Citrus limon seedlings without functional chloroplasts are unable to induce phenylalanine ammonia-lyase in response to inoculation with *Alternaria alternata*

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Summary

A light-dependent bleaching of plastids was induced in lemon seedlings through cultivation in the presence of the herbicide Norfluorazon (NFZ). Also, etiolated seedlings were obtained by cultivation under continuous dark conditions. When both types of seedlings were challenged with *Alternaria alternata* conidia, they were unable to induce phenylalanine ammonia-lyase, PAL (E.C. 4.3.1.5.). Cell ultrastructure of control seedlings showed the presence of chloroplasts, while NFZ-treated lemon seedlings showed altered chloroplasts and in etiolated seedlings only proplastids were observed. In addition, CO_2 assimilation was not detected in NFZ-treated and etiolated seedlings, as compared to controls.

Key words: Rutaceae, Citrus limon, Alternaria alternata, chloroplasts, PAL, Norfluorazon.

Abbreviations: PAL = Phenylalanine ammonia-lyase; NFZ = Norfluorazon; HR = hypersensitive response; PMSF = phenylmethylsulfonyl fluoride.

Introduction

Phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5.) catalyzes the first reaction of the phenylpropanoid pathway; that is the conversion of L-Phenylalanine into trans-cinnamic acid through a non-oxidative deamination. It has been shown that PAL is a key enzyme of the phenylpropanoid metabolism, and its *de novo* synthesis can be induced in response to different elicitors (Jones, 1984; Hahlbrock and Scheel, 1989). Therefore, any change in PAL activity or in the amount of PAL protein would affect this pathway, whose precursors are formed within chloroplasts (Jensen, 1980).

PAL can also be induced by continuous white or UV light (Jones, 1984; Lois et al., 1989; Schulze-Lefert et al., 1989) although it has not been possible to find an involvement of light receptors in PAL gene activation in several plant species (Tobin and Silverthorne, 1985). Light receptors have been described for both the small subunit of ribulose-1,5-biphosphate carboxylase and the light harvesting chlorophyll *a/b* binding protein of photosystem II. The expression of these proteins, coded by nuclear genes, is regulated by light. They are synthesized in the cytoplasm and transported to the chloroplast to exert their function (Simpson et al., 1986; Rapp and Mullet, 1991). Light receptors involved in these processes, and the mechanisms describing how these signals can activate nuclear genes, have been suggested (Simpson et al., 1986; Oelmüller et al., 1986).

Citrus limon seedlings cultivated under a 16/8 h photoperiod develop a hypersensitive response (HR) in response to inoculation with *Alternaria alternata* (Quaas et al., 1993) or treatment with plant-derived or fungal elicitors (Roco et al., 1993; Pérez et al., 1993). We do not know if this HR, which includes activation of the phenylpropanoid pathway (measured as the induction of PAL) and biosynthesis of the phyto-

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alexins umbelliferone and scoparone (Pérez et al., 1994), could be developed in the absence of functional chloroplasts. PAL genes are encoded exclusively in the nuclear genome of *C. limon* and the presence of the PAL enzyme in chloroplasts isolated from this same system has been ruled out (Opitz et al., 1995). Furthermore, PAL is found only in the soluble fraction after subcellular fractionation, and its localization is not modified after fungal attack of lemon seedlings (Carvajal, 1989). Since PAL is a soluble enzyme and is relevant for the development of HR, it is important to determine if its induction requires the participation of chloroplasts.

Chloroplast functions include the biosynthesis of ABA, which is thought to be derived from carotenoids (Parry and Horgan, 1991). ABA has been involved in the wound-induced response of potato and tomato (Peña-Cortés et al., 1989). Also, ABA-deficient plants are unable to accumulate wound-induced proteins after treatment with sistemin (Peña-Cortés et al., 1996). Consequently, if ABA is participating in defense responses, functional chloroplasts would also be needed to allow the development of the HR.

