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**SOMATIC EMBRYOGENESIS AND
ENDOGENOUS HORMONE LEVELS IN ALFALFA**

SHUQUAN JING, B. Sc.

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biology

Carleton University

Ottawa, Ontario

Canada

April, 1997

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**SOMATIC EMBRYOGENESIS AND ENDOGENOUS
GROWTH REGULATOR LEVELS IN ALFALFA**

submitted by **SHUQUAN JING, B. Sc.**

in partial fulfilment of the requirements for the degree of Master of Science

A handwritten signature in cursive script, reading "Daniel C. Brown", written over a horizontal dashed line.

Thesis supervisor

A handwritten signature in cursive script, written over a horizontal dashed line. The signature is partially obscured by a diagonal line drawn across the bottom of the page.

Chairman, Dept. of Biology

ABSTRACT

Somatic embryogenesis has great commercial potential because it is a valuable tool for crop improvement and micropropagation; however, extreme genotype effects and inconsistent *in vitro* responses limit its use. This study attempted to characterize and develop an understanding of *in vitro* regeneration responses. The interaction of genotype and induction protocols and photoperiod on somatic embryogenesis, endogenous hormone levels, and regeneration ability was investigated. Correlations between regeneration trends, the physiological state of plants, and endogenous hormone levels were established. Phenotypes with a range of *in vitro* regeneration abilities, *i.e.* high, middle, low and non-regenerating, from two regenerating populations (A195 and A70) of alfalfa which have different genetic backgrounds, were cultured under 20-h, 8-h and 16-h photoperiods, *i.e.* long, short and standard photoperiod *in vitro* conditions. Endogenous IAA, ABA and cytokinins levels in leaf and petiole explants were determined with HPLC and ELISA. The results show the following: (1) Somatic embryogenesis is affected by the induction media, photoperiod and physiological states of the plant at the time of sampling. The highest level of embryogenesis was observed in those donor explants that were from stock plants cultured under a 16-h photoperiod and collected at the end of the daylight period. However, the relative regeneration trends in both populations were not affected by these factors; (2) Endogenous growth regulator levels varied among different photoperiods, and between dark and light conditions, but their levels did not change in concert with regeneration ability; and (3) the ratios of IAA/ABA and IAA/cytokinins were found to be correlated with the regeneration

trend of the A70 series of plants, *i.e.* the highest ratios of IAA/ABA and IAA/cytokinins were in high-regenerating, followed by middle, low and non-regenerating. Since the A195 series of plants are a cross between a high- and a non-regenerating plant, and the A70 series was selected from a seed lot of cultivar Rangeland, it is speculated that the genes controlling regeneration and endogenous growth regulators are not located on the same loci. Correlations of the ratios of IAA/ABA and IAA/cytokinins and regeneration trend may be a random correlation. It is concluded that genotypic background is the critical factor controlling somatic embryogenesis and endogenous growth regulator levels; the other factors are important modulating factors.

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LIST OF ABBREVIATIONS

ABA	Absciscic Acid
BHT	Butylhydroxytoluene
BSA	Bovine Serum Albumin
CAB	Commonwealth Agricultural Bureau
DHZ	Dihydrozeatin
DHZ-N-G	Dihydrozeatin-O-glucoside
DHZ-O-G	Dihydrozeatin-O-glucoside
DHZR	Dihydrozeatin-riboside
DW	Dry Weight
ELISA	Enzyme Linked Immunosorbent Assay
FW	Fresh Weight
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-Acetic Acid
IPA	Isopentenyladenine
IPAd	Isopentenyladenosine
IP-N-G	Isopentenyladenine-N-glucoside
IP-O-G	Isopentenyladenine-O-glucoside
LP	Long Photoperiod (20/4 h light/dark)
MP	Medium Photoperiod (16/8 h light/dark)
NA	Not Available

P1	Protocol 1
P2	Protocol 2
P3	Protocol 3
P4	Protocol 4
P5	Protocol 5
SE	Standard Error (of the Mean)
SP	Short Photoperiod (8/16 h light/dark)
TBS	Tris-buffer Saline
Z	Zeatin
Z-N-G	Zeatin-N-glucoside
Z-O-G	Zeatin-O-glucoside
ZR	Zeatin-riboside

INTRODUCTION

I. *In Vitro* Somatic Embryogenesis

After a long history of evolution of life on earth, animals and plants, the two most visible kingdoms, dominate the world and provide the main food sources for humans. Two of their significant differences are in movement and differentiation. Unlike animals, plants do not have the ability of movement, and therefore they cannot escape unfavourable environmental changes such as temperature. Instead, they developed internal adaptation systems for survival. Many of these samples are mediated by endogenous hormones. Unlike most animals, differentiation in plant cells, even highly mature and differentiated ones, is reversible if proper conditions are provided. Totipotency, the ability to regenerate a complete organism from any cell, is the foundation of somatic embryogenesis. These two characters, stress mediation through endogenous hormones and totipotency, are the bases of survival of plants.

In vitro somatic embryogenesis is a process by which plant somatic cells develop into whole plants without gametic fusion but undergo a procedure resembling that of zygotic embryogenesis (Ivanova *et al.*, 1994; McKersie and Brown, 1996; Merkle *et al.*, 1995). Somatic embryogenesis is a natural phenomenon and was described by Strasburger in 1878 (cited in Merkle *et al.*, 1995). The first demonstration of *in vitro* somatic embryogenesis

was reported in 1958 by Reinert and Steward, respectively (cited in McKersie and Brown, 1996, Merkle *et al.*, 1995). The concept of embryogenesis has drawn a lot of attention because of its significance in theory and practice. First, compared with zygotic embryos, somatic embryos can be produced easily and quickly (within 21 days), so that it provides an economical and easy way to study plant development. Secondly, synthetic seed developed from somatic embryos opens the possibility for the provision of high quality seed and may allow us to produce seeds from those plants that require a long period for seed production, that produce few seeds in one growth cycle, or for plants in which a seed cycle may cause the loss of a valuable phenotype. Finally, somatic embryogenesis is also useful in plant genetic engineering and selection of genetic or somaclonal variants (McKersie and Brown, 1996). Since the first group of pioneering studies on somatic embryogenesis were reported about 40 years ago (Reinert, 1958; Steward, 1958), many studies have been reported. According to statistics from CAB abstracts, publications from 1976 through 1996 on somatic embryogenesis increased sharply year by year (Fig. 1), indicating an increasing interest in this phenomenon.

All plant cells contain the entire set of genetic information necessary to create a new plant and, if proper conditions are provided, the tightly regulated existing temporal and spatial gene expression can be terminated and the process of embryogenic gene expression can be initiated. There are two developmental routes for embryo induction, one is through callus formation (indirect somatic embryogenesis), and the other is without callus formation (direct somatic embryogenesis). Somatic embryos develop through globular, heart, torpedo

Fig. 1. Publications (1976-1996) on somatic embryogenesis.

¹ Not complete.

and cotyledon stages into a new plant. The understanding of physiological, biochemical and molecular events in somatic embryogenesis have been advanced with a large number of studies (Fig. 2) (For reviews, please refer to: de Jong *et al.*, 1993; Dudits *et al.*, 1995; Emons, 1994; McKersie and Brown, 1997; Nomura and Komamine, 1995; Thorpe, 1995; Zimmerman, 1993); however, the mechanism of initiation of somatic embryogenesis is still not clear because the physiological and biochemical events happening at this stage are rapid and complicated. For example, the critical period is within 4-8 hours after treatment for induction of *Brassica* microspore embryogenesis at 32°C (Pechan *et al.*, 1991). During this short period, the first embryogenic nuclear division happens and a number of mRNAs and proteins are expressed (Pechan *et al.*, 1991). At present, no proper research techniques are available to study the biochemical events occurring during this period. With regard to factors influencing somatic embryogenesis, it is established that the plant genotype and its interaction with the culture media are the most important, followed by explant source and culture conditions (Brown *et al.*, 1995).

i. Genotype Effects

Differences in somatic embryogenesis responses between genotypes have been observed and systematically screened in some plants such as carrot (Schnall *et al.*, 1988), alfalfa (Brown and Atanassov, 1985; Mitten *et al.*, 1984) and soybean (Dhir *et al.*, 1992; Parrott *et al.*, 1989). Although all plant cells contain the entire set of genetic information necessary to create a whole plant and, if proper induction conditions are provided, they

Fig. 2. Morphological, molecular and biochemical events in somatic embryogenesis, arising from studies carried out with the high frequency and synchronous embryogenic single cell system of carrot. (Modified from Thorpe, 1995).

can dedifferentiate into plants (Merkle *et al.*, 1995), their *in vitro* response varies tremendously (0-100%). Genetic analysis suggested that this phenomenon is heritable and that one or more genes control the regeneration response. These genes belong to both nuclear and cytoplasmic genomes (for reviews, please refer to: George, 1993; Henry *et al.*, 1994; McKersie and Brown, 1996), and their number varies among plant species (Henry *et al.*, 1994). In nuclear genomes, the regeneration genes range from 1 to 2 in most tested plants (Henry *et al.*, 1994).

The nuclear genome has quantitative and qualitative influences on somatic embryogenesis. According to experimental results from mono- and dicotyledon plants, the gene action of the nuclear genome is characterized as additive in regeneration and the regenerating-controlling genes vary among the different stages of the regeneration process. For instance, callus induction and somatic embryogenesis are controlled separately by 1 dominant gene and 2 complementary dominant genes in alfalfa (*e.g.* Hernandez-Fernandez and Christie, 1989). These genes can be either recessive such as in wheat (De Buyser *et al.*, 1992) or dominant as in alfalfa (*e.g.* Reisch and Bingham, 1980; Wan *et al.*, 1988). It appears that only a few genes control regeneration ability. However, we do not know the mechanism of their action.

In plants, the cytoplasmic genome consists of genetic material in mitochondria and plastids. They exist and function uniquely in cells, but are partially controlled by the nuclear genome. Their role in somatic embryogenesis is difficult to document, but progress has been

made. It is certain that these organelles take part in callus production and affect regeneration capacity (Henry *et al.*, 1994).

Although genotype plays a main role in somatic embryogenesis, its effects are caused by interactions with the cultural environment, mainly the culture media. Regeneration efficiency varies among media, especially induction media. Meijer and Brown (1987) screened *M. sativa*, *M. caerulea*, *M. falcata* and *M. varia* subspecies of alfalfa plants for their efficiency of somatic embryogenesis with three-(B5h-SH-BOi2Y media) and two-step (UM-MS media) culture protocols. They found differences in both callus and embryo production among the subspecies and culture protocols. For example, in Regen B of *M. sativa*, both B5h and UM induction media induced a similar amount of callus; however, the former produced 13 embryos but the latter no embryos. In F2 of *M. falcata*, both B5h and UM media induced the same amount of callus, but the former produced less embryos than the latter. Chen *et al.* (1987) induced somatic embryogenesis from three cultivars of alfalfa on three medium protocols and observed that medium-specific and explant-specific responses existed in some cultivars. These results were confirmed by several other labs (Kris and Bingham, 1988; Takamizo *et al.*, 1991; Wofford *et al.*, 1992; Yun *et al.*, 1989). Furthermore, somatic embryogenesis also varies among culture methods, *i.e.* direct or indirect somatic embryogenesis, and culture protocols, *i.e.* two, three or multi-step culture. Since media vary with respect to their components in kind and amount, the expression of genes for somatic embryogenesis is assumed to be affected by some of these unidentified factors. However, it is not known to what degree that media and culture protocols can

influence regeneration ability since, up to now, there has been no systematic comparisons of the effects of media and culture protocols and regeneration abilities in a series of plants with different regenerating abilities.

ii. Explant Effects

In a plant, all organs, tissues and cells contain the same genetic information, but the reaction is different to *in vitro* tissue culture because of variation in their physiological state such as age (Takahata *et al.*, 1991) and degree of differentiation (George, 1993; McKersie and Brown, 1996). In monocots, cells differentiate early and rapidly followed by loss of mitotic and morphogenetic ability, and explant source is considered even more important than genotype (Krishnaraj and Vasil, 1995). Somatic embryogenesis has been achieved from such explants as leaves (*e.g.* dos Santos *et al.*, 1980; Mezentsev and Karelina, 1982; Denchev *et al.*, 1991), petioles (*e.g.* Walker *et al.*, 1978, 1979), stems (*e.g.* Walker *et al.*, 1978, 1979), cotyledons (*e.g.* Atanassov and Brown, 1984; Stavarek *et al.*, 1980), hypocotyls (*e.g.* Reisch and Bingham, 1980; Saunders and Bingham, 1975), protoplasts (*e.g.* Atanassov and Brown, 1984; Dijak and Brown, 1987), roots (*e.g.* Vieitez *et al.*, 1991), microspores (*e.g.* Takahata *et al.*, 1991), tuber (Krishnaraj and Vasil, 1995), ovary (Brown *et al.*, 1995), ovule (Dunstan *et al.*, 1995), nucellus (Dunstan *et al.*, 1995) and immature embryos (*e.g.* Walker and Sato, 1981; Walker *et al.*, 1978; 1979). Because of the variation in tissues and organs, different efficiencies of somatic embryogenesis have been noticed among various explants, between young and mature tissues, and between differentiated and undifferentiated tissues (Brown

et al., 1995; George, 1993). Generally speaking, young and undifferentiated cells are easier to regenerate than mature and differentiated cells (Duntan *et al.*, 1995; George, 1993; Krishnaraj and Vasil, 1995). Besides the degree of differentiation, levels of endogenous hormones differ among organs, tissues and cells, and some other unidentified factors may also determine the variation of regenerating responses (Brown *et al.*, 1995; Krishnaraj and Vasil, 1995). It is noteworthy that embryogenic cells of explants that respond to culture environment vary along with the environmental factors (Wenzel and Brown, 1991), possibly because of their different requirements for competence such as exogenous growth regulators (George, 1993; McKersie and Brown, 1996). The difference in regenerating ability among various organs, tissues and cells of a plant suggests that somatic embryogenesis is not only controlled by regenerating genes, but to a certain important degree, also modulated by other factors such as physiological state of explants and culture conditions. The interaction between these factors and genes responsible for regeneration is not understood. In fact, the effect of the donor plant state on *in vitro* regeneration has not been systematically studied.

iii. Medium Effects

The history of research in somatic embryogenesis can be described as a success story of medium development (Brown *et al.*, 1995). Success in somatic embryogenesis from a genotype or explant of a plant usually undergoes a series of experiments in adjusting the components of the medium. MS medium (Murashige and Skoog, 1962) is among media which have been most widely used in somatic embryogenesis (Brown *et al.*, 1995; Dunstan

et al., 1995; Krishnaraj and Vasil, 1995). More than 2,000 medium formulations have been reported, but half of them are modified MS medium (Brown *et al.*, 1995). Other popular media include B5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972), N6 (Chu *et al.*, 1975), and Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1975) media (Brown *et al.*, 1995; Dunstan *et al.*, 1995; Krishnaraj and Vasil, 1995). The culture media vary in both type and concentration of the components, but all media have similar basic classes of components which consist of growth regulators, nitrogen, carbohydrates, inorganic macro- and micronutrients, vitamins, and organic additives (Brown *et al.*, 1995; George, 1993). Because of genotype x medium interaction, changes of components can greatly influence somatic embryogenesis. Among them, carbohydrates, growth regulators, nitrogen and growth regulators play the most important roles.

1. Carbohydrates: Sugar provides cells with carbon and energy, and takes part in osmotic adjustment (Denchev *et al.*, 1993; George, 1993). Its effects on somatic embryogenesis are dependent on the kinds and concentrations of sugar, and the culture protocol. Through comparison and selection, it is now established that sucrose is the most suitable carbohydrate and the most universally used, followed by glucose, maltose, raffinose, fructose, mannitol and some other rarely used sugars (Brown *et al.*, 1995; Denchev *et al.*, 1993; George, 1993; Lippmann and Lippmann, 1993). The effects of these sugars differ among culture protocols and development stages of embryos. In some three-step culture protocols, maltose is applied in medium for embryo development (Denchev *et al.*, 1992), because maltose promoted a more normal morphology than sucrose (McKersie and Bowley, 1992). The optimal

concentration of sugar varies among sugar kinds, plant species and development stage of embryos. In terms of sucrose, optimal concentrations range from 58-88 μM (Brown *et al.*, 1995), or 2-4% for rapid growth of callus (George, 1993). Higher concentrations of sucrose impede embryo development (McKersie and Bowley, 1992).

2. Nitrogen: Nitrogen is indispensable to plant life and to somatic embryogenesis since it is required for key components in the plants structure and function and for materials such as proteins, nucleic acids, and plant hormones. The growth and morphogenesis of callus is greatly affected by the form (reduced or oxidized) and amount of nitrogen provided. Plants utilize reduced nitrogen for their metabolism; ammonia, NH_3 and amino acids (glutamine, proline, glutathione, alanine, serine, *etc.* and casein hydrolysates) are most commonly used in culture medium (Li, 1988; George, 1993). NO_3^- , a highly oxidized form of nitrogen, is required in tissue culture. Plant cells cannot utilize nitrate ion directly, instead, they first reduce nitrate ion into ammonium with energy expense. There are three reasons for the use of nitrate ion. The first is NO_3^- is necessary for embryogenesis, a lack of it in medium results in abnormal embryos or a reversal of embryogenesis to callus formation (George, 1993). The second is that the single application of NH_4^+ or NO_3^- has latent toxicity and a combined application of NO_3^- and NH_4^+ can prevent the toxicity. The third is that, through adjusting pH, nitrate ion promotes the absorption of NH_4^+ (George, 1993). Experiments have confirmed improved embryo results when both are used together rather than separately (*e.g.* Shetty and McKersie, 1993; Walker and Sato, 1981), and a suitable ratio of $\text{NH}_4^+ : \text{NO}_3^-$ is a key factor influencing somatic embryogenesis (McKersie and Brown, 1996). Like

requirements for other nutrients, a suitable concentration of nitrogen is necessary for embryogenesis. A low level of nitrogen cannot meet the requirements of cell activity and growth; whereas, a high concentration of nitrogen inhibits embryogenesis due to the toxic effect of ammonium and nitrate ion (George, 1993).

3. Macro- and microelements: The knowledge about the importance of macro- and microelements in plant life can be dated back as early as the mid-1800s. Carbon and 9 other chemical elements were first established as “essential inorganic nutrients” for plant normal growth (Li, 1988; Raven *et al.*, 1992). By 1954, this family was expended by 6 other members, manganese, zinc, copper, chlorine, boron, and molybdenum (Table 1). These elements vary greatly in concentration, form of absorption and function in plants. However, the absence of any of these elements will result in characteristic abnormalities of growth or deficiency symptoms (Li, 1988; Raven *et al.*, 1992). They are also essential to somatic embryogenesis. For instance, potassium influences the cell K^+ -ATPase activity, which in turn regulates nutrient uptake and metabolite allocation (Shetty and McKersie, 1993). Supplementation of potassium increased the frequency of somatic embryogenesis in wheat tissue culture (Galiba and Yamada, 1988), and a high concentration of potassium has been considered a key factor in high regeneration medium, SH4K for alfalfa (Shetty and McKersie, 1993; McKersie and Brown, 1996).

4. Vitamins: Like animals, plant cells require vitamins as essential intermediates or metabolic catalysts. For instance, thiamine is an essential factor in carbohydrate metabolism,

Table 1. An Overview of Essential Inorganic Macro- and Microelements for Plants

Element	Principal Absorbed Form	Usual Concentration in Plants (% or ppm/DW)	Concentration in Alfalfa	Important Functions
Macronutrients				
Carbon	CO ₂	about 44%	NA	Component of organic compounds
Oxygen	H ₂ O or O ₂	about 44%	NA	Same as above
Hydrogen	H ₂ O	about 6%	NA	Same as above
Nitrogen	NO ₃ ⁻ or NH ₄ ⁺	1-4%	3.12%	Component of amino acids, proteins, nucleotides, nucleic acids, chlorophylls, coenzymes
Potassium	K ⁺	0.5-6%	2.77%	Osmosis, ionic balance, opening and closing of stomata, activator of many enzymes
Calcium	Ca ²⁺	0.2-3.5%	1.7%	Component of cell walls, enzyme cofactor, involved in cellular membrane permeability; component of calmodulin, regulator of membrane and enzyme activities
Phosphorus	H ₂ PO ₄ ⁻ or HPO ₄ ²⁻	0.1-0.8%	0.35%	Component of ATP and ADP, nucleic acids, coenzymes, phospholipids
Magnesium	Mg ²⁺	0.1-0.8%	0.41%	Part of chlorophyll molecule; activator of many enzymes
Sulfur	SO ₄ ²⁻	0.05-1%	0.29%	Component of some amino acids and proteins and of coenzyme A
Micronutrients				
Iron	Fe ²⁺ or Fe ³⁺	25-300 ppm	190 ppm	Chlorophyll synthesis; component of cytochromes and nitrogenase
Chlorine	Cl ⁻	10-10,000 ppm	8800 ppm	Osmosis and ionic balance; photosynthetic reactions
Copper	Cu ²⁺	4030-ppm	9 ppm	Activator or component of some enzymes
Manganese	Mn ²⁺	15-800 ppm	62 ppm	Activator of some enzymes; required for integrity of chloroplast membrane and for oxygen release in photosynthesis
Zinc	Zn ²⁺	15-100 ppm	57 ppm	Activator or component of many enzymes
Molybdenum	MoO ₄ ²⁻	0.1-5 ppm	1.4 ppm	Nitrogen fixation and nitrate reduction
Boron	B(OH) ₃ or B(OH) ₄ ⁻	5-75 ppm	35 ppm	Influences Ca ²⁺ utilization, nucleic acid synthesis, and membrane integrity

nicotinic acid provides the skeleton of NAD and NADP, which are co-factors of dehydrogenase enzymes, and pyridoxine is one of the co-factors for amino acid metabolism (Nie *et al.*, 1989). Plants produce their own vitamins; however, cultured plant tissues and cells can not produce their own requirements. As a result, it is necessary to supply vitamins in culture medium (George, 1993). The commonly applied vitamins are thiamine (Vit. B₁), nicotinic acid (niacin, Vit. PP), pyridoxine (Vit. B₆), and myo-inositol (Vit. B complex) since they play an important role in metabolism but are deficient in cultured cells and tissues (Brown *et al.*, 1995; George, 1993). With applications of these four and other vitamins, improvement in callus production, morphogenesis and cell division have been observed (George, 1993; Lippmann and Lippmann, 1993; Murashige and Skoog, 1962).

