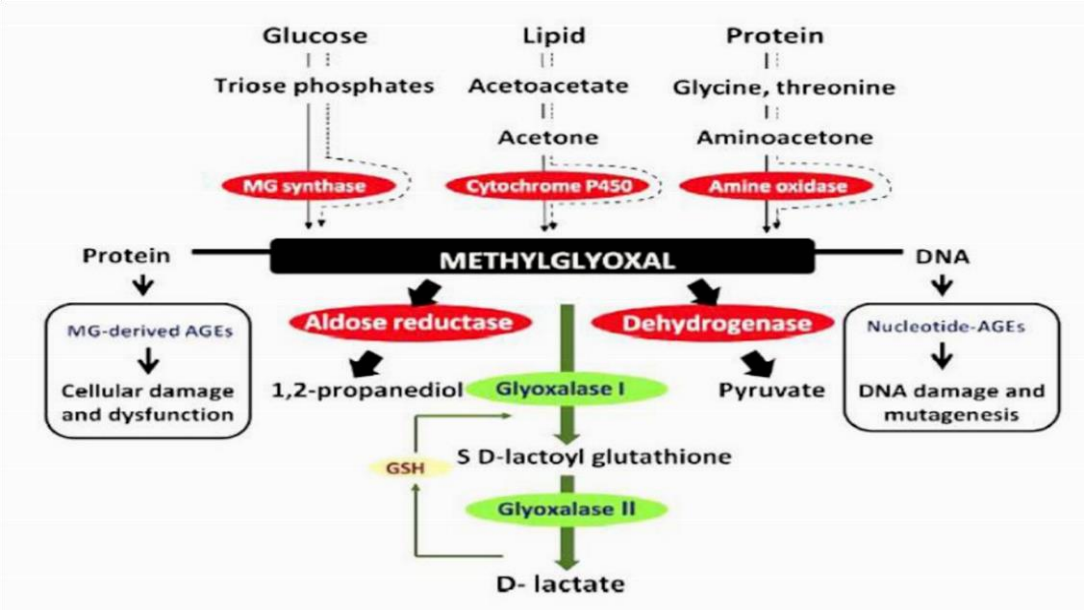


Figure 1: Schematic illustration of pathways involved in MG synthesis (Figure adopted from Bless, 2015).

When MG becomes a toxic molecule due to high concentration, the plants uses pathways to detoxify MG into lower concentrations in order to limit and counteract its harmful effects (Rabbani and Thornalley, 2012). As means of decreasing the toxic MG levels in plants, glyoxalase is the main pathway utilised as it has two enzymes namely glyoxalase I and II which work in together to produce D-lactate with release of glutathione (GSH) from MG (Yamauchi *et al*. 2010). Due to the fact that this metabolite it very reactive, it produces adducts (which contain sugars and lipids) thus resulting in the yield of advanced glycation end-products (AGEs) of nucleotides and other large molecules that can result in great damage (Bless, 2015).

1.5.2 MG synthesis in plants in detail

In plant cells, the cytosol, chloroplasts and mitochondria are all considered to be potential sites of MG production. However, the specific rate and sites of MG production vary depending upon the cell or tissue type, the plant organ (e.g. leaves or roots), and the physiological state of the whole plant (Kaur et al. 2015). Spontaneous production of MG occurs as a consequence of glycolysis, in metabolically active plant cells, from the reaction of the triose sugar phosphates glyceraldehyde3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), both of which are photosynthetic intermediates (Yadav, 2005a; Takagi et al. 2014; Kaur et al. 2015). This reaction is considered to be the principal route for MG formation under normal

physiological conditions (Figure 1). Triose phosphates are unstable metabolites and show a high tendency to release α-carbonyl proton, producing an enediolate phosphate intermediate that has a relatively low energy barrier for the elimination of phosphate groups (Richard, 1984). Thus, MG is formed by the deprotonation followed by the spontaneous β-elimination of the phosphate groups of triose phosphates (Richard, 1993). The enzymatic formation of MG occurs through the triose phosphate isomerase (TPI) that hydrolyses G3P and DHAP, and removes phosphate to yield MG (Phillips and Thornalley, 1993). Methylglyoxal may also be formed by Amadori rearrangement during production of a Schiff base, which involves the reaction of the aldehyde groups of sugars with free amino acids or amino acids of proteins (Vistoli, 2013). Other possible sources for MG formation include the auto-oxidation of sugars, as well as the metabolism of acetone and amino acetone (Kalapos, 1999), although there is little evidence that these routes occur in plants.

1.5.3 MG detoxification in plants via glyoxalase and other metabolic pathways

Methylglyoxal detoxification involves the conversion of MG to less toxic molecules, thus limiting its detrimental effects. The major route for MG detoxification in plants is the glyoxalase pathway, whose presence was demonstrated in plants over 20 years ago (Norton, 1990; Maiti, 1997). In plant cells, the glyoxalase pathway is present in the cytosol and organelles, with high levels of glyoxalase enzyme activity found in chloroplasts and mitochondria (Yadav, 2008; Rabbani and Thornalley, 2012). There are two main enzymes associated with the glyoxalase pathway; glyoxalase I (Gly I; lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase II (Gly II; hydroxyacylglutathione hydrolase; EC 3.1.2.6). These enzymes function in tandem to transform MG, and other 2-oxoaldehydes, to 2-hydroxyacids with the release of glutathione (GSH) (Thornalley, 1990). The detoxification of MG involves two irreversible reactions catalyzed by Pyruvate, the major catabolic product of MG, can enter the tricarboxylic acid (TCA) cycle via acetyl CoA (Figure 2). The availability of cellular GSH is an important factor for MG detoxification via the glyoxalase system as the lack of GSH restricts hemithioacetal formation, resulting in MG accumulation (Hossain et al. 2012). Recently, a novel glyoxalase enzyme, named glyoxalase III (Gly III), was detected in plants, providing a shorter route for MG detoxification (Ghosh, 2016). Gly III contains a DJ-1/PfpI domain, and the presence of this domain has been used to confirm the existence of Gly III like proteins in various plant species. Conventional glyoxalases (Gly I and Gly II) detoxify MG by converting it

to D-lactate, with the help of GSH, but Gly III is able to irreversibly convert MG to D-lactate in a single step, without the need for GSH.

In addition to the glyoxalase system, several other pathways contribute to the detoxification of MG in plants. Other enzymes, including NADPH-dependent reductases, such as the aldoketo reductases and aldehyde/aldose reductase, involved in detoxifying reactive carbonyls (Yamauchi, 2010), can reduce MG to the corresponding alcohol (Simpson, 2009; Narawongsanont, 2012). Another pathway is the irreversible oxidation of reactive aldehydes, including MG, to their corresponding carboxylic acids, which is catalysed by aldehyde dehydrogenases (Kirch, 2005). However, the glyoxalase system is the most efficient MG detoxification system in plants under normal physiological conditions (Ghosh, 2016), and this pathway is very important for plants under stress (Singla-Pareek, 2006; Alvarez Viveros, 2013).

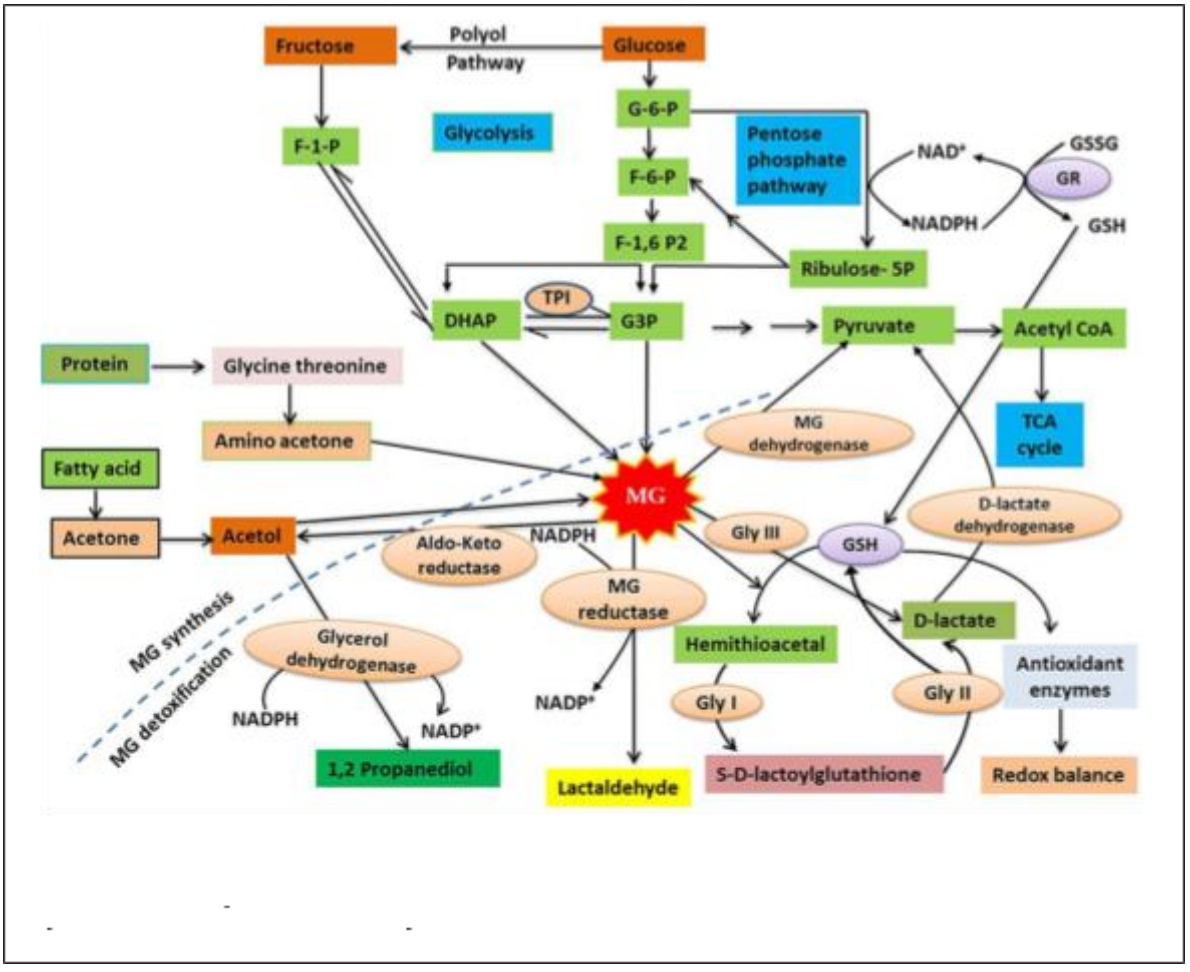


Figure 2: Diagrammatic representation of MG synthesis and detoxification in plants (figure adopted Hossain et al. 2011). Methylglyoxal is primarily produced as a by-product of carbohydrate metabolism with small amounts

produced during protein and lipid metabolism. Cytotoxic MG is efficiently degraded to from D-lactate, D-lactate dehydrogenase finally converts D-lactate into pyruvate which enters the TCA cycle.

1.5.4 Growth and development

In plant cells, MG accumulation has been shown to correlate with increased levels of intracellular oxidative stress, due to the enhanced reactive oxygen species (ROS) production (Maeta, 2005; Kalapos, 2008). Methylglyoxal accumulation may indirectly result in increased ROS production by decreasing available GSH levels and by impairing the function of antioxidant enzymes in plants under oxidative stress. In addition, MG can function as a Hill oxidant and catalyze the photoreduction of O_2 to superoxide (O_2^-) in photosystem I (PSI) (Saito, 2011). The production of O_2^- is deleterious as it can cause oxidative damage to cellular components. Methylglyoxal is an α,β-dicarbonyl compound that can act both as a genotoxic and a glycation agent (Rabbani and Thornalley, 2014). Methylglyoxal has two functional groups; a ketone group and an aldehyde group, the latter being more reactive than the former (Leoncini, 1979). The dicarbonyl group within MG can readily react with the amine groups of proteins and nucleic acids, including DNA and RNA. The accumulation of MG is often called dicarbonyl stress, which has been implicated as a cause of tissue damage and aging (Rabbani and Thornalley, 2014). Methylglyoxal reacts with the amino acid lysine, cysteine and arginine producing glycated proteins, often referred to as advanced glycation end products (advanced glycation ends) (Ahmed and Thornalley, 2007), which can cause inactivation of proteins and oxidative damage to key cellular components (Thornalley, 2006). AGEs and dicarbonyl compounds, including MG, often accumulate in plant leaves upon exposure to high light or elevated CO_2 concentrations (Qiu, 2008; Bechtold, 2009). Thus, it appears that the increase in sugar accumulation and changes in the metabolic flux of sugars, which occur at high CO_2 concentrations, promote the production of MG and other reactive carbonyls, resulting in the accumulation of AGEs. In summary, excessive MG accumulation in plant cells under stress can inhibit cell proliferations, and cause the inactivation and/or degradation of proteins, inactivation of antioxidant defences, leading to disruption of many cellular functions (Hoque, 2010; Hoque , 2012).

Indeed, MG showed toxicity to photosynthesis in the chloroplasts of spinach (*Spinacia oleracea* L.) (Mano, 2009), and the accumulation of MG in the mutant, which lacks the plastid isoform of TPI, exhibited greatly reduced growth and increased chlorosis (Chen and Thelen,

2010). Yadav (2005a) reported that accumulation of MG, as a result of salt stress, directly and adversely influenced plant developmental processes, such as seed germination and seedling growth, in tobacco plants. Similarly, Engqvist (2009) found that Arabidopsis plants grown on MS medium supplemented with MG (0.1 or 1 mM MG) exhibited a significant reduction in shoot and root growth. Later, the same group reported a dose dependent decrease in root and shoot growth of Arabidopsis, tomato and tobacco plants grown on MS medium containing 1 mM MG (Wienstroer, 2012). Furthermore, Hoque (2012b) examined the inhibitory effects of MG on growth and development in Arabidopsis and suggested that 1 mM MG is toxic enough to significantly inhibit seed germination and root elongation in seedlings. However, concentrations lower than 0.1 mM MG had no influence on seed germination, but did reduce the rate of root elongation. In addition, concentrations of 1 mM MG or higher resulted in seedling chlorosis within 4 days of treatment. Recently, Kaur (2015a) also reported that MG exposure caused a significant growth reduction in rice seedlings (*Oryza sativa* cv. IR4), with acute effects on root elongation in a concentration-dependent manner. The above findings highlight the growth inhibitory effects of MG on plants, and indicate that the MG levels causing toxic effects to plants vary depending on plant species, exposure time and perhaps age of plants.

1.6 Nitric oxide (NO)

Nitric oxide (NO) is a small gaseous radical with diverse signalling functions. In plants, NO was first found to play a crucial role in mediating defence reactions against bacterial pathogens and is now well known to influence numerous physiological processes throughout the entire plant life cycle (Gupta and Sinha, 2009). To name a few, NO is involved in germination, leaf expansion, lateral root development, flowering, stomatal closure, cell death and defence against biotic and abiotic stresses. Whereas descriptions of NO-mediated processes are accumulating, the plant signalling pathways governed by NO are still largely unknown. Three routes to yield NO have been described in plants: non-enzymatic conversion of nitrite to NO in the apoplast, nitrate reductase (NR) dependent NO formation and NO synthase (NOS)-like activity that is arginine dependent NO formation (Gupta and Sinha, 2009). In a nutshell: since the enzymatic source(s) of NO in plant stress responses remains elusive, unbiased genetic tools are still lacking for non-invasive manipulations of NO levels in plants. Despite numerous

studies using pharmacological tools demonstrated NOS-like activity in plants, the identity of the enzymes involved remains unknown (Gupta and Sinha, 2009).

Nitric oxide was recently reported to play an important role in immune response against pathogen attack in plants (Besson–Bard, 2015). Similar to ROS, NO triggers signal transduction pathways resulting into expression of stress related genes that directly or indirectly confer stress tolerance in plants (Chaki et.al, 2013; Ding, 2015). Additionally, the communication between ROS and NO signalling pathways, regulate responses that are related to the cell hypersensitive, cell death and senescence in plants (Chu et.al, 2013). That is why NO has been found to provoke both beneficial and harmful effects depending mainly in the localisation and concentration in the site of synthesis, generation pattern , interaction with the cells and other molecules and the balance between NO and ROS along with the antioxidant system (Foresi, 2015).

