TISSUE CULTURE STUDIES ON THE GENUS *ROSA*

WITH SPECIAL REFERENCE TO THE SHOOT TIP

by

GERARD JACOBS

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(Abstract)

A modified Berthelot (1934)-Knop (1865) solution was found to support the growth of rose pith segments while Murashige and Skoog's (1962) medium did not. Possibly the NH_4^+ content of the latter medium is toxic to rose tissue. The best callus growth was obtained with indolebutyric acid (IBA), which was superior to naphthaleneacetic acid (NAA), indoleacetic acid (IAA) and 2,4 dichlorophenoxybutyric acid (2,4-DB) at the concentrations tested, while gibberellic acid (GA) greatly enhanced callus formation in the presence of both IBA and kinetin. Occasional differentiation of shoots was observed both in cultured pith segments and in callus formed at the basal cut surface of the shoot tips. However, the precise culture conditions required for differentiating rose pith and callus tissue remain unknown.

An interaction was found between NAA and kinetin with regard to root and leaf development in shoot tips. Root formation took place only in the absence of kinetin and in the NAA range of 0.5 to 2.0 mg/l, while normal leaf development occurred only in the absence of NAA and in the presence of 4.0 to 18.0 mg/l kinetin. Neither any combination of NAA and kinetin nor the sequential application of growth substances induced both root and shoot growth. Furthermore, shoot tips sampled in late summer formed roots much more readily than tips sampled in late winter.

GA reduced the favourable effect of high kinetin treatments on leaf development. Different species of auxin affected growth of the shoot tip in different ways. IAA did not inhibit growth of the shoot tip in the same manner as was observed for NAA, IBA and 2,4 dichlorophenoxybutyric acid (2,4-D).

It is concluded that further experimentation with different ratios of various species of auxin and cytokinin, as well as the sequential administration of growth substances, may lead to the successful culture of intact plantlets from rose shoot tips and shoot apical meristems, and ultimately to possibly virus-free rose plants.

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INTRODUCTION

Most plants are susceptible to virus infection and it is of major concern to-day that virus-free parent material be established, particularly in respect of those plants which are propagated vegetatively. Morel and Martin (1955) introduced aseptic shoot meristem culture as a means of producing virus-free plants of dahlia and dianthus. This method has subsequently been used to eliminate virus from Chrysanthemum sp. (Holmes, 1956), Dianthus barbatus (Stone, 1968), Dianthus caryophyllus (Quak, 1957; Van Os, 1964; Stone, 1968), Fragaria vesca (Belkengren and Miller, 1962; Miller and Belkengren, 1963; Vine, 1968), Freesia sp. (Brants and Vermeulen, 1965; Brants, 1968), Hyacinthus orientalis (Van Slogteren, 1966), Ipomaea batatas (Nielson, 1960), Iris sp. (Baruch and Quak, 1966), Ribes sp. (Jones and Vine, 1968), Rheum rhaponticum (Walkey, 1968) and Solanum tuberosum (Kassanis, 1957; Manzer, 1959; Quak, 1961; Stace-Smith and Mellor, 1968).

The use of shoot tip culture is particularly appropriate since it appears that meristem tissues may be resistant to virus multiplication (Schneider, 1965). Bawden (1959) is of the opinion that meristematic cells are physiologically capable of either precluding virus infection or, at most, of supporting virus multiplication poorly. Many viruses, if they succeed in penetrating apical meristems, fail to survive. This virus-meristem relation is however a variable one (Esau, 1967). Bennett (1956) and Smith and McWhorter (1957) have reported the presence of virus in apical meristems, in which their behaviour appears to be controlled by metabolic factors (Esau, 1967).

In some instances production of virus-free plant material by shoot meristem culture has reached the commercial stage. The Plant Protection Service, Wageningen, Holland carries out production of virus-free carnations by this means on a large scale (Hakkaart, 1963). In Australia the King Edward cultivar of potato is propagated under a certification scheme, making use of this technique (Bawden, 1967). In South Africa the production of cut rose flowers has become of some importance in the past few years and, with improved facilities for air freight overseas, will possibly become very important in the future. However, a large proportion of the rose bushes in South Africa is infected with one or more of the following viruses: streak, die-back, ring spot, rosette, mosaic and wilt (Klesser, 1967; 1968). The consequences, in some instances, are early death or low productivity of such infected bushes.

It was therefore decided to initiate a research project on the culture of rose shoot tips, initially to find a suitable medium on which to grow shoot tips and shoot apical meristems, and ultimately to attempt production of virus-free plants.

Since callus tissues of different plant species have been successfully differentiated into roots and shoots in a number of cases (Skoog and Miller, 1957; Vasil and Hildebrandt, 1966 b, c; Chen and Galston, 1967; Hill, 1968; Nishi, Yamada and Takahashi, 1968; Winton, 1968; Wolter, 1968) it was considered important to include rose pith tissue in the tissue culture experiments. Information gained from these cultures on the nutritional and hormonal control of growth could then be applied to shoot tip cultures of rose. Although Hollings (1965) states that very little of the

information obtained from pith and callus cultures is applicable to shoot meristem cultures, this cannot be accepted as a general rule.

If success can be obtained with the differentiation of callus tissue derived from pith of rose shoots, then this may also apply to callus derived from the base of shoot meristems. These calli derived from shoot meristems should stand the same chance of being virus-free as the apical meristem itself. Furthermore, these calli can be sub-divided and many rose plants could possibly be regenerated from one single shoot meristem as is done with orchids (Wimber, 1963; Morel, 1964; Scully, 1967).

In this study, particular attention was devoted to the effects of different ratios, and the sequential application of growth substances on growth and differentiation of rose tissue cultured in vitro. The main objective of this work was to gain basic knowledge on the behaviour of rose tissue in culture and also to establish a basis for future study.

1. STANDARD PROCEDURE

1.1. PREPARATION OF STOCK SOLUTIONS AND CULTURE MEDIA

Stock solutions of Murashige and Skoog's (1962) medium (Table 1) were prepared as outlined by Vasil and Hildebrandt (1966a). Stock solutions of Knop's (1865) macro-nutrient solution (half strength) and a modified Berthelot (1934) micro-nutrient solution were prepared as outlined by White (1954), except for the following alterations to the Berthelot solution. Firstly, $Na_2MoO_4 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$ and $CoCl_2 \cdot 6H_2O$ were substituted for MoO_3, $NiSO_4$ and $CoCl_3$, respectively, since these latter salts were not available. Secondly, a more stable Fe-EDTA chelate solution (Murashige and Skoog, 1962) was substituted for $Fe_2(SO_4)_3$ as a source of iron. Thirdly, the H_2SO_4, $Ti(SO_4)_3$ and $BeSO_4$ were omitted; the first because of its effect in depressing the pH, while the other two were not considered necessary. Fourthly, instead of all the ingredients being incorporated into one stock solution, they were divided into two groups viz. solutions V and VI (Table 2), to reduce the possibility of chemical interactions.

All the stock solutions, except Fe-EDTA were prepared by dissolving the salts in the required volume of distilled water. The Fe-EDTA solution of Murashige and Skoog (1962) was prepared as outlined by Vasil and Hildebrandt (1966a).

The auxin and GA solutions were prepared by dissolving them in a few drops of ethanol before making up to volume with distilled water. Kinetin (6 furfurylaminopurine) solutions were prepared by autoclaving the kinetin for 15 minutes at 15 lb. pressure in ca. 250 ml distilled water. After cooling the kinetin solutions were made up to volume with distilled water.

TABLE 1 Murashige and Skoog's (1962) stock solutions for the *in vitro* culture of Nicotiana pith, prepared as outlined by Vasil and Hildebrandt (1966a).

Stock Solution Number	Grams per litre	Constituents
I	82.5	NH_4NO_3
II	95.0	KNO_3
III	1.24	H_3BO_3
	34.0	KH_2PO_4
	0.166	KI
	0.05	$Na_2MoO_4 \cdot 2H_2O$
	0.005	$CoCl_2 \cdot 6H_2O$
IV	88.0	$CaCl_2 \cdot 2H_2O$
V	74.0	$MgSO_4 \cdot 7H_2O$
	4.46	$MnSO_4 \cdot 4H_2O$
	1.72	$ZnSO_4 \cdot 7H_2O$
	0.005	$CuSO_4 \cdot 5H_2O$
VI	7.45	Na_2EDTA
	5.57	$FeSO_4 \cdot 7H_2O$
VII	0.02	Thiamin.HCl
	0.1	Nicotinic acid
	0.1	Pyridoxin.HCl
	0.4	Glycine

TABLE 2 Stock solutions for the in vitro culture of Rosa pith

Stock Solution Number	Grams per litre	Constituents
I	144.0	$Ca(NO_3)_2.4H_2O$
II	25.0	KNO_3
III	51.25	$MgSO_4.7H_2O$
IV	25.0	KH_2PO_4
V	2.958	$MnSO_4.4H_2O$
	0.1448	$ZnSO_4.4H_2O$
	0.078	$CuSO_4.5H_2O$
VI	0.000252	$Na_2MoO_4.2H_2O$
	0.07	$CoCl_2.6H_2O$
	0.1	H_3BO_3
	0.5	KI
	0.0767	$NiCl_2.6H_2O$
VII	7.45	Na_2EDTA
	5.57	$FeSO_4.7H_2O$
VIII	0.02	Thiamin.HCl
	0.1	Nicotinic acid
	0.1	Pyridoxin.HCl
	0.4	Glycine

All the nutrient stock solutions were kept in dark bottles and stored at room temperature (± 22°C). The vitamin and hormone stock solutions were either prepared fresh or stored at 7°C for not more than 2 weeks. The auxin, IAA, was always prepared immediately before use.

In order to obtain the complete and balanced culture medium the stock solutions were combined in the correct proportions (Table 3). The concentrations of the different elements in the two culture media are given in Table 4.

To these solutions, consisting of a mixture of stock solutions, the following supplements were added: (a) for tobacco : sucrose 20 g/l; myo-inositol 100 mg/l; plus the appropriate growth regulators; and (b) for rose : sucrose 10 g/l; glucose 10 g/l; myo-inositol 100 mg/l; cysteine 12 mg/l; ascorbic acid 0.1 mg/l; plus the appropriate growth regulating substances. The pH of the medium was then adjusted to between 5.4 and 5.8 for tobacco, and 4.8 to 5.1 for rose with either HCl or NaOH solutions.

The culture media were sterilized by autoclaving the flasks or test tubes containing an aliquot of the medium at 15 to 18 lb. pressure for 15 minutes.

1.2. PREPARATION OF PLANT TISSUE

1.2.1. Pith Explants

Shoots of Nicotiana tabacum and watershoots of a hybrid tea rose cv. Superstar were cut into pieces four centimetres long and soaked in a 0.35 per cent sodium hypochlorite solution for 12 to 15 minutes. After rinsing twice with sterile water, one-half

TABLE 3. Culture media for Tobacco and Rose

Preparation of culture medium for Nicotiana		Preparation of culture medium for Rosa	
Stock Solution (Table 1)	Volume of stock solution in final medium ml/l	Stock Solution (Table 2)	Volume of stock solution in final medium ml/l
I	20.0	I	5.0
II	20.0	II	5.0
III	5.0	III	5.0
IV	5.0	IV	5.0
V	5.0	V	1.0
VI	5.0	VI	1.0
VII	5.0	VII	5.0
		VIII	5.0

TABLE 4. Nutrient composition of Murashige and Skoog's (1962) and the modified Berthelot-Knop solutions.

Element	Murashige and Skoog's medium for tobacco tissue culture		Modified Berthelot-Knop solutio for rose tissue culture	
	mg/l	millimoles/l	mg/l	millimoles/
N	8.4×10^{2}	6.0×10^{1}	1.02×10^{2}	7.3
K	7.82×10^{2}	2.0×10^{1}	8.39×10^{1}	2.15
Ca	1.21×10^{2}	3.0	1.20×10^{2}	3.0
Mg	3.68×10^{1}	1.5	2.53×10^{1}	1.04
S	5.23×10^{1}	1.6	3.32×10^{1}	1.04
P	3.9×10^{1}	1.25	2.85×10^{1}	9.2×10^{-1}
Cl	3.12×10^{2}	6.0	4.2×10^{-2}	1.2×10^{-3}
Na	4.6	2.0×10^{-1}	4.6	2.0×10^{-1}
Fe	5.5	1.0×10^{-1}	5.5	1.0×10^{-1}
B	1.08	1.0×10^{-1}	4.32×10^{-3}	4.0×10^{-4}
Mn	5.5	1.0×10^{-1}	7.28×10^{-1}	1.33×10^{-2}
Zn	1.92	3.0×10^{-2}	4.18×10^{-2}	6.4×10^{-4}
I	6.4×10^{-1}	5.0×10^{-3}	1.9×10^{-1}	1.5×10^{-3}
Cu	6.4×10^{-3}	1.0×10^{-4}	1.97×10^{-2}	3.1×10^{-4}
Mo	9.6×10^{-2}	1.0×10^{-3}	9.6×10^{-5}	1.0×10^{-6}
Co	6.0×10^{-3}	1.0×10^{-4}	1.65×10^{-2}	2.8×10^{-4}
Ni			1.88×10^{-2}	3.2×10^{-4}

centimetre of tissue was trimmed off either end and the remaining segment divided into three 1 cm pieces. Depending upon their diameter stem segments were then either halved or quartered after removal of the bark. The tissue segments were cultured either in 200 ml erlenmeyer flasks or in specimen tubes. Containers were stoppered with cotton wool and covered with tinfoil caps. Treatments were replicated 10 times.

Culture transfers were carried out in a transfer chamber, which was previously sterilized by wiping the working surface and spraying the atmosphere with 70 per cent ethanol, and by radiating for 30 minutes with a UV germicidal lamp. Cultures were grown at 24 $\pm$ 3°C with a 16 hour photoperiod. Initial experiments were carried out under a mixture of fluorescent and incandescent lights of ca. 3,700 lu/m^2. In later experiments gro-lux fluorescent lights (3,000 lu/m^2) were used. Visual observations of callus development were made at regular intervals.

1.2.2. Shoot Tip Explants

More than one kind of vegetative shoot apex occurs on rose shoots. Apices which develop from sprouting lateral buds on hardwood shoots are usually covered with bud scales (Type 1, Fig. 1). These apices are desirable as explants because they are normally vegetative. However, after extension growth has taken place (Type 3, Fig. 1.) these apices are no longer suitable because in many cases flower differentiation has taken place. A second type of desirable apex develops from lateral buds which sprout on softwood shoots (Type 2, Fig. 1.). Although rose shoot tips have lateral meristems which could be used, as is done with orchids (Scully, 1967), only the apical meristems were used in this study.