The use of herbicides such as Norfluorazon (NFZ) has been effective for the control of weeds, and several commercial products containing this compound are available. It has been reported that NFZ produces an oxidation of chloroplasts in treated plants (Taylor, 1989) and dramatically reduces ABA levels in light-grown cotton (Suttle and Hultstrand, 1993). Hence, the use of herbicides that cause damage in these organelles, producing chemically bleached plants, could alter the defense response of plants against phytopathogens. A similar situation could happen in etiolated seedlings that contain etioplasts.

If functional chloroplasts are required for the induction of PAL in *Citrus limon*, chemically bleached (NFZ-treated) and etiolated seedlings should show an altered defense response to inoculation with *A. alternata*. In this work, we report evidence that supports this hypothesis.

Materials and Methods

Chemicals

All chemicals used were analytical grade. NFZ was a kind gift of Dr. Luis Herrera-Estrella (Centro de Investigación y Estudios Avanzados del IPN, Irapuato-Mexico).

Plant material

Citrus limon seeds were extracted from lemon fruits and surface sterilized with 10 % (v/v) commercial sodium hypochlorite and germinated at 25 °C under the following conditions:

a) Control 1: seeds were germinated and grown in darkness for four weeks on pleated filter paper and then cultivated for another week with a photoperiod of 16/8 h, as described earlier (Roco et al., 1993);

b) Etiolation: seeds were germinated and grown as in a) but in continuous darkness;

c) Control 2: seeds were germinated and grown under continuous white light (128 $W \cdot m^{-2}$), during five weeks, in glass flasks containing half-strength Murashige and Skoog salts (Murashige and Skoog, 1962) with 0.8 % (w/v) agar and 1 % (w/v) sucrose;

d) NFZ-treatment 1: seeds were germinated and grown as in c), but in the presence of 10 µmol L^{-1} Norfluorazon;

e) NFZ-treatment 2: seeds were germinated and grown as in d) during four weeks and using a 16/8 h photoperiod during the fifth week.

Inoculation of seedlings and measurement of PAL activity

Five-week-old seedlings from the different treatments were surface sterilized for one min with 70 % (v/v) ethanol, 15 min with 20 % (v/v) commercial sodium hypochlorite and washed several times with sterile water. Five seedlings were placed per Petri dish containing 10 mL of Sabouraud broth DIFCO and inoculated with 2×10^6 conidia of *A. alternata* prepared in 0.8 % (w/v) NaCl, as described (Roco et al., 1993). After 24 h incubation at 30 °C, seedlings were washed with water, and homogenates were obtained after chopping the seedlings (one g per batch) in 0.1 mol L^{-1} sodium borate pH 8.8, containing 1 mmol L^{-1} PMSF, and 10 mmol L^{-1} β-mercaptoethanol (freshly prepared), using 1 L kg^{-1} wet seedling mass. PAL was assayed spectrophotometrically (Zucker, 1965) in the supernatants obtained after centrifugation at 12,100 g_n for 15 min. Controls of PAL activity were run with seedlings mock-inoculated with the same volume of 0.8 % NaCl solution (w/v). As a positive control, a commercial enzyme (Sigma) was used daily to monitor changes during the assay. Negative controls were performed with boiled enzyme from the corresponding treatment. The direct effect of NFZ on PAL activity was tested using homogenates from control seedlings. The enzyme unit was defined as the amount of enzyme necessary to produce 1 picomole of cinnamic acid from L-Phe, per second (1 pkat). Proteins were measured by the Coomassie blue dye method (Bradford, 1976).

CO_2 assimilation

CO_2 fixation by seedlings from the different treatments was measured with an Infrared Gas Analyzer, IRGA (Ireland et al., 1984). Results are expressed as milligrams of CO_2 fixed per square-decimeter of leaves ($mg \cdot dm^{-2}$). The instrument was previously calibrated with a known CO_2 concentration (360 ± 5 ppm). Light irradiance used was of 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Electron microscopy

Leaves of seedlings from different treatments were fixed in 2 % glutaraldehyde in cacodylate buffer pH 7.4 during 24 h at 4 °C, and postfixed in 1 % OsO_4. The samples were embedded in Epon after dehydration in an acetone series. Thin sections were obtained and stained with lead citrate and uranyl acetate.

