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Author(s): V. Raghavan and John G. Torrey

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## INORGANIC NITROGEN NUTRITION OF THE SEEDLINGS OF THE ORCHID, *CATTLEYA*<sup>1,2</sup>

V. RAGHAVAN AND JOHN G. TORREY

The Biological Laboratories, Harvard University, Cambridge, Massachusetts

### ABSTRACT

When grown *in vitro* in a medium containing  $\text{NH}_4\text{NO}_3$  as the sole source of nitrogen, seeds of the orchid, *Cattleya* (*C. labiata* 'Wonder'  $\times$  *C. labiata* 'Treasure'), germinated readily and proceeded to form small plantlets. Development of the embryos was accompanied by an increase in their total nitrogen and a decline in the percent dry weight. Growth responses of the seedlings in other ammonium salts like  $(\text{NH}_4)_2\text{SO}_4$ ,  $(\text{NH}_4)_2\text{HPO}_4$ ,  $\text{NH}_4\text{Cl}$ , ammonium acetate and ammonium oxalate were similar to that in  $\text{NH}_4\text{NO}_3$ . However, when grown in a medium containing  $\text{NaNO}_3$ , development of the seedlings was drastically inhibited;  $\text{KNO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_2$  and  $\text{NaNO}_2$  also were poor nitrogen sources. Attempts to grow the seedlings in  $\text{NaNO}_3$  by changing the pH or by addition of kinetin, molybdenum or ascorbic acid as supplements were completely unsuccessful. When seedlings growing in  $\text{NH}_4\text{NO}_3$  for varying periods were transferred to  $\text{NaNO}_3$ , it was found that those plants allowed to grow for 60 or more days in  $\text{NH}_4\text{NO}_3$  could resume normal growth thereafter in  $\text{NaNO}_3$ . Determination of the nitrate reductase activity in seedlings of different ages grown in  $\text{NaNO}_3$ , after  $\text{NH}_4\text{NO}_3$ , showed that the ability of the seedlings to assimilate inorganic nitrogen was paralleled by the appearance of the enzyme.

STUDIES of the nutrition of plant embryos *in vitro* are of considerable importance in understanding the biochemical ontogeny of embryogenesis in plants. It is now well established that harmonious development of embryos during their ontogeny and accompanying functional differentiation within them may result from induction by gradients of nutritional substances or from activation of enzymes and hormonal factors in the growing embryos (Needham, 1942; Brachet, 1950; Boell, 1955; Wardlaw, 1955; Raghavan and Torrey, 1963). Evidence in support of this concept has come mainly from work on animal embryos;

few reports exist concerning the causal factors that control organogenesis in plant embryos.

Seeds of *Cattleya*, like those of several other orchids, offer ideal material in which to study development as an integrated system beginning with an undifferentiated embryo. The embryo contained within the seed at the time of shedding is morphologically simple, corresponding to the globular stage of dicotyledonous embryos. Orchid embryo nutrition has been studied quite extensively. The effects of organic and inorganic nitrogen compounds on the growth of embryos of different hybrids of *Cattleya* have been reviewed by Withner (1959). Curtis and Spoerl (1948) found that mature seeds of *C. mollie* and *C. trianaei* used both ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) ions

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for growth, with the former slightly more effective; most of the amino acids inhibited growth. For these species, aspartic acid was as effective as  $\text{NH}_4\text{NO}_3$ . Glycine also promoted growth of embryos of *C. mollie* to some extent (Spoerl and Curtis, 1948). Arginine was readily used by immature embryos of *C. trianaei*, but was less effective for mature embryos of *C. amethystoglossa* (Curtis and Spoerl, 1948; Spoerl, 1948). Peptone and urea have also served as effective nitrogen sources for embryos of *Cattleya* sp., and *C. trianaei*, respectively (Curtis, 1947; Mariat, 1948).

A study of the nutritional requirements of the embryos of *Cattleya* in our laboratory led to an investigation of the comparative utilization of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  by the developing seedlings. During the course of this work, it became apparent that embryos which were unable to utilize inorganic nitrate during germination and early growth, at later stages in their ontogeny showed continued development in a medium containing  $\text{NO}_3^-$ . Data are presented on the utilization of  $\text{NO}_3^-$  by seedlings of different ages and evidence is given for the activation of the enzyme nitrate reductase within the seedling during development.

**MATERIAL AND METHODS**—Seeds of a hybrid *Cattleya*, *C. labiata* "Wonder"  $\times$  *C. labiata* "Treasure," generously supplied by Mr. Rod I. McLellan, S. San Francisco, California, were used for the experiments. The cultures were started in a transfer room previously illuminated by UV-sterilamps. The seeds, while being held in paper gauze, were surface-sterilized for 5 min in a filtered solution of sodium hypochlorite (5% "Pittchlor," Columbia-Southern Chemical Corporation) and then washed repeatedly in sterile distilled water. They were dried between folds of filter paper and sown in standard 10-cm Petri dishes containing 25 ml of the nutrient agar medium. The Petri dishes were sealed with masking tape to prevent desiccation of the cultures. For making germination counts and for chemical analyses, about 200–400 seeds were thinly spread in each Petri dish. For large-scale cultures, the seeds were started in clumps weighing 5–10 mg. This enabled relatively easy handling during subsequent transfers to fresh media, which were made regularly at intervals of 4–6 weeks.

A modification of the medium used by Spoerl (1948) for seeds of *Cattleya* served as the basal medium in these experiments. The composition of this medium is as follows:

I. Macronutrient salts (mg/liter): 270  $\text{KH}_2\text{PO}_4$ ; 240  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 80  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ; 100  $\text{CaH}_4(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$ .

II. Micronutrient salts (mg/liter): 0.60  $\text{H}_3\text{BO}_3$ ; 0.40  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 0.05  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ ; 0.05  $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ ; 0.05  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ; 0.03 KI; 0.05  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ; 3.0 ferric tartrate.

III. Sucrose 20 g/liter; Difco Bacto Agar 9.0 g/liter.

IV. Glass-distilled water to 1 liter.

The pH was adjusted to 5.5 with 0.01 N NaOH before autoclaving. The components of the medium were mixed together and autoclaved at 15 lb/in.<sup>2</sup> for 15 min.  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  salts with equivalent amounts of nitrogen equal to 0.002 M were added separately to the basal medium to study their effects on germination and subsequent growth of the seedlings. All experiments were repeated at least twice, with 4 replicates per treatment.

The cultures were maintained in a culture room at  $25 \pm 1$  C and were given 12 hr illumination daily by a combination of cool-white fluorescent tubes and incandescent bulbs at a distance of 3 ft, giving ca. 150 ft-c at the level of the Petri dishes. The cultures were examined periodically and qualitative aspects of their differentiation noted. For determining the N-content of the plants, seedling samples were collected at biweekly intervals, or at 6 and 14 weeks. The experiments were usually terminated after 14 weeks.

