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Physiology of Tuberization in *Solanum tuberosum* L.

CIS-ZEATIN RIBOSIDE IN THE POTATO PLANT: ITS IDENTIFICATION AND CHANGES IN ENDOGENOUS LEVELS AS INFLUENCED BY TEMPERATURE AND PHOTOPERIOD[1, 2]

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ABSTRACT

Using high pressure liquid chromatography, the cucumber cotyledon bioassay, and mass spectrometry a cytokinin isolated from *Solanum tuberosum* L. cv. Katahdin plant tissues has been identified as *cis*-zeatin riboside. Zeatin riboside (ZR) levels in plants grown under inducing conditions (28 C day and 13 C night with a 10-hour photoperiod) were significantly higher than those in plants grown under noninducing conditions (30 C day and 28 C night with an 18-hour photoperiod). The highest level of ZR was noted in below-ground tissue after 4 days exposure to inducing conditions, with tuber initiation observed after 8 days. A companion study conducted to determine the effect of ZR on *in vitro* tuberization of noninduced rhizomes revealed that after 1 month in culture, controls exhibited 0% tuberization, while ZR treatments of 0.3 and 3.0 milligrams per liter showed 39 and 75% tuberization, respectively.

The tuberization process in the potato (*Solanum tuberosum* L.) is thought to be controlled by environmental factors, mainly photoperiod and temperature, which regulate levels of endogenous growth substances. Short days and cool night temperatures (inducing conditions) have been reported to favor tuberization while long days and high night temperatures delay or inhibit the process (9, 13, 28).

Several workers (7, 8, 21) have suggested the existence of a tuber-forming stimulus which is formed in leaves under inducing conditions and translocated to below-ground tuberization sites. Other workers have demonstrated the movement of a tuber-forming stimulus from an induced scion through a graft union to below-ground rhizomes which subsequently tuberized (17, 25).

Although the exact nature of this tuber-forming stimulus remains unknown, it has been speculated that it may be similar to cytokinins (6). Similarly, Palmer and Smith (26) have demonstrated the requirement for cytokinins for *in vitro* tuberization of excised rhizomes. Results of this laboratory (11, 12, 18) have supported the theory that the tuberizing factor may be cytokinin-like in nature, in that total cytokinin activity was significantly higher in induced than noninduced plants. Two of the four cytokinin compounds separated from potato tissues were significantly higher in induced plants, reaching their highest values in below-ground tissues after 6 days, with tuber initiation noted after 8 to 10 days (11).

In an attempt to elucidate the structure of potato cytokinins, Anstis and Northcote (1) reported isolating a compound which co-chromatographed with ZR.[5] Recently, Van Staden (31) found an N-6-substituted adenyl compound in potato tissue using MS. Accordingly, the objectives of the present study were 2-fold: first, to attempt to isolate and identify the major cytokinin in Katahdin potato tissues; and second, to measure levels of this cytokinin in the plant in an effort to ascertain its possible role in the tuberization process.

MATERIALS AND METHODS

Plant Tissue. Plant material used in this study was obtained by planting tubers of *S. tuberosum* L. cv. Katahdin in flats of moist Perlite. Tubers were incubated in a growth chamber adjusted to 28 C day and 25 C night with a 16-hr photoperiod. Growth chambers provided a light intensity of 660 ft-c at pot level. After 3 weeks, sprout lengths of approximately 9 cm were obtained. Individual sprouts were separated from tubers and planted in 15-cm plastic pots containing a soil-Perlite (2:1) mixture.

Forty-five potted sprouts were selected for each experiment and placed in a growth chamber adjusted to 22 C with a 16-hr photoperiod. They were grown for 2 weeks until plant heights reached 10 to 12 cm. The 40 most uniform plants were transferred to a growth chamber adjusted to 30 C day, 28 C night with an 18-hr photoperiod (noninducing conditions). After approximately 3 weeks, plants had reached a height of 55 to 60 cm and produced rhizomes of suitable length for tuberization. Twenty plants were randomly selected and transferred to a growth chamber adjusted to 28 C day, 13 C night with a 10-hr photoperiod (inducing conditions). The remaining 20 plants were kept in the noninducing chamber. Four plants were harvested from each chamber at 2-day intervals for 10 days, washed in distilled H_2O, separated into above- and below-ground portions, and lyophilized. Samples were ground in a Wiley mill through a 40-mesh screen and stored in glass bottles at −20 C.

