Scientific Paper

The effect of the means of solid support on the biological stability of two strains of Frankia isolated *from* Alnus acuminata *HBK*

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ABSTRACT: *Alnus acuminata* H. B. K. is a tree species which can be incorporated in silvopastoral systems, because it has the capacity to grow on marginal soils and contribute to the conservation of biodiversity and to soil amelioration; which is related to the incorporation of litter, effect of shade, retention of humidity, nutrient recycling and fixation of atmospheric nitrogen, due to its symbiotic association with the actinomycete *Frankia*. The use of Frankia as inoculant is a technology that requires a deep analysis of its performance under controlled conditions. On the other hand, for the commercialization of biofertilizers it is important to preserve their quality as long as possible, on which the acceptance of the product in the productive chain depends. For such reason, a study was conducted in order to evaluate the biological stability of the native *Frankia* strains (Aan17 and Aac49) with different substratum:strain proportions, as well as the effect of storage at 4 ºC. The strains were tolerant to the pH changes and the storage under refrigeration conditions. It is concluded that the inoculants should be stored at 4 ºC during 120 days, with a proportion of 80:20 (inoculant:substratum) in Aan17 and 60:40 in Aac49. In these substrata the microbial protein remained over 0,65 mg/mL in Aan17 and 0,7 mg/mL in Aac49.

Key words: bioinoculants, quality, diazotrophs, biological nitrogen fixation

INTRODUCTION

Farmers, guilds and institutions promote and implement the use of good practices in livestock production to achieve sustainable and productive management of the systems and the ecological certification, as well as to turn conventional livestock production into a sustainable practice. In recent years the studies have proven the importance of the strategic use of trees in silvopastoral systems (SPS), technology which has been increasingly adopted by farmers. In the high tropic, the implementation of SPS is an alternative for the recovery of degraded grasslands that allows to increase animal productivity with a sustainable approach, which is specially promising in specialized dairy systems.

In Colombia, these systems represent 5 % of the national herd (1,2 million heads, approximately) and produce 2 861 million liters of milk per year (around 52 % of the total produced milk). In addition, they generate from seven to eight jobs for every 100 animals and cover an area of 4 906,344 ha (Lafaurie, 2008). In its strategic plan of Colombian livestock production (2019), FEDEGAN stated that the development and implementation of silvopastoral models constitute an opportunity to carry out the modernization processes of the Colombian livestock production sector.

In this sense, a SPS which can be beneficial in the high Colombian tropic includes the native species *Alnus acuminata* H. B. K. This is a multipurpose tree that, in addition to providing shade to grasslands and animals, improves the conditions of soil fertility, which is associated to the incorporation of litter, humidity retention, nutrient recycling and high capacity of atmospheric nitrogen fixation (279-400 kg N/ha/year). This last benefit is due to the fact that its root system is associated to microsymbionts such as the actinomycete *Frankia*, which induces nodule formation (Bautista and Valdés, 2008; Chamorro and Rey, 2008).

The inoculation of *A. acuminata* with *Frankia* strains as biofertilizer is a sustainable alternative that has shown positive responses in dasometric and chemical composition variables. These effects are related not only to nitrogen fixation, but also to the production of indoleacetic acid and the formation

of associations with other microorganisms such as mycorrhizae, phosphate solubilizers and plant growth promoters (Gyaneshwar *et al*., 2000; Rey, 2006; Nain *et al*., 2010).

In Colombia, the Colombian Agricultural Institute (ICA) is responsible for the control of the agricultural inputs that are produced and commercialized in the national territory. Due to the increase of the use of bio-inputs, the ICA emitted the resolution No. 00375 of February 27, 2004, which was substituted by No. 698 of February 4, 2011, in which the requisites for the registration of efficacy essays, as well as of farmers and agricultural bio-input importers, were established. This resolution intends to harmonize the Colombian regulations with the international ones to strengthen the conditions of production, import, commercialization and utilization of bio-inputs, which increases the quality, efficacy and food security to benefit human health, product innocuousness, agricultural health and the environment (ICA, 2013).

