

Liquid fertilizer from nest refuse of leaf-cutter ant nests (*Atta* sp.) in lettuce seedling production

Abstract

The demand for bioinputs for use in agriculture is growing, and liquid biofertilisers have emerged as a practical and affordable tool for small-scale organic vegetable production. The use of liquid biofertilisers in lettuce cultivation represents an alternative to complement the nutritional needs of plants and reduce production costs using commercial formulations. The nest refuse (NR) of leafcutter ants is an agglomerate of discarded plant material along with residues of its symbiotic fungus. For this reason, it is rich in nutrients and has potential as a biofertiliser. This study aimed to evaluate the effect of a liquid formulation of nest refuse (NR), produced by the leafcutter ant *Atta opaciceps* (Hymenoptera: Formicidae), on the production of lettuce seedlings. The experiment was conducted following a randomized design, arranged in two treatments and five replicates. The treatments consisted of the following steps: control, composed only of a commercial substrate, and treatment (commercial substrate + liquid solution containing 10% NR). The seeds were sown in expanded polystyrene trays with 128 cells, each with a volume of 40 cm³. The biological parameters evaluated were fresh and dry weights, stem diameter, total length, number of leaves and root length. Plants treated with biofertiliser showed higher fresh and dry weights, stem diameter, total length and number of leaves. In addition, the levels of macronutrients P, K, Ca and Mg were significantly higher in the biofertiliser when compared to the control substrate. Thus, the addition of biofertiliser to the substrate provided more vigorous plants, in response to the increase in macronutrient levels.

Keywords: biofertiliser, Organic cultivation, Macronutrients, Plant nutrition.

Introduction

Although considered important agricultural pests (Della Lucia and Araújo 2000; Marinho et al., 2006), leafcutter ants also provide important ecological services, such as nutrient cycling in the soil, contributing to its natural fertility. This accumulation of substrate discarded by ants, externally or in underground chambers, is known as nest refuse or nest waste (Del Toro et al., 2012; Farji-Brener and Werenkraut, 2014). The nest refuse (NR) is a cluster composed of the remaining particles of unconsumed plant material, combined with dead ants and unconsumed symbiotic fungi and this substrate is discarded to prevent nest contamination (Bot et al., 2001). Owing to its high nutrient content, it has great potential for use as a natural fertiliser (Guerra et al., 2007; Cerda et al., 2012; Santos et al., 2018), however, studies on their viability in organic agriculture systems are still incipient, although highly promising (Santos et al., 2018; Santos and Sousa-Souto, 2023).

Previous studies have reported that NR is extremely rich in various nutrients, such as phosphorus, nitrogen, calcium, potassium and sulfur compared to soil samples without direct influence of ant colonies (Moutinho et al., 2003; Sousa-Souto et al., 2007; Santos et al., 2018). Leaf-cutter ants generally discard their nest refuse in underground chambers of the nest but it is also common to observe piles of waste deposited externally to the colonies, especially in areas where the soil is shallow or moist (Nascimento et al., 2024) (Figure 1).

