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Potential for Nitrogen Fixation in Maize Genotypes in Brazil

(grass-bacteria associations/*Spirillum lipoferum*/acetylene reduction)

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ABSTRACT **N_2 fixation in field-grown maize (*Zea mays* L.) plants was estimated by a nondestructive acetylene reduction method which permitted the plants to continue growing and produce seeds. Samples from six areas revealed mean nitrogenase activities of 74–2167 nmol of C_2H_4/(g of dry roots × hr) for 10 plants. Among 276 S_1 lines planted in two field experiments, 17 lines were selected for further nitrogenase activity assays after prescreening. Variability within lines was high but significant differences among lines were obtained in one experiment. The best lines showed mean nitrogenase activities of 2026, 2315, and 7124 nmol of C_2H_4/(g of dry roots × hr), whereas the original cultivar reduced only 313 nmol. The highest value approaches the nitrogenase activity of soybean. If the theoretical 3:1 (C_2H_4/N_2 reduced) conversion factor is used, a potential daily N_2 fixation of 2 kg of N_2/hectare can be calculated. Periodic sampling within a brachytic maize cultivar revealed that maximum nitrogenase activity occurred at about the 75% silking stage. Soil effects also were pronounced. N_2-fixing *Spirillum sp.* could be isolated from all active root pieces when they were surface sterilized. These organisms appear to be primarily responsible for root nitrogenase activity in maize.**

Although it has been suggested for some years that the N_2 fixation occurring in tropical grasses is of economic importance, it is (1–3) only since the introduction of the acetylene reduction method that it has been possible to adequately test the suggestion. Numerous forage grasses in Brazil, Nigeria, and the Ivory Coast, mainly *Paspalum notatum, Digitaria decumbens, Pennisetum purpureum, Brachiaria mutica, Panicum maximum,* and *Andropogon gayanus,* have been shown to exhibit nitrogenase activities greater than 200 nmol of C_2H_4/(g of dry roots × hr); about 1000 nmol has been the upper limit (4–6). In the *Paspalum* association, *Azotobacter paspali* appears to be the major responsible microorganism, whereas in *Digitaria decumbens* cv *transvala,* a primitive symbiotic association with *Spirillum lipoferum* has been observed (7). Similar *Spirillum* strains were isolated from *Panicum maximum* and several other N_2-fixing grass roots. Maize (*Zea mays* L.) seedlings grown in water-logged pots in growth chambers were reported to reduce up to 10,000 nmol of C_2H_2/(g of dry roots × hr) under high light intensities (8). Under temperate climatic conditions, maize grown in the field showed variable C_2H_2 reduction; there were short periods of very high activity [3000 nmol of C_2H_4/(g of dry roots × hr)] after rains (9) and the authors suggested that anaerobic N_2-fixing bacteria were primarily responsible, although a role for blue-green algae was not eliminated. Raju *et al.* (10) isolated the facultative N_2-fixing *Enterobacter cloacae* from C_2H_2-reducing maize roots, but activities on intact maize root systems reported were very low (16 nmol of C_2H_4/hr in an entire root system).

Before these findings can be applied in agriculture, it will be necessary to assess the limiting factors involved and to develop agronomic means for increasing N_2 fixation in the grasses. Soil temperatures below 27° and minimum night temperatures below 18° have been identified as major limiting factors in the *Digitaria–Spirillum* association (Abrantes, Day, and Döbereiner, in preparation), but soil humidity also interfered when the wilting point was reached. Phosphate and minor element fertilization increased nitrogenase activity (11). Possibilities for complementing biological N_2 fixation with fertilizer N seem good since NH_4^+ concentrations below 200 ppm in the soil solution did not limit nitrogenase activity in the field and applications of 20 kg N/hectare(ha) every 2 weeks did not affect fixation even after seven such doses (6). N_2 fixation in grasses may be increased by plant breeding; there are significant differences in fixation between *Paspalum notatum* and *Pennisetum purpureum* cultivars (5). The present paper discusses nitrogenase activity in field-grown maize cultivars and S_1 lines, as studied in Brazil, and the isolation of highly efficient N_2-fixing *Spirillum* strains from the most active roots.

