Nitrogen Metabolism in Pepper Plants Applied with Different Bioregulators

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Certain bioregulators were studied in relation to nitrogen metabolism of pepper plants (*Capsicum annuum* L. cv. Lamuyo). Plants were grown under controlled conditions and submitted to regular fertilization with macro- and micronutrients. Treatments were as follows: nontreated control (T0); fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})] (T1); biozyme [GA_3 (32.2 mg L^{-1}) plus IAA (32.2 mg L^{-1}) plus zeatin (83.2 mg L^{-1}) plus chelated micronutrients] (T2); and GA_3 [16 mg L^{-1}] (T3). The concentrations of NO_3^-, organic N, amino acids, and proteins, the activities of nitrate reductase (NR) and nitrite reductase (NiR), and finally the foliar dry weight and yield were analyzed. The results indicated that the application of certain bioregulators, such as fosfonutren (T1), which contain amino acids can cause a negative effect on the efficiency and utilization of NO_3^-, resulting in a drastic loss in growth and yield, even under the control treatment, in which no bioregulator was applied. On the contrary, the application of certain bioregulators based principally on the combination of different hormones, as in the case of biozyme (T2), increased NO_3^- assimilation under our experimental conditions, due possibly to a greater availability of these bioregulators in the leaves and increased NR and NiR activities. This appears to explain why the T2 treatment gave the greatest foliar dry weight and fruit yield per plant in the experiment.

Keywords: *Capsicum annuum L.; nitrogen metabolism; bioregulators; yield*

INTRODUCTION

Plant growth is dependent on an adequate supply of nitrogen (N) required for the formation of the amino acids, proteins, nucleic acids, and other cellular constituents necessary for development. For most plants, inorganic nitrogen is obtained from the soil in the form of NO_3^-. The assimilation of NO_3^- by plants requires the uptake of NO_3^-, reduction to NO_2^-, the conversion of NO_2^- to NH_4^+, and the incorporation of NH_4^+ to organic compounds (Sivasankar and Oaks, 1996; Migge and Becker, 1996). The first step in NO_3^- assimilation, the reduction of nitrate to nitrite catalyzed by nitrate reductase, is highly regulated (Huber et al., 1996; Gojon et al., 1998). Nitrate availability, growth regulators, light, products of nitrate assimilation, and other physiological and environmental parameters are all factors in the regulation of nitrate assimilation (Lillo, 1994; Crawford, 1995; Campbell, 1996; Padgett and Leonard, 1996; Sivasankar and Oaks, 1996; Ruiz et al., 1998, 1999a,b; Ruiz and Romero, 1998).

The activation of NO_3^- assimilation and, more specifically, the reduction of NO_3^- to NO_2^- by nitrate reductase (NR) would avoid or diminish the negative effects produced by the accumulation of NO_3^- in plants that need large amounts of nitrogen for growth.

As we have indicated, one of the factors that directly influence NO_3^- assimilation is the application of bioregulators. Although information on this subject is scant, early studies indicated that the applications of cytokinines, gibberellins, and indolacetic acid boost NR activity in the absence of NO_3^-, whereas the application of Ethephon stimulates NR activity only in the presence of NO_3^- (Hänisch ten Cate and Bretele, 1982). More recent research, by Chanda et al. (1998), confirms the above conclusions, reporting that the effect of the bioregulators giberrellic acid, kinetin, and abscisic acid on nitrogen metabolism is based on the induction of NR activity.

Many of the bioregulators currently used in agriculture contain compounds such as amides and amino acids. These products, as potential end products of NO_3^- assimilation, are known to inhibit the process through NR activity (Padgett and Leonard, 1996; Shankar and Srivastava, 1998). The effect of amino acids and amides on NR activity varies according to the species and nature of the amino acid or the amide. For example, the inhibition of this enzyme activity by glutamine has been reported in tobacco (Vincentz et al., 1993) and maize (Li et al., 1995), although glutamine had no effect in spinach (Sánchez and Heldt, 1990) and even increased in vitro NR activity in *Datura* (Lillo, 1994).

