Strategies in Commercial Micropropagation

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I can trace my learning curve in micropropagation from 1961 and now I can pose questions such as "What is important to the achievement of a successful micropropagation operation? Where should I direct my research?" In a few words, the answer is "to the achievement of high success rates in the acclimatization stage".

In this paper I will be concentrating on my experiences with culture media and my current approach to the selection of media And to start at the end product I will present my latest basal medium and ideas for using it (Table 1) The medium consists of a micronutrient section, a growth factor section, and a gelling agent. Macronutrient, auxin, cytokinin, and carbohydrate sections are treated as experimental variables. Full strength macronutrients are listed in Table 2—and these are diluted (either in groups or as individual components) to prepare different concentrations to suit different purposes. Types of auxins, cytokinins, and carbohydrates and their concentrations are determined by experiments. All media and experimental treatments are dispensed cold into the culture vessels used in my commercial operations—and these vessels receive the same volume of medium and are closed the same way—the point to stress is that whatever methods are used in a commercial operation must also be used in experiments—the emphasis is on reproducibility of results.

THE BASAL MEDIUM

The rationale for my new medium and experimental approach is that macronutrients, auxins, cytokinins and carbohydrates are "important" and worthy of experimentation whereas micronutrients and growth factors are not so likely to yield important results.

MICRONUTRIENTS AND GROWTH FACTORS

It is probably wise to have some growth factors and micronutrients in the medium because their absence may be detrimental with prolonged subculture but, in terms of research effort, we can better concentrate on other ingredients and conditions.

GELLING AGENT

In 1991, the author began testing carrageenan and specifically one called Gelcarin GP812 (FMC Corporation, Marine Colloid Division, Rockland, Maine). This is now used at 6 g/litre and has the clarity of Gelrite and otherwise the properties of agars—it is also inexpensive. Gelcarin GP812 is currently the only gelling agent used in the author's laboratory, and is under test in 15 laboratories in Australia—in particular, the author is waiting to hear whether laboratories which experienced vitrification problems with Gelrite also have this problem with Gelcarin GP812.

Preliminary results, from the laboratory of Dr. Acram Taji (Department of Botany, University of New England, Armidale, NSW), with *Boronia ruppii, Lechenaultia formosa, Ptilotus exaltatus, Rosa* spp , and *Zieria fraseri*, all species

which suffer from vitrification on Gelrite media, indicated that Gelcarin media was similar to agar media with respect to this problem.

Table 1. Basal medium consisting of micronutrients, growth factors, and gelling agent

Chemical	Amount per litre
Micronutrients	
H_3BO_3	100.00 µmol
$CoCl_2$	0 10 µmol
$CuSO_4$	0 10 µmol
$MnSO_4$	100.00 µmol
KI	5 00 µmol
Na_2MoO_4	1.00 µmol
$ZnSO_4$	30 00 µmol
Growth factors	
Inositol	600 00 µmol
Nicotinic Acid	4.00 µmol
Pyridoxine	2 00 µmol
Thiamine	1.00 µmol
Glycine	50 00 µmol
Gelling agent	
GELCARIN GP812	6 g

Ions	Amount per litre
Cl	0.2 µmol
SO_4	130 1 µmol
Na	2.0 µmol
B	100.0 µmol
Mn	100 0 µmol
Zn	30 0 µmol
I	5 0 µmol
Mo	1 0 µmol
Cu	0.1 µmol
Co	0.1 µmol

MACRONUTRIENTS AND IRON

Of course, the Basal Medium would not be used without the addition of at least macronutrients and a carbohydrate supply. In general, experimentation with macronutrients is regarded as being very important and also very difficult to do and to interpret. In general, the (high) concentration of macronutrients in Table 2 appears to be suited to many ferns and herbaceous plants whereas half concentration in Table 2 favours woody species. Experience has shown that research with hormones is not rewarding unless cultures are placed on media without "limiting" factors—an incorrect concentration of macronutrients can be "limiting". In the latest model for experimentation described here, it is suggested that only two

concentrations of macronutrients need to be tested in the initial or "ranging" experiment.

