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ROLE OF CYTOKININ IN WOODY PLANT MICROPROPAGATION

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Abstract. Using what has now become a standard technology, woody plant micropropagation takes advantage of the effect of cytokinins in stimulating growth and causing shoot multiplication under controlled tissue culture conditions. Although the greatest impact of micropropagation involves species in Ericaceae and Rosaceae, a systematic survey of 130 species in 33 families and 16 orders indicated that the method could probably be extended to several woody taxa that are not currently being exploited (e.g. other species in order Ericales and species in families Bignoniaceae and Rubiacaea). The survey also identified taxa that are unresponsive to the cytokinins, N6-isopentenyladenine (i^6Ade), thidiazuron, and N6-benzyladenine. Apparently, woody species can be classified into three groups based on their tissue culture characteristics: 1) inherently responsive to cytokinins, 2) responsive to cytokinins after acclimation (e.g. *Magnolia* spp.), and 3)

unresponsive. To obtain a better understanding of cytokinins in responsive plants, the uptake and metabolism of i^6Ade was studied in *Actinidia arguta* shoot cultures using HPLC methodology. When established cultures were subdivided and individual shoots were recultured on basal medium plus 30 μM i^6Ade, stem zeatin (io^6Ade) levels increased rapidly from 145 nmol/g to >900 nmol/g after 15 days then they declined. It is concluded that during tissue culture growth on i^6Ade, an important aspect of cytokinin metabolism in *Actinidia* involves the conversion of i^6Ade to io^6Ade which accumulates to levels in excess of the critical concentration (150 nmol/g) needed for optimal growth.

INTRODUCTION

In nearly every instance, methods for woody plant micropropagation exploit cytokinin-mediated regulation of shoot growth. By artificially increasing the cytokinin content of shoot explants under *in vitro* conditions, one theoretically supplies sufficient quantities of the phytohormone not only to nourish continued growth but also to overcome apical dominance. The result of this is *shoot multiplication*, a phenomenon that is the foundation of practically all micropropagation technology (10).

Undoubtedly, micropropagation will take on increasing importance as advances are made in biotechnology (2). Because of this, one of the goals of our research involves fundamental studies on processes crucial to micropropagation, especially the role of cytokinins. As more is learned about the scientific basis of micropropagation, the technology can be extended to previously unexploited plant species. In addition, improved understanding means that problems become amenable to logical (i.e. biorational) strategies of solution.

This paper deals with two questions related to cytokinins and micropropagation:

1) To what extent is the classical, cytokinin concept applicable to woody plants, generally? and

2) Do cytokinin levels in shoots increase, as expected, in response to cytokinin treatments and, if so, what are the magnitudes of the changes involved?

To answer the first question, shoot tips from 130 different woody species were surveyed for their cytokinin responses *in vitro*. The results, reported in terms of a framework relating micropropagation potential to systematics (1), suggest that the methodology is probably possible with several additional groups of plants that are not being exploited currently. They also suggest that shoot growth regulation in other groups may not be as easily manipulated by simple cytokinin treatments. With regard to the question of the cytokinin relations in micropropagated shoots, the results with *Actinidia* confirm, for

the first time, the basic assumption that cytokinin treatments elevate internal phytohormone levels. The results further demonstrate that micropropagated shoots are capable of accumulating cytokinin to concentrations far in excess of those present in the medium and of metabolizing supplied cytokinin extensively. The critical concentration of zeatin (io^{6}Ade) for growing *Actinidia* shoots is estimated to be approximately 150 nmol/g.

MATERIALS AND METHODS

Stock plants for tissue culture were greenhouse-grown seedlings (≤1 year old) from the Arnold Arboretum of Harvard University. To screen species for their responses *in vitro*, growing shoot tips (0.5-1 cm) were disinfected and transferred to each of 4 different media: 1) a basal agar medium containing inorganic (11) and organic (8) nutrients but no phytohormone, 2) basal medium plus 30μM N6-isopentenyladenine (i^{6}Ade), 3) basal medium plus 2.2μM thidiazuron (5,9), and 4) basal medium plus 33 μM N6-benzyladenine (bzl^{6}Ade) and 0.6 μM indole-3-acetic acid. Each species was tested on at least 3 different occasions, 4 replications per treatment in each experiment. Cultures (20 ml per tube) were incubated at 27°C usually with a 16 hr light (40-65 $\mu Em^{-2}s^{-1}$) and 8 hr dark photoperiod. A positive (+) response indicates continued growth of the majority of the shoots on at least 1 or more of the 3 different cytokinin media; a negative (−) response indicates no growth or only sporadic growth during the 6 week incubation period. In most cases of positive response, shoots were subcultured for at least 1 passage to demonstrate sustained growth *in vitro*.

