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A TISSUE EMBEDDING TECHNIQUE FOR MEASURING THE STRUCTURE OF HAIRY ROOT MATS OF *Tagetes erecta*

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Abstract – A tissue embedding protocol which preserves root diameter was developed for hairy root mats of *Tagetes erecta*. Embedded root mats were sectioned, stained and the cut surfaces were subjected to image analysis to determine the void fraction of the root mats. Void fractions of mats of different ages, grown from inocula containing different numbers of tips, were analyzed. The method was found to give reproducible values for the void fractions, exhibiting reasonable trends with respect to initial tip number and mat age.

Key words: Hairy Root Mat, *Tagetes erecta*, Plant Tissue Culture, Embedding, Image Analysis

INTRODUCTION

Transformed roots, or hairy roots, are obtained by infection of susceptible plant species with the soil bacterium *Agrobacterium rhizogenes*. Hairy roots are characterized by a high degree of lateral branching, a profusion of meristemic root tips, and high overall growth rates which, in some cases, are comparable to those of plant cell cultures [Sharp and Doran, 1990]. These roots produce the secondary metabolites characteristic of normal roots, at levels comparable to or even exceeding those of the roots of intact plants. However, in contrast to untransformed roots, they grow rapidly in phytohormone-free media [Kamada et al., 1986]. Hairy roots typically produce secondary metabolites at much higher levels than cell cultures but, in contrast to cell cultures, exhibit genetic stability. Because of these characteristics, hairy root culture has been suggested as a system for economic, large-scale production of plant-derived secondary metabolites [Kamada et al., 1986; Toivonen, 1993].

Hairy roots, when grown in suspension culture, form heterogeneous mats of entangled roots in which the branch density varies throughout the mat [Whitney, 1992]. Conditions inside the mat differ from those in the bulk liquid because the entangled root mat impedes mixing and mass transfer [Sharp and Doran, 1990; Prince et al., 1991; Whitney, 1992]. This may severely impair growth, resulting in cell death and necrosis at the core of dense root mats [Yu and Doran, 1994]. Oxygen is one of the least soluble of the nutrients required by plant tissues, and is therefore likely to be the limiting substrate in the interior of root mats. The problem of supplying nutrients, oxygen in particular, to the interior of root mats can be a serious impediment to scale-up [Prince et al., 1991]. Furthermore, mat formation may be related to other root characteristics such as growth rate, production level, and adaptability to liquid culture [Yu and Doran, 1994]. It is therefore evident that understanding the structure of hairy root mats (e.g., void fraction, root thickness, surface area) and how they evolve is essential to comprehending hairy root growth kinetics and to conducting a rational scale-up of hairy root cultures.

Understanding the structure of a hairy root mat requires a method of measuring the porous structure of these mats. In this paper, a technique that is directly applicable to the measurement of the void fraction of hairy root mat is described. Root mats are fixed and dehydrated, then infiltrated with a monomeric compound which is subsequently polymerized to produce a solid block containing the embedded root mat. The block is then sectioned and embedded roots are stained prior to image analysis to measure the void fraction in the mat at the exposed site.

Fixation of the root mat is done to preserve the *in vivo* cell structure in subsequent steps of the procedure. Since different tissues may respond differently to a given fixative, fixation could cause dimensional changes in the intercellular spaces. The pH, total ionic strength, specific ion composition, dielectric constant of the fixative, osmolality, additives, temperature, length of fixation, and method of application of the fixative are critical factors in determining the quality of tissue fixation. The best fixation protocol for hairy roots was determined by testing different fixatives and fixation conditions on single root tips. The protocol giving the least amount of change in root diameter was chosen as the best fixation method for the purpose of measuring the void fraction in root mats.