Due to the lack of information about the production of a *Frankia* inoculant, it is necessary to conduct studies that allow to identify the biological stability of the inoculants; to guarantee that the substratum is compatible with the growth, survival and nodulation capacity of the microsymbiont; as well as to achieve that the storage characteristics avoid contamination by other microorganisms, in correspondence with the dispositions in resolution No. 698.

Taking into consideration that the support is one of the factors that guarantee the quality of the inoculants, as well as that there are previous observations about the benefits associated to the use of peat and rice husk as substrata, the objective of this study was to evaluate the biological stability of the native *Frankia* strains Aac49 and Aan17, with different substratum:strain proportions.

MATERIALS AND METHODS

The strain Aan17 was isolated from a natural population of *A. acuminata* H. B. K., belonging to the settlement Cubijan Alto, of the Pasto municipality (department of Nariño, Colombia), located at 3 070 m.a.s.l. and with an average temperature of 13,5 ºC; while the strain Aac49 was isolated in a population that belonged to the settlement Villa Nataly, Usme locality, department of Cundinamarca, at 3 045 m.a.s.l. and 12 ºC.

These strains were classified at the genus level, through biochemical (growth in sugar and nitrogen sources), morphological (formation of specialized structures: sporangia, vesicles and hyphae) and molecular tests (through the amplification of a variable region V2-V3 of the gen 16S rDNA). Afterwards, they were evaluated in plants of the host, independently (Rey, 2006) and in mixtures with a mixed inoculant of mycorrhizae (*Gigaspora* and *Glomus* sp.).

There was a positive response in the interaction with mycorrhizae; this synergy was proven in the fast growth and higher response in the dasometric and chemical composition variables of *A. acuminata* during the nursery stage (Pintor *et al*., 2007; Chamorro and Rey, 2008). Based on the previous results, these strains were considered promising for the production of biological inoculants of the soil.

The strains were preserved in benzylamine purine (BAP) medium plus glycerol (500 *u*L of BAP + 150 *u*L of 10 % glycerol) at 4 ºC. They were activated and grew during two months in liquid medium BAP –modified by Murry *et al*. (1984)–, which allowed to obtain the stock culture with the required biomass to conduct the micro-essays. The incubation temperature was 36 ºC, with a constant agitation of 150 rpm (Rey, 2006). Staining in fresh was made with trypan blue, as well as Gram staining in order to verify the purity of the isolations.

Preparation of the inoculant of the experimental strains. A pre-inoculant was prepared in 500 mL of liquid medium which contained root extract, yeast and molasses (root extract from *A. acuminata*, 0,1 mL/L: 50 g of root dissolved in 1 L of water; 100 mL of root extract/L of BAP medium; stock solution of micronutrients (g/L): $MnCl_2.4H_2O$ 1,81 $Na_2MoO_4.2H_2O$ 0,025; stock solution of vitamins (mg/100 mL); thiamin HCL 10, pyridoxine HCL 5, nicotinic acid 50, biotin 22,5, folic acid 10, riboflavin 10; buffer solution KH_2PO_4 1 M, K_2HPO_4 1 M). It was maintained in constant agitation (150 rpm), at 36 ºC, during five days. Afterwards, the pre-inoculant was introduced in 1 000 mL of culture medium, for five days, under the same incubation conditions. Likewise, the cultures were homogenized with magnetic agitator and mechanic iron until achieving the breaking up of the colonies. When the incubation time ended a purity analysis was performed by Gram staining (Murry *et al*., 1984; Rey, 2006).

Means of support. In the essays a mix of peat and rice husks was used as support, with a 1:3 proportion, which was ground and sieved in a mesh of 0,16 mm. This support was sterilized in an autoclave, at 121 ºC and 15 pounds of pressure (for 15 min), during three consecutive days (Matos

and Zúñiga, 2003). According to the characteristics of the initial pH of the support (5,3), the correction was made to a pH close to 6,5 through the addition of $CaCO_3$.