Results

PAL activity

Control lemon seedlings, cultivated either under a photoperiod (control 1) during the fifth week or under continuous white light (Fig. 1, A and B: control 2), were fully green and showed 5.2 and 8.4 pkat/mg proteins of basal PAL activity. Control 2 seedlings also increased PAL activity 24 h post-inoculation with conidia of *A. alternata* (Table 1, a and c), as previously described for seedlings cultured under photoperiod (Quaas et al., 1993). This increase in PAL activity was negligible in etiolated seedlings and was absent in lemon

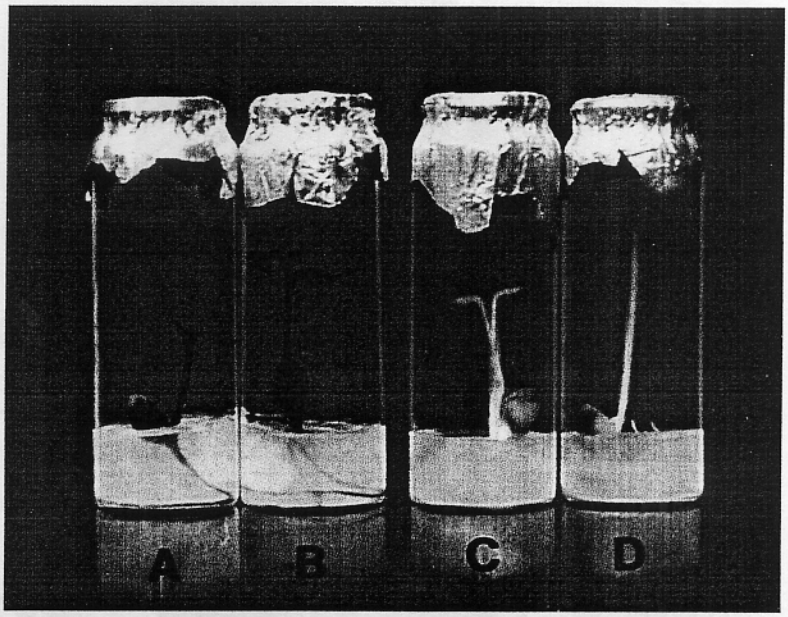

Fig. 1: Lemon seedlings obtained from seeds germinated and grown under continuous white light (128 $W \cdot m^{-2}$), during 5 weeks, in half-strength Murashige and Skoog salts (Murashigue and Skoog, 1962) with 0.8 % (w/v) agar, and 1 % (w/v) sucrose: A and B) in the absence, and C and D) in the presence of 10 $\mu mol\,L^{-1}$ NFZ.

Table 1: PAL induction in *C. limon* seedlings grown under different conditions in response to *Alternaria alternata* inoculation.

Seedling growth conditions	PAL activity (pkat/mg of proteins)		Ratio Basal/ Inoculated
	Basal[a]	Inoculated[b]	
a) Control 1 4 weeks darkness + 1 week photoperiod 16/8	5.2	41.1	7.9
b) Etiolation 5 weeks darkness	5.3	7.4	1.4
c) Control 2 5 weeks continuous white light	8.4	42.8	5.1
d) NFZ-treatment 1 5 weeks continuous white light in the presence of 10 $\mu mol \times L^{-1}$ NFZ	6.7	7.7	1.1
e) NFZ-treatment 2 4 weeks darkness + 1 week photoperiod 16/8 h permanently in the presence of 10 $\mu mol \times L^{-1}$ NFZ	7.8	26.3	3.4

PAL activity measured in crude extracts after 24 h incubation of seedlings with 0.8 % (w/v) NaCl (a) or with 2×10^6 *A. alternata* conidia (b). Results are the mean of three experiments run in triplicates whose standard deviations were less than 10 %.

seedlings bleached through NFZ-treatment 1 (Table 1, b and d). Seedlings in the dark were completely etiolated, while seedlings from NFZ-treatment 1 had some green spots in the cotyledons (Fig. 1, C and D).