1.6.1 NO synthesis pathways

Endogenous NO synthesis is also the perception of plant during stress conditions. Besides being endogenous in origin in plants, it may also be perceived from surrounding environment or soil. In plant cells, NO is biosynthesized in parallel to the ROS accumulation in various organelles like mitochondria, chloroplasts, peroxisomes and cytoplasm. The endoplasmic reticulum and the apoplast have also been reported as source of NO synthesis (Dunner and Frohlich, 2011). Nitric oxide generation carried in oxidative and reductive pathways governed by either the presence or absence of enzymes (Fernie, 2011). The enzymatic reductive pathways includes nitrite-dependent reactions via Xanthine oxidoreductase (XOR), nitrate reductase (NR), Ni:NOR (nitrite dependent nitric oxide reductase) and mtNi (mitochondrial nitrite). While the oxidative route includes the use of $_{L}$ - Arginine (L-Arg)-dependent nitric oxide synthase like enzyme (NOSLE) activity, and polyamines or hydroxylamines reactions (Fernie, 2011). The presence of O_2 differentiates both nitrite- and L-Arg dependent NO synthesis from each other in condition that the absence of O_2 is optimal, whereas the presence of O_2 is obligatory, respectively (Dunner and Frohlich, 2011).

In chloroplast, the one pathway of NO synthesis is NR based nitrite reduction (NiR) where nitrite (NO_2^-) is converted into NO. The other pathway involved in NOSLE activity used L-Arg as substrate and NADPH as electron donor independent of Caþþ/Calmodulin (CaM) presence

(Crane, 2008). The presence of this reaction was determined based on conversion of L - Arginine to Citrulline and NO. In mitochondria, complex I, II and III, located on mitochondrial membrane are the sites where ETC dependant enzymatic nitrite reduction (mtNi:NOR) take place. In this reaction, NR uses nitrite as substrate and NADPH as e^- donor to produce NO. Recently, one enzyme was suggested to have NOS activity in mitochondrial matrix or intermembrane space and was named Arabidopsis thaliana Nitric Oxide Synthase 1 (AtNOS1) which was lately renamed Arabidopsis thaliana Nitric Oxide Associated protein 1 (AtNOA1) (Crane, 2008). However, the role of AtNOS1 in NO synthesis is still debatable as its authenticity was not proved during the cloning experiment conducted by Kwan, 2015. In peroxysomes, XOR, the ubiquitous molybdenum-containing enzyme act in two convertible forms, O form (Xanthine oxidase) and D form Xanthine dehydrogenase (XDH). XOR reduce NO_2^- to NO using NADPH as electron donor or Xanthine as the reducing substrate. NOSLE activity pathway requires NADPH, FMN (Flavin MonoNucleotide), FAD (Flavin Adenine Dinucleotide), Caþþ and CaM to catalyze the reaction for NO production. Root plasma membrane bound nitrate and nitrite: nitric oxide reductase (PM-NR/Ni:NOR) activity uses nitrite as substrate to generate NO and reduced cytochrome c (Cyto c) as e^-donar (Kwan et al, 2015).

Another pathway for NO formation is non-enzymatically carried out under acidic pH where NO_2^- gives rise NO and oxygen. In cytosol, NR enzyme localized in cytosol reduces substrate nitrate (NO_3^-) to NO_2^- in an NAD(P)H dependent manner (Durner and Frohlich, 2011). Nitrate reductase is also able to reduce NO_2^- to NO and O. Polyamine and hydroxylamine are the other forms of oxidative NO formation, which has recently been added in plant cells but the specific sources, sites and specific pathways take place inside the plant cell is uncertain and yet to be explored (Prommer, 2013). Although possible routes have been expected via NR-dependent or L-Arg metabolism and via intermediate process of nitrification, the molecular mechanism underlying is either still unknown or not fully characterized. Nitric oxide is also thought to be generated from other pathways where NO reacts with reduced GSH to produce S-nitrosoglutathione (GSNO), a donor and major reservoir of NO (Durner and Frohlich, 2011). Below is a schematic representation of major functions mediated or affected by NO and ROS.

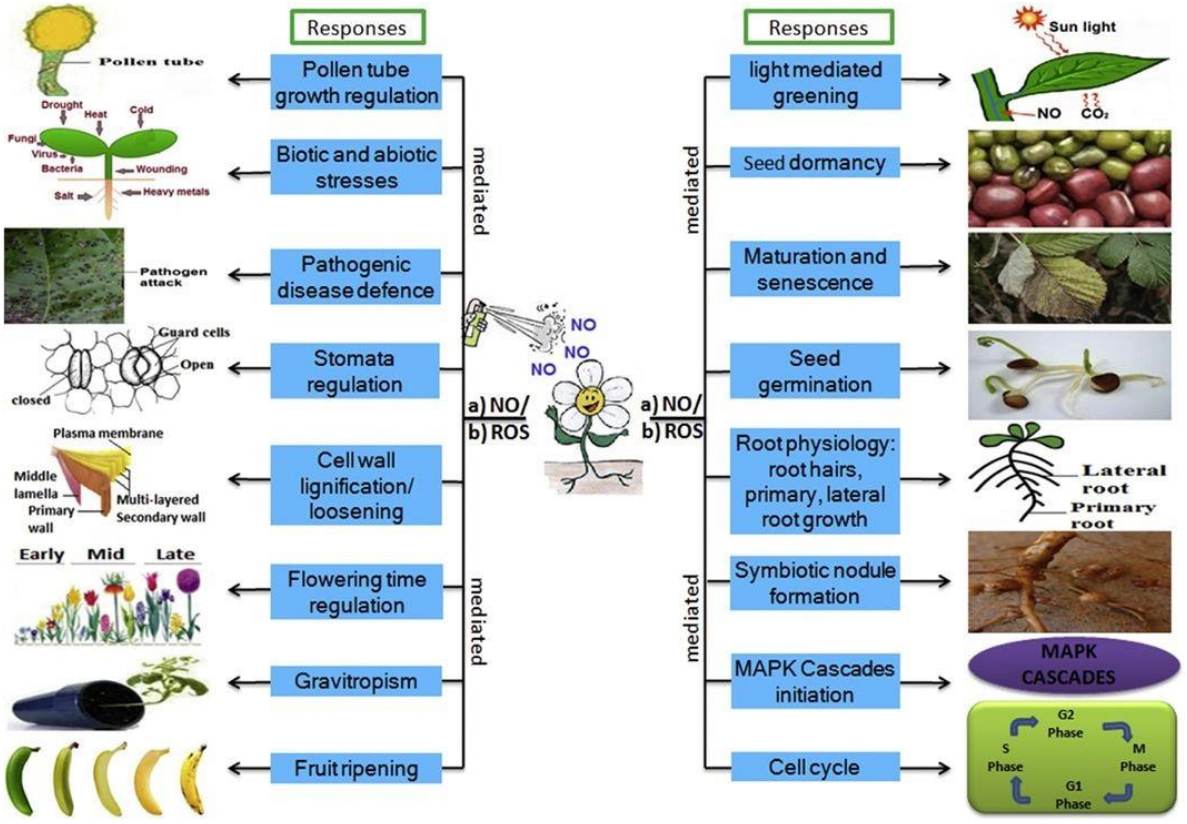


Figure 3: Schematic representation of major functions mediated or affected by NO and ROS recently, has been involved in the following activities for (a) NO in normal growth and developmental processes, including pollen-tube growth regulation, biotic and abiotic stresses, pathogenic defence , controlling stomatal closure, flowering timing, gravitropism or gravitropic bending, fruit ripening, light evoked greening, reduce seed dormancy, maturation and senescence, seed germination, root architecture: primary and lateral root growth, symbiotic relationship in legumes as well and maintaining redox balance, and (b) ROS in metabolic defence against biotic and abiotic stresses, stomatal actions, disease resistance, loosening of cell wall, photosynthesis and photorespiration, seed dormancy alleviation, senescence and fruit ripening, induction of seed germination, root physiological system (figure adopted from Durner and Frohlich, 2011).

1.6.2 The crosstalk between NO and ROS under metal stress

Cell protective property of NO against the metal oxidative stress is post-translational modification (PTM) that has been governed by the Fenton reaction, a process is called metal-nitrosylation. In this reaction, NO reacts and binds with iron/copper/zinc ion (transition metals) and hydroxyl radical (OH) to form metal-nitrosyl (M-NO) complex such as iron-nitrosyl (Fe-NO), copper-nitrosyl (Cu-NO) or zinc-nitrosyl (Zn-NO) complexes and HNO_2 (Ghasemi, 2014). Thus, NO can easily scavenge metal and help to avoid the formation of most powerful and dangerous ROS i.e., the OH radical by preventing Fenton reaction in the cell. In another way, nitric oxide reacts directly with superoxide radical (ROS precursor) to form short lived

and comparatively less toxic NO in place of long lived and highly cytotoxic H_2O_2 . The reason behind it may be that due to the presence of NO in this radical-radical fusion, it does not favour the dismutation of O_2 into H_2O_2 (Radi, 2013; Ghasemi, 2014).

Thirdly, NO can react with lipid radicals such as lipid alcoxy and lipid peroxyl (LO and LOO), and stop the propagation of radical mediated lipid peroxidation, directly. Heavy metal induced accumulation of NO was also found to be responsible for HMs toxicity. Heavy metals-dependent increase in NO concentrations depends upon the synthesis mechanisms, and varies according to the chemical characteristics of the metals exposed to plants (Chen, 2012). For example; lead (Pb) enhances NO levels by enhancing the cytosolic-nitrate reductase (cNR) activity, while Cd is related to Fe-induced deficiency (besson-bard et al. 2015), and mediate NO accumulation by stimulating the GSNOR activity (Airaki, 2012). To counteract the accumulation of ROS caused by heavy metal treatments, the plant cells possess versatile antioxidants defence mechanisms in which NO plays a central role in two different ways. The unpaired e of NO free radical (NO) can directly interact with ROS and modulate the expression of various genes including the redox signalling pathways to sustain/neutralize ROS levels. Another important role of NO was found as signalling molecule. Nitric oxide may stimulate ROS scavenging AOSs and/or mediate reduction of ROS to abate oxidative stress and regulates normal growth in plants (Begara-Morales, 2016). Thus, NO can be categorized as secondary antioxidants during HMs mediated toxicity (Saxena and Shekhawat, 2013).

It has been reported that an active interplay between NO and ROS signal exist and the occurrence of ONOO during $NO^{-}O_2$ (RNSROS) cross talk (Barroso and Corpas, 2013; Fan and Qiao, 2014). The cross talk of NO and ROS together takes place during two phases, first, during its biosynthesis and second, during regulation of gene expression or enzyme activity (Fan and Qiao, 2014). Nitric oxide at higher concentration reacts with ROS and produce imbalance RNS that leads to tyrosine-nitration [addition of a nitro group (NO_2) by ONOO to a Tyr side chain of protein] (Barosso 2013; Begara-Morales, 2013), S-nitrosylation [formation of SNO during NO attachment to a protein thiol (SH) of cysteine residue], and lipid nitration which results into nitrosative stress to plants. The adjustment and existence of NO and ROS in the plant cell is still creating an interest among the researchers to know how interplay between NO and ROS among antioxidants is used by plants to perceive, and respond to environmental cues (Barosso, 2013).

However, it has been believed that both have interplay in protective ways via cooperation or scavenging to one another, and also via competition of attaining toxic concentration that cannot be reverted back. As evident by Arasimowicz-Jelonek *et al*. (2011) the cross-talk of NO and O_2 is required for Cd-induced programmed cell death. Recently, it has been observed that the interaction and fine-tune balance of NO with ROS play an essential role in tolerance of heavy metals and acclimation to other abiotic stresses (Canatto, 2015). Contrary to this, the active interplay of NO and ROS signals when they overlap together in an imbalanced manner of metabolism, resulted in a combination of both nitrosative and oxidative stress in plants exposed to arsenic and selenium treatments (Airaki, 2012; Erdei, 2016), collectively known as secondary stress i.e., "nitrooxidative stress" (Saxena and Shekhawat. 2013) .

1.7 Conclusion

A large number of countries in the African continent are either still developing or under developed and one of the major challenges faced in these countries is poverty due to high population and low crop production. With that being said, phosphate deficiency poses a big threat, not only to food security (which is of utmost importance) but also to the agriculture, economy and nutritional quality of the little food that is produced. Common bean is known to be a stable food all around the world because it is direct protein consumption, affordable compared to animal protein and acts as restorive agent for fertile soil. It is thus of importance that to come up with ways to increase the crop production and that is what this project is aimed at, perusing MG signalling a possible regulator of phosphate deficiency. Methylglyoxal is localised in between metabolic pathways which results in regulatory and cytotoxic effects on the plants cell and as a result of the metabolic pathways, MG production is not controlled but the catabolism of MG is strongly regulated. The small size of MG makes a big impact and it is involved within the cells. On the other hand degrading catalyst are always readily available in all the cell parts illustrating that MG is very important as a signalling mechanism but should be produced in low amounts just to start signalling when cells are under stress and not too much that will cause harm to the cells. With the observed effect of MG from previous studies, it is clear that there is more to be learnt and more research should be done on this product of glycolysis

1.8 Justification

Phosphate deficiency is a drawback in crop production especially in common beans that are very much in demand and so it is very vital that we do research to find ways to modulate phosphate deficiency stress in plants and that is the rationale of the project. Food safety is the main reason for this project, to ensure that sufficient food is produced and not just to be consumed now but food with a long shelf life for the future. Literature has shown MG to be a signalling molecule at low concentrations and so it could be used to help regulate the effects of P stress at an early stage. This point then bring an advantage also in the agricultural sector as the farmers will produce more food per annum than usual. Finally, it will bring about economic benefits because common bean is vital source of protein which is affordable for all people and this will ensure food safety for everyone.

Chapter 2

Materials and method

2.1 Preparation of nutrient solution

A 10 X nutrient solution was first prepared by adding 0.005 mM CoCl, 50 mM K_2SO_4, 200 mM NH_2NO_3, 0.1 mM $NaMoO_4$, 800 mM KH_2PO_4, 0.02 mM $CuSO_4$, 50 mM $MgSO_4$, 3 mM H_3BO_4, 100 Mm $CaCl_2$, 5 mM Fe-EDTA, 1 M KNO_3, 0.07 mM $ZnSO_4$ and 1 mM $MnSO_4$ and made to a final volume of 2 L using deionised water. Just before treatment, the 10 X solution was diluted down to a 5 X. Four treatments were prepared from the 5 X dilution namely; 0.8 mM phosphate, 0.8 mM phosphate 6 µM methylglyoxal, 0.02 mM phosphate, 0.02 mM phosphate and 6 µM methylglyoxal.

2.2 *P. vulgaris* growth parameters

P. vulgaris seeds were germinated in pots containing 500 g grade of sand. The soil makeup was 2:1 ratio of fine sand (0.6 mm grit) and rough sand (1.2 mm grit). The plants were treated three times a week with 200 ml of the nutrient solution that were mentioned above for one month. After a month, the old trifoliate and the roots were harvested and stored at -80^{o}C until needed to assays.

2.3 Dry weight determination in *P. vulgaris* roots and leaves subjected to MG treatment

Sterilised envelopes were prepared in replicates of six per treatments and holes were made on the enveloped to facilitate the drying process. The envelopes contained old trifoliate and the roots from each treatment in replicates of six and then the fresh weight were taken. They were then stored at 50^{o}C for 48 hours after which the dry weights were taken and compared to the fresh weights.

2.4 Cell viability [Evans blue uptake] assay

A modified method of Sanevas (2007) was followed for the cell death assay. Into an Eppendorf tube, 1 ml of Evans blue solution [0.25% (w/v)] was added and for each plant treatment, a 1 cm^3 square from the leaves and 2 cm in length root were cut and placed in the separate tubes with Evans blue solution. The samples were paced at room temperature for 1 hour after which the Evans blue solution was washed off the samples using water. The samples were then placed in water and incubated overnight at room temperature. The following day the water was decanted and into the plant sample, 1 ml SDS [1% (w/v)] was aliquoted and the samples

were crushed using a pestle. The samples were incubated at heating block (65^{o}C) for one hour and pelleted by centrifugation until there was a pellet. The supernatant was used as the final sample and was loaded onto a 96-well microtitre plate and read on a spectrophotometer at wavelength 600 nm.

