

THESIS

CLONAL MULTIPLICATION OF CARNATION BY MICROPROPAGATION

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ABSTRACT OF THESIS

CLONAL MULTIPLICATION OF CARNATION BY MICROPROPAGATION

Clonal multiplication of carnation (*Dianthus caryophyllus* L.) was accomplished in three stages: (1) shoot tip culture initiation, (2) shoot multiplication, and (3) rooting stages.

Shoot tips approximately 1 mm in height, as used in the micropropagation of pathogen-free plants, were grown on a modified Murashige and Skoog medium with 10 μ M kinetin and 1 μ M NAA. This solidified medium supported rapid growth of morphologically normal shoots, while counteracting apical dominance. It was selected after comparing various inorganic salt mixtures, vitamin mixtures, carbohydrates, growth regulators, and additional supplements for their effect on shoot tip growth.

After 3 to 4 wk in the initiation stage, proliferated shoot tip cultures were transferred to the multiplication stage. They were each grown in separate flasks containing the same medium as in the previous stage but without agar and with one fourth the concentration of NAA and kinetin. The flasks were attached to an auxophyton and revolved horizontally at 1 rpm. Growth of axillary shoots was enhanced in this stage, and shoot tip cultures, usually with two or three shoots over 2 cm in length at the beginning of this stage, consistently produced more than 10 shoots of similar length in another 3 wk.

Plantlets were obtained by rooting the individual shoots from the multiplication stage. In the rooting stage conventional techniques

for rooting carnation cuttings were applicable. BR-8 synthetic soil blocks and Jiffy-7 expandable peat pellets were used as supports for root initiation and development. These rooting supports increased survival of plantlets over other types of media conventionally used in propagation.

Micropropagation by the method developed in this research produced some abnormal plants, and selection for plants with desired characteristics may be necessary. Incorporation of this method into a pathogen-free stock program might prove beneficial if large scale use of it is limited by economic considerations.

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Chapter I

INTRODUCTION

Propagation of pathogen-free stock has been the most effective method for controlling carnation diseases (32). The present method for mass production of pathogen-free carnations (*Dianthus caryophyllus* L.) requires multiplication of clonal material for a 3 to 4 yr period using mother blocks (1). Maintaining these plants for this period of time is not only expensive but also increases the probability of pathogen reintroduction.

A more rapid means of obtaining and multiplying pathogen-free carnations has been sought. Shoot tip culture, not only as a means of eliminating pathogens, but also as a method of clonal multiplication, was suggested by Hackett and Anderson (18). Their technique, although successful in multiplying carnations, caused deviations from the normal flower color suggesting a disruption of the periclinal chimeras.

Langhans and Earle (42) have developed a micropropagation program for chrysanthemums (13, 14) and have suggested after preliminary experimentation that their techniques might be applicable to carnation. More recently, Petru and Landa (77) have described an aseptic culture method for multiplying a garden variety of carnation that is not commercially grown in Colorado.

The purpose of this research was to examine the possibility of using micropropagation techniques for the clonal multiplication of carnations with emphasis on developing a method suitable for obtaining pathogen-free plants. A method readily adaptable to commercial utilization was desired.

Chapter II

REVIEW OF LITERATURE

Pathogen-free Propagation

Because of the highly heterozygous nature of most floricultural varieties of carnation, vegetative propagation has long been used to maintain valuable clones (32). Vascular wilt pathogens in cuttings were the first disease producing organisms to become prevalent because of this form of carnation propagation. As early as 1899, Mangin (51) suggested culture indexing to eliminate *Fusarium oxysporum* infected carnation cuttings. His method was to submerge the cut end of cuttings into water and check for mycelia to appear from the vascular region. Not until Dimock (10) in 1941 suggested what is known as the plate method for the elimination of *Verticillium albo-atrum* infected chrysanthemums was culture indexing again considered as a control for wilt organisms (69).

The plate method was utilized by a number of workers in the field of carnation diseases. Their methods have been reviewed by Nelson, Tammen, and Baker (69). Because of the inability of the plate method to consistently detect *Pseudomonas caryophylli*, a bacterial wilt organism, these workers suggested an improved version of a technique called the broth method, which was first developed for the same purpose by Helmers (26).

The maintenance of the pathogen-free status of propagative stock derived from culture indexing was reviewed by Tammen, Baker, and Holley (119). The overall clean stock program as outlined by them was initiated from the healthiest plants obtainable. Cultured cuttings were taken from these plants and a pathogen-free nucleus block was

established. Since it was not feasible to use cultured cuttings for each plant in commercial flower production, the mother block system of propagation was employed. Two types of mother blocks, a nucleus block and an increase block, were used in this operation.

Only 0.3% of the nucleus block plants were found infected in one carnation clean stock program (69). Yearly replacement of the nucleus block plants from cultured cuttings derived from them was suggested to gradually eliminate all pathogens in the program.

The incorporation of shoot tip culture into the clean stock program was reported by Baker and Phillips (1). The shoot tip, as described by Parke (74), is the portion of the shoot which includes the apical meristem and some foliar primordia and can be further defined by stating how many foliar primordia and leaves are present. The shoot tip was first cultured aseptically by Robbins (91) in 1922, and he obtained limited growth from shoot tips of corn, pea, and cotton. In 1936, LaRue (44) described the regeneration of adventitious organs in root tip, hypocotyl segment and shoot tip culture. It was not until 1945 that continuous growth in culture, starting with 5 to 10 mm long asparagus shoot tips, was accomplished (49). One year later, Ball (2) reported the production of whole plants, starting with much smaller shoot tips from *Tropaeolum majus* L. and *Lupinus albus* L.

Following the investigations by Limasset, Cornuet and Gendron (46) which showed that TMV infection was low in tobacco buds but increased as the leaves developed, and the subsequent work of Limasset and Cornuet (45), which demonstrated the failure to obtain TMV transmission from excised apical meristems of infected tobacco plants, the importance of shoot tip culture in obtaining virus free plants

became evident. The first application of shoot tip culture in obtaining virus free plants was accomplished by Morel & Martin (62) working with dahlia. Using a modification of Gautheret's medium, they grew shoot tips into unrooted shoots 1 to 2 cm in length. These were then grafted onto healthy seedlings. Virus free plants were thus obtained, while maintaining desired clonal characteristics.

Norris (73) working with the potato variety Green Mountain was next to report the elimination of a virus by shoot tip culture. He combined shoot tip culture and chemotherapy by malachite green and considered both treatments necessary for the elimination of potato virus X; however, Morel and Martin (63) reported elimination of virus X by shoot tip culture alone.

Potato virus Y proved to be more difficult to eliminate. Shoot tip culture alone was not successful (120). Sheffield (97), contrary to Limasset and Cornuet's work, had shown the presence of tobacco and aucuba mosaic viruses in the meristematic layers of infected tobacco apical meristems. It follows that shoot tip culture or even apical meristem culture alone may not eliminate some viruses. It has been known since the work of Kunkel (39) on curing virus diseases of peach that heat treatment is often a successful virus therapeutant (33). Although heat treatment alone did not eliminate virus Y, the combination of heat treatment followed by shoot tip culture employed by Thomson (120) produced potato plants free from virus Y.

With carnations, symptoms for ringspot virus reappeared in mother block plants 2 to 3 wk after heat treatment; however, 13 of 15 cuttings from these plants which were taken immediately after heat treatment failed to show renewed symptoms (38). Scholten and Belgraven (cited by Quak (84)) were unsuccessful in eliminating viruses from carnation

cuttings after heat treatment. They, however, did show serologically that the upper parts of the carnation cuttings were virus free, while the bases remained infected. Other investigators were unsuccessful in eliminating carnation viruses because of damage caused by the heat treatment (93).

Shoot tip culture was attempted to eliminate carnation viruses. In 1957, Quak (83) found that the low survival rate of shoot tips containing only the apical meristem and one pair of foliar primordia, 0.1 to 0.25 mm in height, was a limiting factor in her attempts to obtain carnations free from a mosaic and mottle virus, and she believed larger tips would not be virus free. In order to overcome this problem, heat treatment was used to "drive" the viruses down the shoot so that a larger, 1 mm, shoot tip containing one or two leaf pairs could be used. The survival rate was increased and virus-free plants were obtained. Since the work of Quak, several other workers have reported successful elimination of carnation viruses by shoot tip culture, whether alone or following heat treatment: Phillips, streak (78); Hollings and Stone, mottle (34), ringspot (35).

Replacing culture indexing by shoot tip culture has advantages other than controlling viruses. *Fusarium* stem rot of carnations (*F. roseum* f. *cerealis*) was endemic in Colorado before the initiation of a large cultured cutting operation (1). Propagative material from that program was found to be largely infected by the stem rot pathogen and consequently "extensive and explosive" losses had occurred. Peterson, Baker and Skiver (76) found that the pathogen was not carried internally in the cuttings but on the surface. Detection of this situation by culture indexing was impossible because of the necessity

of surface sterilization to prevent contamination of the culturing medium. Shoot tip culture by its nature allows non-obligate parasites, including those commonly found on carnations, to be detected. Thus, with the introduction of heat treatment and shoot tip culture, barring reintroduction of pathogens to the mother blocks, clean stock was even cleaner and could be termed pathogen-free.

The present clean stock program requires a 3 to 4 yr period for production and multiplication of pathogen-free material (1). Because of the highly unstable genetic characteristics of carnations (32), it is believed by some workers in the area that shoot tip culture increases mutations. There is no published evidence available supporting or disproving their assumption. Nevertheless, all nucleus block plants are screened by selection procedures before they are used to initiate mother blocks. Maintenance of the clean stock program for this long period is the most important problem now facing investigators (32). One method of resolving this problem would be to accelerate clonal multiplication during the program, possibly through the application of tissue culture techniques.

Micropropagation

Plant tissue culture has many applications, both basic and applied (90, 65). Shoot tip culture for the elimination of pathogens is just one of the applications. Another application is in clonal propagation, and shoot tip culture has also been useful in this area. The propagation of various orchid varieties by shoot tip culture is now routinely used in the orchid industry (61). It was with orchids that clonal propagation by shoot tip culture was first successful.

Morel (59) in an attempt to free *Cymbidium* orchids of viruses by shoot tip culture unexpectedly found that instead of initially obtaining a plantlet from his cultures, a mass of what he termed protocorms developed. He was then able to subculture the protocorms and many more arose. The protocorms eventually developed into plantlets. Morel estimated that over 4×10^6 plantlets could be obtained by this method in 1 yr from a single shoot tip (60). Similar results have been obtained from other orchids (53), bromeliads (65, 52), and gladiolas (28, 98, 147). Murashige (65) in a recent review article on plant propagation through tissue culture has compiled an extensive list of many other plant species which have shown a potential for clonal multiplication: among these is carnation. Murashige suggested that almost any herbaceous plant whose cuttings are easily rooted is multipliable through tissue culture following a minimum of experimentation.

The aseptic culture of plants and plant parts is often termed "tissue culture" in reference to the techniques used (75). Street (112) believes that this use of the term is misleading and suggests that the use be limited to describing any culture consisting of many cells growing in protoplasmic continuity on a solid medium. He distinguishes tissue cultures from suspension cultures, which are cells or aggregates of cells growing in a moving liquid medium, and also from organ cultures, embryo cultures, and other cultures in which the plant or plant parts show the structural and functional homogeneity of that nature. The terms defined by Street, including ones not mentioned, will be used as suggested in this thesis. Another term, "micropropagation," will be used to describe the propagation of plants by tissue culture techniques (43, 114).

Organogenesis, embryogenesis, and the induction of axillary shoot growth are essential morphogenetic phenomena in micropropagation. Each asexually produces organs or entire plants which may be utilized as propagative units. The genetic potential of a particular plant species and the morphogenic control attained during its tissue culture determines which, if any, of the phenomena may be achieved (114).

Selection of a suitable explant is important. Almost every organ or tissue throughout a plant has been the source of explants for tissue culture. However, only explants from specific regions of some species have shown the capacity for organogenesis and/or embryogenesis in culture, while in other species, this capacity has not yet been demonstrated.

The organogenesis of adventitious roots is frequently more easily attained in culture than that of adventitious shoots. Thus, the asexual plant embryo or the shoot is the usual desired initial product of micropropagation. In some instances, the presence of roots directly preceded the initiation of shoots (12, 107).

The shoot tip is often used as a primary explant. It has been the only source of tissue in two tobacco species (*Nicotiana rustica* L. and *Nicotiana glauca* L.) which has produced adventitious shoots (65, 133). While in another species (*N. tabacum* L.) many regions of the plants have been shown to develop adventitious shoots (11, 65, 70, 102, 125). In one investigation, chrysanthemum (*Chrysanthemum morifolium* Ram.) stem explants developed approximately ten adventitious shoots after 4 mo culture (31). A more recent investigation of chrysanthemum propagation of a different variety, using shoot tips as the initial explant, reported approximately five adventitious shoots

were produced every 4 wk (13). A closer look using the same variety would be necessary to determine if one technique is more advantageous than the other, even though indications favor the shoot tip technique.

Cytological studies of asparagus (*Asparagus officinalis* L.) exemplify another problem associated with the choice of explants. Malnassy and Ellison (50) found a high frequency of tetraploid plants developed from suspension and tissue cultures of asparagus. So, even though this plant produces embryos in suspension culture of hypocotyl tissue, and adventitious shoot formation from both hypocotyl and spear slice explants (118, 143), the use of shoot tips was investigated to avoid polyploidy (23, 67). This shoot tip culture technique was successful at producing plantlets and in all plants tested no polyploidy or any other chromosomal aberrations were found. Abundant axillary shoots were formed but not used as separate propagative units.

Reviews dealing with the deviations of nuclear cytology in plant tissue culture are available (11, 75, 116). Torrey (122, 123) demonstrated that the medium may increase polyploidy in tissue cultures and, later, that the medium may be adjusted to select for specific nuclear types. D'Amato (8) demonstrated that polyploidy occurred naturally in mature plant tissues and that the cells of meristems were more uniformly diploid. Possibly, what are considered chromosomal aberrations are only *in vitro* manifestations of a plant's natural *in vivo* tendencies (75).

To what extent chromosomal aberrations will be manifested in micropropagated plants is of extreme importance to clonal multiplication. Mitra, Mapes, and Steward (58) regenerated carrot plants (*Daucus carota* L.) from suspension cultures containing some cells with abnormal chromosome number. They observed that all of the plants were normal. However, Muir (64) observed that organ formation in carrot tissue culture decreased as aneuploids and various polyploids appeared, and tobacco plants regenerated from different aneuploid strains of tissue by Sacristan and Melchers (95) were all infertile and demonstrated different morphological abnormalities.

The choice of explants and the chemical and physical environment in which they are to be cultured appears to be a necessary consideration when a procedure for aseptic multiplication requires the maintenance of desired genetic characteristics of the clone. To complicate matters, the same plant hormones, auxins and cytokinins, which are important in morphogenic regulation in tissue culture (102) have also been shown to influence chromosomal variations (75). Control of morphogenesis is essential to asexual multiplication in culture. The enhancement of chromosomal aberrations can only be detrimental, unless, of course, a mutational breeding procedure is desired (11).

White (138) in 1938 was the first to demonstrate the control of shoot morphogenesis from tissue cultures. This was in the same year that White, Nobécourt, and Gautheret almost simultaneously reported the establishment of the first plant tissue cultures (139). Observations had been made by both White (137) and Nobécourt (72) that leafy tissue and roots respectively arose adventitiously from their tissue cultures.

Induction of shoot morphogenesis by White (138) was accomplished by transferring tobacco tissue cultures from a solid to liquid medium. He hypothesized that lower oxygen availability in the submerged culture brought about the change. These early observations led to the use of tissue cultures as a means of studying morphogenesis and its control in plants.

Among the first investigators to take advantage of White's discovery was Skoog (99). With tobacco tissue cultures supplied by White, he demonstrated the ability of these cultures to still produce buds after 144 subcultures, and he also discovered that indole-3-acetic acid (IAA) in the medium inhibited bud formation while raising the levels of KH_2PO_4 , $\text{Fe}(\text{SO}_4)_3$ and sucrose reversed this inhibition.

Skoog and Tsui (103), using a tobacco variety that differed from that of White by requiring an auxin for rapid growth, showed an interaction between adenine and the auxin, naphthaleneacetic acid (NAA). Supplementing the medium with adenine caused bud initiation to occur. The NAA supplement caused root initiation and tissue growth but inhibited bud initiation. Supplementing the medium with both NAA and adenine caused rapid tissue growth, especially in the pith area of the stem explants, but caused no organ development. Similar results were obtained using IAA as the auxin instead of NAA (55). Further investigations on the stimulatory effect of IAA on pith growth revealed that in the absence of cortical and cambial tissues, IAA stimulated the enlargement of pith cells but they did not divide (36). A cytokinin, kinetin, was purified from herring sperm DNA and then synthesized, causing cell division (56, 57). It was hypothesized that

a substance similar in activity to kinetin was produced in the cambial region of the tobacco stem and then was transferred by some mechanism to the pith region to cause cell division (9). Zeatin, a substance with cytokinin activity, was later purified from corn, showing that such compounds occurred naturally in plants (47).

Shortly after the discovery of kinetin, it was found that it was active in the control of morphogenesis. Skoog and Miller (102) in 1957 demonstrated the interaction of IAA and kinetin in organogenesis of shoots and roots in tobacco tissue cultures. Intermediate levels of IAA and kinetin induced abundant cell proliferation with no organized growth. At a higher IAA to kinetin ratio, abundant roots appeared but no shoots. The opposite effect was obtained by a lower ratio. An observation from the illustrations in their paper showing that shoots did not exhibit apical dominance led to the discovery of the role cytokinins play in counteracting apical dominance (94, 142).

It should not be assumed that organogenesis will occur in any plant tissue culture once the appropriate concentration of an auxin and a cytokinin are found (88). Only a few investigators working with other plant species have found quantitative shifts in the ratio of hormones or other substances regulate organogenesis. Some of these plant species where chemical control of organogenesis has been demonstrated include *Convolvulus arvensis* (4, 30), *Pelargonium hortorum* (82), *Petunia inflata* (85), *Nautilocalyx lynchei* (124), *Gladiolus hortulans* (98, 147), *Arabidopsis thaliana* (17), and *Citrus senensis* (6). In each of these examples, the levels of auxin and cytokinin employed varied. Often a sequential transfer of the tissue

cultures from one hormonal regime to another was necessary. Frequently a tissue culture initiation step was followed by an organ initiation step. In the first step, the dedifferentiation of the explant occurred. This step was then followed by redifferentiation on the subsequent medium.

Once optimal auxin and cytokinin requirements for organogenesis have been met in the tobacco tissue culture system, other additives, such as tyrosine and increased inorganic phosphates, may help to further facilitate organ formation (100). Different cytokinins also may markedly influence tissue growth and organogenesis (21, 100, 101).

The formation of embryoids in cultures from the secondary phloem of carrot roots was reported by Rienert (87), and Steward, Mapes and Mears (107) in 1958. Rienert observed a variety of forms in tissue cultures resembling stages of the development of natural carrot embryos. Steward, *et al.*, periodically withdrew samples from suspension cultures and observed aggregations of cells, many of which also resembled stages of embryo development. Further investigation by Steward, Blakely, Kent and Mapes (104) using a suspension culture of cells derived from a zygotic carrot embryo showed that up to 100,000 embryoids could be counted when a single aliquot was plated out in a petri dish. These results were considered evidence, although indirect, of the totipotency of higher plant cells. Vasil and Hildebrandt (130) later proved unequivocally that higher plant cells could be totipotent when they grew a single tobacco cell in fresh, unconditioned medium and ultimately obtained a mature plant from an adventitious shoot.

Steward's suspension culture apparatus, which is called an auxophyton (106), slowly revolves the tissue around a horizontal

axis at 1 rpm. The culture vessels and revolving apparatus together were designed to alternately expose the tissue to air and immerse it in the medium. This technique was intended to provide the optimum proportions of air, water, and nutrients to the tissue. Detailed descriptions are available of the auxophyton and similar apparatuses, and the special culture vessels used (106, 108, 113).

Steward, *et al.*, (104) concluded that higher plant cells because of their totipotency had the innate ability to form embryoids and that the culture environment could only supply the stimuli for the cells to express this ability. The auxophyton and the particular medium provided a means of obtaining free cells in culture. They pointed out that free cells lack protoplasmic connections, as do natural angiosperm zygotes, and are, therefore, without the epigenetic regulatory influence mutually imposed upon each other by cells in organized tissues. They postulated that free cells and natural embryo nutrients, such as liquid endosperm from coconuts, provided the environment essential for embryogenesis *in vitro*. A subsequent investigation by others has shown cytoplasmic connections between embryoids and other cells in another plant species (121). However, there is still considerable debate as to whether or not physiological isolation is a necessary precondition for embryoid formation (20, 89).

There was also debate concerning the necessity of natural embryo nutrients, but this controversy has been resolved. It was demonstrated that special nutrients such as coconut milk were not essential when carrot embryoids developed in a simple medium containing only inorganic salts, sugar, and thiamine (19).

Other factors in the medium which affect carrot embryoid formation are auxins and the nitrogen source. An auxin is not essential for the production of carrot embryoids, but embryogenesis is vigorous in cultures derived from explants which were induced to proliferate in an auxin containing medium first and then transferred to a medium without auxin (20). Supplementing the primary medium containing auxin with a cytokinin and/or gibberellic acid, suppressed embryoid formation in the subsequent induction medium.

High concentrations of nitrogen, as either ammonium or nitrate, facilitated embryoid formation (87). Under different experimental systems, it was found that ammonium and casein hydrolysate induced embryoid formation while nitrate and glutamine were not as inductive (20).

