1-Naphthyl Acetate-Dependent Medium Acidification by Zea mays L. Coleoptile Segments

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ABSTRACT

Zea mays L. cv INRA 5a coleoptile segments acidify the incubation medium on the addition of 1-naphthyl acetate (1-NA). The buffering capacity of the bathing solution increases during 1-NA stimulated medium acidification. The solution bathing the 1-NA treated coleoptile segment was analyzed by high performance liquid chromatography. A considerable amount of acetic acid was detected in the bathing solution used to measure 1-NA-dependent medium acidification. For the first time, the data demonstrate directly the release of acetic acid from 1-NA. The extent of medium acidification was proportional to 1-NA concentration. Simultaneous measurement of medium acidification and acetate content upon addition of 1-NA showed that both processes were temporally correlated. The stoichiometry of proton equivalents to acetate ion was 0.986. Addition of 50 micromolar N,N'-dicyclohexylcarbodiimide had little effect on 1-NA-dependent medium acidification. The results indicate that 1-NA is hydrolyzed in the extracellular space of coleoptile cells.

The nonauxin compound carboxyester 1-NA² has been shown to stimulate rapid proton efflux with no significant lag phase when applied to sections of lentil roots (26), oat and corn coleoptiles (14, 26), and isolated mesophyll cells of Asparagus sprengeri (9). 1-NA is a common substrate for esterases (12, 13). 1-NA rapidly enters the cells and is hydrolyzed by esterases releasing acetic acid and 1-naphthol (9, 14, 26). Esterase enzymes have been located in several cell organelles (13). Assays of esterase activity in cell wall and cytoplasmic fractions of corn coleoptiles have indicated that by far the largest part (about 90%) of the esterase activity was located in the cytoplasmic fraction (26). An acidification of cell cytoplasm treated with 1-NA has been either directly demonstrated (2, 4, 14, 24, 26) or deduced by the inhibition of dark CO₂ fixation into malate (10).

Three working hypotheses have been put forward to explain 1-NA-stimulated medium acidification:
(a) Vesper and Evans (26) suggested that 1-NA enters the cells rapidly and is hydrolyzed by esterases, releasing acetic acid. The latter serves as an endogenous source of protons. The protons released leave the cell, leading to acidification of the medium and enhanced elongation. 1-NA medium acidification is not primarily caused by the release of acetic acid since the buffering capacity of media from 1-NA treated segments was only slightly greater than media from IAA-treated sections.
(b) Hager and Moser (14) suggested that 1-NA dependent medium acidification is mediated by the continuous formation of acetic acid which lowers cytoplasmic pH (2, 5, 10, 14). This decrease in pH activates proton pumping at the plasmalemma, possibly shifting the cytoplasmic pH closer to the pH optimum of the pump. Plasmalemma ATPase activity showed a pH optimum close to 6.5 (1–3, 8). Since cytoplasmic pH of plant cells tends to be in the 7.0 to 7.6 range (16, 20 and references therein), this means that a decrease of cytoplasmic pH may be expected to result in a significant increase in proton pump activity. 1-NA medium acidification under anoxia or in the presence of ATPase inhibitors, such as diethylstilbestrol or DCCD, is not caused by active proton extrusion but is a result of the activity of esterases set free from broken cells.
(c) Duhaime and Bown (9) have suggested that stimulation of proton efflux results when 1-NA enters the cell and generates acetic acid on being hydrolyzed by esterase enzymes. Equilibration of the electroneutral acetic acid across the plasma membrane and also with internal and external acetate may result in acidification of the medium. This mechanism of acidification is not dependent on ATP. However, a contribution to acidification can be made when the ATPase driving proton efflux is activated in response to increased cytoplasmic proton levels.

So far, corn coleoptile is the only plant tissue in which 1-NA-dependent medium acidification has been examined in any detail. It is evident from these few studies that a systematic study of the mechanism of 1-NA acidification in corn coleoptiles would be valuable. In the present study we focused on the mechanism of 1-NA-dependent medium acidification and report on the release of acetic acid into the bathing medium.

