THE CULTIVATION OF ISOLATED ROOTS OF SUBTERRANEAN CLOVER AND EFFECTS OF AMINO ACIDS ON THEIR GROWTH PATTERN

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[*Manuscript received December* 20, 1960]

Summary

First-passage, isolated roots of subterranean clover (*Trifolium subterraneum* L.) were cultured in a basal medium of macro- and micronutrient salts and sucrose at pH 5·5. Effects of additives to this basal medium on growth rate and growth pattern of these roots and survival and growth of subcultures have been investigated.

A mixture of the vitamins thiamine, pyridoxine, and nicotinic acid doubled the rate of main axis elongation, and the addition of yeast extract further increased elongation rate. This effect of yeast extract was duplicated by casein hydrolysate and a synthetic mixture of amino acids. It was not an unspecific response to nitrogen.

High concentrations of yeast extract and casein hydrolysate inhibited main axis growth, but the latter promoted lateral growth—apparently breaking the dominance of the main axis. The effect of casein hydrolysate upon the growth of laterals could be simulated by a mixture of histidine, tryptophan, and arginine in concentrations corresponding to those in casein hydrolysate. Histidine and tryptophan strongly inhibited the growth of the main axis but they seemed to arouse growth in the lateral roots. Arginine promoted main axis growth and enhanced the growth of initiated laterals. In experiments involving growth of roots in inhibitory concentrations of a wide range of amino acids, no evidence could be found for a protective action of arginine against this inhibition.

In basal medium plus yeast extract and vitamins, the main axis meristem could be subcultured through only three transfers. Lateral roots which had grown to longer than 5 cm have been subcultured through 25 transfers in basal medium plus vitamins, yeast extract, casein hydrolysate, and glutamine. Shorter laterals and the main axis survived only one transfer in this medium.

I. INTRODUCTION

The culture of isolated roots is now a standard laboratory procedure. Over 100 species have now been grown through many transfers, many of them in simple and defined media. But many species have been reported to fail to grow, even in the best media devised (Street 1957).

Of the clovers, white and red clover have been successfully subcultured many times in simple media, without diminution in growth rate, but clones have been established only with difficulty (Bonner 1940; Dawson and Street 1959).

No previous reports of attempts to cultivate isolated roots of subterranean clover are known. Although the present attempt was not completely successful, it has at the same time contributed information on factors involved in the pattern of growth of roots and the nutritional requirements of isolated roots.

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II. Materials and Methods

Seeds of *Trifolium subterraneum* L. cv. Bacchus Marsh, were surface-sterilized by immersion in ethanol, followed by treatment for 10 min with 0·1% mercuric chloride, and by washing alternately with several changes of alcohol and 2% saturated bromine water.

The seeds were spread in sterile petri plates containing basal medium plus 0·7% agar, held at 4°C for 48 hr to break dormancy and give uniform germination, and then incubated at 20°C. When the radicles were 3 cm long, terminal tips 5 or 10 mm long were excised from selected radicles and transferred aseptically to 40 ml of sterile medium in 150-ml conical flasks. The root cultures, one per flask, were incubated in darkness at 25°C. Although 20°C was optimal for germination, subsequent root growth proceeded fastest at 25°C; and this temperature was also optimal for the initiation of growth in subcultured roots.

Basal medium contained Bonner's macronutrient salts (Bonner 1940), White's micronutrient salts (White 1943), with the addition of 0·05 mg/l $CuSO_4.5H_2O$, 0·13 mg/l $(NH_4)_6Mo_7O_{24}.4H_2O$, and ethylenediaminetetraacetic acid (EDTA) equivalent to the heavy metal content. Maximal growth was found to occur when the pH of the medium after autoclaving was 5·5–6·0. Hence the pH of the basal medium was adjusted to 5·5 with $KHCO_3$ before autoclaving. When amino acids were added to media the L-forms were always used.

The concentration of sucrose most favourable to growth was found to be 1·5–2%, and at 1·5%, sucrose was a better carbon source than glucose, fructose, mannose, or soluble starch at equivalent carbon concentration. Basal medium contained 1·5% sucrose.

Due to difficulties in producing clones and in serially subculturing the apical meristems, which will be discussed later, all experiments were based on first-passage roots derived from a single population of seed which was stored at 4°C.

