

Polyamines in Rice Seedlings under Oxygen-Deficit Stress¹

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ABSTRACT

Incubation of 3-d-old seedlings of *Oryza sativa* L. cv Arborio under anaerobic conditions, leads to a large increase in the titer of free putrescine while aerobic incubation causes a slight decrease. After 2 days, the putrescine level is about 2.5 times greater without oxygen than in air. The rice coleoptile also accumulates a large amount of bound putrescine and, to a lesser extent, spermidine and spermine (mainly as acid-soluble conjugates). Accumulation of conjugates in the roots is severely inhibited by the anaerobic treatment. Feeding experiments with labeled amino acids showed that anoxia stimulates the release of ¹⁴CO₂ from tissues fed with [¹⁴C]arginine and that arginine is the precursor in putrescine biosynthesis. After 2 d of anoxia, the activity of arginine decarboxylase was 42% and 89% greater in coleoptile and root, respectively, than in the aerobic condition. The causes of the differences in polyamine metabolism in anoxic coleoptiles and roots are discussed.

Polyamines (Put, Spd, and Spm²) are the subject of numerous studies on account of their involvement in the control of nucleic acid metabolism, protein synthesis, and growth (11). Although the precise biological role of these amines is still unknown, they probably function by interacting with organic polyanions (nucleic acids, phospholipids) (23). Many efforts have been made to clarify the role of Put in conditions of environmental stress, such as K⁺ and Mg²⁺ deficiencies, NH₄⁺ feeding, acidification, high salinity, water deficit and SO₂ fumigation which cause pronounced changes in its concentration (1, 2, 9, 10, 16, 22, 24, 26). Most of the stresses so far considered in the study of PA metabolism induce cytoplasmic acidification and, in this condition, an activation of Put biosynthesis through ADC is hypothesized to occur to balance the excess of protons (10).

Oxygen-deficit stress is known to induce cytoplasmic acidification (20) and it has been suggested that capacity to regulate the cytoplasmic pH is an important factor in determining root-survival differences between species (21). Some anaerobic mechanisms preventing acidosis, for instance, a mainly ethanolic fermentation rather than a lactic fermentation (20) or accumulation of alanine and Gaba (19), have been identified. In this work we investigated the question of

whether, in rice coleoptile and root, Put accumulation is a mechanism adopted in response to oxygen-deficit stress. Rice is one of the few well-known examples of a plant able to germinate in a nitrogen atmosphere (25). In this condition, the rice coleoptile elongates whereas the root requires oxygen in order to grow. In this paper we describe the PA titer in response to anoxic conditions. On 3-d-old seedlings maintained in aerobic condition or transferred to an anoxic environment, we determined the titer of free and bound PAs and the ADC activity. Furthermore, the anaerobic capacity of rice tissues to synthesize PAs was assessed by feeding experiments with radioactive precursors.

MATERIALS AND METHODS

Plant Material

The dehulled seeds of rice (*Oryza sativa* L., cv Arborio) were surface sterilized for 2 min with 70% (v/v) ethanol and for 30 min with 5% (w/v) Ca(OCl)₂, each treatment being followed by several rinsings with distilled water. The seeds were then spread on a net, hung 0.5 cm above the water surface in a 2.5 L jar containing 1.3 L of sterile water, and allowed to germinate for 3, 4, and 5 d in the dark at 25°C. Aerobic germination was assured by a stream of sterile air (passed through a 0.45 μm Millipore filter) flowed through a pumicestone at the bottom of the jar. After 3 d, some aerobic-germinated seedlings were transferred for 1 or 2 d into a jar made anaerobic by flushing nitrogen gas (99.999% nitrogen).

Bacterial contamination was tested by incubating an aliquot of the medium on an agar-nutrient medium as described by Reggiani *et al.* (17). Data from contaminated samples were discarded.

For labeling experiments smaller jars, 25 mL in volume, were used. Excised roots or coleoptiles of 3-d-old seedlings (7 in every sample) were immersed in 3 mL of modified Heller medium (17). Humidified air or nitrogen gas was continuously flushed through the growth medium to effect aerobic or anaerobic treatments. The outlet part of every treatment jar was connected with a jar containing 7 mL of 30% (w/v) KOH to trap the ¹⁴CO₂ released from tissues fed with 0.37 MBq of L-[U-¹⁴C]arginine or L-[U-¹⁴C]ornithine (12.7 GBq/mmol and 10.7 GBq/mmol, respectively).

