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BRIEF COMMUNICATION

Ammonium accumulation in relation to growth of rice cell suspension under phosphate deprivation

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Abstract

Suspension-cultured rice cells growth was markedly inhibited and ammonium content increased when rice cells were deprived of phosphate. When rice cells were cultured at increasing concentrations of ammonium chloride, ammonium content increased, however, no significant inhibition of cell growth was observed. Addition of D-arginine, an inhibitor of putrescine biosynthesis, resulted in a complete recovery of growth in rice cells under phosphate deprivation, but did not decrease the content of ammonium. Our results indicate that the growth inhibition induced by phosphate deprivation is not associated with ammonium accumulation.

Additional key words: D-arginine, *Oryza sativa*.

Toxic accumulation of free ammonium in plant tissues has been considered as one of the factors responsible for growth inhibition of plants (Goyal *et al.* 1982, Ikeda and Oswa 1981, Kaul and Hoffman 1993, Ota and Yamamoto 1989, Puritch and Barker 1967). Availability of phosphorous is known to have a large impact on plant growth. It has been shown that phosphate deficiency induces accumulation of ammonium in *Citrus limon* and *Cucurbita pepo* (Rabe and Lovatt 1986). However, this is the only report relating ammonium accumulation to phosphate deficiency so far. It is not known whether other plants show a similar relationship. Nor do we know the relationship between ammonium accumulation and plant growth under phosphate deficiency.

In the present investigation, we used suspension-cultured rice cells to study the time course of ammonium levels during phosphate deprivation and their correlation with cell growth.

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; f. m. - fresh mass; Pi - inorganic phosphate.
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Rice (*Oryza sativa* cv. Tainan 5) suspension cultures were initiated from immature embryo derived calli (Yu *et al.* 1991). The rice grain was sterilized with 1 % sodium hypochlorite and 1 drop of *Tween 20* for 20 - 30 min and then washed extensively with distilled water. The embryo was excised and placed with scutellum side up on MS agar medium (Murashige and Skoog 1962) containing 0.8 % agar (m/v), 3 % saccharose and 5 µM 2,4-D. The pH was adjusted to 5.8 before autoclaving. The embryo culture was incubated at 25 °C under white light (irradiance 42 µmol m^{-2} s^{-1}). Four weeks later, initiated calli were transferred to liquid Murashige and Skoog medium containing 3 % saccharose and 5 µM 2,4-D.

Approximately 500 mg fresh mass of calli were cultured in 30 cm^3 of medium in a 125 cm^3 Erlenmeyer flasks. The suspension culture was shaken on a reciprocal shaker at 2 rps and incubated at 25 °C under constant irradiance of 42 µmol m^{-2} s^{-1}. Established suspension-cultured cells were subcultured every 7 d by transferring about 0.5 cm^3 of cells into 30 cm^3 of fresh liquid Murashige and Skoog medium in a 125 cm^3 flask. The 7-d-old cultures were used for initiation all the indicated experiments. To cause phosphate deficiency, rice cells were cultured in the absence of phosphorous source. D-Arginine, an inhibitor of putrescine biosynthesis, was filter sterilized before adding to the culture medium. Suspension-cultured cells were collected by filtration through a 400-mesh nylon sieve, and blot-dried on paper towels. The growth of rice cells was measured by fresh or dry mass. Dry mass determination was made after 48 h at 80 °C. Pi was extracted with perchloric acid (2.5 %) and its determination was made by the spectrophotometric method according to Yoshida *et al.* (1972). Ammonium was measured in the crude extract by the Berthelot reaction, modified according to Weatherburn (1967). For ammonium determination, rice cells were homogenized with a motar and pestle using 3 cm^3 0.3 mM sulfuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39 000 *g*. 0.2 cm^3 of clear supernatant were diluted by 0.3 mM sulfuric acid to a final volume of 4 cm^3. For the colour reaction, 0.5 cm^3 of solution A (5 g phenol, 25 mg nitroprusside dissolved in 100 cm^3 water) and then 0.5 cm^3 of solution B (2.5 g NaOH added to 40 cm^3 5 % sodium hypochlorite, and then make up to a final volume of 100 cm^3 with distilled water) were added.

Incubation was carried out in a gently shaking water bath at 37 °C for 20 min. The absorbance was measured at 625 nm against the control without extract. Ammonium level is calculated using extinction coefficient of 3.9982 μmol^{-1} cm^{-1}.

Each treatment was repeated four times and all experiments were repeated three times. Similar results and identical trends were obtained. The data reported here are from a single experiment.

As expected, Pi level in phosphate-deprived cells was always lower than that in the control cells (supplied with sufficient phosphate) during a 8-d culture (Fig. 1*A*). The growth, as judged by fresh or dry mass, of rice cells with sufficient supply of phosphate increased with increasing duration of culture. However, only slight growth was observed in rice cells under phosphate deprivation (Fig. 1*C, D*).

