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Effect of ethylene on adventitious root formation and nicotine content of tobacco callus tissues

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Abstract. We studied the influence of ethylene and medium composition on adventitious root formation and nicotine synthesis in the callus tissue induced from the tobacco *Nicotiana tabacum* cv. Taiyen 5. When ethylene was supplied externally at 10 ppm, adventitious root formation and nicotine synthesis were inhibited. Depletion of ethylene by adding an ethylene absorbent caused a remarkable increase in root formation and nicotine content of calli cultured on Murashige and Skoog medium containing 0.15 ppm α-naphthaleneacetic acid (NAA). When the NAA level was increased to 2 ppm and kinetin was added to 0.2 ppm, with the addition of an inhibitor of ethylene action ($AgNO_3$, 10 μM) or ethylene synthesis (aminoethoxyvinylglycine, 20 μM), or an ethylene absorbent, the calli formed more adventitious root than did the control, but no significant difference in nicotine accumulation was found. These results show that auxins and cytokinins play roles different from that of ethylene in nicotine synthesis, and that root formation is not an obligatory prerequisite for nicotine production in the callus tissue. The influence of different explant sources on the characteristics of induced calli and the potential role of ethylene in the nicotine synthesis are discussed.

Keywords: Adventitious root; Auxin; Cytokinin; Ethylene; Ethylene removal; Ethylene inhibitor; Explant; Nicotine.

Abbreviations: **AVG**, aminoethoxyvinylglycine; **MS**, Murashige-Skoog; **NAA**, α-naphthaleneacetic acid; **SAM**, S-adenosylmethionine.

Introduction

The gaseous plant hormone, ethylene, may be formed in various parts of a plant body, and may also be present in the air as one of the pollutants (Abeles, 1973). So, a plant is under the influence of ethylene from internal and external sources. Ethylene exerts influence on the growth, differentiation, and aging of most, if not all, plants. The synthesis of ethylene is greatly enhanced at the maturation of certain fruits and the aging of flowers and leaves (Kende and Hanson, 1976; Kao and Yang, 1983; Saftner and Baldi, 1990).

Nicotine is one of the specific secondary metabolites of the tobacco plant. It is synthesized mostly in the root, transported to the leaf, and accumulates there with the progress of aging, especially when the nitrogen supply is more than adequate. Although ethylene is a hormone that is physiologically very active at a very low concentration, and nicotine is a metabolically inert secondary metabolite that is accumulated in a rather good quantity in mature leaf tissues, they both share S-adenosylmethionine (SAM) as the common precursor in biosyntheses. How they are related metabolically, other than sharing SAM as the biosynthetic precursor, is not known.

The influences of ethylene on the formation of adventitious root on various plant cuttings and seedlings have been noted, but different studies yielded different results; some found stimulatory (Krishnamoorthy, 1970; Jarvis and Yasmin, 1987), some inhibitory (Biondi et al., 1990; Geneve and Heuser, 1983), and some no (Batten and Mullins, 1978) influence. In studies of tobacco callus cultures, the relationship between adventitious root formation and nicotine content has not been well established (Kinnersley and Dougall, 1982; Miller et al., 1983; Tiburico et al., 1987).

We studied the formation of adventitious roots from the callus tissues induced from the root, pith, and leaf of tobacco seedlings under normal conditions. The influence on adventitious root formation and nicotine accu-

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mulation of externally administered ethylene, depletion of atmospheric ethylene by the ethylene absorbent permanganate, addition of inhibitor of ethylene synthesis—aminoethoxyvinylglycine (AVG), and addition of inhibitor of ethylene action—silver ion, were also studied. This paper reports some of our findings leading to the conclusions that the mechanism of inducing nicotine accumulation is not directly related to adventitious root formation, and that ethylene modulates adventitious root morphogenesis and nicotine accumulation in the tissue culture. A hypothesis of modulatory mechanism based on the availability of SAM as the biosynthetic precursor of ethylene and nicotine has been drawn.

Materials and Methods

Materials

Seeds of tobacco *Nicotiana tabacum* cv. Taiyen 5 were obtained from the Taiwan Tobacco Research Institute. Locally produced ethylene of purity higher than 99.5% was provided by the Department of Horticulture of National Taiwan University. Culture medium components, chemical reagents, etc. were all analytical reagents obtained from Merck (Darmstadt, Germany), Sigma (St. Louis, USA), or Wako (Osaka, Japan).