To obtain a reliable picture of the effects of added substances upon germination and growth of the seeds, the 2 phases were separated arbitrarily. Seeds were considered to be germinated when the embryos were swollen and green, with small papillae protruding from the protocorms. Germination counts were made 6 weeks after sowing, using a dissecting microscope. The different batches of seeds used for this study contained a considerable number of empty ovules (without embryos in them) and sterile ovules (with undeveloped embryos which did not germinate). The values for germination are the percentages of the total number of viable and sterile ovules.

The growth responses of the seedlings to the different compounds were evaluated in terms of the total N-content/mg dry weight of the plants. For this purpose, seedlings were removed from the Petri dishes, washed repeatedly in water, and blotted on folds of filter paper to remove surface moisture, and weighed. Dry weight was obtained after drying for 24 hr at 80 C, and dry weight data (per cent dry weight) are expressed as percentage of fresh weight. Total N was determined according to a method similar to that of Miller and Miller (1948). About 5–20 mg of seedlings were placed in a 10  $\times$  125-mm digestion tube and 0.4 ml of 1:1 sulfuric acid added. The tubes were held in a rack and placed on an aluminum pan containing a heating bath of 10 parts of  $\text{KNO}_3$  and 8.5 parts of  $\text{NaNO}_2$  at 250 C. The tissues were digested for 24 hr using a few drops of 30% hydrogen peroxide ("Superoxol," Merck & Co.) for the final clearing. The extract was diluted with water, and 0.7 ml of 30% NaOH added to bring the total volume to 8 ml. The color intensity developed by adding 2 ml of Nessler's reagent (Fisher Sci. Co.) to this mixture was read in a Beckman DU spectrophotometer at 420  $\mu\text{m}$ . Three to 8 plant samples were analyzed for each treatment, and the values given are the average readings from an experiment.

For determination of nitrate reductase activity, seedlings were harvested in a beaker surrounded by crushed ice. Further operations were carried out in a cold room at 4 C. The tissues were ground with washed and ignited sand in a small volume of 0.001 M glutathione using a chilled mortar and pestle. The homogenate was then pressed through cheesecloth and centrifuged for 30 min at 7000 g. The supernatant fraction was used as the enzyme source. The enzyme activity was determined according to the method of Evans and Nason (1953). The reaction mixture contained in 10 × 125-mm test tubes consisted of 2.5 ml of pyrophosphate buffer at pH 7.0, 50 μM of KNO<sub>3</sub> in 0.5 ml water, and 0.5 μM of reduced nicotinamide-adenine dinucleotide phosphate (NADP) in 1.0 ml water. The reaction was started by the addition of 1.0 ml of enzyme extract. The mixture was incubated at 27 C for 3 hr and the reaction stopped by the addition of 0.5 ml of 1% sulfanilamide in 25%

(v/v) HCl, followed by 0.5 ml of 0.02% solution of N-(1-naphthyl) ethylenediamine dihydrochloride. After 10 min, the absorbency was read in a Beckman DU spectrophotometer at 540 mμ. Control consisted of the reaction mixture complete except for reduced NADP. Enzyme activity was expressed as γ of KNO<sub>2</sub> formed/100 g fresh weight of the tissue in 3 hr.

Nitrate reductase from *Cattleya* was found to be specific for NADP, although with NAD corresponding values were occasionally obtained. Adding flavin nucleotides (FAD and FMN) did not enhance the enzyme activity. Other possible electron donors and activating metals like ascorbic acid-ferrous iron (Vaidyanathan and Street, 1959), succinate (Cheniae and Evans, 1960) and molybdenum (Evans and Hall, 1955) were tried in an attempt to increase the enzyme activity without success. There was no disappearance of enzymatically formed NO<sub>2</sub><sup>-</sup> from the solution,

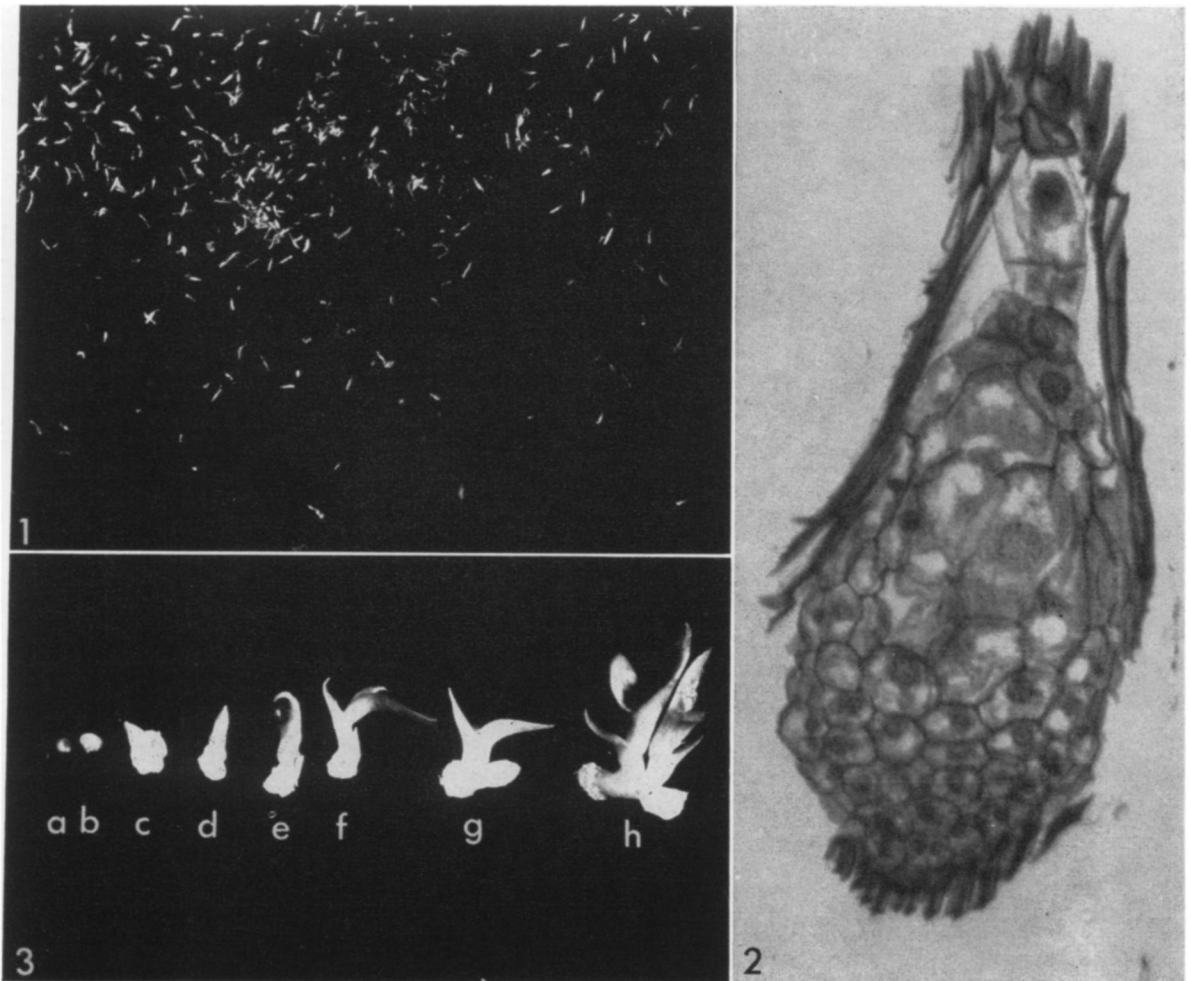


Fig. 1-3.—Fig. 1. Seeds of *Cattleya* before transfer to the medium. ×1.1.—Fig. 2. Longitudinal section through an embryo showing small cells at the anterior end, and larger cells at the micropylar end, terminating in the suspensor. ×143.—Fig. 3. Stages in the development of the seedling when cultured in the basal medium supplemented with NH<sub>4</sub>NO<sub>3</sub>; a-f collected successively at 2-week intervals after sowing; g and h after 14 weeks. ×1.1.