5. Organic additives: Strictly speaking, vitamins should be included in organic additives (Brown *et al.*, 1995). Other organic compounds, mainly amino acids, are beneficial for somatic embryogenesis. Amino acids serve as a supplemental nitrogen source to ammonia and nitrate ion, affect metabolism, and buffer medium pH (George, 1993; Shetty and McKersie, 1993; Stuart and Strickland, 1984; Trigiano and Conger, 1987). The addition of proline and its analogs such as thioproline were reported to be the most effective among amino acids to promote somatic embryogenesis and embryo morphology (Shetty and McKersie, 1993; Stuart and Strickland, 1987). Serine, alanine, glutamine, arginine, lysine, asparagine and ornithine also stimulated the quality and quantity of somatic embryos (Stuart and Strickland, 1984). Casein hydrolysates and yeast extract contain several amino acids, vitamins and other nutrients and are widely used in culture media (Brown *et al.*, 1995).

II. Plant Growth Substances

Plant growth substances are a group of chemicals that are produced in plants and, in small quantity, play very important regulating roles in plant growth and development (Hopkins, 1995; Li, 1988). At present, there are five groups of generally recognized growth substances; that is, auxin, abscisic acid, gibberellins, cytokinins, and ethylene (Gaspar *et al.*, 1996). The synthesis position, transportation and metabolism of these hormones in plants are generally clear, but many details of their synthesis and metabolism is still a question (Table 2). Many of these chemicals and their analogues have been synthesized *in vitro*.

Growth substance is a neutral name, referring to both natural and man-made substances. Those naturally-produced growth substances are also called phytohormones, plant hormones, or endogenous hormones; growth regulators are a synonym for artificial growth substances (Li, 1988). In this paper, hormone and growth regulator will be used hereafter for the natural and artificial growth substances, respectively.

i. Exogenous Application of Growth Regulators in Somatic Embryogenesis

Out of the five groups of growth substances, auxins, cytokinins, gibberellins and ABA and their analogs are important components in culture media since they have been documented to play different roles in the process of embryogenesis from induction through maturation of somatic embryos. Their types, concentrations and the duration of treatment

Table 2 . The Nature, Synthesis and Functions of Plant Hormones.

Hormones	Nature	Sites of Biosynthesis	Transport	Functions	
				Cell & Molecular Level	Plant Level
Auxin	Primarily synthesized from tryptophan. IAA is the most commonly detected auxin, and exists in free & conjugate forms	Leaf primordia, young leaves and developing seeds.	Polar transported from cell to cell.	Activates ATPase H ⁺ pump, promotes RNA synthesis & initiates dedifferentiation.	Keeps apical dominance; tropic responses; vascular tissue differentiation, inhibits leaf and fruit abscission, stimulates ethylene synthesis.
Cytokinins	N ⁶ -adenine derivatives, phenyl urea compounds. Exist as free, glucoside & riboside forms. Zeatin is the most common in plants.	Root tips.	Transported via the xylem from roots to shoots.	Promotes protein synthesis, metabolism of sugar & starch, & cell division.	Promote shoot formation, delays leaf senescence.
Abscisic Acid	Synthesized from mevalonic acid. Exists in cis- & trans- isomeric forms.	Mature leaves & seeds.	Exported from leaves in the phloem.	Promotes storage protein synthesis.	Promotes stomatal closure, photosynthate transport from leaves to developing seeds & seed desiccation & maturation.
Gibberellins	Synthesized from mevalonic acid, >80 kinds.	Young tissues of shoots and developing seeds.	Probably transported in the xylem & phloem.	Stimulates cell elongation & division, & protein hydrolysis in seeds.	Hyperelongates shoots & plant height, & induces seed germination.
Ethylene	Synthesized from methionine.	Ripening fruit & stressed tissues.	Transported by diffusion.	Stimulates enzymes & ripening-related mRNAs.	Promotes fruit ripening, leaf & flower senescence, & leaf & fruit abscission.

Modified from Raven *et al.* (1992).

influence the morphology and efficiency of somatic embryo production (Brown *et al.*, 1995; Finstad *et al.*, 1993; Lazzeri *et al.*, 1987; Levi and Sink, 1991; Lippmann and Lippmann, 1993; McKersie and Bowley, 1993; Phillips and Collins, 1981; Takahata *et al.*, 1993).

1. Auxins: These chemicals are almost indispensable to callus formation and the initiation of somatic embryogenesis (Atanassov and Brown, 1984; Compton *et al.*, 1992; Dhir *et al.*, 1992; Finstad *et al.*, 1993; Hartweck *et al.*, 1988; Liu *et al.*, 1992; Ranch *et al.*, 1985; Shoemaker *et al.*, 1991; Tian *et al.*, 1994; Wenzel and Brown, 1991; Wright *et al.*, 1991). Auxins serve as a stress factor to bind to auxin-binding proteins on the membrane structure, initiate ATPase H⁺ pump, decrease cell pH and change ion concentrations, and initiate somatic embryogenesis (For reviews, please refer to: de Jong *et al.*, 1993; Dutis *et al.*, 1995; George, 1993; Jones, 1994; Thorpe, 1995; Zimmerman, 1993). In most experimental systems, without auxins there would be no induction of somatic embryos. However, the continuous application of auxin can inhibit the development of somatic embryos (Shoemaker *et al.*, 1991). Furthermore, different kinds of auxins have different effects. IAA, the only naturally existing auxin, has only a marginal effect (McKersie and Bowley, 1992). Among the analogues, 2,4-D is considered to be a strong inducer of somatic embryogenesis (*e.g.* Finstad *et al.*, 1993; George, 1993; Lazzeri *et al.*, 1987; Levi and Sink, 1991).

2. Cytokinins: The function of cytokinins is to promote cell division and protein synthesis (George, 1993). They cannot induce embryogenesis by themselves, however, they enhance

the induction effects of auxin (Finstad *et al.*, 1993; Levi and Sink, 1991; Phillips and Collins, 1981). The cytokinin/auxin ratio affects shoot and root formation. A high cytokinin/auxin ratio favours shoot formation, the low ratio promotes root formation, and an intermediate ratio favours callus formation (George, 1993). Among more than 25 naturally existing cytokinins in plants, only kinetin, benzyladenine and few analogues are commercially available. Kinetin is the most commonly used one and the usual concentration is 0.5-2.5 μM (George, 1993). At high concentrations, it inhibits the development of somatic embryos (*e.g.* Levi and Sink, 1991; Lippmann and Lippmann, 1993).

3. ABA: Like auxin, this growth regulator plays important roles in *in vitro* embryogenesis. It seems that ABA affects somatic embryogenesis from the heart or torpedo stage, and functions to induce desiccation tolerance and maturation of embryos by reducing moisture, increasing fatty acids and promoting protein synthesis (Compton *et al.*, 1992; Lippmann and Lippmann, 1993; Phillips and Collins, 1981; Pomeroy *et al.*, 1994; Takahata *et al.*, 1993). The effect of ABA is also dose-dependent. At low concentrations, it promotes embryo development at the heart stage to torpedo stage; a moderate concentration enhances the cotyledon and hypocotyl growth, and high concentration inhibits embryo development (Takahata *et al.*, 1993).

4. Gibberellins: Although more than 80 kinds of gibberellins have been discovered in plants, only GA₃ and a mixture of GA₄ and GA₇ are commercially available, and GA₃ is frequently applied in tissue culture. GA₃ promotes embryo growth and plant recovery by induction of

cell elongation and protein hydrolysis (George, 1993; Li, 1988; Lippmann and Lippmann, 1993; Phillips and Collins, 1981). Gibberellins act at the late stage of embryogenesis. Like the other growth regulators, gibberellins are beneficial for embryo development at optimal concentrations, and inhibit at high concentrations.

ii. Plant Hormones in Somatic Embryogenesis

1. The effect of photoperiod, temperature and water on hormone levels

The levels of plant hormones in plants fluctuate in response to a series of changes of both internal and external factors including various growth and development stages, photoperiod, temperature and water stress. Under the same conditions, different groups of hormones vary with respect to their levels, and a specific group of hormones can have a different response to different environmental factors. Among external factors, photoperiod, temperature and water status are the most important ones to affect a plant's normal growth and development, and hormone levels.

1) Photoperiod: Light is essential to plant life through influence on photosynthesis (Li, 1988; Raven *et al.*, 1992), gene expression (Rees and Atherton, 1987), enzyme activities (*e.g.* Bonzon *et al.*, 1987; Suzuki *et al.*, 1987) and hormone levels. Owing to evolution, plants vary in their requirement for light and photoperiod. The effects of photoperiod on plant growth and hormone levels are multiple and complicated. It has been documented that, in

comparison with long photoperiods, a short photoperiod decreases the levels of IAA, cytokinins and gibberellins levels in chrysanthemum (El-Antably *et al.*, 1991), grape (Kim and Ko, 1986), and tobacco (Lozhnikova *et al.*, 1990), and increases the ABA levels in chrysanthemum (El-Antably *et al.*, 1991), *Betula pubescens* (Rinne *et al.*, 1994), apricot (Kuzina and Kalinina, 1993), and grape (Kim and Ko, 1986). Reverse results, however, have been reported in alfalfa (Chen and Chen, 1988), bean (Bently *et al.*, 1975; Morgan, 1977), soybean (Bhardwaj and Yooder, 1987) and snapbean (Makus and Shannon, 1979). These contradictory results might be due to not only the various experimental plant species and genotypes but also the different experimental conditions.

2) Temperature: In nature, temperature is one of the most critical factors in the physical environment of plants. It exerts a significant influence on plant growth and development by affecting enzyme reactions, metabolic functions and physiological processes, including changes of endogenous hormones. In the literature, the effects of temperature on ABA has been most widely studied. Generally speaking, both high and low temperature stresses lead to a rise in ABA level (Browning, 1980; Eze *et al.*, 1983; Makers and Shannon, 1979; Talanova and Titov, 1994), but the changes of ABA levels differ among genotype and photoperiod (Browning, 1980; Makers and Shannon, 1979). In nature, the effects of temperature on hormones are influenced by other factors such as light. It has been observed that in dark, ABA and IAA levels dropped gradually and rose again after light was provided (Dunlap and Morgan, 1978). In daytime, endogenous hormone levels fluctuated.

2. Plant hormones in somatic embryogenesis

Compared with exogenously applied growth regulators, *i.e.* those synthesized plant growth substances and their analogues, plant endogenous hormones have been less studied in somatic embryogenesis. Research results showed that they are indispensable to somatic embryogenesis. One example is the phenomenon that no matter how high the level of endogenous IAA in explants, exogenous auxin, especially 2,4-D is usually still required for the induction of somatic embryogenesis. On the other hand, when cultured cells were transferred to 2,4-D-free medium for embryo maturation, applications of IAA inhibitors blocked normal embryo development (Fujimura and Komamine, 1979; Rajasekaran *et al.*, 1987; Schiavone and Cooke, 1987). Endogenous ABA also takes part in somatic embryogenesis. If ABA synthesis was inhibited in donor plants, the capacity of explants for somatic embryogenesis was decreased or lost; addition of exogenous ABA enhanced embryogenesis (Rajasekaran *et al.*, 1987). These results suggested the important roles of endogenous IAA and ABA in controlling embryogenic capacity of plants.

Since IAA, ABA and cytokinins play important roles in somatic embryogenesis, their levels in donor plants and somatic embryos were mainly studied in recent years, and were found related to the frequency of somatic embryogenesis and embryo development (Besse *et al.*, 1992; Danin *et al.*, 1993; Ivanova *et al.*, 1994; Michalczyk *et al.*, 1992; Rajasekaran *et al.*, 1987; Spencer and Kitto, 1988). IAA and cytokinins decrease and ABA increases along with the development of somatic embryos (Danin *et al.*, 1993; Ivanova *et al.*, 1994;

Michalczuk *et al.*, 1992). These hormone levels were also correlated with regeneration ability of plants. Ivanova *et al.* (1994) analysed endogenous IAA, ABA and cytokinins levels in three lines of alfalfa of fast-, low- and non-regenerating plants during direct somatic embryogenesis. They reported that IAA was high and ABA low in leaf explants of high-regenerating plants, and their levels reverse along with embryogenesis; in contrast, they showed a reversed trend in low and non-regenerating plants. In all high, low and non-regenerating plants, cytokinins remained at a relatively low and stable level throughout embryogenesis (Ivanova *et al.*, 1994). These results suggested that IAA and ABA play active roles in early and late stages of embryogenesis, respectively; while cytokinins might play a stable but less significant role. Based on these results, a hypothesis was put forward by Ivanova *et al.* (1994): "The levels of endogenous hormones may be correlated to the specific response of different genotypes to embryo-inducing conditions". That is, the endogenous growth regulator profile may be a predictor of the *in vitro* somatic embryo response of any genotype.

III. Research Strategy

The results of genetic studies on somatic embryogenesis showed that the regeneration ability is controlled by 1-2 genes. Studies on this aspect lays the foundation to turn non-regenerating plants into regenerating plants, or low-regenerating plants into high regenerating plants by isolating and transforming the regeneration-controlling genes into cultivars with genetic engineering, or by classical genetic methods. For this purpose, molecular markers that are closely linked to the regeneration-controlling genes are required for isolation and identification of regeneration-controlling genes. Regretfully, there are few such marker genes reported. In alfalfa, there was only one marker gene reported, and even this one was specific to a single cross (McKersie and Brown, 1996).

Genetic analysis suggested that somatic embryogenesis, at least in alfalfa, is not controlled only by the “regenerating” genes. Other genes may play a role in somatic embryogenesis, because even in plants with the same gene arrangement, variation of the regeneration efficiency was observed under different conditions (Henry *et al.*, 1994; Reisch and Bingham, 1980; Wan *et al.*, 1988). Success in screening these genes is of significance to improve our knowledge of somatic embryogenesis. Like marker genes, such genes are also beyond our knowledge at present.

The results from recent experiments on somatic embryogenesis and endogenous hormone levels are encouraging. The correlations of endogenous IAA and ABA with

regenerating ability suggested that there may have some correlations between the genes controlling regenerating ability and endogenous hormone levels. Taking into consideration the interactions of genotype and culture media, these results will be more believable if they are further tested and verified with plants that have a close genotypic background and with multiple culture methods. This consideration served as the starting point of the present research.

i. Research Purpose

These studies were initiated to test the hypothesis put forward by Ivanova *et al.* (1994); *i.e.*, “The levels of endogenous hormones may be correlated to the specific response of different genotypes to embryo-inducing conditions.” A two-step study was set up and is outlined in Fig. 3.

ii. Plant Material

Alfalfa was used in this experiment. This plant was chosen for several reasons. First, it is one of the most valuable forage legumes in the world (Michaud *et al.*, 1988). At present, alfalfa is grown on about 40-45 million acres in North America (Barnes *et al.*, 1988), and provides 60-80% of the nutrients for ruminant animals, which, in turn, provide 36% of the protein in the human diet (McKersie and Bowley, 1988). Secondly, alfalfa is a perennial plant and has no true physiological rest period (McMenzie *et al.*, 1988). This character

contributes to the wide application of alfalfa in lab experiments. Finally, alfalfa is one of the major crop plants that have been regenerated from tissue culture, and was the plant used by Ivanova *et al.* (1994). The first success in somatic embryogenesis with alfalfa was accomplished in 1972 (McCoy and Bingham, 1972). Extensive knowledge in somatic embryogenesis has been accumulated by experiments with alfalfa, including culture media and protocols (*e.g.* Denchev *et al.*, 1991, 1992; Meijer and Brown, 1987; Shetty and McKersie, 1993; McKersie and Brown, 1996), the effects of genotype (Brown and Atanassov, 1985), relations between somatic embryogenesis and endogenous hormone levels (Ivanova *et al.*, 1994), and genetics (*e.g.* Reisch and Bingham, 1980; Wan *et al.*, 1988).

iii. Research Strategy

1. To investigate the effects of genotype, explant source and culture media on the correlations between somatic embryogenesis and endogenous hormone levels, plants with high, mid, low and non-regenerating abilities from two (A70 and A195) populations of alfalfa (*Medicago sativa* L.) were used. The A195 population had a relatively narrow genetic background, being the progeny of a single one-way cross between a high regenerating and non-regenerating plant. The A70 population had a wide genetic background, being selections from a synthetic commercial seed lot. Leaf and petiole tissue of alfalfa were used as donor explants for assessing somatic embryogenesis and measuring endogenous hormone levels. Direct and indirect culture methods, and multiple-step culture protocols were applied. Since there were reports on variation of regenerating rates with different culture media, the

approach required several culture methods and protocols for somatic embryogenesis. This provided a broad-based comparison of somatic embryogenesis and endogenous hormone levels. The levels of endogenous IAA, ABA and cytokinins in leaves and petioles of donor plants were measured and compared with the *in vitro* somatic embryogenesis response in all protocols.

2. The correlations between endogenous hormones and somatic embryogenesis was investigated by attempting to change endogenous hormone levels in the donor plants with different photoperiods. Explants were sampled at three time points of the day period for assessment of somatic embryogenesis and endogenous hormone levels. The levels of endogenous IAA, ABA and zeatin riboside in donor explants were analysed and compared with somatic embryogenesis.

Fig. 3. Research strategy and method of this study.

MATERIALS AND METHODS

PART 1. Analysis of Hormone Levels and Assessment of Somatic Embryogenesis in Leaf and Petiole Tissues

I. Stock Plants

i. Choice of Plants

In order to investigate the effect of genotype on somatic embryogenesis and endogenous hormone levels, the A70 and A195 populations of alfalfa that have different genotypic backgrounds and include plants with high, medium, low and non-regenerating abilities were chosen and used in this study. The A70 population was selected from a seed lot of c.v. Rangelander, and the A195 was a cross of A70.34, a plant of the A70 population with extremely high regenerating ability, and a non-regenerating alfalfa plant. The cross was made by A. McElroy at the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada. The regeneration trend in both populations was established by screening with the B5h-B5M-BOi2Y indirect culture protocol (Table 3). Both populations were established and kept in the greenhouse at the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada.

ii. Culture of Stock Plants

Plants from A70 and A195 populations with high (A70.403, A70.413, A195.406 and A195.407), mid (A70.415 and A195.415), low (A70.409 and A195.416) and non- (A70.406, A195.403, A195.409 and A195.410) regenerating abilities were used for this study. Under aseptic conditions, the stem was detached from stock plants, plunged in sterile distilled water, and cut into 2-cm segments of one node and internode with forceps and surgical knife. Four to five segments, with their internode side down, were then inoculated in 75 ml of either ½ (for A70 plants) or full-strength (for A195 population) MS medium (Murashige and Skoog, 1962) in 4" Magenta containers (Magenta Corp., USA). Each Magenta container was sealed with one layer of Micropore (3M Canada Inc., Canada) and cultured at 25/15°C (day/night temperature) under a 16-h photoperiod and 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 2-2.5 months before their leaves and petioles were collected for induction of somatic embryogenesis and measurement of endogenous hormone levels.

II. Somatic Embryogenesis

One to three top-most trifoliate leaves and petioles were detached from the 2 to 2.5-month-old stock plants and induced for somatic embryogenesis with direct (Denchev *et al.*, 1991; Ivanova *et al.*, 1994) and indirect (Brown, 1995) culture systems, including five different culture protocols (Fig.3, Table 3).

i. Direct System (Direct culture system; Sequence: B50/IV - B50/3M - MS + ABA - MS +GA₃; Fig. 3, Table 3)

This system was developed by Denchev *et al.* (1991) for alfalfa embryogenesis, and was successfully used by Ivanova *et al.* (1994) for analysis of somatic embryogenesis and endogenous hormone levels of alfalfa plants with high, medium and non-regeneration abilities. The components of the media are given in Appendix I and the culture procedure is as follows:

1. Inoculation: Fifteen mg of 1-3 top-most donor trifoliate leaves were taken from stock plants and cut quickly into small pieces in B50 medium (Denchev *et al.*, 1991; Ivanova *et al.*, 1994) with a surgical knife and forceps. Then, the leaf pieces were rinsed with 20-30 ml fresh B50 medium three times, 20 min each.

2. Induction: After rinsing, the leaf material was put into 15 ml of B50/IV medium, which

Table 3. Protocol Scheme for Inducing Somatic Embryogenesis in Alfalfa Explants

Protocols	Induction	Development	Regenerating Media (days)	
	Media (days)	Media (days)	1	2
Direct Culture System (Denchev <i>et al.</i> , 1992)				
P1	B50/IV(30)	B50/3M(21)	MS +ABA(14)	MS + GA ₃ (14)
Indirect Culture System (Brown, 1995)				
P2	B5h (22-24)	B5M (10-11)	BOi2Y (15)	-----
P3	SH4K (22-24)	B5M (10-11)	BOi2Y (15)	-----
P4	B5h (22-24)	-----	BOi2Y (25)	-----
P5	SH4K (22-24)	-----	BOi2Y (25)	-----

were contained in a 125 ml Erlenmeyer flask and autoclaved at 121°C for 22 min. The flasks were sealed with autoclavable foam plugs, capped with aluminium foil, and cultured on a G10 Gyrotory shaker (New Brunswick Scientific, USA) at 130 r.p.m. under the same culture conditions as stock plants; *i.e.*, at 25/15°C (day/night temperature) under a 16/8-h (day/night) photoperiod and 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for one month.

3. Embryo development: The one-month-old culture contained globular-stage embryos. Under sterile conditions, the contents of each flask were put into a 50 ml centrifuge tube, and centrifuged at 600 x g. (International Equipment Company, USA) for 5 min. The supernatant was discarded, and the pipet was washed 2-3 times with 20-30 ml fresh B50 medium. The remaining embryos were then transferred to 15 ml fresh B50/3M medium which was contained in a 125 ml Erlenmeyer flask and autoclaved at 121°C for 22 min, and shake-cultured under the above conditions for 20 days.

4. Embryo maturation: The torpedo-staged embryos were separated from the medium by filtering through a 202 μM nylon screen, and then were transferred onto 20 ml solidified $\frac{1}{2}$ (for A70 plants) or full-strength (for A195 plants) MS + ABA (30 μM) media contained in a 20 x 600 mm Petri dish (Nunc. Inc., USA), sealed with parafilm (American National Can., USA), and cultured for 14 days under the same culture conditions as for stock plants. The $\frac{1}{2}$ and full strength MS + ABA medium was pre-autoclaved at 121°C for 23 min.