Embryogenesis in somatic cells of other plant species of widely differing angiosperm families has been reported (65, 131) and is possibly a fundamental property of many somatic plant cells (88). Embryoid formation in pollen and anther cultures with the production of haploid plants has also been reported (117, 127). As was the situation with organogenesis in tissue cultures, what holds true for the induction of embryogenesis in one plant species may not in another. Sometimes, in contrast to carrot embryogenesis, both a cytokinin and auxin are necessary. In one situation, besides an auxin and cytokinin, adenine was also essential (128). Subsequent transfers from one medium to another with different constituents or the same constituents only in different concentrations is sometimes required (27).

The potential embryogenesis has for clonal propagation, especially when large numbers of plants are desired, is obvious but has not been

explored to any great extent. Again, genetic instability of plant tissue and cell cultures poses a major problem. Finally, although the totipotency may be inherent in higher plant cells, its expression through embryogenesis has on only a limited number of occasions been realized, and then sometimes inconsistently (114).

In addition to the method using organogenesis and the method using embryogenesis, a third method employed in micropropagation, which is also applicable to clonal multiplication, is the enhancement of axillary bud growth followed by the rooting of the axillary shoots as in propagation by cuttings. Although tissue and suspension culture techniques are used, this method employs organ culture in which the maintenance of the same degree of differentiation and organization in culture as is found *in vivo* is desired (65). Some of the advantages of shoot tip as the primary explant in different types of cultures has already been mentioned. One advantage not mentioned is the retention of existing chimeras when shoot tips are cultured as intact organs. It has been shown that tissue culture techniques can be used to separate groups of cells with different genotypes found in plants with chimeras, and subsequent regeneration of genetically uniform plants has been obtained (11).

Since many commercial plants have chimeras, including some chrysanthemums (43) and carnations (32), it may be possible to disrupt these chimeras with some aseptic culture techniques. Hackett and Anderson (18) reported occasional loss of periclinal chimeras when they micropropagated the White Sim carnation variety. This variety has an outer layer of cells, which gives the flower its white color, that

originally mutated from the red William Sim variety. Hackett and Anderson's technique employed shoot tips that were not maintained as intact organs but induced to proliferate into a tissue culture from which plants could be regenerated.

Langhans, Earle and Bush (43) reported that the chrysanthemum variety Indianapolis White retains its chimera after extensive culturing. Their technique uses both shoot tips and leafy tissues obtained from these shoot tips. Phillips (79) laterally split shoot tips of a periclinal carnation variety before regenerating plants from them and observed no change in flower color. Apparently the plant species and micropropagation technique both determine the stability of chimeras in culture.

The micropropagation of gerbera (*Gerbera jamesonii* Bolus) is a successful example of a method producing axillary shoots from which plants can be regenerated (66). Tissue cultures of gerbera inconsistently regenerated small numbers of organs and, thus, were not a good means of multiplication. The successful gerbera multiplication method employed two solid medium formulations differing only in plant hormone supplements. The first medium contained 10 mg of kinetin and 0.5 mg/l IAA and was for the rapid multiplication procedure. The second medium contained 10 mg/l IAA and no kinetin and was for the rooting of axillary shoots prior to transplanting them to soil.

On the rapid multiplication medium, shoot tips produced many axillary shoots which could be continually subcultured with 4 wk passage periods on the same medium for multiplication. Subculturing of axillary shoots eliminated excising shoot tips to initiate subsequent

cultures. This was desirable because of the high number of contaminated shoot tips obtained even after surface sterilization techniques were used and because of the ease with which new cultures could be started. Whenever new plants were desired, axillary shoots were separated from one another and transferred first to the rooting medium for 10 to 14 days and then to soil. Genetic variation in the resultant plants was not observed.

Similar techniques have been used successfully with other plants with only slight modifications. Gloxina (*Sinningia speciosa*) required 30 mg/l of kinetin instead of 10 mg/l as in gerbera for optimum axillary shoot growth (22). Again, the auxin requirement was low for the multiplication step and increased 10 fold in the rooting step. It is interesting to note that with brussel sprouts, only one medium was used for both multiplication and rooting (7). Axillary shoot growth was enhanced by periodically removing the apical shoot. In effect, this treatment replaced the use of kinetin to counteract apical dominance.

Brussel sprouts have also been multiplied in suspension culture (134). This culture method used 2.56 mg/l kinetin and 8 mg/l IAA in the multiplication step. The cultures were either rotated at 6 rpm on an auxophyton or put on a horizontal shaker operated at 80 cycle/min. The propagative units were removed to a filter paper bridge support in stationary liquid medium for rooting. Again, lower levels of kinetin or no kinetin at all in the medium favored root development. Suspension culture had also been used prior to the previously mentioned investigation for clonal multiplication from shoot tips. The same investigators, Walkey and Woolfit (133), had used it for the multiplication of tobacco (*N. rustica*), and Wimber (144) was the first

to use this method when he applied it to *Cymbidium* orchid multiplication. In both instances, the main bulk of the propagative units were adventitiously formed organs, not axillary shoots.

The micropropagation of chrysanthemum is of particular interest, not only because suspension cultures have been used in the procedure, but because the investigators have also reported, following preliminary investigations, that carnations may also be propagated by their technique (43).

An earlier investigation by Ben-Jaacov and Langhans (3) had laid the groundwork for the subsequent, detailed series of two papers describing the micropropagation of chrysanthemum by Earle and Langhans (13, 14). The initial explant was prepared in their investigations by removing most of the primordial leaves surrounding the apical meristem, the sides of the exposed tip were then scraped but not cut, and an approximately 0.5 mm high and 1.0 mm wide shoot tip was then excised with a horizontal cut. The mutilation of the shoot tip in this procedure is similar to the procedure employed by Hackett and Anderson (18) with carnation shoot tips. With both carnations and chrysanthemums, the purpose of removing primordial leaves was to facilitate the formation of multiple shoots. In chrysanthemum, the removal of primordial leaves and the scraping of the sides of the shoot tip also enhanced the formation of "leafy callus" at the base of the multiple shoots. Leafy callus and multiple shoots formed within 1 mo of culture on a modified Murashige and Skoog medium containing 2.0 mg/l kinetin and 0.02 mg/l NAA which had been solidified with agar. The same medium, liquid or solidified, was used on all subsequent multiplication steps. The shoots were either removed surgically and transferred to the rooting medium or used as a source of new shoot tip explants. The leafy callus

was transferred to liquid medium and revolved on an auxophyton for further multiplication. The leafy callus grew rapidly and could be repeatedly subcultured in the liquid medium. Subculture from the liquid medium to a solid, stationary medium supplemented with 10 mg/l gibberellic acid (GA_3) promoted the formation and elongation of leaves and shoots on the tissue. Plantlets were produced by inducing adventitious root formation on shoots obtained from the multiplication steps. The medium used in the multiplication steps, with one-half of the normal inorganic salts and sucrose complement and without any hormones, was found to be satisfactory for root induction. Filter paper bridges were used as the support while roots developed in this liquid medium.

In developing a program to clonally multiply pathogen-free carnations, the investigations into shoot tip culture for the production of pathogen-free carnations should provide useful information on many of the *in vitro* growth requirements of carnations. These requirements can be categorized as nutrient medium characteristics and environmental qualities (65).

Culture Media

Comprehensive reviews of nutrition and metabolism in plant cultures have been written (5, 111, 115, 140, 145). The various aspects of plant culture nutrition can be categorized into two main areas: inorganic and organic nutrition. Inorganic nutrition includes the essential elements in both macro- and micro-amounts which are supplied as mineral salt mixtures. Yeoman (145) has tabulated six widely used mineral salt mixtures. These are the formulations of Heller (24), Nitsch and Nitsch (71), White (141), Murashige and Skoog (68), Hildebrandt, Riker and

Duggar (29), and Gautheret (16) which are used in both basic and modified form. Street (115) has summarized the individual element concentrations in tabular form for 12 mineral salt mixtures commonly used in tissue and suspension cultures.

Widely divergent media, in terms of ion concentrations, are reported to support rapid growth of tissues from the same plant species and part (145). Carnation shoot tip cultures have been successfully established on mineral salt mixtures of Gautheret (1, 83, 109, 126) and Murashige and Skoog (15, 18, 77, 132). The overall mineral salt concentration in Gautheret's mixture is low, and in the Murashige and Skoog mixture it is high (144). However, in all the instances referred to where Gautheret's mixture has been employed, adventitious root formation and shoot growth have been simultaneous, whereas the modified mixture of Murashige and Skoog has been used when rapid shoot development alone is the desired result (15, 77). Sometimes with carnations and other plants shoots alone are formed on a medium with a high concentration of salts, and they are subsequently transferred to a medium which has a low mineral salt concentration for the initiation and growth of roots (13, 14, 77, 130).

In general, the inorganic salt requirement has in most cases remained constant throughout a wide variety of plants and applications. For most purposes, it has only been necessary to compare the effectiveness of a few already existing formulations to find one that is sufficient (65, 115). A half-strength Murashige and Skoog salt mixture was reported by Engvild (15) to support a faster growth rate of carnation stem pith, tissue cultures than the salt mixtures of Heller (24), Gautheret (16), and White (141). It also supported

rapid growth after several subcultures, whereas the full strength Murashige and Skoog salt mixture did not. Another group of investigators reported that the salt mixture of Hildebrandt, Riker, and Duggar (29) supported the continuous subcultures of carnation pith tissue (54). Carnation ovary cultures have been grown using the salt formulations of Nitsch and Nitsch (71), and White (141) for successful *in vitro* pollinations (146).

In contrast to the inorganic requirements, organic constituents vary extensively from medium to medium. Few plant cultures have been shown to be autotrophic with regard to carbonaceous nutrition (5, 145). Sucrose or glucose are included in most media as a source of carbohydrates. A concentration of 2 to 3% sucrose is common in most media (65, 115). Nitsch and Nitsch (71) found that a combination of glucose and sucrose, both in 0.1M concentrations, supported a higher growth rate of Jerusalem artichoke (*Helianthus tuberosus* L.) tissue than when either was used alone in various concentrations. Also, both sugars were more effective after autoclaving. Phillips (80) preferred glucose over sucrose for carnation shoot tip culture and found that 40 g/l glucose supported better root and shoot growth than did 20 g/l glucose. More recent investigations with carnation shoot tip cultures have successfully used sucrose at 20 to 30 g/l (15, 77).

Another important group of organic nutrients in plant cultures are vitamins. All of the essential vitamins can be synthesized by most plant tissues *in vitro*, although some may be produced in suboptimal amounts (5). The need for many vitamin supplements in media still remains equivocal, and they may be used only for precautionary reasons (65). The most commonly used vitamins are thiamine, pyridoxine,

nicotinic acid, and inositol. Alpi (cited in Phillips (80)) demonstrated the need for thiamine in carnation shoot tip culture when all other vitamins were absent from the medium. Thiamine, however, is not an essential constituent of the carnation culture medium because a mixture of calcium pantothenate, inositol, biotin, nicotinic acid, and pyridoxine has also been shown to support carnation shoot tip growth in culture (83).

Many complex natural products have been included in plant culture media to improve growth and morphogenesis. Some of these are: coconut milk, yeast extract, malt extract, tomato juice, maize extract, and casein hydrolysate (5). The synergistic combination of 2,4-dichlorophenoxy-acetic acid (2,4-D) and the liquid endosperm of coconuts (coconut milk) in promoting growth in potato tuber tissue was described by Steward and Chaplin (105). Carnation pith cultures responded favorably to a combination of 2,4-D and coconut milk, but coconut milk was inhibitory when added to a medium containing both an auxin and a cytokinin (115). For pollen and anther culture of many plants, cytokinins can often be replaced by coconut milk (117). The role in cell division of plant endosperm and other fluids or extracts from the environment surrounding plant embryos has been investigated and discussed (105).

Casein hydrolysate has been used in carnation shoot tip cultures and stem pith cultures with beneficial effects on growth (15, 83). Jerusalem artichoke tissues can be grown on casein hydrolysate as the sole source of nitrogen (71). Various amino acids, natural complexes rich in amino acids such as casein hydrolysate and yeast extract, and ammonium salts can provide organic nitrogen for amino acid and

protein synthesis in cultured cells, especially enhancing growth when the reduction of nitrates by plant cells lags behind the rate of protein synthesis (5).

The trend in plant culture media has been toward defined constituents; undefined natural complexes have been losing favor (145). The stimulatory effect of these complexes can often be replaced by a few defined compounds. In tobacco pith cultures, the cell division effect of extracts from the cambial region of tobacco stems has been duplicated by cytokinins (9). A defined medium can replace one containing coconut milk for growth and morphogenesis in carrot tissue cultures (19). The single amino acid, tyrosine, and no others can replace casein hydrolysate in the medium for tobacco cultures (102).

The excessive variability within individual natural complexes has been another factor which has contributed to their disfavor among investigators. Because of this, Murashige (65) feels that natural complexes should only be used when attempts to use a defined medium fail or when additional stimulation might be beneficial.

Another group of organic constituents sometimes found in plant culture media are substances which have been classified as growth regulators. This group includes, among other substances, the plant hormones, both natural and synthetic. The three groups of plant hormones most commonly employed in plant culture media are the auxins, cytokinins and gibberellins (65, 111). Both environmental and genetic control of growth and differentiation in plants is achieved by means which include the participation of growth hormones (135). The regulating activity of auxins and cytokinins in initiating shoot and root growth has been the factor which has made tissue culture techniques applicable to clonal propagation (65).

The gibberellins have found limited use in micropropagation media, because they repress the initiation of organs (65). They have been used to stimulate elongation of already initiated shoots in chrysanthemum micropropagation (13, 14), but were ineffective when used to stimulate adventitious buds of *Convolvulus* to elongate in tissue cultures derived from cell suspensions (12). Engvild (15) used gibberellic acid (GA_3) and a cytokinin in a culture medium for carnation shoot tips.

The auxins NAA, IAA, and 2,4-D, have all been used in various carnation cultures. NAA was employed alone in the media used for obtaining pathogen-free plants from shoot tips when shoot and root development were simultaneously desired (1, 80, 83, 109). 2,4-D alone has been used where the establishment of carnation tissue or suspension cultures have been the objective (15, 54). NAA or IAA and a cytokinin have also been employed in carnation tissue and suspension culture media (15, 77).

Three different cytokinins were unsuccessfully used with auxins in an attempt to regenerate shoots from carnation pith tissue (15). Petru and Landa (77) have successfully regenerated adventitious shoots from carnation tissue cultures using combinations of kinetin and NAA or IAA. The enhancement of axillary carnation shoots in culture has also been demonstrated using 6-benzylaminopurine (BAP) or kinetin (15, 77).

Other growth regulators, with either hormone activity or anti-metabolite activity, have been included in plant culture media. Adenine is probably the most commonly used compound in this category. It has been shown to stimulate adventitious shoot formation (103) and has also been shown to stimulate root initiation (128). It was used in a medium

on which buds were initiated in carnation tissues (77). Phillips (80) believed that it increased the survival rate of carnation shoot tips in culture but reported also that high concentrations caused excess tissue proliferation at the excised base of the shoot tips. Adenine was also shown to enhance the stimulatory effect of casein hydrolysate on the growth of tissue cultures (27).

Antimetabolites such as auxin protectors occur naturally in young plant tissue (110). Cinnamic acid and indole are known to inhibit IAA oxidase activity and when incorporated in the medium for carnation shoot tips, increased their growth (80). In contrast to protecting IAA, the addition of auxin antagonists has been thought to enhance carrot embryoid formation by supposedly lowering the level of endogenously produced auxin (65).

The concentration of hydrogen ions (pH) in culture media affects the growth in plant tissue cultures. Intracellular pH is determined partially by the pH of the medium (29). The general practice is to set the pH of the medium with NaOH or HCl at some value within the range of 5.0 to 6.0 during the preparation of the medium (5, 65). Too high or too low a pH has been shown to affect the availability of some nutrients, either because they precipitate out of the medium or because they become complexed with other constituents and are no longer available to the plant cells. Iron has been shown to complex with sugar acids during autoclaving at pH values above 5.2, and chelating agents, commonly ethylenediamine tetra-acetate (EDTA), have been put into some media to stop this (115). Agar will not solidify at low pH values, and also at these high hydrogen ion concentrations, some organic components may be destroyed (5).

Because the pH of the medium shifts during the culture of plant material (29), Phillips (80) devised a method of telling when the pH had shifted too high for proper growth. He added the pH sensitive dye, brom-thymol blue, to the medium which is yellow below 6.0 pH and turns green to blue as the pH rises above 6.0.

The physical state of the medium is a critical variable. Three main physical states are predominantly used: solid stationary, liquid stationary, and agitated liquid (145). The success in micropropagation of pineapple (*Ananas comosus*) depended upon the sequence of physical states of the medium (36). Only when the shoot tip was induced to form protocorm-like bodies in agitated liquid medium and then transferred to a solid stationary medium did plantlets develop. Similar results were found with *Cattelya* orchids (96). As previously mentioned, transferring tobacco tissue from a solid medium to a stationary liquid medium in which it was submerged elicited a morphogenic response (138). In some plants such as asparagus (143) and chrysanthemum (14), rapid micropropagation is obtained when the culture is initiated on a solid medium and then transferred to an agitated liquid medium for multiplication. With gerbera (66) and gloxina (22), micropropagation procedures were all performed on a solid media.

Agar has been the common solidifying agent used in culture media. The quality and quantity of agar used has been important. It was shown that excised *Picea abies* shoot apical meristems grew best on Difco "purified" agar, second best on Difco "Bacto-agar", and least on Difco "Noble" agar (92). In another investigation, Difco "Bacto-agar" proved superior to washed, shredded agar (71). The quantity of agar which should be used varies with the plant species, plant part, pH of the medium, and quality of agar being used (65).

Often stationary liquid media have been used when critical nutritional studies are impeded by the impurities in agar (25) and when well formed roots are desired (14, 61, 63, 73, 134). Most stationary cultures have been designed so that the organ or tissue being cultured remains mostly in the gas phase while a support acts as a wick to provide nutrients. Pyrex glass wool (49), polyacrylamide gel (61), Sephadex G. 100 (61), and ash-less filter paper (25) have been used as supports in stationary liquid media. Phillips (81) has modified the ash-less filter paper technique of Heller (24) for carnation shoot tip culture and reported better root development on it than on agar.

Temperature and Light

Temperature and the light quality, intensity, and duration are four physical factors of the micropropagation environment which deserve careful consideration (65).

The optimum temperature for the culture of most plant tissues has been 24 to 27 ± 2 to 3 C for different tissues (5). Phillips (80) observed that temperatures between 18 to 22 C had been used for carnation shoot tip culture and elected to use 18 to 20 C. Subsequent investigators working with carnation tissue and shoot tip culture have used 25 C as their approximate standard temperature (15, 77).

Key organogenetic responses in aseptic plant cultures have been photomorphogenic and probably regulated by phytochrome, as indicated by light quality effects (65). Investigations have shown that blue light stimulates shoot initiation, while red light does not, and opposite effects have been observed for root initiation.

Continuous light from cool white fluorescent lamps at approximately 2000 lux was found to enhance carnation shoot tip growth more so than 12 hour photoperiods at the same intensity (80). Adventitious shoot formation in carnation tissue cultures was obtained when the tissue had been initiated on one medium in the dark and then subcultured onto another medium which was maintained in daylight (77). Geranium callus differentiated adventitious shoots when grown under specific photoperiods but not when grown under continuous illumination (82). Micropropagation techniques for gerbera (66), gloxina (22), asparagus (23), and chrysanthemum (13) all have light requirements between 1000 to 4000 lux for the initiation and multiplication stages. Gerbera and asparagus grew better with intermittent dark periods, while gloxina and chrysanthemum responded favorably to continuous illumination. In these investigations Gro-lux or cool white fluorescent lamps were used. No difference in results between the two light sources was observed in asparagus culture. Murashige (65) has observed that day length influences tissue cultures of plants which are normally responsive to photoperiods.

Carnation Micropropagation

Although micropropagation has been employed to produce pathogen-free propagative stock, this micropropagation procedure in itself does not multiply carnations, but produces only one plant for every original explant. The multiplication of pathogen-free plants is then left up to conventional propagative procedures.

The first report of a clonal propagation procedure for carnations using micropropagation techniques was by Hackett and Anderson (18). They put shoot tips on a modified White's medium with five times the

normal inorganic salt concentration. This medium was supplemented with 2 mg/l NAA, and it stimulated unorganized cell proliferation after the primordial leaves of the shoot tip were removed. The tissue culture which resulted from this treatment had many green meristematic areas which could regenerate shoots upon subculture to a Murashige and Skoog medium without NAA. Stock tissue cultures were subcultured and maintained on a modified White's medium with the normal inorganic salt complement and 1 mg/l NAA during an 18 month period without losing the ability to regenerate shoots.

Engvild (15) in attempts to produce carnation plants from single cells first established tissue and suspension cultures from which he hoped to get shoot or embryoid formation. After plating out filtered cell suspensions, only aggregates of cells grew, and single cells died. With tissue cultures, high concentrations of cytokinins were combined with low concentrations of auxins, but adventitious shoot regeneration was not observed. In another experiment, tissues were grown on a medium containing coconut milk and 2,4-D for a month and then transferred to the same medium without 2,4-D. This procedure also failed to stimulate shoot or embryoid formation. Axillary shoot growth, however, was successfully enhanced from 0.2 mm shoot tips. The medium he used for shoot tips was supplemented with casein hydrolysate, gibberellic acid, and BAP and it sometimes stimulated growth of 20 or more 1 to 2 cm shoots in a month from a single shoot tip.

Petru and Landa (77) succeeded in inducing adventitious shoot formation in tissue cultures from the excised hypocotyls of garden variety carnation seedlings. A modified Murashige and Skoog medium,

with 4.0 mg/l IAA and 2.5 mg/l kinetin, was used to culture the hypocotyl explant for one month in the dark. The resultant tissue culture was then subcultured on a medium with lower concentrations of macro-elements, sucrose, NAA (0.25 mg/l), and kinetin (0.5 mg/l) and supplemented with adenine. When grown on this medium under natural light, the tissue was induced to produce adventitious shoots, and these shoots could be induced to form roots when transferred to a medium without hormones.