since addition to the reaction mixture of a few drops of 0.001 M hydroxylamine hydrochloride did not affect the observed nitrite level.

**RESULTS—Seedling development in ammonium nitrate and sodium nitrate**—To consider the effects of the various compounds in their proper relationship, it is necessary to examine growth and development of the seedlings when grown in a basal medium supplemented with  $\text{NH}_4\text{NO}_3$ . At the time of sowing the seeds are fusiform (Fig. 1, 2), 400–500  $\mu$  in length and 80–135  $\mu$  in width. The embryo proper, which is enclosed in the hard testa, consists of small cells at the anterior end and larger cells at the micropylar end (Fig. 2); the latter continue into an elongate suspensor. The first signs of growth were seen 10–12 days after sowing, when the embryos enlarged and gradually turned green. During continued growth, cells at the anterior end gave rise to a meristematic area from which future leaves arose. Aerial roots appeared in about 12–14 weeks. As will be seen from data presented below, growth was accompanied by a steady increase in the total N-content of the seedlings. The different stages in seedling development up to the formation of the aerial roots are shown in Fig. 3; for a more detailed description of the development of the seedling, see Shushan (1959).

Figure 4 shows the development of the seedlings in  $\text{NH}_4\text{NO}_3$  and  $\text{NaNO}_3$ , expressed as total N/mg dry weight. The steady continuous development of seedlings in  $\text{NH}_4\text{NO}_3$  is evident in the linear increase in total N with time. In contrast, the N-content of the seedlings growing in  $\text{NaNO}_3$  was not appreciably different from that of seedlings growing in the absence of N-source (compare values in Fig. 4 with those in Table 1), and was only little more than that of seeds at the time of sowing. The rapid growth of the seedlings in  $\text{NH}_4\text{NO}_3$  was accompanied by a decrease in their percent dry weight; on the other hand, the percent dry weight of seedlings growing in  $\text{NaNO}_3$

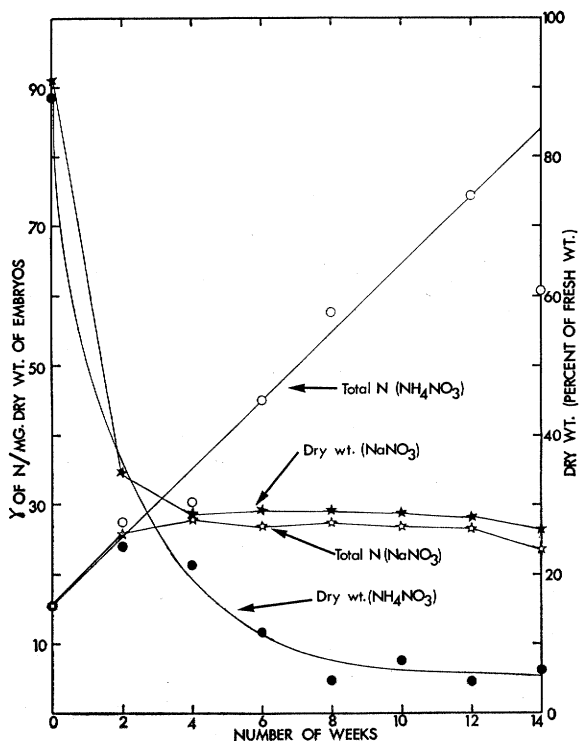


Fig. 4. Effect of  $\text{NH}_4\text{NO}_3$  and  $\text{NaNO}_3$  on the total N-content and dry weight of the seedlings during 14 weeks in culture. Values for seeds before sowing are indicated at 0-time.

remained relatively high following an initial period of hydration (Fig. 4).

**Effects of individual nitrogen compounds on germination and growth**—The effects of several  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  salts on the germination and growth of the seedlings are presented in Table 1. None of the compounds tested had any appreciable effect on germination except  $\text{NaNO}_2$  and  $\text{KNO}_2$  which gave low germination counts. The data

TABLE 1. Effect of different sources of inorganic nitrogen on the germination, dry weight, and total N-content of *Cattleya* seedlings, harvested at 6 and 14 weeks

Nitrogen sources (0.002 M nitrogen)	Percent of germination at 6 weeks	Dry weight as percent of fresh weight		$\gamma$ of N/mg dry weight of seedlings	
		6 weeks	14 weeks	6 weeks	14 weeks
No nitrogen	47.1	27.2	22.9	21.8	23.3
Sodium nitrate	50.9	21.2	18.4	21.7	28.0
Potassium nitrate	49.2	20.8	20.5	21.0	25.6
Calcium nitrate	54.1	17.3	20.8	21.3	29.1
Sodium nitrite	41.4	43.6	24.2	16.4	13.9
Potassium nitrite	36.4	33.4	25.8	12.1	16.3
Ammonium acetate	52.8	9.3	4.3	47.5	62.4
Ammonium chloride	54.6	8.6	6.1	41.2	47.4
Ammonium nitrate	51.6	12.4	5.7	36.9	62.2
Ammonium oxalate	51.0	11.8	5.8	39.5	58.8
Ammonium phosphate	52.5	9.1	4.2	46.8	54.9
Ammonium sulfate	54.9	12.1	5.5	41.9	64.8

confirm that  $\text{NH}_4^+$  salts were better N-sources than nitrates or nitrites; it must be emphasized that  $\text{NH}_4^+$  salts supported continued growth and morphogenesis in the seedlings while nitrates or nitrites did not. Both  $\text{KNO}_3$  and  $\text{NaNO}_2$  appeared to be inhibitory for growth and the N-contents of the seedlings grown in the presence of these salts were much lower than in those growing in a medium with no N.