Polyamine Determination

The seedlings were immediately frozen in liquid nitrogen and the tissues excised and stored at -80°C until extracted. The tissues were ground in a mortar with 0.6 M PCA (0.1 g fresh weight/mL PCA) and the homogenate cleared by centrifugation at 12,000 g for 15 min. The pellet was resuspended

¹ Research work supported by C.N.R. Italy. Special Grant I.P.R.A.-Sub-project 1, paper No. 1692.

² Abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine; PA, polyamine; ADC, arginine decarboxylase; Gaba, γ-aminobutyric acid; PCA, perchloric acid; HPTLC, high performance thin layer chromatography; HCAA, hydroxycinnamic acid amide.

in 1 N NaOH and aliquots (0.2 mL each) of the original supernatant or the resuspended pellet were mixed with 0.3 mL 10 N HCl and sealed in glass ampoules. After 18 h at 110°C, the hydrolysates were dried by speed-vac concentrator (Savant Instruments, Hicksville, NY) and then resuspended in 0.2 mL of 0.6 M PCA. To 0.2 mL of the nonhydrolyzed PCA supernatant, the hydrolyzed PCA supernatant and the hydrolyzed pellet fractions, were added 0.2 mL of saturated Na₂CO₃ and 0.4 mL of dansylchloride (5 mg/mL acetone). The stoppered tubes were stored overnight in the dark at room temperature. Further, the dansylated-amines were extracted into toluene (0.4 mL) by vortex mixing. When the two phases were well separated, 10 to 20 μ L of the toluene layer was loaded with a Linomat IV (Camag, Muttenz, Switzerland) on a HPTLC plate of silica gel 60 with concentration zone (Merck, Darmstadt, West Germany). The plate was run three times using ethylacetate/cyclohexane (4:5, v/v) as solvent. On removal from the tank, the plate was dried for 10 min at 60°C and the fluorescence (excitation 350 nm) of each line read into a densitometer TLC Scanner II (Camag, Muttenz Switzerland). Polyamines, Gaba, ornithine, and arginine were identified by comparison of R_f values for unknowns and standards. Arginine was dansylated with difficulty and migrated with a R_f value greater than the other amines. The data of PAs were quantitated by a D-2000 integrator (Hitachi-Merck, Darmstadt, West Germany).

ADC Assay

Samples for the determination of ADC activity were homogenized in 0.2 M Tris-HCl (pH 8.0), 20 μ M pyridoxal phosphate, 2 mM DTT at 0.1 g fresh weight/mL buffer in a cold bath. The supernatant obtained after centrifugation (12,000 g for 15 min) was used immediately for the assay. Some aerobic and anaerobic samples were desalted on Sephadex G25 disposable columns (Baker Instruments, Allentown, PA) in order to check the presence of ADC activator(s) or inhibitor(s) which could interfere with the enzymatic assay, but the activity of crude and desalted extracts was identical. The activity of ADC (arginine carboxylase, EC 4.1.1.19) in the tissue extract was estimated by measuring the release of ¹⁴CO₂ from L-[U-¹⁴C]arginine. The reaction mixture consisted of 650 μ L crude extract, 300 μ L labeled arginine (10 mM in the mixture containing 0.2 μ Ci L-[U-¹⁴C]arginine), 25 μ L 2 mM pyridoxal phosphate and 25 μ L 80 mM DTT. The assay tubes (Warburg flasks) were incubated for 1 h with gentle shaking at 45°C and the ¹⁴CO₂ trapped onto a paper disc impregnated with 0.15 mL of 30% (w/v) KOH lodged in a central well in the flask. The temperature of 45°C was chosen, being optimal for rice ADC (7). The reaction was stopped, without opening the flask, by adding 0.25 mL of 10% (w/v) TCA contained in the side arm of the flask. Trapping of labeled CO₂ continued for 1 h, after which the disc was removed and dried before being immersed in 3 mL Insta-gel liquid scintillation cocktail (Packard Instruments, Downers Grove, IL) and assayed for radioactivity with a Packard-Tri-Carb 300 spectrometer (Packard). The presence of unspecific decarboxylations was checked by adding 50 μ L 60 mM α -difluoromethylarginine (Merrell-Dow, Cincinnati, OH) to the reaction mixture. In every case, unspecific decarboxylations

represented less than 7% of total activity. Enzyme activity was expressed as pKat/mg protein (1 Katal = 1 mol/s). Proteins were determined by Bio-Rad protein assay (Bio-Rad, München, West Germany), using BSA as a standard.