Petru and Landa grew shoot tips on the hypocotyl tissue culture initiation medium. On this medium, many shoots developed from the initial shoot tip explant. These shoots could be isolated and induced to form roots on a Morel's medium with 0.04 mg/l NAA. Stems 5 to 10 cm long from these rooted shoots were then cut into pieces approximately 2 cm long. Shoots and roots were evoked from these pieces *in vitro*. They believed that this could be an efficient way to multiply pathogen-free carnations.

Chapter III

MATERIALS AND METHODS

Source and Excision of Shoot Tips

Mother block plants maintained in the carnation clean stock facility at Colorado State University were the source of all shoot tips. These plants were originally propagated as shoot tips by the technique developed by Phillips (80) and each plant was grown separately in gravel. Nutrients were proportioned into the watering system and watering was fully automated. The plants were watered simultaneously several times daily at the gravel's surface through leader tubing. To prevent contaminants from being spread by loose water, the foliage was never watered directly and spraying the foliage with fungicides was avoided prior to taking shoot tips.

The mother blocks were periodically pruned. This insured a constant supply of uniformly sized young shoots. To obtain vegetative shoot tips whose apical meristems had not begun to undergo floral morphogenesis, only shoots 10 to 15 cm long were used. The internodes of these shoots had not begun to elongate, an indication that the flowering stage had not started, and only 10 to 14 nodes were present. Shoots smaller than 10 nodes long were difficult to handle while excising the shoot tip. Shoots with over 16 nodes usually had a recognizable floral bud.

Eight to 12 leaf pairs were removed (Fig. 1) from each shoot before exposing the shoot tip. Shoot tips excised for most experiments had one or two leaf pairs surrounding the apical meristems and were ca. 1 mm in height. All leaf pairs over 3 mm in length were removed by hand. The rest were removed with a scalpel. Since the unexposed shoot

Fig. 1. A carnation cutting before removal of any of its leaves, and two cuttings after removal of most of their leaves by hand.



tip was shielded from contaminating organisms by the surrounding leaves, sterilization procedures were not necessary. However, during removal of leaf pairs and after the shoot tip was exposed, precautions were taken to prevent the introduction of contaminants.

Two scalpels (Bard-Parker No. 11 blade) were alternately used during excision of the shoot tips. The scalpels were flame sterilized after dipping the blade end into 95% ethyl alcohol. While one scalpel was used, the other was allowed to cool. Blades were changed often to maintain a sharp cutting edge. The excision process had to be done quickly to prevent desiccation and possible contamination of the exposed shoot tip. By using two scalpels, a clean, sterile scalpel was always ready. Most of the time one scalpel was used to cut away excess tissue and leaves (Fig. 2), and the other was then used to excise the shoot tip. The shoot tips were excised as described by Phillips (80) with a cut perpendicular to the long axis of the shoot. If a smooth cut was made, the shoot tip usually adhered to the scalpel blade and was then easily transferred to the culture medium, and placed upright on the surface of the medium.

In most experiments, a mixture of shoot tips from different carnation varieties (Table 1) was used. These varieties were spread evenly throughout each experiment.

Fig. 2. Carnation shoot tip prior to removing the surrounding leaf bases, lateral shoots, and all but the last one or two remaining leaves. Once the surrounding tissues were removed, the shoot tip was excised for culture.



TABLE 1. Carnation varieties used as a source of shoot tips.

Variety	
CSU Pink	Flamingo
CSU Red	Tangerine
White Sim	Arthur
White Pike's Peak	Linda
Scania	Yellow Dusty

Basal Medium

Throughout the study the composition of the basal medium was changed when results indicated such modifications. Starting with the original basal medium (MS 1) each new modification was consecutively numbered for clarity and convenience in this thesis.

The MS 1 medium was the Linsmaier and Skoog medium (48) as modified for carnation micropropagation (42). Tables 2 and 3 list the inorganic salts (Muraschige and Skoog macro- and micro-elements) of this medium and of other media used throughout this study. Table 4 lists the vitamin mixture of this medium and others also used in the study. The other ingredients were 30 g/l sucrose, 0.5 mg/l NAA, 2.0 mg/l kinetin, and when a gelled medium was desired, 8 g/l Difco "Bacto-agar." The pH was adjusted to 5.5 before autoclaving but after dissolving the agar when it was used. All media were sterilized by autoclaving for 15 minutes at 121 C and 1.27 kg/cm².

Culture Conditions

This study was divided into three stages: (1) the initiation of aseptic cultures, (2) the aseptic multiplication of shoots, and (3)

TABLE 2. Inorganic salts: macro-element mixtures used in various experiments.

Constituents	Macro-elements (mg/l)								
	Nitsch and 1969(70)	Nitsch 1956(71)	Gautheret (16)	White (141)	Heller (24)	Hildebrandt, Riker, and Duggar (29)	THS (129)	Murashige and Skoog (68)	Petru and Landa "B" (77)
KCl	--	1500	--	65	--	65	910	--	150
NaNO ₃	--	--	--	--	600	--	1800	--	--
MgSO ₄ · 7H ₂ O	185	250	125	720	250	180	180	370	125
NaH ₂ PO ₄ · H ₂ O	--	250	--	16.5	125	33	333	--	--
CaCl ₂ · 2H ₂ O	220 ^a	33 ^a	--	--	75	--	--	440	--
KNO ₃	950	2000	125	80	--	80	80	1900	--
Na ₂ SO ₄	--	--	--	200	--	800	800	--	--
(NH ₄) ₂ SO ₄	--	--	--	--	--	--	790	--	--
NH ₄ NO ₃	720	--	--	--	--	--	--	1650	175
KH ₂ PO ₄	68	--	--	--	--	--	--	170	90 ^b
Ca(NO ₃) ₂ · 4H ₂ O	--	--	500	300	--	400	400	--	400

^aConcentration adjusted for waters of hydration by multiplying the given concentration of CaCl₂ by 1.324.

^bSubstituted for KH₂PO₄ · H₂O at the same concentration.

TABLE 3. Inorganic salts: micro-element mixtures used in various experiments.

Constituents	Micro-elements (mg/l)			
	Nitsch and Nitsch 1969 (70)	Murashige and Skoog (68)	Heller (24)	Petru and Landa (77)
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	25	22.3	0.01	0.2
H_3BO_3	10	6.2	1.0	0.3
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10	8.6	1.0	0.03
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.25	---	---
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	0.025	0.03	0.03
KI	---	0.83	0.01	0.02
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	---	0.025	---	---
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	---	---	0.03	---
AlCl_3	---	---	0.03	---
$\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$	---	---	---	0.03
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	---	---	---	0.03
FeEDTA ^a	50	50	50	50
KBr	---	---	---	0.02
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}^b$	---	---	---	0.02
$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	---	---	---	0.02
LiCl	---	---	---	0.02

^aA commercial preparation from Calbiochem, La Jolla, California with 13% iron was used to replace all iron and EDTA containing constituents of the original mixtures.

^bSubstituted for $(\text{NH}_4)\text{MoO}_4$ at the same concentration.

TABLE 4. Compositions of various vitamin mixtures compared for their effect on the growth in shoot tip cultures. An amino acid, glycine, was included in some treatments.

Vitamin mixture	Code	Composition (mg/l)				
		Thiamine-HCl	Pyridoxine-HCl	Nicotinic acid	Myo-inositol	Glycine
Engvild (15)	E	0.5	0.5	0.5	100	---
Linsmaier and Skoog (48)	LS ^a	0.4	---	---	100	---
	LSG	0.4	---	---	100	2.0
White (141)	W	0.1	0.1	0.5	---	2.0 ^b
Murashige and Skoog (68)	MS	0.1	0.5	0.5	---	2.0
	MS50	0.1	0.5	0.5	50	2.0
	MS100 ^a	0.1	0.5	0.5	100	2.0
	MS150	0.1	0.5	0.5	150	2.0
	MS200	0.1	0.5	0.5	200	2.0
	MSNG	0.1	0.5	0.5	---	---

^aOriginal formulation before modification.

^bModified from 3.0 mg/l as given in original formulation.

the rooting of micropropagated shoots. Each stage had a set of culture conditions that differed from the other.

In the initiation stage, the shoot tips were grown on a solid, stationary medium. Each shoot tip was maintained in a separate, 22 x 85 mm vial with 10 ml of medium. Each vial was capped with tin foil and a rubber band was used to increase the tightness of the cap.

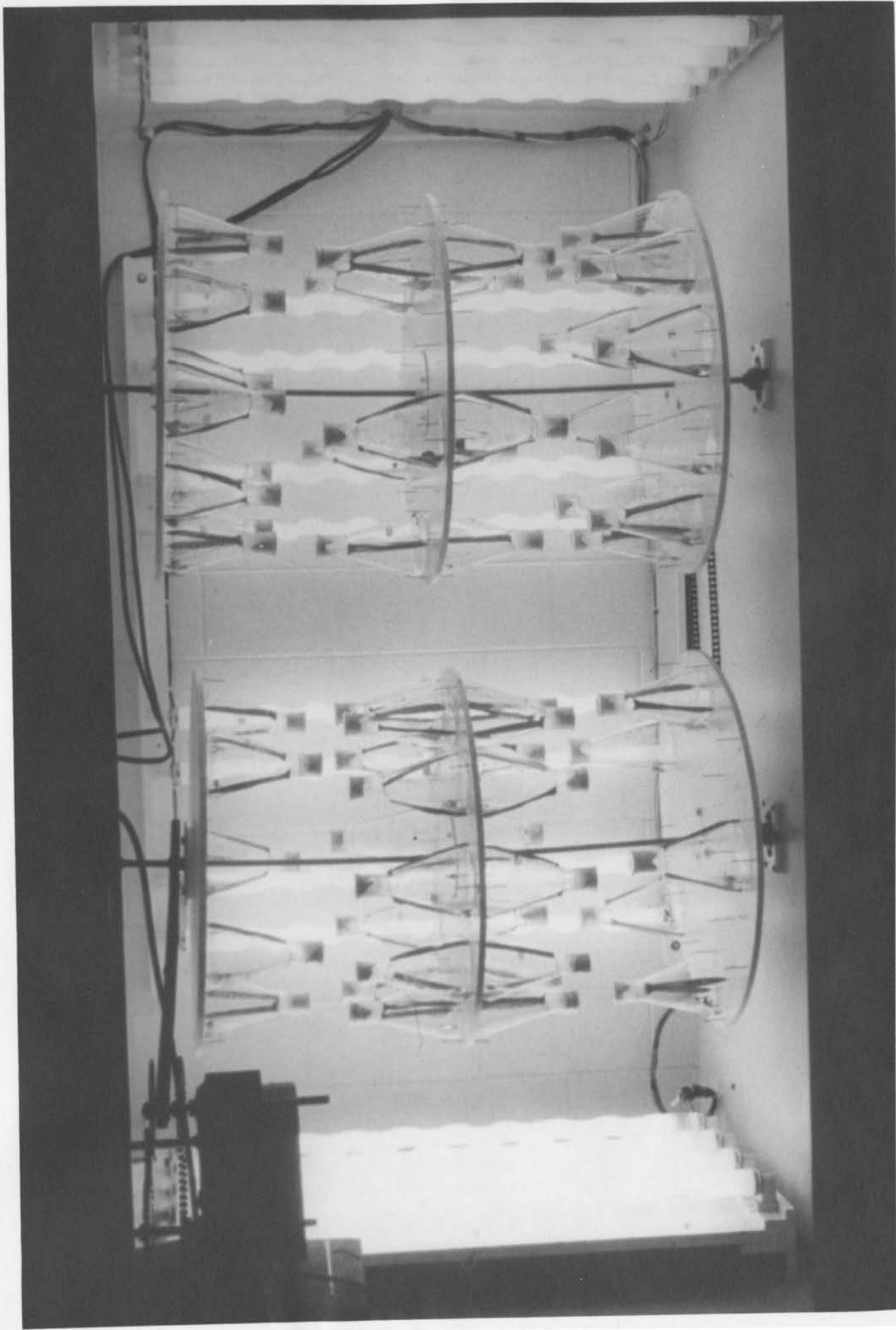
Shoot tips were grown under constant illumination by cool white fluorescent lamps at an intensity of approximately 2,000 lux. The temperature was approximately 22 C. Light intensity and temperature varied little between the different culture rooms used.

After 3 to 4 wk in the initiation stage, the shoots developing from a shoot tip were either sacrificed for taking data or transferred aseptically to the multiplication stage. Dry weights were taken but did not provide any more information than did the fresh weights, thus dry weights are not reported in this thesis. Subjective data based on visual estimations were taken for each treatment, while fresh weight data was taken for each replicate in each treatment.

In the multiplication stage, 1,000 ml Erlenmeyer flasks were used as the culture vessel. Each flask contained 50 ml of liquid medium. In most instances, one culture from a shoot tip was aseptically transferred to one flask, and in a few experiments two were transferred. The flasks were capped with tin foil, and Parafilm used to seal them from contamination.

Each flask was then attached to an auxophyton (Fig. 3) constructed for this study consisting of 80 cm plywood disks attached to iron

Fig. 3. The multiplication stage culture room with the auxophyton.



rods. The iron rods were mounted horizontally to the walls of the culture room in roller bearings and were the axils on which the disks rotated. The axils were revolved by a chain drive mechanism attached to an electric motor. Flasks attached horizontally to the outer perimeter of the disks revolved at approximately 1 rpm.

Continuous illumination was provided by General Electric Power Groove, cool white fluorescent lamps. These were mounted parallel to the axils and were below, above and behind the auxophyton. When all 16 lamps were turned on, the light intensity at the axil was approximately 16,000 lux.

The temperature of the culture room with the auxophyton was maintained at approximately 25 C, and the ballasts for the fluorescent lamps were placed in another room where they would not affect the temperature of the culture room.

After 3 wk the flasks (Fig. 4) were removed from the auxophyton, and the shoots were rinsed with water and then removed from the flasks. Each shoot was then separated from the rest by excision. Only shoots over 2 cm long were transferred to the rooting stage.

Several techniques were used for rooting. In the first, the shoots were rooted in 55 mm wide and 70 mm high glass jars with metal caps. Each jar contained a 2 cm thick layer of perlite (1) or Terragreen (Oil and Dri Corp. of America, Chicago, Ill.) as the rooting supports. Inorganic salt solutions or water with various concentrations of NAA were used to moisten the supports, and each jar, so prepared, was autoclaved for 15 min at 121 C and 1.27 kg/cm².

Three shoots were stuck into the rooting supports of each jar under semi-aseptic conditions. The jars with shoots were then

Fig. 4. Cultures after removal from the auxophyton. The shoots in the flask, and those on the left developed from one shoot tip each after 3 wk in both the initiation and multiplication stage.



maintained for 2 to 3 wk. Under the same physical conditions employed for the initiation stage.

The other rooting techniques employed were all modifications of conventional procedures used for rooting carnation cuttings (32). Propagation benches equipped with intermittent mist systems (41) were used. The cut end of each shoot was dipped into a commercial rooting powder (Stim-Root, Plant Prod. Co. Ltd., Port Credit, Ontario) and the shoots were stuck into different supports for rooting. After a sufficient root system developed data was taken and some plantlets transferred to soil for further tests.

Chapter IV

RESULTS

Stage I - The Initiation of Aseptic Cultures

The concentration ranges of NAA and kinetin regulating the growth of carnation shoot tips were examined. The appropriate ranges were selected after preliminary experiments. Thirty \pm 3 shoot tips/treatment were grown for 4 wk on the MS 1 medium containing the combinations of concentrations shown in Table 5.

Determining the concentrations of the two plant growth regulators, enhancing shoot growth while still counteracting apical dominance, was the purpose of this and other similar experiments. The number of 2 cm or longer shoots produced from each shoot tip (Table 5) was the indicator used to determine the degree to which apical dominance was counteracted, and concentrations from 0.1 to 1 μ M NAA with 5 and 10 μ M kinetin consistently produced the higher numbers of shoots.

TABLE 5. Number of shoots^a, 2 cm or longer, developing in carnation shoot tip cultures in response to various concentrations of NAA and kinetin in the medium.

NAA (μ M)	Kinetin					
	.1	.5	1	5	10	50
0	+	+	+	++	++	-
0.01	+	+	+	++	++	-
0.05	+	+	+	+	++	-
0.1	++	++	++	+	++	-
0.5	+	++	++	++	+++	-
1	+	++	++	++	++	0
5	+	+	+	+	+	0
10	+	+	+	+	+	0
50	0	0	0	0	0	0

^aVisual estimate of number; none (0), one (+), two or three (++), four or more (+++), no growth of cultures or very limited growth (-).

Subjective data was also taken on the presence of leafy tissue (Table 6) growing around the base of the shoots. The tissue contained many leaves and some shoots. Many of these leaves and shoots were morphologically abnormal. The shoots were fasciated and stunted, and the leaves often originated directly from callus forming around the periphery of the shoot tip's base. These leaves were various shapes and sizes, but often they were short and grouped together in rosettes.

TABLE 6. Development^a of leafy tissue at the base of carnation shoot tips in response to various concentrations of NAA and kinetin in the culture medium.

NAA (μ M)	Kinetin (μ M)					
	.1	.5	1	5	10	50
0	0	0	0	+	0	-
0.01	0	0	0	+	0	-
0.05	+	+	+	+	0	-
0.1	+	+	+	+	+	-
0.5	+	+	+	+	++	-
1	+	+	+	++	++	-
5	+	+	+	+	+	-
10	0	0	0	+	+	-
50	0	0	0	0	0	-

^aVisual estimation of presence of leafy tissue; present (+), above average present (++), none present (0), very limited or no growth of cultures (-).

The fresh weight of all individual cultures was taken after 4 wk and the average fresh weight for each treatment is given in Table 7. The individual fresh weights of all cultures, except those with 50 μ M

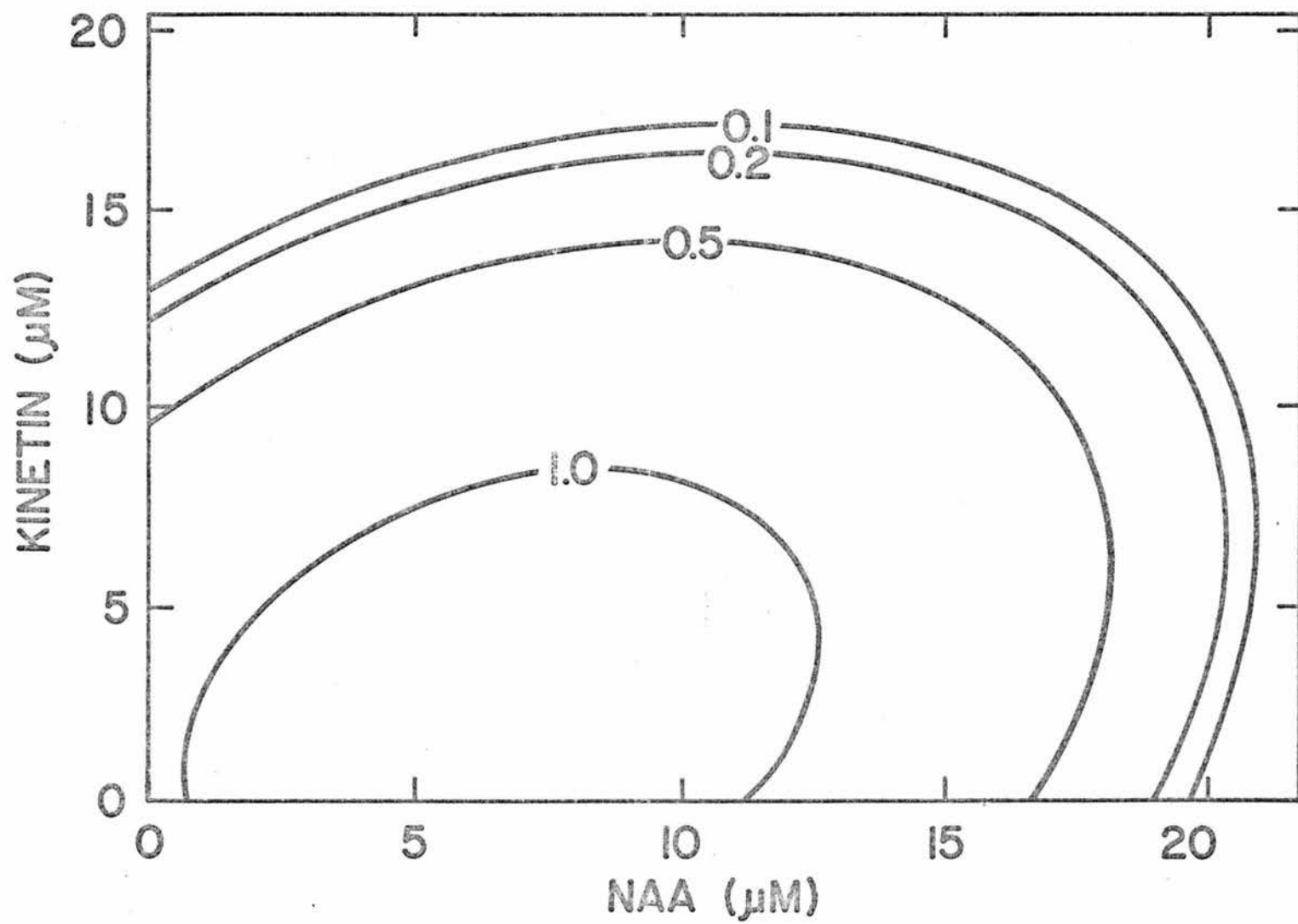
of NAA or kinetin, were used to statistically (Statistical Package for the Social Sciences, Vogelback Computing Center, Northwestern Univ., Ill., used by Colorado State Univ. Statistical Laboratory) derive a second degree polynomial for fresh weight with NAA and kinetin as the independent variables (fresh wt = $0.0651 N + 0.00304 K + 0.005398 N^2 - 0.00545 K^2 + 0.00331 NK + 0.9635$; N = NAA in μM and K = kinetin in μM ; $P = 0.0001$; $R^2 = 0.68281$). This equation was used to plot the fitted response surfaces in Figure 5. Each contour is a response surface for a specific fresh weight and the varying concentrations of NAA and kinetin necessary to obtain this fresh weight in 4 wk.