The lengths of the main axes were measured *in situ* at 3-day intervals, and, where qualitative differences in growth patterns occurred, shadowgraphs were made and dry weights were taken at the conclusion of an experiment and at each subculture. Ten or 20 replications of each treatment were made.

In some experiments the growth of isolated roots was compared with that of roots of intact seedlings. The latter were obtained in sterile culture by supporting sterile seed, 1 cm above the surface of media in conical flasks, on glass slides covered with filter paper.

III. Experimental and Results

(*a*) *Influence on Growth of Supplements of Vitamins and Yeast Extract*

A mixture of the three vitamins thiamine, nicotinic acid, and pyridoxine, one or more of which are commonly required by isolated roots (Street 1957), doubled the length of isolated subterranean clover roots compared with controls in basal medium alone (Fig. 1). However, this increased rate of growth was considerably slower than that produced by the primary root tip of an intact seedling (see Fig. 5).

The addition of yeast extract (Difco, extracted with ether at pH 3) gave greater main axis length growth than the three vitamins (Fig. 1), and its effect was additive to that of the vitamins. Figure 2 shows that higher concentrations of yeast extract were inhibitory, and that the optimal concentration for the promotion of the main axis was 100 mg/l.

The experiment recorded in Table 1 demonstrates the effect of the vitamins and yeast extract upon the growth of roots through three serial subcultures of the primary root tip. Subcultures were made at 14, 24, and 34 days and the experiment concluded 45 days from first transfer. During this time the root becomes

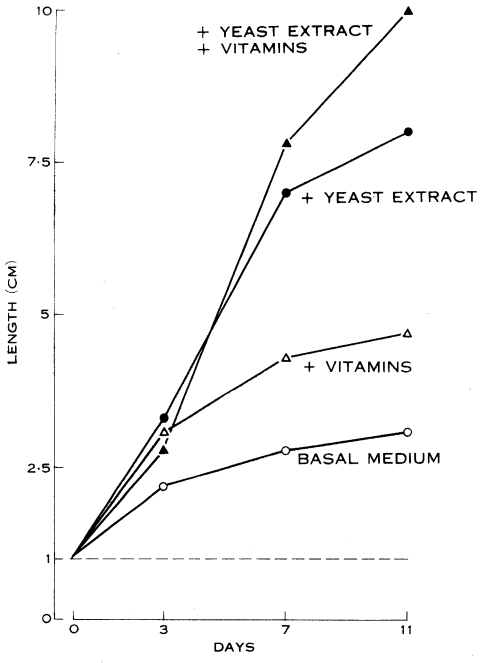

Fig. 1

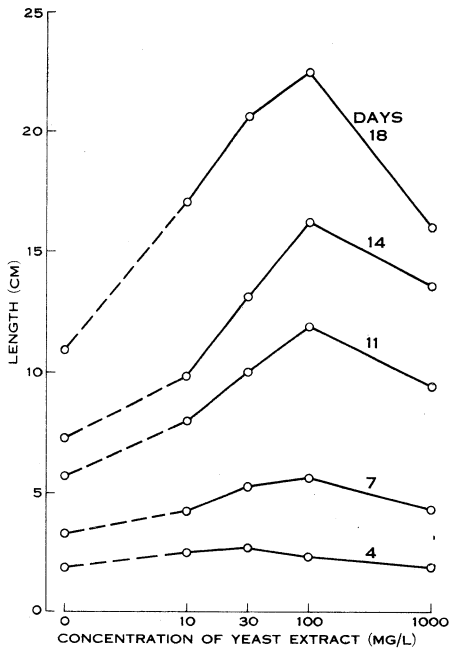

Fig. 2

Fig. 1.—Additive promotion of main axis length of isolated roots of *T. subterraneum* by yeast extract (50 mg/l) and a mixture of the vitamins thiamine (0·1 mg/l), nicotinic acid (0·5 mg/l), and pyridoxine (0·1 mg/l). Least significant differences between mean lengths:

Level	3 Days	7 Days	11 Days
5%	0·80	1·83	2·09
1%	1·08	2·46	2·80

Fig. 2.—Effect of yeast extract on the main axis length of isolated roots of *T. subterraneum*. Least significant differences between concentrations of yeast extract at 18 days are 2·74 at 5% level and 3·67 at 1% level.

more dependent upon externally applied substances. Growth measurements were based on the dry weights of the roots minus the weights of the 10-mm tips that had been cultured. In all treatments the growth rate diminished. Yeast extract alone produced a high initial rate, but no roots survived a second transfer. No group without nicotinic acid survived a second transfer while those with nicotinic acid

survived at least the fourth transfer. Thiamine and pyridoxine both produced a growth response, but their presence was not as essential as nicotinic acid. Figure 3 shows the average, daily, main axis length increments for the fastest (yeast extract, thiamine, nicotinic acid, and pyridoxine) and the slowest (control) groups of roots. The growth rate of the fastest group, during the first passage, is higher than any found in the literature for any species and is at least as great as that of a root attached to the plant.

