Nickel-enriched soybean seeds generate plants more resistant to Asian soybean rust

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ABSTRACT: Asian soybean rust (ASR), triggered by *Phakopsora pachyrhizi*, can cause great yield losses on soybean and nickel (Ni) has potential to control this disease. This study evaluated the effect of using soybean seeds with different Ni contents on ASR control by performing two experiments. In experiment 1, seeds with different Ni contents were obtained by spraying plants three times with solutions containing either 60 or 100 gNi·ha⁻¹. Plants sprayed with water served as the control treatment. In experiment 2, plants originated from seeds obtained in experiment 1 (T1 = 0.9 µgNi·seed⁻¹, T2 = 1.2 µgNi·seed⁻¹, and T3 = 1.6 µgNi·seed⁻¹) were inoculated with *P. pachyrhizi* at the V4 growth stage. The ASR severity was evaluated 16 days after inoculation (DAI) and the chlorophyll (Chl) *a* fluorescence parameters at 8 and 16 DAI. The ASR severity decreased by 64 and 47% for treatments T2 and T3, respectively, in comparison to T1 treatment. The photosynthetic apparatus was negatively affected by ASR. The efficiency of the use of light by photosystem II decreased while the energy dissipated by nonregulated form increased in the infected leaflets. There was no significant difference for Chl *a* fluorescence parameters for T1, T2, and T3 treatments applied to inoculated plants probably due to the biotrophic lifestyle of *P. pachyrhizi* associated with lower ASR severity. In conclusion, the potential of using seeds with higher Ni content as a tool for ASR integrated management control is highlighted in this study.

Key words: *Glycine max*, *Phakopsora pachyrhizi*, rust, photosynthesis, plant nutrition.

Soybean (*Glycine max* [L.] Merr.) is the main source of protein for animal nutrition (Saath and Fachinello 2018). Asian soybean rust (ASR), caused by the biotrophic fungus *Phakopsora pachyrhizi* H. Sydow & P. Sydow, is considered one of the main diseases in soybean. The initial symptoms of this disease appear mainly on the abaxial surface of the leaves as polygonal chloroses. Subsequently, the symptoms evolve into necrotic areas, where uredium containing uredospores are massively formed (Langenbach et al. 2016). The leaf tissue around the uredia acquires a light brown color ("TAN" type lesion), in susceptible cultivars, to a reddish-brown color ("RB" type lesion), in resistant cultivars, that are easily visible on both sides of the leaf (Miles et al. 2011). Under high ASR severity conditions, plants present early defoliation, malformation of pods and reduced grains filling (Goellner et al. 2010). The application of fungicides is the main method of controlling ASR, implying a cost of around US$ 2 billion per year to Brazilian growers (Godoy et al. 2015). However, drastic reductions in the efficiency of chemicals on ASR control have been observed, and this has been related to the high number of mutants present in the *P. pachyrhizi* population associated with the selection pressure imposed on production areas due to the improper use of fungicides (Yokoyama et al. 2013; Schmitz et al. 2014; Klosowski et al. 2016).

Nickel (Ni), a component of the urease enzyme, was the last mineral element that was proven to be essential for plants (Brown et al. 1987). Foliar application of Ni in nonphytotoxic doses has been shown to increase the tolerance of plants to abiotic and biotic stresses (Fabiano et al. 2015). This micronutrient can act on antioxidant metabolism by increasing the activity of enzymes like catalase, peroxidase and superoxide dismutase, enzymes responsible for protecting the plant cell

against free radicals (González et al. 2015). Due to its high mobility in the phloem, up to 70% of the Ni present in the aerial part can be translocated to the seeds in the final period of the cycle of the plant (Cataldo et al. 1978). Studies have shown significant effects of Ni for the control of many diseases, such as scab in pecan (Wood et al. 2012), as well as powdery mildew and ASR in soybean (Barcelos et al. 2018; Einhardt et al. 2020 a). According to Einhardt et al. (2020 a), the foliar application of Ni to soybean plants promoted greater β-1,3-glucanase activity and expression of genes coding for urease, chalcone isomerase and phenylalanine ammonia-lyase, as well lignification of leaf tissues in response to *P. pachyrhizi* infection.