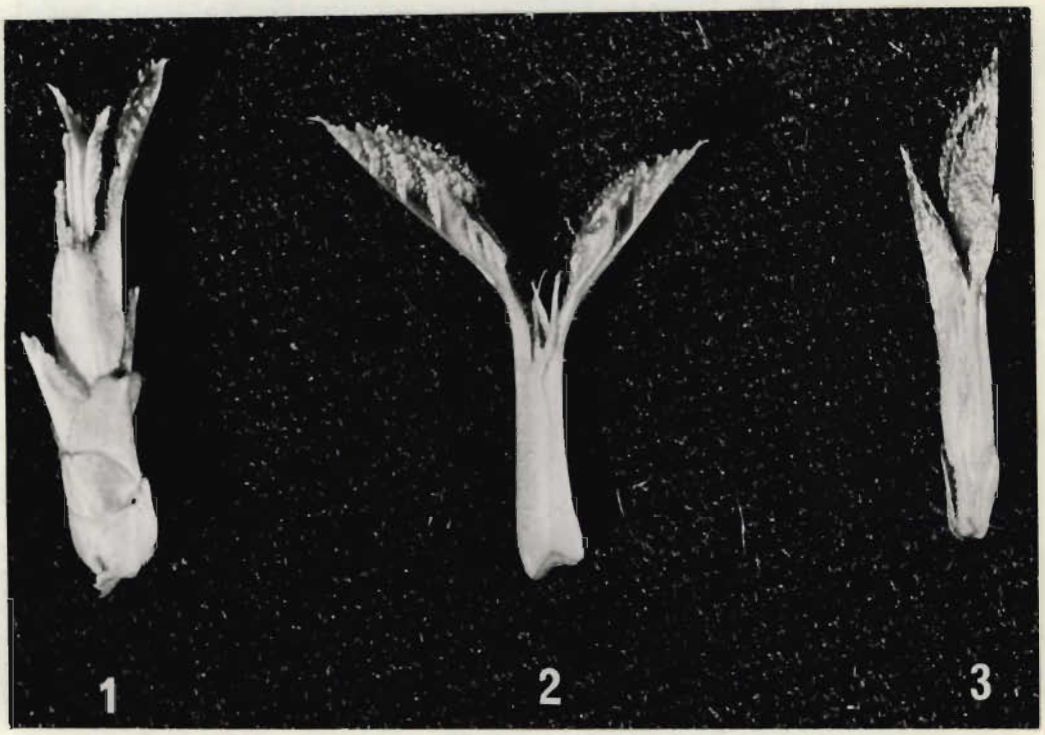

Fig. 1. Types of rose shoot apices developing from:-
1. lateral bud on hardwood, just pushing out;
2. lateral bud on softwood, just pushing out;
3. lateral bud on hardwood after extension growth has taken place.

There still exists some controversy as to exactly how much tissue at the shoot-tip has a fair chance of being free of viruses and what term should be applied to this tissue piece. The most widely used tissue piece for aseptical culture is the meristem dome plus the first pair of leaf primordia (Hollings, 1965). This tissue piece has been called meristem (Quak, 1961), shoot meristem (Sagawa, Shoji and Shoji, 1966) and meristem-tip (Hollings, 1965). To avoid confusion of these terms as well as with shoot-tip (Parke, 1959) and tip cutting and since meristem-tip could refer to either shoot or root apex, Jacobs, Bornman and Allan (1968) preferred the longer but morphologically more correct term of shoot apical meristem. This term is used in its wider sense and includes the tissue piece from immediately below the last node upwards, thus consisting of the apical initials and their derivatives, and one or two leaf primordia depending on the phyllotaxis of the shoot. When it is necessary to distinguish the most distal part of the shoot apex they proposed that the term proto-meristem (Esau, 1965) should be used, whereas the term shoot tip should be reserved for a shoot tip consisting of a shoot apical meristem and variable lengths of shoot proximal to the apex.

Shoot tips ca. 2.0 cm long were collected from the field. After removing some of the outer leaves, tips of the desired length were excised after surface sterilization with sodium hypochlorite as described for pith culture. However, in some of the later experiments surface sterilization was omitted. Shoot tips were cultured in either test-tubes or specimen tubes, with a filter paper wick (ashless, Whatman No. 40, 41 and 42) to support the tip, as describe by Heller (1953). Containers were stoppered with cotton wool and covered with tinfoil caps. Treatments were replicated 10 times.

Transfer procedures and culture conditions were as for the pith cultures. Visual observations were made at regular intervals of callus, root and leaf development. Increases in fresh weight, due mainly to callus formation, were recorded.

2. EXPERIMENTS, RESULTS AND DISCUSSIONS

2.1. PILOT EXPERIMENTS ON PITH OF NICOTIANA AND ROSA

Skoog and Miller (1957) and Murashige and Skoog (1962) developed culture media that cause differentiation of shoots and roots in tobacco callus. Consequently it was decided to repeat this work in order to become acquainted with the basic techniques involved in tissue culture. Results of this work will be referred to where applicable. The purpose of the preliminary experimentation on rose pith was to establish a culture medium suitable for growth - and possibly differentiation - of rose tissue.

2.1.1. Procedures and Results

Experiment 1 - Response to different culture media

The objects of this experiment were (1) to determine whether Murashige and Skoog's (1962) high-salt solution for tobacco pith and callus would support the growth of rose pith tissue; (2) to observe the effect of a low-salt solution (Knop, 1865; Berthelot, 1934) on the growth of tobacco and rose pith; and (3) to study the effects of different auxin : cytokinin ratios on the growth and differentiation of tobacco and rose pith cultures.

Tobacco and rose pith explants were grown separately on both the Murashige and Skoog (1962) and modified Berthelot (1934),-Knop (1865) solutions with varying ratios of the auxin, IAA, and the

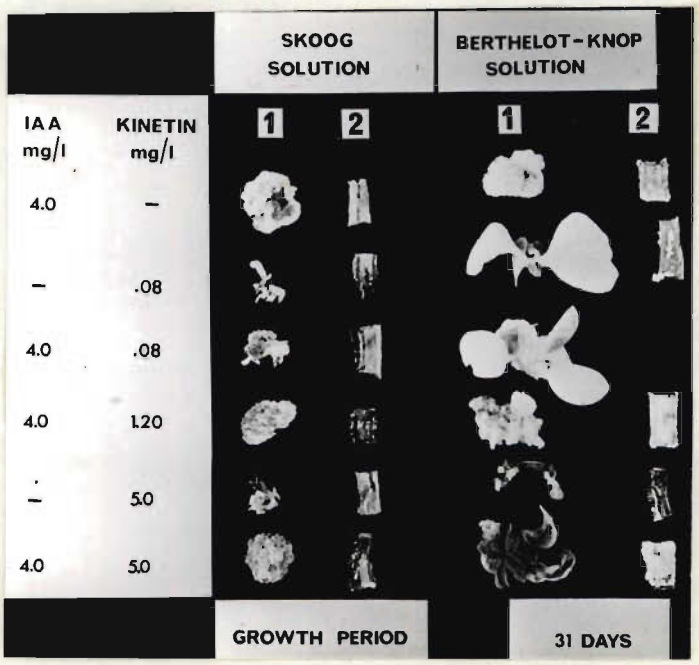

Fig. 2. Influence of two different culture media on growth of tobacco and rose pith tissue:-

1. tobacco; 2. rose.

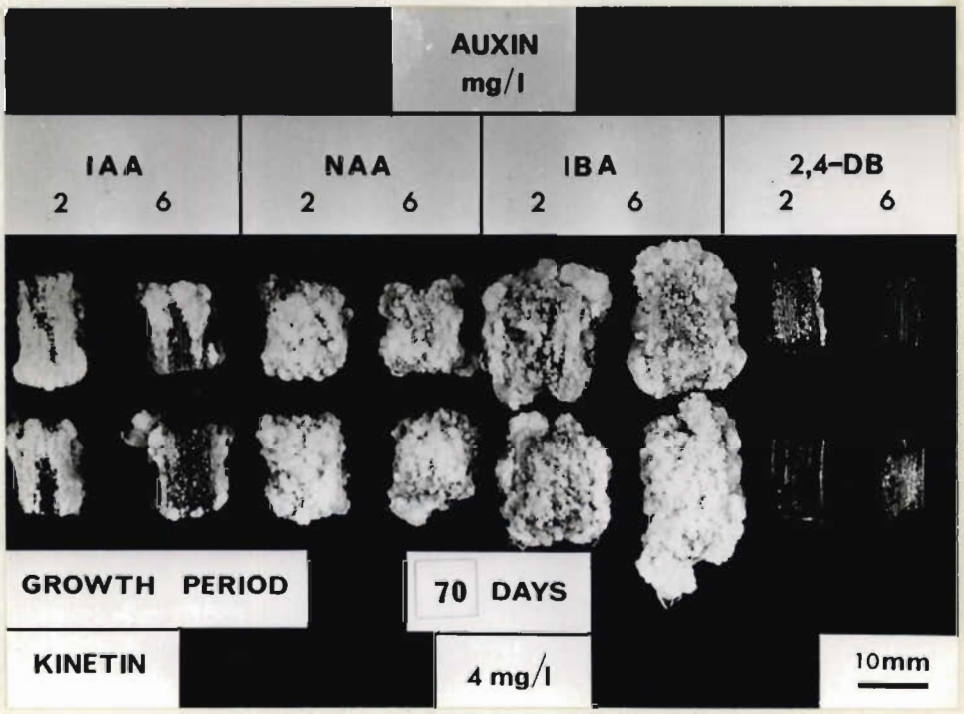

Fig. 3. Influence of different auxins on callus formation from rose pith tissue.

cytokinin, kinetin (Fig. 2). The modifications made to the standard rose medium consisted of: (1) stock solution VII, 7.25 ml/l; (2) stock solution VIII, 5.1 ml/l; (3) ascorbic acid and cysteine omitted; (4) sugars, sucrose, 20 g/l; (5) agar, 10 g/l; with the pH of the solution 4.1. The culture period was 31 days.

Figure 2 shows that tobacco pith segments generated callus tissue rapidly on both media and that differentiation took place as well. However, the rose pith segments formed virtually no callus tissue when cultured on the high salt solution of Murashige and Skoog (1962). When growth did occur the callus tended to migrate from the tissue pieces onto the agar. The less concentrated modified Berthelot-Knop solution resulted in fairly rapid callus formation of the rose tissue, but no differentiation into shoots or roots occurred even at those IAA : kinetin ratios which are normally promotive in the case of tobacco tissue. Blackening of the cells on the cut surfaces of the rose tissue segments occurred in the rose material on both media.

Experiment 2 - Effects of different auxins

The object of this experiment was to observe the effect of four different auxins (IAA, NAA, IBA and 2,4-DB) on callus formation from rose pith segments.

The rose pith explants were grown on the same medium as for Experiment 1, except that ascorbic acid and cysteine were included at concentrations given in the standard procedure. Two concentrations (2.0 and 6.0 mg/l) for each of the four auxins were employed as shown in Fig. 3. The culture period was 70 days. Best growth of callus was given by IBA, followed by NAA and IAA. With 2,4-DB only very slight growth took place after an extended period of

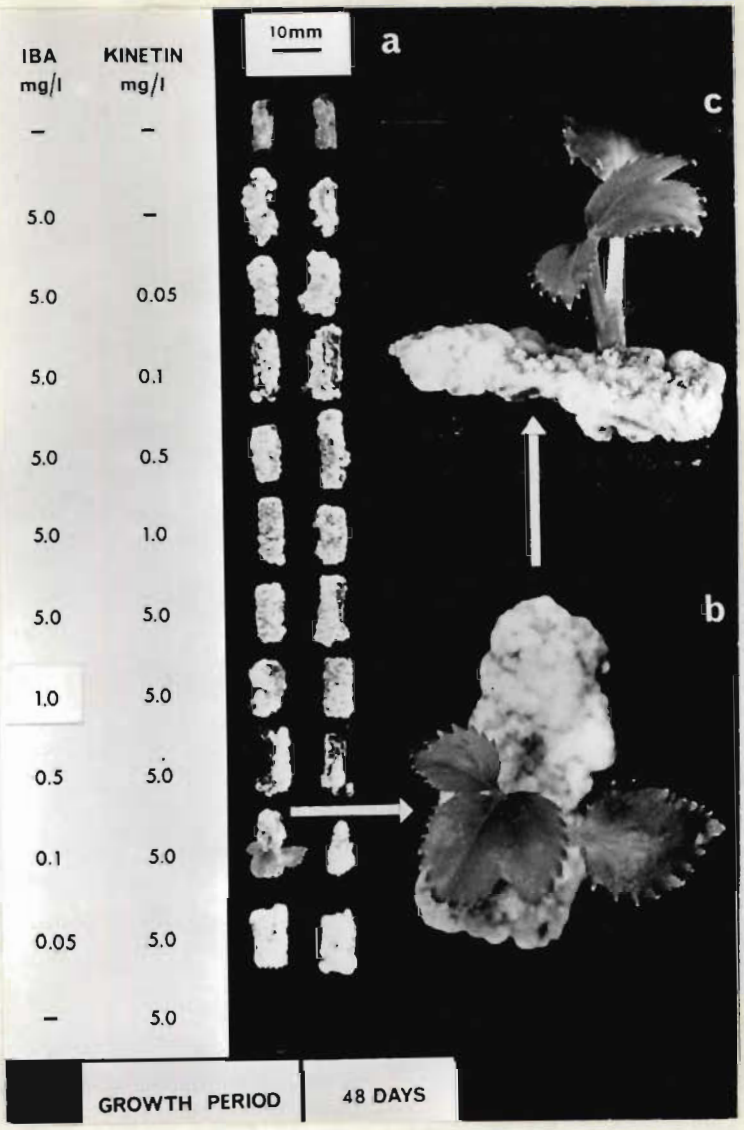

Fig. 4. (a) Influence of different IBA : Kinetin ratios on callus growth from rose pith tissue;

(b) and (c) top and side view of developing rose shoot.