Incorporation Studies

The excised roots and coleoptiles were incubated for 3 h under aerobic and anaerobic conditions. After this period, to each treatment was added 0.37 MBq of L-[U-¹⁴C]arginine (12.7 GBq/mmol), L-[U-¹⁴C]ornithine (10.7 GBq/mmol) or [U-¹⁴C]Put (4.37 GBq/mmol) by means of a microsyringe. The bubbling of the gas in the jars assured a continuous mixing of the solutions. After 3 h of labeling period, the tissues were rinsed with ice cold water and homogenized with 0.4 mL of 0.6 M PCA. After centrifugation (10 min at 12,000g), the pellet was reextracted in 0.2 mL of 0.6 M PCA, centrifuged, and the supernatants combined. An aliquot of PCA-soluble extract or of KOH from the ¹⁴CO₂-trap was treated and counted for radioactivity as described above. Radioactive amines were dansylated and separated by HPTLC as described above. Samples of approximately 10,000 cpm each were loaded on the plate. To locate radioactive amines, the plate was sprayed with En³hance[®] spray (Du Pont Co., Wilmington, DE) and fluorography carried out.

RESULTS

Polyamines and Oxygen-Deficit Stress in Rice

Three-d-old rice seedlings were exposed to aerobic or anaerobic conditions for 1 and 2 d. In coleoptile and root of 3 d, the PAs were present mainly in the free-amine form (about 66–86% of the total) except for Spm in the root of which 69.5% was present as conjugates (Figs. 1 and 2). The bound PAs were found almost all in the hydrolyzed PCA supernatant, with only trace amounts in the pellet (less than 3%, hereafter the two fractions are considered together). During aerobic growth, the level of bound PAs increased (Fig. 1) while the level of free PAs decreased in every tissue as germination proceeded (Fig. 2). Anaerobiosis increased the free Put titer about 2.5 times in 2 d both in coleoptile and in root (Fig. 2). The level of free Spd and Spm in the coleoptile was not affected by the treatment while a slight increase was observed in the root. The anoxic coleoptile accumulated Put, Spd, and Spm as conjugates (Fig. 1), while the stress seems to inhibit conjugation in the root.

In Vivo Labeling

To elucidate the biosynthetic pathway of Put and its further metabolism in anoxia, we fed rice coleoptiles and roots with labeled arginine, ornithine, and Put. The first indication came from the amount of ¹⁴CO₂ released from rice tissues fed with the labeled precursors of Put in air and in anaerobiosis (Table I). In both tissues, anoxia stimulated the uptake of ¹⁴C-labeled arginine and, to a lesser extent, of ¹⁴C-labeled ornithine. Of the labeled arginine taken up, a greater proportion was decarboxylated under anoxia, while the decarboxylation of labeled ornithine was not stimulated. In Figure 3 is shown a chro-

