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DETERMINATION OF THE VIABILITY OF *AZOSPIRILLUM* SYRUPS, MULTIPLIED IN DIFFERENT CULTURE MEDIA

Gabriel Fleck da Rosa, Cristina Rossetti, Priscila Erbice Olin, Yago Müller Alves, Andreia da Silva Almeida

Universidade Federal de Pelotas – UFPel, RS. E-mail: cristinarosseti@yahoo.com.br

Abstract

The use of products of microbial origin, bacteria, for agriculture has grown exponentially in the world, and in Brazil this growth is significant. In recent years, several companies have settled in the country and started to develop different products in this segment. The producer has the possibility of multiplying bacteria on the farms themselves in a process called *on farm*, a homemade way of multiplying bacteria. The objective of this study was to evaluate the efficiency of the mixtures produced through the *on farm* method of multiplication of microorganisms from inoculants based on *Azospirillum* with different culture media. For this, three culture media were used: commercial product Multibacter®, yeast syrup plus sugar and yeast syrup, which were subsequently quantified. The syrups were produced in the *on farm* system, the sterilization of the place of packaging and allocation of the bioreactors was carried out, the samples of the syrups were diluted for incubation and the preparation of the TSA (Trypticasein Soy Agar) also took place. It was possible to conclude that the non-sterile culture media do not present satisfactory results for the multiplication of *on farm* microorganisms with *Azospirillum* syrups. Colony forming units are not parameters to indicate viability for non-sterile culture media.

Keywords: bioinputs; contamination; bacterium; *on farm*.

DETERMINAÇÃO DA VIABILIDADE DE CALDAS DE *Azospirillum*, MULTIPLICADAS EM DIFERENTES MEIOS DE CULTURA

Resumo

O uso de produtos de origem microbiana, bactérias, para a agricultura tem crescido exponencialmente no mundo, e no Brasil este crescimento é significativo. Nos últimos anos várias empresas se instalaram no país e passaram a desenvolver diversos produtos neste segmento. O produtor, tem a possibilidade de multiplicar bactérias nas próprias fazendas em um processo chamado *on farm,* uma forma caseira de multiplicar bactérias. O objetivo deste estudo foi avaliar a

eficiência das caldas produzidas através do método *on farm* de multiplicação de microrganismos de inoculantes a base de *Azospirillum* com diferentes meios de culturas. Para isto foram utilizados três meios de cultura: produto comercial *Multibacter*®, calda de levedura mais açúcar e calda de levedura, os quais posteriormente foram quantificados. As caldas foram produzidas no sistema *on farm*, foi realizada a esterilização do local acondicionamento e alocação dos biorreatores, as amostras das caldas foram diluídas para incubação e também ocorreu o preparo do TSA (Trypticasein Soy Agar). Foi possível concluir que os meios de cultura não estéreis não apresentam resultados satisfatórios para a multiplicação de microrganismos *on farm* com caldas de *Azospirillum*. Unidades formadoras de colônias não são parâmetros para indicar a viabilidade para meios de cultura não estéreis.

Palavras-chave: bioinsumos; contaminação; bactéria; *on farm*.

Introduction

A certain crop may obtain an increase in its yield from the association of several factors, from the use of cultivars adapted to local conditions, good soil fertility, rigorous management of diseases and pests (TEIXEIRA FILHO *et al.*, 2020). One of the outstanding factors for gains in productivity is the proper management of nitrogen fertilization, mainly due to the fact that this element is absorbed in large quantities, thus guaranteeing the best responses in terms of yield (TEIXEIRA FILHO *et al.*, 2020).

A common practice for supplying nitrogen (N_2) to agricultural crops is fertilization with industrialized fertilizers. Despite having high solubility, and consequently slight availability for absorption, it is a nutrient with great potential for loss in the environment (TEIXEIRA FILHO *et al.*, 2020) and, consequently, large amounts of the nutrient are needed by crops, especially grasses, which raises production costs. As an alternative to synthetic N_2, there is the possibility of using inoculants based on N-fixing bacteria, mainly from the genus *Azospirillum* (MILLÉO; CRISTÓFOLI, 2019).