MATERIALS AND METHODS

Plant Material

Zea mays L. cv INRA 5a caryopses were obtained from Lyra S.A. (Madrid, Spain). The kernels were surface-sterilized for 5 min in Clorox (5.25% [v/v] of chlorine) diluted 1:1 with distilled water. This treatment was followed by a 10 min rinse with running tap water. Then the seeds were soaked in 1 mM CaCl₂ for 2 h and set out on filter paper moistened with 1 mM CaCl₂ to germinate for 4 d in darkness at 30°C. Further
treatments were carried out under ambient laboratory light and temperature conditions.

Coleoptiles between 30 and 40 mm were harvested and kept in distilled water. To facilitate proton efflux from coleoptile segments, the cuticle was partially removed by rubbing the segments with an abrasive. The coleoptiles were abraded with a paste of 1 g of aluminium oxide (type T) per 1 mL of distilled water. Emery paste adhering to the coleoptiles was washed off and the coleoptiles were kept in distilled water. When all the coleoptiles had been abraded, they were rinsed with water. Corn coleoptiles were then defoliated and 10 mm sections cut beginning 3 mm below the apical end. Abraded segments were incubated at 4°C for 60 min with 1 mM Hapes and 1 mM CaCl$_2$ (pH 6.0), 10 sections per 200 mL, gently stirred, and bubbled with air using a small aquarium pump.

**Measurement of Medium Acidification**

Ten segments of corn coleoptiles were removed from the pre-incubation medium and placed in a 20 mL vessel containing 10 mL of 1 mM Hapes and 1 mM CaCl$_2$ (pH 6.0). The 10 mL volume was maintained at 30°C in an open water-jacket glass vessel. The contents of the vessel were stirred with a small stir bar. The reaction mixture was aerated by means of an aquarium pump. An Ingold combination electrode (model U402-M6-S7/100) connected to a Crison 2002 pH-meter (Crison Instruments, Alella, Spain) was used to measure medium pH. The output from the pH meter was attenuated with a back voltage to allow adjustment of the recorder to a full scale displacement over a range of 6.1 to 4.5. The coleoptile segments were incubated for 20 min, whereafter 1-NA or IAA was added to the medium.

**Titration Curves**

Inasmuch as the bathing solution was buffered, proton fluxes would be underestimated if calculated directly from pH measurement. We, therefore, titrated the experimental solutions with HCl or NaOH to evaluate the buffering capacity. To calculate the relationship between the buffering capacity and the value of external medium pH, buffering capacity measurements were fitted to the appropriate equation with a nonlinear least-squares analysis computer program. Analysis produces the polynomial coefficients, as well as the correlation coefficient and the standard error of estimate, which indicate goodness-of-fit.

**HPLC Analysis of Acetic Acid**

Fifty microliters of bathing solution were loaded into a Dionex anion exchange column and eluted with a 1.8 mM carbonate and 1.7 mM bicarbonate buffer (pH 10). The concentration of the dissociated form of acetic acid was calculated using a dissociation constant of 1.84 × 10$^{-3}$ M, the value given for a temperature of 25°C (18).

**Statistical Analysis**

Data were analyzed by linear regression analysis, Student’s $t$ test, and correlation analysis, as appropriate (21). In the following text only statistically reliable (P ≤ 0.05) inferences are discussed and “significant(l)" means P ≤ 0.05, even when actual comparisons between data are not shown.

**Enzyme Assay**

Esterase (carboxyl esterase EC 3.1.1.1) was assayed by following the appearance of acetic acid by HPLC. The assay was initiated by the addition of enzyme (0.3–0.01 units) to 10 mL assay mixture containing 1 mM Hapes and 1 mM CaCl$_2$ (pH 6.0) and 0.1 to 0.5 mM 1-NA at 30°C.

**Solutions**

1-NA were dissolved in ethanol and added to the medium to a final concentration of 0.1 to 0.5 mM; the concentration of ethanol in the final solution being 0.02 to 0.1% (v/v). The ethanol concentrations had no significant influence on the 1-NA-stimulated medium acidification within the time course of the experiments. In all solutions, pH was adjusted with HCl or NaOH to a value that would prevent any pH value changes in the incubation media they were added to.

