Plant Physiol. (1977) 59, 827-830

Nitrogen Metabolism in Soybean Tissue Culture

II. UREA UTILIZATION AND UREASE SYNTHESIS REQUIRE Ni^{2+}

Received for publication August 20, 1976 and in revised form December 22, 1976

JOSEPH CARMINE POLACCO
Department of Genetics, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, Connecticut 06504

ABSTRACT

Potassium citrate (10 mM, pH 6) inhibits the growth of cultured (*Glycine max* L.) cells when urea is the sole nitrogen source. Urea-dependent citrate toxicity is overcome by three separate additions to the growth medium: (*a*) NH_4Cl (20 mM); (*b*) high levels of $MgCl_2$ (10 mM) or $CaCl_2$ (5-10 mM); (*c*) low levels of $NiSO_4$ (10^{-2} mM). Additions of 10^{-2} mM $NiSO_4$ not only overcome citrate growth inhibition but the resultant growth is usually better than urea-supported growth in basal medium (neither added citrate nor added nickel). In the *absence* of added citrate, exceedingly low levels of $NiSO_4$ (10^{-4} mM) strongly stimulate urea-supported growth in suspension cultures.

Citrate does not inhibit growth when arginine is sole nitrogen source. However, cells using arginine have no net urease synthesis in the presence of 10 mM potassium citrate. When 10^{-2} mM $NiSO_4$ is added to this medium, urease specific activity is 10 times that observed in basal medium lacking both citrate and added nickel.

Citrate is a chelator of divalent cations. That additional Mg^{2+} or Ca^{2+} alleviates urea-dependent citrate toxicity indicates that citrate is acting by chelation, probably of another trace divalent cation; this is probably Ni^{2+} since at 10^{-2} mM it overcomes citrate toxicity and at 10^{-4} mM it stimulates urea-supported growth in the absence of citrate. That ammonia overcomes citrate toxicity indicates that the trace Ni^{2+} is essential specifically for the conversion of urea to ammonia. Ni^{2+} stimulation of urease levels in arginine-grown cells supports this contention.

In basal medium, soybean cells grow slowly with urea nitrogen source presumably because the trace amounts of Ni^{2+} present ($\leq 10^{-6}$ mM) are growth-limiting.

In a previous communication (11), I reported that citrate blocks the utilization of urea but not ammonia by cultured soybean cells. Citrate does not block urea uptake significantly nor does it inhibit urease activity. However, when citrate is in the growth medium, there is no detectable synthesis of urease. Since citrate can chelate Ni^{2+} (7), and since Ni^{2+} has been reported to be a component of jack bean urease (3), divalent Ni^{+} was added to the growth medium to determine whether it would overcome urea-dependent citrate toxicity. I show here that 10^{-2} mM $NiSO_4$ indeed releases growth inhibition by 10 mM potassium citrate when urea is sole nitrogen source. Further, urease production is higher in the presence of combined 10 mM potassium citrate and 10^{-2} mM $NiSO_4$ than in basal medium containing neither citrate nor added nickel.

MATERIALS AND METHODS

Growth Media and Culture Conditions. Callus and suspension cultures were induced from shoot tips of etiolated soybean seedling (*Glycine max* L., var. Kanrich, Burpee Seed Co., Warminster, Pa.) as previously described (11).

Cultures were induced and maintained on Murashige and Skoog salts (9) supplemented with 1 mg/l thiamine·HCl, 0.5 mg/l pyridoxine·HCl, 0.5 mg/l nicotinic acid, 100 mg/l *myo*-inositol, 30 g/l sucrose, 5 mg/l IAA, 0.5 mg/l 2,4-D, 0.3 mg/l 2-IP,[1] and 8 g/l Difco agar (8). Agar was omitted from shake culture medium. The pH was adjusted to 6 before autoclaving; sucrose was autoclaved separately. To alter the nitrogen source, KNO_3 and NH_4NO_3 were replaced by filter-sterilized additions of urea or arginine to autoclaved medium cooled to 45 C or below. A 1 M potassium citrate stock solution was prepared by adjusting citric acid to pH 6 with KOH and filter-sterilizing. All media where arginine and urea were the nitrogen source were supplemented with 1 mM KCl. Cultures were grown at 25 C in the dark. Callus was maintained by transferring every 3 weeks; shake cultures were transferred every 7 to 10 days by filtering through one layer of cheesecloth and pipetting 10 ml of the filtrate to 65 ml of fresh medium.