Cytokinin Extraction Procedure. The method of Blumenfeld and Gazit (2) as modified by Forsline and Langille (11) was used to extract cytokinins from 2-g samples of potato tissue. Ethyl acetate and aqueous fractions were saved, evaporated to near dryness under reduced pressure at 55 C, and residues redissolved in 5 ml of 80% methanol and 2.5 ml of H_2O, respectively. Prior to HPLC analysis, samples were microfiltered through a 2-μm sieve. One ml of the aqueous phase was diluted 1:9 (v/v). The ethyl acetate phase was not diluted.

Chromatographic Materials and Procedure. A Du Pont model 841 liquid chromatograph equipped with a N_2 gas operated hydraulic pump, an injection port, and a 1-m U-shaped column was

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[5] Abbreviations: HPLC: high pressure liquid chromatography; Rt: retention time; ZIPAX SCX: cation exchange packing—a sulfonated fluorocarbon polymer; ZR: zeatin riboside.

used in this study. The effluent stream was monitored by a dual beam UV detector with a scanning wavelength of 254 nm. The detection system had a maximum full scale of 0.01 optical density units over a range of 1 to 256X. Attenuator setting used was 0.01 *A*. The chromatographic column, 2.1 mm i.d., was prepacked with ZIPAX SCX cation exchange resin (Du Pont Co., Wilmington, Del.). Solvent flow rate was adjusted to 0.5 ml/min at 1,000 p.s.i.

The method of Challice (4) was utilized for cytokinin separations. Two basic operating conditions were followed: for fast eluting cytokinins a mobile phase of 0.05 M KH_2PO_4 was used; while 0.5 M KH_2PO_4 with 10% acetonitrile was used for slower eluting cytokinins. Several pH values for mobile phases between 4.0 and 5.0 were tried in an attempt to optimize resolution. Cytokinin standards were prepared as aqueous solutions adjusted to pH 4.5. Sample loadings ranged from 10 to 200 ng/10 μl, with standards and samples introduced into the column by means of a Hamilton 1701 syringe (Hamilton Co., Reno, Nev.) equipped with a Chaney adapter and disposable needle attachment.

Concentrating and Purifying Extracted Compound. A compound corresponding to the most abundant absorbance peak in the aqueous fraction of potato tissue extracts was collected. Based upon previous results from this laboratory (18), a 4-day, induced above-ground sample was chosen for analysis. Multiple injections were made using HPLC analysis to concentrate the isolated compound.

Combined elutants were purified by the combined methods of Thomas *et al.* (30) and Letham (19). The aqueous fraction was adjusted to pH 7.0 and partitioned seven times against 40-ml volumes of water-saturated 1-butanol. The 1-butanol fractions were combined and evaporated to dryness under reduced pressure at 70 C. The residue was then oven-dried at 45 C for 10 hr to remove any trace of 1-butanol.

Determination of Cytokinin Activity. The dried residue described above was redissolved in 3 ml of 2 mM KH_2PO_4 buffer (pH 5.8) and the cucumber cotyledon bioassay of Fletcher and McCullagh (10) was used to determine cytokinin activity of duplicate samples of this isolated compound. Initially, a standard curve was constructed using known concentrations of ZR. Each value used in the curve was the mean of 12 replications subjected to regression analysis.

