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Title: TECHNIQUES AND MEDIA FOR EXCISED TIP CULTURE OF VINIFERA CULTIVARS

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Excised apical stem tips (2-5 mm) of grape cultivars grew well on Murashige and Skoog's (1962) medium for tobacco callus. Additions of glycine were not essential but gave a slight stimulus to growth. Naphthalene acetic acid slightly reduced growth rates but reductions were not statistically significant. Kinetin and coconut milk were both significantly detrimental to the growth of the excised tips. Tips that developed callus following treatment with IBA (indolebutyric acid) made more growth (significant at .01 probability) than tips without a callus mass at the cut end. Indolebutyric acid was necessary to root production in the second sub-culture on agar medium. Elongated stem tips with roots, transferred to a liquid medium on a filter paper bridge, grew vigorously and produced abundant roots. These young plants were later transferred to greenhouse soils and developed to mature, healthy plants typical of the cultivar.

Techniques and Media for Excised Tip Culture of Vinifera Cultivars

by

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TECHNIQUES AND MEDIA FOR EXCISED TIP CULTURE OF VINIFERA CULTIVARS

INTRODUCTION

In recent years tissue culture and the culture of excised meristems has been useful in direct studies of the nutritional requirements of tissues and organs. It has been demonstrated, with certain tissue, that by appropriate techniques, tissue can be isolated and grown free of diseases--especially some systemic viruses. Where complete plants have been developed from such tissues or meristems, it has been possible to restore vigor and yield previously reduced as a result of the degradative effects of disease.

The technique, media, and art of culturing such plant material thus becomes useful as a means of propagating plants, of aiding in the process of eliminating disease, and providing material for nutritional and other laboratory studies.

The shoot, apical meristem has long been recognized as one of the principal growth centers of plants. During growth, it is supplied by the more mature vascular tissues and organs with the basic materials necessary to sustain meristematic activity. Such materials include essential mineral elements, carbohydrates, amino acids, hormonal and other substances.

Isolated apical shoot meristems fail to develop into normal shoots if the proper sustenance and environment is not provided. It has also been determined that complete plants cannot be grown from the isolated shoot apices of higher plants without the presence of primordia and small amounts of the sub-adjacent tissue.

In the last three decades, much work has been done on the shoot tip culture of various kinds of plants but, for the most part, nutritional problems related to media have not received sufficient attention. A few investigators have reported on the effect of certain organic substances on the growth of the tips of monocotyledonous and herbaceous dicotyledonous plants. This background of information provides a base for the present study.

The objectives of experiments reported here were to determine the techniques, media, and environment necessary to obtain complete plants from excised apical stem tips of specific grape cultivars--particularly of cultivars of Vitis vinifera. The accomplishment of such objectives might then be incorporated into other investigative goals, such as elimination of various systemic diseases that remain with the stock when standard propagation methods requiring larger segments of more mature tissue are used. A successful technique can have important implications in satisfying practical applications and furthering investigations concerned with basic research.

LITERATURE REVIEW

The fundamental objectives in attempting to culture plant stem apices have been summarized into three categories by Street (1969). I. To study specific physiological functions in relation to their anatomical structures. II. To investigate unique techniques in vegetative propagation of certain plants such as some species of orchid. III. To obtain virus-free stock of certain important horticultural species.

As early as the turn of the century, Rechinger (1893) grew isolated buds of Populus nigra, Fraxinus ornus and other plants in sand without nutrients. He observed that some basal callus and a slight extension growth of apical vegetative tissue occurred but that no roots developed. Goebel (1908) grew isolated buds of Ceratopteris thalictroides in moist peat and found that they produced leaves much smaller and more simple morphologically than those of normal plants. These early studies demonstrated the need for more experimentation to satisfy the normal physiological-environmental requirements.

In spite of the continued interest in experimentation by a number of early workers as referred to by White (1936, 1943), it was not until 1922 that shoot tips were first grown in sterile conditions with sugar (Robbins, 1922). He grew stem tips (1.75 to 3.75 cm long) of pea corn, and cotton in the dark on agar media containing glucose. The tips produced shoots with small chlorotic leaves and numerous roots.

In a later study, White (1933) used a culture medium and a hanging drop technique to grow stem tips of Stellaria media 0.1 mm or less in length and width. Tips thus cultured lived up to six weeks. Some tips, produced as above, showed differentiation; i.e., they produced foliar or flower primordia, and others merely enlarged. White considered that the enlargement usually resulted from cell divisions and not from hypertrophy. No complete plants (stem, leaf and root) were formed.

The production of normal plants from bud tissue segments was accomplished by LaRue (1936). He grew tissue segments that included cotyledon bases and plumular meristems of shoot tips of Taraxacum officinale, Chrysanthemum leucanthemum, Lactuca canadensis, Lycopersicum esculentum, and buds from leaf bases of Radicula aquatica (mature plants) in sterile agar and liquid medium. Segments were less than 1 mm long and usually less than 0.5 mm wide. Roots were regenerated basally on these buds and the shoots produced normal plants.

Successful development of complete plants of a monocot resulted from the work of Segelitz (1938) who grew shoot tips of corn (1-4 cm long) on a sterile agar substrate. The tips over 2 cm long rooted in the dark but those of lesser length rooted only in light. Large plants were grown from these cultures. With the increasing background of knowledge and improvements in technique, Smith (1940) grew stem

tips (0.55 to 4.0 mm long) of Helianthus annuus on agar in White's (1933) solution. His studies were concerned with the effects of various accessory growth factors in the medium on the rates of growth.

Loo (1945) grew stem tips (5 mm long) from sterile seedlings of Asparagus officinalis in sterile culture through 20 successive transfers. He found that the tips underwent potentially unlimited growth without giving rise to roots. The culture medium contained the balanced salt solution of Bonner (1943) and 2-4% sucrose. Light was found to be essential for continued growth. In a few cases where adventitious roots were initiated, the growth rate increased three or four times, an effect explained as perhaps following from an increased uptake of nutrients or indicating the more active synthesis of some limiting metabolites or growth regulators. Growth of the cultured stem tips was not enhanced by the addition of B vitamins or of adenine. However, the appearance of cladophylls seemed to enhance the growth rate. The continued growth of non-rooted asparagus stem cultures reported by Loo (1945) remains of considerable interest because excised stem apices of many other angiosperms have either failed to develop in culture or have initiated roots and given rise to plantlets as mentioned by Street (1969). The development of plantlets in this way has proved to be a useful technique for obtaining virus-free stocks of a number of economic plants.

Ball (1946) cultured small stem tips of Tropaeolum majus and

Lupinus albus in a medium containing inorganic salts, glucose and 0.3% agar. The tips included not only the dome of meristematic tissue but also the three youngest leaf primordia and some subjacent tissue. Many such apices when incubated in light gave rise to plantlets bearing roots. Presence of the leaf primordia and subjacent tissue appeared to be important.

