# Changes on Antioxidant Metabolites and Enzymes in Soybean Inoculated and Treated with Fungicides

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**ABSTRACT----** ***Nodules formed in roots of leguminous plants, as result of their interaction with rizobia bacteria generate oxidative stress, due to high respiratory level and the reducing conditions required to fix nitrogen and the leghemoglobinautoxidation process. To avoid oxidative stress nodules need to maintain a high antioxidant activity and therefore they could synthesize antioxidant metabolites which would react with reactive oxygen species (ROS) during symbiosis and biological nitrogen fixation. However, the knowledge about the antioxidant system in soybean plants inoculated with induced Bradyrhizobiumjaponicum is limited. In this work, it was demonstrated that:1) a treatment of soybean seeds with rhizobium bacteria induces nodules formation and this induction was linked with a higher oxidized state of ascorbic acid. 2) The use of fungicidefavors nodulation but provokes a further oxidation of ascorbic acid. 3) The observed increase of ascorbic acid-recycling enzymes is not enough to counteract ascorbic acid oxidation. Taken together, our results suggest that an oxidative stress could occur during nodulation having the infected soybean plants some degree of tolerance to oxidation.***

**Keywords---** Ascorbic acid, nodulation, antioxidants, soybean

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## 1. INTRODUCTION

The biological nitrogen fixation (BNF) is a natural process carried out by bacteria known as diazotrophs. Several of them can establish associations with plants and provide high amounts of fixed nitrogen (N) to the host. The association between the diazotrophic-group rhizobia and plants belonging to legume (Fabaceae) family is the most studied and well characterized symbiosis (Xavier et al. 2010). The rhizobial inoculant is a cheap and non polluting source of bacteria that can provide high rates of nitrogen to the legume, and the use of improved inoculants is a strategy to increase the BNF rates in agroecosystems (Araújoet al. 2011). The symbiosis between legumes and rhizobia is a complex process relying on finely tuned infectious and developmental events. The initial step of the symbiotic interaction is a chemical cross-talk between the plant and the bacterial partners, leading to the production of bacterial nodulation factors (NF) upon sensing of the flavonoids present in root exudates. NF does not only participate in bacterial infection, but also triggers the initiation of a specific developmental program ending in the formation of a new organ, the nodule (Oldroydet al. 2005).

Legume root nodules are sites of intense biochemical activity and consequently are at high risk of damage as a result of the constant generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These can potentially give rise to oxidative and nitrosative damage. But, when their concentrations are tightly controlled by antioxidant enzymes

and metabolites, they also play positive roles as critical components of signal transduction cascades during nodule development and stress (Becanaetal.2010).Reactive Oxygen Species (ROS) include singlet oxygen ($O_2^{\cdot}$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot}$) species. These compounds are highly toxic, since they are able to modify all the primary constituents of the cell such as lipids, DNA, carbohydrates and proteins (Moller et al. 2007).

Both plant and bacterial cells contain an impressive antioxidant defense with enzymatic and non-enzymatic ROSscavengingsystems(Frendoet al. 2005; Chang 2009).This includes: enzymatic systems directly involved in ROSscavenging such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (Prx); the non-enzymatic antioxidant metabolites such as ascorbate (AA), glutathione (GSH), and α-tocopherol; the ascorbate–glutathione pathway, which allows the reduction of these antioxidant metabolites by NAD(P)H; and the enzymes involved in the disulfide reduction, thioredoxin (Trx) and glutaredoxin (Grx). In nodules, several antioxidants such as glutathione havebeen shown to be involved in symbiosis efficiency (Frendoet al. 2005).