Sample Preparation for Mass Spectrographic Analysis. Thirty separation injections were analyzed by HPLC, in conjunction with the aforementioned purification technique, to obtain sufficient dried residue for mass spectra analysis. Residue and a standard compound were desiccated at 4 C prior to use. Analysis was performed on a Du Pont model 491 mass spectrometer set at 400 C probe temperature. Data were presented in mass units as per cent of most abundant ion (M+)

Zeatin Riboside Extraction and Analysis. Two g of lyophilized tissue were extracted as previously described. The aqueous phase was microfiltered and subjected to HPLC analysis as described above. In this case, the mobile phase was 0.05 M KH_2PO_4 (pH 4.0). Endogenous ZR peaks were measured and compared with those of standards of known concentration on the basis of peak height (14). All data were subjected to analysis of variance with Duncan's New Multiple Range Test used to test significance of observed differences between means.

Tissue Culture Techniques. Katahdin potato tubers were sprouted in the dark at 22 C for approximately 2 weeks. Individual sprouts were separated from the tubers and apical and basal nodes were excised. Sprouts were then cut into 1.5-cm sections containing at least one axillary bud and sterilized according to the method of Mingo-Castel *et al.* (23). Following sterilization, 2 mm were aseptically removed from the ends of each section and discarded. Sprout sections were cultured in 125-ml Erlenmeyer flasks containing 40 ml of medium (24) with 2% sucrose. Flasks were stoppered with cotton plugs, sealed with Parafilm, and placed in a nonilluminated incubator at 24 C.

In 2 weeks, buds had elongated into rhizomes of 7 to 12 cm. These were subcultured according to the method of Palmer and Smith (26) in 40-ml vials containing 10 ml of medium with 6% sucrose and ZR at 0, 0.3, and 3.0 mg/l. Vials were stoppered and incubated as described above. Fifteen replicates were prepared/treatment.

Weekly observations were made noting whether the bud had tuberized or elongated. Contaminated cultures were discarded. After 4 weeks in culture, final determinations were made and recorded. Since contamination and bud damage during transfer reduced the number of replicates, a contingency χ^2 determination for a multinomial population (29) was performed to test significance of differences between means.

RESULTS

Cytokinin Separation by HPLC. HPLC analysis of Katahdin potato tissue extracts revealed no detectable compounds under slow eluting conditions (0.5 M KH_2PO_4 with 10% acetonitrile). Hence only fast eluting conditions were used (0.05 M KH_2PO_4 at pH 4.0). Under these operating parameters, five standards eluted within 16 min and separation of zeatin riboside isomers was obtained (Fig. 1).

Separation of four compounds in the aqueous phase of potato tissue is shown in Figure 2. Rt of unknown compounds 1 and 2 compared closely with the adenosine standard (Rt 3.6–3.8 min). Rt of unknown compound 3, however, did not coincide with that of any standard. Compound 4 (Rt 9.22 min) had an Rt which matched that of the *cis* isomer of ZR. Compound 4 was present in quantities 5 to 10 times greater than any other cytokinin in potato tissue extracts.

Bioassay Results. When compared with the standard curve prepared using ZR, unknown compound 4 was found to possess cytokinin activity equivalent to 400 mg of ZR (Table I). This would indicate that compound 4 is a cytokinin and is present in

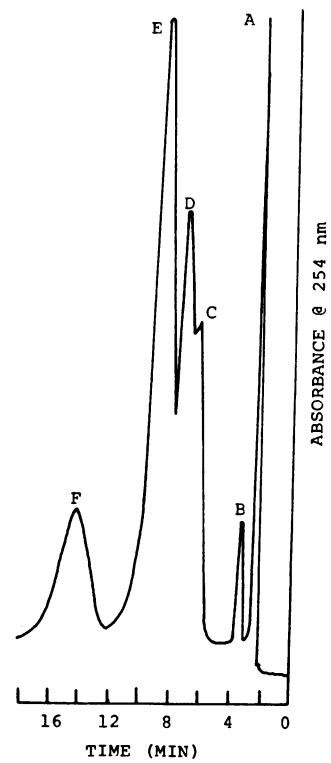

FIG. 1. Separation of fast eluting cytokinin standards on a ZIPAX SCX column eluted with 0.05 M KH_2PO_4 (pH 4.0) at 1,000 p.s.i. A: adenine; B: adenosine; C: kinetin riboside; D and E: *trans* and *cis* isomers of zeatin riboside, respectively; F: benzyl adenosine.

