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Uptake, Distribution, and Metabolism of 1-Naphthaleneacetic Acid and Indole-3-Acetic Acid During Callus Initiation From *Actinidia deliciosa* Tissues

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Abstract. 1-Naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) were required for in vitro callus formation at the basal edge of kiwifruit (*Actinidia deliciosa* [A. Chev] Liang and Ferguson, cv. Hayward) petioles. The uptake, metabolism, and concentration of NAA and indole-3-acetic acid (IAA) content were examined in the explants during the callus initiation period. After 1, 6, 12, 24, 48, and 96 h of culture in the presence of [H^3]NAA, petioles were divided into apical, middle, and basal portions and analyzed. Except for a high IAA level measured at 12 h, IAA content decreased in tissues during a culture period of 96 h. NAA uptake was higher in petiolar edges than in the middle portion, and NAA was rapidly conjugated with sugars and aspartic acid inside the tissues. The amide conjugation was triggered in apical and basal portions from 12 h and in the middle part from 48 h, with α-naphthylacetylaspartic acid being the major metabolite. Free-NAA concentration in cultured petioles achieved an equilibrium with the exogenously applied NAA (0.27 μM) from 12 h, and it remained constant thereafter. The relationships between the role attributed to NAA and BA in the initiation and the maintenance of disorganized growth of callus in kiwifruit cultures are discussed.

Key Words. *Actinidia deliciosa*—Auxin—Callus—Indoleacetic acid—Kiwifruit—Naphthaleneacetic acid

Auxins constitute a small group of plant hormones capable of promoting several aspects of plant growth and development, such as cell division, cell extension, vascular differentiation, adventitious root formation, and apical dominance. Indole-3-acetic acid (IAA) was the first naturally occurring auxin identified, and today it is considered to be the main auxin in plants (Davies 1995). Synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) have demonstrated auxinlike effects in bioassays.

One important use of auxins is their exogenous application for maintenance of plant cell and tissue-culture systems. In such systems, the synthetic compound NAA is often used instead of IAA because it is not photodegraded or oxidized in the medium before entering the tissue (Smulders et al. 1990). Auxins are generally associated with the promotion of growth, proliferation of callus, and the induction of rooting (Ribnicky et al. 1996). Callus cultures thus have a large number of biotechnological applications (Hall 1993). Callus is generated in damaged regions of plant tissues when wounding is followed by aseptic culture in the presence of auxins and cytokinins. Although both classes of plant growth regulators (PGRs) are generally needed to induce and maintain the disorganized growth, knowledge of the hormonal control in the callogenic response is still limited (Krikorian 1995).

Callus formation from *Actinidia deliciosa* (A. Chev) Liang and Ferguson tissues is achieved when young petioles are cultured in the presence of the synthetic auxin NAA and the cytokinin 6-benzyladenine (BA), the callogenic response being dependent on the cell position in the explants; the most basal and immature region has the highest response, and callus is observed just occasionally in apical and more differentiated cells (Centeno et al. 1996). An effort to explain the role of cytokinins in this callogenic response culminated in an established positive

Abbreviations: BA, 6-benzyladenine; CIM, callus induction medium; NAA, 1-naphthaleneacetic acid; NAAsp, α-naphthylacetylaspartic acid; NAGluc, α-naphthylacetyl-β-D-glucose; Met 1, metabolite 1 of NAA; Met 2, metabolite 2 of NAA; Met 3, metabolite 3 of NAA; Z, zeatin.

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correlation between the content of this type of PGR in kiwifruit tissues, mainly BA, and the induction and initiation of basal callus; the onset of cell division, which occurred within the first 96 h of culture on callus induction medium (CIM), was preceded by a high free BA level found in the tissues at 24 and 48 h (Centeno et al. 1998). In relation to the synthetic auxin applied on CIM, it is known that NAA is conjugated into a glucose ester and several amino acid amides. Consequently, the free and active levels of NAA are not the result of NAA uptake, but NAA metabolism must be taken into account (Barendse et al. 1987). On the other hand, to assess the physiologic relevance to studies that use exogenously added auxins, increased knowledge is needed about the naturally occurring distributions and fluctuations of endogenous auxins in tissues (Sitbon et al. 1996).

The objective of this study was to establish the participation of auxins in the callogenic response of kiwifruit petioles and to relate it to the role attributed to cytokinins. Thus, we have studied the uptake, metabolism, and resulting levels of free NAA, as well as IAA content in kiwifruit explants during the first 96 h of culture on CIM. These analyses were made separately in the apical, middle, and basal portions of petioles to test whether the uptake, metabolism, and levels of NAA and/or IAA content were a limiting factor in callus formation in the apical edge of explants.