In all subsequent experiments, basal medium was supplemented with 0·1 mg/l thiamine, 0·5 mg/l nicotinic acid, and 0·1 mg/l pyridoxine.

TABLE 1

EFFECT OF VITAMINS AND YEAST EXTRACT UPON THE GROWTH OF ISOLATED SUBTERRANEAN CLOVER ROOTS

Concentration of thiamine 0·1 mg/l, of nicotinic acid 0·5 mg/l, of pyridoxine 0·1 mg/l, of ether-extracted Difco yeast extract 50 mg/l

Addendum to Basal Medium	Dry Weight Increases (μg/root/day) (average of 10 replicates)			
	1st Passage	2nd Passage	3rd Passage	4th Passage
Control	70	8	—	—
Thiamine	180	10	—	—
Nicotinic acid	180	72	54	54
Pyridoxine	180	13	—	—
Nicotinic acid + thiamine	250	141	100	80
Thiamine + pyridoxine	280	11	—	—
Nicotinic acid + pyridoxine	250	87	80	51
Nicotinic acid + thiamine + pyridoxine	260	87	89	79
Yeast extract	400	24	—	—
Yeast extract + nicotinic acid + thiamine	540	642	204	134
Yeast extract + nicotinic acid + thiamine + pyridoxine	600	536	167	66

(b) Influence upon Growth of Supplements of Casein Hydrolysate or Amino Acids

Yeast extract contains 15% amino acids. An attempt was therefore made to test whether it could be replaced as a root growth supplement by other amino acid mixtures.

Casein hydrolysate (Difco tryptic digest of casein) promoted main axis length growth. Figure 4 shows that, in concentrations up to 100 mg/l of casein hydrolysate, the extension of the main axis may be more than doubled. Beyond this concentration, casein hydrolysate is strongly inhibitory. A synthetic mixture of amino acids in the same proportions as in casein hydrolysate gave an identical result, which ruled out the possibility of the effects of casein hydrolysate being due to peptides or to impurities in the casein or the trypsin.

Even low concentrations of many individual amino acids are notoriously toxic to plant organs (Street 1957), so it seemed fortuitous that the balance of amino acids in casein hydrolysate permitted a growth-promoting effect to manifest itself.

Casein hydrolysate and yeast extract together did not promote main axis growth above that obtained with casein hydrolysate or yeast extract alone (Fig. 5). The same initial growth rate was also obtained for the root main axis of intact seedlings, grown in basal medium plus vitamins.

The particular amino acids in casein hydrolysate which are responsible for stimulating the growth of the root main axis have not been sought exhaustively, but it has been found that arginine and possibly glutamine and asparagine are involved. This is not an unspecific nitrogen response, since the basal medium contains

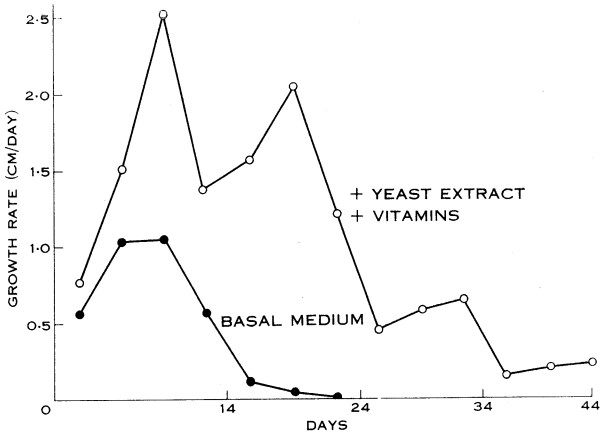

Fig. 3.—Effect of yeast extract plus vitamins on the daily length increment of isolated roots of *T. subterraneum* through three serial subcultures. Subcultures were made 14, 24, and 34 days from first transfer. Concentrations of vitamins are: thiamine, 0·1 mg/l, pyridoxine, 0·1 mg/l, and nicotinic acid, 0·5 mg/l.