Finally, the aim of the present experiment was to analyze the effect of the foliar application of different bioregulators, which are commonly used in agriculture, on the foliar assimilation of NO_3^- and on the growth and yield of pepper plants.

MATERIALS AND METHODS

Crop Design. Seeds of *Capsicum annuum* L. cv. Lamuyo were sown in September 1997. The seedlings were grown in individual peat pots in an experimental greenhouse in southern Spain (Granada) for 45 days and then transferred to a cultivation chamber under controlled environmental conditions with relative humidity of 60−80%, temperature of 30/20 °C (day/night), and 16/8 h photoperiod at a PPFD of 350 μmol

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m^{-2} s^{-1} (measured at the top of the plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE). The plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height; 8 L in volume) filled with vermiculite. Throughout the experiment, each plant received a nutrient solution of 6 mM KNO_3, 2 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 5 μM H_3BO_3. The nutrient solution (pH 5.5−6.0) was renewed every 3 days.

Three bioregulators were applied to plants to runoff as aqueous foliar sprays containing the surfactant Tween 20 (0.5% v/v), using a stainless steel sprayer. The dosages of each bioregulator applied approximated the usual rates applied in the intensive agriculture of southeastern Spain. Treatments were nontreated control (T0), fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})] (T1), biozyme [GA_3 (32.2 mg L^{-1}) plus IAA (32.2 mg L^{-1}) plus zeatin (83.2 mg L^{-1}) plus chelated micronutrients] (T2), and GA_3 [16 mg L^{-1}] (T3). The first application was at the onset of flowering (60 days after sowing), followed by two sprays at intervals of 30 days. The experimental design was a randomized complete block with four treatments, arranged in individual pots with four plants per treatment, each one replicated three times.

Plant Sampling. The plants were sampled at 150 days after sowing. Leaf samples were standardized by using plants with the same size of fully expanded leaves, from the middle part of each replicate plant. The material was rinsed three times in distilled water after it had been disinfected with nonionic detergent at 1% (Wolf, 1982) and then blotted on filter paper. At each sampling, fresh leaf matter was used for the analysis of enzymatic activities [NR and nitrite reductase (NiR)], NO_3^- concentration, amino acids, and proteins. A subsample was dried in a forced air oven at 70 °C for 24 h. Foliar dry weight was recorded and expressed as milligrams of dry weight (dw) per leaf.

Plant Analysis. At each leaf sampling, portions of leaves were ground, with a ratio of 1:10 (w/v), in a mortar at 0 °C in 50 mM KH_2PO_4 buffer, pH 7.5, containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 3000*g* for 5 min, after which time the supernatant was centrifuged at 30000*g* for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure NO_3^- and NR and NiR enzyme activities. The extraction medium was optimized for the enzymatic activities so that these could be extracted jointly according to the same method (Groat and Vance, 1981; Lillo, 1984; Kaiser and Lewis, 1984; Singh and Srivastava, 1986).

For the determination of the NO_3^-, a 100 μL aliquot was taken for nitrate determination and added to 10% salicylic acid solution dissolved in 12 N sulfuric acid (w/v). The NO_3^- concentration was measured by spectrophotometry as described by Cataldo et al. (1975). The results were expressed as micromoles per gram of fresh weight (fw).

The NR assay (EC 1.6.6.1) followed the methodology of Kaiser and Lewis (1984). In a final volume of 2 mL, the reaction mixture contained 100 mM buffer KH_2PO_4, pH 7.5, 100 mM KNO_3, 10 mM cysteine, 2 mM NADH, and enzyme extract. For the NR assay, the incubation was carried out at 30 °C for 30 min and stopped by the addition of 1000 mM zinc acetate. The nitrite formed was colorimetrically determined at 540 nm after azocoupling with sulfanylamide and naphthylethylenediamine dihydrochloride according to the method of Hageman and Hucklesby (1971). The NR activity was expressed as micromoles of NO_2^- formed per gram of fresh weight per hour.