Comparisons of the apices of vascular cryptogams and those of angiosperms cultured in sterile media were made by Wetmore (1949, 1954). He found that apices only 200-250 μ in length of Adiantum pedatum, Selaginella willdenovii, Lycopodium cernuum and Equisetum hyemale grew into plants without difficulty on a medium containing only inorganic ions and sucrose. Growth was, however, very significantly enhanced by incorporating yeast extract (0.5 gm/lt) into the culture medium and was further stimulated by a low concentration of auxin (naphthalene acetic acid 0.05 mg/lt). Apices of a number of angiosperms (Syringa vulgaris, Parthenocissus tricuspidata, and Lupinus albus) grew little on a medium which was excellent for the culture of apices of vascular cryptogams, unless the angiosperm apices taken were as long as 0.5 mm. Angiosperm apices, in contrast to those of vascular cryptogams, could not use nitrate as an effective source of nitrogen. Wetmore, therefore, suggested that angiosperm apices might either be heterotrophic for certain amino acids or might synthesize such amino acids at a critical suboptimal

rate. The study emphasizes the more critical requirements of the higher plants for environment, nutrient, and accessory substances, especially when the apices are small.

Morel and Martin (1952) grew excised shoot tips (approximately 250 μ long) of Dahlia in vitro on media containing Knop's solution[1] supplemented with various vitamins. The tips grew rapidly but did not produce roots and the plantlets were propagated by grafting to healthy Dahlia seedling. They claimed that the cultured plantlets were freed from viruses. Morel and Martin later (1955) used the same technique for potatoes. In this case, roots were eventually formed although no NAA (naphthalene acetic acid) was present in the medium. The plantlets were grafted to potatoes for further growth because many died when transplanted directly to soil. Kassanis (1957) also succeeded in culturing potato apices using an agar medium without NAA and obtained rooting. Quack (1957) claimed that some of the plants resulting from excised carnation tips grown on a modification of Morel's (1955) medium were virus free.

In subsequent studies Morel (1959) cultured the meristems of tropical orchids (particularly hybrid Cymbidium) on a single medium containing only inorganic salts and glucose. Such apices gave rise to a protocorm (a juvenile stage in normal development of the plant) and

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[1] Knop's solution $Ca(NO_3)_2 \cdot 4H_2O$ 1000 KNO_3 250 KH_2PO_4 250 $MgSO_4 \cdot 7H_2O$ 250 mg/lt.

the cultures were serially propagated by division into three or four fragments at each subculture.

Several authors have reported on additional investigations involving carnation. Philips and Danielson (1961) and Baker and Philips (1962) published notes on tip culture with this plant. They used a further modification of Morel's (1955) medium and adapted filter paper bridges developed by Heller (1949, 1956) instead of an agar support. Large tubes containing 15 ml of medium were used to grow tips under continuous light. Stone (1963) found that rooting was affected by the size of the tips and the time of the year at which they were excised. Large sized tips initiated root primordia better than smaller sized tips.

Other investigators employing culture techniques have reported successes in freeing crop plants from virus infections. Walkey (1968) successfully cultivated shoot tips of rhubarb of 300 to 2500 μ diameter in vitro. He reported that most plants grown by the tip-culture technique were free from virus. Gooseberry shoot tips grown in vitro also produced plants apparently free from vein-banding virus (Jones and Vine, 1968). Dissected apices of strawberry stolon tips and axillary buds of less than 0.80 mm length were used to produce new plants (Vine, 1968). Tips excised from heat-treated plants grew more rapidly and more of the cultured plants reached maturity than those from non-heat treated stock. Most of the plants thus derived were free from

virus. Hop shoot tips were cultured by Vine and Jones (1969). They found that the tissue culture technique proved highly effective in eliminating prunus necrotic ringspot and hop mosaic virus from 11 varieties of hops. The technique was successful even when plants were grown from tips as long as 5 mm.

The effects of some accessory substances on the growth of stem tips appears to be of importance. A number of physiologically active compounds have been investigated for their effect on the growth of isolated stem tips in vitro. Loo (1945) found that a mixture of eight active substances used by Van Overbeek, et al. (1941) for the culture of young Datura embryos seemed to be without effect on isolated stem tips of asparagus. The eight substances listed were glycine, thiamine, ascorbic acid, nicotinic acid, pyridoxine, adenine, succinic acid and pantothenic acid.

Some investigators found that many amino acids do not support the growth of the shoot apex of certain plants (Reinert, 1959; Ball, 1960; Mellor, 1969). Others found that some amino acids such as l-arginine stimulated growth (Harris, 1953).

The auxin IAA (indoleacetic acid) and a related compound were found to be detrimental to the growth of the stem tips of asparagus (Loo, 1945), and had no effect on the shoot apex of Lupinus albus (Ball, 1960). However, they were quite necessary for tip rooting of various other kinds of plants (Stone, 1963; Jones and Vine, 1968;

Vine, 1968; Vine and Jones, 1969). Coconut milk and other cytokinin substances seemed to cause detrimental effects to the shoot tips of potato (Mellor, 1969; Elliot, 1969). These same materials were found to be essential to the culture of tips of hops (Vine and Jones, 1969), gooseberry (Jones and Vine, 1968), and strawberry (Vine, 1968).

A review of pertinent literature indicates steady progress in delineating problems relative to the successful culture of apical meristematic tissue. It is clear that limiting factors are related to the special nature of the apical tip in terms of maturity and the presence of subjacent tissue. In addition, the special nature of the media, its composition in terms of nutrients and accessory substances, the presence or absence of light, and other environmental conditions all play significant roles in the successful development of whole plants from tissue segments. Many of the successes also point to a practical method for elimination of virus infections. A single success can thus become an occasion for celebration. The present study is an effort to achieve such a success with excised stem tips of certain vinifera cultivars.

GENERAL MATERIALS AND METHODS

Uniform vinifera grape cultivars from certified, disease-free, foundation stock of a similar physiological age and of the same environmental regimen were chosen for use in the experiments. Outer embryonic leaves of apical buds were removed under a dissecting microscope. Stem apices (tips) with leaf primordia were excised from such buds with a mounted razor blade. All tips selected were within the linear range of 2-5 mm.

Following selection and excision, tips were placed in five percent "Chlorox" solution (sodium hypochlorite) to which two-three drops of absolute alcohol was added to reduce surface tension. Tips were thus treated for a 15 minute period. Transfers to the solution were made within a Tap-A-Line plastic transfer and culturing hood complete with illumination from a cool white, 30 watt, fluorescent light source, and air filtering units. The hood and equipment used were sterilized prior to use with a spray of 6700 parts per million (ppm) Zephiran chloride. Ventilation of the unit was with sterile air filtered through a unit designed to eliminate contaminating organisms.