$2{\cdot}8\times10^{-3}$M nitrate. In Figure 4, growth after 13 days is doubled by casein hydrolysate at 30 mg/l, which is equivalent to $0{\cdot}3\times10^{-3}$M nitrate. Doubling the concentration of nitrogen in the basal medium by adding nitrate, ammonium, urea, or glutamic acid gave no increase in growth.

(*c*) *Influence of Casein Hydrolysate and some Amino Acids upon Growth Pattern*

Casein hydrolysate, in addition to its effect on the length of the main axis, has a striking qualitative effect on the growth pattern of the root. Subterranean clover roots, both in the isolated state and attached to the seedling, strongly exhibit main-tip dominance. No lateral roots emerge within 5–8 cm of the main axis tip, and no laterals within 20–30 cm of the tip grow at a rate comparable with that of the main axis tip. As they become older, laterals seem to undergo a discrete change which permits them to grow more rapidly. In the isolated root, lateral roots are initially

much thinner and grow at only 5–10% of the rate of the main axis, and such lateral meristems when excised, nearly always fail to grow.

As the laterals pass into the phase of rapid growth, they thicken and the zones of cell division and elongation become much longer. Casein hydrolysate has a striking effect in promoting the growth of the laterals (Fig. 6; Fig. 7(*i*)). Figure 6

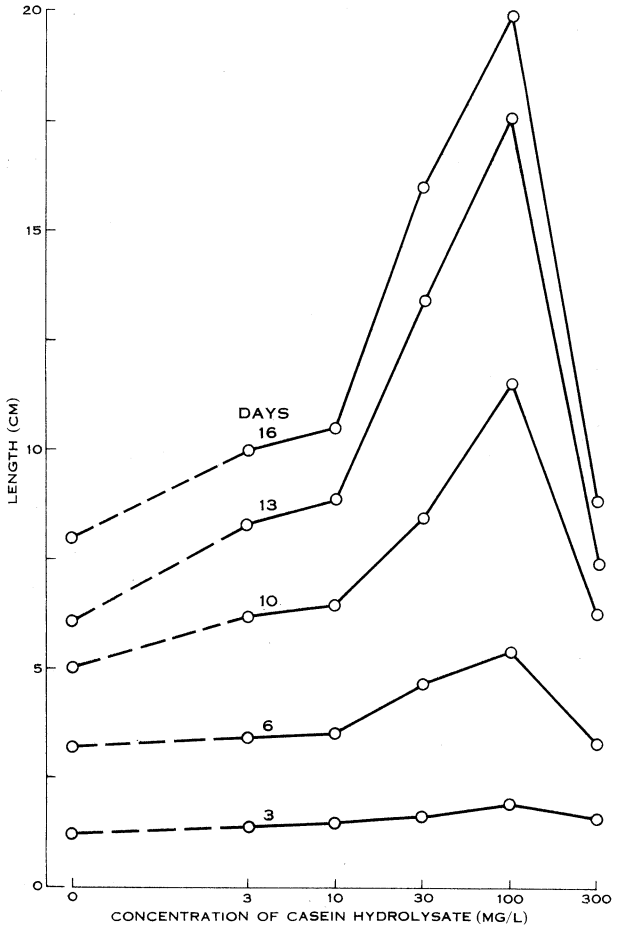

Fig. 4.—Effect of casein hydrolysate (tryptic digest of casein) upon main axis length of isolated roots of *T. subterraneum*. Least significant differences between concentrations of casein hydrolysate at 16 days are 2·72 at 5% level and 3·64 at 1% level.

shows the stimulation of the number of laterals at low casein hydrolysate concentrations. At higher concentrations, supra-optimal for main axis length and lateral number, the total dry weight continues to increase, i.e. the growth of laterals already present is preferentially promoted. Table 2 shows that at 140 mg/l, casein hydrolysate inhibited the growth of the main axis and while the number of emerged laterals is not different, the average total length of laterals is increased fourfold.

A synthetic mixture of amino acids equivalent to casein hydrolysate had an identical effect on the growth of laterals. Identification of the amino acid or combination of amino acids responsible, would involve testing 24 amino acids singly, two at a time, three at a time, etc. and would be a tremendous task. This was not undertaken. Instead, blocks of amino acids were tested in combination, ignoring interactions, and where promoting effects on lateral growth were indicated, the blocks were broken down into individual amino acids and simple combinations of them.