Embedding is the final stage of the preparation of a biological specimen prior to thin-sectioning. In this step, the biological specimen is immersed in a liquid embedding medium which is then polymerized to produce a solid block. The ideal embedding medium should be easily available, preserve tissue structure with minimal extraction of cellular constituents, be soluble in the dehydrating agent, have low viscosity as a monomer, polymerize and harden uniformly with little volume change and yield solid blocks which are easily sectioned [Glauert, 1974]. The requirement of low viscosity is particularly important for root mat embedding because only low viscos-

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ity embedding media can penetrate the mat without changing its structure. None of the embedding media now available possess all of these qualities and therefore compromises must be made when designing the best protocol. In developing the protocol described in this paper, we sought an embedding that i) preserved root diameter, ii) produced polymer blocks with good mechanical properties suitable for sectioning, iii) produced porous blocks to allow for staining of the embedded roots, and iv) was economical and practical.

MATERIALS AND METHODS

1. Plant Material and Culture Methods

The hairy root clone of *Tagetes erecta* T3 was established and maintained as described elsewhere [Mukundan and Hjortso, 1990]. Roots were grown in 50 ml of MS-medium [Murashige and Skoog, 1962] modified with Gamborg's vitamins and iron [Gamborg et al., 1968] in a 125 mL Erlenmeyer flask. The medium was supplemented with 30 g/L sucrose and the pH was adjusted to 5.75 with 0.1 N KOH or 8 % phosphoric acid prior to autoclaving. Flasks were autoclaved for 30 minutes at 15 psig and 121 °C prior to use. Each flask was inoculated with a range (4, 10, 15, 20, 25, 30) of 1-cm-long root tips (lateral branches). The flasks were placed on a rotary shaker at 110 rpm and maintained in the dark at 25 °C.

2. Single Root Tip Protocols

2-1. Fixation

Approximately 0.3-cm-long terminal root tips with uniform thickness were dissected from a growing root and immediately placed into glass vials containing 3 mL of the fixative. The pH of the solutions was adjusted before use if necessary. For room temperature fixation, the vial was placed in an Erlenmeyer flask, on a rotary shaker, and stirred at 75 rpm to facilitate the penetration of fixative. For the low temperature fixation (4 °C), the vial was placed in a refrigerator and stirred for 5 minutes every hour. After fixation, the roots were placed in tap water on a glass slide, photomicrographs at 10× were made and the diameter of the root tip was later measured by comparison of each photograph against a photo of a micro-ruler. The change in diameter was measured after 2 hours and again after 24 hours. Table 1 lists the fixatives which were tested.

2-2. Dehydration and Infiltration

After fixation, the root tip was dehydrated in a series of graded ethanol/water solutions (10 %, 30 %, 50 %, 70 %, 90 % and 100 %, v/v%) at room temperature. The vial was shaken for 10 minutes per step. After dehydration, the tip was transferred to a series of infiltration solutions, graded embedding mixture/ethanol (10 %, 30 %, 50 %, 70 %, 90 %, and 100 %, v/v %), and shaken, at room temperature, for 30 minutes per solution. After each step (fixation, dehydration, and infiltration) the tip was photographed for later determination of diameter.

3. Root Mat Protocols

3-1. Fixation

For fixation of root mats, the MS-medium was removed slowly through a narrow glass pipette attached to a peristaltic pump. Because the hairy root clone used in these experiments is brittle, contact between the glass pipette and the root was avoided. The root mat was carefully rinsed by addition of 50 mL of 0.025 M NaOH-PIPES buffer solution (pH 7.0) and gentle agitation for 10 minutes at 75 rpm on a rotary shaker. The solution was removed by peristaltic pump and 50 mL of fixative solution, 3 % (v/v) glutaraldehyde in 0.025 M PIPES buffer (pH 7.0), was slowly added. The root mat then remained on a rotary shaker at 75 rpm for 2 hours at room temperature.

3-2. Washing

The fixative solution was removed as before and the root mat was rinsed twice for 10 min each with 0.025 M NaOH-PIPES buffer (pH 7.0) while shaken gently at 75 rpm.

3-3. Dehydration

Dehydration was carried out in a graded series of ethanol/water solutions (10 %, 30 %, 50 %, 70 %, 90 % and 100 %, v/v%). The flask was gently shaken at 75 rpm for 10 minutes per step and the solutions were then gently removed.

