# Meristem Tip Culture

**by Gerald G. Weland**

In the summer of 1974 the author conducted a program of Meristem Tip Culture for the purpose of developing a procedure to recover healthy stock from virus infected dahlias in a home environment,. A second similar program was conducted during the summer of 1975 using the same procedure. This article is to document the procedure developed during the conduct of those programs and supply it to the general dahlia public. A step-by-step procedure has been written in report form and made available to several persons who have expressed an interest in the program and the resulting procedure. That detailed procedure is still available to anyone desiring a copy. The following article, although complete in operational details, is hereby edited and condensed for this publication. The original article was first published in the Dahlia Reporter in the fall 1975 edition.

## Forward

Sometime in the mid 1960s the author attended a seminar conducted by Dr. Georges Morel at which he reported on a program of plant tissue culture that he and his partner Dr. Claude Martin had conducted. He reported that their experiments were connected with a program of Meristem Tip Culture of several types of plants including dahlias. For the next several years the author began reading, researching and gathering information about the subject. During the summer of 1974 he was fortunate to be able to attend a workshop program in plant tissue culture at the University of California at Riverside. This article summarizes that experience, the results of two small programs of Meristem Tip Culture which was a fallout of that course, and some conclusions about the future of this process in its application to dahlias.

## Introduction

There are many branches of plant tissue culture. In the workshop course at the University of California. Each student is encouraged to follow their particular branch of interest as well as to work with plant varieties of their choice. The authors choices were to work with dahlias in using the culture process which utilizes the meristem tips of axial shoots. The reason for these choices was primarily to attempt to recover healthy stock from dahlia plants infected with virus.

In 1952 Doctors George Morel and Claude Martin of France showed that certain plants no matter how badly affected with virus diseases, could be recovered and again propagated in a healthy state. Their experiments showed that virus is not equally distributed into all parts of a growing plant, and that the rapidly growing shoot tips have a very good chance of being free of virus pathogens. Fortunately, some of those early experiments were with dahlia. Unfortunately, due to lack of a substantial commercial market for dahlias they were forced to give up that program in favor of crops with a larger commercial demand. There has since been a few small programs with dahlias but none sufficient to recover any large amount of plants or to refine to any large extent, the techniques proposed by Morel and Martin. The first dahlia recovered was *Reve Rose*

*(Pink Dream)* and altogether they succeeded in recovering a total of 15 varieties. The system used for recovering healthy plants from virus infected stock was that of Meristem Tip culture (MTC). By this process a very tiny piece of growing plant issue is removed under extremely sanitary conditions, placed in a culture tube on a nutrient medium and grown under artificial conditions until large enough and mature enough to be grown satisfactorily in the open. Meristem Tip Culture takes advantage of one of the natural developments of plant growth. There are three basic ways in which plants grow; cell division, in which cells are formed; enlargement where existing cells increase in size; and cell differentiation where cells take on the shape and orientation which determines the form and structure of the plant organs. Meristem Tip Culture is concerned with the first of these, i.e. cell division.

In many areas of a plant there are small regions where cell division is nuch more rapid than in others. These areas of rapid cell division are referred to as meristematic regions or simply meristems. Any of the several meristems could be used for culturing but the most practical for dahlias seems to be the one near the tip of the growing shoots. This meristem is not extreme tip of the growing shoot but is located a few rows of cells behind or back of the tip. The meristem is exposed by removing as many leaves as practicable from the growing shoot. The growing tip then appears rounded, shiny and semi-transparent. When the visible leaves have been removed and the shoot tip placed under a fairly strong microscope several protuberances may be seen about the rounded tip. These are leaves in the process of being formed (an example of cell differentiation) and are called primordial leaves. As many of these are removed as possible without damaging the rounded shoot tip:

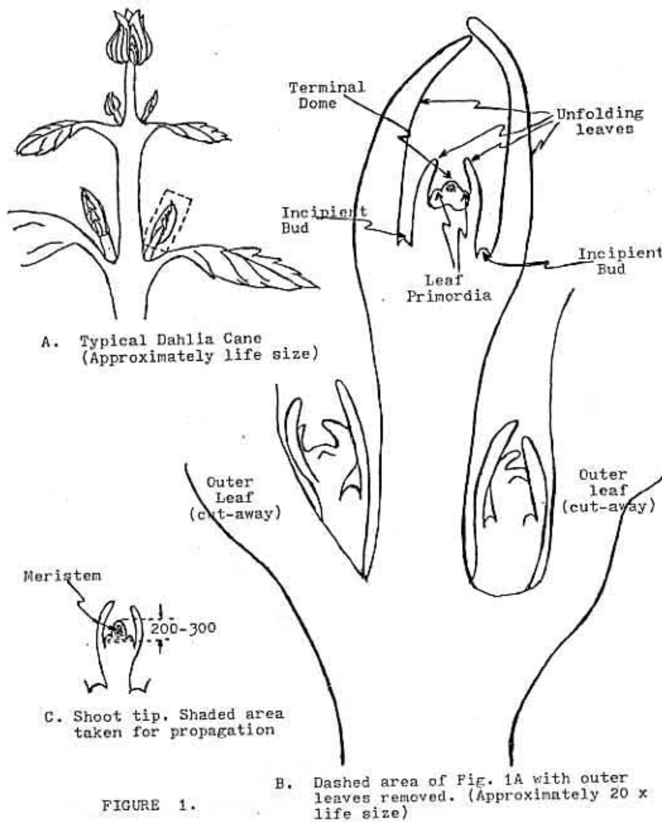


FIGURE 1.

This tip, very tiny (smaller than a period) , is then cut from the shoot and placed in a culture tube containing the nutrient solution. The culture tubes are then capped and placed in an environment with controlled heat and artificial light to facilitate their development into a plant. All this must be done under extremely sanitary conditions to prevent contamination of the cuttings or tube by fungus spores.

As can be noted from this description the cutting used for propagation (called a propagule) includes not only the meristem but also the few rows of cells between it and the tip, some small amount of tissue below or sub-jacent to the meristem and probably the first two primordial leaves. For this reason the process is properly know as Meristem Tip Culture and not simply Meristem Culture as so commonly used in the past. The amount of sub-jacent tissue used , and the number of primordial leaves retained are a matter of compromise. The smaller the amount of tissue used the better the chance of obtaining a virus-free propagule. On the other hand, the smaller the propagule, the less likely to survive in culture.

Although not a recent development, MTC is only recently becoming popular with plant breeders and propagators. There are several uses for MTC: genetic improvement of crops; production of pharmaceutical products; preservation of germ plasm; the rapid increase of such flower crop as Carnations, Gerberas, Orchids, Poinsettias, etc; and most recently as a means of recovery of healthy stock from plants infected with virus.

Most dahlia growers are aware that many of the older varieties (and newer varieties too for that matter) are quite thoroughly infected with various virus diseases. A method of propagating which would assure that a dahlia root or plant for which the growers pay their hard-earned money, was free of those debilitating viruses should be a boon to dahlia circles. This is the promise of MTC and seems definitely within reach.

The basic technique for recovering virus free stock has been developed. It has been applied successfully to many varieties of commercial crops such as potatoes, asparagus, strawberries and tobacco, to name a few. There have been very few programs or efforts designed especially for application to dahlias although it was one of the very first plants to be recovered through MTC.

Dr. Morel and Dr. Martin experienced and reported difficulties in working with dahlias. One such difficulty is that the propagules failed to make roots in the culture tubes. This necessitated grafting the propagules onto a young seedling plant. Since the propagules grown in culture are quite etiolated (spindly) they are generally very delicate, a high mortality rate was experienced. Several hundred cultures were required to get even a very few plants. Additional research and experimentation will be required to perfect the grafting procedure. Recent developments in plant tissue culture techniques has led to enhanced root formation on plants other than dahlias. Through use of those techniques the author has been able to establish a procedure leading to a substantial increase in root formation in his cultures. That procedure will be detailed in the following procedure.

The workshop courses in Plant tissue culture previously referred to has been offered each summer by the Department of Plant Sciences, University of California at Riverside. This course is offered in cooperation with the California Association of Nurserymen and is sufficiently

general to be applicable to most horticultural varieties. Leading Plant Tissue authorities seem to agree that the overall technique of MTC is sound and workable and requires only refinement for each variety of plants. The workshop consists of both formal instruction and practical experience into taking and culturing of various plant tissues. The cultures thus established are propagated for a time in the culture rooms at the University. The research program of the resident students take precedence over workshop programs, so when the academic year begins, the workshop cultures must be removed to make room for controlled research program materials.