5. Conversion: The embryos were further transferred to 20 ml $\frac{1}{2}$ (for A70 plants) or full

strength (for A195 plants) MS + GA₃ (27 µM) medium contained in a 20 x 600 mm Petri dish and sealed with Parafilm, and cultured under the above conditions for conversion of embryos to plants. The ½ and full- strength MS medium was first autoclaved at 121°C for 23 min; GA₃ was dissolved in distilled water, sterilized by filtering through a nylon filter (pore size: 0.22 µm, Micron Separations Inc., USA), and then mixed with the autoclaved media.

ii. Indirect System

This system includes two three-step and two two-step culture protocols based on the protocol described by Brown (1994). The two three-step and two two-step protocols differ with respect to a liquid culture phase in B5M medium, the embryo development medium. Each protocol in the three-step and two-step protocols differs in the induction medium (B5h or SH4K). Briefly, they are described as follows (Fig. 3; Table 3):

1. Three-step protocol (Protocol 2, sequence: B5h - B5M - BOi2Y; and protocol 3, sequence: SH4K - B5M - BOi2Y):

1) Induction: One to three top-most whole petioles and trifoliate leaves were detached from stock plants, and plunged into sterile distilled water. The leaves and petioles were separated. For petioles, 1-2 mm was trimmed from the end of each petiole, and cut into segments, 1-cm each. Then, 6-7 petiole segments or leaves were separately plated on 20 ml solidified B5h

or SH4K medium contained in a 20 x 600 mm Petri dish, wrapped with Parafilm, and cultured at 25/15°C (day/night temperature) under a 16/8-h (day/night) photoperiod of fluorescent light at $50 \mu\text{Em}^{-2}\text{s}^{-1}$ for 22 - 24 days.

2) Induction: One to two grams of calli developed on B5h or 2-3 grams of calli developed on SH4K medium were aseptically transferred into 40 ml liquid B5M medium contained in a 125 ml Erlenmeyer flask. Flasks were closed with autoclavable foam plugs, capped with aluminium foil, and cultured for 10-11 days on an orbital shaker at 130 r.p.m. under the above conditions.

3) The suspension with globular embryos was filtered through a 1000 μm and 200 μm nylon mesh, the embryos were collected on the 200 μm screen, transferred and distributed evenly on 20 ml of solidified BOi2Y medium contained in a 20 x 600 mm Petri dish. The dish was wrapped with Parafilm and cultured under the above conditions.

4) At 15 days culture on BOi2Y, the embryo number was counted in each Petri dish under a microscope, and the embryo production value was calculated on a number/G callus basis.

2. Two - step protocols (Protocol 4, sequence: B5h - BOi2Y; Protocol 5, sequence: SH4K-BOi2Y):

As stated above, the difference between this and the three-step protocol is the omission of culture in B5M medium. The rest of the steps are the same.

III. Measurement of Endogenous Hormone Levels

One to three top-most leaves and petioles were collected at the same time as those that were used to assess somatic embryogenesis, and their fresh weights were recorded. The samples were then frozen in liquid nitrogen, stored at -80°C , freeze-dried at -80°C in a vacuum, and the dry weight was recorded. The dry samples were then air-mailed to Dr. Ivanova in Belgium for measurement of endogenous hormone (IAA, ABA and cytokinins) levels by HPLC in a blind test; *i.e.*, Dr Ivanova analysed the samples without knowing the sources and with no knowledge of the regenerating abilities. Each sample was analysed at the same time for IAA, ABA and cytokinins. The analysis procedures followed that published by Denchev *et al.* (1991) and Ivanova *et al.* (1994). The levels of endogenous IAA, ABA and cytokinins were first determined on pMol g^{-1} DW basis, then changed to pMol g^{-1} FW according to the DW/FW ratio of the samples, so that the comparison between somatic embryogenesis and hormone levels could be made on both fresh and dry weight levels.

IV. Comparison of Somatic Embryogenesis among Various Protocols

In the direct culture method, each 15 mg fresh weight of leaf was induced in 15 ml B50/TV medium contained in one Erlenmeyer flask and was finally cultured on MS + GA₃ medium. The embryo production was calculated as embryo number/flask (15 mg fresh leaf). For the indirect culture, the embryo production was calculated as the number/gram of callus according to the traditional calculation method. Embryos were counted at 15 days after culture on MS + GA₃ medium and BOi2Y medium separately for the direct and three-step indirect protocols, and 25 days after culture on BOi2Y medium for the two-step indirect protocols. The embryo production from both explant sources of both populations with the five culture protocols was also analysed with statistical method outlined in Appendix 4.

V. Comparison of Somatic Embryogenesis and Endogenous Hormone Levels

In order to determine the correlations between the embryogenesis and hormone levels, plant materials were collected at the same time for both procedures. The results of somatic embryogenesis and endogenous hormone levels from the same group of collection were compared. The experiment was repeated three times for A195 leaf samples and twice for A70 leaf materials (except A70.409). Since the amount of petiole-derived material was less than with the leaf-derived material, experimental replicates ranged from one to three.

Part 2. Analysis of Hormone Levels and Assessment of Somatic Embryogenesis in the A195 Plants Cultured in Soil

I. Stock Plants

Alfalfa A195.406, A195.415, A195.416 and A195.403 with high, medium, low and non-regenerating abilities, respectively, grown in MS medium in Magenta containers were transferred into a soil mixture (sand:peat moss:soil:perlite=1:2:2:1) in 13-cm pots with 3 plants per pot. The plants were grown under a 16/8-h day/night photoperiod at 25°C for 7 weeks. During this period, the plants were cut 8 cm above the soil level three times without harvest of the donor explants. Each pot of A195.406, A195.415, A195.416 and A195.403 was cultured as one group in three controlled-environment chambers set separately at 8/16-h, 16/8-h, and 20/4-h day/night photoperiods at 25°C 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The plants were watered daily and fertilized twice a week with Hoaglands (Wed.) and 20:20:20 (Fri.), respectively. During the first month under the new photoperiod, the plants were cut every two weeks and as before, the plant materials were not used for experiments. From then on, at an interval of 15-18 days, the 1-2 top-most trifoliate young leaves were collected at three time points during the day; *i.e.*, at the ends of dark and light, and midday period, for induction of somatic embryogenesis and analysis of hormone levels. After each collection of the explants, the plants were trimmed as before and the plants regrew for the next harvest.

II. Sampling Explants and Somatic Embryogenesis

i. Sterilization of Explants

Freshly collected leaf samples were surface-sterilized with 7% calcium hydrochloride for 9 min, then rinsed in sterile distilled water 3 times, 3 min each.

ii. Culture Protocols

Protocols 2 and 3 were used for induction of somatic embryogenesis in this part. The procedures were the same as that in Part 1 (Fig. 3; Table 3).

III. Analysis of Endogenous Hormone Levels

As described in Part 1, the leaves for hormone analysis were collected at the same time as those used to assess somatic embryogenesis. One fresh gram of each sample was put into a 50-ml centrifuge tube and dipped immediately into liquid nitrogen. The frozen samples were then stored at -80°C for analysis. The ELISA method was used to measure hormones from fresh samples.

i. Extraction

1. The frozen leaf tissues were ground to a fine powder in liquid nitrogen using a chilled mortar and pestle.
2. Ten ml of 80% methanol containing 20 mg L^{-1} BHT was then added to each sample contained in a sterile 50 ml centrifuge tube. The samples were extracted at 4°C in the dark for 2 h using an overhead mixer. The sample solutions were spun at $1,000 \times g$ for 5 min, and the methanolic extracts were collected.
3. Ten ml of 80% methanol containing 20 mg L^{-1} BHT was added to each sample pellet, mixed well and extracted again for 2 h. The methanol extracts were collected as above.
4. The extraction process was repeated a third time. The three extracts of each sample were combined and adjusted to 70% methanol by the addition of water.
5. The extract of each sample was passed through a C-18 Sep-Pak Cartridge (Waters, USA); the column was washed with 3 ml of 70% methanol and the effluent was combined with the

extract.

6. The extract volume was reduced to its aqueous phase to about 2 ml using a rotary evaporator.
7. The volume was equally divided into two parts, one for ZR and ABA analysis, and the other for IAA analysis.
8. The ZR and ABA analysis lot was filtered through a nylon mesh with 0.22 μm pore size (Micron Separations Inc.) and followed by filtration through a centricon (Amicon Inc.) filter.

ii. Methylation of IAA

1. The sample solution was adjusted to pH 9.0, and the volume was doubled with ethyl ether (Fisher Scientific). The solution was extracted in the dark at 4°C with a constant agitation at 120 r.p.m. on an orbitory shaker (Lab-line Instruments Inc.) for 40 min.
2. The ether supernatant was discarded and the remaining volume was doubled with ethyl ether, and extracted again as above.
3. The ether supernatant was removed and the sample solution was adjusted to pH 2.5. The volume was doubled with ethyl ether and extracted as above.
4. The supernatant was collected; the volume was adjusted with ethyl ether and re-extracted twice as above.
5. The supernatants of three extractions were combined and evaporated to dryness with a rotary evaporator.
6. Each sample was dissolved in 0.5 ml methanol, and added with 2 ml diazomethane to

methyrate IAA. Then, 2 ml 0.2 M acetic acid was added into each sample to destroy the excess diazomethane.

7. The solution was evaporated to dryness with a rotary evaporator.

8. Two ml TBS (pH 7.5) was added to each sample and mixed well. The solution was filtered through a nylon mesh with 0.22 μm pore size (Micron Separations Inc.), then through a centricon (Amicon Inc.) filter.

iii. ABA, ZR and IAA Analysis with ELISA

1. ABA analysis

This analysis procedure was based on that of M. Walker-Simmons and Joan Sasing (1987) with a modified extraction method. Briefly, the procedure is as follows:

1) An amount of 18.75 μl of C4-BSA-ABA conjugate (provided by DR D. Dunstan at PBI-NRC, Saskatoon) was added to 20 ml of 0.05 M NaHCO_3 (pH 9.6), and mixed well. 200 μl of the solution was added to each well of a Dynatech Immulon II plate. The plate was sealed with parafilm, and incubated overnight in the dark at 4°C, with constant agitation at 120 r.p.m. on an orbit shaker (Lab-line Instruments, Inc.).

2) ABA standards were prepared with (+)-cis, trans-ABA (Sigma, USA) diluted with TBS, pH 7.8, to concentrations of 10, 25, 50, 100, 200 and 400 pg ABA 100 μl^{-1} .

3) Samples were prepared with a purified sample solution diluted with TBS at 1:10, 1:50

and 1:100 ratios.

4) Mab (Idetek) was prepared to a working solution of about $3.84 \mu\text{g ml}^{-1}$ TBS containing 0.2% (w/v) BSA. The Mab was then mixed in a 1:1 ratio with the ABA standard and sample solutions, and incubated overnight with the plate under the same conditions as outlined above in step 1.

5) The next morning, the C_4 conjugate was removed from the plate, and each well of the plate was rinsed by adding 200 μl TBS containing 0.05 (v/v) Tween 20 and 0.1% (w/v) BSA with a multichannel pipette (Eppendorf) three times, each for 3, 3 and 4 minutes, respectively.

6) 200 μl of the mixed Mab with ABA standards or samples was added to each well of the plate, and incubated for 2 h at room temperature in the dark with constant agitation at 120 r.p.m. on a shaker as outlined above.

7) The plate was emptied of liquid and rinsed as outlined above in step 5.

8) Antimouse IgG alkaline phosphatase (Sigma) was diluted with TBS in a 1:1000 ratio. 200 μl of the solution was added to each well with a multichannel pipette (Costar), and incubated for 2 hours as outlined above in step 6.

9) The plate was emptied and rinsed as outlined above in step 5.

10) One tablet (20 mg) of p-nitrophenyl phosphate disodium salt (Sigma) was dissolved in 20 ml 0.05 M NaHCO_3 (pH 9.6), mixed well. 200 μl of the solution was added to each well with a multichannel pipette (Costar), and incubated as outlined above in step 6 for about 30-40 min.

11) The absorbency of the samples was read at 405 nm with a BioRad Enzyme Immunoassay

Reader.

12) The concentration of IAA in each original sample was calculated with Bio-Tek KinetiCalc EIA application software version 2.12 (Bio-Tek Instruments, Inc., 1992).

2. IAA and ZR analysis

IAA and ZR analyses employed a PHYTODETEK enzyme immunoassay kit (Idetek) with a indole-3-acetic acid methyl ester and zeatin riboside trans-isomer (Sigma) as standards. The procedure of analysis followed the “directions for use” of the company which are as follows:

- 1) One ml distilled water was added to 1 vial of lyophilized Tracer and allowed to dissolve completely. The total contents of reconstituted Tracer were transferred to 4 ml of Tracer diluent and mixed well.
- 2) The standard IAA and ZR was dissolved in absolute methanol to make a 1.0 mM stock solution. From the stock solution, standards was made in 25 mM Tris buffered saline, pH 7.5. For the IAA analysis, the standard solutions were 0.5, 1, 5, 20, 50 and 100 pMol 0.1 ml⁻¹; for ZR, the standard solutions were 0.05, 0.2, 1, 5, 10, 50 and 100 pMol 0.1 ml⁻¹.
- 3) The samples for IAA and ZR analyses were diluted to ratios of 1:10, 1:50 and 1:100 with 25 mM TBS, pH 7.5.
- 4) 100 µl standard of IAA or ZR and corresponding sample solutions were added to each well of the IAA or ZR plate; 100 µl of diluted Tracer solution was added to each well using

a multichannel pipette. The wells were covered with the Plate sealer, and incubated for 3 h in the dark at 4°C with agitation at 120 r.p.m. on a shaker.

5) The solution was decanted and the wells were washed by adding 200 μ l of the Wash solution to each well with a multichannel pipette (Eppendorf) three times, for 3, 3 and 4 min, respectively.

6) One substrate tablet was dissolved in 5 ml Substrate diluent, mixed well. 200 μ l of the solution was added to each well using a multichannel pipette. The wells were covered with the Plate sealer and incubated as outlined above in step 4.

7) The absorbency of the samples was read at 405 nm with a BioRad Enzyme Immunoassay Reader, and the concentrations of IAA and ZR in each original sample were calculated with Bio-Tek KinetiCalc EIA application software version 2.12 (Bio-Tek Instruments, Inc., 1992).

3. Extraction monitoring

One hundred μ g of each standard IAA, ABA and ZR were put into one 50 ml centrifuge tube, and were handled with samples to monitor the recovery rate.

RESULTS

I. Culture of Stock Plants

i. Plants Cultured *in vitro*

After transfer of node explants onto MS medium, the explants remained visibly unchanged for 3-4 days, then axillary shoots began to elongate. The growth was slow in the first month and fast thereafter. At two months, the high regeneration plants, A195.406, A195.407, A70.403 and A70.413, showed slower growth than the other plants, *i.e.* medium-, low- and non-regenerating plants. The leaves of high-regenerating plants were slightly yellow, while the leaves of plants of other regeneration abilities were more green. The petiole morphology also varied among plants. Generally, petioles of the A195 population were thicker than those of the A70 plants, and the petioles varied in length in both populations.

ii. Plant Cultured in Soil

It took several days for the A195 plants to recover after the plants were transferred from MS medium in Magenta containers to soil in pots. Ten to 14 days after transferring into soil, the shoots of plants began to elongate. The three groups of plants grew differently under

various photoperiods. Plants grown under a 16-h photoperiod grew the fastest, and those under a 8-h photoperiod grew the slowest. Plants grown under 16-h and 20-h photoperiod were similar in height of about 75-90 cm at the end of regrowth, but plants grown with a 8-h photoperiod treatment grew much slower and were only about 10-20 cm high at the end of the growth period. During the regrowth period, flower buds in plants appeared in plants under a 16-h photoperiod at about the 18th day, and in those under a 20-h photoperiod at about the 16th day. For plants cultured in a 8-h photoperiod, no flower buds were observed even after 40 days of regrowth. The plants comprising each group showed some difference in growth although they were cultured under the same photoperiod. A195.406, A195.403 and A195.416 showed a similar higher growth rate than A195.415 in all of the three photoperiod treatments. This trend was more obvious in those cultured under the 20-h and 8-h photoperiods than that under the 16-h photoperiod. Under a 20-h photoperiod, A195.403, A195.406 and A195.416 grew as well as those under a 16-h photoperiod, but A195.415 grew poorly, which became more significant with the time of the culture period.

II. Somatic Embryogenesis

i. Embryogenesis from Stock Plants Cultured in MS Medium

1. Embryogenesis induced from leaves

1) The direct culture method: In the first several days after leaves were inoculated into B50/IV induction medium, leaf pieces lost their green colour gradually, and the culture suspension gradually became dense. The density of the cell suspension was negatively correlated with the regenerating ability; *i.e.*, the lower the regeneration ability, the denser the suspension. At 20 - 25 days, embryos became visible. The somatic embryo numbers varied significantly in each replicate for each plant, and there was a great variation in embryo number among plants of different regenerating abilities in both the A70 and A195 populations. The highest mean number of embryos was observed in A195.406 (31 ± 3), A195.407 (46.3 ± 10.5), A70.403 (16.3 ± 5.9) and A70.413 (75.9 ± 11.5), the second highest in A195.415 (15.7 ± 5.6) and A70.415 (10.8 ± 5.8), followed by A195.416 (20 ± 2) and A70.409 (5 ± 4) (Table 4). There was no embryo production in A195.403, A195.409, A195.410 and A70.406 (Table 4). One exception is that somatic embryos were induced in one flask of A195.410 leaf tissue with the direct culture method. Generally, the trend of regeneration was similar to that documented with the P2, and remained unchanged when the embryos were finally counted on the MS + GA₃ medium. However, A195.416, a low-regenerating plant when cultured on B5h medium, showed a higher frequency of embryogenesis than

A195.415, a mid-regenerating plant (Table 4).

2) The indirect culture method: Four protocols of indirect somatic embryogenesis culture methods were used with B5h and SH4K as induction media (Table 3). A distinct difference in callus production both from leaf and petiole tissues between B5h and SH4K induction media was observed. Callus induced by B5h was small in size, yellow in colour, and hard in texture. In contrast, callus on SH4K medium was larger, bright white, soft and easily broken apart. Callus formed on B5h medium was easily picked up by forceps, while those on SH4K medium broke apart. Callus grew more vigorously on SH4K than that on B5h medium (Fig. 4). A difference between the two medium induction protocols was further observed in B5M suspension culture medium. For callus induced by B5h medium, B5M suspension was denser than that of SH4K medium induction. However, there was no obvious difference in the time of embryo appearance on BOi2Y regeneration medium with each pair of two- and three-step culture protocols. Embryos from both three-step culture protocols with B5h and SH4K induction media were visible at about 10 days of culture after being transferred onto BOi2Y medium, and embryos from the two-step culture protocols were obvious at about 20 days of culture after transfer to BOi2Y.

With regard to the efficiency of somatic embryogenesis with the four indirect method protocols, significant differences were observed among B5h and SH4K induction media, and among the two- and three-step culture protocols (Table 4). In the three-step culture

Fig. 4. Difference in callus production after 21 days of the induction on SH4K and B5h media in the A70 regenerable plants. Please note the callus induced by SH4K medium is larger, brighter, softer and more easily broken apart than that induced by B5h medium.

a: A70.403; b: A70.415; c: A70.409.

Left: Calli induced with B5h medium;

Right: Calli induced with SH4K medium.

Table 4. Summary of Somatic Embryo Numbers Induced from Alfalfa Plants Cultured *in vitro*.

Plant	Regene- ration Ability	Mean±SE of Somatic Embryo Numbers from Leaf					Mean±SE of Somatic Embryo Numbers from Petiole				
		P1 ¹	P2	P3	P4	P5	P2	P3	P4	P5	
A195.406	High	31±3	57±21	26±14	8±1	16±5	61±2	51	13	21±4	
A195.407	High	46±11	53±18	45±20	18±8	11±5	100±52	34±11	27±17	37±8	
A195.415	Mid	16±6	16±5	11±4	3±1	7±2	12±6	6	4±2	8±1	
A195.416	Low	20±2	10±3	12±4	6	8±1	7±3	10±3	7±2	12±5	
A195.403	Non	0	0	0	0	0	0	0	0	0	
A195.409	Non	0	0	0	0	0	0	0	0	0	
A195.410	Non	0	0	0	0	0	0	0	0	0	
A70.403	High	16±6	39±19	21±11	27±10	28±6	112	27±8	15±5	20±5	
A70.413	High	76±12	46±6	24±9	40±13	35±10	45±13	86	47±32	49	
A70.415	Mid	11±6	37±2	19±13	22±8	19±8	29±8	36±6	8±2	7	
A70.409	Low	5±4	8±3	4±2	2	6±2	5±2	4±1	2	3	
A70.406	Non	0	0	0	0	0	0	0	0	0	

¹P1: B50IV-B50/3M-MS+ABA-MS+GA3; P2: B5h-B5M-BOi2Y; P3: SH4K-B5M-BOi2Y; P4: B5h-BOi2Y; P5: SH4K-BOi2Y.

P1: the mean value was calculated as embryo number/Flask (15 mg fresh leaf);

P2-P5: the Mean value was calculated as embryo number/gram callus.

protocols, B5h produced more embryos than SH4K medium in all regenerating A70 plants and A195.406, A195.407 and A195.415 plants except A195.416 (Table 4). A similar efficiency of embryogenesis was observed in A195.416 with B5h (10 ± 2.5) and SH4K (11.6 ± 3.5) media with the two three-step culture protocols. The two-step culture protocols with B5h induction medium also resulted in a higher level of embryogenesis than that with SH4K induction medium in all regenerating A70 plants, but a reversed result was noted in regenerating A195 plants (Table 4). In comparing each pair of two- and three-step culture protocols with B5h or SH4K as induction medium, embryo production was higher in the three-step culture protocols with B5h as the induction medium in both A70 and A195 regenerable plants. The three-step culture protocol with SH4K as the induction medium also resulted in a higher level of embryogenesis in A195 regenerable plants; however, a lower level of embryogenesis was documented in A70 regenerable plants than with the two-step culture protocol.

Embryo numbers varied among replicates of each plant, and among plants of both A70 and A195 populations. The use of SH4K induction medium resulted in the same trend of regeneration for the A70 populations as B5h medium (Fig. 5); *i.e.*, the highest embryo number in A70.403, A70.413, followed by A70.415 and A70.409, whereas there was no embryo formation in A70.406 (Table 4). The regenerating trend of the A195 plants documented in the B5h three-step protocol was in contrast to the other three indirect protocols: The former resulted in the same trend as before, while the latter showed some difference with the result that 195.406 and A195.407 maintained a high-regenerating ability,

Fig. 5. Somcttic embryogenesis on BOi2Y medium cultured with P2 from leaf tissue

Sampled from the A70 regenerable plants cultured *in vitro*.

a: A70.403; b: A70.415; c: A70.409.

and A195.403, A195.409 and A195.410 developed no embryos, but A195.416 produced more embryos than A195.415 plants (Table 4). This trend is in line with that documented in the direct method.

