Fast Growing Nitrogen-Fixing Bacteria Engineered for Seedling Production Applied at Post-Mining Restoration Areas

Igor do Vale[1], Vicente Sousa[2], Jonilton Paschoal[3], Maria Alciene dos Santos[4], Cleyton Eduardo Costa Ferreira[5], Francisco de Góes[6], Isamara Silva[7], Athila Leandro Oliveira[8] and Sérgio Miana de Faria[9]
1. Forest Engineer
2, 5. Environmental Engineer
3. Environmental Manager
4. Environmental Coordinator
Hydro Bauxite & Alumina, Paragominas, Brazil
6. Forest Engineer
7. Environment Analyst
EGIS – Engineering and Consultancy, Paragominas, Brazil
8. Post Doc Researcher
9. Researcher
Embrapa Agrobiology, Seropédica, Brazil
Corresponding author: igor.vale.goncalves@hydro.com
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Abstract

Ecosystem restoration following bauxite mining is hampered globally by soil degradation that limits vegetation reestablishment. Our study investigated innovative strategies to promote reforestation by applying of nitrogen-fixing bacteria in seedling cultivation. These bacteria engage in symbiosis with plants, enhancing soil fertility and viability in degraded environments. The research was conducted using the seedling of *Clitoria fairchildiana*, known for its rapid growth. We examined its response under three conditions: (1) control with no inoculation, (2) inoculated with nitrogen-fixing bacteria, and (3) inoculated with bacteria supplemented with sugar. The addition of sugar aimed to provide an immediate energy source to support the symbiotic process. Seedling growth was measured in height and stem diameter. The bacteria inoculation combined with sugar resulted in the tallest seedlings, with a maximum height of 85 cm, compared to 80 and 60 cm for inoculation without sugar and the control group. Moreover, seedlings from the sugar-enhanced inoculation group exhibited a six-fold higher number in maximum stem diameter, peaking at 6 cm versus 1 cm in the control group. These growth parameters are indicators of a seedling's potential for survival and stability, suggesting successful adaptation and nutrient utilization. The pronounced improvements with bacterial inoculation, particularly with added sugar, point out to be a potential method for accelerating the restoration of vegetation on bauxite-mined lands. This research underscores the advantages of integrating nitrogen-fixing bacteria in reforestation, presenting a sustainable process to improve the rehabilitation. These findings contribute critical insights to the field of sustainable land restoration and ecological recovery.

Keywords: Sustainable bauxite mining, Nitrogen-fixing bacteria, Reforestation, Rehabilitation, Seedling production.

1. Introduction

Open-pit mining can considerably alter the environment since it requires the removal of the vegetation along with the most fertile topsoil and the subsurface layers above the minerals [1]. For bauxite extraction, this disruption can be enhanced since the mining progresses horizontally throughout the terrain.

After bauxite extraction and soil reshaping, the land surface undergoes a dramatic transformation, leaving behind a mixture of soil from the original B and C horizons, known as overburden. This soil is deficient in nutrients and organic matter [2]. These physical changes and nutrient deficiencies can severely hinder vegetation reestablishment and growth [3–5]. Therefore, it is imperative to invest in research and develop strategies to restore vegetation on post-mining soils, ensuring effective and cost-efficient forest restoration.

In Brazil, Embrapa, the state-owned agricultural research company, has been studying the symbiotic relationship between forest species and nitrogen-fixing bacteria (NFB) for over 30 years [6,7]. This approach offers several advantages, including reduced planting costs and improved seedling survival in low-fertility and drought conditions [8, 9]. It also enhances water and nutrient absorption, particularly phosphorus [10], boosts disease resistance [11], and facilitates biological nitrogen fixation, thereby reducing the reliance on chemical nitrogen fertilizers [12].

In this context, a partnership between Hydro Paragominas and Embrapa conducted a study to evaluate the growth of seedlings after nitrogen-fixing bacteria inoculation.

2. Materials and Methods

The study was conducted in two locations. The laboratory experiments were conducted at Embrapa Agrobiology, located at Seropédica, State of Rio de Janeiro, Brazil. Nursery experiments were conducted at Hydro Paragominas, located at Paragominas, State of Pará, Brazil. Hydro's nursery is located inside the mining site, and it can produce over 180 000 seedlings per year from more than one hundred native species. The region's climate is classified as "Aw" according to the Köppen-Geiger classification, characterized as hot and humid with well-defined rainy and dry seasons. The average annual temperature is 26.3 ºC, with an average annual relative humidity of around 81 % [13].