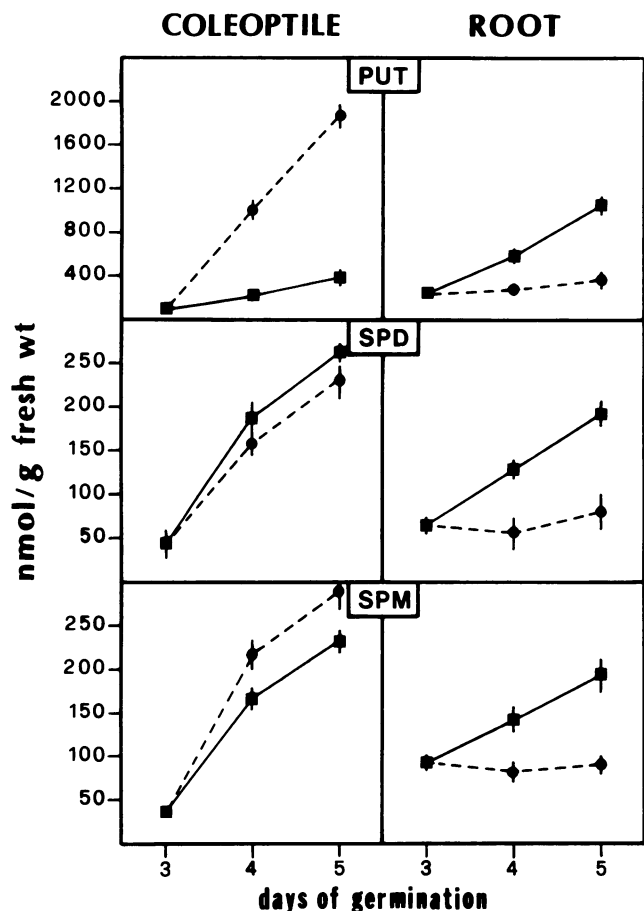


Figure 1. Titer of bound PAs in rice coleoptile and root of 3-d-old seedlings incubated for 2 d in aerobic or anaerobic conditions. Closed square (solid line), aerobic treatment; closed circle (dashed line), anaerobic treatment. Bars represent \pm SE. Each data point is the mean of three independent experiments.

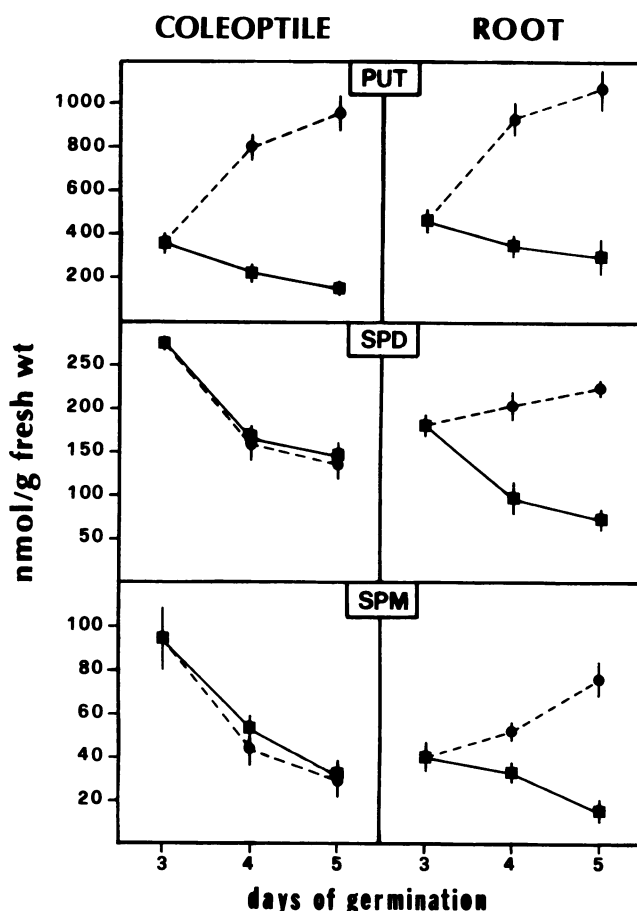


Figure 2. Titer of free PAs in rice coleoptile and root of 3-d-old seedlings incubated for 2 d in aerobic or anaerobic conditions. Symbols as in Figure 1. Each data point is the mean of four independent experiments.

matogram of radioactive dansylated material. Coleoptile and root of rice were able to synthesize Put from arginine under both aerobic and anaerobic conditions. Ornithine appeared to be a precursor for Put biosynthesis in aerobic tissues, while it was not metabolized to Put in the anaerobic coleoptile and only partially in the anaerobic root. In 3 h of labeling, Spd was labeled only in aerobically incubated roots. Gaba, a catabolite of PAs oxidation, was evident in all the aerobic-treated tissues and, surprisingly, also in the anoxic coleoptile and root fed with $[U-^{14}C]$ Put. This last result is of interest because it implies the existence of a degradative PA pathway not involving oxygen. At present, all the enzymes described as degrading PAs are oxidases (23).

ADC Activity in Response to Oxygen-Deficit Stress

The experimental observation that the anaerobic Put biosynthesis from arginine is stimulated while the conversion of ornithine to Put is inhibited, suggests an increase in the specific activity of ADC in tissues exposed to anoxia as reported for other stress conditions (9, 26, 27). This is confirmed by results in Figure 4 where ADC activity increased by 42% in the coleoptile and 89% in the root during a 2 d

Table 1. Uptake of $L-[U-^{14}C]$ Arginine and $L-[U-^{14}C]$ Ornithine and the Release of $^{14}CO_2$ in Coleoptile and Root of Rice in Air and under Oxygen-Deficit Stress

Excised coleoptiles and roots of 3-d-old seedlings were subjected to aerobic or anaerobic conditions for 3 h and then fed with labeled arginine or ornithine for 3 h of pulse. To every treatment jar containing 7 coleoptiles or roots, were applied 0.37 MBq of labeled arginine (12.7 GBq/mmol) or labeled ornithine (10.7 GBq/mmol). Data are mean of two independent experiments. Values are expressed as dpm/mg fresh weight.