The author was fortunate to visit the University when the "house cleaning" was taking place and able to salvage some of his cultures. Out of 24 dahlia meristem tips placed in culture, 8 were recovered and brought home. Because of their immature nature and the lack of an adequate culturing environment, only three of those grew to grafting size. Different types of grafts were used for each propagule.

One graft was established but later the root stock damped off. Efforts to re-graft failed so all propagules of the initial program were lost. Considering the lack of facilities and the conditions under which these first propagules were grown and grafted the loss was understandable. Some improvement in facilities was obtained and the first of these programs was initiated. The nutrient medium used was made up of a combination of both inorganic mineral salts and organic substances. Each is included to promote a particular function and to facilitate organic response in the plant. Since the days of Dr. Morel's and Dr. Martin's experiments much has been learned regarding these substances and their effect on organ growth and differentiation. The second program was conducted employing a process and nutrient medium designed to promote rooting.

After the propagules develop into plants and grow to maturity, they require indexing to see if they are truly free of the specified virus. At the present time, California does not have a program for indexing dahlias nor, in so far as the author has been able to determine, does any other state or agency. Such programs do exist for other crops and according to the District Supervising Biologist of the Department of Agriculture for this part of California, a program could be set up for dahlias with sufficient demand and funding.

In the absence of a State-sponsored program for indexing for the disease-free nature of recovered plants, a blue-ribbon panel of local dahlia experts would probably be a suitable temporary alternative. The dahlias could not in this way carry the designation of being certified by the D. of A, but in the author's opinion would be an acceptable stop gap. Certification by a panel of this type would probably be equally if not more meaningful to those who know dahlias best, the dahlias hobbyists. This seems axiomatic since there are several enthusiasts probably more familiar with the manner in which dahlias virus is manifested than anyone else in the country, and several of them reside in the local area. Various methods of indexing have been developed and reported which would assist the panel in determining the pathogenic condition of the recovered stock. Some of these methods are presently being investigated and can be reported on at a later date.

The experience of the author to date has convinced him that the recovery of virus free dahlias from plants which are completely infected is not only possible but feasible under home conditions, providing proper care and precautions are exercised. Some specialized equipment is

required and considerable time will be needed to develop a stock of virus free dahlias and to perfect the technique which will result in an improved success rate of the overall process. The author believes this is possible and is presently continuing experimentation along such lines. Space suitable for culturing is rather at a premium but at the time of writing something over 1100 cuttings are in various phases of culture.

# Procedure

# Introduction

The process to be covered in this paper is a particular area of the general operation known as Plant Tissue Culture. This process has been referred to as 'Meristem Culture', 'Meristem Tip Culture', 'Shoot Tip Culture', etc, depending on the type and size of the tissue used for culturing . In this paper the term 'Meristem Tip Culture' will be employed as the term best describing the tissue being treated.

Description of this operation is possible only through use of and reliance on certain botanical processes. It will not be an exercise in Botany although certain botanical terms will occasionally be used. In fact, a discussion such as this would not be possible without resorting to botanical terms on occasion. Where such terms are necessary they are explained in a way that the reader, even with a limited knowledge of Botany, will be able to understand and follow the procedure. In order to be as explicit as possible it will be necessary at times to make general statements. The statements may not necessarily be completely true for all specific details. If the reader notices any such statements he is requested to consider the reason and bear along

# Why is Meristem Tip Culture Important?

In an article written as a special publication for the American Dahlia Society in 1959 (1) Mr. Roland A Milner Jr. summarized much of what was known about dahlia virus disease to that time and drew several conclusions regarding the extent, damage and general effect of various virus diseases in dahlia, and stated an urgent need for research into the control and identification of such diseases. The opening paragraph of the article states "Virus diseases are the chief limiting factors in dahlia production. All other diseases and pests, indeed all other problems concerning dahlia growing are insignificant in comparison." Other widely publicized and quoted reports (2,3,4) arrive at substantially the same conclusions.

In the same opening paragraph Mildner states, "moreover the situation is confounded by the refusal of growers, both professional and amateur to admit that the problem exists".

In the years since that report more and more growers are admitting the presence of the problem, and many have expressed concern. A great many have become aware the Meristem Tip Culture process offers a possible solution but appear to be unfamiliar with that process. This paper will describe in detail a program conducted under routine home environmental conditions that has successfully recovered symptomless plants of dahlia from stock which had previously expressed very pronounced symptoms of virus infection.

# History of Plant Tissue Culture

The entire idea may be said to have started in 1902 with a German scientist named Haberlandt who attempted to culture Chlorophyll-containing cells and demonstrate the totopotentiality of cells. (totopotentility means that every cell of an organism has the potential of every other cells or total potential).
To say it another way, each cell of the organism contains factors which determine the final form of the entire organism. (These factors are known today as Genes) Although Dr. Haberlandt's success was not enthusiastically received, he did manage to initiate a new method of plant propagation which has become known as 'plant tissue culture'.

In 1946 another Scientist by the name of Ball (5) obtained complete plants of Lupine through the culture of shoot tips. About this same time several people were studying the distribution of virus proteins in the interior of plants. They found that the presence of virus proteins decreased rapidly when going from the adult leaves to the younger growth. The virus in the terminal buds was so feeble that even with very precise tests its presence could not be determined. Analyzing this thought two French Research Scientists, Dr. Georges Morel and Dr. Claude Martin concluded that it might be possible to recover plants from virus infected stock through the technique used by Ball.

For three years they experimented along that line. We can be thankful that one of the plants chosen for the experiment was dahlia. The results of their experiments were published in a paper presented to the Academie des Sciences in November 1952 (6) Unfortunately dahlia has never been a truly commercial flower. Drs Morel and Martin were forced to abandon their dahlia research in favor of crops of a commercial nature, such as carnation, asparagus, strawberries, potatoes etc.
In the years since 1952 there has been an increasing interest in Plant Tissue Culture for propagating many types of plants. Meristem Tip Culture has been primarily used to increase stock of desired plants which do not reproduce true through sexual means. Other applications include the genetic improvement of crops, the production of pharmaceutical products, preservation of germ plasm and more recently the recovery of disease free plant stock.

The title of the paper describing the experiments of Drs. Morel and Martin when translated to English was "Cure of Dahlias Affected by a Virus Disease Through Culture of Apical Meristems" In a strict sense the dahlias were not cured but rather virus free dahlia cultivars were recovered from a variety known to be totally infected with one or more virus diseases. They succeeded in recovering a small number of plants of the variety *Reve Rose* (Pink Dream) from dahlia mosaic. As reported, the plants "developed normally showing none of the symptoms of dahlia mosaic. On two occasions serological tests were carried out on the plants; both tests gave negative results."

In 1955, F. O. Holmes (7) reported on his success in the elimination of Tomato Spotted Wilt virus (TSWV) from dahlias by propagation of small tip cuttings. In 1962. Michael Hollings (8) reported he had successfully recovered dahlias from Cucumber Mosaic Virus (CMV) by growing the plants under heat for four weeks then taking small tip cuttings. There is no known record of the recovery of dahlias from any other type of virus.

It is difficult to determine exactly how many virus diseases affect dahlias. There have been a dozen or more virus diseases reported; Dahlia Mosaic, Yellow Ringspot, Dahlia Oakleaf, Tomato Spotted Wilt, Cucumber Mosaic , Dahlia Stunt Disease, Dahlia Leaf Curl, Dahlia Leaf Roll, and Aster Yellows, to name the most commonly listed ones. Brierley and Smith writing in 1950 (9) stated that Tomato spotted Wilt induced most of the ringspot and oakleaf patterns in the United States. Dr. John Grainger (10) attributed Oakleaf to a strain of Cucumber Mosaic virus. Consequently there appears to be only a few different viruses which affect dahlias and manifest themselves differently in different varieties of dahlias.

At the present time most authorities agree that there are three or possibly four distinct virus diseases which affect dahlia. Dr. Lawson (4) identified four viruses which he had isolated from different varieties of dahlias: Dahlia Mosaic virus (DMV), Cucumber Mosaic Virus (CMV) , Tomato Spotted Wilt Virus (TSMV) . His method of identification was to compare virus isolates with type viruses. The virus isolates were obtained from various varieties of dahlia; the type viruses are those which have been positively identified through laboratory procedures and maintained as laboratory stock. Features and reactions of two isolates compared favorably with the same features and reactions of CMV and TSWV. No type viruses for DMV was available so his comparisons were made against descriptions in available literature on the subject. The comparison seemed sufficiently accurate to establish DMV as a third definite pathogen. The fourth pathogen was less definite. He summed it up as "a ringspot virus possessing host range and physical properties similar to those of Tomato Ringspot Virus" Other authorities seem to lean to the conclusion that TRV is a mutant or variant of TSWV.