NiR activity (EC 1.7.7.1) was determined by the disappearance of NO_2^- from the reaction medium (Lillo, 1984). The reaction mixture contained 50 mM buffer KH_2PO_4, pH 7.5, 20 mM KNO_2, 5 mM methylviologen, 300 mM $NaHCO_3$, and 0.2 mL of enzyme extract. After incubation at 30 °C for 30 min, the nitrite content was determined colorimetrically as above (Hageman and Hucklesby, 1971) and expressed as micromoles of NO_2^- per gram of fresh weight per hour.

Table 1. Response of the Different Nitrogen Forms to the Foliar Application of Several Bioregulators[a]

treatment	nitrate, μmol g^{-1} of fw	amino acids, mg g^{-1} of fw	proteins, mg g^{-1} of fw	organic N, mg g^{-1} of dw
T0	190.3 ± 9.0	1.65 ± 0.07	6.57 ± 0.24	36.3 ± 2.23
T1	149.1 ± 9.3	2.83 ± 0.09	10.1 ± 0.62	32.8 ± 2.11
T2	224.1 ± 12.2	1.77 ± 0.08	8.13 ± 0.39	41.5 ± 2.42
T3	203.2 ± 10.4	1.72 ± 0.07	8.01 ± 0.28	38.1 ± 2.15
LSD at 0.05	18.54	0.22	0.89	3.03

[a] Data are means ± SE ($n = 3$). The least significant difference (LSD) at 5% probability is provided for comparison of means. T0, control plants; T1, fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})]; T2, biozyme [GA_3 (32.2 mg L^{-1}) + IAA (32.2 mg L^{-1}) + zeatin (83.2 mg L^{-1}) + chelated micronutrients]; T3, GA_3 [16 mg L^{-1}].

Amino acids and proteins were determined by homogenization of 0.5 g of fresh leaf samples in 50 mM cold KH_2PO_4 buffer at pH 7 and centrifugation at 12000*g* for 15 min. The resulting supernatant was used for the determination of total amino acids according to the ninhydrin method of Yemm and Cocking (1955); total free amino acids were expressed as milligrams per gram of fresh weight. Soluble proteins were measured by Bradford G-250 reagent (Bradford, 1976) and expressed as milligrams per grams of fresh weight, using as standard bovine serum albumin (BSA).

Organic N determination was made according to the following procedures: Dry weight (0.1 g) was digested in sulfuric acid in the presence of H_2O_2 (Wolf, 1982). After dilution with deionized water, an aliquot of 1 mL was added to a reaction medium containing buffer [5% potassium sodium tartrate, 100 mM sodium phosphate, and 5.4% (w/v) sodium hydroxide], 15%/0.03% (w/v) sodium salicylate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37 °C for 15 min, and organic N was measured by spectrophotometry (Baethgen and Alley, 1989). The results were expressed as milligrams per gram of dry weight.

Finally, peppers were usually harvested weekly at the mature red stage. Yield was determined by counting and weighing all fruits on each plant.

Statistical Analysis. The data shown are mean values ± standard error (SE). Differences between treatments were compared using the least significant difference (LSD) at the 0.05 probability level. Levels of significance are represented by * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, and ns (not significant).

RESULTS AND DISCUSSION

The application of the bioregulators corresponding to treatments T1 and T2 varied significantly in the foliar NO_3^- concentration in relation to the concentration recorded in control plants ($P < 0.001$) (Table 1); the lowest foliar concentrations appeared for T1, with a decrease of 34% with respect to the highest concentration for T2. It was striking that no statistically significant differences appeared between control plants and those treated with T3 (Table 1).

One of the main effects of the exogenous application of amino acids on N metabolism is the reduction in NO_3^- uptake. Muller and Touraine (1992) demonstrated a 50% decline in NO_3^- uptake after the application of alanine, glutamine, asparagine, arginine, β-alanine, serine, and glutamine. More recently, works such as those of Muller et al. (1995) and Barneix and Causin (1996) have shown that lower NO_3^- uptake caused by excess amino acids is due primarily to an inhibition of NO_3^- input. This inhibition results from the control exerted by certain amino acids imported from the leaves to the roots through the phloem. Finally, Padgett and Leonard (1996) have also indicated that the application

