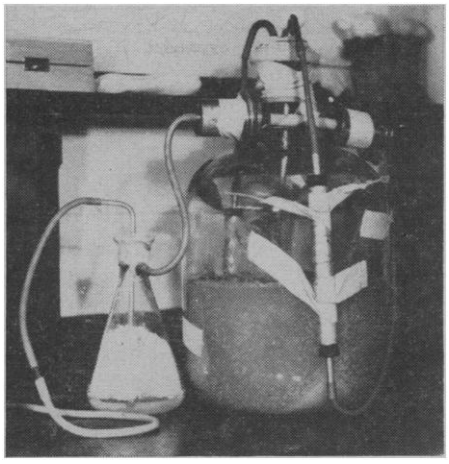

Fig. 1. The carboy culture apparatus. Aeration and agitation are accomplished by the introduction of compressed air (on the left) through a side-arm trap and then through two Seitz filters, which sterilize the air.

Since this approach constitutes part of the tissue-culture program in this laboratory, some of our results on the large-volume growth of plant tissue cultures are presented here.

The principal tissue used for large-volume culture was one obtained from Paul's Scarlet rose stems. This tissue was selected because of its rapid growth rate on solid media and the fact that it grows well in liquid culture. The medium used for carboy studies was made up of White's basal medium (*2*), 2, 4-dichlorophenoxyacetic acid (6 parts per million), yeast extract (0.1 percent), and malt extract (0.1 percent).

The inoculum for the carboy is prepared by taking the tissue culture from a test tube and placing it in liquid medium in a 300-ml erlenmeyer flask. This culture is then agitated on a New Brunswick gyrorotatory shaker. After 1 week's growth, the contents of one flask (the culture in 100 ml of medium) is transferred to a Fernbach flask containing 900 ml of medium. This flask is also aerated and agitated by shaking. After an additional week's growth, the Fernbach culture (1000 ml) is inoculated into a 20-liter carboy containing 9 liters of medium. This is the final stage of this particular scale-up. For purposes of aeration and agitation of the culture during growth, the carboy is fitted with stainless-steel tubing to receive compressed air, which is sterilized by passage through two Seitz filters (Fig. 1). In addition, appropriate connections are included for an air outlet, the addition of medium, and sampling. In all of these manipulations, aseptic procedures must be followed.

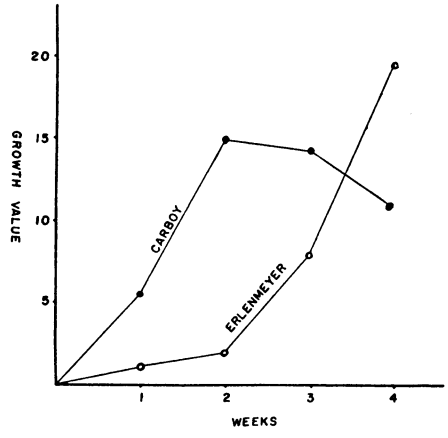

Fig. 2. Growth curves for Paul's Scarlet rose stem tissue grown in carboy culture and in erlenmeyer flasks. The growth value equals the ratio (final fresh weight): (initial fresh weight).

Samples of the carboy culture were made at weekly intervals and compared with results obtained from the same tissue grown on a New Brunswick gyrorotatory shaker in 300-ml erlenmeyer flasks containing 100 ml of culture. These results, shown in Fig. 2, indicate an accelerated rate of growth in the carboy for the first 2 weeks. Growth values (final wet wt./initial wet wt.) of 5.5 and 14.9 are attained by the tissue grown in the carboy system after 1 week and 2 weeks, respectively, as compared to growth values of 1.1 and 1.9 for the same periods with the shaker cultures.

The difference in growth rates between these two systems during the first week is due to the method of aeration. At the end of the first week of growth, 2 liters of medium were added to the carboy; the effect of this supplementation is an increase in rate of growth during the second week. When no further additions are made, the rate of growth drops off sharply, as shown by the growth values for the third and fourth weeks.