Materials and Methods

Plant Material and Culture Conditions

Callus was derived from the basal extreme of young petioles of kiwiplants (*A. deliciosa* [A. Chev] Liang and Ferguson cv. Hayward) as described previously (Centeno et al. 1996). Kiwiplants, developed from cuttings, were already grown in a greenhouse for 5 years at the time of harvesting (August). Petioles from developing leaves were taken and surface sterilized. Then the explants were placed on agar (0.7%, w/v)-solidified MS (Murashige and Skoog 1962) medium (pH 5.6) containing sucrose (2.5%, w/v) for 7 days to eliminate any possibility of contamination.

Callus was observed when petiolar explants were horizontally cultured on CIM consisting of the previously cited medium supplemented with 2.2 μM BA and 0.27 μM NAA. Before the transference from free-hormone medium to CIM, the length of petioles was reduced to 2 cm under aseptic conditions, and longitudinal incisions (approximately 0.5 cm) were made on both extremes of explants. Growth conditions were 25 ± 2°C under a 16-h photoperiod provided by cool-white fluorescent lamps at a photon flux density of 33 μmol m^{-2} s^{-1}.

For analysis of NAA uptake and metabolism during callus initiation, three petioles per flask were cultured in six flasks containing 30 ml of CIM with 1-[^{3}H]NAA (33 KBq per culture vessel, specific activity 836 GBq $mmol^{-1}$, Amersham Ibérica, Spain). Petioles were removed 1, 6, 12, 24, 48, and 96 h from the beginning of CIM culture. The experiment was repeated three times.

Measurements of NAA Uptake

Tissues from each incubation period were washed with distilled water to remove medium residues, dried with filter paper, and divided into three portions of equal length: apical (initially in contact with the leaf blade), middle, and basal (initially in contact with the branch), before being deep-frozen in liquid N_2. Powdered material was weighed and extracted by repeated inversion for 14 h at 4°C in darkness, with 25 ml of 80% (v/v) cold methanol containing 10 mg l^{-1} of butylated hydroxytoluene. Extracts were filtered through GF/A glass filters (Whatman Sci. Ltd.), and the solid residue was re-extracted with 15 ml of the same extraction solvent (7 h). From the combined extracts, three samples of 1 ml were taken, reduced to dryness, and resuspended in 0.5 ml distilled water and 1.5 ml Pico-Aqua (Packard) as scintillating liquid. Radioactivity was measured in a liquid scintillation counter (Packard 2500 TR). Data were corrected according to the extraction volume to determine total NAA uptake.

Analysis of NAA Metabolism

Methanolic extracts were evaporated under vacuum (30°C), and the resulting aqueous solutions were brought to a final volume of 4 ml with distilled water. Samples were cleared by centrifugation (10,000 *g* for 15 min at 4°C) and the supernatants were collected, reduced to dryness by speed-vac concentration (Savant SC-200), and redissolved in 500 μL of 80% (v/v) methanol. After filtration of samples (0.2 μm Anatop-Plus filters, Whatman), aliquots (25 μL) were taken to measure the radioactivity again.

Tentative identification of NAA derivatives in methanol extracts were carried out by silica gel TLC and by alkaline hydrolysis of the separated compounds. Volumes of extracts containing 1 KBq and samples of authentic NAA standard were spotted by triplicate on TLC plates (0.25-mm thickness and 20 × 20 cm plates, Merck $60F_{254}$) and developed with three different solvent systems: S1, chloroform/methanol/acetic acid (75/20/5, v/v/v); S2, chloroform/ethylacetate/formic acid (5/4/1, v/v/v); and S3, isopropanol/ammonium hydroxide/water (8/1/1, v/v/v). Migration profiles and R_f values of the NAA and the other radioactive compounds were determined by autoradiography of the TLC plates (films Kodak XAR Omat). The R_f values obtained in S1, S2, and S3 were compared with those previously reported (Aranda et al. 1984, Caboche et al. 1984, Goren and Bukovac 1973, Smulders et al. 1990, Venis 1972, Vijayaraghavan and Pengelly 1986). Afterwards, the plates were divided according to the R_f values and radioactivity was eluted from each silica fraction with 3 mL of methanol. The extracts were reduced to dryness and radioactivity was counted as previously described. The percentage of the label corresponding to each radioactive spot was calculated for each lane.

NAA metabolites purified by TLC in solvent S1 were hydrolyzed either in 1 mL of 1 mM NaOH at room temperature (ester hydrolysis) or in 1 mL of 7 mM NaOH for 3 h at 100°C (amide hydrolysis). The hydrolysates were reduced to dryness (Savant SC-200) and redissolved in methanol. After centrifugation (8,000 *g*, 10 min), the supernatants were again analyzed by TLC (S1) and autoradiography to determine whether NAA was released from metabolites after either ester or amide hydrolysis.

