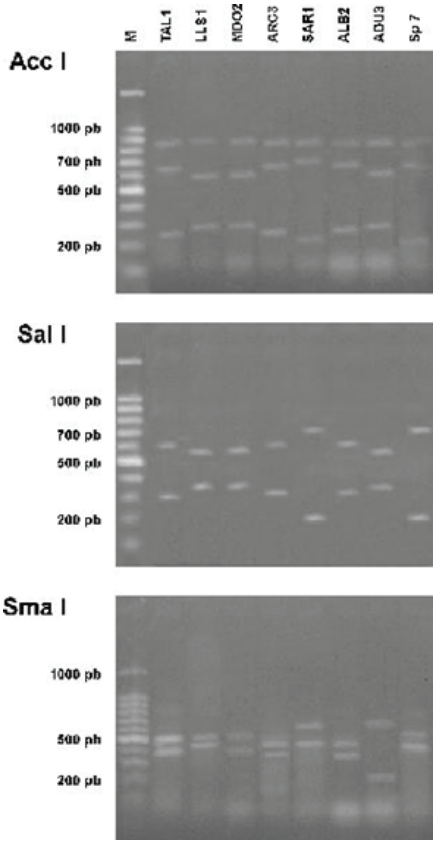

Fig. 3. Electrophoresis of amplified *nir*S fragments digested with *Acc*I, *Sal*I and *Sma*I. 1-1B, 3-2A, 10-2B, 13-2C, 16-2A, 17-2B and 25-2B are designations of *A. brasilense* isolates from the sugarcane area in Tucumán, Argentina. Sp7 is the strain of reference for *A. brasilense*. WM: Molecular weight marker Ladder 100 bp.

Whereas variability in the enzyme digestion profile of the *nir*S fragment was observed, the RAPD technique revealed an intra-specific genomic variability (Fig. 4).

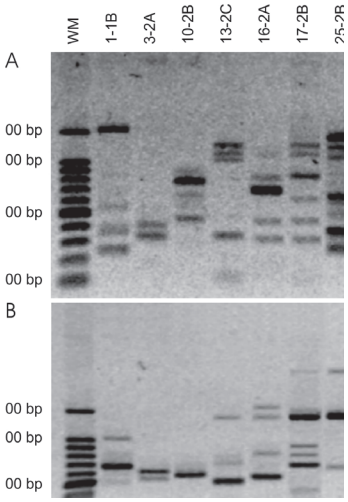

Fig. 4. RAPD profiles of the amplification of genomic DNA of seven A. brasilense local isolates. All showed positive nitrite reductase activity (nir+) and exhibited PCR-*nir*S amplification. A and B correspond to the amplification reaction using the primers A-06 and A-08 respectively..

Discussion

In this work we isolated *Azospirillum* from the rhizoplane of sugarcane plants in two contrasted sugarcane cropping areas of Tucumán, Argentina, and identified the species *A. brasilense*. According to the MPN of denitrifiers, the higher values were found in the wetter area (West) under study, compared with the drier one (East). Considering that only a small percentage of bacteria can be cultivated as yet, the MPN for denitrifiers may represent only a small percentage of bacteria, including the overall denitrifiers. Comparing the two regions of sampling, *A. brasilense* isolates with denitrifying activity was predominant in the West area. Since denitrifying bacteria consume easily available organic matter while using nitrates as their electron acceptor, we speculate that the organic matter content and carbon-nitrogen ratio (Table 1) would not be limiting factors for denitrification. Table 1 shows that these parameters are almost similar in both areas, as well as pH values.

When flooding occurs, as it happens in many parts of the western sugarcane area of Tucumán during summer season, the water covers the soil and hinders the oxygen supply; consequently, bacteria start utilizing nitrogenous compounds as electron acceptors contributing to the denitrification of soils. Thereby, available nitrates can be denitrified when soils are completely saturated, while very little denitrification occurs when soils are well drained. In 1995, Philipott *et al*. studied the role of the dissimilatory nitrate reductase in the competitive abilities of *Pseudomonas* strains, and proved that denitrification can contribute to the persistence and distribution of bacteria in fluctuating soil environments.