MATERIALS AND METHODS

After an initial survey with six different field tests, three field experiments with maize were used to obtain information on how plant genetic variations and variations within the growth cycle influenced N_2 fixation.

Field Selection for N_2 Fixation among S_1 Lines of Maize. Maize was grown in two 12 × 12 simple lattice experiments for evaluation of S_1 lines during the 1974–75 summer season on a low-land area within the experimental fields of the Departamento de Fitotecnia, Universidade Federal Rural do Rio de Janeiro. Planting dates were October 7 for Exp. I, and October 11 and 14 (replications 1 and 2, respectively) for Exp. II. Plots were single rows with 12 hills that were 0.40 m × 0.90 m apart, thinned to two plants per hill. There were 138 inbred S_1 lines per experiment; the remaining six treatments were filled in with bulk seed of the original open-pollinated variety, UR-1. This variety originally was selected from a mixture of lines resistant to rusts (*Seleção Rp Rpp*). It was crossed and backcrossed to *Composto Flint* (developed in the Instituto de Genética, Escola Superior de Agricultura "Luiz de Queiroz," Piracicaba), and underwent one cycle of half-sib family selection. Land was limed in June 1974 to a pH varying from 5.8 to 6.2. Levels of P and K were medium-high before

Abbreviation: ha, hectare.

fertilizing with 35 kg of P and 25 kg of K/ha. No N-fertilizer was applied. On November 25, both experiments were sprayed with 500 g/ha of ammonium molybdate [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$] in solution. Prescreening for nitrogenase activity started on December 18 when one plant per treatment was sampled. Since the results seemed promising, five plants were sampled from both replicates from each of the treatments with highest nitrogenase activity; plants had just passed the 75% silking stage (January 6). Exp. II was prescreened on January 21 and 27, about 23 and 30 days after the 75% silking stage. Further sampling within chosen treatments was done 2 days later, again on five plants per replicate. Analyses of variance were based on the model for a randomized complete block design with subsamples.

Variations of Nitrogenase Activities During the Growth Cycle of Brachytic Maize. A split plot experiment was established in a field of the cultivar *Piranão*, machine-planted at the rate of 50,000 plants per ha on November 27. The variety is a Mexican semi-dwarf maize (br_2br_2) of Tuxpeño background; it is being selected for yield and was released by the Instituto de Genética, ESALQ, Piracicaba. At the time of planting, 35 kg of P and 25 kg of K per ha were applied, but no N-fertilizer was used. Two areas were defined in this field by soil analysis (soils A and B). Soil A supported vigorous plants, whereas growth on soil B was much poorer. Six blocks in each soil consisted of one 10 m row fertilized with Mo and one that was not, separated by one border row. One kg/ha of ammonium molybdate in solution was sprayed on January 22. Sampling for nitrogenase activity was done weekly from January 23 through March 5 on four plants chosen at random in each plot.

Assays of Nitrogenase Activity on Maize Roots. Most of the methods used in these assays were based on the recently developed methodology for field grown *Digitaria* roots (12). Roots were harvested in the late afternoon to allow maximum accumulation of photosynthate. A nondestructive assay was adopted to permit harvest of seeds of any single plant that demonstrated very high C_2H_2 reduction; the corn plants were not removed from the soil, but a few roots were exposed with a hoe and removed at the node. The roots were washed immediately in distilled water to avoid drying and excessive O_2 access, and were placed into 120 ml bottles with rubber closures. In the laboratory, the gas phase in the bottles was replaced by evacuating three times to 340 mm Hg (340 torr) and refilling with N_2 from a cylinder. This left about 10% air (or 2% O_2) in the gas phase which was adequate to support the system overnight. In the morning, the assay vials were reevacuated three times to 340 mm Hg and refilled with N_2; this removed accumulated gases which might interfere in gas chromatographic analyses. Then 5% air and 12% C_2H_2 were added, and the bottles were incubated at 32° for 2 hr. The C_2H_4 produced was measured with a Perkin–Elmer F-11 gas chromatograph fitted with a 2 m × 3 mm Poropak N column at 100°. Assays for C_2H_2-independent C_2H_4 production on more than 50 maize root samples from the same fields were completely negative after 15 hr incubation. Roots were assayed for nitrogenase by cutting the most active roots into 5- to 10-mm pieces and distributing them into 1 ml disposable plastic syringes. The syringes then were filled with a gas mixture of 1% O_2 and 12% C_2H_2 in N_2, and incubated for 6–10 hr with their needles plunged into rubber stoppers. A 0.5 ml sample of the gas mixture then was injected directly from the syringe into the gas chromatograph. The root pieces were surface sterilized after being assayed, i.e., the sterilizing solution or phosphate buffer wash solution was sucked into the syringe and expelled.

