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MINERAL NUTRITION AND PLANT MORPHOGENESIS

CARL M. RAMAGE* AND RICHARD R. WILLIAMS

School of Agriculture and Horticulture, The University of Queensland, Gatton Campus, Queensland 4345, Australia

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Summary

Plant morphogenesis *in vitro* can be achieved via two pathways, somatic embryogenesis or organogenesis. Relationships between the culture medium and explant leading to morphogenesis are complex and, despite extensive study, remain poorly understood. Primarily the composition and ratio of plant growth regulators are manipulated to optimize the quality and numbers of embryos or organs initiated. However, many species and varieties do not respond to this classical approach and require further optimization by the variation of other chemical or physical factors. Mineral nutrients form a significant component of culture media but are often overlooked as possible morphogenic elicitors. The combination of minerals for a particular plant species and developmental pathway are usually determined by the empirical manipulation of one or a combination of existing published formulations. Often only one medium type is used for the duration of culture even though this formulation may not be optimal for the different stages of explant growth and development. Furthermore, mineral studies have often focused on growth rather than morphogenesis with very little known of the relationships between mineral uptake and morphogenesis. This article examines the present knowledge of the main effects that mineral nutrients have on plant morphogenesis *in vitro*. In particular, the dynamics of nitrogen, phosphorus, and calcium supply during development are discussed.

Key words: nitrogen; phosphate; calcium; organogenesis; somatic embryogenesis.

Introduction

The importance of the culture medium, especially the levels of cytokinins and auxins, in callus induction, organ formation and multiplication, has been demonstrated for a large number of plant species. The fact that other species and varieties are recalcitrant and difficult to propagate demonstrates that variation in hormone ratios cannot be the sole mechanism controlling *in vitro* developmental processes. Furthermore, few links have been identified between media components and molecular changes within cells committed to regeneration. Thus, we are driven to identify and explore alternative factors and hypotheses in the control of *in vitro* development.

Very few studies have examined direct effects of mineral nutrients on plant morphogenesis. This is surprising given that mineral composition is a major component of plant tissue culture media, with the combination of minerals for a particular plant species usually determined by the empirical manipulation of one or a combination of existing published formulations. Often only one medium type is used for the duration of culture, even though this formulation may not be optimal for the different stages of explant growth and development. It is presumed that the mineral component of the culture medium has a primarily supportive role in the regeneration process. However, recent investigations have examined the processes involved in the supply of minerals *in vitro* and suggest a complex web of interactions between the explant and culture medium (Williams, 1993, 1995). Furthermore, Preece (1995) makes the comment that if we were to optimize the mineral component of the culture medium, we could reduce the concentrations of plant growth regulators required.

The aim of this article is to review the present knowledge of the main effects of mineral nutrients on plant morphogenesis and to underline the paucity of information that exists regarding the dynamics of mineral nutrition during plant development *in vitro*.

Morphogenesis *In Vitro*

Morphogenesis refers to the development of organs (shoots, roots, or flowers) and overall plant shape and structure. The study of plant development seeks to describe and explain these processes. Research in this field is vast, encompassing studies on the processes of organ morphogenesis and embryogenesis, and extending over a number of disciplines including plant tissue culture, biochemistry, biophysics, physiology, and more recently, molecular biology and genetics.

Plant morphogenesis *in vitro* can be achieved via two pathways, somatic embryogenesis or organogenesis. In somatic embryogenesis, a new plant with both root and shoot axes arises from actively dividing cells, but does not form any direct vascular connections with the original tissue (Hicks, 1980). Once initiated, the somatic embryo develops in a similar manner to a zygotic embryo from the seed (Meinke, 1995).

In contrast, organogenesis involves the separate formation of new organs. Cells within the explant are induced to divide, forming zones of localized cell division, which subsequently leads to the organization of meristematic centers referred to as meristemoids

*Author to whom correspondence should be addressed: Email. ramage@biosci.uq.edu.au

TABLE 1

ESSENTIAL ELEMENTS REQUIRED FOR PLANT GROWTH[a]