2.5 Total protein extraction

Frozen plant material for all the treatments of mass 100 mg was added in Eppendorf tubes (in replicates of three). In the first tube containing plant material 0.5 ml of protein extraction buffer containing 1 mM EDTA, 0.004 M phosphate buffer and 5% PvP (w/v) was transferred and mixed by vortexing. To pellet the plant material, the sample was centrifuged for 5 minutes at 12 000 x g and the resulting supernatant was transferred to the second tube containing 100 mg of plant material. The previous step was done over again for the second and third tube containing fresh material. The resulting supernatant from the third tube was transferred to a fresh tube, quantified using the Bradford assay and stored at -20 ^{o}C.

2.6 Protein quantification

Using the Bradford assay the proteins were quantified using 1 x Quick start Bradford dye reagent. With the use of distilled water the total proteins were diluted to 1:10. Bovine serum albumin (BSA) protein standard ranging from 0 mg.ml^{-1} to 10 mg.ml^{-1} (done in triplicates) were prepared on a 96-well microtitre plate. In addition 10 µl of the protein that was extracted of each treatment was loaded into a 96-well microtitre plate in triplicates and in the standards and sample, the Bradford reagent was added to a final volume of 200 µl. The plate with the sample and standards were incubated on a shaker for 5 minutes before it was read using a spectrophotometer at a wavelength of 595 nm.

2.7 Superoxide [O_2^-] assay

This assay was adapted from the one described by Russo (2008) to determine the superoxide accumulation in *P. vulgaris* old trifoliate and roots. Into 2 ml Eppendorf tubes with 10 mM H_2O_2 (Zn-/Cu and Mn-SOD inhibition), 10 mM potassium cyanide (Cu/Zn-SODs inhibition), 2% SDS (Cu/Zn-SODs and Fe- inhibition) and 80 µM NBT were prepared to a final volume of 800 µl with the use of potassium phosphate buffer (50 mM at pH 7.0). The solution was used to incubate 1 cm^3 leaf blocks and 2 cm length roots (separate tubes) for 20 minutes at room temperature and the samples were crushed with the use of a pestle. In order to pellet the leaf

material, the samples we centrifuged for 5 minutes at 13 000 x g and the obtained supernatant served as the final sample which was loaded in 96-well microtitre plate in triplicated and read the wavelength at 600nm on a spectrophotometer. To determine the superoxide content 12.8 mM^{-1} .cm^{-1} was used as the coefficient. The change in colour was observed indicating variation in O_2^- levels depending on the intensity of the colour.

2.8 Hydrogen peroxide [H_2O_2] assay

A changed technique for Velikova (2002) was taken after to determine the H_2O_2 content in the plant material. The H_2 O 2 standards ranged from 0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM and 25000 nM were set up by using distilled water to dilute H_2 0 2 . The trichloroacetic acid (TCA) extraction which was prepared on frozen material (section 2.5) was used as the samples for H_2O_2 examination. Trichloroacetic acid (50 µl was aliquoted in a 96-well microtitre plate and in addition to each sample, potassium iodide (250 mM) and dipotassium hydrogen phosphate (1.25 mM) were aliquoted. After the preparation, the plate was at room temperature for 20 minutes and read using a spectrophotometer at 390 nm.

2.9 Hydroxyl ion radical [•OH] assay

The method of Halliwell (2006) was used to determine hydroxyl ion concentrations. Into a clean Eppendorf tube, 50 mg of plant frozen material was added, 1 ml of a phosphate buffer (10 mM at pH 7.4) containing 15 mM 2-Deoxy-D-Ribose was added and the solution was mixed using a vortex. The samples were incubated for 2 hours at 37 ^oC and from the samples; 0.7 ml was transferred into a reaction containing 3 ml TBA at 0.5 % (w/v) (which was made up in 1 ml glacial acetic acid and 2 ml of a 5 mM sodium hydroxyl and briefly homogenised with a vortex. The samples were then heated in a heating block at 100 ^oC for 30 minutes and cooled for 5 minutes on ice. The samples were centrifuged to pellet at 10 000 x g for 5 minutes and the obtained supernatant was read using a spectrophotometer at wavelengths 532 nm and 600 nm. The •OH content was determined using 155 mM^{-1} .cm^{-1} as the extinction coefficient.

2.10 Methylglyoxal [MG] assay

A scope of standards (0 mM, 6.5 mM, 45.5 mM, 65 mM and 78 mM) were set up by diluting a suitable volume of methylglyoxal in distilled water. The standards were aliquoted in triplicate. To prepare the samples, 250 mg frozen plant material was aliquoted into clean Eppendorf

tube, 5 volumes of phosphoric acid was added and the was mixed using a vortex for 2 minutes. Samples were incubated for 15 minutes on ice and centrifuged for 10 minutes at 10 000 x g to pellet the plant material. The obtained supernatant was added to a new tube, to the supernatant activated charcoal (10 $mg.ml^{-1}$) followed by 15 minutes incubation at room temperature. The samples were centrifuged at 10 000 x g for 10 minutes and the resulting supernatant was transferred into a fresh Eppendorf tube to which 400 µl of saturation potassium hydroxide was aliquoted to each tube. The resulting samples was placed at room temperature for 15 minutes, after neutralization the sample was centrifuged at 10 000 x g for 10 minutes and the resulting supernatant served as a sample. The samples (130 µl) were loaded on the 96-well microtitre plate and the standards, phosphoric acid (0.5 M), and diaminobenzene (1.8 mM) were added to make up the samples to a final volume of 200 µl. Before taking the absorbance at 405 nm, the plate was incubated on the shaker for 40 minutes at room temperature.

2.11 Malondialdehyde [MDA] assay

A method modified by Zhang *et al*. (2007) was followed for the lipid peroxidation assay. Into an Eppendorf tubes 100mg of leaf and root material were added and to the material 5 volumes [6% (w/v) of Trichloric acid (TCA) was added. The tubes were vortexed in order to mix properly and the material was pelleted by centrifugation the sample for 10 minutes at 1000 x g. The supernatant (200 µl) was decantanted into a clean Eppendorf tube and 300 µl of thiobarbituric acid (TBA) was added and vortexed to mix. The samples were sealed with parafilm and incubated for 20 minutes at 90 ^{o}C on a heating block. After incubation, the samples were placed on ice to cool down and centrifuged for 5 minutes at 13 000 x g. On a 96-well microtitre plate, 200 µl of each sample was loaded and the absorbance value was read at 532 nm and 600nm. The amount of MDA in the sample was obtained using 155 $mM^{-1}.cm^{-1}$ as the extinction coefficient.

2.12 Superoxide dismutase [SOD] activity

Protein were extracted from the plant frozen ground *P. vulgaris* leaves and roots at - 80^{o}C as in section 2.5 and quantified as in section 2.6 and from the protein extract, 10 µl of the protein

extract was loaded in each well to a successive 1 $mg.ml^{-1}$ dilution. Each well was made up to 200 µl with 0.1 mM riboflavin, 10 mM methionine, 20 mM phosphate buffer, 0.1 mM nitrozoliumblue chloride (NBT), 0.1 mM EDTA and distilled water. The well plate was then incubated for 20 minutes at room temperature on a light box before the absorbance values were taken at 560nm. The SOD activity was measured by 50% of how much superoxide is needed to convert NBT to formazan.

2.13 Ascorbate peroxidase [APX] activity

A method by Singh (2007) was used to determine ascorbate peroxidase activity. The protein extract from section 2.5 that was quantified was transferred into Eppendorf tubes and 2 M ascorbate was added and the tube was incubated for 5 minutes. On 96-well microtitre plate, 10 µl of the protein samples were added in triplicates. In addition the sample 71.4 mM K_2HPO_4 and 0.36 mM ascorbate were loaded in each well and just before taking the absorbance value at 290 nm, 0.714 mM H_2O_2 was aliquoted into each well to initiate the reaction (the solution in each well was made up to 200 µl with distilled water). Ascorbate peroxidase activity in the plants was calculated using 2.8 $mM.cm^{-1}$ as the extinction coefficient.

2.14 Catalase activity

This assay was adapted from the methods described by Aebi (1984) to determine catalase (CAT) activity in *P. vulgaris*. A decrease in the CAT absorbance and H_2O_2 dissociation is the main principle of this assay. In a 0.5 ml tube, a 0.5 ml reaction was prepared by adding 100 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 1 mM H_2O_2 and 20 µl protein extract in a measuring tube and the absorbance values were taken at 240 nm. The catalase activity was determined using the extinction coefficient 39.4 $mM^{-1}.cm^{-1}$

2.14 Elemental analysis using ICP-OES

The elemental profile of the different treatments was determined using a modified method for from Zarcinas and colleagues (1987), for the acid digestion of the plant materials. Frozen ground plant material (200 mg) was transferred into 2 mL Eppendorf tubes. 65 % nitric acid was added to the tubes. The tubes were then wrapped using parafilm, this was done to prevent the cap from opening during the incubation period. Then incubation proceeded for 3 hours on a heating block at 90 ˚C this was done to ensure proper digestion of the plant

material. 1 mL of the digested sample and 9 mL of 2 % Nitric acid was added to the respective syringes for each sample digested. The dilution was then filtered into a 15 mL greiner tube. These samples were then subjected to ICP analysis (ICP-OES).

2.15 Spectrophotometric quantification of nitric oxide content

NO content was measured by slight modification of the haemoglobin-based assay (Murphy and Noack 1994). Briefly, the extracts were obtained from leaf tissue (200 mg) by grinding the tissue into fine powder in liquid nitrogen and homogenizing the tissue with 1 ml of ice-cold 5% trichloroacetic acid (TCA). The resulting homogenates were centrifuged at 13 200 X g for 30 min and incubated with 100 Units of catalase and 100 Units of superoxide dismutase for 10 min, followed by addition of oxyhaemoglobin to a final concentration of 10 µM. The mixture was incubated for 2 min, followed by spectrophotometric measurement of NO content by following the conversion of oxyhaemoglobin to methaemoglobin at 401 and 421 nm.

Chapter 3

Effect of exogenous MG treatment on leaf and root morphology and cell damage

3.1 Effect of exogenous MG treatment on leaf and root morphology and cell damage

3.1.1 Exogenous application of MG treatment at low concentrations resulted in improved *P.vulgaris* second youngest and second oldest trifoliate morphology when there is insufficient phosphate

Several indicators such as leaf structure and biomass are utilised to examine the coping ability of plants under various stresses (Acosta-Motos, 2016; Gupta & Sinha, 2009). When plants are under stress, a disturbance in regular metabolism activities of the plant occurs thus resulting in a decrease in biomass and alterations in the physical structure. When plants are exposed to stressful conditions the plants collect phosphate in rich soil patches located in the soil layer on top and this leads to changes in the structure of the roots (Lopez-Aredondo *et al.* 2014). *A. thaliana* exposed to phosphate deficiency produced shorter essential roots and expanded the length and lateral roots (Lopez-Aredondo *et al.* 2014). Looking at the *P. vulgaris* second oldest trifoliate images, the following trend was observed (Figure 3.1). The control (0.8 mM P) trifoliate leaves were bigger in size and had more pigment compared to the 0.02 mM P trifoliate. There was an increase in leaf size in the 0.08 mM + MG and 0.02 mM + MG trifoliate compared to the control. However, there was more green pigmentation in the 0.02 mM + MG compared to the 0.08 mM + MG.

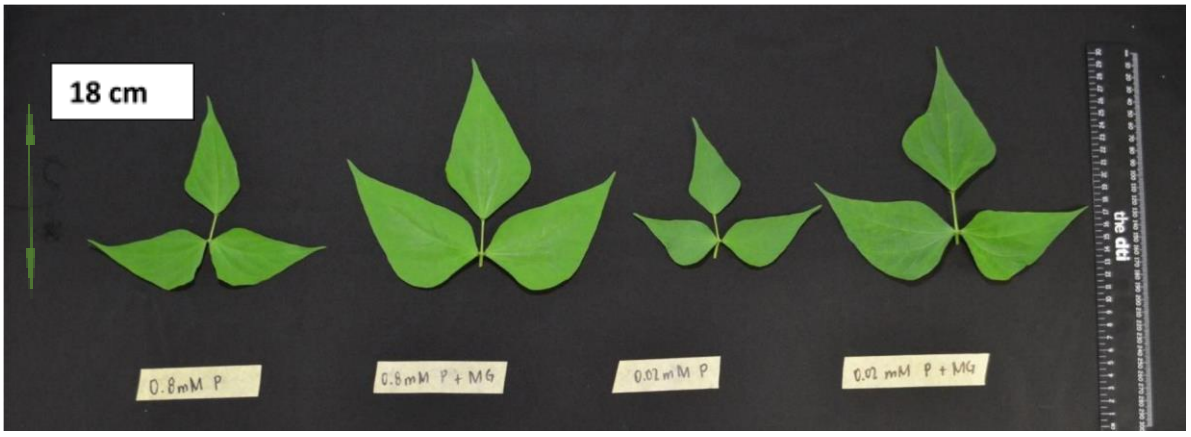


Figure 3.1: The effect of exogenous MG in *P. vulgaris* second youngest trifoliate morphology in response to phosphate deficiency stress. The first trifoliate on the left represents the control treated with 0.8 mM P only. The last three trifoliate are the experimental where some of the leaves were treated with 0.8 mM P + MG, 0.02 mM P and 0.02 mM + MG respectively.

Looking at the *P. vulgaris* second youngest trifoliate images, the following trend was observed (Figure 3.2). The control (0.8 mM P) trifoliate leaves were slightly smaller in size and had less pigment compared to the 0.02 mM P trifoliate. There was an increase in leaf size in the 0.08 mM + MG and 0.02 mM + MG trifoliate compared to the control. However, there was more green pigmentation in the 0.02 mM + MG compared to the 0.08 mM + MG.

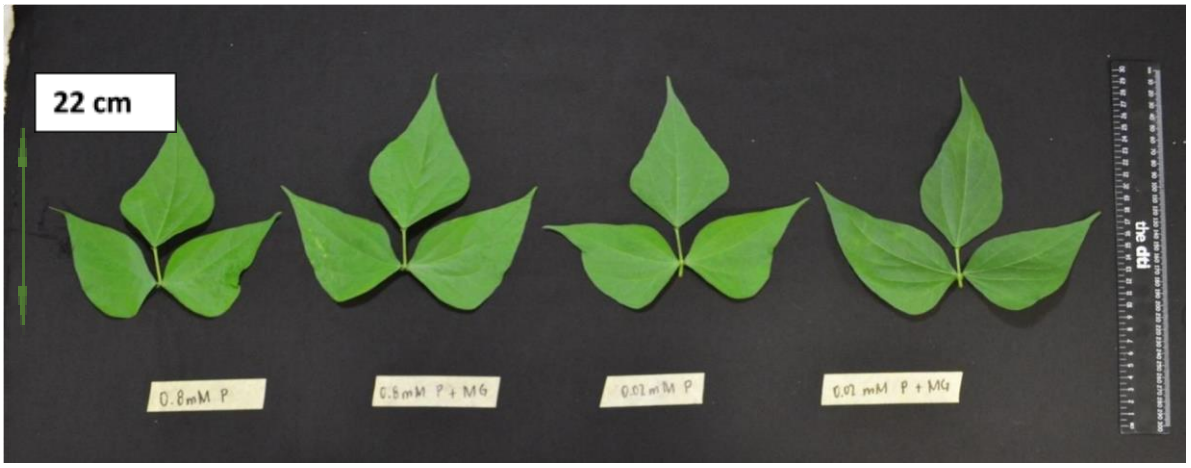


Figure 3.2: The effect of exogenous MG in *P. vulgaris* second oldest trifoliate morphology in response to phosphate deficiency stress. The trifoliate on the left represents the control treated with 0.8 mM P only. The last three trifoliate are the experimental where some of the leaves were treated with 0.8 mM P + MG, 0.02 mM P and 0.02 mM + MG respectively.

Looking at the *P. vulgaris* roots images, the following trend was observed (Figure 3.3). The control (0.8 mM P) had the longest root tap compared to all the other treatments. The experimental treatments (0.02 mM P and 0.02 mM + MG) had a bushier root system compared to the control and the 0.08 mM + MG roots.