The analysis of NAA derivatives in methanol extracts of petiolar portions was carried out by silica gel TLC as described earlier. Volumes of samples containing 0.2 KBq joined to NAA standard were spotted in triplicate on the plates, and samples were routinely developed with S1. Radioactive compounds were extracted from the silica after determination of their R_f values by autoradiography, and radioactivity from each silica fraction was counted. Quantification of NAA and NAA derivatives was done by calculating the percentage of the label associated with each radioactive spot with respect to the total radioactivity measured in each sample.

IAA Analysis

After analysis of NAA metabolites in the petiolar samples, the remaining methanolic extracts were dried, redissolved in 5 ml distilled water,

acidified to pH 3 with diluted HCl, and extracted four times with diethyl ether at 4°C in darkness. Then, the ether was removed under a N_2 stream and samples were methylated with diazomethane. The amount of IAA methylated was measured in triplicate by ELISA with monoclonal antibodies (Sigma, Chemical Co., test kits). Data were corrected according to the volume of each methanolic extract and to the IAA losses. To calculate IAA losses during the extraction process, six culture vessels were prepared without 1-[^{3}H]NAA, and each one of the six corresponding petiolar samples was analyzed separately after addition of [2-^{14}C]IAA (2 GBq mmol^{-1}, Amersham Ibérica) to the initial extracts. The mean of the IAA recovery in these six extracts was 32.42 ± 2.89%.

Results

Levels of IAA

The content of endogenous IAA in the apical, middle, and basal portions of petioles was measured after 1, 6, 12, 24, 48, and 96 h of culture, and for the whole explant it was calculated as the mean of the values found in the three petiolar portions (Fig. 1). Although the IAA content in the whole petiole decreased as culture time increased (4.52 ± 1.02 nmol g^{-1} dry weight at 1 h versus 0.62 ± 0.14 nmol g^{-1} dry weight at 96 h), an accumulation of IAA was observed at 12 h (5.87 ± 2.43 nmol g^{-1} dry weight). This accumulation was due to an increase of the IAA amount measured in the middle portion of explants from 6 to 12 h, which was followed by a marked reduction to levels lower than those found in the basal and apical portions for 24 h. These two petiolar portions showed similar changes in auxin content along the culture (Fig. 1), although IAA levels were slightly lower in the basal portion.

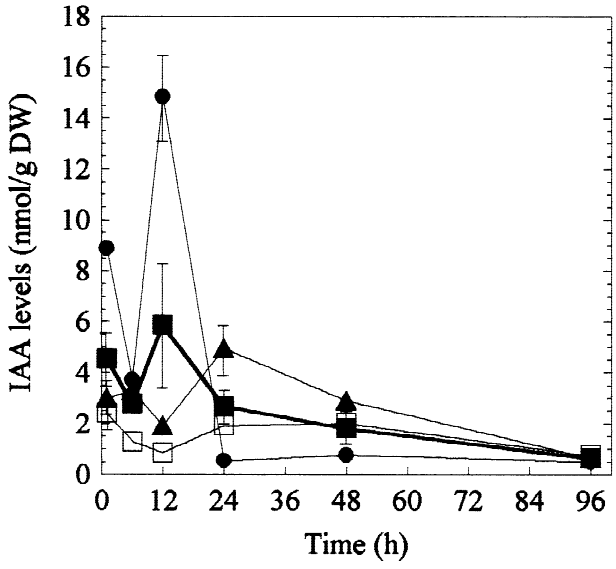

Fig. 1. Endogenous levels of IAA in kiwifruit petioles (■) and in their apical (▲), middle (●), and basal (□) portions along the first 96 h of culture. Values are the mean of three experiments and three analytical replications (n = 9). Standard errors are indicated by vertical bars when larger than the symbols.

NAA Uptake

Kinetics of NAA uptake are shown in Fig. 2. The rate of NAA uptake in the whole petiolar explant was 0.96 nmol g^{-1} dry weight h^{-1} after 1 h of culture, decreased for 12 h, and remained constant from 12 to 96 h with a value close to 0.20 nmol g^{-1} dry weight h^{-1} (Fig. 2*A*). Explants accumulated 27.6% of total radioactivity applied to the medium after 96 h. This accumulation was higher in the apical and basal ends of petioles than in the middle portion (Fig. 2*B*), a difference that became more noticeable during the culture time.

