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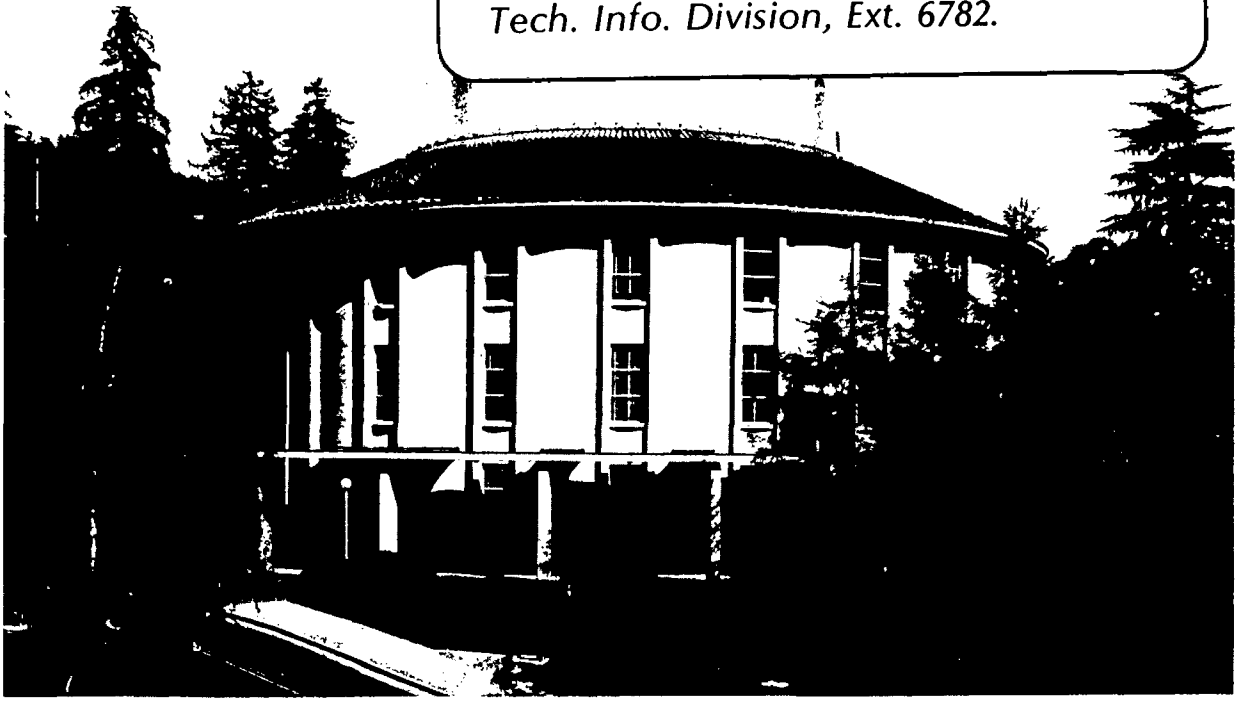

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1-Aminocyclopropane-1-Carboxylic Acid Concentrations in Shoot-Forming and Non-Shoot-Forming Tobacco Callus Cultures

Abbreviations: NAA, naphthalene acetic acid; 2iP, 2-isopentenyl-amino purine; NSF, non-shoot-forming; SF, shoot-forming; ACC, 1-aminocyclopropane-1-carboxylic acid; MCW, methanol:chloroform:water.

ABSTRACT

Shoot-forming tobacco callus tissues contain significantly lower concentrations of the ethylene precursor 1-aminocyclopropane-1- carboxylic acid compared to non-shoot-forming callus tissues. This difference is evident by one day after subculture to shoot-forming or non-shoot-forming medium, and is maintained through the first week of growth. The lack of auxin in shoot-forming medium is the probable cause for this difference in ACC concentrations.

Shoot-formation from tobacco callus tissue was first reported by White in 1939 (14). Several years later Skoog and Miller (12) demonstrated that the relative amounts of exogenously supplied auxin and cytokinin could determine whether tobacco callus remained in the undifferentiated state, or differentiated shoots or roots. Regulation of organ formation from callus by auxins and cytokinins has since been demonstrated with many species, though unfortunately several important crop species (13) remain resistant to attempts to regenerate plants from undifferentiated callus. Furthermore, callus from many plant tissues loses the ability to respond to conditions which normally elict shoot regeneration after only a few months in culture. A thorough understanding of the biochemical events which dictate organogenesis in responsive species, such as tobacco, will elucidate causes for the lack of regenerative ability in other species, and suggest remedies for the problem.