Other tissues also grow well in this carboy system of culture. The data available thus far for tissues from *Ilex* stem, *Lolium* endosperm (*3*), and *Ginkgo* pollen (*4*) indicate growth increases similar to the increase found with the Paul's Scarlet rose stem. The *Ginkgo* pollen tissue, for example, yielded 2 lb of cells after 4 weeks in carboy culture.

Other factors recognized as important in increasing the growth rate of plant tissue cultures are those well known to microbiologists working with large-volume cultures. Besides the necessity for an adequate nutrient medium, which itself may be improved in numerous ways, a number of physical factors are involved. In addition to the method and efficiency of aeration, which have already been stressed, the temperature of incubation and the means of agitation are important. More dependent on the tissue culture itself are such things as the age of the inoculum and the particular strain of tissue selected for growth. Moreover, the maintenance of a maximum growth rate rests on such things as timely addition of medium supplements, minimizing of foam production, and proper buffering of the hydrogen ion concentration.

From a practical standpoint, the carboy system of culturing plant tissues is a step in the direction of decreasing the lag phase of growth and increasing the ultimate yield. Our results emphasize the importance of timely nutritional supplements to the medium and of adequate aeration for the rapid growth of certain plant tissue cultures. The aeration factor receives major consideration in microbial "fermentations," but investigations with plant material have not been concerned with the growth of large amounts of material. In the roller-tube technique employed by Steward, Caplin, and Millar (*5*) and the variable-speed shaker technique used by Muir, Hildebrandt, and Riker (*6*), aeration has been studied, but the aim has not been the production of large yields (*7*). Another difference is that in both of these systems the agitation provides the aeration, whereas in the carboy system the aeration provides the agitation and at the same time furnishes a continuous flow of air.

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Pipecolic Acid in Leaves of Strawberry Plant as Influenced by Treatments Affecting Growth

Abstract. Growth inhibition due to maleic hydrazide spray or to an unsatisfied chilling requirement caused young fully expanded leaves of the strawberry to accumulate pipecolic acid to a very striking degree. It is postulated that the accumulation resulted from blockage in normal metabolic conversion to other intermediary nitrogenous compounds.

Pipecolic acid is a naturally occurring cyclic imino acid that has been isolated from several plants and identified chromatographically in many others (*1*, *2*).

Table 1. Nitrogenous fractions in young fully expanded strawberry leaves, in relation to treatments with maleic hydrazide (MH), 1957.

Treatment and date	Nitrogenous fractions: Total* (μg N / g fresh wt.)	Pipecolic acid: μg N / g fresh wt.	Pipecolic acid: % of total N
Variety: Sparkle			
None			
8 /22	60.1	0.4	0.7
9 /12	73.3	1.3	1.9
MH, 1000 ppm			
8 /22	56.6	0.4	0.8
9 /12	74.3	4.8	6.5
MH, 2000 ppm			
8 /22	54.0	0.5	1.0
9 /12	95.1	6.6	7.0
Variety: Catskill			
None			
8 /25	78.9	1.0	1.4
9 /12	81.3	1.1	0.4
MH, 1000 ppm			
8 /25	65.3	1.4	2.3
9 /12	94.6	3.2	3.4
MH, 2000 ppm			
8 /25	73.1	1.6	2.2
9 /12	78.4	9.5	9.7

* Alcohol-soluble ninhydrin–reacting substances.

Usually present in low concentration, it has been found to accumulate strikingly in the immature fruit and embryo of *Phaseolus vulgaris* (*2*). Working with that species, Grobbelaar and Steward (*3*) have found that lysine is converted to pipecolic acid, which in turn may be metabolized to α-amino adipic acid.

In the strawberry plant (*Fragaria chiloensis* var. *ananassa*) pipecolic acid is usually not found in very significant concentration among the alcohol-soluble ninhydrin-reacting nitrogenous compounds that are separated by two-directional paper chromatography. However, recent studies have indicated that this compound may accumulate dramatically in leaves of strawberry plants which are internally inhibited from active growth in spite of exposure to favorable growing conditions.