2. Embryogenesis induced from petioles

The callus produced from petioles on B5h and SH4K induction media showed similar differences as those from leaves in both A70 and A195 populations. On B5h medium, callus was limited in the origin of its development; *i.e.*, it developed primarily at the cut ends of petioles. The efficiency of somatic embryogenesis also varied among replicates of each plant, among culture protocols and, among plants of both A70 and A195 populations (Table 4, Appendix 2). Generally, the three-step protocol induced more embryos than the two-step protocol, but in some plants, similar embryo numbers were observed such as in A195.416, and A70.415 (Table 4). In A195.416, the three-step and two-step protocols with B5h induction medium produced 6.7 ± 3.1 and 6.5 ± 1.5 embryos, respectively; and the three- and two-step protocols with induction by SH4K medium induced 10.2 ± 2.6 and 11.7 ± 5.4 , respectively. Somatic embryogenesis was also affected by the induction medium; *i.e.*, B5h and SH4K medium. In three-step protocols, B5h induced more embryos than SH4K in A195.406, A195.407, A195.415, and A70.403 and A70.409 plants, but less in A195.416, A70.413 and A70.415 plants. The difference in the induction effect of both media on somatic embryogenesis was also observed in the two-step protocols. In contrast to three-step protocols, SH4K medium induced more embryos than B5h medium in all A195 and A70

regenerable plants except A70.415 (Table 4).

Although variations were observed among culture protocols, between induction media, and among plants of both populations, the regeneration trends of both A195 and A70 populations remained consistent with P2; *i.e.*, the three-step protocol with B5h induction medium (P2, Table 3). However, some differences in regeneration trends in the two populations were observed in the other three protocols. In the A195 population, the high (A195.406 and A195.407) and non-regenerating (A195.403, A195.409, and A195.410) plants remained in their relative rank as those documented by P2, but A195.416, the low-regenerating plant, was reversed with respect to the regeneration rank with A195.415, the mid-regenerating plant (Table 4). This is consistent with the results with the leaf as the explant tissue (Table 4). In the A70 population, the general regenerating trend remained the same as that documented with P2 with one exception: A70.415 ranked superior to A70.403 in the three-step protocol with SH4K as induction medium (Table 4). When both the A70 and A195 populations were analysed statistically, the plant regeneration trend remained the same for all leaf and petiole explants except those with P4 and P5 culture protocols (Appendix 4).

3. Comparison of the five culture protocols in the efficiency of somatic embryogenesis

According to the results of somatic embryogenesis, the efficiency of the five culture protocols were observed (Table 4). As shown in Table 5, the ranking of these protocols was

Table 5. Comparison of the Efficiency of Five Protocols in Somatic Embryogenesis

Plant	Ranking ¹ of the Five Protocols	
	Leaf as Donor Explant	Petiole as Donor Explant ²
A195.406	P2>P1>P3>P5>P4	P2>P3>P5>P4
A195.407	P2>P1>P3>P4>P5	P2>P5>P3>P4
A195.415	P2>P1>P3>P5>P4	P2>P5>P3>P4
A195.416	P1>P3>P2>P5>P4	P5>P3>P2>P4
A195.403	P1=P2=P3=P4=P5	P2=P3=P4=P5
A195.409	P1=P2=P3=P4=P5	P2=P3=P4=P5
A195.410	P1 ³ =P2=P3=P4=P5	P2=P3=P4=P5
A70.403	P2>P5>P4>P3>P1	P2>P3>P5>P4
A70.413	P1>P2>P4>P5>P3	P3>P5>P4>P2
A70.415	P2>P4>P3=P5>P1	P3>P2>P4>P5
A70.409	P2>P5>P1>P3>P4	P2>P3>P5>P4
A70.406	P1=P2=P3=P4=P5	P2=P3=P4=P5

¹ Ranking was based on the mean numbers of embryos produced by each protocol.

² P1 was not available.

³ The somatic embryos induced in the single flask was considered an exception. The regenerating ability of A195.410 was evaluated as non.

Table 6. Main Differences in Composition between B5h and SH4K Media.

Composition		B5h medium	SH4K medium
NH ₄		>50 mM	<50 mM
Ca ²⁺	CaCl ₂ .2H ₂ O	750 mg	200 mg
Mg ²⁺	MgSO ₄ .7H ₂ O	500 mg	400 mg
SO ₄ ²⁻	K ₂ SO ₄	---	8.712 g
	MgSO ₄ .7H ₂ O	500 mg	400 mg
	MgSO ₄ .H ₂ O	10 mg	10 mg
	ZnSO ₄ .7H ₂ O	2 mg	1 mg
	CuSO ₄	0.025 mg	---
	CuSO ₄ .5H ₂ O	---	0.2 mg
	(NH ₄) ₂ SO ₄	134 mg	---
	FeSO ₄ .7H ₂ O	---	15 mg
Gelling agent	Agar	9000 mg	---
	Gelrite	---	2,500 mg
Amino acids	Glutamine	800 mg	---
	Glutathione	10 mg	---
	Serine	100 mg	
	Proline	---	2,300 mg
	Thioprolin	---	53.3 mg
Carbo-hydrate	Sucrose	30,000 mg	10,000 mg
pH		5.5	5.8

complicated. In the A195 population, it is quite clear that P2 was the most efficient, followed by P3. In the A70 population, the efficiency of P1 appeared unstable with variation in leaf tissue of all regenerable A70 plants. That is, its efficiency was the highest in the A70.413, medium in the A70.409, and the lowest in the A70.403 and A70.415 among the five culture protocols. The highest efficiency was observed in P2 in both petiole and leaf tissues of most plants except that in leaf and petiole tissues of the A70.413 and in petiole tissue of the A70.415. In both populations, the efficiency of P4 and P5 was generally lower than the other three protocols. However, no embryogenesis was observed in the non-regenerating plants of both populations with all the five protocols. Since P1 is the direct culture method, the efficiency of embryogenesis by P1 was calculated on the basis of embryo numbers/mg fresh weight of leaf tissue. It is difficult to evaluate its efficiency with the other four indirect culture protocols, for which the efficiency of embryogenesis was calculated according to embryo numbers/gram fresh weight of callus. As the result, the analysis on the effect of induction media focused on SH4K and B5h media. The medium compositions are listed in Table 6.

ii. Embryogenesis in Explants Sampled at Different Times from Stock Plants Cultured in Soil

The results of somatic embryogenesis with P2 and P3, the two three-step culture protocols with B5h and SH4K as induction medium (Table 3), are shown in Tables 7-9. The levels of somatic embryogenesis varied among replicates of each plant (Fig. 6), among

Fig. 6. Somatic embryogenesis on BOi2Y medium cultured with P2 from the leaf tissue sampled from the A195 plants cultured in soil under a 20-h photoperiod.
a: A195.406; b: A195.415; c: A195.416; d: A195.403.

the three photoperiods, among different sampling times with the same photoperiod, and between the two culture protocols. An example of the variation is A195.406. With P2, the highest embryo number induced from leaf sampled at the end of light period was from the A195 plant cultured under a 16-h photoperiod (237 ± 37), followed by a 20-h photoperiod (113 ± 23) and an 8-h photoperiod (49 ± 2); and with P3, the highest embryo number induced from leaf sampled at the same time was from the donor plant treated under an 8-h photoperiod (190 ± 9), followed by a 16-h photoperiod (153 ± 5) and a 20-h photoperiod (89 ± 17) (Table 7). Furthermore, a significant difference was observed between sampling at the end of the dark period and the end of the light period. For A195.406, embryo numbers induced from leaf collected at the end of the dark period from the donor plant cultured under 16-h and 8-h photoperiods decreased significantly, but those from the same plant cultured under a 20-h photoperiod increased significantly in comparison with those sampled at the end of the light period (Tables 7 and 8). For A195.415 and A195.416, their embryo numbers also varied remarkably in explants collected at the end of the dark period and at the end of light period (Tables 7 and 8), but were similar in the midday sampling (Table 9). Although there are great differences between the two culture protocols, among the photoperiods and among the sampling time, no embryos were induced from A195.403, the non-regenerating plant (Tables 7-9). A single regenerating trend was observed in all 9 sampling groups; *i.e.*, A195.406 was high, A195.415 medium, A195.416 low, and A195.403 non-regenerating (Tables 7-9).

As mentioned above, remarkable differences were observed in the frequency of somatic

Table 7. Somatic Embryogenesis, Endogenous Hormone Levels and their Ratios in Leaf Samples Collected at the End of Light Period from Plants Cultured in Soil.

Plant	Somatic Embryo Number/ Gram Callus (Mean±SE)		IAA (pMol/G FW) (Mean±SE)	ABA (pMol/G FW) (Mean±SE)	ZR (pMol/G FW) (Mean±SE)	IAA/ABA ($\times 10^{-3}$)	IAA/ZR
	P2	P3					
A195.406MP ¹	237±37	153±5	471.7±136.4	10080±1015	14.3±3.7	47	33
A195.415MP	11±7	11±5	338.1±140.7	2373±602	21.9±4	142	15
A195.416MP	2±1	9±5	300.6±44.4	5701±1226	20.5±0.2	53	15
A195.403MP	0	0	333.9±20.9	9027.7±794	18.7±0.3	37	18
A195.406SP	49±2	190±9	351±182.8	20758±2027	16.5±3.5	17	21
A195.415SP	2±0.2	14±1	311.6±153.7	9321±2128	15±3.7	33	21
A195.416SP	1±0.2	4±1	302±189.7	7723.9±114.4	13.9±0.9	39	22
A195.403SP	0	0	337.5±162	22115±341	17.7±4.9	15	19
A195.406LP	113±23	89±17	320.5±34.7	27929±848.5	15.8±1.8	11	20
A195.415LP	24±18	10±2	342.6±26.6	13320±1248	14.2±2	26	24
A195.416LP	9±3	2±1	312±7.8	22719±1261	15.6±1.7	14	20
A195.403LP	0	0	441.5±60.7	20322±102	13.5±1.8	22	33

¹MP: Mid-photoperiod (16/8 h day/night); SP: Short-photoperiod (8/16 h day/night); LP: Long-photoperiod (20/4 h day/night).

Table 8. Somatic Embryogenesis, Endogenous Hormone Levels and their Ratios in Leaf Samples Collected at the End of Dark Period from Plants Cultured in Soil.

Plant	Somatic Embryo Number/ Gram Callus (Mean±SE)		IAA (pMol/G FW) (Mean±SE)	ABA (pMol/G FW) (Mean±SE)	ZR (pMol/G FW) (Mean±SE)	IAA/ABA (x10 ⁻³)	IAA/ZR
	P2	P3					
A195.406MP ¹	101±42	111±24	331.7±79.8	5022.5±1167	17.9±1.3	66	19
A195.415MP	9±5	5±0.2	614.8±337.5	2423.9±593.8	17.6±0.3	254	35
A195.416MP	3±1	3±1	288.3±122.8	3119±670	16.7±3.5	92	17
A195.403MP	0	0	508±215.6	3472.7±914.2	17.6±3.1	146	29
A195.406SP	47±2	104±7	253.6±63.2	22505±988	13.3±0.9	11	19
A195.415SP	2±0.2	10±7	376.1	11709.5	16.9±1.4	32	22
A195.416SP	1±0.1	3±1	350.9±103.9	11489±685	17.9±1.8	31	20
A195.403SP	0	0	264±3.2	17593±433	15.6±4.5	15	17
A195.406LP	203±24	144±42	234±3	18110±1896	14.3±3	13	16
A195.415LP	7±2	9±5	336±51.4	10919±1448	11.5±1.1	31	29
A195.416LP	3	1±0.3	331.3±59.5	19661±2331	13.4±1.7	17	25
A195.403LP	0	0	260.2±25.1	7635±410	14.9±1.3	34	17

¹MP: Mid-photoperiod (16/8 h day/night); SP: Short-photoperiod (8/16 h day/night); LP: Long-photoperiod (20/4 h day/night).

Table 9. Somatic Embryogenesis, Endogenous Hormone Levels and their Ratios in Leaf Samples Collected at the Midday Period from Plants Cultured in Soil.

Plants	Somatic Em- bryo Number/ Gram Callus by P2 (Mean±SE)	IAA (pMol/G FW) (Mean±SE)	ABA (pMol/G FW) (Mean±SE)	ZR (pMol/G FW) (Mean±SE)	IAA/ABA ($\times 10^{-3}$)	IAA/ZR
A195.406MP ¹	196±14	194	7408±678	23.3±10.2	26	8
A195.415MP	1±0.28	249.2	5857±989	23.7±10.1	43	10
A195.416MP	1±0.27	265	3875±219	20±9.4	68	13
A195.403MP	0	195.5	5560±145	18.6±7.5	35	10
A195.406SP	25 ±11.2	237.9±27.9	21875±710	20.4±10.1	11	12
A195.415SP	1±0.4	228±56.4	12119±1029	17.5±8.3	19	13
A195.416SP	1±0.2	220.9±53.4	11378±2193	21.2±13	19	10
A195.403SP	0	214.8±8.2	24805±504	19.9±10	9	11
A195.406LP	22±2.9	269.2±55.1	24673±730	16.4±7.3	11	17
A195.415LP	5±0.3	260.5±27.6	12725±328	16±7.2	20	16
A195.416LP	2±0.4	277.2±41.9	21776±1077	16.8±8.1	13	10
A195.403LP	0	259.1±25	23708±166	19.3±8.9	11	14

¹MP: Mid-photoperiod (16/8 h day/night); SP: Short-photoperiod (8/16 h day/night); LP: Long-photoperiod (20/4 h day/night).

embryogenesis with P2 and P3 protocols (Tables 7-9). P3 regenerated more embryos than P2 from leaf tissue sampled either at the end of the dark or at the end of light periods in all regenerable plants (A195.406, A195.415 and A195.416) of the A195 population grown under an 8-h photoperiod (Tables 7 and 8). In comparison, P2 was superior to P3 in embryogenesis of the same tissue harvested at the same daytimes in all regenerable A195 plants with a 20-h photoperiod treatment (Tables 7 and 8), which was consistent with the results in all regenerable plants of the A195 population grown in MS medium (Table 5). With regard to the A195 plants cultured under a 16-h photoperiod, the efficiency of P2 and P3 varied between the sampling times and among plants. For the A195.406, P2 was superior to P3 in explants sampled at the end of the light period, but inferior to P3 in explants sampled at the end of the dark period; for the A195.415, P2 produced more embryos than P3 in explants sampled at both the ends of the light and dark periods; and for the A195.416, P3 did better than P2 in explants collected at both daytimes (Tables 7 and 8). Neither protocol could induce embryos in leaf tissue of the A195.403, a non-regenerating plant. For a convenient comparison, the differences in efficiency of P2 and P3 in somatic embryogenesis in A195 plants cultured in soil were simplified and listed in Table 10.

Table 10. Comparison of the Effects of P2 and P3 on Somatic Embryogenesis

Plant	Sampling Time of the Day	
	At the End of Light	At the End of Dark
A195.406MP ¹	P2>P3	P3>P2
A195.415MP	P2>P3	P2>P3
A195.416MP	P3>P2	P3>P2
A195.403MP	P2=P3	P2=P3
A195.406SP	P3>P2	P3>P2
A195.415SP	P3>P2	P3>P2
A195.416SP	P3>P2	P3>P2
A195.403SP	P2=P3	P2=P3
A195.406LP	P2>P3	P2>P3
A195.415LP	P2>P3	P2>P3
A195.416LP	P2>P3	P2>P3
A195.403LP	P2=P3	P2=P3

¹ MP: Mid-photoperiod (16/8 h day/night);

SP: Short-photoperiod (8/16 h day/night);

LP: Long-photoperiod (20/4 h day/night).

III. Endogenous Hormone Levels

i. Hormone Levels in Plants Cultured *in vitro*

1. DW/FW ratio of leaf and petiole samples

Freeze-dried leaves and petiole samples from A70 and A195 alfalfa populations cultured in $\frac{1}{2}$ and full-strength MS medium were used to measure the levels of endogenous IAA, ABA and cytokinins. For a comparison of endogenous hormone levels with somatic embryogenesis, the endogenous hormone levels were calculated on both a pMol/gram dry and fresh weight sample basis. The ratios of DW/FW of the plant samples are listed in Table 11. In leaf samples, the ratios in each replicate of each plant were close, for example, the ratios of DW/FW from the leaves of A195.406 were 19.84, 20.05 and 18.17. The ratios among plants of both populations varied, but not greatly, with a range from 13.63 (A70.413) to 20.84 (A70.403). In petiole samples, the ratios in each replicate of a given plant differed widely from that of leaves such as the ratios of 195.406, whose petiole DW/FW ratios were 15.41, 19.04 and 16.25. Among plants, the ratios varied more significantly than that of leaves, ranging between 10.27 (A195.410) and 19.04 (A195.406).

2. Hormone levels

The levels of endogenous IAA, ABA and cytokinins in both leaf and petiole tissue

Table 11. The DW/FW% Ratios of Leaves and Petioles from Alfalfa Plants Cultured *in vitro*

Plant	Leaf				Petiole			
	Group 1	Group 2	Group 3	Mean±SE	Group 1	Group 2	Group 3	Mean±SE
A195.406	19.84	20.05	18.17	19.4±0.6	15.41	19.04	16.25	16.9±1.1
A195.407	16.48	13.52	16.04	15.4±0.9	NA	14.69	15.25	15±0.3
A195.415	16.85	17.96	17	17.3±0.3	14.03	15.26	14.16	14.5±0.4
A195.416	13.96	14.42	15.37	14.6±0.4	12.2	10.98	13.32	12.2±0.7
A195.403	16.91	17.01	17.05	17±0.1	15.12	15.81	13.98	15±0.5
A195.409	17.67	17.08	18.06	17.6±0.3	14.54	14.72	14.77	14.7±0.1
A195.410	14.68	16.91	16.03	15.9±0.6	12.53	10.27	13.12	12±0.9
A70.403	NA	NA	20.84	20.8	NA	NA	12.66	12.7
A70.413	13.84	13.63	16.61	14.7±1	12.08	12.37	16.22	13.6±1.3
A70.415	NA	14.22	14.94	14.5±0.4	NA	11.58	12.66	12.1±0.5
A70.409	15.17	14.17	14.88	14.7±0.3	12.15	11.59	13.67	12.5±0.6
A70.406	NA	14.48	18.82	16.7±2.2	NA	13.23	18.97	16.1±2.9

analysed are listed with their respective somatic embryogenesis response, group by group, in Tables 12-15 and Appendix 3. The concentrations of hormones per gram fresh weight were calculated from the dry weight values according to the DW/FW ratio (Table 11).

1) Hormone levels in leaves

A. IAA: In both per gram dry and fresh sample contents, the IAA level varied considerably in most replicates of a given plant and among plants (Tables 12 and 13; Appendix 3). The IAA level ranged from 158 pMol (A70.406, replicate 2) to 7531 pMol (A70.413, replicate 3) per gram dry sample, and from 22.9 (A70.406, replicate 2) to 1250.9 (A70.413, replicate 3). The hormone levels, however, were very close in some plants including A195.407 and A195.409 in terms of dry weight, and A70.409, A195.416 and A195.406 in fresh weight. For example, the analysis results of the 2nd and 3rd replicates of A195.407 were 471 and 482 pMol per gram dry weight, respectively. When compared with the corresponding somatic embryogenesis by the five culture protocols on the bases of both replicate to replicate and mean value, the IAA level, both in per gram dry and fresh weight, and somatic embryogenesis was not correlated in A195 populations (Tables 12 and 13). In the A70 population, the IAA level per gram dry weight was also not correlated with somatic embryogenesis (Table 12), however, a positive correlation was observed between the IAA level in per gram fresh weight and somatic embryogenesis (Table 13). The levels of IAA were high in A70.403 and A70.413, the high-regenerating plants, followed by A70.415, A70.409 and A70.406, mid-, low- and non-regenerating plants.