Enrichment Cultures of N_2-Fixing Microorganisms. Semisolid N-free mineral media are suitable for the enrichment cultures of N_2-fixing organisms from roots, because they simulate the soil-root habitat. Slow diffusion of O_2 and carbon substrate permit the bacteria to grow under optimal conditions; the best conditions are a few mm below the surface for the microaerophyllic *Spirillum* strains. In most of the studies reported here, the following medium was used: KH_2PO_4, 0.4 g; K_2HPO_4, 0.1 g; $MgSO_4\cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.01 g; $NaMoO_4\cdot 2H_2O$, 0.002 g; Na malate, 5.0 g; bromthymol blue 0.5% in ethanol, 5.0 ml; agar (Difco), 1.75 g; H_2O, 1000 ml. Root pieces of 5 mm length were inserted into 2 ml of medium in 6 ml serum bottles and incubated for 1 or 2 days at 30 to 35°. The bottles then were fitted with serum stoppers and 12% C_2H_2 was injected into the bottles (there was no need to change the gas phase). The C_2H_4 formed in 1 hr was determined by gas chromatography. Enrichment of *Spirillum* in this medium is indicated after 2–3 days by the formation of very dense and thin undulating white pellicles and by very high nitrogenase activity (over 100 nmol of C_2H_4/hr from a culture). Care must be taken not to disturb the pellicles since this immediately stops nitrogenase activity. For enrichment of N_2-fixing bacteria other than *Spirillum*, the same semisolid medium was used but malate was replaced by another carbon substrate.

Isolation of N_2-fixing *Spirillum* from cultures enriched on malate is relatively easy. After one or two transfers into new N-free semisolid malate medium, a loop of the dense pellicle was streaked on malate agar complemented with 15 ml of yeast water per liter. After 7–10 days, characteristic *Spirillum* colonies develop. They are small, dry, raised or umbonate, irregular or circular, and their white color, often with green centers, contrasts with the blue agar. Single colonies were transferred into semisolid malate medium to check C_2H_2-reduction and then were streaked for purification on potato agar (200 g of potatoes were cooked in 1 liter of water, and 0.25% sucrose and 0.25% Na-malate was added to the water after filtration). After 1 week, colonies on potato agar are characteristically pinkish, large (0.5 cm), circular or irregular, raised and curled. Some bluish pigmented strains have been found. Highly-enriched cultures obtained from surface-sterilized roots can be isolated directly on this potato agar.

RESULTS

Occurrence of nitrogenase activity

A preliminary survey of the occurrence of C_2H_2 reduction by field-grown maize plants at different developmental stages on red-yellow podzolic and hydromorphic clay soils with various additions of N, P, K, and Mo was encouraging. Although there were large variations, activities in general were higher than those observed in forage grasses (5, 6), and maximum observed values for treatments ranged from 170 to 6846 and means of treatments from 74 to 2167 nmol of C_2H_4/(g of dry roots × hr). The highest values were observed for the S_1 maize lines grown in hydromorphic clay soil treated with P, K, and Mo. Roots were removed from 6- to 10-week-old maize plants

TABLE 1. *Nitrogenase activity in open-pollinated maize cultivar UR-1 and S_1 lines obtained from UR-1**

Experiment I			Experiment II		
	Nitrogenase activity†			Nitrogenase activity†	
Treatment no.	Mean	Max. observed	Treatment no.	Mean	Max. observed
122	7124	13,700	56	313	1084
134	2315	7,655	58	15	72
61	2026	6,846	83	228	1740
130	1850	4,612	115	418	1533
128	1798	6,121	123	177	691
102	1793	6,256	126	280	1114
81	1570	9,856	135	39	266
55	1026	3,036	UR-1	212	528
133	1010	3,909	UR-1	178	738
125	557	2,242	Mean	207	
UR-1	313	1,650			
Mean	1944				

Honestly significant difference (HSD) Tukey 5405 ($P = 0.05$)

* Data were obtained from selected lines after prescreening 276 lines in two 12 × 12 simple lattice field experiments. Values are means of 10 plants, five from each replicate. Maximum values obtained are given to show potential fixation. For nitrogenase activity assays, see *Materials and Methods*.