Surface sterilized tips were washed three times with sterile distilled water to rinse away "Chlorox" residue. Each tip was then placed immediately in a sterile shell vial containing 10 cc's of nutrient media. The shell vials were 95 mm long and 24 mm in diameter. Vial openings were plugged with sterile cotton and covered over with sterile

aluminum foil. The tips were supported either with solidified 0.4 percent agar or a filter paper bridge of the type developed by Heller (1949, 1956) which dipped into the liquid medium in the vial.

The basic medium used was that developed by Murashige and Skoog (1962) for tobacco callus but with some supplemental accessory substances added. The composition of the medium used was as follows:

	Chemical Composition	Concentration (mg/lt)
	Inorganic	
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2 \cdot 2H_2O$	440
	$MgSO_4 \cdot 4H_2O$	370
	KH_2PO_4	170
	Na_2EDTA (disodium salt of ethylene-diamine-tetra acetic acid)	37.3
	$FeSO_4 \cdot 7H_2O$	27.8
Micronutrients	H_3BO_3	6.2
	$MnSO_4 \cdot 4H_2O$	22.3
	$ZnSO_4 \cdot 4H_2O$	8.6
	Kl	0.83
	$Na_2MnO_4 \cdot 2H_2O$	0.25

Continued on next page

	Chemical Composition	Concentration (mg/lt)
	Inorganic	
Micronutrients	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.025
	Organic	
	sucrose	30,000
	glycine	2
	agar	4,000 (liquid medium agar omitted)
	myo-inositol	100
	nicotinic acid	0.5
	pyridoxine.HCl	0.5
	thiamine.HCl	0.1

Accessory substances such as coconut milk, kinetin, naphthaleneacetic acid (NAA), and indolebutyric acid (IBA) at various concentrations were also added in certain of the experiment. The pH's of the various media were adjusted to the range of 5.8-6.2 with 0.1 Normal NaOH. The media were autoclaved at 15-20 psi for 20 minutes prior to being readied for use in the shell vials.

Experiment I. The Effect of Glycine on the Stem Tips of the Grape Cultivar Grenache Grown In Vitro

Glycine is known for its important role in porphyrin biosynthesis and the incorporation of metals into porphyrins which leads to many enzymatic and other organic substances such as peroxidases, catalases, hemoglobin, and cytochrome a, b, and c essential to plant growth (Bogorad, 1965). This experiment was aimed at determining if glycine added to the basic medium would affect the growth of stem tips of the cultivar 'Grenache.'

Materials and Methods

Numerous stem tips of 'Grenache' were excised from the intact plants but only those surviving from contamination during the experimental procedure were used in statistical analysis as indicated in Table 1.

Table 1. The effect of glycine on 'Grenache' stem tips.

Treatment	No. of Tips	No. of Tips Surviving	Initial Length (mm/tip)	Length Increase (mm/tip)
0 mg/lt glycine	15	10	2.3	4.0
2 mg/lt glycine	15	11	2.3	4.1
20 mg/lt glycine	15	11	2.5	5.2
L.S.D. .05				N.S.D.
L.S.D. .01				N.S.D.

All tips were divided into three groups of 15 each and each group cultured on different media.

The media used for groups I, II, and III were as follows:

I. The sterilized Murashige and Skoog (1962) medium for tobacco callus culture minus glycine.

II. The same medium as I but supplemented with 2 mg/lt glycine.

III. The same medium as I but supplemented with 20 mg/lt glycine.

Each tip was cultured in a shell vial containing 10 cc's of liquid nutrient, and supported with a filter paper bridge as developed by Heller (1949, 1956). Vials were placed under illumination of 500 ft-candles of artificial light in a room with an ambient temperature of 68-70^{o}F. Duration of the photoperiod was 16 hours light followed by eight hours of uninterrupted dark.

At five day intervals, in the first three weeks, five tips from each group were aseptically removed from the shell vial and placed in the sterilized petri-dish. Measurements were made under sterile filtered air within the hood. Following the measurements the tips were returned to the shell vials.

Results

The results in Table 1 show no significant differences were

observed among control (no glycine) and other treatments. However the treatment with 20 mg/lt glycine seemed to be slightly better than treatment with 2 mg/lt glycine and the control treatment.

Figure 1 shows the growth rate of the stem tips. The growth rate of the stem tips of all treatments increased rapidly in the five to ten day period and declined after 15 days of culture. The growth of the stem tips nearly ceased after 20 days of culture.

Experiment II. The Effect of Coconut Milk on Excised Stem Tips of Wine Grape Cultivars Grown In Vitro

Coconut milk has been used for some time as an essential nutrient for many tissues. Most of the studies were done on callus culture of tobacco and carrot. Recently, some investigators, Vine (1968), and Vine and Jones (1969) found that the growth of excised stem tips of some monocotyledons and herbaceous dicotyledons was enhanced if coconut milk was present in the medium. However, there is little known about its effect on woody dicotyledons such as the grape cultivars used in the present study.

Materials and Methods

Five cultivars of wine grapes grown in the greenhouse were selected for this study. They were, 'Pinot blanc', 'Gewurztraminer', 'Semillon', 'Sauvignon blanc', and 'Grenache'. Tips from each cultivar were divided into two groups. The first group was cultured on