Callus induction and culture

The tobacco seeds were immersed in 70% (v/v) ethanol for 30 s and transferred into 1% NaOCl for 10 to 20 min. After washing thoroughly with sterile water, the seeds were planted on vermiculite in a 125 ml Erlenmeyer flask and cultured at 25°C under an illumination intensity of 5 J m^{-2} sec^{-1}, 16 h light/8 h dark cycle. After emergence of the 4th leaf, pieces of root, pith, and leaf tissues were cut and placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 2 ppm α-naphthaleneacetic acid (NAA), 0.2 ppm kinetin, 3% sucrose, and 0.8% agar (medium A). After incubating in the dark at 25°C for 3 weeks, callus induction was complete. Subculture passages were carried out under the same conditions every two weeks. Medium A has been known to be not suitable for nicotine synthesis (Ohta et al., 1978; Pinol et al., 1984; Tiburico et al., 1987). To enhance nicotine synthesis, medium A was modified by reducing NAA to 0.15 ppm and omitting kinetin (medium B).

Ethylene level modification treatments

Reduction of ethylene levels or ethylene action in the tissue culture was achieved by adding either AVG at 20 μM (Even-Chen et al., 1982) or $AgNO_3$ at 10μM (Beyer, 1976) to medium A,or by placing an ethylene absorbent $KMnO_4/Na_2CO_3$/vermiculite (Chiang, 1970) in the capped five-liter jar in which the culture dishes were placed. For external ethylene treatment, culture dishes were placed in a five-liter jar containing 10 ppm ethylene. To keep the fluctuation of gas composition to a minimum, the cap of the jar was opened for 30 min everyday. After recapping, ethylene was introduced to 10 ppm through a serum bottle stopper fitted on the cap. A special condition of the atmosphere surrounding the tissue in culture was created by completely sealing the culture dish with two layers of Parafilm. Dishes without such sealing served as the control.

Analytical methods

Nicotine was analyzed by the HPLC method of Saunders and Blume (1981) with some modifications in the sample preparation. Briefly, to 1 g of the wet tissue, 2 ml of 25 mM sodium phosphate (pH 7.8) was added and the mixture homogenized in a Polytron. The homogenate was shaken at room temperature for 24 h, centrifuged at 26,000 *g* for 30 min, the supernatant was vacuum dried, the residue was dissolved in double deionized water, and the solution was passed through a 0.45 μm membrane before HPLC analysis. For the analysis of ethylene emitted from the tissue culture, the tissue was cultured in a 125 ml Erlenmeyer flask. All operations were performed in a laminar flow cabinet. At different time intervals, the silicone plug of the flask was removed and the culture was swept with filtered air for 30 min. The flask was capped with a serum bottle stopper and kept for 6 h. Then 1 ml of the air space was drawn for analysis in a gas chromatograph equipped with a molecular sieve (Porapak Q) column and a flame ionization detector. For the determination of water content, the tissue was dried at 60°C until a constant weight was obtained. It required from 24 to 48 h. The density of adventitious root formation (designated as the root density) was the number of roots grown per one piece of inoculum tissue. The root ratio was the percentage of callus inocula that developed adventitious root. Statistical analysis of the various treatments was done by the Duncan's new multiple range test.

Results and Discussion

Adventitious root formation and nicotine accumulation in calli derived from different tissues

It has been noted during the course of tissue culture system establishment that the leaf explant that was richer in nicotine content than any other tissue explants had developed the highest number of adventitious root. On passage through subcultures, the nicotine content of the leaf-originated callus tissues and the rate of root formation decreased. This trend is obvious from the data presented in Figure 1 and Tables 1 to 3. Tabata and Hiraoka (1976) found no differences in the ability of adventitious root formation and nicotine accumulation in calli derived from different mother tissues, while Kinnersley and Dougall (1982) reported that the callus derived from the pith contained more nicotine than that from the leaf. We may attribute these discrepancies to the differences in the history of tissue culture maintenance, as our data have suggested. Figure 1 and Table 1 also showed that the calli freshly induced from the

Table 1. Adventitious root formation and nicotine content of callus tissue induced from various parts of sterilized seedling[a].