The practical use of inoculants includes the development of formulations that can be combined with fungicides during seed inoculation to avoid diseases (Campo et al. 2010). It has been reported that some fungicides may have negative effects on crop physiology (Petit et al., 2012), but others have shown putative protective role for crops (Dias et al., 2012). Metalaxyl-M is a systemic fungicide which is applied for the control of oomycetes such as *Phytophthora*, it is translocated through the phloem to the aerial parts of the plants (Moulas et al., 2013), generating oxidative stress in some plant species (Teixeira et al., 2011; Souza et al., 2013). Fludioxonilis, a non-systemic contact fungicide, has effects on photosynthesis (Saladin et al., 2003). Previous works demonstrated that the mixture effects of both fungicides when applied to soybean seed treatment have not had impact on the yields induced by seed inoculation with *Bradyrhizobiumjaponicum* (Schulz et al., 2008). However, it is not known if those fungicides applied during seed inoculation could exert any effect on antioxidant metabolism of nodules and roots.

This study aims at determining if the treatment of inoculated seeds with the fungicides fludioxonil and metalaxyl-M affects the antioxidant response of soybean plants. We show that the treatment of soybean seeds with *rhizobium* bacteria induces nodules formation and a higher oxidized state of ascorbic acid. The application of fluodioxonil and metalaxil-M induced higheroxidation of antioxidant metabolitesbut not inhibited nodulation.

## 2. MATERIALS AND METHODS

### 2.1 Seed Treatment and Plant Growth

Two hundredgramsof soybean seeds, variety DM 51i, were carefully selected and inoculated with 0,6mL of aninduced*Bradyrhizobiumjaponicum*culture. A half of the inoculated seeds were further treated with 0.1mL of the fungicidesfludioxonil(1% w/v), 4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrileand metalaxil-M (2% w/v), methyl N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-D-alaninate.Control seeds were neither inoculated nor fungicide treated. Seeds were sowed in plastic pots filled with rhizobia-free soil and plants were grown during thirty days under greenhouse conditions with 5000 lux of illumination, temperature of 25-30°Cand 60-70 % of relative humidity. 20 plants per treatment were used for growth analysis.Thepercentage increase of chlorophyll content, expressed as SPAD unit, was calculated as difference between 30 and 7 days as following:
% increase= (chlorophyll 30 days- chlorophyll 7 days)/chlorophyll 7 days

Shoot and nodules dry weightweretaken at 30 days after sowing. For the analysis of antioxidants, 100 mg roots and nodules per treatment were harvested at 7 and 30 days after sowing and then washed, quickly frozen in liquid nitrogen and kept at -80°C until biochemical analysis.

### 2.2 Measurements of Antioxidant Metabolites

Ascorbic acid (AA) was measured using extracts from roots and/or nodules groundwithtricloroacetic acid (TCA) 3 %. Quantification was done using a HPLC (Shimadzu LC-10ARvp solvent delivery module) fitted with a C-18 column (Variant) and detection carried out at 265nm, as described in Bartoliet al.(2006). The oxidised form (DHA) content was calculated as the difference between the content of total ascorbate (DHA+AA) and AA after reducing DHA with DTT.

Reduced and oxidised forms of glutathione (GSH and GSSG, respectively) were measured following Bartoli et al. (2006). TCA extracts were spun at 17 000 *g* for 10 min and the supernatant used for the assays. Total GSH and GSSG were determined spectrophotometrically before and after derivatization with 2-vinylpiridine. GSH was calculated as the difference between the contents of total and oxidised form.

### 2.3 Activities of Antioxidant Enzymes

Ascorbate peroxidase activity (APX, EC 1.11.1.11)was determined according to Miyake and Asada (1996). For APX analysis extracts were prepared with 0,5mM of ascorbic acid because this enzyme is unstable in absence of substrate. Briefly, frozen tissues(nodule or root) were homogenized in 50 mM potassium phosphate buffer (pH 7,0) containing 0,1 mM EDTA, 0,5 mMAA and 0,1 % (w/v) phenylmethanesulfonyl fluoride and then the homogenate was centrifuged at 13 000 *g* for 15 min. The supernatant was added to $N_2$-bubbling 50 mM potassium phosphate buffer (pH 7,0) containing 10 µM $H_2O_2$. APX activity was measured spectrophotometrically following changes at 290 nm ($\varepsilon$ = 2,8 $mM^{-1}$ $cm^{-1}$).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured as described in Mazorraet al. (2011). The unit of enzymatic activity was defined as the amount of extractthat caused a 50% decrease of the SOD-inhibited nitrobluetetrazolium (NBT) reduction.