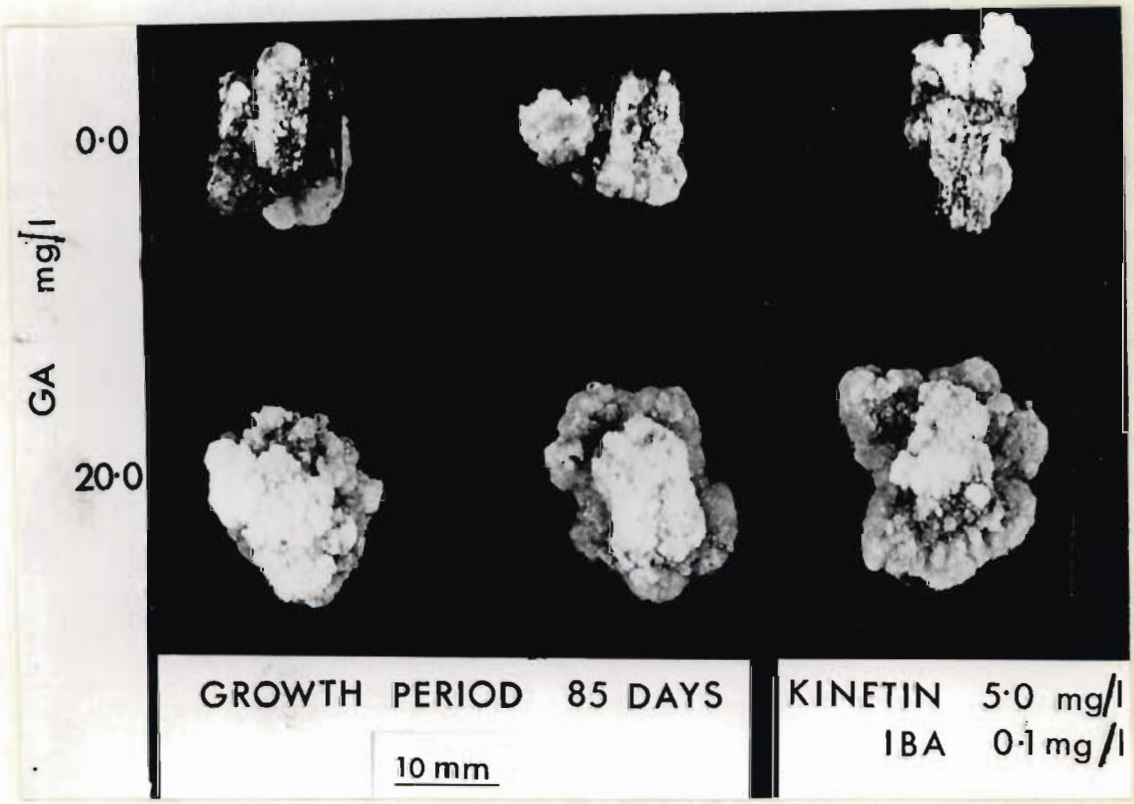

Fig.5. Influence of GA on callus formation from rose pith tissue.

inhibition (Fig. 3). There was no observable difference in the amount of callus produced between treatments receiving either low (2.0 mg/l) or high (6.0 mg/l) concentrations of any of the auxins.

Experiment 3 - Effects of varying auxin : cytokinin ratios

The object of this experiment was to determine the effects of different IBA : kinetin ratios (Fig. 4) on the growth of rose pith tissue. The same medium as for Experiment 2 was used, with a 48 day culture period.

Fairly rapid callus formation took place in all treatments except the control where growth was slow, and in the last treatment where no IBA and 5.0 mg/l kinetin completely inhibited growth (Fig. 4a). The combination 0.1 mg/l IBA plus 5.0 mg/l kinetin resulted in the differentiation of a bud primordium in one of the pith segments. A shoot developed of which the first leaf was simple, followed by a compound leaf (Fig. 4b, 4c). It was found that the bud primordium which gave rise to the shoot had been initiated in the interfasicular parenchyma between two vascular bundles of the original pith segment.

Experiment 4 - Effect of gibberellin

In this experiment the effect of GA (Fig. 5) on callus formation in rose pith in the presence of 5.0 mg/l kinetin and 0.1 mg/l IBA was observed. The standard procedure was adopted, with a culture period of 85 days. In the presence of 20.0 mg/l GA a much greater amount of callus tissue was produced as compared with callus formation in the absence of GA (Fig. 5).

2.1.2. Discussion

The poor callus formation which resulted when rose pith

segments were cultured on the high-salt solution of Murashige and Skoog (1962) could possibly be attributed to one or more of the elements being present at a toxic concentration. A similar inhibitory effect of this high-salt solution on the growth of rose tissue cv. Pink Garnett was observed by Vasil and Hildebrandt (1966a). These workers, however, did not evaluate the toxic element in this culture solution. Considering the difference between the two culture solutions, the NH_4^+, BO_3^{-3} and Cl^- ions in particular were present in far greater amounts in the Murashige and Skoog medium than in the modified Berthelot-Knop medium. Furthermore, Allan (1967) found that a five times concentrated White's (1963) medium gave good growth of rose tissue. This concentrated solution contained no NH_4^+, while the BO_3^{-3} and Cl^- content were almost the same as for Murashige and Skoog's medium. It appears thus that the NH_4^+ ion concentration is the toxic element in Murashige and Skoog's medium, which is in agreement with Nitsch's (1967) opinion. Although the modified Berthelot-Knop solution supports growth of rose tissue reasonably well it is also obvious that it may not be the best medium, and that a much better callus yield would be obtained if a culture solution could be worked out specifically for rose tissue.

The blackening of the cells which occurred on the cut surfaces of the rose tissue segments (Experiment 1) was possibly due to an enzymatic oxidation, presumably of phenolic compounds, in the surface tissue. Cysteine and ascorbate (Palmer and Roberts, 1967) were used in subsequent experiments in an attempt to counteract this oxidation reaction. Although noconclusive results were obtained it was decided to add cysteine and ascorbate to the medium for future experiments.

The lack both in vigour of callus growth as well as differentiation of rose (Experiment 1) could possibly be ascribed to the type of auxin present in the medium. The concept that different species of auxin may vary in their effectiveness in stimulating callus growth as well as organ differentiation, is well known in the field of tissue culture (Wiggans, 1954; Hay, 1962). This concept also applies to the cytokinins (Skoog et al, 1967). It is therefore not surprising that IBA appeared to be superior to IAA, NAA and 2,4-DB over the concentrations tested in regard to rose callus formation (Fig. 3). It should, however, be appreciated that the concentrations employed may not have been the optimum for any of the auxins that were under observation.

Although no differentiated growth occurred (Fig. 3), rapid callus development following on the IBA treatments suggested that with the appropriate IBA : kinetin ratio, differentiation of the tissue could be elicited (Fig. 4). Although only one culture differentiated a shoot (Fig. 4b, 4c) this is evidence that callus tissue of rose could be induced to full totipotency. The author also found occasional bud differentiation in callus tissue derived from rose shoot tips grown in the presence of completely different growth substance combinations. The bud primordia, however, failed to grow out, and this observation was confirmed by Allan (1967) (see Fig. 6).

Thus the precise conditions of culture for differentiating rose callus with certainty still remain unknown.

The growth response of rose pith tissue to GA treatments was exceptional, because generally GA application to tissue cultures of other plant species has given variable results (Murashige, 1961;

Fig. 6. Bud differentiation on callus formed at the base of a rose shoot tip culture grown on Tulecke and Nickell's (1959) medium (Allan, 1967).

Wolter and Skoog, 1966). One of the few, and possibly the only report of GA stimulating organ formation in callus cultures, was that of Hill (1967). He observed bud primordia in callus from stem cultures of the rose cv. The Doctor when cultured on a medium containing 0.5 mg/l NAA; 0.2 mg/l kinetin and 20.0 mg/l GA. The author repeated this with pith tissue from the hybrid tea rose cv. Superstar but did not observe any differentiation of bud primordia. Lack of bud differentiation may be due to varietal differences. However, the callus formed with this treatment was firm with a high chlorophyll content. The implication that GA may be involved in organ differentiation in rose warrants future research.

2.2. PILOT EXPERIMENTS ON SHOOT TIP OF ROSA MULTIFLORA

Due to the initial paucity of material from the hybrid tea rose, cv. Superstar, experimental work was commenced with shoot tips of Rosa multiflora. The objects of these experiments were to develop a standard procedure for culturing rose shoot tips, as well as to observe the manner in which the tissue behaves when cultured in vitro, and possibly to establish some trends.

2.2.1. Procedure and Results

Experiment 5 - Response to different concentrations of auxin

The object of this experiment was to observe the response of rose shoot tips 8.0 mm long to different concentrations of NAA (0.0; 0.1; 0.5; 1.0; 2.0; 4.0 mg/l). The culture solution deviated in the following ways from the standard solution:
(1) Vitamin supplement (mg/l); calcium panthonenate 1.0; pyridoxine.HCl 0.5; thiamine.HCl 0.5; nicotinic acid 0.5;

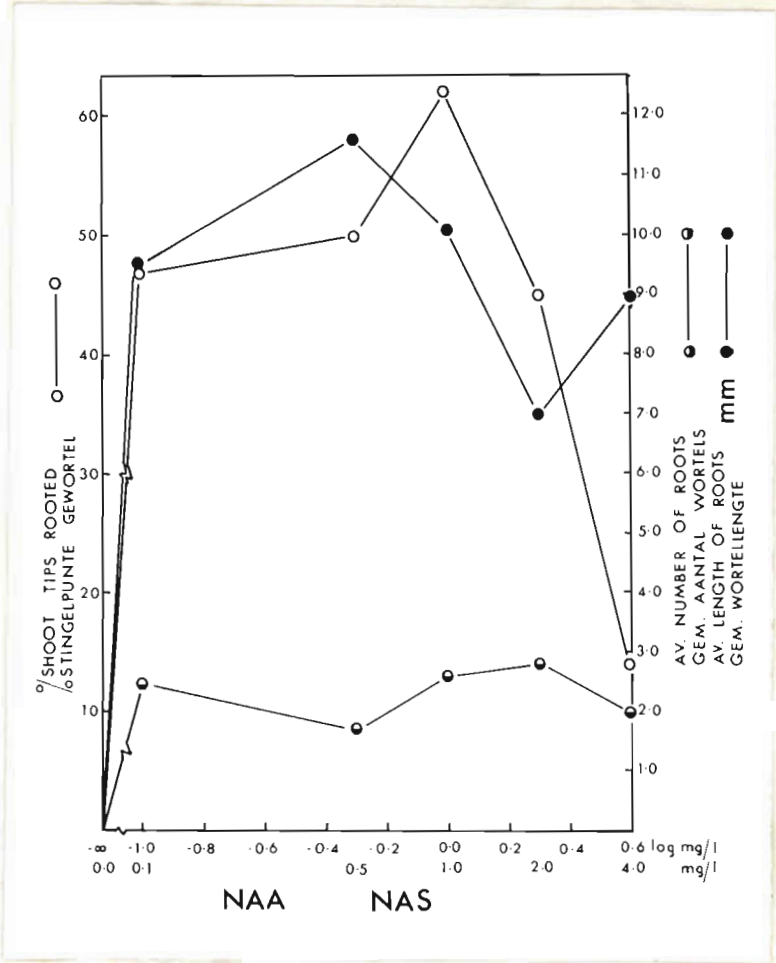

Fig. 7. Influence of NAA on root formation in shoot tips of Rosa multiflora.

i-inositol 100.0; (2) Amino acids (mg/l); glutamine 200.0; (3) sugars (g/l): sucrose 20.0; and (4) cytokinins (mg/l): adenine 10.0. The pH of the culture solution was 4.1. There were 20 replicates per treatment and the culture period was 26 days.

The results of Experiment 5 are given in Fig. 7. Neither roots nor callus were generated at the basal cut surface of the shoot tip in the absence of NAA. When 0.1 mg/l NAA was applied, 47 per cent of the tips formed roots, whereas 62 per cent formed roots when the NAA concentration was increased to 1.0 mg/l. However, further increases in the NAA concentration to 2.0 and 4.0 mg/l caused fewer shoot tips to form roots.

The average length of the roots formed was greater in cultures grown with low NAA concentrations (0.1 to 1.0 mg/l), while there was a tendency towards a greater number of roots per shoot tip of cultures grown on higher NAA concentrations (1.0 to 4.0 mg/l). Although no shoot growth or leaf expansion occurred in the shoot tips grown on the NAA-free medium the tips remained green and appeared to stay alive for extended periods. However, leaves of shoot tips grown on the NAA medium turned brown and died fairly soon.

Experiment 6 - Effects of tip size and liquid vs solid media

The object of this experiment was (1) to establish whether small shoot tips (1.0 to 2.0 mm long) would root when cultured on a medium containing 1.0 mg/l NAA, and (2) to ascertain whether a liquid or a solid 1 per cent agar medium is superior for the growth of the shoot tip. The standard culture medium was used except for sucrose (20.0 g/l), which was the source of sugar, and the pH of

the medium which was 4.1. The culture period was 42 days.

While the control tips failed to root, the frequency with which roots were formed on the tips treated with 1.0 mg/l NAA, as well as the number and length of roots produced, decreased sharply when the size of the shoot tip was reduced from about 8 mm to between 1 and 2 mm. On liquid medium 20 per cent of the tips formed roots whereas on a solid medium no roots were produced, although 30 per cent of these tips had root initials.

Experiment 7 - Effects of varying auxin : cytokinin ratios

The object of this experiment was to observe the growth response of rose shoot tips to (1) kinetin (Table 1) in the presence of 1.0 mg/l NAA; and (2) to different NAA concentrations (0.0; 1.0; 2.0; 4.0; 6.0) in the presence of 5.0 mg/l kinetin. The culture medium was the same as for Experiment 6, with a culture period of 43 days for treatments of object (1) and 55 days for object (2).

Results of Experiment 7 are given in Table 5 and Fig. 8. Table 5 shows that the root inducing ability of NAA was prevented

Table 5. Influence of kinetin in combination with NAA on growth of rose shoot tips after 43 days.