Recently, Huxter, et.al. (4) have demonstrated a role for ethylene in maintaining tobacco callus in the undifferentiated state. This gaseous plant hormone has also been shown to mediate callus formation from cultured citrus buds (3), and to inhibit cytodifferentiation in lettuce pith explants (17). While investigating changes in the sucrose metabolism of shoot-forming and non-shoot-forming tobacco callus cultures, we observed significant differences in the amount of 1-aminocyclopropane-1-carboxylic acid, the precursor to ethylene, in the two types of tissue. These results are reported here, and are interpreted in light of recent findings on the role of auxin in ethylene production, and of ethylene in shoot development from tobacco callus.

MATERIALS AND METHODS

Callus Cultures. Callus was derived from stem pith tissue taken between the fourth and sixth pairs of expanded leaves of vegetative Nicotiana tabacum (var. Wisconsin 38). Pith explants and callus were grown in Petri dishes (100 X 15 cm) on a modified Linsmaier and Skoog medium (9), containing nutrient salts ("Murashige and Skoog Plant Salt Mixture," Flow Labs, Inglewood, Ca.), 100 mg/l myo-inositol, 4.0 mg/l thiamine-HCl, 30 g/l sucrose, and 10 g/l agar, with 3.0 mg/l naphthalene acetic acid, (NAA^2) and 0.3 mg/l 2-isopentenyl-amino purine (2iP). This medium is referred to as non-shoot-forming (NSF) medium. Calli were grown at 24^oC, in continuous light (100-120 $\mu E\ m^{-2}\ sec^{-1}$), and subcultured every three weeks. The average inoculum size was 2 mm^3, and only tissue from the exposed surfaces of the callus was subcultured. Callus grown under these conditions remained undifferentiated for 3 years. Shoot-formation was induced by inoculating callus on the medium described above, but now containing 1.0 mg/l 2iP and lacking NAA. This medium, referred to as shoot-forming (SF) medium, induced the formation of about 50 shoot primordia / cm^3 callus tissue after 3 weeks of growth. Three different lines of callus were used in these experiments. Callus type a was initiated from stem pith explants 9 weeks prior to sampling. Types b and c were initiated 39 and 45 weeks, respectively, prior to sampling.

ACC Concentration Determinations. Intracellular metabolite pools were labelled with radioactive carbon by inoculaing callus at a density of 5 pieces of callus (each piece about 2 mm^3)/ Petri dish to NSF medium containing [U-^{14}C]sucrose (1.0 X 10^{-2} $\mu Ci/\mu gatom$ carbon). After 3 weeks growth on radioactive medium, callus was subcultured at a density of 15

pieces of callus / Petri dish to either NSF or SF medium containing [U-^{14}C]sucrose at the same specific activity.

After 1, 2, 3, 5, or 7 days of growth on NSF or SF medium, the samples were removed and fresh weights determined. Each sample of calAlus consisted of all of the callus from one Petri dish. For callus type a, each data point is the average of 3 samples. For callus types b and c, each data point is the average of duplicate samples. The tissue was homogenized on ice in 1 ml methanol:chloroform:water (MCW) (12:5:3) and transferred to a comical centrifuge tube with 1 ml MCW. The homogenate was centrifuged 4 times, 180 x g, at room temperature, the pellet being resuspended in 2 ml MCW each time. Water, 2 ml, was added to the combined supernatants, and the aqueous layer was removed and reduced to 1 ml by blowing N_2 gas over the surface. The concentrated aqueous phase was fractionated as described previously (7), using cation exchange columns (0.5 X 4 cm, Bio-Rad AG 50W-X8, hydrogen form, 200-400 mesh, Bio Rad Labs, Richmond, CA). Neutral and acidic amino acids were eluted with 4 ml 1N pyridine. This amino acid fraction was chromatoraphed on paper in two dimensions as described by Larsen et al (6), using butanol:acetic acid:water (12:5:3 v/v) in the first dimension followed by butanol:methylethylketone:ammonia:water (5:3:1:1 v/v) in the second dimension. The radioactive ACC was detected by autoradiography, and was eluted and quantified as described previously (7). Metabolite pools were saturated with radioactivity using the conditions described above (data not presented). ACC chromatographed as a distinct spot.