Considering the positive effect of Ni on ASR control, this study aimed to examine the possibility of using Ni-enriched soybean seeds to reduce disease symptoms in soybean leaves.

This study was carried out in two experiments in a greenhouse with temperature of 25 ± 3 °C, relative humidity of 65 ± 5%, and a photoperiod of 13 h. Plants from the cultivar TMG 135, ASR susceptible, were cultivated in 2 L plastic pots containing sand washed with 1.5 N hydrochloric acid (HCl). The plants were irrigated daily, alternating in each day deionized water and nutrient solution of Hoagland and Arnon (1950) (pH 5.8). To remove possible excess of accumulated salts on the surface of the roots, every 4 days the sand of the pots was soaked with deionized water until the formation of a water layer on the pot surface. This practice allowed the excess of volume to be drained via pre-holes established at the bottom of the pots.

In the first experiment, seeds with different Ni contents were obtained by spraying the plants with the solutions of 60 and 100 gNi·ha^{-1}. Plants sprayed with water served as the control treatment. The Ni sulfate (NiSO$_4$.6H$_2$O) was used as the source of Ni. The Ni solutions were sprayed into the plants at the phenological stages of V6, R3 and R5.4, according to Fehr and Caviness (1977). After the physiological maturity, the seeds were obtained from each plant, homogenized in terms of size, and stored in a refrigerator (8 °C) for the second experiment. To evaluate the Ni content in the seeds, a sample of 20 seeds from the replication of each treatment was collected and dried in a drying oven at 60 °C. After that, the dry weight of seeds from each sample was quantified to obtain the dry weight per seed. Seeds were ground in a ball mill (TECNAL TE 350, Piracicaba, SP, Brazil) for 1 min. The Ni was extracted by the nitric-perchloric digestion method and determined by inductive coupled plasma optical emission spectrometry (ICP-OES).

In the second experiment, seeds with different Ni concentrations produced in the first experiment were obtained and corresponded to the following treatments: T1 = 0.9 µgNi·seed^{-1}, T2 = 1.2 µgNi·seed^{-1}, and T3 = 1.6 µgNi·seed^{-1}. The seeds were sown in 2 L plastic pots containing washed sand. Plants were grown as described above. Plants at the V4 growth stage were inoculated with *P. pachyrhizi*, following the procedures reported by Einhardt et al. (2020 a).

The incubation period (IP) was evaluated in the inoculated leaves of each plant per replication of each treatment daily until the disease symptoms appeared. The ASR severity was assessed in the first trifoliate leaf using the diagrammatic scale proposed by Franceschi et al. (2020). Data from ASR severity was used to calculate the area under the disease progress curve (AUDPC) by using the trapezoidal integration of the disease progress curves, according to Shaner and Finney (1997). The first and second trifoliate leaves of each plant per replication of each treatment were collected at 17 DAI, scanned with a resolution of 600 dpi, and the images processed using the QUANT software (Fagundes-Nacarath et al. 2018) to obtain the final ASR severity.

The second trifoliate leaf of each plant per replication of each treatment was collected at 8 and 16 DAI to determine the parameters of chlorophyll (Chl) a fluorescence by using the Imaging-PAM fluorometer and the Imaging Win software MAXI version (Heinz Walz GmbH, Effeltrich, Germany) following the methodology reported by Fagundes-Nacarath et al. (2018). The time of actinic photon irradiance to obtain the steady-state fluorescence yield that was fixed at 5 min.