NAA mg/l	0.0	1.0	1.0	1.0	1.0
Kinetin mg/l	0.0	0.0	0.08	1.2	5.0
% shoot tips rooted	0	20	0	0	0
% shoot tips with callus alone	0	0	70	0	0
% shoot tips alive	70	0	0	30	60

in those treatments receiving kinetin. Shoot tips cultured on

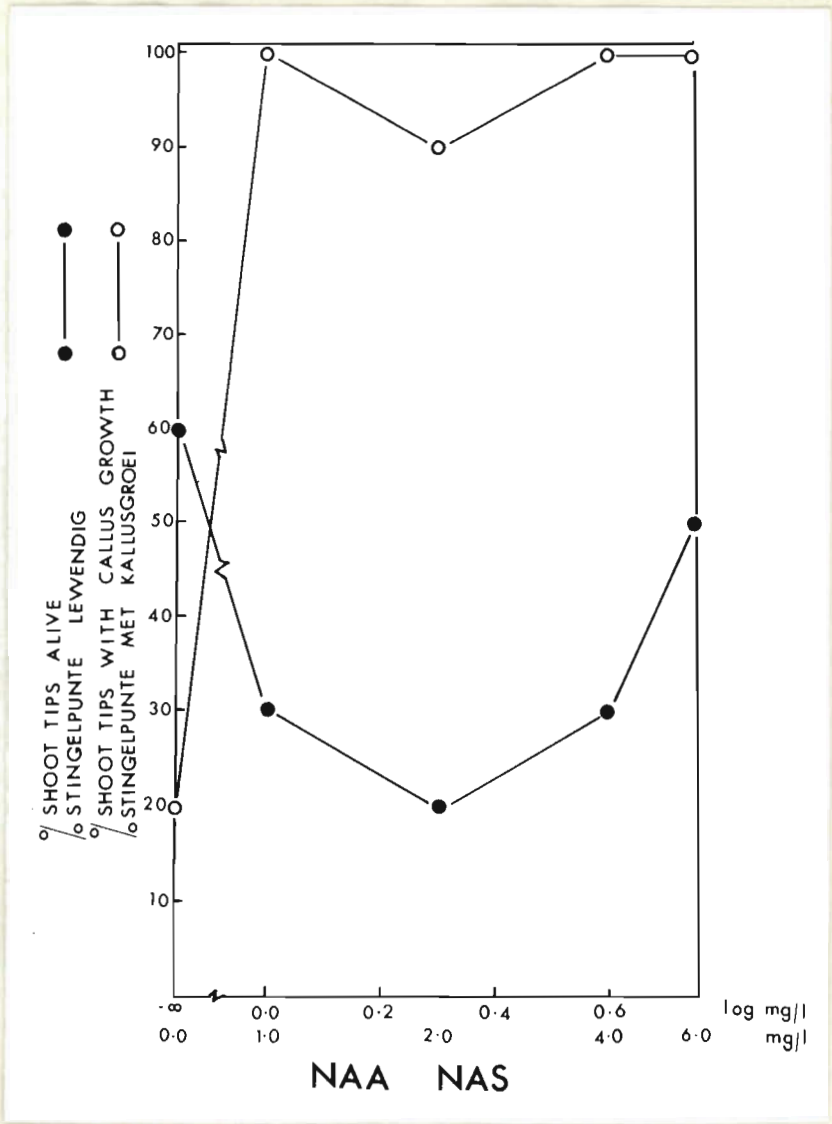

Fig. 8. Influence of NAA on growth of rose shoot tips in the presence of 5.0 mg/l Kinetin.

1.0 mg/l NAA plus 0.08 mg/l kinetin generated callus tissue at the basal cut surface without differentiating roots. However, without kinetin and at higher concentrations of kinetin, callus formation did not take place. The deleterious effect of 1.0 mg/l NAA on leaf survival of the shoot tip was overcome by relatively high concentrations of kinetin (1.2 and 5.0 mg/l).

Figure 8 shows that callus was produced in the presence of NAA. In some of the cultures receiving 5.0 mg/l kinetin without NAA slight callus formation did occur. This was, however, negligible compared with callus production in the presence of NAA. More callus was produced at the higher NAA concentrations. Kinetin supported the longevity of the tip. After 55 days 60 per cent of the shoot tips which received no NAA were still alive, as compared with zero per cent after 43 days where no kinetin was applied and the NAA concentration was 1.0 mg/l (Table 5). The percentage of tips which survived decreased with increasing concentrations of NAA up to 2.0 mg/l. However, at 4.0 and 6.0 mg/l NAA a rise in the percentage of shoot tips alive was observed (Fig. 8).

2.2.2. Discussion

(a) Growth at the basal cut surface: The tendency for root formation to be related to NAA within a certain concentration range is a common phenomenon in cultured plant tissue (Haissig, 1965). This is further supported by the results of Experiment 5 where a greater length of root was formed in the NAA range 0.1 to 1.0 mg/l. The observation that a tendency exists for a greater number of roots to be formed at relatively high NAA levels as compared to relatively low levels could possibly be explained on the basis of the well known fact that auxin levels which favour root initiation may be too high

for root growth.

Small shoot tips (1.0 to 2.0 mm) contain less differentiated tissue than 8.0 mm tips and this is a possible reason for the poor rooting of the small tips (Experiment 6). An apparent interaction exists between NAA and kinetin, since growth induced by NAA was modified to different degrees depending on the concentration of kinetin present in the medium (Table 5). It thus seems that kinetin prevents root formation induced by NAA in rose shoot tips, while even callus formation can be reduced and possibly prevented by increased concentrations of kinetin. The fact that increased NAA concentration (Experiment 7) in the presence of kinetin, could not restore root formation gives further evidence that kinetin inhibits root formation regardless of the NAA concentration. The great differences in the callus production at different concentration combinations of NAA and kinetin suggested that two dimensional grid experiments, with varying NAA and kinetin concentrations, should be conducted, and that results should be evaluated on a more quantitative basis.

The superiority of liquid media on the rooting of rose shoot tips as compared to agar solidified media supports to some degree the finding of Hollings (1965) and Stone (1963). They found that on a liquid medium more roots with root hairs were formed, while on an agar medium more root initials were formed.

(b) Shoot tip survival: The observation in Experiments 5 and 7 that NAA inhibits and kinetin stimulates bud development is in agreement with the generally accepted concept of auxin inhibition and cytokinin stimulation of bud development (Haissig, 1965). It is however difficult to explain the increased longevity of the shoot tips at high NAA concentration (Fig. 8). This may have been due to the growth

substances being present in a more favourable ratio.

Since results with Rosa multiflora shoot tips do not necessarily apply to shoot tips of the hybrid tea rose cv. Superstar the interaction between NAA and kinetin will have to be confirmed with this cultivar. In addition possible GA : NAA and GA : kinetin interactions in shoot tips of 'Superstar' should be investigated.

Another approach to the problem of growing shoot tips into complete plants would be by sequential administration of growth regulating substances.

2.3. EXPERIMENTS ON SHOOT TIPS OF THE HYBRID TEA ROSE cv. SUPERSTAR

2.3.1. Procedure and Results

Experiment 8 - Effects of varying auxin : cytokinin ratios

To test the influence of NAA and kinetin, singly or in combination on the growth of rose shoot tips, a two-dimensional grid (Fig. 9) was established. The standard culture solution was used with a culture period of 70 days.

(a) Callus formation at the basal cut surface: The influences of NAA and kinetin on callus formation are shown in Figs. 9 to 12. Figure 11 shows the fresh weight of callus produced over the concentration range of 0.0 to 8.0 mg/l kinetin, in the absence and presence of NAA. The NAA graph represents the average weight of callus per culture over all the concentrations of NAA tested. No callus was generated in the absence of NAA over the entire kinetin range and the weights recorded in the control curve were those of the tips as such, which were of the order of 5 mg. The NAA curve

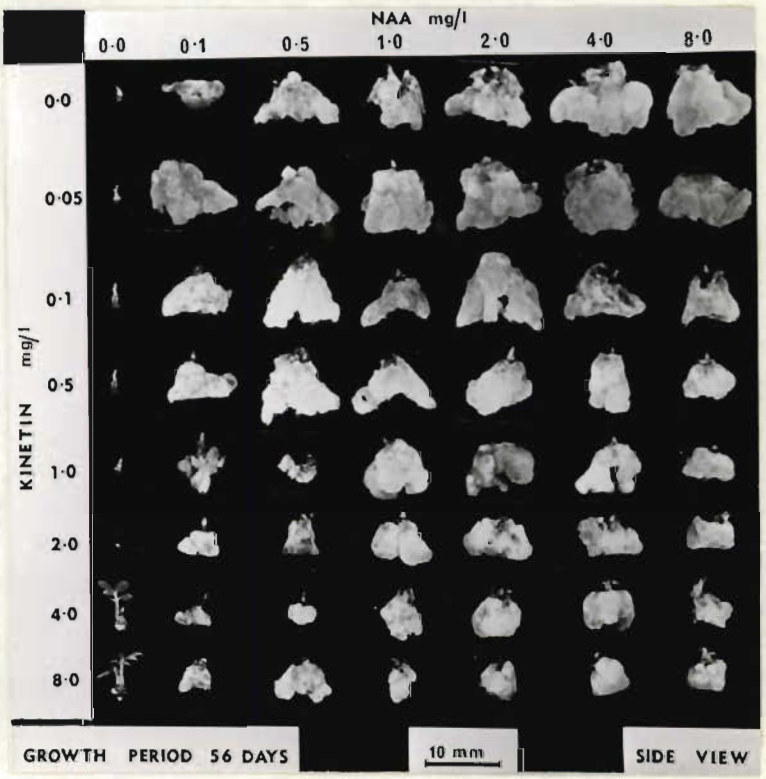

Fig. 9. Influence of different NAA : Kinetin ratios on the growth of rose shoot tips.

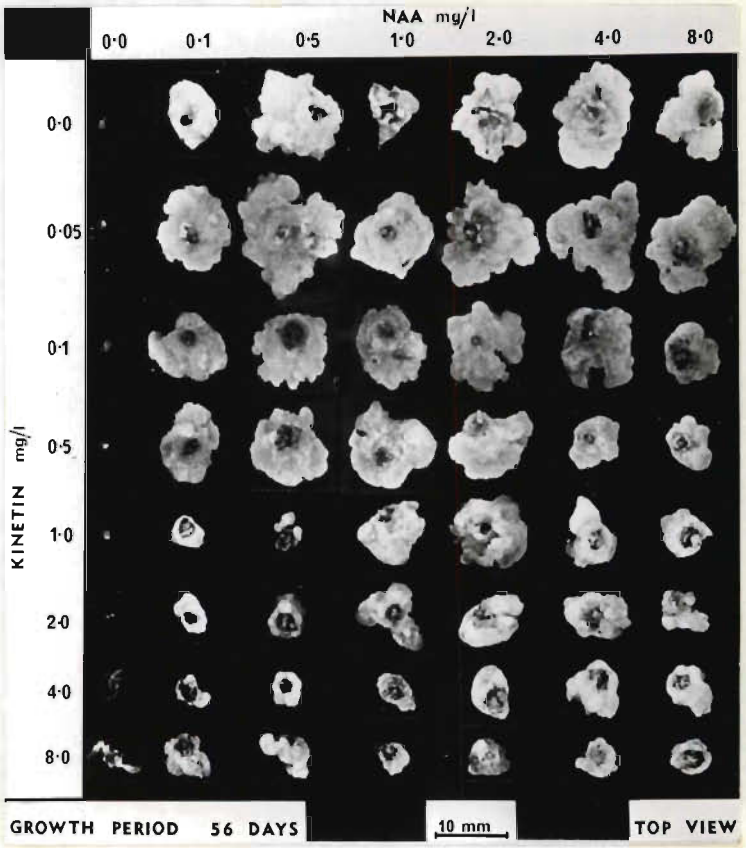

Fig. 10. Influence of different NAA : Kinetin ratios on the growth of rose shoot tips.

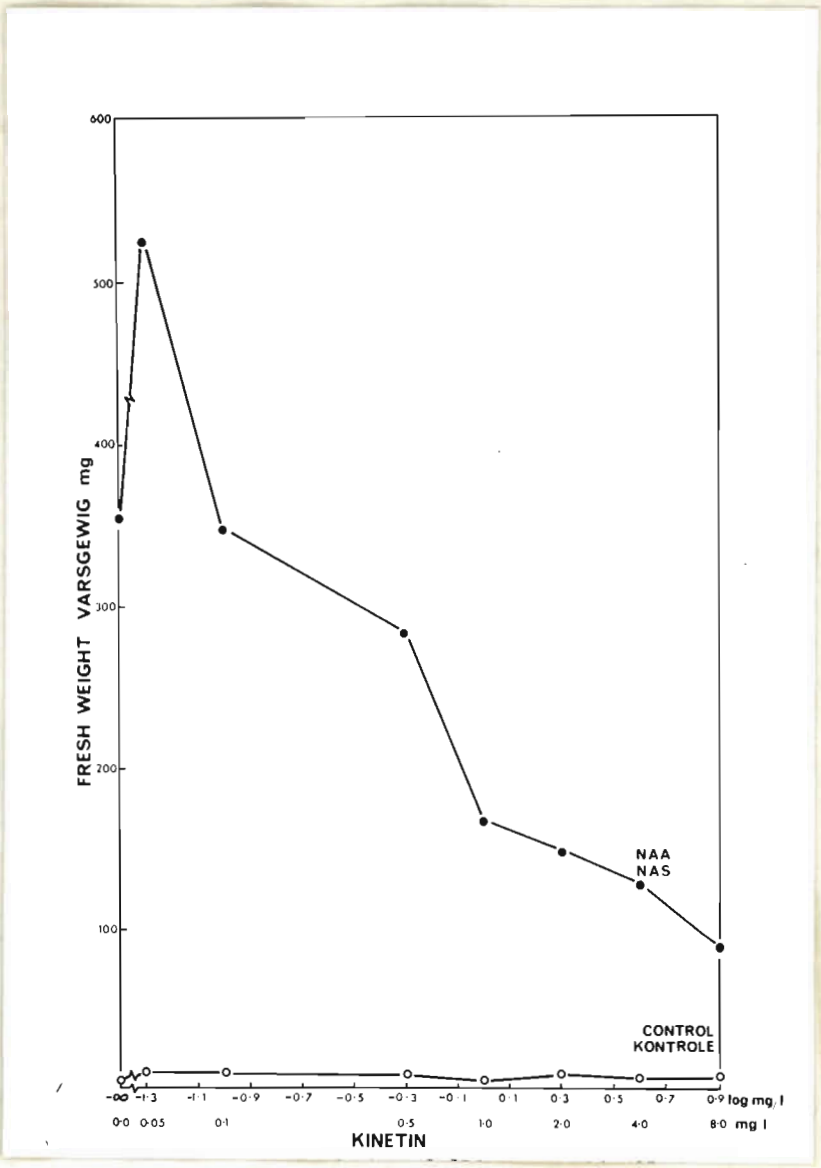

Fig. 11. Influence of Kinetin on callus growth in the absence and presence of NAA.