For growth studies, soybean suspension cultures were shaken in 50 ml of medium in 300-ml Erlenmeyer flasks fitted with side arms (Bellco Glassware, Vineland, N. J.). Periodically, the cells were allowed to settle in the side arm and after 0.5 hr the volume of the settled cells was determined. To determine growth on solid medium, callus was weighed aseptically at 2-week intervals and transferred to fresh medium.

Preparation of Extracts. Three or four g of cells from suspension culture were collected on Miracloth circles (Chicopee Mills, New York) by suction and washed copiously with deionized H_2O. They were ground in about 3 ml of extraction buffer (0.1 M tris-maleate, 1 mM EDTA, 1 mM β-mercaptoethanol, [pH 7] in a mortar containing a small amount of acid-washed sand. The slurry was transferred to 15-ml Corex tubes along with one or two washings of the mortar with fresh buffer. Extracts were sonicated 1.5 to 2 min and spun at 20,000*g* for 15 min. The supernatant fraction was dialyzed for 3 hr with hourly buffer changes. Starting with the grinding step, all manipulations were performed at 0 to 5 C.

Enzyme Assay. Arginase was determined by measuring arginine-dependent production of urea. The procedure is similar to that described by Kollöffel and van Dijke (6). Enzyme extracts were activated by mixing 0.9 ml of extract with 0.1 ml of 20 mM $MnCl_2$ and incubating at 30 C for 5 min. One ml of assay mix contained 180 μmol L-arginine (adjusted to pH 9.7 with KOH), 2 μmol $MnCl_2$, and 0.2 to 0.4 mg extract protein. The reaction was started by adding 0.1 ml of activated enzyme extract to 0.9 ml of assay medium maintained at 30 C. At 15, 30 and 45 min, 1 ml of 2 N H_2SO_4 was added to stop the reaction. One-half-ml aliquots were removed for colorimetric urea determination (10,

[1] Abbreviation: 2-IP: N^6-(Δ^2-isopentenyl) adenine.

11), which was always performed with urea standards. It was not necessary to remove protein before urea was determined. Under these assay conditions, urea production is dependent on arginine and is linear with time to at least 90 min. Arginase activity is expressed as nmol urea released (or arginine consumed)/min·mg protein extract. Urease was assayed as described previously (11). Urease activity is expressed as nmol urea consumed/min·mg protein. Protein was determined by the Biuret method as previously described (11).

RESULTS AND DISCUSSION

Nickel Alleviation of Urea-dependent Citrate Poisoning. In the experiment of Table I, callus cultures which had been grown with 25 mM urea-N were transferred to urea-N medium supplemented with 10 mM potassium citrate. Callus was transferred at 2-week intervals. In agreement with previously reported results (11), the division time of soybean callus using 25 mM urea as N source is about 15 days. Results summarized in Table I indicate that callus growth after 6 weeks with urea-N is seriously slowed in the presence of potassium citrate. Growth beyond the 6th to 8th week of subculture stops almost completely (11). The addition of 10^{-2} mM $NiSO_4$ not only overcomes citrate inhibition but stimulates urea-supported growth over that observed in basal medium (no added citrate or nickel). A 30-fold increase in 0.3 mM $NiSO_4$ also overcomes citrate inhibition (results not shown); however, at 10 mM it is completely toxic (Table I). Addition of 10 mM $MgCl_2$ or 10 mM $CaCl_2$ is not toxic and overcomes citrate inhibition, probably by complex formation with the citrate. The association constants at 25 C between citrate (all carboxylic groups unprotonated) and Mg^{2+}, Ca^{2+}, and Ni^{2+}, respectively, are 3.92×10^3, 4.79×10^4, and 1.29×10^5 (2, 7). At lower levels (5 mM additions, Table I) Ca^{2+} is much more stimulatory than Mg^{2+} in agreement with the greater affinity of Ca^{2+} for citrate. These results indicate a competition for citrate between Ca^{2+} and Mg^{2+} on one hand and Ni^{2+} on the other.