Conditions of evaluation. The evaluation of the inoculant was made in 70-g polyethylene bags (100 g/bag), previously sterilized in an autoclave, at 121 ºC and 15 pounds of pressure, during 30 min (Ramírez, 1992). Then, the substrata were inoculated and for such purpose a syringe was used, according to the proportions of each treatment. The maturation period of the inoculants was 8 days at 24 ºC, to allow the formation of resistance structures –spores and vesicles– (Materon and Weaver, 1985). Afterwards, the mixtures were stored at 4 ºC, during 0, 30, 60, 90 and 120 days.

Design and treatments. To determine the effect of the support on the viability and specialized structures of the native *Frankia* strains, peat:husk and culture medium:strain proportions were evaluated; this proportion was based on the ones used for rhizobia inoculants by Ramírez (1992). The treatments are shown in table 1.

The design was completely randomized, with three replications. Two strains were used, each one with three proportions of support per treatment and five periods of storage, for a total of 30 treatments and 90 evaluations.

Biological stability test of the inoculants. The biological stability was evaluated during storage through the quantification of microbial protein, according to the methodology proposed by Lowry *et al*. (1951) and modified by Rey (2006).

Measurement of the inoculant pH. To measure the pH the peat was diluted in sterile distilled water (1:2), subject to agitation during 20 minutes at 100 rpm and left to rest (Estrada *et al*., 2009).

Calculation of the humidity percentage of the inoculants. Drying was performed in the oven at constant temperature of 60 ºC, until obtaining constant weight. The calculation was made through the difference between the mass of humid sample and the dry sample multiplied by one hundred (Estrada *et al*., 2009).

Statistical processing. The data were subject to a variance analysis (ANOVA) and the means were compared through Tukey's test for 5 % significance, after verifying they fulfilled the normal distribution and variance homogeneity adjustment. All the data were carried out by means of the statistical program Statistic Analysis System (SAS, 1997).

RESULTS AND DISCUSSION

Effect of the means of support on the survival of the Frankia strains in the inoculants

Figures 1 and 2 show the effect of the means of support on the biological stability of the native *Frankia* strains, during the conservation periods.

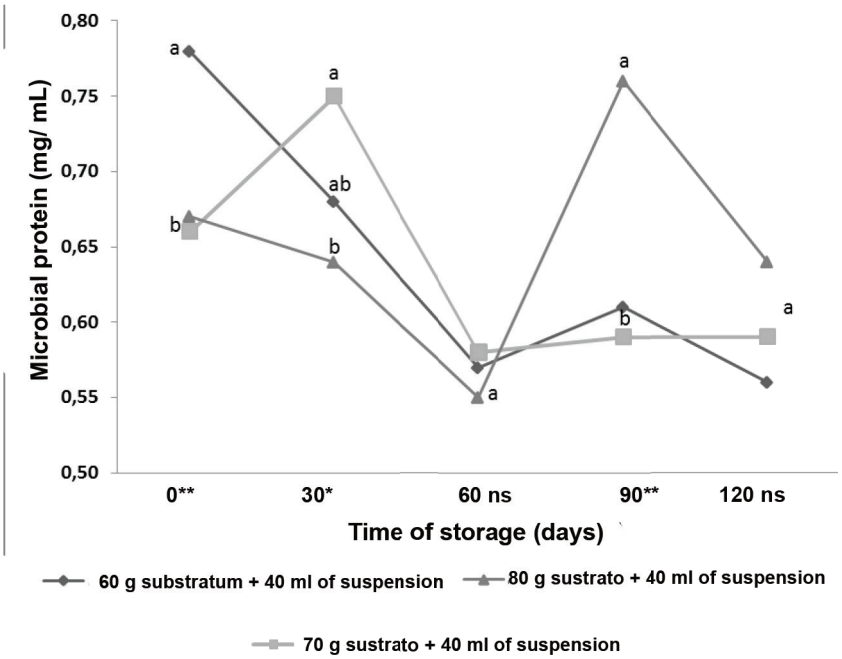
The strain Aan17 started with a concentration of microbial protein of 0,70 mg/mL, with significant differences at 30 and 90 days (fig. 1). In general, such concentration tended to decrease due to conservation. This was maintained in the treatment with the proportion 80:20, with a minimum value of decrease of 0,55 mg/mL at 60 days, which increased at 90 days (0,76 mg/mL).

