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REVIEW

Plant tissue culture media and practices: an overview

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Abstract

This review presents an overview of the culture media and practices used in plant tissue culture and developmental biology. The compositions of the most commonly used basal media, especially Murashige and Skoog (MS) and modified MS (MMS), Gamborg's B5 medium and B5 modifications, Woody Plant Medium (WPM), and Driver and Kuniyuki Woody plant medium (DKW) are discussed, along with typical basal medium manipulations to elicit and support various developmental responses. The most commonly used plant growth regulators and their applications to promote various developmental responses are examined, along with a presentation of the classical phytohormone developmental models for organogenesis and somatic embryogenesis. Elaborated developmental models for both organogenesis and somatic embryogenesis, with emphasis on discrete developmental steps, occasional need for multiple manipulations in culture to achieve a single developmental step, and identification of responsive tissue types in mixed cultures are explored. It is hoped that the information presented here will lead to a deeper understanding of basic tissue culture responses and will assist the reader in the decision-making process by identifying appropriate media and culture conditions for a particular species or application, or by providing a suitable starting point, should further customization be required.

Keywords Basal medium · Plant growth regulator and phytohormone models · Plant tissue culture · Plant regeneration, growth, and development · Somatic embryogenesis and organogenesis

Introduction

Plant tissue culture systems allow for the rearing of whole plants, organs, tissues, or cells under controlled aseptic conditions in the laboratory. The tissue culture system supplies all nutrients, energy, and water necessary for plant or explant growth through the basal medium. In addition, controlled incubation conditions provide optimized light and temperature settings to promote growth. Plant development can then be manipulated by the addition of plant growth regulators (either natural phytohormones or synthetic versions) at particular stages of growth or maturation.

This current review is structured to follow the general sequence of plant tissue culture protocols, beginning with planning and preparation, and commencing through initiation, proliferation, and regeneration steps. Thus, first, a brief review of the considerations for choice of the basal medium and fundamental incubation conditions is presented. Second, commonly used plant growth regulators (synthetic versions of phytohormones) and how they may achieve their physiological effects are briefly surveyed. Third, the classical phytohormone and developmental models for manipulating vegetative development *in vitro* are reviewed. In the final section, more detailed developmental models are explored, which demonstrate additional concepts and principles of basal medium and plant growth regulator manipulation, as related to *in vitro* plant regeneration.

In order to provide relevant context for the discussion of choice of basal medium formulation, the citations related to plant tissue culture regeneration media compiled by Herman (2015) over the period of 2011 to 2015 were surveyed. Although some of these citations were sourced from scientific meeting abstracts, the majority of these citations were

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gathered from the primary original literature. Focusing on the primary original literature citations, there were 123 useful sources, which may or not be comprehensive. Nevertheless, these citations represent a recent 5-y snapshot of plant tissue culture medium usage as documented in the literature and serve as a basis for discussion of recent and current practices in the discipline.

Fundamental Considerations: Basal Medium and Incubation Conditions

Two distinct trends were noted among this recent set of citations (Herman 2015). First, efforts to optimize or customize the basal medium for specific species or genotypes, and for specific developmental steps, such as shoot multiplication or rooting, were ongoing. Response surface methodology, design of experiments (DOE) methodology, and neuro-fuzzy logic software programs were introduced during this time period for basal medium customization. Most of these studies used MS basal medium (Murashige and Skoog 1962) at the start of optimization, but as discussed in Greenway *et al.* (2012), sometimes MS medium components were so inadequate that optimization was not achievable. In those cases, the use of BABI, a B5 (Gamborg *et al.* 1968) formulation modified at Arkansas Biosciences Institute (Greenway *et al.* 2012), might be recommended as an alternative starting point for optimization. The BABI basal medium utilizes a somewhat different set of macronutrient salts than does MS, separating the nitrate from ammonium in different salts, so customization for a particular species or genotype can be fine-tuned to lead to different endpoints. Second, tissue culture protocols were increasingly utilized for high-throughput phenotyping, for example, screening large numbers of *in vitro* regenerated plants or *in vitro* germinated seedlings for a particular transgenic or mutant phenotype, using an automated multi-spectral imaging system such as a ScanAlyzer (LemnaTec GmbH, Aachen, Germany). Published, preferably standardized, protocols for the particular species or genotypes under study should be used for high-throughput phenotyping whenever possible, to allow better comparison of datasets across research groups. Should published or standardized protocols not be available for a particular application, one may be developed using the practices presented in this present review.

Although prepackaged MS, B5, and modifications of these basal media are available commercially, many plant tissue culture labs prepare media using groups of the individual chemical components as stock solutions. Macronutrient salts (N, P, K, Mg, and S) can be prepared together in a 10× or 100× stock solution (Gamborg *et al.* 1976; Gamborg and Phillips 1995). Calcium salts are segregated into a separate stock solution, because insoluble calcium phosphate salts are formed when Ca is included in the macronutrient stock. Iron ethylenediaminetetraacetic acid (FeEDTA) is made as a separate stock, using equimolar amounts of $FeSO_4 \cdot 7H_2O$ and Na_2EDTA, and FeEDTA stock must be autoclaved to force the maximum formation of FeEDTA before use, because EDTA can react with other elements, especially certain micronutrients. Micronutrient salts (B, Mn, Zn, Mo, Cu, Co, and I) can be grouped into a single 100× or 1000× stock solution. Macro- and micronutrient salt stocks can be autoclaved for storage or frozen in aliquots. Double-distilled or reverse osmosis water is preferred for preparation of chemically defined media.

Vitamins and some organic compounds (typically thiamine, pyridoxine, nicotinic acid, and myo-inositol) can be grouped together into a single stock solution (100×), but this stock solution should be refrigerated for storage and should not be autoclaved prior to medium preparation. Growth regulators can also be made into individual stock solutions, at 1 mg mL^{-1}, and stored in the refrigerator (these organic compounds should not be autoclaved, except in the final prepared medium). Some heat-sensitive organic compounds may be added to the autoclaved medium after filter sterilization. Carbohydrates, such as sugars, typically are added dry to the basal medium, but some labs prefer to filter-sterilize the carbohydrate because autoclaving can lead to breakdown or carmelization. Gelling agents, such as different types of agars, agarose, gellan gum, or calcium alginate, do have distinct effects on plant tissue culture responses. When it comes to medium preparation, the most important principle is consistency; if a customized tissue culture protocol identifies a particular gelling agent, the specific type and brand should be used for all experiments of a given kind.

Commonly used basal media Not surprisingly, the most widely used plant tissue culture basal medium is MS (Murashige and Skoog 1962; see Table 1) and modifications thereof, such as half-strength MS (½-MS); MS-based media were used in 82% of the 5-y set of citations (Herman 2015). While at one time in the past, B5 medium (Gamborg *et al.* (1968; Table 1) or its modifications were widely used, in this 5-y set of citations, B5-related media were used in only 5% of the cases. Numerous citations identify the use of MS salts with B5 vitamins. Schenk and Hildebrandt medium (SH; 1972) was mentioned only once (< 1%); it and other specialty basal media are documented in Gamborg and Phillips (1995). Woody plant basal media (see Table 1) such as Woody Plant Medium (WPM; Lloyd and McCown 1980) and Driver and Kuniyuki Woody plant medium (DKW; Driver and Kuniyuki 1984) were used in 6% of the 5-y set of citations. Another 7% of the citations used ½-MS or MMS (modified MS; ½-macro MS salts + full-strength micro MS salts + B5 vitamins) for woody plant applications, so altogether woody plant basal media comprised a total of 13% of the citations. Half-strength MS was used in another 6% of the citations for

Table 1. The nutrient concentrations of commonly used plant tissue culture basal media

Basal medium	B5	BDS	BABI	MS	MMS	WPM	DKW
Macronutrient components (mg L^{-1})							
KNO_3	2500	2500	2500	1900	950	–	–
K_2SO_4	–	–	–	–	–	990	1559
NH_4NO_3	–	320	320	1650	825	400	1416
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	–	–	–	556	1948
$NH_4H_2PO_4$	–	230	230	–	–	–	–
$NaH_2PO_4{\cdot}H_2O$	150	150	150	–	–	–	–
$(NH_4)_2SO_4$	134	134	134	–	–	–	–
$MgSO_4{\cdot}7H_2O$	250	250	250	370	185	370	740
KH_2PO_4	–	–	–	170	85	170	265
$CaCl_2{\cdot}2H_2O$	150	150	440	440	220	96	149
Micronutrient components (mg L^{-1})							
H_3BO_3	3	3	3	6.2	6.2	6.2	4.8
KI	0.75	0.75	0.75	0.83	0.83	–	–
$MnSO_4{\cdot}H_2O$	10	10	10	16.9	16.9	22.3	33.5
$ZnSO_4{\cdot}7H_2O$	2	2	2	10.6	10.6	8.6	–
$Zn(NO_3)_2{\cdot}6H_2O$	–	–	–	–	–	–	17
$CuSO_4{\cdot}5H_2O$	0.039	0.039	0.039	0.025	0.025	0.25	0.25
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.39
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025	0.025	0.025	–	–
$NiSO_4{\cdot}6H_2O$	–	–	–	–	–	–	0.005
$FeSO_4{\cdot}7H_2O$	27.8	27.8	27.8	27.8	27.8	27.8	33.8
Na_2EDTA	37.3	37.3	37.3	37.3	37.3	37.3	45.4
Vitamins and organics (mg L^{-1})							
Myo-inositol	100	100	100	100	100	100	100
Nicotinic acid	1	1	1	0.5	1	0.5	1
Pyridoxine HCl	1	1	1	0.5	1	–	0.5
Thiamine HCl	10	10	10	0.1	10	1.6	2
Glycine	–	–	–	2	–	–	20
L-Glutamine	–	–	–	–	–	–	250
Sucrose (g/L)	20	30	30	30	30	20	30
pH	5.5	5.8	5.8	5.8	5.8	5.6	5.5

rooting applications. The remaining 6% of the citations were orchid applications using Knudson (1925), VW (Vacin and Went 1949), or chemically non-defined media.