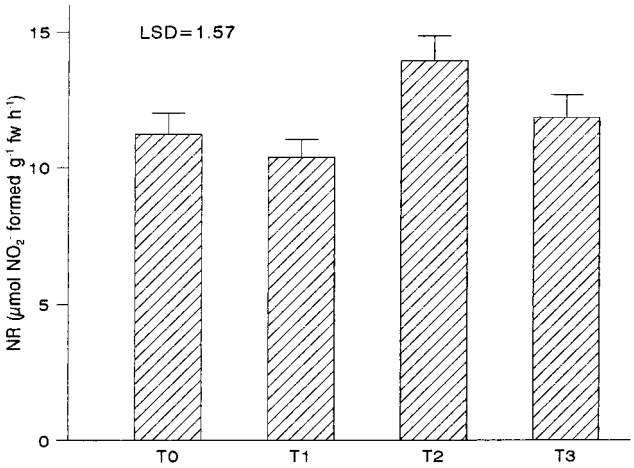

Figure 1. Response of the NR activity to the foliar application of several bioregulators. Data are means ± SE (n = 3). The LSD at 5% probability is provided for comparison of means. T0, control plants; T1, fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})]; T2, biozyme [GA_3 (32.2 mg L^{-1}) + IAA (32.2 mg L^{-1}) + zeatin (83.2 mg L^{-1}) + chelated micronutrients]; T3, GA_3 [16 mg L^{-1}].

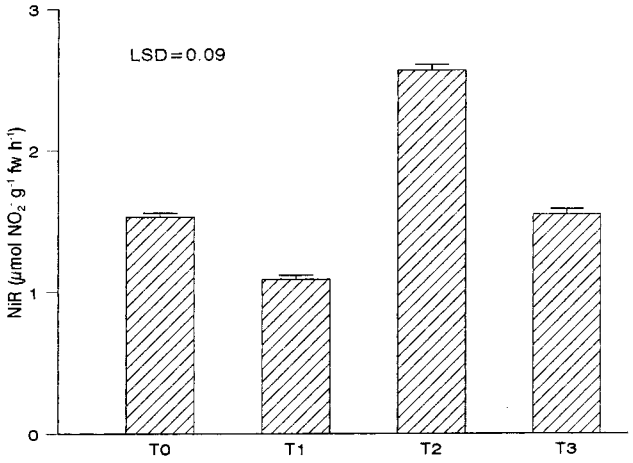

Figure 2. Response of the NiR activity to the foliar application of several bioregulators. Data are means ± SE (n = 3). The LSD at 5% probability is provided for comparison of means. T0, control plants; T1, fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})]; T2, biozyme [GA_3 (32.2 mg L^{-1}) + IAA (32.2 mg L^{-1}) + zeatin (83.2 mg L^{-1}) + chelated micronutrients]; T3, GA_3 [16 mg L^{-1}].

of amino acids individually at a concentration of 2 mM inhibits NO_3^- uptake in maize cells. All of these results would account for the lowest foliar NO_3^- concentrations in our experiment being found for the application of the bioregulator fosfonutren (T1).

In addition, NR is the principal limiting enzyme in the process of NO_3^- assimilation (Campbell, 1996; Huber et al., 1996; Gojon et al., 1998). The trends of NR activity varied in the same way as that of NO_3^-, depending on the bioregulator applied ($P < 0.001$) (Figure 1). The highest NR activity occurred for T2, with an increase of 34% with respect to the lowest for T1. Again, no statistically significant differences were found between control and T3 plants. The main effect of the bioregulators on the nitrogen metabolism derives, according to the literature available, primarily from the induction or, on the contrary, the inhibition of NR activity.

As indicated in the Introduction, different works have reported that the joint application of cytokinines, gibberellins, IAA, and kinetin induces NR activity (Hänisch ten Cate and Bretele, 1982; Chanda et al., 1998). Our experiment gave results similar to those of these authors, in that the joint application of GA_3, IAA, and Zeatina (T2: biozyne) led to the highest NR activity, whereas, on the contrary, the individual application of GA_3 (T3: gibberellin) failed to stimulate NR (Figure 1). Finally, the lowest NR activity was recorded for T1, possibly due to the action of the amino acids applied, because, as described in several other works, another effect of the application of these compounds on the nitrogen metabolism is the inhibition of NR activity (Barneix and Causin, 1996; Shankar and Srivastava, 1998).