In the strain Aac49 there were not significant differences in the concentration of microbial protein with regards to the days of storage. The minimum value of microbial protein was obtained at 60 days, while at 90 days its increase was observed in all the proportions. If the characteristics of this micro-symbiont are taken into consideration, it can be supposed that the effect is due to the production of specialized structures, such as spores, which was corroborated at 120 days of storage.

In the last period of storage the presence of spores and sporangia was observed as a mechanism

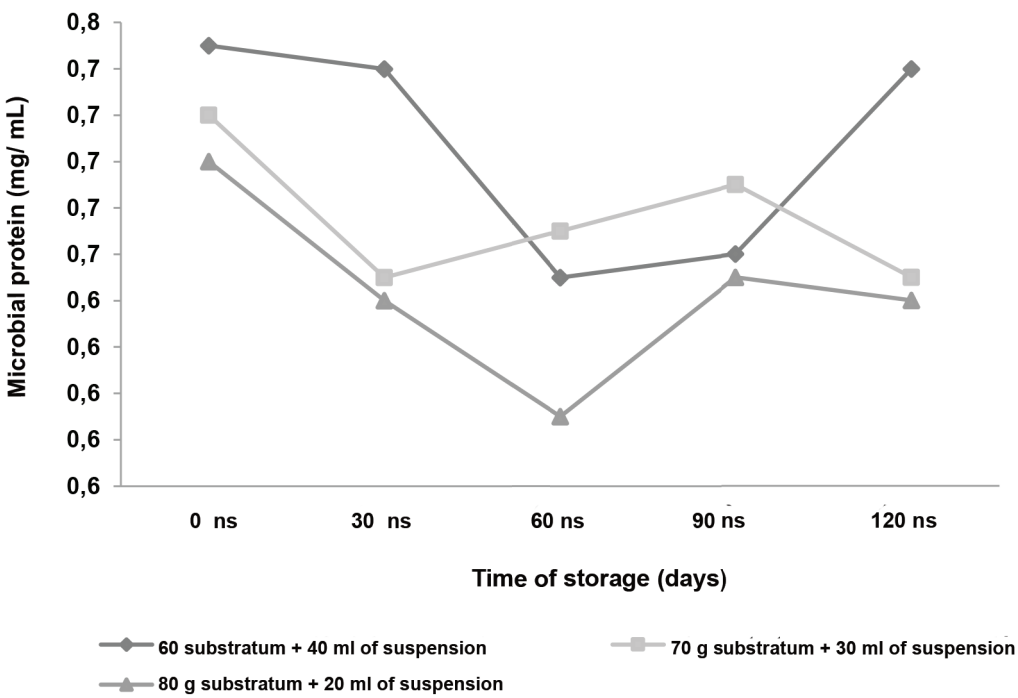
Table 1. Proportions used in the essay.

Strain	Substratum:suspension
Aan17	T1: 60 g of substratum + 40 mL of strain suspension
	T2: 70 g of substratum + 30 mL of strain suspension
	T3: 80 g of substratum + 20 mL of strain suspension
Aac49	T4: 60 g of substratum + 40 mL of strain suspension
	T5: 70 g of substratum + 30 mL of strain suspension
	T6: 80 g of substratum + 20 mL of strain suspension



** Highly significant differences ($p < 0,01$), * significant differences ($p < 0,05$), n. s.: there are no differences.

Figure 1. Effect of the substratum on the survival of the strain Aan17.