Murashige and Skoog medium is widely used for both dicots and monocots, and it makes a good plant regeneration medium, because of the high levels of nitrogen in both nitrate and ammonium forms, with a relatively high ratio of ammonium to nitrate (Table 1). However, sometimes MS medium is not the best medium for optimal growth, precisely because it does contain high levels of ammonium ions (Gamborg *et al.* 1976). In contrast, B5 medium may be too low in ammonium (Table 1) for some plant species. Dunstan and Short (1977) found deficiencies in B5 medium for *Allium cepa* L. (onion) tissue culture, and modified B5 by adding a modest amount of ammonium and phosphate and called the medium BDS (B5 as modified by Dunstan and Short; Table 1). The BDS formulation recently was modified with the addition of more calcium, and this version, called BABI (Table 1), which works well with a wide variety of plant species and tissue culture applications, including *Nicotiana tabacum* L. (tobacco), other dicots, monocots, and some woody plants (Greenway *et al.* 2012). Although the extra calcium in BABI does not have a strong effect on biomass growth compared with BDS, in several cases, an advantage in plant regeneration productivity was reported with the additional calcium (Greenway *et al.* 2012).

Woody plant basal media generally contain lesser amounts of the macronutrient salts, so ½-MS, MMS (Modified MS), and WPM are now widely used for woody plants. With only half of the MS ammonium, the ½-MS or MMS media do not appear to induce ammonium toxicity. Similarly, WPM has less total nitrogen and less ammonium than MS. In contrast, DKW has a higher ammonium to nitrate ratio similar to MS, but less total nitrogen than MS and it uses different salt sources, resulting in an increase in sulfate concentration (Table 1). Some woody plants may benefit from the greater sulfate in the medium.

There are other basal medium formulations that have been developed for specialty applications. For example, the Nitsch and Nitsch (1969) medium was optimized for tobacco anther cultures to produce haploid plants, and this basal medium has served as a good starting point for androgenesis of other plants. It has approximately half as much total nitrogen as MS, but with a similar ammonium to nitrate ratio. The reduction in nitrogen favors induction of androgenesis (Ferrie and Caswell 2011). Similarly, the NLN (Nitsch and Nitsch as modified by Lichter) basal medium described by Lichter (1982) was developed for isolated microspore culture of *Brassica napus* L. and contains very low total nitrogen with no ammonium salts. Another example of a specialty basal medium is the Kao and Michayluk (1975) medium developed for protoplast culture, which has been a useful starting point for protoplast cultures of many plant species. This medium contains the long version of the B5 organic supplements (vitamins and amino acids), plus additional vitamins, organic acids, sugars, and sugar alcohols. The Kao and Michayluk (1975) medium, rich in organic supplements, provides the numerous metabolic precursors that allow protoplasts to regenerate their cell walls and initiate cell divisions.

In summary, unless a customized system has been devised for a specific application and species, MS or BABI basal media may be recommended for most herbaceous plant tissue culture applications. Alternatives, such as MMS, WPM, or DKW basal media, may be recommended for woody plants.

Incubation conditions Most plant tissue cultures are incubated in reach-in growth chambers or growth rooms. Growth facilities should provide temperature control, variable light

intensity of appropriate wavelengths, photoperiod control, and in some cases humidity control (Gamborg and Phillips 1995). Temperatures should be controlled to ± 1°C. Temperature typically remains constant, most frequently in the range of 22–28°C, depending on the plant species. However, specialty situations may call for short-term (days to weeks) cold or heat treatments, with a lower limit of 4°C and an upper limit of 35°C being most common.

Typically, light intensity is set between 50 and 100 μmol m^{-2} s^{-1} photosynthetically active radiation, but photosynthetic photon flux as low as 5, or as high as 150 μmol m^{-2} s^{-1}, have been reported depending on the species and application. Photoperiod is often set to 16 h, resulting in 8 h dark periods per d, but may be adjusted for the species of interest. Some protocols identify longer day-lengths, up to continuous light incubation. Humidity controls may be necessary in extremely dry environments to reduce evaporation of the medium during incubation. Ventilation of culture vessels, culture densities, and other fundamental considerations are discussed in Gamborg and Phillips (1995).

Plant tissue cultures are subcultured to fresh medium on a regular basis, depending on their growth rates (Gamborg and Phillips 1995). Callus cultures, shoot proliferation cultures, and rooting cultures on semi-solid media are typically subcultured at 3–4 wk intervals, but subculture frequency may be as often as weekly or bimonthly, or as seldom as every 8 wk, depending on the culture system and incubation conditions. Cell suspensions in batch cultures may be subcultured to fresh medium at biweekly, weekly, or bimonthly intervals, or even continuously, using certain bioreactors. Most plant tissue culture scientists attempt to time the subculture interval to coincide with mid-exponential or early-linear growth phases, in order to maximize growth potential, or time subcultures to preclude the growth-limiting depletion of any nutrient in the basal medium.

Plant Growth Regulators

Synthetic *vs.* natural Once the basal nutrient demands of plant tissues are met, further developmental responses are stimulated through the addition of growth regulators such as auxins, cytokinins, gibberellins, ethylene (or, more accurately, anti-ethylene), or abscisic acid. For the majority of applications, auxins and cytokinins are the most important of these growth regulators. However, synthetic growth regulators, especially synthetic auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (PIC), are more potent by perhaps 10–1000 times than natural auxins, such as indole-3-acetic acid (IAA); therefore, amounts used are relative to potency. Auxin potency also varies among plant species. For these reasons, it is impossible for classical physiologists to compare the action of different natural and synthetic auxins on an equimolar basis, because a nanomolar amount of PIC may have similar activity to a micromolar amount of IAA. It is therefore common to report growth regulator concentrations in mg L^{-1}, the actual amount of the specific chemical used during media preparation, rather than molarity. Commonly used plant growth regulators, as surveyed from Herman (2015), are presented in Table 2 and discussed in more detail below.

Auxins The 5-y set of media citations (Herman 2015) indicated that α-naphthaleneacetic acid (NAA; 15% of citations) and indole-3-butyric acid (IBA; 9% of citations) are the most frequently used auxins, followed by 2,4-D, IAA, and PIC. Natural auxin, IAA, is light sensitive and degrades easily, while the other auxins mentioned are synthetic and chemically more stable. Commonly, NAA, IBA, and IAA are used in shoot multiplication cultures and in rooting cultures. Dicamba (DCA), 2,4-D, and PIC are auxinic herbicides and tend to exhibit activity at lower concentrations than do IAA, IBA, and NAA. Any of these auxins can be used to stimulate callus or cell proliferation. For induction of somatic embryogenesis, 2,4-D has been used in many species; however, in some species, DCA and PIC can also be used to induce somatic embryogenesis. Other auxinic herbicides, such as ρ-chlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid have been employed as well, but as they do not seem to have any unique properties compared to 2,4-D, they are rarely used in current protocols. Following induction, 2,4-D is known to suppress somatic embryo development, which leads to a decline in regeneration capacity over time. Dunstan and Short (1978) reported that regeneration from onion callus was only possible for callus up to 6 mo of age, when 2,4-D was used for induction, whereas, Phillips and Hubstenberger (1987) demonstrated that PIC sustains regeneration capacity over longer periods of time by regenerating plants from 20-mo-old onion callus. Additionally, brassinosteroids have been evaluated as alternative auxins or auxin stimulators, along with several other auxin variants and inhibitors available for specialty situations, and new generation auxinic herbicides are continuously under development (George *et al.* 2008).

Cytokinins The 5-y set of media citations (Herman 2015) indicated that N^6-benzyladenine (BA) is the most frequently used cytokinin (31% of citations), followed by kinetin (KIN; 7.5% of citations). Thidiazuron (TDZ) and 2-isopentenyladenine (2-IP) were cited less frequently (4% of citations each). Natural cytokinins such as zeatin (ZEA) are expensive and less chemically stable than synthetic versions, such as BA. Thidiazuron is more frequently used in woody plant applications (Huetteman and Preece 1993); it appears to have properties in common with both auxins and cytokinins and was originally developed as an herbicide by virtue of its inhibition of cytokinin oxidase. Adenine hemisulfate (ADE) is used as a cytokinin precursor and has weak cytokinin activity. Manipulation of auxin and

Table 2. Phytohormones and growth regulators commonly used in plant tissue culture

Phytohormone or growth regulator	Activity	Abbreviation
Indole-3-acetic acid	Natural auxin	IAA
Indole-3-butyric acid	Synthetic auxin	IBA
α-Naphthaleneacetic acid	Synthetic auxin	NAA
2,4-Dichlorophenoxyacetic acid	Synthetic auxinic herbicide	2,4-D
Picloram (4-amino-3,5,6-trichloropicolinic acid)	Synthetic auxinic herbicide	PIC
Dicamba (3,6-dichloro-σ-anisic acid)	Synthetic auxinic herbicide	DCA
Relative activity of auxins: PIC > > 2,4-D, DCA ≥ NAA, IBA > IAA		
Zeatin	Natural cytokinin	ZEA
Kinetin (6-furfurylaminopurine)	Natural/synthetic cytokinin	KIN
N^6-Benzyladenine (6-benzylaminopurine)	Synthetic cytokinin	BA
2-Isopentenyladenine [6-(γ,γ-dimethylallylamino)purine]	Natural cytokinin	2-IP
Adenine hemisulfate	Cytokinin precursor	ADE
Thidiazuron [N-phenyl-N′-(1,2,3-thiadiazol-5-yl)urea]	Synthetic cytokinin regulator	TDZ
Relative activity of cytokinins: TDZ ≥ BA > 2-IP, KIN, ZEA > > ADE		
Gibberellic acid	Natural gibberellin	GA3
Paclobutrazol	Synthetic anti-gibberellin	PBZ
Uniconazole	Synthetic anti-gibberellin	UNI
Abscisic acid	Natural abscisic acid	ABA
Silver nitrate	Blocks ethylene action	$AgNO_3$
Silver thiosulfate	Blocks ethylene action	AgS_2O_3
Jasmonic acid	Natural jasmonate, used as an elicitor for defense responses	JA
Ascorbic acid	Antioxidant	ASA
Polyvinylpyrrolidone	Antioxidant	PVP

cytokinin alone was sufficient to accomplish culture goals in 89% of the cases cited by Herman (2015).

