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Internal Zinc Accumulation Is Correlated with Increased Growth in Rice Suspension Culture

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Abstract. A high zinc concentration of 520 μM, approximately 100 times that used most often in standard plant tissue culture media, was found to be superior in liquid callus cultures of japonica rice, increasing growth to 146% compared with standard N6 medium. At the same time, the internal zinc concentration increased 40 times in fast growing cells; soluble protein doubled, and free amino acids decreased. Under zinc-free conditions the cultures slowed in growth, and several free amino acids such as aspartic acid, glutamic acid, asparagine, and glutamine accumulated. We suggest that zinc acts as a direct regulatory factor in inducing auxin activity, but not auxin levels, making high internal zinc accumulation mandatory if high auxin concentrations are required as in rice callus cultures.

Key Words. Amino acid—Auxin—*Oryza sativa*—Rice—Zinc

For many years, the functional aspects of zinc nutrition in plants and the possible effects of its deficiency have been investigated in correlative studies coupled with auxin levels (Mengel and Kirkby 1987). Skoog (1940) reported that tomato plants, grown hydroponically in a zinc-deficient nutrient solution, failed to elongate and that their internal auxin concentration was extremely low. Resumption of growth and an increase in auxin content were observed after the addition of zinc. Indole-3-acetic acid (IAA), either added to the nutrient solution or sprayed on leaves, was shown to repair stem growth, but only during the early stages of zinc deficiency.

Previously, Cakmak et al. (1989) reported an increase in the concentration of free tryptophan under zinc deficiency in beans and its decline after the addition of zinc. Also, in these zinc-deficient plants stem growth as well as the internal concentrations of IAA and soluble protein could be increased to control levels by reapplying zinc within 96 h. Takaki and Kushizaki (1970, 1972, 1976) also reported high levels of tryptophan in stunted zinc-deficient plants coupled with an increase in tryptamine. Tryptophan is known to be converted to IAA either via indolepyruvic acid or via tryptamine followed by indoleacetaldehyde, which appears to be the immediate precursor of IAA (Gibson et al. 1972).

Domingo et al. (1992) did not find a decrease in internal free IAA in zinc-deficient radish plants, and we found the same to be true of conjugated (ester + peptidic) IAA (in preparation), indicating that external zinc does not affect the internal IAA concentration in stunted radish shoots. Furthermore, stunted growth caused by zinc deficiency can be corrected during early stages, when zinc is still abundant, through the external application of IAA.

In the present study we investigated the effect of external zinc in callus cultures of japonica rice. These cultures need auxin for proliferation. The micronutrient content of most plant tissue culture media seems to have been modeled after dry weight analysis of mature plants rather than physiologic function. Ohira et al. (1975) and Ojima et al. (1977) have shown that zinc deficiency reduces cell growth *in vitro* remarkably. We used liquid N6 (Chu et al. 1975) medium to grow rice callus in suspension. N6 medium contains 1 mg/liter of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and Zn^{2+} at 5.2 μM delivered as $ZnSO_4$ (1.5 mg/liter). This concentration range is typical for most plant tissue culture

Abbreviations: IAA, indoleacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; HPLC, high performance liquid chromatography; PTC, phenylthiocarbamyl.

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media in use today. We found that significantly higher levels of Zn^{2+} are required to optimize callus growth.

Materials and Methods

Induction of Callus

Seeds of japonica rice (*Oryza sativa* L.), cv. Nipponbare, were used as explants. After removal of the husks, the seeds were surface sterilized by stirring in 70% ethanol for 30 s and 3% sodium hypochlorite for 15 min followed by washes in sterile water. The sterilized seeds were placed on MS medium (Murashige and Skoog 1962), containing 2 mg/liter 2,4-D, 30 g/liter sucrose, and 8 g/liter agar, for callus induction. Proline and casein hydrolysate were changed to 10 mM and 300 mg/liter, respectively, and the pH of the medium was brought to 5.8 before autoclaving. Cultures were kept at 25°C in the dark.