The first experiment was arranged in a completely randomized design with three treatments and three replications. The second experiment was arranged in a completely randomized design using a 3 × 2 factorial scheme (three Ni concentrations in the seeds [0.94, 1.21 and 1.59 µgNi·seed^{-1}]) and noninoculated or inoculated plants with *P. pachyrhizi* with four replications. For each experiment, the experimental unit consisted of a plastic pot with four plants. Data from the variables and parameters evaluated were checked for normality and homogeneity of variance and then submitted to analysis of variance (ANOVA). Means of treatments were compared by Tukey's test (p ≤ 0.05) by using the Minitab v.19 software (Minitab Inc.).

For the first experiment, the Ni content in the seeds was higher by 30 and 70% for plants of treatments T2 and T3, respectively, compared to plants from treatment T1 (Fig. 1). Plants from T3 treatment showed visual symptoms of phytotoxicity. However, no physiological disturbance was noticed on plants from treatment T3 in the second experiment, indicating no phytotoxicity in the plants generated from seeds containing high Ni content.
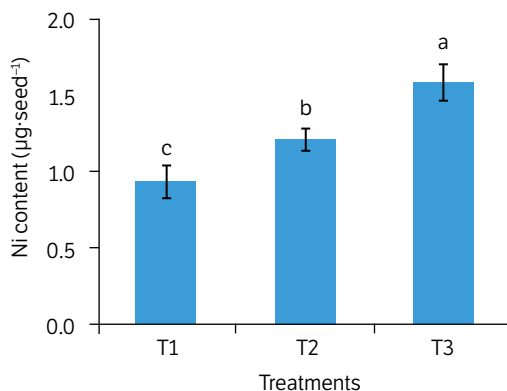
Figure 1. Nickel content in seeds of soybean plants sprayed with water (T1), sprayed three times with 60 gNi·ha^{-1} (T2), and sprayed three times with 100 gNi·ha^{-1} (T3). Means for each treatment followed by different letters are significantly different (p ≤ 0.05) according to Tukey's test. The bars represent the standard error of the means. n = 3.

For the second experiment, there was no significant difference in the IP of ASR among the treatments. The first ASR symptoms were noticed at 6 DAI. The ASR progressed much faster in plants from treatment T1 compared to plants for the other treatments (Fig. 2a). The AUDPC was significantly lower by 30 and 19% for plants from treatments T2 and T3, respectively, compared to plants from treatment T1 (Fig. 2b). There was no significant difference in the AUDPC values among the treatments T2 and T3. There were less necrotic lesions on the leaflets of plants from treatments T2 and T3 than in the leaflets of plants from treatment T1 (Fig. 3a-f). The ASR severity at 16 DAI was reduced by 61 and 51% for plants from treatments T2 and T3, respectively, compared to plants from treatment T1 (Fig. 4).
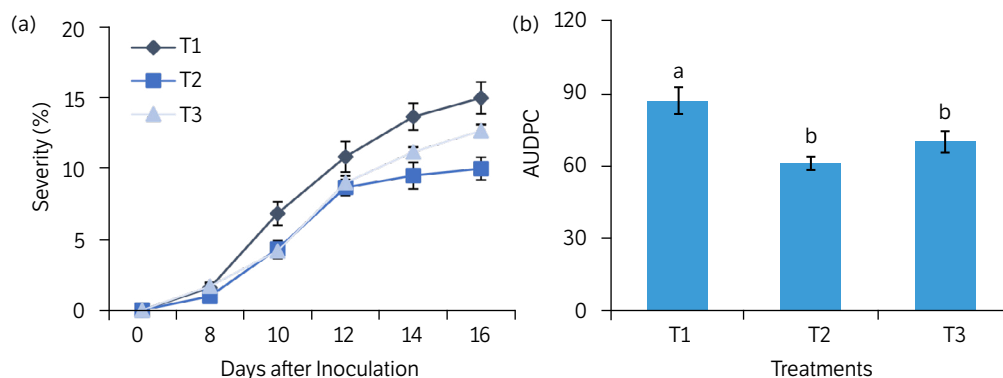

Figure 2. Asian soybean rust severity progress curves (a) and area under the disease progress curve (AUDPC) (b) determined in the leaflets of soybean plants obtained from seeds containing different contents of Ni (T1: 0.94 µgNi·seed^{-1}, T2: 1.21 µgNi·seed^{-1} and T3: 1.59 µgNi·seed^{-1}). Means for each treatment followed by different letters are significantly different (p ≤ 0.05) according to Tukey's test. The bars represent the standard error of the means. n = 8.