Gibberellin, abscisic acid, ethylene, and others Gibberellic acid (GA_3) was cited in 4% of the cases summarized by Herman (2015) and is commonly used in shoot elongation protocols, but it can also influence seed development and overcome dormancy. Anti-gibberellins, such as paclobutrazol (PBZ) and uniconazole (UNI) are available; they are used to prevent GA_3 synthesis and thus reduce stem elongation. Interestingly, PBZ and UNI were reported in the Herman (2015) citations for use in stimulating rooting of woody plants. Abscisic acid (ABA), commonly used to regulate somatic embryo development, was employed in 2% of the cases referenced by Herman (2015). Silver nitrate or silver thiosulfate was used in 5% of the cases in the Herman 5-y survey (2015); silver compounds are frequently used to inhibit the action of ethylene. The synthesis of ethylene can be promoted by 1-aminocyclopropane-1-carboxylic acid (ACC) or 2-chloroethylphosphonic acid (ethephon) (Biddington 1992). Jasmonic acid (JA) is often used as an elicitor in hairy root cultures. While not phytohormones, ascorbic acid (ASA) or polyvinylpyrrolidone (PVP) have been used as antioxidants in 4% of the Herman (2015) 5-y references.

Phytohormone receptors and differential activity Plant tissue culture scientists may spend significant time identifying genotypes that are responsive in culture or determining the explant types that will support the type of growth or developmental response under study. Many have observed that the same growth regulator treatment applied to different explants (tissue types) of the same species or genotype may result in different responses, suggesting possible tissue specificity of phytohormone receptors or effectors (Phillips 1988) or the interaction of endogenous phytohormones within the tissues and the exogenously supplied growth regulators in tissue culture.

Phytohormones achieve their effects through signal transduction and transcriptional activation mediated initially by binding to specific receptors. At one time, it was thought that there would be only a single receptor per phytohormone class, *e.g.*, only one auxin-binding receptor. However, recent research has identified multiple receptors per phytohormone class. For instance, there has been a 23-member family of auxin response factor (ARF) proteins, a 29-member family of Aux/IAA co-receptor proteins, and a 5-member family of auxin signaling F-Box proteins identified to be associated with auxin activity (Mockaitis and Estelle 2008). Similarly, three cytokinin receptors (histidine kinases) localized to the endoplasmic reticulum (ER) have been identified (Wulfetange *et al.* 2011). Gibberellin

signaling involves two F-Box receptors (Daviere and Achard 2013). Abscisic acid (ABA) receptors include 14 members of the Pyrabactin Resistance (PYR)/PYR-Like (PYL)/Regulatory Components of ABA Receptors (RCAR) family (Kline *et al.* 2010). There are five structurally and functionally distinct ER-localized receptors for ethylene (Gallie 2015). The last of these authors asked: "Why so much complexity?"

In contrast to plants, animal hormone models tend to be discretely compartmentalized in organs, with little direct interaction among them; thus, the answer to the query above might simply be: there is so much complexity among phytohormone receptors, because phytohormones initiate or mediate so many different physiological processes and interact with each other in various ways during these processes (illustrated in Table 3). A single receptor per phytohormone would seem unable to mediate such different responses; while a single phytohormone binding to a single receptor may have a particular allosteric result and physiological effect, it is difficult to imagine how that would lead to multiple responses. Table 3 illustrates that different physiological responses do occur, at least in part, because of phytohormone interactions. Another illustration of phytohormone interactions is the fact that cytokinin is synthesized in root apices and then transported to the shoot, whereas auxin is synthesized in the shoot apices and transported to roots, thus creating reciprocal gradients throughout the plant tissues.

Another way in which a single phytohormone can affect multiple responses is by subcellular compartmentation (Spartz and Gray 2008); abscisic acid binds to distinct receptors in the plasmalemma, in the nucleus, and in the chloroplast, which may explain some of the different physiological functions of the phytohormone. Similarly, auxin is known to bind to distinct receptors in the cytoplasm and in the nucleus (Mockaitis and Estelle 2008). Compartmentation readily explains how a single phytohormone can have more than one physiological effect. While animal hormones and their receptors tend to be compartmentalized discretely by organ or tissue type, phytohormone activity appears to be compartmentalized by subcellularly located receptors with distinct signal transduction pathways.

In addition, auxin receptor–ligand interaction studies have shown that electrostatic differences in binding of IAA *vs.* PIC lead to differential binding affinities (Calderon Villalobos *et al.* 2012). This explains why a nanomolar amount of PIC may have similar effect as a micromolar amount of IAA. With multiple receptors and co-receptors for auxin, and differential binding affinities for the various versions of synthetic growth regulators and the phytohormone itself, it can easily be envisioned that different combinations of these components lead to multiple physiological responses. At any given time during development, some of the co-receptors and effectors available vary depending upon tissue and environmental cues. If plant growth and development is based, at least in part, on the allosteric interaction of each phytohormone (or its synthetic counterparts) with its subcellular-specific compartment or situation-specific (environmentally cued) receptors and/or co-receptors/effectors, then these differential interactions may explain the distinct effects of growth regulators observed both *in vivo* and *in vitro*. Perhaps such differential allosteric/electrostatic binding effects might explain why 2,4-D used as an auxin seems to have the almost unique ability to induce somatic embryogenesis in tissue cultures of many plant species.

Classical Developmental Models

Tobacco organogenesis The classic demonstration of plant tissue response to growth regulators was reported by Skoog and Miller (1957), using tobacco as a model system to study the effects of IAA and KIN (see Fig. 1). Using a factorial experiment, Skoog and Miller (1957) noticed that shoots formed when cytokinin was high and auxin low, whereas roots formed and proliferated when auxin was moderate to high and cytokinin was absent. Callus (undifferentiated mass of cells) proliferated when moderate amounts of cytokinin were combined with high auxin (Fig. 1).

Table 3. Examples of plant developmental processes involving six classes of phytohormones (data extracted from Curaba *et al.* 2014)

Plant developmental stage	Phytohormone involvement					
	Auxin	Cytokinin	Abscisic acid	Gibberellic acid	Ethylene	Jasmonic acid
Embryo	Yes	Yes	No	Yes	Yes	No
Germination	Yes	Yes	Yes	Yes	Yes	No
Stress response	Yes	Yes	Yes	Yes	Yes	Yes
Root	Yes	Yes	Yes	Yes	Yes	Yes
Leaf	Yes	Yes	No	Yes	Yes	Yes
Phase transition	No	No	Yes	Yes	No	No
Flower	Yes	Yes	No	Yes	Yes	Yes
Senescence	Yes	Yes	Yes	Yes	Yes	Yes

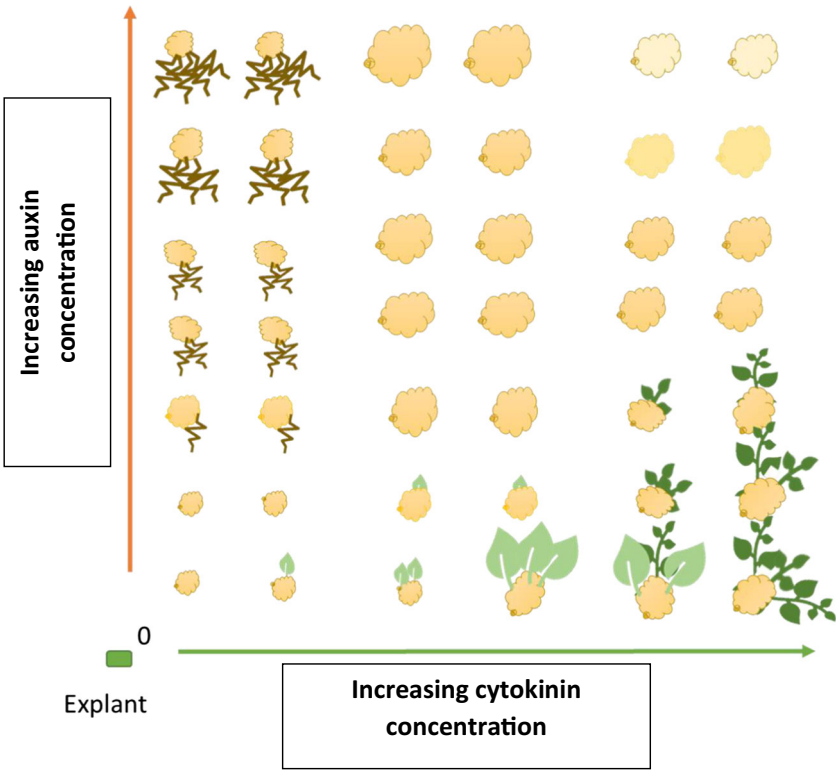

Figure 1. Schematic illustration of the Skoog and Miller (1957) *Nicotiana tabacum* L. (tobacco) phytohormone model for organogenesis. Auxins and cytokinins used in combination elicit an array of tissue responses based on their relative concentrations. Higher relative auxin concentrations increase root formation, whereas higher relative cytokinin concentrations induce shoot formation. When both auxin and cytokinin concentrations are moderate to high, callus is induced.

