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Characterization of plant growth-promoting rhizobacteria from maize under low temperature
Caractérisation de rhizobactéries promotrices de la croissance du maïs à basse température

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Introdução

The maize is an essencial culture to Brazil as food source as exchange value. The brazilian maize production occupies a prominence position so much in national ambit as international being placed in the third place after United States and China. Although the production amount is high, 32 million tons, the volume for cultivated area is low, 2.275 kg/ha, when compared with other producing countries as United States, France, Yugoslavia and Romenia, with medium revenues of grains of the order of 7.000 kg/ha (IBGE, 1991-92).
When sowed in soils with high humidity and lower temperature, it happens the retardation in the germination and in the seedling development, doing with that the same ones stay in a susceptibility state the soil pathogens for a longer period (Galli, 1980).
The main tecnhiques used in the control of soil pathogens are the application of the chemical products in the seeds and the use of resistant varieties. Due to these they be pointed as ineffective in some cases, the search of alternative methods as the biocontrol becomes necessary (Kloepper, 1991).
The use of plant growth-promoting rhizobacteria (PGPR) has been considered as a good method for the control of soil pathogens and it presents a great promise in agricultural crop production.
The objective of this study was the isolation, characterization and identification of microorganisms capable to improve the seedling emergence of maize under low temperature.

Material and Methods

Bacterial isolation and purification

For the isolation of the rhizobacteria, the seeds of the maize were sown in the plastic pots with 500 grams of soil samples from the different sites in Brazil (table 1). After thirty days of the planting, the roots were harvested. To remove the bacterial cells from the root surface of maize, the root system was rinsed with sterile water several times until adhering soil was removed. Five grams of root segments were put in a flask, containing 5ml of sterile phosphate buffer pH 7,0 and were macerated. Ten-fold serial dilution (10^{-1} to10^{-9}) of the suspension was prepared assepticalIy for inoculation. The isolation and purification were carried out in two different media. 0,1ml samples were spread onto Nutrient agar plates (Difco) and King's medium B (King et al. ,1954) and incubated at 15°C for 48 h. Well developed colonies were selected and subjected to purification. The isolates were stored in King's medium B at 15°C.

Table 1. Soil samples

Samples	Locality
1	Caxias do Sul , Bairro Torqueta
2	CNPMA/EMBRAPA
3	Chile, contaminated by *Fusarium* sp
4	Limeira/São Paulo, Sítio Primavera
5	Eng. Coelho, Fazenda São Pedro
6	Iracemapolis, Sítio Santo Antonio
7	Ponta Grossa/PR, Fazenda Escola da Universidade
8	Piracicaba/ São Paulo, ESALQ
9	Limeira/São Paulo, solo de painço

Identification and characterization of the bacteria isolates

To obtain data on the description and identification of the isolated strains, cultures were grown on Nutrient agar medium. Gram-staining and motility by the hanging drop technique were examined by following method described by Case and Johnson (1984). Cell shapes were observed in Gram-stained smear under a light microscope. Cytochrome-oxidase production was tested using paper oxidase test (Difco). Catalase activity was determined by the production of bubles after adding one drop of 3% hydrogen peroxide. For the oxidative-fermentative test (OF test), a test tube containing 8ml of Hugh -Leifson's glucose medium (Cowan and Steel's, 1995) was inoculated with the fresh culture by stabbing.

ID EB-20 and NF-18 (Nissui SA, Japan) test procedure

Bacteria were removed from a 24 hour culture on the Nutrient agar plate. The cells were suspended in 5 ml of 0,85% sterile NaCl solution to an opacity of the MacFarland 0,5 standard. The cell suspensions were distributed into the wells containing the test media. Cultures of the trays and observations were performed by following the directions of the system. Identification was conducted by consulting the Nissui profile index and when results could not be consulted with the index, traditional biochemical tests were done.