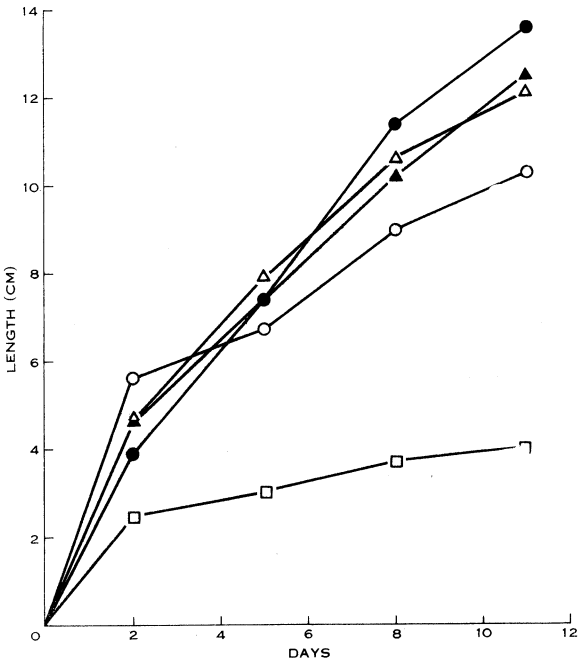

Fig. 5.—Comparison of the effect of yeast extract and casein hydrolysate, alone and together, on the main axis length of isolated roots of *T. subterraneum*, with the growth of roots of intact seedlings. □ Isolated roots in basal medium plus vitamins. ● Isolated roots in basal medium plus vitamins plus yeast extract (50 mg/l). ▲ Isolated roots in basal medium plus vitamins plus casein hydrolysate (30 mg/l). △ Isolated roots in basal medium plus vitamins plus yeast extract (50 mg/l) and casein hydrolysate (30 mg/l). ○ Intact seedlings in basal medium plus vitamins. Least significant differences between treatments:

Level	2 Days	11 Days
5%	1·37	3·49
1%	1·83	4·69

Although other possibilities exist, it was found that the effect of casein hydrolysate on laterals could be simulated by a mixture of histidine, tryptophan, and arginine in concentrations corresponding to their concentrations in casein hydrolysate (see Table 3 and Fig. 7). Histidine and tryptophan are both strongly inhibitory to the growth of the main axis, but they seem to initiate rapid growth in the laterals. Added arginine not only partially overcomes the suppression of growth of the main apex by tryptophan and histidine, but it also enhances growth in the initiated laterals

(cf. Fig. 7). Figure 8 shows that arginine can promote the growth of the main axis inhibited by histidine plus tryptophan and can increase the dry weight of roots under these conditions.

Goldacre (1957) found that 2,6-diaminopurine, at concentrations of 3×10^{-9}M, and below, promoted the growth of the main axis of *isolated* subterranean clover roots without any change in growth pattern. In *whole* seedlings the growth of the root main

