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Indole-3-Acetic Acid and Rice Coleoptile Elongation Under Anoxia

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Abstract. To investigate the presence of a possible synergistic effect of IAA and anaerobiosis on rice coleoptile elongation, excised coleoptiles grown in aerobic and anaerobic conditions were tested and compared with intact seedling aerial parts for response to exogenous IAA and for levels of endogenous IAA. Excised coleoptiles were fed with ^{3}H-IAA to study aerobic and anaerobic IAA metabolism. Our results can be summarized as follows. (1) IAA and anaerobiosis have no synergistic effect on rice coleoptile elongation. (2) This behavior is due not to an inhibition of IAA uptake but probably to a reduced and different IAA metabolism in coleoptile grown in the absence of oxygen. (3) In anaerobic rice coleoptiles, the conversion to inactive conjugate (IAA-Asp) could be adopted as means of detoxification in the case of abnormally high and unutilized IAA levels. (4) The increase in IAA level found in coleoptiles of intact seedlings during anaerobic treatment could be due, as in the roots, to a translocation from the endosperm, in which the hormone is contained in a great quantity.

The involvement of auxins in plant cell extension growth is well known (Zeroni and Hall 1980, Cleland 1981), and from the beginning the use of cereal coleptiles has played an important part in these studies (Went 1926, Went and Thimann 1937). Among cereals, only rice has the peculiar characteristic of promoting coleoptile cell elongation when seeds are germinated under water with lack of oxygen (Taylor 1942, Yamada 1954, Bertani et al. 1980, Hoson and Wada 1980, Menegus et al. 1984).

Several analogies between the effects induced by auxin and anaerobiosis in the mechanism that could control cell elongation have been reported. The primary action of indole-3-acetic acid (IAA) is to lower the cytosolic pH, which stimulates the proton pump (Brummer and Parish 1983, 1985, Felle et al. 1986). A low oxygen tension induces a lactic fermentation which lowers the cytoplasmic pH (Roberts et al. 1984). The acidification would lead to changes in membrane potential and transmembrane ion gradients; as a consequence, wall loosening would occur.

Analogies are also known at the level of the structure and metabolism of the cell wall polysaccharides. The decrease of wall hexosamine content and the regulation of the level of hydroxyproline-containing glycoprotein in the cell wall have been found to be controlled by means of different mechanisms, but producing similar results, by auxin (Cleland and Karlsnes 1967, Masuda 1978, Cleland 1981, Kato and Fujii 1982, Hoson 1987) and by the absence of oxygen (Fujii 1978, Hoson and Wada 1980, 1983).

In the present work, the effects of exogenous auxin and the endogenous IAA level and metabolism were investigated in rice coleoptiles during anaerobic growth in order to obtain information concerning a possible interaction of anaerobiosis and IAA.

Materials and Methods

Plant Material, IAA Application

Rice seeds (*Oryza sativa* cv. Arborio) were sterilized for 2 min with 70% (v/v) ethanol and for 5 min with commercial diluted (1:5) NaClO. For the experiments using excised organs, germination was carried out for 3 days on sterile wet Whatman 3M paper in Petri dishes at 25°C in the dark. Coleoptiles were cut approximately 1 cm from the apices, including tips, under dim green light and incubated for 2 days in closed microjars (20 coleoptiles/jar) containing IAA in 10 ml of a modified Heller medium (Reggiani et al. 1985).

For the experiments using intact seedlings, the seeds were placed on a net hung 1 cm above the water surface in 2-L closed jars containing 300 ml of sterile water. An air stream bubbled into the water through a pumice stone at the bottom of the jars provided the seeds with both moisture and aeration. When germination occurred, the roots were immersed in water. After 3 days of germination, 50 seedlings with 10 ± 0.5 mm coleoptile length were selected and used for IAA treatments. The treatments were carried out in the same jars with roots immersed in 300 ml of IAA dissolved in sterile water.

Seedlings or excised coleoptiles were treated for 2 days with IAA at concentrations ranging between 1 μM and 1 mM. At the selected time, the length of coleoptiles was measured.