The content of ammonium in the control cells increased at day 3 and subsequently decreased (Fig. 1*B*). Phosphate-deprived rice cells had higher level of NH_4^+ than the

control cells as was reported in *Citrus limon* and *Cucurbita pepo* (Rabe and Lovatt 1986).

To determine if inhibition on growth of rice cells in the absence of phosphate is caused by NH_4^+ accumulation, we cultured rice cells under conditions of various concentrations of NH_4Cl. Rice cell cultured in higher concentrations of NH_4Cl had high concentration of NH_4^+ in cells, however, no significant inhibition of cell growth was observed.

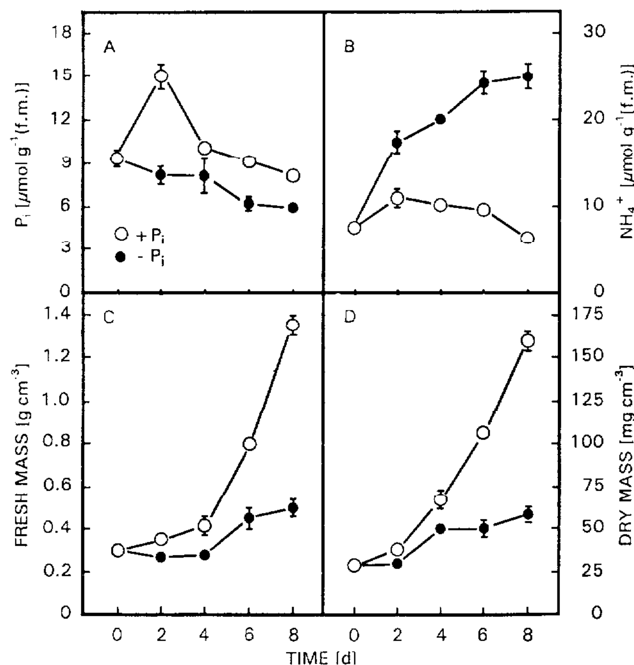

Fig. 1. Changes in Pi and NH_4^+ content and fresh and dry mass in rice cells cultured with sufficient phosphate (+Pi) and under phosphate deprivation (-Pi). *Vertical bars* represent standard errors (n = 4). Only those standard errors larger than symbol size are shown.

In a recent work, we demonstrated that phosphate deprivation resulted in a higher putrescine level and concluded that putrescine accumulation is a factor causing growth inhibition of rice cells under phosphate deprivation (Shih and Kao 1996). Addition of D-arginine, an inhibitor of putrescine biosynthesis, resulted in a decrease in putrescine level and a complete recovery of growth in rice cells under phosphate deprivation (Table 1). If ammonium accumulation is responsible for the growth inhibition in rice cells under phosphate deprivation, then addition of D-arginine is expected to decrease ammomiun level in phosphate deprived cells. However, no such decrease was observed by the addition of D-arginine (Table 1).

In view of the findings reported here, we believe that ammonium accumulation is not associated with the growth inhibition induced by phosphate deprivation, but is part of the overall expression of phosphate deprivation in rice cells.

Table. 1 Effect of D-arginine on the growth of and ammonium content in rice cells under phosphate deprivation (-P). The concentration of D-arginine was 5 mM. Cell growth and ammonium level were determined 4 d after treatment. Means ± S.E. (n = 4).

Treatment	Fresh mass [mg cm^{-3}]	Dry mass [mg cm^{-3}]	NH_4^+ [μmol g^{-1}(f.m.)]
+Pi	15.00 ± 2.33	2.23 ± 0.10	10.2 ± 0.7
-Pi	10.00 ± 0.33	1.63 ± 0.03	19.8 ± 0.8
-Pi + D-arginine	14.67 ± 0.33	2.23 ± 0.07	19.4 ± 1.2

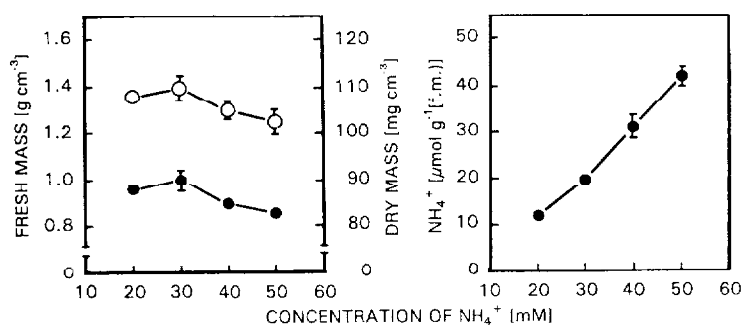

Fig. 2. Effect of ammonium chloride concentrations on growth of and ammonium level in rice cells. Fresh (*closed circles*) and dry (*open circles*) masses and ammonium level were measured after 8 d of treatment. See Fig. 1 for details.

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