3-4. Embedding Medium

A glycol methacrylate (GMA) mixture was used as an embedding medium. The stock solution was prepared by dissolving 1 g of benzoyl peroxide (catalyst) in a mixture of 66.5 mL of glycol methacrylate (2-hydroxymethyl methacrylate or GMA), 3 mL of 2-butoxyethanol (ethylene glycol monobutyl ether), (a softener), 28.5 mL of N-butyl methacrylate (a plasticizer), and 5 mL of ethylene dimethacrylate (ethylene glycol dimethacrylate), (a hardener or crosslinker). All reagents were obtained from Sigma Chemical.

To accelerate dissolution of the catalyst, the mixture was heated to 40-45 °C with continuous stirring until it became clear and transparent, a process taking from 2 to 5 hours.

3-5. Infiltration and Polymerization

The embedding medium was diluted with absolute ethanol to make the infiltration solutions, a graded embedding mixture/ethanol series (10 %, 30 %, 50 %, 70 %, 90 %, 100 %, v/v%). Stock solutions were stored in tightly capped bottles and kept

Table 1. Fixative solutions tested

Fixative solution	Buffer solution
3 % (v/v) Formaldehyde (Sigma Chemical)	Millonig's phosphate buffer[a)]
1-5 % (v/v) Glutaraldehyde (Polyscience, 25 % biological grade)	Sorensen's phosphate buffer (0.025 M, 0.1 M)
1-5 % (v/v) Glutaraldehyde	PIPES (Fisher Scientific) buffer (0.025 M, 0.1 M)
3 % (v/v) Glutaraldehyde	Cacodylate (Sigma Chemical) buffer (0.025 M, 0.1 M)
3 % (v/v) Glutaraldehyde	Collidine (Sigma Chemical) buffer (0.025 M, 0.1 M)
10 % (v/v) Acrolein (Sigma Chemical)	PIPES buffer (0.025 M)
3 % (v/v) Acetaldehyde (Sigma Chemical)	PIPES buffer (0.025 M)
3 % (w/v) Paraformaldehyde	PIPES buffer (0.025 M)

[a)]modified Millonig's formalin, pH 7.2

in the refrigerator to prevent pre-polymerization but were brought to room temperature before use [Cole, 1982]. Samples were infiltrated for 30 minutes in each solution while being shaken at 75 rpm.

To facilitate the infiltration of the embedding medium, 60 mL of fresh 100% embedding medium was added to the flask which was then agitated at 75 rpm for 24 hours. To accelerate the polymerization reaction, 0.1 mL of N, N-dimethyl aniline was added and the flask was flushed with nitrogen (99.9 %) and sealed to prevent oxygen from inhibiting the polymerization reaction. The flask was gently agitated until polymerization began. As soon as the temperature of the mixture started rising rapidly, the reaction was quenched by placing the flask in ice for 10 minutes with vigorous shaking and was subsequently stirred for 1 minute at room temperature. This was done because excess temperatures produce gas bubbles in the plastic that impair the cutting properties of the block. This cycle was repeated for 2-5 hours until complete polymerization occurred and the sample was encased in a transparent block of polymer. After complete polymerization, remaining monomer was rinsed away with 95% ethanol. Further hardening at room temperature for 2-3 days was done to improve sectioning quality and prevent the block from sticking to the glass. The Erlenmeyer flask was then broken carefully with a hammer and the polymer block was dried in a hood for an additional 2-3 days at room temperature. The complete procedure was carried out in a fume hood due to the toxicity of the embedding mixtures.

3-6. Sectioning

A fine diamond saw (THE Co.) was used to slice the polymer block at a distance of 1-cm from the bottom, yielding a circular section, approximately 3 mm thick and 6 cm in diameter. This section was washed with distilled water and dried for several hours at room temperature.