**Presentation of Data**

All experiments were performed at least three times, with similar results. Our pH tracings, obtained with a chart recorder and redrawn for appropriate magnification, represent typical rather than average data, since pH is based on a logarithmic scale making average data difficult to interpret.

**Chemicals**

Hapes, porcine liver esterase, 1-NA, 1-naphthol, and acetic acid were purchased from Sigma Chemical Co., and CaCl$_2$, DCCD, ethanol, HCl, and NaOH from Merck.

**RESULTS**

**1-NA Dependent Medium Acidification**

When abraded corn coleoptile segments were suspended in 1 mM Hapes and 1 mM CaCl$_2$ (pH 6.0), proton efflux was not observed until 90 min (Fig. 1A). On adding 0.5 mM 1-NA, acidification of the bathing medium rapidly ensues (Fig. 1E). After approximately 120 min, during which the pH dropped from 5.985 to 4.991, acidification ceased. The lag phase between 1-NA addition and detectable pH response varied from 1 to 2 min for different experiments. Various control experiments were performed to eliminate facile explanations of the results described (Fig. 1E). When 0.5 mM 1-NA was added to the bathing solution in the absence of coleoptile segments, acidification was not detected (Fig. 1B). In the presence of 20 μL of ethanol or 0.5 mM 1-naphthol, neither external pH changes nor acidification was observed (Fig. 1, traces C and D).

Figure 2 shows changes in the pH of solutions bathing coleoptile tissue at different concentrations of 1-NA. Coleoptile segments acidified the medium at all concentrations used. Figure 3 shows a comparison of 1-NA-induced medium acidification in intact versus abraded coleoptile segments. Using abraded segments, 1-NA-induced medium acidification is
Figure 1. 1-NA-dependent medium acidification in abraded coleoptile segments. Changes in pH were measured in a medium containing 1 mM Hepes, 1 mM CaCl₂, and either 0 coleoptiles (trace B) or 10 coleoptile segments/10 mL (traces A, C, D, and E). At zero times, the samples received 0.5 mM 1-NA (traces B and E), 20 μL ethanol (trace C), or 0.5 mM 1-naphthol (trace D). The number next to the lines shows the initial pH value. The initial pH of each trace is offset from the others so that the different traces can be distinguished.

Figure 2. Effect of varying the 1-NA concentration on the acidification of the bathing solution. Medium acidification was measured under standard conditions, except for the 1-NA concentration, which was varied. Each trace represents a separate experiment.

Figure 3. Influence of abrasion on 1-NA medium acidification. Segments were prepared by abrasion or no treatment (intact). 1-NA (0.4 mM) was added at the arrow in each curve. The medium was both aerated and stirred. Curves are from representative experiments. Each experiment was repeated at least four times.

Medium Buffering Capacity

Time-dependent changes in bathing solution pH have been interpreted by Hager and Moser (14) to indicate proton fluxes from cells in coleoptile segments treated with 1-NA. Nevertheless, weak organic acids, mainly acetic acid generated from 1-NA hydrolysis, may be released from the tissue during the monitoring period.

Bathing solution buffering capacities were estimated to determine if 1-NA-dependent medium acidification was due to proton extrusion rather than leakage of organic acids from coleoptile cells. The buffering capacity of the external medium (1 mM Hepes, 1 mM CaCl₂, 40 μM IAA, and 10 coleoptile segments) is dependent upon pH values (Fig. 4); only negligible changes in the more acid pH range of bathing medium buffering capacity being induced by IAA-dependent proton extrusion. The buffering capacity of the bathing solutions measured at the end of the acidification period increased gradually as concentration of 1-NA increased (Fig. 4). Control experiments indicated that this increase in medium buffering capacity was not a result of the presence of 1-NA, because the addition of 0.5 mM 1-NA to a reaction mixture without coleoptile segments had no effect on the buffering power. Additional experiments were conducted to examine the effect of 1-naphthol. Experiments with 0.5 mM 1-naphthol showed that buffering capacity was not modified (data not shown). These results indicate the release of compounds with buffering capacity in the pH range of 4.8 to 6.0 during 1-NA mediated medium acidification.
Figure 4. Medium buffering capacity (neq H⁺/pH x 10 mL reaction mixture) as a function of pH values. The reaction mixture contained 10 mL 1 mM HEPES, 1 mM CaCl₂, and 10 abraded segments, and when added 40 μM IAA, 0.2, 0.4, or 0.5 mM 1-NA. Buffering capacity was determined at the beginning (IAA-dependent proton extrusion or without addition) or at the end of the acidification period (1-NA treated samples). The curves were polynomial fittings (r ≥ 0.98). The inset shows the neq H⁺ for titration from 6.0 to 5.0 against the concentration of 1-NA. The results shown are from representative experiments.