AUTHOR'S NOTE: ( In the five years preceding 1998 several other viruses have been reported as affecting dahlia. They are not included here as definite viruses since no information on method of determining their presence or identification has been published).

From the results of experiments with these diseases on other plants it appears highly likely that the basic method used by Dr. Morel and Dr. Martin, can be readily used for recovery of plants afflicted by DMV. Furthermore, since it appears from the Holmes and Hollings operations that TSWV and CWV respectively can be overcome by relatively large cuttings, it certainly seems likely that any process which will accomplish recovery of stock from plants infected with DMV will also eliminate the other pathogens, if present. Since Meristem Tip Culture has been used successfully to recover healthy plants from varieties infected with DMV and presumably any other known type of virus present in the specimens used, it appears that MTC offers a definite promise of recovering healthy dahlia plants under any conditions of virus infection.

# Getting Acquainted

By definition the 'meristem' is "plant tissue in process of formation; vegetable cells in a state of active division and growth."

There are therefore several meristematic regions in plants. The region of interest in this paper is the one at the tip (apex) of the very young shoots which develop from the areas where the leaf and stem meet (the leaf axis) (See Figure 1). The meristem is enclosed in a tiny terminal dome which is the actual end of the growing shoot. In the very youngest state this area of rapid cell

division has not yet developed into elongated growth but is simply an area of incipient growth. Once elongation is initiated the terminal dome becomes encased in leaves. These leaves generally extend beyond the meristematic dome making it appear that the leaves rather than the dome is the tip of the shoot. However these leaves which enfold the dome may be removed until the dome is exposed. The closer we approach the dome the smaller and less identifiable the leaves become. The very youngest leaves appearing as no more than bumps protruding from the domed region just slightly back from (below) the extreme tips. These leaves, still in the incipient state are called leaf primordia. The section of plant tissue just below the meristematic region, and the area from which the leaf primordia project is called sub-jacent tissue.

The true meristem in dahlias is in the order of 50 to 80 micrometers (microns) in diameter. Dr. Morel and Dr. Martin in their experiments used sections of tissue approximately 250 microns in size. All other known operations including the programs being discussed herein have used comparable sized sections.

There are certain advantages to be derived from the use of smaller sections, for example, the smaller the section the better the probability that it will be free of virus. On the other hand the smaller the section the more difficult it is to work with and the lower the survival rate in culture. It has been found that even with the larger sections (ie approximately 250 microns) the probabilities of obtaining pathogen free explants (the small plant that grows from the cutting) are acceptable. The sections that have almost always been used in the past and which will most probably be used in the future are in the order of 250 microns. Sections of dahlia shoots of this magnitude will include one or two leaf primordia, the few rows of cells between the meristem and the actual tip of the shoot and some sub-jacent tissue.

The recent interest in Meristem Tip Culture for propagation of dahlias has been stimulated by the growing concern over the apparent spread of virus disease in these plants and the realization that 75 to 90 percent, if not more, of all dahlias being gown at the present time are infected with one or more virus diseases. Although new varieties of dahlias of excellent quality are constantly being developed and introduced, many people believe that a large number of the older varieties are still the best of the class. The results at the annual dahlia shows will usually bear this out, the debilitating effects of virus notwithstanding. Unfortunately most of the older varieties are probably 100 percent infected. Brierley(2)put it this way, "any variety that has been field grown for five years or more has a very high likelihood of being infected with some virus disease".

It has become almost axiomatic with dahlia growers that seedling dahlias are free of virus. Many authors mention in their writings that dahlia virus is not transmitted through seed. However they either fail to lay any basis for this statement or simply cite Dr. Brierley's article. It is interesting to note that Dr. Brierley makes no such claim that such is the case. In discussion of Dahlia Mosaic he has only this to say, "the writer has found no evidence suggestive of persistence in soil, and no evidence of seed transmission." His only statement on the subject is, "no mosaic appeared in 44 seedling dahlias grown from seed of *Catherine Wilcox.* Three types of seedling dahlias grown from commercial seed proved free of mosaic". In summarizing the article he says simply, "Mosaic persists in vegetative parts of affected plants but has not been found to pass through seed." The idea that virus does not pass through seed is not only virtually unfounded but definitely in error as shown in the article 'Dahlia Virus and Seedlings' in the Dahlia Reporter Vol

10, No. 2, 1980(12). That article based on records and experiments over the years 1976 through 1978 established that virus is indeed transmitted through seed. Growing and comparing over 800 seedlings from known diseased varieties and the same varieties that had been recovered through Meristem Tip Culture showed that virus transmission does occur in a surprisingly high percentage of seedlings from diseased dahlias. Distribution according to type of virus was not determined.

Since only a short and limited program was carried out by Dr. Morel and Dr. Martin, much experimentation and refinement of their technique and procedures will be necessary before an acceptable level of success may be expected. An acceptable level of success is envisioned as one which would make the program commercially feasible. In addition to the Morel and Martin program there have been a few other experiments carried out along the same line. These programs have in general only verified the possibilities of recovery of healthy plant material and have not greatly improved or modified the basic technique.

The scientific and operational requirements of a program to recover healthy dahlia cultivars from diseased stock are not highly demanding nor complicated. They do require a reasonable knowledge of organic chemistry and botany of dahlias as well as a fairly high degree of manual dexterity. The expense of setting up a facility suitable for conducting a commercially successful program may prove to be the limiting factor although the program to be described later has shown that such a program can be performed successfully in a normal home environment.

In the United States at the present time the dahlia is primarily a hobby crop and not a highly demanded cut flower. The popularity of dahlias as cut flowers seems to be increasing but would appear to still lag the popularity of roses and carnations as example. Therefore the present commercial value of dahlias is mostly as roots, pot roots, or plant starts sold to dahlia hobbyists, for home garden and exhibition purposes. When the dahlia does attain status as a popular cut flower, top exhibition quality will probably not be a requisite. In fact top exhibition quality would not be practical in a cut flower. The cost of producing top quality blooms would price them out of the cut flower market. Many new varieties coming on the market will be suitable for cut flowers. These new varieties will generally be reasonably tolerant of virus for two or three years. Even after becoming infected the general purchaser of cut flowers would not be aware of such infection, nor would they be concerned since they are purchasing blooms to be enjoyed for a few days and thrown away, and have no concern about further infection of other stock. Another prime consideration for a cut flower variety is prolificness. This is generally not an important characteristic of exhibition varieties where number of blooms are held to but a few on a plant anyway in order to attain that final measure of perfect form and size. Thus it appears that if a program to recover healthy cultivars from diseased stock is to be undertaken it would be primarily for the benefit of the dahlia hobbyist and the producer of exhibition variety stock

It has been variously stated that planting of anywhere from one thousand to ten thousand seedlings is required to have a reasonable probability of producing one variety equal to those already in existence. If this ratio could be reduced to one out of ten or even one out of 20 it would be a great boon to the world of dahlias. This is in effect the promise of Meristem Tip Culture. Even this percentage may be greatly improved after experimentation and development of an ultimate program.

Unlike experimenting with seedlings the quality of the dahlia obtained through Meristem Tip Culture is known beforehand. If we obtain a clone of *Juanita* or *Kidd's Climax* or of *Golden Heart* we know we have a clone of superb quality. We may be pleasantly surprise at just how high the quality turns out to be. We are accustomed to seeing blooms of these varieties with certain characteristics, which we recognize as outstanding. However these blooms are outstanding in spite of probable debilitation from a virus disease. When virus free clones of these varieties are recovered the resulting blooms, unaffected by the debilitating effect of virus may be better than we were to expect.

In the spring of 1978 catalogs of commercial growers there was over 4000 different dahlia cultivars listed in the catalogs published in the United States alone. How many of these varieties are wholly or in part diseased is not known, From the author's experience over the years 1967 through 1978, for which records were kept the number has proven to be considerably higher than might be generally believed. During that period a total of 63 orders were placed with 21 different sources around the world. It was necessary to rogue out from 47 percent of those from one source to 100 percent of those from another the first year grown.