TABLE 7. Fresh weight of shoot tip cultures in response to various concentrations of NAA and kinetin in the culture medium.

NAA (μM)	Kinetin (μM)					
	.1	.5	1	5	10	50
0	1.17 ^a	1.14	0.81	0.94	0.41	0.01
0.01	1.15	1.04	1.06	0.89	0.28	0.01
0.05	0.96	0.75	1.04	0.94	0.45	0.01
0.1	0.89	0.89	0.78	0.57	0.46	0.01
0.5	1.02	1.00	0.89	0.89	0.59	0.01
1	1.08	1.05	1.05	0.98	0.68	0.04
5	1.01	1.34	1.06	1.04	0.89	0.04
10	1.08	1.37	0.76	1.37	0.75	0.04
50	0.47	0.33	0.17	0.24	0.21	0.04

^aFresh wt (g); each wt is the average of 30 ± 3 replications after 4 wk of culture.

Fig. 5. Constant contour plots of the fitted response surfaces for fresh weights (g). Each fresh weight is represented by an individual response surface. Fresh weight data from shoot tips that were grown for 4 wk on the MS 1 medium with various concentrations of NAA and kinetin were used to derive the equation for plotting the response surfaces.



These response surfaces indicate that NAA and kinetin interacted as evidenced by the skewed nature of the surfaces toward the upper right hand corner ($P = 0.0001$). This interaction was directly proportional to the concentration of NAA and kinetin used; thus, as the concentration of one growth regulator was increased, an increase in the other's concentration was necessary to obtain the highest growth rate possible with any one concentration of the two.

The concentric nature of the response surfaces indicates that optimum concentration in regards to fresh weight obtained would have been somewhere between 2.5 and 7.5 μM NAA and 1 and 5 μM kinetin.

The fresh weight data alone in this and subsequent experiments was often misleading. In some treatments most of the fresh weight was due to unorganized tissue (Table 8). This tissue grew in areas where the surface had been damaged either by cutting or some other way during the shoot tip excision procedure. By comparing the data in Tables 5 through 8, it was observed that, in general, the highest fresh weight did not come from the treatments with the most shoots and leafy tissue, but came from treatments where unorganized tissues and/or single shoots accounted for most of the growth.

A subsequent experiment was executed using concentrations of NAA and kinetin in the range where the most shoots were produced in the previous experiment. The same type of data was taken, and a second degree polynomial (fresh wt = $0.23211 N - 0.09049 K - 0.03169 N^2 + 0.00139 K^2 + 1.64288$; N = NAA in μM and K = kinetin in μM ; $P = 0.0001$; $R^2 = 0.7331$) and fitted response surface plots (Fig. 6A) from this equation were also obtained.

TABLE 8. Unorganized tissue growth² from carnation shoot tips in response to various concentrations of NAA and kinetin in the culture medium.

NAA (μ M)	Kinetin (μ M)					
	.1	.5	1	5	10	50
0	+	+	0	+	0	-
0.01	0	+	+	+	0	-
0.05	0	0	0	+	0	-
0.1	0	0	0	0	0	-
0.5	0	0	0	0	0	-
1	+	0	0	++	+	-
5	++	++	++	+++	++	-
10	++	+++	++	+++	++	-
50	+++	+++	+++	+++	+++	-

^aVisual estimation of % total fresh weight per treatment; 0 to 25% (0), 26 to 50% (+), 51 to 75% (++), 76 to 100% (+++), very limited or no growth of cultures (-).

Unorganized tissue growth (Table 9) was less throughout this experiment; however, in addition to the plots (Fig. 6A) of the fitted response surface for fresh weight, the polynomial (fresh wt = $0.23661 N - 0.0862 K - 0.04367 N^2 + 0.00118 K^2 + 0.00262 NK + 1.61502$; N = NAA in μ M and K = kinetin in μ M; $P = 0.0001$; $R^2 = 0.778$) for fresh weight was adjusted for the difference in fresh weight and due to unorganized tissue growth, and another set of plots (Fig. 2B) were derived.

Fig. 6. Constant contour plots of the fitted response surfaces for fresh weights (g). Each fresh weight is represented by an individual response surface. The plots were derived in the same manner as those in Fig. 5, except the NAA and kinetin concentrations in the medium for growing the shoot tips were varied over the range in which only the greatest number of shoots were observed, and, in addition to the plots (A) not adjusted for the difference in fresh weight due to unorganized tissue growth, another set of plots (B) was adjusted.

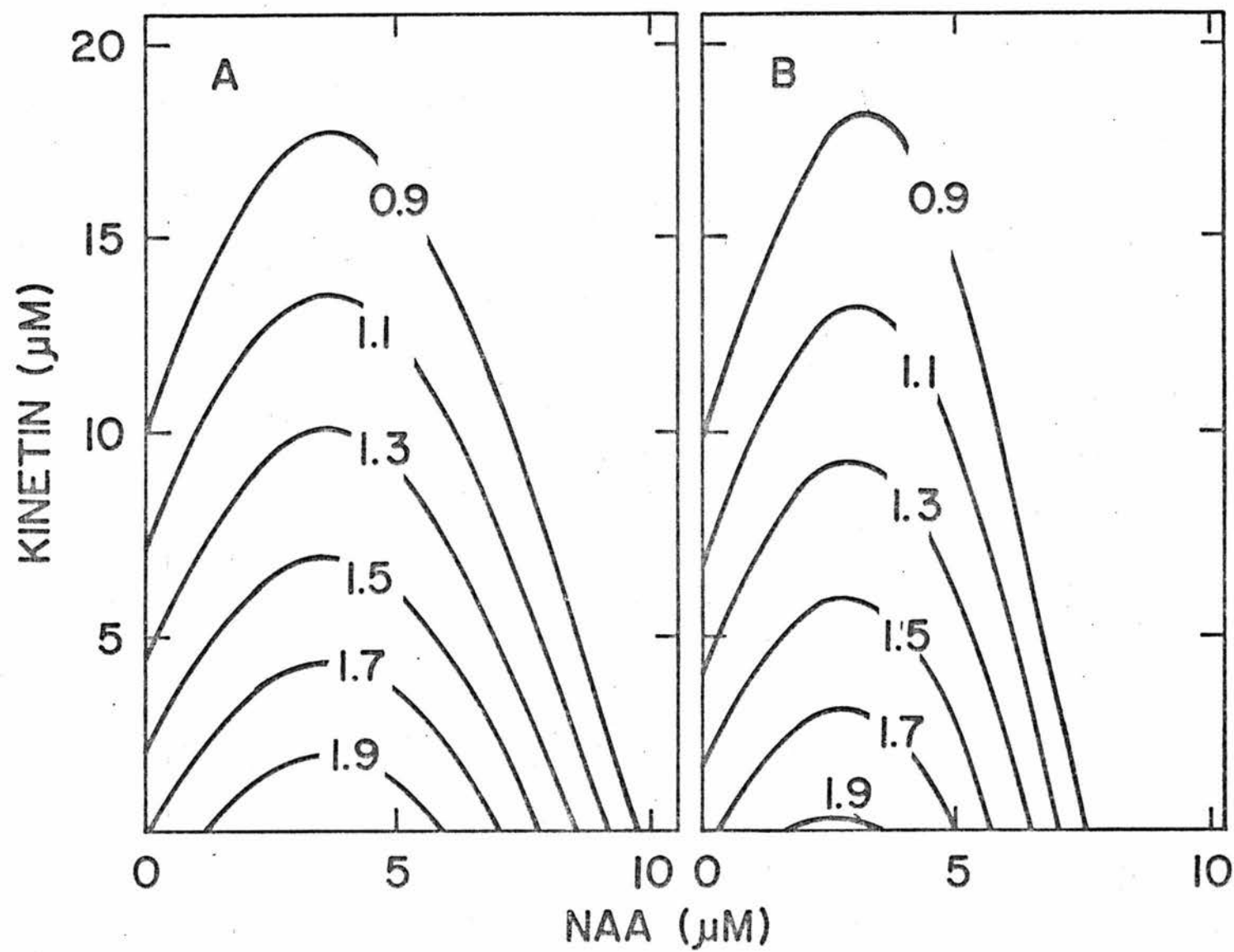


TABLE 9. Unorganized tissue growth^a from the cut surface of carnation shoot tips in response to various concentrations of NAA and kinetin in the culture medium.

NAA (μ M)	Kinetin (μ M)				
	2.5	5	7.5	10	25
0.25	++	++	++	+	0
0.5	+	+	+	0	0
0.75	0	0	0	0	0
1	0	0	0	0	0
2.5	0	+	+	0	0

^aVisual estimate of % total fresh weight per treatment; 0 to 25% (0), 26 to 50% (+), 51 to 75% (++), 76 to 100% (+++).

Comparison of the plots in Figs. 6A and 6B emphasizes what was observed in this and the previous experiment concerning the relationship of fresh weight, NAA concentration, and unorganized tissue growth. This was, as the NAA concentration above optimum increased so did the development of unorganized tissue and the amount of fresh weight that was accounted for by unorganized tissue. The opposite was observed for the kinetin concentration as also indicated by comparison of the plots in Fig. 6A and 6B.

A contradiction arises when comparing the average fresh weights in Table 10 with the plots in Fig. 6A and 6B. The same fresh weight data was used to derive the plots and compute the average. The plots indicate the maximum fresh weight was not obtainable using kinetin in the medium and the fresh weight decreased with increasing concentrations of kinetin; however, the average fresh weights (Table 7) showed kinetin at 5 μ M consistently supported the highest growth rates, and concentrations below and above this were inferior with respect to growth rates.

TABLE 10. Fresh weight of shoot tip cultures after 4 wk in response to various concentrations of NAA and kinetin in the medium.

NAA (μ M)	Kinetin (μ M)				
	2.5	5	7.5	10	25
0.25	1.79 ^a	1.89	1.59	1.09	0.84
0.5	1.52	1.75	1.50	1.25	0.79
0.75	1.51	1.85	1.79	1.33	1.03
1	1.41	1.48	1.29	1.33	0.46
2.5	1.59	1.46	1.21	0.68	0.63

^aFresh wt (g); each wt is the average of 24 ± 2 replications.

Although fresh weight was higher at the lower kinetin concentrations, the number of shoots produced (Table 11) increased with kinetin concentration to the point where kinetin became too inhibitory for growth. The most shoots developed with the second highest concentration, 10 μ M, tried.

TABLE 11. Number of shoots^a, 2 cm or longer, developing in carnation shoot tip cultures in response to various concentrations of NAA and kinetin in the medium.

NAA (μ M)	Kinetin (μ M)				
	2.5	5	7.5	10	25
0.25	0	0	0	+	+
0.5	+	++	+	++	+
0.75	++	+	++	++	++
1	+	++	++	++	0
2.5	0	0	++	+	0

^aVisual estimate of number; none (0), one (+), two or three (++), four or more (+++).

The development of leafy tissue (Table 12) was greater with the concentrations of kinetin, 7.5 μM and 25 μM , on both sides of the 10 μM concentration that was best for producing the greatest number of shoots. Using a 0.75 μM concentration of NAA, the development of leafy tissue was above average for all but one concentration of kinetin used.

TABLE 12. Development^a of leafy tissue at the base of carnation shoot tips in response to various concentrations of NAA and kinetin in the culture medium.

NAA (μM)	Kinetin (μM)				
	2.5	5	7.5	10	25
0.25	0	+	+	+	++
0.5	+	+	++	+	++
0.75	+	++	++	++	++
1	+	++	++	+	++
2.5	+	+	+	+	++

^aVisual estimation of presence of leafy tissue; present (+), above average present (++), none present (0).

A NAA concentration of 0.75 μM was consistently as good or better than 2.5 μM for supporting the growth of the greatest number of shoots (Table 11). The plots in Fig. 6A and 6B indicated that ca. 2.5 μM NAA was optimum for maximum fresh weight attainment.

A modification of the MS 1 medium was made following analysis of the results of the NAA and kinetin concentration tests. A 10 μM concentration was adopted for kinetin because of the consistently high number of shoots produced and a 1 μM concentration was adopted for NAA. The NAA concentration chosen was a compromise between the concentration

that produced the most shoots and the concentration that was indicated as optimum for fresh weight attainment. The new basal medium (MS 2) was used in subsequent experiments.

Further experiments were completed using different auxins or cytokinins in the MS 2 medium.

The effect of various concentrations of IAA (Table 13) was examined. The highest two concentrations of IAA supported growth similar to that supported by the basal medium with NAA as the auxin. At a 50 μ M IAA concentration shoot production and leafy tissue development was good.

TABLE 13. Response of shoot tips^a to various concentrations of IAA in the culture medium after 4 wk of growth. The kinetin concentration was held constant at 10 μ M.

IAA (μ M)	Unorganized tissue	Leafy tissue	Shoots	Fresh weight (g)
0.5	0	+	+	0.42
1	0	+	+	0.59
5	0	+	+	0.74
10	0	+	+	0.64
23	0	+	++	0.90
50	0	++	++	1.09

^aSee tables 5 to 8 for explanation of symbols. There were 24 ± 2 replications per treatment.

Shoot tip response to different cytokinins was compared to that of kinetin (Table 14). Apparently BAP and 6-(γ,γ -dimethylallylamino) purine (DMAAP) were more active in enhancing the growth rate. As

far as the greatest number of shoots produced was concerned, DMAAP and BAP were as active as kinetin at a ten times lower concentration; however, it was observed that DMAAP and BAP at the 1 μ M concentration also enhanced the abnormal broadening and thickening of the leaves (Fig. 7) and the leaves were often chlorotic.

TABLE 14. Response of shoot tips^a to various concentrations of cytokinins in the culture medium after 4 wk of growth. The NAA concentration was held constant at 1 μ M.

Cytokinin (μ M)		Unorganized tissue	Leafy tissue	Shoots	Avg. fresh wt (g)
Kinetin	10	0	+	++	0.81
BAP	1	+	+	++	1.39
	5	+	+	+	1.48
	10	+	++	+	1.05
	50	0	+	0	0.30
DMAAP	1	++	+	++	1.57
	5	++	++	+	1.90
	10	++	++	+	1.70
	50	0	++	0	2.92

^aSee tables 5 to 8 for explanation of symbols. There were 24 ± 2 replications per treatment.

Many small shoots and much leafy tissue was formed when DMAAP at 50 μ M was used. Very few shoots over 2 cm developed, but the growth rate of the shoot tips was the highest observed for the initiation stage in this study. As was generally the situation when growth of this nature occurred, the small shoots and leaves were for the most part morphologically aberrant.

Fig. 7. Two initiation stage cultures contrasting the morphogenic responses obtained when different growth regulators were used in the medium. The culture on the left was grown on the MS 2 medium, while the one on the right was grown on a modified MS 2 medium with 5 μ M BAP substituted for kinetin. Both cultures were 4 wk old at the time the picture was taken.



Fourteen inorganic salt mixtures were examined for their ability to support the growth of shoots in the initiation stage. Seven macro-element mixtures (Nitsch and Nitsch (71), Nitsch and Nitsch (70), Gautheret (16), White (141), Heller (24), Hildebrandt, Riker, and Duggar (29), and Murashige and Skoog (68)) whose formulations as used have been listed in Table 2 were combined with either the micro-element mixture of Heller (24) or Murashige and Skoog (68). The formulations of these mixtures is in Table 3. The experiment was repeated twice with 30 ± 3 replicates/treatment each time, and consistent results were obtained. The inorganic salt solution of Murashige and Skoog (68) with both micro- and macro-element complements was consistently superior to the other mixtures. The results of one experiment is given in Fig. 8.

Mixtures of vitamins in their original formulation or slightly modified (Table 4) were compared. The main difference between the various mixtures tried was in the rate of growth they supported (Fig. 9). The mixture of Murashige and Skoog (68) with 50 mg/l *myo*-inositol and supplemented with 2 mg/l glycine was consistently best. White's vitamins (141) with 2 mg/l glycine also gave good results, and was better than Murashige's and Skoog's mixture with glycine but without *myo*-inositol. Therefore, a subsequent experiment was completed that compared White's, and Murashige's and Skoog's mixtures both with 2 mg/l glycine and 50 mg/l *myo*-inositol. Again, Murashige's and Skoog's vitamin mixture as modified was superior.

Myo-inositol, although not necessary for shoot tip growth, did enhance the growth rate. Experiments using the MS 2 medium and 0, 50,

Fig. 8. Effect of different inorganic salt mixtures. Each salt mixture was a combination of both macro- and micro-elements. Each graph represents the average fresh weight of 24 to 25 shoot tip cultures after 4 wk. Murashige and Skoog's macro- and micro-elements together were significantly better than Murashige and Skoog's macro-elements with Heller's micro-elements ($P = 0.05$), and the other combinations ($P = 0.01$).

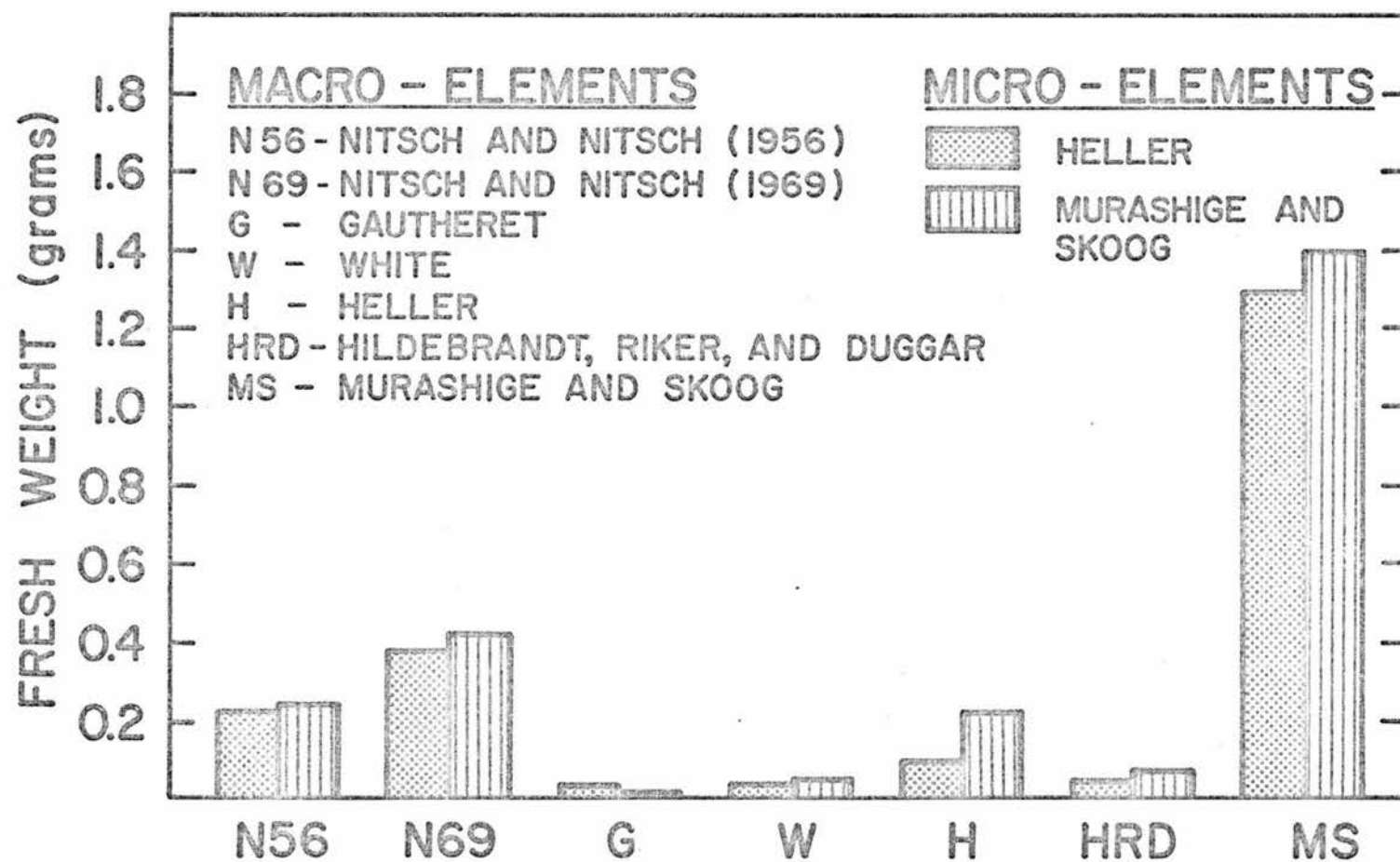
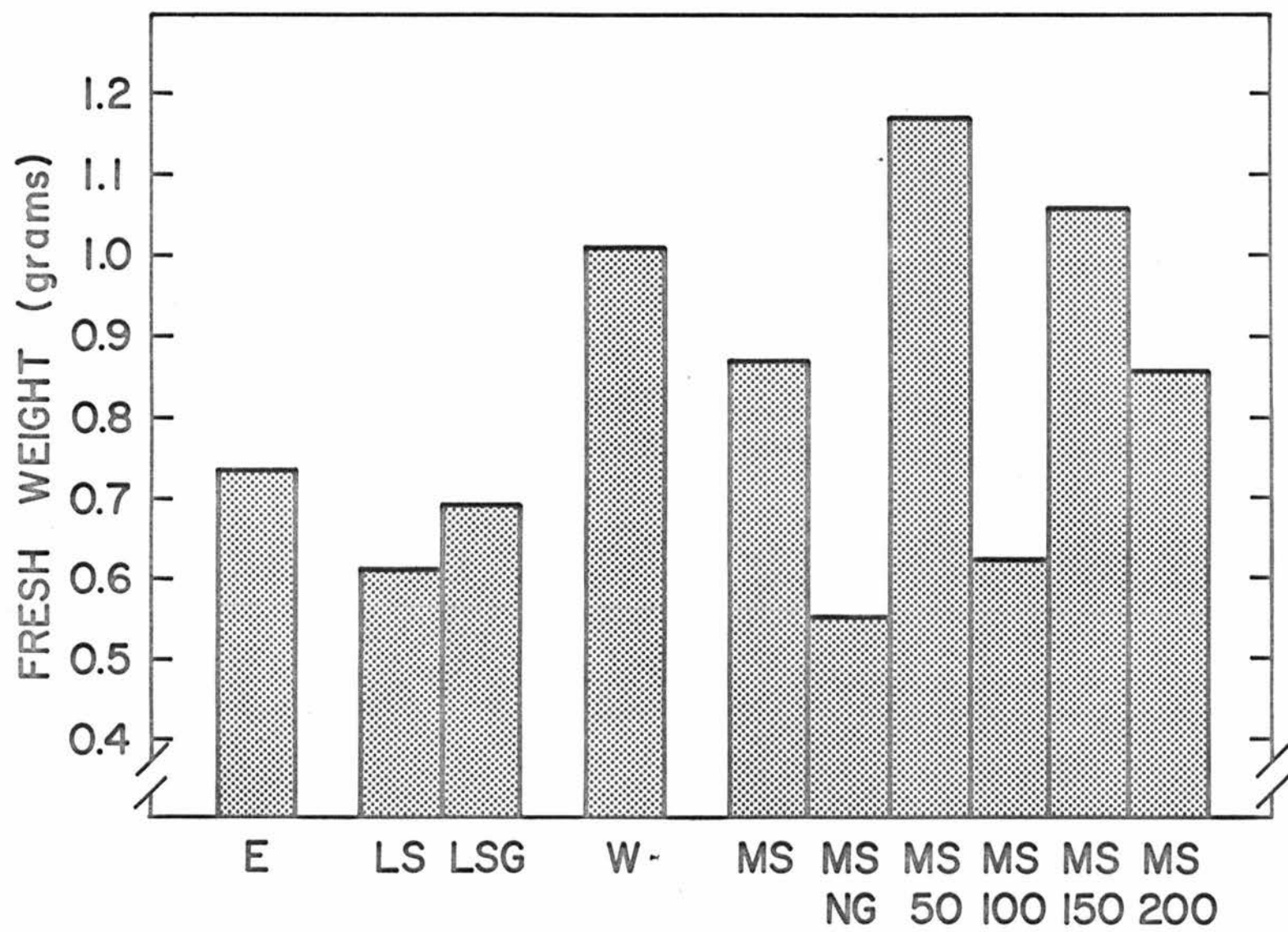