Induction of Suspension Culture and Zinc Application

After about 4 weeks of incubation friable callus could be removed from the seeds and transferred to 100-mL Erlenmeyer flasks containing 25 mL of liquid N6 medium supplemented with 1 mg/liter 2,4-D and 30 g/liter sucrose. The cultures were placed on a rotary shaker at 105 rpm in continuous light at 25°C and subcultured weekly. Beginning with the third subculture, 0.5-g cell clusters were subcultured into 25 mL of N6 medium modified to contain different zinc concentrations (0, 5.2, 52, 520, and 780 μM). These media were replaced weekly for 4 weeks and cultures analyzed for callus yield, zinc, free amino acid, and soluble protein content at the end of each culture period (four measurements). Callus growth was measured as fresh weight increase in weekly intervals with surface moisture removed by suction. An independent experiment consisted of three probes/test medium, and three independent experiments were run to explore the effect of zinc.

Zinc Measurement

For analysis of internal zinc content, callus (5 g), harvested for fresh weight determination, was washed on a Buchner funnel and dried at 110°C overnight for dry weight determination followed by 550°C for 4 h in a muffle furnace. The ash was extracted in 20 mL of 1 N HCl, the extract filtered, and the filtrate analyzed for zinc content using an atomic absorption spectrophotometer (Shimadzu model AA-646).

Sulfate Measurement (HPLC)

Inorganic sulfate concentration was determined by anion exchange column HPLC [TSK-GEL, IC-Anion-pw 4.6 × 50 mm (Tosoh Ltd.)], the detector was electric conductivity.

Amino Acid Measurement (HPLC)

One or 0.5 g, fresh weight, of callus was homogenized in 50 mL of 80% methanol, and the cellular debris was removed by suction filtration. The extracts were evaporated under reduced pressure at 40°C using a rotary evaporator. The residue was dissolved in 2–3 mL of 80% methanol after dispersion using a flash mixer and filtered through a 0.45-μm membrane filter. To be analyzed by hydrophobic HPLC, a portion of the extract was phenylthiocarbamylated, and 10 or 20 mL of the solution containing the PTC derivatives was separated under the following conditions: column TSK-GEL ODS-80TM, 6 × 150 mm (Tosoh Ltd.); eluent A (45 mL of acetonitrile + 955 mL of sodium acetate buffer, pH 6.0); eluent B (600 mL of acetonitrile + 400 mL of sodium acetate buffer, pH 6.0); flow rate 1 mL/min; column temperature 40°C. The PTC derivatives were eluated successively with increasing concentrations of eluent B (0 min, 0%; 45 min, 100%; 50 min, 100%; 55 min, 0%). The amino acid content was calculated from peak area calibrated with the authentic amino acid (Shimadzu model C-R1B Chromatopac).

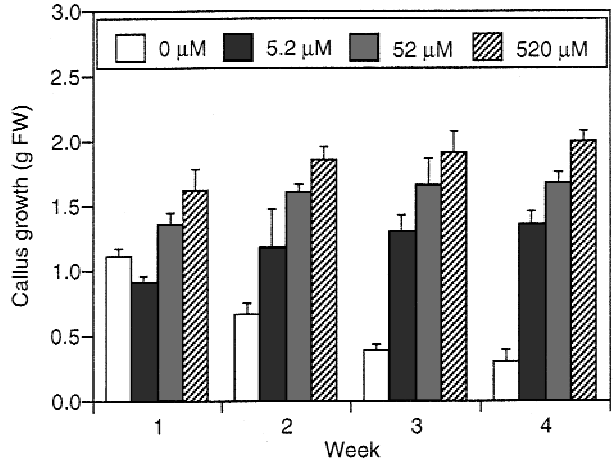

Fig. 1. Effect of different zinc concentrations (in μM Zn^{2+}) in N6 medium on callus growth in rice.

Protein Measurement

For determination of the soluble protein content, callus (1 or 2 g, fresh weight) was homogenized with 10 mL of distilled water for 20 min on ice. The homogenate was filtered, and a concentration series of five 1-mL samples was prepared by dilution with distilled water. The Coomassie Brilliant Blue binding method was applied (Bio-Rad Laboratories) with bovine serum albumin as standard.