Cellular NO_3^- levels constitute one of the determining factors in the assimilation of NO_3^- (Zhang and Mackown, 1993; Crawford, 1995; López-Cantarero et al., 1997; Ruiz and Romero, 1998; Ruiz et al., 1998, 1999a). Changes in the quantity and activity of NR parallel the accumulation and disappearance of NO_3^- in plant tissues. In our experiment, the relationship between NO_3^- and the activity of NR was positive and significant (r = 0.83***). NiR activity, which converts NO_2^- to NH_4^+ (Migge and Becker, 1996; Sivasankar and Oaks, 1996), showed a trend similar to that of NR ($P < 0.001$) (Figure 2), presenting the highest activity for T2 and the lowest for T1, with a decline of 57%. As in the case of NR, the NiR activity is induced mainly by NO_3^- (Oaks, 1994; Ruiz et al., 1998, 1999a), the relationship between the two parameters in our experiment being positive and significant (r = 0.86***). In light of our results, we suggest that the action of bioregulators, mainly T1 and T2, on NR and NiR activities could be indirect, resulting from the direct action of bioregulators on the NO_3^- uptake and translocation toward the aerial part.

The end products of NO_3^- assimilation are amino acids and proteins (Barneix and Causin, 1996). The trend of these nitrogenous compounds was completely contrary to that of the enzymes NR and NiR, given that the highest foliar concentrations were presented for the application of the bioregulated fosfonutren (T1) and the lowest in control (T0) (amino acids, $P < 0.05$; proteins, $P < 0.001$) (Table 1). As expected, the application of fosfonutren, which has amino acids in its composition, augmented the accumulation of these compounds in the leaves. The increased foliar content of these nitrogenous compounds for T1 and their possible translocation toward different organs of the plant, such as the roots, could be the reason for the decline in both the uptake and translocation of NO_3^- toward the aerial part (Table 1), as well as the decline in the NR (Figure 1) and NiR (Figure 2) activities. On the other hand, the fact that the foliar concentrations of amino acids were similar in T0, T2, and T3 suggests that the principal action of treatment T2 was based either on stimulating absorption and translocation of NO_3^- toward the shoot, or, as proposed by other authors (Hänisch ten Cate and Bretele, 1982; Chanda et al., 1998), or on stimulating NR and NiR activities.

Another of the nitrogenous compounds increased by NO_3^- assimilation is organic N (Vicentz et al., 1993). As opposed to amino acids and proteins, organic N followed a trend similar to that of NR, registering the highest foliar concentrations for T2 and the lowest for T1 ($P < 0.001$) (Table 1). The relationship between the two nitrogenous parameters was positive and significant

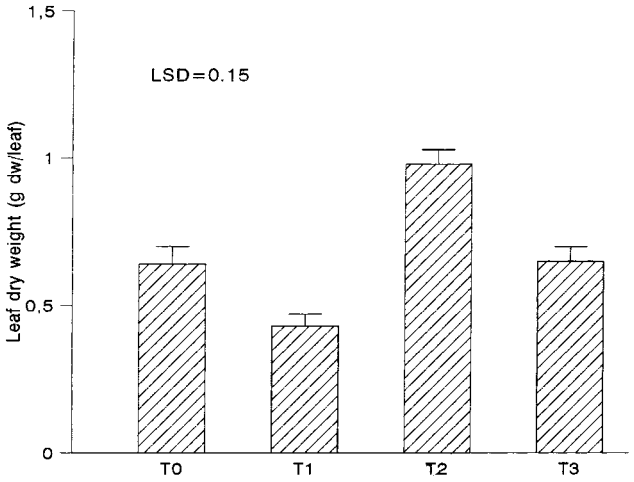

Figure 3. Response of the foliar dry weight to the foliar application of several bioregulators. Data are means ± SE ($n = 3$). The LSD at 5% probability is provided for comparison of means. T0, control plants; T1, fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})]; T2, biozyme [GA_3 (32.2 mg L^{-1}) + IAA (32.2 mg L^{-1}) + zeatin (83.2 mg L^{-1}) + chelated micronutrients]; T3, GA_3 [16 mg L^{-1}].