Data on the dry weight changes of the seedlings (Table 1) reflect the effectiveness of N-compounds for seedling development. The lower the per cent dry weight, the greater the development of the seedling in these studies. The changes reflect the progressive hydration of the embryo and the development of organized structure of high water content. The percent dry weight of the seedlings grown for 14 weeks in ammonium salts was only 4.15–6.07% of their fresh weight, while the corresponding values for  $\text{NO}_3^-$  and  $\text{NO}_2^-$  grown seedlings were 18.42–20.77% and 24.15–25.78%, re-

spectively. Representative samples of the plants grown in the different compounds for 14 weeks are shown in Fig. 5–16; these photographs are all at the same magnification so that direct comparison of the effects of the compounds may be made. In a more detailed experiment,  $\text{NaNO}_2$  was tested at a range of concentrations; as is evident from the data in Fig. 17, at no level did this compound serve as an effective N-source. Concentrations higher than 0.01 M were toxic, causing browning and death of the seedlings soon after their germination.

Attempts were also made to determine whether growth of the seedlings in  $\text{NaNO}_3$  was improved by changing the pH of the medium or by adding supplements. To test the effect of pH, medium was prepared with pH values between 4.3 and 8.1 after autoclaving (Table 2). The results of these experiments showed that pH's near or above 7 were inhibitory to germination and at no pH were the seedlings able to grow well in  $\text{NaNO}_3$ . Our

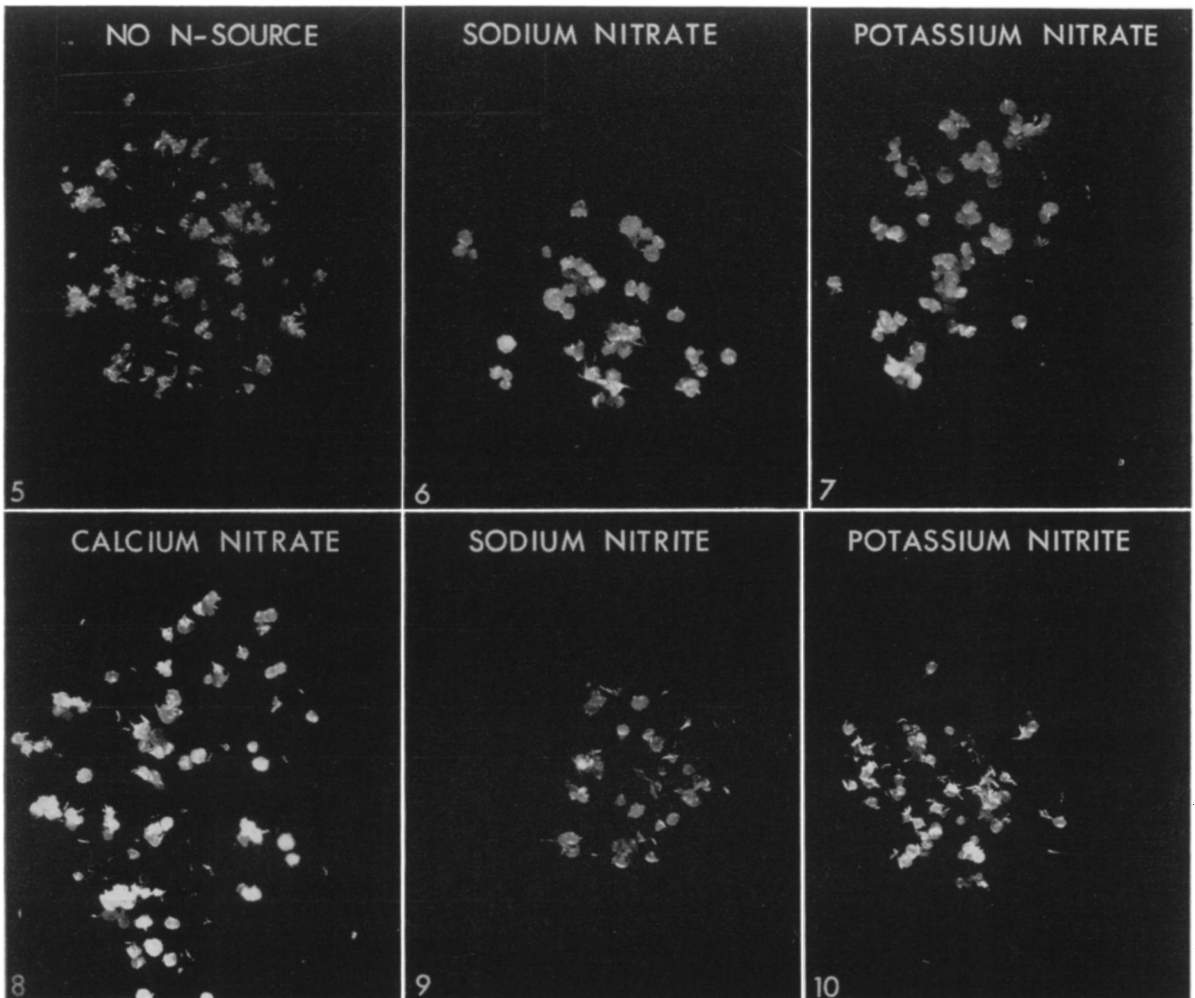


Fig. 5–10. Seedlings of *Cattleya* cultured on medium containing different nitrate and nitrite salts for 14 weeks. All  $\times 1.0$ .

results are in agreement with those of Curtis and Spoerl (1948) who found that regardless of pH,  $\text{NH}_4\text{NO}_3$  was superior as a N-source for seedlings of *C. mollie*.

In another experiment, the effects of ascorbic acid, molybdenum, and kinetin on the utilization of  $\text{NaNO}_3$  by the seedlings were investigated. All the supplements failed to stimulate growth of the seedlings in  $\text{NaNO}_3$ .

*Growth of the seedlings of different ages in sodium nitrate*—These results clearly showed that embryos of *Cattleya* required a source of ammonium nitrogen for their continued growth and development. Burgeff (1936) stated that  $\text{NH}_4^+$  was most favorable to orchid embryos in their "saprophytic stage," before they had developed green leaves, while  $\text{NO}_3^-$  was well utilized by epiphytic photosynthetic plants. In view of this observation, it was interesting to test whether seedlings of *Cattleya* could utilize  $\text{NaNO}_3$  at later stages in their ontogeny.

Seeds were initially grown in  $\text{NH}_4\text{NO}_3$  medium and after 20, 40, 60 or 80 days they were transferred to fresh media containing  $\text{NH}_4\text{NO}_3$ ,  $\text{NaNO}_3$ , or one without a source of N. At intervals of 2 weeks thereafter, dry weight and total N-content of representative samples from each treatment were determined. This experiment was terminated 14 weeks after transfer to new media. The results are presented in Fig. 18–21.

It is seen that while 20- and 40-day-old seedlings failed to grow either in  $\text{NaNO}_3$  or in a medium lacking N, 60- and 80-day-old seedlings resumed normal growth if transferred to  $\text{NaNO}_3$ . The absence of any appreciable growth of 60- and 80-day-old seedlings in a N-free medium rules out the possibility that the response of these plants to  $\text{NaNO}_3$  was a carry-over effect. Aerial roots were profuse in 60- and 80-day-old seedlings growing in  $\text{NH}_4\text{NO}_3$  and  $\text{NaNO}_3$ , while those growing in a medium lacking N were characterized by an almost complete absence of root formation (Fig.