Based on Fig. 5, it is possible to notice an apparent effect of *P. pachyrhizi* infection in the parameters of Chl *a* fluorescence, especially at 16 DAI. The ANOVA showed no significant difference for the Chl *a* fluorescence parameters among the Ni concentrations (Fig. 6). However, significant differences in the photosynthetic parameters occurred between noninoculated and inoculated plants, mainly at 16 DAI. The maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) at 16 DAI was lowered by 5, 4, and 3% for inoculated plants of treatments T1, T2, and T3, respectively, in comparison to noninoculated plants of these same treatments (Fig. 6a-b). At 16 DAI, the effective quantum yield of PSII (Y(II)) values were lower by 32, 32, and 35% for inoculated plants of the treatments T1, T2, and T3, respectively, in comparison to noninoculated plants of these same treatments (Fig. 6c-d). For the yield of energy dissipated by down-regulation (Y(NPQ)), there was no significant difference between noninoculated and inoculated plants regardless of Ni contents on seeds (Fig. 6e-f). The yield for other

nonphotochemical (nonregulated) losses (Y(NO)) was higher by 10, 7, and 10% at 8 DAI and by 21, 17, and 20% at 16 DAI for inoculated plants of the treatments T1, T2, and T3, respectively, in comparison to noninoculated plants of these same treatments (Fig. 6g-h). The electron transport rate (ETR) was lower by 24, 24 and 28% at 8 DAI and by 53, 45 and 46% at 16 DAI for inoculated plants of treatments T1, T2 and T3, respectively, in comparison to noninoculated plants of these same treatments (Fig. 6i-j).
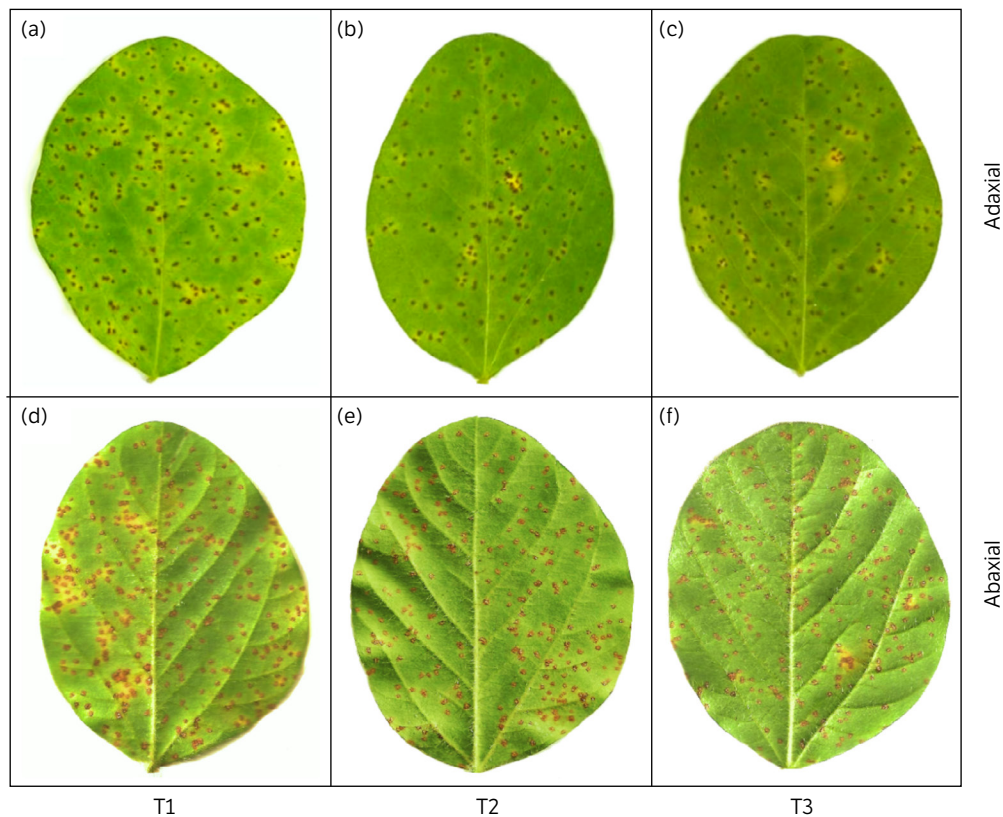