Verification of ACC. [^{14}C]ACC was identified by cochromatography with authentic ACC on the 2-dimensional paper chromatograms. The darkened region of the autoradiogram coincided precisely with ninhydrin-positive ACC.

Cochromatography of the radioactive spot with ACC was also observed in high pressure liquid chromatography using a modification of the method of Radjai and Hatch (10), with 0.125% (w/v) sodium dodecyl sulphate, 0.1% (v/v) H_3PO_4, pH 2.45 as the mobile phase, postcolumn derivatization with o-phthalaldehyde, and detection by fluorescence. The eluted paper chromatoraphy spot was further verified as ACC by the assay of Lisada and Yang (8), in which ACC is degraded using NaOCl in the presence of Hg^{2+}, and ethylene production assayed by gas chromatography.

RESULTS AND DISCUSSION

The amount of ACC present in shoot-forming tobacco callus is significantly less than that found in non-shoot-forming callus tissue (Fig. 1). This difference is evident by 1 day after subculture to the respective media, and is maintained through the first week of growth. Our investigations of the concentrations of over 20 intermediary metabolites in the two types of tissue revealed no other significant differences which repeatedly occur in less than 5 days after subculture (these results will be published in a separate paper). Furthermore, ACC is the only compound we found which accumulates preferentially in the NSF callus.

Our NSF medium contains 3.0 mg/l auxin, whereas the SF medium supplies no auxin. The stimulation of ethylene production by auxin is often attributed to an increased activity of ACC synthase (5,15). In contrast, the enzyme system responsible for the conversion of ACC to ethylene appears to be constitutive in most vegetative plant tissues (2). It is therefore reasonable to suggest that the relatively large amounts of ACC found in NSF callus are a response to the auxin supplied in the growth medium, and that these levels reflect the relative amounts of ethylene synthesized by the tissues.

This hypothesis is supported by the findings of Huxter, et. al. (4), who showed that the amount of ethylene evolved from NSF tobacco callus is significantly greater than that evolved from SF callus tissue. Exogenous ethylene supplied to light-grown SF callus significantly reduced the number of calli which formed shoots, depending on the concentration and time of application. Addition of ACC to SF medium also reduced the formation of shoots. Though we believe it would be premature to evoke a cause-and-effect

hypothesis concerning ACC levels and shoot-formation, the correlation is striking.

There is a steady increase in the concentration of ACC in NSF callus during the first 3 days after subculture (Fig.1). A similar increase in the rate of ethylene production in NSF callus has been reported (4). Both the time-scale of this response (11,16), and the lack of a similar change in SF tissue suggest that the increasing concentrations of ACC in NSF tissue are not the result of wound-formation during subculture. Possibly subculture of tissue to freshly-prepared NSF media exposes callus to higher levels of auxin than were present after 3 weeks of growth on similar media, thereby stimulating an increase in ACC concentration.

It is interesting to note that the 3 different callus lines contained almost identical amounts of ACC at 3 and 7 days after subculture to NSF medium (Table 1). The amount of ACC present in the different SF calli was more variable, though in all cases significantly less than that found in NSF callus of identical origin. This might be related to the observation that the absolute timing and number of shoots formed in different lines of callus varies considerably. The unorganized state, however, is quite reproducibly maintained on NSF media.