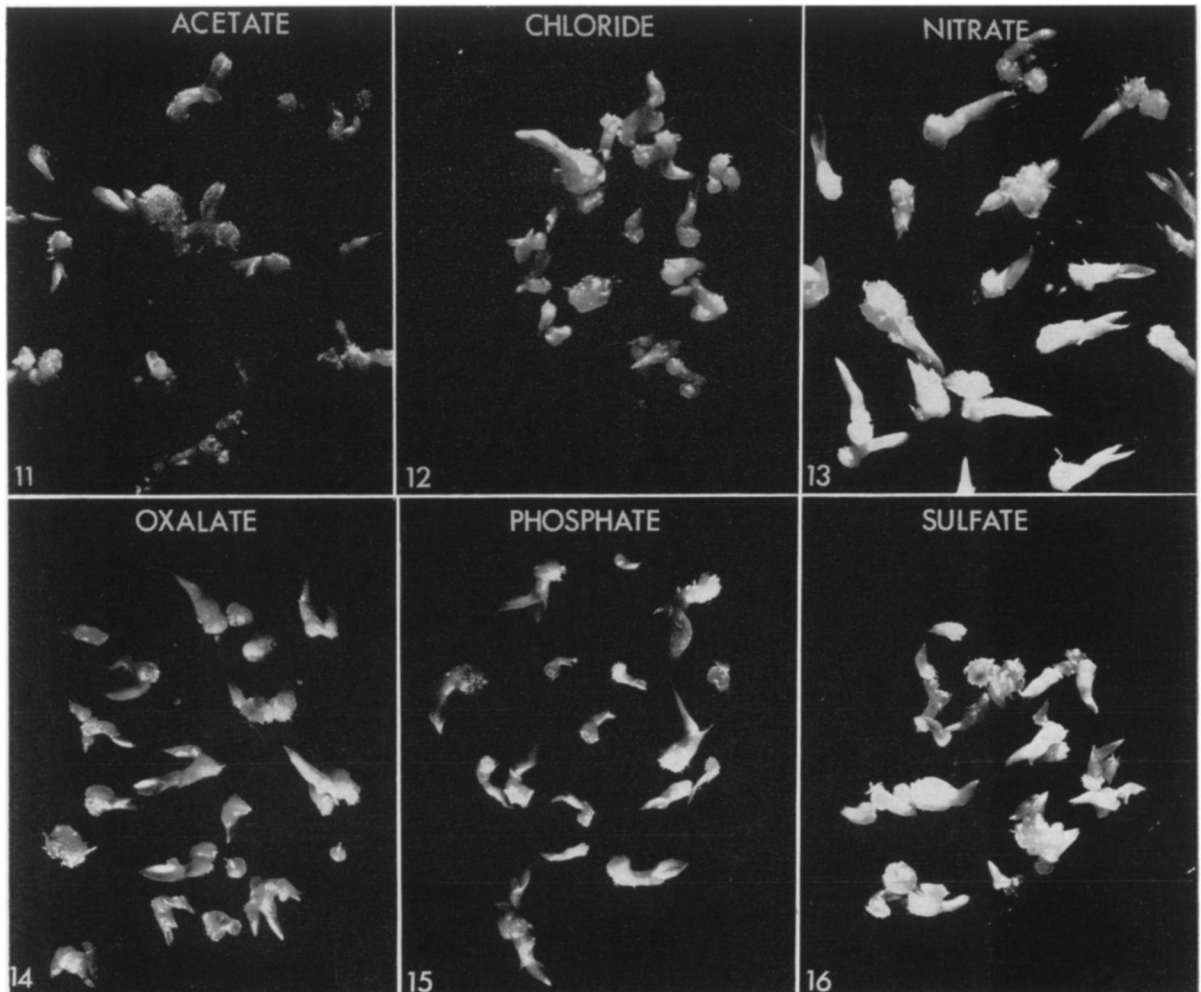


Fig. 11–16. Seedlings of *Cattleya* cultured in media containing different ammonium salts for 14 weeks. All  $\times 1.0$ .

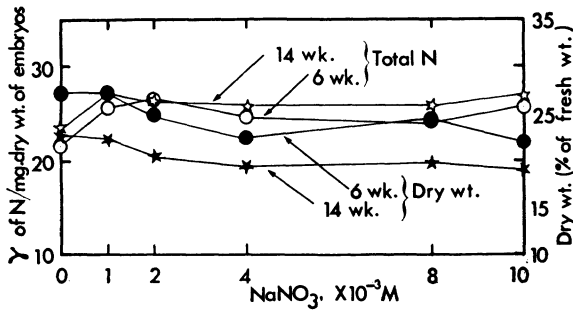


Fig. 17. Effect of  $\text{NaNO}_3$  concentrations on the total N-content and percent dry weight of the seedlings at 6 and 14 weeks.

20, 21). The average number of leaves in the plants transferred to  $\text{NaNO}_3$  after 60 and 80 days was nearly the same as those growing in  $\text{NH}_4\text{NO}_3$ , but the leaves in the former were small and yellowish-green.

The values for the N-content of the 20-, 40-, 60- and 80-day-old seedlings growing in  $\text{NH}_4\text{NO}_3$ ,  $\text{NaNO}_3$  and in a medium lacking N during a subsequent period of 14 weeks are shown in Fig. 22. It should be noted that each group of seedlings is 20 days older at transfer than the preceding one; this age difference is reflected in the increasing N-values at zero time, which correspond to values seen in Fig. 4. Limited growth occurring in the 20- and 40-day-old seedlings in  $\text{NaNO}_3$  can be explained as an effect of  $\text{NH}_4\text{NO}_3$  carried over in the medium. It was noted that N-content of 60- and 80-day-old plants growing in  $\text{NaNO}_3$  was nearly the same as those growing in  $\text{NH}_4\text{NO}_3$ , but that of seedlings growing in the absence of N was well below that of either one.

The percent dry weights of the 20- and 40-day-old seedlings growing in  $\text{NaNO}_3$  and in N-free medium were higher than those growing in  $\text{NH}_4\text{NO}_3$ , and in this respect they followed the pattern observed earlier (Fig. 23). Although 60- and 80-day-old seedlings did not grow appreciably in a N-free medium, their percent dry weight did not show any change from those growing in  $\text{NH}_4\text{NO}_3$  or  $\text{NaNO}_3$  (Fig. 23).