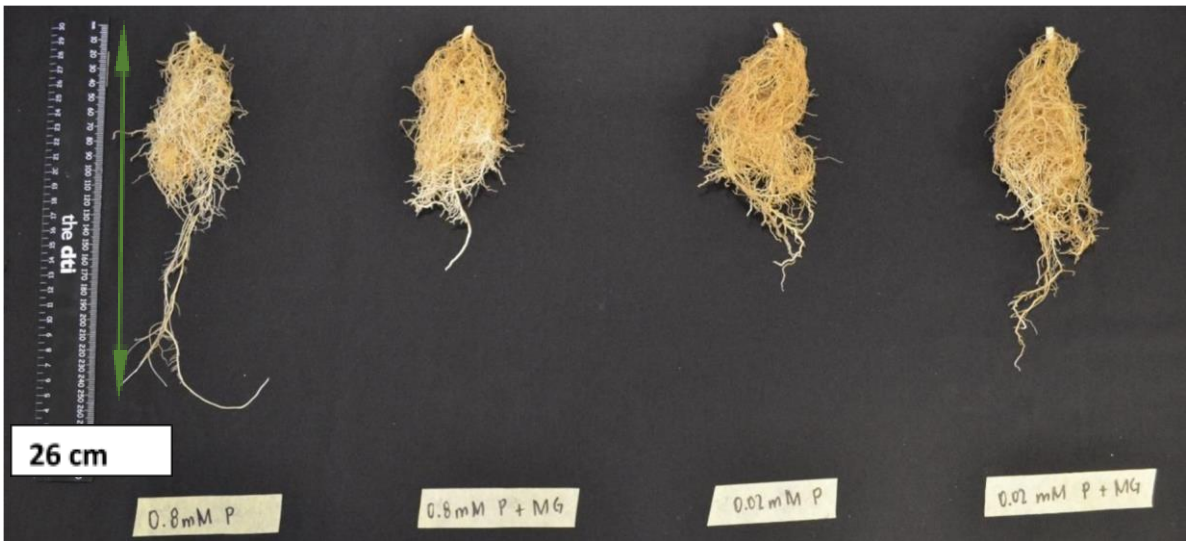


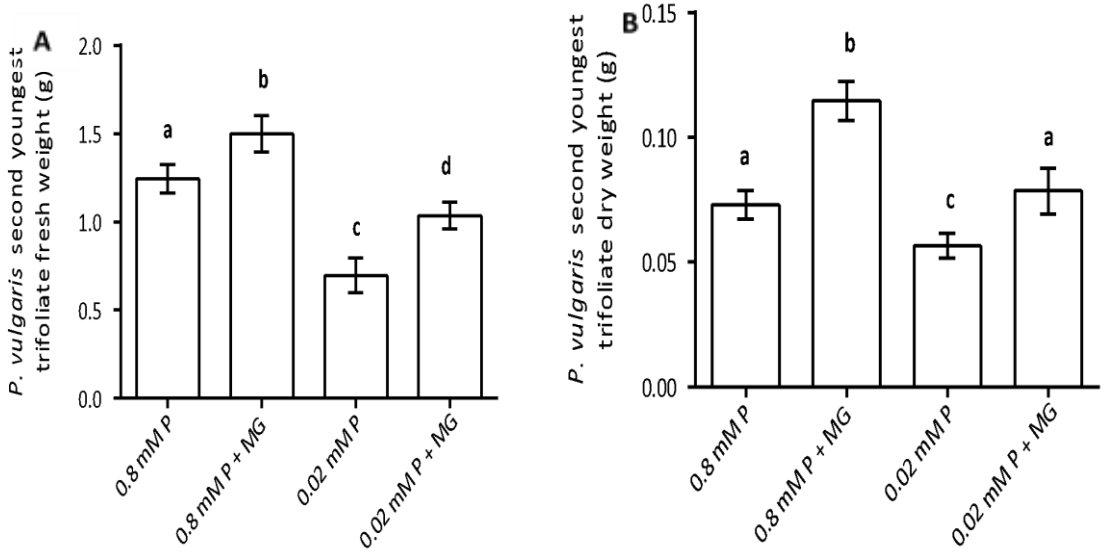
Figure 3.3: The effect of exogenous MG in *P. vulgaris* root morphology in response to phosphate deficiency stress. The first root on the left represents the control treated with 0.8 mM P only. The last three roots are the experimental where some of the leaves were treated with 0.8 mM P + MG, 0.02 mM P and 0.02 mM + MG respectively.

3.1.2 Exogenous application of MG treatment result in increased second youngest and oldest trifoliate fresh and dry weight in *P. vulgaris* when there is insufficient P

Scientists use many indicators to assess how well plants cope under different conditions. These indicators include the associated biomass, the leaf area and morphology as well as length (Acosta-Motos, 2016; Gupta and Sinha, 2009).The disruption in normal metabolism may lead to reduction in plant biomass as well as changes in physiological characteristics such as leaf formation.

Looking at the *P. vulgaris* second youngest trifoliate fresh weight, the following trend was observed (Figure 3.4). When the 0.8 Mm P + MG , 0.02 mM P and 0.02 mM P + MG fresh weight samples were compared to the control **(figure 3.4 A)**, there was an 86 % increase, 55 % decrease and 83 % decrease, respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 67 % increase. A similar trend was observed in the dry weight of the second youngest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG dry weight samples were compared to the control **(figure 3.4 B)**, there was a 64 % increase, 76 % increase and 93 % increase, respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 71 % increase. The second oldest *P. vulgaris* trifoliate fresh weight and dry weights were also compared. When the 0.8

Mm P + MG , 0.02 mM P and 0.02 mM P + MG fresh weight samples were compared to the control **(figure 3.4 C)**, there was a 71 % increase, 57 % decrease and 90 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 64 % increase. A similar trend was observed in the dry weight of the second youngest trifoliate. When the 0.8 mM P + MG , 0.02 mM P and 0.02 mM P + MG dry weight samples were compared to the control **(figure 3.4 D)**, there was a 73 % increase, 77 % decrease and 91 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 64 % increase.



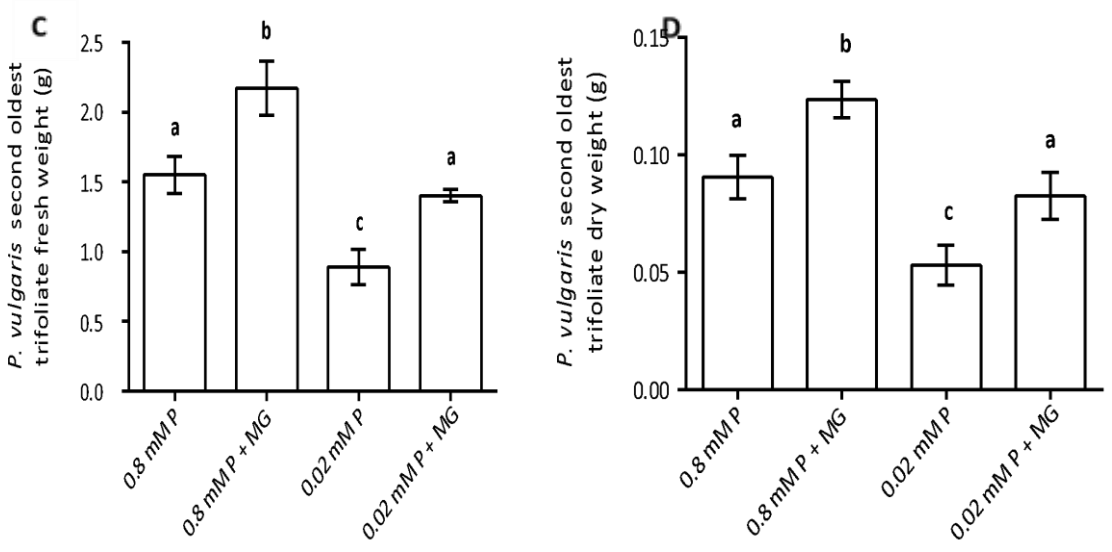


Figure 3.4: The effect of MG treatment in *P. vulgaris* fresh and dry weight in response to phosphate deficiency. *P. vulgaris* second youngest trifoliate fresh and dry weight **(A and B respectively)** and second oldest trifoliate **(C and D respectively)** were exposed to 8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG, combination respectively and after 4 weeks of growing the dry weight on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=4).

3.1.3 Exogenous application of MG treatment reduced cell death *P. vulgaris* second youngest and oldest trifoliate when there is insufficient phosphate

Due to the relationship between cell death and stress, cell death can be used as an indicator for the damage in plants. Cell death can be analysed by using an Evans blue assay, which works on the principle that the Evans blue reagent will only enter and be retained in dead cells (Baker and Orlandi, 1995).

Looking at the *P. vulgaris* second youngest trifoliate cell death, the following trend was observed. When the 0.8 Mm P + MG , 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 3.5 A)**, there was an 82 % decrease, 67 % increase and 75 % increase respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 85 % decrease. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG , 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 3.5 B)**, there was a 74 % decrease, 71 % increase and 93 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 76 % increase.

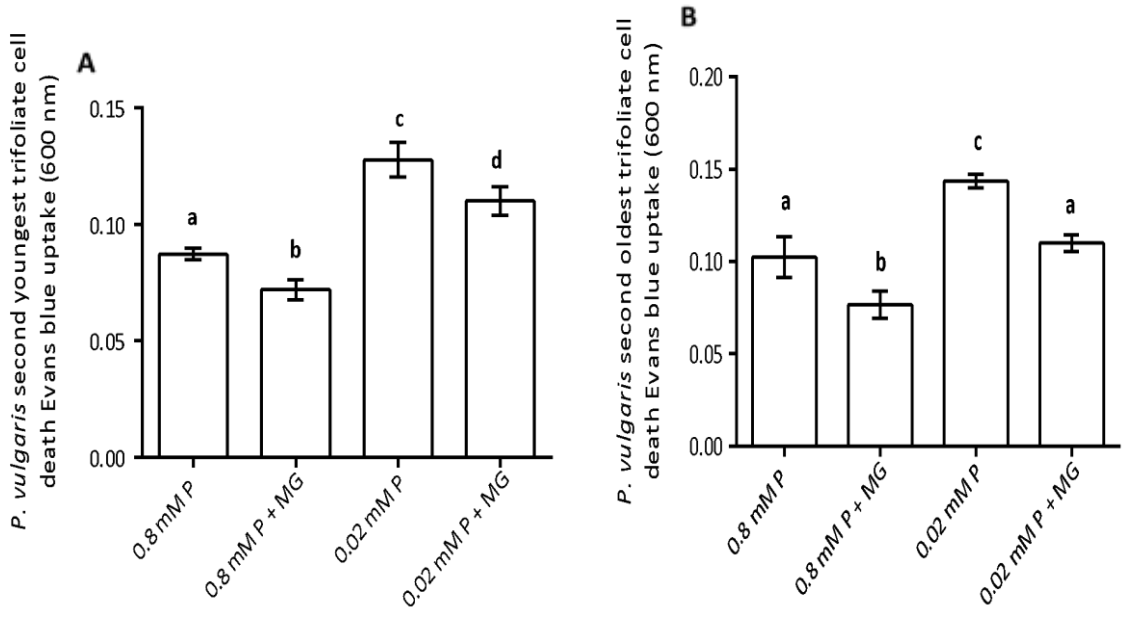


Figure 3.5: The effect of MG treatment in *P. vulgaris* cell viability in response to phosphate deficiency. *P. vulgaris* second youngest trifoliate **(A)** and second oldest trifoliate **(B)** were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing the level of cell death on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

Discussion

Due to the fact that phosphate deficiency is known to have harmful effect on the plant resulting in impaired growth and oxidative stress, the intention of this project was to observe the extent of damage the plants health caused by phosphate deficiency and how the effects can be modulated when MG is administered at low concentrations and the coping mechanisms the plant uses. Looking at the morphological characteristics observed in *P. vulgaris* second youngest and second oldest trifoliate (figure 3.1 and figure 3.2), when comparing the 0.8 mM P + MG to the control (0.8 mM P), the trifoliate treated with the MG combination shows a slightly greener pigment and the leaves increased in size. However more chlorosis is observed in the 0.02 mM P trifoliate but the greener pigment appears in the 0.02 mM + MG combination and the leaves are bigger in size. Then we looked and compared the roots morphology for all the four treatments (figure 3.3). The control roots had the longest tap root and quite a few short lateral root, however, the 0.08 mM P + MG roots were more bushy meaning there was more lateral roots than then control. The root tap for the MG treated root was damaged during harvest and so we could not compare it to that of the control. The 0.02 mM P root had a shorter tap root and it also had fewer and shorter lateral roots compared to the control. However, the MG was added to this experimental treatment, the roots appeared to be more bushy meaning that there was an increase in the number and length of lateral roots compared to the 0.02 mM P treated roots. As mentioned above in the literature review, plants exposed to P stress make use of phosphate patches in the soil as to make up for the unavailable P, and this results to an alteration in the root morphology (Lopez-Aredondo *et al*. 2014). Studies done show that under phosphate stress conditions in *Arabidopsis thaliana*, the number of lateral root and hairs is increased and shorter essential roots are formed (Lopez-Aredondo *et al*. 2014). Additionally *A. thaliana* produced shoots and leaves that were reduced in size, and this resulted in an increase shoot to root to weight ratios. The same trends were observed in our study and the reasoning could be that P was not supplied to the plants and no P was in the soil because we used sand thus eliminating any P patched in the soil for the roots to reach, the morphology of the plants was altered. As a result of this smaller leaves, shorter and fewer roots are observed. However, it seems that adding low doses

of MG counteracts this effect possibly by inhibiting any antioxidant production that will scavenge and kill the plants. Another theory could be the in the absence of P, MG takes the role of finding a way into the plants metabolic activities or up regulating certain proteins that will ensure the plants survival.

An investigation that was carried out by Lopez-Aredondo *et al.* (2014) illustrated that when *A. thaliana* was subjected to P deficiency, smaller and shorter leaves were produced due to an increase in root to shoot weight proportion. A study done by Du Qi (2017) showed that under potassium deficiency among maize (*Zea mays* L) inbred lines, there was a significant decrease in leaf thickness. They also measured the chlorophyll content and found that at the tasselling and flowering stages there was a significant decrease in the chlorophyll content. On the other hand, the positive effect of MG was reported by Bless *et al*.(2017) where it was found that in *Brassica* seedlings, MG treatment at low concentrations improved seed germination and seedling growth under zirconium stress. Although the papers mentioned above were about different types of plants and stresses, they are still in correlation with my hypothesis that under low MG treatments in a plants under stress, plant morphology is improved

When a plant is under biotic or abiotic stress, a lot of damage may occur in the plant itself, such as delayed growth, slower germination and cell death. When plants undergo stress they produce ROS and they subsequently alter their metabolic activity as protective mechanisms (Sharma, 2016). The overproduction and accumulation of ROS leads to oxidative damage of DNA, lipids and proteins ultimately resulting in cell death (Sharma, 2016; Maruta et al. 2012).

Looking at the *P. vulgaris* second youngest biomass (figure 3.4 A and B), the 0.02 mM P decreased when compared to the control, whereas the 0.8 mM P + MG combination and 0.02 mM combination increased; respectively. The *P. vulgaris* old trifoliate (figure 3.4 C and D) biomass also exhibited the same trend at the roots with a decrease in 0.02 mM P compared to the control and increase in the 0.8 Mm P + MG. It has been reported under P deficiency stress, an increase in cell death is prone to be observed due to ROS molecules accumulating at high levels and destroying the cells (Sharma, 2016; Maruta et al. 2012). A decrease in biomass was observed at 0.02 mM P *P. vulgaris* second youngest and second oldest trifoliate (figure 3.4) in comparison to the 0.8 mM P control; however 0.02 mM + MG combination showed a decrease in cell death compared to the 0.02 mM P. Then we proceeded to look a cell death in *P. vulgaris* second youngest and second oldest trifoliate (figure 3.5) and the

trend was similar to of biomass. A decrease in cell death was observed in both the second youngest and oldest trifoliate when treated with MG when compared to both the control and the experimental treatments without MG. According to this trend, it is evident that the addition of low MG concentration somehow increases plant biomass and reduces cell death in *P. vulgaris* plants under P deficiency. This decrease in biomass observed in plants under P deficiency may be the result of cell damage and the effect ROS production has on the plants such as slow growth and slow germination. The morphological alterations on the plant will also have a great effect on the reduce biomass observed under P deficiency. However the addition of low MG doses increases the biomass of the plants, the reason for this could be that MG activates the production of antioxidants and inhibits that of ROS thus reducing cell death and reducing the effect on the plants morphology thus resulting in improved biomass. Our results are in agreement with that observed by Gokul (2018) which showed metal stress (vanadium at concentration over 240 µM) decreased the biomass of leaves and roots, therefore concluded that biomass of Agamax plant is determined by the level of metal stress.