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Table 1. Comparison of ACC concentrations in 3 types of tobacco callus after 3 and 7 days of growth on non-shoot-forming (NSF) and shoot-forming (SF) media. Data presented as averages ± standard error of the mean for callus type a. For types b and c, data presented as average of duplicate samples.

callus type	a	b	c
3 days			
NSF	311 ± 20	275	293
SF	69 ± 4	37	67
7 days			
NSF	285 ± 20	322	321
SF	98 ± 13	trace	158

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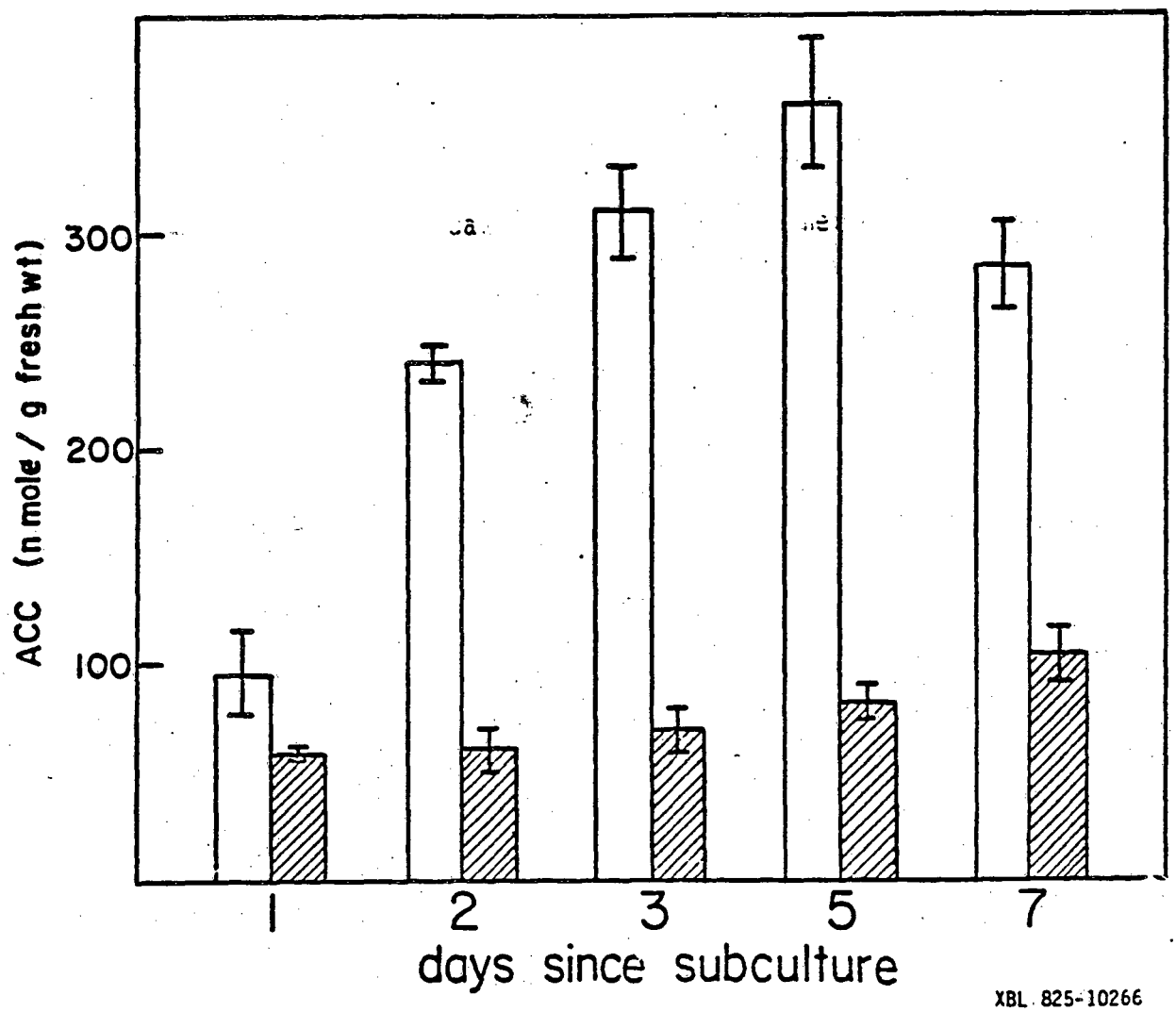

Figure 1. Average ACC concentrations in non-shoot-forming (open bars) and shoot-forming (hatched bars) tobacco callus type a during the first week after subculture to the respective media. Error bars represent standard error of the mean. Differences between mean values for NSF and SF callus were significant at 5% level on day 1, and at 1% level on days 2,3,5, and 7 according to Student's t-test.

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