The effect of citrate on urea-supported growth was studied in greater detail (Fig. 1) in soybean suspension cultures. At low levels (10^{-2} to 1 mM), citrate actually stimulates growth. A possible explanation is that at low citrate levels citrate-nickel complexes are more efficiently transported than free Ni^{2+}, and that within the cell, citrate metabolism releases nickel. This explanation is supported by the observation (Fig. 2) that in the presence of 1 mM citrate, exceedingly low levels of added nickel are toxic. At higher citrate levels (3–10 mM), there is increasing inhibition of growth. Ten mM citrate has no effect on the utilization of the Murashige and Skoog (9) nitrogen source (first order growth constant of 19.8×10^{-3} hr^{-1} in the presence and absence of citrate). Figure 1 also shows that the addition of 20 mM NH_4Cl releases the cells from citrate inhibition. Thus, citrate growth inhibition involves a block in the conversion of urea to ammonia.

Table I. Effects of Divalent Cation Supplementation on Growth of Soybean Callus with Urea-N[a] in the Presence of Citrate

Urea (25mM) was used in place of the Murashige and Skoog (7) nitrogen source of 18.8 mM KNO_3 and 20.6 mM NH_4NO_3.

Addition	Initial Dry Weight[1]	Final Dry Weight[2]	Callus Division in 6 weeks
	mg		No.
None	10.0	120	3.6
Citrate	17.9	74	2.0
Citrate plus 10^{-2}mM $NiSO_4$	13.7	864	6.0
Citrate plus 10mM $NiSO_4$	10.0	13	0.4
Citrate plus 10mM $CaCl_2$	14.8	293	4.3
Citrate plus 10mM $MgCl_2$	10.0	176	4.1
Citrate plus 5mM $CaCl_2$	13.2	1290	6.4
Citrate plus 5mM $MgCl_2$	11.4	72	2.7

[1]Initial dry weights were calculated from dry weight: fresh weight ratio of urea-grown callus and the initial fresh weights.
[2]Dry weights were determined by overnight drying of callus tissue at 50 C.

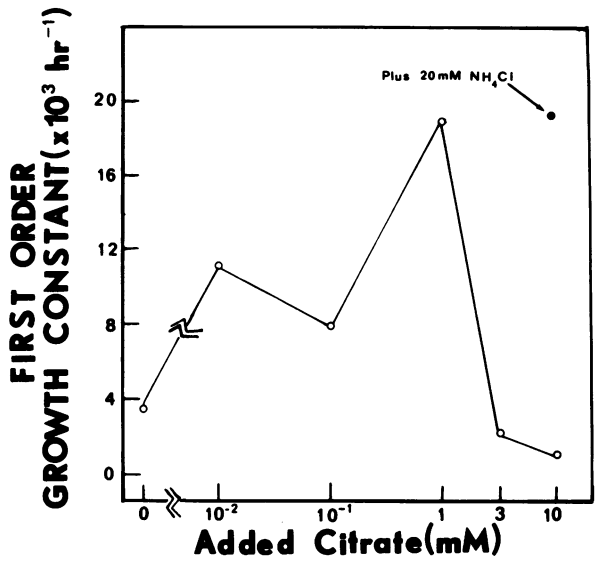

FIG. 1. Effects of citrate on the growth of soybean suspension cultures using urea-N source. Cells were transferred from Murashige and Skoog N source, cultured on 25 mM urea medium supplemented with different levels of citrate for 4 days and retransferred to the same media for growth determinations. First order constants (k) were determined from slopes of semilogarithmic growth plots. k = .693/doubling time.

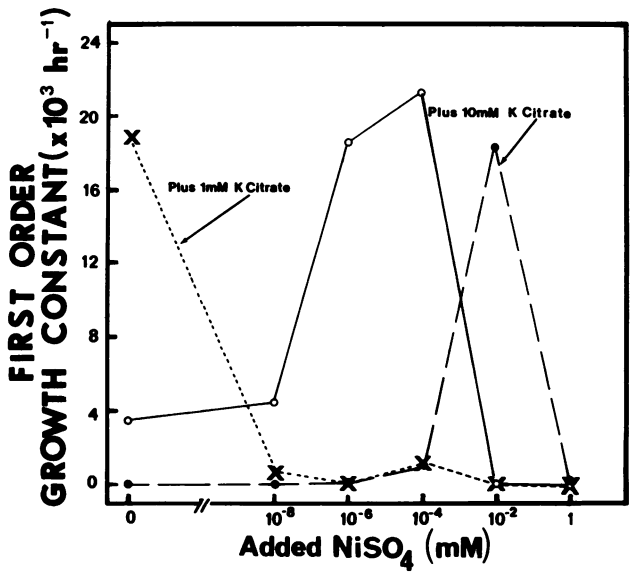

FIG. 2. Effects of added Ni on the growth of soybean suspension culture using urea N source. $NiSO_4$ was added to cultures containing no added citrate (○——○), 1 mM citrate (×-----×), or 10 mM citrate (●- - -●). Growth conditions and determinations were as described in the legend to Figure 1.