HPLC Analysis of Total Acetic Acid Released

To demonstrate directly the release of the proposed organic acids, the bathing solution was separated by HPLC after medium acidification was complete. When IAA-dependent medium acidification was analyzed, it was found that there was only one single peak at the point where a calcium chloride standard was known to elute (Fig. 5A). Incubation of coleoptile segments with 1-NA resulted in one additional peak on the HPLC elution profile (Fig. 5, traces B, D, and E). Its height increased when the 1-NA concentration was increased from 0.1 to 0.5 mM. Omission of 1-NA from the acidification assay medium resulted in an elution profile without the additional peak. The retention time (1.93–1.97 min) of this additional peak was identical to that of the reference acetic acid. Confirmation of peak identity was made by prior mixing of an aliquot of the reaction mixture and a reference standard and by analyzing the mixture by HPLC. The peak to be identified and that of the reference standard showed a complete overlap on the elution profile (Fig. 5C). In the concentration range we used, detector responses were directly proportional to the acetic acid concentration. The two peaks (retention times 5.56–5.65 and 7.78–7.82 min) appearing on the HPLC chromatogram of a bathing solution containing 0.5 mM 1-NA were not identified (occurrence of the unidentified compounds being variable). Other organic acids were not found in the bathing solution. Thus, the acetic acid expected to be released into the bathing solution could be detected by HPLC.

The total amount of acetic acid released into the bathing solution measured at the end of the acidification period was significantly correlated with the concentration of 1-NA added (regression line: acetic acid = −49.57 + 0.42 × 1-NA, r = 0.98). A similar relationship was obtained from intact coleoptiles (regression line: acetic acid = −51.66 + 0.446 × 1-NA, r = 0.98). The slopes of these two lines were not significantly different from each other, as is shown by a significance test between two linear regression lines. The stoichiometry of acetic acid/1-NA was 0.42:1.00.

1-NA Medium Acidification: Dose-Response

Medium acidification is directly proportional to the concentration of 1-NA (regression line: medium acidification (neq H⁺) = −12.87 + 0.24 × 1-NA (nmol), r = 0.99). The stoichiometry eqH⁺/mol 1-NA was 0.24:1.00. It has previously been shown (using a different acidification assay) that medium acidification by coleoptile segments was linear for 1-NA lower than 0.8 mM (26). Differences in the assays and the presentation of results make it difficult to compare the results presented here with the latter study.

Figure 5. HPLC chromatograms of bathing solutions. Tracing A: 40 μM IAA (12 h); tracing B: 0.2 mM 1-NA (2 h); tracing C: 0.2 mM 1-NA (2 h) + 100 μM acetic acid; tracing D: 0.3 mM 1-NA (2 h); tracing E: 0.5 mM 1-NA (2 h). The elution peaks and corresponding retention time in min for identical compounds are for chloride (2.65) and acetate (1.96). Other compounds separated from the bathing solution shown in tracing E have not been identified.
Acetic Acid and Medium Acidification

The time course of acetic acid appearance in the bathing solution and medium acidification was investigated using 0.4 mM 1-NA (Fig. 6). Acetic acid appearance from abraded coleoptiles showed two phases: it was nearly linear in the first 60 min and was then followed by a phase of declining rate of appearance. The segments maintain a fairly constant acidification rate over a period of 60 min; the rate decreasing progressively thereafter. The concentration of acetate correlates with medium acidification (r = 0.97 and regression coefficient (neq H+/nmol acetate) = 0.94). It is also interesting to note that the time course of acetic acid appearance and medium acidification show a similar pattern both for abraded and for intact segments (data not shown).