3-7. Staining

The roots on the surface of the thin section were stained for 5 minutes with a 1% (w/v) aqueous toluidine blue O solution and excess dye was then rinsed off with running water. A final rinse was done with distilled water after which the sections were air-dried.

3-8. Image Analysis

The stained section was placed on a white paper and partitioned into 30 to 40 rectangles of approximately 0.4 mm by 0.3 mm. The image of each rectangle was displayed on a video raster screen through a CCM camera (Olympus OVM100n) with 20× magnification. The screen image was then captured and transferred to a graphic file by a video digitizing board (Jandel Scientific). The stained hairy roots appearing on the image file were painted manually by using an image analysis software (Mocha, Jandel Scientific). The total area of hairy roots in each polymer section was determined and the void fraction at the cut was calculated.

RESULTS AND DISCUSSION

1. Single Root Tip: Variation of Root Diameter

1-1. Effect of Fixatives

Since the diameter or volume of a root may change during fixation, dehydration, infiltration, or embedding, it was necessary to monitor the diameter during each step. Results of the tests of various protocols are summarized in Table 2. The best fixative was found to be 3% glutaraldehyde used in conjunction with PIPES buffer, Sorensen's phosphate buffer or collidine buffer. Paraformaldehyde, acetaldehyde, modified Millonig's formalin and acrolein gave rise to significant changes, 2% to 8%, in the root diameter, which is in agreement with the results for other plant tissues [Sabatini et al., 1963; O'Brien et al., 1973; Coetzee and Merwe, 1985].

Table 2. Effect of fixatives on the preservation of hairy root diameter, T=25 °C, duration=24 hours

Fixatives	Buffer	pH	Diameter change (%)
3% Glutaraldehyde	0.025 M PIPES	7.0	0.0
	0.1 M PIPES	7.0	0.0
	0.025 M Phosphate	7.0	0.0
	0.1 M Phosphate	7.0	7.0
	0.025 M Collidine	6.8	0.0
	0.1 M Collidine	7.0	8.2
	0.025 M Cacodylate	7.0	5.0
	0.1 M Cacodylate	7.0	5.3
3% Paraformaldehyde	0.025 M PIPES	7.0	6.7
Millonig's modified formalin		7.2	2.2
3% Acetaldehyde	0.025 M PIPES	7.0	3.8
10% Acrolein	0.025 M Phosphate	7.0	3.6

Table 3. Effect of fixative concentration on the preservation of hairy root diameter, GA=glutaraldehyde, pH=7.0, T=25 °C

Fixative (v/v%)	Buffer	Diameter of hairy root tip (mm)		
		Initial state	After 2 hr	After 24 hr
1% GA	0.025 M PIPES	0.48	0.45	0.50
2% GA	0.025 M PIPES	0.46	0.46	0.46
3% GA	0.025 M PIPES	0.44	0.44	0.44
4% GA	0.025 M PIPES	0.51	0.48	0.47
5% GA	0.025 M PIPES	0.45	0.42	0.41
1% GA	0.1 M PIPES	0.48	0.42	0.47
3% GA	0.1 M PIPES	0.43	0.43	0.43
5% GA	0.1 M PIPES	0.49	0.47	0.45

1-2. Effect of Fixative Concentration

The effect of fixative concentration is shown in Table 3. The optimal range of glutaraldehyde concentration was 2-3 % (v/v%), in agreement with the range determined for other plant tissues [Scala et al., 1968; O'Brien et al., 1973; Lawton and Harris, 1978].