Characterization of NAA Metabolites

The tentative identification of NAA metabolites was carried out in extracts from callus cultures grown during fifth subcultures in a maintenance medium (Centeno et al. 1996) with labeled NAA to obtain a high amount of radioactive compounds in the tissues. The NAA derivatives formed by callus were separated by TLC with three different solvent systems: S1, chloroform/methanol/acetic acid (75/20/5, v/v/v); S2, chloroform/ethylacetate/formic acid (5/4/1, v/v/v); and S3, isopropanol/ammonium hydroxide/water (8/1/1, v/v/v). The R_f values of radioactive compounds, which were visualized by autoradiography of the TLC plates, were compared with the R_f obtained for authentic NAA and with the R_f values previously reported (Aranda et al. 1984, Caboche et al. 1984, Goren and Bukovac 1973, Smulders et al. 1990, Venis 1972, Vijayaraghavan and Pengelly 1986) because of the lack of standards for NAA metabolites (Table 1).

In system S1, which gave the best separation, six compounds were detected and their tentative identification was based on the following facts: (1) NAA: Compound with R_f 0.85–0.95 in S1, 0.62–0.7 in S2, and 0.48–0.59 in S3 matched exactly with the NAA standard and accounted for 5.7–7.79% of the total radioactivity in all the three solvents (Table 1). (2) α-Naphthylacetylaspartic acid (NAAsp): The amount of radioactivity associated with the most abundant compound in S1 (R_f 0.35–0.45) was also similar after analysis with S2 (R_f 0.34–0.42) and S3 (R_f 0.06–0.17), and the R_f coincided with the values reported for NAAsp (Aranda et al. 1984, Caboche et al. 1984, Goren and Bukovac 1973, Smulders et al. 1990, Venis 1972, Vijayaraghavan and Pengelly 1986); moreover, Aranda et al. (1984) and Caboche et al. (1984) identified NAAsp by mass spectrometry. In our study, the ester hydrolysis of this compound did not release NAA, although the amide hydrolysis did. (3) α-Naph-

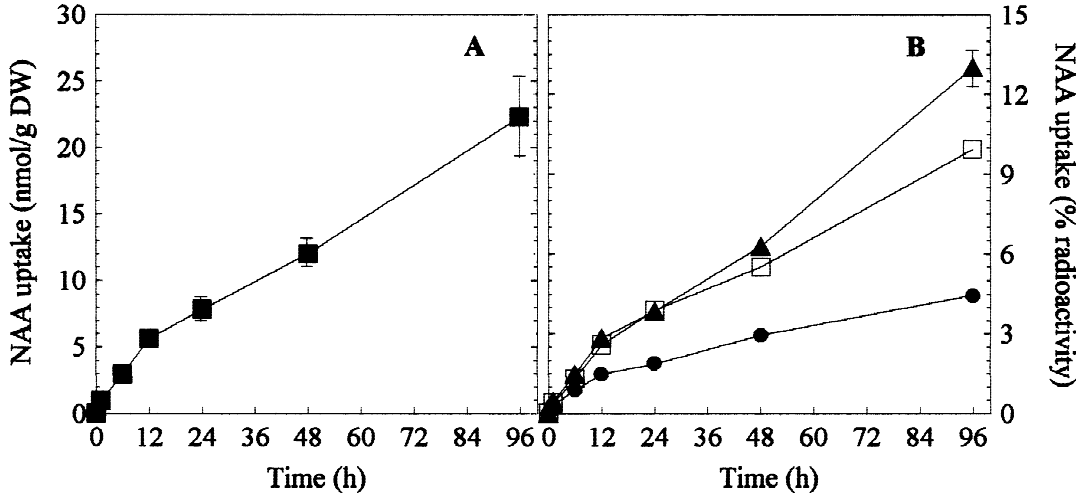

Fig. 2. 96-h time-course of [^{3}H]NAA uptake by kiwifruit petioles cultured on callus induction medium (*A*) and radioactivity distribution between apical (▲), middle (●), and basal (□) portions of explants (*B*). The results are extrapolated and expressed as nmol of total NAA (radioactive + nonradioactive) in *A* and as percentage of total radioactivity per portion in *B*. Values are the mean of three experiments and three analytical replications ($n = 9$). Standard errors are indicated by vertical bars when larger than the symbols.

Table 1. Radioactivity distribution detected in an extract of kiwifruit tissues cultured in presence of [^{3}H]NAA after separation by TLC using three different solvent systems.