Nitrogen fertilization on sugarcane crops in Tucumán is carried out between October and November; consequently, the availability of nitrates is guaranteed during summer season, coinciding with the raining period. In this way, the amount of nitrates in soil and the input of rainfall water, plus the estival temperatures, do contribute to favor the denitrification process.

It is well know that soil texture (*e.g.* loamy in West area and clay-loam, silty-loam in East area, Table 1) and soil type (e.g. typic argiudoll-fluvenitc in the West and typic ustifluvent-thaptoargic in the East, Table 1) are characteristics that affect moisture retention and aeration of the soil. Likewise, the clay content contributes to increase surface area and chemical properties of soils, affecting therefore the microbial activity. We speculate that in our case, these factors may surely

influence the differential occurrence of *Azospirillum* within the sugarcane cropping region studied. Eaton (2001) demonstrated that significant variations exist in the soil composition of different and closely situated habitats in the subtropical forest of Belize, associated with the relative richness of the vegetation. Considering this latter, as in our study the sampled area has only sugarcane plants, the variations observed in the MPN of denitrifiers would not be related to vegetation type but to environmental conditions

Another important aspect that supports the influence of water (rainfall) in the distribution of *Azospirillum* in the sugarcane cropping region is the evapotranspiration process between the two areas under study. While in the West the mean annual evapotranspiration (potential) is 900 mm or less, in the East area it is 1046 mm. Comparing these values with the mean annual rainfall volumes in both areas, we can see a positive hydric balance (rainfall minus evapotranspiration) in the West area and a negative hydric balance in the East area. According to the higher occurrence of denitrifying isolates in the West area, we suggest that there is a clear influence of the rainfall and also soil type and texture, since the rate of drainage depends on these soil features. In a previous work, we observed a similar behavior about the environmental influence on the genetic diversity of *A. brasilense* isolates and hypothesized that the genetic diversity observed in different isolates can be a result of the selective pressure exerted by local chemical and physical-chemical properties of the soil over a certain period of time, giving place to a heterogeneity of environmental niches (Pedraza and Diaz-Ricci, 2003).

Although we have carried out PCR amplification of the *nir*S and *nir*K genes of all the isolates, regardless if they were denitrifiers or not, when nitrite reductase activity in semisolid NFb medium was assessed, only those that displayed nir^+ activity showed amplification bands of the gene *nir*S. The latter confirms that the denitrifying isolates of *A. brasilense* belong to the *nir*S type of the nitrite reductase, which contains cytochrome cd_1 as reported in other works (Bothe *et al*., 1994; Braker *et al*., 1998; Braker *et al*., 2000, Kloos *et al*., 2001). Our results also show that the variability observed in the nir^+ activity of the isolates (number and size of bubbles) may be associated with the genetic variability detected in the *nir*S fragment through enzyme digestion.

According to our results, since nir^+ isolates showed intragenic diversity within a functional nitrite reductase, we may speculate that the *nir*S fragment amplified does not correspond to a conserved domain of the nitrate reductase enzyme. Furthermore, environmental differences in nutrient availability and humidity may also affect the kinetics of bacterial enzymes such as nitrite reductase. If we consider that these factors can work as selective agents on gene controlling enzyme systems, then we should accept that "a relationship does exist between various components of genetic diversity among populations of bacteria and variability associated with their environment" as suggested by McArthur *et al*. (1988).

RAPD analysis of the denitrifying *A. brasilense* isolates showed that these strains not only presented intragenic diversity within the *nirS* gene, but also differences at genomic level. Nevertheless, comparison of restriction profiles of the 16SrDNA fragment amplified by PCR of all isolates showed that they were similar, including the reference strain Sp7 of *A. brasilense*. However, from our results we can not infer that the genomic differences observed and presented in Fig. 4 can be associated with the denitrifying activity, nor to the variability detected in the gene *nir*S. The RAPD method amplifies at random DNA fragments that may not be necessarily the same corresponding to the *nir*S gene assessed in this study.

According to results presented in this work, effective non-denitrifying wild-type *Azospirillum* strains can be isolated from sugarcane roots that could be used as microbial inoculants as they are believed to be more beneficial for plant growth promotion. The knowledge of the ecology of denitrifiers (in this case, *Azospirillum* strains), represents a step forward to the rational utilization of the microbial diversity in agriculture.

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