Aerobic and anaerobic conditions were obtained by fluxing humidified air or nitrogen gas (99.999%, SIAD) through the medium at the rate of 30 L/h and 5 L/h in 2-L jars and microjars, respectively. The anoxia condition was checked by a Clark oxygen electrode (Biological Oxygen Monitor) and GasPack anaerobic systems (BBL). Anoxia was obtained in not more than 20 min both in 2-L jars and in the microjars.

IAA Extraction and HPLC Analysis

Excised coleoptile, incubated for 1 or 2 days in a modified Heller medium in aerobic or anaerobic conditions, were weighed, frozen, and subsequently ground in a mortar with 80% (v/v) cold methanol, and IAA extraction and

HPLC analysis were performed according to the methods described by Sandberg et al. (1981). Excised coleoptile growth solutions were concentrated under vacuum to a reduced aqueous volume, used for the extraction and HPLC analysis of IAA released into the medium.

Feeding with ^{3}H-IAA and Labeled Compound Identification

Excised coleoptiles were incubated in 5 ml of Heller medium containing μ 50 Ci of ^{3}H-IAA (28 Ci/mmol). After a labeling period of 150 min, the growth solution was removed, and the coleoptiles were rinsed three times with fresh Heller medium and then incubated in the same solution for 15 h. To maintain the anaerobic condition, the ^{3}H-IAA removal and rinses were carried out with a syringe under continuous nitrogen flux and without opening the microjars.

After the selected period, labeled coleoptiles were frozen, lyophilized, and extraced with 70% (v/v) acetone; aqueous residue was fractionated on anionic exchange column (LC-SAX, Supelco) to separate acidic substances. The acidic substance fraction was then concentrated on Sep-Pack C18 (Waters Associates), eluted with ether, and analyzed by cochromatography with an appropriate mixture of unlabeled reference compounds on TLC silica gel plates (Merck). Two solvent systems were used: (1) $CHCl_3$:CH_3COOH:H_2O (90:9.5:0.5) and (2) CH_3CN:CH_3COOH:H_2O (84:1:15). The plates, treated with EnHance (Du Pont), were exposed with XAR5 Kodak films.

To estimate ^{3}H-IAA total uptake and recovery during the extraction procedure, the radioactivity was determined by mixing aliquots of samples with Insta-gel cocktail (Packard) and using a Packard-Tri-Carb scintillation counter.

Chemicals

Indole-3-acetic acid (IAA) and other indole derivates were purchased from Sigma USA; ^{3}H-IAA (28 Ci/mmol) was from Amersham U.K. Indole-3-acetyl-L-aspartate (IAA-Asp) was synthesized as described previously (Cohen 1981).

Results

Effect of IAA Application on Rice Coleoptile Growth

In aerobic conditions, IAA, whether applied to the excised coleoptiles or to the roots of the seedlings, stimulated coleoptile elongation (Fig. 1A). The maximum length was obtained with a concentration of 0.1 mM in the excised organs; at a higher IAA concentration, the coleoptile length diminished. This was accompanied by coleoptile death, visualized with tetrazolium salt stain. In intact seedlings, the coleoptiles continued to grow with 1 mM IAA, but their length did not reach that of excised organs.