Chapter 4

Effect of exogenous MG treatment on reactive oxygen species production, antioxidant activity and nutrient profile in *P. vulgaris* leaves

4.1.1 Exogenous MG treatment at low concentration reduced internal methylglyoxal content in *P. vulgaris* second youngest and second oldest trifoliate when there is insuss

Methylglyoxal is a reactive molecule that is known to be toxic in plants when it accumulates at high levels; however it is also known to act as a signalling molecule at low concentrations between 30 – 75 µM (Yadav*,* 2005). Looking at the *P. vulgaris* second youngest trifoliate internal MG content, the following trend was observed. When the 0.8 mM P + MG , 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(**f**igure 4.1A)**, there was a 91 % increase, 82 % increase and 99 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 83 % decrease. A similar trend was observed in the second youngest trifoliate. When the 0.8 Mm P + MG , 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.1B)**, there was an 86 % increase, 76 % increase and 91 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 82 % decrease.

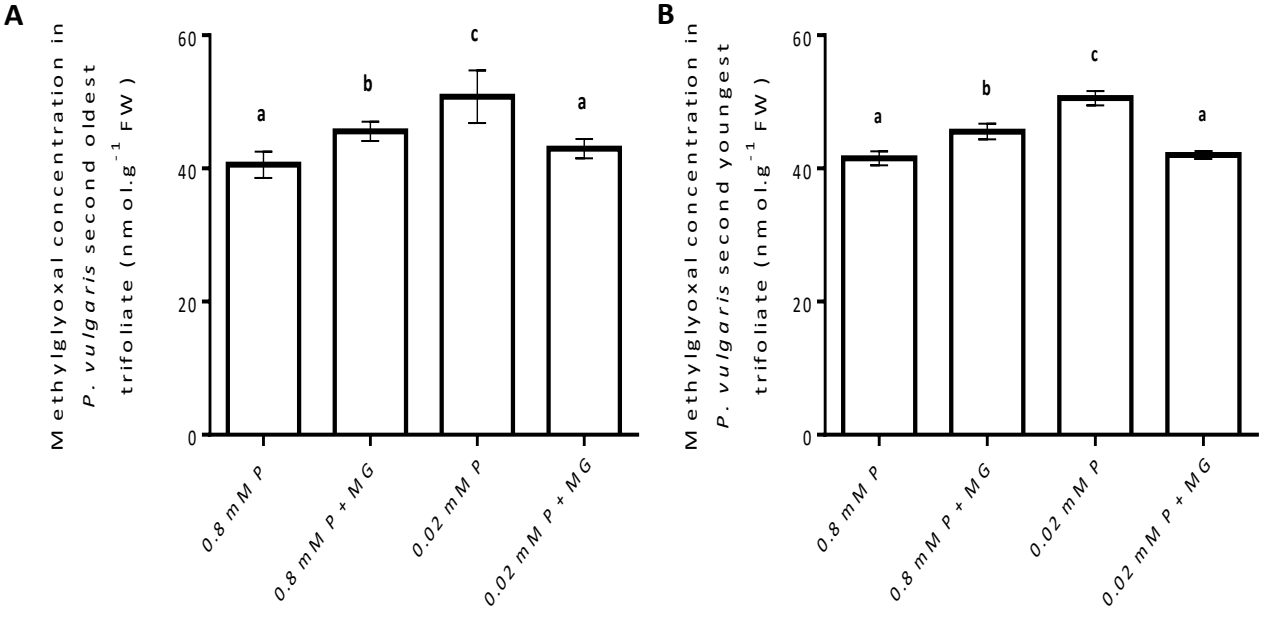


Figure 4.1: The effect of MG treatment in *P. vulgaris* methylglyoxal content in response to phosphate deficiency. *P. vulgaris* second youngest trifoliate **(A)** and second oldest trifoliate **(B)** were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing methylglyoxal content on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.1.2 Exogenous MG treatment at low concentration reduced superoxide content in *P. vulgaris* second youngest and second oldest trifoliate under P deficiency

Literature shows that when ROS is available at low concentrations, it has impacts that are beneficial to the plant, however high level of ROS can lead to harmful damage to the plant and so it was interesting to observe O_2^- content in *P. vulgaris* under stress and under low doses of exogenous MG. Looking at the *P. vulgaris* second youngest trifoliate superoxide content, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.2A)** , there was a 68 % decrease, 52 % increase and 64 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 83 % decrease. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.2B)** , there was an 81 % decrease, 82 % increase and 97 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 85 % decrease.

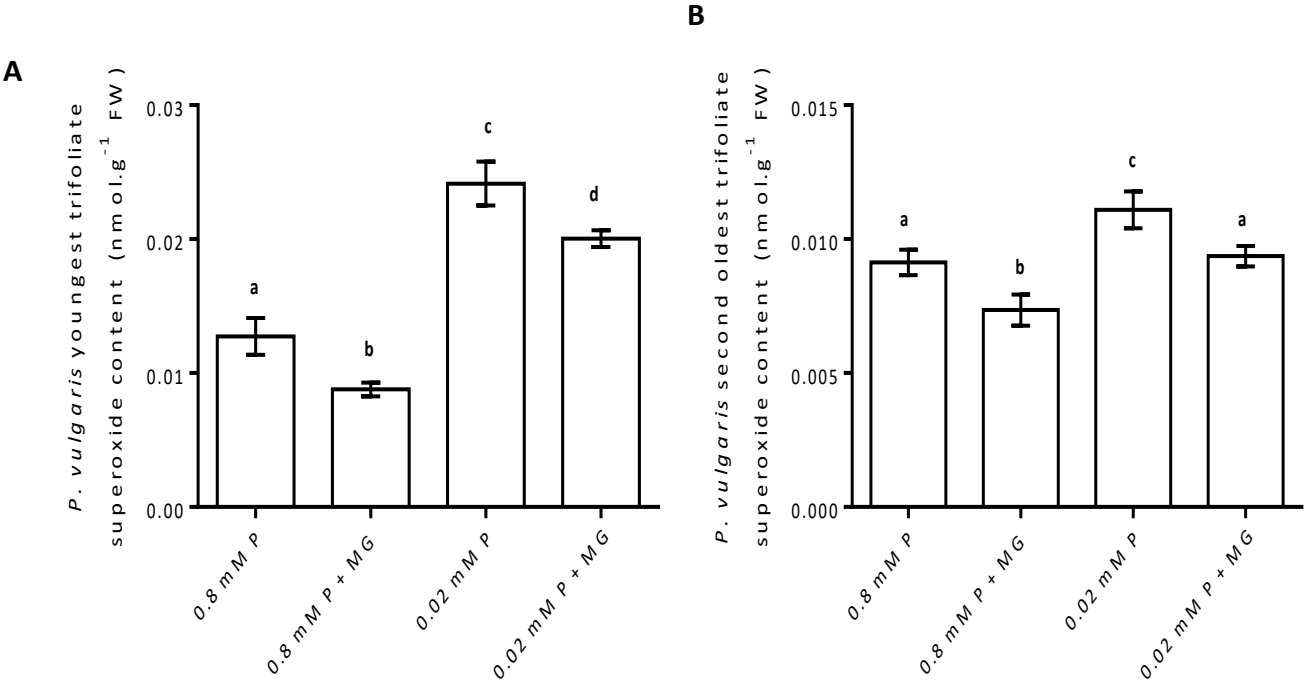


Figure 4.2: The effect of MG treatment in *P. vulgaris* superoxide content in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of

growing superoxide content on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.1.3 Exogenous MG treatment at low concentration reduced hydrogen peroxide content in *P. vulgaris* second youngest and second oldest trifoliate when there is insufficient P

The least damaging and reaction ROS species is H_2O_2known to be a signalling molecule in cells but it has also been reported that high accumulation of H_2O_2 level, increases ROS production and causes a lot of damage to the plants (Singla-Pareek, 2006; Hossain, 2012). H_2O_2 can only act as a signalling molecule when it is kept at low to intermediate levels (Cheeseman, 2007; Hossain et al. 2012; Hossain, 2012). Looking at the *P. vulgaris* second youngest trifoliate hydrogen peroxide content, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.3A)**, there was a 76 % decrease, 63 % increase and 94 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 67 % decrease. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 3.4B)**, there was an 88 % decrease, 74 % increase and 93 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 79 % decrease.

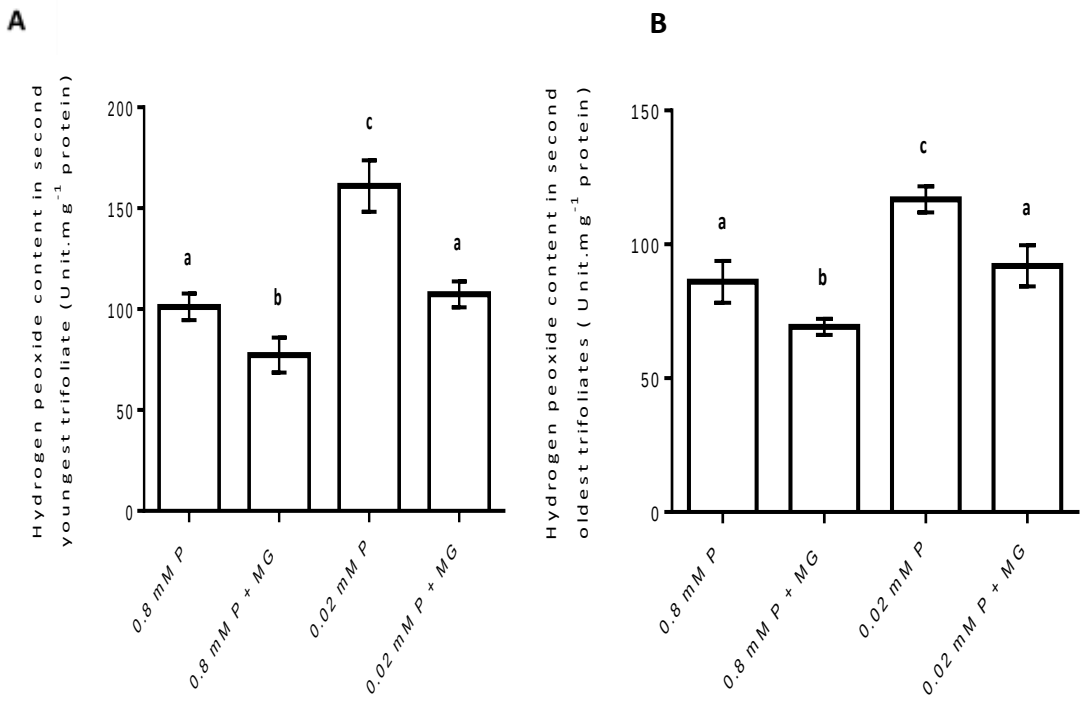


Figure 4.3: The effect of MG treatment in *P. vulgaris* hydrogen peroxide content in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing hydrogen peroxide content on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.1.4 Exogenous MG treatment at low concentration reduced hydroxyl radicle content *in P. vulgaris* second youngest and second oldest trifoliate when there is insufficient P

•OH production is usually under normal condition at a basic level, however a significant increase can be observed in •OH production when the plant is under stress (Bhattacharjee, 2011) thus leading to detrimental cell damage in the plant (Schopfer, 2001). Looking at the *P. vulgaris* second youngest trifoliate hydroxyl radicle content, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.4A)**, there was a 40 % decrease, 62 % increase and 99 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 62 % decrease. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.4B)**, there was a 60 % decrease, 71 % increase and 80 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 57 % decrease.

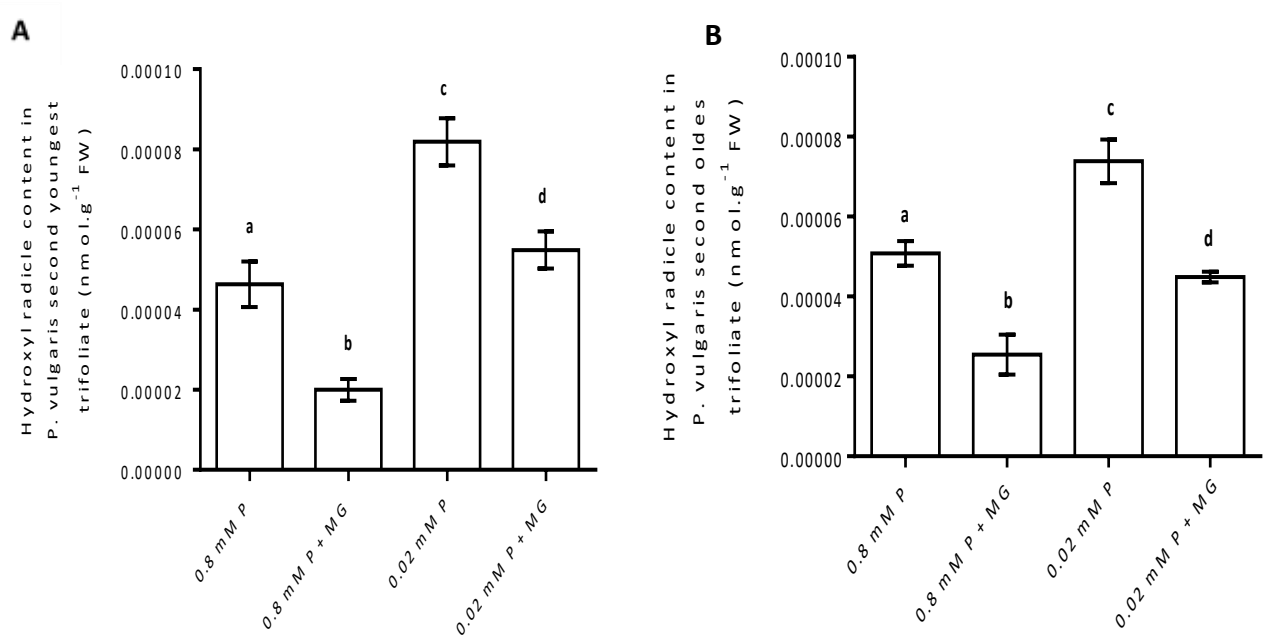


Figure 4.4: The effect of MG treatment in *P. vulgaris* hydroxyl radicle content in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing hydroxyl radicle content on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.1.5 Exogenous MG treatment at low concentration reduced lipid peroxidation in *P. vulgaris* second youngest and second oldest trifoliate when there is insufficient P

Based on literature, an increase in the amount of MDA is often observed in plants under oxidative stress suggesting that there is greater lipid peroxidation activity. However, it also has been observed that when plants under stress were treated with exogenous MG, lipid membrane damage reduced. Looking at the *P. vulgaris* second youngest trifoliate MDA content, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.5A)**, there was a 70 % decrease, 65 % increase and 95 % decrease; respectively. In addition, when 0.02 mM P + MG sample

was compared to the 0.02 mM P sample, there was a 61 % decrease. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.5B)**, there was a 69 % decrease, 84 % increase and 94 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 79 % decrease.