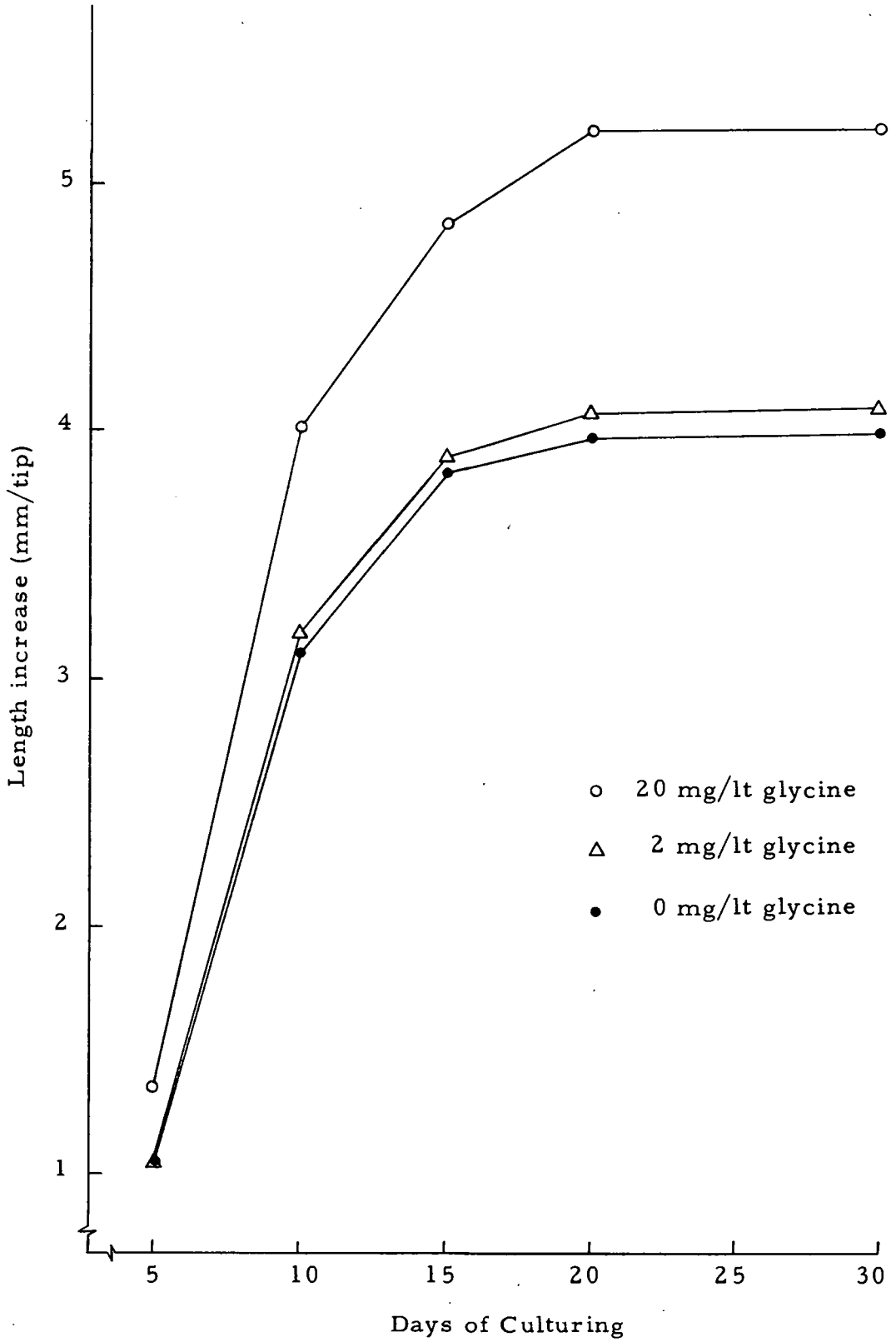

Figure 1. The effect of glycine on the growth of 'Grenache' tips.

Murashinge and Skoog medium (1962) for tobacco callus supplemented with 18 mg/lt glycine. the second group was cultured on the same medium but supplemented with 10% coconut milk.[2]

The treatments were arranged in a 5 x 2 factorial experiment (5 cultivars x 2 levels of coconut milk) in completely randomized design with ten replications of one tip each. At five day intervals in the first three weeks, five tips from each treatment were measured to determine the growth rate.

Results

Table 2 shows the increase in length of the stem tips (mm/tip) of all treatments at the end of the experiment (30 days after culture). They were significantly different at the 1% probability level. Tips from the cultivar 'Gewurztraminer' on medium, minus coconut milk, grew more vigorously than the tips from other treatments (Figure 2). The tips from 'Pinot blanc', on medium, minus coconut milk, grew poorly when compared to those of the same cultivar grown with coconut milk 100 cc's/lt. (Figure 2)

Table 3 shows the effect of main effects (coconut milk and varieties) on the increase in length of the stem tips. There were significant

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[2] For further detail, see in General Material and Methods.

Table 2. The effect of coconut milk on stem tips of five cultivars of vinifera grape.

Cultivars	Levels of Coconut Milk (cc/lt)	No. of Tips	Initial Length (mm/tip)	Average of Length Increase After 30 Days (mm/tip)
Grenache	0	10	2.8	5.4
	100	10	2.7	3.8
Sauvignon blanc	0	10	3.0	5.8
	100	10	2.9	5.7
Semillon	0	10	3.2	3.9
	100	10	3.3	3.3
Gewurztraminer	0	10	3.0	8.5
	100	10	2.8	6.6
Pinot blanc	0	10	3.2	3.1
	100	10	3.1	3.6
L.S.D. .05				1.1
L.S.D. .01				1.5

Table 3. Main effect of cultivars and coconut milk on the excised stem tips.

Source	Level (cc/lt)	Average Length Increase After 30 Days (mm/tip)
Grenache	-	5.0
Sauvignon blanc	-	5.7
Semillon	-	3.6
Gewurztraminer	-	7.5
Pinot blanc	-	3.4
L.S.D. .05		0.8
L.S.D. .01		1.0
Coconut Milk	0	5.3
	100	4.6
L.S.D. .05		0.5
L.S.D. .01		0.7

The effect of interaction (cultivars x coconut milk) is significant at 5% probability level.