Element	Chemical symbol	Atomic weight	Form in which the element is absorbed	Dry weight in healthy plants
Macronutrients				
Calcium	Ca	40.08	Ca^{2+}	0.2–3.5%
Carbon	C	12.01	CO_2	≈44%
Hydrogen	H	1.01	H_2O	≈6%
Magnesium	Mg	24.32	Mg^{2+}	0.1–0.8%
Nitrogen	N	14.01	NO_3^- or NH_4^+	1–4%
Oxygen	O	16	H_2O or O_2	≈44%
Phosphorus	P	30.98	$H_2PO_4^-$ or HPO_4^{2-}	0.1–0.8%
Potassium	K	39.1	K^+	0.5–6%
Sulfur	S	32.07	SO_4^{2-}	0.05–1%
Micronutrients				
Boron	B	10.82	BO_3^- or $B_4O_7^{2-}$	5–75 ppm
Copper	Cu	63.54	Cu^{2+}	4–30 ppm
Chlorine	Cl	35.46	Cl^-	100–10 000 ppm
Iron	Fe	55.85	Fe^{2+} or Fe^{3+}	25–300 ppm
Manganese	Mn	54.94	Mn^{2+}	15–800 ppm
Molybdenum	Mo	95.95	MoO_4^{2-}	0.1–5 ppm
Zinc	Zn	65.38	Zn^{2+}	15–100 ppm

[a] Adapted from Taji et al. (1993).

(Brown and Thorpe, 1986). These in turn give rise to small meristems, the growth and development of which gives rise to a new organ. These organs arise either directly from the parental tissues (direct organogenesis, e.g. tobacco thin cell layers) or indirectly through a callus phase (indirect organogenesis, e.g. tobacco callus) (Hicks, 1980). The process of organogenesis has been reviewed extensively (Reinert, 1973; Street, 1973; Meins and Binns, 1979; Hicks, 1980; Thorpe, 1980; Tran Thanh Van, 1980; Wareing, 1982; Brown and Thorpe, 1986), indicating that the process is complex due to the large number of external and internal variables identified.

Mineral Transport and Uptake *In Vitro*

Plants grown *in vitro* differ markedly from those grown *in vivo*. The most basic difference is related to the plant/nutrient interphase. The root system of *in vivo*-grown plants provides a large surface area and hence a greater potential for mineral uptake. Plants grown *in vitro* often lack root systems and rely solely on unspecialized cells for nutrient uptake (Leifert et al., 1995). In addition, the natural environment provides a certain amount of replenishment through mineralization and ion exchange. Therefore, *in vivo* plants have a relatively continuous supply of mineral nutrients. Generally, *in vitro* culture media contains a defined level of mineral nutrients that are not usually replaced unless the plant is subcultured onto fresh media. Whilst there exists a number of fundamental differences between *in vivo*- and *in vitro*-grown plants, the underlying principles of mineral uptake and transport appear to be the same (reviewed by Leifert et al., 1995; Williams, 1995).

An important point raised by Williams (1995) is that a distinction must be made between the depletion of minerals from the medium and deficiency within the explant. The rate of mineral uptake for a specific ion may exceed its rate of utilization. As a result of this, pools of available ions may form within the explant, allowing continued growth, and possibly affecting development, well after the uptake of the ion has ceased (Williams, 1995). For example, Velikey et al. (1976) demonstrated that under conditions of high magnesium supply and high culture medium pH, *Ipomoea* cells absorb large amounts of magnesium in excess of the requirement for growth.

Very few investigations into the relationship between mineral uptake, transport, metabolism, and growth *in vitro* have been reported (Gautheret, 1955; Schmitz and Lörz, 1990a, b; Williams, 1991, 1992, 1993, 1995; Cousson and Tran Thanh Van, 1993; Debergh et al., 1994; Leifert et al., 1995). Furthermore, even less is known of the relationship between mineral uptake and morphogenesis.

Minerals Essential for Morphogenesis

An essential mineral is defined as an element that is critical or essential for a plant to complete its life cycle (Epstein, 1972; Marschner, 1995). To be classified as 'essential' the mineral must fulfill the following criteria: (1) an obligatory requirement for normal growth and reproduction; (2) it is not possible to replace the mineral with another mineral or substance; (3) have a direct or indirect role in plant metabolism (Arnon and Stout, 1939). Essential mineral nutrients are classified into two groups, macro- or micronutrients, depending on the relative amount of each element required for growth (Table 1). Macronutrients such as nitrogen, phosphorus, and sulfur are important components of macromolecules such as proteins and nucleic acids, as well as constituents of many small molecules. Micronutrients are required in much smaller quantities than macronutrients and function in various roles such as enzyme cofactors or components of electron transport proteins (Marschner, 1995). Given these important roles that 'essential' minerals play in cellular processes, it is not difficult to imagine that the supply of these minerals is important for optimal morphogenesis.