From the growth data summarized in Figure 2 it is obvious that extremely low levels of added $NiSO_4$ greatly stimulate urea-dependent growth (optimum [$NiSO_4$] is at or near 10^{-4} mM). In the presence of 10 mM citrate, there is almost an absolute requirement for added $NiSO_4$, and the optimum concentration of added $NiSO_4$ is increased roughly 100-fold. Thus, the evidence strongly suggests that citrate blocks the conversion of urea to ammonia because it chelates Ni^{2+} cations.

In contrast to suspension cultures growing on urea nitrogen

(Fig. 3A), soybean cell suspensions using 9 mM arginine-N source (Fig. 3B) are not inhibited by citrate. There is considerable arginase in these cells (Fig. 4). The products of the arginase reaction are urea and ornithine. Since urea has been shown to stimulate urease synthesis (11) and since citrate does not inhibit arginine-supported growth (Fig. 3B), it is possible to study the effects of citrate on urease production in arginine-utilizing cells free from the interference of citrate-induced nitrogen starvation and from any putative citrate effects on uptake of extracellular urea.

Effects of Citrate and Nickel on Urease Production. Cells (Fig. 4) were taken from mid-log phase and cultured for 18 hr in a medium containing no nitrogen. At zero time they were transferred to medium with arginine-N or 9 mM arginine plus 10 mM potassium citrate (pH 6). Citrate has no effect on the pattern of arginase synthesis in these cells. However, urease production appears to cease in the presence of citrate. The decrease in urease levels is much greater than expected from simple dilution of existing enzyme during growth. At 46 hr, cells grown on both types of medium are just starting out of lag phase. By 142 hr, cells on both media had undergone about one division (70% of each medium was changed for fresh medium at 106 hr to avoid nitrogen starvation). I conclude that urease is broken down and not resynthesized in cells growing in citrate-containing medium while there is net urease synthesis in cells growing on citrate-free medium.

In a separate experiment when 10^{-2} mM nickel was added to cells growing on arginine-N plus 10 mM potassium citrate, there was no stimulation of growth. Arginase levels were little changed in these cells in the presence of Ni^{2+} (Fig. 5). However, Ni^{2+} stimulated the urease-specific activity 7- to 10-fold over citrate-depressed levels (Fig. 5). Urease production in cells grown in the

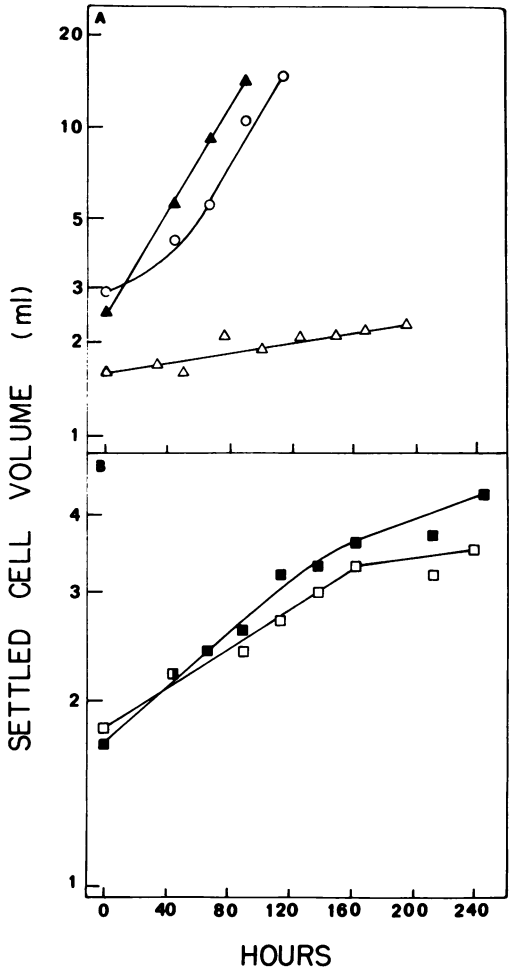

FIG. 3. A. Effects of citrate and Ni^{2+} on growth of soybean suspension cultures using urea-N source. Cells were grown with 25 mM urea (O——O); 25 mM urea plus 10 mM potassium citrate (Δ——Δ); 25 mM urea, 10 mM potassium citrate, and 10^{-2} mM $NiSO_4$ (▲——▲). Cells were transferred from Murashige and Skoog N source (9), cultured on the new media for 2 days and retransferred to the respective media for growth determinations. The basal growth rate with urea-N is unusually high in this experiment. B. Effect of citrate on growth of soybean suspension cultures using arginine-N. Cells growing with 9 mM arginine as nitrogen source (□——□); cells growing with 9 mM arginine plus 10 mM potassium citrate (■——■).