The genus *Azospirillum* has the ability, when associated with grasses, to fix atmospheric nitrogen and help with the solubilization of inorganic phosphate, in addition to being widely present in tropical and subtropical soils (ELMERICH; NEWTON, 2017; HUERGO *et al.*, 2018, ALMEIDA *et al.*, 2021). According to Cantarella (2017), these organisms assimilate atmospheric nitrogen and transform it into NH_3 (ammonia) being responsible for biological fixation through the enzymatic complex nitrogenase.

The process of biological nitrogen fixation is the second most important biological process in plants, after photosynthesis. Nitrogen operates in the plant metabolism, as it participates directly

in the biosynthesis of proteins and chlorophyll, as well as in the initial development of the plant (ANDRADE *et al.*, 2020). Therefore, the use of ammonium and urea-based fertilizers ends up causing soil acidification, especially with the increase in doses in the production system (CAIRES *et al.*, 2018), burdening production and enhancing environmental impacts (FERNANDES *et al.*, 2017).

According to Bartchechen *et al.* (2021), the main barrier to the use of *Azospirillum* on a large scale has been the inconsistency of the results presented in the studies. These variations have been associated with the genetic diversity of different cultivars, edaphoclimatic conditions and methodologies used in research. Bashan *et al.* (2020) reported greater root development, water and mineral absorption, and greater tolerance to stress in relation to drought and salinity as results from inoculation with *Azospirillum brasilense*.

However, Correa *et al.* (2018) pointed out that with the increase of root growth and nutrition by the practice of inoculation with *A. brasilense*., the plants develop a greater tolerance to the pathogenic agents. Sales *et al.* (2021) observed, in the inoculation of rice seeds with *A. brasilense* added to 50% of the recommended dose of N_2, there was an increase in plant height, number of panicles and grain yield (SALES *et al.*, 2021). Rampim (2021) found no differences between treatments in the first corn crop, even with doses higher than the recommended inoculant. The greatest root development of the seedlings was obtained only from the reinoculation for two consecutive seasons, however the development of the aerial part of the corn plants was restricted. In the research carried out by Novakowski *et al.* (2021), the association of nitrogen fertilization in the winter pasture (ryegrass and black oats) and the inoculation of *A. brasilense* in corn was evaluated. They also concluded that, in this production system, there is a residual effect of the nitrogen (N_2) applied in the pasture on the corn crop, as well as an increase in corn productivity by inoculation (NOVAKOWSKI *et al.*, 2021).

For Dartora *et al.* (2021), when evaluating the inoculation response of *A. brasilense* and *Herbaspirillum seropedicae* in relation to nitrogen fertilization in corn, verified that nitrogen fertilization favoured the development of the crop. The inoculation of microorganisms provided a 12% increase in shoot dry matter and a 7% increase in productivity. According to Araújo *et al.* (2020), inoculation with *A. brasilense* in maize increased the number and mass of commercial husked ears. Furthermore, the combination of inoculation and nitrogen fertilization increased ear production by approximately 30%. In the experiment conducted by Dias *et al.* (2018), inoculation with *A. brasilense* promoted an increase in grain productivity and oil content. This interaction has enabled a reduction in the application of fertilizers to crops and a reduction in production costs, as well as providing less environmental contamination (HUNGRIA *et al.*, 2020). A viable alternative for pest and disease control is biological control. It is defined as the use of living organisms to

suppress the population of a specific pest or disease, making it less abundant or less harmful. This is a natural phenomenon, as almost all species have natural enemies that regulate their populations (HUNGRIA *et al.*, 2020).

Thus, it is important for producers to be guided about the risks that the multiplication of these microorganisms inside the farms, called *on farm*, may cause and to show the use of bioreactors to carry out this multiplication, which are sterilizing equipment, which control the pH, aeration and temperature of the medium, aiming to multiply only the microorganisms that promote growth and those that will be used in the biological control of pests, without contamination of the handlers of crops and products (HUNGRIA *et al.*, 2020).

Therefore, based on the above and the need for research, the present study aimed to evaluate the efficiency of the mixtures produced through the *on farm* method of multiplication of microorganisms from inoculants based on *Azospirillum* with different culture media.