1-3. Effect of Buffer

Several buffers have been suggested in the literature [Salema and Brandao, 1973; Lawton and Harris, 1978; Luft and Wood, 1963] and their effects on hairy root diameter are shown in Table 4. PIPES, Sorensen's phosphate, and collidine buffers were effective in preserving root diameter with 3% glutaraldehyde, the desired fixative, while cacodylate buffer was not. The 0.025 M PIPES buffer (pH 6.8-7.2) and 0.025 M phosphate buffer (pH 6.8-7.0) exhibited good preservation at wider

Table 4. Effect of buffer type on the preservation of hairy-root diameter, fixative=3 % (v/v%) glutaraldehyde, T= 25 °C

Buffer	Diameter of hairy root tip (mm)			
	pH	Initial state	After 2 hr	After 24 hr
0.025 M PIPES	6.8	0.42	0.42	0.42
	7.0	0.44	0.44	0.44
	7.2	0.45	0.45	0.45
0.025 M Sorensen's	6.8	0.43	0.43	0.43
Phosphate	7.0	0.44	0.44	0.44
0.025 M Collidine	6.8	0.47	0.47	0.47
0.025 M Cacodylate	7.0	0.45	0.44	0.44
	7.2	0.56	0.57	0.59

pH ranges than the 0.025 M Collidine buffer (pH 7.0). The efficiency of the phosphate buffer is probably due to its physiological property: phosphate buffers are found in living systems in the form of inorganic phosphates and phosphate esters.

1-4. Effect of Buffer Concentration

PIPES buffer concentrations of 0.025 M [Lawton and Harris, 1978] and 0.1 M and a phosphate buffer concentration of 0.025 M [O'Brien et al., 1973] have previously been used in fixation of plant tissues. Our results, in Table 5, indicate that a concentration of 0.025 M was also best for preservation of the diameter of the root. Changes in the molality of the PIPES buffer from 0.025 M to 0.1 M at pH 7.0 did not significantly change the quality of fixation with 3 % glutaraldehyde. Molality increase in other buffers (Sorensen's phosphate, collidine, cacodylate buffer) resulted in obvious changes in the root diameter.

1-5. Effect of pH

It has been suggested that the optimal pH for fixation is near the physiological pH of plant cells, which is 6.8 ± 0.2 [Sabatini et al., 1963] and this suggestion was confirmed for preservation of root diameter. The optimal pH was found to be in the range 6.8-7.2 for 0.025 M PIPES buffer, 6.8-7.0 for 0.025 M Sorensen's phosphate buffer, and 6.8 for 0.025 M collidine buffer, all used with a 3 % glutaraldehyde fixative at 25 °C.

1-6. Effect of Temperature

It is known that the use of low temperatures during fixation decreases extraction of cellular components and autolysis,

Table 5. Effect of buffer concentration on the preservation of hairy root diameter, fixative=3 % (v/v%) glutaraldehyde, T=25 °C

Buffer	Diameter of hairy root tip (mm)			
	pH	Initial state	After 2 hr	After 24 hr
0.025 M PIPES	7.0	0.44	0.44	0.44
0.1 M PIPES	7.0	0.43	0.43	0.43
0.025 M Phosphate	7.0	0.44	0.44	0.44
0.1 M Phosphate	7.0	0.41	0.42	0.44
0.025 M Collidine	6.8	0.47	0.47	0.47
0.1 M Collidine	6.8	0.50	0.52	0.50
0.025 M Cacodylate	7.0	0.45	0.44	0.44
0.1 M Cacodylate	7.0	0.44	0.42	0.42

Table 6. Effect of temperature on the preservation of hairy root diameter, fixative=3 % (v/v%) glutaraldehyde

Buffer	Temp.	Diameter of hairy root tip (mm)			
		pH	Initial state	After 2 hr	After 24 hr
0.025 M PIPES	25 °C	7.0	0.44	0.44	0.44
	4 °C	7.0	0.43	0.42	0.41
0.1 M PIPES	25 °C	7.0	0.43	0.43	0.43
	4 °C	7.0	0.45	0.44	0.47

but low temperature fixation did not preserve root diameter (Table 6). Fixation at low temperature resulted in severe changes in the diameter of roots while fixation at room temperature did not.

1-7. Effect of Dehydration and Infiltration

Although shrinkage or swelling of hairy roots mainly occurs during the fixation step, the diameter of hairy roots also changes during subsequent dehydration, infiltration, and embedding. Although dehydration and infiltration resulted in shrinkage of the root, the diameter always returned to its original measurement after the infiltration step.