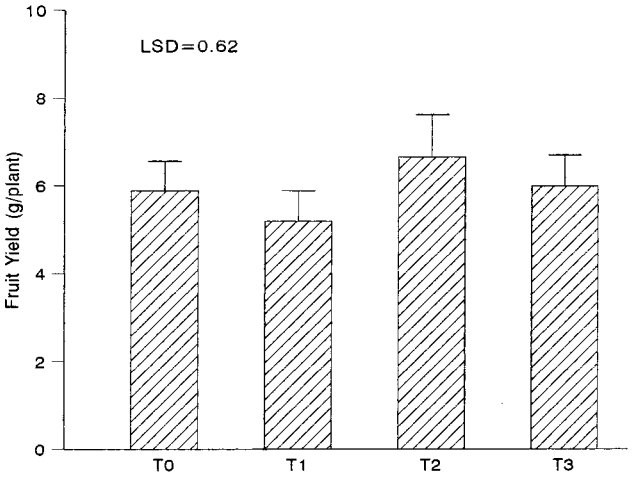

Figure 4. Response of the fruit yield to the foliar application of several bioregulators. Data are means ± SE ($n = 3$). The LSD at 5% probability is provided for comparison of means. T0, control plants; T1, fosfonutren [essential amino acids and micronutrients (46.9 mg L^{-1})]; T2, biozyme [GA_3 (32.2 mg L^{-1}) + IAA (32.2 mg L^{-1}) + zeatin (83.2 mg L^{-1}) + chelated micronutrients]; T3, GA_3 [16 mg L^{-1}].

($r = 0.94$***), indicating that the NO_3^- assimilation was favored for T0, T3, and especially T2, in contrast to T1. The highest concentrations of organic N for T2 may have resulted because, although this treatment presented the greatest NO_3^- assimiltion, the foliar amino acid and protein levels were similar to those of T0 and T3, both of these showing lower NO_3^- reduction rates (Table 1).

In other works, it has been demonstrated that greater efficiency in the use of N leads to a general increase in foliar dry weight and final yield of a plant (Mattson et al., 1991; McDonald et al., 1996; López-Cantarero et al., 1997; Ruiz and Romero, 1998, 1999a,b; Ruiz et al., 1999b). In our experiment, both foliar biomass and foliar dry weight ($P < 0.01$) (Figure 3) as well as fruit yield ($P < 0.01$) (Figure 4) were highest for T2, whereas the lowest values were found for T1. On the other hand, as occurred with other parameters of N metabolism, foliar biomass and fruit yield were similar in control and T3 plants (Figures 3 and 4). Taking NO_3^- assimilation to organic N as a reference, we confirm the directly proportional relationship with the growth parameters analyzed (organic N−foliar biomass, $r = 0.96$***; organic N−yield, $r = 0.98$***). Finally, the highest fruit yield in treatment T2 may be due to amino acid translocation toward the fruits (Ruiz and Romero, 1999b), a situation that would also explain the comparable levels between T2 and T3 in amino acids and proteins (Table 1).

In conclusion, the application of certain bioregulators, such as fosfonutren (T1), that contain amino acids can negatively affect the efficiency and utilization of NO_3^-, resulting in a drastic loss in growth and yield, even under the control treatment, in which no bioregulator was applied. On the contrary, the application of certain bioregulators based principally on the combination of different hormones, as in the case of biozyme (T2), increased NO_3^- assimilation under our experimental conditions, due possibly to a greater availability of these bioregulators in the leaves and increased NR and NiR activities. This would explain why this treatment gave the greatest foliar dry weight and fruit yield per plant in our experiment. Finally, under our experimental conditions, the application of hormone GA_3 (T3) did not stimulate N metabolism nor yield in foliar biomass or fruit, as compared with control values. These results confirm the effectiveness of the application of bioregulators that combine different hormones (T2).

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