In plant tissue culture, the basis of all nutrient media is a mixture of mineral salts combining the essential macro- and micronutrients

TABLE 2

COMPOSITION OF SELECTED PLANT TISSUE CULTURE MEDIA

Media Abbreviation	Hoagland and Snyder (1933) H&S	White (1954) W	Murashige and Skoog (1962) MS	Gamborg et al. (1968) B5	Schenk and Hilderbrandt (1972) S&H	Chu et al. (1975) N_6
Macronutrients[a]						
KNO_3	506	80	1900	2500	2500	2830
NH_4NO_3	–	–	1650	–	–	–
$Ca(NO_3)_2.4H_2O$	1180	300	–	–	–	–
$CaCl_2.2H_2O$	–	–	440	150	200	166
$MgSO_4.7H_2O$	493	720	370	250	400	185
KCl	–	65	–	–	–	–
KH_2PO_4	136	–	170	–	–	400
$NH_4H_2PO_4$	–	–	–	–	300	–
$NaH_2PO_4.H_2O$	–	16.5	–	150	–	–
Na_2SO_4	–	200	–	–	–	–
$(NH_4)_2SO_4$	–	–	–	134	–	463
Micronutrients[a]						
$MnSO_4.H_2O$	–	7.0	22.3	–	–	–
$MnSO_4.4H_2O$	–	–	–	10.0	10.0	4.4
$ZnSO_4.7H_2O$	–	3.0	8.6	2.0	1.0	1.5
H_3BO_3	–	1.5	6.2	3.0	5.0	1.6
KI	–	0.75	0.83	0.75	1.0	0.8
$CuSO_4.5H_2O$	–	–	25 μg	25 μg	0.2	–
$Na_2MoO_4.2H_2O$	–	–	250 μg	250 μg	0.1	–
$CoCl_2.6H_2O$	–	–	25 μg	25 μg	0.1	–
$FeSO_4.7H_2O$	–	–	27.8	–	15.0	27.85
NaFeEDTA	–	–	–	28.0 S[b]	–	–
$Na_2EDTA.2H_2O$	–	–	37.3	–	20.0	37.25
$Fe_2(SO_4)_3$	–	2.5	–	–	–	–
Vitamins[a]						
myo-Inositol	–	–	100	100	1000	–
Thiamine-HCl	–	–	0.1	10.0	5.0	1.0
Nicotinic acid	–	–	0.5	1.0	5.0	0.5
Pyridoxine-HCl	–	–	0.5	1.0	0.5	0.5
Ca D-pantothenate	–	–	–	–	–	–
Biotin	–	–	–	–	–	0.05
Folic acid	–	–	–	–	–	0.5
Choline chloride	–	–	–	–	–	–
Riboflavin	–	–	–	–	–	–
Ascorbic acid		–	–	–	–	–

[a] Expressed in $mg\,l^{-1}$ unless otherwise stated.
[b] S = sequestrene 330 Fe (FeDTPA).

supplemented with a source of carbon; usually sucrose. However, due to the stresses associated with explanting, few cultures can be maintained on these simple nutrient media. Therefore, other supplements such as vitamins, amino acids, and plant growth regulators are required (Table 2). The question is, are all 'essential' minerals also essential for morphogenesis?

Lee and DeFossard (1977) conducted a series of mineral omission experiments during bud formation of strawberry nodal cultures (*Fragaria ananassa* Duchesne). The selective omission of iron, phosphate, potassium, nitrate, and chloride resulted in a significant reduction in explant growth. The omission of other minerals, such as calcium, magnesium, sulfate, ammonium, sodium, and several micronutrients did not significantly affect explant growth. Mineral omission also affected the mean number of leaves produced by nodal cultures. The omission of nitrate, phosphate, and potassium significantly reduced leaf number, whereas the omission of calcium, sulfate, ammonium, and magnesium had no significant effect (Lee and DeFossard, 1977). Similarly, in our own studies using liquid shoot induction media and tobacco leaf discs, the selective removal of nitrate or phosphate completely inhibited shoot meristem formation. In contrast, whilst the frequency of meristem formation was reduced when other minerals were removed, initiation was not completely inhibited (Table 3).