*Determination of nitrate reductase activity in the seedlings*—The data presented above indicate that seedlings cultured in media lacking  $\text{NH}_4^+$  but containing  $\text{NO}_3^-$  fail to develop due to lack of ability to reduce  $\text{NO}_3^-$ . It is also evident that if seeds are first cultivated in  $\text{NH}_4\text{NO}_3$  and then transferred to  $\text{NO}_3^-$  alone, there is a progressive development of capacity to reduce  $\text{NO}_3^-$ . Further work in this system was designed to shed light on the relation between growth of the seedlings in  $\text{NaNO}_3$  and activity of nitrate reductase in them. Enzyme activity was determined in seedlings cultured for various periods of time in  $\text{NH}_4\text{NO}_3$  (20, 40, 60, 80 or 100 days) and then transferred for 12–14 weeks' further development in medium containing only  $\text{NaNO}_3$ . Data on the activity of nitrate reductase in all replicates of plants of various ages analyzed are shown in Table 3. Seedlings growing exclusively in  $\text{NH}_4\text{NO}_3$  analyzed at various intervals showed no enzyme activity. As seen from the table, there was no detectable activity of nitrate reductase in plants cultured only 20 and 40 days on  $\text{NH}_4\text{NO}_3$ , even after subsequent prolonged periods of growth in  $\text{NaNO}_3$ . Seedlings first cultured for 60 days or more on  $\text{NH}_4\text{NO}_3$  and thereafter in  $\text{NaNO}_3$  alone showed nitrate reductase activity. The appearance of nitrate reductase in the homogenates of these seedlings parallels closely their nearly normal growth in  $\text{NaNO}_3$ .

*DISCUSSION*—The interpretation that emerges from this study is that the embryos of *Cattleya* require a source of ammonium nitrogen for their germination and early growth. Although the embryos would germinate in a medium containing  $\text{NO}_3^-$  or even in the absence of added N, they failed to grow further without a supply of  $\text{NH}_4^+$ . That  $\text{NH}_4^+$  is directly utilized by the seedlings is shown by the increase in their N-content during growth in ammonium salts.

The failure of  $\text{NO}_3^-$  to promote growth of the seedlings is somewhat surprising, since  $\text{NO}_3^-$  is known to be the major source of N for many higher plants and microorganisms. In the excised and cultured embryos of *Anagallis*, *Capsella*, *Arabidopsis* and *Sisymbrium*, Rijven (1958) has also noted that the response to  $\text{NO}_3^-$  was slight

TABLE 2. Effect of changing the pH of the medium on the extent of germination, per cent dry weight and total N-content of *Cattleya* seedlings grown in media supplemented with 0.002 (M) sodium nitrate

Germination (Exp. 1)		Percent dry weight and N-content (Exp. 2)				
pH	Percent of germination at 6 weeks	pH	Dry weight as percent of fresh weight		$\gamma$ of N/mg dry weight of seedlings	
			6 weeks	14 weeks	6 weeks	14 weeks
5.2	53.6	4.3	22.9	18.1	22.4	19.4
5.3	57.8	5.1	21.7	19.3	18.9	24.4
6.2	62.1	5.8	24.7	19.1	15.1	27.6
7.0	45.3	5.9	21.2	19.1	15.9	25.4
7.8	39.2	7.7	22.8	16.6	18.9	26.9
7.8	43.5	7.8	20.0	17.4	18.9	28.8
8.1	48.8	8.0	22.7	20.4	20.8	20.9

or absent in all the species. On the other hand, mature embryos of barley (Brown, 1906), oats (Harris, 1956), and wheat (Rijven, 1960) apparently utilized nitrate nitrogen and were able to meet their amino acid requirements satisfactorily by synthesis from inorganic nitrate.

It is, however, a striking fact that after completion of a certain phase of morphological development, as represented by growth in  $\text{NH}_4\text{NO}_3$  for

60 days, the seedlings were able to assimilate  $\text{NO}_3^-$  and to continue normal growth in that medium. At 60 days, the seedlings were characterized by the formation of the first 1-2 leaves (Fig. 3d,e), while at 20-40 days they were still in the protocorm stage or, at best, had formed leaf primordia. It seemed reasonable to assume that during germination and early growth, the seedlings were unable to synthesize nitrate reductase