Figure 3. Symptoms of ASR on the adaxial (a-c) and abaxial (d-f) surfaces of leaflets obtained from soybean plants at 16 DAI. These leaflets were from plants whose seeds contained different contents of Ni (T1: 0.94 µgNi·seed^{-1}, T2: 1.21 µgNi·seed^{-1} Ni and T3: 1.59 µgNi·seed^{-1}).
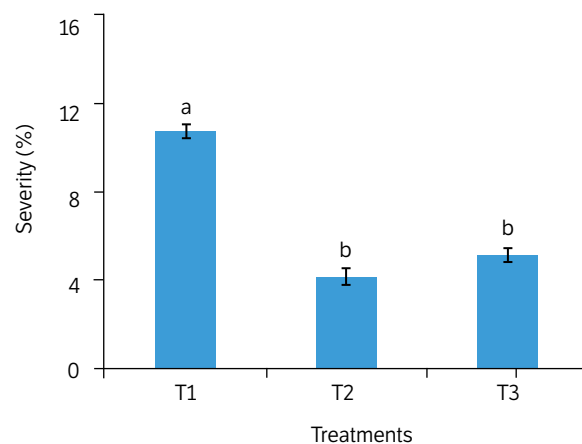

Figure 4. Asian soybean rust severity in the leaflets of soybean plants obtained from seeds containing different contents of Ni (T1: 0.94 µgNi·seed^{-1}, T2: 1.21 µgNi·seed^{-1} and T3: 1.59 µgNi·seed^{-1}). Means for each treatment followed by different letters are significantly different (p ≤ 0.05) according to Tukey's test. The bars represent the standard error of the means. DAI = days after inoculation. n = 8.
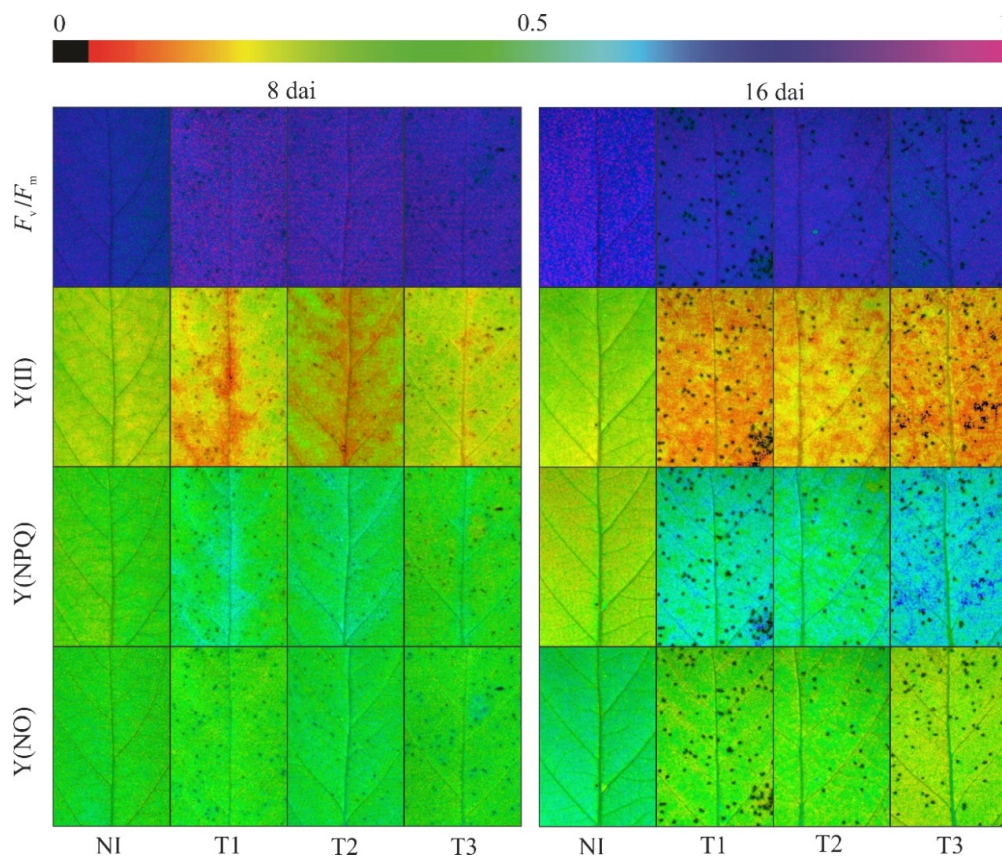
Figure 5. Images of the Chl *a* fluorescence parameters: maximum photochemical efficiency of PSII (F_v/F_m), effective quantum yield of PSII (Y(II)), yield of energy dissipated by down-regulation (Y(NPQ)), and yield for other nonphotochemical (nonregulated) losses (Y(NO)) determined in the leaves of soybean plants obtained from seeds containing different contents of Ni (T1: 0.94 µgNi·seed⁻¹, T2: 1.21 µgNi·seed⁻¹ and T3: 1.59 µgNi·seed⁻¹) and noninoculated (NI) or inoculated with *P. pachyrhizi*. dai = days after inoculation.

The foliar application of increasing doses of Ni generated seeds with a higher content of this micronutrient, confirming, therefore, its high mobility in the phloem and translocation to the reproductive parts of the plants and corroborating with the findings of Cataldo et al. (1978). The present study shows the positive effect of using Ni-enriched seeds for ASR control without any apparent damage to their photosynthetic apparatus. According to Einhardt et al. (2020a), on soybean supplied with Ni, ASR severity was reduced due to an increase in the *β*-1,3-glucanase activity, high lignin concentration, and a great expression of the *URE* gene and the defense-related genes *PAL1.1*, *PAL2.1*, *CHI1B1* and *PR-1A*.