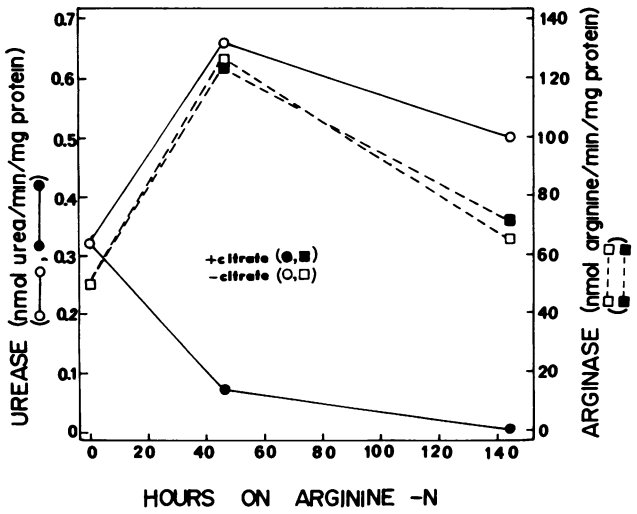

FIG. 4. Effect of citrate on urease and arginase production in soybean suspension cultures growing with arginine-N. After 18 hr culture in the absence of any nitrogen source, cells were transferred to several flasks containing arginine nitrogen source in the presence (●, ■) or absence (○, □) of 10 mM potassium citrate. Urease (○——○, ●——●) and arginase (□---□, ■---■) were determined at 0, 46, and 144 hr after transfer to arginine-N.

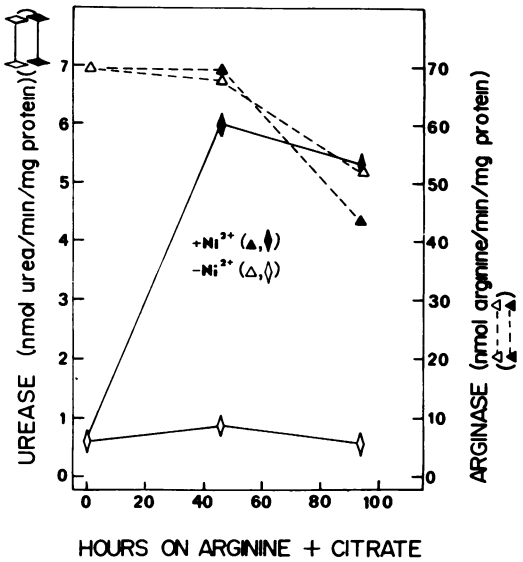

FIG. 5. Effect of Ni^{2+} on citrate-inhibition of urease production. Cells were prepared as in the experiment of Figure 4 and transferred to medium containing 9 mM arginine as nitrogen source plus 10 mM potassium citrate. Half of the flasks were supplemented with 10^{-2} mM $NiSO_4$ (▲, ◆). Urease (◇——◇, ◆——◆) and arginase (△---△, ▲---▲) were determined at 0, 45.5, and 93.5 hr after transfer to arginine-citrate medium.

presence of added Ni^{2+} plus citrate (Fig. 5, ◆) is 10 times that in basal medium (free of added nickel and citrate; Fig. 4, ○). The antagonistic effects of citrate and Ni^{2+} on urease production indicate that free nickel (Ni^{2+}) is essential for urease production in soybean cells.

The report of Dixon *et al.* (3) that jack bean urease contains Ni^{2+} at the active site prompted this investigation. I had previously observed (11) that 10 mM potassium citrate blocked the utilization of urea but not that of NH_4Cl (1–15 mM) nor of the Murashige and Skoog (9) nitrogen source of 18.8 mM KNO_3 and 20.6 mM NH_4NO_3. Further, citrate did not seriously inhibit urea uptake or urease activity *in vitro* (11). The results presented here demonstrate that citrate completely blocks the synthesis of urease and this appears to explain its inhibition of urea utilization by cultured soybean cells. The mode of action of citrate is most likely chelation of Ni^{2+} (7) since adding Ni^{2+} to the growth medium results in 10-fold increases in urease over the levels observed in basal medium. The Murashige and Skoog (9) salts employed in this work do not contain added Ni^{2+}. Thus, urease production is dependent upon trace Ni^{2+} which can be "scavenged" by adding citrate.