Fig. 9. Effect of different mixtures of vitamins with or without glycine. The composition of each mixture is given in Fig. 4, and each figure in the graph represents the fresh weight average of 24 to 25 cultures. There was a significant difference between the mixture of Linsmaier and Skoog (LS), and that of Murashige and Skoog with 50 mg/l of *myo*-inositol (MS 50) ($P = 0.05$), Linsmaier and Skoog's mixture was not significantly different from the other mixtures ($P = 0.05$).



100, and 150 mg/l of *myo*-inositol and the experiments using the MS 2 medium modified with Murashige and Skoog's vitamins and glycine, and *myo*-inositol at 0, 50, 100, and 150 and 200 mg/l (Figure 4), generally indicated that fresh weight increased with increasing concentrations of *myo*-inositol up to 150 mg/l, but unorganized tissue growth also increased over the same range. No apparent reason was found for the decrease in fresh weight observed in the experiment in Fig. 9, with *myo*-inositol at 100 mg/l, and other experiments did not support this trend.

Following the examination of vitamin mixtures, a modified Murashige and Skoog mixture with 2 mg/l glycine and 50 mg/l *myo*-inositol was adopted, and the new basal medium (MS 3) was used in subsequent experiments.

Using the MS 3 medium, different carbohydrate sources and concentrations were examined. The three used were sucrose, glucose, and fructose in different concentrations either alone or in combination. The results of one experiment generally represented those observed in preliminary experiments (Table 15 and Fig. 10). Sucrose, or glucose alone, or in combination, supported the highest growth rates and best all around growth. The highest concentration of sucrose tried was 50 g/l, the best concentration for the development of leafy tissue and more than one shoot over 2 cm. The growth rate was as good or better than those of other concentrations.

Glucose supported more leafy tissue development throughout the concentrations tested than did other sugars. Shoots grown on a medium with glucose often appeared darker green than some cultures with sucrose or fructose alone in the medium.

Of the combinations of carbohydrates tested, sucrose and glucose at 20 g/l each was best, and comparable in quality and rate of growth to that of the best concentrations of glucose or sucrose alone.

TABLE 15. Response of shoot tips^a to various concentrations of carbohydrates in the culture medium after 4 wk of growth.

Carbohydrate ^b	Concentration (g/l)	Unorganized tissue	Leafy tissue	Shoots	Avg. fresh wt (g)
Sucrose	20	0	0	+	0.48
	30	0	+	+	0.95
	40	0	+	++	0.70
	50	0	++	++	0.93
Glucose	20	0	++	+	0.45
	30	0	++	+	0.40
	40	0	++	++	0.91
	50	0	++	++	0.75
Sucrose and glucose	20				
	20	0	++	++	0.84
	30				
	10	0	++	++	0.79
Glucose and fructose	20				
	20	0	++	+	0.64
Fructose	40	0	0	0	0.34

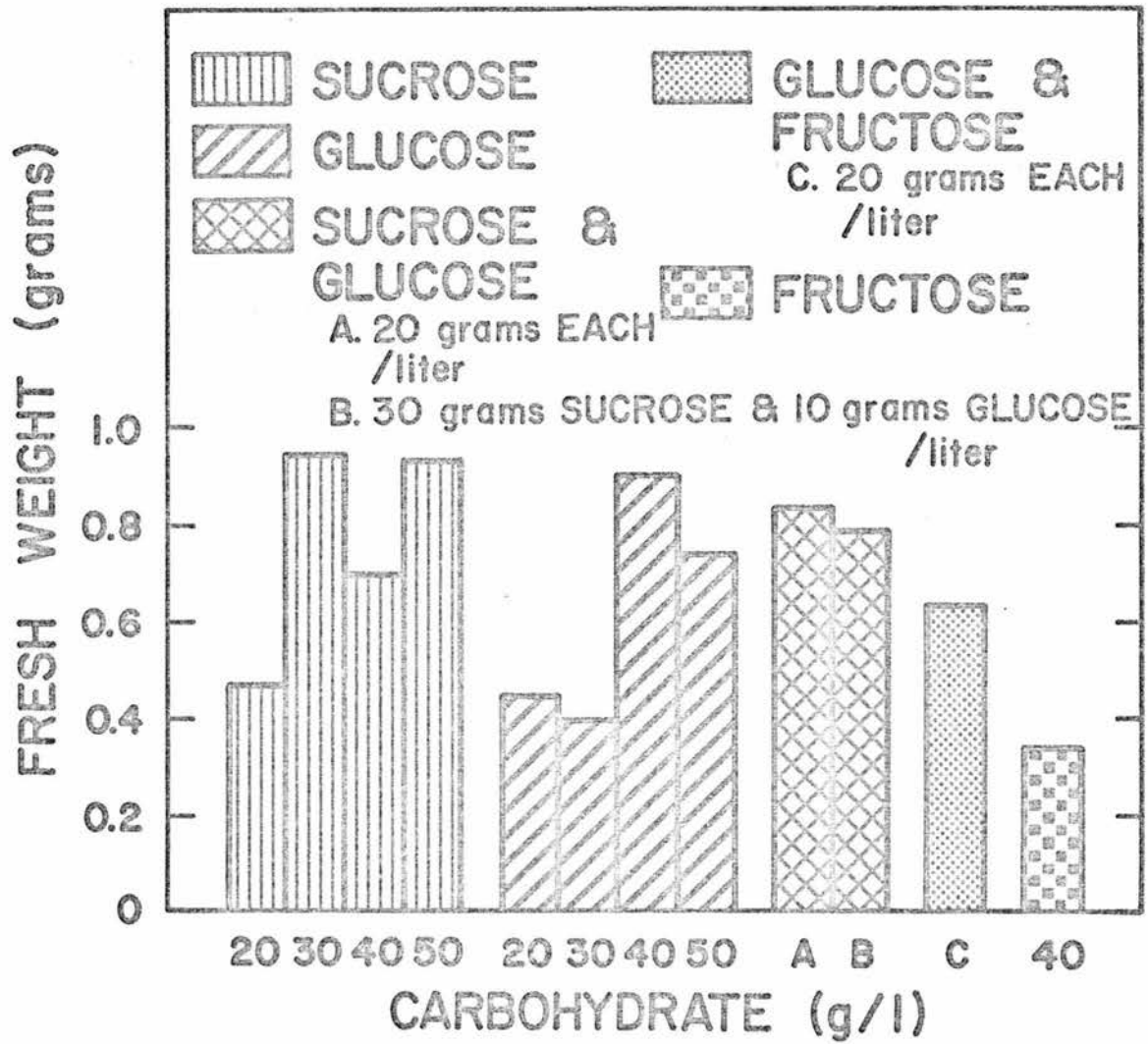
^aSee tables 2 to 5 for explanation of symbols. There were 24 ± 2 replications per treatment.

^bAll stereoisomers were in the D configuration.

Fructose alone was used at only one concentration, 40 g/l, and supported slow growth of shoots and chlorotic leaves. Fructose in combination with glucose was an inferior carbohydrate source, but the leaves were not chlorotic.

In preliminary and repeat experiments with sucrose, the 40 g/l concentration supported a growth rate as great as or better than the

Fig. 10. Effect of different carbohydrate sources. Each fresh weight is the average of 24 to 25 shoot tip cultures after 4 wk. There was not a significant difference ($P = 0.05$) between the effect of sucrose at 30 g/l, and that of sucrose at 50 g/l, glucose at 40 or 50 g/l, or either of the two combinations of sucrose and glucose.



30 g/l concentration, and the quality of growth was as good as that of 50 g/l.

Casein hydrolysate was reported by Engvild (15) to enhance the growth of carnation tissue and shoot tip cultures. After several preliminary experiments (two illustrated in Fig. 11), a concentration of 3 g/l was selected for a further test. Casein hydrolysate at this concentration significantly ($P = 0.01$) enhanced the growth of shoots as indicated by the fresh weight data. An average fresh weight of 0.253 g was obtained when just the MS 3 medium was used, and with casein hydrolysate supplementing this medium the average fresh weight was 0.866 g.

Figure 12 shows the effect of adenine sulfate. Although the fresh weight increased as a result of supplementing the medium with adenine sulfate, the number of shoots over 2 cm developing from each shoot tip decreased and apical dominance appeared to be enhanced. Also, at higher concentrations, adenine sulfate enhanced abnormal growth, which was especially evident in the broad leaves that developed.

The MS 3 medium supplemented with 5 μ M cinnamic acid was significantly ($P = 0.01$) better with than without cinnamic acid at supporting a faster rate of growth. The quality of growth on both media was similar, except that cinnamic acid appeared to slightly induce more unorganized tissue growth and leafy tissue development at the base of the shoot tips.

Other supplements to the MS 3 medium were examined. These are listed in Table 16 with the concentrations used and the overall effects on growth after 4 wk. The casein and casein hydrolysate with

Fig. 11. Effect of casein hydrolysate (vitamin and salt free; a acid hydrolyzed). The two curves represent the same experiment completed at different times, and each point is for the average of 24 to 25 cultures after 4 wk.

Fig. 12. Effect of adenine sulfate. Each point represents the average of 10 shoot tip cultures after 4 wk growth ($P = 0.01$).

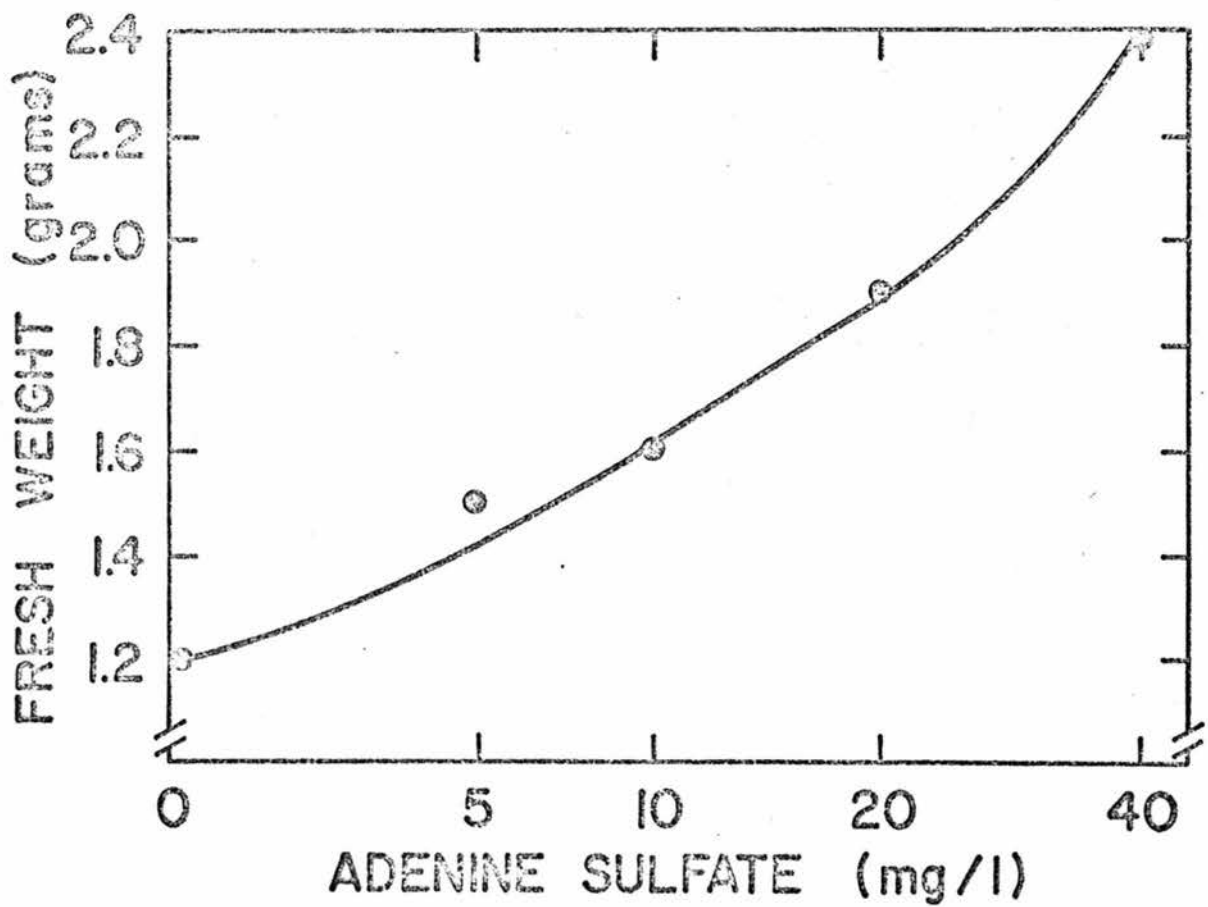
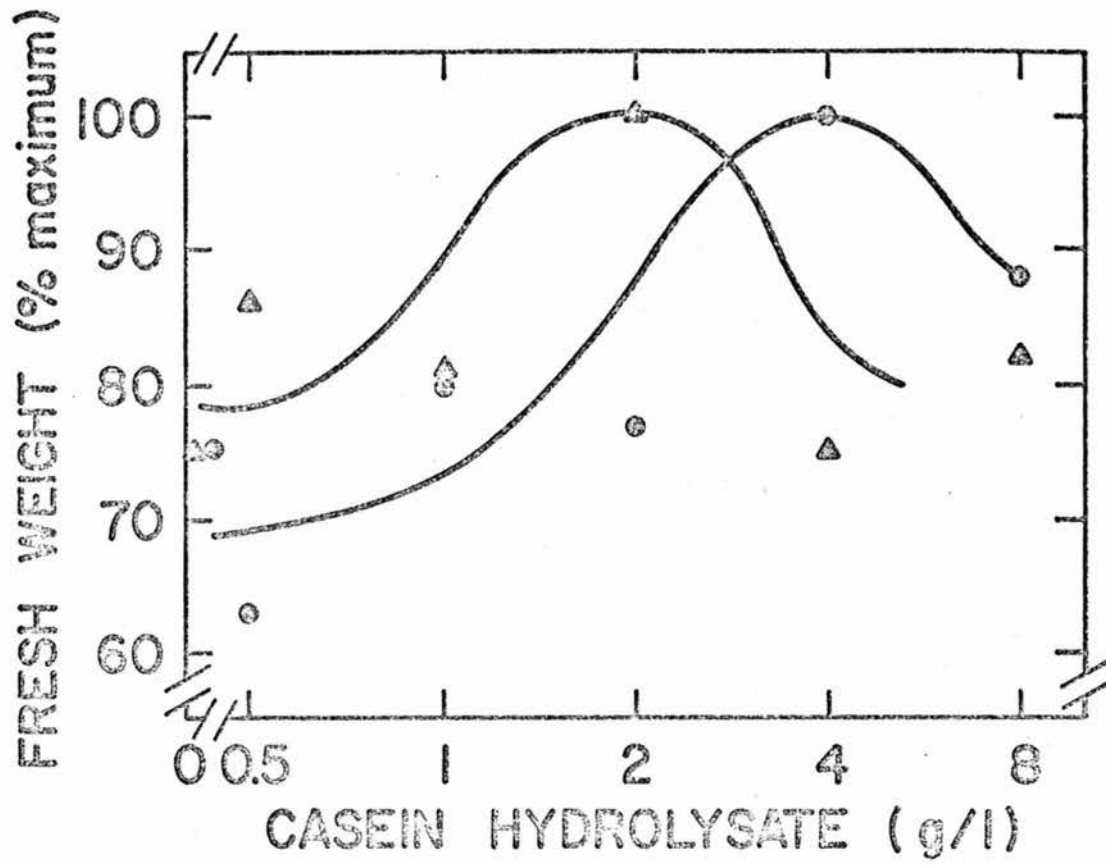


TABLE 16. Additional supplements examined for their effect on the growth of carnation shoot tips in culture.

Supplement	Concentration (g/l)	Growth ²
Casein hydrolysate (35% NaCl; acid hydrolyzed)	0.1	+
	0.2	0
	0.3	0
	0.4	0
	0.5	0
	0.6	0
	0.7	0
	0.8	0
Casein (vitamin-free; micropulverized)	0.7	-
	1.3	+
	2.7	0
	5.3	-
	10.6	-
Lactalbumin (enzymatic digest)	0.7	-
	5.3	-
Difco "Proteose-peptone No. 3"	0.1	0
Difco "Bacto-peptone"	0.07	-
	0.7	-
	5.3	-
Difco "Bacto-tryptone"	0.7	-
	5.3	-
Difco "Bacto-nutrient Broth"	0.07	0
	0.7	-
	5.3	-
L-tyrosine	0.1	0
L-tyrosine and L-phenylalanine	0.1	
	0.025	0
Nucleic acid (yeast)	0.01	0
Vitamin B ₁₂	0.016	0
	0.012	0
	0.008	0
	0.004	0
	0.002	0
	0.001	0
	0.008	0

^aEnhanced growth (+); no effect on growth (0); inhibited growth (-).

35% NaCl enhanced growth at certain concentrations but the increase was not as consistent as was that of acid hydrolyzed casein (vitamin and salt-free), and the results of experiments with this casein hydrolysate were previously reported in this thesis.

Standardization of the basal medium with regards to pH was necessary. Certain supplements such as adenine sulfate increased the acidity of the medium, yet other supplements such as casein hydrolysate had the opposite effect. The Difco "Bacto-agar" showed a buffering capacity at ca. 5.8 pH.

When casein hydrolysate and adenine sulfate were added to the medium, the pH range for obtaining the best growth rates was lower and narrower (Fig. 13) than when the MS 3 medium alone was used. In separate experiments, similar results were obtained when the concentrations of the supplements were different. In the first experiment, the concentrations were 40 mg/l adenine sulfate and 200 mg/l casein hydrolysate, while in the second experiment 4 mg/l adenine sulfate and 2 g/l casein hydrolysate were used. The standard pH used in all the media had been 5.5 and the fresh weights obtained at various pH's (Fig. 13) indicated that no change was necessary.

Different concentrations of three Difco agars (Fig. 14) were examined for their effect on shoot tip growth. At 6 g/l Difco "Noble" agar supported a higher growth rate than Difco "Bacto-agar" and Difco "purified" agar did at the same concentration. In general, the concentrations used above 6 g/l produced almost identical results. Always, as the concentration of the agars increased, the growth rates decreased.

Fig. 13. Effect of pH. Each curve represents the results of separate experiments, and each point was derived from the average fresh weight of 18 to 20 shoot tip cultures after 4 wk. The MS 3 initiation stage medium was used in both experiments, and the medium was (●—●) or was not (▲—▲) supplemented with 2 g/l casein hydrolysate and 4 mg/l adenine sulfate.

