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Nitrogen Compounds Responses in Two Cultivars of Common Bean Inoculated with *Colletotrichum lindemuthianum*

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Abstract: The aim of this study was to investigate the responses of the nitrogen compounds in the cultivars México 222 (resistant) and Widusa (susceptible) of *Phaseolus vulgaris* L. plants inoculated with *Colletotrichum lindemuthianum* race 23. The experimental design used was entirely randomized in factorial scheme, with 2 cultivars (México 222 and Widusa) and 2 treatments (control and inoculated). The compounds evaluated were free ammonium, amino acids, proline and total soluble proteins. The proline level suffered significant increase only in cultivar Widusa, which the increase showed was at 18.9% in plants under pathogen inoculation, when compared with the control plants. The total soluble proteins level in Mexico 222 had significant increase, with the control and inoculated plants presented 4.77 and 6.60 mg g DM^{-1}, respectively. The results revealed that the Widusa cultivar (susceptible) suffered significant changes in free ammonium, total soluble amino acids and proline, however the total soluble proteins level was kept stable. The Mexico 222 cultivar (resistant) presented not significant modification only in proline level. In addition, this study proved the strong influence of the *Colletotrichum lindemuthianum* pathogen on the nitrogen metabolism of *Phaseolus vulgaris* plants.

Key words: *Phaseolus vulgaris* L., *Colletotrichum lindemuthianum*, biotic stress, nitrogen, resistance

INTRODUCTION

The species *Phaseolus vulgaris* L. is known as common bean, as well as is considered one of the more important leguminous crops in world (Broughton *et al*., 2003), due it presents great amount of protein and carbohydrates (Vieira, 2005). Moreover, this crop is economically favorable to Brazil, because it is the higher producer and consumer in worldwide, with annual production of approximately 3 million tons (Sartorato and Rava, 1994; FAO, 2006).

The anthracnose is one of mains diseases of the common bean crop, which the etiologic agent *Colletotrichum lindemuthianum* (Sacc and Magn.) can infect the plant in all the stages and several regions as root, stem, leaf, pod and seed (Sartorato and Rava, 1994; Dalla *et al*., 2003). The disease might be considered as a limiting factor, because under favorable conditions as temperature and air relative humidity the pathogen promotes reduction of the quality grain due to occurrence of the tags, besides to decrease the productive potential of this species (Pastor-Corrales *et al*., 1995).

The nitrogen is an essential element to plant growth and development, which can be absorbed in the forms of nitrate and ammonium (Lea and Azevedo, 2006; Lobato *et al*., 2008a), besides the adequate plant nutrition and consequent maintenance of the pathway of nitrogen assimilation and translocation is fundamental to protection and plant defense against pathogen infections (Walters and Bingham, 2007). Study conducted by Maringoni (2003) revealed reductions in foliar nitrogen levels of 5 *Phaseolus vulgaris* cultivars after infection by *Curtobacterium flaccumfaciens*. Jesus *et al*. (2001) showed decreases in yield of *Phaseolus vulgaris* plants inoculated with *Uromyces appendiculatus* and *Phaeoisariopsis griseola*.

The aim of the study was to investigate the responses of the nitrogen compounds in the cultivars México 222 (resistant) and Widusa (susceptible) of *Phaseolus vulgaris* L. plants inoculated with *Colletotrichum lindemuthianum* race 23.

MATERIALS AND METHODS

Experiment location: The study was conducted in greenhouse and mist chamber located in the Núcleo de Pesquisa Aplicada à Agricultura (Nupagri), which is a research station belonging to Universidade Estadual de

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Maringá (UEM), Maringá city, Paraná state, Brazil (23°26'S and 51°53'W). The biochemical analyses were carried out in the Laboratório de Fisiologia Vegetal Avançada (LFVA) of the Universidade Federal Rural da Amazônia (UFRA).

Plant material: The seeds of the Mexico 222 and Widusa cultivars were obtained from the seed bank of the Nupagri. The seeds used are resistant (Mexico 222) and susceptible (Widusa) to pathogen *Colletotrichum lindemutianum* race 23.