In anaerobiosis, rice coleoptile elongation is greater than under aerobic con-

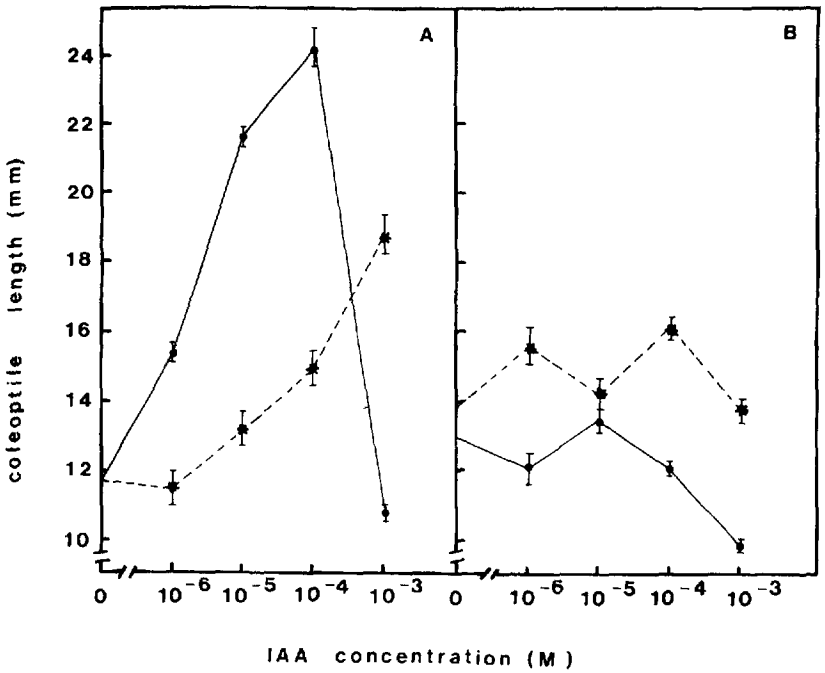

Fig. 1. Effect of IAA application on elongation of excised (●) and intact (*) rice coleoptiles grown in aerobic (A) or anaerobic (B) conditions. Length measured 2 days after application of IAA.

ditions. When IAA was applied in anoxia (Fig. 1B), it revealed no synergistic effect with anaerobic elongation in either excised or intact coleoptiles.

Following IAA application, the rice coleoptile, which normally opens in aerobic conditions, tended to remain closed, and leaf growth was progressively inhibited (Fig. 2), as in coleoptiles under anaerobic conditions. In air, the increase in coleoptile length was accompanied by similar changes of the fresh weight both in IAA treated and untreated coleoptiles and seedlings. In anoxia, in spite of length increase, no significant increase of coleoptile fresh weight was observed during the 2-day experiment; the coleoptile weight was not modified by presence of IAA in the medium.

Endogenous IAA Levels in Rice Coleoptiles

The absolute value of endogenous IAA quantities decreased in excised rice coleoptiles during growth in aerobic and anaerobic conditions (Fig. 3). However, an increase of IAA released into the medium was observed in both conditions. IAA total quantities (inside plus released) after 1 and 2 days of coleoptile culture were similar to those observed at the beginning of the treatment in aerobic or anaerobic conditions. In a previous work (Mapelli et al. 1986) an increase of IAA in rice seedling coleoptiles during aerobic growth was reported: this increase was higher when seedlings were subjected to anoxia.

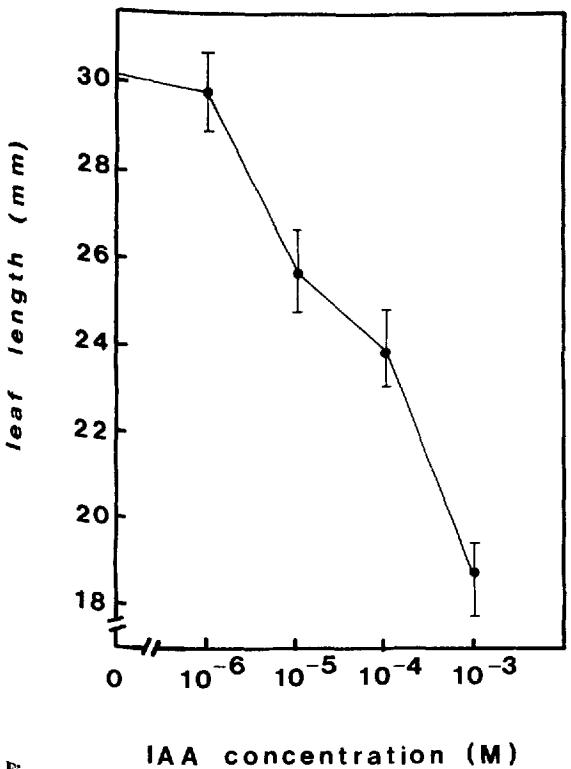

Fig. 2. Effect of IAA application on growth of the first leaf of rice seedling grown in aerobic conditions.