One of the problems associated with Lee and DeFossard's study is that culture media were solidified with agar. In terms of mineral composition, this can best be described as an unknown factor in their experiments. The concentration and type of gelling agent used in plant tissue culture can have dramatic effects on the growth and development of explants (Williams, 1993). Agar contributes to the matrix potential, the humidity of culture vessels, and affects the availability of water and dissolved media components (Debergh, 1983). In addition, constituents of the media may not necessarily be evenly distributed throughout the medium or equally available to the explant (Debergh, 1983). Furthermore, agar contains a considerable

TABLE 3

SHOOT FORMATION OF *NICOTIANA TABACUM* zz100 LEAF DISCS CULTURED WITHOUT INDIVIDUAL MACRONUTRIENTS

Mineral removed[z]	Callus formation (%)	Meristem formation (%)	Shoot formation (%)	Mean shoot number[y]	Mean dry weight[y]
Control	100	100	100	87.3 ± 13 c	312.6 ± 24.8 a
No nitrogen[w]	0	0	0	0	15.5 ± 1.5
NO_3^-	5.3 ± 5.3	0	0	0 a	11.6 ± 2 d
Ca^{2+}	100	14.3 ± 14.3	0	0 a	116.2 ± 11.2 c
PO_4^{3-}	13.3 ± 13.3	0	0	0 a	5.8 ± 0.4 e
K^+	33.3 ± 8.3	20.8 ± 15	0	0 a	2.9 ± 0.4 f
Mg^{2+}	95.2 ± 4.8	75 ± 25	0	0 a	8.9 ± 1.2 de
NH_4^+	93.8 ± 5.1	72.3 ± 12.4	65.2 ± 18.2	67.5 ± 15.5 b	215.7 ± 19.8 b

[z] Explants were cultured on liquid MS media supplemented with 5 μ*M* N^6-benzyladenine, 3% (w/v) sucrose but without individual macronutrients. Where a nutrient was removed, the ionic balance of the medium was maintained using Na^+ and Cl^- ions. Data are the means from three independent experiments of $n = 8$, ± 1 SE.

[y] Mean number of shoots per explant ± 1 SE; means with the same *letter* are not significantly different ($P \geq 0.05$).

[x] Mean dry weight (mg per explant) ± 1 SE; means with the same *letter* are not significantly different ($P \geq 0.05$).

[w] Not part of the statistical analysis.

amount of contaminating minerals, for example Bi-Tek agar contains 885 mg g^{-1} of potassium and 2100 mg g^{-1} of phosphorus (cf. Murashige and Skoog, 1962 (MS), 783.96 and 38.71 mg l^{-1}, respectively; Williams, 1993). The presence of these contaminants complicates the interpretation of Lee and DeFossard's results. These agar-induced variations in the composition of the culture medium were avoided in our study by the use of liquid media with the ionic balance maintained using Na^+ and Cl^- as carrier ions.

From our results and those of Lee and DeFossard, it appears that not all minerals are required for meristem initiation but all are 'essential' for growth. In particular, nitrate and phosphate supply seems to be critical. Furthermore, just like plant growth regulators, these effects may vary for different plant species and explant types.

Changes in Explant Response to Plant Growth Regulators

Several studies have explored the relationship between mineral composition and plant growth regulators in culture (reviewed by Preece, 1995). It appears that mineral composition may affect the sensitivity of explants to plant growth regulators. For example, the elevation of calcium in suspension cultures of *Daucus carota*

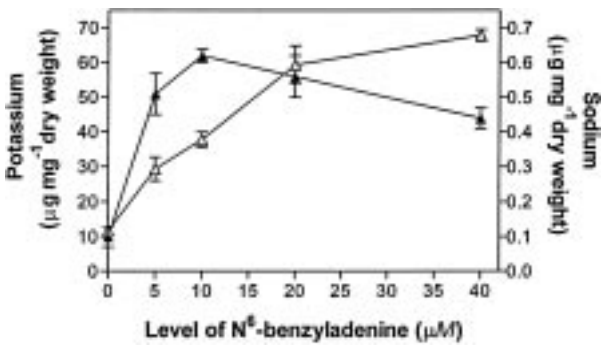

FIG. 1. Concentration (μg mg^{-1} dry weight) of potassium (△) and sodium (▲) within explants cultured with various levels of the cytokinin N^6-benzyladenine for 35 d. Mineral concentrations are the means of three independent samples ± 1 SE.

appeared to counteract the inhibitory effect of 2,4-D on somatic embryogenesis (Jansen et al., 1990). The amount of the cytokinin thidiazuron (TDZ) used to induce shoots in olive petiole explants could be reduced to half by culturing on full-strength instead of half-strength MS medium, without significant differences in shoot numbers (Mencuccini and Rugini, 1993). Similarly, Gomez and Segura (1994) eliminated the requirement for 1-naphthaleneacetic acid (NAA) in high-frequency shoot formation of *Juniperus oxycedrus* leaf explants, simply by changing from MS minerals to Schenk and Hildebrandt (1972) minerals. The Schenk and Hildebrandt mineral formulation contains almost 50% less calcium and 50% more phosphate than MS medium (Table 2).