Although each of the 4000 plus varieties obviously has its admirers, to attempt to recover stock of all diseased varieties would be economically impractical. On the other hand hit or miss selection of a few favorite varieties would do little to alleviate the problem of virus disease in dahlias. A middle road where efforts were concentrated on two or three varieties of each classification group would seem to be a most sensible approach. This would result in 250 to about 400 cultivar recovery candidates. A program of this size is well within the capabilities of a well-organized Meristem Tip Culture operation. It would probably require 4 to 5 years to develop sufficient stock to provide starts for all the growers desiring them. Even then it would not be a one-time operation. To be effective it must be on a continuing basis. Since field grown dahlias will rapidly become re-infected (unless growers become sufficiently interested and conscientious as to eliminate all other types and grow only stock from recovered varieties), a steady on-going program will be required to continuously re-culture those varieties which again become infected.

There are several separate phases involved in the conduct of a program to recover pathogen free cultivars from diseased stock. Each phase has specific requirements of space, equipment, techniques and timing. Some of these phases are: developing and selecting stock for the the recovery program; performing the propagule excising operation; culturing the propagules; hardening off; grafting and/or rooting and planting out the developed explant; maturing the primary clone; developing stock of the primary clone; and certification of the pathogen free nature of the recovered cultivar. Each of these phases will be covered in detail in the following paragraphs.

# Operational Considerations

The basic method for recovering dahlia plants from virus infection is detailed in the following paragraphs. By basic method is meant the method used most successfully in the past and the one which would most likely be used as a reference or starting point for a Meristem Tip Culture program in the future. This program has proven satisfactory for use in a home atmosphere and

through its use 83 plants of 63 different varieties (35 plants of 26 varieties in the first program; 48 plants of 37 varieties in the second program) were satisfactorily recovered. The reference program is a revision of the Morel and Martin program in several respects. There are two primary areas of revision that may be pointed out at this time. The first is the nutrient medium used. Dr. Morel and Dr. Martin used the standard Knop medium. In a personal correspondence in 1967 Dr. Morel recommended use of a nutrient medium developed by Drs. Munishige and Skoog with certain slight variations. The nutrient medium used for this program was generally that recommended by Dr. Morel with several other small changes developed during the course of the programs. The second change is one of culturing technique. Dr. Morel and Dr. Martin reported that dahlias did not grow roots in vitro. They therefore found it necessary to graft the recovered explant onto a seedling plant. The etiolated nature of the explant makes this a very delicate operation with a high incidence of failure. The program being discussed here includes a re-culturing phase in which the nutrient medium was altered to induce rooting. Rooting resulted in approximately 35 percent of the propagules so treated, thereby eliminating the need for grafting, which is not only diffcult, but given the high percentage of seed transmission of virus, is highly questionable. Other minor revisions will be pointed out and their rationale given in the appropriate area of the discussion. This discussion may prove to be more detailed than required by an experienced operator. It is simply a step by step documentation of the programs conducted. It is not expected that the experienced operator will find it necessary to follow the step by step procedure. However any interested grower wishing to undertake a program of their own will find that the step by step procedure, if followed faithfully, will allow them to conduct a successful Meristem Tip Culture program.

It is in no way suggested that this is the ultimate program. The reference program may be expected to yield from 8 to 15 percent overall success over a period of 2-3 years. Variations in technique, sizes, amounts, quantities and ratios of medium ingredients or any other variable factor of the procedure must be attempted and results noted to determine the optimum method or value for the ultimate program. The experiments should include varying the constituents of the nutrient medium, varying light intensities and durations, varying temperatures, varying propagule size, varying methods of culture, etc. Varying each of these factors, or any other variable of the program above and below the values of the reference program, should establish an optimum value for that particular variable. Once optimums are established for individual elements, combinational variation could be attempted until an optimum program is derived. The degree of increased success rate cannot be estimated but could be substantial. The program described here has more than quadrupled the survival rate over that which relies on grafting as a part of the propagating procedure. Certainly any future Meristem Tip Culture program would not be adequate without additional effort to determine the optimum conditions and an ultimate program.

Meristem Culture has been defined as, a method of propagating plant life by aseptically isolating an apical meristem and implanting it on a nutrient medium to grow in vitro. We can therefore readily define Meristem tip culture by simply substituting Meristem Tip for Meristem in both instances in the above definition. Aseptic isolation is the handling and removal of the propagule under conditions which will prevent infection or contamination during the operation. The nutrient medium is a mixture of inorganic salts, organic compounds, hormones and growth

regulators, and a gelifying agent. This nutrient medium supplies all the known elemental requirements for growth of the propagule. In vitro means simply, in a glass container.

# Selecting the Plant Part to be Used

As shown by Haberlandt, every cell of an organism contains the total genetic potential of that organism. The older (more mature) cells have ceased dividing, or rather greatly decreased their rate of division, so it is very difficult to induce differentiation in them. The actively dividing cells of the meristematic regions are more apt to differentiate readily into the functional components of the organism. There are several meristematic regions in a growing dahlia plant suitable for propagating. The one that has been used in the past and the one still considered best for culturing is the apical meristem of axillary shoots. (Figure 1) These shoots are normally well above the ground, the tip is generally tightly enclosed by enveloping leaves and are quite free of fungus, dirt or any other contaminating agent. They are reasonably plentiful on a growing plant. Care must be exercised in selecting the shoots. A shoot that has already formed a flower bud is less suitable than a vegetative shoot. There is no sure way to tell whether a flower bud has formed until several sets of enfolding leaves have been removed. A good guide is to select only shoots that do not have unfolded leaves. A normal dahlia cane will have three to five internodes before the flower bud is formed. However it is not uncommon for this many sets of leaves (each marking an internode) to form, at least in a rudimentary manner, before the first set of leaves are fully unfolded. So, if the leaves are unfolding there is a high probability that the flower bud has already formed. Conversely, where the leaves are still tightly folded over the shoot tip there is a good probability that the flower bud has not yet formed. The flower bud is easily recognized once the first two or three sets of leaves are removed, but this is a loss of valuable time. With a little experience the operator will soon learn which size shoot will likely have flower buds and which size to expect vegetative tips.

Although the flower bud is less suitable than the vegetative buds, they can still be used if desired or necessary. The flower bud terminates development of a shoot so will not itself develop into new shoot growth, However if leaf primordia are included with the cutting there will be at least one incipient vegetative shoot meristem in the axis of these primordial leaves. If we remember the morphology of a dahlia the terminus of each cane has three growing buds - one a crown bud which will develop into a bloom, one a second flowering bud and the third having an additional set of leaves before the flowering bud. These latter two growths issue from axes of the crown bud and the uppermost set of leaves. The latter of the three buds will in turn have a meristemic region at its own leaf axis. These will be slower to develop in culture and the longer the propagule takes to develop, the greater the chance of contamination of the medium.

# Preparing the Shoot Tip

Removal of the shoot tip to be used for culture does not require any special aseptic conditions. Reasonable care should be used of course, but general home environmental cleanliness will suffice at this stage.

Since, it is impractical to sterilize the entire cane or even large cuttings, the area immediately surrounding and including the shoot tip is cut away from the dahlia cane in advance. A small block of tissue, approximately one-fourth inch cubed is left with the shoot tip to provide an area for holding the shoot tip while removing the outside leaves (Figure 2):

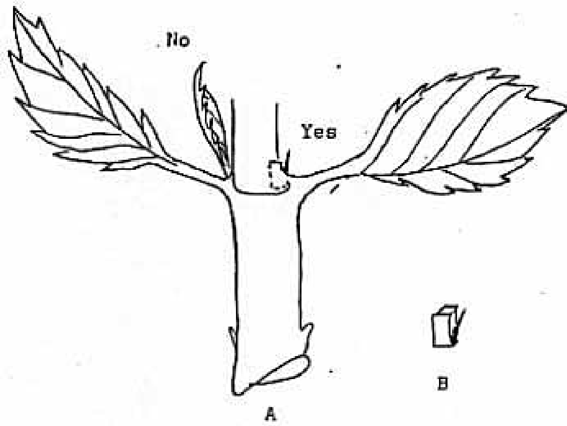



FIGURE 2.

To remove the small blocks of material a surgical scalpel or razor blade is used. Make four incisions around the shoot tip as shown by the dashed lines in the figure, being careful not to damage the shoot tip. The cuts should be made about an eighth inch above, below and on either side of the selected shoot. By sliding the point of the scalpel through the cane behind the block of tissue thus delineated, the block of tissue may be lifted out on the point of the of the scalpel. It should appear somewhat like the small section shown in (Figure 2).