	Leaf		Pith		Root	
	open[b]	closed[b]	open	closed	open	closed
Root density[c]	6.89	0	0.25	0	1.00	0
Nicotine (mg/g dry weight)	5.735	0.158	2.342	_[d]	4.965	0.158

[a] The callus tissue was induced on medium A in the dark at 25°C for 3 weeks. The numbers of leaf, pith and root explants were 16, 7 and 10, respectively.
[b] 'Closed' representes a plastic petri dish that was completely sealed by two layers of Parafilm; 'open' representes a plastic petri dish that was not sealed by Parafilm.
[c] Average number of root per callus derived from an inoculum.
[d] Not determined.

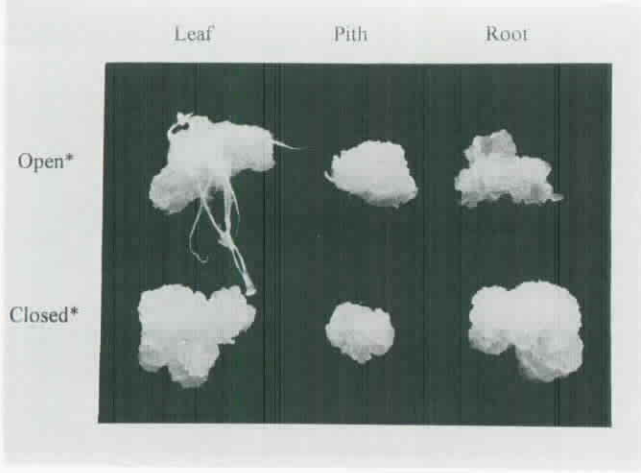

Figure 1. The appearance of tobacco callus tissue induced from various parts of sterilized seedling. The notation is the same as in Table 1.

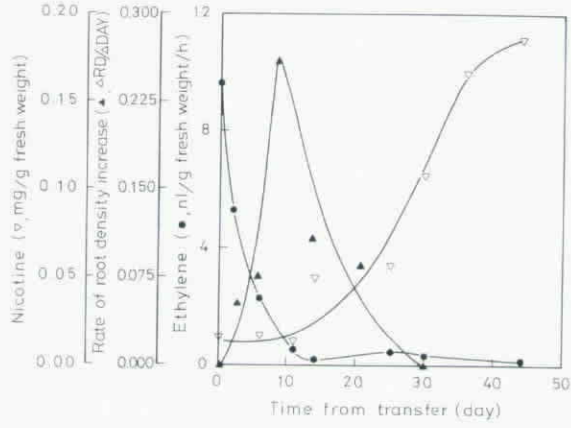

Figure 2. The time course of ethylene production, rate of root density increase, and nicotine content of tobacco callus tissue. Three pieces (each about 0.2 g fresh weight) of a stock culture tissue induced from leaf after four to eight subculturings were transferred to a sealed 125 ml flask containing 25 ml agar medium B kept in the dark at 25°C.

leaflet had distinctly higher root density than those derived from either root or pith under the normal conditions (6.89 vs. ≤ 1). The nicotine contents also showed the same tendency. When the culture dishes were sealed to prevent gas diffusion, the adventitious root formation was completely abolished and the nicotine content decreased in all types of callus. Whether the difference in the nicotine content and abolishment of root formation are due to the accumulation of ethylene or CO_2, the depletion of O_2, or to a combination of these factors, is not known. Nevertheless, these data show that the type of mother tissue and the gaseous environment in which the calli grow strongly influence their morphology and biochemistry. The formation of adventitious roots is positively related to nicotine accumulation, but as Table 3 shows, nicotine is present in a culture without adventitious root. Thus we may say that root formation is not an obligatory prerequisite for nicotine synthesis in the tissue culture.

Influence of ethylene

We have not found a definite answer to the question "is ethylene essential to the formation of adventitious roots?" in a search of the literature. From our results, however, it is quite evident that adventitious root formation is greatly influenced by ethylene.

When ethylene depletion was achieved by adding alkaline permanganate to the callus growing environment, regardless of whether the callus tissue was growing in the medium favoring (B) or not favoring (A) nicotine accumulation, the growth rate in terms of fresh weight gain was not significantly different. The data of root density, root ratio, and nicotine content, however, showed the same increasing tendency under ethylene depletion, especially in medium B (Table 2).