To study the uptake and metabolism of cytokinins, shoots were extracted and analyzed by HPLC as described (3,4). Tissues were fixed overnight at 5° C in 95% ethanol (2.5 ml ethanol/g) and then the mixtures were homogenized and filtered. After concentrating filtrates by evaporation *in vacuo*, samples were suspended in 10% ethanol and fractionated by chromatography using a Varian 5000 liquid chromatograph equipped with a C_{18} micropak MCH-5 column and a 254 nm uv detector. Cytokinins, eluted with successive linear gradients of methanol from 15-70% followed by 70-15%, were quantitated by uv absorbance. The identities and purities of HPLC peaks have been confirmed for *Actinidia* based on characteristic uv and mass spectra (3,4).

RESULTS AND DISCUSSIONS

Micropropagation potential in relation to systematics. In spite of its demonstrated importance, micropropagation is still a fairly recent innovation. As a result, the technology has only

been applied to a limited diversity of plant taxa. Table 1 summarizes an extensive survey involving over 6000 cultures and nearly 3 years of investigation to determine the relationship between *in vitro* response of shoots and systematics. As indicated, explants from growing seedlings were tested on basal, phytohormoneless medium as well as on three cytokinin-containing media representing a natural cytokinin (i.e. i^6Ade) and two types of synthetic cytokinins (i.e. bzl^6Ade, an adenine analogue and thidiazuron, a phenylurea cytokinin).

Table 1. Taxa of woody plants tested for cytokinin response in tissue cultures.

Superorder	Order	Family	Number of species	Response
Magnoliidae	Magnoliales	Magnoliaceae	7	−
		Annonaceae	1	−
	Laurales	Calycanthaceae	3	−
		Lauraceae	3	±
	Ranunculales	Ranunculaceae	1	−
Hamamelidae	Fagales	Betuclaceae	7	+
Dilleniidae	Theales	Theaceae	4	±
		Guttiferae	2	+
	Malvales	Malvaceae	1	+
		Tiliaceae	8	−
		Sterculiaceae	3	−
	Urticales	Ulmaceae	7	−
		Moraceae	3	±
	Ericales	Actinidiaceae	5	±
		Ericaceae	3	+
		Clethraceae	1	+
Rosidae	Rosales	Rosaceae	5	+
		Saxifragaceae	1	+
	Fabales	Leguminosae	14	+
	Cornales	Cornaceae	7	±
		Nyssaceae	1	−
	Sapindales	Aceraceae	10	−
		Staphyleaceae	2	−
		Sapindaceae	1	−
		Rutaceae	7	+
		Anacardiaceae	5	±
Asteridae	Gentianales	Oleaceae	7	+
	Lamiales	Verbenaceae	2	+
	Scrophulariales	Scrophulariaceae	1	+
		Bignoniaceae	6	+
	Rubiales	Rubiaceae	2	+

Figure 1 shows clearly that cytokinin responsiveness varies according to the systematic placement of species. Only a single species out of 15 tested in superorder Magnoliidae (i.e. *Sassafras albidum*), for example, exhibited a positive response while all 18 species of superorder Asteridae that were examined could be grown as shoot cultures. In other superorders the response of species were more variable. Within superorder Rosidae, for instance, orders Fabales and Rosales were both consistently positive but order Sapindales contained negative as well as positive species. Likewise, superorder Dilleniidae

could be subdivided into positive and negative systematic groupings.

