WILLIAM J. ROBBINS
Columbia University and New York Botanical Garden

Chapter 7

Hybrid Nutritional Requirements

Hybrid vigor has been recognized for more than a century. It has been considered from a genetic, morphological, developmental, physiological, and commercial standpoint. Although a great deal of information has been accumulated about the phenomenon, we are still unable to define exactly why a hybrid grows better than the parents from which it comes.

It is obvious that the cause is physiological—the hybrid functions more effectively or for a longer period of time, and accumulates a greater mass of cell substance. Its metabolic efficiency is greater (East, 1936). It would be illuminating if we could locate specifically the physiological processes which are responsible for the greater vigor of the hybrid—recognizing that they may be numerous and complex rather than single and simple, and that they may not be the same for all examples of hybrid vigor.

For many years I have been interested in the factors which determine why one plant species, variety, or strain grows slowly in a given environment where another flourishes. I have dealt mainly with microorganisms, especially the filamentous fungi, because the external environment can be more easily controlled and photosynthesis is not a complicating factor. From my experience, as well as from the work of others, it is clear that in many instances growth—the accumulation of cell substance—is limited by the efficiency of the organism's metabolic machinery, especially the activity of one or more enzyme systems. Whether this concept can be applied also to the phenomenon of hybrid vigor is still to be determined. However, it is a hypothesis which deserves exploration.

Let us begin with a simple example of growth-limitation. *Aspergillus niger* grows well in a liquid medium of sugar, mineral salts, and asparagine. In the same medium *Phycomyces Blakesleeanus* will not grow at all.

Does Phycomyces fail to grow in the basal solution because of the absence of something essential which it needs for growth, or because of the presence of something detrimental? Does *Aspergillus niger* grow in the basal solution because it does not need to be furnished with the "essential" substance, or because it is more resistant to the supposed injurious ingredient?

For the example cited, we have a definite and well demonstrated explanation. Phycomyces fails to grow in the basal medium because it requires the vitamin, thiamine—which it is unable to make from sugar, mineral salts, and asparagine. *Aspergillus niger* also needs thiamine, but it constructs the vitamin from the elementary materials present in the basal solution. In this instance, therefore, the failure to grow is due to the lack of something essential for growth; namely, thiamine, the precursor of co-carboxylase.

This is not an isolated example. Many species of fungi grow slowly, or not at all, in a basal medium because of their inability to make one or more of the essential metabolites. These metabolites may include various vitamins, purine and pyrimidine bases, amino acids, fatty acids, or substances as yet unidentified.

ESSENTIAL METABOLITES—RELATION TO GROWTH

It may be assumed that the complex chemical compounds which make up the cell substance of a living organism are constructed by the organism from simpler compounds. A series of intermediate chemical compounds are formed between the original simple foods and nutrients and the final product, cell substance. This step-wise progression from simple to complex is made possible by a series of enzymes, also made by the organism, which operate on each stage as that stage is completed. Although synthesis is likely to be emphasized in considering growth, there are other subsidiary processes—necessary concomitants for the building up of new cell substance. The catabolic processes of digestion and respiration also occur in steps, and are made possible by the action of a series of enzyme systems.

Any substance playing a necessary part directly or indirectly in the chain of reactions which end in the synthesis of new cell substance is an essential metabolite. Unless each essential metabolite, each chemical substance in the step-wise process of growth, each enzyme which facilitates the chemical reactions concerned, is made within the organism or supplied from without, the series is interrupted. New cell substance is not made, and growth does not occur. If not enough of an essential metabolite is made, growth will be slowed.

Of course, this is an oversimplified statement of a very complicated process. The reactions concerned in growth probably do not occur in a straight line. Some steps may be bypassed and side reactions may occur, all of which may affect the speed and character of the growth which results.

It would be difficult to estimate the number of essential metabolites in-

volved in the growth of even the simplest organism, or to put a limit on the number for which some organism may not eventually be found to exhibit a deficiency.