Urea-N-dependent inhibition of growth by citrate strongly suggests that Ni^{2+} is not necessary for the synthesis of any other enzyme essential to soybean cells under the growth conditions described. Citrate does not inhibit growth when arginine (Fig. 3B) or ammonia (4, 11) is sole nitrogen source. Indeed, citrate *stimulates* utilization of ammonia-N in soybean (4) and tobacco (1) suspension cultures.

If the ureases produced by soil microorganisms also contain Ni^{2+}, this metal would be an essential micronutrient in urea-fertilized fields. I have found that the growth of several air-borne fungi is severely restricted by EDTA when urea but not NH_4Cl is the N source (unpublished experiments). Indeed, Gauthier *et al.* (5) have reported that 3-amino-1,2,4-triazole, a herbicide which forms metal complexes, inhibits the production of surface soil urease activity by 38 to 87% at 2 μM.

Surprisingly, citrate does not inhibit the growth of soybean cells utilizing arginine-N (Fig. 3B). Since the cells have considerable arginase levels (Figs. 4 and 5), one may postulate the following scheme for the assimilation of arginine:

$$\text{urea} \xrightarrow{\text{urease}} 2\ NH_3 + \text{bicarbonate}$$

$$\text{arginine} \xrightarrow{\text{arginase}} \text{ornithine} + \text{urea}$$

$$\text{ornithine} \longrightarrow \longrightarrow 2\ NH_3 + \alpha\text{-ketoglutarate}$$

If the above scheme is valid, citrate is depriving arginine-using cells of half the nitrogen in the growth medium (that contained in the urea molecule). Growth with arginine-N is much slower than growth with urea-N (Fig. 3, A and B), suggesting that urea is either not produced from arginine or that, if produced, it is unavailable to the cells. The unusually low urease levels, citrate-insensitivity, and lack of Ni^{2+} stimulation of arginine-grown cells are consistent with both possibilities. A study of urea levels and the effects of urease inhibitors (11) in arginine-grown cells is necessary to help resolve the pathway of arginine utilization in cultured soybean cells.

Acknowledgments—It is a pleasure to acknowledge the expert technical assistance of C. Forte, J. Colacino, and C. Nowie. I thank S. Anagnostakis, P. R. Day, R. Sparks, and I. Zelitch for critical reading of the manuscript, and W. Norvell and M. Volz for helpful discussions on chelation chemistry.

LITERATURE CITED

1. Behrend J, RJ Mateles 1976 Nitrogen metabolism in plant cell suspension cultures. II. Role of organic acids during growth on ammonia. Plant Physiol 58: 510–512
2. Davies CW, BE Hoyle 1955 The interaction of calcium ions with some citrate buffers: a correction. J Chem Soc 1038
3. Dixon NE, C Gazzola, RL Blakeley, B Zerner 1975 Jack bean urease (EC 3.5.1.5) a metalloenzyme. A simple biological role for nickel? J Am Chem Soc 97: 4131–4133
4. Gamborg OL, JP Shyluk 1970 The culture of plant cells with ammonium salts as the sole nitrogen source. Plant Physiol 45: 598–600
5. Gauthier SM, SS Ashtakala, JA Lenoir 1976 Inhibition of soil urease activity and nematocidal action of 3-amino-1,2,4-triazole. HortScience 11: 481–482
6. Kollöffel C, HD van Duke 1975 Mitochondrial arginase activity from cotyledons of developing and germinating seeds of *Vicia faba* L. Plant Physiol 55: 507–510
7. Li NC, A Lindenbaum, JM White 1959 Some metal complexes of citric and tricarballylic acids. J Inorg Nuclear Chem 12: 122–128
8. Linsmaier EM, F Skoog 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100–127
9. Murashige T, F Skoog 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
10. Ormsby AA 1943 A direct colorimetric method for the determination of urea in blood and urine. J Biol Chem 146: 595–604
11. Polacco JC 1976 Nitrogen metabolism in soybean tissue culture. I. Assimilation of urea. Plant Physiol 58: 350–357