Figure 2 shows the changes in nicotine content and rates of ethylene production and root density increase during the 45-day period of a subculture on medium B. It seems that the initial high rate of ethylene production induces the formation of adventitious root which in turn starts synthesizing nicotine after the root reaches a certain stage of maturation, or when the number of roots stops increasing. These results seem to conflict with the data of ethylene depletion as shown in Table 2, but ethylene is only a trigger, and its extended presence in the culture, even at a low concentration, exerts an inhibitory influence. This view is in accordance with the finding that less root and nicotine are found in the tissue

Table 2. Influence of ethylene on growth, root formation, and nicotine content of tobacco callus tissue*.

	Fresh weight (g)	Root density	Root ratio (%)	Nicotine (mg/g dry weight)
Med. A	7.62±1.00[a]	1.00	25	0.882±0.032[b]
Med. A + $KMnO_4$	8.44±1.05[a,b]	2.12	67	0.918±0.013[b]
Med. B	6.46±0.93[a]	1.12	50	2.634±0.172[c]
Med. B + $KMnO_4$	7.91±0.46[a]	3.12	75	4.066±0.161[d]
Med. B + C_2H_4	6.35±0.67[a]	0.88	42	0.436±0.112[a]

* Four pieces (each about 0.2 g fresh weight) of a stock culture tissue induced from leaf after five subculturings were transferred into a 9-cm plastic petri dish containing 20 ml agar medium in the dark at 25°C for 4 weeks. The petri dish was not sealed by Parafilm chamber. The air was exchanged once each day. Three petri dishes were used for each treatment. Data represent means ± SE of at least two replicates.

[a,b,c,d] Means followed with the same letter are not significantly different at the 5% level by the Duncan's multiple range test.

Table 3. Influence of AVG and silver on growth, root formation, ethylene production and nicotine content of tobacco callus tissue*.

	Fresh weight (g)	Root density	Root ratio (%)	Nicotine (nl/g fresh wt/h)	Ethylene (mg/g dry weight)
Med.A	10.12±0.48[a]	0	0	0.80±0.26[a]	0.983±0.114[a]
Med.A +AVG	9.55±0.14[a]	3.15	83	0.08±0.01[b]	2.216±0.622[a]
Med.A +Ag^+	9.30±0.39[a]	0.42	17	1.16±0.08[a]	1.784±0.800[a]

* Three pieces (each about 0.2g fresh weight) of a stock culture tissue induced from leaf after over ten subculturings were transferred into a covered 125 ml flask containing 25 ml agar medium kept in the dark at 25°C for 4 weeks. Four flasks were used for each treatment. Data represent means ± SE of at least three replicates.

[a,b] Means followed with the same letter are not significantly different at the 5% level by the Duncan's multiple range test.

Table 4. Influence of added ethylene on tobacco callus tissue grown on an AVG containing medium A*.

	Fresh weight (g)	Root density	Root ratio (%)	Nicotine (mg/g dry weight)
Med.A + AVG	7.90±0.03[a]	3.62	75	1.687±0.166[b]
Med.A + AVG + C_2H_4	8.10±0.33[a]	0.17	8	0.257±0.049[a]

* The culture conditions were the same as in Table 3, except that the flask was covered with a super-type silicosen and put into a 5L closed chamber. Everyday, the air was exchanged once and ethylene was applied to give 10 ppm within the chamber. Four flasks were used for each treatment. Data represent means ± SE of at least two replicates.

[a,b] Means followed with the same letter are not significantly different at the 5% level by the Duncan's multiple range test.

grown on medium A, which contains more ethylene inducing hormones. Addition of AVG or $AgNO_3$ also caused the same effects as that of depleting atmospheric ethylene (Table 3). Although the fresh weights of calli were not noticeably different among the control and the AVG and $AgNO_3$ added cultures, the calli cultured on AVG-containing medium had a longer lag phase than did the others (data not shown). This phenomenon is probably due to the interference of AVG with the pyridoxal phosphate requiring system that is essential for the initial growth of calli. Silver ion stimulated ethylene production. This is consistent with many other reports (Penarrubia et al., 1992; Pua, 1993). When ethylene concentration was increased in the atmosphere surrounding calli grown on medium B (Table 2), or AVG-added medium A (Table 4), retardation of both adventitious root formation and nicotine accumulation was evident.