Tissue	Condition	Labeled	Uptake	$^{14}CO_2$	Total Uptake %
Coleoptile	Aerobiosis	Arg	15619	406	2.6
		Orn	5316	69	1.3
	Anaerobiosis	Arg	89318	4345	4.9
		Orn	11890	143	1.2
Root	Aerobiosis	Arg	29998	719	2.4
		Orn	5276	74	1.4
	Anaerobiosis	Arg	65263	2781	4.3
		Orn	8562	121	1.4

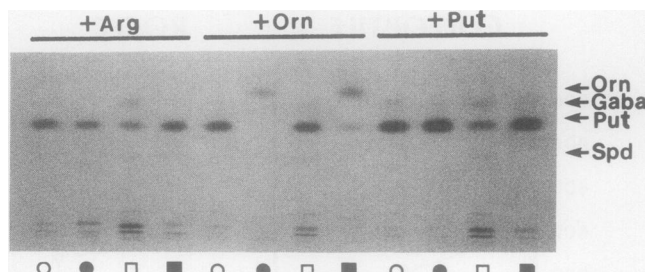


Figure 3. An autoradiogram of dansylated ^{14}C -amines separated on a HPTLC silica gel 60 plate. Rice coleoptiles and roots of 3 d were excised and incubated for 3 h in aerobic and anaerobic conditions. The plant material was then fed with 0.37 MBq of [^{14}C]arginine or [^{14}C]ornithine or [^{14}C]Put for 3 h. Samples of approximately 10,000 cpm each were loaded on the plate. Open circle, aerobic coleoptile; closed circle, anaerobic coleoptile; open square, aerobic root; closed square, anaerobic root.

anoxic treatment. This increase supports the hypothesis that ADC is the most important biosynthetic enzyme in the anaerobic-induced increase in Put concentration.

DISCUSSION

Rice tissues exposed to oxygen-deficit stress showed a pattern of PA metabolism response similar to that produced by other stress conditions such as K^+ deficiency, osmotic shock, and low pH (9, 26, 27). The common events of this response are the activation of the ADC-mediated pathway of Put biosynthesis and a massive increase in the diamine titer (10). This phenomenon is undoubtedly important for balancing the anoxic production of organic acids. After 4 h of lack of oxygen, lactic acid reaches a concentration of $0.9 \mu\text{mol/g}$ fresh weight in the rice coleoptile (14) and of $1.5 \mu\text{mol/g}$ fresh weight in the rice root (18). Succinic acid, which is accumulated exclusively by the coleoptile, reaches $1.4 \mu\text{mol/g}$ fresh weight after 4 h (14). As shown in Figures 1 and 2, the concentration of Put after 24 h of anaerobiosis was 0.8 and $1.0 \mu\text{mol/g}$ fresh weight (free and bound) in the coleoptile and 1.0 and $0.3 \mu\text{mol/g}$ fresh weight (free and bound) in the root. These concentrations are comparable in magnitude to the concentrations of organic acids in anoxic tissue. Putrescine is among the compounds described as increasing in anaerobic-incubated tissues, and is the one able to produce more basic equivalents for counteracting stress-induced acidosis. The synthesis of Put is accompanied by a simultaneous disappearance of arginine. We previously determined (19) that the anaerobic level of arginine in rice roots increases from $0.17 \mu\text{mol/g}$ fresh weight in air to $0.33 \mu\text{mol/g}$ fresh weight after 1 d of lack of oxygen. There are two processes active in anoxia for maintaining adequate levels of arginine for PA biosynthesis: amino acid translocation from the endosperm (3, 13), which was also confirmed by the anaerobic stimulation of [^{14}C]arginine uptake (Table I), and proteolysis of protein present in the tissue (19).