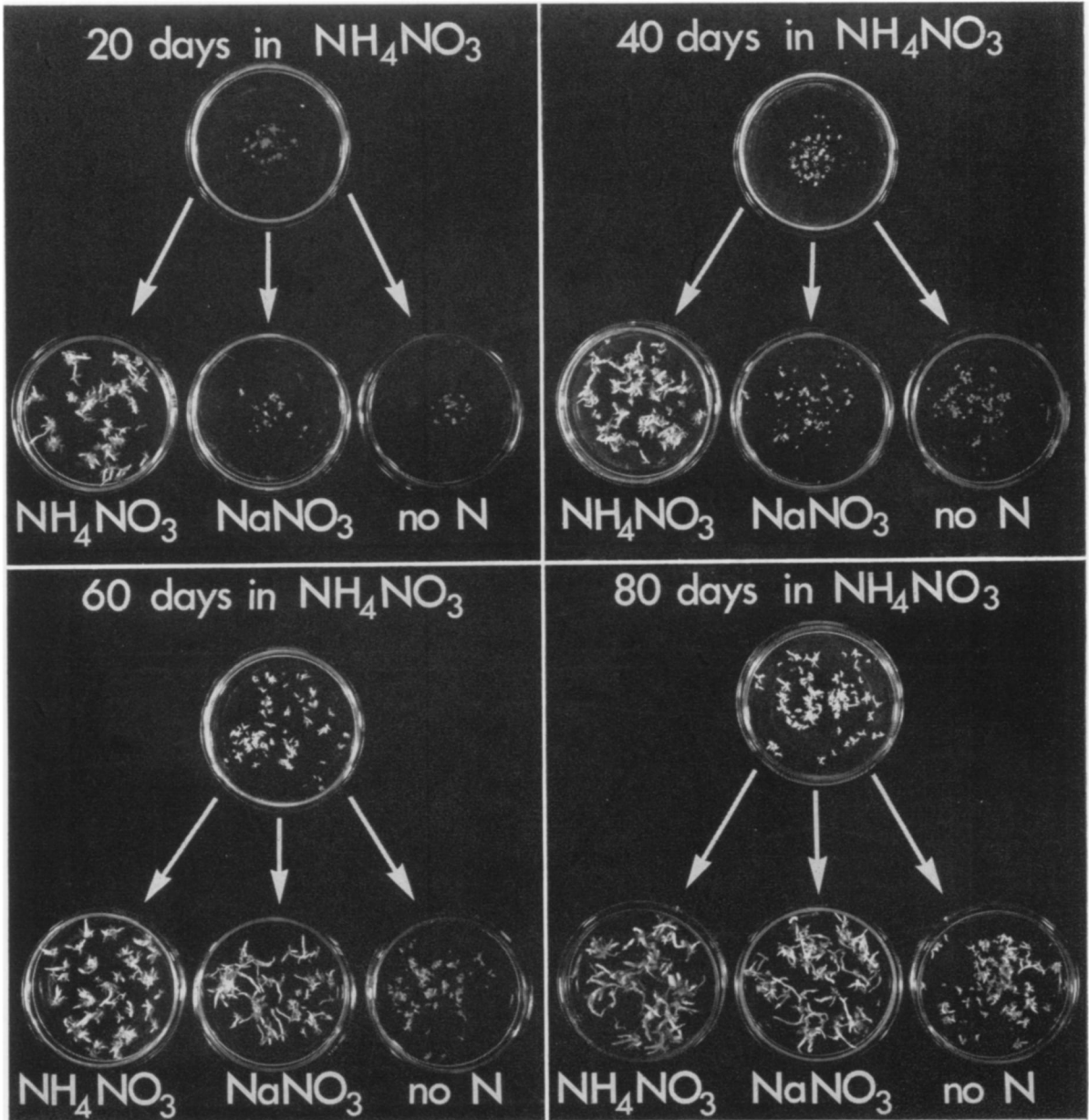


Fig. 18-21. Growth of seedlings of *Cattleya* of different ages in  $\text{NaNO}_3$  and in medium lacking N. Before transfer to new media, seedlings were grown for different periods in  $\text{NH}_4\text{NO}_3$  and are shown at the top of each figure. In each figure, the sample at bottom left represents plants growing in  $\text{NH}_4\text{NO}_3$  for the same length of time as those in  $\text{NaNO}_3$  or in medium lacking N. Both  $\text{NH}_4\text{NO}_3$  and  $\text{NaNO}_3$  were used at 0.002 M N-equivalent. All photographed 14 weeks after transfer to new media.—Fig. 18 (top, left). Seedlings 20 days old at transfer.—Fig. 19 (top, right). Seedlings 40 days old at transfer.—Fig. 20 (bottom, left). Seedlings 60 days old at transfer.—Fig. 21 (bottom, right). Seedlings 80 days old at transfer. All  $\times 0.2$ .

but formed this enzyme only at later stages of development. However, at no time did the plants show any detectable nitrate reductase activity when they were grown continuously in  $\text{NH}_4\text{NO}_3$ . This fact, besides showing the absence of de novo synthesis of the enzyme, indicates that  $\text{NO}_3^-$  is not utilized in the presence of  $\text{NH}_4^+$ .

On the other hand, it seems that at a certain stage of morphological development, probably with the appearance of the leaves, the seedlings acquired the capacity to form nitrate reductase in the presence of a substrate, while younger plants were unable to do so. Support for this view comes from the inability of 20- and 40-day-old seedlings to grow in  $\text{NaNO}_3$  or to show any detectable enzyme activity even after prolonged periods of culture in  $\text{NaNO}_3$ . Although no "accumulation"

of  $\text{NO}_3^-$  was noted in these seedlings, qualitative tests with diphenylamine have indicated the presence of  $\text{NO}_3^-$  in them, thus showing that there were no barriers to permeability of  $\text{NO}_3^-$  into the plants. Since the 60- and 80-day-old seedlings did not grow *as well* in  $\text{NaNO}_3$  as those growing in  $\text{NH}_4\text{NO}_3$ , one is tempted to think that even the older plants were habitually conditioned to utilize  $\text{NH}_4^+$ , but could adapt to grow in  $\text{NaNO}_3$  only in the absence of  $\text{NH}_4^+$ . The low level of enzyme activity in the seedlings is also suggestive of this. Indeed, the activity of nitrate reductase was surprisingly low in *Cattleya* in comparison to other systems like soybean seedlings (Evans and Nason, 1953), tomato roots (Vaidyanathan and Street, 1959), corn shoots (Hageman and Flesher, 1960), and nodules of leguminous plants (Cheniae and

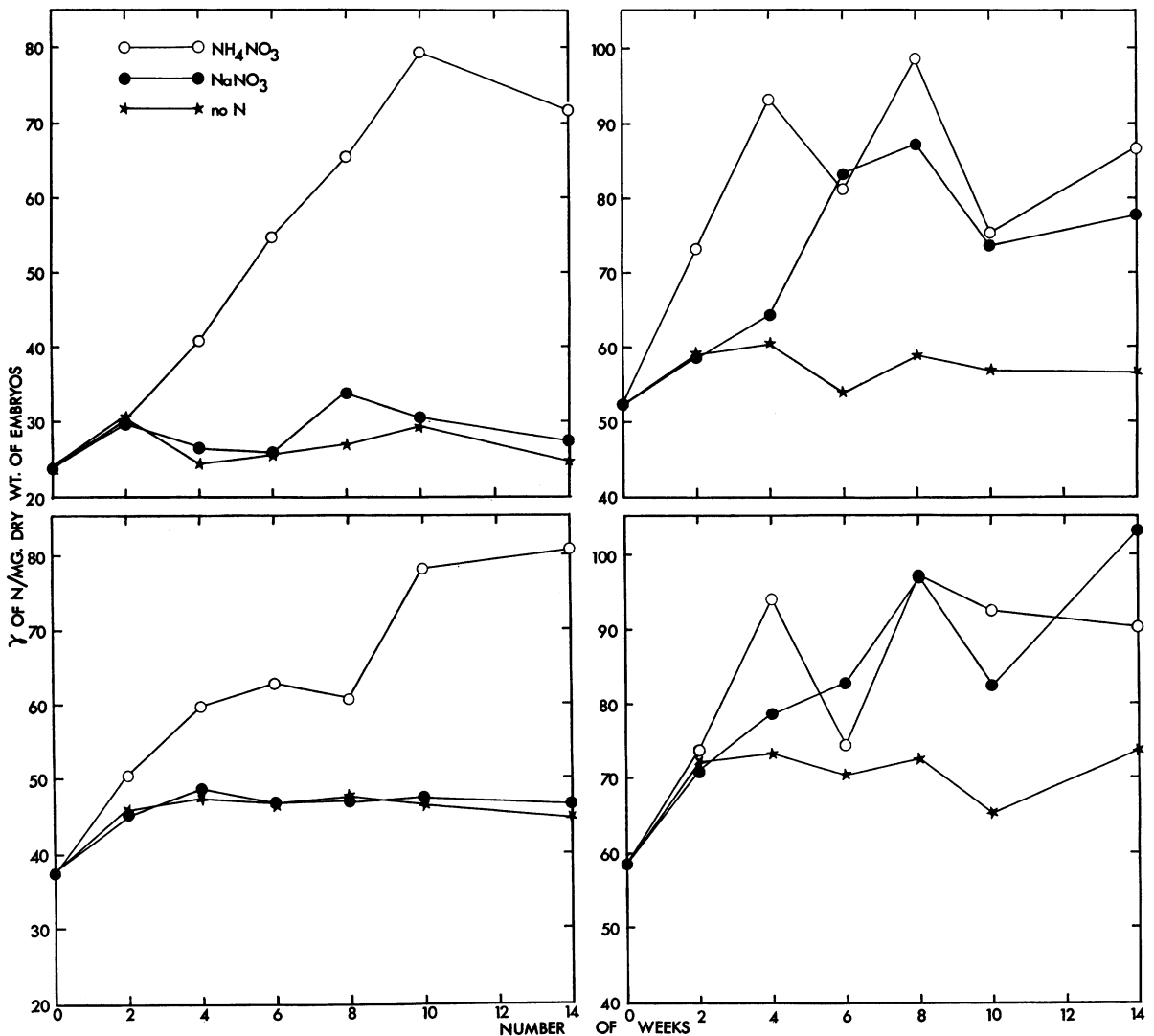


Fig. 22. Total N-content of seedlings of *Cattleya* of different ages growing in  $\text{NH}_4\text{NO}_3$ ,  $\text{NaNO}_3$  and in a medium lacking N. Before transfer to these media, seedlings had been grown for different periods in  $\text{NH}_4\text{NO}_3$ ; values at 0-time indicate the N-content of seedlings at the time of transfer. Top, left: plants 20 days old at transfer; bottom, left: plants 40 days old at transfer; top, right: plants 60 days old at transfer; bottom, right: plants 80 days old at transfer.