MATERIAL AND METHODS

The experiment was conducted during the 2021/2022 agricultural season in the municipality of Jaguari/RS. The experimental area is located at Latitude: 29° 30′ 22″ South and longitude 54° 40′ 31″ West and altitude of approximately 110 m. The climate of the region was considered humid subtropical climate, according to the Köppen-Geiger climate classification (Cfa). The interpretation of the results was performed according to the color developed by the colonies.

Site sterilization and bioreactor storage: The site and equipment were sterilized to prevent any type of contamination. Water was added up to the maximum capacity of the bioreactor (Figure 1), in which it was bleached with an active chlorine tablet (400 g) for 30 minutes in the aeration system. After this cleaning, the water was discarded.

Figure 1. Venturi model bioreactor for multiplication of *A. brasilense* bacteria in the *on farm* system.



Afterwards, 20l of water were added to the bioreactor, and two chlorine tablets of 5 g were dissolved, which had disinfectant action of the water that was used in the bacterial multiplication. Along with this process, 40 ml of antifoam was added, the aeration system was activated for 15 minutes to treat the water and activate the product.

Soon after this treatment period, Multibacter® medium was added, in the amount of 300 g, and 200 g of sucrose was added to 20l of water. 100 ml of *A. brasilense* inoculum was added, and thus the bioreactor was kept on for 24 hours. At the end of the bacterial multiplication stage, the product was removed with the equipment turned on, so that the bacteria could be concentrated.

The bioreactor and its connections were cleaned, 1l of disinfectant was added to 700l of water for 15 minutes for a deeper cleaning. The sterilization process adopted by the Multibacter® product (Figure 2) was added to all other multiplication systems with different culture media.

In the bacteria multiplication process, through the yeast culture medium, 20l of water were added to the bioreactor, and soon after the addition of 200 g of the medium, keeping the bioreactor turned on for 24 hours. The yeast multiplication closure process occurred in the same process as the other media, keeping the syrup stored in a suitable place until the time of use.

The multiplication of the syrup through the yeast/sugar medium, the amount of 20l of water was added in the bioreactor, and then 200 g of the yeast medium and 200 g of sugar were added, the bioreactor was kept on for 24 hours. The multiplication process of the yeast/sugar culture medium followed the same methodology as the previous ones.

Figure 2. Materials used to prepare the culture medium and subsequent multiplication of *A. brasilense.*



Dilution of incubation solution samples: To carry out serial dilutions, it was started with organisms in liquid medium. 1 ml of medium was added to 9 ml of saline to create a 1:10 dilution; 1 ml of a 1:10 dilution was added to 9 ml of saline to create a 1:100 dilution; and so on. With each dilution, the number of bacteria per milliliter of liquid was reduced by 9/10. Subsequent dilutions of 1:1000, 1:10,000, 1:100,000, 1:1,000,000 or even 1:10,000,000 were performed on an original culture which contained many microorganisms.

A saline solution was used to carry out the dilution, in which 1l of distilled water was prepared, 8.5 g of Sodium Chloride was added, and 1ml of Tween 80, in which they were autoclaved and transferred to test tubes, remembering that all glassware was also completely autoclaved, together with the saline solution, for 20 minutes and at a temperature of 121 °C.

From each dilution, 1:100, 100 µl of the syrup was transferred to an agar plate. The transfer was carried out by the spreading plate method. A plate was prepared by adding 1 ml of the diluted syrup obtained in the serial dilution to 9 ml of nutrient agar called TSA (Trypticasein Soy Agar). Then, the medium was mixed, poured into an empty petri dish. After the agar medium cooled, solidified, and incubated, colonies formed on the medium.

When live bacteria settled on the agar plate, it divided and formed a colony. Each bacterium represented a colony forming unit (CFU). To count the actual number of colonies, present, the plate was placed under the colony counter loupe, and the colonies on the entire plate were counted. To determine the number of colonies forming units in the original culture, the number of colonies on the plate was multiplied by the dilution factor; a dilution factor of 1000 was expressed as 1:1000 or 1/1000, and a dilution factor of 10000 was expressed as 1:10000.