Preparation of bacterial DNA

Bacteria cultures were grown on the Nutrient agar plates for 48 hours at 15°C. The cells were carefully suspended with saline-EDTA and centrifuged at 10,000 rpm for 20min at 4°C. The cells were suspended with saline- EDTA for lysing. Methods for extraction are based upon Marmur's procedure (Marmur, 1962).

Determination of guanine plus cytosine (G+C) content

The DNA dissolved in 0,1x SSC (saline-citrate) was heated at 100°C for 10 min and cooled rapidly in an ice bath. 10 ul of a nuclease P1 solution was added to 10 ul of the denaturated DNA solution. Then 10 ul of the mixture was applied to HPLC after one hour of incubation at 50°C. Analysis Standard was applied as the standard quantification. HPLC was performed by using a model LC-6A apparatus, chromatopac C-R4A (Shimadzu Corp.), under as follows conditions: column, Cosmosil 5C18 4.6x150mm; eluent: 20mM $NH_4H_2PO_4$-CH_3CN (20:1, v/v); flow rate, 1ml/min; detection at UV 270nm.

Teste of antagonism *in vitro* against *Fusarium moniliforme*

The test was done by pairing of cultures on Petri dishes containing 20 ml of potato dextrose agar (PDA) medium. Previously 7 mm of well grown *Fusarium* sp discs on PDA medium for 5 dias at 24°C were put in one side of Petri dish. One day after, at the opposite side of Petri dish, bacterial strain was inoculated and incubated at 15°C for 96 h. After incubation period, the results were evaluated by the distance of inhibition halo (cm) from the margin of fungi to bacterial colony.

Rhizobacteria effect in the germination and seedling growth

In this test 58 isolated colonies from maize rhizoplane were tested. Previously maize seeds were surface sterilized by immersion in 1% aqueous hypochlorite sodium by 10 minutes, throughly rinsed in sterile distilled water. Then the seeds were put into a bacterial suspension (10^{-8} ufc/ml) with sacarose 5% for 10 minutes. Ten seeds were sown on the 500 ml pots, four pots for each treatment. The pots were incubated in a growth chamber at 15°C, photoperiod 12/12h. After 15 days the seedling emergence were counted and 30 days after the seedlings were removed from the pots, the roots were cut and rinsed in sterile distilled water and placed to dry under 60°C.
The result was determined with comparison of averages by Tukey test.

Results and Discussion

Characterization and grouping of strains

From the total of 58 colonies isolated 6 Gram-negative bacterial strains that promoted seed germination and seedling emergence were characterized.and identified. Some of the characteristics of the strains are listed in table 2, 3, 4 and 5. Cells of the strains were mostly rod-shaped and differed in size. One strain presented a fluorescence yellow pigment on the King's medium B.

Table 2. Characterization and grouping of selected strains on the basis of the motility , OF-test (oxidative/fermentative), oxidase and fluorescence pigment production on King B medium

Test \ Isolates	MIR	M7I	M3G	MIT	M4L	M3A
OF-test -	F	F	O	I	O	I
Motility	-	+	+	-	+	-
Oxidase	-	-	+	-	+	+
Fluorescence pigment	-	-	-	-	+	-

F= fermentative; O= oxidative; I= inert

Table 3. Main phenotypic characters of differenciation between OF-fermentative isolates

Test\ Isolates	MIR	M7I
Motility	-	+
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	+
Nitrate to nitrite	+	-
Voges Proskauer test (37°C)	+	-
Fermentation:		
Raffinose	+	-
Rhamnose	-	+
G + C mol%	53,2	48,7

Table 4. Main phenotypic characters of differenciation between OF- oxidative isolates

Test\ Isolates	M3G	M4L
Pigment	violet	yellow
ONPG	+	-
Aesculin hydrolysis	+	-
Fluorescence King's medium B	-	+
Carbohydrates acid from:		
Maltose	+	-
Raffinose	+	-
Sucrose	+	-
G+C %mol	60,7	57,9