	Compound	R_f reported[a]	R_f in extracts of kiwifruit tissues[b]	Radioactivity distribution (%)
	Met 3	0–0.12	0–0.12	13.55
	Met 2	0.12–0.16	0.14–0.21	5.28
S1	Met 1	0.25–0.29	0.24–0.30	5.57
	NAAsp	0.4–0.45	0.35–0.45	60.76
	NAGluc	0.52–0.63	0.5–0.6	2.39
	NAA	0.96	0.85–0.95	7.79
	Met 3	0–0.06	0–0.06	12.88
	NAGluc+Metl	0.05–0.12	0.08–0.14	8.25
S2	NAAsp	0.4	0.34–0.42	59.99
	Met 2?		0.48–0.55	5.45
	NAA	0.85	0.62–0.7	5.7
	NAAsp	0.1–0.2	0.06–0.17	68.61
			0.17–0.27	3.48
			0.27–0.34	3.28
S3	Met 1?	0.38	0.34–0.41	2.48
			0.41–0.48	4.76
	NAA	0.55–0.65	0.48–0.59	7.41
	NAGluc	0.7–0.8	0.71–0.79	3.71

[a] See reference cited in the text.
[b] Range recorded in several experiments.

thylacetyl-β-D-glucose (NAGluc): Compounds with R_f values 0.5–0.6 in S1 and 0.71–0.79 in S3 coincided with the migration reported for authentic NAGluc (Goren and Bukovac 1973, Smulders et al. 1990), and the radioactivity percentage found in both solvent systems was similar (2.39% and 3.71%, respectively, Table 1). (4) The radioactivity associated with the compound with R_f 0.08–0.14 in S2 (8.25%) was close to the sum of radioactivities of NAGluc and metabolite 1 of NAA (Met 1) in S1 (5.57 ± 2.39%). Thus, in agreement with Caboche et al. (1984) and Smulders et al. (1990), NAGluc and Met 1 could co-chromatograph in S2 giving a single compound. (5) NAGluc, Met 1, and metabolite 2 of NAA (Met 2) (R_f 0.14–0.21 in S1) released NAA after ester hydrolysis; therefore all these compound are ester conjugates of NAA. Metabolite 3 of NAA (Met 3) (R_f 0–0.12 in S1), also found by Caboche et al. (1984) and Smulders et al. (1990), released NAA after ester hydrolysis also.

Metabolism of NAA

The analysis of NAA and its metabolites was done separately in apical, middle, and basal portions of kiwi petioles for different periods of time during the first 96 h of culture on CIM in the presence of [^{3}H]NAA. NAA was rapidly metabolized into the earlier tentatively identified compounds: NAGluc, NAAsp, Met 1, Met 2, and Met 3 (Fig. 3). The NAA metabolism changed during the course of culture, so that, at different stages, the relative amounts of metabolites were different. To clarify Fig. 3, Met 1 and Met 2 are represented as the sum of both compounds because their evolution was similar.

Kinetics of NAA metabolism were similar in apical and basal portions of explants (Fig. 3*A* and *C*). Despite the fact that free NAA accumulated during the first 6 h (about 40% of total radioactivity taken up), the proportion of NAA decreased quickly, paralleling the increase of the radioactivity associated with NAAsp. This amide