† nmol of C_2H_4/(g of dry roots × hr).

for testing, and 94 plants were examined in these preliminary tests.

Field selection for N_2-fixation among S_1 lines of maize

Tentative sampling within Exp. I revealed appreciable nitrogenase activity (C_2H_2 reduction) in roots and justified a more ample screening. Prescreening of replicate 1 at the early flowering stage yielded values ranging from zero to 2619 nmol of C_2H_4/(g of dry roots × hr). More ample sampling in chosen treatments at the late flowering stage yielded values (Table 1) ranging from zero to 13,700 nmol of C_2H_4/(g of dry roots × hr). F values were significant at $P = 0.01$ for replications and at $P = 0.05$ for treatments. Means were classified into two groups by using Tukey's HSD. Line 122 of maize showed consistently high values with only one plant lower than 2687 nmol of C_2H_4/(g of dry roots × hr). The original variety UR-1 showed the lowest values. There was no way to correct for soil differences within replications. Acetylene reduction was much less in Exp. II than in I. Prescreening on January 21 and 27, 1975 yielded values ranging from zero to 2141 nmol of C_2H_4/(g of dry roots × hr). Results of further sampling from chosen treatments (January 29) are summarized in Table 1. In this experiment, 50% silking occurred about December 26, 1974. The general mean was only 207 nmol of C_2H_4/(g of dry roots × hr) as compared with 1944 nmol obtained in Exp. I. Differences among replications were highly significant, but differences between means of treatments were nonsignificant.

Nitrogenase activity during part of the growth cycle

The large variation of nitrogenase activity observed in the S_1 line selection seemed associated partially with the stage of plant growth. Therefore, an experiment was performed on 2-month-old brachytic maize plants which showed lower, but reasonably constant, nitrogenase activity. Results are presented in Fig. 1. The analysis of variance revealed highly significant ($P = 0.01$) differences that were associated with the sampling date; the maximum nitrogenase activity measured occurred close to the 75% silking stage. During this period, ovules are fertilized and rapid cell division follows. A second increase in activity was observed at the last sampling date which coincided with rapid cell multiplication in the endosperm and the beginning of grain filling (13). The classical figures on growth cycle variations are similar to N_2 fixation in legumes such as the soybean (*Glycine max*, L.) Soil temperatures during this experiment (Fig. 1) did not correlate well with C_2H_4 reduction; near optimal temperatures for N_2 fixation prevailed. Moisture conditions also were ideal except at sampling 5 when the plants started to wilt after 10 days of intense heat without rain.

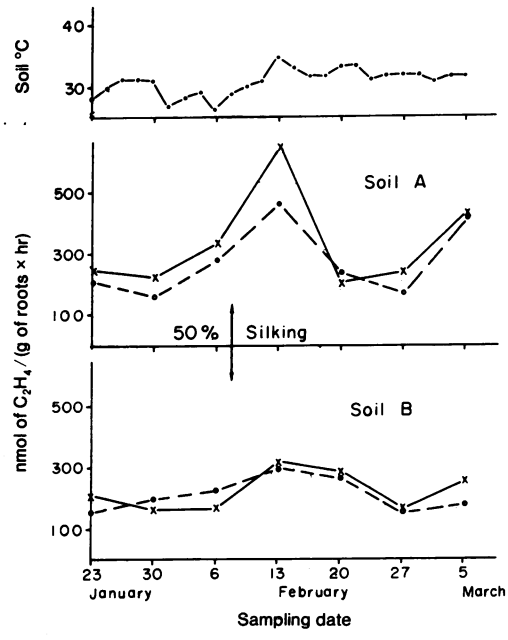

FIG. 1. Variation in nitrogenase activity on maize roots as influenced by the plant growth cycle. Maize cultivar Piranão (brachytic) was planted November 27 with P and K fertilizer. On January 22, half of the plots were sprayed with 1 kg/ha of ammonium molybdate (——); the other half were not sprayed (– – –). Nitrogenase activity was determined, as described in *Materials and Methods*, in root samples from four plants in each plot every week. Points represent means of six plots of 4 plants each. Analysis of variance was based on a split plot model of randomized complete blocks. Differences among soils and sampling times were significant at $P = 0.01$, but the effect of Mo and the interactions were not significant. Soil temperatures given in the top section of the figure were taken 10 cm below the soil surface at 3:00 p.m.