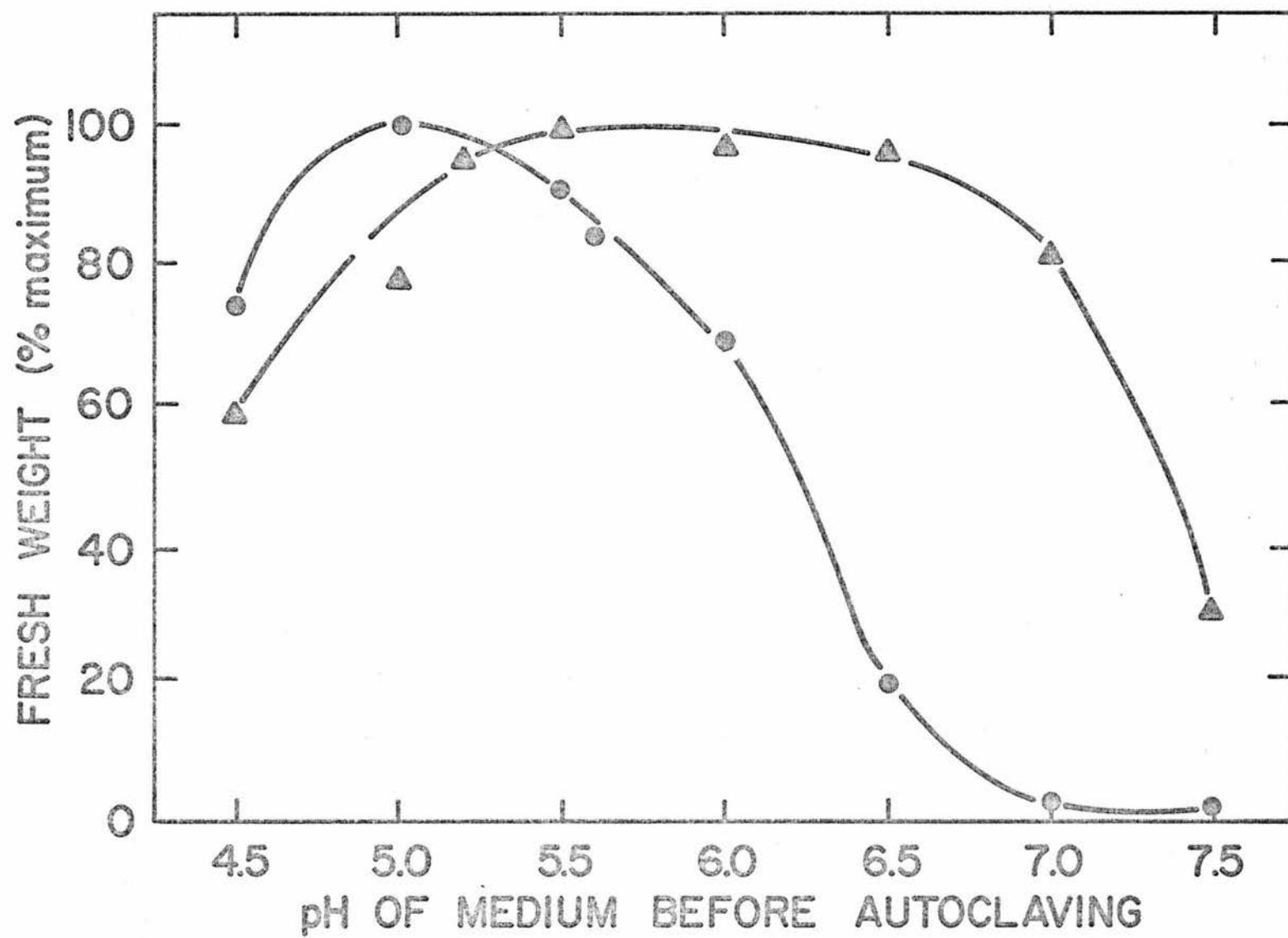
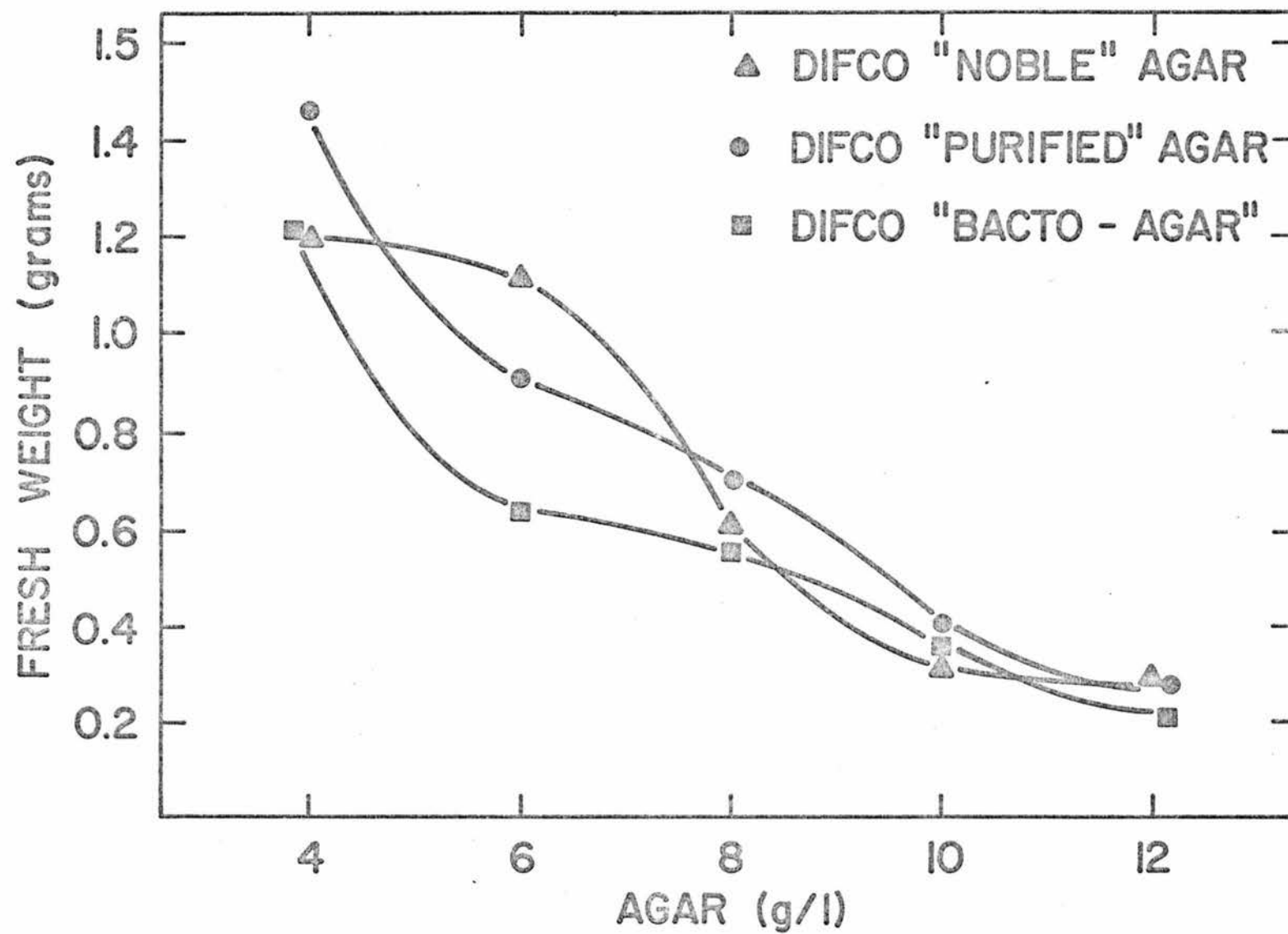


Fig. 14. Effect of agar. Each point represents the average fresh weight of 24 to 25 shoot tip cultures after 4 wk on the initiation stage medium. There was a significant difference ($P = 0.05$) between the three agars at the 6 g/l concentration.



The "purified" and "Bacto" agars did not gel as well as "Noble" agar at 4 g/l. A rapid growth rate was observed when the agar did not gel properly, and shoot tips often sank below the surface in this situation. Shoot tips also fell over on extremely soft agar. Leaves in contact or submerged in the medium often grew abnormally broad and thick, especially when the agar was very soft.

When ashless-filter paper bridges were used to replace agar in the medium, the increase of weight during growth often caused the bridge to collapse. Again, when the leaves came in contact with the medium, abnormal growth was observed. Shoot tips on bridges that did not collapse grew well but not as well as those on agar at 6 g/l or less. An average fresh weight of 0.43 g was obtained from 24 shoot tips growing on filter paper bridges. The shoot tips were grown at the same time and on the same medium without agar as those in Fig. 14 with agar.

The media developed by Engvild (15) and Petru and Landa (77) were compared with the MS 3 medium, but considerable unorganized growth developed from the base of the shoot tips, and the leaves were all abnormally broad and short. The leaves were also chlorotic and resembled those obtained on the basal medium with BAP as the cytokinin.

On Petru and Landa's medium, the growth rate and quality of growth were comparable to that obtained on the MS 3 medium. Petru and Landa's medium is very similar in composition to the MS 2 medium with IAA as the auxin.

After 3 to 4 wk of growth in the initiation stage, the rate of growth subsided (Fig. 15). The shoots that had developed were attached to the base of the main shoot and usually less than five shoots

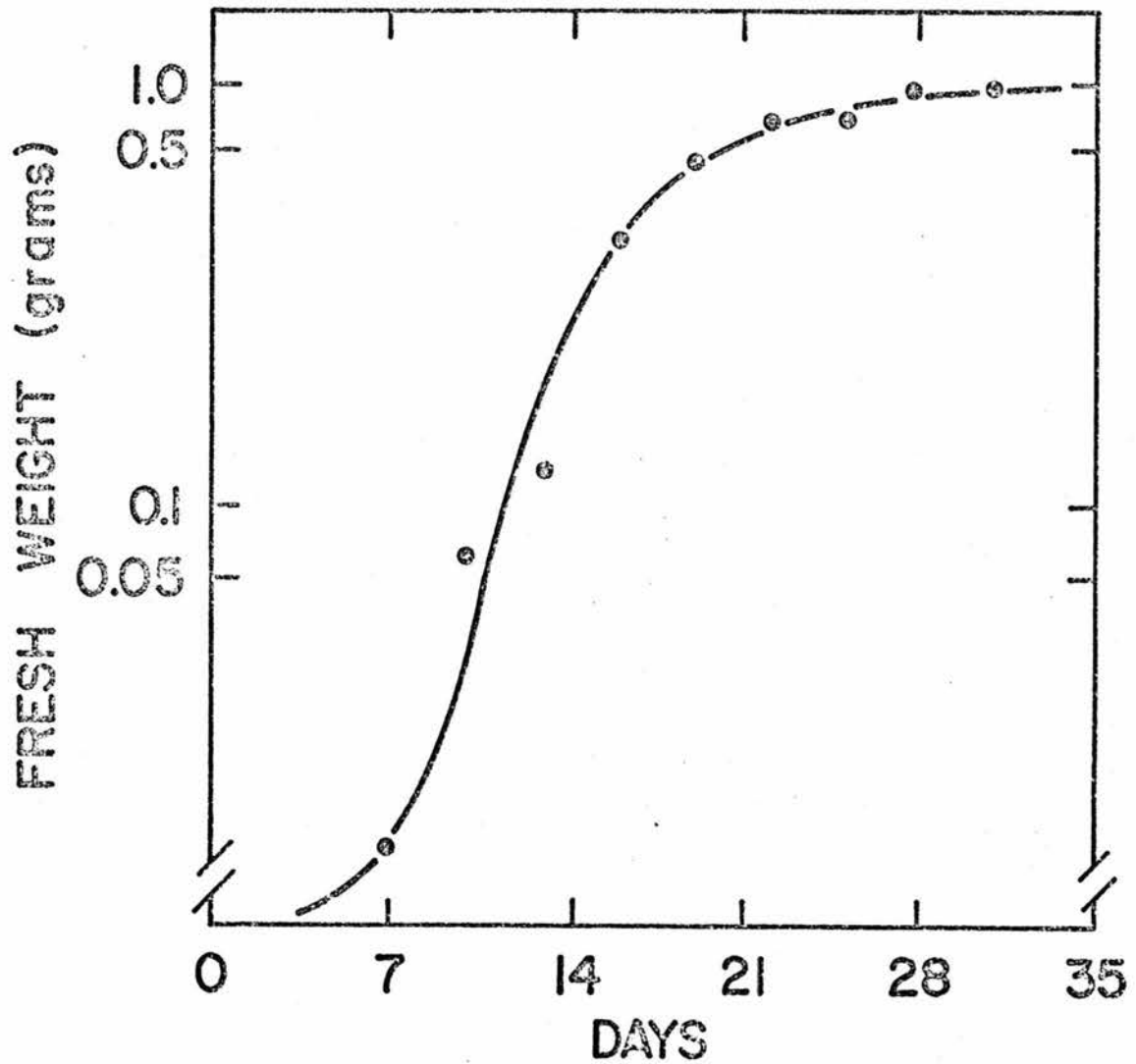


Fig. 15. Growth curve for the initiation stage. The fresh weight averages are plotted on a log scale. Shoot tips were grown on the MS 1 medium. Each point represents the average fresh weight of 20 cultures.

were over 2 cm long. Occasionally, lateral shoots were evident on the centrally affixed shoots.

Stage II - Multiplication of Shoots

In preliminary experiments a light intensity of 16,000 lux was used to illuminate the revolving flask cultures. It was observed that even at this light intensity the shoots were not autotropic. They did not survive without a carbohydrate in the medium, and at low concentrations of sucrose they grew poorly. When sucrose was included in the medium at 10, 20, 30, and 40 g/l, only the cultures with 30 g/l or more had leaves with normal greenness and consistency. Those grown with 10 and 20 g/l sucrose were chlorotic and the consistency was mushy.

The high light intensity often bleached portions of the leaves more directly hit by the light. This problem was solved by turning off one half of the fluorescent lamps. Following this adjustment, the leaves were darker green and had no bleached areas.

The number of shoots over 2 cm in length produced when adenine sulfate and casein hydrolysate were either included in the first or second stage medium or not included has been shown in Table 17. The MS 2 medium without agar was used in these cultures. In none of the treatments where supplements were added to the medium was the number of shoots as great as when the supplements were omitted. Also, the leaves grew abnormally broad when the supplements were added to the second stage medium.

Preliminary experiments indicated the concentrations of NAA and kinetin were too high in the second stage medium, and they were then reduced to a fourth of that in the MS 3 medium for the first stage.

TABLE 17. Number of shoots over 2 cm long produced during 3 wk of stationary culture and a subsequent 3 wk culture on the auxophyton. The medium was solidified with agar in the first stage, and in both stages the effect of supplements in the medium was examined. Three carnation varieties were used as the source of shoot tips.

Variety	Supplements		No. of shoot tip cultures/flask	No. of flasks	Shoots produced	
	Stage one	Stage two			Total no.	Avg/flask
White Pike's Peak	none	casein hydrolysate (1 g/l) adenine sulfate (4 mg/l)	2	10	120	12
CSU Red	none	Casein hydrolysate (1 g/l) adenine sulfate (4 mg/l)	2	8	58	7
CSU Pink	none	casein hydrolysate (1 g/l) adenine sulfate (4 mg/l)	2	7	71	10
	none	none	1	29	390	13
	casein hydrolysate (2 g/l) adenine sulfate (4 mg/l)	none	1	17	173	10
	casein hydrolysate (2 g/l) adenine sulfate (4 mg/l) L-tyrosine (100 mg/l)	none	1	18	136	8
	casein hydrolysate (2 g/l) adenine sulfate (4 mg/l) L-tyrosine (100 mg/l) NaH ₂ PO ₄ (85 mg/l)	none	1	5	44	9

Thus, a modified first stage medium with 0.25 μM NAA and 2.5 μM kinetin and without supplements produced the results shown in Table 18.

The lowering of the growth regulator concentrations resulted in more shoots over 2 cm in length being produced for each original shoot tip. The quality of the shoots increased also. Their leaves were not as thick as those previously grown in stage two, and the stems were more elongated.

TABLE 18. Number of shoots produced during 3 wk of stationary culture and a subsequent 3 wk culture on the auxophyton. The medium used for both stages was the same except the agar was ommitted, and the hormone concentrations were reduced to a fourth for the second stage. Three carnation varieties were used as the source of shoot tips.

Variety	No. of shoot tip cultures/flask	No. of flasks	Shoots produced	
			Total no.	Avg/flask
CSU Pink	1	23	535	23
CSU Red	1	29	280	13
White Pike's Peak	1	9	119	13
	2	17	280	16

The number of shoot tip cultures transferred to each revolving flask culture was increased to two in some situations. The increase in the final number of shoots obtained from each flask however was not proportionally increased (Table 17) and the number of shoots from each shoot tip was considerably decreased.

After 2 to 3 wk on the auxophyton, the growth rate began to decrease rapidly. Also, the longer the shoots remained in those conditions, the more they became damaged. Shoots managing to remain out of the liquid medium during most of their growth on the auxophyton were

less often damaged and were often more normal in appearance. Some groups of shoots rolled in response to the movement of the flask and some of their leaves became broken as they formed a ball of shoots. Other groups of shoots slid along the sides of the flasks, always remaining in the lowest portion of the flask, and their shoots underwent negative geotropism growing up and out of the medium. Thus, many different shapes as well as sizes of shoots were produced in the same treatment but in different flasks.

Stage III - Rooting of Micropropagated Shoots

The shoots, once removed from the flasks, were excised from the central tissue and those over 2 cm long were kept for rooting and preparation for greenhouse conditions.

The shoots had been growing in the very humid atmosphere of the culture vessels and became desiccated very easily if proper precautions were not taken. Also they had no root system to provide them with water and nutrients. To overcome these problems, several rooting procedures were tried.

In one test for the best rooting conditions, water and three inorganic salt solutions with varied concentrations of NAA were used. These were poured into jars with either a perlite or Terragreen layer at the bottom. The shoots were then stuck in the moistened supports for rooting and the caps put on the jars.

The three inorganic salt solutions consisted of macro-element mixtures whose formulations are listed in Table 2 and are the Murashige and Skoog (68) mixture, a half strength Murashige and Skoog mixture, and the THS (129) mixture.

In water with NAA, 72% of the shoots developed roots at their bases. The shoots, however, during the 2 to 3 wk period required for rooting became very chlorotic. Chlorosis was not as severe when inorganic salt solutions were used but roots did not develop as readily. In the THS and half strength Murashige and Skoog solutions, 61% of the shoots developed roots while only 45% rooted in the full strength Murashige and Skoog solution.

Concentrations of NAA varied in each of the previous treatments. The concentrations were 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/l NAA, and rooting powder was also tried. The powder was used as recommended for cuttings. The highest percentages of rooting were with 0.8 and 1.6 mg/l NAA and were 76% and 72% respectively. Rooting powder was only 33% successful. Whether perlite or Terragreen was used as the support made little difference.

The biggest problem with this procedure was transplanting the small plantlets to greenhouse conditions. Less than half of the plantlets survived transplanting on numerous occasions. Many plantlets were produced in this manner throughout the summer of 1974 and many transplanting conditions were tried. When planted in a soil mixture (1:1:1; sand, peat, and soil), intermittent mist and screening from full sunlight increased survival. A better survival rate (approximately 85%) was obtained when the plantlets were planted in a mixture of peat moss and Terragreen (1:1), screened from full sunlight, and misted periodically throughout the day by hand.

A more efficient procedure for rooting was developed. Shoots from the multiplication stage were transferred directly to greenhouse

conditions but maintained throughout rooting under intermittent mist. A variety of rooting supports were used including sand, perlite, peat moss, BR-8 synthetic soil blocks and Jiffy-7 expandable peat pellets. The bases of the shoots were dipped into rooting powder and then stuck into the supports.

BR-8 blocks and Jiffy-7 peat pellets (Fig. 16) were advantageous because the rooted shoots could be transferred to soil while remaining in them. This reduced death due to transfer shock.

Table 19 shows the percentage rooting of shoots of different varieties in either BR-8 blocks or Jiffy-7 peat pellets. Jiffy-7 peat pellets were the best in this experiment; however, in a different propagative bench at a different greenhouse the opposite results were observed. In this situation, 26 out of 30 CSU pink shoots rooted in BR-8 blocks while only 18 out of 30 rooted in Jiffy-7 peat pellets. At the same time, 26 out of 30 also rooted when stuck into perlite.

In the past two years, over 500 plants produced by this micropropagation technique have been transferred to soil for mutation comparison studies with normally propagated plants from the same mother block plants. These studies have not been completed at this time.

Fig. 16. Micropropagated plantlets rooted in different supports. The shoots were approximately the same size when placed in the two supports, a Jiffy-7 peat pellet and a BR-8 synthetic soil block, 6 wk earlier and rooted under the same conditions.



TABLE 19. Comparison of rooting supports for their effect on the initiation of roots on shoots developing from shoot tips from three carnation varieties in aseptic culture. Two supports, BR-8 synthetic soil blocks^a and Jiffy-7 peat pellets^b were used, and rooting was under intermittent mist.

Substrate	Variety	No. of shoots	% rooted
BR-8 blocks	CSU Pink	232	53
	CSU Red	86	53
	White Pike's Peak	<u>102</u>	<u>61</u>
	Total	420	55
Jiffy-7 peat pellets	CSU Pink	48	65
	CSU Red	5	100
	White Pike's Peak	<u>127</u>	<u>86</u>
	Total	180	81
Combined Total		600	63

^aAmerican Can Co., New York, N.Y.

^bJiffy Products, Grorud, Norway

Chapter V

DISCUSSION

Clonal multiplication of carnation was accomplished through enhancement of axillary shoot growth in aseptic culture. The extent of abnormal plant production by this method has not yet been ascertained. Possibly by avoiding the regeneration of shoots from unorganized tissue, both the disruption of chimeras as was observed by Hackett and Anderson (18) and the enhancement of genetic aberrations will not be excessive.