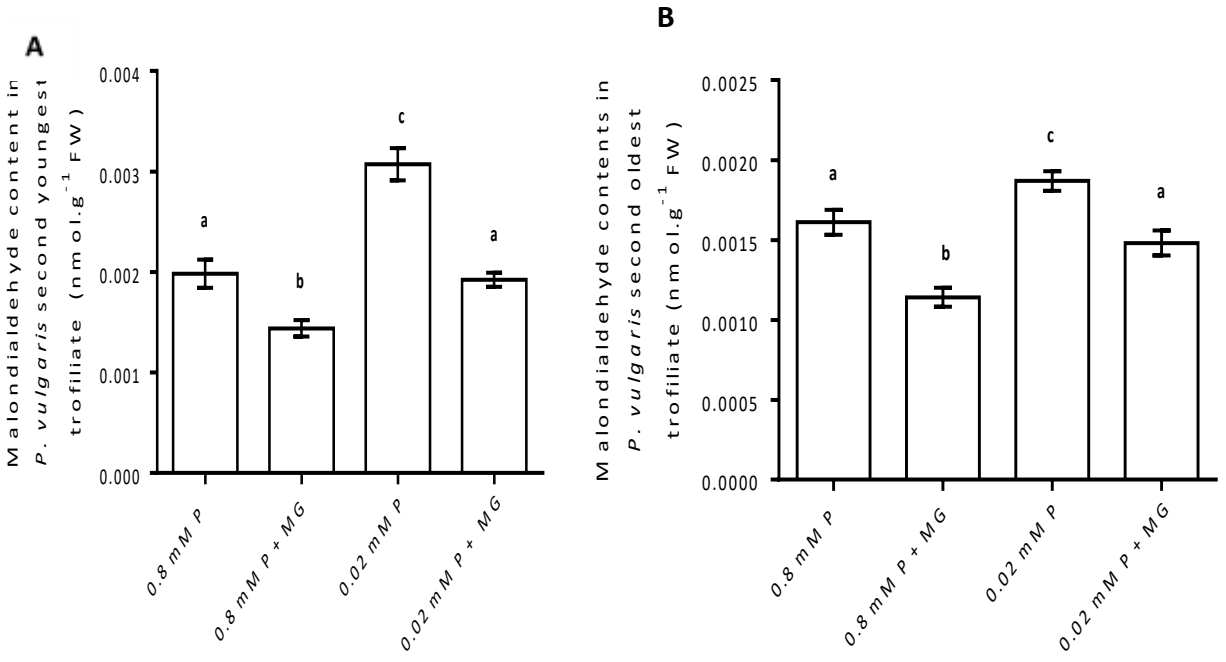


Figure 4.5: The effect of MG treatment in *P. vulgaris* malondialdehyde content in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing malondialdehyde content on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.2 Antioxidants

4.2.1 Exogenous MG treatment at low concentration increased superoxide dismutase activity in *P. vulgaris* second youngest and second oldest trifoliate when there is insufficient P

When the plant is under stress, the first antioxidant to respond is SOD as it can convert superoxide into hydrogen peroxide and water (Wang et al. 2016). The cytoplasm is the location of Cu/ZnSODs which allow the scavenging of superoxide. Thus it seems necessary to examine the SOD activity in plants under oxidative stress. Looking at the *P. vulgaris* second youngest trifoliate SOD activity, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.6A)**, there was an 85 % increase, 81 % decrease and 89 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 72 % increase. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control (f**igure 4.6B**) , there was an 82 % increase, 78 % decrease and 96 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 71 % increase.

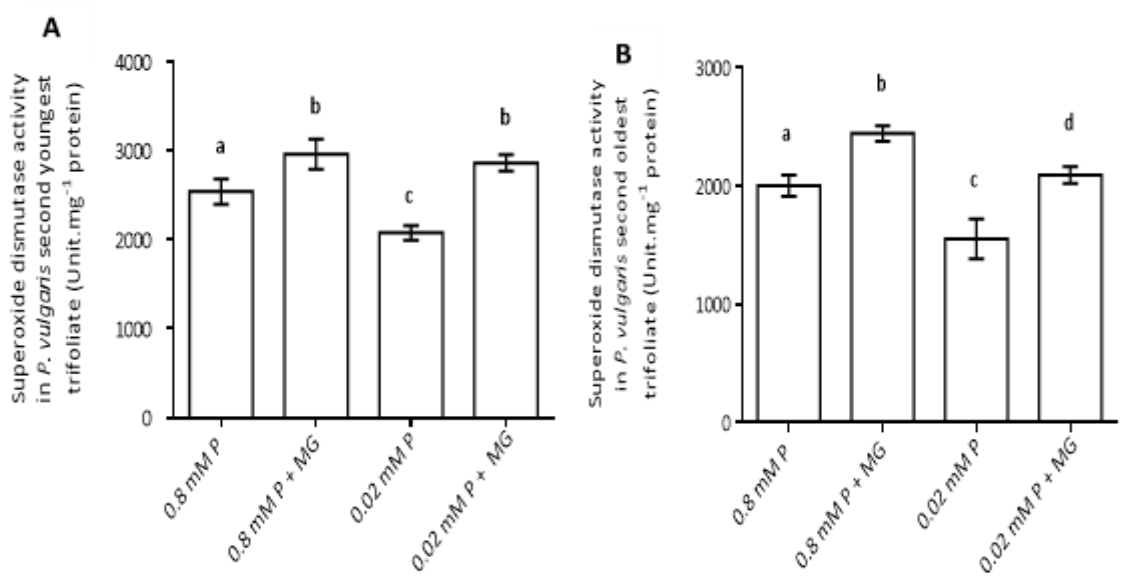


Figure 4.6: The effect of MG treatment in *P. vulgaris* superoxide dismutase activity in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing superoxide dismutase activity on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.2.2 Exogenous MG treatment at low concentration increased ascorbate peroxidase activity in *P. vulgaris* second youngest and second oldest trifoliate when there is insufficient P

When the plant is exposed to biotic and abiotic stress, APX converts H_2O_2 to $2H_2O$ with the use of the ascorbate electron donor (Sarowar *et al.* 2005). A study done by Duan *et al.* (2016) showed that when tomatoes were exposed to cold stress, the level of APX increased. Looking at the *P. vulgaris* second youngest trifoliate APX activity, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.7A)**, there was a 65 % increase, 79 % increase and 54 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 86 % increase. A similar trend was observed in the oldest youngest trifoliate. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.7B)**, there was a 57 % increase, 78 % increase and a 44 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 56 % increase.

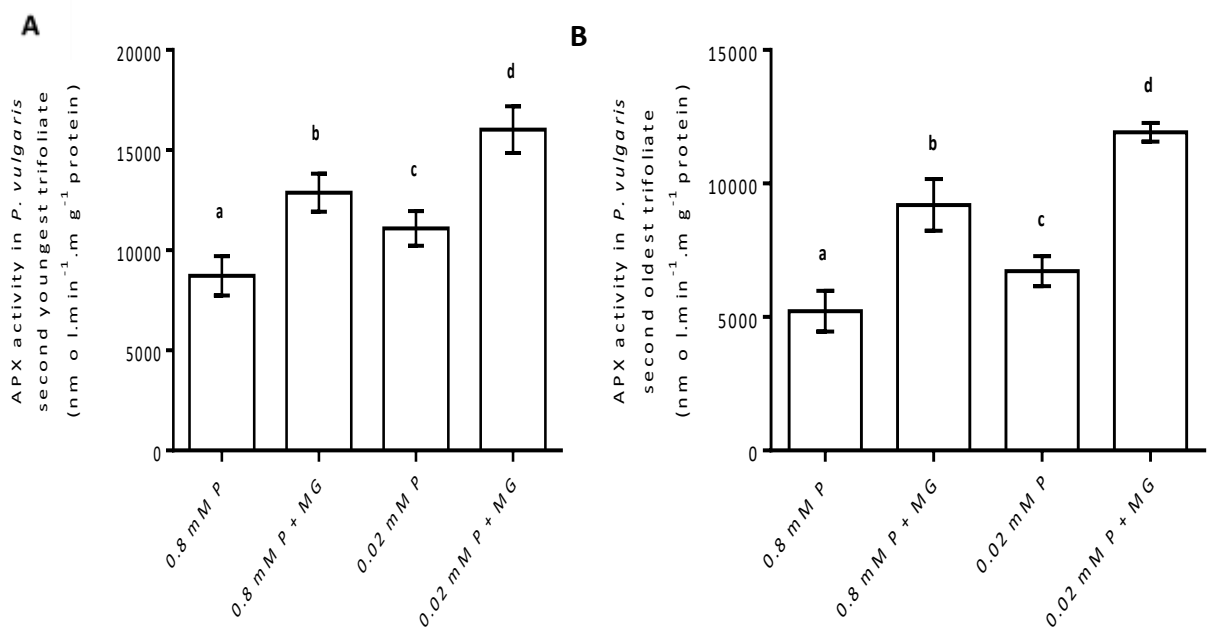


Figure 4.7: The effect of MG treatment in *P. vulgaris* ascorbate peroxidase activity in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing ascorbate peroxidase activity on each treatment was determined. The different letters indicate a significant change across means at $P< 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

4.2.3 Exogenous MG treatment at low concentration increased catalase activity in *P. vulgaris* second youngest and second oldest trifoliate when there is insufficient P

Catalase is also involved in the scavenging mechanism that converts H_2O_2 to water and oxygen and play a vital role as H_2O_2 is their major substrate (Scandalios, 1997; Mhamdi *et.al* 2010). Although APX is the first antioxidant to scavenge H_2O_2, CAT is also important as it scavenges any excess H_2O_2 that was left by the APX (Scandalios, 1997). Looking at the *P. vulgaris* second youngest trifoliate CAT activity, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.8A)**, there was an 85 % increase, 871 % decrease and 98 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 71 % increase. A similar trend was observed in the second oldest trifoliate. When the 0.8 mM P + MG, 0.02 mM was an 84 % increase, 66 % decrease and 98 % decrease; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 64 % increase. P and 0.02 mM P + MG samples were compared to the control **(figure 4.8 B)**,there was an 81 % decrease, 82 % increase and 97 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 85 % decrease.

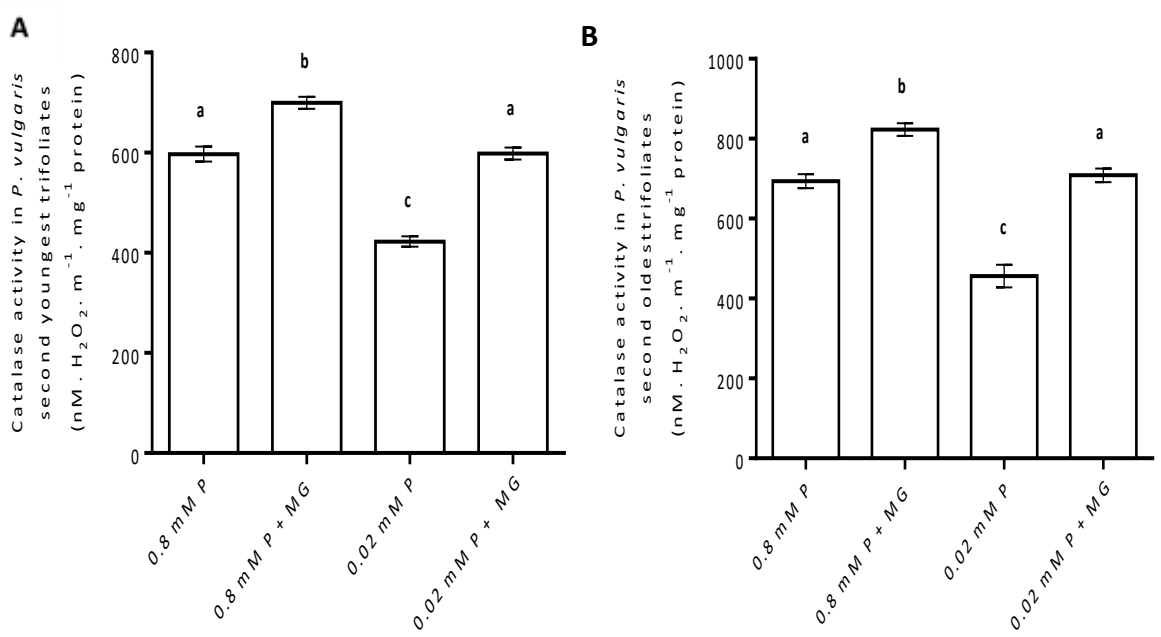


Figure 4.8: The effect of MG treatment in *P. vulgaris* catalase activity in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination;respectively and after 4 weeks of growing catalase activity on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.3 Nitric oxide and nutrient profile

4.3.1 Exogenous MG treatment at low concentration increased NO content in *P. vulgaris* second youngest and second oldest trifoliate under P deficiency

Literature shows that when the plant is under biotic and abiotic stress, NO acts as a signalling molecule. Looking at the *P. vulgaris* second youngest trifoliate NO content, the following trend was observed. When the 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.9A)**, there was a 78 % increase, 81 % decrease and 82 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 71 % increase. A similar trend was observed in the second oldest trifoliate. When the 0.8 Mm P + MG , 0.02 mM P and 0.02 mM P + MG samples were compared to the control **(figure 4.9B)**, there was an 82 % increase, 90 % decrease and 90 % increase; respectively. In addition, when 0.02 mM P + MG sample was compared to the 0.02 mM P sample, there was a 65 % increase.

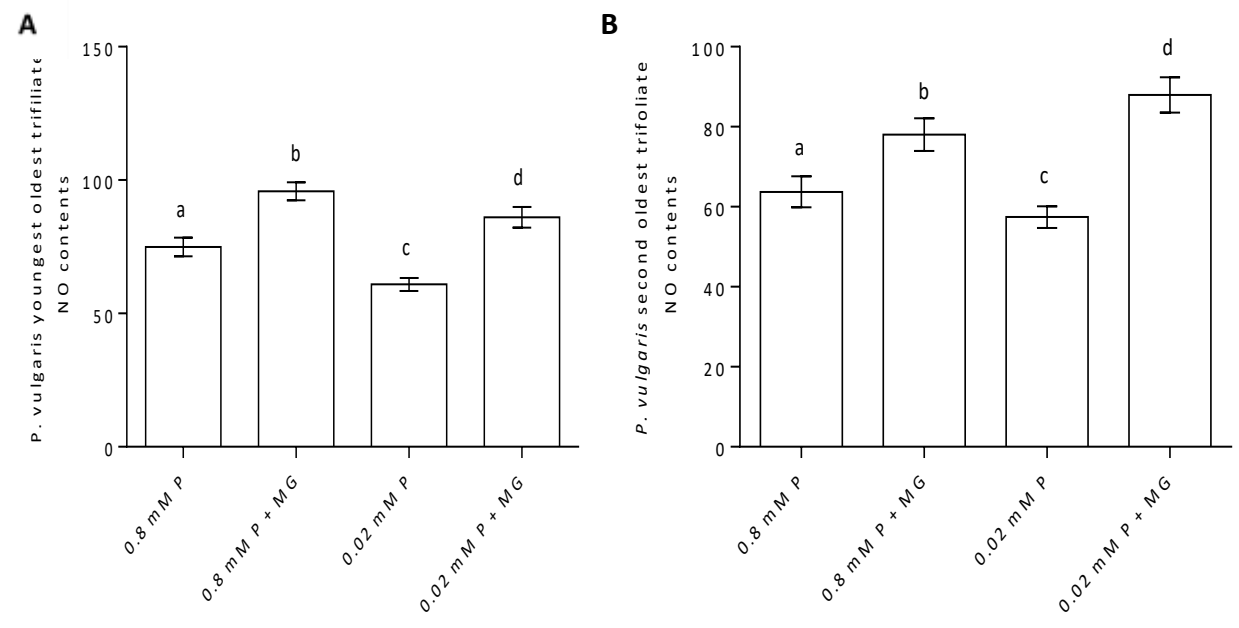
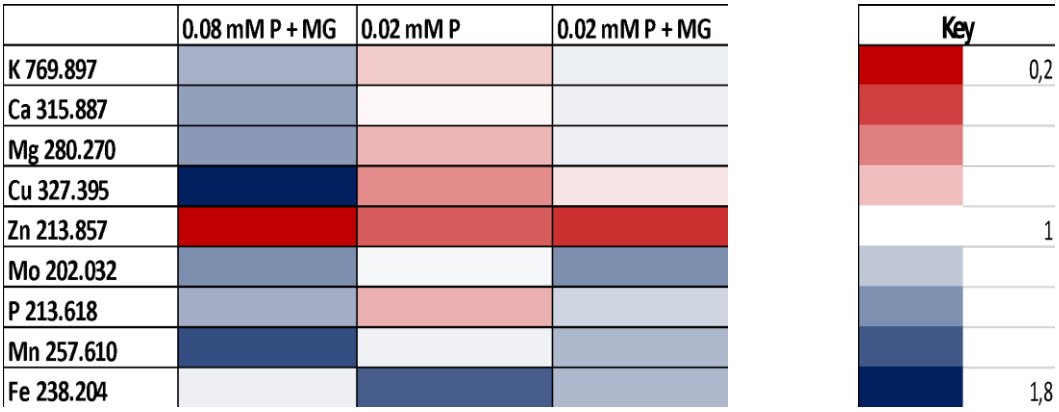


Figure 4.9: The effect of MG treatment in *P. vulgaris* increased NO content in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing catalase activity on each treatment was determined. The different letters indicate a significant change across means at P< 0.05 (Tukey-Kramer test). Values are means ± S.E (N=3).