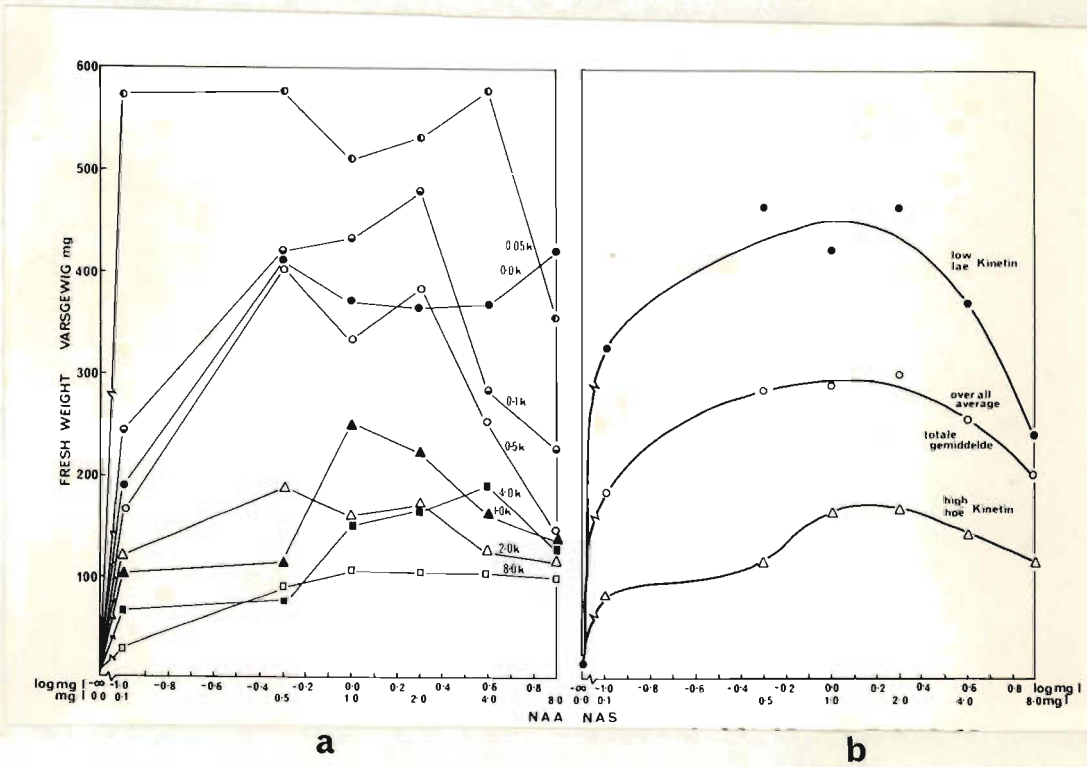

Fig. 12. (a) and (b). Influence of NAA on callus growth at various concentrations of Kinetin.

shows that in the absence of kinetin callus formation was stimulated by NAA. An even greater stimulation occurred when 0.05 mg/l kinetin was combined with NAA, whereas higher kinetin concentrations resulted in a steady reduction of callus production.

The overall effect of NAA (alone and in combination with kinetin) is represented by the "overall average" curve in Fig. 12a. There was a rapid increase in callus production from zero NAA up to 0.5 mg/l, but between 0.5 and 1.0 to 2.0 mg/l the increase was less pronounced, whereas at concentrations from between 1.0 and 2.0 up to 8.0 mg/l NAA, callus growth was reduced. The control curve (zero kinetin, Fig. 12a) deviated considerably from this overall trend. From zero NAA up to 0.5 mg/l it followed the normal pattern but between 1.0 and 4.0 mg/l the curve dropped and rose again at 8.0 mg/l. At low kinetin levels (0.05 to 0.5 mg/l) the callus produced exceeded or virtually equalled that of the control (Fig. 12a) over the lower levels of the auxin range, but at the higher levels callus production was greatly reduced. This effect is represented by the low kinetin curve in Fig. 12b, which is the average of the three curves 0.05, 0.1 and 0.5 mg/l kinetin. With further increases of kinetin to the higher levels (1.0 to 8.0 mg/l) the general trend of the curve was maintained but the amount of callus produced was greatly reduced over the whole NAA range. This is represented by the high kinetin curve in Fig. 12b, which is the average of the four curves 1.0, 2.0, 4.0 and 8.0 mg/l kinetin.

(b) Root formation: Root formation as affected by NAA and kinetin is given in Figs. 13 and 14a. With the exception of a few isolated cases root formation was observed only in the absence of kinetin and in the NAA range of 0.5 to 2.0 mg/l. Maximum root development

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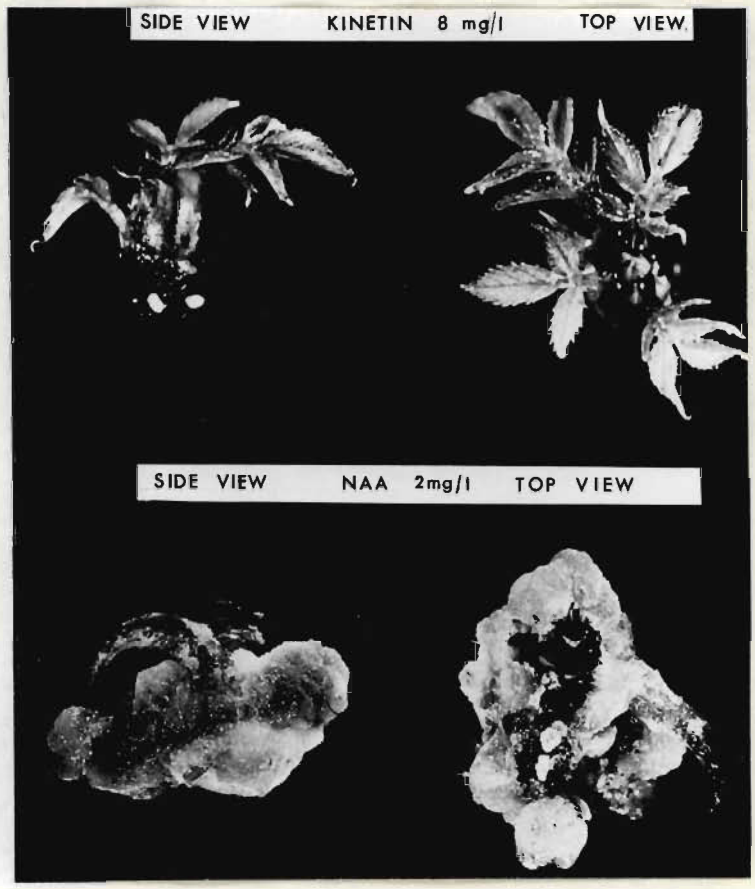

Fig. 13. Leaf and root development in excised rose shoot tips.

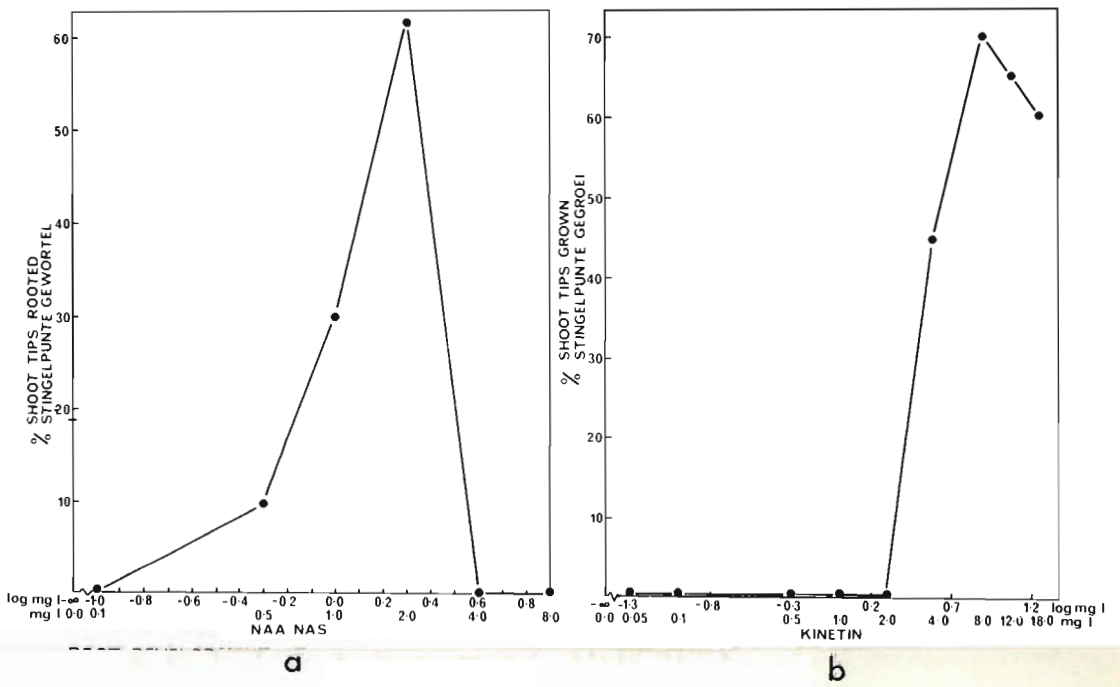

Fig. 14. (a) Root development at various concentrations of NAA, and
(b) shoot growth at various concentrations of Kinetin.

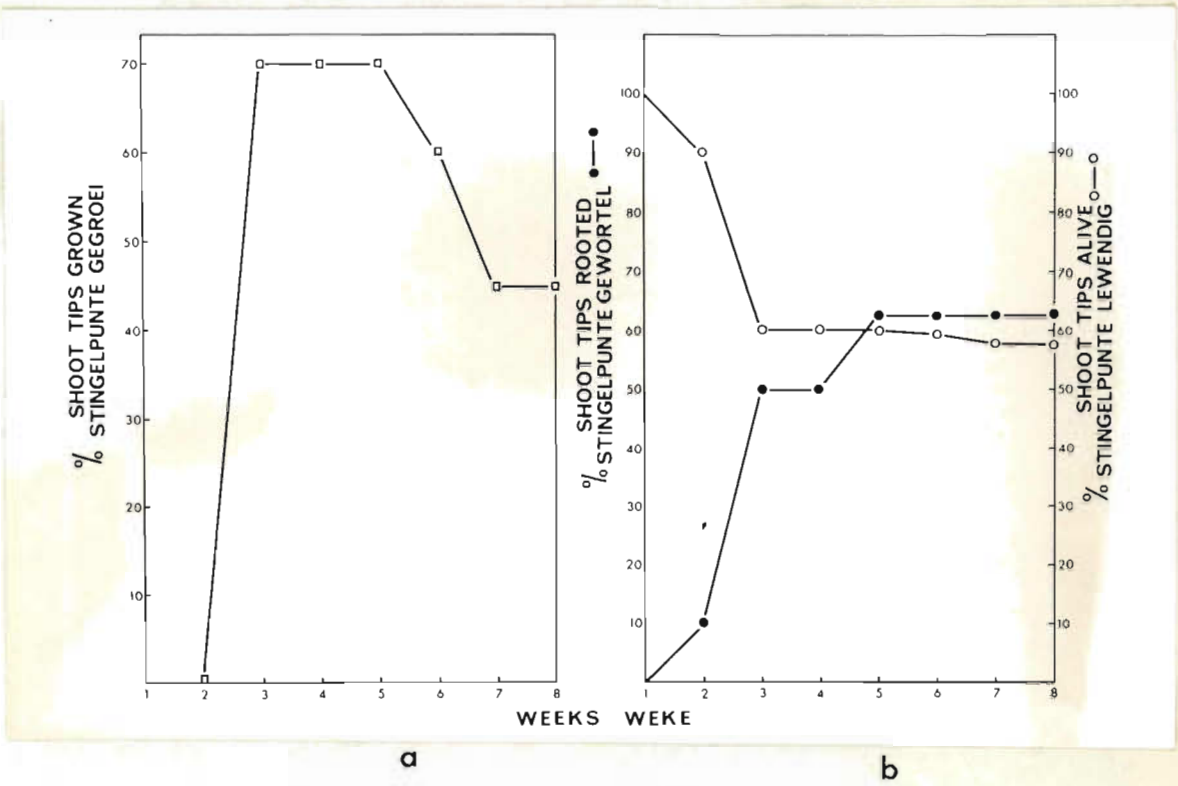

Fig. 15. Time required for:-
(a) shoot growth (Kinetin 8 mg/l),
(b) root development (NAA 2.0 mg/l).

occurred at 2.0 mg/l NAA. The roots appeared to develop at the junction between the shoot tip and the callus mass. The developing roots grew to a length of approximately 1 to 2 cm and then stopped growth.

(c) Shoot and leaf growth: In Figs. 9, 10, 13 and 14b the influences of NAA and kinetin on shoot and leaf growth are shown. Normal leaf growth occurred only in the absence of NAA and at the higher concentration levels of kinetin (4.0 mg/l and higher) with 8.0 mg/l as the optimum. Either no leaf growth or severe distortions of the leaves occurred, in combination with some degree of callus growth, when NAA and kinetin were applied jointly. It was difficult to decide whether or not a certain amount of shoot growth accompanied leaf development.

(d) Change in growth response of shoot tips to NAA and kinetin with time: The changes in leaf development, percentage shoot tips rooted and shoot tip survival with time are shown in Figs. 15a and b. Within three weeks 70 per cent of the tips on 8.0 mg/l kinetin medium had shown leaf expansion. However, after the fifth week the tips started to die back rapidly. Maximum root formation was obtained after five weeks with 2.0 mg/l NAA but, as shown in Fig. 15b, 40 per cent of the tips died during the first three weeks after which the percentage of shoot tips alive remained almost constant.

Experiment 9 - Effects of varying cytokinin : gibberellin ratios

This experiment was conducted to determine the influence of kinetin and GA, singly or in combination, on the growth of rose shoot tips (Fig. 16). The standard culture solution was used with a culture period of 25 days.

(a) Shoot tip survival: Figure 17 shows the influence of kinetin

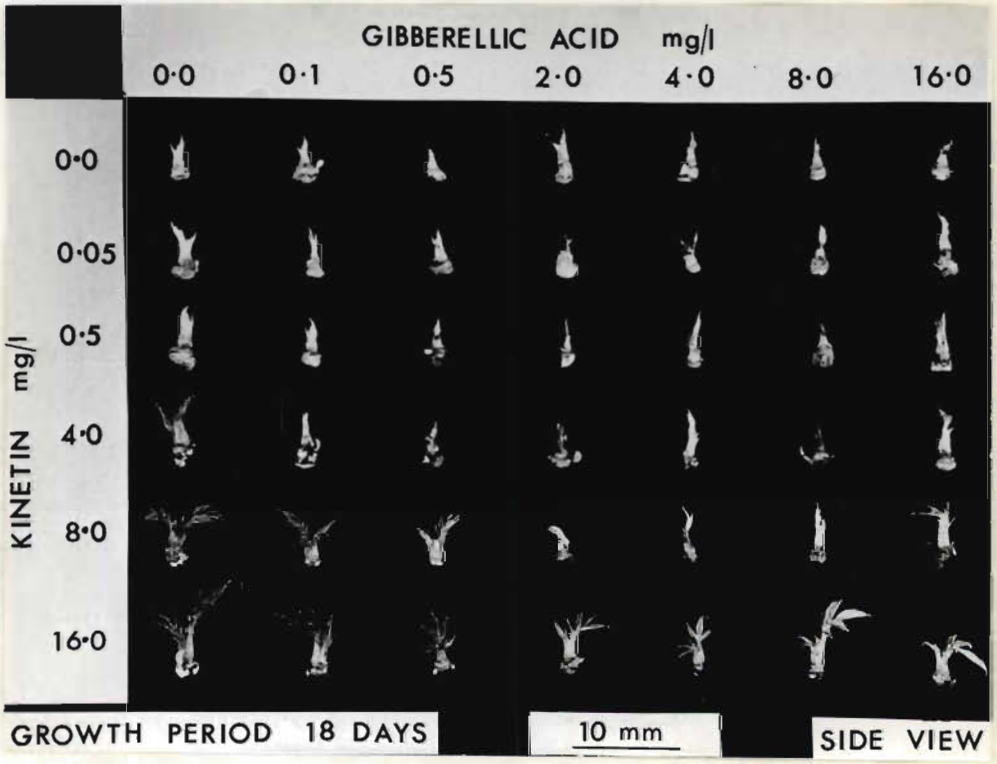

Fig. 16. Influence of different GA : Kinetin ratios on the growth of rose shoot tips.