These observations formed the basis of shoot and root organogenesis phytohormone models and of biomass (callus) growth in tissue culture, and essentially established the modern concept of plant growth regulators. Induction and development of the organ can occur on the same medium. It is noteworthy that shoots are induced and developed on one medium treatment, while roots are induced and developed on another medium treatment (within typical subculture intervals), so that at least two culture steps are required to obtain a whole plant: at least one induction/development treatment for each organ (shoot, root). Some plants are induced for rooting on an auxin-containing medium, then transferred to growth regulator-free medium for development (Gamborg and Phillips 1995), thereby adding an extra culture step.

***Daucus carota* L. (carrot) somatic embryogenesis** Not long after Skoog and Miller's (1957) discovery of shoot and root formation in tobacco, Reinert (1958) and Steward *et al.* (1958) observed carrot cells in culture develop into embryos rather than simple organs (Fig. 2). These so-called somatic embryos follow the characteristic developmental stages of zygotic embryos. Somatic embryos are induced by high auxin, and 2,4-D seems to have an almost unique capacity to stimulate somatic embryogenesis in many plant species. In some cases, direct somatic embryogenesis occurs without additional cycles of embryogenesis, whereas in other cases, the somatic embryos will proliferate additional series of somatic embryos in an adventitious manner (arising from an unusual location other than a pre-existing meristem). In yet other species, an embryogenic callus proliferates, which is then manipulated to recover mature somatic embryos. Developing somatic embryos are bipolar structures, containing both shoot- and root-meristems. Typically, high auxin (usually 2,4-D) induction is followed by phytohormone-free development and maturation of the somatic embryos, so at least two culture steps are required to obtain the whole plant: one induction step for the bipolar embryo, and at least one development (maturation) step.

Origins and modes of development The easiest and most direct mode to regenerate plants from tissue cultures is by enhanced axillary branching, which is the basis of most micropropagation or cloning protocols (Fig. 3*A*). Axillary buds are preformed meristems, and stimulation of axillary branching typically results in a 10-fold increase in shoot number per monthly culture passage, with as many as 1000,000 shoots in 6 mo proliferating from a single explant. A proportion of these shoots can be separated and rooted for commerce, while others are used to maintain the shoot proliferation stock. Proliferation of axillary branches or nodes tends to minimize spontaneous mutations in the stock material (Chu 1992).

As illustrated above in the classical developmental models, plants also can be regenerated *via* organogenesis (Fig. 3*B*, *C*) or through somatic embryogenesis (Fig. 3*C*, *D*; Gamborg and Phillips 1995). Either of these modes can occur from an adventitious origin (Fig. 3*B*, *D*) or from a *de novo* origin (Fig. 3*C*). Adventitious and *de novo* organogenesis and *de*

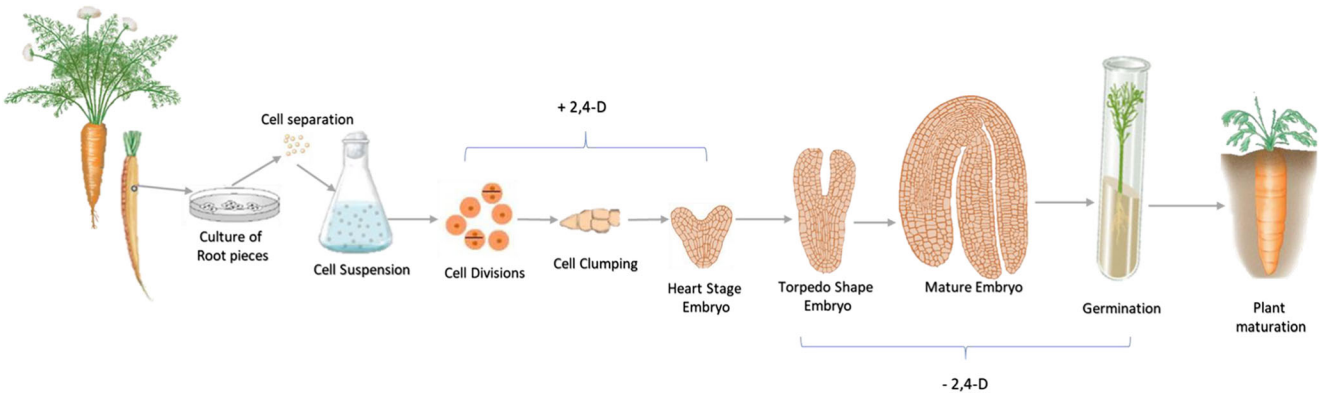

Figure 2. Schematic illustration of the Reinert (1958) and Steward *et al.* (1958) *Daucus carota* L. (carrot) phytohormone model for somatic embryogenesis. The auxin 2,4-dichlorophenoxyacetic acid (*2,4-D*) is used to proliferate cells and to induce somatic embryogenesis. Upon removal of *2,4-D*, the small somatic embryos develop into mature embryos and plantlets, following the same developmental stages as zygotic embryogenesis.

novo somatic embryogenesis systems tend to accumulate more spontaneous mutations, compared to enhanced axillary branching and adventitious somatic embryogenesis (Chu 1992). Adventitious shoots, roots, or somatic embryos arise from organized tissues, but not from preformed meristems (Gamborg and Phillips 1995). Often the presence of vascular or dermal tissues is required for new adventitious structures to form, and adventitious responses are typically shorter-lived, persisting for perhaps 30–60 d of culture. In contrast, *de novo* shoots, roots, or somatic embryos "arise anew" from unorganized proliferating tissues such as callus, and *de novo* responses typically persist for months or even a few years in culture. The *de novo* systems of plant regeneration are necessary for cell selection and other applications requiring longer-term culture manipulation. Adventitious systems can be useful for micropropagation and other shorter-term applications. Either adventitious or *de novo* systems are used to recover plants following gene transfer, depending upon the species.

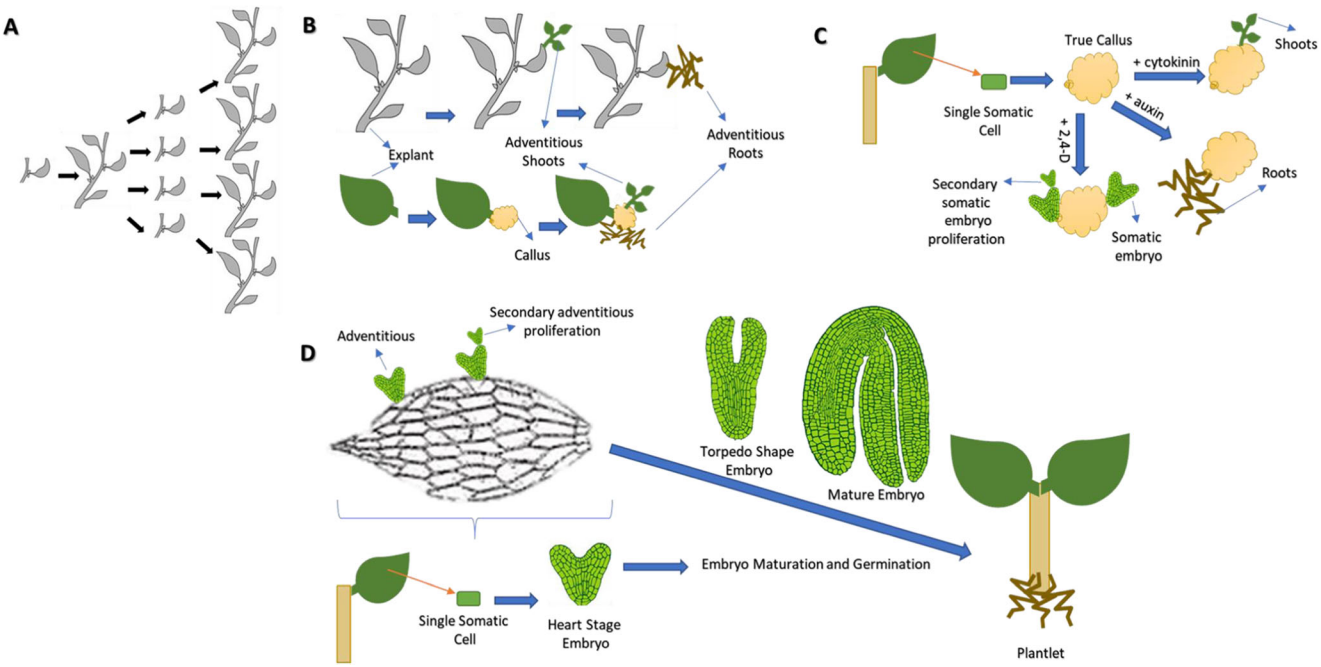

Figure 3. Origins and modes of development during *in vitro* plant regeneration. (*A*) Mode of axillary branching, originating from preformed meristems. (*B*) Adventitious origin of the mode of organogenesis, giving rise to *shoot* organs or *root* organs occurring at an unusual location on the plant, not from preformed meristems. (*C*) *De novo* origin of regeneration from unorganized *callus*, illustrated for the mode of organogenesis (*shoots*, *roots*) and for the mode of *somatic embryogenesis via* 2,4-dichlorophenoxyacetic acid (*2,4-D*). (*D*) Adventitious origin of the mode of somatic embryogenesis.