4.3.2 Exogenous MG treatment at low concentration improved nutrient content in *P. vulgaris* second youngest and second oldest trifoliate under P deficiency

Looking at the *P. vulgaris* second oldest trifoliate nutrient content, the following trend was observed. When comparing the 0.02 mM P crops K, Ca, MG, Cu, Mo, P and Mn content to 0.8 mM P + MG, an increase of 74 %, 80 %, 67 %, 67 %, 80 %, 69 % and 73 % was observed; respectively. When comparing the 0.02 mM P + MG crops K, Ca, MG, Cu, Mo, P and Mn content to 0.02 mM P, a decrease of 85 %, 94 %, 81 %, 83%, 80%, 75% and 83% was observed respectively. When comparing the 0.02 mM P + MG crops K, Ca, MG, Cu, Mo, P and Mn content to 0.8 mM P + MG, an increase of 88 %, 83 %, 84 %, 84 %, 97 %, 92 % and 83 % was observed; respectively. However, on the other hand, Zn and Fe showed a different trend, when comparing the 0.02 mM P crops Zn and Fe content to 0.8 mM P + MG, a decrease of 67 % and 75 % was observed respectively. When comparing the 0.02 mM P + MG crops Zn and Fe content to 0.02 mM P, anincrease of 83 % and 84 % was observed respectively. When comparing the 0.02 mM P + MG crops Zn and Fe content to 0.8 mM P + MG, a decrease of 81% and 89% was observed respectively.

Table 1: Heat map illustrating the effect of MG treatment in *P. vulgaris* nutrient content in response to phosphate deficiency. *P.vulgaris* second youngest trifoliate (A) and second oldest trifoliate (B) were exposed to 0.8 mM P treatment and some to a 0.8 mM P + MG, 0.02 mM P and 0.02 mM P + MG combination; respectively and after 4 weeks of growing nutrient content on each treatment was determined.


0.08 mM P + MG
0.02 mM P
0.02 mM P + MG
K 769.897
Ca 315.887
Mg 280.270
Cu 327.395
Zn 213.857
Mo 202.032
P 213.618
Mn 257.610
Fe 238.204
Key
0,2
1
1,8

Discussion

Historically, MG was known a toxic by-product of cellular metabolism especially glycolysis and photosynthesis. Excessive accumulation of MG in plants leads to carbonyl stress (also known as MG stress) (Kaur, 2016; Li, 2016; Mostofa *et al.* 2018). Today, MG among hydrogen peroxide, nitric oxide and hydrogen sulphide has been found to be a signalling molecule (at low concentrations) with multifunction in plants, participating in cellular metabolism, plant growth, development and adaptation to environmental stress.

Looking at the *P. vulgaris* second youngest and second oldest trifoliate (figure 4.1 A and B), there was a small increase in 0.8 mM + MG combination in comparison to the 0.8 mM control, but 0.02 mM P had higher MG levels compared to the 0.8 mM control and no decrease was observed in the 0.02 mM + MG treatments. The high levels of MG seen in 0.02 mM P occur because the plant is under stress and could be the reason for the high lipid peroxidation that was seen in the same treatment but also the trend is maintained and cell death level are lower when MG was administered at low doses to the plant under stress. When *P. vulgaris* was under stress, MG contents increased and this is possible because when a plant is under stress MG is over produced, making it one of the ROS in the plant and that leads lipid peroxidation we observed in the results. However when low exogenous doses of MG are added to a P deficient plant that has high levels of toxic endogenous MG, the exogenous low MG does inhibit the production of ROS production including the toxic MG that is produced under P deficiency stress. This then result in the inhibition of cell death, reduced biomass and lipid peroxidation. Another hypothesis could be that the exogenous low MG works in tandem with glyoxylase I and II, thus detoxifying the toxic MG in the plant and then keep it at concentrations where it benefits the plant rather than destroy it. Our work is in agreement with a study done by YueWang et.al (2019) showed how MG triggered heat resistance. There was an irrigation of maize seedlings under heat stress with different concentration of (0 µM, 50 µM, 100 µM, 150 µM) for 6 hours. The results firstly showed that an increase in the MG concentration used to irrigate the maize led to an increase in internal MG (toxic). Furthermore

a decrease in internal MG content was seen when the plants were not exposed to heat stress but just treated with MG compared to plants exposed to heat stress. A slight increase in MG was still seen as the exogenous treatment increased in the non-heat stressed plants.

From these results we can then hypothesise the following about *P. vulgaris* plants exposed to P deficiency stress. If low exogenous doses of MG are administered to a P sufficient plant, it improves the internal MG content and still keeps it under the ratio helpful to plants. However exogenous MG administered to a P deficient plants reduces the toxic internal MG possibly by activating enzymes that detoxify MG.

Among many O_2^- is one of the first ROS species in the cells to accumulate from the reduction of O_2^- (Pua and Douglas, 2004; Hung and Kao, 2007). During the conversion of O_2^- to H_2O , four electrons are required to interact in a stepwise manner where only one electron is recognised principally through ETC in the mitochondria, oxygen is produced (Ahsan*,* 2003). An increase in lipid membrane damage and physiological stress are a result of the dangerous impacts that O_2^- has on the plants (Ahsan*,* 2003; Yadav *,* 2005; Held*,* 2012; Ahuja*,* 2015). Based on the results of superoxide content *P. vulgaris* second youngest and second oldest trifoliate (figure 4.2 A and B), there is a slight decrease in the 0.8 mM P + MG in comparison to the 0.8 mM control; on the other hand 0.02 mM P had a greater increase compared to the control and that is due to the stress it is under but a decrease is seen in the 0.02 mM + MG when compared to the 0.02 mM P due to the low exogenous MG concentrations. A possible explanation for the trends could be that when the plant is under P deficiency stress, the production of ROS is much greater than that of antioxidants; hence we see an increase in O_2^- content and a reduction in antioxidant activities. We see the effect of O_2^- in the increase in lipid peroxidation seen in *P. vulgaris* under P deficiency. The damage in the cell membrane of the plant cells then means that the cell metabolism pathways or normal activities are disrupted eventually resulting in cell death. However when low doses of MG were added to the plants, it inhibited the production of ROS and activated or increased the production rate of antioxidants such as SOD, APX and CAT which scavenge O_2^- thus eliminating and reducing lipid peroxidation, reducing cell death in the plants and improving the plant biomass. A study done by Du Qi (2017) showed that under potassium defiency among maize *(Zea mays* L) inbred lines, showed that there was an increase in superoxide content in some of the inbred lines exposed to potassium defiency compared the control that has sufficient nutrients. Additionally, a study

done by Gokul (2018) showed that Agamax plants exposed to 350 µM vanadium resulted in an increase in leaf superoxide content due to heavy metal stress. From these results we can then hypothesise that *P. vulgaris* plants exposed to P deficiency stress could have high superoxide content. Although there is not a lot of work on MG regulating superoxide, we still add to our hypothesis that under low exogenous MG treatments, superoxide content may be reduced.

Hydrogen peroxide is not only toxic to the plant at high levels but it also aids in the plants defence when the plant is under abiotic stress at low levels thus known as a signalling molecule (Cheeeseman, 2007). When looking at the H_2O_2 content in *P. vulgaris* roots and old trifoliate (figure 4.3 A and B), a decrease was observed in 0.8 mM + MG in comparison to the control but the 0.02 mM P was higher than the 0.8 mM + MG treatments meaning that the 0.2 mM P was under oxidative stress. Keeping in mind that we are investigating the effect of low MG concentrations, it was interesting to see the decrease in 0.8 mM + MG and 2 mM + MG levels in comparison to the 2 mM P second youngest and second oldest trifoliate which are under stress, and this indicates MG response to oxidative stress. As mentioned already that when *P. vulgaris* is under stress, ROS production greatly exceeds that of antioxidants hence we see a great increase in H_2O_2 and previously discussed the increase in O_2^-. Hydrogen peroxide at high concentration poses great damage to the plant and that is why we see alterations in morphology, plant biomass and cell death (Gill, 2010). Overproduction of this ROS molecule in a plant results in lipid peroxidation due to O_2^- could also lead to disruptions in plant cell metabolic pathways and enzyme function. However, when low MG doses reduce and detoxify H_2O_2 by increasing the production of SOD first as the main or rather the first antioxidant to respond to damage by this ROS molecule. As a result of this antioxidant activity which is up- regulated by MG. To prove that MG reduces ROS production, we see a significant decrease in ROS content after the treatment. A study done by Du Qi (2017) showed that under potassium deficiency among maize *(Zea mays* L) inbred lines, showed that there was an increase in hydrogen peroxide content in some of the inbred lines exposed to potassium defiency compared the control that has sufficient nutrients. Additionally, a study done by Phillips (2012) showed a drastic increase in superoxide in soybean plants exposed to three weeks of drought compared to untreated control. The papers above are in correlation with my findings showing that abiotic stress increase superoxide to the plant being under stress.

We hypothesised that nutrient deficiency in *P. vulgaris* resulted in an increase in superoxide content but the low doses of exogenous MG reduce superoxide possibly by triggering antioxidant activity.

Hydroxyl radicals (•OH), have obtained their notoriety by being highly reactive in cells, interacting with every kind of macromolecule viz. lipids, DNA, RNA, protein and carbohydrates. There is an interesting correlation between the impact of accumulated •OH and increased MG in cells. Methylglyoxal is a metabolite whose presence leads to irrevocable AGEs being formed (Hossain *et al*. 2012). Methylglyoxal attacks PUFAs, lipids and acts directly on O_2 , reducing it to O_2^-. Similarly •OH readily interacts with biomolecules causing irreversible damage in DNA, proteins and RNA (Ahsan*,* 2003; Hossain *et al*. 2012). If upon MG-treatment, and the resulting accumulation of MG in cells, the •OH levels drastically increases with the MG increase, it may have devastating consequences that impact plants in a plethora of ways. Hydroxyl radicle is prone to be formed even when the plant is not under stress and this is due to the fact that it is produced from metal ions constantly interacting with H_2O_2 , and so if O_2 is over produced, the formation of •OH from H_2O_2 could be inhibited by ascorbate peroxidase or catalase to further inhibit low H_20_2 contents in the plant. Looking at the •OH content in the *P. vulgaris* second youngest and second oldest trifoliate (figure 4.4 A and B) of this study, there was a slight decrease in the 0.8 mM P + MG when compared to the 0.8 mM P control. The increase in •OH content in 0.02 mM P in comparison to 0.8 mM P control clearly indicate that the *P. vulgaris* second youngest and second oldest trifoliate are under stress which leads to an extent of cell damage, however, a decrease was seen in the 0.02 Mm P + MG trifoliate when compared to the 0.02 mM P treatment. Hydroxyl radicle is another dangerous ROS molecule to the plant as it attacks the plants DNA and lipids especially in the presence of toxic MG. The increased •OH contents we see together with the increased toxic MG are a result levels of P deficiency and have played a role in lipid peroxidation and cell death. It is also the reduction we see in APX activity that allows this great damage to the plant to occur. However when low MG doses are administered to the plant it activates the production of APX which will detoxify and inhibit further •OH production thus reducing damage. As seen in the results with reduced •OH content, there is reduced less lipid peroxidation. A study done by (Venkateshwaran *et al.* 2013) also showed that under phosphate deficiency conditions, an increase in •OH content and reduced biomass under those conditions was observed in *P.*

vulgaris. Additionally, a study by (Bless, 2015) illustrated that when MG was administered at low concentrations in plants under heavy metal stress, the •OH content decreased and biomass increased. The finding stated above are in agreement with our results and support our hypothesis.

Lipid peroxidation is often used as a measure of oxidative damage due to the formation of radicles and H_2O_2 (Cherif*,* 1997). Lipid peroxidation has been reported in literature to be a stimulator of ROS accumulation through lipid membrane peroxidation (Cherif*,* 1997; Ahuja*,* 2015). Due to the effect the ROS poses to the plant, damage on the membrane can be seen by determining the MDA content. In this study, there was an increase in lipid peroxidation in the 0.02 mM P in comparison to the 0.8 mM control, but a decrease was seen in the 0.02 mM P + MG combination in comparison to the 0.02 mM P with the most membrane damage. This means that the P phosphate deficient plant had high lipid damaged but when exogenous MG is applied at low concentrations, the membrane damage on the cells is reduced. Lipid peroxidation is damage to the cell membrane (lipids) of the plants, under P deficiency we see an increase in O_2^- and H_2O_2 which are the main contributed to lipid peroxidation through destroying lipids in the membrane, leave the cell's metabolic pathway in harm's way. However, the MG added to the plant at low doses activates and increases the production rate of APX and CAT making sure it exceeds that of these ROS molecules that lead to lipid peroxidation. In this way the ROS molecules are inhibited the lipid peroxidation does nor or occur or is reduced, cell metabolic pathways are not disturbed, cell death is reduced and plant biomass is increased. A study done by Venkateshwaran *et al.* (2013) also showed that under phosphate deficiency conditions, an increase in lipid peroxidation and decrease in cell death was observed in *P. vulgaris*. Additionally, a study by Bless (2015) illustrated that when MG was administered at low concentrations in plants under heavy metal stress, the MDA levels were decreased and biomass also decreased. Our results with the work stated above suggest that lipid peroxidation is reduced in *P. vulgaris* under P deficiency stress when MG is exogenously administered at low concentrations.

There is growing evidence plants subjected to environmental stress leads to significant crop losses. The stresses are numerous and often crop- or location-specific. They include increased UV-B radiation, water, high salinity, metal toxicity, herbicides, fungicides, air pollutants, light,

temperature, topography and hypoxia (restricted oxygen supply in waterlogged and compacted soil) (Cherif, 1997). Since higher plants are immobile, they can't escape from environmental stresses. The ability of higher plants to scavenge the toxic effects of active oxygen seems to be very important determinant of their tolerance to these stresses (Bless, 2015). Antioxidants are the first line of defence against free radical damage. They are critical for maintaining optimum health of plant cells (Venkateshwaran et al. 2013). There are several antioxidant enzymes, peptides and metabolites involved in the scavenging of active oxygen in plants, and their activation are known to increase upon exposure to oxidative stress. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Bless, 2015). Antioxidant metabolites include phenolic and nitrogen compounds.

Superoxide dismutase is said to be the most important antioxidant as it is the first line of defence in scavenging O_2^- in the cells so this means that an increase in the O_2^- component will result in an increase in SOD activity (Ahsan, 2003). Superoxide dismutase is also important because it reduces lipid peroxidation that is caused by O2- when it targets lipid molecules (Ahsan, 2003; Hossain et al. 2012; Mourato *et al.* 2012). Looking at the SOD activity in *P. vulgaris* second youngest and second oldest trifoliate (figure 4.6 A and B) there is increase in the 0.8 mM P + MG and 0.02 mM + MG in comparison to the 0.8 mM P control meaning that MG stimulated SOD activity when the *P. vulgaris* second youngest and second oldest trifoliate were under stress. There was also an increase in in the 0.02 mM P + MG compared to the 0.02 mM P treatments. Under P deficiency, an increase in all the ROS molecules but the antioxidant activity was decreased; this is because under P stress the production of ROS exceeds that of antioxidant activity leaving the plant in a state where it is vulnerable to cell death lipid peroxidation which ultimately results in reduced biomass and bad morphology. However low MG doses not only inhibit O_2^- (which is mainly detoxified by SOD) it ensures that the production of SOD exceeds O_2^- leaving the plant healthy with no cell death or lipid peroxidation.

APX is another crucial antioxidant that scavenges ROS and is known to be the first antioxidant that responds to convert H_2O_2 to $2H_2O$ to when it is produced at high levels (Ahsan, 2003; Held *et al.* 2012). Although CAT also scavenges H_2O_2 , APX has a greater ability of scavenging H_2O_2 . APX works in tandem with ascorbate and the amount of APX activity in plants will determine the H_2O_2 content present (Blokhina et al. 2013). The *P. vulgaris* APX activity in the second oldest and second youngest oldest (figure 4.7 A and B) from this investigation illustrates a slight increase in both combination treatments (0.8 Mm P + MG and 0.02 mM P + MG) in comparison to the control but a drastic decrease in the 0.02 mM P compared to the control. This finding verifies that although the 0.2 mM P is under stress, there is little antioxidant activity hence we see poor morphology in these trifoliate. The 0.02 mM P + MG treatments showed an increase in comparison to the 0.02 Mm P meaning that the low MG activated APX activity as a decrease is seen in the *P. vulgaris* ROS contents graphs. The decrease in APX activity is a result of the plant being under P deficiency where the plant is in a state where H_2O_2 is being produced at a higher rate than APX so it can't keep up when trying to detoxify this ROS molecule. However low MG doses inhibited and reduced the production of H_2O_2 and at the same time activated the production of APX which ensures the detoxification of H_2O_2 ensures that the effects mentioned above are improved and that the plant is in a good state. Catalase (CAT) is an enzyme that only uses H_2O_2 as a substrate when there is excess H_2O_2 available in the plant, CAT will scavenge it (Cheeseman, 2007; Held, 2011; Hossain et al. 2012). There are several antioxidants that are activated or stimulated in response to H_2O_2 accumulation because it can diffuse across the membrane and resulting in detrimental •OH formation in the membrane (Ahsan, 2003). Although CAT has less ability to reduce H_2O_2, it is still of great importance in scavenging excess H_2O_2 which would cause great damage on the plant without CAT (Ahsan, 2003). The results on *P. vulgaris* second youngest and second oldest trifoliate CAT activity (figure 4.8 A and B) showed that there was an increase in 8 mM P + MG and 2 mM + MG second oldest and second youngest trifoliate in comparison to the control treatments, but on the other hand, the 2 mM P showed a decrease in comparison to the 8 mM P. In instances where there is too much H_2O_2 content in the plants, CAT is activated as a secondary scavenger. In our case of P deficiency, just like APX activity was reduced, so was the CAT activity. The reason for low activity would still be that there is an overproduction of ROS in comparison to CAT production, thus leaving the plant in unfavourable conditions such as cell death and lipid peroxidation which ultimately lead to reduced biomass and poor

morphology. However, the low doses of MG will again in this case as in APX inhibit and reduce ROS while on the other hand ensuring more production of CAT is increased. This change in trends then ensures that the plant does not go through the damages mentioned above.