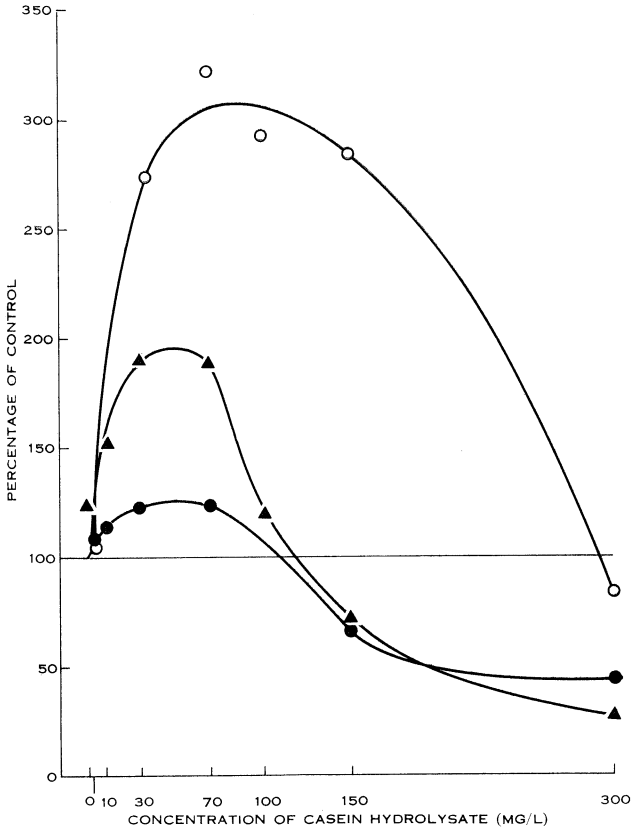

Fig. 6.—Effect of casein hydrolysate upon dry weight (○), number of lateral roots (▲), and main axis length (●) of isolated roots of *T. subterraneum* grown for 11 days in basal medium plus vitamins.

axis was stimulated by low concentrations while the laterals were stimulated by concentrations supra-optimal for main axis growth. As this differential promotion occurs only in the intact seedling it was suggested that substances normally produced in the aerial portions of the plant may be necessary for the stimulation of secondary root meristems by 2,6-diaminopurine. Because of their effect on lateral root development discussed above it seemed possible that these substances may in fact be tryptophan, histidine, and arginine. Therefore first-transfer roots were grown in medium supplemented with 16·8 mg/l tryptophan, 2·98 mg/l histidine, and 6·24 mg/l

arginine, with and without 3×10^{-9}M or 10^{-7}M 2,6-diaminopurine. The amino acids at these concentrations stimulate lateral growth, but they had no effect upon the growth of laterals in concentrations of 2,6-diaminopurine which are supra-optimal for main axis growth. That is, the amino acids did not substitute for the tops.

TABLE 2

EFFECT OF CASEIN HYDROLYSATE ON THE GROWTH OF THE MAIN AXIS AND THE GROWTH OF LATERAL ROOTS OF SUBTERRANEAN CLOVER

Incubated 13 days at 25°C

Treatment	Average Length of Main Axis (cm)	Average Number of Emerged Laterals	Average Total Length of Laterals (cm)
Control	15·3	30·2	7·5
Casein hydrolysate (140 mg/l)	11·0	36·0	27·7

(d) Growth in the Presence of Individual Amino Acids and the Effects of Arginine

It was observed that arginine promoted subterranean clover root growth, alone and in the presence of other amino acids although the effect was variable. Harris (1953) had observed the interaction of amino acids whereby one amino acid

TABLE 3

EFFECT OF AMINO ACIDS UPON THE GROWTH OF ISOLATED SUBTERRANEAN CLOVER ROOTS

Incubated 10 days at 25°C. The concentrations of amino acids are equivalent to those found in 140 mg/l casein hydrolysate

Amino Acid added to Basal Medium plus Vitamins	Dry Weight per Root (mg)		Length of Main Axis (cm)*	
	Alone	With Arginine (6·24 mg/l)	Alone	With Arginine (6·24 mg/l)
Control	4·01	5·86	22·7	24·3
Tryptophan (16·8 mg/l)	4·23	7·96	11·2	15·2
Histidine (2·98 mg/l)	2·85	4·61	8·0	14·0
Tryptophan + histidine	3·86	9·88	6·5	15·8
Synthetic casein hydrolysate (140 mg/l)	11·19	—	25·3	—

* L.S.D. between mean lengths of main axis: 4·15 at 5% level, 5·52 at 1% level.

relieved the inhibition by another amino acid of the growth of roots of *Avena* embryos. Gullino *et al.* (1955) found that the toxicity of individual amino acids to rats was reduced by arginine. The inhibition of growth of tobacco pith callus by valine has been found to be relieved by isoleucine (Sandstedt and Skoog 1960).

It was of interest then to measure the main axis elongation and dry weight increases of first transfer roots after 14 days growth in a series of concentrations (2×10^{-6}M–128×10^{-6}M) of amino acids without and with 10^{-3}M arginine. Arginine alone consistently increased the main axis length and dry weight of roots, but the amount of this increase varied between 5 and 100% on different occasions No inhibition of growth was obtained with arginine at concentrations up to 10^{-3}M.