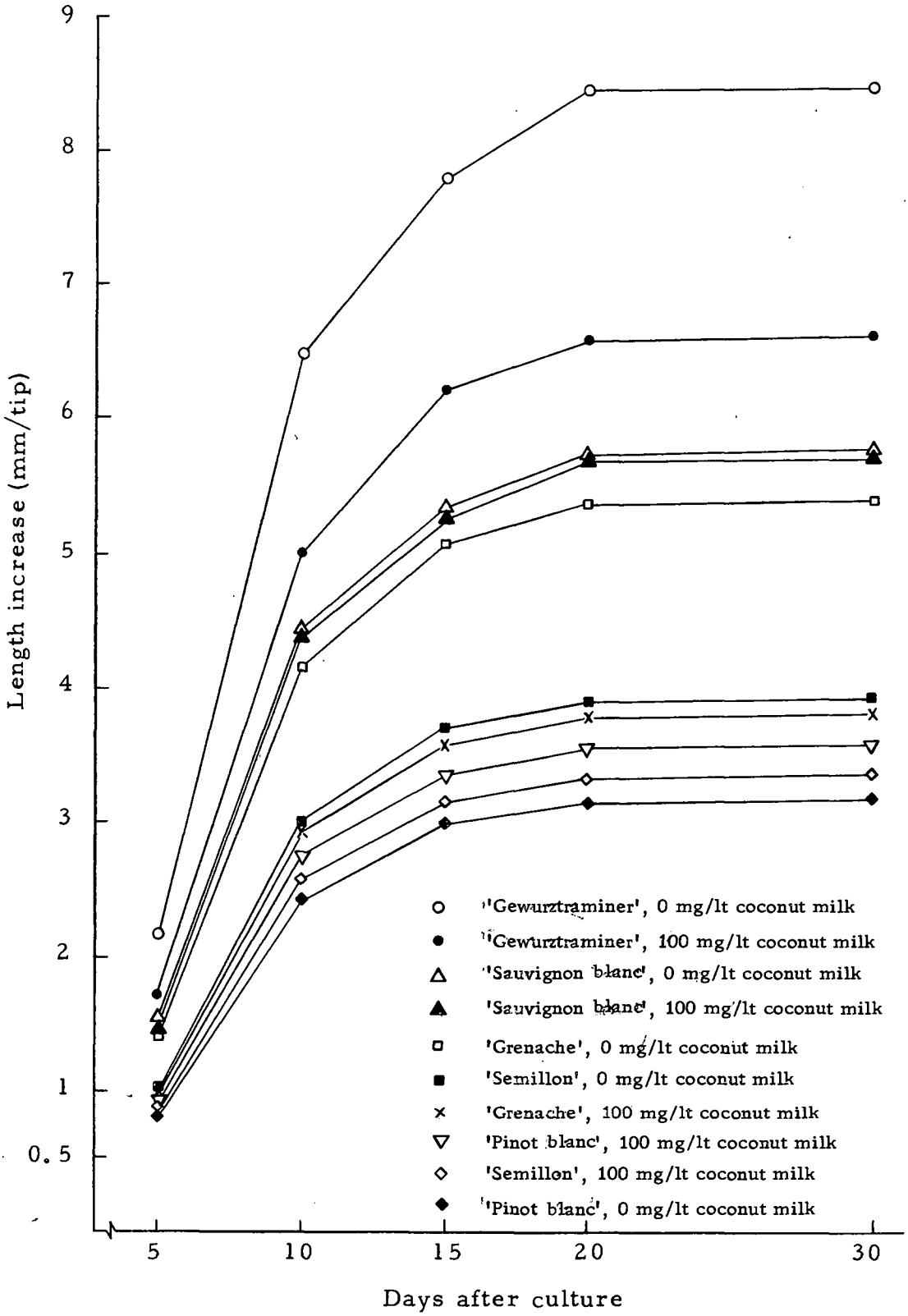

Figure 2. The effect of coconut milk on the growth of the five cultivars of vinifera grape.

differences among varieties and levels of coconut milk at the 1% probability level. The interaction effect was also significant but at the 5% probability level.

The cultivar Gewurztraminer grew more vigorously than other varieties. The cultivar Pinot blanc grew much less vigorously and made less than half the growth of 'Gewurztraminer'. The addition of coconut milk retarded the growth of the stem tips in all cultivars except 'Pinot blanc'. With 'Grenache' and 'Gewurtztraminer' the reductions were significant at . 01 probability level.

Figure 2 presents the growth rate of the stem tips as affected by the various treatments. They grew rapidly during the five to ten day period and seemed to decline after 15 days of culture. Almost no growth was observed from such tips after 20 days of culture.

Experiment III. The Effect of NAA and Kinetin Applied on Stem Tips of Grape, Cultivar Grenache in Vitro

'Grenache' is one of a number of wine grape cultivars being considered for growth in Oregon. It was selected for this experiment because of its vigorous growth in the greenhouse.

Materials and Methods

Forty-two tips were taken from healthy 'Grenache' grown in the greenhouse. They were cultured on sterilized medium as used by

Murashige and Skoog (1962) supplemented with 18 mg/lt glycine in shell vials containing 10 cc's of nutrient. The medium was sterilized by autoclaving at 15-20 psi for 20 minutes.

All treatments were arranged in 2 x 3 factorial experiment in a completely randomized design with seven replications of one tip each. Two levels of auxin, NAA, at 0 and 0.1 mg/lt of nutrient, and three levels of kinetin, 0, 0.1, 1 mg/lt of nutrient were added to the original media. All shell vials were placed under 16 hour day length of artificial light with light intensity of 500 ft-candle. The ambient temperature of the control room was held at 68-70°F.

At five day intervals, five tips from each group were removed aseptically from the shell vials and the length of each determined. Following the measurements, the tips were returned to the same shell vial.

Results

The results of this experiment are presented in Tables 4 and 5. Table 4 shows the average increase of the length of tips of all treatments but no significant differences were observed among them. However, the highest rate of NAA and kinetin (0.1 mg/lt of NAA and 1 mg/lt of kinetin) appeared to be detrimental to the growth of the stem tips. The control treatment (0 mg/lt NAA and 0 mg/lt kinetin) gave the best result among all treatments.

Table 4. The effect of NAA and kinetin on stem tips of the grape cultivar Grenache.

NAA (mg/lt)	Kinetin (mg/lt)	No. of Tips	Initial Length (mm/tip)	Length Increase (mm/tip)
0	0	7	2.5	5.5
0	0.1	7	2.4	4.3
0	1	7	2.3	3.9
0.1	0	7	2.3	4.7
0.1	0.1	7	2.4	4.0
0.1	1	7	2.5	3.3
L.S.D. .05				N.S.D.
L.S.D. .01				N.S.D.

Table 5. Main effects of NAA and kinetin on stem tips of 'Grenache'.

Source	Level	Average Length Increase (mm)
NAA	0	4.6
	0.1	4.0
L.S.D. .05		N.S.D.
L.S.D. .01		N.S.D.
Kinetin	0	5.1
	0.1	4.2
	1	3.60
L.S.D. .05		1.1
L.S.D. .01		N.S.D.

The effect of interaction (NAA x Kinetin) is not significant.

Table 5 shows the main effect on the length of the stem tips. There were significant differences at 5% probability levels among different levels of kinetin but there were no significant differences on the effect of NAA. The effect of interaction (NAA x kinetin) was also not significant. The addition of kinetin reduced the growth of the stem tips.

Figure 3 presents the average increase of the length of stem tips of all treatments at four different intervals from culturing date. The growth rate of all treatments increased rapidly at the five to ten day periods and seemed to decline after 15 days of culture.

Experiment IV. Comparison of Two Methods of Applying IBA at the Second Subculture of Stem Tips of Five Cultivars of Vinifera Grapes, Grown In Vitro

IBA (indolebutyric acid) has long been known as an effective rooting hormone. It is less subject to inactivation by light and high temperature than IAA (indoleacetic acid).

This experiment was carried on with plant material from the second experiment for the purpose of obtaining rooted plantlets.

Materials and Methods

After four weeks, all tips of five cultivars used in the second experiment, were transferred to other fresh media. Two methods were adopted to continue culture of the tips.