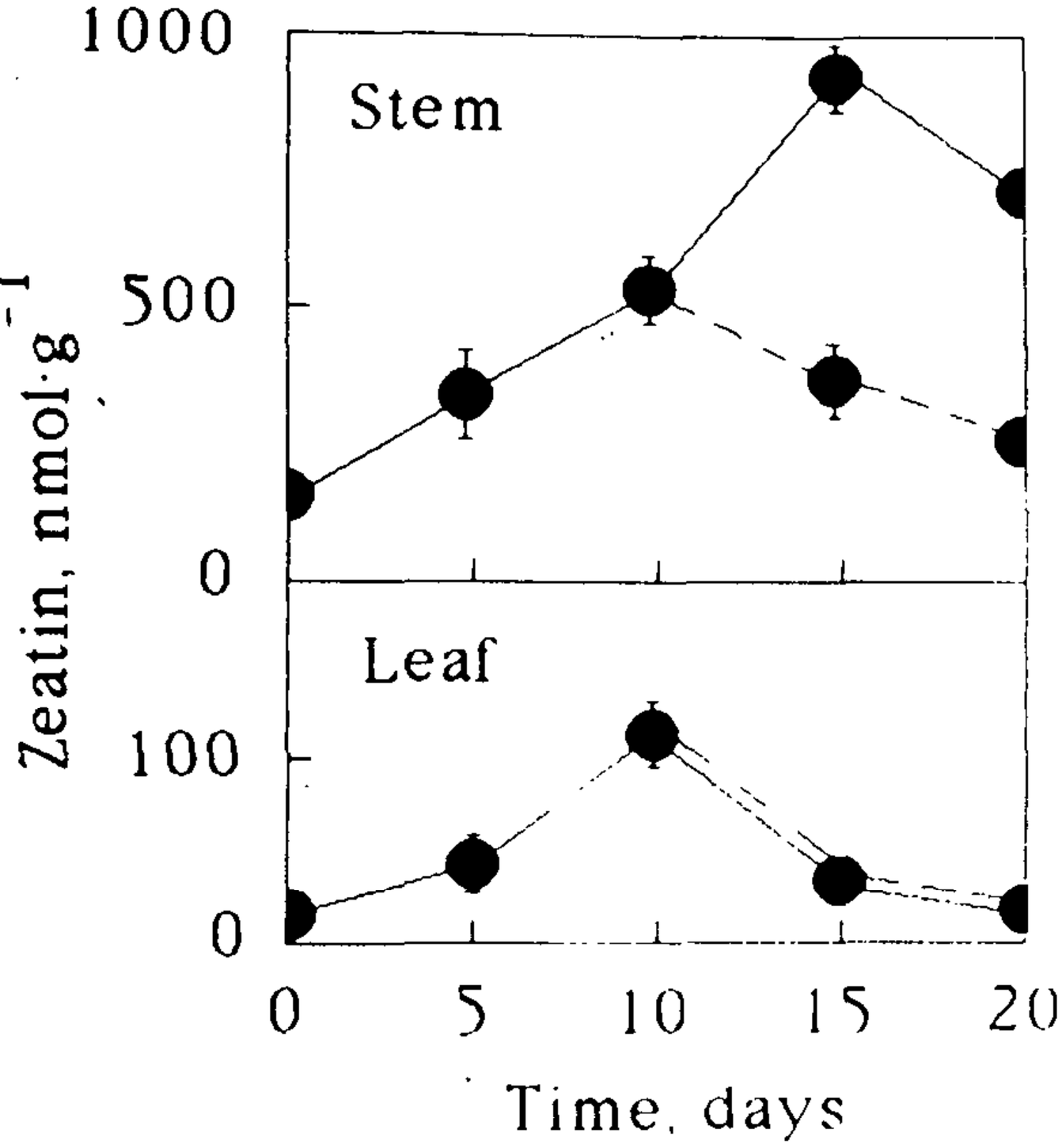

Figure 1. Framework relating the cytokinin response of shoots to systematics. Numbers indicate the different woody species within each order that were tested while stippling shows the percent of these species giving a postive growth response. Superorders: 2+, 3±; Orders: 7+, 7±, 2−, Families: 15+, 5±, 11−; Species: 72+, 58−.

Apparently, woody species fall into three different groups depending on the tissue culture characteristics of their shoots. The first group contains those species that exhibit an inherent response as explants; i.e. positive (+) species in our screen. Examples of plants in this group that are not being micropropagated currently include species in the important tropical tree families Bignoniaceae and Rubiaceae. The second group contains species that respond sporadically as explants or only after repeated subculturing, a phenomenon that has been referred to as *acclimation*, *phase transition*, or *habituation*. An example in this group is *Magnolia* × *soulangiana* which can be grown slowly from explants at a low frequency (approximately 5% of cultures responding) on the medium containing 30 μM i^6Ade. Finally, the third group consists of species that are unresponsive to cytokinin treatments.