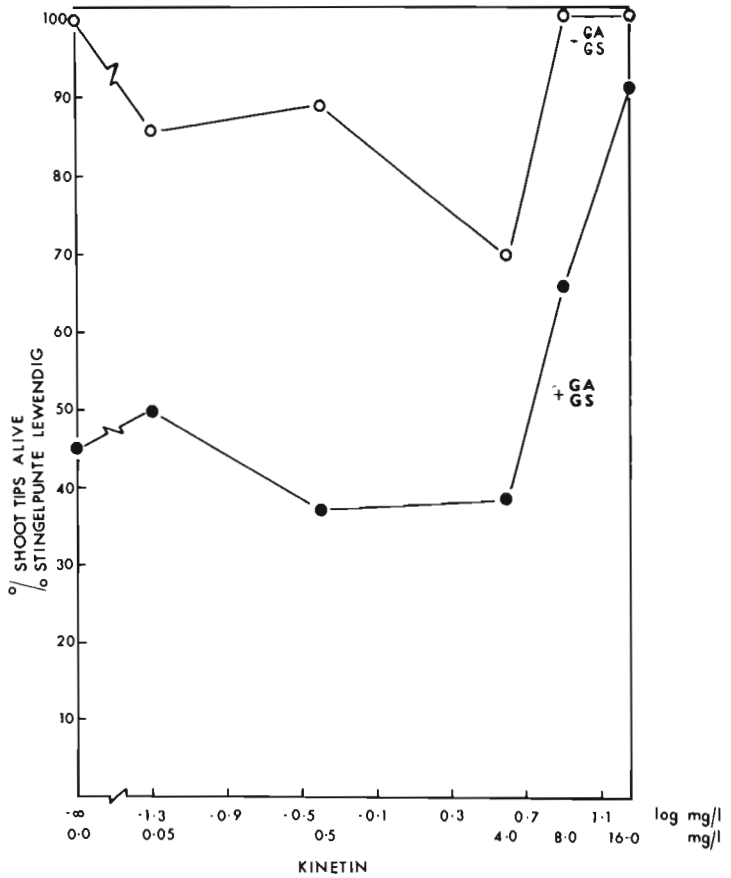

Fig. 17. Influence of Kinetin alone and in combination with GA on shoot tip survival after 18 days.

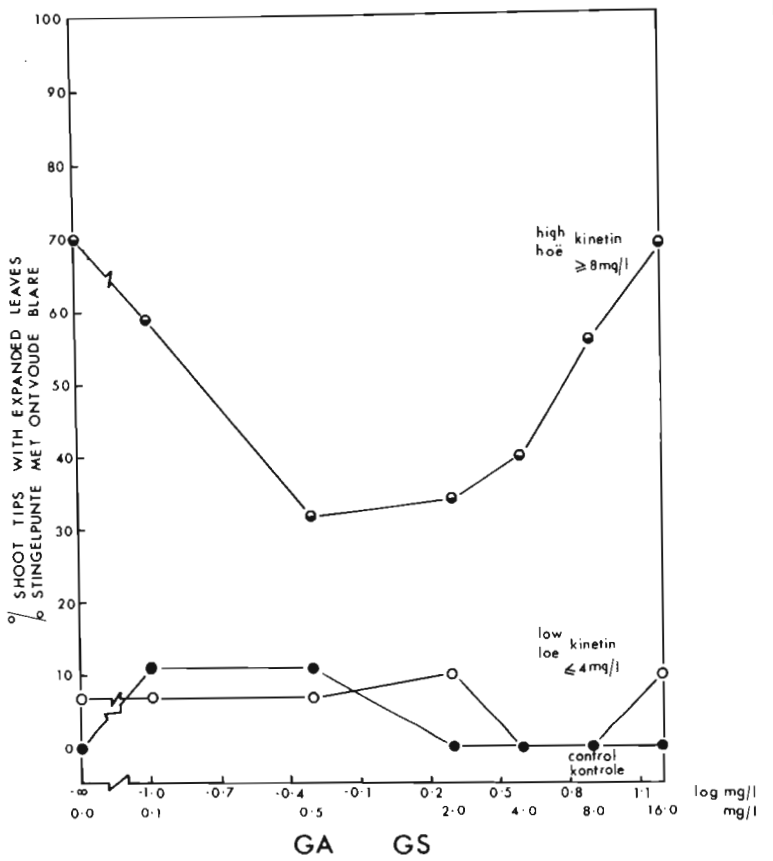

Fig. 18. Influence of GA alone and combined with Kinetin on leaf growth after 18 days.

on the survival of the shoot tips in the presence and absence of GA. The GA graph represents the average number of shoot tips alive over all the concentrations of GA tested. In the absence of GA no significant difference in the percentage tip survivals was observed after 18 days, at the different kinetin concentrations tested. There was, however, a tendency for some of the tips to die more readily at low to medium-high (0.05 to 4.0 mg/l) kinetin concentrations as compared with the control (zero kinetin) and high (8.0 and 16.0 mg/l) kinetin concentrations. However, after 25 days significantly more tips were alive at high (8.0 and 16.0 mg/l) kinetin as compared with lower concentrations.

In the presence of GA significantly more tips died within the first 18 days especially at zero or low (up to 4.0 mg/l) kinetin concentrations, whereas in the presence of high kinetin concentrations (8.0 and 16.0 mg/l) far more tips remained alive, as shown by the sharp rise in the GA curve. No definite trend regarding shoot tip survival after 18 days could be established between different GA concentrations, except that intermediate concentrations (0.5 to 4.0 mg/l) appeared to be more detrimental to the tip than low (0.1 mg/l) or high (8.0 and 16.0 mg/l) concentrations.

(b) Shoot and leaf growth: Figure 18 shows the percentage of shoot tips with leaf growth after 18 days at various levels of GA, and in the absence and presence of low and high kinetin concentrations. The low kinetin curve represents the average number of shoot tips with leaf growth cultured on 0.05, 0.5 and 4.0 mg/l kinetin, whereas the high kinetin curve represents those cultured on 8.0 and 16.0 mg/l kinetin. No significant differences in the number of shoot tips with leaf growth were observed between treatments

receiving GA, either alone or plus low concentrations of kinetin (low K curve) over all concentrations of GA. However significantly more shoot tips survived in the presence of high kinetin (high K curve). In the presence of high kinetin levels the same effect of GA was observed as with shoot tip longevity, namely, that intermediate concentrations of GA (0.5 to 4.0 mg/l) reduced the percentage of shoot tips with leaf development as opposed to zero, low (0.1 mg/l) and high (8.0 and 16.0 mg/l) GA concentrations.

Experiment 10 - Effects of varying auxin : gibberellin ratios

The object of this experiment was to determine the influence of NAA and GA, singly and in combination, on the growth of rose shoot tips (Fig. 19). The standard culture solution was used with a culture period of 49 days.

(a) Callus formation at the basal cut surface: The influence of varying NAA and GA concentrations on callus production is shown in Fig. 20. In the absence of GA callus production was stimulated by NAA, and reached a maximum at 2.0 mg/l. Eight mg/l NAA resulted in a reduction of callus produced as opposed to 2.0 mg/l NAA. In the presence of 0.1, 0.5, 8.0 and 16.0 mg/l GA, callus production increased with increasing concentrations of NAA. At 2.0 mg/l GA the amounts of callus produced in the presence of 2.0 and 8.0 mg/l NAA were virtually equal, whereas at 4.0 mg/l GA more callus was produced in the presence of 2.0 mg/l NAA than of 8.0 mg/l.

Since callus production in the presence of 1.0 mg/l NAA and less was very low these results will not be described in detail. The effect of GA on callus yield can be seen best by

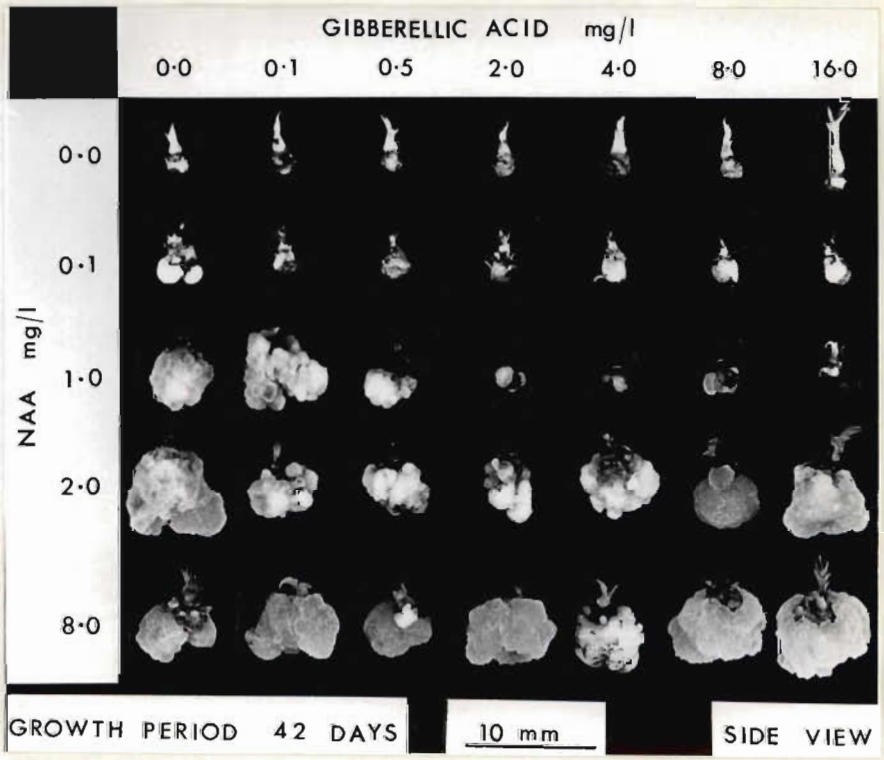

Fig. 19. Influence of different GA : NAA ratios on the growth of rose shoot tips.

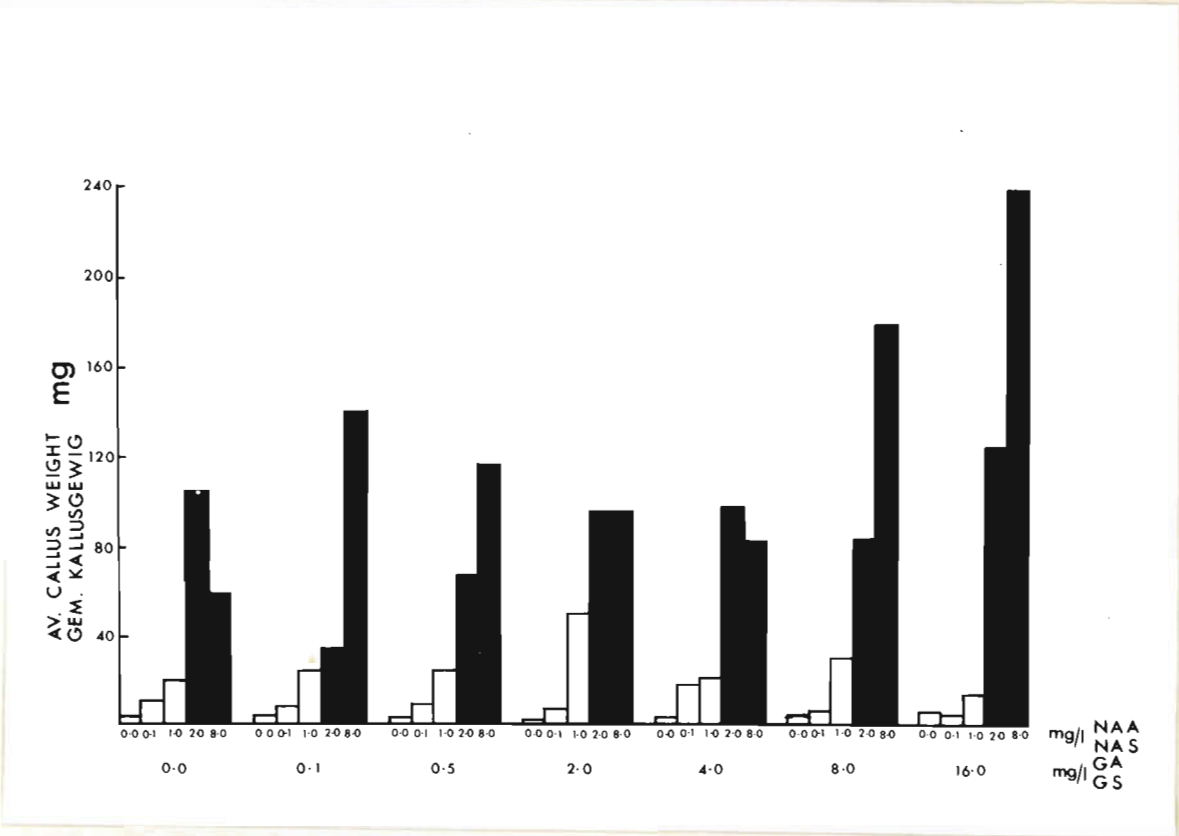

Fig. 20. Influence of GA on callus formation at various levels of NAA.