2. Whole Root Mat

2-1. Fixation of Whole Root Mats

After growth for 30 or 40 days in MS-medium, the root mats were fixed for 2 hours with 3 % glutaraldehyde in 0.025 M PIPES buffer, pH 7.0. Gentle shaking at 75 rpm facilitated fixation.

2-2. Dehydration and Infiltration

Since GMA is miscible with both ethanol and water, dehydration of tissues is not always a prerequisite for infiltration by the embedding mixtures [Cole, 1982]. Some tissues can be directly dehydrated in GMA/ethanol solutions without previous treatment with ethanol. Although the use of a water-soluble embedding medium as a dehydrant is considered as an advantage, problems such as severe shrinkage of specimens, disruption of cell cytoplasm, and the inherent extraction of cell components are inevitable. We found that direct use of embedding medium/ethanol solutions as a dehydrant caused severe shrinkage of the hairy root mat. Dehydration by the progressively more concentrated ethanol/water solutions followed by infiltration with progressively more concentrated embedding medium/ethanol solutions preserved the hairy roots well.

2-3. Selection of Embedding Medium

Since the hairy roots are grown in aqueous media, water-soluble (hydrophilic) embedding media such as polyethylene glycol, Durcupan, GMA (glycol methacrylate), JB4 (commercial embedding mixture based on GMA), hydroxypropyl methacrylate (HPMA), Lowicryl K4M, K11M and LR White can be used.

Polyethylene glycol 400 is water-soluble, easily dissolves in water with heating at 50 °C, and has been frequently used as an embedding medium. Due to its viscosity, PEG compressed the root mat when added to the flask and thus completely changed the original morphology of the mat. Furthermore, the PEG solution did not penetrate into the root mat and caused visible shrinkage in root volume due to severe extraction.

Durcupan (aliphatic polyepoxide) is a colorless resin of re-

Table 7. Composition of modified embedding medium

Spaur and Moriarty [1979]	Modified embedding medium	
2-Hydroxyethyl methacrylate (GMA): 66.5 ml	2-Hydroxyethyl methacrylate (GMA): 66.5 ml	Monomer
Water: 3.5 ml	2-Butoxy ethanol: 3 ml	Softener
N-Butyl methacrylate: 28.5 ml	N-Butyl methacrylate: 28.5 ml	Plasticizer
Ethylene dimethacrylate: 5.0 ml	Ethylene dimethacrylate: 5.0 ml	Crosslinker or hardener
Benzoyl peroxide: 1.5 g	Benzoyl peroxide: 1.0 g	Catalyst
	N,N-dimethyl aniline: 0.1 ml	Accelerator

latively low viscosity and is completely miscible with water. Under our conditions, it frequently failed to harden to a sufficient degree, even without the use of plasticizer.

Hydroxypropyl methacrylate (HPMA) is one of the water-soluble methacrylates with properties similar to those of GMA, but with better cutting properties. However, HPMA penetrates tissues more slowly than GMA and thus the procedures involved are relatively tedious and time-consuming.

Other embedding media such as Lowicryl K4M, K11M, and LR White are also possible candidates for embedding of hairy roots. However, they were originally designed for low temperature embedding and a special cooling apparatus is required for their use [Ashford et al., 1986]. They are also more expensive than GMA.

Using GMA as an embedding medium has many advantages. It is a relatively inexpensive water-soluble methacrylate, and the ultimate shrinkage after embedding in GMA is far less than shrinkage in water-insoluble media such as paraffin. Also, the high infiltration temperature required for paraffin or polyethylene glycol (PEG or Carbowax) is not necessary for embedding with GMA. GMA is easily thin-sectioned and the morphological preservation of specimens is excellent.

GMA has several advantages: (1) it is able to infiltrate tissue and polymerize without complete removal of water, (2) it is compatible with aqueous fixatives (e.g. glutaraldehyde), (3) it hardens enough to permit sections to be cut without distortion of the specimen, (4) sections are easy to cut, thus improving resolution, and (5) GMA is porous enough to permit easy staining of the root.