Drop the block of tissue into a Petri dish about half filled with an anti-oxidant solution. Either of the two following solutions are satisfactory.

| | | |
|---|---|---|
| 100 Mg. of Ascorbic acid in 1000ml. of distilled water | OR | 150 Ml. of Citric Acid in 1000 ml. of distilled water |
| (mg. = Milligrams) | | (ml. = Milliliters) |

Immersion in the anti-oxidant will prevent the sections from drying out or turning brown. Fill as many Petri dishes as will be required for the up-coming operating session. Limit the sections in each dish to about fifty. More than this number may result in damage to the shoots. These sections may be stored in a refrigerator at 35 to 38 degrees Fahrenheit overnight if desired with no apparent deterioration. The number of sections needed for the Meristem Tip excision

operation will vary with the operator. An experienced operator can excise a Meristem Tip and place it in culture in an average of 2 to 3 minutes.

If desired, broken razor blades may be substituted for the scalpels for preparing the sections. The razor blades should be mounted in a handle made of small (about 1/4 inch) dowel four or five inches in length. Split or saw into one end about 1 inch; insert the broken razor blade into this opening or slot, pinch the dowel tightly and wrap or tape so the blade is held securely. (For this a good grade double-edged blade works well.) Break the blade into two parts along the longitudinal axis. This amounts to breaking the small supporting sections at each end of the blade since large openngs usually exist in the center of the blades. Next, carefully twist each section of blade until it snaps in two. If properly twisted the blade will break at a quite sharp angle giving a very fine point. Two pairs of pliers may be used for holding the blade while twisting. Holding the blades with one pair of pliers and twisting with the fingers and thumb of the other hand also seems to work quite well. This seems to give a better feel for the way the blade is reacting to the twist, but does require caution to prevent injury. After the blade sections are broken into scalpel blades the protruding edges, originally the center spacers of the blade may be broken off with the pliers. Slide the butt end of the broken blade section into the split previously made in one end of the dowel. Bind dowel about blade in two places using very fine stainless steel wire (fishing leader).

## When ready to begin the excising operation, sterilize the plant sections as follows:

**First** - dip and agitate for 5 to 10 seconds in a surfactant such as a solution of 75% Ethanol (grain alcohol), TWEEN, (a stock surfactant), or plain detergent (Lux, Joy, etc)

**Second** - Plunge into a solution of 6 % Calcium Hypoclorite or Sodium Hypoclorite (Clorox ) for about 10 minutes.

**Third** - Drain and rinse in distilled water three or four times.

The reason for dipping and agitating in the surfactant is to facilitate sterilization. The surface of the sections, especially the areas other than the excision surfaces are covered with tiny hair like protuberances normally visible only under a microscope. The pre-dipping is to reduce the surface tension and allow more completes wetting by the sterilizing agent and hence more positive sterilization. (This is the wetter-water principle).

To make it easier to handle the sections of plant material during the dipping, soaking and rinsing they can be wrapped loosely in a piece of cheese cloth. Use a piece of cheese cloth about six inches square. Place the sections from the Petri dish on the cheese cloth, fold the cheese cloth loosely over the sections to form a small sack like container. Gently press the sack of material into a test tube, which has been half filled with the surfactant solution. Swirl the tube gently for a few seconds. Pour off the surfactant and add the sterilizing agent to cover the pieces of tissue. Allow the tissue to remain in the sterilizing agent for approximately 10 minutes. Drain and rinse three or four times with distilled water. Thoroughly drain off water after final rinse.

It is not necessary, nor desirable to remove the material from the tube until after the final rinse and when ready to begin the excising operation. At completion of rinsing, replace the closure (stopper, lid, kaput. cover, etc.) on tubes, leaving the pieces wrapped in the moist cheese cloth until needed later. (The nearly 100 percent humidity in the tube will prevent any drying out of the material.) If necessary the tubes may again be stored in the refrigerator (35 - 38 degrees F.) until needed. If stored again, cover with the anti-oxidant. When ready to resume the excising operation, the sterilizing process should be repeated.

All these precautions may seem superfluous since the shoot to be used probably came from a positon relatively high above the ground. However the percentage of successful culturing will be disappointingly low under the best of condition - it is certainly worthwhile to observe all forseeable rules of cleanliness.

# Material Required for Excising Operation

**In addition to a properly lighted work area, the following items will be required:**
Adjustable stool, or comfortable chair.
Binocular Microscope, variable 6 to 25 power
Adjustable spotlight for lighting operating area (unless built into the microscope)
Quantity of sterilized Petri dishes. (about one dozen) 15 x 100 mm.
Pack of sterile filter paper (9 cm. diameter).
2 surgical scalpels, Size 7 handles with size #10 & #11 blades.
An alternate to the scalpels, somewhat more economical and the tools used during this program are two scalpels made from broken razor blades as described in the section titled PREPARING THE SHOOT TIP.

Two sets (4 cutting tools can be easily and rapidly made. Two sets allow one to be soaking in a sterilizing solution while the other set is in use.
3 pairs of forceps (tweezers)
1 9 1/2 inch fine tip.
1 4 inch micro, fine tip.
1 4 inch micro, extra fine
Test tube stand for holding large tweezers
Tray with sterilizing agent for holding scalpels and forceps between uses.
Test tube racks - 40 position, 1-4 or as required.
Prepared culture tubes with closures (200 or as required).
Marking Pen

# Familiarization

Before beginning the operator should be completely familiar with the morphology (form and structure) of the shoot tip. The shoot tip of concern is depicted in Figure 3:

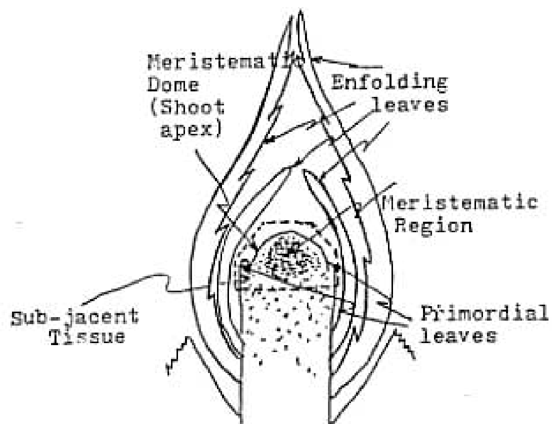


FIGURE 3.

This is a greatly expanded drawing of the small shoot tip shown in Figure 2B. To help understand this drawing, think of standing the small shoot on its base (area that attaches to the block of the stem) and then with a sharp knife cutting downwards through the center of the shoot. If the two halves thus formed are then laid apart, each section would look somewhat like Figure 3. The part to be taken for culturing is shown by the dashed lines. Notice it includes the first two primordial (rudimentary) leaves. The area depicted by the dashed line is approximately 1/2 to 1/4 the size of a period (.).

# Culture Medium Preparation

There are several nutrient solutions, which have been used successfully in plant tissue culture. Some of these solutions may be bought as stock items from Laboratory Supply Houses throughout the country. In most cases the nutrient solutions (mediums) have been developed for, and constituents varied, to determine the best combination for culturing specific tissues of specific plants. There is no instance known to the author where experimentation has been carried out to determine the optimum medium for dahlia Meristem Tip Culture. Probably the best such solution presently known is the one developed during these programs. Although the successful program reported by Dr. Morel and Dr. Martin used a solution called KNOP's Medium with various vitamins and organic substances added, Dr. Morel has since recommended the use of a different medium for dahlias. The nutrient medium recommended uses as its base inorganic mineral salts medium developed by Drs. Toshio Murishige and Folke Skoog for tobacco tissue culture. To that formula, organic substances and hormones (growth regulators) believed to be specifically beneficial to culture of dahlia shoot tip cuttings has been added. The program discussed here has proven the advantage of the following medium:

# The following nutrient medium is suggested as the reference medium for dahlia Meristem Tip Culture:

## Onorganic Salts

Mineral salts solution according to Murashige and Skoog as reported in PHYSIOLOGIA PLANTARIUM, Vol, 15, 1962, Pg. 485. (Instructions for preparation are included later in this paper. Those preparations will show two deviations from the M-S nutrient medium)

## Organic Substances

| | |
|---|---|
| Sucrose | 30 g/l of medium |
| Vitamins: Thiamine HCl | 0.4 mg/l of medium |
| I - inositol | 100 mg/l of medium |
| Amino Acid: L - tyrosine | 100 mg/l of medium |
| Hormonal substances: Kinetin | 1 mg/l of medium |
| Indole - 3 acetic acid, IAA | 1mg/l of medium |
| Agar | 7 g/l of medium |

## Operating Procedure

The following procedures are based on use of `non-clean' (for want of a better description) facilities. What is intended is normal household cleanliness, not a laboratory clean room and all the attendant sterile conditions.

Assemble all tools and equipment listed under the section titled Materials Required For Excising Operation.