^{3}H-IAA Uptake and Metabolism

Data given in Table 1 show that IAA was absorbed by excised coleoptiles in both the presence and absence of oxygen. The anaerobic treatment reduced the ^{3}H-IAA absolute uptake by 50% in comparison with the aerobic. During a subsequent 15-h incubation in the absence of ^{3}H-IAA, about 50% of the ^{3}H-IAA taken in by the coleoptiles was released into the medium in both conditions. The radioactivity taken up by coleoptiles was always found mainly in the acetone-soluble fraction extracts. The acetone extracts were purified on SAX column and the eluted acidic fraction, in which IAA was included, and the fraction of neutral and basic compounds were analyzed by TLC. In the neutral and basic substance fraction, no differences between air and nitrogen samples were seen in the compounds formed by the ^{3}H-IAA. These compounds did not cochromatograph with any of the indolyl standard substances available (data not shown).

TLC analysis of the acidic fraction with the solvent system 1 (Fig. 4A) showed, for the aerobic condition, a decrease of intensity of the spot corresponding to IAA (spot No. 3) after the pulse period; the disappearance of the same spot was evident after the chase period. This was accompanied by the

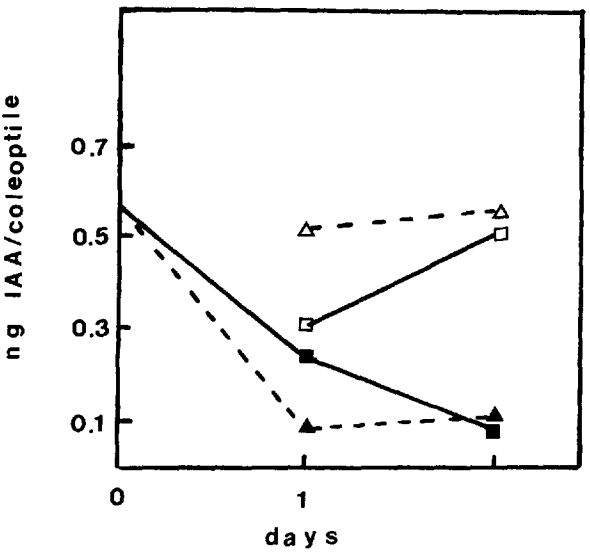

Fig. 3. Variation of endogenous IAA in excised coleoptiles during aerobic (■) or anaerobic (▲) treatment and released in the medium (□, △).

appearance of lower intensity spots and the increasing of intensity of the polar sustances spot (No. 1) at the beginning of TLC. In anoxia, the decrease in intensity of the IAA spot and the simultaneous enlargement of spot No. 1 occurred again and was clearly revealed by comparing the 2.5-h-labeled and the chase samples. Spot No. 2, present in all samples, was due to an impurity of the labeled IAA material.

The TLC of the acidic fractions developed with the second solvent system (Fig. 4B) confirmed the decrease of intensity of the IAA spot and gave indication of the nature of spot No. 1. In this solvent system, it was distributed in two spots (Nos. 4 and 5). In air samples, the No. 5 was always larger; in nitrogen treatment, spots No. 4 and 5 were absent at the end of the labeling period but present later, and spot No. 4, comigrating with the IAA-Asp standard, was the larger. The presence of IAA-Asp was the only clearly evident difference between the air- and nitrogen-treated samples.

Discussion

In this work the absence of a synergistic effect of exogenous IAA and anaero-

Table 1. Rice coleoptile ^{3}H-IAA uptake and partitioning between acetone-soluble and solid residue fractions.

	Total cpm ($\times 10^6$) in coleoptiles	cpm ($\times 10^6$) in acetone extract	cpm ($\times 10^6$) in solid residue
Air 2.5-h ^{3}H pulse	7.15 (100%)	5.66 (79.2%)	1.49 (20.9%)
Air 15-h chase	4.16 (58.1%)	3.02 (42.2%)	1.14 (15.9%)
N_2 2.5-h ^{3}H pulse	3.68 (100%)	2.80 (76.1%)	0.88 (23.9%)
N_2 15-h chase	1.97 (53.5%)	1.52 (41.3%)	0.45 (12.2%)