Catalase (CAT, EC 1.11.1.6)activity was determined in phosphatebufferextracts through monitoring of the decrease of hydrogen peroxide ($H_2O_2$), (Mazorraet al. 2011). The unit of catalase activity was defined as the enzyme amountwhichdecomposes 1µmol $H_2O_2$per minute at 25°C.

Dehydroascorbatereductase(DHAR, EC 1.8.5.1), monodehydroascorbatereductase(MDAR, EC1.6.5.4)and glutathione reductase(GR, 1.8.1.7) were extracted using phosphate buffer and the enzyme activities in the resulting extracts were measured, according to Bartoliet al. (2005).
The protein content was determined by the Bradford method (Bradford 1976).

### 2.4 Statistical Analysis

The means were statistically analysed by the Tukey test, significance determined at $p \leq 0.05$. The software Assistat version 7.7 Beta was used for analysis.

## 3. RESULTS

### 3.1 Effect ofinoculationand fungicide application on the redox states of ascorbic acid and glutathione in root and nodules

As shown in table I, no differences in ascorbic acid content were detected between inoculated and non-inoculated plantsin roots measured at 7 days after sowing. Nevertheless, this antioxidant was more oxidized in roots of plants inoculated. In fact, we detected ~10.1-11.5% of total ascorbic acid (AA+DHA) in the oxidized state for the inoculated plants. The inoculant alone was able to stimulate the ascorbic acid content in roots evaluated at 30 days after sowing. However, this stimulating effect lacked in inoculated seeds treated with the fungicides fludioxoniland metalaxil-M. Also, the ascorbic acid was more oxidized in roots of 30-days-old plants with independence of the fungicide treatment. Interestingly, the presence of fungicides provoked a greater oxidation of ascorbic acid (22.78%) in the infected roots.

Table 1:Effect of Inoculation on Root Contents of Ascorbic and DehydroascorbicAcidsin 7 and 30 Days-Old Plants

| Treatments | AA¥ | DHA¥ | AA¥ | DHA¥ |
|---|---|---|---|---|
| | 7 days | | 30 days | |
| Control | 0,293±0,05a | 0,02±0,001b (6,38%) | 0,68±0,05b | 0,07±0,003b (9,3%) |
| Inoculant | 0,246±0,02a | 0,032±0,004a (11,5%) | 1,07±0,06a | 0,14±0,02a (11,57%) |
| Inoculant+fungicides | 0,267±0,03a | 0,03±0,005a (10,1%) | 0,61±0,06b | 0,18±0,014a (22,78%) |

¥Units expressed as µmol.$g^{-1}$ FW.Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tukey'stest ($p<0.05\%$). Numbers in bracket represent the percentages of oxidized form respect to total ascorbic acid (DHA/AA+DHA). AA: ascorbic acid, DHA: dehydroascorbic acid

Given previous studies indicated that GSH is essential for the proper development of the root nodules resulting from the symbiotic interaction (Frendoet al. 2005), we tested the amount of this antioxidant in roots and nodules.Here, treatments with the inoculant alone did not show any effect on the reduced glutathione or its oxidized form in roots (Table 2). Nevertheless, the glutathione content of 30-days post-inoculated roots increased in presence of these fungicides. The proportion of oxidized glutathione was similar among treatments.