Select a location with good lighting in a room that is as dust free as possible. A day with no wind and low humidity is preferable.

Keep scalpels and forceps in sterilizing agent when not in use.

Place a piece of sterile filter paper in a Petri dish. Saturate with anti-oxidant.

Before proceeding it is recommended the operator wash hands in the sterilizing solution. Rinse under running tap water.

Assemble a 40 - position rack of tubes prepared as describe later under the heading of

Preparation of the Final Nutrient Solution.

Remove one of the sacks of plant material from its test tube and place in a sterilized Petri dish. The large tweezers will be required for this operation. With two pair of tweezers unfold the cheesecloth and remove one of the shoot setions. Replace the cover on the top of the Petri dish containing the remaining shoot sections to prevent their drying out. Place the removed section in the Petri dish with the saturated filter paper.

Center under the viewing area of the microscope. At the 6 power setting bring shoot section into the field and focus. Adjust spot light for best lighting angle and field of illumination. While holding shoot section (block portion) with the tweezers, remove outer leaves by pulling out and down until the leaf tears away. (Only experience will show the best way to do this). If it becomes difficult to distinguish the form of the remaining shoot tip, re-adjust microscope to the 25 power setting and focus for best viewing. Remove next set of leaves until the round shiny dome of the tip is seen. After the first sets of leaves have been removed, it was found that two scalpels or razor blades, or one scalpel or razor blade and a needle (mounted in a piece of doweling) was more convenient than using tweezers. Remove all primordial leaves possible without damaging the meristematic dome of the shoot. With microscope at the 25X setting and focused for clearest viewing, make four incisions, one along each quadrantal surface of the tip. Push the tip of the scalpel under the shoot tip, excising as small a section as practicable. This small section will be sufficiently sticky to adhere to the tip of the scalpel. Rest the scalpel with the tiny cutting across the Petri dish until ready for further use.

Take up the first culture tube to be used, turn upside down or at an angle so the opening of the tube is below the bottom (the nutrient will be sufficiently gelled to hold its positon). Remove the closure and stand it in the dish of sterilizing agent. Take up the scalpel with the cutting in place and place the cutting on the medium, shake off the excess sterilizing agent from the closure and replace on the tube. Mark the tube with indelible marker to identify as necessary and replace in the rack or a second rack.

Any contaminating dust particles, fungus, bacteria, etc. floating in the air will have a less tendency to enter the opened tube when it is partially inverted; standing the closure in the in sterilizing agent will help prevent contamination by particles that may settle on or in the closure while removed from the tube. (Flaming the tube opening and cover would be more desirable than dipping if such facilities are available.)

Marking is for positive identification at a later date. Mark with the variety name, date taken, medium code, and any other data which may be pertinent to the culturing or later data recording or other identification.

After each operation return the tweezers and scalpels used to the sterilizing tray and using the second set of tools repeat the process for the next shoot. Continue to alternate tools and repeat the operation for the quantity of Meristem Tip Cuttings to be taken.

If desired, four or five propagules, of the same variety of course, may be placed in each tube. The advantage being that 4-5 times as many propagules may be cultured in the same space, and the chances of getting a culture to develop in each tube is increased. The disadvantage being that if the tube becomes contaminated it may result in loss of four or five propagules rather than one. Usually however, not all four or five will grow so only those that are trying to grow will in effect be lost.

## Culturing

Upon completion of the excising operation or when a rack has been filled the test tubes should be immediately transferred to the culture room. Here are they are placed in the culturing environment and the long period of waiting begins. No change will be noticeable for several days. After two to three weeks the propagules will be seen to be enlarging and becoming greener. The white and yellow varieties will generally show an almost translucent light green color; the pink, red, purple and blend varieties will generally show somewhat darker green and be more opaque. The propagules will be seen to continue to enlarge for six to eight weeks and then for some yet unexplained reason will stop enlarging and seem to become dormant. During these first six to eight weeks of enlargement the propagule may show varying responses. Some will show definite shoot growth and some only callous. The callous may at a later time begin to show shoot growth, but unless ample space is available it is probably wise to discard any propagule not showing shoot growth within the first 6 to 8 week period. The tubes must be monitored daily for fungus, bacteria or other contamination. These growths will generally manifest themselves as a film on the surface of the nutrient medium. This film usually seems to be growing out from the propagule but sometimes may be entirely separate from the propagule indicating that contamination apparently took place while the tube was opened to implant the propagule(s). Again unless ample space is available it is probably wise to discard these propagules. It may be possible to kill the fungal or bacterial growth by dropping a small amount of the clorox sterilizing mixture on the area were it is seen. This is not always successful and quite often damage to the propagule may be experienced - but if no replacement propagules are available it may be worth a try. Allow the sterilizing agent, mixed one part clorox to 19 parts distilled water to remain on infected area for five to ten seconds and pour off immediately. Flush the area gently with distilled water and pour off, being careful not to dislodge the propagules.

Dr. Morel and Dr. Martin reported that the dahlia propagules would not form roots in culture. It was therefore necessary for them to attempt to graft the propagules onto a dahlia seedling. Because of the etiolation of the explant (the small plant that develops from the propagule) and their very delicate nature, attempts to graft on seedlings during this program were not successful. This necessitated a different approach. The approach used involved a reculturing process by which approximately 35 percent of the propagules developed roots adequate to sustain the propagule upon transplanting to a growing medium.

At the end of the approximately six to eight week period, in which growth took place, the propagules were transferred to a revised medium. This medium was identical to the original except the amount of Kinetin was decreased. The Kinetin was decreased to 1/2, 1/4, and one eighth that of the initial medium and even to zero. A level of about 1/4 seem to give the best results. It is believed that increasing the Auxin (IAA) may further promote rooting. At best rooting seems to depend on a balance between the Kinetin and the Auxin. The relationship for best rooting has not been definitely established. It is recommended that further experimentation be undertaken to attempt to establish this relationship.

After the several weeks of dormancy, the propagules whether re-cultured or remaining in the original culture medium again began to show growth . Some to grew rapidly, others agonizingly slow. Those developing roots grew more rapidly and also were more vigorous and sturdy.

# Transplanting

After the propagule (explant) reaches approximately four inches and has developed 2 to 3 sets of of leaves it may be transferred to a medium to further enhance root development. For the explants with out roots, best success was obtained by treating just like a normal green plant cutting rather than trying to graft on to a seedling. That is they were cut off just below a node dipped in a rooting hormone "Rootone" and placed in rooting medium. For this sterilized builders sand is used. The sand was the sterilized by sprinkling about 1/2 in. deep on a cookie sheet and baking for approximately two hours in a 250 degree oven. Explants which had developed a good root system are placed directly in the growing medium to be described later. Explants with only a few or small roots were also placed in the rooting medium. For this they were carefully removed from the culture tubes, the nutrient medium washed off by a gentle flow of the boiled and cooled distilled water. The explant then suspended in the pots and dry sand drifted around and over the roots. As soon as placed in the sand the pots are watered until the sand is saturated, the excess water poured off and then placed in a shallow tub and covered with a clear glass. Small jelly glasses, dried beef glasses or cheese spread glasses work very well. Water is then added to the tub until pots stand approximately 1/2 in. deep. From now on no more water is added to the pots. The water level in the tub is maintained. In this way the pots absorb just about the optimum amount of moisture through the porous clay.

One and one-half inch pots are ideal for rooting. The bottom is sealed with a small piece of plastic and glue. A piece of a plastic marking stake and Weldwood work quite well. After sealing the pots are also sterilized in the oven two hundred fifty degrees F. for two hours.

Return the tub and pots to the same culture environment used for initial culturing (See section on Culture Room Requirements)

After 2 to 4 weeks or when the explants show new growth. They are transplanted to a growing medium. The medium used for this program was sterilized worm castings. Any good sterile potting mix should work equally well. The transplanting technique is similar to that used for rooting green plants. The plant is suspended in a 4 or 6 inch pot and the potting mix is drifted around and over the roots. Six inch pots work best. The sand may not be completely removed from the rooted plant. However the loose sand may be readily removed by shaking gently. A mound made in the center of the plot to allow the roots to spread over and around works quite well. Place the pot in a shallow container a coffee can cover works quite well and water until the soil is saturated and seeps out the bottom. A very mild solution of Vitamin B1in ordinary tap water is used for this first watering. Pour out the excess water and return to the culture environment. Cover the plants with a pint mason jar. Water as required or as would be done for any green plant. When new growth is again noted begin fertilizing with a very weak fish emulsion. When worm castings are used no fertilizing is required during this phase. If fertilizing is necessary use only about one teaspoon of fish fertilizer per gallon of water. Also begin increasing the light intensity either by adding more lights, stronger lights or moving pots closer to the light source. This is the beginning of a hardening off process preparatory to moving the plants from the culture environment. When the plants again gain vigor and color they will appear quite similar to a rather spindly green plant as developed from ordinary cuttings and are ready for transferring to the greenhouse.