Plant conduction: The seeds of the Mexico 222 and Widusa cultivars were placed in containers (length×width × height; 40×30×10 cm, respectively) that contained the substrate Plantmax®. Twenty seeds were placed in each container and the seedlings were thinned after the 8th day allowing only 12 seedlings to remain in the container.

Growth conditions: The plants remained in the greenhouse environment under natural conditions day/night (the air temperature minimum/maximum and relative humidity were 27.8/7.6°C and 42/71%, respectively during the experimental period). The photoperiod medium was of 12 h of light and a Photosynthetic Active Radiation (PAR) was 1860 μmol/m^2/s (at 12:00 h).

Fungal material and inoculum preparation: The isolate was obtained from Nupagri and was transferred into tubes that contained culture medium and immature pod beans. The tubes were then incubated at 20±2°C for 15 days (Mathur *et al*., 1950).

Plant inoculation: The plants were inoculated with suspension of spores adjusted to 1.2×10^6 spores mL^{-1}. After inoculation, the plants were transferred to a mist chamber and incubated for a period of 96 h at temperature of 20±2°C, relative humidity of 100% and photoperiod of 12 h of light. The control and inoculated plants of both the cultivars that were kept in the greenhouse for 25 days, which it were divided in control and inoculated plants. All plants remained in mist chamber for three days under the conditions described previously, as well as the leaves were harvested on the 8th day after pathogen inoculation.

Experimental design and treatments: The experimental design used was entirely randomized in factorial scheme, with 2 cultivars (Mexico 222 and Widusa) combined with 2 treatments (control and inoculated). The experiment was composed by 6 repetitions and 24 experimental units, as well as 1 plant in each unit.

Leaf dehydration: The leaves were harvested and placed in an oven with forced air circulation at 70°C by 96 h. After this period, the leaf dry matter was triturated and the powder was kept in glass containers. The containers remained in the dark at temperature of 15°C until the moment to carry out biochemical analysis.

Free ammonium, amino acids and proline: The free ammonium, amino acids and proline were determined with 50 mg of leaf dry matter powder, which was incubated with 5 mL of sterile distilled water at 100°C by 30 min, after the homogenized was centrifuged to 2.000 g by 5 min at 20°C and the supernatant was removed. The quantification of the free ammonium was carried out at 625 nm in agreement with Weatherburn (1967), as well as was used $(NH_4)_2$ SO_4 (Sigma chemical) as standard. The quantification of the total soluble amino acids was carried out at 570 nm according to Peoples *et al*. (1989) and was used L-asparagine + L-glutamine (Sigma Chemicals) as standard. The quantification of proline was carried out at 520 nm according to Bates *et al*. (1973), in which was utilized L-proline (Sigma chemicals) as standard.

Total soluble proteins: The determination of the total soluble proteins was carried out with 100 mg of powder, in which was incubated with 5 mL of extraction buffer (Tris-HCl at 25 mM and pH 7.6). The homogenized was kept in agitation by 2 h, after this period centrifuged to 2000 g by 10 min at 20°C and subsequently the supernatant was removed. The quantification of the total soluble proteins was carried out at 595 nm in agreement with Bradford (1976), as well as was used albumin bovine (Sigma chemicals) as standard.

Data analysis: The data were submitted at variance analysis and when significant differences occurred were applied to Tukey test at 5% level of error probability, as well as the standard errors were calculated in all evaluated treatments (Gomes, 2000). The statistical analysis were carried out with the software SAS Institute (1996).

RESULTS

Free ammonium: The amounts of free ammonium suffer significant changes after inoculation in both cultivars, which the resistant cultivar (Mexico 222) presented 0.258 and 0.221 μmoL NH_4^+ g DM^{-1} in control and inoculated plants, respectively (Fig. 1). The susceptible cultivar (Widusa) had reduction significant at 7.4% in free ammonium level, when compared with the plants of the control treatment. The results reveal that in normal and infected conditions the cultivar Widusa has higher amount of free ammonium that the cultivar Mexico 222.

Total soluble amino acids: The amino acids level in Mexico 222 was significantly different (Fig. 2), as well as

the treatment under inoculation presents increase at 18.3%, when compared with control treatment. The control and inoculated plants of México 222 present 157.5 and 186.4 μmol g DM^{-1}, respectively. The cultivar Widusa had 151.8 and 214.9 μmol g MS^{-1}, in control and infected treatments, respectively. As well as the inoculated plants present total increase at 41.5%, when compared with control treatment.