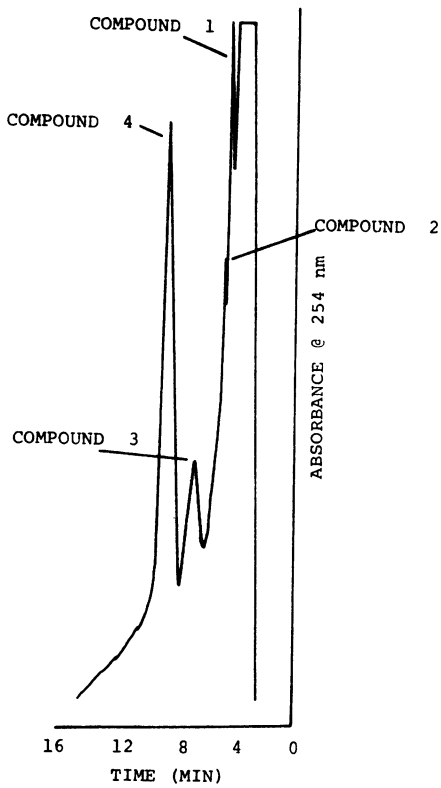

FIG. 2. Separation of four cytokinins in the aqueous phase of potato tissue extract on a ZIPAX SCX column eluted with 0.05 M KH_2PO_4 (pH 4.0) at 1,000 p.s.i. Injection volume: 10 μl.

Table I. Bioassay results comparing zeatin riboside standards with unknown cytokinin from 'Katahdin' plant tissue

Zeatin Riboside (ng)	Absorbance 665 nm x 103
0	95.8
4	125.5
40	155.1
400	184.8
Unknown	184.0

Katahdin potato tissue after four inductive cycles in a concentration of 8.34 μg/g dry tissue.

Mass Spectrographic Analysis. Mass spectrographic analysis was performed on dried residue of unknown compound 4 and a 1-mg sample of ZR standard (Fig. 3). The most abundant peak (m/e 181) was set at 100%. All other peaks were presented as a proportion of this base peak. The base peak was identical for both compounds. In fact, fragmentation patterns to the left and right of the base peak in the case of each of the standard and compound 4 compared favorably.

Endogenous ZR Levels Prior to and During Tuber Formation. For all harvest dates and tissues combined, ZR was significantly higher in induced than noninduced tissue (Table II). Furthermore, highest ZR levels occurred in both above- and below-ground portions of induced plants 4 days after the initiation of inductive conditions (Fig. 4), with tuber initiation noted after 8 days. Although levels in above-ground tissue were somewhat higher than below-ground, no such dramatic changes were observed in ZR levels of noninduced tissues.

The portion of the study designed to determine if ZR would stimulate tuberization *in vitro* demonstrated that this compound does indeed have a promotive effect (Table III). The highest concentration (3.0 mg/l) was associated with 75% tuberization, while no evidence of tuberization was observed for the control (0 mg/l) treatment after 4 weeks in culture. The lower ZR concentration (0.3 mg/l), although less effective than the higher concentration, nevertheless produced 38.5% tuberization.

DISCUSSION

The foregoing results make a rather convincing case for the presence of *cis*-zeatin riboside in Katahdin potato tissues. Results of the bioassay establish that the previously unidentified compound is indeed a cytokinin, since this assay procedure has been reported (10) to be specific for this class of compounds. The Rt and MS data provide good agreement between the unknown and the ZR standard. It should be noted that the MS obtained in the present study differed from those reported by Letham (20). It is suggested that this difference was due to the high probe temperature (400 C) used in the present study which altered the normal fragmentation patterns.