Ilan (1971) reported that cytokinin treatments changed the K^+ and Na^+ selectivity in cells of sunflower leaves and cotyledons. The affinity of cells for K^+ as compared with Na^+ increased with kinetin in detached cotyledons, and treatment with benzyladenine caused a significant elevation in the ratio of K^+:Na^+ in attached cotyledons. Similarly, we have observed a linear relationship between exogenous N^6-benzyladenine supply and the concentration of K^+ in tobacco leaf discs (Fig. 1). The level of Na^+ was also greater in leaf discs cultured with N^6-benzyladenine but no significant differences were observed between explants cultured with increasing levels of cytokinin. In contrast, Jacoby and Dagan (1970) demonstrated benzyladenine inhibition of Na^+ uptake in intact bean leaves.

A common feature of these examples is that the requirement for, or sensitivity to, the plant growth regulator changed when mineral formulations were changed. Preece (1995) suggested that the supply of plant growth regulators might partially compensate for less than optimal nutrient media. The hypothesis that minerals affect cell sensitivity to plant growth regulators is further supported by regeneration studies on *Oryza sativa* L. somatic embryogenesis (Koetje et al., 1989). Immature embryo-derived callus differed in regeneration capacity depending upon whether N_6 (Chu et al., 1975) or MS minerals were used (Table 2). Increasing the level of 2,4-dichlorophenoxyacetic acid (2,4-D) in N_6 media resulted in a dramatic reduction in the number of somatic embryos induced, while on MS media, regeneration was unaffected by 2,4-D supply (Koetje et al., 1989). Further insight into this phenomenon was provided by

Grimes and Hodges (1990) who reported that the morphogenic response of *Oryza* to 2,4-D appeared to be related to the NO_3^-:NH_4^+ ratio.

Nutritional Factors Affecting Morphogenesis

The importance of nitrogen. Influences of the absolute and relative amounts of nitrate and ammonium on the induction and differentiation of plant cell cultures have been reported for a number of *in vitro* systems (Halperin and Wetherell, 1965; Wetherell and Dougall, 1976; Chaleff, 1983; Grimes and Hodges, 1990; Cousson and Tran Thanh Van, 1993). In typical regeneration media the nitrogen pool is usually comprised of two major components, inorganic nitrate and ammonium. Drew et al. (1973) showed that localized nitrate, ammonium, and inorganic phosphorus applications could stimulate root branching in barley (*Hordeum vulgare*). Hardy and Thorpe (1990) identified enhanced nitrogen metabolism during *de novo* shoot organogenesis of tobacco callus. In a follow-up study, enzyme and labeling data indicated that this occurred prior to meristemoid formation (Joy et al., 1994).

Nitrate has been regarded as the principal form of nitrogen for the culture of plant tissue (Sathyanarayana and Blake, 1994) and in several studies could serve as the sole source of nitrogen for morphogenesis (Bayley et al., 1972; Wetherall and Dougall, 1976; Cousson and Tran Thanh Van, 1993; Ramage, 1999).

The importance of nitrate in developmental processes led Trewavas (1983) to put forward a case for the NO_3^- ion to be reclassified as a true plant growth regulator. Whilst an argument could be generated for many compounds, nitrate remains a mineral of interest in the control of developmental processes. Scheible et al. (1997a, b) provided convincing evidence of a role of nitrate in regulating plant development. Using tobacco (*Nicotiana plumbaginifolia*) mutants deficient in nitrate reductase, the authors correlated accumulation of nitrate in the shoot with a dramatic reduction in root growth. Furthermore, this effect may, in part, be due to changes in the redistribution of carbon as starch synthesis and metabolism was severely inhibited in the leaves. More recently, a nitrate-inducible MADS box gene (*ANR1*) was identified in *Arabidopsis* (Zhang and Forde, 1998). Products of MADS box genes share homology with DNA binding domains and a number of MADS box genes have been shown to be critical in determining organ identity during floral organogenesis (Weigel and Meyerowitz, 1994). *ANR1* is a component of the signal transduction pathway linking external nitrate supply to the increases in lateral root elongation observed by Drew et al. (1973) (Zhang and Forde, 2000). In addition, the authors provide evidence of an overlap between auxin and nitrate response pathways in the control of root elongation.