2.1 Selection of the Seedling Species

First, Embrapa conducted a floristic survey in the conserved forest fragments, areas in rehabilitation, and the seedling nursery of Hydro Paragominas to identify species capable of associating with nitrogen-fixing bacteria. In Paragominas, 37 floristic samples were collected, and nodulation was observed in ten species: *Bowdichia nitida, Campsiandra laurifolia, Clitoria fairchildiana, Enterolobium maximum, Inga edulis, Inga laurina, Inga capitata, Inga splendens, Ormosia paraensis* and *Samanea tubulosa*. The collected nodules were brought to Embrapa's laboratory in Rio de Janeiro, Brazil, where the bacteria were isolated. Along with the collections, geographic coordinates were recorded, and the parent plants were marked for seed collection and subsequent seedling production in the nursery (Figure 1).

Clitoria fairchildiana from the Fabaceae family was the first species to be chosen for a nursery experiment. *Clitoria fairchildiana* is a fast-growing tree [14], commonly known as sombreiro, palheteira and butterfly pea. It is found in the states of Amazonas, Pará, Maranhão and Tocantins, in Brazil's Amazon rainforest. *C. fairchildiana* is used for reforestation [14], as it can grow in substrates consisting solely of Yellow Latosol [15]. Additionally, *Clitoria fairchildiana* was identified as a key species for seedling planting at the Hydro Paragominas site due to its high incidence in areas under rehabilitation [16].

Figure 1. Parent plant marking. A - Example of a marked and georeferenced parent tree at the Hydro Paragominas site; B - Floristic material collection using a pruning saw by a technician from Hydro Paragominas.

2.2 Selection and Development of Strains in the Laboratory

To select a highly efficient nitrogen-fixing rhizobia strain for *Clitoria fairchildiana*, inoculation was first tested with broad-spectrum strains, meaning rhizobia that interact with various plant species. It also tested more specific strains, such as those isolated from *Clitoria fairchildiana*. After purification on Petri dishes with YM medium and pH indicator, the strains were grown and isolated in a liquid 79% medium (Figure 2). Liquid media are also known as broths. From the broths, the inoculum is prepared [17].

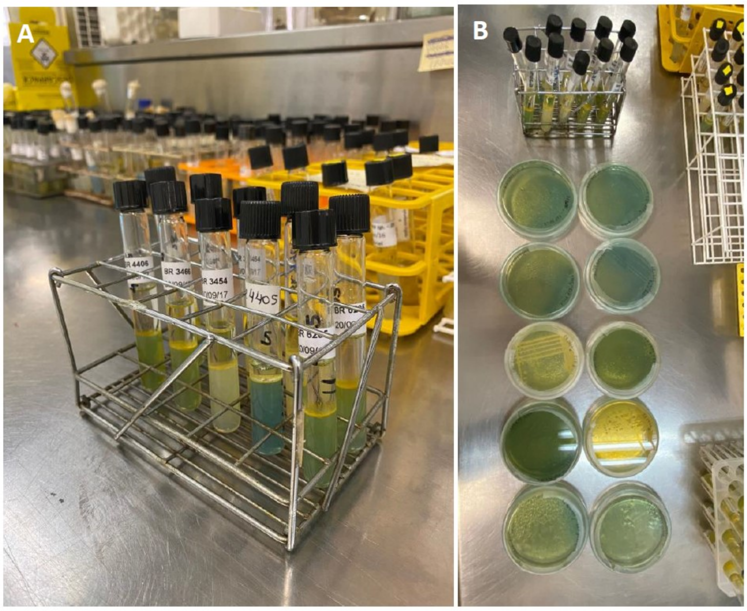

Figure 2. Left: A) Strains that were tested, Right: B) growth in solid YMA.

2.3 Selection of Rhizobia Strains in Greenhouse

After the selection of the strains, an experiment is carried out in sterilized conditions (Figure 3), where the bacterial isolates are tested to confirm if they are rhizobia and show a preliminary approach to determining whether they are efficient. Then, the bacteria are tested in non-sterilized soil, where they are tested for their competitiveness against the native rhizobia, and finally, they are tested on the nursery bed [18]. For *Clitoria fairchildiana*, *Bradyrhizobium* sp., variation BR 8007, was the bacterium with the highest efficiency in BNF.

Figure 3. Preliminary test on sterilized conditions of bacterial isolates in *Clitoria fairchildiana.*

2.4 Seed Inoculation and Seedling Production in Nursery

The growth of *Clitoria fairchildiana* seedlings was evaluated under three treatments:

1. Control, without bacterial inoculation.
2. Inoculation with nitrogen-fixing bacteria is achieved by seed contact with the isolated strains of the bacteria (Figure 4).
3. Inoculation with nitrogen-fixing bacteria with 4g of sugar [18].