Results

Effect of Zinc on Callus Growth and Internal Zinc Accumulation

Different zinc concentrations in the culture medium had a marked effect on callus morphology. In zinc-free medium, callus, although still proliferating, became brown and appeared swollen, whereas the control (5.2 μM Zn^{2+}) was white with smaller cell clusters. At 520 μM Zn^{2+}, callus remained white, and clusters were even smaller.

Callus growth was monitored at 0, 5.2, 52, 520, and 780 μM Zn^{2+}. The highest concentration (780 μM) turned out to inhibit callus growth after 2 weeks in culture, leading to high internal zinc concentrations at the end of the 4-week experimental period (6,958 μg/g, dry weight, of callus). Data obtained for all lower concentrations are given in Fig. 1 (growth) and Table 1 (zinc content).

Table 1. Effect of different concentrations of zinc supplement to N6 medium on the internal zinc concentration in cells of rice suspension culture. Values are means ± S.E. ($n = 3$).

Zinc treatment (μM Zn^{2+})	Culture time and internal zinc content (μg/g, DW,[a] of callus)			
	1 week	2 weeks	3 weeks	4 weeks
0	26.2 ± 1.86	20.5 ± 2.60	16.1 ± 2.35	13.8 ± 2.38
5.2	50.0 ± 0.63	54.3 ± 2.35	56.5 ± 3.82	57.5 ± 3.45
52	222.3 ± 3.18	250.3 ± 9.74	354.0 ± 85.03	397.0 ± 100.34
520	1,774.0 ± 350	2,054.7 ± 479	1,976.3 ± 496	2,339.7 ± 430

[a] DW, dry weight.

Under zinc deficiency (0 μM) callus growth is negatively affected from the 2nd week on. Increasing zinc concentrations, up to 100 times (520 μM) the level of standard plant tissue culture media (5.2 μM), significantly stimulate callus proliferation and raise internal zinc to approximately 2,000 μg/g, dry weight, of callus. In zinc-deficient, slow growing cultures the internal zinc concentration drops as well. In standard medium, the zinc concentration remains stable over 4 weeks at approximately 55 μg/g, dry weight, of callus, whereas a dramatic zinc accumulation (up to 2,340 μg/g) takes place in cultures growing in the presence of high zinc concentrations.

Effect of Sulfate on Callus Growth and Internal Sulfate Accumulation

With increasing zinc concentrations the amount of sulfate in the medium also increases. In addition to $ZnSO_4$, the sulfate is applied as $(NH_4)_2SO_4$, $MgSO_4$, $MnSO_4$, and $FeSO_4$. The concentration of sulfate in the standard medium is 422 mg/liter, increasing to 472 mg/liter in medium containing 520 μM zinc. The internal concentration of sulfate in callus grown on control medium was found to be 0.95 mg/g, fresh weight, and was almost identical on high zinc medium.

Effect of Zinc on Content of Free Amino Acids

HPLC chromatograms of free amino acids produced the quantitative data given in Table 2. Under zinc deficiency conditions, a general increase in free amino acids is visible with a remarkable specific accumulation of aspartic acid, glutamic acid, asparagine, and glutamine. Compared with the control, representing a typical plant tissue culture medium (5.2 μM Zn^{2+}), increased media concentrations of zinc (10 × and 100 ×) led to a further reduction of free amino acids such as asparagine and glutamine.

Table 2. Effect of different concentrations of zinc supplement to N6 medium on the amino acid content of cells of rice suspension culture.