Soil B had markedly lower levels of P, K, Ca, and Mg than soil A, and nitrogenase activity also was lower. No significant difference arising from Mo spraying could be detected on either soil, although soil A showed greater activity in Mo-treated plots. The high coefficient of variability (41%) can be explained by soil variations within plots and probably by genetic variability of nitrogenase activity within this cultivar.

Isolation and identification of bacteria

Repeated observations on numerous high- and low-activity maize root systems indicated that thick (3–5 mm) roots with

TABLE 2. *Effect of surface sterilization of maize roots on nitrogenase activity of enrichment cultures**

Sterilizing solution	nmol of C_2H_4/(culture × hr) Exp. I	Exp. II
Check (H_2O)	34	—
H_2O_2, 10%	70	—
$HgCl_2$, 0.1%	6	42
Ethanol, 70%	450	170
Household disinfectant†	142	127
HSD Tukey $P = 0.01$	42	55

* Maize roots from field-grown plants were selected for high nitrogenase activity. Roots 5–7 mm thick with many laterals were sterilized for one sec (Exp. I) or 30 sec (Exp. II), washed six times in phosphate buffer (pH 7), and cut into pieces of 5–10 mm in length. These were placed into semisolid N-free malate medium (2 ml), and C_2H_2 reduction was assayed after 1 day (Exp. I) or 3 days (Exp. II) in the bottles without disturbing the pellicles. Values are means of 10 enrichment cultures.

† Quaternary nitrogen disinfectant.

many laterals are usually the most active. Pieces of such roots, 5–10 mm long, were placed into N-free semisolid malate medium. After 24 hr, enrichment cultures were obtained which in an hr reduced about 100 nmol of C_2H_2 per culture. Some acid and gas production was observed, and the cultures consisted of a variable mixture of organisms. The sugar medium usually supported lower nitrogenase activity, but produced more acid and gas. The nitrogenase activity of root pieces measured before they were placed into the culture media did not correlate well with the nitrogenase activity of the enrichment cultures they produced in malate, glycerol, mannitol or sucrose media.

It seemed desirable to suppress N_2-fixing organisms, which multiply rapidly in culture but are not very active on the roots, by surface sterilization of the thick corn roots (Table 2). Rapid submersion (1 sec) of entire roots in disinfectant increased the nitrogenase activity of the enrichment cultures, and 93% of these cultures showed the typical *Spirillum* pellicle in 3 days. Cultures from nonsterilized root pieces had pellicles in only 63% of the cultures. After 30 sec sterilization, 3-day enrichment cultures showed characteristic *Spirillum* pellicles. Microscopic examinations revealed characteristic curved, highly motile rods with prominent refractive fat globules. To assess whether enrichment cultures obtained from surface-sterilized roots represented the microflora responsible for root nitrogenase activity, we assayed 60 root pieces from each of two different active maize root systems in syringes. Half of them were surface-sterilized for 1 sec in 1% household disinfectant and half in 10% H_2O_2. Ethanol could not be used because it produced odd peaks in the chromatographic analyses even after six washings. The root pieces were placed into semisolid malate medium for 24 hr at 35°, and then the C_2H_4 that was produced in 1 hr was measured. There were highly significant positive correlations of root piece activity with that of the enrichment cultures for the two root systems when household disinfectant was used for surface sterilization, but not when H_2O_2 was used.

Isolation of N_2-fixing *Spirillum* strains from the most active enrichment cultures yielded 25 pure cultures which resembled the *Spirillum lipoferum* strains isolated from *Digitaria* roots (7). However, variability in cell size and pigment formation seem more common in the maize strains. Nitrogenase activity of three of these strains and the type strain from *Digitaria* were tested by measuring C_2H_2 reduction in semisolid malate cultures after 24 hr incubation at 35°. In 1 hr, 519, 628, and 717 nmol of C_2H_4 per culture (2 ml) were produced, whereas the type strain from *Digitaria* produced 489 nmol.