Table 2:Effect of Inoculation on Root Contents of Reduced and Oxidized Glutathione in 7 and 30 Days-Old Plants

| Treatments | GSH[¥] | GSSG[¥] | GSH[¥] | GSSG[¥] |
|---|---|---|---|---|
| | 7 days | | 30 days | |
| Control | 0,15±0,009a | 0,02±0,001a (11,7%) | 0,22±0,02b | 0,027±0,003ab (10,9%) |
| Inoculant | 0,16±0,015a | 0,02±0,0003a (11,1%) | 0,21±0,006b | 0,022±0,002b (9,5%) |
| Inoculant+fungicides | 0,15±0,02a | 0,015±0,002a (9,0%) | 0,36±0,03a | 0,03±0,001a (7,7%) |

[¥] Units expressed as µmol.g$^{-1}$ FW.Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tukey'stest ($p<0.05\%$). Numbers in bracket represent the percentages of oxidized form respect to total glutathione (GSG/GSH+GSSG). GSH: Reduced glutathione, GSSG: Oxidised glutathione

The behavior of ascorbic acid in nodules was similar to that obtained in roots at 30 days.Content of ascorbic acid was higher in thetreatment with the inoculant alone (Table 3). The amount of DHA was unchanged in nodules; nevertheless, a higher proportion of AA remained in oxidized form as compared to roots. In fact, ~16-29% of total AA was oxidized in nodules. Apparently, nodules from plants treated with fludioxonil and metalaxil-M showed higher AA oxidation. This fungicide mixture stimulated the accumulation of reduced and oxidised glutathione, similar to that occurring in roots at the same time. Also, a higher oxidation degree of glutathione seems to take place in presence of these fungicides (Table 3).

Table 3: Effect of Inoculation with or without Fungicides on contents of ascorbic and dehydroascorbicacidsin Nodules

| Treatments | AA | DHA | GSH | GSSG |
|---|---|---|---|---|
| Inoculant | 0,57±0,09a | 0,11±0,02(16,2 %)a | 0,17±0,017b | 0,016±0,003b (8,6%) |
| Inoculant+fungicides | 0,29±0,06b | 0,12±0,02(29,2 %)a | 0,26±0,015a | 0,038±0,001a (12,8%) |

[¥]Units expressed as µmol.g$^{-1}$ FW. Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tuckey'stest ($p<0.05\%$). Numbers in bracket represent the percentages of oxidized form respect to total glutathione (GSG/GSH+GSSG) or total ascorbic acid (DHA/AA+DHA). AA: ascorbic acid, DHA: dehydroascorbic acid, GSH: Reduced glutathione, GSSG: Oxidised glutathione

### 3.2 Effect of inoculation and fungicide application on antioxidant enzymes in root and nodules

The inoculation increased the activities of ascorbate peroxidase, dehydroascorbatereductaseandmonodehydroascorbatereductasein roots at 7 days; ascorbate peroxidase still being higher when fluodioxonil and metalaxil-M were present (Table 4). Nevertheless, glutathione reductase and superoxide dismutase were stimulated only in response to inoculation in presence these fungicides.Noeffect on catalase activity was observed (Table 4).Whereas the inoculation stimulateddehydroascorbatereductase,monodehydroascorbatereductase and catalase in 30-days-old roots, the addition of fungicides reduced ascorbate peroxidase, monodehydroascorbatereductase, glutathione reductaseand catalase (Table 5).

Table4:Effect of Treatments of Seeds on Antioxidant Enzyme Activities in 7-Days Old Roots

| Treatments | APX | DHAR | MDAR | GR | SOD | CAT |
|---|---|---|---|---|---|---|
| Control | 0,08±0,006 c | 0,019±0,002 b | 0,009±0,0006 b | 0,6±0,058 b | 12,3±0,9 b | 18,3±1,2 a |
| Inoculant | 0,12±0,006 b | 0,044±0,002 a | 0,011±0,0006 a | 0,57±0,12 b | 12,7±1,45 b | 18,7±2,9 a |
| Inoculant+ fungicides | 0,17±0,012 a | 0,043±0,003 a | 0,011±0,0013 a | 1,33±0,09 a | 25,0±1,53 a | 19,0±1,53 a |

APX: ascorbateperoxidase, DHAR: dehydroascorbatereductase, MDAR: monodehydroascorbatereductase, GR: glutathionereductase, SOD: superoxidedismutase and CAT: catalase. Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tukey'stest ($p<0.05\%$).