Ammonium used as the sole source of nitrogen appears to have a negative effect on growth and morphogenesis (Kirkby and Hughes, 1970; Cousson and Tran Thanh Van, 1993; Raab and Terry, 1994, 1995; Walch-Liu et al., 2000), although several studies have identified plants that can tolerate ammonium nutrition equally as well as nitrate [e.g. *Oryza sativa* L. (Grimes and Hodges, 1990); *Calluna vulgaris* (L.) Hull (Troelstra et al., 1995)].

Morphogenic studies of tobacco thin cell layers (TCL) by Cousson and Tran Thanh Van (1993) showed that when NH_4^+ was the sole nitrogen source, the culture medium became acidified to a pH of approximately 4.2 during incubation. The authors suggest that this acidification possibly increases K^+ and Na^+ influxes by the lowering of explant intracellular pH. An increase in the concentration of K^+ from 20 to 80 m*M* resulted in the suppression of morphogenesis in 70% of the TCL explants.

It is widely accepted that the form of nitrogen nutrition influences the pH of culture media (Kirkby and Mengel, 1967). Tobacco cells are unable to utilize ammonium as a sole nitrogen source unless the medium is also supplemented with citrate, malate, or pyruvate (Gamborg, 1970; Behrend and Mateles, 1975). In our studies we have also demonstrated that shoot meristems can be initiated from tobacco leaf discs using ammonium as the sole nitrogen source when the medium is supplemented with the organic acid 2[*N*-morpholino]ethanesulfonic acid (MES) (Ramage and Williams, unpublished results). In contrast, several studies have demonstrated that cell cultures can proliferate on media with ammonium as the sole nitrogen source if the pH of the medium is adjusted throughout the culture period and in the absence of an organic acid (*Ipomoea* and soybean, Martin et al., 1977; wild carrot, Dougall and Weyrauch, 1980).

Changes in medium pH as a result of nitrogen nutrition may be explained by a differential uptake of NH_4^+ and NO_3^-. The preferential uptake of NH_4^+ at high pH causes medium acidification, which in turn results in the preferential uptake of NO_3^- and an increase in medium pH (Martin and Rose, 1975; Dougall, 1980; Congard et al., 1986). Perhaps as a consequence of this, the amount of available nitrogen appears to be less important for morphogenesis than the ratio between NO_3^- and NH_4^+. Grimes and Hodges (1990) investigated the effect of varying the ratio of NO_3^- to NH_4^+ on the regeneration of somatic embryos from rice. Somatic embryogenesis was achieved with a wide range of ratios, and lowering the ratio from the control 80:20 (36 m*M* NO_3^- and 9 m*M* NH_4^+) to 70:30 (31.5 m*M* NO_3^- and 13.5 m*M* NH_4^+) resulted in a 1.5-fold increase in the mean number of plants induced per embryo (Grimes and Hodges, 1990). Similarly, Reinbothe et al. (1990) found that the optimal ratio of NO_3^- to NH_4^+ was 11:1 in the production of somatic embryos from suspension cultures of *Digitalis lanata*. Wetherell and Dougall (1976) also found that a reduced source of nitrogen (NH_4Cl) was required to supplement nitrate levels for rapid growth and somatic embryogenesis of *Daucus carota*.

Phosphorus. Balanced phosphorus nutrition is important for both explant growth (Lee and DeFossard, 1977) and morphogenesis (Ramage, 1999). Phosphorus is rapidly consumed by tobacco suspension cultures (Bellamy and Bieleski, 1966; Ueki and Sato, 1971; Kato et al., 1977) and may be the limiting mineral in many plant tissue cultures (Lumsden et al., 1990). In our studies with tobacco leaf discs, phosphate was rapidly consumed by shoot-forming but not non-shoot-forming explants, corresponding to both the initiation and the growth of shoots in the former (Fig. 2). Over 50% of the phosphorus pool was consumed by day 20 of culture, during shoot meristem initiation, indicating that this process requires high-energy inputs. The remaining 50% of the phosphorus pool was consumed over the following 15 d during the growth of meristems into leafy shoots.