Table 12. Mean of Somatic Embryos, IAA, ABA, Total Cytokinins, ZR and Ratios (pMol/G DW Leaves)

Plant	Rege- nera- tion ¹	Somatic Embryo Numbers ² (Mean±SE)					IAA (Mean±SE)	ABA (Mean±SE)	Total Cytokinins (Mean±SE)	ZR (Mean±SE)	IAA/ ABA	IAA/ Cytokinins	IAA/ ZR
		P1	P2	P3	P4	P5							
A195.406	High	31±3	57±21	26±14	8±1	19±6	616±115	2413±28	1255±87	40.7±11.8	0.26	0.49	15.1
A195.407	High	46±11	53±18	45±11	22±9	14±5	477±5.5	4331±60	1158±257	40.8±1.8	0.11	0.41	11.7
A195.415	Mid	16±6	16±5	11±4	8±6	9±1	572±104	3522±430	1067±168	45.3±9.8	0.16	0.54	12.6
A195.416	Low	20±2	10±3	12±4	6	8±1	402±44	3160±238	1166±39	49.3±13.9	0.13	0.34	8.2
A195.403	Non	0	0	0	0	0	419±42	2961±205	1126±43	62.2±28.5	0.14	0.37	6.7
A195.409	Non	0	0	0	0	0	278±24	3683±308	1084±64	44.3±10.1	0.08	0.26	6.3
A195.410	Non	0	0	0	0	0	434±100	2401±308	1319±237	158.9±84	0.18	0.33	2.7
A70.403	High	22±3	42±33	32	19±9	33±5	4157±3375	2247±524	1993±27	33.1±6.9	1.85	2.09	125.6
A70.413	High	66±10	52±14	32	47±15	34±15	610	2740	1022	41.7	0.22	0.60	14.6
A70.415	Mid	15±7	37±4	31	14	20±11	819±84	5370±222	1984±9	31.9±19.1	0.15	0.41	25.7
A70.409	Low	9	11±3	6	2	8±1	771±279	5259±427	2158±146	76.4±44.3	0.15	0.36	0.1
A70.406	Non	0	0	0	0	0	181±23	5848±348	1778±18	43.3±36.3	0.03	0.1	4.2

¹Regenerating ability;² P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Table 13. Mean Somatic Embryo Numbers, IAA, ABA, Cytokinins, ZR and Ratios (pMol/G FW Leaves)

Plant	Rege- nera- tion ¹	Somatic Embryo Numbers (Mean±SE) ²					IAA (Mean±SE)	ABA (Mean±SE)	Total Cytokinins (Mean±SE)	ZR (Mean±SE)	IAA/ ABA	IAA/ Cytokinins	IAA/ ZR
		P1	P2	P3	P4	P5							
A195.406	High	31±3	57±21	26±14	8±1	19±6	118.5±21	469.5±57	242.5±17	7.8±2.2	0.25	0.49	15.19
A195.407	High	46±11	53±18	45±20	22±9	14±5	70.5±6	640.8±63	168±23	6.4±4.6	0.11	0.42	11
A195.415	Mid	16±6	16±5	11±4	8±6	9±1	99.5±20	605.7±64	185.3±33	7.7±2	0.16	0.54	12.92
A195.416	Low	20±2	10±3	12±4	6	8±1	58.3±5	461.5±41	169.7±3	7.1±2	0.13	0.34	8.21
A195.403	Non	0	0	0	0	0	71.2±7	503.2±35	191.3±8	10.6±5	0.14	0.37	6.72
A195.409	Non	0	0	0	0	0	48.8±4	646.8±45	190.5±9	7.8±2	0.08	0.26	6.26
A195.410	Non	0	0	0	0	0	69.7±18	383.7±58	207±32	25±14	0.18	0.34	2.78
A70.403	High	22±3	42±33	32	19±9	33±5	127.1	571	213	8.7	0.22	0.6	14.61
A70.413	High	66±10	52±14	32	47±15	34±15	679±572	331.9±46	302±34	3.3±2	2.05	2.25	205.7
A70.415	Mid	15±7	37±4	31	14	20±11	120±15	783.7±52	289±9	4.9±3	0.15	0.41	24.43
A70.409	Low	9	11±3	6	2	9±1	114.8±43	777.6±78	318.8±26	11.5±7	0.15	0.36	9.98
A70.406	Non	0	0	0	0	0	30.6±8	981±185	295.5±36	8±7	0.03	0.1	3.83

¹Regenerating ability;² P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Table 14 . Mean Somatic Embryos Numbers, IAA, ABA, Cytokinins, ZR and Ratios (pMol/G DW Petiole)

Plant	Rege- nera- tion ¹	Somatic Embryo Numbers ² (Mean±SE)					IAA (Mean±SE)	ABA (Mean±SE)	Total Cytokinins (Mean±SE)	ZR (Mean±SE)	IAA/ ABA	IAA/ Cytokinins	IAA/ ZR
		P1	P2	P3	P4	P5							
A195.406	High	NA	61±2	51	13	21±4	473	623±585	2818	51.9	0.76	0.17	9.11
A195.407	High	NA	100±52	34±11	27±17	37±8	546	1480	1785	42.7	0.37	0.31	12.79
A195.415	Mid	NA	12±6	6	4±2	8±1	1563±322	6550±5260	1798.5±604	74.4±45.4	0.24	0.87	21
A195.416	Low	NA	7±3	10±3	6±2	12±5	494±256	947±31	1593±344	137.1±66	0.52	0.31	3.6
A195.403	Non	NA	0	0	0	0	1023	1437	503	36.5	0.71	2.03	28.03
A195.409	Non	NA	0	0	0	0	563±329	1775±612	2005±198	23.4±2	0.32	0.28	24.06
A195.410	Non	NA	0	0	0	0	1251±241	1969±41	1801.5±196	72.7±44.7	0.64	0.69	17.21
A70.403	High	NA	112	27±8	15±5	20±5	798	2065	2911	386.6	0.39	0.27	2.06
A70.413	High	NA	45±13	86	47±32	49	754	635	2669	189.1	1.19	0.28	3.99
A70.415	Mid	NA	29±8	36	8±2	7	653.5±62	2054±358	3111±858	98.6±71.6	0.32	0.21	6.63
A70.409	Low	NA	6±3	4±1	2	3	686±21	1809±32	3960±1755	270.4±144	0.38	0.17	2.54
A70.406	Non	NA	0	0	0	0	735	3448±336	1797.5±203	225.4±114	0.21	0.41	3.26

¹Regenerating ability;² P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Table 15. Mean Somatic Embryo Numbers, IAA, ABA, Total Cytokinins, ZR, and their Ratios (pMol/G FW Petiole)

Plant	Rege- nera- tion ¹	Somatic Embryo Numbers ² (Mean±SE)					IAA (Mean±SE)	ABA (Mean±SE)	Total Cytokinins (Mean±SE)	ZR (Mean±SE)	IAA/ ABA	IAA/ Cytokinins	IAA/ ZR
		P1	P2	P3	P4	P5							
A195.406	High	NA	62±2	51	13	21±4	76.9	458.3±262	457.9	8.4	0.17	0.17	9.15
A195.407	High	NA	100±52	34±101	27±17	37±8	83.3	225.7	272.2	6.5	0.37	0.31	12.82
A195.415	Mid	NA	12±6	6	4±2	8±1	228.1±39	992±809.8	261.3±78.9	10.7±6.3	0.23	0.87	21.32
A195.416	Low	NA	7±3	10±3	7±2	12±5	57.1±25.4	114.7±7.3	189.6±23.2	17.5±9.7	0.75	0.3	3.26
A195.403	Non	NA	0	0	0	0	143	200.9	70.3	5.1	0.71	2.03	32.5
A195.409	Non	NA	0	0	0	0	82.2±47.6	29.9±71.9	294.1±28	3.4	0.32	0.28	24.18
A195.410	Non	NA	0	0	0	0	142.9±11	229.7±23	207.9±2.8	9.2±/-6.3	0.62	0.69	15.53
A70.403	High	NA	112	27±8	15±5	20±5	101	261.4	368.5	48.9	0.39	0.27	2.07
A70.413	High	NA	45±13	86	47±32	49	122.3	103	472.2	30.7	1.19	0.26	3.98
A70.415	Mid	NA	29±8	36	8±2	7	79.6±11	247±32.3	381.7±121	12.3±9.2	0.32	0.21	6.47
A70.409	Low	NA	6±2	4±1	2	3	86.9±9.8	228.1±14.8	518.4±263	35.7±21	0.38	0.17	2.43
A70.406	Non	NA	0	0	0	0	139.4	545.4±45	295.2±84.2	39.6±25	0.26	0.47	3.52

¹ Regenerating ability;² P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

B. ABA: As with the IAA levels, the ABA levels in both populations varied from replicate to replicate and among plants in both per gram dry and fresh weight (Tables 12 and 13; Appendix 3). With regard to each replicate, the ABA levels ranged from 1723 pMol (A70.413) to 6195 pMol (A70.406) per gram dry weight, and between 271.4 pMol (A195.410) and 1165.9 pMol (A70.406) per gram fresh weight. In terms of mean value, the ABA levels ran from 2413 pMol (A195.406) to 5848 pMol (A70.406) per gram dry weight, and between 331.9 pMol (A70.413) and 981.2 pMol (A70.406) per gram fresh weight (Tables 12 and 13). With respect to somatic embryogenesis with the five culture protocols and ABA levels both per gram dry and fresh weight, there was no correlations observed between the regenerating trend and ABA levels in the A195 population, but a general negative correlation between them in A70 populations (Tables 12 and 13). The ABA levels were the lowest in A70.403 and A70.413 (high regeneration), the highest in A70.406 (non-regeneration) with A70.415 (mid regeneration) and A70.409 (low regeneration) between them without significant difference.

C. Cytokinins: In contrast to IAA and ABA levels, the levels of total cytokinins were generally relatively stable among replicates in most plants of both populations, except that the levels of this group of hormones were lower in the third replicate analysis of A195.407, A195.415 and A195.410 and A70.413, and in the first replicate analysis of A70.409 (Tables 12, 13; Appendix 3). Compared with the corresponding IAA and ABA levels of each plant, cytokinins ranked in the middle in terms of pMol per gram dry and fresh weight, with mean values ranging from 1022 pMol (A70.413) to 2158 pMol (A70.409) in dry weight value, and

between 168 pMol (A195.407) and 318.8 pMol (A70.409) per gram fresh weight (Tables 12 and 13). With regard to various forms of cytokinins, they differed greatly among replicates and plants of both populations, although the level of total cytokinins remained stable in each replicate and close among plants of both populations (Tables 12 and 13; Appendix 3).

2) Hormone levels in petioles

Since the amount of petiole material was less than leaf samples in each collection, the analysis of endogenous hormone levels was performed with 1-3 replicates. The primary results were as follows:

A. IAA: As in leaf tissues, IAA levels varied among replicates and plants, ranging between 234 pMol (A195.409) and 1884 pMol (A195.415) per gram dry weight, and from 31.7 pMol (A195.416) to 879.2 pMol (A70.415) per gram fresh weight (Tables 14 and 15; Appendix 3). With respect to the efficiency of somatic embryogenesis with the four culture protocols, the IAA levels were not correlated with the regenerating trend in both A70 and A195 populations, and this was most obvious in the A195 population. The IAA level was about twice as high in the A195.403 and A195.410 (non-regeneration) than that in the A195.406 (high-regeneration) plant (Tables 14 and 15). The IAA level in petiole samples was higher than that in leaf samples in all plants with exception of A195.406, A70.403, A70.415 and A70.409 on the basis of dry weight (Table 14). In contrast, the hormone levels in leaf samples were higher in A195.406 and A195.416, and all A70 plants except A70.406 on a per

gram fresh weight basis (Table 15).

B. ABA: As in the leaf samples, the ABA level in petioles fluctuated among replicates and plants, and ranged between 635 pMol (A70.413) and 3783 pMol (A195.406 and A70.406), and between 72 pMol (A195.406) and 590 pMol (A70.406) in terms of per gram dry and fresh weight, respectively (Tables 14 and 15; Appendix 3). There was no correlation observed between the ABA level and the regeneration trend in both A70 and A195 populations (Tables 14, 15). The ABA level in petiole samples was lower than that observed in leaf samples in all plants of both populations except A195.415 (Tables 12-15).

C. Cytokinins: This group of hormones fluctuated considerably among replicates and among plants of both populations. The level of total cytokinin ranged from 503 pMol and 70.3 pMol (A195.403) to 5714 pMol and 781.1 pMol (A70.409) on the bases of per gram dry and fresh weight, respectively (Tables 14 and 15; Appendix 3). The total cytokinin content was higher in petioles than that in leaves on a dry weight basis comparison except for A195.403, which showed the opposite result. In fresh weight concentration comparisons, the total cytokinin level in petiole tissue was still low in A195.403, and similar in A195.410 and A70.406, and high in the rest of the plants. Compared with the corresponding mean IAA and ABA levels, the total cytokinins level was the highest in all plants except A195.415 (medium regenerating ability), A195.403 and 195.410, and A70.406 (non-regenerating ability) plants. As in leaf tissues, each form of cytokinin fluctuated among replicates and plants (Tables 14 and 15; Appendix 3).

3. Ratios of endogenous hormone levels

The ratios of mean values of IAA to ABA and total cytokinins were calculated and compared with regeneration trends in petiole and leaf tissues of both A70 and A195 populations. In the leaf tissue of the A70 populations, there was a distinct positive correlation of the ratio IAA/cytokinins with the regenerating abilities; *i.e.*, the ratio increased along with the increase in regeneration ability, and a general positive correlation was also observed between the ratio of IAA/ABA and regeneration abilities (Tables 12 and 13). These correlations were not documented in petiole tissue from the A70 population and in neither petiole nor leaf tissue of the A195 plants. The analysis of the correlations of each form of cytokinin with the regeneration ability showed that the ratios of IAA/ZR in leaf tissue were more than 8.2 in regenerating plants, and less than 6.7 in non-regenerating plants of both populations (Tables 12 and 13); however, this correlation was not observed in petiole tissue (Tables 14 and 15).

The ratios of hormone levels were further analyzed statistically and the results are shown in Appendix 4. There was no significant correlations among the hormone levels.

ii. Hormone Levels in Plants Cultured in Soil

1. Hormone levels

1) IAA: The IAA levels in all replicates and in all plants of three groups with various

photoperiod treatments and sampled at different daytimes fluctuated and were higher than that in leaf and petiole tissues of plants cultured in MS medium (Tables 7-9, 12-15). The mean value of the IAA level ranged from 300.6 pMol (A195.416MP) to 471.7 pMol (A195.406MP), 234 pMol (A195.406LP) to 614.8 pMol (A195.415MP), and from 193.6 pMol (A195.406MP) to 277.2 pMol (A195.416LP) per gram fresh weight collected at the end of light, dark and at the midday, respectively (Tables 7-9). Generally, the IAA level was lower but varied more significantly in samples harvested at midday, and was the most stable in samples collected at the end of the light period (Tables 7-9). With respect to somatic embryogenesis, a positive correlation was observed between the IAA level and the regeneration trend; *i.e.*, the IAA level ranged from high to low in A195.406, A195.415, A195.416 and A195.403, plants with high-, medium-, low- and non-regeneration abilities, respectively, in the group of samples collected at midday from stock plants cultured under an 8-h photoperiod (Table 9). There was a similar trend of IAA level in two groups of samples that were sampled at the end of the dark period from plants treated with 8-h and 20-h photoperiod (Table 8). In this pair of groups, the IAA level ran from high to low in A195.415, A195.416, A195.403 and A195.406. The ranking of the IAA level was the same in the group sampled at the end of the light period from plants cultured under a 16-h photoperiod and those in leaf tissue of corresponding plants cultured in MS medium, that is, the IAA ranged from high to low in 195.406, A195.415, A195.403 and A195.416 plants (Tables 7, 12, 13).

2). ABA: The ABA level fluctuated and varied significantly in all groups of samples, and

was extremely high compared to the level in leaves of plants cultured on MS medium (Tables 7-9, 12, 13). The ABA level ranged between 2373 pMol (A195.415MP) and 27929 pMol (A195.406LP), 2423.9 pMol (A195.415MP) and 19661 pMol (A196.416LP), and from 3875 pMol (A195.416MP) to 24805 pMol (A195.403SP) in samples collected at the end of light and dark periods, and at the midday period, respectively (Tables 7-9). Unlike the IAA level, the ABA level was not correlated with the regeneration trend in all groups (Tables 7-9). The levels of the hormone, however, ranked in the same order in two groups of samples, one was from high to low in A195.406, A195.403, A195.416 and A195.415 in the three groups of samples collected at the end of light and dark periods from plants under the 16-h photoperiod treatment, and at midday from plants cultured under a 20-h photoperiod. In the other case, the order ran from high to low in A195.403, A195.406, A195.415 and A195.416 in the two groups of samples harvested at the end of the light period and at midday from plants cultured under an 8-h photoperiod (Tables 7-9). There was no similar conclusion of ABA levels between any group of plants cultured in soil and those cultured in MS medium (Tables 7-9, 12, 13).

3). ZR: As was observed with ABA and IAA levels, the ZR level also varied among replicates and among plants of all groups, and was the lowest concentrations but had the most stable values, ranging from 13.5 pMol (A195.403LP) to 21.9 pMol (A195.415MP), 11.5 pMol (A195.415LP) to 17.9 pMol (A195.406MP and A195.416SP), and 16 pMol (A195.415LP) to 23.7 pMol (A195.415MP) per gram fresh weight in groups collected at the end of the light period, the end of the dark period, and at midday (Tables 7-9). In contrast

to the trends observed for ABA and IAA, ZR was not correlated with the regeneration trend in all groups, and there was no consistency in the order of ZR levels among the groups of samples (Tables 7-9). The ZR levels, however, ranked in the same order in the group of samples collected at the end of the light period from plants cultured under an 8-h photoperiod with those cultured in MS medium. In these two groups, the ZR levels ranked from high to low in A195.403, A195.406, A195.415 and A195.416 (Tables 7, 12-13).

2. The ratios of hormone levels

Based on the calculated mean values of IAA, ABA and ZR, the ratios of IAA/ABA and IAA/ZR were calculated to determine if there was a correlation with the plant regeneration abilities (Tables 7-9). In leaf tissue of plants cultured in MS medium, the ratio of IAA/ABA was very low due to the extremely high level of ABA, and the ratio of IAA/ZR was high owing to the high level of IAA (Tables 7-9). No correlation was observed between any of the ratios and the regeneration trend in all groups. The ratio of IAA/ABA ranked in the same way in two groups of samples collected at the end of the light period from plants with a 20-h photoperiod treatment, and at the end of the dark period harvested from plants cultured under a 16-h photoperiod (Tables 7 and 8). In these two groups, the ratio of IAA/ABA ranked from high to low in A195.415, A195.403, A195.416 and A195.406 (Tables 7 and 8).

3. The recovery rates of hormones: The recovery rates of IAA, ABA and ZR were 51%, $70 \pm 8.8\%$ and $62 \pm 5.4\%$, respectively.

DISCUSSION

I. Somatic Embryogenesis and Regenerating Trend are Determined by Genotype, and Modulated by the Culture Protocol, Explant Source, and Physiological State of Donor Plants

The results of somatic embryogenesis of the A70 and A195 populations with five culture protocols confirmed that genotype is the controlling factor in somatic embryogenesis of alfalfa. Since the first report of somatic embryogenesis with alfalfa 25 years ago (Saunders and Bingham, 1972), this plant has been used successfully in many studies in somatic embryogenesis, including development of culture methods (*e.g.*, Denchev *et al.*, 1990; Meijer and Brown, 1987), culture medium (Shetty and McKersie, 1993), and elucidation the role of genotype (Brown and Atanassov, 1985; Chen *et al.*, 1987). It has been established that genotype is the controlling factor among various factors that affect somatic embryogenesis (Bingham *et al.*, 1988; Brown and Atanassov, 1985; Meijer and Brown, 1985; Sugimoto *et al.*, 1991; Thorpe, 1995). The genotype determines not only the frequency of somatic embryogenesis but also the callus production (Brown and Atanassov, 1987; Sugimoto *et al.*, 1991; Takamizo *et al.*, 1990). Reisch and Bingham (1980) first reported that two dominant genes controlled bud differentiation from callus of diploid alfalfa germplasm. Later, Wan *et al.* (1988) published their conclusions that somatic embryogenesis in tetraploid alfalfa was also controlled by two dominant complementary genes. These

conclusions were followed by confirmations from experiments with various genotypes of alfalfa from other labs (Crea *et al.*, 1995; Hernandez-Fernandez and Christie, 1989; Kielly and Bowley, 1992; Yu and Paul, 1993). It is also accepted that callus formation was controlled by one dominant gene (Hernandez-Fernandez and Christie 1989). Both callus formation and somatic embryogenesis is genotype specific and a highly heritable trait (Bingham *et al.*, 1988). The results of the somatic embryogenesis from the A70 and A195 populations in the present study confirmed the effects of genotype on callus formation and somatic embryogenesis. Remarkable observations were that there were no embryos produced in the non-regenerating plants, and the regenerating trend remained the same although the embryogenesis level varied among the regenerating plants of both A70 and A195 populations and among the five culture protocols in the present study (Tables 4, 7-9). According to Kielly and Bowley (1992), the non-regenerating plants do not carry dominant regenerating genes. As the result, somatic embryogenesis cannot be induced in these plants; *i.e.*, A195.403, A195.409, A195.410 and A70.406. However, this conclusion is not completely supported by the results in the present study because somatic embryos were induced with P1 in one replicate from leaf tissue of A195.410, a non-regenerating plant documented with P2 (Table 4). This rare case is perplexing (and not reproducible) but interesting because it suggests that A195.410 may be not an absolute non-regenerating plant. On the basis of cross results between A70.34, a highly embryogenic genotype selected from the cultivar Rangelander by Dr. Brown, Agriculture and Agri-Food Canada, and R3, a non-embryogenic plant, Hernandez-Fernandez and Christie (1989) suggested that somatic embryogenesis in alfalfa is controlled by two complementary loci, *A* and *B*, with additive

effects within each locus. The minimum number of alleles for embryogenesis in the proposed genotypes is either one *A* allele and two *B* alleles (*A*—*BB*—); or three *A* alleles and one *B* (*AAA*-*B*—). The genotypes for A70.34 and R3 were speculated to be *AAaa BBbb* (double duplex) and *aaaa Bbbb* (nulliplex), respectively. They proposed the genotypes for their offsprings were: *AAA- BB—*, *AAaa BBBB* (high- regeneration),; *AAAA Bbbb*, *AAaa BB-b*, *Aaaa BBB-* (mid-regeneration); *AAAa Bbbb*, *Aaaa BBbb* (low-regeneration); *AAA- bbbb*, *AAaa Bbbb* (abnormal-regeneration); and *A-aa bbbb*, *aaaa —* (non-regeneration). Since the A195 population is the results of a cross between A70.34 and a non-regenerating alfalfa plant, the genotypes of the A195 plants may be classified as the above. A195.410 may be in the class of abnormal regeneration. The product of embryos in this plant demonstrates that a “non-regenerating” plant documented under certain conditions, especially culture medium, can change into a “regenerating” plant when both internal and external conditions are suitable for regeneration. These types of observations support the concept of totipotency; *i.e.*, with the proper conditions any plant cell is capable of complete regeneration.

The effects of genotype on the regenerating trend of plants was shown in the present study. One distinct regenerating trend was documented from both leaf and petiole donor explants by all five culture protocols in the A70 population, and from leaf tissue from the A195 plants cultured in soil (Tables 4, 7-9). In the interactions of genotype and culture medium, the former is the decisive factor. The culture media and protocols can change the regenerating abilities, but cannot reverse the regenerating trend in the A70 and A195 populations. However, the effects of culture media and protocols may become significant

when the genetic background of plants are narrow. Two regenerating trends were shown in the A195 plants cultured in MS medium (Table 4), which are assumed to be the results of several factors. First, the genotypic backgrounds of the A195 population are very close, as these plants are the progeny of a single one-way cross. Their embryogenesis response varied even in the same protocol (Table 4). There was no clear difference in the somatic embryo numbers in each replicate among regenerating plants. Secondly, different culture protocols resulted in variation in somatic embryogenesis. Their effects were minor when the plants of different regenerating abilities have a wider genotype background, such as in the A70 population. However, the effects of different protocols appear to become significant when the genotypic background is narrow such as in the A195 plants. An interaction between genotype x medium was responsible for the change in rank for A195.416 and A195.415 regenerating abilities. Finally, the physiological state of stock plants at sampling may also contribute to the reversed rank between A195.415 and 416 regenerating abilities. Like the effect of medium, the effect of physiological state is not remarkable in somatic embryogenesis from plants with wide genetic backgrounds, but is significant in those plants with a close genetic backgrounds. As shown in Tables 7-9, somatic embryogenesis varied among explants that were sampled at different day times from plants cultured in soil and under various photoperiods. However, the same regenerating trend was documented by both P2 and P3 in all groups, which was consistent with the trend documented with P2 in the A195 population cultured in MS medium (Table 4). It is noteworthy that the embryo production was similar between the explants from A195.415 and A195.416 that were cultured in soil under an 8-h and a 16-h photoperiods (Table 9). As far as we know, this

experiment is the first assessment for the effects of genotype and culture medium and protocols on regenerating trend, the relations of regenerating trend with genotype and culture medium deserves further exploration in future.