Evans, 1960), in which this enzyme has been characterized. Although 60- and 80-day-old seedlings were able to assimilate  $\text{NO}_3^-$  at 4 weeks after transfer to  $\text{NaNO}_3$  (Fig. 22), relatively longer periods of incubation (12–14 weeks) were necessary to obtain measurable nitrate reductase activity in homogenates of these plants. A more sensitive method for enzyme assay will be needed to study the time course of induction of nitrate reductase in the seedlings.

Induced enzyme synthesis is widely known in bacteriological literature, but attempts to provoke such induced synthesis in embryonic cells have usually been unsuccessful (Brachet, 1960). Induction of nitrate reductase in embryos of wheat and *Capsella* and of glutamyl transferase in germinating wheat embryos has been shown by Rijven

TABLE 3. Nitrate reductase activity of *Cattleya* seedlings of different ages grown in medium containing sodium nitrate

Age of seedlings (Number of days in $\text{NH}_4\text{NO}_3$ before transfer to $\text{NaNO}_3$ )	Sample Number	Number of days of growth in $\text{NaNO}_3$	Fresh weight g	$\gamma$ of $\text{KNO}_2$ formed in 3 hr/100 g fresh wt of tissue
	1	85	21.4	nil
	2	86	18.3	15.4
20	2	98	19.6	nil
	3	99	36.0	nil
	1	85	29.7	nil
40	2	98	22.3	nil
	3	99	43.2	nil
	1	84	38.0	17.6
60	2	88	29.6	14.6
	1	85	42.0	22.5
80	2	85	16.2	32.9
	1	86	25.3	21.9

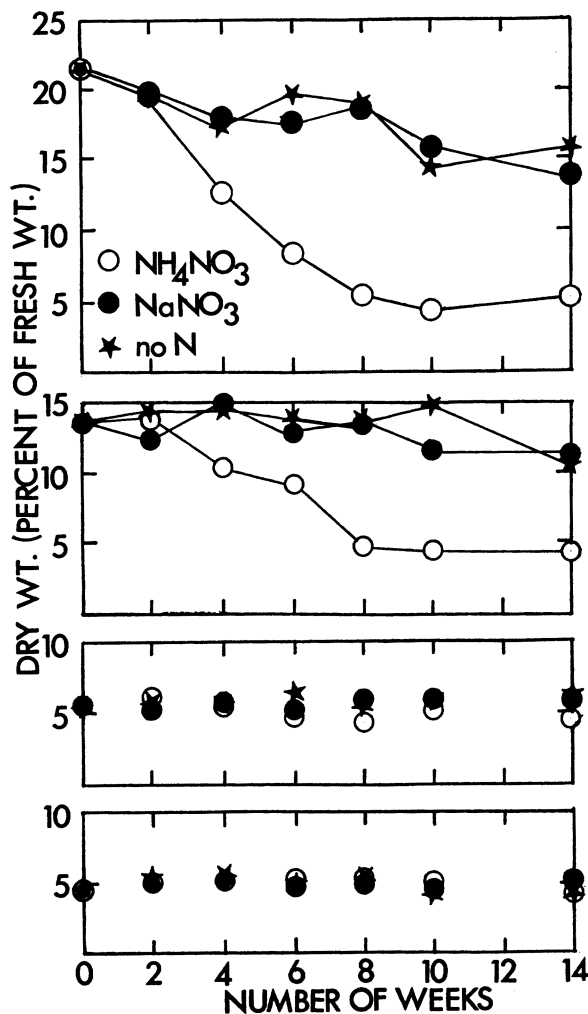


Fig. 23. The percent dry weight of seedlings of *Cattleya* of different ages growing in  $\text{NH}_4\text{NO}_3$ ,  $\text{NaNO}_3$ , and in a medium lacking N. Before transfer to these media, seedlings had been grown for different periods in  $\text{NH}_4\text{NO}_3$ ; values at 0-time indicate the dry weight at the time of transfer; (from top to bottom) 20-, 40-, 60- and 80-day-old seedlings, respectively, at time of transfer.

(1958, 1961). Our results on the induction of nitrate reductase in the seedlings of *Cattleya* at later stages of development are consistent with the ideas of Burgeff on the ability of seedlings to assimilate inorganic nitrogen during periods after they have developed their photosynthetic capacities.

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## ADVENTIVE EMBRYONY IN TISSUE CULTURES OF THE WILD CARROT, *DAUCUS CAROTA*<sup>1,2</sup>

W. HALPERIN<sup>3</sup> AND D. F. WETHERELL

Department of Botany, University of Connecticut, Storrs, Connecticut

### A B S T R A C T

Cultures of callus tissue derived from roots, petioles, or umbellet peduncles of the wild carrot have been observed to form large numbers of adventive embryos which closely resemble ovular embryos of the same species. In the presence of coconut milk, which is not otherwise required in the nutrient medium, these embryos germinate and produce plants which may be normal in all respects. The sequence of forms in embryogenesis has been traced back to embryos of fewer than 10 cells, and the pattern of development has been found in many cases to correspond closely to that of ovular embryos, especially with respect to the early filamentous stages. The accumulation of granular starch in both the parenchymatous cells of the callus and in the embryos at certain stages of their development is a marked characteristic of differentiating callus. Investigation of the nutrient requirements of the callus tissue undergoing differentiation of embryos reveals that a wide latitude is possible in the composition of the basal medium, consisting of mineral salts, vitamins, and sucrose. Embryos form readily in tissue which has been extensively subcultured on a simple defined medium which consists of the basal medium plus adenine and 2,4-D. Tissues cultured on coconut milk and 2,4-D, or kinetin and 2,4-D, are more heterogeneous in composition and may contain, in addition to embryos, idioblastic tracheids, anthocyanin-pigmented cells, and complex vascularized nodules. Chemical control of alternative morphogenetic pathways, and of embryogenesis itself, is partially demonstrable.

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THE STUDY of developmental processes in the simple, relatively homogeneous tissue of callus cultures, in the absence of preformed specialized tissues and organs, has made it clear that the parenchymatous cells of the callus have remarkable regenerative powers. The reappearance of