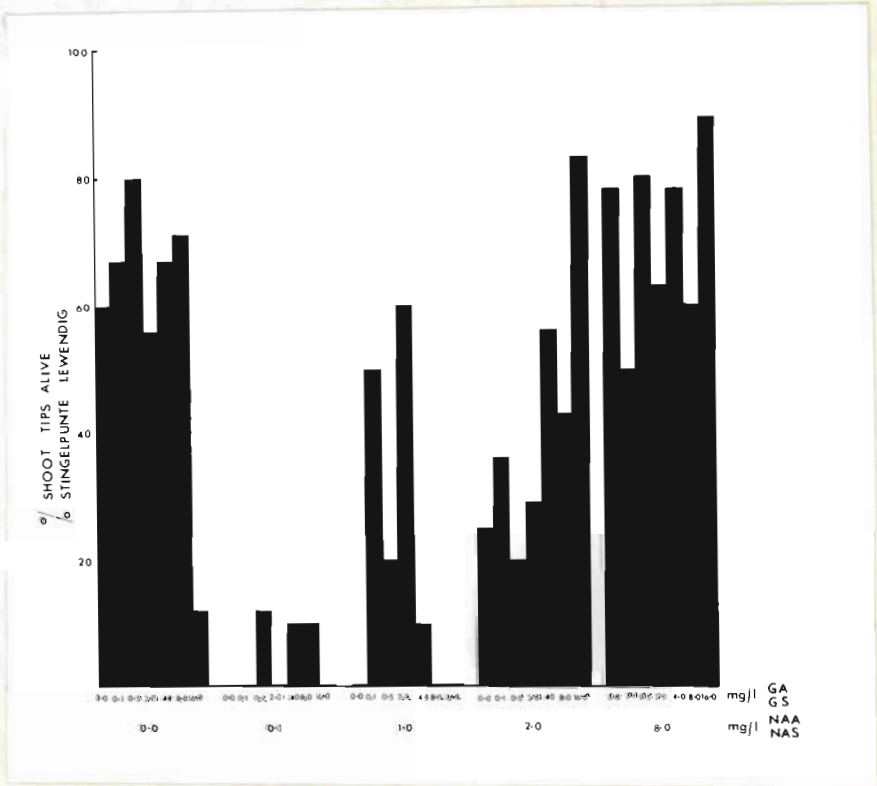

Fig. 21. Influence of NAA on shoot tip survival at various levels of GA after 28 days.

comparing the amount of callus produced at 2.0 and 8.0 mg/l NAA at the various GA levels (Fig. 20). The addition of 0.1 mg/l GA to the 2.0 mg/l NAA medium gave less than a quarter of the callus yield. However with further increases in the GA concentration there was a gradual increase in callus production, and at 16.0 mg/l the callus yield exceeded that of the 2.0 mg/l NAA without GA. Callus production was doubled when 0.1 mg/l GA was added to the 8.0 mg/l NAA medium. However with further increases in the GA concentration up to 4.0 mg/l there was a steady decrease in callus production, followed by a sharp increase at 8.0 and 16.0 mg/l.

(b) Shoot tip survival: Figure 21 shows the effect of NAA and GA on shoot tip survival after 28 days. In the absence of NAA a greater proportion of the shoot tips survived the four week period than tips cultured on a medium containing 0.1 mg/l NAA. However, as the NAA level increased from 0.1 to 8.0 mg/l an increase in the percentage of shoot tip survivors was observed, with the average at 8.0 mg/l NAA being equally as good as with zero NAA. No consistent trend regarding shoot tip survival at different GA concentrations was found.

Experiment 11 - Response to different auxins

This experiment was designed to determine the influence of the auxins : IAA; NAA; IBA and 2,4-D on the growth of rose shoot tips. The concentrations in mg/l employed were:- IAA (0.0; 0.5; 1.0; 3.0 and 6.0); IBA (0.0; 0.5; 1.0; 2.0 and 4.0); NAA (0.0; 0.1; 1.0: 2.0 and 8.0); 2,4-D (0.0; 0.1; 0.5; 1.0 and 2.0). The standard culture solution was used with a culture period of 35 days.

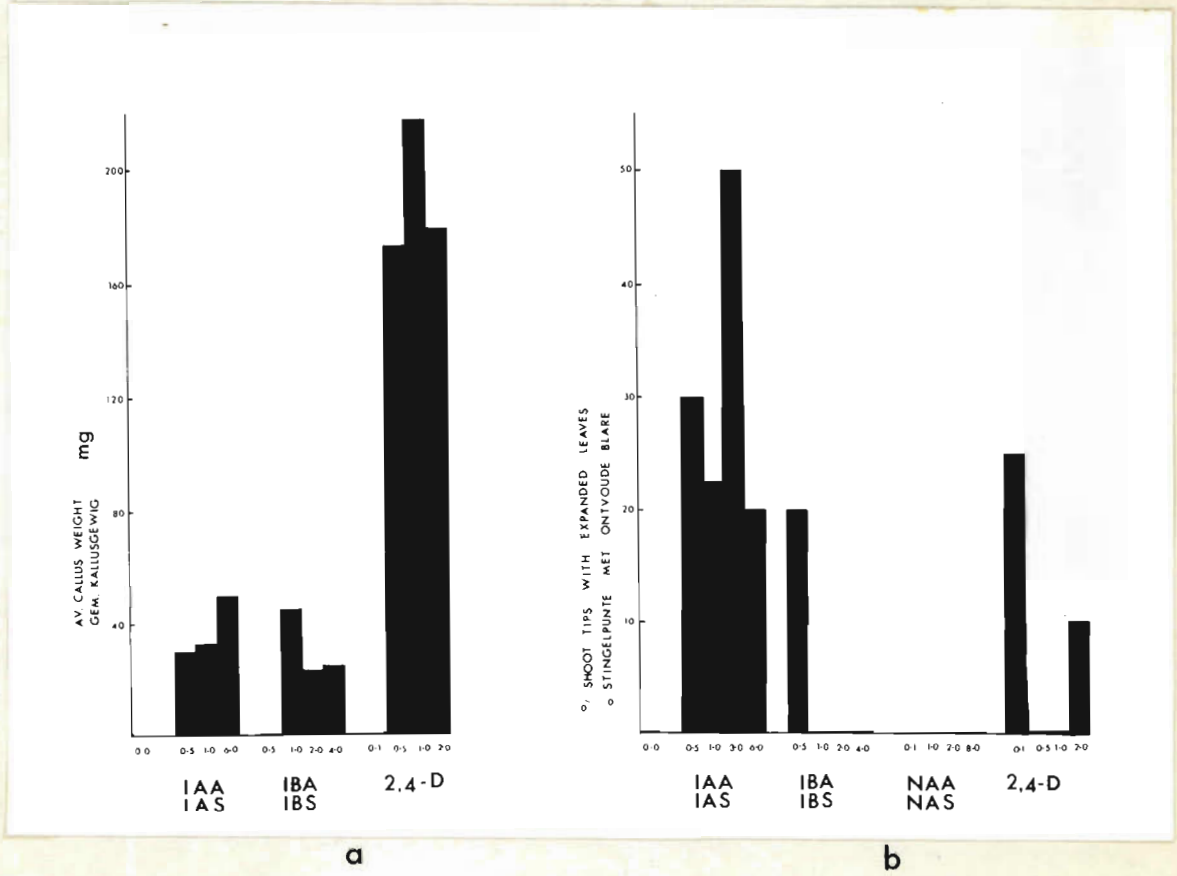

Fig. 22. Influence of different auxins on the growth of rose shoot tips.

(a) Callus formation;

(b) leaf growth, after 21 days.

(a) Callus formation at the basal cut surface: No roots were formed. The average weight of callus produced per culture, with the various auxin species and concentrations, is presented in Fig. 22a. Weights were not recorded for the NAA treatments nor for 3.0 mg/l IAA. The latter were sub-cultured for another purpose. No callus tissue was formed in the absence of an auxin or in the presence of 0.5 mg/l IBA or 0.1 mg/l 2,4-D. Callus production increased with increasing concentrations of IAA, with IBA or 2,4-D 1.0 mg/l gave maximum yield of callus, while lower or high concentrations of these auxins resulted in less callus formation. At stimulatory levels 2,4-D produced four to five times more callus than IAA or IBA.

(b) Shoot and leaf growth: The effect of the different auxins on leaf development is shown in Fig. 22b. A greater percentage of the shoot tips grown on the range of IAA concentrations had leaf growth as compared to the other auxins. Three mg/l IAA appeared to have the most favourable effect. In a few instances leaf development occurred in the presence of 0.5 mg/l IBA and 0.1 and 2.0 mg/l 2,4-D. No leaf growth was observed with NAA treated shoot tips or at the other concentrations of IBA and 2,4-D.

Experiment 12 - Effects of sequential administration of growth regulating substances

The objects of this experiment were (1) to observe the effect of different concentrations of IBA (0.0; 1.0; 2.0 and 4.0 mg/l), GA (0.0; 0.1; 1.0; 2.0; 4.0 and 8.0 mg/l) and coconut milk (0.0; 1.0; 5.0; 10.0; 20.0 and 40.0 per cent) on growth of shoot tips which were precultured for 18 days on

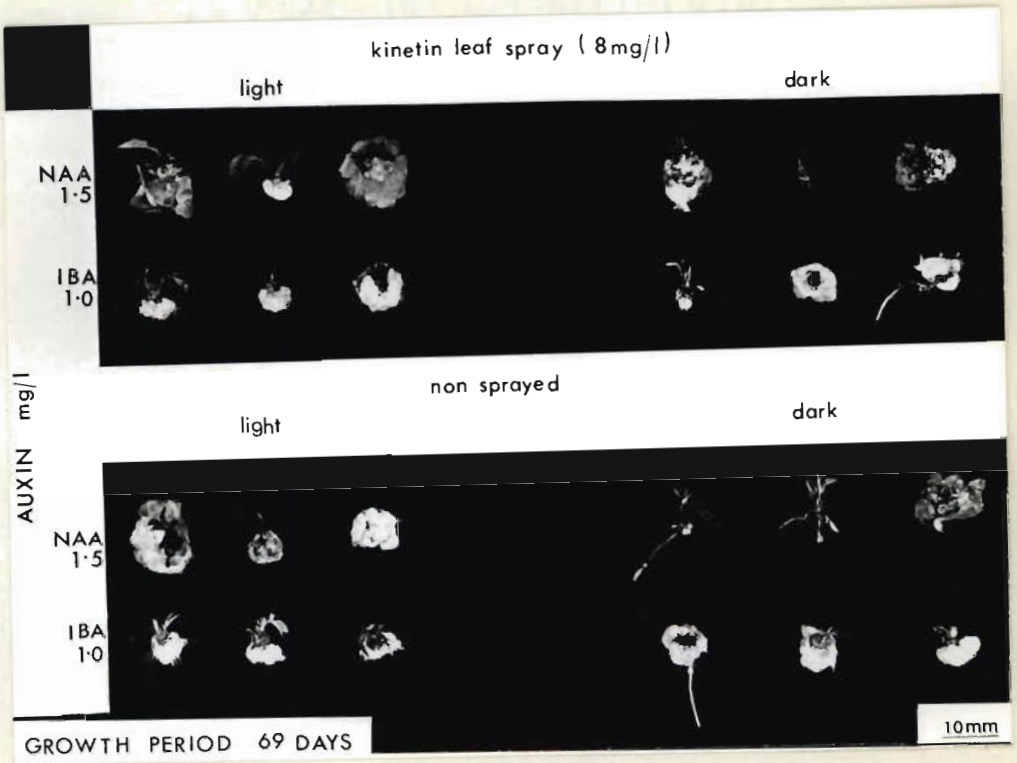

Fig. 23. Variation in growth response of rose shoot tips to different treatments, after preculture for 8 days in Kinetin medium.

a 8.0 mg/l kinetin medium; (2) to observe the effect of an 8.0 mg/l kinetin leaf spray on the growth of shoot tips which were precultured for 8 days on a 8.0 mg/l kinetin medium, and then transplanted to either 1.5 mg/l NAA or 1.0 mg/l IBA media; and (3) to compare the growth of shoot tips in (2) grown either in light or darkness.

The standard culture solution was used and treatments were repeated 10 times as in all previous experiments, but for cultures grown in the light for object (3) the treatments were repeated 30 times. The culture period in the case of (1) was 28 days, while it was 69 days for cultures of (2) and (3).

None of the treatments supported or induced further growth of the leaves or shoots after transfer. On the coconut milk medium tips survived for a longer period than on the IBA, NAA or GA media. No difference was observed between light and dark as well as sprayed and non-sprayed cultures. (Fig. 23). In the presence of NAA and IBA, callus formation was stimulated inconsistently while root formation was observed in only a few cases (Fig. 23).

2.3.2. Discussion

(a) Callus formation at the basal cut surface

(i) NAA : Kinetin effects: The results of Experiment 1 demonstrated the dependence of callus initiation and growth on the presence of an auxin. The general pattern of the curve of fresh weight of callus versus NAA concentration (Fig. 12b, overall average), viz. a sharp rise in callus production at low NAA concentrations, a levelling off at intermediate and a decline

at high NAA concentration, is well established in tissue cultures of other plant species.

Part of the deviation of the control curve (zero kinetin, Fig. 12a) from the overall trend can be attributed to the development of roots at 1.0 and 2.0 mg/l NAA. It appeared that where roots were formed callus growth was checked to some extent, thus giving a somewhat different curve compared with the normal pattern.

Figure 12b illustrates an interesting phenomenon. Over the NAA range 2.0 to 8.0 mg/l the rate of decrease in callus production (fresh weight) was more rapid at low than at high kinetin concentrations, although the former actually gave a higher callus yield over this range of NAA. Considering the growth substances individually it was shown that low NAA concentrations had a stimulating effect on callus production (Fig. 12a), whereas at high NAA concentrations it was concluded that NAA had a retarding effect on callus growth. No callus growth occurred when kinetin alone was applied (Fig. 11), whereas kinetin in combination with NAA was stimulatory at low levels and inhibitory at high levels. It therefore appears that the ratio between the two growth substances is important when they are applied jointly. At low NAA and kinetin concentrations, where maximum callus growth occurred (Fig. 12b), the ratio of NAA to kinetin was from 0.5 : 1 to 5 : 1. When both NAA and kinetin concentrations were high the ratio was 0.5 : 1 to 2 : 1, which is within the same range. However at high NAA and low kinetin concentrations the ratio was from 10 : 1 to 40 : 1 and it was presumably this rapidly widening ratio which accounted for the rapid decrease in callus production, as shown by the slope of the low kinetin curve in Fig. 12b over

the high NAA range.

However, despite this apparently unfavourably wide ratio of high NAA : low kinetin, compared with high NAA : high kinetin, the actual amount of callus produced was greater in the former case. This suggests that at high NAA and low kinetin concentrations only NAA was inhibitory, whereas at high NAA and high kinetin, where the ratios were more favourable, both were inhibitory. The concentration effect of the individual growth substances was presumably more important than the wide ratio effect in this experiment. However, if the NAA concentration had been increased further and if there is no basic change in the trends of the low and high kinetin curves (by extrapolation), a point would be reached beyond which a higher callus yield should be obtained with the high NAA : high kinetin treatment as compared to the high NAA : low kinetin. This would imply that the ratio between the two growth substances would then be more important than the effect of their individual concentrations on the growth of the calli.

(ii) NAA : GA effects: The conclusion that the control curve (Fig. 12a, zero kinetin) would have had the normal pattern if no roots were formed is justified by the results of Experiment 10 (Fig. 20). Since no roots were generated in cultures of Experiment 10 treated with 1.0 and 2.0 mg/l NAA alone, the callus production curve had the normal pattern. Although the initial rise in the curve was not as sharp as was found in Experiment 8, this is due to other factors which will be discussed later.