Elaborated Developmental Models

***Trifolium pratense* L. (red clover)** A comprehensive system for the culture of cells, tissues, and organs of red clover, *Trifolium pratense* L., is presented here, because it illustrates a number of principles and concepts that elaborate on the classical developmental models and medium manipulation.

Basal media were evaluated for callus proliferation using five cultivars of red clover (Phillips and Collins 1979a). A series of factorial and split-plot design experiments were performed using visual ratings of callus proliferation. First, the macronutrients were optimized, then the micronutrients were optimized using MS, SH, and B5 as initial control media. More than 60 formulations were assessed to optimize the macro- and micronutrients. More than 20 formulations were compared for carbohydrate and vitamin requirements. More than 45 formulations were compared for growth regulator composition, and these were tested additionally with multiple genotypes of *Glycine max* (L.) Merr. (soybean) and *Medicago sativa* L. (alfalfa). This tedious process resulted in the L2 (Legume version 2; Phillips and Collins 1979a) basal medium developed specifically to support growth of forage and grain legumes (Table 4). Biomass fresh weight increases were measured in a final comparison of L2, MS, and SH (B5 was not used because in previous experiments, it had yielded responses identical to those of SH). The L2 basal medium resulted in statistically superior callus growth for alfalfa and soybean over MS and SH. Red clover performed equally well on MS or L2, but L2 was superior to SH for callus growth. The Phillips and Collins (1979a) L2 basal medium is similar to MS, but with increased amounts of some nutrients and less ammonium nitrogen. Callus cultures proliferating on high PIC or 2,4-D concentrations regenerated shoots, some of which spontaneously rooted, when transferred to medium augmented with 0.005 mg L^{-1} PIC + 1.0 mg L^{-1} BA.

The red clover tissue culture system was then adapted for virus elimination from cultivar parental clones (Phillips and Collins 1979b). Full-strength L2 basal medium using 0.004 mg L^{-1} PIC + 1.0 mg L^{-1} BA appeared to be best for stimulating shoot growth and shoot multiplication by enhanced axillary branching from explants containing the meristematic dome and one primordial leaf (0.1 to 0.4 mm shoot meristem tips). The auxin content had to be slightly reduced in order to eliminate excess callusing. These shoots rooted on half-strength L2 basal medium containing 0.2 mg L^{-1} IAA (RL; Rooting on modified L2) shown in Table 4. The rooting medium was developed by comparing full-strength, ½-strength, and ¼-strength versions of L2, with ½-strength being best. Also, different auxins were tested before arriving at 0.2 mg L^{-1} IAA (Phillips and Collins 1979b).

The callus to plant regeneration system was elaborated further in a subsequent study using cell suspensions of red clover (Phillips and Collins 1980). The basal medium for cell suspension culture, SL2 (Suspensions in modified L2; Table 4), was adjusted by lowering the amounts of macronutrient salts (approximately 90% of L2) and especially ammonium nitrogen, because full-strength L2 appeared to be borderline toxic to liquid cell suspension cultures (Phillips and Collins 1979a). By taking cultures through a cycle from fine cell suspension back to callus and using 0.06 mg L^{-1} PIC + 0.1 mg L^{-1} BA for both steps, it was apparent that somatic embryogenesis was the mode of plant regeneration (Phillips and Collins 1980). That is, cotyledons emerged, followed by the appearance of a unifoliate leaf, which in turn was followed by the development of trifoliate leaves—the typical legume embryo–to–plant developmental stages. Although a range of concentrations of PIC or 2,4-D supported the induction of somatic embryogenesis, Phillips and Collins (1980) determined that the best induction of somatic embryos occurred using 0.01 mg L^{-1} 2,4-D + 2 mg L^{-1} ADE. Subsequently, somatic embryos were transferred to shoot pole promotion medium containing 0.001 mg L^{-1} PIC + 0.2 mg L^{-1} BA and were eventually rooted as before. Later this strategy was

Table 4. The nutrient compositions of the Legume version 2 (*L2*) basal medium (Phillips and Collins 1979a) and its modifications (Phillips and Collins 1979b, 1980) used for tissue culture of *Trifolium pratense* L. (red clover)

Basal medium	L2	SL2	RL	SGL
Macronutrient components (mg L^{-1})				
KNO_3	2100	2100	1050	210
NH_4NO_3	1000	600	500	100
$NaH_2PO_4{\cdot}H_2O$	85	–	42.5	8.5
$MgSO_4{\cdot}7H_2O$	435	400	217.5	43.5
KH_2PO_4	325	250	325	32.5
$CaCl_2{\cdot}2H_2O$	600	550	300	60
Micronutrient components (mg L^{-1})				
H_3BO_3	5	4.5	2.5	–
KI	1	0.9	0.5	–
$MnSO_4{\cdot}H_2O$	15	13.5	7.5	–
$ZnSO_4{\cdot}7H_2O$	5	4.5	2.5	–
$CuSO_4{\cdot}5H_2O$	0.1	0.09	0.05	–
$Na_2MoO_4{\cdot}2H_2O$	0.4	036	0.2	–
$CoCl_2{\cdot}6H_2O$	0.1	0.09	0.05	–
$FeSO_4{\cdot}7H_2O$	25	25	25	2.5
Na_2EDTA	33.5	33.5	33.5	3.4
Vitamins and organics (mg L^{-1})				
Myo-inositol	250	250	125	62.5
Pyridoxine HCl	0.5	0.5	0.25	0.13
Thiamine HCl	2	2	1	0.5
Nicotinic acid	–	–	1	–
Sucrose (g/L)	25	25	15	10
Plant tissue culture agar (g/L)	8	–	6.5	6.5
pH	5.8	5.8	5.8	5.8

extended to regeneration of plants from clover protoplasts (Grosser and Collins 1984; Myers *et al.* 1989).

The tissue culture system was then adapted to rescue immature heart-staged hybrid zygotic embryos from interspecific crosses (Phillips *et al.* 1982). Immature zygotic embryos were rescued and developed initially using L2 basal medium + 12.5% (*w/v*) sucrose (to inhibit precocious root germination) + 0.006 mg L^{-1} PIC + 2 mg L^{-1} ADE, designated LIH (L2 for Interspecific Hybrid embryos). These "matured" embryos were transferred to a version of the shoot pole promotion medium (as used with somatic embryos), the best version in this case being 0.001 mg L^{-1} PIC + 0.15 mg L^{-1} BA. The BA concentration had to be adjusted downward from 0.2 mg L^{-1} to eliminate excess callusing. Once shoots emerged on this medium, they were transferred to a version of the shoot multiplication medium (as used with shoot meristem tips), the best version in this case being 0.003 mg L^{-1} PIC + 0.5 mg L^{-1} BA. Shoots were rooted as before. This strategy also worked for a second interspecific hybrid (Phillips *et al.* 1992).

As can be observed from the history above, some developmental steps were optimized in an evolving manner as the system was applied to other situations or explants. All of these developmental steps were tied together into a model system (Collins and Phillips 1982; Phillips and Collins 1984), illustrated in Fig. 4. This model (Fig. 4) identifies discrete developmental stages and their relationships: Seedlings may be germinated under sterile conditions on SGL (Seed Germination modified L2; Table 4) medium to provide explants. Explants produce callus on L2 medium. Callus may be used to establish cell suspension cultures using SL2 medium, and suspensions may be cycled back to callus (using L2 medium), prior to regeneration. Also, protoplasts from cell suspensions or from leaf explants are recovered as cell suspensions and cycled to callus for regeneration. Callus from any of these sources may be induced for somatic embryogenesis on LSE (L2 for Somatic Embryogenesis) medium. Somatic embryos are transferred to LSP2 (L2 for Shoot Pole promotion v.2) medium for embryo shoot pole promotion and germination. Immature hybrid heart-staged zygotic embryos are matured on LIH medium, and then the shoot poles are promoted using LSP2 medium. Whether derived from somatic embryos or immature zygotic embryos, shoots are multiplied by enhanced axillary branching using ML8 (Multiplication on L2 v.8) medium. Shoot tips of any clone of red clover or shoot meristem tips as used for virus elimination also proliferate multiple shoots using ML8 medium. Regardless of the source of shoots, they are rooted using RL medium. The resulting plantlets are ready for acclimatization in a growth chamber or greenhouse, and eventual transfer into a field for further study.

The red clover model illustrates two distinct ways in which the medium can be manipulated to achieve specific developmental or growth responses. First, the basal medium can be adjusted for specific developmental steps (Table 4), and, second, the growth regulators can be adjusted for specific developmental steps (Table 5).

The L2 Basal medium is the full-strength version used for tissue and organ proliferation (Table 4). The SL2 medium has less ammonium nitrogen and a little less of the other macronutrient salts to support growth of cell suspensions, whereas the RL medium is a half-strength version of L2 used specifically for rooting of shoots (Table 4). Seed Germination modified L2 (SGL) medium, used for *in vitro* seed germination, is approximately a 1/10-strength version of L2 major salts with no micronutrient salts, but with ¼-strength vitamins and 40% of the sucrose (Table 4).