The results of somatic embryogenesis in leaf and petiole tissues from both A70 and A195 populations cultured *in vitro* showed that explant sources affect somatic embryogenesis but are not a determining factor. In theory, somatic embryos can be induced from any somatic cells in any tissue of plants, because they contain the entire set of genetic information necessary to develop a whole plant. Each tissue and cell of a plant should carry the same type of regenerating gene. Study results have showed that somatic embryogenesis can be induced in almost all tissues of plants (Brown *et al.*, 1995; Dunstan *et al.*, 1995; Krishnaraj and Vasil, 1995). As one of the better plants for studies on somatic embryogenesis, many tissues such as leaf, petiole, cotyledon, shoot apex, hypocotyl, root and stem can be used to induce somatic embryogenesis in alfalfa (Brown *et al.*, 1995). Somatic embryos are formed from specific cell types in different tissues. The epidermis or subepidermis or both seem to be the origin of somatic embryos in immature cotyledon explants (Barwale *et al.*, 1988), immature embryo hypocotyls (Maheswaran and Williams, 1985) and petioles (Wenzel and Brown, 1991). The somatic embryos seem to be induced from epidermis or mesophyll cells or both in leaf explants (Wernicke and Brettell, 1980). The variations in the origin of somatic embryos, in differentiation and development states among different tissues and cells in a plant may result in differences in the frequency of somatic embryogenesis. Several labs have performed the comparison on the efficiency of

somatic embryogenesis among various explant sources including leaf, cotyledon, hypocotyl, root and shoot apex (Brown and Atanassov, 1985; Chen and Marowitch, 1987; Levi and Sink, 1991; Suginobu *et al.*, 1991; Takamizo *et al.*, 1991; Yun *et al.*, 1989). It was reported that both callus formation and somatic embryos varied among explants sources. Cotyledons had a tendency to produce more callus than hypocotyls and shoot apex (Brown and Atanassov, 1985; Yun *et al.*, 1989), followed by petioles and root tips (Suginobu *et al.*, 1991). The highest embryo production was observed in leaf, followed by hypocotyl, cotyledon, petiole and shoot tip and ovary (Brown and Atanassov, 1985). It should be pointed out that callus formation and embryo production vary among plant genotypes. With different cultivars of alfalfa, there may be no significant differences among these explant sources (Suginobu *et al.*, 1991). By contrast, significant differences in both callus production and frequencies of embryo formation were observed among cultivars of alfalfa (Suginobu *et al.*, 1991). In order to perform a convenient comparison of endogenous hormone levels, the present study took leaves and petioles as explant sources for analysis of somatic embryogenesis and endogenous hormone levels. The results showed that the callus production was similar in both leaf and petiole tissues from a given plant, which was consistent with those reported (Suginobu *et al.*, 1991). Noteworthy is the difference in frequency of somatic embryogenesis between the two explant sources. For example, in A70.415, P5 induced 19.7 ± 10.7 and 7 embryos in leaf and petiole tissues, respectively (Tables 12-15). Even so, the regenerating trend remained the same in both explant sources in the A70 population; *i.e.*, A70.403 and A70.413 are high, A70.415 mid, A70.409 low and A70.406 non-regenerating (Tables 12-15; Appendix 4). In the A195 population, however,

in addition to the variation in somatic embryogenesis between petiole and leaf sources, a reversed trend in regeneration between A195.415 and A195.416 was observed; that is, there was a higher embryo production in petiole tissue with P3, P4 and P5 in A195.416 than in A195.415 (Tables 14 and 15). This may be due to the embryogenesis variation in each replicate. It is noteworthy that the rank for high and non-regenerating A195 plants did not change (Tables 12-15). The results of present study indicate that although somatic embryo cells originate from different cell sources in leaf and petiole tissues, and the frequency of somatic embryogenesis varied between the two explants, the trend in regeneration of a given population will remain the same. It is concluded that explant source affects somatic embryogenesis, but is not a decisive factor. This conclusion should be tested further with more explant sources.

The results of somatic embryogenesis in leaf tissue sampled from the A195 plants cultured in soil demonstrate that the physiological state of explants is another important internal factor affecting somatic embryogenesis (Tables 7-9). These explants varied in that they were sampled at the end of night-dark period, at the end of day-light period and at the midday period from donor plants cultured under various photoperiods. The results indicate that the optimal photoperiod for alfalfa growth and somatic embryogenesis was 16-h, followed by 20-h and 8-h photoperiods. The regenerating abilities were lower at the end of dark than at the end of light for both 8-h and 16-h photoperiod cultures, and decreased at the end of light compared to that at the end of dark for the 20-h cultures (Tables 7 and 8). These results suggest that long daylight (>16 h) and long dark (>8 h) photoperiods repress somatic

embryogenesis. Although somatic embryos varied, all 9 sample groups showed the same trend in regeneration; *i.e.*, A195.406 is high, A195.415 medium, A195.416 low, and A195.403 non-regenerating (Tables 7-9). These results demonstrate that photoperiod and sampling time influence somatic embryogenesis, but can not change the non-regenerating state of A195.403, and did not affect the trend in regeneration of the A195 plants, suggesting that they are modulating factors and exert their effects through genotype. It is not clear how light affects somatic embryogenesis. As we know it, light is indispensable to normal plant growth, both because it provides energy for photosynthesis and because it provides many of the environmental signals that regulate plant development. Light interacts with endogenous developmental programs to influence metabolism and structure of plant cells, including the changes in DNA expression (Quail, 1991; Raj and Chinoy, 1980), protein synthesis (Wang *et al.*, 1991), mitochondria number (Gukasyan *et al.*, 1994), H⁺ATPase activity (Bellamine *et al.*, 1994) and length and number of epidermal cells (Nishizawa, 1994). The reaction of gene expression to light is divided into positive (*e.g.*, nitrate reductase), negative (*e.g.*, calmodulin and β -tubulin), or just fluctuating following a diurnal or circadian rhythm (*e.g.* α -tubulin and heat shock proteins) (Quail, 1991; Thompson, 1991); and light may affect translation, protein folding and transport, post-translational modification, association with cofactors, and protein degradation such as Chl a/b-binding proteins (CAB proteins) (Thompson, 1991). Light may affect the acquisition of competence and dedifferentiation of plant cells for somatic embryogenesis through some unknown mechanism on the regeneration-controlling gene and other regeneration-related genes, and/or through its influence on cell organelles, cytoskeleton, H⁺ATPase activity and cell wall. Even though

there is no evidence in this aspect, some studies on the effect of light on somatic embryogenesis favour this idea. Aleith and Richter (1991b) reported that the gene DC9.1 was rapidly and transiently expressed in somatic embryos after blue-light irradiation. This gene encodes a glycine-rich protein which was postulated to play some role in somatic embryogenesis (Aleith and Richter, 1991a). Further study in this aspect may elucidate the relationship between the light and regenerating ability.

The results of somatic embryogenesis with the five culture protocols showed that the protocol affects somatic embryogenesis, and its efficiency varies among explant sources and the physiological state of donor plants. Although medium and protocols change regenerating ability of regenerating plants, they did not affect the non-regenerating plants and trend in regeneration of the A70 and A195 population (Tables 4, 7-9). In the genotype x medium interaction, genotype is the determining factor, and medium plays a modulating role. This conclusion is consistent with those reported before (Chen *et al.*, 1986; Suginobu *et al.*, 1991; Yun *et al.*, 1989). Culture protocols are an important external factor in somatic embryogenesis. A success in induction of somatic embryogenesis from a plant usually is the result of optimization in the composition of culture medium and protocol. In somatic embryogenesis in alfalfa, both direct and indirect culture methods, and two- and three-step indirect culture protocols have been established (Denchev *et al.*, 1991, 1992; Meijer and Brown, 1987; Walker *et al.*, 1978, 1979). The frequency of somatic embryogenesis was reported to be dependent upon not only the induction medium and protocol but also the genotype of the donor plant (Brown and Atanassov, 1985; Chen *et al.*, 1987; Suginobu *et al.*,

1991; Takamizo *et al.*, 1991). Generally speaking, a two-step protocol produces less somatic embryos than the three-step protocol with the same induction medium, but may produce the similar or higher embryos than the three-step protocol in some genotypes such as Rangeland and Heinrichs (Brown and Atanassov, 1985; Chen *et al.*, 1987). The variation in somatic embryogenesis with different protocols was further shown in such aspect that one protocol could induce somatic embryos but others could not in the same genotypes such as *Citation* (Brown and Atanassov, 1985). The direct culture protocol for alfalfa was developed recently (Denchev *et al.*, 1991, 1992) and there is no comparison between this direct and indirect culture methods. To test whether differences in somatic embryogenesis are significant enough to change the regenerating trends, five protocols were used in the present study. The results of the present experiment indicate that all five protocols varied in their efficiency in somatic embryogenesis, but they documented the same trend in regeneration in both A70 and A195 populations, and none of them induced embryos in the non-regenerating plants A70.406, A195.403, A195.409 and A195.410, with one exception that embryos were produced in one replicate of A195.410 with the direct culture method (P1), which deserves further exploration. Noteworthy is that three-step protocols were more efficient but required a longer time for somatic embryogenesis than the two-step protocols (Table 4). The higher efficiency with three-step protocols may be a result from the liquid environment in B5M medium that provided an increased access to nutrients and growth regulators. In contrast, the direct transfer of embryos in callus on the induction medium to the solid BOi2Y medium does not provide an equal opportunity for all embryos to take up nutrients. This outcome favours the suggestion that two-step protocols be used in studies

such as evaluation of regenerating ability and three-step protocols in synseed production, because the former is quicker and the latter more productive (McKersie and Brown, 1996; Meijer and Brown, 1987; Takamizo *et al.*, 1991).

The results showed that the efficiency of callus production and somatic embryogenesis varied between the two induction media, B5h and SH4K (Tables 4, 7-9). SH4K induced more callus than B5h medium in the A70 plants (Fig. 3), but a similar amount (size) of callus induced by both media was observed in the A195 population (data not shown). Furthermore, both media showed a difference in callus quality (Fig. 3). The variation in somatic embryogenesis between the two media differed among the plants, photoperiods and sampling time (Tables 4, 7-9). Although both induction media have such differences, they did not change the regenerating trend of the plants. These results demonstrate that induction media affect callus production and somatic embryogenesis as a modulating factor in combination with genotype, explant physiological state and sampling time. The variation in callus formation and embryo production among induction media have been reported by several labs (Brown and Atanassov, 1985; Chen *et al.*, 1987; Sugimoto *et al.*, 1991; Yun *et al.*, 1989). However, there was no systematic comparison on the trend in regeneration among induction media. Considering that SH4K (Shetty and McKersie, 1993) and B5h (Meijer and Brown, 1984) media are very efficient and the most common induction media used in somatic embryogenesis in alfalfa, and were not compared in their efficiency in somatic embryogenesis (Chen and Marowitch, 1987; Sugimoto *et al.*, 1991; Takamizo *et al.*, 1991; Yun *et al.*, 1989), the present study provides a more systematic comparison and

thus provides reliable information.

SH and B5 media are basically the same in composition (Yun *et al.*, 1989), and SH4K and B5h were modified from them, respectively (Meijer and Brown, 1984; Shetty and McKersie, 1993). A comparison between them indicates that many elements may be responsible for their different effects in callus and embryo production, including the concentrations of nitrogen, macro- and micronutrients, gelling agents, amino acids, pH, and carbohydrate (Table 6). Overall, many factors may contribute to the difference in callus induction and embryogenesis frequency between B5h and SH4K media. Each of them appears to be important. However, it is difficult to simply conclude that a higher embryogenesis can be achieved by changing one or several factors, because a complicated interaction may exist among the components in medium. A systematic evaluation of these aspects is required.

II. The Levels and Ratios of Endogenous Growth Regulators Are Not Correlated with the Trend in Regeneration

The analysis of endogenous IAA, ABA and cytokinins in leaf and petiole tissues from the A70 and A195 populations cultured *in vitro* showed that the correlations between the endogenous hormone levels and trend in regeneration is a random phenomenon. As shown in Tables 12-15, only the IAA level in leaf tissue of the A70 population was distinctly correlated with the regenerating trend of the plants; *i.e.*, high in A70.403 and A70.413, mid in A70.415, low in A70.409 and the lowest in A70.406. However, this correlation was not observed in the petiole tissue of the A70 population and none of the leaf and petiole tissues of the A195 population. These results are attributed to the role of genotype and the distribution of endogenous hormones in plant tissues. The variation in genotype that determines the endogenous hormones may be dependent upon the genetic backgrounds. In the A195 plants, the genetic backgrounds are very narrow, and the genes that determine endogenous growth regulators may be widely separated from the genes that control the regenerating ability. That would result in the non-correlation between the endogenous hormone levels and regenerating abilities. In the A70 populations, the genetic backgrounds may be wide enough to keep the loci of IAA and the regenerating gene together, so that a correlation between them was observed. However, the genetic backgrounds of the A70 plants are still wide enough to separate ABA and cytokinin genes from the regenerating genes to the degree that they were not correlated. If the genetic backgrounds were much wider, the correlations between the regenerating trend and ABA and cytokinins might be

observed.

The levels of the endogenous growth regulators varied among the plants and fluctuated between dark and light and among photoperiods (Tables 7-9). For example, under a 16-h photoperiod, the IAA level in A195.406, A195.415 and A195.403 was higher at the end of the light period than at the end of the dark period, but the hormone level showed a reversal in A195.416. Under a 8-h photoperiod, A195.406 and A195.403 had their endogenous IAA levels increased at the end of the light period, but A195.415 and A195.416 showed a decrease in the IAA levels at the end of the light period. Varying reactions of plants to different photoperiods in terms of endogenous hormone levels have also been observed in other plants such as soybean, chrysanthemum, tobacco and *Betula pubescens* (Bhardwaj and Yooder, 1987; El-Antably *et al.*, 1991; Lozhnikova *et al.*, 1990; Rinne *et al.*, 1994). Noteworthy is the extremely high levels of ABA, which may have resulted from the photoperiod and temperature stress. Besides light, temperature affects plant hormone levels, especially ABA. In the present study, plants cultured in growth chambers were under a constant 25°C. This temperature is suitable for plant growth at daytime, but not optimum at night, since a difference of approximately 10°C between daytime and night is required for optimal growth of plants (Hopkins, 1995). A temperature of 25°C at night may be a stress condition for alfalfa plants, resulting in water evaporation, accumulation of toxic substrates of some enzymes, and injuries to cellular structure (McMenzie *et al.*, 1988). As a protective reaction, the levels of ABA increased to prevent high temperature damage due to water loss. Morphological events triggered are closure of stoma and rolling of leaves along their long

axis (Hopkins, 1995; Raven *et al.*, 1992). High levels of ABA induced by high temperature have also been reported in other plants (Eze *et al.*, 1983; Rinne *et al.*, 1994). These results suggest that light and photoperiod are not the key factor affecting endogenous hormone levels.

The levels of the endogenous growth regulators in leaf tissue from the A195 plants cultured in soil further showed that they are not correlated with the trend in regeneration. Comparison of the regenerating trend and the endogenous IAA, ABA and zeatin riboside levels from 9 groups of leaf tissue sampled at various times of the day from A195 plants cultured in soil under various photoperiods showed that there was only one positive correlation between IAA and the regenerating trend in leaf tissue that was sampled at midday from the plants cultured under a 8-h photoperiod (Table 9). The ratios of IAA/ABA and IAA/ZR were also not correlated with the regenerating trend (Tables 7-9). Photoperiods resulted in variation in the endogenous hormone levels, but the regenerating trend of the A195 plants remained the same. These results support the conclusion drawn from the *in vitro*-cultured A70 and A195 populations that the correlation between endogenous hormone levels and regenerating trend is a random phenomenon. These results favour the opinion that the IAA and ABA levels are not a key factor in determining regenerating ability, and genes controlling their levels are possibly not located on the same loci with the regeneration-controlling gene. The correlations between the endogenous hormone levels and regenerating ability reported by others in literature (Danin *et al.*, 1993; Ivanova *et al.*, 1994; Michalczyk *et al.*, 1992; Rajasekaran *et al.*, 1987; Takeno *et al.*, 1983) is considered to be a random

correlation which is applicable only in some specific plants and cannot be extended to a general plant population.

The conclusion of this study on the relationship between endogenous hormone levels and regenerating trend is different than those reported previously. The role of endogenous growth regulators has drawn more attention in recent years and were reported to be correlated with regenerating ability of plants (Danin *et al.*, 1993; Hu *et al.*, 1990; Ivanova *et al.*, 1994; Michalczuk *et al.*, 1992; Rajasekaran *et al.*, 1987; Takeno *et al.*, 1983). It was found that application of IAA inhibitors would block normal somatic embryo maturation (Fujimura and Komamine, 1979; Rajasekaran *et al.*, 1987; Schiavone and Cooke, 1987). Endogenous ABA is indispensable to induction of somatic embryos (Rajasekaran *et al.*, 1987). It is essential to apply exogenous growth regulators in tissue culture to induce somatic embryogenesis in most plants. The expression of many embryo-associated genes are found to be related to the application of growth regulators (McKersie and Brown, 1997; Thorpe, 1995). All these facts impressed researchers that hormone involvement in tissue culture competence is of major importance, and the genetic basis of tissue culturability could therefore depend upon genes involved in plant hormone metabolism (Henry *et al.*, 1994). A hypothesis has been put forward that "genes involved in the phytohormone signals are involved in plant regeneration" (Henry *et al.*, 1994). On the basis of analysis of endogenous IAA, ABA and cytokinins levels in three alfalfa lines (high, mid and non-regeneration), Ivanova and her colleagues hypothesized that "the levels of endogenous hormones may be correlated to the specific response of different genotypes to embryo-inducing conditions" (Ivanova *et al.*, 1994).

Unfortunately, the hypothesis proposed by Henry and his colleagues (1994) was based on the report of Mathias and Fukui (1986) that modification of the cellular hormonal metabolism initiated the role of the 4B wheat chromosome in somatic embryogenesis; and the three alfalfa lines used by Ivanova and her colleagues (1994) were independent isolations from *M. falcata* L. and their genetic backgrounds were unknown (Atanassov *et al.*, 1986; Brown, personal comm., 1997). Both hypotheses seem to lack a solid genetic basis compared with the present study. The conclusion drawn from this study appears to be much stronger: The correlations between the endogenous hormone levels and regenerating ability observed in this study and reported in literature is a random correlation.

CONCLUSION

A series conditions that affect somatic embryogenesis and endogenous hormone levels of alfalfa A70 and A195 populations were tested in this study. It was found that *in vitro* somatic embryogenesis and endogenous hormone levels are controlled by the genotype of plants, and affected by the explant sources and the physiological state of donor plants (including light, photoperiod and sampling time in the present study), and by the induction medium and culture protocols. Except genotype, all the other conditions tested in this study are modulating factors, which exert their effects along with the genotype.

Although the regenerating abilities of the alfalfa plants changed under different conditions, the regenerating trend remained the same in both A70 and A195 populations. The endogenous hormone (IAA, ABA, cytokinins) levels, however, did not follow the same trend along with regeneration, except IAA that was noted in the leaf tissue of the A70 plants cultured *in vitro* and of the A195 population cultured under an 8-h photoperiod and sampled at midday. It is concluded that the correlations of the regenerating ability of plants with the endogenous hormone levels and their ratios is a random correlation and may apply only in specific cases. The hypothesis that the regenerating ability of a plant can be predicted with endogenous hormone levels and their ratios was not supported by the experimental results.

One unique discovery in this study is the effects of sampling time on the efficiency of

somatic embryogenesis. The results showed that more somatic embryos were produced in leaf tissue sampled at the end of light from donor plants cultured under 16-h and 8-h photoperiods, and sampled at the end of dark from donor plants cultured under 20-h photoperiod. The mechanism regulating this phenomenon deserves further exploration. A proper sampling time is suggested to be chosen along with culture photoperiod and induction medium in somatic embryogenesis for both research and production.

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Appendix I. Media for Somatic Embryogenesis.

I. Media for the direct regeneration method¹:

1. Inoculation, induction and development media:

Composition	B50 Medium (/L)	B50/IV Medium (/L)	B50/3M Medium (/L)
Salts ²	100 ml ³	100 ml ³	100 ml ³
Micronutrients ²	100 ml ³	100 ml ³	100 ml ³
Vitamines ²	100 ml ³	100 ml ³	100 ml ³
Sucrose	30 g	30 g	-
Maltose	-	-	30 g
Casein hydrolysate	500 mg	500 mg	500 mg
Myo-inositol	500 mg	500 mg	500 mg
2,4-D	-	3.978 mg	-
Kinetin	-	0.2 mg	-
Adenine	-	0.6 mg	-
Glutathione	-	9.987 mg	-
PEG - 6000	-	-	25 g
pH	5.5+/-0.1	5.5+/-0.1	5.5+/-0.1

¹ Denchev *et al.*, 1991; Ivanova *et al.*, 1994;

² Gamberg *et al.*, 1968;

³ 10X stock.

2. Media for embryo maturation and shoot elongation:

Composition	MS + ABA Medium (/L)	MS + GA3 Medium (/L)
Salts ¹	100 ml ²	100 ml ²
Vitamines ¹	100 ml ²	100 ml ²
Sucrose	30 g	30 g
ABA	7.929 mg	-
GA3	-	9.3528 mg
Agar	8 g	8 g
pH	5.8+/- 0.1	5.8+/- 0.1

¹ Murashige and Skoog, 1962;

² 10X stock.

II. Media for the indirect regeneration method and protocols for somatic embryogenesis¹:

1. Induction media:

Composition	B5h Medium (/L)	SH4K Medium (/L)
Salts	100 ml ²	100 ml ³
Micronutrients	100 ml ²	-
Vitamines	100 ml ²	-
Sucrose	30 g	10 g
Calcium hydrochloride	600 mg	-
Glutamine	800 mg	-
Glutathione	10 mg	-
Serine	100 mg	-
2,4-D	1mg	1 mg
Kinetin	0.2 mg	0.2 mg
Adenine	1 mg	-
Potassium sulphate	-	8.712 g
Proline	-	2.3 g
Thioprolin	-	53.3 mg
Agar	8.5 g	-
Gelrite	-	2.5 g
pH	5.5+/-0.1	5.8+/-0.1

¹ Brown, 1995;

² Gamborg *et al.*, 1968;

³ Including micronutrients and vitamins, Shenk and Hildebrandt, 1972.

2. Somatic embryo proliferation medium:

Composition	B5M Medium (/L)
Salts ¹	100 ml ²
Micronutrients ¹	100 ml ²
Vitamines ¹	100 ml ²
Sucrose	20 g
2,4-D	1mg
NAA	0.1 mg
pH	5.5+/- 0.1

¹ Gamborg *et al.*, 1968;

² 10X stock.