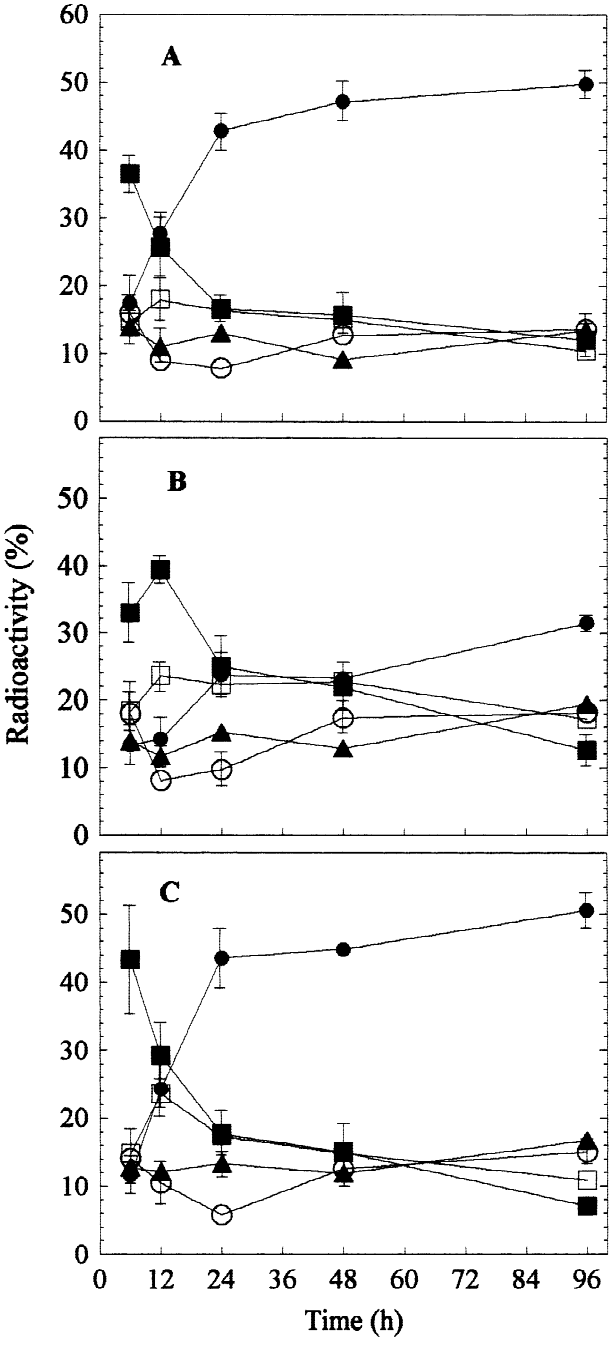

Fig. 3. Distribution of radioactivity in NAA metabolites in apical (*A*), middle (*B*), and basal (*C*) portions of kiwifruit petioles along the first 96 h of culture. Values are expressed as percentage of the total radioactivity extracted and they are the mean of three experiments and three analytical replications ($n = 9$). Standard errors are indicated by vertical bars when larger than the symbols. ■, NAA; ●, NAAsp; ○, Met 3; ▲, Met 1 + Met 2; □, NAGluc.

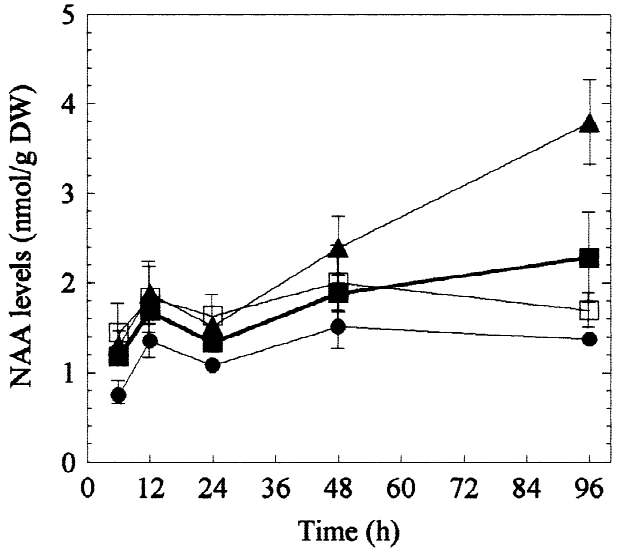

Fig. 4. NAA content in kiwifruit petioles (■) and in their apical (▲), middle (●), and basal (□) portions along the first 96 h of culture. The amount of free NAA for each sample was calculated on the basis of the total label found in the tissue, the percentage of label corresponding to the free NAA radioactive spot after TLC, and the specific activity of the labeled compound. Values for each sample are the mean of three experiments and three analytical replications ($n = 9$). Standard errors are indicated by vertical bars when larger than the symbols.

conjugate reached 45% of total radioactivity from 24 to 96 h, being the major NAA metabolite formed in the tissues. The proportion of NAGluc was also high during the first 12 h of CIM culture, mainly at the basal portion of petioles, but it decreased slowly for this time. The percentage of Met 3 changed in an opposite way to NAGluc. The radioactivity associated with Met 1 and Met 2 remained relatively constant, about 12%, along the whole study period.

The NAA derivatives found in the middle portions of kiwi petioles were the same as those found in petiole edges, but the NAA metabolism was slower (Fig. 3B). As free NAA accumulation (39.3%) was extended over 12 h, its decrease, as well as the increase of NAAsp formation, showed a delay of 6 h in relation to the apical and basal portions. Moreover, the proportion of these two compounds not only remained constant and similar from 24 to 48 h at about 22%, but the amide conjugate also reached only 31.46% of total radioactivity taken up after 96 h.

Concentration of NAA in Kiwi Tissues—Relationships Between NAA and BA During Callus Initiation

As a result of NAA uptake and metabolism, free NAA content in the tissues changed as shown in Fig. 4. Free NAA levels evolved similarly in the three petiolar portions, although the middle portion always had a lower free NAA amount than the petiolar ends. Thus, the free NAA amounts oscillated between 1.30 ± 0.16 and 2.39 ± 0.38 nmol g^{-1} dry weight in apical and basal edges, with the exception of a higher level found in the apical end at

Table 2. Concentration (μM) of exogenously applied NAA and BA in the medium, endogenous concentration (μM) of free NAA, and free BA found in the petioles of kiwifruit along the first 96 h and after 30 days of culture in callus induction medium and relationship between both plant growth regulators in the medium and in the tissues.