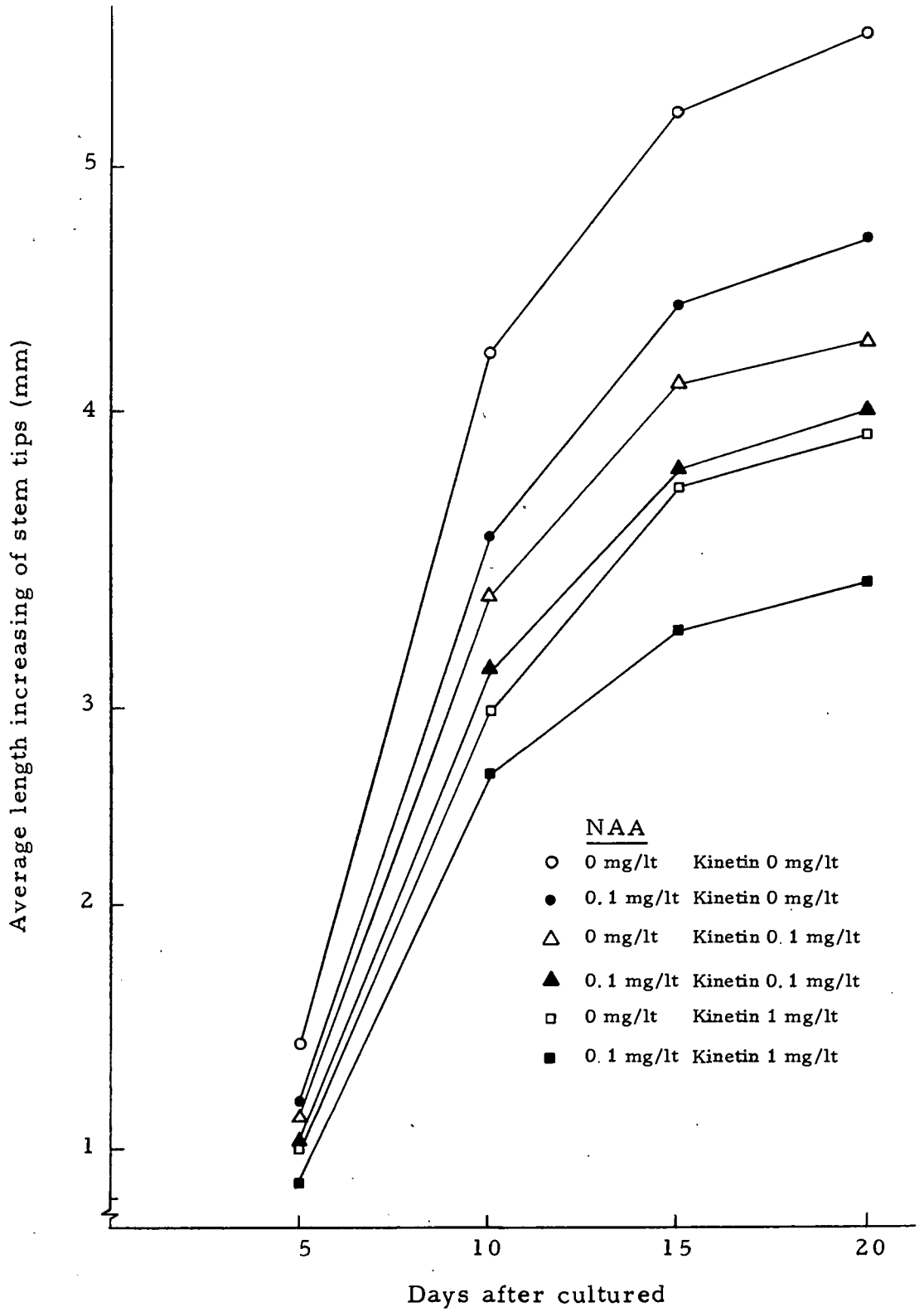

Figure 3. The effect of NAA and kinetin on 'Grenache' shoot tips.

Method I. Cultures were started on Murashige and Skoog medium (1962) for tobacco callus culture supplemented with 18 mg/lt glycine, 0.1 mg of IBA (indolebutyric acid) and 0.4% of bacto agar. The tips were grown in this medium for four weeks. Some tips initiated roots, then were transferred to a medium of one-half concentration of macro inorganic nutrient plus the full concentration of micronutrient and organic nutrient of Murashige and Skoog medium (1962), supplemented with 18 mg/lt glycine but without IBA and agar. The unrooted tips were transferred to the original medium, without IBA and agar.

Method II. The tips were treated with IBA before being cultured on the medium without coconut milk, described in the second experiment. This involved dipping the basal 2-3 mm of the tips in a 10 ppm aqueous IBA solution for ten minutes under sterile conditions. After four weeks, the rooted and unrooted tips were sub-cultured on the media, described in Method I.

When the rooted tips, cultured from Methods I and II, had grown vigorously and developed abundant roots, they were transferred to small pots, kept in a control room under 16 hour day length of 500 ft-candle artificial light, as described in Experiment I. The plants were protected for four-five weeks with plastic bags. The pots used were small waxy cups 6 cm tall and 4 cm rim diameter filled with Perlite. A drainage hole was punched at the base. The tips were

treated every evening with liquid Murashige and Skoog medium using the 1/2 macronutrient modification minus sugar. When 6-9 cm tall, the plants were transferred to 6" pots containing 1:1:1:1 vermiculite, Perlite, peat and sand. The potted plants thus developed from stem tips were then cared for routinely as small potted plants.

Results

The results of this experiment are presented in Tables 6, 7, 8, and 9. Table 6 shows that the excised tips from 'Grenache', 'Sauvignon blanc', 'Semillon' and 'Pinot blanc', when treated with 0.1 mg/lt IBA, rooted 20, 30, 40 and 10 percent respectively. Those treated by dipping the basal 2-3 mm of the shoot in a ten ppm aqueous IBA for ten minutes rooted 0, 10, 20 and 10 percent respectively. Ten percent of the tips from 'Gewurztraminer', dipped with ten ppm aqueous IBA for ten minutes, initiated roots but in the other treatment method no roots occurred when tips were left in Murashige-Skoog medium containing 0.1 mg/lt IBA.

The tips from 'Semillon', treated with 0.1 mg/lt IBA rooted better than other varieties. The highest percentage of the tips surviving in both treatments were the tips of 'Sauvignon blanc'. Some tips from cultivars, 'Gewurztraminer' and 'Pinot blanc' developed small roots but the plants failed to survive.

Table 7 shows that the tips with callus at the cut end grew

Table 6. Influence of IBA on rooting and development of excised tips of five cultivars of vinifera grape.

Cultivars	Level of IBA (mg/lt)	No. of Tips Cut	Tips Rooting			Tips Surviving		
			No.	%**	Avg.	No.	%***	Avg.
Grenache	0.1	10	2	20		1	50	
	10*	10	0	0	10	0	0	25
Sauvignon blanc	0.1	10	3	30		3	100	
	10*	10	1	10	20	1	100	100
Semillon	0.1	10	4	40		1	25	
	10*	10	2	20	30	0	0	12.5
Gewurztraminer	0.1	10	0	0		0	0	
	10*	10	1	10	5	0	0	0
Pinot blanc	0.1	10	1	10		0	0	
	10*	10	1	10	10	0	0	0

*Dip of basal 2-3 mm in aqueous IBA for ten minutes and return to fresh medium of Murashige and Skoog (1962). Supplemented with 18 mg/lt glycine.

**Percentage rooting based on tips rooting/tips cut.

***Percentage surviving based on tips surviving/tips rooting.

Table 7. Growth comparison of callus and non-callused tips after two weeks on agar medium.

Treatment	Length Increase (mm/tip)
Tips with callus	3.3
Tips without callus	0.8
L.S.D. .01	1.0
L.S.D. .05	0.6

Table 8. Influence of IBA treatments on rooting and development of excised tips of five cultivars of vinifera grape (statistical procedure).