3. Somatic embryo development medium:

Composition	BOi2Y (/L)
Salts, micronutrients & vitamins ¹	100 ml
Sucrose	20 g
Yeast extract	2 g
Meso-inositol	100 mg
Agar	10 g
pH	5.9+/-0.1

¹ Modified Blaydes' medium stock (1966) by Saunders and Bingham, 1975;

² 10X stock.

Appendix 2. Somatic Embryogenesis of A70 and A195 Plants Cultured in Vitro.

Table A2.1. Somatic Embryo Number Induced by the Direct Regeneration Method (P1)

Plant	Rege- nera- tion ¹	Group 1		Group 2		Group 3		Final Mean±SE (/Flask)
		Embryos/Flask	Mean±SE	Embryos/Flask	Mean±SE	Embryos/Flask	Mean±SE	
A195.406	High	45, 17, 22	28±9	43, 37, 22	34±6	NA	NA	31±3
A195.407	High	35, 41, 44, 12	33±7	29, 49	39±10	67	67	46±11
A195.415	Mid	31, 17	24±7	6, 15, 26, 24	18±5	5	5	16±6
A195.416	Low	13, 27, 32, 6, 19, 11	18±4	21, 27, 18	22±3	NA	NA	20±2
A195.403	Non	0, 0, 0	0	0, 0, 0	0	0, 0	0	0
A195.409	Non	0, 0, 0	0	0, 0, 0	0	0, 0	0	0
A195.410	Non	31, 0, 0	0 (31?)	0, 0, 0	0	0, 0	0	0 (31?)
A70.403	High	11, 27	19±8	19, 31	25±6	5	5	16±6
A70.413	High	41, 63, 64	56±8	103, 49	76±27	36, 17, 234	96±69	76±12
A70.415	Mid	29, 21, 14, 24	22±3	8	8	2, 3	3±1	11±6
A70.409	Low	9	9	NA	NA	1	1	5±4
A70.406	Non	0, 0, 0	0	0, 0, 0	0	0, 0	0	0

¹ Regenerating ability.

Table A2.2. Somatic Embryo Numbers Induced from Leaf by the B5h-B5M-BOi2Y Protocol (P2)

Plant	Regene- ration ¹	Group 1		Group 2		Group 3		Final Mean±SE(/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	
A195.406	High	37, 35	36±1	NA	NA	73, 84	79±6	57±21
A195.407	High	11, 40, 33	28±9	89	89	10, 75	43±33	53±18
A195.415	Mid	6, 6	6	22	22	30, 13	21±9	16±5
A195.416	Low	5, 9	7±2	8	8	15	15	10±3
A195.403	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0	0
A195.409	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0	0
A195.410	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0	0
A70.403	High	5, 14	9±4	74	74	34	34	39±19
A70.413	High	24, 53	38±15	65	65	53, 17	35±18	46±6
A70.415	Mid	17, 49	33±16	41	41	3, 74	38±35	37±3
A70.409	Low	8	8	13	13	2	2	8±3
A70.406	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0	0

¹ Regenerating ability.

Table A2.3. Somatic Embryo Numbers Induced from Leaf by the SH4K-B5M-BOi2Y Protocol (P3).

Plant	Regene- ration ¹	Group 1		Group 2		Group 3		Final Mean±SE (/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	
A195.406	High	5, 7, 109	40±34	NA	NA	13, 13	13	26±14
A195.407	High	NA	NA	65	65	49, 2	25±24	45±20
A195.415	Mid	21, 15, 8	15±4	NA	NA	8, 6	7±1	11±4
A195.416	Low	7, 14	11±4	18	18	5, 7	6±1	12±4
A195.403	Non	0, 0, 0	0	0, 0	0	0, 0	0	0
A195.409	Non	0, 0, 0	0	0, 0	0	0, 0	0	0
A195.410	Non	0, 0, 0	0	0, 0	0	0, 0	0	0
A70.403	High	49, 15, 56, 6	32±12	NA	NA	10	10	21±11
A70.413	High	NA	NA	20, 45	33±13	25, 6	16±10	24±9
A70.415	Mid	19, 37	31±6	NA	NA	4, 8	6±2	19±13
A70.409	Low	NA	NA	6	6	2, 4	3±1	6
A70.406	Non	0, 0, 0	0	0, 0	0	0, 0	0	0

¹ Regenerating ability.

Table A2.4. Somatic Embryo Numbers Induced from Leaf by the B5h-BOi2Y Protocol (P4).

Plant	Regeneration ¹	Group 1		Group 2		Group 3		Group 4			Final Mean±SE (/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Mean±SE	
A195.406	High	9, 5	7±2	NA	NA	6, 10	8±2	NA	NA	8±1	
A195.407	High	2, 20	11±9	39	39	16	16	4	4	18±8	
A195.415	Mid	2	2	NA	NA	13	13	3, 2, 8	4±2	3±1	
A195.416	Low	NA	NA	6	6	6	6	NA	NA	6	
A195.403	Non	0, 0	0	0, 0	0	0, 0	0	NA	NA	0	
A195.409	Non	0, 0	0	0, 0	0	0, 0	0	NA	NA	0	
A195.410	Non	0, 0	0	0, 0	0	0, 0	0	NA	NA	0	
A70.403	High	NA	NA	10	10	20, 34	27±7	20, 65	43±22	26±10	
A70.413	High	20	20	72	72	49	49	5, 29	17±12	40±13	
A70.415	Mid	9, 19	14±5	14	14	NA	NA	25, 50	38±13	22±8	
A70.409	Low	NA	NA	2	2	2	2	NA	NA	2	
A70.406	Non	0, 0	0	0, 0	0	0, 0	0	NA	NA	0	

¹ Regenerating ability.

Table A2.5. Somatic Embryo Numbers Induced from Leaf by the SH4K-BOi2Y Protocol (P5).

Plant	Rege- nera- tion ¹	Group 1		Group 2		Group 3		Group 4		Final Mean±SE (/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	
A195.406	High	17	17	30	30	11	11	8, 3	5±3	16±5
A195.407	High	12	12	23	23	7	7	1	1	11±5
A195.415	Mid	8	8	NA	NA	10	10	2, 1	2±1	7±3
A195.416	Low	NA	NA	8	8	7	7	NA	NA	8±1
A195.403	Non	0	0	0	0	0	0	NA	NA	0
A195.409	Non	0	0	0	0	0	0	NA	NA	0
A195.410	Non	0	0	0	0	0	0	NA	NA	0
A70.403	High	31	31	27	27	42	42	10, 4, 25,	13±7	28±6
A70.413	High	5	5	45	45	52	52	64, 17, 31	37±14	35±11
A70.415	Mid	9	9	9	9	41	41	31, 7, 7	15±8	19±8
A70.409	Low	NA	NA	7	7	8	8	2	2	6±2
A70.406	Non	0	0	0	0	0	0	0	0	0

¹ Regenerating ability.

Table A2.6. Somatic Embryo Numbers Induced from Petiole by the B5h-B5M-BOi2Y Protocol (P2)

Plant	Rege- nera- tion ¹	Group 1		Group 2		Group 3			Finalal Mean±SE(/ G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Mean±SE	
A195.406	High	76, 99, 70, 3, 49, 56	59±13	NA	NA	52, 72	63±11		61±2
A195.407	High	83, 49, 17, 18	42±16	203	203	54	54		100±52
A195.415	Mid	3, 10	7±4	NA	NA	18	17.7		12±6
A195.416	Low	4, 6	5±1	13, 12	13±1	2, 3	3±1		7±3
A195.403	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0		0
A195.409	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0		0
A195.410	Non	0, 0, 0	0	0, 0, 0	0	0, 0, 0	0		0
A70.403	High	NA	NA	112	112	NA	NA		112
A70.413	High	27, 38	32±6	58	58	NA	NA		45±13
A70.415	Mid	27, 47	37±10	21	21	NA	NA		29±8
A70.409	Low	4, 12	8±4	3	3	NA	NA		5±2
A70.406	Non	0, 0,	0	0, 0	0	NA	NA		0

¹ Regenerating ability.

Table A2.7. Somatic Embryo Numbers Induced from Petiole by the SH4K-B5M-BOi2Y Protocol (P3)

Plant	Regeneration Ability	Group 1		Group 2		Group 3		Final Mean±SE (/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	
A195.406	High	77, 41, 34	51±13	NA	NA	60, 40	51±10	51
A195.407	High	19, 33, 18	24±5	56	56	23	23	34±11
A195.415	Mid	NA	NA	6	6	4, 7	6±1	6
A195.416	Low	5.4, 10	8±2	NA	NA	14, 12	13±1	10±3
A195.403	Non	0, 0, 0	0	0	0	0, 0	0	0
A195.409	Non	0, 0	0	0	0	0, 0	0	0
A195.410	Non	0, 0	0	0	0	0, 0	0	0
A70.403	High	35, 35	35	19	19	NA	NA	27±8
A70.413	High	NA	NA	86	86	NA	NA	86
A70.415	Mid	30, 43	36±6	NA	NA	NA	NA	36
A70.409	Low	3	3	5	5	NA	NA	4±1
A70.406	Non	0, 0	0	0, 0	0	NA	NA	0

Table A2.8. Somatic Embryo Numbers Induced from Petiole by the B5h-BOi2Y Protocol (P4).

Plant	Regeneration Ability	Group 1		Group 2		Group 3		Final Mean±SE (/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	
A195.406	High	13	13	12	12	8, 18	13±5	13
A195.407	High	12	12	61	61	9	9	27±17
A195.415	Mid	5	5	1	1	6	6	4±2
A195.416	Low	NA	NA	5	5	8	8	7±2
A195.403	Non	0, 0	0	0	0	0	0	0
A195.409	Non	0, 0	0	0	0	0	0	0
A195.410	Non	0, 0	0	0	0	0	0	0
A70.403	High	9	9	21	21	NA	NA	15±5
A70.413	High	15	15	79	79	NA	NA	47±32
A70.415	Mid	9	9	6	6	NA	NA	8±2
A70.409	Low	NA	NA	2	2	NA	NA	2
A70.406	Non	0, 0	0	0, 0	0	NA	NA	0

Table A2.9. Somatic Embryo Numbers Induced from Petiole by the SH4K-BOi2Y Protocol (P5).

Plant	Regene- ration Ability	Group 1		Group 2		Group 3		Final Mean±SE (/G Callus)
		Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	Embryos/G Callus per Dish with BOi2Y	Mean±SE	
A195.406	High	18	18	17, 15	16±1	29	29	21±4
A195.407	High	43	43	35, 59	47±33	9, 31	20±11	37±8
A195.415	Mid	NA	NA	9	9	11, 3	7±4	8±1
A195.416	Low	22	22	4	4	9	9	12±5
A195.403	Non	0, 0	0	0	0	0	0	0
A195.409	Non	0	0	0	0	0	0	0
A195.410	Non	0	0	0	0	0	0	0
A70.403	High	12	12	25	25	NA	NA	20±5
A70.413	High	NA	NA	49	49	NA	NA	49
A70.415	Mid	7	7	NA	NA	NA	NA	7
A70.409	Low	NA	NA	3	3	NA	NA	3
A70.406	Non	0, 0	0	0, 0	0	NA	NA	0

Appendix 3. Endogenous Hormone Levels Analysed with HPLC in Plants Cultured *in vitro*.

Table A3.1. Endogenous IAA, ABA and Cytokinins Levels of Donor Leaves Analysed by HPLC (pMol/G DW)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers(Mean±SE) ²					IAA	ABA	Total Cytokinins	Z	ZR	IPA	DHZ	IPAd
			P1	P2	P3	P4	P5								
A195.406	High	1	28	36	40	7	17	388	2419	1379	520	53	350	14.8	167
		2	34	NA	NA	NA	30	701	2805	1088	149	17	45	34	27
		3	NA	78	13	8	11	759	2015	1297	33.8	52.1	14.5	13.9	39.9
A195.407	High	1	33	28	NA	11	12	NA	NA	NA	NA	NA	NA	NA	NA
		2	39	89	65	39	23	471	4271	1415	257	13	79	79	30
		3	67	43	25	16	7	482	4390	901	63.3	68.9	28.5	20.6	90.9
A195.415	Mid	1	24	6	15	2	8	423	4334	1038	430	63	139	19.7	120
		2	18	22	NA	NA	NA	773	2870	1372	163	29	133	89	78
		3	5	21	7	13	10	519	3361	792	63.9	43.8	8.9	11.7	60.5
A195.416	Low	1	18	7	11	NA	NA	468	3360	1184	570	77	207	16.5	141
		2	22	8	18	6	8	421	2685	1223	237	33	67	46	50
		3	NA	15	6	6	7	318	3435	1090	56.9	38	22.5	31.3	24.8
A195.403	Non	1	0	0	0	0	0	411	2854	1061	460	50	150	11.9	69
		2	0	0	0	0	0	351	3358	1110	190	20	79	21	78
		3	0	0	0	0	0	495	2671	1207	58.5	116.5	16.7	9.5	52.6
A195.409	Non	1	0	0	0	0	0	254	3859	1001	517	62	141	5.7	98
		2	0	0	0	0	0	326	4106	1210	262	44	77	34	49
		3	0	0	0	0	0	253	3085	1041	21.1	26.9	5.6	14.7	14.7
A195.410	Non	1	31	0	0	0	0	368	1849	1736	750	109	259	30	137
		2	0	0	0	0	0	630	2440	1305	289	45	78	33	56
		3	0	0	0	0	0	303	2914	916	37.7	322.6	7.8	14.7	26.4

Table A3.1. (Cont'd)

Plant	Rege- nera- tion	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N- G	DHZ- N-G	Z-o-G	ZR-o-G	DHZ- o-G	DHZ R-o-G
			P1	P2	P3	P4	P5								
A195.406	High	1	28	36	40	7	17	2.8	4.9	4	49.7	59	44.5	49.7	59
		2	34	NA	NA	NA	30	20	6	2.9	4.9	156	261	56	309
		3	NA	78	13	8	11	4	44.4	0	4.7	411	376	303	0
A195.407	High	1	33	28	NA	11	12	NA	NA	NA	NA	NA	NA	NA	NA
		2	39	89	65	39	23	3.8	13.7	2.9	11.7	227	310	79	308
		3	67	43	25	16	7	3.4	28.1	0	4.7	257	331	3.9	0
A195.415	Mid	1	24	6	15	2	8	2.8	11	4	6.1	138	29.7	49	25
		2	18	22	NA	NA	NA	10	20	8	4.6	306	238	54	239
		3	5	21	7	13	10	6.8	57.8	0	8.5	183	0	347	0
A195.416	Low	1	18	7	11	NA	NA	2.1	16.7	4	3	138	22	3	37
		2	22	8	18	6	8	2.1	17	6.5	3	178	274	57	252
		3	NA	15	6	6	7	5.4	39.3	0	14.2	1.5	534.2	323.6	0
A195.403	Non	1	0	0	0	0	0	2.8	3.8	9.1	3.8	83	89	10	118
		2	0	0	0	0	0	2.8	3.8	4	3.8	158	197	79	273
		3	0	0	0	0	0	5.3	32.1	0	8.7	485.2	0	423.8	0
A195.409	Non	1	0	0	0	0	0	1.4	6.8	1.8	1.9	59.4	30	30	46
		2	0	0	0	0	0	25	8.7	1.8	4.9	49	310	36	308
		3	0	0	0	0	0	4.1	30	0	4.8	112.6	264.8	37.7	0
A195.410	Non	1	31	0	0	0	0	7.1	8.7	2.9	2.3	277	37	66	50
		2	0	0	0	0	0	7	8.7	4.7	4.6	187	231	69	291
		3	0	0	0	0	0	13.8	29.8	0	4.1	56.9	0	0	0

Table A3.1. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cytokinins	Z	ZR	IPA	DHZ	IPAd	
			P1	P2	P3	P4	P5									
A70.403	High	1	19	9	32	NA	31	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	25	74	NA	10	27	NA	NA	NA	NA	NA	NA	NA	NA	NA
		3	NA	NA	NA	27	42	610	2740	1022	51.5	41.7	45.5	28.4	39.8	
A70.413	High	1	56	38	NA	20	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	76	65	32	72	45	782	2770	1966	457	40	194	50	88	
		3	NA	NA	NA	49	52	7531	1723	2020	7.2	26.1	0.1	6.3	32.5	
A70.415	Mid	1	22	33	31	14	9	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	8	41	NA	14	9	735	5148	1975	341	51	66	61	48	
		3	NA	NA	NA	NA	41	902	5591	1993	17.7	12.7	2.8	66	10.4	
A70.409	Low	1	9	8	NA	NA	NA	1329	6042	2202	670	163	249	25.7	503	
		2	NA	13	6	2	7	506	4572	1887	289	16.8	230	68	68	
		3	NA	NA	NA	2	8	478	5164	2386	14.9	49.4	2.7	8.5	41.5	
A70.406	Non	1	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	0	0	0	0	0	158	5500	1795	63	7	18	10	8	
		3	NA	NA	NA	NA	NA	203	6195	1760	15.9	79.6	1.9	2.6	59.2	

Table A3.1. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N-G	DHZ- N-G	Z-o-G	ZR-o- G	DHZ- o-G	DHZR- o-G
			P1	P2	P3	P4	P5								
A70.403	High	1	19	9	32	NA	31	NA	NA	NA	NA	NA	NA	NA	NA
		2	25	74	NA	10	27	NA	NA	NA	NA	NA	NA	NA	NA
		3	NA	NA	NA	27	42	6.2	8.7	0	13.2	143.6	0	134.2	0
A70.413	High	1	56	38	NA	20	5	NA	NA	NA	NA	NA	NA	NA	NA
		2	76	65	32	72	45	4.9	158	19	49	316	217	178	195
		3	NA	NA	NA	49	52	1.4	28.2	0	4.5	224.3	0	79.9	0
A70.415	Mid	1	22	33	31	14	9	NA	NA	NA	NA	NA	NA	NA	NA
		2	8	41	NA	14	9	8.7	29	4	20	625	289	148	284
		3	NA	NA	NA	NA	41	2.4	59.6	0	15.7	228.3	0	189.2	0
A70.409	Low	1	9	8	NA	NA	NA	3.5	19.8	2.5	10.3	287	18.6	258	70
		2	NA	13	6	2	7	8	23	1.8	12	346	338	148	338
		3	NA	NA	NA	2	8	3.7	24.8	0	6.8	912.8	601	379.1	342.1
A70.406	Non	1	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		2	0	0	0	0	0	2.1	8.7	2.9	4.9	49	302	20	299
		3	NA	NA	NA	NA	NA	1.2	26.8	0	2.9	845.3	533.5	21.2	175.1

¹Regenerating ability.²P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Table A3.2. Endogenous IAA, ABA and CKs Levels of Donor Leaves Analysed by HPLC (pMol/G FW)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cy- tokinins	Z	ZR	IPA	DHZ	IPAd
			P1	P2	P3	P4	P5								
A195.406	High	1	28	36	40	7	17	77	480	273.6	103.2	10.5	69.4	2.9	33.3
		2	34	NA	NA	NA	30	140.6	562.4	218.1	29.9	3.4	9	6.8	5.4
		3	NA	78	13	8	11	137.9	366.1	235.7	6.1	9.5	2.6	2.5	7.2
A195.407	High	1	33	28	NA	11	12	NA	NA	NA	NA	NA	NA	NA	NA
		2	39	89	65	39	23	63.7	577.4	191.3	34.7	1.8	10.7	10.7	4.1
		3	67	43	25	16	7	77.3	704.2	144.5	10.2	11	4.6	3.3	14.6
A195.415	Mid	1	24	6	15	2	8	71.3	730.3	174.9	72.5	10.6	23.4	3.3	20.2
		2	18	22	NA	NA	NA	138.8	515.5	246.4	29.3	5.2	23.9	16	14
		3	5	21	7	13	10	88.2	571.4	134.6	10.9	7.4	1.5	2	10.3
A195.416	Low	1	18	7	11	NA	NA	65.4	469.3	165.3	79.6	10.7	28.9	2.3	19.7
		2	22	8	18	6	8	60.7	387.2	176.4	34.2	4.8	9.7	6.6	7.2
		3	NA	15	6	6	7	48.9	528	167.5	8.7	5.8	3.5	4.8	3.8
A195.403	Non	1	0	0	0	0	0	69.5	482.9	179.4	77.7	8.5	25.4	2	11.7
		2	0	0	0	0	0	59.7	571.2	188.8	32.3	3.4	13.4	3.6	13.3
		3	0	0	0	0	0	84.4	455.4	205.8	10	19.9	2.8	1.6	9
A195.409	Non	1	0	0	0	0	0	44.9	681.9	176.9	91.4	11	24.9	1	17.3
		2	0	0	0	0	0	55.7	701.3	206.7	44.7	7.5	13.2	5.8	8.4
		3	0	0	0	0	0	18.1	557.2	188	3.8	4.9	1	2.7	2.7
A195.410	Non	1	31	0	0	0	0	54	271.4	254.8	110.1	16	38	4.4	20.1
		2	0	0	0	0	0	106.3	412.6	220.7	48.9	7.6	13.2	5.6	9.5
		3	0	0	0	0	0	48.6	467.1	146.8	6	51.7	1.3	2.4	4.2

Table A3.2. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N-G	DHZ- N-G	Z-o- G	ZR-o- G	DHZ- o-G	DHZR- o-G
			P1	P2	P3	P4	P5								
A195.406	High	1	28	36	40	7	17	0.6 4 0.7	1 1.2 8.1	0.8 0.6 0	9.9 1 0.9	11.7 31.3 74.7	8.8 52.3 68.3	9.9 11.2 55.1	11.7 62 0
		2	34	NA	NA	30									
		3	NA	78	13	8	31								
A195.407	High	1	33	28	NA	11	12	NA 0.5 0.5	NA 1.9 4.5	NA 0.4 0	NA 1.6 0.8	NA 30.7 41.2	NA 41.9 53.1	NA 10.7 11.9	NA 41.6 0
		2	39	89	65	23									
		3	67	43	25	16	7								
A195.415	Mid	1	24	6	15	2	8	0.5 1.8 1.2	1.9 3.6 9.8	0.7 1.4 0	1 0.8 1.5	23.3 55 31	5 42.7 0	8.3 9.7 59	4.2 42.9 0
		2	18	22	NA	NA									
		3	5	21	7	13	10								
A195.416	Low	1	18	7	11	NA	NA	0.3 0.3 0.8	2.3 2.5 6	0.6 0.9 0	0.4 0.4 2.3	19.3 25.7 0.2	3.1 39.5 82.1	0.4 8.2 49.7	5.2 36.3 0
		2	22	8	18	6	8								
		3	NA	15	6	6	7								
A195.403	Non	1	0	0	0	0	0	0.5 0.5 0.9	0.6 0.6 5.5	1.5 0.7 0	0.6 0.6 1.5	14 26.9 82.7	15 33.5 0	1.7 13.4 72.3	20 46.4 0
		2	0	0	0	0	0								
		3	0	0	0	0	0								
A195.409	Non	1	0	0	0	0	0	1.2 1.5 5.4	0.3 0.3 0	0.3 0.8 0.9	0.3 0.8 0.9	10.5 8.4 20.3	5.3 52.9 47.8	5.3 6.1 6.8	8.1 52.6 0
		2	0	0	0	0	0								
		3	0	0	0	0	0								
A195.410	Non	1	31	0	0	0	0	1 1.2 2.2	13 1.5 4.8	0.4 0.8 0	0.3 0.8 0.7	40.7 31.6 9.1	5.4 39.1 0	9.7 11.7 0	7.3 49.2 0
		2	0	0	0	0	0								
		3	0	0	0	0	0								