To rule out that NFZ was not affecting PAL induction at a different level than the chloroplast, the herbicide was added: a) directly to the incubation medium for the measurement of PAL activity, and b) to the cultivation media of seedlings. In this case, seedlings were grown in darkness for four weeks and under photoperiod during the fifth week (NFZ-treatment 2). These seedlings were then inoculated with *A. alternata,* in order to test the effect of NFZ on their PAL activity. Results showed that: a) PAL activity was not affected by the presence of NFZ in the assay medium; and b) basal PAL activity was not affected by the presence of NFZ in the cultivation media of seedlings. Furthermore, these latter plantlets had the ability to increase PAL activity after fungal inoculation (Table 1, e). In this case, seedlings were as green as controls (Fig. 1, A and B), suggesting that they contained some functional chloroplasts necessary for the induction of the response. Nevertheless, their increase in PAL activity was lower than that observed in controls, probably due to the effect of NFZ on chloroplasts during the light period.

Electron microscopy

To correlate the increase in PAL activity with the morphology of chloroplasts, electron microscopy of leaves from seedlings with different treatments was performed.

Control 1 and control 2 seedlings contained chloroplasts with well-organized thylakoid membranes and grana (Fig. 2, A and B). They assimilated CO_2 at the rates of 6.0 ± 0.43 and 4.86 ± 0.33 $mg \cdot dm^{-2}$ of leaf, respectively.

Etiolated seedlings contained no chloroplasts but only some plastids that presented electron-dense droplets and no membrane organization (Fig. 2, C). CO_2 assimilation could not be detected in these seedlings.

NFZ-treated lemon seedlings cultivated under continuous white light showed two different types of plastids: i) those that contained electron-dense droplets, similar to those observed in etiolated seedlings (Fig. 3, A and C); and ii) those with several membranes, which probably correspond to thyla-