Some species or strains exhibit a complete deficiency for one or more essential metabolites. They are unable to synthesize any of the substances in question and do not grow unless the substances are supplied in the medium in which they are cultivated (Robbins and Ma, 1942). Others suffer from partial deficiencies, that is, they grow slowly in the absence of a particular

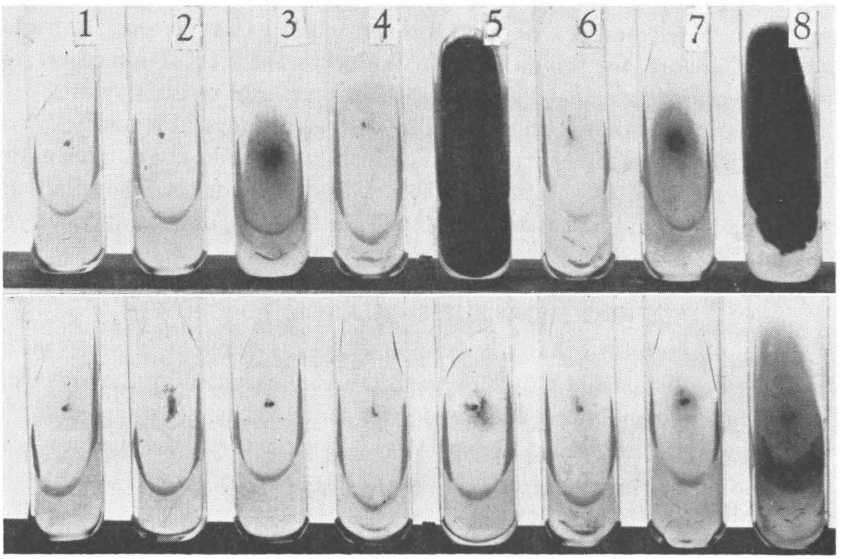

FIG. 7.1—Growth affected by complete and partial deficiencies for essential metabolites. Fungi grown on mineral-dextrose medium containing asparagine and purified agar and supplemented as follows: (1) no addition; (2) thiamine; (3) pyridoxine; (4) biotin; (5) thiamine and pyridoxine; (6) thiamine and biotin; (7) pyridoxine and biotin; (8) all three vitamins. Above, *Ceratostomella multiannulata*, complete deficiency for pyridoxine, partial for thiamine; below, *C. microspora*, complete deficiency for thiamine, biotin, and pyridoxine.

essential metabolite but more rapidly if it is added to the medium (Fig. 7.1).

For example, the clone of excised tomato roots, with which we have worked for many years, suffers from a complete deficiency of thiamine and a partial deficiency of pyridoxine. It will not grow unless the medium contains thiamine or its equivalent. When pyridoxine is added to a medium containing thiamine, the growth of the excised roots is markedly increased.

In a sugar, mineral-salt solution, the growth of our clone of excised tomato roots is limited by its ability to synthesize thiamine. In a thiamine solution, growth is limited by the ability of the roots to synthesize pyridoxine (Robbins, 1946). We have not been able to define what limits the growth of the root in a solution which contains both thiamine and pyridoxine. Other examples

of partial deficiencies could be cited. Their effect is to decrease the rate of growth but not to inhibit it entirely.

As a result of investigations which have extended over the past decade or two, we know of many examples in which poor growth or failure to grow in a specific environment is due to the inability of the organism to synthesize adequate quantities of one or more essential metabolites. The metabolic machinery lacks a part, or some part works slowly, with the result that the organism does not make sufficient quantities of one or more growth essentials, and unless supplied with the missing materials from without, grows slowly, or not at all.

Not all instances of failure to grow or of poor growth in a given environment are explainable on the basis of deficiencies of essential metabolites. In some instances growth may be limited by autogenic growth inhibitors.

AUTOGENIC INHIBITORS

Zalokar (1948), Emerson (1947, 1948), and others have described a mutant strain of Neurospora which grows poorly at high temperatures. Growth occurs if sulfonamide is added to the medium. One might conclude that sulfonamide acts for this organism as an essential metabolite. It appears, however, that this mutant produces growth inhibitors which are antagonized in some way by the sulfonamide. This seems to be an example of poor growth caused by the accumulation of autogenic growth inhibitors, and not because of the lack of an essential metabolite.

Information on the role of autogenic inhibitors in limiting growth is less specific and more difficult to obtain than evidence for the limitation of growth due to a deficiency of an essential metabolite. How commonly do internally produced inhibitors reduce growth? What is the nature of these substances?

From the investigation of antibiotic substances we know that many organisms form metabolic products, highly inhibitory for organisms other than themselves. Do they also produce substances which limit their own growth? The role of autogenic inhibitors in limiting growth deserves much more attention than it has received.