Preparation of TSA: After sterilizing the glassware in all processes for incubating the specific syrup, TSA Nutrient Agar (Trypticasein Soy Agar) was prepared. 40 g of the medium was used in 1l of distilled water, being kept in high agitation, and the medium at high temperature was dissolved, in which the dissolution process occurred between 1 minute and 2 minutes of boiling, using the micro-waves. It was dispensed in an appropriate container and sterilized in an autoclave at 121 °C for 15 minutes.

Right after the autoclave process, it was necessary to monitor the temperature of the medium, until it reached approximately 40 °C, in which 2 ml of Nystatin, the recommended antifungal agent, was used, so that contamination did not occur in the culture medium for the incubation.

After mixing the medium, 10 ml of Nutrient Agar was inserted into each Petri dish, in which cooling, solidification and the time of incubation of the specific syrup were temporarily awaited 100 µl of the already dissolved solution was added to each Petri dish, which was spread with the

Drigalski Strap, and then attached to the microprocessed oven for bacteriological culture for 48 hours.

Results and Discussion

The basic premise of biological control is to maintain the population density of pest species associated with agriculture at economically and ecologically acceptable levels. The results found for the three-culture media multiplied *on farm* revealed that the Multibacter® medium obtained an average of 10^8 UFC (colony forming unit) for four repetitions and an average of 10^7 UFC for only one repetition (Table 1). Thus, making it a totally viable means when related to commercial products.

The Multibacter® is a culture medium created to multiply microorganisms (mainly bacteria for agricultural use) through aerated and controlled multiplication systems. Its technology offers the producer different forms of production controls, directly reducing the use of pesticides in the crop, by developing low-cost biological products on his property.

The multiplication of microorganisms on Farm is regulated, through the legislation of the Ministry of Agriculture, Livestock and Supply (MAPA), by Decree No. produced exclusively for their own use". However, national agriculture bodies are working towards more specific legislation for biofactories, with the implementation of registration and inspection of properties that use the technique, in addition to promoting research and training in the area.

Official data from the Ministry of Agriculture, Livestock and Supply (MAPA) show that from 2006 to 2018 there was a jump from 1 to 79 companies in this segment in Brazil. The same trend is found for the registration of biological products (BORSARI; CLAUDINO, 2018). The expectation is that this scenario will be even more favored with the Decree (10,375 of May 26, 2020) establishing the National Bioinputs Program which aims, among other things, to stimulate the development of the sector in the country with a focus on small and medium-sized biofactories.

During the industrial process for the production of products based on microorganisms, strict procedures are followed to guarantee the final quality of the product. It is even necessary, during the registration of any product based on biological agents for pest control, to be informed about the possible presence of microbial toxins and other metabolites, mutant strains, allergenic substances, etc. (BRASIL, 2002).

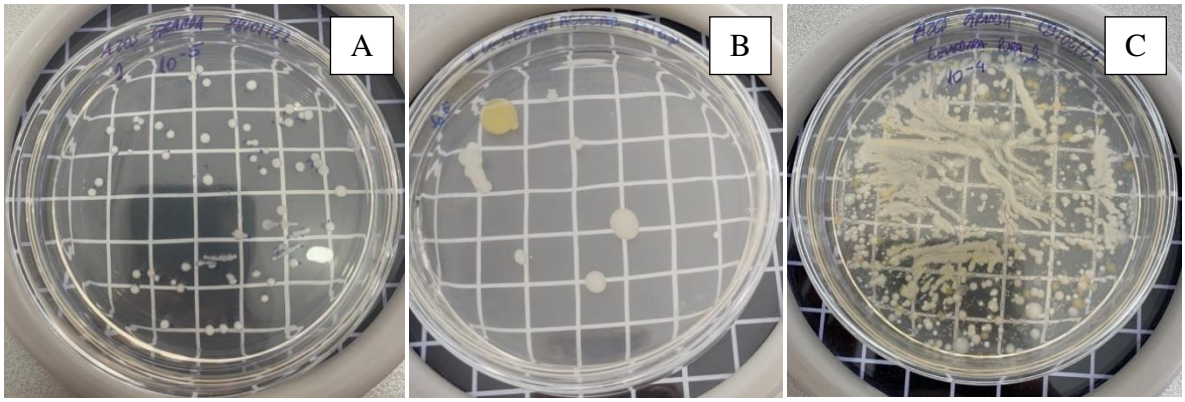
The National Health Surveillance Agency (ANVISA) requires a detailed study on residues when the product is indicated for use in crops for human or animal consumption, or even when the use of the product may result in the presence of residues in human or animal food (BRASIL, 2012). There is still the requirement of the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) to carry out damage assessments on non-target organisms and environmental