Preliminary experiments on temperature and oxygen requirements for nitrogenase activity on maize roots showed 31° and a pO_2 of 3.8–7.6 torr as optimal (Nery, Day, Neves, and Döbereiner, in preparation). Temperature optima of three cultures from maize were as high as those from *Digitaria;* 31–40° gave maximal activity. Mean maximum and minimum soil temperatures at 10 cm depth were 31.1 and 24.3° in January and 31.0 and 26.0° in February 1975, as measured at the local weather station (Fig. 1).

DISCUSSION

Nitrogenase activities in maize roots described in this paper are higher than any reported before for grass–bacteria associations under field conditions. This was unexpected, but high activities will be necessary if N_2 fixation in maize is to become of economic importance. Converting C_2H_2 reduction into kg of N_2 fixed is subject to criticism, and the assays used here on roots removed from the soil put further limitations on such estimates. However, comparisons of root activity of maize with that of intact soil-plant systems in *Paspalum notatum* (5) and *Digitaria decumbens* (12) indicate underestimates by root assays even when the roots are harvested on sunny afternoons. Recognizing these limitations, approximations from the analytical data still are helpful in understanding the potential of N_2 fixation in maize. The mean nitrogenase activity of the best S_1 line (an hourly production of 7124 nmol of C_2H_4/g of dry roots), if converted by the theoretical 3:1 factor (C_2H_2/N_2) and assuming a root mean dry weight of 1500 kg/ha (14), corresponds to a daily fixation of 2.394 kg of N_2/ha. Nitrogenase activity in the original cultivar, if calculated in the same way, corresponds to only 0.105 kg of N_2. Well-nodulated soybeans, which can obtain all of their N by fixation of N_2, when grown with similar soil and fertilizer treatments, reduced 30,000, 36,000, and 29,000 nmol of C_2H_2/(g of dry nodules × hr) at the blooming stage (Nery and Döbereiner, in preparation). If the whole root system, including the nodules is considered, these values corresponded to 9137, 12,544, and 9792 nmol of C_2H_2/g of dry roots. Specific activities of soybean nodules cited in the literature are in the same range (15). These data indicate that the potential nitrogenase activity in maize roots approaches that of soybeans. It is not known to what extent it will be possible to exploit this potential. As in forage grasses, in cooler regions soil temperatures may be a limiting factor (Abrantes, Day, and Döbereiner, in preparation). In tropical regions, the possibilities appear favorable for plant breeding to achieve more efficient N_2 fixation in maize–bacteria associations. It also may be possible to adapt the associations to function in temperate climates. Nitrogenase activity in roots results from a complementary interaction between plant and bacteria, and many factors are involved, such as photosynthetic efficiency, translocation, root anatomy and physiology, and bacterial characteristics,

As in the legume–bacterial symbioses, the growth stage of the plant is of major importance. Initial observations in the

maize system (Fig. 1) have indicated maximal nitrogenase activity during the flowering stage, but reasonably high activity occurs during the whole period of active growth and grain filling. It generally is accepted that in maize the reservoir rather than the source of photosynthates is the limiting factor for dry matter production in grain (16). If so, N_2 fixation would not necessarily compete with the grain for carbon substrates.

We cannot now define categorically the kind of bacterial association in maize. However, the consistent isolation of highly efficient N_2-fixing *Spirillum* from the most active root pieces of maize strongly indicates that *Spirillum* is of primary importance among the microorganisms responsible for high nitrogenase activity in maize roots. There is, however, a poorly defined N_2-fixing microbial population even on root pieces with low N_2-fixing capability; this has not been observed in the *Digitaria* association (7). The highly significant correlations of root activity with *Spirillum* enrichment culture activity, after surface sterilization of the roots, supports the existence of an interior inter- or intracellular maize–*Spirillum* association in roots that is similar to that reported for *Digitaria* (7). The extremely high activities for C_2H_2 reduction would be difficult to explain by a casual rhizosphere association.

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