It is well known that minute amounts of specific chemical compounds materially modify the amount and nature of growth in plants. Zimmerman and Hitchcock (1949) treated Kalanchoe plants with small amounts of the ortho, para, and meta forms of chlorophenoxyacetic acid. The para form caused the apical meristem to develop into a spathe-like organ which could be cut off and rooted. It had little resemblance to Kalanchoe. The ortho and meta forms of this compound did not have this effect. This modification was not a mutation. The effect wore off as the chemical in the plant disappeared, and the Kalanchoe eventually returned to its normal growth pattern. If the change had been permanent, we would have been inclined to call it a mutation and look for a genic explanation; i.e., look for a gene which controlled the

production of para-chlorophenoxyacetic acid. We might say that this compound and the Kalanchoe plant acted temporarily as linked genes.

Many other kinds of abnormal growth in plants are probably the result of the effect of minute amounts of specific chemical compounds. Insect galls are characterized by an abnormal but specific growth pattern superimposed on normal tissue by the presence of a foreign living organism. It seems very likely from the observations of Boysen Jensen that the abnormal growth of insect galls is caused by specific chemical compounds produced by the larvae which inhabit the galls.

It must be emphasized that growth is an extremely complex process, not just a series of chemical reactions. To consider it as such is admittedly an oversimplification giving no thought to the organization in which these reactions occur, or to the structural elements, physical processes, and chemical reactions which must play a role.

The concept of growth as a series of catalyzed reactions is useful and stimulating, however, in considering the role of essential metabolites—especially enzymes—and the action of inhibitors and minute amounts of specific chemical compounds.

HYBRID VIGOR

Some years ago I attempted to determine whether hybrid corn contains a greater quantity of substances which stimulate the early growth of *Phycomyces Blakesleeanus* than the inbred parents. The effect of extracts of air dry grains and of partially germinated grains of the hybrid corn and its inbred parents was determined on the growth of Phycomyces in the presence of thiamine (Robbins, 1940, 1941a).

When compared on the basis of extract per grain, I found that the extracts of the grains of the hybrid corn gave a greater dry weight of mycelium of Phycomyces than those of either of the inbred parents (Fig. 7.2). The stimulating material seemed to be present in both the embryo and the endosperm. Since the solution in which the beneficial effects of the extracts were exhibited contained sugar, asparagine, mineral salts, and thiamine, it appeared that the effect was produced by unidentified growth substances. These were termed for convenience, factor *Z*.

After estimating the amount of factor *Z* present—from the effects of the extracts of the corn grains on the early growth of Phycomyces in the presence of thiamine—the following generalities seemed permissible. The amount of factor *Z* increased with the time of the germination of the corn grains, at least up to seventy-two hours' germination. The quantity of *Z* was greater per endosperm than per embryo, and was greater in the grains of the hybrid than in those of either parent. The amount of thiamine and its intermediates in the embryo and endosperm of the grains of the hybrid and its parents was not correlated with the amount of factor *Z*, nor did the amount of biotin in the extracts appear to be correlated with the amount of factor *Z*.

These results suggest that there is present in the grains of corn, material which stimulates the early growth of Phycomyces in the presence of thiamine, and that there is more of this material per grain in heterotic hybrids than in those of the inbred parents.

Interpretation of these results depends in part on the identity of factor *Z*.