** Highly significant differences ($p < 0,01$), * significant differences ($p < 0,05$), n. s.: there are no differences

Figure 2. Effect of the substratum on the survival of the strain Aac49.

of reproduction and defense from the conditions of storage. The treatments with proportions 70:30 and 80:20 showed a higher trend to generate sporangia with different forms. Seemingly, the lowest humidity had incidence on the formation of this type of structures, which is possibly associated to the stress conditions that appeared in the media.

Since 120 days of storage there was no significant difference in the cell concentrations of the strains Aac49 and Aan17, for which it can be concluded that the inoculants based on these microorganisms behaved stably under the storage conditions and with the proportions used.

The results of this study proved that the *Frankia* strains are tolerant to storage in peat and with refrigeration; this can be associated to the fact that they come from *A. acuminata* forests located in the Andes of the high Colombian tropic of Nariño and Cundinamarca, where the presence of peat is common and there are soils originated by volcanic ashes. In addition, in the sites of collection there was a high production of litter due to the effect of *A. acuminata*. Likewise, the formation of specialized structures (such as sporangia) is very important, because they act as a protection mechanism against adverse conditions. In microorganisms the effect of low temperatures on the cells produces the inactivation of growth due to the loss of fluidity of the plasmatic membrane, which generates decrease in the metabolic functions, in the transport of nutrients and the concentration gradients (Sylvia *et al*., 2005). For such reason, this effect can be taken into consideration for the storage and conservation of this type of inoculants.

At international level, the quality standards of inoculants have been defined for nitrogen-fixing bacteria, such as *Rhizobium*, *Azotobacter* and *Azospirillum*, with minimum viable recounts from 5 x 10^7 to 1*10^9 CFU/g or mL of inoculant (Benintende, 2010). However, there are no indicators, in Colombia or worldwide, regarding the minimum effective concentration of inoculants prepared from *Frankia* strains.

Studies have been conducted in strains of other nitrogen-fixing microorganisms (*Rhizobium*, *Azospirillum* and *Azotobacter*) using peat as substratum. Isora and Rondón (1998), when using three types of peat as carriers during the production of an *Azospirillum*-based inoculant, observed that brown peat maintained the highest viability of the strain until the end of the evaluation period (180 days).

On the other hand, Estrada (2002) evaluated the effect of the quality of the inoculants stored at different temperatures, including peat as substratum. This author stated that the storage temperature of the inoculant has incidence on the conservation of the population of diazotrophic bacteria, and that the best results are obtained at 19-26 ºC. At 4 ºC, two of the studied strains reached adequate conservation, over 10^8 CFU/g of inoculant, during the 150 days of evaluation.

In this study it was proven that the efficiency of the inoculation with *Frankia* is mainly determined by the early stages of plant growth during the first weeks after optimum root colonization. Diverse studies have been conducted on *A. acuminata* species about the effect of inoculation from macerated nodules (Nickel *et al*., 2001; Ridgway *et al*., 2004) and from strains that grew in enriched culture media, methods which can only be viable for research purposes. However, in this type of experiments *Frankia* is one of the least studied microorganisms for inoculant production.

Effect of storage on inoculant humidity

The conditions of storage and temperature (4 ºC) generated a loss of humidity of the inoculant between 0,5 and 6 % (table 2).

The isolation Aan17 showed significant humidity losses, which was mainly shown, in the proportions 60:40 and 80:20; while the proportion 70:30 maintained more stable conditions. On the other hand, in Aac49 there was higher humidity loss in the three inoculants at the end of the evaluation.