In addition, maximum shoot numbers were obtained with phosphate concentrations at the standard MS levels (1.25 m*M*). Culture media with higher levels of phosphate had a large amount of precipitate and explants produced a lower number of shoots (Ramage and Williams, unpublished results). Increased phosphate concentrations may have limited the availability of other mineral ions such as Fe^{3+} and/or Ca^{2+} (Loneragan and Asher, 1982). For

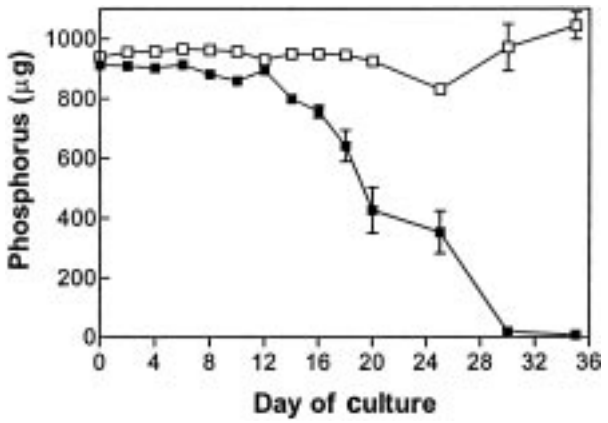

FIG. 2. Phosphorus depletion from shoot-forming (SF, ■) and non-shoot-forming (NSF, □) tobacco leaf disc cultures. Explants were cultured on liquid MS media supplemented with 3% (w/v) sucrose with (SF) or without (NSF) 5 µ*M* N^6-benzyladenine. Concentrations are the means of three replications representing µg of phosphorus per culture jar (volume 25 ml) with ± 1 SE determined at 14 harvest intervals over a 35-d culture period.

example, the growth of *Ocimum* cell cultures was reduced due to the precipitation of iron phosphate from liquid MS medium (Dalton et al., 1983). Mineral precipitation was attributed to an imbalance in the ratio of iron to EDTA in the culture medium and was overcome by increasing the EDTA: iron ratio to 3:1. However, high concentrations of EDTA were toxic to *Ocimum* cells, so it was necessary to reduce the level of iron in the culture medium.

The precipitation of minerals may be facilitated by autoclaving. Schnek et al. (1991) demonstrated that the precipitation of minerals during autoclaving was due to the inclusion of Fe-EDTA and KH_2PO_4. During autoclaving, Fe-EDTA reacted with micronutrients, and KH_2PO_4 reacted with calcium to form insoluble mineral complexes that were no longer available to the plant for growth and development.

Calcium. Calcium alone has been proposed as a major mediator of various physiological processes in plant cells. Saunders (1992) highlighted the interaction between calcium and cytokinin signal transduction. Increases in the internal concentrations of Ca^{2+} of cytokinin-treated *Funaria* cells led to cell division. Since these increases were detected in both bud-forming and non-bud-forming cells, the author suggested that hormonal stimulation of bud formation must lie further down the signal transduction pathway.

Jansen et al. (1990) reported a two-fold increase in the number of somatic embryos from *Daucus carota* suspension cultures due to an increase in medium calcium concentration. The authors also suggest that the elevation in calcium concentration counteracts, to a certain extent, the inhibitory effect of 2,4-D on somatic embryogenesis. Similar responses were seen with cytokinin-induced shoot bud formation in *Torenia* stem segments (Tanimoto and Harada, 1986).

It has been proposed that, in the absence of calcium, cultured plants may continue to grow for some time, utilizing endogenous calcium (Eklund and Eliasson, 1990; Williams, 1995). Furthermore, calcium transport may be a limiting factor in plant tissue culture (Williams, 1995). Tanimoto and Harada (1986) investigated the effect of various chemicals related to the transport and/or action of calcium during adventitious bud formation in *Torenia* stem segments. The application of the calcium ionophore A23187 induced meristematic divisions in the absence of cytokinin, but leafy shoots were not formed. The incubation of explants on media containing the calcium chelator EGTA, the calcium antagonist lanthanum, or the channel inhibitor verapamil prevented cytokinin-induced shoot formation. The authors proposed that cytokinin-induced adventitious bud formation in *Torenia* may be partially mediated by an increase in intracellular calcium. This role for calcium is supported by calcium influx studies during organogenesis in *Graptopetalum* leaves (Hush et al., 1991). Using a vibrating probe, ionic fluxes in the organogenic zone at the petiole base of detached leaves were measured. Calcium influx (2.4 µÅ cm^{-2}) was observed within 2 h of leaf detachment and preceded the first signs of organogenesis (Hush et al., 1991). However, after 12 h the influx had declined to 1.2 µÅ cm^{-2} and by 24 h was no longer detectable, with a net efflux of -1.54 µÅ cm^{-2}.