Most of the developmental manipulation is accomplished through the use of growth regulators (Table 5): Seed germination on SGL does not require any growth regulators. Callus or

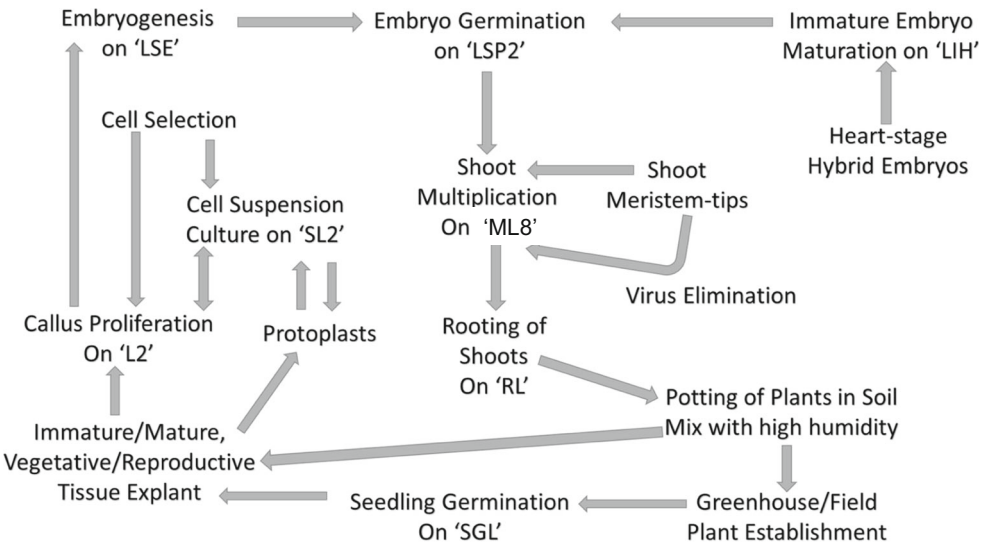

Figure 4. Discrete developmental steps in *Trifolium pratense* L. (red clover) tissue culture (adapted from Phillips and Collins 1984). *LSE*, *L2* modified for somatic embryogenesis; *LSP2*, *L2* for shoot pole promotion version 2; *LIH*, *L2* modified for interspecific hybrid embryos; *SL2*, suspension culture modified *L2*; *ML8*, multiplication modified *L2* version 8; *L2*, legume medium version 2; *RL*, rooting modified *L2*; *SGL*, seed germination modified *L2*.

Table 5. Growth regulator manipulations used in tissue culture of *Trifolium pratense* L. (red clover) adapted from Phillips and Collins (1984)

Medium designation	Use	Basal medium (from Table 3)	Auxin		Cytokinin	
			Source, mg L^{-1}	Relative activity	Source, mg L^{-1}	Relative activity
SGL	Seedling germination	SGL	–	–	–	–
L2	Callus growth	L2	PIC, 0.06	High	BA, 0.1	Moderately low
SL2	Cell suspension growth	SL2	PIC, 0.06	High	BA, 0.1	Moderately low
LSE	Induction of somatic embryogenesis	L2	2,4-D, 0.01	Low	ADE, 2	Very low
LIH	Rescue of heart-staged hybrid embryos	L2 + 12.5% sucrose	PIC, 0.006	Moderately low	ADE, 2	Very low
LSP2	Shoot development from sexual or somatic embryos	L2	PIC, 0.001	Very low	BA, 0.15	Moderate
ML8	Shoot multiplication by axillary branching	L2	PIC, 0.003	Low	BA, 0.5	High
RL	Rooting of shoots	RL	IAA, 0.2	Moderately low	–	–

2,4-D, 2,4-dichlorophenoxyacetic acid; *PIC*, picloram (4-amino-3,5,6-trichloropicolinic acid); *IAA*, indole-3-acetic acid; *BA*, N6-benzyladenine (6-benzylaminopurine); *ADE*, adenine hemisulfate

cell suspension proliferation on L2 or SL2 medium involves a relatively high level (0.06 mg L^{-1}) of the auxin PIC along with a moderately low level (0.1 mg L^{-1}) of BA as cytokinin. Somatic embryogenesis is induced on LSE medium using a very low level of (0.01 mg L^{-1}) the auxin 2,4-D, plus very weak cytokinin activity using ADE (2 mg L^{-1}; Table 5). Heart-staged hybrid embryos are cultured on LIH medium with 12.5% (*w/v*) sucrose to prevent precocious germination of the root pole, supplemented with a low level (0.006 mg L^{-1}) of the auxin PIC, plus very weak cytokinin activity, using 2 mg L^{-1} ADE (Table 5). Both somatic embryos and immature zygotic embryos are encouraged to express the shoot pole using a very low level (0.001 mg L^{-1}) of PIC, plus a moderate level (0.15 mg L^{-1}) of BA in LSP2 medium. Shoots are multiplied on ML8 medium using a moderately low level (0.003 mg L^{-1}) of PIC as auxin plus a high level (0.5 mg L^{-1}) of BA as cytokinin. Shoots are rooted on RL medium using a moderate auxin level of 0.2 mg L^{-1} IAA (Table 5).

This is one optimized model or approach; there are other variations that are equally effective in other systems. For example, in the Finer–Parrott soybean system (Finer and Nagasawa 1988; Samoylov *et al.* 1998), somatic embryos are induced from primary explants, and then those primary somatic embryos are proliferated in large numbers in suspension culture. Individual somatic embryos are matured and germinated into plants. Both the clover and soybean methods include a cloning step: shoots from somatic embryos are cloned in the red clover approach, while somatic embryos themselves are cloned in the soybean approach.

There are intriguing parallels between induction of somatic embryogenesis and development of heart-staged hybrid embryos in the red clover system: both need a low level of auxin (though a different source in each case) and a weak cytokinin signal. Also, the trend of auxin and cytokinin signals from induction through expression shows a pattern: induction involves low auxin + weak cytokinin; then, shoot pole promotion requires a very low auxin + moderately low cytokinin; finally, shoot multiplication benefits from a moderate level of auxin + high cytokinin. The cytokinin signal starts low and increases with each step to a higher level, while the auxin signal fluctuates with each step. It is known that auxin plays a critical role in shoot apical organization (Wang and Jiao 2018); thus, these auxin fluctuations from induction through development might be interpreted as likely to be related to apical organizational steps.

Extension to onion and *Oryza sativa* L. (rice) Although the clover model was developed for a dicot, elements of the clover model were tested on the monocot *Allium* spp., (onion). Basal media were screened and BDS (Dunstan and Short 1977) was found to be superior to others for onion tissue culture growth (Phillips and Luteyn 1983). Callus production was better using 2.0 mg L^{-1} BA + 0.75 mg L^{-1} PIC compared to NAA or 2,4-D as auxin, and this combination also induced somatic embryogenesis. This callus developed somatic embryos when transferred to 0.03 mg L^{-1} PIC + 0.35 mg L^{-1} BA (Phillips and Luteyn 1983; Phillips and Hubstenberger 1987). As somatic embryos developed their shoot poles, they were transferred to 0.03 mg L^{-1} PIC + 0.5 mg L^{-1} BA for shoot multiplication. This regeneration system also applied to onion protoplasts (Hansen *et al.* 1995). In that case, isolated protoplasts from cell suspensions initiated on either 2,4-D- or PIC-based medium were maintained on 2.0 mg L^{-1} NAA + 0.5 mg L^{-1} BA. Cell colonies and calluses were recovered from protoplasts using 1.0 mg L^{-1} NAA + 0.5 mg L^{-1} ZEA. These calluses were transferred to 0.03 mg L^{-1} PIC + 0.35 mg L^{-1} BA for development of somatic embryos.

Subsequently, *Oryza sativa* L. (rice) was tested in a manner similar to onion. Once again, BDS was found to be superior to MS or N6 (Chu 1978) as the basal medium (Chowdhury *et al.* 2006). Callus cultures were started on 2.2 mg L^{-1} 2,4-D or 5.0 mg L^{-1} PIC and were transferred to 0.03 mg L^{-1} PIC + 0.35 mg L^{-1} BA for development of somatic embryos and shoot poles (Chowdhury *et al.* 2006). The resulting rapid rice regeneration system used the same shoot pole promotion medium as was used in onion, with similar success (Dabul *et al.* 2009).

Onion and rice, both monocots, seem to be about 10× less sensitive to PIC, compared to red clover, a dicot plant. Callus formation in onion used 0.75 mg L^{-1} PIC (Phillips and Luteyn 1983) *vs.* 0.06 mg L^{-1} PIC for red clover (Phillips and Collins 1980). Clover shoot pole promotion used 0.001 mg L^{-1} PIC (Phillips *et al.* 1982), but for onion or rice shoot pole promotion, 0.03 mg L^{-1} PIC had to be used (Phillips and Hubstenberger 1987; Dabul *et al.* 2009). Shoot pole promotion in onion and rice also used a higher amount of BA, 0.35 mg L^{-1} (Phillips and Hubstenberger 1987; Dabul *et al.* 2009) *vs.* 0.15 mg L^{-1} for clover (Table 5; Phillips and Collins 1984). The results from these contrasting plant systems lead to the suggestion that a range of shoot pole promotion media using 0.001 to 0.03 mg L^{-1} PIC + 0.15 to 0.35 mg L^{-1} BA might be used to screen other species.