Addition of small amounts of hardener results in a much more stable section [Leduc and Holt, 1965], but addition of too much hardener makes the polymer block very brittle. Two hardeners or crosslinkers were tested: triglycol dimethacrylate and ethylene dimethacrylate. Ethylene dimethacrylate was found to be the better of the two. A softener such as PEG 400, 2-butoxy ethanol or water can be added to obtain a softer, less brittle, and more porous plastic. Conversely, hard blocks can be prepared by limiting the amounts of softeners. The addition of softener to the embedding medium results in a medium that is relatively insensitive to ambient temperature in the range 18-28 °C. However, addition of too much softener should be avoided because it results in soft polymer block which is very sticky, difficult to remove from the Erlenmeyer flask, and hard to section.

The composition of the embedding medium that was chosen as the best for this study is shown in Table 7. N-Butyl methacrylate was added as a plasticizer to increase sectioning quality.

2-4. Polymerization Method

The GMA embedding medium is usually polymerized by either i) heating in an oven (50-60 °C), ii) irradiation with ultraviolet (UV) light, or iii) addition of accelerator (N,N-dimethyl aniline). Great care should be taken when carrying out the polymerization reaction. The polymerization of GMA is an exothermic reaction and if the reaction is too fast, a substantial amount of heat is generated. When the heat of polymerization is not removed effectively, the evolved heat may result in uneven block hardness and formation of gas bubbles in the plastic, and it can seriously impair or even destroy the polymer block by forming cracks inside the polymer. The risk of forming gas bubbles is greater with larger blocks than with small. Prevention of bubble formation depends on regulating the rate of polymerization, providing adequate cooling, and varying the amounts of accelerator and/or catalyst so as to avoid excess rise of temperature.

Heating in an oven is a convenient method for polymerization, though temperature control is difficult to achieve with this method. Polymerization by UV irradiation can be carried out at room temperature or in the cold. An ordinary black-light UV lamp (>360 nm) is generally used, with α,α-azobisisobutyronitrile (azonitrile) as catalyst. The specimens are usually placed a few inches away from the lamp. A specially designed apparatus should be used to control the heat produced during polymerization [Ashford et al., 1972].

In our work, the embedding medium was polymerized by the addition of accelerator. The appropriate concentration of the accelerator should cause the medium to polymerize at an acceptable rate. If the polymerization is too fast, the heat evolved will cause formation of bubbles and cracks; if it is too slow, incomplete polymerization results, even after 48 hours. In our experiments, one or two drops (less than 0.1 mL) of accelerator was added, since more than this amount generated so much heat that it became impossible to control the temperature. After polymerization, the polymer block was further hardened at room temperature for 2-3 days to improve sectioning quality. The resulting polymer block was easily separated from the Erlenmeyer flask, had an amber color, and had enough hardness to section.

2-5. Sectioning

Section thickness is important for the exact measurement of the total hairy root area because the thickness is directly related to image contrast. In principle, the higher the resolution desired, the thinner the section required. With thicker sections, more of a structure will be included in the image and opaque bodies may give an erroneously large measurement; less-opaque material in thinner sections will be obscur-

ed and may give reduced area measurement [Anderson, 1982]. We found that a 2-3 mm thick section was most appropriate from both the standpoint of contrast and of image analysis.

We tested various tools (razor blade, fine hack saw, diamond knife, glass knife, and diamond saw) to obtain thin polymer sections. Only a diamond-coated saw (THE Co.) successfully produced sections of uniform thickness and clean surfaces without any wrinkles or breaks. A clean, uniform surface is important for staining and image analysis. A section containing wrinkles or breaks hinders good staining and subsequent image analysis becomes very difficult.

GMA blocks must be sliced slowly, with uniform speed, or they will become hot and liquefy around the saw blade, refilling the cut with a sticky resin that may seize the blade. This can be avoided by supplying enough coolant and by reducing cutting speed. The thin sections obtained must be handled carefully because the GMA tends to be brittle.