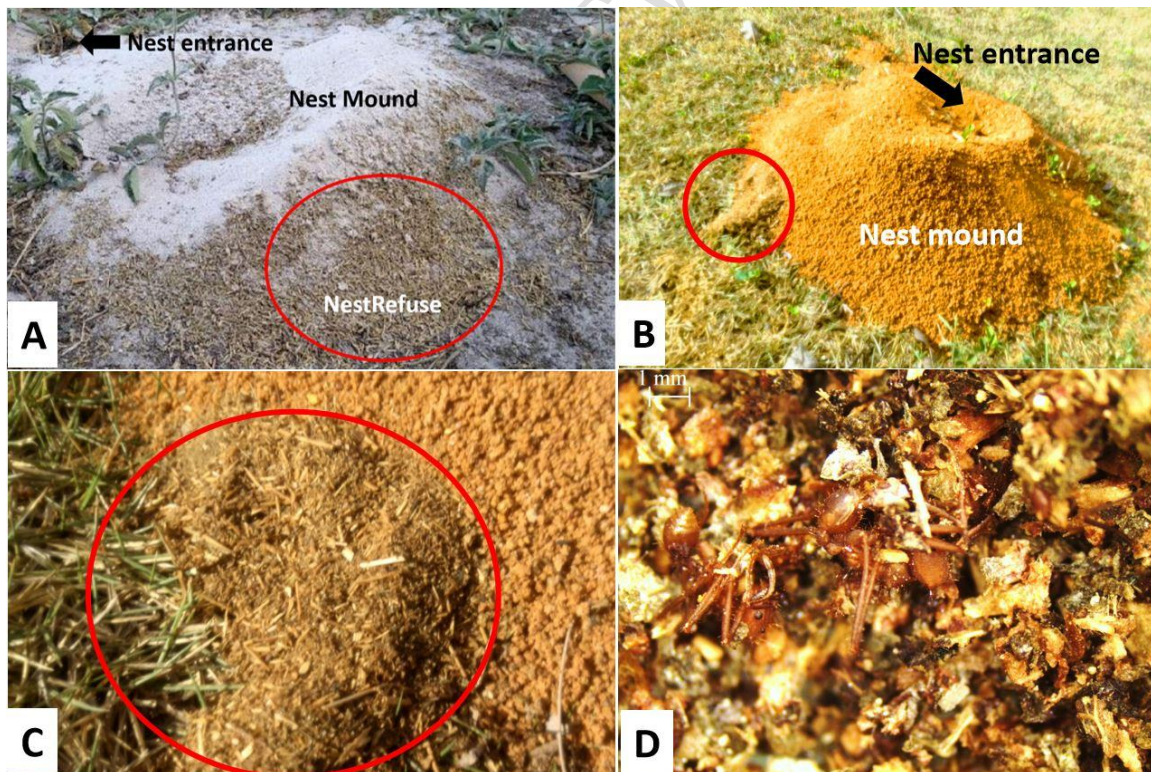


Figure 1. Nests of two species of leaf-cutter ants: A: Nest of *Acromyrmex balzani*. B: Nest of *Atta opaciceps*. The NR, composed of plant fragments and other debris, is discarded

externally, around the nest (red circles). C: Detail of the NR deposited externally in colony B.
D: NR seen under a stereomicroscope at 10x magnification.

Thus, NR from leafcutter ant nests can represent an organic compound easy to obtain and with high potential for composing alternative substrates (Cerdeira et al. 2012). One possible application could be as a liquid fertiliser, although there are currently no available studies on this type of formulation or its possible applicability.

Lettuce (*Lactuca sativa* L.) is produced regardless of the season or time of year in various regions of the country (Freitas et al., 2013; Echer et al., 2016). It has over 100 commercial varieties and is of significant economic importance owing to its adaptability, affordability for the population, mineral content, and low calorie count (Freitas et al., 2013). There are several known cultivation systems practiced, but technical and methodological challenges need to be overcome. One such challenge is to choose cost-effective fertilisers for the producers. Considering the increasing societal pressure for techniques and products with less environmental impact to maintain biodiversity, the use of alternative products has gained strength, such as in biodynamic agriculture (Silva et al., 2023), which applies biofertilisers to soil and plants, or those inoculated in compost or organic fertilisers, such as urban waste leachate (Miller and Inacio, 2009).

This study aimed to verify, on a small scale, the effect of a liquid formulation of the substrate produced by the leafcutter ant species *Atta opaciceps* (Hymenoptera: Formicidae) on lettuce seedling production.

Material and Methods

The experiment was conducted in a greenhouse, located in the Department of Ecology of the Federal University of Sergipe (UFS), in São Cristóvão, Sergipe, Brazil (10° 55' 46" S and 37° 06' 13" W). The experiment involved planting lettuce seeds in polystyrene trays, with each tray containing 128 cells. Each tray was divided into groups of 36 cells corresponding to two treatments: control (substrate) and substrate with NR. To eliminate possible edge effects, the lateral cells and central strip of the tray dividing the two groups were left empty, resulting in 72 cells per tray. In this design, the tray was considered a randomized block containing the two treatments (substrate and control). Thus, five trays (blocks) were set up, with two treatments in each tray and 360 cells sown. The trays were filled with commercial substrate, composed of sphagnum peat, expanded vermiculite, dolomitic limestone, agricultural gypsum, and NPK fertiliser (trace amounts).

The nest refuse was obtained from six colonies of *Atta opaciceps*, located in a wooded area on the Campus of the Federal University of Sergipe. The species *A. opaciceps* has a generalist habit, and can cut leaves of monoyledons or dicoyledons Santos and Sousa-Souto, 2023). For this reason, determining the origin of the plant material collected by the ants is difficult. However, during the NR collection period, ants were constantly observed cutting grass and leaves of *Hibiscus rosa-sinensis* L. and *Hibiscus tiliaceus* L. (Malvaceae), as well as leaves and ripe fruits of *Terminalia catappa* (Combretaceae), tree

species with high frequency in the study area. The NR was collected daily from piles outside the colonies (Figure 1), using a spoon. The material collected from these six ant colonies was mixed, deposited in plastic pots (250 mL) and later stored in the freezer until the biofertiliser was prepared.