Since very little callus tissue was formed in Experiment 10 at NAA concentrations of 1.0 mg/l and lower, even in the absence of GA, the effects of GA on callus formation in the

presence of 2.0 and 8.0 mg/l NAA will be discussed (Fig. 20). Considering the ratios between NAA and GA the following arises:

NAA : GA ratios with NAA kept constant at 2.0 mg/l

NAA	:	GA	Average weight in mg of callus/culture
1	:	8	125
1	:	2	98
1	:	1	96
1	:	4	84
1	:	0.25	67
1	:	0.05	34

NAA : GA ratios with NAA kept constant at 8.0 mg/l

NAA	:	GA	Average weight in mg of callus/culture
1	:	2	241
1	:	2	180
1	:	0.0125	140
1	:	0.0625	117
1	:	0.25	96
1	:	0.5	83

In both instances a greater yield of callus was produced when the ratio between NAA and GA was 1 : 1 or greater in favour of GA, as compared to yields obtained when NAA was present in greater amounts than GA. The amount of callus produced by 2.0 mg/l NAA (which is close to the optimum concentration for callus production) was sharply reduced by the addition of 0.1 mg/l GA (Fig. 20). This decrease in callus yield can be attributed to the NAA : GA ratio being unfavourable. The steady increase in callus production with increasing concentrations of

GA could be ascribed to the NAA : GA ratio becoming more favourable for callus formation. It therefore appears that GA applications to cultures in the presence of the near optimum concentration of NAA for callus formation had a depressing effect on callus formation. Only when the GA was present in much greater amounts than the NAA was this adverse effect overcome.

The unfavourable effect of high NAA concentrations (8.0 mg/l) on callus formation (Fig. 20) was overcome in various degrees by the addition of different concentrations of GA. It therefore appears that the depressing effect of high NAA concentrations on callus formation can be overcome by GA. It is however difficult to explain why a greater yield of callus was obtained when the NAA : GA ratio was 1 : 0.0125 and 1 : 0.625 as compared to 1 : 0.25 and 1 : 0.5.

(iii) Effect of different auxin species: The differences between various auxin species both in their ability to initiate and stimulate callus formation as well as their differences in optimum concentrations for callus growth, show the important differences that exist between these substances even though they belong to the same class of growth regulators. Thus preliminary tests should be conducted to study both the quantitative and qualitative effects of the different species of growth regulating substance on a specific tissue. This effect was confirmed by the work of Skoog et al (1967) on cytokinin species. For this reason care should be taken when considering different species of auxins since, although they belong to the same class of growth regulating substance, their effects on tissue behaviour can differ a great deal.

(iv) Influence of sampling dates of explants: Great differences in the ability of auxin to induce callus formation at the basal cut surface, as well as differences in amount of callus produced, were observed between shoot tips taken at different times of the year. Shoot tips sampled in late summer (Experiment 8) responded positively to NAA concentrations of 0.1 and 0.5 mg/l by forming relatively large amounts of callus tissue, whereas with tips sampled in late winter (Experiment 10) 0.1 and 1.0 mg/l NAA resulted in only very slight callus formation. A greater variation among shoot tips in their ability to generate callus was also observed, as shown in Table 6.

Table 6. Seasonal influence on ability of shoot tips to generate callus when treated with an auxin.

	NAA mg/l						
	0.0	0.1	0.5	1.0	2.0	4.0	8.0
% shoot tips with callus							
Late summer	0	100	100	89	100	100	100
Late winter	10	50	-	67	100	-	78

(b) Root formation:

(i) NAA : Kinetin effects: Root initiation and development as stimulated by an auxin such as NAA in rose shoot tips is in agreement with the rooting of cuttings in general (Hartmann and Kester, 1968) as well as in stem tissue segments and undifferentiated callus tissue in culture (Winton, 1968; Wolter, 1968). The observation that root initiation did not occur at very low

(0.1 mg/l) or high concentration (4.0 and 8.0 mg/l) of NAA (Fig. 14a) can be ascribed to the concentration effect, low concentrations not being stimulatory enough, and high concentrations inhibiting root initiation and development. These findings are in agreement with the findings of other workers such as Alleweldt and Radler (1962), who found that grape tissue varies in its root-initiating capacity according to the quantity of NAA employed.

It is clear from results in Experiment 8 that an interaction between NAA and kinetin exists, since the root initiating capacities of 1.0 and 2.0 mg/l NAA were completely abolished by kinetin at concentrations as low as 0.05 mg/l. This finding is in agreement with the generally accepted belief that cytokinins inhibit root formation (Haissig, 1965). However this statement is not always valid as in the case of tobacco for example (Skoog and Miller, 1957) root formation takes place in the presence of kinetin. Numerous cases can however be quoted where cytokinins did in fact inhibit root formation (De Ropp, 1956; Schaeffer and Smith, 1963). Skoog and Miller (1957) established a critical ratio between auxin and cytokinin where root and shoot formation both took place in tobacco callus. Such a ratio between NAA and kinetin could not be established for rose shoot tips. It is possible that such a ratio may exist but at a different ratio or concentration of NAA and kinetin to those tested, or that other species of auxin or cytokinin may have the desired effect.

In view of the beneficial effect of IAA on leaf expansion compared with the negligible effect of NAA (Experiment 11), further experimentation with an IAA : kinetin grid is indicated. The seasonal effect on root formation as well as on IBA : kinetin grid

must also be considered in view of the generally more favourable effect of IBA on rooting of cuttings.

Two possible reasons may account for the fact that the rose roots initiated in Experiment 8 grew to a length of 1 to 2 cm and then stopped growth. Possibly the NAA concentration of 1.0 and 2.0 mg/l was favourable for root initiation but was too high for further growth of the roots. It was however observed that roots ceased to grow before they reached the culture solution. In separate experiments by the author on root formation in rose shoot tips it was found that roots that reached the culture solution kept growing. The possibility therefore exists that the roots stopped growth due to starvation of certain essential growth requirements which could not be supplied by the callus. Whether this is due to the callus tissue being poor in such requirements, or whether the requirements were not transportable down the root due to its polar orientation is difficult to say.

(ii) NAA : GA effects: No roots were formed in cultures of Experiment 10 despite the application of 1.0 and 2.0 mg/l NAA, presumably due to the time of year the explants were sampled. Thus no information regarding the influence of GA on root formation could be obtained.

(iii) Influence of sampling dates of explants: The date of explant sampling had an even greater effect on the ability of the shoot tip to form roots than on its ability to generate callus. Shoot tips sampled in late summer (Experiment 8) formed roots readily when cultured on a medium containing 1.0 and 2.0 mg/l NAA. No roots were formed by shoot tips sampled

in late winter (Experiments 10 and 11) and given an auxin treatment. This variation in rooting ability due to sampling dates is a common phenomenon in cuttings (Hartman and Kester, 1968). Such a variation also exists in tissue cultures (Fallot, 1955). Although no variation could be observed between the different auxins in their ability to stimulate root formation due to the inability of the shoot tips to form roots, the author is convinced that differences would have occurred if the shoot tips were sampled in summer. It has been shown with other plant species that different auxins vary in their effectiveness to induce roots (Wiggans, 1954; Jacquiot, 1955; Hay, 1962; Wolter, 1968).

(c) Shoot tip survival: This criterion was the most unreliable of the criteria used in this study since it relied solely on visual judgment whether the tip was alive or dead. Such a classification of the tips into two distinct groups was hampered by the tips being in different stages of the die-back process. The general effects of the growth substances involved will however be discussed.

The tendency for low (0.1 and 1.0 mg/l) NAA concentrations to reduce the percentage shoot tip survivals more than either zero or high concentrations (Fig. 21, Experiment 10) was also observed in Experiment 8. This phenomenon is difficult to explain. It is also not known whether the superiority of IAA on shoot tip longevity as opposed to NAA, IBA and 2,4-D could be attributed to it being a natural auxin while the others are synthetic auxins.

The favourable effects of kinetin on bud formation is a well established phenomenon. In both Experiments 8 and 9 where kinetin was employed the favourable effect of kinetin on shoot tip survival and leaf expansion was observed, especially at the higher

concentrations of 4.0 to 18.0 mg/l (Fig. 21). Gibberellic acid in general had a depressing effect on shoot tip survival (Fig.17). No consistent trend could however be established between different GA concentrations either singly or in combination with either kinetin (Experiment 9) or NAA (Experiment 10). These results add to the existing controversies regarding GA effects in tissue culture. Smith (1968) for example found that GA overcame the inhibitory effect of kinetin on shoot apical meristems of wheat.

(d) Shoot and leaf growth:

(i) Auxin : Kinetin effects: The interaction which existed between NAA and kinetin in relation to root formation was also observed in regard to leaf development. The favourable features of high kinetin (4.0 to 18.0 mg/l) on leaf development were counteracted by the presence of NAA (Experiment 8). It was difficult to decide whether a certain proportion of shoot growth accompanied leaf growth. These findings agree well with the generally accepted idea that NAA inhibits bud formation while kinetin stimulates bud formation (Haissig, 1965). As mentioned earlier tobacco callus differentiates both roots and shoots when cultured on a combination of auxin and cytokinin, provided the ratio between the two substances is correct. As in the case with root formation no such ratio could be established for rose shoot tips. Evidence in Experiment 11 suggested this may be due to differences between auxin species and that if the correct auxin and cytokinin species are combined in the correct proportion such a ratio may be established.

Malformed leaves (Experiment 8, Fig. 9) which did develop in some instances could have been due either to the unfavourable effect of the NAA as such, or to the NAA : kinetin ratio being out

of balance. The possibility exists that malformed leaves may be linked to the inherent nature of the NAA and that other auxins may not show the same degree of malformation of the leaves. That great differences between different species of auxin exist was clearly shown by Experiment 11 (Fig. 21). The hope of obtaining normal leaf development in the presence of an auxin was almost abandoned after considering the results of Experiment 8 where NAA was used. Although the leaf growth which took place in the presence of IAA was by no means as good as in the presence of high kinetin (Experiments 8 and 9), it was sufficiently good to state that IAA does not inhibit leaf development in the same manner as NAA. It would be most interesting to know how an IAA : kinetin grid experiment will effect the growth of rose shoot tips.

It was observed that some shoot tips underwent a second phase of leaf growth after the first leaf initials that grew and had died (Figs. 24a and b), when cultured on 8 mg/l kinetin for extended periods. In addition a slight formation of callus took place at the basal cut surface. This suggested that the endogenous auxin content of such tips was high enough to account for the callus growth, but more important, the endogenous auxin did not inhibit leaf development. This observation gives further support to the view that auxins other than NAA may have a more desirable effect on leaf development.

(ii) GA : Kinetin effects: The existence of an interaction between kinetin and GA in relation to leaf development is clear from Figs. 16 and 17 (Experiment 9). The favourable effects of high kinetin (8.0 and 16.0 mg/l) was diminished by intermediate concentrations of GA (0.5 to 4.0 mg/l). The phenomenon suggests

Fig. 24. (a) and (b) Shoot tips which underwent a second phase of leaf growth.

that ratio effects may possibly be involved, although no unequivocal explanation could be given for this peculiar behaviour of the shoot tips when treated with different concentrations of GA in the presence of high kinetin. Although not similar, a related behaviour of certain plant tissues to some amino acids was reported by other workers (David, 1963; Riker and Gutsche, (1948). These workers found that low concentrations of certain amino acids inhibit growth of plant tissue cultures whereas higher concentrations did not, but they could not explain this peculiar situation either. The observation (Experiment 9) that leaf growth was slightly slower at 8.0 mg/l kinetin than at 16.0 mg/l and that tips remained alive for longer periods at 8.0 mg/l may be an advantage, especially when sub-culturing.

(e) Sequential administration of growth regulating substances:

Very little information was gained from Experiment 12 on the sequential administration of growth regulators. It is difficult to give an account of the great variation observed with the same treatment when sub-cultured. This suggests that the tips at the time of transferring were in different physiological conditions, and their variable response to the treatments when sub-cultured is an outcome of the physiological variation. It is also possible that the time of year in which the tips were sampled may affect the degree of variation. Similar variations in tissue cultures were observed by Vasil and Hildebrandt (1966b, c.).

GENERAL CONCLUSIONS

Although the inorganic fraction of a culture solution may prove suitable for the culture of tissues of many different species, over-generalization should be avoided. A culture solution which may support excellent growth of tissue cultures of one species may inhibit or retard growth of another. A case in point is the inhibition of rose pith tissue when cultured on the medium developed for tobacco callus by Murashige and Skoog (1962). This inhibition is possibly due to NH_4^+ content. With regard to rose tissue cultures the modified Berthelot (1934)-Knop (1865) solution is superior to that of Murashige and Skoog (1962).

The precise culture media and conditions for differentiating rose callus into shoots and roots is still unknown. Nevertheless the occasionally observed instances of differentiation in rose calli, as reported in this study, is good evidence that cultured rose tissue is capable of becoming fully totipotent, but further work, for example in manipulating the applications of growth substances, is necessary.

Large shoot tips (8 mm) root much more readily than small ones (1 to 2 mm) and should stand a better chance of growing into an intact plant. This is possibly due to more reserves being present in the larger tip, and also that the tissue has reached a higher degree of differentiation which favours root formation.

The fact that different species of growth substances belonging to the same class of example auxins may vary greatly in their effects on the quantitative and qualitative expressions of

growth in tissue cultures is often overlooked. This study re-emphasises the importance of examining the effects on growth of the various species of growth substances belonging to each of three major classes.

This step is important since the success of future experimentation along the lines of varying ratios and sequential applications of growth substances may depend on the selection of the appropriate species of growth substances. The selection of the appropriate growth substance will depend on the type of growth which is to be investigated. For example if leaf growth and development of shoot tips in culture is to be investigated the auxin species which has the most desired effect on leaf growth should be selected, regardless of whether it gives the best callus growth or root formation. That inter-species differences do exist in regard to growth and development of plant tissue cultures has been clearly shown by this study as well as by other workers (Skoog et al, 1967; Wolter, 1968).

The use of different ratios and sequential application of growth substances, developed by workers as an experimental tool for investigating growth and development of pure tissue cultures, have proved very useful in this study on rose shoot tips, and the author finds no justification for the statement made by Hollings (1965) that very little information gained by true tissue cultures is applicable to shoot tip and shoot apical meristem cultures.

The successful culture of rose shoot tips and shoot apical meristems into independent plantlets is a prerequisite for possibly eliminating virus diseases from rose. This study

has only partially solved this problem, since shoot tips could be induced to develop roots only or leaf growth only, with possibly some degree of shoot growth. Until such time as both shoot and root growth can be combined in one and the same shoot tip, thus giving rise to a normal intact plant, indexing for the presence of virus diseases will not be feasible. Combining different concentrations and species of auxins and cytokinins in two dimensional grid experiments as done in this study, may lead to the establishment of a culture solution which could induce both root and shoot growth in rose shoot tips. Alternatively, if the above approach fails, further experimentation with the sequential administration of growth substances may prove worth while.

As information is increasing in this field it is likely that more basic concepts will be explored and combined with the existing few, namely, that (i) growth substances individually, (ii) different ratios of growth substances, (iii) synergistic phenomena between growth substances and possible other substances, and (iv) their sequential administration to tissue cultures, will aid in solving one of nature's most complex and intricate mysteries, that of differentiation.

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