Proline: The proline level suffered significant increase only in cultivar Widusa, which the increase showed was at 18.9% in plants under pathogen inoculation, when compared with the control plants. However, in Mexico 222 were showed 6.82 and 6.88 μmol g DM^{-1} in control and inoculated plants, respectively (Fig. 3). These results reveal that occurred increase not significant in resistant cultivar after inoculation.

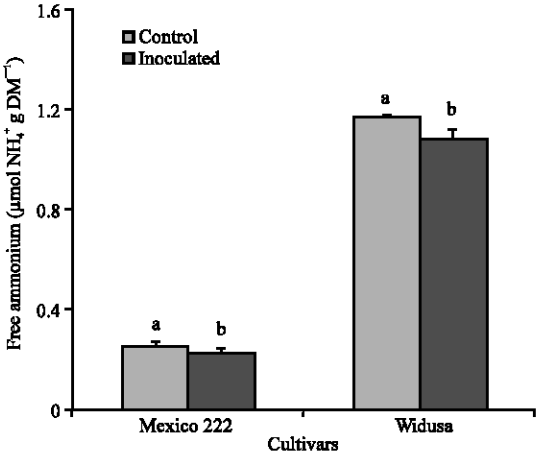

Fig. 1: Free ammonium in *P. vulgaris* cultivars México 222 and Widusa inoculated with *C. lindemuthianum* race 23. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

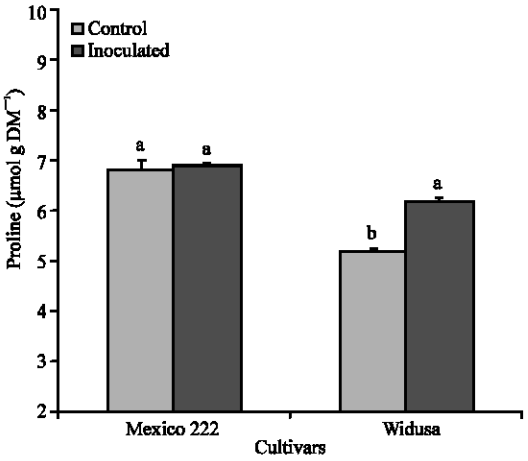

Fig. 3: Proline in *P. vulgaris* cultivars México 222 and Widusa inoculated with *C. lindemuthianum* race 23. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

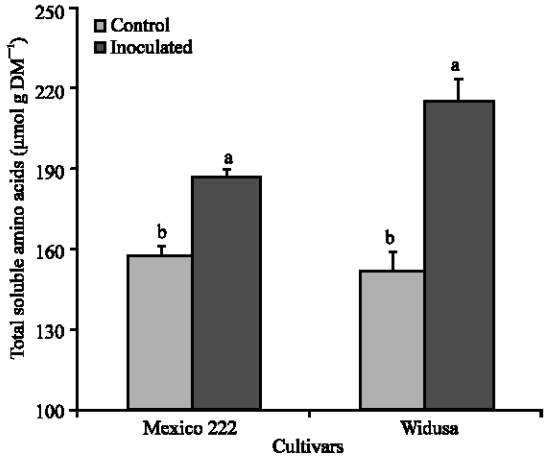

Fig. 2: Total soluble amino acids in *P. vulgaris* cultivars México 222 and Widusa inoculated with *C. lindemuthianum* race 23. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

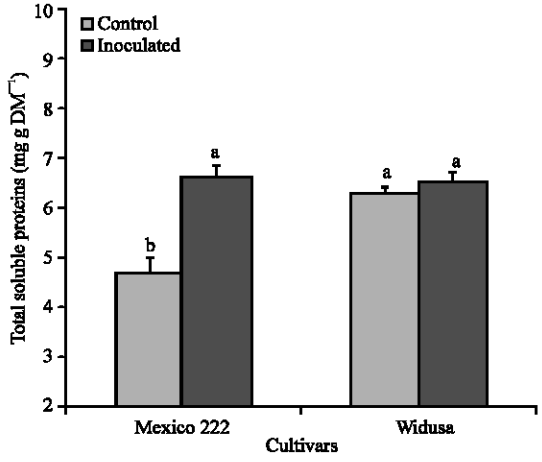

Fig. 4: Total soluble proteins in *P. vulgaris* cultivars México 222 and Widusa inoculated with *C. lindemuthianum* race 23. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