Estrada *et al*. (2009) studied the effect of the storage temperature on the quality of the inoculants, using peat as solid support, in populations of *Azospirillum brasilense* Sp245 (BR11005), *Azospirillum amazonense* Y2 (ATCC35120), *Herbaspirillum seropedicae* ZAE94 (BR11417) and *Rhizobium tropici* BR322 (CIAT 1988). Additionally, they included the variables pH and inoculant humidity until 150 days of storage. The results indicated that for this type of nitrogen fixers the ambient temperature between 19 and 26 ºC was the most adequate to preserve the inoculants, during 150 days of storage.

Effect of inoculant conservation on the pH dynamics

The pH dynamics was affected during the storage time. Significant differences were observed in all the treatments, except in the proportion 80:20 of Aac49 (table 3).

The pH decrease in the inoculants could have occurred due to the fact that the culture means in which the inoculants was multiplied contained

Table 2. Humidity percentage of the inoculants prepared with two isolations of *Frankia*.

Isolation	Treatment	Time (days)				
		0	30	60	90	120
Aan17	60 g of substratum + 40 mL of suspension *	78,0^{a}	74,5^{b}	72,5^{b}	75,0^{ab}	72,5^{b}
	70 g of substratum + 30 mL of suspension *	62,0^{a}	58,5^{b}	62,5^{a}	65,0^{a}	62,5^{a}
	80 g of substratum + 20 mL of suspension **	63,0^{a}	59,5^{b}	57,5^{b}	60,0^{ab}	57,5^{b}
Aac49	60 g of substratum + 40 mL of suspension **	69,0^{a}	66,0^{ab}	64,0^{b}	66,5^{ab}	64,0^{b}
	70 g of substratum + 30 mL of suspension *	66,5^{a}	62,5^{b}	60,5^{b}	63,0^{ab}	60,5^{b}
	80 g of substratum + 20 mL of suspension*	61,5^{a}	58,5^{ab}	56,5^{b}	59,0^{ab}	56,5^{b}

**Highly significant differences ($p < 0,01$), *significant differences ($p < 0,05$)

Averages in the same rows with equal letters do not differ significantly.

Table 3. Effect of storage on the pH of the inoculants.

Isolation	Treatment	Time (days)				
		0	30	60	90	120
Aan17	60 g of substratum + 40 mL of suspension*	6,60^{a}	6,07^{bc}	6,10^{b}	5,87^{bc}	5,75^{c}
	70 g of substratum + 30 mL de suspension**	6,65^{a}	5,94^{b}	5,92^{b}	5,93^{b}	5,94^{b}
	80 g of substratum + 20 mL de suspension**	6,49^{a}	6,07^{b}	6,17^{b}	6,26^{ab}	6,21^{b}
Aac49	60 g of substratum + 40 mL of suspension**	6,60^{a}	6,49^{ab}	6,27^{bc}	6,14^{c}	6,13^{c}
	70 g of substratum + 30 mL of suspension**	6,71^{a}	6,38^{a}	5,44^{b}	6,06^{ab}	6,07^{ab}
	80 g of substratum + 20 mL of suspension^{ns}	6,40	6,20	6,32	6,20	6,13

**Highly significant differences ($p < 0,01$), *significant differences ($p < 0,05$), n. s.: there are no differences.

Averages in the same rows with equal letters do not differ significantly.

molasses, root extract and yeast, which were metabolized by the strains to form specialized structures (spores and sporangia) as consequence of the conservation temperature and humidity of the support means (Swan and Karalazos, 1990.

CONCLUSIONS

- Diversity was found in the strains.
- The support with peat:husk at 4 ºC was the adequate one to preserve the inoculants during 120 days of storage; the most convenient proportion for Aan17 was 80:20 and for Aac49, 60:40.
- By storing the inoculants at 4 ºC less humidity was lost in the treatments; the strain Aan17 had the highest loss (5 %) in the proportions 60:40 and 80:20, while Aac 49 showed losses between 3,5 and 5 % in the three proportions.
- The pH was affected during the storage time, with a minimum of 5,75; but these decreases did not influence significantly the biological stability of the strains.

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