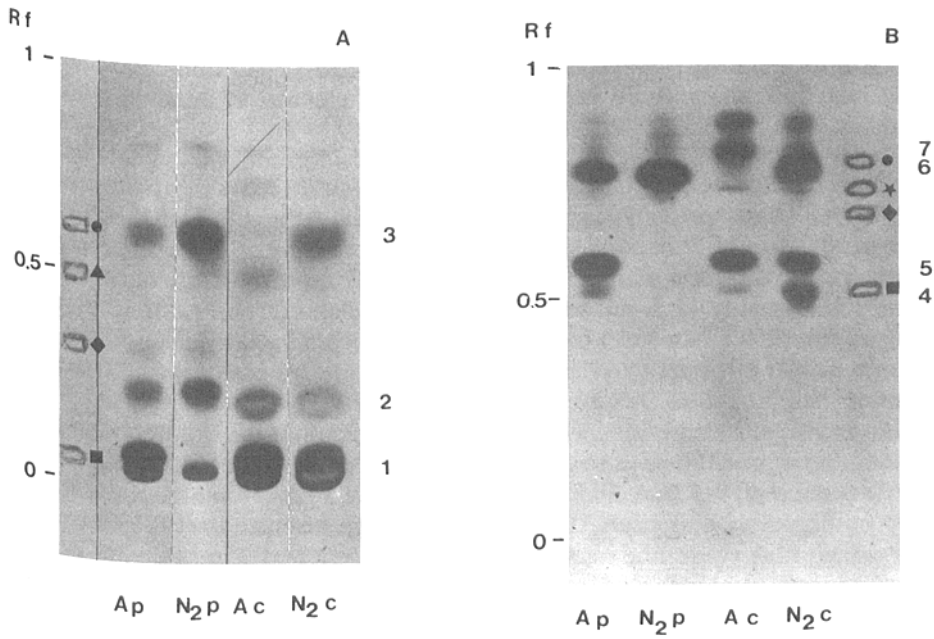

Fig. 4. TLC fluorography of ^3H-labeled metabolites extracted from excised rice coleoptiles: air-treated after 150-min pulse (Ap) and after 15-h chase (Ac); anaerobiosis-treated after 150 min labeling period (N_2p) and after 15-h anaerobiosis chase (N_2c). (A) solvent system 1 and (B) solvent system 2. Migration of standard indole: ●, indole-3-acetic acid; ▲, indole-3-ethanol; ◆, oxindole-3-acetic acid; ■, indole-3-acetylaspartic acid; ★, indole-3-acetamide.

biosis on rice coleoptile elongation is shown, although, singly, both IAA and anoxia are able to induce elongation. The different intensity in the growth response of intact and excised coleoptiles to increasing IAA concentrations (Fig. 1A) is probably due to IAA absorption, more immediate in the excised organ, immersed in IAA solution, than in the seedling, where the phytohormone, taken up from the roots, must be transported to the coleoptiles in order to have its stimulatory effect. In addition, root growth was totally inhibited by 10 μM or higher IAA concentrations, and this could affect the rate of water and solute uptake. Root growth inhibition did not interfere with IAA effect on coleoptiles, which continued to elongate with the increase of IAA concentration in the solution.

The difference of elongation in IAA air and nitrogen treatments (Fig. 1) is due to a reduced general metabolism in anoxia, but in this condition the coleoptile is able to continue to elongate for several days, reaching a length similar to that observed during air IAA treatment. The absence of a synergistic effect of IAA and anaerobiosis on coleoptile elongation could be due to the absence of absorption or to the different metabolism of IAA when seedlings are cultivated in anoxia.

That the lack of IAA-induced elongation in anoxia was not due to the inhibition of IAA uptake is clear from Table 1, in which the absorption by the rice coleoptile in anaerobic conditions, even though at a reduced rate, is evident.

The hypothesis of a different IAA metabolic rate under anoxia is supported by TLC fluorographic analysis (Fig. 4). Under anaerobic conditions, IAA metabolism was slower, and there was accumulation of a compound that can be identified as IAA-Asp.