Amino acid	Amino acid content (μmol/g, FW[a])			
	0 μM[b]	5.2 μM	52 μM	520 μM
Asp	8.64	1.72	0.60	0.32
Asn	8.93	1.60	0.01	0.01
Glu	9.24	6.10	3.26	2.55
Gln	38.15	2.08	0.38	0.11
Gly	4.39	1.96	1.49	1.10
Ser	9.26	1.36	1.36	0.86
Arg	1.73	0.32	0.93	0.07
Lys	0.97	0.31	0.30	0.06
Trp	0.49	0.16	0.19	0.01
Ile	0.59	0.34	0.01	0.01
Leu	0.72	0.73	0.34	0.06

[a] FW, fresh weight.
[b] Zinc treatment.

Table 3. Effect of different concentrations of zinc supplement to N6 medium on the soluble protein concentration in cells of rice suspension culture.

Zinc treatment (μM)	Soluble protein (mg/g, FW[a])
0	5.2
5.2	24.4
52	29.8
520	48.4

[a] FW, fresh weight.

Effect of Zinc on Content of Soluble Proteins

Soluble protein content is doubled at 100 × the zinc concentration compared with standard medium (Table 3). An effect is not detectable at 10 ×; but under zinc deficiency the content of the soluble protein is low.

Discussion

Zinc is an essential element in higher plants and thus is included in all plant tissue culture media commonly

used. As a micronutrient, zinc is usually supplied as $ZnSO_4 \times 7H_2O$ in amounts between 1 and (rarely) 10 mg/liter, which results in a Zn^{2+} content of approximately 3–30 μM. The N6 medium was used successfully in numerous rice tissue culture studies (Okamoto et al. 1996) as well as in other grass species (Akashi et al. 1993). It contains Zn^{2+} at 5.2 μM. In this study we demonstrate that a 100-fold increase of the zinc concentration of N6 medium furthers the growth of suspended cell clusters of japonica rice.

When the medium was completely deprived of zinc, growth retardation and accumulation of some free amino acids such as asparagine, aspartic acid, glutamic acid, and glutamine and a decrease in soluble protein were found. This is in agreement with several studies in a number of plant species (Fujiwara and Tsutsumi 1959, Kitagishi and Obata 1986, Possingham 1956), indicating that zinc is one of the key elements in protein synthesis in plants.

In calli generated in N6 at 100 × zinc (520 μM), free amino acids were reduced compared with the standard medium and soluble protein was found to accumulate, i.e. with increasing concentration of zinc in the medium synthesis of soluble protein is accelerated in callus, and as a result, the free amino acid content is exhausted. While the zinc content of the medium was increased to 100 × the standard condition the cellular concentration of zinc rose approximately 40 times to above 2,000 μg/g, dry weight, of callus. In standard medium the internal concentration is stable at approximately 55 μg. This indicates that an internally high concentration of zinc needs to be maintained for rapid cell proliferation. Our data also indicate that a concentration above 3,500 μg/g, dry weight, of callus is correlated with a decline in callus growth and thus may be toxic. Ishizuka and Tanaka (1962) reported that deficient and excess critical levels of zinc in shoots of rice plants were below 15 μg and above 600 μg of zinc/g of dried tissue, respectively, and that the yield of the rice plants remained constant at that range.

Nonhabituated cell cultures do not grow without an external auxin source. The same is true for our rice culture. Auxin, most efficient 2,4-D at a high concentration, here 1 mg/liter, is essential. We suggest that the high concentration of zinc used in this study may be acting by participating in the regulation of the biological activity of auxin. The zinc concentration applied and shown to be beneficial in our cultures is clearly too high to be explained only in terms of its role as essential micronutrient. Pavletich and Pabo (1991) discovered the zinc finger protein in plants. This zinc-containing protein is a DNA-binding transcription factor. Sakai et al. (1986) found evidence for an auxin-binding protein in plants which specifically binds to RNA polymerase. Domingo et al. (1992) reported that two-dimensional thin layer chromatography and gas chromatography/mass spectrometry revealed the presence of IAA in zinc-deficient stunted radish shoots to be identical with the concentration in healthy shoots. Our investigation (in preparation) confirmed this for conjugated IAA in zinc-deficient and normal radish shoots. These results suggest that zinc nutrition does not affect the amount of IAA in radish significantly but does affect its activity. This suggests the involvement of zinc in its regulation.

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