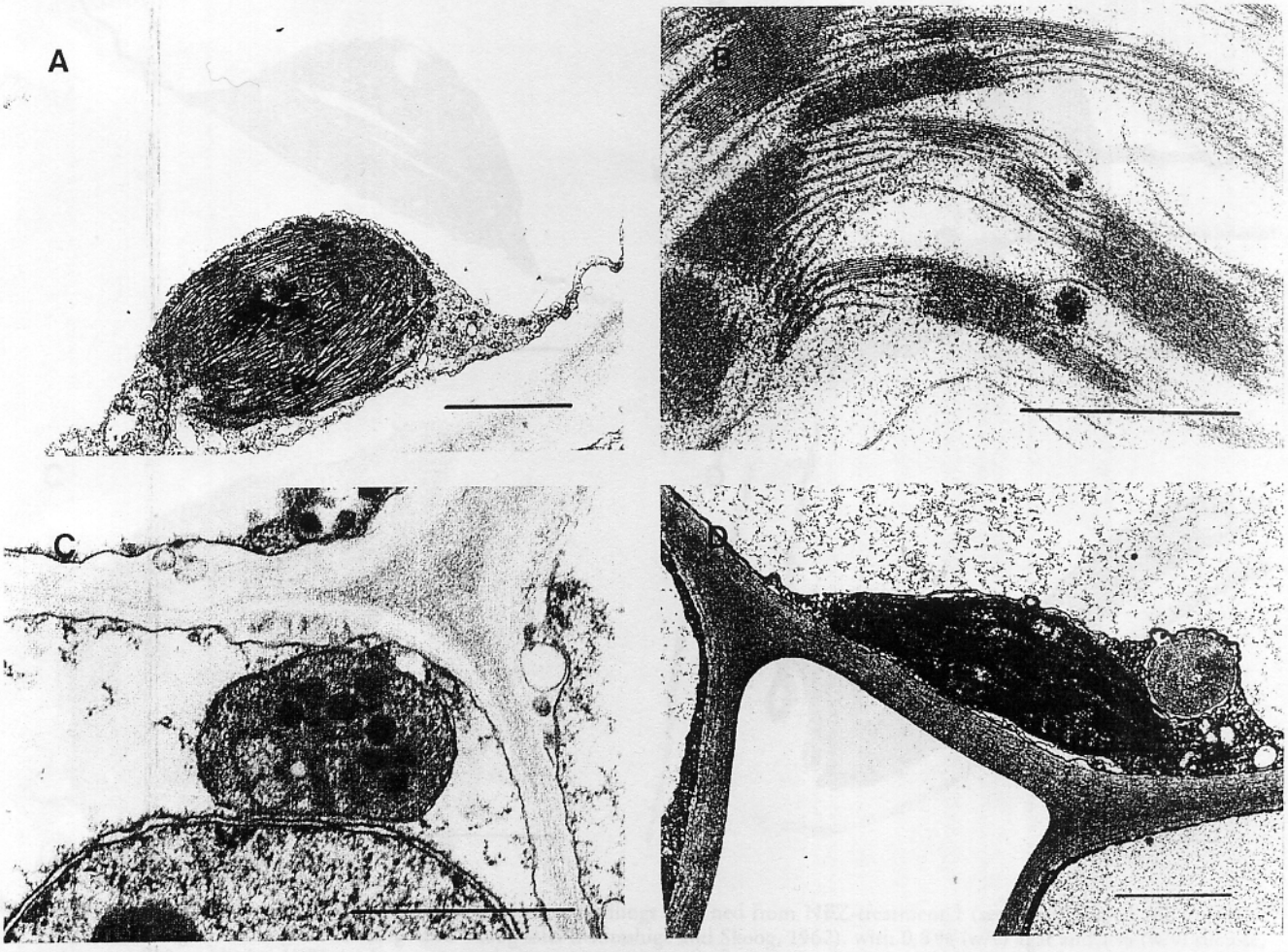

Fig. 2: Transmission electron microscopy of leaves from lemon seedlings obtained from seeds germinated and grown: A) Control 1 seedlings (darkness: 4 weeks and 16/8 h photoperiod for one week); B) Control 2 seedlings (continuous white light: 128 $W \cdot m^{-2}$, during 5 weeks); C) Etiolation; D) NFZ-treatment 2 (darkness: 4 weeks and 16/8 h photoperiod for one week, in the continuous presence of 10 $\mu mol \cdot L^{-1}$ NFZ). All bars represent 1 µm.

koids but are not organized into grana (Fig. 3, A, B and D). Also, in some plastids, the presence of starch-like material could be observed, as well as electron-light areas of different shapes (Fig. 3, B). Some unusually long chloroplasts could also be observed (Fig. 3, D), although CO_2 assimilation could not be detected.

Seedlings treated with NFZ, but cultivated during the fifth week under photoperiod, showed essentially normal chloroplasts with well-established membrane systems but also with some electron-dense droplets (Fig. 2, D).

Discussion

Control lemon seedlings cultivated under 16/8 h photoperiod (control 1) showed a basal PAL activity similar to that already described (Quaas et al., 1993). This basal activity was slightly higher in seedlings cultivated in half-Murashige and Skoog medium, suggesting that the salts contained in the media could be affecting the level of basal activity. As expected, both types of control seedlings that contained functional chloroplasts increased their PAL activity 24 h post-inoculation with conidia of *A. alternata,* increase that has been shown to occur through *de novo* synthesis of the enzyme (Quaas et al., 1993). The ultrastructure of plastids was that of normal chloroplasts and similar in both control seedlings, indicating that the culture media did not affect their morphology. Also, it correlated with the ability of the seedlings to increase PAL activity in response to fungal inoculation, which does not modify the soluble distribution of the enzyme (Carvajal, 1989) discarding that the observed increase in PAL activity could be due to the expression of a chloroplastic PAL. The small difference in CO_2 assimilation between controls could be attributed to the culture media and/or to the continuous illumination system in control 2 seedlings.