The formation of IAA-Asp as an IAA metabolic product has been found in legumes (Andreae and van Ysselstein 1960, Tsurumi and Wada 1980), tobacco crown gall (Raush et al. 1986), and *Coleus blumei* (Brennan and Jacobs 1983). The production of IAA conjugates has been interpreted as a protection against oxidation (Cohen and Bandurski 1978) or as a form of detoxification induced by an abnormally high concentration of exogenous IAA (Andreae and van Ysselstein 1960, Tsurumi and Wada 1980). In *Coleus blumei,* Brennam and Jacobs (1983) attributed IAA-Asp formation to the generally reduced metabolism of the plant.

In anaerobic conditions, IAA-Asp production cannot be a protection against oxidation but could be a detoxification form adopted by the rice coleoptile as a consequence of the IAA high concentration and reduced metabolic rate. Recent studies have shown that intact rice seedlings react to anaerobic stress by increasing IAA content in all seedling organs, particularly in roots and coleoptiles (Mapelli et al. 1986). However, excised roots grown in anoxia do not reveal an increase in IAA levels (Bertani and Mapelli 1987). It has been postulated that the IAA increase in intact roots subjected to anaerobic treatment could be due to a translocation of this phytohormone from the endosperm, in which quantities of more than 100 ng per endosperm have been reported (Mapelli et al. 1986).

In excised coleoptiles cultivated in the presence or absence of oxygen, the total IAA levels, inside plus outside coleoptile, did not increase (Fig. 3). It is possible that this organ is also unable to synthesize IAA and that the IAA increase, detected previously during the anaerobic treatment in intact coleoptiles, is due to a translocation from the endosperm, as in the roots. The results here presented throw new light on results previously reported.

Yamada (1954) observed that the rice coleoptile tip was not necessary for coleoptile elongation in hypoxia, whereas it was for elongation in the air condition. This suggests that the IAA is not necessary for elongation in hypoxia or that the tip is not the main IAA source. Our data confirmed that both situations are present. Exogenous IAA does not increase anoxia-induced elongation, and IAA in coleoptiles increases only in intact seedlings, presumably because it is transported from the endosperm. That the endosperm is the main IAA source for the coleoptile was recently reported also for oat seedlings, where endosperm removal strongly reduced IAA level and elongation in the coleoptile (Kamisaka et al. 1987).

Furthermore, Yamada (1954), measuring the level of IAA absorption and destruction in the rice coleoptiles grown in 2.8 mM IAA, concluded that in hypoxia the coleoptile absorbed less IAA, but the reduced rate of IAA destruction was the consequence of coleoptile elongation. In the present experiments with ^{3}H-IAA, used at a more physiological concentration of 340 nM, the data concerning the IAA absorption rate and catabolism reduction in anoxia conditions were confirmed. Considering the working theory proposed by Bandurski (1986) that IAA is destroyed, primarily oxidized, at the moment it commits the

growth-promoting act, we infer that this is the situation in rice coleoptiles. Bandurski et al's. theory explains very well the rapid IAA metabolism and coleoptile elongation in air growth condition and the absence of interaction between anoxia and IAA in the elongation and the concomitant reduced IAA metabolism.

We have proposed that IAA-Asp, formed in the rice coleoptile, serves to detoxify cells suffering from a high IAA concentration, as reported for legumes (Andreae and van Ysselstein 1960, Tsurumi and Wada 1980). Considering that IAA was not utilized for cell elongation in anoxia, IAA could be toxic and converted to IAA-Asp even when coleoptiles were grown in medium containing 340 nM ^{3}H-IAA. That a high abnormal IAA level could be toxic may be deduced from rice coleoptile death observed when they are cultivated in air in 10 mM IAA solution. IAA-Asp is probably also formed in the air growth condition but at a higher IAA concentration than is necessary for formation of IAA-Asp in anaerobiosis.

In conclusion, our results can be summarized as follows: (1) IAA and anaerobiosis do not show a synergistic effect on rice coleoptile elongation. (2) This behavior is not due to an inhibition of IAA uptake but is probably related to a reduced and different IAA metabolism in the coleoptile grown in the absence of oxygen. (3) In the anaerobic rice coleoptile, IAA conversion to inactive conjugate (IAA-Asp) could be adopted as a means of lowering the abnormally high and unutilized IAA level. (4) The IAA increase detected in coleoptiles of intact seedlings during growth, an increase enhanced by anaerobic treatment, is due to a translocation from the endosperm, as in the roots.

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