	Into the medium	Into the kiwifruit tissues					
		6 h	12 h	24 h	48 h	96 h	30 days[a]
BA, μM[b]	2.2	2.10 ± 0.09	2.90 ± 0.37	4.39 ± 0.39	4.26 ± 0.18	3.37 ± 0.39	0.07 ± 0.01
NAA, μM	0.27	0.15 ± 0.02	0.21 ± 0.02	0.17 ± 0.01	0.24 ± 0.03	0.29 ± 0.06	0.29 ± 0.01
BA/NAA	8.15	14.41 ± 0.63	13.81 ± 1.77	26.41 ± 2.39	18.11 ± 0.76	11.83 ± 1.36	0.24 ± 0.03

[a] Centeno et al. (1996).
[b] Centeno et al. (1998).

96 h. Free NAA levels in the middle portion oscillated between 0.75 ± 0.09 and 1.51 ± 0.23 nmol g^{-1} dry weight.

Free NAA levels in the whole petiole were calculated as the mean of the values found in the three petiolar portions (apical, middle, and basal) (Fig. 4). Once the free NAA achieved a level near 2 nmol g^{-1} dry weight at 12 h inside the explants, the hormone was present in similar amounts during the remaining period of study. Taking into account that the fresh weight/dry weight ratio for kiwifruit petioles was 8 ± 0.02, these data could be represented as endogenous concentrations of free NAA (Table 2), being almost equal to the initial exogenously applied NAA concentration for 12 h.

Table 2 also shows the relationships between the free NAA and free BA concentrations in kiwifruit explants during the culture period of callus initiation, the cytokinin concentration being established in a previous work (Centeno et al. 1998). The BA/NAA ratio was favorable for the cytokinin in kiwifruit tissues, as well as in the medium at the initial time, but changed during CIM culture. The ratio increased in petiolar explants for 12 h to reach a maximum value at 24 h and then decreased so that when the callus was developed after 30 days of culture (Centeno et al. 1996), the BA/NAA rate was now favorable for the auxin and 100 fold lower than that found at 24 h.

Discussion

Culture conditions for callus formation from kiwifruit petioles were previously established in our laboratory (González et al. 1995). Longitudinal incisions made in both edges of explants were a required inductive stimulus for cell division, as well as the presence of auxin and cytokinin in the medium. Cellular division surrounding the wounded tissues took place either in apical or basal petiolar ends, although the proliferation led to callus development in the basal extreme alone. This callogenic response has been shown in previous reports (Centeno et al. 1996, 1998): the beginning of cell division occurred within the first 96 h of CIM culture and differences among the two petiolar edges were visible for 10 days, when the basal callus was already in a developing state. We also studied the uptake, metabolism, and levels of BA in kiwifruit explants during the earlier period of culture (Centeno et al. 1998). In this study, similar analyses were carried out for applied auxin.

The higher NAA uptake by apical and basal ends in relation to the middle part of petioles was probably associated with the deep wound made in both edges (Fig. 2). NAA enters easily through the damaged tissues because of the absence of physical barriers such as cuticle and hairs in the cut areas, which also exposes a greater surface area for direct contact with the medium and therefore for NAA diffusion.

Another result related to wounds may be the initial reduction of IAA content observed in petioles (Fig. 1). In agreement with our data, Thornburg and Li (1991) found a twofold decline of IAA levels in tobacco leaves 6 h after the wound compared with unwounded tissues. Because the IAA decrease lasted longer in the damaged edges of kiwifruit explants (12 h) than in the middle part (6 h), it is possible that IAA was released through wounded areas. On the other hand, it is known that wounding can activate IAA catabolism (Normanly 1997).

Further modifications of IAA levels observed in kiwifruit petioles could be explained by the presence of applied PGRs. The manipulation of cytokinin levels in plants by exogenous application results in more auxin (Coenen and Lomax 1997). Also, it has been proven that NAA application greatly alters the metabolism and levels of IAA in several plant culture systems (Liu et al. 1997, Oetiker and Aeschbacher 1997). Furthermore, the synthetic auxin may disturb the endogenous IAA amount by altering its transport because applied NAA is able to interfere with the basipolar transport of IAA (Lomax et al. 1995), even when cultured tissues are entirely in direct contact with the medium (Smulders et al. 1988).