Cytokinin dynamics of tissue-cultured shoots. Despite the fact that over 5 billion shoots have been produced by micro-

propagation in the last decade, very little is known about the fate of cytokinins during tissue culture growth. Because of this, and especially in view of the crucial role of cytokinins in micropropagation, studies of cytokinin uptake and metabolism *in vitro* are integral to improving the scientific understanding of micropropagation and, ultimately, to developing new technology.

Figure 2 shows the cytokinin content of *A. arguta* shoots subcultured on basal medium plus 30 μM i^6Ade. As indicated, at the time of transfer shoots already contained 145 nmol/g io^6Ade from the previous passage *in vitro*. Upon subculture to fresh medium, i^6Ade was taken up from the medium and converted to io^6Ade which increased in stems to 930 nmol/g during the initial 15 days of incubation and then declined. Interestingly, the io^6Ade contents of leaf tissues were significantly lower than those of stems even though leaves also exhibited an initial increase followed by a decrease in io^6Ade content.

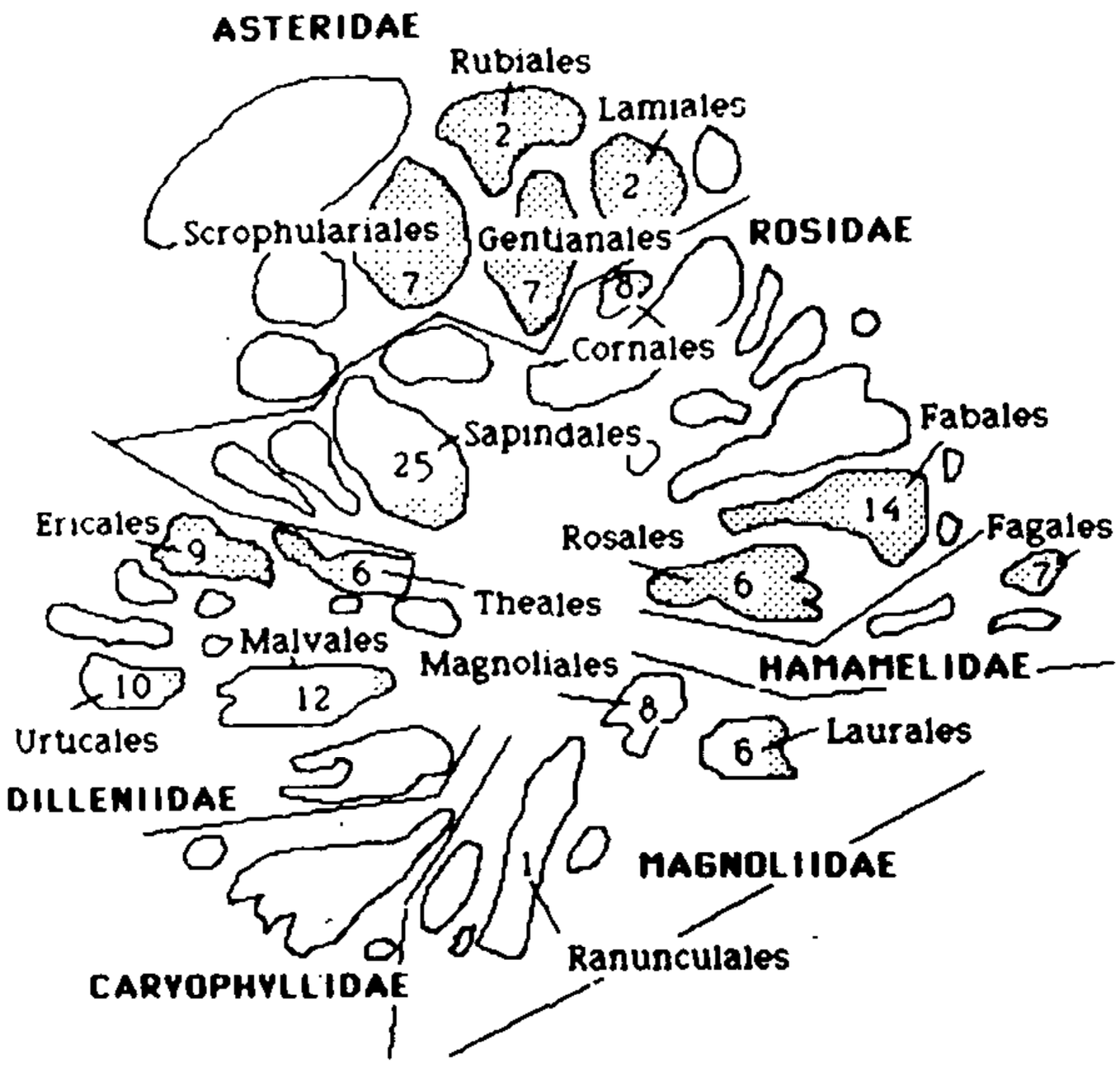

Figure 2. Zeatin contents of stem and leaf tissues in micropropagated *Actinidia arguta*. Shoots were transferred from established 4-week old cultures to medium containing 30 μM i^6Ade. The dotted lines show the effect of shoot transfer to basal medium on the subsequent zeatin contents of stems and leaves. All points are means calculated from 4 independent determinations ± SE.