The biofertiliser was obtained as follows: 100 mL of NR material was blended and sieved (using a sieve with a diameter of 0.5 mm) and then added to 900 mL of distilled water. The solution was transferred to a 1.2-liter beaker and agitated for 1 min every 30 min for 2 hours. After this process, the solution was transferred to a 2-liter bottle and kept at room temperature for 48 h before use. After the 48-hour period, the solution was filtered using filter paper and transferred again to a 2-liter bottle, remaining in a light-protected environment at room temperature throughout the experiment. Chemical analyses of the substrate consisted of determining the mass fractions of macronutrients (N, P, K, Ca, Mg, S), using a sample of the substrate used, following the procedures described in Santos et al., (2018). To measure pH, 10 g of dry substrate material was diluted in 25 mL of deionized water and stirred with a glass rod for 2 minutes. The samples were left to rest for 1 hour and potentiometric pH measurement was performed.

In each cell, 3–4 lettuce seeds of the Crespa Grand Rapids variety (Feltrin ®) were sown, watered daily, and exposed to sunlight. Germination occurred within 4–5 days, and the seedlings were watered for another 10 days until the emergence of true leaves. Thinning was then performed, leaving only the well-developed seedlings in each cell. The application of treatment solution began on the third day after thinning, except for the control group plants. In total, four applications of the *A. opaciceps* extract were performed, with 2.5 mL per cell every 5 days. Two days after the final application, the experiment was concluded. The treatments consisted of the following: a) "Control Group": Seedlings grown in commercial substrate without the addition of NR, irrigated daily with filtered water, twice a day; b) "Treatment Group": Seedlings grown in commercial substrate, irrigated daily with filtered water twice a day + 4 applications of 2.5 mL per plant, of NR extract on days 5, 10, 15, and 20 after thinning.

About 22 days after the start of the experiment, the seedlings were collected and the roots were cleaned to remove the commercial substrate by rinsing them in water. Each seedling was then gently dried using a paper towel to measure the total plant length, root length, and stem diameter using a digital caliper. Subsequently, the fresh weight of the plants was measured using a balance (Model: Shimadzu BL320H) with a precision of three decimal places (0.001 g). After measuring the fresh weight, the seedlings were individually placed in paper bags, dried in an oven at 60 °C until constant weight. After this, the dry weight was measured.

Differences in the evaluated plant variables among treatments were compared using t-tests or Wilcoxon (Mann-Whitney) tests depending on the normality of the data. Normality was assessed using the Shapiro–Wilk test ($\alpha = 0.05$). The analyses were conducted using R software (R Core Team, 2025).

Results and Discussion

Physicochemical characteristics of the substrate used are shown in Table 1, while contents obtained for pH, macronutrients measured from the nest refuse are presented in Table 2.

Table 1 - Chemical and physical characteristics of the commercial substrate used in the production of lettuce seedlings (Cruz et al., 2018 and Wieth et al., 2019)

Physical-chemical characteristics	Warranty values
pH	5.5
C (%)	20.4
N (%)	0.11
M.O (%)	35.00
P	0.006 g kg ⁻¹
K	0.0013 mg kg ⁻¹
Ca	0.14 mg kg ⁻¹
Mg	0.63 mg kg ⁻¹
EC (dS m ⁻¹)	0.46
wet density (kg m ⁻³)	262.57
dry density (kg m ⁻³)	122.41
TP %	87.73
AS %	38.91
WHC (10) %	8.82

Note: EC = electrical conductivity obtained in a 1:5 (v/v) solution, pH = determined in H₂O, dilution 1:5 (v/v), Carbon (C), Nitrogen (N), Organic matter (M.O), phosphorus (P), potassium (K), Calcium (Ca), Magnesium (Mg), TP = total porosity, AS = aeration space, WHC10 = water holding capacity under suction of 10cm of water column determined on a volumetric basis - v/v

Comparing the values of the macronutrients P, K, Ca and Mg present in both the substrate and the biofertiliser, we can observe higher levels in the biofertiliser. For example, Ca or Mg levels in biofertiliser exceed the levels in commercial substrate by 26 and 40.5 times, respectively. In relation to P and K contents, the difference is greater, indicating the contribution of the biofertiliser to the nutrient input to the cultivation substrate of these seedlings.