behaviour of the microbiological control agent (BRASIL, 2012). Bureaucratic procedures must be followed to guarantee a product with 100% reliability.

As for the yeast plus sugar medium (Table 1), artisanal culture medium plus sugar, the results indicated an average of 10^6 UFC for four repetitions and 10^7 UFC for only one repetition. These results revealed that the samples were considered viable, however, the averages were below those obtained in commercial products. This may have occurred due to the fact that, when sugar was added, more conditions were provided in the medium for *A. brasilense* to develop and, consequently, less undesirable bacteria would be present. It is important to emphasize that one of the greatest risks of *on farm* multiplication is caused by contamination of the system, which is something that can easily happen in any of the production phases, whether from materials, equipment, culture medium, water, air and inputs used. Carelessness in the sterilization and disinfection of the system or even the use of strains and products of dubious origin can be decisive in the development of contaminating microorganisms, reaching the point of losing the batch of the product or, if there is no identification, a wasted application.

The results for the pure yeast medium (Table 1), totally artisanal culture medium (Figure 3), it was verified that high results were found (10^9 UFC and 10^8 UFC), however, when the sugar was removed from the medium, we increased the multiplication of undesirable bacteria which could not be identified. An important fact to be highlighted is that the difference between commercial and artisanal products is the high presence of contaminants. Therefore, we should not use colony-forming units to indicate the superiority of the culture medium, mainly because it is not sterile.

Figure 3. UFC (colony forming unit) resulting from plating the different culture media used for *A. brasilense* (A) Multibacter® (B) Yeast+sugar (C) Yeast.



A problematic point is the fact that the system is not sterile, that is, when adding the target microorganism source, there will be the presence of several others from water, air, sugar, antifoam and the culture medium itself. Sterilization is a physical or chemical process that eliminates all

forms of life (vegetative and spore-forming). Biochemical reactors and piping are generally sterilized by applying moist heat (saturated steam) (SCHMIDELL *et al.*, 2021).

Table 1. Quantification of the multiplication of culture media carried out on the farm of the bacterium *A. brasilense* in Multibacter® culture medium, yeast plus sugar and pure yeast.

Culture Medium	Repetitions (UFC - colony forming unit)				
	1	**2**	**3**	**4**	**5**
Multibacter®	$2.03x10^8$	$3.29x10^8$	$1.6x10^8$	$1.3x10^8$	$7.3x10^7$
Yeast + Sugar	$5.0x10^6$	$4.68x10^6$	$7.2x10^6$	$8.4x10^6$	$1.2x10^7$
Pure Yeast	$1.8x10^9$	$2.03x10^9$	$8.6x10^8$	$4.1x10^8$	$3.7x10^8$

Today's agriculture, within the N-P-K concept, which has commendably brought us to high levels of productivity, charges high prices, with soils taken to the extreme. Since even the most current practices such as direct planting and crop rotation are not stopping soil deterioration.

The practice of multiplication of microorganisms "*on farm*" brings the possibility of inverting this situation, due to the fact of flooding the soil with beneficial bacteria and fungi, with safe multiplication processes, directly reflecting on the return of life in the soil and allowing that, with costs smaller, we can reach healthy soils, thus requiring a significant reduction in the use of chemical inputs, and thus, consequently, bringing more profitability and sustainability to the field.

Conclusion

It was concluded that the non-sterile culture media did not show strong results for the multiplication of microorganisms *on farm* with *A. brasilense* syrups.

And the colony forming units are not parameters to indicate the viability of non-sterile culture media.

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