Table 5. Main phenotypic chacters of differenciation between OF- inert isolates

Test\ Isolates	M3A	MIT
Catalase	-	+
Oxidase	+	-
Haemolysis	-	B
Carbohydrates acid from:		
Lactose	+	-
Mannitol	-	+
Raffinose	-	+
Sorbitol	-	+
Sucrose	-	+
Xylose	+	-
G+C% Mol	39,6	64,7

The six selected strains which had been classified into fermentative, oxidative and inert group were further identified using the ID test-EB-20 and NF-18 (Nissui SA, Japan) identification strips. The results were confirmed by DNA base composition of the isolates. It is well known that in soil there are various kinds of bacteria which are functionally and taxonomically diverse. This may sometimes account for the low applicability of ID system, which has been mainly used for medical or food isolates that are not diverse compared to those from soil. So it is very important to do additional tests to confirm the identification. Therefore the knowledge of bacteriological characteristics of the soil isolates, as well as a modification in usage of the system, we can use the ID system and confirm the applicability of the system on the soil bacteria.
According to Bergey's Manual of Determinative Bacteriology (Krieg et al., 1984 and 1994); Cowan and Steel's (1995), the strains were identified as:

MIR - *Serratia plymuthia*
M7I - *Buttiauxella agrestis* (Ferragut *et al.*, 1981)
M3G -*Janthinobacterium lividum*
M4L - P*seudomonas fluorescens*
M3A - *Sphingobacterium multivorum* (*Flavobacterium multivorum* grupo IIk-2)
MIT - *Acinetobacter haemolyticus*

Test of antagonism *in vitro* against *Fusarium moniliforme*
The selected isolates were tested for inhibition of *Fusarium moliniforme* growth on PDA medium. Six isolates were capable to inhibit the pathogen fungi growth, being obseved inhibition halo varying among 0,28 to 2,50 cm (table 6). Although there was a decrease in the growth, none of isolates inhibited completely the growth as observed by Gells & Schippers, 1983. The capacity of antibiotics production in the soil by the introduced microorganisms has been inquired due to the lack of methods to evaluate the importance of these in the inhibition of another microorganisms (Fravel, 1988).

Table 6. Effect of rhizobacteria on the ***Fusarium moniliforme*** (Fm) growth *in vitro.*

Strains	Fm growth (cm)	inhibition halo(cm)
M3G	2,95	1,95
MIR	2,08	2,50
M4L	3,60	1,10
M3A	2,50	1,80
M7I	2,90	0,88
MIT	4,55	0,28

Effect of plant growth promoting-rhizobacteria in the maize seedling

In the germination test (table 7) some of the isolates increased the germination rate of maize seeds in up to 31% and the best treatment was with the isolate M3G that provided 100% of germination.
It was also observed that all the selected isolates, although the difference has not been significant (Tukey 1%), the dry weight of seedling increases in up to 24% when compared to the control without bacterial inoculation).
Coating of maize seeds with these isolates some ones strongly promoted the germination of the seed and the subsequent establishment of the seedling under low temperature.
The decrease or increase on the growth of plants can be influenced by the type of microorganism introduced (Schippers *et al.*, 1987; Alstron & Gerhardeson, 1989; Chanway *et al.*, 1991; Hofte *et al.*, 1991).

Table 7. Effect of plant growth promoting-rhizobacteria in the maize seedling

Isolates	dry weight(g)	germination (%)
M3G	0,1775ab	100,0
M IR	0,1800b	52,5
M4L	0,1950b	90,0
M3A	0,1925b	92,5
M7I	0,1675a	97,5
MIT	0,1850b	92,5
Control	0,1502a	53,5

CV 8,449%, Tukey test (1%)

Seed inoculation with plant growth-promoting rhizobacteria can be expected in agricultural systems. In the same line of research the identification of rhizobacteria that promote the seed germination and seedling emergence of some tropical agricultural species in low temperature has been considered as new approach.

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