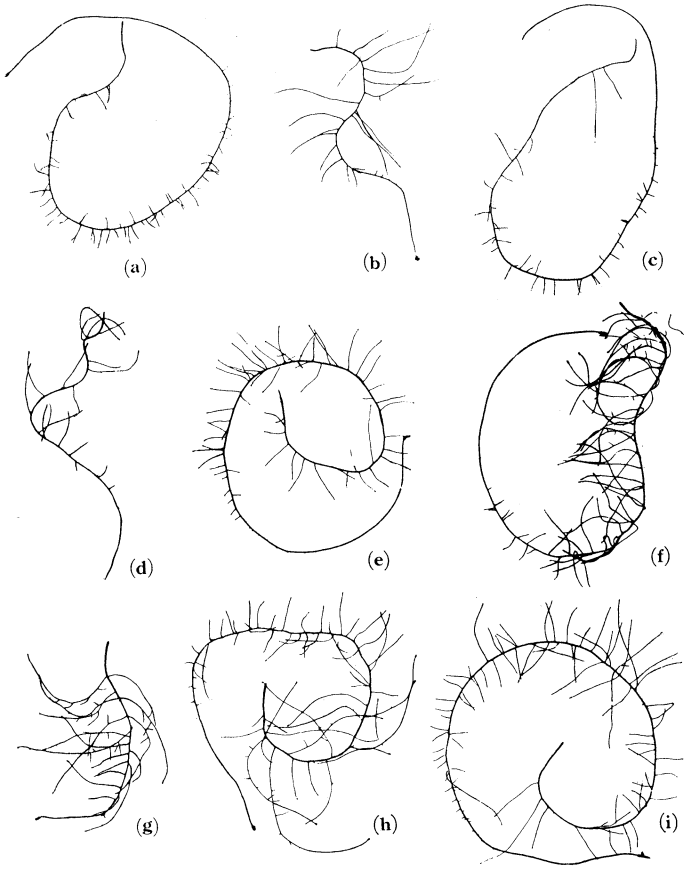

Fig. 7.—Shadowgraphs of isolated roots of *T. subterraneum* grown for 12 days in basal medium containing vitamins plus: (*a*) no addendum; (*b*) histidine, 2·98 mg/l; (*c*) arginine, 6·24 mg/l; (*d*) tryptophan, 16·8 mg/l; (*e*) histidine, 2·98 mg/l, plus arginine, 6·24 mg/l; (*f*) tryptophan, 16·8 mg/l, plus arginine, 6·24 mg/l; (*g*) histidine, 2·98 mg/l, plus tryptophan, 16·8 mg/l; (*h*) histidine, 2·98 mg/l, plus tryptophan, 16·8 mg/l, plus arginine, 6·24 mg/l; (*i*) casein hydrolysate, 140 mg/l. The concentrations of amino acids are equivalent to those found in casein hydrolysate at a concentration of 140 mg/l.

Those amino acids which did not influence growth were: glutamic acid, glutamine, glycine, and asparagine. Phenylalanine, isoleucine, hydroxyproline, tryptophan, aspartic acid, and serine inhibited at all concentrations or were ineffective at low concentrations and inhibited at high concentrations. Lysine, methionine, leucine, histidine, threonine, alanine, cysteine, valine, tyrosine, and proline slightly promoted

growth at a low concentration and inhibited at high concentrations. Arginine had the same general effect in all experiments. The promotion of growth obtained in the presence of arginine alone was more or less maintained in all concentrations of another amino acid. There was no evidence for synergism or of a protective effect of arginine from the inhibition by other amino acids.