The micropropagation techniques employed in this study did produce some abnormal plants. Before rooting, many of the abnormal shoots were observed and after rooting more were apparent. Observations in one experiment showed that 4% of the 378 plantlets which survived rooting were abnormal with regards to stem and leaf morphology, and they were then discarded before planting the rest in soil. Similar abnormal growth was often observed in shoots growing from damaged nodes of mother block plants. Excision of the shoot tips may have also damaged nodal areas and their axillary meristems. The disruption of these meristems could be the cause of abnormal shoot development. Often leafy tissue was observed around the base of growing shoot tips. Sometimes shoots developed from this tissue. Assuming that adventitious leaves and shoots did not develop, this leafy tissue must have been from axillary meristems.

Leafy tissue development in chrysanthemum was induced by slightly damaging the surface of shoot tips before culture (13). This tissue was used, however to produce normal plants. If leafy carnation tissue is the

source of abnormal shoots, possibly it can be eliminated by refinements in the excision of the shoot tip or adjustments to the medium.

When cultures from the initiation stage were left in the dark for a few days before transfer to the multiplication stage, they underwent etiolation. Normal shoots appeared to etiolate more so than abnormal shoots, and if this is consistent, then etiolation might be used to select for normal shoots. With chrysanthemum, gibberellic acid (GA_3) is used to induce the elongation of shoots from the leafy tissue (13). A gibberellin might also affectively cause elongation of carnation shoots and the adverse effects accompanying etiolation might be avoided.

Mutations not readily recognized until the carnations are more mature present a more difficult problem. Not only are they difficult to detect, but also their cause may not easily be determined. Remedying this situation might require only a minor adjustment or might be next to impossible.

The mutation comparison studies should provide information on the extent that abnormal plants are obtained. Without considering the economic feasibility of a propagation program using the techniques developed in this study, the excessive production of abnormal plants alone might limit the program's use, because all plants produced in the program might have to undergo selection procedures, as do the plants that are produced from shoot tips in the present clean stock program.

Combined with heat-treatment, the techniques developed in this research might be employed by commercial growers to replace the present

shoot tip culture technique. The size of the shoot tips that were used is comparable to the size suggested for production of pathogen-free stock (1). It was observed that larger shoot tips with several leaf pairs initially grew faster than smaller ones with only one leaf pair; however, after completion of the multiplication stage, there was no consistent difference in the number of shoots that were produced.

From the data in Tables 18 and 19, it is evident that at least 10 plantlets from one shoot tip is a fair estimate of the clonal multiplication that was obtained. Only 8 wk were required to complete the entire process, while 6 to 8 wk were required to produce a comparable plantlet in the present clean stock program (1). The survival rate of shoot tips during clonal multiplication was at least as great as the rate for shoot tip culture following Phillips' (80) technique for the production of pathogen-free stock.

Much attention was focused on improving the medium for the initiation of the aseptic cultures. Consistent results were sought in this stage before the subsequent stages were examined. Improvement of clonal multiplication by comparing the end results from modifications in either of the first two stages was attempted only on a few occasions because of the limited space on the auxophyton.

Of the three cytokinins tried in the initiation stage, kinetin was the best. DMAAP and BAP often stimulated better growth rates, but the shoots were usually more deformed and not as green as when kinetin was used. Comparison of the three cytokinins in a tobacco tissue culture assay showed that DMAAP was the most active throughout a wide

range of concentrations, and BAP was the next most active (101). The same order of activity was found using carnation shoot tips. If the mass of short shoots and leafy tissue which was produced with 50 μ M of DMAAP in the MS 3 medium could be induced to produce normal shoots, the rate of clonal multiplication might be increased, since unlike kinetin, DMAAP enhanced the growth rate at concentrations that completely counteracted apical dominance.

Further investigations using the number of plantlets produced from each shoot tip as the basis of comparison might more clearly indicate what the optimum concentration is of NAA and kinetin in the first two stages. The same procedure could be used to determine which auxin, NAA or IAA, is best.

The concentrations of kinetin and NAA were reduced in the medium for the multiplication stage with good results. The purpose of the auxophyton in this stage was to provide optimum proportions of air, nutrients, and water (106). Since the nutrients are more available to the plant cells, an adjustment in the concentration of other constituents of the medium in addition to the hormones may be beneficial. Whether the adjustment would mean an increase or decrease in concentration of certain ingredients would depend not only on availability under these conditions, but also on any change in metabolic rates brought about by the new conditions.

In an effort to simplify studies on the effect various types of constituents in the initiation medium had on growth, mixtures of inorganic salts and vitamins that had been selected from different culture media were compared in either basic or modified forms rather

than examining each individual ingredient. For the most part, variations in constituents other than hormones affected only the growth rate and not the morphology of the shoots. Adenine sulfate and cinnamic acid, however, were exceptions to this generalization. Both compounds exhibited growth regulator activity. Adenine sulfate was shown to have weak cytokinin activity in regulating bud regeneration in tobacco stem tissue cultures (100). Cinnamic acid was believed to inhibit IAA oxidase activity in carnation shoot tips causing an increase in growth (80).

In this study both adenine sulfate and cinnamic acid increased the proliferation of cells at the base of carnation shoot tips, and affected the development of leafy tissue. Adenine sulfate also caused abnormalities in leaf morphology. Both compounds increased the growth rate.

A synergism between adenine sulfate and casein hydrolysate increasing the rate of growth as in grape tissue cultures (27) was not observed in these studies. Combinations of the two reduced the number of shoots obtained for rooting. Together the pH range best suited for shoot growth was lower than when they were not added to the medium. The effect on the pH range may have had to do with the hardening of agar. It was noticed that when the pH in the medium was 5.0, the agar gelled after autoclaving; however, when casein hydrolysate and adenine sulfate were added to the medium and the pH was 5.0, the agar did not gel. It was determined that adenine sulfate lowered the pH during autoclaving by comparison after autoclaving of the pH's from the media

with adenine sulfate and/or casein hydrolysate or without the supplements. This lowering of the pH means that the optimum pH range of growth was actually even lower.

Since agar at lower concentrations was shown to allow a higher growth rate and was softer, it is possible that the addition of supplements to the medium affecting the hardening of the agar, either by lowering the pH or by other means, may cause an increased growth rate without actually being beneficial themselves. Thus, although agar was a convenient support for the shoot tip cultures, inconsistency in some results may have been due to its use. Also, it has not been determined to what extent the shifting of pH during autoclaving due to various treatments other than casein hydrolysate or adenine sulfate affected results.

Increasing the sucrose concentration from 30 g/l to 50 g/l increased the number of shoots developing during the initiation stage. Similar results were found by increasing the concentration of glucose up to 40 g/l when it was used as the carbohydrate source. At optimum concentrations neither carbohydrate was superior while they both were superior to fructose at 40 g/l. The superiority of sucrose and glucose over fructose was reported by Nitsch and Nitsch (71) using Jerusalem artichoke tissue. They also reported that a combination of sucrose and glucose gave the best results. When 20 g/l of both carbohydrates were used in the media for either the initiation or multiplication stages, the growth of the shoots was good. In the initiation stage, this modification of the medium resulted in more shoots developing than when the medium with 30 g/l sucrose was not modified, but when either sucrose at 50 g/l or glucose at 40 g/l

was used, the growth rates and quality of growth were essentially the same as when the combination was used. With apical meristems of *Picea abies* a combination of glucose and fructose supported the best growth rates in culture (92). This was not found to be so with carnation shoot tips.

Often shoots grown on medium containing glucose were greener than if other carbohydrates were used, suggesting that although the same growth rates were obtained using different carbohydrates, the carbohydrate sources may differentially affect chlorophyll production or chloroplast development. Wetmore and Rier (136) were able to show that combinations of an auxin (IAA or NAA) and sucrose or glucose at various concentrations controlled the differentiation of vascular tissue in plant tissue cultures. The use of different carbohydrates in carnation shoot tip culture might result in different concentrations being available to the various tissues within the growing shoots resulting in different rates of growth and differentiation of these tissues. In tobacco tissue cultures, the differentiation of chloroplasts was related to the growth rate (40) and this may also be so with carnation shoot tip cultures.

The vitamin mixture of Murashige and Skoog (68) and the addition of glycine as suggested by these same investigators supported the best growth rate when compared to other similar mixtures. In other studies, carnation shoot tip cultures were grown with thiamine alone (1) or without thiamine but with other vitamins (83). The present study showed that the combination of pyridoxine, nicotinic acid, *myo*-inositol and thiamine was superior to the combination of only

thiamine and *myo*-inositol used by Linsmaier and Skoog (48). Linsmaier and Skoog reported that *myo*-inositol was not necessary for growth of tobacco tissue cultures, but enhanced growth when combined with thiamine alone. The same situation is apparently true for carnation shoot tips, and the addition of nicotinic acid and pyridoxine enhances growth even more.

Investigations on improving the medium for the multiplication stage were limited. Much of the time the 80 flasks on the auxophyton were employed for producing shoots to be rooted and grown in the mutation comparison studies being conducted by the Department of Horticulture here at Colorado State University. Many preliminary investigations on the multiplication stage were hindered by inconsistent results obtained early in the study from the initiation stage.

These preliminary investigations did suggest lowering the concentration of hormones in the multiplication medium and decreasing the light intensity. Both adjustments later proved beneficial. There were many problems created by the culture conditions in the second stage which have not been solved.

The main problem with the use of the auxophyton for culturing carnation shoots was the effect on leaf morphology, possibly caused by growing the shoots submerged for part of the time. After removal of the shoots from the flasks, many of their leaves were highly turgid and easily damaged. It is likely that the readily available water, due to the high humidity and the liquid medium in the culture vessel, created the conditions favoring the excessive uptake of water by the shoots. This situation might be rectified by adjusting the osmotic potential of the medium.

Abnormal leaf growth is another problem associated with the multiplication stage. Shoot tips accidentally placed upside down on the initiation medium were observed on numerous occasions to grow at a rapid rate while taking up nutrients through their leaves' surfaces, and these leaves were often abnormal when compared to leaves not in contact with the medium. It can be assumed that abnormal leaf growth in the multiplication stage was also related to uptake of nutrients, especially growth regulators, by the leaves. Theoretically, these nutrients were not as subject to the regulatory devices during normal uptake and have disrupted controlled growth by their excess availability.

Success in this research depended on a combination of natural growth regulation and growth regulation induced by constituents in the media. Exogenous growth regulators were included to counteract apical dominance and promote shoot growth. When they interfered with the desired endogenous regulation of growth, a compromise had to be made.

As many as 50 small shoots resembling rosettes were produced from a shoot tip in response to one set of conditions in the multiplication stage in a preliminary experiment (16,000 lux light intensity; the medium had Linsmaier and Skoog inorganic salts, vitamins, and sucrose (48) and 0.5 mg/l NAA, 1 mg/l BAP, 10 mg/l adenine sulfate and 200 mg/l casein hydrolysate (35% NaCl)). Although there was a large number of shoots, they were abnormal. A compromise was made between shoot number and quality by adopting the basal medium used in this study. Further experimentation has improved the basal medium but the

number of shoots from any single shoot tip has never been as great as before the compromise.

A stationary, solidified medium for the multiplication stage was used for gerbera (66) and might be used with carnation. Supplying enough hormones to the axillary meristems once the shoot has begun to elongate may create a problem. Possibly, with the right growth regulators in the initiation medium, apical dominance could be completely counteracted and many shoots produced. A second stationary medium might then be used to regenerate normal shoots from the highly proliferated mass of shoots.

Rooting of the shoots obtained from the multiplication step can be accomplished in aseptic culture as has been done in another study on clonal multiplication of carnation (77). In large scale propagation, however, this procedure is not likely to be economically feasible.

Results in this study have indicated that modification of conventional procedures for rooting carnation cuttings might be more practical.

Often, older leaves of the micropropagated shoots died during rooting. These leaves were subjected to longer periods under the conditions of the multiplication step, and possibly were more damaged during the procedure. Another possibility is that the small shoots did not have enough nutrients stored for use during rooting, and the nutrients from the older leaves were transported at the leaves' expense to younger tissues. Nutrients fed to the shoots in high pressure mist might eliminate this problem.

The use of BR-8 synthetic soil blocks or Jiffy-7 peat pellets for rooting supports increased the survival rate following transplanting of

the plantlets to soil. These supports retain water and, therefore, might be advantageous for pathogens if proper precautions are not taken. High water content in these supports during rooting increased the incidence of saprophytic microorganisms on the dead leaves and, when this occurred, the survival rate of the shoots was decreased.

The effect of light quality, duration, and intensity were not examined to any extent in this study. The light conditions suggested by Phillips (80) were used for most initiation stage experiments. When one culture room was first used the light intensity was initially ca. 5000 lux, and appeared to inhibit growth. The intensity was decreased to the standard ca. 2,000 lux and growth appeared to resume a normal rate. Growth also slowed when the duration of the light was decreased from continuous illumination to 16 hour photoperiods, and without light the shoots etiolated.

A lower light intensity than was initially used for the multiplication stage was found to be beneficial. Murashige (65) has suggested that more attention should be paid to light effects, and has found that various species of plants have different requirements for different stages in their micropropagation. Observations made in this study indicate that the three stages have different light requirements and further examination of these requirements is warranted.

The temperature range in which each stage of this research was maintained fluctuated considerably due to different cooling systems. Temperatures from 18 to 25 C were sometimes used without much difference in growth being observed. Further examination of what the optimum temperature is for each stage might be beneficial.

The comparisons made of Engvild's (15) and Petru and Landa's (77) media for the initiation of aseptic culture, with the initiation

medium developed in this study, showed that this study's medium was best for the type of growth desired. Most of this study had been completed by the time Petru and Landa's work was published, and further comparison of their clonal multiplication technique was not possible. They used different varieties of the Grenadin carnation; a garden carnation not grown for commercial cut flower production. Engvild used a Sim variety that is used for cut flower production, but was not available for comparison studies.

In this research attempts were made to make the technique universal for commercially grown carnation varieties by using a mixture of varieties for most experiments. Only on few occasions were the individual varieties followed through their development. This follow-up indicated that there were clonal differences as well as varietal differences in aseptic culture; however, of the carnation varieties and their clones used, all responded favorably to micropropagation.

APPENDIX

CARNATION TISSUE AND SUSPENSION CULTURE

I. EXPLANT: STERILIZATION AND SOURCE

A. Basal Medium

1. Linsmaier and Skoog (48) medium modified by substituting 6 mg/l 2,4-D for the kinetin and IAA constituents and supplementing the medium with 0.1 g/l each of malt and yeast extracts.

B. Explant

1. Approximately 2 mm thick segments of carnation stem internodes were cultured with one cut surface on the medium.

C. Culture Conditions

1. 85 x 20 mm vials with 10 ml of medium each; capped with tin foil.
2. Continuous illumination at 2000 lux
3. Temperature 22 to 25 C

D. Experiment Ia: Sterilization time

1. Internodes were dipped in 95% EtOH completely coating the surfaces.
2. Internodes were submerged and periodically agitated in a 20% vol/vol chlorox in sterile, distilled water.
3. 2, 4, 6, 8 and 10 minute sterilization periods were used.
4. Immediately following sterilization, the internodes were rinsed twice with sterile, distilled water.
5. Internodes were sliced, end segments discarded, and

remaining segments cultured on the basal medium.

6. Results: Surface sterilization was complete in the 4 to 6 minute periods, while causing the least damage to the internodes. A standard sterilization time of 5 minutes was selected.

E. Experiment Ib: Explant source

1. Ca. 25 cm axillary shoots with approximately 11 partially elongated internodes were used.
2. Segments from each internode were grown in culture.
3. Results: The youngest internodes grew faster and were easier to slice. Tissue cultures were pale yellow to white and very friable.

II. LIGHT REQUIREMENT: CONTINUOUS ILLUMINATION OR DARK

A. Medium: Same as in Ia

B. Explant: Same as in Ib

C. Experiment IIa

1. Stem segments were cultured either in the dark or under continuous illumination from cool white fluorescent lamps at approximately 2000 lux.
2. Results: The tissue grew about twice as fast in the light and was very friable and pale yellow to white under both conditions.

III. NUTRIENT REQUIREMENTS FOR STEM SEGMENT GROWTH: INORGANIC AND ORGANIC

A. Experiment IIIa

1. Inorganic salts: varied

- a. Murashige and Skoog (68) inorganic salts
 - b. Nitsch and Nitsch (71) inorganic salts
 - c. Murashige and Skoog macro-elements and Nitsch and Nitsch micro-elements.
 - d. Nitsch and Nitsch macro-elements and Murashige and Skoog micro-elements.
2. Organic constituents: Held constant at 1 mg/l kinetin, 0.08 mg/l IAA, 0.02 mg/l 2,4-D, 0.1 g/l yeast extract, 30 g/l sucrose, 12 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.
 3. Results: All treatments grew. Those with Murashige and Skoog inorganic salts grew best. The growth was lateral and the tissue was pale green and compact.

B. Experiment IIIb

1. Inorganic salts: Varied
 - a. Murashige and Skoog inorganic salts
 - b. Murashige and Skoog inorganic salts and 15 mg/l $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 15 mg/l KNO_3 and 10 mg/l $\text{NH}_4\text{H}_2\text{PO}_4$ (129)
 - c. THS inorganic salts (129)
 - d. Hackett and Anderson (18) inorganic salts (5X White's).
2. Organic Constituents: Held constant at 1 mg/l BAP, 0.08 mg/l IAA, 0.02 mg/l 2,4-D, 40 g/l sucrose, 20 mg/l adenine sulfate, 0.04 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 mg/l agar.
3. Results: Murashige and Skoog inorganic salts followed by THS inorganic salts were the best for growth, and the rate

of growth was approximately twice that of the two other mixtures.

C. Experiment IIIc

1. Inorganic salts: Varied
 - a. Murashige and Skoog inorganic salts
 - b. THS inorganic salts
 - c. One-half concentration of Murashige and Skoog inorganic salts.
2. Organic Constituents: Held constant at 0.4 mg/l BAP, 5.6 mg/l NAA, 200 mg/l casein hydrolysate, 30 g/l sucrose, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol and 10 g/l agar.
3. Results: Murashige and Skoog inorganic salts were best. THS and one-half Murashige and Skoog inorganic salts were more favorable to adventitious root initiation. The tissue formed was green, compact and grew in all directions.

D. Experiment IIId

1. Inorganic salts: Varied as in Experiment IIIc.
2. Organic constituents: Held constant at 2 mg/l DMAAP, 0.56 mg/l NAA, 125 mg/l L-tyrosine, 25 mg/l L-phenylalanine, 30 g/l sucrose, 20 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol and 10 g/l agar.
3. Results: Murashige and Skoog inorganic salts were best and were followed by THS mineral salts. Growth was rapid in all directions away from the medium and the tissue was green and compact.

E. Experiment IIIe

1. Inorganic salts: Varied as in Experiment IIIb.
2. Organic constituents:

a. Held constant: 1 mg/l BAP, 0.08 mg/l IAA, 0.02 mg/l 2,4-D, 40 g/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol and 10 g/l agar.

b. Varied

(1) no supplements

(2) 0.1 g/l yeast extract

(3) 200 mg/l casein hydrolysate (35% NaCl)

3. Results: Casein hydrolysate enhanced growth while yeast extract inhibited growth. Murashige and Skoog inorganic salts and casein hydrolysate was the best combination and were followed by THS inorganic salts with casein hydrolysate.

F. Experiment IIIf

1. Inorganic salts: Varied as in Experiment IIIa

2. Organic constituents

a. Held constant: Same as in Experiment IIIa

b. Varied

(1) no supplements

(2) 125 mg/l L-tyrosine and 25 mg/l L-phenylalanine

3. Results: L-tyrosine and L-phenylalanine were stimulatory to tissue growth.

G. Experiment IIIg

1. Inorganic salts: Murashige and Skoog inorganic salts modified by substituting 1.55 g/l NaNO_3 for NH_4NO_3 (preliminary experiments resulted in a slight increase in growth rate when NaNO_3 was used replacing NH_4NO_3).

2. Organic constituents

a. Held constant: 3 mg/l 2,4-D, 0.1 g/l yeast extract,

0.1 g/l malt extract, 40 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol and 10 g/l agar

b. Varied:

(1) Sucrose at 10, 25, 40, 55, or 70 g/l

(2) Glucose at 10, 25, 40, 55, or 70 g/l

3. Results: 40 g/l sucrose was the best carbohydrate source.

Glucose was best at 25 g/l. The top three concentrations of sucrose were better than glucose at those concentrations while the opposite was true for the bottom two concentrations of both sugars.

H. Experiment IIIh

1. Inorganic salts: Murashige and Skoog inorganic salts

2. Organic constituents

a. Held constant: 0.25 mg/l kinetin, 2 mg/l IAA, and 10 g/l agar

b. Varied:

(1) 40 g/l sucrose or glucose in all treatments

(2) Thiamine-HCl at 0.187, 0.375, 0.75 or 1.5 mg/l in all treatments

(3) *myo*-inositol at 50, 100, 150 or 200 mg/l in all treatments

3. Results: Different concentrations of thiamine-HCl and *myo*-inositol in combination did not affect tissue growth differently due to the sugar used. *Myo*-inositol was best at 100 mg/l. Tissue growth was better and became more

friable with increasing concentrations of thiamine-HCl; however, 0.375 mg/l was best for both growth and adventitious root formation. The tissue was generally green and compact.