In the first of these studies, field-grown plants of the varieties Sparkle and Catskill were sprayed with aqueous solutions containing 1000 and 2000 parts of maleic hydrazide (MH) per million on 20 August and again on 4 September 1957. By 6 September, the growth-inhibiting effects of the MH sprays were visible, but there was no apparent injury to the leaf surface. Between 20 August and 15 September samples of young fully expanded leaves were taken at intervals of 2 to 3 days and preserved in 80-percent alcohol. The alcohol extracts were subsequently analyzed for amino acids by two-directional chromatography, with phenol and butanol-acetic acid used as the solvents. The pipecolic acid spots were identified by position and by their fluorescence in ultraviolet light. Quantitative estimation was accomplished by the procedure of Thompson and Morris (*4*). Table 1 shows the pipecolic acid concentrations (micrograms of N per gram fresh weight) in young leaves from untreated and MH-treated plants early and late in the experimental period. The rise in pipecolic acid due to MH was apparent in the Sparkle leaf samples by approximately 12 days after the first treatment, and accumulation continued progressively until the last sampling time.

In the second study, young Sparkle strawberry daughter plants were potted in late August and held outside until early November. They were then divided into two lots, one of which was held in the greenhouse at 65° to 75°F under 10-hour days, and the other subjected to chilling at 35° to 45°F continuously, also under short-day conditions. Approximately 2 months later the chilled plants were brought to the greenhouse. At that time both the chilled plants and those that had been held continuously at warm temperatures were separated into long (16-hour) day and short (12-hour) day lots. After a month, these four lots of plants were again divided, and half of each lot was sprayed with 100 parts of gibberellic acid (Gibrel) per million.

The unchilled plants grew more slowly and their leaves were smaller and darker green than those from chilled plants in all lots. In addition to the growth stimulus from chilling, there were additive stimulatory effects of long days and Gibrel. Thus, the unchilled, short-day plants that received no Gibrel grew least, and the chilled, long-day plants that received Gibrel grew most. Table 2 shows the effects of these treatments on pipecolic acid content, as micrograms per gram of dry weight, of the young fully expanded leaves sampled about 2 weeks and about 5 weeks after the chilled plants were brought to the greenhouse. At the first sampling the growth stimulus resulting from chilling was accompanied by a very great rise in ninhydrin-reacting nitrogenous compounds. As a result, although no consistent difference in the micrograms of N as pipecolic acid was apparent, that present amounted to 13.8 and 14.9 percent of the total soluble nitrogen accounted for in leaves of unchilled plants and only 3.6 and 4.9 percent of that found in leaves from chilled plants. Three weeks later, and about 2 weeks after Gibrel treatment, ninhydrin-reacting N had increased markedly in all the leaf samples from nonchilled plants and had decreased in the leaves from chilled plants. In addition, there was a striking increase in pipecolic acid N in all four leaf samples from unchilled plants, and this became the dominant form of ninhydrin-reacting N in those samples, rising to 39.6 percent of the total in the nonchilled plants that were under long days but did not receive Gibrel.

Table 2. Influence of chilling, day length, and gibberellic acid treatment on nitrogenous fractions in young, fully expanded leaves of the Sparkle strawberry, 1959.

Date and treatment	Nitrogenous fractions: Total* (μg N / g dry wt.)	Pipecolic acid: μg N / g dry wt.	Pipecolic acid: % of total N
Short day			
(1 /30)			
Chilled	1283	46	3.6
Nonchilled	398	59	14.9
(2 /19)			
Chilled	654	16	2.4
Nonchilled	769	146	18.9
(2 /19)			
Chilled + Gibrel	733	14	1.9
Nonchilled + Gibrel	833	193	23.2
Long day			
(1 /30)			
Chilled	1091	54	4.9
Nonchilled	279	39	13.8
(2 /19)			
Chilled	485	11	2.4
Nonchilled	757	300	39.6
(2 /19)			
Chilled + Gibrel	529	14	2.7
Nonchilled + Gibrel	570	96	16.8

* Alcohol-soluble ninhydrin–reacting substances.

Lysine was low in all samples and did not vary consistently as a result of treatments affecting growth. If α-amino adipic acid was present, it did not appear at the expected position on any of the two directional chromatograms in sufficient concentration to react with ninhydrin. It seems possible, therefore, that the increase in pipecolic acid associated with internal growth inhibition resulted from blockage in metabolic conversion to other compounds. (*5*).

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