Etiolated lemon seedlings which presented basal levels of PAL activity did not contain chloroplasts. The few plastids observed had electron-dense droplets, similar to those found

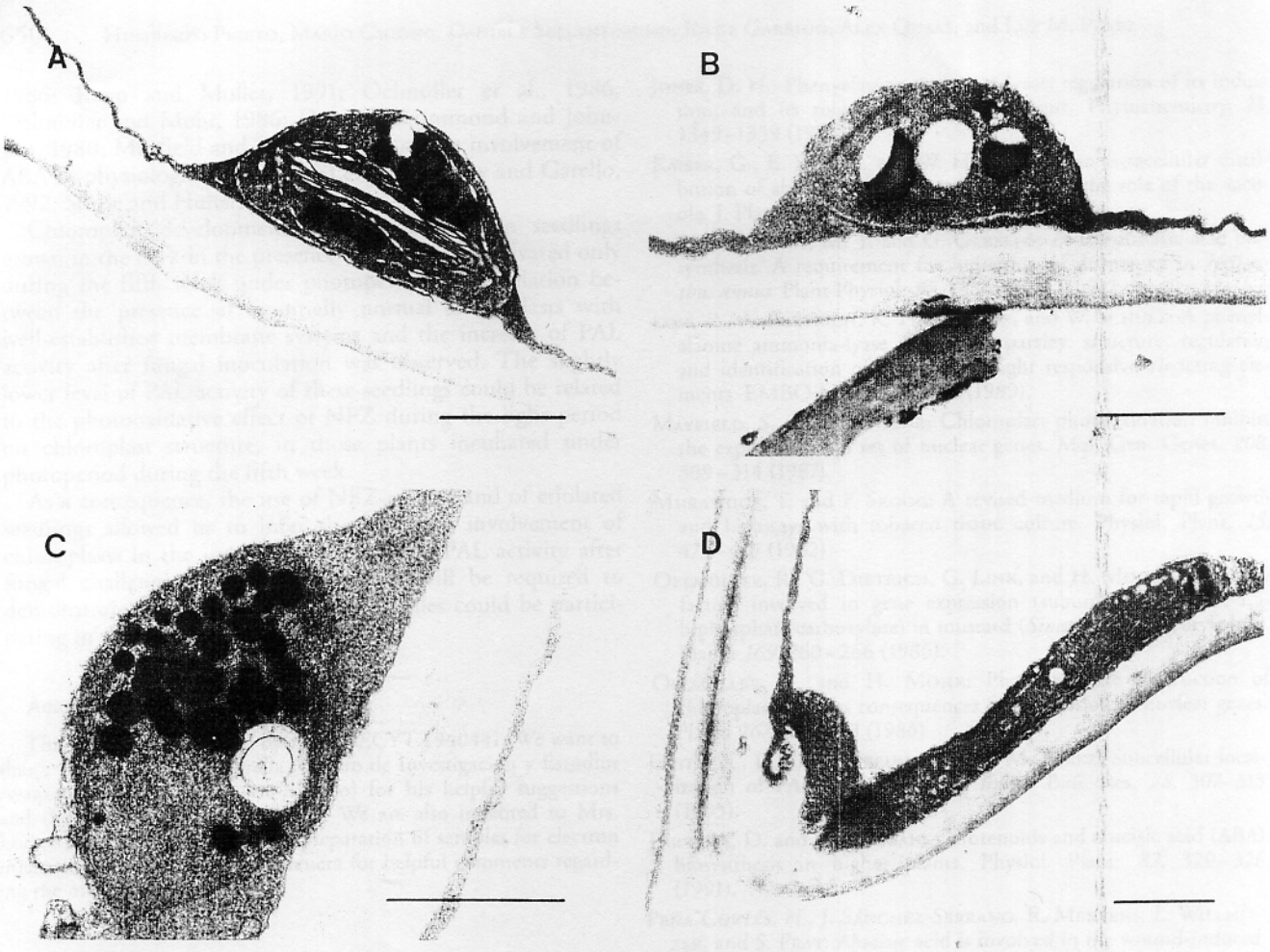

Fig. 3: Transmission electron microscopy of leaves from lemon seedlings obtained from NFZ-treatment 1 (seeds germinated and grown in glass flasks containing half-strength Murashige and Skoog salts (Murashige and Skoog, 1962), with 0.8% (w/v) agar and 1% (w/v) sucrose, in the presence of $10\,\mu mol \times L^{-1}$ NFZ, under continuous white light ($128\,W \cdot m^{-2}$) during 5 weeks): A, B, C and D. All bars represent 1 µm.

in chromoplasts from *C. sinensis* flavedo cells, previously described (Pérez and Garrido, 1985). PAL activity did not increase in response to fungal inoculation and CO_2 assimilation could not be detected. These results are to be expected if functional chloroplasts are involved in PAL induction in lemon seedlings. Etiolation of dark-grown plants results in an increase of ABA in the apoplast rather than in the etioplast (Kaiser et al., 1985), and this lower ABA concentration could be affecting the induction of PAL. The severely diminished induction of PAL activity in response to fungal inoculation could not be attributed to the absence of its substrate, since it has been reported that etioplasts contain all the enzymes necessary to synthetize the precursors for the phenylpropanoid pathway (Jensen, 1980). This suggests that the presence of the observed plastids is not enough to develop the defense response, and that the presence of mature and metabolically active chloroplasts is required for the defense of the plant against *A. alternata.*

PAL activity did not increase in response to inoculation with *A. alternata* in chemically bleached seedlings. These showed an alteration in the morphology of chloroplasts, correlating well with the absence of PAL induction in response to fungal challenge, and also with the fact that CO_2 assimilation could not be detected. It has been reported that seeds germinated under continuous white light in the presence