Table5:Effect of Treatments of Seeds on Antioxidant Enzyme Activities in 30-Days Old Roots

| Treatments | APX | DHAR | MDAR | GR | SOD | CAT |
|---|---|---|---|---|---|---|
| Control | 1,00±0,06 A | 0,35±0,03 b | 0,1±0,01 b | 1,0±0,06 a | 13,67±1,2 a | 51,7±2,19 b |
| Inoculant | 0,93±0,13 A | 0,6±0,06 a | 0,16±0,04 a | 1,03±0,12 a | 12,7±1,45 a | 64,0±2,65 a |
| Inoculant +fungicide | 0,73±0,09 B | 0,67±0,07 a | 0,11±0,02 b | 0,4±0,06 b | 13,0±1,15 a | 57,0±3,6 b |

APX: ascorbate peroxidase, DHAR: dehydroascorbatereductase, MDAR: monodehydroascorbatereductase, GR: glutathione reductase, SOD: superoxide dismutase and CAT: catalase. Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tukey's test ($p<0.05\%$).

Nodules of inoculated plants treated with the mixture of fluodioxonil and metalaxil-M showed lower activities of ascorbate peroxidase, dehydroascorbatereductase and monodehydroascorbatereductase. However, the enzyme activities of glutathione reductase, superoxide dismutase and catalase were increased in presence of these fungicides (Table 6).

Table 6: Effect of Treatment of Seeds on Antioxidant Enzymes from Nodules

| Treatments | APX | DHAR | MDAR | GR | SOD | CAT |
|---|---|---|---|---|---|---|
| Inoculant | 0,9±0,058a | 0,22±0,02a | 0,11±0,009a | 0,32±0,023b | 2,7±0,56b | 64,3±3,38b |
| Inoculant +fungicide | 0,33±0,04b | 0,1±0,014b | 0,07±0,003b | 0,73±0,05a | 6,4±0,87a | 129,0±9,54a |

APX: ascorbate peroxidase, DHAR: dehydroascorbatereductase, MDAR: monodehydroascorbatereductase, GR: glutathione reductase, SOD: superoxide dismutase and CAT: catalase. Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tuckey'stest ($p<0.05\%$).

To verify the effect of the fungicide mixture on plant growth and nodulation, assessments of chlorophyll and dry weight of shoots and nodules were performed. As expected, inoculation of soybean seeds with the induced *Bradyrhizobium* culture promoted the formation and development of nodules (Table 7). The chlorophyll content of non-inoculated plants decreased during the growth, however, it increased in inoculated plants. It may likely suggest a higher contribution of biological nitrogen fixation in inoculated plants. We observed that these positive effects of inoculation did not lead to a higher shoot growth. The application of fungicides increased the weight of nodules but not shoot greening and biomass. In fact, no differences in biomass accumulation and chlorophyll content between inoculated treatments with or without fungicides were detected (Table 7).It suggests that the application of fungicides increased the weight of nodules but not greater greening and biomass.

Table 7:Effect of Inoculation on Plant Growth and Nodule Formation

| Treatment | Chlorophyll (% increase) | Shoot dry weight ($mg.plant^{-1}$) | Dry weight nodules ($mg\ nodule.plant^{-1}$) |
|---|---|---|---|
| Control | -14 % | 802,0±43,9a | 0c |
| Inoculant | +9,5 % | 826,2±45,4a | 1,16±0,06b |
| Inoculant+fungicide | +6,8 % | 853,9±36,8a | 1,40±0,15a |

Percentage of increase of chlorophyll was calculated during the period of nodule development between 7-30 days. Data indicate mean ±standard error and Letters represent significant differences among treatments as determined by Tukey'stest ($p<0.05\%$).

## 4. DISCUSSION

The results of this work highlight two key points: The treatment of soybean seeds with *rhizobium* bacteria induces nodules formation, which linked with a higher oxidized state of ascorbic acid, and application of fluodioxonil and metalaxil-Mfavors nodulation despite generating a further oxidation of antioxidant metabolites.