The two tissues of rice showed a great difference in their capacity to form conjugates (Fig. 2). The bound-soluble forms of PAs are widely distributed in higher plants and are mostly found as HCAAs. The physiological significance of these

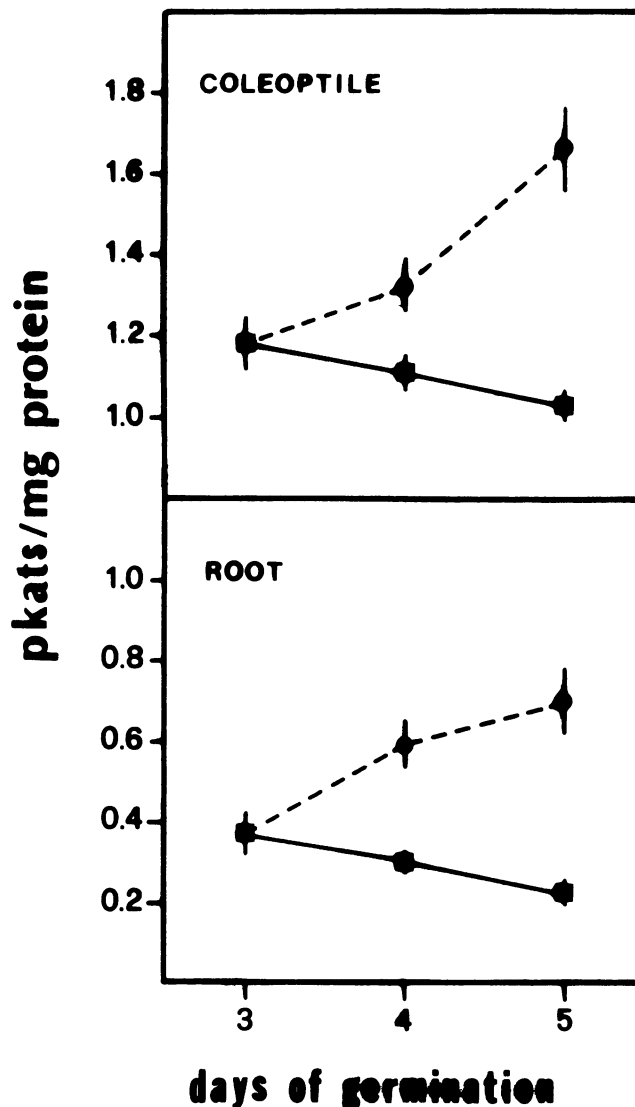


Figure 4. Changes in ADC activity in rice coleoptile and root of 3-d-old seedlings incubated for 2 d in aerobic or anaerobic conditions. Symbols as in Figure 1. Each data point is the mean of three independent experiments.

HCAAs is not known. They increase during various processes such as flowering, virus infection, and mineral deficiencies (6, 8, 12). The observation that the level of free Put is fairly similar in coleoptile and root (Fig. 2), but that Put conjugates are formed mainly in the coleoptile (Fig. 1) would seem to suggest that the titer of free Put is not the limiting factor in conjugate formation. More likely is the hypothesis that the reaction(s) involved in the synthesis of conjugates is the limiting step of their accumulation in the root. In the coleoptile, HCAAs could represent, during stress, either a storage form (of PAs or nitrogen) or compounds with a specific role of their own. The similar increase of bound PAs in the aerobic and anaerobic coleoptile (Fig. 1), however, seems to indicate that the development of young rice seedlings both in aerobic and anaerobic conditions is characterized by the increase of conjugates. It is significant that the only enzyme described as

affecting this type of reaction (agmatine coumaroyl CoA transferase) shows maximum activity at 3 to 4 d of germination (4). The difference between root and coleoptile in the rate of synthesizing PA conjugates could find a metabolic explanation in terms of energy. If this process involves step(s) requiring energy in the form of ATP, the rate of synthesis in the two tissues would be dramatically different. The rice coleoptile, in fact, shows high adenylate energy charge values both in aerobic and anaerobic conditions (15) while in the rice root the energy charge declines from 0.85 to 0.34 in 24 h of treatment (5).

The different behavior of the rice coleoptile and root in anoxia (the former can grow while the latter cannot) is associated with marked differences in PA metabolism. In the anoxic condition, the root reveals, in comparison with the coleoptile, a higher level of free Spd and Spm (Fig. 2), and inability to form conjugates (Fig. 1). The relationship between these factors and anaerobic growth is not clear and is currently under investigation.

ACKNOWLEDGMENTS

We thank Prof. P. P. McCann (Merrell-Dow Research Institute, Ohio, USA) for a gift of α -difluoromethylarginine.

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