Table 2 - Chemical composition of the nest refuse (NR) of the leafcutter *Atta opaciceps*

pH	P	K	Ca	Mg	S
	gKg ⁻¹				
5.3	0.5	6.0	3.7	25.5	0.8

Considering plant variables measured, with the exception of root length ($p > 0.05$), significant differences were observed for the evaluated biological parameters, with seedlings cultivated with liquid biofertiliser showing the highest values (Figure 2A-D).

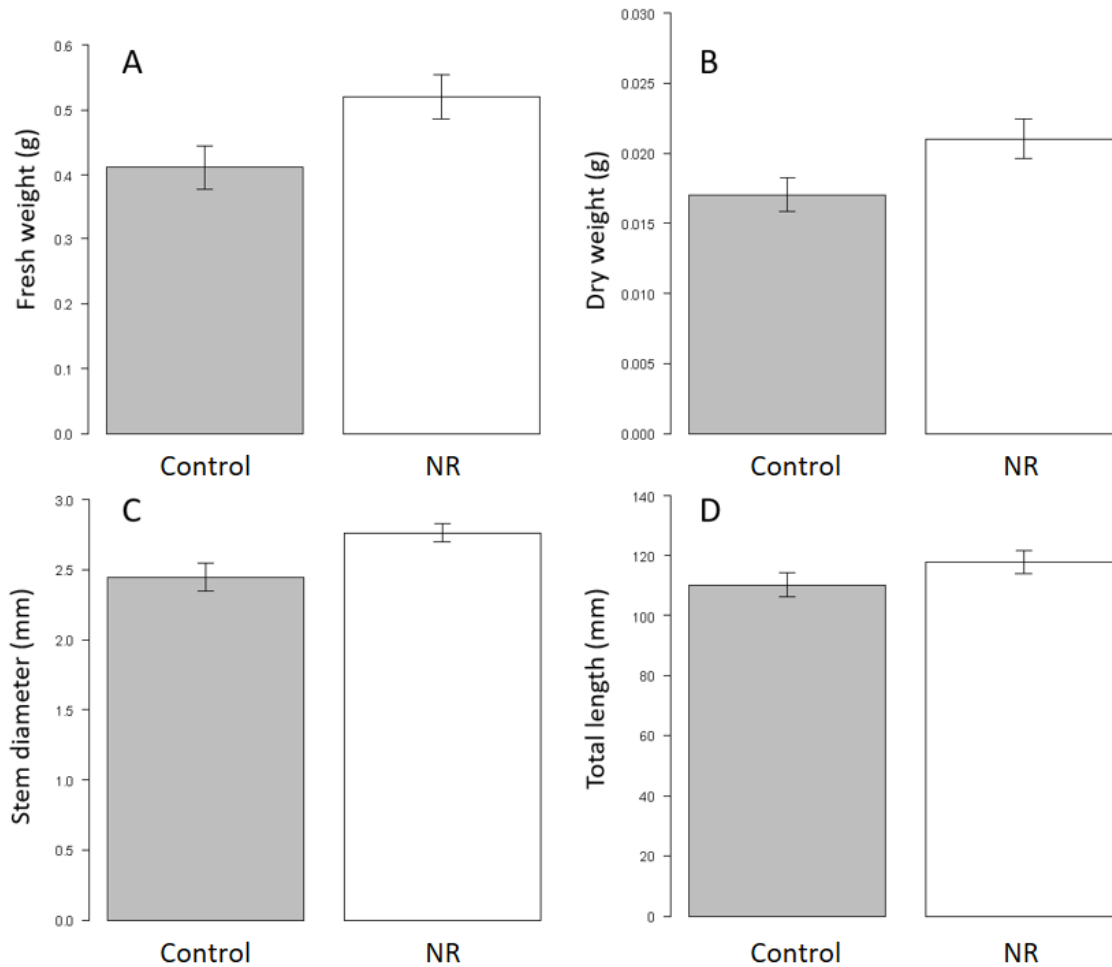


Figure 2 - Mean (± 1 SE) values of comparisons using t-tests of A: fresh weight ($t = 2.95$, $p = 0.003$); B: dry weight ($t = 2.77$, $p = 0.006$); C: stem diameter ($t = 3.02$, $p = 0.003$); and D: total length of seedlings ($t = 1.96$, $p = 0.052$) of plants treated with NR biofertiliser or with only substrate and water (control).