To determine whether changes in the concentration of the dissociated form of acetic acid were responsible for pH decrease, medium acidification and total acetate content were measured at the end of the acidification period for several concentrations of 1-NA. Figure 7 shows that correlation between medium acidification and acetate concentration was 0.97 and the regression line slope 0.966.

Addition of esterase to the stirred and aerated assay medium resulted in a rapid drop in pH. Concomitant with this medium acidification was the increase of buffering capacity and the appearance of acetic acid (data not shown). The H+/acetate ratio 1.02 is not significantly different to the ratio measured in the presence of coleoptile segments (H+/acetate = 0.966).

1-NA Medium Acidification and Plasmalemma ATPase

DCCD in the millimolar range is a powerful inhibitor of plasma membrane ATPase (17, 22). DCCD at 30 μM completely inhibits the auxin dependent proton extrusion within 10 min and continues to block any pH decrease for the next 2 h (11). Figure 8 shows pH traces depicting the effect of 50 μM DCCD on 1-NA-dependent medium acidification. Coleoptiles were treated for 40 min with 0.4 mM 1-NA and tested at the end of this period for their ability to acidify the medium when exposed to 50 μM DCCD. 1-NA-dependent medium acidification was, on average, 10% lower in the presence of DCCD. A decline in the acidification rate on application of DCCD can be equated with a decline in the rate of medium acidification, since the ATPase inhibitor does not increase medium buffering capacity. In another experiment, intact coleoptile segments were treated with 0.4 mM 1-NA both in the presence and absence of DCCD; 50 μM DCCD caused a medium acidification inhibition of almost 10% (data not shown).

Esterase Activity in the Bathing Solution

When coleoptile segments exhibiting an active acidification rate in the presence of DCCD at the external pH of 5.60 were transferred to a vessel with the same reaction mixture but omitting 1-NA, the remaining coleoptile-free medium showed a slow alkalinization (Fig. 8C). When, after 30 min, coleoptile segments were readded the external pH was not affected by this addition and, after a few seconds a high rate of acidification was observed a new. Thus, esterase activity in the presence of DCCD was not detected in the bathing solution.
were observed that reported increased The medium in study, this too rapid equilibration of carbonate and CO₂, system open of organic acids and/or other buffering substances, (b) bicarbonate uptake, (c) formation of carbonic acid from respiratory CO₂, and (d) proton efflux.

Experiments in which medium was shaken gently in an open system demonstrated that CO₂ escapes into the atmosphere too rapidly to allow significant acidification (19). In this study, the medium was stirred and bubbled to ensure rapid equilibration with the air being used.

It is important to determine bathing solution buffer capacity in order to be able to correlate external pH modification to the intensity of exchange of protons or proton-equivalents. Buffering capacity of the bathing solution from 1-NA treated coleoptiles increased during medium acidification (Fig. 4). The failure of Vesper and Evans (26) to find an increase in medium buffering capacity may be due to the low concentration of 1-NA used. Results from our experiments are in agreement with the data of Duhaime and Bown (9), who reported that a substantial buffering power in the range of pH 4 to 5 was observed when isolated Asparagus sprengeri cells were incubated with 1-NA. The greater buffering capacity observed in the presence of coleoptiles treated with 1-NA is consistent with the view that some compound/compounds must be released into the external medium.

Various possibilities concerning 1-NA-dependent increase in buffering capacity were investigated. Bathing solution assays in vitro demonstrated that different concentrations of 1-NA and 1-naphthol did not influence buffering capacity. HPLC analysis of the bathing solution showed that a significant amount of an anion compound was present (Fig. 5). This compound was identified as acetate since it coeluted with the standard acetic acid. It should be pointed out that the acetic acid is not considered to be present in the bathing solutions (9, 26). Therefore, to the authors' knowledge, this is the first report of such an occurrence.