The photosynthetic apparatus of soybean leaflets was significantly affected only at advanced stages of fungal infection (16 DAI), similar to what was observed by Rios et al. (2017). This result may be related to the biotrophic nature of *P. pachyrhizi* that did not cause any cytological damage in the infected leaflets. Considering that F_v/F_m is an indicator of the photosynthetic performance of plants, with optimal values close to 0.8 for most species (Krause and Weis 1991), and that Y(II) is the effective quantum yield of PSII, reductions in the values of these parameters for inoculated plants indicated losses in photosynthetic efficiency of infected plants. The nonsignificant difference in Y(NPQ) between noninoculated and inoculated plants, regardless of the treatments in the second experiment, indicated that the fungal infection was not able to provoke any physiological alteration in the mechanism of photoprotection by dissipating the excess of energy through heat (Kramer et al. 2004). For Y(NO), there was a significant difference between noninoculated and inoculated plants at 8 and 16 DAI. According to Klughammer and Schereiber (2008), progressive increases in the Y(NO) values were associated with photooxidative damage in leaf tissues, suggesting that protective regulatory mechanisms become physiologically inefficient. These changes probably reflect the inability of the plants to regulate their photoprotection mechanisms, resulting in greater photooxidative damage in the infected tissues (Rolfe and Scholes 2010).
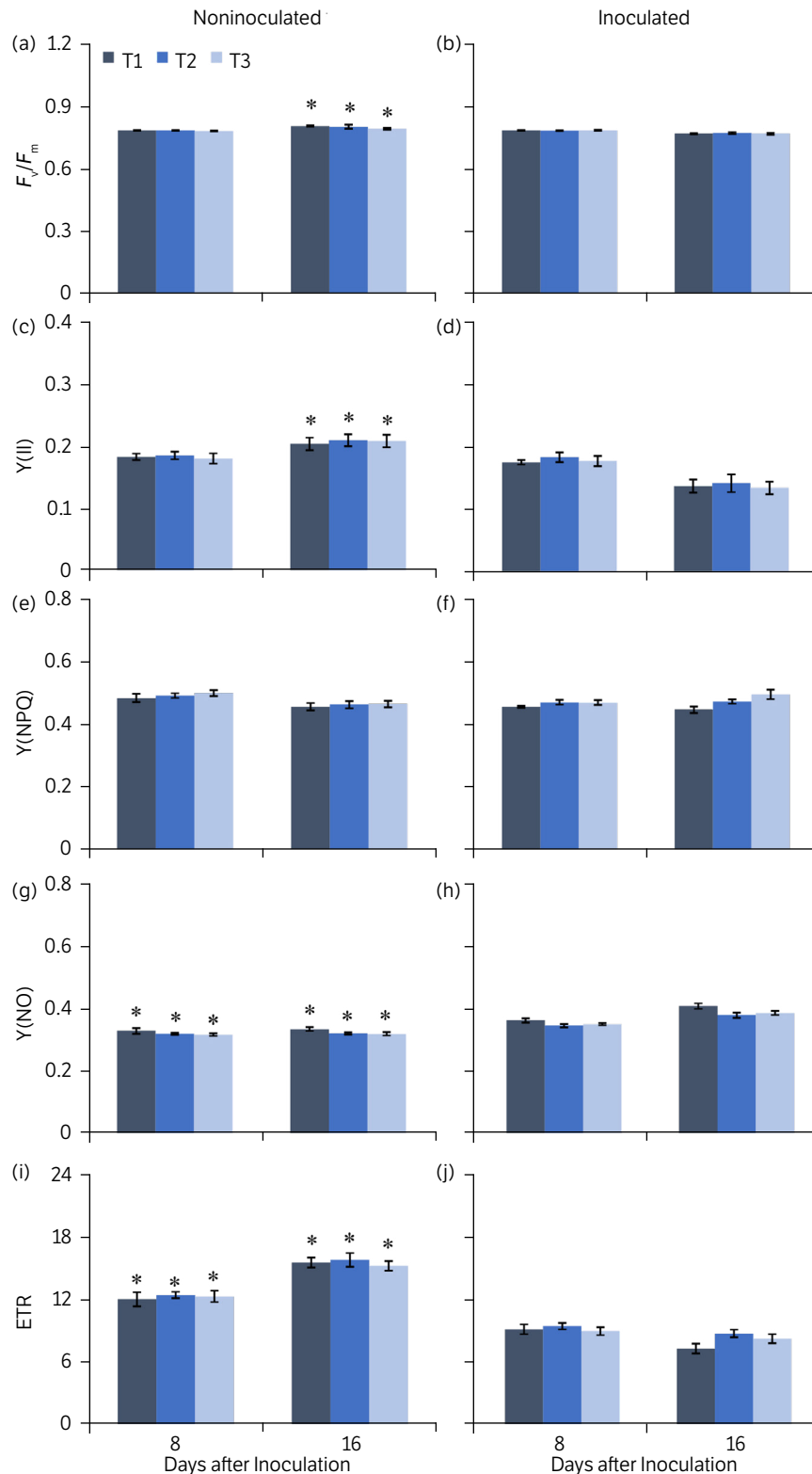
Figure 6. Chlorophyll *a* fluorescence parameters: maximum photosystem II quantum efficiency (F_v/F_m), photochemical yield (Y(II)), yield for dissipation by down-regulation (Y(NPQ)), and yield for other nonphotochemical (nonregulated) losses (Y(NO)) determined in the leaves of soybean plants obtained from seeds containing different contents of Ni (T1: 0.94 µgNi·seed^{-1}, T2: 1.21 µgNi·seed^{-1} and T3: 1.59 µgNi·seed^{-1}) and noninoculated (a, c, e, g and i) or inoculated (b, d, f, h and j) with *P. pachyrhizi*. For each evaluation time, means for noninoculated and inoculated plants followed by an asterisk (*) are significantly different (p ≤ 0.05) according to F test. The ANOVA showed no significant difference for the Chl *a* fluorescence parameters among the Ni concentrations. The bars represent the standard error of the means. n = 4.