Our study is in contrast with a study done by Du Qi (2017) which showed that under potassium deficiency among maize (*Zea mays* L) inbred lines, there was an increase in CAT, SOD and APX activity compared to nutrient sufficient maize. In contrary with their findings because plant nutrients are vital for its survival so under nutrient stress, plants undergo morphological changes that interfere with the plants day to day metabolism pathways and lead to alleviated ROS production. Their study shows that under potassium deficiency, an increase in ROS was seen but they also show that under potassium deficiency, there is an increase in antioxidant activity. We would expect to see a decrease in SOD, APX and CAT activity, which would explain the increased ROS contents (superoxide, hydrogen peroxide and lipid peroxidation) showed in their results or vice versa (reduced ROS content vs high antioxidant activity). However on the other hand, a study done by Wang (2019) showed that SOD, APX and CAT activity was increased and ROS activity was decreased in maize seedling exposed to stress through MG triggering by AsA-GSH cylcle and methylglyoxal scavenging system. We agree with this paper because we also saw increased antioxidant activity when low MG doses were administered to *P. vulgaris* under P deficiency; therefore our hypothesis is that MG somehow activate or triggers the ROS scavenging system activating antioxidant production.

Nitric oxide is said to play a key role to counteract heavy metal induced ROS, whether by directly scavenging ROS or by stimulating antioxidant defence team in plants. Looking at the nitric oxide content in *P. vulgaris* second youngest and second oldest trifoliate (figure 4.9 A and B) there is increase in the 0.8 mM P + MG and 0.02 mM + MG in comparison to the 0.8 mM P control meaning that NO signalling might be inhibited in the *P. vulgaris* second youngest and second oldest trifoliate were under stress but activated when low MG doses were administered. There was also an increase in the 0.02 mM P + MG compared to the 0.02 mM P treatments. A possible hypothesis for the trend observed could be that under P deficiency, NO signalling is inhibited due to the overproduction of ROS molecules leaving the plant damaged. However the low MG doses administered work together with NO as signalling molecules or NO regulates MG and that reduces ROS and activate the production of antioxidants leaving the plants in a healthy state. Literature paper written by Gupta and Sahay

(2017) reported that the cell protective property of NO against the metal oxidative stress is post transcriptional modification that has been governed by the Fenton reaction, a process called metal nitrosylation. In this reaction, NO react and binds with iron/ zinc/ copper ion (transition metals) and hydroxyl radicle to form metal nitrosly complex such as iron nitrosyl, zinc nitrosyl complexes. Thus NO can easily scavenge metal to help avoid the formation of the most dangerous ROS i.e. the hydroxyl radicle by preventing Fenton reaction in the cell. These findings agree with our results as they show that when antioxidant activity was increased under low MG in *P. vulgaris* under P stress, NO content was also increased. It agrees with our hypothesis that NO signals MG to activate antioxidant activity thus lowering the ROS content.

Looking at the nutrient content in *P. vulgaris* second oldest trifoliate (Table 1) there is an increase in the 0.8 mM P + MG and 0.02 mM + MG in comparison to the 0.02 mM P meaning that MG improved nutrient content in the *P. vulgaris* second oldest trifoliate were under stress. With the plants under phosphate stress, their germination rate, growth rate, root length and numbers are reduced; the plants take a while or cannot properly absorb the nutrients provided to the plant. In addition, due to the lipid peroxidation and cell death the plant's normal day to day pathways that contribute to acquiring nutrition are not done. However, with the advantageous features of low MG doses in the plant (no lipid peroxidation), the plant is able to absorb and nutrient provided and still do its day to day cell metabolic pathways that may also contribute to getting nutrients. A study done by Ding et al. (2017) showed that under P deficiency, results from ICP experiment on the leaves in tea plant showed a decrease in Cu, Zn, Mn, P and Fe compared to the P sufficient tea plants. These results are in correlation with our results also showing a poor nutrient profile in *P.vulgaris* under P deficiency. Although there was no study correlating the results with the MG combination, our hypothesis could be that MG improves the nutrient profile.

Chapter 5

Conclusion and future perspectives

We first conducted a pre-trial experiment where *P. vulgaris* was treated with different concentrations of phosphate to determine a nutrient sufficient and deficient P concentration. The plants with the poorest concentration were those treated with 0.02 mM P and plants with the best morphological characteristics were those treated with 0.8 mM P. After four weeks of treating the four batches of *P. vulgaris* with different P concentrations and low MG concentrations, all the morphologies were compared. The results basically showed that under P deficiency, there was poor plant morphology, increased ROS content and reduced antioxidant activity compared to the P sufficient plants. On the other hand, P deficient plants treated with low MG doses had improved morphology, reduced ROS and increased antioxidant activity. The hypothesis is that MG somehow regulated P deficiency in *P. vulgaris* .

As mentioned earlier on, phosphorous is one of the main nutrient sources for plants due to the fact that it plays a role in a number of plant pathways such as genetic transfer, phospholipid cell membrane and nutrient transportation. The lack of phosphate in *P. vulgaris* beans resulted in alteration in morphology such as reduced leaf size, chlorosis and reduced roots. All these alterations then affect the plants normal activities such as photosynthesis. Additionally, these alterations in roots and leaves disturb the nutrient transport system from the soil to the leaves of the plants and due to the fact that little nutrients are not being passed to the plants, this results in a decrease in the biomass of the plants.

Another key observation that we saw was that under P deficiency in *P. vulgaris*, an increase in ROS content was observed. First we see an increase in superoxide content and this shows that the plant is under stress. Superoxide is said to be a medium reactive molecule because it cannot diffuse through the membrane and so the only way the plant can detoxify it or try to lower its concentration is by using SOD which will convert it to hydrogen peroxide. Hydrogen peroxide is also a ROS molecule that accumulated when *P. vulgaris* was under P deficiency, but an increase in hydrogen peroxide also signals the plant that it is under P deficiency stress. Constant increase in hydrogen peroxide in a *P. vulgaris* under P stress becomes so toxic that it causes lipid peroxidation to the plant cells. We saw an increase in lipid peroxidation in our crop under P deficiency, this means that the lipids that make up the cell membrane of the plants were damaged, this results in a damage in cell membrane permeability resulting in leakage and loss

of vital ions such as potassium and others required in the day to day metabolic pathways that occur inside the plant cells.

A ROS molecule called hydroxyl radicle which is said to be the most reactive ROS was also seen to increase in content when *P. vulgaris* was exposed to P deficiency stress. We see an increase of this molecule under P deficiency stress because it is formed through Fenton reactions from hydrogen peroxide and superoxide which are also seen to increase in content in *P. vulgaris* under P deficiency stress. The accumulation of hydroxyl radicle in *P. vulgaris* lead to damage in proteins, lipids of *P. vulgaris* .

Antioxidant activity also seems to be really low under P deficiency in *P. vulgaris*. Superoxide dismutase is the first line of defence followed by ascorbate peroxidase and catalase, however, under P deficiency a decrease in all these antioxidants activities was observed. From this we can then conclude that P deficiency not only negatively alter *P. vulgaris* morphology but induces the production of ROS molecules and inhibits the production of antioxidant enzymes. This leads to both internal and external damage to the plants resulting in wilting and death of the plant.

After treating *P. vulgaris* under P deficiency stress with low concentrations of MG an improvement in the plants was observed under both P sufficient and P deficient conditions. First we saw better morphology, meaning bigger leaves, better pigment with no chlorosis, and longer roots. This means that the plant transportation system was improved and necessary nutrients were being transported to all parts of the plant where needed, resulting in an increase in the biomass of the P deficient *P. vulgaris* crop.

Low concentrations of MG also reduced superoxide and hydrogen peroxide content in *P. vulgaris* under P deficiency and under normal conditions and this leads to a reduction in lipid peroxidation, meaning that the cell membranes are kept intact and no vital ions are lost. All this is explained by the high SOD activity that we observed, this would mean that SOD detoxifies superoxide by converting it to hydrogen peroxide. We also observed a decrease in hydroxyl radicles content in our crops under P deficiency, however the increase in SOD explains this result. On the other hand we see an increase in APX, which is a secondary scavenger of hydrogen peroxide and hydroxyl radicle. We also observed an increase in CAT activity and we hypothesise that there was a lot of ROS accumulation and that CAT and APX work in tandem to detoxify the plant of toxic molecules. Although APX and CAT work together, APX is the primary scavenger as

our results show that it has a greater activity than CAT. From this we concluded that when MG is administered to *P. vulgaris* under P deficiency it regulates this stress by activating antioxidant activity thus reducing ROS content.

In addition, to ROS molecules, MG is also a reactive molecule when it accumulates at toxic concentrations which can lead to inhibition of root growth and root germination. Under P deficiency in *P. vulgaris* we see an increase in internal MG content and this contributes to the root damage and also disturbing the nutrient transport of the plant. However, when low doses of exogenous concentration of MG were used to treat *P. vulgaris* under P deficiency, a decrease in internal MG was observed. We then hypothesised that exogenous MG treatment that was used to trigger or activated glyoxalase I and glyoxalase II which are the primary scavengers that detoxify toxic MG molecules in the plants. Another interesting observation that we saw was that when low MG concentrations were administered to *P. vulgaris* under P deficient conditions, a slight increase in MG content was observed. However, this increase lead to reduced ROS content, increase in antioxidant activity and improved morphology. From these results we concluded that exogenous MG treatment that was used to treat *P. vulgaris* under normal P conditions kept the internal MG content at the concentration where the molecule can be considered as a signalling molecule.

Our results clearly show that MG regulates the effect of P deficiency stress. We looked at NO content since it is known to be one of the signalling molecules and also a secondary antioxidant. Our results showed that when MG was administered to *P. vulgaris* under P deficiency stress and increase in NO was observed and a decrease was observed in the P deficient plant with no MG. Even under P sufficient conditions, the treatment of low doses of MG increased the NO content of the plant. Because there has been some work showing NO to be signalling molecule in plants under stress, we then hypothesised that NO is the main signalling molecule that triggers or activates MG to regulate or counteract the effect of P deficiency.

When looking at the nutrient profile of the plant which showed an improved nutrient profile when MG was administered to common bean under P deficiency stress, the results correlated with the rest of our results. From our results, the hypothesis is; MG improves morphology, activated antioxidants and reduces ROS. Improved morphology allows proper transportation of nutrients and that is why we observe a better nutrient profile. For all this process to

happen, NO acts as a signalling molecule which activates MG which then regulates P deficiency in *P. vulgaris* .

Looking at future perspectives, first thing to prove our MG theory for the trend that we observe, this could be done by conducting a glyoxalase I and II assay and this would show enzyme activity in *P. vulgaris* when there is insufficient P and when low MG concentration doses are administered. With the elucidated role of MG as a signalling molecule and NO as its trigger, further molecular and proteomic research must be done in order to further understand the mechanism and to observe which proteins are up regulated and down regulated when *P. vulgaris* is under P deficiency stress and when low concentration doses of MG are administered to a P deficient crop. Molecular research should also be done on NO in order to further understand its mechanisms as a signalling molecules and we could understand the exact pathway of NO triggering MG. Below is a diagram that summarises all the results and which explains both the effect of P deficiency in *P. vulgaris* and how MG regulates this stress.

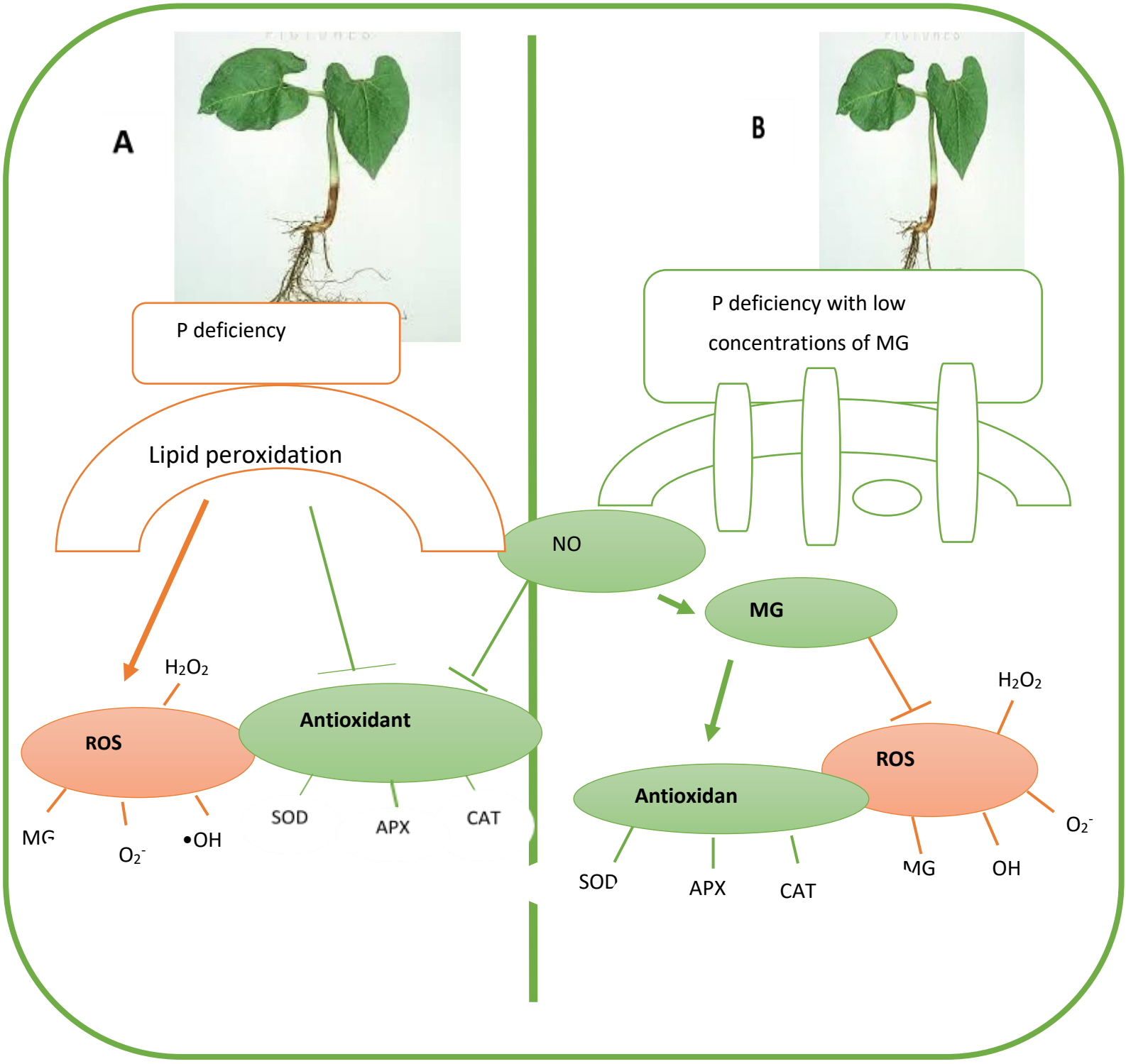


Figure 5: Schematic representation of **(Figure 5A)** *P. vulgaris* under P deficiency with ROS overproduction and inhibition of antioxidant activity. The right side **(Figure 5B)** is a representation of how MG regulates P deficiency through NO signalling, thus activating antioxidant activity and inhibiting ROS production.

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