It is unlikely that 1-NA-induced medium acidification reflects death of cells and loss of membrane integrity. 1-NA-induced medium acidification stops or slows spontaneously after about 50 to 100 min (Fig. 2). The quantity of free amino acids released into the medium has been used as a measure of membrane integrity (25). To determine whether 1-NA medium acidification was partially caused by a loss of membrane permeability, amino acids were measured by reverse-phase HPLC. The results of HPLC analysis showed that 1-NA had no effect on the release of amino acids from the tissue up to 0.5 mm concentration (data not shown).

Evidence is accumulating to the effect that the normal process of proton extrusion is driven by an ATPase located at the plasmalemma (6, 23). The stimulation of 1-NA-dependent medium acidification has been interpreted as a result of decreased cytoplasmic pH which, in turn, stimulates plasma membrane-bound ATPase (14). If this interpretation is correct, 1-NA-dependent medium acidification must be sensitive to plasma membrane ATPase inhibitors. However, when corn coleoptiles were incubated with 1-NA in this study, DCCD addition did not prevent stimulation of medium acidification (Fig. 8), indicating that this type of medium acidification is not ATPase dependent.

The detection of acetic acid, as presented in this paper, has important implications for the mechanism of 1-NA-dependent medium acidification. The presence of acetic acid implies that this medium acidification may be due to acetic acid dissociation. Three experimental observations support the hypothesis that 1-NA-dependent medium acidification is due to the production of acetic acid. First, acetic acid is detected in the solution bathing coleoptile segments (Fig. 5). The second experimental evidence concerns the time dependence of acetic acid appearance and medium acidification (Fig. 6); both processes having been properly correlated in time. Finally, the H⁺/acetate stoichiometry was found to be 0.966, whereas the corresponding ratio with porcine esterase was 1.02, indicating a 1:1 stoichiometry between medium acidification and acetate content in both cases.

The cuticle offers substantial resistance to the entry and exit of protons (7) and, thus, one would expect that it also restricts the diffusion of 1-NA and acetic acid. As a result, when nonabraded segments were used, 1-NA could be hydrolyzed by those cells, or cell walls, at or near the cut surface and, therefore, one should expect it to have a decreased rate of medium acidification (Fig. 3). It is also possible that aluminum oxide abrasion may induce a new response to 1-NA (15). However, several experimental results contradict this
possibility. First, HPLC analysis of the solution bathing intact coleoptiles during 1-NA medium acidification showed that a significant amount of acetic acid was present when measured at the end of the acidification period. Second, the stoichiometry of medium acidification to acetate ion was not affected by abrasion. Finally, the buffering powers of the medium bathing coleoptiles treated with 1-NA, both intact and abraded, were similar (data not shown). Although abrasion causes some damage to the tissue, we think it unlikely that it generates a 1-NA response anew.

Agreeing with Vesper and Evans (26), we found that esterase activity was not released into the medium containing live coleoptile segments. Our results show that first, when coleoptile segments were removed from the medium after 120 min, subsequent addition of 1-NA did not result in acidification (data not shown); and second, there was a lack of medium acidification when coleoptiles were removed (Fig. 8).

The experimental results reported in this paper question the validity of earlier working hypotheses about how 1-NA induces extracellular acidification of plant cells.

Based on the results reported, we propose a tentative model of the 1-NA-dependent medium acidification mechanism. 1-NA is hydrolyzed to 1-naphthol and acetic acid by extracellular esterases. The acetic acid dissociates releasing protons, increasing the buffering capacity and lowering the external pH. Although 1-NA also may be hydrolyzed by intracellular esterases (14, 26), acetic acid release from cytoplasm to cell wall is bound to be very low because of the low concentration of the undissociated form at the cytoplasmic pH value (16, 20). It is accepted that acetic acid permeates only in its protonated form. The tentative model is consistent with these experimental observations and may also explain the lack of sensitivity of 1-NA medium acidification to cycloheximide and KCN (26), and to oligomycin and anoxia (14). It needs to be said that more experimental work must be done to prove or disprove this working hypothesis.

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LITERATURE CITED