Similarly to the findings of Einhardt et al. (2020 b), the decrease in the ETR values for inoculated plants accompanied the lower F_v/F_m and Y(II) values, indicating the occurrence of photochemical dysfunctions and that the photoprotection mechanism was inefficient for avoiding damage in the photosynthetic apparatus, as reported by Klughammer and Schereiber (2008).

Although the reduction in ASR severity for plants, originated from Ni-enriched seeds, occurred in contrast to plants originated from seeds with low Ni content in the second experiment, there was no significant difference for Chl *a* fluorescence parameters regardless of the treatments and plant inoculation. This result may be related to the biotrophic nature of the fungus and the lower disease severity occurring in the plants. It is well-known that biotrophic fungi do not cause any apparent damage to the infected plant tissues during their infection process, as well as when the disease reaches low levels of severity (Langenbach et al. 2016). Reduced damage to the photosynthetic apparatus soybean plants due to *P. pachyrhizi* infection was sufficient to promote significant differences between noninoculated and inoculated for the Chl *a* fluorescence parameters examined, regardless of the treatments imposed on the plants.

According to Rodak et al. (2015), an initial content of 0.15 μg·Ni·seed^{-1} met the soybean requirement of the plants for this element during their life cycle. The content of Ni in the seeds of plants from treatments T1, T2, and T3 was considerably higher than those indicated as ideal by Rodak et al. (2015). Brown et al. (1987) grew barley plants in nutrient solution and showed that the Ni content in the seeds of 0.25 μg·Ni·g^{-1} was sufficient to supply this micronutrient up to three generations of plants without any sign of its deficiency. Therefore, it is acceptable to hypothesize that low levels of Ni in the seeds may not generate deficiency symptoms in plants, but may predispose them to greater susceptibility to diseases. In the present study, it was verified that low Ni content in soybean seeds was sufficient to avoid Ni deficiency symptoms, but this suboptimal Ni content resulted in more ASR symptoms.

In conclusion, the results of the present study pointed out the potential of using Ni-enriched seeds as a tool for ASR management. However, additional studies are needed to determine the ideal concentrations of this micronutrient in the seeds obtained from soybean cultivars with different resistance levels to ASR.

AUTHORS' CONTRIBUTION

Conceptualization: Ferreira S. and Einhardt A. M.; **Methodology:** Ferreira S., Einhardt A. M., and Rodrigues F. A.; **Investigation:** Ferreira S., Picanço B. B. M., Fontes B. A., and Einhardt A. M.; **Writing – Original Draft:** Ferreira S. and Einhardt A. M., and Rodrigues, F. A.; **Writing – Review and Editing:** Rodrigues F. A.; **Funding Acquisition:** Rodrigues F. A.; **Resources:** Rodrigues F. A.; **Supervision:** Rodrigues F. A.

DATA AVAILABILITY STATEMENT

Data will be available upon request.

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