Kiwifruit explants formed several NAA conjugates from the NAA taken up (Fig. 3), which confirms again conjugation as the main metabolic path for regulation of levels of exogenously provided auxins to plant tissues

(Kleczkowski and Schell 1995). NAA conjugation was fast in apical and basal edges of petioles; after only 24 h of culture, more than 80% of NAA was present as ester conjugates but mostly as the amide conjugate NAAsp. NAA, as with IAA, has been shown to induce its own conjugation by increasing the capacity for formation of NAAsp. Moreover, the enzyme (L)-aspartic acid *N*-acylase is specific for compounds with auxin activity and depends on the substrate concentration (Smulders et al. 1990, Venis 1972). This may explain the slower NAAsp formation in the middle part of petiolar explants; NAA conjugation with (L)-aspartic acid was actually triggered only when tissues reached levels of free NAA as high as 1.5 nmol g^{-1} dry weight, which were achieved from 6 to 12 h in petiolar ends and from 48 h in the middle part (Fig. 4). However, the ester conjugation seemed to be independent of the NAA content because it occurred in a similar magnitude along the whole length of explants for 6 h (Fig. 3) despite differences in NAA uptake among the edges and middle parts of explants.

NAA conjugation seemed to play a regulatory role in maintaining a particular level of free hormone in kiwi tissues because free NAA concentration was almost unchanged from 12 to 96 h (Table 2) and was the same even after 30 days of CIM culture (0.29 ± 0.01 μM, Centeno et al. 1996). Furthermore, NAA conjugation allowed the tissue to establish an equilibrium of NAA concentration inside and outside the tissues (0.27 μM). The role of conjugation in auxin homeostasis has been shown for IAA using mutants that accumulate IAA precursors and auxin-overproducing transgenic plants that generally accumulate conjugated IAA to a much greater degree than free IAA (Bartel 1997, Hobbie and Estelle 1994). In relation to the synthetic auxin, Smulders et al. (1990) probed the breakdown of NAA metabolites, including NAAsp, and their interconversion in cultures of tobacco mesophyll and proposed these processes as a mechanism to maintain the level of free NAA.

The latter results allowed us to correlate the maintenance of a free NAA content in kiwifruit tissues with both the initiation and the further support of cellular proliferation. As was observed in kiwifruit explants, NAA levels measured in carrot hypocotyls during callus formation were similar after 1 and 4 weeks of culture (Ribnicky et al. 1996). Furthermore, both the presence of a synthetic auxin in the media (NAA or 2,4-D) and the maintenance of a level of the applied compound in the tissues were required to initiate and to maintain callus growth.

The behavior of IAA was different from NAA because its content fluctuated in kiwi petioles throughout CIM culture, reaching levels 10-fold greater than NAA after 30 days when basal callus had formed (Centeno et al., 1996). So, natural auxin seems to participate at least, even to a larger extent than NAA, in the growth of the established calli.

Contrasting with the role proposed for NAA and IAA, in a previous report (Centeno et al. 1998) we established that BA and natural cytokinins are more involved in the onset than in the maintenance of disorganized growth of kiwifruit callus because they reached their highest tissue levels at 24 and 48 h of CIM culture, coinciding with first cell divisions, and decreased afterwards. These observations are also supported by the BA/NAA ratio, its maximum value being measured at 24 h (Table 2). In agreement with us, Walden et al. (1993) found that untransformed protoplasts of tobacco require auxin and cytokinin in the media for callus formation, whereas protoplasts overexpressing *rol B* and *rol C* genes form callus in absence of applied auxin and cytokinin, respectively. Nevertheless, the long-term callus growth showed an absolute requirement for auxin in the three callus lines, with the exogenous cytokinin not being so essential. Therefore, although auxin and cytokinin regulate cell division synergistically in callus cells and protoplasts (Coenen and Lomax 1997), the significance of each kind of PGR could change during the growth response.

Cellular division was initiated in apical cells of kiwifruit petioles in the same way as in basal tissues because wounds were sealed on both petiolar extremes; however, apical cells were unable to form callus. Despite the fact that auxin gradients or asymmetric distribution is often invoked to explain differential responses by cells in proximity (Normanly 1997), we did not find differences either in NAA or in IAA levels among the apical and basal edges of explants during the period of callus initiation. The lack of apical response could be related to at least two possibilities: (1) developmental status of apical cells is more advanced than basal cells (Wernicke and Milkovits 1987) and (2) conditions in the apical extreme became unfavorable at later stages for maintenance of cellular proliferation. Because auxins are probably involved in this last function, a probe for hypothesis 2 was the IAA content found in the basal edge of kiwifruit petioles after 30 days of culture on CIM being double that found in the apical edge (Centeno et al. 1996).

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