Table 2. Full strength (×1) macronutrients and iron compound The concentrations of these nutrients are used as experimental variables either individually or as a group(s)

Chemical	Amount per litre
Macronutrients and iron	
$NH_4H_2PO_4$	2.00 mmol
NH_4NO_3	18 00 mmol
$CaCl_2$	2 00 mmol
$Ca(NO_3)_2$	1 00 mmol
$MgSO_4$	1 50 mmol
KNO_3	20.00 mmol
FeNaEDTA	100 00 µmol
Ions	**Amount per litre**
NO_3	40 0 mmol
NH_4	20 0 mmol
N	60 0 mmol
P	2.0 mmol
K	20.0 mmol
Ca	3 0 mmol
Mg	1 5 mmol
Cl	4.0 mmol
Fe	100 0 µmol
SO_4	1500 0 µmol
Na	100 0 µmol

CARBOHYDRATES

In initial experiments, I would not anticipate that a species would prefer a carbohydrate other than sucrose but I would expect that, in some species and with some objectives, experiments with different concentrations of sucrose may be rewarding. My experience with *Chenopodium rubrum* made me aware that a species could have a requirement for a very low concentration of sucrose. Most of my culture media have either 60 mM or 90 mM (approximately 2% and 3%,w/v, respectively) sucrose but, for the preparation of cultures for planting out, it is worth testing 120 mM, 150 mM or higher concentrations of sucrose—these higher concentrations are used with *Lilium* and *Begonia* Rex Cultorum Hybrid [*Begonia rex*] leading to stronger cultures and high acclimatization success rates.

AUXINS AND CYTOKININS

In the mid 1970s, I decided to test, as a group, six substances in the auxin category and two substances in the cytokinin category with the idea that a species may regard this as a smorgasbord and select whichever auxin and cytokinin it preferred and ignore the rest. By and large, this approach was quite successful, it really did

appear as if plants were able to select just the hormones they needed, for example, *Eriostemon australasius* used BAP and ignored kinetin in a mixture of the two cytokinins. But, as part of the investigation of riboflavin action, it became clear that the auxins, NOA, pCPA and 2,4-D, tended to induce callus formation in our cultures whereas IAA, IBA and NAA, tended to keep cultures in an organized state We also learned that with at least three woody species (*Simmondsia chinensis* [jojoba], *Eucalyptus*, and *Eriostemon*) that either BAP or PBA stimulated multiplication whereas kinetin, 2iP, and zeatin did not.

The strategy today is to test three auxins (IAA + IBA + NAA) as a group and two cytokinins (kinetin + BAP) as a group with initial research with untried species. If research has been published on a species and other auxins and cytokinins had been used, then these other hormones would also be included in initial experiments. For micropropagation, zero hormone control treatments would always be included in experiments.

SUGGESTED INITIAL EXPERIMENTS

The following initial experiment would probably yield interesting information with many species:

1) Initiate cultures on a medium consisting of:
   Basal Medium
   Half-strength macronutrients
   60 mM sucrose
2) Subculture apparently aseptic cultures to the following treatments:
   Basal Medium
   Macronutrients: ×½ and ×1 strength
   Sucrose: 60 mM and 90 mM
   Auxins (IAA + IBA + NAA): 0, 0.1 µM, and 1.0 µM
   Cytokinins (kinetin + BAP): 0, 1 µM, and 10 µM

This is an experiment with 2 × 2 × 3 × 3 treatments, that is 36 treatment combinations. If insufficient material for 36 treatments were available, consideration could be given to using only one concentration of macronutrients (for example, ×1 for herbaceous species, ×½ for woody species) and one of sucrose (for example, 60 mM for woody species, 90 mM for herbaceous species)—this would give an experiment with 9 treatments.

3) Follow up experiments would concentrate on simplifying media for different objectives. For example, for multishoot formation, for yielding microcuttings, and so on. The aim of this research is to develop a satisfactory multiplication system, to minimize technical losses and to yield plants which have a high planting out success rate. While initial experiments need not involve planting out the cultures, later experiments should **ALWAYS** involve evaluation of treatments in terms of planting out success rates—all costs in the laboratory ride on the cultures sent to the planting-out facility and all losses in acclimatization have to be accommodated by plants which are to be sold.