Treatments	Root Initiation (Percentage)	Survivors (Percentage)
0.1 mg/lt IBA	20	35
10 mg/lt IBA*	10	20
L.S.D. .01	N.S.D.	N.S.D.
L.S.D. .05	9.2	N.S.D.

*Dipping the basal 2-3 mm of the shoot in a 10 ppm aqueous IBA for ten minutes.

Table 9. Growth comparison of rooted and unrooted tips after two weeks in fresh medium.

Treatments	Length Increase (mm/tip/day)
Rooted plants	0.8
Unrooted plants	0

Table 10. Simple correlation coefficient between top growth and tip rooting of five grape cultivars.

Cultivars	Average Top Growth (mm)	Percentage Tip Rooting
Grenache	5.0	10
Sauvignon blanc	5.7	20
Semillon	3.6	30
Gewurztraminer	7.5	5
Pinot blanc	3.4	10

$r = -.4995$

$r\ .05 = .878$ N.S. $\qquad r\ .01 = .959$ N.S.

better than the tips without callus at the cut end. The difference was statistically significant at the 1% probability level.

Table 8 shows that the tips treated with 0.1 mg/lt IBA initiated roots proportionally better than those treated with 10 mg/lt IBA. The difference was statistically significant at the 5% probability level. No significant difference was observed on the percentage of the surviving tips of either treatment after they successfully initiated roots.

When the rooted plants were transferred to the fresh medium on the filter paper bridges, they grew at an average rate of 0.82 mm/day. Essentially no growth was observed on the unrooted tips after transferring to the fresh medium (Table 9).

DISCUSSION

The present investigation is concerned with developing techniques of culturing small (2-5 mm) apical bud segments of vinifera grape cultivars. Many investigators have tried to establish the ideal medium for all tissues cultured in vitro. The review of literature indicates that this has not yet been achieved.

Various tissues respond differently to the same medium. The differences have been attributed by Butenko (1968) as mainly due to the organic substances added to the base medium. The differences that relate to the diversity of family, genera and species as well as the complexities of genetic background would appear also to be factors of considerable importance.

The experiments reported have involved only five cultivars of Vitis vinifera, which limits diversity. The tissue selected was from (2-5 mm) sections of apical bud tissue to limit possible differences in organic substances that may develop with physiological aging.

For methods of culture employing both liquid and solidified agar media, the widely used medium developed by Murashige and Skoog (1962) for culture of tobacco callus was used with several modifications. Individual experiments were directed at developing workable techniques and modifications of the basic medium that would permit evaluations of apical growth, callus, root development and ultimately result

in the successful development of complete plants.

Statistical analyses were made of some of the experiments but in general these were made on quite limited observations due to insufficient quantities of material for desired replications.

Light source, photoperiod, temperature and tissue employed were similar for all experiments. Variables were related to cultivars, media modifications and accessory substances.

Experiment I established that excised apical buds of the cultivar, 'Grenache', can make extension growth on the Murashige-Skoog (1962) medium for tobacco callus culture with or without glycine additions. The data show that more extension growth developed in the presence of glycine and that the higher concentration of glycine gave the most growth response. The statistical analysis does not show that the observed differences were significant. This is attributed to the effect of considerable variations in growth that result from a high degree of non-uniformity in size within the 2-5 mm range.

These results were similar to the work reported by Loo (1945) with the stem tips of asparagus; Elliott (1969) with sweet potato; and Stone (1963) with carnation meristem tips. Street (1969) in his review concluded that glycine was not essential to the growth of most excised root clones. However, the present experiments with grape benefited somewhat from the presence of 20 mg/lt glycine, therefore later experiments were carried on by supplementing this substance to the

basic medium.

The growth curves plotted in Figure 1 show that growth is rapid for the first five to ten days but that it becomes limited after 15 days and nearly ceases at 20 days. This suggests that some factor or factors affecting growth have become limited in this time period. No roots were developed on material used in this experiment, indicating that the tissue was unable to synthesize substances necessary for the initiation and development of roots. This may be attributed to lack of suitable substrate, environment, or time.

The addition of coconut milk (Experiment II) at 100 mg/lt or kinetin (Experiment III) at 1 mg/lt to the basic medium restricted the growth of the excised stem tips (Tables 3 and 5). The reduction of growth attributed to the coconut milk was significant at the 1% probability level, that of kinetin at the 5% probability level. The interaction effect of cultivars x coconut milk was significant at the 5% probability level.

The effects of these materials might suggest that such tips already had cytokinins at the optimum level before they were excised from the intact plant. The applied kinetin may have resulted in tissue levels of these materials in excess of that required for normal growth and thus caused a detrimental effect to the tips. The detrimental effect of kinetin was previously reported by Norris (1954), Elliot (1969) and Mellor (1969) on excised potato buds.

Addition of another accessory substance, NAA, had no effect in stimulating the growth of the stem tips. The data do show that there is a slight reduction in growth of the tips but the differences were not statistically significant at the 5% probability level. Effects similar to this were reported for excised potato buds (Mellor, 1969). An explanation similar to that described for the cytokinins to account for growth reductions may be applicable. In this case NAA may be over-balancing endogenous auxin levels. NAA obviously is not necessary to the growth of grape stem tips.

An examination of Figures 2 and 3, which relate growth rate to time, show that stem tip growth increased rapidly during the first five to ten day period and declined after 15 days of culturing. Limitations for growth apparently are inflicted by the environment or due to the inability of the tissue to synthesize components vital to growth.

Growth rates of different cultivars are shown in Table 3. 'Gewurtztraminer' grew more vigorously than all other cultivars and was 2.24 times greater than that of 'Pinot blanc', the slowest growing cultivar. This very likely is due to a particularly good adaptation of tips of this cultivar to the special environment.

In an attempt to cause root development and develop complete plants, the tips from Experiment II were transferred to agar medium supplemented with either IBA 0.1 mg/lt or treated by dipping the basal 2-3 mm of the shoot in a 10 ppm aqueous IBA for ten minutes

prior to placing in the Murashige and Skoog (1962) medium for tobacco callus culture. Some tips from both treatments produced callus masses at the cut end. Such tips continued growth far better than those without callus masses at the cut end for a short period of time. Growth of the tips that failed to produce callus masses at the cut end was very little.

It has been suggested that some essential substances in the medium might be absorbed through the callus proliferation and upward to the shoot apices. According to Ball (1946), tips without callus masses at the cut end fail to supply these essential substances to the meristematic region.