## Rationale for Foregoing the Procedure

Plugging the hole in the bottom of the pots used for rooting and placing the pots in the partly filled tub allows the sand to remain damp but not saturated. Placing the small glasses and/or mason jars over the plants keeps a high level of humidity around them. When in the tube the humidity was near 100 percent. Retaining the high humidity helps keep the plants from drooping until they can become acclimated to ambient conditions.

The light intensity and duration should be gradually increased to that which will be experienced in the green house (approximately 2000 to 2100 microwatts per square meter.)

# Growing on in the Greenhouse

When the explants have been hardened off they can be transplanted into 4 or 5 gallon nursery tubs. For this two different planting mixes were used, but any good commercial mix should do just as well. Both worm casting and a standard planting mix were used. The worm castings were not sterilized and considerable fungus growth took place on some tubs. A low concentration of clorox solution (19 parts water to 1 part Clorox) can be sprayed on the soil at weekly intervals to help inhibit fungus growth. Some of the plants were placed in a mix regularly used as a standard planting medium. The mix consists of equal parts of:

#3 Perlite Builders sand
Peat moss Redwood soil Amendment

To each gallon of this mix, 1 tablespoon of Osmocote (14-14-14) was added.

From this point on growth is quite rapid and comparable to that of regular green plants. As soon as side laterals develop they can be used to make green plants for stock increase. The crown bloom is allowed to develop to check that the plant is indeed true to type and did not mutate. Green plants developed in this manner are treated just like any other green plant.

Both the original clone and green plants grown for stock increase are grown under artificial light of approximately 2100 microwatts per square centimeter, using Sylvania Gro-Lux fluorescent lamps, unless the available light is sufficiently strong. When grown in this manner in the early spring or late fall the Gro-Lux lamps can be used to augment the available light to provide a minimum of 14 hours of light per day.

# Certification

At present there is no known certification program for dahlias at the U. S. or State level. Certification programs are conducted by the U.S. Department of Agriculture for many crops. Some crop Associations and private nurseries have their own certification programs. The cost of such programs are quite high. It appears questionable whether the dahlia market can ever support any government program for certification. This should be the most pressing concern for any future ADS research programs.

Although the probability of obtaining healthy plants is high, it is not certain. All together Dr. Morel and Dr. Martin reported they had recovered healthy stock of fifteen different varieties, all of which indexed negative by a serological test. These two programs recovered sixty three different varieties. Of these, 3 were thrown out while still in the transplant stage as possibly diseased. The rogueing was based on visual symptoms not on indexing. It is believed that in the absence of a proven indexing program, the recovered stock should be grown in an isolated area (at least 1/2 half mile from any standard varieties for a minimum of two and preferably three full seasons and carefully monitored before assuming they are in fact free of any virus.

For three years following the Meristem programs the author experimented with an indexing program. Literature is available describing different programs using various pproaches,(12,13,14,15,16) all stating to give quite positive results. Out of these different methods a progam employing the bio-assay method(12,13) was selected as the one best within the means and experience of the operator for experimentation. This method involves the use of fast growing plants sensitive to dahlia viruses, When quite young, the indicator plants are inoculated with plant sap from the dahlia being indexed. Several species of plants have proven reasonably sensitive to dahlia viruses. By bio-assay a dahlia can be indexed in from 3 to 7 weeks depending on the type virus. The advantage of this type program over growing out for 2 or 3 seasons is obvious. The colormetric program described by Martin(17)is reported to be almost immediate since it gives a sort of litmus paper indication upon exposure. Either should prove not only a time saver but more positive than visual observation. Many varieties of dahlias are tolerant or semi-tolerant to certain of the virus diseases. These may grow rather vigorously with few or no obvious symptoms. It is believed that an indexing plant, which has proven sensitive to dahia viruses will not demonstrate tolerance.

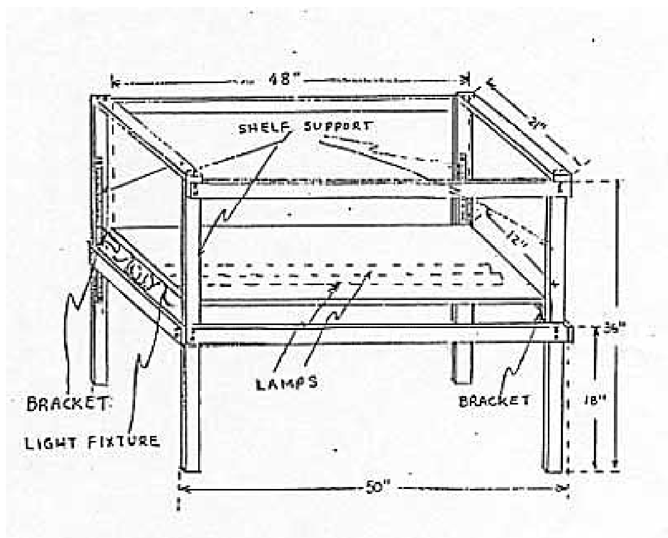
## Culture Room Requirements

As with all plant life, growth and development of the propagules is dependent on the light and temperature to which they are exposed. However, the requirements for tissue culture under laboratory condition are considerably different from that of most plant life. In so far as known, there has been no experimentation to develop optimum environment for propagation of dahlia tissue culture. Light intensity of 200-700 microwatts per square centimeter, for a period of 14 to 16 hours per day have been used successfully. A constant temperature of 80 degrees F., or a varying temperature of 80-85 degrees F. during the lighted period and 60-65 degrees F. during the darkened period was used satisfactorily during a part of this program. Humidity requirements have not been evaluated but a relatively high humidity would appear best especially in the early phases of the program. This high humidity is taken care of during the in vitro propagation. The humidity within the sealed tubes remains quite high and constant. High humidity during the rooting operation was assured by covering the pots with small glasses. The quality of light is of equal importance with intensity and duration. Plant propagules in vitro utilize primarily the red and blue bands of the radiant energy spectrum. These particular wavelength regions trigger and control certain growth factors. Blue light is essential for shoot formation; red light is essential for root formation. By the proper balance of blue and red light, vegetative growth is stimulated and, enzymatic and chemical processes are controlled. For the reference program the Sylvania Standard Gro-Lux flourescent lamps are recommended. For experimental purposes, after other

factors of the optimum program are determined, use of Wide Spectrum lamps with different filtering may be undertaken.

Later phases of the program will require a much higher intensity of light. Prior to planting out in the green house, the propagules should be exposed to a gradually increasing intensity of light from 700 to 2100 microwatts per square centimeter. The standard Gro-Lux fluorescent lamps are capable of supplying this amount of light and is recommended for this application. In its new environment the propagule will begin relying on photosynthesis to an increasing degree. The ratio of blue and red light in the Gro-Lux lamps produces an energy distribution designed to produce a maximum photosynthetic process in plants. While in vitro, on the nutrient medium, photosynthesis was less important because the medium supplies sucrose and other elements required for respiration, in a state which is readily assimilate-able by the plant tissue. The increased light intensity during the second phase of the program is essential to promote the photosynthetic process.
In Southern California, where this program was conducted the temperature is quite moderate. The culturing was done in an inside room so light could be controlled, both in duration and intensity. It was found that by operating the light from about seven o'clock in the evening until nine o'clock in the morning and turning it off during the normal daylight period, the temperature could also be adequately regulated, except during the winter months, and for a few short periods of warm weather during the hotter months of the year. Short periods of 3-4 days of 90 degree F. temperature in the culture room produced no noticeable deterioration or damage to the propagules.

The light stand shown in Figure 4.




is similar to that used for this program. In addition to the table shown, a standard 24-inch indoor plant light stand was mounted on the top shelf to give two tiers of culture space and a way to allow two different light intensities for the different phases of the culturing process. This stand provided space for approximately 200 tubes below the shelf, and 120 tubes on top of the shelf. The space was initially filled to capacity. By the time space was needed for phase two (rooting),

enough of the tubes had been emptied to provide space for the plants for that phase while still allowing continued phase one operation.

For the hardening off phase, the plants are placed under the shelf and the shelf lowered to increase light intensity as desired.