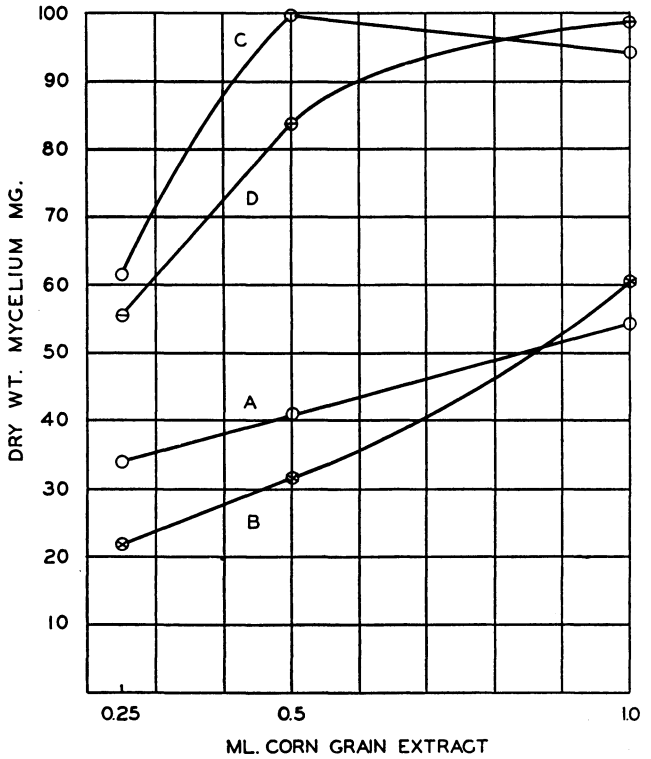

FIG. 7.2—Increase in dry weight of *Phycomyces* produced by extracts of air dry grains of maize. Extracts added to medium of sugar, minerals, asparagine, and thiamine. *A* = line 4–8; *B* = line 187; *C* = 985, 4–8 × 187; *D* = 995, 187 × 4–8. 1 ml. extract = 1 grain.

Unfortunately, we do not know what factor *Z* is. We succeeded in dividing it. We demonstrated that factor *Z* is multiple, and separated it into a fraction adsorbed on charcoal, factor Z_1, and a filtrate fraction, factor Z_2. Factor Z_1 was identified as hypoxanthine. Factor Z_2 may be a mixture of amino acids.

Although this problem is left in an uncertain and unsatisfactory condition, it suggests a line of attack. This would be an investigation of heterosis by studying the effect of extracts of parents and of heterotic hybrids on the growth of other organisms. This may serve as a means of bioassay for favorable or unfavorable growth factors.

Vigor in Heterocaryons

Observations of Dodge (1942) on heterocaryosis in Neurospora are of interest to the general problem of heterosis. Dodge inoculated three petri dishes, one with his Dwarf 16 strain of *Neurospora tetrasperma*, one with race C-8, and the third with mixed mycelium or conidia of both the dwarf and the C-8 races. He observed that the mycelium of the mixed culture grew much more rapidly and produced more abundant conidia than the mycelium of either the dwarf or the C-8 races (Fig. 7.3).

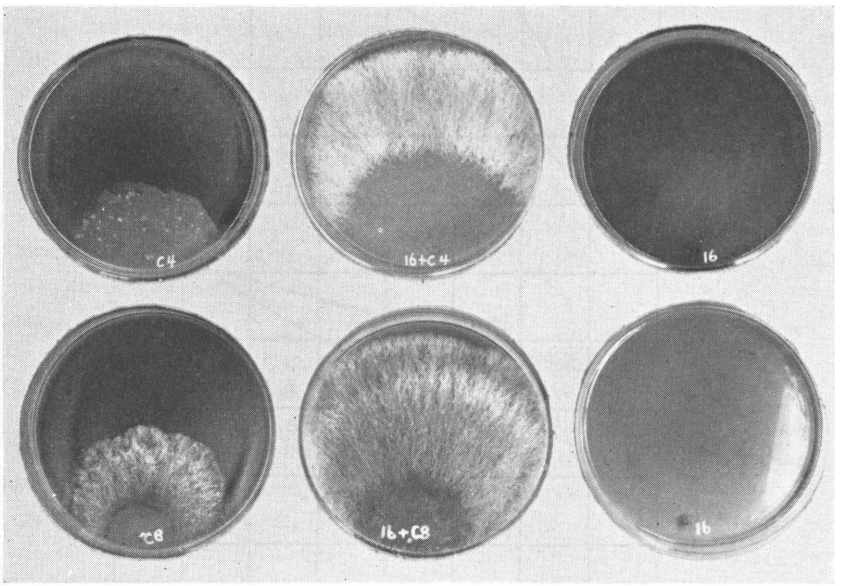

FIG. 7.3—Heterocaryotic vigor in *Neurospora tetrasperma*. Growth in 34 hours at room temperature in petri dishes. The mycelium of the two heterocaryotic races (*16* + *C 4* and *16* + *C8*) has nearly covered the medium in the dishes; *C4* and *C8* have not grown halfway across the medium and *Dwarf 16* has made no visible growth.