2-6. Measurement of the Void Fraction of Hairy Root Mats

After the formation of a slightly linked root mat, the hairy root cultures grew well with a perfusion of branches. It was evident, from visual inspection, that 40-day old cultures had more crowded root mats than 30-day old cultures, and that cultures inoculated with a large number of root tips (20 to 30 tips) were more crowded than cultures inoculated with a small number of root tips (4 or 10 tips). After 40 days, the cultures inoculated with a larger number of root tips approached the death phase, as indicated by a browning of the tissue and medium.

The image analysis system available for this study was not sufficiently powerful to automatically distinguish root cross-sections from the surrounding polymeric matrix. However, to the human eye, root cross-sections were easily identifiable, and the image was therefore modified by manual painting of each root cross-section. This modified image was then analyzed by computer to obtain the void fraction. A large amount of time was required to manually paint all the images, making this step the most time consuming part of the protocol. Systematic studies of root mat structure as a function of growth conditions will require image analysis of a large number of embedded root mats. It is therefore clear that an extensive use of the embedding technique to study root mat structure will require a sophisticated and powerful image analysis system.

The measured void fraction in the mats is shown versus culture age and inoculum tip number in Fig. 1. All data are for sections 1 cm from the bottom of the Erlenmeyer flask. As indicated by the figure, variations in porosity with different growth conditions are clearly evident. The small variation in void fraction between mats grown and measured under identical conditions is a strong indication that the embedding procedure did not significantly distort the mat structure. A least square fit of the void fraction data to a linear function gives the following expression:

$$\varepsilon = 1.144 - 0.001n - 0.006d$$

where

ε=void fraction of hairy root mat (cm^2/cm^2)

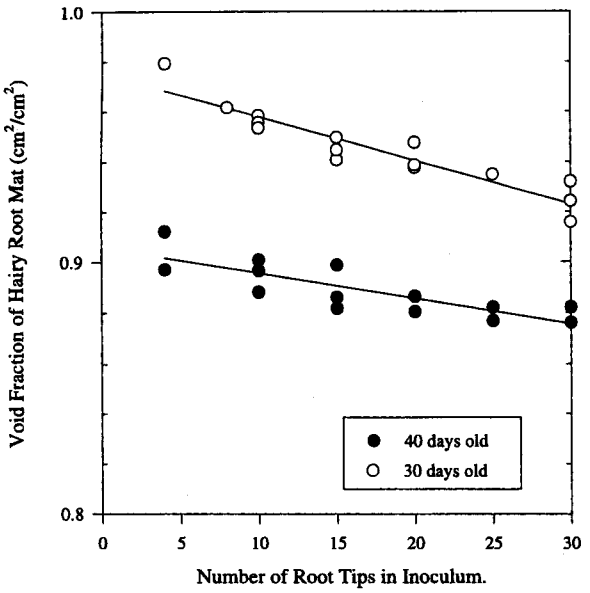

Fig. 1. Void fraction in *Tagetes erecta* hairy root mats, measured 1 cm from the bottom of a 150 mL Erlenmeyer flask, as a function of age and number of tips in the inoculum.

n=number of root tips in the inoculum

d=growth time (days)

As expected, cultures inoculated with a larger number of root tips have a smaller void fraction than those inoculated with a smaller number of root tips and the void fraction decreases with respect to the age of the culture.

CONCLUSION

An embedding protocol which preserves the diameter of *T. erecta* hairy roots was developed. Embedded root mats were sectioned and analyzed to obtain the void fraction in the root mat. The data clearly demonstrate that reproducible and reasonable results for the root mat void fraction are obtained with this technique.

The technique can be used to measure the structure and void fraction of root mats as a function of time, position in mat and growth conditions. Such studies will lead to a greater understanding of the nutrient and oxygen transport processes in the mat and of how nutrient and oxygen limitations affect root growth and development. Thus, better models of root growth kinetics in shake flasks and reactors will result.

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