I. Experiment IIIi

1. Inorganic salts: same as in Experiment IIIg
2. Organic constituents:
 - a. Held constant: same as in Experiment IIIh except for IAA and kinetin.
 - b. Varied:
 - (1) Kinetin at 0.01, 0.10, 1 or 10 mg/l in all treatments
 - (2) IAA at 0.1, 2, 8, or 32 mg/l in all treatments
3. Results: The tissue color and growth, and adventitious root formation all varied because of different hormone levels in combination with different sugars (see Fig. 17).

J. Experiment IIIj

1. Inorganic salts: Same as in Experiment IIIg.
2. Organic constituents
 - a. Held constant: Same as in Experiment IIIh except only 40 g/l was used
 - b. Varied:
 - (1) Kinetin at 0, 0.1, 0.10, 1, 5, 10, 15, 20, or 30 mg/l in each treatment
 - (2) IAA at 0.01, 0.05, 0.10, 0.50, 2, 8, 30, 50 or 70 mg/l in all treatments

3. Results: Table 20 shows the results of different hormone concentrations and combinations on growth. Adventitious shoot formation was never observed.

K. Experiment IIIk

1. Inorganic salts: Same as in Experiment IIIg
2. Organic constituents
 - a. Held constant: Same as in Experiment IIIg except IAA and kinetin omitted
 - b. Varied: 2,4-D at 1.5, 3, 6, 9, or 12 mg/l.
3. Results: The best tissue growth was at the lowest 2,4-D concentrations. At 3 mg/l 2,4-D, the initial growth was rapid, but at 1.5 mg/l 2,4-D the growth was eventually greater after a slower start. The tissue was whiter and more friable as the concentration of 2,4-D increased until the 2,4-D became toxic.

L. Experiment IIIl

1. Inorganic salts: Murashige and Skoog inorganic salts modified by substituting 1.55 g/l NaNO_3 for NH_4NO_3 .
2. Organic constituents
 - a. Held constant: same as in Experiment IIIg except only sucrose at 40 g/l was used.
 - b. Varied:
 - (1) No supplements
 - (2) 0.5 mg/l kinetin
3. Results: Kinetin was inhibitory to growth. The tissue was friable and pale yellow.

Fig. 17. Effect of IAA and kinetin with different carbohydrate sources on carnation stem segment cultures. The carbohydrate source was either sucrose (—) or glucose (----) both at 40 g/l. Each set (A-C) of graphs is for separate culture characteristics that were rated on a scale from 1 to 4 and the rating was derived from visual estimations after 4 wk growth:

A. Color of the tissue

1. Brown
2. Browning
3. Light green
4. Dark green

B. Adventitious root development

1. None
2. Few primordia
3. Average
4. Above average

C. Growth

1. None
2. Limited
3. Average
4. Above average

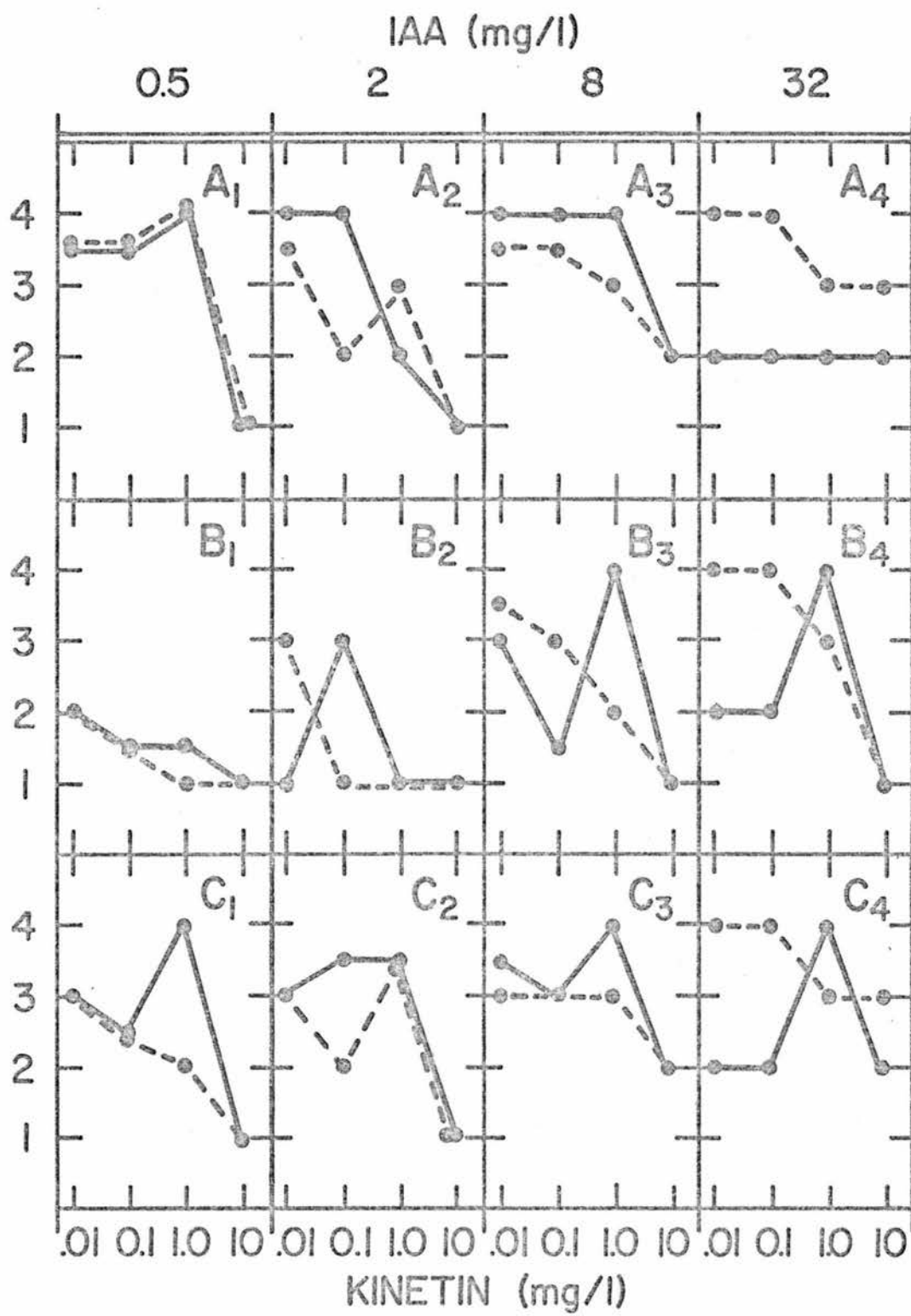


TABLE 20. Relative growth^a of carnation stem segment explants in response to combinations of different concentrations of IAA and kinetin.

IAA (mg/1)	Kinetin (mg/1)								
	0	0.01	0.10	1	5	10	15	20	30
0.01	-	+	+	+	+				
0.05	+	+	++	+	+				
0.10	++	++	++	++	+				
0.50	++	+++	++	+++	+	-			
2		+++	++	+++	+	-			
8		+++	+++	+++	++	++			
30		+++	+++	+++	++	++	++	++	+
50							++	+	-
70							-	-	-

^aNo growth (-), limited growth (+), avg growth (++), above avg growth (+++).

M. Experiment III_m

1. Inorganic salts: Murashige and Skoog inorganic salts.
2. Organic constituents:
 - a. Held constant: 0.1 g/l yeast extract, 0.1 g/l malt extract, 30 g/l glucose, 12 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.
 - b. Varied:
 - (1) 0.2 mg/l IAA and 0.2 mg/l 2,4-D
 - (2) 0.2 mg/l IAA, 0.2 mg/l 2,4-D, 125 mg/l L-tyrosine, and 25 mg/l L-phenylalanine.
 - (3) 0.2 mg/l NAA and 0.2 mg/l 2,4-D
3. Results: None of the three treatments were consistently better than others. Tissue growth was rapid and uniform in all directions away from the agar, and the tissue was dark green, and slightly compact.

N. Experiment III_n

1. Inorganic salts: Murashige and Skoog inorganic salts.
2. Organic constituents:
 - a. Held constant: Murashige and Skoog organic constituents modified by omitting edamin, kinetin, and IAA.
 - b. Varied: NAA and 2,4-D together or alone
 - (1) NAA at 5×10^{-6} M, 5×10^{-7} M or 5×10^{-8} M
 - (2) 2,4-D at 5×10^{-6} M, 5×10^{-7} M or 5×10^{-8} M
3. Results: Table 21 summarizes the relative growth obtained in all treatments. All combinations with 5×10^{-6} M 2,4-D

produced friable, pale yellow tissue. All other combinations produced tissue which was green and compact, and induced adventitious root formation.

TABLE 21. Relative growth^a of carnation stem segment explants in response to two auxins, NAA and 2,4-D, at various concentrations in combination or alone.

NAA (μ M)	2,4-D (μ M)			
	0	0.05	0.50	5
0	-	-	++	+++
0.05	+	+	+++	+++
0.50	+	++	+++	+++
5	++	++	++	+++

^aNo growth (-); limited growth (+); avg growth (++); above avg growth (+++).

IV. OVULE CULTURE

- A. Explant: Ovules excised from ovaries which were removed from the flower bud just prior to opening.
- B. Culture support: Ash-less filter paper bridges.
- C. Experiment IVa
 - a. Held constant: 30 g/l sucrose, 0.4 mg/l thiamine-HCl, and 100 mg/l *myo*-inositol.
 - b. Varied:
 - (1) 0.06 mg/l 2,4-D
 - (2) 0.6 mg/l 2,4-D
 - (3) 6 mg/l 2,4-D

- (4) 2 mg/l IAA and 1 mg/l kinetin
- (5) 0.6 mg/l 2,4-D and 1 mg/l kinetin
- (6) 0.6 mg/l 2,4-D and 0.5 mg/l kinetin

2. Results: Combinations of 2,4-D and kinetin supported growth. White, friable tissue was obtained.

D. Experiment IVb

1. Inorganic salts: Murashige and Skoog inorganic salts.

2. Organic constituents:

a. Held constant: Same as in Experiment IVa.

b. Varied:

- (1) 0.6 mg/l 2,4-D and 1 mg/l kinetin.
- (2) 0.6 mg/l 2,4-D, 1 mg/l kinetin, and 0.1 g/l yeast extract.
- (3) 0.6 mg/l 2,4-D, 1 mg/l kinetin, 0.1 mg/l yeast extract, and 10 mg/l adenine sulfate.
- (4) 0.6 mg/l 2,4-D, 1 mg/l kinetin, 0.1 g/l yeast extract, 125 mg/l L-tyrosine, and 25 mg/l L-phenylalanine.
- (5) 0.2 mg/l 2,4-D, 0.2 mg/l IAA, and 1 mg/l kinetin.
- (6) 0.6 mg/l 2,4-D and 1 mg/l kinetin.
- (7) 0.2 mg/l 2,4-D and 1 mg/l kinetin.

3. Results: All treatments with yeast extract failed to support growth. Combinations with 0.6 mg/l 2,4-D and 1 or 2 mg/l kinetin grew best and were followed by the combination with 2,4-D, IAA, and kinetin. The tissue derived from this was white and very friable.

E. Experiment IVc

1. Inorganic salts: Varied
 - a. Murashige and Skoog inorganic salts
 - b. Nitsch and Nitsch inorganic salts
2. Organic constituents: Held constant at 0.6 mg/l 2,4-D, 1 mg/l kinetin, 30 g/l sucrose, 0.4 mg/l thiamine-HCl, and 100 mg/l *myo*-inositol.
3. Results: Nitsch and Nitsch inorganic salts supported growth almost as well as Murashige and Skoog inorganic salts. The tissue derived was white and very friable.

V. SUBCULTURE

A. Experiment Va

1. Inorganic salts: Hackett and Anderson inorganic salts (5 x White's).
2. Organic constituents
 - a. Held constant: 30 g/l sucrose, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.
 - b. Varied:
 - (1) 30 mg/l IAA and 30 mg/l kinetin
 - (2) 40 mg/l adenine sulfate
 - (3) 40 mg/l adenine sulfate, 125 mg/l L-tyrosine, and 25 mg/l L-phenylalanine.
3. Tissue source: Approximately 5 mm³ pieces of tissue from Experiment Ia.
4. Results: The treatment with both IAA and kinetin supported little growth, and the tissue turned brown and died early.

While the other two treatments also did not support growth, the tissue did remain alive without any apparent changes for several months.

B. Experiment Vb

1. Inorganic salts: Varied
 - a. Murashige and Skoog inorganic salts
 - b. Nitsch and Nitsch inorganic salts
2. Organic constituents: Held constant at 30 g/l sucrose, 20 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar
3. Tissue source: Approximately 5 mm³ pieces of tissue from Experiment Ib.
4. Results: Tissue grew slowly but continuously for about seven months. It remained pale yellow during this period except for some green near the top.

C. Experiment Vc

1. Inorganic salts: Murashige and Skoog macro-elements, and Nitsch and Nitsch micro-elements.
2. Organic constituents: 1 mg/l IAA, 0.2 g/l kinetin, 30 g/l sucrose, 40 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.
3. Tissue source: Approximately 5 mm³ pieces of tissue from Experiment Ib.
4. Results: Same as Experiment Vb.

D. Experiment Vd

1. Inorganic salts: Hackett and Anderson inorganic salts (5 x White's)

2. Organic constituents: 2 mg/l NAA, 30 g/l sucrose, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.
3. Tissue source: Approximately 5 mm³ pieces of tissue from Experiment Ib.
4. Results: The tissue grew slowly, turned light green and adventitious root primordia appeared.

E. Experiment Ve

1. Inorganic salts: Murashige and Skoog inorganic salts
2. Organic constituents:
 - a. Held constant: 40 g/l sucrose, 20 mg/l adenine sulfate, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.
 - b. Varied:
 - (1) 0.05 mg/l BAP and 0.01 mg/l 2,4-D
 - (2) 0.10 mg/l BAP and 0.01 mg/l 2,4-D
 - (3) 0.05 mg/l BAP and 0.10 mg/l IAA
 - (4) 0.10 mg/l BAP and 0.10 mg/l IAA
3. Tissue source: Approximately 5 mm³ pieces of tissue from Experiment IIIg.
4. Results: Whether sucrose or glucose was in the medium on which the tissue was initially grown made no difference. The first and fourth treatments were best. The growth was slow, and the tissue was friable with the area near the medium's surface turning pale green.

F. Experiment Vf

1. Inorganic salts: Murashige and Skoog inorganic salts.
2. Organic constituents:

a. Held constant: 20 g/l sucrose, 20 mg/l adenine sulfate, 125 mg/l L-tyrosine, 25 mg/l L-phenylalanine, 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, and 10 g/l agar.

b. Varied:

(1) 2 mg/l DMAAP and 0.5 mg/l NAA

(2) 1 mg/l DMAAP

3. Tissue source: Approximately 5 mm³ pieces of tissue from several previous experiments: IIIc, IIId, IIIg, IIIm, IVb.
4. Results: All the subcultures continued to grow on the medium with DMAAP and NAA. The medium with DMAAP alone was more selective and only tissue cultures initiated on 2,4-D with one exception continued to grow on this medium. The one exception was tissue grown on a medium containing Murashige and Skoog inorganic salts and a high NAA concentration (Experiment IIIc). Tissue from ovules grown on a 2,4-D and kinetin containing medium grew quite well on the medium with DMAAP alone. In both treatments, the tissue obtained became green in regions and adventitious roots often appeared.

G. Experiment Vg

1. Media: Petru and Landa's (77) medium A and medium B.
2. Tissue source: Tissue cultures derived from the excised hypocotyls of carnation seedlings (Double Marguerite and Dwarf Fragrance) which were grown on Petru and Landa's medium A in the dark or under continuous illumination.
3. Results: The hypocotyl tissue was subcultured onto either medium A or medium B and grown either under continuous

illumination, 16 hour photoperiods or daylight, without adventitious shoot formation. Tissue transferred to medium A grew well and was green with root primordia. Continuous illumination was best for growth. Tissue transferred to medium B did not grow and died after a while.

H. Experiment Vh

1. Media: Petru and Landa's media A and B modified by substituting Murashige and Skoog's micro-elements for those of Petru and Landa.
2. Tissue source: Same as experiment Vg.
3. Results: Same as in Experiment Vg, except for slightly faster growth of the tissue on medium A.

I. Experiment Vi

1. Media: Same as Experiments Vg and Vh.
2. Tissue source: Carnation stem segment tissue grown as was the hypocotyl tissue in Experiment Vg, and tissue from suspension cultures obtained in Experiment VIg.
3. Results: Same as in Experiments Vg and Vh.

VI. SUBCULTURE: SUSPENSION CULTURES

A. Culture Conditions

1. 250 ml erlenmeyer flasks with 50 ml of medium each.
2. Rotated on a horizontal shaker at approximately 120 cycle/min.
3. 16 hr photoperiods at 2000 lux.

4. Temperature at 22 to 27 C.

B. Experiment VIa

1. Media: Linsmaier and Skoog medium modified by omitting agar and replacing IAA and kinetin as given by:
 - a. 1 mg/l IAA and 0.1 mg/l kinetin
 - b. 0.6 mg/l 2,4-D
 - c. 0.6 mg/l 2,4-D and 1 mg/l BAP
2. Tissue source: Approximately 1 cm³ pieces of tissue from Experiment Ia.
3. Results: The tissue grew little but did turn pale green. A suspension culture was not established.

C. Experiment VIb

1. Media: Hackett and Anderson inorganic salts (5 x White's) and the organic constituents of Linsmaier and Skoog modified by omitting agar, IAA, and kinetin and supplementing the medium with:
 - a. 40 mg/l adenine sulfate
 - b. 40 mg/l adenine sulfate, 125 mg/l L-tyrosine, and 25 mg/l L-phenylalanine.
 - c. As in b with 0.6 mg/l 2,4-D
 - d. 40 mg/l adenine sulfate and 0.6 mg/l 2,4-D
 - e. 0.6 mg/l 2,4-D
2. Tissue source: Same as in Experiment VIa.
3. Results: Treatment "c" was the best and was followed by treatment "d". Growth occurred in all treatments but was very slow and in large lumps of cells without any green

coloration. Although growth temporarily was good, it ceased prematurely before a good suspension culture was established.

D. Experiment VIc

1. Media: Linsmaier and Skoog medium supplemented with 24 mg/l adenine sulfate, 125 mg/l L-tyrosine, 25 mg/l L-phenylalanine, and modified by omitting the agar and replacing IAA and kinetin as given by:
 - a. 0.05 mg/l kinetin
 - b. 0.05 mg/l kinetin and 0.08 mg/l IAA
 - c. 0.08 mg/l IAA and 1 mg/l BAP
 - d. 0.08 mg/l IAA and 1 mg/l BAP
 - e. 0.05 mg/l kinetin
 - f. 0.08 mg/l IAA and 0.05 mg/l kinetin
2. Tissue source: Approximately 1 cm³ pieces of tissue from Experiment IIIa.
3. Results: The treatment with 0.05 mg/l kinetin was best. The first three treatments supported growth, and the cell aggregates were large and pale green. Many roots developed from the cell aggregates while in suspension. Most of the growth in the cultures was from the cell aggregates. Small aggregates of cells appeared to break off the larger ones and grow, but a cell suspension was not formed.

E. Experiment VIId

1. Media: Linsmaier and Skoog medium without agar and supplemented with 12 mg/l adenine sulfate, 200 mg/l casein

hydrolysate (35% NaCl) and modified by substituting 1.55 g/l NaNO_3 for NH_4NO_3 and two treatments for the IAA and kinetin constituents:

- a. 0.08 mg/l IAA, 0.02 mg/l 2,4-D, and 1 mg/l kinetin
- b. 0.05 mg/l kinetin

2. Tissue source: Same as Experiment Vc
3. Results: Growth was slow in the first treatment and the cells died early. Many roots formed in the second treatment, and were from aggregates of cells as in Experiment VIe. A cell suspension was not formed.

F. Experiment VIe

1. Media: Same as Experiment IIIb without agar
2. Tissue source: Approximately 1 cm^3 pieces of tissue from Experiment IIIa
3. Results: Murashige and Skoog inorganic salts and THS inorganic salts supported the fastest growth of cell aggregates among the treatments; however, rapidly growing roots soon developed, especially in the medium with THS inorganic salts, and unorganized growth ceased. A cell suspension was not established.

G. Experiment VIg

1. Media: Murashige and Skoog medium supplemented with 10 mg/l adenine sulfate, 400 mg/l casein hydrolysate (35% NaCl), 1 mg/l 2,4-D; omitting edamin, IAA and agar; kinetin included at 1 mg/l; 125 mg/l L-tyrosine and 25 mg/l L-phenylalanine was supplemented to one half the treatments.

2. Tissue source: Approximately 1 cm³ pieces of tissue from stem segments grown on a Murashige and Skoog medium with 1 mg/l 2,4-D substituted for IAA and kinetin and edamin omitted.
3. Results: Rapidly growing suspension cultures were established. The cultures with tyrosine and phenylalanine grew faster and had smaller aggregates of cells. The cultures were lumpy and pale green. Small groups of cells cleaved off the large aggregates and appeared to grow and divide very slowly.

H. Experiment VIg

1. Medium: Murashige and Skoog inorganic salts, 1 mg/l kinetin, 1 mg/l NAA, 30 g/l sucrose, 100 mg/l L-tyrosine, 1.5 mg/l thiamine-HCl, 200 mg/l *myo*-inositol, 4 mg/l adenine sulfate, 1 g/l casein hydrolysate, and pH at 6.0.
2. Tissue source: Same as in Experiment VI f.
3. Results: A rapidly growing lumpy, pale green culture of carnation cells was obtained.

I. Experiment VIh

1. Media: Petru and Landa's (77) medium A and medium B modified by omitting the agar and substituting Murashige and Skoog's micro-elements for those of Petru and Landa.
2. Tissue source: Subcultured from Experiment VIg
3. Results: Medium A supported the same type of growth as found in Experiment VIg. Medium B did not support growth, but the cells remained alive for a couple of months.

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