When two races of *Neurospora tetrasperma* are grown together, there is a migration of nuclei through the openings at the points of hyphal anastomoses. The races need not be of opposite sex. After nuclear migration, the cells of the resulting mycelium are heterocaryotic. They contain two kinds of haploid nuclei. The greater vigor of the mixed culture referred to above appears to be the result of the presence in a common cytoplasm of two kinds of nuclei.

Heterocaryotic vigor does not always accompany heterocaryosis. Dodge (1942) observed heterocaryotic vigor when the two races, Dwarf 16 and C-4, were grown together. But heterocaryosis for races C-4 and C-8 did not result in increased vigor in the mixed culture. Not all dwarf races act as race 16 does. Some of them evidence heterocaryotic vigor with both C-4 and C-8,

others with C-4 but not with C-8, and still others develop none with either C-4 or C-8.

Dodge has suggested that the heterocaryotic hybrid may synthesize a full quantity of growth substances or essential metabolites. Whereas the growth of each of the parents is limited by their inability to synthesize adequate quantities of one or more essential metabolites.

Dwarf 16, for example, may be able to make adequate quantities of essential metabolites 1, 2, 3, and 4, but unable to construct enough of 5, 6, 7, and 8. On the other hand, race C-4 may be unable to synthesize enough of 1, 2, 3, and 4, but be capable of producing an adequate supply of 5, 6, 7, and 8. When nuclei of the two races are brought together in a common cytoplasm, the essential metabolites synthesized by one of the nuclear components supplement those synthesized by the other component. The heterocaryotic mycelium is then supplied with adequate quantities of all the essential metabolites necessary for rapid growth.

We have tried to test this hypothesis by supplementing with various substances the medium on which race 16 and other dwarf races were grown. If it were possible to increase materially the growth rate of the dwarf race by supplements in the medium, without introducing the heterocaryotic condition, the limiting factors for dwarfness could be identified and the stimulus involved in the heterocaryotic condition identified.

A basal agar medium containing mineral salts, dextrose, asparagine, neopeptone, and thiamine was supplemented by a mixture of purine and pyrimidine bases; by a vitamin mixture containing PAB, calcium pantothenate, inositol, nicotinic acid, pyridoxine, riboflavin, thiamine, guanine, hypoxanthine, and 2-methyl-1, 4-naphthohydroquinone diacetate; by malt extract, casein hydrolysate, cow's milk, dried yeast, choline, *a*-tocopherol, hemin, oleic acid, ascorbic acid (filtered sterile), coconut milk, Taka-diastase (filtered sterile), water extracts of the mycelium of Neurospora, liver extracts (both filtered sterile and heated), adrenal cortical extract (unheated), estrogenic substance, progesterone, anterior pituitary extract, posterior pituitary extract, whey, or potato extract.

None of the substances or combinations of them as used increased the growth rates of any of the dwarf races to an extent adequate to explain heterocaryotic vigor. Some beneficial effects, usually noted only in older cultures, were obtained from cow's milk and from liver extract. These effects were not sufficiently marked to suggest that either supplement supplied the missing factors.

We were unsuccessful, therefore, in defining the factors limiting the growth of the dwarf races and conversely those effective in inducing more rapid growth in the heterocaryotic mycelium.

Our failure may be explained in various ways. We may not have included in our various supplements the missing essential metabolites. These metabo-