The fact that ZR was isolated from potato plant tissues is not altogether surprising. This compound has been found in other plant tissues, such as cotton ovules (27), pinto bean fruit (16), sycamore sap (15) and crown gall tumor tissue (22). Results of this laboratory (18) revealed that induced potato tissue was significantly higher in total cytokinin activity than noninduced tissue. Although two of the four cytokinins separated by paper chromatography were higher in induced tissue, no attempt was made to elucidate their structures (11). Also using paper chromatographic techniques, Antis and Northcote (1) and Van Staden (31) have isolated a cytokinin from potato tuber tissue which co-chromato-

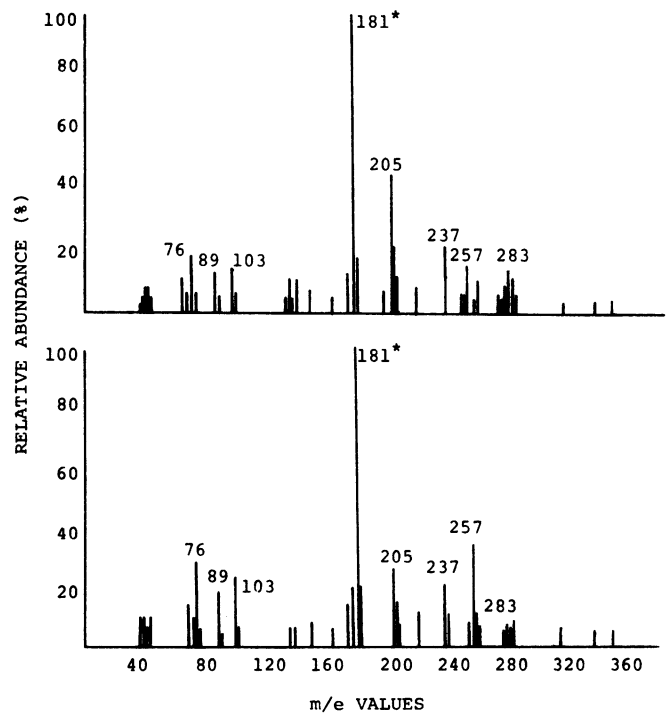

FIG. 3. Mass spectra of zeatin riboside (top) and an unknown compound isolated from the aqueous phase of potato tissue extract (bottom).

Table II. Effect of induction on levels of zeatin riboside in potato tissue extracts. Means are the average of 30 determinations

Type of Induction	Zeatin Riboside Eq (ng/g dry wt.)*
Induced	633.6 a
Non-Induced	490.0 b

*Means followed by the same letter were not significantly different by Duncan's New Multiple Range Test at 5% level.

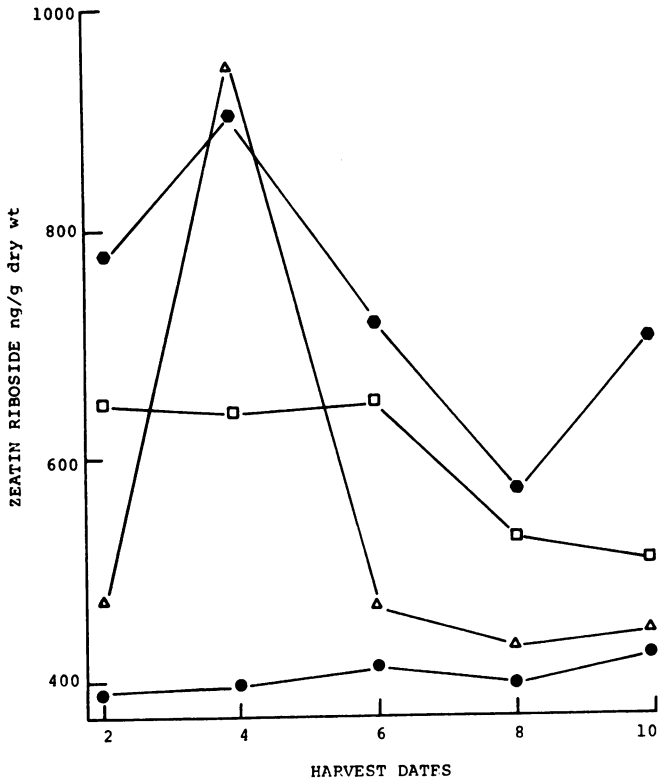

FIG. 4. Levels of endogenous zeatin riboside in Katahdin potato plants as influenced by type of tissue, induction, and days after transfer to inducing conditions. (△): induced, below-ground tissue; (▲): induced, above-ground tissue; (●): noninduced, below-ground tissue; (□): noninduced, above-ground tissue.

graphed with ZR. The latter paper (31) reported using MS to establish the compound as an N-6-substituted purine. More specific identification could not be made.