of the herbicide NFZ generate bleached plants due to the photooxidation of their chloroplasts. This photooxidation, in addition to destroying chlorophyll, also affects other chloroplast components, producing total blocking of chloroplast development (Taylor, 1989). NFZ inhibits phytoene desaturase, which catalyzes one of the steps for the biosynthesis of carotenoids (Sanderman and Böger, 1989). Carotenoids normally protect chlorophyll from photooxidation (Anderson and Robertson, 1960; Frosh et al., 1979), and the inhibition of carotenoid biosynthesis also affects ABA content (Parry and Horgan, 1991). NFZ has been widely used to demonstrate the participation of chloroplasts in the induction of nuclear genes such as the small subunit of ribulose-1,5-biphosphate carboxylase, the light harvesting chlorophyll *a/b* binding protein of photosystem II, and the nitrate reductase (Simpson et al.,

1986; Rapp and Mullet, 1991; Oelmüller et al., 1986; Oelmüller and Mohr, 1986; Deanne-Drummond and Johnson, 1980; Mayfield and Taylor, 1987); or the involvement of ABA in physiological processes (LePage-Degivry and Garello, 1992; Suttle and Hultstrand, 1993).

Chloroplast development was not affected in seedlings grown in the dark in the presence of NFZ and cultivated only during the fifth week under photoperiod. A correlation between the presence of essentially normal chloroplasts with well-established membrane systems and the increase of PAL activity after fungal inoculation was observed. The slightly lower level of PAL activity of these seedlings could be related to the photooxidative effect of NFZ during the light period on chloroplast structure, in those plants incubated under photoperiod during the fifth week.

As a consequence, the use of NFZ-treated and of etiolated seedlings allowed us to infer the necessary involvement of chloroplasts in the increase of the soluble PAL activity after fungal challenge. Further experiments will be required to demonstrate at which level these organelles could be participating in the induction of PAL activity.

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