Other macronutrients. The importance of potassium, magnesium, and sulfur in morphogenesis appears to be supportive. However, nutritional imbalance may have significant downstream effects on morphogenesis. Potassium plays a predominantly osmotic role in plants and is highly mobile throughout the plant body through selective potassium transport mechanisms (Marshall and Porter, 1991; Marschner, 1995). Although it is not a substrate for growth, potassium influences the flux of other minerals such as nitrogen, carbon, and phosphorus, and enhances the translocation of photosynthates (Mengel and Kirkby, 1987).

In tobacco TCLs, organogenic pathways may be determined by changes in the concentration of Na^+ and K^+ ions. Cousson and Tran Thanh Van (1993) found that when Na^+ concentrations were increased from 0.3 m*M* (control level) to 78.9 m*M*, 100% of explants failed to form vegetative buds. At 41.5 m*M*, the ratio of floral to vegetative bud formation changed to favor vegetative induction. Similarly, an increase in the concentration of K^+ from 20 to 80 m*M* resulted in the suppression of morphogenesis in 70% of the TCL explants. In contrast, the concentration of Na^+ and K^+ ions was not critical for adventitious shoot formation in *N. tabacum* zz100 leaf discs (Ramage and Williams, unpublished results).

Using cell suspension cultures of *Ipomoea* sp. (Morning Glory), Veliky et al. (1976) demonstrated that the uptake of magnesium increased with an increase in the supply of magnesium in the culture medium and with an increase in the medium pH. The uptake of magnesium by plants grown under field conditions is also influenced by the pH and calcium content of the surrounding soil (Christenson et al., 1973; Marschner, 1995).

Micronutrients. The role of micronutrients in the control of regeneration has received little attention, although a few micronutrients have been shown to enhance regeneration significantly. For example, nickel and cobalt stimulated morphogenesis in *Daucus carota* callus cultures (Roustan et al., 1989) and increasing the level of $CuSO_4$ in the culture medium significantly enhanced shoot regeneration from the calluses of wheat, and triticale, as well as of tobacco leaf discs (Purnhauser and Gyulia, 1993).

In our studies with tobacco leaf discs, iron was removed from both shoot-forming and non-shoot-forming media earlier than any other mineral (Ramage and Williams, unpublished results). Over 50% of iron was taken up by shoot-forming explants in the first 4 d of culture. Similarly, 75% of the exogenous iron was removed from non-shoot-forming media and therefore its consumption does not appear to be related to morphogenesis. Interpretation of the role of iron is complicated by interactions between iron and other micronutrients

(Williams, 1995). For example, an excess of manganese can cause a deficiency in iron while excess iron or EDTA can reduce the uptake of zinc (Williams, 1995).

Conclusions and Future Directions

In summary, minerals do appear to play an important role in the regulation of plant morphogenesis as opposed to just growth. The relative supply, uptake, transport, and metabolism of different minerals appear to vary between stages of organ and meristem initiation and subsequent growth. Balances in nitrogen, phosphorus, and calcium nutrition are essential for both morphogenesis and growth, whereas ions such as potassium, magnesium, and sulfur appear to play supportive roles. Of particular note is the apparent stimulatory effect of some micronutrients. Clearly there is a need to explore their morphogenic role more thoroughly.

Final resolution of the respective roles of minerals and other factors in the regulation of the pattern of development versus subsequent growth is dependent on a more precise means of determining just when embryo and meristem induction actually occurs. The expanding use of molecular genetic tools for understanding the interplay between genes during plant development is rapidly advancing our understanding of these processes (for reviews see Weigel and Meyerowitz, 1994; Meinke, 1995; Evans and Barton; 1997; Poethig, 1997; Sinha, 1999). Furthermore, the study of mineral transporters, such as those identified for phosphate and sulfur (reviewed by Smith et al., 2000), nitrate and ammonium (reviewed by Forde and Clarkson, 1999), and potassium (reviewed by Schachtman, 2000) in conjunction with molecular genetics and *in vitro* technology offers some exciting possibilities for elucidating the role of individual minerals in plant morphogenesis.

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