# Preparation of the Culture Medium

The mineral salts used to make up the nutrient medium for Meristem Tip Culture of dahlias are:

Salt Quantity (mg per liter medium)

| | |
|---|---|
| Ammonium Nitrate, $NH_4NO_3$ | 1650 |
| Potassium Nitrate, $KNO_3$ | 1900 |
| Calcium Chloride, $CaCl_2$•$2H_2O$ | 440 |
| Magnesium Sulphate, $MgSO_47H_2O$ | 370 |
| Potassium Phosphate, $KH_2PO_4$ | 170 |
| DiSodium-Ethylenediamminetetraacetic Acid, $Na_2$•EDTA | 37.3 |
| Ferrous Sulphate, $FeSO_47H_2O$ | 27.8 |
| Boric Acid, $H_3BO_3$ | 6.2 |
| Manganous Sulphate, MnSO4•$H_2O$ | 16.9* |
| Zinc Sulphate, ZnSO4•$7H_2O$ | 8.6 |
| Potassium Iodide, KI | 0.83 |
| Sodium Molybdate, $Na_2MoO_4$•$2H_2O$ | 0.25 |
| Cupric Sulphate, $CuSO_4$•$5H_2O$ | 0.025 |
| Cobalt Chloride, $CoCl_2$•$6H_2O$ | 0.025 |

*Quantity specified somewhat different from the basic M-S Revised Medium.

In the case of those mineral salts where mixture with distilled water is indicated, such mixing should be accomplished prior to measuring into the medium or stock solution.

# Preparation of Stock Solutions of Inorganic Substances

Stocks of the the various groups of inorganic salts may be made up in advance and stored at laboratory temperatures indefinitely, Since salts are used in relatively small amounts, pre-mixing is preferred and facilitates both handling and more accurate measuring of the small quantities used. The mineral salts can be compatibly grouped into 5 categories for stock solution purposes. These categories and the quantity of each mineral salt is shown in the following tables:

| NITRATES | Amount (grams / liter of stock) |
|---|---|
| Ammonium Nitrate, $NH_4$ $NO_3$ | 165 |
| Potassium Nitrate, K $NO_3$ | 190 |

| | |
|---|---|
| SULFATES | |
| Magnesium Sulfate, Mg $SO_4 . 7H_2O$ | 37 |
| Manganous Sulfate, Mn $SO_4 . H_2O$ | 1.69 |
| Zinc Sulfate, Zn $SO_4$ •$7H_2O$ | 0.86 |
| Cupric Sulfate, Cu $SO_4$ $5H_2O$ | 0.0025 |
| HALIDES | |
| Calcium Cloride, Ca $Cl_2 . 2H_2O$ | 44 |
| Potassium Iodide, KI | 0.083 |
| Cobalt Chloride, Co $Cl_2 . 6H_2O$ | 0.0025 |
| $PO_4$, $BO_3$, $MoO_4$ | |
| Potassium Phosphate, $KH_2$ $PO_4$ | 17 |
| Boric Acid, $H_3$ $BO_3$ | 0.62 |
| Sodium Molybdate, $Na_2$ $MoO_4$ •$2H_2O$ | 0.025 |
| Na Fe EDTA | |
| Ferrous Sulphate, Fe $SO_4 . 7H_2O$ | 2.78 |
| Ethelendiaminetetraaceticacid, Disodium Salt, $Na_2$ EDTA | 3.72 |

In preparing stock solutions only re-agent grade chemicals and distilled water should be used.

To prepare the stock solutions, measure the groups of elements (ie Nitrates, Sulfates, Halides, etc) into separate dry flasks. Add necessary quantity of distilled water and shake vigorously for several minutes (to insure thorough mixing. Cover and store on laboratory shelf. (The stock solution may be stored in regular mason jars if desired)

Although the solutions will be autoclaved later it is a worthwhile caution to sterilize the containers. Wrap the stock solution of Na Fe-EDTA in a double thickness of aluminum foil. Maintain in the wrapped condition at all times except when dispensating to prevent photodecomposition. If the amount dispensed is not to be mixed in the basal solution immediately, wrap it also.

# Preparation of Stock Solutions of Organic Substances

Organic substances will generally require refrigeration. It is therefore important that they be mixed just before use in so far as practicable. Surplus quantities may be stored in the refrigerator for limited periods (one to two weeks), but some deterioration may be expected. In preparing stock solutions of organic substances it is sometimes necessary to dissolve the weighed or measured crystals in a very small quantity of an organic co-solvent prior to dilution with distilled water. Ethanol, $C_2 H_6 O$ (Ethyl Alcohol) is the most commonly used co-solvent. However, it is somewhat toxic to plant tissue. DSMO (Dimethylsuloxide) is recommended as the co-solvent used with the stock solutions listed to follow. DMSO has as good or better dissolving capability as Ethyl alcohol and is less toxic to plant tissue

**IAA (Indole - 3 acetic Acid)**
Weigh 20 mg. crystals into a 250 ml. flask. Add 0.5 ml. DSMO. Warm gently to dissolve

completely. Add 75 ml. distilled water briskly. Transfer to a 100 ml. volumetric flask; fill to 100 ml. level and mix well. Cover and store in refrigerator

**KINETIN**
Weigh 20 mg. crystals into a dry 250 ml. Flask. Add 0.5 ml. DSMO. Warm gently to dissolve completely. Add 75 ml. Distilled water briskly. Transfer to a 1000 ml.. volumetric flask; fill to 1000 ml. level and mix well. Cover and store in refrigerator.

**THIAMINE HYDROCLORIDE (Vitamin $B_1$ )**
weigh 40 mg. Crystals into a 1000 ml. volumetric flask. Fill with water to 1000 ml. levcl and mix well. Cover and store in refrigerator.

As with the organic stock solutions these are at 100 times the final medium concentration. Hence all stock solutions $1/100^{th}$ liter (10ml.) will be required. Organic substances used in large quantities such
as i - inositol and sucrose need not be prepared in stock solution. However great care must be taken when they are added to the final medium solution to insure they are thoroughly and completely dissolved.

# Preparation of Basal Solution

In a 500 ml. volumetric flask combine:

Nitrate Stock solution……………………………………………………………….10 ml.
Sulfate Stock solution……………………………………………………………….10 ml.
Halides Stock solution…………………………………………………...…….10 ml.
$PO_4$, BO3, MoO4, Stock solution……………………………………………....…..10 ml.
Na Fe-EDTA Stock solution……………………………………………….…..10 ml.
Thiamine - Hcl Stock solution…………………………………………………….10 ml.
i- inostol…………………………………………………………………...…….100 mg.
Sucrose (sugar)……………………………………………………………….…… 30 g.

Add distilled water to the 500 ml. level and mix well, until all components, especially the sucrose is dissolved

# Preparation of the Final Nutrient Solution

Transfer the 500 ml. of basal solution to a 1000 ml. volumetric flask.
Add: 5ml. IAA (indole - 3 Acetic Acid) stock
: 1.5 ml. Kinetin Stock
Dilute to 800 ml. , mix well.

Adjust ph to 5.0 plus or minus 0.1. For this operation an Electronic ph indicator should be used. Color matching can be used, but is not recommended for controlled research. If too acid use

Sodium Hydrochloride; if too alkaline use Hydrochloric Acid . Add drop by drop and test after each addition.

Dilute to 1000 ml. and mix well. To each liter of the final nutrient solution must be added 7.0 g. of Bacto-Agar to cause the solution to form a gel. In the laboratory this is done by heating in an autoclave for approximately 10 minutes at 121 degrees C. (210 degrees F.). For this program the following procedure was used and proved sufficiently dependable and accurate: When ready to mix, pour the nutrient solution into a wide mouth pyrex pot - a coffee carafe works well. Bring the solution to a near boil. When small bubbles rise in the solution, add the agar while stirring constantly with a long handled spoon. Reduce heat and continue stirring until the liquid becomes clear.

While the solution is still hot distribute into 25 x 150 mm. Culture tubes at the rate of about 16 ml/tube. A liter thus fills about 60 tubes. Cap tubes with kaputs. Mount the tubes in 40 position metal cultrure tube racks. Place racks, tubes and medium in autoclave . Sterilize 15 minutes at 15 pounds pressure . (121 degrees C./250 degrees F.). Remove racks from autoclave. Place on a slant of approximately 30 degrees and allow medium to cool.
A simple method for home use (where an autoclave large enough to accommodate the 40 position racks is not available) a small rack of 1 inch chicken netting can be easily fabricated. The rack can be made to fit a standard home pressure cooker. After removal of tubes from the pressure cooker they can be transferred to metal racks for cooling on a slant.

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