Table A3.2. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cy- tokinins	Z	ZR	IPA	DHZ	IPAd	
			P1	P2	P3	P4	P5									
A70.403	High	1	19	9	32	NA	31	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	25	74	NA	10	27	NA	NA	NA	NA	NA	NA	NA	NA	NA
		3	NA	NA	NA	27	42	127.1	571	213	10.7	8.7	9.5	5.9	8.3	8.3
A70.413	High	1	56	38	NA	20	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	76	65	32	72	45	106.6	377.6	268	62.3	5.5	26.4	6.8	12	12
		3	NA	NA	NA	49	52	1250.9	286.2	335.5	1.2	1	0	1	5.4	5.4
A70.415	Mid	1	22	33	31	14	9	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	8	41	NA	14	9	104.5	732	280.8	48.5	7.8	9.4	8.7	6.8	6.8
		3	NA	NA	NA	NA	41	134.8	835.3	297.8	2.6	1.9	0.4	9.9	1.6	1.6
A70.409	Low	1	9	8	NA	NA	NA	201.6	916.6	334	101.6	24.7	37.8	3.9	76.3	76.3
		2	NA	13	6	2	7	71.7	647.9	267	50	2.4	32.6	9.6	9.6	9.6
		3	NA	NA	N	2	8	71.1	768.4		2.2	7.3	0.4	1.3	6.2	6.2
A70.406	Non	1	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		2	0	0	0	0	0	22.9	796.4	260	9.1	1	2.6	1.4	1.2	1.2
		3	NA	NA	NA	NA	NA	38.2	1165.9	331	3	15	0.4	0.5	11.1	11.1

Table A3.2. (Cont'd)

Plant	Rege- nera- tion ¹	Gro up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N- G	IP-N- G	DHZ- N-G	Z-o-G	ZR-o- g	DHZ- o-G	DHZR- o-G
			P1	P2	P3	P4	P5								
A70.403	High	1	19	9	32	NA	31	NA	NA	NA	NA	NA	NA	NA	NA
		2	25	74	NA	10	27	NA	NA	NA	NA	NA	NA	NA	NA
		3	NA	NA	NA	27	42	1.8	1.8	0	2.8	30	0	28	0
A70.413	High	1	56	38	NA	20	5	NA	NA	NA	NA	NA	NA	NA	NA
		2	76	65	32	72	45	0.7	21.5	2.6	6.7	43.1	29.6	24.3	26.6
		3	NA	NA	NA	49	52	0.2	4.7	0	0.7	37.2	0	13.3	0
A70.415	Mid	1	22	33	31	14	9	NA	NA	NA	NA	NA	NA	NA	NA
		2	8	41	NA	14	9	1.2	4.1	0.6	2.8	88.9	41.1	21	40.4
		3	NA	NA	NA	NA	41	0.4	8.9	0	2.3	34.1	0	28.3	0
A70.409	Low	1	9	8	NA	NA	NA	0.5	3	0.4	1.6	43.5	2.8	39.1	10.6
		2	NA	13	6	2	7	1.1	3.3	0.3	1.7	49	47.9	21	47.9
		3	NA	NA	NA	2	8	0.6	3.7	0	1	135.8	89	56.4	50.9
A70.406	Non	1	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		2	0	0	0	0	0	0.3	1.3	0.4	0.7	7.1	43.7	2.9	43.3
		3	NA	NA	NA	NA	NA	0.2	5	0	0.5	159.1	100.4	4	32.9

¹Regenerating ability;² P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Table A3.3. Endogenous IAA, ABA and CKs Levels of Donor Petioles Analyzed by HPLC (pMol/G DW)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cytokinins	Z	ZR	IPA	DHZ	IPAd
			P1	P2	P3	P4	P5								
A195.406	High	2	NA	NA	NA	12	16	NA	3783	NA	NA	NA	NA	NA	NA
		3	NA	63	51	13	29	473	1208	2818	25.1	51.9	1.9	12.7	47.3
A195.407	High	3	NA	54	23	9	20	546	1480	1785	23.9	42.7	2.3	11.6	24.1
A195.415	Mid	2	NA	NA	6	1	9	1241	11810	1195	150	29	95	48	27
		3	NA	18	6	6	7	1884	1290	2402	31.6	119.8	2.2	44.4	92.2
A195.416	Low	2	NA	13	NA	5	4	750	978	1937	539	71	207	31	126
		3	NA	3	13	8	9	238	916	1249	66.7	203.2	0.9	67.1	97.6
A195.403	Non	3	NA	0	0	0	0	1023	1437	503	16.6	36.5	1.7	11.5	34.3
A195.409	Non	1	NA	0	0	0	0	892	2712	2253	510	25	140	19.8	84
		2	NA	0	0	0	0	NA	1608	2148	524	25	130	26	80
		3	NA	0	0	0	0	234	1006	1615	8.6	20.1	0.5	10.7	6
A195.410	Non	2	NA	0	0	0	0	1492	2009	1997	471	28	104	29	76
		3	NA	0	0	0	0	1010	1928	1606	27.5	117.4	2.2	44	89.5

Table A3.3. (Cont'd)

Plant	Rege- nera- tion'	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N- G	DHZ- N-G	Z-o-G	ZR-o- G	DHZ- o-G	DHZ R-o-G
			P1	P2	P3	P4	P5								
A195.406	High	2 3	NA NA	NA 63	NA 51	12 13	16 19	NA 2.5	NA 24	NA 0	NA 3	NA 1978	NA 0	NA 671	NA 0
A195.407	High	3	NA	54	23	9	20	3.8	10.4	0	3.3	1578	0	84	0
A195.415	Mid	2 3	NA NA	NA 18	6 6	1 6	9 7	8 5.9	13 78.2	8.2 0	1.9 9.7	138 1572	302 0	84 446.5	291 0
A195.416	Low	2 3	NA NA	13 3	NA 13	5 8	4 9	8 7.7	8 49.8	5.2 0	4.9 6.9	287 674	302 0	57 74.3	291 0
A195.403	Non	3	NA	0	0	0	0	2.6	14.9	0	4.8	379	0	4.1	0
A195.409	Non	1 2 3	NA NA NA	0 0 0	0 0 0	0 0 0	0 0 0	8 8 2.4	16 17 10.7	6.9 6.2 0	6.8 6.8 2.5	1034 247 1221	84 498 0	189 85 327.9	129 495 4.3
A195.410	Non	2 3	NA NA	0 0	0 0	0 0	0 0	7.7 8.6	16 39.5	5.9 0	5.7 9.7	138 1149	498 0	118 118.7	499 0

Table A3.3. (Cont'd)

Plant	Rege- nera- tion ¹	Grou- p	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cytokins	Z	ZR	IPA	DHZ	IPAd
			P1	P2	P3	P4	P5								
A70.403	High	3	NA	NA	NA	NA	NA	798	2065	2911	196	387	13.8	29.6	188
A70.413	High	3	NA	NA	NA	NA	NA	754	635	2669	39.1	189	3	17.7	94.9
A70.415	Mid	2	NA	21	NA	6	NA	592	2411	2253	330	2717	128	146	63
		3	NA	NA	NA	NA	NA	715	1696	3969	92.9	0	16.1	42.1	130
A70.409	Low	2	NA	3	5	2	3	665	1840	2205	141	127	414	42	337
		3	NA	NA	NA	NA	NA	707	1777	5714	101	413.8	2.9	45.3	238
A70.406	Non	2	NA	0	0	0	0	NA	3783	1595	414	112	204	42	123
		3	NA	NA	NA	NA	NA	735	3112	2000	104	338.7	10.3	69.6	202

Table A3.3. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N- G	DHZ- N-G	Z-o-G	ZR-o- G	DHZ- o-G	DHZ R-o-G
			P1	P2	P3	P4	P5								
A70.403	High	3	NA	NA	NA	NA	NA	11.7	64.9	0	21.1	1707	98	194	0
A70.413	High	3	NA	NA	NA	NA	NA	18.8	93.3	0	37.5	1720	155	293	7.4
A70.415	Mid	2 3	NA NA	21 NA	NA	6 NA	NA NA	15 11.4	19.8 172.8	27 0	19.8 19.4	733 2833	305 366	148 115	291 0
A70.409	Low	2 3	NA NA	3 NA	5 NA	2 NA	3 NA	9.8 11.3	6.8 107.2	8.9 0	6.8 10.7	644 2802	105 0	237 133	126 225.1
A70.406	Non	2 3	NA NA	0 NA	0 NA	0 NA	0 NA	8 11.9	6 47.4	8.3 0	5.7 10.3	109 994	162 60	237 104.7	164 46.7

¹Regenerating ability;²P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Table A3.4. Endogenous IAA, ABA and Cytokinins Levels of Donor Petioles Analysed by HPLC (pMol/G FW)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cytokinins	Z	ZR	IPA	DHZ	IPAd
			P1	P2	P3	P4	P5								
A195.406	High	2	NA	NA	NA	12	16	NA	72	NA	NA	NA	NA	NA	NA
		3	NA	63	51	13	29	76.9	196.3	457.9	4.1	8.4	0.3	2.1	7.7
A195.407	High	3	NA	54	23	9	20	83.3	226	272	3.6	6.5	0.4	1.8	3.7
A195.415	Mid	2	NA	NA	6	1	9	189.4	1802	182.4	22.9	4.4	14.5	7.3	4.1
		3	NA	18	6	6	7	266.8	182.7	340.1	4.5	17	0.3	6.3	13.1
A195.416	Low	2	NA	13	NA	5	4	82.4	107.4	212.7	59.2	7.8	22.7	3.4	13.8
		3	NA	3	13	8	9	31.7	122	166.4	8.9	27.1	0.1	8.9	13
A195.403	Non	3	NA	0	0	0	0	143	200.9	70.3	2.3	5.1	0.2	1.6	4.8
A195.409	Non	1	NA	0	0	0	0	129.7	394.3	327.6	74.2	3.6	20.4	2.9	12.2
		2	NA	0	0	0	0	NA	236.7	316.2	77.1	3.7	19.1	3.8	11.8
		3	NA	0	0	0	0	34.6	148.6	238.5	1.3	3	0.1	1.6	0.9
A195.410	Non	2	NA	0	0	0	0	153.2	206.3	205.1	48.4	2.9	10.7	3	7.8
		3	NA	0	0	0	0	132.5	253	210.7	3.6	15.4	0.3	5.8	11.7

Table A3.4. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N- G	DHZ- N-G	Z-o-G	ZR-o- G	DHZ- o-G	DHZ R-o-G
			P1	P2	P3	P4	P5								
A195.406	High	2 3	NA NA	NA 63	NA 51	12 13	16 29	NA 0.4	NA 3.9	NA 0	NA 0.5	NA 321.4	NA 0	NA 109	NA 0
A195.407	High	3	NA	54	23	9	20	0.6	1.6	0	0.5	241	0	12.8	0
A195.415	Mid	2 3	NA NA	NA 18	6 6	1 6	9 7	1.2 0.8	2 11.1	1.3 0	0.3 1.4	21.1 222.6	46.1 0	12.8 63.2	44.4 0
A195.416	Low	2 3	NA NA	13 3	NA 13	5 8	4 9	0.9 1	0.9 6.6	0.6 0	0.5 0.9	31.5 89.8	33.2 0	6.3 9.9	32 0
A195.403	Non	3	NA	0	0	0	0	0.4	2.1	0	0.7	53	0	0.6	0
A195.409	Non	1 2 3	NA NA NA	0 0 0	0 0 0	0 0 0	0 0 0	1.2 1.2 0.4	2.3 2.5 1.6	1 0.9 0	1 1 0.4	150.3 36.4 180.3	12.2 73.3 0	27.5 12.5 48.4	18.8 72.9 0.6
A195.410	Non	2 3	NA NA	0 0	0 0	0 0	0 0	0.8 1.1	1.6 5.2	0.6 0	0.6 1.3	14.2 150.7	51.1 0	12.1 15.6	51.2 0

Table A3.4. (Cont'd)

Plant	Rege- nera- tion ¹	Gro up	Somatic Embryo Numbers (Mean±SE) ²					IAA	ABA	Total Cytokinins	Z	ZR	IPA	DHZ	IPAd
			P1	P2	P3	P4	P5								
A70.403	High	3	NA	NA	NA	NA	NA	101	261	369	24.8	48.9	1.7	3.7	23.8
A70.413	High	3	NA	NA	NA	NA	NA	122	103	472	6.3	30.7	0.5	2.9	15.4
A70.415	Mid	2	NA	21	NA	6	NA	879.2	279.2	260.9	38.2	3.1	14.7	16.9	7.3
		3	NA	NA	NA	NA	NA	90.5	214.7	502.5	11.8	21.5	2	5.3	16.5
A70.409	Low	2	NA	3	5 NA	2	3	77.1	213.3	255.6	16.3	14.7	48	4.9	39.1
		3	NA	NA		NA	NA	96.6	242.9	781.1	13.8	56.6	0.4	6.2	32.5
A70.406	Non	2	NA	0	0	0	0	NA	500.5	211	54.8	14.8	27	5.6	16.3
		3	NA	NA	NA	NA	NA	139.4	590.3	379.4	19.7	64.3	2	13.2	38.3

Table A3.4. (Cont'd)

Plant	Rege- nera- tion ¹	Gro- up	Somatic Embryo Numbers (Mean±SE) ²					DHZR	Z-N-G	IP-N- G	DHZ- N-G	Z-o-G	ZR-o- G	DHZ- o-Z	DHZ R-o-G
			P1	P2	P3	P4	P5								
A70.403	High	3	NA	NA	NA	NA	NA	1.5	8.2	0	2.7	216	12.4	24.5	0
A70.413	High	3	NA	NA	NA	NA	NA	3.1	15.1	0	6.1	279	25.1	47.6	1.2
A70.415	Mid	2	NA	21	NA	6	NA	1.7	2.3	3.1	2.3	84.9	35.3	17.1	33.7
		3	NA	NA	NA	NA	NA	0.1	21.9	0	2.4	358.7	46.3	14.6	0
A70.409	Low	2	NA	3	5	2	3	1.1	0.8	1	0.8	74.6	12.2	27.5	14.6
		3	NA	NA	NA	NA	NA	1.5	14.7	0	1.5	383	0	18.2	30.8
A70.406	Non	2	NA	0	0	0	0	1.1	0.8	1.1	0.8	14.4	21.4	31.4	21.7
		3	NA	NA	NA	NA	NA	2.3	9	0	1.9	188.6	11.4	19.9	8.9

¹Regenerating ability;²P1: the mean value was calculated as embryo number/15 mg fresh leaf;

P2-P5: the mean value was calculated as embryo number/gram callus.

Appendix 4. Statistical Analysis of Somatic Embryogenesis of the A70 and A195 Alfalfa Populations with Various Culture Protocols and the Ratios of Hormone Levels.

Table A4.1. Statistical Analysis of Embryo Production from Different Explant Sources¹.

Plants	Number of Somatic Embryos (Mean±Deviance)		
	Leaf with P1	Leaf with P2-P5	Petiole with P2-P5
A195.406 and 407	37.69986±4.84588	33.41556±2.94587	48.14062±3.36876
A195.415	18.35313±4.39094	11.64449±2.32141	7.34484±2.14107
A195.416	19.36077±3.85921	9.46918±2.35382	8.02947±1.85961
A195.403, 409 and 410	1.30370±0.61451	0.00017±0.00239	0.00018±0.00271
A70.403 and 413	53.64730±5.32103	33.68075±2.71154	40.30590±4.29332
A70.415	14.47586±3.79286	26.34883±3.50501	24.06771±4.63804
A70.409	4.88755±4.05585	5.46324±1.97781	4.48897±2.09023
A70.406	0.00018±0.00761	0.00018±0.00434	0.00017±0.00492

¹ The analysis was performed with Genstat 5 Release 3.2 (IBM-PC 80386/DOS), and the data was transformed from Appendix 2. The predictions have been standardized by averaging over the levels of some factors:

Factor Weighting policy Status of weights

group Marginal weights Adjusted to exclude combinations not present

protocol marginal weights Adjusted to exclude combinations not present

Table A4.2. Statistics of Embryo Production with Various Culture Protocols¹.

Explant Sources	Protocols	Number of Somatic Embryos (Mean±Deviance)			
		High ² Regeneration	Mid-Regeneration	Low Regeneration	Non-Regeneration
Leaf	P1	43.11174 ±3.65848	17.03735 ±3.16084	14.48784 ±2.90075	0.86134 ±0.40598
	P2	50.99312 ±5.05486	25.29314 ±4.27150	9.38249 ±3.20004	0.00016 ±0.00333
	P3	33.10494 ±4.26823	13.27119 ±3.12476	9.39738 ±3.14114	0.00017 ±0.00393
	P4	19.64611 ±2.94891	11.04691 ±2.63647	3.95349 ±2.61213	0.00019 ±0.00450
	P5	21.87057 ±3.24691	8.03102 ±2.48521	7.53419 ±3.65435	0.00017 ±0.00611
Petiole	P1	0	0	0	0
	P2	76.46867 ±6.48010	18.81135 ±4.48875	6.71467 ±2.29502	0.00016 ±0.00349
	P3	43.63355 ±4.97178	16.62200 ±4.75816	7.28610 ±2.63023	0.00018 ±0.00490
	P4	21.12335 ±3.51926	3.98342 ±2.02212	4.98094 ±3.39335	0.00020 ±0.00565
	P5	26.61907 ±3.95608	7.70557 ±3.69441	9.41887 ±4.04935	0.00017 ±0.00546

¹ The analysis was performed with Genstat 5 Release 3.2 (IBM-PC 80386/DOS), and the data was transformed from Appendix 2. The predictions have been standardized by averaging over the levels of some factors:

Factor Weighting policy Status of weights

group Marginal weights Adjusted to exclude combinations not present

protocol marginal weights Adjusted to exclude combinations not present

² The plants with the same regenerating abilities in both A70 and A195 populations were combined in this analysis. For example, the high regenerating plants include A195.406, A195.407, A70.403 and A70.413.

Table A4.3. Accumulated Statistical Analysis of Deviance¹.

Change	Degree of Freedom	Deviance	Mean Deviance	Deviance Ratio
Plant	1	459.623	459.623	66.77 ²
Regenerating Abilities	3	9110.846	3036.949	441.18 ²
Plant. Regeneration	3	194.899	64.966	9.44 ²
Protocol	4	733.669	183.417	26.65 ²
Explant Source	1	69.762	69.762	10.13 ²
Explant Source. Protocol	3	27.296	0.099	1.32
Plant.Protocol	4	105.122	26.281	3.82 ²
Regeneration. Explant Source	6	207.787	34.631	5.03 ²
Protocol.Group	12	514.350	42.863	6.23 ²
Plant.Explant Source	1	7.754	7.754	1.13
Regeneration.Protocol	9	56.788	6.31	0.92
Plant.Regen.Explant Source	6	99.808	16.635	2.42 ²
Regeneration.Explant Source.Protocol	9	25.616	2.846	0.41
Residual	435	2994.409	6.884	----

¹ The data given in Appendix 2 and 3 were analyzed using generalized linear models (McCullagh and nelder, 1989), since neither the count of embryos not the biochemical quantities have homogeneous variances. The embryo count was analysed using a compound Poisson distribution with a log link.

² Significant with $P < 0.05$ when the deviance ratios were referred to the F-distribution.

Table A4.4. Correlations of the Endogenous Hormone Levels with Statistical Analysis¹.

	IAA	ABA	Cyto ²	Z	ZR	IPA	DHZ	IPAd	DHZ R	ZNG	IPNG	DHZ NG	ZoG	ZRoG	DHZo G	DHZ RoG
IAA	1.00															
ABA	0.14	1.00														
Cyto	0.27	0.44	1.00													
Z	-0.06	0.25	0.23	1.00												
ZR	0.02	0.07	0.27	0.12	1.00											
IPA	20.03	0.35	0.29	0.71	0.12	1.00										
DHZ	0.09	0.38	0.47	0.21	0.61	0.31	1.00									
IPAd	0.15	0.36	0.61	0.47	0.17	0.65	0.3	1.00								
DHZR	-0.1	-0.02	0.16	0.17	0.22	0.03	0.36	0.08	1.00							
ZNG	0.19	0.23	0.70	0	0.12	0.01	0.35	0.35	0.15	1.00						
IPNG	-0.05	-0.01	-0.05	0.20	0.34	0.23	0.19	0.01	-0.03	-0.03	1.00					
DHZNG	0.03	-0.18	-0.11	-0.17	0.03	0.01	0.03	-0.05	-0.02	0.13	-0.08	1.00				
ZoG	0.13	0.11	0.82	-0.09	0.21	-0.05	0.28	0.41	0.15	0.69	-0.13	-0.02	1.00			
ZRoG	-0.07	0.47	0.28	0.21	0.1	0.13	0.25	0.01	0.16	0.19	0.07	-0.15	0.07	1.00		
DHZZoG	0.16	0.26	0.56	-0.05	0.12	0.08	0.20	0.32	0	0.44	-0.1	-0.05	0.55	0.12	1.00	
DHZRoG	0.04	0.51	0.37	0.38	0.18	0.38	0.46	0.16	0.18	0.03	0.23	-0.12	0.03	0.66	0.05	1.00

¹ The analysis was performed with Genstat 5 Release 3.2 (IBM-PC 80386/DOS), and the data was transformed from Appendix 3, which consist of various biochemical substances, all of which are non-negative by definition, so that it is likely that the variance is dependent on the mean. As a consequence, the data were analysed using generalized linear models assuming a constant coefficient of variation rather than a constant variance. This is achieved by specifying a gamma distribution and a log link for a generalized linear model. The coefficient of variation is estimated by the square root of the residual mean deviance. The correlations among the biochemical quantities given in the above table require further multivariate analysis.

² Total cytokinins.