Total soluble proteins: The soluble proteins concentration in Mexico 222 presented significant increase at 38.3% after pathogen inoculation, which was showed in control and inoculated treatments 4.77 and 6.60 mg g DM^{-1}, respectively (Fig. 4). In Widusa showed not significant changes after inoculation of *C. lindemuthianum* race 23, as well as the control and inoculated plants presented the levels of 6.38 and 6.57 mg g MS^{-1}, respectively.

DISCUSSION

The ammonium free levels were reduced in both cultivars, which the reduction in ammonium free in Widusa cultivar (susceptible) occurs due to damages provoked by the pathogen in the pathway of nitrogen assimilation. However, the reduction in Mexico 222 cultivar (resistant) reveals that the ammonium free is converted in proteins and amino acids with the objective to active and express the resistance gene presents in this cultivar. The ammonium is the final form of inorganic nitrogen into of the production route of nitrogen organic compounds as proteins, amino acids and nucleotides (Nelson and Cox, 2000; Wu *et al.*, 2007), as well as the process of ammonium assimilation occurs through of the ammonium incorporation into carbon skeleton during the photosynthesis (Lancien *et al*., 2000).

The increase in total soluble amino acids level of the cultivar Mexico was probably provoked by the protease enzymes, which these enzymes breakdown the proteins with the objective to synthesize others amino acids that are utilized in biochemical pathway of activation of the resistance gene (Wu *et al.*, 2007). Similar results on increase in amino acids level was reported by Scarpari *et al.* (2005). Whereas amino acids accumulation in Widusa occurred due to mechanism of programmed cellular death, in which it provokes protein breakdowns (Hurst and Clark, 1993), besides it to reveal that these compounds can be utilized as biochemical indicators due to be very responsibles after to plant infection process.

The increase in proline level of the Widusa cultivar after the inoculation with *Colletotrichum lindemuthianum* race 23 occurred probably due to oxidative stress, in which the pathogen invasion provokes the formation of free radicals that are controlled by the osmoprotection mechanism. This amino acid has capacity to minimize the damaging effects provoked during biotic and abiotic stresses imposed to plant, besides it is utilized to intermediate the gene activation (Fabro *et al*., 2004; Lobato *et al*., 2008b). Results similars on proline accumulation were reported by Grote *et al.* (2006) investigating *Lycopersicon esculentum* plants inoculated by *Phytophthora nicotianae*.

The increase in amount of total soluble proteins is due the synthesis of proteins responsibles by the resistance to pathogen *Colletotrichum lindemuthianum* race 23, in which the resistance gene Co-3 (Gonçalves-Vidigal *et al*., 2008) presents in this cultivar promotes the synthesis of Pathogenesis-Related proteins (PR-proteins), as well as quinases and β-1, 3-glucanases that it induces the fortification of the cellular wall and increase the resistance against the pathogen invasion (Wu *et al*., 1997). Results similars were found by Junqueira *et al.* (2004) in *Zea mays* plants infected with bushy stunt phytoplasma. Whereas, the maintenance of the total soluble proteins in Widusa cultivar indicates that susceptible plants to anthracnose probably suffer 2 events simultaneous and opposites, in which the primary response is correlated with the strategy to keep the plant metabolism under normal conditions and it to survive the pathogen invasion increasing the amount of proteins (Wu *et al*., 2007). However, the secondary response occurs through of the protein breakdown induced by the protease enzymes that promotes the reduction of this nitrogen compound (Lobato *et al*., 2008a).

CONCLUSION

The results revealed that the Widusa cultivar (susceptible) suffered significant changes in free ammonium, total soluble amino acids and proline, however the total soluble proteins level was kept stable. The Mexico 222 cultivar (resistant) presented not significant modification only in proline level. In addition, this study proved the strong influence of the *Colletotrichum lindemuthianum* pathogen on the nitrogen metabolism of *Phaseolus vulgaris* plants.

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