EXPERIMENTAL METHODS

Refining a culture medium is best done by using equal increment dose-response type experiments (Fig. 1) Equal-increment dose-response experiments are recommended where single substances such as hormones, carbohydrates and micronu-

trients are tested. They can also be used with macronutrients but interpretation of results is more difficult—if the growth of cultures increases with increase in concentration of KNO_3, is this response due to K^+ ions or to NO_3^- ions?

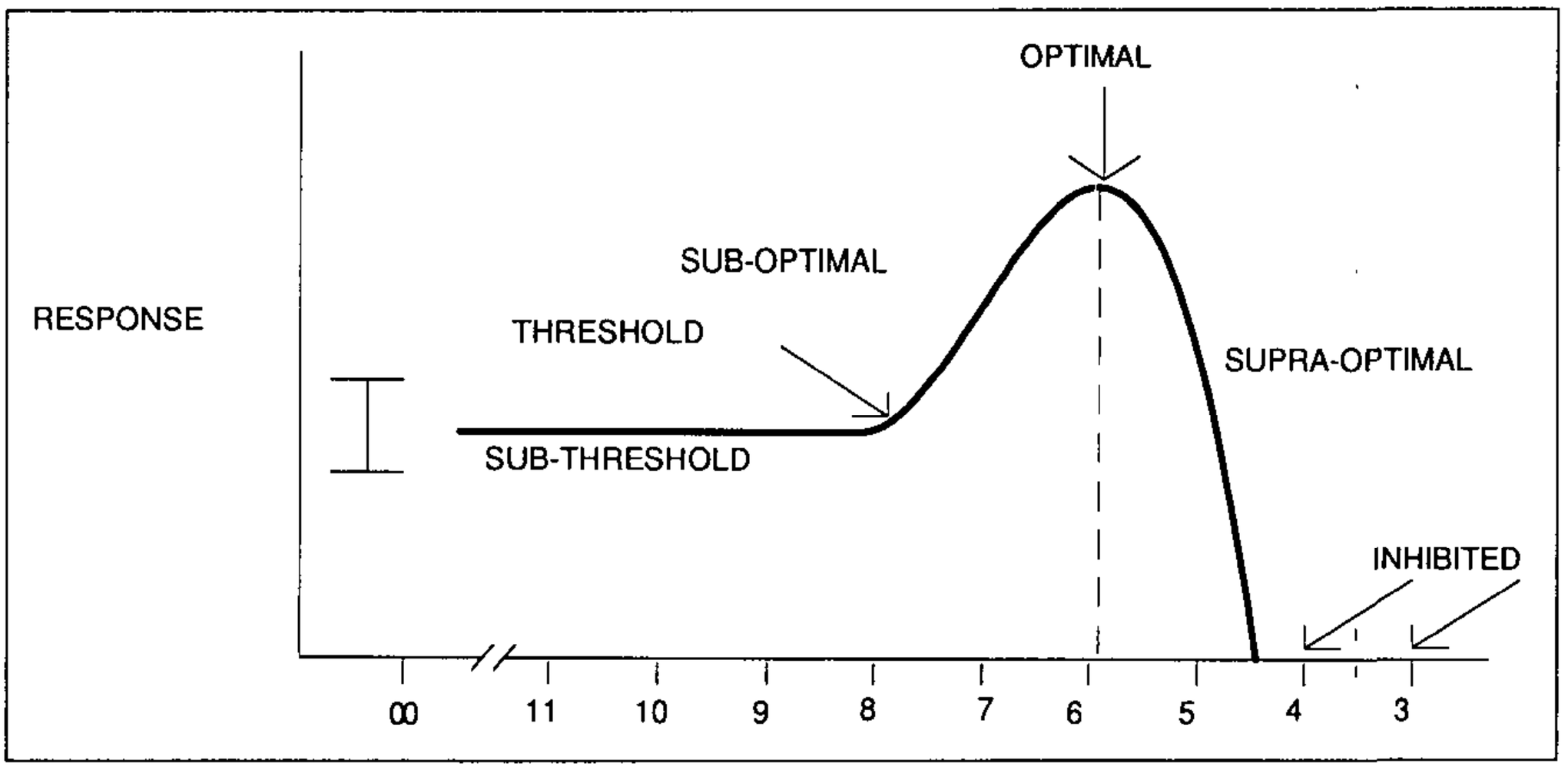

Figure 1. Graph showing sub-threshold, threshold, sub-optimal, optimal, supra-optimal, and inhibited responses of cultures to increasing concentrations of a substance

IMPLICATIONS OF THE PARABOLIC RESPONSE OF CULTURES TO COST CUTTING

One outcome of receiving formal training in science is to be precise and accurate with measurements, one disadvantage of this is that one balks at not being precise and accurate. Yet if less accurate methods were to reduce the cost of production in a commercial operation, can one rationalize one's use of them? Is there any way to feel "comfortable" with less than precise methods? I think it is important to be accurate when making concentrated chemical stock solutions for culture media and when doing experiments but, when a "good" medium has been evolved through experimentation, I think that cultures will not "notice" small variations in chemicals, and this thought leaves the way open to use cheaper methods of production. And the reason for this conclusion can be found by looking at any "parabola" of response. Say a culture medium's recipe calls for 10 ml of 10,000 µM FeNaEDTA per litre of medium and that a cheaper method for the preparation of media results in sometimes 9 5 ml and other times 10.5 ml being added per litre. If the accurate method were followed, then the medium would have a final concentration of 100 µM FeNaEDTA—with the less accurate method, the final concentration of FeNaEDTA would be in the range 95 µM to 105 µM FeNaEDTA. The question comes down to this : "Would cultures respond in a significant way to such differences in concentration?" By answering "not likely" to this question, has allowed me to develop rapid methods for the preparation of culture media and for its dispensation to culture vessels and, thus, minimizing my cost of production in this area

USING A PUBLISHED MEDIUM IN A COMMERCIAL OPERATION