***Pinus brutia var. eldarica* (Medw.) Silba (Eldarica pine)**

Differences in tissue culture requirements are not limited to dicots and monocots; gymnosperms have their special stipulations as well. *Pinus brutia var. eldarica* (Medw.) Silba (Eldarica pine) presents an interesting case study. Basal media were screened, and it was found that MMS (Table 1), with half-strength macronutrient salts and full-strength micronutrient salts, was most suitable (Gladfelter and Phillips 1987). A *de novo* organogenesis regeneration system from up to 3-yr-old callus, maintained on medium containing 0.5 to 1.0 mg L^{-1} NAA + 0.5 to 1.0 mg L^{-1} BA (Fig. 5; Gladfelter and Phillips 1987), was developed. The interesting aspect of this regeneration system was that four distinct culture manipulations were required to obtain shoots (Fig. 5): The first step was bud induction, using 0.05 mg L^{-1} IBA + 1.0 mg L^{-1} KIN. The second step was a resting phase on growth regulator-free MMS medium, needed for bud maturation. Callus could be recycled between these two media to enhance competence for regeneration. Once the buds had matured sufficiently on MMS, the third step was apical organization, including early needle development (shoot pole promotion), using 0.05 mg L^{-1} IBA + 0.1 mg L^{-1} KIN. These buds were advanced to step four, shoot elongation using growth regulator-free MMS. Some shoots were rooted using a 1-d pulse of a high level (5.0 mg L^{-1}) of NAA. Once again, a low level of auxin (0.05 mg L^{-1} IBA) and a moderate level of cytokinin (0.1 mg L^{-1} KIN) encouraged shoot apical organization in this *de novo* regeneration system. The same medium as used for bud induction, MMS + 0.05 mg L^{-1} IBA + 1.0 mg L^{-1} KIN, was used successfully for short-term adventitious shooting from explants and for enhanced axillary branching of Eldarica pine, as well as for *de novo* shoot organogenesis (Fig. 5).

This Eldarica pine model developed by Gladfelter and Phillips (1987) demonstrates that cycling between defined media may enhance acquisition of competence for regeneration, as well as shows that multiple steps of growth regulator manipulation may be needed to achieve individual organ or plant recovery. These concepts were also being developed along independent lines of evidence by Christianson (1987)

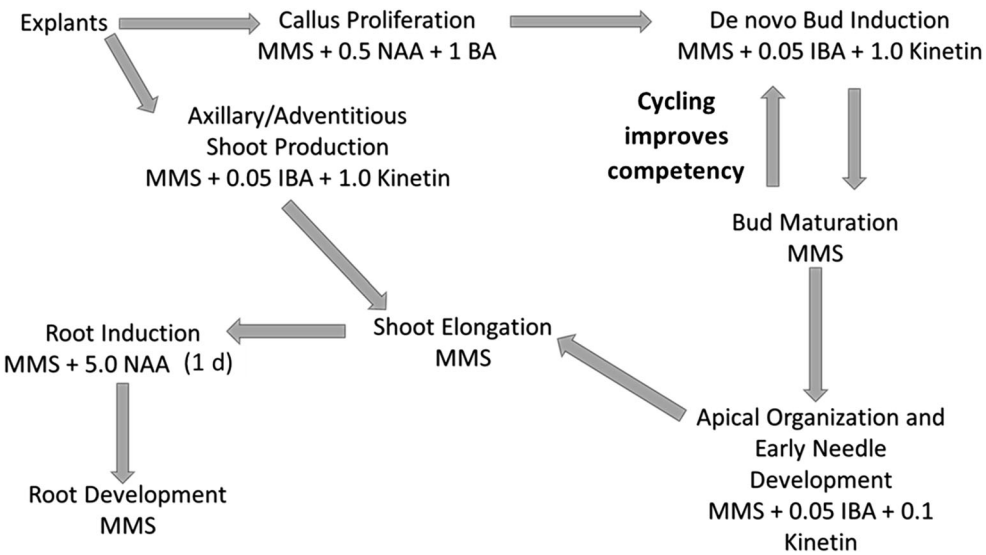

Figure 5. Discrete developmental steps in *Pinus brutia var. eldarica* (Medw.) Silba (Eldarica pine) *de novo* organogenesis. Growth regulators shown in mg L^{-1} (Gladfelter and Phillips 1987, reproduced with permission from Springer Nature, Switzerland AG). *MMS*, modified Murashige and Skoog medium; *NAA*, α-naphthaleneacetic acid; *BA*, N^6-benzyladenine; *IBA*, indole-3-butyric acid.

using *Convolvulus arvensis* L. (field bindweed). Christianson (1987) demonstrated that pluripotency (*i.e.*, capability of multiple organogenic responses, such as shoot *vs.* root *vs.* callus) was a short-term phenomenon, that cycling between defined media may enhance acquisition of competence for a particular organogenic response, and that the regeneration process can be arrested at several distinct points of time by different treatments. Both the Eldarica pine and the *Convolvulus* system appear to be consistent with each other in these respects, especially if the assumption is made that the multiple steps of growth regulator manipulation observed in Eldarica pine cultures correspond to overcoming points of organogenic arrest in Christianson's model.

There is also a parallel between the Eldarica pine multi-step regeneration process and the soybean somatic embryogenesis system developed by the aforementioned Finer (Finer and Nagasawa 1988) and Parrott (Samoylov *et al.* 1998) labs. The soybean induction medium was optimized primarily by increasing the ammonium to nitrate ratio and total nitrogen amounts, along with high 2,4-D (Finer and Nagasawa 1988). Once induced, somatic embryos were proliferated using lower levels of 2,4-D and lower total nitrogen. Histo-differentiation and maturation of these somatic embryos into whole plants required another two culture treatments, involving the use of maltose as a carbohydrate source and activated charcoal, followed by removal of the activated charcoal (Samoylov

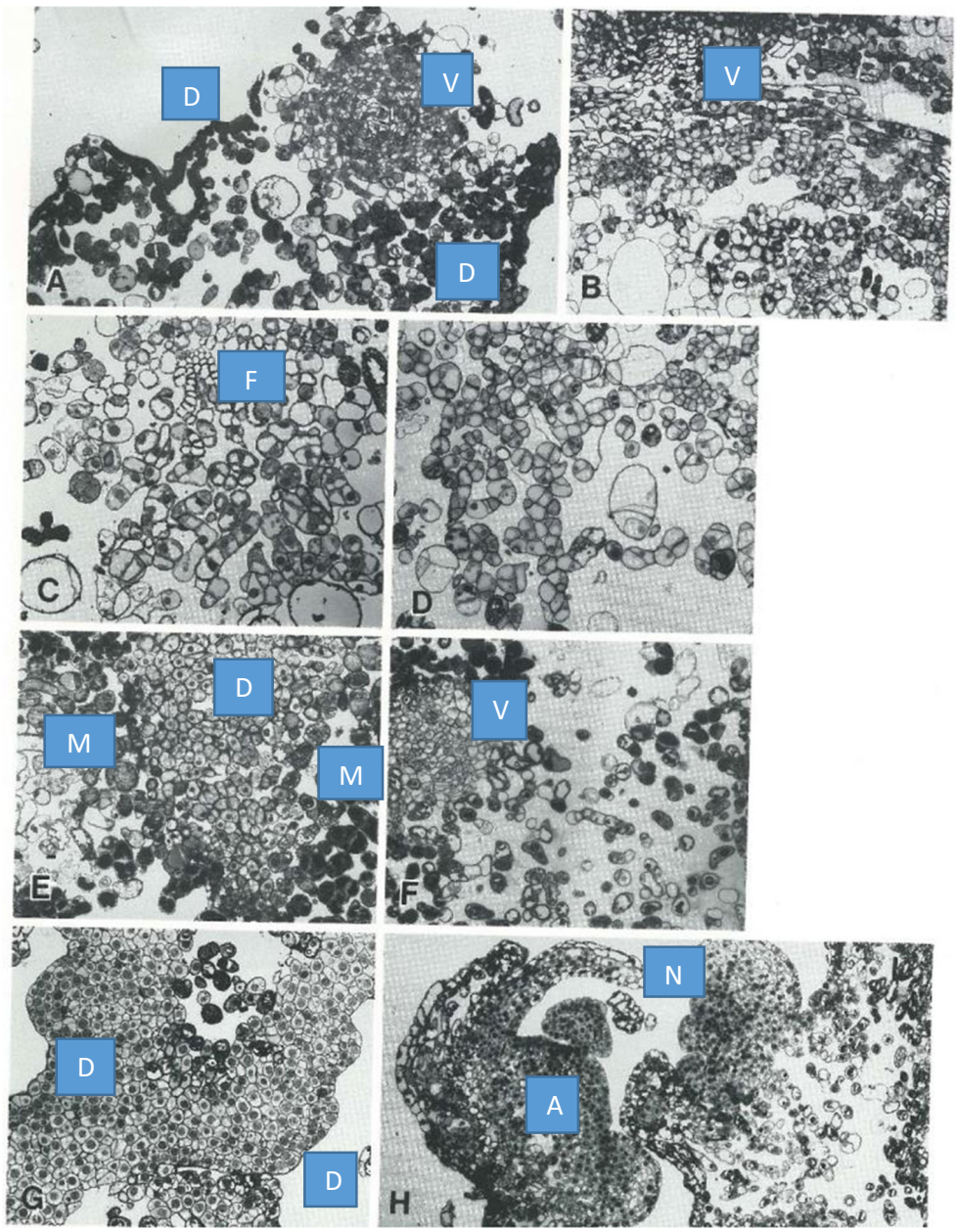

Figure 6. Photomicrography of callus development and *de novo* shoot organogenesis in *Pinus brutia var. eldarica* (Medw.) Silba (Eldarica pine). (*A*) Longitudinal section of cotyledon explant showing vascular organization (*V*) and dermal layers (*D*). (*B*) Callus proliferation after 2 mo, exhibiting remnants of the vascular trace (*V*). (*C*) Callus 2 mo of age illustrating random location of a file (*F*) of specialized cells. (*D*) Unorganized callus after 6 mo of culture. (*E*) Early stage of *de novo* bud induction in callus 18 mo of age; note the subsurface concentration of dividing cells (*D*), surrounded by moribund cells (*M*). (*F*) Note nonrandom planes of cell division and vascular development (*V*) during *de novo* bud induction. (*G*) Evidence of dermal layer formations (*D*) deep within the callus undergoing *de novo* bud induction. (*H*) Advanced stages of apical organization (*A*) and early stages of needle development (*N*) in *de novo* shoot buds from 2-yr-old callus (Wagley *et al.* 1987).

et al. 1998). An alternative method involved a histodifferentiation and maturation medium followed by desiccation of somatic embryos, prior to germination into plants. Thus, recalcitrant species may need multiple steps to induce regeneration and plant recovery.