The addition of sugar aimed to provide protection and an immediate energy source to support the symbiotic process. After bacterial inoculation, the seeds were planted in plastic bags measuring 15 cm × 20 cm × 0.10 mm filled with a substrate composed of 70 % topsoil collected from the vegetation suppression areas of Hydro Paragominas + 30 % white sand (Figure 5). Each treatment consisted of five blocks with 15 seedlings each, totaling 75 seedlings per treatment and 225 seedlings overall. Bacterial inoculation and seed planting occurred in June 2023. After eight months, in February 2024, seedlings were measured in height and stem diameter to compare growth between treatments.

Figure 4. Inoculation of seed of *Clitoria fairchildiana* with nitrogen-fixing bacteria.

Figure 5. Seed planting after nitrogen-fixing bacteria inoculation.

3. Results and Discussion

The treatment in which the seeds of *Clitoria fairchildiana* were inoculated with bacteria and sugar resulted in the tallest seedlings, with a maximum height of 85 cm, compared to 80 and 60 cm for inoculation without sugar and the control group, respectively. Moreover, seedlings from the sugar-enhanced inoculation group exhibited a six-fold higher number in maximum stem diameter, peaking at 6 cm, while the control treatment reached a maximum of 1 cm. Bacterial inoculation with sugar also presented the highest average stem diameter reaching 1.09 cm, compared to 0.65 and 0.69 cm for inoculation without sugar and control treatment, respectively (Table 1).

Although the non-inoculated seedlings produce nodules with native bacterial strains, they were not as efficient as the selected rhizobia strain using the procedure described by James Vincent [18] (Figure 6). The use of this procedure can result in productivity gains, as it delivers an enhanced seedling development, and financial benefits, as it reduces the need for chemical fertilizers.

Table 1. Average, maximum and minimum values of height and stem diameter for seedling of *Clitoria fairchildiana*.

Treatment	Average height (cm)	Max. height (cm)	Min. height (cm)	Average diameter (cm)	Max. diameter (cm)	Min. diameter (cm)
Control	41.72	60.00	26.00	0.69	1.00	0.60
Bacterial inoculation	42.76	80.00	21.00	0.65	1.20	0.60
Bacterial inoculation with sugar	40.96	85.00	15.00	1.09	6.00	0.60

Figure 6. Left: A)Tallest seedling from (1) control treatment, (2) inoculation without sugar and (3) inoculation with sugar, Right: B) Presence of nitrogen-fixing bacteria nodules in seedling roots.

The large-scale use of nitrogen fertilizer significantly increases plant production costs and has negative environmental impacts because some of the nutrient is lost through volatilization without being used by plants, and about 90 % of it is imported [19]. Although nitrogen is not readily available in the soil, 78 % of the atmosphere is composed of nitrogen, and some microorganisms can use atmospheric nitrogen through the process of biological nitrogen fixation. In this process, atmospheric N_2 is converted into NH_3 by an enzymatic complex known as nitrogenase, which is present in these organisms [20].

A great example of the agronomic benefit generated from this symbiotic relationship is soybean cultivation, which in Brazil practically does not use nitrogen fertilization. This allows Brazilian soybeans to be competitive in the international market [21].

Some plant species in the legume family, such as *Clitoria fairchildiana*, engage in a mutualistic symbiosis with nitrogen-fixing bacteria, known as rhizobia, and benefit from the potential of these bacteria to fix atmospheric nitrogen [22]. In return, the plants provide the bacteria with part of the photosynthates obtained through the photosynthetic process.

It is also important to note that, despite the encouraging results in maximum height and diameter growth of *Clitoria fairchildiana* after bacterial inoculation with sugar, the average height growth was lower than the treatments without inoculation and with bacterial inoculation without sugar. This result can be explained by the high genetic variation found in forest tree species; an intrinsic trait that serves as raw material for adaptation to the ever changing and variable natural environments in which these species reproduce [23].

At Hydro Paragominas alone, there are more than fifteen *Clitoria fairchildiana* parent trees from which the seeds used in this experiment were collected. Therefore, it may be essential that, in addition to the use of nitrogen-fixing bacteria, a genetic material selection process occur to meet the reforestation objectives.

4. Conclusion

The pronounced improvements in seedling growth with bacterial inoculation, particularly with added sugar, make it a potential method for accelerating vegetation restoration on bauxite-mined lands. This research underscores the advantages of integrating nitrogen-fixing bacteria in reforestation efforts, presenting a sustainable method to improve rehabilitation in post-mined lands. These findings contribute critical insights to sustainable land restoration and ecological recovery with gains in productivity and cost reduction.

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