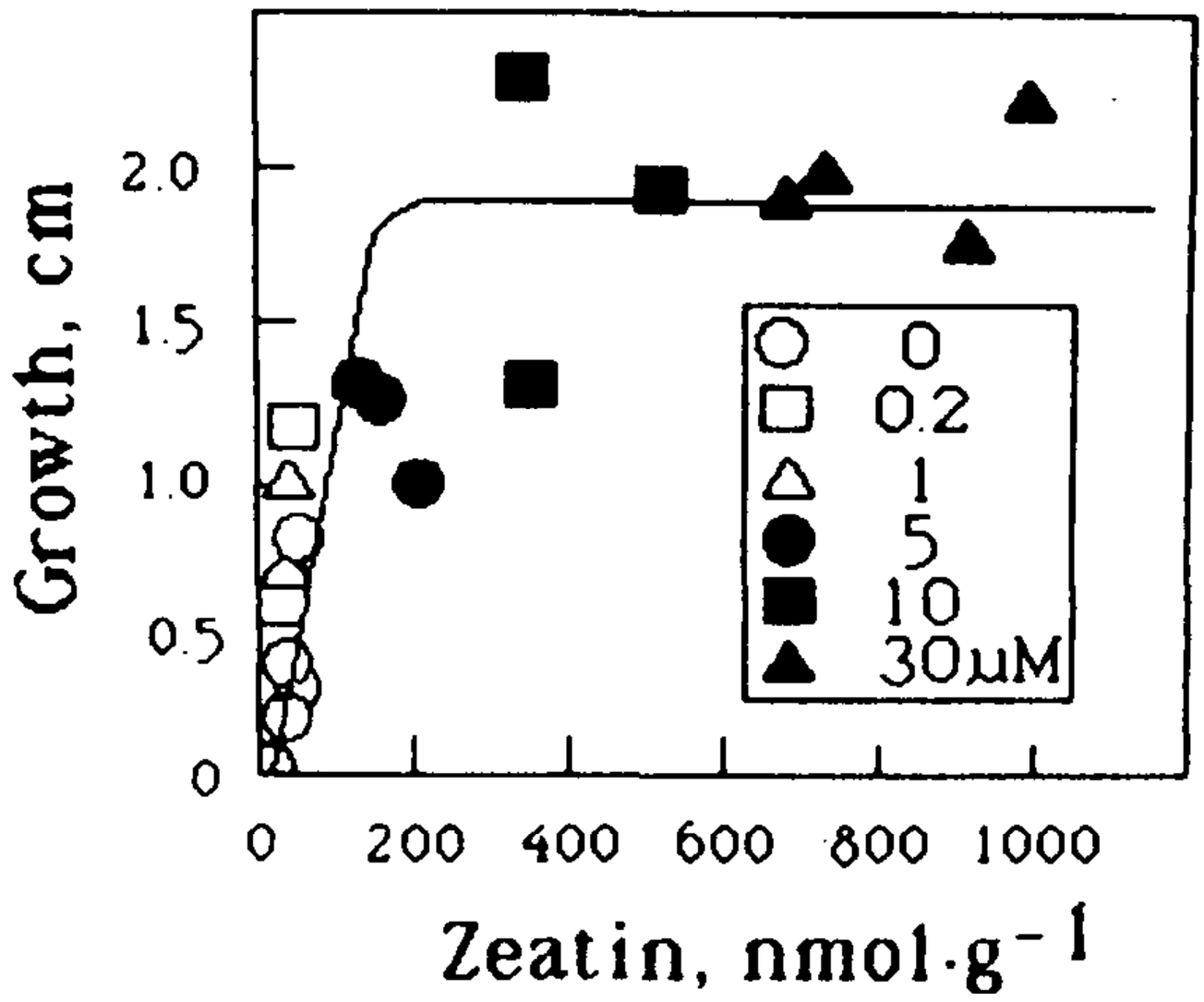

Figure 3. Growth of *Actinidia arguta* shoots in tissue cultures in relation to their internal cytokinin content. Shoots (1.0 cm) from established cultures were transferred to media containing the designated i^6 Ade concentrations (symbols) and incubated for 20 days. After measuring the growth increment, stems were extracted and analyzed for cytokinin contents by HPLC.

SUMMARY

The common objective of both areas considered in this paper is to provide fundamental information as the basis for a biorational approach to micropropagation. It is apparent, for example, that taxa vary with respect to their responsiveness to cytokinin-controlled manipulation of shoot growth. Designation of taxa into groups according to their tissue culture characteristics, therefore, is a way to predict whether or not a given species is a prime target for micropropagation. The process also identifies systematic groupings where further research is needed.

Likewise, an improved understanding of phytohormone assimilation during *in vitro* growth can serve as the foundation for studies to determine why shoots of other species do not grow. Are negative species, for example, able to generate a critical cytokinin concentration within their stems? If they are not, is it because of poor cytokinin uptake and/or transport, or are these plants especially active in destroying cytokinin? Answers to these simple but crucial questions about micropropagation will advance further our knowledge of the scientific basis underlying the technology.

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MICHAEL MARCOTRIGIANO: Question for John Einset. Do you think that some of the plant families that are not responsive are not responsive because you chose not to screen enough cytokinin sources? Possibily their ability to transform the synthetic cytokinin into zeatin is impared.

JOHN EINSET: It is possible. I tried to select media that were representative of media that are used in micropropagation. We had to set limits on the number of media and plants to screen.

RALPH SHUGERT: Question for John Einset. Have you had any experience with the genus *Taxus*? A concern of a propagator is the trueness-to-type when you have a cultivar of a species, such as *Taxus cuspidata* 'Capitata', and propagate it. We know what happens when you take lateral cuttings of that plant. What will we get if we do micropropagation of 'Capitata'?