Related to the fact that inoculant-increased ascorbic acid oxidation in soybean nodules and roots, the results revealed that the redox state of ascorbic acid changed to a higher oxidation in response to nodulation. However, the increases in the DHAR and MDHAR enzymes did not counteract the AA oxidation.It is well known that both enzymes play important roles in maintaining the normal level of AA by recycling oxidized AA (Qin et al. 2011), although MDHAR has sometimes shown to negatively regulate AA content (Gest et al., 2013). Moreover, the lack of increase in GSH content and GR activity could also contribute to the greater AA oxidation. DHAR utilizes GSH as substrate during DHA reduction to AA (Gallie 2013), whereas GR efficiently maintains the reduced pool of glutathione (Gill et al.2013). The higher AA oxidation in roots and nodules could suggest that the maintaining of AA pool in reduced redox state is not a

critical need for nodule formation and *Bradyrhizobium*-mediated nitrogen fixation. In addition, this AA redox state could regulate the establishment of interaction given the redox transduction pathway plays an important role in nodule development (Chang et al. 2009).Despite AA has been hypothesized to be produced in nodules (Matamoros et al. 2006), it is still unclear the role of this antioxidant in nodulation and biological nitrogen fixation.

Our results indicate that the antioxidant activity does not remain constant. Consistently, Matamoros et al., 2013 showed that the capacity to regenerate AA is reduced in oldest nodules and it is apparently critical for the normal nodule functioning. Likely, a higher AA oxidation could be related to anover-production of reactive oxygen species (ROS) during nitrogen fixation.

An intriguing fact was the difference in the antioxidant capacity between nodules and roots in inoculated plants. Some antioxidants can be up-regulated in roots while are down-regulated in nodules. It could indicate a different need for protection against oxidative stress in these organs. It is thought the infection with the nitrogen-fixing bacteria *Bradyrhizobium* generates an oxidative stress in roots and nodules, but also antioxidants, which may reduce the stress and allow a success symbiotic interaction (Frendoet al. 2005; Becanaet al. 2010). Likely, there is a tight communication between root and nodules in the regulation of oxidative stress. Further measurements of ROS production in nitrogen-fixing nodules and roots are required to test this hypothesis.

The AA and GSH are more oxidized in response to fluodioxonil and metalaxil-M: This work also revealed that this fungicide mixture led to a greater oxidation of AA and GSH in both roots and nodules. Clearly, this effect was associated with an inhibition of the AA-recycling antioxidant enzymes (APX, DHAR and MDAR) in nodules. In addition, the oxidation of GSH could not diminished by GRactivity. It suggests that the presence of these fungicides that would apparently generate oxidative stress,it did not prevent the nodulation and the plant growth improvement. It supports the hypothesis of that inoculated plants can have tolerance against a putative oxidative stress generated by this fungicide mixture. It should be highlighted that the systemic component of this mixture, metalaxil-M, can be translocated through plant tissues and provokes oxidative stress (Teixeira et al, 2011; De Souza et al., 2013). However, further studies are required to reveal the direct effects of these fungicides on nodulation considering that other physiological effects, including photosynthesis could be altered by several fungicides (Petit et al. 2012; Dias et al. 2014).

A higher tolerance to oxidative stress of inoculated and treated plants could be related to the tolerance to multiple types of stress (cross-tolerance) (Bartoliet al. 2013). In fact, previous studies reported an increased tolerance of inoculated plants to drought, salinity and other environmental stressors (Freixas et al. 2011; Kim et al. 2012; Egamberdieva et al. 2014). If oxidative stress in nodules and roots could have a role in a potential cross tolerance of inoculated plants remain to be tested.

## 5. CONCLUSIONS

In summary, this work demonstrates that 1) The inoculation with rhizobium induced changes on the antioxidant system in soybean nodules and roots. 2) The treatment of seeds with the fungicides fluodioxonil and metalaxil-M impaired the antioxidant system but it did not affect nodulation.

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