All data obtained in this study indicated that when the ethylene level, of internal or external origin, is elevated, adventitious root formation and nicotine synthesis are retarded. Since the known biosynthetic pathways of ethylene and nicotine share the same intermediate, SAM, it may be hypothesized that ethylene serves as a feedback modulator that influences SAM metabolism to bring about a compensatory change in ethylene and nicotine levels. Ethylene has been reported to be either a positive or negative feedback modulator of its own synthesis (Riov and Yang, 1982). From the data we obtained, we tend to speculate that a higher atmospheric level of ethylene has an autocatalytic effect, enhancing its own synthesis. Thus its removal by an absorbing agent abolishes the enhancement, and causing an effect similar to that of the addition of AVG. AVG is known to inhibit the conversion of SAM into ethylene and to bring about the accumulation of SAM (Even-Chen et al., 1982). The excess SAM accumulated because of the inhibitory influence may be diverted to nicotine synthesis, resulting in a compensatory change of ethylene and

nicotine levels. One way to test this hypothesis is to see whether the SAM level in callus tissue is influenced by the ethylene absorbent. Another way of testing is to set up an experiment to measure the kinetics of ethylene release from tissue cultures that are kept under varied levels of atmospheric ethylene. If air containing a low level of ethylene is slowly injected from a large syringe into a small chamber in which a tissue culture is kept, and the outflow from the chamber collected by a synchronously moving syringe, ethylene emitted from the tissue culture over the experimental period can be measured from the difference in the ethylene concentrations of the input and outflow of the air. Preconditioning of the tissue in a large chamber containing the input concentration of ethylene will increase the reproducibility of the experiment.

Medium A has higher NAA and kinetin concentrations than medium B. Auxins are known to stimulate ethylene production in many plant tissues (Yu and Yang, 1979), and cytokinins have synergistic influence on auxins-induced ethylene production (Lau and Yang, 1973). Based on these reports and our results that simultaneous addition of AVG and ethylene to medium A resulted in the retardation of adventitious root formation and nicotine accumulation (Table 4), ethylene can be seen as a suppresser of nicotine synthesis in medium A, but neither simple removal of ethylene from (Table 2) nor addition of inhibitor of ethylene synthesis or ethylene action (Table 3) to calli grown on medium A had any significant influence on the nicotine content. Why could externally added ethylene affect the nicotine status in medium A only in the presence of AVG is an intrigueing but difficult to answer question. We can only say that nicotine synthesis in the tissue culture is governed by auxins and cytokinins in modes different from that of ethylene. We think only measurements of rates of ethylene emission and the concentrations of ethylene precursors, especially 1-aminocyclopropane-1-carboxylic acid and SAM, in the tissues grown in media A and B may yield answers to the questions raised in this study.

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乙烯對菸草癒合組織不定根形成和尼古丁含量的影響

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本文探討乙烯及培養基成份對菸草癒合組織形成不定根與合成尼古丁的影響。外加乙烯 (10 ppm) 批式處理，則會抑制癒合組織不定根生成與尼古丁累積。當癒合組織培養在含有 0.15 ppm α-naphthaleneacetic acid (NAA) 的 Murashige-Skoog (MS) 培養基中，經由過錳酸鉀吸收降低乙烯濃度，造成不定根生成及尼古丁含量顯著增加。如果荷爾蒙改爲 2 ppm NAA， 0.2 ppm kinetin 並加入抑制乙烯合成 (aminoethoxyvinylglycine，20 μM) 或乙烯作用 ($AgNO_3$，10 μM) 的抑制劑，或乙烯吸收劑，雖然不定根較控制組更易形成，但是尼古丁含量並未明顯增加。由這些結果顯示，癒合組織合成尼古丁時，auxins 和 cytokinins 所扮演的角色異於乙烯， 而且生成不定根並不是癒合組織生產尼古丁的必要條件。在本文，亦討論不同來源的培殖體會影響剛誘起之癒合組織的特性以及乙烯對生成尼古丁可能擔任的角色。

關鍵詞：不定根；Auxin；Cytokinin；乙烯；乙烯抑制劑；培殖體；尼古丁。