JOHN EINSET: I have no experience with 'Capitata'. With regards to your second question, research with other plants has shown that if you start with organized structures and force laterals, it should be true-to-type. If you go through a callus you can generate variability, which is called somaclonal variation.

PAUL READ: This is a further response to Ralph's question. Cytokinins are a little tricky in that if you supply high cytokinin levels you may generate a bushier plant in the initial phases. That does not mean that you have modified the genetic make up of the plant. It does mean that you will have to find hormone levels that will give you a single stem plant if that is what you want.

RALPH SHUGERT: My question is, will you get a 'Capitata' type if you micropropagate from a lateral bud?

PAUL READ: I doubt that very much based on our current technology. If you look at something in the same category, *Araucaria*, you do not get good vertical growth from a lateral shoot. I have worked some on spruces and there is a similar concern.

JOHN EINSET: I think that we should remember that a micropropagated shoot is a small shoot and if you have problems with a cutting you will probably have trouble in a test tube.

EVALUATION OF A HOME TISSUE CULTURE MEDIUM

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Abstract. A comparison between variations of a proposed Home Tissue Culture Medium to the Murashige and Skoog basal medium is described. African violets, Boston ferns and variegated wandering Jew were able to be micropropagated on each medium. Although growth occured on all media, the Home Tissue Culture Medium supplemented with a vitamin tablet produced the best growth. Results with this medium were comparable to those obtained with Murashige and skoog's medium. The Home Tissue Culture Medium supplemented with coconut milk had the worst growth. By using the medium described, plant micropropagation can be performed at home using household items and simplified procedures.

REVIEW OF LITERATURE

Plant tissue culture is used by over 267 commercial nurseries and greenhouse growers in the United States for plant propagation (4). Although technical procedures for micropropagation are diverse, the popularity of this microtechnique is spreading to plant hobbyists (1). Bridgen and Veilleux (2,3) proposed simplified procedures and a culture medium for

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