During a two to four week period of culturing, some tips with or without callus masses at the cut end initiated roots. By the end of the fourth week the rooted tips were transferred to modified Murashige and Skoog medium in which the concentration of macronutrients were diluted by half. The tips grew rapidly at the average rate of 0.82 mm/tip/day during the two week period of culturing. The unrooted tips, which did or did not produce callus masses at the cut end, were also transferred to the fresh medium of Murashige and Skoog (1962) to bring about rooting and survival. These tips showed no sign of further development on the fresh medium and eventually died. Similar results have been explained by DeRopp (1946), Clowes (1961), and Street (1969) as probably due to the ability of roots to synthesize and

transport from the medium to the shoot meristem certain substances necessary for shoot development. It appeared from observations made of the present work with grape that physiological aging represented by generations and maturation of cells, coupled with environmental limitations occurring at critical times through subculture transfers were also contributing factors to growth and survival.

There were no correlations between the length of the shoot tips and their abilities to initiate roots at the 5% probability level (Table 10, Figure 4). This suggests that the root initiation was probably due to certain physiological changes within the cells near vascular tissues which were stimulated by the exogenous IBA regardless of the length of the tips, rather than any effect of length.

Table 6 shows that the cultivar differences were the main factors influencing tip rooting and tips surviving. However within the same cultivar, few tips initiated roots and gave rise to plantlets. The results are very likely due to critical sensitivities related to the physiological stage of development of the tips and their particular biochemical status. Confirmation of such a hypothesis would require a major research effort.

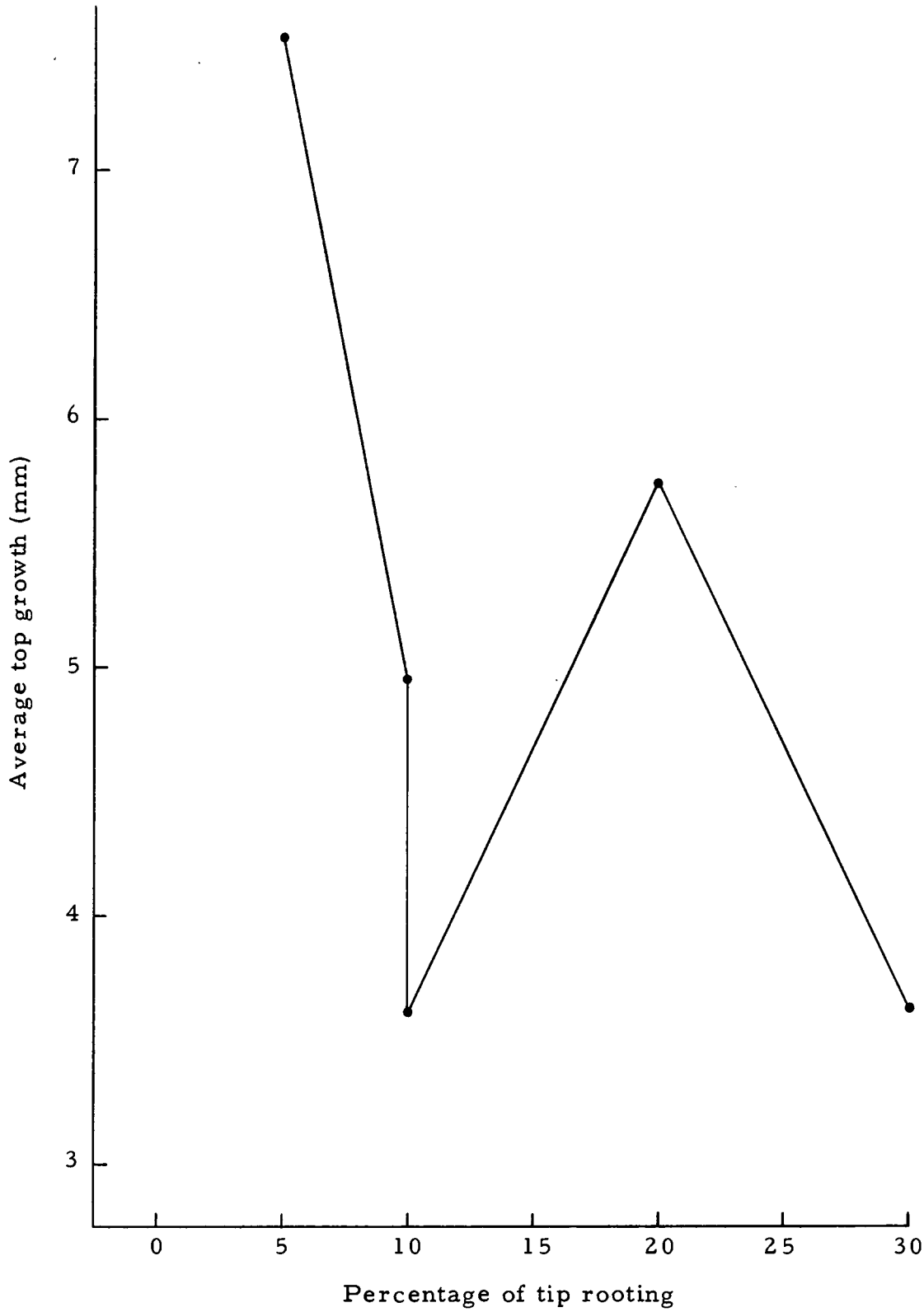

Figure 4. The average of top growth in relation to the percentage of tips rooting.

CONCLUSIONS AND SUMMARY

The present study was successful in accomplishing the major objective, that of producing complete plants from 2-5 mm segments of apical stem tissue of vinifera grape cultivars. The segments were first induced to elongate on Murashige-Skoog (1962) medium modified with additional glycine. Elongated tissue was then induced to develop roots by treatment with IBA. Developed plants were subsequently potted off and grown into mature vines in standard greenhouse culture.

Although the percentage of successes and surviving plants was rather small, the results clearly indicate that cultivars of vinifera can be developed in this way. The technique coupled with heat therapy could be important to the development of virus free stock from infected material.

The results and observations derived from the various tests indicate that limitations relate to the linear size of the tissue segment, the particular medium, the cultivar, and the environment.

The results obtained are summarized as follows:

1. Statistically, the amino acid, glycine, added to the basal medium was not essential to the growth of the stem tips of grape, but it did have some minor effect on the growth rate of the tips.
2. Auxins and cytokinins such as NAA, kinetin and coconut

milk were more detrimental than beneficial to the excised grape tips, at least in the early culture. But the effect of NAA was not statistically significant at the 5% probability level.

3. The tips, regardless of their size, cultivar differences, and various treatments, grew rapidly at the first five to ten days and seemed to decline after 15 days of culturing.
4. Tips with callus at the cut end grew better than those without callus at the cut end.
5. The auxin, IBA, was necessary for rooting of tips at the second subculture. The tips supplemented with 0.1 mg/lt IBA in the agar medium initiated roots better than the tips dipped with 10 ppm aqueous IBA for ten minutes prior to placement on the medium.
6. The amount of growth extension of tips was not correlated with the percent of tips rooting.
7. The cultivar was a principal influencing factor in differences of growth, rooting, survival and development of apical stem tips.

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