The fact that ZR was significantly higher in induced than noninduced tissue (Table I) reinforces earlier findings from this laboratory (18) which showed total cytokinin activity to be higher in the induced tissue. Since ZR levels in Katahdin tissue were 10 to 100 times greater than any other cytokinin detected, ZR may have accounted for most of the total cytokinin activity measured in the earlier study.

These results are consistent with reports by earlier workers (5, 13, 25) who suggested that under rather specific conditions of temperature and photoperiod (inducing conditions) a tuber-forming stimulus is elaborated at above-ground growing points and moves throughout the plant. Although Courdoroux (6) speculated that the tuber-forming stimulus might be cytokinin-like in nature, it was Palmer and Smith (26) who demonstrated that cytokinin was required for *in vitro* tuberization of isolated potato rhizomes.

Results of the present study differed somewhat from others conducted in this laboratory (18). In induced plants the high cytokinin value previously noted in below-ground tissue after 6 days was observed at 4 days for ZR in the present study. This difference may have been due to the length of the sampling interval. If plants had been sampled more frequently, it is possible that the previously noted movement of cytokinin from tops to below-ground region would have become apparent. However, since the ZR content of below-ground portions of induced plants was more than twice that of noninduced plants, it would suggest that Gregory's (13) "state to tuberize" had been achieved. This would tend to be substantiated by the fact that tuberization was noted after 4 more days of inducing conditions. These results are supported by previous studies (3, 18) where tuberization was observed 8 to 10 days after initiation of inducing conditions.

Table III. Effect of zeatin riboside concentration on in vitro tuberization of non-induced potato rhizomes. Observations made after 4 weeks in culture

Zeatin riboside Concn (mg/L)	No of Replications	% Tuberization*
0	10	0.0 c
0.3	13	38.5 b
3.0	12	75.0 a

*Means followed by the same letter were not significantly different by contingency X^2 test at 5% level.

Further evidence that this rise in ZR levels signaled entrance into the state to tuberize comes from an earlier study (12) where we observed that percentage tuberization of nodal stem segments from induced plants was significantly higher than noninduced plants when cultured *in vitro*. With the addition of kinetin to the medium, however, this relationship was no longer apparent, *i.e.* noninduced segments receiving kinetin tuberized as well as induced segments.

Since ZR was significantly higher in induced than noninduced tissue and since tuberization was noted only 4 days after the increase in ZR in the below-ground portion of induced plants, it is tempting to speculate that this compound may be intimately involved in the tuberization process.

Further evidence for this involvement came from the results of the *in vitro* study (Table III). Since the cytokinin requirement for *in vitro* tuberization has been demonstrated (26), it is not altogether surprising that addition of ZR to the culture medium promoted tuberization. What is noteworthy, however, is that we have isolated and identified ZR from potato tissue and found it to be significantly higher in induced tissue. Since noninduced tissue will tuberize to some extent *in vitro* (12), the subculturing method of Palmer and Smith (26) was employed to reduce endogenous cytokinin levels in the cultured tissue. Lack of tuberization in the 0 mg/l treatment indicated that endogenous cytokinin content had been reduced and the tuberization noted at the higher concentrations (0.3 and 3.0 mg/l) was due to the presence of ZR.

Results of this study along with others recently published from this laboratory (11, 12, 18) support the case for the causative role of cytokinins in the tuberization process. Inasmuch as ZR seems to be the principal cytokinin in potato tissue, it is tempting to suggest that this compound may indeed be the tuber-forming stimulus previously reported in earlier literature. Experiments are presently in progress to elucidate further the role of ZR in tuberization of the potato.

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