There was a significant difference in fresh weight between the treatments ($t = 2.95$, $p = 0.003$). Plants treated with NR extract had 33% higher fresh weight than plants grown only with substrate and water (control) (Figure 2A). There was also a significant difference in the dry weight ($t = 2.77$, $p = 0.006$) between the treatments. The dry weight of the plants treated with biofertiliser increased by 27% compared that with of the control plants (Figure 2B). There was a significant difference in stem diameter ($t = 3.02$, $p = 0.003$), with the biofertiliser-treated plants having a 13% larger diameter than that of the control plants (Figure 2C). A significant difference was also observed in the total length ($t = 1.96$, $p = 0.052$), which was 8.5% greater than that of the control (Figure 2D). Additionally, there was

a significant difference in the number of leaves ($U = 1415.5$, $p = 0.04$), with a 15% increase in this variable in plants treated with NR extract.

The present study tested the hypothesis that lettuce plants cultivated with liquid fertiliser based on discarded substrate (NR) from *A. opaciceps* would exhibit better development than control plants (grown only with commercial substrate). The results supported this hypothesis, with the exception of one variable (root length). Plants subjected to four applications of the fertiliser (10 mL in total) showed superior development compared to the control plants, confirming the feasibility of this method for producing more vigorous seedlings.

Previous studies have already demonstrated the effectiveness of NR from different species of leafcutter ants for the production of lettuce and rocket (*Eruca sativa*) seedlings (Santos et al., 2018) and for studying its effects on herbaceous plant communities (Cerdeira et al., 2012; Santos and Sousa-Souto, 2023). Santos et al. (2018) observed that plants treated with a mixture containing 25% dry NR incorporated into the soil showed 42-53% superior growth, 10% larger stem diameter, and twice the dry weight compared to control plants. The same authors did not find a significant difference in root length between the treatments.

Attributes such as larger size and dry weight are indicative of more vigorous and early seedlings, which can be transplanted to the final location in a shorter time, optimizing production and increasing profitability for the grower. However, previous studies used nest refuse in its dry form, incorporated into the soil, whereas the present study is the first to test a liquid formulation for the development of a species of agronomic importance. Nonetheless, both the present study and previous work demonstrate that the main factor responsible for the increased vigor of the plants is the extraordinary nutrient increment provided by NR.

For comparison purposes, other studies have presented urban waste leachate as a biofertiliser, promoting the replacement of mineral fertilisers (Miller and Inacio, 2009; Peixe and Hack, 2014). However, the same authors considered that when leachate is not properly treated, it can pose risks of environmental contamination. Risks of contamination by possible pathogens present in the NR cannot be neglected either. However, such risks are reduced due to the presence of antiseptic substances, produced by the ants themselves, to prevent contamination within the colony (Peixe and Hack, 2014).

One difficulty related to the use of alternative substrates is that production costs are not always reduced. For example, Diniz et al., (2006) acknowledged that a substrate with humus represents an additional cost of 2.5 times compared to the commercial substrate alone. Furthermore, Smiderle et al., (2001) verified that using only commercial substrate resulted in more vigorous plants compared to other alternative mixtures, but recognized the viability and importance of these formulations for the production of high-quality seedlings. Thus, the present study presents a considerable advantage, considering that the

cost of adding the biofertiliser is practically negligible, as it can be mixed with the irrigation water itself, and the substrate for extract production is directly collected from field nests.

This study was planned for use on a small scale, however, because of the wide geographic distribution and high density of leafcutter ants in most regions of Brazil (Sousa-Souto et al., 2013), other studies can be conducted to implement a simple, inexpensive, and efficient method of collecting and processing NR for use on a large scale and make it cost-effective for producers.

Our results demonstrate the viability of using NR extracts from leafcutter ants in the production of lettuce seedlings, and that its use may be viable in the production of other vegetable species, allowing future studies on the application of this method in field conditions. Here, considering the dosage applied to the plants and the responses obtained in the laboratory, it was possible to use this amount of extract to fertilize 360 seedlings under field conditions.

In this study, a concentration of 10% was used because, according to Santos et al., (2018), it was one of the concentrations in the preliminary tests, and the best result found in that study was at a concentration of 25% (NR-Dry). Thus, the tests were started at a concentration of 10%, and satisfactory results were obtained. It is possible that different and perhaps more promising results are obtained at concentrations above 10%, always with the caveat that certain dosages can be toxic to seedlings, and the results can vary depending on the ant species or even the plant species.

Conclusion

Lettuce seedlings cultivated with a commercial substrate (Carolina soil®) + liquid solution containing 10% of NR showed better development compared to the control treatment (commercial substrate only). The best plant development is due to the vigorous increase in macro and micronutrient levels, promoted by biofertiliser. Thus, we can conclude that the application of 10 mL of this fertiliser per plant, divided into four stages (10, 15, 20, and 25 days after germination), is an inexpensive and effective alternative for the production of more vigorous lettuce seedlings.

Conflict of Interest

The authors declare no conflict of interest.

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