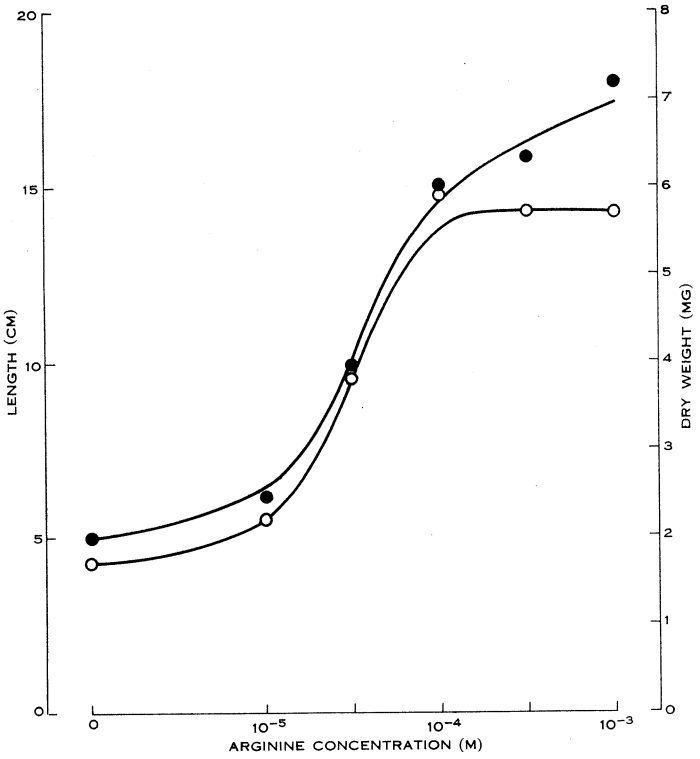

Fig. 8.—Effect of arginine upon the main axis length (●) and dry weight (○) of isolated roots of *T. subterraneum* cultured for 14 days in basal medium plus vitamins, histidine (2·98 mg/l), and tryptophan (16·8 mg/l). In absence of amino acids, weight was 0·5 g and length was 12·5 cm. Least significant differences between arginine concentrations:

Level	Length	Dry Weight
5%	1·57	0·74
1%	2·09	0·99

(*e*) *Subculturing of Subterranean Clover Roots*

As was seen in Figure 3, the addition of yeast extract plus the three vitamins thiamine, pyridoxine, and nicotinic acid did not sustain the main axis meristem beyond three transfers. The following attempts were made to increase the number of viable transfers.

Street (1954) had shown that the substance α-(1-naphthylmethylsulphide)-propionic acid (NMSP) overcomes the "aging" of the main axis meristem of tomato

roots. Although NMSP increased the growth of the main axis of first-transfer subterranean clover roots, it did not improve the growth of subsequent subcultures. The possibility that substances in seeds, contributing to root growth, would diffuse out of seeds and influence the growth of excised roots, was investigated. Diffusates of subterranean clover seeds were found to be inhibitory to isolated roots. Other substances which did not increase the growth of subcultured main axis meristems were pantothenic acid, riboflavin, biotin, inositol, folic acid, and *p*-aminobenzoic acid.

Street and Roberts (1952) found that the main axis meristem of tomato could be subcultured a limited number of times and obtained clones by excising laterals alternately with the main axis meristem. This procedure was not effective with subterranean clover because the growth of the laterals is also unreliable. As pointed out previously, the laterals of subterranean clover roots undergo an abrupt change from very slow to rapid growth. The ability of laterals in these two states to be subcultured has been studied and compared with the performance of the main axis meristem. The supplements to the basal medium plus vitamins were 50 mg/l yeast extract, 70 mg/l casein hydrolysate, and 50 mg/l glutamine. It was found that the main axis and laterals less than 5 cm long could be subcultured only once. Laterals longer than 5 cm, however, were subcultured 25 times over a period of two years, but the chance of an individual tip surviving a transfer was only 50%. Experience showed that the lateral root tips which survived were those which floated on the medium after excision.

IV. Discussion

Excised root culture is still mainly in the descriptive, comparative phase of investigation. This paper adds to the descriptive facts with particular reference to a peculiarity of subterranean clover roots: the strong dominance of the main axis tip over the laterals.

Other workers have shown similar effects of exogenous amino acids upon the growth of isolated roots, particularly groundsel and red clover roots (Skinner and Street 1954; Charles and Street 1959; Dawson and Street 1959; and Harris 1959).

The results emphasize the complexity of the isolated root as a growth system. Although isolated roots are sometimes classified as "tissue cultures", they are more accurately described as organ cultures, and these experiments suggest that the isolated subterranean clover root is an organ of changing synthetic capacity. Lateral meristems, either on the intact plant or on an isolated root in the best medium available, grow slowly and make no further growth when subcultured. After a time these laterals undergo an abrupt change which produces rapid growth both on the intact plants and on an isolated root and allows a reasonable chance of successful subculture. A combination of histidine, tryptophan, and arginine hastened the transformation of lateral meristems from sluggish to active growth but no clues could be found concerning the reason for the transformation *in situ*. Experiments on subculturing the two types of lateral and the primary root suggested that the state of the meristem at the time of excision, rather than the exogenous nutritional conditions determines growth behaviour.

V. Acknowledgment

The statistical treatment of the data was done by Mr. G. A. McIntyre, Division of Mathematical Statistics, C.S.I.R.O.

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