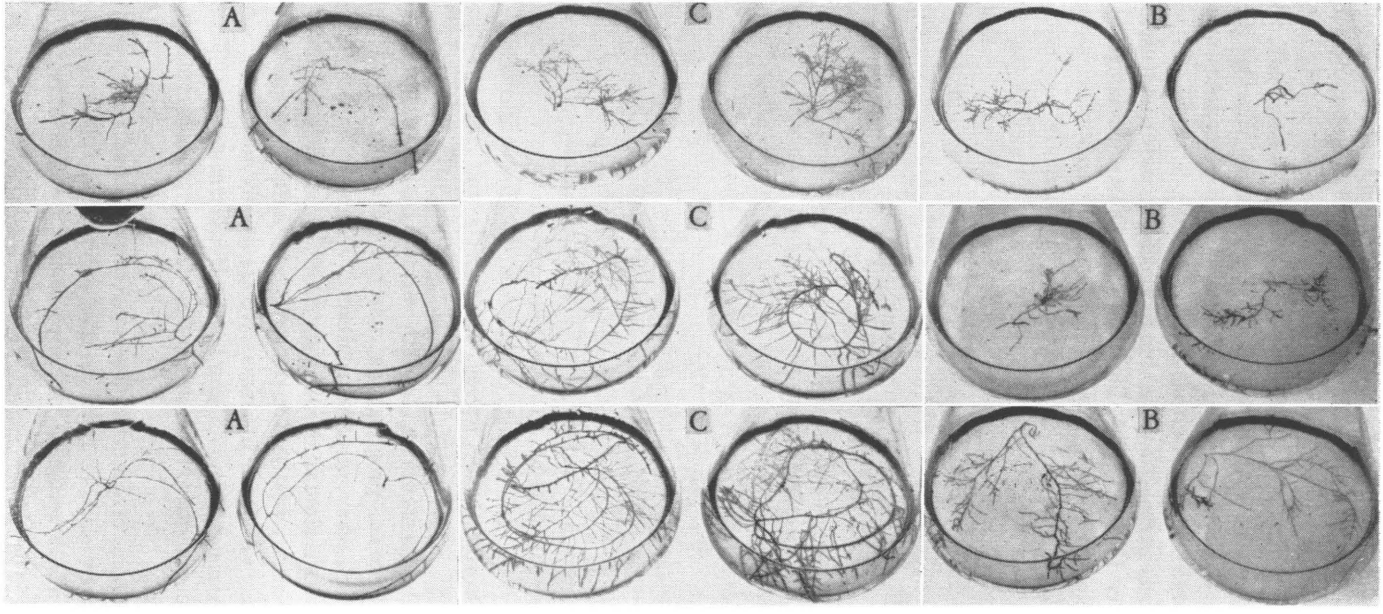

FIG. 7.4—Heterotic vigor in excised tomato roots. *A*, Johannesfeur; *B*, Red Currant; *C*, the heterotic hybrid. Above, grown in solutions supplemented with thiamine; center, thiamine and pyridoxine; bottom, thiamine, pyridoxine, and nicotinamide.

lites may be non-diffusible or very labile substances such as enzyme proteins, which could only be introduced into the cell through inserting a nucleus and its genes. The original hypothesis may be in error. We may not be dealing with limiting quantities of essential metabolites but with inhibitors. We might assume that the growth of one or both of the parents is limited by autogenic inhibitors, and the presence of both kinds of nuclei in a common cytoplasm results in the neutralization in some fashion of the inhibitors.

Emerson (1948) has succeeded in producing heterocaryons in which one kind of haploid nucleus neutralizes the effect of the other. The augmented growth of the heterocaryon, as compared to that of strains which are homozygous, reminds one, says Emerson, of instances of single gene heterosis in maize reported by Jones.

The importance of internal factors in heterosis is suggested by the results I obtained on the growth of the excised roots of a heterotic tomato hybrid and its inbred parents (Robbins, 1941b). The hybrid roots and the roots of the two inbred parents were grown in liquid culture which contained mineral salts and cane sugar. This basal medium was supplemented with thiamine, with thiamine and pyridoxine, and with thiamine, pyridoxine, and nicotinamide.

Growth of the roots of the hybrid exceeded that of either of the inbred parents in all three types of media (Fig. 7.4). Growth of one parent was improved by the addition of pyridoxine to the thiamine solution, but a further supplement of the medium with nicotinamide had little effect. Growth of the second inbred parent was little affected by the addition of pyridoxine to the thiamine medium, but was improved by the further addition of nicotinamide to the thiamine and pyridoxine solution.

These results suggest that the greater vigor of growth of the heterotic hybrid is determined in part by its greater ability to synthesize pyridoxine and nicotinamide. That is evidently not the whole story, because its growth exceeded that of the inbred parents in media containing all three vitamins.

Although heterosis may be considered and should be considered from the genetical standpoint, it should also be studied from the physiological standpoint. I have suggested that it may be important to devote attention to the question of what the internal factors are which limit growth, what they are in inbreds, and how they are removed in heterotic hybrids. We should consider in such investigations the role of essential metabolites, of growth inhibitors, and of other specific chemical compounds which materially modify growth. Microorganisms might be utilized as tools for the detection of growth stimulators or growth inhibitors.