The companion paper on Eldarica pine by Wagley *et al.* (1987) showed the correlation of external culture features with internal (histological) features (Fig. 6). Axillary branching was maintained for up to 4 mo in culture, and adventitious shooting from explants occurred for up to 5 mo, then both responses disappeared. Callus production occurred during the initial 3 mo of culture (Fig. 6*B*), with a gradual disappearance of vascular tissues and specialized cells (Fig. 6*A*), and proliferation of non-specialized meristematic clusters of cells (Fig. 6*C*). Unorganized callus (Fig. 6*D*) was predominant in 3 to 6-mo-old cultures. Cultures 6 to 20 mo old were treated with the bud induction medium (Fig. 6*E*). The first visible internal change following bud induction was that the most actively dividing cells were associated with layers of dark moribund (denoted by (*M*); Fig. 6*E*) cells. The next visible internal change was the formation of specialized cells, such as vascular cells, and organization of vascular bundles (Fig. 6*F*). Then, the formation of dermal layers of cells (Fig. 6*G*) and primordial needles (Fig. 6*H*) was observed. By this time, primordial buds were evident in the external features of the callus. As the cultures were cycled through the other regeneration media, needles became better formed and shoot buds with clear apical structure emerged on the surface of the cultures. This study by Wagley *et al.* (1987) illustrates how dedifferentiation into callus occurs, and how it is then followed by redifferentiation back into new organized structures.

***Capsicum* spp.** Another plant genus recalcitrant in tissue culture are the chile peppers, *Capsicum* spp. (Kothari *et al.* 2010).

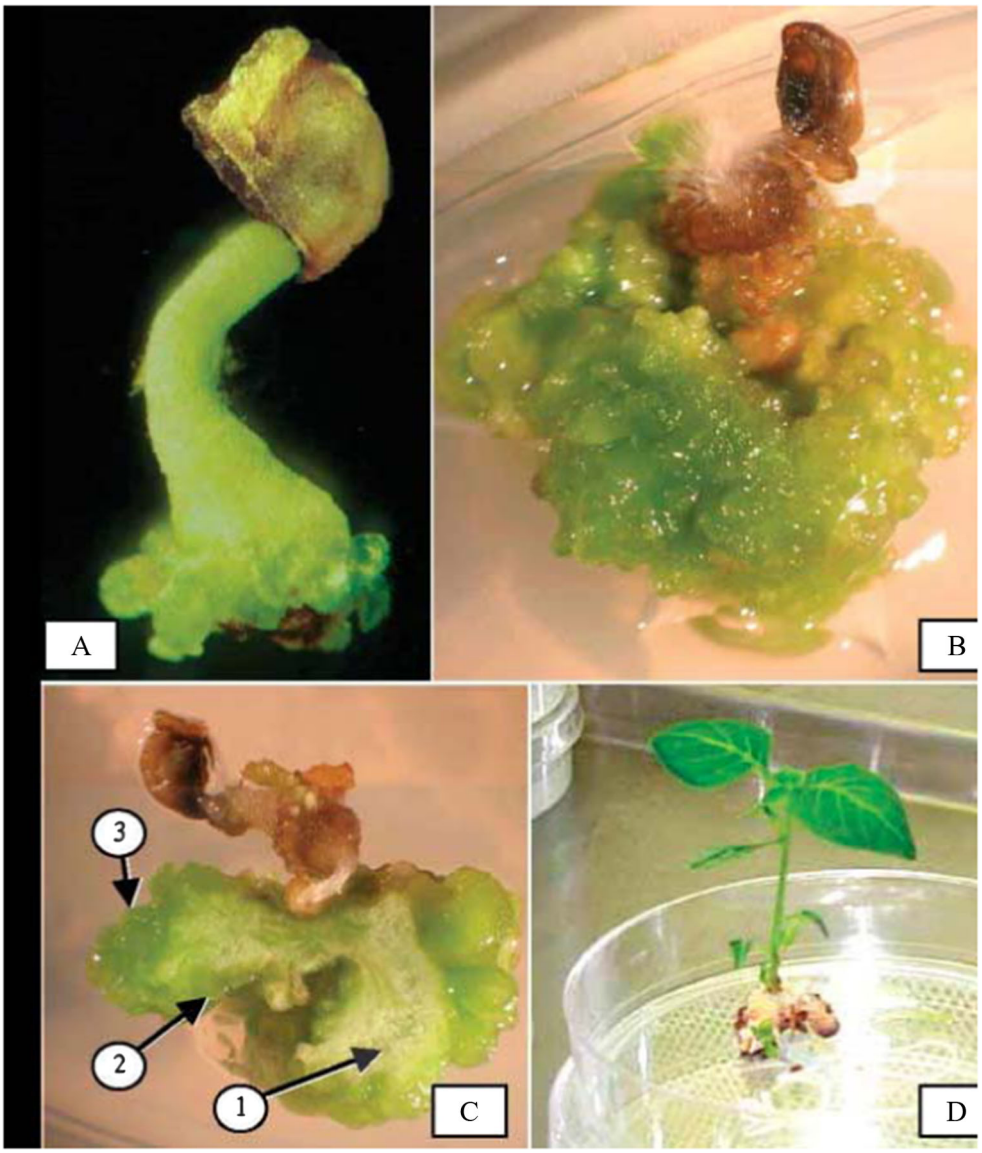

Figure 7. Depiction of complex tissue of *Capsicum baccatum* L. (chile pepper) from half-seed explants containing the radicle and hypocotyl end of the explant, showing adventitious buds transitioning to organogenic callus, with subsequent plant regeneration: (*A*) 5-wk-old explant with adventitious buds, (*B*) 4-mo-old organogenic callus, (*C*) section of a 4-mo-old callus, (*arrow 1*, soft whitish core; *arrow 2*, green portion of hard tissue; *arrow 3*, cluster of buds), and (*D*) plant regeneration from organogenic callus (Valera-Montero and Phillips 2005, reproduced with permission from the Society for In Vitro Biology).

Even though peppers are in the same family as tobacco, *Solanum lycopersicum* L. (tomato), and *Petunia* spp., peppers are considerably more challenging to adapt to tissue culture than other members of the Solanaceae family. Most pepper tissue culture responses have been adventitious and short-term in nature, with few, if any, long-term callus *de novo* regeneration responses reported. A major limitation seems to be recalcitrance for establishing shoot apical organization and shoot elongation (Hyde and Phillips 1996), which was addressed in the same study with the use of silver nitrate, showing modest improvement in successful shoot elongation.

In search of a long-term callus regeneration system for peppers, the half-seed explant of *Capsicum baccatum* L. was evaluated (Fig. 7*A*; Valera-Montero and Phillips 2005). Explants were cultured on MS medium + 5.0 mg L^{-1} BA + 1.0 mg L^{-1} IAA + 2.0 mg L^{-1} GA_3, where they proliferated a mass of semi-organized tissue (Fig. 7*B*) for up to 3 y, while retaining the ability to regenerate plants. Shoot buds elongated into plantlets (Fig. 7*D*) when transferred to growth regulator-free medium. These semi-organized tissues were composed of three distinct tissue types shown in Fig. 7*C*: (*1*), friable, whitish, non-organogenic tissue, which could be eliminated upon transfer; (*2*), hard green tissue with vascular tissues, few or no buds; and (*3*), amorphous hard green tissue with many primordial shoot buds. This latter type (*3*) was the organogenic callus, but it could only be maintained in the presence of some of the (*2*) type tissue. If all (*2*) type tissue was eliminated, then bud formation or development stopped. Apparently, the vascular structures in the (*2*) type tissue were necessary to sustain the formation of buds observed in the (*3*) type tissue.

This kind of regeneration system appears to have parallels with the woody plant nodule system reported by McCown *et al.* (1988) for *Populus* spp. Both cases (Valera-Montero and Phillips 2005; McCown *et al.* 1988) involved semi-organized tissues, which could be proliferated for extended periods of time, while retaining the ability to regenerate plants. Because there was not a complete loss of organization in the callus prior to shoot bud induction, and a certain level of tissue organization was necessary to support the proliferation of these structures, it would seem that these systems are a type of extended adventitious regeneration rather than a *de novo* system.

There is a further parallel between the *C. baccatum* system and the development of the *Zea mays* L. (maize) somatic embryogenesis system (Armstrong and Green 1985). Both of these systems are dependent upon the recognition of specific tissue types in complex cultures in order to isolate proliferating tissues capable of long-term plant regeneration.

Conclusions

The field of plant tissue culture has matured to the point where many scientists are seeking to use well-defined "cookbook" approaches to apply tissue culture systems to current studies. For a number of plant species, such cookbook recipes are available and can be applied successfully, without adaptation or special training. However, there are still many species where little tissue culture research has been conducted and available recipes may or may not be optimal. It does not make sense to customize the medium for a given species or situation, unless the correct starting point is identified (*e.g.*, starting with MS *vs.* BABI *vs.* MMS *vs.* WPM *vs.* DKW), nor does it make sense to conduct high-throughput phenotyping using tissue culture systems, when non-optimized protocols are used for the given species or situation. The basic principles of how to manipulate the basal medium and plant growth regulators to achieve better-optimized tissue cultures in little-researched plant species have been outlined in this present review. These principles assume that responsive genotypes and responsive explants can be identified within the given species, and that the operator can recognize tissue organizational progression through developmental steps.

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