The type of "fuzzy logic" described above has other uses in my laboratory Most published research in micropropagation comes from universities and research institutes, and cultures are tested under various conditions and with various media using, mostly, test tubes or other small vessels. Larger vessels are used in commercial operations and, for published research to be relevant to commercial operations, ideally the same type of container, the same type of closure, and the same volume of medium should be used, as well as the same types of preparation procedures. These factors do influence cultural responses—at least, it is better to assume that they do. This is where cost of production analyses again enters the equation. In the author's laboratory, all media are dispensed cold and, for both experimentation and production, 250 ml polycarbonate vessels fitted with polypropylene screw on lids (Techno-Plas Pty Ltd., P.O.Box 239, Melrose Park, South Australia 5039) are used—volume of medium per vessel is 50 ml.

The problem is that different commercial laboratories use different procedures and containers. The lesson is that if researchers do not at least approximate commercial conditions, then their work is largely irrelevant—and the next part of the message is that commercial laboratories must therefore do their own research, research appropriate to their own commercial conditions.

CONCLUSIONS

Using the types of approximations described in this paper for the technical aspects of micropropagation, I have been able to "translate" most published media into forms which I can prepare from the stock solutions used to prepare the medium in Tables 1 and 2, with a minimal amount of other stock solutions. The impetus for this is partly (largely?) to do with minimizing my cost of production in a commercial micropropagation laboratory but, amongst other things, the studies involved have led to a clear ranking of objectives in commercial as distinct from academic micropropagation.

Top ranking goes to achieving a high success rate on planting out cultures and next ranking goes to getting the greatest number of suitable plants per culture and to maximizing the rate of deflasking. These objectives are affected by having a suitable planting-out facility/methods and by getting the right sort of cultures in the laboratory. In turn, that involves finding the best culture medium and testing other pre-deflasking treatments, such as exposing cultures in vessels to ambient conditions in the planting-out facility prior to deflasking.